Banisteriopsis caapi, a unique combination of MAO inhibitory and antioxidative constituents for the activities relevant to neurodegenerative disorders and Parkinson's disease

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Abstract

Aim of the study—Parkinson's disease is a neurological disorder mostly effecting the elder population of the world. Currently there is no definitive treatment or cure for this disease. Therefore, in this study the composition and constituents of the aqueous extract of *B. caapi* for monoamine oxidases (MAO) inhibitory and antioxidant activities were assessed, which are relevant to the prevention of neurological disorders, including Parkinsonism.

Materials and methods—The aqueous extract of *B. caapi* stems was standardized and then fractionated using reversed-phase (RP) chromatography. Pure compounds were isolated either by reversed-phase (RP) chromatography or centrifugal preparative TLC, using a Chromatotron®. Structure elucidation was carried out by 1D and 2D NMR, Mass, IR and Circular Dichroism spectroscopy and chemical derivatization. Chemical profiling of the extract was carried out with RP-HPLC. The inhibitory activity of MAO-A, MAO-B, acetylcholinesterase, butyrylcholinesterase and catechol-*O*-methyl transferase enzymes, as well as antioxidant and cytotoxic activities of both *B. caapi* extract and isolated compounds were evaluated.

Results—An examination of the aqueous extracts of *B. caapi* cultivar Da Vine yielded two new alkaloidal glycosides, named banistenoside A (**1**) and banistenoside B (**2**), containing "azepino[1,2-a]tetrahydro-β-carboline" unique carbon framework. One additional new natural tetrahydronorharmine (**4**), four known β-carbolines harmol (**3**), tetrahydroharmine (**5**), harmaline (**6**) and harmine (**7**), two known proanthocyanidines (−)-epicatechin (**8**) and (−)-procyanidin B2 (**9**), and a new disaccharide β-D-fructofuranosyl-(2→5)-fructopyranose (**14**) together with known sacharose (**15**) and β-D-glucose (**16**) were also isolated. In addition, the acetates of **1, 2, 8, 9, 14** and

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15 (compounds **10**–**13, 17, 18**) were also prepared. Harmaline (**6**) and harmine (**7**) showed potent *in vitro* inhibitory activity against recombinant human brain monoamine oxidase (MAO) -A and -B enzymes (IC$_{50}$ 2.5 and 2.0 nM, and 25 and 20 μM, respectively), and (−)-epicatechin (**8**) and (−)-procyanidin B2 (**9**) showed potent antioxidant and moderate MAO-B inhibitory activities (IC$_{50}$ <0.13 and 0.57 μg/mL, and 65 and 35 μM). HPLC analysis revealed that most of the dominant chemical and bioactive markers (**1, 2, 5, 7**–**9**) were present in high concentrations in dried bark of large branch. Analysis of regular/commercial *B. caapi* dried stems showed a similar qualitative HPLC pattern, but relatively low content of dominant markers **1, 2, 7**, and **9**, which led to decreased MAO inhibitory and antioxidant potency.

Conclusion—Collectively, these results give additional basis to the existing claim of *B. caapi* stem extract for the treatment of Parkinsonism, including other neurodegenerative disorders.

Keywords

Banisteriopsis caapi (Spruce ex Griseb.); β-carboline alkaloids; Proanthocyanidines; Monoamine oxidase inhibitors; Antioxidants; Parkinson's disease

1. Introduction

Banisteriopsis (Family: Malpighiaceae) is a tropical South American genus with 92 species distributed mainly in Brazil, Bolivia, Colombia, Ecuador, and Peru (Mabberley, 1997; Schultes, 1970). *B. caapi* (Spruce ex Griseb.) Morton (Vine of the Soul) (Schultes and Raffauf, 1992) is an ingredient of the popular sacred and psychoactive drinks Ayahuasca, also known as Caapi, Pinde, Natema or Yaje, which is widely used for prophecy, divination, and as a sacrament in the northern part of South America (Schultes, 1970; Schultes and Siri von Reis, 1995). However, to the best of our knowledge, no traditional drink prepared only from *B. caapi* has been consumed for such uses. Earlier chemical investigation have reported the presence of β-carboline alkaloids (β-CA) harmine, harmaline and tetrahydroharmine (THH) as the principal MAO inhibitors, together with other β-CA's, from *B. caapi* (Hochstein and Paradies, 1957; Hashimoto and Kawanishi, 1975; *ibid* 1976; Callaway et al., 2005). In addition, two pyrrolidines, shihunine and (*S*)-(+)-dihydroshihunine (Kawanishi et al., 1982), and terpenoids (Aquino et al., 1991) were also reported. The alkaloid content of *B. caapi* was determined previously by GC/MS (Rivier and Lindgren, 1972), LC/MS (Kawanishi et al., 1998), and HPLC (Serrano-Duenas et al., 2001), suggesting the content of harmine is highest among β-CA's, followed by THH and harmaline.

Parkinson's disease (PD) is caused by a loss of neurons from *substantia nigra* of the brain. Once damaged, these neurons stop producing dopamine and compromise the brain's ability to control movement. It is not known what damages certain neurons in PD patients. One reason is that free radicals/ toxic particles normally deactivated in the body are responsible, which can be controlled by antioxidants as adjuvant with dopamine agonist or MAO inhibitors. The usefulness of *B. caapi* was established for alleviating symptoms of PD (Serrano-Duenas et al., 2001), which contains MAO inhibitor harmine as active constituent used in PD treatment (Sanchez-Ramos, 1991). A double-blind, randomized placebo-controlled trial of *B. caapi*, using a single dose, revealed a significant improvement in motor function of PD patients (Serrano-Duenas et al., 2001). Tests for MAO inhibition using liver homogenate showed that *B. caapi* stem extract and harmine showed a concentration-dependent inhibition of MAO-A, and an increase in release of dopamine from rat striatal slices (Schwartz et al., 2003).

It should be noted that the identities of different *Banisteriopsis* species are incompletely known due to the paucity of fertile collections and lack of detailed taxonomic study. There are at least thirty different *B. caapi* that natives of Amazon have knowledge of and have different uses

(Schultes and Hofman, 1992). During the course of chemical and biological standardization of *B. caapi* under an NIH funded investigation at the NCNPR for neurological disorders relevant to Parkinsonism, an extract of *B. caapi* cultivar Da Vine, collected in Oahu, Hawaii, demonstrated potent *in vitro* MAO-A inhibitory and antioxidant activities. This led to the bioassay guided isolation of two new β-carboline alkaloidal glycosides, banistenoside A (**1**) and banistenoside B (**2**), a new tetrahydronorharmine (**4**), four known β-carbolines harmol (**3**), tetrahydroharmine (**5**), harmaline (**6**) and harmine (**7**), two proanthocyanidines (−)-epicatechin (**8**) and (−)-procyanidin B2 (**9**), and a new disaccharide β-D-fructofuranosyl-(2→5)-fructopyranose (**14**) (Figure 1), using regular and RP silica gel chromatography. In this paper, we report the isolation, characterization and bioactivities of isolated compounds, and HPLC analysis of *B. caapi* cultivar Da Vine and two regular/ commercial samples of *B. caapi*.

2. Materials and methods

2.1. General experimental procedures

Optical rotations were measured in $CHCl_3$ or MeOH using an AUTOPOL IV® instrument at ambient temperature; IR spectra were obtained using a Bruker Tensor 27 FTIR instrument; Circular Dichroism (CD) spectra were recorded on a Olis DCM 20 CD spectrometer. The HPLC system consisted of a Model 2695 Alliance Separations Module equipped with a 2996 photodiode array detector, and a computerized data station equipped with Waters Empower 2 software (Waters, Milford, MA), using a Gemini C18 110 Å column (Phenomenex, 150×4.6 mm I.D.; 5 µm particle size; Phenomenex Inc., Torrance, CA, USA) and operated at 30 °C. The column was equipped with a 2 cm LC-18 guard column (Phenomenex Inc., Torrance, CA, USA). The NMR spectra were acquired on a Bruker Avance DRX-400 instrument at 400 MHz (^{1}H), 100 (^{13}C) in $CDCl_3$ or CD_3OD, using the residual solvent as int. standard; multiplicity determinations (DEPT) and 2D NMR spectra (COSY, HMQC, HMBC and NOESY) were obtained using standard Bruker pulse programs; HRMS were obtained by direct injection using a Bruker Bioapex-FTMS with electro-spray ionization (ESI). Plant material was extracted by either Coffee Maker (Mr. Coffee®, ISX-43) or Accelerated Solvent Extractor (Dionex®, ASE-200) using H_2O as a solvent. Water extracts were freeze-dried using Freeze Dry System (Labconco®, Freezone 4.5). TLC was carried out on either reversed phase silica gel (Analtech®, RP18 SiO_2, 150 µm, UV254) with $MeCN$-H_2O (9:1) or acetone-H_2O-$NH_3 \cdot H_2O$ (7:3:0.1), silica gel (EMD® Chemicals Inc., SiO_2 60 F_{254}) using $CHCl_3$-MeOH (7:3) or alumina plates (EMD® Chemicals Inc., Al_2O_3 60 F_{254}) using CH_2Cl_2-MeOH (9:1) as solvent system; centrifugal preparative TLC (CPTLC) was performed by a Chromatotron® (Harrison Research Inc., model 8924), tagged with a fraction collector (SpectralChrom® CF-1) on a 1, 2 or 4 mm silica gel rotors (Analtech®, SiO_2 coated with F_{254} indicator); flash CC was carried out over reversed phase silica gel (J.T. Baker, Bakerbond® C18 prep LC SiO_2, 40 µm). Samples were dried using a Savant Speed Vac Plus SC210A Concentrator. The isolated compounds were visualized by observing under UV light at 254 or 365 nm, followed by spraying separately with Dragendorff's and/or 1% vanillin-H_2SO_4 spray reagents. The reference standards of harmol, harmine, harmaline, and (−)-epicatechin were purchased from Sigma-Aldrich (St. Louis, MO), while others were available in our laboratories.

2.2. Plant material

Fresh leaves, stems, and large branches of *B. caapi* cultivar Da Vine (Miller, 1986) were collected from the island of Oahu, Hawaii, in August and November 2007, and June 2008, as well as from Hilo (Big island), Hawaii, USA, in October, 2007. A reference specimen was collected from Oahu, and a voucher specimen (HLA # 7835) was deposited at the Herbarium of Harold Lyon Arboretum, University of Hawaii. The collector of the voucher specimen of the plant at Lyon Arboretum was Dr. Kenneth M. Nagata (Accession # L-81.0727; collector #

2789; dated 03/13/1984). The regular *B. caapi* (mature stems) samples analyzed in this study (BCEx-1–BCEx-4) were procured/obtained from commercial (*via* internet) and NCNPR sources during 2005–2009. In addition, all samples used in this work are preserved using the standard procedures for collection, drying, grinding and packaging at the NCNPR.

2.3. Extraction of plant material

2.3.1. Preparation of extracts from different plant parts—The different plant parts (leaves, young and mature stems, bark and debarked stems, and large branches (diameter: 3–8 cm)) of *B. caapi*, fresh and dried, were extracted using hot water by two different methods. A portion of each fresh sample was dried at 45 °C for 2–4 days, to get dried material. Before processing, the fresh plant material was cut into small pieces, while dried plant material was ground.

A. "Hot maceration". Water extracts were prepared by simmering with boiled water using a Coffee Maker. Plant material was placed onto a coffee filter and extracted successively with distilled H_2O for three times (30–70 g/ 100 mL; $H_2O \times 3$). This gave strong yellow colored extracts.

B. "ASE extraction". Water extracts were prepared by a semi-automated Accelerated Solvent Extractor (ASE-200). Plant material was packed into a steel 33 mm cells and extracted successively with distilled H_2O for four times (15–20 g/ 5 mL $H_2O \times 4$; 100 °C, 3.45 MPa of N_2, 30 min). Next optimal method was developed and used: preheat time = 0 min, pressure = 3.45 MPa, heat time = 5 min, T = 100 °C, static time = 30 min, flush = 100% vol, purge time = 2 min, cycles = 4. This gave strong yellow-brown colored water extract (ca 20 mL in total).

All water extracts were cooled, frozen at −20 °C and freeze-dried by Freeze Dry System (−44 °C, 1.6 Pa). After freeze-drying all samples were transferred into the vials and sealed with Parafilm®.

2.3.2. Preparation of standardized extract of B. caapi (Da Vine) whole stem—Based on the MAO-A and antioxidant activity of initial extracts of different plant parts, prepared by using different extraction methods, the "most active" extracts were identified (Table 1). These are hot aqueous extracts from dried powdered samples of large branch, whole mature/big stem and mature stem bark prepared by method A (using a coffee maker). In order to proceed for bioassay-guided isolation of active constituents and markers, bulk extraction of dried powdered large branch (471 g) was executed using the standardized method, as described above, which yielded 60 g of dried pale-yellow powder.

2.4. Bioassay-guided fractionation and characterization of isolated compounds

The freeze-dried powdered aqueous extract (53.7 g), obtained by bulk isolation from large branch, was subjected to RP-CC over C-18 silica gel (1:15) and eluted with MeCN (8 L) and then MeCN-H_2O mixture with increasing concentrations of H_2O (1% → 10%), and 50 mL fractions were collected. Fractions were pooled by TLC, combined, and then evaporated under reduced pressure (total 18.65 g from 14 combined fractions, i.e. fractions A-N). Fraction A (325 mg) was purified by CPTLC, using 2 mm silica gel rotor with CHCl$_3$-MeOH (9:1) as a solvent, which afforded **8** (110 mg), followed by **9** (24 mg), while fraction B (125 mg) yielded **5** (38 mg), followed by **4** (24 mg), by using a similar CPTLC procedure. Fraction D (118 mg) was also separated by CPTLC, using 1 mm silica gel rotor with CHCl$_3$-MeOH (9:1) as a solvent, which afforded **7** (56 mg), followed by **6** (17 mg), while fraction H (276 mg) was separated by a 2 mm silica gel CPTLC rotor, using CHCl$_3$-MeOH (9:1) as solvent, which gave **14** (20 mg), and a mixture H-1 (90 mg), containing **9** and **14**. Fraction I (489 mg) was also separated by CPTLC, using 4 mm silica gel rotor [CHCl$_3$-MeOH (9:1) as solvent], which yielded **7** (29

mg), followed by a another mixture I-1 (317 mg), which was further purified by 2 mm silica gel CPTLC rotor [CHCl$_3$-MeOH (9:1) as solvent] to yield compounds **15** (sacharose; 79 mg) and **16** (β-D-glucose; 55 mg). Finally, fraction L (2.0 g) was separated by RP-flash chromatography over C-18 silica gel, eluted with increasing concentrations of H$_2$O in acetone (1:9 → 1:1, 2 L), which afforded **1** (60 mg), followed by **2** (20 mg) as well as fractions L-1 (0.3 g) and L-2 (1.3 g). Fraction L-2 consisted of mixtures of **1, 2** and **15**, while fraction L-1 was the mixture of **6** and **7**.

Using the above procedures, bulk quantities of **1**–**9** (i.e. 80–100 mg) were isolated from aqeuous stem extracts of samples collected in 2007 and 2008. Compound **3** was identified by HPLC using direct comparison with an authentic sample of harmol. Compounds **5**–**9, 15** and **16** were identified by comparison of physical and spectral data with those reported in the literatures, and by direct comparison with their authentic samples as THH, harmaline, harmine, epicatechin and procyanidin B2, sacharose and glucose, respectively.

Banistenoside A (1)—Colorless solid; UV (MeOH) λ$_{max}$, nm: 226, 269 and 290; IR (film) ν$_{max}$, cm^{-1}: 3382 (NH), 2920, 2850, 1804, 1752, 1631, 1225, 1037, 733; ^{1}H and ^{13}C NMR data, see Table 2; HRESIMS *m/z* 367.1522 [MH+H$_2$O-Glucosyl]$^+$ (calcd. for C$_{17}$H$_{23}$N$_2$O$_7$, 367.1505); 529.2013 [MH+H$_2$O]$^+$ (calcd. for C$_{23}$H$_{33}$N$_2$O$_{12}$, 529.2033).

Banistenoside B (2)—Colorless solid; UV (MeOH) λ$_{max}$, nm: 226, 269 and 290; IR (film) ν$_{max}$, cm^{-1}: 3448 (NH), 3021, 2923, 2851, 1749, 1692, 1632, 1221, 1044, 757; HRESIMS *m/z* 367.1491 [MH+H$_2$O-Glucosyl]$^+$ (calcd. for C$_{17}$H$_{23}$N$_2$O$_7$, 367.1505); 529.2028 [MH+H$_2$O]$^+$ (calcd. for C$_{23}$H$_{33}$N$_2$O$_{12}$, 529.2033).

Tetrahydronorharmine (4)—Colorless solid; UV (MeOH) λ$_{max}$, nm: 226, 269 and 290; ^{1}H NMR (CDCl$_3$, J in Hz) δ$_H$ 7.10 (1H, d, 7.5, H-5), 6.67 (1H, brs, H-8), 6.49 (1H, brd, 7.5, H-6), 4.15 (2H, m, H-1), 3.58 (3H, s, OCH$_3$), 3.30 (2H, m, H-3), 2.81 (2H, m, H-3); ^{13}C NMR (CDCl$_3$) δ$_C$ 156.9 (s, C-7), 137.8 (s, C-8b), 124.0 (s, C-9a), 120.8 (s, C-4b), 118.2 (d, C-5), 109.2 (d, C-6), 105.8 (s, C-4a), 94.5 (d, C-8), 54.7 (q, OCH$_3$), 42.6 (t, C-3), 41.0 (t, C-1), 18.5 (t, C-4); HRESIMS *m/z* 203.1176 [MH]$^+$ (calcd. for C$_{12}$H$_{15}$N$_2$O, 203.1184).

(−)-2*R*,3*R*–Epicatechin (8)—The physical and spectral data of **8** were identical to those reported in the literature (Sun et al., 2006; Balde et al., 1991). The CD data [(in MeOH); λ$_{max}$, nm ([θ] in mdeg): 244 (21.58), 269 (−15.83), 290 (−3.46)] was in agreement with those recorded for an authentic sample of (−)-2*R*,3*R*-epicatechin, and (Korver and Wilkins, 1971).

(−)-2*R*,3*R*-epicatechin-4β,8-(−)-2*R*,3*R*-epicatechin [(−)-procyanidin B2] (9)—The physical and spectral data of **9** were identical to those reported in the literature (Korver and Wilkins, 1971; Barrett et al., 1979; Khan et al., 1997). In addition, the CD data [(in MeOH) λ$_{max}$, nm ([θ] in mdeg): 221 (−16.24), 243 (52.58), 276 (−3.64)] was also in agreement with those reported in the literatures (Barrett et al., 1979)

(−)-β-D-Fructofuranosyl-(2→5)-fructopyranose (Di-D-Fructose) (14)—Colorless solid; [α]$_D^{23.5}$ −55.8 (c 0.78, MeOH); ^{1}H NMR (Pyridine-D$_5$, *J* in Hz) δ$_H$ 5.60 (brs, OH), 5.12 (1H, d, 8.0, H-3), 5.04 (1H, t, 8.0, H-4), 4.82 (1H, brd 12.0, H-4'), 4.62 (1H, dd, 8.0, 12.0, H-3'), 4.59 (1H, m, H-5), 4.49 (1H, dd, 1.0, 12.0, H-6'), 4.39 (1H, d, 8.0, H-1'), 4.38 (1H, m, H-5'), 4.29^a (m, H-1'), 4.29^a (m, H-6), 4.24–4.26^a (m, H-1), 4.24 (1H, d, 12.0, H-6), 4.11 (1H, dd, 4.0, 12.0, H-6'); ^{13}C NMR (Pyridine-D$_5$) δ$_C$ 103.6 (s, C-2), 99.3 (s, C-2'), 84.3 (d, C-5), 78.0 (d, C-3), 77.0 (d, C-4), 73.3 (d, C-3'), 71.7 (d, C-5'), 70.0 (d, C-4'), 66.2 (t, C-1'), 64.9 (t, C-1), 64.2 (t, C-6'), 63.8 (t, C-6); ^{1}H NMR (CD$_3$OD, *J* in Hz) δ$_H$ 5.51 (brs, OH), 4.62^b (m, H-1'), 4.62^b (m, H-1), 4.49^c (d, 8.0, H-1), 4.49^c (d, 8.0, H-1'), 4.06^d (m, H-3), 4.06^d (m, H-5'), 4.02 (1H, dd, 4.0, 12.0, H-6'), 3.86 (1H, brs, H-3'), 3.78^e (m, H-4), 3.78^e (m, H-4'), 3.73 (1H,

m, H-5), 3.62^e (dd, 4.0, 12.0, H-6), 3.62^e, (dd, 4.0, 12.0, H-6'), 3.49 (1H, brd, 8.0, H-6) ($^{a–e}$signals are superimposed on each other); ^{13}C NMR (CD$_3$OD) δ_C 101.7 (s, C-2), 97.8 (s, C-2'), 81.3 (d, C-5), 76.6 (d, C-5'), 75.4 (d, C-3), 70.4 (d, C-4), 69.8 (d, C-3'), 68.3 (d, C-4'), 64.5 (t, C-6'), 62.9 (t, C-1'), 62.9 (t, C-1), 61.2 (t, C-6).

Acetylation of Fraction L-2—Fraction L-2 (355 mg) containing the mixture **1**, **2** and **15** was dissolved in pyridine (1 mL) and treated with acetic anhydride (2 mL). The reaction mixture was stirred overnight at room temperature, and then dried under vacuum to give the solid residue. The residue was subjected to CPTLC over 2 mm silica gel rotor [CHCl$_3$-MeCN (9:1) as solvent], which afforded **10** (28 mg) and **18** (127 mg), together with fraction L-2-1 (127 mg), which was further separated by CPTLC, using 1 mm silica gel rotor with CHCl$_3$-MeCN (19:1) as a solvent to yield compounds **11** (20 mg) and additional amounts of **18** (37 mg). Compound **18** was identified as sacharose octaacetate by physical and NMR data.

Banistenoside A heptaacetate (10)—Colorless solid; [α]$_D^{28}$ −8.7 (c 0.6, CHCl$_3$); CD (in MeOH) λ_{max}, nm ([θ] in mdeg): 316 (13.03), 361 (−0.85), 369 (−0.79); IR (film) ν_{max}, cm^{-1}: 3455 (OH, NH), 3382 (OH, NH), 2920, 2850, 1804, 1752, 1631, 1426, 1371, 1225, 1072, 1037, 910, 733; ^{1}H and ^{13}C NMR data, see Table 2; HRESIMS m/z 805.2635 [MH]$^+$ (calcd. for C$_{37}$H$_{45}$N$_2$O$_{18}$, 805.2667); 827.2454 [MNa]$^+$ (calcd. for C$_{37}$H$_{44}$N$_2$O$_{18}$Na, 827.2487).

Banistenoside B heptaacetate (11)—Colorless solid; [α]$_D^{28}$ +31.1 (c 0.4, CHCl$_3$); CD (in MeOH) λ_{max}, nm ([θ] in mdeg): 311 (21.39); IR (film) ν_{max}, cm^{-1}: 3448 (OH, NH), 3021, 2923, 2851, 1749, 1692, 1434, 1371, 1221, 1044, 757; ^{1}H and ^{13}C NMR data, see Table 2; HRESIMS m/z 805.2693 [MH]$^+$ (calcd. for C$_{37}$H$_{45}$N$_2$O$_{18}$, 805.2667); 827.2518 [MNa]$^+$ (calcd. for C$_{37}$H$_{44}$N$_2$O$_{18}$Na, 827.2487).

Acetylation of (−)-epicetechin (8)—Epicatechin **8** (27 mg, 93 mmol) was dissolved in pyridine (1 mL). Acetic anhydride (1 mL) was added dropwise to the solution while stirring. The reaction mixture was stirred overnight at ambient temperature. The reaction mixture was dried under vacuum to give the solid residue which was further purified by flash chromatography over silica gel to yield compound **12** (39 mg, yield 84 %) as a pale solid. Physical and Spectral data of **12** were identical to those reported in the literature (Castillo et al., 2004).

Acetylation of Fraction H-1—Fraction H-1 (77 mg), containing the mixture **9** and **14**, was dissolved in pyridine (1 mL) and treated with acetic anhydride (2 mL). The reaction mixture was stirred overnight at room temperature, and dried under vacuum to give the solid residue, which was subjected to CPTLC, using 1 mm silica gel rotor with CHCl$_3$-MeCN (98:2) as solvent system to afford acetates **13** (29 mg) and **17** (47 mg).

(−)-Procyanidin B2 acetate (13)—Colorless solid; [α]$_D^{235}$ −80 (c 0.025, MeOH); CD (in 1,4-dioxane) λ_{max}, nm ([θ] in mdeg): 233 (−5.14), 245 (73.43), 274 (−12.35), 285 (−10.86); IR (film) ν_{max}, cm^{-1}: 2963, 2917, 2855, 1767, 1621, 1596, 1208, 1122, 1049, 901; ^{1}H and ^{13}C NMR data were identical to those reported in the literature (Korver and Wilkins, 1971; Barrett et al., 1979; Khan et al., 1997); HRESIMS m/z 1016.2859 [MNH$_4$]$^+$ (calcd. for C$_{50}$H$_{50}$O$_{22}$N, 1016.2824); 1021.2346 [MNa]$^+$ (calcd. for C$_{50}$H$_{46}$O$_{22}$Na, 1021.2378); 1037.2160 [MK]$^+$ (calcd. for C$_{50}$H$_{46}$O$_{22}$K, 1037.2118).

β-D-fructofuranosyl(2→5)fructopyranose octaacetate (17)—Colorless solid; [α]$_D^{235}$ +30.4 (c 0.1, CHCl$_3$); IR (film) ν_{max}, cm^{-1}: 2942, 1746, 1433, 1372, 1222, 1050; ^{1}H NMR (CDCl$_3$, J in Hz) δ_H 5.72 (1H, d, 4.0, H-3), 5.63 (1H, dd, 2.0, 8.8, H-3'), 5.44 (1H, d, 2.0, H-4'), 5.15 (1H, m, H-2'), 5.07 (1H, dd, 4.0, 6.0, H-4), 4.86 (1H, brd, 17.6, H-6'), 4.63 (1H,

brd, 17.6, H-6'), 4.55 (1H, d, 9.6, H-1), 4.45 (1H, m, H-5), 4.35 (1H, dd, 2.8, 12.4. H-1'), 4.30 (1H, d, 9.6, H-1), 4.22 (1H, dd, 5.6, 12.4, H-6), 4.10 (1H, dd, 5.2, 12.4, H-1'), 4.05 (1H, dd, 4.8, 12.4, H-6), 2.15, 2.10, 2.05, 2.04 ($\times$3), 2.02, 2.01 (8s, CH_3); ^{13}C NMR ($CDCl_3$) δ_C 197.9 (s, C-2'), 179.9, 170.4, 169.8, 169.6, 169.5, 169.4, 169.02, 169.0 (8s, C=O), 107.9 (s, C-2), 80.6 (d, C-5), 78.4 (d, C-3), 76.1 (d, C-4), 74.2 (d, C-3'), 68.3 (d, C-4'), 68.0 (d, C-5'), 66.6 (t, C-1'), 62.8 (t, C-6'), 61.7 (t, C-6), 61.5 (t, C-1), 20.7 ($\times$2), 20.6 (x2), 20.5, 20.4, 20.3, 20.2 (8q, CH_3); HRESIMS m/z 696.2330 $[MH_2O]^+$ (calcd. for $C_{28}H_{42}O_{19}N$, 696.2351); $C_{28}H_{38}O_{19}$; 701.1903 $[MNa]^+$; (calcd. for $C_{28}H_{38}O_{19}Na$, 701.1905).

2.5. Inhibition kinetics assay using recombinant human MAO-A and MAO-B

Recombinant human Monoamine Oxidase A (MAO-A) and Monoamine Oxidase B (MAO-B) were obtained from BD Biosciences (Bedford, MA). To investigate the effect of extracts on MAO-A and MAO-B, the kynuramine deamination assay was adapted for 96-well plates. The inhibition kinetics of MAO-A and MAO-B by inhibitors/extracts were determined in a range expected to produce 30% to 90% inhibition. The IC_{50} values for extracts, pure compounds and reference standards were determined at five concentrations of 1, 0.1, 0.01, 0.001 and 0.0001 µg/mL for MAO-A, where as for MAO-B, percent inhibitions of all crude extracts were evaluated at three concentrations, 100, 10 and 1 µg/mL. All experiments were carried out in duplicate. A fixed substrate concentration and varying inhibitor concentrations were used to determine the IC_{50} value at the point where 50% inhibition of the catalytic activity of the enzyme occurred. For MAO-A, the substrate concentration of 80 µM kynuramine was chosen because the apparent K_m value for substrate binding has been reported previously was approximately 40 µM (Parikh et al., 2002). Since K_m is the substrate concentration at half V_{max}, therefore, $2 \times K_m$ ($2 \times 40 = 80$ µM), was selected for determining IC_{50} values. Similarly, for MAO-B, substrate concentration of 50 µM kynuramine was chosen. The assay was performed with the addition of inhibitor. Inhibition was calculated as percent of product formation compared to the corresponding control (enzyme-substrate reaction) without the inhibitors. The reactions were carried out in 0.1 M potassium phosphate buffer at pH 7.4. Incubations mixtures contained 5 µg/mL of MAO-A (50 µL in buffer) and 10 µg/mL of MAO-B (50 µL in buffer). The inhibitor was dissolved in DMSO or in buffer (if not dissolved in DMSO). The total reaction volume was 200 µL yielding a final DMSO concentration of 1.0% in the reaction mixture. The reaction mixtures were pre-incubated for 10 minutes at 37 °C followed by the addition of MAO-A / MAO-B to initiate the reactions. Reactions were incubated for 20 minutes at 37 °C and were stopped immediately by the addition of 75 µL of 2N NaOH. The formation of 4-hydroxyquinoline was determined fluorometrically by SpectraMax M5 fluorescence plate reader (Molecular Devices, Sunnyvale, CA) with an excitation and emission wavelength of 320 nm and 380 nm, respectively, using the SoftMax Pro program.

2.6. In vitro cytotoxicity assay

The *in vitro* cytotoxic activity was determined against four human cancer cell lines (SK-MEL, KB, BT-549 and HepG$_2$), monkey kidney fibroblasts (VERO) and pig kidney epithelial cells (LLC-PK$_{11}$). All cell lines were obtained from the American Type Culture Collection (ATCC, Rockville, MD). The assay was performed in 96-well tissue culture-treated microplates. Cells were seeded at a density of 25,000 cells/well and incubated for 24 h. Samples at different concentrations were added and plates were again incubated for 48 h. The number of viable cells was determined using Neutral Red according to a modification of the procedure of Borenfreund *et al* (1990). IC_{50} values were determined from dose response curves of percent growth inhibition against test concentrations. Doxorubicin was used as a positive control, while DMSO was used as the negative (vehicle) control.

2.7. Cell Based Assay for Antioxidant Activity (Rosenkranz et al., 1992; Scudiero et al., 1988)

The effect of samples on the generation of ROS in Myelomonocytic HL-60 cells is determined by the DCFH-DA (2',7'-dichlorofluorescin diacetate) method. HL-60 cells (ATCC) were cultured in RPMI-1640 medium with 10% fetal bovine serum, penicillin (50 units/mL) and streptomycin (50 µg/mL). 125 µL of the cell suspension (1×10^6 cells/mL) was added to the wells of a 96-well plate. After treatment with different concentrations (i.e., six concentrations of 62.50, 31.25, 15.63, 7.82, 3.91 and 1.95 µg/mL were used) of the test samples for 30 min, cells were exposed to 100 ng/mL phorbol 12-myristate-13-acetate (PMA, Sigma) for 30 min. DCFH-DA (Molecular Probes, 5 µg/mL) was added and cells were further incubated for 15 min. Levels of fluorescent DCF (produced by ROS catalyzed oxidation of DCFH) was measured on a PolarStar Galaxy plate reader with excitation wavelength at 485 nm and emission at 530 nm. Inhibition of ROS generation by test samples is determined in terms of % decrease in DCF production compared to the vehicle control. Vitamin C and trolox were used as the positive control in each assay. DCFH-DA is a non-fluorescent probe that diffuses into cells, where cytoplasmic esterases hydrolyse the DCFH-DA to 2',7'- dichlorofluorescin (DCFH). The ROS generated within HL-60 cells oxidize DCFH to the fluorescent dye 2',7'- dichlorofluorescein (DCF). The ability of the test materials to inhibit exogenous cytoplasmic ROS-catalysed oxidation of DCFH in HL-60 cells is measured in comparison to PMA treated vehicle control. The cytotoxicity of samples to HL-60 cells was also determined after incubating the cells (2×10^4 cells/well in 225 µL) with test samples for 48 hours by XTT method. Briefly, 25 µL of XTT-PMS solution (1 mg/ml XTT solution supplemented by 25 µM of PMS) was added to each well. After incubating for four hours at 37 °C, absorbance was measured at a dual wavelength of 450 – 630 nm on a Bio-Tek plate reader.

2.8. Inhibition assay for acetylcholinesterase, butyrylcholinesterase and catechol-O-methyl transferase enzymes activity

Acetylcholinesterase, butyrylcholinesterase and catechol-*O*-methyl transferase enzymes assays were carried out as described previously (Ellman et al., 1961; Learmonth et al., 2004).

3. Results and discussion

The hot aqueous extracts of fresh and dried large branches of *B. caapi* demonstrated significant MAO-A inhibitory and antioxidant activity (Table 1). A bioassay-guided fractionation of crude aqueous extract of *B. caapi* by reversed-phase column chromatography (RPCC) afforded a polar non-alkaloidal fraction by eluting with MeCN, which showed potent antioxidant and moderate MAO-B inhibitory activities. This was followed by an alkaloidal fraction, eluted with MeCN-H_2O as solvent, which showed potent MAO-A and moderate MAO-B inhibitory activites. Subsequent RPCC of polar alkaloidal fraction yielded two new alkaloidal glycosides **1** and **2**, as major markers, while polar non-alkaloidal fractions afforded the potent antioxidants **8** and **9**. Furthermore, centrifugal preparative TLC (CPTLC) of alkaloidal fractions yielded the tetrahydronorharmine (**4**), and four known MAO inhibitors β-CA's **3** and **5**–**7**.

3.1. Structure elucidation

Compound **1** was analyzed for $C_{23}H_{30}N_2O_{11}$ by ESIHRMS. The UV spectrum of **1** demonstrated absorption bands at λ_{max} 226, 269 and 290 nm, typical of THH chromophore (Hashimoto and Kawanishi, 1975; *ibid* 1976), and IR spectrum showed absorption bands at ν_{max} 3550 and 1740 cm^{-1}, attributed to hydroxyl and amide (δ_{C-14} 175.3) groups, respectively. The NMR spectrum of **1**, recorded in $CF_3COOD+D_2O$, revealed the presence of three aromatic protons δ_{H-5} 6.90, δ_{H-6} 6.19 and δ_{H-8} 6.35, a methine proton at δ_{H-1} 4.44, two methylene groups at δ_{H-3} 3.05 and 3.31 (each 1H), and δ_{H-4} 2.51 (2H), and a methoxyl group at δ_H 3.23, suggested the presence of a C-1 substituted THH nucleus. It also showed four oxymethine protons at

$\delta_{\text{H-10}}$ 4.23, $\delta_{\text{H-11}}$ 4.44, $\delta_{\text{H-12}}$ 4.09 and $\delta_{\text{H-13}}$ 4.04, either as broad singlets or multiplets, which showed 2D NMR COSY correlations for the system [-CH(OH)-CH(OH)-CH(OH)-CH(OH)-]. The ^{13}C NMR spectrum showed the presence of a glucose moiety ($\delta_{\text{C-1}'}$ 94.4, $\delta_{\text{C-2}'}$ 71.8, $\delta_{\text{C-3}'}$ 71.7, $\delta_{\text{C-4}'}$ 66.1, $\delta_{\text{C-5}'}$ 72.3 and $\delta_{\text{C-6}'}$ 61.5), and a methoxyl group ($\delta_{\text{C-7-OMe}}$ 54.3). In addition, the ^{13}C NMR showed signals for C-1 – C-9a of THH base skeleton, four oxymethine carbons and a quaternary amide carbonyl, which were assigned by using 2D NMR HMQC and HMBC experiments of **1**. Compound **2** was also analyzed for $C_{23}H_{30}N_2O_{11}$ by ESIHRMS and its UV spectrum demonstrated absorption bands at λ_{max} 226, 267 and 294 nm, as observed for **1**. The presence of amide carbonyl and hydroxyl groups was suggested from the IR spectrum. Compound **2** was highly insoluble in most of the NMR solvents, like **1**, and therefore, the spectroscopic analyses were performed on the acetates for both the compounds.

Acetylation of a fraction (L-1) containing mixture of **1** and **2** afforded the corresponding heptaacetates **10** and **11**, which were subsequently separated and purified by CPTLC (see Materials and Methods Section). Compound **10** was analyzed for $C_{37}H_{44}N_2O_{18}$ by ESIHRMS, and found to be levorotatory; $[\alpha]_D$ −8.7 (c 0.6, CHCl$_3$). It's ^{1}H and ^{13}C NMR spectra displayed signals for seven acetyl groups, a methoxyl and a glucose moiety (Table 2). The ^{1}H NMR spectrum of **10**, in CDCl$_3$, demonstrated multiplicities for all the protons, previously not observed for **1** in CF$_3$COOD+D$_2$O, and structure **1** was fully substantiated by 2D NMR COSY, HMQC, HMBC and NOESY experiments on **1**-Ac (**10**). The presence of tetra-*O*-acetyl-β-D-glucose moiety (Baddeley et al., 2005) was suggested from its ^{13}C and ^{1}H NMR [$\delta_{\text{H-12}}$ 96.7, 71.4, 72.1, 68.6, 72.8, 62.2; C-1′-C-6′, respectively; $\delta_{\text{H-1}'}$ 4.54 (d, J=4.8 Hz)]. A 2D NMR COSY spectrum showed correlations between four acetoxy protons at $\delta_{\text{H-10}}$ 4.32, $\delta_{\text{H-11}}$ 5.20, $\delta_{\text{H-12}}$ 5.61 and $\delta_{\text{H-13}}$ 5.72, together with the correlation between $\delta_{\text{H-10}}$ 4.32 (brd, J=4.3 Hz) and $\delta_{\text{H-1}}$ 5.98 (d, J=4.3 Hz), suggesting C-10 of the system [$-^1$CH-^{10}CH(OR)-^{11}CH(OAc)-^{12}CH (OAc)-^{13}CH(OAc)-] was connected with C-1 of THH base skeleton. The structure was confirmed by a 2D NMR HMBC experiment (Table 2), which showed key correlations between H-1 and C-3, C-4a, C-9a, C-10, C-11 and C-14, and H-10 and C-1, C-1' (i.e., anomeric carbon), C-9a and C-12, confirming the formation of a seven- member ring between C-1 and N-2 of THH skeleton (-^{1}C-^{10}C-^{11}C-^{12}C-^{13}C-^{14}C-^{2}N-), and the linkage of C-1′ of glucose moiety at C-10. HMBC further showed cross peaks between N$_9$-H ($\delta_{\text{N-H}}$ 8.45) and C-4a, C-4b, C-8a and C-9a, and between C-7-OMe ($\delta_{\text{H-7-OMe}}$ 3.84) and C-7 (δ_{C} 156.8), confirming the presence of a free indole N-H proton and -OMe group at C-7. Finally, the configuration of compound **10** was assigned by a Circular Dichroism spectra and NOESY experiment (*vide infra*).

Compound **11** was also dextrorotatory {$[\alpha]_D$ +31.1 (c 0.4, CHCl$_3$)}, and analyzed for $C_{37}H_{44}N_2O_{18}$ by ESIHRMS. The ^{1}H and ^{13}C NMR (Table 2) showed the presence of seven acetyl groups, and its THH carbon skeleton fused with a seven-member ring, *via* five oxygenated carbons C-10 – C-14, was found to be similar to those of acetate **10**. The ^{1}H NMR spectrum of **11** was generally similar to those observed for **10** except for significant shieldings of chemical shift values for protons H-1, H-10 and H-12, and deshieldings of H-11, H-13 and H-1', compared to those observed in **10** ($\delta_{\text{H-1}}$ 5.08, $\delta_{\text{H-10}}$ 3.98, $\delta_{\text{H-11}}$ 5.77, $\delta_{\text{H-12}}$ 5.32, $\delta_{\text{H-13}}$ 5.87, $\delta_{\text{H-1}'}$ 4.91 *vs.* $\delta_{\text{H-1}}$ 5.98, $\delta_{\text{H-10}}$ 4.32, $\delta_{\text{H-11}}$ 5.20, $\delta_{\text{H-12}}$ 5.61, $\delta_{\text{H-13}}$ 5.72 and $\delta_{\text{H-1}'}$ 4.54, respectively, for **10**). A COSY spectrum showed correlations between $\delta_{\text{H-10}}$ 3.98, $\delta_{\text{H-11}}$ 5.77, $\delta_{\text{H-12}}$ 5.32 and $\delta_{\text{H-13}}$ 5.87, and between $\delta_{\text{H-10}}$ 3.98 (brd, *J*=10.0 Hz) and $\delta_{\text{H-1}}$ 5.08 (d, *J*=10.0 Hz), suggesting a similar system [−CH-CH(OR)-CH(OAc)-CH(OAc)-CH(OAc)-] as observed for **10**, although the chemical shift values for these protons were different to those of **10**. Likewise, the ^{13}C NMR also displayed significant deshielding of C-10 and C-1' ($\delta_{\text{C-10}}$ 81.2, $\delta_{\text{C-1}'}$ 101.6 *vs.* $\delta_{\text{C-10}}$ 72.9, $\delta_{\text{C-1}'}$ 96.7), shielding of C-11 and C-14 ($\delta_{\text{C-11}}$ 69.5, $\delta_{\text{C-14}}$ 164.4 *vs.* $\delta_{\text{C-11}}$ 77.2, $\delta_{\text{C-14}}$ 170.6, respectively, for **10**), compared to those observed for **10**, suggesting compound **11** appeared to be a diastereoisomer of **10**. The gross structure was established by HMBC experiments, which revealed ^2J and ^3J-correlation of protons and carbons of ring A – C, and glucose moiety involving H-1 and C-3, C-4a, C-9a, C-10, C-11 and C-14, and H-10

and C-1, C-1', C-9a and C-12. The presence of a free indole N-H proton and C-7-OMe group was also confirmed by HMBC, which showed correlation between N$_9$-H (δ_{N-H} 8.45) and C-4a, C-4b, C-8a and C-9a, and between C-7-OMe ($\delta_{H-7-OMe}$ 3.84) and C-7 (δ_C 156.8), respectively, as observed for **10**.

The absolute stereochemistry of C-1 stereocenter of compound **10** and **11** was established by Circular Dichroism spectra and NOESY correlations. The CD spectra of the two compounds displayed a high amplitude strong positive Cotton effect in the 310–320 nm regions, which is explicable to C-1(*R*) chiral center with α-orientation of H-1. These data was in agreement with C-1(*R*) mitragynine and analogs (Lee et al., 1967) with strong positive Cotton effect at 270–300 nm regions, while C-1(*S*)-speciogynine (Lee et al., 1967) and C-1(*S*)-cadambine (Brown and Fraser, 1974) exhibited strong negative Cotton effect in these regions in their CD spectra. In addition, the ^{1}H NMR spectrum showed small coupling constant (*J* = 4.3 Hz) of **10**, compared to large value (*J* = 10 Hz) for **11**, suggesting *cis*- and *trans*-relationships between H-1 and H-10 protons in these two compounds, respectively. Based on this assumption, the spatial orientation of the relevant protons was confirmed by NOESY spectrum of **10** and **11** (Figure 2). Compound **10** showed correlation between H-1, H-3a, H-11 and H-12, and H-10 and H-3a, indicating that these protons are α-cofacially oriented, thereby C-10-glucosyl, and C-11 and C-12-acetoxy groups were on the β-face of the molecule. In addition, NOESY spectra also showed cross peaks between H-1' and H-1 and H-5', and H-5' and H-11, suggesting they are in close spatial proximity among themselves. A close comparison of the NOESY spectra of **11** with those of **10** revealed clear differences for the stereocenters C-10 and C-13, which was earlier indicated by the differences in ^{1}H NMR chemical shift values for H-10 – H-13 between the two compounds. A NOESY experiment of **11** (Figure 2) showed correlation between H-1, and H-11 and H-13, as well as H-11 and H-12, suggesting these protons are *cis* and placed at the α-face of the molecule, since H-1 had α-disposition due to C-1(*R*) absolute configuration, like **10**, determined by CD spectra. Therefore, the acetyl groups at C-11, C-12 and C-13 were placed on the β-face of the molecule. The placement of C-10 glucosyl moiety at the α-face of the molecule was evident from the large *trans* coupling ($J_{1,10}$ = 10 Hz) between H-1 and H-10, as well as NOESY correlations between H-10 and H-1', suggesting their close spatial relationships in **11**. Analyses of the molecular models further confirmed the above observations. In addition, NOESY spectra also showed cross peaks between the protons H-1', H-5', and H-11, and H-6'a and H-11, suggesting their close spatial proximity. Collectively, these data permitted the assignment of configuration for compound **10** and **11**, and unequivocally confirmed the structures of banistenoside A (**1**) and banistenoside B (**2**).

Compound **4** was isolated as gum and analyzed for $C_{12}H_{14}N_2O$ by ESIHRMS. This compound was homogenous on TLC [R*f* 0.40, silica gel/solvent: CHCl$_3$-MeOH (2:8)] and not reactive to Dragendorff's reagent. The ^{1}H and ^{13}C NMR spectra of **4** was generally similar to those observed for THH (**5**), except for the differences associated with the presence of a methylene group at C-1 (δ_{H-1} 4.15, 2H, m; δ_c 41.1, t), instead of a methine substituted with a methyl group (δ_{H-1} 1H, m; δ_c 50.9, d; δ_{H-10} 1.69, d, *J*=6.4 Hz; δ_c 18.1) of **5**. In addition, a close comparison between the NMR spectra of **4** and other THH analogs (Faizi and Naz, 2002), led to the conclusion that **4** contained a methylene group instead of the methine group at C-1 of THH, suggesting **4** was a C-1 *nor*- derivative of THH (**5**). Complete assignment of the spectral data was carried out using 2D NMR experiments, due to unavailability of spectral data in the literatures. The HMBC experiment showed three bond correlations between H$_2$-1, C-3, C-4a and two bond correlations between H$_2$-1 and C-9a. It also showed correlations between H-5, C-6 and C-7, and the H$_7$-OMe group showed cross peak with C-7, suggesting the substitution of the OMe was at C-7. Based on the foregoing data compound **4** was established as THNH.

During the course of the investigation, three sugars, β-D-fructofuranosyl (2→5) fructopyranose (D-difructose) (**14**), sucrose and β-D-glucose were isolated as major primary metabolites.

Compound **14** was isolated as gum and homogenous on TLC [Rf 0.35, silica gel/solvent: CHCl$_3$-MeOH (3:7)]. Its ^{13}C NMR spectrum was generally similar to those observed for fructan-type disaccharide with two fructofuranosyl units (Timmermans et al., 2001), which displayed 12 major carbon signals consisting four triplets, six doublets and two singlets, the latter assigned to anomeric carbons at C-2 and C-2' (i$_{C-2}$ 101.7, $\delta_{C-2'}$ 97.8 in CD$_3$OD). Comparison of the ^{13}C NMR data of **14** with those of β-D-fructofuranosyl(2→2)β-D-fructofuranose, isolated from *Morinda citrifolia* (Lin, 2004), suggested the presence of a non-symmetrical dimmer for **14**, instead of a symmetrical dimmer, which reported only six ^{13}C NMR signals due to symmetrical nature of the molecule. On acetylation fraction (H-1) containing **14** as a major compound afforded the corresponding octaacetate **17** by using CPTLC (see Materials and Method section). Compound **17** was analyzed for C$_{28}$H$_{38}$O$_{19}$ by positive ion ESIHRMS, and found to be dextrorotatory. Its ^{1}H and ^{13}C NMR spectra (in CDCl$_3$) displayed signals for eight acetyl groups, in addition to 12 carbon signals consisting four triplets, six doublets and two singlets, the latter assigned to one anomeric carbon (δ_{C-2} 107.9) and a carbonyl (δ_{C-2} 197.9), suggesting one of the anomeric center of **14** was opened during acetylation. The ^{1}H NMR spectrum of **17** demonstrated multiplicities for all the protons, previously not observed for **14**, and structure **14** was fully substantiated by 2D NMR COSY, HMQC, HMBC and NOESY experiments on **14**-Ac (**17**). The HMBC experiment showed key ^{2}J and ^{3}J- correlations between C-2 and H$_2$-1, H-3 and H-4, and C-5 and H-3 and H$_2$-6 for the fructofuranose unit. It also showed correlations between C-2' and H$_2$-1', H-3' and H-4', and C-5' and H-4', and H$_2$-6', attributable to acetylated acyclic fructose unit of **17**. Based on the foregoing data compound **14** was established as β-D-fructofuranosyl-(2→5)-fructopyranose.

The structures of harmol (**3**), THH (**5**), harmaline (**6**) (Coune et al., 1980; Atta-ur-Rehman and Tayyaba, 1972), harmine (**7**), (−)-2*R*,3*R*–epicatechin (**8**) (Balde et al., 1991; Sun et al., 2006) and (−)-2*R*,3*R*–epicatechin-4β8-(−)-2*R*,3*R*–epicatechin (procyanidin B2) (**9**) (Barrett et al., 1979; Korver and Wilkins, 1971; Khan et al., 1997) were determined by physical and spectroscopic data (^{1}H and ^{13}C NMR, 2D NMR experiments, and MS) as well as by direct comparison, except for **9**, with their respective authentic samples. The ^{1}H and ^{13}C NMR spectra of harmaline (**6**) in CD$_3$OD exhibited an equilibrium between imine-enamine tautomeric forms (Coune et al., 1980; Atta-ur-Rehman and Tayyaba, 1972). The imine was predominant in DMSO-d$_6$ (aprotic solvent), while both the tautomers were detected in CD$_3$OD/ trifluroacetic acid (1 drop) as mixtures in the NMR spectra. The structures of **8** and **9** were confirmed by chemical derivatization to their corresponding peracetate **12** and **13**. Compound **9** and **13** adopt two conformations in solution, in which the interflavan dihedral angles are roughly ±90°. The preferred conformation of the pyran ring is a half-chair with the 3,4-dihydroxyphenyl substituents in *pseudo*-equatorial positions (Khan et al., 1997). The absolute configuration of **8** and **9** was confirmed by optical rotation and Circular Dichroism experiments, which is consistent with the CD spectra of reference (−)-2*R*,3*R*-epicatechin (for **8**) and literature data (for **9**) (Korver and Wilkins, 1971; Barrett et al., 1979).

3.2. Biological activity

The hot aqueous extracts of fresh and dried large branches of *B. caapi* and isolated compounds **1–9** were evaluated for MAO inhibitory and antioxidant activities, as well as cytotoxicity against selected human cancer and mammalian (VERO) cells. Assays for MAO-A and MAO-B inhibitions were carried out fluorometrically at 460 nm emission wavelength using recombinant human brain MAO-A and B enzymes, which showed strong inhibitory activities against MAO-A (IC$_{50}$ ~0.01–0.4 µ*g*/mL), and weak activities against MAO-B (i.e., ~25% inhibition at 5 µg/mL) for most of the extracts. During the measurement of MAO-B activity, strong fluorescence interferences at 460 nm emission wavelength were observed at a concentrations of >5 µg/mL, which interfered with IC$_{50}$ determinations. To resolve

fluorescence interference, a modified standardized protocol with an emission wavelength of 380 nm was employed (Parikh et al., 2002). Using this protocol, percent inhibitions were determined at 1, 10 and 100 µg/mL for MAO-B, while the MAO-A inhibitions remained closely similar to those measured at 460 nm. The extracts of fresh and dried samples of bark/ debarked large branch demonstrated potent MAO-A inhibition (IC_{50} 0.02–0.05 µg/mL) and modest MAO-B inhibitory activities (IC_{50} ~100 µg/mL) (Table 1). MAO-A activity of extracts of fresh/ dried stem or bark/ debarked large branch was found to be 2500-fold more potent than MAO-B. The activity of most potent MAO-A inhibitors harmine (**7**) or harmaline (**6**) was found to be >10,000-fold more potent than MAO-B (IC_{50} 2.0 nM/ 2.5 nM *vs*. 20 µM/ 25 µM), which was proportionately consistent with those observed for the crude extract. The activity of **3** and **5** was found to be much weaker than **6** or **7** against MAO-A, while compounds **1, 2** and **4** were inactive (Table 1).

In addition, strong antioxidant activity (Rosenkranz et al., 1992; Scudiero et al., 1988) for inhibition of cellular Reactive Oxygen Species (ROS) generation by phorbol-12-myristate-13-acetate (PMA) was observed for large branches (IC_{50} 0.4–3.0 µg/mL) (Table 1). (−)-Epicatechin (**8**) and (−)-procyanidin B2 (**9**) demonstrated potent antioxidant activity; both found to be more potent than vitamin C (IC_{50} <0.13 and 0.57µg/mL *vs*. 1.34 µg/mL), while **8** was also more effective than trolox (IC_{50} 0.14 µg/mL). Both the compounds also showed MAO-B inhibitory activity (IC_{50} 65 and 35 µM, respectively) and weak MAO-A inhibitions (IC_{50} 51.7 and 8.5 µM for **8** and **9**, respectively). However, epicatechin and catechin had previously been reported as MAO-B inhibitors (IC_{50} 58.9 µM) (Hou et al., 2005). Evaluation of regular sample of *B. caapi* dried stem (BCEx-1 and BCEx-4) showed consistent MAO-A inhibitory and antioxidant activity, but was relatively less potent than bark of large branch of Da Vine for MAO-A inhibitory (IC_{50} 0.32–0.39 *vs*. 0.02–0.03 µg/mL) and antioxidant (IC_{50} 1.0–7.8 µg/mL *vs*. 1.9–2.0 µg/mL) activity. Finally, extracts from different parts of large branch, together with regular/ commercial samples, were tested against acetylcholinesterase (AChE), butylcholinesterase (BuChE) and catechol-O-methyl transferase (COMT) enzymes (100 µg/mL), and cytotoxic activities (Borenfreund et al., 1990) against human breast, melanoma, skin and prostate cancer, and mammalian VERO and kidney cell lines (at 10 µg/mL), and were found to be inactive.

3.3. HPLC analyses and quantification

The isolated markers (**1**–**9**) were employed for chemical profiling of *B. caapi* Da Vine, thereby markers **1**–**9** were consistently assigned and quantified from aqueous extracts using a reverse phase C-18 column by HPLC according to published methods for β-carboline alkaloids (Abourashed et al., 2003) (using harmine, harmaline, harmol and harmane as standards) and green tea catechins/ proanthocyanidines (Khokhar et al., 1997) (using catechin and epicatechin as standards). HPLC analysis revealed that most of the dominant chemical and bioactive markers were present in high concentrations in dried bark of large branch [**1** (0.71%), **2** (1.93%), **5** (0.10%), **7** (0.67%), **8** (0.43%) and **9** (0.16%)]. Analysis of regular/commercial *B. caapi* dried stems showed a similar qualitative HPLC pattern, but relatively low- content of dominant markers **1** (0.15, 0.17%), **2** (0.07, 0.15%), **7** (0.04, 0.13%) and **9** (0.03, 0.38%) in samples BCEx-1 and BCEx-4, respectively, which led to decreased MAO and antioxidant potency in these samples. However, two other regular/ commercial samples (BCEx-2 and BCEx-3) were found to be inactive and devoid of markers **1**–**9**.

4. Conclusion

This appears to be the first report of banistenoside A (**1**) and banistenoside B (**2**), containing an unique "azepino[1,2-a]tetrahydro-β-carboline" carbon framework, either from natural or synthetic sources. Presumably **1** and **2** appears to be biogenetically analogous to mitragynine-

type alkaloid dihydrocadambine (Brown and Fraser, 1974) with the unusual N-4 –C-18 bond (i.e., using mitragynine carbon numbering system) in a seven-membered ring. Therefore, the biogenetic pathway for 1(*R*)-banistenoside A and −B can be envisioned from the precursor of indole alkaloids 3α-H strictosidine *via* 3α-H epoxystrictosidine, followed by trioxoaglycone (Szabó, 2008), which undergoes extensive decrboxylation, cyclyzation, oxidation and glycoxylation to **1** and **2**. Interestingly, a synthetic azepinotetrahydro-β-carboline [CAS 610782-531] related to the compounds **1** or **2** demonstrated potent α2-adrenoceptor activity (Din Belle et al., 2003), therefore, further biological work on **1** and **2** is warranted. In addition, THNH (**4**) and β-D-fructofuranosyl-(2→5)-fructopyranose (**14**) were isolated for the first time from a natural source, and (−)-epicatechin (**8**) and (−)-procyanidin B2 (**9**) for the first time from the genus *Banisteriopsis*. During the course of the investigation, 6-methoxy-THH (pinoline) or its corresponding C-5 oxygenated tryptamine analogs, which have potential hallucinogenic or behavioral properties (Rothchild, 2005; Pahkla et al., 1996), were neither identified nor isolated from this cultivar, or regular/ commercial samples. It is also noteworthy that all the isolated β-carbolines **1**–**7** are oxygenated at C-7 position, suggesting they are biogenetically derived from corresponding C-6 oxygenated tryptamine precursor.

Inhibition of MAO-B activity by β-carbolines harmine (**7**) and harmaline (**6**), in addition to potent MAO-A inhibition responsible for antidepressant activity, provide protection against neurodegeneration, and has a potential therapeutic value for the treatment of Parkinson's diseases. In addition, oxidative stress induced by ROS has been strongly associated with the pathogenesis of neurodegenerative disorders, including Parkinson's and Alzheimer's disease (Barnham et al., 2004). Therefore, the presence of two potent antioxidants, (−)-epicatechin (**8**) and (−)-procyanidin B2 (**9**), have significant added value for the protection of neuronal cells damage by oxidative free radicals. Their selective MAO-B inhibitory activity (Hou et al., 2005) benefits them even more. Usefulness of compounds **8** and **9** was reported for amyloid diseases (Castillo et al., 2002 and 2004), as well as protection against PC12 cells from Aβ-induced neurotoxicity by compound **8** (Cho et al., 2008; Heo and Lee, 2005). In addition, proanthocyanidin dimers, including **9**, were recently reported for treating amyloid, α-synuclein or NAC fibrillogenesis in a mammalian subject (Castillo et al., 2004). Collectively, these results give additional basis to the existing claim of *B. caapi* stem extract for the treatment of Parkinsonism, including other neurodegenerative disorders.

Acknowledgments

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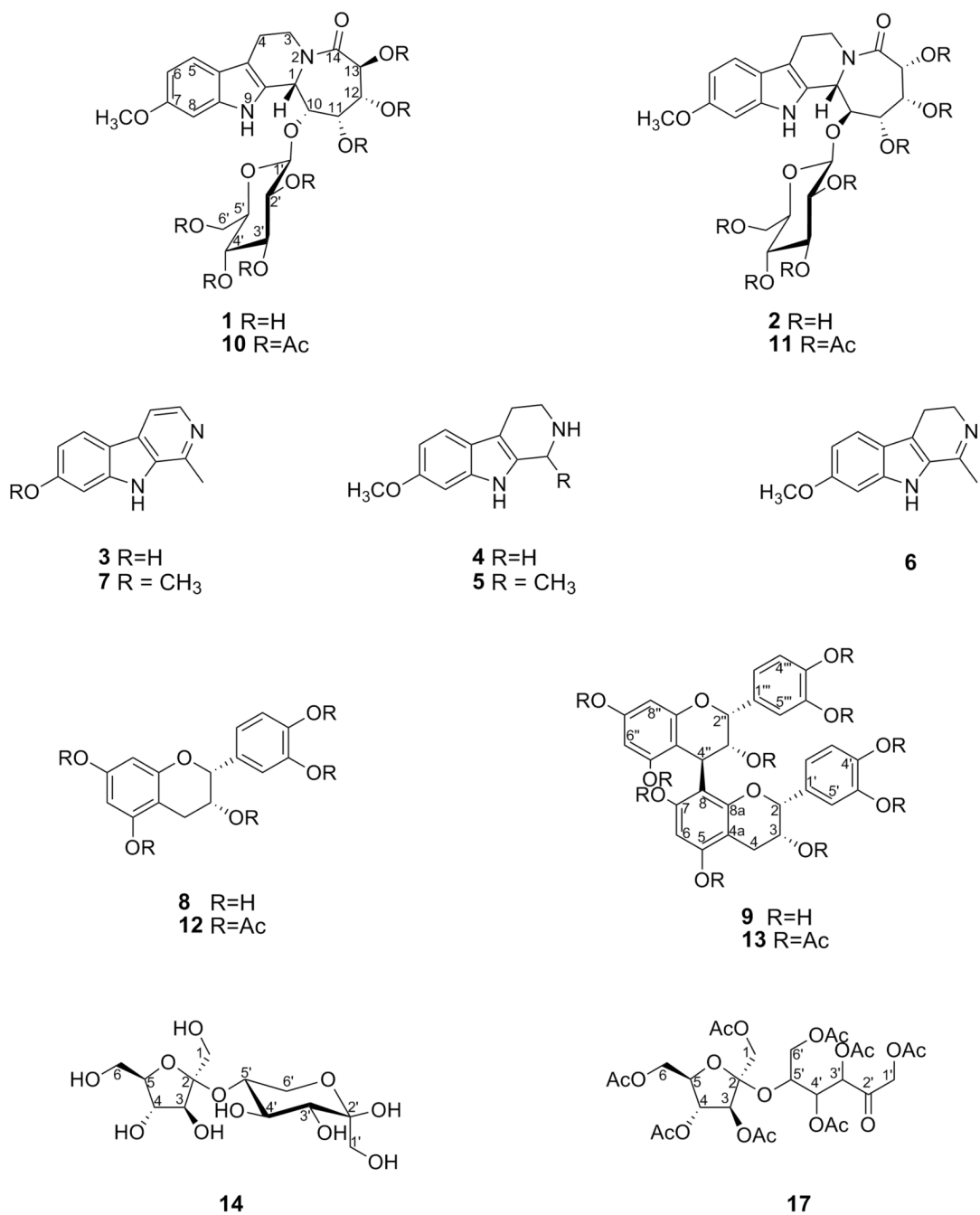
Figure 1.
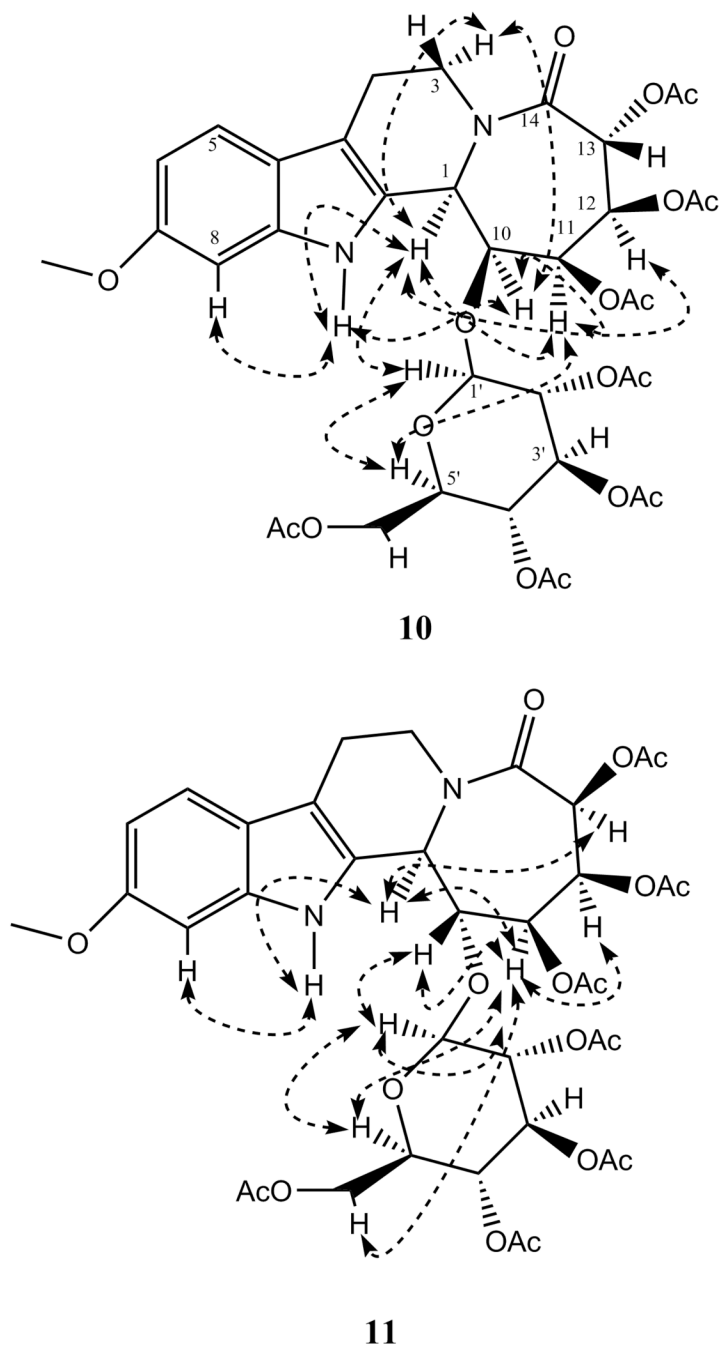Chemical structures of compounds isolated from Banisteriopsis caapi.

Figure 2.
2D NMR NOESY correlations of 10 and 11.

Table 1

MAO-A and MAO-B inhibitory, and antioxidant and cytotoxic activities of representative *B. caapi* extracts and compounds.

Sample Name	Samples collected	Code #	Yield^a %	MAO-A IC$_{50}$ (μg/mL)	MAO-B		Antioxidant IC$_{50}$ (μg/mL)	Cytotoxicity IC$_{50}$ (μg/mL)
					μg/mL	% Inhibition		
Fresh stem		fBCDVS-1	0.85	0.2 – 0.67	100	43	NT	NT
Dried stem		BCDMS	12.74	0.04	100	38	NT	NT
Dried young stem bark		BCBYS	10.08	0.1	100	46	1.6	NC
Fresh mature stem bark		fBCDVBm-1	2.16	0.015 – 0.025	100	23	NT	NT
Dried mature stem bark		BCBMS	14.29	0.033	100	45	2.2	NC
Fresh debarked young stem	August, 2007; Oahu	fBCDVDB-1	2.67	0.025 – 0.035	100	27	NT	NT
Dried debarked young stem		DDYS	9.76	0.08 – 0.043	100	45	4.0	NC
Dried debarked mature stem		BCDMDS	10.12	0.05	100	57	4.0	NC
Fresh leaves		fBCDVL-1	0.49	0.125	100	41	NT	NT
Dried leaves		BCDL	10.06	0.6	100	39	5.9	NC
Fresh stem^b		fBCDVS-2	11.7	0.9	100	16	NT	NT
Fresh leaves^b		fBCDVL-2	5.28	0.2	100	30	NT	NT
Fresh young stem		BCDVFYSO	0.80	0.58	>100	ND	NT	NT
Dried young stem	October, 2007; Big Island^d	BCDVDYSO	7.28	1.13	>100	ND	NT	NT
Fresh leaves		BCDVFLO	0.60	1.35	>100	ND	NT	NT
Dried leaves		BCDVDLO	4.08	0.33	>100	ND	NT	NT
Fresh young stem^b		BCDVFYSAO	6.29	0.24	>100	ND	NT	NT
Fresh leaves^b		BCDVFLAO	8.35	0.22	>100	ND	NT	NT
Fresh bark large branch		BCfBS	3	0.045	100	51	0.4	NC
Fresh debarked large branch	November, 2007; Oahu	BCfDS	5.2	0.035	100	55	3.0	NC
Dried bark large branch		BCDBS	18.1	0.03	100	34	1.9	23
Dried debarked large branch		BCDDS	7.6	0.02	100	46	2.0	NC

Sample Name	Samples collected	Code #	Yield[a] %	MAO-A IC$_{50}$ (µg/mL)	MAO-B		Antioxidant IC$_{50}$ (µg/mL)	Cytotoxicity IC$_{50}$ (µg/mL)
					µg/mL	% Inhibition		
Fresh big stems	July 2008; Big Island	fBCDVSbig	2.9	0.034	>100	ND	NT	NT
Dried big stems		BCDVSbig	9.56	0.028	>100	ND	NT	NT
Dried matured stem[c]		BCEx-1	15.43	0.32	>100	ND	7.8	NC
Dried matured stem[c]		BCEx-4	9.80	0.385	>100	ND	1.0	29

Pure compd/ Standards	MAO-A IC$_{50}$ (nM)	MAO-B IC$_{50}$ (µM)	Antioxidant IC$_{50}$ (µg/mL)	Cytotox. IC$_{50}$ (µg/mL)	Pure compd/ Standards	MAO-A IC$_{50}$ (nM)	MAO-B IC$_{50}$ (µM)	Antioxidant IC$_{50}$ (µg/mL)	Cytotox. IC$_{50}$ (µg/mL)
Banistenoside A (1)	4.9×10^3	>100	NA	>12.5	Epicatechin (8)	51.7×10^3	65	<0.13	6.3
Banistenoside B (2)	21.5×10^3	>100	NT	7.8	Procyanidin B2 (9)	8.5×10^3	35	0.57	NC
Harmol (3)	18	NT	NT	NT	Clorgyline	1.6	1.8	NT	NT
THH (5)	74	>100	NA	NC	Deprenyl	9.0	0.040	NT	NT
Harmaline (6)	2.5	25	NA	25	Vitamin C	NT	NT	1.34	NT
Harmine (7)	2.0	20	NA	NC	Doxorubicin	NT	NT	NT	0.2 – 0.35

[a]Yield was measured by weight.

[b]Extracted with water by automated ASE-200 extractor.

[c]Regular/Commercial *B. caapi* sample.

[d]Plants from this collection are 3 years old.

NT = Not tested. ND = Not detected (tested up to maximum concentration of 100 µg/mL). NA = No antioxidant activity upto 31.25 µg/mL. NC = No cytotoxicity to HL-60 cells up to 31.25 µg/mL.

Table 2

^{1}H and ^{13}C NMR data of **1**, **10** and **11**.

#	1 ^{1}H	1 ^{13}C	10 ^{1}H	10 ^{13}C	10 HMBC	11 ^{1}H	11 ^{13}C	11 HMBC
1	4.44 m	56.0 (d)	5.98 d (4.3)	49.4	C-3, 4a, 9a, 10, 11, 14	5.08 d (10.0)	52.2	C-3, 4a, 9a, 10, 11, 14
3	3.31 brs 3.05 m	40.8 (t)	4.06 dd (2.0, 12.4), 3.42 m	41.9	C-14	4.98 dd (5.6, 11.6), 2.81 m **	38.3	-
4	2.51 m	16.0 (t)	2.81 m	21.8	C-4a	2.81 m **	20.7	C-4a
4a	-	109.6 (s)	-	109.6	-	-	110.2	-
4b	-	119.9 (s)	-	120.4	-	-	120.0	-
5	6.90 *	118.0 (d)	7.34 d (8.8)	118.9	C-4a, 4b, 7, 8a	7.34 d (8.4)	119.1	C-4a, 4b, 7, 8a
6	6.19 brd (8.8)	108.3 (d)	6.77 dd (2.0, 8.8)	109.1	C-4b, 7, 8	6.76 dd (2.4, 8.8)	109.8	C-4b, 7, 8
7	-	158.5 (s)	-	156.8	-	-	156.9	-
8	6.35 d (2.8)	94.6 (d)	6.81 d (2.0)	94.8	C-4b, 6, 7, 8a	6.92 d (2.4)	95.2	C-4b, 6, 7, 8a
8a	-	137.0 (s)	-	137.2	-	-	137.1	-
9a	-	120.0 (s)	-	126.7	-	-	126.7	-
10	4.23 d brs	73.0 (d)	4.32 d (4.3)	72.9	C-1, 1´, 9a, 12	3.98 d (10.0)	81.2	C-1, 1´, 9a, 11, 12
11	4.44 m	79.3 (d)	5.20 d (8.4)	77.2	C-10, 12, 13	5.77 d (4.4)	69.5	C-10, 12, 13
12	4.09 brs	68.9 (d)	5.61 t (8.0.8.4)	72.5	C-10, 11, 13	5.32 t (4.4, 4.8)	71.1	C-10, 11, 13
13	4.04 brs	70.9 (d)	5.72 d (8.0)	71.2	C-12	5.87 d (4.8)	73.4	C-12, 14
14	-	175.3 (s)	-	170.6	-	-	164.4	
1´	4.44 m	94.4 (d)	4.54 d (4.8)	96.7	C-2´, 10	4.91 d (3.6)	101.6	C-2´, 10
2´	4.36 m **	71.8 (d)	4.94–5.0 m **	71.4	-	5.11–5.17 m **	71.5	-
3´	4.38 m **	71.7 (d)	4.94–5.0 m **	72.1	-	5.11–5.17 m **	73.3	-
4´	4.31 m	66.1 (d)	4.94–5.0 m **	68.6	C-6´	5.11–5.17 m **	67.4	C-6´
5´	3.82 m **	72.3 (d)	3.55 m	72.8	-	3.81 dd (2.4, xx)	72.5	-
6´	3.81 m ** 3.31 m	61.5 (t)	4.30 dd (5.2, 12.0), 4.12 m	62.2	C-4´	4.38 dd 4.0,12.4), 4.0 dd (1.2, 12.4)	61.5	C-4´

#	1 (¹H)	1 (¹³C)	10 (¹H)	10 (¹³C)	10 (HMBC)	11 (¹H)	11 (¹³C)	11 (HMBC)
#	**¹H**	**¹³C**	**¹H**	**¹³C**	**HMBC**	**¹H**	**¹³C**	**HMBC**
7-OMe	3.23 s	54.3 q	3.83 s	55.6 q	C-7	3.86 s	55.7	C-7
OH/NH	-	-	8.45 s (NH)	-	C-4a, 4b, 8a, 9a	7.9 brs (NH)	-	C-4a, 4b, 8a, 9a
OCOMe	-	-	2.23, 2.15, 2.14, 2.07, 1.98, 1.94, 1.91 (7 × s)	22.0, 20.7, 20.5 ×3, 20.4, 20.2 (7q)	-	2.21, 2.17, 2.09 × 2, 2.01, 2.0, 1.53, (7 × s)	20.9,20.6, 20.6 ×3, 20.5 ×2 (7q)	-
CO-Me	-	-	-	170.7, 170.1, 169.9, 169.5, 169.4, 169.2 ×2	-	-	170.4 ×2, 170.1, 170.0, 169.4 ×2, 169.2	-

NMR Spectra of **1** (CF₃COOD/D₂O), and **10** and **11** (in CDCl₃) were recorded at 400 (¹H) and 100 (¹³C) MHz, using a Varian spectrometer. The coupling constants (J values, in Hz) are in parentheses. The multiplicities of carbon signals were determined by ¹³C NMR DEPT experiments. Assignments were made using 2D NMR COSY, HMBC and HMQC experiments.

*
Signals superimposed with solvent.

**
Signals superimposed on each other.