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Isolation and Structure Elucidation of Highly Antioxidative 3,8″-Linked Biflavanones and Flavanone-*C*-glycosides from *Garcinia buchananii* Bark

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ABSTRACT: The aim of this study was to identify antioxidants from *Garcinia buchananii* bark extract using hydrogen peroxide scavenging and oxygen radical absorbance capacity (ORAC) assays. LC-MS/MS analysis, 1D- and 2D-NMR, and circular dichroism (CD) spectroscopy led to the unequivocal identification of the major antioxidative molecules as a series of three 3,8″-linked biflavanones and two flavanone-*C*-glycosides. Besides the previously reported (2*R*,3*R*,2″*R*,3″*R*)-naringenin-C-3/C-8″ dihydroquercetin linked biflavanone (GB-2; **4**) and (2*R*,3*S*,2″*R*,3″*R*)-manniflavanone (**3**), whose stereochemistry has been revised, the antioxidants identified for the first time in *Garcinia buchananii* were (2*R*,3*R*)-taxifolin-6-*C*-*β*-D-glucopyranoside (**1**), (2*R*,3*R*)-aromadendrin-6-*C*-*β*-D-glucopyranoside (**2**), and the new compound (2*R*,3*S*,2″*S*)-buchananiflavanone (**5**). The H_2O_2 scavenging and the ORAC assays demonstrated that these natural products have an extraordinarily high antioxidative power, especially (2*R*,3*S*,2″*R*,3″*R*)-manniflavanone (**3**) and GB-2 (**4**), with EC_{50} values of 2.8 and 2.2 μM, respectively, and 13.73 and 12.10 μmol TE/ μmol. These findings demonstrate that *G. buchananii* bark extract is a rich natural source of antioxidants.

KEYWORDS: *3,8″-linked biflavanones, flavanone-6-C-glycosides, Garcinia buchananii, (2R,3S,2″R,3″R)-manniflavanone, (2R,3S,2″S)-buchananiflavanone, antioxidants*

INTRODUCTION

Oxidative stress is a serious condition that leads to chronic metabolic and degenerative diseases. It is caused by endogenous free radicals, generated in the human body as a metabolic byproduct, or free radicals from exogenous sources like ultraviolet light, ionizing radiation, chemotherapeutics, environmental toxins, and inflammatory cytokines, which attack various substrates in the body evoking irreparable injuries, cell death, and necrosis. The targets of these free radicals are all cellular components, e.g., proteins, carbohydrates, nucleic acids, and polyunsaturated fatty acids. The destructive power of these free radicals causing oxidative damage is associated with coronary heart diseases, atherosclerosis, aging, cancer, and inflammatory conditions.[1,2]

Natural phytochemicals having antioxidant activity, such as vitamins (ascorbic acid and vitamin E), carotenoid terpenoids (carotenoids), flavonoid polyphenolics, and alkyl sulfide reduce risks of many degenerative and metabolic diseases. The antioxidative power of phenolic compounds is mainly due to their redox properties, which play an important role in absorbing and neutralizing free radicals, quenching singlet and triplet oxygen, or eliminating peroxides. In the past few years, several investigations have shown that the antioxidant activity of a plant extract is highly correlated with the extract's phenolic content.[3−6]

About 400 species are known of the genus *Garcinia*, familiy Guttiferae, in which extracts and pure isolates from *Garcinia* species exhibited forms of biological activity such as anticancer,[7] anti-inflammatory,[8] antimicrobial,[9] and antioxidant properties.[10−12]

Stem and root bark extracts of *G. buchananii* are traditionally used to treat diarrhea, dysentery, abdominal pain, and a range of infectious diseases in Sub-Saharan Africa.[13] It is thought that the extract's antidiarrhea effects reduce colon motility by inhibiting neurotransmission and possibly 5-HT_3 and 5-HT_4 receptors.[14,15] Furthermore, the extract has anti-inflammatory, antinociceptive, and antidiarrheal effects.[16,17] Initial compound screening using standard chemical tests and preparative thin layer chromatography methods suggest that the compounds having antidiarrheal properties are flavonoids, or a combination of flavonoids with alkaloids or steroids. Whether the flavonoids found in *G. buchananii* bark extract have antioxidant activities has never been determined.

Therefore, the purpose of the present study was to use antioxidative activity-guided screening to determine if the stem bark extract of *G. buchananii* has fractions that have antioxidative activity and to isolate and elucidate the chemical structures of compounds showing the most powerful antioxidant activity.

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Table 1. Antioxidant Activities of *Garcinia b.* Extract and Fractions M1−M8

		H_2O_2 assay[a,b]		ORAC assay[c,d]	
	each amount (mg)	EC_{50} (dilution degree)		(μmol TE/each amount)	
EtOH extract	100.0	11320.5	(10061.5−12721.7)	1359.15	(±14.84)
recombination of M1−M8		8912.8	(7840.1−10040.1)	1102.95	(±46.09)
calculated sum of M1−M8		9879.8		1251.78	
M1	16.3	609.4	(534.1−702.0)	102.38	(±3.05)
M2	7.1	673.1	(600.6−748.9)	80.89	(±1.38)
M3	41.0	5679.3	(5008.0−6473.8)	744.83	(±27.87)
M4	6.4	722.3	(639.9−812.1)	86.89	(±1.61)
M5	10.3	1039.1	(894.4−1207.7)	141.68	(±3.44)
M6	5.2	382.4	(331.2−444.7)	44.89	(±1.56)
M7	8.3	572.2	(509.9−641.2)	42.34	(±2.97)
M8	5.3	202.1	(180.5−226.4)	7.89	(±0.20)

[a]Each sample was analyzed by means of the H_2O_2 assay by triplicate studies. [b]The range in parentheses represents 95% confidence interval. [c]Each sample was analyzed by means of the ORAC assay by quadruplicate studies. [d]The numerical value in parentheses represents SD.

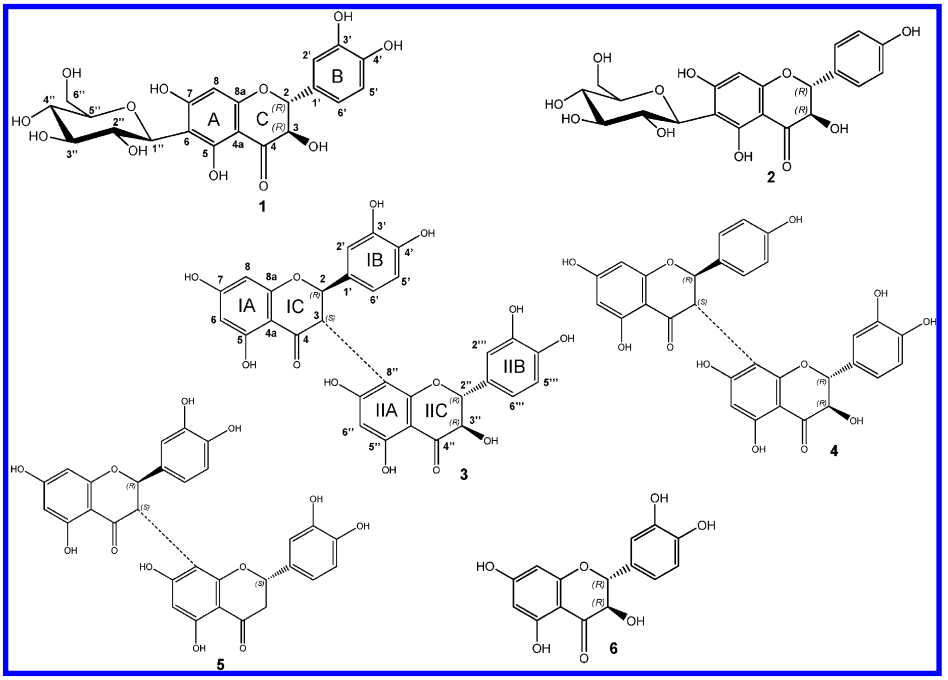

Figure 1. Chemical structures of compounds **1**−**6**.

MATERIALS AND METHODS

Chemicals. The following reagents were obtained commercially: hydrogen peroxide (Merck, Hohenbrunn, Germany); 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), peroxidase from horseradish (HRP), (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), fluorescein sodium salt (FL), 2,2′-azobis(2-methylpropinamidine) (AAPH), quercetin, (−)-epicatechin, (±)-naringenin, methyl iodide, ascorbic acid (Sigma-Aldrich, Steinheim, Germany), rutin, and (+)-(2*R*,3*R*)-taxifolin (AppliChem, Darmstadt, Germany). Water for chromatographic separations was purified with a Milli-Q Gradient A10 system (Millipore, Schwalbach, Germany), and solvents used were of HPLC-grade (Merck, Darmstadt, Germany). Deuterated solvents were obtained from Euriso-Top (Gif-sur-Yvette, France).

General Experimental Procedure. 1D and 2D NMR spectroscopy ^{1}H, ^{1}H-^{1}H-*g*COSY, *g*HSQC, *g*HMBC, and ^{13}C, ^{1}H-^{1}H rotating frame nuclear Overhauser enhancement spectroscopy (phase-sensitive ROESY) NMR measurements were performed on an Avance III 500 MHz spectrometer with a CTCI probe or an Avance III 400 MHz spectrometer with a BBO probe (Bruker, Rheinstetten, Germany). Mass spectra of the compounds were measured on a Waters Synapt G2 HDMS mass spectrometer (Waters, Manchester, UK) coupled to an Acquity UPLC core system (Waters, Milford, MA, USA). For CD spectroscopy, methanolic solutions of the samples were analyzed by means of a Jasco J810 Spectro polarimeter (Hachioji, Japan). HPLC separations were performed using a preparative HPLC system (PrepStar, Varian, Darmstadt, Germany). MPLC separations were performed on a Büchi Sepacore (Flawil, Switzerland) system using PP cartridges (id. 40 mm, l. 150 mm) and LiChroprep RP18, 25−40 μm mesh material (Merck, Darmstadt, Germany).

Plant Material. *Garcinia buchananii* stem bark was collected from plants in their natural habitats in Karagwe, Tanzania, and processed as described previously.[14] A sample of bark powder was deposited at the University of Idaho Stillinger herbarium (voucher # 159918).

Extraction and Isolation. *G. buchananii* bark powder (10 g) was suspended in a mixture of ethanol/water (50 mL, 70/30, v/v), sonicated (10 min), stirred at RT (20 min), and filtered. The filtrate was extracted with hexane (50 mL), the ethanol/water extract was evaporated, and then the sample was freeze-dried. Aliquots (1 g) of the freeze-dried ethanol/water extract were dissolved in a water/methanol mixture (10 mL, 50/50, v/v) and fractionated using MPLC. Chromatography was performed starting with a mixture (65/35, v/v) of aqueous formic acid (0.1% in water, pH 2.5) and MeOH, increasing

the MeOH content up to 55% within 25 min, and in 5 min to 100%. Eight fractions (M1−M8, 160, 70, 401, 63, 101, 51, 51, and 52 mg, Table 1) were collected, concentrated under reduced pressure, and freeze-dried.

■ ANTIOXIDANT ASSAYS

Hydrogen Peroxide Scavenging Assay. Hydrogen peroxide scavenging assay was performed in accordance with the method of Ichikawa et al.[18] Sample solutions at appropriate concentrations were prepared using phosphate buffer (100 mM, pH 6.0). Sample solution (100 μL), phosphate buffer (30 μL, 100 mM, pH 6.0), and hydrogen peroxide solution (10 μL, 500 μM) were mixed in a 96-well clear micro plate (VWR, Ismaning, Germany). Then peroxidase (40 μL, 150 U/mL) and ABTS (40 μL, 0.1%) were added. The microplate was incubated at 37 °C for 15 min. The absorbance (A) of each well was measured at 414 nm with FLUOstar OPTIMA (BMG LABTECH, Offenburg, Germany). The scavenging effect (E) was calculated as shown using the formula below (blank stands for solution without hydrogen peroxide, and control did not include a test compound) and EC_{50} was calculated by the probit method. After freeze-drying in triplicate, MPLC fractions M1−M8 were analyzed in natural ratios.

$$E = [(A - A_{\text{blank}})_{\text{control}} - (A - A_{\text{blank}})_{\text{test}}] / (A - A_{\text{blank}})_{\text{control}} \times 100$$

Oxygen Radical Absorbance Capacity (ORAC) Assay. The ORAC assay was carried out according to the method of Ou et al.[19] with some modifications. Trolox and FL were used as a standard and a fluorescent probe, respectively. Free radicals were produced by AAPH to oxidize FL. Different dilutions of Trolox (200, 100, 50, 25, and 12.5 μM) and appropriate dilutions of the tested sample were prepared with phosphate buffer (10 mM, pH 7.4). Trolox dilution (25 μL) or sample solution were pipetted into a well of a 96-well black microplate (VWR), and then FL (150 μL, 10 nM) was added. The reaction mixture was incubated at 37 °C for 30 min. Afterward, fluorescence was measured every 90 s at the excitation of 485 nm, and the emission of 520 nm using FLUOstar OPTIMA. After 3 cycles, AAPH (25 μL, 240 mM) was added quickly, and then the measurement was resumed and continued up to 90 min (60 cycles in total). The background signal was determined using the first 3 cycles. The ORAC values were calculated according to the method of Cao et al.[20] and expressed as the Trolox equivalent (μmol TE/μmol). After freeze-drying in triplicate, MPLC fractions M1−M8 were analyzed in natural ratios.

Isolation and Structural Characterization of Compounds with Antioxidant Activity. Fractions that showed higher levels of antioxidant activities were subjected to the identification and characterization of chemical compounds.

M3 afforded (2*R*,3*R*,2″*R*,3″*R*)-manniflavanone (**3**, Figure 1), and MPLC fractions (M1 and M4−M5) were further purified by means of HPLC. M1: Chromatography was performed using a RP column (21.2 × 250 mm, Phenylhexyl, 5 μm; Phenomenex, Aschaffenburg, Germany) as the stationary phase. The effluent (18 mL/min) was monitored at 290 nm. The separation started with a mixture (83/17, v/v) of aqueous formic acid (0.1% in water, pH 2.5) and MeOH, and the MeOH content was increased up to 40% within 12 min. Collected fractions were concentrated under reduced pressure and freeze-dried twice, affording (2*R*,3*R*)-taxifolin-6-*C*-β-D-glucopyranoside (**1**, Figure 1) and (2*R*,3*R*)-aromadendrin-6-*C*-β-D-glucopyranoside (**2**, Figure 1). M4: Using the same column and flow rate as those described above, chromatography was performed starting with a mixture (70/30, v/v) of aqueous formic acid (0.1% in water, pH 2.5) and ACN, and increasing the ACN content up to 43% within 13 min. The collected fraction was concentrated under reduced pressure and freeze-dried twice, affording (2*R*,3*R*,2″*R*,3″*R*) GB-2 (**4**, Figure 1). M5: Using the same column and flow rate as those described above, chromatography was performed starting with a mixture (68/32, v/v) of aqueous formic acid (0.1% in water, pH 2.5) and ACN, and increasing the ACN content up to 43% within 13 min. The collected fraction was concentrated under reduced pressure and freeze-dried twice, affording (2*R*,3*S*,2″*S*) buchananiflavanone (**5**, Figure 1).

Methylation of Manniflavanone. Manniflavanone (**3**) (0.17 mmol) was dissolved in dry acetone (100 mL), and methyl iodide (32 mmol) and K_2CO_3 (14.5 mmol) were added. The mixture was refluxed for 24 h, with the addition of further methyl iodide (8 mmol) and K_2CO_3 (7.2 mmol) after 8 h. The mixture was evaporated to dryness and then taken up in a water/methanol mixture (2 mL, 50:50, v/v) and purified by solid phase extraction (SPE) (Strata Gigatube C18, Phenomenex, Aschaffenburg, Germany). The SPE cartridge was flushed with water, and the methanol eluate was further purified by means of HPLC. Monitoring the effluent (4.2 mL/min) at 290 nm, chromatography was performed using a RP column 10 × 250 mm, Phenylhexyl, 5 μm (Phenomenex, Aschaffenburg, Germany) as the stationary phase and starting with a mixture (20/80, v/v) of aqueous formic acid (0.1% in water, pH 2.5) and MeOH. The MeOH content was increased to 100% within 20 min. Collected fractions were concentrated under reduced pressure and freeze-dried twice, affording (2*R*,3*R*,2″*R*,3″*R*)-nonamethylmanniflavanone (**3a**) and a mixture of two octamethylmanniflavanones (**3b**,**c**).

*(2R,3R)-Taxifolin-6-C-β-D-glucopyranoside (**1**, Figure 1).* Colorless powder; UV (MeOH/H_2O, 5/5, v/v) λ_{max} = 225, 290, 345 nm; (−) HRESIMS m/z 465.1035 $[M - H]^-$ (calcd for $C_{21}H_{21}O_{12}$, 465.1033). CD (MeOH, 0.67 mmol/L): $\lambda_{max}(\Delta\varepsilon)$ = 333 (+2.0), 296 (−6.5), 252 (+0.9), 222 (+6.6).^{1}H NMR (500 MHz, DMSO-d_6, COSY): 3.10 [m, 1H, J = 9.0 Hz, H−C(4″)], 3.14 [m, 1H, J = 6.0 Hz, H−C(5″)], 3.16 [dd, 1H, J = 8.4, 8.6 Hz H−C(3″)], 3.40 [d, 1H, J = 11.1 Hz, H−C(6″α)], 3.67 [d, 1H, J = 11.1 Hz, H−C(6″β)], 3.99 [pt, 1H, J = 9.1 Hz, H−C(2″)], 4.45 [dd, 1H, J = 5.3 Hz, H−C(3)], 4.49 [d, 1H, J = 9.8 Hz, H−C(1″)], 4.97 [d, 1H, J = 10.8 Hz, H−C(2)], 5.74 [d, 1H, J = 5.7 Hz, HO-C(3)], 5.92 [s, 1H, H−C(8)], 6.74 [s, 2H, H−C(5′,6′)], 6.87 [s, 1H, H−C(2′)], 8.98 [2xbrs, HO-C(3′,4′)], 12.47 [s, HO-C(5)]. ^{13}C NMR (125 MHz, DMSO-d_6, HSQC, HMBC): δ 61.6 [C-6″], 70.3 [C-2″], 70.7 [C-4″], 71.6 [C-3], 73.0 [C-1″], 79.1 [C-3″], 81.6 [C-5″], 82.9 [C-2], 94.7 [C-8], 100.1 [C-4a], 106.0 [C-6], 115.2 [C-5′], 115.3 [C-2′], 119.3 [C-6′], 128.0 [C-1′], 145.0 [C-4′], 145.8 [C-3́], 161.3 [C-8a], 162.6 [C-5], 166.1 [C-7], 197.9 [C-4].

*(2R,3R)-Aromadendrin-6-C-β-D-glucopyranoside (**2**, Figure 1).* Colorless powder; UV (MeOH/H_2O, 5/5, v/v) λ_{max} = 213, 228, 293, 347 nm; (−) HRESIMS m/z 449.1100 $[M - H]^-$ (calcd for $C_{21}H_{21}O_{11}$, 449.1084). CD (MeOH, 0.74 mmol/L): $\lambda_{max}(\Delta\varepsilon)$ = 329 (+1.5), 291 (−3.8), 248 (+1.3), 233 (+3.2), 218 (+7.0).^{1}H NMR (500 MHz, DMSO-d_6, COSY): 3.11 [m, 1H, J = 9.1 Hz, H−C(4″)], 3.12 [m, 1H, H−C(5″)], 3.16 [pt, 1H, J = 8.4, 8.7 Hz H−C(3″)], 3.41 [1H, H−C(6″α)], 3.66 [d, 1H, J = 10.7 Hz, H−C(6″β)], 4.00 [pt, 1H, J = 9.2 Hz,

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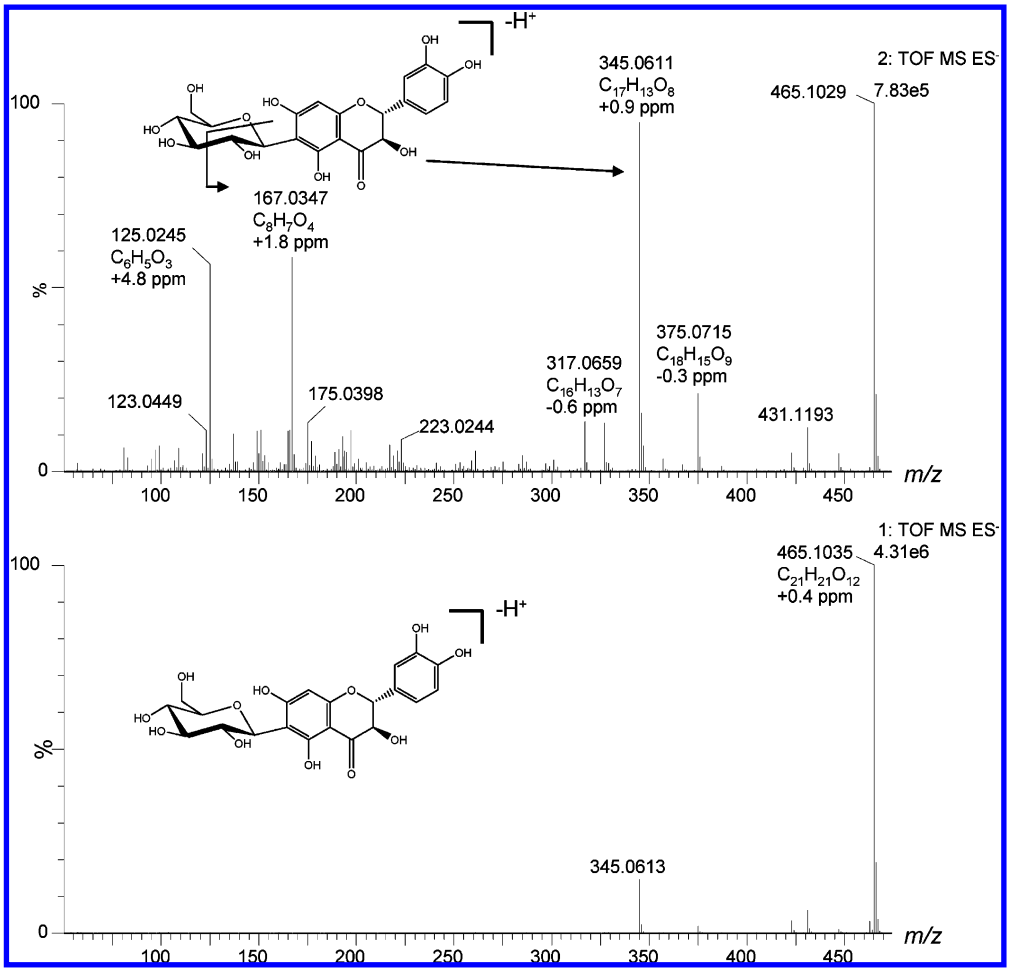

Figure 2. UPLC-ESI$^-$-HRMS spectrum of **1**.

H−C(2″)], 4.48 [d, 1H, J = 9.8 Hz, H−C(1″)], 4.50 [d, 1H, J = 11.0 Hz, H−C(3)], 4.99 [d, 1H, J = 11.0 Hz, H−C(2)], 5.74 [brs, HO-C(3)], 5.83 [s, 1H, H−C(8)], 6.78 [d, 2H, J = 8.6 Hz, H−C(3′,5′)], 7.29 [d, 2H, J = 8.6 Hz, H−C(2′,6′)], 9.57 [brs, HO-C(4′)], 12.50 [brs, HO-C(5)]. ^{13}C NMR (125 MHz, DMSO-d_6, HSQC, HMBC): δ 61.5 [C-6″], 70.3 [C-2″], 70.6 [C-4″], 71.4 [C-3], 73.1 [C-1″], 79.1 [C-3″], 81.4 [C-5″], 82.7 [C-2], 95.2 [C-8], 99.5 [C-4a], 106.2 [C-6], 114.9 [C-3′,5′], 127.6 [C-1′], 129.3 [C-2′,6′], 157.7 [C-4′], 161.2 [C-8a], 162.7 [C-5], 167.5 [C-7], 197.0 [C-4].

*(2R,3S,2″R,3″R)-Manniflavanone (**3**, Figure 1).* Colorless powder; UV (MeOH/H_2O, 6/4, v/v) λ_{max} = 210, 290, 346 nm; (−) HRESIMS m/z 589.0989 [M − H]$^-$ (calcd for $C_{30}H_{21}O_{13}$, 589.0982). CD (MeOH, 0.46 mmol/L): $\lambda_{max}(\Delta\varepsilon)$= 341 (+1.3), 321 (−0.7), 303 (−5.6), 283 (+7.6), 240 (−1.3), 218 (−11.7). ^{1}H NMR (400 MHz, acetone-d_6+DMSO-d_6, 9/1, 8 °C, COSY): δ 4.05 [dd, J = 5.4, 11.7 Hz, H−C(3″)], 4.30 [dd, J = 5.4, 11.3 Hz, H−C(3″)], 4.55 [d, J = 12.1, H−C(3)], 4.71 [d, J = 12.1, H−C(3)], 4.91 [d, J = 11.7 Hz, H−C(2″)], 5.09 [d, J = 11.3 Hz, H−C(2″)], 5.45 [d, J = 12.1 Hz, H−C(2)], 5.64 [d, J = 5.9 Hz, HO-C(3″)], 5.68 [d, J = 12.1 Hz, H−C(2)], 5.80 [d, J = 5.9 Hz, HO-C(3″)], 5.81−5.94 [4xs, H−C(6″,6,8)], 6.03 [s, H−C(6″)], 6.60 [dd, J = 1.8, 7.9 Hz, H−C(6′)], 6.66−6.71 [m, H−C(6′,5′,5‴)], 6.77−6.80 [2xdd, J = 1.8, 8.4 Hz, H−C(6‴)], 6.83−6.89 [m, H−C(5′,5‴,2′)], 6.89−6.94 [m, H−C(2′,2‴)], 8.80−9.00 [4xbrs, HO-C(3′,4′,3‴, 4‴)], 10.64, 10.68, 11.00, 11.24 [brs, HO-C(7″,7)], 11.89, 11.97 [s, HO-C(5″)], 12.29, 12.36 [s, HO-C(5)]. ^{13}C NMR (100 MHz, acetone-d_6 + DMSO-d_6, 9/1, HSQC, HMBC): δ 48.2 [C-3], 73.0, 73.3 [C-3″], 82.3, 82.7 [C-2], 83.8 [C-2″], 95.4, 95.5, 96.1, 96.4, 96.6 [C-6″,6,8], 100.4, 101.0 [C-4a″], 101.9, 102.0 [C-4a,8″], 115.4, 115.5, 115.6, 115.9, 116.1 [C-5‴,5′,2‴,2′], 118.1 [C-6‴], 119.2, 119.4, 119.5 [C-6‴,6′,6′], 129.0, 129.1, 129.3, 129.5 [C-1′,1‴], 145.4, 145.5, 146.2, 146.3, 146.4, 146.5 [C-3′,4′,3‴,4‴], 160.4, 161.1 [C-8a″], 162.9, 163.2 [C-5″], 163.5, 163.6 [C-8a], 164.6, 164.7 [C-5], 165.4, 166.0 [C-7″], 167.2, 167.3 [C-7], 197.3, 197.5 [C-4], 198.2 [C-4″].

*(2R,3S,2″R,3″R)-GB-2 (**4**, Figure 1).* Colorless powder; UV (MeOH/H_2O, 6/4, v/v) λ_{max} = 204, 292, 347 nm; (−) HRESIMS m/z 573.1037 [M − H]$^-$ (calcd for $C_{30}H_{21}O_{12}$, 573.1033). CD (MeOH, 0.47 mmol/L): $\lambda_{max}(\Delta\varepsilon)$= 341 (+2.0), 320 (−1.6), 303 (−9.1), 281 (+12.0), 246 (−2.3), 237 (0.0), 214 (−16.4). ^{1}H NMR (400 MHz, acetone-d_6 + DMSO-d_6, 9/1, 8 °C, COSY): δ 4.10 [dd, J = 4.9, 11.7 Hz, H−C(3″)], 4.31 [dd, J = 4.7, 11.2 Hz, H−C(3″)], 4.61 [d, J = 12.2, H−C(3)], 4.77 [d, J = 12.2, H−C(3)], 4.93 [d, J = 11.7 Hz, H−C(2″)], 5.06 [d, J = 11.2 Hz, H−C(2″)], 5.43 [m, HO-C(3″)], 5.53 [d, J = 12.2 Hz, H−C(2)], 5.57 [m, HO-C(3″)], 5.77 [d, J = 12.2 Hz, H−C(2)], 5.82 [s, H−C(6″)], 5.91−6.03 [4xs, H−C(6,8)], 6.73 [m, H−C(3′,5′)], 6.79 [d, J = 8.0 Hz, H−C(6‴)], 6.86−6.90 [m, H−C(5‴)], 6.92 [s, H−C(2‴)], 7.01 [d, J = 1.5 Hz, H−C(2‴)], 7.21 [m, J = 8.9 Hz, H−C(2′,6′)], 8.63−8.86 [4xbrs, HO-C(3‴, 4‴)], 9.33, 9.40 [brs, HO-C(4′)], 10.57, 10.91, 11.19 [brs, HO-C(7″,7)], 11.84, 11.92 [s, HO-C(5″)], 12.32, 12.39 [s, HO-C(5)]. ^{13}C NMR (100 MHz, acetone-d_6 + DMSO-d_6, 9/1, HSQC, HMBC): δ 48.1 [C-3], 72.8, 73.3

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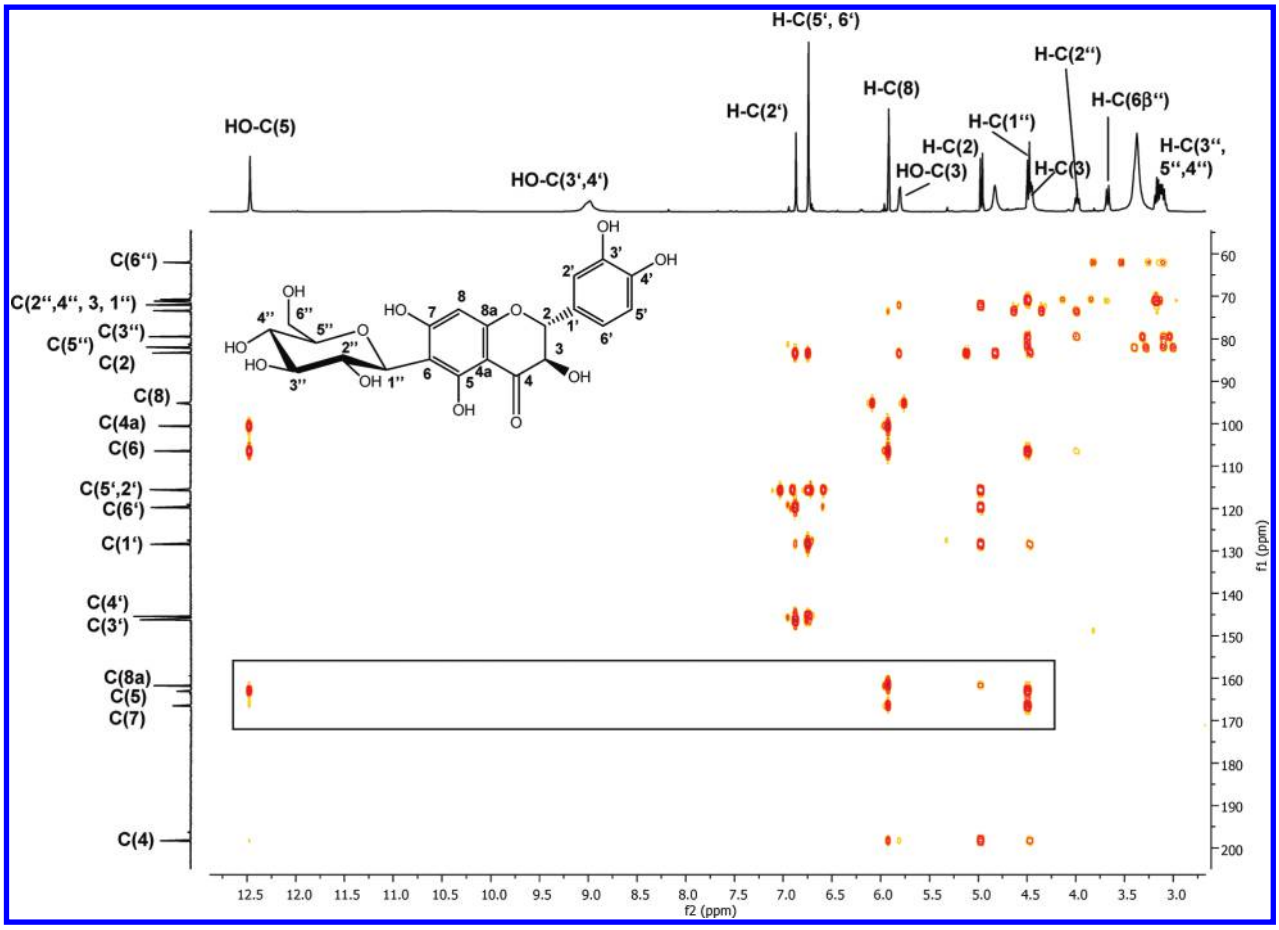

Figure 3. HMBC spectrum (500 MHz, d_6-DMSO) of (2*R*,3*R*)-taxifolin-6-*C*-*β*-D-glucopyranoside (**1**).

[C-3″], 82.1, 82.5 [C-2], 83.8 [C-2″], 95.3, 95.4, 96.0, 96.4, 96.5 [C-6″,6,8], 100.3, 100.8 [C-4a″], 101.9 [C-4a], 101.8, 102.0 [C-8″], 115.2, 115.3 [C-3′,5′], 115.4, 115.5 [C-5‴],115.6, 115.7 [C-2‴], 118.1, 119.8 [C-6‴], 128.4, 128.5 [C-1‴], 128.6, 128.8 [C-1′], 129.1 [C-2′,6′], 145.2, 145.6, 146.0, 146.5 [C-3‴,4‴], 158.5, 158.6 [C-4′], 160.3, 161.0 [C-8a″], 163.0, 163.3 [C-5″], 163.5, 163.6 [C-8a], 164.7, 164.8 [C-5], 165.5, 166.0 [C-7″], 167.1, 167.2 [C-7], 197.3, 197.4 [C-4], 198.2 [C-4″].

*(2R,3S,2″S)-Buchananiflavanone, (2R,3S,2″S)-2-(3,4-Dihydroxyphenyl)-2,2′,3,3′-tetrahydro-5,5′,7,7′-tetrahydroxy-2′-(3,4-dihydroxyphenyl)-[3,8′-Bi-4H-1-benzopyran]-4,4′-dione (**5**, Figure 1).* Colorless powder; UV (MeOH/H_2O, 6/4, v/v) λ_{max} = 215, 225, 291, 347 nm; (−) HRESIMS *m*/*z* 573.1037 $[M - H]^-$ (calcd for $C_{30}H_{21}O_{12}$, 573.1033). CD (MeOH, 0.37 mmol/L): $\lambda_{max}(\Delta\varepsilon)$= 341 (+1.5), 315 (−1.2), 298 (−5.6), 282 (+5.8), 239 (−2.5), 218 (−13.1). ^{1}H NMR (400 MHz, DMSO-d_6, COSY): *δ* 2.60 [dd, *J* = 15.2 Hz, H−C(3″*α*)], 2.69 [2xdd, *J* = 14.7 Hz, H−C(3″*αβ*)], 2.96 [dd, *J* = 13.6, 13.8 Hz, H−C(3″*β*)], 4.50 [d, *J* = 12.1, H−C(3)], 4.63 [d, *J* = 12.0, H−C(3)], 5.29 [d, *J* = 12.1 Hz, H−C(2″)], 5.39 [d, *J* = 12.2 Hz, H−C(2)], 5.42 [d, *J* = 12.1 Hz, H−C(2″)], 5.64 [d, *J* = 12.0 Hz, H−C(2)], 5.80, 5.87, 5.90 [3xs, H−C(6,8,6″)], 6.49 [d, *J* = 12.1 Hz, H−C(6′)], 6.58−6.76 [m, H−C(5′,5‴,2′,2‴)], 6.62 [m, H−C(6‴)], 6.63 [m, H−C(6′)], 6.71 [m, H−C(6‴)], 6.81 [m, *J* = 7.7 Hz, H−C(2′)], 6.83 [m, *J* = 7.7 Hz, H−C(2‴)], 8.93−9.00 [brs, HO-C(3′,4′,3‴, 4‴)], 10.83 [brs, HO-C(7″,7)], 12.05, 12.15 [s, HO-C(5″)], 12.17, 12.21 [s, HO-C(5)]. ^{13}C NMR (100 MHz, DMSO-d_6, HSQC, HMBC): *δ* 43.1 [C-3″], 47.4 [C-3], 78.5, 78.6 [C-2″], 81.6, 81.9 [C-2], 94.9, 95.0, 95.2, 95.5, 96.1 [C-6″,6,8], 101.1, 101.2 [C-4a,4a″], 101.6 [C-8″], 113.4 [C-2′], 114.0 [C-2‴], 114.7, 114.9, 115.1, 115.3, 115.6, 115.7 [C-2′,5′,2‴,5‴], 116.4, 117.2 [C-6‴], 118.5, 118.9 [C-6′], 128.5, [C-1′], 129.9 [C-1‴], 144.7, 145.1, 145.3, 145.5, 145.6, 145.7 [C-3′,4′,3‴,4‴], 159.9, 160.8 [C-8a″], 162.0, 162.4 [C-5″], 162.6, 162.8 [C-8a], 163.6, 163.7 [C-5], 164.6, 165.0 [C-7″], 166.3, 166.4 [C-7], 196.1 [C-4″], 196.6 [C-4].

*(2R,3R,2″R,3″R)-Nonamethylmanniflavanone (**3a**).* Slightly brownish powder; UV (MeOH/H_2O, 9/1, v/v) λ_{max} = 210, 290, 346 nm; (−) HRESIMS *m*/*z* 715.2390 $[M - H]^-$ (calcd for $C_{39}H_{39}O_{13}$, 715.2391). ^{1}H NMR (500 MHz, $CDCl_3$, COSY): *δ* 3.70−3.93 [9xs, CH_3O−C(5,7,3′,4′,3″,5″,7″,3‴,4‴)], 4.48 [d, 1H, *J* = 12.5, H−C(3)], 4.73 [d, 1H, *J* = 12.6, H−C(3″)], 5.65 [d, 1H, *J* = 12.4 Hz, H−C(2)], 5.83 [d, 1H, *J* = 12.7 Hz, H−C(2″)], 6.15−6.19 [3xs, 3H, H−C(6,8,6″)], 6.56 [d, *J* = 7.9 Hz, H−C(6′)], 6.61−6.91 [H−C(2′,5′,2‴,5‴)], 6.91 [d, *J* = 8.2 Hz, H−C(6‴)]. ^{13}C NMR (125 MHz, $CDCl_3$, HSQC, HMBC): *δ* 49.1 [C-3″], 51.2 [C-3], 55.6−56.3 [CH_3O-(5,7,3′,4′,3″,5″,7″,3‴,4‴)], 81.4 [C-2″], 81.6 [C-2], 93.2−93.6 [C-6″,6,8], 100.7, 100.8 [C-4a″,4a], 105.8 [C-8″], 109.4−110.8 [C-2′,5′,2‴,5‴], 119.8 [C-6′], 120.2 [C-6‴], 130.4 [C-1‴], 130.7 [C-1′], 147.7−149.4 [C-3′,4′,3‴,4‴], 162.5−170.1 [C-5,7,8a,8a″,5″,7″], 188.0 [C-4], 188.3 [C-4″].

*Octamethylmanniflavanones (**3b,c**).* Slightly brownish powders; UV (MeOH/H_2O, 9/1, v/v) λ_{max} = 210, 290, 346 nm; (−) HRESIMS *m*/*z* 701.2225 $[M - H]^-$ (calcd for $C_{38}H_{37}O_{13}$, 701.2234). ^{13}C NMR (125 MHz, $CDCl_3$): *δ* 55.5−56.1 [CH_3O-(5,7,3′,4′,3″,5″,7″,3‴,4‴)].

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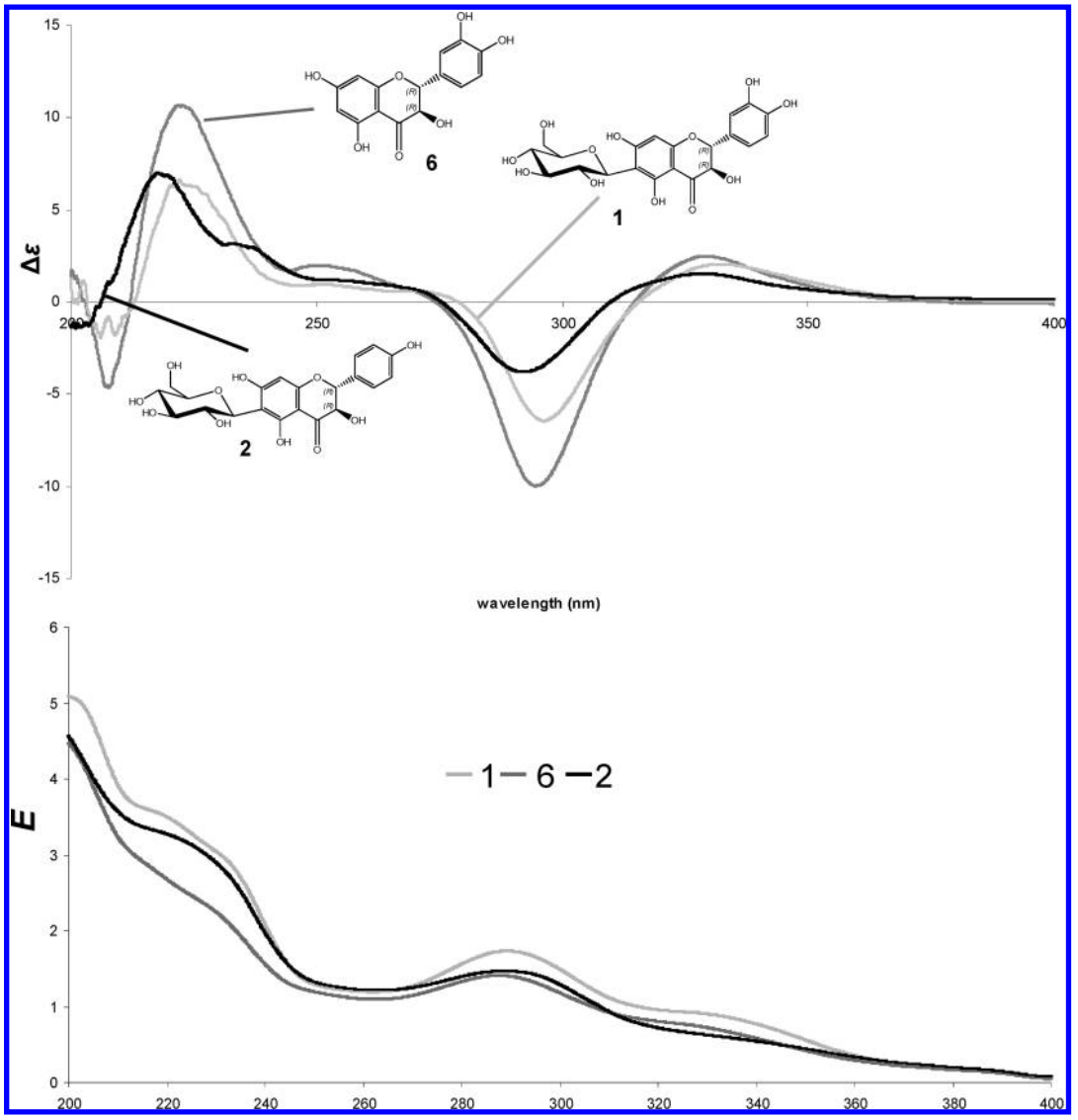

Figure 4. CD-spectra of (+)-(2*R*,3*R*)-taxifolin (**6**), (2*R*,3*R*)-taxifolin-6-*C*-*β*-D-glucopyranoside (**1**), and (2*R*,3*R*)-aromadendrin-6-C-*β*-D-glucopyranoside (**2**).

*(+)-(2R,3R)-Taxifolin (**6**, Figure 1).* CD (MeOH, c = 0.43mmol/L): $\lambda_{max}(\Delta\varepsilon)$ = 330 (+2.5), 295 (−10.0), 252 (+2.0), 244 (+1.7), 222 (+10.6), 208 (−4.7).

RESULTS AND DISCUSSION

In a first antioxidative screening, the aqueous ethanolic extract of *Garcinia buchananii* was analyzed by means of ORAC and H_2O_2 assays (Table 1). The ethanolic extract of *Garcinia buchananii* revealed an extraordinarily high antioxidant value of 1359 *μ*mol TE/100 mg. This is higher than or comparable to that of natural product extracts known to have high antioxidative activities, such as bilberry, elderberry, red wine extract, and grape seed extract. These have ORAC values of 265, 222, 694, and 1189 *μ*mol TE/100 mg,[19] respectively.

To get a deeper insight into the chemistry of this highly antioxidative aqueous ethanolic extract of *Garcinia buchananii*, as well as to focus on the highly antioxidative compounds, the sample was fractionated using medium pressure liquid RP-18 chromatography. Eight fractions (M1−M8) were obtained and then analyzed in their natural ratios for antioxidant activities using the ORAC and H_2O_2 scavenging assays. By far, the highest antioxidant activity was observed both in the ORAC and hydrogen peroxide scavenging assays for fraction M3. In both cases, the activity of this fraction represented about the half of the whole activity of the crude extract (Table 1). This was followed by fraction M5, which showed the second highest activity in both assays, and by fractions M1, M2, and M4, which all had a similar range of antioxidative activities. Fractions M6−M8 had the lowest activities. Therefore, fractions M1−M5 were further purified by means of RP-HPLC as described above.

The antioxidative compound no. **1** isolated from fraction M1 was obtained as a colorless amorphous powder. This compound showed the typical absorption maxima expected for flavanones. Results from electrospray ionization (ESI) MS indicated that this compound forms an $[M - H]^-$ ion with m/z 465, as well as a fragment ion with m/z 345, as expected for a *C*-glycoside. As shown in Figure 2, high resolution LC-MS analysis confirmed the target compound to have the molecular formula $C_{21}H_{22}O_{12}$ and the fingerprint fragment $C_{17}H_{14}O_{18}$. The ^{1}H NMR spectrum of compound **1** showed an aromatic singlet for H−C(8) at 5.92 ppm and three aromatic protons H−C (5′,6′, 2′) resonating at 6.74 and 6.87 ppm. In addition,

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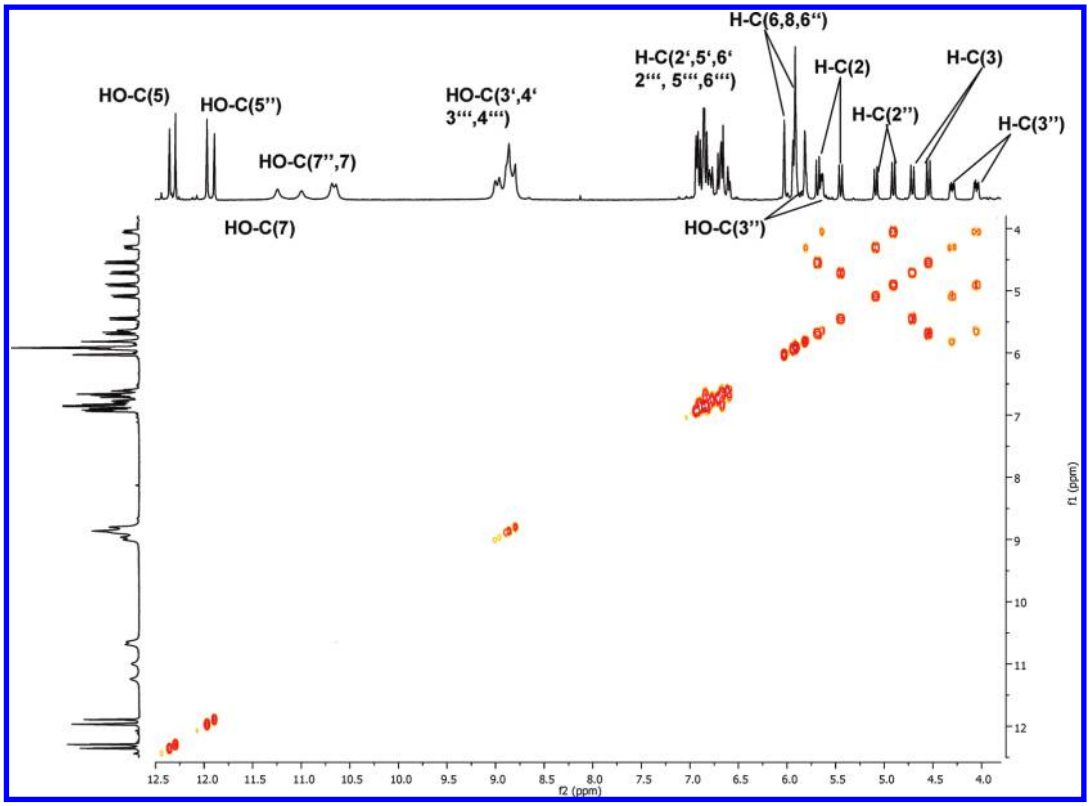

Figure 5. COSY-NMR (400 MHz, d_6-acetone + d_6-DMSO, 9/1, v/v) of (2*R*,3*S*,2″*R*,3″*R*)-manniflavanone (**3**).

the two aliphatic protons H−C(3) and H−C(2) were observed coupling with each other at 4.45 and 4.97 ppm suggesting a taxifolin aglycone. Besides the signals of the flavanone aglycone, the ^{1}H NMR spectrum also exhibited seven aliphatic protons resonating at 3.10 ppm [H−C(4″)], 3.14 ppm [H−C(5″)], 3.16 ppm [H−C(3″)], 3.40 ppm [H−C(6″α)], 3.67 ppm [H−C(6″β)], 3.99 ppm [H−C(2″)], and 4.49 ppm [H−C(1″)] as expected for a hexose unit.

Considering all the coupling constants of the sugar moiety in the molecule, and, in particular, the coupling constant of $J \sim 9$ Hz observed for the protons H−C(1″) and H−C(2″), and comparing these values with the H−C(1)/H−C(2) coupling constants reported for β-D-glucopyranosides and α-D-glucopyranosides,[21−24] the D-glucopyranose moiety was proposed and the β-linkage undoubtedly identified.

A comparison of the ^{13}C NMR spectrum, in which 21 signals appeared, with the results of the heteronuclear single-quantum correlation spectroscopy (HSQC) experiment showing 12 signals, revealed 9 signals corresponding to quaternary carbon atoms. Unequivocal assignment of these quaternary carbon atoms and the hydrogen-substituted carbon atoms, respectively, could be successfully achieved by means of heteronuclear multiple bond correlation spectroscopy (HMBC) and HSQC. The typical ^{13}C chemical shifts of the sugar part confirmed the D-glucopyranose. Additionally, the HMBC experiment revealed a correlation between the sugar proton H−C(1″) resonating at 4.49 ppm and neighboring carbon atoms C(5), C(7) and C(6), as well as no correlation to C(8a), thus demonstrating clearly the intramolecular 6-C-linkage of the β-D-glucopyranose to its aglycone (Figure 3). The characteristic chemical shift of the carbon atom C(1″) at 73.0 ppm confirmed the C-linkage of the sugar part and the taxifolin-6-*C*-β-D-glucopyranoside.[25] The same strategy resulted in the structure of aromadendrin-6-*C*-β-D-glucopyranoside,[26] isolated also from fraction M1 and detected as the major constituent in fraction M2.

To clarify the configuration of the carbon atoms C(2) and C(3) present in the aglycone taxifolin and aromadendrin of compounds **1** and **2**, circular dichroism (CD) spectroscopic measurements were performed using the commercially available reference isomer (+)-(2*R*,3*R*)-taxifolin (**6**) as well as the isolated *C*-glycosides **1** and **2** (Figure 4). The CD spectra of (+)-(2*R*,3*R*)-taxifolin (**6**) was well in line with literature data.[27,28] The data obtained clearly demonstrated that the spectra of *C*-glycosides **1** and **2** isolated from fraction M1 were similar to the spectrum of (+)-(2*R*,3*R*)-taxifolin; therefore, the stereochemistry could be deduced as (2*R*,3*R*)-taxifolin-6-*C*-β-D-glucopyranoside (**1**) and (2*R*,3*R*)-aromadendrin-6-*C*-β-D-glucopyranoside (**2**).

The antioxidative compounds **3**−**5** isolated from fractions M3−5 showed the typical absorption maxima expected for flavanones, and high resolution LC-MS analysis confirmed the target compound to have the molecular formula $C_{30}H_{22}O_{13}$ for **3** and $C_{30}H_{22}O_{12}$ for **4** and **5**, respectively. The ^{1}H NMR measurements of **3**−**5** in d_3-MeOD (RT), DMSO-d_6 (RT, 45 °C), and acetone-d_6 (RT) as well as mixtures of acetone-d_6 + DMSO-d_6 (9/1, v/v, RT, 8 °C) showed two series of signals typical for biflavanoids and the duplication of nearly all signals, indicating the presence of two main rotational isomers.[28,29] The sharpest signals were obtained at 8 °C (Figure 5). The ^{1}H NMR spectrum of compound **3** showed four sharp exchangeable signals at 12.36 and 12.29 for HO-C(5) and 11.97 and 11.89 ppm for HO-C(5″), four broad exchangeable singlets from 10.64 to 11.24 ppm for the OH-groups of 7 and 7″, several broad exchangeable signals between 8.80 and 9.00 ppm for HO-C(3′,4′,3‴,4‴), and two exchangeable doublets for the protons HO-C(3″) at 5.64 and 5.80 ppm. Typical A-ring

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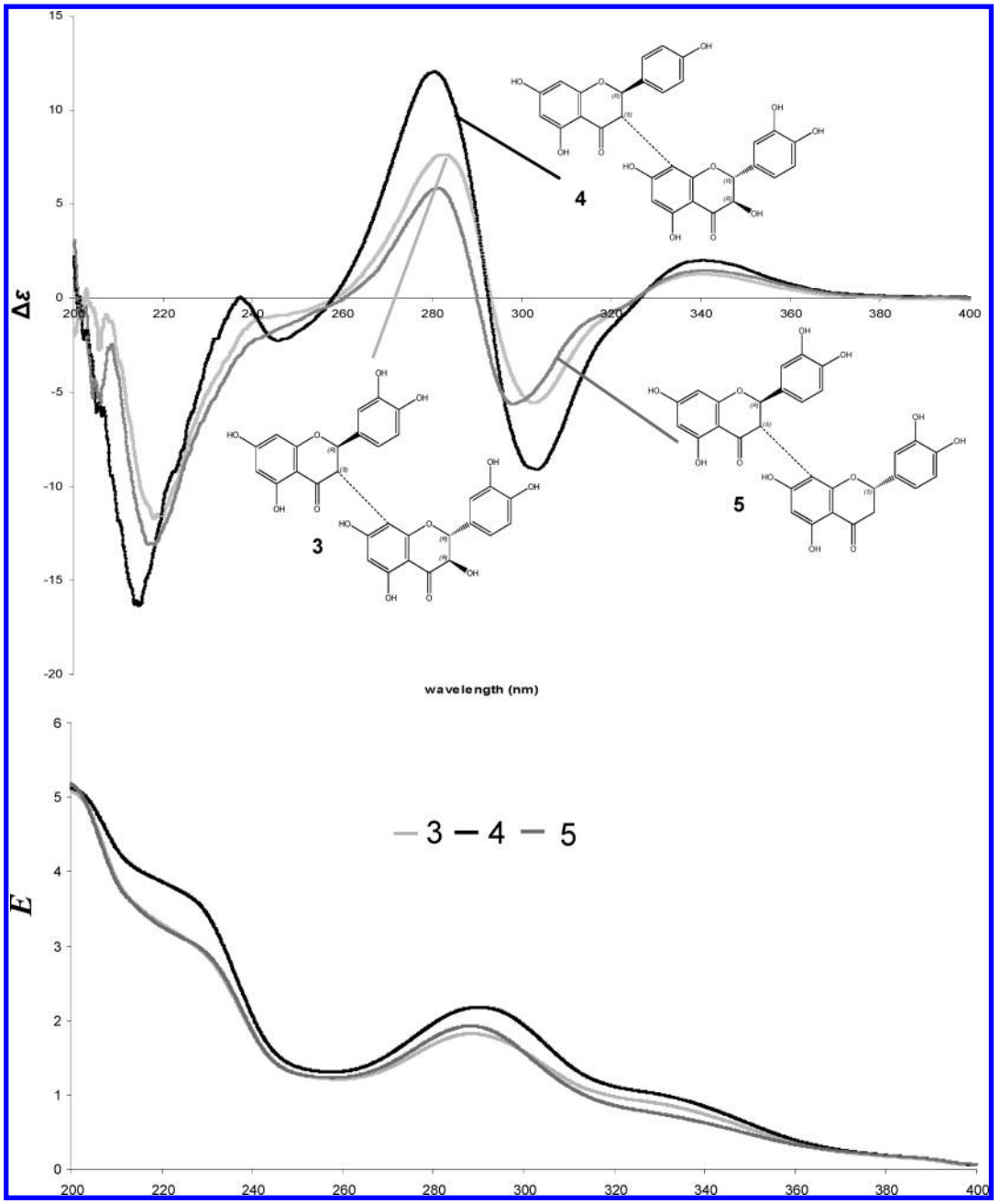

Figure 6. CD-spectra of (2*R*,3*S*,2″*R*,3″*R*)-manniflavanone (**3**), (2*R*,3*S*,2″*R*,3″*R*)-GB-2 (**4**), and (2*R*,3*S*,2″*S*)-buchananiflavanone (**5**).

aromatic singlets for H−C(6,8,6″) from 5.81 to 5.94 ppm as well as characteristic C-ring aromatic protons resonating at 6.03−6.94 ppm could be observed. In addition, the two sets of aliphatic protons H−C(3,3″) and H−C(2,2″) coupling with each other were observed suggesting a GB-type biflavanoid (Figure 5). The vicinal coupling constants of 11.3−12.1 Hz indicated their *trans*-diaxial relative configuration.

Unequivocal assignment of carbon atoms was successfully achieved by means of HMBC and HSQC. The HMBC experiment revealed a correlation between the proton H−C(3) resonating at 4.55 and 4.71 ppm and neighboring carbon atom C(8a″) and C(7″), as well as no correlation to C(5″), thus demonstrating clearly the intramolecular C-3/C-8″-linkage of the two flavanone monomers. Additionally, methylation of **3** was performed,[30] whereas after HPLC cleanup a mixture of two 8-fold methylated manniflavanone derivatives (**3b**,**c**) and nonamethylmanniflavanone (**3a**) was obtained. It is known from the literature[27,31] that the ^{13}C chemical shifts of sterically hindered OMe substituents of flavonoids occur between 59 and 61 ppm as compared with 55−57 ppm for non *ortho*-disubstituted methoxyl groups. In **3a**−**c**, all aromatic OMe substituents were observed between 55.5 and 56.3 ppm. If the intramolecular linkage had been through C-3/C-6″, then C(5″)-OMe would have been sterically hindered and consequently deshielded. NMR data of compounds **3** and **3a** are in line with partially described data for manniflavanone.[30]

The 1/2D-NMR data of compound **4** was very similar to manniflavanone (**3**), besides the substitution pattern of the naringenin B-ring giving doublets (J = 8.9) H−C(2′/6′) and H−C(3′/5′) at 7.21 and 6.73 ppm. Compound **4** was identified as GB-2, naringenin-C-3/C-8″ dihydroquercetin linked biflavanone.[29,32]

Table 2. Antioxidant Activities of Isolated Compounds 1−5 and Reference Compounds

	H_2O_2 assay[a,b]	ORAC assay[c,d]	literature[e,f,g]
	EC_{50} (μM)	(μmol TE/ μmol)	
(2*R*,3*R*)-taxifolin-6-*C*-β-D-glucopyranoside (**1**)	11.0 (9.0−11.4)	9.57 (±0.50)	n.r.[g]
(2*R*,3*R*)-aromadendrin-6-*C*-β-D-glucopyranoside (**2**)	10.9 (9.5−12.9)	4.23 (±0.08)	n.r.[g]
(2*R*,3*S*,2″*R*,3″*R*)-manniflavanone (**3**)	2.8 (2.4−3.2)	13.73 (±0.43)	n.r.[g]
(2*R*,3*S*,2″*R*3*R*)-GB-2 (**4**)	2.2 (1.9−2.6)	12.10 (±0.26)	n.r.[g]
(2*R*,3*S*,2″*S*)-buchananiflavanone (**5**)	14.4 (12.8−16.5)	10.50 (±0.43)	n.r.[g]
(+)-taxifolin (**6**)	11.3 (9.7−13.2)	7.63 (±0.68)	9.74[f]
ascorbic acid	16.5 (15.0−18.3)	0.34 (±0.10)	0.95[e]
rutin	6.9 (5.9−8.0)	6.45 (±0.28)	6.01[e];13.70[f]
quercetin	6.1 (5.3−7.1)	5.61 (±0.07)	7.28[e];8.04[f]
(−)-epicatechin	4.1 (3.7−4.6)	9.65 (±0.53)	9.14[f]
(±)-naringenin	8.6 (6.8−11.9)	3.96 (±0.19)	9.23[f]

[a]Each sample was analyzed by means of the H_2O_2 assay by triplicate studies. [b]The range in parentheses represents the 95% confidence interval. [c]Each sample was analyzed by means of the ORAC assay by quadruplicate studies. [d]The numerical value in parentheses represents the SD. [e]Values are from Ou et al. [f]Values are from Wolfe and Liu in which the stereochemistry of naringenin and taxifolin is not stated. [g]n.r.: not reported.

In comparison to manniflavanone (**3**), 1/2D-NMR measurements of compound **5** revealed a methylene group at carbon atom C(3″) resonating at 43.1 ppm and therefore diastereotopic protons H−C(3″$\alpha\beta$). Again, unequivocal assignment of carbon atoms could be successfully achieved by means of HMBC and HSQC, and the linkage between the eridictyol monomers was confirmed via HMBC experiment, revealing a correlation between the proton H−C(3) resonating at 4.50 and 4.63 ppm and neighboring carbon atom C(8a″) and C(7″), as well as no correlation to C(5″), thus demonstrating clearly the intramolecular C-3/C-8″-linkage of the two eridictyol monomers. To the best of our knowledge, compound **5**, which we have named buchananiflavanone, 3′,3‴,4′,4‴,5,5″,7,7″-octahydroxy-3,8″-biflavanone, has never been described before.

To clarify the configuration of the carbon atoms C(2) and C(3) in compounds **3**−**5**, the following CD spectroscopic measurements were performed with the commercially available (+)-(2*R*,3*R*)-taxifolin (**6**) as well as compounds **3**−**5**. The CD spectrum of GB-2 (**4**) (Figure 6) was well in line with literature data.[28] Consequently, the stereochemistry of GB-2 (**4**) could be deduced as (2*R*,3*S*,2″*R*,3″*R*). Since the CD spectrum of manniflavanone (**3**) was identical to GB-2 (**4**), the absolute configurations must be the same, and thus, the absolute configurations of manniflavanone must be revised to (2*R*,3*S*,2″*R*,3″*R*)-manniflavanone (**3**) (Figure 6). The CD spectrum of buchananiflavanone (**5**) (Figure 6) was also very similar to (2*R*,3*S*,2″*R*,3″*R*)-GB-2 (**4**) and (2*R*,3*S*,2″*R*,3″*R*)-manniflavanone (**3**), showing typical CD bands with corresponding signs for 3−8″-biflavanones[28] and/or mono flavanones like naringenin.[34] In combination with Gaffield's rule,[35] the results from Duddeck et al.[27] and the detailed investigations from Ferrari et al.[28] for $n \rightarrow \pi^*$ and $\pi \rightarrow \pi^*$ transitions of 3−8″-biflavanones consisting of either flavanones and hydroxyflavanones or flavanones and benzofuranones, the stereochemistry of buchananiflavanone (**5**) could be deduced as (2*R*,3*S*,2″*S*).

Antioxidative Activity of Isolated Compounds 1−5. Compounds **1**−**5** as well as known reference compounds with high antioxidative activity,[19,33] quercetin, rutin, (−)-epicatechin, ascorbic acid, and (±)-naringenin, were analyzed by means of ORAC and hydrogen peroxide scavenging assays (Table 2). In comparison to known very antioxidative single compounds, generally compounds **1**−**5** revealed relative high activity. By far, the highest activity in both assays was observed for (2*R*,3*S*,2″*R*,3″*R*)-manniflavanone (**3**) and (2*R*,3*S*,2″*R*,3″*R*)-GB-2 (**4**), which showed outstanding activity in comparison to that of quercetin, rutin, (−)-epicatechin, ascorbic acid, and (±)-naringenin as well as available literature data.[19,33] Also, all EC_{50} values of the hydrogen peroxide scavenging activity of compounds **1**−**5** are lower than that of ascorbic acid. The newly isolated compound (2*R*,3*S*,2″*S*)-buchananiflavanone (**5**) revealed high activity in the hydrogen peroxide scavenging assay and also a very strong activity in the ORAC assay.

Conclusions. The application of the newly developed method for screening of natural antioxidative compounds by means of the hydrogen peroxide scavenging assay dilution analysis in combination with the ORAC assay on *Garcinia buchananii* extracts, revealed (2*R*,3*R*)-taxifolin-6-*C*-β-D-glucopyranoside (**1**), (2*R*,3*R*)-aromadendrin-6-*C*-β-D-glucopyranoside (**2**), (2*R*,3*S*,2″*R*,3″*R*)-manniflavanone (**3**), (2*R*,3*S*,2″*R*,3″*R*)-GB-2 (**4**), and the previously unreported compound (2*R*,3*S*,2″*S*)-buchananiflavanone (**5**) as highly antioxidative active constituents. When taken together, our findings indicate that *G. buchananii* bark extract is a rich natural source of antioxidants with the potential to be utilized as food supplements in the future.

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Notes

The authors declare no competing financial interest.

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