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A New Cytotoxic and Antioxidant Amentoflavone Monoglucoside from *Cycas revoluta* Thunb

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Abstract

Normal and reversed phase silica gel together with Sephadex LH_{20} column chromatography of *Cycas revoluta* Thunb extracts of the leaflets led to the isolation and identification of eight compounds (**1-8**. Compound **6** is a new amentoflavone glucoside to be reported**.** Compounds **1**, **7** and **8** were reported for the first time from *Cycas* genus. Compound **2** was isolated for the first time from *C. revoluta* Thunb. Strikingly, compounds **5**, **6** and **7** showed antioxidant activity nearly two to four folds higher than that of quercetin at a dose of 12.5 µg/ml. Compounds **6** and **7** showed very strong cytotoxic activity against MCF 7 cell line (IC_{50} = 6.12, 4.73 µg/ml, respectively) compared to doxorubicin (IC_{50} = 4.13 µg/ml), while compound **5** showed strong cytotoxic activity (IC_{50} = 18.70 µg/ml). The cytotoxic and antioxidant activities are most likely due to the biflavonoid content, especially the new compound **6**.

Key Words: amentoflavone-4'-*O*- α- D- glucoside, antioxidant activity, *Cycas revoluta* Thunb, cytotoxic activity.

INTRODUCTION

Cycadaceae is a family rich in flavonoids, flavonoid glycosides and biflavonoids [1]. The only recognized genus of this family is *Cycas* which includes 100 species of which *Cycas revoluta* is the most widely cultivated [2]. *Cycas revoluta*, known as Sago palm, is an ornamental plant native to Southern East Asia [3, 4] and exhibits cytotoxic, antioxidant, antimicrobial and antileismanial activities [5, 6, 7]. The seeds of *C. revoluta* are used in Chinese medicine as expectorant and antirheumatic [8]. Phytochemical investigation of the plant leaflets in previous studies led to the isolation of flavonoids (naringenin; [6] prunin; [5] vitexin-2″-rhamnoside [5]), biflavonoids (amentoflavone; [9] 2,3-dihydroamentoflavone; [6] 2, 3, 2″, 3″-tetrahydroamentoflavone; 2, 3-dihydro- 7- *O*- β- D-glucopyranosylamentoflavone; [5] 2, 3- dihydro- 7, 7´´- di-*O*- β-D-glucopyranosylamentoflavone; [5] hinokiflavone; [6, 9] 2, 3-dihydrohinokiflavone; [9] 2, 3, 2″, 3″-tetrahydrohinokiflavone; [6] podocarpusflavone A), [6] phenolic and alcoholic compounds ((+)-lariciresinol; [6] protocatechuic acid; [5] vomifoliol [6]). Little data were found in literature regarding the cytotoxic activity and no data were found about the antioxidant activity of the active principles from *C. revoluta* leaflets [5, 6]. Here we report the isolation of one new and four known compounds from *C. revoluta* leaflets for the first time from the genus *Cycas* and explore the antioxidant and cytotoxic activities of the studied extracts and some purified compounds.

MATERIALS AND METHODS

GENERAL EXPERIMENTAL PROCEDURES

Authentic β-sitosterol was purchased from Merck, Darmstadt, Germany. Authentic amentoflavone was kindly provided by Prof. Abdel-Rahim Sayed Ibrahim, Professor of Pharmacognosy, Faculty of Pharmacy, Tanta University. Naringenin, anhydrous Na_2SO_4, glucose, rhamnose, $ALCl_3$, NaOA, and boric acid were obtained from were obtained from Sigma Chemical Co., USA. Melting points were measured using melting point apparatus "Galenkamp type" and were uncorrected. Thin layer chromatography (TLC) was performed using silica gel G F_{254} plates (E. Merk). Column chromatography was accomplished using silica gel (E. Merck, 70-230 mesh). Sephadex LH-20 was purchased from Sigma- Aldrich chemical Co., USA. Reversed phase octadecylsilyl-silica gel (RP-C18) was obtained from Merck, Germany and Diaion™ HP20SS from Mitsubishi Chemical, Japan. Sheets of Whatman No. 1 filter paper for chromatography were obtained from Whatman Ltd., England. NMR spectra were measured using Bruker High Performance Digital FT-NMR Spectrometer Avance III operating at 400 MHz. IR spectra were recorded as KBr discs using Jasco, FT/IR-6100 spectrophotometer, Japan. UV spectra were obtained using Shimadzu UV spectrophotometer UV- 1800, Japan. EI-MS spectra were recorded using mass Spectrometer Thermo Scientific ISQ Single Quadrupole MS, USA and mass Spectrometer Thermo Scientific ISQ Quantum Access MAX triple Quadrupole, USA.

BIOLOGICAL ACTIVITY

2, 2- Diphenyl-1- picrylhydrazyl (DPPH); dimethyl sulfoxide (DMSO); trichloroacetic acid; sulforhodamine B (SRB) stain; ethylenediaminetetraacetic acid buffer (EDTA) and acetic acid were purchased from Sigma Chemical Co. St. Louis, Mo. Quercetin was purchased from Fluka Chemical Crop, USA. Breast carcinoma cell line (MCF-7) and liver carcinoma cell line (HepG2) were obtained from the American Type Culture Collection (ATTC). Absorbance in the antioxidant assay was measured using a UNICO spectrophotometer (UV/Vis) UV-2000 (UNICO Instrument CO. LTD, USA) and an ELISA processor II microplate reader was used in the cytotoxic activity assessment.

Plant material

Cycas revoluta Thunb leaflets were collected in August 2011 from local garden in Tanta city, Gharbia Governorate, Egypt. The plant was kindly identified by Prof. Kamal Shaltout, professor of plant ecology, botany department, Faculty of Science, Tanta University. They were shade-dried, pulverized and stored in amber-colored glass bottles for further investigation.

Extraction and isolation

The shade dried powder of *Cycas revoluta* Thunb leaves (2 kg) was extracted by cold maceration with ethanol (95%) until exhaustion. Alcohol extract was evaporated under reduced pressure to yield 70 gm of crude total extract, which was resuspended in aqueous methanol (50%) and successively fractionated with petroleum ether (40-60 °C), methylene chloride, ethyl acetate and n- butanol saturated with water.

Pet. ether extract (2 g) was saponified by boiling under reflux with 10% alcoholic KOH (30 ml) for 5 hrs. The major part of alcohol was then distilled off and the aqueous liquid left was diluted with 20 ml water and extracted with several portions of ether till exhaustion. [10] The combined ethereal extract was washed with distilled water, dehydrated over anhydrous Na_2SO_4 and distilled off under reduced pressure to give the unsaponifiable matter (0.37 g). The unsaponifiable matter was subjected to column chromatography on silica gel (1.5 x 35 cm, 30 g) gradiently eluted using pet. ether-methylene chloride mixtures of increasing polarity. Fractions (52-61) eluted with pet. ether-methylene chloride (50:50) yielded white powder (90 mg) which was recrystallised from methanol to afford compound **2** as white needle crystals (25 mg). Fractions (20-32) collected with pet. ether- methylene chloride (80:20) were pooled, evaporated to dryness (20 mg) and rechromatographed on silica gel column eluted with methylene chloride- methanol (2% increment) to yield compound **1** (4 mg).

Methylene chloride fraction (4 g) of the crude total extract was chromatographed on silica gel (3 x 60 cm, 105 g) gradiently eluted with methylene chloride- methanol mixtures of increased polarity. Fractions (4-10) eluted with methylene chloride- methanol (90:10) were pooled and evaporated to dryness to yield 800 mg and rechromatographed on silica gel column (2.5 × 47 cm, 25 g). The column was eluted with n-hexane- ethyl acetate mixtures of which the 50:50 eluate was further purified on sephadex gel column (1.25 x 25 cm, 10g) to yield compound **3**. Fractions (11-20) eluted with methylene chloride- methanol (86:14) were pooled, evaporated to dryness to yield 360 mg and rechromatographed on silica gel column (1.25 x 25 cm, 15 g) eluted with methylene chloride and increasing polarity using methanol of which the methylene chloride- methanol 94:6 and 90:10 eluates were pooled separately. The methylene chloride- methanol 94:6 eluate (54 mg) was further chromatographed on silica gel mini column (0.7× 10 cm, 2 g) using methylene chloride- methanol (95:5) to afford compound **4** (21 mg). The methylene chloride- methanol 90:10 eluate (110 mg) was further purified on a Sephadex LH_{20} gel column eluted with methanol to yield compound **5** (60 mg).

Ethyl acetate fraction (2 g) of the crude total extract was chromatographed on a silica gel column (3 x 60 cm,75 g) gradiently eluted with methylene chloride- methanol mixtures of increasing polarity. Fractions eluted with methylene chloride- methanol (88:12) and (84:16) were pooled separately for further purification. The methylene chloride- methanol 88:12 eluate (fractions 23-30, 174 mg) was rechromatographed on a reversed phase C-18 silica gel column (1.5 x 32 cm, 8 g) eluted with 10% MeOH in water for HPLC and decreasing polarity by HPLC methanol of which the 40:60 eluate provided compound **6** (44 mg). The Fractions 31-40 obtained with methylene chloride-methanol (84:16) of ethyl acetate fraction was rechromatographed on silica gel column (0.7 x 25 cm, 2.5 g) gradiently eluted with methylene chloride- methanol mixtures of increasing polarity of which the 88:12 to 84:16 eluates yielded compound **7** (20 mg) after evaporation to dryness.

n- Butanol fraction (7.5 gm) of crude total extract was suspended in deionized water and applied on top of a Diaion HP-20 column (3 × 60 cm, 80 g). The column was initially eluted with deionized water (1 L) to remove free sugars and salts followed by water and methanol mixtures of decreasing polarity of which 60% methanol eluate (500 mg) was rechromatographed on a silica gel column (1.5 x 60 cm, 15 g) and eluted with methylene chloride and increasing polarity using methanol of which the methylene chloride-methanol (75:25) eluate provided compound **8** (16 mg) after repeated crystallization from methanol.

Acid hydrolysis of the isolated glycosides [11]

Complete acid hydrolysis was carried out by refluxing the glycoside (5 mg) in aqueous methanol with 5% H_2SO_4 (10 ml) for 2 hrs. The hydrolysate was extracted with ethyl acetate and washed with water, then evaporated to obtain the aglycone. The mother liquor of hydrolysate was neutralized with Ba_2CO_3, filtered, concentrated and used to study the sugar moiety using paper chromatography.

BIOLOGICAL SCREENING

Antioxidant activity

The antioxidant activity was assessed by measuring the free radical scavenging activity of the different extracts and some isolated pure compounds using DPPH according to literature procedure [12, 13, 14]. Tested solutions were prepared in ethanol. The test was carried out, in triplicate, by mixing 0.2 ml of 1 mM DPPH solution, 0.2 ml of tested sample and 4.6 ml ethanol. The mixture was incubated at 30 °C for 30 min in the dark. The absorbance was measured at 517 nm using ethanol as a blank. Quercetin was used as a positive control and negative control was prepared by omitting the addition of test sample. The percent of inhibition of free radical DPPH (I %) was calculated.

Cytotoxic activity

Potential Cytotoxicity of the different extracts and isolated pure compounds was investigated according to Skehan *et al* [15] using MCF-7 and HepG2 cell lines. Doxorubicin was used as a standard and the IC_{50} was calculated and assessed according to Ayyad classification [16].

CHARACTERIZATION

Compound 1

White crystal; mp 188-189°C; IR (KBr) ν_{max} 3443, 2934, 2865, 1715, 1642, 1460, 1372 cm^{-1}; EI-MS *m/z* 426 [M^+], 411 [M^+- CH_3], 408 [M^+-H_2O], 381 [M^+- $3CH_3$].

Compound 2

White crystals; mp 137-139°C; IR (KBr) ν_{max} 3449, 2936, 2861, 2359, 2069, 1640, 1461, 1377, 1329, 1244, 1187, 1107, 1056, 961, 924, 877, 833, 800, 619 cm^{-1}; EI-MS *m/z* 414 [M^+], 396 [M^+-H_2O], 381[M^+-(CH_3+H_2O)], 329 [M^+-C_6H_{13}]; ^{1}H NMR [DMSO-d_6, 400 MHz] δ_H: 5.26 (1H, t, H-6), 3.50 (1H, m, H-3), 1.24 (3H, s, H-19), 0.90 (3H, d, *J* = 6 Hz, H-21), 0.84 (3H, d, *J* = 6.4 Hz, H-26), 0.81 (3H, d, *J* = 6.8 Hz, H-27), 0.65 (3H, s, H-18), 0.65-2.24 (m, H-1, 2, 4, 7-9, 11, 12, 14-17, 20, 22-25, 28, 29).

Compound 3

White amorphous granules; UV λ_{max} (MeOH) 293, 325 (sh) nm; IR (KBr) ν_{max} 3957, 3780, 3290 , 3119, 2918, 2831, 2709, 1634 cm^{-1}; ^{1}H NMR [DMSO-d_6, 400 MHz] δ_H: 5.45 (1H, dd, *J* = 12.8, 3.4 Hz, H-2), 2.69 (1H, dd, *J* = 17.2 , 3,4 Hz, H-3 axial), 3.30 (1H, dd, *J* = 17.0 ,12.8 Hz, H-3 equatorial), 5.88 (2H, d, *J* = 2.1 Hz, H-6, 8), 7.31 (2H, d, *J* = 8.0 Hz, H-2`, 6`), 6.81 (2H, d, *J* = 8.0 Hz, H-3`, 5`), 12.15 (1H, s, OH).

Compound 4

Yellow amorphous granules; UV λ_{max} (MeOH) 269, 280, 305 (sh) and 332 nm; IR (KBr) ν_{max} 3858, 3450, 2927, 2859, 2353, 2319, 2072, 1644, 1995, 1430,1364, 1287, 1247, 1170, 1109, 1042, 947, 835, 643, 593 cm^{-1}; ^{1}H NMR [DMSO-d_6, 400 MHz] δ_H: 6.76 (1H , s, H-3"), 6.81 (1H, s, H-3), 6.18 (1H, d, *J*=1.8 Hz, H-6), 6.28 (1H, s, H-6"), 6.41 (1H, d, *J*= 1.8 Hz, H-8), 7.60 (2H, d, *J*= 8.6 Hz, H-2‴, 6‴), 6.68 (2H, d, *J*= 8.6 Hz, H-3‴, 5‴), 7.98 (1H, dd, *J*= 8.6, 2.3 Hz, H-6′), 8.08 (1H, d, *J*= 2.3 Hz, H-2′), 7.07 (1H, d, *J*= 8.6 Hz, H-5′), 10,77 (1H, s, OH at C7), 10.21(1H, s, OH at C7"), 13.02 (1H, s, OH at C5), 13.14 (1H, s, OH at C5"); ESI-MS m/z 553 $[M+H]^+$.

Compound 5

Yellow amorphous powder; UV λ_{max} (MeOH) 270, 332 nm; IR (KBr) ν_{max} 3963, 3426, 1654, 1610, 1500, 1429, 1360, 1285,1244, 1172,1108, 1040, 836, 754, 560 cm^{-1}; ^{1}H NMR [DMSO-d_6, 400 MHz] δ_H: 6.79 (1H , s, H-3"), 6.83 (1H, s, H-3), 6.19 (1H, d, *J*=1.8 Hz, H-6), 6.40 (1H, s, H-6"), 6.47 (1H, d, *J*= 1.8 Hz, H-8), 7.58 (2H, d, *J*= 8.0 Hz, H-2‴, 6‴), 6.73 (2H, d, *J*= 8.0 Hz, H-3‴, 5‴), 7.15 (1H, d, *J*= 8.8 Hz, H-5′), 8.00 (1H, d, *J*= 1.5 Hz, H-2′), 7.99 (1H, dd, *J*= 8.8, 1.5 Hz, H-6′), 10.86 (1H, s, OH at C7), 10.31(1H, s, OH at C7"), 10.65 (2H, brs, OH at 4′and 4‴), 12.97 (1H, s, OH at C5), 13.10 (1H, s, OH at C5"); ^{13}C NMR [DMSO-d_6, 100 MHz] δ_C: 164.3 (C-2), 103.4 (C-3), 182.2 (C-4), 161.9 (C-5), 99.1 (C-6), 164.2 (C-7), 94.5 (C-8), 157.8 (C-9), 104.2 (C-10), 120.5 (C-1`), 128.2 (C-2′), 121.9 (C-3`), 160.9 (C-4`), 116.7 (5`), 131.8 (C-6′), 164.6 (C-2"), 103.1 (C-3"), 182. 6 (C-4"), 160.1 (C-5"), 99.3 (C-6"), 162.4 (C-7"), 104.5 (C-8"), 154.9 (C-9"), 104.1 (C-10"), 121.4 (C-1‴), 128.6 (C-2‴,6‴), 116.2 (C-3‴, 5‴), 161.5 (C-4‴); ESI-MS m/z 539 $[M+H]^+$.

Compound 6

Yellow amorphous powder; UV λ_{max} (MeOH) 271, 332 nm; IR (KBr) ν_{max} 3928, 3782, 3420, 2926, 2860, 2305, 1723, 1650, 1609, 1498, 1429, 1362, 1285, 1245, 1172, 1106, 948, 836, 744, 637, 560, 513 cm^{-1}; ^{1}H NMR [DMSO-d_6, 400 MHz] δ_H: 6.80 (1H , s, H-3"), 6.82 (1H, s, H-3), 6.34 (1H, s, H-6"), 6.19 (1H, d, *J*= 2.1 Hz, H-6), 6.44 (1H, d, *J*= 2.1 Hz, H-8), 7.6 (2H, d, *J*= 8.4 Hz, H-2"', 6"'), 6.77 (2H, d, *J*= 8.4 Hz, H-3"', 5"'), 8.00 (1H, d, *J*= 2.1 Hz, H-2'), 7.12 (1H, d, *J*= 8.4 Hz, H-5'), 7.99 (1H, dd, *J*= 8.4, 2.1 Hz, H-6'), 10.85(1H, s, OH at C7), 10.30 (1H, s, OH at C7"), 13.11 (1H, s, OH at C5), 12.99 (1H, s, OH at C5"), 4.47 (1H, d , *J*= 4.4 Hz, anomeric proton); ^{13}C NMR [DMSO-d_6, 100 MHz] δ_C: 164.4 (C-2), 103.0 (C-3), 182.2 (C-4), 161.4 (C-5, 5"), 99.3 (C-6), 161.9 (C-7), 94.5 (C-8), 157.3 (C-9), 104.1 (C-10), 127.8 (C-1`), 128.0 (C-2`,6'), 121.9 (C-3`), 161.0 (C-4`), 121.8 (5`), 164.6 (C-2"), 103.2 (C-3"), 182.5 (C-4"), 99.5 (C-6"), 164.0 (C-7"), 104.1 (C-8"), 154.9 (C-9"), 104.0 (C-10"), 121.9 (C-1‴), 128.6 (C-2‴, 6"'), 116.2 (C-3‴, 5‴) , 161.7 (C-4‴), 99.9 (Glu1), 70.0 (Glu2), 72.9 (Glu3), 67.9 (Glu-4), 70.2 (Glu5), 63.5 (Glu6); ESI-MS *m/z* 701 $[M+H]^+$.

Compound 7

Yellow amorphous powder; UV λ_{max} (MeOH) 227, 283, 340 (sh) nm; IR (KBr) ν_{max} 3449, 2958, 2042, 1639, 1586, 1506, 1270, 1178,1128, 1127,1022, 875, 818, 745, 647, 547 cm^{-1}; ^{1}H NMR [DMSO-d_6, 400 MHz] δ_H: 5.52 (1H, dd, J=12.6, 3.2 Hz, H-2), 2.55 (1H, dd, J= 16.3, 3.2 Hz, H-3 axial), 3.78 (1H, dd, *J*= 16.3,12.6, H-3 equatorial), 6.12 (1H, d, *J*= 3.6, H-6), 6.14 (1H, d, *J*= 3.6 Hz, H-8), 6.96 (1H, d, *J*=1.6 Hz, H-2'), 6.91 (1H, d, *J*= 8.4 Hz, H-5'), 6.94 (1H, dd, *J*= 1.6,8.4 Hz, H-6'), 3.9 (3H, s, OCH_3), 4.97 (2H, d, *J*= 7.4 Hz, glucose anomeric proton), 4.69 (H, d, *J*= 2.8 Hz, rhamnose anomeric proton), 1.09 (3H, d, *J*= 6.2 Hz, CH_3 of rhamnose), 9.12 (1H, s, OH at C-3'), 12.01 (1H, s, OH at C-5); ^{13}C NMR [DMSO-d_6, 100 MHz] δ_C: 78.5 (C-2), 40.9 (C-3), 197.5 (C-4), 163.3 (C-5), 96.8 (C-6), 165.6 (C-7), 95.8 (C-8), 162.5 (C-9), 104.6 (C-10), 131.3 (C-1`), 112.5 (C-2`), 146.9 (C-3`), 148.4 (C-4`), 114.6 (C-5`), 118.4 (C-6`), 99.9 (Glu1), 76.7 (Glu2), 76.0 (Glu3), 71.2 (Glu-4), 73.5 (Glu5), 59.8 (Glu6), 101.1 (Rha1), 70.1 (Rha 2), 70.7 (Rha 3), 72.5 (Rha 4), 68.8 (Rha5), 18.3 (CH_3 of Rha 6), 56.2 (OCH_3); ESI-MS *m/z* 611 $[M+H]^+$.

Compound 8

Yellow granules; UV λ_{max} (MeOH) 229, 260, 357 nm; UV λ_{max} (NaOH) 258, 288 , 408; ($ALCl_3$) 272, 305 (sh), 430; UV λ_{max} ($AlCl_3$/ HCl) 302, 370, 400; UV λ_{max} (NaOAc) 274, 412; UV λ_{max} (NaOAc/Boric acid) 264, 374; IR (KBr) ν_{max} 3419, 2925, 2855, 1627, 1383 cm^{-1}; ^{1}H NMR [DMSO-d_6, 400 MHz] δ_H: 6.14 (1H ,d , *J*=2.0 Hz, H-6), 6.33 (1H, d, *J*=2.0 Hz, H-8), 6.83 (1H, d, *J*=8.4 Hz, H-5'), 7.54 (2H, m, H-2' ,6'), 4.39 (1H, d, *J*=2.0 Hz, rhamnose anomeric proton), 5.32 (1H, d, *J*=7.0 Hz, glucose anomeric proton), 0.99 (3H, d, *J*=6.0 Hz, CH_3 of rhamnose), 12.58 (1H , s, OH at C-5).

RESULTS AND DISCUSSION

PHYTOCHEMICAL INVESTIGATION

β- Amyrin (**1**) and β- sitosterol (**2**) were identified by comparing their physical and spectral data to that of authentic samples and published literature [17-20].

Compound 3

UV spectrum showed λ_{max} characteristic for flavanones at 293 and 340 (sh) nm [21]. ^{1}H-NMR data confirmed the flavanone nature of compound **3** as it exhibited signals for methylene protons at C-3 resonating at δ_H 2.69 (dd, *J*=17.2, 3.4 Hz) and at δ_H 3.3 (dd, *J*= 17.0, 12.8). Additionally, ^{1}H NMR spectrum indicated an AA'BB' system by signals at δ_H 7.31 (d, *J* = 8.0 Hz) for H-2`, 6` and 6.81 (d, *J* = 8.0 Hz) for H-3`, 5`. A signal at δ_H 5.88 (2H, d, *J*= 2.1 Hz) is attributed to *meta*-coupled equivalent protons H-6, 8. The UV and ^{1}H NMR data were found matching those of naringenin in literature [22, 23]. The structure of compound **3** was confirmed by direct comparison with naringenin authentic sample through TLC and superimposable IR & UV spectra.

Compound 4

UV spectrum showed λ_{max} at 269 and 332 nm, which suggested a flavonoid structure. ^{1}H NMR data confirmed the biflavonoid nature of compound **4** and suggested an amentoflavone pattern [24]. The ^{1}H NMR spectrum showed an AA'BB' system as indicated by the signal at δ_H 7.60 (d, 8.6 Hz) for H-2‴, 6‴ and at δ_H 6.68 (d, 8.6 Hz) for H-3‴, 5‴. An ABX system was also shown by the signals at δ_H 8.08 (d, *J*= 2.3 Hz) for H-2', δ_H 7.07 (d, *J*= 8.6 Hz) for H-5' and δ_H 7.98 (dd, *J*= 8.6, 2.3 Hz) for H-6'. A singlet at δ_H 6.28 and *meta*-coupled protons at δ_H 6.18 and 6.41 (*J*= 1.8 Hz) were assigned for H-6", H6 and H-8, respectively. The NMR data indicated that C-8″ and C-3' are involved in the biflavonoid linkage. The ESI$^+$-MS of compound **4** showed a pseudomolecular ion at *m/z* 553.0 for $[M+H]^+$ which is consistent with an amentoflavone methoxy derivative. Inspection of the ^{1}H NMR spectrum revealed the absence of 4′, 4‴-OH signal and showed a new signal suggesting 4′-methoxy amentoflavone structure at either site. The UV spectrum of compound 4 in methanol exhibited λ_{max} at 269, 280, 305 (sh) and 332 nm which is consistent with 4‴- and not 4′- methoxy derivative [25]. In the ^{1}H NMR the methoxy signal was buried under the residual water peak. Based on all the data discussed above, compound 4 was determined as podocarpus flavone A which was previously isolated from the plant [6].

Compound 5

UV spectrum showed λ_{max} at 270 and 332 nm which suggested a flavonoid structure. ^{1}H-NMR data confirmed the biflavonoid nature of compound **5** and suggested an amentoflavone pattern. The ^{1}H NMR spectrum showed an AA'BB' systems as indicated by δ_H 6.72 (d, 8.0 Hz) for H-3‴, 5‴ and δ_H 7.58 (d, *J*= 8.0 Hz) for H-2"', 6"'. Additionally, the ^{1}H NMR spectrum showed signals for *meta*-coupled protons at δ_H 6.19 and 6.46 (*J*= 1.8 Hz) for H-6 and H-8, respectively, also a singlet at δ_H 6.40 was assigned to H-6". An ABX system was also shown by the signals at δ_H 8.00 (d, *J*= 1.5 Hz) for H-2', δ_H 7.15 (d, *J*= 8.8 Hz) for H-5' and δ_H 7.99 (dd, *J*= 8.8, 1.5 Hz) for H-6'. These data indicated that C-8″ and C-3' are involved in the biflavonoid linkage which is consistent with amentoflavone in literature. The structure was confirmed by ^{13}C NMR which showed 30 carbons and confirmed the conjugation linkage between C-8″ and C-3' due to the downfield shift of C-8" by 10 ppm and of C-3" by 6.00 ppm, respectively [26, 27, 28]. The ESI$^+$-MS of compound **5** showed a pseudomolecular ion at *m/z* 539.34 for $[M+H]^+$ which is consistent with an amentoflavone structure. The NMR data were consistent with those in literature for amentoflavone [24, 26-29].

Compound 6

The results of chemical tests, Molisch's and NaOH tests, of compound **6** suggested a flavonoid glycoside nature. IR spectrum showed a strong band at 3420 cm^{-1} for hydroxyl stretching, 1650 cm^{-1} for carbonyl group, 2926 cm^{-1} for aliphatic methylene stretching, 1362 cm^{-1} for methylene bending and 1285 cm^{-1} for (C-O) stretching. UV spectrum showed λ_{max} at 271, 299 and 332 nm which are characteristic of flavonoids [27]. ^{1}H NMR data confirmed the biflavonoid nature of compound **6** and suggested an amentoflavone pattern as compared to compound **5** data discussed above. ^{1}H NMR spectrum established the presence of two hydroxyl groups resonating at δ_H 13.11, 12.99 indicating the presence of two chelated hydroxyl groups at C-5, 5", respectively. The signals of *meta*-coupled protons at δ_H 6.19 and 6.44 (d, *J*=2.1 Hz) can be assigned for H-6 and H-8, respectively while the signal at δ_H 6.34 (1H, s) accounts for H-6". The signals at δ_H 6.82 ppm (s) and 6.80 (s) are for H-3 and H-3", respectively. The ^{1}H NMR spectrum also showed an ABX system represented by signals at δ_H 8.00 (d, *J*=2.1 Hz) for H-2', 7.12 (d, *J*=8.4. Hz) for H-5', 7.99 (dd, *J*= 8.4, 2.1 Hz) for H-6'. There is an AA'BB' system confirmed by the signals at δ_H 6.77 (d, *J*=8.4 Hz) for H-3"', 5"' and δ_H 7.60 (2H, d, *J*=8.4 Hz) for H-2"', 6"'. Acid hydrolysis of compound **6** yielded amentoflavone as the aglycone part which was confirmed by direct comparison to the isolated amentoflavone in this work on TLC. Paper chromatography of the sugar part resulting from acid hydrolysis showed that glucose is the glycone moiety. The glycosidic linkage was identified as α as indicated by the signal at δ_H 4.47 (*J*= 4.4 Hz) for the anomeric proton. ^{13}C NMR signals at δ_C 99.9, 72.9, 70.2, 70.0, 67.9, 63.5 ppm confirmed the presence of one sugar moiety (*O*-glucose) and matched those for α anomer [30]. The ESI$^+$-MS of compound **6** showed an *m/z* at 701 for $[M+H]^+$, which is a solid evidence for amentoflavone glucoside. The glucosidation of the 5, 5"– OH was ruled due to the presence of signals in the ^{1}H NMR spectrum for free hydroxyl groups at these sites as mentioned above and thus suggested the conjugation of either 7-OH, 7"-OH, 4'-OH or 4‴-OH. ^{1}H NMR spectrum showed also signals at δ_H 10.30 (brs) and 10.85 (brs) which are assigned for 7- OH and 7"- OH, respectively [31]. However the signals for 4′, 4‴-OH were absent indicating possible conjugation at either position. Markham *et al* in 1978 indicated that glucosylation causes upfield shift of the *ipso* carbon and downfield shift of the *ortho* and *para* carbons, with greater impact on the *para* position, however only one example of glycosylation at C-4′ was studied and the results were considered preliminary [26]. Literature of apigenin 4'-*O*-α-

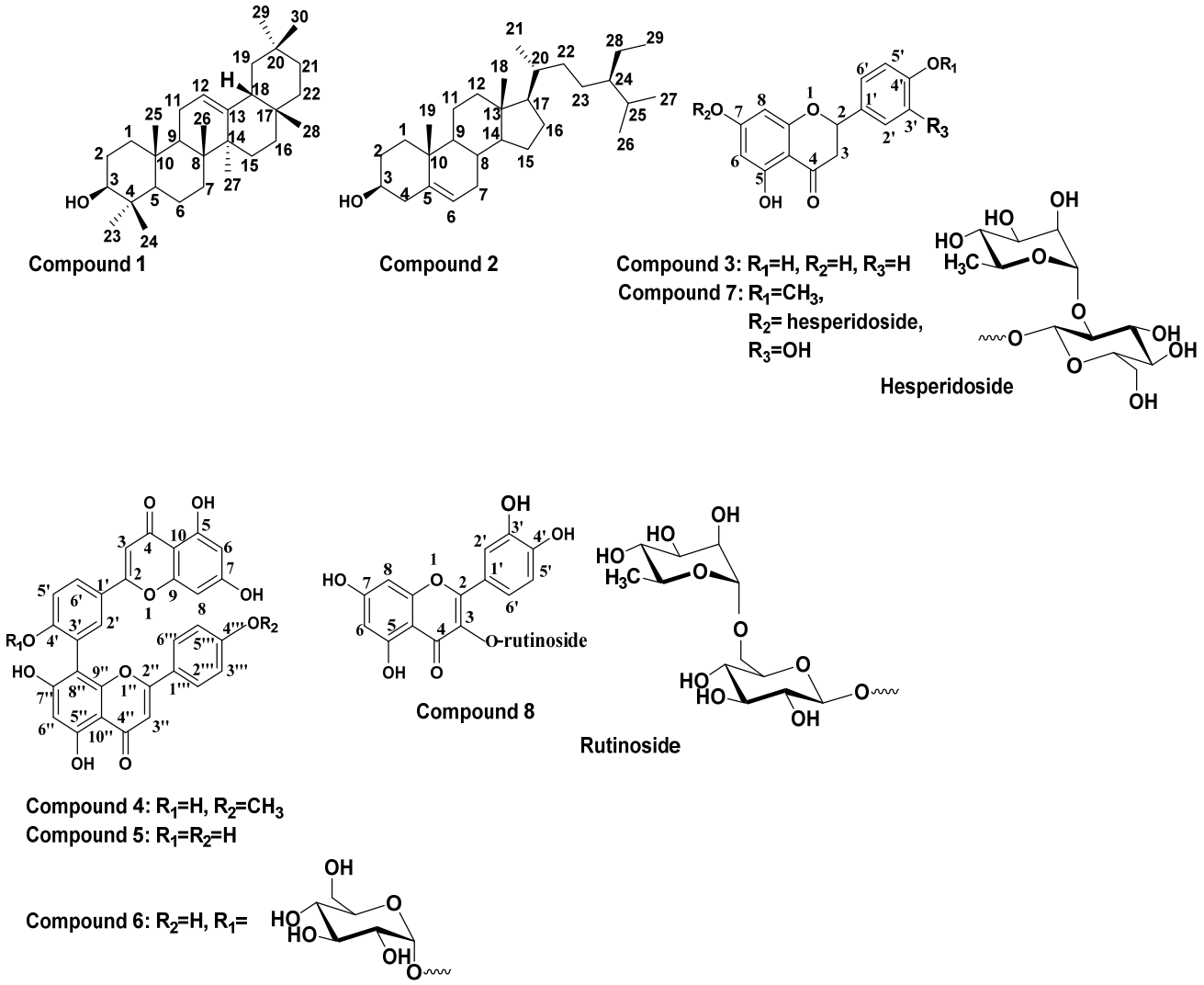

Figure 1: Structures of compounds 1-8.

D-glucopyranoside indicated that the glucosylation has no significant impact on the *ipso*, *ortho* and *para* carbons [24, 33] and thus the glycosylation rules are questionable at this site. ^{13}C NMR data of compound **6** showed that C-4′ was shifted downfield (*ipso* carbon, δ_C 161.0) accompanied by downfield shift of the *ortho* carbons C-3', 5' (δ_C 121.9, 121.8, respectively), and the *para* carbon C-1' (δ_C 128.5) compared to the respective sites of amentoflavone isolated in this work (δ_C 159.6, 121.7, 116.4, and 120.5, respectively) and in literature [26, 29]. However, there were no significant differences in the chemical shift of C-4‴, C-3‴, C-5‴, and C-1‴ resonating at δ_C 161.7, 116.7, 116.17, and 121.9, respectively compared to the respective sites in compound **5** observed at δ_C 161.5, 116.2, 116.2, 121.4, respectively and also to the literature data [29]. Thus, the ^{13}C NMR augments the conclusion to name compound **6** as amentoflavone- 4'- *O*- α- D- glucopyranoside indicating that glycosylation of biflavonoids could behave differently from simple flavonoids (Figure 1). To our knowledge, this is the first report of monoglucosidated amentoflavone from plants elucidated by NMR and MS studies. Enzymatic synthesis of amentoflavone monoglycoside by Chaudhary *et al* was accomplished by using recombinant oleandomycin glycosyltransferase [33]. However, the authors did not determine the site of glycosylation and only used LC-MS/MS for detection of the glycoside.

Compound 7

Compound **7** gave positive Molisch's test. UV spectrum showed λ_{max} at 283 and 340 (sh) nm, which suggested a flavonoid glycoside structure. ^{1}H-NMR spectrum showed the characteristic signals for flavanone structure represented by the signals at δ_H 2.55 (1H, dd, *J*= 16.3, 3.2 Hz) and 3.78 (1H, dd, *J*= 16.3, 12.6 Hz) for C-3 protons and δ_H 5.52 (1H, dd, *J*= 12.6, 3.2) for H-2. Signals for *meta*-coupled protons at δ_H 6.12 and 6.14 (*J*= 3.6 Hz) are consistent with signals due to H-6 and H-8. An ABX system was displayed by signals resonating at δ_H 6.96 (d, *J*= 1.6 Hz) for H-2′, 6.94 (dd, *J*= 1.6, 8.4 Hz) for H-6′ and 6.91 (d, *J*= 8.4 Hz) for H-5′. A signal at δ_H 3.9 integrated for 3 protons is consistent with a methoxy group. Signals for two anomeric protons resonating at δ_H 4.69 (d, *J*= 2.8 Hz) and 4.97 (d, *J*= 7.4 Hz) suggested the presence of α-L-rhamnose and β-D-glucose. The presence of two hydroxyls

at C-3′ and C-5 was evidenced by signals at δ_H 9.12 and 12.01, respectively, which indicated that compound **7** is an *O*-glycoside of a disaccharide at either C-7 or C-4′. The ^{13}C NMR data established the site and the linkage of the disaccharide conjugation. The upfield shift of C-7 by 2.2 ppm and downfield shift of C-6, C-8 and C-10 by 1.7, 1.5 and 1.5 ppm, respectively in comparison with the aglycon data in literature confirmed the site of conjugation at C7 [32, 34]. The inter-linkage of rhamnose and glucose is determined to be (1→2) and not (1→6), which was supported by the downfield shift of C-2 signal of glucose at δ_C 76.7 while C-6 was represented by the signal at δ_C 59.8 which is consistent with hesperidoside disaccharide [30]. ESI^+-MS of compound **7** showed an $[M+H]^+$ peak at *m/z* 611 which is in concordance with the determined strucrure. The NMR and MS data of compound **7** confirmed the structure as neohesperdine which matches those in literature [23, 34-36].

Compound 8

Compound **8** gave positive Molisch's test. UV spectrum showed λ_{max} at 260 and 357 nm which suggested a flavonoid glycoside structure. UV spectra of compound **8** with different shift reagents suggested a flavonol structure with free hydroxyls at positions 5, 7, 3′ and 4′. ^1H-NMR spectrum confirmed the flavonol structure by the absence of the H-3 singlet. Signals for *meta*-coupled protons at δ_H 6.16 and 6.33 (*J*= 2.0 Hz) are consistent with H-6 and H-8 assignment. Signals at δ_H 7.54 (m) and 6.83 (d, *J*= 8.4 Hz) were assigned for H-2′, H-6′ and H-5′, respectively. Signals at δ_H 4.39 (d, *J*= 2.0 Hz) and 5.32 (d, *J*= 7.0 Hz) matches those in literature for α-L-rhamnose and β-D-glucose, respectively [35]. Direct comparison of compound **8** with rutin authentic sample by TLC and superimposable IR alongside the literature data [37, 38] confirmed the structure as rutin.

BIOLOGICAL ACTIVITY

The antioxidant activity of the methanol extract and its fractions (pet. ether, methylene chloride, ethyl acetate and n-butanol) was investigated using DPPH method. Of the tested extracts, methylene chloride and ethyl acetate fractions gave the best results (Table 1). The activity of methylene chloride fraction can be attributed to its content of amentoflavone and naringenin. Amentoflavone is a well known antioxidant and free radical scavenger [39]. Naringenin exhibits substantial free radical scavenging and DNA protection properties as shown by Cavia –Saiz *et al* [40]. The activity of the ethyl acetate fraction was investigated further by testing the activity of the pure compounds isolated from this fraction (amentoflavone glucoside **6** and neohesperidin **7**) alongside amentoflavone **5**. The SC_{50} of amentoflavone glucoside and neohesperidin were found 14.8 and 13.2 µg/ml, respectively which are lower than that of quercetin (18 µg/ml). Amentoflavone exhibited an SC_{50} of 24.2 µg/ml. The results indicated that neohesperidin and amentoflavone glucoside are potent free radical scavengers, three to four times more potent than quercetin at low dose levels (6.25 µg/ml) (Table 2) and even more potent than amentoflavone itself.

Cytotoxic activity of the methanol extract and its fractions (pet. ether, methylene chloride, ethyl acetate and n-butanol) against MCF7 breast cancer cell and HepG2 liver cancer cell line was investigated using SRB assay. Ethyl acetate fraction showed the highest inhibitory activity (69.06%) against MCF7 cell line followed by n-butanol and methylene chloride fractions (66.58% and 65.93%, respectively) (Table 3). n-Butanol fraction was the most active extract against HepG2 cell line (68.4%) followed by pet. ether and methylene chloride (64.12% and 62.15%, respectively). The percentage inhibition of n-butanol fraction against HepG2 cell line can be justified by its rutin content. Cristina *et al* investigated the cytotoxicty and protective activity of rutin against the pro-carcinogen benzo(a)pyrene and their results suggested that rutin is a cytotoxic agent against HTC hepatic cells and has a protective effect against DNA damage [41]. Naringenin and amentoflavone possess anticancer action, [42, 43] which can account for the cytotoxic activity displayed by the methylene chloride fraction in our study. Amentoflavone alongside amentoflavone glucoside and neohesperidin were tested for their cytotoxic activity against MCF7 cell lines to explain the activity of the ethyl acetate fraction. Neohesperidin showed an IC_{50} of 4.73 µg/ml, which is comparable to that of doxorubicin (IC_{50} of 4.13 µg/ml) followed by amentoflavone glucoside (IC_{50} of 6.12 µg/ml), whilst amentoflavone exhibited an IC_{50} of 18.70 µg/ml (Table 4). Our results of antioxidant and cytotoxic activity investigation demonstrated that amentoflavone glucoside and neohesperidin are promising antioxidant and cytotoxic agents which awaits further studies towards understanding the molecular mechanism of their action.

Table 1: Antioxidant activity of different extracts

Sample	% Scavenging activity ±SE				
	Conc. (mg/ml)				
	10	7.5	5	2.5	1.25
Quercetin	100	100	100	97.5±0.03	76.3±0.04
Alcohol extract	64±0.07	60.7±0.03	58.9±0.03	44.8±0.03	38.4±0.03
Pet. ether extract	69.2±0.05	63.5±0.04	59.1±0.05	54±0.02	39.4±0.04
Methylene chloride extract	74.2±0.05	68.5±0.05	66.5±0.04	52.5±0.06	43.3±0.05
Ethyl acetate extract	74.2±0.04	73.3±0.03	70.1±0.04	52.5±0.05	47.1±0.03
n-Butanol	63.4±0.04	56.3±0.03	55.1±0.05	48.8±0.04	46.4±0.05

SE= standard error, n=3

Table 2: Antioxidant activity of compounds 5, 6 and 7.

Compound	% Scavenging activity ±SE					
	Conc. (µg/ml)					
	100	50	25	12.5	6.25	SC_{50}
Quercetin	77.0±0.05	76.1±0.03	65.7±0.04	30.0±0.02	10±0.04	18±0.05
Amentoflavone	60.9±0.03	60.1±0.05	59.3±0.05	33±0.02	19.4±0.05	24.2±0.06
Amentoflavone glucoside	66.2±0.02	63.8±0.03	62.5±0.052	45.3±0.06	43.2±0.02	14.8±0.05
Neohesperidin	64.5±0.03	63.1±0.05	60.9±0.04	45.3±0.02	33.4±0.04	13.2±0.05

SE= standard error, n=3

Table 3: Cytotoxic activity of the tested extracts using single dose (100 µg/ml)

Extract	% Inhibition ±SE	
	MCF7	HepG2
Alcohol	62.16±0.4	58.9±0.08
Pet. ether	63.89±0.22	64.12±0.12
Methylene chloride	65.93±0.18	62.15±0.14
Ethyl acetate	69.06±0.14	59.85±0.18
n-Butanol	66.58±0.14	68.4±0.18

SE: standard error, n=3

Table 4: Cytotoxic activity of compounds 5, 6 and 7 using MCF-7 cell line.

Compound	Surviving fraction±SE				
	Conc. (µg/ml)				
	5	12.5	25	50	IC_{50}
Doxorubicin	0.40±0.031	0.44±0.029	0.37±0.035	0.40±0.031	4.13
Amentoflavone	0.75±0.014	0.58±0.036	0.42±0.016	0.43±0.037	18.70
Amentoflavone glucoside	0.52±0.051	0.32±0.029	0.22±0.018	0.20±0.016	6.12
Neohesperidin	0.49±0.013	0.51±0.021	0.44±0.025	0.41±0.029	4.73

SE: Standard error, n=3.

CONCLUSION

Chemical investigation of *C. revulota* leaflets indicated that flavonoids and biflavonoids are the main principals. A new amentoflavone glucoside was reported for the first time in this paper based on NMR and MS analyses. β amyrin (**1**), neohesperidin (**7**), and rutin (**8**) were reported for the first time from *Cycas* genus. β sitosterol (**2**) was isolated for the first time from *C. revoluta* Thunb.

Biological screening of the isolated compounds showed an interesting activity profile for amentoflavone-4'-*O*-α-D-glucopyranoside and neohespredin as antioxidant and cytotoxic agents which requires further studies for better understanding and possible harnessing in medicine.

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