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Phytochemical Investigation of *Cycas circinalis* and *Cycas revoluta* Leaflets: Moderately Active Antibacterial Biflavonoids

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Abstract

Chemical examination of the methanolic extract of the leaflets of *Cycas circinalis* L. led to the isolation of one new biflavonoid, (2*S*, 2″*S*)-2,3,2″,3″-tetrahydro-4′,4‴-di-*O*-methylamentoflavone (tetrahydroisoginkgetin; **2**), and 15 known compounds, 11 of which are reported for the first time from *C. circinalis*. Chromatographic separation of the chloroform extract of *C. revoluta* Thunb. leaflets afforded 12 compounds, seven of which are reported for the first time from this species. The isolated compounds from both species include 14 biflavonoids, three lignans, three flavan-3-ols, two flavone-*C*-glucosides, two *nor*-isoprenoids, and one flavanone. This is the first report of NMR and CD data of 2,3,2″,3″-tetrahydro-4′-*O*-methyl- and 2,3-dihydro-4′-*O*-methyl-amentoflavone (**6**) and (**7**). The effect of *O*-methylation on the chemical shifts of the neighboring carbons in the ^{13}C NMR spectra of the dihydro- and tetrahydro-amentoflavone skeletons provides a tool to identify the location of the methoxy groups. Compounds **2**, **6**, and **18** displayed moderate antibacterial activity against *Staphylococcus aureus* (IC_{50} values of 3.8, 9.6, and 8.2 μM, respectively) and methicillin-resistant *S. aureus* (MRSA; IC_{50} values of 5.9, 12.5, and 11.5 μM, respectively).

Keywords

Cycadaceae; *Cycas circinalis*; *Cycas revolute*; biflavonoid; lignin; flavan-3-ol; *nor*-isoprenoid; flavone-*C*-glucoside; antibacterial activity

Introduction

Cycas is the only currently known genus of the family Cycadaceae, order Cycadales. *Cycas revoluta* Thunb is the most widespread species of the genus *Cycas* and is known as sago Cycas or king sago palm [1], while *C. circinalis* L is known as queen sago palm. This genus is native to eastern and southeastern Asia and is cultivated in many tropical and subtropical areas for ornamental purposes [2]. The Chinese utilize the seeds of *C. revoluta* as an antirheumatic, expectorant, and tonic. The terminal shoots are utilized as an astringent

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diuretic [3]. The very young leaves are edible and the juice of tender leaves is useful for the treatment of flatulence and vomiting [4]. It was also reported that a tincture of *C. revoluta* leaves contains inhibitors of cytochrome P-450 aromatase and thus may be efficacious in treating estrogen-dependent carcinoma [5]. Most of the research on the title plants focused on the seeds which produce neurotoxic metabolites [6–8]. Little information could be traced regarding the isolation of secondary metabolites from the leaves. In a previous chemical investigation of the leaves, a series of biflavonoids including amentoflavone (**11**), hinokiflavone (**25**), their dihydro derivatives (**18**, **8**), podocarpusflavone A (**19**), isoginkgetin (**9**), and bilobetin (**10**) were identified [9]. Some phenolic acids were detected by TLC [10]. In this paper, we report the isolation of a new biflavonoid, (2*S*,2″*S*)-2,3,2″,3″-tetrahydro-4′,4‴-di-*O*-methylamentoflavone (2,3,2″,3″-tetrahydroisoginkgetin) (**2**), together with 24 known compounds belonging to the biflavonoid (**4, 6 – 11, 17 – 19, 21, 22, 25**), lignan (**5, 20, 23**), flavan-3-ol (**12 – 14**), flavone-*C*-glucoside (**15, 16**), *nor*-isoprenoid (**1, 24**), and flavanone (**3**) classes of plant secondary metabolites. We also report the antimicrobial activity of the three biflavonoids **2**, **6,** and **18**. Compounds **6** and **7** were reported as new compounds from *Selaginella uncinata* [11], but ours is the first report of their NMR, CD, and antimicrobial data.

Materials and Methods

General experimental procedures

NMR spectra were recorded on a Bruker DRX NMR spectrometer operating at 400 MHz for ^{1}H and 100 MHz for ^{13}C with a 3 mm direct carbon probe. NMR samples were dissolved in acetone-d_6 (biflavonoids), DMSO-d_6 (flavone glycosides), methanol-d_4 (flavan-3-ols), and $CDCl_3$ (lignans and *nor*-isoprenoids). Chemical shifts were standardized to the solvent resonances ($CDCl_3$ 7.24 ppm, CD_3OD 4.78, 3.31ppm, acetone-d_6 2.05 ppm, and DMSO-d_6 2.5ppm). UV spectra were recorded on a Varian Cary 50 Bio UV-Visible spectrometer and CD on Olis DSM 20 instrument. IR spectra were measured in $CHCl_3$ on an ATI Mattson Genesis series FT-IR spectrophotometer, whereas optical rotations were acquired with a 589–546 Rudolph Research Analytical Autopol IV automatic polarimeter. Accurate mass measurements were carried out on an Agilent HPLC 1100 series instrument equipped with a diode array detector and a mass detector in series (Agilent Technologies). The time-of-flight mass detector (model G1969A) was equipped with an electrospray ionization interface and controlled by Aligent software (Aligent Mass Hunter Work Station, A.02.01). HPLC was done using a Delta Prep 4000 (Waters Corporation) equipped with a dual wavelength detector Model 2487 adjusted at 210 and 330 nm.

Materials for CC were silica gel (32–63 μm; Dynamic Adsorbents Inc.), Sephadex LH-20 (40 – 70 μm; GE Healthcare Bio-Science AB), polyamide resin, and C-18 silica gel (40 – 63 μm; Sorbent Technology Co.). The preparative HPLC column was from Phenomenex Luna C18 (2) (100 A 250 × 15.00 mm, 5 μ).

Plant material

C. circinalis and *C. revoluta* leaflet*s* were collected in May–August 2007 in the National Research Center in Giza, Egypt and identified by Mrs T. Labib, head specialist for plant identification, El-Orman Public Garden, Cairo, Egypt. They were also identified by the herbarium of the Faculty of Sciences, Cairo University.

Extraction and isolation

The shade-dried and powdered leaflets of *C. circinalis* (700 g) and *C. revoluta* (1500 g) were separately extracted with 80% MeOH by percolation. The solvent was evaporated under

reduced pressure at 40°C to yield 100 and 170 g of crude extracts, respectively. The aqueous MeOH extracts were suspended in H_2O and partitioned with petroleum ether, $CHCl_3$, EtOAc, and *n*-BuOH saturated with H_2O. The *C. circinalis* $CHCl_3$ extract (A, 3 g) was subjected to CC on silica gel (90 g, 30 × 3.8 cm) and eluted successively with a gradient of *n*-hexane - $CHCl_3$ then $CHCl_3$ - MeOH mixtures of increasing polarities. The *n*-hexane - $CHCl_3$ (20:80) eluate (A1, 900 mg) was rechromatographed on silica gel (30g, 15 × 3.5 cm), eluted with $CHCl_3$ – MeOH, then purified on RP-HPLC using H_2O + 0.05% formic acid (A) and MeOH + 0.05% formic acid (B) in a gradient mode: A/B 60/40 - 50/50; 10 min, 50/50 - 25/75; 5 min, 25/75 - 0/100; 10 min, 0:100; 10 min with a flow rate of 10 mL/min to give **1** (t_R = 10.43 min, 6.5 mg), **2** (t_R = 23.35 min, 15 mg), **3** (t_R = 20.01 min, 14 mg), and **4** (t_R = 23.52 min, 12 mg). The $CHCl_3$- MeOH (90:10) eluate (A2, 0.8 g) was purified on RP-HPLC using the same method to give **5** (t_R = 12.99 min, 5mg), **6** (t_R = 20.83 min, 35 mg), **7** (t_R = 21.90 min, 18 mg), **8** (t_R = 22.80 min, 60 mg), and **9** (t_R = 24.50 min, 28 mg). The *C. circinalis* EtOAc extract (B, 2 g) was subjected to CC on silica gel (60g, 30 × 3cm) eluted successively with gradient $CHCl_3$ - EtOAc mixtures and rechromatographed over silica gel with *n*-hexane - EtOAc and Sephadex LH-20 with MeOH to afford **10** (4 mg), **11**(30 mg), **12** (15 mg), **13** (6 mg), and **14** (40 mg). The *C. circinalis n*-BuOH extract (C, 8 g) was fractionated on polyamide with H_2O - MeOH mixtures and then on a C-18 silica gel column and RP-HPLC to obtain **15** (6.5 mg) and **16** (20 mg). The *C. revoluta* $CHCl_3$ extract (D, 8 g) was subjected to CC on silica gel (150 g, 60 × 3cm) and eluted successively with gradient *n*-hexane - EtOAc in 5% increments to afford four subfractions. The *n*-hexane-EtOAc (50:50, D1, 200 mg) eluate was rechromatographed on silica gel with gradient $CHCl_3$ - MeOH and then purified on Sephadex LH-20 with MeOH to give **3** (45 mg). The *n*-hexane - EtOAc (40:60 - 30:70, D2, 630 mg) eluate was rechromatographed on silica gel with gradient $CHCl_3$ – MeOH, then purified on Sephadex LH-20 with MeOH to afford **17** (16 mg) and **18** (30 mg). The *n*-hexane - EtOAc (25:75, D3, 500 mg) eluate was rechromatographed on silica gel with gradient $CHCl_3$ - MeOH, then RP-HPLC using H_2O + 0.05% formic acid (A) and MeOH + 0.05% formic acid (B) in a gradient mode: A/B 80/20 - 60/40; 15 min, 60/40 - 20/80; 25 min with a flow rate of 10 mL/min to afford **18** (t_R = 23.52 min, 8 mg), **19** (t_R = 27.28 min, 6 mg), and **8** (t_R = 28.49 min, 24 mg). The *n*-hexane - EtOAc (20:80 - 0:100, D4, 800 mg) eluate was rechromatographed on silica gel with gradient CH_2Cl_2 - Me_2CO in 5% increments to afford three subfractions. The CH_2Cl_2 - Me_2CO (65:35, D4.1, 150 mg) eluate was repurified on silica gel with $CHCl_3$ - MeOH, then RP-HPLC to give **20** (t_R = 12.71 min, 13 mg), **21**(t_R = 32.09 min, 3 mg), and **22** (t_R = 30.22 min, 2 mg). The CH_2Cl_2 - Me_2CO (55:45, D4.2, 350 mg) eluate was repurified on silica gel with $CHCl_3$ - MeOH, Sephadex LH-20 with MeOH, and RP-HPLC to afford **23** (t_R = 13.52 min, 6 mg) and **24** (t_R = 7.34 min, 12 mg). The CH_2Cl_2 - Me_2CO (35:65, D4.3, 100 mg) eluate was filtered through Sephadex LH-20 with MeOH, then purified with RP-HPLC to give **11**(t_R = 22.07 min, 60 mg) and **25** (t_R = 27.96 min, 5 mg).

Antimicrobial assay

All organisms were obtained from the American Type Culture Collection (ATCC) and included the fungi *Candida albicans* ATCC 90028, *C. glabrata* ATCC 90030, *C. krusei* ATCC 6258, *Cryptococcus neoformans* ATCC 90113, and *Aspergillus fumigatus* ATCC 204305, as well as the bacteria *Staphylococcus aureus* ATCC 29213, methicillin-resistant *S. aureus* ATCC 33591 (MRSA), *Escherichia coli* ATCC 35218, *Pseudomonas aeruginosa* ATCC 27853, and *Mycobacterium intracellulare* ATCC 23068. Susceptibility testing was performed using a modified version of the Clinical and Laboratory Standards Institute (CLSI; formerly National Committee for Clinical Laboratory Standards - NCCLS) methods [12–15]. *M. intracellulare* was tested using a modified method of Franzblau et al. [16]. Samples were serially-diluted in 20% DMSO/saline and transferred in duplicate to 96 well flat bottom microplates. Microbial inocula were prepared by correcting the OD_{630} of

microbe suspensions in incubation broth to afford final target inocula. Drug controls [ciprofloxacin (ICN Biomedicals) for bacteria and amphotericin B (ICN Biomedicals) for fungi] were included in each assay. All organisms were read at either 530 nm using the Biotek Powerwave XS plate reader (Bio-Tek Instruments) or 544ex/590em (*M. intracellulare, A. fumigatus*) using the Polarstar Galaxy Plate Reader (BMG LabTechnologies) prior to and after incubation. Minimum fungicidal or bactericidal concentrations were determined by removing 5 μL from each clear well, transferring to agar, and incubating. The Minimum Fungicidal Concentration/Minimum Bactericidal Concentration (MFC/MBC) is defined as the lowest test concentration that kills the organism and allows no growth on agar.

Characterization

(2*S*,2″*S*)-2,3,2″,3″-tetrahydro-4′,4‴-di-*O*-methylamentoflavone [(2*S*,2″*S*)-2,3,2″,3″-tetrahydroisoginkgetin] (**2**): yellowish white powder; $[\alpha]^{25}_{D}$ −28.0 (MeOH, c 0.20), UV (MeOH) λ_{max} = 290 and 330 nm; CD (MeOH) $[\theta]_{293.2}$ −6.79 × 10, $[\theta]_{330}$ +1.49 × 10; IR (KBr) ν_{max} 3326, 2926, 2853, 1713,1638, 1515, 1462, 1341, 1307, 1253, 1181, 1159, 1087, 1028 cm^{-1}; HR-MSD-TOF (ES negative-ion mode): *m/z* 569.1590 $[M-H]^-$ and 1139.3056 $[2M-H]^-$ (calculated for *m/z* 569.1447 $[M(C_{32}H_{26}O_{10})-H]^-$ and 1139.2973 $[2M(C_{64}H_{52}O_{20})-H]^-$, respectively; ^1H and ^{13}C NMR data: see Tables 1 and 2.

(2*S*,2″*S*)-2,3,2″,3″-tetrahydro-4′-*O*-methylamentoflavone [(2*S*,2″*S*)-2,3,2″,3″-tetrahydrobilobetin] (**6**): yellowish white powder; $[\alpha]^{25}_{D}$ −2.0 (MeOH, c 0.15); UV (MeOH) λ_{max} = 291 and 332 nm; CD (MeOH) $[\theta]_{291.5}$ - 5.46 × 10, $[\theta]_{330}$ + 1.1 × 10; HR-MSD-TOF (ES negative-ion mode): *m/z* 555.1467 $[M-H]^-$ and 1111.2777 $[2M-H]^-$ (calculated for *m/z* 555.1291 $[M(C_{31}H_{24}O_{10})-H]^-$ and 1111.2660 $[2M(C_{62}H_{48}O_{20})-H]^-$, respectively). ^1H and ^{13}C NMR data: see Tables 1 and 2.

(2*S*)-2,3-dihydro-4′-*O*-methylamentoflavone [(2*S*)-2,3-dihydrobilobetin] (**7**): yellow powder; $[\alpha]^{25}_{D}$ +11.2 (MeOH, c 0.29); UV (MeOH) λ_{max} = 289 and 330 nm; CD (MeOH) $[\theta]_{293.2}$ - 3.17 × 10, $[\theta]_{331}$ + 8.07; HR-MSD-TOF (ES negative-ion mode): *m/z* 553.1273 $[M-H]^-$ and *m/z* 1107.2427 $[2M-H]^-$ (calculated for *m/z* 553.1134 $[M(C_{31}H_{22}O_{10})-H]^-$ and 1107.2347 $[2M(C_{62}H_{44}O_{20})-H]^-$, respectively). ^1H and ^{13}C NMR data: see Tables 1 and 2.

Supporting Information

^1H NMR, ^{13}C NMR, and HR-MS spectra of compounds **2**, **6**, and **7** are available as Supporting Information.

Results and Discussion

The $CHCl_3$ soluble fraction of the MeOH extract of the leaflets of *C. circinalis* was subjected to various chromatography steps to afford (−)-loliolide (**1**) [17, 18], the new (2*S*,2″*S*)-2,3,2″,3″-tetrahydroisoginkgetin) (**2**), (2*S*)-naringenin (**3**) [19], (2*S*,2″*S*)-2,3-dihydro-4′,4‴-di-*O*-methylamentoflavone (**4**) [20], (+)-(7*S*,8*R*)-dihydrodehydrodiconiferyl alcohol (3′-methylcedrusin) (**5**) [21], (2*S*,2″*S*)-2,3,2″,3″-tetrahydro-4′-*O*-methylamentoflavone (**6**), (2*S*)-2,3-dihydro-4′-*O*-methylamentoflavone (**7**), (2*S*)-2,3-dihydrohinokiflavone (**8**) [22], and 4′,4‴-di-*O*-methylamentoflavone (isoginkgetin) (**9**) [22]. The EtOAc soluble fraction was subjected to column chromatography sequentially over silica gel and Sephadex LH-20 to obtain 4′-O-methylamentoflavone (bilobetin; **10**) [22, 23], amentoflavone (**11**) [22], epicatechin (**12**) [24], epigallocatechin (**13**) [25, 26], and gallocatechin (**14**) [26]. Fractionation of the *n*-BuOH soluble fraction on polyamide-6, RP-18, and RP-HPLC afforded vicenin-2 (violanthin) (**15**) [27], and 2″-glucosylvitexin (**16**) [28, 29]. The $CHCl_3$ soluble fraction of the methanolic extract of *C. revoluta* leaflets was

subjected to column chromatography over silica gel followed by Sephadex LH-20 and RP-HPLC to afford (2*S*)-naringenin (**3**), (2*S*)-2,3-dihydrohinokiflavone (**8),** amentoflavone (**11**), (2*S*,2″*S*)-2,3,2″,3″-tetrahydroamentoflavone (**17**) [30], (2*S*)-2,3-dihydroamentoflavone (**18**) [22], podocarpusflavone A (**19**) [22, 31], (+)-lariciresinol (**20**) [32], (2*S*)-2,3-dihydroisocryptomerin (**21**) [33], (2*S*,2″*S*)-2,3,2″,3″-tetrahydrohinokiflavone (**22**) [34], (+)-isolaricerisinol (**23**) [35], (6*S*,7*E*,9*S)*-6,9-dihydroxy-4,7-megastigmadien-3-one (vomifoliol) (**24**) [36], and hinokiflavone (**25**) [22]. The structures of these compounds were established by analysis of their physical and spectroscopic data in comparison with reported data.

Compound 2

The MSD-TOF spectrum of compound **2** displayed ions at *m/z* 569.1590 $[M-H]^-$ and *m/z* 1139.3056 $[2M-H]^-$ which were consistent with a molecular formula $C_{32}H_{26}O_{10.}$ The IR spectrum of **2** indicated a broad OH absorption band at 3326 cm^{-1} and C=O at 1638 cm^{-1}, while the UV spectrum showed absorption maxima at 290 and 330 nm, characteristic of a flavanone derivative [37]. The ^1H and ^{13}C NMR spectra of compound **2** at 294° K showed a pair of rotamers (1:1) due to the restricted rotation about the interflavanyl bond. At this temperature the signals were duplicated hence rendering the assignment of resonances to the individual rotamers not feasible. The biflavanone structure of **2** was evident from the presence of two duplicated benzylic oxymethine carbon signals in the ^{13}C NMR spectrum at δ_C 78.58, 78.45 (C-2), and 79.07 (C-2″), two duplicated methylene carbon signals at δ_C 42.45, 41.87 (C-3) and 43.01, 42.79 (C-3″), as well as two carbonyl carbon signals at δ_C 196.28 (C-4) and 196.71, 196.73 (C-4″). The ^1H NMR spectrum further supported the biflavanone structure of **2** via two sets of ABX spin patterns of rings C and F at δ_H 2.60–2.91 (m, H-3$_{eq}$, H-3″$_{eq}$), 3.07–3.23 (m, H-3$_{ax}$, H-3″$_{ax}$) and 5.41–5.58 (m, H-2, H-2″), and two D_2O-exchangeable hydrogen-bonded hydroxy groups at δ_H 12.21(1H, s, OH-5) and 12.30 (1H, s, OH-5″). The ^1H NMR spectrum also showed three signals corresponding to two methoxy groups at δ_H 3.77, 3.79, and 3.82, signals corresponding to H-6 and H-8 of ring A at δ_H 5.96, 5.98, 6.01, and H-6″ of ring D at δ_H 6.13 and 6.12. The AA′ BB′ spin pattern of ring E resonated at δ_H 6.90 (t, *J* = 8.7 Hz, H-3‴, H-5‴) and 7.37 (d, *J* = 8.7 Hz, H-2‴, H-6‴). The ABM spin pattern of ring B resonated at δ_H 7.04 (dd, *J* = 8.7, 4.9 Hz, H-5′) and 7.47 – 7.40 (m, H-2′, H-6′). The assignment of these protons was confirmed by Heteronuclear Multiple Quantum Correlation (HMQC) and Heteronuclear Multiple Bond Correlation (HMBC) experiments. The HMBC spectrum of **2** confirmed the involvement of C-3′ (B-ring) and C-8″ (D-ring) in the interflavanyl linkage via the $^3J_{CH}$ correlations of H-5′ (δ_H 7.04) with C-3′ (δ_C 121.75) and the $^3J_{CH}$ correlations of H-6″ (δ_H 6.13 and 6.12) and H-2′ (δ_H 7.40–7.47) with C-8″ (δ_C 105.83 and 105.95) thus indicating compound **2** as a member of the amentoflavone class of biflavonoids. The location of the methoxy groups was confirmed by the $^3J_{CH}$ correlations between 4′-OCH_3 (δ_H 3.82) and 4‴-OCH_3 (δ_H 3.79) with C-4′ (δ_C 157.98, 158.36) and C-4‴ (δ_C 159.76, 159.87), respectively. It was also confirmed by the Nuclear Overhauser Effect (NOE) between the 4‴-OCH_3 (δ_H 3.79) and H-3‴ and H-5‴ (δ_H 6.90), as well as between 4′-OCH_3 (δ_H 3.82) and H-5′ (δ_H 7.04). The CD spectrum of **2** showed high amplitude negative and low amplitude positive Cotton effects for the $\pi \rightarrow \pi^*$ and n → π^* transitions at 293 and 330 nm, respectively, which permitted the assignment of (2*S*,2″*S*) absolute configuration [38, 39]. Based on this data, compound **2** was unambiguously assigned as (2*S*,2″*S*)-2,3,2″,3″-tetrahydro-4′,4‴-di-*O*-methylamentoflavone [(2*S*,2″*S*)-2,3,2″,3″-tetrahydroisoginkgetin].

Compound 6

The MSD-TOF spectrum of compound **6** displayed ions at *m/z* 555.1467 $[M-H]^-$ and *m/z* 1111.2777 $[2M-H]^-$ which were consistent with a molecular formula $C_{31}H_{24}O_{10}$. The compound showed similar structural features to compound **2** except for possessing only one

methoxy group. The location of the methoxy group was defined at C-4′ since the methoxy protons at δ_H 3.97 showed NOE correlations to H-5′ (δ_H 7.04). The presence of rotational isomers was also evident in both the ^{1}H and ^{13}C NMR spectra. The CD spectrum showed high amplitude negative and low amplitude positive Cotton effects for the $\pi \rightarrow \pi^*$ and n → π^* transitions at 291 and 330 nm, respectively, which permitted the assignment of (2*S*,2″*S*) absolute configuration. Compound **6** was thus identified as (2*S*,2″*S*)-2,3,2″,3″-tetrahydro-4′-*O*-methylamentoflavone [(2*S*,2″*S*)-2,3,2″,3″-tetrahydrobilobetin].

Compound 7

The molecular formula $C_{31}H_{22}O_{10}$ was established via MSD-TOF as it showed ions at *m/z* 553.1273 $[M-H]^-$ and *m/z* 1107.2427 $[2M-H]^-$. The compound showed similar structural features to compound **18** except for possessing an additional methoxy group which was located at C-4′ from HMBC and NOE data. The CD spectrum showed high amplitude negative and low amplitude positive Cotton effects for the $\pi \rightarrow \pi^*$ and n → π^* transitions at 293 and 331 nm, respectively, which permitted the assignment of an (2*S*) absolute configuration. Compound **7** was thus identified as (2*S*)-2,3-dihydro-4′-*O*-methylamentoflavone ((2*S*)-2,3-dihydrobilobetin). The *O*-methylation induced shifts in the carbon resonances in the ^{13}C NMR spectra of the dihydro- and tetrahydro-amentoflavone skeletons (Fig. 3, Table 3) are similar to those previously reported for amentoflavone [22]. In addition, 4′-*O* –methylation induced a shift of + (1.5 – 2.0) ppm for C-3′. The absolute configurations reported for compounds **1** and **24** [40, 41] were confirmed by X-ray crystallography.

The isolated biflavonoids were tested for antimicrobial activity. None showed antifungal, antimalarial, or antileishmanial activity. Compounds **2**, **6**, and **18** displayed moderate antibacterial activity against *Staphylococcus aureus* (IC_{50} values of 3.8, 9.6, and 8.2 μM, respectively) and methicillin-resistant *S. aureus* (MRSA; IC_{50} values of 5.9, 12.5, and 11.5 μM, respectively). All three compounds possess the amentoflavone-type skeleton. Compound **2** is approximately 11 times less potent than ciprofloxacin against *S. aureus* and 21 times less potent than ciprofloxacin against MRSA.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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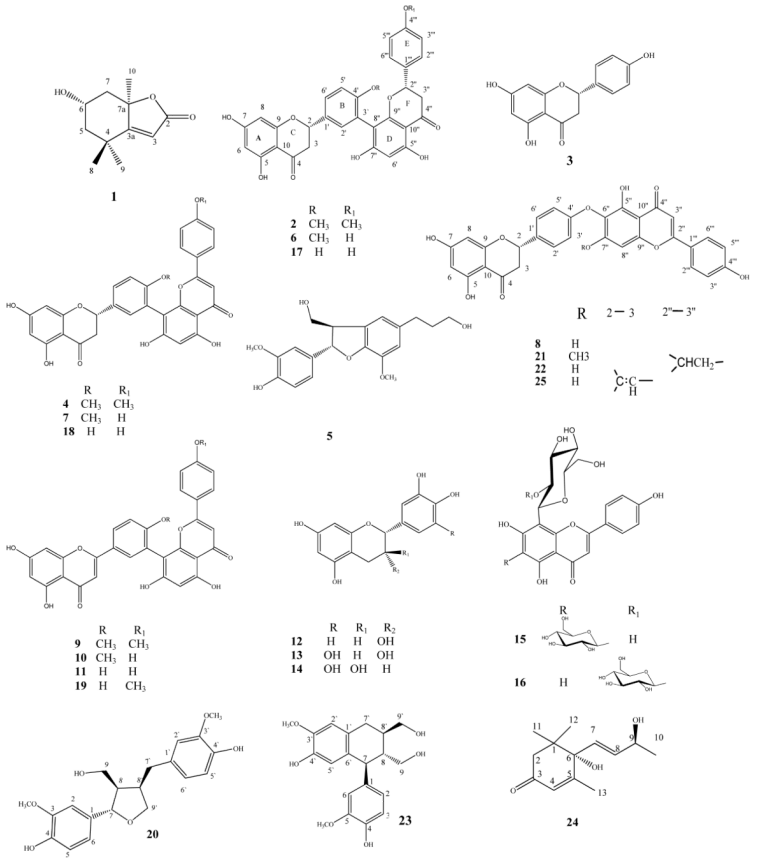

Fig. 1.
Chemical structures of compounds from *C. circinalis* and *C. revoluta* leaflets.

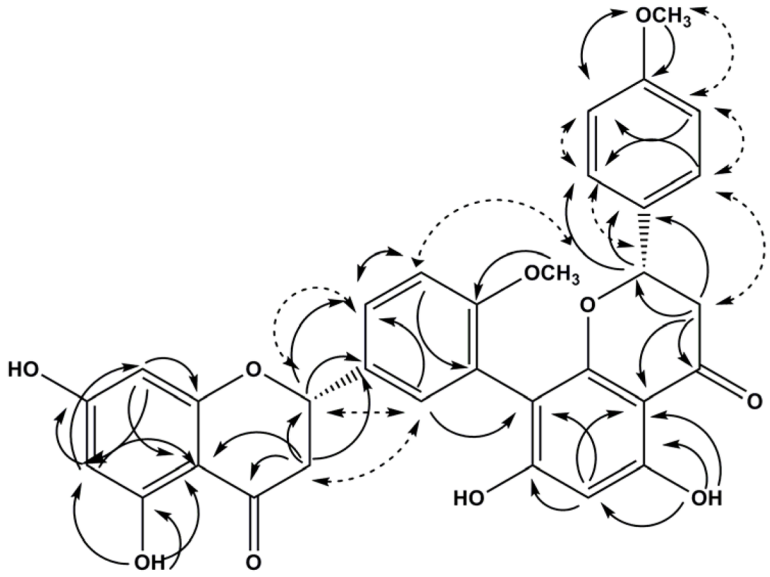

Fig. 2.
Important HMBC (single-head arrow) and NOESY (double-head dashed arrow) correlations of compound **2.**

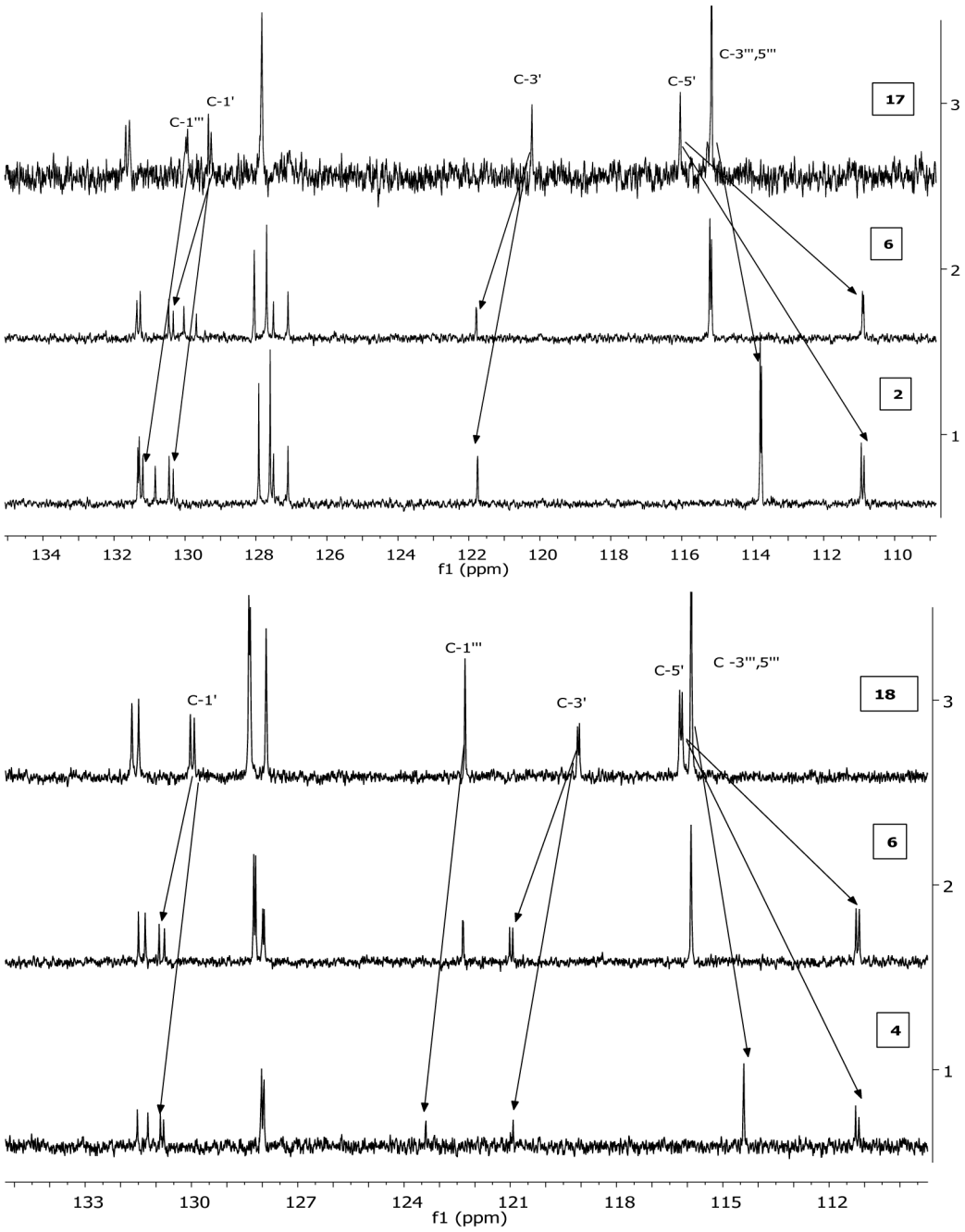

Fig. 3.

Comparison between the ^{13}C NMR spectra (110–135 ppm) of some of the isolated biflavonoids.

Table 1

The ^{13}C NMR (100 MHz) data of compounds **2**, **6**, and **7** (Me_2CO-d_6)

No.	2 δ^*_C	6 δ_C	7 δ_C
2	78.57, 78.44[a]	78.79, 78.53	79.08, 79.00
3	42.46, 41.87	42.53, 41.79 [a]	43.06, 42.70
4	196.2	196.2	196.23, 196.20
5	164.44, 164.42	164.4	164.4
6	95.9	95.79, 95.96	96.1
7	166.65, 166.67	166.8	166.5
8	95.07	95.04, 95.07	95.1
9	163.5	163.5 [b]	163.5[a]
10	102.25, 102.29	102.22,102.25 [c]	102.3
1′	130.33, 130,44	130.45, 130.33	130.91, 130.76
2′	131.28, 131.32	131.26, 131.35	131.50, 131.31
3′	121.8	121.8	121.02, 120.93
4′	158.36, 157.98	158.0, 158.3	158.30, 158.26
5′	110.86, 110.94	110.90, 110.90	111.23, 111.15
6′	127.09, 127,50	127.50, 127.09	127.99, 127.95
2″	79.1[a]	79.1	164.01, 163.98
3″	42.78, 43.01	43.03, 42.72 [a]	102.68, 102.63
4″	196.7	196.9	182.6
5″	163.7	163.4 [b]	161.5
6″	96.1	96.02,96.06	98.8
7″	164.09, 164.17	164.01, 164.11	161.4[a]
8″	105.82, 105.95	105.82, 105.95	104.7
9″	160.3	160.4	154.8
10″	102.51, 102.54	102.52, 102.57 [c]	104.5
1‴	130.83, 131.19	130.03, 129.67	122.3
2‴	127.92, 127.60	127.70, 128.04	128.24, 128.19
3‴	113.78, 113.75	115.20, 115.15	115.9
4‴	159.76, 159.77	157.53, 157.64	161.0
5‴	113.78, 113.75	115.20, 115.15	115.9
6‴	127.92, 127.60	127.70, 128.04	128.24, 128.19
4′-O-CH_3	54.65, 54.63	55.11, 55.03	55.2
4‴-OCH_3	55.12, 55.05	----------	----------

*Chemical shift values in ppm.

Values having similar superscripts in the same column may be interchanged.

Table 2

The ^{1}H NMR (400 MHz) data of compounds **2**, **6**, and **7** (Me_2CO-d_6)[a,b]

No.	2* δ_H	6* δ_H	7 δ_H
2	5.58 – 5.41 m	5.57 – 5.41 m	5.60 dt (13.0, 2.8)
3	3.23 – 3.07 m, H-3*ax* 2.91 – 2.60 m, H-3*eq*	3.24 – 3.06 m, H-3*ax* 2.89 – 2.62 m, H-3*eq*	3.38 – 3.21 m, H-3*ax* 2.90 – 2.73 m, H-3*eq*
6	5.96–6.01 m	5.99 – 5.92 m	5.96 s
8	5.96–6.01 m	6.01 d (2.1)	5.99 s
2′	7.47 – 7.40 m	7.49 – 7.40 m	7.71 – 7.62 m
5′	7.04 dd (8.7, 4.9)	7.04 dd (8.8, 4.2)	7.24 d (9.1)
6′	7.47 – 7.40 m	7.49 – 7.40 m	7.71 – 7.62 m
2″	5.58 – 5.41 m	5.57 – 5.41 m	----------
3″	3.23 – 3.07 m, H-3*ax* 2.91 – 2.60 m, H-3*eq*	3.24 – 3.06 m, H-3*ax* 2.89 – 2.62 m, H-3*eq*	6.65 s
6″	6.12 d (4.6)	6.12 d (4.9)	6.43 s
2‴	7.37 d (8.7)	7.28 dd (8.5, 3.2)	7.60 dd (8.0, 5.2)
3‴	6.90 t (8.7)	6.87 – 6.75 m	6.97 – 6.85 m
5‴	6.90 t (8.7)	6.87 – 6.75 m	6.97 – 6.85 m
6‴	7.37 d (8.7)	7.28 dd (8.5, 3.2)	7.60 dd (8.0, 5.2)
5-OH	12.21 s	12.21 s	12.18 s
5″-OH	12.30 s	12.31 s	13.12 s
4′-OCH3	3.82 s, 3.77 s	3.79 d (13.2)	3.79 s
4‴–OCH3	3.79 s, 3.77 s	----------	----------

[a] Chemical shift values in ppm and J values (in Hz) are presented in parentheses.

[b] The assignments are based on DEPT, HMQC, HMBC and NOE experiments.

* Multiplicity is complicated because of rotational isomerism.

Table 3

Shifts in the ^{13}C NMR data observed in compounds **2**, **4**, **6**, and **7** compared to the phenolic derivatives **17** and **18**

Compound	^{13}C NMR Shifts Observed (ppm)					Indication
	C-5′	C-1′	C-3‴	C-3‴, 5‴	C-1‴	
2	−5	+1	+1.6	−1.4	+1.2	4′- and 4‴-*O*-methylation
4	−5	+0.8	+1.9	−1.5	+1	4′- and 4‴-*O*-methylation
6	−4.8	+1	+1.6			4′-*O*-methylation
7	−5	+0.8	+1.9			4′-*O*-methylation

Table 4

Antibacterial activity displayed by isolated biflavonoids

Compound	*Staphylococcus aureus*			MRSA		
	IC_{50}	MIC	MBC	IC_{50}	MIC	MBC
2	3.9	17.5	NA	5.9	NA	NA
6	9.7	35.9	NA	12.5	35.9	NA
18	8.2	37.0	NA	11.5	37.0	NA
Ciprofloxacin	0.3	0.8	3.0	0.3	0.8	3.0

IC_{50} = the test concentration (μM) that afford 50% inhibition relative to controls.

MIC (minimum inhibitory concentration) is the lowest test concentration (μM) that allows no detectable growth.

MBC (minimum bactericidal concentration) is the lowest test concentration (μM) that kills the organism.

NA = not active at the highest test concentration of 20μg/mL (35.0, 35.9, and 37.0 μM, respectively).