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Antibacterial and cytotoxic biflavonoids from the root bark of *Ochna kirkii*

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ABSTRACT

The new isoflavonoid kirkinone A (**1**) and biflavonoid kirkinone B (**2**) along with six known compounds (**3**–**8**) were isolated from the methanolic extract of the root bark of *Ochna kirkii*. The compounds were identified by NMR spectroscopic and mass spectrometric analyses. Out of the eight isolated natural products, calodenin B (**4**) and lophirone A (**6**) showed significant antibacterial activity against the Gram-positive bacterium *Bacillus subtilis* with MIC values of 2.2 and 28 μM, and cytotoxicity against the MCF-7 human breast cancer cell line with EC_{50} values of 219.3 and 19.2 μM, respectively. The methanolic crude extract of the root bark exhibited cytotoxicity at EC_{50} 8.4 μg/mL. The isolated secondary metabolites and the crude extract were generally inactive against the Gram-negative *Escherichia coli* (MIC ≥400 μg/mL). Isolation of biflavonoids and related secondary metabolites from *O. kirkii* demonstrates their chemotaxonomic significance to the genus *Ochna* and to other members of the family Ochnaceae.

1. Introduction

The genus *Ochna* belongs to the family Ochnaceae, which consists of 86 species of ornamental shrubs occurring widely in Africa, Asia and Madagascar [1,2]. Several species in this genus are used in folk medicine to treat various ailments, such as lumbago, malaria, ulcer, epilepsy, dysentery, asthma, snake bite, menstrual pain or as abortifacient agents [3,4]. Phytochemical studies of *Ochna* species indicated the genus to produce a diverse collection of bi-, tri-, and pentaflavonoids, anthranoids, steroids and fatty acids [5,7]. The crude extracts and the isolated constituents of this genus have been reported to exhibit potent antiinflammatory, antimalarial, antibacterial, analgesic and cytotoxic activities [4].

Ochna kirkii Oliv., commonly named Mickey Mouse Plant or Bird's Eye Bush, is native to tropical Africa and occurs mainly in eastern Tanzania and Kenya, and in southern Mozambique [3,7,8]. It grows as a shrub or small tree of height up to 6 m tall, producing colorful ornamental flowers and roundish-glossy black fruit, which protrude from the swollen base of the bright red calyx associated with its common name [9]. *O. kirkii* has not been previously phytochemically investigated or explored for biological activity. The phytochemical investigation of this species was inspired by previous work on other members of genus *Ochna* as well as by the current need for discovery of new antibacterial agents [5,10]. The World Health Organization (WHO) has identified antimicrobial resistance (AMR) as a global challenge so serious that it threatens the fundamental achievements of modern medicine [11]. If not tackled quickly and radically, millions of AMR-related deaths are expected the coming decades [12]. Plant metabolites have historically played a key role in the discovery of antibacterial agents [13,14], which motivated us for the investigation of the antibacterial effect of the secondary metabolites of *O. kirkii*. Thereto, we aimed for evaluation of the cytotoxicity of the exracts and of the isolates to assess their therapeutic applicability. Accordingly, we evaluated the methanolic extract of the root bark and its isolated constituents for antibacterial activity against *Escherichia coli* and *Bacillus subtilis*, representative Gram-negative and Gram-positive bacteria species, respectively, and for cytotoxicity against the MCF-7 human breast cancer cell line.

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2. Experimental

2.1. General experimental procedures

IR (KBr disks) spectra were recorded on a PerkinElmer Spectrum FT-IR spectrometer. Optical rotations were determined using a 341 LC OROT polarimeter (589 nm, $T = 23$ °C), ECD experiments were performed on a Jasco Model *J*-810, Rev. 1.00 spectropolarimeter. UV spectra were acquired on a Specord S600 (Analytik Jena AG) spectrophotometer. NMR spectra were acquired on Agilent 400 MHz (OneNMRProbe), Bruker Avance NEO 500 MHz (TCI cryogenic probe) and 600 MHz (TXI cryogenic probe) spectrometers, and were processed using the MestreNova (v14.0.0) software. Chemical shifts were referenced to the carbon and residual proton signals of the deuterated solvents (CD_3OD δ_H 3.30 and δ_C 49.0, $CDCl_3$ δ_H 7.26 and δ_C 77.16). Assignments were based on 1D (^1H and ^{13}C), and 2D (COSY, HSQC, HMBC, TOCSY and NOESY) NMR spectra. LC-ESIMS data were obtained on a Micromass GC-TOF micro mass spectrometer (Micromass, Wythenshawe, Waters Inc. UK), using direct inlet, and 70 eV ionization voltage and eluting with a gradient mobile phase (H_2O/CH_3CN 95:05–5:95) with a run time of 6 min. HRESIMS spectra were acquired with a Q-TOF-LC/MS spectrometer using a 2.1 × 30 mm 1.7 μm RPC_{18} column and a H_2O/CH_3CN gradient (5:95–95:5 in 0.2% formic acid) at Stenhagen Analysis Lab AB, Gothenburg, Sweden. TLC was carried out on silica gel 60 F_{254} (Merck, Darmstadt, Germany) using pre-coated aluminum plates and visualized under UV light (254 and 366 nm). The TLC plates were sprayed with anisaldehyde reagent followed by heating to detect the UV negative compounds and observe the color change of the UV positive spots (the reagent was prepared by mixing 3.5 mL of 4-anisaldehyde with 2.5 mL concentrated H_2SO_4, 4 mL of glacial CH_3COOH and 90 mL of CH_3OH). Gravitational column chromatography was carried out on silica gel 60 (230–400 mesh). Gel filtration was carried out on Sephadex LH-20 (GE Healthcare), eluted in CH_2Cl_2-CH_3OH (1:1). Preparative RP-HPLC was performed on an Interchim Ultra Performance Flash Purification (PF-430) system, using the Interchim v. 5.1d.02 software, and an RP-C_8 Kromasil® column (250 mm × 25 mm, 5 μm) with H_2O-CH_3OH gradient (60:40 to 0:100) for 15–60 min with a flow rate of 5–10 mL/min.

2.2. Plant material

The roots of *Ochna kirkii* were collected in November 2018 from the coastal forest at Mkwanyula village along the road to Somanga Kivinje (GPS location S 08° 51′54.9″ E 39° 29′ 00″ at an elevation of 14 m), Kilwa District, Lindi region, Tanzania. The plant was identified and authenticated by Mr. F. M. Mbago, a senior taxonomist of the Herbarium, Botany Department, University of Dar es Salaam, where a voucher specimen (FMM 3907) was deposited.

2.3. Extraction and isolation

The root bark of *O. kirkii* was air-dried under the shade for three weeks and ground to fine powder to obtain 1453 g of plant material. The ground material was soaked three times in 3 L of methanol at room temperature for 72 h, yielding a total of 58.0 g of crude extract after evaporation using a rotary evaporator at 40 °C. A 20 g portion of this crude extract was adsorbed on silica gel (1:1, sample-silica gel) and loaded on a silica gel column. Gravitational elution was performed with a gradient of increasing polarity, using EtOAc (0–100%) in isohexane, followed by 10% CH_3OH in EtOAc. A total of 198 fractions were collected and combined to sub-fractions based on TLC analyses. Fractions 39–49 were combined and eluted on a Sephadex LH-20 column (eluent: CH_2Cl_2-CH_3OH; 1:1) to afford 4,5-dimethoxy-6,7-methylenedioxy-isoflvone (**3**, 8 mg) [15], as white solid. On the basis of TLC analyses, fractions 66–69 were combined and purified on Sephadex LH-20 column (eluent: CH_2Cl_2-CH_3OH; 1:1) to afford kirkinone B (**2**, 6 mg).

Table 1
^1H (500 MHz) and ^{13}C (125 MHz) NMR spectroscopic data for Kirkinone A (**1**), obtained for CD_3OD solution.

Position	δ_H, m (*J* in Hz)	δ_C, type
2	8.21, s	156.3, CH
3		123.7, C
4		177.3, C=O
5	8.08, d (8.9)	128.2, CH
6	7.02, dd (8.9, 2.4)	116.7, CH
7		164.9, C
8	6.92, d (2.4)	103.3, CH
9		159.7, C
10		117.3, C
1′		131.0, C
2′/6′	7.16, AA’	132.0, CH
3′/5′	6.74, XX’	116.1, CH
4′		156.9, C
1″		113.8, C
2″		166.9, C
3″	6.33, d (2.3)	103.6, CH
4″		166.7, C
5″	6.38, dd (8.9, 2.3)	109.2, CH
6″	7.90, d (8.9)	133.9, CH
1‴		205.0, C=O
2‴	5.38, dd (8.5, 6.6)	43.6, CH
3a’‴	3.38, dd (13.7, 8.5)	38.5, CH_2
3b’‴	3.07, dd (13.7, 6.6)	–

Table 2
^1H NMR (500 MHz) and ^{13}C NMR (125 MHz) spectroscopic data for Kirkinone B (**2**), obtained for $CDCl_3$ solution.

Position	δ_H, m (*J* in Hz)	δ_C, type
2/2″	5.26, br m	81.6, CH
3/3″	3.78, br m	49.0, CH
4/4″		190.7, C=O
5/5″	7.86, d (8.9)	129.3, CH
6/6″	6.59, dd (8.8, 2.4)	110.4, CH
7/7″		166.1, C-O
8/8″	6.33, d (2.4)	100.6, CH
9/9″		163.4, C-O
10/10″		114.2, C
1′/1‴		129.2, C
2′/6′, 2‴/6‴	7.11, AA’	129.8, CH
3′/5′, 3‴/5‴	6.81, XX’	115.7, CH
4′/4‴		155.5, C-O
2/2″	5.26, br m	81.6, CH
3/3″	3.78, br m	49.0, CH
4/4″		190.7, C=O
OMe-11/11″	3.77, s	55.6, CH

The combined fractions 86–95 were further separated on a Sephadex LH-20 column (eluent: CH_2Cl_2-CH_3OH; 1:1) to yield calodenin B (**4**, 78 mg),[16] as well as two other subfractions. The first of these was further purified on Sephadex LH-20 (eluent: 100% CH_3OH) to yield kirkinone A (**1**, 6 mg) as white solid. The second sub-fraction was purified on Sephadex LH-20 (eluent: 100% CH_3OH) to afford afzelone (**8**, 16 mg). [17] Fractions 121–136 were combined and purified on a silica gel column (eluent: CH_2Cl_2-EtOAc; 1:1), followed by repeated chromatography on Sephadex LH-20 (eluent: CH_2Cl_2-CH_3OH; 1:1) to afford calodenone (**7**, 5 mg) [7] as an amorphous brown solid. The combined fractions 144–146 were purified on Sephadex LH-20 (eluent: CH_2Cl_2-CH_3OH; 1:1) followed by silica gel chromatography (eluent: CH_2Cl_2-EtOAc; 1:1), to yield 4′,6,6′-trihydroxy-2′-(4-hydroxyphenyl)-7′-((*E*)-3-(4-hydroxyphenyl)acryloyl)-2′H,3H-2,3′-spirobi[benzofuran]-3-one (**5**, 6 mg).[18] Purification of fractions 147–158 on a Sephadex LH-20 column (eluent: 100% CH_3OH) gave lophirone A (**6**, 20 mg).[19]

2.4. Physical and spectroscopic data of Kirkinones A and B

Kirkinone A (1): white solid; m.p. 100.9 °C; $[\alpha]^{23}_D + 61.5$ (c 0.00013,

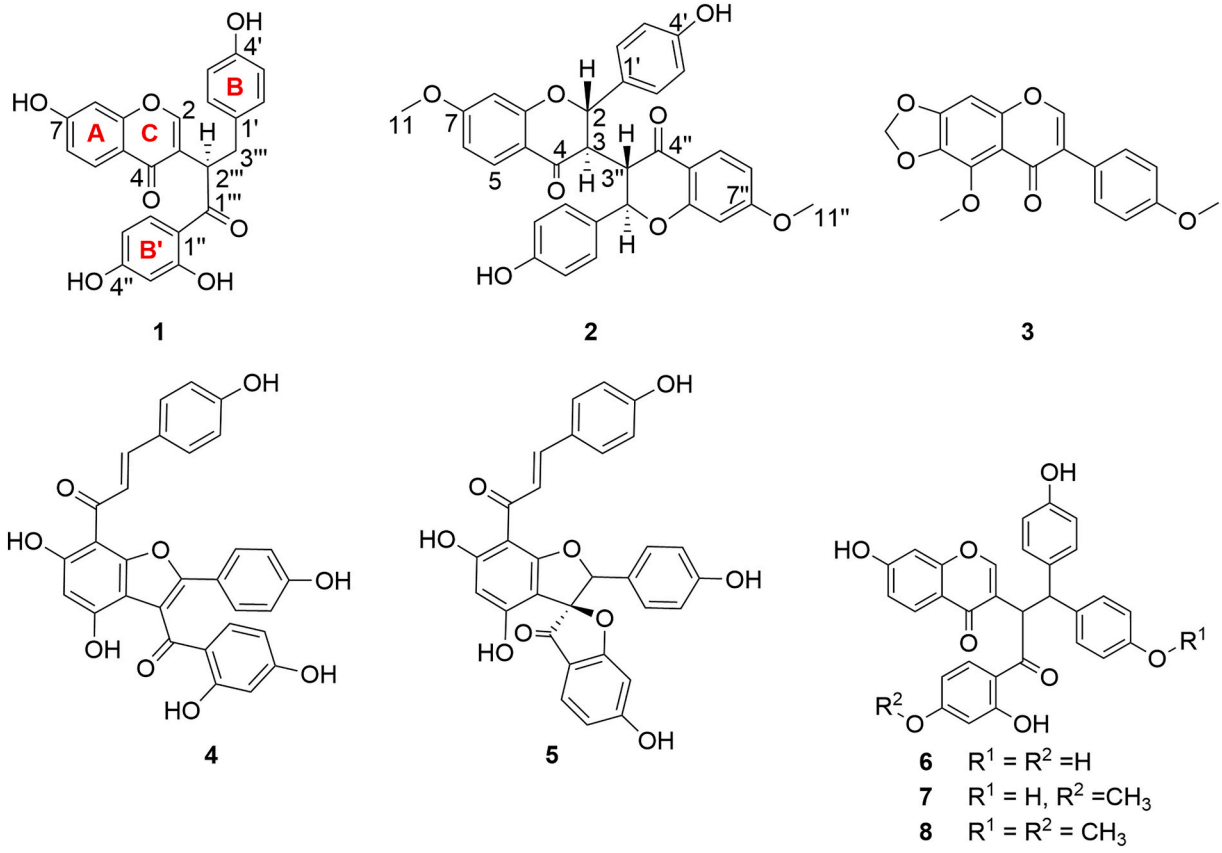

Fig. 1. The structures of compounds **1**–**8** isolated from *Ochna kirkii*.

CH_3OH); UV (CH_3OH) λ_{max} (log ε) 324 (3.7), 286 (3.4) nm; ECD (*c* 0.03, CH_3OH) λ_{max} ($\Delta\varepsilon$) 340 (7.0), 290 (−18.0), 240 (4.0), 210 (−40.0); IR ν_{max} 3250, 2927, 1698, 1625, 1452, 1231, 1139, 956, 816 cm^{-1}; ^1H and ^{13}C NMR, see Table 1. HRESIMS *m/z* 419.1158 $[M + H]^+$ (calcd 419.1131 for $[C_{24}H_{18}O_7 + H]^+$).

Kirkinone B ***(2):*** white solid; $[\alpha]^{23}_D$ + 7.69 (c 0.00013 CH_3OH); UV (CH_3OH) λ_{max} (log ε) 324 (3.7), 286 (3.4) nm; IR ν_{max} 3296, 2927, 1698, 1627,1598, 1515.1363, 988, 833 cm^{-1}; ^1H and ^{13}C NMR, see Table 2. HRESIMS *m/z* 539.1697 $[M + H]^+$ (calcd 539.1706 for $[C_{32}H_{26}O_8 + H]^+$).

2.5. Antibacterial assays

The antibacterial activity of the isolated natural products and the crude extract was tested against Gram-positive *Bacillus subtilis* and Gram-negative *Escherichia coli*. The samples were first dissolved at 10 mg/mL in 100% DMSO, then further diluted 30 × in H_2O and stored at −20 °C. For *in vitro* antibacterial assay, *B. subtilis* and *E. coli* were cultured by previous described standard procedure [20–22]. Briefly, a culture of bacterial cells was grown to OD $600_{nm} = 0.5$, then diluted 10× with pre-warmed medium. The substances to be tested were added to the culture medium for a final concentration of 30 μg/mL, each at 100 μL in a 96-well microtiter plate. For 18 h the culture of the substance to be tested were incubated for 37 °C without agitation. Cell viability was measured by resazurin-based assay, following a previously described standard procedure. Subsequently, 12 μL of AlarmarBlue solution was added to each well and incubated at 37 °C continuously for 1 h. Next, the fluorescence was measured using a POLARstar Omega microplate reader from BMG Labtech with the excitation filter set to 544 nm and emission filter to 590 nm. Ampicillin, a standard antibiotic was used as a positive control while DMSO, a solvent used to dissolve the test substances was used as negative control. The assay set up in microtiter format was performed following bleed-through of fluorescence from resorufin between wells in the microtiter plate fluorescence reader measured about <1% between adjacent wells. Quenching of fluorescence of the compound under investigation was checked by grown bacteria cultures being mixed after 1 h of incubation with resazurin and the compound of interest at the highest concentration to be evaluated, and the immediately measured fluorescence was compared with sample without addition of compound. Each test of compound activity was performed in three independent replicates. The compound that expressed a reduction of fluorescence by at least 50% relative to the solvent control in any of the species was followed up by additional tests.

2.6. Cytotoxicity assay

The cytotoxicity of the isolated compounds and crude extract was evaluated against human MCF-7 cells grown in Dulbecco's modified Eagles medium supplemented with 10% fetal calf serum and kept in exponential growth following standard procedure, as previously described [23]. Briefly, cells were reseeded into 96-well microtiter plates at a density allowing continued exponential growth and allowed to settle for 24 h. Stock solution in DMSO containing isolated compounds were added for final concentration of 0.3% *v*/v of the solvent in the culture medium. Cell viability was assayed using Prestoblue cell viability reagent (ThermoFischer) accordingly to the manufacturer's instructions following 24 h of incubation in the presence of the compounds. A row of the plate containing only DMSO, a solvent used to dissolve the test substances was used as negative control. A polar Star Omega plate reader (BMG Lab Tech) was used to measure resorufin fluorescence at 544 nm excitation/590 nm emission. The survival was expressed as percentage of solvent-only control. EC_{50} value for each compound was calculated for independent three replicate experiments, using 2-fold dilution intervals.

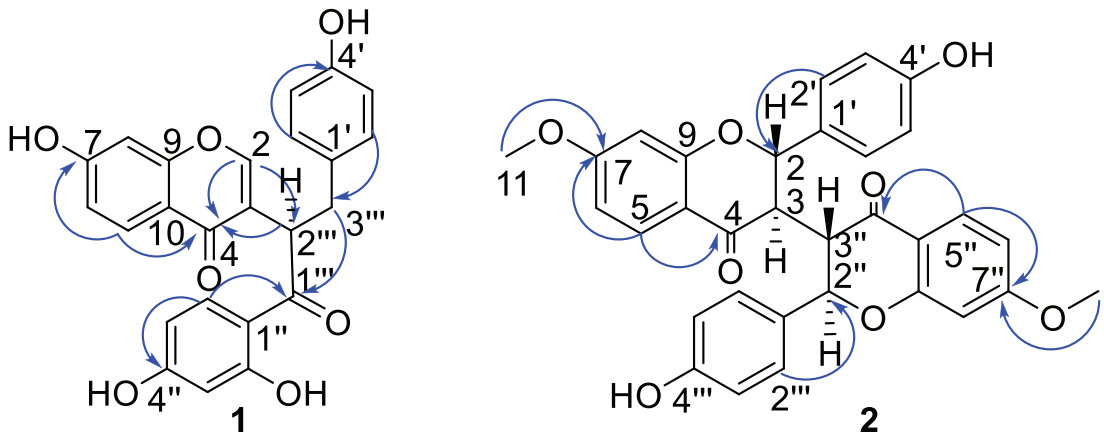

Fig. 2. Key ^{1}H to ^{13}C HMBCs (via ^{3}J) for **1** and **2**.

3. Results and discussion

Systematic gravitational chromatographic separation of the methanolic extract of the root bark of *O. kirkii* using silica gel 60 and Sephadex LH-20 columns as well as by preparative reverse-phase HPLC yielded the new isoflavone kirkinone A (**1**), the new biflavonoid kirkinone B (**2**) along with the six known compounds 4′,5-dimethoxy-7,7-methylenedioxyisoflavone (**3**) [15], calodenin B (**4**) [16], 4′,6,6′-trihydroxy-2′-(4-hydroxyphenyl)-7′-((*E*)-3-(4-hydroxyphenyl)acryloyl)-2′H,3H-2,3′-spirobi[benzofuran]-3-one (**5**) [18], lophirone A (**6**) [19], calodenone (**7**) [7] and afzelone D (**8**) [24], (Fig. 1, and Figs. S1-63, Supplementary Material). The structures of the isolated metabolites were determined by NMR spectroscopic and mass spectrometric analyses, and the known secondary metabolites were further identified by comparison of their observed and reported spectroscopic data.

Compound **1** was obtained as a white solid. It was assigned the molecular formula $C_{24}H_{18}O_7$ based on HRESIMS ($[M + H]^+ m/z$ 419.1158, calcd. 419.1131, Fig. S8, Supplementary Material) and NMR (Table 1, Figs. S2-S7, Supplementary Material) analyses. It showed specific optical rotation, $[\alpha]^{23}_D$ + 61.5 (*c* 0.00013, CH_3OH) revealing it to be chiral. Its UV absorption maxima at λ 324 and 286 nm suggested it to possess aromatic moieties, whereas its IR absorptions at 3250, 1652, 1231 cm^{-1} corresponded to vibrations typical of O—H, C═C and C—O functionalities, respectively. The ^{1}H NMR data of **1** contained a singlet at δ_H 8.21 (H-2) attached to δ_C 159.7 (C-2), consistent with the expected chemical shifts of a proton attached to an oxygenated olefinic carbon of an isoflavone skeleton. As expected, this proton (H-2) did not give any COSY cross-peaks, but showed HMBC correlations to C-3 (δ_C 123.7), C-4 (δ_C 177.3) and C-9 (δ_C 159.7) of ring A, and in addition to C-2‴ (δ_C 43.6) connecting ring C to the 1,3-diarylated propanone bridge (Fig. 2 and Fig. S6, Supplementary Material). The position of the hydroxy group of ring A was deduced from the coupling pattern of H-5, H-6 and H-8, i.e. $^{3}J_{5,6}$ = 8.9 Hz and $^{4}J_{6,8}$ = 2.4 Hz. The methine proton H-2‴ (δ_H 5.38) showed COSY cross-peaks to the diastereotopic protons resonating at δ_H 3.38 (H-3a'‴), and 3.07 (H-3b'‴); however, with unequal ^{3}J-couplings (8.5 Hz and 6.6 Hz), which revealed hindered rotation around the C-2‴–C-3‴ bond. This proton (H-2‴) showed HMBC correlations to C-1‴ (δ_C 205.0) and C-3‴ (δ_C 38.5), and to C-2 (δ_C 156.3), C-3 (δ_C 123.7) and C-4 (δ_C 177.3) of ring C, as expected, and to C-1′ (δ_C 131.0) of ring B. The ^{1}H NMR signals of the ring (B) at δ_H 7.16 (2H, H-2′/6′) and 6.74 (2H, H-5′/3′) were diagnostic for a higher order AA'XX' coupling pattern, indicative for a *p*-disubstituted aromatic ring. The H-2′/6′ (δ_H 7.16) showed HMBC correlation to C-3‴ (δ_C 38.5), corroborating the attachment of ring B to the aliphatic propanone bridge. The location of the 1,2,4-trisubstituted aromatic ring B′ was indicated by the HMBC cross-peak of H-6″ (δ_H 7.90) to C-1‴ (δ_C 205.0). Dioxygenation of this ring was suggested by the high chemical shift of its C-2″ (δ_C 166.9) and C-4″ (δ_C 166.7), and the position of the two hydroxy groups was derived from the scalar coupling pattern of the ring ($^{3}J_{5'',6''}$ = 8.5 Hz and $^{4}J_{3'',5''}$ = 2.3 Hz), and supported by HMBC correlations (Table 1, Fig. S4 and S6). The NMR data of **1** is similar to that of lophirone A (**6**) [19], but lacks signals for an additional *p*-disubstituted aromatic ring at C-3‴. To determine the configuration at C-2‴, the CD spectrum of **1** was acquired (Fig. 3). It showed a weak positive Cotton effect for the π → π* transition at ca 340 nm, a negative Cotton effect for the n → π * transition at ca 290 nm, and a negative Cotton effect at ca 210 nm for the ^{1}La electronic transition, which indicated a (*2‴R*) absolute configuration for the flavanone moiety [25]. Based on the above spectroscopic data, the new compound, kirkinone A (**1**), was characterized as (2‴*R*)-3-(1-(2,4-dihydroxyphenyl)-3-(4-hydroxyphenyl)-1-oxopropan-2-yl)-7-hydroxy-4*H*-chromen-4-one. The biogenesis of kirkinone A (**1**) is expected to follow the pathway previously reported for related isoflavonoids [26,27]. A plausible biosynthetic route for **1** is shown in Fig. 4.

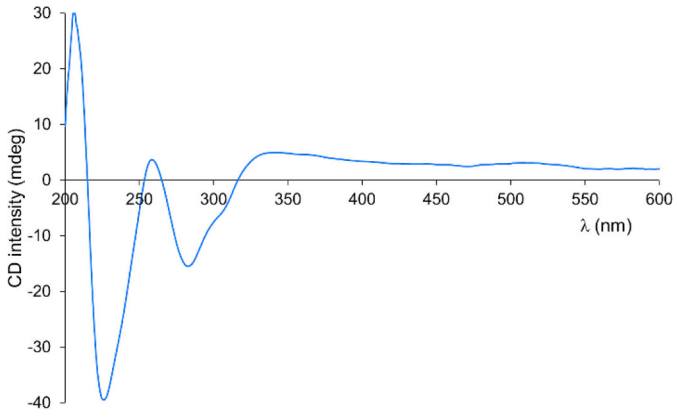

Fig. 3. The ECD spectrum of kirkinone A (**1**).

Compound **2** was isolated as a white solid. It was assigned the molecular formula $C_{32}H_{26}O_8$ based on HRESIMS ($[M-H]^+ m/z$ 539.1697, calcd 539.1706) and NMR data (Table 2, Figs. S9-S10, Supplementary Material). It showed the specific rotation $[\alpha]^{23}_D$ + 7.69 (c 0.00013, CH_3OH), and IR absorption at 3296 cm^{-1}, the latter being compatible with the O—H stretch frequency. The IR absorption at 1698 cm^{-1} is in line with a conjugated carbonyl system, whereas those at 1627 cm^{-1} and 1598 cm^{-1} suggest the presence of aromatic double bonds. The presence of aromatic groups was further confirmed by the UV absorptions at 324 and 286 nm. The ^{1}H NMR data (Table 2, Fig. S9, Supplementary Material) displayed an ABX spin system resonating at δ_H 7.86 (H-5/5″), 6.59 (H-6/6″) and 6.33 (H-8/8″), two *p*-disubstituted aromatic spins at δ_H 7.11 (H-2′/6′, and H-2‴/6‴) and 6.81 (H-3′/5′, 3‴/5‴), methoxy

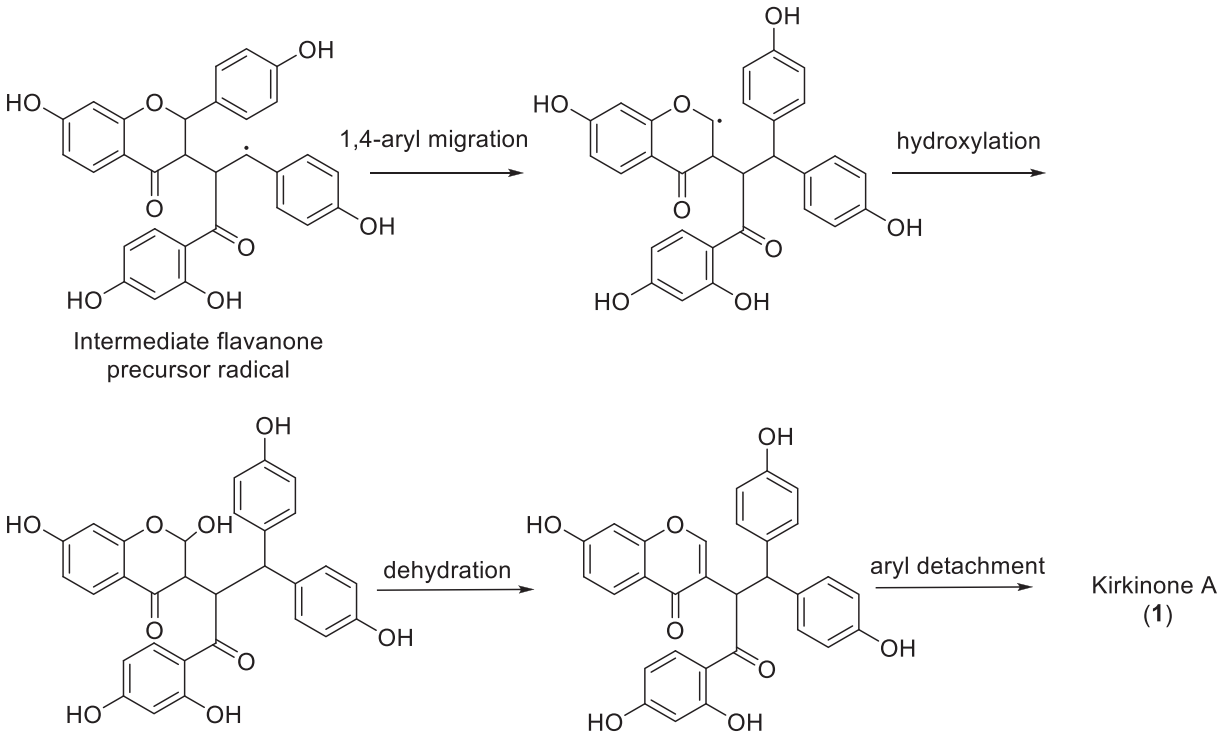

Fig. 4. A plausible biogenetic route to kirkinone A (**1**), based on reference [26].

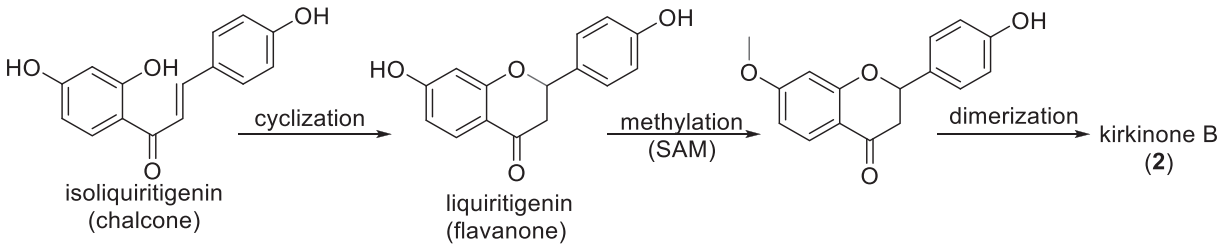

Fig. 5. The biogenetic derivation of kirkinone B (**2**).

groups at δ_H 3.77 (H-11 and H-11″), oxygenated and benzylic methine protons at δ_H 5.26 (H-2 and H-2″), and methine protons at δ_H 3.78 (H-3 and H-3″). The signals of H-2/2″ and H-3/3″ were broadened, similarly to that reported for some biflavonoids [5]. The NMR spectroscopic features of **2** resemble those of isocampylospermone A [3], a dimeric flavonoid previously reported along with its diastereomer campylospermone A [3] from *Campylospermum mannii*, and their *meso* isomer 3,3″-di(7,4′-dihydroxyflavanone-3-yl) that was isolated from *Ochna integerrima* [5,28]. The minor difference in the NMR data of **2** to the latter compounds suggests that methoxy groups (δ_H 3.77) are attached to its C-7/7″ (δ_C, 166.1), instead of hydroxy groups, which was confirmed by HMBC (Figs. 2 and S15, Supplementary Material). The ^{1}H NMR integrals of **2** in combination with its molecular mass suggested it to be a symmetric dimer. We reasoned that the broadening of the signals of H-3/3″ (δ_H, 3.78) and H-2/2″ (δ_H, 5.26), which hampered the detection of HSQC and HMBC cross-peaks from these protons, was caused by hindered rotation around the C-3–C-3″ axis. This also caused broadening of the δ_C 81.6 (C-2) and δ_C 49.0 (C-3) signals. Our observations are akin to those reported for isocampylospermone A and for the *meso* form of 3,3″-di(7,4′-dihydroxyflavanone-3-yl) [5,28]. The HMBC cross-peak of H-2′/H-6′, H-2‴/H-6‴ (δ_H, 7.11) to C-2/C-2″ (δ_C, 81.6) indicated the position of the *p*-disubstituted ring B'/B'‴ at C-2/C-2″ (δ_C, 81.6) (Fig. S13, Supplementary Material). The relative configuration at the C-3–C-3″ bond was concluded based on the small optical rotation, +7.69, that is comparable to that of the *meso* isomer of structurally closely related biflavonoids [3,5]. Based on the above spectroscopic data, this new compound, kirkinone B (**2**), was characterized as (2*R**,2″*S**,3*S**,3″*R**)-2,2′-bis(4-hydroxyphenyl)-7,7′-dimethoxy-[3,3′-bichromane]-4,4′-dione. The biosynthesis of flavonoids is well established [29,30]. Accordingly, kirkinone B (**2**) is expectably derived from isoliquiritigenin [31], a chalcone, through a sequence of cyclization, methylation, and oxidative dimerization (Fig. 5).

The six known compounds were identified as 4′,5-dimethoxy-7,7-methylenedioxyisoflavone (**3**) [15], calodenin B (**4**) [16], 4′,6,6′-trihydroxy-2′-(4-hydroxyphenyl)-7′-((*E*)-3-(4-hydroxyphenyl)acryloyl)-2'H,3H-2,3′-spirobi[benzofuran]-3-one (**5**) [18], lophirone A (**6**) [19], calodenone (**7**) [7], and afzelone D (**8**) [24], based on comparison of their spectroscopic data (Fig. S17–S63, Supplementary Material) to that reported in the literature.

Compounds **3**, **4**, **7** and **8** have been previously isolated from *Iris tingitana* (Iridaceae) [15], *Brackenridgea zanguebarica* [16], *Ochna afzelii* [24], and *O. calodendron* (Ochnaceae) [7], respectively; however, without their bioactivity having been investigated. Compound **6**, previously isolated from *B. zanguebarica,* exhibited strong antifungal activity against *Cladosporium cucumerinum* and antibacterial activity against *Bacillus subtilis* [18]. Compound **5**, previously reported from *Lophira lanceolate* (Ochnaceae) and from *O. holstii*, possesses strong antibacterial activity against *Pseudomonas aeruginosa* and *Salmonella*

Table 3
The antibacterial activity of compounds **4–8** against Gram-positive *Bacillus subtilis*. The EC_{50}, EC_{90} and MIC values (in μM) determined for each compound are shown.

Compound	4	5	6	7	8
EC_{50} (μM)	1.6	1049.7	9.0	2543.0	120.0
SD	1.3	352.2	1.7	209.2	62.7
SE range	0.8–2.4	846.3–1253.0	8.0–10.0	2422.2–2663.8	83.8–156.2
EC_{90} (μM)	2.0	1511.0	11.4	3980.3	2214.0
SD	1.4	272.1	1.3	216.0	1663.4
SE range	1.2–2.8	1353.9–1668.1	10.7–12.2	3855.6–4105.1	1253.7–3174.3
MIC (μM)	2.2	N/A	28.0	>4578.8	>4459.6

Table 4
The cytotoxicity of isolated compounds and crude extract against MCF-7.

Compound	EC_{50} [μg/mL]	EC_{50} [μM]
1, **3**, **5**, **7** and **8**	>200	not determ.
4	114.90	219.3
6	9.79	18.44
Crude	8.4	–

typhi [1,18]. Inspired by the antibacterial activity of the above mentioned compounds, we tested the isolated natural products **1**, **3–8** and the crude root bark methanol extract for antibacterial activity against the Gram-negative *E. coli* and the Gram-positive *B. subtilis* (Table 3) as well as for cytotoxicity against the MCF-7 human breast cancer cell line (Table 4). While *B. subtilis* is itself nonpathogenic and served as a model bacterium for Gram-positive species, many of *E. coli* strains are human pathogens and are therapeutically pertinent. Out of the eight isolated natural products, calodenin B (**4**) and lophirone A (**6**) showed significant antibacterial activities against the Gram-positive bacterium *B. subtilis* with MIC values of 2.2 and 28 μM, respectively. They also showed cytotoxicity with EC_{50} values of 19.2 and 219.3 μM against the MCF-7 human breast cancer cell line, respectively. The methanolic crude extract of the root bark exhibited cytotoxicity at EC_{50} 8.4 μg/mL, and inhibitory effect (37% viability) against *E. coli* (Gram negative, $EC_{50} = 256.2$ μg/mL and MIC >402 μg/mL, ~100× less potent than ampicillin). The positive control ampicillin showed an $EC_{50} = 11.5$ and $EC_{50} = 17.2$ μM against *E. coli* and *B. subtilis*, respectively (Fig. S64, Supplementary Material). None of the eight compounds were active against *E. coli* (MIC ≥400 μg/mL). The observed moderate effect of the crude extract on *E. coli* could be attributed to the synergistic effects of either the eight isolated constituents, or along with those that could not been isolated. On the other hand, the crude extract and compounds **4–8** were active against *B. subtilis* (Gram positive), amongst which compounds **4**, **6**, and **8** displayed the lowest EC_{50} values. Compound **4** was as active as the crude extract and potentially not toxic (Table 4). Hence, compound **4** is the most promising against Gram positive bacteria. Additionally, compounds **6**, **7** and the crude extract were potent, but had cytotoxic effect. Compounds **4** and **5**, although being structurally closely related, behave differently towards the tested bacteria and MCF-7 cells. We could not correlate the differences in their potencies to the subtle differences in molecular structure. Out of **6**, **7** and **8**, compound **6** is the most active against *B. subtilis* (and also the most cytotoxic). Antibacterial activity dose-response curves for compounds **4–8** against *B. subtilis* are given in the Supplementary Material (Fig. S65).

4. Conclusion

Chemotaxonomically, the genus *Ochna* (and generally Ochnaceae family) is known to be rich in biflavonoids, many possessing a hydrogen bonded *peri*-hydroxy group. Therefore, the isolation of the new isoflavonoid kirkinone A (**1**) and biflavonoid kirkinone B (**2**) as well as the previously known biflavonoids **4–8** from *O. kirkii* has chemotaxomic importance, as this supports the taxonomic placement of *O. kirkii* to the genus *Ochna*, and to the Ochnaceae family in general. The crude extract of the root bark of *O. kirkii* and its isolated constituents, notably calodenin B (**4**) and lophirone A (**6**), showed promising antibacterial activities. This is in good agreement with the previous studies of other members of the genus *Ochna* [1,32,34,35].

Authors contributions

The contribution of authors to this work is as follows: M.M.N., S.S·N. and M.E. conceptualized the work. Extraction and isolation of compounds was performed by T.M.K. under the supervision of M.M.N., J.J.E. M., S.S.N. and M.E.; NMR analyses was performed by T.M.K. with the help of Y.A. and M.E.; Spectroscopic characterization of the compounds was carried out by T.M.K., Y.A., P.J.G., S.S.N. and M.E.; antibacterial and cytotoxicity assays were performed by A.R., C.B. and P.S.. All authors contributed to the preparation of the manuscript.

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Declaration of Competing Interest

The authors have no conflicts of interest to declare.

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Appendix A. Supplementary data

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