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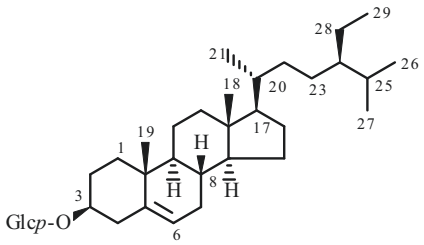
COMPOUNDS ISOLATED FROM *Tinospora crispa*

Muhammad Ismail[1*] and Muhammad Iqbal Choudhary[2]

The genus *Tinospora* (L.) Miers., belonging to the family Menispermaceae, comprises 75 genera with 520 species [1]. *Tinospora* have resulted in the isolation of a variety of compounds, including furanoditerpenes, steroids, flavonoids, lignanoids, alkaloids, and different phenolic constituents. Among them clerodane-type furanoditerpenes are commonly found in this genus [2–4].

In continuation to our previous study [5, 6], an authentic sample of *T. crispa* of Malaysian origin was thoroughly investigated to isolate and identify secondary compounds. We report the isolation and structures of compounds **1–4** from this plant for the first time while compound **5** was isolated as a known secondary metabolite. The structures of these compounds were deduced by comparing the EI-MS, UV, IR, and ^{1}H and ^{13}C NMR data with those reported in the literature.

The present study on *Tinospora crispa* collected from Malaysia led to the isolation and characterization of a different class of secondary metabolites **1–5**. The medium polar compounds **1**, **3**, and **4** were isolated from the least polar fractions of the ethyl acetate extract of *T. crispa*, whereas compound **2** was isolated from the polar fractions of the ethyl acetate extract by CC followed by recycling preparative HPLC. Compound **5**, which was characterized as cycloeucalenol, was obtained from the *n*-hexane extract of *T. crispa*. Compound **1** showed similar spectral data as that of methyl-3,4-dihydroxybenzoate earlier reported from *Schisandra verruculosa* [7]. Compound **2** was identified as a known compound syringin, previously isolated from *Stellera chamaejasme*, on the basis of comparison of spectral data with that of the reported data [8]. The structure of compound **3** was deduced as apigenin, reported in the literature from many fruits and vegetables [9]. The ^{1}H and ^{13}C NMR spectra of compound **4** showed characteristic signals for 3-*O*-*β*-D-glucopyranosyl-*β*-sitosterol, which was isolated earlier from several plants [10]. On the basis of spectroscopic data, the structure of compound **5** was deduced to be the known cycloeucalenol, previously isolated from *Tinospora crispa*. It is known for its cardiac contractility and hepatoprotective activities [11].



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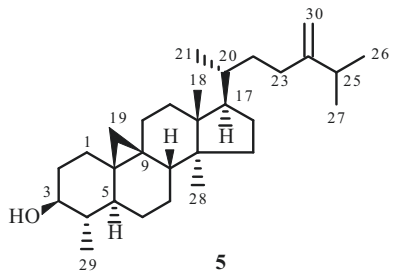

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General Procedures. Optical rotations were measured on a JASCO DIP-360 digital polarimeter in methanol unless otherwise stated. UV spectra were recorded on a Shimadzu UV-240 spectrophotometer in MeOH solutions and presented as λ_{max} nm (log ε), and IR spectra were recorded as KBr discs on a JASCO A-302 spectrophotometer and presented in cm^{-1}. 1D NMR spectra were recorded on Bruker Avance spectrometer operating at either 400 (^{1}H NMR) or 100 (^{13}C NMR) MHz, unless otherwise stated. Chemical shifts are given in δ (ppm), referenced to the residual solvent signal ($CD_3OD/CDCl_3$), while coupling constants (J) are measured in Hz. 2D NMR spectra were taken on a Bruker AMX 500 NMR spectrometer. Electron-impact mass spectra (EI-MS) were taken at 70 eV on a Finnigan MAT-112 or MAT-312 instrument. Fast-atom bombardment mass spectra (FA-BMS) were measured as glycerol matrix on a JEOL HX-110 mass spectrometer.

1) Department of Chemistry, Karakoram International University, University Road, 15100, Gilgit, Pakistan, e-mail: dr.ismail@kiu.edu.pk; 2) H.E.J. Research Institute of Chemistry, International Center for Chemical and Biological Sciences, University of Karachi, 75270, Karachi, Pakistan. Published in *Khimiya Prirodnykh Soedinenii*, No. 6, November–December, 2016, pp. 989–990. Original article submitted March 27, 2015.

TLC was performed on pre-coated silica gel plates (DC-Alugram 60 UV-254 of E. Merck), and spots were observed first under UV (254 nm) and then stained with ceric (IV) sulfate spray reagent and heated until appearance of color. Diaion HP-20 (Mitsubishi Chem. Ind., Tokyo, Japan), ODS C-18 (63-212 μm, Wako Pure Chemical Industries Ltd., Japan), polyamide-6 DF (Riedel-De Haen AG), and silica gel (E. Merck, 230–400 μm mesh) were used as adsorbents. Recycling preparative HPLC separation was performed on a JAI LC-908W instrument (Japan Analytical Industry) with YMC ODS H-80, L-80, and GS-320 columns (YMC Ltd., Kyoto, Japan).

Plant Material. The authentic sample of *Tinospora crispa* was collected from the herbal garden of the Laboratory of Natural Products (LHS), University of Putra Malaysia (UPM). The plant was identified by Mr. Shamsul Khamis, a resident botanist at LHS, and a specimen (SK 1537/08) was deposited at the Herbarium of the Institute of Bioscience, UPM.

Extraction and Isolation. Dried and ground aerial parts of *T. crispa* (16 kg) were extracted three times with distilled methanol, and the solvent was evaporated on a rotary evaporator. The crude methanolic extract (ca. 284 g) was then successively partitioned by solvent-solvent fractionation into four major fractions, *n*-hexane (60.31 g), ethyl acetate (69.43 g), *n*-butanol (72.61 g), and water (81.65 g). The ethyl acetate extract was subjected to column chromatography (silica gel, 200–400 μm mesh) and eluted with gradients of *n*-hexane–acetone (95:5) and *n*-hexane–ethyl acetate (90:10, 80:20, 70:30) and finally washed with ethyl acetate (100%). A total of 15 fractions (EA–EO) was collected. Fractions H–K from the ethyl acetate extract were combined on the basis of the same TLC pattern and loaded on a silica gel column and eluted with 10–15% ethyl acetate in *n*-hexane to afford fine yellowish crystals of compound **1** (7.2 mg, 4.5×10^{-5} % yield). Compound **1** was recrystallized in chloroform and characterized as methyl 3,4-dihydroxybenzoate. The purification of fraction ET (300 mg) on an ODS column (RP-18, water–methanol, 3:1) using recycling HPLC (L-80, 1:1 H_2O–MeOH, 3.5 mL/min) afforded compound **2** (125 mg, 7.8×10^{-4} % yield, T_R = 41 min), which was later characterized as syringin. Fraction EF (4 g) from the ethyl acetate extract was subjected to repeated column chromatography on silica gel (3–10% acetone in *n*-hexane). Compound **3** (8.3 mg, 5.2×10^{-5} % yield) was thus obtained as yellow needle-like crystals characterized as a known compound, apigenin. Fractions EL (3 g) from the ethyl acetate extract on column chromatography (silica gel) using 10–15% ethyl acetate in *n*-hexane as eluent yielded compound **4** (22 mg, 1.4×10^{-4}% yield) as a white powder. Comparison of spectral and physical data indicated the compound to be 3-*O*-*β*-D-glucopyranosyl-*β*-sitosterol. The hexane extract (60.31 g) was fractionated into 50 fractions (H-1 to H-50) by column chromatography on normal-phase silica gel, eluting with *n*-hexane along with a gradient of chloroform (1–10%). Repetitive CC of fractions H-33 to H-37 (6% $CHCl_3$ in *n*-C_6H_{12}) produced compound **5** (13.6 mg, 8.5×10^{-5}% yield), characterized as cycloeucalenol.

Acid Hydrolysis of Compounds 2 and 4. Compounds **2** and **4** (2 mg each) were separately dissolved in 10% aqueous HCl (1 mL) at 90°C for 4–5 h and cooled to room temperature. The reaction mixture was concentrated *in vacuo*, and the residue was subjected to TLC and GLC analysis in order to confirm the nature of the sugar moiety in the hydrolysate. TLC on silica gel developed with BuOH–Me_2CO–H_2O (4:5:1) indicated glucose at R_f 0.33. The residue was trimethylsilylated with 0.1 mL silylating agent (pyridine–trimethylchlorosilane, 5:1, by vol.) for 30 min at room temperature and subjected to GLC (1.5% silicon SE-30, 3 mm × 2 m, column temp. 150°C, N_2 1.0 kg/cm^2), which showed the presence of D-glucose with t_R 10.4 min, $[\alpha]_D^{22}$ +51.4° (*c* 0.021, H_2O).

Methyl 3,4-Dihydroxybenzoate (1). Yellowish crystals from chloroform, mp 135°C. IR ($CHCl_3$, ν_{max}, cm^{-1}): 3395, 3235 (O–H), 1695 (C=O), 1615, 1490, 1440 (aromatic C=C). HR-MS *m/z* 168.0417 ($C_8H_8O_4$, calcd 168.0423). FAB-MS (–ve) *m/z* 167 $[M - 1]^-$. EI-MS (70 eV) *m/z* (%) 168 $[M]^+$ (2.04).

Syringin (2). Colorless crystals from methanol, mp 190°C. IR (KBr, $CHCl_3$, ν_{max}, cm^{-1}): 3564, 3433, 3391 (O–H), 1589–1420 (aromatic C=C). UV (MeOH, λ_{max}, nm): 265 and 222. HR-MS *m/z*: 374.1421 ($C_{17}H_{24}O_9 + H_2$, calcd 374.1420). EI-MS *m/z* (%): 374 (7), 357.4 (43.6), 205.2 (100.0), 187.2 (17.5), 121.2 (24.1), 81.1 (64.4).

Apigenin (3). Yellow needle crystals from acetone, mp 351°C. HR-MS *m/z* 270.0522 ($C_{15}H_{10}O_5$, calcd 270.0528). EI-MS (70 eV) *m/z* (%): 270 (100), 242 (16), 152 (30), 121 (23), 91 (15), 69 (28), 55 (27).

3-*O*-*β*-D-Glucopyranosyl-*β*-sitosterol (4). IR (KBr, ν_{max}, cm^{-1}): 3430 (O–H), 1635 (C=C). ^{1}H NMR (500 MHz, pyridine-d_5, δ, ppm, J/Hz): 5.33 (1H, s, H-6), 5.06 (1H, d, J = 7.5, H-1′), 0.63 (3H, s, H-28), 0.68 (3H, s, H-29), 0.83 (3H, s, H-18), 0.94 (3H, s, H-26), 1.05 (3H, s, H-19), 1.11 (3H, s, H-21).

Cycloeucalenol (5). Yellow needle-like crystals from acetone, mp 141°C. ^{1}H NMR (300 MHz, $CDCl_3$, δ, ppm, J/Hz): 4.64 (2H, d, $J_{30a,30b}$= 15.0, H-30), 3.19 (1H, m, H-3), 1.60 (1H, t, $J_{17,16}$ = 7.2, H-17), 0.95 (1H, overlap, H-4), 1.17 (1H, m, H-5), 1.31 (2H, m, H-12), 1.59 (2H, t, $J_{15,16}$ = 7.2, H-15) 1.02 (3H, d, $J_{26,25}$ = 1.1, H-26), 0.99 (3H, d, $J_{27,25}$ = 1.1, H-27), 0.36 (1H, d, $J_{19a,19b}$ = 3.0, H-19a), 0.12 (1H, d, $J_{19b,19a}$ = 3.0, H-19b). ^{13}C NMR (100 MHz, CD_3OD, δ, ppm): 30.8 (C-1), 34.8 (C-2), 76.5 (C-3), 44.6 (C-4), 43.3 (C-5), 24.6 (C-6), 28.1 (C-7), 46.8 (C-8), 23.5 (C-9), 29.5 (C-10), 25.1 (C-11), 35.3 (C-12),

45.3 (C-13), 48.9 (C-14), 32.9 (C-15), 26.9 (C-16), 52.2 (C-17), 17.7 (C-18), 27.2 (C-19), 36.1 (C-20), 18.3 (C-21), 35.0 (C-22), 31.3 (C-23), 156.9 (C-24), 33.8 (C-25), 22.0 (C-26), 21.8 (C-27), 19.1 (C-28), 14.3 (C-29), 105.9 (C-30). HR-MS *m/z* $C_{30}H_{50}O$, 426.3863 (calcd 426.3862). EI-MS (70 eV) *m/z* (%): 426 $[M]^+$ (22), 408 (4), 393 (60), 383 (9), 324 (10), 309 (15), 300 (23), 285 (14), 273 (9), 256 (13), 245 (20), 233 (10), 227 (13), 216 (21), 201 (25), 189 (20), 175 (36), 159 (36), 147 (50), 133 (45), 121 (53), 107 (53), 95 (72), 81 (56), 69 (79), 55 (100).

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