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Non-cannabinoid constituents from a high potency *Cannabis sativa* variety

Mohamed M. Radwan[a], **Mahmoud A. ElSohly**[a,b,*], **Desmond Slade**[a], **Safwat A. Ahmed**[a], **Lisa Wilson**[c], **Abir T. El-Alfy**[c], **Ikhlas A. Khan**[a,d], and **Samir A. Ross**[a,d,*]

[a]National Center for Natural Products Research, School of Pharmacy, The University of Mississippi, University, MS 38677, USA

[b]Department of Pharmaceutics, School of Pharmacy, The University of Mississippi, University, MS 38677, USA

[c]Department of Pharmacology, School of Pharmacy, The University of Mississippi, University, MS 38677, USA

[d]Department of Pharmacognosy, School of Pharmacy, The University of Mississippi, University, MS 38677, USA

Abstract

Six new non-cannabinoid constituents were isolated from a high potency *Cannabis sativa* L. variety, namely 5-acetoxy-6-geranyl-3-*n*-pentyl-1,4-benzoquinone (**1**), 4,5-dihydroxy-2,3,6-trimethoxy-9,10-dihydrophenanthrene (**2**), 4-hydroxy-2,3,6,7-tetramethoxy-9,10-dihydrophenanthrene (**3**), 4,7-dimethoxy-1,2,5-trihydroxyphenanthrene (**4**), cannflavin C (**5**) and β-sitosteryl-3-*O*-β-D-glucopyranoside-2'-*O*-palmitate (**6**). In addition, five known compounds, α-cannabispiranol (**7**), chrysoeriol (**8**), 6-prenylapigenin (**9**), cannflavin A (**10**) and β-acetyl cannabispiranol (**11**) were identified, with **8** and **9** being reported for the first time from cannabis. Some isolates displayed weak to strong antimicrobial, antileishmanial, antimalarial and anti-oxidant activities. Compounds **2–4** were inactive as analgesics.

Keywords

Cannabis sativa L.; Cannabaceae; High potency; Non-cannabinoid; Antimicrobial; Antileishmanial; Antimalarial; Anti-oxidant

1. Introduction

Cannabinoids are phenolic compounds possessing a C_{21} terpenophenolic structure uniquely found in *Cannabis sativa* L. (ElSohly and Slade, 2005). Currently, 86 cannabinoids have

*Corresponding authors. Tel.: + 1-662-915-1031; fax: + 1-662-915-7989 (S.A. Ross), tel.: + 1-662-915-5928; fax: + 1-662-915-5587 (M.A. ElSohly). ; Email: sross@olemiss.edu (S.A. Ross), ; Email: melsohly@olemiss.edu (M.A. ElSohly)

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been isolated from cannabis (Ahmed et al., 2008; ElSohly and Slade, 2005; Radwan et al., 2008). Non-cannabinoid constituents isolated from cannabis include flavonoids, spiroindans, dihydrostilbenes, dihydrophenanthrenes, sterols and alkaloids, among others (Ross and ElSohly, 1995; Turner et al., 1980). As part of our program to study the constituents of high potency cannabis and their pharmacology (Ahmed et al., 2008; Radwan et al., 2008), we herein report the isolation and structure elucidation of eleven non-cannabinoid constituents including six new (**1–6**) and five known (**7–11**) compounds as well as their antimicrobial, antileishmanial, antimalarial and antioxidant activities. The analgesic activities of **2–4** are also reported.

2. Results and Discussions

Compound **1** was obtained as an orange amorphous powder. Its positive mode HRESIMS exhibited a pseudomolecular ion at *m/z* 373.2425 $[M+H]^+$ corresponding to a molecular formula of $C_{23}H_{32}O_4$. The IR spectrum of **1** revealed the presence of an α,β-unsaturated ketone and ester carbonyl groups at v_{max} 1663 and 1780 cm^{-1}, respectively. The ^1H NMR spectrum of **1** displayed three olefinic methyl singlets (δ_H 1.56, 1.63 and 1.69), one primary methyl triplet (δ_H 0.87, H_3-5'), one acetoxy methyl singlet (δ_H 2.32), one aromatic singlet (δ_H 6.53, H-2) and 7 methylene resonances (δ_H 1.32–2.39). The ^{13}C NMR, DEPT and HMQC spectra of **1** revealed 23 resonances including five methyl, seven methylene, one aromatic methine, two olefinic methine and eight quaternary carbons. The two carbonyl carbons resonating at δ_C 187.1 and 180.8 are characteristic for a benzoquinone skeleton (Mossa et al., 1999), while NMR analysis suggested an acetoxy (δ_H 2.32, δ_C 168.1, 20.5), a geranyl (Radwan et al., 2008) and an *n*-pentyl substituent (Ahmed et al., 2008; Radwan et al., 2008), indicating that **1** is a trisubstituted-1,4-benzoquinone derivative. HMBC analysis (Fig. 1) placed the geranyl substituent at C-6 due to the correlation of H_2-1" (δ_H 3.10) with C-6 (δ_C 135.6), C-1 (δ_C 187.1) and C-5 (δ_C 149.1). The three bond HMBC correlation of H-2 (δ_H 6.53) with C-6 (δ_C 135.6) and C-4 (δ_C 180.8), and the correlation of H_2-1' (δ_H 2.39) with C-4 (δ_C 180.8) and C-2 (δ_C 132.7) placed the *n*-pentyl moiety at C-3. The location of the acetoxy group at C-5 was determined by the four bond HMBC correlation between the acetoxy methyl (δ_H 2.32) and C-5 (δ_C 149.1). The presence of the *n*-pentyl and geranyl groups were confirmed by COSY correlations (Fig. 1), establishing **1** as 5-acetoxy-6-geranyl-3-*n*-pentyl-1,4-benzoquinone.

Compound **2** was isolated as a brown amorphous powder. Its molecular formula was determined as $C_{17}H_{18}O_5$ from the positive mode HRESIMS ion at *m/z* 325.1100 $[M+Na]^+$ and ^{13}C NMR spectrum. The ^1H and ^{13}C NMR spectra of **2** (Table 1) displayed two sets of methylene protons [δ_H 2.64 (4H, *s*, H_2-9 and H_2-10) correlated to δ_C 30.6 (C-9) and 31.8 (C-10) in the HMQC spectrum], a pair of signals for an *ortho*-coupled AB spin system [δ_H 6.74 (1H, *d*, $J = 8.0$ Hz, H-7) and 6.79 (1H, *d*, $J = 8.0$ Hz, H-8) correlated to δ_C 109.2 (C-7) and 119.1 (C-8), respectively in the HMQC spectrum], and an isolated aromatic proton [δ_H 6.50 (*s*, H-1)] suggesting that **2** is a 2,3,4,5,6-pentasubstituted 9,10-dihydrophenanthrene (Crombie et al., 1979; Leong et al., 1997; Stermitz et al., 1983). The ^{13}C NMR (Table 1) and APT spectra indicated the presence of five oxygenated quaternary aromatic carbons. Three of these carbons have methoxyl substituents as revealed by three sharp singlets in the ^1H

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NMR [δ_H 3.85, 3.86 and 3.89 (3H each)], while the remaining two carbons have hydroxyl substituents as was confirmed by the presence of a characteristic absorption band at ν_{max} 3420 cm^{-1} in the IR spectrum. The ^3J HMBC correlations between H-8 (δ_H 6.79) and C-6 (δ_C 147.6), C-4b (δ_C 121.1) and C-9 (δ_C 30.6); between H-7 (δ_H 6.74) and C-8a (δ_C 132.6) and C-5 (δ_C 141.7) and between the methoxyl group at δ_H 3.85 and C-6 (δ_C 147.6), located this methoxyl group at C-6, as was confirmed by ROESY correlation (Fig. 2). The ^3J HMBC correlation between the isolated aromatic proton at δ_H 6.50 and C-10 (δ_C 31.8) and C-4a (δ_C 114.9) determined its location at C-1. The ROESY correlation between the remaining two methoxyl groups at δ_H 3.86 and 3.89 in conjunction with the ROESY correlation between the methoxyl group at δ_H 3.89 and H-1 (δ_H 6.50) fixed their positions at C-3 and C-2, respectively, which was confirmed by HMBC correlations (Fig. 2), establishing **2** as 4,5-dihydroxy-2,3,6-trimethoxy-9,10-dihydrophenanthrene.

Compound **3** was isolated as a pale brownish, amorphous powder. The molecular formula of **3** was determined as $C_{18}H_{20}O_5$ from the positive mode HRESIMS at *m/z* 339.1279 [M +Na]$^+$, a mass difference of 14 amu compared to **2**. The ^{1}H and ^{13}C NMR spectra of **3** (Table 1) were similar to **2** except for the replacement of one hydroxyl by a methoxyl group (δ_H 3.92, δ_C 56.1). Therefore, **3** exhibited four methoxyl groups [δ_H 3.88 (3H, *s*), 3.89 (3H, *s*) and 3.92 (6H, *s*); δ_C 56.0, 56.1, 56.2 and 61.3] and one hydroxyl group. Their locations on the dihydrophenanthrene skeleton were determined by comparison to the ^{1}H and ^{13}C NMR data of **2** (Table 1) and confirmed by HMBC and ROESY correlations, placing two methoxyl groups at C-2 and C-3 and the hydroxyl at C-4. The remaining methoxyl groups are therefore attached to ring B. The presence of a pair of isolated aromatic singlets [δ_H 6.79 (H-8) and 8.02 (H-5)] in the ^{1}H NMR assigned the two methoxyl groups to C-6 (δ_C 147.2) and C-7 (δ_C 147.1) (Leong et al., 1997), which was confirmed by ROESY [δ_H 3.92 (OMe-6 and OMe-7)/H-5 and H-8; H-8/H$_2$-9] and HMBC (H-5/C-7, C-4a; H-8/C-6, C-8a, C-9) correlations. Thus, the structure of **3** was established as 4-hydroxy-2,3,6,7-tetramethoxy-9,10-dihydrophenanthrene.

Compound **4** was isolated as a reddish brown powder. Its molecular formula was determined as $C_{16}H_{14}O_5$ from negative mode HRESIMS (*m/z* 571.1630 [2M–H]$^-$) and ^{13}C NMR data. The UV spectrum of **4** (λ_{max} 258, 282 and 303 nm) is characteristic for a phenanthrene skeleton (Leong et al., 1999). The ^{1}H NMR (Table 1) displayed a pair of signals for an *ortho*-coupled AB spin system [δ_H 8.06 (1H, *d*, J = 8.4 Hz, H-9), 8.12 (1H, *d*, J = 8.4 Hz, H-10)], two *meta*-coupled protons [δ_H 6.82 (1H, *d*, J = 2.0 Hz, H-8), 6.93 (1H, *d*, J = 2.0 Hz, H-6)] and an isolated aromatic singlet [δ_H 6.15 (1H, *s*, H-3)] indicative of a pentasubstituted phenanthrene (Rethy et al., 2006; Leong et al., 1999). The ^{1}H and ^{13}C NMR spectra of **4** showed two aromatic methoxyl groups [δ_H 3.90 (3H, *s*) and 3.96 (3H, *s*)] and five oxygenated quaternary carbons, indicating that **4** has three hydroxyl groups. The ROESY correlation of the methoxyl group at δ_H 3.90 with the protons at δ_H 6.93 (H-6) and δ_H 6.82 (H-8) established its location at C-7 (δ 161.0), which was confirmed by HMBC [δ_H 3.90 (OMe-7)/C-7; H-8/C-7, C-6, C-8a, C-9; H-6/C-7, C-8] correlations. The ROESY and HMBC correlations of the methoxyl group at δ_H 3.96 with H-3 (δ_H 6.15) and C-4 (δ_C 161.5), respectively, assigned its location at C-4. Therefore, the structure of **4** was determined as 4,7-dimethoxy-1,2,5-trihydroxyphenanthrene.

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Compound **5** was obtained as a yellow amorphous powder. Its positive mode HRESIMS displayed an $[M+Na]^+$ ion at *m/z* 459.1766 suggesting $C_{26}H_{28}O_6$ as the molecular formula and 13 degrees of unsaturation. The IR spectrum showed absorption bands at v_{max} 3421 and 1662 cm^{-1} due to hydroxyl and carbonyl groups, respectively, while the UV absorption maxima at λ_{max} 275 (band I) and 340 (band II) nm were indicative of a flavone skeleton (Mabry et al., 1970). The ^1H NMR spectrum of **5** revealed a chelated hydroxyl group [δ_H 13.05 (*s*, HO-5)] which was confirmed by the bathochromic UV shift (+ 25 nm) of band II upon the addition of $AlCl_3$ to a methanolic solution of **5**. Bathochromic UV shifts upon the addition of NaOMe (+61 nm) and NaOAc (+ 5 nm) suggested hydroxylation at C-4' and C-7, respectively (Mabry et al., 1970). The ^1H NMR displayed two sharp singlets at δ_H 6.66 (1H, H-3) and 6.36 (1H, H-6), one methoxyl group (δ_H 3.98) and an ABX spin system of ring B [δ_H 7.01 (1H, *d, J* = 8.0 Hz, H-5'), 7.58 (1H, *d, J* = 2.0 Hz, H-2'), 7.64 (1H, *dd, J = 2.0*, 8.0 Hz, H-6')]. The presence of a geranyl group was deduced from the three methyl singlets at δ_H 1.48, 1.53 and 1.82 and two olefinic proton triplets at δ_H 5.02 and 5.35 in the ^1H NMR spectrum (Ahmed et al., 2008; Radwan et al., 2008). The ^{13}C NMR, DEPT-135 and HMQC spectra displayed 26 resonances including three methyl, one methoxyl, three methylene, seven methine and twelve quaternary carbons. The location of the methoxyl group was determined to be at C-3' from HMBC (OMe-3'/C-3'; H-5'/C-3'; H-2'/C-3', C-4') and ROESY (OMe-3'/H-2') correlations (Fig. 2). The carbon resonance at δ_C 98.9 corresponding to a proton singlet at δ_H 6.36 in the HMQC spectrum indicated an unsubstituted C-6 position (Agrawal, 1989). The location of the geranyl group at C-8 was confirmed by the HMBC correlation of the benzylic protons [δ_H 3.57 (2H, *d*, *J* = 6.8 Hz, H_2-1'')] with C-8 (δ_C 106.5), C-7 (δ_C 162.4) and C-9 (δ_C 155.4) (Fig. 2). The spectroscopic data of **5** are similar to those reported for cannflavin A (**10**) (Agrawal, 1989; Choi et al., 2004) except for the location of the geranyl group at C-8 instead of C-6, establishing **5** as 8-geranyl-5,7,4'-trihydroxy-3'-methoxyflavone (cannflavin C).

Compound **6** was obtained as an optically active white amorphous powder. Its molecular formula was deduced from the positive mode HRESIMS $[M+Na]^+$ ion at *m/z* 837.6621 as $C_{51}H_{90}O_7$. The ^1H NMR displayed two tertiary [δ_H 0.75 (Me-18) and 0.99 (Me-19)], three secondary [δ_H 0.63 (Me-26 and Me-27) and 0.90 (Me-21)] and one primary [δ_H 0.63 (Me-29)] methyl groups in addition to an olefinic proton at δ_H 5.33 (*bs*, H-6), indicating a sitosterol skeleton (Kovganko et al., 2000; Takemoto et al., 1967). The presence of an anomeric proton [δ_H 4.33 (*d*, *J* = 7.6 Hz, H-1')] and carbon [δ_C 101.6 (C-1')] in the HMQC spectrum indicated monoglycosylation at C-3 (δ_C 80.1) (Ishii et al., 1977) that was confirmed by HMBC (H-3/C-1'; H-1'/C-3) and ROESY (H-1'/H-3) correlations (Fig. 2). The sugar moiety was identified as P-D-glucopyranose by acid hydrolysis of **6** and TLC comparison with authentic sugar samples. The ^1H and ^{13}C NMR data of **6** were similar to those reported for β-sitosterol-3-*O*-β-D-glucopyranoside (Chang et al., 1981) with the addition of 16 resonances characteristic for a palmitate moiety (Segre and Mannina, 1997), which was confirmed by methylation of the alkaline hydrolysis product of **6** followed by GCMS analysis. The downfield esterification shift of C-2' (+ 2 ppm), upfield shifts of C-1' (-4 ppm) and C-3' (-3 ppm) and the four bond HMBC correlation of H-1' (δ_H 4.33) and C-1'' (δ_C 174.6) (Fig. 2) placed the palmitate moiety at C-2' (Terui et al., 1976; Yamasaki et al.,

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1977). Thus, the structure of **6** was established as β-sitosteryl-3-*O*-β-D-glucopyranoside-2'-*O*-palmitate.

Compound **7** was obtained as colorless prisms. Its positive mode HRESIMS gave an $[M+H]^+$ ion at *m/z* 249.2393 corresponding to a molecular formula of $C_{15}H_{20}O_3$. The GCMS showed a molecular ion at *m/z* 248 (33%) and two characteristic ions at *m/z* 189 (100%) and 176 (65%) suggesting that **7** is a spiroindan derivative (El-Feraly et al., 1986). The ^{13}C NMR, DEPT and HMQC spectra of **7** displayed 15 resonances including one methoxyl, six methylene, two aromatic methine, one sp^3 oxymethine and five quaternary carbons. The spectroscopic data of 7 are similar to those reported for β-cannabispiranol (Boeren et al., 1977; Shoyama and Nashioka, 1978; Radwan et al., 2008) except for the downfield shift of the oxymethine carbon (+5.6 ppm), indicating a 4'α-configuration. Although 7 is a known cannabis constituent (Crombie et al., 1982), this is the first report of the full NMR assignments.

The flavones **8** and **9** were isolated as yellow amorphous powders. Their molecular formulae were determined from the HRESIMS as $C_{16}H_{12}O_6$ and $C_{20}H_{18}O_5$, respectively. Their spectroscopic data (UV and NMR) were in agreement with reported values for chrysoeriol (**8**) (Toth et al., 1980; Agrawal, 1989) and 6-prenylapigenin (**9**) (Abegaz et al., 1998). This is the first report of their isolation from cannabis. The NMR spectra of **10** and **11** were identical with those of cannflavin A (Agrawal, 1989; Choi et al., 2004) and β-acetyl cannabispiranol (Shoyama and Nashioka, 1978), respectively.

The antimicrobial, antileishmanial, antimalarial and anti-oxidant activities of the isolated compounds were tested. Compound **1** displayed weak anti-MRSa (IC_{50} 15.0 μg/mL), moderate antileishmanial (IC_{50} 13.0 μg/mL) and mild antimalarial activity against *Plasmodium falciparum* (D6 clone) and *P. falciparum* (W2 clone) with IC_{50} values of 2.8 and 2.6 μg/mL, respectively. Compound **5**had moderate antileishmanial activity (IC_{50} 17.0 μg/mL). Compound **9**showed moderate anti-MRSa (IC_{50} 6.5 μg/mL), weak anticandidal (IC_{50} 20.0 μg/mL) and mild antimalarial activity against *P. falciparum* (D6 clone) and *P. falciparum* (W2 clone) with IC_{50} values of 2.8 and 2.0 μg/mL, respectively. Compound **10** exhibited strong antileishmanial activity (IC_{50} 4.5 μg/mL). Compound **11** displayed weak antileishmanial activity (IC_{50} 31.0 μg/mL).

Compounds **2, 5** and **11** displayed strong, **1, 7** and **10** moderate and **8** and **9** weak anti-oxidant activities in the DPPH assay, with **6** being inactive.

Compounds **2, 3** and **4** exhibited no antinociceptive action in both tail-flick and hot-plate assays up to 120 min following injection (Supplementary data).

3. Experimental

3.1. General

^1H NMR (400 MHz), ^{13}C NMR (100 MHz), DEPT-135, APT and 2D–NMR spectra were recorded using the residual solvent signal as internal standard on a Varian AS 400. IR spectra were measured on a Bruker Tensor 27. UV spectra were obtained on a Varian Cary

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50 Bio UV-Visible spectrophotometer. Optical rotation was measured on an Autoplot IV automatic polarimeter. High resolution mass spectra were measured using a Bruker BioApex. HPLC was performed on a Waters Delta Prep 4000 Preparative Chromatography System connected to a Waters 486 Tunable UV Absorbance detector using Phenomenex Luna C18 and Si columns (250 × 21.2 mm, 5 μm, 100 Å). GCMS analysis was carried out on a HP 6890 series GC, equipped with a split/splitless capillary injector, a HP 6890 series injector autosampler and an Agliant DB-5ms column (30 m × 0.25 mm × 0.25 μm), interfaced to a HP 5973 Mass Selective Detector (MSD). The injector temperature was 250°C and 1 μL injections were performed in the splitless mode, with the splitless time set at 60 s, the split flow set at 50 ml/min and the septum purge valve set to close 60 s after the injection occurred. The oven temperature was raised from 70 to 270°C (hold 20 min) at a rate of 5°C/min, for a total run time of 60 min; the transfer line temperature was 280°C.

3.2 Plant material

C. sativa plants were grown from high potency Mexican seeds. The seeds and plants were authenticated by Dr. Suman Chandra, The University of Mississippi, and a specimen (S1310V1) was deposited at the Coy Waller Complex, The University of Mississippi. Whole buds of mature female plants were harvested, air-dried, packed in barrels and stored at low temperature (–24°C).

3.3. Extraction, isolation and characterization

The plant material (9.0 kg) was sequentially extracted with hexanes (48 L), CH_2Cl_2 (40 L), EtOAc (40 L), EtOH (40 L), EtOH/H_2O (36 L, 1:1) and H_2O (40 L) at room temperature. The extracts were evaporated under reduced pressure at 40°C to afford hexanes (1.48 kg), CH_2Cl_2 (0.15 kg), EtOAc (0.13 kg), EtOH (0.09 kg), EtOH/H_2O (0.77 kg) and H_2O (0.54 kg) extracts for a total extract of 3.16 kg (35.1%, w/w). Portions of the CH_2Cl_2, EtOAc and EtOH extracts were combined (191.0 g) since they showed similar TLC profiles (EtOAc/*n*-hexane, 4:6) and were subjected to silica gel VLC, eluting with EtOAc/*n*-hexane [0:100, 10:90, 20:80, 30:70, 40:60, 50:50, 75:25, 100:0 (2 L of each mixture)] followed by EtOH (4 L), yielding 9 fractions (A-I). Fraction B (10 g) was fractionated on a silica gel column (EtOAc/petroleum ether, 90:10) to give 16 subfractions ($B_{1–16}$). Subfraction B_1 (265 mg) was purified by Si HPLC (EtOAc/*n*-hexane, 5:95, 25 mL/min, UV 270 nm) to afford **1**(12.3 mg, rt. = 4.0 min). Subfraction B_3 (236.0 mg) was purified by Si-SPE column eluting with MeOH/CH_2Cl_2 (1:19) yielding **6**(21.6 mg). Fraction D (14.3 g) was subjected to silica CC (EtOAc/petroleum ether, 5:95 to 20:80) followed by C18 flash chromatography (MeOH/H_2O, 8:2) and C18 HPLC (MeCN/H_2O, 50:50, 25 mL/min, UV 270 nm) to afford **9**(5.0 mg, rt. = 3.8 min), **10**(1.2 mg, rt. = 4.6 min), **7**(15.0 mg, rt. = 6.7 min) and **2**(11.6 mg, rt. = 8.7 min). Fraction F (28.5 g) was chromatographed on silica gel (EtOAc/*n*-hexane, 10:90 to 60:40), yielding 42 fractions ($F_{1–42}$, 200 mL each). Fraction $F_{32–35}$ (5.5 g) was subjected to silica gel CC (MeOH/CH_2Cl_2, 3:97) to yield 24 subfractions ($SF_{1–24}$). $SF_{2–3}$ (210 mg) was chromatographed on Si-SPE column (EtOAc/*n*-hexane, 10:90) followed by C18 HPLC purification (MeOH/H_2O, 85:15, 25 ml/min, UV 279 nm) to yield **3**(2.3 mg, rt. = 6.3 min) and **4**(3.9 mg, rt. = 8.8 min). $SF_{3–6}$ (1.9 g) was purified by Sephadex LH-20 CC (MeOH) followed by C18-SPE purification (MeOH/H_2O, 75:25) to give **5**(12.9 mg), **8** (284.5 mg) and **11** (15.0 mg).

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3.3.1. 5-Acetoxy-6-geranyl-3-n-pentyl-1,4-benzoquinone (1)—Orange amorphous powder; UV λ_{max} (MeOH): 205, 270, 384 nm; IR ν_{max} (neat): 1663 (C=O, ketone), 1780 (C=O, ester), 1610 (C=C) cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz): δ_H 0.87 (3H, *t, J* = 6.4 Hz, H_3-5'), 1.32 (4H, *m*, H_2-3' and H_2-4'), 1.56 (3H, *s*, H_3-8"), 1.57 (2H, *m*, H_2-2'), 1.63 (3H, *s*, H_3-9"), 1.69 (3H, *s*, H_3-10"), 1.94 (2H, *m*, H_2-4"), 2.05 (2H, *m*, H_2-5"), 2.32 (3H, *s*, OCO*CH₃*), 2.39 (2H, *t, J* = 7.6 Hz, H_2-1'), 3.10 (2H, *d, J* = 7.2 Hz, H_2-1"), 4.99 (1H, *t, J* = 7.2 Hz, H-2"), 5.02 (1H, *t, J* = 7.2 Hz, H-6"), 6.53 (1H, *s*, H-2); ^{13}C NMR ($CDCl_3$, 100 MHz): δ_C 187.1 (C-1), 132.7 (C-2), 148.3 (C-3), 180.8 (C-4), 149.1 (C-5), 135.6 (C-6), 28.9 (C-1'), 27.6 (C-2'), 31.6 (C-3'), 22.6 (C-4'), 14.1 (C-5'), 23.0 (C-1"), 118.4 (C-2"), 138.4 (C-3"), 39.8 (C-4"), 26.7 (C-5"), 124.1 (C-6"), 131.8 (C-7"), 17.9 (C-8"), 25.9 (C-9"), 16.4 (C-10"), 20.5 (OCOCH_3), 168.1 (*OCO*CH_3); HRESIMS *m/z* 373.2425 $[M+H]^+$ (Calc. for $C_{23}H_{33}O_4$, 373.2379).

3.3.2. 4,5-Dihydroxy-2,3,6-trimethoxy-9,10-dihydrophenanthrene (2)—Brown amorphous powder; UV λ_{max} (MeOH): 220, 267, 310 nm; IR ν_{max} (neat): 3420 (OH), 1610, 1537, 1462 (benzene ring) cm^{-1}; ^1H and ^{13}C NMR: Table 1; HRESIMS *m/z* 325.1100 $[M+Na]^+$ (Calc. for $C_{17}H_{18}O_5Na$, 325.1052).

3.3.3. 4-Hydroxy-2,3,6,7-tetramethoxy-9,10-dihydrophenanthrene (3)—Pale brownish amorphous powder; UV λ_{max} (MeOH): 220, 267, 310 nm; IR ν_{max} (neat): 3420 (OH), 1610, 1537, 1462 cm^{-1}; ^1H and ^{13}C NMR: Table 1; HRESIMS *m/z* 339.1279 $[M+Na]^+$ (Calc. for $C_{18}H_{20}O_5Na$, 339.12887).

3.3.4. 4,7-Dimethoxy-1,2,5-trihydroxyphenanthrene (4)—Reddish brown amorphous powder; UV λ_{max} (MeOH): 258, 282, 303 nm; IR ν_{max} (neat): 3413 (OH), 1610, 1533, 1462 cm^{-1}; ^1H and ^{13}C NMR: Table 1; HRESIMS *m/z* 571.1630 $[2M–H]^-$ (Calc. for $C_{32}H_{27}O_{10}$, 571.1604).

3.3.5. 8-Geranyl-5,7,4'-trihydroxy-3'-methoxyflavone (Cannflavin C) (5)—Yellow amorphous powder; UV λ_{max} (MeOH): 275, 340, (+NaOMe) 280, 342, 401, (+NaOAc) 280, 340, (+$AlCl_3$) 300, 346, 360, (+$AlCl_3$+HCl) 300, 346, 360 nm; IR ν_{max} (neat): 3421 (OH), 1662 (C=O) cm^{-1}; ^1H NMR (acetone-d_6, 400 MHz): δ_H 1.48 (3H, *s*, H_3-9"), 1.53 (3H, *s*, H_3-8"), 1.82 (3H, *s*, H_3-10"), 1.96 (2H, *m*, H_2-4"), 2.05 (2H, *m*, H_2-5"), 3.57 (2H, *d, J* = 6.8 Hz, H_2-1"), 3.98 (3H, *s*, OMe-3'), 5.02 (1H, *t, J* = 6.8 Hz, H-6"), 5.35 (1H, *t, J* = 6.8 Hz, H-2"), 6.36 (1H, *s*, H-6), 6.66 (1H, *s*, H-3), 7.01 (1H, *d, J* = 8.0 Hz, H-5'), 7.58 (1H, *d, J* = 2.0 Hz, H-2'), 7.64 (1H, *dd, J* = 2.0, 8.0 Hz, H-6'), 13.05 (1H, *s*, HO-5); ^{13}C NMR (acetone-d_6, 100 MHz): δ_C 164.1 (C-2), 103.3 (C-3), 182.7 (C-4), 160.5 (C-5), 98.9 (C-6), 162.4 (C-7), 106.5 (C-8), 155.4 (C-9), 104.5 (C-10), 124.4 (C-1'), 109.7 (C-2'), 148.3 (C-3'), 151.1 (C-4'), 115.8 (C-5'), 120.7 (C-6'), 21.7 (C-1"), 123.0 (C-2"), 135.1 (C-3"), 39.7 (C-4"), 26.7 (C-5"), 124.4 (C-6"), 131.0 (C-7"), 17.9 (C-8"), 25.1 (C-9"), 16.0 (C-10"), 55.8 (OMe-3'); HRESIMS *m/z* 459.1766 $[M+Na]^+$ (Calc. for $C_{26}H_{28}O_6Na$, 459.1784).

3.3.6. β-Sitosteryl-3-O-β-D-glucopyranoside-2'-O-palmitate (6)—White amorphous powder; $[\alpha]_D$: + 91.3 (*c* 0.05, $CHCl_3$); IR ν_{max} (neat): 1736 (OH), 1610 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz): δ_H 0.63 (9H, *m*, H_3-26, 27, 29), 0.75 (3H, *s*, H_3–18), 0.83 (3H, *t, J* = 7.2 Hz, H_3–16"), 0.90 (3H, *d*, 6.8 Hz, H_3-21), 0.99 (3H, *s*, H_3-19), 1.24 (18H, *bs*, H_2-5" to

H_2-13"), 1.28–1.30 (4H, *m*, H_2-4", 15"), 2.34 (2H, *m*, H_2-2"), 3.49 (1H, *m*, H-3), 3.35–3.82 (4H, H-2' to H-5'), 4.33 (1H, *d, J* = 7.6 Hz, H-1'), 4.1–4.2 (2H, *m*, H_2-6') 5.33 (1H, *bs*, H-6); ^{13}C NMR ($CDCl_3$, 100 MHz): δ_C 37.6 (C-1), 29.6 (C-2), 80.1 (C-3), 39.2 (C-4), 140.6 (C-5), 122.2 (C-6), 32.2 (C-7), 32.1 (C-8), 50.3 (C-9), 36.9 (C-10), 21.3 (C-11), 40.0 (C-12), 42.5 (C-13), 57.0 (C-14), 24.5 (C-15), 28.3 (C-16), 56.4 (C-17), 12.1 (C-18), 19.6 (C-19), 36.4 (C-20), 19.0 (C-21), 34.2 (C-22), 26.4 (C-23), 46.0 (C-24), 29.4 (C-25), 19.2 (C-26), 20.0 (C-27), 23.3 (C-28), 12.2 (C-29), 101.6 (C-1'), 73.5 (C-2'), 76.6 (C-3'), 70.8 (C-4'), 73.8 (C-5'), 64.1 (C-6'), 174.6 (C-1"), 34.5 (C-2"), 25.2 (C-3"), 29.6 (C-4" to C-13"), 30.0 (C-14"), 22.9 (C-15"), 14.3 (C-16"); HRESIMS *m/z* 837.6621 $[M+Na]^+$ (calc. for $C_{51}H_{90}O_7Na$, 837.6585).

3.3.7. α-Cannabispiranol (7)—

Colorless prisms (MeOH/H_2O); mp 182°C; UV λ_{max} (MeOH): 210, 222 nm; IR v_{max} (neat): 3410, 3180 (OH), 1610, 1596 cm^{-1}; ^{1}H NMR (C_5D_5N, 400 MHz): δ_H 1.75 (4H, *m*, H_2-2', 6'), 2.12 (2H, *t, J* = 7.2 Hz, H_2-2), 2.25 (4H, *m*, H_2-3', 5'), 2.90 (2H, *t, J* = 7.2 Hz, H_2-3), 3.70 (3H, *s*, OMe-5), 4.10 (1H, *m*, H-4'), 6.52 (1H, *bs*, H-4), 6.67 (1H, *bs*, H-6); ^{13}C NMR (C_5D_5N, 100 MHz): δ_C 49.2 (C-1), 36.4 (C-2), 31.9 (C-3), 102.0 (C-4), 161.1 (C-5), 101.3 (C-6), 156.7 (C-7), 129.9 (C-8), 146.9 (C-9), 34.6 (C-2', 6'), 34.3 (C-3', 5'), 70.8 (C-4'), 55.6 (OMe-5); HRESIMS *m/z* 249.2393 $[M+H]^+$ (Calc. for $C_{15}H_{21}O_3$, 249.2379); GCMS *m/z* (rel. int.): 248 (M^+, 33%), 230 (13%), 215 (12%), 201 (12%), 189 (100%), 176 (65%), 161 (18%).

3.4. Acid hydrolysis of 6

Compound **6** (5 mg) was refluxed with 3% H_2SO_4 in MeOH (4 mL) for 8 hours. The reaction mixture was neutralized with Na_2CO_3 and extracted with EtOAc (3 × 10 mL). TLC comparison of the aqueous layer with authentic sugar samples (CH_2Cl_2/MeOH/H_2O, 6:4:1; *n*-BuOH/AcOH/H_2O, 5:5:1), identified the glycone as β-D-glucose.

3.5. Alkaline hydrolysis of 6 and identification of the fatty acid

Compound **6** (5 mg) was added to 10% KOH in MeOH (10 mL) and H_2O (5 mL), and after refluxing for 30 minutes, H_2O (10 mL) was added, followed by extraction with $CHCl_3$ (3 × 10 mL). The aqueous layer was acidified with dil. HCl to pH 5 and extracted with $CHCl_3$ (3 × 10 mL). The $CHCl_3$ layer was dried (Na_2SO_4), filtered and concentrated *in vacuo*. The filtrate was dissolved in dry ether and MeOH (4 drops), followed by methylation with trimethylsilyl-diazomethane (200 μL). The reaction mixture was left open at room temperature for 30 minutes, concentrated under N_2 and analyzed by GCMS. The fatty acid methyl ester was identified as methyl palmitate via a library search (NIST).

3.6. Antimicrobial, antileishmanial and antimalarial bioassay

The isolated compounds were evaluated for antimicrobial (*Candida albicans* ATCC 90028, *Escherichia coli* ATCC 35218, *Pseudomonas aeruginosa* ATCC 27853, *Mycobacterium intracellulare* ATCC 23068, *Aspergillus fumigat* ATCC 90906, Methicillin Resistant *Staphylococcus aureus* ATCC 43300) (Bharate et al., 2007; Babu et al., 2006), antileishmanial (Radwan et al., 2008) and antimalarial activity [*P. falciparum* (D6 clone) and *P. falciparum* (W2 clone)] (Bharate et al., 2007).

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3.7. Anti-oxidant activity

A TLC autographic assay for DPPH (1,1-diphenyl-2-picrylhydrazyl) radical-scavenging effect was used to determine anti-oxidant activity (Takamatsu et al., 2003). The isolated compounds were dissolved in DMF (2 mg/mL) and applied in the form of a spot (4 μl, 4–5 mm in diameter) on silica gel GF plates. The residual DMF was removed under vacuum (15–20 min). A similar amount of vitamin E in DMF was used as positive anti-oxidant control. The radical-scavenging effects of the compounds were detected on the TLC plate using DPPH spray reagent (0.2% w/v in MeOH). The plate was observed 30 min after spraying. Active compounds are observed as yellow spots against a purple background. Relative radical-scavenging activity was assigned as "strong" (compounds that produce an intense bright yellow zone), "medium" (compounds that produce a clear yellow spot), "weak" (compounds that produce a weakly visible yellow spot), or "not active" (compounds that produce no sign of any yellow spot). Vitamin E produced an intense bright yellow zone.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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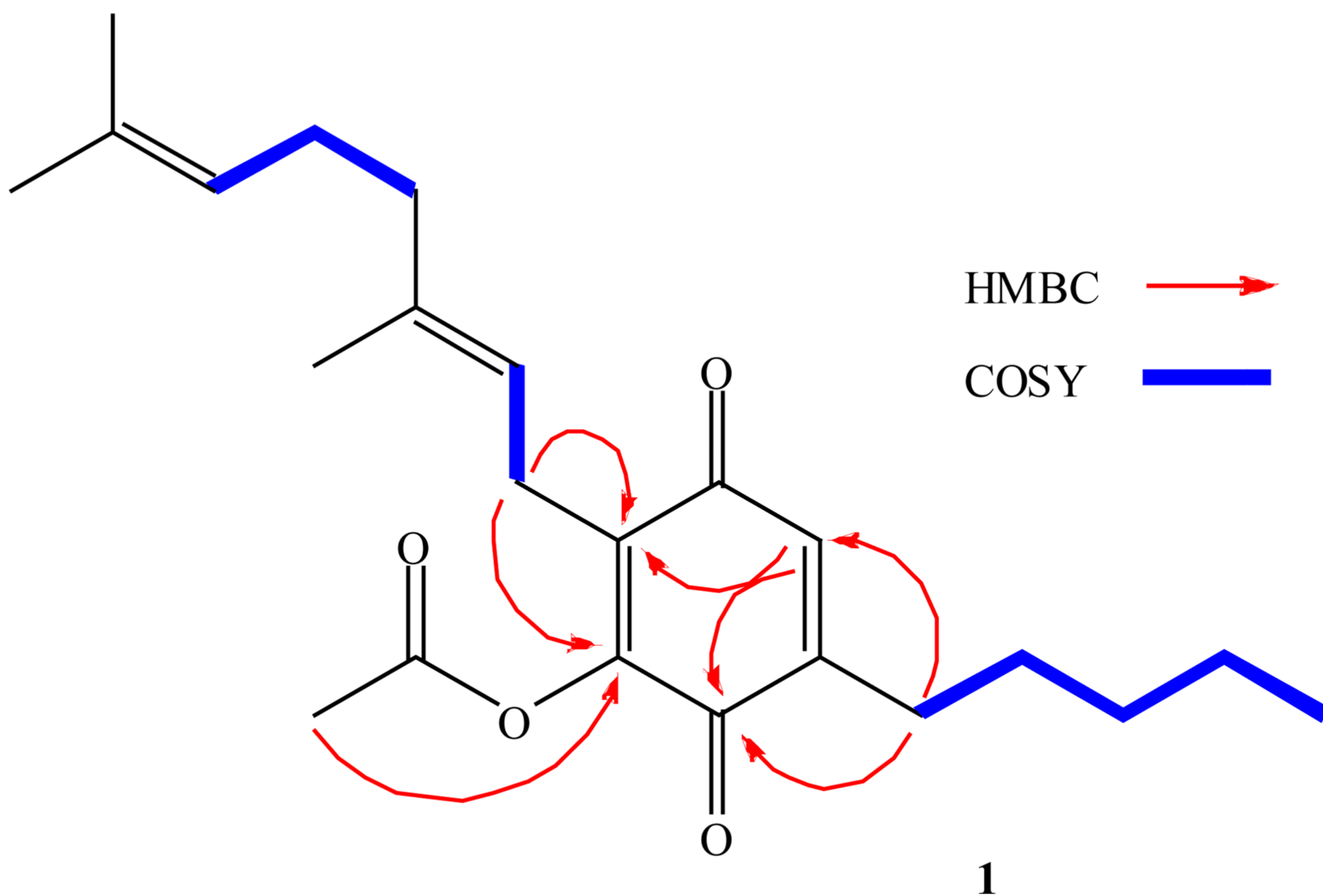

Fig. 1.
HMBC and COSY correlations of **1**.

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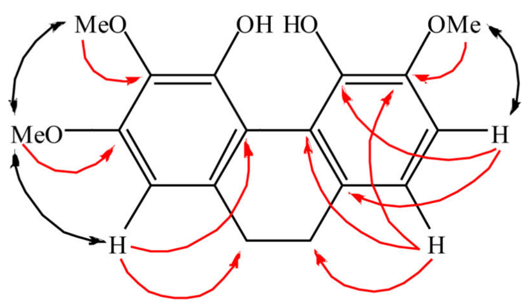

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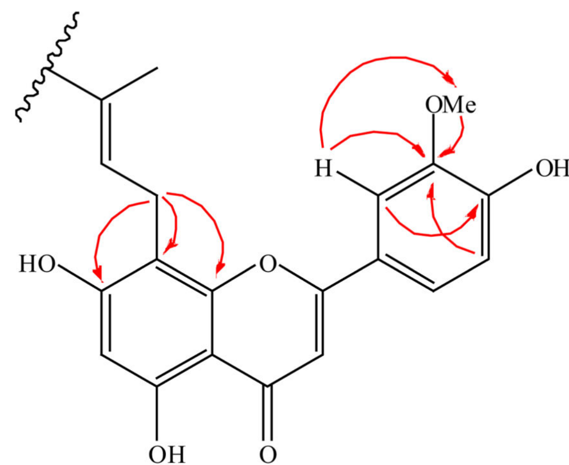

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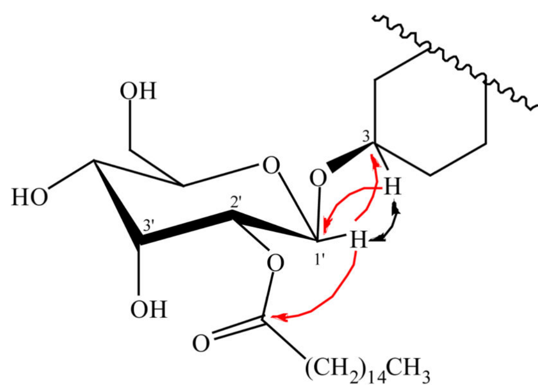

6

HMBC
ROESY

Fig. 2.

HMBC and ROESY correlations of **2, 5** and **6**.

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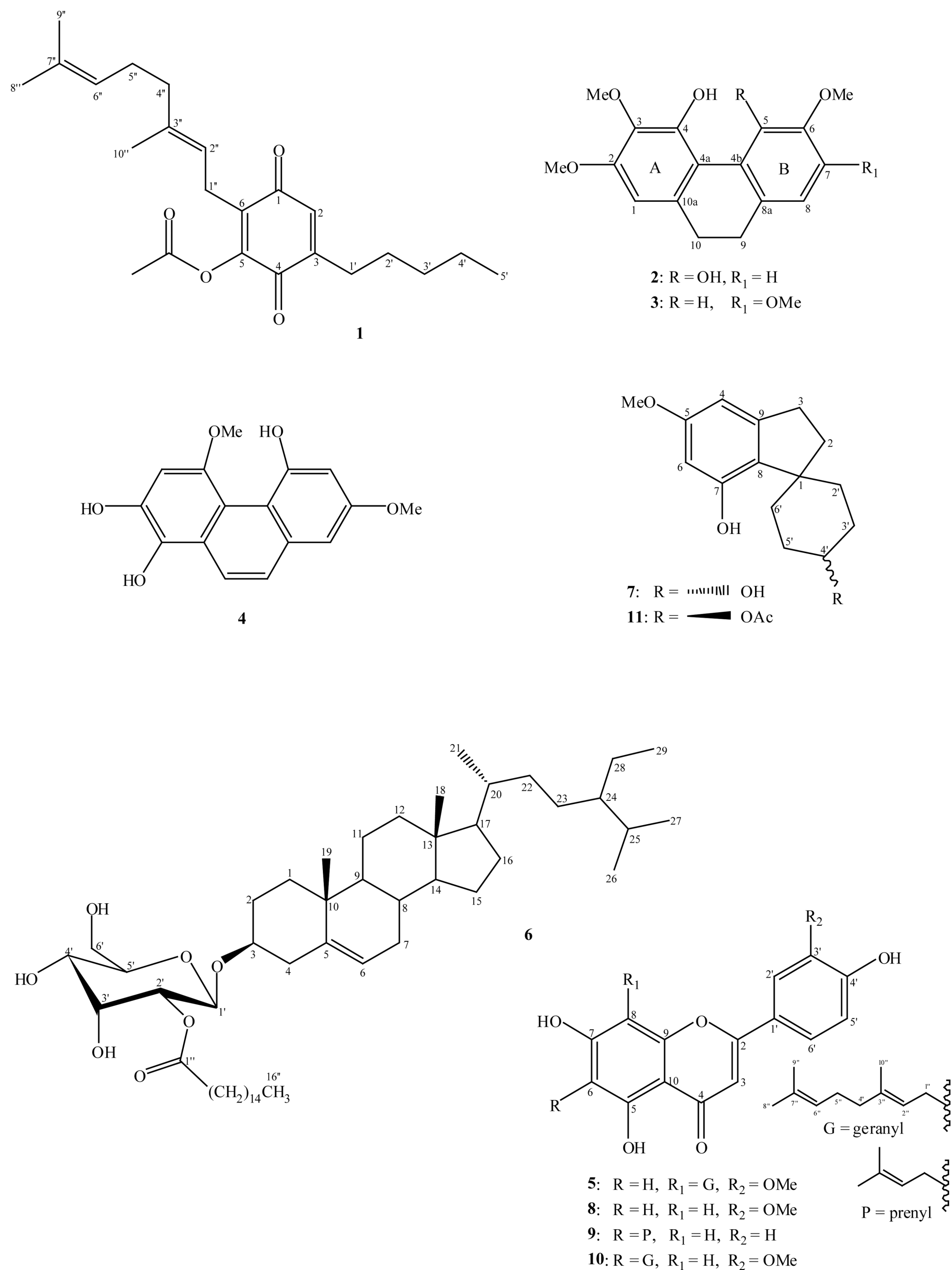

Fig. 3.

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Table 1

[1]H (400 MHz) and ^{13}C (100 MHz) NMR spectroscopic data of **2–4** ($CDCl_3$, δ in ppm, *J* in Hz)[a]

Position	**2**		**3**		**4**	
	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C
1	6.50 *s*	104.4	6.39 *s*	103.9	-	140.2
2	-	152.0	-	150.3	-	140.2
3	-	136.4	-	134.5	6.15 *s*	107.6
4	-	147.2	-	146.8	-	161.5
4a	-	114.9	-	114.7	-	124.2
4b	-	121.1	-	107.8	-	117.4
5	-	141.7	8.02 *s*	111.5	-	156.6
6	--	147.6	--	147.2	6.93 *d* (2.0)	108.9
7	6.74 *d* (8.0)	109.2		147.1	-	161.0
8	6.79 *d* (8.0)	119.1	6.79 *s*	111.1	6.82 *d* (2.0)	102.0
8a	-	132.6	-	125.4	-	129.0
9	2.64 *s*	30.6	2.74 *s*	29.5	8.06 *d* (8.4)	137.7
10	2.64 *s*	31.8	2.74 *s*	30.9	8.12 *d* (8.4)	122.9
10a	-	136.4	-	130.1	-	132.7
OMe-2	3.89 *s*	56.3	3.88 *s*	56.2	-	-
OMe-3	3.86 *s*	61.0	3.89 *s*	61.3	-	-
OMe-4	-	-	-	-	3.96 *s*	57.2
OMe-6	3.85 *s*	56.1	3.92 *s*	56.0	-	-
OMe-7	-	-	3.92 *s*	56.1	3.90 *s*	55.7

[a]Assignments confirmed by APT, gHMQC, gCOSY and gHMBC experiments