Laevifins A–G, clerodane diterpenoids from the Bark of *Croton oblongus* Burm.f.

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1. Introduction

The genus *Croton* is the second largest of the Euphorbiaceae family, comprising about 1300 species distributed in tropical and subtropical world regions (Salatino et al., 2007). Several species are used worldwide as traditional medicines for various conditions, including gastric diseases (Craveiro et al., 1980), snake bites (Lima et al., 2010), wound healing (Pieters et al., 1993; Rao et al., 2007), rheumatism and ulcers (Rao et al., 2007; Nardi et al., 2003), diarrhoea and cancer (Rao et al., 2007), malaria (Thuong et al., 2012), diabetes, gastrointestinal disturbances and high cholesterol (Campos et al., 2002). In East Africa, *Croton* species are used traditionally as dietary additives and the smoke is inhaled to treat chest complaints (Aldaher et al., 2017). The genus *Croton* is known to produce a range of compounds including terpenoids, alkaloids and flavonoids, some of which have been shown to possess anti-cancer, anti-inflammatory, anti-ulcer, anti-malarial and anti-oxidant activities (Salatino et al., 2007).

A phytochemical investigation of the stem barks of the Malaysian *Croton oblongus* Burm.f. (Syn. *Croton laevifolius* Blume) (Euphorbiaceae) yielded seven previously undescribed *ent-neo*-clerodane diterpenoids, laevifins A–G and the known crovatin (3). Structures were established by a combination of spectroscopic methods including HRESIMS, NMR spectroscopy and X-ray crystallography. The absolute configuration of crovatin and laevifins A–G was established by comparison of experimental ECD and theoretical TDDFT ECD calculated spectra. This is the first report on the occurrence of the sesquiterpenoid cryptomeridiol in a *Croton* species. In vitro cytotoxicity assays on laevifins A, B and G showed moderate activities against the MCF-7 cancer cell line (IC50 102, 115 and 106 μM, respectively) while β-amarin and acetyl aleuritolic acid showed good anti-inflammatory activity on the LPS-induced NF-κB translocation inhibition in RAW 264.7 cells assay with IC50 values of 23.5 and 35.4 μM, respectively.

2. Results

The n-hexane and CH2Cl2 extracts were separated using chromatographic methods. The CH2Cl2 extract yielded seven previously undescribed *ent-neo*-clerodane-type diterpenoids (1, 2, 4–8) and the known crovatin 3 (Moulis et al., 1992), acetyl aleuritolic acid, β-
The HMBC spectrum (Fig. 1a) showed correlations between the H-12 odane diterpenoids with a 12, 20-ether linkage and a 19, 20-lactone NMR chemical shifts were in good agreement with those of furocler-

The 1H NMR spectroscopic data (500 MHz, CDCl3) of compounds 1–4 (J in Hz).

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* Peaks superimposed.

amyrin, β-amyrone, cryptomeriol, pachypodol and β-sitostenone from the n-hexane extract. The structures and absolute configurations of the compounds were established using spectroscopic methods including HRESIMS, NMR spectroscopy, X-ray crystallography and ECD measurements coupled with TDDFT ECD calculations and structures of previously reported compounds were confirmed by comparison against literature data.

Compound 1, laevifin A, was isolated as colourless crystals. HRESIMS showed a [M + H]+ peak at m/z 389.1600 (calcd. 389.1600) establishing the molecular formula as C20H24O7 with ten degrees of unsaturation. The IR spectrum showed absorption bands for a hydroxyl group (3504 cm−1), carbonyl (1749 and 1714 cm−1) and cyclic ethers (1096 and 1030 cm−1) and a β-substituted furan ring (3145, 1505, 875 cm−1). The 1H NMR spectrum (Table 1) showed the characteristic resonances of a β-substituted furan ring at δH 7.39 (bs, 2H) and δH 6.33 (bs, W0/2 = 3.9 Hz). The 13C NMR spectrum (Table 3) showed twenty-one carbon resonances including one methyl (δC 16.4), one methoxy (δC 53.0), four methylene (δC 25.3, 29.7, 29.8, 38.8), two methane (δC 36.8, 39.3), an oxymethine (δC 74.9), a fully substituted oxygenated carbon (δC 73.2), two quaternary carbons (δC 49.1 and 50.6), two carbonyls (δC 171.2 and 174.8), a β-substituted furan ring (δC 108.1, 127.4, 139.1 and 143.9), a disubstituted alkene (δC 126.2 and 129.4) and one unusual downfield signal of a dioxygenated methine carbon at δC 105.0. The 1H and 13C NMR chemical shifts were in good agreement with those of furocler-dane diterpenoids with a 12, 20-ether linkage and a 19, 20-lactone ring, accounting for the downfield shift of the C-20 resonance (δC 105.0), as reported for compounds such as croverin (Fujita et al., 1980), crotonolide D (Liu et al., 2014) and sonderianin (Cravoira et al., 1981).

The HMBC spectrum (Fig. 1a) showed correlations between the H-12 (δH 5.66) and C-9 (δC 127.4, 129.4) and C-10 (δC 126.2) and C-16 (δC 139.1) and C-9 (δC 49.1) resonances. In addition, C-12 (δC 74.9) showed a correlation with H-20 (δH 5.66, brs) which, in turn, correlated with C-

The COSY spectrum (Fig. 1a) showed the following coupled systems: H-10/H2-1/H-2 (δH (6.02)/H-3 (δH 6.03), indicating the presence of an alkene double bond at C-2; two H-6/two H-7/H-8/3H-17; and two H-11/H-12. Finally, the HMBC spectrum confirmed the presence of a methyl ester and hydroxy group at C-4 by correlations observed between the C-18 and H-3 and the downfield C-4 (δC 73.2) and H-2 resonances. Compound 1 has a similar structure to that of croverin, an ent-neo-clerodane from C. levati (Fujita et al., 1980) but the alkene double bond occurs at C-3 in croverin as compared to C-2 in 1. Moreover, 1 has an additional hydroxy group at C-4. The relative configuration of C-12 was deduced to be R* by the NOESY correlation observed between H-12 and H2-1 (Fig. 1b). The H-20 resonance showed correlations with the H-7β and Me-17 resonances indicating that the methyl group is in the β-orientation which placed H-8 in α-orientation, enabling assignment of the configuration at C-20 as S*. Furthermore, H-8 showed correlations with H-7α and H-10 proton resonances, while C-10 also showed a correlation with the C-4-OH proton which determined the configurations at C-10 and C-4. The relative configuration of 1 was determined by ECD studies. The measured ECD spectrum of 1 compares well with the theoretical calculated spectrum for ent-neo-clerodane, with a negative Cottons' effect at (λ = (λ c) 236 (−0.3) and a positive Cottons' effect at (λ = (λ c) 196 (+ 20.2), thus establishing the configurations at the chiral centres as 4R,5R,8S,9S,10S,12R,20S and confirming the structure as an ent-neo-clerodane (Fig. 3).

The HRESIMS of compound 2, laevifin B, isolated as white solid gave a [M + H]+ peak at m/z 391.1772 (calcd. 391.1757) indicating a molecular formula of C21H26O7. Compound 2 differed from 1 only in the structure of ring A. In 2, the C-2 double bond was not present, the C-4 hydroxy group was absent, a 3β-hydroxy group was present and the
carbomethoxy group at C-4 was now alpha. The COSY spectrum for compound 2 showed a H-10 (δ_H 2.27, dd, J = 13.5, 3.0) and H2-1 (δ_H 1.83, m) coupled system. The NOESY spectrum showed correlations between H-20 (δ_H 5.60 brs) and H-6β (δ_H 1.78, m), H-7β (δ_H 1.26, m) and 3H-17 (δ_H 0.99 d, J = 6.5 Hz) and between the H-12 (δ_H 5.35, t, J = 8.5 Hz) and both H2-1 resonances, as expected. In addition, H-4 was α-oriented, as indicated by its correlations with the H-3 and H2-6 resonances. Furthermore, the methyl group of the methyl ester (δ_H 3.68) showed a strong correlation with H-10 indicating that this group was α-oriented and hence H-4 was β-oriented. Thus, the structure of 2 was established as the ent-neo-clerodane diterpenoid as shown in Fig. 9.

The HRESIMS of compound 3 showed a [M + H]^+ peak at m/z 375.1819 (calcd. 375.1808), indicating a molecular formula of C_{21}H_{26}O_{6} and nine degrees of unsaturation. The IR spectrum showed the presence of furan ring with absorptions at 3138 cm\(^{-1}\), 1505 cm\(^{-1}\), 873 cm\(^{-1}\), and 802 cm\(^{-1}\), ester carbonyl stretch (1741 cm\(^{-1}\)), ester C=O stretch (1285, 1209 and 1126 cm\(^{-1}\)) and cyclic ethers (1073 and 1026 cm\(^{-1}\)). NMR analysis (Tables 1 and 3, Fig. 4) indicated the compound was crovatin (Moulis et al., 1992), the known clerodane diterpenoid whose relative conformation was determined by single-crystal X-ray diffraction. This compound has a carbomethoxy group at C-4α, a β-substituted methyl group at C-8, and two acetal groups in a structural moiety CH–O–CH–O–CH–O–CH, accounting for the C-3 (δ_C 75.7), C-19 (δ_C 104.4), C-20 (δ_C 100.7) and C-12 (δ_C 74.9) tertiary carbons, confirmed by HMBC analysis. We have corrected the C/H-6 and C/H-7 NMR assignations in the literature by using HMBC analysis (Table 1). The NOESY spectrum showed correlations between Me-17 and H-20; H-4 and H-3, H-6α and β; and, importantly, between H-4 and H-19, confirming the conformation at C-4 to be the same as in 2. This was confirmed by single-crystal X-ray diffraction which showed that the relative configuration was the same as in crovatin (Fig. 5). The optical rotation of compound 2 has negative value as was previously reported for crovatin. Herein, we confirmed the absolute configuration by comparing the experimental and calcd. ECD spectra. The experimental ECD spectrum of 3 showed a negative Cottons’ effects at λ (Δε) 231 (−2.9) and a positive one at λ (Δε) 209 (+1.1) confirming the presence of the ent-neo-clerodane (Fig. S75, Supporting Information). Thus, compound 3 was confirmed to be crovatin and the absolute configurations of the chiral centres were determined as 3S,4S,5R,8S,9S,10S,12R,20S.

HRESIMS of compound 4, laevifin C, showed a M + H]^+ peak at m/z 373.1623 (calcd. 373.1606) indicating a molecular formula of C_{21}H_{24}O_{6}. A base peak was noted at m/z 355.1517 [M + H - H2O]^+. The NMR spectra (Tables 1 and 3) showed that compound 4 only differed from compound 3 at C-8. The Me-17 (δ_H 0.93, d, J = 7.0 Hz) and
H-8 (δH 1.71, m) resonances seen in the 1H NMR spectrum of 3 were replaced by broad singlets in 4 due to the H2-17 protons (δH 4.75; δH 4.85) of a δ8,17 exocyclic methylene group.

Compound 5, laevif D, was isolated as white solid and had a molecular formula of C21H22O6, as established by a [M + H]+ peak at m/z 389.1607 (calcld. 389.1600) in the HRESIMS. The NMR spectra were very similar to those of 4 but H-4β was replaced by a hydroxyl group, accounting for the extra oxygen in the molecular formula and the downfield shift of C-4 from δC 53.7 in 4 to δC 84.7 in 5.

Compound 6, laevif E, was isolated as colourless crystals. Its HRESIMS showed a [M + Na]+ peak at m/z 413.1582 (calcld. 413.1576), giving a molecular formula of C20H24O7 with nine degrees of unsaturation. The IR spectrum showed absorption signals for hydroxyl group stretch (3453 cm⁻¹), C=O stretching of the esters (1275, 1203 and 1182 cm⁻¹) and C−O stretching of the cyclic ethers (1051 cm⁻¹) and a furan ring (3155, 1505 and 777 cm⁻¹). Compound 6 possessed a similar furanoclerodane skeleton to 3. However, instead of the two acetals C-19 and C-20 present in 3, a hemiacetal C-19 (δC 96.6) and a 20,12-lactone (δC 72.1, C-12; δC 178.4, C-20) were present in 6. The HMBC correlations from the hemiacetal proton H-19 (δH 6.06, s) to C-3 (δC 75.5, C-4 (δC 52.7), C-10 (δC 43.6) (Fig. 6) confirmed the position of H-19 while correlations from H-12 (δH 5.34, t, J = 9.0) to C-9 (δC 52.7), C-20, C-14 (δC 108.1) and C-16 (δC 139.5) and from H2-11 to C-8 (δC 40.2), C-10 (δC 43.6) and C-13 (δC 125.3) established the presence of the 20,12-lactone moiety. The relative configurations of 6 were established by single-crystal X-ray diffraction (Fig. 7). The β-orientations of H-4 and H-19 were supported by the NOESY correlations seen between H-4 (δH 3.06, t, J = 5.5 Hz) with H-3 (δH 4.56, t, J = 5.5) and H-6β (δH 1.76, m) as well as correlations seen between H-19 with H-1β (δH 1.80, m) and H-7β (δH 1.92, m) (Fig. 6b). The ECD spectrum of 6 (Fig. 8) showed two negative Cottons’ effects at λ (Δε) 256 (−0.7) and λ (Δε) 216 (−0.9) as well as two positive Cottons’ effects at λ (Δε) 235 (+1.2) and λ (Δε) 195 (+6.2) which were in accordance with the TDDFT calcld. ECD profile for the 3S,4S,5R,9S,10S,12R-ent-neo-clerodane.

The structures of compounds 7 and 8 were likewise established as ent-neo-clerodanes and, in addition to the 19,20- and 12,20- oxygen linkages seen in compounds 1 and 2, NMR spectra showed an additional 18,19-lactone ring. For compound 7, laevif F, the HMBC spectrum showed correlations from H-19 (δH 5.41, s) to C-5 (δC 44.2), C-6 (δC 30.8), C-10 (δC 43.7), C-18 (δC 175.6) and C-20 (δC 102.2), thus confirming the presence of the additional 18,19-γ-lactone. The NOESY spectrum showed correlations between H-19 and H-6β (δH 1.88, m), H-7β (δH 1.87, m) and Me-17 (δH 1.02, d, J = 6.5 Hz) confirming the configuration at C-5 as R*. The COSY spectrum showed the coupling system of H-10 (δH 1.57, m)/H2-1 (δH 2.44, m; δH 2.60, m)/H-2 (δH 6.17, bs)/H-3 (δH 5.75, dd, J = 10.0, 3.0), confirming the presence of a

![Fig. 6. (a) Selected COSY and HMBC correlations of compound 6 (b) Selected NOESY correlations of compound 6.](image-url)
double bond at C-2. The HRESIMS of 7 gave a molecular ion peak at m/z 359.1476 (calcd. 359.1494) [M + H]^+ indicating a molecular formula of C20H22O6. The IR spectrum showed the presence of a hydroxy group (3429 cm⁻¹), β-substituted furan ring (3044, 1504, 874 cm⁻¹), carbonyl group (1776 cm⁻¹, γ-lactone) as well as C=O stretch of an ester (1203-1129 cm⁻¹) and cyclic ether (1088-1019 cm⁻¹). The downfield shift of C-4, and molecular formula indicated the presence of a hydroxyl group at this position. This type of 18,19-γ-lactone has been found to occur in compounds such as swassin (Roengsumran et al., 1982; Takahashi et al., 1983; Premprasert et al., 2013) as well as methyl-15,16-epoxy-3,13 (16),14-ent-clerodatrien-18,19-olide-17-carboxylate (Youngsa-ad et al., 2007).

Compound 8, laevin G, was isolated as yellow needles. Its molecular formula was determined as C20H22O6, by HRESIMS with a [M + Na]^+ ion at m/z 381.1297 (calcd. 381.1314). The IR spectrum showed the presence of carbonyl groups (γ-lactone, 1785 cm⁻¹ and aliphatic ketone, 1718 cm⁻¹) and C=O stretch of an ester (1162 and 1133 cm⁻¹) and cyclic ethers (1025 cm⁻¹). Compound 8 differed from 7 in the structure of ring A. Instead of the alkene double bond at C-2 in 7, a keto group (δC 202.0) occurred at C-3 in 8. This was confirmed by correlations observed in the COSY spectrum of H-10 (δH 1.48, dd, J = 13.0, 1.5 Hz)/H2-1 (δH 2.47, m; δH 2.16, m)/H2-2 (δH 2.31 m; δH 2.75, dd, J = 19.0, 6.0 Hz) and the HMBC correlation between H2-1 and C-3. The H-4 proton (δH 3.25, s) was confirmed as alpha by a correlation seen in the NOESY spectrum with the H-10 (δH 1.48, dd, J = 13.0, 1.5 Hz) resonance. The structures of compounds 1–8 are given in Fig. 9.

2.1. Biological activities of Croton oblongus Burm.f. Bark

It is known that inflammation is closely linked to tumour promotion and almost all tumours have inflammatory cells reside in them regardless of the underlying cause of the tumour (Rosenberg et al., 1991; Medzhitov, 2008). Hence, extracts and selected pure compounds from the bark of C. oblongus were evaluated for their cytotoxicity and the closely related anti-inflammatory activity via inhibition of LPS-induced NF-κB translocation in RAW 264.7 cells evaluation.

Crude extracts of C. oblongus were screened against a panel of cell lines consisted of normal liver cell line (WRL-68), lung cancer cell line (A549), prostate cancer cell line (PC-3), melanoma cell line (A375), colon cancer cell line (HT-29) and human breast cancer cell lines (MCF-7). IC50 values of the hexane extract against these cell lines ranged between 35.0 and 66.1 μg/mL while no activity recorded against HT-29 cell line. The CH2Cl2 extract showed more selective activity, affecting only A549 (IC50 57.6 μg/mL) and A375 (IC50 63.6 μg/mL) cell lines. The methanol extract did not show activity.

All purified compounds were isolated from hexane and CH2Cl2 extracts, which in the initial screen had shown potential cytotoxicity. However, most compounds were found to have very weak activity with IC50 > 100 μM. Only β-amyrone and β-sitostenone exhibited IC50 < 100 μM, 73 and 94 μM respectively. Compounds 1, 3, β-amyrin and acetyl aleuritolic acid were also tested in the LPS-induced NF-κB translocation inhibition in RAW 264.7 cells assay. Compounds 1 and 3...
showed no activity while β-amyrin and acetyl aleuritolic acid showed good anti-inflammatory activity with IC50 values of 23.5 and 35.4 μg/mL respectively.

3. Experimental

3.1. General experimental procedures

Melting points were measured on Sinososuge SW X-4 melting point apparatus. The UV spectra were obtained using a BioChrom Libra UV–vis spectrophotometer. NMR spectra were acquired using Bruker AV 500 and AV 600 (Bruker BioSpin, Rheinstetten, Germany) NMR spectrometers using CDCl3 as solvent. Chemical shifts (δ) for 1H and 13C NMR spectra are expressed in ppm. HRESIMS was performed on LCMS-TOF (6224 TOF LC/MS, Agilent Technology, USA) mass spectrometer. Single crystal X-Ray crystallography was performed using an Agilent SuperNova Dual diffractometer with an Atlas detector. Electronic circular dichroism (ECD) spectra were measured on JASCO J-815 (Jasco Corporation, Tokyo, Japan) and Chirascan V100 (Applied Photophysics, Surrey, UK) circular dichroism spectrometers using a 1 mm cell and CH3CN as the solvent. Medium pressure liquid chromatography (MPLC) was carried out using a Yamazen W-prep 2XY system with Hi-Flash silica cartridge (Yamazen Corporation, Osaka, Japan). Radial chromatography (RC) was performed on a HRS5257 Chromatotron (Harrison Research, California, USA). High performance liquid chromatography (HPLC) analysis was performed on an Agilent 1200 series instrument (Agilent Technologies, USA) equipped with a photodiode array detector, eluted through an XBRide analytical column (4.6 × 150 mm, 5 μm particle size) from Waters (Waters Corporation, U.S.A.). Recycling preparative high performance liquid chromatography (RP-HPLC) was carried out on a LC-908W preparative system (JAI Co. Ltd., Tokyo, Japan) equipped with a ultra-violet (UV) and refractive index (RI) detectors, eluted through JAI-ODS-L80, JAI-ODS-320, JAI-1H and JAI-2H preparative columns (JAI Co. Ltd., Tokyo, Japan). Column chromatography was carried out using silica gel of 70–230 mesh. Aluminium sheets precoated with silica gel 60 F254 (20 × 20 cm, 0.2 mm thick; Merck, Germany) were used for TLC to check the purity of the compounds and were visualized under UV light (254 and 366 nm) followed by spraying with vanillin/sulfuric acid spraying reagent and heating. Conformational analysis was performed using Spartan’14 (Wavefunction) software using a Molecular Mechanics Force Field (MMFF), Time-Dependent Density Functional Theory (TD-DFT) simulation for Electronic Circular Dichroism (ECD) spectra was carried out using Gaussian09 software at 6-31 + G (d,p) in CH3CN.

3.2. Plant material

The bark of Croton oblongus Burn.f. (Euphorbiaceae) was collected from Hutan Simpan Bukit Bauk, Dungun province of Terengganu, Malaysia in April 1998. The samples were authenticated by Mr. Teo Leong Eng and a voucher specimen (KL 4775) has been deposited in the herbarium of the Department of Chemistry, University of Malaya, Kuala Lumpur, Malaysia.

3.3. Extraction and isolation

The air-dried, powdered stem bark of Malaysian Croton oblongus (1.02 kg) was successively extracted with n-hexane, CH2Cl2 and MeOH by leaving the plant material to soak for 2 × 48 h with each solvent with occasional stirring. The CH2Cl2 extract (19.67 g) was chromatographed using MPLC and eluted with n-hexane/ EtOAc (0–100% EtOAc) followed by EtOAc/MeOH (0–10% MeOH) gradient solvent systems. A total of 135 fractions (18 mL) were collected. Fractions were then pooled to give 23 combined fractions (A - W). Fraction H (563 mg) was re-chromatographed on MPLC using n-hexane/EtOAc (2–50% EtOAc) gradient solvent system to obtain subfractions H1 – H10. A mixture of two compounds in subfraction H5 (85 mg) was subjected to Recycling Preparative HPLC (RHPLC) [JAI-ODS-AP column, solvent system MeOH/water (85:15), after 3 recycles] to give compound 3 (45 mg) and 4 (13 mg). Fraction M (1.48 g) was subjected to silica gel column chromatography (CC) using n-hexane/CH2Cl2 (0–20% CH2Cl2) gradient system to give subfractions M1 - M16. Subfraction M5 was eluted through silica gel CC using n-hexane/EtOAc (20–50% CH2Cl2) gradient system to afford 8 (21 mg). The combined subfractions M6-M9 were chromatographed over CC eluted with n-hexane/EtOAc (10–50% EtOAc) gradient solvent system to yield compound 1 (135 mg) and a small fraction of 8 (7 mg). Repeated CC of subfraction M10 [n-hexane/ EtOAc (10–40% EtOAc)] yielded 7 (2 mg). Subfraction M11 was subjected to CC using n-hexane/EtOAc (10–50% EtOAc) solvent system and fractionated into five subfractions, labeled as M11A-M11E. Subfractions M11C and M11E were subjected to RHPLC to afford 2 (7 mg) [JAI-GE- GS-320 column, solvent system MeOH/water (4:1), after 1 recycle] and 5 (7 mg) [JAI-ODS-L80 column, solvent system MeOH/water (4:1), after 3 recycles]. Meanwhile, 6 (28 mg) was obtained from the purification of subfraction M13 via RHPLC equipped with JAI-1H and JAI-GEL-2H size exclusion preparative columns connected in parallel using CHCl3 (100%) mobile phase, without recycling. Further purification of subfraction M16 via RHPLC gave the sesquiterpene, cryptomeri diol (5 mg) (Ahmad et al., 1992; Egharevba et al., 2012) [JAI-ODS-L80 column, solvent system MeOH/water (4:1), after 7 recycles].

The n-hexane crude extract (0.98 g) was chromatographed on MPLC through a Hi-Flash silica cartridge eluted with n-hexane/EtOAc gradient solvent system (0–100% EtOAc) to give eleven fractions, combined according to their TLC profiles and labeled A-K. Fraction B (24 mg) was subjected to PTLC with n-hexane/EtOAc (94:6) to afford β-amyrone (8 mg) (Shan et al., 2014; Singab et al., 2012). Fraction E gave crystalline white needles of acetyl aleuritolic acid (24 mg) (Cuong et al., 2002). The n-hexane-soluble residue of subfraction E was then chromatographed on RC using gradient n-hexane/EtOAc (1:3–50% n-hexane) solvent system to afford β-sitost erol (6 mg) (Kan et al., 2011). The residue of this RC were re-chromatographed using n-hexane/acetone/ MeOH (95:4:1) isocratic solvent system to give β-amyrin (27 mg) (Kajikawa et al., 2005). Fraction F of MPLC gave a mixture of stigmasterol and β-sitosterol (73 mg). Purification of fraction I using PTLC (n-hexane/acetone 7:3) yielded a flavonoid, pachypodol (7 mg) (Kumari et al., 1986). Structures of known compounds were determined using NMR spectroscopy and confirmed by comparison against literature data as referenced above.

3.3.1. Laevifin A (1)

Colourless crystals, m.p. 158–160 °C; [α]20 D −11 (c 0.01, CHCl3); IR (KBr) νmax 3504, 3145, 1749, 1714, 1505, 1249, 1163, 1096, 1030 and 875 cm−1. UV (CH3CN) λmax = 209 nm; ECD (CH3CN) λ (Δε) 236 (−0.3), 196 (+20.2); 1H and 13C NMR data see Tables 1 and 3. HRE-SIMS m/z = 389.1609 [M + H]+ (calcd. for C21H24O7, 389.1600).

3.3.2. Laevifin B (2)

White amorphous powder; [α]20 D −20 (c 0.01, CHCl3); IR (KBr) νmax 3498, 3144, 1732, 1719, 1505, 1226, 1150, 1100, 1049 and 875 cm−1. UV (CH3CN) λmax = 206 nm; ECD (CH3CN) λ (Δε) 222 (−10.0), 191 (−41.8); 1H and 13C NMR data see Tables 1 and 3. HRE-SIMS m/z = 391.1772 [M + H]+ (calcd. for C22H22O7, m/z 391.1757).

3.3.3. Crovatin (3)

Colourless crystals, m.p. 162–164 °C; [α]20 D −19 (c 0.01, CHCl3); IR (KBr) νmax 3504, 3138, 1741, 1505, 1285, 1209, 1126, 1073, 1026 and 873 cm−1. UV (CH3CN) λmax = 209 nm; ECD (CH3CN) λ (Δε) 231 (−2.9), 209 (+1.1); 1H and 13C NMR data see Tables 1 and 3. HRE-SIMS m/z = 375.1819 [M + H]+ (calcd. for C23H22O8, m/z 375.1808).
3.3.4. Laviycin C (4)

Colourless crystals, 13 mg (CHCl₃), m.p. 163–165 °C; [α]D⁰ -18 (c 0.01, CHCl₃); IR (KBr) νmax 3504, 3134, 1736, 1642, 1505, 1277, 1201, 1161, 1074, 1018 and 876 cm⁻¹; UV (CH₃CN) λ max = 208, 213 nm; ECD (CH₃CN) λ (Δε) 209 (–1.0), 213 (+2.6); ¹H and ¹³C NMR data see Tables 1 and 3. HRESIMS m/z = 373.1623 [M + H]⁺ (calcd. for C₂₁H₂₆O₇).

3.3.5. Laviycin D (5)

White solid; [α]D⁰ -17 (c 0.01, CHCl₃); IR (KBr) νmax 3406, 3144, 1740, 1643, 1270, 1182, 1159, 1091 and 1030 cm⁻¹; UV (CH₃CN) λ max = 192, 213 nm; ECD (CH₃CN) λ (Δε) 231 (–1.1), 213 (+2.6); ¹H and ¹³C NMR data see Tables 1 and 3. HRESIMS m/z = 389.1607 [M + H]⁺ (calcd. for C₂₁H₂₆O₈).

3.3.6. Laviycin E (6)

Colourless crystals, 28 mg (CHCl₃); [α]D⁰ -17 (c 0.01, CHCl₃); IR (KBr) νmax 3453 (OH group), 3155, 1755, 1737, 1644, 1505, 1277, 1203, 1181, 1051, 777 cm⁻¹; UV (CH₃CN) λ max = 209 nm; ECD (CH₃CN) λ (Δε) 209 (+10.9); ¹H and ¹³C NMR data see Tables 2 and 3. HRESIMS m/z = 389.1607 [M + Na]⁺ (calcd. for C₂₁H₂₆O₈).

3.3.7. Laviycin F (7)

White solid, 2 mg (CHCl₃); [α]D⁰ -22 (c 0.01, CHCl₃); IR (KBr) νmax 3429, 3044, 1776, 1644, 1504, 1152, 1130, 1088, 1019 and 874 cm⁻¹. UV (CH₃CN) λ max = 217, 234 nm; ECD (CH₃CN) λ (Δε) 231 (–30.4), 196 (–16.9); ¹H and ¹³C NMR data see Tables 2 and 3. HRESIMS m/z = 359.1476 [M + H]⁺ (calcd. for C₂₁H₂₆O₈).

3.3.8. Laviycin G (8)

Colourless crystals, 28 mg (CHCl₃), m.p. 180–182 °C; [α]D⁰ -14 (c 0.01, CHCl₃); IR (KBr) νmax 1785, 1718, 1162, 1133, 1025 cm⁻¹. UV (CH₃CN) λ max = 198, 209 nm; ECD (CH₃CN) λ (Δε) 209 (–1.0), 213 (+1.4), 219 (+6.1); ¹H and ¹³C NMR data see Tables 2 and 3. HRESIMS m/z = 373.1623 [M + H]⁺ (calcd. for C₂₁H₂₆O₇).
using MTT method (Looi et al., 2013), as described in the Supporting Information.

3.7. Anti-inflammatory assay

The extracts and selected pure compounds were tested for inhibition of LPS-induced NF-κB translocation in RAW 264.7 cells. In this evaluation, RAW264.7 cells were treated with samples prior to stimulation with lipopolysaccharide (LPS); a potent inducer and activator of NF-κB. NF-κB acts as a central mediator of inflammatory responses and compounds that inhibit NF-κB activation are potential anti-inflammatory agents. Immunofluorescence staining of NF-κB p65 was performed. In the absence of LPS, NF-κB p65 was seen in the cytoplasm of RAW264.7 cells. In response to LPS stimulation, NF-κB p65 was translocated from cytoplasm into the nucleus, implying NF-κB activation (Looi et al., 2013). Details of the analyses are provided in the Supporting Information.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.phytochem.2018.10.002.

References