2019

Antibacterial and Inhibitory Activities against Nitric Oxide Production of Coumaronochromones and Prenylated Isoflavones from Millettia extensa

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**Publication Details**

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Abstract
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This journal article is available at Research Online: https://ro.uow.edu.au/smhpapers1/905
Antibacterial and Inhibitory Activities Against Nitric Oxide Production of Coumaronochromones and Prenylated Isoflavones from Millettia extensa


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ABSTRACT: A chemical investigation of leaf and root extracts of *Millettia extensa* led to the isolation and structural elucidation of four new prenylated isoflavones, millexatins G-J (1-4), and three new coumaronochromones, millexatins K-M (5-7), along with 16 known compounds. The structures of the new compounds were determined on the basis of NMR and MS data. Compound 4 is rare isoflavone in having a 2-hydroxyethyl moiety at C-8, whereas the structures of compounds 5-7 formally arise from a ring closure through HO-2′ and C-2. The absolute configurations at the C-2 and C-3 positions of 5 and 6 were determined from their ECD spectra through comparison with those of previously reported compounds. Most of compounds were evaluated for the inhibitory effects against nitric oxide (NO) production on RAW264.7 macrophages and their antibacterial activities. Compounds 18 and 19 inhibited NO production with IC_{50} values of 8.5 and 14.3 μM, respectively. Compounds 13 and 14 showed antibacterial activity against various Gram-positive bacteria with MIC values ranging from 2-8 μg/mL.
*Millettia extensa* (Benth.) Baker is a tropical medicinal plant belonging to the family Leguminoseae. The bark has been used to treat sprains and scabies, whereas the roots have been used as a contraceptive and used as a protective medicine for women after childbirth.\(^1\) Recently, we reported the isolation of prenylated isoflavones from the stems of *M. extensa* and their antibacterial activity.\(^2\) To complete the chemical study of this plant as well as continue the investigation of the bioactivities of the natural products obtained, seven new compounds (1-7) along with 16 known compounds (Chart 1) were isolated and identified from the leaf and root extracts of this plant. Most of the isolated compounds were evaluated for their antibacterial activities as well as their anti-inflammatory activity by assessing their inhibitory effects on LPS-induced nitric oxide (NO) production in RAW264.7 macrophages.

Millexatin G (1) was isolated as a yellow viscous oil and its protonated molecular ion peak at *m/z* 465.1917 ([M + H]\(^+\), calcd 465.1913) in the HRESITOFMS corresponded to the molecular formula of C\(_{27}\)H\(_{28}\)O\(_{7}\). Analysis of the \(^{13}\)C NMR and DEPT data (Table 2) of 1 indicated 27 carbon resonances, corresponding to four methyls (\(\delta_c\) 27.8 (2), 25.4, and 17.8), two methoxy groups (\(\delta_c\) 56.3 and 56.2), seven methines (\(\delta_c\) 154.1, 127.6, 119.4, 115.1, 114.8, 99.4, and 94.4), one sp\(^3\) methylene (\(\delta_c\) 65.6), 12 quaternary carbons (\(\delta_c\) 158.9, 156.8, 156.4, 151.3, 148.9, 143.0, 137.4, 120.1, 110.4, 105.7, 105.1, and 77.5), and one conjugated carbonyl carbon (\(\delta_c\) 180.4). The \(^1\)H and \(^{13}\)C NMR spectroscopic data (Tables 1 and 2) showed characteristic resonances of a pentaoxygenated isoflavone with a *gem*-dimethylpyran group [\(\delta_H/\delta_c\) 6.73 (1H, d, \(J = 9.9\) Hz, H-1′′)/115.1, 5.62 (1H, d, \(J = 9.9\) Hz, H-2′′)/127.6, 1.47 (6H, s, H-4′′ and H-5′′)/27.8, \(\delta_c\) 77.5 (C-3′′)], an isoprenyloxy unit [\(\delta_H/\delta_c\) 5.53 (1H, t, \(J = 6.7\) Hz, H-2′′′)/119.4, 4.63 (2H, d, \(J = 6.7\) Hz, H-1′′′)/65.6, 1.79 (3H, s, H-4′′′)/25.4, 1.76 (3H, s, H-5′′′)/17.8, \(\delta_c\) 137.4 (C-3′′′)], an olefinic proton [\(\delta_H/\delta_c\) 7.83 (1H, s, H-2)/154.1], and three aromatic protons [\(\delta_H/\delta_c\) 6.86 (1H, s, H-
These spectroscopic data were similar to those of millexatin C previously isolated from the stem of *M. extensa*. The difference between these two compounds was that the OMe-5 substituent of millexatin C was replaced by a OH-5 substituent in 1, which displayed a resonance for a H-bonded hydroxy proton at $\delta_H$ 13.20 (1H, s, OH-5). The NOESY correlations between H-3′ and OMe-2′ ($\delta_H$ 3.76) and H-6′ and OMe-5′ ($\delta_H$ 3.84) confirmed the locations of these two methoxy groups. The HMBC correlations of OH-5 to C-4a ($\delta_C$ 105.7), C-5 ($\delta_C$ 156.4), and C-6 ($\delta_C$ 105.1) and of H-1′′ ($\delta_H$ 6.73) to C-5 ($\delta_C$ 156.4), C-7 ($\delta_C$ 158.9), and C-3′′ ($\delta_C$ 77.5) (Figure 1) further supported this assignment.

The HRESITOFMS of millexatin H (2), a yellow viscous oil, exhibited an ion peak at $m/z$ 435.1804 [M + H]$^+$ (calcd for C$_{26}$H$_{27}$O$_6$, 435.1808), corresponding to the molecular formula C$_{26}$H$_{26}$O$_6$. The NMR spectroscopic data (Tables 1 and 2) were very close to those of isoauriculasin (8), except for the disappearance of a H-bonded C-5 hydroxy proton in the $^1$H NMR spectrum of 2. The additional methoxy group ($\delta_H$ 3.89) in 2 was placed at C-5 due to the HMBC correlations (Figure 1) between OMe-5 and C-5 ($\delta_C$ 155.3) and between H-1′′ ($\delta_H$ 6.75) to C-5 ($\delta_C$ 155.3), C-7 ($\delta_C$ 157.6), and C-3′′ ($\delta_C$ 77.3).

Millexatin I (3), a yellow viscous oil, gave a protonated molecular formula of C$_{25}$H$_{25}$O$_5$, which was deduced from its HRESITOFMS data ([M + H]$^+$ $m/z$ 405.1703, calcd 405.1702). When a comparison was made of the $^1$H and $^{13}$C NMR spectroscopic data of 3 (Tables 1 and 2) with those of millexatin D (13), the major difference found was that compound 3 showed a resonance for a H-bonded hydroxy proton at C-5 [$\delta_H$ 13.24 (1H, s) while 13 had a OMe group resonance associated with the C-5 position. In addition, the two singlet resonances for the para-aromatic protons on the B ring of compound 13 were replaced with a set of ABC spin-coupled aromatic protons [$\delta_H$/\(\delta_C\) 7.46 (1H, d, $J = 1.6$ Hz, H-6′)/126.7, 7.37 (1H, d, $J = 8.4$, 1.6 Hz, H-
2′)/128.4, and 6.97 (1H, d, J = 8.4 Hz, H-3′)/117.5) in 3. These assignments were supported by
the HMBC correlations (Figure 1) of OH-5 to C-4a (δC 105.7), C-5 (δC 156.5), and C-6 (δC
105.1), of H-2′ to C-3 (δC 123.4), C-4′ (δC 154.7), and C-6′ (δC 126.7), and of H-6′ to C-3, C-2′
(δC 128.4), C-4′ (δC 154.7), and C-1′′ (δC 39.9).

Millexatin J (4) was obtained as a yellow solid that gave a protonated molecular ion peak
at m/z 397.1292 [M + H]+ (calcd, 397.1287) in its HRESITOFMS, suggesting a molecular
formula of C22H20O7. The NMR spectroscopic data of 4 (Tables 1 and 2) indicated it to be
related structurally to auriculatin (16), except that compound 4 displayed resonances for a 2-
hydroxyethyl moiety at δH 3.69 (2H, t, J = 7.3 Hz, H-3′′)/δC 60.7, and 2.95 (2H, t, J = 7.3 Hz,
H-2′′)/δC 25.8 instead of prenyl unit as in 16. The location of this group at C-8 was confirmed
by the HMBC correlations (Figure 1) of H-2′′ with C-7 (δC 157.1), C-8 (δC 104.5), and C-8a (δC
155.3).

Millexatin K (5), a yellow viscous oil, showed a protonated molecular ion peak at m/z
451.1760 [M + H]+ (calcd for 451.1757) in the HRESITOFMS, corresponding to a molecular
formula of C26H26O7. Comparison of its 1H and 13C NMR data (Tables 1 and 2) with those of
(2R,3S)-3,7,4′-trihydroxy-5-methoxycoumaronochromone indicated that the structure of 5 is a
coumaronochromone derivative. Compound 5 displayed additional resonances for a gem-
dimethylpyran group [δH/δC 6.49 (1H, d, J = 10.0 Hz, H-1′′)/115.0, 5.56 (1H, d, J = 10.0 Hz, H-
2′′)/128.7, 1.41 (3H, s, H-5′′)/27.9, 1.40 (3H, s, H-4′′)/28.2, and δC 78.1], and an isoprenyloxy
unit [δH/δC 5.42 (1H, t, J = 6.1 Hz, H-2′′′)/118.6, 4.42 (2H, d, J = 6.1 Hz, H-1′′′)/64.8, 1.77 (3H,
s, H-4′′′)/25.5, 1.71 (3H, s, H-5′′′)/17.8, δC 138.7 (C-3′′′)]. The HMBC correlations of H-1′′ with
C-5 (δC 156.2), C-6 (δC 110.4), and C-7 (δC 160.5) confirmed the location of the gem-
dimethylpyran unit at C-6/C-7. An isoprenyloxy unit was placed at C-4′ due to the HMBC
correlation of H-1'' with C-4' (δc 161.4). The (2R,3S) absolute configuration of 5 was identified from the comparison of its ECD spectrum (Figure 2) and specific rotation with those of (2R,3S)-3,7,4'-trihydroxy-5-methoxycoumaronochromone.³

The molecular formula of millexatin L (6), a yellow viscous oil, was deduced as C_{26}H_{26}O_{7} based on a sodium adduct molecular ion peak at m/z 459.1422 [M + Na]^+ (calcd for 459.1420) in the HRESITOFMS. The NMR spectroscopic data (Tables 1 and 2) of 6 were comparable to those of 5, except that the resonance for the OMe-5 in 5 was replaced by a H-bonded hydroxy proton resonance in 6 at δH 11.75 (1H, s, OH-5). The (2R,3S) absolute configuration of 6 was assigned from a comparison of its ECD spectrum and specific rotation with those of 5.

Millexatin M (7) was isolated as a yellow solid. The molecular formula C_{25}H_{22}O_{6} was deduced from the protonated molecular ion peak at m/z 419.1490 [M + H]^+ (calcd for 419.1495) in the HRESITOFMS. The NMR spectroscopic data of 7 (Tables 1 and 2) were closely related to those of 5 except that compound 7 displayed a resonance for an OH-4' substituent, instead of resonances for a 4'-oxyprenyl group, and resonances for a prenyl unit at C-8 rather than a resonance for an aromatic proton in 5. The HMBC data confirmed the position of the prenyl group in 7 at C-8 from the correlation of H-1'' with C-8. Additionally, a formal loss of water from compound 5 is postulated to generate the Δ²⁴(3) double bond in 7, resulting in two sp² carbons at C-2 and C-3.

The known compounds were identified as isoauriculasin (8),² 5-O-methyl-4’-O-(3-methyl-2-butenyl)alpinumisoflavone (9),² 2'-deoxyisoauriculatin (10),² isoauriculatin (11),² millexatin B (12),² millexatin D (13),² millipurone (14),⁴ millexatin A (15),² auriculasin (16),² auriculatin (17),² scandenone (18),² 5,7,3’,4’-tetrahydroxy-6,8-diprenyl isoflavone (19),⁵
millexatin F (20), 1 5-hydroxy-7-methoxy-4′-O-(3-methylbut-2-enyl)isoflavone (21), 1 7,4′-di-O-prenylgenistein (22), 1 and erycristagallin (23). 6

Nitric oxide (NO), a highly reactive radical, represents an important signaling molecule that plays a key role in several physiological and pathological processes. 7 This mediator is synthesized endogenously mainly under the catalysis of the inducible (i-), constitutive (c-), or endothelial (e-) isoforms of nitric oxide synthase (NOS), which catalyze NO and L-citrulline formation from O2 and L-arginine. 8 iNOS is a key mediator or regulator in the development and progress of different inflammatory diseases and plays a certain role in carcinogenesis. 9,10 It has become a target for drug development in the treatment of chronic inflammatory diseases. 8 Most of the isolated compounds (1-7, 9, 15, 17-19, and 21-22) were tested for their NO inhibitory effects in RAW264.7 macrophage cells. Among these, compounds 18 and 19 inhibited NO production with IC50 values of 8.5 and 14.3 μM, respectively, more potently than the positive control, indomethacin (IC50 = 62.9 μM). None of these compounds showed cytotoxicity at 10 μM against RAW264.7 macrophage cells (Table S1, Supporting Information). The other compounds were not active against NO production. These results suggest that the para-disubstituted aromatic ring of 18 might improve inhibition of NO production, compared to compounds 16 and 17. The open side chain of the isoprenyl substituent at C-5 and C-6 in 19 seems important for activity when compared with 16.

Thirteen compounds (1-7, 12-14, 19, and 21-22) were tested for their antibacterial activities (Table S2, Supporting Information) against Gram-positive (Micrococcus luteus, Streptococcus mutans, Staphylococcus epidermidis, Bacillus cereus, and Staphylococcus aureus) and Gram-negative (Escherichia coli, Pseudomonas aeruginosa, Salmonella typhimurium, and Shigella flexneri) bacteria. Compound 13 showed strong antibacterial activity against B. cereus
and *S. aureus* with the same MIC value of 8 μg/mL, while 14 exhibited more potent antibacterial activity against *M. luteus*, *S. epidermidis*, and *S. aureus* with MIC values of 2-4 μg/mL. Compound 19 demonstrated more modest antibacterial activities against *M. luteus*, *S. mutans*, and *B. cereus* with the same MIC value of 16 μg/mL. All remaining compounds were found to have weak activities (MIC 32-128 μg/mL) or no activity against the tested bacterial strains. The results of preliminary antibacterial activity suggested that millipurone (14) may have a potential as lead compounds for further evaluation and development of antibacterial agents.

**EXPERIMENTAL SECTION**

**General Experimental Procedures.** The general information on instruments and materials was the same as in previous reports.²

**Plant Material.** The leaves and roots of *M. extensa* were collected, identified, and deposited as described earlier.²

**Extraction and Isolation.** Air-dried leaves of *M. extensa* (856.6 g) were extracted with EtOAc (3 × 6 L, for 3 days) at room temperature. After removal of the solvent by evaporation, the crude extract (72.6 g) was subjected to quick column chromatography (QCC) over silica gel using a gradient of hexanes-acetone to give seven fractions (LA-LG). Fraction LB (154.6 mg) was purified by CC using EtOAc-hexanes (3:17, v/v) to give compounds 1 (1.2 mg), 23 (1.4 mg), and a sub-fraction LB-1. Sub-fraction LB-1 (77.8 mg) was further separated by CC using acetone-hexanes (1:4, v/v) to give 8 (1.7 mg) and 10 (1.9 mg). Fraction LC (2.1 g) was applied to a CC eluting with EtOAc-hexanes (3:17, v/v) to afford 9 (2.1 mg), 17 (2.6 mg), and 20 (1.6 mg). Fraction LE (1.6 g) was similarly subjected to CC using EtOAc-hexanes (3:7, v/v), then followed by CC eluting with acetone-hexanes (1:4, v/v) to give 14 (2.8 mg), 16 (324.6 mg), and 19 (6.0 mg). Fraction LF (451.3 mg) was subjected to a RP-18 CC using MeOH-H₂O (7:3, v/v),
then followed by CC eluting with MeOH-CH₂Cl₂ (1:49, v/v) to yield 2 (4.7 mg) and 15 (0.5 mg). Compound 4 (2.5 mg) was isolated from fraction LG (278.7 mg) by Sephadex LH-20 CC with 100% MeOH and followed by CC using MeOH-CH₂Cl₂ (1:24, v/v).

Air-dried roots of *M. extensa* (3.0 kg) were extracted with EtOAc (3 × 15 L, for 3 days) at room temperature. Evaporation of the solvent under reduced pressure to give the EtOAc extract (153.8 g). This crude extract was subjected to QCC on silica gel using a gradient of hexanes-acetone to provide seven fractions (RA-RG). Fraction RB (7.19 g) was further separated by QCC using EtOAc-hexanes (1:49, v/v), then followed by CC eluting with CH₂Cl₂-hexanes (1:9, v/v) to give 10 (13.0 mg) and 22 (10.2 mg). Fraction RD (496.8 mg) was purified by Sephadex LH-20 CC with MeOH-CH₂Cl₂ (8:2, v/v) and followed by CC using acetone-hexanes (1:19, v/v) to yield compounds 11 (2.3 mg) and 21 (6.3 mg). Fraction RE (173 mg) was subjected to Sephadex LH-20 CC using MeOH-CH₂Cl₂ (8:2, v/v) to give 18 (12.5 mg). Fraction RF (5.6 g) was applied to a QCC eluting with acetone-hexanes (1:9, v/v) to afford 5 (2.0 mg) and 12 (49.0 mg). Fraction RG (1.76 g) was applied to Sephadex LH-20 CC with MeOH-CH₂Cl₂ (8:2, v/v) and followed by CC using acetone-hexanes (1:4, v/v) to give 3 (1.6 mg), 6 (1.7 mg), 7 (3.6 mg), and 13 (1.3 mg).

*Millexatin G* (1): Yellow viscous oil; UV (CHCl₃) \( \lambda_{\text{max}} \) (log \( \varepsilon \)) 275 (4.07), 339 (3.87) nm; IR (KBr) \( \nu_{\text{max}} \) 3442, 2928, 1654, 1511, 1462 cm⁻¹; \(^1\)H and \(^{13}\)C NMR, see Tables 1 and 2; HRESITOFMS \( m/z \) 465.1917 [M + H]⁺ (calcd for C₂₇H₂₉O₇, 465.1913).

*Millexatin H* (2): Yellow viscous oil; UV (CHCl₃) \( \lambda_{\text{max}} \) (log \( \varepsilon \)) 220 (3.97), 265 (4.05), 333 (3.47) nm; IR (KBr) \( \nu_{\text{max}} \) 3384, 2974, 1603, 1509, 1466 cm⁻¹; \(^1\)H and \(^{13}\)C NMR, see Tables 1 and 2; HRESITOFMS \( m/z \) 435.1804 [M + H]⁺ (calcd for C₂₆H₂₇O₆, 435.1808).
Millexatin I (3): Yellow viscous oil; UV (CHCl₃) λmax (log ε) 286 (3.93), 336 (3.32) nm; IR (KBr) νmax 3411, 2974, 1607, 1468 cm⁻¹; ¹H and ¹³C NMR, see Tables 1 and 2; HRESITOFMS m/z 405.1703 [M + H]⁺ (calcd for C₂₅H₂₅O₅, 405.1702).

Millexatin J (4): Yellow solid, mp 190-191 °C; UV (CHCl₃) λmax (log ε) 257 (3.92), 330 (3.39) nm; IR (KBr) νmax 3388, 2932, 1652, 1520, 1437 cm⁻¹; ¹H and ¹³C NMR, see Tables 1 and 2; HRESITOFMS m/z 397.1292 [M + H]⁺ (calcd for C₂₂H₂₁O₇, 397.1287).

Millexatin K (5): Yellow viscous oil; [α]²²D − 28 (c 0.1, CHCl₃); ECD (c 0.05, CHCl₃) λmax nm (Δε) 212 (2.91), 236 (−2.00), 262 (0.87), 285 (−0.14); UV (CHCl₃) λmax (log ε) 237 (2.89), 284 (2.95) nm; IR (CHCl₃) νmax 3435, 2924, 1600, 1470, 1269 cm⁻¹; ¹H and ¹³C NMR, see Tables 1 and 2; HRESITOFMS m/z 451.1760 [M + H]⁺ (calcd for C₂₆H₂₇O₇, 451.1757).

Millexatin L (6): Yellow viscous oil; [α]²²D −36 (c 0.1, CHCl₃); ECD (c 0.05, CHCl₃) λmax nm (Δε) 211 (3.61), 248 (−0.19), 271 (1.22), 323 (−0.34); UV (CHCl₃) λmax (log ε) 211 (3.15), 273 (3.39), 322 (3.08) nm; IR (KBr) νmax 3431, 2978, 1623, 1493 cm⁻¹; ¹H and ¹³C NMR, see Tables 1 and 2; HRESITOFMS m/z 459.1422 [M + Na]⁺ (calcd for C₂₄H₃₀O₇Na, 459.1420).

Millexatin M (7): Yellow solid; mp 249-250 °C; UV (CHCl₃) λmax (log ε) 247 (3.95), 296 (4.01), 351 (3.42) nm; IR (CHCl₃) νmax 3257, 2920, 1609, 1503, 1424 cm⁻¹; ¹H and ¹³C NMR, see Tables 1 and 2; ESITOFMS m/z 419.1490 [M + H]⁺ (calcd for C₂₅H₂₃O₆Na, 419.1495).

Nitric Oxide Production Inhibitory Assay. The nitric oxide production in the medium was previously described by Joo et al.¹¹ Briefly, RAW 264.7 cells were plated at a density of 5.0 × 10⁵ cells/mL in 96-well plates and incubated for 24 h at 37 °C and 5% CO₂. Then culture supernatant of each well was discarded and stimulated with 1 μg/mL LPS in the presence or absence of the compounds. After incubation at 37 °C for 24 h, 50 μL of the culture supernatant
was incubated with 50 μL of a Griess reagent at room temperature for 10 min. The absorbance was measured at 570 nm against a calibration curve with sodium nitrite standards.

**Cell Viability Assay.** Cell viability studies were carried out by the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide; Millipore Sigma) assay, as previously described.\(^\text{12}\)

**Antibacterial Assay.** All bacterial strains used in these experiments were obtained from the Microbiological Resources Center, Bangkok, Thailand. The antibacterial assays were determined as recommended by Clinical and Laboratory Standards Institute.\(^\text{13-15}\)

**ASSOCIATED CONTENT**

**Supporting Information**
The Supporting Information is available free of charge on the ACS Publications website at DOI:

HRESITOFMS, 1D and 2D NMR spectra of new compounds 1–7 (PDF).

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**Notes**
The authors declare no competing financial interest.

**ACKNOWLEDGMENTS**
We are grateful to the Thailand Research Fund and Mae Fah Luang University for financial support (BRG5980012). We thank Mr. M. Van de Bult for the plant identification. University of British Columbia is also acknowledged for certain laboratory facilities.

REFERENCES


Chart 1.
Figure 1. Key HMBC and NOESY correlations for compounds 1-7.

Figure 2. ECD spectra for compounds 5 and 6.
Table 1. \( ^1 \text{H} \) NMR (600 MHz) Spectroscopic Data of Compounds 1–7 in CDCl\(_3\) (\( \delta \) in ppm, \( J \) in Hz)

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\(^a\) Data of 4 and 7 were measured at 400 MHz.

\(^b\) Recorded in acetone-d\(_6\)

\(^c\) Recorded in DMSO-d\(_6\)

\(^d\) Interchangeable
Table 2. $^{13}$C NMR (150 MHz) Spectroscopic Data of Compounds 1–7 in CDCl$_3$ ($\delta$ in ppm)$^a$

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$^a$ Data of 4 and 7 were measured at 100 MHz.

$^b$ Recorded in acetone-$d_6$.

$^c$ Recorded in DMSO-$d_6$.

$^d$ Interchangeable.
Table of Content