Supplementary Material

Chemical constituents of *Clausena lenis*

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Abstract: Phytochemical examination of *Clausena lenis* Drake (Rutaceae), collected in Thailand, led to the isolation of seven coumarins, four furoquinolines, two amides, and one flavonoid glycoside. Four of these compounds, one coumarine derivative named as gravelliferone A (3), two furoquinoline derivatives (kokusagenin A (8) and B (9)) and one amide, clausenalansamide H (13), are reported for the first time. Compound 3 was isolated from the root bark, compound 8 from the stem bark and compounds 9 and 13 from the leaves. The molecular structures of all isolated compounds were established by means of NMR experiments combined with mass spectrometry. Preliminary tests of the lipophilic stem bark extract against various human pathogenic gram positive and gram negative bacteria strains revealed promising effects against *Staphylococcus aureus* ATCC 43300.

Keywords: *Clausena lenis*; Coumarin; Aurantioidae; furoquinoline; Kokusagenin A; Kokusagenin B; Rutaceae
List of content
General experimental procedures
NMR data of 3 and 13
Table S1. NMR data of the 8 and 9
Table S2. Antibacterial activities against human pathogenic bacteria strains.
Figure S1. HMBC (left) and NOESY (right) correlations of gravelliferone A (3)
Figure S2. \(^1\)H-NMR spectrum of 3
Figure S3. \(^1\)H-NMR spectrum of 3, expansion
Figure S4. \(^1\)H-NMR spectrum of 3, expansion
Figure S5. \(^13\)C-NMR spectrum of 3
Figure S6. COSY spectrum of 3
Figure S7. HSQC spectrum of 3
Figure S8. HMBC spectrum of 3
Figure S9. NOESY spectrum of 3
Figure S10. HR-MS of 3
Figure S11. \(^1\)H-NMR spectrum of 8
Figure S12. \(^1\)H-NMR spectrum of 8, expansion
Figure S13. \(^1\)H-NMR spectrum of 8, expansion
Figure S14. \(^13\)C-NMR spectrum of 8
Figure S15. \(^13\)C-NMR spectrum of 8, expansion
Figure S16. HSQC spectrum of 8
Figure S17. HMBC spectrum of 8
Figure S18. NOESY spectrum of 8
Figure S19. HR-MS of 8
Figure S20. \(^1\)H-NMR spectrum of 9
Figure S21. \(^1\)H-NMR spectrum of 9, expansion
Figure S22. \(^1\)H-NMR spectrum of 9, expansion
Figure S23. \(^13\)C-NMR spectrum of 9
Figure S24. COSY spectrum of 9, expansion
Figure S25. HSQC spectrum of 9
Figure S26. HMBC spectrum of 9
Figure S27. NOESY spectrum of 9
Figure S28. HR-MS spectrum of 9
Figure S29. \(^1\)H-NMR spectrum of 13
Figure S30. \(^1\)H-NMR spectrum of 13, expansion
Figure S31. \(^13\)C-NMR spectrum of 13
Figure S32. \(^13\)C-NMR spectrum of 13, expansion
Figure S33. HSQC spectrum of 13
Figure S34. HMBC spectrum of 13
Figure S35. HR-MS of 13
Figure S36. HR-MS of 13
1. General experimental procedures

HPLC analyses were performed on Agilent 1100 Series equipped with an UV-DAD detector, Hypersil BDS-C18 column (250 x 4.6 mm, 5 µm particle size), eluted with Methanol (MeOH) in aq. Buffer consisting of 15 mM ortho-H$_3$PO$_4$ and 1.5 mM Bu$_4$NOH with a flow rate of 1.0 mL min$^{-1}$, injection volume of 10 µL and linear gradient starting from 60% MeOH to 90% at 17 min to 100% at 20 min kept for 8 min. The detection signal was set at 230 nm. Thin layer chromatography (TLC) analyses were performed on silica gel 60 F$_{254}$ plates (Merck) using solvent systems consisting of hexane and ethyl acetate (EtOAc) (60:40) or chloroform/methanol (80:20) used sprayed with anisaldehyde reagent. For preparative TLC silica gel 60 F$_{254}$ plates of 0.5 mm thickness were used. The stationary phases for column chromatography (CC) were either Sephadex LH-20 eluted with MeOH or silica gel 60 (40-63 µm particle size) eluted with mixtures of petrol ether (PE) and EtOAc. Medium pressure liquid chromatography (MPLC) was performed on silica gel 60 columns (40–63 µm particle size). All the preparative separation steps were monitored by analytical TLC and HPLC.

1.1. NMR spectroscopy and mass spectrometry

For NMR spectroscopic measurements, each compound was dissolved in 0.6 mL of CDCl$_3$ or CD$_3$OD. All spectra were recorded on a Bruker Avance II 400 (resonance frequencies 400.13 MHz for $^1$H and 100.63 MHz for $^{13}$C) equipped with a 5 mm observe broadband probe head (BBFO) with z–gradients at room temperature with standard Bruker pulse programs. Chemical shifts are given in ppm, referenced to residual solvent signals (CDCl$_3$: 7.26 ppm for $^1$H, 77.0 ppm for $^{13}$C; CD$_3$OD: 3.31 ppm for $^1$H, 49.0 ppm for $^{13}$C).

Mass spectra were recorded on a high-resolution time-of-flight (HR-TOF) mass spectrometer (maXis, Bruker Daltonics) by direct infusion electrospray ionization (ESI) in positive ionization mode (mass accuracy ± 5 ppm) as well as in negative mode (mass accuracy ± 10 ppm). HR-TOF MS measurements have been performed within the selected mass range of m/z 100–2500. ESI was made by capillary voltage of 4 kV to maintain a (capillary) current between 30 and 50 nA. Nitrogen temperature was maintained at 180°C using a flow rate of 4.0 L min$^{-1}$ and the N$_2$ nebulizer gas pressure at 0.3 bar.

2. Extraction and isolation

The preparation of the crude methanolic leaf, stem bark and root bark extracts were done as described previously (Wongthet et al., 2018). Each crude extract was portioned between petrol ether (PE), chloroform (CHCl$_3$) and water. The aqueous phase was subsequently washed with ethyl acetate and n-butanol.

Root bark—The CHCl$_3$ fraction (1.0 g) from root bark was separated into 16 fractions by silica gel column chromatography (CC) using a step gradient elution of hexane, diethyl ether and MeOH (v/v/v, 95:5:0 to 0:0:100). Fraction 6 to 9 (478 mg) were combined and were
separated by CC over silica gel 60 eluted with mixtures of petroleum ether PE/ EtOAc/ MeOH. This step afforded 20 fractions. Fraction SG01-F01 contained 16.7 mg of pure 1. The fractions containing 3 and 4 (39.4 mg) were combined and subjected to Sephadex LH-20 eluted with MeOH. This step afforded 3.3 mg of 3 and 3.9 mg of 4. The fractions containing 2, 5 and 6 (35 mg) were combined and subjected to Sephadex LH-20 eluted with MeOH. This yielded 38 fractions. Fractions 7–9 contained 1 mg of pure 5. The pooled fractions containing impure 2 and 6 (26 mg) were purified by preparative TLC developed with PE and EtOAc (8:2). This gave 7.7 mg of 2 and 2.2 mg of 6. The pooled fractions containing 7 were purified over a Sephadex LH-20 eluted with MeOH. This afforded 8.2 mg of 7.

Stem bark—The CHCl3 phase (466 mg) was separated by CC over silica gel 60 eluted with mixtures of petroleum ether PE/ EtOAc/ MeOH. Fraction 10 (7.8 mg) was finally purified by MPLC leading to 1.6 mg of 8.

Leaves—The CHCl3 phase (196 mg) was separated by CC over silica gel 60 eluted with mixtures of 5% isopropanol in petrol ether and MeOH. This step afforded 30 fractions. Fractions containing 9 and 10 were pooled and subjected to Sephadex LH-20 eluted with MeOH. This led to 8.3 mg of 9 and 3.1 mg of 10. Compound 11 (5.4 mg) was purified over Sephadex LH-20 eluted with MeOH. The PE phase (1470 mg) was separated by CC over silica gel 60 eluted with mixtures of isopropanol in petrol ether. This step afforded twenty fractions. The pooled fractions containing impure 12 and 13 were purified over a Sephadex LH-20 eluted with MEOH and followed by MPLC. These both steps led to 4.7 mg of 12. Compound 13 (1.2 mg) was obtained by preparative TLC. The water phase (365 mg) was subjected to Sephadex LH-20 eluted with MeOH. This step yielded 5.2 mg of 14.

2.1. Antibacterial assay

This screening was conducted against the four-gram positive bacteria strain Staphylococcus aureus ATCC 25923, S. aureus ATCC 43300, Enterococcus faecium UCLA192, and E. faecalis ATCC 29212 and eight-gram negative bacteria strains Escherichia coli ATCC 25922, Pseudomonas aeruginosa DMST 37166, Klebsiella pneumonia ATCC-BAA 1705, K. pneumoniae ATCC-BAA 1706, K. pneumoniae ATCC 700603, Acinetobacter baumannii ATCC 19606, Stenotrophomonas maltophilia DMST 19079, and Salmonella choleraesuis ATCC 10708, obtained from the Department of Medical Sciences, Ministry of Public Health in Thailand. The strains were stored in Muller-Hinton broth (MHB) (Oxoid, Hamshire, UK) and adjusted to a turbidity of 0.5 McFarland standard and then plated thoroughly on Muller-Hinton agar (MHA) (Oxoid). The minimum inhibitory concentrations (MIC) were determined using a broth microdilution technique, following the recommendations of the CLSI 2014. Stock solutions of 40 mg/mL prepared by dissolving each lipophilic extract in dimethylsulfoxide (DMSO) Sigma-Aldrich, USA) were serially
diluted in Mueller Hinton Broth (MHB) to achieve in-test concentrations ranging from 1.56 to 3.200 µg/mL. Each bacterial inoculated was prepared in MHB. Each inoculum was standardized in order to obtain a concentration of $5 \times 10^5$ CFU/mL in each inoculated well of the microtiter plate. Each experiment was repeated twice. The microtiter plates were incubated at 37 °C in incubator (Biobase, BJPX-H50) and read at 18-20 hours. The commercially available antibiotics ciprofloxacin and ampicillin were used as positive control.
NMR data of 3 and 13

Gravelliferone A (3): white amorphous powder; HR-ESI-MS \( m/z = 329.1754 \ [M+H]^+ \) (calcd. 329.1747) and \( m/z = 351.1575 \ [M+Na]^+ \) (calcd. 351.1567); UV max \( \text{MeOH/H}_2\text{O} \) 222, 296 sh, 238 nm; \(^1\)H (CDCl\(_3\), 400 MHz), \( \delta \) [ppm]: 1.34 (3H, s, H-5′′), 1.40 (3H, s, H-4′′), 1.47 (6H, s, H-4′, H-5′), 2.77 (1H, m, H-1′a), 2.98 (2H, m, H-1′b, H-2′′), 3.89 (3H, s, OMe), 5.08 (1H, d, \( J = 17.4 \), H-3′a), 5.09 (1H, d, \( J = 11.0 \), H-3′b), 6.17 (1H, dd, \( J = 17.4, 11.0 \), H-2′), 6.78 (1H, s, H-8), 7.29 (1H, s, H-5), 7.52 (1H, s, H-4). \(^{13}\)C (CDCl\(_3\), 100 MHz), \( \delta \) [ppm]: 19.0 (C-4′′), 24.8 (C-5′′), 26.1 (C-4′, C-5′), 29.1 (C-1′), 40.4 (C-1′), 55.9 (OMe), 58.9 (C-3′′), 63.4 (C-2′′), 98.1 (C-8), 112.1 (C-3′), 112.5 (C-4a), 123.7 (C-6), 128.4 (C-5), 131.8 (C-3), 137.8 (C-4), 145.6 (C-2′), 154.0 (C-8a), 159.9 (C-7), 160.1 (C-2).

Clausenalansamide H (13): white amorphous powder; HR-ESI-MS \( m/z = 338.0917 \ [M+Na]^+ \) (calcd. 338.0918); UV max \( \text{MeOH/H}_2\text{O} \) 214 sh, 262 nm; \(^1\)H (CDCl\(_3\), 400 MHz), \( \delta \) [ppm]: 3.03 (3H, s, NCH\(_3\)), 3.82 (1, d, \( J = 7.5 \), OH), 4.70 (1H, dd, \( J = 7.5, 3.5 \), H-8), 5.27 (1H, d, \( J = 3.5 \), H-7), 6.24 (1H, d, \( J = 8.6 \), H-11), 6.30 (1H, d, \( J = 8.6 \), H-12), 7.25-7.40 (8H, m, H-3,4,5,14-18), 7.44 (2H, dd, \( J = 7.9, 2.0 \), H-2,6). \(^{13}\)C (CDCl\(_3\), 100 MHz), \( \delta \) [ppm]: 35.2 (NCH\(_3\)), 63.4 (C-7), 73.2 (C-8), 126.8 (C-11/12), 126.9 (C-11/12), 127.8 (C-2,6), 128.5 (C-3,5 or C-15,17 or C-14,18), 128.7 (C-4 or C-16), 128.8 (C-3,5 or C-15,17 or C-14,18), 128.9 (C-4 or C-16), 129.0 (C-3,5 or C-15,17 or C-14,18), 133.4 (C-13), 137.9 (C-1), 170.9 (C-9).
Table S1. NMR shifts of compounds 8 and 9 recorded in CDCl$_3$.

| position | $^1$H (ppm) | $^{13}$C (ppm) | $^1$H (ppm) | $^{13}$C (ppm) |
|----------|-------------|----------------|-------------|----------------|
| 1a       | -           | 163.09         | -           | 162.83         |
| 2        | 7.57 (d, $J = 2.7$) | 142.47         | 7.59 (d, $J = 2.7$) | 142.93         |
| 3        | 6.98 (d, $J = 2.7$) | 104.80         | 6.96 (d, $J = 2.7$) | 104.16         |
| 3a       | -           | 102.98         | -           | 103.09         |
| 4        | -           | 155.09         | -           | 154.48         |
| 4a       | -           | 113.56         | -           | 113.33         |
| 5        | 7.51 (s)    | 100.57         | 7.51 (s)    | 100.11         |
| 6        | -           | 147.81         | -           | 148.02         |
| 7        | -           | 152.66         | -           | 152.76         |
| 8        | 7.35 (s)    | 106.81         | 7.39 (s)    | 106.74         |
| 8a       | -           | 142.76         | 4.82 (dd, $J = 10.7$, 4.0) | 142.71         |
| 1'       | 5.17 (d, $J = 6.7$) | 68.27         | 4.69 (dd, $J = 10.7$, 6.1) | 70.60         |
| 2'       | 5.64 (t, $J = 6.7$) | 119.30         | 3.35 (dd, $J = 6.1$, 4.0) | 61.14         |
| 3'       | -           | 139.18         | -           | 58.28          |
| 4'       | 1.85 (s)    | 25.84          | 1.45 (s)    | 24.60          |
| 5'       | 1.81 (s)    | 18.43          | 1.42 (s)    | 19.16          |
| 6-OMe    | 4.02 (s)    | 56.04          | 4.03 (s)    | 56.06*         |
| 7-OMe    | 4.03 (s)    | 56.04          | 4.03 (s)    | 56.04*         |

*…..exchangeable
Table S2. Antibacterial activities against human pathogenic bacteria strains. The MIC values are given in µg/mL.

| Strain                              | leaves | stem bark | CIP | AMP |
|-------------------------------------|--------|-----------|-----|-----|
| *S. aureus* ATCC 25923 (methicillin-susceptible strain) \(^1\) | 256    | 256       | 18  | 11  |
| *S. aureus* ATCC 43300 (methicillin-resistant strain) \(^1\) | 64     | 24        | 25  | nt  |
| *E. faecium* UCLA192 (vancomycin-resistant strain) \(^1\)    | 96     | 48        | 6   | 6   |
| *E. faecalis* ATCC 29212 \(^1\)    | 128    | 48        | 23  | 8   |
| *E. coli* ATCC 25922 \(^2\)       | 256    | 256       | nt  | nt  |
| *P. aeruginosa* DMST 37166 \(^2\) | 256    | 256       | 44  | 14  |
| *K. pneumoniae* ATCC–BAA 1705 \(^2,3\) | 384    | 512       | nt  | 6   |
| *K. pneumoniae* ATCC–BAA 1706 \(^2,4\) | 384    | 512       | nt  | 6   |
| *K. pneumoniae* ATCC 700603 \(^2,5\) | 256    | 384       | 19  | 6   |
| *A. baumannii* ATCC 19606 \(^2\)  | 256    | 256       | 24  | 6   |
| *S. maltophilia* DMST 19079 \(^2\) | 80     | 160       | 27  | 6   |
| *S. choleraesuis* ATCC 10708 \(^2\) | 256    | 256       | nt  | nt  |

\(^1\) gram positive; \(^2\) Gram negative, \(^3\) KPC-producing strain, \(^4\) carbapenem-resistant strain, \(^5\) SHV-18 producing strain. CIP: Ciprofloxacin; AMP: Ampicillin; nt = not tested.
Figure S1. Significant HMBC (left) and NOESY (right) correlations of gravelliferone A (3).
Figure S2. $^1$H-NMR spectrum of 3.
Figure S3. $^1$H-NMR spectrum of 3, expansion.
Figure S4. $^1$H-NMR spectrum of 3, expansion.
Figure S5. $^{13}$C-NMR spectrum of 3.
Figure S6. COSY spectrum of 3.
Figure S7. HSQC spectrum of 3.
Figure S8. HMBC spectrum of 3.
Figure S9. NOESY spectrum of 3.
Figure S10. HR-MS of 3.
Figure S11. $^1$H-NMR spectrum of 8.
Figure S12. $^1$H-NMR spectrum of 8, expansion.
Figure S13. $^1$H-NMR spectrum of 8, expansion.
Figure S14. $^{13}$C-NMR spectrum of 8.
Figure S15. $^{13}$C-NMR spectrum of 8, expansion.
Figure S16. HSQC spectrum of 8.
Figure S17. HMBC spectrum of 8.
Figure S18. NOESY spectrum of 8.
Figure S19. HR-MS of 8.
Figure S20. $^1$H-NMR spectrum of 9.
Figure S21. $^1$H-NMR spectrum of 9, expansion.
Figure S22. $^1$H-NMR spectrum of 9, expansion.
Figure S23. $^{13}$C-NMR spectrum of 9.
Figure S24. COSY spectrum of 9, expansion.
Figure S25. HSQC spectrum of 9.
Figure S26. HMBC spectrum of 9.
Figure S27. NOESY spectrum of 9.
Figure S28. HR-MS spectrum of 9.
Figure S29. $^1$H-NMR spectrum of 13.
Figure S30. $^1$H-NMR spectrum of 13, expansion.
Figure S31. $^{13}$C-NMR spectrum of 13.
Figure S32. $^{13}$C-NMR spectrum of 13, expansion.
Figure S33. HSQC spectrum of 13.
Figure S34. HMBC spectrum of 13.
Figure S35. HR-MS of 13.
Figure S36. HR-MS of 13. The m/z peak at 340 represents the isotope peak with $^{37}\text{Cl}$.