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Chemical constituents of *Clausena lenis*

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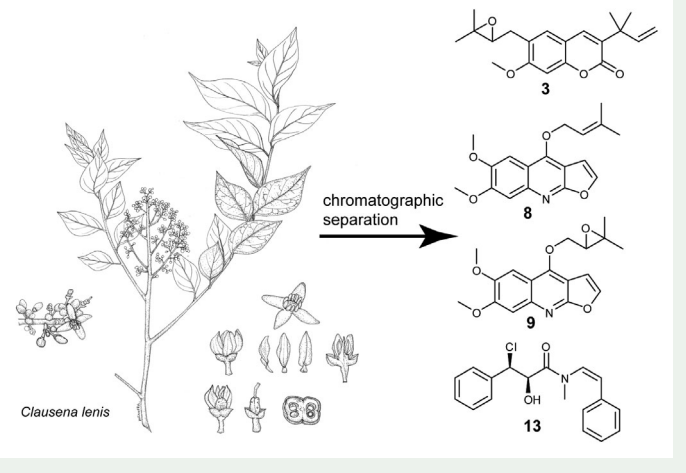
ABSTRACT

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 bacteria strains revealed promising effects against *Staphylococcus aureus* ATCC 43300.



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1. Introduction

The small palaeotropically distributed genus *Clausena* (Aurantioideae, Rutaceae) consists of twenty-five woody species occurring in various forest types (Molino 1994). In Thailand, eight taxa occur (Molino 1994). Several species, such as *C. excavata* Burm. f., *C. harmandiana* (Pierre) Guillaumin and *C. anisata* (Willd.) Hook. f. ex Benth., are widely used by local people for ethnomedicinal and other purposes (Albaayit et al. 2016; Mukandiwa et al. 2016a, 2016b). Phytochemical studies on *Clausena* species revealed the preponderance of prenylated/geranylated coumarins and carbazole alkaloids with interesting bioactivities (Ito et al. 2000; Sripisut et al. 2012; Cao et al. 2018; Yan et al. 2019). Generally, prenylation of specialised metabolites seems to be common within rutaceous species and might be of chemotaxonomic importance for the subfamily Aurantioideae (Lukaseder et al. 2009; Sakunpak et al. 2013; Ma et al. 2018a). The studied species *Clausena lenis* occurs in the border region between China, Thailand and Burma. Phytochemical data are only available from individuals occurring in China (He et al. 2003a, 2006; Liu et al. 2019; Yan et al. 2019) so far. Herein we report four hitherto undescribed compounds from *C. lenis* and screening results of antibacterial activities.

2. Results and discussion

Chromatographic separation of leaf, stem bark and root bark extracts afforded 14 compounds, four of them (**3**, **8**, **9** and **13**) being described for the first time (Figure 1). The root bark yielded the coumarins 3-(1,1-dimethylallyl) xanthyletin (**1**), xanthyletin (**2**), gravelliferone A (**3**), gravelliferone (**4**), 5-isopentenyloxy-8-(2′,3′-epoxyisopentenyloxy)-psoralen (**5**), imperatorin (**6**), and heliettin (**7**). The stem bark afforded the furoquinoline kokusagenin A (**8**) and the leaf extract the furoquinolines kokusagenin B (**9**), kokusagenin (**10**), 1-[(6,7-dimethoxyfuro[2,3-*b*]quinolin-4-yl)oxy]-3-methylbutane-2,3-diol (**11**) together with the amides (2*S**,3*R**)-*N*-methyl-*N*-[(*Z*)-styryl]-3-phenyloxirane-2-carboxamide (**12**), clausenalansamide F (**13**) and the flavonoid glucoside myricitrin (**14**).

The presence of bioactive *C*- and *O*-prenylated pyrano- and furocoumarins is quite common within *Clausena* species (Ouyang et al. 2016; Ma et al. 2018b; Wongthet et al. 2018) and was also reported for *C. lenis* (Liu et al. 2018). In contrast, furoquinoline derivatives (**8**–**11**) seem to be an extraordinary feature of this species, since there has been only one report of such compounds within the genus *Clausena* (He et al. 2003b). Within Aurantioideae, furoquinoline derivatives are reported only for *Aegle marmelos* (Linn.) Correa (Mohammed et al. 2016). They are also known to occur in *Vepris* (Kouam et al. 2018) and *Melicope* species (Chen et al. 2003; Rasamison et al. 2016), which both belong to the subfamily Toddalioideae.

2.1. Structure elucidation of the new compounds

The molecular formula of **3** was determined as $C_{20}H_{24}O_4$ based both on the $[M+H]^+$ peak at $m/z = 329.1754$ (calcd 329.1747 for $C_{20}H_{25}O_4$) and the $[M+Na]^+$ peak at $m/z = 351.1575$ (calcd 351.1567 for $C_{20}H_{24}O_4Na$). The ^1H NMR spectra revealed three

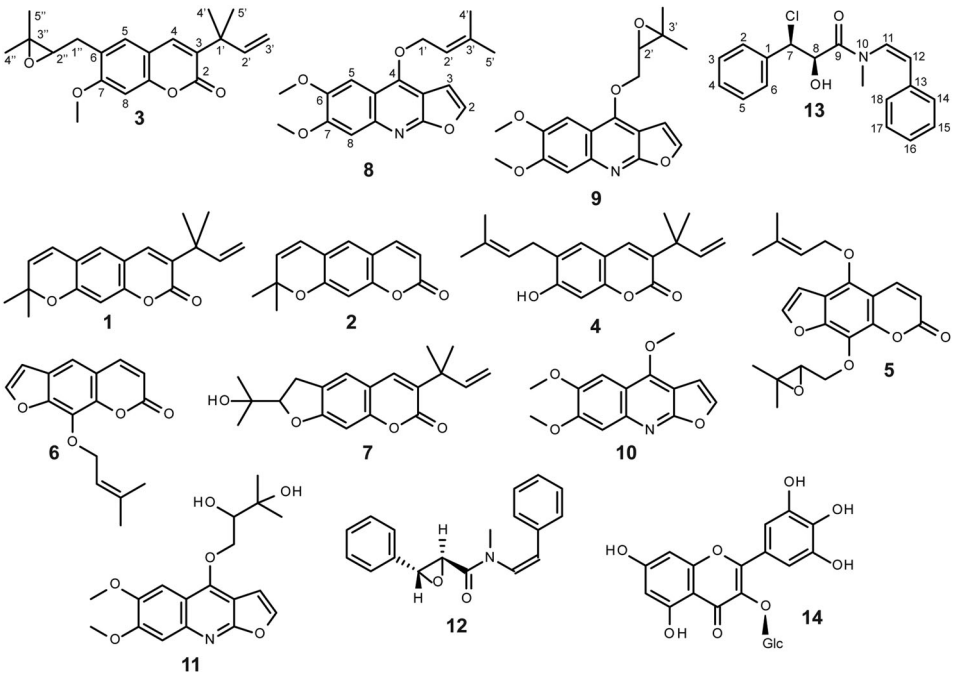

Figure 1. Structural formulae of the isolated compounds from *Clausena lenis* (1–14). 3-(1,1-dimethylallyl) xanthyletin (**1**), xanthyletin (**2**), gravelliferone A (**3**), gravelliferone (**4**), 5-isopentenyloxy-8-(2′,3′-epoxyisopentenyloxy)-psoralen (**5**), imperatorin (**6**), heliettin (**7**), kokusagenin A (**8**), kokusagenin B (**9**), kokusagenin (**10**), 1-[(6,7-dimethoxyfuro[2,3*b*]quinolin-4-yl)oxy]-3-methylbutane-2,3-diol (**11**), (2*S**,3*R**)-*N*-methyl-*N*-[(*Z*)-styryl]-3-phenyloxirane-2carboxamide (**12**), clausenalansamide H (**13**) and myricitrin (**14**).

aromatic singlets at δ_H 7.52, 7.29 and 6.78 ppm lacking the typical doublet of ca $J = 10$ Hz for H-3 and H-4 between 6.0–6.5 ppm (H-3) and 7.7–8.2 ppm (H-4) of coumarins (Szabó et al. 1985). This indicated (1) a substitution at one of these two positions and (2) the presence of a trisubstituted coumarin. Based on the aromatic protons being singlets only a 3,6,7- or 4,6,7-substitution pattern was possible. A NOESY crosspeak between the signals at δ_H 7.52 and 7.29 ppm eliminated the 4,6,7-type, and allowed the assignment of these resonances to H-4 and H-5, respectively. As substituents, one methoxyl (δ_H 3.89 ppm), one epoxyprenyl and one dimethylallyl group were identified. The epoxyprenyl sidechain is characterised by two methyl groups at δ_H 1.34 and 1.40 ppm which show HMBC crosspeaks to one quarternary oxygenated carbon at δ_C 58.9 and an oxymethine carbon at δ_C 63.4 ppm. These two carbons show additional long-range crosspeaks to the remaining CH_2-protons at δ_H 2.77 & 2.98 ppm. The dimethylallyl group is characterised by its vinyl signals at δ_H/δ_C 6.17/145.6 ppm and δ_H/δ_C 5.08 & 5.09/112.1 ppm, a 6H-singlet at δ_H/δ_C 1.47/26.1 ppm and a quarternary carbon at δ_C 40.1 ppm. Analyses of NOESY and HMBC spectra resolved the subsitution pattern: in the NOESY spectra the methoxyl group showed a crosspeak to the singlet at δ_H 6.78 ppm (H-8), whereas the CH_2-group of the epoxyprenyl moiety had a correlation to δ_H 7.29 ppm (H-5) and finally both methyl groups of the dimethylallyl sidechain gave crosspeaks to H-4. This pattern was also proved by relevant HMBC correlations, e.g.,

crosspeaks δ_H/δ_C 7.52/160.1 (H-4/C-2), δ_H/δ_C 7.52/128.4 (H-4/C-5) or δ_H/δ_C 2.77 & 2.98/128.5 (H-1″/C-5). Because no X-ray data were available the absolute stereochemistry of the epoxy substructure remains unclear. Therefore, the structure of compound **3** was elucidated as given in Figure 1 and denominated gravelliferone A. Significant HMBC and NOESY correlations of **3** are presented in Figure S1.

The molecular formula of **9** was determined as $C_{18}H_{19}NO_5$ based on its $[M+Na]^+$ peak at $m/z = 352.1155$ (calcd 352.1155 for $C_{18}H_{19}NO_5Na$) and the $[M+H]^+$ peak at $m/z = 330.1334$ (calcd 330.1336 for $C_{18}H_{20}NO_5$). The ^1H as well as the ^{13}C NMR spectra revealed the typical signals of a furan moiety with resonances at δ_H 7.59/δ_C 142.93 ppm and δ_H 6.93/δ_C 104.16 ppm, where the protons showed the characteristic small doublet coupling of $J = 2.7$ Hz (Nunes et al. 2005). Additionally two aromatic singlet signals at δ_H 7.51 and 7.39 ppm were observed, suggesting the presence of a trisubstituted furoquinoline. This assumption was supported by the detection of two methoxyl groups (both δ_H 4.03 ppm) and one oxy-epoxyprenyl group, the latter characterised by two methyl groups, epoxycarbons at δ_C 58.3 and 61.4 ppm, and the oxymethylene signal at δ_H/δ_C 4.69 & 4.82/70.6 ppm. The substitution pattern was determined with the information extracted from 2 D NMR spectra, allowing complete signal assignment. The methoxyl groups showed NOESY crosspeaks to δ_H 7.51 ppm (H-5) and 7.39 ppm (H-8), locating them at positions C-6 and C-7, whereas H-3 (δ_H 6.96 ppm) gave a NOESY correlation to the OCH_2-protons of the epoxyprenyl sidechain. Therefore, the structure of compound **9** was established as given in Figure 1 and denominated kokusagenin B.

Compound **8** had a molecular formula of $C_{18}H_{19}NO_4$ based on its HR-ESI-MS data at $m/z = 336.1223$ $[M+Na]^+$ (calcd. 336.1206 for $C_{18}H_{19}NO_4Na$) and showed ^1H NMR spectra similar to **9**, with the only difference in the isoprenyl sidechain. The epoxide functionality was replaced by a double bond, characterised by the olefinic signal at δ_H/δ_C 5.64/119.3 ppm. NOESY correlations from the methoxyl groups to H-5 and H-8 and from the prenyl sidechain to H-3 revealed the same substitution pattern as for compound **9**. Similar to **3**, the absolute configuration of the oxirane remains unclear. Compound **8** was shown to be a new furoquinoline alkaloid as well, and was denominated kokusagenin A. The ^1H and ^{13}C NMR data of **8** and **9** are listed in Table S1.

Compound **13** was denominated as clausenalansamide H. This compound showed NMR spectra similar to those of clausenalansamide E (Shen et al. 2017) and gave also the same molecular formula of $C_{18}H_{18}NO_2Cl$, calcd. from its $[M+Na]^+$ peak at $m/z = 338.0917$ (calcd 338.0918 for $C_{18}H_{18}NO_2ClNa$). In the HMBC spectrum, a hydroxyl proton at δ_H 3.82 ppm showed a long-range correlation to the carbonyl carbon at δ_C 170.93 ppm, the oxymethin proton at δ_H 4.70 ppm gave a correlation to only a quarternary aromatic carbon at δ_C 137.92 ppm, whereas the chloromethin proton at δ_H 5.27 ppm had also long range crosspeaks to protonated aromatic carbons. These data confirmed **13** to be also a 3-hydroxy-4-chloro derivative, but compared to clausenalansamide E there are significant shift differences in the ^1H as well as in the ^{13}C spectra. So, the oxymethin resonances reported for clausenalansamide E are at δ_H/δ_C 4.86/72.6 ppm (for **13**: 4.70/73.2 ppm) whereas those of the chloromethin are at δ_H/δ_C 5.04/62.5 ppm (for **13**: 5.27/63.4 ppm). Therefore we assume that **13** is a diastereomer of clausenalansamide E with either *R*,*R*- or *S*,*S*-configuration. As we were also able to

isolate the epoxide **12**, it is hence likely that **13** was formed by addition of HCl, which is often present in traces in $CHCl_3$ used in the isolation process.

2.2. Antibacterial assays

Lipophilic extracts from leaves and stem bark of *C. lenis* were tested against *Staphylococcus aureus* ATCC 25923, *S. aureus* ATCC 43300, *Enterococcus faecium* UCLA192, and *E. faecalis* ATCC 29212 (all gram-positive) as well as the gram-negative 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. The antibiotics ciprofloxacin and ampicillin were used for positive control. The leaf extract showed only moderate activities, but the stem bark extract showed good results against *S. aureus* ATCC 43300 (IC_{50} 24 μg/mL), *E. faecium* UCLA192 (IC_{50} 24 μg/mL) and *E. faecalis* ATCC 29212 (IC_{50} 24 μg/mL) (Table S2) but lack of plant material prevented identification of the active compounds.

3. Experimental

3.1. General experimental procedures

Technical details are provided in the supplementary material.

3.2. Plant material

The plant material was collected and identified by W. Aiyakool and N. Wongthet near Mueang Khong, Chiang Dao, Chiang Mai province, Thailand, in 2017. A voucher specimen (Aiyakool, W. No. 2017-186, BKF No. 194891) was deposited at the Bangkok Forest Herbarium (BKF) in 10900 Bangkok, Thailand.

3.3. Extraction and isolation

A detailed description is provided in the supplementary material.

3.4. Isolated compounds

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 $max_{(MeOH/H_2O)}$ 222, 296 sh, 238 nm; NMR data are given in the supplementary material.

Kokusaginine A (**8**): White amorphous powder; HR-ESI-MS $m/z = 336.1223$ $[M + Na]^+$ (calcd. 336. 1206). UV $max_{(MeOH/H_2O)}$ 246, 250, 308 and 322 nm; NMR data see Table S1.

Kokusaginine B (**9**): White amorphous powder; HR-ESI-MS $m/z = 330.1334$ $[M + H]^+$ (calcd. 330.1336) and $m/z = 352.1155$ $[M + Na]^+$ (calcd. 352.1155); UV $max_{(MeOH/H_2O)}$ 246, 250, 308 and 322 nm; NMR data see Table S1.

Clausenalansamide H (**13**): White amorphous powder; HR-ESI-MS $m/z = 338.0917$ $[M + Na]^+$ (calcd. 338.0918); UV $max_{(MeOH/H_2O)}$ 214 sh, 262 nm; NMR data are given in the supplementary material.

4. Conclusion

Thirteen lipophilic compounds, belonging to coumarins, furoquinolines, amides and one flavonoid glucoside, were isolated from root bark, stem bark and leaves of *Clausena lenis*. All these compounds were herein described for the first time for this plant species. Except the furoquinoline derivatives, the described compounds fit well to the array of compounds known from *Clausena* and related species from this subfamily. The antibacterial activity of the tested lipophilic extract from the stem bark suggests the presence of potential antibacterial agents but identification of these compounds was prevented by lack of plant material.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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