A New Dinor-*cis*-Labdane Diterpene and Flavonoids with Antimycobacterium Activity from *Colebrookea oppositifolia*

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Abstract

The new 14,15-dinor-*cis*-labdane diterpene, named (+)-14,15-dinor-9α-hydroxy-*cis*-labd-11(E)-en-13-one (**1**), was isolated from the acetone extract of the aerial parts of *Colebrookea oppositifolia*, along with the known compounds alnustin (**2**), mosloflavone (**3**), flindulatin (**4**), 5,6,7-trimethoxy baicalein (**5**), tanetin (**6**), scutellarein 4′-methyl ether (**7**), apigenin (**8**), caffeic acid (**9**), anisofolin A (**10**), apigetrin (**11**), and forsythoside A (**12**). Structures of the new and known compounds were established by detailed analysis of 1D and 2D nuclear magnetic resonance studies. The isolated compounds **1**–**12** were evaluated for their antimycobacterium activity against *Mycobacterium tuberculosis* H37Ra and *Mycobacterium bovis* in both dormant and active phases. Compounds **1**, **7**, and **8** exhibited inhibitory activity against *M. tuberculosis* with IC_{50} values in the range of 8.1–55.0 µM (MIC 14.4–119.7 µM) in the active phase and 7.4–43.5 µM (MIC 11.5–123.3 µM) in the dormant phase. Similarly **1**, **7**, and **8** exhibited inhibitory activity against *M. bovis* with IC_{50} values in the range of 4.1–98.5 µM (MIC 13.7–161.0 µM) in the active phase and 4.1–111.1 µM (MIC 13.0–166.4 µM) in the dormant phase.

Key words

Colebrookea oppositifolia · Lamiaceae · 14,15-dinor-*cis*-labdane diterpene · antimycobacterium · flavonoids

Supporting information available online at http://www.thieme-connect.de/products

Colebrookea (Lamiaceae) is a monotypic genus represented by *Colebrookea oppositifolia* Sm. (syn. = *Colebrookea ternifolia* Roxb.), commonly known as Panrasa, and is distributed in hilly parts of India and China [1, 2]. The roots of this shrub are used for epilepsy and the leaves are applied for wound healing and bruises [1–3]. It is used for the treatment of fractures, traumatic injuries, and rheumatoid arthritis in China [2]. Some other traditional uses are: the decoction of its roots is given as an abortifacient; the juice of the leaves is used to stop bleeding and as an eye and ear drop; and the paste of the leaves is applied to toothaches and mouth and tongue sores [4]. Different extracts of this shrub are reported to exhibit antibacterial [5–7], antimycobacterial [8], antioxidant [7], and antifertility [9] activities. Acteoside, a constituent from the aerial parts, exhibited an *in vitro* potent synergistic fungicidal effect in combination with amphotericin B [10]. Different parts of this plant have been studied phytochemically to isolate flavonoids [2, 11–14], acteoside [10], sterols [15], and fatty compounds [11, 15].

Despite the availability of treatment, tuberculosis (TB) continues to be a deadly disease [16–18]. We are continuously involved in the process of the isolation of novel phytochemicals with promising anti-TB activity [19–21]. During our program for the isolation of anti-TB compounds from plants found in Western Ghats of Maharashtra, India, a phytochemical analysis of the acetone extract of the aerial parts of *C. oppositifolia* was performed. Herein we report the isolation and structure elucidation of compounds **1**–**12** (**Fig. 1**) and their evaluation for antimycobacterium activity against two microbial strains, *Mycobacterium tuberculosis* H37Ra and *Mycobacterium bovis* in both active and dormant phases.

Compound **1** was obtained as yellow gum. Analysis of the ^{13}C NMR and DEPT-135 spectra revealed 18 resonances along with a pseudomolecular peak $[M + Na]^+$ at *m/z* 301.2135 in the high-resolution electrospray ionization mass spectrometry (HRESIMS; **Fig. 1 S**, Supporting Information) and allowed for the determination of the molecular formula as $C_{18}H_{30}O_2$, corresponding to four indices of the hydrogen deficiency. The ^1H NMR data (**Table 1**) showed the presence of four tertiary methyl singlets at δ_H 0.86, 0.90, 1.06, and 2.27, and one secondary methyl at δ_H 0.72 with coupling constant 6.8 Hz. Two methine protons at δ_H 6.35 and 6.80 with coupling constant 15.9 Hz suggested the presence of a *trans* double bond. ^{13}C NMR data (**Table 1**) showed the presence of a carbonyl carbon at δ_C 197.8 and two methine carbons at δ_C 130.1 and 151.4 accounted for two indices of hydrogen deficiencies, suggesting that **1** was a bicyclic diterpenoid. The NMR data of **1** was similar to previously reported dinor-labdane diterpene, 8-hydroxy-14,15-dinor-11-labden-13-one [22, 23], except that a tertiary carbon at δ_C 73.5 at position C-8 was replaced by a methine carbon at δ_C 34.0, and a methine carbon at δ_C 67.0 at position C-9 was replaced by the oxygenated tertiary carbon at δ_C 79.6 in compound **1**. These observations established the 14,15-dinor diterpene skeleton for **1**. The structure was confirmed by 2D NMR studies as follows: The methyl protons at δ_H 0.86 and 0.90 showed the heteronuclear multiple bond correlation (HMBC; **Fig. 6 S**, Supporting Information) with a methylene carbon at δ_C 41.6 and a quaternary carbon at δ_C 33.4. A methine proton at δ_H 1.50 showed the HMBC correlation with a methylene carbon at δ_C 30.6 and with a methyl carbon at δ_C 17.2. Methyl protons at δ_H 0.72 and 1.06 and a methine proton at δ_H 6.35 showed the HMBC correlation with an oxygenated tertiary carbon at δ_C 79.6. This suggested the placement of an oxygenated tertiary carbon at position C-9 and a methyl (δ_H 0.72) at position C-8 unequivocally. Other HMBC correlations from protons at δ_H 6.35 and 6.80 with the ketonic carbonyl carbon at δ_C 197.8 and a proton at δ_H 6.35 with a methyl carbon at δ_C 27.9 were also observed (**Fig. 2**). The correlation spectroscopy (COSY; **Fig. 7 S**, Supporting Information) correlations were observed between δ_H 1.38 and 1.43, δ_H 1.43 and 1.17, δ_H 1.50 and 1.31, δ_H 1.31 and 1.52, δ_H 1.52 and 2.04, and δ_H 2.04 and 0.72, and suggested a H_2-1-H_2-2-H_2-3 and H_1-5-H_2-6-H_2-7-H_1-8-H_3-17 linkage, which confirmed the presence of a decalin ring skeleton in **1** (**Fig. 2**).

Comparison of NMR values of **1** with those reported for 8-hydroxy-14,15-dinor-11-labden-13-one [22, 23] revealed a deviation in the chemical shift at the carbon 5, i.e., upfield shift by 10 ppm at C-5 in **1**. This upfield shift of C-5 (~ 10 ppm) can be explained by the *cis*-fused A/B ring junction [24, 25]. The fused rings assume a nonsteroidal conformation (*cis* A/B ring junction),

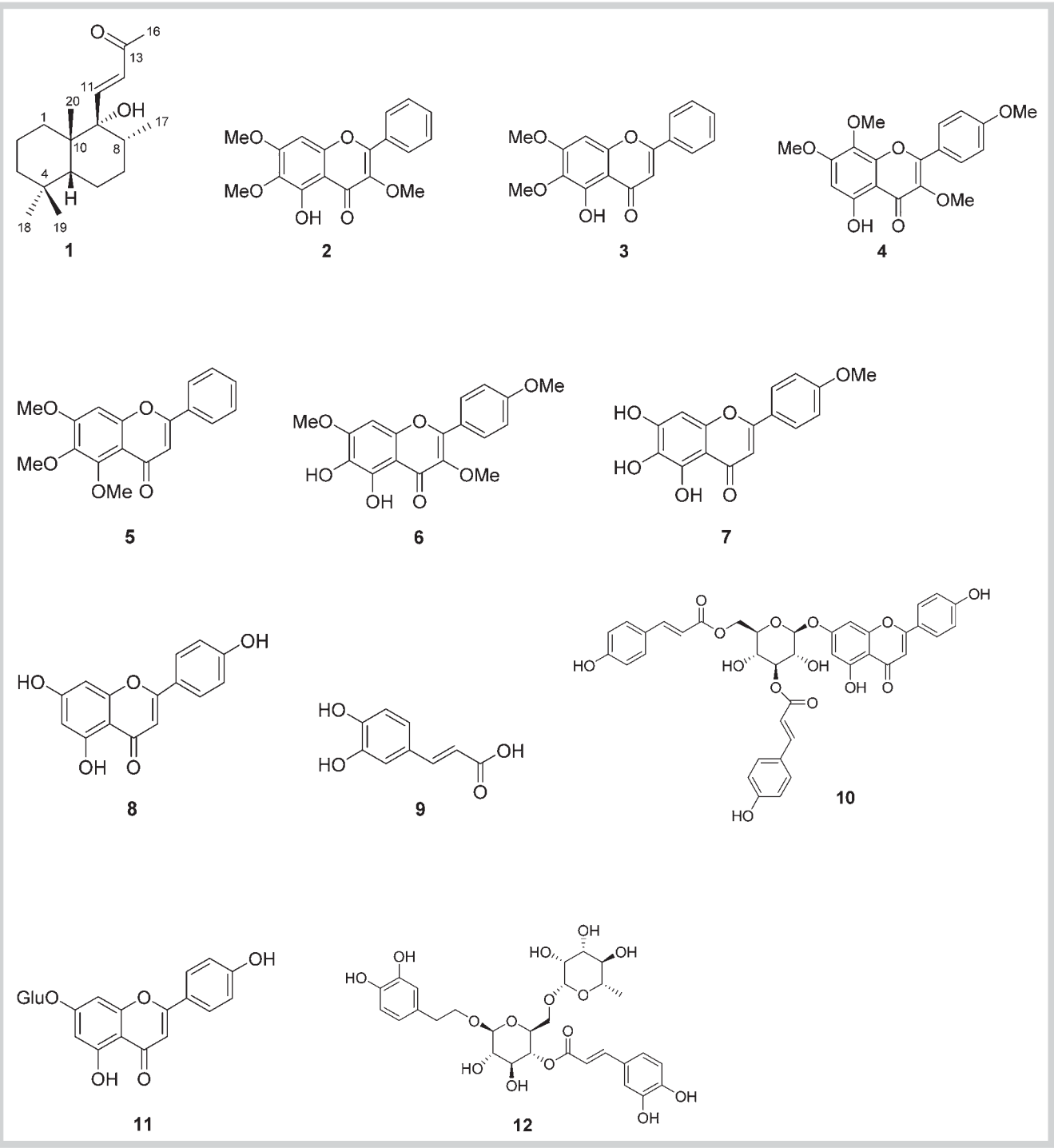

Fig. 1 Compounds **1**–**12** isolated from the acetone extract of the aerial parts of *C. oppositifolia*.

as revealed by the strong nuclear overhauser effect spectroscopy (NOESY; **Fig. 8 S**, Supporting Information) correlation observed between H_3-20 (δ_H 1.06) and H-5 (δ_H 1.50) (**Fig. 2**). Other observed NOESY correlations were between δ_H 1.06 and 6.80 and between δ_H 1.06 and 2.04, and indicated that the presence of the side chain at position C-9 was *β* orientated and a methyl at position C-8 was *α* orientated. Thus, based on a combination of detailed analysis of the 2D NMR data and comparison of observed and literature NMR data with the reported compound [22,23], **1** was identified as a new natural product, (+)-14,15-dinor-9*α*-hydroxy-*cis*-labd-11(E)-en-13-one, and belongs to the rare class of 14,15-dinor-diterpenes with a *cis* A/B ring junction.

Compounds **2**–**12** (**Fig. 1**) were identified by comparison with observed and literature NMR data, and supported by liquid chromatography electrospray ionization mass spectrometry (LCESIMS) data. Compounds **2**–**8** were identified as flavonoids alnustin (**2**) [26], mosloflavone (**3**) [27], flindulatin (**4**) [28], 5,6,7-trimethoxy baicalein (**5**) [29], tanetin (**6**) [30], scutellarein 4′-methyl ether (**7**) [31], and apigenin (**8**) [32]. Compound **9** was

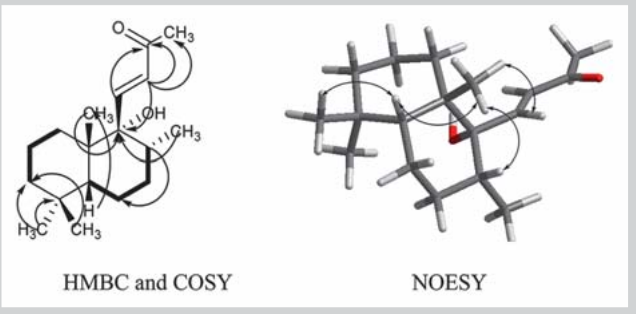

Fig. 2 Key HMBC (H→C), H^1-H^1COSY (–) and NOESY (↔) correlations of compound **1**.

identified as a caffeic acid [33]. Compounds **10** and **11** were identified as flavonoid glycosides anisofolin A [34] and apigetrin [35], respectively. Compound **12** was identified as forsythoside A [36]. Compounds **2**–**4** and **6**–**12** are reported for the first time from the genus *Colebrookea*, except compound **5** was already reported from *C. oppositifolia* [12]. Anisofolin A (**10**), which was previously reported from *Leucas mollissima* Wall. ex Benth. (Lamiaceae) by Chinchansure and coworkers, with its antimycobacterium activity, is also reported in this study [21].

Table 1 ^{1}H and ^{13}C NMR data for compound **1** in $CDCl_3$ (500 MHz for ^{1}H and 125 MHz for ^{13}C, δ in ppm).

Position	δ_H	δ_C	HMBC
1a	1.38 (m)	21.4	–
1b	1.59 (m)		
2a	1.43 (m)	18.4	–
2b	1.52 (m)		
3a	1.17 (m)	41.6	C-18, C-19
3b	1.36 (m)		
4	–	33.4	–
5	1.50 (m)	45.4	C-6, C-20
6a	1.31 (m)	30.6	C-8
6b	1.65 (m)		
7a	1.12 (m)	32.8	C-20
7b	1.52 (m)		
8	2.04 (m)	34.0	C-6
9	–	79.6	–
10	–	42.4	–
11	6.80 (d, *J* = 15.9 Hz)	151.4	C-13
12	6.35 (d, *J* = 15.9 Hz)	130.1	C-9, C-13, C-16
13	–	197.8	–
16	2.27	27.9	–
17	0.72 (d, *J* = 6.8 Hz)	16.2	C-9
18	0.90 (s)	33.7	C-3, C-4
19	0.86 (s)	22.0	C-3, C-4
20	1.06 (s)	17.2	C-5, C-7, C-9

Material and Methods

General experimental procedures, chemical, and biochemicals: Melting points were measured on Buchi B-540 instrument. Optical rotations were measured with a JASCO P-1020 polarimeter. UV spectra were measured with a SpectraMax plus 384 Microplate Reader. The IR spectrum was measured with a Bruker ALPHA FT-IR Spectrometer. ^{1}H and ^{13}C NMR spectroscopic data were recorded on a Bruker Avance Ultra Shield NMR instrument (^{1}H: 500 MHz, ^{13}C: 125 MHz). LCESIMS data were recorded with an API-QSTAR-PULSAR spectrometer. HRESIMS data was recorded with a Thermo-scientific Q-Exactive spectrometer. The silica gel (100–200 and 230–400 mesh) was purchased from Thomas Baker Pvt. Ltd., Mumbai, India. Preparative TLC was carried out using TLC plates supplied by Merck Ltd. MTT and rifampicin were purchased from Sigma-Aldrich, USA. *M. tuberculosis* H37Ra (ATCC No. 25 177) and *M. bovis* (ATCC 35 734) were obtained from AstraZeneca, India.

Plant material: C. oppositifolia aerial parts were collected from Mulshi, District Pune, India on January 12, 2013 and were identified by Dr. Swati Joshi, CSIR-NCL, Pune. A herbarium (Voucher No. HVT-1) was deposited in the Botanical Survey of India, Western Circle, Pune. The plant was cleaned, shade dried, cut, and pulverized.

Extraction and isolation: Pulverized aerial parts, 2.9 kg, were extracted with acetone, 6 L × 3 × 14 h, at room temperature. The acetone solubles were filtered and concentrated under reduced pressure to yield a greenish extract (165.7 g, 5.7% based on dry plant weight), 100 g of which was separated by column chromatography (CC) using a gradient of acetone in petroleum ether from 10 to 100% as an eluent to collect 150 fractions. Fractions showing a similar TLC pattern were combined to afford 16 fractions (COA1–COA16). The isolation of compounds **1**–**12** is provided in Supporting Information.

(+)-14,15-Dinor-9α-hydroxy-cis-labd-11(E)-en-13-one (**1**): yellow gum; $[\alpha]_D^{26}$ + 14.8 (*c* 0.55, $CHCl_3$); UV (MeOH) λ_{max}: 240 nm; IR (Nujol) ν_{max}: 3508, 2924, 1736, 1710, 1673, 1456, 1270, 986 cm^{-1}; ^{1}H NMR ($CDCl_3$, 500 MHz) and ^{13}C NMR ($CDCl_3$, 125 MHz), see **Table 1**; HRESIMS *m*/*z*: 301.2135 $[M + Na]^+$ (calculated for $C_{18}H_{30}O_2$, 278.2246).

Antimycobacterial assay: M. tuberculosis H37Ra (ATCC No. 25 177) and *M. bovis* (ATCC 35 734) strains were tested for their susceptibility to compounds **1**–**12** in active and dormant phases by using the XRMA protocol. All the experiments were performed in triplicate, and IC_{50} and MIC values were calculated from their dose-response curves. The MIC was defined as the lowest concentration of the anti-tubercular agents that prevented visible growth with respect to the growth control [37–39]. Rifampicin was used as a positive control.

Compounds **1**, **7**, and **8** exhibited inhibitory activity against *M. tuberculosis* with IC_{50} values in the range of 8.1–55.0 µM (MIC 14.4–119.7 µM) in the active phase and 7.4–43.5 µM (MIC 11.5–123.3 µM) in the dormant phase (**Table 2**). Similarly **1**, **7**, and **8** exhibited inhibitory activity against *M. bovis* with IC_{50} values in the range of 4.1–98.5 µM (MIC 13.7–161.0 µM) in the active phase and 4.1–111.1 µM (MIC 13.0–166.4 µM) in the dormant phase (**Table 2**).

Supporting information

HRESIMS and 1D and 2D NMR data of compound **1**, dose dependence curves for compound **1**, the isolation of compounds **1**–**12**, and NMR and other characterization data for compounds **2**–**12** are available as Supporting Information.

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Compound	*Mycobacterium tuberculosis* H37Ra		*Mycobacterium bovis*	
	Active	Dormant	Active	Dormant
	IC_{50}* (MIC*)	IC_{50}* (MIC*)	IC_{50}* (MIC*)	IC_{50}* (MIC*)
1	55.0 ± 0.3 (119.7 ± 0.4)	43.5 ± 0.2 (123.3 ± 0.1)	98.5 ± 0.2 (161.0 ± 0.1)	111.1 ± 0.4 (166.4 ± 0.3)
7	23.7 ± 0.2 (80.3 ± 0.3)	32.6 ± 0.3 (77.6 ± 0.4)	16.6 ± 0.2 (46.3 ± 0.4)	13.0 ± 0.4 (27.6 ± 0.1)
8	8.1 ± 0.1 (14.4 ± 0.2)	7.4 ± 0.2 (11.5 ± 0.4)	4.1 ± 0.1 (13.7 ± 0.1)	4.1 ± 0.3 (13.0 ± 0.3)
Rifampicin	0.0021 ± 0.0004 (0.019 ± 0.003)	0.021 ± 0.005 (0.031 ± 0.003)	0.0065 ± 0.0003 (0.034 ± 0.004)	0.018 ± 0.001 (0.037 ± 0.002)

Table 2 Antimycobacterial activity of compounds **1**, **7**, and **8** against *M. tuberculosis* H37Ra and *M. bovis*.

* µM = micro molar

Conflict of Interest

The authors declare no conflict of interest.

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