Vietnam Journal of Chemistry, International Edition, **55**(4): 509-513, 2017
DOI: 10.15625/2525-2321.2017-00500

Chemical constituents and their biological activity of *Pinus dalatensis*

Part 1. Terpenoids from the leaves

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Received 15 July 2017; Accepted for publication 28 August 2017

Abstract

Seven terpenoids were isolated from the dried leaves of *Pinus dalatensis* Ferré, including one caryolane sesquiterpenoid (**1**), five labdane diterpenoids (**2**, **3**, **4**, **5**, **6**) and one serratane triterpenoid (7). The structures of the isolated compounds were determined on the basic of spectroscopic methods (ESI-MS, NMR) and comparison with reported data. Among these compounds, the mixture of compounds **2** and **3** showed inhibitory effects on the growth of acute myeloid leukemia (OCI-AML) cells. This is the first report on the chemical compositions of *Pinus dalatensis* in Vietnam.

Keywords. *Pinus dalatensis*, serratane triterpenoid, caryolane sesquiterpenoid, labdane diterpenoid, acute myeloid leukemia, antiproliferative effect.

1. INTRODUCTION

Pinaceae is the largest family of conifers comprised of 11 genera including 225 species. There are about 114 species, which belong to the genus *Pinus* [1]. *P. dalatensis* is an endemic plant mostly distributed in Lam Dong province of Vietnam. It is a large tree growing up to 40 m high. The needles are finely serrated, 4–10 cm long and in bundles of five. The cones are slender, 6 to 23 cm long and 2 to 4 cm broad (closed), opening to 3 to 9 cm broad [2, 3]. This is a member of the white pine group (subgenus *Strobus* of *Pinus*). In the traditional medicine, the turpentine oils have been used for the treatment of rheumatic and neuralgic ailments, muscle pain, toothaches, bronchitis, and leukemia [4]. Moreover, terpenoids and some other aromatic constituents contribute a large percentage to pine essential oils, which have exhibited significant biological activities, such as antioxidant, antimicrobial [5], antiplatelet [6], and anticancer activities [7]. This study aims to identify the chemical constituents and to evaluate the antiproliferative activity of the major compounds from *P. dalatensis* leaves.

2. EXPERIMENTAL

2.1. General

Optical rotation was measured with a JASCO P2000 Polarimeter at 25 °C and D-line of the sodium spectrum. NMR spectra were recorded on Bruker Avance 500. ESI-MS: Agilent LC-MSD-Trap SL. TLC: Silica gel 60 F_{254} (0.25 mm, Merck); CC: Silica gel 60 (230-400 mesh, Merck) for the first column, silica gel 60, 40-63 μm (Merck) and Sephadex LH-20 for the following columns. Biotests were carried out at University of Perugia, Italy.

2.2. Plant material

Leaves of *P. dalatensis* was collected in January 2013 in Lam Dong province of Vietnam and determined by Dr. Nguyen Tien Hiep, Viet Nam

National Museum of Nature, Vietnam Academy of Science and Technology. A voucher specimen (VNMN. B000005006) is deposited at Viet Nam National Museum of Nature, Hanoi, Viet Nam.

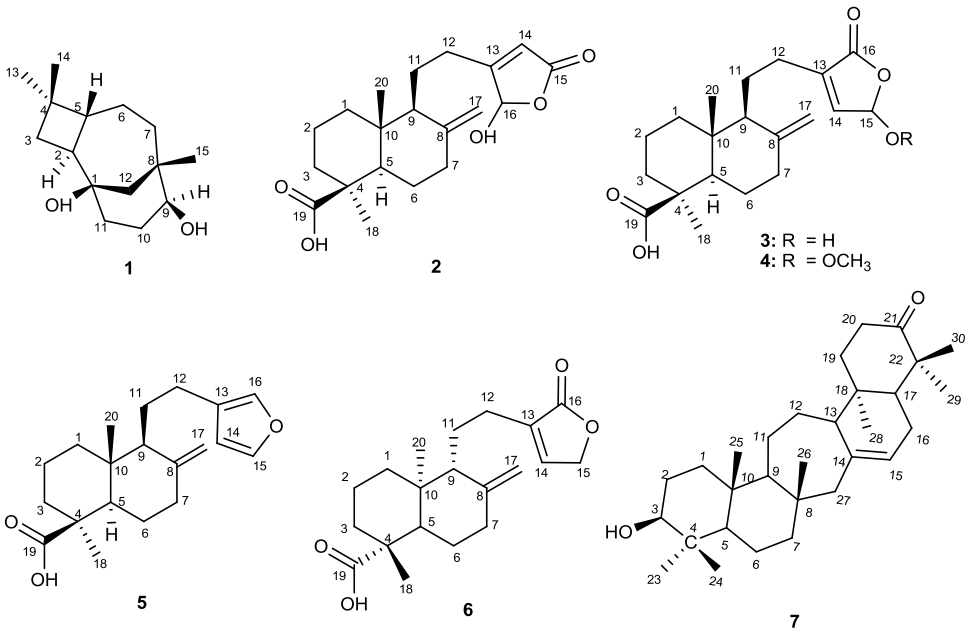

Figure 1: Structures of compounds **1–7** from the leaves of *Pinus dalatensis*

2.3. Extraction and isolation

The air-dried powder of *P. dalatensis* leaves (830 g) was extracted three times (each in 3 days) with 90 % aqueous methanol at room temperature. The MeOH extract was evaporated under reduced pressure and the aq. solution was extracted with *n*-hexane (9.2 g), ethyl acetate (16 g) and *n*-butanol (40 g), successively.

The EtOAc (16 g) fraction was subjected to silica gel CC using a gradient elution of *n*-hexane-EtOAc-MeOH (100:5:0 → 0:80:20) to afford fifteen fractions (F1-F15). The fraction F6 (110 mg) was rechromatographed over a silica gel column using *n*-hexane-EtOAc-MeOH (10:10:1) as eluent to give compound **7** (10 mg). The fraction F7 (390 mg) was chromatographed over silica gel column using *n*-hexane-CH_2Cl_2-EtOAc (increasing amounts CH_2Cl_2 from 15 to 100 %, ethyl acetate from 0 to 95 %) as eluent to give four subfractions (F7.1 to F7.4). The subfraction F7.4 was purified on Sephadex LH-20 column (MeOH) to afford compound **5** (4 mg). The fraction F8 (218 mg) was submitted to Sephadex LH-20 column (MeOH) to afford six subfractions (F8.1→F8.6). The subfraction F8.1 was rechromatographed on sillica gel column eluted with *n*-hexane-CH_2Cl_2-EtOAc (2:2:1) to give compound **4** (6 mg). The fraction F10 (715 mg) was purified by silica gel column with *n*-hexane-CH_2Cl_2-acetone (15:50:2) as eluent to yield five fractions (F10.1→ F10.5). The subfraction F10.5 was rechromatographed on slilica gel column eluted with CH_2Cl_2-EtOAc (1:1) to give compound **1** (12 mg).

The *n*-hexane extract (9.2 g) was chromatographed on silica gel column and eluted with increasing polarity of *n*-hexane-ethyl acetate (97:3→90:10) to yield twelve main fractions (F1→F12). The fraction F8 (312 mg) was rechromatographed on silica gel column eluted with *n*-hexane-EtOAc (20:80→80:20) to give three subfractions (F8.1→F8.3). The subfraction F8.3 was re-chromatographed on silica gel column, eluted with *n*-hexane-CH_2Cl_2 (80:20→15:85) to give compound **6** (15 mg). The fraction F9 (609 mg) was chromatographed over silica gel column using *n*-hexane-CH_2Cl_2-MeOH (30:10:1) as eluent to give seven subfractions (F9.1→F9.7). The subfraction F9.2 was chromatographed over silica gel column, eluting with CH_2Cl_2-MeOH (99:1) to yield the mixture of two isomers (**2**+**3**, 52 mg).

2.4. Spectra data of isolated compounds

Caryolane-1*β*,9*β*-diol (1): yellowish oil, $[\alpha]^{25}_D$: +0.7 (*c*1.0, $CHCl_3$); ESI-MS (*m/z*): 261.2 $[M+Na]^+$; ^{1}H NMR ($CDCl_3$, 500 MHz): δ_H 3.44 (1H, t, *J* = 3.5 Hz, H-9), 2.19-2.23 (1H, m, H-2), 1.89 (1H, m, H-5) 1.19 (3H, br s, H-15), 1.02 (3H, br s, H-14), 1.00 (3H, br s, H-13); ^{13}C NMR ($CDCl_3$, 125 MHz): δ_C 70.7 (C-1), 38.2 (C-2), 34.1 (C-3), 35.1 (C-4), 44.0 (C-5), 20.5 (C-6), 35.5 (C-7), 39.4 (C-8), 72.3 (C-9),

28.2 (C-10), 33.5 (C-11), 42.5 (C-12), 20.8 (C-13), 30.6 (C-14), 26.6 (C-15).

16-Hydroxy-8(17),13-labdadien-15,16-olid-19-oic acid (2): ESI-MS (*m/z*): 371.2 $[M+Na]^+$; ^1H NMR ($CDCl_3$, 500 MHz): δ_H 5.97 (1H, br s, H-16), 5.84 (1H, br s, H-14), 4.89 (1H, br s, H-17a), 4.50 (1H, br s, H-17b), 1.24 (3H, s, H-18), 0.60 (3H, s, H-20); ^{13}C NMR ($CDCl_3$, 125 MHz): δ_C 39.2 (C-1), 21.1 (C-2), 37.9 (C-3), 44.2 (C-4), 56.2 (C-5), 26.0 (C-6), 38.6 (C-7), 147.3 (C-8), 55.6 (C-9), 40.6 (C-10), 21.8 (C-11), 26.8 (C-12), 171.5 (C-13), 117.1 (C-14), 170.3 (C-15), 99.2 (C-16), 106.8 (C-17), 29.0 (C-18), 183.0 (C-19), 12.9 (C-20).

15-Hydroxypinusolidic acid (3): ESI-MS (*m/z*): 371.2 $[M+Na]^+$; ^1H NMR ($CDCl_3$, 500 MHz): δ_H 6.82 (1H, br s, H-14), 6.10 (1H, br s, H-15), 4.89 (1H, br s, H-17a), 4.56 (1H, br s, H-17b), 1.24 (3H, s, H-18), 0.61 (3H, s, H-20); ^{13}C NMR ($CDCl_3$, 125 MHz): δ_C 39.2 (C-1), 19.9 (C-2), 37.9 (C-3), 44.2 (C-4), 56.2 (C-5), 26.0 (C-6), 38.0 (C-7), 147.4 (C-8), 55.7 (C-9), 40.5 (C-10), 21.8 (C-11), 24.3 (C-12), 138.6 (C-13), 143.3 (C-14), 97.3 (C-15), 171.9 (C-16), 106.8 (C-17), 29.0 (C-18), 183.0 (C-19), 12.9 (C-20).

15-Methoxypinusolidic acid (4): yellow oil; $[\alpha]^{25}_D$: +51.8 (*c* 1.0, $CHCl_3$); ESI-MS (*m/z*): 363.2 $[M+H]^+$; ^1H NMR ($CDCl_3$, 500 MHz): δ_H 6.77 (1H, s, H-14), 5.73 (1H, d, *J* = 2.0 Hz, H-15), 4.89 (1H, s, H-17a), 4.57 (1H, d, *J* = 6.0 Hz, H-17b), 3.57 (3H, br s, 15-OCH_3), 1.24 (3H, br s, H-18), 0.60 (3H, br s, H-20); ^{13}C NMR ($CDCl_3$, 125 MHz): δ_C 39.2 (C-1), 19.9 (C-2), 38.0 (C-3), 44.1 (C-4), 56.2 (C-5), 26.0 (C-6), 38.6 (C-7), 147.2 (C-8), 55.7 (C-9), 40.5 (C-10), 21.8 (C-11), 24.6 (C-12), 139.2 (C-13), 141.5 (C-14), 102.5 (C-15), 171.4 (C-16), 106.8 (C-17), 29.0 (C-18), 182.5 (C-19), 12.8 (C-20), 57.0 (15-OCH_3).

Lambertianic acid (5): colorless oil; $[\alpha]^{25}_D$: +80.0 (*c* 1.0, MeOH); ESI-MS: *m/z* 315.2 $[M-H]^+$, *m/z*: 339.3 $[M+Na]^+$; ^1H NMR ($CDCl_3$, 500 MHz): δ_H 7.34 (1H, m, H-15), 7.19 (1H, br s, H-16), 6.25 (1H, d, *J* = 0.5 Hz, H-14), 4.88 (1H, s, H-17a), 4.57 (1H, s, H-17b), 1.23 (3H, br s, H-18), 0.60 (3H, br s, H-20); ^{13}C-NMR ($CDCl_3$, 125 MHz): δ_C 39.0 (C-1), 19.9 (C-2), 37.8 (C-3), 44.2 (C-4), 56.3 (C-5), 26.0 (C-6), 38.7 (C-7), 147.9 (C-8), 55.2 (C-9), 40.4 (C-10), 23.6 (C-11), 24.3 (C-12), 125.4 (C-13), 110.9 (C-14), 142.7 (C-15), 138.7 (C-16), 106.5 (C-17), 29.0 (C-18), 184.4 (C-19), 12.8 (C-20).

8(17),13-*ent*-Labdadien-15→16-lactone-19-oic acid (6): yellowish oil; $[\alpha]^{25}_D$ –44.6 (*c* 1.2, MeOH); ESI-MS (*m/z*): 333.2 $[M+H]^+$; ^1H NMR ($CDCl_3$, 500 MHz): δ_H 7.10 (1H, s, H-14), 4.89 and 4.59 (1H, s and 1H, s, H-17), 4.67 (2H, d, *J* = 1.5 Hz, H-15), 1.24 (3H, br s, H-18), 0.60 (3H, br s, H-20); ^{13}C NMR ($CDCl_3$, 125 MHz): δ_C 39.2 (C-1), 19.9 (C-2), 37.9 (C-3), 44.2 (C-4), 56.3 (C-5), 26.0 (C-6), 38.6 (C-7), 147.4 (C-8), 55.7 (C-9), 40.5 (C-10), 21.9 (C-11), 24.7 (C-12), 134.9 (C-13), 143.9 (C-14), 70.1 (C-15), 174.4 (C-16), 106.8 (C-17), 29.0 (C-18), 183.2 (C-19), 12.8 (C-20).

3*β*-Hydroxy-14-serraten-21-one (7): white powder, mp 247 °C; ESI-MS (*m/z*): 441.4 $[M+H]^+$; ^1H NMR ($CDCl_3$, 500 MHz): δ_H 5.38 (1H, s, H-15), 3.19 (1H, dd, *J* = 11.5 Hz, 4.5 Hz, H-3*α*), 1.09 (3H, br s, H-30), 1.04 (3H, br s, H-29), 0.97 (3H, br s, H-23), 0.92 (3H, br s, H-28), 0.83 (3H, br s, H-26), 0.80 (3H, br s, H-25), 0.77 (3H, br s, H-24); ^{13}C NMR ($CDCl_3$, 125 MHz): δ_C 38.6 (C-1), 27.5 (C-2), 78.8 (C-3), 38.2 (C-4), 51.2 (C-5), 18.9 (C-6), 45.1 (C-7), 37.1 (C-8), 62.7 (C-9), 39.0 (C-10), 25.5 (C-11), 27.2 (C-12), 56.5 (C-13), 138.3 (C-14), 122.0 (C-15), 24.5 (C-16), 55.7 (C-17), 36.2 (C-18), 34.8 (C-19), 38.4 (C-20), 217.0 (C-21), 47.7 (C-22), 28.1 (C-23), 15.4 (C-24), 15.7 (C-25), 19.8 (C-26), 55.9 (C-27), 12.9 (C-28), 24.5 (C-29), 21.6 (C-30).

2.5. Cell line culture and characterization

OCI/AML3 (OCI) were maintained in RPMI medium with 10 % fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin at 37 ºC, 5 % CO_2. OCI cell line was obtained from ATCC and maintained in logarithmic growth and seeded in 24-well plates to evaluate its relative growth and morphology. The culture was maintained at 2×105 cells/mL and treated with varying concentrations of DMSO or compounds (**2**+**3**), were harvested after 24 hours and counted with a hemacytometer.

2.6. Analysis of cell viability and cell cycle progression

Cell viability and cell cycle progression were analyzed by flow cytometry to determine DNA content of cell nuclei stained with propidium iodide (PI). Briefly, cells were collected by centrifugation and washed in PBS. DNA was stained by incubating the cells in PBS containing 50 μg/mL PI and incubated for 30 min at 4 ºC. Fluorescence was measured and analyzed using a Becton Dickinson FACScan and Cell Fit software.

3. RESULTS AND DISCUSSION

Compound **1** was obtained as yellowish oil. The molecular formula was determined as $C_{15}H_{26}O_2$ by a combination of the ^1H-, ^{13}C-NMR and HSQC spectra. The NMR spectra gave 15 skeletal carbons,

including three methyls, six methylenes, three methines, and three quaternary carbons. One oxyquaternary carbon and one oxymethine appeared at δ_C 70.7 (C-1) and 72.3 (C-9), respectively. The ^{1}H NMR spectrum of **1** showed three methyl groups at δ_H 1.19 (3H, br s, H-15), 1.02 (3H, br s, H-14) and 1.00 (3H, br s, H-13), together with the characteristic signals for oxymethine proton at δ_H3.44 (1H, t, *J* = 3.5 Hz, H-9). The NMR spectra and specific rotation value of **1** were similar to the caryolane-1*β*,9*β*-diol from *Sindora sumatrana* [8]. Based on the above evidence, the structure of **1** was assigned as caryolane-1*β*,9*β*-diol.

Compounds **2** and **3** were isolated as an inseparable mixture (2:3) of two isomers. They have the same molecular formula ($C_{20}H_{28}O_5$) based on their ESI-MS (*m/z* 371.2 $[M+Na]^+$) and NMR data. The NMR data showed that **2**&**3** were labdane diterpenes. All ^{13}C-NMR and DEPT spectra contained two sets of resonances attributable to two carbonyl groups (δ_C 171.5/171.9 and 183.0, 2xC), four olefinic carbons (δ_C 171.5/138.6, 117.1/143.3, 147.3/147.4 and 106.80/106.75), one oxygen-bearing methine (δ_C 99.2/97.3), two methine (δ_C 56.23/56.20 and 55.6/55.7) and two methyl groups (δ_C 29.0, 2xCH_3 and 12.8, 2xCH_3). The NMR spectra also showed the presence of an *α*,*β* unsaturated *γ*-lactone moiety with two coupled ^{1}H-NMR signals at δ_H 5.84 (H-14), 5.97 (H-16) for **2** along with at δ_H 6.82 (H-14), 6.10 (H-15) for **3**, and with ^{13}C-NMR at δ_C 171.5 (C-13), 117.1 (C-14), 170.3 (C-15), 99.2 (C-16) for **2** along with δ_C 138.6 (C-13), 143.3 (C-14), 97.3 (C-15), 171.9 (C-16) for **3**. Cross-peaks on the HSQC spectrum helped to assume the position of each hydrogen attached to corresponding carbon in **2**/**3**. Crosspeaks in the HMBC spectrum from H-5 to C-19 and C-20, from H-9 to C-20 accord with the assignment of the *trans-anti-trans-anti* configuration between C-19, H-5, C-20 and H-9. Comparison of these data with those in the literature [9, 10] indicated that **2**/**3** were to be 16-hydroxy-8(17),13-labdadien-15,16-olid-19-oic acid and 15-hydroxypinusolidic acid, respectively.

The NMR spectral data of **4** were similar to those of **3** except the additional signals of a methoxy group (δ_C 57.0 and δ_H 3.57) attached on C-15 (δ_C 102.5). By means of the analysis of NMR spectra and comparing with the data in the reference [11], compound **4** was identified as 15-methoxypinusolidic acid.

Compound **5** was obtained as colorless oil. On the basis of the spectroscopic data (NMR and ESI-MS at *m/z* 339.3 $[M+Na]^+$), its molecular formula $C_{20}H_{28}O_3$ was proposed. ^{13}C-NMR, DEPT and HSQC confirmed the presence of 20 carbons with 28 directly attached protons, including two methyls, eight methylenes, five methines and five quaternary carbons (containing a carbonyl group). The ^{1}H NMR spectrum included resonances consistent with a 3-substituted furan moiety at δ_H 7.34 (1H, m, H-15), 7.19 (1H, s, H-16) and 6.25 (1H, d, *J* = 0.5 Hz, H-14) and two ankenyl proton at δ_H 4.88 (1H, s, H-17a), 4.57 (1H, s, H-17b). These spectral characteristics revealed that compound **5** was a $\Delta^{8,17}$-labdane. Moreover, these spectroscopic data were similar to those of lambertianic acid [12], therefore structure of **5** were deduced as lambertianic acid.

Compound **6** was obtained as yellowish oil. The molecular formula, $C_{20}H_{28}O_4$, was inferred from ESI-MS (*m/z* 333.2 $[M+H]^+$). Its 1D and 2D-NMR spectra were typical of a labdane skeleton and its characteristic to the *ent* types was supported by a negative optical rotation. All NMR data of **6** were in good agreement with those reported for 8(17),13-*ent*-labdadien-15→16-lactone-19-oic acid [13] showing that they are identical, therefore **6** was identified to be 8(17),13-*ent*-labdadien-15→16-lactone-19-oic acid based on NMR spectral analysis.

Compound **7** was obtained as white powder. The ESI-MS spectrum of **7** showed a pseudo-molecular ion peak at *m/z* 441.4 $[M+H]^+$, corresponding to the molecular formula $C_{30}H_{48}O_2$. The ^{1}H-NMR spectrum showed the presence of seven tertiary methyl groups at δ_H 0.97 (3H, s, H-23), 0.77 (3H, s, H-24), 0.80 (3H, s, H-25), 0.83 (3H, s, H-26), 0.92 (3H, s, H-28), 1.04 (3H, s, H-29) and 1.09 (3H, s, H-30), an axial oxygenated methine proton at δ_H 3.19 (1H, dd, *J* = 11.5 Hz and 4.5 Hz, H-3) and proton of double bond at δ_H 5.38 (1H, s, H-15). The ^{13}C-NMR and HSQC spectra indicated 30 carbon signals, including one oxygenated methine (δ_C 78.8), one ketone group (δ_C 217.0), six quaternary carbons, five methines, ten methylenes and seven methyls. The NMR spectra of **7** and 3*β*-methoxy-14-serraten-21-one [14] are nearly identical except for the differences caused by the absence of a methoxy group at C-3. Upon examination of the ^{1}H-, ^{13}C-NMR and HSQC spectra, **7** can be determined as 3*β*-hydroxy-14-serraten-21-one [15].

The effects of the major compounds (the mixture of **2**+**3,** 52 mg) on the growth of OCI-AML cells were investigated by evaluating cell counts, cell death, and the cell cycle. Results show that the higher (25 µg/mL) and intermediate concentrations (12.5 µg/mL) decreased the number of cells, although with different mechanisms since a significant increase of cell death was seen only with the higher concentration that also significantly decreased both G_0/G_1 and the S phases and increased G_2/M phases. On the contrary, the intermediate

concentration significantly increased G_0/G_1 and decreased both the S and the G_2/M phases of the cell cycle. Even the lower concentration had a significant effect in impairing the cell cycle (significant increased the G_0/G_1 and decreased the S and G_2/M phases), although these changes were not sufficient to decrease the OCI cell number. The cell death effect obtained with the higher concentration of the compounds was due to a significant increase in caspase-3-dependent apoptosis, whereas the altered cell cycle progression was due to a slightly increased p53 (for the higher and intermediate compound's concentrations) and a strong increased p21 expression.

Acknowledgment. *We would like to thank the National Foundation for Science & Technology Development (NAFOSTED) of Vietnam (code: 104.01-2013.62) and the Italian Ministery of Foreign Affairs and International Cooperation (MAECI) for financial support for this research.*

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