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CHEMICAL CONSTITUENTS OF THE LEAVES OF *ALNUS NEPALENSIS* D. DON. (BETULACEAE)

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SUMMARY

*Taraxeryl acetate (**1**), physcion (**2**), 1-nonacosanol (**3**), β-sitosterol (**4**), quercetin (**5**), β-sitosterol 3-O-β-D-glucopyranoside (**6**), and quercitrin (**7**) were isolated from the leaves of Alnus nepalensis D. Don. (Betulaceae). Their structures were determined by spectroscopic methods.*

Keywords: *Alnus nepalensis*; Betulaceae; flavonoid; anthraquinone; phytosterol; triterpenoid.

I - INTRODUCTION

Woody plants have been known to produce many biologically active metabolites. In addition to the studies on chemical constituents of herbal medicinal plants, our phytochemical program also targets woody plants as potential sources of useful natural compounds. *Alnus nepalensis* D. Don. (Betulaceae) (Vietnamese name: Tống quán sủi) is a woody plant that reaches up to 10 - 15 m in height. The bark of *A. nepalensis* is used to treat diarrhoea, bacillary dysentery, and inflammatory diseases [1]. The study on *A. nepalensis* should be of interest since the constituents of *Alnus* species have been demonstrated to possess antioxidant [2], anti-inflammatory [3 - 5], anticancer [6] and hepatoprotective effects [2, 7]. In this paper the isolation and structural elucidation of seven compounds, taraxeryl acetate (**1**), physcion (**2**), 1-nonacosanol (**3**), β-sitosterol (**4**), quercetin (**5**), β-sitosterol 3-*O*-β-D-glucopyranoside (**6**), and quercitrin (**7**) (Fig. 1) from the MeOH extract of the leaves of *A. nepalensis* D. Don. (Betulaceae) collected in mountainous areas of northern Vietnam were reported.

II - EXPERIMENTAL

General Procedure

Electron-impact (EI) mass spectra (70 eV) were measured on a Hewlett-Packard 5989B mass spectrometer. Electrospray Ionization (ESI) mass spectra were recorded on a LC/MSD Trap Agilent Series 1100 system with an ESI source. ^{1}H-NMR (500 MHz) and ^{13}C-NMR (125 MHz) with DEPT program spectra were obtained on a Bruker Avance 500 NMR spectrometer. Tetramethyl silane (TMS) was used as zero reference. Silica gel 60 (63-200 μm, Merck, Darmstadt, Germany) was used for open column (CC) and silica gel 60 (15-40 and 40-63 μm, Merck, Darmstadt, Germany) for flash column (FC) chromatography. TLC was performed on precoated DC Alufolien 60 F_{254} sheets (Merck, Darmstadt, Germany) and detected by UV light (λ 254 nm) or by spraying with 1% vanillin in conc. H_2SO_4.

Plant Material

The fresh leaves of *A. nepalensis* were identified and collected by Dr. Tran Ngoc Ninh, Institute of Biological Resources and Ecology, Vietnamese Academy of Science and Technology, Hanoi, Vietnam in Dong Van, province Ha Giang, Vietnam in June 2007.

Extraction and Isolation

The air-dried leaves of *A. nepalensis* (432.7 g) were oven-dried at 40ºC, then powdered and extracted with MeOH by percolation (6 times) at room temperature. The combined MeOH extract was concentrated under reduced pressure. The resultant MeOH extract was suspended in H_2O and partitioned successively with *n*-hexane and EtOAc. After removal of solvents *n*-hexane- (**AH**, 22.8 g) and EtOAc- (**AE**, 8.1 g) soluble fractions were obtained. Part of the *n*-hexane-soluble fraction (22 g) was chromatographed on silica gel CC using a gradient *n*-hexane-EtOAc solvent system (*n*-hexane; *n*-hexane-EtOAc 7:1, 4:1, 2:1, and 1:1; and EtOAc). Fifteen pooled fractions were collected on the basis of the volumes of eluents and TLC analysis; fraction **AH0** (0.41 g) was eluted with *n*-hexane; fractions **AH1** (0.38 g), **AH2** (3.71 g), **AH3** (0.44 g), and **AH4** (0.84 g) with *n*-hexane-EtOAc 7:1; fractions **AH5** (1.45 g), **AH6** (0.88 g), **AH7** (0.29 g), and **AH8** (0.7 g) with *n*-hexane-EtOAc 4:1; fractions **AH9** (0.79 g) and **AH10** (0.7 g) with *n*-hexane-EtOAc 2:1; fractions **AH11** (0.77 g), **AH12** (0.31 g), **AH13** (0.86 g), and **AH14** (0.54 g) with *n*-hexane-EtOAc 1:1; and fraction **AH15** (0.66 g) with EtOAc. Separation of fr. **AH1** on silica gel CC (*n*-hexane-EtOAc 90:1) gave **1** (21.3 mg). Separation of fr. **AH2** on silica gel CC (gradient *n*-hexane-EtOAc 90:1, 70:1, and 49:1) gave **1** (50 mg), **2** (22 mg) after recrystallization from *n*-hexane-EtOAc 9:1, and **3** (30 mg). **3** (28 mg) was also obtained from fr. **AH3** on column separation on silica gel (gradient *n*-hexane-EtOAc 30:1, 15:1, and 7:1) and recrystallization (*n*-hexane-EtOAc 7:1). Fr. **AH4** was washed with EtOAc and then recrystallized from CH_2Cl_2 to afford **3** (49 mg). Fr. **AH6** was chromatographed by silica gel CC (gradient *n*-hexane-EtOAc 30:1, 15:1, 7:1 and 4:1) and one of the fractions obtained was recrystallized from CH_2Cl_2 to give **4** (8 mg). Part of the EtOAc-soluble fraction (8 g) was subjected to silica gel column chromatography and eluted with a gradient CH_2Cl_2-MeOH solvent system (CH_2Cl_2; CH_2Cl_2-MeOH 90:1, 70:1, 19:1, 9:1, 7:1, 4:1, 2:1, and 1:1; and MeOH). Based on TLC profile, ten fractions were collected, fraction **AE1** (70 mg), **AE2** (20 mg), **AE3** (60 mg), **AE4** (0.27 g), **AE5** (0.7 g), **AE6** (0.88 g), **AE7** (0.98 g), **AE8** (0.98 g), **AE9** (3.29 g), and **AE10**. Fr. **AE5** was rechromatographed on silica gel CC (gradient CH_2Cl_2-$(CH_3)_2CO$ 19:1 and 1:1). Two of the fractions obtained gave crystalls on standing at room temperature, one of which was purified by FC on silica gel (*n*-hexane-EtOAc 1:1) to give **5** (26 mg) and the other was washed with $(CH_3)_2CO$ and recrystallized from a $CHCl_3$-MeOH mixture to give **6** (25 mg). **7** (141,5 mg) was obtained from fr. **AE9** on repeated separation on silica gel CC (gradient CH_2Cl_2-$(CH_3)_2CO$ 2:1 and 1:7) and FC (CH_2Cl_2-EtOAc 1:9) followed by recrystallization from $(CH_3)_2CO$.

*Taraxeryl acetate (**1**):* White rods. R_f 0.46 (*n*-hexane-EtOAc 4:1). EI-MS: *m/z* 468 ($M^{+\cdot}$, $C_{32}H_{52}O_2$). ^{1}H-NMR ($CDCl_3$): δ 0.82 (3H, s, 17-CH_3), 0.86 (3H, s, 4-CH_3), 0.88 (3H, s, 4-CH_3), 0.90 (3H, s, 20-CH_3), 0.91 (3H, s, 13-CH_3), 0.95 (6H, s, 10-CH_3, 20-CH_3), 1.09 (3H, s, 8-CH_3), 2.04 (3H, s, 3-OAc), 4.46 (1H, dd, *J* = 11.0 Hz, 5.0 Hz, H-3), 5.53 (1H, dd, *J* = 8.0 Hz, 3.0 Hz, H-15). ^{13}C-NMR ($CDCl_3$): δ 15.5 (q, C-25), 16.6 (q, C-24), 17.5 (t, C-11), 18.7 (t, C-6), 21.3 (q, C-30), 23.5 (t, C-2), 25.9 (q, C-26), 27.9 (q, C-23), 28.8 (s, C-20), 29.8 (q, C-27), 29.9 (q, C-28), 33.1 (t, C-7), 33.4 (q, C-29), 33.7 (t, C-16), 35.1 (t, C-21), 35.8 (s, C-17), 36.7 (t, C-12), 37.4 (t, C-22), 37.6 (s, C-10), 37.7 (t, C-1), 37.7 (s, C-13), 37.9 (s, C-4), 39.0 (s, C-8), 41.3 (t, C-19), 48.8 (d, C-18), 49.2 (d, C-9), 55.7 (d, C-5), 81.0 (d, C-3), 116.9 (d, C-15), 158.0 (s, C-14), 21.3 (q) and 170.9 (s, 3-OAc).

*Physcion (**2**):* Orange needles. R_f 0.54 (*n*-hexane-EtOAc 15:1). EI-MS: *m/z* 284 ($M^{+\cdot}$,

$C_{16}H_{12}O_5$). ^{1}H-NMR ($CDCl_3$): δ 2.45 (3H, s, 6-CH_3), 3.94 (3H, s, 3-OCH_3), 6.69 (1H, d, *J* = 2.0 Hz, H-7), 7.08 (1H, br s, H-2), 7.37 (1H, d, *J* = 2.0 Hz, H-5), 7.63 (1H, br s, H-4), 12.1 (1H, s, 1-OH), 12.3 (1H, s, 8-OH). ^{13}C-NMR ($CDCl_3$): δ 22.2 (q, C-15), 56.1 (q, 3-OCH_3), 106.8 (d, C-2), 108.2 (d, C-4), 110.3 (s, C-13), 113.7 (s, C-7), 121.3 (d, C-12), 124.5 (d, C-5), 133.3 (s, C-14), 135.3 (s, C-11), 148.5 (s, C-6), 162.5 (s, C-8), 165.2 (s, C-1), 166.6 (s, C-3), 182.1 (s, C-10), 190.8 (s, C-9).

*1-Nonacosanol (**3**):* White amorphous powder. R_f 0.5 (*n*-hexane-EtOAc 4:1). EI-MS: *m/z* 364 ($M^{+\cdot}$, $C_{29}H_{60}O$, – 60). ^{1}H-NMR ($CDCl_3$): δ0.88 (3H, t, *J* = 7.0 Hz, H-29), 1.26 50H, br s), 1.58 (4H, m (2H-2 → 2H-28), 3.64 (2H, t, *J* = 6.5 Hz, H-1).

*β-Sitosterol (**4**):* White amorphous powder. R_f 0.37 (*n*-hexane-EtOAc 4:1). The co-TLC analysis is superimposable with that of our authentic sample.

*Quercetin (**5**):* Yellow needles. R_f 0.54 (CH_2Cl_2-$(CH_3)_2CO$ 2:1). ESI-MS: *m/z* 302.9 $[M+H]^+$ (positive mode), m/z 301.0 $[M–H]^-$ (negative mode). ^{1}H-NMR (CD_3OD): δ 6.2 (1H, d, *J* = 2.0 Hz, H-8), 6.4 (1H, d, *J* = 2.0 Hz, H-6), 6.90 (1H, d, *J* = 8.5 Hz, H-5′), 7.64 (1H, dd, *J* = 8.5 Hz, 2.5 Hz, H-6′), 7.75 (1H, d, *J* = 2.5 Hz, H-2′). ^{13}C-NMR (CD_3OD): δ 94.4 (d, C-8), 99.2 (d, C-6), 104.5 (s, C-10), 116.0 (d, C-2′), 116.2 (d, C-5′), 121.7 (s, C-6′), 124.1 (s, C-1′), 137.2 (s, C-3), 146.2 (s, C-3′), 148.0 (s, C-2), 148.7 (s, C-4′), 158.2 (s, C-9), 162.5 (s, C-5), 165.5 (s, C-7), 177.3 (s, C-4).

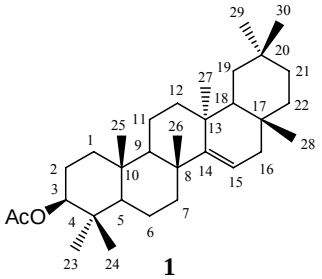
*β-Sitosterol 3-O-β-D-glucopyranoside (**6**):* White amorphous powder. R_f 0.54 (CH_2Cl_2-$(CH_3)_2CO$ 1:3). The ^{1}H-NMR (CD_3OD) is identical with that of our authentic sample.

*Quercitrin (**7**):* Yellow needles. R_f 0.57 (CH_2Cl_2-$(CH_3)_2CO$ 1:3). EI-MS: *m/z* 302 ($M^{+\cdot}$, $C_{21}H_{20}O_{11}$, – 146). ^{1}H-NMR (CD_3OD): δ 0.96 (3H, d, *J* = 6.0 Hz, 5″-CH_3), 3.33 (1H, m, H-4″), 3.44 (1H, m, H-3″), 3.77 (1H, dd, *J* = 8.0 Hz, 3.5 Hz, H-2″), 4.24 (1H, m, H-5″), 5.37 (1H, d, *J* = 1.0 Hz, H-1″), 6.22 (1H, d, *J* = 2.0 Hz, H-8), 6.39 (1H, d, *J* = 2.0 Hz, H-6), 6.93 (1H, d, *J* = 8.5 Hz, H-5′), 7.32 (1H, dd, *J* = 8.5 Hz, 2.0 Hz, H-6′), 7.36 (1H, d, *J* = 2.0 Hz, H-2′). ^{13}C-NMR (CD_3OD): δ 17.6 (q, C-6″), 71.9 (d, C-5″), 72.0 (d, C-3″), 72.1 (d, C-2″), 73.3 (d, C-4″), 94.7 (d, C-8), 99.8 (d, C-6), 103.6 (d, C-1″), 105.9 (s, C-10), 116.4 (d, C-2′), 117.0 (d, C-5′), 122.9 (s, C-6′), 123.0 (s, C-1′), 136.3 (s, C-3), 146.4 (s, C-3′), 149.8 (s, C-4′), 158.5 (s, C-2), 159.3 (s, C-9), 163.2 (s, C-5), 165.8 (s, C-7), 179.6 (s, C-4).

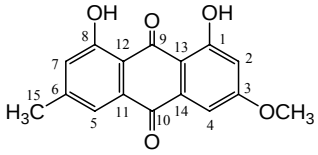
III - RESULTS AND DISCUSSION

The dried leaves of *A. nepalensis* were extracted with MeOH, and the resultant MeOH extract was partitioned between H_2O and solvents of increasing polarity to give *n*-hexane- and EtOAc-soluble fractions. Fractionation of the *n*-hexane- and EtOAc-soluble fractions by silica gel open column (CC) and flash column (FC) chromatography resulted in the isolation of seven compounds **1**-**7** (Fig. 1). Compounds **1**-**7** have so far not been reported from the genus *Alnus* (Betulaceae).

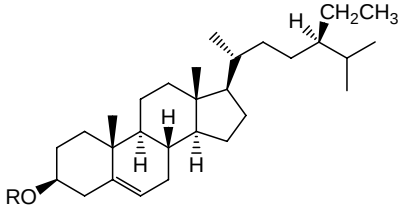
Compound **1** was isolated as white rods [R_f 0.46 (*n*-hexane-EtOAc 4:1)] from the *n*-hexane-soluble fraction. The electron impact mass spectrum (EI-MS) of **1** showed the molecular ion peak at *m/z* 468 ($M^{+\cdot}$, $C_{32}H_{52}O_2$). The ^{1}H-NMR spectrum of **1** indicated the presence of eight tertiary methyl groups (all singlets) [[δ_H 0.82 (3H), 0.86 (3H), 0.88 (3H), 0.91 (6H), 0.95 (6H), 1.09 (3H)], an oxygenated methine [[δ_H 4.46 (1H, dd, *J* = 11.0 Hz, 5.0 Hz)], and an olefinic proton [δ_H 5.53 (1H, dd, *J* = 8.0 Hz, 3.0 Hz)]. The ^{13}C-NMR and DEPT spectra of **1** supported the ^{1}H-NMR data; the occurence of 30 ^{13}C signals was suggestive for a triterpenoid structure. The presence of an acetoxy group was shown by the NMR signals at δ_H 2.04 (3H, s) and δ_C 21.3 (s) and 170.9 (s). Thus, the structure of **1** was determined to be taraxeryl acetate by comparing their ^{1}H- and ^{13}C-NMR spectroscopic data with those reported in literature [8].



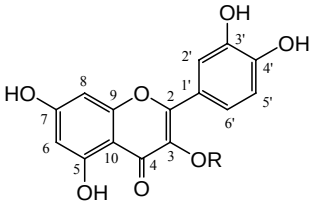
1



2



4 R=H
6 R=Glc



5 R=H
7 R=Rha

Fig. 1: Chemical Structures of Compounds **1**-**7**

Compound **2** was isolated as orange needles [R_f 0.54 (*n*-hexane-EtOAc 15:1)] from the *n*-hexane-soluble fraction. **2** was suggested to have a molecular formula $C_{16}H_{12}O_5$ from its EI-MS spectrum. The ^{1}H-, ^{13}C-NMR, and DEPT spectra of **2** exhibited the presence of two *meta*-coupled proton pairs [δ_H 6.69 (1H) and 7.37 (1H) (each d, J = 2.0 Hz)] and [δ_H 7.08 (1H) and 7.63 (1H) (each br s)], a methoxy group [δ_H 3.94 (3H, s), δ_C 56.1 (q)], and a methyl group bonded to an aromatic ring [δ_H 2.45 (3H, s), δ_C 22.2 (q)]. In addition, the ^{13}C NMR chemical shifts were indicative of the presence of two substituted benzene rings and two carbonyl groups [δ_C 190.8 (s) and 182.1 (s)]. Taken together, a 9,10-anthraquinone skeleton of emodin-type compounds was suggested for **2** [9]. The chemical shifts of two *peri*-hydroxyl protons at C-1 [δ_H 12.1 (s)] and C-8 [δ_H 12.3 (s)] and two carbonyl groups at C-9 [δ_C 190.8 (s)] and C-10 [δ_C 182.1 (s)] [9] were used to unequivocally determine the structure of **2** to be 1,8-dihydroxy-3-methoxy-6-methylanthraquinone (physcion).

Compound **3** was isolated as a white amorphous powder [R_f 0.5 (*n*-hexane-EtOAc 4:1)] from the *n*-hexane-soluble fraction. **3** was determined to be 1-nonacosanol from its ^{1}H-NMR spectroscopic data. In the ^{1}H-NMR spectrum of **3** the terminal methyl group δ_H 0.88 (3H, t, J = 7.0 Hz)], methylene chains [δ_H 1.26 (50 H, br s) and 1.58 (4H, m)], and a methylene group bearing a hydroxy group [δ_H 3.64 (2H, t, J = 6.5 Hz)] were observed. The number of methylene groups was deduced to be 28 from the ^{1}H-NMR integration. The EI-MS spectrum of **3** showed the highest peak at *m*/*z* 364, which was probably derived from simutlaneous loss of H_2O and ethylene, and a methylene group ($M^{+\cdot}$, $C_{29}H_{60}O$, – 18 – 28 – 14). 1-Nonacosanol was found as constituent of several species, *Agave*, *Sisalana*, *Citrulus*, and *Rhizophora* [10].

Compound **5** was isolated as yellow needles [R_f 0.54 (CH_2Cl_2-$(CH_3)_2CO$ 2:1)] from the EtOAc-soluble fraction and was suggested to to have a molecular formula $C_{15}H_{10}O_7$ from its electrospray ionization mass spectrum (ESI-MS) (*m*/*z* 302.9 $[M+H]^+$, m/z 301.0 $[M–H]^-$). The ^{1}H-NMR

spectrum of **5** exhibited a flavonoid pattern; two *meta*-coupled protons [δ_H 6.2 (1H) and 6.4 (1H) (each d, J = 2.0 Hz)] of the flavonoid A ring and a 1,3,4-trisubstituted benzene ring [δ_H 6.9 (1H, d, J = 8.5 Hz), 7.64 (1H, dd, J = 8.5 Hz, 2.5 Hz), and 7.75 (1H, d, J = 2.5 Hz)] of the flavonoid B ring. Furthermore, the ^{13}C-NMR signals [δ_C 137.2 (s, C-3), 148.0 (s, C-2), and 177.3 (s, 4-CO)] were indicative for a flavonol skeleton. Comparison of the ^{1}H- and ^{13}C-NMR spectroscopic data of **5** with literature data [11] determined the structure of **5** to be quercetin.

Compound **7** was isolated as yellow needles [R_f 0.57 (CH_2Cl_2-$(CH_3)_2CO$ 1:3)] from the EtOAc-soluble fraction. The ^{1}H- and ^{13}C-NMR spectra of **7** indicated that quercetin was the aglycon of **7**. The presence of an α-L-rhamnopyranosyl unit was evidenced by the anomeric NMR signal at δ_H 5.37 (1H, d, J = 1.0 Hz), δ_C 103.6 (d) and the secondary methyl group at δ_H 0.96 (3H, d, J = 6.0 Hz), δ_C 17.6 (q). Comparison of the ^{13}C-NMR signals of **7** and **5** showed the upfield shift at C-3 ($\Delta\delta_C$ −0.9) and pronounced downfield shift at C-2 ($\Delta\delta_C$ +10.5) suggesting the attachment of the rhamnopyranosyl unit at C-3. The EI-MS spectrum of **7** showed the peak at *m/z* 302 which was resulted from the loss of a deoxyhexose unit ($M^{+\cdot}$, $C_{21}H_{20}O_{11}$, − 146). Thus, the structure of **7** was determined to be quercetin 3-*O*-α-L-rhamnopyranoside (quercitrin) by comparison of its spectroscopic data with literature data [11].

Compound **4**, β-sitosterol, was identified on the basis of co-TLC analysis. Compound **6**, β-sitosterol 3-*O*-*β*-D-glucopyranoside, was identified by comparison of its ^{1}H-NMR spectrum with that of our authentic sample.

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