Biflavones from *Chamaecyparis obtusa*

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Z. Naturforsch. **60 c**, 679–685 (2005); received January 3/March 14, 2005

From the leaves of *Chamaecyparis obtusa* several biflavones were isolated and identified, namely: sciadopitysin, ginkgetin, isoginkgetin, podocarpusflavone B, 7,7″-*O*-dimethylamentoflavone, bilobetin, podocarpusflavone A, and 7-*O*-methylamentoflavone. The presence of amentoflavone and hinokiflavone was also confirmed. The composition of biflavones in other *Chamaecyparis* species – *Ch. lawsoniana*, *Ch. thyoides* – and cultivar varieties – *Ch. pisifera* "Squarrosa", *Ch. pisifera* "Boulevard" – was compared using the HPLC method. It was stated, that podocarpusflavone A and 7-*O*-methylamentoflavone in addition to amentoflavone and hinokiflavone may be classified as chemotaxonomic markers of the genus *Chamaecyparis*.

Key words: Biflavones, HPLC, *Chamaecyparis*

Introduction

Biflavones are chemotaxonomic markers of the genus *Chamaecyparis* (Gadek and Quinn, 1983, 1985, 1987). Gadek and Quinn (1985) analysed, using the TLC method, the chemical composition of biflavones in some species from *Chamaecyparis*, including *Ch. obtusa.* It was suggested, that the presence of di- and tri-methoxylated derivatives of amentoflavone differentiates the genus *Chamaecyparis* from *Cupressus* within the subfamily Cupressoideae (Gadek and Quinn, 1985, 1987). However, no reports confirm the presence of these compounds in *Ch. obtusa.* Until now, the chemical composition of *Chamaecyparis obtusa* was rarely investigated. Apart from the biflavones amentoflavone and hinokiflavone (Gadek and Quinn, 1985), cryptomerin, isocryptomerin, chamaecyparin and the other flavonoids 3-*O*-glucoside taxifolin and 4,4′-dihydroxychalcone (Harborne and Baxter, 1999) were also analysed in leaves. Furthermore, diterpenes, lignans and steroids were separated from the wood of cultivar varieties – *Chamaecyparis obtusa* var. *formosana* (Kuo *et al.*, 1998). Most plants from the genus *Chamaecyparis* are cultivated as ornaments, only some of them – *Ch. nootkatensis*, *Ch. pisifera* and *Ch. thyoides* – are used in folk medicine as analgesic and antirheumatic remedia (Johnson, 1999).

Regarding the chemotaxonomic significance of methoxylated derivatives of amentoflavone, the objective of this work was to isolate and identify these compounds in the leaves of *Chamaecyparis obtusa*, as well as to perform comparative analysis of biflavone complexes occurring in leaves of four species and cultivar varieties of the genus *Chamaecyparis.*

Material and Methods

Plant material

The leaves of *Chamaecyparis obtusa* Endl., *Chamaecyparis lawsoniana* (A. Murray bis) Parl., *Chamaecyparis thyoides* B.S.P, and cultivar varieties, *Chamaecyparis pisifera* (Siebold et Zucc.) Endl. "Squarrosa", *Ch. pisifera* (Siebold et Zucc.) Endl. "Boulevard" (Cupressaceae), were collected from the Medicinal Plants Garden of the Medical University of Gdańsk (Poland) in September 1997. The voucher specimens have been deposited in the Herbarium of the Department of Pharmacognosy of the Medical University of Gdańsk (Poland) with the following numbers: 97–012 (*Ch. obtusa*), 97–013 (*Ch. lawsoniana*), 97–014 (*Ch. thyoides*), 97–015 (*Ch. pisifera* "Squarrosa"), 97–016 (*Ch. pisifera* "Boulevard").

Extraction and isolation

Dried and pulverized leaves of *Ch. obtusa* (0.5 kg) were exhaustively extracted with petroleum ether in a Soxhlet apparatus. The flavonoids were extracted with chloroform and next with

0939–5075/2005/0900–0679 $ 06.00

methanol. The chloroform extract was concentrated and chromatographed over a polyamide column (50 g, 40 × 2 cm) with $CHCl_3$/MeOEt (4:3 v/v) (eluates 1–10, 15 ml each), $CHCl_3$/MeOH/MeOEt (4:2:3 v/v/v) (eluates 11–30), (4:8:6 v/v/v) (eluates 31–51). From eluates 3–9 compound **1** (10 mg) was precipitated. Compounds **2** (8 mg, eluates 8–10), **3**, **4** were separated from eluates 10–25 over a Sephadex LH-20 column (20 g, 40 × 1.5 cm) with MeOH (eluates 1–30, 4 ml each). From the obtained eluates 6–10 compounds **3** (6 mg) and **4** (6 mg) were isolated by preparative TLC on a polyamide column with $CHCl_3$/MeOH/MeOEt (4:1:2 v/v/v). Chromatograms were developed twice, the first to a distance of 6 cm, and after drying the second to a distance of 9 cm. From eluates 32–51 compounds **7** (2 mg), **8** (2 mg) and **9** (7 mg) were purified in the same way. Compounds **5** (10 mg, eluates 15–19) and **6** (6 mg, eluates 31–33) were obtained from eluates 26–29 by chromatography over a Sephadex LH-20 column (20 g, 40 × 1.5 cm) with MeOH and rechromatography over a column packed with the same adsorbent (5 g, 9 × 1 cm). The methanol extract was concentrated and chromatographed over a polyamide column (100 g, 45 × 3cm) with MeOH/H_2O (70:30 v/v) (eluates 1–12, 25 ml each) and MeOH (eluates 13–19, 25 ml each). Compound **10** (30 mg) was precipitated from the obtained eluates 13–15. Compounds **11** (8 mg) and **12** (4 mg) were separated from eluates 3–5 and 7–11, respectively, over a Sephadex G-10 column (10 g, 17 × 1 cm) with MeOH and next by preparative TLC on cellulose with IsoPrOH/HCOOH/H_2O (2:5:5 v/v/v).

TLC analysis was done as described earlier (Krauze-Baranowska and Malinowska, 2005). Separation was performed on cellulose F_{254} plates (Merck, Darmstadt, Germany, 20 × 20 cm, 0.10 mm) with the following mobile phases: $CHCl_3$/MeOH/MeOEt (4:2:3 v/v/v) (A), (4:8:6 v/v/v) (B) (Krauze-Baranowska and Malinowska, 2005), IsoPrOH/HCOOH/H_2O (2:5:5 v/v/v) (C), BuOH/H_2O/CH_3COOH (4:1:5 v/v/v) (D). HPTLC analysis was carried out on HPTLC RP-18 F_{254} plates with the mobile phases: MeOH/H_2O/HCOOH (70:30:6 v/v/v) (E), MeOH/THF/H_2O/HCOOH (52.5:17.5:30:6 v/v/v/v) (F) in a horizontal chamber DS II (saturation 10 min) (Lublin, Poland). Column chromatography was performed with a polyamide (Roth) and a Sephadex LH-20 column (Pharmacia). ^1H and ^{13}C NMR spectra, NOE and 2D–COSY, ROESY, HMBC, HSQC spectra were recorded on a Unity Plus 500 instrument (Varian, Inc., Palo Alto, USA) at 500 MHz in DMSO-d_6 using TMS as an internal standard. EI-MS (70 eV) and LSI-MS (+) (NBA, Cs^+, 6 keV) mass spectral data were obtained using an AMD-Intectra spectrometer. FAB-MS (+) (thioglycerol) spectra were recorded on a Trio-3 VG instrument (Masslab, Manchester, UK).

HPLC

The separation of biflavones was performed as described in the literature (Krauze-Baranowska *et al.*, 1999) employing Spherisorb ODS II (250 × 4 mm, 5 μm) (Knauer, Berlin, Germany) and Lichrospher RP-18 (250 × 4 mm, 5μm) (Merck) columns. Aquisition of data was carried out by means of HPLC 211a (Knauer) (Lichrospher RP-18 column) and Eurochrom 2000 (Knauer) (Spherisorb ODS II column) softwares.

Identification

7,4′,4‴-O-Trimethylamentoflavone (sciadopitysin) (**1**): TLC polyamide: R_f: A – 0.90, B – 0.90. – HPTLC RP-18: R_f: F – 0.12. – HPLC: t_R = 35.1 min. – FD-MS: *m/z* (rel. int.) = 580 $[M]^+$ (50). – UV, ^1H NMR and ^{13}C NMR data were consistent with those in the literature (Konda *et al.*, 1995; Markham *et al.*, 1987; Wollenweber *et al.*, 1998).

4′,4‴-O-Dimethylamentoflavone (isoginkgetin) (**2**): TLC polyamide: R_f: A – 0.71, B – 0.75. – HPTLC RP-18: R_f: E – 0.12. – HPLC: t_R = 24.3 min. – UV as described in the literature (Baker *et al.*, 1963). – FAB-MS (+): *m/z* (rel. int.) = 567 $[M+H]^+$ (100). – ^1H NMR (DMSO-d_6): see Table I; the δ values of protons H-3, H-3″ and the positions of OCH_3 groups were elucidated from ROESY, ^1H-^1H COSY and NOE spectra.

7,7″-O-Dimethylamentoflavone (**3**): TLC polyamide: R_f: A – 0.85. – HPTLC RP-18: R_f: F – 0.23. – HPLC: t_R = 22.4 min. – UV (MeOH): λ_{max} = 270, 333 nm; ($AlCl_3$): λ_{max} = 280, 317sh, 340, 368sh nm; ($AlCl_3$/HCl): λ_{max} = 279, 316sh, 340, 369sh, 388 nm; (CH_3ONa): λ_{max} = 278, 400 nm; (CH_3COONa): λ_{max} = 270, 333 nm; (CH_3COONa/H_3BO_3): λ_{max} = 271, 333 nm. – FAB-MS (+): *m/z* (rel. int.) = 567 $[M+H]^+$ (100). – EI-MS: *m/z* (rel. int.) = 568 $[M+H]^+$ (60). – ^1H NMR (DMSO-d_6): δ = 6.36 (1H, s, H-6″), 6.64 (1H, s, H-6), 6.70 (2H, d, *J* = 8.8 Hz, H-3‴,5‴), 6.71 (1H,

s, H-8), 6.80, 6.85 (1H, s, H-3,3″), 7.14 (1H, d, J = 8.3 Hz, H-5′), 7.54 (2H, d, J = 8.7 Hz, H-2‴,6‴), 7.96 (1H, s, H-2′), 8.00 (1H, d, J = 8.3 Hz, H-6′), 3.80, 3.78 (3H, s, OCH_3-7,7″).

7,4′-O-Dimethylamentoflavone (ginkgetin) (**4**): TLC polyamide: R_f: A – 0.77, B – 0.80. – HPTLC RP-18: R_f: E – 0.12, F – 0.28. – HPLC: t_R = 27.8 min. – UV (MeOH): λ_{max} = 271, 335 nm; ($AlCl_3$): λ_{max} = 280, 315sh, 343, 386 nm; ($AlCl_3$/HCl): λ_{max} = 279, 315sh, 343, 374sh, 385 nm; (CH_3ONa): λ_{max} = 281, 333, 345sh, 385 nm; (CH_3COONa): λ_{max} = 271, 308, 339 nm; (CH_3COONa/H_3BO_3): λ_{max} = 270, 295sh, 335 nm. – FAB-MS (+): *m/z* (rel. int.) = 567 $[M+H]^+$ (100), 535 $[M+H-32]^+$ (12). – ^1H and ^{13}C NMR data are in agreement with the literature data (Markham *et al.*, 1987; Sun *et al.*, 1997).

7,4‴-O-Dimethylamentoflavone (podocarpusflavone B) (**5**): TLC polyamide: R_f: A – 0.60. – HPTLC RP-18: R_f: F – 0.22. – HPLC: t_R = 34.2 min. – UV (MeOH): λ_{max} = 270, 333 nm; ($AlCl_3$): λ_{max} = 279, 315sh, 346, 400 nm; ($AlCl_3$/HCl): λ_{max} = 280, 315sh, 343, 400 nm; (CH_3ONa): λ_{max} = 281, 377 (the decrease of intensity), 400 nm; (CH_3COONa): λ_{max} = 270, 334 (the decrease of intensity), 400sh nm; (CH_3COONa/H_3BO_3): λ_{max} = 270, 333 nm. – FAB-MS (+): *m/z* (rel. int.) = 567 $[M+H]^+$ (100). – ^1H NMR (DMSO-d_6): δ = 6.18 (1H, s, H-6), 6.32 (1H, s, H-6″), 6.56 (1H, s, H-8), 6.80 (1H, s, H-H-3″), 6.83 (1H, s, H-3), 6.91 (2H, d, J = 8.8 Hz, H-3‴,5‴), 7.01 (1H, d, J = 8.7 Hz, H-5′), 7.69 (2H, d, J = 8.8 Hz, H-2‴,6‴), 7.95 (1H, d, J = 8.3 Hz, H-6′), 8.17 (1H, s, H-2′), 3.64 (3H, s, OCH_3-4‴), 3.76 (3H, s, OCH_3-7), 13.01, 13.11 (1H, s, OH-5, OH-5″). – ^{13}C NMR (DMSO-d_6): δ = 182.1 (C-4″), 181.7 (C-4), 164.1 (C-2), 163.8 (C-2″), 165.4 (C-7), 162.5 (C-4‴), 162.1 (C-7″), 161.2 (C-5), 160.8 (C-5″), 160.2 (C-4′), 157.9 (C-9), 154.6 (C-9″), 131.9 (C-6′), 128.3 (C-2‴,6‴), 128.1 (C-2′), 123.2 (C-1‴), 120.6 (C-1′), 120.3 (C-3′), 116.3 (C-5′), 114.7 (C-3‴,5‴), 105.1 (C-10), 104.1 (C-3), 103.8 (C-10″) 103.7 (C-8″), 103.4 (C-3″), 98.9 (C-6″), 98.2 (C-6), 93.1 (C-8), 56.1 (OCH_3-7″), 55.8 (OCH_3-4‴); the δ values of protons and carbon atoms were elucidated from ^1H-^1H COSY, ROESY, NOE, HMBC, HSQC spectra.

4′-O-Methylamentoflavone (bilobetin) (**6**): TLC polyamide: R_f: A – 0.43, B – 0.56. – HPTLC RP-18: R_f: E – 0.23, F – 0.50. – HPLC: t_R = 17.9 min. – FD-MS: *m/z* (rel. int.) = 552 $[M]^+$ (100). – UV, ^1H and ^{13}C NMR data are in agreement with the literature data (Markham, 1982; Markham *et al.*, 1987; Silva *et al.*, 1995; Wollenweber *et al.*, 1998).

7-O-Methylamentoflavone (**7**): TLC polyamide: R_f: A – 0.16. – HPTLC RP-18: R_f: F – 0.41. – HPLC: t_R = 25.8 min. – UV, EI-MS, ^1H NMR and ^{13}C NMR data were consistent with those in the literature (Krauze-Baranowska *et al.*, 1999, 2002).

4‴-O-Methylamentoflavone (podocarpusflavone A) (**8**): TLC polyamide: R_f: A – 0.11. – HPTLC RP-18: R_f: E – 0.45. – HPLC: t_R = 20.9 min. – UV, EI-MS and ^1H NMR data were consistent with those in the literature (Krauze-Baranowska *et al.*, 1999; Sun *et al.*, 1997).

Hinokiflavone (**9**): TLC polyamide: R_f: A – 0.03, B – 0.06. – HPTLC RP-18: R_f: E – 0.43. – HPLC: t_R = 38.1 min. – FAB-MS (+): *m/z* (rel. int.) = 539 $[M+H]^+$ (100). – UV, ^1H NMR and ^{13}C NMR data were consistent with those in the literature (Geiger and Markham, 1996; Geiger *et al.*, 1993; Silva *et al.*, 1995).

Amentoflavone (**10**): TLC polyamide: R_f: A – 0.06. – HPTLC RP-18: R_f: F – 0.60. – HPLC: t_R = 16.4 min. – UV, EI-MS, ^1H NMR and ^{13}C NMR data were consistent with those in the literature (Krauze-Baranowska *et al.*, 1999; Markham *et al.*, 1987; Wollenweber *et al.*, 1998).

Quercetin (**11**): TLC R_f values in used systems (polyamide: A; cellulose: C, D) were in agreement with presented earlier (Krauze-Baranowska, 2004). – UV data: as described in the literature (Markham, 1982). – LSI-MS (+) NBA: *m/z* = 307 $[M+H]^+$ (60); EI-MS: *m/z* = 302 $[M]^+$ (30).

Kaempferol (**12**): TLC R_f values in used systems (polyamide: A; cellulose: C, D) were in agreement with presented earlier (Krauze-Baranowska, 2004). – UV data: as described in the literature (Markham, 1982). – LSI-MS (+) NBA: *m/z* = 287 $[M+H]^+$ (28); EI-MS: *m/z* = 302 $[M]^+$ (100).

Results and Discussion

The methanol and chloroform extracts from the leaves of *Chamaecyparis obtusa* were subjected to phytochemical analysis. The flavonoids were separated by preparative column chromatography on polyamide (compounds **1**–**10**) and Sephadex LH-20 columns (**2**, **5**–**9**, **11**–**12**), and by preparative TLC on cellulose (**11**, **12**) and polyamide (**3**, **4**) columns.

The structures of isolated flavonoids were elucidated by spectroscopic methods: UV, MS (com-

pounds **1**–**12**), ^{1}H NMR (**1**–**10**), ^{13}C NMR (**1**, **4**–**7**, **9**, **10**), 2D NMR (COSY, HMBC, HSQC, ROESY) (**2**, **4**) and co-chromatography with standards (**11**, **12**). Compound **1** was identified as sciadopitysin, **2** as isoginkgetin, **3** as 7,7″-*O*-dimethylamentoflavone, **4** as ginkgetin, **5** as podocarpusflavone B, **6** as bilobetin, **7** as 7-*O*-methylamentoflavone, **8** as 4‴-*O*-methylamentoflavone, **9** as hinokiflavone, **10** as amentoflavone, **11** as quercetin, and **12** as kaempferol.

The structure of compound **2** was established as 4′,4‴-*O*-dimethylamentoflavone (isoginkgetin). In the ^{1}H NMR spectrum of **2**, the δ values were assigned to individual protons on the basis of the following correlated signals from COSY: H-3‴,5‴/H-2‴,6‴, H-2′/6′, H-6′/5′, H-6/H-8 and from ROESY spectrum: H-3″/H-2‴,6‴, H-3/H-2′, H-3/H-6′, OCH_3-4‴/H-2‴,6‴, OCH_3-4‴/H-3‴,5‴, OCH_3-4′/H-5′. To our knowledge, the ^{1}H NMR spectrum of isoginkgetin has not been published previously. The presence of a C-C/3′–8″ linkage was confirmed by a NOE experiment. After irradiation of a proton of the OH-5″ group at δ 12.98 the NOE effect was observed with the proton H-6″ at δ 6.16. Moreover, in the ^{1}H NMR spectrum of isoginkgetin, recorded at 97 °C, the signals of protons H-6″, H-3, H-3″ and H-6′ were upfield shifted, respectively about $\Delta\delta$ = 0.14 ppm, $\Delta\delta$ = 0.18 ppm, $\Delta\delta$ = 0.20 ppm and $\Delta\delta$ = 0.08 ppm, in comparison to the spectrum obtained at 22 °C (Table I). The differences dependent on the temperature of the sample in ^{1}H NMR spectra of C-C/3′–8″ biflavones were noticed earlier for 7-*O*-methylamentoflavone (Krauze-Baranowska *et al.*, 2002). In NMR analyses, the temperature of samples affects dynamic and conformational processes leading to changes in the geometry of a molecule (Ejchart and Kozerski, 1988). Biflavones are an interesting object for conformational analysis because of restricted rotation around C-C linkages and molecular dissymmetry (Rahman *et al.*, 1982; Zhang *et al.*, 1995). Konda *et al.* (1995) elucidated molecular conformations of sciadopitysin. It was established, that one conformer of sciadopitysin exhibited free rotation around all linkages and another showed partially restricted rotation around C-3′ and C-8″ linkage.

Table I. ^{1}H NMR data of compound **2**, isoginkgetin, isolated from *Ch. obtusa* recorded in DMSO-d_6 at 22 °C and 97 °C at 500 MHz; the δ values of protons were assigned from ^{1}H-^{1}H COSY, ROESY, HMBC, HSQC spectra.

H	**5** (22 °C)	**5** (97 °C)
H-3	6.78 s	6.60 s
H-6	6.08 s	6.05 brs
H-8	6.36 s	6.29 s
H-2′	7.92 s	7.87 s
H-3′	–	–
H-5′	7.28 d (8.8)	7.36 d (8.8)
H-6′	8.07 d (8.8)	7.99 d (8.8)
H-3″	6.72 s	6.52 s
H-6″	6.16 brs s	6.02 s
H-8″	–	–
H-2‴,6‴	7.56 d (8.8)	7.52 d (8.8)
H-3‴,5‴	6.91 d (8.8)	6.90 d (8.8)
OH-4′,4‴	3.76 s, 3.74 s	–
OH-5,5‴	12.85 s, 12.98 s	–

In the FAB-MS spectrum of compound **5**, the molecular ion at *m/z* 567 is in accordance with the molecular weight of dimethylbiapigenin. The UV spectrum of **5** suggests the presence of substituted OH groups in positions C-7 (or C-7″) and C-4‴ (or C-4′) (the lack of bathochromic shift of II maximum with CH_3COONa and the decrease of intensity of I maximum with CH_3ONa) (Markham, 1982). Moreover, in the ^{1}H NMR spectrum of **5**, two three protons signals were observed in the range of 3.64–3.75 ppm, characteristic for OCH_3 groups (Markham, 1982). Furthermore, the signals of protons characteristic for a *para*-substituted phenyl group of system AA′BB′ at δ 6.91 (H-3‴,5‴), 7.69 (H-2‴,6‴), and system ABX at δ 7.95 (H-6′), 8.17 (H-2′), 7.01 (H-5′) were observed. The δ values of protons were attributed on the basis of COSY and ROESY spectra, including the chemical shifts of H-3 and H-3″ at 6.83 and 6.80, respectively. The type of linkage between two flavonyl moieties was deduced as C-C/3′–8″ from HMBC and HSQC spectra. In the HSQC spectrum of **7** the following C/H correlations were present: H-6/C-6 (δ 6.18/δ 98.2), H-6″/C-6″ (δ 6.32/δ 99.3) and H-8/C-8 (δ 6.56/δ 93.0). These δ values are in accordance with the literature data for carbon atoms in positions C-6, C-6″ and C-8 of amentoflavone (Geiger and Markham, 1996; Geiger *et al.*, 1993; Markham, 1982; Markham and Geiger, 1994). The signals of OCH_3 groups at δ 3.64 and δ 3.75 were assigned to carbon atoms in positions C-7 and C-4‴ from ROESY and HSQC spectra. In the ROESY spectrum the correlated signals were observed between protons H-8 (δ 6.56), H-6 (δ 6.18) and the signal of the OCH_3 group at δ 3.64 (C-7), protons H-2‴,6‴ (δ 7.69), H-3‴,5‴ (δ 6.91) and the signal of the OCH_3 group at δ 3.75 (C-4‴).

From these results, therefore, the structure of compound **5** was established as 7,4‴-*O*-dimethylamentoflavone (podocarpusflavone B). This compound was earlier identified only in some species of the genus *Podocarpus* (Podocarpaceae) (Geiger, 1994; Geiger and Quinn, 1988) and *Putranjiva roxburghii* (Euphorbiaceae) (Harborne and Baxter, 1999).

The next isolated biflavone, compound **3**, under the used chromatographic conditions gave values of R_f and t_R typical for dimethyl derivatives of amentoflavone (Krauze-Baranowska and Malinowska, 2005; Krauze-Baranowska *et al.*, 1999). The presence of two OCH_3 groups was confirmed by mass spectral analysis – the molecular ion at *m/z* 568 in the FAB-MS spectrum – and also by

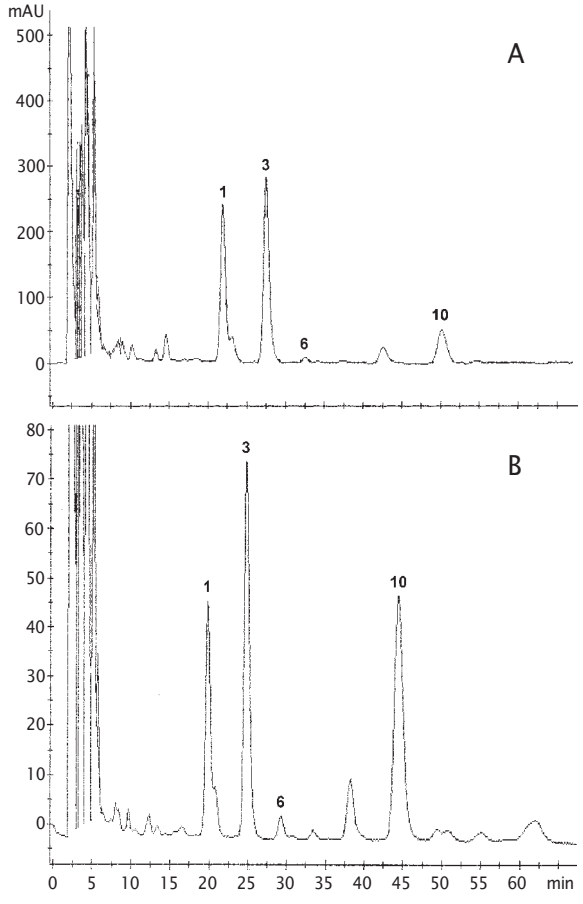

Fig. 1. HPLC chromatograms of the methanol extracts from *Chamaecyparis pisifera* "Squarrosa" (A) and *Chamaecyparis pisifera* "Boulevard" (B): 1, amentoflavone; 3, 4‴-*O*-methylamentoflavone; 6, 7-*O*-methylamentoflavone; 10, hinokiflavone.

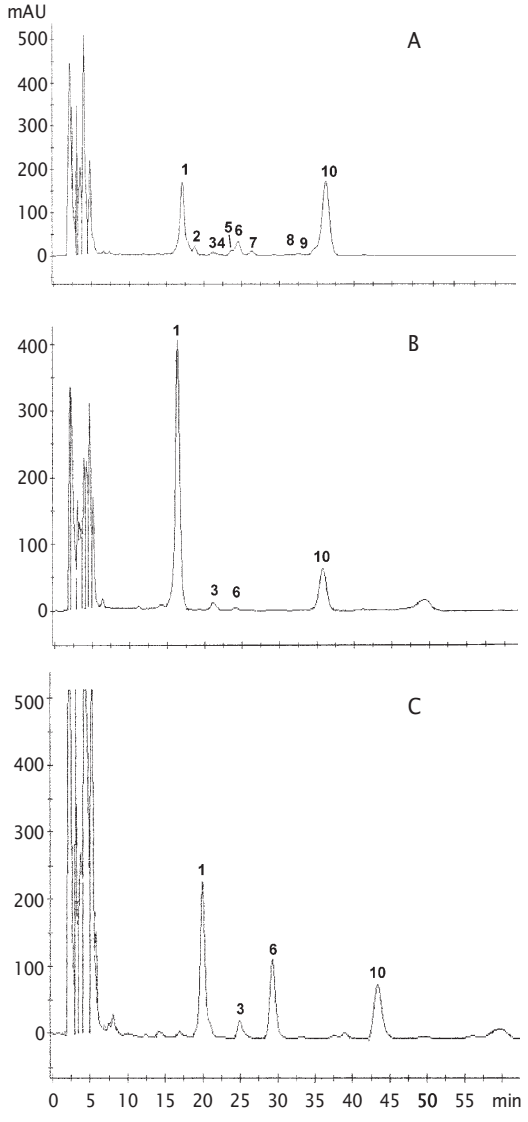

Fig. 2. HPLC chromatograms of the methanol extracts from *Chamaecyparis obtusa* (A), *Chamaecyparis thyoides* (B), and *Chamaecyparis lawsoniana* (C): 1, amentoflavone; 2, bilobetin; 3, 4‴-*O*-methylamentoflavone; 4, 7,7″-*O*-dimethylamentoflavone; 5, isoginkgetin; 6, 7-*O*-methylamentoflavone; 7, ginkgetin; 8, sciadopitysin; 9, 7,4‴-*O*-dimethylamentoflavone; 10, hinokiflavone.

^{1}H NMR analysis – two signals of OCH_3 groups at δ 3.80 and 3.78. The UV spectrum of **3** suggested the lack of free OH groups in positions C-7 and C-7″ (Markham, 1982). In comparison to amentoflavone, the downfield shifts of protons H-6, H-6″ and H-8 in the ^{1}H NMR spectrum of **3** is a diagnostic feature for biflavones substituted in

positions C-7 and C-7″ (Geiger and Markham, 1996; Geiger *et al.*, 1993; Markham *et al.*, 1990; Markham and Geiger, 1994).

As result conclusively, compound **3** can be identified probably as 7,7″-*O*-dimethylamentoflavone. This compound was recognized earlier in other species of the family Cupressaceae, namely *Cupressus lawsoniana* (Ahmad *et al.*, 1984) and *Thuja javanica* and *Thuja gigantea* (Harborne and Baxter, 1999).

The results of the study demonstrate, that leaves of *Ch. obtusa* contain a complex composition of biflavones, especially derivatives of amentoflavone. In contrast to earlier reports (Harborne and Baxter, 1999), no methoxylated derivatives of hinokiflavone have been separated and identified. It is worth to notice, that podocarpusflavone B was for the first time described in the family Cupressaceae (Geiger, 1994; Geiger and Quinn, 1988; Harborne and Baxter, 1999).

The biflavone composition in the leaves of two further species from *Chamaecyparis* – *Ch. lawsoniana*, *Ch. thyoides* – and two cultivar varieties – *Ch. pisifera* "Squarrosa" and "Boulevard" – were compared by employing the HPLC method. In both cultivars of *Ch. pisifera*, 4‴-*O*-methylamentoflavone was determined, besides amentoflavone and hinokiflavone (Fig. 1). In contrast to *Ch. pisifera* "Squarrosa", in the leaves of *Ch. pisifera* "Boulevard" hinokiflavone was determined in significantly lower concentration. It was concluded, that a high amount of 4‴-*O*-methylamentoflavone discriminates *Ch. pisifera* among other investigated cypress (Fig. 2). Until now, this compound was chromatographically (TLC) identified in the complexes of *Ch. nootkatensis*, *Ch. formosansis* and *Ch. lawsoniana* "Erecta" (Gadek and Quinn, 1985). In *Chamaecyparis lawsoniana* 4‴-*O*-methylametoflavone was detected besides dominating amentoflavone, 7-*O*-methylamentoflavone and hinokiflavone. Furthermore, in the leaves of *Chamaecyparis thyoides*, 4‴-*O*-methyloamentoflavone was recognized instead of bilobetin, that was earlier identified only in this one species within the *Chamaecyparis* genus (Gadek and Quinn, 1985) (Fig. 2). The obtained results confirm the chemotaxonomic description of the genus *Chamaecyparis* given by Gadek and Quinn (1985). On the other hand, it seems that mono-methoxylated derivatives of amentoflavone, namely 4‴-*O*-methylamentoflavone and 7-*O*-methylamentoflavone, may be regarded as the chemotaxonomic markers of this species, in addition to amentoflavone and hinokiflavone.

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