Biflavones from *Chamaecyparis obtusa*

Miroslawa Krauze-Baranowska, Loretta Pobłocka, and Atef Ahmed El Hela

*a* Department of Pharmacognosy, Medical University of Gdańsk, Gen. J. Hallera 107 str., 80-416 Gdańsk, Poland. Fax: +48 583 49 32 06. E-mail: krauze@amg.gda.pl

*b* Department of Pharmacognosy, Faculty of Pharmacy, Al-Azhar University, Cairo, Egypt

* Author for correspondence and reprint requests

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 anti-rheumatic remedies (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
methanol. The chloroform extract was concentrated and chromatographed over a polyamide column (50 g, 40 x 2 cm) with CHCl₃/MeOH (4:3 v/v) (eluates 1–10, 15 ml each), CHCl₃/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 x 1.5 cm) with MeOH (eluates 1–30, 4 ml each). From the obtained elutes 6–10 compounds 3 (6 mg) and 4 (6 mg) were isolated by preparative TLC on a polyamide column with CHCl₃/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 x 1.5 cm) with MeOH and re-chromatography over a column packed with the same adsorbent (5 g, 9 x 1 cm). The methanol extract was concentrated and chromatographed over a polyamide column (100 g, 45 x 3 cm) with MeOH/H₂O (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 x 1 cm) with MeOH and next by preparative TLC on cellulose with IsoPrOH/HCOOH/H₂O (2:5:5 v/v/v).

TLC analysis was done as described earlier (Krauze-Baranowska and Malinowska, 2005). Separation was performed on cellulose F₂₅₄ plates (Merck, Darmstadt, Germany, 20 x 20 cm, 0.10 mm) with the following mobile phases: CHCl₃/MeOH/MeOEt (4:3:2 v/v/v) (A), (4:8:6 v/v/v) (B) (Krauze-Baranowska and Malinowska, 2005), IsoPrOH/HCOOH/H₂O (2:5:5 v/v/v) (C), BuOH/H₂O/CH₃COOH (4:1:5 v/v/v) (D). HPTLC analysis was carried out on HPTLC RP-18 F₂₅₄ plates with the mobile phases: MeOH/H₂O/HCOOH (70:30:6 v/v/v) (E), MeOH/THF/H₂O/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 ¹³C 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-d₆ 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 x 4 mm, 5 µm) (Knauer, Berlin, Germany) and Lichrospher RP-18 (250 x 4 mm, 5 µm) (Merck) columns. Acquisition 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** (sciadopytin) (1): TLC polyamide: Rf: A – 0.89, B – 0.90. – HPTLC RP-18: Rf: F – 0.12. – HPLC: t_R = 35.1 min. – FD-MS: m/z (rel. int.) = 580 [M⁺] + (50). – UV, ¹H NMR and ¹³C 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: Rf: A – 0.71, B – 0.75. – HPTLC RP-18: Rf: 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-d₆): see Table I; the δ values of protons H-3, H-3″ and the positions of OCH₃ groups were elucidated from ROESY, ¹H–¹H COSY and NOE spectra.

**7,7″–O–Dimethylamentoflavone** (3): TLC polyamide: Rf: A – 0.85. – HPTLC RP-18: Rf: F – 0.23. – HPLC: t_R = 22.4 min. – UV (MeOH): λ_max = 270, 333 nm; (AlCl₃): λ_max = 280, 317sh, 340, 368sh nm; (AlCl₃/HCl): λ_max = 279, 316sh, 340, 369sh, 388 nm; (CH₃ONa): λ_max = 278, 400 nm; (CH₃COONa): λ_max = 270, 333 nm; (CH₃COONa/H₂BO₃): 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-d₆): 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, OCH3-7,7′).

7′-O-Dimethylaminofoflavone (ginkgetin) (4): TLC polyamide: Rf; A: 0.77, B: 0.80. – HPTLC: \( R_f \) = 0.12, F = 0.28. – HPLC: \( t_R \) = 27.8 min. – UV (MeOH): \( \lambda_{max} \) = 271, 335 nm; (AlCl3): \( \lambda_{max} \) = 280, 315sh, 343, 386 nm; (AlCl3/HCl): \( \lambda_{max} \) = 279, 315sh, 343, 374sh, 385 nm; (CH3ONa): \( \lambda_{max} \) = 271, 333, 345sh, 385 nm; (CH2COONa): \( \lambda_{max} \) = 271, 308, 339 nm; (CH2COONa/H3BO3): \( \lambda_{max} \) = 270, 295sh, 335 nm. – FAB-MS (+): \( m/z \) (rel. int.) = 567 [M+H]+ (100). – 1H and 13C 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-Methylaminofoflavone (7): TLC polyamide: \( R_f \); A: 0.16. – HPTLC RP-18: \( R_f \); E: 0.41. – HPLC: \( t_R \) = 25.8 min. – UV, EI-MS, 1H NMR and 13C NMR data were consistent with those in the literature (Krauze-Baranowska et al., 1999, 2002).

4′-O-Dimethylaminofoflavone (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: 0.22. – HPLC: \( t_R \) = 8.7 Hz, H-2, 8.00 (1H, s, H-2), 6.80, 6.85 (1H, s, H-3,3′). – 1H and 13C NMR data are in agreement with the literature data (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 13C NMR data were consistent with those in the literature (Krauze-Baranowska et al., 1999; Markham, 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-
1H NMR (1–10), 13C 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 querce- 
ins, and 12 as kaempferol.

The structure of compound 2 was established as 4’,4”-
O-dimethylamentoflavone (isoginkgetin). In the 
1H NMR spectrum of 2, the δ values were as- 
signed to individual protons on the basis of the 
following correlated signals from COSY: H-3,5’,5”/ 
H-3/H-6’, OCH3-4”/H-2’,6”, OCH3-4”/H-3’,5” 
and OCH3-4’/H-5’. To our knowledge, the 
1H 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 1H 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 Δ δ = 0.14 ppm, Δ δ = 0.18 ppm, 
Δ δ = 0.20 ppm and Δ δ = 0.08 ppm, in comparison 
to the spectrum obtained at 22 °C (Table I). The 
differences depend on the temperature of the 
sample in 1H NMR spectra of C-C/3’–8’ biflav-
ones were noticed earlier for 7-O-methylamentoflav-
one (Krauze-Baranowska et al., 2002). In NMR 
analyses, the temperature of samples affects dy-
amic 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 re-
stricted rotation around C-C linkages and molecu-
dissymmetry (Rahman et al., 1982; Zhang et al., 
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.

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 CH3COONa and the decrease of inten-
sity of I maximum with CH3ONa) (Markham, 
1982). Moreover, in the 1H NMR spectrum of 5, 
two three protons signals were observed in the 
range of 3.64–3.75 ppm, characteristic for OCH3 
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 
oberved. 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 
flavonol moieties was deduced as C-C/3’–8’ from 
HMBC and HSQC spectra. In the HSQC spec-
trum 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 car-
bon atoms in positions C-6, C-6” and C-8 of amen-
toflavone (Geiger and Markham, 1996; Geiger 
et al., 1993; Markham, 1982; Markham and Geiger, 
1994). The signals of OCH3 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 OCH3 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 OCH3 group at δ 3.75 (C-4”).

Table 1. 1H NMR data of compound 2, isoginkgetin, isolated from Ch. obtusa recorded in DMSO-d6, at 22 °C 
and 97 °C at 500 MHz; the δ values of protons were assigned from 1H-1H COSY, ROESY, HMBC, HSQC 
spectra.

<table>
<thead>
<tr>
<th>H</th>
<th>5 (22 °C)</th>
<th>5 (97 °C)</th>
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<tbody>
<tr>
<td>H-3</td>
<td>6.78 s</td>
<td>6.60 s</td>
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<tr>
<td>H-6</td>
<td>6.08 s</td>
<td>6.05 brs</td>
</tr>
<tr>
<td>H-8</td>
<td>6.36 s</td>
<td>6.29 s</td>
</tr>
<tr>
<td>H-2’</td>
<td>7.92 s</td>
<td>7.87 s</td>
</tr>
<tr>
<td>H-3’</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>H-5’</td>
<td>7.28 δ (8.8)</td>
<td>7.36 δ (8.8)</td>
</tr>
<tr>
<td>H-6’</td>
<td>8.07 δ (8.8)</td>
<td>7.99 δ (8.8)</td>
</tr>
<tr>
<td>H-3”</td>
<td>6.72 s</td>
<td>6.52 s</td>
</tr>
<tr>
<td>H-6”</td>
<td>6.16 brs</td>
<td>6.02 s</td>
</tr>
<tr>
<td>H-8”</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>H-2”,6”</td>
<td>7.56 δ (8.8)</td>
<td>7.52 δ (8.8)</td>
</tr>
<tr>
<td>H-3”,5”</td>
<td>6.91 δ (8.8)</td>
<td>6.90 δ (8.8)</td>
</tr>
<tr>
<td>OH-4”,4’</td>
<td>3.76 s, 3.74 s</td>
<td>–</td>
</tr>
<tr>
<td>OH-5,5”</td>
<td>12.85 s, 12.98 s</td>
<td>–</td>
</tr>
</tbody>
</table>
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 Podsoparas (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 $^1$H NMR analysis — two signals of OCH$_3$ groups at $\delta$ 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-dimethylametoflavone. 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-methylametoflavone 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-methylametoflavone discriminates *Ch. pisifera* among other investigated cypress (Fig. 2). Until now, this compound was chromatographically (TLC) identified in the complexes of *Ch. nootkatensis*, *Ch. formosanensis* and *Ch. lawsoniana* “Erecta” (Gadek and Quinn, 1985). In *Chamaecyparis lawsoniana* 4’-O-methylametoflavone was detected besides dominating amentoflavone, 7-O-methylametoflavone and hinokiflavone. Furthermore, in the leaves of *Chamaecyparis thyoides*, 4’’-O-methylolametoflavone 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-methylametoflavone and 7-O-methylametoflavone, may be regarded as the chemotaxonomic markers of this species, in addition to amentoflavone and hinokiflavone.


