

## Antifungal Activity of Biflavones from *Taxus baccata* and *Ginkgo biloba*

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Bilobetin and 4''-O-methylamentoflavone were isolated and identified in the needles of *Taxus baccata*, for the first time in this species. The antifungal activity of biflavones from *T. baccata* and *Ginkgo biloba*, namely amentoflavone, 7-O-methylamentoflavone, bilobetin, ginkgetin, sciadopitysin and 2,3-dihydrosciadopitysin towards the fungi *Alternaria alternata*, *Fusarium culmorum*, *Cladosporium oxysporum* was determined employing computer-aided image analysis coupled to a microscope. Bilobetin exhibited a significant antifungal activity with values of ED<sub>50</sub> 14, 11 and 17 µM respectively. This compound completely inhibited the growth of germinating tubes of *Cladosporium oxysporum* and *Fusarium culmorum* at a concentration 100 µM. Activity of ginkgetin and 7-O-methylamentoflavone towards *Alternaria alternata* was stronger than that of bilobetin. Moreover, slight structural changes in the cell wall of *Alternaria alternata* exposed to ginkgetin at concentration of 200 µM were observed.

**Key words:** Biflavones, Isolation, Antifungal Activity

Biflavonoids are the chemotaxonomic markers in a majority of families from Gymnospermae, including the family Taxaceae and Ginkgoaceae (Geiger and Quinn, 1988). The first biflavone, ginkgetin was separated from *Ginkgo biloba* by Furukawa in 1929 (Lin *et al.*, 1997). Like ginkgetin, all other biflavones isolated from *G. biloba* as well as from species of the genus *Taxus*, are C-8/C-3'''apigenin dimers (Geiger and Quinn, 1988) with the exception of 2,3-dihydrosciadopitysin that belongs to a group of flavanone-flavones (Wollenweber *et al.*, 1998; Krauze-Baranowska and Sowiński, 1999). The following biflavones in *Taxus* species were investigated by other authors: sciadopitysin, ginkgetin in needles and stems bark of *T. baccata* by Khan *et al.* (1976), Das *et al.* (1995), Reddy and Krupadanam (1996), in *T. wallichiana* by Parveen *et al.* (1985), Singh *et al.* (1997) and in *T. cuspidata* by Konda *et al.* (1995), kayaflavone, amentoflavone in needles and stems bark of *T. baccata* by Das *et al.* (1994; 1995), in *T. wallichiana* by Parveen *et al.* (1985), Singh *et al.* (1997), 7-O-methylamentoflavone in *T. baccata* by Khan *et al.* (1976), in *T. wallichiana* by Parveen *et al.* (1985), 7''-O-methylamentoflavone by Di Modica *et al.* (1962) and 4',7''-O-dimethylamentoflavone, 7,4', 7''-O-trimethylamentoflavone by Das *et al.* (1994) in needles of *T. baccata*. Some of the above

data concerning the structures of biflavones in *T. baccata* are contradictory (Di Modica *et al.*, 1962; Khan *et al.*, 1976; Das *et al.*, 1994; Reddy and Krupadanam, 1996).

Dimeric flavones possess antiviral (Hayashi *et al.*, 1992; Lin *et al.*, 1997; 1999; Zembower *et al.*, 1998; Ma *et al.*, 2001) and antibacterial activity (Majinda *et al.*, 1997). The antifungal action of these compounds is not well known and was confirmed only for amentoflavone (Królicki and Lamer-Zarawska, 1984), cupressuflavone and 4'-O-methylcupressuflavone (Krauze-Baranowska *et al.*, 1999).

The aim of this work was to recognize and identify biflavones present in the needles of *T. baccata* growing in Poland. Simultaneous studies have also been performed on antifungal activity of biflavones isolated from *T. baccata* and *G. biloba* towards species of the fungi used in our previous studies (Krauze-Baranowska *et al.*, 1999), namely *Alternaria alternata*, *Fusarium culmorum* and *Cladosporium oxysporum*.

### Material and Methods

#### Plant material

The needles of *Taxus baccata* L. were collected from the Medicinal Plants Garden of the Medical

University of Gdańsk (Poland) in January 1997. A voucher specimen of the plant (97-001) is deposited at the Herbarium of the Department of Pharmacognosy of Medical University of Gdańsk (Poland).

NMR spectra were recorded on a Bruker MSL 300 instrument at 500 MHz (for  $^1\text{H}$ ) and 75.5 MHz (for  $^{13}\text{C}$ ) in DMSO- $d_6$  using TMS as an internal standard. EI (70 eV) and FD-MS [(8 + 3) kV  $J_E = 20$  mA] mass spectral data were obtained using an AMD-Intectra spectrometer and Varian MAT 711 spectrometer, respectively.

Analytical and preparative TLC were carried out on precoated plates with polyamide 11 F<sub>254</sub> (Merck, 20 × 20, 0.25 mm thickness) using mobile phases: CHCl<sub>3</sub>-MeCOEt-MeOH (4:2:3 v/v/v) (A), (4:8:6 v/v/v) (B), (4:2:1 v/v/v) (C), CHCl<sub>3</sub>-MeCOEt (4:3 v/v) (D). Column chromatography was performed with polyamide (Roth, Karlsruhe, Germany) and Sephadex LH-20 (Pharmacia, Uppsala, Sweden). HPLC analysis was made according to Krauze-Baranowska *et al.* (1999).

#### Extraction and isolation

Dried and pulverized needles of *T. baccata* (0.5 kg) were extracted in Soxhlet apparatus with petroleum ether, chloroform and methanol. The methanol extract was concentrated (50 ml) and chromatographed over a polyamide column (100 g, 45 × 3 cm, 15 ml each eluate) using methanol/water mixtures with increasing concentration of MeOH: 30%, 60%, 80% (eluates 1–51 containing flavonoid O-glycosides and aglycones) and the next with MeOH (eluates 52–71 containing biflavones). Compound **1** was purified from eluates 57–59 from a polyamide column eluted with mobile phases D and the next A (eluates 26–29). Finally, compound **1** (eluates 13–15, 6 mg) was obtained from the last eluates chromatographed over Sephadex LH-20 column (5 g, 8 × 1 cm, each eluate 1 ml) with MeOH. Compound **2** (eluates 14–16, 5 mg) was isolated from eluates 62–65 by preparative TLC on polyamide (chromatograms were twice developed with mobile phase C, the first time to a distance 6 cm and after drying the second time to a distance of 10 cm) and next was additionally purified over Sephadex LH-20 column (5 g, 8 × 1 cm, each eluate 1 ml) with MeOH.

Compound **1**: bilobetin. TLC R<sub>f</sub>: A – 0.42, B – 0.57; HPLC t<sub>R</sub> min –21.8; FD-MS *m/z* (rel. int.):

552 [M]<sup>+</sup> (100); UV,  $^1\text{H}$  NMR and  $^{13}\text{C}$  NMR data were consistent with those in the literature (Joly *et al.*, 1980; Markham, 1982; Markham *et al.*, 1987; Silva *et al.*, 1995).

Compound **2**: 4'''-O-methylamentoflavone TLC R<sub>f</sub>: A – 0.12, B – 0.21; HPLC t<sub>R</sub> min –24.1; UV, EI-MS data identical with those described in the literature (Markham, 1982),  $^1\text{H}$  NMR and  $^{13}\text{C}$  NMR data as reported by Sun *et al.* (1997).

2,3-dihydrosciadopitysin was isolated from the leaves of *Ginkgo biloba* according to procedure described earlier (Krauze-Baranowska and Sowiński, 1999).

#### Determination of the antifungal activity

The fungi *Alternaria alternata* (Fr.) Kiessler, *Cladosporium oxysporum* Berk. Curt and *Fusarium culmorum* W. G. Smith (Sacc.) were used in this study. Isolates of the above fungi were obtained from natural infected soft wheat grains and identified according to Ellis (1971) and Nelson *et al.* (1983). Spores of *A. alternata* and *C. oxysporum* ( $5 \times 10^5$  per ml) were inoculated in Czapek-Doxa liquid medium (Nelson *et al.*, 1983) whereas *F. culmorum* was suspended in Armstrong liquid medium (Booth, 1971). A 100  $\mu\text{l}$  of each spore suspension was transferred to an application well of microtiter plates (Sarstedt, Nümbrecht, Germany) and next 25  $\mu\text{l}$  of biflavone solutions in mixture methanol/liquid medium were added to obtain the concentrations of: 20, 40, 100 and 200  $\mu\text{M}$ . Amphotericin B (Sigma) was used as a positive control. Another control was carried out by addition of methanol to spore suspensions (the final concentration of methanol was less than 20%). After incubation at 25 °C (4 h for *A. alternata*, 7 h for *F. culmorum* and 24 h for *C. oxysporum*) the images of germinating tubes were recorded using a CCD camera (Evi-1011p Sony) connected to a microscope Nikon Labofot 2A and computer PC with card of frame grabber Aver 2000 Pro I and pocket of measuring software MultiScan 4.01 (CSS Scan, Warsaw, Poland). The measurements (n-21) of the length of germinating tubes (by ordinary visual counting) were performed by three different fields of visions. For statistical analysis the Student-Newman Kuels test was used.

## Results and Discussion

From the methanolic extract from the needles of *Taxus baccata* the biflavones bilobetin (**1**) and 4'''-O-methylamentoflavone (**2**) together with the known ones: sciadopitysin, ginkgetin, amentoflavone and 7-O-methylamentoflavone were isolated. The structures of compounds were established by co-chromatography with standards and spectroscopic methods – UV, MS, NMR (Joly *et al.*, 1980; Markham *et al.*, 1987; Silva *et al.*, 1995; Sun *et al.*, 1997). It is the first report about the occurrence of bilobetin and 4'''-O-methylamentoflavone in the family *Taxaceae* (Geiger and Quinn, 1988).

The potential of biflavones isolated from *T. baccata*-amentoflavone, 7-O-methylamentoflavone (sequoiaflavone), bilobetin, ginkgetin, sciadopitysin and 2,3-dihydrosciadopitysin from *Ginkgo biloba* to inhibit the growth of fungal spores and germinating tubes were determined using a computer-aided image analysis. An image analysis system enabled to observe the morphological changes of the spores during their germination in liquid medium, and assessing the antifungal activity of compound against fungal spore germination using automated discrimination of non-germinated spores from germinated (Oh *et al.*, 1996). This method makes it possible to check the homogeneity of the fungal cell culture, that influences

population growth rates. Variation of the fungal cell morphology is widely encountered in cell culture, therefore it is difficult to measure fungal cell growth cultured under various conditions (Oh *et al.*, 1996). Bioassays of biflavones were performed towards three fungi: *Alternaria alternata*, *Fusarium culmorum*, *Cladosporium oxysporum*. Bilobetin exhibited the significant antifungal activity with values of ED<sub>50</sub> 14, 11 and 17 μM respectively (Table I). This compound at a concentration 100 μM fully inhibited the growth of germinating tubes of *Cladosporium oxysporum* and *Fusarium culmorum*. Ginkgetin and 7-O-methylamentoflavone were stronger towards *Alternaria alternata* than bilobetin totally inhibiting the growth of fungal spores at a concentration 100 μM (Table I). Moreover, slight structural changes in the cell wall of *Alternaria alternata* exposed to ginkgetin at concentration 200 μM were observed (Fig. 1). Both biflavones- ginkgetin and 7-O-methylamentoflavone (sequoiaflavone) demonstrated activity towards other fungi similar to that of bilobetin with one exception – 7-O-methylamentoflavone was inactive against *Cladosporium oxysporum* (Table I). However, against this latter fungus sciadopitysin – a compound with three methoxyl groups, exhibited the strongest antifungal effect (ED<sub>50</sub> 9 μM, Table I). Biflavones without a methoxyl group such as amentoflavone and biflavo-

Table I. The inhibitory effect on the growth fungal germ tubes by biflavones.

Compound	Fungus					
	<i>Alternaria alternata</i>		<i>Cladosporium oxysporum</i>		<i>Fusarium culmorum</i>	
	% inhibition of growth at concentration 100 μM ± SEM	ED <sub>50</sub>	% inhibition of growth at concentration 100 μM/ml ± SEM	ED <sub>50</sub>	% inhibition of growth at concentration 100 μM/ml ± SEM	ED <sub>50</sub>
Amentoflavone	54 ± 12	72	41 ± 22	–	74 ± 8	15
Bilobetin	80 ± 5	14	100	17	100	11
Sequoiaflavone	100	18	12 ± 22	111	100	12
Ginkgetin	100	23	63 ± 8	19	100	12
Sciadopitysin	59 ± 9	27	100	9	78 ± 7	14
2,3-Dihydrosciadopitysin	46 ± 11	116	61 ± 13	68	78 ± 6	13
Amphotericin B	100	2	100	1	100	3

Inhibitory effect at 100 μM is represented as% of inhibition, mean ± standard error of n = 21, and its significant difference from the control p < 0.01.

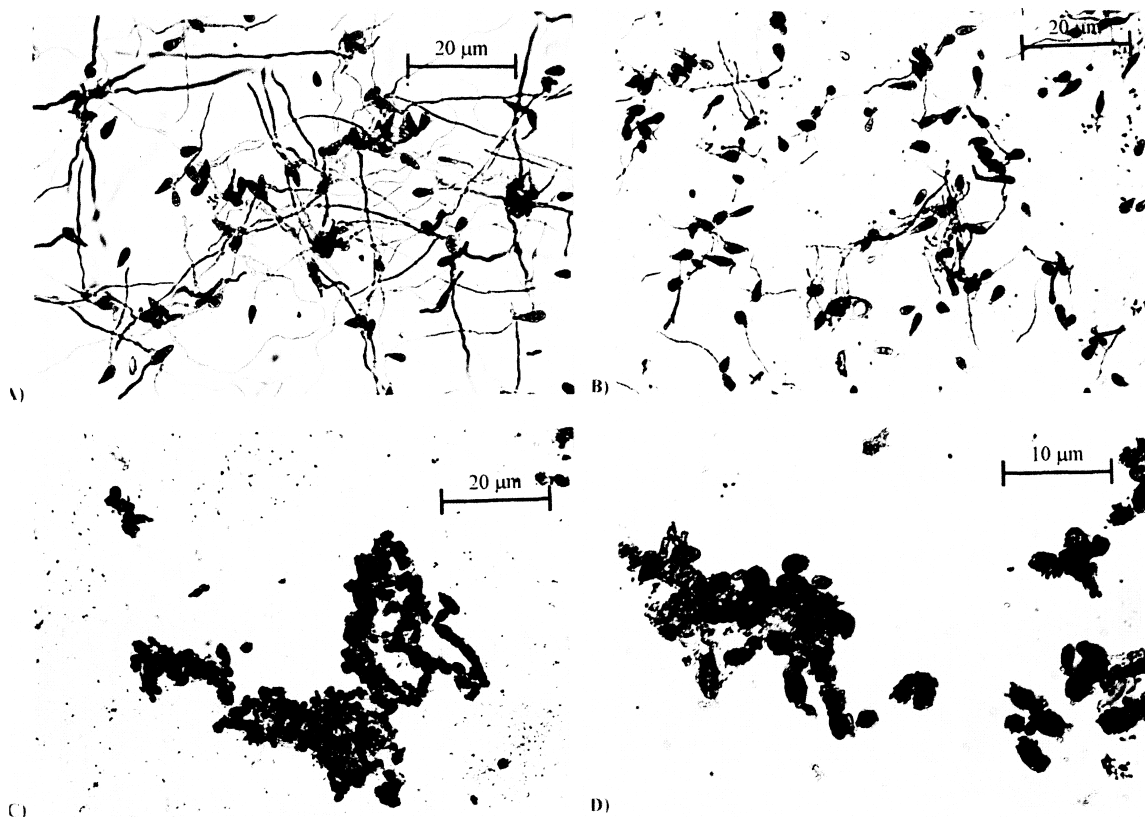


Fig. 1. The antifungal action of ginkgetin towards *Alternaria alternata*: A) the germ tubes of the fungus-control, B) and C) the inhibition of growth of germ tubes at doses of 40  $\mu\text{M}$  and 100  $\mu\text{M}$ , respectively D) the effect on cell wall of germ tubes at dose 200  $\mu\text{M}$ .

noid consisting of flavanone-flavone units like 2,3-dihydrosciadopitysin were inactive or weakly towards *Alternaria alternata* and *Cladosporium oxysporum* (Table I). A significant effect of a methoxyl group on increased antifungal activity of biflavones, has been found especially with *Cladosporium oxysporum*. In contrast, the increase of a number of methoxyl groups decreased antifungal action of biflavones towards *Alternaria alternata*. On the other hand is interesting, that all biflavonoids assayed had a similar activity against *Fusarium culmorum* expressed by very close values of  $\text{ED}_{50}$  in the range of 11–15  $\mu\text{M}$ . The above results show the specific fungal sensitivity towards

biflavones and confirm our earlier observations of antifungal activity with C-8/C-8'-biapigenin derivatives (Krauze-Baranowska *et al.*, 1999). In conclusion, a computer-aided image analysis coupled to a microscope in comparison with conventional antimicrobial assays might be successfully used for screening of antifungal agents isolated from plants even in small amounts – as little as 0.2 mg of a compound is sufficient for the test.

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