ACTEOSIDE AND RELATED PHENYLETHANOID GLYCOSIDES IN *BYBLIS LINIFLORA* SALISB. PLANTS PROPAGATED IN VITRO AND ITS SYSTEMATIC SIGNIFICANCE

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ABSTRACT

From plantlets of *Byblis liniflora* Salisb. (Byblidaceae), propagated by in vitro culture, four phenylethanoloid glycosides – acteoside, isoacteoside, deshamnosylacteose and deshamnosylisoacteoside were isolated. The presence of acteoside substantially supports a placement of the family Byblidaceae in order Scrophulariales and subclass Asteridae. Moreover, the genera containing acteoside are listed; almost all of them appear to belong to the order Scrophulariales.

KEY WORDS: *Byblis liniflora*, Byblidaceae, Scrophulariales, chemotaxonomy, phenylethanoloid glycosides, acteoside, in vitro propagation.

INTRODUCTION

Byblidaceae are a small family of essentially Western and Northern Australian (extending to Papuasia) herbs with exstipulate, linear sticky leaves spirally arranged along a more or less upright or sprawling stem and solitary, ebracteolate, pentameros, weakly sympetalous, very weakly zygomorphic (stamens and style slightly bent) flowers in the axils of the upper leaves. Pollen is shed as individual grains with smooth exine. The superior ovary is 2-locular with a single style terminating in a knob-shaped stigma, the axile placentas bear numerous unitegmic ovules. Chalazal and micropylar endosperm haustoria are present. Chromosome counts of 2n=14, 2n=16, 2n=18, 2n=24, and 2n=32 have been reported (Conran et al. 2002a). Fossils are unknown.

The only genus of the family Byblidaceae – *Byblis* Salisb. was described for the first time by English botanist and gardener – R.A. Salisbury in “The Paradisus Londinensis” in 1808 (Fessler 1982) and until recently it involved only two species – *B. liniflora* Salisb. and *B. gigantea* Lindl., but Lowrie and Conran (1998) and Conran et al. (2002b) established four new species – *B. aquatica* Lowrie et Conran, *B. filifolia* Planch., *B. rorida* Lowrie et Conran, and *B. lamellata* Conran et Lowrie.

*Byblis liniflora* Salisb. grows erect to 15-20 cm. Its leaves are alternate, involute in vernalation, simple, linear with a clavate apical swelling, and with stipitate, adhesive and sessile, digestive glands on the lamina (Huxley et al. 1992; Lowrie 1998). The species is an annual in its native habitats where soils dry out part of the year. In situations of permanently wet soils it is a perennial (Pietropalo and Pietropalo 1996).

The chemical composition of *Byblis* is rather hardly known. Absence of naphthoquinones recorded for *B. gigantea* supported the assumption that Byblidaceae are not related to Drosoraceae (Zenk et al. 1969; Juniper et al. 1989).

Here we present our phytochemical investigations on *Byblis liniflora* obtained by in vitro culture, which led to isolation and identification of four phenylethanoloid glycosides for the first time found in the family Byblidaceae. One of them – acteoside (verbascoside) was previously found in numerous species including many medicinal plants; it is also known for many biological activities and generally considered as important chemotaxonomic marker (Jimenez and Riguer 1994). For the latter reason we discuss the sy-
soteric significance of acetyls in Byblidaceae and other taxa containing this compound.

MATERIAL AND METHODS

General
UV spectra were recorded in methanol on a Specord M-40 (Zeiss, Jena) using previously described procedures (Mabry et al. 1970). NMR spectra were recorded on a Varian Unity 300, at 300 MHz for $^1$H NMR and 75 MHz for $^{13}$C NMR, in CD$_2$OD solutions with TMS as standard. Analytical thin-layer chromatography (TLC) was carried out on pre-coated silica gel and cellulose plastic-backed sheets (Merck, Darmstadt) and self-made polyamide (Woelm, Eschwege, Germany) or polyamide DC6 (Macherey-Nagel, Düren) plates. For detection, the developed chromatograms were viewed under UV$_{365}$nm and UV$_{254}$ nm before and after spraying with 0.1% Naturstoffreagens A (NA) or 1% aluminum chloride in ethanol followed by warming. Preparative thin layer chromatography (PTLC) was performed on self-made polyamide (Woelm, Eschwege) and PF$_{254}$ silica gel (Merck, Darmstadt) (0.5 or 1 mm thickness) plates. Open column chromatography (CC) was carried out with polyamide SC-6 (Macherey-Nagel, Düren) and Sephadex LH-20 (Pharmacia, Uppsala).

Plant material
In vitro cultured plantlets of Byblis liniflora Salisbl. were originally received from the Micropropagation Unit of Royal Botanic Gardens, Kew, United Kingdom, in 1987. In vitro cultures
RM medium (Reinert and Mohr 1967) and MS medium (Murashige and Skoog 1962) with half-strength concentration of mineral salts (1/2 MS) were used. Initial explants – shoots and nodal segments – were taken from plantlets of Byblis liniflora. Response to auxin – IBA (1 mg/l), alone or in combination with cytokinins – kinetin (1-2 mg/l) and/or BA (0.5-1 mg/l) was investigated with single shoot fragments placed in small test tubes (20 explants per each experiment). For further mass-propagation, the aggregates consisting of 2-3 shoots with callus tissue formed at the ba-

| TABLE 1. $^1$H and $^{13}$C NMR data for phenylethanoid glycosides from Byblis liniflora. |
|------------------------|------------------------|------------------------|------------------------|------------------------|
| Position               | $^{13}$C 1            | $^1$H 1                | $^{13}$C 2            | $^1$H 2                |
|                        | 1            | 2            | 3            | 4            |
| aglycone               | 131.5        | 131.5        | 131.5        | 131.6        |
|                        | 117.1        | 6.71 d (2.1) | 117.2        | 6.67 d (2.1) |
|                        | 146.1        | 146.8        | 146.2        | 146.2        |
|                        | 144.6        | 144.7        | 144.7        | 144.7        |
|                        | 116.4        | 6.70 d (8.4) | 116.4        | 6.63 d (8.1) |
|                        | 121.3        | 6.57 dd (2/1.8/4) | 121.3        | 6.53 dd (1.8/8.1) |
|                        | 36.5         | 2.79 r (9.6) | 36.7         | 2.79 r (7.5) |
|                        | 72.3         | 4.05 m       | 72.4         | 3.96 m       |
|                        | 3.74 m       | 3.71 m       | 3.71 m       | 3.74 m       |
| caffeoyl               | 127.7        | 127.8        | 127.8        | 127.8        |
|                        | 114.7        | 7.03 d (2)   | 114.9        | 7.03 d (2)   |
|                        | 146.8        | 146.4        | 146.8        | 146.9        |
|                        | 144.8        | 149.6        | 149.7        | 149.8        |
|                        | 116.6        | 6.79 d (8.1) | 116.6        | 6.76 d (8.1) |
|                        | 123.3        | 6.89 dd (2/8.1) | 123.2        | 6.88 dd (2/8.1) |
|                        | 148.1        | 7.56 d (15.9) | 147.5        | 7.56 d (15.9) |
|                        | 115.3        | 6.28 d (15.9) | 115.2        | 6.29 d (15.9) |
|                        | 168.4        | 169.2        | 169.2        | 168.7        |
| glucose               | 104.2        | 4.38 d (7.8) | 104.5        | 4.33 d (7.5) |
|                        | 76.2         | 3.40 dd (7/8/9.3) | 75.8        | **          |
|                        | 81.7         | 3.82 r (9.3) | 84.1         | **          |
|                        | 70.4         | 4.94 r (9.6) | 70.1         | **          |
|                        | 76.0         | 3.60-3.50 m  | 75.5         | **          |
|                        | 62.3         | 3.60-3.50 m  | 64.7         | 4.49 dd (1.9/11.4) |
|                        | 3.60-3.50 m  | 4.35 dd (5.7/11.4) | 64.7         | 4.50 dd (2.1/12.0) |
| rhamnose              | 103.0        | 5.19 d (1.5) | 102.8        | 5.18 d (1.3) |
|                        | 72.8         | 3.93 dd (1/3.5/3.3) | 72.47        | **          |
|                        | 72.0         | 3.60-3.50 m  | 72.3         | **          |
|                        | 73.8         | 3.30 r (9.6) | 74.0         | **          |
|                        | 70.6         | 3.60-3.50 m  | 70.5         | **          |
|                        | 18.5         | 1.09 d (6.0) | 17.9         | 1.24 d (6.0) |

* signals sequenced by $^1$H-$^1$H-COSY spectrum
** signal pattern unclear due to overlap
sal part of shoots were placed onto a fresh RM medium every 2-3 months in 200 ml Erlenmeyer flasks. The cultures were maintained in a growth chamber at 22-24°C with 24 h light of 30 μm x m^2 x s^1.

**Plant material for phytochemical analyses**

The plantlets of *B. liniiflora* obtained on RM medium were harvested in August 1995.

**Extraction and isolation of phenylethanoid glycosides 1-4.**

The fresh, whole plants (246 g) were plunged into boiling methanol (1L) and left for maceration at ambient temperature, which was repeated twice and lasted six months in total. The methanol extract was concentrated to dryness in vacuo, suspended in water (40 ml) and extracted with chloroform (3×100 ml) and 1-butanol saturated with water (5×50 ml). The extract in vacuo butanolic fraction (1.9 g) was separated over Sephadex LH20 column by sequential elution with 50%, 80% and 100% methanol to give 15 fractions. Fraction 10 yielded compound 1 (361.2 mg), after PTLC on polyamide in chloroform-methanol-butanol-2-acetyloacetone (9:4:2:1), followed by CC on Sephadex LH20 with methanol. Fraction 12, was separated by PTLC on silica gel in ethyl acetate-ethanol-water (30:3:2) to afford compounds: 2 (7.6 mg), 3 (25.1 mg) and 4 (7.4 mg), after final purification by CC on polyamide and Sephadex LH20 in methanol, respectively.

*acteoside (=verbascoside) (1)*

UV: 248, 292, 335; +AlCl₃: 263, 298, 365. ¹H and ¹³C NMR: Table 1.

*isacteoside (2)*

UV: 248, 291, 330; +AlCl₃: 257, 296, 350. ¹H and ¹³C NMR: Table 1.

*desrhamnosyl isacteoside (3)*

UV: 247, 291, 329; +AlCl₃: 257, 296, 350. ¹H and ¹³C NMR: Table 1.

*desrhamnosyl acteoside (4)*

UV: 247, 291, 330; +AlCl₃: 260, 299, 359. ¹H and ¹³C NMR: Table 1.

**RESULTS AND DISCUSSION**

Efficient growth, proliferation and rooting of plantlets of *B. liniiflora* in vitro conditions was obtained on the basal RM medium and 1/2 MS medium. Supplementation of the RM medium with growth regulators slightly improved shoot propagation from initial explants when BA was used (approximately from 7 to 12 shoots per explant). However, the best explants for mass propagation were aggregates consisting of 2-3 shoots with the green-white callus tissue formed at their base part from which adventitious shoots regenerated (approximately 20 shoots per explant) within 2-3 months of culture. Therefore, one can conclude that basal RM medium is satisfactory for mass propagation of *B. liniiflora*. Efficient propagation in in vitro culture on RM or MS media without exogenous growth regulators have been earlier observed in the case of other carnivorous species like those of the genera *Drosera* and *Dionaea* of the family Drosiceraceae (Kukulczanka 1991; Kukulczanka and Budzianowski 2002). Similarly, stem cuttings of *Byblis liniiflora* can be rooted in vivo (Fessler 1982; Slack 1985; Huxley et al. 1992).

The methanol extract of the fresh plantlets of *Byblis liniiflora* obtained on RM medium was separated into chloroform, butanol and water soluble fractions. Preliminary analyses by thin-layer chromatography on silica gel of the chloroform fraction, according to Budzianowski (1995), showed absence of napthoquinones (plumbagin or 7-methyljuglone) previously also reported for *Byblis gigantea* (Juniper et. al. 1989), whereas two-dimensional thin-layer chromatography (2D TLC) on cellulose (Budzianowski and Skrzypczak 1995) of butanolic and water fractions, suggested presence of caffeic acid derivatives in butanol fraction. The latter was separated by combination of column and thin layer chromatography to afford compounds 1-4. The isolates exhibited very similar UV spectra with bathochromic shift with aluminium chloride (AlCl₃), indicative of the free ortho-diphenolic grouping, and were typical for the caffeic acid esters (Harborne 1984). The chemical structures of compounds 1-4 (Fig. 1) were identified by comparison of their ¹H and ¹³C NMR data (Table 1) with those published in the literature as: acteoside (verbascoside) (1) (Budzianowski and Skrzypczak 1995; Debrauwer et. al. 1989; Xiong et. al. 1996), isoacteoside (isoverbacoside) (2) (Kobayashi et.al. 1987), desrhamnosylisacteoside (calcelarioside B) (3) (Shimomura et al. 1987) and desrhamnosylacteoside (calcelarioside A) (4) (Nishimura et. al. 1991). The ¹³C NMR assignments of resonances for carbon atoms C-2, C-3, C-5 of glycone moieties as well as those for C-3', C-4' and C-8' of caffeoyl acyl groups, pre-

![Fig. 1. Chemical structures of phenylethanoid glycosides isolated from *Byblis liniiflora.*](image-url)
sented in Table 1, are assigned according to data verified recently by Xiong et al. (1996). All compounds found are new for the genus *Byblis* and the family Byblidaceae and they appear to be very helpful (especially acteoside) chemotaxonomical characters for solving problems of systematics of those taxa discussed below.

The genus *Byblis* has been of controversial systematic placement since its discovery. A number of authors (e.g. Planchon 1848) supposed a proximity to Droseraceae mainly due to the presence of glandular trichomes on the leaf surface. But as already pointed out by Diels (1906), the gland structures of *Byblis* and *Drosera* (or indeed any member of Nepenthales) are fundamentally different. An affinity to *Roridula*, essentially inspired by a common placement in Droseraceae, was assumed for similar reasons (Domin 1922) and can likewise be discounted due to ultrastructural differences. Similarities in floral structure already mentioned by Planchon (1848) led a large number of

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authors (cf. Diels 1906) to assume an affinity of Byblis to Pittosporaceae. But missing resin ducts, different ovule structure and the stalked glands that lack any equivalent in Pittosporaceae separate Byblis from the latter family. A “sympetalous” ovule structure in combination with the insect-capturing habit inspired Lang (1901) to compare Byblis with Lentibulariaceae, but the very weak sympetaly of the spurless flowers (always bilabiate and spurred in Lentibulariaceae), lacking reduction in the androecium (two stamens and no staminodes in L.), and the sepal ovary (free central in L.) clearly indicate otherwise. The presence of iridoid compounds in Byblidaceae (positive Ehrlich test, Gibbs 1974) does, however, suggest a placement in Asteridae.

Gene sequence homology comparisons (rubL: Albert et al. 1992, 18S rRNA: Conran & Dowd 1993) support an Asterid placement of Byblidaceae clearly separate from Drosoraceae (Nepenthales, “Caryophyllales s.lat.”), Roridulaceae (Ericalles), and Pittosporaceae (Araliales, Apiales), and inclusion in a common monophyletic clade with Lamiaeae, Scrophulariaceae, and Lentibulariaceae comprising the order Scrophulariales (syn. Lamiales, Bignoniaceae). This placement was more recently confirmed by homology comparisons of six chloroplast DNA markers (Bremner et al. 2002).

Although the initial enthusiasm about the systematic usefulness of phytochemical (particularly flavonoid) characters was largely disappointed in past decades, some classical examples of characteristic secondary metabolites in selected taxonomic contexts (betalains in Caryophyllales, glucosinolates in Capparales, seed coat phytomelans in Asparagales) clearly demonstrate the validity of the chemotaxonomic concept. Recently, some phylogenetic lineages established primarily on the basis of gene sequence homology comparison have been substantiated by phytochemical data, e.g. in the case of Nepenthales, in which the majority of families (Polygonaceae, Plumbaginaceae, Drosoraceae, Nepenthaceae, Drosophyllaceae, Dioncophyllaceae, Ancistrocladaceae) contain acetylenic quinones like plumbagin (Schlauer 1997).

In this context it is remarkable that acteoside (verbascone, kusaginin) was established as a characteristic constituent of Scrophulariales (Scogin 1992). While other caffeoyl-dihydroxyphenethylic glycosides have been identified in a number of not closely related families throughout the plant kingdom (Hegnauer 1990; Jimenez and Riguera 1994; Wada et al. 1995, Braca et al. 2001), acteoside has so far been detected in only five genera not belonging to Scrophulariales, viz. Cissusnopsis, Craterocapsa, Echinacea, Magnolia, and Momordica. On the other hand, all families of Scrophulariales investigated so far did contain at least one genus yielding acteoside (cf. Table 2).

Because of the obvious chemosystematic value of acteoside, its identification in Byblis liniflora, as reported in this paper, is of primary importance considering the systematic placement of Byblidaceae. It fully supports a placement in order Scrophulariales and subclass Asteridae and confirms corresponding hypotheses based on gene sequence homology. Like in Nepenthales this is another example for the usefulness of chemotaxonomy especially in cases where morphological and ultrastructural characters are ambiguous or misleading.

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