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PRODUCTION OF LIGNANS BY *HAPLOPHYLLUM PATAVINUM IN VIVO* AND *IN VITRO*

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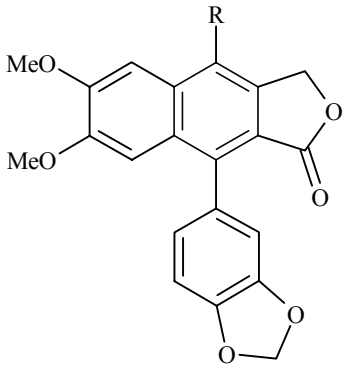
Abstract - Three known arylnaphthalene lignans (justicidin B, diphyllin and tuberculatin), and one dibenzylbutyrolactone (arctigenin) were isolated from native plants, callus and suspension cultures of *Haplophyllum patavinum*. The present paper represents the first report on arylnaphthalene lignan production by *in vitro* cultures; *in vitro*, the biogenetic potential is strictly dependent on the cell strain.

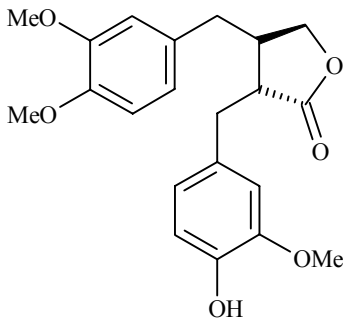
Haplophyllum patavinum (L.) G. Don fil. (Rutaceae) is a perennial endangered herb, which faces extinction in its Italian range, as the result of habitat modification by man's activities and the peculiar propagation of the species.

No use of this species in folk medicine is reported, in spite that several biologically active compounds of potential pharmaceutical interest are known to occur in the genus *Haplophyllum* namely coumarins, lignans and alkaloids.[1] Systematic chemical investigation of *H. patavinum* has been seriously restricted by the very limited plant biomass available in the natural habitat.[2] Nevertheless *in vitro* cultures provided additional plant material for chemical analysis. Moreover, several workers[1] have studied the secondary metabolites in intact plant organs of the genus *Haplophyllum*, but the biosynthetic potentialities of *in vitro* cultures have never been investigated before. In a previous research a number of coumarin compounds (simple coumarins, linear and angular furocoumarins) were isolated from *H. patavinum* either *in vitro*

than *in vivo* conditions.[1,2]

Because of the biological importance of lignans as antitumoural and antiviral compounds, our phytochemical investigations were focused on this class of metabolites. As a result of our research we isolated and identified four lignans (**1-4**) from two cell strains of calli, cell suspensions and native plants.





1 R= H
2 R= OH
3 R= O-β-D-apiofuranosyl

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The bluish fluorescence of lignans (**1-3**) under UV light as well as their similar UV spectra indicated that all these compounds bear an arylnaphthalene nucleus.[3] The known compounds (**1-3**) were identified as justicidin B (**1**), diphyllin (**2**) and tuberculatin (**3**), by comparison of their spectral data (UV, IR, ^{1}H and ^{13}C NMR and ESI-MS spectra) with those previously reported.[4-6] The compound (**4**), present only in traces, was identified as the dibenzylbutyrolactone lignan (arctigenin) by the comparison with an authentic sample.

The amount of lignans recovered from native plants from the Euganean Hills sites, tissue and suspension cultures are reported in Table 1.

Table 1. *In vivo* and *in vitro* lignan contents (mg/100 g dry wt).

		NATIVE PLANTS	TISSUE CULTURES				SUSPENSION CULTURES	
			Strain A		Strain B		Strain A	
			callus	medium	callus	medium	cell	medium
JUSTICIDIN B	(**1**)	28.6 ± 0.4	nd	nd	9.3 ± 0.1	nd	nd	nd
DIPHYLLIN	(**2**)	317 ± 2.8	nd	nd	55.3 ± 0.2	nd	nd	nd
TUBERCULATIN	(**3**)	423 ± 6.9	nd	nd	586 ± 6.5	nd	nd	nd
ARCTIGENIN	(**4**)	nd	traces	nd	traces	nd	traces	nd

nd = not detected

The arylnaphthalene lignans [justicidin B (**1**), diphyllin (**2**) and tuberculatin (**3**)] were isolated both from

cell cultures and native plants. Conversely, the dibenzylbutyrolactone lignan (arctigenin) (**4**) was detected only *in vitro* conditions. However, the occurrence of trace amounts of arctigenin (**4**) *in vivo* cannot be excluded with absolute certainty, as a consequence of the very limited plant biomass from the native populations available for chemical analysis. At our knowledge, arctigenin (**4**) has been hitherto isolated only from one *Haplophyllum* species.[7]

In vitro, the biogenetic potential is strictly dependent on the cell strain: the white-yellow friable calli (strain A) showed poor biosynthetic potential and only arctigenin was detected in both callus and cell suspension cultures. Conversely the dark-yellow hard calli (strain B), that are not able to afford suspension cultures, produced both arctigenin (**4**) and the arylnaphthalene lignans (**1**-**3**). *In vitro*, no excretion of lignan compounds into the medium was observed.

The arylnaphthalene lignans are rather widespread in the genus *Haplophyllum* (seventeen species have been hitherto investigated). Diphyllin (**2**) and justicidin B (**1**) were isolated from different species of the genus,[6,8-15] tuberculatin (**3**) was detected only in five species.[10,15-17]

At our knowledge, the present paper represents the first report on arylnaphthalene lignan production by *in vitro* cultures, biosynthesis of these compounds having been observed hitherto only under *in vivo* conditions. In fact at the moment, research on lignan production by plant cell cultures has been mainly addressed towards the aryltetralin lactones as podophyllotoxin,[18-21] that is the starting compound for the semisynthesis of the anticancer drugs etoposide and teniposide.

Since dibenzylbutyrolactone and arylnaphthalene lignans have been isolated and identified in *H. patavinum*, it will be very interesting to establish the biosynthetic correlations among these different compounds. At the moment the biosynthetic pathway to the dibenzylbutyrolactones has been deeply investigated and broadly defined in *Forsythia.*[22] The dibenzylbutyrolactone lignans are biosynthetic precursors of the aryltetralin lactone lignans by oxidative cyclization in *Podophyllum.*[23]

Conversely the pathway to arylnaphthalene lignans is not complitely elucidated. Arylnaphthalene may be synthesised chemically by oxidation of aryltetralins,[24-25] therefore potentially a similar sequence could occur in nature. Broomhead and coll.[26] supposed that matairesinol, analogue of arctigenin (**4**), might be anticipated to function in the biosynthesis of diphyllin (**2**). If the dibenzylbutyrolactones might represent an early stage in the biosynthetic pathway to the arylnaphthalene lignans, arctigenin (**4**) occurrence *in vitro* by *H. patavinum* might be regarded as the consequence of failure of expression (or inadequate production) of enzymes involved in the some biosynthetic steps. For this reason, further studies on differentiated cultures are in progress. In fact different experiments have demonstrated that gene(s) responsible for key steps in a biosynthetic pathway may be intact in callus and suspension cultures but expressed only in differentiated or organized tissues.[27]

EXPERIMENTAL

General. UV spectra in EtOH were obtained on a UV Perkin-Elmer mod. Lambda 5 spectrophotometer. IR spectra on a KBr disk were obtained on Perkin-Elmer 1600-FT IR spectrophotometer. Fluorescence spectra were recorded on Perkin-Elmer LS-5 spectrofluorimeter equipped with 3700 Data Station.

^{1}H NMR spectra of compounds (**1**) and (**4**) were recorded on a Varian 200 MHz spectrometer, with TMS as internal standard and in $CDCl_3$ as solvent. A Bruker DXR-600 spectrometer operating at 599.19 MHz for ^{1}H and 150.86 MHz for ^{13}C with the UXNMR software package, used in $CDCl_3$ and CD_3OD solutions of compounds (**2**) and (**3**). In 2D experiments, inverse detected ^{1}H-^{13}C HSQC (Heteronuclear Single Quantum Coherence) and HMBC (Heteronuclear Multiple Bond Correlation) were obtained as described previously.[28] MS spectra were performed on an LCQ (Finnigan, San José, CA, USA) ion trap mass spectrometer equipped with an electrospray ion source. Multiple MS spectrometry experiments were performed by isolating either $[M+H]^+$ or fragments ions.

Plant material. Native plants of *H. patavinum* from Euganean Hills (a voucher is deposited at the Botanical Garden of the University of Padova, PAD 3703) and tissue and cell cultures were analysed.

Tissue and cell cultures. Tissue and cell cultures were obtained and maintained as previously described.[2]

Extraction and isolation. Samples of dried plant material (10 g) from native specimens grown in the natural habitat, frozen-dried callus tissues (50 g), solid media and frozen-dried cells (100 g) from suspension cultures, were extracted with MeOH in a Soxhlet extraction apparatus for 48 h, the solvent was distilled, the residue was dissolved in MeOH and analysed. The liquid media, obtained by centrifuging (5000 rev./min) the suspension cell cultures, were extracted with Et_2O (48 h) in a liquid extraction apparatus, the solvent was distilled, the residue was dissolved in MeOH and analysed. The separation of compounds were carried out by TLC on preparative and analytical silica gel plates (Merck) using $CHCl_3$ or EtOAc/cyclohexane in different ratios (1:2, 1:1, 2:1, 3:1 v/v) as eluents.

Quantitative determination of lignans. Plant material (10 g) and frozen-dried cells (20 g) were extracted with MeOH in a Soxhlet extraction apparatus under the same conditions as described above. The lignan's content was performed by HPLC using a Jupiter column (RP C18, 5µ, 300 Å, 250 x 4.60 mm, Phenomenex) with a small precolumn (C18, 4 x 3 mm I.D.). The flow rate was 1 mL/min. The multistep linear solvent gradient was performed using steadly 5% MeOH and varying the percentage of H_2O and CH_3CN: at time 0 min 90% H_2O; at time 1 min 85% H_2O; at time 5 min 70% H_2O; at time 18 min 40% H_2O; at time 25 min 20% H_2O; at time 30 min 20% H_2O. Lignans elution was routinely monitored at 260 nm. Calibration curves were determined using a series of standard solutions containing varying amounts of stock justicidin B, diphyllin and tuberculatin. Limits of detection: justicidin B (**1**) 0.13 µg/mL (R = 0.9995), diphyllin (**2**) 0.38 µg/mL (R = 0.9970) and tuberculatin (**3**) 1.12 µg/mL (R =

0.9982). Quoted data are the average values of quantitative determinations performed on three different samples; standard deviation was less than 4%.

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