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HYDROXYCINNAMIC ACID ESTERS FROM CELL SUSPENSION CULTURES AND PLANTS OF *LEONTOPODIUM ALPINUM*

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Key Word Index—*Leontopodium alpinum*; Compositae; Edelweiss; micropropagation; cell culture; hydroxycinnamic acid esters; chlorogenic acid; 3,4-di-*O*-caffeoylquinic acid; β-sitosterol.

Abstract—Cell suspension cultures of *Leontopodium alpinum* were established in a modified Murashige and Sköog medium. The cultures were found to produce sitosterol and high concentrations of chlorogenic acid and 3,4-di-*O*-caffeoylquinic acid. Leaves and roots of Edelweiss plants obtained through micropropagation were also found to contain small amounts of these hydroxycinnamic acid esters together with sitosterol.

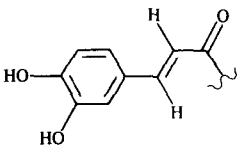
INTRODUCTION

The European alpine Edelweiss, *Leontopodium alpinum*, though not regarded as a traditional medical plant, has been reported as useful in the treatment of cancer [1] and diarrhoea [2], as well as showing toxicity to animals [3]. Phytochemical evaluation of *Leontopodium* and the related genus *Gnaphalium* has indicated the presence of flavonoids [4]. Limited analyses of the aerial parts of *L. alpinum* have shown the presence of luteolin and its 4′- and 7-β-glucosides [5], as well as caffeic acid [6]. In accord with our interest in plant cell cultures and their potential as a source of natural products with therapeutic value, a phytochemical analysis of the metabolites produced by cell suspension cultures of *L. alpinum* was undertaken and the results are reported here.

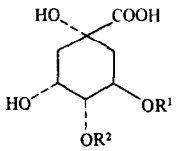
RESULTS AND DISCUSSION

Cell suspension cultures of *L. alpinum* were established and maintained on a modified Murashige and Sköog (M + S) medium. Cultures were repeatedly subcultured at monthly intervals until sufficient biomass was obtained. The fresh cell cultures, which fluoresced under UV (350 nm), were dried and extracted with methanol. The concentrated extract was chromatographed on a Sephadex LH 20 column and two major compounds were isolated.

Compound **1** was identified as chlorogenic acid (3-*O*-caffeoylquinic acid) on the basis of its physical and chemical properties. The FAB mass spectrum of (M^+ 516, $C_{25}H_{24}O_{12}$) together with duplication of the cinnamic acid resonances in its ^1H NMR spectrum and its previously unreported ^{13}C NMR spectrum (see Experimental) indicated a close structural resemblance to **1**. The identification of **2** as 3,4-di-*O*-caffeoylquinic acid was based on its physical properties coupled with a full spectroscopic analysis and by comparison with literature values [7] [8]. HPLC quantitation of extracts from 28-day-old cultures of *L. alpinum* gave yields of 300 mg chlorogenic acid and 350 mg of 3,4-di-*O*-caffeoylquinic acid per litre of medium. This represents a concentration of 2% (based on dry wt) for both **1** and **2**. HPLC analyses of methanolic extracts of dried *L. alpinum* leaves (collected at the pre-flowering stage) showed **1** to be present at a concentration of 0.45% and at 0.55%. Roots obtained from the same plants yielded 0.05% of **1** and 0.07% of **2**.



Caffeoyl



1 R^1 = Caffeoyl, R^2 = H
2 R^1 = R^2 = Caffeoyl

The presence of chlorogenic acid (**1**) in *L. alpinum* is not surprising because of its widespread occurrence in the plant kingdom. 3,4-, 3,5- and 4,5-dicaffeoylquinic acids are frequently isolated as a mixture (isochlorogenic acid), for example from coffee [9] [10] and the composite plant *Chrysothamnus paniculatus* [8]. Only one previous report on the isolation of 3,4-dicaffeoylquinic acid as a single product has been found, from *Pterocaulon virgatum* [7] (Compositae), where 1% of this isomer was recorded. There have been no previous reports of this compound or any of its isomers from *L. alpinum* plants or tissue cultures. Sitosterol was isolated from the petrol ether extracts of Edelweiss leaves, roots and suspension cultures and identified on the basis of its spectroscopic data, physical properties and co-chromatography with an authentic reference [11, 12].

EXPERIMENTAL

Plant material. Seeds of *L. alpinum*, supplied by W. J. Unwin Ltd, Cambridge, were germinated and grown to maturity in a peat-based potting compost at the School of Pharmacy, Dublin.

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Micropropagation. Apical buds from mature, overwintering plants were surface sterilized by soaking in a 10% aq. soln. of a commerical NaOCl concd for 20 min and rinsing × 5 with sterile dist. H_2O. Shoots developed and rooted on an agar-solidified Murashige and Sköog (M + S) salt medium [13] containing NAA (0.1 mg/l), kinetin (0.05 mg/l), thiamine HCl (0.4 mg/l), mesoinositol (80 mg/l), casein hydrolysate (100 mg/l) and sucrose (30 g/l). Rooted plantlets were subdivided and subcultured at monthly intervals onto 20 ml fresh medium in culture tubes and grown in continuous fluorescent light at 25° (±2°. Some were transplanted into potting compost and grown to maturity.

Suspension cultures. Callus cultures were developed from leaves of sterile, micropropagated plants placed on a modified M + S medium [14] and grown in continuous dim fluorescent light at 25°. Suspension cultures were initiated by transferring callus portions into liquid modified M + S medium. Flasks were shaken on an orbital shaker (120 rpm, 1 cm throw). Suspensions were subcultured at monthly intervals by transferring 10 ml of suspension culture into 90 ml fresh liquid medium.

Extraction and separation. Cells were dried in a fan oven (below 40°). Powdered material (25 g) was refluxed in MeOH (2 × 500 ml) for 30 min; filtered and the combined filtrates evapd to dryness. The residue was resuspended in MeOH (15 ml) and applied to a column of Sephadex LH20 (50 g). This was eluted with MeOH (1 l) and fractions (10 ml) were collected. All fractions were examined by TLC, System I: *n*-BuOH–HOAc–H_2O (4:1:5 upper phase) and like fractions were combined. System II: Et_2O–EtOAc (3:2) was also used with $KHSO_4$–silica gel (1:14) [17]. Two major compounds, **1** and **2**, were recovered and further purified by PLC (System I).

Identification. Mps are uncorr. ^1H NMR spectra were recorded at 270 MHz ($CDCl_3$, TMS, δ values), ^{13}C NMR (67.8 MHz). High resolution MS were recorded at 70 eV direct inlet. TLC was performed on precoated silica gel plates (60 F_{254}, Merck). PLC was performed on glass plates (20 × 20 cm) coated with silica gel (GF_{254}, Merck). HPLC was carried out on a Spherisorb 10 ODS, 250 × 4.6 mm column; solvent system AcCN–HOAC–H_2O (18:1:82), flow rate 2 ml/min, with detection by UV at 340 nm [15].

3-O-*Caffeoylquinic acid* (chlorogenic acid) (**1**). Amorphous solid, mp 202–205° (lit. mp 208°) [11]; $[\alpha]_D^{25°}$ −17° (H_2O, *c* 1.0) (lit $[\alpha]_D^{16}$ −35° (H_2O) [11]. NH_3, green; $FeCl_3$, black; R_f: 0.52 (system I) and 0.30 (system II); $[M]^+$ 336 [$C_{16}H_{18}O_9 - OH$].

3,4-*Di*-O-*caffeoylquinic acid* (**2**). Grey amorphous powder; mp 192–194° (lit. mp 194–195° [7];$[\alpha]_D^{23}$ −295(H_2O, *c* 0.9) (lit $[\alpha]_D^{20}$ −307 (H_2O) [7]; NH_3, green; $FeCl_3$, grey; R_f: 0.80 (system I) and 0.64 (system II). FABMS (+ve ion) $[M]^+$ 516; $C_{25}H_{24}O_{12}$ ^{13}C NMR (CD_3OD) 37.7 (*t*, C-6), 40.9 (*t*, C-2), 72.5 (*d*, C-4), 73.2 (*d*, C-3), 74.5 (*d*, C-5), 76.3 (*s*, C-1), 115.1 (*d*, C-5′/C-8′), 115.6 (*d*, C-5′/C-8′), 116.1, 116.5 (*d*, C-2′), 122.9 (*d*, C-9′), 127.9, 128.1 (*s*, C-4′), 146.8 (*d*, C-3′), 149.3, 149.5 (*s*, C-6′/C-7′), 169.0, 169.4 (*s*, C-1′), 179.5 (*s*, C-7).

Sitosterol was isolated from petrol extracts, separated on a silica gel column, gradient eluted with petrol: EtOAc, R_f: 0.73, [cyclohexane–EtOAc (1:1)], mp 136–137° (needles, MeOH) (lit. mp 136°) [11]; $[M]^+$ 414 ($C_{29}H_{50}O$).

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