Free Radical Scavenging Activity and Secondary Metabolites from *in vitro* Cultures of *Sanicula graveolens*

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An *in vitro* propagation system was developed to obtain shoot and root cultures from the Andean spice *Sanicula graveolens* (Apiaceae). Propagation of shoots, roots and plantlets was achieved by the temporary immersion system. The free radical scavenging effect of the methanol/water (7:3 v/v) extracts was determined by the discoloration of the 1,1-diphenyl-2-picrylhydrazyl radical (DPPH). Total phenolic, flavonoid, chlorogenic acid (CA) and quercetin 3-*O*-glucoside content in the samples was assessed by spectrophotometry and DAD-HPLC analysis, respectively. On a dry weight basis, the crude extracts showed total phenolic values ranging from 3.57 to 6.93%, with highest content for the root culture sample. Total flavonoid content ranged from 1.23 to 2.23% and was lower for the root culture. Chlorogenic acid and neochlorogenic acid were identified by TLC in all samples. Highest free radical scavenging effect was observed for the root culture which also presented the highest CA content. Two of the shoot culture samples, with similar IC_{50} values in the DPPH discoloration assay, also presented close quercetin-3-*O*-glucoside content.

Key words: In vitro Propagation, Secondary Metabolite Content, *Sanicula graveolens*

Introduction

The small herb *Sanicula graveolens* Poepp. ex DC (Apiaceae) is an aromatic plant that can be found in the Andean slopes at 1000–2000 m above sea level. The herb, with an aroma that closely resembles that of coriander (*Coriandrum sativum* L.), is used as a spice in some rural areas and was shown to display free radical scavenging effect (Viturro *et al*., 1999). It is known as “wild coriander” and is adapted to large temperature differences which can range from below 0 °C during the night to higher than 40 °C in sunny days.

Little has been done on the micropropagation and secondary metabolite production by native Andean plant cultures. The aim of this work was to develop a methodology for callus production and for micropropagation of *S. graveolens* and to assess the free radical scavenging effect and phenolic content in the shoot and root cultures. The *in vitro* regeneration, as well as growth conditions for callus production of this species have not been reported so far.

Materials and Methods

Chemicals

All solvents used were of analytical grade. Chloroform and methanol were obtained from J. T. Baker (Phillipsburg, NJ, USA). HPLC-grade acetonitrile and formic acid from Merck (Darmstadt, Germany) were used. Thin layer chromatography analysis was carried out on aluminum-coated silica gel (Sigma-Aldrich, St. Louis, MO, USA) and cellulose F_{254} plates from Merck. 1,1-Diphenyl-2-picrylhydrazyl (DPPH) was purchased from Sigma Chemical Co. (St. Louis, USA). Plant growth regulators, standard media and agar used for tissue culture were obtained from Duchefa Biochemie BV (Haarlem, The Netherlands) or Sigma Chemical Co.

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Plant material

The samples of wild growing *S. graveolens* including the seeds used for germination and callus cultures were collected in Chile at Las Trancas, Chillán, VIII Region, in the western Andean slopes, 1200 m above the sea level in November–December 2000. A voucher herbarium specimen has been deposited at the Herbario de la Universidad de Talca and was identified by Prof. José San Martin.

Establishment of callus cultures

Several growth regulators, *i. e.* dichlorophenoxyacetic acid (2,4-D), naphthaleneacetic acid (NAA), benzyladenine (BA) and thiodiazuron (TDZ), in various combinations were tested to induce morphogenic responses in leaf and stem sections of *S. graveolens* plants grown in the greenhouse. The nutrient medium assayed was Murashige and Skoog (MS) (Murashige and Skoog, 1962) provided with 30 g/l sucrose. The culture conditions to induce callus initiation are summarized in Table I.

In vitro propagation

Collected seeds were surface-sterilized with 70% ethanol for 30 s and sodium hypochlorite (3%) for 15 min under constant agitation, then washed three times in sterile distilled water and aseptically cultivated under *in vitro* conditions.

Shoot cultures (1 cm long) and leaf explants (1 cm^2) from plants obtained from the seeds were performed on MS basal medium containing macroelements, microelements and vitamins according to Murashige and Skoog (1962) in microcontainers (clear containers in size of 250 cm^3 and made from polypropylene). For shoot propagation the MS medium was additionally supplemented with 100 mg/l myo-inositol, 0.5 mg/l thiamine · HCl, 0.5 mg/l indoleacetic acid (IAA), 1.5 mg/l 6-benzylaminopurine (BAP) and 30 g/l sucrose. Solid medium was prepared by addition of 7.5 g/l agar, the pH value was adjusted to 5.8 before autoclaving. The culture temperature was 26 °C, the light intensity was 50 μmol s^{-1} m^{-2} for 16 h per day (Philips TLT 40W/54 R. S.). Roots were obtained from *in vitro* plantlets and cut in pieces of about 1 cm length. They were grown on a medium containing macroelements and microelements according to Schenk and Hildebrandt (1972) supplemented with 1 g/l myo-inositol, 5 mg/l thiamine · HCl, 0.5 mg/l pyridoxine, 5 mg/l nicotinic acid and 30 g/l sucrose. The cultures were performed in the dark and the culture temperature was as given above.

Biomass production in temporary immersion systems

Biomass production was performed using a bioreactor system working according to the temporary immersion system (TIS) (Etienne and Berthouly, 2002). This culture system allows the optimization of gas and nutrients exchange. Two different devices were used. The first device was a standard RITA system. The second device consisted of 1 l glass bottles as cultivation vessels. For shoot cultures they were equipped with culture rafts. Under standard conditions the system was run with 100 ml medium that was changed every 2 weeks and plants were immersed every 4 h for 5 min. The inoculum amount was 2.5 g per vessel and the culture temperature and the light intensity were as given above.

Applying different cultivation systems, the following samples were obtained:

Sample 1. Shoot culture in RITA systems with culture rafts on liquid medium; special *Pelargonium* medium (Applegren *et al.*, 1991).

Sample 2. Shoot culture in 1 l-TIS systems with culture rafts on liquid medium; modified Murashige and Skoog medium (as described above).

Sample 3. Shoot culture in microcontainers on semisolid modified Murashige and Skoog medium (as described above).

Sample 4. Shoot culture in micro containers on semisolid modified Murashige and Skoog medium (as described above).

Sample 5. Root culture in 1 l-TIS-systems with liquid medium; modified Schenk and Hildebrandt medium (Schenk and Hildebrandt, 1972).

Determination of total phenolic and flavonoid content

The total phenolic content of the extracts was determined using the method described by Singleton *et al.* (1999). Briefly, the appropriate extract dilutions were oxidized with the Folin-Ciocalteu reagent and the reaction solution was neutralized with sodium carbonate. The absorbance of the resulting blue color was measured at 700 nm after 30 min using a Heλios α V-3.06 UV-VIS spectrophotometer. Quantification was done on the basis of a standard curve of gallic acid. Results were expressed as gallic acid equivalent (%). Data are

reported as mean ± standard deviation (SD) for at least three replicates.

The total flavonoid content in the samples was determined by the methodology of Chan *et al.* (2002). Quercetin was used as a reference for the calibration curve. The absorbance of the reaction mixture was measured at 415 nm. Results were expressed as quercetin equivalent (%). Data are reported as mean ± SD for at least three replicates.

Extraction and isolation of the active compounds

The freeze-dried material was extracted with MeOH/H_2O 7:3 in a Soxhlet apparatus for 2 h with a 70 ml solvent/2 g sample ratio. The resulting extracts were taken to dryness under reduced pressure and then lyophilized. Some 0.5 g of each crude extract was partitioned with $CHCl_3$ (3 × 5 ml) to afford a $CHCl_3$-soluble and a $CHCl_3$-insoluble fraction. Representative samples of the $CHCl_3$-insoluble fraction, which correspond to the more polar extract constituents, were permeated on a Sephadex LH-20 column (26 cm column length, 3 cm internal diameter) using MeOH/H_2O 7:3 as the eluent. Some 30 fractions of 7 ml each were collected and pooled together according to the TLC patterns (silica gel, EtOAc/acetic acid/H_2O, 10:2:3, upper phase). The extract amount used from each extract was as follows: sample 2, 364 mg; sample 3, 370 mg; sample 4, 400 mg; sample 5, 428 mg; sample 6, 485 mg. From all the samples, chlorogenic acid (3-caffeoylquinic acid, CA) and neochlorogenic acid (NCA) were isolated, the last compound in minute amounts. For further differentiating the metabolite pattern of the different extracts, TLC fingerprints as well as DAD-HPLC traces were used. TLC analysis (silica gel, EtOAc/acetic acid/H_2O, 10:2:3, v/v/v) showed the caffeic acid derivatives and a flavonoid glycoside in all except for sample 5. The ^1H and ^{13}C NMR data of chlorogenic acid were in agreement with those of a commercial reference sample and nearly identical to those erroneously reported by Jin *et al.* (2005) and Merfort (1992) for 5-caffeoylquinic acid, while the spectroscopic information for neochlorogenic acid agrees with the data of Sefkow *et al.* (2001) for synthetic caffeoylquinic acid derivatives.

HPLC analysis

The determination of quercetin-3-*O*-β-D-glucoside and chlorogenic acid in the samples was performed according to Sánchez-Rabaneda *et al.* (2003) and Cheel *et al.* (2005) with some modifications. HPLC analysis was performed using an HPLC-DAD Merck-Hitachi (LaChrom, Tokyo, Japan) equipment consisting of an L-7100 pump, an L-7455 UV diode array detector and a D-7000 chromato-integrator. A C18-RP column (250 × 4.6 mm i.d., 5 μm; Phenomenex, Torrence, CA, USA) was used. The compounds were monitored at 256 nm and the absorbance was measured between 200 and 400 nm. Gradient elution was carried out with water/0.1% formic acid (solvent A) and 20% solvent A in 80% acetonitrile (solvent B) at a constant flow rate of 1 ml/min. A linear gradient was used. Under our experimental conditions, the R_t (min) of the isolated compounds was as follows: quercetin-3-*O*-β-D-glucoside 47.8 min. and chlorogenic acid 37.4 min. Calibration curves were performed to estimate the main active compounds content in the samples. The correlation between concentration/peak area was assessed by the ordinary least square regression model. The amount of the active principles was expressed as mg per 100 g of dry material.

Structural identification of the compounds

Nuclear magnetic resonance (NMR) spectra were obtained using a Bruker spectrometer, operating at 400 MHz for ^1H and 100 MHz for ^{13}C NMR. Dimethyl sulfoxide (DMSO-d_6) was used as solvent. UV spectra were obtained using a Heλios α V-3.06 UV/VIS spectrophotometer; MeOH was used as solvent. HPLC-DAD was used for identification and quantification of the main compounds in the crude extracts, fractions and as a purity criterion of the isolated compounds before NMR measurements and assays.

DPPH discoloration assay

The free radical scavenging effect of the extracts and compounds was assessed by the discoloration of a methanolic solution of DPPH as previously reported (Cheel *et al.*, 2005). Crude extracts were assayed at 100, 33 and 11 μg/ml. The scavenging of free radicals by extracts was evaluated spectrophotometrically at 517 nm against the absorbance of the DPPH radical.

The percentage of discoloration was calculated as follows:

$$\% \text{ of discoloration} = 1 - \frac{\text{absorbance of compound/extract}}{\text{absorbance of blank}} \times 100.$$

The degree of discoloration indicates the free radical scavenging efficiency of the substances. For extracts, values are reported as mean ± SD of three determinations. For the compounds, the half-maximum inhibitory concentration (IC_{50}) was calculated by linear regression analysis and expressed as mean of three determinations. Quercetin was used as a free radical scavenger reference compound.

Statistical analysis

To determine whether there was any difference between the activity of phenolics and flavonoids of samples, a one-way analysis of variance (ANOVA) was applied. Values of $P < 0.05$ were considered as significantly different. The differences between means were determined using the Tukey's multiple comparison test. To assess the relationship between the activities and the phenolic compounds, Pearson's correlation coefficients were calculated with 95% confidence. The Statistical Package S-Plus 2000 for Windows was used to analyze the data.

Results and Discussion

Only shoot sections but not leaf explants initiated growth responses. After 2 months abundant friable yellow callus (about 1–5 cm in diameter) was observed in the stem sections in the presence of 0.1 mg/l 2,4-D, 1.0 mg/l NAA and 0.5 mg/l TDZ. 2,4-D seemed to be necessary to initiate cell proliferation with callus induction. Only the presence of this hormone resulted in the reduction of browning that experimented several explants in several hormone combinations. The presence of TDZ, instead of BA, was more effective for callus induction, although this response was only achieved in approx. 60% of the explants. Other hormone combinations also led to callus formation, with the exception of 2,4-D levels higher than 1.0 mg/l that resulted toxic. Under these conditions with a light regime of 48 μmol m^{-2} s^{-1} at 25 °C, callus proliferation continued for about 3 months. However, no shoot or root initiation was observed after transferring callus pieces into the different growth regulator combinations. It is probably that the long-term presence of 2,4-D, necessary for callus initiation, could have affected both morphogenic responses as it has been reported for many species (Zaer and Mapes, 1982; Bergfeld *et al.*, 2003) (see Table I).

Table I. Best growth regulator treatments to induce callus initiation in stem explants of *S. graveolens* plants grown *in vitro* after 2 months.

Plant growth regulators [mg l^{-1}]				Callus initiation (%)
2,4-D	NAA	BA	TDZ	
0.1	1.0	1.0	0.0	15
0.1	0.5	0.2	0.0	10
0.1	0.5	0.0	0.5	5
0.1	1.0	0.0	0.5	60
1.0	1.0	1	0.0	0 (browning)

Results from initial 20 explants/treatment.

Under the described seed germination conditions, a rather low germination rate was observed. All seedlings were transferred to the described culture medium for induction of the shoot propagation after development of the first leafs. The shoots were subcultured every four weeks and stored in a culture collection. After several subcultures they reached a propagation ratio of 1:1.25.

The shoots and roots obtained under the conditions described above were transferred to the liquid culture system. Shoots and roots grown in microcontainers were used as inoculum for the cultivation in bioreactors. For shoot propagation, the following vessels were used: RITA®system (0.25 l) and TIS-twin-vessels (1 l).

The RITA®system was not suitable for the *in vitro* cultivation of *S. graveolens.* All shoots showed hyperhydricity due to the high humidity in the cultivation chamber. Only a low shoot multiplication from 5.6 g fresh weight to 55.5 g fresh weight within 139 days (multiplication rate of 9.9) was observed. Biomass cultivated in TIS-twin-vessels showed much lower hyperhydricity effects. There was a biomass production from 2.5 g fresh weight to 73.9 g fresh weight (multiplication rate of 29.6) within 38 days. Roots were cultivated only in TIS-twin-vessels (1 l). They showed a good growth and were of a light brown color. All cultures were produced in small scale for analytical evaluation only.

Crude extracts showed total phenolic values from 3.57 to 6.93 g%, with highest content for the root culture sample (sample 5). Total flavonoid content ranged from 1.23 to 2.23 g% and was lower for the root culture. Chlorogenic acid (CA) and neochlorogenic acid (NCA) were identified by TLC in all samples. Chlorogenic acid and quercetin-3-*O*-β-D-glucoside were quantified and identified by DAD-HPLC by the retention time and UV

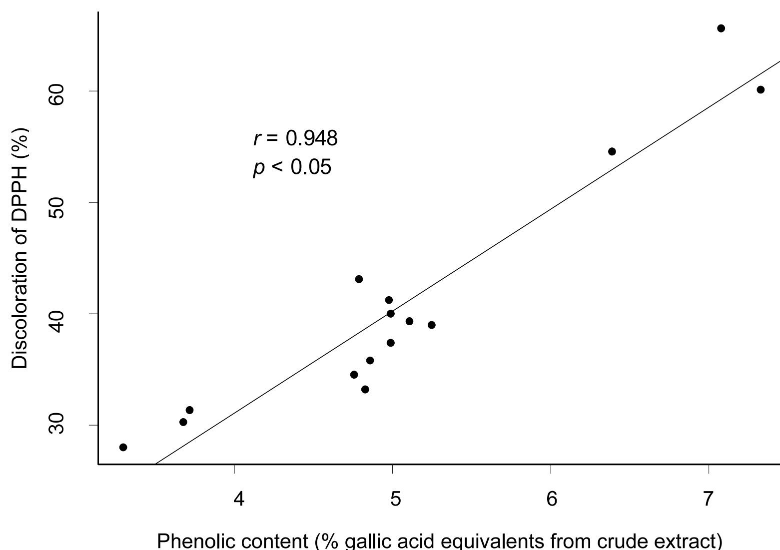

Fig. 1. Correlation between DPPH activity of *S. graveolens* samples and percentual phenolic content.

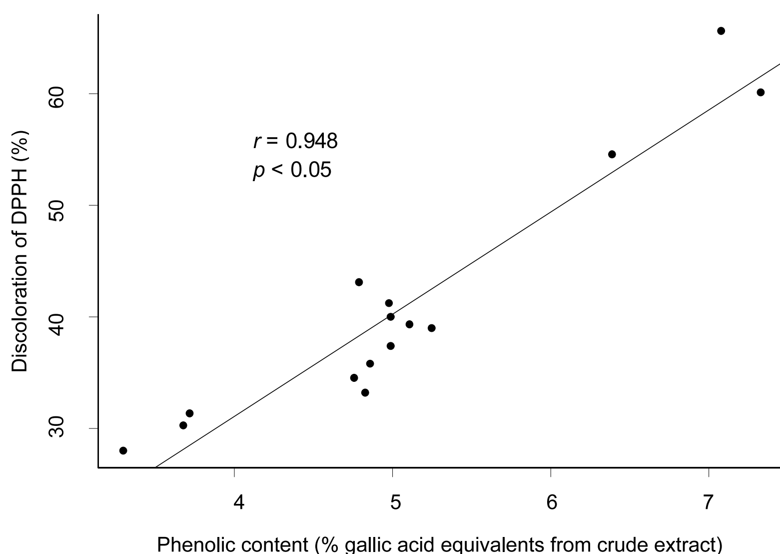

Fig. 2. Correlation between DPPH activity of *S. graveolens* samples and percentual flavonoid content.

spectrum in comparison with standard samples. Highest free radical scavenging effect was observed for the root culture (sample 5) which also presented the highest CA content. The shoot culture samples 1 and 4, with nearly similar IC_{50} values in the DPPH discoloration assay also presented close quercetin-3-*O*-D-glucoside content. Under our assay conditions, the IC_{50} values for chlorogenic acid, neochlorogenic acid, quercetin-3-*O*-D-glucoside and quercetin were 16, 17, 115 and 2.6 μg/ml, respectively.

The total phenolic and flavonoid content of the samples is summarized in Table II. In our study with the crude *S. graveolens* extracts a high and positive correlation between phenolic content and DPPH activity ($r = 0.948$, $P < 0.05$) was observed (Fig. 1). That correlation presented a determination coefficient of $R^2 = 0.889$ which suggested that

89% of the free radical scavenging activity of the studied extracts mainly resulted from the contribution of phenolics.

Regarding the flavonoid content, a weak inverse correlation was observed between total flavonoid content and DPPH activity ($r = -0.688$, $P < 0.05$) (Fig. 2). The negative correlation in *Sanicula* extracts could be due to the very high content of phenolics not including flavonoids, which could account for most of the DPPH value. A similar association was found in a study on strawberries (Scalzo *et al.*, 2005) using the TEAC (Trolox equivalent antioxidant capacity) assay and on different berries using a LDL oxidation system (Heinonen *et al.*, 1998). The fact that the free radical scavenging activity shows a negative correlation with the content of flavonoids in the samples assayed does not mean that these do not contribute to the overall antioxidant capacity of the evaluated extract, but that could be the result of antagonisms, still unknown as reported by García-Alonso *et al.* (2004). Besides, it must be noted that the efficiency of antioxidants depends strongly on the oxidation conditions, and thus DPPH assay used in this study gives only one approximation of the possibilities of an extract to act as an antioxidant. Therefore, it is likely that flavonoids from *Sanicula* extracts could have a different or a positive response in other antioxidants assays.

In a previous study, the free radical scavenging effect of wild growing *S. graveolens* plants was related with the occurrence of chlorogenic acid, dicaffeoylquinic acid and quercetin derivatives in the polar extracts. In the present work, the cell cultures contained mainly caffeic acid derivatives, with chlorogenic acid being the main constituent in the extracts. On the other hand, large variations both in the phenolic content and free radical scavenging effect were observed. Moreover, a clear distinction was possible within the samples 1–4 which presented a main active compound, characterized as quercetin-3-*O*-*β*-D-glucoside as well as chlorogenic acid, and the sample 5 which only contained detectable levels of CA (see Table II). The antioxidant activity of CA was reported by several authors, including Wang *et al.* (1999) and Cheel *et al.* (2005). Caffeic acid, the aglycone of CA and quercetin as well as its 3-*O*-glucoside are well known antioxidant and free radical scavenging compounds (Wagner *et al.*, 2006; Hu *et al.*, 2006). Quercetin represents one of the most abundant flavonoids in foods, being present in fruits, vegetables, wine and tea in the form of glycosides. It exhibits a variety of biological activities, including cardiovascular protection, anti-cancer, anti-inflammatory and antioxidant activities (Hertog *et al.*, 1993, 1995; Knekt *et al.*, 1996).

In previous studies on the bioactive secondary metabolites content in native Chilean plant cultures, CA was the main phenolic in the cell suspension of *Solidago chilensis* with only traces detected in the callus culture (Schmeda-Hirschmann *et al.*, 2005) and also occurred in callus and TIS-grown roots of *Fabiana imbricata* (Schmeda-Hirschmann *et al.*, 2004). The potential of plant cell cultures in the search for new active compounds has been outlined by Schripsema *et al.* (1996). A comparison of secondary metabolite production in callus culture, cell suspensions and temporary immersion system was recently reported by Wilken *et al.* (2005) and showed the challenge of this technique in changing both the content and composition of secondary metabolites in medicinal and aromatic plants.

Table II. Dry weight (g) and % w/w extraction yield of the *S. graveolens* samples under investigation, DPPH discoloration activity (IC_{50} values), percentual total phenolics and flavonoids (g%), quercetin-3-*O*-*β*-D-glucoside and chlorogenic acid content (mg%) in terms of dry starting material.

Sample[a]	Dry weight [g] (% w/w extraction yield)	DPPH IC_{50} [μg ml^{-1}]	Total phenolics (g%)	Total flavonoids (g%)	Quercetin-3-*O*-*β*-D-glucoside (mg%)	Chlorogenic acid (mg%)
1	6.97 (22.57)	47.7 ± 1.3[b]	4.96 ± 0.16[b]	1.87 ± 0.04[b]	4.52 ± 0.03[b]	0.52 ± 0.02
2	4.53 (23.83)	75.7 ± 3.9	3.57 ± 0.23	1.81 ± 0.10[b]	3.70 ± 0.02	0.81 ± 0.01
3	3.19 (31.30)	58.7 ± 1.7	4.82 ± 0.05[b]	1.83 ± 0.04[b]	4.45 ± 0.02	0.92 ± 0.03
4	5.00 (33.00)	50.1 ± 1.7[b]	5.07 ± 0.15[b]	2.23 ± 0.15	4.57 ± 0.01[b]	1.28 ± 0.01
5	2.30 (31.96)	36.0 ± 1.4	6.93 ± 0.48	1.23 ± 0.05	ND	1.93 ± 0.02

[a] See biomass production in Materials and Methods.
[b] Not significantly different; $P < 0.05$.
ND, not detected.

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