SUPPLEMENTARY MATERIAL

Phenolic acids, antioxidant and antiproliferative activities of Naviglio[®] extracts from *Schizogyne sericea* (Asteraceae)

Giovanni Caprioli^a, Romilde Iannarelli^a, Gianni Sagratini^a, Sauro Vittori^a, Christian Zorzetto^b, Candelaria C. Sánchez-Mateo^b, Rosa M. Rabanal^b, Luana Quassinti^a, Massimo Bramucci^a, Luca A. Vitali^a, Dezemona Petrelli^c, Giulio Lupidi^a and Filippo Maggi^{a,*}

^a*School of Pharmacy, MS 015, University of Camerino, Camerino, Italy*

^b*Departamento de Medicina Física y Farmacología, Universidad de La Laguna, Tenerife, Spain*

^c*School of Biosciences and Veterinary Medicine, University of Camerino, Camerino, Italy*

^d*Department of Chemistry, Sapienza University, Rome, Italy*

^e*Department of Environmental Biology, Sapienza University, Rome, Italy*

*Corresponding author: Filippo Maggi: E-mail: filippo.maggi@unicam.it

Abstract

Schizogyne sericea, well-known as ‘salado’, is a halophytic shrub widespread on coastal rocks of Tenerife (Canary Islands). This plant is used traditionally as analgesic, astringent, anti-inflammatory and vulnerary agent. In the present work we have analysed the aqueous and ethanolic extracts of *S. sericea* for the content of phenolic acids by HPLC-DAD. The dynamic solid-liquid Naviglio[®] extractor was used to extract the flowering aerial parts. Water extracts showed higher levels of phenolics than ethanolic extracts. *S. sericea* extracts were rich in chlorogenic and isochlorogenic acids. The Naviglio[®] extracts obtained were assayed for *in vitro* biological activities, namely antioxidant, antimicrobial and cytotoxicity on tumor cells by DPPH, ABTS, FRAP, agar disc-diffusion, and MTT methods, respectively. Results showed that water extracts,

being richer in phenolic acids, are endowed with relevant radical scavenging activity (TEAC values in the range 208-960 $\mu\text{mol TE/g}$) while ethanolic extracts exhibited noteworthy antiproliferative effects on tumor cells.

Keywords: *Schizogyne sericea*, Naviglio[®] extracts, polyphenols, cytotoxic, antioxidant, antimicrobial.

3. Experimental section

3.1. Plant materials

Flowering aerial parts of *S. sericea* were collected from three different collection sites of Tenerife (Canary Islands, Spain) in November/December 2014. These locations include Palm Mar (Arona, arid environment, N 28°01'17", W 16°41'45", 71 m a.s.l.), Los Roques (Fasnia, semi-arid environment, N 28°13'10", W 16°26'53", 26 m a.s.l.) and La Barranquera (Valle de Guerra, humid environment, N 28°32'12", W 16°23'49", 2 m a.s.l.). Voucher specimens were identified by Dr. Consuelo Hernández Padrón, Department of Plant Biology, University of La Laguna (Tenerife, Spain) and deposited in the Herbarium of the same Institution (included in the online edition of Index Herbariorum by the New York Botanical Garden: <http://sweetgum.nybg.org/ih/>) under the codes TFC 51136 (for sample from Palm Mar), TFC 52126 (Los Roques) and TFC 52125 (La Barranquera). Once collected, the aerial parts were dried at room temperature for one week, cut into small pieces and stored in a dark box at room temperature.

3.2. Reagents and standards

The analytical standards of gallic acid (CAS Number 149-91-7), trans-ferulic acid (CAS Number 537-98-4), caffeic acid (CAS Number 331-39-5), chlorogenic acid (3-O-caffeoylquinic acid, CAS Number 327-97-9), isochlorogenic acid (3,5-di-O-caffeoylquinic acid, CAS Number 2450-53-5), neochlorogenic acid (5-O-caffeoylquinic acid, CAS Number 906-33-2), were purchased from Sigma-Aldrich (Milano, Italy). The stock standard solution was prepared by dissolving 10 mg of the analyte in 10 ml of methanol and stored in a glass-stoppered bottle at 4°C in the dark. Standard working solutions, at various concentrations, were daily prepared by appropriate dilution of aliquots of the stock solutions in water. HPLC-grade methanol and acetonitrile were purchased from Sigma-Aldrich (Milan, Italy), while HPLC-grade formic acid 99-100% was bought from J.T. Baker B.V. (Deventer, Holland). For sample preparation and chromatographic analysis, deionized water of 18 M Ω cm resistivity purified with a Milli-

Q system (Millipore, Bedford, USA) was used. All solvents and solutions were filtered through a 0.45- μ m PTFE filter from Supelco (Bellefonte, PA, USA) before use. ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid), DPPH (2,2-diphenyl-1-picrylhydrazyl), TPTZ (2,4,6-tripyridyl-s-triazine), Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) and Folin-Denis' reagent were purchased from Sigma Aldrich (Milan, Italy), anhydrous FeCl₃ from J.T. Baker D.V. (Deventer, Holland) and NaCO₃ from Carlo Erba reagents (Milan, Italy). Ethanol, gallic acid and activated MnO₂ were purchased from Fluka (Buchs, Switzerland). Flat-bottomed 96-well microplates (FALCON 96, BD Biosciences) were used to do the colorimetric measurements with a FluoSTAR omega spectrophotometer, BMG Labtech (Offenburg, Germany).

3.3. Preparation of extracts by Naviglio®

Dried aerial parts of *S. sericea* were extracted by Naviglio Extractor®, Mod. 1000 cc. (Atlas Filtri Engineering, Limena, Italy) using two different solvents, i.e. water and ethanol. Fifty g of *S. sericea* aerial parts were crushed into a mortar and put into a socket in the cylinder of the Naviglio Extractor®. Afterwards, 500 ml of distilled water or ethanol were added. For each sample twelve extraction cycles were performed (the static phase lasted 10 min, followed by 3 min of dynamic phase) for a total time of 2.6 h. The extracts were concentrated under reduced pressure at 35/40 °C using the Buchi rotavapor R-200. Afterwards, they were lyophilized with an Edwards Pirani 1001 (West Sussex, UK). Before use, samples were stored in the refrigerator at 4 °C. For HPLC analysis, the samples were prepared by re-dissolving 10 mg of each extract in 1 ml of methanol. The sample solutions were filtered through a 0.45 μ m pore size nylon membrane filter (Phenex, Phenomenex, Torrance, CA, USA) before injection into HPLC-DAD. Each sample was analyzed in triplicate.

3.4. HPLC-DAD analysis

HPLC-DAD studies were performed using a Hewlett-Packard HP-1090 Series II (Palo Alto, CA, USA), equipped with a vacuum degasser, a binary pump, an autosampler and a model 1046A HP photodiode array detector (DAD) ([Zorzetto et al. 2015](#)). Chromatographic separation was accomplished on a Synergi Polar-RP C18 (4.6 mm x 150 mm, 4 μ m) analytical column from Phenomenex (Cheshire, UK). The column was preceded by a security cartridge. The mobile phase for HPLC-DAD (diode array detector) analyses was a mixture of (A) water with 0.1% formic acid (v/v) and (B) methanol, flowing at 0.7 ml/min in isocratic conditions: 60% A, 40% B. The injection volume was 5 μ l. UV spectra were recorded in the range 210-350 nm for the seven

compounds: 210 nm was used for quantification of gallic acid, 325 nm for caffeic acid, trans-ferulic acid, chlorogenic acid, neochlorogenic acid and isochlorogenic acid (Figure S1).

3.5. Analytical method validation

The method was validated by determining linearity, repeatability, recovery, limits of detection (LODs) and limits of quantification (LOQs) (Table S2). Calibration curves of the analyzed compounds were constructed injecting 0.5-100 mg/l of standard solutions at six different concentrations, i.e. 0.5, 1, 5, 10, 50 and 100 mg/l in HPLC/DAD technique. Five replicates for each concentration were performed and the relative standard deviation (RSDs) ranged from 1.3 to 4.6% for run-to-run precision and from 1.9 to 8.8% for day-to-day precision. All the calibration curves of the analyzed phenolic compounds showed a correlation coefficient greater than 0.993. The obtained recoveries for all compounds, evaluated spiking the samples at two different levels of concentration (10 and 50 mg/l) with a standard mixture of the seven compounds, were in the range 92-97% and 99-102%, respectively, with a % RSDs < 12% (n = 5) in all cases. LODs and LOQs were in the range 0.01–0.15 mg/l and 0.05–0.4 mg/l, respectively. Retention time stability was utilized to demonstrate the specificity of the HPLC-DAD method. Reproducibility of the chromatographic retention time for each compound was examined five times per day over a 5-day period (n=25). The retention times using this method were stable with a percent RSD value of $\leq 1.89\%$.

3.6. Determination of total phenolic content

Spectrophotometric methods were employed to evaluate the amounts of total phenols in Naviglio[®] extracts using Folin–Ciocalteu reagent in NaHCO₃ solution by measuring the absorbance at 765 nm (Srinivasan et al. 2007). This method gives a general measurement of phenolic content, as it is not completely specific for phenolic compounds and not all phenolic compounds exhibit the same level of activity in the assay. The total phenolic content was expressed as mg gallic acid equivalents (GAE) per g of extract. All tests were conducted in triplicate.

3.7. Antioxidant activity

Free radical scavenging activity (DPPH assay) of Naviglio[®] extracts was assessed on a microplate analytical assay according to the procedures described by Srinivasan et al. (2007), while the total radical scavenging capacity of the same products was measured by the ABTS assay modified as by Iqbal et al. (2012), for application to a 96-well microplate assay. Determination of antioxidant activity by FRAP assay was carried out according to the procedure described by Firuzi et al. (2005), by monitoring the

reduction of Fe³⁺-tripyridyl triazine (TPTZ) to blue-coloured Fe²⁺-TPTZ. The ability of Naviglio[®] extracts to scavenge the different radicals in all assays was compared to Trolox used as standard and expressed as Trolox-equivalent antioxidant capacity $\mu\text{mol TE/g}$ of product. Antioxidant activity of the samples was also expressed as IC₅₀, defined as the concentration of the extract required to cause a 50% decrease in absorbance. Each experiment was repeated at least three times.

3.8. Agar disc diffusion test

Naviglio[®] extracts were tested by disc diffusion method against a panel of bacterial species including *Staphylococcus aureus* ATCC 25923 (American Type Culture Collection, Rockville, MD, USA), *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Enterococcus faecalis* ATCC 29212 and *Candida albicans* ATCC 24433. Bacterial strains were cultured overnight at 37 °C in blood agar plates (Oxoid, Basingstoke, UK). For *C. albicans* a Petri dish with solid RPMI medium (thickness 4 mm) was used. Tests were performed following the Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI 2009). Briefly, a suspension of the tested microorganism ($1-2 \times 10^8$ cells per ml in saline) was spread on the solid media plates using a sterile cotton swab. Sterile paper discs (6 mm in diameter) were placed on the surface of inoculated plates and spotted with 10 μl of extract (20 mg/ml). The plates were incubated 24 h at 35 ± 1 °C. The diameters of zone inhibition (including the 6 mm disc) were measured with a calliper. A reading of more than 6 mm indicated growth inhibition. Ciprofloxacin (5 μg disc) and Nystatin (100 Units disc) were used as reference antimicrobials against bacteria and fungi, respectively.

3.9. MTT assay

Human colon carcinoma cell line HCT116 was cultured in RPMI1640 medium with 2 mM L-glutamine, 100 IU/mL penicillin, 100 $\mu\text{g/mL}$ streptomycin, and supplemented with 10% heat-inactivated fetal bovine serum (HI-FBS) (PAA Laboratories GmbH, Austria). Human breast adenocarcinoma cell line MDA-MB 231, and human malignant melanoma cell line A375 were cultured in Dulbecco's Modified Eagle's Medium (DMEM) with 2 mM L-glutamine, 100 IU/mL penicillin, 100 $\mu\text{g/mL}$ streptomycin, and supplemented with 10% HI-FBS. Cells were cultured in a humidified atmosphere at 37°C in the presence of 5% CO₂. The MTT assay was used as a relative measure of cell viability. Cell-viability assays were carried out according to a previously reported procedure (Quassinti et al. 2013). Cytotoxicity was expressed as the concentration of extract inhibiting cell growth by 50% (IC₅₀) after 72 h of incubation. The IC₅₀ values

were determined with the GraphPad Prism 4 computer program (GraphPad Software, S. Diego, CA, USA).

References

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Table S1. Antimicrobial activity of Naviglio[®] extracts from *Schizogyne sericea* determined by the agar disk diffusion test. Values indicate the diameter of the growth inhibition zone (mm).

	<i>S.</i> <i>aureus</i>	<i>E.</i> <i>faecalis</i>	<i>E.</i> <i>coli</i>	<i>P.</i> <i>aeruginosa</i>	<i>C.</i> <i>albicans</i>
Ethanollic extracts					
<i>S. sericea</i> Palm Mar	6	6	6	6	7-9
<i>S. sericea</i> Los Roques	7	8	6	6	10
<i>S. sericea</i> La Barranquera	8	8	6	6	6
Aqueous extracts					
<i>S. sericea</i> Palm Mar	7	6	7	6	6
<i>S. sericea</i> Los Roques	7	6	7	6	6
<i>S. sericea</i> La Barranquera	6	6	7	6	6
Reference antibiotics					
Ciprofloxacin	28	23	30	31	n.r.
Nystatin	n.r.	n.r.	n.r.	n.r.	27-28

n.r.: Not recommended for this species.