SUPPLEMENTARY MATERIAL

Essential oil composition and biological activity from *Artemisia caerulescens* subsp. *densiflora* (Viv.) Gamisans ex Kerguélen & Lambinon (Asteraceae), an endemic species in the habitat of La Maddalena Archipelago.

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Abstract

The purpose of this study was to investigate the composition of the essential oil obtained from a population of *A. caerulescens* subsp. *densiflora* growing in Razzoli, an island in the La Maddalena Archipelago (Sardinia, Italy). *Artemisia caerulescens* supsp. *densiflora* Viv. (Asteraceae), a wild herb, seldom studied in the Mediterranean, represents one of the many rare endemic species growing in North Sardinia. The essential oil composition was analysed by means of GC/MS analysis, which showed davana ethers as the major volatile components, accounting together for 17.5%, followed by (*E*)-nerolidol (4.5%), β -oplophenone (3.3%), *cis*-sabinene hydrate (5.2%) and terpinen-4-ol (4.7%). The oil was tested for antioxidant activity by means of DPPH test, by inhibition of lipid oxidation test, and hypochlorous acid test which showed a quite interesting scavenger capacity. For the first time we reported the cytotoxic activity of the essential oil of *A. caerulescens* subsp. *densiflora*, against three human tumor cell lines (A375, MDA-MB231 and HCT116), with IC₅₀ values in the range 5.20-7.61 μ g/ml, which deserved further studies to support its use as chemopreventive agent. Finally, the antimicrobial activity of the essential oil, displayed on a panel of human pathogens, was very low.

Key word: *Artemisia caerulescens* subsp. *densiflora* Viv., davana ethers, davanone, (*E*)-nerolidol, β -oplophenone, *cis*-Sabinene, terpinen-4-ol.

1. Experimental

1.1 Plant Material.

A. caerulescens subsp. *densiflora* were collected at the beginning of April 2014 from a protected area in the La Maddalena archipelago, at south-west of Razzoli island. (GPS coordinates: lat. N 41°18' 0.6" long. E 9° 20' 48"). The samples was identified by Dr.ssa Cinzia Sanna (Co.S.Me.Se.) and Dr.Yuri Donno (National Park of La Maddalena Archipelago). A voucher specimen of the plant (Herbarium CAG 736) has been retained in the General Herbarium of the botanical garden of the University of Cagliari, deposited with the herbarium of this department.

1.2 Extraction of Essential Oil.

Aerial parts (685.0 g) of *A. caerulescens* subsp. *densiflora* were subjected to hydrodistillation a Clevenger-type apparatus for 4 h using 1000 ml of dist. H₂O. At the end of each distillation, the oils were collected in a Clevenger trap, dried (anh. Na₂SO₄), stored in hermetically sealed glass containers with rubber lids, covered with aluminum foil to protect the content from light, and kept at 48 until analysis. The oil samples from plants harvested at the three different growth periods were obtained with the same protocol. The oil yield, calculated on the dry-weight basis of the plant material was 0.15% (w/w).

1.3 Chemicals

For identification of volatiles the following analytical standards purchased from Sigma Aldrich (I-Milan) were used: *p*-cymene, 1,8-cineole, γ -terpinene, terpinolene, linalool, *trans*-pinocarveol, terpinen-4-ol, α -terpineol, carvacrol, eugenol, (*E*)-caryophyllene, α -humulene, (*E*)-nerolidol, caryophyllene oxide.

Viridiflorol was kindly provided by Michael Russell, Department of Primary Industries, Industry and Investment NSW, Wollongbar, NSW, Australia. For retention-index (RI) determination, a mixture of hydrocarbons, ranging from octane (C₈) to triacontane (C₃₀) (Supelco, USA), was used and run under the experimental conditions reported below. All compounds were of analytical standard grade. Analytical-grade hexane solvent was purchased from Carlo Erba (I-Milan); it was successively distilled through a Vigreux column before use. For antioxidant assays, 1,1-diphenyl-2-picrylhydrazyl radical (DPPH), butylated hydroxytoluene (BHT), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), Tween 20, β -carotene, linoleic acid, sodium hypochlorite, taurine, phosphate buffer saline (PBS) and potassium iodide were purchased from Sigma-Aldrich-Fluka (Milan, Italy).

1.4 GC-MS analysis

GC-MS Analysis was performed on an Agilent 6890N gas chromatograph coupled to a 5973N mass spectrometer. Separation of volatiles was achieved by using an HP-5 MS (5% phenylmethylpolysiloxane, 30 m, 0.25 mm i.d., 0.1-mm film thickness; J & W Scientific, Folsom) capillary column. The oven temperature programme was the following: 5 min at 60°C, subsequently 4°C/min up to 220°C, then 11°C/min up to 280°C, held for 15 min, for a total run of 65 min. Injector and detector temperatures were 280°C. He was used as the carrier gas, at a flow rate of 1 ml/min. Split ratio, 1:50; acquisition mass range, m/z 29–400. All mass spectra were acquired in electron-impact (EI) mode with an ionization voltage of 70 eV. Oil samples were diluted to 1 : 100 in *n*-hexane, and the volume injected was 2 μ l. Whenever possible the essential oil constituents were identified by co-injection with authentic standards (see chemicals). Otherwise, the peak assignment was carried out according to the recommendations of the International Organization of the Flavour Industry (<http://www.iofi.org/>), i.e. by the interactive combination of chromatographic linear retention indices that were consistent with those reported in the literature (Adams, 2007; NIST 08, 2008; FFNSC2, 2012) for apolar and polar stationary phases, and MS data consisting in computer matching with the WILEY275, NIST 08, ADAMS, FFNSC2 and home-made (based on the analysis of reference oils and commercially available standards) libraries.

Quantification of essential oil components was achieved by peak-area internal normalisation without using correction factors.

1.5 Antioxidant Activity

1,1-diphenyl-2-picrylhydrazyl (DPPH) radical-scavenging activity

The antioxidant activity of *A. caerulescens* subsp. *densiflora* essential oil was determined by the DPPH radical-scavenging method. Each sample (different concentration at final volume of 10 μ l) was mixed with 900 μ l of 100 mM Tris-HCl buffer, pH 7.4, 40 μ l ethanol, and 50 μ l 0.5% w/w Tween 20 solution, and then added to 1 ml 0.5 mM DPPH in ethanol (250 μ M in the reaction mixture). Tween 20 was used as an oil-in-water emulsifier. The control sample was prepared using ethanol instead of essential oils. Trolox was employed as a standard antioxidant to examine the radical-scavenging activities. 50 μ L of 1 mM Trolox in ethanol was added to the reaction mixture (final concentration of 25 μ M). Absorbances of the mixtures were measured at 517 nm. The activity was calculated as Trolox equivalents. All tests and analyses were run in triplicate and averaged.

β -Carotene/linoleic acid assay

In this assay, the antioxidant capacity was determined by measuring the inhibition of volatile organic compounds and conjugated diene hydroperoxides arising from linoleic acid oxidation. A stock solution of β -carotene/linoleic acid mixture was prepared as follows: 0.5 mg of β -carotene was dissolved in 1 ml of chloroform, then 25 μ l of linoleic acid and 200 mg of Tween 40 were added. Then, 100 ml of distilled water saturated with oxygen (30 min, 100 ml/min) was added with vigorous shaking; 2.5 ml of this reaction mixture were dispensed into test tubes and 10 μ l of the different concentrations of essential oil were added; the emulsion system was incubated for up to 48 h at room temperature. The same procedure was repeated with the synthetic antioxidant butylated hydroxytoluene (BHT) as positive control, and a blank. After this incubation period, absorbances of the

mixtures were measured at 490 nm. The activity was calculated as BHT equivalents. All tests and analyses were run in triplicate and averaged.

Hypochlorous acid scavenging (HOCl)

The amount of HOCl was measured by the chlorination of taurine (Weiss et al., 1982; Gressier et al., 1994; Rakotoarison et al., 1997). 100 µl of sodium hypochlorite (600 mM) were added to 100 µl of taurine (150 mM) and 10 µl of different concentrations of essential oil in Tween 20 in a final volume of 1 ml PBS, pH 7.4. Absorbance was measured at 350 nm after the addition of 10 µl of 2 M potassium iodide. The activity was calculated as Trolox equivalents. Trolox was used as the positive control. All tests and analyses were run in triplicate and averaged.

Cytotoxic activity

A375 (human malignant melanoma cells) and MDA-MB 231 cells (human breast adenocarcinoma cells) were cultured in Dulbecco's Modified Eagle's Medium (DMEM) with 2 mM L-glutamine, 100 IU/mL penicillin, 100 µg/mL streptomycin, and supplemented with 10% heat-inactivated fetal bovine serum (HI-FBS). HCT116 cells (human colon carcinoma cells), were cultured in RPMI1640 medium with 2 mM L-glutamine, 100 IU/mL penicillin, 100 µg/mL streptomycin, and supplemented with 10% HI-FBS. Cells were cultured in a humidified atmosphere at 37° C in presence of 5% CO₂. The MTT assay was used as a relative measure of cell viability. Cell-viability assays were carried out as described (Quassinti et al. 2013). Briefly, cells were seeded at the density of 2×10^4 cells/ml. After 24 h, samples were exposed to different concentrations of essential oil (1.56-200 µg/ml). Cells were incubated for 72 h in a humidified atmosphere of 5% CO₂ at 37 °C. Cisplatin was used as the positive control (0.05-20 µg/ml) . At the end of incubation, each well received 10 µl of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazoliumbromide (MTT) (5 mg/ml in phosphate-buffered saline, PBS) and the plates were incubated for 4 h at 37 °C. The extent of MTT reduction was measured spectrophotometrically at 540 nm using a Titertek

Multiscan microElisa (Labsystems, FI-Helsinki). Experiments were conducted in triplicate. Cytotoxicity is expressed as the concentration of compound inhibiting cell growth by 50% (IC₅₀). The IC₅₀ values were determined with GraphPad Prism 4 computer program (GraphPad Software, S. Diego, CA, USA).

Antimicrobial activity

The essential oil from *A. caerulea* subsp. *densiflora* was tested against a panel of microorganisms including *Staphylococcus aureus* ATCC (American Type Culture Collection) 25923, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Enterococcus faecalis* ATCC 29212, *Candida albicans* ATCC 24433. Bacterial strains were cultured overnight at 37 °C in blood agar plates. *C. albicans* was grown in Sabouraud Dextrose Agar. Tests by the paper disc diffusion method were performed following the Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2009). Briefly, a suspension of the tested microorganism (1-2 x 10⁸ cells per mL in saline-10⁶ per mL for *Candida*) 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 either the essential oil or 1 mg of extract dissolved in DMSO. The plates were incubated 24 h at 35 ± 1 °C (48 h for *C. albicans*). 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. No zone inhibition was observed using DMSO alone. Ciprofloxacin (5 µg disc) and Nystatin (100 Units disc) were used as reference antimicrobials against bacteria and fungi, respectively. Each test was done in triplicate.