

SUPPLEMENTARY MATERIAL

Phytochemical profile of three *Ballota* species essential oils and evaluation of the effects on human cancer cells

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Three *Ballota* species, *B. undulata*, *B. saxatilis* and *B. nigra* ssp. *foetida*, were investigated for their cytotoxicity against two human cancer cells, hepatoma HepG2 cell line and breast cancer MCF-7 cell line, and for their antioxidant activity. The chemical composition of essential oils was studied by GC and GC-MS. Sesquiterpenes were the main constituents. The most antiproliferative essential oil against HepG2 cells was *B. undulata* with a percentage of inhibition of 81.36 ± 3.54 at a concentration of 100 $\mu\text{g/ml}$ while against MCF-7 cells essential oil from *B. saxatilis* was the most active with a percentage of inhibition of 24.18 ± 1.13 at a concentration of 100 $\mu\text{g/mL}$. The antioxidant activity were investigated by DPPH test for all the oils. *B. undulata* showed the highest antiradical effect, with IC_{50} value of 529.7 ± 37.4 $\mu\text{g/mL}$.

Keywords *Ballota* species; essential oil; Antiproliferative Activity; Sesquiterpenes; Hepatoma; Breast cancer.

1. Experimental

1.1 Plant materials

The flowering aerial parts of the following *Ballota* species were collected: *B. nigra*: Brac (Croazia) at 400 m a.s.l., August 2013; *B. saxatilis* and *B. undulata*: Kfardin, Racheye El Wadi (Lebanon), on rocky soul at 1000 m. a.s.l.. Voucher specimens (NAP#237, NAP#238 and NAP#239) are deposited at the Herbarium Neapolitanum (NAP), Dipartimento di Biologia Vegetale, Università degli Studi di Napoli "Federico II", Naples, Italy.

1.2 Extraction procedure

The essential oils from air-dried aerial parts of plants were isolated by hydrodistillation for 3 h, using a Clevenger-type apparatus according to the method recommended in the *European Pharmacopoeia* (2004). The oils were dried over anhydrous sodium sulphate and stored under N_2 at $+4^\circ\text{C}$ in the dark until tested and analysed. All the oils were yellowish with a pleasant smell. The yields (w/w) were the following: 0.15% for *B. nigra* ssp. *foetida* (**Bn**); 0.17% for *B. undulata* (**Bu**); 0.10% for *B. saxatilis* (**Bs**).

1.3 GC and GC-MS analysis

Analytical gas chromatography was carried out on a Perkin-Elmer Sigma 115 gas chromatograph fitted with a HP-5 MS capillary column (30 m x 0.25 mm i.d.; 0.25 μm film thickness). Helium was the carrier gas (1 mL min⁻¹). Column temperature was initially kept at 40°C for 5 min, then gradually increased to 250°C at 2°C min⁻¹, held for 15 min and finally raised to 270°C at 10°C min⁻¹. 1 μL of diluted samples (1/100 v/v, in *n*-hexane) was injected manually at 250°C, and in the splitless mode. Flame ionization detection (FID) was performed at 280°C. Analysis was also run by using a fused silica HP Innowax polyethyleneglycol capillary column (50 m x 0.20 mm i.d.; 0.20 μm film thickness).

GC-MS analysis was performed on an Agilent 6850 Ser. II apparatus, fitted with a fused silica HP-1 capillary column (30 m x 0.25 mm i.d.; 0.33 μm film thickness), coupled to an Agilent Mass Selective Detector MSD 5973; ionization voltage 70 eV; electron multiplier energy 2000 V. Gas chromatographic conditions were as reported above; transfer line temperature, 295°C. Analysis was also run by using a fused silica HP Innowax polyethyleneglycol capillary column (60 m x 0.25 mm i.d.; 0.33 μm film thickness).

1.4 Qualitative and quantitative analyses

Most constituents were identified by gas chromatography by comparison of their retention indices (*I*) with either those of the literature or with those of authentic compounds available in our laboratories. The retention indices were determined in relation to a homologous series of *n*-alkanes (C₈-C₂₄) under the same operating conditions. Further identification was made by comparison of their mass spectra on both columns with either those stored in NIST 02 and Wiley 275 libraries or with mass spectra from the literature and our home made library. Component relative concentrations were calculated based on GC peak areas without using correction factors.

1.5 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay

The absorbance was measured using a Perkin Elmer Lambda 40 UV/VIS spectrophotometer at 517 nm against blank, which was without DPPH. All tests were run in triplicate and averaged. Ascorbic acid was used as a positive control. Decreasing of DPPH solution absorbance indicates an increase of DPPH radical scavenging activity. This activity is given as % DPPH radical-scavenging that is calculated in the equation:
% DPPH radical-scavenging = [1 - (sample absorbance with DPPH - sample absorbance without DPPH / control absorbance)] x 100.

1.6 Cell line and cell culture

Two cancer cell lines, human hepatoma HepG2 cells and breast cancer MCF-7 cells, were used in this experiment. The cells, purchased from the American Type Culture Collection (ATCC), were grown in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10 % fetal bovine serum (1 %), 1% L-glutamine, 1% penicillin/streptomycin. Cultured cells were maintained at 37 °C in a humidified atmosphere

of 5 % CO₂. The cells were subcultured every 2–3 days before cell confluence using 0.25 % trypsin–EDTA, and medium was changed after an overnight incubation.

Cell counts and viability were performed using a standard trypan blue cell counting technique. The cell concentration was adjusted to $2 \cdot 10^4$ cells/ml. 100 μ l of this cell concentration were cultured in a 96-well plate for 1 d to become nearly confluent. Concentrations ranging from 5 – 100 μ g/ml of the samples were prepared from stock solutions by serial dilution in the medium to give a volume of 100 μ l in each well of a microtiter plate (96-well). Then cells were cultured with vehicle and essential oils for 48 h.

1.7 Cytotoxic activity assay

Cytotoxicity was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma) assay. The assay for each concentration of samples was performed in quadruplicate, and the culture plates were kept at 37 °C and 5% (v/v) CO₂ for 1 d. After incubation, 100 μ l of medium were removed from each well. Subsequently, 100 μ l of 0.5% w/v MTT (Sigma, Italy), dissolved in phosphate buffered saline, were added to each well and allowed to incubate for further 4 h. Then, 100 μ l of DMSO were added to each well to dissolve the formazan crystals. Absorbance at 550 nm was measured with a microplate reader (GDV DV 990 B/V, Roma, Italy). Cytotoxicity was expressed as IC₅₀ which is the concentration to reduce the absorbance of treated cells by 50% with reference to the control (untreated cells).

1.8 Statistical analyses

Biological assays for the evaluation of the cytotoxic activity were run in quadruplicate, while DPPH test was carried out in triplicate. Data were expressed as means \pm S.E. The 50% inhibitory concentration (IC₅₀) was calculated from the Prism dose response curve (GraphPad Prism Software, San Diego, CA, USA) obtained by plotting the percentage of inhibition versus concentrations. Data were checked for normality (D'Agostino-Pearson test) and tested for homogeneity of variances (Levene's test). Statistical significance was assessed by one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test ($P \leq 0.05$), using SigmaStat Software (Jantel Scientific Software, San Rafael, CA).