SUPPLEMENTARY MATERIAL

Change in Caco-2 cells following treatment with various lavender essential oils

Donadu M. G.[1], Usai D.[1], Mazzarello V.[1], Molicotti P.[1], Cannas S.[1], Bellardi M.G.[2], Zanetti S.[1*]

[1] Department of Biomedical Science, University of Sassari, 07100 Sassari, Italy

[2] Department of Agricultural Sciences, University of Bologna, 40127 Bologna, Italy

*Corresponding author: Dott. Matthew Gavino Donadu, Department of Biomedical Sciences, University of Sassari, Viale San Pietro 43b, 07100 Sassari, Italy; tel: 3458593956, email: mdonadu@uniss.it

Abstract

Lavender is an aromatic evergreen shrub diffused in the Mediterranean basin appreciated since antiquity. The genus *Lavandula* is part of the Lamiaceae family and includes more than 20 species, among which true lavender (*L. vera* D.C.), (*L. angustifolia* Miller.) and spike lavender (*L. latifolia* Medikus); there are also numerous hybrids known as lavandins (*L. hybrida* Rev.). *L. vera,* spike lavender and several hybrids are the most intensely used breeding species for the production of essential oils. Lavender and lavandin essential oils have been applied in food, pharmaceutical and other agro-industries as biological products. In their chemical composition, terpenes linalool and linalyl acetate along with terpenoids such as 1,8-cineole are mostly responsible for biological and therapeutic activities. This study evaluates cytotoxic activity of essential oils derived from four lavender species on human epithelial colorectal adenocarcinoma cells. Analysis of pre- and post-treatment cell morphology has been performed using scanning electron microscope.

Key words: colon cancer, essential oils, morphology, lavender, electron microscopy, drugs

Experimental

Essential oil distillation

In the second week of August 2013 the apical parts of the three cvs of *L. hybrida* and *L. vera* D.C. were hand-collected and immediately used (as fresh leaf and flower material) to obtain EO by 2 hours steam distillation with a commercial Clevenger apparatus; a unique voucher specimen number was assigned to each EO and inserted into the laboratory register: *L. vera* D.C. cod n° MICRO0345; *L. angustifolia* Miller cod n°MICRO0346; *L. latifolia* Medikus cod. n°MICRO0347; *L. hybrida* cod n° MICRO0348 . Approximately 4-5 kg of fresh collected material for each lavender blotch were weighted; EO samples were stored at −5 ◦C until gas chromatography analysis, in glass vials in the absence of light.

Gas chromatography (GC)

GC analysis was performed on a Fisons (Rodano, Milano, Italy) 9130–9000 series gas-chromatograph equipped with a Fisons EL980 processor, a FID detector and a MEGA SE52 (Mega, Legnano, Italy) 5% polydiphenyl 95% dimethylsiloxane bonded phase column (i.d. ¼ 0.32 mm, length 30m, film thickness ¼ 0.15 mm).

Operating conditions were as follows: injector temperature 280 °C; FID temperature 280 °C, carrier (helium) flow rate 2 ml/min and split injection with split ratio 1:40. The oven temperature was initially 45 °C and then raised to 100 °C at a rate of 1 °C/min, then elevated to 250 °C at a rate of 5 °C/min and finally held at that temperature for 10 min. One microliter of each sample dissolved in CH_2Cl_2 was injected. The percentage composition of the EOs was computed by the normalization method from the GC peak areas, without any correction factors.

Gas chromatography mass spectrometry (GC-MS). Essential oil constituents were analyzed by a Hewlett Packard HP5890 series II plus gas chromatograph equipped with a HPMS 5989b mass spectrometer operating on EI mode. The GC conditions were the same reported for GC analysis and the same column was used. The MS conditions were as follows: ionization voltage, 70 eV; emission current, 40 μA; scan rate, 1 scan/s; mass range, 35–300 Da; and ion source temperature, 200 °C.

Identification of compounds

The MS (Mass Spectrometry) fragmentation patterns were checked with those of other EOs of known composition, with pure compounds and by matching the MS fragmentation patterns with NBS75K mass spectra libraries and with those in the literature. The relative amounts of the individual components were obtained from GC analysis based on peak areas without FID factor

correction. The constituents of the volatile oils were also identified by comparing their GC retention indices. A mixture of aliphatic hydrocarbons (C_8–C_{24}) in hexane (Sigma, Saint Louis, MO, USA) was injected under the above mentioned temperature program to calculate the retention indices using the generalized equation by Van del Dool and Kartz.

We analyzed the cytotoxic effect of four lavender EOs on human epithelial colorectal adenocarcinoma cells (Caco-2) cell line. The cells were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% foetal bovine serum and 100 U/ml penicillin/streptomycin, and incubated at 37°C, 5% CO_2 air atmosphere. $1{,}5x10^5$/mL cells were further seeded in 96-well plates and incubated overnight at 37°C, 5% CO_2. Dilutions (from 16% to 0,004% V/V) of the EO were prepared in culture medium with the addition of Tween 80 (0.5%) and assessed for 30 minutes. The cytotoxicity assay (*in vitro* toxicology assay kit MTT based, Sigma-Aldrich) was performed following the manufacturer's instruction. Wells were washed twice with PBS and 100 µl of culture medium without serum plus 1/10 MTT solution (3-[4,5- dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide)/PBS was added. After 4 hours of incubation, M-8910 MTT solubilisation solution - 10% Triton X-100 plus 0,1N HCl in anhydrous isopropanol was added. The quantity of formazan (presumably directly proportional to the number of viable cells) was measured by recording changes in absorbance at 570 nm using a plate reading spectrophotometer. The percentage of viability was calculated according to the following formula: (OD [570 nm] sample assessed/(OD [570 nm] negative control) = R; R x 100 = % cells viability. If the percentage is greater than 50%, the oil has no cytotoxicity; for the values lower than 50%, the oil is considered cytotoxic (Cannas et al, 2015). Subsequently, cells were seeded on coverslips for observation under a scanning electron microscope (SEM). Each sample was tested for three concentrations of lavender EO: non cytotoxic, superior and inferior. The cells, to be observed by SEM, were fixed in a 2% glutaraldehyde solution in a 0.1 M cacodylate buffer (1 h). After three washes, 5 min each in the same buffer, the samples were dehydrated through graded alcohol solutions, air-dried with hexamethyldisilazane for 10 min (Forge et al., 1992), examined and photographed in low vacuum using SEM FEI Quanta 200.

Table S1: Major compounds are listed in order of elution from a SE-52 column

Compound	***L. vera* D.C.**	***L. angustifolia* Miller.**	***L. latifolia* Medikus**	***L. hybrida* Rev.**
3 Octanone	1.56[a]	0.37	0.5	1.32
Myrcene	3.09	1.13	2.36	2.84
Hexyl acetate	Tr	0.12	1.05	1.88
1,8 cineole	Tr	8.7	6.49	6.74
Cis- ocimene	0.89	0.16	0.39	0.84
Trans - ocimene	2.41	0.38	0.89	4.32
linalool	36.15	56.57	34.43	39.24
Allo-ocimene	1.15	0.22	0.61	1.32
Canfor	0.98	10.01	8.84	4.26
borneol	2.39	1.83	1.6	1.12
Terpinen – 4-ol	16.13	4.82	0.63	0.1
Hexyl butanoate	0.04	2.25	1.94	1.99
Lynalil acetate	17.08	Tr	24.36	22.88
Lavandulyl acetate	2.5	Tr	1.93	1.34
Geranil acetate	1.58	Tr	1.51	Tr
Caryophyllene	1.77	2.26	1.47	1.14

[a] RA%, Relative area percentage

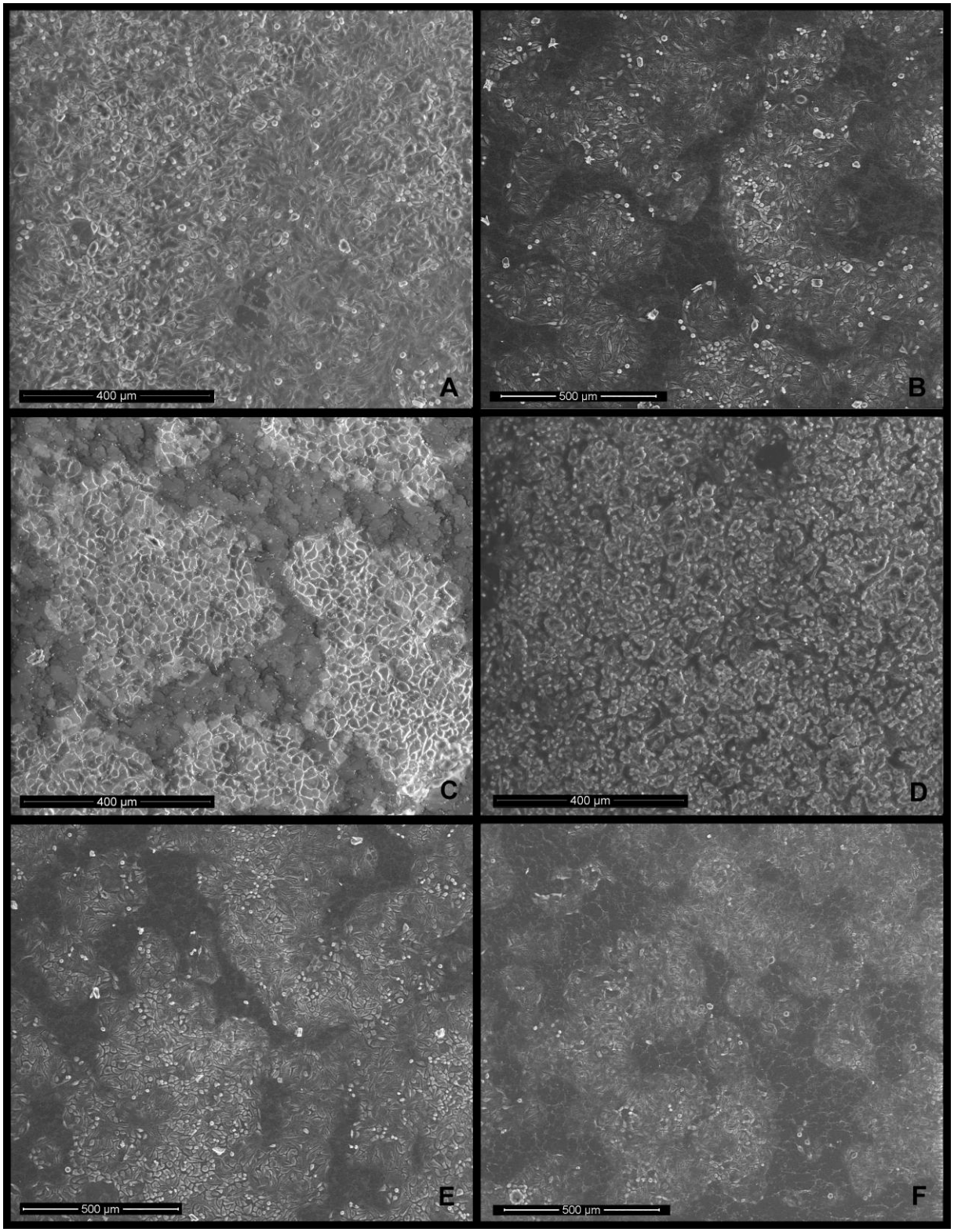

Figure S1 : Scanning Electron Microscopy micrographs of Caco-2 cells. Figure S1 (a) Control. Figure S1 (b) Caco-2 cells treated with Lavandula hybrida at a non cytotoxic concentration of 0.0005%. Figure S1 (c) Caco-2 cells treated with L. hybrida at a cytotoxic concentration of 0.06%. Figure S1 (d) Caco-2 cells treated with L. vera at a cytotoxic concentration of 0.015%. Figure S1 (e) Caco-2 cells treated with L. vera at a non cytotoxic concentration of 0.008%. Figure S1 (f) Caco-2 cells treated with L. hybrida at a non cytotoxic concentration of 0.008%

References

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