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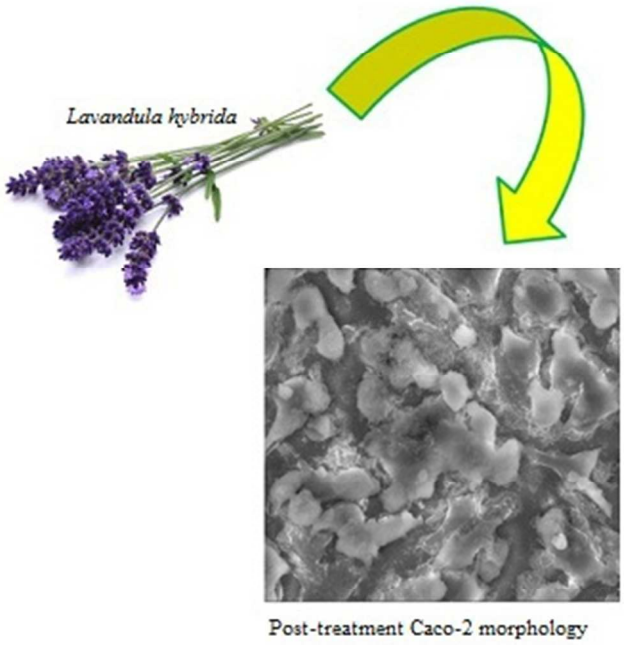

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Abstract

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. or *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

1. Introduction

Phytotherapy is one of the most antique practices used by human to cure health problems; its traces have been found among ancient civilizations including Egypt, Indus Valley, Greek, Chinese and Roman Empire (Campanini, 2004). Lavender essential oils (EO) present important pharmacological properties and low toxicity, and are promising candidates to be used as food supplements or in pharmaceutical applications.

Even though lavender's antiseptic activity is less pronounces compared to that of EO derived from other plants of the Lamiaceae family such as thyme, it presents important anti-inflammatory and decongestant effects without causing irritations. These features make lavender EO extremely useful as a treatment of low grade inflammations. While numerous scientific studies describe the composition and antimicrobial activity of EO extracted from multiple lavender species and hybrids, the action on human epithelial colorectal adenocarcinoma (Caco-2) cells is poorly reported.

The study reported here presents a comparative screening of four medicinal plants including three different samples of *L. hybrida* and one sample of *L. vera* cv Selection, cultivated at the Herb Garden of Casola Valsenio (Emilia Romagna region, northern Italy). Oxygenated monoterpenes, such as 1,8-cineole, lavandulol and necrodane derivatives, are the main components of EO. They present a significant antioxidant activity with a high ability to inhibit lipid peroxidation and showed an outstanding effect against a wide spectrum of microorganisms including gram-positive and gram-negative bacteria and pathogenic yeasts (Ait Said L et al, 2015) (Cavanagh et al, 2002). Moreover, we analyzed the cytotoxic activity of the four EOs on Caco-2 cells by evaluation of cell morphology before and after treatment with the aid of scanning electron microscope.

2. Results and Discussion

In this study we analyzed four lavender EOs on Caco-2 cells. The EOs tested showed no cytotoxic effect at very low concentrations, ranging from 0.03% (*L. hybrid Rev*), 0.015% (*L. latifolia Medikus*), 0.008% (*L. vera D.C.*), to 0.001% (*L. angustifolia Miller*). The finding that lavender essential oil a medicinal plant-derived natural multicomponent preparation may be a source of pharmacological active substances that interfere with Caco-2 cells. EOs are considered more potent than their constituents (Table S1) due to their synergistic and more selective effect. In addition, EOs from plants growing in varied environments differ in their composition and hence have different uses (Prusinowska R et al, 2016) (Carrasco et al, 2016).Microscopic evaluation of cell monolayers

highlighted their heterogeneous morphology confirmed even by visual inspection. As a result, over the years the characteristics of the cells used in different laboratories around the world have diverged significantly, which makes it difficult to compare results across laboratories (Sambuy et al, 2005). The SEM appearance of tumor cells in the control group (Figure S1 (a)) showed well-preserved cells forming a monolayer (Meunier et al, 1995) and (Hidalgo et al, 1989). (Figure S1 (b)) showed Caco-2 cells treated with Lavandula hybrida at a non cytotoxic concentration of 0.0005%. Caco-2 cells express tight junctions, microvilli, and a number of enzymes and transporters characteristic of such enterocytes: peptidases, esterases, P-glycoprotein, uptake transporters for amino acids, bile acids carboxylic acids, etc. They appear flat, of variable and mostly lengthened shape, closely collocated with a narrow intercellular space however a few rounded cells, probably detached from the monolayer, were identified. Smooth surface with tiny microvilli, thin filamentous pattern inside the cells and wide nuclear relief were observed. Groups treated with cytotoxic EO concentrations presented the loss of uniform cell monolayer with wide empty spaces and few cell islands (Figure S1(c)). In the group where EO showed more cytotoxicity towards Caco-2 cells changes in cell morphology were observed (Figure S1(d)). The cells appeared bulgy and separated by wide intercellular spaces, with cell surface smoothened following the loss of microvilli and irregular due to the presence of numerous blebs. Moreover, cell debris was randomly revealed between cells. These phenomena probably result from apoptosis induced by lipophilic compounds such as terpenes. The composition of lavender EOs suggest (Table S1) that *L. angustifolia Miller* has a high cytotoxicity, due to the large presence of terpenes that cause apoptotic mechanisms. The anti-cancer activity of plant essential oils has been mostly ascribed to terpenoids as the majors compomponds. Also, many of isolated terpenoids have been shown to possess anti-proliferative and chemopreventive activities in various models (Kuttan et al, 2011).

EOs probably affect cell membrane permeability and act on different cellular targets involved in various pathways. EOs increase intracellular ROS/RNS levels which results in apoptosis in cancer cells. Inhibition of Akt, mTOR, and MAPK pathways at different steps by EOs leads to corresponding up-/downregulation of various key biomolecules. Alteration in expression of NF-κB caused by exposure to EOs and further binding of NF-kB to DNA result as well in apoptosis in Caco-2 cells. Yet, dephosphorylation of Akt by the action of EOs leads to overexpression of p21, which either induces apoptosis by increasing caspases level or results in cell cycle arrest by binding to cyclins. In addition, EOs-induced mitochondrial stress leads to activation of Bcl-2 and membrane depolarization resulting in enhanced release of cytochrome-C to the cytoplasm, which activates apoptotic cell death. EOs also modulate DNA repair mechanisms by acting as DNA polymerase inhibitors and lead to PARP cleavage which also results in apoptosis in cancer cells (Gautan N et

al, 2014) It is plausible that the action of EOs determines minor cytotoxic effect as the cells represented in (Figure S1(c)) seem to have undergone less damage. In fact, they are visibly smaller, flattened due to the loss of nuclear relief, characterized by cell surface covered by thick microvilli and tend to assume rounded shape. When lower doses of EOs were applied, damaging effect on Caco-2 cells was only modest; cell monolayer presented a few empty spaces (Figure S1(e)), while the cells appeared flattened and outdistanced ones from the others(Figure S1(f)).

In eukaryotic cells, EOs can change the fluidity of membranes, which become abnormally permeable resulting in leakage of radicals, cytochrome C, calcium ions and proteins, as in the case of oxidative stress and bioenergetic failure (Alizadeh A et al, 2016) (Yoon et al, 2000) (Armstrong 2006). Cytotoxic activities of EOs or their major components, sometimes activated by light, have been also demonstrated in mammalian cells *in vitro* by short-term viability assays using specific cell staining including MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) Test (Carvalho de Sousa et al 2004). EO's cytotoxicity in mammalian cells is caused by induction of apoptosis and necrosis.

3. Conclusions

The present study was designed to evaluate the effect of EOs of lavender on cancer cell lines to see if these cells were suitable for treatment with natural products. Caco-2 cell line showed different susceptibility. Data presented here are related to a preliminary study; further study are necessary to understand the mechanism. Lavender EO components as linalool and linalyl acetate can induce cell death (Table S1). Although further work is needed for more elucidation, these preliminary data show that lavander EO could be developed as therapeutic agent on cancer cell lines. Further research is needed to clarify other relevant activities and to confirm "*in vivo*" our findings.

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Conflicts of Interest

The authors declare no conflict of interest.

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SUPPLEMENTARY MATERIAL

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Abstract

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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

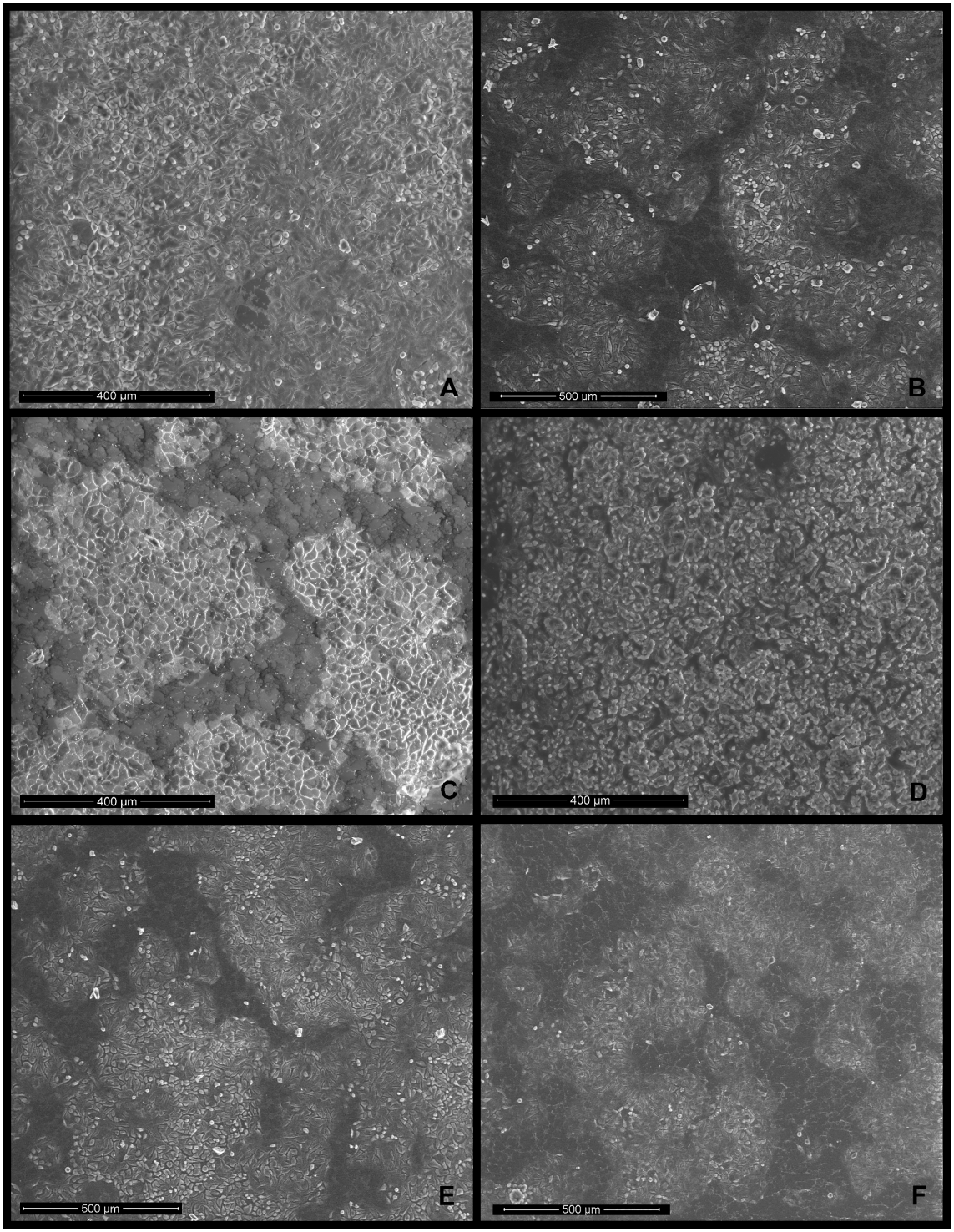

Figure S1 : Scanning Electron Microscopy micrographs of Caco-2 cells. Figure S1 (a) Control. Figure S1 (b) Caco-2 cells treated with Lavandula hybrida at a non cytotoxic concentration of 0.0005%. Figure S1 (c) Caco-2 cells treated with L. hybrida at a cytotoxic concentration of 0.06%. Figure S1 (d) Caco-2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

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