Assessing the cytotoxic/genotoxic activity and estrogenic/antiestrogenic potential of essential oils from seven aromatic plants.

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Abstract

Alternative therapies with new drugs are needed because the clinical efficacy of conventional chemotherapy is often reduced due to collateral effects. Many natural products of plant origin, including essential oils (EOs) have proved to be effective in prevention and therapy of several diseases such as bacterial infections, chronic diseases and cancer. In the present study, we investigated some biological activities of EOs extracted from seven plants: *Rosmarinus officinalis, Salvia somalensis, Thymus vulgaris, Achillea millefolium, Helichrysum italicum, Pistacia lentiscus, Myrtus communis*. In particular, we evaluated the cytotoxic and genotoxic activity using the cytochalasin B-blocked micronucleus assay (CBMN) in human peripheral lymphocytes, cytotoxicity in a human ovarian carcinoma cell line, and the estrogenic/antiestrogenic activity using a yeast strain expressing the human estrogen receptor alpha (ERα). Our results show that most EOs can have a strong cytotoxic and a slight/moderate genotoxic effect on human peripheral lymphocytes, and also a pronounced cytotoxic effect in a ovarian cell line. In addition, some EOs seem to have a marked antiestrogenic activity that could potentially perturb the estrogen-dependent tissues.

1. Introduction

Plant-derived natural products have a long-standing application in cosmetics and prevention and therapy of human disease. An important fraction of those products is represented by essential oils, which are concentrated hydrophobic liquids with a specific fragrance (Ríos, 2016). Essential oils (EOs) are complex mixtures of organic compounds, characterized by the presence of two or three components, generally responsible for the biological activity, and more than 20 minor components present in traces (Bakkali *et al*., 2008). Several studies documented that EO composition and yield can qualitatively and/or quantitatively vary depending on physiological conditions of the plants and the environment (*e.g*. geographic location and climate) (Barra, 2009; Figueiredo *et al*., 2008). Major constituents of essential oils are terpenes and their derivatives, terpenoids, followed by minor amounts of low molecular weight aliphatic and aromatic compounds. Terpenes are a class of molecules that are synthetized by plant secondary metabolism of isoprenoids, and they play a role in plant physiology as hormones, photosynthetic pigments and electron carriers (Theis and Lerdau, 2003). As volatile compounds, terpenes contained in EOs play an important role in plant communication, they attract pollinating insects and/or protect plant by repelling herbivores (Pichersky and Raguso, 2018). Moreover, due to their lipophilic nature, terpenes confer to EOs the potential for targeting and disrupting membranes of pathogenic bacteria (Burt, 2004).

Over the years, beneficial biological activities of EOs and their components have been identified and demonstrated also regard to human health (Bakkali *et al*., 2008; Elshafie and Camele, 2017). For example, EOs are described as potent antimicrobial agents against many foodborne bacteria such as *S. enterica, S. aureus* (Silva *et al*., 2013; Zengin and Baysal, 2014) as well as pathogenic fungi such as *Candida* spp. and *Aspergillus* spp. (Ebani *et al*., 2018). Phenolic terpenoids such as thymol and carvacrol also display important antioxidant properties (Prieto *et al., 2007;* Youdim *et al*., 2002). The scavenging activity of EOs might counteract the overproduction of reactive oxygen species (ROS), thus exerting protective effects against cellular oxidative stress, present in chronic inflammatory diseases, cancer or aging. EO extracted from *Melaleuca alternifolia* showed an

antioxidant and anti-inflammatory activity, by modulating leukocytes ROS production, thus reducing the release of pro-inflammatory cytokines (Caldefie-Chézet et al., 2006). Purified terpenes also display radical scavenging activity and regulate cytokine release by suppressing the NF-κB signaling pathway, a key transcription factor in the pathogenesis of inflammatory diseases (Marques et al., 2019). EOs also exert an anti-cancer activity by an opposite mechanism: once penetrated into cells, the components of EOs may react with ROS and generate new radical species that promote cell death, and several authors reported a pro-apoptotic activity of EOs in cancerous cells, (Jo et al., 2012; Navarra et al., 2015). EOs were also proved to show antimutagenic effects in bacterial and mammalian cells, by inhibiting the activation of indirect mutagens (Idaomar et al., 2001) or by promoting the repair of DNA lesions (Nikolić et al., 2011). However, administration to mammalian cells of high doses of EO constituents like the monoterpenes camphor, eucalyptol and thujone produced DNA breakage due to oxidative damage (Nikolić et al., 2015).

Furthermore, EOs are also recognized to exhibit either a weak estrogenic or anti-estrogenic activity both in vivo and in vitro (Bartoňková and Dvořák, 2018; Howes et al., 2002; Simões et al., 2018). The classical (anti)estrogenic activity of plant derived - as well as man-made - compounds is determined by their ability to bind the estrogen receptors alpha (ERα) and/or beta (ERβ). Interaction with these receptors confers the potential to act as "endocrine disruptors". Thus, both activities must be taken into account when analyzing the chemo-preventive and chemo-therapeutic potential of EOs.

In the present study we investigated the biological activities of EOs extracted from seven aromatic plants: Rosmarinus officinalis L., Salvia somalensis Vatke, Thymus vulgaris L., Achillea millefolium L., Helichrysum italicum Roth (G. Don), Pistacia lentiscus L., Myrtus communis L.. In particular, we evaluated: 1) the cytotoxic and genotoxic activity using the cytochalasin B-blocked micronucleus assay (CBMN) in human peripheral lymphocytes, 2) cytotoxicity in the human ovarian carcinoma cell line A2780, and 3) the estrogenic/antiestrogenic potential in a recombinant yeast strain expressing the human Erα.

2.   Materials and Methods

2.1 Test compounds

The essential oils of *R. officinalis* (REO), *S. somalensis* (SEO), *A. millefolium* (AEO), *T. vulgaris* (TEO), *H. italicum* (HEO) and *M. communis* (MEO) were obtained from plants cultivated at CREA (Centro di Ricerca Orticoltura e Florovivaismo, Sanremo, Italy). The voucher specimens are deposited in the herbarium of Giardini Botanici Hanbury (La Mortola–Ventimiglia, Imperia, Italy): *R. officinalis* (HMGBH.e/7219.2018.001), *S. somalensis* (HMGBH.e/7290.2018.001), *A. millefolium* (HMGBH.e/9332.2018.001), *T. vulgaris* (HMGBH.e/7319.2018.001), *H. italicum* (HMGBH.e/9006.2018.001), *M. communis* (HMGBH.e/5558.2018.001). All plants were vegetatively propagated, planted and grown under uniform conditions while *P. lentiscus* plants were harvested on Elba island (Laconella collection site, 42.759333,10.2962719). The aerial parts were dried and hydrodistillated to obtain the respective EOs, that were maintained at 4°C in dark glass vials and microbiologically tested before use. All cell systems used in the study were then treated with various *v/v* concentrations of each EO that were obtained dissolving and appropriately diluting the extracts in DMSO. Supplementary Table 1 shows the corresponding *w/v* final concentrations. These values were calculated after having weighed three times an equal amount (200 µl) of each EO.

2.2   Gas chromatography – mass spectrometry analyses

The chemical composition of each essential oil was determined by Gas chromatography coupled to mass spectrometry. The GC/EI-MS analyses were performed with a Varian CP-3800 apparatus equipped with a DB-5 capillary column (30 m × 0.25 mm i.d., film thickness 0.25 µm) and a Varian Saturn 2000 ion-trap mass detector. The oven temperature was programmed from 60° C to 240° C at 3° C/min; injector temperature, 220°C; transfer-line temperature, 240° C; carrier gas, He (1 mL/min). The acquisition parameters were as follows: full scan; scan range: 35-300 m/z; scan time: 1.0 sec; threshold: 1 count. The identification of the constituents was based on the comparison of their retention times (tR) with those of pure reference samples and their linear retention indices

(LRIs) determined relatively to the tR of a series of n-alkanes. The mass spectra were compared to those listed in the commercial libraries NIST 14 and ADAMS and in a homemade mass-spectral library built up from pure substances and components of known oils, and MS literature data (Adams, 1995; Adams *et al.*, 1997; Davies, 1990; Jennings and Shibamoto, 1980; Masada, 1976; Swigar and Silverstein, 1981).

2.3 CBMN assay

Cell cultures, treatment and harvesting

The assay was performed according to the OECD guideline (2016). Heparinized whole blood samples were obtained by venipuncture from healthy 20- to 35-year-old donors. The study was approved by the Pisa University Ethical Committee.

At least two independent experiments, each consisting of two replicates, were performed for each treatment. Culture tubes were set up with 300 µl of whole blood and 4.7 ml of RPMI-1640 medium (Gibco Thermo Fisher Scientific, Milan, Italy) supplemented with 15% foetal bovine serum (Euroclone, Milan, Italy), 1% antibiotic/antimicotic (Euroclone, Milan, Italy) and 1.5% phytohaemagglutinin (Euroclone, Milan, Italy) and incubated at 37 °C for a total time of 72 h. In order to maximize the probability to detect genotoxic activity, peripheral lymphocytes were exposed to the EOs for 48 h (extended treatment) or for 3 h (short treatment). To evaluate whether or not the observed cytotoxic/genotoxic effects depend on the presence of direct, indirect mutagens, or a combination of both, short treatment was performed in absence or presence of an exogenous metabolizing system. This consists of the post-mitochondrial fraction from rat livers (S9) supplemented with appropriate co-factors (S9-mix, where S9 is present in the mix at a final concentration of 7.5% (*v/v*)) (Trinova Biochem, Giessen, Germany). Concentrations of the seven EOs were selected, for each type of treatment, among a dose range which was proven to be non- or moderately toxic for PHA-stimulated lymphocytes (*i.e.* when at the maximum dose tested no more than a 50% reduction in cell proliferation was observed). Control cultures received DMSO not

exceeding 0.01% (*v/v*) final concentration. The clastogenic compound mitomycin-C (MMC, 0.2 µg/ml) (Sigma-Aldrich, Milan, Italy), the spindle poison nocodazole (NOC, 0.15 µg/ml) (Sigma-Aldrich, Milan, Italy) and the indirect-acting mutagen cyclophosphamide (CP, 45 µg/ml) (Sigma-Aldrich, Milan, Italy) were used as positive controls. In the extended or short treatment, cytochalasin B (cytB, Sigma-Aldrich, Milan, Italy, 6 µg/ml final concentration) was added to block cell cytodieresis at 44 h or 51 h, respectively. Cell harvesting was carried out at 72 h. Briefly, after a 5-min centrifugation at 2100 rpm, the cell pellet was treated with 5 ml of 0.075 mM KC1 for a few min to lyse erythrocytes, pre-fixed in methanol/acetic acid (3:5), fixed in 100% methanol for at least 30 min, washed twice in 7:1 methanol:acetic acid, and dropped onto clean glass slides. The air-dried slides were then stained with 5% Giemsa.

Evaluation of cytotoxicity and genotoxicity

Slides were analyzed using an optical microscope equipped with a $40 \times$ objective ($400 \times$ final magnification). Cytotoxicity was evaluated by the cell proliferation index (CBPI) according to the following formula: $(M + 2B + 3P)/(M + B + P)$, where M, B and P were the number of cells that had still not entered the first mitosis (M, mononucleated) and cells that had divided once (B, binucleated) and twice (P, plurinucleated; the latter cells comprise both tri- and tetranucleated), respectively. $(M + B + P)$ represents a total of at least 500 scored cells per culture. Genotoxicity was evaluated scoring 1000 cells per culture for the presence of MN in both binucleated (BMN) and mononucleated cells (MMN). MN frequency and CBPI were then expressed as the mean ± SEM.

2.4 Cytotoxicity assay in cancer cells

The human ovarian carcinoma cell line A2780, was grown in RPMI 1641 supplemented with 10% fetal bovine serum, 1% L-Glutamine (Sigma-Aldrich, Milan Italy). Cytotoxicity was evaluated by the tetrazolium colorimetric water-soluble tetrazolium-1 assay (Roche Diagnostics, Milan, Italy) following the manufacturer's instructions. A2780 cells were plated in 96 multiwells at a density of 15000 cells for each well, after 24 h they were treated with different concentrations of EOs (from

0.001 to 1.0 µl/ml) for additional 24 h. Then 10 µl of water-soluble tetrazolium-1 was added to each well and after 4 h of incubation, cells were analyzed at 450 nm (Victor[3] 1420 multilabel counter; Perkin-Elmer, Waltham, MA). At least two independent experiments with three replicates each were conducted. Cell survival is expressed as percentage of cell density respect to negative control (cultures receiving DMSO alone). The effective concentrations inhibiting the cell growth by 50% (IC_{50}) and 70% (IC_{70}) were also calculated according to the concentrations reported in Supplementary Table 1.

2.5 Screening for estrogenic and antiestrogenic activity *in-vitro*

The (anti)estrogenic activity was determined using an estrogen-inducible yeast screen on *Saccharomyces cerevisiae* (yeast strain RMY326) expressing the human (ERα) and the reporter gene lacZ encoding the β-galactosidase enzyme. Transcription of the reporter gene by the complex receptor-ligand was detected and quantified in a microplate reader (Victor[3], 1420 Multilabel Plate Counter, PerkinElmer Italia Spa, Milan). As previously described (Garritano *et al*., 2006; Pinto *et al*., 2004), yeast cells were incubated at 28°C for 7 h in an orbital shaker. After incubation, OD at 595 nm was measured and adjusted to < 0.1 nm by diluting with fresh medium. To test for agonistic activity, yeast cultures were incubated overnight in the presence of increasing concentrations (final concentration range: 0.00001 µl/ml to 0.1 µl/ml) of essential oils. Positive control was represented by 17 β-estradiol (E_2) at final concentration 10 nM (Sigma-Aldrich, Milan, Italy), negative control by 0.1% vehicle (DMSO, Sigma-Aldrich, Milan, Italy). The enzymatic reaction was started by adding O-nitrophenyl β-D-galactopyranoside (ONPG) (Sigma-Aldrich, Milan, Italy) and incubating at 30°C for 10 min. The reaction was stopped by adding Na_2CO_3, the absorbance was measured at 415 nm. To test for antagonistic activity, yeast cells were treated with 1 nM E_2. Samples able to inhibit the activity of the natural ligand E_2 is expected to show a dose-dependent decrease in β-gal expression. E_2 and essential oils were dissolved in DMSO and added to the yeast culture so that the concentration of solvent did not exceed 0.2% (v/v).

The β-galactosidase (β-gal) activity was normalized to the number of cells assayed in the test (OD595 nm). The final part of the assay was conducted using phthalate-free disposable laboratory equipment. At least two independent experiments with three replicates were conducted. The results are expressed as percent of the β-gal activity obtained with E_2. Each value represents the mean ± SEM.

2.6 Statistical analyses

All the data of the CBMN assay were analyzed by one-way analysis of variance (ANOVA) using the STATGRAPHICS Plus 5.1 software (Statistical Graphics Corporation, 2001, Rockville, USA). The Dunnett's multiple comparison tests were used to compare data of each concentration. When necessary, Bonferroni's multiple range test was used to perform a 2x2 comparison among the dose groups. For the (anti)estrogenic activity, statistical analyses were performed by the GraphPad Prism software v.5 (GraphPad Software, Inc. CA, USA). Dose-dependent activity was measured by first order regression analysis, and each activity was considered significant when it reached, at the maximum concentration tested, at least a 20% increase or a 40% decrease. A p-value smaller than 0.05 was considered to be statistically significant.

3.   Results

3.1 Chemical composition of essential oils

The composition (%) of the main components of the seven essential oils are summarized in Table 1 (complete data in Supplementary Table 2). Almost all the oils show monoterpenes and sesquiterpenes as major components either in their oxygenated or hydrocarbon forms. HEO shows a prevailing presence of neryl-acetate (31.83%) which is not (or barely) detected in the other analyzed oils. Major components of SEO were instead bornyl acetate (29.59%) and camphor, (20.93%) which was also detected in AEO (7.44%) and REO (7.57%). PEO shows high content in myrcene (35.99%) and α-pinene (11.98%). EO of *A. millefolium* does not show a predominant compound,

and it is rather a mixture of three main compounds: β-pinene (8.16%) 1,8-cineole (13.12%), globulol (11.10%). 1,8-cineole was also found as a major component in MEO (28.95%) along with a very high percentage of tricyclene (49.04%). More than 50% of REO composition is represented by α-pinene and 1,8 cineol (37.89% and 22.01% respectively). Finally, TEO presents elevate concentrations of thymol (52.61%) and p-cymene (15.25%).

Table 1. Composition of the tested EOs (the main values are highlighted in bold character) and summary of the major classes of the identified constituents

Compound	L.R.I.[a]	L.R.I.[b]	H. italicum (HEO)	S. somalensis (SEO)	P. lentiscus (PEO)	A. millefolium (AEO)	R. officinalis (REO)	T. vulgaris (TEO)	M. communis (MEO)
					Relative percentage (%)[c]				
α-Thujene	932	930	**7.24**	1.78	0.57	0.22	0.24	0.11	0.55
Tricyclene	938	935			1.49	0.13	0.21		**49.04**
α-Pinene	940	939			**11.98**	2.80	**37.89**	0.85	
Camphene	955	954		2.35	2.50	3.53	**5.36**	0.28	0.24
β-Pinene	981	979	1.00	0.95	2.65	**8.16**	**5.01**		0.87
Myrcene	993	991	0.51	0.31	**35.99**	0.59	1.63	0.66	0.55
p-Cymene	1028	1025	1.10	1.53		2.52		**15.25**	2.66
Limonene	1032	1029	**6.97**	2.75	**6.28**	1.03	3.26	0.41	**5.94**
1,8-Cineole	1038	1031	2.25			**13.12**	**22.01**	0.66	**28.95**
Camphor	1148	1146		**20.93**		**7.44**	**7.57**	0.51	
Bornyl acetate	1287	1286		**29.59**	0.17	4.85	3.32		
Thymol	1290	1290						**52.61**	
Neryl acetate	1368	1362	**31.83**			1.50			
β-Caryophyllene	1418	1419	3.11	2.18	2.13	0.57	4.06	**6.77**	0.53
ar-Curcumene	1484	1481	**5.56**						
Globulol	1584	1585				**11.10**			
5-epi-7-epi-α-Eudesmol	1606	1608	**5.47**	0.29		0.77			
Monoterpene hydrocarbons			19.16	11.79	78.04	28.90	56.52	21.68	61.22
Oxygenated monoterpenes			35.47	56.58	2.58	40.68	36.67	64.14	31.11
Sesquiterpenes hydrocarbons			29.40	17.41	12.41	10.80	4.38	9.20	1.18
Oxygenated sesquiterpenes			9.35	12.72	0.58	14.51	0.25	0.00	0.00
Unknowns			2.61	0.30	0.22	1.99	0.12	1.69	0.34
EO yields (% w/w)			0.3	0.4	0.15	0.8	1.3	1.2	0.3
TOTAL IDENTIFIED			94.25	98.92	94.17	96.88	97.94	97.31	93.85

[a]L.R.I. indicates the Linear retention indices on a DB5 column, and [b]L.R.I. Linear retention indices from the literature (Adams, 1995)

[c]Only compounds present at a concentration ≥ 5% are included in the table.

3.2 Evaluation of cytotoxicity/genotoxicity of EOs in human peripheral cells

Extended treatment

CBPI of treated cultures decreased significantly with increasing EOs concentration, thus indicating a dose-dependent cytostatic effect for each EO, with the sole exception of the lowest doses of *R. officinalis*, *S. somalensis* and *M. communis*. Three out of seven EOs displayed a strong cytotoxicity as their lowest CBPI values were observed at 0.1 µl/ml for *S. somalensis*, (1.33 ± 0.07), *T. vulgaris* (1.09 ± 0.06) and *M. communis* (1.24 ± 0.07). Likewise, the genotoxic activity of EOs also led to a dose-dependent increase in MN frequencies (Table3). Compared to the control (2.00 ± 0.56), treatment with REO led to a four-fold increase of BMN frequencies for all the intermediate doses tested, reaching a four-fold increase at the highest dose of 0.2 µl/ml (8.00 ± 1.37). On the other hand, MMN frequencies did not differ significantly from the basal level. SEO, MEO and TEO led to a similar dose-response patterns: BMN frequencies increased significantly at intermediate doses as compared to the respective control (SEO 0.05 µl/ml: 10.75 ± 1.30 *vs*. 2.63 ± 0.92; *M. communis* 0.05 µl/ml: 19.00 ± 2.11 *vs*. 4.30 ± 1.34; TEO 0.025 µl/ml: 13.25 ± 1.56 *vs*. 4.0 ± 0.99). At the highest doses, probably due to the concomitant decrease in cell proliferation, we observed a reduction of BMN levels. In the case of mononucleated cells, the highest MN frequencies, as compared to the respective control value, were obtained for TEO at 0.1 µl/ml (7.25 ± 0.72 *vs*. 2.00 ± 0.46), SEO at 0.08 µl/ml (3.25 ± 0.63 *vs*. 0.88 ± 0.45) or MEO at 0.05 µl/ml (5.00 ± 0.79 vs. 2.10 ± 0.50).

Both BMN and MMN frequencies from cultures treated with AEO increased with increasing the dose, but statistical significance was reached only for BMN levels at doses ≥ 0.1 µl/ml. Compared to control cultures (2.33 ± 1.13), BMN frequencies of peripheral cells treated with HEO at concentrations ≥ 0.025 µl/ml stand around approximately a 4-fold increase, reaching the highest value at 0.15 µl/ml (17.25 ± 1.96). MMN frequency displays a significant increase only at the highest dose tested of 0.15 µl/ml (10.00 ± 1.68 *vs*. 1.42 ± 0.97). Finally, PEO increased BMN frequencies from 2.44- to 3.31-fold as compared to control cultures at all the tested doses, with the

maximum level obtained at 0.1 µl/ml (10.17 ± 0.63 *vs*. 3.07 ± 0.58); induction of MN in mononucleated cells was observed only at this dose (2.75 ± 0.36 *vs*. 1.29 ± 0.33).

The magnitude of the EOs genotoxic response was slightly increased if compared to the positive controls, NOC 0.15 µg/ml (mean induction of 63.55 ± 0.71 MN in mononucleated cells) and MMC 0.2 µg/ml (mean induction of 80.55 ± 0.53 MN in binucleated cells).

Table 2. Proliferation index (CBPI) of human peripheral lymphocytes after 48 h treatment with EOs. Data represents means ± SEM of at least two independent experiments

Concentration (µl/ml)	*R. officinalis* (REO)	*S. somalensis* (SEO)	*T. vulgaris* (TEO)	*A. millefolium* (AEO)	*H. italicum* (HEO)	*P. lentiscus* (PEO)	*M. communis* (MEO)
0.01				1.44 ± 0.04^b			
0.0125			1.49 ± 0.06^a				
0.02	1.68 ± 0.09	1.64 ± 0.09					
0.025			1.38 ± 0.06^b		1.52 ± 0.08^a	1.65 ± 0.05^a	1.58 ± 0.07
0.05	1.32 ± 0.06^b	1.43 ± 0.07^b	1.28 ± 0.05^b	1.35 ± 0.05^b	1.41 ± 0.08^b	1.61 ± 0.04^b	1.38 ± 0.05^b
0.08		1.39 ± 0.07^b					
0.1	1.39 ± 0.06^b	1.33 ± 0.07^b	1.09 ± 0.06^b	1.26 ± 0.06^b	1.30 ± 0.05^b	1.52 ± 0.03^b	1.24 ± 0.07^b
0.15	1.24 ± 0.06^b				1.22 ± 0.08^b		
0.2	1.13 ± 0.09^b			1.19 ± 0.09^b		1.39 ± 0.04^b	
DMSO (0.01%)	1.84 ± 0.04	1.80 ± 0.05	1.73 ± 0.04	1.84 ± 0.04	1.79 ± 0.05	1.81 ± 0.03	1.70 ± 0.04
NOC (0.15 µg/ml)	1.28 ± 0.06^b	1.31 ± 0.04^b	1.21 ± 0.05^b	1.28 ± 0.06^b	1.26 ± 0.04^b	1.27 ± 0.04^b	1.19 ± 0.03^b
MMC (0.2 µg/ml)	1.18 ± 0.06^b	1.15 ± 0.06^b	1.11 ± 0.04^b	1.18 ± 0.06^b	1.15 ± 0.06^b	1.20 ± 0.05^b	1.10 ± 0.05^b

NOC: nocodazole, MMC: mytomicin C.

[a], significantly different ($p < 0.05$, Dunnett test) from control cultures (DMSO, 0.01%).

[b], significantly different ($p < 0.01$, Dunnett test) from control cultures (DMSO, 0.01%).

Table 3. MN frequencies in mononucleated (**A**) or binucleated (**B**) human peripheral lymphocytes after 48 h treatment with EOs. Data represents means ± SEM of at least two independent experiments

Concentration (µl/ml)	R. officinalis (REO)	S. somalensis (SEO)	T. vulgaris (TEO)	A. millefolium (AEO)	H. italicum (HEO)	P. lentiscus (PEO)	M. communis (MEO)
A							
0.01				1.00±0.45			
0.0125			3.50±0.72				
0.02	1.50±0.74	1±0.90					
0.025			4.75±0.72[a]		2.50±1.68	2.25±0.63	2.50±0.79
0.05	1.75±0.53	2.75±0.63	4.50±0.59[a]	1.33±0.52	0.75±1.68	1.83±0.51	5.00±0.79[a]
0.08		3.25±0.63[a]					
0.1	2.75±0.53	1.5±0.63	7.25±0.72[b]	2.25±0.63	3.13±1.18	2.75±0.36[a]	2.90±0.56
0.15	1.75±0.53				10.00±1.68[b]		
0.2	3.00±0.74			3.00±0.90		2.33±0.51	
DMSO (0.01%)	1.17±0.30	0.88±0.45	2.00±0.46	1.30±0.40	1.42±0.97	1.29±0.33	2.10±0.50
NOC (0.15 µg/ml)	65.31±0.51[c]	62.12±0.89[c]	63.23±0.77[c]	61.01±0.75[c]	64.99±0.69[c]	64.69±0.61[c]	63.50±0.70[c]
B							
0.01				4.13±0.75			
0.0125			7.00±1.56				
0.02	2.50±1.37	2.00±0.92					
0.025			13.25±1.56[b]		9.25±1.96[a]	8.50±1.09[b]	10.50±2.11
0.05	6.00±0.97[a]	10.75±1.30[b]	9.83±1.27[b]	4.50±0.87	9.50±1.96[a]	7.50±0.89[b]	19.00±2.11[b]
0.08		8.50±1.30[b]					
0.1	6.75±0.97[b]	7.75±1.30[a]	9.25±1.56[a]	8.75±1.07[b]	8.75±1.38[b]	10.17±0.63[b]	15.63±1.49[b]
0.15	6.25±0.97[a]			11.50±1.51[b]	17.25±1.96[b]		
0.2	8.00±1.37[b]					8.00±0.97[b]	
DMSO (0.01%)	2.00±0.56	2.63±0.92	4.00±0.99	2.50±0.68	2.33±1.13	3.07±0.58	4.30±1.34
MMC (0.2 µg/ml)	83.23±0.65[c]	79.86±0.54[c]	81.40±0.57[c]	78.95±0.74[c]	80.01±0.67[c]	79.85±0.54[c]	80.55±0.62[c]

NOC: nocodazole, MMC: mytomicin C.

[a],significantly different (p < 0.05, Dunnett test) from control cultures (DMSO, 0.01%).

[b],significantly different (p < 0.01, Dunnett test) from control cultures (DMSO, 0.01%).

[c],significantly different (p < 0.001, Dunnett test) from control cultures (DMSO, 0.01%).

Short treatment (with or without metabolic activation)

Since EOs are a complex mixture of potentially bioactive molecules, which can also include indirect-acting mutagens, we exposed peripheral lymphocytes to EOs for 3 h, either in presence or absence of S9-mix. As shown in Table 4, the treatment of cells with the seven EOs resulted in a consistent and dose-dependent decrease of CBPI, especially at the highest doses tested. As expected, these decreases were, in general, less marked if compared to those observed after 48 h treatment. The results of genotoxicity assay are shown in Table 5. BMN frequencies in cultures treated with REO were significantly higher both in presence or absence of metabolic activation. Conversely, we did not detect significant increase in MMN levels in both conditions. In absence of metabolic activation, SEO led to an increase in BMN frequencies at all the tested doses, while MMN frequencies did not differ from control condition. Interestingly, neither BMN nor MMN levels differed significantly from the spontaneous frequency when S9-mix was applied. No significant increase in MN frequencies was observed for TEO in absence of exogenous metabolic activation, for both mononucleated and binucleated cells. A more evident dose-dependent effect in the two populations of cells was observed in presence of S9-mix, reaching a significant increase for dose 0.1 µl/ml in binucleated cells (13.50 ± 1.59; control: 5.0 ± 1.12). AEO treatment induced similar results with or without metabolic activation. Although both BMN and MMN frequencies increased with the dose, a statistical significance was obtained at 0.1 µl/ml and 0.15 µl/ml only for BMN levels. Compared to the control values, (2.56 ± 0.68 or 3.50 ± 0.74 for MMN or BMN frequencies, respectively) treatment with HEO in absence of S9-mix provoked a significant increase in BMN frequencies at 0.05 µl/ml (7.13 ± 0.95), followed by a reduction at higher doses, probably linked to cytotoxic effects. Likewise, after treatment with HEO in presence of S9-mix, we obtained a 2.3-fold increase in BMN frequency at 0.05 µl/ml (8.00 ± 1.04), respect to the control value (3.50 ± 0.74), followed again by a decline for the two highest doses. Cultures treated with PEO and MEO did not increase BMN and MMN frequencies with or without metabolic activation.

Table 4. Proliferation index (CBPI) of human peripheral lymphocytes after 3 h treatment with EOs in the presence/absence of S9-mix. Data represents

means ± SEM of at least two independent experiments

Concentration (µl/ml)	*R. officinalis* (REO)		*S. somalensis* (SEO)		*T. vulgaris* (TEO)		*A. millefolium* (AEO)		*H. italicum* (HEO)		*P. lentiscus* (PEO)		*M. communis* (MEO)	
	3h-S9	3h+S9	3h-S9	3h+S9	3h-S9	3h+S9	3h-S9	3h+S9	3h-S9	3h+S9	3h-S9	3h+S9	3h-S9	3h+S9
0.025	1.52±0.06	1.59±0.03		1.53±0.05	1.59±0.05	1.63±0.04							1.58±0.04	1.59±0.03
0.05	1.47±0.04	1.45±0.04[b]	1.52±0.04	1.47±0.05	1.52±0.03	1.55±0.04	1.50±0.04	1.54±0.04	1.53±0.04	1.52±0.05	1.49±0.04[a]	1.63±0.03	1.52±0.04	1.45±0.04
0.1	1.36±0.04[a]	1.33±0.04[b]	1.37±0.04[b]	1.41±0.05[a]	1.40±0.04[a]	1.16±0.04[b]	1.42±0.04[b]	1.45±0.05	1.51±0.04		1.45±0.03[b]	1.49±0.05	1.47±0.04[b]	1.33±0.04[b]
0.15							1.33±0.04[b]	1.33±0.05[a]	1.37±0.08[a]	1.54±0.05				
0.2			1.26±0.06[b]						1.31±0.06[b]	1.14±0.07[a]	1.22±0.06[b]	1.28±0.07[a]		
DMSO (0.01%)	1.59±0.03	1.67±0.04	1.56±0.03	1.59±0.04	1.60±0.03	1.62±0.03	1.56±0.03	1.59±0.05	1.62±0.03	1.63±0.03	1.64±0.03	1.63±0.03	1.61±0.03	1.67±0.04
NOC (0.15 µg/ml)	1.32±0.04[b]		1.29±0.05[b]		1.33±0.04[b]		1.29±0.05[b]		1.34±0.03[b]		1.36±0.02[b]		1.34±0.04[b]	
MMC (0.2 µg/ml)	1.42±0.02[b]		1.40± 0.04[b]		1.43±0.02[b]		1.40±0.04[b]		1.43±0.01[b]		1.45±0.02[b]		1.44±0.01[b]	
CP (45 µg/ml)		1.39±0.02[b]		1.32±0.08[b]		1.35±0.06[b]		1.33±0.04[b]		1.36±0.03[b]		1.34±0.05[b]		1.39±0.06[b]

NOC: nocodazole, MMC: mytomicin C, CP: cyclophosphamide.

[a],significantly different (p < 0.05, Dunnett test) from control cultures (DMSO, 0.01%).

[b],significantly different (p < 0.01, Dunnett test) from control cultures (DMSO, 0.01%).

[c],significantly different (p < 0.001, Dunnett test) from control cultures (DMSO, 0.01%).

Table 5. MN frequencies in mononucleated (**A**) or binucleated (**B**) human peripheral lymphocytes after 3 h treatment with EOs in the presence/absence of S9-mix.

Data represents means ± SEM of at least two independent experiments

Concentration (µl/ml)	*R. officinalis* (REO)		*S. somalensis* (SEO)		*T. vulgaris* (TEO)		*A. millefolium* (AEO)		*H. italicum* (HEO)		*P. lentiscus* (PEO)		*M. communis* (MEO)	
	3h-S9	3h+S9	3h-S9	3h+S9	3h-S9	3h+S9	3h-S9	3h+S9	3h-S9	3h+S9	3h-S9	3h+S9	3h-S9	3h+S9
A														
0.025	5.25±1.44	4.50±0.74		3±0.90	2.75±0.77	2.50±0.94							3.50±0.69	3.00±1.14
0.05	3.75±1.01	3.00±0.74	2.38±0.71	2.75±0.90	1.5±0.54	3.50±0.94	2.50±0.90	4.50±0.91	2.13±0.58	4.25±0.80	2.25±0.49	3.50±0.90	1.50±0.60	4.50±1.14
0.08														
0.1	4.38±1.01	3.50±0.74	3.44±0.67	2.25±0.90	2.33±0.63	4.25±0.94	4.18±0.77	3.00±0.91			1.08±0.40	1.63±0.64	1.75±0.60	2.75±1.14
0.15							3.25±0.90	4.25±0.91	1.75±0.82	1.75±0.80				
0.2			2.00±1.01						1.00±1.16	3.00±1.13	2.25±0.69	3.00±1.28	1.00±1.19	
DMSO (0.01%)	2.00±0.68	2.83±0.61	2.00±0.47	2.13±0.64	2.42±0.44	2.75±0.66	2.00±0.60	2.13±0.64	1.44±0.41	2.00±0.66	1.57±0.37	2.00±0.64	1.88±0.42	2.75±0.81
NOC (0.15 µg/ml)	37.85±0.46[c]		36.90±0.56[c]		39.57±0.61[c]		39.98±0.60[c]		38.12±0.54[c]		36.28±0.78[c]		38.12±0.59[c]	
CP (45 µg/ml)		29.64±0.30[c]		31.11±0.37[c]		30.06±0.35[c]		31.91±0.33[c]		30.68±0.32[c]		31.29±0.37[c]		30.78±0.34[c]
B														
0.025	8.50±1.41[b]	15.5±1.13[b]		7.50±1.44	6.25±2.25	4.75±1.59							9.67±1.91	5.50±2.75
0.05	6.75±1.00[a]	11.00±1.13[a]	7.88±1.49[a]	6.25±1.44	5.38±1.59	9.00±1.59	6.88±1.33	9.75±1.21	7.13±0.95[b]	8.00±1.04[b]	4.38±0.73	5.50±1.10	5.50±1.66	11.50±2.75
0.08														
0.1	9.13±1.00[b]	10.75±1.13[a]	9.11±1.40[b]	7.50±1.44	8.67±1.84	13.5±1.59[b]	8.00±1.13[b]	10.75±1.21[a]			3.33±0.60	3.25±0.78	4.75±1.66	6.75±2.75
0.15							9.5±1.33[b]	12.75±1.21[b]	6.25±1.35	4.25±1.04				
0.2			9.25±2.11[a]						4.00±1.91	7.00±1.47	4.25±1.03	4.00±1.55		
DMSO (0.01%)	3.50±0.66	5.67±0.92	3.33±1.00	5.67±1.18	4.25±1.30	5.00±1.12	3.33±0.88	5.67±0.99	2.56±0.68	3.50±0.74	2.57±0.55	3.50±0.78	3.88±1.17	5.00±1.94
MMC (0.2 µg/ml)	44.13±0.49[c]		41.90±0.60[c]		41.75±0.55[c]		45.23±0.50[c]		44.15±0.60[c]		42.73±0.70[c]		43.32±0.55[c]	
CP (45 µg/ml)		38.98±1.00[c]		38.00±0.82[c]		42.60±0.70[c]		40.02±0.89[c]		36.67±0.98[c]		39.16±0.53[c]		39.24±0.82[c]

NOC: nocodazole, MMC: mytomicin C, CP: cyclophosphamide.

[a],significantly different (p < 0.05, Dunnett test) from control cultures (DMSO, 0.01%).

[b],significantly different (p < 0.01, Dunnett test) from control cultures (DMSO, 0.01%).

[c],significantly different (p < 0.001, Dunnett test) from control cultures (DMSO, 0.01%).

3.3 Cytotoxicity in A2780 cell line

The results of the cytotoxicity assay in the human ovarian carcinoma cell line A2780 are shown in Figure 1. With the exception of SEO which was ineffective at all the tested doses (less than 10% at 1.0 µl/ml), the others EOs significantly reduced survival of the cancer cells. In particular, the most active extracts were HEO, MEO and PEO, which caused about a 90% toxicity at 0.1 µl/ml. Interestingly, cell proliferation was seen to drastically drop (50%) for the three extracts when their concentration increased from 0.01 to 0.05 µl/ml. Compared to the previous EOs, REO resulted moderately toxic, as the sharp decrease in cell survival was observed approximately at 0.5 µl/ml. Regarding TEO and AEOs, cytotoxicity against the cell line increased slightly, as at 1.0 µl/ml (maximum dose tested) about 34% and more than 50% of cells were alive, respectively. Approximately 90% of the cells treated with SEO proliferated at this concentration.
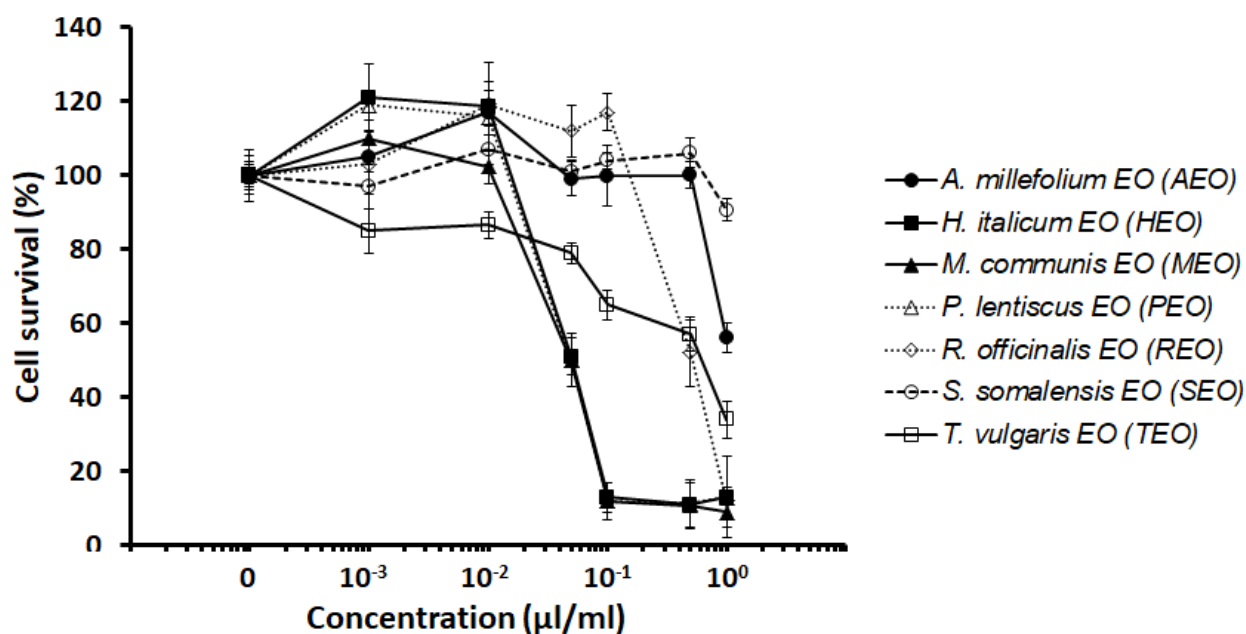

Figure 1. Evaluation of EOs cytotoxicity in the human ovarian carcinoma cell line A2780. Cells were exposed for 24 h to different concentrations of EOs (from 0.001 µl/ml to 1.0 µl/ml). Viability was evaluated by tetrazolium-1 assay. Cell survival was expressed as percentage of cell density. Values are the means ± SEM obtained from at least two independent experiments with three replicates each.

The ability to impair proliferation of the cancer cell line by the seven EOs is confirmed by their IC_{50} and IC_{70} values (see Table 6). HEO (39.94 µg/ml and 48.83 µg/ml), PEO (40.04 µg/ml and 42.97 µg/ml) or MEO (40.98 µg/ml and 48.74 µg/ml), in fact, exerted a strong inhibitory activity, whereas REO, TEO, AEO (values much higher than 100 µg/ml) and SEO (values not determined) did not.

Table 6. Effective concentrations inhibiting proliferation of the A2780 cancer cell line by 50% (IC_{50}) or 70% (IC_{70}) for the seven EOs.

Extract	IC_{50}[a] (µg/ml)	IC_{70}[a] (µg/ml)
H. italicum (HEO)	39.94	48.83
P. lentiscus (PEO)	40.04	42.97
M. communis (MEO)	40.98	48.74
R. officinalis (REO)	426.1	459.7
T. vulgaris (TEO)	621.2	> 1000
A. millefolium (AEO)	999.4	> 1000
S. somalensis (SEO)	not determined	not determined

[a]Values are calculated according to the concentrations reported in Supplementary Table 1.

3.4 *In vitro* estrogenic/antiestrogenic activity

All EOs were found to be cytotoxic on *S. cerevisiae* cells at the highest concentration tested (0.1 µl/ml), markedly inhibiting the yeast cell growth in both the agonistic and antagonistic assay. TEO and HEO strongly inhibited cell growth even at 0.1 µl/ml in the estrogenic assay, whereas in the antagonistic assay, toxicity at this dose was observed only for HEO (data not shown). At the lower concentration, PEO, REO and SEO showed a slight increase in cell proliferation, although not

significant (data not shown). The majority of EOs tested showed no estrogenic activity in the estrogen-responsive yeast screen. As shown in Figure 2, TEO and PEO showed weak estrogenic activity (higher than 20% of the control) (maximum β-gal activity $28.5 \pm 1.3\%$ and $29.3 \pm 1.5\%$ of E_2, respectively) whereas MEO showed a positive activity only at lower concentrations. PEO showed a dose-dependent increase in estrogenic activity with a maximum of β-gal expression at 0.001 µl/ml concentration. When EOs were tested for their ability to inhibit β-galactosidase expression induced by 1nM E_2, all the samples showed a dose-dependent antagonistic activity (Figure 3). HEO, SEO and AEO exerted the highest antiestrogenic activity. SEO reached a maximum inhibition of 90.7% at 0.1 µl/ml, while AEO and HEO reduced the E_2-mediated activity by 77.2% and 75.0%, at 0.1 µm/ml and 0.01 µl/ml, respectively.

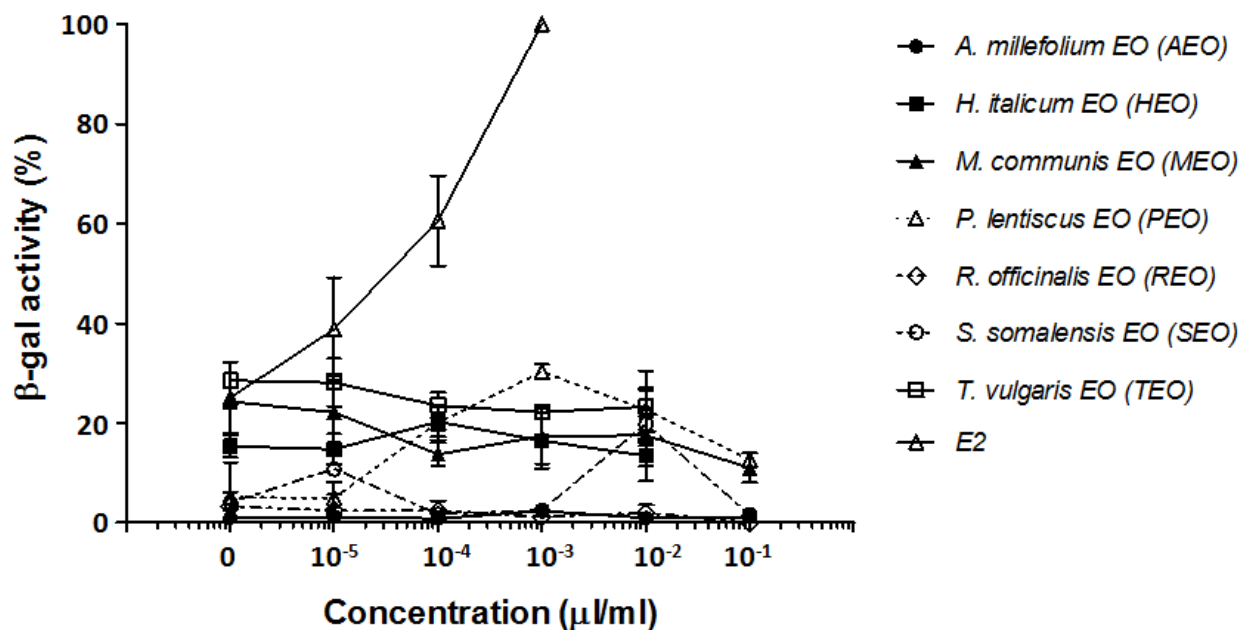**Figure 2.** Evaluation of EOs agonistic activity on the human estrogen receptor ERα. Yeast strains expressing the human ERα were incubated with different concentrations of EOs (from 0.00001µl/ml to 0.1 µl/ml) or with 10 nM E_2. Results are expressed as percentage of the β-gal activity induced by E_2 (100%). Values are the means ± SEM obtained from at least two independent experiments with three replicates each.
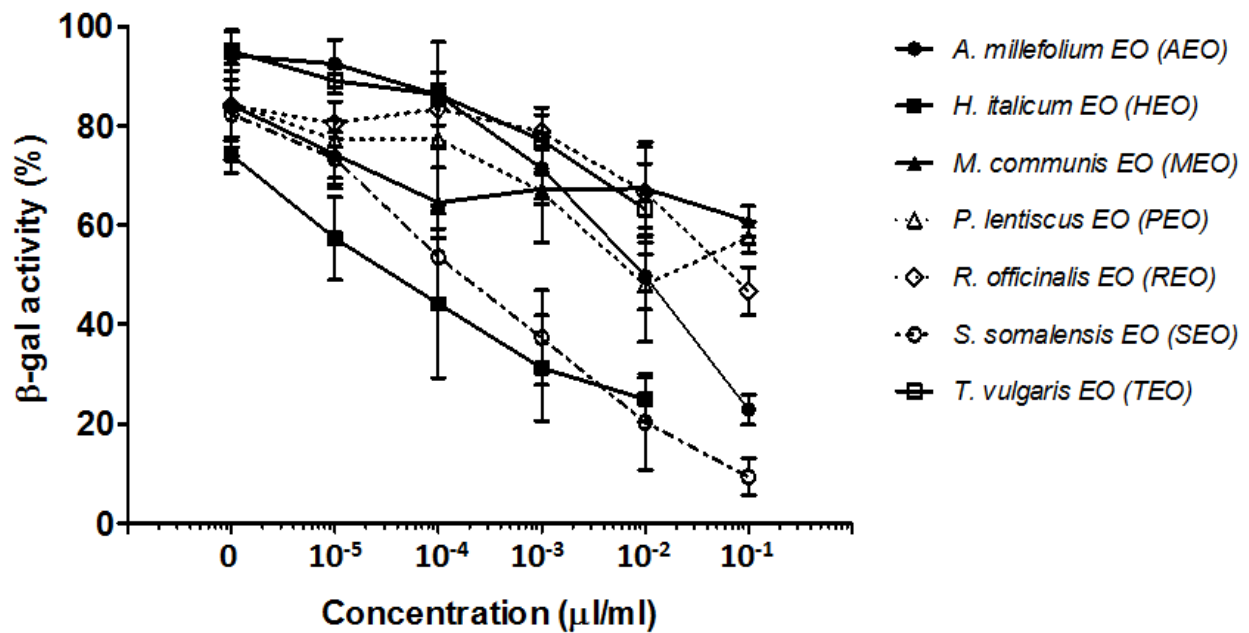
Figure 3. Evaluation of EOs inhibitory activity on the human estrogen receptor ERα. Yeast strains expressing the human ERα were co-incubated with different concentrations of EOs (from 0.00001µl/ml to 0.1 µl/ml) and 1 nM E_2. Results are expressed as percentage of the β-gal activity induced by E_2 alone (100%). Values are the means ± SEM obtained from at least two independent experiments with three replicates each.

4.   Discussion

EOs are natural compounds present in cosmetics and widely used in the food industry, mainly as dietary supplements, which are also known to show pharmaceutical and therapeutic potential. Thus, it is important to understand their biological activity, especially in light of the impact they can exert on human health. This work analyzed the cytotoxicity, genotoxicity and estrogenic activity of seven essential oils extracted from aromatics plants endemic to Somalia (SEO) or the Mediterranean area (REO, TEO, AEO, PEO, HEO, MEO). Concerning the cytostatic and cytotoxic activity, the results presented in this work indicate that all EOs affect, in a dose-dependent manner, proliferation and viability of human peripheral lymphocytes. Interestingly, a strong decrease in cell proliferation was

observed for the majority of EOs (REO, TEO, AEO, HEO and MEO) also in presence of the exogenous metabolizing system. It is well known that the functioning of enzymes involved in the xenobiotic metabolism, depending on the cell type, can lead to the production of harmless or toxic metabolites. Thus, our results suggest that some components of EOs are converted into cytotoxic metabolite(s).

Only few works characterized the effect of EOs on the proliferation of a healthy cell system such as immune cells. For example, Rivas da Silva *et al.* reported cytotoxicity of α-pinene, the main component of REO and to a minor extent of PEO, on murine macrophages (Silva et al., 2012). Similarly, thymol, the main component of TEO, induced a dose-dependent decrease of proliferation in human peripheral lymphocytes (Buyukleyla and Rencuzogullari, 2009). The essential oils of palmarosa, citronella, lemongrass and vetiver showed a dose-dependent cytotoxic activity in human lymphocytes, arguably linked to the induction of oxidative stress and apoptosis (Sinha et al., 2014). Some of the toxic effect observed by these authors were correlated to the presence of citral, one of the major components of lemongrass essential oil, whereas geraniol, the major component of palmarosa and citronella essential oils did not show any toxicity. Regarding the correlation between cytotoxicity of EOs and their chemical composition, it has been shown that such activity is greater for the total EOs compared to the individual compounds, highlighting the synergy between the different components (Wang et al., 2012).

Regarding genotoxic activity, this work showed that the seven EOs tested induced an increase in MN frequencies of cultured human peripheral lymphocytes in binucleated cells and, to a lesser extent , also in mononucleated cells. The strongest activity was observed when cells were exposed to EOs for 48 h (extended treatment). The observed effects can be attributed to the presence of the predominant compounds in the EOs mixtures that were previously proved to possess clastogenic, aneugenic or both activities. For example, thymol (52.61% in TEO) was demonstrated to be a genotoxic compound in rat bone marrow cells where it induced structural and numerical chromosomal aberrations (Azirak and Rencuzogullari, 2008). Similar results were obtained on

human peripheral lymphocytes where thymol increases the frequency of chromosomal aberrations, sister chromatid exchanges (SCEs) and MN in binucleated cells (Buyukleyla and Rencuzogullari, 2009). Another compound, 1,8-cineole (also known as eucalyptol), which was detected in appreciable amount in REO, AEO and MEO), is a monoterpene epoxide capable of inducing cytotoxicity and genotoxicity through oxidative damage (*e.g.* formation of 8-oxiguanine) in the colorectal cancer cells HCT116 (Dörsam *et al*., 2015). Nikolic *et al*. (2011) also showed that this compound induces DNA breaks in Vero cells. A third example is represented by (αβ)-pinene (both compounds present at a concentration $\geq 5\%$ in REO, PEO or AEO), a bicyclic monoterpene, which was showed to compromise genomic stability either by altering the mitotic spindle causing chromosome malsegregation or by producing ROS in Chinese hamster V79-C13 cells (Catanzaro *et al*., 2012). Finally, limonene, which is present in all of the tested EOs, caused nuclear abnormalities in V79 Chinese hamster cells (Mauro *et al.* (2013).

However, some of the compounds detected in our EOs are reported not to cause adverse effects. For example, β-myrcene (35.99% in PEO) did not induced chromosomal aberrations and SCEs in human lymphocytes (Kauderer et al., 1991), had no clastogenic activity in mouse bone marrow cells (Zamith et al., 1993), and did not cause genotoxic damage in Hep2G (human hepatoma) and NC-NC (human β-lymphoid cell) cells (Mitić-Ćulafić et al., 2009). Rather, this compound had the ability to reduce the DNA damage induced by tert-butyl hydroperoxide. Furthermore, some compounds were shown to induce an hormetic response in several human cell lines, thus acting as anti-mutagenic or genotoxic agents at low or high concentrations, respectively. This is the case of camphor (20.93% in SEO, 7.57% in REO and 7.44% in AEO), 1,8-cineole (Nikolić *et al*., 2015, 2011), and limonene, which was able to reduce the genotoxic and oxidative damage induced by cadmium in human peripheral lymphocytes (Verma *et al*., 2019).

Regarding the formation of compounds active against the genetic material, as the endogenous metabolic activity of peripheral lymphocytes is barely expressed, treatment of the cells with or without an exogenous metabolizing system (S9-mix) allowed us to infer about the presence of direct

or indirect mutagens. The genotoxic activity detected in cultures treated with PEO and SEO without S9-mix alone indicated the presence of direct mutagens. On the contrary, TEO showed pro-mutagenic activity as it induced MN in cultures received also metabolic activation. We suggest that thymol, the predominant compound detected in TEO might be responsible of the observed genotoxic effect. In fact, thymol can be obtained from p-cymene (the two compounds differ in a meta-OH group), and the presence of a metabolizing system would facilitate this conversion (Bagamboula *et al.*, 2004). In the case of HEO, we observed an equivalent genotoxic effect in both conditions, this suggesting the presence of direct-acting mutagenic metabolite(s) which are not detoxified by S9-mix. The genotoxic damage induced by AEO and REO in cultures supplemented with exogenous metabolic activation was higher than that obtained without S9-mix, thus indicating the presence of both direct and indirect mutagens. As aneugens are generally considered direct-acting compounds, all EOs did not increase the basal levels of MMN when peripheral lymphocytes were co-treated with the exogenous metabolizing system. Collectively, we can hypothesize that the moderate genotoxic activity observed in the present work can be due to a synergistic, additive and/or competitive effect of the various terpenes contained in the EOs, rather than to the action of a single component.

In the context of tumor cells, HEO, PEO and MEO caused a strong and dose-dependent inhibition of cell proliferation in the human ovarian carcinoma cell line A2780. According to the ISO 10993-5 guidelines (2009), these extracts are to be considered cytotoxic agents, as they caused 70% of cell inhibition at concentration well below the cut-off value of 100 µg/ml, while the remaining EOs resulted ineffective against this cancer cells. So far, most of the literature dealing with cytotoxicity of EOs points to their antiproliferative activity on human tumor cell lines. For example, the EO of *P. lentiscus* was able to induce apoptosis in thyroid carcinoma cell lines but not in healthy fibroblasts (Catalani et al., 2017). The antiproliferative activity towards different tumor cell lines (MDA-MB231, A375 and HCT116 from adenocarcinoma, malignant melanoma and colon cancer,

respectively) has also been demonstrated for the essential oil of *Helichrysum* (Ornano et al., 2014), and of *R. officinalis* (Jardak et al., 2017; Melušová et al., 2014; Wang et al., 2012).

Attention has mainly focused on identifying chemopreventive or chemoactive phytochemicals that could be used in complementary therapies as chemotherapeutic agents for breast cancers unresponsive to endocrine treatment (Hoai *et al*., 2015) or to manage side effects of estrogen replacement therapy (ERT) used in cancer and neurodegenerative disease treatment (Howes *et al*., 2002; Simões *et al*., 2018). Indeed, EOs and their constituents are also being recognized as able to exhibit either weak estrogenic or anti-estrogenic activity both *in vivo* and *in vitro* (Bartoňková and Dvořák, 2018; Howes et al., 2002; Simões et al., 2018). The ability to interact with these receptors can results as "endocrine disruptors". The results of the present study suggest that all the tested EOs showed a weak or no estrogenic activity, while SEO, HEO and AEO displayed appreciable antiestrogenic activity. Analogous findings were obtained for extracts of pollens from two Mediterranean species (*Salix alba* L. and *Cystus incanus* L.) that were found to be effective estrogen inhibitors (Pinto et al., 2010).

In conclusion, this study showed that the EOs analyzed can be considered strong cytotoxic agents characterized by a slight or moderate genotoxic activity for peripheral human lymphocytes. Some EOs exhibit a marked antiestrogenic activity that could potentially perturb the estrogen-dependent tissues, showing also cytotoxicity against the human cancer cell line A2780. These results confirm literature data indicating that some compounds of EOs are known for their anticancer effects.

This work can provide a useful contribution to better delineate the biological activities of essential oils against human cells and to direct future studies towards the identification of more specific synergies between terpenes and their molecular targets. On the other hand, due to their growing use in the cosmetic and medicinal fields (e.g. massages and aromatherapy) (Bagetta *et al*., 2015; Scuteri *et al*., 2017), it would be advisable to investigate whether or not these compounds show genotoxic/cytotoxic activity.

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