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Cytotoxic Activity of Essential Oils of Aerial Parts and Ripe Fruits of *Echinophora spinosa* (Apiaceae)

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The cytotoxic effects of the essential oils obtained from the flowering aerial parts (APO) and ripe fruits (RFO) of *Echinophora spinosa* L. (Apiaceae) from central Italy toward human U937 promonocytoid cells were studied; the contribution of each of the major constituents to the whole cytotoxic activity of either APO or RFO was also characterized. The major components of APO were β-phellandrene (34.7%), myristicin (16.5%), *p*-cymene (16.3%), δ^3-carene (12.6%), α-pinene (6.7%) and α-phellandrene (6.2%); those of RFO *p*-cymene (50.2%), myristicin (15.3%), α-pinene (15.1%) and α-phellandrene (8.1%). Both oils tested were toxic to U937 cells, but RFO was much more cytotoxic: indeed, the IC_{50} values calculated from the linear regression curves of RFO and APO were 14.5 ± 0.85 and 43.4 ± 2.81 μg/mL, respectively. α-Pinene and α-phellandrene were identified as the most toxically relevant constituents: however, they did not completely account for the toxic effects of genuine APO and RFO. Interestingly, we found that *p*-cymene, although *per se* devoid of toxicity within the tested range of concentrations, was capable of significantly sensitizing U937 cells to the cytotoxic activity of α-pinene and α-phellandrene, and that specific mixtures of these three terpenes were as toxic as genuine APO and RFO.

Keywords: Apoptosis, Cytotoxicity, *Echinophora spinosa*, Essential oil, *p*-Cymene, U937 cells.

Echinophora spinosa L. (Apiaceae) is an herbaceous perennial plant from the Mediterranean region, growing mainly on maritime sands. The plant is edible with a pleasant taste: thornless young and tender leaves are used for salads and the roots as carrots [1]. The literature shows very few studies of this species; one concerned the alleged content of phytosterols in the roots [2], and much more recently Kubeczka *et al.* [3] characterized, for the first time, the chemical composition of the essential oils of *E. spinosa* of unspecified origin. They reported that the main constituents of root oil were terpinolene (77.2%), myristicin (9.1%), limonene (4.3%) and falcarinol (2.9%), a widespread acetylenic compound in Apiaceae, while those of the aerial parts were α-phellandrene (36.8%), *p*-cymene (27.3%), α-pinene (15.0%), β-phellandrene (6.9%), limonene (2.6%) and cosmene (5.0%). Subsequently, only two works - namely those of Glamoclija *et al.* [4] and Fraternale *et al.* [5] - dealt with the biologically relevant effects of the essential oils of this plant, reporting their chemical composition and antimicrobial activity against some human and animal pathogens (bacteria and fungi). Indeed, several plants of the genus *Echinophora* are used in folk medicine to heal wounds and to treat gastric ulcers [6]. Interestingly, over the last decade, some essential oils from plants belonging to the Apiaceae have been tested for their cytotoxicity against tumor cell lines [7-10]. *Ridolfia segetum* and *Oenanthe crocata* oils were cytotoxic toward K562 cells [7]; the oil of *Bupleurum marginatum*, a herb indigenous to southern and southwestern China, exhibited a strong cytotoxicity to different cancer cell lines (CCRF, CEM, HePG2) [8]; *Prangos asperula* oil was active against human renal adenocarcinoma [9]; the essential oils from the fruits of four different chemotypes of *Angelica archangelica* growing in Iceland were shown to exert cytotoxic activity toward PANC-1 human pancreas cells [10]. Interestingly, with *A. archangelica*, this last study reported that the oils from two very similar chemotypes showed a markedly different cytotoxic activity [10], a finding suggesting that the cytotoxic capacity was independent of the specific amount of their main constituents, which were very similar in these two oils. In an attempt to interpret these data, the authors hypothesized that more components act together, synergistically or cumulatively. Such a hypothesis implies that, with regard to the cytotoxic activity, complex interactions may occur between the constituents of a given essential oil.

Since the essential oils from some Apiaceae plants seem to have some potential in anticancer therapy, we investigated the cytotoxic activity of the hydrodistilled APO and RFO of *Echinophora spinosa* collected in central Italy on the beach (Fano-Marche) of the Adriatic sea, as well as that of their major components.

The composition of these essential oils has been reported in a recent work [5] and is shown in Table 1. Twenty-three and twenty-six compounds, representing 98.7% of the total in both cases, were identified in APO and RFO oils, respectively. The major compound in APO is β-phellandrene (34.7%), followed by myristicin (16.5%), *p*-cymene (16.3%), δ^3-carene (12.6%), α-pinene (6.7%) and α-phellandrene (6.2%), while the major compound in RFO is *p*-cymene (50.2%), followed by myristicin (15.3%), α-pinene (15.1%) and α-phellandrene (8.1%);

Both oils were toxic to human promonocytoid U937 cells (Figure1A-1B): 48 h exposure to increasing concentrations of APO caused a marked decrease of U937 cell survival and, under the same conditions, RFO was much more cytotoxic. Indeed, the IC_{50} values calculated from the linear regression curves of RFO and APO cytotoxicity were 14.5 ± 0.85 and 43.4 ± 2.81 μg/mL, respectively. It is worth noting that the numbers of viable cells in samples treated for 48 h with the highest doses of RFO ($9.2 \pm 1.11 / 10^{-4}$) and APO ($1.42 \pm 0.13 / 10^{-5}$) were lower as compared to those at the beginning of the treatment stage ($4.0/10^{-5}$): such an extensive cell demise suggests that high doses of both oils are capable of causing cell death in treated cultures, rather than a simple growth arrest. The increased release of LDH, which depends on cell membrane disruption and might be suggestive of cell necrosis, in cultures exposed to the highest concentration (25 μg/mL) of RFO for 48 h (165.6% ± 7.2 as compared with control cells) strengthens this hypothesis. Using the Fast Halo Assay (FHA) [11] we next tested

the type of cell death, i.e. apoptosis *vs* necrosis, induced by APO (not shown) or RFO (Figure 1D) in U937 cells. Using FHA, apoptotic cells, unlike viable ones, are characterized by the presence of large DNA halos surrounding small nuclear remnants: this shape can be appreciated in Figure 1E, showing cells exposed to staurosporine, a non DNA-damaging, apoptogenic compound, included as a positive control; these halos cannot be observed in either RFO (Fig 1D) or APO (not shown) intoxicated cells. Thus, the absence of apoptotic shapes, the strong reduction of viable cells' number (Figure 1A and B), the increased LDH release in RFO- and APO- treated cells led us to the conclusion that cell death was largely dependent on necrotic processes, at least under the exposure paradigm used throughout this study.

The three fold difference between the IC_{50}s of the two oils prompted us to search for the component(s) of RFO responsible for its higher cytotoxic activity. The compositions of RFO and APO (Table 1) indicate that the major differences between the two oils refer to *p*-cymene (50.2% in RFO *vs* 16.3% in APO), α-pinene (15.1% *vs* 6.7%), α-phellandrene (8.1% *vs* 6.2%) and β-myrcene (2.1% in RFO, absent in APO). On the contrary, higher concentrations of δ^3-carene, β-phellandrene and β-pinene can be found in APO (12.6, 34.7 and 2.6%, respectively) compared with RFO (1.1, 1.1 and 0.2%); the amounts of other relevant compounds, such as myristicin and α-phellandrene are similar in both oils. Thus we focused on *p*-cymene, α-pinene, α-phellandrene, and β-myrcene as the most likely candidates for the higher cytotoxic potency of RFO. Our experimental approach consisted of preparing "reconstituted oils" containing the same percentages present in RFO of each – or of mixtures – of the above compounds in dimethylsulfoxide (DMSO) used as vehicle (for the sake of clarity, the detailed composition of the reconstituted oils used in the experiments shown in Figure 2 is given in Table 2). We then evaluated the cytotoxic activity of these reconstituted oils toward U937 cells: the mixtures were given to cells at concentrations equivalent to those of RFO (see Figure 1) and using identical exposure conditions (the concentrations in Fig. 1 and 2 are expressed as µg of the mixtures per mL of culture medium). The first tested mixture was that containing 50.2% *p*-cymene in DMSO: indeed *p*-cymene, the most abundant constituent of RFO, could likely represent the more toxic compound. However, *p*-cymene (up to the same concentration corresponding to that present in the higher dose tested of RFO, i.e. equivalent to 12.5 µg/mL of *p*-cymene) was devoid of any cytotoxic effect on U937 cells (not shown). This finding is not surprising because a very recent study by Ferraz *et al*. [12] reported IC_{50} values for the cytotoxic activity of *p*-cymene towards various cultured cell lines always higher than those of the concentrations of *p*-cymene tested herein.

Also, β-myrcene (2.1% in DMSO) did not affect U937 cells' survival (not shown): accordingly, Tavares *et al.* [13] reported that the essential oil of *Distichoselinum tenuifolium*, containing up to 79% myrcene, is non-toxic to cultured human macrophages. Interestingly, unlike the above two terpenes, α-pinene (15.1% in DMSO) caused a dose dependent inhibition of U937 cell survival (Figure 2A); however, α-pinene/DMSO cytotoxic activity was far lower than that of the genuine RFO.

α-Phellandrene was then tested (8.1% in DMSO): notably, α-phellandrene inhibited the cell survival in a dose dependent fashion (Figure 2B), but, again, to an extent lower than that of genuine RFO. Other relevant compounds possibly contributing - by virtue of their high concentrations in both oils - to the toxicity of RFO and APO, such as δ^3-carene and myristicin, were tested for cytotoxicity up to the same concentrations as those present in the higher dose tested of both oils. Doses up to 75 µg/mL of a mixture of 16.5% myristicin/83.5% DMSO or 25 µg/mL of 12.5% δ^3-carene/87.5% DMSO were not toxic to U937 cells (not shown). As to myristicin, this finding is in keeping with the weak cytotoxic profile reported by Martins *et al*. [14] and Lee *et al.* [15] in EM9 and SK-N-SH cells, respectively. As to δ^3-carene, not all literature reports are in keeping with ours: indeed this terpene was reported as non-toxic by Gminski *et al*. [16] and Jeong *et al*. [17], but significantly cytotoxic by Johansson *et al*. [18]. However, it is worth noting that these data were obtained in experimental systems radically different from ours, in such a way that a direct comparison can hardly be drawn.

On the whole, the results illustrated so far would suggest that α-pinene and α-phellandrene are likely to represent the compounds directly responsible for RFO cytotoxicity.

Thus we mixed α-pinene (15.1%) and α-phellandrene (8.1%) in DMSO (76.8%), and treated U937 cells with increasing concentrations of this reconstituted oil. Unexpectedly, the cytotoxic effect of this mixture was still significantly lower than that of genuine RFO (Figure 2C). This would indicate that, although α-pinene and α-phellandrene are the most toxic components, they fail to resemble the cytotoxic activity of genuine RFO which, consequently, should also depend on further compounds. Then we tested a mixture containing 15.1% α-pinene, 8.1% α-phellandrene, 50.2% *p*-cymene and DMSO (26.6%), as vehicle: surprisingly, the toxicity of this mixture was virtually superimposable on that of genuine RFO (Figure 2D). In fact, addition of *p*-cymene caused a shift in the IC_{50} value from 24.8 ± 1.05 to 13.7 ± 0.64 µg/mL, i.e. a nearly doubled sensitivity of U937 cells (compare Figure 2 C and D). Such a finding would indicate that, although *p*-cymene is *per se* non-toxic in our system, it significantly sensitizes cells to the toxicity of α-pinene and α-phellandrene.

To see whether α-pinene and α-phellandrene were both, or not, susceptible to the sensitizing effect of *p*-cymene, we tested the cytotoxicity of α-pinene (15.1%) and α-phellandrene (8.1%) alone and in combination with *p*-cymene (50.2%). Results shown in Figure 2A and B indicate that *p*-cymene sensitizes cells to the toxicity of α-pinene and α-phellandrene. Finally, addition of δ^3-carene, myrcene or myristicin to reconstituted APO or RFO, both without *p*-cymene, did not affect their cytotoxic activity toward U937 cells (not shown), a finding strengthening the peculiarity of the *p*-cymene sensitizing effect, at least in the oils tested herein.

The effect of adding increasing concentrations of *p*-cymene on the cytotoxicity induced by the mixture of 15.1% α-pinene, 8.1% α-phellandrene in DMSO (i.e. the same concentrations of these two terpenes in genuine RFO) is shown in Figure 3A. As expected (Figure 3B), *p*-cymene dose-dependently augmented the cytotoxicity of the reconstituted oil containing the same concentrations of α-pinene and α-phellandrene as APO (6.7 and 6.2%, respectively). The concentrations of *p*-cymene capable of doubling the sensitivity of U937 cells to the cytotoxicity caused by 17.5 µg/mL of the "*p*-cymene-free, RFO-like" mixture in Figure3A or by 25.0 µg/mL of the "*p*-cymene-free, APO-like" mixture in Figure3B were 8.95 ± 0.66 and 12.3 ± 1.05 µg/mL, respectively. Notably, addition of 50.0 or 15.0 %, w/w, of *p*-cymene to the mixtures shown in Figure 3A or B, caused a cell demise remarkably similar to that induced by 17.5 µg/mL of genuine RFO or 25 µg/mL of genuine APO, respectively (compare Figure 3A and B with Figure 1A and B).

To the best of our knowledge, this is the first report dealing with the cytotoxic activity of the hydrodistilled essential oils (from ripe

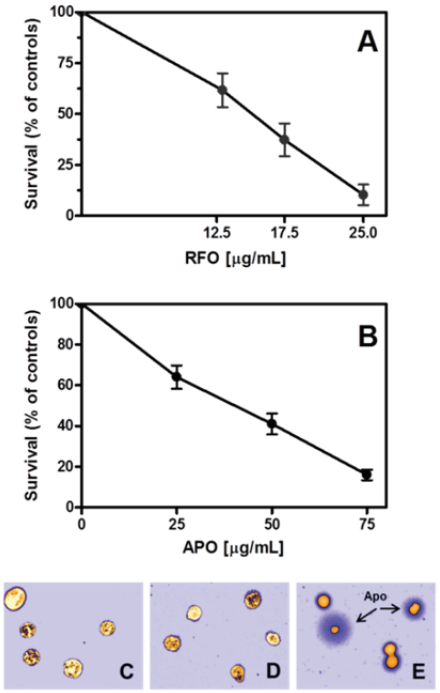

Figure 1: Cytotoxic effect of increasing concentrations of the essential oils of *E. spinosa* extracted from aerial parts and ripe fruits on U937 cell growth. U937 were treated with essential oil of either ripe fruits (**A,** 12.5, 17.5 and 25µg/mL, RFO) or of aerial parts (**B,** 25, 50 and 75 µg/mL APO) for 48 h in culture medium. The cytotoxic response was then determined with the trypan blue exclusion assay. Results represent the means ± SEM from at least 5 separate experiments.*p<0,05, **$p \leq$ 0,01, ***$p \leq$0,001 as compared with respective controls (one-way ANOVA). Also shown are representative, digitally pseudocolored micrographs of Giemsa-stained cells analysed with the fast halo assay to determine the presence of apoptotic cells. (**C**) control cells, (**D**) 25 µg/mL RFO for 24 h, (**E**) 5 µM staurosporine for 3 h and post-incubated in drug-free medium for a further 21 h. Arrows indicate apoptotic cells in panel **E**.

fruits and aerial parts) of *E. spinosa* with a specific regard to their composition. Experiments aimed at determining the relative contribution of each of the major compounds, namely α-pinene, α-phellandrene, *p*-cymene and β-myrcene to the cytotoxic activity of RFO and APO indicate that α-pinene and α-phellandrene were the toxic species. This finding is in keeping with others dealing with the cytotoxic activity of essential oils from different plants. For example, α-pinene has been reported as a cytotoxic monoterpene contributing to the antiproliferative activity of various essential oils [19]. The essential oils of *Salvia leriifolia* Benth. and *S. acetabulosa* L. were rich in α-pinene (4.7% and 52.3% respectively). Both oils exerted a strong antiproliferative activity against COR-L23 cells, and *S. acetabulosa* was also found to possess cytotoxic activity against renal adenocarcinoma [20].

High amounts of α-pinene were also found in *Platycladus orientalis* essential oil, which exerted an interesting activity on renal adenocarcinoma cells [9]. Previous studies also reported antitumor activity of some *Thuja* species, and some isolated constituents, including α- and β-pinene [21,22]. In agreement with the antiproliferative activity shown by pinene on different tumor cell lines, the bioguided fractionation of the dichloromethane extract of *Tilia x viridis* flowers yielded a fraction rich in limonene, α-pinene and β-pinene, which presented antiproliferative action on tumor lymphocytes and each of these monoterpenes alone, including α- and β-pinene, exhibited this activity [23]. Finally, Lampronti *et al*. [24] found that α-pinene inhibited K562 cell growth with an IC_{50} of 117.3 ±14.4 µM, along with α-terpineol, caryophyllene and carvacrol, three compounds not found in the RFO tested herein.

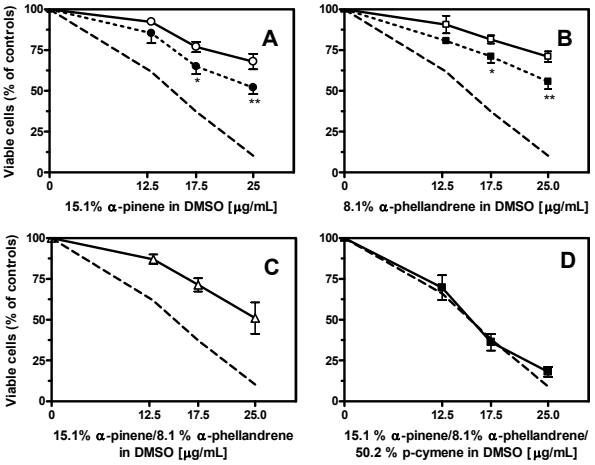

Figure 2: Cytotoxic effect of the major compounds of RFO on U937 cell growth. U937 cells were treated for 48 h with 12, 17.5 and 25 µg/mL of reconstituted oils containing 15.1% α-pinene with (closed circles) or without (open circles) 50.2% *p*-cymene (**A**); 8.1% α-phellandrene with (closed squares) or without (open squares) 50.2% *p*-cymene (**B**); 15.1% α-pinene/8.1% α-phellandrene in DMSO (**C**); or 15.1% α-pinene/8.1% α-phellandrene/50.2% *p*-cymene (**D**). All mixtures were adjusted to 100%, w/w, adding proper amounts of DMSO; for the sake of clarity the quantitative composition of each of the above reconstituted oils is detailed in Table 2. The cytotoxic response was determined with the trypan blue exclusion assay. In each panel the cytotoxic effect of the reconstituted oil is compared with that of genuine RFO (striped line). Results represent the means ± SEM from at least 5 separate experiments. * $p \leq 0.05$ and ** $p \leq$ 0.01 compared with either α-pinene or α-phellandrene without *p*-cymene (panels A and B).

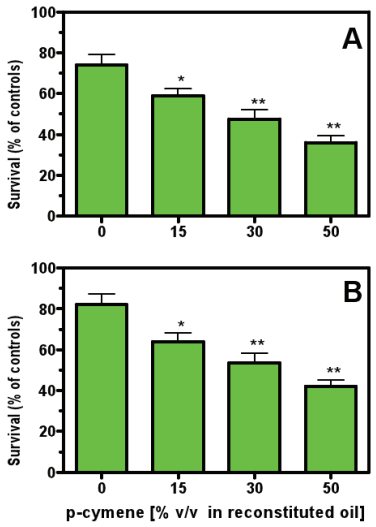

Figure 3: Effect of increasing concentrations of *p*-cymene on the cytotoxic effect of mixtures of α-pinene and α-phellandrene. U937 cells were treated for 48 h with 17.5 µg/mL of a mixture of 15.1% α-pinene, 8.1% α-phellandrene (the same as in RFO) (A) or 25 µg/mL of 6.7% α-pinene, 6.2% α-phellandrene (the same as in APO) (B), both supplemented with the indicated concentrations of *p*-cymene. All mixtures were adjusted to 100%, w/w, adding proper amounts of DMSO. The cytotoxic response was then determined with the trypan blue exclusion assay. Results represent the means ±SEM from at least 5 separate experiments. *$p \leq 0.05$ and **$p \leq 0.01$ compared with the reconstituted oil devoid of *p*-cymene (one-way ANOVA followed by Dunnett's *post hoc*).

To the best of our knowledge there is no literature data describing the cytotoxic activity of pure α-phellandrene towards cultured human cells, and this is the first study demonstrating that it inhibits the growth of cultured human cells, namely U937 promonocytoid cells. However, α-phellandrene represents one of the major

Table 1: Constituents identified in essential oils of flowering aerial parts and ripe fruits of *E. spinosa* L.

Compound	RI	% APO	% RFO
Tujene	916	0.2	0.2
α-Pinene	931	6.7	15.1
Verbenone	966	0.1	0.1
Sabinene	970	0.1	0.1
β-Pinene	981	2.6	0.2
β-Myrcene	988	-	2.1
α-Phellandrene	1005	6.2	8.1
δ^3-Carene	1010	12.6	1.1
α-Terpinene	1016	0.3	0.5
p-Cymene	1026	16.3	50.2
β-Phellandrene	1029	34.7	1.1
cis-β-Ocimene	1040	0.1	0.1
trans-β-Ocimene	1049	0.1	0.3
γ-Terpinene	1059	-	0.1
Terpinolene	1085	1.1	1.4
Perillene	1102	0.1	0.1
Trans-Sabinol	1119	0.1	0.2
Eucarvone	1150	0.1	0.1
m-Cymen-8-ol	1181	0.1	0.3
p-Cimen-8-ol	1185	0.1	0.2
Verbenone	1207	0.2	0.4
β-Elemene	1389	0.1	0.2
γ-Muurolene	1440	0.1	0.2
α-Selinene	1495	0.2	0.4
δ-Cadinene	1516	-	0.6
Myristicin	1520	16.5	15.3

RI= Retention Indices relative to C_7-C_{25} *n*- alkanes on Polar CP-Sil 88 column. APO (flowering aerial parts essential oil). RFO (ripe fruit essential oil).

Table 2: Composition of the reconstituted oils utilized in the experiments shown in Figure2 A-D.

Figure 2 panel	α-pinene*	β-phellandrene*	*p*-cymene*	DMSO*[§]
A (open circles)	3.77	-	-	21.23
A (closed circles)	3.77	-	12.5	8.73
B (open squares)	-	2.02	-	22.98
B (closed squares)	-	2.02	12.5	10.48
C	3.77	2.02	-	19.21
D	3.77	2.02	12.5	6.71

* Values are expressed in μg/mL and are referred to the final concentration of 25.0 μg/mL of each reconstituted oil in treatment medium.
[§] DMSO was neither cytotoxic nor cytostatic *per se* at any of the concentrations used.

constituents of a number of cytotoxic essential oils such as those from *Myrica gale* L., *Schinus molle* L., *S. terebinthifolius*, *Solanum erianthum* and *S. macranthum* [25-27], a notion which strengthens the role of this terpene in the toxic activity of RFO and APO studied herein. Also, both oils tested, in particular RFO, exhibit cytotoxicity that could deserve future research and exploitation. However, the most attractive finding of our study is the peculiar effect of *p*-cymene: in fact, this terpene displays no toxicity within the tested range of concentrations, but is capable of sensitizing target cells to the cytotoxic activity of the other constituents, namely α-pinene and α-phellandrene. Although further studies will be needed to unravel the mechanism of this peculiar effect of *p*-cymene, the fact that more compounds within the same essential oil act together, cooperatively, synergistically or cumulatively, is a notion that has been recognized and discussed elsewhere [10,28]. In particular, Buhagiar *et al.* [28] concluded that "*The cytotoxic activity of the essential oils was independent of the quantity of their main components.", i.e. that the net toxicity of an essential oil does not necessarily coincide with the simple sum of the intrinsic toxicity of each active component, but rather depends on positive (or negative) interactions occurring at specific proportions*." Essential oils are known to change the fluidity of membranes, which become abnormally permeable resulting in leakage of radicals, cytochrome C, calcium ions, proteins and cytokines, such as IL-1α, which is reputed as the most relevant trigger of inflammation in this context [22]. Permeabilization of cells and organelle membranes is likely to promote extensive necrotic response, i.e. the type of cell death observed in our study. From a clinical and toxicological point of view, permeabilization of cell membranes and disruption of skin barrier function represent the main events leading to skin irritation and/or favoring the onset of skin allergy upon topical exposure to potentially irritating ingredients used in cosmetics and in aromatherapy, including essential oils [29,30]. Notably, Schmitt *et al.* [31] reported that each single component of complex mixtures of a given essential oil can profoundly affect the skin permeation of the other constituents in a cooperative and hardly predictable fashion. In conclusion, our data raise the question of the safety of using mixtures of different essential oils or essential oil components - *per se* scarcely toxic. but potentially harmful when combined together - in skin care and aromatherapy products. Thus, more attention should be paid in designing mixtures of different essential oils or essential oil components in the formulation of the above products and, as a final remark, our data might raise the issue of the safety evaluation of essential oil mixtures and of their legislative regulation.

Experimental

Chemicals and reagents: Reagent grade chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Cell culture media, sera, trypsin and antibiotics were from Cambrex Corporate (East Rutherford, NJ, USA). *p*-Cymene, β-myrcene, α-phellandrene and α-pinene were from Sigma Aldrich (St.Louis, MO, USA).

Plant materials: Flowering aerial parts of *Echinophora spinosa* L. were collected during the flowering stage in August 2011 and the ripe fruits in October 2011 from "Riserva Floristica Baia del Re" Fano (PU)- Marche region-Italy. The samples were identified by Dr Daniele Fraternale and voucher specimens, ESP09 and ESF09 respectively for aerial parts (branches, leaves and flowers) and ripe fruits, were deposited in the Botanical Garden Herbarium of the University of Urbino "Carlo Bo". Fresh plant materials were chopped and subjected to hydrodistillation for 3 h using a Clevenger apparatus to obtain the essential oils from aerial parts or from ripe fruits, and subsequently they were dried over anhydrous sodium sulfate and, after filtration, stored at 4°C until analysis and use in the biological tests. The oil yields were 0.83%, v/w, for the aerial parts and 1.13%, v/w, for the ripe fruits.

Identification of essential oil constituents: Essential oils from flowering aerial parts: branches, leaves and flowers (APO) and ripe fruits (RFO) were analyzed by GC and GC/MS [5].

Cell culture and treatment conditions: U937 human promonocytoid cells were grown in RPMI-1640 medium supplemented with antibiotics (50 units/mL penicillin and 50μg/mL streptomycin), 1.2 mM glutamine and 10% fetal bovine serum in an atmosphere of 95% air and 5% CO_2 at 37°C. For experiments, U937 cells were resuspended at a number of $4.0/10^{-5}$ cells/treatment ($2.0/10^{-5}$ cells/mL) in 35 mm culture plastic dishes. Cells were treated in culture medium with increasing concentrations of both essential oils and incubated for 48 h. To increase their solubility in water, essential oils were diluted 1:1 in DMSO. At the used concentrations, DMSO was not cytotoxic *per se*. In some experiments, cells were treated with selected components – alone or in mixture – of APO or RFO at the same concentrations produced by dissolving each of the essential oils in culture medium for their cytotoxicity testing.

Cytotoxicity studies: In all experiments the cytotoxicity was determined after 48 h of treatment with APO, RFO or of selected constituents with the trypan blue exclusion assay or with the lactic dehydrogenase (LDH) release assay. Trypan blue assay: an aliquot of cell suspension was diluted 1:1 with 0.4% trypan blue and the cells were counted with a hemocytometer. Results are expressed as

''percent survival'' that is the percent ratio between viable (unstained) cells in treated and untreated samples. LDH release assay: samples were centrifuged at 250 x *g* for 5 min and the activity of LDH was detected spectrophotometrically in the supernatants by monitoring at 340 nm the disappearance of NADH during LDH-catalysed conversion of pyruvate to lactate [32].

Detection of apoptotic cells with the fast halo assay (FHA): Cells were treated with APO, RFO or reconstituted oils for 24 h and then assayed for apoptosis using FHA. FHA was conducted under non denaturing conditions, pH 10.1 [11]. Giemsa stained nuclei were visualized with a fluorescence microscope at 200X. Resulting images were digitally acquired, processed and pseudocolored with ImageJ freeware (http://rsb.info.nih.gov/nih-image/); representative micrographs are shown in Fig 1C-E.

Statistical analysis: The results are expressed as mean values ± SEM from 3 to 6 determinations. The effects of treatments were tested using one-way ANOVA analysis and Dunnett's test (Figure 3) as *post hoc*. The significance threshold was set to p=0.05.

References

[1] http://www.actaplantarum.org/floraitalie/viewtopic.php?t=985

[2] Tarbouriech LJ, Hardy J. (**1909**) A phytosterol from *Echinophora spinosa* L. *Bulletin of Science Pharmacological*, ***14***, 387-392

[3] Kubeczka KH. (**1982**) Chemical investigations of essential oils of umbellifers. *World Crops: Production, Utilization, Description, (Aromatic Plants)*, *7*, 165-173

[4] Glamoclija JM, Sokovic MD, Siljegavic JD, Ristic MS, Ciric AD, Grubisic DV. (**2011**) Chemical composition and antimicrobial activity of *Echinophora spinosa* L. (Apiaceae) essential oil. *Records of Natural Products*, ***5***, 319-323.

[5] Fraternale D, Genovese S, Ricci D. (**2013**) Essential oil composition and antimicrobial activity of aerial parts and ripe fruits of *Echinophora spinosa* L. (Apiaceae) from Italy. *Natural Product Communications*, ***8***, 527-530.

[6] Baytop T. (**1984**) *Therapy with medicinal plants in Turkei (past and present).* Istambul University Publication No. 3255, Istambul, Turkei.

[7] Bicchi C, Rubiolo P, Ballero M, Sanna C, Matteodo M, Esposito F, Zinzula L, Tramantano E. **(2009**) HIV-1-inhibiting activity of the essential oil of *Ridolfia segetum* and *Oenanthe crocata*. *Planta Medica*, ***75***, 1331-1335.

[8] Ashour ML, El-Readi M, Youns M, Mulyaningsih S, Sporer F, Efferth T, Wink M. (**2009**) Chemical composition and biological activity of the essential oil obtained from *Bupleurum marginatum* (Apiaceae). *Journal of Pharmacy and Pharmacology*, ***61***, 1079-1087.

[9] Loizzo MR, Tundis R, Menichini F, Saab AM, Statti GA, Menichini F. (**2008**) Antiproliferative effects of essential oils and their major constituents in human renal adenocarcinoma and amelanotic melanoma cells. *Cell Proliferation*, ***41***, 1002-1012.

[10] Sigurdsson S, Ogmundsdottir HM, Gudbjarnason S. (**2005**) The cytotoxic effect of two chemotypes of essential oils from the fruits of *Angelica archangelica* L. *Anticancer Research*, ***25***, 1877-1880.

[11] Sestili P, Martinelli C, Stocchi V. (**2006**) The fast halo assay: An improved method to quantify genomic DNA strand breakage at the single-cell level. *Mutation Research - Genetic Toxicology and Environmental Mutagenesis*, ***607***, 205–214.

[12] Ferraz RPC, Bomfim DS, Carvalho NC, Soares MBP, da Silva TB, Machado WJ, Prata APN, Costa EV, Moraes VRS, Nogueira PCL, Bezerra DP. (**2013**) Cytotoxic effect of leaf essential oil of *Lippia gracilis* Schauer (Verbenaceae). *Phytomedicine*, ***20***, 615-621.

[13] Tavares AC, Goncalves MJ, Cruz MT, Cavaleiro C, Lopes MC, Canhoto J, Salgueiro LR. (**2010**) Essential oils from *Distichoselinum tenuifolium*: chemical composition, cytotoxicity, antifungal and anti-inflammatory properties. *Journal of Ethnopharmacology*, ***130***, 593-598.

[14] Martins C, Doran C, Laires A, Rueff J, Rodrigues AS. (**2011**) Genotoxic and apoptotic activities of the food flavourings myristicin and eugenol in AA8 and XRCC1 deficient EM9 cells. *Food and Chemical Toxicology*, ***49***, 385-392.

[15] Lee BK, Kim JH, Jung JW, Choi JW, Han ES, Lee SH, Ko KH, Ryu JH. (**2005**) Myristicin-induced neurotoxicity in human neuroblastoma SK-N-SH cells. *Toxicology Letters*, ***157***, 49-56.

[16] Gminski R, Tang T, Mersch-Sundermann V. (**2010**) Cytotoxicity and genotoxicity in human lung epithelial A549 cells caused by airborne volatile organic compounds emitted from pine wood and oriented strand boards. *Toxicology Letters*, ***196***, 33-41.

[17] Jeong JG, Kim YS, Min YK, Kim SH. (**2008**) Low concentration of 3-carene stimulates the differentiation of mouse osteoblastic MC3T3-E1 subclone 4 cells. *Phytotherapy Research*, ***22***, 18-22.

[18] Johansson A, Lundborg M. (**1997**) Effects of low concentrations of 3-carene on alveolar macrophages *in vitro*. *Toxicology*, ***120***, 99-104.

[19] Wang W, Li N, Luo M, Zu Y, Efferth T. (**2012**) Antibacterial activity and anticancer activity of *Rosmarinus officinalis* L. essential oil compared to that of its main components. *Molecules*, ***17***, 2704-2713.

[20] Loizzo MR, Menichini F, Tundis R, Bonesi M, Nadjafi F, Saab AM, Frega NG, Menichini F. (**2010**) Comparative chemical composition and antiproliferative activity of aerial parts of *Salvia leerifolia* Benth and *Salvia acetabulosa* L. essential oils against human tumor cell *in vitro* models. *Journal of Medicinal Food*, ***13***, 62-69.

[21] Iwamoto M, Minami T, Tokuda H, Ohtsu H, Tanaka R. (**2003**) Potential antitumor promoting diterpenoids from the stem bark of *Thuja standishii*. *Planta Medica*, ***69***, 69-72.

[22] Sunila ES, Kuttan G, (**2006**) A preliminary study on antimetastatic activity of *Thuja occidentalis* L. in mice model. *Immunopharmacology Immunotoxicology*, ***28***, 269-280.

[23] Manuele MG, Ferraro G, Anesini G. (**2008**) Effect of *Tilia x viridis* flower extract on the proliferation of a lymphoma cell line and on normal murine lymphocytes: contribution of monoterpenes, especially limonene. *Phytotherapy Research*, ***22***, 1520-1526.

[24] Lampronti I, Saab AM, Gambari R. (**2006**) Antiproliferative activity of essential oil derived from plants belonging to the Magnoliophyta division. *International Journal of Oncology*, ***29***, 989-995.

[25] Sylvestre M, Legault J, Dufour D, Pichette A. (**2005**) Chemical composition and anticancer activity of leaf essential oil of *Myrica gale* L.. *Phytomedicine*, ***12***, 299-304.

[26] Bendaoud H, Romdhane M, Souchard JP, Cazaux S, Bouajila J. (**2010**) Chemical composition and anticancer and antioxidant activities of *Schinus molle* L. and *Schinus terebinthifolius* Raddi berries essential oils. *Journal of Food Sciences*, ***75***, 466-472.

[27] Essien EE, Ogunwande IA, Setzer WN, Ekundayo O. (**2012**) Chemical composition, antimicrobial, and cytotoxicity studies on *S. erianthum* and *S. macranthum* essential oils. *Pharmaceutical Biology*, ***50***, 474-480.

[28] Buhagiar JA, Podesta MT, Wilson AP, Micallef MJ, Ali S. (**1999**) The induction of apoptosis in human melanoma, breast and ovarian cancer cell lines using an essential oil extract from the conifer *Tetraclinis articulata*. *Anticancer Research*, ***19***, 5435-5443.

[29] Welss, T, Basketter DA, Schröder KR. (**2004**) *In vitro* skin irritation: facts and future. State of the art review of mechanisms and models. *Toxicology in Vitro*, ***18***, 231-43.

[30] Bakkali F, Averbeck S, Averbeck D, Idaomar M. (**2008**) Biological effects of essential oils-a review. *Food and Chemical Toxicology*, ***46***, 446-475.

[31] Schmitt S, Schaefer UF, Doebler L, Reichling J. (**2009**) Cooperative interaction of monoterpenes and phenylpropanoids on the *in vitro* human skin permeation of complex composed essential oils. *Planta Medica*, ***75***, 1381-1385.

[32] Beutler E. (**1975**) *Red Cell Metabolism, a Manual of Biochemical Methods*, 3rd ed. Grune and Stratton, New York.