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Encapsulation of Hemp (*Cannabis sativa* L.) Essential Oils into Nanoemulsions for Potential Therapeutic Applications: Assessment of Cytotoxicological Profile

Cristina Aguzzi [1,†], Diego Romano Perinelli [2,†], Marco Cespi [2], Laura Zeppa [1], Eugenia Mazzara [2], Filippo Maggi [2], Riccardo Petrelli [2], Giulia Bonacucina [2,*] and Massimo Nabissi [1,*]

[1] Department of Experimental Medicine, School of Pharmacy, University of Camerino, Via Madonna delle Carceri 9, 62032, Camerino, Italy

[2] Chemistry Interdisciplinary Project (ChIP) Research Center, School of Pharmacy, University of Camerino, Via Madonna delle Carceri, 62032, Camerino, Italy

* Correspondence: giulia.bonacucina@unicam.it; massimo.nabissi@unicam.it

† These authors equally contributed .

Abstract: Industrial hemp (*Cannabis sativa* L.), due to its bioactive compounds (terpenes and cannabinoids), has gained an increasing interest in different fields, including medical purposes. The evaluation of the safety profile of hemp essential oil (EO) and its encapsulated form (nanoemulsion, NE) is a relevant aspect for potential therapeutic applications. This study aimed to evaluate the toxicological effect of hemp EOs and NEs from cultivars Carmagnola CS and Uso 31, on three cell lines selected as models for topical and inhalant administration, by evaluating the cytotoxicity and the cytokine expression profiles. Results show that EOs and their NEs have comparable cytotoxicity, if considering the quantity of EO present in the NE. Moreover, cells treated with EOs and NEs showed, in most of the cases, lower levels of pro-inflammatory cytokines compared to Etoposide used as positive control, and the basal level of inflammatory cytokines was not altered, suggesting a safety profile of hemp EOs and their NEs to support their use for medical applications.

Keywords: MTT assay; cell toxicity; cytokine gene expression; nanoencapsulation; dynamic light scattering; topical formulation

1. Introduction

In recent years, the essential oil (EO) from industrial hemp (*Cannabis sativa* L.) has received a great scientific and commercial attention as a valuable resource potentially exploited in different fields [1,2]. Hemp EO is predominantly composed of monoterpenes (α-pinene, myrcene, and terpinolene) and sesquiterpenes (α-humulene, and caryophyllene), with a lower content of the cannabinoid fraction, among which cannabidiol (CBD) generally represents the main component [3,4]. Therefore, hemp EO configures as a non-psychotropic natural product endowed with plenty of biological activities (e.g., insecticidal, parasiticidal, antimicrobial, and anti-inflammatory) which make it attractive for agrochemical, cosmeceutical, nutraceutical, and pharmaceutical applications [5–7]. Regarding human health, hemp EO is reported to exert several benefits related to the pharmacological actions of the single compounds or the entire phytocomplex (cannabinoids and terpenes), which include immunomodulatory, anticonvulsant, anxiolytic, neuroprotective, spasmolytic, immunomodulatory, anti-inflammatory, and anticancer effects [8–10]. The use of hemp EO in topical formulations as a functional ingredient is constantly increasing [11]. Indeed, hemp EO has been largely investigated for its beneficial effect on inflammatory skin diseases, such as acne, seborrhoea, dermatitis, and psoriasis [12,13]. As a primary anatomical barrier, skin is continuously subjected to a variety of external physical stimuli, responding through the modulation of specific pathways. Keratinocytes represent the epidermis's main cellular population, and they are involved in activating and maintaining inflammation and immunological responses [14,15]. Fibroblasts, in the

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epidermis, are involved in the wound healing process, and act synergistically with keratinocytes in maintaining homeostatic conditions [16]. Keratinocytes and fibroblasts release several proinflammatory mediators, including interleukin-1 beta (IL-1 beta), IL-6, IL-8, and tumor necrosis factor-alpha (TNF alpha), under inflammatory stimuli. Traditionally, several plants' EOs have long-time been used in medicine as expectorant, bronchodilator, anti-inflammatory, antiviral, antibacterial, and antiseptic agents, mainly in the treatment of the upper respiratory system affections [17,18]. More recently, CBD-enriched extracts have been demonstrated to have an immunomodulatory effect on pulmonary cell lines due to a downregulation of the cytokine storm or a modulation of gene expression, which can be beneficial for the treatment of lung inflammatory chronic diseases [19,20]. Anyway, due to the poor water solubility and the possibility of chemical degradation and biological inactivation of hemp EO into the environment, effective and safe formulations for the administration and delivery to the target are required. An efficient strategy to overcome these issues is offered by the encapsulation into nanoemulsions (NEs) [21,22]. According to these premises, the main objective of this study was to compare the cytotoxicity of pure hemp EOs and NEs from the cultivars Carmagnola CS and Uso 31 and their effect on inflammation markers. Male inflorescences of the dioecious variety Carmagnola CS usually represent a waste of hemp product processing, and for this reason they were chosen for this work in order to exploit their still underestimated potential. These studies were carried out in human keratinocytes, fibroblasts, and bronchial cell lines, selected as a model for a topical administration on the skin or for a pulmonary vehiculation of hemp EO-based nanoformulations.

2. Results

2.1. EOs chemical composition

Both hemp inflorescences provided a comparable yield in terms of the amount of the EO obtained after the extraction procedure. Specifically, Uso 31 and Carmagnola CS yielded 0.18% and 0.20% w/w of EO on a dry weight basis, respectively. The determined density values were also similar, being 0.884 and 0.896 g/mL for Uso 31 and Carmagnola CS EO, respectively. Table 1 contains the main chemical constituents of the two EOs, in terms of relative abundance with respect to the whole composition, which is reported in Table S1 in the Supplementary material. In the case of Uso 31 EO, the predominant compound was the sesquiterpene (E)-caryophyllene (25.93%), followed by the monoterpene myrcene (15.28%). Other components present in significant percentages were especially α-pinene (11.07%), α-humulene (8.92%), caryophyllene oxide (7.23%), and (E)-β-ocimene (6.71%). On the other hand, the monoterpene myrcene dominated in Carmagnola CS EO, accounting for 37.57% of the chemical profile. The second most abundant constituent was (E)-caryophyllene (16.99%), followed by α-pinene (13.45%), and α-humulene (6.11%). The content of the other detected compounds was under 6.0%. Specifically, compounds of the cannabinoid fraction (including CBD) are present in the EOs only at trace levels.

Table 1. Main chemical constituents of Uso 31 and Carmagnola CS EOs.

Component[a]	RI Calc.[b]	RI lit[c]	% Uso 31	% Carmagnola CS
α-pinene	926	932	11.07	13.45
β-pinene	968	974	3.59	5.45
myrcene	989	988	15.28	37.57
limonene	1025	1024	1.59	5.37
(*E*)-β-ocimene	1047	1044	6.71	1.79
terpinolene	1085	1086	5.46	2.97
(*E*)-caryophyllene	1409	1417	25.93	16.99
α-humulene	1443	1452	8.92	6.11
caryophyllene oxide	1571	1582	7.23	2.95

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Total identified (%)	99.03	97.72
Monoterpene hydrocarbons (%)	44.24	67.27
Oxygenated monoterpenes (%)		0.43
Sesquiterpene hydrocarbons (%)	45.31	26.18
Oxygenated sesquiterpenes (%)	9.04	3.58
Cannabinoids (%)	0.43	0.23

[a] Order of compounds according to their elution from HP-5MS column. [b] Linear retention index calculated with a mixture of n-alkanes (C_8–C_{30}) with respect to HP-5MS column. [c] Retention index for non-polar columns taken from ADAMS library.

2.2. Characterization and physical stability over time of C. sativa EO-based NEs

A preliminary screening was carried out to select the best C. sativa EO – based NE formulation in terms of total oil (EO plus ethyl oleate) incorporation (%) and surfactant (polysorbate 80) concentration (%) to have a mean droplet size (Z-average, diameter) and PDI lower than 200 nm and 0.3, respectively. The addition of ethyl oleate in the formulation was required for a better solubilization of the C. sativa EO, thereby ensuring a longer stability time as reported in a previous work [21]. As illustrated in Figure 1A, the particle size distribution of the prepared 5% w/w Uso 31 EO-based NE and 6% w/w Carmagnola CS EO-based NE is below 1000 nm with the maximum centered at around 100-150 nm. A slightly larger width of the droplet distribution (PDI values 0.27 vs. 0.24, respectively) and a slightly higher Z-average value (131 nm vs. 117 nm) was determined for Carmagnola CS EO-based NE with respect to the Uso 31-based NE, thereby reflecting the different percentage of the incorporated EO (6% w/w vs. 5% w/w). Both NEs did not display any macroscopic physical instability phenomena (e.g., creaming or phase separation) over the observation time up to six months. Moreover, Z-average and PDI values remained for both NEs in the range between 110-160 nm and 0.24-0.27, respectively, indicating the good stability at room temperature of the nanosystems, without appreciable variation of the droplet size of the internal oil phase (Figure 1B,C).

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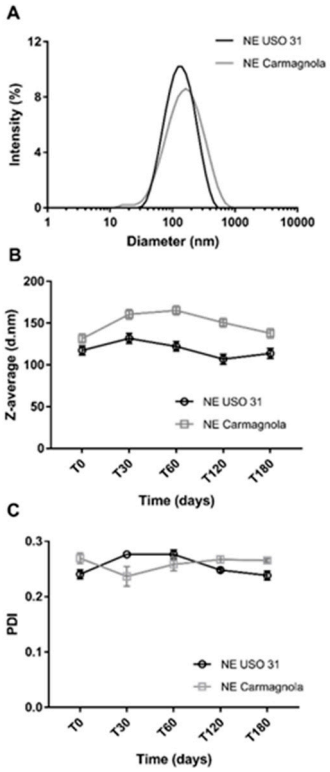

Figure 1. Particle size distribution by Intensity (%) as recorded by DLS for Uso 31 EO and Carmagnola CS EO-based NEs after preparation (A) and variation of Z-average (diameter, d., nm) (B) and polidispersity index (PDI) (C) values as measured over time up to six months.

2.3. Evaluation of C. sativa EO-based NEs safety profile in human cell lines

The safety profile of C. sativa EO-based NEs was investigated on human keratinocytes (HaCaT), human fibroblasts (NHF A12), and human bronchial cell (HBEpC) lines, to assess at which concentrations these nanoformulations can be administered topically on the skin or by inhalation testing HBEpC as bronchial model. As such, cells were treated with different dilutions (%v/v) of C. sativa EO-based NEs and pure EOs for comparison. Results show that HBEpC cells were more sensitive to all formulations showing lower viability values (%) than HaCaT and NHF A12 both for the pure EOs and NEs (Figure 2). Notably, C. sativa EO-based NEs induced a lower cytotoxic effect compared to C. sativa EOs for all tested cell lines, since the two NEs contain only 5% or 6% w/w EO with respect to their total composition, while the main constituent is water. A much lower cytotoxic effect than C. sativa EO-based NEs has been observed for control NEs against all tested cell lines. Specifically, the residual effect on cell viability for controls can be mainly attributed to the presence of polysorbate 80 used as an emulsifier in the formulations.

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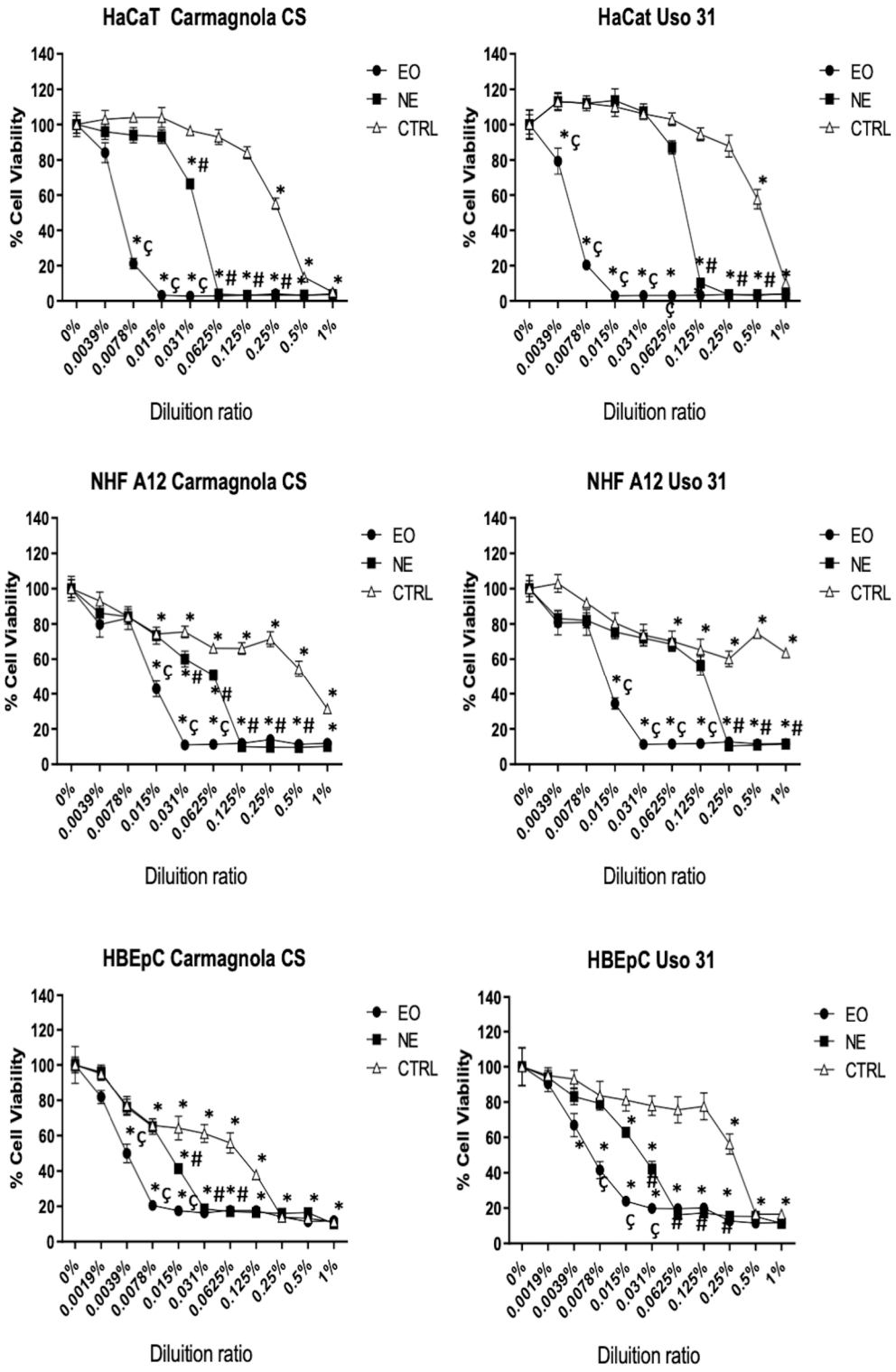

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Figure 2. Cell cytotoxicity of Uso 31 EO and Carmagnola CS EO-based NEs in HaCaT, NHF A12 and HBEpC cell lines was determined by MTT assay. All cell lines were treated with different dilutions and cell viability was evaluated after 72 h post-treatment. $^{*}p < 0.05$ vs vehicle, $^{\#}p < 0.05$ *C. sativa* EO-based NEs vs CTRL, $^{\varphi}p < 0.05$ *C. sativa* EO-based NEs vs *C. sativa* EO.

A thorough comparison between C. sativa EO-based NEs and the corresponding EOs can be performed by calculating the IC_{50} values (Table 2). Similar IC_{50} values were determined for the two C. sativa EOs against the three tested cell lines, highlighting that the two hemp varieties exert the same cytotoxic effect. Regarding NE formulations, their IC_{50} values were much higher than those of the corresponding pure EOs against the same cell lines, as expected from the composition of the formulation. Nevertheless, for a direct comparison of the cytotoxic effect exerted by NEs with that of pure EOs, IC_{50} values of NEs after normalization by the percentage of EO contained in the formulation are reported in brackets in Table 2. The normalized IC_{50} values for NEs are slightly lower but still comparable to those calculated for pure EOs, suggesting that the incorporation of the EOs in the formulations does not markedly affect the cytotoxic profile of C. sativa EOs. On the other hand, the slight difference in IC_{50} of the NEs can be ascribed to the different percentages of the other two components as polysorbate 80 and ethyl oleate. Indeed, Carmagnola CS NE, formulated with a 3% w/w of polysorbate 80, and 2% w/w of ethyl oleate showed lower IC_{50} values than Uso 31 NE containing, instead 2% w/w of polysorbate 80, and 1% w/w of ethyl oleate. The possible effect exerted by polysorbate 80 and ethyl oleate on cytotoxicity is also evident by comparing the IC_{50} values of the two control NEs.

Table 2. Calculated IC_{50} values (mg mL^{-1}) for Carmagnola CS and Uso 31 EO and NE in comparison with control NE against different cell lines (HaCaT, NHF A12 and HBEpC).

	IC_{50} mg mL^{-1}		
	HaCaT	NHF A12	HBEpC
Carmagnola CS EO	0.052 ± 0.002	0.119 ± 0.009	0.034 ± 0.002
Carmagnola CS NE	0.354 ± 0.018 (0.021± 0.001)	0.448 ± 0.020 (0.023± 0.001)	0.154 ± 0.010 (0.009± 0.001)
CTRL Carmagnola CS NE	2.606 ± 0.050	9.666 ± 0.600	1.221 ± 0.500
Uso 31 EO	0.049 ± 0.002	0.103 ± 0.008	0.041 ± 0.003
Uso 31 NE	0.857 ± 0.030 (0.043± 0.002)	1.505 ± 0.058 (0.075 ± 0.003)	0.245 ± 0.010 (0.012 ± 0.001)
CTRL Uso 31 NE	5.388 ± 0.100	>> 10.04	3.255 ± 0.090

2.4. Evaluation of C. sativa EO-based NEs effect on inflammatory condition of human cell lines

According to the results obtained from the cytotoxic assay, HaCaT, NHF A12 and HBEpC cells were treated for 24 h with 0.0039% (%v/v) of C. sativa EOs and 0.015% (%v/v) of C. sativa EO-based NEs and their respective control. Cells were also treated with ETO 2.5 μM used as a positive control to induces inflammation. The gene expression of pro-inflammatory cytokines, such as IL-1beta, IL-6, TNF-alpha, and STAT3, were measured by RT-PCR. From a general point of view, C. sativa EOs and their NEs did not induce an inflammatory state compared to ETO in all cell types used. In HaCaT cell line, Carmagnola CS EO and Uso 31 EO slightly increased IL-1beta expression, and the effect is partially reduced for C. sativa EO-based NEs. C. sativa EO-based NEs can revert the basal condition as demonstrated especially for IL-6 and TNF-alpha in HaCaT model. Regarding STAT3, C. sativa EOs, under the encapsulated and non-encapsulated form, did not alter their basal expression, (Figure 3).

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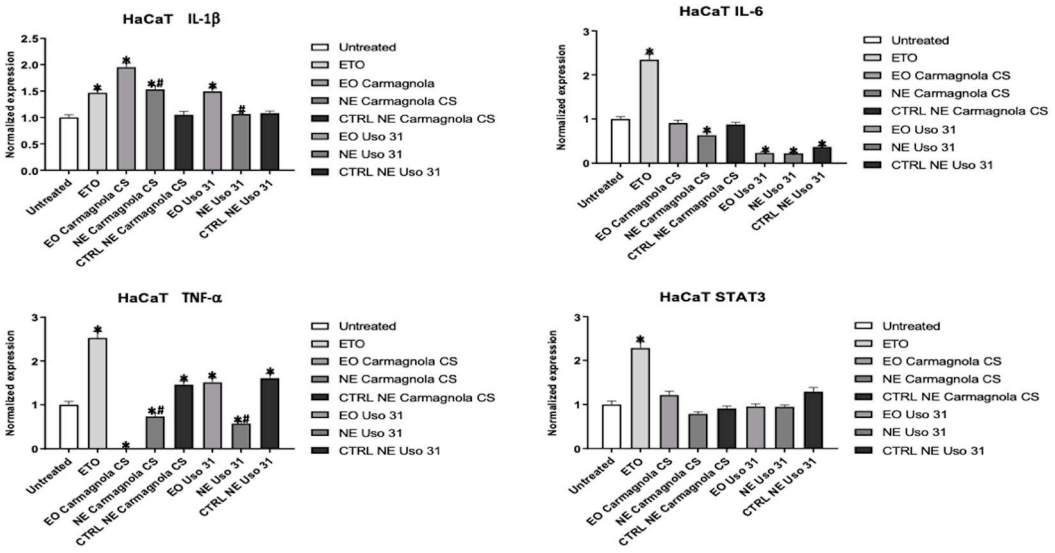

Figure 3. Effect of Uso 31 EO and Carmagnola CS EO-based NEs on the inflammatory state was evaluated in HaCaT cell line. *IL-1beta*, *IL-6*, *TNF-alpha*, and *STAT3* mRNA levels were determined by RT-PCR. Cells were treated with 0.0039% (% v/v) of *C. sativa* EOs and 0.015% (% v/v) of *C. sativa* EO-based NEs and their control, compared with cells treated with ETO 2.5 μM used as a positive control, after 24 h. $^{*}p < 0.05$ vs. untreated, $^{\#}p < 0.05$ *C. sativa* EO-based NEs vs. *C. sativa* EO.

In NHF A12, among all pro-inflammatory genes analyzed, the larger reduction in the expression was observed for TNF-alpha when cells were treated with Uso 31 EO-based NE. Other statistically significant reductions in comparison to the untreated cells and the pure EO were observed for Uso 31 EO-based NE in the case of STAT3 and IL-6 expression. Moreover, a statistically significant reduction of IL-1β expression was observed for Carmagnola CS EO-based NE in comparison to the pure EO (Figure 4).

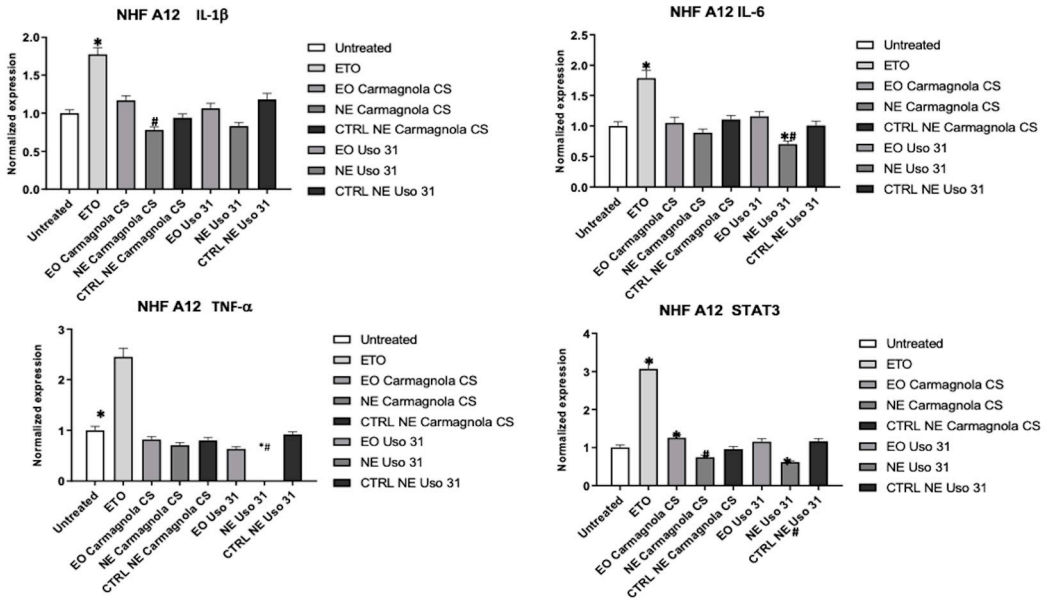

Figure 4. Effect of Uso 31 EO and Carmagnola CS EO-based NEs on inflammatory state was evaluated in NHF A12 cell line. *IL-1beta*, *IL-6*, *TNF-alpha* and *STAT3* mRNA levels were determined by RT-PCR. Cells were treated with 0.0039% (% v/v) of *C. sativa* EOs and 0.015% (% v/v) of *C. sativa* EO-based NEs

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and their control, compared with cells treated with ETO 2.5 µM used as positive control, after 24 h. *p < 0.05 vs. untreated, #p < 0.05 *C. sativa* EO-based NEs vs. *C. sativa* EO.

Similar results were obtained on human bronchial HBEpC cell line in which the basal expression was not hampered by the treatments but, additionally, IL-6 expression was reduced by Carmagnola CS EO-based NE (Figure 5).

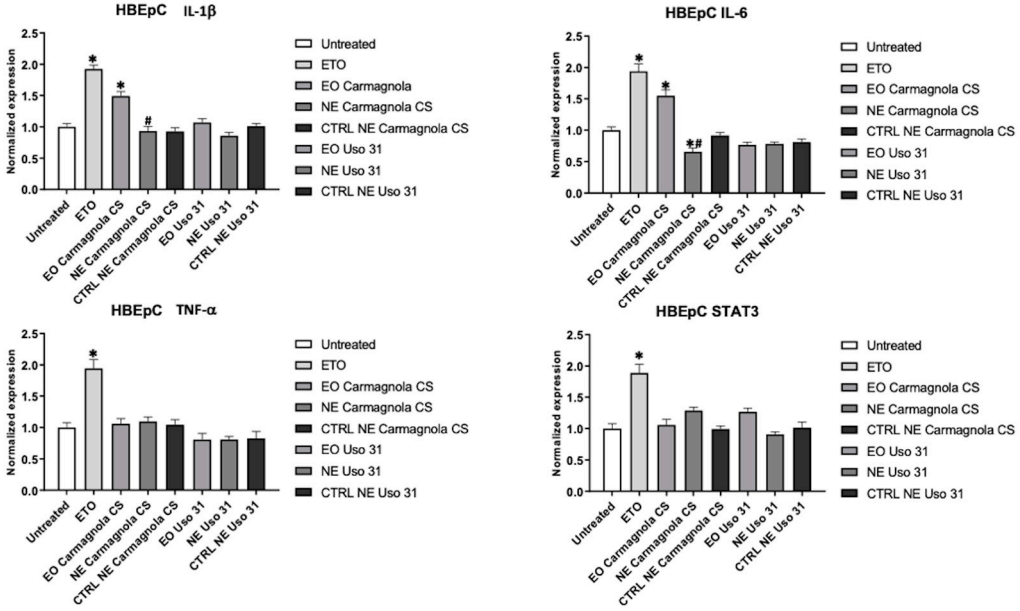

Figure 5. Effect of Uso 31 EO and Carmagnola CS EO-based NEs on inflammatory state was evaluated in HBEpC cell line. *IL-1beta*, *IL-6*, *TNF-alpha* and *STAT3* mRNA levels were determined by RT-PCR. Cells were treated with 0.0039% (% v/v) of *C. sativa* EOs and 0.015% (% v/v) of *C. sativa* EO-based NEs and their control, compared with cells treated with ETO 2.5 µM used as positive control, after 24 h. *p < 0.05 vs. untreated, #p < 0.05 *C. sativa* EO-based NEs vs. *C. sativa* EO.

3. Discussion

The use of *C. sativa* as a medicinal plant has a long history in the field of traditional medicine [27]. Indeed, the medical interest around this botanical species has renewed in the last century thanks to the discovery of phytocannabinoids, which are among the main constituents of cannabis, to which are ascribed the well-known anti-inflammatory, analgesic, myorelaxant, and psychoactive effects [28]. Most of *C. sativa* varieties, known as "industrial hemp", and available for cultivation, are poor in the cannabinoids fraction, but they contain a noticeable fraction of terpenes and terpenoids. These compounds are also recognized to exert several pharmacological activities as antifungal, antiviral, anticancer, anti-inflammatory, anti-hyperglycemic, antiparasitic, and antioxidant [29]. In this work, Carmagnola CS and Uso 31 were selected as "industrial hemp" varieties to extract the EOs which contain more than 95% (according to GC analysis) of terpenes/terpenoids fraction, and only traces of phytocannabinoids. NEs have been formulated to encapsulate these EOs with the aim of investigating the toxicological profile and understanding the impact of their encapsulation in terms of inflammatory cytokines expression. Regarding the chemical composition, quali-quantitative differences in the relative abundances of hemp EOs main constituents can occur, due to several parameters affecting EO profile, such as genetics, cultivation practice, harvesting, storage, and drying conditions [30]. Few studies can be found in the literature regarding the chemical composition of Uso 31 hemp EO [3,31]. By comparing our research work with those by Pieracci et al., 2021 [3], and Ascrizzi et al., 2020 [31], components content variability could depend especially on the different plant material status (fresh in our study, and dry in that by [3]), and distillation method (the innovative MAE here, and the conventional HD in both Ascrizzi et al., 2020 [31], and Pieracci et al., 2021 [3] papers). Similar considerations can be made concerning the EO from Carmagnola CS male

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inflorescences. In our previous work on the steam-distilled EO of the same variety coming from the same hemp farm [21], sesquiterpenes, particularly (*E*)-caryophyllene, were predominant in contrast to the current study. Such differences could be a consequence of the diverse harvesting periods, and extraction techniques. The encapsulation of EOs with potential therapeutic applications could contribute to the development of formulations with a practical use not only by increasing their stability and bioavailability, possibly helping to overcome the drawbacks associated with the occurrence of side effects related to therapy. The incorporation of Carmagnola CS and Uso 31 EOs in the formulated NEs brought to achieve physically stable systems with a mean droplets size in the nanometric range, and a narrow size distribution. Both EOs after encapsulation into NEs showed similar toxicological profiles, compared to pure EOs, toward the investigated cell lines (HaCaT, NHF A12, and HBEpC), as revealed by the normalized IC_{50} values. By considering the not normalized IC_{50} values directly determined from MTT assay, NEs revealed a safety profile from a toxicological point of view. Indeed, the calculated IC_{50} values for NEs (containing 5-6% w/w of EO) were much higher than those from pure EOs despite they are influenced by the amount of EOs and NE composition in terms of content of surfactant (polysorbate 80), and co-solvent (ethyl oleate). To corroborate the safety profile of EOs and their NEs, in the light of potential applications as therapeutics, analysis of inflammatory genes expression profiles on the same cell lines was performed. The results, reported here, are coherent with those previously obtained for *C. sativa* EOs (Felina 32 and male Carmagnola CS varieties) for whom an anti-inflammatory effect on the tested human cell lines was observed [21]. In this study, we confirmed that the EOs and their NEs at the doses tested do not induce an inflammatory condition, in terms of inflammatory genes expression, in comparison to ETO. However, the three cell lines showed different results with respect to EOs and NEs. In general, the encapsulation of the EO leads to a better modulation of the analyzed cytokine levels as it can be observed for Uso 31 EO-based NE, particularly towards HaCaT and NHF A12 cell lines or for *C. sativa* EO-based NEs towards HBEpC cell line. As already observed in previous studies regarding other EOs [32], the encapsulation into nanosystems could potentially improve the safety profile in human cell lines also in terms of cytokine expression profile.

4. Materials and Methods

4.1. Plant material

The monoecious inflorescences of Uso 31, and the male inflorescences of Carmagnola CS hemp varieties were grown by La Biologica farm (Fiuminata, central Italy, 43°10'40" N, 12°56'59" E, 451 m a.s.l.), and harvested at the beginning of August 2020. Then, all the hemp biomass was frozen until use. The moisture content of plant material was evaluated by using a hot air oven (BINDER GmbH, Germany) at 105°C for 24 h, and the average water content was 70.2 and 80.3% for Uso 31 and Carmagnola CS inflorescences, respectively.

4.2. Microwave assisted extraction (MAE)

Uso 31 and Carmagnola CS male inflorescences were processed through Milestone ETHOS X microwave equipment (Milestone, Italy), used at the maximum irradiation power of 1800 W for 100 min. About 2 kg of frozen hemp inflorescences, defrosted for 30 min, were placed in a glass reactor inside the microwave apparatus, equipped with a stainless-steel Clevenger. The volatile compounds of interest were collected in the form of an EO, due to a circulating cold water flow, maintained at 8°C by a Chiller Smart H150-2100S by Labtech srl (Sorisole, Bergamo, Italy). The obtained EOs were collected by the collection burette after separation from water, and kept in a sealed vial at 4°C until further analysis. The EOs yield was measured on dry matter (w/w). The density of the two hemp EOs was calculated at 20 °C with an oscillating U-tube density meter (DA-100M, Mettler Toledo, Greifensee, Switzerland).

4.3. EOs GC-MS characterization

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The chemical composition of the two hemp EOs was assessed according to a previously GC-MS published method [23], using an Agilent 8890 (GC) equipped with an Agilent 5977B Mass Spectrometer (Santa Clara, CA, USA). Compounds were identified by comparing mass spectra with those present in commercial libraries, namely ADAMS [24], NIST17 [25], and FFNS C3 [26].

4.4. C. sativa EO-based nanoemulsions (NEs) formulation and characterization

NEs were prepared through a high-pressure homogenization method. Firstly, an emulsified system was achieved by adding the required amount of EO, previously solubilised in ethyl oleate, into a polysorbate 80 aqueous solution under high-speed stirring (Ultra Turrax T25 basic, IKA® Werke GmbH & Co.KG, Staufen) applied for 5 min at 9500 rpm. Then, the emulsions were homogenised at the pressure of 130 MPa using a French Pressure Cell Press (American Instrument Company, AMINCO, MY, USA) apparatus for 4 cycles. The final composition of the prepared NEs was 5% w/w EO, 2% w/w polysorbate 80, 1% ethyl oleate, and 92% distilled water for Uso 31 EO-based NE, and 6% w/w EO, 3% w/w polysorbate 80, 2% ethyl oleate and 89% of distilled water for Carmagnola EO-based NE. Droplet size (Z-average, diameter) and polydispersity index (PDI) were assessed by dynamic light scattering (DLS) (Zetasizer nanoS, Malvern, Worcestershire, UK) after preparation (T0) and at different time points (one month, T30; two months T60; four months, T120 and six months, T180) to check the physical stability of the formulations. Analyses were performed in three replicates.

4.5. Cell lines

Immortalized Human Keratinocytes cell line (HaCaT) and Human Fibroblasts (NHF A12), provided by IFO (Istituti Fisioterapici Ospitalieri, Rome, Italy), were cultured in Dulbecco's modified Eagle's medium (DMEM; Lonza Bioresearch, Basel, Switzerland), enriched with 10% fetal bovine serum (FBS, Thermo Fisher Scientific Inc., Waltham, MA, USA), 100 IU mL^{-1} penicillin/streptomycin, 2 mM L-glutamine and kept at 37°C with 5% CO_2 and 95% humidity. Human Bronchial Epithelial Cells (HBEpC), obtained from the surface epithelium of normal human bronchi (Sigma Aldrich, Milan, Italy), were grown in Bronchial Epithelial Cell Growth medium (Sigma Aldrich, Milan, Italy), following the manufacturing protocol. Medium was specifically designed to promote attachment, spreading and proliferation of HBEpC cells in culture. This medium is serum-free, and it was fully supplemented with growth factors, trace elements, and antibiotics. Media were changed every 48 h until cells were 90% confluent. Cells were used between passages three and six. Aliquots of passage three were frozen in liquid nitrogen and cultured until passage six.

4.6. Cell viability assay

Cell lines (3^10^4 cells/mL) were seeded in 96-well plates, in a final volume of 100 µL/well and after one day of incubation, EOs, EO-based NEs, and respective controls, were added. At least six replicates in each experiment were used for each treatment. After 72 h, cell viability was assessed by adding 0.8 mg/mL of 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) (Sigma-Aldrich, Milan, Italy) to the media. The absorbance of samples, solubilized in dimethyl sulfoxide (DMSO), against a background control (medium alone) was measured at 570 nm using a reader microliter plate (BioTek Instruments, Winooski, VT, USA).

4.7. RNA Isolation, Reverse Transcription and Quantitative Real-Time PCR and TaqMan Array

Total RNA from cells treated with Etoposide (ETO), EOs, EO-based NEs and respective controls, was extracted with the RNeasy Mini Kit (Qiagen, Milan, Italy), and cDNA was synthesized using the iScript Advanced cDNA Synthesis Kit for RT-qPCR (Bio-Rad, Milan, Italy) according to manufacturer's protocol. Quantitative real-time polymerase chain reactions (qRT-PCR) were performed with TaqMan® Array, containing 14 genes and 2 assays to candidate endogenous control genes, which was purchased (Thermo Fisher, Grand Island, NY, USA), and used to evaluate the safety profile of each treatment compared to ETO-treated cells used as a positive control. Measurement of housekeeping gene glyceraldehydes-3-phosphate dehydrogenase (*GAPDH*) on the samples was used

to normalize mRNA content. The gene expression levels of treated cell lines were expressed as normalized fold to untreated cells. For the detection, the iQ5 Multicolor Real-Time PCR Detection System (Bio-Rad, Milan, Italy) was used. The PCR parameters were 10 min at 95 °C followed by 40 cycles at 95 °C for 15 s and 60 °C for 40 s. Target gene levels were calculated by the $2^{-\Delta\Delta Ct}$ method.

4.8. Statistical analysis

The presented data represent the mean with standard deviation (SD) of at least 3 independent experiments. The statistical significance was determined by Student's t-test and by OneWay-Anova and Two Way-Anova with Bonferroni's post-test. Determination and statistical analysis levels of the inhibitory concentration causing 50% of cell death (IC_{50}) were performed using Prism 5.0a (GraphPad Software, San Diego, CA, USA).

5. Conclusions

Results from this study highlighted the cytotoxicological profile of hemp (*C. sativa*) EOs, specifically Carmagnola CS and Uso 31 varieties, and their formulated Nes,on three different cell lines, human keratinocytes (HaCaT), fibroblasts (NHF A12) and bronchial epithelial cells (HBEpC). The encapsulation of EOs into NEs does markedly affect the safety profiles of EOs, which are comparable among all the tested cell lines. The prepared NEs were stable over time for at least 4 months, and they showed IC_{50} values apparently higher than those from the corresponding pure EOs, despite formulation parameters (EO, surfactant, and co-solvent concentration) can slightly affect cytotoxicity, at least at the investigated experimental conditions. Cytokines expression analysis showed a safe profile , since cytokines levels were almost lower than those induced by the positive control ETO and, in most of the cases, comparable to the basal level (untreated cells), although some differences have been observed among the expression of the investigated cytokines (IL-1 beta, IL-6, TNF-alpha, and STAT3). Overall, the present study supports the possible safe use of hemp EO formulations for therapeutic applications.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org. Table S1: Complete GC-MS characterization of Uso 31 and Carmagnola CS EOs.

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