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Anticancer Effect of Solid-Lipid Nanoparticles Containing *Mentha longifolia* and *Mentha pulegium* Essential Oils: *In Vitro* Study on Human Melanoma and Breast Cancer Cell Lines

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Abstract: Breast cancer and melanoma are common cancers with several treatments such as surgery, radiotherapy, chemotherapy, and their side effects. Therefore, new drugs using plant-derived substances (especially essential oils) have received more attention in recent years; however, they are generally less effective than synthetic drugs. Therefore, the preparation of essential oil-based nanoformulations is considered a promising approach to improving their efficiency. In this study, ingredients of *Mentha longifolia* and *Mentha pulegium* essential oils were first identified by GC-MS analysis. Their anticancer effects were then evaluated against one melanoma cell line (A-375) and two breast cancer cell lines (MDA-MB-468 and MCF-7). The cytotoxic effect of the essential oil on all cell lines at even the highest concentration, 1200 µg/mL, was not proper (viability > 55%). After that, solid lipid nanoparticles containing each essential oil with particle sizes of 107 ± 9 (PDI 0.274) and 191 ± 8 (PDI 0.174) nm and zeta potential -7.10 and -4.81 mV were prepared. Interestingly, both prepared nanoformulations reduced the viability of all three cell lines to around 10% at half the mentioned concentration, 600 µg/mL. Thus, the prepared nanoformulations could be introduced as proper candidates for investigation in-vivo research and supplementary medicine.

Keywords: anticancer effect; A-375; MCF-7; MDA-MB-468.

1. Introduction

Uncontrolled or abnormal growth and proliferation of cells (in any organ of the body) is called cancer; melanoma and breast cancer are common cancers worldwide [1,2]. The prevalence of melanoma is increasing worldwide; it is the cause of 75% of skin cancer-related deaths [3,4]. A-375 cells are among the most widely used melanoma cell lines in research; it is more aggressive with high metastatic potential and low responsiveness to chemotherapy [5,6].

Breast cancer is the first common cancer in females, impacting over 1.5 million women annually [7,8]. Amongst breast cancer cell lines, MCF-7 is the most commonly studied due to hormone sensitivity and estrogen receptor expression [9,10]. MDA-MB-468, without estrogen and progesterone receptors and HER2 non-expression (triple-negative), is another commonly studied cell line [11,12].

Plant-derived substances (especially EOs) have recently received more attention due to occurring resistance in cancers against chemotropic drugs. Essential oils (EOs) are naturally secreted oil as secondary metabolites in aromatic plants [13]. They have been widely used in the cosmetic, food, and pharmaceutical industries due to their bioactive compounds [14,15]. *Mentha* is a genus of the *Lamiaceae* family, which contains approximately 220 genera and 3300 species [16]. *Mentha longifolia* and *Mentha pulegium* are two important medicinal plants from this family, which have achieved a special place in the pharmaceutical industry [17,18]. *M. longifolia*, commonly known as "wild mint," could be useful for the treatment of bronchitis, headache, cough, nausea, asthma, liver diseases, digestive disorders, stomach, abdominal disorders, etc. [19,20]. *M. pulegium*, commonly known as "pennyroyal" could be useful for the treatment of colds, sinusitis, cholera, food poisoning, abdominal cramps, smallpox, bronchitis and tuberculosis [21,22]. Other biological actives such as antispasmodic, anticancerous, antimicrobial, antioxidative, anticandidal, insect repellent, anticholinergic, antidiabetic, and neuroprotective effects have also been reported for their EOs [23,24].

Nowadays, formulating EOs as nanoformulations such as nanoemulsions and polymeric or lipidic nanoparticles is a promising approach to improving their effectiveness and adjusting their concentration to achieve proper efficiency [25,26]. Lipid nanoparticles, also known as solid lipid nanoparticles (SLNs) with lipophilic ingredients, are proper carriers for loading EOs; they have unique features such as small size, large surface area, high drug loading, and low toxicity [27,28].

In this study, the cytotoxic effect of EOs of *M. longifolia* (MLEO) and *M. pulegium* (MPEO) was first investigated on A375, MCF7, and MDA-MB-468 cancer cell lines. By formulating them as solid lipid nanoparticles, we then tried to improve their efficiency.

2. Materials and Methods

2.1. Materials.

MCF-7 (ATCC HTB-22) and MDA-MB-468 (ATCC HTB-22) breast cancer cell lines, as well as melanoma cell line A-375 (ATCC CRL-1619), were provided by the Pasteur Institute of Iran. Sigma-Aldrich (USA) provided tetrazolium salt, 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazo-lium bromide (MTT) and phosphate-buffered saline (PBS) tablets. Shellmax (China) supplied penicillin-streptomycin, trypsin, Dimethyl Sulfoxide (DMSO), and Dulbecco's Modified Eagle's Media (DMEM) cell culture medium. Gibco (USA) provided fetal bovine serum (FBS). Merck Co. (Germany) supplied the stearic acid, Tween 80, and Span 60. A Milli-Q water system (Milli-pore, Direct-Q) was used to purify deionized water.

2.2. GC-MS analysis.

For identifying constituents of MLEO and MPEO, GC-MS analysis was used as described in our previous study [29].

2.3. Preparation of SLN containing EOs.

The high-pressure homogenizer method was used to prepare MLEO and MPEO containing SLNs (ML-SLNs and MP-SLNs), as described in our previous research (30). Briefly, MLEO and MPEO (1% v/v) was first dissolved in the melted solid lipid (stearic acid 4% v/v, 85 ºC) and lipophilic surfactant (Span 60, 2% v/v). After that, the mixture was distributed in a heated aqueous surfactant solution, tween 80 4% v/v; subjected to high-shear homogenizer (D-91126 Schwabach, Heidolph, Germany) for 1 minute at 8000 rpm. The obtained pre-emulsion was then homogenized at high pressure (3 cycles, 500 bar) using an APV Micron Lab 40 (APV Systems, Unna, Germany) thermostated at 90ºC.

2.4. Characterization of SLNs.

The particle size, polydispersity index, and zeta-potential of the nanoparticles were determined using Malvern zetasizer (Malvern Instruments, UK). The sample was calibrated at 25°C with an angle detection of 90 in this process. The samples' concentration for analysis on the Zeta sizer was 20–400 kilo counts per second (KCPS), and 100000 counts per second were the intensity of diffraction.

2.5. MTT assay.

MTT assay was used to investigate the anticancer functions of MLEO and MPEO (as bulk samples) as well as ML-SLNs and MP-SLNs (as nanoformulations). MLEO and MPEO were dissolved (4800 µg/mL) in PBS solution containing 0.5% DMSO; required concentrations were also prepared using the same solvent.

The A-375, MCF-7, and MDA-MB-468 cell lines were cultured at 75 cm2 culture flasks (37 °C, 5% CO2) in DMEM perfect medium containing FBS (10%) and Penicillin-streptomycin (1%). Separated cells from a flask, using trypsin, were seeded in 96-well plates and incubated for 24 hours. The culture media was then discarded, and each well was filled with 75 µL of full fresh medium. Concentrations were set at 1200, 600, 300, 150, and 75 µg/mL after applying the samples' desired amount. After 24 h of incubation, the plate contents were discarded, and the wells were washed with PBS to clear the milky color of the nanoformulations. Every well was then filled with 100 µL MTT solution (0.5 mg/mL), and plates were incubated for 4 hours. Finally, 100 µL/well of DMSO was applied to each well to dissolve the formed formazan crystals. Using an ELISA Plate Reader, the absorbance of each well was measured at 570 nm. Dividing the mean absorbance of each sample's concentration by the mean absorbance of the control group, cell viability was calculated. The test was performed in triplicate; six wells were considered the control in each plate, filled with PBS solution containing 0.5% DMSO (25 µL) and DMEM (75 µL).

3. Results and Discussion

3.1. Ingredients of CSEO.

Constituents of MLEO and MPEO were identified using GC-MS analysis comprising more than 1% (see Table 1). All ingredients have been arranged by retention time (RT) from smaller to larger. Five major components of MLEO are pulegone (% 47.6), 1,8-cineole(%12.6), piperitenone (%5.6), menthofuran (%4.6) and arvone (%4.1). Five major components of

MPEO are pulegone (%69.0), trans-cyclohexanone, 5-methyl-2-(1-methylethyl) (%9.4), limonene (%4.0), 8-hydroxy-p-menthan-3-one (%3.7) and Iso-pulegone (%3.4).

Table 1. Identified ingredients (>1%) in the MLEO and MPEO using GC-MS analysis.

No.	[a]RT	Compound	[b]RI	MLEO		MPEO	
				Area	%	Area	%
1	9.4	α-pinene	622	29138914	1.8	--	--
2	11.1	sabinene	689	19211346	1.2	--	--
3	11.2	β-pinene	693	36089576	2.3	--	--
4	12.2	3-octanol	721	--	--	31660011	1.3
5	13.6	limonene	756	61759470	3.9	96878221	4.0
6	13.7	1,8-cineole	759	197657002	12.6	--	--
7	19.2	5,7-octadienoic acid, methyl ester	879	35260468	2.2	--	--
8	19.5	*trans*-cyclohexanone, 5-methyl-2-(1-methylethyl)	884	--	--	223515392	9.4
9	19.9	menthofuran	892	71977135	4.6	--	--
10	19.9	*cis*-cyclohexanone, 5-methyl-2-(1-methylethyl)	893	--	--	33275891	1.3
11	20.1	borneol	896	20202046	1.2	--	--
12	20.5	Iso-pulegone	904	26990916	1.7	80850780	3.4
13	21.5	Iso-dihydrocarvone	923	18371508	1.1	--	--
14	23.7	pulegone	963	746264316	47.6	1642627212	69.0
15	23.8	carvone	965	64391028	4.1	--	--
16	24.2	8-hydroxy-p-menthan-3-one	972	--	--	88487891	3.7
17	24.8	8-hydroxy-p-menthan-3-one	984	--	--	31372778	1.3
18	28.0	piperitenone	1045	89158439	5.6	30048157	1.2
19	29.1	piperitenone oxide	1066	27012819	1.7	--	--
20	31.2	*trans*-caryophyllene	1106	19455003	1.2	--	--

[a] retention time, [b] retention index.

3.2. Characteristics of the prepared nanoformulation (ML-SLNs and MP-SLNs).

DLS analysis and potential zeta profile of ML-SLNs and MP-SLNs are depicted in Figures 1 and 2. Their particle sizes were 107 ± 9 and 191 ± 8 nm, polydispersity indexes (PDI) were 0.274 and 0.174, and zeta potentials were -7.10 and -4.81 mV. In phospholipid vesicles, a PDI < 0.3 is considered a homogenous formulation; thus, both prepared nanoformulations had satisfying properties [30,31].

The current research is the first study to deal with MLEO or MPEO (ML-SLNs and MP-SLNs) preparation as the anticancer agent to the authors' best knowledge. One research on the preparation of MP-SLNs with a particle size of 202 nm, PDI 0.76, and zeta potential −26.7 as an antibacterial agent against dental caries was reported previously [32].

From the literature, some reports on the preparation of SLNs containing other EOs have also been found. For instance, in our previous report, *Zataria multiflora* essential oil was loaded in SLNs with a particle size of 134 ± 7 nm, PDI 0.24, and zeta potential -9.82. The prepared nanoformulation's repellent effect against the main malaria vector, *Anopheles stephensi*, was 93 min compared to 29 min for no-formulated EO [33]. By another group, *Z. multiflora* essential oil was loaded in SLNs with a particle size of around 255 nm, PDI 0.369, and zeta potential -37.8 mV; its antifungal properties were investigated [34]. There have also been reports of the preparation of nanoparticles containing other EOs. For example, *Yuxingcao* EO was loaded in SLNs with a particle size of 171 nm and zeta potential of 17.1 mV [35]. Essential oils of *frankincense* and *myrrh* (FMO) were loaded in SLNs with a mean size of 113.3 ± 3.6 nm and zeta potential −16.8 ± 0.4 mV [36].

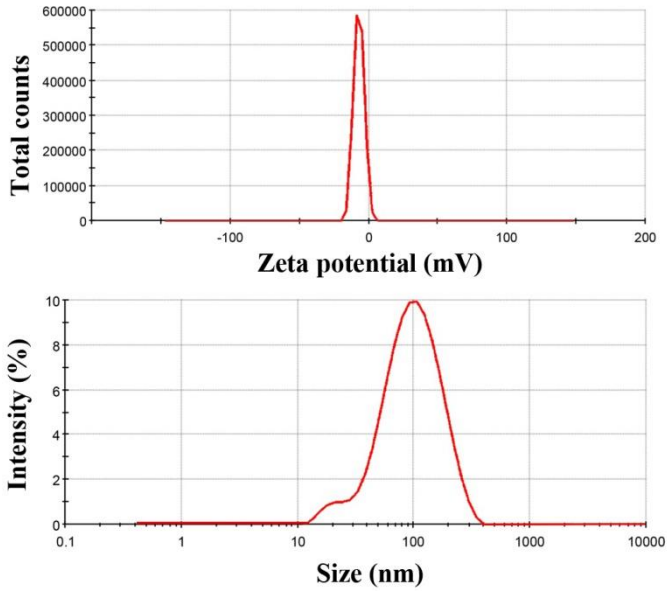

Figure 1. Zeta potential, -7.10 mV (top), and DLS analysis (down) of solid-lipid nanoparticles containing *M. longifolia* EO (ML-SLNs) with a particle size of 107 ± 9 nm and PDI 0.274.

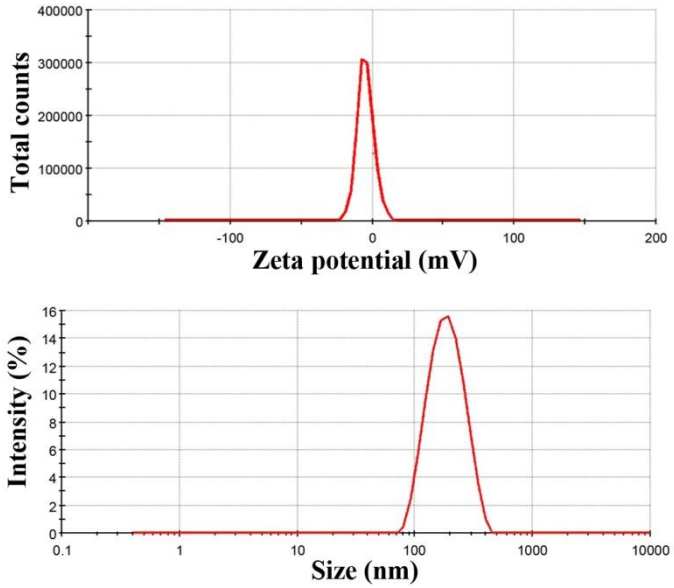

Figure 2. Zeta potential, -4.81 mV (top), and DLS analysis (down) of solid-lipid nanoparticles containing *M. pulegium* EO (MP-SLNs) with a particle size of 191 ± 8 nm and PDI 0.174

3.3. Comparison of cytotoxic effect of the EOs and their nanoformulated forms.

Anticancer effects of MLEO and MPEO and their nanoformulated forms, ML-SLNs and MP-SLNs, against A-375 cells are depicted in Figure 3. MP-SLNs showed significantly more potent than MPEO at all examined concentrations ($p < 0.001$), and ML-SLNs at four concentrations, including 150, 300, 600, and 1200 µg/mL was significantly more potent than MLEO. The cell's viability at the highest concentration (1200 µg/mL) of MLEO and MPEO was decreased to 89 and 73%. Our findings from the MTT assay interestingly provide that the highest anticancer activity was observed after 24 h treatment of the A-375 cells with ML-SLNs and MP-SLNs in 600 and 1200 µg/mL concentrations; the viability of A-375 was reduced to < 5%.

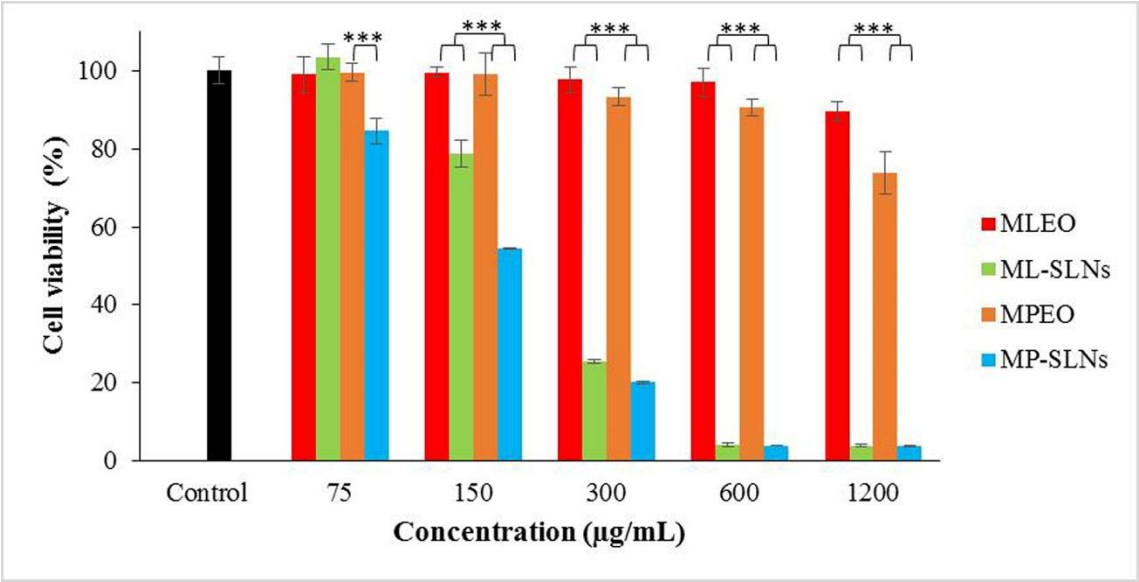

Figure 3. Comparison of cytotoxic effect of EOs of *M. longifolia* and *M. pulegium* (MLEO and MPEO) and their nanoformulated forms (ML-SLNs and MP-SLNs) on A-375. Data are presented as mean ± standard deviations (n = 3). ***: $p < 0.001$.

Anticancer effects of MLEO, MPEO, ML-SLNs, and MP-SLNs, against MCF-7 cells are illustrated in Figure 4. ML-SLNs at all examined concentration was more potent ($p < 0.001$) than its no-formulated form MLEO, and cytotoxic effect of MP-SLNs was more potent ($p < 0.001$) than MLEO at four concentration including 150, 300, 600, and 1200 µg/mL. Cell viability after treatment with MLEO and MPEO 1200 µg/mL were decreased to 83 and 74%. Notably, the present results show that MCF-7 cells (for 24 h) treatment with ML-SLNs and MP-SLNs with 600 and 1200 µg/mL reduced cell viability to < 7%. These results suggested that ML-SLNs and MP-SLNs exerted a more significant inhibitory effect on the breast cancer cell line.

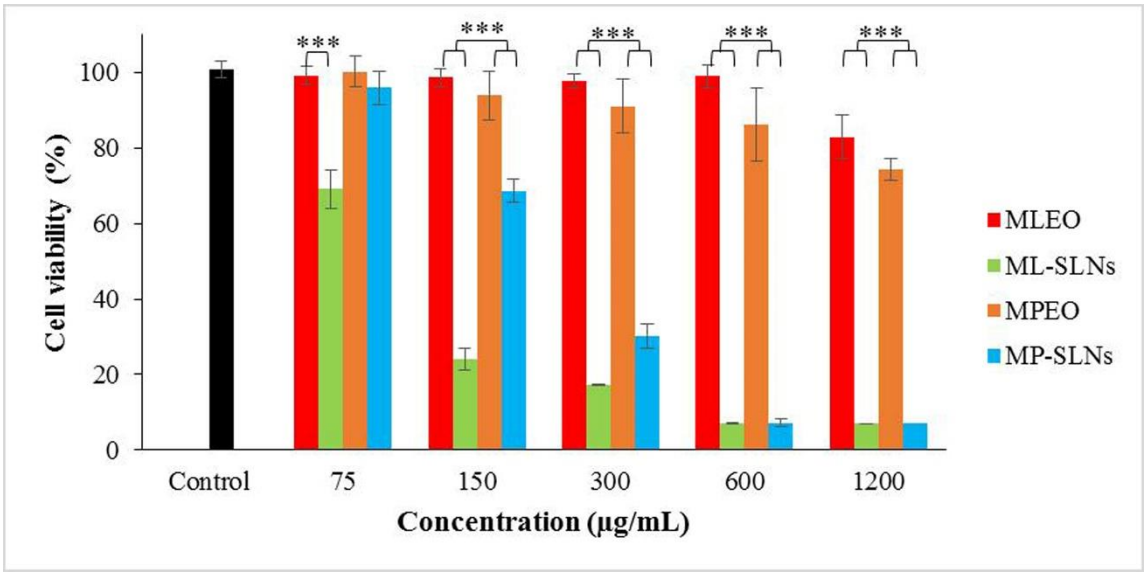

Figure 4. Comparison of cytotoxic effect of EOs of *M. longifolia* and *M. pulegium* (MLEO and MPEO) and their nanoformulated forms (ML-SLNs and MP-SLNs) on MCF-7. Data are presented as mean ± standard deviations (n = 3). ***: $p < 0.001$.

Anticancer effects of MLEO and ML-SLNs, as well as MPEO and MP-SLNs on MDA-MB-468, are compared in Figure 5. Both nanoformulations (ML-SLNs and MP-SLNs) at all examined concentrations were more potent ($p < 0.001$) than their no-formulated forms (MLEO

and MPEO). The cells' viability at 1200 µg/mL of MLEO and MPEO (highest concentration) was decreased to 57 and 88%. Interestingly, the results showed that ML-SLNs and MP-SLNs therapies at three concentrations of 300, 600, and 1200 µg/mL had reduced the cells' viability to < 15%.

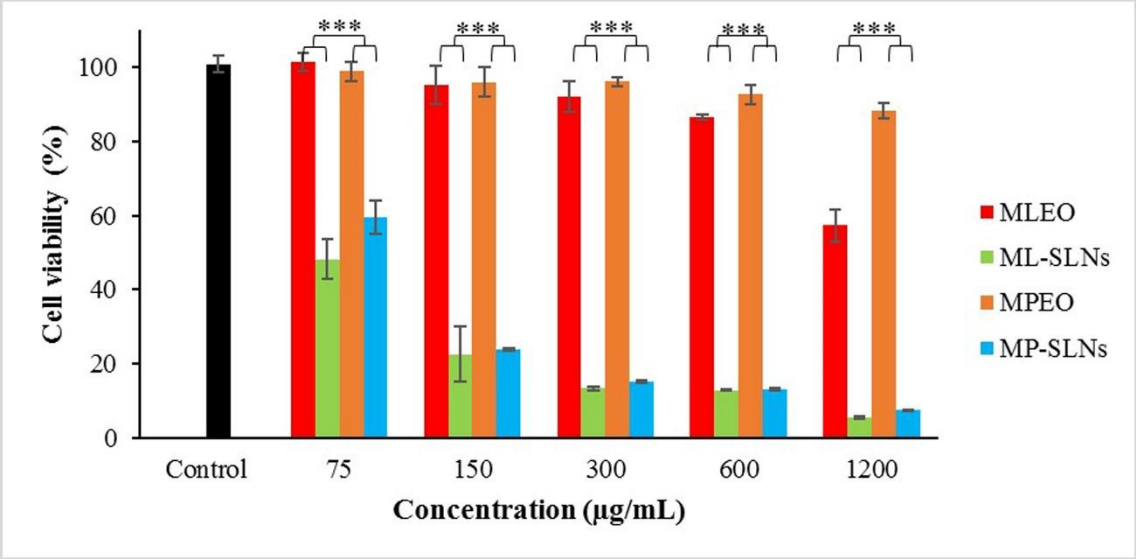

Figure 5. Comparison cytotoxic effect of EOs of *M. longifolia* and *M. pulegium* (MLEO and MPEO) and their nanoformulated forms (ML-SLNs and MP-SLNs) on MDA-MB-468. Data are presented as mean ± standard deviations (n = 3). ***: $p < 0.001$.

The use of nanocarriers (niosome, nanoemulsions, SLNs, nanofibers, and polymeric nanoparticles) to improve pharmacokinetics and the bioavailability of therapeutic agents has recently received more attention [37,38]. For example, in vitro cytotoxicity assay of HepG2, MCF-7, and A549 cell lines showed that D-limonene-loaded niosome had a noticeable anticancer effect compared to D-limonene and empty niosome; it reduced viabilities of the cells to ~ 40% at a concentration of 20 µM [37]. Moreover, by preparing EO-based nanoformulations, the droplets' dispersion is uniform in the aqueous medium. Therefore, it leads to more and better interactions with the cell; improved nanoformulation efficiency compared to the non-formulated state is more likely [39,40]. This improvement also results from decreasing EOs droplet size and better penetration into the cells [41,42]. In our previous study, the anticancer effect of nanoemulsion of *Mentha piperita* EO with a mean droplet size of 136 ± 2 nm (PDI 0.3) was significantly better ($p < 0.001$) than that of non-formulated EO; the obtained effect with an exposure time of 24 h was significantly better than non-formulated EO within 72-h exposure time [43]. From the literature, SLNs containing limonene reduced growth percentages in cancer cells with a low toxic effect on the non-tumoral cell line; however, no comparison was performed with non-formulated limonene [44]. In another study, the antioxidant activity of limonene emulsion with a particle size of 339.5 nm showed more potent than bulk limonene; concluded that encapsulation could solve its low solubility in water and enhance its bioactivity [45].

4. Conclusions

Ingredients of *M. longifolia* and *M. pulegium* were first identified using GC-MS analysis; pulegone was the major constituent of both. They showed no proper cytotoxic effect on A-375 (melanoma cell line), MDA-MB-468, and MCF-7 (breast cancer cell lines). However, solid-lipid nanoparticles containing them at concentrations of 600 and 1200 600

µg/mL reduced cell viability to ~ 10%. The prepared nanoformulations could thus be considered for further investigation on other cell lines and in-vivo studies.

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Not applicable.

Conflicts of Interest

There is no conflict of interest amongst the authors.

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