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Microemulsions of essentials oils – Increase of solubility and antioxidant activity or cytotoxicity?

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A B S T R A C T

Essential oils (EO) possess a wide range of biological activities. However, their application in aqueous media is often limited due to their hydrophobicity and volatile character. This study was designed to prepare stable, water-dilutable microemulsions (ME) containing essential oils of citronella (*Cymbopogon nardus* (L.) Rendle), mint (*Mentha x piperita* L. 'Multimentha') and eucalyptus (*Eucalyptus globulus* Labill.) and to evaluate their *in vitro* antioxidant and cytotoxic properties. The comparison of cytotoxicity of EO solubilised in microemulsions and in dimethyl sulfoxide as well as the recovery of volatiles from cells culture medium over time was also performed. The clear ME were obtained in a range between 10% and 50% of aqueous phase for citronella EO and up to 60% of aqueous phase for mint and eucalyptus EO, in all ratios of Tween 80 to oil phase (from 5:1 to 9:1). Microemulsions of EO (EO/ME) showed higher antioxidant activity compared to EO. The increase in activity was 13.96%, 22.25% and 45.60% for eucalyptus, mint and citronella EO, respectively. The analysis of cytotoxicity profiles of EO/ME and EO/DMSO in Vero and HeLa cell lines showed differences in activity, however, they were statistically significant only in case of mint EO. Furthermore, it can be concluded that after 24 h of incubation ME vehicle itself was responsible for the observed cytotoxic effect. At the same time ME provided good solubility of constituents of EO and diminished evaporation of volatiles from culture medium.

1. Introduction

Essential oils (EO) possess a wide range of biological activities. However, their application in aqueous media is often limited due to their hydrophobicity and volatile character. Encapsulation prevents rapid evaporation of highly volatile constituents and enables mixing of larger amounts of EO with aqueous solvents without separation of phases (Leimann et al., 2009). Microemulsions (ME) are one of the possible forms of encapsulation. These are transparent, thermodynamically stable mixtures of oil and water phases stabilized with emulsifiers. ME form spontaneously with relatively simple starting ingredients, because components are at a higher energetic state than that of the final microemulsion product (Anton and Vandamme, 2011; McClements, 2012). The process of ME formation is forward-driven, what makes these formulations inexpensive and stable during a long storage. What is more, the physical characteristics of a product containing ME is usually unaffected because of small nanoparticles size ranging from 10 to 100 nm (McClements, 2012).

Antimicrobial activity of ME containing EO is the most studied one with the application in food products (Basak and Guha, 2017; Shaaban et al., 2015; Wang et al., 2014; Zhang et al., 2014), infected wounds healing (Ghosh et al., 2013, 2015; Valizadeh et al., 2018), acne treatment (Jantrawut et al., 2018; Viyoch et al., 2006) and others (Taufik et al., 2018; Raffaella et al., 2017). Also larvicidal activity of eco-friendly ME formulations with EO was recently reported (Pavela et al., 2019). ME systems containing EO were shown to enhance the shelf-life

Abbreviations: DPPH, 2,2-diphenyl-1-picrylhydrazyl; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; CITR, citronella essential oil; DMSO, Dimethyl sulfoxide; DMF, Dimethylformamide; DLS, Dynamic light scattering; EO, Essential oils; EO/DMSO, Essential oils dissolved in dimethyl sulfoxide; EO/ME, Essential oils dissolved in microemulsions; EUC, eucalyptus essential oil; RI^{ex}, Experimental retention index; FBS, Foetal bovine serum; GC-MS, Gas chromatography – mass spectrometry; GRAS, Generally recognized as safe; RI^{lit}, Literature retention index; ME, Microemulsions; MINT, mint essential oil; MEM, Modified Eagle Medium; MLS, Multiple light scattering; CC_{50}, Percentage (%) of tested formulation in cell media causing the 50% decrease of cell viability; PBS, Phosphate buffer saline; Rt, Retention time; SD, Standard deviation

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and processability of essential oils (Cespi et al., 2017). Moreover, ME were also found to improve antimicrobial properties (Valizadeh et al., 2018) or not to affect the activity of EO (Leimann et al., 2009). Antioxidant potential of ME containing EO has been reported (Chaiyan et al., 2014; Deng et al., 2015; Hamed et al., 2012; Karami et al., 2019). However, very little is known about *in vitro* cytotoxicity of these formulations. ME are considered as useful carriers of EO diminishing their irritating properties (Leimann et al., 2009) but although the components usually used for preparation of ME are generally recognized as safe (GRAS), the toxicity of surfactants has been reported (Arechabala et al., 1999). Because ME formulations require the presence of emulsifiers at a quite high concentration (McClements, 2012), the evaluation of ME cytotoxicity should not be omitted during biological testing. Hence, in this study we prepared stable, water-dilutable microemulsions containing essential oils of citronella (*Cymbopogon nardus* (L.) Rendle), mint (*Mentha x piperita* L. 'Multimentha') and eucalyptus (*Eucalyptus globulus* Labill.) and we evaluated their *in vitro* antioxidant and cytotoxic properties. The comparison of cytotoxicity of EO solubilised in ME and in dimethyl sulfoxide as well as the recovery of volatiles from cells culture medium over time was also performed.

2. Materials and methods

2.1. Materials

Tween 80, soybean oil, polypropylene glycol, dimethyl sulfoxide (DMSO), 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich (St. Louis, Missouri, United States). Methanol and dimethylformamide (DMF) were obtained from Avantor Performance Materials (Gliwice, Poland).

Cell lines Vero (ECACC, No. 84113001) and HeLa (ATCC, No. CCL-2) were acquired from the American Type Culture Collection (ATCC) and the European Collection of Cell Cultures (ECACC). Foetal bovine serum (FBS) was purchased from Biowest (Nuaillé, France). Modified Eagle Medium (MEM), Penicillin-Streptomycin Solution and phosphate buffer saline (PBS) were obtained from Corning (Tewksbury, MA, USA).

Citronella essential oil was purchased from Pollena Aroma (Nowy Dwór Mazowiecki, Poland). *Eucalyptuss globules* BIO leaves were purchased from NAT (Długołęka, Poland), whereas *Mentha piperita* var. multimentha plant material was harvested in June 2017 in the Garden of Cosmetic Plants and Raw Materials, Research and Science Innovation Center in Wola Zdybska (51° 44′ 49″ N 21° 50′ 38″ E). The collection and identification was made by A. Kiełtyka-Dadasiewicz. Eucalyptus and mint essential oils were obtained in 3 h hydrodistillation process in Deryng-type apparatus. The quality of all essential oils was checked by means of gas chromatography coupled to mass spectrometry (Baj et al., 2017).

2.2. Preparation of microemulsions

The oily phase was a mixture of the essential oil and soybean oil in a volume ratio of 3:1. Tween 80 was mixed with an oil phase in 5 vol ratios varying from 5:1 to 9:1 in each series. The added aqueous phase, being a mixture of water and polypropylene glycol in a 1:1 vol ratio, ranged from 10% v/v to 90% v/v. Formulations were vigorously mixed using a laboratory shaker. The essential oils constituted from 0.8% to 11% of the microemulsions.

2.3. Evaluation of microemulsions stability

2.3.1. Visual evaluation

The turbidity of prepared formulations was evaluated visually. The transparent ME were incubated in ambient temperature and in 37 °C for two weeks and then were checked for the signs of delamination.

2.3.2. Evaluation of particle size distribution

The diameter of the ME droplets was measured by means of dynamic light scattering (DLS) technique using Zetasizer Nano-ZS (Malvern, UK). From each series of microemulsions, those containing 50% of the aqueous phase and a Tween 80 to oil phase ratio of 5:1 were selected to compare the particle sizes in these systems. The content of EO in selected ME was 6.2%. The undiluted samples were measured a day after preparation and after 7 days. The samples diluted 10 times with deionized water were evaluated one day after preparation and then after 7 and 17 days. Measurements were conducted at 25 °C with a fixed angle of 90°. Between measurements the samples were stored in the darkness and in the ambient temperature. Sizes quoted are the z-average mean of the microemulsion hydrodynamic diameter (nm) obtained from 3 measurements.

2.3.3. Evaluation of stability over time

The acquisition of transmission and backscattering of the light in undiluted ME system was done for ME containing eucalyptus EO. The formulation of ME was similar to that used for DLS measurements. For multiple light scattering (MLS) in the sample TurbiscanLAB^{Expert} (Formulaction, France) was used. The detection head was acquiring transmission and backscattering data every 40 μmon a distance of 13 mm sample height. The experiment was performed in 25 °C, on 2, 13, 23 and 29 days after preparation of ME. Between measurements the sample was stored in the darkness and in the ambient temperature.

2.4. Evaluation of antioxidant properties of EO and EO in ME

The antioxidant activity was determined using 2,2-diphenyl-1-picrylhydrazyl assay according to the method previously described by Baj et al. (2018). From each series of ME, those containing 50% of the aqueous phase and a Tween 80 to oil phase ratio of 5:1 were selected to compare antioxidant properties of ME and EO. The content of EO in selected ME was 6.2%. The final concentration of EO incorporated into ME and EO dissolved in methanol was 5 mg/ml in all studied samples. The free radical scavenging properties of each EO incorporated into ME and EO dissolved in methanol were calculated as percent inhibition according to Eq.:Inhibition (%) = $(A_{contol} - A_{sample})/A_{control} \times 100$. All experiments were performed in triplicate.

2.5. Cell cultures and media

Cytotoxicity evaluation was carried out on Vero and HeLa cell lines. Vero is a normal cell line established from the kidney of an adult African green monkey, whereas HeLa is a human cancer cell line established from cervical adenocarcinoma. Cells were grown in T25 flasks, 48-well plates and 96-well plates (NUNC) using MEM. Cell media were supplemented with FBS and antibiotics (Penicillin-Streptomycin Solution). For cell passaging the culture media was supplemented with 10% FBS, whereas the media used for experiments contained 2% of FBS. All experiments with cell cultures were carried out at 37 °C in the 5% CO_2 atmosphere (Lab-Line incubator, USA).

2.6. Evaluation of cytotoxic properties of ME and EO

Microculture tetrazolium (MTT) assay was used to evaluate cytotoxicity. It is a quantitative colorimetric toxicity test based on the transformation of yellow, soluble tetrazolium salts (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) into purple-blue insoluble formasane by cellular dehydrogenases (Rajtar et al., 2017). Since this process may occur only in viable, metabolically active cells, the level of enzyme activity is the measure of viability.

Cells were seeded into 96-well plates at a density of 1.5×10^4 cells/well (Vero) or 2.5×10^4 cells/well (HeLa) in the appropriate medium containing 10% FBS to produce semi-confluent monolayer. After overnight incubation, the growth medium was removed and the cells were

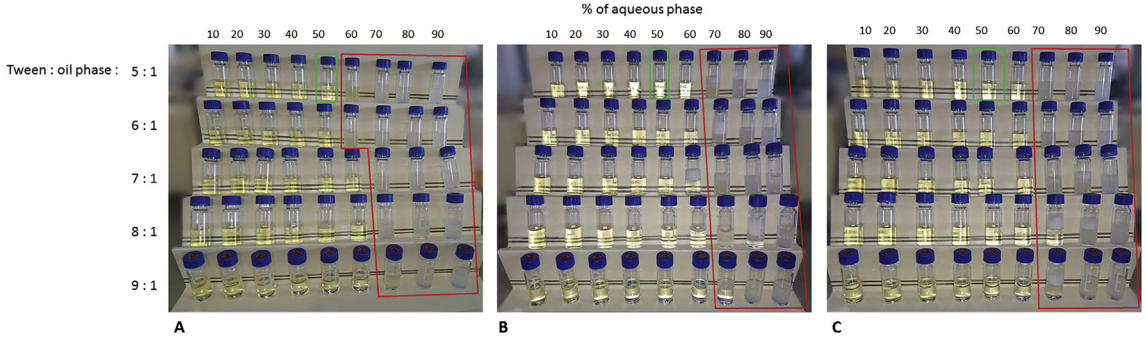

Fig. 1. Photos of microemulsions containing citronella EO (A), mint EO (B) and eucalyptus EO (C). A red rectangle indicates cloudy samples which are macroemulsions. The green rectangle indicates the samples selected for following experiments. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

treated with media containing twofold, starting from 2%, dilutions of ME containing essential oil or DMSO containing the same amount of essential oil and further incubated. Simultaneously, the cytotoxicity of ME and DMSO was evaluated. Control cells were supplemented only with medium containing 2% of FBS.

After 24 h incubation, the culture media was removed from the plates, cells were washed with PBS, and 100 µl of the culture media containing 10% of MTT solution (5 mg/ml) was added to each well and the plates incubated for the next 4 h at 37 °C. Afterwards, 100 µl of SDS/DMF/PBS solution (14% SDS, 36% DMF, 50% PBS) per well was added and after an overnight incubation the absorbance was read. Absorbance was measured at 540 and 620 nm using a microplate reader (Epoch, BioTek Instruments, Inc., Winooski, VT, USA). Data evaluation was performed using Gen5 software (ver. 2.01.14; BioTek Instruments) and GraphPad Prism (version 7.04, GraphPad Software, Inc., La Jolla, CA, USA). The CC_{50} (percentage (v/v) of tested formulations - EO/ME, EO/DMSO and vehicle – ME and DMSO, decreasing cell viability by 50%) were calculated from four-parameter dose-response curves (nonlinear regression, variable slope) using GraphPad Prism. Experiments were conducted in triplicate. In case of Vero cells, the cytotoxicity was also evaluated after 4, 8 and 12 h of incubation.

2.7. Recovery of volatiles from culture medium and GC-MS analysis

Vero cells were seeded (1.5×10^4 cells/well) into 48-well plates and incubated for 24 h. Afterwards, cell medium was removed and the cells were treated with media supplemented with 2% of ME containing essential oil or 2% of DMSO containing the same amount of essential oil and further incubated. The cells supplemented with medium containing 2% of FBS were used as blanks. After 4, 8 12 and 24 h samples were collected and 500 µl of each sample was extracted thrice with portions of 200 µl of n-hexane. Collected n-hexane fractions were dried over anhydrous sodium sulphate and stored in 4 °C until further analysis. The initial amount of volatiles extracted from media containing 2% of ME with essential oil or 2% of DMSO containing the same amount of essential oil was also analysed.

The volatiles extracted to the hexane were analysed by gas chromatography – mass spectrometry (GC–MS). Shimadzu GC–2010 Plus coupled to a QP2010 Ultra mass spectrometer with Phenomenex capillary column ZB–5 MS (30 m, 0.25 mm inside diameter, and 0.25 µm coating thickness) was used for GC–MS separation. The initial column temperature was set at 50 °C, held for 3 min, and then heated to 250 °C at a rate of 8 °C/min; this temperature was held for 2 min. The injector temperature was 250 °C. Helium was used as the carrier gas with a flow rate of 1 mL/min. Split ratio was set at 1:20. Ionization was performed by electron impact at 70 eV. The interface and ion source temperature were 250 and 220 °C, respectively. Mass spectral data were acquired in the scan mode in the m/z range 40–500 with the scan rate 0.20 s per scan. Volatiles were identified based on their MS spectra and retention indices determined with reference to a homologous series of C8–C24 n-alkanes using a computer-supported spectral library (MassFinder; NIST, National Institute of Standards and Technology, 1999.).

2.8. Statistical analysis

Statistical analysis was carried out using GraphPad Prism. All the data were expressed as mean ± SD ($n \geq 3$). One-way analysis of variance (ANOVA) was performed using GraphPad Prism software at a significance levels of $p < 0.01$ (significant) and $p < 0.001$ (highly significant).

3. Results

3.1. Evaluation of microemulsions stability

The clear ME were obtained in a range between 10% and 50% of aqueous phase for citronella EO and up to 60% of aqueous phase for mint and eucalyptus EO, in all ratios of Tween 80 to oil phase (from 5:1 to 9:1) (Fig. 1). The appearance of the formulations did not change during two weeks monitoring in 37 °C and in ambient temperature. For all following experiments the samples with the highest aqueous phase content and the lowest amount of surfactant were used (50% of the aqueous phase and a Tween 80 to oil phase ratio of 5: 1; 6.2% of EO in ME).

During DLS measurements performed one day after preparation of formulations the undiluted samples of ME showed high polydispersity described by the polydispersity index (PDI) and visualized as two or three peaks on histograms of size distribution by intensity and high values of standard deviations (Fig. 2). The small peak of particles with size around 10 nm was accompanied by presence of larger particles in the system. The particle size distribution observed after one week was slightly and individually changed for every EO/ME, however the systems were still characterized by presence of different particles sizes (Fig. 2).

The evaluation of the stability of undiluted ME was difficult because of the presence of high number of larger particles in the system. Hence turbiscan measurements were performed for eucaluptus EO loaded ME as the exemplary one. This experiment can detect sedimentation, creaming or coalescence. The results presented on Fig. 3 indicate that no signs of a microemulsion fracture were found. High density of the mixture and the quite large amount of Tween 80 were the cause of prolonged persistence of air bubbles in the tested system what resulted in visible fluctuations in the graphs over 29 days.

The 10-fold dilution of the formulations strongly changed the particle size distribution. The samples became monodisperse, containing only one type of particles (between 10 and 20 nm) with low values of standard deviations. No significant changes in particle size distribution after 7 and 17 days were observed (Fig. 4).

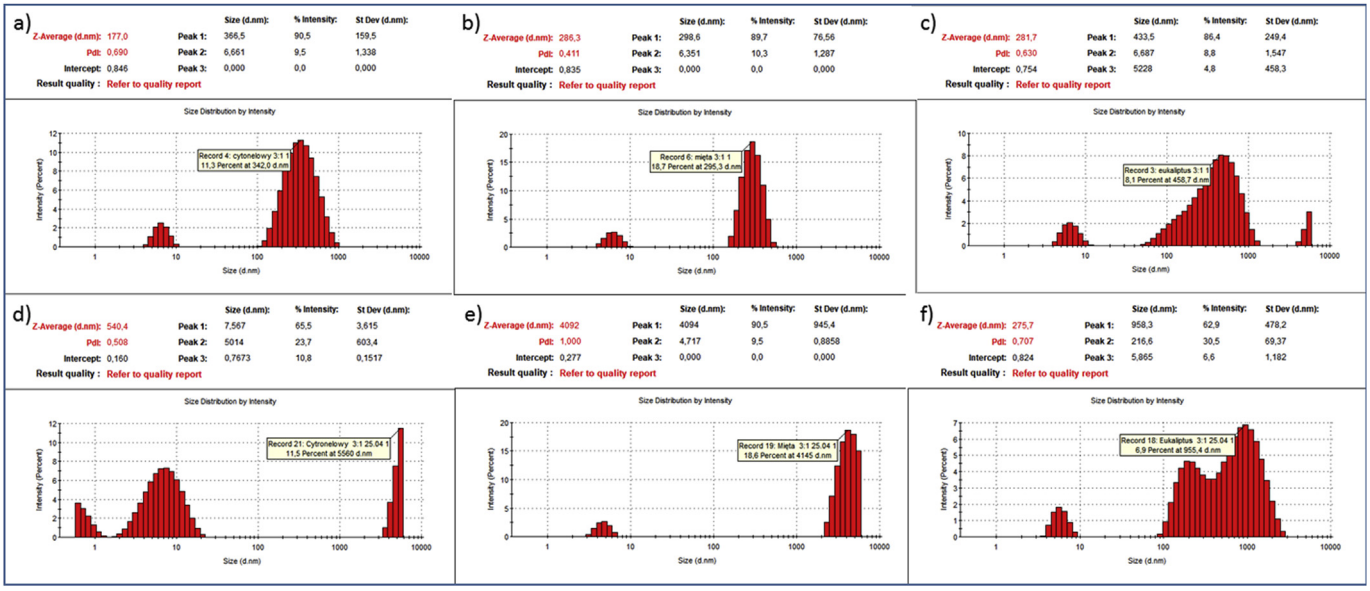

Fig. 2. Particle size distribution in undiluted microemulsions: a) and d) refer to citronella EO; b) and e) refer to mint EO; c) and f) refer to eucalyptus EO. The studies were performed after: 1 day of incubation a), b), c); 7 days d), e), f).

3.2. Evaluation of antioxidant properties of EO and EO in ME

Antioxidant activity evaluated for EO dissolved in methanol at concentration of 5 mg/ml and expressed as percent of inhibition ranged from 2.76% to 32.15% and from 8.28% to 77.75% for EO in microemulsions indicating the enhanced antioxidant activity of EO/ME. The increase in activity was 13.96%, 22.25% and 45.60% for eucalyptus, mint and citronella EO, respectively (Fig. 5).

3.3. Evaluation of cytotoxic properties of ME and EO

The evaluation of *in vitro* cytotoxicity of ME formulations containing EO (EO/ME) in comparison to EO dissolved in DMSO (EO/DMSO) on Vero and HeLa cells after 24 h incubation is shown on Fig. 6 and Fig. 7, respectively. In case of Vero cells, EO/ME showed similar cytotoxicity regardless of the tested EO. When EO/DMSO were tested noticeable differences could be observed, e.g. EUC/DMSO and CITR/DMSO showed identical cytotoxicity profile and MINT/DMSO was noticeably less toxic. Whereas, in case of HeLa cells, CITR/ME was more toxic than other EO/ME and the results of EO/DMSO were comparable to those obtained for the Vero cells. In both cell lines the cytotoxicity of ME used as a solvent was noticeably higher than DMSO. What is more, the ME and EO/ME showed an inhibitory effect on tested cells in all tested series of 2-fold dilutionsin the cell media with the exception of the last concentration (0.0019%). The analysis of cytotoxicity profiles of ME and EO/ME in both cell lines indicates that the vehicle (ME) contribute

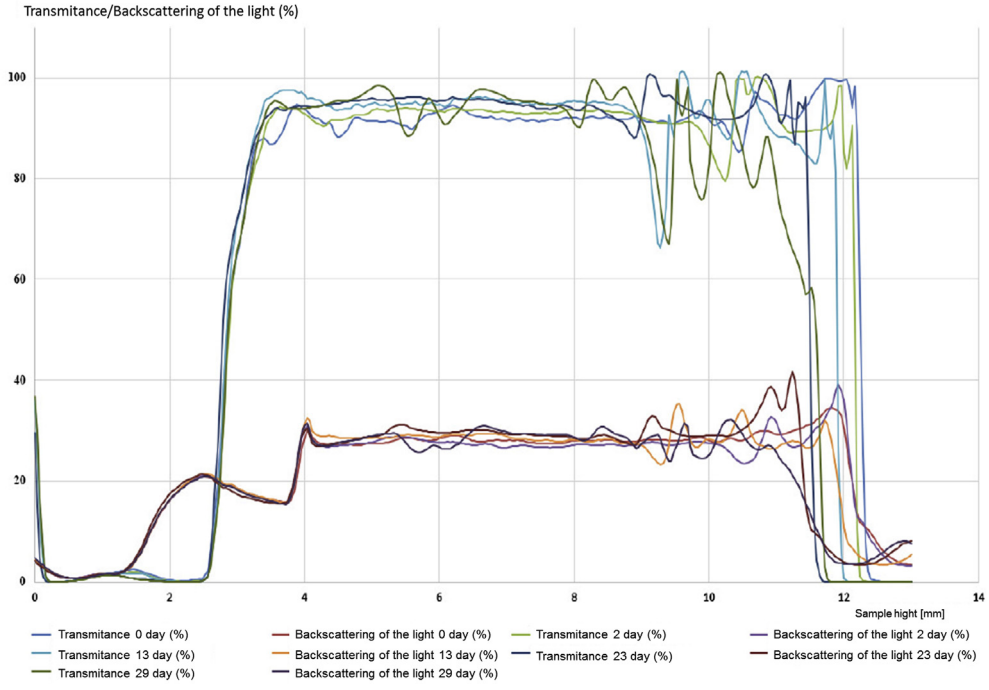

Fig. 3. Depicts the transmittance and backscattering of the light in undiluted ME of eucalyptus EO during 30 days period of time.

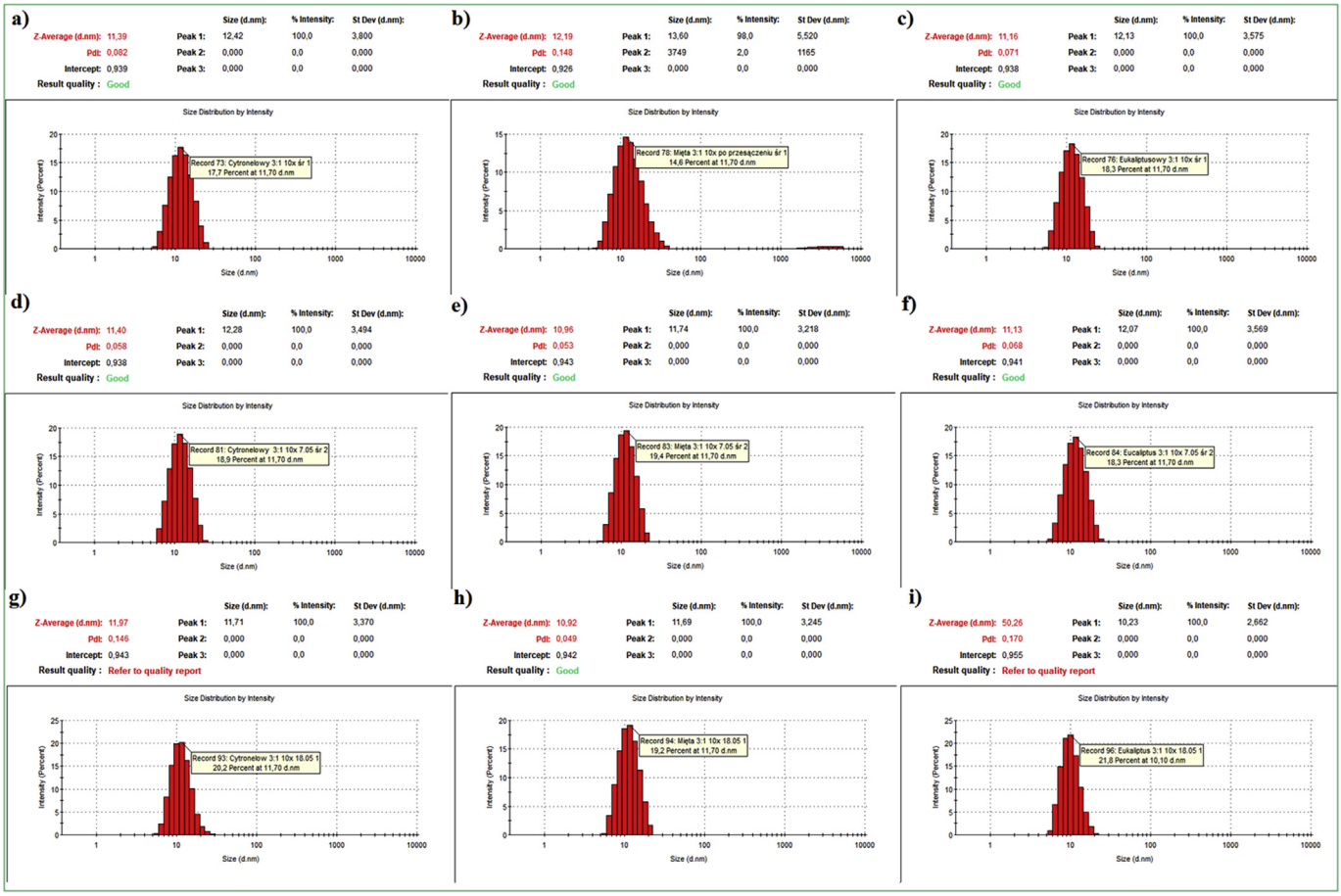

Fig. 4. Particle size distribution in diluted microemulsions: a), d) and g) refer to citronella EO; b), e) and h) refer to mint EO; c), f) and i) refer to eucalyptus EO. The studies were performed after: 1 day of incubation a), b), c); 7 days d), e), f); 17 days g), h), i).

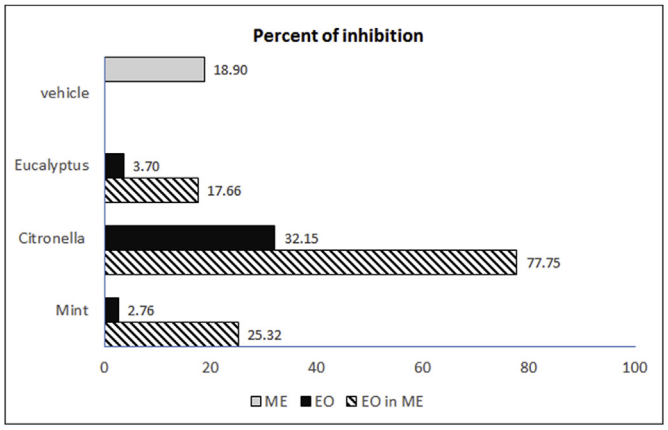

Fig. 5. Antioxidant activity of vehicle, EO and EO incorporated into ME, evaluated at 5 mg/ml concentration and expressed as percent of inhibition.

to the observed cytotoxic effect. The only exception was CITR/ME on HeLa cells where a probable synergistic effect between EO and ME could be responsible for higher toxicity towards this cancer cell line. The cytotoxic influence of DMSO on Vero cells was observed in 2% concentration while in case of HeLa cells it was noticeable beginning from 1% of DMSO.

The CC_{50} values presented in Table 1 are the percentage of tested formulations (EO/ME, EO/DMSO) decreasing cell viability by 50%. Statistically highly significant differences were found for MINT/ME and MINT/DMSO on VERO cells as well as for ME and DMSO on both tested cell lines. All EO/ME showed similar CC_{50} on VERO cells but in case of HeLa the CC_{50} of CITR/ME was noticeably lower compared to other EO/ME, but the difference was not statistically significant.

In case of Vero cells, the cytotoxicity was also evaluated after 4, 8 and 12 h of incubation (Fig. 8). Interestingly, after 4 h of incubation, the citronella and eucalyptus EO showed similar toxicity regardless of the tested vehicle. Whereas, in case of mint EO after 4 h, the MINT/ME was significantly more toxic than MINT/DMSO. After 8 and 12 h incubation all samples showed higher cytotoxicity when ME was used as a solvent. What is more, the cytotoxicity of ME after 12 h was noticeably lower than those observed for all EO/ME. It can be concluded, that during the first 12 h of incubation ME increases cytotoxicity of EO when used as a solvent. This can be clearly seen when analysing the profiles of cytotoxicity at lower concentrations, below 0.25%. After 24 h, the cytotoxicity of ME itself determines the cytotoxicity of all EO/ME on Vero cells (Fig. 6).

3.4. Recovery of volatiles from culture medium

For the recovery of volatiles from cells culture medium the ME containing citronella and eucalyptus EO were selected to cover wide range of studied compounds. At the initial time, the composition of citronella EO constituents dissolved in cell culture medium using ME and DMSO was rather similar (Table 2). The noticeable difference observed at initial time was the higher content of limonene obtained via ME. The content of limonene in a medium with ME was decreasing during 24 h, however when limonene was applied in DMSO it was not detected after 4 h of incubation. Linalool and citronellal presented decreasing trend in concentration regardless of the carrier used, but their

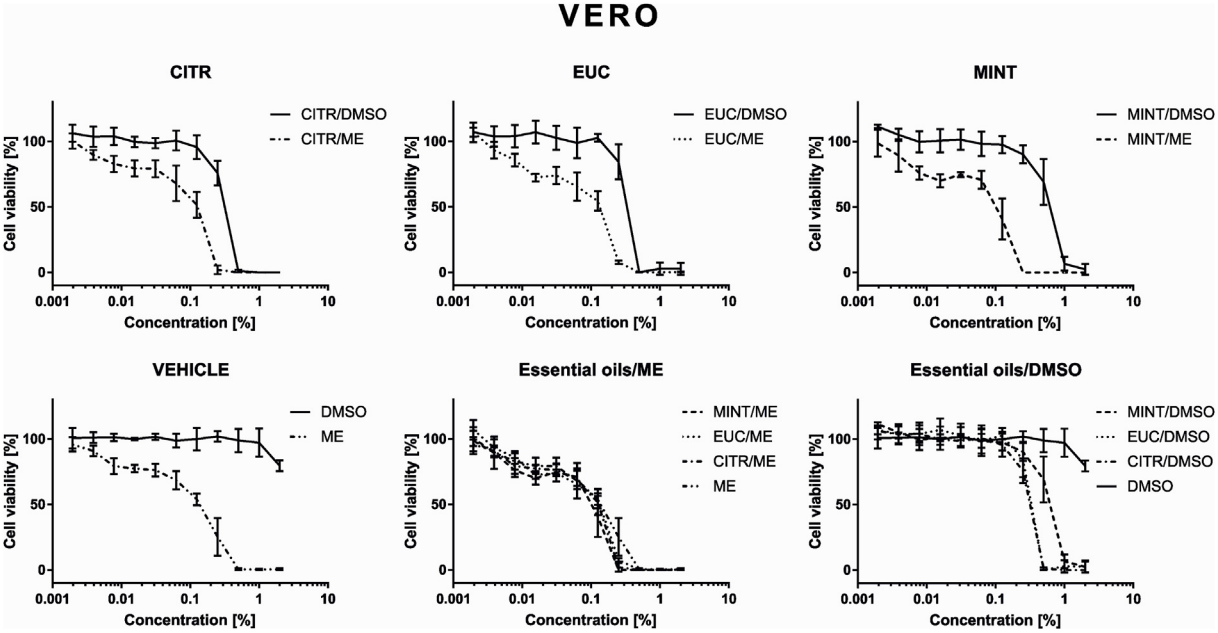

Fig. 6. Cytotoxicity of tested microemulsions containing essential oils on Vero cells after 24 h incubation. CITR – citronalla EO; EUC – eucalyptus EO; MINT – mint EO.

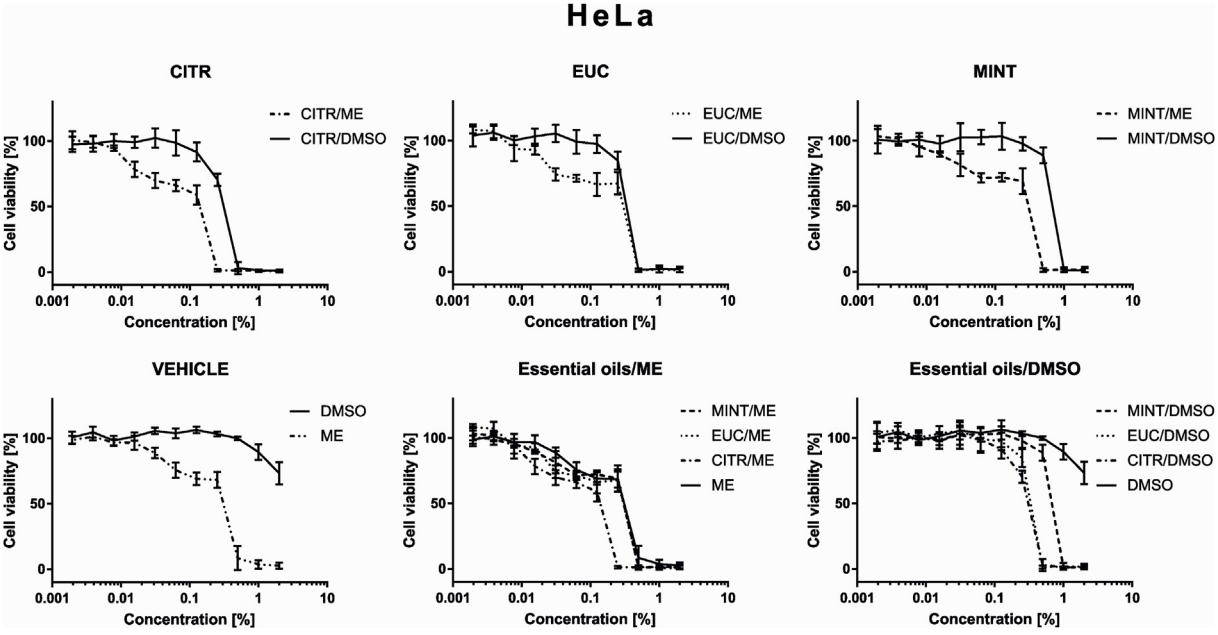

Fig. 7. Cytotoxicity of tested microemulsions containing essential oils on HeLa cells after 24 h of incubation. CITR – citronalla EO; EUC – eucalyptus EO; MINT – mint EO.

percentages in the sum of detected compounds at the final time (24 h) were higher in case of CITR/ME. The majority of constituents dissolved in DMSO decreased their concentration or disappeared in time (Table 2), with the exception of citronellol, geraniol, eugenol and elemol, for which the peak area percentages were higher over time in both samples (ME and DMSO), but for DMSO only in case of eugenol this tendency has continued until the end of incubation time. ME provided better solubility of constituents of citronella EO in cell culture medium (Table 2), which were recovered in similar (*trans*-citral, citronellol acetate, geranyl acetate, β-elemene, 4-*epi*-cubedol) or higher (citronellol, geraniol, eugenol, germacrene D and elemol) amounts over incubation time.

The constituents of eucalyptus EO dissolved in ME or DMSO and recovered from cells culture medium at the initial time were comparable (Table 3). When ME was used as vehicle the amounts of majority of compounds detected were higher over incubation time indicating slow release of volatiles from ME. At the last time point (24 h) the peak area percentages of *trans*-pinocarveol, pinocarvone, α-terpineol acetate and aromadendrene decreased, however they were still higher than at initial time (Table 3). When DMSO was used as solubiliser of eucalyptus EO the area percent content of α-pinene, eucalyptol and α-terpineol acetate started to decrease from the second time point (4 h). After 24 h, α-pinene, α-terpineol acetate and aromadendrene were not detected in the medium, while the majority of other constituents decreased their percentages comparing to starting time point. Globulol and β-eudesmol kept their content at a level higher that at initial time, but only in case

Table 1
The CC50 (%) values of tested formulations on VERO and HeLa cells after 24 h incubation.

	CC_{50} (SD) [%]			
	VERO	*p*	HeLa	*p*
MINT/ME	0.121 (0.011)	*	0.289 (0.048)	*ns*
MINT/DMSO	0.588 (0.042)		0.563 (0.021)	
EUC/ME	0.116 (0.021)	*ns*	0.315 (0.075)	*ns*
EUC/DMSO	0.269 (0.021)		0.275 (0.013)	
CITR/ME	0.129 (0.012)	*ns*	0.111 (0.016)	*ns*
CITR/DMSO	0.291 (0.020)		0.290 (0.013)	
ME	0.158 (0.022)	*	0.286 (0.050)	*
DMSO	> 2		> 2	

ns – not significant; *p < 0.0001; CC_{50} are percentage (%) of tested formulation in cell media causing the decrease of cell viability by 50%, SD – standard deviation.

of β-eudesmol the area percent was similar to this provided by ME.

The other interesting observation was the presence of several constituents of essential oils in the blanks. The blank which was placed on the same experimental plate as citronella EO (dissolved in ME or DMSO) contained linalool, citronellal, citronellol and geraniol (Fig. S1). The intensity of peaks characteristic for these compounds was increasing till the fourth time point (12 h) and then decreased (Table S1). Similarly, in case of eucalyptus EO, the blank contained eucalyptol, *trans*-pinocarveol, pinocarvone and α-terpineol acetate. The largest peak area was observed for eucalyptol in the third time point (Table S1 and Fig S2). In the final time point (24 h) detected volatiles were on a quite low level. The presence of EO constituents in the control samples was caused by their highly volatile properties and underlines the limitations of *in vitro* studies of EO and other volatile substances.

Besides essential oils constituents, the volatile metabolites of Vero cells were also recovered from the studied samples. The abundance of these metabolites (Table S2) can be correlated with cells viability in the experimental conditions. Compounds present in blanks had higher percentage peak areas comparing to samples under the direct influence of EO suggesting that their metabolic activity was not disturbed. The highest abundance of these volatiles was observed at the final time point (24 h) what can be explained by metabolites accumulation. Comparing the carrier used, the application of ME as solubiliser for EO caused the reduction of content of cells metabolites in the samples

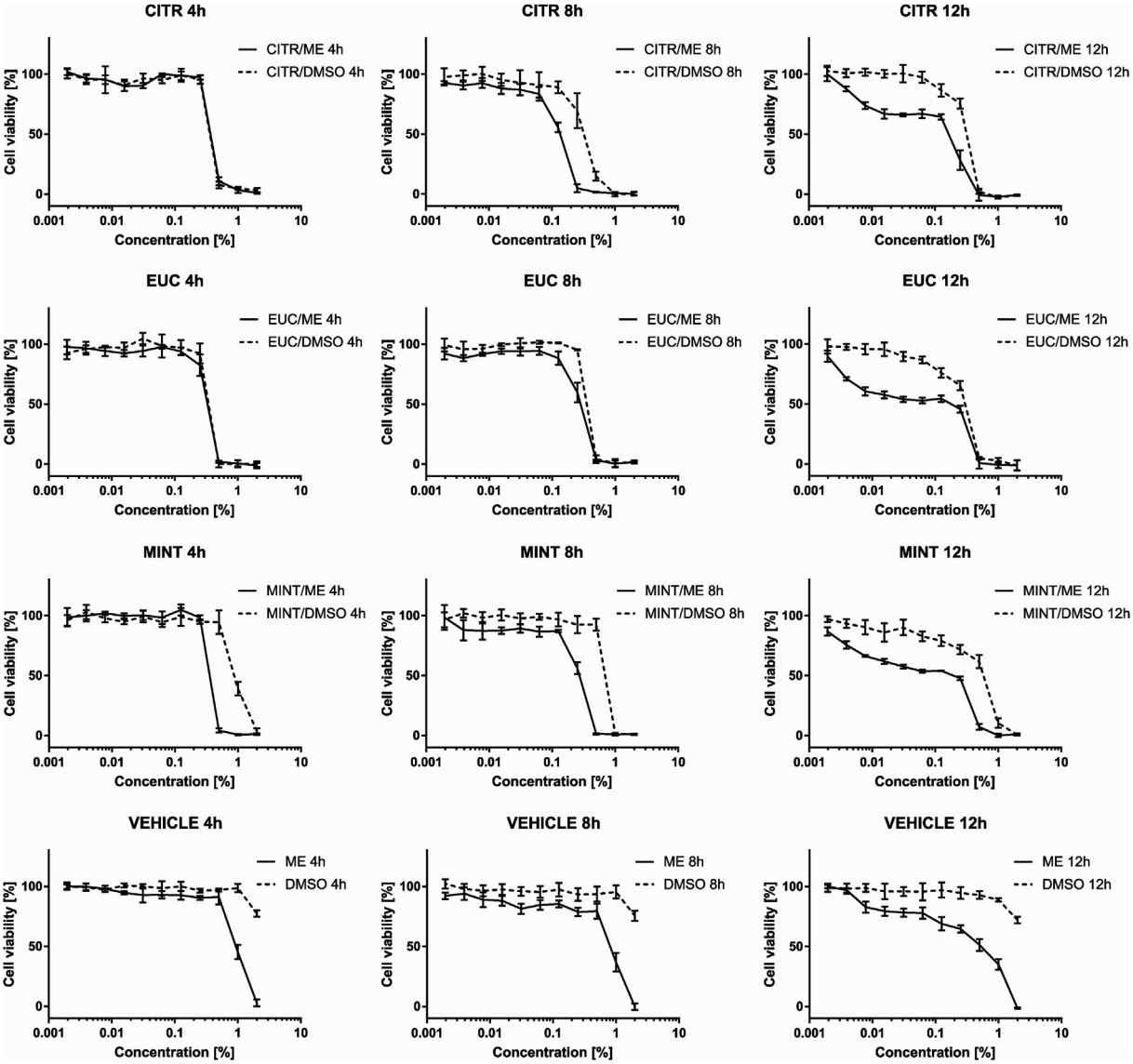

Fig. 8. Comparison of cytotoxicity of tested microemulsions containing essential oils on Vero cells after 4, 8 and 12 h of incubation. CITR – citronalla EO; EUC – eucalyptus EO; MINT – mint EO.

Table 2
The recovery of citronella EO constituents solubilised in cell culture medium in ME and DMSO.

	RI^{ex}	RI^{lit}	CITR/ME peak area (%)					CITR/DMSO peak area (%)				
			0 h	4 h	8 h	12 h	24 h	0 h	4 h	8 h	12 h	24 h
Limonene	1029	1018	3.7	1,8	1.1	0.8	–	0.9	–	–	–	–
Linalool	1099	1082	0.7	0.6	0.7	0.6	0.5	0.8	0.8	0.8	0.7	0.3
Citronellal	1151	1125	39.0	32.1	29.6	23.1	5.5	38.1	15.1	13.2	9.2	2.6
Citronellol	1226	1179	15.0	16.5	18.1	20.5	24.0	16.7	22.1	24.1	24.3	16.1
Geraniol	1250	1228	23.8	26.2	28.7	31.7	37.1	28.0	43.8	48.2	51.7	45.7
trans-Citral	1266	1174	1.7	1.6	1.7	1.6	1.4	2.0	1.7	1.7	1.6	0.7
Citronellolacetate	1344	1302	2.5	2.9	2.8	3.2	2.4	1.8	0.7	0.7	0.7	–
Eugenol	1354	1392	0.7	0.6	0.8	0.9	1.1	0.9	1.4	1.5	1.7	1.8
Geranylacetate	1372	1352	3.3	3.6	3.6	3.9	2.9	2.9	1.3	1.1	1.7	0.4
β.-Elemene	1390	1398	1.0	1.3	1.0	1.3	1.1	0.6	0.3	–	–	–
4-*epi*-cubedol	1486	1454	0.4	0.6	0.4	0.6	0.6	0.3	–	–	–	–
Germacrene D	1520	1515	0.7	1.0	0.8	1.1	1.2	0.4	0.2	–	–	–
Elemol	1552	1522	2.8	3.3	3.3	4.2	5.4	2.8	3.1	3.2	3.7	3.4

RI^{ex} – experimental retention index; RI^{lit}-literature retention index; CITR/ME – citronella EO in microemulsion; CITR/DMSO - citronalla EO in DMSO.

Table 3
The recovery of eucalyptus EO constituents solubilised in cell culture medium in ME and DMSO.

	RI^{ex}	RI^{lit}	EUC/ME peak area (%)					EUC/DMSO peak area (%)				
			0 h	4 h	8 h	12 h	24 h	0 h	4 h	8 h	12 h	24 h
α-Pinene	933	948	3.6	4.1	3.0	2.0	–	1.8	0.3	–	–	–
Eucalyptol	1033	982	69.7	48.7	47.6	26.5	1.2	74.6	53.4	51.8	31.2	1.5
trans-Pinocarveol	1145	1131	3.5	4.3	5.9	7.3	6.4	4.1	7.4	9.6	11.5	7.2
Pinocarvone	1165	1132	1.3	1.4	1.8	1.8	0.7	1.5	1.7	2.0	1.9	0.5
α-Terpineol	1198	1143	1.6	2.2	2.9	3.8	4.2	1.9	4.2	5.5	7.2	6.6
α-Terpineol acetate	1364	1333	2.5	4.6	5.0	7.2	4.4	2.1	1.8	1.9	1.6	–
Aromadendrene	1444	1386	1.5	3.8	3.0	5.4	4.0	1.0	1.0	0.7	0.8	–
Epiglobulol	1570	1530	0.8	1.5	1.5	2.7	3.5	0.5	0.9	1.0	1.6	1.3
Globulol	1594	1530	4.1	7.6	8.0	13.8	17.5	3.6	6.4	7.5	12.5	12.6
Cubenol	1603	1580	0.9	1.9	1.9	3.3	4.4	0.7	1.3	1.1	2.1	1.8
β-Eudesmol	1664	1593	1.5	2.7	2.8	5.2	6.5	1.4	2.6	3.1	5.3	6.6

RI^{ex} – experimental retention index; RI^{lit}-literature retention index; EUC/ME – eucalyptus EO in microemulsion; EUC/DMSO - eucalyptus EO in DMSO.

(Table S2).

4. Discussion

Microemulsions have a significant potential to increase dispersion of hydrophobic molecules in water environment and this property has been successfully used in various pharmaceutical products. The formulation of EO/ME enables to achieve concentrations of EO unattainable for simple dispersions. Incorporation of EO into nanoparticles ranging from 10 to 100 nm can provide a significant increase in the surface of the oil phase. At the same time the presence of soybean oil ensures better dilution of ME with water (Ma and Zhong, 2015) and protection of volatiles from evaporation (Kim et al., 1995). In this work stable formulations were obtained for a maximum 60% of aqueous phase (Fig. 1), however, Ma and Zhong (2015) reported a clear ME wit thymol containing 90% of aqueous phase. Although studied ME were similar in composition, various volatiles present in essential oils, which are complex mixtures comparing to the single component thymol resulted in different characteristics. Our studies of EO/ME, as well as these performed by Ma and Zhong (2015), confirmed stability of undiluted ME over time. The particle size distributions measured in undiluted ME showed the presence of larger particles in the system, which were aggregates of nanoparticles (Fig. 2). Such cylindrical, hexagonal and cubic aggregates can be present in undiluted systems where water occupies only 25% of volume (Brinker et al., 1999). After dilution with water aggregates were no longer detected and diluted formulations were also stable (Fig. 4). The obtained stable formulations of EO in ME were then tested for their antioxidant and cytotoxic activity in order to compare it with the activity of EO solubilised in typical solvents.

Since volatile character of EO constituents is an important issue during activity testing the good solubilisation is crucial to make tested substances available for interactions with cells. The data presented in this study (recovery tests) confirmed that ME provide good solubility of constituents of EO and diminished evaporation of volatiles what is in agreement with previous reports (Kim et al., 1995). Although EO solubilised with ME and DMSO presented similar chemical profiles at the initial time, ME prevented evaporation of more volatile constituents (e.g. α-pinene, limonene) and ensured prolonged interaction with tested cells.

Determination of antioxidant activity of EO and ME containing EO showed that incorporation of EO into ME formulation enables much easier reaction of antioxidants with DPPH free radical. This condition is due to the fact that the oil phase in the ME system has a larger surface area as a result of the decrease of the size of oil droplets. With the distribution of one drop into 8 minor droplets a two-fold increase in the surface of the oil phase is achieved. Antioxidant activity of ME systems is also strongly dependent on the amount of water phase and significantly increases with an increase of water phase even though the concentration of the EO decreases (Deng et al., 2015). This suggests a significant influence of microemulsion structure on the reactivity with DPPH reagent. During studies an antioxidant activity of ME formulation without EO (vehicle) was also observed (Fig. 5). This could contribute to increased activity of EUC/ME. Observation similar to ours regarding increase in antioxidant activity of EO/ME against DPPH free radical was reported by Hamed et al. (2012) for ME containing eugenol and clove bud essential oil comparing to individual components. Whereas the opposite results were obtained by Kim et al. (2009) who evaluated antioxidant potential of tea tree EO and its ME, showing a decrease of

antioxidant activity of ME. This scatter of results was previously explained by different chemical character of EO constituents encapsulated in ME and their varied diffusion within nanoparticles causing preferable interactions of compounds placed in the interfacial layer (Hamed et al., 2012; Kim et al., 2009). However, an additional factor, such as a surfactant and co-surfactant used should also be considered. The increase of antioxidant potential was observed for formulations with Tweens (this study and Hamed et al., 2012) while decrease occurred for formulations with sucrose laurate (Kim et al., 2009). The significantly larger interfacial layer area formed by tweens may contribute to higher attainability of incorporated compounds for interactions with DPPH radical. Thus, in order to achieve high antioxidant activity of ME formulations, the ME structure and appropriate selection of surfactants should be taken into consideration.

The surfactants should be carefully selected also because of their possible cytotoxic effects. As was shown by Arechabala et al. (1999) increasing cytotoxicity of surfactants evaluated against human fibroblast cultures was placed in the following order: Tween 80 < Texapon N40 < Tween 60 < Texapon K1298 < Triton 3 × 100 < benzethonium chloride (Arechabala et al., 1999). Since the Tween 80 was described as the least toxic compound, it was also used in our experiments. Our data revealed that EO/ME had higher cytotoxicity than EO/DMSO, however, the results were statistically significant only in case of mint EO on Vero cell line. Statistically highly significant differences were observed between vehicles (ME and DMSO) on both studied cell lines. The evaluation of cytotoxicity against Vero cells after 4, 8 and 12 h of incubation, showed that during the first 4 h of incubation the EO strongly contributed to the reduction of cell viability. Later, until 12 h of incubation, ME increased cytotoxicity of EO when used as a solvent, whereas after 24 h, the cytotoxicity of ME itself determined the cytotoxicity of all EO/ME on Vero cells.

Data related to the non-cytotoxic effects of Apiaceae essential oils in ME against invertebrates was recently reported (Pavela et al., 2019). However, reports related to cytotoxicity of ME of EO against eukaryotic cells are quite limited. The study performed by Mektrirat et al. (2016) showed mild cytotoxic effect of clove essential oil ME on peritoneal macrophages. Also Cespi et al. (2017) reported a decrease in cell viability of HCT116 human colon carcinoma cell line after the exposure to *Smyrnium olusatrum* essential oil in ME. Our results are in accordance with above cited references, additionally indicating that the vehicle in essential oil ME based on Tween 80 determines its cytotoxicity after 24 h of incubation. The intrinsic cytotoxicity of unloaded microemulsion observed previously (Cespi et al. (2017) was confirmed in our study but differences in activity between EO and EO/ME were statistically significant only in one case (mint EO). ME of EO were studied extensively against different microorganisms, proving their usefulness in inhibiting the growth of bacteria and molds (Basak and Guha, 2017; Ghosh et al., 2013; Ghosh et al., 2015; Jantrawut et al., 2018; Raffaella et al., 2017; Shaaban et al., 2015; Taufik et al., 2018; Valizadeh et al., 2018, Viyoch et al., 2006, Wang et al., 2014; Zhang et al., 2014). What is more, even ME prepared without EO showed ability to inhibit microbial growth (Zhang et al., 2008, 2009). This indicates the cellular toxicity of ME formulations. This activity can be mainly attributed to the presence surfactants. The cellular toxicity of Tweens and Tween based microemulsion formulations may be a result of either solubilisation or osmotic lysis, depending on the concentration of the surfactant. In low concentrations surfactant molecules are intercalated into the membrane and change the membrane permeability and at high concentrations they cause cell lysis via solubilisation. What is more, the absorption enhancing mechanism described for ME is known to be closely related to the damage of lipid membranes and proteins (Kaur and Mehta, 2017). In case of EO/ME, the activity of EO constituents is based on the similar mechanism of action (changing the permeability of the outer membrane) (Andrade-Ochoa et al., 2015) resulting in the potentiation of cytotoxic activity. Importantly, further studies, preferably also using animal models, are needed to confirm the safety of EO/ME, which can develop different reactions in living organisms.

Summing up, the data presented in this study indicate that ME formulations influence the cytotoxic activity of tested EO, however, to different extent, depending on the EO used. At the same time ME provided good solubility of constituents of EO and diminished evaporation of volatiles from culture medium.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.fct.2019.04.038.

Transparency document

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