Low energy nanoemulsions as carriers for essential oils in topical formulations for antioxidant skin protection

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Abstract

In this study several essential oils (EOs): basil – BA, lemon balm – LB and oregano – OR were incorporated into nanoemulsions (NEs) as prospective carriers for natural and sensitive bioactives. NEs were prepared *via* the phase inversion composition (PIC) method, which is an energy-efficient cold process. Physicochemical stability of NEs was confirmed by particle size distribution analysis, electrical conductivity and pH value measurements, as well as by optical microscopy observations. The type of EO and the surfactant and oil mix concentration were found to be crucial factors governing the NE properties and stability. Raman spectra of the EOs confirmed main active ingredients and provided detection of interactions with the nanocarrier, which is a novel application of this technique. The antioxidant activity towards DPPH radical in methanol was concentration-dependent with a similar trend for individual oils and oil-loaded NEs (OR> LB> BA). However, the ABTS test in an aqueous medium revealed notable change in the order of activity after EO nanonisation at higher EO concentrations. Overall, it was found that OR-NE was the most effective and stable system, since OR acted as a co-stabiliser in the NE formulation, and its remarkably high antioxidant activity was successfully preserved during 6 months of storage.

Keywords: oregano; basil; lemon balm; nanonisation; Raman spectroscopy; stability.

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1. INTRODUCTION

Aromatic plants have been used since ancient times as a source of essential oils (EOs), which are known as natural remedies in traditional medicine systems – phytotherapy and aromatherapy. They are also widely used for everyday purposes, for example, as fragrant components in skincare preparations and perfumery, and as flavouring agents and natural preservatives in food industry [1−3]. With respect to chemical composition, EOs represent complex mixtures of various volatile and lipophilic molecules that can be classified as terpenoids (monoterpenes, sesquiterpenes, and diterpenes), phenylpropanoids, and other low molecular weight molecules (aliphatic hydrocarbons, acids, alcohols, aldehydes, and esters). In each EO, there are usually two or three principal compounds which can be used for the EO identification and these molecules are usually linked to the oil bioactivity and application (*i.e.*, antimicrobial, anti-inflammatory, antioxidant, and anticarcinogenic activities, *etc.*) [1,4,5].

Despite the renewed popularity of EOs in personal care and pharmaceutical products, their volatile nature and sensitivity to heat, light and air require optimised formulations to preserve the activity and to avoid side effects related to the usage [2−4,6]. Since EOs have to be diluted for the use, different carrier systems such emulsions [7] and nanoemulsions [8,9] are proposed as a convenient solution for oil solubilization into the aqueous-based, oil-in-water

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(O/W) products. Nanoemulsions (NEs), particularly those produced by low energy methods, can fulfil the above-mentioned requirements and ensure optimal characteristics for topical application. NEs of the O/W type represent colloidal dispersions of oil in the aqueous phase, in the form of ultra-fine nano-droplets (preferably < 200 nm), stabilised by a surfactant (and/or co-surfactant) monolayer. Due to fine droplet sizes, NEs have a pleasant visual appearance with characteristic bluish shine (they can be milky white, or semi-transparent, if small droplet sizes of < 100 nm are achieved) [10−12]. NEs can provide modified release and stabilisation of delicate ingredients, so that improved physical stability is expected as compared to conventional emulsions, while at the same time NEs have better safety profiles compared to microemulsions, which are typically produced with higher surfactant concentrations [12,13]. Due to the good spreadability of NEs, homogenous and compact film of nano-droplets is formed on the skin surface, enabling better skin hydration and enhanced penetration of active substances as compared to conventional emulsions [12,14]. Moreover, an important property of O/W NEs is that they can be diluted with water without damaging the emulsion structure: for example, they can be used as concentrated products (*i.e.*, NEs with orange or lemon essential oil), which can be incorporated in beverages and other food systems, acting as flavouring agents [15,16]. It is also possible to convert NEs into nanoemulgels either by adding rheology modifiers directly into the NE aqueous phase [17] or by adding the NE into the gel [18]. Therefore, NEs are particularly suitable for potential applications in pharmaceutical or cosmetic products of different consistencies (*i.e.*, sprays, lotions, creams or gel-nanoemulsions) [12,19].

It is known that many EOs contain molecules with considerable antioxidant activity, such as phenolic compounds, flavonoids and terpenoids, acting alone or synergistically [20,21]. Besides antimicrobial action [22], EOs prepared from basil−BA (*Ocimum basilicum*), oregano – OR (*Origanum vulgare*) and lemon balm − LB (*Melissa officinalis*) leaves are reported to scavenge free radicals [9,23−25]. Therefore, these particular EOs and their bioactives could be good candidates for skin-protective formulations. However, there is a lack of literature data on the concentration-dependent and substrate-dependent (type of free radical) behaviour of these EOs. Moreover, comparative studies done with neat EOs and EO-loaded NEs are scarce, although such studies could reveal important information regarding potential usage of these systems in pharmaceutics and cosmetics.

Having all the above mentioned in mind, this research was organized into three main parts.

1. Preparation of stable NEs containing 1 wt.% EOs (BA, OR or LB) with polyethylene glycol free (PEG-free) natural surfactant mixture (containing polyglycerol-4 laurate as the main surfactant) suitable for cold processing *via* the phase inversion concentration (PIC) method [26].
2. Screening of chemical compositions of EOs and EO-loaded NEs *via* Raman spectroscopy, as a suitable and quick method that can identify chemical compositions of plant and essential oils [26,27] and track the interactions between formulation ingredients [10]. These investigations aimed to reveal additional information on the composition/ structure of EOs before and after nanoemulsification, which can be linked to the stability and performance of EO-loaded NEs.
3. Screening study of antioxidant activity of neat EOs and EO-loaded NEs was performed by using different sample concentrations and methods (DPPH and ABTS) to gain insight into free radical scavenging profiles of EOs before and after nanoemulsification, as a proof of concept that NEs can be used as effective carriers of EOs with antioxidant properties.

2. MATERIALS AND METHODS

2. 1. Chemicals and reagents

2. 1. 1. Essential oils

For this study several EOs prepared from leaves of plants from the *Lamiaceae* family were used: *Ocimum basilicum* (basil – BA), *Origanum vulgare* (oregano – OR) and *Melissa officinalis* (lemon balm – LB). EOs were produced by Aromaaz International, New Delhi, India, for domestic brand Eterra/company Terra Co, Novi Sad, Serbia, and they were obtained from a local distributor with the certificates of analysis.

2. 1. 2. Nanoemulsion ingredients

In this study a commercially available mixture of PEG-free surfactant and oil (SO mix) was used, consisting of polyglyceryl-4 laurate and dilauryl citrate, diethylhexyl carbonate, phenoxyethanol (Tego®Wipe DE PF, Evonik Goldschmidt GmbH, Essen, Germany) without parabens. The water phase was ultra-purified water (obtained by a GenPure apparatus, TKA Wasseranfbereitungssysteme GmbH, Neiderelbert, Germany) without any additives.

2. 1. 3. Reagents for antioxidant assays

The following reagents were used: 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), potassium persulfate, (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox) and methanol (HPLC grade), all produced by Sigma-Aldrich, Steinheim, Germany. Phosphate buffered saline (PBS buffer, pH 7.4) was prepared fresh before the ABTS assay.

2. 2. Nanoemulsion preparation and characterisation

2. 2. 1. Phase inversion composition (PIC) method

In order to preserve the stability and activity of EOs, EO-loaded NEs were prepared by using the phase inversion composition (PIC) method at room temperature (RT), which is an energy-efficient cold process [11,12,26]. Firstly, one of the selected EOs – BA, OR or LB (1 wt.%) was added to the commercially available SO mix (5 or 10 wt.% in the final NE) and vortex mixed for 3 min at 1300 rpm. Then, ultra-purified water was added gradually to the SO-EO mix (at continuous vortex-mixing at 1300 rpm), until the total water content of 89 or 94 wt.% was reached, and the obtained NEs were shortly homogenized (3 min at the same stirring speed). All samples were prepared in triplicate.

2. 2. 2. Nanoemulsion particle size distribution

Z-average droplet size (*Z*-ave) and polydispersity index (PDI) as crucial parameters describing particle size distribution were obtained by a dynamic light scattering (DLS) device (Zeta Sizer Nano ZS, Malvern Instruments, Malvern, UK) applied to NEs freshly diluted with ultra-purified water (1:100 v/v), as a standard procedure to avoid multiple light scattering [9,16]. Measurements were performed in triplicate, initially 24 to 48 h after preparation, then after 30 days, and finally after 6 months of storage at RT (stability study).

2. 2. 3. pH value and electrical conductivity

Electrical conductivity of undiluted intermedium/transient phases and NEs was measured by using the Sension+EC71 apparatus (HACH, Loveland, CO, USA). The pH value of the undiluted NEs was measured by using a HI9321 microprocessor pH meter (Hanna Instruments Inc., Ann Arbor, Michigan, USA). The measurements were performed in triplicate at 25±2 °C.

2. 2. 4. Optical microscopy

Optical microscopy was employed to investigate the signs of anisotropy in the intermedium phases and the corresponding EO-loaded nanoemulsions. Micrographs of undiluted samples were taken by an Olympus BX53P polarizing microscope, at 100x magnification. The obtained images were analysed by the Olympus cellSens software Entry version 1.14 (Olympus, Tokyo, Japan).

2. 3. Raman spectroscopy

Raman spectra excited with a diode-pumped solid-state laser (excitation wavelength 532 nm) were collected on a DXR Raman microscope (Thermo Scientific, Waltham, MA, USA), equipped with a research optical microscope, motorized X–Y stage and CCD detector. The Raman spectra were recorded for the small volume (5 µl) samples (EOs or EO-loaded NEs) transferred onto a gold support (Gold EZ-Spot Micro Mount sample slide from Thermo Scientific), which was placed on an X-Y motorized sample stage and the laser beam was focused on the sample by using a 10× objective

magnification. The laser power applied to the sample was kept at 8 mW. The spectra were recorded using an exposure time of 10 s and 10 exposures per spectrum.

2. 4. In vitro antioxidant activity

2. 4. 1. The DPPH method

The DPPH assay was performed in methanol according to our previous work [10], with small modifications. Free radical scavenging activity was tested by mixing different sample amounts with 5 ml of 0.004 % DPPH test solution in methanol, and methanol was added up to 10 ml to obtain total sample concentrations of 0.5, 2.5, 5 and 10 µL ml^{-1} for EOs, or 10, 20, 30 and 50 µL ml^{-1} for EO-loaded NEs in the reaction vials. The samples were kept in dark glass volumetric flasks, with slight shaking every 5 min, at RT. After 30 min, the absorbance was measured at 514 nm by a spectrophotometer (Beckman DU 650, Beckman Coulter, California, USA). The obtained results were expressed as INH, % (DPPH inhibition) according to the equation (1):

$$\mathrm{INH}_{\mathrm{DPPH}} = \frac{A_{\mathrm{DPPH}} - A_{\mathrm{SAMPLE}}}{A_{\mathrm{DPPH}}} 100 \quad (1)$$

where A_{DPPH} is the absorbance of the DPPH standard sample (5 ml of DPPH standard solution mixed with 5 ml of methanol) and A_{SAMPLE} is the absorbance of samples after 30 min. Trolox was used as a standard compound to validate the method, and methanol was used as a blank.

2.4.2. The ABTS method

The ABTS assay was performed in PBS buffer as described in our previous work [10], with small modifications. Free radical scavenging was tested by mixing the necessary sample amounts with 9.9 ml of the activated ABTS^{+} radical solution (absorbance of 0.7±0.05 units), and the reaction vial was filled up to 10 ml with PBS buffer (pH of 7.4). Therefore, the final tested sample concentrations in the reaction vials for the EOs were 0.1, 0.5, 2.5, 5 and 10 µL ml^{-1}, while for the EO-loaded NEs, only 10 µL ml^{-1} could be used because there was an increase in turbidity of the reaction mixtures at higher concentrations. Trolox was used as a standard compound to validate the method, and PBS buffer was used as a blank, except for the EO-loaded NEs (blank was NE in PBS buffer). The mixtures were kept for 20 min at RT (with slight shaking repeated every 5 min), and the decrease in absorbance was measured at 734 nm. The obtained results were expressed as INH, % (ABTS^{+} inhibition) according to the equation (2):

$$\mathrm{INH}_{\mathrm{ABTS}} = \frac{A_{\mathrm{ABTS}} - A_{\mathrm{SAMPLE}}}{A_{\mathrm{DPPH}}} 100 \quad (2)$$

where A_{ABTS} is the absorbance of the ABTS^{+} solution (0.7) and A_{SAMPLE} is the absorbance of samples, after 20 min of reaction time.

2. 5. Statistical analysis

Student's t-test was performed to compare the antioxidant activity of pure EOs before and after nanoemulsification. Two-way ANOVA with Tukey post hoc test was performed for three and more normally distributed data groups when the two factors (for example, EO type and SO concentration, or EO type *vs*. EO concentration) were varied at the same time, with the significance level set to $p<0.05$ (OriginPro8.5, Originlab Corporation, Northampton, MA, USA).

3. RESULTS AND DISCUSSION

3. 1. Nanoemulsions as potential carriers for essential oils

In this study, three different essential oils prepared from the leaves of aromatic plants were incorporated into a natural nanoemulsion carrier system suitable for topical application. Besides antimicrobial action [22], EOs prepared from basil - BA, oregano - OR and lemon balm - LB leaves are reported to scavenge free radicals [9,23−25]. Thus, these particular EOs and their bioactives could be good candidates for topical formulations intended for antioxidant skin protection.

Since EOs are typically natural products, to create natural final formulations it is necessary to use natural surfactants as well. Polyglycerol esters of vegetable fatty acids are naturally-derived, biodegradable and biocompatible surfactants suitable for production of nanoemulsions by using low energy methods [26,29]. It was previously reported that the commercially available PEG-free surfactant and oil (SO) mix consisting of polyglyceryl-4 laurate and dilauryl citrate (main surfactant and cosurfactant), diethylhexyl carbonate (carrier oil) and phenoxyethanol (preservative/cosurfactant) can form O/W NEs by simple dilution with water *via* a low energy mechanism termed as the phase inversion concentration (PIC) method [26]. Since the PIC process does not require heating or high-shear/pressure devices to obtain NE, it is particularly suitable for natural and sensitive ingredients (such as those in EOs), aiming to preserve their bioactivity [10,11,19].

In brief, this eco-friendly and cost-efficient preparation procedure starts from the appropriate surfactant-oil (SO) mixtures, and as water is gradually added, several characteristic intermedium phases appear depending on the water content: W/O emulsion or microemulsion (at low water content), liquid crystalline (LC) phase/ microemulsion (ME)/ /O/W/O multiple emulsion (at the phase transition point), before the system turns into the final O/W NE (at higher water contents) [10−12,26,30]. The crucial intermedium phase at the phase transition point is always characterised by low interfacial tension, thus enabling the formation of nanodroplets [12]. Therefore, it was important to investigate the phase behaviour of the SO-EO mixtures during water titration to detect mutual ratios of the key ingredients, which provided formation of homogenous EO-loaded NEs. It was found that all tested EOs exhibited similar phase behaviour, since they were all found to be compatible with the SO mix used in this study. As it can be seen from the representative phase diagram of OR-loaded NE (Fig. 1), the system passes through different types of liquid phases (W/O emulsion and a bicontinuous emulsion phase) prior to its conversion into the O/W NE, at ~75 wt.% of water in the SOW system. This was in line with the electrical conductivity measurements during the water titration, where a sharp increase in electrical conductivity appeared at ≥45 wt.% of water, indicating a transition from W/O emulsion to a bicontinuous transient phase [26,30]. At this point the excess amount of oil, which was not solubilized yet, was still visible *via* the optical microscope, while at ≥ 75 wt.% of water in the system the oil droplets were not visible anymore since they were fully incorporated into the nanoemulsion submicron system (Fig. 2).

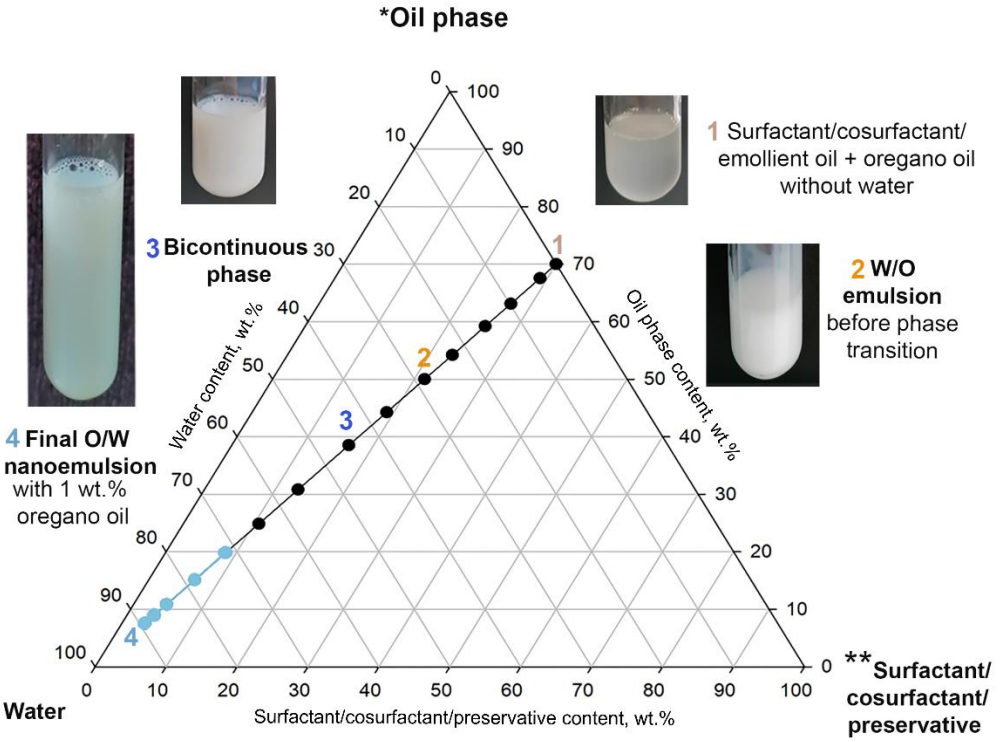

*Figure 1. Phase behaviour of the surfactant/cosurfactant/preservative and oil phase mixture with oregano oil at the optimal ratio 70:30 w/w during the nanoemulsion formation by water titration (according to the phase inversion composition - PIC method). The characteristic intermedium phases are marked with numbers while the nanoemulsion region is marked in blue. *diethylhexyl carbonate: oregano oil = 6.6 : 1 w/w; **polyglyceril-4 laurate: dilauryl citrate: phenoxyethanol = 23.4 : 1 : 13.3 w/w*

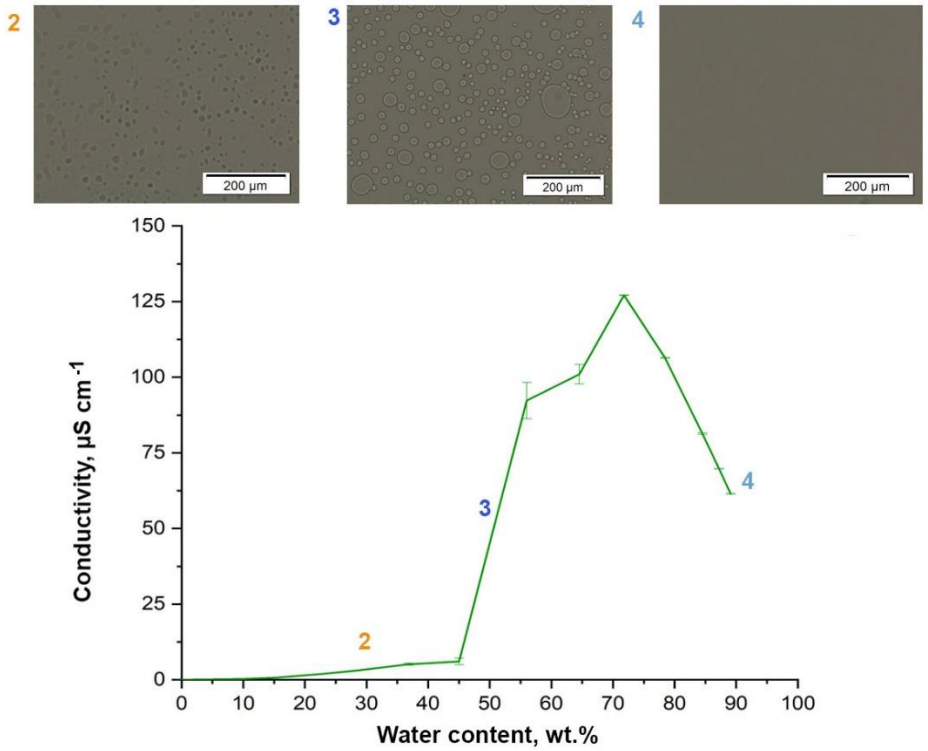

Figure 2. Electrical conductivity curve along the optimal 70:30 w/w surfactant/cosurfactant: oil phase line (the PIC method) and op tical micrographs of the characteristic intermedium phases during the nanoemulsions formation (2,3) and the final nanoemulsion (4) containing 1 wt.% oregano oil

After the NE region was detected, two important factors were investigated simultaneously to optimise the formulation: the type of EO and the SO mix concentration. These parameters were analysed regarding the effects on the *Z*-average droplet size (*Z*-ave) and mean polydispersity index (PDI) of the EO-loaded NEs and the blank NE. It was found that there was a significant difference in *Z*-ave between all EO-loaded NEs and between the EO-loaded NEs and the blank NE (Fig. 3). Influence of the SO mix concentration on the *Z*-ave was also very prominent, with higher SO mix concentration leading to the significant reduction in *Z*-ave in all EO-loaded NEs, contrary to the blank NE. PDI values (Fig. 4) were insignificantly different between the NEs prepared with different EOs or between the NEs prepared with the same EO but at different SO concentrations, since the obtained PDI values were all below 0.15, implying narrow droplet size distribution leading to system stability [23].

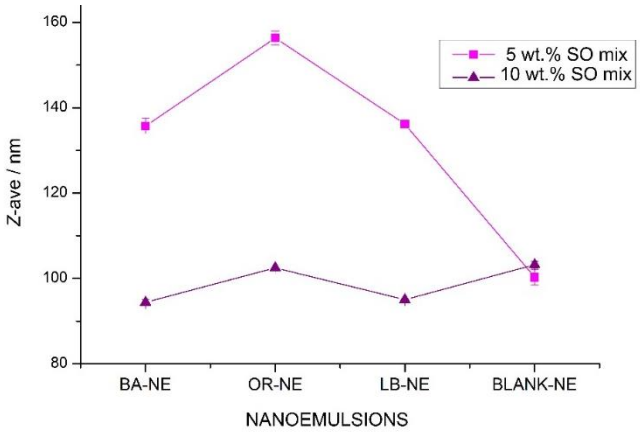

Figure 3. Z-average droplet sizes (Z-ave) in nanoemulsions prepared with different essential oils (BA – basil, OR – oregano, LB – lemon balm) and in the blank nanoemulsion at different concentrations of the surfactant and oil (SO) mix

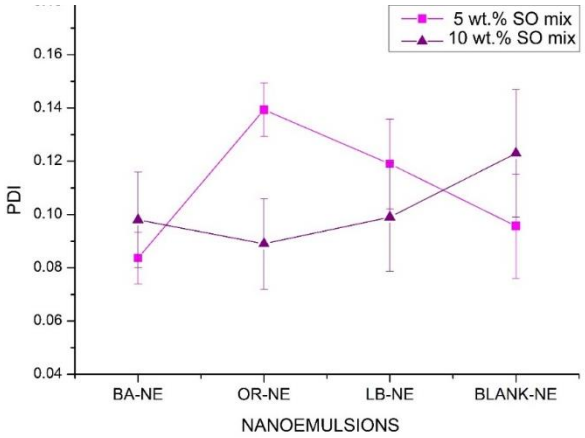

Figure 4. Mean polydispersity index (PDI) of the nanoemulsions prepared with different essential oils (BA – basil, OR – oregano, LB – lemon balm) and the blank nanoemulsion at different concentrations of the surfactant and oil (SO) mix

To conclude, all EO-loaded NEs had uniform droplet size distribution and droplet sizes were in the desired nanorange (*Z*-ave ≤200 nm), confirming the possibility for the formation of NEs with all studied EOs. However, since the EO-loaded NEs prepared with 10 wt.% of the SO mix had significantly lower droplet sizes (*Z*-ave ≤102.5 nm) than the ones prepared with 5 wt.% SO mix (*Z*-ave ≤156.3 nm), for all further investigations the NEs containing 1 wt.% EOs and 10 wt.% of SO mix were chosen.

The physico-chemical properties (*Z*-ave, PDI, pH value and electrical conductivity) of the NEs prepared with 10 wt.% SO mix were measured 24 h and one month after preparation, during storage at RT (Table 1). It was concluded that all EO-loaded NEs were preliminary stable since major signs of instability (*i.e*., creaming or phase separation as a result of a rapid droplet growth) or some other critical changes in the studied physicochemical parameters were not found after one-month storage at RT. According to the literature, the upper limit for the nanoemulsion droplet sizes varies between 100 and 500 nm while better stability is expected in the ones with smaller droplets (preferably ≤ 100 nm) or lower PDI values (preferably < 0.2) [12,13].

Table 1. Mean droplet size (Z-ave), polydispersity index (PDI), pH value and electrical conductivity of essential oil-loaded low energy nanoemulsions, 24 h and one month after preparation, stored at room temperature. The results represent mean ± standard deviation of the measured parameters, with each measurement repeated 3 times

Sample name/ measured parameter	*Z*-ave / nm		PDI		pH		Conductivity, μS cm^{-1}	
	24 h	1 month	24 h	1 month	24 h	1 month	24h	1 month
BA-loaded NE	94.39± 0.64	97.23± 1.73	0.098± 0.018	0.109± 0.025	5.67± 0.01	5.07± 0.02	82.70± 0.26	92.43± 0.12
LB-loaded NE	95.02± 0.20	95.87± 0.64	0.089± 0.017	0.073± 0.21	5.36± 0.07	5.03± 0.06	89.53± 0.06	104.90± 0.26
OR-loaded NE	102.50± 0.79	98.86± 1.25	0.099± 0.021	0.106± 0.025	5.74± 0.09	5.36± 0.01	94.73± 0.65	92.97± 0.06
Blank NE	103.2± 0.81	104.8± 1.02	0.123± 0.02	0.088± 0.018	5.56± 0.03	5.27± 0.05	79.53± 0.70	88.77± 0.31

However, after 6 months of storage at RT, significant differences in the stability were apparent among NEs prepared with different EOs. It was observed that only OR-loaded NEs remained stable during the prolonged storage (*Z*-ave ~97 nm, PDI ~0.033, pH ~5.4 and electrical conductivity ~136.6 μS cm^{-1}), without the signs of droplet aggregation or phase separation (as observed by using optical microscopy). Moreover, this formulation showed superior stability compared to the blank NE, which exhibited a dramatic increase in the droplet size and some moderate changes of the other relevant parameters (*Z*-ave ~187 nm, PDI ~0.056, pH ~5.38 and electrical conductivity ~128.8 μS cm^{-1}). Thus, the EO type and the SO mix concentration were both found to be crucial factors governing the NE properties and stability. The optimal SO mix concentration of 10 wt.% (containing ~2.2 wt.% of surfactant) can be considered favourable having in mind that potential skin irritation is typically associated with high surfactant concentrations in topical formulations [19,30].

4. 2. Raman spectroscopy study of the essential oils before and after nanoemulsification

It is known that each EO can contain up to 100 different molecules, with usually two or three principal compounds comprising up to 80–90 wt.% of the EO. Therefore, it is particularly important to identify more precisely the oil composition, having in mind that it can vary significantly depending on the plant genetics, harvesting time, production process, *etc*. [31,32]. Principal compounds in the EOs we used were reported in the producer specifications as follows: basil oil – methyl chavicol (syn. estragole), oregano oil – carvacrol and thymol, and lemon balm oil – geranial (citral a), neral (citral b) and β-caryophyllene oxide. This was in accordance with the literature data [8,9,23,24,31−33].

Raman spectra of the studied EOs are presented in Figure 5. As expected, the most intense bands in the Raman spectrum of OR observed at: 1623, 1460, 1445, 1380, 1261, 1180, 1067, 869 and 760 cm^{-1} are the ones characteristic for carvacrol, since it is the main component of OR [27,34]. Additionally, the presence of the weak band at 740 cm^{-1} (visible as a shoulder on the band at 760 cm^{-1}), which is characteristic for the ring vibration of thymol [27,34], confirms the presence of thymol in OR. Thus, the Raman spectrum of OR was in line with the producer's specification and the literature.

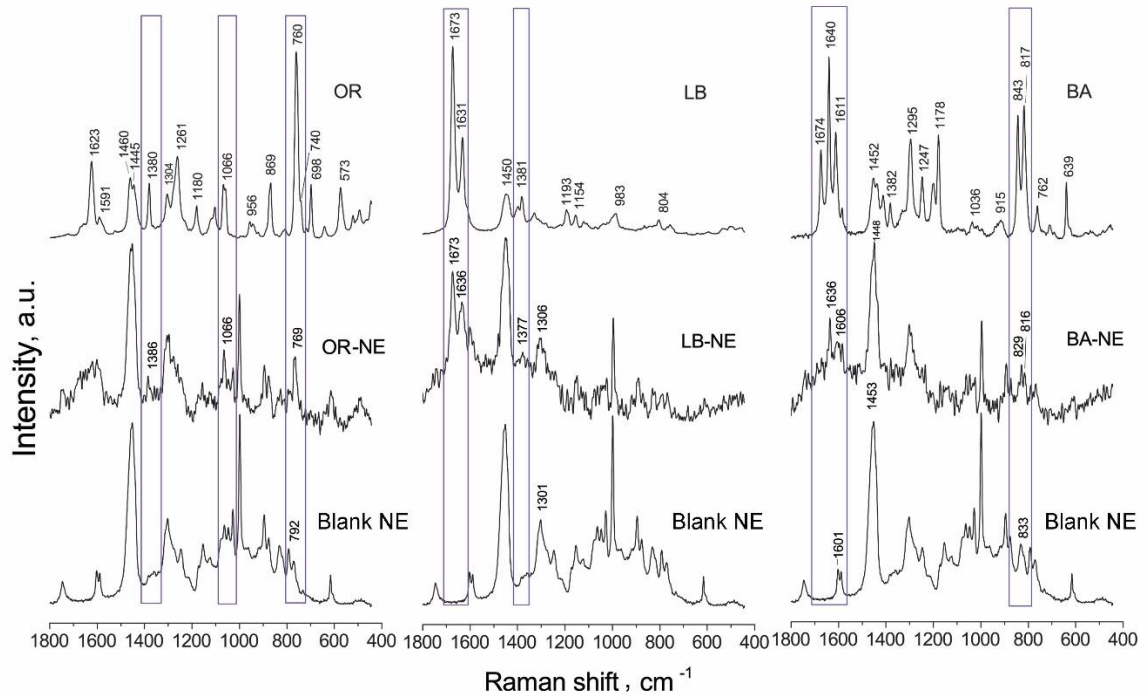

Figure 5. Comparison of Raman spectra of the blank nanoemulsions – Blank NE (bottom row), the EO-loaded nanoemulsions – BA-NE, OR-NE, LB-NE (middle row) and neat essential oils – BA, OR, LB (upper row). Each spectrum represents the average of 3 measurements acquired from a different place in the sample

Raman spectroscopy is suitable to detect different chemotypes of plant oils, such as in the case of BA, with methyl chavicol and linalool being the usual principal components, although eugenol and 1,8-cineole have been also reported [28,31]. According to the declared composition, the principal component of BA used in our study is methyl chavicol. The bands observed in the Raman spectrum of BA (Fig. 5) are in line with the earlier reported Raman spectrum of methyl chavicol at 1640, 1611, 1295, 1178, 843, 817, and 639 cm^{-1} [28]. The additional bands observed at 1674, 1640, 1452, and 1382 cm^{-1} suggest the significant presence of linalool in BA [28] as the second principal component, which was not stated by the producer. Still, this component can significantly influence antioxidant activity of BA, due to the synergism with methyl chavicol [31].

To the best of our knowledge, the Raman spectrum of LB has not been published yet. The bands observed in the Raman spectrum of LB (Fig. 5) can be identified as the characteristic peaks of the main components of this oil – geranial and neral (two geometric isomers of citral) at 1673, 1631, 1445 and 1382 cm^{-1} [28,35]. Since the lemon balm EO also has the same two main components as lemongrass, one can easily notice great similarity between the LB Raman spectrum obtained here and the one reported for lemongrass [35]. However, the LB Raman spectrum exhibited following distinguishable differences: a band at 1397 cm^{-1} assigned to the CH_2, scissoring (deformation) mode due to the presence of *β*-caryophyllene oxide, as well as a complex band at 1450/1441 cm^{-1} which probably includes a contribution from the antisymmetric methyl deformation mode of the CH_3-C group [36]. Since the LB Raman spectrum did not show intensive bands in the range between 600 and 800 cm^{-1}, it was confirmed that components containing aromatic rings were not present, unlike BA and OR oils containing aromatic molecules as their principal compounds.

Recently, novel applications of Raman spectroscopy were reported, for example, to detect structural changes in nanosystems, when oils and curcumin [37] or proteins [38] are incorporated into nanodroplets, as well as to study interactions among nanoemulsion components. Therefore, after the main components of the tested EOs were confirmed by the Raman spectroscopy, the next goal was to track potential interactions of these oils with the proposed NE carrier, by using the same approach as in our previous work investigating the interactions of red raspberry seed oil in polysorbate 80-stabilised NEs [10]. Raman spectra of the EO-loaded nanoemulsions (OR-NE, BA-NE and LB-NE) are shown in Figure 5. Although the EO concentration was low (1 wt.%), bands characteristic for each EO were still visible in the Raman spectra of all EO-loaded NEs. Still, the Raman spectra of the EO-NEs were not informative in the whole

region recognized for the qualitative differentiation of the tested EOs because the blank nanoemulsion had a very pronounced spectrum. However, some differences in the position of Raman bands of EO-loaded NEs as compared to the corresponding spectra of EOs and the blank NE were observed. This implies occurrence of some interactions in the NE after addition of the EOs. It should be noted that the Raman spectrum of OR-NE remained without significant changes after 6 months of storage (Supplementary Fig. 1), which could be attributed to the observed physicochemical stability of this particular system.

3. 3. *In vitro* antioxidant activity of EOs before and after nanoemulsification

Antioxidant activity investigations aimed to gain an insight into free radical scavenging profiles of EOs before and after nanoemulsification, as a proof of concept that nanoemulsions can be used as effective carriers for EOs exhibiting antioxidant properties. It is particularly beneficial to test the concentration dependence of the antioxidant activity and to combine different assays to gain a broader picture of the antioxidant profiles of the tested molecules/systems, given the fact that the activity of EOs depends not only on the composition but also on the concentration and the type of substrate, physical state of the system, temperature, *etc.* [16,18,24]. Therefore, two different methods (DPPH and ABTS) were employed in our study.

DPPH test is a standard assay for antioxidant activity screening, a fast, reliable and sensitive procedure suitable for studies of neat EOs and EO-containing emulsions dissolved in an organic solvent (usually methanol). To address the activity of more hydrophilic compounds in the EOs and the influence of conversion of EOs into O/W NEs, the $ABTS^+$ assay was chosen, since it can be performed in an aqueous medium (*e.g*. in PBS buffer).

Test results of the DPPH assay revealed that there are significant differences in the antioxidant activity between all EOs used at the same concentration, as well as for each EO used at different concentrations (Fig. 6). Interactions among the type of EO and concentration were also significant (two-way ANOVA, Tukey post hoc test). The order of EO antioxidant activity was: OR > LB > BA (Fig. 6). A very high percentage of free radical inhibition (INH_{DPPH} > 80 %) was observed for neat oregano oil (even at the smallest test concentration of 0.5 µl ml^{-1}). In previous studies in literature high antioxidant activities of OR were also reported by using the DPPH test in methanol (*e.g*. [23], INH over 80 % [26]). This is a consequence of the presence of carvacrol and thymol in OR, acting as excellent free radical scavengers due to the free hydroxyl group [20,23,25]. BA oil we used in this study contained mainly methyl chavicol and linalool, and it exhibited intermediate antioxidant activity, which was in line with the results of the DPPH study in ethanol performed with a similar BA chemotype, from Ararat and Napoletano cultivars [31]. In the case of LB, a slightly lower antioxidant activity was observed compared to the work of de Souza *et al.*, who reported an IC50 value of 2 µl ml^{-1} (DPPH in ethanol) for LB with similar main compounds as in our study (citral a and citral b) [17].

As can be seen in Figure 7 and Table 2, according to the DPPH test, the order of EO activity remained the same after nanoemulsification. However, there was a significant decrease in antioxidant activities of BA and LB in the NE carrier, on the contrary to the OR-loaded NE, which successfully preserved the activity (INH_{DPPH} of 87.8 to 88.5 %). This implies some significant interactions between each EO and the nanocarrier, inducing changes in the EO antioxidant activity after nanoemulsification.

However, results of the ABTS test revealed a more complicated concentration-dependent behaviour of antioxidant molecules in the EOs when tested in the aqueous medium (Fig. 8). The statistical analysis once more confirmed that all EOs exhibited significantly different INH of ABTS radical when used at the same concentrations, as well as when different concentrations of the same oil are used, with the interactions of these two factors also being significant (two-way ANOVA, $p < 0.05$). The observed order of antioxidant activity of the EOs at lower concentrations (0.1, 0.5 and 2.5 µl ml^{-1}) was the same as in the case of the DPPH test (OR> LB> BA), but with a higher INH ABTS (up to 98.5 % in the case of OR). However, when 5 µl ml^{-1} of EOs were employed the order of activity changed to LB> OR> BA, and finally, at 10 µl ml^{-1} another change in order occurred: BA> OR> LB, with all three oils exhibiting very high INH_{ABTS} (>87 %). Therefore, when these EOs were employed at higher concentrations, slightly hydrophilic antioxidant molecules in the BA (*e.g*., linalool) and LB (*e.g*., beta-caryophyllene oxide) or some other components not stated in the product specifications, could be dispersed in the aqueous phase. The result of this combined activity among the EO components is reflected in the

effective scavenging of the ABTS free radical. It is not unusual that the biological activity cannot be attributed only to the principal compound in an EO, because of naturally occurring synergistic interactions among oxygen-containing compounds in EOs [23,31].

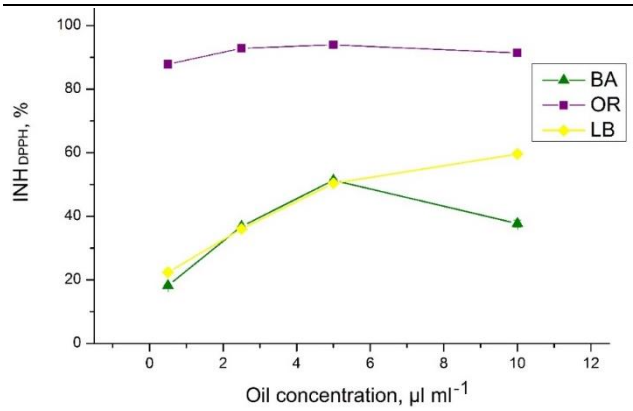

Figure 6. Antioxidant activity of neat basil (BA), lemon balm (LB) and oregano (OR) essential oil at different concentrations, measured by the DDPH assay in methanol. The results are expressed as inhibition of the DPPH free radical (INH_{DPPH})

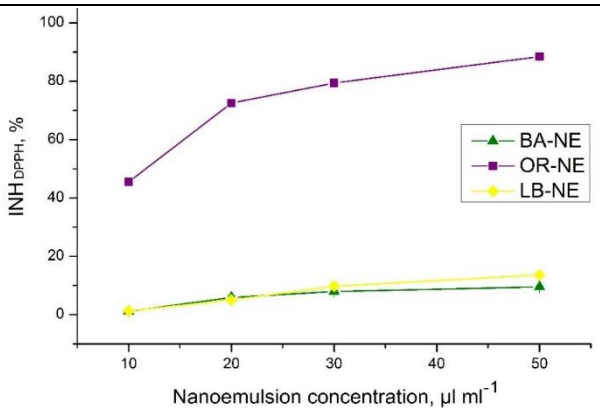

Figure 7. Antioxidant activity of nanoemulsions containing 1 wt.% of basil (BA), lemon balm (LB) or oregano (OR) essential oil at different concentrations, measured by the DDPH assay in methanol. The results are expressed as inhibition of the DPPH free radical (INH_{DPPH})

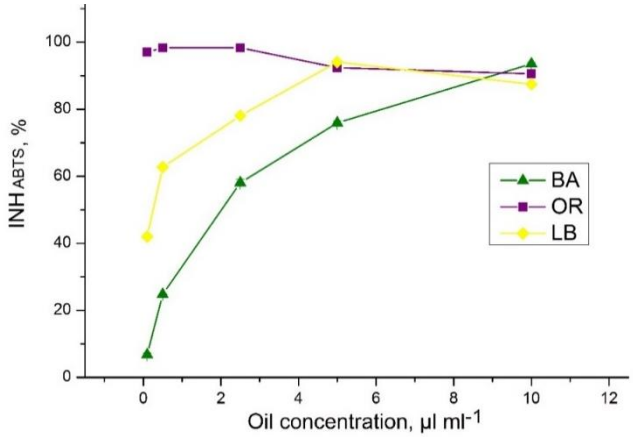

Figure 8. Antioxidant activity of basil (BA), lemon balm (LB) and oregano (OR) essential oil at different concentrations, measured by the ABTS assay in an aqueous medium (PBS buffer). The results are expressed as inhibition of the ABTS free radical (INH_{ABTS})

Table 2. Antioxidant activity of neat oils vs. essential oil-loaded nanoemulsions obtained using two different assays (DPPH in methanol and ABTS in an aqueous medium). The results are presented as INH (free radical inhibition), for the maximum nanoemulsion sample concentrations compared to the corresponding neat oil concentrations (Student's t-test, $p < 0.05$).

	INH, %			
	DPPH		ABTS	
Sample name (concentration)	Neat oil (0.5 µl ml^{-1})	Oil-loaded nanoemulsion (50 µl ml^{-1})	Neat oil (0.1 µl ml^{-1})	Oil-loaded nanoemulsion (10 µl ml^{-1})
Basil	18.22 ± 0.11	9.49 ± 0.53	6.76 ± 0.56	32.01 ± 0.99
Lemon balm	22.38 ± 0.06	13.62 ± 0.27	42.02 ± 0.47	5.97 ± 0.18
Oregano	87.85 ± 0.04	88.46 ± 0.27	97.05 ± 0.04	95.09 ± 0.16

Essential oil-loaded nanoemulsion composition: 1 wt.% essential oil, 10 wt.% SO mixture, 89 wt.% ultra-purified water while Blank nanoemulsion had the same composition, except the EO;
The results represent mean ± standard deviation of the INH of the free radicals, with each measurement repeated three times;
INH values for Blank nanoemulsions did not exceed 2 % for all tested concentrations.

In the studies of the antioxidant activity of EO-loaded NEs, the DPPH test could be performed at several different test concentrations (since both, the carrier and EO were dissolved in methanol), while the concentration range for the ABTS

assay was more limited due to the increase in turbidity when higher concentrations of these non-transparent NEs were employed. Therefore, only 10 µl ml^{-1} of EO-loaded NE was used for comparison with the results obtained for the corresponding concentration of pure EOs (*i.e*. 0.1 µl ml^{-1}) in which case the order of activity changed from OR > LB > BA for neat EOs to OR > BA > LB for EO-loaded NEs. The increase in activity of BA in the NE system (INH_{ABTS} from 6.8 to 32.0 %) can be associated with the increased solubility of the hydrophilic EO component (linalool) due to oil emulsification, *i.e*. increased total contact area with the aquatic surrounding. In fact, several EOs are reported to act as cosurfactants in nanoemulsion systems due to their positioning at the oil-water interface [39−40], which could also explain their changed antioxidant activity compared to the neat oil. The opposite behaviour, *i.e*., decrease in the activity of the LB in NE (INH_{ABTS} from 42 to 6 %) implies a different type of EO-SO mix interaction. In this case, LB constituents were packed in such a way that they became unavailable for the reaction with the ABTS radical (probably due to their positioning deep in the nanodroplet oil core). Since LB was the only oil with the aliphatic main components without free hydroxyl groups, some other structural arrangement in the NE compared to the phenolic, bulkier molecules (in BA and OR) is somewhat expected. Finally, in the case of OR, the antioxidant activity remained remarkably high in the nanoemulsion carrier (INH_{ABTS} 97 *vs*. 95 %) implying its optimal packing within the NE, which preserved its high antioxidant performance.

Having in mind the potential application in cosmetic/pharmaceutical products, storage stability is an important property to analyse during the formulation development. All EO-loaded NEs remained stable (Table 1) and without significant changes in the antioxidant activity after 1 month storage at RT (data not shown). However, with time droplet size growth and creaming started to appear even in the case of the blank NE, and the only remaining stable system after six months of storage at RT was the OR-loaded NE (a milky-white fluid emulsion with droplet sizes ~100 nm and a very narrow PDI value of ~0.1), implying that OR served as a co-stabiliser for the studied NE. Since this formulation also maintained a very high INH of DPPH and ABTS free radicals (>85 %), it was concluded that oregano oil was the most compatible with the investigated polyglycerol-ester based nanoemulsion carrier among tested essential oils. Therefore, based on the preliminary stability study and the results of antioxidant assays combined with the Raman investigations, it can be concluded that OR-loaded NEs can be proposed for antioxidant skin protection.

4. CONCLUSIONS

This work represents a feasibility study of polyglycerol-ester based nanoemulsion carrier for several EOs (basil, lemon balm and oregano) in the view of their application for antioxidant skin protection. Since the chemical composition of each EO is complex, at least two different assays were necessary to screen their antioxidant potential as well as the concentration-dependence study of the antioxidant activity. Most importantly, our study revealed that interactions of EOs with NE carriers can have a positive or negative impact on the NE stability, while at the same time nanonisation can increase or decrease the EO effectiveness as a free radical scavenger. Overall, it was found that OR-NE was the most effective and stable system, since OR acted as a co-stabiliser in the NE formulation, and its remarkably high antioxidant activity was successfully preserved during 6 months of storage. The results of this study confirm recent findings that some EOs (or their isolated compounds) could become multifunctional additives in nanoemulsions in the future, due to their cosurfactant/costabilising effect, in addition to their antioxidant and antimicrobial activities.

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Niskoenergetske nanoemulzije kao nosači za etarska ulja u topikalnim formulacijama za antioksidantnu zaštitu kože

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(Naučni rad)

Izvod

U ovoj studiji nekoliko različitih etarskih ulja (EU): bosiljak – BO, matičnjak – MA i origano – OR inkorporirano je u nanoemulzije (NE) kao potencijalne nosače za prirodne i osetljive bioaktivne sastojke. NE su pripremljene pomoću metode inverzije faza (engl. *phase inversion composition – PIC method*) koja predstavlja niskoenergetski i ekonomičan postupak izrade. Fizičkohemijska stabilnost nanoemulzija potvrđena je analizom raspodele veličina kapi, merenjem električne provodljivosti i pH vrednosti, kao i optičkom mikroskopijom. Nađeno je da su vrsta EU i koncentracija smeše surfaktanata i uljane faze dva ključna faktora koja su uticala na karakteristike i stabilnost dobijenih emulzija. Tehnikom Ramanske spektroskopije potvrđeni su glavni aktivni sastojci etarskih ulja i detektovane su moguće interakcije sa nanonosačem, što predstavlja noviju primenu pomenute tehnike. Pokazano je da antioksidantna aktivnost prema DPPH radikalu u metanolu zavisi od koncentracije, sa sličnim trendom za čista etarska ulja i za nanoemulzije sa uljima (OR> MA> BO). Međutim, ABTS test u vodenoj sredini pokazao je izrazite promene u redosledu aktivnosti sa povećanjem koncentracije EU i nakon nanonizacije EU. Generalno, dokazano je da je OR-NE najefikasniji i najstabiliniji sistem, s obzirom da je ulje origana ispoljilo ulogu kostabilizatora u formulaciji, uz istovremeno očuvanje njegove veoma visoke antioksidantne aktivnosti u obliku nanoemulzije, tokom 6 meseci čuvanja.

Ključne reči: origano; bosiljak; matičnjak; nanonizacija; Ramanska spektroskopija; stabilnost