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Mediterranean essential oils as precious matrix components and active ingredients of lipid nanoparticles

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ARTICLE INFO

Chemical compounds studied in the article:
Tegin O (Gliceryl Monooleate)
Tween® 80 (Polysorbate 80)
Kolliphor RH40 (Polyoxyl 40 hydrogenated castor oil)
Labrafil (Oleoyl Macrogol-6 Glycerides)
Softisan 100 (Hydrogenated Coco-Glycerides)
Rosmarinus officinalis
Lavandula x intermedia "Sumian"
Origanum vulgare subsp. *hirtum*
Thymus capitatus

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ABSTRACT

Essential oils are recognized as valuable active pharmaceutical ingredients attributed to a set of biological properties, which include antibacterial, antifungal, antiviral, antioxidant, anticancer, immune-modulatory, analgesic and anti-inflammatory activities. Their use in pharmaceutics is however compromised by their limited water solubility and low physicochemical stability (i.e. volatility, oxidation). In order to overcome these limitations, we aimed to develop nanostructured lipid carriers (NLC) as delivery systems for Mediterranean essential oils, in particular *Rosmarinus officinalis* L., *Lavandula x intermedia* "Sumian", *Origanum vulgare* subsp. *hirtum* and *Thymus capitatus* essential oils, selected on the basis of their antioxidant and anti-inflammatory activities. NLC composed of Softisan (as solid lipid) have been produced by phase inversion temperature (PIT) and high-pressure homogenization (HPH), using two different emulsifiers systems. Particles have been further characterized for their mean particle size, polydispersity, zeta potential, morphology and chemical interactions. Best NLC formulations were obtained with Kolliphor/Labrafil as surfactants, and using *Rosmarinus*, *Lavandula* and *Origanum* as essential oils (PDI between 0.126 and 0.141, Zave < 200 nm). Accelerated stability studies have also been carried out to estimate the effect of the production method and surfactant composition on the long-term stability of EOs-loaded NLC. *In vitro* biological cell viability and anti-inflammatory activities were evaluated in Raw 264.7 cells (macrophage cell line), while *in vitro* antioxidant activity was checked by DPPH assay. *Lavandula* and *Rosmarinus* NLC were shown to be the most biocompatible formulations up to a concentration of 0.1% (v/v), whereas they were able to induce a dose-dependent anti-inflammatory activity in the order *Lavandula* > *Rosmarinus* ≥ *Origanum*.

1. Introduction

In the last decades, a renewed interest in plants and traditional medicine is diffusing all over the world, with particular attention to essential oils (EOs) as promising natural compounds for their different activity (e.g. antibacterial, antifungal, antiviral, antioxidant, anticancer, immune-modulatory, analgesic and anti-inflammatory actions) (Bakkali et al., 2008; Bona et al., 2016). Their complex chemical composition and high concentration of terpenes (monoterpenes, sesquiterpenes, and diterpenes) and other oxygenated compounds (esters, aldehydes, ketones, alcohols, phenols, and oxides) are responsible for their ability in modifying the microorganisms' membrane and cell wall, with consequent release of cell contents

Abbreviations: BS, back scattering; ΔT, variation in transmission; DLS, dynamic light scattering; DMEM, Dulbecco's modified eagle medium; DPPH, 2,2-diphenyl-1-picrylhydrazyl; EOs, essential oils; FBS, fetal bovine serum; FT-IR, Fourier transmission infrared spectroscopy; GRAS, generally recognised as safe; HPH, high pressure homogenization; L, *Lavandula* essential oil; LPS, lipopolysaccharide; NLC, nanostructured lipid carriers; NO, nitric oxide; O, *Origanum* essential oil; PDI, polydispersity index; PIT, phase inversion temperature; R, *Rosmarinus* essential oil; ROS, reactive oxygen species; SD, standard deviation; SLN, solid lipid nanoparticles; T, *Thymus* essential oil; Tt, transmission; TAGS, Turbiscan® Ageing Station; TEAC, trolox equivalent antioxidant capacity; TEM, transmission electron microscopy; TSI, Turbiscan stability index; Zave, mean particle size; ZP, zeta potential

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(Altintas et al., 2013). Based on their composition, EOs result particularly interesting for the treatment and prevention of diseases related to inflammation or ROS production (Severino et al., 2015). Furthermore, recent studies highlight the possibility to use these components as active agents for the treatment of candidiasis, for wound healing or even for solid cancers (Bona et al., 2016; Gonzalez-Vallinas et al., 2014; Hammer et al., 1998; Petiwala et al., 2014; Pozzatti et al., 2008; Tampieri et al., 2005; Wang et al., 2012). Currently, EOs and most generally medicinal and aromatic plants, are worldwide considered as one of the most important field for the revalidation of traditional products. Numerous EOs present in plants from the Mediterranean area, such as *Origanum* spp., *Thymus* spp. and *Rosmarinus* spp., commonly used in the food industry and generally recognised as safe (GRAS), have been reported to be effective against several microorganisms, particularly against *Candida* spp., as recently reviewed by Bona et al. (Bona et al., 2016). However, their hydrophobicity and insolubility in water, the high volatility and instability due to oxidation and hydrolysis, still represent a challenge for the effective use of EOs in food, cosmetic and pharmaceutical industries (Hosseini et al., 2013a; Trinetta et al., 2017). In order to overcome these drawbacks, nanoencapsulation in delivery systems has been suggested as a valid approach for preserving the properties of EOs, increasing their effectiveness (Severino et al., 2015).

With the purpose of exploiting the beneficial effects of EOs as antioxidant and anti-inflammatory ingredients, we developed nanostructured lipid carriers (NLC) using EOs extracted from Mediterranean plants as oily components of the lipid matrix.

In particular, *Rosmarinus officinalis* L., *Lavandula x intermedia* "Sumian", *Origanum vulgare* subsp. *hirtum* and *Thymus capitatus* (*Coridothymus capitatus*) were used both as oily liquid component for the preparation of NLC and active ingredients, prepared by phase inversion temperature (PIT) and high pressure homogenization (HPH) methods. All developed NLC formulations were physicochemically characterised to evaluate the effect of each EO on nanoparticles features, with particular attention to the mean particles size and polydispersity (by dynamic light scattering (DLS), morphological structure (by transmission electron microscopy (TEM), chemical interactions (by Fourier transmission infrared spectroscopy FT-IR) and long-term stability (Turbiscan AGS). *In vitro* biological cell viability and anti-inflammatory activity of EOs both as pure compounds and as matrix components of NLC, were evaluated in RAW 264.7 cells (macrophage cell line), considered as early stage of inflammation (Kim et al., 2017a,b). Furthermore, *in vitro* antioxidant activity was studied using the DPPH assay.

2. Materials and methods

2.1. Materials

Tegin O (Gliceryl Monooleate) was bought from ACEF (Piacenza, Italy). Tween® 80 (Polysorbate 80), was purchased from Sigma Aldrich Co (St. Louis, MO, USA). Kolliphor RH40 (Polyoxyl 40 hydrogenated castor oil) was kindly provided by BASF Italia S.p.a. (Cesano Modena, Italy) while Labrafil (Oleoyl Macrogol-6 Glycerides) was a gift from Gattefossé Italia s.r.l. (Milano, Italy). Softisan 100 (Hydrogenated Coco-Glycerides) was purchased from IOI Oleo GmbH (Oleochemicals, IOI group). *Rosmarinus officinalis*, *Lavandula x intermedia* "Sumian", *Origanum vulgare* subsp. *hirtum* and *Thymus capitatus* were kindly provided by Exentiae s.r.l. (Catania, Italy). All solvents were of HPLC grade and were bought from VWR International (Milano, Italy). The cell culture media (DMEM), supplements (FBS, L-glutamine, antibiotics) and trypsin were from Gibco (Alfagene, Portugal). Alamar Blue was from Invitrogen (Alfagene, Portugal). The cell culture flasks and multiwells were from Orange Scientific (Frilabo, Portugal).

2.2. Nanoparticles preparation

2.2.1. Phase inversion temperature method (PIT)

NLC were prepared using the previously reported PIT method (Carbone et al., 2014a,b; Carbone et al., 2012). Aqueous phase (containing 6% w/w of the mixture Tween 80/Tegin O or Kolliphor R40/Labrafil) and 8% w/w of lipid phase consisting of the mixture solid lipid/EO were separately heated to 70 °C, then the aqueous phase was added dropwise to the oily phase and the mixture cooled to room temperature under stirring for 1 h.

2.2.2. High pressure homogenization (HPH)

The selected NLC based on the same amount of the surfactants mixture Kolliphor R40/Labrafil, were produced by HPH using the Ultra-Turrax® (IKA, model T25, impeller 10 G, Germany). For all formulations, the hot aqueous phase was slowly added to the hot lipid phase (8% w/w). The formulation was mixed for 1 min at 11,000 rpm. An external water bath heated at approximately 70 °C was used to maintain the sample temperature. The hot O/W nanoemulsion was further processed using a high-pressure homogenizer (GEA Niro Soavi, model NS1001L2K, PANDA 2 K, Italy) at 70 °C for three cycles. The final formulation was then cooled to room temperature leading to the lipid phase recrystallization and finally the lipid nanoparticles were formed (Severino et al., 2012).

2.3. Dynamic light scattering

Mean particle size (Zave) and the polydispersity index (PDI) of all prepared NLC were determined by Dynamic Light Scattering (DLS) using a Zetasizer Nano S90 (Malvern Instruments, Malvern, UK). Each value was measured at least in triplicate. For measurements, samples were properly diluted (50 µL) in 1 mL of ultrapurified water. Results are shown as mean ± standard deviation (SD).

2.4. Turbiscan® AG Station

Stability studies were carried out using an optical analyzer Turbiscan® Ageing Station (TAGS, Formulaction, L'Union, France) which is the TLAB equipped with an ageing station. Turbiscan® technology is based on Static Multiple Light Scattering for the analysis of concentrated dispersions. TAGS consists in a robot with three thermo-regulated blocks for the storage of 54 samples. A volume of 20 mL NLC, prepared by PIT or HPH methods, was placed in a cylindrical glass cell and positioned in the Turbiscan® at room (21.0 ± 1.0 °C) and body (35.5 ± 1.0 °C) temperatures. The detection head was composed of a pulsed near-infrared light source (λ = 880 nm) and two synchronous transmission (Tr) and back scattering (BS) detectors. The T detector receives the light, which crosses the sample (at 180° from the incident beam). The detection head scanned the entire height of the sample cell (65 mm longitude), acquiring T each 40 µm (1625 acquisitions in each scan). Its application is useful to evaluate and detect possible processes of dispersions destabilization, giving also information of the type of destabilization. The measuring is based on the variation of the particle volume fraction (migration) or diameter (coalescence), resulting in a variation of T signals.

2.5. Fourier transform-infrared (FT-IR) analysis

Fourier transform-infrared (FT-IR) characterizations of pure lipid and EO, physical mixtures and NLC prepared by using different essential oils were performed using a FT-IR spectrophotometer (Perkin Elmer Spectrum RX I, USA) equipped with an ATR accessory of diamond Zn/Se. For each sample, 16 scans at a resolution of 2 cm^{-1} were obtained from a wave number 650–4000 cm^{-1}, using a speed of 0.50 cm/s and a force gauge of 100 (Hosseini et al., 2013b). In the case of NLC, nanosuspensions were freeze-dried for 24 h using a LyoQuest laboratory freeze-dryer (Telstar Life Science, Spain).

2.6. Transmission electron microscopy (TEM)

NLC dispersions (5 µL) were placed on a 200-mesh formvar copper grid (TAAB Laboratories Equipment, Berks, UK) for negative-staining