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# Thyme essential oil loaded in nanocochleates: Encapsulation efficiency, *in vitro* release study and antioxidant activity

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ABSTRACT

Essential oils (EOs) are widely used as natural preservatives in foods to control lipid oxidation and spoilage by microorganisms. In this study nanocochleates (NCs), based on phosphatidylcholine, cholesterol and calcium ions were loaded with 1 mg or 0.5 mg/100 ml of TEO and fully characterised.

Particle size of TEO NCs were ca. 250 nm and 210 nm, respectively, and all were negatively charged. Thymol and carvacrol encapsulation efficiencies were, 46.3 ± 0.01 and 50.9 ± 0.02 respectively, using 1 mg/ml of TEO. *In vitro* release studies of thymol and carvacrol indicated a steady release in the first h and after 6 h ca 41% and 31% for thymol and carvacrol were released. Free TEO showed a strong antioxidant activity (75.2%) at the concentration of 1 mg/ml and also TEO-loaded NCs maintained this high scavenging activity (72.2%), indicating that this innovative formulation was able to preserve the antioxidant activity of TEO constituents. Nanochocleates could represent an innovative, completely biodegradable approach for the prolonged and sustained release of the EO, preserving functional properties.



## 1. Introduction

Nowadays there is a growing interest in bioactive natural compounds, which have a key role in many fields ranging from medicine to food chemistry. In particular, many consumers are demanding foods without what they perceive as artificial and harmful chemicals, including those used as antimicrobials and preservatives. Typically, essential oils (EOs) are the characteristic constituents of spices and aromatic herbs used from the ancient by humankind as natural preservative of food. EO is a complex mixture of a heterogeneous class of secondary metabolites, which include terpenoid hydrocarbons, oxygenated terpenoids, phenol derivatives, amines, and sulphides (Bilia et al., 2014). Their antioxidant and antimicrobial activities control lipid oxidation and spoilage by microorganisms. Hence, lipid oxidation is one of the main causes of chemical deterioration in food and this undesired consequence is a common risk wherever a lipid or perishable organic substrate is present, with a consequent development of undesirable off-flavours, creating eventual toxicity and severely affect the shelf-life of many goods. Thyme essential oil (TEO), from *Thymus vulgaris* L. (Lamiaceae family), is composed mainly of phenolic compounds such as thymol and carvacrol and it represents a paradigmatic essential oil with huge antioxidants, antiradicals and antimicrobial properties (Sacchetti et al., 2005).

We have recently reported noticeable efficacy of TEO against five enterotoxin producers of *Staphylococcus aureus* and five *Listeria monocytogenes* strains as ingredient of meatballs (Pesavento et al., 2015) and the strong inhibition of the growth of 18 bacterial type strains belonging to the 18 known species of the *Burkholderia cepacia complex* (Bcc) (Maida et al., 2014).

However, despite these interesting properties, TEO usage has many limitations. TEO components as well as those of the other essential oils are very volatile with a consequent limited efficacy. Mostly of them are sensitive compounds that can degrade easily by the action of heat, oxygen, and light. They can interact with the environment with a consequent inactivation of their efficacy. Therefore, a suitable formulation for dosing and protection of the essential oils must be developed to achieve a practical and rational application (Varona, Martín, & Cocero, 2011; Liolios, Gortzi, Lalas, Tsaknis, & Chinou, 2009).

To the best of our knowledge only a nanoemulsion based on TEO

*Abbreviations:* DLS, dynamic light scattering; EE, encapsulation efficacy; EOs, essential oils; NCs, nanocochleates; PBS, phosphate buffered saline; PD, polydispersity; PDI, polydispersity index; P90G, phospholipon 90G; TEM, transmission electron microscope; TEO, thyme essential oil.

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was previously developed as potential antimicrobial delivery system (Ziani, Chang, McLandsborough, & McClements, 2011). Briefly, thyme oil-in-water nanoemulsions were stabilized by the nonionic surfactant Tween 80. The nanoemulsions were highly unstable to droplet growth and phase separation, and in order to increase stability other ionic surfactants were added. A cationic surfactant (lauric arginate) or an anionic surfactant (sodium dodecyl sulfate). The antifungal activity of nanoemulsions containing positive, negative, or neutral thymol droplets were less active than the solely surfactants because oil droplets decreased their efficacy (Ziani et al., 2011).

Aim of this study was to encapsulate TEO in nanocochleates (NCs) which are unique lipid nanocarriers, composed of simple, naturally occurring materials such as phosphatidilcholine, cholesterol and calcium ions. NCs are stable phospholipid-calcium precipitate, structurally different from liposomes. Their unique structure consists of a large, continuous, solid, lipid bilayer sheet rolled up in a spiral, with no internal aqueous space. Calcium ions maintain the cochleate in its rolled form, bridging each successive layer, through ionic interaction (Gould-Fogerite and Mannino, 1997). NCs are very attractive nanocarriers because biodegradable, nontoxic, non-immunogenic and biocompatible.

This study focuses on the preparation, optimisation and characterization of NCs prepared from a liposomal preparation. The effects of TEO on morphology and physicochemical properties of NCs (mean size, polydispersity and ζ-potential) were investigated. Encapsulation efficiencies (EE %) of thymol and carvacrol, the main and characteristics constituents of TEO, are calculated by HPLC analysis. A stability study at room temperature of empty NCs was also carried out during two months after lyophilisation and, finally, the DPPH-scavenging activity of TEO encapsulated in this innovative nanocarrier was investigated.

## 2. Materials and methods

### 2.1. Materials

Phospholipon 90G (phosphatidilcoline 90%) was purchased from Lipoid GMBH (Cologne, Germany); cholesterol, phosphate buffered saline pH 7.4, calcium chloride were supplied from Sigma-Aldrich (Milano, Italy) and *Thyus vulgaris* essential oil (TEO, containing 56 ml/100 ml carvacrol and 38 ml/100 ml thymol by GC) was purchased from FLORA s.r.l. (Italy). Thymol European Pharmacopoeia (EP) Reference Standard was used for the calibration curve. All solvents used were HPLC grade: $CH_3CN$, $CH_2Cl_2$, MeOH and MeOH were from Sigma-Aldrich (Milano). HCOOH (85 ml/100 ml) was by Carlo Erba (Milan, Italy). Phosphotungstic acid was by Società Italiana Chimici (Rome, Italy). Water was purified by Milli-Qplus system from Millipore (Milford, MA, USA).

### 2.2. Methods

#### 2.2.1. Preparation of PC based-liposomes and nanocochleates

As a first step of the preparation of NCs, the liposomes were prepared using the film hydration method (Righeschi et al., 2014). TEO-loaded liposomes were formulated as follows: the required amounts of phospholipids (6 mg/ml) and cholesterol (1.2 mg/ml), TEO (0.5 mg/ml and 1 mg/ml) were dissolved in $CH_2Cl_2$ (5 ml) by stirring. The obtained organic solution was then vacuum evaporated and the lipid film was hydrated by addition of phosphate buffered saline (10 ml PBS). The dispersion was stirred with a mechanical stirrer (RW 20 digital, IKA) for 30 min in a water bath at the constant temperature of 37 °C. The liposomal suspension loaded with TEO was used as such to prepare the cochleates. $CaCl_2$ 0.1 mol/L was added to liposomal formulation according to the *trapping method*. Briefly, 80 μl of calcium chloride solution were added dropwise under magnetic stirring (150 rpm, 15 min, TA). The immediate clouding indicates the NCs formation.

#### 2.2.2. Stability study of freeze dried NCs

NCs can be freeze dried, which provides the potential to be stored for long periods of time at room temperatures. Accordingly, both TEO-loaded NCs suspensions obtained from liposomes were freeze dried without any cryoprotectant. The stability of lyophilised NCs was evaluated after reconstitution of the suspension to the original volume using PBS every week during two-month period. After the addition of PBS, the samples were mixed using a vortex mixer at room temperature until they were dispersed. DLS was used to evaluate their size, ζ-potential and polydispersity, while TEM analysis assessed their morphological shape.

#### 2.2.3. Characterization of TEO loaded-NC: size, polydispersity index and ζ-potential

Liposomes and NCs' particle sizes were measured by dynamic light scattering (DLS) (Zetasizer® Nano ZS90; Malvern Instruments, Malvern, UK) equipped with a JDS Uniphase 22 mW He–Ne laser operating at 632.8 mm, an optical fibre-based detector, a digital LV/LSE-5003 correlator and a temperature controller (Julabo waterbath) set at 25 °C. Time correlation functions were analysed to obtain the hydrodynamic diameter of the particles and the particle size distribution (polydispersity index [PD]) using ALV-60X0 software V.3.X (Malvern, UK). Autocorrelation functions were analysed by the cumulant method fitting a single exponential to the correlation function to obtain the mean size, PD, and distribution method (to fit a multiple exponential to the correlation function to obtain particle size distributions). In particular, polydispersity values were calculated for each peak as peak width/mean diameter. Scattering was measured in an optical quality 4 ml borosilicate cell at a 90° angle, diluting the samples in $H_2O$ Millipore.

The superficial charge (ζ-potential) of the NCs systems was measured using a Malvern Instruments Zetasizer Nano series ZS90 (Malvern, UK). For all samples, an average of three measurements at stationary level was taken. The temperature was kept constant at 25 °C by a Haake temperature controller. The ζ-potential was calculated from the electrophoretic mobility (μE) by means of the Henry correction to Smoluchowski's equation (eq (1)).

$$\zeta = \frac{3\mu E\eta}{2\varepsilon_0\varepsilon_r}\frac{1}{f(ka)} \quad (1)$$

where $\varepsilon_0$ is the vacuum permittivity, $\varepsilon_r$ is the relative permittivity of the solvent, $a$ is the particle radius, $\kappa^{-1}$ is the Debye length, and $\eta$ is the solvent viscosity (water, in our case).

#### 2.2.4. Morphological characterization by transmission electron microscopy (TEM)

NCs were analysed in terms of morphology, shape and dimensions by transmission electron microscope (TEM, Jeol Jem 1010, USA). NCs dispersion diluted 10-times was applied to a carbon film-covered copper grid. Most of the dispersion was blotted from the grid with filter paper to form a thin film specimen, which was stained with a phosphotungstic acid solution 1 g/100 mL in sterile water. The samples were dried for 1 min and then were examined under a JEOL 1010 electron microscope and photographed at an accelerating voltage of 64 kV.

#### 2.2.5. Determination of EE of TEO components by HPLC

The encapsulation efficiency (EE%) is defined as the percentage amount of drug entrapped in the *cigar-like* structure in relation to the total amount of drug present during the carrier formation and