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New findings on the incorporation of essential oil components into liposomes composed of lipoid S100 and cholesterol

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Abstract:

The encapsulation of essential oil components into liposomes was demonstrated to improve their solubility and chemical stability. The aim of the present study is to investigate the effect of chemical structure, henry constant (H_C) value and aqueous solubility of essential oil components on their liposomal encapsulation. Based on ethanol injection method, the essential oil components (estragole, eucalyptol, isoeugenol, pulegone, terpineol, and thymol) were encapsulated in lipid S100-liposomes. Static headspace gas chromatography method showed that H_C of estragole, isoeugenol and eucalyptol was approximately 10 times greater than that of pulegone, terpineol and thymol. Besides, dynamic light scattering and diffraction technique revealed that all liposomal vesicles were of micrometric in size and the presence of phenols, isoeugenol and thymol, promoted enlargement of vesicles. Incorporation in liposomes was better (encapsulation efficiency > 90 %) for the essential oil components exhibiting low aqueous solubility, estragole, isoeugenol, and pulegone. Besides, efficient entrapment in liposomes (loading rate > 18 %) was obtained for isoeugenol, terpineol and thymol. This suggests that the presence of hydroxyl group in the structure and exhibiting lower H_C ameliorated the entrapment of essential oil components into liposomes. Furthermore, release rate from liposomes varied among the investigated essential oil components and it was controlled by loading rate of essential oil components into liposomes, size of liposomal batches, location of essential oil components within lipid bilayer, and cholesterol incorporation rate of liposomes. Finally, liposomal encapsulation of isoeugenol, pulegone, terpineol, and thymol was suitable to protect them as a considerable concentration was retained in liposomes after 10 months with respect to initial concentration.

Keywords: aqueous solubility, encapsulation, essential oil components, henry constant, liposomes, release

1. Introduction

Essential oils are complex mixtures composed mainly of monoterpenes and phenylpropenes and biosynthesized in nature by aromatic plants for their protection against various pathogens (Bakkali et al. 2008). Also, essential oils received remarkable attention in food, cosmetics, and pharmaceutical industries. For instance, they are widely used as flavouring agents, food preservatives, and in manufacturing several dosage forms including suppositories, capsules, soap, perfumes, and others (Asbahani et al. 2015). In the present study, the phenyl propenes; estragole and isoeugenol, and the monoterpenes; eucalyptol, pulegone, terpineol, and thymol were chosen as models of essential oil components. These components exhibit considerable biological effects including antimicrobial, antifungal, antinociceptive, anti-inflammatory (Dogan et al. 2017; Kfoury et al. 2016; de Sousa et al. 2011; Melo Júnior et al. 2017; de Oliveira et al. 2012; Wattanasatcha, Rengpipat, and Wanichwecharungruang 2012; Riella et al. 2012) antioxidant (Kfoury et al. 2014), and anesthetic (Reiner, Perillo, and García 2013) activities.

The selected essential oil components differ by their octanol/water partition coefficient (Log P) value, their aqueous solubility, and their henry constant (H_c) value. According to literature, log P values varied between 2.6 (Pubchem) and 3.4 (Pubchem) for isoeugenol and estragole respectively (Table 1). Also, their aqueous solubility ranged between 178 for estragole (Yalkowsky and Dannenfelser 1992) and 7100 mg/L for terpineol (Li and Perdue 1995) (Table 1). Besides, H_c values of the chosen essential oil components were determined in literature as estimated from the vapour pressure and aqueous solubility at 25 °C using the vapour pressure/aqueous solubility method (US EPA. 2012). The values ranged between 2.23×10^{-6} atm m^3 /mol for terpineol and 4.62×10^{-4} atm m^3 /mol for estragole (Table 2). However, to our knowledge there are no experimental H_c values determined for the essential oil components we are studying.

The use of essential oils components is hindered by their volatility, poor water solubility and instability in presence of heat, light and oxygen (Turek and Stintzing 2012). Hence, their encapsulation in different carrier systems including liposomes, cyclodextrins, solid lipid nanoparticles, micelles, and others, was demonstrated to overcome these drawbacks and preserve their activities.

Liposomes are spherical microscopic vesicles comprising a central aqueous compartment enclosed by a membrane constituted mainly of phospholipids and may contain cholesterol. They are biocompatible,

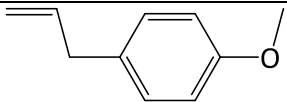
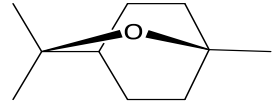
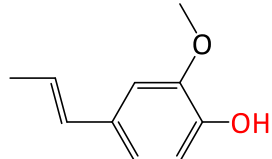
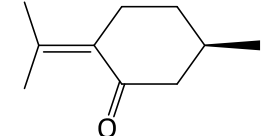
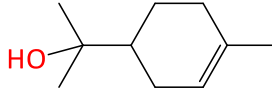
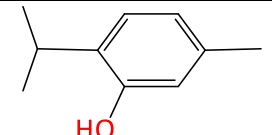
biodegradable, non-immunogenic and non-toxic (Gharib et al. 2015). Thus, liposomes are suitable for delivery of bioactive compounds in which hydrophobic substances can be entrapped within the lipid bilayer, hydrophilic molecules within the aqueous internal cavity and amphiphilic ones at the water-bilayer interface (Laouini et al. 2012). It had been reported that liposomal incorporation represents an efficient opportunity for improving solubility and chemical stability of essential oils components (Detoni et al. 2012).

The interaction of the selected essential oil components with dipalmitoylphosphatidylcholine (DPPC) membrane has been studied by Raman spectroscopy, differential scanning calorimetry and fluorescence anisotropy (Gharib et al. 2017, 2018). Results demonstrated that the essential oil components exhibited a membrane fluidizing effect. Besides, their incorporation, except eucalyptol, decreased the transition enthalpy (ΔH_m) value and increased the gauche conformers leading to an increase in the disorder of lipid bilayer.

Several studies prepared liposomes containing various essential oil components and characterized them in terms of their size, drug encapsulation efficiency (EE) and loading rate (LR), release kinetics, storage stability, and biological activities (Sebaaly et al. 2015; Gharib, Auezova, et al. 2017; other references). Furthermore, few studies discussed the effect of drug properties on liposomes characteristics. Zhigaltsev et al. (Zhigaltsev et al. 2005) compared the characteristics of egg sphingomyelin/cholesterol liposomes containing three structurally closely related vinca alkaloids; vincristine, vinorelbine and vinblastine and demonstrated that lipophilicity of drug, determined by its log P value, influences its loading and release kinetics from liposomes. Nevertheless, our study is the first to deal with a series of components and estimate the factors that modulate their liposomal encapsulation and release.

In the present study, liposomal formulations containing estragole, eucalyptol, isoeugenol, pulegone, terpineol and thymol were prepared by ethanol injection method using lipoid S100 and cholesterol as liposomes constituents. The liposomal batches were characterized for their size, drug encapsulation efficiency (EE), loading rate (LR), and drug release from liposomes. Phospholipids and cholesterol incorporation rates were also determined. Finally, the stability of liposomal suspensions was evaluated after storage for 10 months at 4 °C. The effect of bearing a hydroxyl group in essential oil components structure, the physiochemical properties of essential oil components including Henri constant value and aqueous solubility, and the final liposomal composition experimentally determined (phospholipid: cholesterol: drug molar ratio) on liposome characteristics is discussed.

Table 1: Structure, log P, and aqueous solubility of the studied essential oil components

Essential oil component	Structure	Log P	Aqueous solubility (mg/L)
Estragole		3.4 (Pubchem)	178 (Yalkowsky and Dannenfelser 1992)
Eucalyptol		2.74 (Griffin, Wyllie, and Markham 1999)	3500 (Yalkowsky, He, and Jain 2010)
Isoeugenol		2.6 (Pubchem)	665; 810 (Kfoury et al. 2014; HERA 2012)
Pulegone		3.08 (Griffin, Wyllie, and Markham 1999)	276 (US EPA. 2012)
Terpineol		2.98 (Li and Perdue 1995)	7100 (Li and Perdue 1995)
Thymol		3.3 (Pubchem)	900 (Yalkowsky and Dannenfelser 1992)

2. Materials and methods

2.1. Materials

Non-hydrogenated soybean phosphatidylcholine lipid S100 (94% soybean phosphatidylcholine, 3% lysophosphatidylcholine, 0.5% N-acyl-phosphatidylethanolamine, 0.1% phosphatidylethanolamine, 0.1% phosphatidylinositol, 2% water, 0.2% ethanol) was supplied by lipid GmbH, Germany. 4-Amino-3-hydroxy-1-naphthalenesulfonic acid was purchased from Fluka, India and hydrogen peroxide from Fisher Scientific, UK. Cholesterol (94%) was purchased from Sigma-Aldrich, Japan; ammonium molybdate, eugenol, isoeugenol, potassium dihydrogenophosphate and thymol from Sigma-Aldrich, Germany; pulegone and terpineol from Sigma-Aldrich, Switzerland; estragole from Sigma-Aldrich, China; eucalyptol and triton X-100 from Sigma-Aldrich, USA; and methanol HPLC grade from Sigma-Aldrich, France. Absolute ethanol and sulfuric acid were purchased from VWR Pro-labo chemicals, France. Cholesterol assay kit was purchased from Spin react Company, Spain.

2.2. Preparation of liposomes

Ethanol injection method was applied to prepare the liposomal formulations. The required amounts of lipid S100 (10 mg/ml) and cholesterol (5 mg/ml) were dissolved in absolute ethanol. 10 ml of the obtained organic phase was later injected using a syringe pump (Fortuna optima, GmbH- Allemagne) into 20 ml aqueous phase at a flow rate of 1 ml/min, under magnetic stirring at 400 rpm, and at room temperature. As soon as the organic phase was in contact with the aqueous phase, spontaneous liposomes formation occurred. The liposomal suspensions were then kept for 15 min under stirring (400 rpm) at room temperature. Finally, ethanol was removed by rotary evaporation (Heidolph instruments GmbH and co., Germany) at 41 °C and 60 rpm under reduced pressure. Both blank liposomes and drug loaded liposomes were formed in which the essential oil component (estragole, eucalyptol, isoeugenol, pulegone, terpineol or thymol) was added to the organic phase at a concentration of 2.5 mg/mL. Each of the batches was prepared in triplicate. The liposomal formulations were stored at 4 °C prior to analysis.

2.3. Characterization of liposomal formulations

2.3.1. Measurement of liposome particle size

Particle size of liposomes was determined by a Laser granulometer (Partica Laser scattering, particle size distribution analyser LA-950V2; HORIBA, Japan) designed for measuring particle size between 0.01 and 3000 µm. Liposomal particle size and percentage of each population in the suspension were measured and data were expressed as mean ± standard deviation.

2.3.2. Determination of phospholipids: cholesterol: drug molar ratio in the liposome structures

The un-retained components in preparations were separated from the retained ones by submitting liposomal suspension to centrifugation at 15,000 rpm and 4 °C for 1 hour using a vivaspin 500 centrifugal concentrator (Sartorius Stedim Biotech, Germany, MW cut off=10,000 Da). Aliquots were removed from liposomal suspensions then sonicated for 10 minutes in ice to determine the total concentration of liposomal constituents (phospholipids, cholesterol, and drugs). Also, aliquots were taken from filtrate to determine the free concentration of liposomal constituents. The phospholipids: cholesterol: drug molar ratio for each formulation was later calculated.

2.3.2.1. Assay of phospholipids

The phospholipids concentration in the liposomal suspension and liposomal filtrate was quantified through Bartlett's method. The organic phosphates in the samples (500 µL from filtrate, total liposomal suspension, and standard solution of phosphorus) were digested by the addition of 400 µL sulfuric acid

(5M) at 200 °C for 1h. Then, the organic phosphates were oxidized to inorganic phosphates through incubating the samples in the presence of 100 µL H₂O₂ (30 %) for 30 min at 200 °C. Through interaction with ammonium molybdate (4.6 ml), the phosphomolybdic complex was formed. The latter was reduced to a blue product upon interaction with 4-amino-3-hydroxy-1-naphthalenesulfonic acid (200 µL) at 100 °C for 15 min and the absorbance of this blue compound was later measured at 815 nm using a UV-vis spectrophotometer (U-2900, Hitachi High-Tech Science Corporation, Japan). Stock solution of potassium dihydrogenophosphate was formed in ultrapure water at a concentration of 3.2 mM and diluted to obtain final concentrations of phosphorus ranging from 0.016 to 0.416 mM. The corresponding absorbance against the concentration of phosphorus was plotted. The phospholipids incorporation rate (IR %) was calculated as follows

$$IR_{PO_4^{3-}} (\%) = \frac{M_{PO_4^{3-} T} - M_{PO_4^{3-} F}}{M_{PO_4^{3-} \text{ organic phase}}} \times 100 \quad (\text{Equation 1})$$

in which $M_{PO_4^{3-} T}$ is the mass of phospholipids in the liposomal suspension, $M_{PO_4^{3-} F}$ is the mass of phospholipids in the liposomal filtrate and $M_{PO_4^{3-} \text{ organic phase}}$ is the initial mass of phospholipids added to the organic phase during liposomes preparation.

2.3.2.2. Dosage of cholesterol

For quantifying the total and free concentrations of cholesterol in suspension, enzymatic colorimetric method was utilized. 1 ml of the cholesterol assay kit containing cholesterol esterase, cholesterol oxidase, peroxidase, and 4-aminophenazone, was added to samples (10 µl of cholesterol standards, filtrate and total liposomal suspension). Cholesterol in the samples was oxidized by cholesterol oxidase into 4-cholestenona and hydrogen peroxide. The latter reacts with 4-aminophenazone in the presence of peroxidase to form a coloured complex, quinonimine, of a colour intensity proportional to the cholesterol concentration in the sample. The absorbance of the compound was measured at 505 nm using a UV-vis spectrophotometer (U-2900, Hitachi High-Tech Science Corporation, Japan). Stock solution of cholesterol was prepared in triton X-100 at a concentration 2 mg/ml and diluted to final concentrations ranging between 0.05 and 1.5 mg/ml. The incorporation rate IR (%) was determined based on the following equation:

$$IR_{CHO} (\%) = \frac{M_{CHO T} - M_{CHO F}}{M_{CHO \text{ organic phase}}} \times 100 \quad (\text{Equation 2})$$

where M_{CHO_T} is the cholesterol mass in the liposomal suspension, M_{CHO_F} is the mass of cholesterol in filtrate and $M_{\text{CHO}_{\text{organic phase}}}$ is the initial mass of cholesterol added to the organic phase during liposomal preparation.

2.3.2.3. Determination of drug encapsulation efficiency and loading rate

The EE (%) and LR (%) values of the selected essential oil components were calculated after HPLC analysis of the samples. The analysis was performed using an analytic column (C 18: 15 cm× 4.6 mm (Agilent)) at a wavelength of 204 nm. The mobile phase was made up of methanol and water (70:30) for all samples except for pulegone (65:35). The flow rate was set at 1 ml/min. The injection volume was 20 µl.

Stock standard solutions of the selected essential oil components were prepared in methanol at a concentration of 1 mg/ml then diluted to desired concentrations. Linearity was proved between 1 to 250 µg/mL for estragole; 50 to 1000 µg/mL for eucalyptol; 0.2 to 100 µg/mL for isoeugenol and thymol; 2.5 to 100 µg/mL for pulegone; and 1 to 100 µg/mL for terpineol. Besides, stock standard solutions (1 mg/ml) of the internal standards were prepared in methanol and diluted to desired concentrations. Thymol (100 µg/ml) was used as an internal standard for the dosage of estragole and isoeugenol. Eugenol was used at a concentration of 1 µg/ml for eucalyptol dosage and 2 µg/ml for pulegone, terpineol and thymol dosages.

100 µl of each solution (standard solution, liposomal suspension, or liposomal filtrate) was added to a solution of internal standard (100 µl) and methanol (200 µl). The EE (%) value of each formulation was calculated as follows:

$$\text{EE (\%)} = \frac{[\text{Drug}]_{\text{total}} - [\text{Drug}]_{\text{free}}}{[\text{Drug}]_{\text{total}}} \times 100 \quad (\text{Equation 3})$$

where $[\text{Drug}]_{\text{total}}$ and $[\text{Drug}]_{\text{free}}$ correspond to the total and free drug concentrations in liposomal suspension respectively.

Besides, the LR (%) values were calculated according to the following equation:

$$\text{LR (\%)} = \frac{m_{\text{liposomal suspension}} - m_{\text{filtrate}}}{m_{\text{organic phase}}} \times 100 \quad (\text{Equation 4})$$

Where $m_{\text{liposomal suspension}}$ and m_{filtrate} correspond to the mass of drug in liposomal suspension (total) and liposomal filtrate (free) respectively. $m_{\text{organic phase}}$ is the initial mass of drug added to the organic phase during liposome preparation.

2.3.3. In vitro drug release Kinetics

The dynamic release of drugs from liposomes was studied by multiple headspace extraction (MHE) coupled to gas chromatography. 1 ml of each drug loaded liposome sample was added to 9 ml of water in 22 ml sealed vials; the corresponding free drug (estragole and thymol at 1 ppm; isoeugenol at 200 ppm; terpineol at 5 ppm; pulegone at 2 ppm and eucalyptol at 0.5 ppm) was also introduced in 22 ml sealed vials. After equilibrium, vials were submitted to 30 successive gas extractions at constant time interval (8 min), oven temperature of 60°C, transfer line temperature of 250°C and using nitrogen as a carrier vector. 1 ml of the vapour present in the gaseous phase was then transferred to gas chromatography (GC) for analysis. All measurements were conducted using an Agilent G1888 headspace sampler coupled to a Perkin Elmer Autosystem XL gas chromatography equipped with a flame ionization detector and a DB624 column. GC conditions were set as follows: GC cycle of 8 min and column temperature of 160°C for estragole, eucalyptol, terpineol, pulegone and thymol and of 180°C for isoeugenol. The percentage of essential oil component remaining at time t was calculated as follows:

$$\text{Percentage of remaining essential oil component} = \frac{A_t}{A_1} \times 100 \quad (\text{Equation 5})$$

where, A_t and A_1 corresponds to the area of the chromatographic peak of studied essential oil component at time t and at the first extraction, respectively.

2.3.4. Determination of loading capacity

The amount of essential oil component in liposome was quantified using MHE. The concentration of the analyte in the gas phase of the vial decreases at each extraction steps. Thus, the sum of the analyte amounts removed in the individual extractions will be equal to the total amount of analyte present in the original sample (Kolb and Ettre 2006).

A linear regression was constructed for the standard and the samples using the following equation:

$$\ln A_t = -q(t-1) + \ln A_1$$

where A_1 is the area of the chromatographic peak of essential oil component at the first extraction; A_t is the peak area of essential oil component at the extraction time t and $-q$ is the slope of the regression curve that illustrates $\ln A_t$ as a function of $(t - 1)$.

The sum of peak areas corresponding to the total amount of essential oil component present in the sample or in the standard are obtained as follows:

$$\sum A_t = \frac{A_1}{1-q}$$

Loading capacity (LC) is expressed as μg of essential oil component / mg of liposomes.

$$LC = \frac{\text{drug}_{\text{exp}} (\mu\text{g})}{\text{mass of liposomes (mg)}} \quad (\text{Equation 6})$$

2.3.5. Study of liposome storage stability

After 10 months of storage at 4 °C, the hydrodynamic particle size of the liposomal formulations was measured by dynamic light scattering and diffraction technique. Besides, the free and encapsulated concentration of each essential oil component in liposomal suspension was assessed after 10 months via ~~through~~ HPLC analysis and the deduced values were compared to those obtained immediately during preparation.

2.4. Determination of Henry constant

Henry's constant law (H_C) is the vapor-liquid partition coefficient value of the various essential oil components which was determined by static headspace–gas chromatography (SH-GC). H_C is expressed as follow (Kolb and Ettre 2006):

$$H_C = \frac{C_G}{C_L} \quad (\text{Equation 7})$$

where C_G is the concentration of drug in the vapor phase and C_L , the concentration of drug in the aqueous phase

For the determination of H_C in water, several headspace vials were prepared containing different amounts of water (0.5; 0.6; 1, 2, 3 and 5mL for estragole, pulegone, eucalyptol and 0.1; 0.2; 0.3; 0.4; 0.5 and 0.6 mL for thymol, isoeugenol and terpineol) to which the same amount of drug was added (10 μL from a standard solution of 10000 ppm). The headspace settings were as follows: an oven temperature of 30 or 60 °C and an equilibrium time of 30 min. The transfer line temperature was set at 250 °C. The GC settings were a described above.

Using the phase ratio variation method described by (Kolb and Ettre 2006), the values of H_C were determined by the relationship between the reciprocal chromatographic peak areas and the vapour–liquid volumetric ratio (V_G/V_L , with V_G and V_L being the vapour volume and the liquid volume, respectively):

$$\frac{1}{AV_L} = \frac{1}{\alpha} \frac{V_G}{V_L} + \frac{1}{\alpha H_C} \quad (\text{Equation 8})$$

where A is the peak area, α is a constant incorporating several parameters and H_C is the vapour-liquid partition coefficient, directly calculated from the ratio of the slope and intercept of the fit of the experimental data obtained after allowing a vapour-liquid equilibrium time of 30 min in the oven.

3. Results and discussion

3.1. Henry constants of the studied essential oil components

Applying the static head space-gas chromatography method, H_C of the selected essential oil components was determined and results are presented in table 2. First, H_C values increased upon increasing temperature from 30 to 60 °C. Thus, increasing temperature induced essential oil component to reside more in the gaseous phase. This is in agreement with Copolovici et al study (Copolovici and Niinemets 2005) which demonstrated strong influence of temperature on H_C value of each of the ten plant monoterpenes, including terpineol, at temperature range 25-50°C. Moreover, H_C of the selected essential oil components followed the same order at 30 and 60 °C except isoeugenol; which was more affected by temperature increase in comparison to other essential oil components. Furthermore, H_C of estragole, eucalyptol and isoeugenol were approximately 10 times greater than that pulegone, terpineol and thymol at 30 °C. Finally, we could notice that the experimental H_C values, determined in our study at 30 °C, were consistent with the predicted H_C values present in literature ($R^2 > 0.99$) except for isoeugenol. It was demonstrated that temperature increase greatly affect H_C of isoeugenol so it may represent great variation between 25 °C (temperature used for estimating H_C in literature) and 30 °C (temperature used to determine H_C values in our study) in comparison to the other components.

Table 2: Estimated and experimental Henry constants of the selected essential oil components at 30 and 60 °C

Drug	Henry constant (atm m ³ /mol at 25 °C)	Henry constant (30 °C)	Henry constant (60 °C)
Estragole	$4.62 \times 10^{-4}_a$	0.03	0.07
Eucalyptol	$1.1 \times 10^{-4}_a$	0.01	0.05
Isoeugenol	$3.6 \times 10^{-6}_b$	0.03	0.1
Pulegone	$5.87 \times 10^{-5}_a$	0.005	0.009
Terpineol	$2.23 \times 10^{-6}_c$	0.001	0.003

Thymol	$3.5 \times 10^{-6}_a$	0.003	0.009
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a. (US EPA. 2012); b. (US EPA 2011); c. (Copolovici and Niinemets 2005)

3.2. Determination of phospholipids: cholesterol: drug molar ratio

3.2.1. Phospholipids

The total and free phospholipids concentrations in liposomal suspension were assessed through Bartlett's method and the incorporation rates (IR) values were calculated according to equation 1 and results are summarized in table 3. Standard curve was constructed by plotting the corresponding absorbance against the concentration of phosphorus in mM. The linear relationships were evaluated by regression analysis with the least squares method and the correlation coefficient was found to be 0.999.

For blank liposome, phospholipids IR value was high (94.2 ± 6.45 %). Thus, most of the initial mass of phospholipids used for liposome preparation was embedded in liposomes. The effect of essential oil components addition on phospholipids retention in liposomes differed among the tested components. Compared to initial phospholipids: cholesterol: drug molar ratio used for liposome preparation and to blank liposomes, it is obvious that the addition of estragole and eucalyptol did not widely affect the loading of phospholipids in liposomes. However, the presence of isoeugenol, pulegone, terpineol and thymol significantly reduced phospholipids retention in liposomes and their effect decrease in order of pulegone > thymol > isoeugenol > terpineol. Hence, isoeugenol, pulegone, terpineol and thymol replaced phospholipids molecules in lipid bilayer.

Upon interaction with phospholipids bilayer, certain molecules cause the variation of both transition temperature T_m and enthalpy difference (ΔH), thus act as "substitution impurities" of membrane taking place of lipid molecules. Instead, other molecules cause T_m , but not ΔH , variation, thus act as "interstitial impurities" by insertion among the flexible acyl chains of lipids (Cristani et al. 2007). In particular, Gharib et al. (Gharib et al. 2017, 2018) studied respectively the interaction of several phenylpropenes and monoterpenes, including the ones selected in this study, with DPPC liposomes. As a result, it had been investigated that the essential oil components, except eucalyptol, act as substitution impurities, taking place of lipid molecules. On the other hand, eucalyptol acts as interstitial impurity and intercalate in the bilayer thus does not affect phospholipids loading in membrane. Moreover, it had been proven that the presence of estragole induces less decrease in T_m and ΔH_m in comparison to different phenylpropenes studied and ΔH_m decreased significantly in presence of estragole from molar ratio of 100:5 in a concentration-dependent manner. Thus, the phospholipid: cholesterol: estragole molar ratio (113:110:2) determined in our study could provide a logical reasoning for the lower effect of estragole on phospholipids incorporation rate compared to other components studied.

3.2.2. Cholesterol

Using the enzymatic colorimetric method, cholesterol concentration in liposomal suspension and filtrate was determined and cholesterol IR values were calculated according to equation 2. Results are shown in table 3. For blank liposomes, IR of cholesterol was 77.6 ± 1.18 %. Compared to empty vesicles, a significant increase in the cholesterol IR was only obtained upon the addition of estragole and terpineol to liposomes. Gharib et al. 2017, 2018 demonstrated that the selected essential oil components are able to fluidize the membrane by decreasing the phase transition temperature of DPPC vesicles. Hence, we may propose an increase in cholesterol incorporation into estragole and terpineol loaded liposomes with increasing membrane fluidity induced by the presence of estragole and terpineol respectively. On the other hand, loading of eucalyptol, isoeugenol, pulegone, and thymol into liposomes reduced cholesterol incorporation in the formulations. Hence, eucalyptol, isoeugenol, pulegone, and thymol replaced cholesterol in lipid bilayer. This agree with Frang et al study (Fang et al. 2001) which reported that certain lipid soluble drugs compete with cholesterol molecules for the hydrophobic space in liposomal membrane, thus decrease cholesterol incorporation in liposomes.

3.2.3. Drugs

The retention times on HPLC were 8.4 ± 0.04 , 6.9 ± 0.09 , 3.5 ± 0.00 , 7.8 ± 0.06 , 6.3 ± 0.01 , 5.6 ± 0.01 for estragole, eucalyptol, isoeugenol, pulegone, terpineol and thymol respectively. Besides, the retention time of eugenol was 3.4 ± 0.00 with all drugs, except with pulegone it was 4.3 ± 0.005 . These results are due to the different methanol: water ratio (65:35) used for HPLC analysis of pulegone compared to that used for analysis of the other molecules (70:30). The calibration curves were constructed by plotting $AUC_{drug}/AUC_{internal\ standard}$ against the drug concentrations in $\mu\text{g/ml}$. The linear relationships were evaluated by regression analysis with the least squares method and the correlation coefficient (R^2) ranged between 0.989 and 0.999. After HPLC analysis, the EE and LR values of estragole, eucalyptol, isoeugenol, pulegone, terpineol and thymol into lipoid S100- liposomes were calculated according to equations 3 and 4 respectively.

The entrapment of drugs in liposomes, determined by their LR values, varied among the studied essential oil components. LR values of essential oil components bearing a hydroxyl group, isoeugenol, terpineol and thymol were high (> 18 %) and decrease in order of $LR_{thymol} > LR_{isoeugenol} > LR_{terpineol}$. However, the non-hydroxylic essential oil components, estragole, eucalyptol, and pulegone represented lower LR values (< 5 %) and $LR_{eucalyptol} > LR_{pulegone} > LR_{estragole}$. Therefore, the presence of hydroxyl group

in essential oil component structure enhanced its incorporation in liposomes. This implies that isoeugenol, terpineol, and thymol would be incorporated within the lipid bilayer with their hydroxyl group located toward the polar groups of phospholipids and hydrophobic parts are buried inside the lipid bilayer. Our findings were in good agreement with other studies in literature reported by Reiner et al. (Reiner, Perillo, and García 2013) where phenols, mainly thymol, interact and insert in egg phosphatidylcholine vesicles due to the presence of free hydroxyl group in their structure and their incorporation decrease viscosity of monolayers. Besides, it had been reported that the presence of hydroxyl group in monoterpenes structure enhances its membrane fluidizing effect (Gharib et al. 2018). Therefore, increasing fluidity of membranes promotes the incorporation of essential oil components.

On the other hand, the lower LR values of estragole, eucalyptol and pulegone may be attributed to the greater loss of these components due to their higher H_C compared to other selected components. Concerning the estimated H_C present in literature, it was demonstrated that H_C of estragole, eucalyptol and pulegone are significantly greater than those of isoeugenol, terpineol and thymol. However, regarding the experimental H_C values, results were different. Isoeugenol exhibits high H_C (0.03 at 30 °C) with a high LR value (19.86 ± 2.16 %) and pulegone exhibit low H_C (0.005 at 30 °C) with a low LR value (2.91 ± 0.32 %). In fact, liposomes were prepared at room temperature while H_C values were determined at 30 °C and temperature was demonstrated to be factor that influence H_C of essential oil components. Hence, exhibiting high H_C could be a factor that causes reduced incorporation of essential oil components into liposomes but other factors could affect the retention of EO component in liposome.

With respect to drugs EE in liposomes results were different. For essential oil components exhibiting lower aqueous solubility, incorporation in liposomes was better. Very high EE values (> 90 %) into liposomes were obtained for estragole, isoeugenol and pulegone (table 4) of aqueous solubility 178, 665, 276 mg/L respectively. However, eucalyptol, terpineol and thymol, of aqueous solubility 3500, 7100 and 900 mg/ml respectively, exhibited lower EE (< 80 %) values. Therefore, eucalyptol, terpineol and thymol are able to partition better between aqueous and lipid compartments of liposomes. The correlation coefficient (R^2) between EE of essential oil components into liposomes and aqueous solubility of the components was calculated and its value was 0.85, with the exception of terpineol. Besides, hydrophobicity is another factor that may influence drug encapsulation in liposomes (Zhigaltsev et al. 2005). Indeed, hydrophobicity of essential oil components determined by their log P values increased in order of isoeugenol (2.376, 2.6) < eucalyptol (2.74) < terpineol (2.98) < pulegone (3.08) < estragole (3.13, 3.4) ~ thymol (3.34). Thus, our results demonstrated that hydrophobicity of essential oil components did not influence their encapsulation into liposomes.

From the above results, bearing a hydroxyl group in the chemical structure of essential oil components, exhibiting low aqueous solubility and low H_C value are the main parameters that improve essential oil components encapsulation in liposomes, as evidenced by the high EE and LR values.

3.3. Loading capacity of drugs

Through MHE method, amount of drugs in formulations was quantified for calculating loading capacity (LC) of essential oil components. Table 3 represents the LC value, expressed as μg of essential oil components / mg of liposome. It is important to note that loading capacity represents the total mass of drug in suspension rather than the mass encapsulated in liposomes so it would not correlate with LR and EE values determined before.

Based on LC values, we could demonstrate that isoeugenol represents the highest retention level in liposomal suspension (LC was $4.9 \mu\text{g}/\text{mg}$) with respect to other components; LC of isoeugenol was about 10 times greater than that of other components studied. Pulegone, terpineol and thymol showed moderate levels of encapsulation in suspension with LC being in order of pulegone ($0.1 \mu\text{g}/\text{mg}$) < terpineol ($0.6 \mu\text{g}/\text{mg}$) ~ thymol ($0.6 \mu\text{g}/\text{mg}$) respectively. Estragole and eucalyptol were less retained in suspension (LC < $0.02 \mu\text{g}/\text{mg}$). The difference in LC could be related to variation in total mass of liposomes affected by final liposomal composition (phospholipid: cholesterol: drug molar ratio) and amount of water accumulated in vesicles. Also it may be related to the difference in H_C of the components used in which components with less H_C value were better retained in suspension.

Table 3: Phospholipids and cholesterol incorporation rates (IR), drug encapsulation efficiency (EE), drug loading rate (LR), phospholipids: cholesterol: drug molar ratio and loading capacity for the various essential oil components loaded liposomes

Liposomal formulation	Liposomal components				Pho: CHO: drug molar ratio	Pho: CHO: drug molar percentage in liposome structure	Loading capacity (µg/mg)
	IR (%) of Phospholipids (pho)	IR (%) of Cholesterol (CHO)	EE (%) of essential oil components	LR (%) of essential oil components			
Blank liposome	94.2 ± 6.45	77.6 ± 1.18	-	-	125:129:0 118:95:0	55:45:0	0
Drug loaded liposomes							
Estragole	98.1 ± 0.85	84.8 ± 2.78	90.9 ± 0.74	1.12 ± 0.28	125:129:168 113:110:2	50:49:1	0.014
Eucalyptol	88.3 ± 0.63	44.4 ± 1.69	61.9 ± 7.05	4.99 ± 0.07	125:129:162 111:57:8	63:32:5	0.0068
Isoeugenol	68.5 ± 8.85*	50.4 ± 3.39	96.4 ± 3.25	19.86 ± 2.16	125:129:152 96:73:30	48:37:15	4.9
Pulegone	72.2 ± 5.52*	39.6 ± 5.09	90.5 ± 0.32	2.91 ± 0.32	125:129:164 90:51:5	62:35:3	0.1
Terpineol	85.4 ± 4.36*	95.2 ± 4.41	73.7 ± 4.13	18.67 ± 0.62	125:129:162 107:116:30	42:46:12	0.6
Thymol	81.3 ± 2.55*	68.4 ± 5.09	79.1 ± 3.86	20.54 ± 1.89	125:129:166 94:78:34	46:38:16	0.6

For Pho: Cho: drug ratio normal font is based on initial moles of liposomal component used for liposome preparation, bold is based on liposomal components embedded in liposomes.

3.4. Liposomal particle size measurement

Table 4: Percentage distribution and mean particle size of populations for blank liposomes and the drug loaded liposomes at day of preparation and after 10 months of storage at 4 °C.

Italic: after 10 months of storage at 4 °C

Liposomal formulations	Population 1		Population 2		Population 3	
	(%)	Mean size (µm)	(%)	Mean size (µm)	(%)	Mean size (µm)
Blank liposomes	-	-	100.0 ± 0 <i>100.0 ± 0</i>	6.16 ± 0.51 <i>7.13 ± 1.51</i>	-	-
Estragole loaded liposomes	19.3 ± 5.5 <i>14.6 ± 3.78</i>	0.23 ± 0.02 <i>0.23 ± 0.02</i>	64.6 ± 6.42 <i>64.0 ± 14.79</i>	7.74 ± 1.04 <i>11.14 ± 1.81</i>	16.0 ± 4.35 <i>21.3 ± 16.28</i>	65.4 ± 13.05 <i>68.8 ± 17.13</i>
Eucalyptol loaded liposomes	-	-	100 ± 0.00 <i>100 ± 0.00</i>	6.43 ± 0.49 <i>9.45 ± 0.64</i>	-	-
Isoeugenol loaded liposomes	-	-	22.6 ± 12.42 <i>13.4 ± 5.77</i>	12.12 ± 0.96 <i>16.10 ± 3.42</i>	77.3 ± 12.42 <i>86.6 ± 5.77</i>	70.76 ± 5.65 <i>81.08 ± 6.49</i>
Pulegone loaded liposomes	-	-	100 ± 0.00 <i>.177 ± 6.35</i>	6.43 ± 0.49 <i>16.82 ± 0.95</i>	- <i>82.3 ± 6.35</i>	- <i>81.08 ± 6.49</i>
Terpineol loaded liposomes	26.7 ± 7.09 <i>25.5 ± 9.19</i>	0.24 ± 0.01 <i>0.23 ± 0.04</i>	64.3 ± 9.01 <i>59.0 ± 2.82</i>	7.04 ± 0.56 <i>7.67 ± 0.00</i>	9.00 ± 2.64 <i>15.5 ± 12.02</i>	49.23 ± 3.75 <i>68.14 ± 13.0</i>
Thymol loaded liposomes	-	-	74.0 ± 6.00 <i>82.0 ± 7.81</i>	27.7 ± 5.89 <i>23.9 ± 1.91</i>	26.0 ± 6.00 <i>18.0 ± 7.81</i>	184 ± 27.7 <i>175 ± 23.8</i>

Through laser granulometry, the particle size distribution of the liposomal batches was measured and compared between the various batches. Table 3 represents the number, percentage distribution and mean particle size of populations obtained in each formulation. For blank liposomes, a single micrometric sized ($6.16 \pm 0.51 \mu\text{m}$) population appeared. Thus, larger vesicles were obtained in comparison to literature. This difference can be ascribed to the mechanism by which vesicles are formed during the selected method (Lasic 1988). During ethanol diffusion into water phase, phospholipids dissolved in the organic phase precipitate at the water/organic boundary phase forming bi-layered phospholipids fragments (BPFs). BPFs seal off to form vesicles and the short distance between them enhances the possibility of forming larger vesicles by facilitating their coalescence probability (Yang et al. 2012). Moreover, this difference (in what? In particle size?) could be related to the different methods used for size characterization. Indeed, some are determined using dynamic light scattering (DLS) apparatus (Zetasizer Nano ZS; Malvern Instruments Ltd, France) allow measurement of only submicron size range populations (Storti and Balsamo 2010). However, laser granulometry technique used in this study is designed for measuring particle size between 0.01 and 3000 µm.

Regarding the number of populations obtained, eucalyptol and pulegone loaded liposomes were the most similar to blank liposomes in which one micrometric sized population appeared. However, the presence of other essential oil components induced an increase in number of populations obtained.

For isoeugenol and thymol loaded liposome two micrometric sized populations appeared. For estragole and terpineol loaded liposomes three populations arose; one in nanometric and two in micrometric sizes (table 4).

The effect of adding the selected essential oil components on particle size varied among the selected essential oil components (table 4). In comparison to blank formulation, the addition of estragole and terpineol promoted the production of nanometric batches. On the other hand, the incorporation of estragole, isoeugenol, terpineol and thymol boosts the formation of larger vesicles (and accordingly reduced the percentage of smaller micrometric vesicles) compared to blank liposomes. It might be that the accumulation of lipophilic drugs in the hydrophobic part of the membrane affects the interactions between the acyl chains of phospholipids and induce swelling of the membrane, leading to formation of larger vesicles (Sikkema, de Bont, and Poolman 1995). Moreover, large vesicles were reported to be ~~clusters or~~ aggregates of smaller particles (Domazou and Luigi Luisi 2002).

Besides, the mean particle size of estragole, eucalyptol, pulegone and terpineol loaded liposomes did not significantly differ from that of blank batches; the size of the major population obtained was close to size of blank liposome. However, the encapsulation of the phenolic components, isoeugenol and thymol, in liposomes increased liposome particle size in which the size of the major population (70.76 ± 5.65 and $27.7 \pm 5.89 \mu\text{m}$ for isoeugenol and thymol respectively) was significantly greater than that of blank liposome ($6.16 \pm 0.51 \mu\text{m}$). It had been demonstrated that the essential oil "Eucalyptus camaldulensis" (Moghimipour et al. 2012) and the essential oil component, eugenol, induce a considerable increase in vesicles size (Sebaaly et al. 2015). Moreover, Reiner et al. (Reiner, Perillo, and García 2013) had demonstrated that five phenol compound, including thymol, insert in egg phosphatidylcholine-liposomes in regions between the polar groups (choline molecule), the glycerol backbone and the first atoms of the acyl chains. This arrangement induces a reduction of the phospholipids head group repulsive force and decreases the mobility of hydrocarbon chains, thus induce an increase in particle size. Therefore, the presence of phenolic hydroxyl group in thymol and isoeugenol structure promotes an increase in liposome vesicle size.

3.5. Drug release study

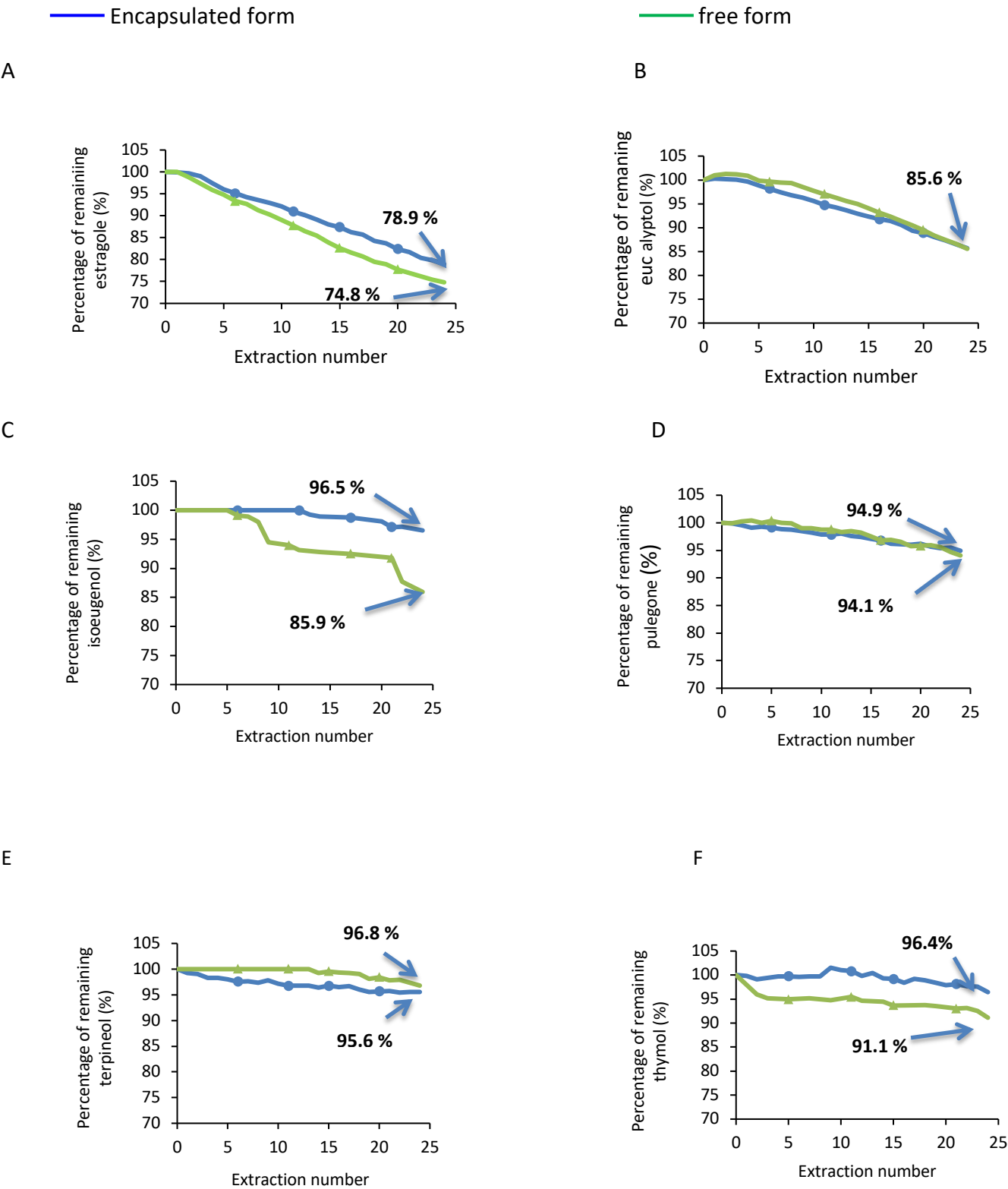


Figure 1: The remaining percentage of various essential oil components, in their free and liposome encapsulated form after different extractions. (A) estragole; (B) eucalyptol; (C) isoeugenol; (D) pulegone; (E) terpineol; (F) thymol .

The experiment of drug release was conducted at 60 °C using MHE method. Figure 1 depicts, at each extraction step, the remaining percentage of essential oil components as free molecules or when encapsulated in liposomes.

We can notice based on figure 1 that liposomal encapsulation of estragole, isoeugenol, and thymol reduced their volatility and permitted their sustained release. At each extraction step, the remaining percentage in liposomal suspension was considerably greater than that in the free form. However, liposomal encapsulation of eucalyptol, pulegone, and terpineol did not influence their release.

In their free form, the studied essential oil components exhibited different release pattern and the remaining percentage at the last extraction differed among the various essential oil components (figure 1). The latter increased in order of estragole (74.8 %) < eucalyptol (85.6 %) < isoeugenol (85.9 %) < thymol (91.1 %) < pulegone (94.1 %) < terpineol (96.8 %). This order correlates with that of H_c determined at 60 °C, except for isoeugenol (R^2 were 0.53 and 0.89 with and without isoeugenol respectively).

Moreover, when loaded in liposomes, essential oil components represented different release profiles. Isoeugenol and thymol manifested biphasic release patterns in which they remain unreleased for a period of time (first phase), followed by a slow release over the second phase. The other essential oil components exhibited one phase release pattern with different release rates. Terpineol and pulegone showed slow release rate while estragole and eucalyptol showed rapid release rate. In addition, the remaining percentage at the last extraction increased in order of estragole (78.9 %) < eucalyptol (85.6 %) < pulegone (94.9 %) < terpineol (95.6 %) < isoeugenol (96.5 %) < thymol (96.4 %).

Based on the above results, we could notice that EE of essential oil components into liposomes, chemical structure of components, their aqueous solubility values, and their log P values did not seem to influence their release rate from liposomes. Moreover, release of components was not affected by their H_c so liposomes efficiently retained them and consequently reduced their volatility.

Indeed, LR of essential oil components into liposomes controlled their release rate. Linear relationship was found between the percentage of essential oil components determined at the last extraction step and LR of essential oil components into liposomes, except for pulegone; R^2 was 0.54 with pulegone while 0.95 without pulegone.

Besides, results of release rate from liposomes may be attributed to the size of formulations and the location of essential oil components within the lipid bilayer (Juliano, Stamp, and McCullough 1978). Based on results of particle size measurement (batches have size greater than 0.5 μm) and method of liposomes preparation, it can be stated that the formulations are multi-lamellar vesicles (Rongen, Bult,

and van Bennekom 1997; Jaafar-Maalej et al. 2010). Also, size of isoeugenol and thymol loaded liposomes was significantly greater than that of other formulations. Therefore, isoeugenol and thymol may be encapsulated within the internal lamellas of liposomes and must pass several barriers before being released to the outer environment. This explains the biphasic release pattern of isoeugenol and thymol when loaded in liposome. Moreover, regarding the location of essential oil components within lipid bilayer, (Gharib et al. 2017, 2018) studied the interaction of the studied essential oil components with DPPC liposomes and specified their location within the bilayer. As a result, it had been proven that position of double bond in propenyl group of phenylpropenes control its incorporation in liposomes where isoeugenol, possessing the double bond at position C7–C8 in the propenyl side chain, inserted deeply in the bilayer in comparison to estragole. Also, the presence of hydroxyl group allows a deep bilayer incorporation of terpineol and thymol in comparison to pulegone and eucalyptol.

Pulegone exhibited low LR value (2.91 ± 0.32 %) and previous study reported that it did not deeply incorporate in DPPC bilayer (Gharib et al. 2018). Nevertheless, it exhibited a slow release rate (94.9 % remained at the final extraction step). This could be ascribed to the lower cholesterol incorporation rate ($39.6 \pm 5.09\%$) for pulegone loaded liposomes in comparison to the other prepared vesicles. It had been confirmed that liposomal retention of floxuridine was enhanced by decreasing cholesterol content in the formulation (Tardi et al. 2007) and idarubicin is retained better in cholesterol free vesicles in comparison to cholesterol containing ones (Dos Santos et al. 2002). Cholesterol helps to decrease the membrane fluidity and allows at the phase transition temperature (T_m) of phospholipids the switching from fluid phase of phospholipids bilayer to gel phase (Liu et al. 2017). Besides, it was demonstrated that cholesterol may induce a reduction in permeability of liposomal membrane (Sezer, Akbuğa, and Baş 2007). Hence, the presence of lower cholesterol content in the formulation may delay the release of some essential oil components from liposomes.

Hence, size of liposomal batches, IR of cholesterol into liposomes, LR of essential oil components, and the location of components within lipid bilayer are the main factors that affect essential oil components release rate from lipoid S-100 liposomes.

2.3. Storage stability

After 10 months of storage at 4 °C, the stability of the formulations was examined. All of the prepared batches, except thymol loaded liposomes, showed a significant increase in liposome particle size (table 3). These results were similar to those obtained by Sebaaly et al (Sebaaly et al. 2015) in which eugenol loaded lipid-S100 liposomes particle size increased after storage for 2 months at 4 °C.

For each formulation, the total and free concentrations of essential oil components in liposomal suspensions were determined at day of preparation and after 10 months. Figure 2 summarizes the obtained results. In general, a considerable amount of essential oil components remained in liposomal suspension (> 50 %), except for estragole loaded liposomes, in comparison to initial amount. Besides, with respect to initial concentration of essential oils incorporated in liposomes; a noticeable concentration of isoeugenol, pulegone, terpineol, and thymol was retained in liposomes after 10 months. Those findings correlate with that of release study in which isoeugenol, pulegone, terpineol, and thymol exhibiting a delayed release were satisfactory incorporated in liposomes after 10 months. Thus, the encapsulation of isoeugenol, pulegone, terpineol and thymol in lipid S-100 liposomes is efficient for protecting them.

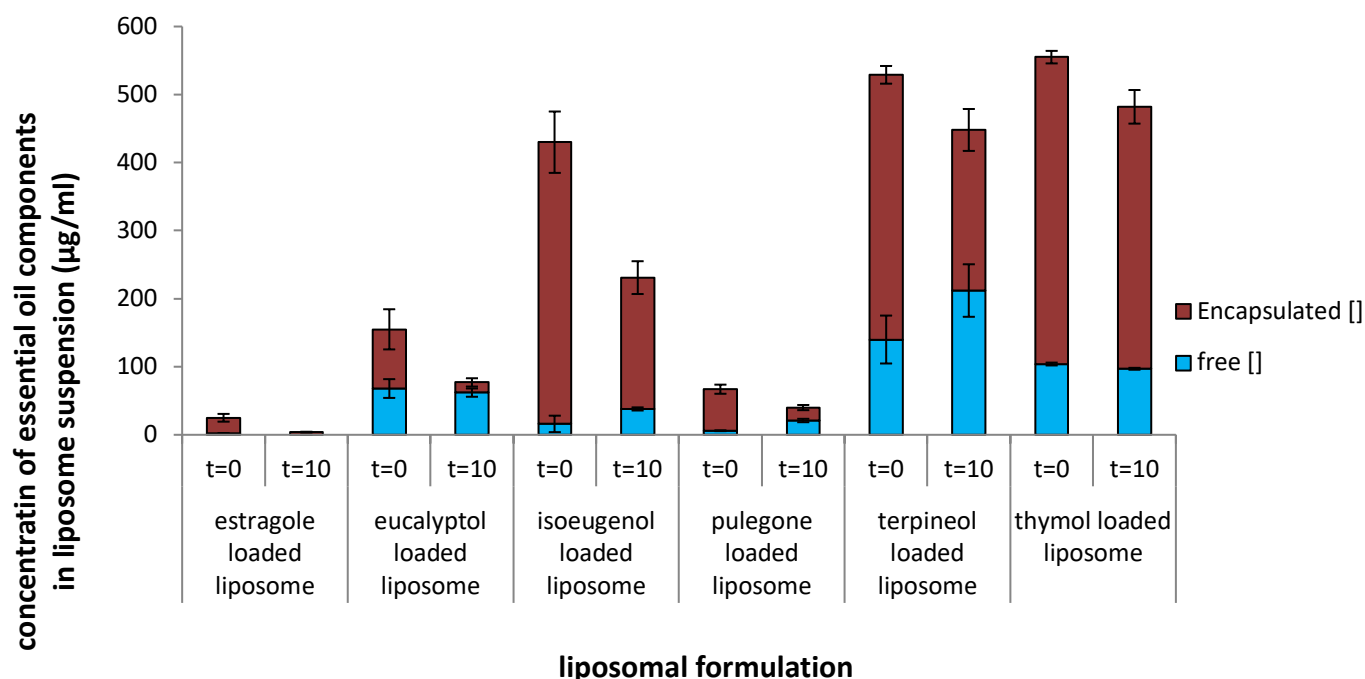


Figure 2: Storage stability of the various prepared liposomal batches: Remaining concentrations of free and encapsulated drugs in liposome suspension

4- Conclusion

Drug loaded lipid S-100 liposomes encapsulating diverse essential oil components were prepared by ethanol injection method and characterized via several techniques. The parameters that affect liposomal particle size, EE and LR, release kinetics and liposome storage stability had been discussed. The addition of the phenolic essential oil components, isoeugenol and thymol, induces enlargement of vesicles in comparison to blank liposomes. Besides, EE values into liposomes were greater for essential oil components exhibiting lower aqueous solubility. Regarding the LR values, results were different. Hydroxylated essential oil components were better entrapped in liposomes than non hydroxylated ones and incorporation was better for essential oil components with low henry constants. Furthermore, the release profiles were variable among the various formulations. Liposome particle size, LR of essential oil components into liposomes, location of components within liposome bilayer, and IR of cholesterol into liposomes were the key parameters that affect release of essential oil components from liposomes. Finally, liposomes loading isoeugenol, pulegone, terpineol, and thymol were stable after long term storage at 4°C. Hence, liposomes could be useful for the encapsulation of essential oil components extending their various applications. However, several factors control their incorporation into liposomes must be considered in future studies.

Highlights to review

- At 30 °C, H_C of estragole, eucalyptol and isoeugenol were approximately 10 times greater than that pulegone, terpineol and thymol
- Isoeugenol, pulegone, terpineol and thymol replaced phospholipids molecules while eucalyptol, isoeugenol, pulegone, and thymol replaced cholesterol in lipid bilayer.
- The presence of hydroxyl group in essential oil component structure enhanced its incorporation in liposomes
- Exhibiting high H_C could be a factor that causes reduced incorporation of essential oil components in liposomes
- Incorporation in liposomes was better for the essential oil components exhibiting low aqueous solubility
- The incorporation of estragole, isoeugenol, terpineol and thymol boosts the formation of larger vesicles
- The presence of phenolic hydroxyl group in thymol and isoeugenol structure promotes an increase in liposome vesicle size

- Size of liposomal batches, cholesterol IR of liposomes, LR of essential oil components into liposomes, and location of essential oil components within lipid bilayer are the main factors that affect essential oil components release rate from liposomes.

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