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Studies concerning the entrapment of *Anethum graveolens* essential oil in liposomes

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Abstract

The influence of liposome composition, size, lamellarity on the entrapment efficiency on essential oil was studied. For this purpose, both multillamelar (MLV) and unilamellar (SUV) liposomes were prepared using the thin film hydration method. The stability of the liposomal formulations over 1 year was also investigated. Vesicle dispersions were characterized for their oil content and average size distribution. Results showed that the incorporation of Anethum graveolens essential oil in liposomes is in good amounts when appropriate formulations are used. From the stability point of view, the liposomes content of incorporated oil was stable almost the entire period, and the size distribution showed only slight modifications at the end of the year, especially SUV formulations.

Keywords: essential oil, *Anethum graveolens,* liposomes, stability

Introduction

Essential oils (aetherolea) are volatile, natural, complex compounds, characterized by a strong odour, formed by aromatic plants as secondary metabolites. They are mixtures of different chemical constituents, possessing interesting therapeutically properties. An extensive body of research has demonstrated that essential oils and their main components possess a wide spectrum of biological activity, which may be of great importance in several fields, from food chemistry to pharmaceutics (M. Cristiani, et al.) [1]. Several studies investigated and demonstrated the potential of *Anethi aetheroleum* (the essential oil of *Anethum graveolens)* as antifungal and antibacterial agent (G. Singh et al, L. Jirovetz et al.) [2, 3].

It is well known that most essential oils are biologically instable, poorly soluble in water and they distribute defectively to target sites. For these reasons, some new methods have been developed in order to improve their stability and their bioavailability. Among of these is the use of liposomal encapsulation (Y. Shoji & H. Nakashima), [4], which reduces reactivity with the environment (water, oxygen, light), decreases the evaporation or the transfer rate to the outside environment, promotes handling ability, masks taste and enhances dilution to achieve a uniform distribution in the final product when used in very small amounts (B. F. Gibbs et al., R. J. Versic et al.) [5, 6].

As liposomes have been reported to be promising drug carriers in antimicrobial therapy, due to their capability to give slow drug release, cutaneous targeting and low transdermal delivery of a drug, the aim of our study was to prepare *Anethi aetheroleum* - loaded liposomes and to determine the influence of some parameters, such as vesicle size and composition, on the entrapment efficiency and stability of these formulations.

Materials and methods

Phosphatidylcholine from fresh egg yolk (PC), dipalmitoylphosphatidyl choline (DPPC), dimiristroylphosphatidyl choline (DMPC), dioleoylphosphatidyl choline (DOPC), dimiristroylphosphatidyl glycerol (DMPG), chloroform p.a., methanol p.a. were obtained from Sigma. Cholesterol p.a. was purchased from Fluka. The essential oil was extracted from *Anethi fructus* in the laboratory.

Preparation of Liposomes

Multilamellar vesicles (MLV) were prepared according to the thin film hydration method. Lipid solutions were prepared by dissolving precise amounts of phosphatidyl choline, cholesterol and essential oil in chloroform. 5.0 mL from each solution was introduced in a 100 mL round-bottomed flask. The solvent was evaporated in a Heidolph Laborota 4000 rotaevaporatory**,** at 35°-40°C, under reduced pressure (13-14 mm Hg). The obtained dry lipid film was hydrated with 5 mL distilled water. The mechanical stirring of the lipids in aqueous medium was performed with the rotaevaporatory equipment at 37°C and by manual stirring in the water bath, for 2 h, at the same temperature. This suspension was allowed to hydrate for 2 h in order to anneal any structural defects.

Unilamellar vesicles (SUV) were obtained by sonication of the MLV liposomes, in a bath-type sonicator (Sono Swiss SW 6L) for 30 min. (6x5min.). The sonication temperature was above T_c of the lipids (a temperature under T_c determines structural defects in the bilayers of the liposomes, which conducts to the fusion of vesicles).

Purification of liposomes.

The compound incorporated vesicles were separated from the unincorporated compounds by centrifugation. Vesicular dispersions were spun in a laboratory centrifuge Hettich Universal 320 R, at 10ºC, 10000 rpm, for 60 min. The supernatant was removed and the liposomes were reconstituted with 5 mL distilled water.

Determination of the essential oil of *Anethi fructus* quantity encapsulated in liposomes

We measured the quantity of essential oil encapsulated both in MLV and SUV vesicles. In case of purified liposomes, after the centrifugation, the supernatant was removed by the sediment. The quantity of essential oil was measured using a Perkin-Elmer Lambda 2 Spectrophotometer at $\lambda = 236$ nm.

The incorporation efficiency (E %) of the essential oil was calculated using the following formula:

$$E = \frac{T - S}{T} 100$$

where: T – the total amount of essential oil from supernatant and sediment (measured after disruption of liposomes with methanol) and S – the quantity of essential oil from supernatant (C. Holvoet et al) [7].

Stability evaluation of the liposomes formulations

The stability of the vesicles dispersions was monitored for 1 year. The liposomal formulations were stored at 4 ± 1ºC. At certain time intervals, both MLV and SUV liposomes were evaluated for the essential oil loss and size distribution. The evaluation of size distribution was performed with a Mastersizer 2000R equipment, with 6 samples of liposomal formulation, in the same experimental conditions.

Results and discussions

The essential oil of *Anethi fructus* is a lipophillic substance, soluble in organic solvents and its encapsulation in liposomes were performed by passive entrapment method. Because the activity of the active principle encapsulated in liposomes depends on the size and lipid composition of liposomes, for this study we monitored the influence of type, composition and concentration of lipids used in liposomes formulations on the entrapment efficiency and time stability of liposomes.

The influence of lipids type on the entrapment efficiency

The characteristics of liposomes (entrapment efficiency, stability, release rate of active principle) are strongly influenced by the type of lipids used for preparation. For this reason, we selected for the experiments a number of phospholipids with different characteristics, such as: PC – a natural phospholipid common for liposomes preparation, DPPC and DMPC – saturated synthesis phospholipids, DOPC – a DMFG – an unsaturated synthesis phospholipids, negatively charged and cholesterol (C).

For these determinations we prepared several series of MLV liposomes, using the thin film hydration method described in "Material and method". In these formulations, we were varying the phospholipids type. The weight ratio of phospholipids, essential oil and cholesterol was kept constant: 100 : 25 : 10 (phospholipid : essential oil : cholesterol). The results are presented in Table 1.

Sample	Phospholipids	Entrapment efficiency (%)
LFR1	FC	98
LFR2	DPFC	97
LFR3	DMFC	58.9
LFR4	DOFC	77
LFR5	DMFG	55,2

Table 1.The influence of phospholipids type on entrapment efficiency of essential oil of *Anethi fructus*

From the data presented in the table we can observe that good entrapment efficiency has the formulations in which we used PC and DPPC. However, the use of DPPC was more difficult, requiring higher temperatures because of the high T_c of the lipids. If we are using DMPC, DMPG and cholesterol in the formulations of liposomes, the entrapment efficiency is low, 58.9%, respectively 55.2%. In case of using an unsaturated phospholipid, the entrapment efficiency didn't exceed 80%; besides, the stability of the liposomes was not so good, probably because of the existent unsaturation in the molecule (A. D. Sezer and J. Akbuga) [8]. Considering all this, we selected for the next studies the use of PC for the preparation of the *Anethi aetheroleum* – loaded liposomes.

Quantification of essential oil of *Anethum fructus* by UV spectrophotometrical analysis.

It is well known that most of the biological molecules don't have absorption in VIS, but in UV. This capacity is used in estimating the concentration of the essential oil of *Anethi fructus*, by measuring the absorbance at the corresponding wavelength.

For the quantitative determination of the essential oil we took into consideration the fact that, PC in methanol doesn't present characteristic peaks at the same wavelength as the essential oil (λ = 236 nm); besides, the same value is registered also for the pure carvone, the main component of the essential oil of *Anethi fructus*.

The absorbance spectrum of carvone in methanol solutions between 0 – $2.4 \cdot 10^{-4}$ g/100 mL emphasizes the wavelength λ = 236 nm as the maximum absorbance. The value of specific absorbance from the regression equation obtained from the experimental data Abs = 571.95C – 0.0022 (R^2 = 0.9996) is

$$A_{1cm}^{1\%} = 572$$

By analyzing the absorption spectrum of a methanol solution of essential oil (c = $4 \cdot 10^{-4}$ g / 100 ml) we determined an absorbance of 0.1716 at λ = 236 nm. The hypothesis that this maximum is due to the carvone component which is present in the essential oil, we determined the concentration of carvone:

$$c = \frac{0.1716}{572} = 0.0003 \text{ g / 100 mL}$$

Gas chromatographic analysis of the essential oil (these results will be presented in future article) showed that the oil has a content of 75.21 % carvone. For the analyzed solution, the carvone concentration is approximately 0.0003 g / 100 mL, value also found by spectrophotometrical analysis.

Both the validation of the spectrophotometrical method and the value of the specific absorbance were used in the determination of the entrapment efficiency of the essential oil in liposomes.

Purification studies

The purification of the liposomes implies the separation of the liposomes incorporating essential oil from the unincorporated one, by centrifugation. The supernatant was removed and analyzed spectrophotometrically, at λ = 236 nm, in order to quantify the existent essential oil. The sediment was dissolved in methanol and the entrapped quantity of the essential oil was determined spectrophotometrically. Essential oil recovery from supernatant and sediment was ascribed to the used dose in the preparation process.

In order to establish if the method is reproducible and so to be used in the determination of the incorporation efficiency we prepared a series of parallel samples of 2 lots of MLV liposomes. The results of these determinations are presented in table 2.

Lot	Determination	Quantity of essential oil from supernatant (mg / mL)	Percentage of recovered essential oil from sediment and supernatant (%)	Incorporation efficiency (%)	Medium incorporation efficiency (%)
LFR 6	1	$25 \cdot 10^{-5}$	95.85	87.5	87.5
	2	$23 \cdot 10^{-5}$	96.23	89.58	
	3	$25.5 \cdot 10^{-5}$	95.76	85.42	
LFR 7	1	$18 \cdot 10^{-5}$	97.22	90.08	91.29
	2	$17.5 \cdot 10^{-5}$	96.35	91.42	
	3	$17 \cdot 10^{-5}$	96.85	92.38	

Table 2.The incorporation efficiency and the percentage of the recovered quantity of the essential oil of *Anethi fructus* after purification of liposomes

The results presented showed that the essential oil is in traces in supernatant. This observation, correlated with the percentages obtained for the recovered oil from supernatant and sediment ascribed to the used dose (above 95%) demonstrate that the loss of essential oil

in the preparation and purification processes was very low. It can be also observed that the entrapment efficiency calculated for each determination of each liposomes series has similar values, which qualify the method for determination of incorporation efficiency.

The influence of lipid composition on entrapment efficiency

In order to obtain liposomes with higher entrapment efficiencies we conduct an experiment in which we prepared some series of MLV and SUV liposomes, using the thin film hydration method, as described in "Material and method". The ratio PC: essential oil was varied (C.C.Liolios et al) [9]. The compositions of the tested liposomes are presented in table 3, the same for both MLV and SUV vesicles. We have to specify that the presented compositions represent the result of an extensive study of formulation, in which several ratios PC: essential oil was investigated. We used defined, quantities of PC, cholesterol (C) and essential oil extracted from *Anethi fructus* (OFR), dissolved in 5 mL chloroform.

	Formulations							
Components	**LFR9**	**LFR10**	**LFR11**	**LFR12**	**LFR13**	**LFR14**	**LFR15**	**LFR16**
FC	19.5	19.5	19.5	19.5	39	39	19	39
C	2.6	2.6	2.6	5.2	2.6	5.2	5.2	2.6
OFR	6	3	9	9	9	9	6	6

Table 3. Sample Composition (mg/mL)

The essential oil content was spectrophotometrically assayed at λ = 236 nm, after the disruption of the vesicles with methanol. We calculated the entrapment efficiency for all the liposomal formulations. The results are presented in table 4.

Liposomal formulation	**Absorbance**		**Dilution coefficient**	**Concentration* $\cdot 10^{-4}$ g/100 mL**		**Incorporation efficiency (E%)**	
	MLV	**SUV**		**MLV**	**SUV**	**MLV**	**SUV**
LFR9	0.247	0.245	1000	5,75	5.7	92	90
LFR10	0.125	0.120	1000	2.9	2.8	95.5	91.5
LFR11	0.374	0.370	1000	8.7	8.6	88	85
LFR12	0.266	0.262	1000	6.2	6.1	70	66.5
LFR13	0.378	0.380	1000	8.8	8.85	90.5	86.5
LFR14	0.253	0.247	1000	5.9	5.75	67.5	60.5
LFR15	0.234	0.230	1000	5.45	5.35	87	82
LFR16	0.251	0.249	1000	5.85	5,8	93	90.5

Table 4. Incorporation efficiency of essential oil of *Anethi fructus* in different liposomal formulations

* represents the total concentration of the recovered essential oil from supernatant and sediment after centrifugation

The results showed that both MLV and SUV dispersions had a good incorporation efficiency; nevertheless. SUV dispersions always gave a lower content in incorporated essential oil than MLV, because of their smaller size and unilamellar structure. It could be observed that for the same ratio of PC: C a slight decrease of oil content appeared with the increase of essential oil concentration from the formulation, yet the total amount of essential oil being greater in LFR 11 formulation. Slight differences in the values of the incorporation efficiencies were present, depending on the phospholipids concentration (higher concentrations, higher entrapment efficiencies). As far as the dependence of the incorporation efficiency on cholesterol content is concerned, we observed a decrease of oil content with the increase of cholesterol content.

In conclusion, we can assume that a higher content of essential oil of *Anethi fructus* is obtained from MLV dispersions, with the formulation proposed for LFR11.

Stability measurements

Stability of vesicle dispersions was studied for one year; they were assayed for their content in essential oil after disruption of vesicles with methanol and for modification of size distribution in this time interval. The liposomal formulations were stored at 4 ± 1ºC and were evaluated at fixed time intervals (1 day, 7 days, 1 month, 2 months, 6 months and 12 months after preparation).

All the proposed formulations were assayed (table 3), both MLV and SUV. The results showed that a good stability was present for the first 6 months, oil leakage was very low (at least 95% of the incorporated oil was still in the liposomes), while vesicles size increased slightly (5 - 10% for MLV and a higher percentage for SUV), after one year, oil retention was still good (at least 90%), while size distribution was considerable modified (at least 40% bigger), especially in SUV dispersions, suggesting that fusion of vesicles occurred (Fig. 1, 2).

Figures 1 and 2 present the percentages of liposomes of certain medium sizes a function of medium size of liposomes for MLV, respectively SUV dispersions for LFR 11 formulation, at considered time intervals. The values represent the medium of 6 identical series of liposomes, monitored in the same experimental conditions.

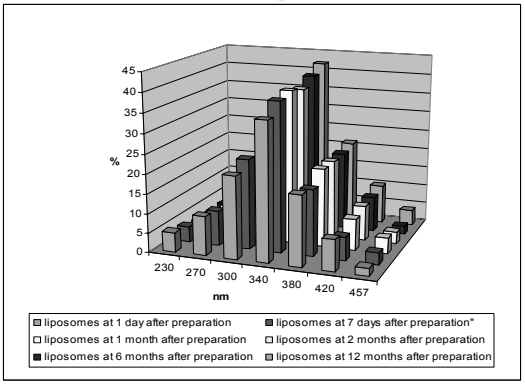

Fig.1. Size distribution of MLV dispersions for 1 year.

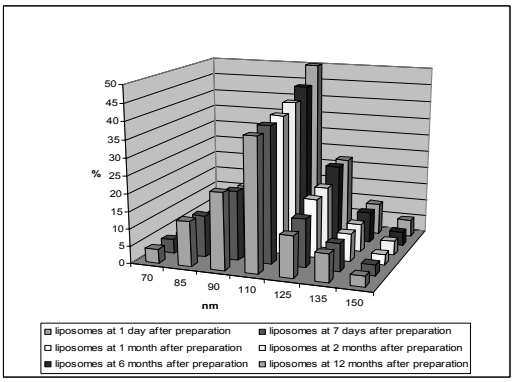

Fig.2. Size distribution of SUV dispersions for 1 year.

The graphics show a polidispersion of the vesicles, mean size ranging from about 70 to about 150 nm for SUV, respectively 230 – 457 nm for MLV. The two histograms show a majoritar percentage in interval 90 – 125 nm, respectively 300 – 380 nm.

Conclusions

The conducted experiments showed that the molar ratios of essential oil, phosphatidyl choline (PC) and cholesterol influence the drug entrapment of the liposomes. Studies show a good incorporation efficiency of the essential oil for all the considered formulations.

After sonication, the dimensions of liposomes population were between 70 - 150 nm, and for MLV between 230 – 457 nm; these values were approximately constant also after 2 months, but considerably increased after 12 months, especially in SUV dispersions. The stability measurements also showed that liposomal dispersions maintained the content of volatile oil. This conduct to the conclusion that the entrapment of the essential oil in liposomes increased the oil stability.

The validation of the spectrophotometrical method for essential oil dosing by determination of carvone content is an important step in monitoring the *Anethum graveolens* essential oil, because this method is easy to use, fast and require small quantities of sample.

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