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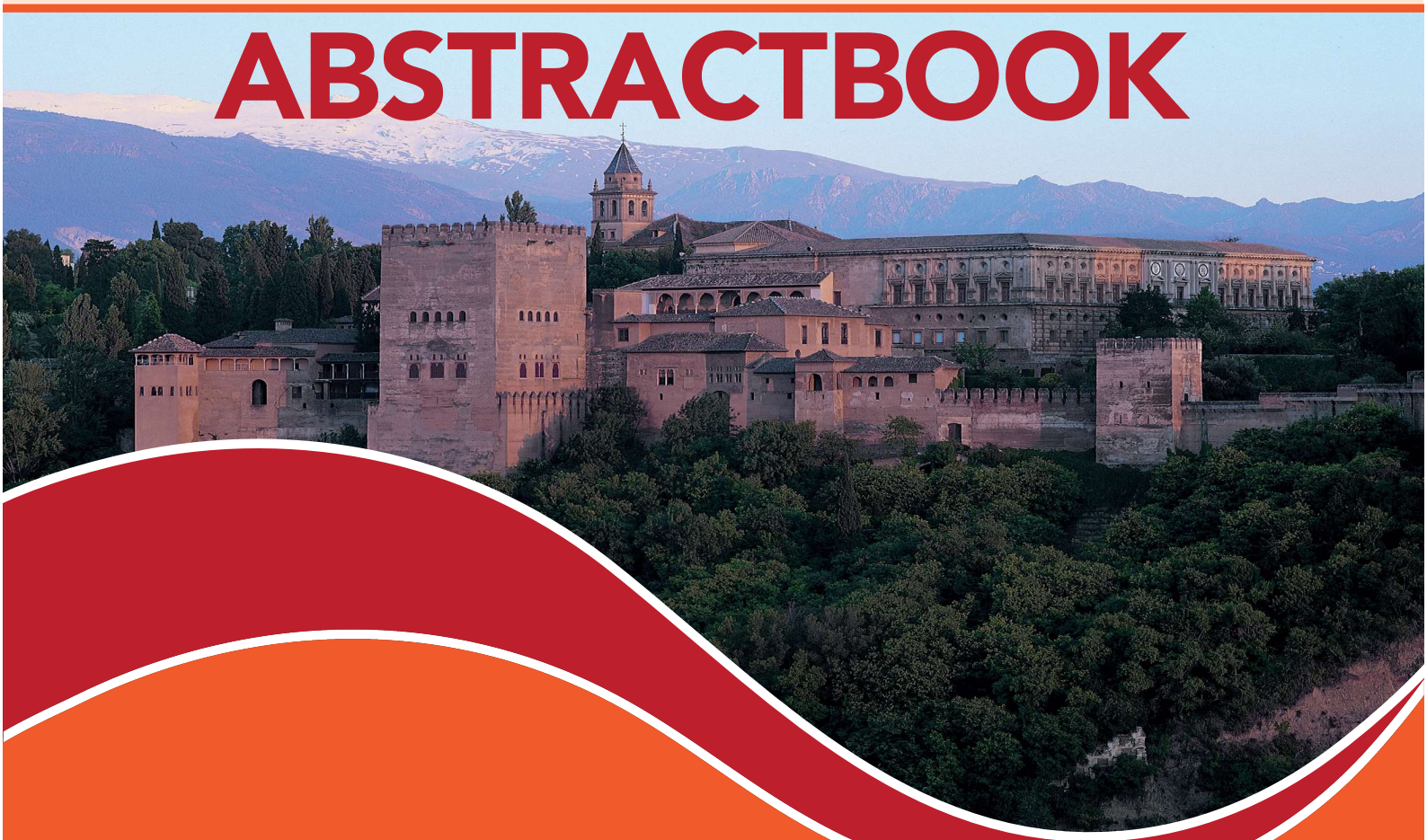

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Antioxidant activity of PEGylated liposomes loaded with rosemary dry extract for treatment of Alzheimer's disease

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INTRODUCTION

Over the last two decades, oxidative stress and inflammation have been identified as leading causes of brain aging. The use of antioxidant and anti-inflammatory molecules, such as polyphenols, is recommended as a useful strategy to prevent the aging of the brain and the appearance of several neurodegenerative diseases, including Alzheimer's disease [Obulesu et al., 2011].

Lipid peroxidation, widely recognized as a primary toxicological event, is caused by the generation of free radicals from a variety of sources including organic hydroperoxides, redox cycling compounds and iron-containing compounds.

In addition to the numerous effects, the extract of *Rosmarinus officinalis* L. (fam. Lamiaceae) also exhibits an antioxidant activity. Rosmarinic acid (RA) is characterized as compound with the strongest antioxidant activity among the other hydroxycinnamic acids [Fadel et al., 2011].

Its efficacy has been confirmed *in vitro* relating to the prevention of molecular disorders such as: oligomerization and the formation of Aβ plaques, Aβ-oligomer induced synaptic toxicity, and neurotoxicity, oxidative destabilization caused by Aβ fibrils (lipid peroxidation, DNA fragmentation, activation of caspase-3) and hyperphosphorylation of tau proteins [Obulesu et al., 2011].

However, for many polyphenolic compounds of natural origin, bioavailability is limited by their low solubility in biological fluids, as well as by the rapid metabolism *in vivo*. Therefore, liposomes are appropriate carriers for drug delivery purposes, due to their biocompatibility, wide choice of physico-chemical properties and easy preparation.

The aim of this study was to determine the antioxidative potential of PEGylated nanoliposomes (NLs) loaded with rosemary extract (RO-E) using different antioxidant assays.

MATERIALS AND METHODS

Materials

Soybean lecithin (SL) was purchased from Vitalia, Macedonia. LIPOID PE 18:0/18:0-PEG 2000 (PEG) was kindly donated from Lipoid, Germany. Rosmarinic extract (RO-E) was a gift from the Institute of Pharmacognosy, Faculty of Pharmacy, Skopje, Macedonia. Cholesterol (CH), rosmarinic acid (RA), butylatedhydroxyanisole (BHA), β-carotene, Tween 20, linoleic acid, iron (II) sulfate, acetic acid, thiobarbituric acid (TBA), sodium dodecyl sulphate (SDS) and trichloroacetic acid (TCA) were purchased from Sigma Aldrich (Germany). All the other chemicals and reagents were of the highest purity grade commercially available and used as received.

Methods

Preparation of NLs

In order to obtain formulations with desired physicochemical and biopharmaceutical characteristics, after preliminary studies [Cambuleva et al., 2016; Shalabalija et al., 2017], an experimental design approach was implemented and optimal formulations were selected.

Samples were prepared by the modified lipid film hydration technique [Cambuleva et al., 2016]. Briefly, required amounts of SL, CH and PEG (mass ratio SL:CH:PEG = 8.7:1:1.7 and 9:1:0.17 for samples 1 and 2 respectively) and 400 mg RO-E (for samples 1a and 2a) were dissolved in chloroform/methanol mixture 4:1 (v/v). Afterwards, the organic solvents were removed by evaporation under vacuum using a rotavapor (25 °C, 50 rpm; Buchi 215, Switzerland). Thus obtained dried lipid film was hydrated with phosphate buffer pH7.4. Obtained liposomes were submitted to high shear homogenization (24 000 rpm, 5 min; Ultra-Turrax T25, Ika-Werke, Germany) and were allowed to stand for 24 h at 4 °C.

NLs characterization

Mean particle size (D_{50}) and particle size distribution (SPAN factor) of NLs were determined using laser diffractometry (Mastersizer 2000, Hydro 2000S, Malvern Instr. Ltd., UK).

In order to determine the drug loading (DL, mg drug/100 mg lipid) and encapsulation efficiency (EE%), the vesicle suspensions were centrifuged (15 min, 4500 rpm; 4 cycles) and the supernatants were removed. The concentration of the drug was determined by previously validated HPLC method [Cambuleva et al., 2016].

Antioxidant activity of RO-E loaded NLs

TBARS assay

A modified thiobarbituric acid reactive species (TBARS) assay established by Dorman et al. [1995] was used to measure the potential antioxidant capacity of the PEGylated NLs with dry RO-E, using egg yolk as a lipid-rich media. Briefly, 500 µl of 10% (w/v) homogenate and 100 µl of sample, solubilized in buffer (pH = 7.4) or methanol, were

added to a test tube and made up to 1.0 ml with distilled water. 50 µl of iron (II) sulfate (0.07 M) was added to induce lipid peroxidation after incubation at 37 ºC for 30 minutes. Afterwards 1.5 ml of each of the following reagents: 20% acetic acid (pH 3.5), 0.8% (w/v) TBA prepared in 1.1% (w/v) SDS and 20% TCA were added. The resulting mixture was stirred with a vortex, and then heated at 95 ºC for 60 minutes. After cooling, 5.0 ml butan-1-ol was added to each tube, stirred, and centrifuged at 3000 rpm for 10 min. The absorbance of the organic layer was measured at 532 nm using Agilent 8453 UV-VIS spectrometer. The concentration that exhibits 50% inhibition of lipid peroxidation ($IC_{50,}$ mg/ml) was determined for all samples.

β-Carotene bleaching assay (β-CBA)

The β-carotene method was carried out according to the method developed by Wettasinghe and Shahidi [1999]. Briefly, 2 ml of β-carotene solution (0.2 mg/ml in chloroform) were pipetted into a round-bottom flask containing 40 mg linoleic acid and 400 mg Tween 20. The mixture was evaporated at 40 ºC to remove the solvent, followed by the addition of distilled water (100 ml). After agitating vigorously the mixture, aliquots of the resulting emulsion (5 ml) were transferred into test tubes containing 200 µl of different formulations of NLs. The mixture was placed in a water bath at 37 ºC or 50 ºC for 2 h and the absorbance of the tested samples was measured at 470 nm using a UV–VIS spectrophotometer. All determinations were performed in duplicates and $IC_{50\%}$ was calculated.

RESULTS AND DISCUSSION

The physicochemical properties of RO-E loaded PEGylated NLs are presented in Table 1.

Samples	D_{50} (nm±SD)	SPAN factor	EE (%)	DL
1a	126 ± 2.13	1.32	85.93	4.10
2a	118 ± 2.09	1.83	93.15	4.28

Table 1. Physicochemical properties of PEGylated NLs

Considering $IC_{50\%}$ values (Table 2) for TBARS and β-CBA and intended NLs` application (Alzheimer's disease), β-CBA at 37 °C was selected as most appropriate. It can be seen that sample 2a compared to 1a exhibited higher antioxidant activity and hence, similar to RO-E. In addition higher antioxidant activity was determined for blank (without RO-E) sample 2 compared to sample 1, 0.3789 mg/ml and 0.5985 mg/ml $IC_{50\%}$ values, accordingly. So, the observed difference between samples 1a and 2a are most likely related to the physicochemical properties (D_{50}, EE and DL) and drug release rate (data not presented) associated with differences in the formulations` composition (SL:CH:PEG ratio of 8.7:1:1.7 (samples 1 and 1a) and 9:1:0.17 (samples 2 and 2a).

Samples	TBARS	β-CBA	
		50 °C	37 °C
1a	0.1122	0.2812	0.2884
2a	0.1861	0.1003	0.0962
RO-E	1.6164	0.0832	0.0969

Table 2. $IC_{50\%}$ value for the tested samples

Although prepared samples showed significantly lower antioxidant activity compared to BHA $IC_{50\%}$ (0.0028 mg/ml for TBARS, 0.0125 mg/ml for β-CBA at 50 °C and 0.00017 mg/ml for β-CBA at 37°C) according to the classification established by Kaur & Kapoor [2002], RO-E belong to the group of agents with high antioxidant activity potential.

CONCLUSION

In this study, RO-E loaded PEGylated NLs were prepared and characterized. The results indicated the potential of designed formulations to inhibit the oxidation thus favoring the perspectives for efficient Alzheimer's disease treatment. β-CBA at 37 °C was selected for further studies because the performance conditions are most similar to the *in vivo* ones.

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