ORIGINAL ARTICLE

DEVELOPMENT AND EVALUATION OF THE ANTIOXIDANT ACTIVITY OF LIPOSOMES AND NANOSPHERES CONTAINING ROSMARINIC ACID

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Abstract

Rosmarinic acid (RA) is a strong antioxidant compound that is present in many natural plant extracts. Despite its strong antioxidant activity, it had a limited use because of the low water solubility and chemical instability. Encapsulation technologies have been used for improving the solubility and long-term stability of bioactive molecules. Liposomes (LPs) and nanospheres (NSs) can encapsulate many types of molecules and can provide high bioavailability and are able to assure a sustained release for a long time. In this study, RA-loaded LPs and NSs were developed and characterized *in vitro*. Encapsulation efficiencies (EE) % of formulations were found 55.6% and 43.4% respectively. Release studies of RA, performed using pH 7.4 phosphate buffer for 24 hours through dialyse membrane were investigated and 74.2 ± 2.11% and 83.2 ± 1.91% drug release rates were obtained respectively. The stability studies were performed when the formulations were stored at three different temperatures for 3 months. The significant particle size increase and the zeta potential decrease were not observed in LPs and NSs after 3 months when stored at 4ºC ($p > 0.05$). Antioxidant activities were measured using $DPPH^{\bullet}$ and $ABTS^{\cdot+}$ radical scavenging effect and ascorbate-iron(III)-catalysed phospholipid peroxidation and the results were found to be favourable. Generally, RA could load the drug carrier systems successfully and it was concluded that the prolonged antioxidant activity could be sustained for 24 hours.

Rezumat

Acidul rosmarinic (RA) este un compus puternic antioxidant, care este prezent în multe extracte naturale din plante. În ciuda activității sale antioxidante puternice, utilizarea sa a fost limitată, din cauza solubilității scăzute și a instabilității chimice. Tehnologiile de încapsulare au fost utilizate pentru îmbunătățirea stabilității pe termen lung a moleculelor bioactive. Lipozomii (LPs) și nanosferele (NSs) pot să încapsuleze multe tipuri de molecule, pot oferi o biodisponibilitate ridicată și pot asigura o eliberare susținută pentru mult timp. In acest studiu, au fost dezvoltate și caracterizate *in vitro,* LPs și NSs încărcate cu RA, demonstrând o eficiență la încapsulare (EE%) de 55,6% și respectiv 43,4%. Studiile de eliberare a RA, efectuate în tampon de fosfat pH 7,4, timp de 24 de ore prin membrană de dializă, au arătat rate de eliberare a RA de 74,2 ± 2,11% și respectiv 83,2 ± 1,91%. Studiile de stabilitate au fost efectuate atunci când formulările au fost depozitate la trei temperaturi diferite, timp de 3 luni. Nu au fost observate creșteri semnificative ale dimensiunilor particulelor și nici scăderea potențialul zeta la LPs și NSs, după 3 luni de păstrare la 4°C ($p > 0,05$). Activitatea antioxidantă a fost evaluată prin testele $DPPH^{\bullet}$ și $ABTS^{\cdot+}$ și prin peroxidarea fosfolipidelor sub influența ionilor ascorbat-fier (III), iar rezultatele s-au dovedit a fi favorabile. În general, RA ar putea fi utilizat cu succes în sistemele medicamentoase de transport și s-a ajuns la concluzia că activitatea antioxidantă poate fi menținută timp de 24 de ore.

Keywords: Rosmarinic acid, liposome, nanosphere, prolonged antioxidant activity

Introduction

Rosmarinic acid (RA) (a-O-caffeoyl-3,4-dihydroxyphenillactic acid) is a phenolic compound and a strong natural antioxidant, found in many species of the *Lamiaceae* family such as rosemary that has an antiviral, antibacterial and antiinflammatory properties [9]. Phenolic compounds play an important role by neutralizing free radicals and oxidants [16]. LPs are spherical, single or multiple-layer vesicles that are spontaneously formed when phospholipids are dispersed in water. They are biocompatible, non-toxic, can be formulated in small (nano) size and can entrap both hydrophobic and hydrophilic compounds within their structure, protect the compounds from degradation, and release the entrapped compounds at designated targets [7, 20]. Polymeric NSs are ideal vehicles for many controlled delivery applications due to their ability to encapsulate a variety of drugs, biocompatibility, bioavailability and sustained drug release. NSs were prepared with synthetic biodegradable polymers such as poly(lactid-*co*-glycolic acid) (PLGA), thus being widely studied because of its superior biodegradability and regulatory physiochemical properties [15, 22].

Encapsulation technologies have been used for improving low water solubility and long-term stability and enhancing effectiveness of active compounds using lipids and biodegradable polymers [12]. Two different free radicals were used to assess the potential free radical-scavenging activities of released RA from LP and NS, namely the 2,2-diphenyl-1-picryl-hydrazyl ($DPPH^{\bullet}$) and 2,2'-azino-bis(3-ethylbenzo-thiazoline-6-sulphonic acid ($ABTS^{+\bullet}$) synthetic free radicals. The stable nitrogen-cantered free radicals, $DPPH^{\bullet}$ and $ABTS^{+\bullet}$ are frequently used for the estimation of free radical-scavenging ability [8, 21]. In this study, we aimed to develop and characterize new formulations containing RA and we investigated the antiradical activity and sustained antioxidant efficiency against lipid peroxidation of RA *via* LPs and NSs.

Materials and Methods

Materials

Rosmarinic acid (RA), 1,2-Dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), Cholesterol (Ch) and Poly lactid-*co*-glycolic acid (PLGA) were obtained from Sigma, USA. Polyvinyl alcohol (PVA) was provided by Wecker-Germany. All standards and reagents were of the highest purity available and obtained from the Sigma, USA.

Methods

Analytical method and calibration. High-performance liquid chromatography (HPLC, Agilent 1200) equipped with C18 column (150 x 4.6 mm, 5 µm) was used to measure drug content in formulations. Methanol: water:acetic acid (43:52:2) mixture was used as mobile phase and degassed ultrasonically before use. The flow rate of mobile phase was adjusted to 1 mL/min [14]. For quantification, standard calibration curve was done ranging from 30 to 1.25 µg/mL of RA. The linearity plotting at 306 nm was ($y = 204.7x + 16.618$) ($r^2 = 0.9997$) for working solutions, where *x* is RA the concentration as µg/mL and *y* is the peak area.

Preparation of liposome and nanosphere

RA loaded LPs were prepared using dry film hydration method [4]. DPPC and Ch were added the round-bottomed flask in 1:1 molar ratios. They were dissolved with chloroform and evaporated in a rotary evaporator (Heidolph) under a vacuum at 44□C until the observation of a dry film. The lipid film was kept in desiccators to remove traces of organic solvent. The dry film was hydrated by RA solution and vortexed. LP suspension was ultra-centrifuged at 15,000 rpm at 4°C for 60 min. Supernatants and LPs were separated. The solvent evaporation method has been used to prepare NSs [18]. PLGA was used as a polymer. Polymer (400 mg) was dissolved in dichloromethane:methanol (9:1) mixture at room temperature, then injected slowly (0.5 mL/min) into aqueous phases containing RA and 0.4% PVA. The mixture was stirred at 11,000 rpm by Ultraturrax® for 10 minutes. After organic phase evaporation, NS suspensions were ultra-centrifuged at 10,000 rpm for 30 min at 4ºC. Supernatants and NSs were separated.

Liposome and nanosphere characterization and stability

The mean particle size (PS), polydispersity index (PDI) and zeta potential (ZP) of LPs and NSs were measured by using a Malvern Zetasizer Nano ZS (Malvern Instruments, Worcestershire, UK). EE% of RA was determined after centrifugation of the LP and NS dispersions and measurement of the drug content at the supernatant phase was subtracted from the total.

The stability of two formulations was investigated for 3 months under three different conditions (4ºC, 25ºC + 60% and 40ºC + 75% relative humidity). RA contents, PS and ZP of formulations were determined periodically.

In vitro release studies of RA from liposome and nanospheres formulations

Release studies were performed for RA using Franz diffusion cells with a 12,000 Dalton pore size dialysis membrane. A 2.0 mL RA-loaded LP and NS suspensions were placed in the donor compartment of the diffusion cells. The receiver compartment was filled with 2.0 mL phosphate buffer (pH 7.4). RA release studies were performed for 24 h at 37ºC. Samples of 2.0 mL were withdrawn at determined time points and fresh buffer was immediately replenished at the same volume. The samples were analysed by HPLC as described above and released RA was then studied for anti-oxidant activity.

$DPPH^{\bullet}$ scavenging activity

The ability of the formulations to scavenge $DPPH^{\bullet}$ was determined by the method of Gyamfi *et al.* [11]. The percentage inhibition was calculated using Eq. 1.

$$\% \text{ inhibition} = [(Abs_{control} - Abs_{sample}) / Abs_{control}] \times 100 \qquad \text{(Eq. 1)}$$

$ABTS^{\bullet+}$ radical scavenging

To further confirm the free radical scavenging activity of the formulations, an alternative synthetic radical $ABTS^{\bullet+}$ model was used, following the method of Re *et. al.* Absorbance was measured on a UV spectrophotometer at 734 nm [21].

Ascorbate-iron(III)-catalysed phospholipid peroxidation

The ability of the formulations to scavenge hydroxyl radicals was determined by the method of Aruoma *et al*. [2]. We have estimated the lipid peroxidation in terms of quantifying malon-

dialdehyde (MDA) level. Amount of thiobarbituric reacting substances formed is calculated from standard curve prepared, using 1,1′,3,3′ tetra-methoxy propane, and the values were expressed as nanomoles *per* millilitre.

Statistical analysis

All data in this study were considered as means ±SD, and one-way ANOVA was used for statistical analysis. GraphPad InStat ver. 2 was used for the analysis program. Significant differences between means were determined by Tukey's pairwise comparison test.

Results and Discussion

In this study, LP and NS formulations were prepared containing RA for evaluation of sustained antioxidant activity. For characterization of formulations PS, ZP, PDI and EE% were measured and given Table I. We found that mean PSs of RA-LPs and NSs 0.426 ± 0.004 µm and 0.736 ± 0.007 µm, ZPs of RA-LPs and NSs were -0.130 ± 2.3 mV and -1.48 ± 1.01 mV, and PDIs of RA-LPs and NSs were 0.319 ± 0.067 and 0.340 ± 0.023 respectively. Preparation, characterization and antioxidant activity of LP containing RA as an active compound has not been studied. In literature, antioxidant efficiency of RA against lipid peroxidation, its coactions with lipid membranes and effect on long-term stability at LPs were investigated and the results revealed that RA may increase the oxidative and physical stability of LPs [9, 20]. In other study, authors prepared nanoliposomes with *Orthosiphon stamineus* (OS) ethanol extract including RA. OS liposomes were prepared with conventional film method and characterized for PS, ZP, EE%, release and antioxidant activity. PS, ZP and EE% were found 152.5 ± 1.1 nm, -49.8 ± 1.0 mV and 54.1 ± 0.2% respectively [1]. In another study, Kim *et al.* prepared and characterised RA-loaded poly-caprolactone (PCL) microspheres using emulsion solvent evaporation method. PS and EE% were found within the range of 5 to 15 µm and 22 to 78% with different ionic surfactants. Their results suggest that RA may be stably and efficiently encapsulated into polycaprolactone microspheres [12]. In recent study, PDI values were under 0.5 in both formulations and it is concluded that distributions has been found to be monodisperse. A monodisperse distribution of colloidal systems is required and it means that the particle size distribution is narrow [5, 10]. Therefore, our findings from characterization were in accordance with the literature.

Table I

Characterisation of RA liposome and nanosphere formulations (n = 3)

Formulations	PS ± SD (µm)	ZP ± SD (mV)	PDI ± SD	EE ± SD (%)
RA-LP	0.426 ± 0.004	-0.130 ± 2.3	0.319 ± 0.067	55.6 ± 1.25
RA-NS	0.736 ± 0.007	-1.48 ± 1.01	0.340 ± 0.023	43.4 ± 2.76

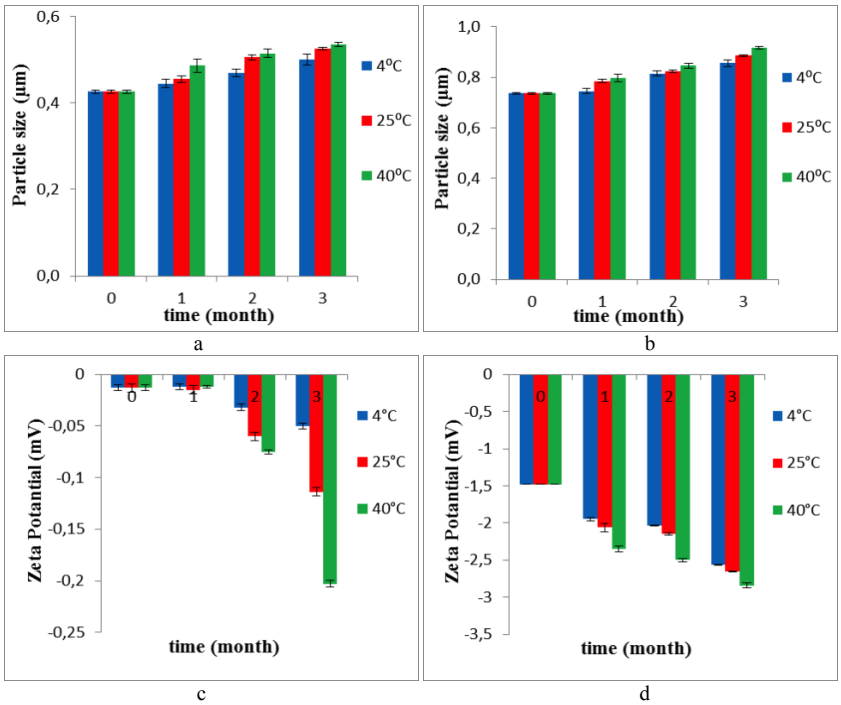

Figure 1.

The mean particle size and zeta potential of RA LPs (a, c) and RA NSs (b, d) stored at 4ºC, 25ºC and 40ºC (error bars represent standard deviations, n = 3)

Stability studies were performed using different condition for LPs and NSs. Mean PS, ZP, drug content and degradation kinetics of the formulations were determined for 3 months (Figure 1). The particle size increase and the zeta potential decrease were not observed in RA LPs and NSs after 3 months when stored at 4ºC ($p > 0.05$). When stored at 25ºC and 40ºC for 3 months, the particle size increase and zeta potential decrease were observed significantly ($p < 0.005$). Therefore, the physical stability of LPs and NSs depends considerably on storage conditions and the aggregation process may be observed at high temperatures [6]. The degradation of RA LPs and NSs followed first order kinetics, and shelf life were calculated as 12 days at 4ºC ($r^2 = 0.964$), 9 days at 25ºC ($r^2 = 0.947$) and 6 days at 40ºC ($r^2 = 0.940$) for RA LPs and 10 days at 4ºC ($r^2 = 0.943$), 8 days at 25ºC ($r^2 = 0.937$) and 6 days at 40ºC ($r^2 = 0.942$) for RA NSs respectively (Figure 2). In literature, two cosmetic formulations, emulsions, containing RA-PCL microspheres and only RA were prepared in order to observe the long-term stability of RA, at two different temperatures (25ºC and 50ºC). RA-loaded PCL microspheres showed a better long-term stability of the RA compared with those containing only RA ($p < 0.05$) [12]. Therefore, our stability results suggest that RA may be stably and efficiently encapsulated into LPs and NSs.

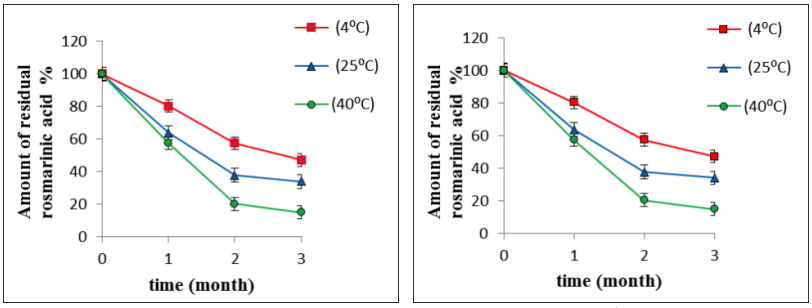

Figure 2.
Degradation of RA-LP (a) and RA-NS (b) stored at 4ºC, 25ºC and 40ºC (error bars represent standard deviations, n = 3)

The *in vitro* RA release experiment from LPs and NSs were performed at 37ºC with pH 7.4 phosphate buffer (Figure 3).

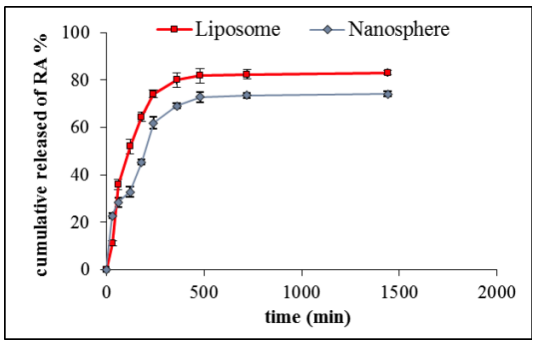

Figure 3.
In vitro release profiles of RA from LP and NS at pH 7.4 phosphate buffer (error bars represent standard deviations, n = 3)

RA release from LPs and NSs were found to be with RRSBW (Rosin-Rammler-Sperling-Bennet-Weibull) kinetics, and correlation coefficients were 0.9616 and 0.9589 respectively. In this RRSBW kinetic, steeper initial slope followed by a flattened tail in the final part was obtained [19]. There is no literature data to compare our release study results, so this is the first study of nanocarriers containing RA as a bioactive molecule.

Numerous information have indicated that free radicals play a critical role in a variety of pathological conditions such as brain dysfunction, cancer, heart diseases and inflammation by damaging cellular components of DNA, proteins and lipids [13, 17]. Recent studies have been focused recently on natural sources of phenolic antioxidants, not only for the prevention and treatment of diseases, but also for protecting quality of pharmaceutical formulations by preventing oxidative deterioration of lipids. In this study, we chose to investigate the antiradical activity and antioxidant efficiency against lipid peroxidation of RA-loaded formulations. It is generally admitted that RA is an antioxidant as it may act as free radical scavenger [9], but there are not enough studies that reveals the RA formulations. In our study, DPPH$^\bullet$ radical-scavenging effect was measured from samples taken at specified time periods, throughout release studies. RA solution's DPPH$^\bullet$ radical-scavenging effect was found IC_{50} 4 µg/mL. We could not calculate IC_{50} values of released RA at specified times, because of high EE% and high release of formulations from 30th minute to 24th hour. RA LPs inhibited the DPPH$^\bullet$ radical 41 ± 0.23% in 30 min. After 30 min, the inhibition increased to 89 ± 0.15% at the end of 24 hours. RA NSs

inhibited the DPPH$^{\bullet}$ radical 82% ± 0.25 in 30 min and the inhibition reached to 85 ± 0.31% at the end of the 24 hours. In a previous report, release study of OS liposome showed 62.4 ± 0.1% and the released extract was then studied for DPPH$^{\bullet}$ scavenging effect. Stronger free radical scavenging effect was obtained in OS liposomes that in non-formulated extract [1].

From the Trolox equivalent antioxidant capacity (TEAC) data, the concentration of 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) (mmol/L) having the ABTS$^{\bullet+}$ scavenging activity equal to cumulative released amounts from both LPs and NSs and the standard 20 µg/mL concentrated butylated hydroxy toluene (BHT) as shown in Figure 4. It can be seen that the calculated TEAC values for RA formulations are smaller than the TEAC values of BHT and displayed statistically similar activities to the ABTS$^{\bullet+}$ radical scavenger.

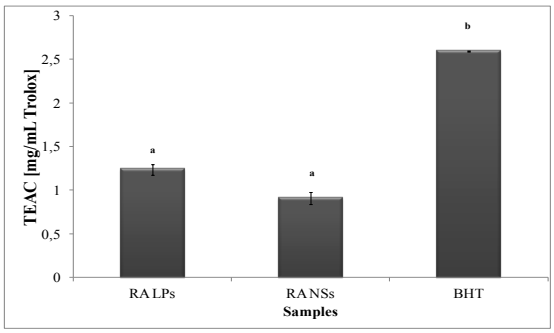

Figure 4.
The ABTS$^{\bullet+}$radical scavenging activity of RA-LPs and RA-NSs and standard BHT. Values (mg/mL) expressed as mean ± standard errors, bars with the same lower case letter (a-b) are not significantly ($p > 0.05$) different.

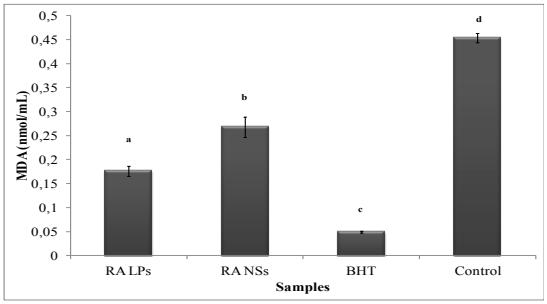

Figure 5.
The effects of the RA-LPs and RA-NSs and BHT on MDA formation in Ascorbate-Iron(III)-Catalysed Phospholipid Peroxidation. Values (nmol/mL) expressed as mean ± standard errors, bars with the same lower case letter (a-d) are not significantly ($p > 0.05$) different.

The lipid peroxidation is the accumulated effect of reactive oxygen species, and it is a chain of reactions causing the dysfunction of biological systems. During the oxidation process, peroxides are gradually decomposed to lower molecular weight compounds such as MDA [3]. As shown in Figure 5, RA-LPs inhibited the formation of MDA compared to control group and also was found more active than RA-NSs. Both RA-LPs and RA-NSs were not as active as the 20 µg/mL concentrated BHT solution.

Conclusions

It was concluded that LPs and NSs can encapsulate active compounds such as RA and also can release them at controlled rates for relatively long periods of time. This developed formulations with RA are suitable and effective nanocarriers for sustained protection effect against different oxidative stress related diseases and also these formulations were proposed for the cosmetic and topical formulation industry, based on the antioxidant and antiradical properties of RA.

Conflict of interest

The authors declare no conflict of interest.

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