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Delivery systems for natural antioxidant compounds: Archaeosomes and archaeosomal hydrogels characterization and release study

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ABSTRACT

The aim of this study was to use archaeosomes, a novel kind of liposomes made up by archaeal polar lipids, both multilamellars (MLVs) and unilamellars (SUVs), as a topical delivery system for natural antioxidant compounds recovered from olive mill waste. For comparative purpose an analogue formulation of phosphatidylcholine liposomes was prepared. SUVs were smaller than MLVs ones, showing size values smaller than 200 nm, which was maintained during the stability study. Transmission electron microscopy showed spherical morphology for conventional liposomes while archaeosomes had more irregular membranes. Vesicle encapsulation efficiency was quite similar in both formulations and was enough to ensure a good antioxidant activity. Stability studies were performed one month after the preparation of formulations, which showed a high stability with no change in the initial characteristics of the suspensions. Furthermore, the possibility of incorporating the liposomal suspensions in different excipients (Carbopol-940® and Pluronic-127®) for topical administration was studied. In order to evaluate the release behaviour of the different systems prepared, in vitro diffusion studies were carried out using vertical diffusion Franz cells. In both cases the incorporation of the vesicles into the gels lead in a sustained release for 24 h. Archaeosome gels released a similar amount of phenolic compounds regardless the excipient used, while in liposomal gels great release differences were found between carbopol and pluronic gel.

1. Introduction

Skin ageing is associated with physiological processes and is inevitable; however there is an exogenous ageing which is caused by extrinsic harmful environment factors and can be prevented (Kaur et al., 2007). One of the most harmful extrinsic factors for skin is UV radiation, either A and B radiation (UVA and UVB). Exposition to these radiations leads the formation of reactive oxygen species (ROS), which are involved in phototoxicity reactions and inflammation, aggravating previous skin diseases and being involved in the development of malignant tumors (Sakurai et al., 2005).

Among the approaches used to protect skin from these degenerative effects, use of antioxidant has been adopted as an important strategy, being incorporated in cosmetic and pharmaceutical formulations to scavenge free radicals in skin (Lupo, 2001).

Phenolic compounds found in olives have shown a high antioxidant activity (Visioli et al., 2002), but due to their partition coefficient (K_p) during the obtention of olive oil these compounds tend to accumulate in the waste product (Rodis et al., 2002). For this reason the waste product of the olive oil industry could be a source of natural antioxidants that provide an alternative to the use of synthetic antioxidants in the industry that we are referring to (cosmetics and pharmaceuticals) (Obied et al., 2005a).

However, delivery of these antioxidants using the conventional dosage forms is a challenge due to various reasons like poor solubility and permeability, instability and extensive first pass metabolism before reaching systemic circulation (Ratnam et al., 2006). To overcome these disadvantages of antioxidant orally administration, topically administration could be a more effective alternative because of the accumulation of the antioxidant compounds just in the area which needs to be protected (Pinnell, 2003). However, by applying an antioxidant directly on the skin surface, it will find a number of difficulties to penetrate through the stratum corneum, and therefore the use of a delivery system, such as liposomes, could have a doubly beneficial effect in one hand it will protect the antioxidant compound of phenomena that can alter its chemical structure or biological activity, and on the other one it will facilitate the passage through the skin and the formation of a deposit in deeper layers of the stratum corneum (Sinico and Fadda, 2009).

Despite the many advantages of liposomes, their instability constitutes one of the main drawbacks associated with their therapeutic application (Grit and Crommelin, 1993). It is remarkable

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that lipid composition of liposome membrane is one factor with a high influence on liposomes stability (Betageri, 1993). In this sense, archaeal membrane lipids are an excellent raw material to form liposomes. Archaea consist of single-celled organisms distinct from eubacterial and eukaryotic cells. Archaeosomes are vesicles made with membrane polar lipids extracted from species in this Domain. Archaeal membranes are composed of polar ether lipids consisting of branched isopranoid chains (fully saturated) that are uniquely attached via ether bonds to the glycerol backbone carbons at the sn-2,3 positions, feature that is unique to this Domain (Kates, 1993; Sprott, 1992). In contrast, conventional phospholipids found in Bacteria and Eukarya consist of unbranched unsaturated fatty acyl chains and are attached via ester bonds to the sn-1,2 glycerol carbons (Patel et al., 2007). Archaeal polar lipids are composed by a lipid core and polar head groups. The core structures can be a glycerol diether with 20 carbons per isopranoid chain (diphytanyl glycerol diether) called archaeol, and a dymer formed by two covalently linked diether molecules (dibiphytanyl diglycerol tetraether) containing 40 carbons per isopranoid chain, which is called caldarchaeol, and modifications thereof (Koga and Morii, 2005; Nishihara et al., 1987).

This particular chemical structure confers to these lipids some advantages for liposomes preparation if compared with the phospholipid usually used with this purpose. The ether linkages are more stable than esters over a wide range of pH, and the branched structure helps both to reduce crystallization and membrane permeability; the saturated alkyl chains impart stability toward oxidative degradation, and the unusual stereochemistry of the glycerol backbone ensures resistance to enzyme attack (e.g., phospholipases) (Choquet et al., 1992; Kates, 1992; Koga and Morii, 2005). Consequently, archaeosomes have been reported to be more stable to oxidative stress, high temperature, alkaline or acidic pH, action of gastrointestinal tract enzymes, and thus proposed as an alternative to conventional liposomes for the enhanced delivery of drugs or as novel vaccine delivery systems (Patel et al., 2000; Sprott et al., 1997, 2003). Furthermore, the delivery of betamethasone dipropionate to skin was more effective when the drug was encapsulated in archaeosomes than in conventional liposomes, which suggest that suitably developed archaeosomes may hold great promise as delivery vehicles for topical applications (Gonzalez-Paredes et al., 2010).

However, the major disadvantage in using liposomal formulations for topical drug delivery or other mucosal routes is the liquid nature of the preparation (Pavelic et al., 2001). Suitable viscosity and application properties of liposomes can be achieved by their incorporation in an appropriate vehicle. It has been confirmed that liposomes are fairly compatible with viscosity increasing agents such as methylcellulose, as well as polymers derived from acrylic acid (Carbopol®) (Foldvari, 1996; Skalko et al., 1998). In addition, liposomal Carbopol gels were found to enhance the skin retention of drugs, i.e. they provide higher and sustained skin concentrations of drugs compared to conventional gels and creams, without enhancing the systemic absorption of drugs (Dragicevic-Curic et al., 2009). Higher skin retention of the drug may be due to closer contact of the gel, more viscous than the liposomal suspension, with the epithelial layer of the skin (Seth et al., 2004).

Another excipient which is suitable for the incorporation of liposomes is Poloxamer® or pluronic® block copolymers, which have been proposed for diverse pharmaceutical applications (Dumortier et al., 2006; Ruiz et al., 2008). Pluronic® are the series of difunctional triblock copolymers of non-ionic nature. They comprise of a central block of relatively hydrophobic polypropylene oxide surrounded on both sides by the blocks of relatively hydrophilic poly ethylene oxide (Alexandridis and Alan Hatton, 1995). Pluronic® copolymers are excipients widely used in a variety of pharmaceutical applications, being particularly useful in topical, rectal and ocular formulations because of its low toxicity and irritation and its behaviour as drug permeation enhancer (Lee et al., 1997).

The main purpose of the present study was to evaluate the incorporation of antioxidant phenolic compounds in archaeosomes formulation, to characterize them and to incorporate antioxidant archaeosomes in a proper excipient, Carbopol® and Pluronic® gels, in order to obtain a dosage form for topical application. As well, equivalent liposomal formulation and dosage forms were prepared with phosphatidylcholine for comparative purpose. Furthermore, the release of all the formulations was evaluated in order to ascertain the most suitable one for topical administration of antioxidant phenolic compounds.

2. Material and methods

2.1. Materials

Archaeal lipids (AL) were extracted from Archaea *Halobacterium salinarum CECT 396*. Soy phosphatidylcholine (Phospholipon® 90, P90G) were kindly obtained from Natterman Phospholipids, (Cologne, Germany). Antioxidant phenolic extract was obtained from olive mill waste. Cholesterol (Chol), chloroform, methanol and Trolox were purchased from Sigma–Aldrich (Spain). Carbopol-940® and Pluronic-127® were obtained from Fagron (Spain). All solvents used were HPLC grade, while all other materials were of analytical grade.

3. Methods

3.1. Extraction of antioxidant phenolic compounds

5 g of freeze-dried olive mill waste were treated with 25 ml of methanol (1:5, w/v), stirred and centrifuged during 10 min at 450 g. This process was repeated six times. Then the solvent was evaporated in a rotatory evaporator until complete elimination of the solvent. The solid residue was resuspended in bidistilled water and filtered through 0.45 μm filter to eliminate fat residues and particles in suspension. The concentration of this phenolic aqueous solution was adjusted to a total phenols content of 321 ± 11 μg CAE/ml (Caffeic Acid Equivalents) which was determined by the method of Folin–Ciocalteau (calibration curve $y = 1.2158x - 0.0184$, $R^2 = 0.9942$) (Singleton and Rossi, 1965).

3.2. Growth of H. salinarum and lipid extraction

The obtention of *H. salinarum* biomass and the extraction of polar lipids were performed as previously described (Gonzalez-Paredes et al., 2010). Briefly, the microorganism was grown in mineral salt medium OS (Oesterhelt and Stoeckenius, 1974) supplemented with 0.1 ml/l of a 0.1-N HCl solution with different trace metals (6.6 g/L $ZnSO_4 \times 7H_2O$, 1.7 g/L $MnSO_4 \times H_2O$, 3.9 g/L $Fe(NH_4)2SO_4 \times 6H_2O$, and 0.7 g/L $CuSO_4 \times 5H_2O$) at it optimum pH (7.3) in a glass bioreactor (working volume: 1 l). Total lipid extracts (TL) were obtained by a modification of the Bligh and Dyer procedure described by Kates (Bligh and Dyer, 1959; Kates, 1986). Polar lipids (PL) were collected from the total lipid extract by precipitation with cold acetone (1:20, v/v), as described by Choquet et al. (1992). The lipids were stored in chloroform:methanol (2:1, v/v) at −20 °C until use.

3.3. Identification and characterization of archaeal polar lipids

Polar lipids had been previously identified (Gonzalez-Paredes et al., 2010). Differential scanning calorimetry (DSC) was carried out to determine the transition temperature of the polar lipids

(Shimadzu DSC-50Q). A slope of 2 °C/min was used. The starting temperature was 20 °C and it was raised until 100 °C.

3.4. Vesicle preparation

One liposomal formulation was designed for both archaeosomes and conventional liposomes of phosphatidylcholine (P90G). The composition of the formulations, with the molar ratio and quantities of each component are shown in Table 1.

Multilamellar vesicles (MLVs), were prepared according to the thin film hydration method (Bangham et al., 1974). Briefly, PL or P90G and cholesterol were dissolved in chloroform; the lipid mixture was deposited as a thin film in a round-bottom flask by roto-evaporating the chloroform under vacuum. The vacuum was applied for 1 h to ensure total removal of trace solvents. The phenolic aqueous solution was poured into the flask in order to hydrate the lipid film, which was achieved by stirring this mixture for 1 h at a temperature above the gel–liquid transition temperature of the amphiphiles (Tc). Sonicated vesicles (SVs) were prepared by sonicating MLVs dispersions at 80% of amplitude during 4 min with a Hielscher UP50H ultrasonic disintegrator (Germany).

3.5. Vesicle characterization

The average size, polydispersity index (P.I.) and zeta potential of the archaeosomal and liposomal vesicles were measured by dynamic light scattering using a Zetasizer Nano ZS (Malvern Instrument, UK). Samples were backscattered by a helium–neon laser (633 nm) at an angle of 173° and a constant temperature of 25 °C. The nano-ZS systematically and automatically adapts to the sample by adjusting the intensity of the laser and the attenuator of the photomultiplier, thus ensuring accuracy and reproducibility of the experimental measurement conditions.

Vesicle formation and morphology were checked by transmission electron microscopy (TEM) using a Zeiss 902 (Germany) microscope operating at an accelerating voltage of 80 kV. Vesicles were examined using a negative staining technique: samples were adsorbed on a carbon film-covered copper grid and stained with 2% (w/v) uranyl acetate.

3.6. Incorporation efficiency (E%)

The incorporation efficiency (*E*%) was determined after purification of the vesicles from untrapped drug by dialysis during 4 h in bidistilled water (200 ml) using a cellulose membrane of 12–14000 Da (SpectraPor). Later, vesicles were disrupted with 0.025% non ionic Triton X-100, and total phenols were quantified by Folin–Ciocalteau method (Singleton and Rossi, 1965). Incorporation efficiency was expressed as a percentage according to the following formula:

$$E\% = \left(\frac{C_{\text{purified}}}{C_{\text{total}}}\right) \times 100$$

where C_{purified} was the phenolic content in the purified vesicles and C_{total} was the total phenolic content in the non-purified vesicles.

3.7. Antioxidant activity

Antioxidant activity of liposomal dispersions was evaluated using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging method (Brand-Williams et al., 1995), which is widely use by many authors to measure antioxidant activity of phenolic compounds (Foti and Daquino, 2006; Laghari et al., 2011; Villano et al., 2007; Visioli et al., 1998). Hydrogen atoms or electrons donation ability of encapsulated phenolic compounds was measured by their bleaching capability of purple colored DPPH methanol solution. Reaction mixture was comprised of 3.9 ml of DPPH in methanol solution (25 mg/l), 0.1 ml of each kind of liposomal formulation and it was kept in the dark for 60 min at room temperature, which was enough time to ensure that the reaction reached the steady state. The absorbance was measured on Cintra 10 UV/VIS Spectrophotometer at 515 nm. Decreasing amplitude of signal at the selected wavelength confirmed the radical scavenging activity. The antioxidant activity of Trolox as standard reference was assayed. Methanol was used as blank and the measurement of solutions without sample were used as the control.

The inhibition of DPPH radicals by the samples was calculated as follows:

$$\text{DPPH inhibition\%} = \left[\left(\frac{A_{\text{control}} - A_{\text{sample}}}{A_{control}}\right)\right] \times 100$$

where A_{control} is the absorbance without extract and A_{sample} is the absorbance with the liposomal formulation.

3.8. Preparation of liposomal and control gels

Two kinds of gels were prepared using 1% Carbopol 940® or 20% Pluronic 127®. On suspension of archaeosomes or liposomes already prepared, the adequate amount of Carbopol 940® or Pluronic 127® to achieve the final concentration of 1% and 20% respectively was added and stirred slowly until it was completely dissolved. In carbopol gels, triethanolamine was added to achieve pH ≈ 6. To check the influence of liposomal incorporation on extract release, liposome-free gels were prepared with non encapsulated phenolic extract. Control gels were prepared for each kind of gel, in which there were no phenolic extract. Both gels were left overnight to ensure the complete formation of the gel structure.

3.9. Organoleptic study

The organoleptic characteristics of the prepared gels may vary depending on their composition. The gels were scored for appearance, texture, odour, consistency and presence of exudates 24 h after preparation. Organoleptic characteristics were classified with descriptive terms as thick, hard, creamy, smooth, soft, dry, thin, spreadable, cool or warm (Rigano and Siguri, 1996).

3.10. Stability study

Stability of formulation was evaluated one month after their preparation. Liposome dispersions were stored at 4 ± 1 °C and size, polydispersity index and zeta potential were measured after this period, as well as entrapment efficiency and antioxidant activity. Organoleptic characteristics of gels were also evaluated after one month of storage at 4 ± 1 °C.

3.11. In vitro release studies and Kinetic evaluation

In vitro release studies were performed using vertical diffusion Franz cells (FDC-400) which were supplied by Vidra-Foc (Barcelona, Spain). It consists of two compartments with a membrane clamped between the donor and receiver chambers. The receiver compartment had a volume of 11 ml and was filled with bidistilled water (pH ≈ 5.5) as receptor phase. It was constantly stirred with a small magnetic bar and thermostated at 37 ± 1 °C throughout the experiments. 0.3 g of gel was placed on the membrane surface. The membranes characteristics were 47 mm in diameter and 0.45 μm in pore size. Two types of membranes were tested: HA (cellulose ester) and HVLP (polyvinylidene fluoride) (Millipore, Spain).

Table 1
Composition of liposomal formulations.

Formula	Molar ratio lipid:chol:antioxidant	mg/ml			
		PL	P90G	Chol	Antioxidant extract (CAE)
Archaeosomes	1:1:0.1	20	–	8.6	0.321
Conventional liposomes	1:1:0.1	–	20	10	0.321

Samples of the receiver compartment were extracted after elapsed times of 0.25, 0.75, 1, 1.25, 1.5, 2, 3, 4, 5, 6, 7, 8 and 24 h, replaced with an equal volume of fresh solution to ensure sink conditions, and analysed by UV-spectrophotometry at 280 nm (λ_{max}). The method was previously validated and verified for accuracy, precision and linearity.

The data obtained from in vitro release studies were fitted to various kinetic equations to find out the mechanism of phenolic extract release from Carbopol and Pluronic gels, archaeosomes and liposomes suspensions and archaeosomes and conventional liposomes gels.

Three different kinetic models (zero order, first order and Higuchi) were used to fit the experimental data obtained in the drug release experiments. In order to better characterize the drug release behaviour Korsmeyer–Peppas model was further applied.

$$\frac{\%R_t}{\%R_\infty} = K_0 \times t \quad \text{zero order}$$

$$\frac{\%R_t}{\%R_\infty} = 1 - e^{-K_1 \times t} \quad \text{first-order}$$

$$\frac{\%R_t}{\%R_\infty} = KH \times t^{1/2} \quad \text{Higuchi's equation}$$

$$\frac{\%R_t}{\%R_\infty} = K \times t^n \quad \text{Korsmeyer–Peppas's equation}$$

where $\%R_t$ is the percentage drug released at time t, $\%R_\infty$ is the total percentage drug released, $\%R_t/\%R_\infty$ is the fraction of drug released at time t, K is the release rate constant, n is the diffusion release exponent that could be used to characterize the different release mechanism $n < 0.5$ (Fickian diffusion), $0.5 < n < 1.0$ (anomalous transport), and >1.0 (case II transport; i.e. zero-order release (Costa et al., 2001).

A nonlinear least-squares regression was performed using the WinNonLin® software (WinNonlin® Professional edition version 3.3, Pharsight Corporation, USA), and the model parameters calculated. Also the Akaike Information Criterion (AIC) was determined for each model as it is an indicator of the model's suitability for a given dataset (Yamaoka et al., 1978).

3.12. Statistical analysis

All tests have been run in triplicate and mean values have been reported.

The analysis of variance (ANOVA) intra and inter group was used to compare different parameters. The results were considered statistically significant when $p < 0.05$.

4. Results and discussion

4.1. Identification and characterization of archaeal polar lipids

The polar lipids found in this strain were previously identified as archaeol analogs of phosphatidylglycerophosphate (PGP) or the monomethylated derivative (PG-Me), and phosphatidylglycerosulfate (PGS) (Gonzalez-Paredes et al., 2010).

These results are in agreement with the early findings of Kates, who reported as the major and minor phospholipid components of extreme halophiles PGP, PGP-Me, PGS, and phosphatidic acid (PA), which was later confirmed by other authors (Corcelli et al., 2002; Renner et al., 2005).

The DSC analysis showed that in the range of temperature tested no phase transition was observed for polar lipids, as shown in Fig. 1. It could be conclude that the phase transition temperature for this kind of lipids is lower than 20 °C, which was the starting temperature for this analysis. These results are in agreement with previous studies which revealed for archaeal lipids phase transition temperatures much lower than those found for ester lipids such as phosphatidylcholine (Blocher et al., 1984; Yamauchi et al., 1993).

The chemical composition of archaeal polar lipids, which have fully satured isopranoid chains, may affect the fluidity of the liposomal membrane, which is dependent on insaturation degree and environmental temperature (Gabrijelcic et al., 1990). In general terms, low phase transition temperature and satured alkyl chains helps to reduce crystallization, membrane permeability and oxidative degradation (Choquet et al., 1992; Koga and Morii, 2005). For this reason, archaeosomes appear to offer some advantages with respect to conventional liposomes.

4.2. Characterization of vesicular formulations and stability study

Archaeosomes and conventional liposomes, both MLVs and SUVs, were prepared for the entrapment of the antioxidant extract. Table 2 summarizes the main characteristics of both types of vesicular carriers.

The DLS analysis showed a bimodal distribution in the particle size which was later confirmed by the polidispersity index, which was around 0.5. Archaeosomes and conventional liposomes

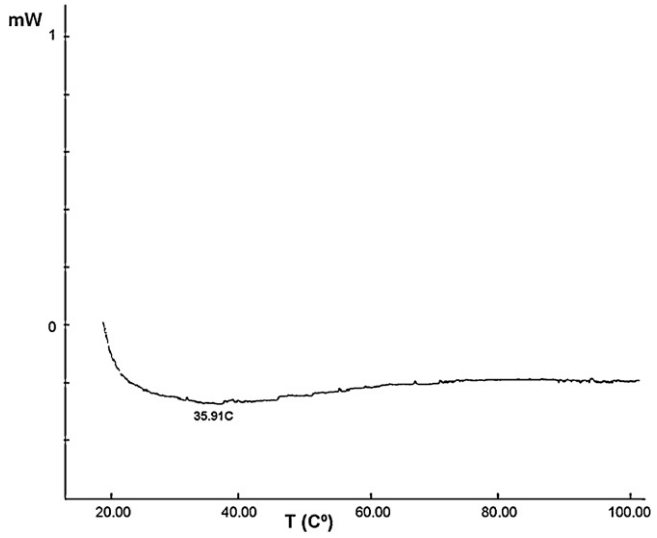

Fig. 1. Archaeal polar lipids, DSC analysis.

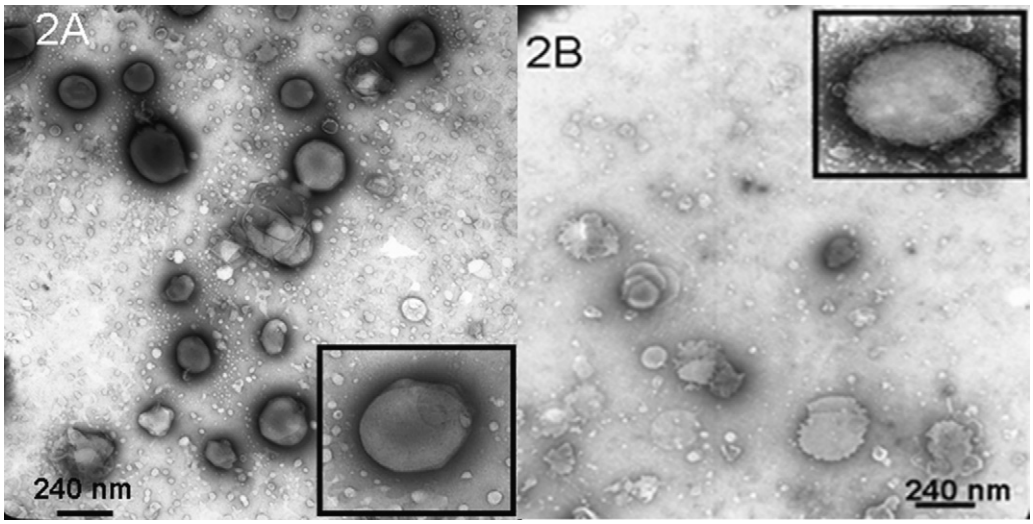

Fig. 2. SUVs microphotographs (TEM): conventional liposomes (A) and archaeosomes (B). A zoom of each kind of vesicle is showed in the inset.

were not significantly different in size in the case of SUVs, but archaeosomes MLVs were bigger than conventional liposomes ones. Anyway, the mean diameter in both archaeosomes and conventional liposomes MLVs did not exceed 400 nm.

As it could be expected, unilaminar vesicles (SUVs) were smaller than multilaminar ones (MLVs), showing size values smaller than 200 nm, which make them more suitable for topical administration. In fact, different authors have investigated the influence of liposome size on the skin penetration of liposomes when entrapping a hydrophilic substance. These studies indicated that for hydrophilic compounds, the small liposomes showed an enhanced penetration into the skin if compared with large ones (Duplessis et al., 1994; Verma et al., 2003).

TEM microphotographs are shown in Fig. 2. Conventional liposomes appeared as spherical vesicles, with a smooth and uniform membrane (Fig. 2A), while archaeosomes had more irregular membranes (Fig. 2B). These morphological characteristics remained unaltered after one month of storage.

The zeta potential is a good index of the magnitude of the repulsive interaction between colloidal particles, closely related to the stability of colloidal systems. If the particles have a large negative or positive zeta potential, they will repel each other and the suspension will be stable. In particles with low zeta potential, the repulsion forces would be low and the particles could eventually aggregate, resulting in suspension instability. Under the tested conditions, the zeta potential was negative in both kinds of formulations. Previous studies showed that liposomes negatively charge exhibited high physical stability, small particle size and relatively high percentage of drug entrapment when compared with positive charge ones (Manosroi et al., 2002), while other authors concluded that the negative charge improves permeation and retention of liposomes in the skin (Contreras et al., 2005; Ogiso et al., 2001). For these reasons, these values of zeta potential represent an advantage when using these vesicles as drug carriers and will facilitate their therapeutic use. It is remarkable that archaeosomes were highly charged if compared with conventional liposomes, so a better stability behaviour for archaeosomal formulations may be expected, and it can be reasonably assumed that it is due to the chemical features of polar lipids comprising these vesicles, which have a prevalence of polar groups with negative charge (Patel et al., 2007). In fact, no aggregation phenomena were observed for archaeosomes formulation, which could be concluded by the maintenance of the mean diameter during the stability study, while in conventional liposomes formulation a slight increasement of the main diameter was observed.

The incorporation efficiency of the antioxidant extract is quite similar in both carriers. The efficiency was slightly higher in the case of SUVs when compared with MLVs. Anyway, in both SUVs and MLVs archaeosomes showed higher values of entrapment efficiency than conventional liposomes, although this difference was not statistically significant.

Both formulations showed a high stability one month after the preparation and under storage at $4 \pm 1\,^{\circ}C$. Mean size, polidispersity index and zeta potential values did not change appreciably during this period of time, and no leakage of antioxidant extract was observed (Table 3).

For the previously characteristics described, SUVs of archaeosomes and conventional liposomes were selected for the incorporation in two different topical excipients.

4.3. Antioxidant activity

DDPH assay was used to evaluate the antioxidant capability of liposomal formulations. Both kinds of formulations, archaeosomes and conventional liposomes, showed a high antioxidant activity, around 85% of DPPH radical inhibition and significant statistical differences between both formulations were not found. These values of inhibition are slightly lower than those obtained with a 1 mM Trolox solution. Furthermore, the inhibition capacity of DPPH radical in both formulations was maintained after a month of

Table 2
Mean diameter (size), polidispersity index (PI), zeta potential and incorporation efficiency (*E*%) of archaeosomes and conventional liposomes.

Formula		Size (nm ± SD)	PI	Zeta potential (mV ± SD)	*E*%
Archaeosomes	MLVs	331 ± 16	0.576 ± 0.07	−61 ± 1	33.55 ± 3.1
	SUVs	171 ± 3	0.392 ± 0.014	−60 ± 2	38.74 ± 2.51
Conventional liposomes	MLVs	272 ± 2	0.45 ± 0.03	−26 ± 2	28 ± 2.8
	SUVs	150 ± 4	0.309 ± 0.05	−28 ± 1	33.03 ± 3.84

Table 3
Mean diameter (size), polidispersity index (PI), zeta potential and incorporation efficiency (*E*%) of archaeosomes and conventional liposomes (SUVs) one month after the preparation.

Formula	Size (nm ± SD)	PI	Zeta potential (mV ± SD)	*E*%
Archaeosomes	182 ± 20	0.472 ± 0.023	−65 ± 2	38.59 ± 5.67
Conventional liposomes	166 ± 3	0.318 ± 0.072	−23 ± 1	30.25 ± 7.42

preparation (Fig. 3). Indeed, the encapsulation of this kind of active compounds has a double benefit; on the one hand their antioxidant capacity reinforces the stability of the vesicles obtained (Grit and Crommelin, 1993; Hincha, 2008) and on the other one the liposomal system protects the phenolic compounds of possible degradation phenomena, prolonging their antioxidant activity during the time (Mozafari et al., 2006). These results support that independently of the value of entrapment efficiency both formulations encapsulated an amount of phenolic extract that guarantee the radical scavenging activity.

It is remarkable that this antioxidant activity is comparable to that of the free phenolic extract, as shown in Fig. 3, so it could be conclude that the incorporation of it into the vesicles did not affect its antioxidant capability. We can compare this results with those obtained by Gopinath et al. (2004), which measured the antioxidant potency of ascorbic acid, the most effective antioxidant in human blood plasma, which is widely used in cosmetic and dermatological preparations because its large number of favorable effects on the skin (Frei et al., 1990; Silva and Maia Campos, 2000). This study compared the antioxidant activity of an ascorbic acid solution, an ascorbyl palmitate aqueous dispersion and bilayered vesicles of ascorbyl palmitate, which revealed a similar antioxidant capacity for all three samples, with values of DPPH inhibition comparable to that of our formulations.

The encapsulation of olive phenolic compounds in different carriers has been previously described showing values of antioxidant activity similar to those obtained in the present study (Mourtzinos et al., 2007; Paiva-Martins et al., 2003). Furthermore, the biological activity of phenolic compounds recovered from olive mill waste has been widely study by others authors (Mulinacci et al., 2005; Obied et al., 2005b). The phenolic composition of the extract, and therefore their antioxidant activity, is highly dependant on the olive variety, the harvesting time and seasonal factors (Mulinacci et al., 2001; Obied et al., 2008). Nevertheless, the kinetics of DPPH radical scavenging activity of two different Australian olives mill waste was comparable to those obtained with our phenolic extract and the formulations (Obied et al., 2007).

Scientific literature dealing with liposomal or archaeosomal encapsulation of phenolic compounds recovered from olive mill waste has not been found. However, a large variety of studies show that the encapsulation of different antioxidants enhance the effect against diseases in which oxidative stress is involved; among these it could be highlight the enhancement of skin deposition of

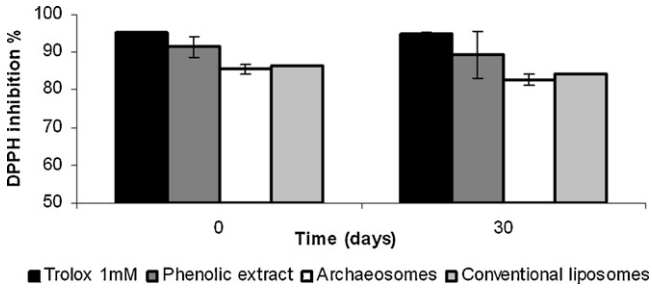

Fig. 3. DPPH radical-scavenging activities of liposomal formulations and phenolic extract at 0 and 30 days of storage. A Trolox solution 1 mM was used as a positive control.

catechins when incorporated in liposomes (Fang et al., 2006), the improvement of plasma antioxidant activity following oral administration of curcumin-loaded liposomes (Takahashi et al., 2009), the prevention of lipidic peroxidation and brain edema formation in rats during ischemia process by administration of alpha tocopherol and ascorbic acid loaded liposomes (Sinha et al., 2001) and the increase in the protective effect of resveratrol in Parkison's disease when a liposomal system was used (Wang et al., 2011).

4.4. Organoleptic study

Carbopol gel: Conventional liposomes gel appeared clearer than archaeosomes one. In both case, the color of the gels were slightly dun, due to the characteristic color of the phenolic extract, which confer this color to liposomal formulations. We observed that archaeosomes gel preparation was easier than conventional liposomes one, because the solubization of Carbopol® was reached immediately when archaeosomes suspension was used. Several mechanisms were suggested to account for the formation of Carbopol gels. In acid conditions a small proportion of the carboxyl groups present on the polymer chain dissociate, producing a flexible coil structure. Base causes further dissociation resulting in electrostatic repulsion between the charges on the polymer, thus a gel forms as the molecules extend and become rigid (Barry and Meyer, 1979). The differences found between archaeosomes and conventional liposomes pH values, 6.5 and 4.5 respectively, may be the reason for an easier gel formation when archaeosomes were used.

Both gels appearance was homogeneus, without a characteristic odour and spreadable with an adequate texture and viscosity. These properties remained after 1 month of storage at 4° ± 1 °C.

Pluronic gels: Initially the Pluronic® copolymers, in presence of water, formed micelles once the critical concentration and temperature were reached; as temperature increased, the micelles interacted and fused to form packed micelle (Bromberg and Ron, 1998; Sharma and Bhatia, 2004). Interaction among micelles gave rise to a fluid structure that was initially cuboid and became hexagonal at high temperatures. The system became more rigid as a result and thus showed gelling (Zhou et al., 1996). For this reason Pluronic gels were in liquid state at 4 °C, becoming a spreadable gel at 25 °C. Archaeosomes gels were clearer than conventional liposomes ones, which had a milky appearance. Moreover, pluronic gels were homogeneus, absent of odour and with appropriate texture and viscosity, properties which remained also during the period of storage at 4 ± 1 °C.

4.5. Membrane selection

In order to evaluate the drug release from gels, we selected the most suitable membrane as that which offered the least resistance to the diffusion of the antioxidant extract, in order to minimize the influence of membrane type in the test. For this study, a 355 ± 12 μg CAE/ml (10 mg/ml) solution of the phenolic extract was used as the donor phase. Samples were taken at predetermined time intervals from 0.25 to 2 h. As shown in Fig. 4, both membranes allowed the passage of the phenolic extract in good yields, however, after 30 min of test the HA membrane has reached 90% of accumulation in the recipient compartment, in contrast with 70%

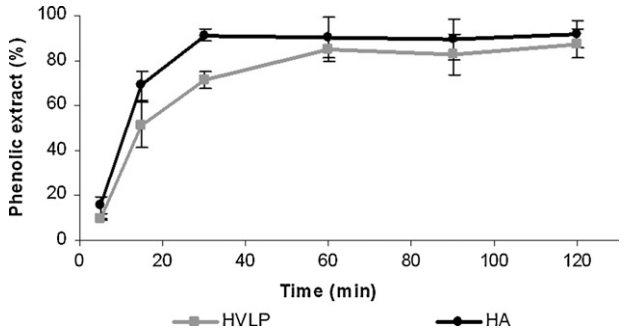

Fig. 4. Membrane selection for in vitro release studies: amount of phenolic extract (%) transferred through each type of membrane (HA – cellulose ester; HVLP – polyvinylidene fluoride).

of accumulation through the HVLP membrane in the same period of time. Because of lower resistance showed by the HA membrane to the crossing of phenolic extract, we selected this membrane for further studies.

4.6. In vitro release studies and kinetic evaluation

Two comparatives studies were carried out. The first study was performed for each kind of gel, Carbopol and Pluronic, in order to highlight the differences in the release of phenolic extract liposome-free gel, liposomal gels and liposomal suspensions. The second one aimed to select the best gel for each kind of liposomal formulation, in terms of release of phenolic extract.

4.6.1. Kinetic evaluation

In order to study the mechanism of antioxidant phenolic extract release from formulations, data obtained from the in vitro release study were fitted to various kinetic equations, which is summarizes in Table 4. The smaller the value of AIC, the better the model adjusts the data. For the model selection also were taken into account the parameter accuracy, expressed as % coefficient of variation (%CV) and the residual distribution plots.

By kinetic modelling it was found that all formulations followed first order release pattern, except for carbopol gel, with a release constant (K) similar for both vesicular suspensions, with values of 1.16 h^{-1} and 1.23 h^{-1} for liposomes and archaeosomes, respectively. It means that the active release from the formulations follows a concentration gradient pattern, based on the first Ficks law, where the released amounts are directly proportional to the amount remaining into the dosage form. These results are similar to that observed for the release of other hydrophilic compounds (Ali et al., 2010; Bochot et al., 1998; Harashima and Kiwada, 1996). However, zero-order kinetics has also been reported (Fadel et al., 2009; Glavas-Dodov et al., 2002; Liu et al., 2007; Zhang et al., 2002). This discrepancy might be due to differences in experimental conditions or in drug gel properties and vesicles. In fact, the mathematical description of the entire drug release process is rather difficult because of the number of physical characteristics that must be taken into consideration. There are too much factors involved. Among these it could be include the percentage, solubility and drug particle size; the type of polymer, the percentage incorporated, its degree of viscosity and the polymer particle size; the type of vesicle, its size and composition. In our study, phenolic extract was very water soluble, which differs from those poorly water soluble compounds previously reported.

On the other hand, carbopol gel was found to follow the Higuchi model. This describes several theoretical models to study the release of water soluble and low soluble drugs incorporated in semi-solid and/or solid matrixes, indicating the drug release from matrix as a square root of time dependent process based on Fickian diffusion.

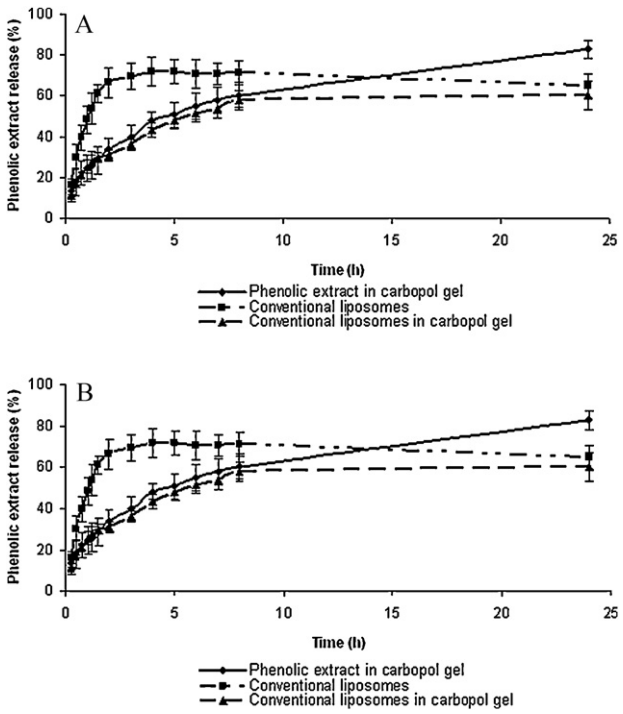

Fig. 5. Carbopol gels in vitro release study: archaeosome formulations (A) and conventional liposome formulations (B).

According to Korsmeyer–Peppas model, we will show how the diffusional exponent, n, can be used to obtain important information about the mechanism of drug release. Taking into account the value of the diffusional exponent obtained (Table 4), a Fickian diffusion seemed to be involved in the release mechanism of phenolic extract from formulations ($n < 0.5$). On drug release kinetic modeling it was found that there was not much difference in the release pattern of drug from all formulations, and a Fickian diffusional release occurs by the usual molecular diffusion of the drug due to a chemical potential gradient. Therefore, it can be considered that transport of drug from gels, vesicles and vesicles in gels was concentration dependent and Fickian, indicating diffusion-controlled drug release. Nevertheless, similar to other authors, the value of the release exponent obtained $n < 0.45$ (Table 4) is beyond the limits of Korsmeyer model so-called power law and thus the power law can only give limited insight into the exact release mechanism of the drug (Shoaib et al., 2006).

4.6.2. Release from carbopol gels

Fig. 5 represents the release of archaeosomes and conventional liposomes carbopol gels.

As it could be expected, the release rate constant (K_d) of the different vesicle suspensions ($K_{liposomes-suspension} = 1.16 \pm 0.056\ h^{-1}$; $K_{archaeosomes-suspension} = 1.23 \pm 0.070\ h^{-1}$) are higher if compared with liposomes ($K = 0.48 \pm 0.058\ h^{-1}$) and archaeosomes ($K = 0.73 \pm 0.081\ h^{-1}$) in carbopol gels, which confirm a slower release of the antioxidant phenolic compounds when the vesicles are incorporated into the gel. Also, the release of vesicles-free gels was higher if compared with encapsulated phenolic extract, reaching values of 80–90% after 24 h of study.

Phenolic extract delivery when archaeosomes and conventional liposomes suspensions were used achieved values about 70% after 2 h of release, which was maintained until the end of the study.

Table 4
Kinetic evaluation of in vitro release studies.

Formulation	Parameter	Zero order	First order	Higuchi	Korsmeyer–Peppas
Phenolic extract carbopol gel	k	2.84 (h^{-1}) ± 0.438	0.29 (h^{-1}) ± 0.049	16.74 (% × $h^{-1/2}$) ± 1.007	26.31 (h^{-n}) ± 0.887
	$\%R(_\infty$	–	71.89 ± 5.035	–	–
	$\%R_t$	27.03 ± 3.280	–	–	–
	n	–	–	–	0.38
	CV (%)	15.41 (K) 12.14 ($\%R_t$)	16.50 (K) 7.00 ($\%R_\infty$)	6.02	3.37 (K) 4.16 (n)
	AIC	102.40	90.52	78.94	67.40
Archaeosomes suspension	k	1.43 (h^{-1}) ± 0.646	1.23 (h^{-1}) ± 0.070	10.51 (% × $h^{-1/2}$) ± 2.726	50.58 (h^{-n}) ± 2.845
	$\%R(_\infty$		72.45 ± 1.058	–	
	$\%R_t$	53.40 ± 4.838	–	–	
	n		–	–	0.18
	CV (%)	45.13 (K) 9.06 ($\%R_t$)	5.69 (K) 1.46 ($\%R_\infty$)	25.92	5.63 (K) 17.93 (n)
	AIC	113.28	67.09	106.83	98.44
Archaesosomes carbopol gel	k	1.38 (h^{-1}) ± 0.543	0.73 (h^{-1}) ± 0.081	9.87 (% × $h^{-1/2}$) ± 2.175	30.55 (h^{-n}) ± 2.366
	$\%R(_\infty$		52.62 ± 1.781	–	–
	$\%R_t$	32.49 ± 4.065	–	–	–
	n		–	–	0.24
	CV (%)	39.28 (K) 12.51 ($\%R_t$)	11.10 (K) 3.39 ($\%R_\infty$)	22.05	7.74 (K) 16.98 (n)
	AIC	108.41	77.10	100.51	93.47
Liposomes suspension	k	1.22 (h^{-1}) ± 0.764	1.16 (h^{-1}) ± 0.056	9.86 ± 3.445	48.76 (h^{-n}) ± 3.919
	$\%R(_\infty$	–	70.95 ± 0.898	–	–
	$\%R_t$	52.04 ± 5.716	–	–	–
	n	–	–	–	0.16
	CV (%)	62.67 (K) 10.98 ($\%R_t$)	4.82 (K) 1.27 ($\%R_\infty$)	34.93	8.04 (K) 25.73 (n)
	AIC	117.95	62.12	113.38	107.40
Liposomes carbopol gel	k	1.94 ± 0.492	0.48 (h^{-1}) ± 0.058	12.37 ± 1.676	26.59 (h^{-n}) ± 1.729
	$\%R(_\infty$		55.74 ± 2.349	–	–
	$\%R_t$	27.83 ± 3.681	–	–	–
	n		–	–	0.31
	CV (%)	25.39 (K)13.23 ($\%R_t$)	12.12 (K) 4.22 ($\%R_\infty$)	13.55	6.50 (K) 10.50 (n)
	AIC	105.63	78.50	93.20	85.20
Phenolic extract pluronic gel	k	1.40 (h^{-1}) ± 0.889	1.37 (h^{-1}) ± 0.067	11.26 (% × $h^{-1/2}$) ± 4.040	65.20 (h^{-n}) ± 4.561
	$\%R(_\infty$	–	90.13 ± 1.075	–	–
	$\%R_t$	69.34 ± 6.658	–	–	–
	n	–	–	–	0.16
	CV (%)	63.46 (K) 9.60 ($\%R_t$)	4.87 (K) 1.19 ($\%R_\infty$)	35.89	7.00 (K) 25.38 (n)
	AIC	122.22	68.26	117.84	111.64
Archaeosomes pluronic gel	k	1.37 (h^{-1}) ± 0.448090	1.06 (h^{-1}) ± 0.118	9.29 (% × $h^{-1/2}$) ± 1.738	35.41 (h^{-n}) ± 1.710
	$\%R(_\infty$	–	53.88 ± 1.610		–
	$\%R_t$	37.20 ± 3.355	–		–
	n	–	–		0.21
	CV (%)	32.71 (K) 9.02 ($\%R_t$)	11.15 (K) 3.03 ($\%R_\infty$)	18.72	4.83 (K) 12.74 (n)
	AIC	103.09	78.18	94.22	84.25
Liposomes pluronic gel	k	0.35 (h^{-1}) ± 0.182	0.19 (h^{-1}) ± 0.044	2.73 (% × $h^{-1/2}$) ± 1.179	7.39 (h^{-n}) ± 1.977
	$\%R(_\infty$	–	19.01 ± 1.936	–	–
	$\%R_t$	10.49 ± 2.054	–	–	–
	n	–	–	–	0.30
	CV (%)	51.34 (K) 19.59 ($\%R_t$)	23.22 (K) 10.19 ($\%R_\infty$)	43.18	26.74 (K) 38.11 (n)
	AIC	25.67	19.39	24.52	23.92

Archaeosomes carbopol gel release was 20% lower than when used the archaeosomal suspension, as well as conventional liposomes carbopol gel. These results showed that the incorporation of the vesicles inside the matrix of a polymeric gel such as carbopol, lead in a retention of the phenolic extract and in a sustained release. In fact, the formation of liposome-gel complex modifies the release of the active compounds, resulting in a slower release if compared with the administration of free liposomes (Mourtas et al., 2007). In contact with water, carbopol® polymer swells forming a hydrated matrix, which may control the flux of water through it and the diffusion of the active compounds. To be released from the gel, phenolic compounds have to get out of the vesicles and mix with the water in the gel matrix. Is for this reason that the delivery from the vesicles incorporated in carbopol gels is slower than when the liposomal suspension is used, and it may allow to used then as an antioxidant reservoir (Varma et al., 2004).

4.6.3. Release from pluronic gels

Fig. 6 represents the delivery of phenolic compounds from archaeosomes and conventional liposomes pluronic gels. As in the previous part of the study, the delivery of phenolic extract liposome-free gels, liposomal gels and liposomal suspensions were compared.

The difference in the kinetic constant between liposomal ($K = 0.19 \pm 0.044\ h^{-1}$) or archaeosomal ($K = 1.06 \pm 0.118\ h^{-1}$) pluronic gel system and phenolic extract pluronic gel also confirmed that the release of drug is lower from a liposomal or archaeosomal gel than from a pure micellar gel system. In this case the release of liposome-free gels was really fast, with a high release constant, $K = 1.3727 \pm 0.067\ h^{-1}$ (CV = 4.87%), reaching 60% of deliverance after the first hour, and 90% after 4 h. This value is sustained until 8 h of delivery study, suffering a slight decrease after 24 h. The surfactant character of this polymer enhance the solubility of some active compounds, thus the high delivery percentage of phenolic extract achieved by liposome-free pluronic gel may be due to this phenomenon (Florence and Attwood, 1998). This release is noticeable higher than those achieved with archaeosomes suspension and archaeosomal pluronic gel, which reached a maximum release of 60% after 24 h. In this case, the differences between the suspension and the gel are narrower than in the case of carbopol gel after 24 h, with 10% of difference.

Table 5
Phenolic extract release (%) depending on formulation: archaeosome formulations: archaeosomes suspension (AS), archaeosomes carbopol gel (ACG) and archaeosomes pluronic gel (APG). Conventional liposome formulations: conventional liposomes suspension (CLS), conventional liposomes carbopol gel (CLCG) and conventional liposomes pluronic gel (CLPG).

Formulation	Phenolic extract release (%) in time (h)							
	0.25	0.75	1.25	2	4	6	8	24
AS	23.7 ± 5.7	45.8 ± 8.4	54.2 ± 6.5	64 ± 6	72.3 ± 6.1	73.5 ± 6	76.3 ± 5.5	72 ± 7
ACG	11.9 ± 5.8	23.4 ± 4.3	33.3 ± 5	36 ± 6	47 ± 6	53.3 ± 6.3	57.6 ± 6.2	60.9 ± 3.5
APG	14.7 ± 3.6	34 ± 0.1	35.9 ± 1.2	43.1 ± 0.6	53.5 ± 6.3	55.1 ± 2,7	57.5 ± 0.6	59.6 ± 2.9
CLS	16.3 ± 3	39.7 ± 6.1	53.6 ± 7.7	72.1 ± 5.1	77.1 ± 5.8	76.2 ± 4.2	75.8 ± 4.6	79.6 ± 5.6
CLCG	11.1 ± 0.6	21.2 ± 1.3	28.1 ± 2.6	31.2 ± 2.5	43.3 ± 3.4	51.4 ± 3.8	58.2 ± 4.6	60.1 ± 6.5
CLPG	–	–	–	–	8.5 ± 3.2	12.9 ± 3.6	17.7 ± 2.6	18 ± 0.5

In the case of conventional liposomes, greater differences were found between the suspension form and the gelled one, being outstanding the slow release constant for liposomes Pluronic gel ($K = 0.19 \pm 0.044\ h^{-1}$), respect to liposomes suspension ($K = 1.16 \pm 0.056\ h^{-1}$). In this way, the pluronic gel containing conventional liposomes had a poor release, with 20% as maximum, while conventional liposomes suspension released 70% at the end of the study. This phenomenon may be explained because when liposomes are included in the gel, part of the phosphatidylcholine which form the liposomal membrane could interact with pluronic® molecules and water, resulting in a ternary system of the type pluronic lecithin organogel (Ruiz et al., 2008). This is a type of organogel with a vesicular structure much more consistent and stable than polymeric micelles or liposomes, thus hindering the delivery of active compounds. Studies performed with light microscopy revealed the formation of micellar structures by the addition of lecithin, which may account for changes in rheological properties, thus the presence of micelles of lecithin could be changing the equilibrium of the drug in the gel matrix and modifying its release mechanism and rate. In fact, the increasement of lecithin concentration led to a permeation reduction (Bentley et al., 1999).

It seems that this phenomenon does not occur when archaeosomes are used, showing a value of $K = 1.06 \pm 0.118\ h^{-1}$ (CV = 11.15%) and a total release drug of 53.87% (CV = 3.03%). It has been established that unsaturation in phospholipid molecules is a desired property for the formation of lecithin organogels, because it may affect on the nature of self-assembly to form these microstructures. In contrast to the saturated hydrogenated phospholipids, unsaturation in phospholipid molecules would result in better hydration of the polar head group, thereby increasing the area per lipid polar head group. Consequently, larger area and relatively smaller volume would favorably alter the spontaneous curvature of lipid monomers for the formation of micelles and subsequently their self-assembly to form the micellar network (Shchipunov, 2001). In case of using archaeosomes, whose membrane lipids are satured, the formation of organogel does not occur, thus the delivery of phenolic compounds is easier than when using conventional liposomes.

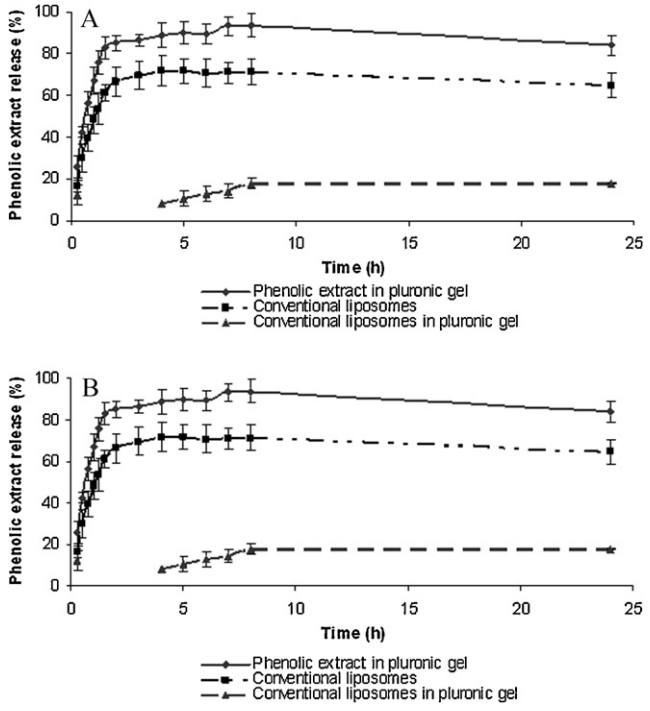

Fig. 6. Pluronic gels in vitro release study: archaeosome formulations (A) and conventional liposome formulations (B).

4.6.4. Selection of a gel for each liposomal suspension

Table 5 summarizes the phenolic extract release (%) achieved by the different formulations studies.

When archeosomes were employed, both kind of gelling excipient produced similar release results at 24 h study. It is interesting to highlight that pluronic gel produced a sustained release since the early stages of the study, while carbopol delivery was increasing progressively until the end.

In the other hand, when using conventional liposomes only carbopol® may be useful as gelling agent, because of the great retention of phenolic extract produced when conventional liposomes were incorporated in pluronic gel, presumably due to the formation of an organogel. Just one advantage could be obtained using pluronic® for this formulation, and it is to have a long-lasting effect on skin due to the reservoir effect.

5. Conclusions

Archaeosomes appeared as a suitable carrier for topical delivery of antioxidant phenolic compounds due to their characteristics of stability, entrapment efficiency and antioxidant activity, which were comparable with those obtained with classical phosphatidylcholine liposomes. Moreover, archaeosomes seemed to be more versatile than conventional liposomes for incorporation into gels. Thus, the release using carbopol® or pluronic® is quite similar when using archaeosomes, so both excipients could be used indiscriminately, while it does not occur when conventional liposomes were used and so the selection of one excipient may be based on the desired effect. Nevertheless, further studies may be carried out to confirm the benefits of this new archaeal lipid-based delivery system.

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