Design and *in vitro* effectiveness evaluation of *Echium amoenum* extract loaded in bioadhesive phospholipid vesicles tailored for mucosal delivery

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Abstract

The *Echium amoenum Fisch. and C.A. Mey.* (*E. amoenum*) is an herb native from Iranian shrub, and its blue-violet flowers are traditionally used as medical plants. In the present study, an antioxidant phytocomplex was extracted from the flowers of *E. amoenum* by ultrasounds-assisted hydroalcoholic maceration. The main components, contained in the extract, have been detected using HPLC-DAD, and rosmarinic acid was found to be the most abundant. The antioxidant power of the extract along with the phenolic content were measured using colorimetric assays. The extract was loaded in liposomes, which were enriched adding different bioadhesive polymers (i.e., mucin, xanthan gum and carboxymethyl cellulose sodium salt) individually or in combination. The main physico-chemical properties (i.e. size, size distribution, surface charge) of the prepared vesicles were measured as well as their stability on storage. The viscosity of dispersion and the ability of vesicles to interact with mucus were evaluated measuring their stability in a mucin dispersion and mobility in a mucin film. The biocompatibility and the ability of the formulations to protect keratinocytes from damages caused by hydrogen peroxide and to promote the cell migration were measured *in vitro*.

Keywords: Plant extract; liposomes; mucin; xanthan gum; carboxymethyl cellulose; keratinocytes; viscosity; mucoadhesion; scratch assay.

1. Introduction

Echium genus belongs to the Boraginaceae family, several species of which occur in different biogeographic regions of the world and are mainly distributed in Macaronesian islands, Mediterranean region and Irano-Turanian area (Nadi, 2016). In Iran grown many species but among all, only *E. amoenum* has medicinal properties (Hooper, 1937; Mehrabani et al., 2005; Mozaffarian, 1996). The plant is native in these area , and it is known as "Gol e Gav Zaban" in Persia or as "Lesanalsour" in Iran. It has been widely used in traditional medicine, especially, its blue-violet petals, in the form of a decoction due to their various benefits, including sedative, anxiolytic, demulcent, anti-inflammatory, analgesic and antioxidant effects (Bekhradnia, S Ebrahimzadeh, 2016; Danaei et al., 2018; Heidari et al., 2006; Jafari et al., 2016; Mikaili, P et al., 2012; Shafaghi et al., 2010). These beneficial properties are mainly due to the variety of active molecules contained in petals such as gallic acid, catechin, hydroxybenzoic acid, chlorogenic acid, caffeic acid, vanillic acid, epicatechin, ρ-coumaric acid, ferulic acid, rosmarinic acid, carotenoids and anthocyanins such as cyanidin-3-glucoside, cyanin chloride, cyanidin-3-rutinoside, and pelargonidin-3-glucoside (Zannou et al., 2022, 2021). The association of these beneficial molecules can exert antimicrobial, antiviral, antioxidant, and anti-inflammatory activities, making the extract a valuable source of a phytocomplex, that, if ad hoc formulated, can be of interest for pharmaceutical, food, and cosmetic industries (Jawed et al., 2019). Polar extracts of *E. amoenus*, usually obtained by decoction or maceration in a hydro-ethanolic mixture, are considered the highest antioxidant and they are able to inhibit some key enzymes related to the occurrence of important health problems (Asghari et al., 2019). Another preliminary study confirmed the safety of this extract (up to 50 mg/kg) *in vivo*, suggesting that it did not cause significant damage to the heart tissue in mice model and its hematotoxicity should be considered at high doses (Sadighara

et al., 2019). In recent research, the stability and functional characteristics of *E. amoenun* extract was ameliorated by its microencapsulation in solid particles of maltodextrin and modified maize starch obtained by spray drying. The loading increased the bioavailability of anthocyanins contained in the extract, which reached the small intestine, while the ability of extract to penetrate and be absorbed along the gut mucosa was not evaluated (Mehran et al., 2020).

Therefore, considering its natural abundance in Iran, as well as its promising, under explored antioxidant activity, the present study was focused on obtaining by water-ethanol maceration an extract from petals of *E. amoenum* rich in active molecules and, for the first time, loading it into phospholipid vesicles enriched with different bioadhesive polymers (mucin, xanthan gum and carboxymethyl cellulose).

The use of bioadhesive polymers was addressed for improving the residence time of vesicles at the application site thus enhancing their passage through the biological barriers and the beneficial properties of the phytocomplex. The main components, the antioxidant capacity and the total phenolic content of the extract were measured along with the physico-chemical and technological properties of formulated vesicles. The stability of vesicles in mucin dispersion and their mobility on mucin film were tested. The biocompatibility of selected formulations, along with their ability to counteract cell damages caused in keratinocytes by hydrogen peroxide, were evaluated. Finally, the ability of the vesicles to promote proliferation and migration of keratinocytes was assayed *in vitro*.

2. Materials and methods

2.1. Materials

Lipoid S75 (S75), a mixture of soybean phospholipids (70% phosphatidylcholine, 9% phosphatidylethanolamine, and 3% lysophosphatidylcholine), triglycerides, and fatty acids, was

purchased from AVG S.r.l. (Garbagnate Milanese, Milan, Italy) the Italian supplier of Lipoid GmbH (Ludwigshafen, Germany). Tween 80, 2,2-diphenyl-1-picrylhydrazyl (DPPH), ferric sulfate, Folin–Ciocalteu reagent, gallic acid, TPTZ (2,4,6-Tris(2-pyridyl)-s-triazine), tetrazolium salt, sodium acetate, ferric chloride, hydrochloric acid, trihydrated sodium acetate and sodium carbonate, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), xantan gum with molecular weight approximately 3×10^5 g/mol, mucin from porcine stomach (molecular weight ~640 kDa), carboxymethyl cellulose sodium salt with molecular weight around 262.19 g/mol and degree of polymerization from 80 to 1500, ethanol, acetonitrile, methanol, and all other solvents and reagents of analytical grade were purchased from Sigma-Aldrich (Milan, Italy). Life Technologies Europe provided reagents and plastics for cell culture (Monza, Italia). Ultrapure water (18 MW cm) was obtained using a Milli-Q Advantage A10 System apparatus (Millipore, Milan, Italy).

2.2. Plant material collection and extraction

The flower of *E. amoenum* were collected in June 2017 from the north of Iran (Sari, Mazandaran) and immediately dried. The dried material (25 g) was macerated in 500 mL of extractive solution containing 70% ethanol and 30% water (v/v). The dispersion was left at room temperature (25 °C) for 72 h under constant stirring (200 rpm) and sonicated for 5 min every 24 h, using a Soniprep 150 ultrasonic disintegrator (MSE Crowley, London, UK). At the end of the extraction process (72 h) the extractive dispersion was centrifuged twice (30 min, 8000 rpm) and the precipitate was separated. The extractive solution was then maintained in an oven to promote the evaporation of ethanol and then was diluted with water (1:100) and freeze-dried using an Operon FDU8606 freeze-drier (Nuova Criotecnica Amcota, Rome, Italy) thus obtaining a green-dark powder, which was vacuum packed in a dark glass container until its use (Manca et al., 2016; Sedlak et al., 2018).

2.3. Qualitative-quantitative analysis of the extract by HPLC-DAD

An Agilent HPLC 1100 coupled with a diode array detector (DAD) and a computerized data integration system (ChemStation-Agilent) were used for recognition and quantification of polyphenols. The column was a Phenomenex C18 (5 μm. 150 mm × 4.6 mm) working at room temperature. A binary solvent gradient was employed: (A) phosphoric acid 0.22 M, and (B) acetonitrile and methanol (1/1, v/v), with the following multistep gradient: 0 min, 4% B; 40 min, 50% B; 45 min, 60% B; 70 min 100% B. The initial condition was hold for 12 min. The injection volume was 20 μL, and the flow rate was 0.3 mL/min. The monitoring wavelengths for UV analysis were 225 nm for flavan 3-ols, 313 nm for hydroxycinnamic acid derivates, 360 nm for flavonols, and 520 nm for anthocyanins. The polyphenols were recognized by the retention times, in comparison with commercial standards and UV-Vis spectra. All data were reported as mg of compound/g of dry extract and performed in triplicate.

2.4. Antioxidant power, radical scavenger activity and total phenolic content of the extract

The antioxidant and radical scavenger power of the extract along with its total phenolic content have been evaluated using different colorimetric assays. For all assays, the extract was diluted in water (250, 500 and 1000 μg/mL) and filtered (0.45 μm) prior analyses.

The total phenolic content of the extract was determined with a modified Folin-Ciocalteu's method mixing 600 μL of water, 10 μL of extract dispersion or blank solution or gallic acid solution and 50 μL of Folin-Ciocalteu's phenol reagent. After 10 min, 150 μL of sodium carbonate (20% w/v) was added, and the mixture was shaken and diluted with water to the final volume of 1 mL. After 2 h of incubation in the dark and at room temperature, the absorbance was read at 760 nm against blank. The total phenolic content was expressed as mg of gallic acid equivalent/g of dry extract

(GAE) using the calibration curve made of freshly prepared gallic acid standard solutions (5–150 µL/mL). All experiments were performed in triplicate.

DPPH test was used to measure the antioxidant activity by means of the extract ability to scavenge the DPPH• radical. Briefly, extract dispersions or gallic acid solution (40 µl) were dissolved in 1960 µl of DPPH• methanolic solution (40 µg/mL) and incubated for 30 min at room temperature, in the dark. Then, the absorbance was measured at 517 nm. The antioxidant activity was expressed as GAE using the calibration curve made of freshly prepared gallic acid standard solutions (5–125 µg/mL). All experiments were performed in triplicate.

The FRAP assay was performed preparing a ferric complex of 2,4,6-tris(pyridin-2-yl)-1,3,5-triazine (TPTZ) and Fe^{3+} mixing 15.62 mg of TPTZ and 16.22 mg of ferric chloride hexahydrate in 50 mL acetate buffer pH 3.6. The extract dispersion (40 µL) or blank solution (40 µL) or gallic acid solution (40 µL) were dissolved in 250 µL of ferric complex and, after 4 min of incubation in the dark, absorbance was measured at 593 nm. For FRAP assays, a ferric sulfate calibration curve in the range of 0.09–1.045 µM was prepared and the data were expressed as antioxidant capacity of mMol of ferric sulfate equivalents/g dry extract. All the measurements were performed in triplicate.

2.5. Vesicle preparation

Lipoid S75 (180 mg/mL) and the extract (40 mg/mL) were weighed in a glass vial and hydrated with water to obtain liposomes. Mucin (MU), xanthan gum (XG) and carboxymethyl cellulose (CMC) (each 5 mg/mL) were added, individually or in combination, to the mixture of phospholipid and extract (Table 1). The dispersions were immediately sonicated (13 microns, 25 cycles, 5 s on and 2 s off) using a Soniprep 150 ultrasonic disintegrator (MSE Crowley, London, UK) to obtain small and homogeneous vesicles. All formulations were prepared in triplicate.

Table 1. Composition of the *E. amoenum* extract loaded liposomes prepared with Lipoid S75 (S75), mucin (MU), xanthan gum (XG), carboxymethyl cellulose sodium salt (CMC).

Sample	S75 (mg/ml)	Extract (mg/ml)	MU (mg/ml)	XG (mg/ml)	CMC (mg/ml)
Liposomes	180	40	-	-	-
MU-liposomes	180	40	5	-	-
XG-liposomes	180	40	-	5	-
CMC-liposomes	180	40	-	-	5
MU-XG-liposomes	180	40	5	5	-
MU-CMC-liposomes	180	40	5	-	5
XG-CMC-liposomes	180	40	-	5	5
MU-XG-CMC-liposomes	180	40	5	5	5

2.6. Vesicle characterization

Vesicle formation and morphology were evaluated by cryo-TEM observation. A thin film of each sample was formed on a carbon grid, which was vitrified keeping it at 100% humidity and room temperature into ethane, using a Vitrobot (FEI Company, Eindhoven, The Netherlands). The obtained films were transferred to a Tecnai F20 TEM (FEI Company), and the samples were observed in low-dose mode. Images were acquired at 200 kV and ~−173 °C, using a CCD Eagle camera (FEI Company).

The average diameter, polydispersity index (a measure of the width of size distribution) and zeta potential of the vesicles were measured by dynamic and electrophoretic light scattering using a Zetasizer ultra (Malvern In-struments, Worcestershire, UK). Samples were diluted with water (1:100) and analyzed at 25 °C immediately after their preparation.

To evaluate the amount of phytocomplex effectively incorporated into the vesicles, samples (1 mL) were purified from the non-incorporated components by dialyzing them (Spectra/Por® membranes 12–14kDa MW, cut-off, 3 nm pore size; Spectrum Laboratories Inc., DG Breda, Netherlands) against water (1 L) for 2 h, refreshing the water after 1 h. Entrapment efficiency was

176 determined by HPLC-DAD, according to the method described in Section 2.3. Since a derivative
177 of rosmarinic acid was found to be the most abundant compound in the extract, its concentration
178 before and after dialysis was used to determine the entrapment efficiency.
179 The average size, the polydispersity index and the surface charge of the vesicle dispersions were
180 monitored for a period of 90 days by storing them at room temperature (~25 °C).
181 All measurements were made in triplicate.

182 **2.7. Interaction of extract loaded liposomes with mucin**

183 **2.7.1 Stability of extract loaded liposomes in saturated mucin dispersion**

184 The stability of liposomes was evaluated in a saturated mucin dispersion (0.8% w/v) prepared as
185 previously described (Conte et al., 2018). Variations of mean diameter and polydispersity index
186 were assessed after their preparation (0 h) and at 2, 4 and 24 h. Vesicle dispersions were diluted in
187 the mucin dispersion 1:100 and maintained at 37 °C throughout the test, to better simulate *in vivo*
188 conditions.

189 **2.7.2. Mobility of extract loaded liposomes on mucin film**

190 The distance travelled by extract loaded liposomes was assessed on vertical mucin film. Briefly, 3
191 ml of the mucin dispersion (0.8% w/v) were added to each well of six-well plates. The plates were
192 stored at 50 °C and allowed to dry overnight in order to obtain a thin film. Samples (20 ml) were
193 added on the upper part of the film at the same time and in the same line for each row by using a
194 multichannel pipette. 6-well plates were then vertically positioned, and pictures were taken at four
195 different time points (0, 5, 10 and 15 min). The distance between the drop and the border of each
196 well was measured using GIMP 2.10.24 software in order to calculate distance travelled (%) at
197 each time point. The distance at the border of the well, calculated from the drop, was considered

as the 100% of the distance travelled. The duration of the experiment was chosen according to the sample with the highest distance travelled, that was liposomes.

2.8. Viscosity measurements

The viscosity of formulations was measured using a Brookfield Programmable LVDV-II + Viscometer (AMETEK GB LTD T/A Brookfield Technical Centre, Essex, UK) coupled with a FE2 HAAKE thermostatic bath. The studies were carried out using a spindle 18 at controlled temperature (~25 ± 2°C) and the viscosity (mPas) was measured as a function of Torque (torsion force expressed as a percentage), Shear Rate (1/s) and Shear Stress (dyne/cm^2).

2.9. Biocompatibility of the extract loaded liposomes

Human immortalized keratinocytes (HaCaT) were grown as monolayers in 75 cm^2 flasks, incubated with 100% humidity and 5% carbon dioxide at 37 °C. They were cultured with phenol red-free Dulbecco's Modified Eagle Medium (DMEM) with high glucose, supplemented with 10% fetal bovine serum and penicillin and streptomycin. The cells were seeded into 96-well plates at a density of 7.5×10^3 cells/well and after 24 h of incubation, they were treated for 48 h with the extract in dispersion or loaded in vesicles. All the dispersions were properly diluted to reach the desired concentrations (40, 4, 0.4, 0.04 µg/mL of extract in the medium). The possible toxic effect has been assessed measuring the viability of HaCaT after incubation using the MTT (tetrazolium salt, 3- (4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide) colorimetric assay. At 48 h, the medium was removed and MTT (100 µl) was added to each well and incubated at 37 °C for 2-3 h. The formazan crystals formed in viable cells were dissolved in dimethyl sulfoxide, and the absorbance measured at 570 nm with a microplate reader (Synergy 4, BioTek Instruments, AHSI S.p.A, Bernareggio, Italy). All the experiments were repeated at least three times and each time in

triplicate. The results are expressed as the percentage of live cells compared to untreated cells (100% viability).

2.10. Protective effect of extract loaded liposomes against cell damages induced by hydrogen peroxide in keratinocytes

HaCaT cells ($5x10^4$ cells/well) were seeded in 96-well plates with 250 µl of culture medium, incubated at 37 °C for 24 h, then stressed for 4 h with hydrogen peroxide (1:40.000 dilution) and simultaneously treated to the extract in dispersion or loaded in the vesicles (4 mg/mL final concentration). Unstressed cells were used as positive control (100% viability) and hydrogen peroxide-stressed cells, treated with extract-free medium were used as negative control. After 4 h of incubation the medium was removed and the viability of the cells was determined with the MTT colorimetric assay, adding 100 µl of reagent in each well. After 2-3 h, the formed formazan crystals were solubilized adding dimethyl sulfoxide and their concentration was measured spectrophotometrically at 570 nm as above (Paragraph 4.5).

2.11. Scratch assay

The ability of the formulations to stimulate migration and proliferation of keratinocytes was evaluated using the scratch assay test. Cells were seeded in 6-well plates and kept in culture until the complete confluence was reached. Subsequently, a thin wound was generated on the cell monolayer using a sterile plastic tip. Cell fragments were removed by gently washing each well with the medium preheated at 37 °C. Immediately after the generation of the wound (0 h) cells were treated with the extract in dispersion or loaded in vesicles (4 mg/mL of extract, final concentration) and incubated up to 48 h. Untreated cells were used as negative control. At each time point (0, 8, 24, 32 and 48 h) cells were observed using an optical microscope (10x objective) to monitor cell proliferation and migration along with wound closure.

2.12. Statistical analysis of data

Results are expressed as means ± standard deviations. Analysis of variance (ANOVA) was used to evaluate multiple comparison of means and Tukey's test and Student's t-test were performed to substantiate differences between groups using Excel Statistics for Windows. The differences were considered statistically significant for $p < 0.05$.

3. Results and Discussion

3.1 Extract characterization

The antioxidant phytocomplex obtained from the petals of *E. amoenum* was extracted by ultrasound-assisted maceration using a mixture of ethanol and water (70:30) as extractive medium. The dried extract was obtained by evaporation of ethanol and subsequently freeze-dried.

The main polyphenols contained in *E. amoenum* extract were evaluated by HPLC-DAD. Hydroxycinnamic acid family was the most represented (7 compounds) followed by flavonoids (3) and flavan 3-ols (1) (Figure 1). The total polyphenol concentration was 57.81 mg/g (Table 1). The most concentrated polyphenol was a derivative of rosmarinic acid (46% of the total polyphenols). Rosmarinic acid was also previously reported as the most abundant compound in *Echium amoenum* extract (24mg of rosmarinic acid / g of dry extract), similar concentration that found in the present study after applying the extraction procedure (26,58 mg rosmarinic acid/g of dry extract) (Asghari et al., 2019). In the present extract, a derivative of epicatechin (17.8%), verbascoside (5.73%) and a derivative of verbascoside (9.35%) were also found in high amount (Table 3). Verbascoside and its derivative were also previously identified in other *Boraginaceae* species in high amount (Katanić et al., 2020). Additionally, several p-coumaric and luteolin derivatives were also detected according to previous reported analyses (Aouadi et al., 2021)

Table 3. Main polyphenols contained in *E. amoenum* extract: [a] indicates the compounds expressed as equivalent in epicatechin; [b] those expressed as equivalent in p-coumaric acid; [c] those expressed as luteolin 7-O-rutinoside; [d] those expressed as equivalent in verbascoside; [e] those expressed ad equivalent in rosmarinic acid.

	Compounds	R.t. (min)	mg/g
1	Gallic acid	13.13	0.48
2	Epicatechin derivative[a]	16.36	10.31
3	p-Coumaric acid derivative[b]	24.72	0.78
4	p-Coumaric acid derivative [b]	26.35	0.90
5	p-Coumaric acid derivative [b]	31.05	2.65
6	Flavonoid derivative[c]	31.61	4.74
7	p-Coumaric acid	31.88	0.36
8	Verbascoside derivative [d]	32.16	5.40
9	Verbascoside	32.91	3.31
10	Luteolin 7-O-glucoside	34.73	1.60
11	Luteolin 7-O-glucuronide	35.50	0.70
12	Rosmarinic acid derivative [e]	38.89	26.58
	Total		57.81

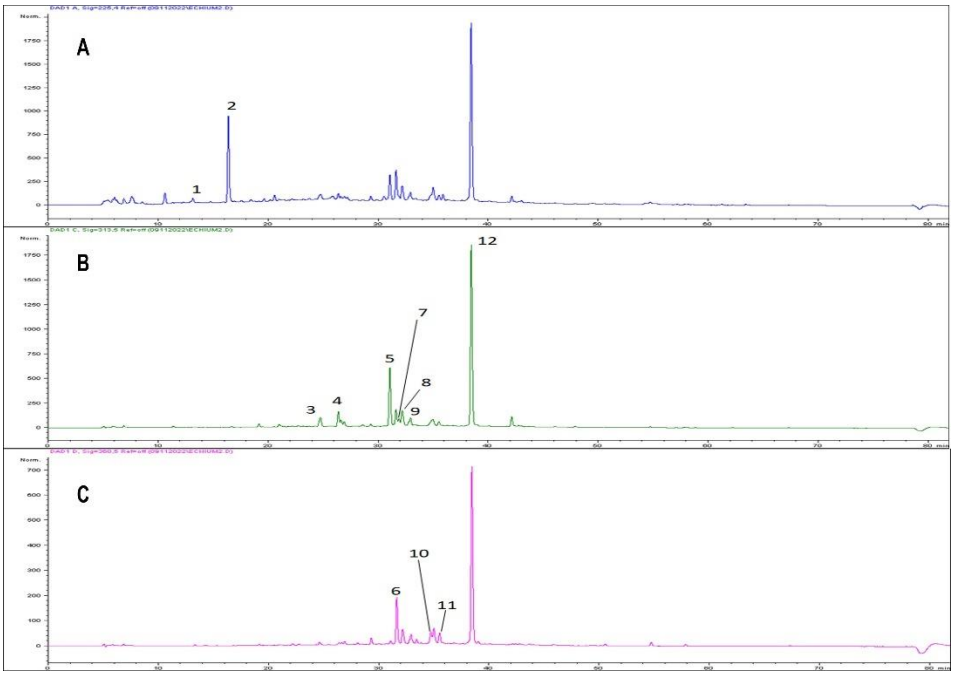


Figure 1. Chromatogram of *E. amoenum* extract recorded at 225 nm (A), 313 nm (B) and 360 nm (C).

The antioxidant activity and the total phenolic content of the extract was evaluated using different colorimetric assays (Folin-Ciocalteu, DPPH and FRAP, Table 2). The total phenolic content of extract was 104 mg gallic acid equivalents/g extract. This value was higher than that previously obtained by ultrasound assisted extraction in water (26.5 mg gallic acid equivalents/g extract) or in ethanol (10.08 mg gallic acid equivalents/g extract) (Zannou et al., 2021). Additionally, Zannou et al. also performed a conventional extraction using natural deep eutectic solvents based on choline chloride and glycerol. They obtained an extract containing 85 mg gallic acid equivalents/g extract, higher than those previously obtained by aqueous and ethanolic extractions (Zannou et al., 2022). The greater phenol content found in the present extract obtained by maceration in hydroalcoholic mixture should be related to the long extraction time (72 h), the used solid-liquid ratio and the ultrasound-assisted process. The antioxidant capacity was also tested using the DPPH

radical scavenging test (Table 2) and 21 mg gallic acid equivalents/g extract was found. Using the FRAP assay, the antioxidant capacity of extract was lower (0.69 mmol ferric sulfate/g of dry extract) than that previously measured in the *E. amoenum* extracts obtained in natural deep eutectic solvent (1.5 and 0.93 mmol ferric sulfate/g of dry extract) (Zannou et al., 2022, 2021). On the other hand, when water and ethanol were used as extraction solvents, the antioxidant capacity of the obtained hydroalcoholic extract measured by FRAP (0.69 mmol $FeSO_4$/g of dry extract) was higher than those obtained by Zannou *et al* using water or ethanol (0.66 and 0.44 mmol $FeSO_4$/g of dry extract, respectively) (Zannou et al., 2021). These results are in accordance with the total phenol content of the present extract, which was higher since the extraction was performed in water and ethanol, and phenolics, being polar components, are recovered in higher amount. These results underline the efficacy of extraction in natural deep eutectic solvent in improving the total phenol content and the antioxidant activity of *E. amoenum* extracts. Even if the extraction with water and ethanol, performed in the present study, permitted to obtain an extract from *E. amoenum* with a higher total phenol content, the antioxidant capacity was comparable. As added values, the former method led to use only food-grade solvents (water and ethanol) and low dissipative methodologies, ensuring the safety and sustainability of final product.

Table 2. Total phenol content (TPC) and antioxidant activity of *E. amoenum* extract measured using DPPH and FRAP assays.

	TPC (mg gallic acid equivalents/g extract)	DPPH (mg gallic acid equivalents/g extract)	FRAP (mmol$FeSo_4$ equivalents/g extract)
Echium Aqueous Extract	104±1	21±1	0.69±0.04

3.2 Preparation and characterization of extract loaded liposomes

303 The extract rich in active molecules has been loaded at increasing concentrations (10, 20, 30, 40,
304 50 mg/mL) in liposomes aiming at founding the highest one capable of leading the formation of a
305 stable and homogeneous system composed of small vesicles. 40 mg/mL was selected as the more
306 suitable concentration, since using 50 mg/mL, the extract partially precipitated immediately after
307 the sonication process. Moreover, to find the best composition of the final formulation, different
308 kinds and concentrations of phospholipids were used and hydrated with water or buffer solution at
309 pH 7.4. The smaller and more homogenous vesicles were those obtained using water as dispersant
310 medium and 180 mg/mL of a commercial mixture of soybean phospholipids mainly composed of
311 phosphatidylcholine (S75). This starting formulation was then modified adding different polymers
312 individually or in combination: mucin, xanthan gum and carboxymethyl cellulose sodium salt (5
313 mg/mL each). These polymers have been selected aiming at improving the viscosity and the
314 stability of dispersions and their adhesion to the mucosae. Mucin is a highly glycosylated protein
315 secreted by specialised goblet cells and represents the main component of mucus, which lines the
316 epithelia acting as the first barrier against a variety of agents for all organs exposed to the external
317 environment (Bansil and Turner, 2006). Depending on the location, mucins are classified as cell-
318 associated mucins and gel-forming mucins. Nevertheless, all mucins share the same structure,
319 which essentially consists of a peptide backbone that is highly glycosylated in the centre and non-
320 glycosylated in the C- and N-terminal regions (Collado-González et al., 2019). Because of their
321 capability of generating a 3-dimensional viscoelastic biopolymeric network, at specific pHs and
322 their adhesive properties, they have been exploited in several pharmaceutical health-care products
323 (Authimoolam and Dziubla, 2016; He et al., 2019; Manca et al., 2021a).
324 Xanthan gum (XG) is a bacterial exo-polysaccaride, composed of a main chain based on a linear
325 backbone of 1,4-linked β-D-glucose, with a charged trisaccharide side chain at C3 position of

every alternate glucose residue, containing acetate groups. It is industrially obtained by fermentation and its use has been approved as food additive by U.S. Food and Drug Administration (Habibi and Khosravi-Darani, 2017). In addition, its valuable pseudoplasticity and solid-like or gel-like behaviour provide adequate rheological properties allowing this polymer to be used as suspending, thickening, and stabilizing agent in the pharmaceutical field (Casas et al., 2000; Li et al., 2022).

Carboxymethyl cellulose (CMC) is a copolymer of β-d-glucose and β-d-glucopyranose-2-O-(carboxymethyl)-monosodium salt, connected through β-1,4-glycosidic bonds (Han and Wang, 2017). It has an internal ring structure with a degree of substitution (the number of carboxymethyl groups substituted per monomer) that varies out of a maximum of 3 (Lan et al., 2018). As sodium salt, this derivative of cellulose is characterized by water solubility and due to its rheology modifying properties, it is widely utilized in cosmetic, pharmaceutical, and other industries (Lopez et al., 2015).

Extract loaded liposomes were small (~97 nm), highly homogeneously dispersed (polydispersity index ~0.02) and highly negatively charged (zeta potential ~-53 mV) due to the deprotonation of phosphate group of phosphatidylcholine at this pH (~5.5), Table 3.

Table 3. Mean diameter (MD), polydispersity index (PI), zeta potential (ZP) and entrapment efficiency (EE) of the extract loaded liposomes modified with mucin (MU), xanthan gum (XG), carboxymethyl cellulose sodium salt (CMC). Mean values ± standard deviations are reported (n ≥ 6). The same symbol (*,#,§,@,°,+,ς,^,”) indicates the same value (p>0.05).

Sample	MD (nm)	PI	ZP (mV)	EE (%)
Liposomes	97±1*	0.02	-53±3°	93±4ς

MU-liposomes	113±10§	0.12	-55±2°	88±7ϛ
XG-liposomes	87±1#	0.15	-46±7+	87±6ϛ
CMC-liposomes	102±6*	0.18	-45±2+	83±7”
MU-XG-liposomes	142±2&	0.07	-51±1°	92±4ϛ
MU-CMC-liposomes	131±2@	0.07	-52±2°	89±1ϛ
XG-CMC-liposomes	122±7§	0.18	-43±3+	78±5”
MU-XG-CMC-liposomes	118±10§	0.18	-51±3°	93±4ϛ

The addition of the different polymers, individually or in combination, led the formation of vesicles with modified properties, as each of them affected one of the parameters tested (i.e. mean diameter, polydispersity index and surface charge). Being mucin a large hydrophilic molecule, its addition to the vesicles caused an increase of their mean diameter (~113 nm, $p<0.05$ versus that of liposomes) and polydispersity index (~0.12), while the zeta potential remained unchanged (~-55 mV, $p>0.05$ versus that of other vesicles), thus suggesting its preferential localization inside the vesicles, especially in the aqueous compartments. The addition of xanthan gum, which is a polysaccharide with a high molecular weight, highly soluble in water, addressed a decrease of the mean diameter (~87 nm, $p<0.05$ versus that of liposomes), and a slightly increase of polydispersity index (~0.15) and zeta potential (~-46 mV, $p<0.05$ versus that of liposomes), which became less negative than that of liposomes and mucin-liposomes. Carboxymethyl cellulose sodium salt, which derived from cellulose, is highly soluble in water as well. The addition of this polymer did not modify the mean diameter of the formed vesicles (~102 nm, $p>0.05$ versus that of liposomes), while the polydispersity index (~0.18) and the zeta potential (~-45 mV) slightly increased, in particular the polydispersity index approached to that of liposomes prepared with xanthan gum. The combination of mucin with xanthan gum or with carboxymethyl cellulose sodium salt caused the highest increase of the mean diameter of liposomes up to ~131 and 142 nm, respectively

(p<0.05 versus that of liposomes and other vesicles), while the zeta potential was still similar to that of vesicles containing mucin alone (~-52 mV). The combination of xanthan gum and carboxymethyl cellulose affected in a lesser extent the physico-chemical properties of the vesicles as only a small increase of the mean diameter (~122, p<0.05 versus that of liposomes) and a slightly decrease of zeta potential (~-45 mV) were detected. The simultaneous addition of the three polymers allowed the formation of vesicles having the same parameters of those prepared by adding mucin only, indicating a higher tendence of this polymer in affecting their physico-chemical properties.

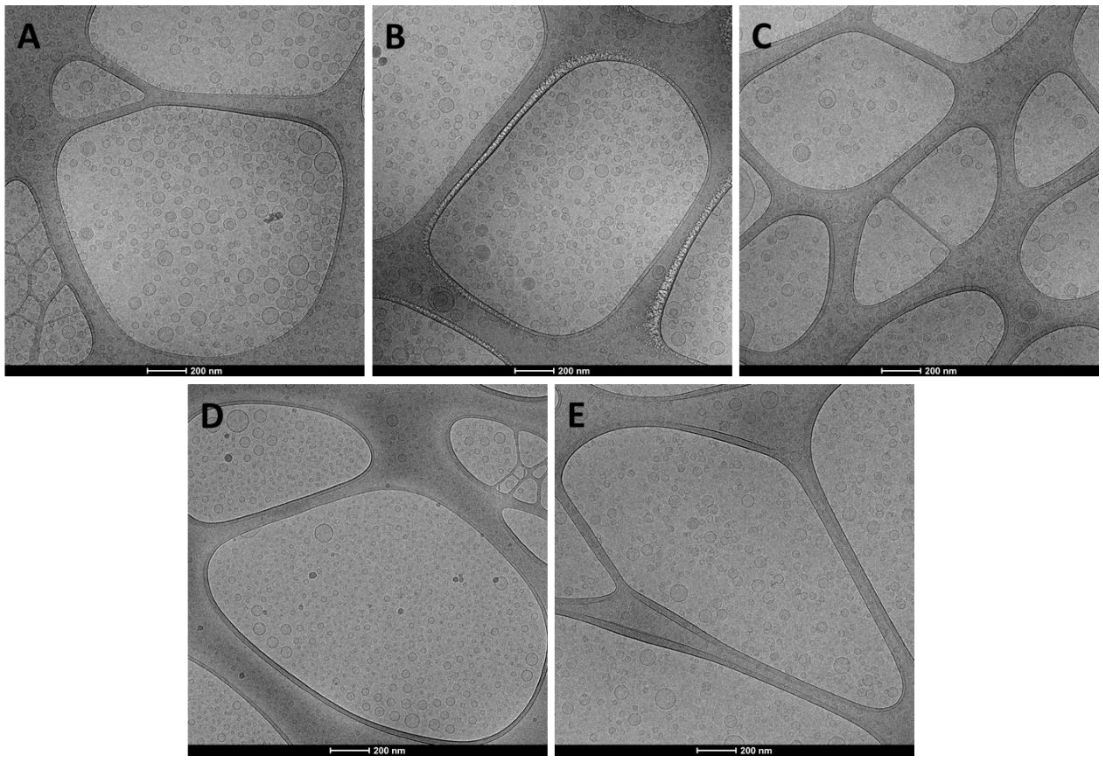


Figure 2. Representative cryo-TEM images of liposomes (A), liposomes modified with mucin (B), xanthan gum (C), carboxymethyl cellulose sodium salt (D) and mucin, xanthan gum and carboxymethyl cellulose together (E).

375 The entrapment efficiency of extract in the liposomes was determined measuring. in dialyzed and
376 non-dialyzed formulations, the amount of rosmarinic acid by HPLC-DAD method, being it the
377 most abundant compound in the extract. The entrapment efficiency of all formulation was ~90%.
378 However, that of liposomes modified with xanthan gum and those modified with xanthan gum,
379 carboxymethyl cellulose was lower (~70%).
380 The long-term stability of the liposomes in dispersion was evaluated storing the samples for a
381 period of 3 months at 4 °C and measuring their mean diameter, polydispersity index and zeta
382 potential at scheduled time points (**Figure 3**). The storage at 4 °C was selected to avoid or at least
383 slow-down the thermic variations, which may promote aggregation phenomena (Sennato et al.,
384 2008). Mean diameter of liposomes, which was ~97 nm immediately after preparation, increased
385 up to ~120 nm ($p<0.05$ versus the starting value) at 3 months of storage, denoting a low stability
386 of vesicles in dispersion. The addition of the polymers individually (mucin or xanthan gum or
387 carboxymethyl cellulose) did not improve this instability, since the vesicle size progressively
388 increased. On the contrary, the combination of xanthan gum and carboxymethyl cellulose, with
389 and without mucin, effectively stabilized the vesicles as their mean size remained constant over
390 the storage period (~122 nm, $p>0.05$ among the values of these two samples at different time
391 points). Despite the observed increase of size of liposomes modified with the polymers
392 individually, the polydispersity index slightly increased but remained always lower than 0.23,
393 indicating that the samples were still monodispersed. The zeta potential remained strongly negative
394 during all the storage and stability check, even if some minor changes were detected.
395

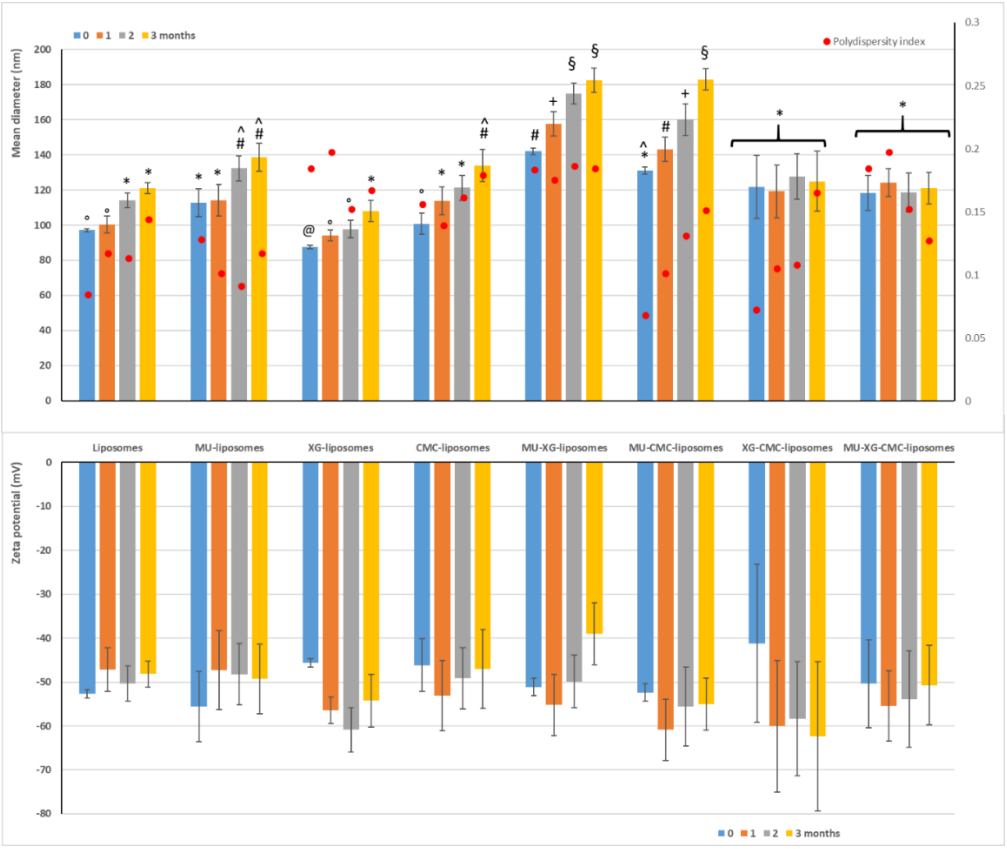
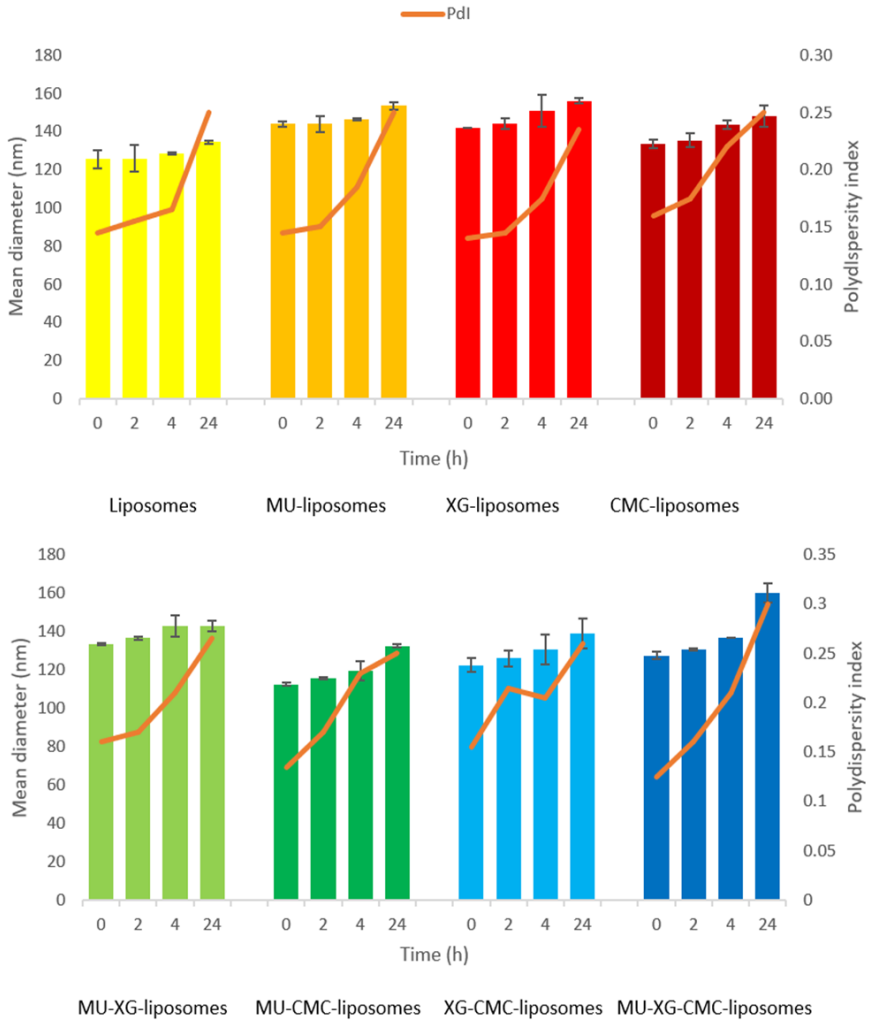


Figure 3. Mean diameter, polydispersity index (upper panel) and zeta potential (lower panel) of the extract loaded liposomes modified with mucin (MU), xanthan gum (XG), carboxymethyl cellulose sodium salt (CMC), measured during 3 months of storage at 4 °C. Data represent the mean values ± standard deviations of at least three replicates. The same symbol (*,§,+,#,°,^,@) indicates the same value.

3.3. Stability and interaction of extract loaded liposomes with mucin

Vesicles' stability in a mucin dispersion was evaluated as these systems were designed to be applied in the buccal mucosa, which is a site responsible for the production of mucus whose major component indeed mucin (Pelin and Suflet, 2020). As a consequence, mean diameter and polydispersity index were monitored up to 24 h after incubation with the aforementioned mucin dispersion. (**Figure 4**). Even if slightly changes in the mean diameter of all the vesicles were detected, overall, all the liposomes were stable after dilution in the saturated mucin dispersion

409 (0.8% w/v), and their mean diameter remained unchanged (for each formulation $p>0.05$ among
410 the values measured at 2, 4, 24 h). On the contrary the polydispersity index of all dispersions
411 slightly increased up to 0.30, suggesting the formation of a slight polydisperse system due to little
412 aggregation phenomena. Despite this, overall results confirmed their possible application and
413 suitability on the mucosae because the vesicles kept intact their structure under the aforementioned
414 conditions. A plausible explanation of this can be found in the stabilizing effect of the polymers.
415 For example, Wang and co-workers were able to fix liposomes instability against high ionic
416 strength by adding carboxymethyl cellulose (Wang et al., 2019). The same way, Manca and
417 colleagues increased the stability of liposomes coating them with xanthan gum and Toniazzo et
418 al. were able to stabilize β-carotene-loaded liposomes up to three months (Manca et al., 2012;
419 Toniazzo et al., 2014).
420

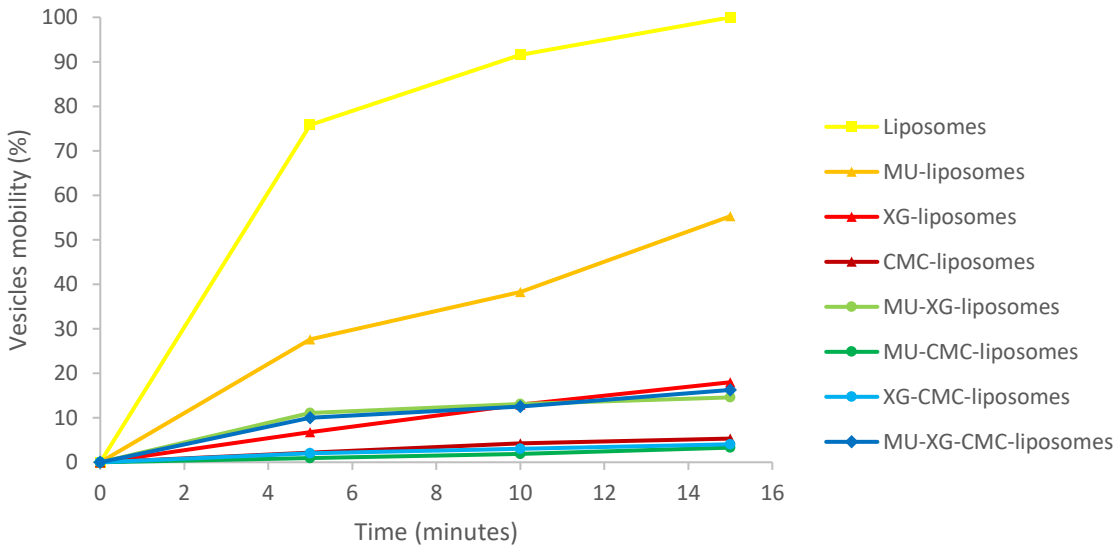


421

422 **Figure 4.** Mean diameter and polydispersity index of extract loaded liposomes modified with
423 mucin (MU), xanthan gum (XG), carboxymethyl cellulose sodium salt (CMC), diluted in mucin
424 dispersion (0.8% w/v). Values were measured at 0, 2, 4 and 24 h. Data represent the mean values
425 ± standard deviations of at least three replicates.

426 In order to test the effect of polymers on improving vesicles adhesion to the mucosal surface, the
427 distance traveled by each formulation placed on a vertical film of mucin was also measured at
428 different times (**Figure 5**). Liposomes travelled the highest distance at each time point, suggesting
429 low interaction and adherence properties to mucus as early as 5 minutes (76% at 5 min and even

100% at 15 min). Comparing enriched liposomes, those modified with mucin traveled a higher distance (55% at 15 min). A similar behavior was reported by Berry and colleagues, who addressed the lack of adhesion of mucin-coated formulations due to charge repulsion between mucins (Berry et al., 2001). The best performances were instead achieved by three out of four liposomes modified with carboxymethyl cellulose sodium salt, alone or in combination with xanthan gum or mucin. These vesicles travelled the shortest distance at each time point, reaching 5%, 4% and 3% at 15 min, respectively, thus confirming strong interactions with the mucin film suggesting an improved adherence. Three out of four liposomes modified with xanthan gum, alone or in combination with mucin or with both mucin and carboxymethyl cellulose sodium salt (MU-XG-CMC-liposomes), travelled slightly less than the previous ones reaching 18%, 15% and 16% of travelled distance at 15 min, respectively. The addition of carboxymethyl cellulose or xanthan gum on the vesicles resulted in reduced travelled distance, suggesting the formation of interactions between vesicle surfaces and the mucin film due the presence of these polymers. This type of interactions has been known for a long time (Mansuri et al., 2016). As a result, both polymers have been adopted extensively to treat buccal cavity related diseases in form of buccal patches, films or tablets (Alhakamy et al., 2022; Chen et al., 2019; Mohammed and Khedr, 2003; Pleguezuelos-Villa et al., 2019; Shiledar et al., 2014).

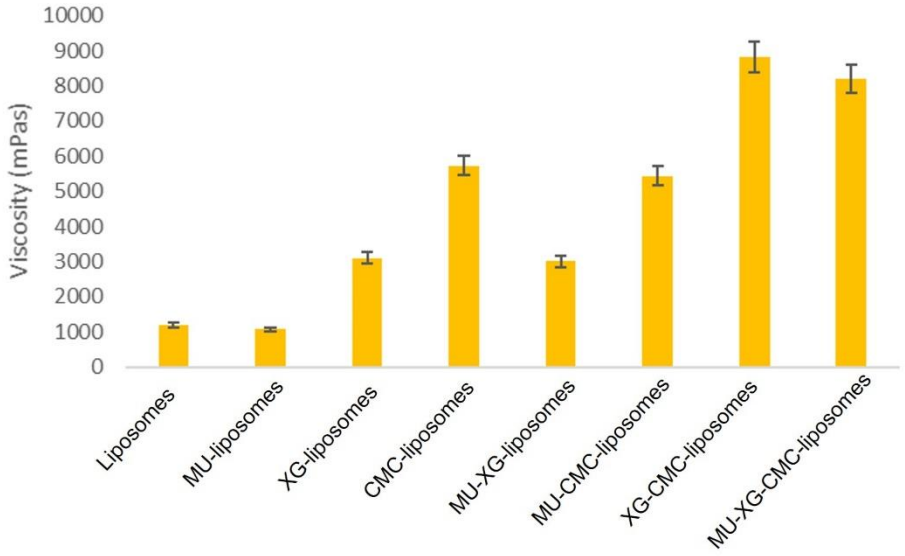


447

448 **Figure 5.** Distance (%) traveled by the extract loaded liposomes modified with mucin (MU),
449 xanthan gum (XG), carboxymethyl cellulose sodium salt (CMC) on vertical film of mucin at 0, 5,
450 10 and 15 min. Data represent the mean values ± standard deviations of at least three replicates.

451 **3.4. Viscosity of vesicle dispersions**

452 The addition of the polymers caused a change in the viscosity of the dispersions, which was a
453 function of the used polymer or their combination (Figure 6).



454

455 **Figure 6.** Viscosity of extract loaded liposomes modified with mucin (MU), xanthan gum (XG),
456 carboxymethyl cellulose sodium salt (CMC). Data represent the mean values ± standard deviations
457 of at least three replicates.

458 Liposomes and liposomes modified with mucin were the less viscous systems suggesting an
459 irrelevant effect of the glycoprotein as thickening agent. This effect was also evident when it was
460 combined with other polymers. Differently, xanthan gum and carboxymethyl cellulose
461 significantly improved the viscosity of the dispersions, being the former the most effective
462 thickening agent. Liposomes simultaneously modified with xanthan gum and carboxymethyl
463 cellulose had the highest viscosity, probably because of the synergistic effect of these two
464 polymers, while as above, the presence of mucin in liposomes modified with mucin, xanthan gum
465 and carboxymethyl cellulose, slightly reduced the consistency of the final system. The low
466 thickening effect of mucin can be mainly connected with both concentration of the glycoprotein
467 and pH of the final system. In particular, only at acidic pH (~2) the interaction between the mucin
468 and the media changes as the chain conformation of the glycoprotein may change inducing a
469 mucin-mucin interaction stronger than mucin-media, resulting in a physical network capable of
470 forming gels. At moderate acidic pH (between 4 and 6), as in the case of the tested formulations,
471 the chain conformation is still in favour of mucin-media interaction, which in turn led the formation
472 of a low viscous system (Caicedo and Perilla, 2015; Moreno et al., 2015; Münstedt, 2014).

473 **3.5. Evaluation of the biological activity of the extract loaded in liposomes**

474 The evaluation of the cytotoxicity of new formulations is the initial step that allow to assess their
475 suitability as systems for the delivery of active molecules. These studies are usually performed *in*
476 *vitro* using immortalized cell lines, rather linked to the target tissue (Kirkpatrick et al., 1998).
477 These techniques are useful in evaluating the toxicity or irritancy potential of both materials and

chemicals and they provide an excellent way to screen materials prior to *in vivo* tests. The direct contact procedure is recommended with soft materials like liposomes followed by the quantitative or qualitative analysis of cell grown (Ciapetti et al., 1994). Taking into account that formulations were designed for mucosal delivery, in the present study the extract loaded in liposomes or in liposomes enriched with polymers (individually or combined) were incubated with keratinocytes, which are the main representative cells of epidermis. The tested formulations were not toxic, since the cell viability at 48 h was always higher than 95% (**Figure 7**) (ISO, 2009; Ozdemir et al., 2021). Any statistical difference was detected in the viability of cells treated with the extract in dispersion or loaded in liposomes, confirming the high biocompatibility of both, extract and carriers.

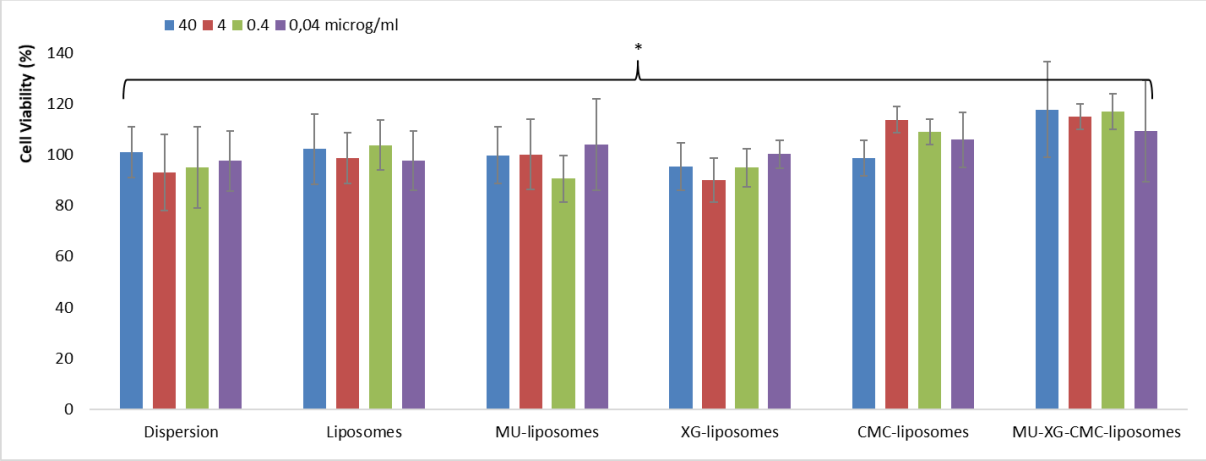


Figure 7. Viability of keratinocytes incubated for 48 h with the extract in dispersion or loaded in liposomes modified with mucin (MU), xanthan gum (XG), carboxymethyl cellulose sodium salt (CMC) and properly diluted to reach 40, 4, 0.4, 0.04 µg/mL of extract (final concentration). Mean values ± standard deviations are reported. Each symbol (*) indicates the same value ($p > 0.05$).

Considering the high biocompatibility of formulations, their ability to protect the cells from damages caused by the oxidative stress was evaluated. Cells were stressed with hydrogen peroxide and co-incubated with the extract in dispersion or loaded in liposomes (**Figure 8**).

The treatment with hydrogen peroxide only, caused a mortality of 30% of cells (viability ~70%), suggesting a toxic effect of this stressing agent (Leyva-Jiménez et al., 2020). The treatment with the extract in dispersion did not reduce the damages as the cell viability remained unchanged (~70%, $p>0.05$ versus the viability of stressed and untreated cells). The loading of the extract in liposomes, irrespective of their composition, usefully affected its efficacy thanks to their carrier ability, indeed, the cell viability was ~100%. Thus, extract loaded liposomes completely avoid the negative effects of hydrogen peroxide and restored the health conditions (Manconi et al., 2018).

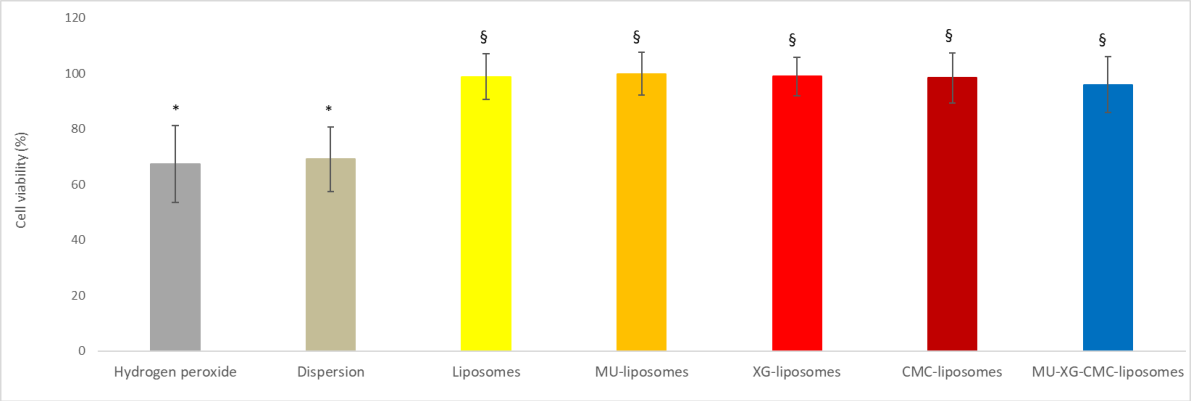


Figure 8. Viability of keratinocytes stressed with hydrogen peroxide and protected with the extract in dispersion or loaded in liposomes modified with mucin (MU), xanthan gum (XG), carboxymethyl cellulose sodium salt (CMC) and properly diluted to reach 4 µg/mL of extract (final concentration). Data are reported as mean values (n = 9) ± standard deviations (error bars) of cell viability expressed as the percentage of untreated cells (100% viability). Each symbol (*,§) indicates the same value ($p>0.05$ versus others).

In vitro scratch assay was performed to evaluate the ability of formulations to promote cell migration in the wounded area (Sağıroğlu et al., 2021). Taking into account that all the

formulations were highly biocompatible and avoid in the same manner the oxidative damage of cells, only the liposomes and liposomes modified with xanthan gum and carboxymethyl cellulose sodium salt or with the combination of the three polymers were tested. The formers were selected as the most promising from a physico-chemical and technological point of view, because they were the most stable during storage and among the most bioadhesive to the mucin film. As previously reported in other studies, the extract in dispersion did not effectively promote the migration of cells as the speed of the closure of the wounded monolayer was slightly higher (~0.8 cm at 24 h) than that of untreated cells (~1.1 cm at 24 h), denoting that its action is only partially promoted (Manca et al., 2021b). On the contrary, the effectiveness of the extract upon loading into liposomes significantly increased and its was evident already after 24 h of treatment, as the length of the wounded area was significantly reduced and was ~0.6 cm using liposomes and ~0.3 cm using liposomes modified with the three polymers (**Figure 9**). The same behaviour was observed at 32 and 48 h, as the migration increased as much as the complete closure was reached at 48h of treatment for MU-XG-CMC-liposomes and XG-CMC-liposomes, for liposomes the length of the scratched area was very small (~0.4 cm at 32 h and ~0.1 cm at 48 h), while for both untreated and cells treated with the extract in dispersion the length of the scratched area was still big (~0.4 and 0.6 cm at 48 h, respectively). As previously reported, this can be achieved due to the ability of vesicles to interact with cells favouring the entrance/uptake of the active molecules that are then capable of exerting their beneficial effect.

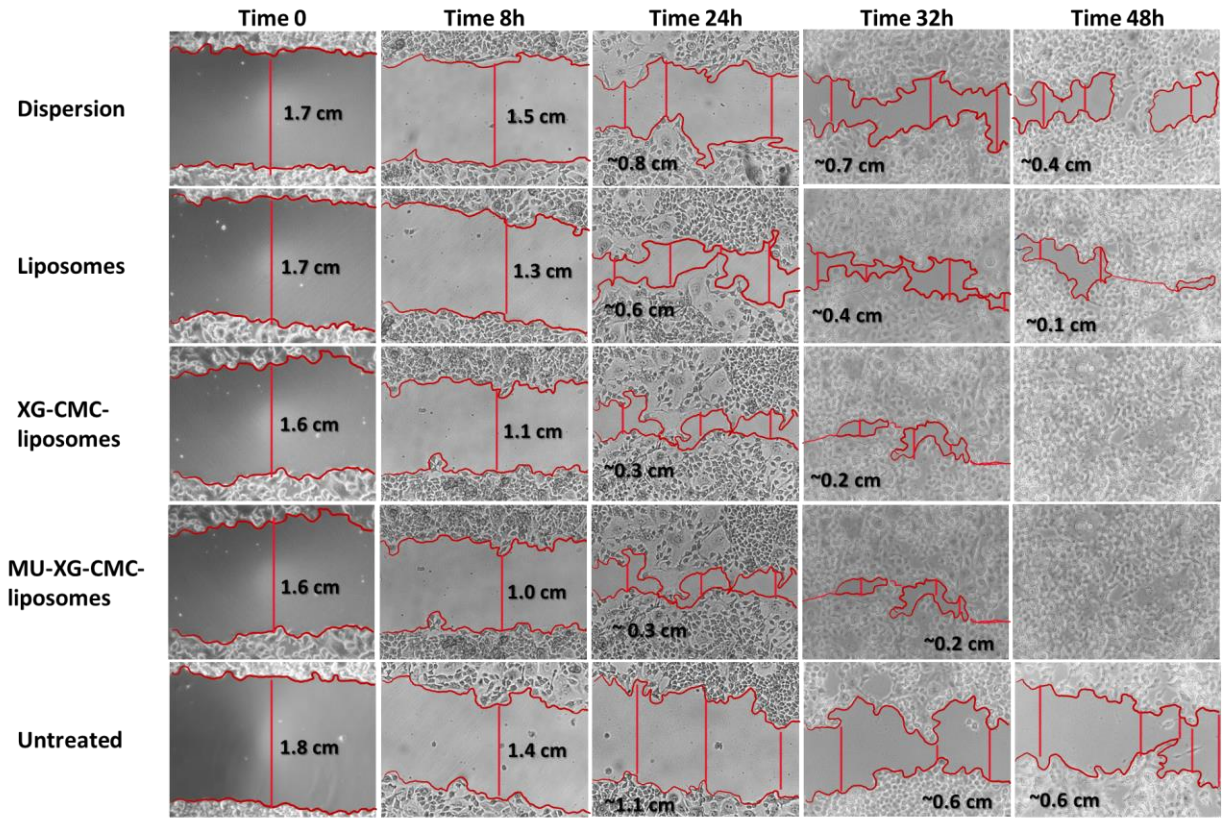


Figure 9. Representative images of a scratched area treated for 48 h, with the extract in dispersion or loaded in liposomes properly diluted to reach 4 μg/mL of extract (final concentration).

4. Conclusions

The antioxidant extract obtained from the blue flowers of *E. amoenum* was successfully incorporated into liposomes, which have also been enriched with the addition of mucin, xanthan gum, carboxymethyl cellulose. Their combination allowed to formulate delivery systems specifically tailored to be applied to the buccal mucosa. In particular, the addition of the polymers, and especially their association, led to the formation of small and homogeneous vesicles, stable on storage (4°C for 3 months) but also in mucin-rich environments, which exerted great muco-adhesive properties. The formulations were highly biocompatible, effectively counteracted the oxidative damages induced in keratinocytes and promoted their migration.

Regarding these promising results, these formulations might be incorporated into innovative oral gels or mouthwashes, beyond other self-care products, to be applied to buccal mucosa wounds. Indeed, inserted into these systems, they may facilitate the treatment of oral ulcers or sores. Despite outstanding biocompatibility results of these nanosystems, further studies are needed to evaluate the effectiveness of the extract loaded liposomes *in vivo*.

Acknowledgments: This research was financially supported by Fondazione di Sardegna 2018

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