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Tackling the challenges of the oral administration of a fermented flour extract with liposomes: Effect on intestinal HT-29 cells

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ABSTRACT

LisosanG is a fermented flour that contains polyphenols, polyunsaturated fatty acids, vitamins, and alpha-lipoic acid with antioxidant/anti-inflammatory properties. These compounds are often characterized by instability, low solubility/bioavailability, and poor absorption that restrict their application in therapy. These problems can be solved by using delivery systems among which liposomes have emerged as prominent candidates. Liposomes can load compounds with varied physico-chemical properties, providing protection from degradation, increased solubility, modulation of release, and facilitated diffusion across biomembranes. Lisosan G liposomes tailored for oral administration were prepared. Eudragit® enteric polymer was added to protect the liposomes from acidic pH. The Eudragit-liposomes were around 100 nm and unilamellar. They were resistant to acidic conditions and did not alter cell viability, nor intracellular ROS levels. These findings confirm that liposomes are an efficient system for the loading of multicomponent-based extracts and that, when properly formulated, can offer protection from physiological degradation, and be safely applied to cells.

1. Introduction

In recent years, there has been growing interest in fermented foods. Lisosan G is a fermented flour from organic grain registered at the Italian Ministry of Health as a food supplement. Fermentation improves the nutritional value and the functional properties of grain (Balli et al., 2019; Ozdemir et al., 2007). Lisosan G was proved to have antioxidant properties owing to a rich variety of compounds, such as polyphenols, gallic acid, polyunsaturated fatty acids, alpha-lipoic acid, and vitamins (Longo et al., 2007). Lisosan G modulated antioxidant and detoxifying enzymes in rat primary hepatocytes (La Marca et al., 2013). Antioxidant and anti-inflammatory activities were detected in human endothelial progenitor cells, whose functionality was improved through the down-regulation of pro-inflammatory factors and the strengthening of anti-oxidant defenses (Gabriele et al., 2018; Lubrano et al., 2012; Lucchesi et al., 2014). Lisosan G downregulated the expression of inflammation biomarkers and raised the cellular antioxidant defenses in human colon adenocarcinoma HT-29 cells (Giusti et al., 2017). Despite the wide array of biological activities of dietary compounds, their application in therapy is often limited by their low bioavailability and poor absorption in the gastrointestinal tract (Hendawy, 2021; Stillhart et al., 2020). Nanotechnologies represent a leading strategy to overcome these limitations. In particular, liposomes have attracted the interest of researchers due to their versatility, biocompatibility, ability to load compounds with varied physico-chemical properties while offering protection from degradation, enhancing the bioavailability, controlling the release, and promoting the transport across biomembranes (Hendawy, 2021; Zylberberg & Matosevic, 2016). In the frame of oral administration, liposomes can be adjusted for composition, charge, coating to enhance their stability and transit in the gastrointestinal tract (He et al., 2019; Nguyen et al., 2016). A range of functional materials, such as polysaccharides, dextrins, Poloxamers and Eudragits have been explored to protect liposomes and their cargo from gastrointestinal degradation (Hua, 2014). Eudragit® enteric polymers are polyacids unionized at low pH, thus being insoluble. As pH increases, the acidic groups ionize, and the Eudragit® becomes soluble (Nikam et al., 2023).

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Hence, the integrity of liposomes in the stomach and the release of their cargo in the intestines rely on pH variations in the gastrointestinal tract.

A previous study (Gabriele et al., 2022) described the preparation of Eudragit-liposomes for the loading of Lisosan G aqueous extract. The encapsulation of the extract into the Eudragit-liposomes was demonstrated to be a promising strategy to preserve the extract's antioxidant activity, which was assayed *in vitro* (via Folin Ciocalteu, DPPH, FRAP assays) and *ex vivo* (in human erythrocytes) in comparison with the raw aqueous extract. Differently from what has been proposed previously, in the present study, the Lisosan G extract Eudragit-liposomes are compared with empty Eudragit-liposomes, Lisosan G extract liposomes, and empty liposomes. The structural features (i.e., size, polydispersity, charge, entrapment efficiency, lamellar structure) of the vesicles and their physical stability in fluids mimicking the gastrointestinal environment are also reported. Furthermore, the non-toxicity of the vesicle formulations in human intestinal cells and the effects on intracellular reactive oxygen species (ROS) levels are herein reported.

2. Materials and methods

2.1. Materials

Lisosan G (LG), which was produced by the fermentation of *Triticum aestivum* flour, was provided by Agrisan Srl (Larciano, Pistoia, Italy). Soy lecithin (Lecinova®, Céréal, Nutrition & Santé Italia S.p.A., Lainate, Italy) was bought from a pharmacy. Eudragit® L100 (1:1 methacrylic acid-methyl methacrylate copolymer; weight average molar mass (Mw) approximately 125,000 g/mol) was donated by Evonik Industries AG (Essen, Germany). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; ≥98%) and 2′-7′-dichlorodihydrofluorescein diacetate (DCFH-DA; ≥97%) and other chemicals were from Sigma-Aldrich/Merck (Milan, Italy), unless otherwise reported.

2.2. Preparation of Lisosan G aqueous extract

LG aqueous extract was produced following a procedure described previously (Gabriele et al., 2022). In short, LG powder was dispersed in water (8.3 mg/mL), sonicated (3 cycles, 10 s on/10 s off; Soniprep 150, MSE Crowley, London, UK), and centrifuged (10 min, 2300×*g*, 4 °C; Jouan CR3i centrifuge, Thermo Fisher Scientific, Basingstoke, UK). The supernatant was filtered and stored at 4 °C.

2.3. Preparation and characterization of Eudragit-liposomes and liposomes

To produce LG Eudragit-liposomes, the procedure reported by Gabriele et al. (2022) was applied. Briefly, Lecithin (90 mg/mL) and Eudragit (2 mg/mL) were dispersed in a 1:1 LG aqueous extract:water blend and sonicated (15 cycles, 5 s on/2 s off + 20 cycles, 3 s on/2 s off) with an ultrasound disintegrator (Soniprep 150 plus, MSE Crowley, London, UK). Empty Eudragit-liposomes (i.e., without LG extract), LG liposomes (i.e., without Eudragit) and empty liposomes (i.e., without LG extract and without Eudragit) were produced following the same procedure used to prepare LG Eudragit-liposomes. The pH of the formulations was measured using a calibrated pH meter at 25 °C.

The mean diameter (i.e. intensity-based average size weighed according to the scattering intensity of each particle fraction), polydispersity index (i.e. dimensionless number calculated from the Cumulants analysis of the Dynamic Light Scattering-measured intensity autocorrelation function; it describes the width of particle size distribution), and zeta potential of the vesicles were measured using a Zetasizer nano-ZS (Malvern Panalytical, Worcestershire, UK). The vesicle dispersions ($n > 15$) were diluted with water and analysed by dynamic and electrophoretic light scattering.

Both the LG Eudragit-liposomes and the LG liposomes were dialysed to remove the non-encapsulated LG extract active compound. The vesicle dispersions were loaded into dialysis membranes (12–14 kDa MWCO; Spectrum Laboratories Inc., Breda, The Netherlands) and dialysed against water (Gabriele et al., 2022). Both non-purified and purified vesicles were diluted with 20:80 methanol:water and analysed by liquid chromatography to quantify gallic acid, a major component of LG extract. An Alliance 2690 HPLC system (Waters, Milan, Italy) was used, with a Waters SunFire column (3.5 μm, 4.6 × 150 mm), a mobile phase consisting of acetonitrile:water:acetic acid (94:5.8:0.2 %*v/v*), and a flow rate of 0.3 mL/min. Gallic acid was quantified at 259 nm (Gabriele et al., 2022). The entrapment efficiency was calculated as the percentage of gallic acid detected in purified *vs.* non-purified samples.

2.4. Small-Angle X-ray scattering

Small-Angle X-ray Scattering (SAXS) analyses were performed to gain a structural characterization of the vesicles. An S3-MICRO (Hecus X-ray Systems GmbH, Graz, Austria) coupled to a GENIX-Fox 3D X-ray source (Xenocs, Grenoble, France) and a PSD-50 detector (Hecus X-ray Systems GmbH) was used. Working conditions were previously reported (De Luca et al., 2023). The scattering curves were recorded every 20 min for 2 h, including the pertinent backgrounds. After background subtraction, SAXS patterns were fitted to the Fourier Transform of bilayer electronic profiles based on a Gaussian description of the profiles. The fitting was performed using a home-made fitting routine using a Levenberg-Marquardt minimization scheme and incorporating the appropriate detector-signal convolution functions for detector width, detector depth and beam profile (Caddeo et al., 2014, 2018; Heftberger et al., 2014; Pabst et al., 2000; Pedersen, 1997).

2.5. Stability in mimicked gastrointestinal fluids

Their behavior of LG Eudragit-liposomes in mimicked gastrointestinal fluids was studied. To evaluate the protective effect of the gastroresistant Eudragit, LG liposomes were also tested.

The mean diameter, polydispersity, and zeta potential of the vesicles were evaluated after dilution (1:100 *v:v*) with a mimicked gastric fluid (0.1 M HCl, pH 1.2) or a mimicked intestinal fluid (disodium hydrogen phosphate buffer, pH 7.0; Carlo Erba Reagents Srl, Cornaredo, Milan, Italy), and after incubation for 2 or 6 h (37 °C), respectively. 0.3 M sodium chloride was added to the fluids to increase the ionic strength.

2.6. Cell culture

Human colon adenocarcinoma HT-29 cells (DSMZ, ACC299, Braunschweig, Germany) were grown in Dulbecco's Modified Eagle's Medium/Ham's nutrient mixture F-12 (DMEM/F-12; EuroClone S.p.A., Pero, Milan, Italy) plus foetal bovine serum (FBS), penicillin and streptomycin (Life Technologies, Carlsbad, CA, US) in a humidified 5% CO_2 atmosphere (37 °C). For the experiments, the cells were seeded into 96-well plates and DMEM/F-12 medium without FBS was used.

2.7. Cell viability

The viability of HT-29 cells was evaluated via the MTT assay, which measures the mitochondrial activity in living cells. After 24 h of exposure to 0.04 and 0.08 mg/mL of LG solution (1:1 LG aqueous extract: water blend) or LG liposomes or LG Eudragit-liposomes, the cells (2.5×10^4 cells/well) were incubated with MTT reagent (0.5 mg/mL). After 1 h, the medium was removed, the cells were rinsed with 1X PBS, and the formazan crystals were solubilized in dimethyl sulfoxide:isopropanol (10:90 %*v/v*; 100 μL). The amount of formazan produced, which is directly correlated to metabolically active cells, was determined by measuring the absorbance at 540 nm using a multiplate reader.

Empty liposomes and empty Eudragit-liposomes were tested at the same dilutions used for LG solution and LG liposomes/Eudragit-liposomes to reach the selected LG extract concentrations. The

viability test was performed at least three times independently, each time in triplicate.

2.8. Determination of intracellular ROS

The endogenous cellular ROS were detected after exposure of HT-29 cells to a cell-permeant fluorogenic dye that measures the redox state of cells (DCFH-DA). In short, the cells (2.5×10^4 cells/well) were seeded into 96-well black plates and incubated with DCFH-DA (15 μM/well) for 60 min. After rinsing with 1X PBS, the cells were treated with LG solution (1:1 LG aqueous extract:water blend) or LG liposomes or LG Eudragit-liposomes (0.04 and 0.08 mg/mL of LG extract) for 24 h. ROS levels were measured by reading the fluorescence (excitation: 495 nm - emission: 527 nm) with a spectrophotometer. The ROS test was performed at least three times independently, each time in quadruplicate.

2.9. Statistical analysis

Results are presented as means ± standard deviations. The difference between independent groups was performed by ANOVA, while single comparisons were made via the Tukey's test. p values < 0.05 were considered statistically significant. GraphPad Prism software was employed for all the analyses.

3. Results

3.1. Characterization of the vesicles

The Eudragit-liposomes and the liposomes were characterized by determining the mean diameter, the polydispersity, and the zeta potential. The effect of LG extract on these three parameters was evaluated by comparing the Eudragit-liposomes and liposomes loaded with LG extract respectively with empty Eudragit-liposomes and empty liposomes (Table 1). The effect of Eudragit was evaluated as well (Table 1).

The empty liposomes displayed a mean diameter of 94 nm. The size increased markedly upon encapsulation of the LG extract (126 nm), and the PI improved significantly, reaching the value of 0.15, which indicates an excellent size homogeneity of these vesicles.

The mean diameter of the empty Eudragit-liposomes was markedly larger than that of the empty liposomes (116 *vs.* 94 nm) and the homogeneity decreased, though the PI value was 0.25, thus below the critical limit of 0.3 (Table 1). The encapsulation of the LG extract in the Eudragit-liposomes induced a further enlargement of the vesicles (126 nm), which was accompanied by an improvement of the homogeneity (PI 0.22; Table 1).

The zeta potential of both the empty liposomes and empty Eudragit-liposomes was highly negative (approx. −45 mV; Table 1) and became less negative upon incorporation of the extract (approx. −30 mV; Table 1), which evidently induced an alteration of the assembly of the phospholipids.

The pH of the formulations was also measured. The values were around 5.7 for the empty vesicles (Table 1) and decreased slightly in LG extract-loaded vesicles (approx. pH 5; Table 1) due to the inner acidity of LG caused by the formation of short chain fatty acids during the fermentation process.

The entrapment efficiency was calculated as a function of gallic acid, a major compound of the LG extract detected in purified *vs.* non-purified LG vesicle formulations. The values were above 70% in both Eudragit-liposomes and liposomes, which demonstrates that the polymer did not affect the ability of the vesicles to encapsulate the extract (Table 1).

A structural characterization of the Eudragit-liposomes and the liposomes was carried out by SAXS analyses. The vesicle bilayers were fitted using a model based on a modified Caillé Gaussian description of the bilayer (Caddeo et al., 2018; Pabst et al., 2000). The model incorporates the possibility of asymmetric bilayers. Since the vesicle composition corresponds to a complex mixture of polar heads and chains, we restricted the mean electronic density of the system to that of water (the major component) and the electronic density of the CH_2 chains to that of liquid methylene chains. No other restrictions were used for the polar or apolar parts of the bilayers. As a consequence, some of the usual parameters of bilayers could not be determined, such as the area per polar head. With those restrictions, the fits were quite good (Fig. 1a and b), and produced reduced chi squared values below 2 (χ^2; Table 2).

We systematically fitted a symmetric model first and once optimized, let the model parameters of the bilayer split in the two leaflets. Although the fitting improved somewhat, unrealistic parameters were obtained for the asymmetric models. In all of them the results corresponded to a doubling of the headgroup electron density and a halving of the width. This corresponds to the conservation of the total electron contribution of the headgroup, and the improvement of the fit is based only on the reduction of the minima in the spectra due to the presence of asymmetry. Therefore, the symmetric model was considered. The SAXS profiles shown in Figs. 1 and 2 can be fitted with a unilamellar model for both the liposomes and the Eudragit-liposomes. This implies that either multilamellarity is absent in the samples or that such multilamellarity correspond to non-correlated lamellae. The fitting parameters are shown in Table 2. Since σ_c is large for all the vesicles, and as evident from the electron density profiles (Figs. 1b and 2b), we can confirm that there is no segregation of the methylene groups at the center of the bilayers. From Figs. 1 and 2 and Table 2 we cannot observe relevant effects of LG extract or Eudragit on the bilayer characteristics. However, the combined effect of the two components produced slight changes, that is, a small but still significant reduction in Z_H (which corresponds to the position of the maximum in the polar Gaussian position) and a slight increase in σ_H (which corresponds to the polar headgroup Gaussian width). This increase is also accompanied by a slight reduction of the polar head contrast (σ_H; Table 2).

Table 1
Mean diameter (MD), polydispersity index (PI), zeta potential (ZP), pH, and entrapment efficiency (EE) of liposomes and Eudragit-liposomes. Mean values ± standard deviations (SD; $n > 10$) are presented. *** values different ($p < 0.001$) from empty liposomes; °°° values different ($p < 0.001$) from empty Eudragit-liposomes; §§§ values different ($p < 0.001$) from LG liposomes.

Formulation	MD nm ± SD	PI ± SD	ZP mV ± SD	pH	EE % ± SD
Empty liposomes	94 ± 4.3	0.20 ± 0.02	−47 ± 3.5	5.7 ± 0.03	
LG liposomes	***126 ± 5.9	***0.15 ± 0.04	***−34 ± 3.0	5.1 ± 0.02	71 ± 4.2
Empty Eudragit-liposomes	***116 ± 5.1	***0.25 ± 0.02	−45 ± 2.5	5.6 ± 0.06	
LG Eudragit-liposomes	°°°126 ± 4.9	°°°§§§0.22 ± 0.02	°°°−31 ± 3.8	5.0 ± 0.02	75 ± 7.1

3.2. Stability in mimicked gastrointestinal fluids

The stability of the vesicles under conditions that mimic the gastrointestinal environment was assessed. After 2 h at pH 1.2, LG liposomes displayed a marked increase in size (175 *vs.* 126 nm; Table 3 *vs.* Table 1), along with a much greater polydispersity (PI 0.38 *vs.* 0.15; Table 3 *vs.* Table 1). Under the same conditions, LG Eudragit-liposomes were unchanged: 132 nm and PI 0.26 *vs.* 126 nm and PI 0.22. After 6 h at pH 7.0, both LG liposomes and LG Eudragit-liposomes did not vary significantly. Zeta potential values fluctuated due to the protons or salts present in the acidic and neutral fluids (Table 3).

These results point to the key role of Eudragit, which increased the physical stability of the vesicles by providing resistance to acidic conditions.

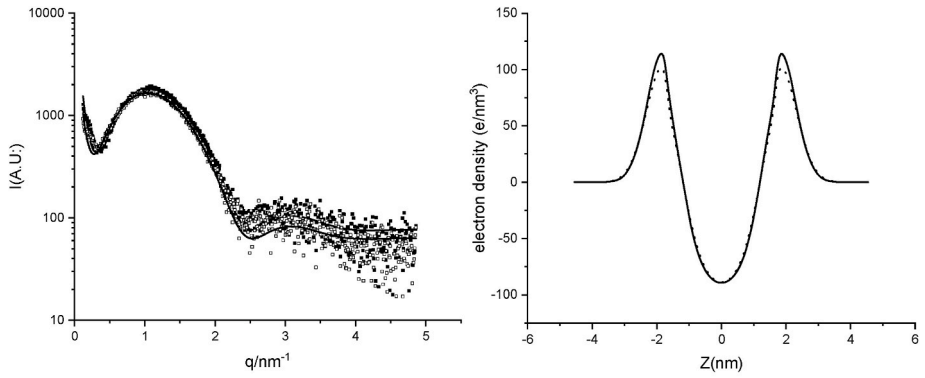

Fig. 1. (**a**) SAXS curves of empty liposomes (full symbols) and LG liposomes (open symbols). The lines correspond to the fit of Gaussian bilayer model. (**b**) Electron density profiles corresponding to the fits of empty liposomes (full line) and LG liposomes (dotted line).

Table 2
Fitting parameters and derived parameters (± estimated error from the fit) for SAXS curves of the vesicle formulations. χ^2 (reduced chi squared), Z_H (polar head Gaussian center), σ_H (polar head Gaussian amplitude), ρ_H (polar head Gaussian electronic density contrast), and σ_C (methyl Gaussian amplitude).

	Empty liposomes	LG liposomes	Empty Eudragit-liposomes	LG Eudragit-liposomes
χ^2	1.9	1.5	1.5	2.0
Z_H (Å)	17.8 ± 0.3	17.7 ± 0.2	17.6 ± 0.2	16.9 ± 0.3
σ_H (e/nm^3)	5.0 ± 0.3	5.4 ± 0.2	5.4 ± 0.2	6.2 ± 0.3
σ_C (Å)	9 ± 1	9 ± 1	9.6 ± 1	9.8 ± 1

3.3. Evaluation of cell viability and intracellular ROS

The absence of cytotoxicity of the prepared formulations was demonstrated. Both the free LG extract in solution and encapsulated in liposomes or Eudragit-liposomes showed no negative impact on the viability of HT-29 cells, which was well above 85% (Fig. 3a).

The production of endogenous ROS induced by the formulations in HT-29 cells was determined by using a cell-permeant redox sensitive dye. After 24 h of application of LG solution, LG liposomes or LG Eudragit-liposomes, or of empty liposomes or empty Eudragit-liposomes, the intracellular ROS levels were not affected, since the fluorescence intensity was comparable to that of non-treated control cells (Fig. 3b).

4. Discussion

Lisosan G (LG) is obtained from the fermentation of flour. Various studies indicate that the fermentation process increases both phenolic content and antioxidant activity (Adebo & Medina-Meza, 2020). We have previously demonstrated that the antioxidant activity of an LG aqueous extract was preserved when it was formulated in Eudragit-liposomes (Gabriele et al., 2022). Liposome technology was proposed to increase the therapeutic impact of the LG extract.

The use of liposomes has proved to be a leading strategy to facilitate the application of bioactive compounds from food, especially with regards to controlled release and increased stability (Ajeeshkumar et al., 2021). Liposomal encapsulation has also been reported as an efficient strategy to prevent a decrease in antioxidant activity of food extracts' compounds due to degradation/digestion processes (Rudrapal et al., 2022; Zylberberg & Matosevic, 2016).

In this study, in order to investigate the potential of Eudragit-liposomes for the oral delivery of the LG extract, we deepened their structural features, their behaviour in simulated gastrointestinal fluids, and their non-toxicity in intestinal cells. We compared the stability and biocompatibility of LG Eudragit-liposomes and LG liposomes. The presence of Eudragit was essential to resisting acidic conditions. Recently, Kumeria et al. (2022) demonstrated that Eudragit provided protection to immunoglobulin A-2 loaded into porous silicon nanoparticles under acidic conditions and a release of the protein >50% at neutral pH. Similarly, *in vitro* drug release studies showed that Eudragit protected superparamagnetic iron oxide nanoparticles (SPIONs) from digestion in acidic environment and allowed release of carmofur at

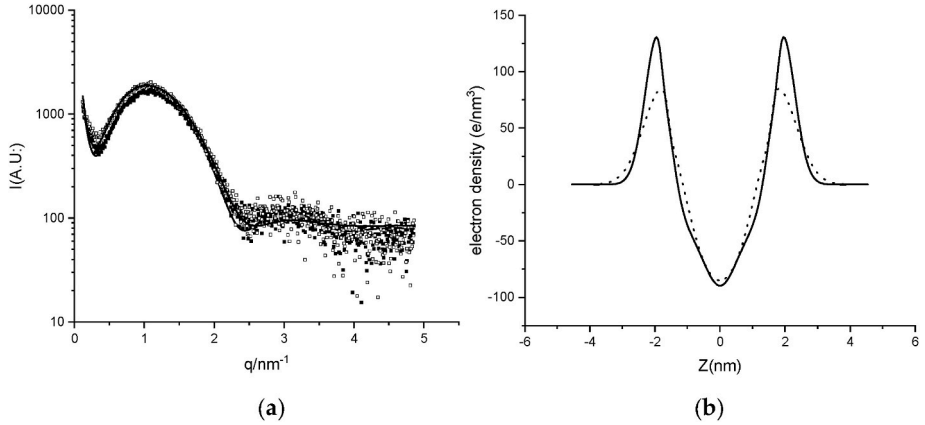

Fig. 2. (**a**) SAXS profiles of empty Eudragit-liposomes (full symbols) and LG Eudragit-liposomes (open symbols). The lines correspond to the fit of Gaussian bilayer model. (**b**) Electron density profiles corresponding to the fits of empty Eudragit-liposomes (full line) and LG Eudragit-liposomes (dotted line).

Table 3
Mean diameter (MD), polydispersity index (PI) and zeta potential (ZP) of LG liposomes and LG Eudragit-liposomes measured after dilution (t_0) and after 2 (t_{2h}) or 6 h (t_{6h}) of incubation at pH 1.2 or 7.0, respectively. Mean values ± standard deviations (SD) are presented ($n = 4$).

Formulation	pH	Time	MD nm ± SD	PI ± SD	ZP mV ± SD
LG liposomes	1.2	t_0	163 ± 7.0	0.21 ± 0.06	−10 ± 0.3
		t_{2h}	175 ± 7.1	0.38 ± 0.03	−10 ± 1.1
	7.0	t_0	124 ± 3.2	0.13 ± 0.03	−26 ± 0.6
		t_{6h}	140 ± 9.2	0.19 ± 0.09	−23 ± 0.9
LG Eudragit-liposomes	1.2	t_0	136 ± 5.4	0.28 ± 0.07	−9 ± 1.3
		t_{2h}	132 ± 3.7	0.26 ± 0.06	−9 ± 0.1
	7.0	t_0	121 ± 4.6	0.17 ± 0.03	−26 ± 1.0
		t_{6h}	123 ± 4.7	0.19 ± 0.01	−25 ± 0.9

near-neutral pH values, giving the formulations potential for oral administration (Zhang et al., 2020). In addition, we found that the LG Eudragit-liposomes did not affect neither cell viability nor intracellular ROS balance of the intestinal cells.

5. Conclusions

The proposed Eudragit-liposomes were small, unilamellar, and capable of encapsulating LG extract efficiently. They were proved to be resistant to acidic conditions and cytocompatible with intestinal cells, thus suitable for oral administration. *In vitro* digestion studies would be valuable to investigate whether the Eudragit-liposomes reach the intestine intact, proving to be an ideal vehicle for substances that may be degraded during oral intake.

CRediT author statement

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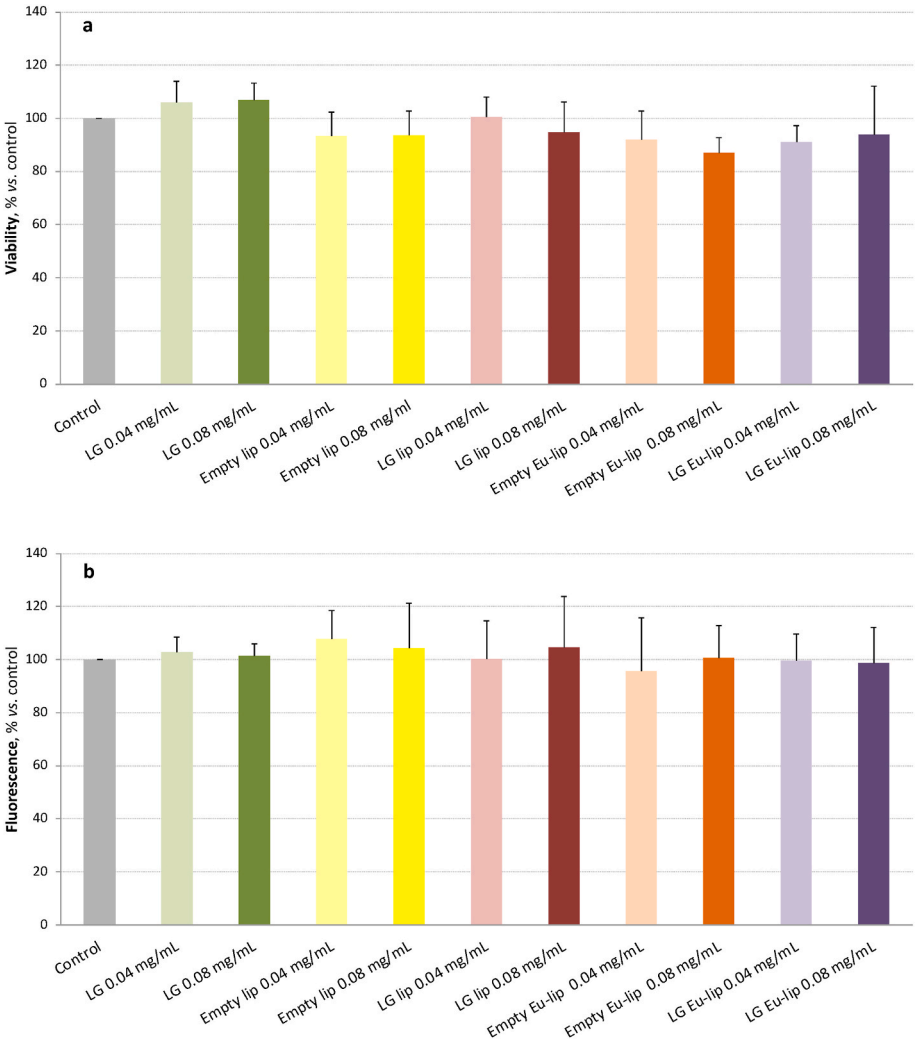

Fig. 3. **(a)** Viability of HT-29 cells after application of empty liposomes and empty Eudragit-liposomes, LG solution, LG liposomes and LG Eudragit-liposomes for 24 h. **(b)** Effects of LG solution, LG liposomes, LG Eudragit-liposomes, and empty liposomes or empty Eudragit-liposomes on ROS levels in HT-29 cells after 24 h of application. Mean values ± standard deviations are presented; $n \geq 3$. ANOVA p = not significant.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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