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Encapsulation of bioactive fermented wheat (Lisosan G) in Eudragit-liposomes

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ABSTRACT

Liposomes are one of the most studied and most promising nanocarriers to date, representing a biocompatible, safe, and efficient delivery system for both hydrophilic and lipophilic compounds.

This work aimed to evaluate the efficacy in encapsulating an aqueous extract of Lisosan G (LG), a *Triticum aestivum* whole grains fermented powder with strong antioxidant activity, into Eudragit-liposomes designed for oral delivery.

The total phenolics and the antioxidant activity of LG extract and LG extract encapsulated in Eudragit-liposomes were evaluated by the Folin-Ciocalteu, DPPH, and FRAP colorimetric assays. Finally, the biological activity of both LG formulations was assessed as human erythrocytes protection from oxidative stress through the cellular antioxidant activity (CAA) assay.

Our results demonstrate that the encapsulation into Eudragit-liposomes preserved the phenolics content of LG extract and retained its antioxidant properties both *in vitro* and *ex vivo*. Furthermore, LG Eudragit-liposomes exhibited increased ferric reducing capacity and protection of human erythrocytes from oxidative insult, probably due to the antioxidant properties of liposome constituents.

Therefore, the encapsulation into Eudragit-liposomes represents an excellent strategy to prevent possible reduction and loss of activity of LG bioactive compounds, and to deliver them via oral administration.

1. Introduction

Lisosan G (LG) is a fermented product obtained from *Triticum aestivum* (whole wheat) registered as a nutritional supplement at the Italian Ministry of Health. The production process is performed by Agrisan srl (Italy) through the following steps: 1) whole grains are grounded to a coarse powder, and wheat bran and germ are collected; 2) water is added to moisten the powder, and *Lactobacillus* and natural yeast strains are inoculated to start fermentation; 3) the product is dried.

It was found that LG, which contains vitamins, minerals, and polyunsaturated fatty acids, did not interfere with the drug-metabolizing system (Longo, Chirulli, Gervasi, Nencioni, & Pellegrini, 2007).

More recently, we demonstrated that LG protects the retina from oxidative stress and reduces inflammation and retinal damage associated with diabetic retinopathy (Amato et al., 2018). Moreover, the protective effects of LG in glaucoma, likely attributable to its antioxidant properties, have been highlighted (Amato et al., 2021), which suggests the potential use of LG as a treatment for different retinal pathologies.

Good antioxidant and anti-inflammatory properties were also observed in inflamed human endothelial progenitor cells and intestinal cells, where LG down-regulated the expression of inflammation biomarkers, by limiting the translocation of the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB), and raised the cellular antioxidant defenses via the nuclear factor (erythroid-derived 2)-like 2 (Nrf2) signaling pathway (Gabriele Pucci, Árvay, & Longo, 2018; Giusti et al., 2017; Lucchesi et al., 2014). The antioxidant properties of LG are likely due to key compounds, such as gallic acid, 3-hydroxybenzoic acid, vanillic acid, and alpha-lipoic acid (Gabriele et al., 2018; La Marca, Beffy, Pugliese, & Longo, 2014; Lucchesi et al., 2014).

In the present study, we evaluated whether the bioactivity of LG

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Table 1
Composition of LG Eudragit-liposomes.

Formulation	Lecinova	Eudragit L100	LG aqueous extract	H_2O
LG eudragit-liposomes	180 mg	4 mg	1 mL	1 mL

could be enhanced by its encapsulation into liposomes. Among the types of nanocarriers investigated to date, liposomes are one of the most successful systems for the delivery of bioactive compounds (Sercombe et al., 2015). Thanks to their unique composition and structure similar to biological membranes, which make them biocompatible and safe, liposomes can carry either hydrophilic or lipophilic compounds. They represent an efficient delivery approach, through different administration routes, for medical applications, cosmetics and food industry, as they provide a number of advantages, such as payload protection, modified/controlled pharmacokinetics and distribution, facilitated transport through biological membranes, increased dose delivered to the target (Zylberberg & Matosevic, 2016).

With the aim of assessing the efficacy of liposomes in encapsulating LG, delivering it by the oral route, and enhancing its antioxidant activity, Eudragit-liposomes were produced.

Eudragit® is a methacrylic acid and methyl metacrylate copolymer, insoluble at acidic pH and soluble at neutral pH. Hence, Eudragit-liposomes are expected to protect LG from acidic degradation in the stomach, allowing pH-driven absorption in the intestine, and ultimately facilitate the entry of LG components in cells, thus potentiating its bioactivity. The phenolic content and the antioxidant properties of LG extract and LG extract encapsulated in Eudragit-liposomes were assessed *in vitro*. Furthermore, the protection of LG and LG Eudragit-liposomes from oxidative stress was evaluated *ex vivo* on oxidized human erythrocytes.

2. Materials and methods

2.1. Materials

Lecinova® (Céréal, Nutrition & Santé Italia S.p.A., Origgio, Italy), granular soy lecithin, was purchased in a local pharmacy. Eudragit® L100, an anionic 1:1 methacrylic acid-methyl methacrylate copolymer of 135 kDa (Cetin, Atila, & Kadioglu, 2010), was kindly supplied by Evonik Industries AG (Essen, Germany). Folin-Ciocalteu reagent, sodium carbonate, gallic acid, quercetin, 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2′,7′-dichlorofluorescein diacetate (DCFH-DA), 2,2-azobis (2-amidinopropane) dihydrochloride (AAPH), phosphate buffer saline (PBS), chloridric acid, 2,4,6-tri(2-pyridyl)-*s*-triazine (TPTZ), ferrous sulfate heptahydrate, and ferric chloride hexahydrate were purchased from Merck (Spruce St, Saint Louis, MO, USA). Absolute ethanol and methanol were purchased from VWR (Radnor, PA, USA).

2.2. LG extraction

LG produced by fermenting and drying *Triticum aestivum* whole wheat grains flour, was kindly supplied by Agrisan srl (Larciano, PT, Italy). LG aqueous extract was obtained by dispersing LG powder in water (8.3 mg mL^{-1}), sonicating and centrifugating at 2300×*g* for 10 min, at 4 °C (Jouan CR3i centrifuge, Newport Pagnell, UK). Then, the supernatant was collected, filtered (0.2 μm), and stored in the dark at 4 ± 2 °C until use.

2.3. Liposome preparation and characterization

To produce eudragit-liposomes, Lecinova® and Eudragit® were weighed in a vial, dispersed in a 50:50 LG aqueous extract:water blend (Table 1), and sonicated (15 cycles, 5 s on/2 s off + 20 cycles, 3 s on/2 s off) with a Soniprep 150 (MSE Crowley, London, UK).

The average diameter, polydispersity index (PI, a measure of the width of size distribution), and zeta potential of the liposomes were determined via dynamic and electrophoretic light scattering using a Zetasizer nano-ZS (Malvern Panalytical, Worcestershire, UK). Prior to analysis at 25 °C, the samples (n > 10) were diluted with water (1:100).

The storage stability of the formulations was assessed by analyzing the mean diameter, PI and zeta potential of the vesicles for 3 months at 4 ± 2 °C.

The vesicle dispersions were purified from the non-encapsulated LG active compounds by dialysis. The dispersions (1 mL) were loaded into 12–14 kDa MWCO Spectra/Por® membrane tubing (Spectrum Laboratories Inc., DG Breda, The Netherlands) and dialysed for 2 h against water (2 L) to allow the removal of the non-encapsulated compounds. After disruption of unpurified and purified liposomes with 20:80 methanol:water, the entrapment efficiency (E), expressed as the percentage of gallic acid detected in unpurified samples, was determined using an Alliance 2690 HPLC system (Waters, Milan, Italy). Gallic acid was quantified using a Sunfire C_{18} column (3.5 μm, 4.6 × 150 mm, Waters), an acetonitrile:water:acetic acid (94:5.8:0.2 %v/v) mobile phase, and an 0.3 mL/min flow rate. The absorbance at 259 nm (A_{259}) was measured for gallic acid quantification.

2.4. Total phenolics

Total phenolics were estimated as Folin-Ciocalteu (FC) reducing capacity as described by Gabriele et al. (2015) and expressed as mg of gallic acid equivalents per g of dry weight (mg GAE/g dw). Briefly, 100 μL of LG formulations or gallic acid solution, used as a reference, were added to 500 μL of Folin-Ciocalteu reagent (0.2 eq L^{-1}, diluted in water) and kept for 5 min in the dark at room temperature. Thereafter, 400 μL of 0.7 mol L^{-1} sodium carbonate was added. After 2 h of incubation in the dark at room temperature, A_{760} was measured.

2.5. In vitro and ex vivo antioxidant assays

2.5.1. DPPH assay

The antioxidant activity of LG formulations was assessed as a function of their ability to scavenge $DPPH^{\bullet}$, a stable nitrogen-centered free radical. 2 ml of $DPPH^{\bullet}$ methanolic solution (25 μmol L^{-1}) was mixed with 40 μL of LG Eudragit-liposomes or LG aqueous extract and stored in the dark at room temperature. After 30 min, A_{517} was measured against blank. The degree of discoloration of the violet-colored $DPPH^{\bullet}$ solution, which depends on the radical scavenging/antioxidant capacity and the concentration of a sample, is quantified as a decrease in absorbance (A). The percent antioxidant activity (AA) of the samples was calculated according to Equation (1):

$$AA = \left(\frac{A_{DPPH} - A_{sample}}{A_{DPPH}}\right) x\ 100 \quad (1)$$

The antioxidant activity was expressed also as Trolox Equivalents (TE). TE values were calculated using a calibration curve built with different concentrations (0.5–3 mg mL^{-1}) of Trolox (antioxidant reference). Results were expressed as μg TE mL^{-1} solution.

2.5.2. FRAP assay

The antioxidant capacity of LG formulations to reduce Fe^{3+} ferric iron to Fe^{2+} ferrous iron was determined by the FRAP (Ferric Reducing Antioxidant Power) assay (Colosimo et al., 2020). Briefly, a mixture of 300 mmol L^{-1} acetate buffer (pH 3.6), 20 mmol L^{-1} $FeCl_3{\cdot}6H_2O$, 10 mmol L^{-1} TPTZ in 40 mmol L^{-1} HCl was added to each sample. After 30 min at room temperature, A_{593} was measured against blank. The results were expressed as μmol Fe^{2+} L^{-1} using a ferrous iron standard curve.

2.5.3. Cellular antioxidant activity in human erythrocytes

Human red blood cells (RBC) were obtained from healthy donors

Table 2
Characteristics of LG Eudragit-liposomes. Mean diameter (MD), polydispersity index (PI), zeta potential (ZP), and entrapment efficiency (E) are reported. Each value represents the mean ± SD ($n > 6$).

LG eudragit-liposomes	
MD (nm)	132 ± 4.4
PI	0.24 ± 0.03
ZP (mV)	−29 ± 1.9
E (%)	75 ± 3.7

upon informed consent for the use of residual blood samples for research purposes, according to Italian regulations and "Fondazione G. Monasterio CNR-Regione Toscana" regulations.

Blood samples, collected in ethylene-diamine-tetra acetic acid (EDTA)-treated tubes, were centrifuged at 2300×*g* for 10 min, at 4 °C. After plasma and buffy coat removal, erythrocytes were washed twice with PBS (pH 7.4).

The cellular antioxidant activity (CAA) of LG formulations was tested on human erythrocytes under mild oxidation conditions (Frassinetti, Gabriele, Caltavuturo, Longo, & Pucci, 2015). Erythrocytes were 100-fold diluted with PBS (pH 7.4) and incubated for 1 h at 37 °C with 2′,7′-dichlorofluorescein diacetate (DCFH-DA, 15 μmol L^{-1}) and LG formulations at different concentrations (41.5 and 415 μg mL^{-1}), quercetin (8 μmol L^{-1}) used as a reference standard, or PBS used as a blank and control sample. Then, erythrocytes were washed twice and resuspended in cold PBS. Finally, the 2,2-azobis (2-amidinopropane) dihydrochloride (AAPH, 1.2 mmol L^{-1}), a peroxyl radicals generator, was added to the cell suspension, with the exclusion of blank sample, and the fluorescence of ROS-oxidized 2′,7′-dichlorofluorescein (DCF) was read at λ_{ex} = 485 nm and λ_{em} = 535 nm using a Victor TM X3 Multilabel Plate Reader (Waltham, MA, USA). CAA values were expressed according to Equation (2) (Wolfe & Liu, 2007):

$$\text{CAA unit} = 100 - (\int SA/\int CA) \times 100 \quad (2)$$

where $\int SA$ and $\int CA$ represent the integrated area of the sample curve and the control curve, respectively.

2.6. Statistical analysis

Results, expressed as mean values ± standard deviation (SD), were analyzed by one-way analysis of variance (ANOVA) with Dunnett's test and unpaired Student's t-test for single comparisons using GraphPad Prism 5.00 (GraphPad Software, San Diego, CA). p values ≤ 0.05 were considered statistically significant.

3. Results and discussion

3.1. Vesicle preparation and characterization

In the present study, a vesicular formulation was developed for the oral delivery of LG extract. Eudragit-liposomes were produced to protect LG extract from degradation by stomach acid and allow pH-driven absorption in the intestine, since Eudragit® L100 dissolves above pH 6, and to enhance its antioxidant activity at cellular level. Eudragit-liposomes were made of Lecinova®, a cheap dietary supplement of gluten-free, non-GMO soy lecithin containing phospholipids, ω-3 and ω-6 fatty acids, vitamin E and B6, marketed as an aid to lowering cholesterol. Therefore, it is worth noting that, in addition to functional properties, including acidic protection and improvement of the bioavailability of the incorporated compounds from LG extract, the produced liposomes have a food-grade composition, ideal for oral administration.

LG Eudragit-liposomes were produced via an easy, organic solvent-free, and scalable method, and the main physico-chemical and technological features were evaluated. As displayed in Table 2, light scattering results show that LG Eudragit-liposomes were relatively small in size (~130 nm). These liposomes were also characterized by good homogeneity (PI 0.24) and negative zeta potential (~−30 mV), due to the charge of soy lecithin and Eudragit.

The entrapment efficiency (E%) was calculated based on the content of gallic acid, one of the most abundant components of LG extract (Gabriele et al., 2018). E% was 75% (Table 2) and did not diminish significantly (70% ± 2.6; $p > 0.05$) during a three-month-storage period. The stability of the formulations was assessed for three months by analyzing mean diameter, PI, and zeta potential of the vesicles, as well. No statistically significant changes were found among the three examined parameters ($p > 0.05$).

Table 3
Total phenolics content of LG extract, as such or formulated in liposomes. Results are expressed as mg of gallic acid equivalents (GAE) per g on a dry weight (dw) basis.

Formulation	Total phenolics (mg GAE/g dw)
LG extract	9.19 ± 0.20
LG eudragit-liposomes	9.32 ± 0.21

Results are reported as mean value ± SD of 3 independent determinations.

Table 4
In vitro antioxidant activity of LG extract in the vesicle formulations in comparison with an aqueous solution (50:50 %v/v). DPPH results are expressed as AA (%) and as μg TE mL^{-1} concentration, whereas FRAP results are expressed as μmol Fe^{2+} L^{-1}. Results are reported as the mean value ± SD of 3 separate experiments, each performed in triplicate.

	DPPH		FRAP
Formulation	AA (%)	(μg TE mL^{-1})	(μmol Fe^{2+} L^{-1})
LG extract	81 ± 1.8	307 ± 8.9	162.5 ± 3.6
LG eudragit-liposomes	79 ± 2.3	299 ± 10.8	***215.5 ± 2.1

*values statistically different from LG extract, ***$p < 0.001$.

3.2. Total phenolics

The total phenolics of LG formulations were estimated as Folin-Ciocalteu (FC) reducing capacity. Table 3 reports the results of the FC assay showing that LG Eudragit-liposomes contanined comparable phenolics content to the LG extract, which indicates that the encapsulation process preserved LG bioactive compounds.

3.3. In vitro antioxidant assays

The antioxidant activity of LG formulations was firstly assessed by the DPPH assay, which is based on the reduction of the free radical, and secondly by the FRAP assay, which is based on the reduction of ferric iron to ferrous iron, by antioxidant molecules. Table 4 reports the results of both assays. As anticipated by the composition of LG extract, which includes many known antioxidant molecules such as gallic acid, vanillic acid, quercetin, *trans*-ferulic acid, alpha-lipoic, acid 3-hydroxybenzoic acid (Gabriele et al., 2018), the DPPH radical was almost completely scavenged (~80%). It is worth noting that the antioxidant activity of LG extract in Eudragit-liposomes was essentially the same ($p > 0.05$) and corresponded to ~300 μg mL^{-1} of Trolox equivalents. This demonstrates that encapsulation in the vesicle system did not alter the inner capability of the extract to scavenge free radicals (Caddeo et al., 2019). Conversely, the ferric reducing capacity of LG extract in Eudragit-liposomes, corresponding to ~215 μmol L^{-1} of Fe^{2+} equivalents, was significantly higher than that of LG extract (~162 μmol L^{-1} of Fe^{2+} equivalents, $p < 0.001$; Table 4). The higher value found for LG in liposomes is likely due to a contribution from Lecinova®, which contains phospholipids, polyunsaturated fatty acids (PUFAs such as linolenic acid and linoleic acid), and vitamin E. Indeed, PUFAs, which cannot be synthesized by the human body and must be taken in through diet (Rydlewski et al., 2017),

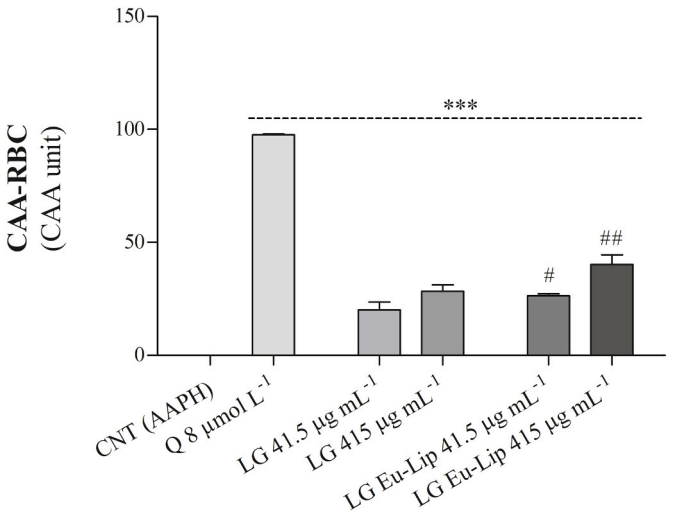

Fig. 1. Effects of increasing doses (41.5 and 415 μg mL^{-1}) of LG extract (LG) and LG extract in Eudragit-liposomes (LG Eu-lip) on the cellular antioxidant activity (CAA) of red blood cells (RBC) under oxidative conditions. Quercetin (Q 8 μmol L^{-1}) was used as a reference standard. Results represent the means ± SD ($n \geq 3$). *significantly different from CNT (AAPH-treated cells; CAA = 0), ***$p \leq 0.001$. #significantly different from the corresponding LG: #$p \leq 0.05$; ##$p \leq 0.01$.

are known to exert antioxidant activities (Gülçin, 2012; Oppedisano et al., 2020), and vitamin E, owing to its antioxidant properties, protects PUFAs from oxidation (Valk & Hornstra, 2000).

3.4. Ex vivo biological activity

The biological activity of increasing concentrations of raw LG extract and LG extract in Eudragit-liposomes was screened *ex vivo* on human erythrocytes under oxidative conditions using the CAA assay. Erythrocytes, which lack both nucleus and mitochondria, represent a reliable *ex vivo* cellular system for assessing the radical scavenging activity of natural compounds (Honzel et al., 2008).

The erythrocytes were exposed to AAPH, whose thermal decomposition in peroxyl radicals caused oxidative insult, after 1 h pre-treatment with different dilutions (41.5 and 415 μg mL^{-1}) of LG formulations. As displayed in Fig. 1, all pre-treatments improved the antioxidant activity of the erythrocytes significantly, as compared to AAPH-treated control cells (CAA unit = 0, ***$p < 0.001$), with values lower than those obtained with quercetin standard (8 μmol L^{-1}). Indeed, when compared to oxidized erythrocytes, both doses of the LG extract in Eudragit-liposomes revealed a significant increase of CAA unit, providing further evidence of the positive effect of the vesicle encapsulation preserving the inner properties of the extract. Moreover, as shown in Fig. 1, human erythrocytes pre-treated with both doses of LG extract in Eudragit-liposomes showed a significant increase in CAA values in comparison with LG extract ($p < 0.05$). As supposed for the FRAP results (Section 3.3), the increased antioxidant activity observed for LG in Eudragit-liposomes may be explained by the antioxidant power possessed by Lecinova® constituents, which represents an added value to the liposomal formulation of LG extract. Overall, the findings of this study point to the crucial role that nanoencapsulation can play in developing new functional products that enhance the bioactivity and bioaccessibility of food matrices. Furthermore, the nanoencapsulation can increase the stability of bioactive compounds through digestion after oral administration, as well as through industrial processing, thus avoiding degradation and loss of biological efficacy.

4. Conclusions

There are many potential applications of liposomes in the food industry and, among these, the protection of ingredients with a proven action on human health plays a fundamental role.

Liposomal encapsulation is an excellent strategy to avoid a reduction of the antioxidant, anti-inflammatory, and antimicrobial properties of the bioactive components of food extracts, caused by degradation or digestion processes. It also allows the delivery through the oral route.

The *in vitro* and *ex vivo* results of our study showed that the encapsulation in liposomes made it possible to preserve the antioxidant properties of LG aqueous extract. Further *in vivo* studies are needed to demonstrate a significant role in enhancing the transport of bioactive ingredients across biological membranes and in prolonging or controlling their release.

CRediT authorship contribution statement

Morena Gabriele: Investigation, Formal analysis, Writing – original draft, Writing – review & editing. **Carla Caddeo:** Investigation, Writing – original draft, Writing – review & editing. **Valter Lubrano:** Resources. **Donatella Valenti:** Investigation. **Laura Pucci:** Conceptualization, Supervision, Funding acquisition, Writing – original draft, Writing – review & editing.

Declaration of competing interest

None.

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