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Valorisation of wild cardoon leaf by-product: Extraction, bioactive compounds, antioxidant activity and nanoformulation

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ABSTRACT

Wild cardoon (*Cynara cardunculus* subsp. *cardunculus* L.) is an endemic plant of the Mediterranean basin with nutritional and health properties. Herein, fresh wild cardoon leaf by-product was extracted with either a 20:80% *v/v* EtOH:H_2O or an 80:20% *v/v* EtOH:H_2O mixture (WCE1 and WCE2, respectively). The quali-quantitative profiles of the two extracts were analysed by LC-ESI-QTOF MS/MS and HPLC-PDA, revealing hydroxycinnamic acids as the predominant phenolic compounds, followed by flavonoids. WCE2, the extract richest in phenolic compounds was incorporated into nanosized enteric polymer-coated liposomes, which exhibited a high entrapment efficiency. The extract's nanoformulation showed antioxidant properties in *in vitro* cell-free and cell-based models as well as good stability during storage and in both simulated and *ex vivo* gastrointestinal fluids. Overall, the wild cardoon leaf extract incorporated into polymer-coated liposomes for oral delivery was demonstrated to be a valuable source of antioxidants, thus offering opportunities for their valorisation into functional foods.

1. Introduction

Vegetable and fruit by-products are the underutilised residues of the processing, distribution, and consumption of the edible and non-edible parts of the plants that can be reintegrated into further productive use (Halim et al., 2025). In fact, the term “by-products” denotes secondary outputs of a production process that retain functional or economic value, in contrast to “waste” that refers mainly to material that is discarded or intended for discarding. Recently, plants by-products have received significant interest concerning their conversion into new value-added products, which may contribute to a sustainable circular economy by reducing disposal volume and enhancing resource efficiency. In this context, wild cardoon (*Cynara cardunculus* L., syn. *Cynara cardunculus* subsp. *cardunculus* L. or *C. cardunculus* var. *sylvestris* (Lam.) Fiori) (WFO, 2025), a member of the Asteraceae family endemic to the Mediterranean basin, shows interesting potential for food and health applications.

Wild cardoon is highly valued for its ecological significance, traditional applications and botanical traits. It is a resilient perennial herb that manifests with a typical rosette of acerate leaves and branched blooming stalks (Ammar et al., 2014). Its pivotal role in the fields of botany and agriculture is evidenced by its identification as the wild progenitor of cultivated variants, including globe artichoke and cardoon (Casadevall et al., 2011; Silva et al., 2022). The wild cardoon's adaptability and evolutionary history have yielded a significant degree of physical diversity, with distinct gene pools developing primarily in the eastern Mediterranean and the Iberian Peninsula (Ammar et al., 2014;

Abbreviations: Cryo-TEM, cryogenic-Transmission Electron Microscopy; DPPH, 2,2-diphenyl-1-picrylhydrazyl; DMSO, Dimethyl Sulfoxide; EE, Entrapment Efficiency; Eu-liposomes, Eudragit®-coated liposomes; FE, Ferrous Equivalents; FRAP, Ferric Reducing Antioxidant Power; H_2-DCF-DA, 2′,7′-dichlorofluorescin diacetate; MTT, (3-(4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide); PBS, Phosphate Buffered Saline; ROS, Reactive Oxygen Species; SIF, Simulated Intestinal Fluid; SGF, Simulated Gastric Fluid; TBH, tert-butyl hydroperoxide; TE, Trolox Equivalents; USP, United States Pharmacopoeia; WCE1, extract 20:80% v/v EtOH:H_2O; WCE2, extract 80:20% *v*/v EtOH:H_2O.

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Gatto et al., 2013).

Throughout history, the wild cardoon has been used for a variety of traditional purposes due to its nutritional and therapeutic properties. Conceição et al. (2018) reported that the leaves and flower heads of the plant are used in many culinary contexts, particularly in Mediterranean cuisine. Moreover, the plant is a valuable source of herbal medicines and dietary supplements due to its high content of bioactive components, including polyphenols (especially di-caffeoylquinic acids and their derivatives), which possess anti-inflammatory and antioxidant properties (Pandino, Courts, Lombardo, Mauromicale and Williamson, 2010). Furthermore, polyphenols exhibit a wide range of physiological properties, including anti-allergic, anti-atherogenic, antimicrobial, antithrombotic, cardioprotective and vasodilator properties (Durazzo et al., 2013). Additionally, the potential health benefits of wild cardoon are underscored by the presence of cynaropicrin (Ramos et al., 2013), a sesquiterpene lactone of the guaianolide type that shows several biological activities, such as anti-inflammatory and anti-tumoral activity, as well as the capacity to protect against photoaging and to combat the hepatitis C virus (Elsebai, Mocan and Atanasov, 2016).

The significant volumes of by-products generated by packing houses and food-processing facilities constitute a substantial environmental concern. It has been demonstrated that up to 60% of the harvested vegetables can be utilised by the industry, as evidenced by the case of the industrial manipulation process of artichokes and cardoons (Amoriello et al., 2022). The recent focus on wild cardoon has been prompted by the potential of its by-products to contribute to circular economy principles and sustainable farming practices. The investigation into the potential non-food uses of wild cardoon by-products, including its leaves and stems, focuses on the production of biofuels and valuable phytochemicals, which are of interest to the cosmetics and pharmaceutical industries (Dufour et al., 2013; Rana et al., 2022). In addition, wild cardoon by-products can be utilised as ingredients in nutraceuticals and functional foods due to their high inulin content (Rana et al., 2022). Recent research has indicated that optimising the utilisation of wild cardoon can enhance environmental sustainability and resource efficiency (Graikou et al., 2021; Rana et al., 2022).

Among the most valuable phytochemicals are phenolic compounds, a group of secondary metabolites found in plants, that are of great interest due to their antioxidant activities and potential health benefits. These compounds, which include hydroxycinnamic acids and flavonoids, have shown remarkable bioactivities, including antimicrobial, anti-inflammatory, and anti-cancer properties. However, their low bioavailability often limits their therapeutic value. This issue is caused by several factors, such as fast clearance from the body after consumption, extensive phase I/II and microbial metabolism into conjugated forms, and poor absorption in the gastrointestinal tract (Borges et al., 2020; Muller et al., 2019). Novel strategies have been applied to increase the stability and delivery of phenolic compounds, such as encapsulation in polymeric nanoparticles, which have shown enhanced antioxidant properties by preserving the bioactivity of the compounds (Muller et al., 2019). Furthermore, interactions between phenolic compounds and matrix components in food or supplements may affect their stability, which in turn can influence their bio-accessibility and, ultimately, their bioavailability *in vivo* (Cosme et al., 2020; Ribas et al., 2019). To improve the medicinal and preventive potential of phenolic compounds through increased stability and bioavailability, developments in food technology and formulation techniques are essential (Chen et al., 2017). Considering the poor water solubility of most natural extracts, which results in reduced bioavailability, several studies developed and assessed the delivery of natural extracts in liposomes. Liposomes are expected to increase the solubility and bioavailability of the bioactive compounds of the extracts, and consequently their biological activities. This is due to the fact that liposomes are an aqueous-based formulation that can be safely administered orally (Shishir et al., 2019). The success of liposomes lies in their high biocompatibility and biodegradability as well as in their versatile, tuneable structure/composition, which allows for the loading of compounds with diverse chemical properties and for various strategies for targeted or controlled release (Alizadeh et al., 2025). In the case of oral administration, enteric polymer-coated liposomes were proved to be effective in delivering phytoproducts, offering protection and modulating release in the gastrointestinal environment (De Leo et al., 2023; Pani et al., 2024).

In this context, the aim of this study was to verify the effect of two different hydro-alcoholic mixtures for extracting bioactive compounds from wild cardoon leaves and to develop liposomes for the oral delivery of the obtained extracts. To this purpose, two EtOH:H_2O mixtures (20:80% and 80:20% *v/v*) were used, and the obtained extracts, WCE1 and WCE2 respectively, were characterised by (high-resolution) liquid chromatography electrospray ionisation quadrupole time-of-flight mass spectrometry (HR) LC-ESI-QTOF MS/MS and high-performance liquid chromatography with photodiode array detector HPLC-PDA. WCE2, the 80:20% *v/v* EtOH:H_2O extract, which showed the highest content of bioactive compounds, was formulated in oral Eudragit-coated liposomes. These Eudragit-coated liposomes were characterised for size, surface charge, entrapment efficiency, stability during storage and in fluids that simulate the stress conditions of the gastrointestinal tract. Moreover, the antioxidant and antiradical activities of the extract alone or formulated in Eudragit-coated liposomes were investigated in *in vitro* experimental systems. These included the Ferric Reducing Antioxidant Power (FRAP) assay, 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay, and the cell-based 2′,7′-dichlorofluorescin diacetate (H_2-DCF-DA) test carried out in Caco-2 intestinal cell monolayers.

2. Materials and methods

2.1. Chemicals and reagents

Phospholipon 90G (≥ 94% soy phosphatidylcholine) was supplied by Lipoid GmbH (Ludwigshafen, Germany). Stearylamine, phosphate-buffered saline (pH 7.4) and other reagents were obtained from Merck (Milan, Italy), unless otherwise reported. Eudragit® L100 (poly(methyl methacrylate-*co*-methacrylic acid; soluble above pH 6.0) was supplied by Evonik Industries AG (Essen, Germany). All the chemicals used were of analytical grade. LC-MS grade acetonitrile, methanol, and 85% *w/w* phosphoric acid were purchased from Merck KGaA (Darmstadt, Germany). Standards of bioactive compounds were purchased from Extrasynthese (Genay Cedex, France) and TransMIT (Giessen, Germany). Ultrapure water (18 MΩ·cm) was obtained with a Milli-Q Advantage A10 System (Millipore, Milan, Italy). All cell culture materials were purchased from Euroclone (Milan, Italy).

2.2. Sample and extracts preparation

Wild cardoon leaf by-products (40–80 cm length) corresponding to the non-valuable edible parts of the plant were collected in Serrenti (Sardinia) in May 2024. The specimens were identified by Prof. Cinzia Sanna (University of Cagliari, Italy), and a voucher sample (number DISVA.ALI.03.2024) was deposited at the Department of Life and Environmental Sciences of the University of Cagliari (Italy). The fresh samples were carefully cleaned, the harder parts were removed and discarded, and the remaining parts were extracted as reported by Cacciola et al. (2025) using two EtOH:H_2O mixtures (20:80% *v/v* EtOH:H_2O to produce WCE1, and 80:20% *v/v* EtOH:H_2O to produce WCE2) in a 2:5 *w/v* plant:solvent ratio using initially an immersion blender followed by an Ultra Turrax® T18 digital (IKA-Werke GmbH & Co. KG, Staufen, Germany). The obtained extracts were filtered, centrifuged and the supernatants were concentrated under vacuum (Rotavapor® R-210 BUCHI, Cornaredo, Italy) to eliminate the EtOH. The extracts thus obtained were centrifuged to separate the less polar compounds (*e.g.* chlorophylls, waxes, *etc.*) that possibly separated due to the increase of water amount, and the supernatants were then collected, frozen and freeze-dried using a Lio5P freeze-dryer (5Pascal, Trezzano sul Naviglio,

Italy), and stored at −20 °C until use (within 2 months).

2.3. Instrumental conditions

For the quali-qualitative assessment of bioactive compounds in WCE1 and WCE2 extracts, the LC-MS and HPLC-PDA methods outlined by De Luca et al. (2023), Masala et al. (2024) and Cacciola et al. (2025) were used with some modifications. The investigation into the qualitative analysis the extracts was conducted utilising a QToF LC-MS system, including a 1290 Infinity II UPLC equipped with an autosampler (G7167B), a quat pump (G7120A), a column comp (G7116B) and a 6560 IM-QTOF (Agilent Technologies Inc., Palo Alto, CA, USA). The instrument performance was assessed prior to analysis using the G1969–85000 Agilent tuning solution mix and during the analysis, two reference masses at *m/z* 112.9855 and *m/z* 966.0007 were continuously infused into the system to ensure constant mass correction. The electrospray ionisation (ESI) source in negative and positive ion mode was conducted in two separated runs, with the source parameters optimised as following: drying gas 300 °C, flow 5 L/min; sheath gas 250 °C, flow 12 L/min; nebuliser 45 psi; capillary voltage 3500 V, nozzle voltage 500 V. The automatic acquisition MS/MS experiments were executed by employing a formula to calculate the collision energy by linear interpolation, as outlined in the following equation: collision energy = [slope (5) × *m/z* of precursor mass)/100 + Offset (2)]. The MS spectra were acquired by full range acquisition in a 40–1300 *m/z* range.

The chromatographic separation was carried out on a Kinetex EVO C18 column (150 × 2.1 mm, 1.7 µm, 100 Å; Phenomenex, Castel Maggiore, Bologna, Italy) maintained at 55 ± 1 °C. The mobile phase was constituted of a mixture of solvent A (0.1% formic acid) and solvent B (acetonitrile +0.1% formic acid), with a 0.3 mL/min flow rate at the following gradient elution: 0–20 min (99–80% A), 20–35 min (80–70% A), 35–40 min (70–1% A), 40–45 min (1–1% A), 45–46 min (1–99% A) and 46–50 min (99–99% A). The injection volume was 4 µL. The ESI/QToF MS data was then assessed using the MassHunter Workstation Qualitative Analysis program *v.* 10.0 (Agilent Technologies). In order to tentatively identify the metabolites and predict fragmentation and molecular formulae, the MassHunter METLIN metabolite PCDLdatabase B.08.00 (Agilent Technologies) was utilised, in conjunction with a comparison between the experimental MS/MS spectra and spectra from a publicly available mass spectral data repository (*e.g.* Sirius® software *v.* 5.5.5 (Dührkop et al., 2019; Hoffmann et al., 2022) and MZmine® *v.* 4.3 (Schmid et al., 2023)), fragmentation patterns that have been published in the literature and other natural products databases (KNApSAcK®, PubChem®, Coconut®). For the quantification by HPLC-PDA of bioactive compounds in WCE1 and WCE2 extracts and in the nanoformulations, the analysis was performed using a 1260 Infinity II HPLC system equipped with a pump (G7111A), an autosampler (G7129A), a thermostated column compartment (G7116A; 30 ± 1 °C), and a photodiode array detector (G4212B) (Agilent Technologies). The chromatographic separation was conducted on a Kinetex EVO C18 column (150 × 4.60 mm, 2.6 µm, Phenomenex) using a mobile phase of 0.22 M phosphoric acid (solvent A) and acetonitrile (solvent B) with a 0.8 mL/min flow rate at the following gradient elution: 0–20 min (100–80% A), 20–35 min (80–70% A), 35–45 min (70–0% A), 45–50 min (0–100% A), and 50–55 min (0–100% A). The injection volume was 10 µL. The chromatograms and spectra were processed using an OpenLab CDS software *v.* 2.51 (Agilent Technologies), and phenolic compounds were detected and quantified based on absorption at characteristic wavelengths as flavonoid at 360 nm, caffeoyl derivatives at 313 nm, and cynaropicrin at 210 nm. Stock standard solutions and working standard solutions were prepared in methanol and in 0.22 M phosphoric acid, respectively. The calibration curve was plotted using the external standard method: the peak area was correlated with the concentration by means of the least-squares method, with a coefficient of determination (r^2) = 0.9998 in the range of 20.0–0.5 mg/L and the amount was expressed as mg/g of dry extract mass (dm). Reference standards used for the calibration curves were luteolin 7-*O*-rutinoside, luteolin 7-*O*-glucoside, luteolin, apigenin, neochlorogenic acid, chlorogenic acid, cynarin (1,5-dicaffeoylquinic acid), and cynaropicrin. Calibration curves of luteolin, chlorogenic acid and cynarin were used for the quantification of luteolin derivatives, monocaffeoylquinic derivatives, and dicaffeoylquinic derivatives, respectively (Table 1). For the quantitative analysis of the nanoformulations, they were first diluted (1:50 *v/v*) with methanol. Raw WCE2 demonstrated adequate solubility in methanol, while WCE1 was subjected to ultrasonication to enhance solubilisation. Then, all the samples were diluted (1:1 *v/v*) with 0.22 M H_3PO_4. The solutions were then filtered with 0.22 µm cellulose acetate syringe filters (Ø = 13 mm) before injection.

Table 1
Quantification of target compounds by LC-PDA method (mg/g dm).

Peak No.	RT min	COMPOUND	Identification*	WCE1 mg/g dm	WCE2 mg/g dm
				mean ± SD	mean ± SD
		Flavonoids			
1	21.2	luteolin 7-*O*-rutinoside	rt, UV–Vis, MS	1.03 ± 0.00^a	31.56 ± 0.38^b
2	21.3	luteolin 7-*O*-glucoside	rt, UV–Vis, MS	1.91 ± 0.04^a	nd
3	23.9	luteolin acetyl-glucoside [A]	UV–Vis, MS	1.48 ± 0.00^a	4.81 ± 0.02^b
4	29.0	luteolin	rt, UV–Vis, MS	8.35 ± 0.06^a	1.50 ± 0.00^b
5	33.1	apigenin	rt, UV–Vis, MS	1.02 ± 0.00^a	nd
		Total flavonoids		13.79 ± 0.11^a	37.87 ± 0.40^b
		Hydroxycinnamic acids			
6	8.2	monocaffeoyl-quinic acid isomer I [B]	UV–Vis, MS	0.60 ± 0.00^a	2.20 ± 0.01^b
7	8.7	neochlorogenic acid	rt, UV–Vis, MS	0.12 ± 0.00^a	0.75 ± 0.00^b
8	12.7	chlorogenic acid	rt, UV–Vis, MS	2.43 ± 0.01^a	31.33 ± 0.16^b
9	13.1	monocaffeoyl-quinic acid isomer II [B]	UV–Vis, MS	0.41 ± 0.00^a	0.86 ± 0.01^b
10	22.6	dicaffeoylquinic acid isomer I [C]	UV–Vis, MS	0.16 ± 0.00^a	2.77 ± 0.01^b
11	23.2	dicaffeoylquinic acid isomer II [C]	UV–Vis, MS	1.65 ± 0.01^a	20.50 ± 0.08^b
12	23.6	dicaffeoylquinic acid isomer III [C]	UV–Vis, MS	0.16 ± 0.00^a	1.46 ± 0.02^b
13	24.2	dicaffeoylquinic acid isomer IV [C]	UV–Vis, MS	nd	0.55 ± 0.00^a
14	24.8	dicaffeoyl-succinoyl quinic acid isomer I [C]	UV–Vis, MS	5.43 ± 0.06^a	38.64 ± 0.21^b
15	27.5	dicaffeoyl-succinoyl quinic acid isomer II [C]	UV–Vis, MS	1.10 ± 0.01^a	14.77 ± 0.17^b
16	28.6	dicaffeoyl-disuccinoyl quinic acid [C]	UV–Vis, MS	0.75 ± 0.02^a	5.49 ± 0.10^b
		Total hydroxycinnamic acids		10.60 ± 0.11^a	119.33 ± 0.77^b
		Others			
17	25.8	cynaropicrin	rt, UV–Vis, MS	43.65 ± 0.02^a	45.00 ± 0.02^b

[A] expressed as luteolin equivalents; [B] expressed as chlorogenic acid; [C] expressed as cynarin. * identification: rt.: comparison with retention time of pure standard; UV–Vis: comparison with typical UV–Vis spectra of pure compound or similar pure standards; MS: comparison with MS and MS/MS spectra of pure compound or literature data (see Table S2). nd: not determined. Data are given as means ± standard deviations (SD; $n = 3$). Mean values within a line with different letters are significantly different at $p < 0.05$. WCE1: 20:80% *v/v* EtOH:H_2O, WCE2: 80:20% *v/v* EtOH:H_2O.

2.4. Liposomes preparation

Firstly, uncoated liposomes were prepared by dispersing phosphatidylcholine (P90G), stearylamine and WCE2 in phosphate-buffered saline (**Table S1**) and sonicating this dispersion for 28 alternating cycles (18 cycles at five seconds on plus 10 cycles at three seconds on) using an ultrasonic disintegrator. Secondly, liposomes were coated with a pH-responsive polymer by adding the liposome dispersion dropwise to an equal volume of 0.1% *w/v* Eudragit® L100 in phosphate buffered-saline while stirring. Empty uncoated liposomes and empty Eudragit-coated liposomes were prepared following the above procedure, in the absence of WCE2.

2.4.1. Liposomes characterization

WCE2 Eudragit-coated liposomes were imaged *via* cryogenic-Transmission Electron Microscopy (cryo-TEM) in comparison with WCE2 uncoated liposomes, empty uncoated and Eudragit-coated liposomes. The vesicle dispersions (4 μL) were deposited onto glow-discharged EM grids, blotted, and vitrified by freezing into ethane using an automatic plunge freezer (Vitrobot MarkIV, FEI Company/ ThermoFisher Scientific, Waltham, MA, US). The vitrified samples were observed on a 200 kV electron microscope (Glacios2, FEI Company). Micrographs were acquired under low electron dose conditions on a Falcon 4i direct electron detector (ThermoFisher Scientific).

WCE2 Eu-liposomes were also characterised by measuring their mean diameter, polydispersity index, and zeta potential *via* light scattering (Zetasizer nano-ZS; Malvern Panalytical, Worcestershire, UK). WCE2 uncoated liposomes, empty uncoated liposomes and empty Eu-liposomes were characterised as well.

The entrapment efficiency of WCE2 uncoated liposomes and Eu-liposomes was determined. Firstly, the vesicle dispersions were dialysed against water to remove the non-incorporated extract's components. Secondly, non-dialysed and dialysed dispersions were diluted (1:50 *v/v*) with methanol and processed by HPLC-PDA (see section 2.3). The percentage entrapment efficiency was calculated using quantitative data of target compounds of WCE2, according to the following formula:

$$Entrapment\ efficiency = \left(\frac{amount\ of\ extract's\ compound\ in\ dialysed\ vesicles}{amount\ of\ extract's\ compound\ in\ non-dialised\ vesicles}\right) x\ 100$$

2.5. In vitro antioxidant activity by DPPH and FRAP assays

The antioxidant activity of WCE1 and WCE2 was studied by means of 2,2-diphenyl-1-picrylhydrazyl (DPPH) and Ferric Reducing Antioxidant Power (FRAP) colorimetric assays. For the DPPH assay, WCE1 or WCE2 in methanol and WCE2 in Eu-liposomes (1 mg/mL) were added (25 μL) to a 25 μM DPPH methanol solution and stored in the dark for 30 min. The decrease in absorbance was detected at 517 nm and the antioxidant activity was estimated by calculating the percentage of the absorbance of the samples relative to the absorbance of the DPPH solution. Trolox (reference antioxidant) equivalents (μg TE/mL solution) were calculated by using a calibration curve of Trolox (linearity range: 12.5–125 μg/mL). For the FRAP assay, WCE1 or WCE2 in DMSO and WCE2 in Eu-liposomes (1 mg/mL) were added (50 μL) to the FRAP solution and stored in the dark for 4 min. The increase in absorbance was detected at 593 nm and the ferrous equivalents (μg FE/mL solution) were calculated by using a standard calibration curve of 10 mM ferrous sulphate (linearity range: 13.9–556 μg/mL).

2.6. Cytotoxicity and ROS production in Caco-2 cells

The cytotoxicity of WCE2 and nanoformulations was tested on intestinal Caco-2 cells by a spectrophotometric MTT (3-(4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide) test as a measure of cell viability. The antioxidant activity of WCE2 and nanoformulations was assessed by fluorescent probe 2′,7′-dichlorofluorescin diacetate (H2-DCF-DA) assay. Caco-2 cells (ECACC Salisbury, Wiltshire, UK) were cultured as previously reported by Deiana et al. (2019). Briefly, cells (5×10^4 cells/well), at passage 51–60, were grown in 96-well plates for 14–21 days before treatments to allow spontaneous differentiation into enterocyte-like cells, a process known to occur upon prolonged culture (Ferruzza et al., 2012). In the MTT assay, WCE2 in DMSO and the nanoformulations diluted with the culture medium +2.5% serum were added at different concentrations (0.1–50 μg/mL) for 24 h. The concentration range of 0.1–50 μg/mL for the extract was chosen based on literature reports indicating that these levels are sufficient to elicit biological effects in Caco-2 cells without inducing cytotoxicity. For instance, studies on polyphenol-rich food extracts have shown modulation of antioxidant defences, enzyme activities, and gene expression within similar concentration ranges (Barrera-Chamorro et al., 2024; Cerulli et al., 2024; Elejalde et al., 2025). After incubation, the diluted samples were replaced with 100 μL of MTT reagent (5 mg/mL in medium +2.5% serum), and the cells were incubated for an additional 4 h. Then, MTT was removed, and the resulting formazan crystals were dissolved in 100 μL of DMSO. The absorbance was measured at 570 nm using an Infinite 200 microplate reader (Tecan, Salzburg, Austria). Viability was reported as % of control (untreated cells) for each sample.

Intracellular ROS production in Caco-2 cells and the antioxidant effects of WCE2 and nanoformulations were evaluated as previously reported (Gil et al., 2023). Briefly, cells were washed with PBS 1× solution and incubated for 30 min with H_2-DCF-DA (10 μM) dissolved in PBS 1×. H_2-DCF-DA was then removed, and cells were treated with the extract at different concentrations (0.5–50 μg/mL) together with the pro-oxidant tert-butyl hydroperoxide (TBH 2.5 mM), or with TBH alone. ROS levels were measured using the microplate reader at a controlled temperature of 37 °C. The measurement was performed using an excitation of 490 nm and an emission of 520 nm taking fluorescence values every five minutes for 2 h. ROS production was expressed as % of fluorescence emitted by untreated cells (Control).

2.7. Liposomes stability assessment

The storage stability (at 4 °C) of WCE2 Eudragit-coated liposomes was assessed *via* periodic measurements of their mean diameter, polydispersity index, and zeta potential (see section 2.4.1). The stability of WCE2 Eudragit-coated liposomes was also assessed *in vitro* in simulated gastrointestinal fluids containing pepsin or pancreatin and in *ex vivo* gastrointestinal fluids from rats. Firstly, WCE2 extract Eu-liposomes were diluted (1:50 *v:v*) with United States Pharmacopoeia (USP) simulated gastric fluid (SGF) with pepsin from porcine stomach mucosa (pH 1.2) or with USP simulated intestinal fluid (SIF) with pancreatin from porcine pancreas (pH 6.8) and incubated at 37 °C for 2 h or 6 h, respectively. Secondly, WCE2 Eu-liposomes were diluted (1:50 *v:v*) with gastric or intestinal fluid from rats and incubated at 37 °C for 2 h or 6 h, respectively. For the collection of the *ex vivo* gastrointestinal fluids, eight Sprague–Dawley rats (Envigo-Inotiv, S. Pietro al Natisone, Udine, Italy) weighing 300–325 g were used. Rats were housed 4 per cage under a 12-

h light/dark cycle (lights on at 08:00 h), with food and water available *ad libitum*. Procedures were performed according to the European guidelines for animal experimentation (Directive, 2025Directive 2010/63/EU 22/09/2010) and to the guidelines approved by the Animal Welfare Body of the University of Cagliari. Appropriate procedures were followed to minimise animal discomfort and the number of subjects used. Specifically, to reduce the use of experimental animals in compliance with the 3R principles, the rats used in the present study were taken from a larger cohort of rats that was part of another investigation involving the repeated parenteral administration of amphetamine (1 mg/kg, intraperitoneal). The rats received the last amphetamine administration 4 h before being euthanised with an overdose of sodium pentothal (100 mg/kg, intraperitoneal). After injection, the rats were monitored for the onset of respiratory depression, and as soon as no signs of respiratory activity were observed, a vertical incision was made in their abdomen, and the stomach and small intestine were identified. Then, the oesophagus (approx. 1 cm upstream of the stomach) and the small intestine (approx. 30 cm downstream of the stomach) were ligated with silk suture thread to prevent leakage of gastrointestinal fluids. Afterwards, the stomach and a portion of the small intestine (approx. 30 cm) were gently detached from the connective tissue, harvested from the abdomen and immediately frozen at −20 °C, pending further experiments. The stomach of each rat was flushed with 10 mL of SGF (without pepsin, pH 1.2) and the content was collected from the pylorus. The intestine (approx. 30 cm) of each rat was flushed with 10 mL of SIF (without pancreatin, pH 6.8) through the upper portion of the small intestine and the content was collected at the opposite side. Both gastric and intestinal fluids were centrifuged twice at 4200 rpm for 20 min, and the supernatants were centrifuged at 15,000 rpm for 10 min, at 5 °C. The supernatants were pooled, stored at 4 °C and used within 48 h.

Both the WCE2 Eudragit-coated liposomes diluted with the *in vitro* simulated gastrointestinal fluids containing pepsin or pancreatin and the WCE2 Eudragit-coated liposomes diluted with the *ex vivo* gastrointestinal fluids from rats were analysed by measuring their mean diameter, polydispersity index and zeta potential (see section 2.4.1) after incubation for 2 h or 6 h. In addition, WCE2 uncoated liposomes were analysed to determine the effects of the fluids in the absence of the Eudragit coating.

2.8. Statistical analysis

All data are expressed as mean ± standard deviation (SD) of at least $n = 3$ independent experiments (the specific n for each experiment is reported in the legends). Statistical analysis was carried out using one-way analysis of variance (ANOVA) to evaluate differences among multiple treatment groups, followed by Tukey's Honest Significant Difference (HSD) *post hoc* test for pairwise comparisons. Prior to ANOVA, data distribution and homogeneity of variances were verified. Differences were considered statistically significant at $p < 0.05$. Statistical analyses were performed using GraphPad Prism *v.* 5.0 software.

3. Results and discussion

The two wild cardoon extracts obtained with different EtOH:H_2O mixtures, WCE1 and WCE2, were evaluated for their quali-quantitative composition in terms of bioactive compounds before the development of a nanoformulation for oral delivery. In this context, taking into account that green extraction of bioactive compounds from plant by-products requires solvents characterised by efficacy, safety, cost-effectiveness and environmental sustainability (Shahbaz et al., 2025), and that water and ethanol meet these requirements and are widely recognized GRAS (Generally Recognized As Safe) solvents for food and flavourings, they were the first choice for extracting bioactive compounds from wild cardoon leaves. Considering that the ratios of EtOH:H_2O mixtures can modulate the extraction of specific bioactive compounds (Lajoie et al., 2022), including botanical sources such as plants belonging to Asteraceae family and specifically to *Cynara* genus (Lee et al., 2024; Masala et al., 2024), two EtOH:H_2O mixtures, namely EtOH:H_2O 20:80% and 80:20% *v/v*, were selected for the present extraction leading respectively to WCE1 and WCE2.

3.1. Quali-quantitative analysis of target compounds by LC-MS/MS and HPLC-PDA methods

HR LC-ESI-QTOF MS/MS was utilised for the qualitative evaluation of wild cardoon leaf extracts, employing both positive and negative ion modes. Seventeen compounds were identified by comparing the *m/z* values with those reported in the literature, as well as by comparing the experimental MS/MS spectra with the fragmentation patterns of both pure standard compounds and those reported in the literature and public repository of mass spectral data (Hoffman et al., 2022; Sirius; Masala et al., 2024). Furthermore, the analysis of the compounds was completed by measuring their UV–Vis absorption in the range 200–600 nm and comparing the spectra with those of pure compounds or with the literature data.

Fig. S1 reports the MS chromatogram in negative and positive ion modes of WCE1 (20:80% *v/v* EtOH:H_2O extract) and WCE2 (80:20% *v/v* EtOH:H_2O extract), and peaks were identified as reported in **Table S2**. The compounds were identified as flavonoids (1–5), hydroxycinnamic acids (6–16) and a sesquiterpene lactone of the guaianolide type (17). The presence of hydroxycinnamic acids and flavonoids, characteristic secondary metabolites of *C. cardunculus spp.* was confirmed, thereby corroborating previous findings in the literature (Acquaviva et al., 2023; Cacciola et al., 2025). Table 1 reports the quantification of target compounds by the HPLC-PDA method (mg/g dm). Overall, WCE2 showed a higher amount of total flavonoids (37.87 ± 0.40 *vs.* 13.79 ± 0.11 mg/g dm) and total hydroxycinnamic acids (119.33 ± 0.77 *vs.* 10.60 ± 0.11 mg/g dm) than WCE1, with the exception of luteolin 7-*O*-glucoside, which was found only in WCE1, and luteolin aglycone, which was higher in WCE1 (8.35 ± 0.06 mg/g dm). Among flavonoids, the most abundant in WCE2 was luteolin 7-*O*-rutinoside (31.56 ± 0.38 mg/g dm), while luteolin aglycone was higher in WCE1. In the study conducted by Pandino et al. (2011), the presence of luteolin rutinoside and glucoside was identified in wild cardoon leaves. However, the glucoside was found to be present in a higher concentration compared to the rutinoside. It is interesting to note that neither caffeoylquinic acid nor succinylcaffeoylquinic acid was detected, and that the quantities were found to be negligible, which may be attributable to variations in genotype. Furthermore, apigenin was exclusively detected in WCE1 (1.02 ± 0.00 mg/g dm), and literature data indicate that this compound was previously identified in wild cardoon (Ucar Turker et al., 2024). Among hydroxycinnamic acids, the most abundant was dicaffeoyl-succinoyl quinic acid isomer I (38.64 ± 0.21 and 5.43 ± 0.06 mg/g dm for WCE2 and WCE1, respectively), followed by chlorogenic acid (31.33 ± 0.16 and 2.43 ± 0.01 mg/g dm for WCE2 and WCE1, respectively). Notably, cynaropicrin was the most abundant compound in both extracts (45.00 ± 0.00 and 43.65 ± 0.02 mg/g dm for WCE2 and WCE1, respectively) and it was detected in positive ion mode (Masala et al., 2024). The presence of mono- and di-succinoylquinic derivatives in *C. cardunculus spp.* leaves has been previously reported (Cacciola et al., 2025; Silva et al., 2022). To identify these compounds, it was essential to compare the *m/z* values with those reported in the literature, especially for more complex compounds such as dicaffeoyl-succinoylquinic acid (such as *m/z* 615 + 515, and *m/z*: 615 + 453) and dicaffeoyl-disuccinoylquinic acid (such as *m/z* 615 + 515, and *m/z* 615 + 453) (Clifford et al., 2007), as shown in **Table S2**.

Given the phytochemical profile of WCE2, which was far richer in flavonoids and hydroxycinnamic acids than WCE1 (see Table 1), WCE2 was used for further studies.

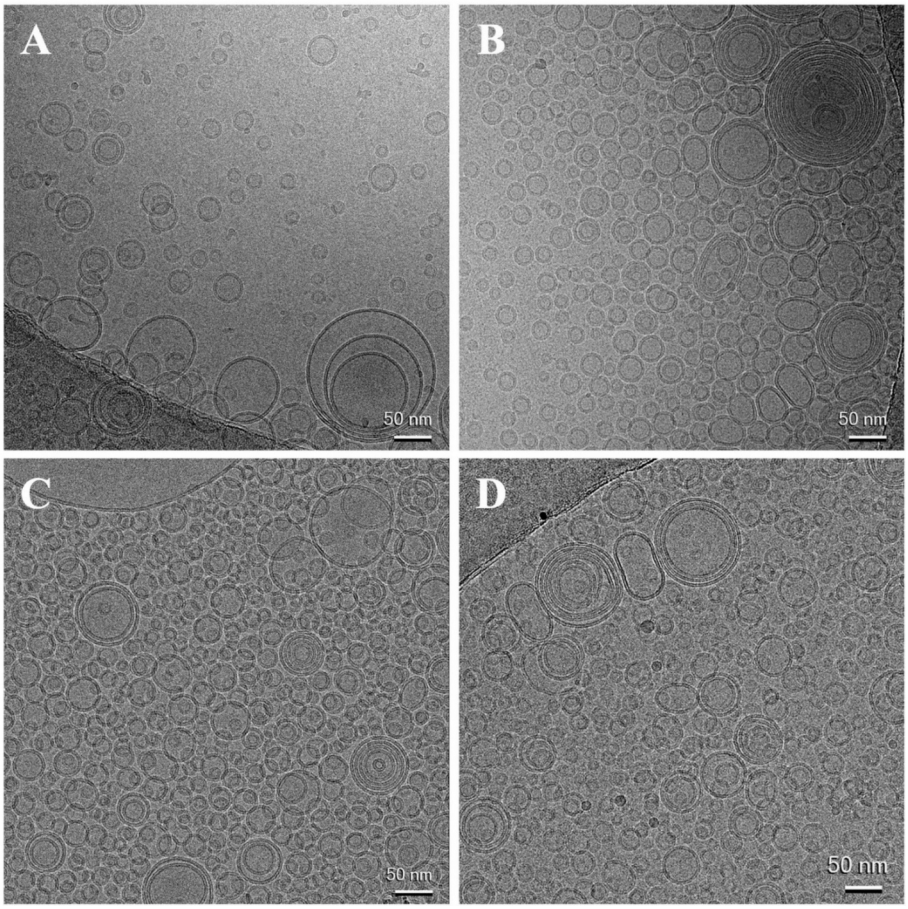

Fig. 1. Cryo-TEM micrographs of WCE2 uncoated liposomes (A) and WCE2 Eu-liposomes (B) *vs.* empty uncoated liposomes (C) and empty Eu-liposomes (D) at 92,000× magnification.

3.2. Liposomes characterization

Eudragit-coated liposomes were developed for the oral delivery of WCE2, the wild cardoon extract richest in flavonoids and hydroxycinnamic acids. The pH-responsive Eudragit polymer, which is soluble at a pH > 6, was used to protect the liposomes against degradation due to low pH encountered in the gastric tract. The liposomes, in turn, are expected to protect the extract against the stress conditions of the gastrointestinal tract (*e.g.* enzymes, bile salts).

The WCE2 Eu-liposomes were visualized using cryo-TEM compared with WCE2 uncoated liposomes, empty uncoated liposomes and empty Eudragit-coated liposomes to evaluate possible structural differences induced by the Eudragit coating and/or the extract. Representative micrographs are shown in Fig. 1**.** The four samples appeared very similar structurally. The micrographs displayed spherical vesicles, predominantly unilamellar, around 100 nm in size. A higher number of lamellae and a greater inhomogeneity were visible in the micrographs of Eudragit-coated liposomes (**Fig. 1B** and D), which can reasonably be ascribed to a rearrangement of the phospholipids during the coating process. The loading of the extract (**Fig. 1A** and **B** *vs.* **C** and **D**) had no apparent impact on the vesicles' morphology.

The WCE2 Eu-liposomes were further characterised for mean diameter, polydispersity and surface charge and compared with WCE2 uncoated liposomes to evaluate the changes induced by the Eudragit coating (Table 2). A comparison was also made with empty uncoated and Eudragit-coated liposomes to evaluate the changes induced by the extract (Table 2). The WCE2 uncoated liposomes showed basically the same physicochemical characteristics as empty uncoated liposomes: the mean diameter was 86 nm, the polydispersity index was below 0.30, and the zeta potential was above +30 mV (Table 2). Hence, the

Table 2
Mean diameter (MD), polydispersity index (PI) and zeta potential (ZP) of WCE2 Eu-liposomes *vs.* uncoated liposomes.

	MD nm ± SD	PI ± SD	ZP mV ± SD
WCE2 uncoated liposomes	86 ± 3.9	0.28 ± 0.01	+33 ± 3.2$^{\#}$
WCE2 Eu-liposomes	117 ± 5.0*	0.34 ± 0.03*	+2 ± 1.0*
Empty uncoated liposomes	86 ± 2.6	0.29 ± 0.01	+39 ± 4.6
Empty Eu-liposomes	117 ± 2.3°	0.31 ± 0.01°	+4 ± 2.2°

Values are the means ± standard deviations (SD; $n > 10$). * statistically different ($p < 0.001$) from WCE2 uncoated liposomes; $^{\circ}$ statistically different ($p < 0.001$) from empty uncoated liposomes. $^{\#}$ statistically different ($p < 0.001$) from empty uncoated liposomes.

Table 3
Entrapment efficiency (EE) of WCE2 uncoated and Eudragit-coated liposomes.

Compound	% EE ± SD	
	WCE2 uncoated liposomes	WCE2 Eu-liposomes
luteolin 7-*O*-rutinoside	80.3 ± 10.9^{a}	89.0 ± 3.7^{a}
luteolin acetyl-glucoside	81.9 ± 10.8^{a}	91.1 ± 5.7^{a}
dicaffeoylquinic acid isomer II	84.7 ± 8.2^{a}	86.0 ± 8.3^{a}
dicaffeoyl-succinoyl quinic acid isomer I	83.1 ± 10.8^{a}	96.3 ± 4.5^{a}
cynaropicrin	63.5 ± 11.4^{a}	70.4 ± 5.2^{a}

Data are given as means ± standard deviations ($n = 4$). Mean values within a line with same letter are not significantly different ($p \geq 0.05$).

incorporation of the extract had no remarkable effect on the liposomes, which aligns with the morphological similarities displayed by cryo-TEM (**Fig. 1A** and **C**). However, the coating with Eudragit led to the formation of larger and more inhomogeneous vesicles (117 nm, with a polydispersity index >0.30; Table 2), which were also characterised by a near neutral charge (approx. +2–4 mV; Table 2) because of the distribution of the Eudragit's anionic chains on the vesicles' surface.

The entrapment efficiency (EE, Table 3) of uncoated liposomes and Eu-liposomes was calculated based on the amounts of five bioactive compounds characteristic of WCE2. Given their abundance in the extract, two flavonoids, two hydroxycinnamic acids and cynaropicrin could be accurately and reliably quantified by LC-PDA, which is a robust and effective method for confirming the compounds' identity and assessing their peak purity, reducing the risk of misquantification due to coeluting substances (a common issue in complex extracts) or low-level concentrations. Both formulations showed good EE values, which were > 80% for phenolic compounds and > 63% for cynaropicrin. No significant statistical difference ($p \geq 0.05$) was observed in the entrapment efficiency between the two nanoformulations.

3.3. Antioxidant activity: DPPH and FRAP assays

Given the phytochemical composition of WCE2, which was rich in flavonoids and hydroxycinnamic acids (see Table 1), a strong antioxidant activity was anticipated. The presence of these compounds is indeed correlated with an extract's ability to reduce oxidative stress in experimental assays and biological systems. Hence, DPPH and FRAP assays were performed and the antioxidant activity of WCE2 was determined based on its radical scavenging and ferric reducing abilities. A DMSO solution of the extract scavenged the DPPH radical completely (94%; Table 4). As anticipated by the LC-PDA quantitative analysis showing a greater content of bioactive compounds in WCE2 than WCE1, the antioxidant activity of WCE2 was much stronger than that of WCE1, which was 23% (Table 4).

The nanoformulated WCE2 exhibited an antioxidant activity as high as that of WCE2 in solution (91%, Table 4; $p \geq 0.05$), corresponding to approx. 100 mg of Trolox equivalents/mL (Table 4). Similarly, the ferric reducing ability was greater for WCE2 than WCE1 and was maintained in the Eu-liposomes (> 450 μg of ferrous equivalents/mL; Table 4). The results of the two assays confirm the antioxidant potential of WCE2, which was not affected by the nanoformulation process.

3.4. Cytotoxicity and antioxidant activity in cells

Regarding the behaviour of WCE2 in Caco-2 cells, we preliminarily tested different concentrations to determine whether it was toxic in the selected experimental model. The extract was tested alone or formulated in Eu-liposomes, and empty Eu-liposomes served as a control. As shown in Fig. 2, the extract exhibited no cytotoxicity at any of the concentrations tested (0.1–50 μg/mL). Furthermore, it is important to note that cells treated with the higher concentrations of the nanoformulated extract or with empty Eu-liposomes showed a statistically significant increase in viability with respect to untreated cells (Fig. 2B and C). It is accepted that the composition (*e.g.*, type and amount of lipids and/or additives) and charge of the liposomes (*e.g.*, cationic, anionic, or neutral) significantly influence their interaction with cells and their overall effect on cell viability. Furthermore, different cell lines have varying sensitivities and uptake mechanisms, which can affect the response to liposomal treatment. It has also been suggested that liposomes can interfere with MTT reduction to formazan by metabolically active cells, which is the basis of the MTT cell viability assay, by increasing or decreasing formazan storage in cells, corresponding to increased or decreased viability values (Angius and Floris, 2015). Therefore, the observed viability values should be interpreted exclusively as indicative of the absence of cytotoxicity.

The antioxidant activity of the wild cardoon extract at concentrations of 0.5–50 μg/mL was then studied. As shown in Fig. 3, TBH

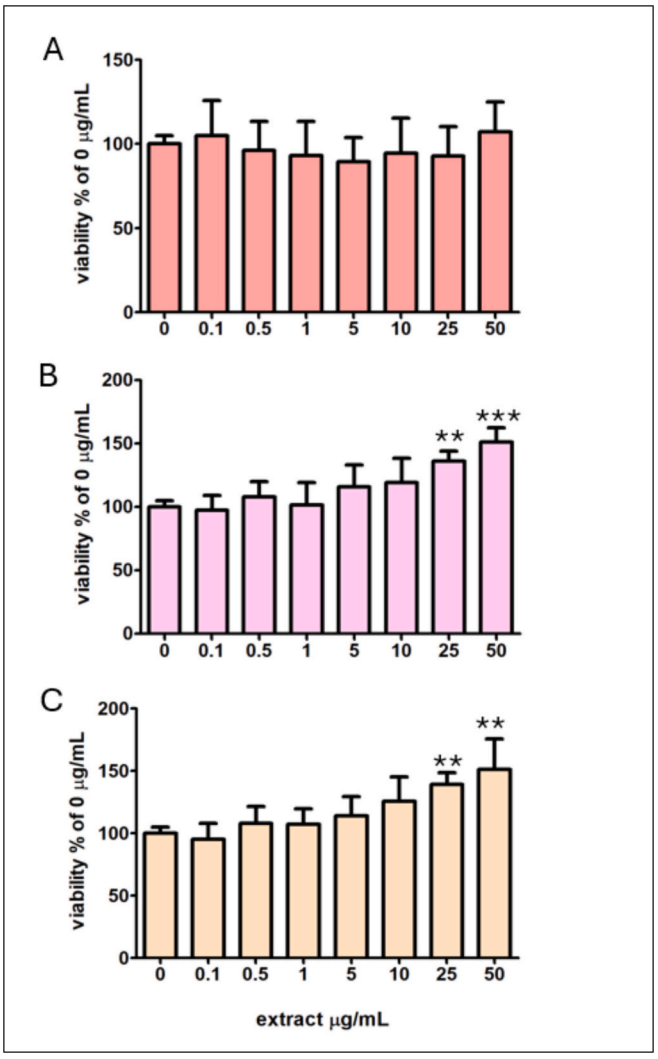

Fig. 2. Percentage of cell viability compared to untreated cells (0 μg/mL, 100%) of differentiated Caco-2 cells incubated for 24 h with different concentrations of WCE2 in DMSO (A), WCE2 Eu-liposomes (B) and empty Eu-liposomes (C) (0.1–50 μg/mL). Each bar represents the mean ± SD of independent experiments ($n = 16$). ** = $p < 0.01$ *vs.* 0 μg/mL; *** = $p < 0.001$ *vs.* 0 μg/mL. The increase in cell viability above 100% observed at high concentrations might be attributed to interference of the lipid-based formulations with the MTT assay rather than to a true proliferative effect.

Table 4
Antioxidant activity of WCE1 and WCE2 (1 mg/mL) in DMSO or in WCE2 Eu-liposomes.

	DPPH assay		FRAP assay
	AA % ± SD	TE μg Trolox equivalents/mL ± SD	FE μg Fe^{2+} equivalents/mL ± SD
WCE1 solution	23 ± 3.4	19 ± 2	101 ± 6
WCE2 solution	94 ± 9.5	109 ± 12	498 ± 29
WCE2 Eu-liposomes	91 ± 5.7	105 ± 8	455 ± 33*

Results are expressed as AA (%) and TE (μg Trolox equivalents/mL) for the DPPH assay, and as FE (μg ferrous equivalents/mL) for the FRAP assay. Values are the means ± standard deviations (SD) of three experiments, each carried out in triplicate. * $p < 0.01$: statistically different from extract solution.

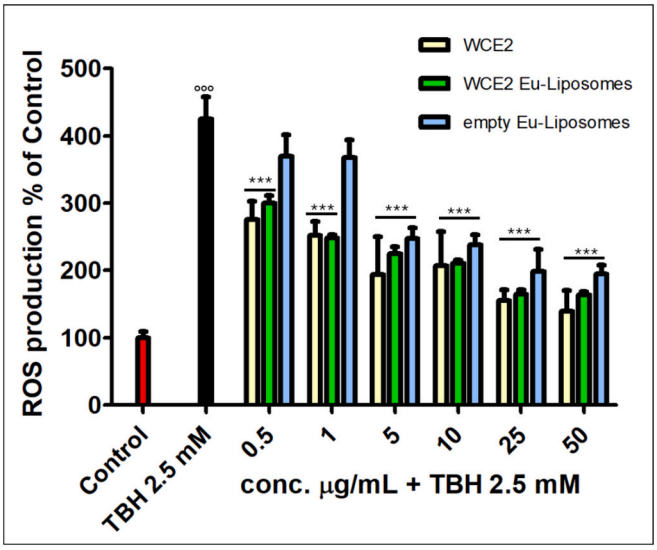

Fig. 3. ROS levels, visualized as H_2-DCF-DA fluorescence and expressed as % of the control (non-oxidized non-pretreated, 100% of ROS production) cells, in differentiated Caco-2 after 30 min incubation with TBH 2.5 mM and WCE2, WCE2 Eu-liposomes and empty Eu-Liposomes (0.5–50 µg/mL). Each column represents the mean ± SD of independent experiments ($n = 16$). °°° = $p < 0.001$ *vs.* control; *** = $p < 0.001$ *vs.* TBH 2.5 mM. The figure summarizes the dose-dependent effects of the tested formulations under the experimental conditions described in the Materials and methods section.

significantly induced ROS production in cells (+320%, $p < 0.001$ *vs.* control). In cells stressed with TBH and treated with either WCE2 in DMSO or nanoformulated WCE2 (0.5–50 µg/mL), ROS production decreased significantly in a dose-dependent manner and was comparable between the two treatments. Empty Eu-liposomes also limited TBH-induced ROS production, albeit to a lesser extent. This is most likely due to the ability of liposomes to get oxidized in place of the biological membranes of Caco-2 cells, as already observed in previous studies (De Luca et al., 2022; Pinilla et al., 2020). Overall, these results confirm previous findings, since extracts from wild cardoon leaves and stems, especially those obtained with water or ethanol, have shown high total antioxidant capacity and strong free radical scavenging activity. This was closely linked to their high total phenolic content, making them promising natural sources of antioxidants (Chihoub et al., 2019; Nabih et al., 2023; Ucar Turker et al., 2024).

3.5. Storage stability

The WCE2 Eu-liposomes displayed good medium-term storage stability based on the values of the mean diameter, polydispersity index and zeta potential. After four months, the WCE2 Eu-liposomes showed an increase in size (from 117 ± 2.3 to 127 ± 4.5 nm; $p < 0.05$) and in the polydispersity index (from 0.34 ± 0.03 to 0.42 ± 0.01; $p < 0.001$), while the zeta potential remained unchanged (from +2 ± 1.0 to +1 ± 0.2 mV; $p \geq 0.05$).

3.5.1. Stability under simulated gastrointestinal stress conditions

Because the WCE2 Eu-liposomes were developed for oral administration, this study examined their stability, in terms of average diameter, polydispersity and surface charge, in fluids that simulate the stress conditions of the gastrointestinal tract. The stability of WCE2 uncoated liposomes was also studied to discriminate between the role of the phospholipid vesicle and the Eudragit coating. The stability of the nanoformulations was first assessed *in vitro* using USP simulated gastrointestinal fluids routinely used for dissolution and disintegration testing of oral formulations under conditions of the stomach/intestine in a fasted state. After 2 h in simulated gastric fluid, the WCE2 uncoated liposomes displayed an increase in size and polydispersity index (99 nm and 0.39, respectively; Table 5), in comparison with the freshly prepared vesicles (86 nm, with a polydispersity index of 0.28; Table 2). After 6 h in simulated intestinal fluid, the WCE2 uncoated liposomes showed a further increase in size (108 nm, with a polydispersity index of 0.31; Table 5). These data suggest aggregation of the vesicles, likely due to the decrease in zeta potential from +33 mV to +10 mV in simulated gastric fluid and to −8 mV in simulated intestinal fluid induced by the ions in the fluids: H^+, Na^+, Cl^- in the former and Na^+, K^+, Cl^-, OH^-, HPO_4^{2-} in the latter.

However, WCE2 Eu-liposomes incubated with simulated gastric fluid were smaller than the freshly prepared vesicles, being at around 101 nm in size *vs.* 117 nm and more polydispersed (polydispersity index of 0.41 *vs.* 0.34; Table 5 *vs.* Table 2). This shrinking effect could be due to the initially weak surface charge of WCE2 Eu-liposomes (+2 mV), which suggests incomplete coating of their surface by the anionic Eudragit. By diluting and incubating the WCE2 Eu-liposomes with the simulated gastric fluid, which contains mainly protons/cations, the zeta potential increases to +11 mV (Table 5) and adjacent vesicles are more distant, resulting in a smaller size. A similar decrease in size was detected when the WCE2 Eu-liposomes were incubated with the simulated intestinal fluid. The smaller size (104 nm *vs.* 117 nm of the freshly prepared vesicles; Table 5 *vs.* Table 2) and the high homogeneity (polydispersity index of 0.27 *vs.* 0.34 of the freshly prepared vesicles; Table 5 *vs.* Table 2) can be explained by two phenomena: one is the complete inversion of the zeta potential (from +2 to −13 mV) due to the anions in the simulated intestinal fluid, which favours the repulsion between vesicles; the other is the dissolution of the Eudragit at a pH of 6.8 that leads to a loss of the protective coating. Apart from the alterations correlated with pH and ionic strength, no signs of significant degradation were detected after incubation with both fluids. Thus, it can be assumed that the effect of the enzymes was negligible under the described experimental conditions.

3.5.2. Stability in gastrointestinal fluids from rats

To further elucidate the protective properties of Eudragit-coated liposomes against the stress conditions of the gastrointestinal tract, gastric and intestinal fluids collected from rats were used in the stability studies. Gastrointestinal fluids from rats and humans show significant similarities, such as enzyme activity, microbiota activity, and digestion of lipids. The *ex vivo* approach was preferred over static models of digestion (*e.g.*, INFOGEST protocol, Brodkorb, Egger, Alminger, et al., 2019) since the major endpoint of the present study was to evaluate key

Table 5
Mean diameter (MD), polydispersity index (PI) and zeta potential (ZP) of WCE2 Eu-liposomes *vs.* uncoated liposomes in USP simulated gastrointestinal fluids and in gastric and intestinal fluids from rats.

	USP simulated fluid	MD nm ± SD	PI ± SD	ZP mV ± SD	*Ex vivo* fluid	MD nm ± SD	PI ± SD	ZP mV ± SD
WCE2 uncoated liposomes t_2	SGF	99 ± 6.6	0.39 ± 0.06	+10 ± 0.9	Gastric	101 ± 3.0	0.38 ± 0.01	+3 ± 0.6
WCE2 Eu-liposomes t_2		101 ± 6.8	0.41 ± 0.03	+11 ± 1.0		117 ± 8.9	0.41 ± 0.04	+2 ± 0.3
WCE2 uncoated liposomes t_6	SIF	108 ± 1.6	0.31 ± 0.04	−8 ± 0.8	Intestinal	261 ± 10.5	0.43 ± 0.03	−10 ± 0.6
WCE2 Eu-liposomes t_6		104 ± 3.8	0.27 ± 0.01	−13 ± 2.4		339 ± 6.0	0.29 ± 0.02	−12 ± 0.5

t_2: incubation for 2 h; t_6: incubation for 6 h. SGF: USP simulated gastric fluid with pepsin. SIF: USP simulated intestinal fluid with pancreatin. Values are the means ± standard deviations (SD; $n = 4$).

characterizing parameters of the nanoformulations upon incubation with gastrointestinal fluids, rather than their sequential oral, gastric and intestinal digestion. Moreover, the *ex vivo* procedures were done in compliance with the 3R principles. With such an approach, we could maximize the experimental results obtained from individual animals, since they were taken from a cohort of rats belonging to another investigation.

Similarly to what was observed *in vitro* in simulated gastric fluid, the WCE2 uncoated liposomes diluted and incubated with the *ex vivo* gastric fluid showed increased mean diameter and polydispersity index (101 nm and 0.38, respectively; Table 5) in comparison with the freshly prepared vesicles (86 nm and 0.28; Table 5). This result again indicates a moderate level of vesicle aggregation likely due to the decrease in zeta potential from +33 mV to +3 mV. The WCE2 Eu-liposomes, however, remained unaltered, with a size of around 117 nm, a polydispersity index of 0.41, and a zeta potential of +2 mV (Table 5). These results demonstrate an effective protection provided by the polymer coating against both acidic conditions (pH 2.9 ± 0.8) and digestive enzymes, including proteases and lipases, from the rat stomach.

The most pronounced changes were observed in the *ex vivo* intestinal fluid. The mean diameter of both the WCE2 uncoated liposomes and the WCE2 Eudragit-coated liposomes tripled (261 nm and 339 nm, respectively; Table 5), and the same inverted zeta potential value was detected (approx. −10 mV; Table 5). The key difference was the polydispersity index, which increased further for the uncoated liposomes (0.43; Table 5) but it decreased markedly for the Eu-liposomes (0.29; Table 5), indicating aggregation and clustering of inhomogeneous vesicles for the former and homogeneous vesicles for the latter. In the WCE2 Eudragit-coated liposomes, solubilisation of the polymer due to the *ex vivo* intestinal fluid pH (6.4 ± 0.2) is expected to occur, followed by an alteration of membrane integrity caused by the intestinal enzymes (*e.g.* lipases, phosphatases) and bile salts, leading to a localised release of the vesicles' cargo.

4. Conclusions

The findings of this study demonstrated that the use of green cost-effective extraction with an ethanol-water mixture is optimal for obtaining an extract from wild cardoon leaf by-products rich in phenolic compounds and cynaropicrin. The use of Eudragit-coated liposomes offers the possibility to valorise the wild cardoon extract for nutraceutical or pharmaceutical purposes, as the investigated nanoformulation ensures stability during storage and under stress conditions of the gastrointestinal tract. Additionally, this study provides compelling evidence that the extract from *C. cardunculus* subsp. *cardunculus* L. can be a promising nutrient for preventing oxidative stress-induced damage, especially when incorporated into nanosized enteric polymer-coated liposomes. This oral delivery system represents a valuable approach for developing functional foods or nutritional supplements based on wild cardoon extracts.

CRediT authorship contribution statement

Valentina Masala: Writing – review & editing, Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Data curation. **Simona Demuro:** Software, Investigation, Data curation. **Gabriele Serreli:** Writing – review & editing, Visualization, Methodology, Investigation, Formal analysis, Data curation. **Marina Kranjac:** Writing – review & editing, Visualization, Supervision, Investigation. **Nicola Simola:** Writing – review & editing, Investigation. **Monica Deiana:** Writing – review & editing, Visualization, Methodology, Investigation. **Carlo I.G. Tuberoso:** Writing – review & editing, Writing – original draft, Visualization, Validation, Resources, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Carla Caddeo:** Writing – review & editing, Writing – original draft, Validation, Supervision, Resources, Methodology, Investigation, Data curation.

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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2026.148585.

Data availability

Data will be made available on request.

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