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Chemical characterization of liposomes containing nutraceutical compounds: Tyrosol, hydroxytyrosol and oleuropein

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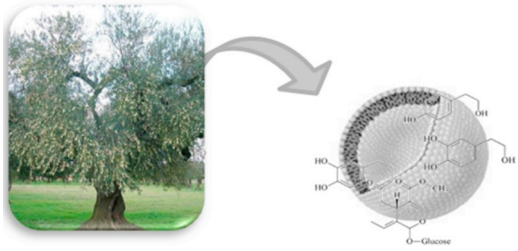
HIGHLIGHTS

- DOPC/DOPE liposomes loaded with antioxidants from *Olea europaea* L were synthesized.
- Plain and loaded liposomes were chemically characterized.
- Loaded liposomes encapsulation efficiency percentages were determined.
- Plain and loaded liposomes were assayed on human chondrocyte cells.

GRAPHICAL ABSTRACT



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ABSTRACT

Tyrosol, hydroxytyrosol and oleuropein are among the major phenolic compounds in fruits, leaves and oils from *Olea europaea* L. These natural antioxidants molecules revealed several beneficial effects on human health, but a low bioavailability and accessibility to targeted site. Liposomes are drug/nutraceutical delivery carriers, used for driving bioactive molecules to desired target tissues, decreasing potential side effects and protecting the encapsulated molecule from enzymatic metabolic processes.

In this study, zwitterionic liposomes containing tyrosol, hydroxytyrosol and oleuropein were synthesized and characterized for their size and surface charge. Particular attention was devoted to the determination of encapsulation efficiency (EE%), quantifying the loaded Tyr, HTyr and Ole amount, by using three different techniques: direct UV spectrophotometry, High Performance Liquid Chromatography and Trolox Equivalent Antioxidant Capacity assay. The results revealed higher EE% for oleuropein. Cyto-toxicity and cyto-compatibility of liposomes were also tested on human chondrocyte cells.

1. Introduction

Liposomes have found use and application in a vast number of sectors, most importantly as drug-delivery systems, in the pharmaceutical industry [1–4].

Liposomes are spherical in shape (15–1000 nm), made of a bilayer of phospholipids, within the bilayer, the hydrophobic tails of the phospholipid groups face each other, while the hydrophilic heads face the inner core and the outer boundary of the liposome. This structure allows the possible encapsulation of hydrophilic substances within the

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core of the liposome, while the hydrophobic ones are partitioned within the bilayer [5]. The ability to carry both hydrophilic and lipophilic moieties, in addition to their biocompatibility and biodegradability, are the main reasons for liposomes uses for pharmaceutical and cosmetic applications [6]. Liposomes increase the efficacy and therapeutic index of carried-drugs [7,8], increase stability and reduce toxicity of encapsulated agents [9], improve pharmacokinetic effects (reduced elimination, increased circulation life times) [10–13]. Their use becomes essential to increase the bioavailability of natural compounds [14].

Olea europaea L. is the botanical name of the olive tree. The tree is mainly found in the Mediterranean basin and the olive fruit and its oil represent among the main ingredients of the Mediterranean diet. Several vegetables used in the ordinary diet, contains a combination of active ingredients useful in the prevention of different degenerative diseases [15–17]. Extra virgin olive oil is obtained from mechanical treatment of fresh ripen fruits without any physical and/or chemical process (heat, extraction, …) [18]. The relationship between the Mediterranean diet and low incidence of coronary heart disease (CHD) and cancer has been provided and supported by numerous studies. A study by Marta Guasch-Ferré et al. [19] reported that for each 10 g/day increase in extra-virgin olive oil consumption, cardiovascular disease and mortality risk decreased by 10% and 7%, respectively [19]. Mainly, this evidence has been related to the high content of monounsaturated fatty acids and polyunsaturated fatty acids. Olive oil, being the main source of fats in Mediterranean diet, has shown to possess a high monounsaturated fatty acids content, which ranges from 56 to 84% of total fatty acids (expressed as oleate) and high levels of polyunsaturated fatty acids, which ranges from 3 to 21% of total fatty acids content (expressed as linoleate). In addition, olive oil contains other non-triglyceride components as well as non-glyceride components including hydrocarbons, monoglyceride esters, tocopherols, alkanols, flavonoids, anthocyanins, hydroxyl- and dihydroxy-terpenic acids, sterols, polyphenols and phospholipids. Tyrosol, hydroxytyrosol and oleuropein are among the phenolic compounds found in extra virgin olive oil, which were suggested to contribute to the low incidence of CHD and some types of cancers [20–22]. Similarly to resveratrol [15,23] these polyphenols act as caloric restriction in skeletal and cardiac muscles. Moreover it was previously suggested [24], that the protective contributions of both tyrosol and hydroxytyrosol, against lipid peroxidation, may be due to their copper chelating properties. Cu(II) and Cu(I) complexes, revealed high antioxidant potentials and radical scavenger effects, because the synergic contributions of the ligands (organic molecules, such as tyrosol, hydroxytyrosol, glutathione, …) and the redox active metal center [25–28].

Recent studies on animal and human demonstrated a beneficial effect of olive and its derivatives (tyrosol, hydroxytyrosol and oleuropein) on the development of osteoarthritis. In vitro studies suggested that the augmentation of autophagy and suppression of inflammation by olive oil polyphenols could contribute to the chondroprotective effects of these compounds [29,30]. Many studies also reported the use of liposome preparations, as carrier, to overcame side effects and enhance the pharmacological effects of the drugs/nutraceuticals [31] and particularly the use of liposome formulations for intra-articular (IA) delivery. As an example, VX- 745, chondroitin sulfate and celecoxib showed an improved drug residence time, when loaded in liposomes with respect to the use as free drugs [32,33]. Other studies reported the effects by natural bioactive molecules (i.e. oleuropein) on the articulate inflammations [34].

Tyrosol (Tyr, 2-(4-hydroxyphenyl) ethanol) is a phenethyl alcohol present in different natural sources as a natural phenolic antioxidant (Fig. 1a). It is mostly found in the fruit and oil [35] of olive tree. It has been shown to inhibit the oxidation of LDL and to prevent the risk of reactive oxygen metabolite-mediated diseases such as inflammatory bowel disease, inhibiting leukocyte 5-lipooxygenase and protecting the Caco-2 intestinal mucosal cells against the cyto-static and cyto-toxic

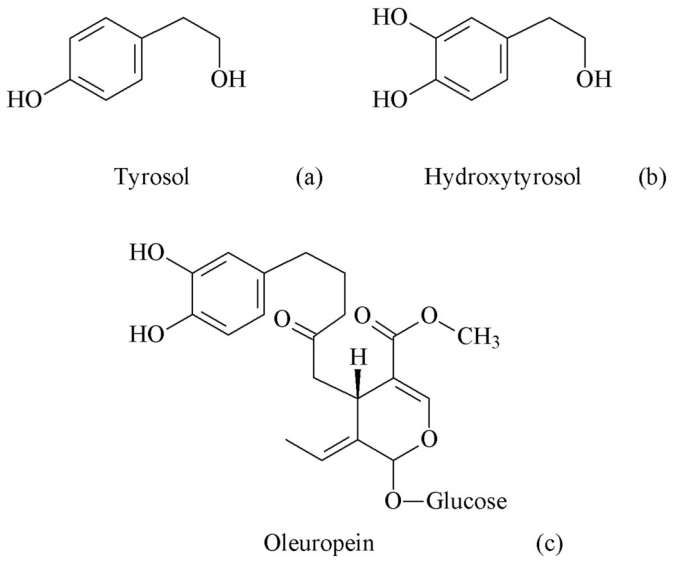


Fig. 1. Structure of (a) tyrosol (Tyr), (b) hydroxytyrosol (HTyr), and (c) oleuropein (Ole).

effect of oxidized LDL [36] modifying the cell redox potential. The antioxidant activity relationship has shown that Tyr exerts its effects only as a hydroxyl radical scavenger or at most an α-tocopherol regenerator [37,38], but, as it only carries one hydroxyl group in *para*-position, its action as an antioxidant is somewhat limited with respect to the other phenolic antioxidants present in the olive fruit and oil.

In the hydroxytyrosol (HTyr, 4-dihydroxyphenylethanol; Fig. 1b) the OH group at the *ortho*-position contributes to higher antioxidant potency and chemotherapeutic efficacy than Tyr [39]. It is well-known that the antioxidant properties of *ortho*-diphenols are related to hydrogen-donation and their ability to improve radical stability by forming an intramolecular hydrogen bond between the free hydrogens of their hydroxyl group and their phenoxyl radicals [40]..

HTyr also showed to be highly protective against nitration of tyrosine and DNA damage caused by peroxynitrite in vitro [20,21,41]. Apart from its antioxidant effect, HTyr has been classified as an anti-infective agent and as a platelet aggregation inhibitor as it was proven to inhibit the chemically induced aggregation, the accumulation of the pro-aggregant agent thromboxane in human serum, the production of the pro-inflammatory molecules leukotrienes by activated human leukocytes and the inhibition of arachidonate lipoxygenase [22].

Oleuropein (Ole, methyl (4S,5E,6S)-4-[2-[2-(3,4-dihydroxyphenyl) ethoxy]-2-oxoethyl]-5-ethylidene-6-[(2*S*,3*R*,4*S*,5*S*,6*R*)-3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl]oxy-4H-pyran-3-carboxylate, Fig. 1c) is the major phenolic constituent of the oil leaf (*Olea europaea* L.) and it is also present in olive oil and fruit (up to 14% of the dry weight of olives). Ole do not only occurs in the *Olea* genus, but also in many other genera belonging to the *Oleaceae* family [42].

Several studies have been carried out on Ole and they have shown promising results as regards anti-microbial, anti-infective, anti-hypertensive, antioxidant, vasodilator properties. Moreover, the antioxidant activities of Ole, as well as of HTyr, have been proven to be more effective than vitamin E, which was confirmed, using a metal-independent oxidative systems and stable free radicals, such as DPPH in a series of experiments which showed both a strong metal-chelation and free-radical scavenging action. According to a study carried out by Manna and coworkers [43] *ortho*-diphenolic components of olive fruits and by-products (olive pomace), mainly hydroxytyrosol and its esterified form (oleuropein aglycone), were responsible for the protection against ROS-induced oxidative injury to human cells. A good protection against hemolysis of red blood cells was also observed and reported, after pretreatment by *ortho*-diphenols compounds [44]. In addition,

oleuropein showed to increase nitric oxide synthase expression which leads to the increase in the nitric oxide released by cultured macrophages after endotoxin challenges which as a result holds an anti-infective and anti-parasitic capacity [22].

The present work will be focused primarily on the preparation of zwitterionic liposome loaded with antioxidants to increase their bioavailability and to optimize the delivery processes. The vesicles size, chemical release of tyrosol, hydroxytyrosol and oleuropein, encapsulation efficiency and stability of the liposomes will be studied.

2. Materials and methods

2.1. Reagents, standards and solvents

DOPE (1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine) and DOPC (1,2-dioleoyl-*sn*-glycero-3-phosphocholine) were purchased from Lipoid GmbH (Germany).

All reagents, standards and solvents for the liposome preparation and the subsequent analyses were purchased from Sigma-Aldrich (Milan): tyrosol (Tyr, 2-(4-hydroxyphenyl)ethanol, ≥98.0%), hydroxytyrosol (HTyr, 4-dihydroxyphenylethanol, ≥98.0%), oleuropein (Ole, methyl(4S,5E,6S)-4-[2-[2-(3,4-dihydroxyphenyl)ethoxy]-2-oxoethyl]-5-ethylidene-6(2*S*,3*R*,4*S*,5*S*,6*R*)-3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl]oxy-4H-pyran-3-carboxylate; ≥98.0%), resveratrol (Rsv, 3,4′,5-trihydroxy-*trans*-stilbene, 5-[(1*E*)-2-(4-hydroxyphenyl) ethenyl]-1,3-benzenediol, ≥99.0%), trolox (Trx, (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid, 97%), ABTS (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid), ≥98.0%), $K_2S_2O_8$ (potassium persulfate, ≥99.0%). All the used solvents were HPLC grade: methanol (MeOH, 99.9%), ethanol (EtOH, 99.9%), acetonitrile (CH_3CN, 99.9%), acetic acid (CH_3COOH, 98%). The distilled water was produced by a distiller (Acquinity P/7).

Primary Human Chondrocytes (HC), chondrocytes growth medium, trypsin solution, and all the reagents and solvents used for cell culture were purchased by Sigma Aldrich (Germany). Mouse immortalized fibroblasts NIH3T3 were from American Type Culture Collection.

2.2. Liposomes preparation

Thin-film hydration method [45] was utilized for liposomes preparation according to the standard solutions of DOPE and DOPC were prepared maintaining (1:1) mole ratio. Then standard solutions of Tyr, HTry and Ole were prepared and then added to total phospholipids to obtain liposome with a final molar ratio (1:1).

All samples were mixed in a round bottom vial, then were dried under nitrogen for evaporating the solvent and under vacuum overnight. Finally, the samples were rehydrated with Milli-Q grade H_2O, yielding multilamellar dispersion. Freeze/thaw cycles were carried out 9 times and liposome were extruded (LiposoFast apparatus, Avestin, Ottawa, Canada) by a 100 nm polycarbonate membrane to control size and to obtain unilamellar in the final suspension. The liposome were dialyzed using a dialysis membrane (molecular weight cut-off = 14.000) for 3 h. All liposomes were stored at 4 °C, before subsequent analyses.

2.3. Dynamic light scattering and ζ -potential

All sizing and ζ-potential measurements were made on a Zetasizer Nano ZS90 (Malvern Instrument Ltd., UK), at 25 °C. All measurements were made on the liposome samples without any dilution. The Nano ZS incorporates non-invasive backscatter (NIBS™) optics for sizing measurements. The detection angle of 173°enables size measurements of concentrated, turbid samples to be made. However, the scattered light detected from samples during a ζ-potential measurement is made at the forward angle of 12°.

As the radii of liposomes were always large enough compared with the Debye–Huckel parameters, the ζ-potentials were calculated directly from the Helmoholtz–Smolowkovski equation (by the zetasizer) [46]. Sizes were also calculated in the same experiments, according to the procedure described by Langley [47].

2.4. IR spectroscopy

IR spectra were acquired with a Thermo FT-IR spectrometer Nicolet 5700, operating between 4000 and 700 cm^{-1}. An MCT detector was used, and the apparatus was purged with dry nitrogen. Typically, 300 scans at a resolution of 2.0 cm^{-1} were averaged. The frequency scale was internally calibrated with a reference He—Ne laser to an accuracy of 0.01 cm^{-1}. An attenuated total reflection (ATR) cell for liquid with germanium crystal was used to record the spectra. Each sample was scanned by using three replicates. ATR-FTIR spectra were recorded and managed by the software OMNIC 4.1b (Thermo Nicolet).

2.5. UV–Visible spectroscopy

UV–Vis spectrophotometer used was a dual-beam Perkin Elmer Lambda EZ 201, equipped with software PESSW 1.2 (Perkin Elmer, Monza, Italy). UV–Vis spectra range was 190–1100 nm, against reference (corresponding sample solvent). The cuvettes were 10 mm optical pathway, in quartz for UV analysis, or PMMA/UV grade (Kartell) for colorimetric assay (Vis analysis).

2.6. HPLC-UV

Liquid chromatography analyses were carried out on isocratic HPLC Varian ProStar 210 machine (Varian, Inc., USA) equipped with UV detector (Varian 9050) and Varian software Star-6.41 for instrument management and data collection.

2.7. Encapsulation efficiency

Encapsulation efficiency is a parameter that indicates the amount of a substance that the carrier system can encapsulate and consequently transport to the target site. It can be expressed as the ratio between the amount of the actual encapsulated compound with respect the total amount of the compound used at the synthesis stage and theoretically enclosed in the carrier during the synthesis:

$$EE\% = E_{drug}/T_{drug}{}^{*}\,100$$

where T_{drug} is the total amount of the compound added during the carrier synthesis (μM) and E_{drug} is the amount of the experimental encapsulated compound (μM).

The E_{drug} can be measured by using several analytical procedures (based on direct or indirect spectrophotometric tests, as well as chromatographic analysis), on the liposome previously disrupted in order to get rid of the scattering background (scaling as λ^{-4}), due to large aggregates in solution, which can affect precise intensity evaluation. To disrupt liposomes and release the entrapped antioxidants, samples underwent several cycles of freezing (−32 °C) and after methanol solution was added to them. These solutions were then filtered through a Whatman RC 0.20 μm syringe filter (Sigma-Aldrich, Milan) and properly diluted before the analyses.

2.8. UV direct quantification

UV direct quantification of Tyr, HTyr and Ole was carried out via UV spectrophotometer and calibration curves were built by measuring the absorbance of standard solutions at 280 nm, chosen to maximize the detection of the tree studied compounds.

Pretreated liposome samples were properly diluted and each sample was analyzed in triplicate.

2.9. TEAC/ABTS assay

The TEAC/ABTS assay is an analytical photometric method used to evaluate the scavenging ability of antioxidant compounds quenching the radical cation, $ABTS^{\bullet+}$, and having as reference trolox standards solutions (TEAC, Trolox Equivalent Antioxidant Capacity). The present study optimized an indirect TEAC/ABTS method to evaluate the EE% of the liposomes, looking at their TEAC value, compared with standard solution of pure antioxidants.

The protocol was optimized according to the procedure reported by Re (1999), with some modifications. Briefly, the $ABTS^{\bullet+}$ free radical cation, was prepared by treating a solution of ABTS (7 mM) with a $K_2S_2O_8$ solution (140 mM) and the mixing was allowed to incubate overnight (12–16 h; in the darkness at 4 ± 1 °C) and then properly diluted in absolute EtOH before use. A known volume of this diluted solution was treated with Trolox standard solutions ranging 0.20–20.00 μM for calibration. After 30 min of incubation the adsorption at 734 nm, was read, against EtOH. Calibration curves were constructed reporting the relative decreasing in absorbance (A_{734}%) of the $ABTS^{\bullet+}$ solution treated with standards or samples, with respect to the blank solution ($ABTS^{\bullet+}$ solution not treated): $Abs_{734}\% = \{[1 - (Abs_{Trolox/Sample}/Abs_{Blank})]*100\}$.

Calibrations showing correlation factors $R^2 > 0.990$ were accepted for analyses, and the results were expressed as μmolTrxEq/L of sample (TrxEq, Trolox equivalent). This calibration was then used to determine the TEAC/ABTS values for standard solutions of Tyr, HTyr, and Ole to obtain a secondary calibration of TEAC/ABTS value for each of the three studied antioxidants. Secondary calibrations showing correlation factors $R^2 > 0.990$ were accepted for intercepting the value obtained for pretreated liposome samples properly diluted, and final results were expressed as μM of each relevant compound (Tyr, HTyr or Ole). Triplicate analyses were performed on each sample.

2.10. HPLC analysis

Liquid chromatographic analysis of the pretreated samples, was also carried out, for comparison reasons. The HPLC protocols was optimized using a C18 column (Phenomenex Luna C18, 5 U, 250 × 4.6 mm, 5 μm particles, 100 Å pores) with a safe guard pre-column (Phenomenex C18, 4.0 × 3.0 mm) and a isocratic elution by 0.2% CH_3COOH/CH_3CN (70:30, *v*/v), at 0.5 mL/min flow rate, and 21 ± 2 °C. Injection volume was 20 μL and UV spectrophotometer detector was set at 280 nm. The external calibration method was used for the analytical quantification, using resveratrol as an internal standard. Calibration curves were designed injecting standard solutions of HTyr, Tyr and Ole in MeOH having retention times: t_R 6.46, 7.5, and 10.60 min, respectively (Fig. 1S). The three analytes showed a range of linear calibration by 0.10–2.00 mM, showing correlation factors, $R^2 > 0.990$. The values for limit of quantification (LOQ) and limit of detection (LOD) were also defined as 0.04 and 0.01 mM for Tyr and Ole, and 0.06 and 0.02 mM for HTyr. Each sample was analyzed in triplicate and average and standard deviation parameters were calculated.

2.11. In vitro cyto-toxicity and cyto-compatibility: NIH3T3 and HC viability

To evaluate the in vitro cyto-toxicity and cyto-compatibility of products, the direct contact tests, proposed by ISO 10995-5:2009 [48] was used. This test is suitable for sample with various shapes, sizes or physical status (i.e. liquid or solid).

The evaluation of in vitro acute toxicity does not depend on the final use for which the product is intended, and the document ISO 10995-5:2009 recommends many cell lines from American Type Collection. Among them, to test liposomes cyto-toxicity, NIH3T3 mouse fibroblasts were chosen.

Cells for cyto-compatibility tests are usually chosen according to the end-use of the products under investigation. In order to evaluate the use of the liposomes loaded with different antioxidants in the treatment of osteoarthritis, primary Human Chondrocytes derived from normal human articular cartilage were selected.

The percentage of cell viability was calculated by referring to the cell viability of NIH3T3 and HC in contact with the control, which is considered equal to 100%.

Fibroblasts NIH3T3 and Human Chondrocytes (HC) were propagated, respectively, in DMEM supplemented with 10% fetal calf serum, 1% L-glutamine-penicillin-streptomycin solution, and 1% MEM non-essential amino acid solution, and in chondrocytes growth medium at 37 °C in a humidified atmosphere containing 5% CO_2. Once at confluence, the culture medium was removed, the cells were washed with PBS 0.1 M, separated with trypsin-EDTA solution and centrifuged at 1000 rpm for 5 min. The pellet was re-suspended in complete medium (dilution 1:15). 1.5×10^3 cells suspended in 1 mL of complete medium were seeded in each well of a 24 well round multiwells and incubated at 37 °C in humidified atmosphere of 5% CO_2. After 24 h of culture, the culture medium was discharged and the test compounds, properly diluted in completed medium, were added to each well. All samples were set up in triplicate. Complete medium was used as negative control. Fibroblast viability was evaluated after 24 h of incubation and HC viability after 1, 7, 14 and 21 days of incubation by Neutral Red uptake (Sigma-Aldrich, Switzerland) following the procedure previously described [9].

The in vitro cyto-toxicity and cyto-compatibility assays were performed on empty liposomes and liposomes loaded with Tyr, HTyr and Ole at different concentrations. The liposomes concentrations tested were 0.1, 1 and 5% (*v*/v) corresponding to the following antioxidants concentrations:

Tyr 4.0×10^{-4}, 4.0×10^{-3} and 2.0×10^{-2} mM
HTyr 1.2×10^{-3}, 1.2×10^{-2} and 6.0×10^{-2} mM
Ole 2.8×10^{-3}, 2.8×10^{-2} and 1.4×10^{-1} mM.

The in vitro cyto-toxicity of Tyr, HTyr and Ole free solution in ethanol was also determined, for comparison reasons, using the following range of concentrations: Ole: 4.5×10^{-2}-9.0×10^{-1} mM; HTyr: 1.4×10^{-1}-5.4 mM,; Tyr: 2.8×10^{-1}-5.5 mM. These values were chosen according to the antioxidants concentrations determined as encapsulated into the liposomes.

2.11.1. Statistical data treatment

All measurements were carried out in triplicate and mean values and estimated standard deviations (esd) were calculated and reported. The analysis of variance was carried out to verify the variation between samples and the Tukey's test was used to determine significant differences. Data showing *p*-values < .05 were considered statistically significant. Calculation were carried out by using Microsoft Office Excel 2007, implemented with regression analysis subroutine, and Origin Pro8 SR2 (ver.0891, B891; OriginLab Corporation, Northampton, USA).

3. Results and discussion

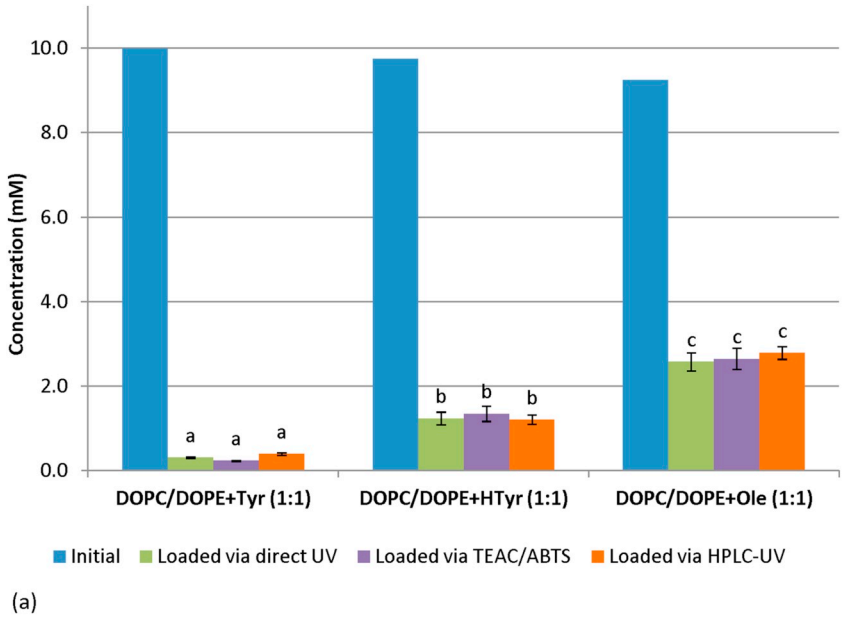
3.1. Liposomes chemical characterization

Table 1 shows the results obtained by Dynamic Light Scattering (DLS) measurements for the size distribution of DOPC/DOPE liposomes loaded with Tyr, HTyr and Ole antioxidant species (Fig. 2S).

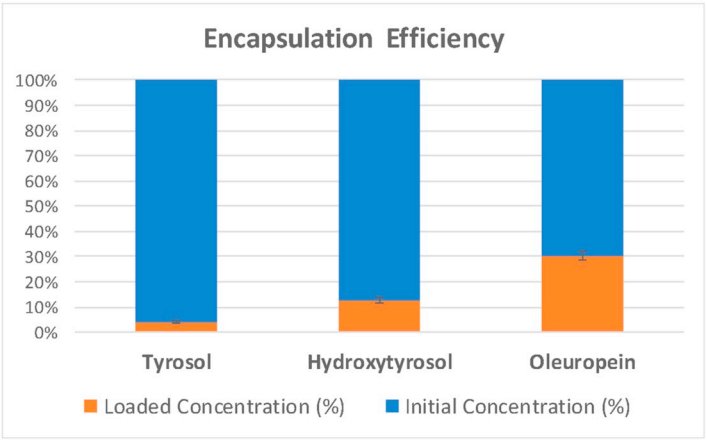
As reported in Table 1, there is a slight increase in liposome mean diameter when Tyr and HTyr are encapsulated in the vesicles, with not ~~low~~ statistical significance (Tukey's test): the difference in mean diameter between ~~of~~ drug incorporating vesicles with respect to ~~and~~ non-incorporating ones, Δ_{max} being 8.6 nm, to be compared with an averaged esd of 6.5 nm. The third case of liposomes encapsulating Ole, showed a statistically significant decrease of mean diameter (Δ being 18.3 nm, with respect esd of 7.0 nm, $p < 0.05$, Tukey's test). This can be considered a first indication that Tyr and HTyr may be packed in the

Table 1
Particle size distribution with polydispersity index (P.I.), ζ-potential and encapsulation efficiency (EE%) for all liposomes. The values are the average of three measurements (mean ± esd) and different letters in the same column indicate significant differences ($p < 0.05$, Tukey's test).

Composition	Mean diameter (nm)	P.I.	ζ-potential (mV)	Encapsulation efficiency EE%
DOPC/DOPE (1:1)	124.4 ± 7.0a	0.22	−10.2 ± 9.7a	–
DOPC/DOPE + Tyr (1:1)	132.3 ± 6.1a	0.19	−11.7 ± 5.7a	4.0 ± 0.3a
DOPC/DOPE + HTyr (1:1)	133.0 ± 6.5a	0.23	−18.4 ± 6.5a	12.4 ± 1.2b
DOPC/DOPE + Ole (1:1)	106.1 ± 7.0b	0.18	−19.9 ± 9.7a	30.2 ± 1.6c



(a)



(b)

Fig. 2. (a) Initial and loaded concentrations (expressed as mM) in liposomes for Tyr, HTyr and Ole as determined via UV direct photometric quantification, TEAC/ABTS assay and HPLC-UV analysis (different letters indicate significant differences, $p < 0.05$, Tukey's test). (b) Graphical representation of the encapsulation efficiency (EE%) for the liposomes loaded with Tyr, HTyr and Ole, as determined via HPLC-UV analysis.

lipid bilayer. On the other hand, the encapsulation of Ole determines a decrease in average size of liposomes and this behavior may suggest that Ole changes the normal conformation of empty liposomes, signifying that its incorporation occurred successfully and with a preferential disposition through the lipid bilayer, modifying its properties. These results are also in agreement with values obtained for EE% (see below).

The low polydispersity indexes (P.I.) showed by all the systems revealed that liposomes were not deeply altered upon interaction with antioxidants. The synthesized liposomes remained, in fact, fairly monodispersed. Since aggregation may be used as a measure of liposome physical stability, we may conclude that the incorporation of Tyr, HTyr and Ole in DOPC/DOPE liposome bilayer does not modify their physical stability.

Analyzing the data reported in Table 1 it can be observed that empty DOPC/DOPE liposomes have a small negative ζ-potential, though the net polar head charge of zwitterionic phospholipids is zero. The encapsulation of Tyr, HTyr and Ole changes the net surface charge of the liposome and, in particular, the charge of DOPC/DOPE + Ole become more negative (−19.9 mV, while not statistically different, Tukey's test) decreasing the aggregation process in solution. The ζ-potential allows to evaluate the aggregation processes in solution. It is well known that

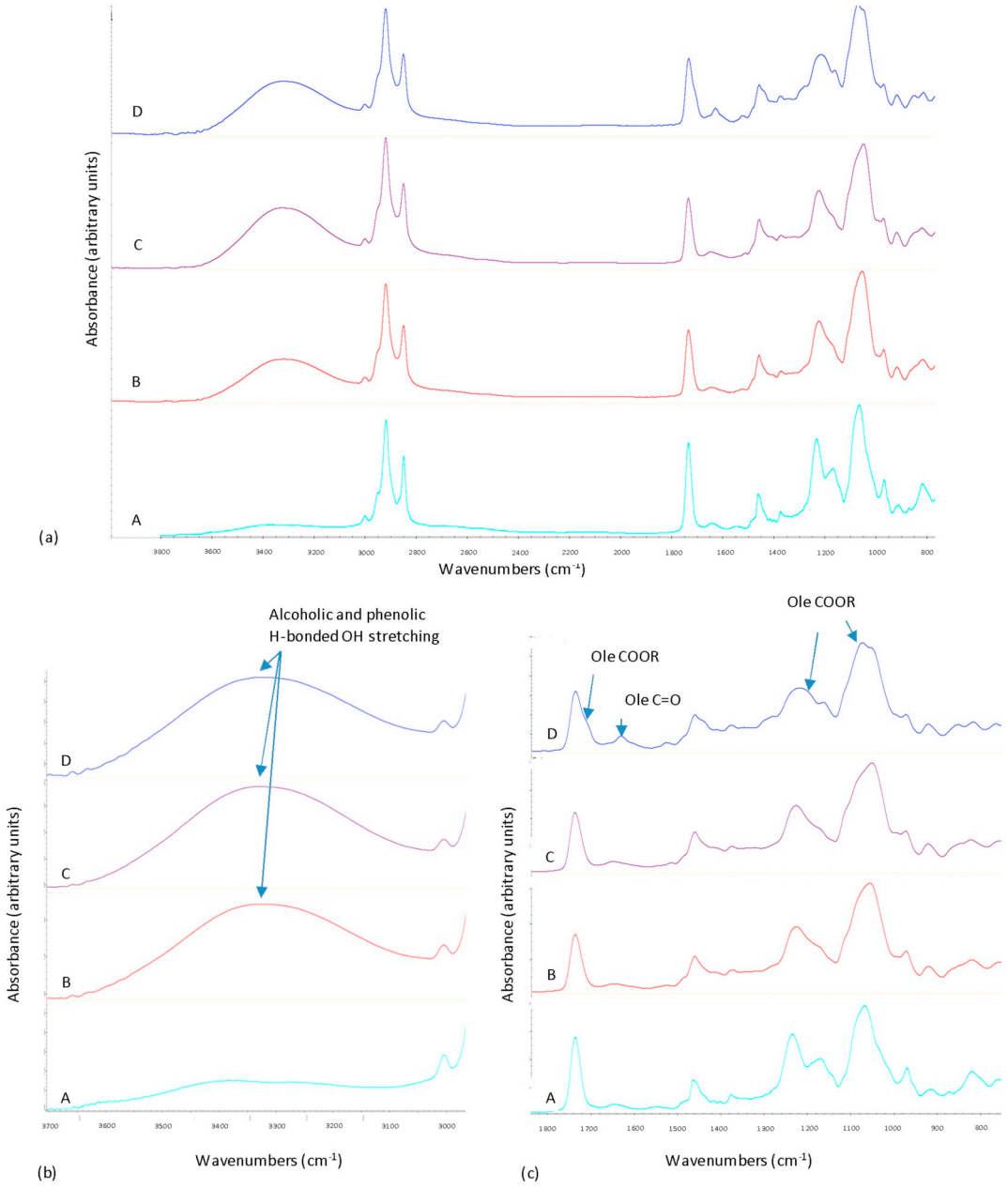


Fig. 3. (a) ATR-FTIR spectra of: A) DOPC/DOPE liposomes, B) DOPC/DOPE liposomes loaded with HTyr, C) DOPC/DOPE liposomes loaded with Tyr, and D) DOPC/DOPE liposomes loaded with Ole. (b) Magnification of the 3700–3000 cm^{-1} spectral region, highlighting the contribution of the nutraceuticals OH groups. (c) Magnification of the 1800–800 cm^{-1} spectral region, highlighting the contribution of the Oleuropein ester group.

systems with ζ-potentials more positive than + 30 mV or more negative than − 30 mV are usually considered stable [49]. This information suggests that the DOPC/DOPE + Ole liposome is the most stable over time by presenting less aggregation processes in solution than the other liposomes.

3.2. Encapsulation efficiency

The encapsulation efficiency (Table 1, EE%) was evaluated by means of different analytical techniques and the results obtained were compared. For this purpose, photometric UV direct quantification, TEAC/ABTS assay and HPLC-UV analyses were carried out.

Preliminary tests performed for the quantification of the amount of Tyr, HTyr and Ole loaded in liposomes showed comparable results (Fig. 2a). However, the HPLC-UV technique resulted the most suitable for the quantitative analysis, since both UV direct quantification and TEAC/ABTS assay gave a positive response for the plain liposomes to be subtracted to the results obtained for the loaded liposomes. Moreover, the readapted TEAC/ABTS assay for the quantification of Tyr, HTyr and Ole showed linearity in a narrow range of concentration of the three compounds (from about 1.0 to 6.0 μM). On the contrary, the HPLC-UV quantification method showed linearity in a larger range of concentration, covering more than an order of magnitude (from about 0.1 to 2.0 mM). For the reasons mentioned above the HPLC-UV method was chosen for the determination of the encapsulation efficiency.

As shown in Table 1, the HPLC-UV analyses revealed concentration of 0.40 ± 0.03 for Tyr, 1.21 ± 0.11 mM for HTyr and 2.79 ± 0.15 mM for Ole (Fig. 2a), corresponding to an encapsulation efficiency of 4.0 ± 0.3%, 12.4 ± 1.2% and 30.2 ± 1.6% respectively (Fig. 2b). Fig. 3S reports the relevant chromatograms.

The preparation procedure of liposomes followed in the present work brought to a relatively low incorporation rate for Tyr and HTyr,

Table 2
Main wavenumbers observed in the ATR-IR spectra of plain and loaded liposomes together with their assignments.

Liposome samples	Wavenumbers (cm^{-1})	Assignments
Plain	2920–2850	CH_3 and CH_2 stretch.
	1735	Phospholipids ester C=O stretch.
	1465–1410	CH_3 and CH_2 bending
	1250–1050	C=O + C-O-C stretch.
	970	Phospholipids P=O stretch.
		$(CH_3)_3$-N^+ stretch.
Loaded with Tyr	3700–3000	O-H stretch. of H-bonded alcohols
	2920–2850	and phenols
	1735	CH_3 and CH_2 stretch.
	1465–1410	Phospholipids ester C=O stretch.
	1250–1050	CH_3 and CH_2 bend.
	970	C=O + C-O-C stretch.
		Phospholipids P=O stretch.
		$(CH_3)_3$-N^+ stretch.
Loaded with HTyr	3700–3000	O-H stretch. of H-bonded alcohols
	2920–2850	and phenols
	1735	CH_3 and CH_2 stretch.
	1465–1410	Phospholipids ester C=O stretch.
	1250–1050	CH_3 and CH_2 bend.
	970	C=O + C-O-C stretch.
		Phospholipids P=O stretch.
		$(CH_3)_3$-N^+ stretch.
Loaded with Ole	3700–3000	O-H stretch. of H-bonded alcohols
	2920–2850	and phenols
	1735	CH_3 and CH_2 stretch.
	1710	Phospholipids ester C=O stretch.
	1650	Oleuropein ester C=O stretch.
	1465–1410	Oleuropein carbonyl C=O stretch.
	1250–1050	CH_3 and CH_2 bend.
	970	C=O + C-O-C stretch.
		Phospholipids P=O stretch.
		$(CH_3)_3$-N^+ stretch.

while it gave good results for Ole, suggesting that the two types of phospholipids (DOPE/DOPC) employed in the synthesis are more suitable for this last, that is the most lipophilic compound, than for Tyr and HTyr, that are smaller and more hydrophilic compounds. This evidence confirms that Ole is mainly incorporated through the lipid bilayer, and it suggests that the incorporation of this class of compounds occurs preferentially through this way.

3.3. Infrared spectroscopy

The ATR-FTIR spectra of dried samples of both plain and loaded (Tyr, HTyr and Ole) liposomes were very similar (Fig. 3). Table 2 reports the main wavenumbers observed in the collected spectra together with their assignments [50]. The spectral regions relative to the most characteristic functional groups are: 2920–2850 cm^{-1} and 1470–1410 cm^{-1} for hydrocarbon chains; 1750–1700 cm^{-1} and 1250–1090 cm^{-1} for ester groups, 1230–1050 cm^{-1} for phospholipids. The absorption band centered at 970 cm^{-1} is characteristic of $(CH_3)_3$-N^+ stretching of DOPC choline head.

Despite the similarity of the general pattern given by the functional groups of phospholipids, there is a significant difference in the IR spectra of loaded liposomes with respect to the plain ones, in the 3700–3000 cm^{-1} spectral region (Fig. 3) due to the presence of the broad band in the spectra of loaded liposomes that is assigned to the O—H stretching of alcoholic and phenolic groups of nutraceutical compounds [51]. Due to the surface sensitivity of ATR/IR spectroscopy, this finding suggests that tyrosol, hydroxytyrosol and oleuropein were loaded in liposomes. In particular, the presence of IR absorption bands attributed to the OH groups, characteristic of two water soluble nutraceuticals compounds, supports the hypothesis that such molecules are present in the hydrophilic cavity of the purified vesicles.

Moreover, in the spectrum of Oleuropein loaded liposome the characteristic absorption bands of the nutraceutical at 1710 cm^{-1} (ester C=O) and 1650 cm^{-1} (carbonyl C=O) appeared and the intensity of the bands at 1210 and 1090 cm^{-1} increases due to the Oleuropein ester group contribution. These data confirm that oleuropein is inserted within the phospholipidic bilayer.

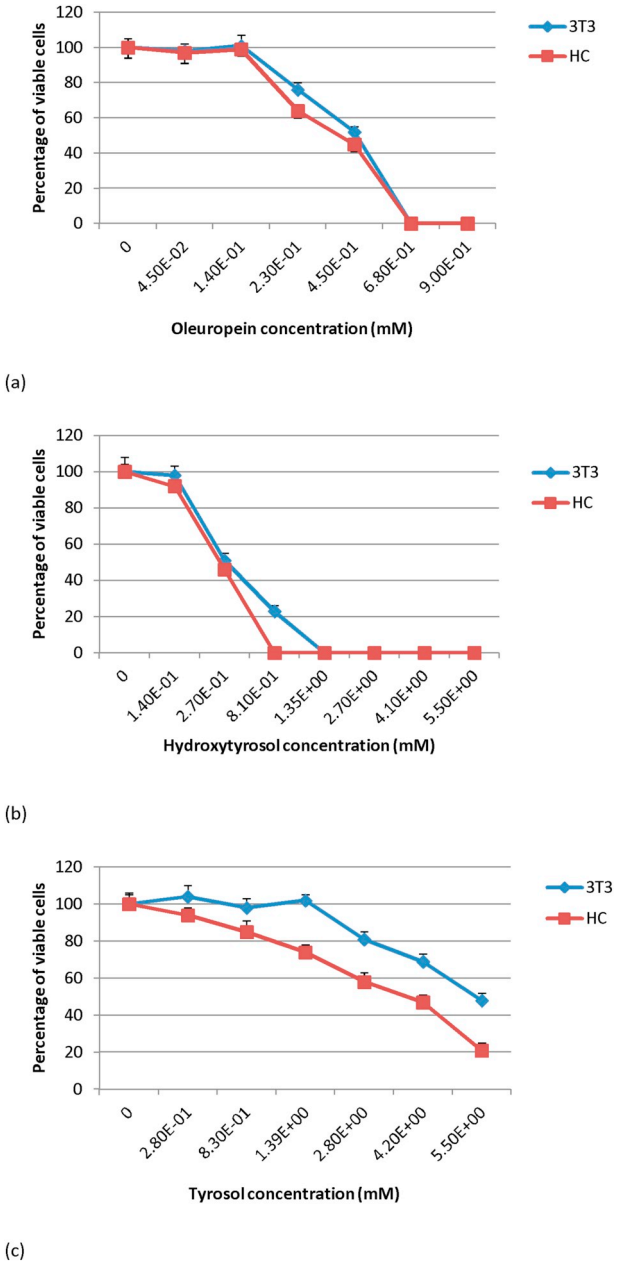


Fig. 4. Fibroblasts and chondrocytes viability percentage after 24 h of contact with different concentrations of antioxidants: (a) oleuropein (the concentration of 4.5×10^{-1} mM reduced cell viability by 50%; (b) hydroxytyrosol (the concentration of 2.7×10^{-1} mM reduced cell viability by 50%); (c) tyrosol (the concentration of 5.5 mM and 4.3 Mm reduced, respectively, NIH3T3 and HC viability by 50%).

3.4. In vitro cyto-toxicity and cyto-compatibility: NIH3T3 and HC viability

Assessment of cyto-toxicity and cyto-compatibility was based on the amount of neutral red incorporation by fibroblasts NIH3T3 and HC after 24 h of contact with different concentrations of Ole, HTyr and Tyr. Fig. 4a shows the effect of Ole concentration ranging from 4.5×10^{-2} up to 9.0×10^{-1} mM, with respect to the 100% viability of untreated control samples (medium; reported as first point in the graph in

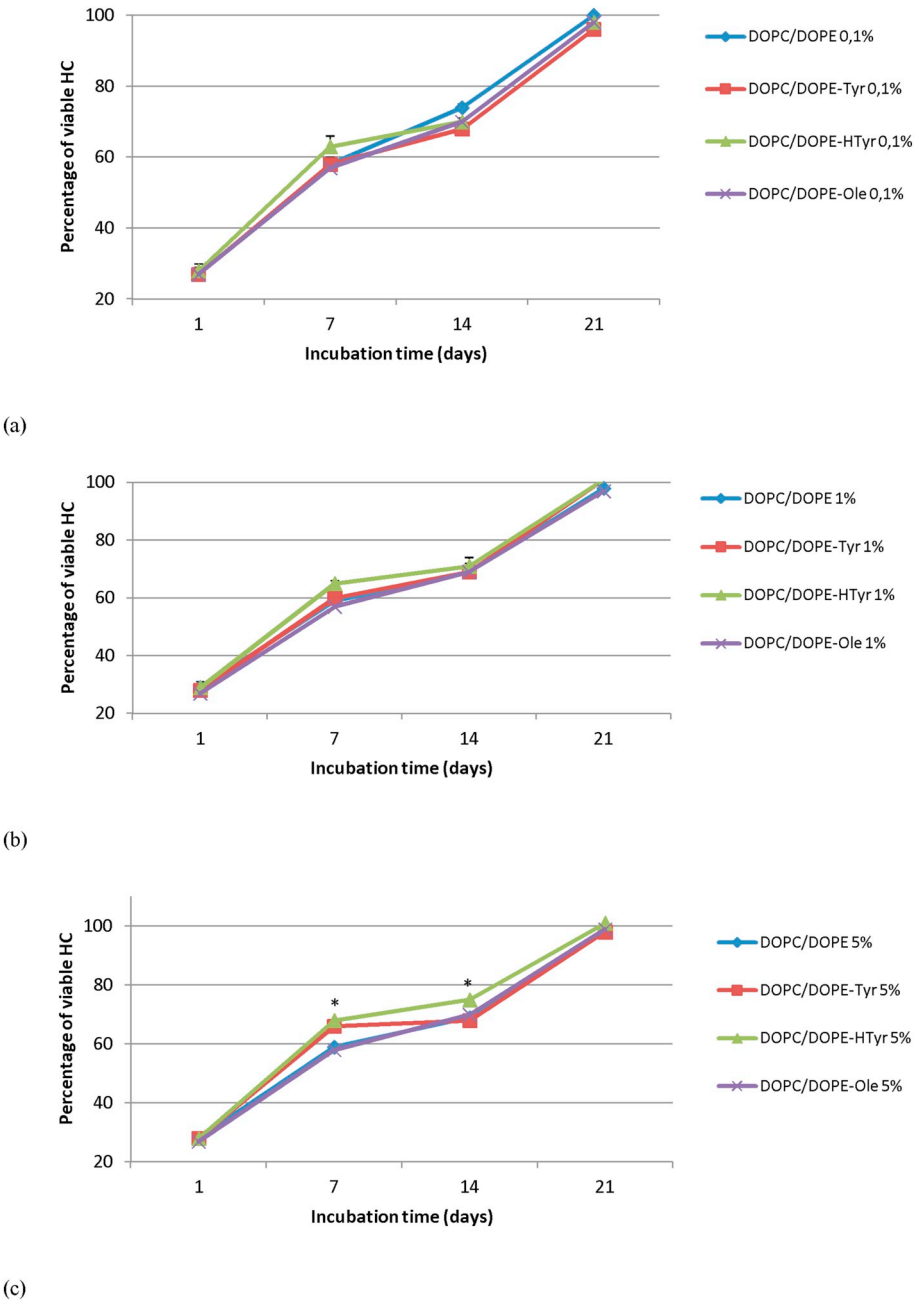


Fig. 5. HC viability at 1, 7, 14 and 21 days of incubation time with different concentrations of DOPC/DOPE, DOPC/DOPE + Tyr, DOPC/DOPE + HTyr, and DOPC/DOPE + Ole: a) 0.1% (*v*/v); b) 1% (v/v); c) 5% (v/v). Data are mean ± sd of three experiments run in six replicates. *Values are statistically different versus unloaded liposomes, $p < 0.05$.

Fig. 4a). A strong reduction in cell viability was observed for concentrations higher than 1.4×10^{-1} mM and the 50% cell viability reduction was estimated at a oleuropein concentration by 4.5×10^{-1} mM for both fibroblasts and chondrocytes.

Similarly, the effect of HTyr is shown in Fig. 4b, that shows the percentage of cell viability decreasing with increasing the antioxidant molecule concentration with respect to the untreated control samples. The concentration of 2.7×10^{-1} mM reduced NIH3T3 and HC viability by 50%.

Also the percentage of cell viability decreases by increasing the Tyr concentration with respect to the untreated control samples, as shown in Fig. 4c. The concentration of 5.5 mM and 4.3 mM reduced, respectively, NIH3T3 and HC viability by 50%.

The cyto-toxicity of the liposome systems, empty and loaded with Ole, HTyr and Tyr, was also evaluated with NIH3T3 cells and the results were reported in Fig. 4S. All liposomes were tested at three different concentration 0.1, 1 and 5% (*v*/v) corresponding to the following antioxidants concentrations: 4.0×10^{-4}, 4.0×10^{-3} and 2.0×10^{-2} mM for Tyr; 1.2×10^{-3}, 1.2×10^{-2} and 6.0×10^{-2} mM for HTyr and 2.8×10^{-3}, 2.8×10^{-2} and 1.4×10^{-1} mM for Ole.

Empty and antioxidant loaded DOPC/DOPE liposomes resulted not cyto-toxic towards fibroblasts NIH3T3 and cell density was not statistically different ($p < 0.05$) for all the test carriers, when compared with the negative control. It is important to underline that although liposome carriers favor cell internationalization of the antioxidant molecules, they did not show cyto-toxic activity towards fibroblasts.

The cyto-compatibility towards HC was also assessed for plain and antioxidant loaded DOPC/DOPE liposomes. The test was carried out by

evaluating cell incorporation of neutral red, as a function of both concentration and incubation time, and results are reported in Fig. 5. HC viability resulted not reduced by any of the tested concentrations for all the four products and a time dependent proliferation rate was revealed. In fact, the number of viable cells increased by increasing the incubation time and any toxic effect was observed. The percentage of viable cells in contact with DOPC/DOPE-Tyr, DOPC/DOPE-HTyr and DOPC/DOPE-Ole was not statistically different, with respect to that of empty liposomes, both for concentrations by 0.1 and 1% (Fig. 5a and b). In the case of 5% HTyr liposomes concentration, the percentage of viable HC increased after 7 and 14 days incubation (Fig. 5c).

These data suggested that zwitterionic liposomes loaded with natural antioxidants (such as Tyr, HTyr and Ole) have a great efficacy as delivery systems into HC cells. Moreover, the biocompatibility of vesicles allows the optimized releasing directly into the cells or in the specific receptor sites.

As well as for fibroblasts, the loading inside the liposomes favors cell internationalization of the antioxidant molecules, when compared to free molecules in solution, but they did not show any cyto-toxic activity also towards chondrocytes.

4. Conclusion

In the present work zwitterionic liposomes preparation containing three different nutraceutical compounds found in the fruit, leaf and oil of *Olea europaea* L. (tyrosol, Tyr; hydroxytyrosol, HTyr, and oleuropein, Ole) have been synthesized and characterized.

Determination of the average size of liposomes showed a slight change, statistically not significant, in the mean size of diameter of liposomes loaded with tyrosol and hydroxytyrosol. These data evaluated together to EE% suggestes that both molecules where successfully integrated with in the liposomes while a decrease in the mean size of liposomes loaded with oleuropein has been noted which could suggest that the incorporation of oleuropein in liposomes occurred trough the bilayer membrane, changing the typical bilayer conformation of the liposomes. The overall charge of liposomes, which showed to be more negative for oleuropein suggest the better physical stability for this preparation, indicating a less probability of aggregation of liposomes, versus time, containing oleuropein than liposomes loaded with hydroxytyrosol and tyrosol.

The comparison of the values of the encapsulation efficiency (EE%) using Ultraviolet spectroscopy, TEAC assay and HPLC-UV analysis, confirm the better incorporation of oleuropein with respect of hydroxytyrosol and tyrosol within the liposomes.

The difference in ATR-FTIR spectra between empty and loaded liposome preparations, suggest that the three nutraceutical compounds expose the hydroxylic groups on the surface of the liposomes, hence confirming their preferential incorporation within the lipid bilayer.

Cyto-toxicity assay of the different liposomal preparation by human chondrocytes shows that liposomes are not cyto-toxic at any concentration. Therefore, the zwitterionic liposomes loaded with natural compounds are important *drug-delivery system* to increase the bioavailability of antioxidants in the osteoarthritic pathologies and to reduce the collateral effect of classical drugs.

Declarations of interest

The authors report no declarations of interest.

Conflict of interest

The authors declare that they have no conflicts of interest.

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