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Hydroxytyrosol oleate: a promising neuroprotective nanocarrier delivery system of oleuropein and derivatives

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Abstract

Olive Phenols (OPs) are known to be potent antioxidants and possess various bioactivities and health benefits. Epidemiological studies suggested that consumption of olive oil reduces the risk of different diseases exerting a protective effect against certain malignant tumors (prostate, breast, digestive tract, endothelium, etc.). However, extremely low absorption rate of olive phenolic compounds restricts their bioactivity. In this context, solid lipid nanoparticles (SLNs) are a promising solution because they provide higher drug stability and can incorporate both lipophilic and hydrophilic drugs. Interesting experimental results have been obtained using hydroxytyrosol oleate (HtyOle) as a main component of a nanoparticle delivery system containing oleuropein (OL), oleuropein aglycone (3,4-DHPEA-EA), or hydroxytyrosol itself (Hty). In this work, hydroxytyrosol oleate (HtyOle) and hydroxytyrosol oleate (HtyOle)-based solid lipid nanoparticles were prepared and characterized. In addition, we evaluated*in vitro* their antioxidant activity by DPPH assays and by ROS formation using the SH-SY5Y cell line.

Keywords

Hydroxytyrosyl oleate, Olive Phenols, Oleuropein, 3,4-DHPEA-EA, Hydroxytyrosol, nanoparticles

1. Introduction

The food industries produce large amounts of waste, which represent disposal and potentially environmental pollution problem. Efficient, inexpensive, and environmentally friendly utilization of wastes is becoming more important. New methods for the handling and treatment of these materials have been introduced in the recovery, bioconversion, and utilization of valuable constituents from these wastes (Okonko et al. 2009).

Olive oil is an important component of the Mediterranean diet, containing variable amounts of triacylglycerols and small quantities of free fatty acids, glycerol, pigments, aroma compounds, sterols, tocopherols, phenols, unidentified resinous components and others. It is recognized that the well-known pharmacological properties of micro-components of olive oil, olive fruit, and leaves are ascribable to their phenolic content. During the olive oil production process, the major proportion of the phenolic compounds [oleuropein (OL) and its metabolites as 3,4-dihydroxyphenylethanol-elenolic acid dialdehyde (3,4-DHPEA-EDA), 3,4-dihydroxyphenylethanol-elenolic acid (3,4-DHPEA-EA) and hydroxytyrosol] are found in the waste. Several *in vitro* and *in vivo* studies confirm that phenolic compounds exert multiple biological activities, such as antioxidant (Visioli et al. 1998, Nardi et al. 2014, Rizzo et al. 2017), antiatherogenic (Karantonis et al. 2006), angiotensin-converting enzyme (ACE) inhibitory (Kiss et al., 2008; Alcaide- Hidalgo et al. 2020), antimicrobial (Guo et al. 2019) anti-inflammatory (Sindona et al. 2012), and anticancer (Giada et al. 2019, Campolo et al. 2012). However, the hydrophilicity of olive phenolic compounds restricts to a great extent their application in lipophilic systems.

In the last decade, Procopio et al. (Procopio et al., 2011) reported that several derivatives of oleuropein, the predominant phenolic component in the olive leaves, such as oleuropein aglycone, hydroxytyrosol, and their acetylated lipophilic forms, can be prepared by simple and environmentally friendly semisynthetic protocols. Thus, lipophilic oleuropein aglycone derivatives were synthesized and evaluated in terms of their lipophilicity and antioxidant capacity. The biological activities of these

derivatives were directly related to their level of lipophilicity, with the maximum antioxidant and neuroprotective activity reported for the oleyl derivative (Nardi e al. 2017).

Another excellent strategy to increase the bioavailability of biologically active compounds could be encapsulation in nanoparticles (NPs) (Yang et al. 2020). These spherical particles are an alternative drug delivery system to classical carriers and an excellent candidate for the encapsulation of drugs with poor bioavailability. The solid lipid nanoparticles (SLNs) are typically spherical with an average diameter between 10 and 1000 nanometers and contain a hydrophobic solid matrix core with one layer of a lipid coating (Ekambaram et al. 2012; Pardeike et al. 2009).

In this way, drugs are co-processed with a surfactant to be formulated into stabilized nanosuspensions and are made suitable for pharmacological treatments (Scioli Montoto et al. 2020). Clinical use of SLNs, however, required toxicological risk assessment, and the toxicological and inflammatory potential of SLNs was investigated using *in vitro, ex vivo,* and *in vivo* methods (Nassimi et al. 2010) that have indicated the cancer treatment as the most relevant field of application (Hare et al. 2017).

However, the low efficacy and lack of selectivity can cause severe side effects for health issues. Furthermore low drug loading capacity, the carrier's toxicity (the carrier is usually the major component of the formulation) and its biodegradation can be a potential problem for pharmaceutical therapy (Tapeinos et al. 2017).

Considering our wide experience and our research studies on the phenolic compounds of olive (Procopio et al. 2009; Impellizzeri et al. 2011; Nardi et al. 2020; Mazzei et al. 2020; Mancuso et al. 2021), we synthesized a new carrier-free drug system using hydroxytyrosol oleate as solid lipid in the synthesis of nanoparticles containing phenolic compounds (PCs) as OL, 3,4-DHPEA-EA and Hty (Figure 1).

Please Insert the Figure 1 here

The hydroxytyrosyl esters are known for their ability to reduce nitric oxide (NO) production, especially in the case of the oleyl derivative HtyOle. This conjugate was an excellent topical therapeutic agent to treat skin diseases due to his permeation profile through the human stratum corneum and viable epidermis membranes (Procopio et al. 2011).

The HtyOle was identified in olive oil by-products by high performance liquid chromatography coupled with mass spectrometry (Plastina et al. 2019) and its antioxidant capacity was tested in human keratinocytes (Benincasa et al. 2020). The compound was synthesized by the modified Lewis catalysis method proposed by Procopio et al. (Procopio et al. 2011) using 2-Me THF as green solvent. The 2-Me THF can be produced from natural sources (corncobs or bagasse) and recently tested as an excellent reaction medium for the synthesis of organic molecules involving the use of Lewis acid catalysis (Nardi et al. 2015; Nardi et al. 2017).

The nanoparticles obtained (SLN-HtyOle, SLN-HtyOle-OL, SLN-HtyOle-3,4-DHPEA-EA and SLN-HtyOle-Hty respectively), were tested on neuroblastoma SH-SY5Y cell line evaluating the antioxidant proprieties by ROS formation and using the DPPH (1,1-diphenyl-2-picrylhydrazyl) assay (Xie et al. 2014).

Aim of the work was to show the greater bioavailability of encapsulated phenolic compounds using hydroxytyrosyl oleate-based solid lipid nanoparticles and the greater capacity for scavenging radicals in the biological environment.

2. Materials and methods

2.1. Materials

Hydroxytyrosol was purchased from TCI AMERICA (Purity: >98.0% (GC)). The DPPH was purchased from Eastman Organic Chemical (Rochester, NY) and all other chemicals were purchased from Sigma (Sigma-Aldrich, Milan, Italy), unless otherwise specified.

2.2. Synthesis of HytyOle

The hydroxytyrosol oleate was prepared using a slightly modified synthesis protocol reported by Procopio et al. (Procopio et al. 2011). To a solution of hydroxytyrosol (1.62 mmol) in 2-MeTHF (8 mL) 1 equiv of oleyl acid chloride (1.62 mmol) and $Er(OTf)_3$ (0.0162 mmol) were added under stirring (Heidolph 505-20000-00 Hei-Standard, Kintail House, Inverness-shire, IV2 3BW, United Kingdom). The mixture reacted for 12 h at room temperature (25 °C). After completion, the reaction mixture was extracted with H_2O and then, the organic phase was dried over Na_2SO_4. The crude material, dried under vacuum (Hei-VAP Advantage Rotary Evaporator, Heidolph, Germany) (∼1 mmHg), was purified by flash chromatography (mobile phase $CHCl_3$/MeOH, 9.5/0.5, 90% yield) and identified by TLC and NMR analyses (Bruker Avance 500 MHz NMR spectrometer equipped with a QNP (^{31}P, ^{13}C, ^{15}N and ^{1}H) cryoprobe). ^{1}H-NMR and ^{13}C-NMR showed the HtyOLe

Hydroxytyrosol oleate (HtyOLe): ^{1} H-NMR (500 MHz, $CDCl_3$) δ= 0.88 (3H, CH_3, t, J = 5.0 Hz), 1.30 (14H, $7CH_2$, m), 1.60 (2H, CH_2, m), 1.98 (4H, CH_2-C=C-CH_2, dd, J=5, J=10), 2.28 (1H, CHCO, t, J = 7.6 Hz), 2.35 (1H, CHCO, t, J = 7.6 Hz), 2.82 (2H, CH_2Ph, t, J = 7.2 Hz), 4.23 (2H, CH_2OCO, t, J = 7.3 Hz), 5.35 (2H, CH=, m), 6.65 (1H, CH_{ar}, dd, J = 3.1, 1.9 Hz), 6.76 (2H, CH_{ar}, dd, J = 8.0, 1.9 Hz); ^{13} C-NMR (500 MHz, $CDCl_3$) δ= 14.1, 22.7, 25.0, 27.7, 29.3, 29.4, 29.7, 29.9, 31.9, 33.9, 34.8, 115.9, 116.4, 122.8, 130.6, 144.5, 145.6.

2.3. Oleuropein Extraction

The MW-assisted extraction of oleuropein (Procopio et al. 2009) was performed on several samples of olive leaves from Coratina cultivar of *Olea europaea* L, dried for 48 h at 50 °C, milled, and kept at r.t. until use (Costanzo et al. 2017).

141 *2.4. Synthesis of 3,4-DHPEA-EA.*

142 Oleuropein (740 mg, 1.34 mmol) was dissolved in aqueous CH_3CN (12.7 mL) in the presence of
143 $Er(OTf)_3$ (123.5 mg, 0.20 mmol) and refluxed at 80 °C for 8 h. At the end of the conversion, the
144 hydrolysate was cooled, 5 mL of water was added, and the mixture was extracted with CH_2Cl_2. After
145 drying on Na_2SO_4, the organic solvent was removed in vacuo and the crude product was purified by
146 flash chromatography (mobile phase CH_2Cl_2/MeOH 8:2 v/v) and identified by TLC, and NMR
147 analyses (Procopio et al. 2009).

148 *2.5. Preparation of Oleuropeine (SLN-HtyOle-OL), Hydroxytyrosol (SLN-HtyOle-Hty), 3,4-*
149 *DHPEA-EDA (SLN-HtyOle-3,4-DHPEA-EA) loaded Hydroxytytrosol oleato nanocarriers.*

150 Nanoformulations were prepared employing a modified thin-film hydration method (Zupanĉiĉ et al.
151 2014). OL, Hty and 3,4-DHPEA-EDA (5 mg) within the presence of HtyOle (37.5 mg) and Lutrol®
152 micro 68 (37.5 mg) were individually dissolved in 10 mL of CH_2Cl_2 using flask (50 mL). Each
153 mixture wassonicated for a 1 min employing a VWR ultrasonic cleaner bath USC300T (VWR
154 International Limited, UK). The mixture was evaporated under vacuum employing a rotary
155 evaporator (Hei-VAP Advantage Rotary Evaporator, Heidolph, Germany) until a skinny film was
156 obtained. The resultant thin film, present within the flask, was immediately dispersed in 10 mL of
157 H_2O at temperature (25 °C) for 1-2 minutes, then sonicated for an extra 1 min until the film was fully
158 removed and dispersed within the water.

159 After the purification by means of Amicon® Ultracentrifugal Filters (cut-off 10 KDa, 4000 rpm for
160 60 min), the SLN-HtyOle-OL, SLN-HtyOle-HTy and SLN-HtyOle-3,4-DHPEA-EA dispersions
161 were dried by freeze-drying process employing a Virtis AdVantage 2.0 BenchTop freezedryer (SP
162 Industries, UK) for further analysis. A measurement of PCs recovery during filtration has been made
163 (See supporting information). SLN-HtyOle nanoformulations were prepared as described above and
164 used as an impact treatment within the cellular assays.

165 *2.6. Particle size distribution and zeta potential measurements*

166 The particule size and zeta potential of SLN were measured using Malvern Zetasizer NanoZS
167 (Malvern Instruments, UK). Three independent measurements were performed on each sample. The
168 samples were diluted appropriately with the aqueous phase of the formulation at 25° C.for the
169 measurements. Size distribution was measured via photon correlation spectroscopy (PCS) as Z-Ave
170 hydrodynamic diameter and polydispersity index (PDI). Each formulation was prepared at least six
171 times and measurements were taken in triplicate for each sample (Petkar et al. 2018).

172 *2.7. Percentage drug content into solid nanoparticles.*

173 The loading efficiency of the formulations was determined spectroscopically and, subsequently,
174 confirmed using HPLC (wavelength: 255 nm; solvent: acetonitrile/water (70/30, v/v). The
175 encapsulation efficiency (EE) and the loading capacity (LC) of the formulations were
176 calculadeterminated using the following equations.

177

178 EE (%) = (weight of loaded compound/ weight of compound in feed)) x 100 (1)

179 where

180 (weight of loaded compound) is the weight of encapsulated PC detected spectroscopically within
181 the nanocarriers after 0.22 μm filtration to remove unencapsulated material

182 (weight of compound in feed) is the weight of PC originally added to the formulation.

183

184 LC (%) = (weight of loaded compound / weight of SLNs) x100 (2)

185 where

186 weight of SLNs is total weight of HtyOle, surfactant and PC

187 SLN-HtyOle-OL, SLN-HtyOle-Hty and SLN-HtyOle-3,4-DHPEA-EA formulations (8 mg of
188 nanoparticles) were diluted to 5 mL with methanol (0.1 mg of PC in 1ml) and were analyzed by

reverse-phase HPLC on a Jasco LC-NetII/ADC, UV-2075 detector to estimate OL, Hty or 3,4-DHPEA-EA content. A Luna Altech 4.6 × 150 mm Adsobosphere C18 column with 5 μm particles of bonded silica gel with a guard column (4.6 × 7.4 mm Adsobosphere C18) was used. Absorbance chromatograms were obtained at 255 nm and every one the measurements were performed at room temperature. A binary mixture of acetonitrile/water (70/30, v/v) was applied as mobile phase with a rate of flow 1 mL/min and 20 µl injection loop. The retention time was 1.2 min for OL, 1.4 min for Hty and 4.3 for 3,4-DHPEA-EA. The strategy was validated for linearity, precision and recovery employing a standard solution (200µg/mL; 140µg/mL; 100µg/mL; 70µg/mL;) of OL, Hty and 3,4-DHPEA-EA. All calibration curves (See Supporting Information) showed good linear regression (R^2 = 0.974; R^2 = 0.962; R^2 = 0.972 respectively) over the wide test ranges. The calibration curve was linear within the used solvent. the typical recovery was 99.4% for OL, 98,8% for 3,4-DHPEA-EA and 99.2% for Hty with relative variance (RSD) 0.326 %, 0,187% and 0.521 % (n = 3) (See Supporting Information, Figure S3, S4, S5).

The PCs release was quantified in *vitro* by a membrane dialysis tube method. 75 mg of freeze-dried samples was dissolved in 1 mL of 0.1 M Phosphate buffered saline (PBS, pH 7.4) and was placed into a cellulose acetate dialysis tube (12 kDa, diameter 6,5 mm, 0,32 mL/cm, area surface=3,30 cm^2) (Spectra/Pro, Spectrum Lab, Breda, Netherlands). The dialysis bag was immersed in a flask containing 100 mL of buffer solution and stirred (1000 rpm) a 37 °C. Samples (1 mL of the dissolution medium) were taken at determined time point (1, 2, 4, 6, 8, 10, 16, 24, 36 and 48 h), diluted by adding 4 mL of buffer solution and quantified by HPLC method above described. 1 mL of fresh PBS was adding to flask to the receptacle. This experiment was performed three times.

2.8. DPPH radical scavenging activity.

DPPH reagent standard solution: 0.0039 g of DPPH was dissolved in 100 mL of methanol to generate a 0.1 mM DPPH stock solution; working solutions were prepared fresh daily by diluting the stock solution with solvent sufficiently to reduce the absorbance at 517 nm to 1.00 (±0.02). Stock solutions

(100 mM) of test antioxidants were prepared in methanol, then serially diluted for progressively lowering the concentrations for testing. The stock solution of molecules encapsulated, were prepared dissolving 10 mg of SLN in 2.5 mL of water. 1 mL solution of DPPH (0.1 mM) was added to 1 mL of 100, 50, 25, 12.5, 6.25, 3.125, 1.562 and 0.78 mM of PC-loaded SLNs or free PCs (the concentration was calculated based on the respective encapsulated phenolic compound). An equal amount of methanol and DPPH served as control. After 40 min of incubation in the dark, absorbance was recorded at 517 nm and the experiment was performed in triplicate, using a Jenway 7310/7315 UV Vis Scanning Spectrophotometers. The percentage scavenging was calculated using the formula:

$$\text{Inhibition (\%)} = [(\text{Control} - \text{Test}) / \text{Control}] \times 100 \quad (3)$$

where control is the absorbance of the negative control (DPPH solution) and test is the absorbance of the compounds at different concentrations. The sample concentration providing 50% inhibition (IC_{50}) was calculated by plotting the inhibition percentage vs. the sample concentration.

2.9 Biological Tests

2.9.1 Cell Culture.

The human SH-SY5Y neuroblastoma cell line was purchased from ATCC, The Global Bioresource Center, and maintained in Dulbecco′s modified Eagle′s medium (DMEM) supplemented with 5 mg/mL penicillin/streptomycin, 10 mM L-Glutamine, and 10% (v/v) fetal bovine serum (FBS). Cells were kept in an incubator at 37 °C with a 5% CO_2 atmosphere. After two passages, the SH-SY5Y cells were plated at a density of $2x10^4$ cells/well in a 96-well microplate for the sulforhodamine B assay (SRB) and reactive oxygen species (ROS) assays

2.9.2 Sulforhodamine B (SRB) Cell Proliferation Assay.

The *in vitro* cytotoxicity of the plain and PC-loaded SLNs (OL, HTy and 3,4-DHPEA-EA) against SH-SY5Y cells were evaluated using a SRB assay (Vichai et al. 2006). The neuroblastoma cells,

inoculated in 96-well microtiter plates (200 µL at $2x10^4$ cells/well) were allowed to attach overnight. A stock solution of the nanoparticules (0.1 M in dimethylsulfoxide (DMSO) was diluted to 0,5 µM, 1 µM, 10 µM and 100 µM with the culture medium. The cell media was removed and the PC solutions added to well to achieve a final volume of 200 µL. The obtained plates were incubated under standard conditions for 24 h. The supernatant was discarded and the cells were fixed *in situ* gently via the addition of 100 µL of cold trichloroacetic acid (TCA) 10% (w/v) and incubated for 60 min at 4 °C. The plates were washed 5 times with double distilled water (DDW) and then air-dried. A Sulforhodamine B (SRB) solution (50 µL) at 0.4% (w/v) in 1% acetic acid was added to each well and the plates were incubated at room temperature for 30 min. When the color appears, the excess dye was collected and the plates were washed four times with 1% acetic acid and air-dried. The bound dyes were then solubilized in a 10 mM Tris base (200 µL of 10 mM unbuffered solution, pH 10.5, Sigma) and the plate was shaken for 15 min. The absorbance was measured at 492 nm using a Spectra-Max-190 (Molecular Devices, Sunnydale, USA) microplate reader (Skehan et al. 1990). The plate-by-plate analysis of the test wells relative to control wells was used to determine percent growth, and the ratio of the absorbance in the test well to the absorbance in the control wells × 100 was calculated.

2.9.3 ROS Formation

The cells were plated at $2x10^4$ cells/well dilution in 250 µL, in a black, flat-bottom 96-well plate (Thermo Fisher Scientific) and incubated overnight. The DMSO PC solution was added at the appropriate concentrations diluted in 100 µL fresh culture medium and incubated for 2.5 h. 50 µL of stock solution of 2,7-dichlorofluorescin diacetate in EtOH (DCFH-DA (5000 µM) was added to the wells to obtain a final concentration of 5 µM. The plates were wrapped in aluminum foil and incubated at 37 °C. After 30 min, 100 µL of 6-hydroxy dopamine (6-OHDP, 10000 µM stock solution in fresh medium) was added to achieve a final concentration of 25 µM (Zihua et al 2012) and the

plates wrapped in aluminum foil, incubated for an additional 24 h period, after which DCF fluorescence was read at 530 nm.

ROS formation data were calculated with the following equation :

ROS formation (%) = [(M.F.U. drug DMSO + 6-OHDP cells) – (M.F.U. drug DMSO blank) - (M.F.U. DMSO cells) (M.F.U. 6-OHDP cells) - M.F.U. DMSO cells)] x 100 (4)

where

M.F.U. is the Mean Fluorescence Units

2.9.4 Statistical analysis

The experiments were repeated thrice and the results are expressed as mean ± standard error of the mean (SEM). The data were evaluated by one-way analysis of variance (ANOVA) followed by Tukey's test. The in *vitro* procedures were carried out with n = 3, while cell culture experiments were performed with n = 6 (6 wells per group). The differences among the data were evaluated by the analysis of variance (ANOVA) followed by Tukey-test according to the statistical program SigmaStat1 (Jandel Scientific, Chicago, IL, USA). A *p*-value less than 0.001 was regarded as significant.

3. Results and discussion

The Mediterranean diet, that includes olive oil as one of the major fatty food components, has been reported to prevent and improve neurological diseases. Oleuropein is the major phenolic component present in different parts of the Olive (*Olea europaea* L.) tree. The protective role of oleuropein in preventing neurodegenerative diseases has been reported in several studies (Hornedo-Ortega et al. 2018) and has been related to several mechanisms such as the enhancing of the antioxidant pool of the cerebral region and the decreasing of the release of proinflammatory cytokines and chemokines that prevents the occurrence of neuroinflammation. Oleuropein is a natural phenolic compound

obtained from olive leaves (waste material from the olive production chain), potentially valuable for employment as an extraordinary starting material for the generation of other natural OPs (oleuropein aglycone, oleacein and hydroxytyrosol) considered potential phenolic compounds of interesting therapeutic utility (Cavaca et al. 2017). One of the main restrictions to the therapeutic use of OPs in free radical related disorders, including neurodegenerative diseases, is their poor bioavailability (D'Archivio et al. 2010). In the current work HytOle, of more lipophilic nature than the parent compound Hty, was synthesized using an environmental-friendly and fast procedure and used as solid lipid in the formulation of SLN-HytOle, containing OL, 3,4-DHPEA-EA or Hty. The obtained nanoparticles) were characterized and tested for determining the antioxidant concentration required for the 50% reduction in the DPPH and tested as able antioxidant agents to perform scavenging activities in a biological environment, evaluating the ROS scavenging capacities using the SHSY-5Y tumor cell line.

3.1 Synthesys of HytOle

Procopio et al. developed a protocol for the synthesis of lipophilic hydroxytyrosol fatty acid conjugates using lanthanoid salts as Lewis acid catalysts (Procopio et al. 2009). The method involved the use of erbium triflate in anhydrous conditions and a solvent not environmentally sustainable as acetonitrile. The synthetic method proposed in the present work was more convenient using 2-MeTHF as a green solvent in non-anhydrous conditions with still excellent yield, selectivity of the desired product (93% yield) and an easy work-up process. The HtyOle was characterized using ^{1}H-NMR and ^{13}C-NMR spectroscopy (see Supporting Information).

3.2 Particle size, zeta potential and stability of SLN-HtyrOle based.

The hydrodynamic diameter and zeta potential were detected by DLS.

Please Insert the Table 1 here

As shown in Table 1, the mean particle size of HtyOLe conjugate nanoparticles increased after oleuropein and oleuropein derivatives incorporation, indicating the successful encapsulation of OPs inside the HtyOle nanoparticle.

The SLN-HtyOle particle size was 141 nm, and the PDI value was 0.177. The addition of oleuropein, its suitably synthesized aglycone and hydroxytyrosol increased ($p < 0.05$) particle size of the formed nanoparticles. The measured PDI of SLNs was in all cases approximately 0.2, demonstrating the excellent dispersion of prepared nanoparticles.

The SLNs were stable at 4 °C over a period of 60 days, unchanging mean particle size and PDI at day 1, 30 and 60 (Table 1, gray entries). When the same SLNs were stored at 25 °C, the mean particle size increased (Table 1, white entries) compared to samples stored at 4-8 °C.

3.3 Encapsulation efficiencies, loading capacity and in vitro release of OL, 3,4-DHPEA-EA and Hty

The encapsulation efficiencies *(*EE) and loading capacity (LC) of SLN-HtyOle based containing OL, 3,4-DHPEA-EA and Hty reported in Table 2.

The loading efficiency of all formulations was evaluated through HPLC by comparison with a standard curve obtained using OL, 3,4-DHPEA-EA and Hty respectively solution in methanol at known PC concentrations (Table 2). The results showed that both formulations have a loading efficiency of 99.25%, 98.81% and 99.21% for SLN-HtyOle-OL, SLN-HtyOle-3,4-DHPEA-EA and SLN-HtyOle-Hty ($n = 3$) respectively. The PC concentrations were used as an abscissa and the absorbance was used as the ordinate for standard curve construction (See Supporting Information).

Please Insert the Table 2 here

As shown in Table 2, the EE of PCs in SLN-HtyOle was very high and the SLN-HtyOle-OL and SLN-HtyOle-Hty showed the best encapsulation efficiency. The PCs loading amount were approximately 6 %.

Release curves (Figure 2) showed that free PCs were released rapidly while the encapsulated PCs tended to be released slowly showing better sustained-release properties. Particularly interesting werethe results obtained for the SLN-HtyOle-Hty.

Please Insert the Figure 2 here

The excellent sustained-release behavior might increase the bioavailability of PCs *in vivo*.

3.4. Antioxidant Activity measurated using DPPH Assay.

The significant antioxidant potentials of SLNs-HtyOle based obtained were evaluated by DPPH radical scavenging assay having IC_{50} ranging between 1.13 μM (for SLN-HtyOle-3,4-DHPEA-EA) and 26.19 μM (for OL) (Table 3).

Please Insert the Table 3 here

As expected, the test determining the antioxidant concentration required a 50% reduction in the DPPH as it was performed in an organic environment (namely, in MeOH) and it has been observed an IC_{50} at a concentration 10 times lower for formulated nanoparticles (Table 3, entries 2, 3 and 4) than the corresponding free polyphenol (Table 3, entries 5, 6 and 7). Particularly interesting was the data obtained for SLN-HtyOle which showed an IC_{50} value equal to 6.32 μM (Table 3, entry 1) compared to the value of non-conjugated hydroxytyrosol and not in the form of nanoparticles which showed an IC_{50} value equal to 13.05 μM (Table 3, entry 7). Also, Hty itself has an even lower IC_{50} value (3.22 μM) if it is encapsulated (Table 3, entry 4). The encapsulated OL (Table 2, entry 2) showed a value almost fourteen times lower (1.92 μM) than the free OL (Table 2, entry 5) which showed an IC_{50} value equal to 26.19 μM. Even more significant were the differences in the results regarding 3,4-DHPEA-EA. The encapsulated aglycone (Table 2, entry 3) showed an IC50 value twenty-two times lower (1.13 μM) than the same free aglycone that showed an IC_{50} value equal to 25.23 μM (Table 2, entry 6).

354 *3.5 In vitro antioxidant activity determination*

355 For the evaluation of the antioxidant activity of OPs, a combination of several chemical and cell
356 culture assays are available. Cell culture assays are used to determine the physiological functions and
357 to measure cytotoxic effects in response to oxidative stress or cytoprotection by antioxidants (Gülden,
358 Jess, Kammann, Maser, & Seibert, 2010). Thus, to evaluate the protective effects of the formulated
359 nanoparticles in a cellular system (insulted by a pro-oxidant agent), we employed human neuron-like
360 cells SH-SY5Y as an in vitro model. These cells elicit a functional response like human neurons, such
361 as outgrow neurites and undergo morphological changes when challenged by oxidative stress *in vitro*
362 (Rabelo et al., 2012).
363 In a recent review, R Huang et al. (Huang et al, 2021) clearly showed that ROS play a dual role in the
364 initiation, development, suppression, and treatment of cancer. Furthermore, ROS inhibition is a
365 practical test useful for determining whether an antioxidant agent is able to perform its scavenging
366 activities in a biological environment, thus correlating the results with the bioavailability of the tested
367 compounds. To comprehend whether the increased bioavailability of the encapsulated PCs could
368 reflect stronger antioxidant activities in the cells than the free PCs, we evaluated the ROS scavenging
369 capabilities of the synthesized nanoparticles by determining their ROS levels in a biological
370 environment using the SHSY-5Y tumor cell line. (Figure 3). After exposed SHSY-5Y cells to
371 extracellular ROS attack by 6-OHDP (6-hydroxydopamine) and pretreated with different
372 concentrations of free PCs (OL, Hty, 3,4-DHPEA-EA) and the respective SLN-HtyOle based
373 nanoparticles with and without PCs (SLN-HtyOle-OL, SLN-HtyOle-Hty, SLN-HtyOle-3,4-DHPEA-
374 EA an SLN-HtyOle) (0.1, 0.05, 0.01 and 0.005 µM), % ROS formation was determined by the
375 previously described fluorescence method. In the presence of SLN-HtyOle based nanoparticles, ROS
376 generation was significantly reduced, and a 50% reduction in ROS formation was observed at only
377 0.01 µM. Otherwise, in the presence of free PCs ROS generation was significantly reduced, and a
378 50% reduction in ROS formation was observed at 0.05 µM.Treating SHSY-5Y cells with formulated
379 nanoparticle PC free (SLN-HtyOle), a 25.13% reduction in ROS formation was observed at the same

concentration (0.05 µM) and ~19% ROS formation with formulated nanoparticles containing OL, Hty and 3,4-DHPEA-EA (SLN-HtyOle-OL, SLN-HtyOle-Hty, SLN-HtyOle-3,4-DHPEA-EA respectivelyCell proliferation tests were performed on the SH-SY5Y cells using an SRB assay (Figure 4). The Figure 4 (SRB Assay) showed that using 0.1 µM of all formulations the percentage of control cell growth is almost 100%.

Please Insert the Figure 3 here

Please Insert the Figure 4 here

50% reduction in ROS formation was observed at only 0.01 µM when the cells were pretrattated with formulated nanoparticles. This demonstrates the hypothesis of the correlation between cellular bioavailability and antioxidant activity.

4. Conclusions

In conclusion, SLN-HtyOle and SLN-HtyOLe-OL(Hty)(3,4-DHPEA-EA) were synthetized and characterized for loading efficiency, size and antioxidant activity.

The results showed that both formulations have a great loading efficiency and dimensions over the range of 141-173 nm. Furthemore, the present paper proposes a new, simple and sustainable method for the synthesis of HtyOle and new nanoparticles HtyOle based containing OL, Hty and 3,4-DHPEA-EA. The obtained nanoformulations showed greater antioxidant activity and an excellent sustained-release. . In particular, this paper clearly demonstrated that the increased bioavailability of polyphenolic compounds as oleuropein derivatives is responsible for the compounds' augmented radical-scavenging capacity in an organic medium as well as in a biological environment. The encapsulated OPs slowly released and showed a much lower ROS formation value than the respective free Ops.This demonstrates the effectiveness of the proposed nanoformulations which could be used in the future on *in vivo* models.

Aknowledgements

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Appendix A. Supplementary data associated with this article can be found, in the online version, at http//.....

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Figure and Table Captions

Figure 1. Molecular structure of hydroxytyrosol oleate, Oleuropein, 3,4-DHPEA-EA and Hydroxytyrosol

Figure 2. In vitro release profiles of SLN-HtyOle-OL, SLN-HtyOle-3,4-DHPEA-EA, SLN-HtyOle-Hty and PCs free. Data expressed as means ± SD (n = 3). *** $p < 0.001$ versus corrisponding free drugs

Figure 3. ROS formation: SHSY-5Y cells were exposed to the extracellular ROS attack by 6-OHDP 25 μM (and pretreated with free PCs as OL, Hty and 3,4-DHPEA-EA and the corrispondents nanoparticles ancapsulated in SLN-HtytOle to concentrations 0.1, 0.05 and 0.01 and 0.005 μM). Data expressed as means ± SD of three independent observations. ***$p < 0.001$ versus SLN-HtyOle; ### $p < 0.001$ versus Control.

Figure 4. SRB assay: assay SRB for determination % of control cell growth. Data expressed as means ± SD of three independent observations. ***$p < 0.001$ versus SLN-HtyOle; ### $p < 0.001$ versus Control. **$p < 0.01$ versus SLN-HtyOle; § $p < 0.1$ versus Control.

Table 1. Size distribution and surface charge of SLN-HtyOle, SLN-HtyOle-OL, SLN-HtyOle-3,4-DHPEA-EA, SLN-HtyOle-Hty stored at 25 °C and 4 °C at day 1, 30 and 60.[a]

Table 2. Drug loading and drug entrapment efficiencies of SLN-HtyOle-OL , SLN-HtyOle-3,4-DHPEA-EA and SLN-HtyOle-Hty (n = 3).

Figure 1

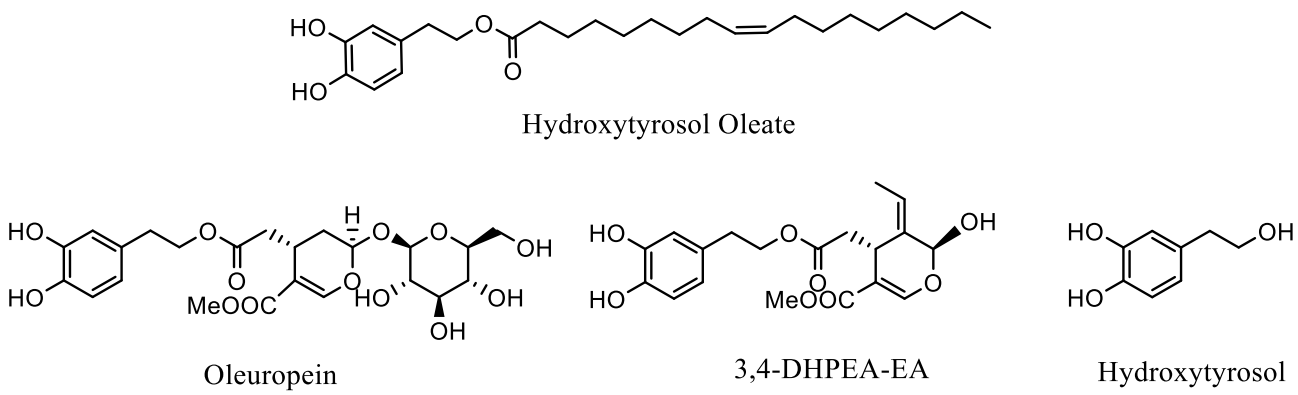

Figure 2

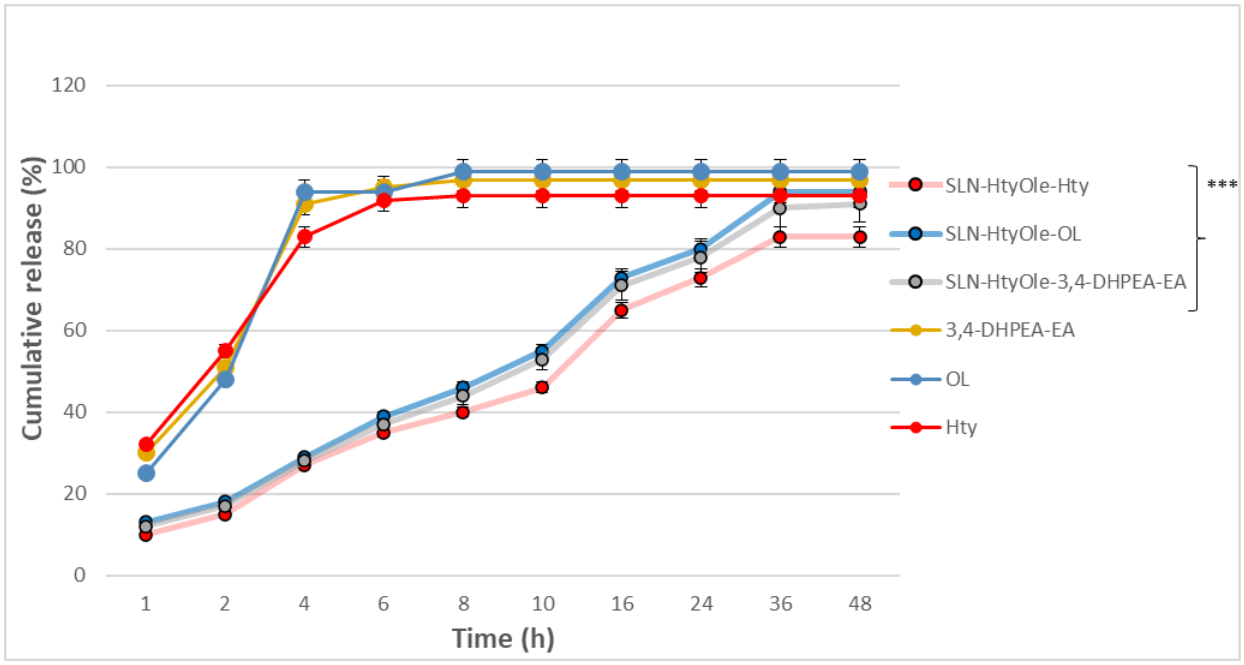

Figure 3

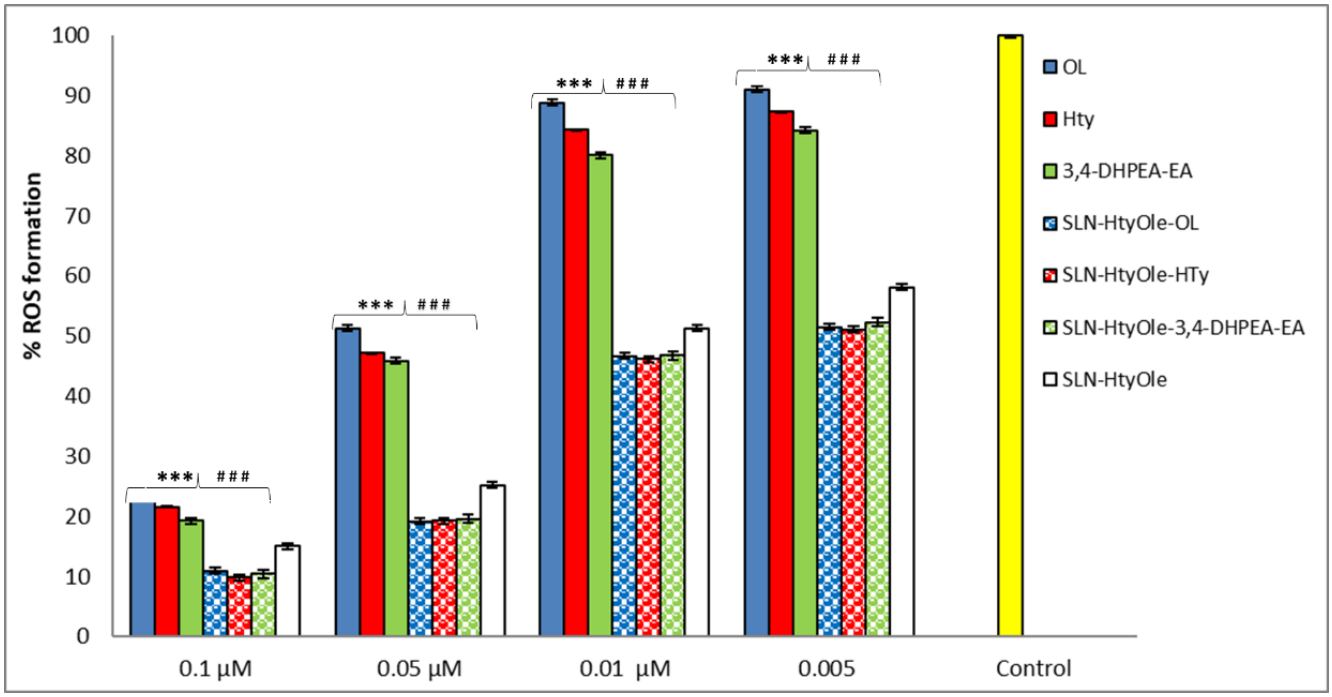

Figure 4

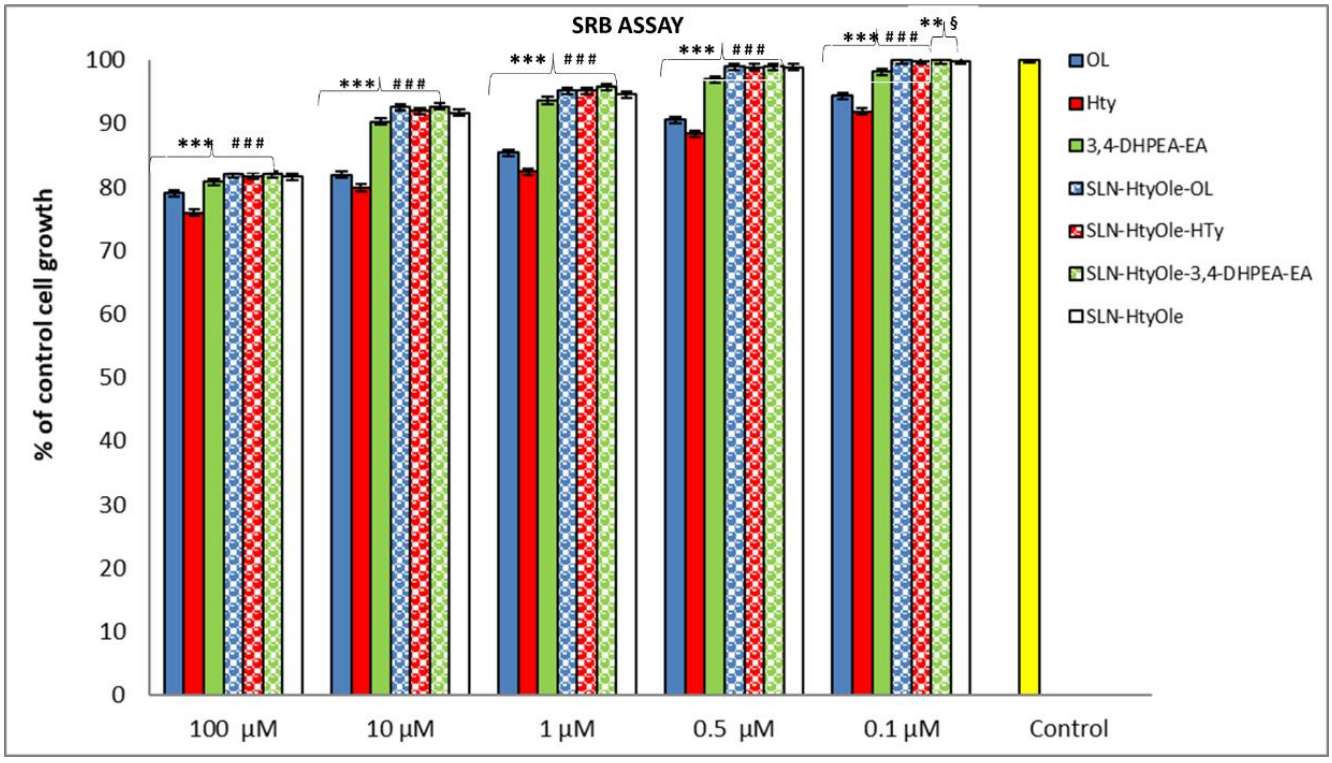

Table 1.

Storage condition	SLN-HtyOle	SLN-HtyOle-OL	SLN-HtyOle-3,4-DHPEA-EA	SLN-HtyOle-Hty
Day 1 (Initial)				
Particle size (nm)	141.3 ± 0.139	147.5 ± 0.153	173.4 ± 0.261	162.8 ± 0.764
PDI	0.177 ± 0.017	0.181 ± 0.027	0.229 ± 0.018	0.233 ± 0.032
Zeta potential (mV)	−41.3 ± 0.349	−10.3 ± 0.599	−23.7 ± 0.643	-28.6 ± 0.586
Day 30 at 4 °C				
Particle size (nm)	***153.3 ± 0.220	***160.7 ± 1.051	***179.1 ± 0.218	***170.0 ± 0.534
PDI	0.170 ±0.012	0.183 ±0.011	0.230 ±0.018	0.235 ± 0.022
Zeta potential (mV)	−41.4 ± 0.151	−10.1 ± 0.112	−23.1 ± 0.206	-28.7 ± 0.570
Day 30 at 25 °C				
Particle size (nm)	***265.0 ± 0.120	***272.3 ± 1.140	***297.2 ± 0.204	***187.8 ± 0.214
PDI	0.191 ±0.012	0.193 ±0.036	0.237 ±0.074	0.240 ± 0.030
Zeta potential (mV)	−43.4 ± 0.254	−12.7 ± 0.312	−25.01 ± 0.276	-29.8 ± 0.554
Day 60 at 4 °C				
Particle size (nm)	***152.9 ± 0.200	***160.9 ± 0.951	***178.9 ± 0.198	***170.6 ± 0.333
PDI	0.171 ±0.012	0.184 ±0.031	0.230 ±0.018	0.235 ± 0.022
Zeta potential (mV)	−41.4 ± 0.151	−10.1 ± 0.112	−23.1 ± 0.206	-28.7 ± 0.570
Day 60 at 25 °C				
Particle size (nm)	***268.2 ± 1.016	***278.5 ± 1.034	***298 .1 ± 1.126	***190.8 ± 0.654
PDI	0.188 ±0.017	0.117 ±0.043	0.225 ±0.011	0.250 ± 0.073
Zeta potential (mV)	−43.99 ± 0.201	−12.9 ± 0.134	−26.19 ± 0.161	-30.2 ± 0.750

[a]Each formulation was prepared at least six times and measurements were taken in triplicate for each sample. (***$p < 0.001$ versus Day 1).

Table 2

	Entrapment efficiency (Mean %, ± SD)	**LC (Mean %, ± SD)**
SLN-HtyOle-OL	99.248 ± 0.327	6.10 ± 0.04
SLN-HtyOle-3,4-DHPEA-EA	98.815 ± 0.187	6.01 ± 0.05
SLN-HtyOle-Hty	99.212 ± 0.521	6.08 ± 0.03

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Table 3. Free radical-scavenging activities measured using the DPPH assay.[a]

Entry	**Compound**	**IC_{50}±SD [µM]**
1	SLN-HtyOle	6.32±0.01
2	***SLN-HtyOle-OL	1.92±0.02
3	***SLN-HtyOle-3,4-DHPEA-EA	1.13±0.05
4	***SLN-HtyOle-Hty	2.67±0.05
5	***OL	26.19±0.24
6	***3,4-DHPEA-EA	25.23±1.06
7	***Hty	13.05±0.15

[a]Data are expressed as the means ± SD of three independent observations. ***$p < 0.001$ versus SLN-HtyOle).

625

Reviewers' comments:

Reviewer #1:

1. Not all the materials used in the study are listed. Please declare the purity of Hydroxytyrosol

   A: In accordance with the Reviewer's suggestion, in the line 117 we reported "Hydroxytyrosol was purchased from TCI AMERICA (Purity: >98.0% (GC)

2. Filtration is not the correct method to remove the free drug for the determination of EE. Dialysis method under cold condition should be used.

   A: It has been a mistake reporting the filtration type. We purified the sample by means of Amicon® Ultracentrifugal Filters (cut-off 10 KDa, 4000 rpm for 60 min). In accordance with the Reviewer's suggestion, we reported the correct used method.

3. What is the surface area of the dialysis membrane used in the release study? The more the area, faster/higher will be release. Please define the area of the membrane exposed to the formulation.

   A: In accordance with the Reviewer's suggestion, in the line 204 we reported "a cellulose acetate dialysis tube (12 kDa, diameter 16 mm, 2ml/cm, A=29,14 cm^2) (Spectra/Pro, Spectrum Lab, Breda, Netherlands)"

4. What is the rationale for dispersing the formulation in 75mg/1 ml of the buffer?

   A: The saturated solubility of Hydroxytyrosol in the water is 5gr/100ml (50mg/ml). The solubility of oleuropeine (less soluble) in the water is 0,73mg/ml. In the formulations it is 10mg of the drugs in the 75mg. However, in order to operate under sink conditions, the investigation of drug release profiles was performed using 1 ml of solution buffer containing 10 mg of the drug, placed into dialysis bags moved into beaker (100 ml of the release medium).

5. Why were cell uptake studies not conducted? Current cell culture-based assays appear to be inadequate make strong conclusions?

   A: The authors' goal was to test the antioxidant activities of the new nanoformulations using neuroblastoma SH-SY5Y cell line. From these results we could start to subsequently carry out cell uptake studies of promising neuroprotective nanocarrier delivery system.

6. Why did authors not select accelerated conditions? Current studies provide only 4 and 25 deg C?

   A: In this work, our aim is to demonstrate the nanoformulations stability at storage temperatures and room temperature even after 60 days. Therefore, we did not consider the use of further drastic conditions appropriate.

Reviewer #2:

This paper deals with the preparation of a new carrier-free drug system using hydroxytyrosol oleate as solid lipid for the synthesis of nanoparticles containing phenolic compounds (PCs) as OL, 3,4-DHPEA-EA and Htyr. The synthesis of hydroxytyrosol oleate was also described. The aim of this work was to evaluate the bioavailability of the encapsulated polyphenolic compounds using hydroxytyrosyl oleate-based solid lipid nanoparticles, their antioxidant activity and ROS formation on neuroblastoma SH-SY5Y cell line. A combination of techniques was used. However, there are some major issues to be addressed before its publication.

1. The article needs extensive English editing. Expression and syntax must be improved throughout the manuscript. Also, there are plenty of typos in the text that should be corrected.

A: In accordance with the Reviewer's suggestion, we reviewed the article making the appropriate corrections.

2. A review of the highlights is recommended.

A: In accordance with the Reviewer's suggestion, we reviewed the highlights.

3. The use of extensive sentences should be avoided.

A: In accordance with the Reviewer's suggestion, we have reduced the length of the sentences

4. Line 126: "1 mol % of $Er(OTf)_3$ (0.0162 mmol)". What does the % refer to? Present just the mmol.

A: In accordance with the Reviewer's suggestion, we reported just mmol

5. Line 141: "The MW-assisted extraction of oleuropein (1)". The number 1 in the bracket to what corresponds to?

A: In accordance with the Reviewer's suggestion, in the line 141, we eliminate the number 1.

6. The results section should be carefully checked and re-edited as many text-flaws were found.

A: In accordance with the Reviewer's suggestion, we revised the results section.

7. 2-MeTHF is a biogenic solvent deriving from biomass, thus its production is indeed environmentally friendly. However, it is highly toxic when used as a solvent. Please make a comment on this, because it is a bit confusing when you describe an environmentally friendly approach for the preparation of hydroxytyrosol oleate.

A: 2-MeTHF is often suggested as a suitable replacement for more traditional solvents because it derives from renewable sources and is more easily recovered in reactions involving an aqueous medium, thus facilitating the recycling and reuse of this solvent. For these reasons 2-MeTHF is generally considered as a greener alternative to some traditional solvents, and its use is advocated by the ACS Green Chemistry Pharmaceutical Roundtable.

However, since to date only limited toxicological information has been disclosed on 2-MeTHF it is currently not included in the International Conference on Harmonisation (ICH) Q3C residual solvent guideline. ICH Q3C reports the Permitted Daily Exposure (PDE) values for a number of solvents which establish the limits below which there would be negligible safety concerns to patients exposed to them as residual impurities in drug products. Nevertheless, preliminary toxicological investigations suggest that exposure to 2-MeTHF is not linked to mutagenicity or genotoxicity [1]. Moreover, since 2-MeTHF is increasingly used as a solvent in the pharmaceutical industry, Parris et al. published data following the ICH methodology to enable calculation of a PDE for 2-MeTHF [2]. Their study, conducted in accordance with United Kingdom Good Laboratory Practice regulations, included multiple dose levels, an appropriate control group, a reversibility phase, and a comprehensive assessment of safety endpoints enabling the calculation of a PDE to support the safe use of 2- MeTHF in the pharmaceutical industry. Taken together these new data depict a low toxicity profile of 2-MeTHF as demonstrated in the current 3-month oral toxicity study in rats, so that should be considered appropriate to attribute a level of 1 (the lowest level, applies to agents that usually pose a minimal potential threat to laboratory workers and the environment and do not consistently cause disease in healthy adults) to safety factor F4 (no serious toxicity observed), resulting in a calculated PDE of 50 mg/day for 2-MeTHF.

[1] Toxicological Assessment of 2-Methyltetrahydrofuran and Cyclopentyl Methyl Ether in Support of Their Use in Pharmaceutical Chemical Process Development V. Antonucci John Coleman,‡ J. B. Ferry, N. Johnson, M. Mathe, J. P. Scott, J. Xu Org. Process Res. Dev. 2011, 15, 939-941. dx.doi.org/10.1021/op100303c

[2] Calculation of a permitted daily exposure value for the solvent 2-methyltetrahydrofuran P. Parris, J. N. Duncan, A. Fleetwood, W. P. Beierschmitt Regulatory Toxicology and pharmacology, 2017, 87, 54-63. http://dx.doi.org/10.1016/j.yrtph.2017.04.012

8. Section 3.2: more concise. The authors repeat some sentences (e.g., Lines 310-312 and Lines 322-324).

A: In accordance with the Reviewer's suggestion, the authors deleted the repeated sentences,

9. Line 332: The authors state: "Interesting is the negative value of zeta potential for all nanoformulations". Can the authors elucidate that and explain why this is important for these nanoformulations and their application?

A: In accordance with the Reviewer's suggestion, the authors deleted the sentence.

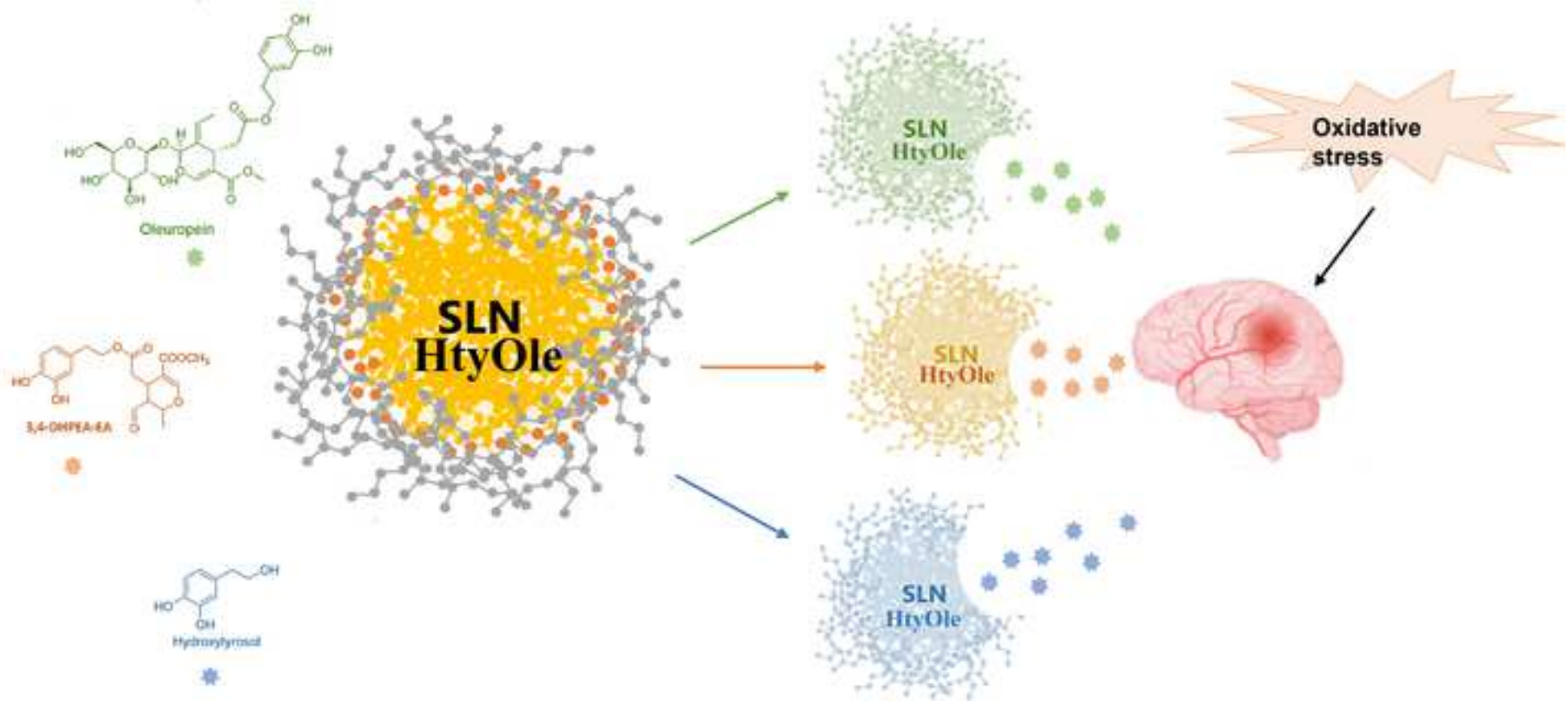
10. In figure 2, the results are expressed as means ± SD of three independent observations? Has a statistical analysis been performed?

A.: The authors reported the statistical analysis in the *2.9.4 Statistical analysis section.* In accordance with the Reviewer's suggestion, the authors reported in the Figure Legends, Figure 2, the sentence ". Data expressed as means ± SD(n = 3). *** $p < 0.001$ versus free drugs. In accordance with the Reviewer's suggestion, the authors modified figure 2

11. Table 3: How do the authors explain that the IC_{50} of free Hydroxytyrosol was the lowest among Ole derivatives whereas the IC50 of Hydroxytyrosol incorporated in SLN nanoparticles was the highest amongst the incorporated compounds? EE and LC were almost the same in any case.

A: In accordance with the Reviewer's suggestion, the authors revised the experimental data and we realized that the % inhibition value, at the concentration of 1.03 uM (see supporting material), was not considered. This caused an IC_{50}±SD [µM] calculation error regarding the encapsulated Hyt (as can be seen from the graph of the supporting material which has been replaced). The new value is 2.67 uM which is not very different from calculated value for other encapsulated PCs.

Graphical Abstract (for review)



COVER LETTER to reviewers

TITLE: Hydroxytyrosol oleate: a promising neuroprotective nanocarrier delivery system of oleuropein and derivatives

The manuscript submitted has been prepared according to the journal's 'Instructions for Authors',

On submission of the manuscript, the authors agree not to withdraw the manuscript at any stage prior to publication.

In this work hydroxytyrosyl oleate (HtyOle)- and hydroxytyrosyl oleate (HtyOle)-based solid lipid nanoparticles containing OL, 3,4-DHPEA-EA and Hty (SLN-HtyOle, SLN-HtyOle-OL, SLN-HtyOle-3,4-DHPEA-EA and SLN-HtyOle-Hty respectively), were prepared and characterized for size and loading efficiency. In addition, we evaluated *in vitro* the antioxidant activity of these formulations by DPPH assays and by ROS formation using the SH-SY5Y cell line.

The manuscript was resubmitted after making the corrections.

The authors answered point by point to the questions and suggestions posed by the reviewers

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Conflicts of Interest

The authors declare no conflict of interest.

Author Statement

☒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.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Hydroxytyrosol oleate: a promising neuroprotective nanocarrier delivery system of oleuropein and derivatives

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Highlights

- We synthesize hydroxytyrosyl oleate (HtyOle)-based solid lipid nanoparticles containing OL, 3,4-DHPEA-EA and Hty
- The nanoparticles were characterized for loading efficiency, size and antioxidant activity.
- We demonstrated that the increased bioavailability of polyphenolic compounds causes an increase in the antioxidant activity of the compounds themselves