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Original article

Fishroesomes as carriers with antioxidant and anti-inflammatory bioactivities

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ABSTRACT

The great diversity of marine habitats and organisms renders them a high-value source to find/develop novel drugs and formulations. Therefore, herein, sardine (*Sardina pilchardus*) roe was used as a lipidic source to produce liposomes. This fish product presents high nutritional value, being its lipidic content associated with important health benefits. Consequently, it can be advantageously used to produce therapeutically active delivery devices. Roe lipids were extracted using the Matyash method. After lipid film hydration and extrusion, sardine roe-derived large unilamellar liposomes (LUVs), designated as fishroesomes, presented a size of ≈330 nm and a significant negative surface charge (≈−27 mV). Radical scavenging assays demonstrated that fishroesomes efficiently neutralized peroxyl, hydroxyl and nitric oxide radicals. Moreover, fishroesomes significantly reduced the expression of pro-inflammatory cytokines and chemokines by LPS-stimulated macrophages at non-toxic concentrations for L929 and THP-1 cells. Consequently, the developed liposomes exhibit unique properties as bioactive drug carriers for inflammatory diseases treatment.

1. Introduction

Marine products are a well-recognized source of pharmaceuticals, nutritional supplements and cosmetic additives [1]. Particularly in inflammation, marine compounds are considered valid alternatives to aid or to treat inflammatory disorders. Fish, for instance, contains antioxidants and anti-inflammatory compounds. Its antioxidants include enzymes (superoxide dismutase, catalase, glutathione peroxidase and glutathione S-transferase), amino acids, peptides, reduced glutathione, gadusol, ascorbic acid, carotenoids, tocopherols, lipoic acid and ubiquinones [2–4]. Compounds with anti-inflammatory action include fatty acids, proteins and polysaccharides [5]. In fish, antioxidant and immune-relevant activities have also been described for vitellogenin, a glycolipophosphoprotein, and its derived yolk proteins, lipovitellin and phosvitin [6]. Consequently, fish can be advantageously used for the development of novel nutraceuticals. Indeed, the search of novel and natural compounds with biological activity has been extensively performed.

Oxidative stress and inflammation are closely interrelated and have a major impact on the development of several and serious inflammatory diseases (e.g., autoimmune diseases). Oxidative stress results from the imbalance between the formation and neutralization of reactive oxygen species (ROS) and reactive nitrogen species (RNS) by endogenous antioxidants, resulting in an excessive accumulation of these species [7]. ROS include peroxyl radicals ($ROO^{\bullet}$), hydroxyl radicals ($^{\bullet}OH$), superoxide anion radicals ($O_2^{\bullet-}$), singlet oxygen, alkoxyl radical, hydrogen peroxide and lipid hydroperoxide, whereas RNS comprise nitric oxide ($NO^{\bullet}$), and species derived from $NO^{\bullet}$, such as peroxynitrite [4]. The production of ROS begins with the uptake of oxygen, activation of NADPH oxidase, and the production of $O_2^{\bullet-}$. This reactive species is then converted to hydrogen peroxide and oxygen, which can interact with iron chelates to produce the highly reactive $^{\bullet}OH$. Conversely, $ROO^{\bullet}$ is formed during oxidative damage of lipids, proteins, DNA and carbohydrates [8]. $NO^{\bullet}$ is continuously produced in large amounts by the enzyme nitric oxide synthase. The reaction of $NO^{\bullet}$ with $O_2^{\bullet-}$ produces peroxynitrite, a very potent oxidant. These radicals can react immediately and virtually with all biomolecules of vital cellular components and lead to the onset of the inflammation signalling. Inflammation is a

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complex process and a necessary protective response of the immune system against a noxious stimulus, such as pathogens or damaged cells. However, when inflammation is excessive, it can lead to numerous pathological conditions [9]. Several chemical mediators regulate this process, including cytokines (e.g., interleukins – IL-1, IL-6 –, tumour necrosis factor – TNF-α), chemokines (e.g., CCL2, CCL5, CXCL8), lipid mediators (eicosanoids and platelet-activating factors), and reactive species that are generated by inflammatory cells [10,11]. Consequently, antioxidants and anti-inflammatory agents are critical to treat chronic inflammation.

Groups of antioxidants and anti-inflammatory agents already available in the clinic or under development include, for instance, vitamins, scavenging compounds and food supplements (e.g., flavonoids) for the former set of compounds [7], whereas the latter group comprises nonsteroidal anti-inflammatory drugs (NSAIDs), glucocorticoids, anti-cytokine therapies, small molecules that block the activity of kinases, resolvins, statins, and small RNAs, for instance [12]. However, some of these therapeutic agents present serious side effects and, consequently, there is still a need for new anti-inflammatory agents with minimal organ toxicity or for new approaches to limit their effect at the diseased tissue. Indeed, nanoparticles can be the solution to increase the therapeutic index of drugs with recognized and strong anti-inflammatory activity.

Considering the beneficial effects of fish, this work explores sardine (*Sardina pilchardus*) roe as a source to produce novel liposomes – fishroesomes - with improved properties as carriers for drug delivery. In fact, it has been reported that sardine consumption reduces oxidative and inflammatory conditions [13] and it has beneficial effects in cardiovascular diseases [14]. Moreover, benefits related to attenuated hyperglycaemia, improved insulin sensitivity and reversed cholesterol transport in type 2 diabetic rats were also described [15]. As sardine, its roe also presents high nutritional value. Indeed, it is an excellent source of ω3 polyunsaturated fatty acids (PUFAs), as recently demonstrated by gas chromatography/mass spectrometry (GC/MS) analyses [16], as well as essential amino acids (e.g., taurine and histidine) and B vitamins (unpublished work). Despite multilamellar liposomes (MLVs) value in particular applications [16], the most commonly used type of liposomes is large unilamellar liposomes (LUVs; formed by a single phospholipid bilayer and a large aqueous core, with a size higher than 50 or 100 nm, depending on the author [17]) due to their advantages, including higher homogeneity and lower size than MLVs. Indeed, these lipid-based nanovesicles are currently in the market [18]. Therefore, the present work aims to develop and characterize fishroesomes with antioxidant and anti-inflammatory properties. To produce fishroesomes, roe lipidic extracts were obtained using a green approach, namely the Matyash or methyl-*tert*-butyl ether (MTBE) method [19]. They were characterized in terms of size distribution, surface potential, stability and morphology. Their cytocompatibility was evaluated using L929 cells and THP-1 cells stimulated or not with lipopolysaccharides (LPS). L929, a mouse lung fibroblast cell line, is widely used for the early cytocompatibility evaluation of biomaterials [20]. THP-1 is a human monocytic cell line commonly used as a cellular model of inflammation, after its stimulation with LPS [21]. The antioxidant activity of the developed liposomes was assessed in vitro against different ROS ($ROO^{\bullet}$, $^{\bullet}OH$ and $O_2^{\bullet-}$) and RNS ($NO^{\bullet}$), using protocols well established in the literature [22–25]. Moreover, the anti-inflammatory activity of the produced liposomes was evaluated by quantifying the amount of pro-inflammatory cytokines (IL-6, IL-1β, TNF-α and IL-23) and chemokines (CCL2) produced by the LPS-stimulated macrophages.

2. Materials and methods

2.1. Materials

1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) was supplied by Avanti Polar Lipids, Inc., Alabaster, AL, USA. 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH), β-Nicotinamide adenine dinucleotide, reduced disodium salt hydrate (NADH), ammonium iron (II) sulfate, dexamethasone, Dulbecco's modified Eagle's medium (DMEM), ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA), fluorescein sodium salt, gallic acid, L-α-phosphatidylcholine from egg yolk, L-Ascorbic acid, LPS from *Escherichia coli* O26:B6, magnesium sulfate, *N*-(1-Naphthyl)ethylenediamine dihydrochloride (NED), N,N,N-Trimethyl-4-(6-phenyl-1,3,5-hexatrien-1-yl)phenylammonium p-toluenesulfonate (TMA-DPH), nitrotetrazolium blue chloride (NBT), phenazine methosulfate (PMS), phorbol 12-myristate 13-acetate (PMA), phosphate buffer saline (PBS), phosphoric acid, sodium bicarbonate, sodium nitroprusside (SNP) dihydrate, sulfanilamide (SA), *tert*-butyl methyl ether, trolox were obtained from Sigma-aldrich, Saint Louis, MO, USA. Antibiotic-Antimycotic, fetal bovine serum (FBS), Roswell Park Memorial Institute (RPMI) 1640 Medium and quant-iT™ Pico Green® dsDNA Assay Kit were acquired from Life Technologies, Paisley, United Kingdom. Celecoxib was obtained from abcr GmbH, Karlsruhe, Germany. CellTiter 96® AQueous One Solution Cell Proliferation Assay was supplied by Promega, Madison, USA. Dimethyl sulfoxide (DMSO) was acquired from AMRESCO, Solon, USA. Ethanol and methanol were acquired from Fisher Chemical, Loughborough, Leicestershire. Glacial acetic acid was supplied by VWR International LLC, Radnor, USA. Hydrogen peroxide 30% (w/w) was obtained from Panreac Química, Castellar del Vallès, Barcelona, Spain. LabAssay™ Phospholipid was supplied by Wako, Osaka, Japan. Micro BCA™ Protein Assay kit was acquired from Thermo Fisher Scientific, Kalamazoo, MI.

2.2. Roe lipids extraction

Roes of sardine (*Sardina pilchardus*), captured at the Portuguese mainland coast, were kindly donated by a local fisherman in October 2017. Samples were weighed, freeze-dried (LyoQuest Plus ECO, Telstar) and stored at − 20 °C until lipids extraction.

Lipids from sardine roe were extracted using the Matyash or methyl-*tert*-butyl ether (MTBE) method, as previously described [16,19]. Briefly, 3 g of a freeze-dried roe sample was homogenized using an Ultra Turrax (T18 Basic, IKA) for 2 min with 120 mL of ice-cold 75% methanol. 300 mL of *tert*-butyl methyl ether was then added, and the resulting homogenized was shaken for 1 h at room temperature (RT). Then, 75 mL of water was added and 10 min later, the solution was centrifuged (5810R, Eppendorf) at 12,000 *g* for 15 min, at 4 °C. The organic phase was collected, dehydrated with magnesium sulfate and then dried under vacuum (RE-301, Stuart) or under a stream of nitrogen to obtain a lipid film.

2.3. LUVs production

The dried lipid film was hydrated with PBS and strongly vortexed to produce MLVs. Then, the MLVs suspension was extruded (Avanti Mini Extruder, Avanti Polar Lipids, Inc) through polycarbonate membranes of 400 and 100 nm of pore size, 43 times, resulting in a LUVs suspension. This procedure was performed at 37 °C.

Liposomes composed of 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) or of egg yolk phosphatidylcholine (EPC) were also produced and used as controls. 4 mM DPPC and 4 mM EPC solutions, both in ethanol, were used to obtain liposomes as described above but, instead of 43, it was performed 21 passages through the polycarbonate membranes. DPPC and EPC MLVs and subsequent LUVs preparation were also carried out above the respective phase transition temperature (45 °C and RT, respectively).

To evaluate the antioxidant activity of the produced liposomes, an appropriate amount of the fluorescent probe, namely TMA-DPH, was added to LUVs suspension in a 35:1 lipid to probe ratio.

2.4. LUVs characterization

2.4.1. Phosphatidylcholine quantification

The phosphatidylcholine (PC) concentration of LUVs was determined by LabAssay™ Phospholipid, following the recommendations of the manufacturer. Briefly, 2 μL of sample or standard solution and 300 μL of colour reagent were mixed and incubated at 37 ºC for 5 min. The absorbance at 600 nm was measured using a microplate reader (Synergy HT, BioTek). PC concentration for each sample was calculated using a standard curve of concentration (ranging from 0 to 596.1 mg/dL) versus absorbance. As phosphatidylcholine is the predominant phospholipid in fish eggs [26], its content was generally referenced as the fishroesomes concentration. Additionally, it was recently demonstrated that this phospholipid accounted for about 87.6% of the total phospholipids in the MTBE extracts [16].

2.4.2. Size distribution and zeta potential

The size and polydispersity index (PDI) of the LUVs suspension were evaluated by dynamic light scattering (DLS), and the zeta potential was determined by laser Doppler micro-electrophoresis using a zetasizer (Nano ZS, Malvern Instruments Ltd). The measurements were performed at 37 ºC and the liposomes concentration was 500 μM.

2.4.3. LUVs stability

The storage stability of the LUVs, kept at 4 ºC and under static conditions, was assessed for one month through the regular determination of their size, PDI and zeta potential, as just described.

2.4.4. LUVs morphology

The morphology of the produced liposomes was assessed by scanning transmission electron microscopy (STEM) using a high-resolution field emission scanning electron microscope (Auriga Compact, Zeiss). LUVs concentration (PC content) was adjusted to 10 μM and the suspension was disposed into the surface of a support film of carbon (type-B 400 mesh Cu). Microscope observation was carried out at 25 kV.

2.5. In vitro antioxidant activity

The LUVs scavenging activity against $ROO^{\bullet}$ and $^{\bullet}OH$ was assessed by measuring over time the fluorescence of two probes with different solubilities, namely fluorescein (hydrophilic) and TMA-DPH (amphiphilic), as described elsewhere [23]. Both probes lose their fluorescence when oxidized by $ROO^{\bullet}$ or $^{\bullet}OH$.

In the fluorescein assays, the decay on the fluorescence intensity was monitored at excitation and emission wavelengths of 485 ± 20 nm and 528 ± 20 nm, respectively. For TMA-DPH, the fluorescence was recorded at excitation and emission wavelengths of 355 ± 20 nm and 460 ± 20 nm, respectively. Fluorescence measurements were performed using a microplate reader (Synergy HT, BioTek), at 37 ºC, for 180 or 30 min for $ROO^{\bullet}$ or $^{\bullet}OH$ scavenging assays, respectively. Blank was only composed of the radical initiator and fluorescent probe (without the evaluated compounds). Additionally, to guarantee the stability of the probes in the presence of the different tested compounds, the following controls were made and compared: probes with the antioxidant and without the initiator, and probes in the buffer (without neither antioxidant nor initiator). As the fluorescence of the probes was similar in both cases throughout the experiment, it was possible to conclude that the tested compounds do not interfere with the probe behaviour.

2.5.1. $ROO^{\bullet}$ scavenging assay

$ROO^{\bullet}$ were generated in the aqueous phase by the thermodecomposition of AAPH, at 37 ºC.

For fluorescein assays, the reaction mixture contained the following final concentrations of reactants: 48 nM fluorescein, 15 mM AAPH and different concentrations of liposomes (0, 0.5, 1.0, 2.5, 5.0, 12.5, 25.0 and 50.0 μM) or trolox (used as reference antioxidant; 0, 2.5, 5.0, 10.0, 15.0 and 20.0 μM). APPH solution was added immediately before starting the measurements. Data were converted to relative fluorescence values by dividing the fluorescence intensity at a given time by the fluorescence intensity at 0 min. The $ROO^{\bullet}$ scavenger efficiency was determined using Eq. (1):

$$\frac{AUC_{evaluated\ compound} - AUC_{blank}}{AUC_{blank}} \times 100 \quad (1)$$

where AUC is the area under the curve obtained for the fluorescence intensity decay in the presence or absence (blank) of the evaluated compounds. The resulting data were plotted versus the concentration of the tested compounds in order to obtain the profile of their $ROO^{\bullet}$ scavenging activity. Moreover, the scavenger efficiency of the tested LUVs against $ROO^{\bullet}$ was expressed as IC_{50} values, which were calculated as the concentration of each compound required to obtain 50% in the Eq. (1).

In TMA-DPH experiments, the final mixture contained: 15 mM AAPH, and different concentrations of LUVs incorporating the amphiphilic probe in a ratio of 35:1 (0.05, 0.10, 0.18, 0.25, 0.50 and 1.00 mM).

2.5.2. $^{\bullet}OH$ scavenging assay

$^{\bullet}OH$ were generated by Fenton reaction [23]. Fe^{2+}-EDTA solution was added to the samples, followed by the addition of the hydrogen peroxide solution.

For fluorescein assays, the reaction mixture contained the following final concentrations of reactants: 48 nM fluorescein, 1 mM Fe^{2+}-EDTA (1:1.1), 4 mM hydrogen peroxide and different concentrations of liposomes (0, 0.5, 1.0, 2.5, 5.0, 12.5, 25.0 and 50.0 μM) or trolox (0, 2.5, 5.0, 10.0, 15.0 and 20.0 μM). The scavenger efficiency of the tested compounds in the presence of $^{\bullet}OH$ was expressed as previously referred.

For TMA-DPH assays, the final mixture contained: 1 mM Fe^{2+}-EDTA (1:1.1), 4 mM hydrogen peroxide and different concentrations of LUVs with TMA-DPH in a ratio of 35:1 (0.05, 0.10, 0.18, 0.25, 0.50 and 1.00 mM).

2.5.3. $NO^{\bullet}$ scavenging assay

SNP spontaneously produces $NO^{\bullet}$ at physiological pH. Then, $NO^{\bullet}$ interacts with oxygen to produce nitrite ions that can be detected by the Griess reagent. The detection involves the deionization of SA, in an acidic medium, to a diazonium salt, which couples with NED to produce a purple product. The experiment was adapted from that described by Pardau and collaborators [24]. The reaction mixture contained 8 mM SNP and different concentrations of liposomes (0, 5.0, 12.5, 25.0, 50.0, 100.0, 200.0 μM) or gallic acid (used as reference antioxidant; 0, 1.0, 2.0, 5.0, 10.0, 15.0, 20.0, 30.0, 40.0 and 50.0 μM). After incubation of these mixtures for 15 min at 37 ºC and under light [25], 1% SA in 20% glacial acetic acid was added. 10 min later and addition of 0.1% NED in 2.5% phosphoric acid, the absorbance at 540 nm was measured in a microplate reader (Synergy HT, BioTek).

The scavenger efficiency of the tested compounds against $NO^{\bullet}$ was calculated using the Eq. (2):

$$\frac{A_{blank} - A_{tested\ compound}}{A_{blank}} \times 100 \quad (2)$$

where A is the absorbance in the presence or absence (blank) of the evaluated compounds. The resulting data were also plotted against the concentration of the compounds. Additionally, the $NO^{\bullet}$ scavenging activity was expressed as IC_{25} values, which were determined as the concentration of each tested compound required to obtain 25% in the Eq. (2).

2.5.4. $O_2^{\bullet-}$ scavenging assay

$O_2^{\bullet-}$ were generated in the NADH/PMS mixture, in the presence of dissolved oxygen. Then, these radicals reduce NBT to a purple formazan

[25]. The reaction mixture contained the following reagents at the indicated final concentrations: 166 μM NADH, 43 μM NBT, 2.7 μM PMS, different concentrations of liposomes (0, 5.0, 12.5, 25.0, 50.0, 100.0, 200.0 μM) or ascorbic acid (used as reference antioxidant; 0, 10.0, 20.0, 40.0, 80.0 and 160.0 μM). After incubation for 2 min at RT, the absorbance at 560 nm was measured in a microplate reader (Synergy HT, BioTek). The inhibition of the $O_2^{\bullet-}$ production in percentage was determined using Eq. (2).

2.6. Biological assays

2.6.1. Cell culture and seeding

A mouse fibroblast cell line, L929, was cultured in DMEM, supplemented with 10% FBS and 1% antibiotic/antimycotic solution (100 U/mL penicillin and 100 mg/mL streptomycin), and incubated at 37 °C in a humidified 5% CO_2 atmosphere. For the assessment of the biological effects of fishroesomes, cells were seeded on 24-well plates at a density of 1×10^5 cells. L929 cells were used between passage number 19 and 23. Different concentrations of liposomes (0, 0.06, 0.12, 0.25, 0.50 and 1.00 mM) were added to the cell cultures 24 h later. LUVs were sterilized by filtration through membranes of 0.2 μm pore size. 10% DMSO in culture medium was used as the positive control for cell death. Cells were collected after 24, 48 and 72 h of incubation.

Human monocytic cell line, THP-1, was obtained from American Type Culture Collection (ATCC® TIB-202™). These cells, at passage number 11–15, were maintained in RPMI 1640 medium with 2 mM glutamine, 25 mM HEPES, 1% antibiotic/antimycotic solution (100 U/mL penicillin and 100 mg/mL streptomycin) and 10% FBS (complete RPMI), and incubated at 37 ºC with 5% CO_2 in air atmosphere. THP-1 cells differentiation into macrophages was performed as described elsewhere [27]. Briefly, 0.5×10^6 cells were seeded in complete RPMI with 100 nM PMA for 24 h, in 24-well plates. Non-adherent cells were then removed, and the adherent cells were washed twice with complete RPMI. Then, they were incubated for additional 48 h in complete RPMI without PMA. THP-1 cells were incubated, or not, for 2 h with 100 ng/mL of LPS in fresh medium for their stimulation. Afterwards, cells were incubated with different concentrations of LUVs (0, 0.01, 0.02, 0.05, 0.10 and 0.20 mM) or positive controls, namely dexamethasone (10 μM) or celecoxib (10 μM). After 22 h of incubation, cells were retrieved and supernatants were collected and stored at − 80 ºC until further analyses.

2.6.2. Cell metabolic activity

The metabolic activity of the cells was evaluated using the MTS assay (CellTiter 96® AQueous One Solution Cell Proliferation Assay), according to the manufacturer's instructions. At all time-points, cells were incubated with a mixture of culture medium, without FBS and phenol red, and MTS reagent (ratio of 5:1) for 3 h at 37 °C in a humidified 5% CO_2 atmosphere. The absorbance of the MTS reaction medium was then measured in triplicate at 490 nm in a microplate reader (Synergy HT, BioTek). Absorbance is directly proportional to the number of metabolically active cells in culture.

2.6.3. Cell proliferation

Cell proliferation was assessed using the Quant-iT™ Pico Green® dsDNA Assay Kit, in accordance with the manufacturer's instructions. Briefly, 1 mL of ultrapure water was added to the cells and then they were collected and stored at − 80 ºC until further analyses. Samples were defrosted and sonicated for 15 min, before DNA quantification. 28.7 μL of sample or standard solution, 71.3 μL of PicoGreen solution, and 100 μL of Tris-EDTA buffer were mixed and incubated for 10 min at RT. The fluorescence intensity was measured in triplicate at excitation and emission wavelengths of 485 ± 20 nm and 528 ± 20 nm, respectively, in a microplate reader (Synergy HT, BioTek). The DNA concentration for each sample was calculated using a standard curve of DNA concentration (ranging from 0 to 2 μg/mL) versus fluorescence intensity.

2.6.4. Total protein

The total protein content of the cells was determined using the Micro BCA™ Protein Assay kit, following the manufacturer's instructions. Samples were treated as described above for DNA quantification. Then, 150 μL of sample or standard solution and 150 μL of working reagent were mixed and incubated for 2 h at 37 ºC. The absorbance at 562 nm was measured in triplicate using a microplate reader (Synergy HT, BioTek). The protein content for each sample was determined using a standard curve of albumin concentration (ranging from 0 to 40 μg/mL) versus absorbance.

2.6.5. Cell morphology

For the assessment of cell morphology, cells were observed at all time-points under an inverted microscope (AxioVert A1 FL LED, Zeiss), and optical images were acquired at magnifications of 10 and 20x.

2.7. Anti-inflammatory activity

The anti-inflammatory activity of the LUVs was evaluated by their ability to reduce the amount of pro-inflammatory cytokines and chemokines that are produced by LPS-stimulated THP-1 cells after 22 h of incubation. Dexamethasone and celecoxib were used as positive controls. The quantification of IL-6, IL-1β, TNF-α, IL-23 and CCL2 in the culture supernatants was performed by commercially available ELISA kits (R&D Systems, Minneapolis, MN, USA), following the recommendations of the manufacturer. Briefly, a 96-well plate was coated with the respective capture antibody overnight at RT. Then, the plate was washed and blocked with 1% bovine serum albumin in PBS for 1 h, at RT. After washing, 100 μL of culture supernatants or standards were added to each well and incubated during 2 h, at RT. The plate was then washed, and the respective detection antibody was added, being this mixture incubated for 2 h, at RT. After washing, the incubation with streptavidin conjugated to horseradish peroxidase was performed for 20 min, at RT. The plate was washed again and the substrate solution was added to each well for 20 min, at RT. Finally, the stop solution was added to each well and the absorbance was measured at 450 and 540 nm using a microplate reader (Synergy HT, BioTek). Readings at 450 nm were subtracted from readings at 540 nm for wavelength correction. The concentration of cytokines and chemokine for each sample was determined using a standard curve of concentration versus absorbance.

2.8. Statistical analysis

Data are shown as arithmetic means ± standard deviation of at least three independent measurements. Statistical analyses were performed using GraphPad Prism software v5.01 (GraphPad Software, Inc., USA). The statistical significance of the difference between the conditions assessed was analysed using the non-parametric Kruskal-Wallis test followed by Dunn's multiple comparison test. Differences were considered statistically significant when $p < 0.05$.

3. Results

3.1. Characterization of LUVs

The size, PDI and zeta potential of the liposomes produced in this

Table 1
Size, PDI and zeta potential of fishroesomes, DPPC LUVs and EPC LUVs.

Formulations	Size (nm)	PDI	Zeta Potential (mV)
Fishroesomes	331.3 ± 76.8	0.34 ± 0.09	-27.1 ± 3.2
DPPC LUVs	142.9 ± 12.6	0.15 ± 0.07	-2.9 ± 3.8
EPC LUVs	143.9 ± 11.3	0.13 ± 0.07	-3.9 ± 2.5

work are presented in Table 1. Fishroesomes are bigger and present a higher heterogeneity in terms of size (higher PDI values) than the liposomes used as controls (DPPC and EPC LUVs). Moreover, while DPPC and EPC LUVs present slightly negative zeta potential values, fishroesomes have a stronger negative surface charge (Table 1).

In Fig. 1A it is possible to observe that DPPC and EPC LUVs keep their size during the studied period (one month), while the size of the sardine roe-derived LUVs changed few days after their preparation. This illustrates the importance of using these LUVs in a short period of time after their production, without the need to resort to additional procedures. PDI values were constant throughout the experiment only for EPC LUVs, as illustrated in Fig. 1B. Variations in zeta potential over time were also obtained for fishroesomes, whereas this parameter was more constant for the other formulations (Fig. 1C).

In Fig. 1D it is possible to analyse the fishroesomes. The small LUVs seem having a circular cross-section, but the larger ones seem to have some fluidity in its shape having oval cross-sections. They also present a heterogeneous distribution in terms of size, which corroborates DLS measurements.

3.2. Antioxidant activity

3.2.1. $ROO^{\bullet}$ scavenging assay

In the present work, the antioxidant capacity of the tested formulations against $ROO^{\bullet}$, generated by the thermo-decomposition of AAPH at 37 °C, was evaluated using fluorescein and TMA-DPH. In the presence of an antioxidant, the radicals are scavenged and the decay of the probe fluorescence is delayed. The assays performed with the hydrophilic probe demonstrated that increasing concentrations of the liposomes (considering their content in phosphatidylcholine) and the reference antioxidant, trolox, retarded the decay of the fluorescein fluorescence (Fig. 2A-D). Fig. 2E presents the results determined by Eq. (1). It is possible to observe that fishroesomes presented a higher antioxidant activity than trolox as well as EPC and DPPC liposomes, as demonstrated in Fig. 2A-D. Indeed, DPPC LUVs did not present a quantifiable antioxidant activity against these radicals, while EPC LUVs were much less active than sardine roe-derived LUVs for all the tested concentrations (Fig. 2). Table 2 shows the IC_{50} values (calculated as the concentration of each compound required to obtain 50% in Eq. (1)) of the scavenger activity of the tested compounds against $ROO^{\bullet}$. Fishroesomes presented the lowest IC_{50} value, and thus this formulation had a higher antioxidant activity even than trolox, because a lower LUVs concentration is needed to obtain equivalent antioxidant capacity. Consequently, Fig. 2 demonstrates that $ROO^{\bullet}$ are efficiently scavenged by fishroesomes.

In the TMA-DPH assays, liposomes were also able to prevent the oxidation of the probe in a concentration-dependent manner (Fig. 3). Increasing the concentration of all formulations avoided the immediate decay of the probe fluorescence. Fishroesomes were much more active against $ROO^{\bullet}$ than the other LUVs formulations. These results show that sardine roe-derived LUVs act as potent $ROO^{\bullet}$ scavenger even when the active part of the fluorescence probe is in the hydrophobic region of the lipid bilayer.

3.2.2. $^{\bullet}OH$ scavenging assay

The antioxidant efficiency of the liposomes as scavengers of $^{\bullet}OH$, generated by the Fenton reaction, was also assessed by their ability to avoid fluorescein and TMA-DPH oxidation and, consequently, fluorescence decay. The antioxidant activity of the tested formulations was also expressed as IC_{50} values calculated using Eq. (1) (Table 2).

Fig. 4A shows that increasing concentrations of the fishroesomes resulted in an enhanced delay in the fluorescence decrease of fluorescein. Consequently, these liposomes presented antioxidant activity against $^{\bullet}OH$ in a concentration-dependent manner. A similar conclusion was obtained for the reference antioxidant trolox (Fig. 4D). Conversely, DPPC and EPC LUVs did not present ability to scavenge these radicals (Fig. 4B and C). In Fig. 4E and Table 2 is possible to observe that the scavenging activity of fishroesomes in the presence of $^{\bullet}OH$ is comparable to that observed for trolox.

All LUVs formulations prevented the oxidation of TMA-DPH induced

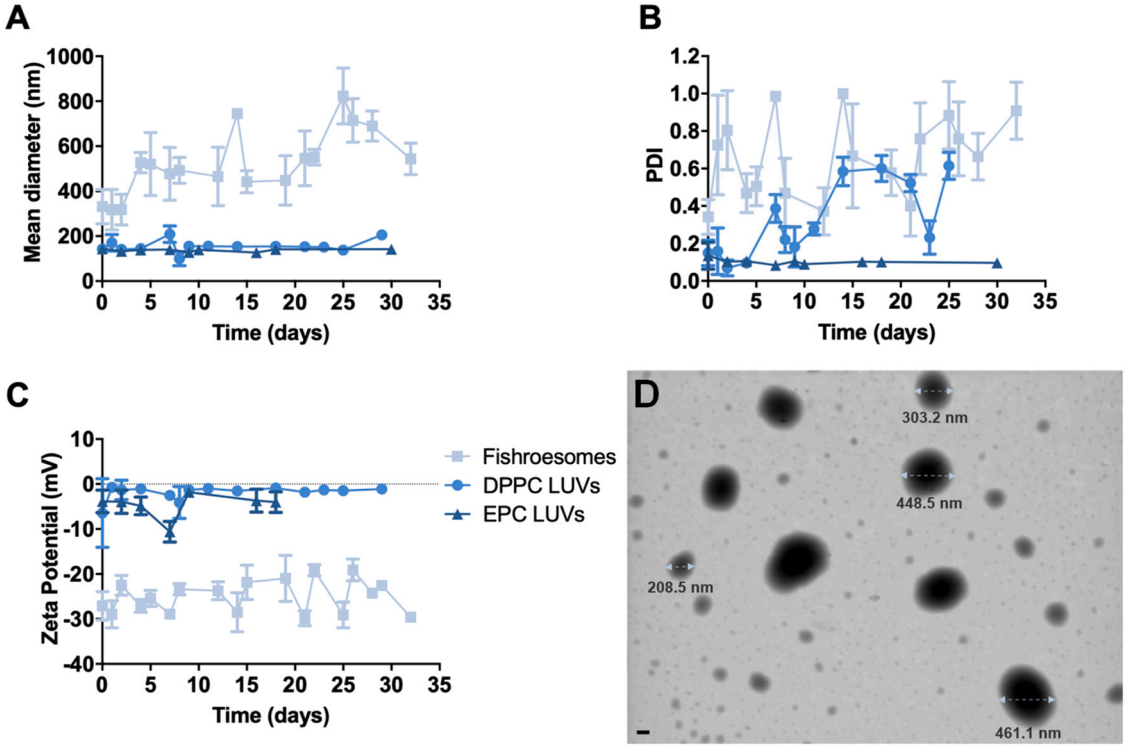

Fig. 1. Size (A), PDI (B) and zeta potential (C) of fishroesomes, DPPC LUVs and EPC LUVs suspensions in PBS, stored at 4 °C for one month. STEM micrograph (D) of the fishroesomes. Scale bar = 100 nm.

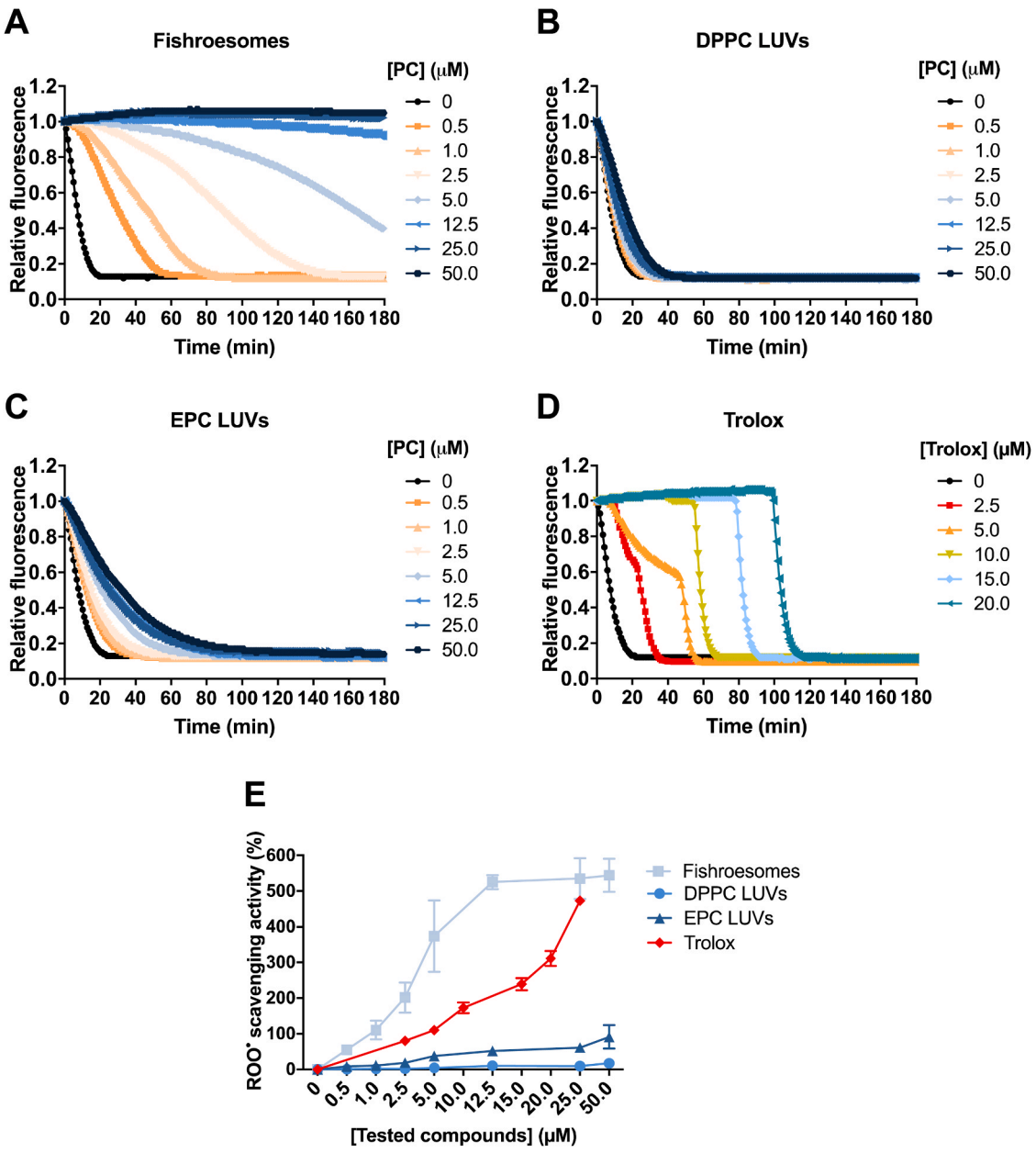

Fig. 2. Relative fluorescence intensity of fluorescein in the presence of ROO• and different concentrations of fishroesomes (A; 0, 0.5, 1.0, 2.5, 5.0, 12.5, 25.0 and 50.0 μM), DPPC LUVs (B; 0, 0.5, 1.0, 2.5, 5.0, 12.5, 25.0 and 50.0 μM), EPC LUVs (C; 0, 0.5, 1.0, 2.5, 5.0, 12.5, 25.0 and 50.0 μM) or trolox (D; 0, 2.5, 5.0, 10.0, 15.0 and 20.0 μM), at 37 °C. Scavenging activity of the tested compounds against ROO• using Eq. (1) versus their concentration (E).

Table 2
Scavenging activities of the tested compounds and respective reference antioxidants against ROO•, •OH, NO• and $O_2^{\bullet-}$.

	ROO•	•OH	NO•	$O_2^{\bullet-}$
Tested compounds	IC_{50} (μM)		IC_{25} (μM)	
Fishroesomes	0.81 ± 0.64	10.35 ± 8.27	83.57 ± 28.15	n.r.
DPPC LUVs	n.r.	n.r.	n.r.	n.r.
EPC LUVs	15.06 ± 3.21	n.r.	n.r.	n.r.
Trolox	1.66 ± 0.22	11.47 ± 8.60		
Gallic acid			7.80 ± 0.31	
Ascorbic acid				13.67 ± 2.44

n.r. — not reached.

by •OH in a dose-dependent manner (Fig. 5). Fishroesomes had a more potent effect against these radicals, since the decay of fluorescence intensity was more retarded compared to the same tested PC concentrations of the other LUVs formulations.

3.2.3. NO• scavenging activity

NO• neutralization by LUVs formulations was evaluated using SNP, which spontaneously generates NO•. When antioxidants are present, they compete with oxygen leading to reduced production of nitrite ions and, thus, lower formation of the coloured product is also observed.

The antioxidant activity of the tested formulations and gallic acid (used as reference antioxidant) against NO• was expressed as IC_{25} values using Eq. (2). Fig. 6 and Table 2 demonstrate that the antioxidant efficiency of fishroesomes was smaller than that observed for the reference antioxidant, since a higher concentration of these liposomes was necessary to obtain the same effect. Moreover, DPPC and EPC LUVs were

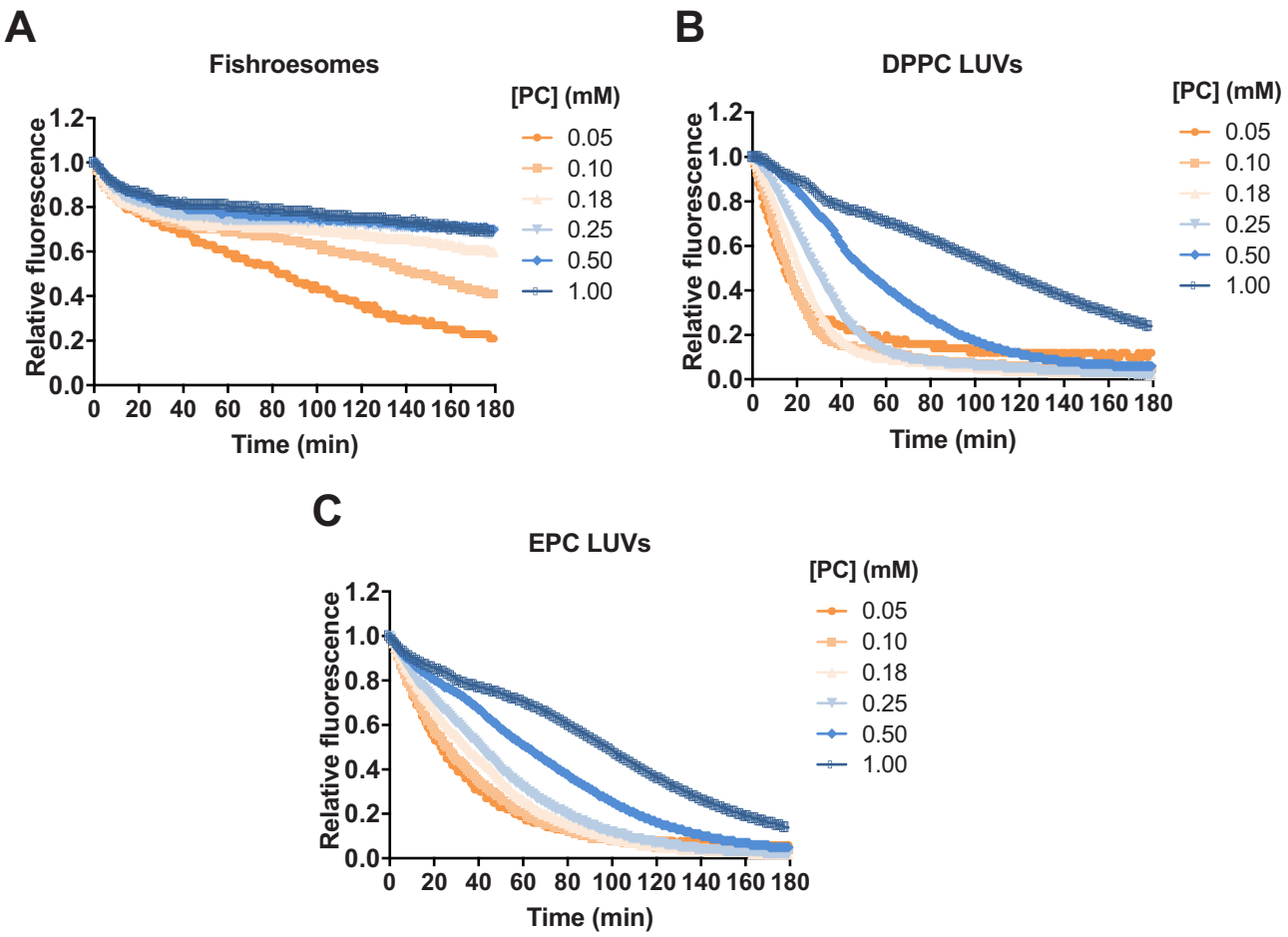

Fig. 3. Relative fluorescence intensity of TMA-DPH in the presence of $ROO^{\bullet}$ and different concentrations of fishroesomes (A; 0.05, 0.10, 0.18, 0.25, 0.50 and 1.00 mM), DPPC LUVs (B; 0.05, 0.10, 0.18, 0.25, 0.50 and 1.00 mM) or EPC LUVs (C; 0.05, 0.10, 0.18, 0.25, 0.50 and 1.00 mM), at 37 ºC.

not able to achieve 25% of $NO^{\bullet}$ scavenging activity (Fig. 6).

3.2.4. $O_2^{\bullet-}$ scavenging assay

In the $O_2^{\bullet-}$ scavenging assay, radicals were produced in the NADH-PMS reaction. When antioxidants are present, they neutralize the radicals leading to a reduced production of the coloured product. The LUVs formulations studied did not present ability to reduce the coloured product and, consequently, they show no activity as scavengers of $O_2^{\bullet-}$ (Table 2).

3.3. Biological assays

Different cell biology protocols were performed to evaluate the effect of fishroesomes on the metabolic activity, proliferation, protein synthesis and morphology of L929 cells and macrophages.

MTS assay results demonstrated that fishroesomes are cytocompatible for concentrations until 0.25 mM in the presence of L929 cells, at all time-points tested (Fig. 7A). However, L929 cell proliferation significantly decreased for LUVs (PC) concentration equal or higher than 0.25 mM when compared to the control, in the time-points tested (Fig. 7B). Regarding the protein concentration, L929 cells were able to synthesize more protein in the presence of 0.06 and 0.12 mM of fishroesomes, at 24 and 72 h, respectively (Fig. 7C). The opposite was observed for the concentrations 0.25, 0.50 and 1.00 mM. The evaluation of the cell morphology corroborated these results. As it is possible to observe in Fig. 7D-F, only the highest tested concentrations (0.50 and 1.00 mM) altered the morphology of L929 cells.

Considering the effect of fishroesomes on the biological performance of the L929 cells, lower concentrations were tested in the in vitro assays performed with non- or LPS-stimulated macrophages. Indeed, a preliminary assay was performed to assess the metabolic activity and morphology of non-stimulated macrophages in the presence of LUVs formulations. This assay demonstrated that only the highest tested concentration of fishroesomes affected the metabolic activity of the non-stimulated THP-1 cells (Fig. 8A). Conversely, the effect of DPPC LUVs was comparable to the control for all tested concentrations (Fig. 8B). The morphology of these cells was not altered by any formulation (Fig. 8C-F).

Similarly, for LPS-stimulated macrophages, only fishroesomes at a concentration equal to 0.20 mM had a negative impact, reducing the metabolic activity of cells by about 50%, and their amount (DNA concentration) and protein synthesis by 40% (Fig. 9A1, B1 and C1, respectively). The morphology of LPS-stimulated macrophages was also not significantly altered by these LUVs (Fig. 9E-G). The effect of DPPC LUVs on macrophages behaviour presented again a good cytocompatibility at all tested concentrations (Fig. 9A2, B2, C2, H and I).

3.4. Anti-inflammatory activity

LPS-stimulated THP-1 cells were used to assess the anti-inflammatory activity of the liposomes. It is possible to observe in Fig. 10 that non-stimulated macrophages do not produce pro-inflammatory mediators. Importantly, this figure also demonstrates that fishroesomes cause a decrease in the production of all the proteins assessed. The decrease on the amount of IL-1β and CCL2 was dose-dependent. For IL-6, the greatest decrease in its content was observed

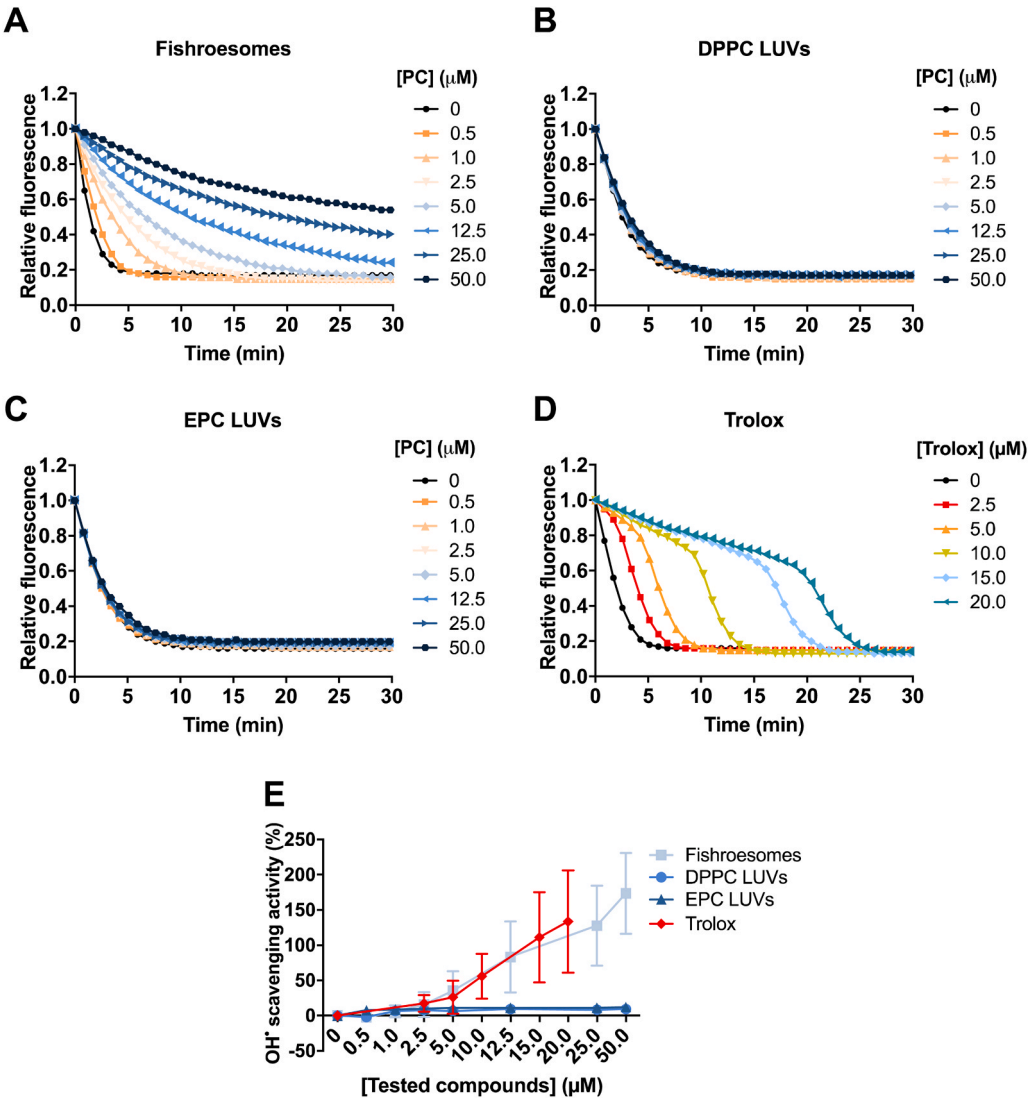

Fig. 4. Relative fluorescence intensity of fluorescein in the presence of •OH and different concentrations of fishroesomes (A; 0, 0.5, 1.0, 2.5, 5.0, 12.5, 25.0 and 50.0 μM), DPPC LUVs (B; 0, 0.5, 1.0, 2.5, 5.0, 12.5, 25.0 and 50.0 μM), EPC LUVs (C; 0, 0.5, 1.0, 2.5, 5.0, 12.5, 25.0 and 50.0 μM) or trolox (D; 0, 2.5, 5.0, 10.0, 15.0 and 20.0 μM), at 37 ºC. Scavenging activity of the tested compounds against •OH using Eq. (1) versus their concentration (E).

for the highest tested concentrations of fishroesomes. Moreover, a high decrease in the amounts of IL-6 (60%), IL-1β (68%), IL-23 (47%) and CCL2 (46%) in the presence of 100 μM fishroesomes is observed. The effect of these liposomes at 12.5 μM on the levels of IL-6 (Fig. 10A) and CCL-2 (Fig. 10E) was similar and superior than that observed for celecoxib, respectively. DPPC LUVs do not affect significantly the amount of cytokines or chemokine. These data suggest that fishroesomes exert a significant anti-inflammatory effect by decreasing the production of some pro-inflammatory cytokines and chemokine.

4. Discussion

The main objective of this work was the preparation of LUVs from sardine roe lipidic extracts, designated as fishroesomes. Their physicochemical characterization, cytocompatibility assessment, as well as their antioxidant and anti-inflammatory activities evaluation were performed to demonstrate the potential of these new drug delivery devices.

LUVs prepared from sardine roe phospholipids presented a higher size (331.3 ± 76.8 nm) and heterogeneity (PDI of 0.34 ± 0.09) than liposomes made of DPPC and EPC (Table 1). Despite the same method was used to prepare the three liposomal formulations, fishroesomes presented a higher size compared to EPC and DPPC LUVs. However, it is well known that the lipidic composition (e.g., presence and percentage of sterols) affects several properties of the liposomes, such as stability, curvature of the bilayer, phase-transition temperature and formation of lipid domains. Moreover, over time, LUVs can suffer modifications in their leaflet compositions to decrease the curvature energy to a minimum. This is probably due to a directional "flip-flop" movement of the phospholipid molecules, since the starting number of phospholipids in the outer leaflet is greater than in the inner leaflet [28]. Therefore, as in this work a mixture of natural lipids was used to produce fishroesomes, after extrusion, they can reorganize to achieve a high stability, and consequently, a higher size was obtained. Zeta potential measurements also demonstrated a significant difference between the liposomes with different compositions. The slight negative zeta potential values observed for DPPC and EPC LUVs are in agreement with those reported elsewhere [29,30]. Conversely, fishroesomes presented a more negative surface charge (− 27.1 ± 3.2 mV), possibly due to the presence of phospholipids with anionic polar head groups, such as phosphatidic acid, phosphatidylserine and phosphatidylinositol [31]. Indeed, sardine roe-derived LUVs present a higher variety of lipids, mainly when compared to DPPC LUVs, which have a homogeneous composition. Lipid composition and also the presence of other fat-soluble biomolecules in the fishroesomes can explain the differences observed in these

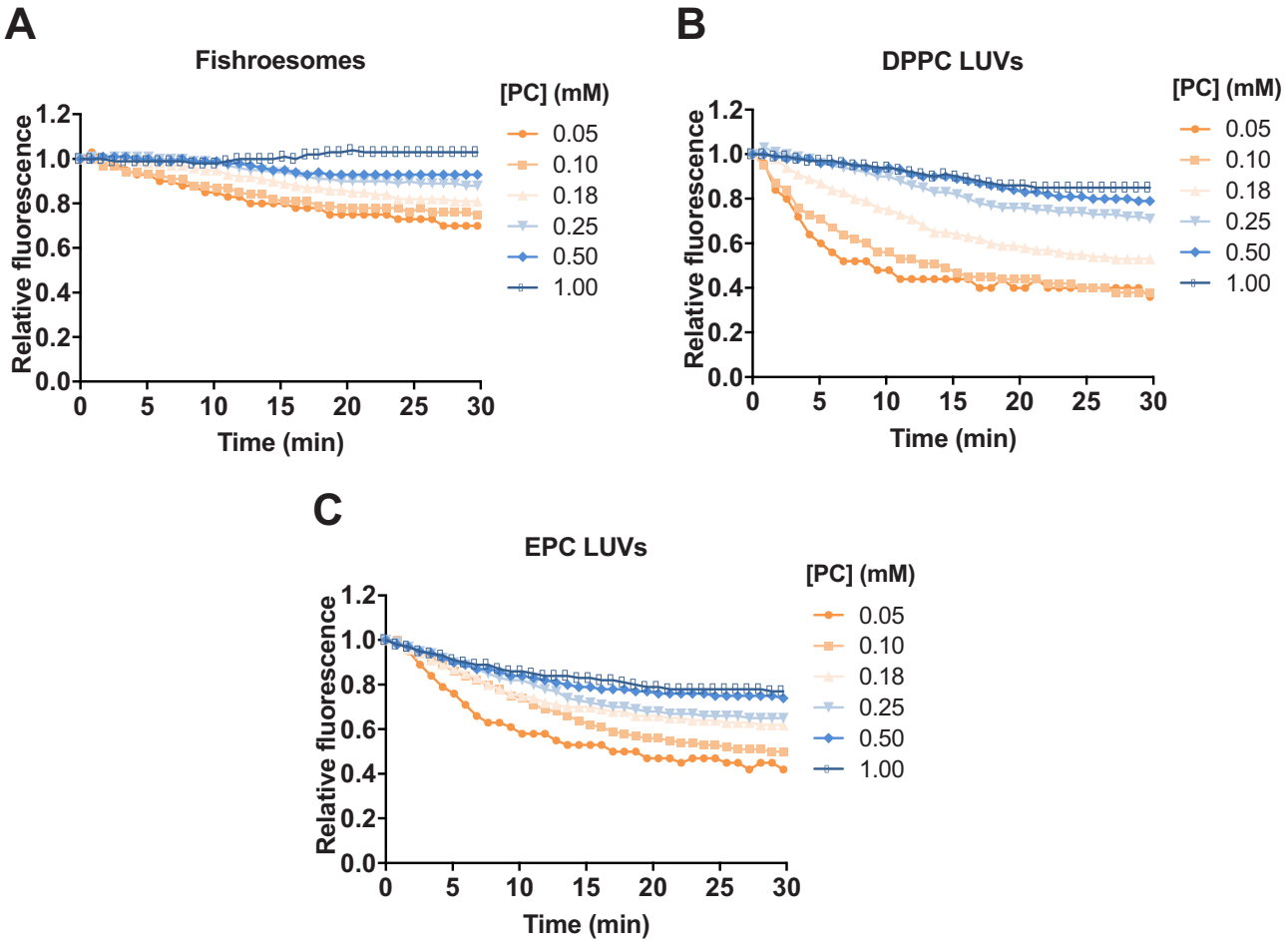

Fig. 5. Relative fluorescence intensity of TMA-DPH in the presence of •OH and different concentrations of fishroesomes (A; 0.05, 0.10, 0.18, 0.25, 0.50 and 1.00 mM), DPPC LUVs (B; 0.05, 0.10, 0.18, 0.25, 0.50 and 1.00 mM) or EPC LUVs (C; 0.05, 0.10, 0.18, 0.25, 0.50 and 1.00 mM), at 37 °C.

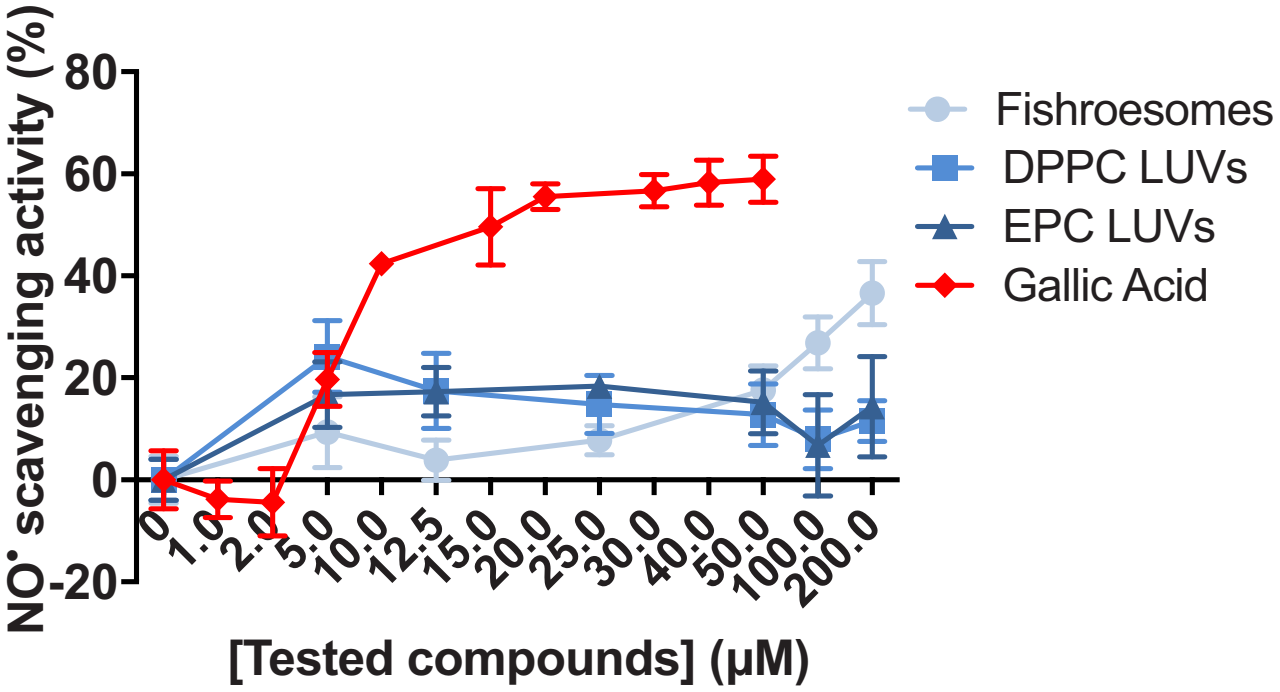

Fig. 6. Scavenging activity of the tested compounds against NO• using Eq. (2) versus their concentration.

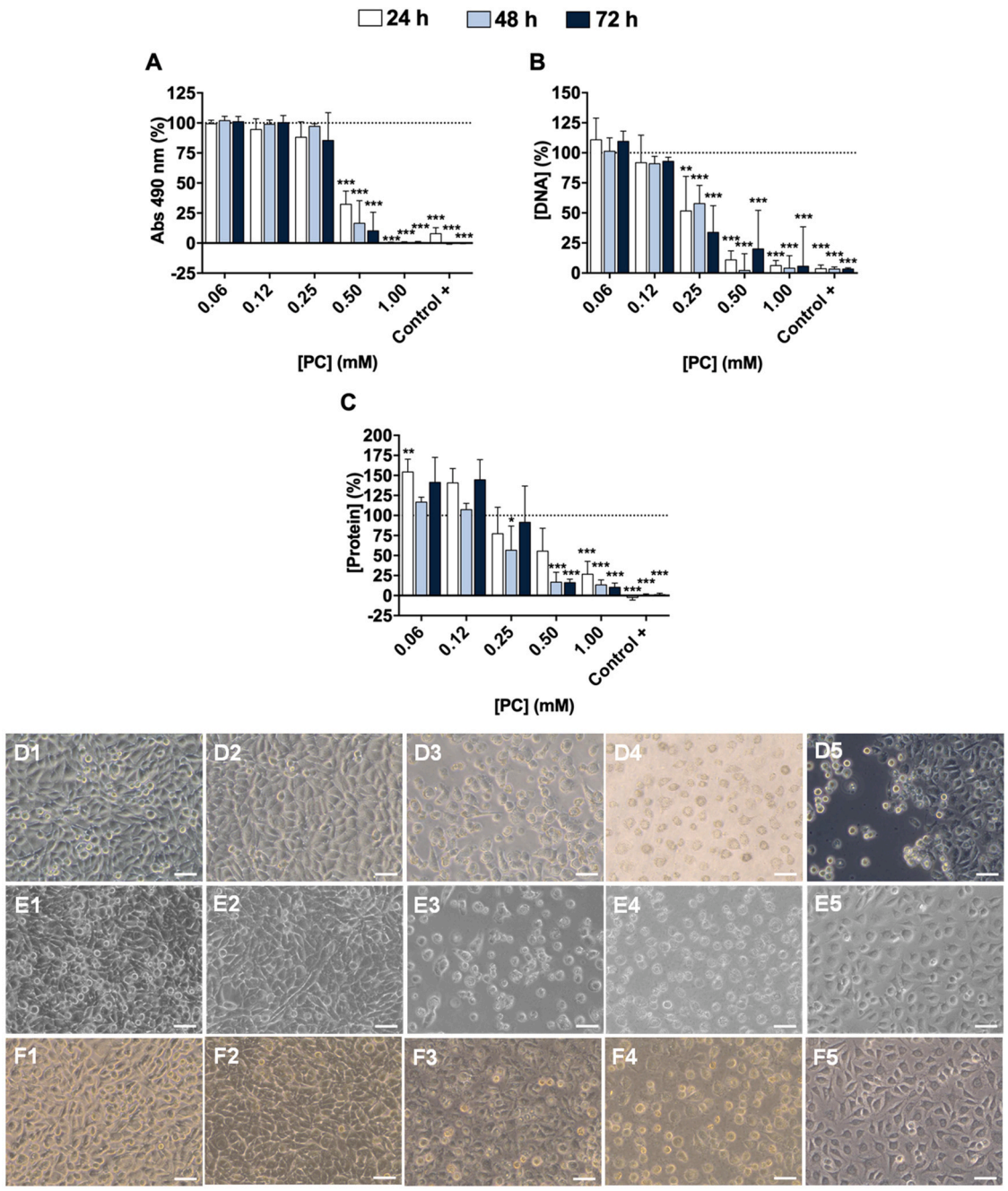

Fig. 7. Effect of fishroesomes and 10% DMSO (positive control for cell death) on the L929 cells metabolic activity (A), DNA concentration (B) and total protein content (C). Cells were or not (control; dashed line corresponding to 100%) incubated with different concentrations of fishroesomes (0.06, 0.12, 0.25, 0.50 and 1.00 mM) or 10% DMSO, for 24, 48 and 72 h. *$p < 0.05$ versus control; **$p < 0.01$ versus control; ***$p < 0.001$ versus control. Optical images of L929 cells incubated with fishroesomes at 0 (1), 0.25 (2), 0.50 (3) and 1.00 mM (4) or 10% DMSO (5) for 24 (D), 48 (E) and 72 h (F). Scale bar = 50 μM.

properties.

The stability is also one of the most important features when envisioning the use of those liposomes after a storage period. The stability of liposome suspensions is influenced by their physical, chemical and biological stability and also by the extent of electrical and/or steric repulsive forces operating between them (zeta potential) [32]. During the storage of the liposomes, phospholipid membranes may degrade, aggregate and fuse, which, in turn, can lead to changes in the mean size and size distribution towards higher values. The stability of the liposomes is evaluated by the changes of these physical properties. The values of size, PDI and zeta potential for fishroesomes are not constant, while for DPPC and EPC LUVs they did not change significantly during the period considered, with the exception of the PDI values of DPPC LUVs from day 4 (Fig. 1A-C). Puskás and Csempesz reported that both the mean size and the PDI of the DPPC liposomes increased after one week of storage at 25 ºC [32]. Although the stability of fishroesomes was lower than that of the other formulations, this can be improved by adding cholesterol, since it has been reported that this molecule can enhance membrane stability [18]. Moreover, these liposomes can be stored in the lyophilized form and reconstituted immediately before use.

The assessment of the antioxidant activity of sardine roe-derived LUVs was carried out against different ROS ($ROO^{\bullet}$, $^{\bullet}OH$ and $O_2^{\bullet-}$) and RNS ($NO^{\bullet}$). At low levels, ROS and RNS contribute to cell signalling, mitochondrial respiration and biogenesis, but at high levels, they can damage essential biomolecules, leading to, for instance, lipid peroxidation, protein oxidation and DNA strand scission [33]. These

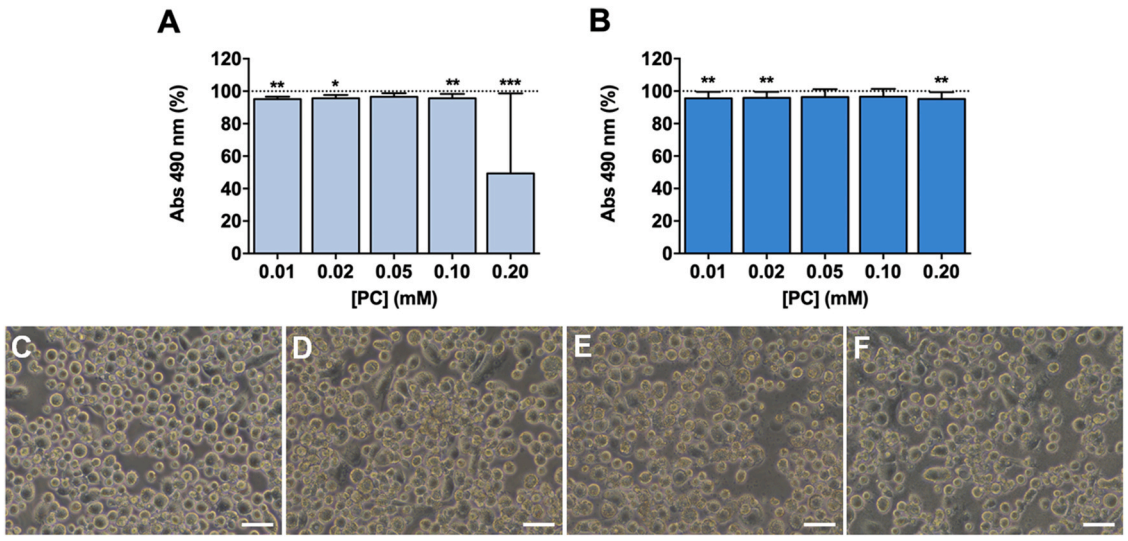

Fig. 8. Effect of fishroesomes (A) and DPPC LUVs (B) on the metabolic activity of non-stimulated macrophages. Cells were or not (control; dashed line corresponding to 100%) incubated with different concentrations of LUVs (0.01, 0.02, 0.05, 0.10 and 0.20 mM), for 24 h. *$p < 0.05$ versus control; **$p < 0.01$ versus control; ***$p < 0.001$ versus control. Optical images of non-stimulated macrophages incubated with fishroesomes at 0 (C), 0.10 (D) and 0.20 mM (E) or DPPC LUVs at 0.01 mM (F), for 24 h. Scale bar = 50 μM.

modifications impair different physiological functions and lead to the development of several disorders, including inflammatory diseases. The protection of organisms against the negative effects of reactive species is therefore extremely important. Several endogenous antioxidants can impair the negative effects of the reactive species, but they may be decreased during oxidative stress and inflammation. In this case, exogenous antioxidants with effective scavenging action against reactive species have an added importance. Consequently, there is a growing need to study the antioxidant ability of different compounds, envisioning their application in the prevention or therapy of these disorders.

The antioxidant capacity of the tested formulations against $ROO^{\bullet}$ and $^{\bullet}OH$ was evaluated in the presence of probes with different solubilities and, consequently, they have stronger interactions with different compartments of the liposomes. Fluorescein is in the aqueous media surrounding the liposomes, while the active part of the TMA-DPH is located in the LUVs hydrophobic region, and thus the interaction between this probe and free radicals occurs within the lipid bilayer [34]. Moreover, TMA-DPH is almost nonfluorescent in the aqueous media [35]. Despite $ROO^{\bullet}$ and $^{\bullet}OH$ being produced in the aqueous phase, they are able to diffuse easily through the bilayer and react with hydrocarbon chains promoting their oxidation [36]. The results obtained in this study demonstrate that $ROO^{\bullet}$ and $^{\bullet}OH$ can be efficiently scavenged by fishroesomes, regardless of the location of the fluorescent probe. Indeed, the existence of a plateau in the fluorescence assays (Figs. 2–5) indicates that the tested formulations react much more rapidly with the radicals than the probe [23]. Once the antioxidant compounds are consumed in the reaction, the decay of the signal is similar to that of the blank.

In the fluorescein assays, the effect of fishroesomes against $ROO^{\bullet}$ and $^{\bullet}OH$ was superior (Fig. 2), or similar (Fig. 4), to that observed for trolox, respectively. Besides trolox, DPPC and EPC LUVs were also used as controls, since these synthetic and natural phospholipids, respectively, have been extensively used in liposomes preparation. DPPC LUVs were used as negative control for the assessment of LUVs antioxidant and anti-inflammatory properties, because it was previously shown that DPPC does not have antioxidant effect [37]. EPC LUVs were used as positive control due to their improved antioxidant activity, when compared with other phospholipids [2,38]. However, in this work, DPPC and EPC LUVs presented less antioxidant efficiency against $ROO^{\bullet}$ than fishroesomes and trolox, and they do not act as $^{\bullet}OH$ scavengers.

Regarding TMA-DPH assays, sardine roe-derived LUVs were more potent as $ROO^{\bullet}$ (Fig. 3) and $^{\bullet}OH$ (Fig. 5) scavengers than DPPC and EPC LUVs. Unexpectedly, the effect of DPPC LUVs as $ROO^{\bullet}$ scavenger was similar to that observed for EPC LUVs. This result may be explained by the fatty acids composition of DPPC LUVs. The presence of saturated fatty acids decreases the membrane fluidity of liposomes, in comparison with unsaturated fatty acids [39]. Consequently, radicals may have limited access to initiate peroxidation along the hydrocarbon chains of the DPPC LUVs. In addition, the chain reaction of lipid peroxidation is unable to propagate efficiently [36].

The present study demonstrated that fishroesomes can act as scavengers of $NO^{\bullet}$, although they are less potent than the reference antioxidant used, gallic acid (Fig. 6). Some studies showed that lipidic extracts from various tissues of seafood species reduced significantly the production of $NO^{\bullet}$ and the expression of the inducible nitric oxide synthase (iNOS) gene, an isoform of the enzyme that synthesizes $NO^{\bullet}$ [40–42]. This effect can be caused by the presence of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), as suggested by Allam-Ndoul and co-authors [43]. Moreover, DHA-loaded liposomes inhibited the activation of nuclear factor kappa B, which plays a crucial role in inducing $NO^{\bullet}$ production via iNOS [40]. Additionally, herein it was shown that fishroesomes, as well as DPPC and EPC LUVs, do not exhibit $O_2^{\bullet-}$ scavenging activity under the experimental conditions tested (Table 2).

The antioxidant activity of fishroesomes can be due to the presence, as just referred, of ω3 PUFA, but also of others lipophilic antioxidants, such as tocopherols (e.g., vitamin E), carotenoids (e.g., β-carotene, astaxanthin, canthaxanthin), ubiquinones (e.g., coenzyme Q10), lipoic acid and phospholipids [2]. Indeed, coenzyme Q10 and vitamin E were identified in the muscle tissue of the sardine species used in this work [44]. The lipophilic antioxidants mentioned can have different mechanisms of action [2]. Tocopherols, carotenoids and ubiquinones can inhibit the propagation of free radical reactions, such as lipid peroxidation. Tocopherols, carotenoids and phospholipids can reinforce the activity of other antioxidants. Phospholipids can also act as metal scavengers, inhibiting metals to catalyse the production of free radicals. In addition, carotenoids can inactivate singlet oxygen, and it has been proposed that ubiquinones and phospholipids are able to recycle other antioxidants, such as vitamin E. All the antioxidants present in the fishroesomes may exert a synergistic effect in the scavenging of the radicals. There are studies that report an effective antioxidant activity of phospholipids in synergy with α-tocopherol extracted from different marine species, including sardine [37,45].

Since both ROS and RNS are produced by immune cells, such as

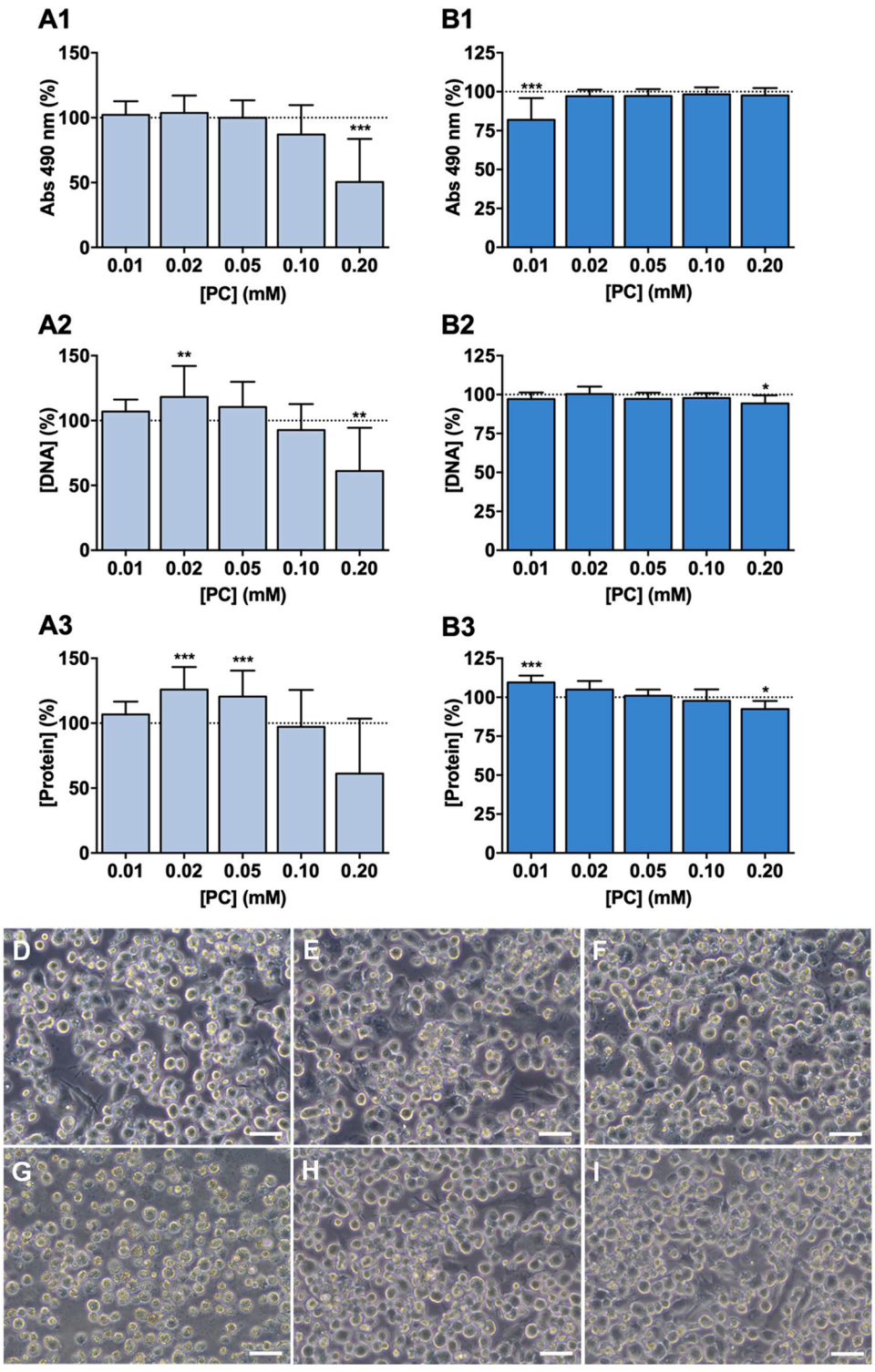

Fig. 9. Effect of fishroesomes (1) and DPPC LUVs (2) on the LPS-stimulated macrophages metabolic activity (A), DNA concentration (B) and total protein content (C). Cells were or not (control; dashed line corresponding to 100%) incubated with different concentrations of LUVs (0.01, 0.02, 0.05, 0.10 and 0.20 mM), for 22 h. *$p < 0.05$ versus control; **$p < 0.01$ versus control; ***$p < 0.001$ versus control. Optical images of LPS-stimulated macrophages incubated with fishroesomes at 0 (D), 0.05 (E), 0.10 (F) and 0.20 mM (G) or DPPC LUVs at 0.01 (H) and 0.20 mM (I), for 22 h. Scale bar = 50 μM.

activated macrophages, during the inflammatory process [46], they are considered reliable markers for the screening of new anti-inflammatory treatments [42]. The observed ability to scavenge these inflammatory mediators may also contribute to the anti-inflammatory effects of fishroesomes.

The effect of the produced LUVs on the biological performance of L929 and THP-1 cells stimulated, or not, with LPS was concentration-dependent (Figs. 7–9). Some studies evaluated the cytocompatibility of lipidic extracts and/or liposomes composed of phospholipids obtained from fish or fish oil. Similarly to what was observed in the present work, these authors describe that their formulations do not impair the performance of the cells or have slight negative effects. For instance, salmon liposomes did not present any toxic effect on the neuronal metabolic activity [47]. Another study demonstrated that liposomes encapsulating DHA extracted from fish oil reduced the viability of LPS-stimulated THP-1 cells by about 30%, at the highest tested concentration (0.5 mM of total lipid) [40]. Considering non-stimulated RAW264.7 macrophages, lipids extracted from *Arctoscopus japoniscus* eggs did not

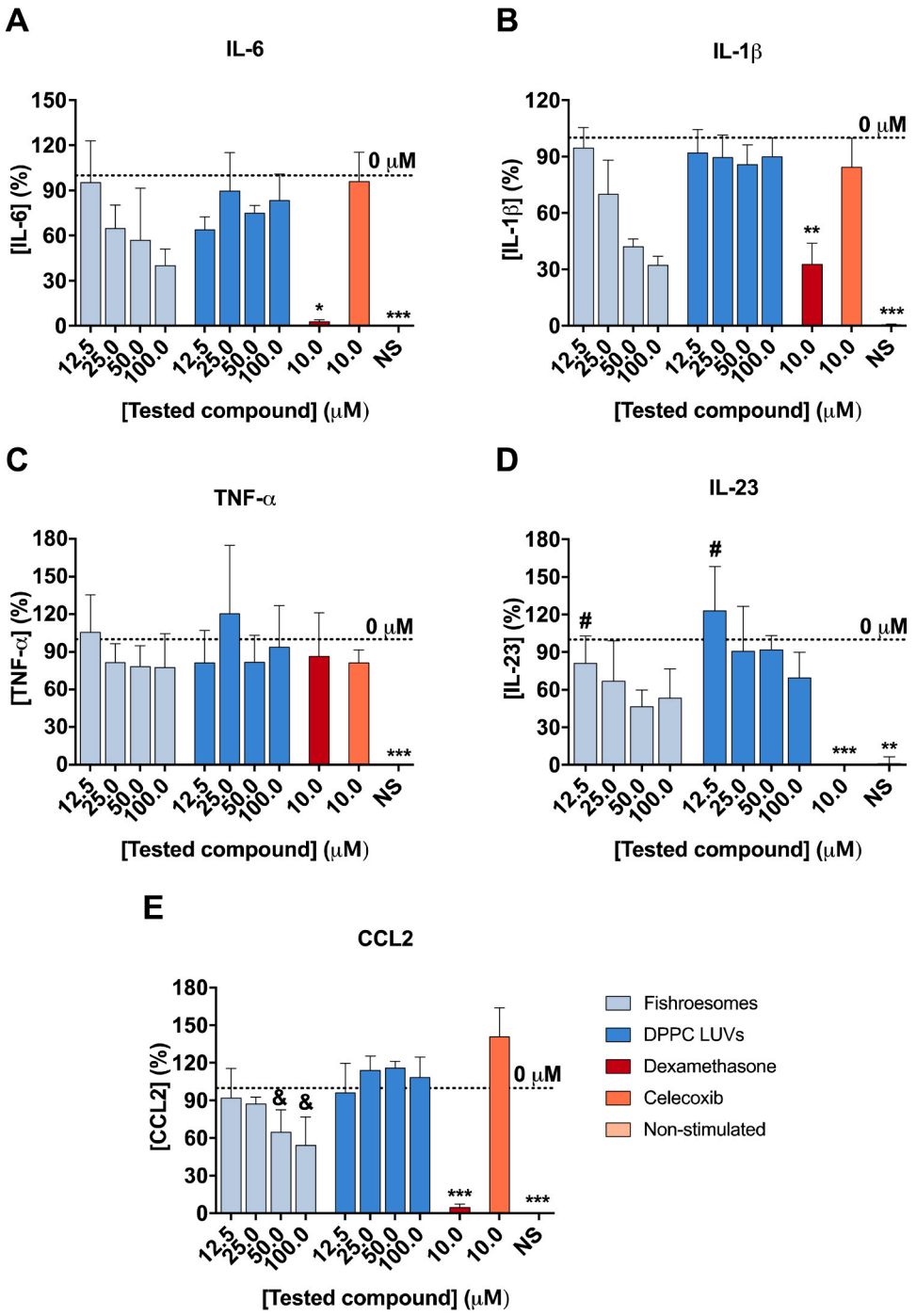

Fig. 10. Effect of fishroesomes, DPPC LUVs, dexamethasone and celecoxib on the production of IL-6 (A), IL-1β (B), TNF-α (C), IL-23 (D) and CCL2 (E) by THP-1 cells stimulated, or not (NS), with LPS. Cells were incubated with different concentrations of LUVs (12.5, 25.0, 50.0 and 100.0 μM), dexamethasone (10 μM) or celecoxib (10 μM), for 22 h. The dashed line represents the nontreated condition (0 μM; LPS positive control) for each assay. *$p < 0.05$ versus control; **$p < 0.01$ versus control; ***$p < 0.001$ versus control; # $p < 0.05$ versus dexamethasone; & $p < 0.05$ versus celecoxib.

decrease their proliferation (2% lipids was the highest tested concentration) [41]. Finally, none of the lipid extracts from common Australian seafood by-products at 50 μg/mL reduced the cell viability of 3T3-ccl-92 fibroblasts and non-stimulated RAW 264.7 macrophages [42]. The highest concentrations of fishroesomes negatively affected the cell behaviour, possibly due to the presence of free fatty acids, which have toxic effects on mammalian cells [48]. However, this can be improved by submitting the lipid extracts to a chemical extraction before sardine roe-derived LUVs preparation [16].

The anti-inflammatory effects of sardine roe-derived LUVs and DPPC LUVs were evaluated using LPS-stimulated macrophages. Macrophages are very sensitive to LPS, expressing many inflammatory cytokines (e.g., IL-1β, IL-6, TNF-α and IL-23) and chemokines (e.g., CCL2) [11,21]. The pro-inflammatory cytokines and chemokines selected in this work play important roles in the inflammatory process, including the initiation of the inflammatory response and the recruitment and activation of immune cells to the site of injury. Fishroesomes reduced LPS-induced cytokines production in a dose-dependent manner (Fig. 10). The anti-inflammatory effect of these liposomal formulations was compared with that of clinically used anti-inflammatory drugs, namely dexamethasone and celecoxib. As Fig. 10 illustrates, the treatment of LPS-stimulated macrophages with the highest tested concentration of fishroesomes resulted in a similar decrease in IL-1β and TNF-α levels compared to dexamethasone. Moreover, the inhibitory effects of these LUVs, at all tested concentrations, were similar or superior than those observed for celecoxib. This effect may be related to the high levels of ω3

PUFAs, namely DHA and EPA, present in the lipidic extracts of sardine roe, previously analysed by our group using a GC/MS technique [16]. Indeed, these fatty acids are well known for their anti-inflammatory properties. Several mechanisms may explain these actions. DHA and EPA suppress leukocytes and macrophages infiltration, inhibit adhesion molecule expression and leukocyte-endothelial adhesive interactions, reduce the production of eicosanoids (e.g., prostaglandins and leukotrienes) from arachidonic acid and inflammatory cytokines (e.g., TNF-α and IL-6), modify T cell reactivity, and increase the production of anti-inflammatory endocannabinoids and inflammation resolving mediators (e.g., resolvins, protectins, and maresins) [5]. Moreover, these fatty acids effectively inhibit the expression of several cytokines by LPS-stimulated macrophages. For instance, DHA-loaded liposomes inhibited the production of TNF-α and CCL2 in LPS-stimulated THP-1 cells [40]. The ability of DHA to inhibit the expression of IL-1β, IL-6 and TNF-α genes in LPS-stimulated THP-1 cells was also already reported [49]. Additionally, *A. japoniscus* egg lipids suppressed the expression of these genes by LPS-stimulated RAW264.7 macrophages [41]. Lipidic extracts from commonly consumed Australian seafood also reduced the levels of TNF-α in this murine macrophage cell line [42].

Importantly, the antioxidant and anti-inflammatory activities of fishroesomes can also be attributed to the fact that these fatty acids are in the phospholipids, instead of being free, which will protect them against lipid peroxidation, increasing their oxidative stability [50]. The presence of vitamin E may also contribute to this. Additionally, it has been reported that liposomes formed by marine phospholipids have advantageous features, including better oxidative stability, higher bioavailability, and superior fluidity when compared to other types of liposomes [51].

Finally, it is important to emphasize that fishroesomes have remarkable antioxidant and anti-inflammatory effects at concentrations of phosphatidylcholine in which they are not toxic for the two types of cells studied.

5. Conclusions

The search for natural origin compounds with antioxidant and anti-inflammatory properties, low toxicity and low cost is of great interest for the development of new formulations for the treatment of inflammatory diseases. In the present work, liposomes were produced from the lipidic extracts derived from sardine (*Sardina pilchardus*) roe, in the past considered a by-product of the fish industry. Fishroesomes demonstrated cytocompatibility for concentrations in which they exhibit antioxidant and anti-inflammatory activities, and frequently with a higher efficacy than the positive controls. Consequently, the developed sardine roe-derived LUVs may be considered as a valid alternative for the treatment or as a carrier for the treatment of inflammatory conditions, such as osteoarthritis and rheumatoid arthritis.

Conflict of interest 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.

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References

[1] S.A. Pompani, The oceans and human health: the discovery and development of marine-derived drugs, Oceanography 14 (2001) 78–87.
[2] M. Bragadóttir, Endogenous Antioxidants in Fish, Department of Food Science, University of Iceland, 2004.
[3] E.M. Arbeloa, M.J. Uez, S.G. Bertolotti, M.S. Churio, Antioxidant activity of gadusol and occurrence in fish roes from Argentine Sea, Food Chem. 119 (2) (2010) 586–591.
[4] S.B. Nimse, D. Pal, Free radicals, natural antioxidants, and their reaction mechanisms, RSC Adv. 5 (35) (2015) 27986–28006.
[5] R.C.F. Cheung, T.B. Ng, J.H. Wong, Y.C. Chen, W.Y. Chan, Marine natural products with anti-inflammatory activity, Appl. Microbiol. Biotechnol. 100 (4) (2016) 1645–1666.
[6] C. Sun, S. Zhang, Immune-relevant and antioxidant activities of vitellogenin and yolk proteins in fish, Nutrients 7 (10) (2015) 8818–8829.
[7] S. Chatterjee, Chapter two - oxidative stress, inflammation, and disease, in: T. Dziubla, D.A. Butterfield (Eds.), Oxidative Stress and Biomaterials, Academic Press, 2016, pp. 35–58.
[8] P. Arulselvan, M.T. Fard, W.S. Tan, S. Gothai, S. Fakurazi, M.E. Norhaizan, S. S. Kumar, Role of antioxidants and natural products in inflammation, Oxid. Med. Cell. Longev. 2016 (2016), 5276130.
[9] D. Pereira, P. Valentão, P. Andrade, Lessons from the sea: distribution, sar and molecular mechanisms of anti-inflammatory drugs from marine organisms, in: R. Atta ur (Ed.), Studies in Natural Products Chemistry, Elsevier, 2013, pp. 205–228.
[10] R. Medzhitov, Origin and physiological roles of inflammation, Nature 454 (7203) (2008) 428–435.
[11] G.A. Duque, A. Descoteaux, Macrophage cytokines: involvement in immunity and infectious diseases, Front. Immunol. 5 (2014) 1–12.
[12] C.A. Dinarello, Anti-inflammatory agents: present and future, Cell 140 (6) (2010) 935–950.
[13] Z. Madani, K. Louchami, A. Sener, W.J. Malaisse, D. Ait Yahia, Dietary sardine protein lowers insulin resistance, leptin and TNF-alpha and beneficially affects adipose tissue oxidative stress in rats with fructose-induced metabolic syndrome, Int. J. Mol. Med. 29 (2) (2012) 311–318.
[14] M. Balfego, S. Canivell, F.A. Hanzu, A. Sala-Vila, M. Martinez-Medina, S. Murillo, T. Mur, E.G. Ruano, F. Linares, N. Porras, S. Valladares, M. Fontalba, E. Roura, A. Novials, C. Hernandez, G. Aranda, A. Siso-Almirall, G. Rojo-Martinez, R. Simo, R. Gomis, Effects of sardine-enriched diet on metabolic control, inflammation and gut microbiota in drug-naïve patients with type 2 diabetes: a pilot randomized trial, Lipids Health Dis. 15 (2016) 78.
[15] N. Benaicheta, F.Z. Labbaci, M. Bouchenak, F. Boukortt, Effect of sardine proteins on hyperglycaemia, hyperlipidaemia and lecithin:cholesterol acyltransferase activity, in high-fat diet-induced type 2 diabetic rats, Br. J. Nutr. 115 (2015) 1–8.
[16] M. Guedes, A.R. Costa-Pinto, V.M.F. Gonçalves, J. Moreira-Silva, M.E. Tiritan, R. L. Reis, H. Ferreira, N.M. Neves, Sardine roe as a source of lipids to produce liposomes, ACS Biomater. Sci. Eng. 6 (2) (2020) 1017–1029.
[17] R. Silva, H. Ferreira, A. Cavaco-Paulo, Sonoproduction of liposomes and protein particles as templates for delivery purposes, Biomacromolecules 12 (10) (2011) 3353–3368.
[18] N. Grimaldi, F. Andrade, N. Segovia, L. Ferrer-Tasies, S. Sala, J. Veciana, N. Ventosa, Lipid-based nanovesicles for nanomedicine, Chem. Soc. Rev. 45 (23) (2016) 6520–6545.
[19] V. Matyash, G. Liebisch, T.V. Kurzchalia, A. Shevchenko, D. Schwudke, Lipid extraction by methyl-tert-butyl ether for high-throughput lipidomics, J. Lipid Res. 49 (5) (2008) 1137–1146.
[20] ISO, ISO 10993–5:2009 Biological evaluation of medical devices, Part 5: Tests for in vitro cytotoxicity, 2009.
[21] M. Guha, N. Mackman, LPS induction of gene expression in human monocytes, Cell. Signal. 13 (2) (2001) 85–94.
[22] D. Granato, F. Shahidi, R. Wrolstad, P. Kilmartin, L.D. Melton, F.J. Hidalgo, K. Miyashita, Jv Camp, C. Alasalvar, A.B. Ismail, S. Elmore, G.G. Birch, D. Charalampopoulos, S.B. Astley, R. Pegg, P. Zhou, P. Finglas, Antioxidant activity, total phenolics and flavonoids contents: should we ban in vitro screening methods? Food Chem. 264 (2018) 471–475.
[23] M. Lucio, H. Ferreira, J. Lima, S. Reis, Use of liposomes as membrane models to evaluate the contribution of drug-membrane interactions to antioxidant properties of etodolac, Redox Rep. 13 (5) (2008) 225–236.
[24] M.D. Pardau, A.S.P. Pereira, Z. Apostolides, J.C. Serem, M.J. Bester, Antioxidant and anti-inflammatory properties of Ilex guayusa tea preparations: a comparison to Camellia sinensis teas, Food Funct. 8 (12) (2017) 4601–4610.
[25] E. Fernandes, D. Costa, S.A. Toste, J. Lima, S. Reis, In vitro scavenging activity for reactive oxygen and nitrogen species by nonsteroidal anti-inflammatory indole, pyrrole, and oxazole derivative drugs, Free Radic. Biol. Med. 37 (11) (2004) 1895–1905.
[26] M.D. Wiegand, Composition, accumulation and utilization of yolk lipids in teleost fish, Rev. Fish. Biol. Fish. 6 (3) (1996) 259–286.
[27] S.F. Vieira, H. Ferreira, N.M. Neves, Antioxidant and anti-inflammatory activities of cytocompatible salvia officinalis extracts: a comparison between traditional and soxhlet extraction, Antioxidants 9 (11) (2020).
[28] D.D. Lasic, Liposomes: From Physics to Applications, Elsevier Science Limited, 1993.
[29] M.M. Mady, M.M. Darwish, Effect of chitosan coating on the characteristics of DPPC liposomes, J. Adv. Res. 1 (3) (2010) 187–191.

[30] H. Ferreira, T. Matama, R. Silva, C. Silva, A.C. Gomes, A. Cavaco-Paulo, Functionalization of gauzes with liposomes entrapping an anti-inflammatory drug: a strategy to improve wound healing, React. Funct. Polym. 73 (10) (2013) 1328–1334.
[31] J.C. Valverde, M.D. Hernandez, S. Garcia-Garrido, C. Rodriguez, J. Estefanell, J. I. Gairin, C.J. Rodriguez, A. Tomas, B.G. Garcia, Lipid classes from marine species and meals intended for cephalopod feeding, Aquacult. Int. 20 (1) (2012) 71–89.
[32] I. Puskás, F. Csempesz, Influence of cyclodextrins on the physical stability of DPPC-liposomes, Colloids Surf. B 58 (2) (2007) 218–224.
[33] O.R. Justo, P.U. Simioni, D.L. Gabriel, W.M. Tamashiro, T. Rosa Pde, A.M. Moraes, Evaluation of in vitro anti-inflammatory effects of crude ginger and rosemary extracts obtained through supercritical CO_2 extraction on macrophage and tumor cell line: the influence of vehicle type, BMC Complement. Altern. Med. 15 (2015) 390.
[34] D. Bonarska-Kujawa, H. Pruchnik, H. Kleszczynska, Interaction of selected anthocyanins with erythrocytes and liposome membranes, Cell. Mol. Biol. Lett. 17 (2) (2012) 289–308.
[35] C. Bronner, Y. Landry, P. Fonteneau, J.G. Kuhry, A fluorescent hydrophobic probe used for monitoring the kinetics of exocytosis phenomena, Biochemistry 25 (8) (1986) 2149–2154.
[36] R.M. Cordeiro, Reactive oxygen species at phospholipid bilayers: distribution, mobility and permeation, Biochim. Biophys. Acta Biomembr. 1838 (1, Part B) (2014) 438–444.
[37] T. Ohshima, Y. Fujita, C. Koizumi, Oxidative stability of sardine and mackerel lipids with reference to synergism between phospholipids and α-tocopherol, J. Am. Oil Chem. Soc. 70 (3) (1993) 269–276.
[38] N.M. Bandarra, R.M. Campos, I. Batista, M.L. Nunes, J.M. Empis, Antioxidant synergy of α-tocopherol and phospholipids, J. Am. Oil Chem. Soc. 76 (8) (1999) 905–913.
[39] A. Jacquot, G. Francius, A. Razafitianamaharavo, F. Dehghani, A. Tamayol, M. Linder, E. Arab-Tehrany, Morphological and physical analysis of natural phospholipids-based biomembranes, PLoS One 9 (9) (2014), 107435.
[40] A. Alaarg, N.Y. Jordan, J.J. Verhoef, J.M. Metselaar, G. Storm, R.J. Kok, Docosahexaenoic acid liposomes for targeting chronic inflammatory diseases and cancer: an in vitro assessment, Int. J. Nanomed. 11 (2016) 5027–5040.
[41] W. Rod-In, C. Monmai, S.M. Lee, S.K. Jung, S. You, W.J. Park, Anti-inflammatory effects of lipids extracted from arctoscopus japonicus eggs on LPS-stimulated RAW264.7 cells, Mar. Drugs 17 (10) (2019).
[42] T.B. Ahmad, D. Rudd, M. Kotiw, L. Liu, K. Benkendorff, Correlation between fatty acid profile and anti-inflammatory activity in common australian seafood by-products, Mar. Drugs 17 (3) (2019).
[43] B. Allam-Ndoul, F. Guénard, O. Barbier, M.-C. Vohl, Effect of n-3 fatty acids on the expression of inflammatory genes in THP-1 macrophages, Lipids Health Dis. 15 (2016) 69.
[44] S. Passi, S.F. Cataudella, P. Di Marco, F. De Simone, L. Rastrelli, Fatty acid composition and antioxidant levels in muscle tissue of different Mediterranean marine species of fish and shellfish, J. Agric. Food Chem. 50 (25) (2002) 7314–7322.
[45] H. Moriya, T. Kuniminato, M. Hosokawa, K. Fukunaga, T. Nishiyama, K. Miyashita, Oxidative stability of salmon and herring roe lipids and their dietary effect on plasma cholesterol levels of rats, Fish. Sci. 73 (3) (2007) 668–674.
[46] E.M. Conner, M.B. Grisham, Inflammation, free radicals, and antioxidants, Nutrition 12 (4) (1996) 274–277.
[47] M. Hasan, S. Latifi, C.J.F. Kahn, A. Tamayol, R. Habibey, E. Passeri, M. Linder, E. Arab-Tehrany, The positive role of curcumin-loaded salmon nanoliposomes on the culture of primary cortical neurons, Mar. Drugs 16 (7) (2018).
[48] S. Kigoshi, R. Ito, High levels of free fatty acids in lymphoid cells, with special reference to their cytotoxicity, Experientia 29 (11) (1973) 1408–1410.
[49] G. Zhao, T.D. Etherton, K.R. Martin, J.P. Vanden Heuvel, P.J. Gillies, S.G. West, P. M. Kris-Etherton, Anti-inflammatory effects of polyunsaturated fatty acids in THP-1 cells, Biochem. Biophys. Res. Commun. 336 (3) (2005) 909–917.
[50] R. Lordan, A. Tsoupras, I. Zabetakis, Phospholipids of animal and marine origin: structure, function, and anti-inflammatory properties, Molecules 22 (11) (2017).
[51] F.S. Lu, N.S. Nielsen, M. Timm-Heinrich, C. Jacobsen, Oxidative stability of marine phospholipids in the liposomal form and their applications, Lipids 46 (1) (2011) 3–23.