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Comparison of antioxidant efficiencies in oil-in-water emulsion using extracellular vesicles from olive co-products or liposomes as antioxidants carriers

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Abstract

Olive extracellular vesicles and synthetic liposomes were evaluated as carriers of antioxidants to stabilize oil-in-water emulsions against oxidative degradation. For this, hydroxytyrosol, rosmarinic acid and their lipophilic counterparts, (hydroxytyrosyl dodecanoate esters or eicosyl rosmarinate esters) were loaded into these carrier vesicles and the antioxidant efficiencies of these formulations were compared with those of the corresponding antioxidants alone. Using the conjugated autoxidizable triene assay (CAT assay), our results shows that loaded synthetic liposome mimicking the lipid membrane composition of olive extracellular vesicle allowed to enhance the antioxidant effect of the loaded antioxidant especially with the two lipophilic hydroxytyrosol and rosmarinic acid esters. On the contrary, the loading of the studied antioxidant into the olive extracellular vesicles did not result in an improvement of the antioxidant activity. The antioxidant effects of loaded vesicles were also evaluated in rapeseed oil (1% w/w)-in-water emulsions that were stored at 40°C for 21 days and for which oxidative status was monitored by the quantification of primary and secondary oxidation compounds. In that case, the boosting effect of liposomal carriers was not confirmed. This could be due to a different type of emulsions compared to the one used with the CAT assay as different surfactants and oxidation inducers were employed. Additionally, the limited physical stability of the carrier could be involved as liposomes loaded with the most lipophilic antioxidants, namely hydroxytyrosyl dodecanoate and eicosyl rosmarinate were shown to be instable for period exceeding 10 days of storage.

KEYWORDS
antioxidant, emulsions, extracellular vesicles, liposomes, olive, polyphenols

Abbreviations: AAPH, 2,2′-azobis(2-amidino-propane) dihydrochloride (AAPH); CAT, conjugated autoxidizable triene assay (CAT assay); DGDG, 1,2-diacyl-3-O-(α-D-galactosyl1-6)-α-D-galactosyl-*sn*-glycerol (Digalactosyldiacylglycerol); DPPC, 2-dipalmitoyl phosphatidylcholine; DSPC, 1,2-distearoyl phosphatidylcholine; EV, extracellular vesicle; HT, hydroxytyrosol; HTC12, hydroxytyrosyl dodecanoate; MDA, malondialdehyde; MGDG, 1,2-diacyl-3-O-β-D-galactosyl-*sn*-glycerol (Monogalactosyldiacylglycerol); OPC, olive phenolic compounds; PBS, phosphate buffer solution; PC, 1,2-dioleoyl-*sn*-glycero-3-phosphatidylcholine; PE, 1,2-dioleoyl-*sn*-glycero-3-phosphatidylethanolamine; PV, peroxide value; RC, rosmarinic acid; RC20, eicosyl rosmarinate (RC20); SDS, sodium dodecyl sulfate; TBARS, 2-thiobarbituric acid reactive substances.

INTRODUCTION

The use of unsaturated lipids in food or cosmetic industries implies the risk of their oxidation during processing or storage of final products. Such an oxidative degradation results in off-flavors development, rancidity, loss of essential fatty acids or liposoluble vitamins, and appearance of new oxidized chemical species with potential toxicity (Skibsted, 2010). Lipid oxidation is a complex reaction, generally involving radical species, that leads to the appearance of many diverse molecules such as primary and secondary oxidation products (Frankel, 1984; Schaich, 2013, 2020). While the oxidative degradation of oils and fats has been documented as early as the early 19th century (Hammond & White, 2011), and the chemical composition of the resulting species was further elucidated in the 20th century, the exact chemical pathways involved are not yet definitively established and remain subject to ongoing scrutiny, particularly regarding the methods and techniques for measuring and quantifying lipid oxidation (Schaich, 2020, 2023).

A variety of strategies have been proposed to mitigate lipid oxidation, ranging from the development of packaging with oxygen or UV light barriers to the incorporation of antioxidants. Concerning these latter, in the food or cosmetic industry, the synthetic or natural antioxidants that are added to the final products generally belongs to the class of (poly)phenolic molecules. Indeed, phenolic compounds are known for their antioxidant properties which are essentially due to their radical scavenging activity (through H donation or electron transfer mechanism) or their capacity to chelate prooxidant transition metals (Decker et al., 2010). In the beginning of their extended study and subsequent use in food or cosmetic products, phenolic compounds where considered efficient antioxidants whatever the nature of the system they would be added in. In particular, no distinction was made regarding the effectiveness and mechanisms of action of antioxidants employed in either bulk oils or emulsion systems. However, in the 1990s, some key publications appeared, showing the difficulty of extrapolating results from one system to another (Frankel et al., 1994; Porter et al., 1989). These studies were pivotal in lipid oxidation research and introduced the concept of the antioxidant polar paradox. This theory suggests that polar antioxidants are more effective in bulk oils, whereas non-polar antioxidants exhibit greater efficacy in lipid dispersions, such as oil-in-water emulsions. The explanation of this so-called paradox was attributed to the interfacial location of antioxidants that would optimize their activity because oxidation in emulsions occurs at the surface of lipid droplets where both lipid hydroperoxides and transition metals can accumulate (Frankel et al., 1994). Interestingly, similar interfacial phenomenon can also explain lipid oxidation in bulk oils as these latter contain small associations colloids that introduce an interfacial dimension to the system (Chen et al., 2011). These structures have a water-lipid interface that can influence the partitioning and activity of both prooxidants and antioxidants in bulk oils. Further on, a more detailed explanation was given to explain the behavior of antioxidants in emulsified systems with the notion of the cut off effect (Laguerre et al., 2009, 2010; Laguerre et al., 2015) and the pseudo-phase model (Losada Barreiro et al., 2013) that also emphasized the importance of the interfacial location of the antioxidants in order to guarantee an optimized antioxidant activity (Shahidi & Zhong, 2011). However, although the location of an antioxidant at the interface of the emulsion is important, recent studies suggested that this fact alone may not be sufficient and that the way oxidation spreads in between lipid droplets is a crucial factor to consider when designing effective antioxidant strategies (Berton-Carabin et al., 2014; Budilarto & Kamal-Eldin, 2015; Decker et al., 2017; Hennebelle et al., 2024; Laguerre et al., 2017; Villeneuve et al., 2023). Indeed, it is now speculated that the diffusion of "oxidation information" from droplet to droplet, could be made by the transport of primary oxidation compounds (hydroperoxides) and secondary ones (aldehydes) (Klooster et al., 2023). The way such transport is performed is still unclear but recent evidence suggests that surfactant micelles that are present in the concerned emulsion could serve as carriers of oxidative species or that primary oxidation compounds could themselves aggregate into micellar structures at a lipid droplet interface.

Similarly, some recent studies showed that the vectorization of some antioxidants through their micellization or loading into vesicles could increase their activity in heterogenous systems (Villeneuve et al., 2023). For example, Kiralan et al. (2014) observed that the partitioning of tocopherols into the aqueous phase via the formation of surfactant (Tween 20)–tocopherol comicelles could enhance their antioxidant activity. In order to explain these data, the authors hypothesized that tocopherol co-micelles could form a reservoir of antioxidants that could replace oxidized tocopherols in the emulsion droplets to maintain an optimum level of antioxidants. Alternately, they also suggested that reactive oxygen species could exist in the aqueous phase of the O/W emulsion, and the presence of the Tween 20–tocopherol co-micelles could scavenge these radicals before they could oxidize the fatty acids in the oil droplets. A comparable study was performed using sodium dodecyl sulfate as surfactant (Inchingolo et al., 2021). They showed that when α-tocopherol was added post-emulsification, SDS concentrations above 4 mM, lead to a marked increase in α-tocopherol in the aqueous phase, suggesting that SDS micelles were solubilizing α-tocopherol out of the emulsion droplet. Concomitantly, the oxidative stability of the emulsion increased

with increasing concentrations of SDS. Similarly, Feng et al. (2017) used niosomes as nanocarriers for catechin and epigallocatechin laurates and tested them as antioxidants for liposomes preservation. Results showed that such assemblies had considerably higher lipid-oxidation inhibitory ability than the non-vectorized molecules due to their preferential location in the liposome membrane. These studies suggest that the associations of antioxidants with assemblies such as micelles or nanovesicles can improved their efficiency in heterogenous systems such as emulsions.

In that context, the recent discovery of natural extracellular vesicles (EV) from plants is of great interest (Cao et al., 2023; Cong et al., 2022; Mu et al., 2023; Subha et al., 2023). Such vesicles have indeed been characterized in various plants such as barley leaf (An et al., 2007), lemon (Pocsfalvi et al., 2018; Yang et al., 2020), carrot (Mu et al., 2014), ginger (Chen et al., 2019) sunflower (Regente et al., 2009), grape fruit (Pérez-Bermúdez et al., 2017) or tea flowers (Chen et al., 2022). Beside their role in intercellular communications role, plant EVs are also believed to allow small RNA trafficking between plant hosts and pathogens (Cai et al., 2019) and were also found to contain antimicrobial compounds and defense related proteins and deliver their cargo to invading fungi (An et al., 2006; Meyer et al., 2009). Some studies have shown that EVs can be used as new drug delivery systems for bioactive cargo molecules (Bashyal et al., 2022; Chavda et al., 2023; García-Manrique et al., 2018; Gudbergsson et al., 2019; Johnsen et al., 2014; Reiner & Somoza, 2019; Sun et al., 2010). Plant EVs are around 50–110 nm size and may naturally contain some phenolic compounds. For example, naringin and naringenin have been detected in grape fruit extracellular vesicles (Wang et al., 2014), gingerol and shogaol in ginger (Zhang et al., 2016), quercetin and quercetin derivatives in tea leaves (Zu et al., 2021). In our recent study, we described the presence of phenolic compounds in EVs from olive pomaces or wastewaters (Barouh et al., 2024) and characterize them in terms of lipid membranes molecular composition and phenolic compounds load. In the present work, we evaluate the potentiality of these olive EVs naturally rich in polyphenols as new antioxidants systems in emulsions. Results are also compared to the one obtained with synthetic vesicles such as liposomes enriched in pure antioxidants molecules.

MATERIALS AND METHODS

Chemicals

Phosphate buffer solution (PBS, pH 7.2), Tung oil from *Aleurites fordii* seeds (average MW = 872 g/mol), lauryl ether (Brij 35 estimated MW = 1198 g/mol), hydroxytyrosol (≥98%), rosmarinic acid (≥98%), 2-butanone, octanoyl chloride, n-eicosanol, sodium dodecyl sulfate (SDS), iron sulfate, ammonium thiocyanate, 1,1,3,3-tetramethoxypropane (TEM), Amberlite IR-120H resin, cumene hydroperoxide, trichloroacetic acid, thiobarbituric acid, mono and dibasic sodium phosphate, and all solvents (HPLC or analytical grade) were all purchased from Sigma-Aldrich (Saint Quentin Fallavier, France). 1,2-dioleoyl-*sn*-glycero-3-phosphatidylcholine (PC), 1,2-dioleoyl-*sn*-glycero-3-phosphatidylethanolamine (PE), 1,2-diacyl-3-O-β-D-galactosyl-*sn*-glycerol (Monogalactosyldiacylglycerol: MGDG) and 1,2-diacyl-3-O-(α-D-galactosyl1-6)-α-D-galactosyl-*sn*-glycerol (Digalactosyldiacylglycerol: DGDG) were purchased from Avanti Polar Lipids (Alabaster, USA). Trolox (97%) was obtained from Accros Organic (Geel, Belgium). 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH) was purchased from Wako Chemical (Neuss, Germany).

Refined Rapeseed (*Brassica napus* L.) oil was obtained from local supermarkets in Montpellier (France) and used without further purification. The oil quality was as follows: peroxide value 1.47 meq 0_2/kg oil, α-tocopherol: 326 mg/kg oil (756 μM) and γ-tocopherol: 433 mg/kg oil (1039 μM). Fatty acids composition was as follows: 16:0, 4.4%; 16:1 n-7, 0.2%; 18:0, 1.8%; 18:1 n-9, 62.3%; 18:2 n-6, 19.7%; 18:3 n-3, 9.9%; 20:0, 0.5%; 20:1 n-9, 1.2%. Total percentage of polyunsaturated fatty acids = 29.6%.

Hydroxytyrosyl dodecanoate (HT12) and eicosyl rosmarinate (RC20) were synthesized according to the following procedure: hydroxytyrosol (0.653 mmol) and dodecanoic acid (3.202 mmol) were mixed in 2 mL of anhydrous 2-butanone until complete dissolution of both compounds. Then 60 milliequivalents of octanoyl chloride were added and the reaction medium was stirred at 350 rpm in an orbital shaker (KS 4000i control, IKA-Werke, Staufen, Germany) at 60°C. The formation of hydroxytyrosyl dodecanoate was followed over time by HPLC: for this, aliquot fractions were diluted at 1% in methanol, filtered (Millex 0.45 μm, Millipore) and 20 μL were injected into HPLC (U3000 Thermo Fisher, San Jose, CA, USA) pump Surveyor MS pump, DAD detector at 280 nm. ACE C18 column (5 μm, 250 × 4.6 mm, AIT, Houilles, France), water: acetic acid (99.9:0.1, v:v) (solvent A) and methanol: acetic acid (99.9:0.1, v:v) (solvent B). Gradient: solvent B from 50% to 100% in 3 min, 10 min in isocratic conditions, back to 50% solvent B in 1 min then 10 min. Solvents flow: 1.3 mL/min. Final product was then purified by Flash Chromatography (Reveleris X2) type system (Butchi, Switzerland). Two silica columns (4 g Silica, SERLABO) were used in series with hexane: acetic acid (99.9:0.1, v:v) and diethyl ether: acetic acid (99.9:0.1, v:v) as solvent A and B respectively. Elution: From B 0% for 4 min to B 60% in 5 min, then from B 60% to 100% in 1 min, and plateau at 100% for 5 min: Flow: 5 mL/min. Detection: 280 nm. Fractions containing hydroxytyrosyl dodecanoate were collected, and evaporated to dryness under vacuum at 55°C.

For eicosyl rosmarinate, the procedure already described by Lecomte et al. (2010) was used: Typically, rosmarinic acid (56 μmol) and n-eicosanol (5 mL, 13.6 mmol) were placed in sealed flasks and stirred (orbital shaker, 250 rpm, 55°C) prior to the addition of sulfonic resin Amberlite IR-120H resin (5% w/w—total weight of both substrates) previously dried at 110°C for 48 h. The water generated during the reaction was removed by absorption on molecular sieves (40 mg/mL) added to the medium. Samples (20 μL) were regularly withdrawn from the reaction medium and analyzed using the same procedure as for hydroxytyrosyl dodecanoate (HT12) with UV detection at 328 nm. After complete conversion (>15 days) of rosmarinic acid into the corresponding ester, the latter was purified by Flash Chromatography in a two-step procedure as previously described for HT12.

Structure and purity (>99%) of isolated products were confirmed by NMR ^{1}H and ^{13}C: the ^{1}H and ^{13}C NMR spectra were obtained with a 500 MHz Bruker Advance III spectrometer (Bremen, Germany) equipped with a helium cryoprobe type BBFO (wideband) operating at 500 MHz and 298 K. The compounds were diluted in deuterated DMSO (DMSO-d6), and the chemical shifts were expressed relative to DMSO-d6 at 2.5 and 39.5 ppm for ^{1}H and ^{13}C, respectively.

Preparation of antioxidants loaded vesicles

All antioxidants loaded vesicles were prepared with 92% membrane lipids and 8% of antioxidants on dry mass. Pure antioxidants molecules namely hydroxytyrosol (HT), hydroxytyrosyl dodecanoate (HTC12), rosmarinic acid (RC), eicosyl rosmarinate (RC20) and Olive Phenolic Compounds (OPC) were independently loaded into three kinds of lipid vesicles. Detailed loading methods are provided below (Section 2.2). The OPC extract was first obtained according to the following procedure: In a 5 mL flask, 80 μL HCl 0.1 N, 1.2 mL Folch solvent (chloroform/methanol/water 8:4:3 v/v/v) and 100 μL of olive extracellular vesicles samples (previously obtained as described by Barouh et al., 2024) were mixed. Then, 300 μL Folch mixture and 25 μL NaCl 0.73% were added. Samples were vortexed for 30 seconds after each step then centrifuged at 2000 rpm for 5 min (CR4-12 refrigerated centrifuge, Jouan, Winchester, UK). The methanolic phase containing the antioxidants extract was then recovered.

Nature and preparation of vesicles

The two types of lipid vesicles into which the antioxidants were loaded as follows.

Olive extracellular vesicles

These vesicles were obtained from wastewaters according to the procedure described by Barouh et al. (2024): Typically, two clarifying centrifugations at 3000 × g for 30 min, then 10,000 × g for 1 h (Avanti J-C JA 40, Beckman Coulter, California, USA) were performed to separate large particles, plant fibers and intact organelles. Then, a first preparative centrifugation at 16,500 × g for 1 h at 4°C (Avanti JXN-26/rotor JA-14.50, Beckman coulter, California, USA) followed by a preparative ultracentrifugation at 100,000 × g for 1 h at 4°C (Optima L80 XP/rotor 45 Ti, Beckman coulter, USA) were done to pellet the extracellular vesicles (EVs). EVs obtained after the last centrifugation were collected and further characterized in terms of lipid composition and phenolic content (Barouh et al., 2024).

Artificial membrane vesicles

These vesicles were obtained from pure polar lipids mixed in specific proportions in order to mimic the typical lipid composition of the membrane of natural olive oil extracellular vesicles (Barouh et al., 2024). Accordingly, the detailed composition of these artificial membranes was a mixture of the following polar lipids in a chloroform/methanol solution (9:1 v/v): phosphatidylcholine (PC) (1.24 mg/mL), phosphatidylethanolamine (PE) (0.40 mg/mL), monogalactosyldiacylglycerol (MGDG) (0.37 mg/mL) and digalactosyldiacylglycerol (DGDG) (0.91 mg/mL).

Antioxidant loading

Antioxidant methanolic solution were individually prepared for each antioxidant at the following concentrations: hydroxytyrosol (0.25 mg/mL), hydroxytyrosyl dodecanoate (0.57 mg/mL), rosmarinic acid (0.25 mg/mL) and eicosyl rosmarinate (0.44 mg/mL) or the whole recovered OPC from 100 μL of vesicles (corresponding to 0.014 mg/mL of antioxidant concentration). These solutions were first diluted to one-tenth. 100 μL of each individual diluted solution were introduced in a 25 mL round-bottom flask and then dried over vacuum. Then, for artificial vesicles formation, 100 μL of the corresponding lipid fractions were added. Solvent was then evaporated under vacuum for 1 h. The obtained dried lipid film was hydrated in 1 mL of potassium phosphate buffer (pH 7.4). The obtained mixture was vortexed for 30 s to obtain multilamellar vesicles and the solution was then left in the fridge (4°C) overnight. Extrusion was then performed using a mini-extruder (Avanti) through 1000 nm then through 200 nm pore size polycarbonate filters (Whatman) to form large unilamellar vesicles.

For the loading of olive extracellular vesicles, these vesicles were diluted to one-tenth in potassium phosphate buffer (pH 7.4), then 100 μL were recovered and added to the dried phenolic compounds. The mixture was then vortexed and let in contact for 60 min before analysis.

The Encapsulation Efficiency was determined as followed. The liposomes suspension was centrifuged at 12.000 g (Thermo scientific, Heraeus Pico 21 centrifuge) for 60 min. The supernatant was recovered and analyzed by HPLC. Encapsulation efficiencies were >80% for all vesicles or liposomes suspensions.

Physical characterization of antioxidants loaded vesicles

A Nicomp DLS/ZLS Nanoparticle Size Analyzer (Nicomp, Nano Z3000, USA) was used for characterization of particles sizes and charges. For size determinations, vesicles were suitably diluted three hundred times in PBS and placed in a clear disposable zeta cell. Size measurements were performed during 10 days of storage at 4°C to evaluate the vesicles stability. Each sample was measured five times in succession to obtain a mean-size distribution curve according to the distribution-volume. Charges were measured on the same diluted vesicles solutions at pH = 7.

Evaluation of antioxidant activity of antioxidants loaded vesicles in oil-in-water emulsions

Conjugated autoxidizable triene (CAT) assay

Extracellular and artificial vesicles loaded in HT, HTC12, RC, RC20, or OPC were evaluated for their antioxidant capacity in oil-in-water emulsions, first using the CAT assay. Nude extracellular vesicles and artificial vesicles (not loaded in antioxidants), were also tested. In parallel, the antioxidant efficiencies of the same antioxidants not loaded in vesicles were also measured. Blank control was made with PBS only and Trolox was used as reference antioxidant.

The method, originally developed by (Laguerre et al., 2008), was slightly modified according to the following procedure.

Preparation and physical characterization of nanoemulsions

40 mg of tung oil were added to 200 mL of PBS solution (pH 7.2) containing 70 μM Brij 35 (neutral emulsifier, estimated MW = 1198 g/mol). Afterward, the mixture was homogenized using a high-speed blender (Janke & Kunkel, Staufen, Germany) at 15,000 rpm for 90 s. Then, the coarse emulsion was processed through a microfluidizer (LM20 Microfluidizer Processor, Microfluidics, USA) configured with 75 μm Y-type chamber at 20000 psi (2 passes) to produce nanoemulsions. The particle size distribution of the emulsion was measured by static light scattering (Nicomp Nano Z3000, USA). All prepared emulsions had similar droplet sizes (mean diameter = 200 ± 75 nm) and were physically stable over the course of the experiment.

CAT assay procedure

In 1.5 mL brown flasks, vesicles samples or non-loaded antioxidants methanolic solutions were introduced in the emulsions and compared at the same antioxidant concentrations. Controls with neat methanol (antioxidant-free) and Trolox were also made. Solutions were stirred for 5 s using a vortex apparatus. Afterward 50 μL of Tung oil-in-PBS emulsions, with different concentrations of tested samples, were transferred into a UV-star-96 well microplate (Greiner, Frickenhausen, Germany). Then, 150 μL of a freshly prepared AAPH solution in PBS (1.3 mM) were added to each well using multichannel micropipette. Each well contained 200 μL of a solution having final concentrations of 0.05 mg/mL in Tung oil, 17.5 μM in Brij 35, 1 mM in AAPH and 0.06 to 0.83 mg/mL for antioxidants. The progress of reactions was immediately monitored after AAPH addition, by recording the decrease in absorbance at 273 nm, using an infinite 200 PRO microplate reader (Tecan, Gröedig, Austria) equipped with Magellan software. Measurements were performed each minute for 9 h at 37°C with 5 s agitation before each measure.

Expression of the results

To normalize the results, raw absorbance was converted into relative absorbance (Equation 1):

$$\text{Relative Absorbance} = A_t/A_0 \quad (1)$$

where A_t and A_0 are the absorbances measured at time t and 0 min, respectively. The area under the curve (AUC) corresponding to the relative decrease in absorbance at 273 nm was calculated after 540 min (9 h) according to Equation 2:

$$\text{AUC} = 1 + A_1/A_0 + A_2/A_0 + \ldots + A_{539}/A_0 + A_{540}/A_0 \quad (2)$$

Finally, Trolox was used as a reference antioxidant, and the antioxidant activity (CAT value) of a sample was given in moles of Trolox equivalent per mole of tested compound (TE) according to Equation 3.

$$\begin{aligned}\text{CAT value(TE)} = {} & [(\text{AUC}_{\text{sample}} - \text{AUC}_{\text{control}})/ \\ & (\text{AUC}_{\text{Trolox}} - \text{AUC}_{\text{control}})] \\ & \times [(\text{mol of Trolox})/ \\ & (\text{mol of tested antioxidant})]\end{aligned} \quad (3)$$

Evaluation of antioxidant efficiency in oil in water emulsion

Oil-in-Water emulsions were prepared by homogenization of 1% rapeseed oil with 99% aqueous phase (w/w). The aqueous phase consisted of phosphate buffer solution (10 mM, pH 7.0) and SDS at a 5 mM concentration in the final emulsion. Both phases were mixed and pre-emulsified twice for 5 min at 5000 rpm with a L5M Silverson (Silverson, Longmeadow, USA). The emulsion was then homogenized with 4 cycles at 350/40 bars using an APV-1000 High Pressure Homogenizer (SPXflow, Charlotte, North Carolina, USA) resulting in emulsion droplet of around 0.4–0.5 μm. Vesicles samples or non-loaded antioxidants methanolic solutions were introduced in the aqueous phase of the emulsions after the homogenization step (i.e., post-homogenization) to give a 50 μM final concentration of antioxidant. Ethanol was added to control (no antioxidants) treatments. Ethanol concentration in the final emulsions was negligible (<0.1%). For the oxidation experiments, 20 mL of emulsions were transferred into 25-mL screw capped amber vials, and incubated at 40°C for 21 days under gentle orbital stirring (110 rpm) using an IKA KS 4000 i-control incubator (IKA, Staufen, Germany). Samples were collected every 2 days for analysis. Three replicates of oxidation for each antioxidant treatment were carried out.

The evaluation of lipid oxidation in the emulsions was carried out by monitoring the formation of both primary (lipid hydroperoxides) and secondary (2-thiobarbituric acid reactive substances, TBARS) oxidation products. Peroxide value (PV) was determined according to the following procedure: Briefly, 300 mg of samples were mixed with 1.5 mL of isooctane/isopropanol solution (3:1 v/v) and vortexed three times for 10 sec. Then, an aliquot of this mixture was added to methanol/butanol (3:7 v/v) to give a final volume of 260 μL in the microplate well. Then, 2.5 μL of aqueous ammonium thiocyanate (300 mg/mL) and ferrous solution (0.144 mol/L) were added to give a final volume of 265 μL in the microplate UV star 96 well COC F-bottom (Greiner Bio-One, Courtaboeuf, France). Each microplate was first incubated at 25°C for 10 min with a stirring of 1000 rpm in PHMP-4 microplate thermoshaker (Grant instruments ltd Shepreth, Cambridge, England) and then placed in an Infinite M200 microplate reader (Tecan, Gröedig, Austria). Absorbances were measured at 500 nm. Data acquisition was made using Magellan software (Tecan). PV were determined using a standard calibration curve of cumene hydroperoxide and were expressed as mmmol of hydroperoxides/kg oil.

TBARS were determined according to the following procedure. Briefly, 50 μL of samples were mixed with 200 μL of the reagent solution (150 mg/mL of trichloroacetic acid, 3.75 mg/mL of thiobarbituric acid and 0.25 mol/L of HCL) and heated at 95°C for 15 min. Samples were then cooled in an ice bath for 5 min and centrifuged (10,000 rpm) for 10 min using a Pico 21 centrifuge (ThermoFisher Scientific, USA). The absorbance of the supernatant was read using an Infinite M200 microplate reader Tecan, Gröedig, Austria) at 532 nm with the Magellan software. TBARS were determined using a standard calibration curve of 1,1,3,3-tetramethoxypropane and were expressed as mg malondialdehyde per kilogram oil (MDA/kg oil. Analyses were performed in triplicate.

Statistical analysis

Results are presented as mean ± SD. Statistical analysis were performed using the statistics software R (version 3.5–1). Statistical differences were evaluated using a one-way ANOVA test and considered significant for $p < 0.05$.

RESULTS AND DISCUSSION

The efficiency of antioxidants in heterogenous systems such as emulsions is governed by their chemical reactivity and their physico-chemical properties (Decker et al., 2017; Laguerre et al., 2017). While much information is already available regarding the chemical reactivities of phenolic compounds, much less is known about the impact of their physico-chemical behavior on their resulting efficiency as antioxidants in emulsions. Indeed, in such heterogeneous systems, oxidation occurs primarily at oil–water interface where pro-oxidant agents come into contact with oxidizable lipid substrate and initiate the reaction. In this context, various studies on model oil-in-water emulsions have demonstrated the importance of using antioxidant molecules able to locate predominantly at interface to optimize their activity (Costa et al., 2015; Laguerre et al., 2009, 2010; Panya et al., 2012). Up to now, however, the transport and diffusion of radicals and antioxidant species within emulsions is only partially described. Yet, a better understanding of these dynamics of transfer between the various components of the food systems (micellar phase-interface-lipid substrate) would lead to a better use of antioxidant molecules and an optimized stabilization of lipids. For example, it has been suggested that the physical location of antioxidants in emulsions can be modulated by surfactant micelles and the impact of the role of micelles in lipid oxidation in emulsions has been recently reviewed (Villeneuve et al., 2023). Similarly, the mode of incorporation of antioxidants in dispersed lipids can also play a role in their efficiency. The distribution of an antioxidant is influenced by its partitioning behavior in the different regions of the emulsion and also by the type of

transport. Therefore, depending on the phase in which the antioxidants are initially incorporated, their capacity to reach the interface or to reach their dynamic equilibrium within the system may significantly differ between antioxidants, thus affecting their efficiency (Barden et al., 2015; Durand et al., 2019; Ferreira da Silveira et al., 2021). In that context, Schröder et al. (2020) proposed the use of Pickering particles loaded in antioxidants (tocopherols or carnosic acid) in order to favor their locations at the lipid/water interface of emulsions and consequently, optimize their antioxidant efficiency. They showed that both tested antioxidants were largely more effective when loaded within Pickering particles than when solubilized in the oil droplet interior, thus confirming the importance of the way of incorporation of antioxidants into emulsified systems. Accordingly, in the present work, we aimed at evaluating the potential of antioxidant loaded vesicles as antioxidants carriers and checked whether such antioxidant formulations could improve their efficiency to combat lipid oxidation in emulsions. These vesicles corresponded to natural ones obtained from olive by-products or to artificial vesicles prepared by the thin-layer hydration technique (Figure 1). All were individually loaded with synthetic antioxidants varying in polarity: hydroxytyrosol (HT), hydroxytyrosyl dodecanoate (HT12), rosmarinic acid (RC) and eicosyl rosmarinate (RC20) or in the natural antioxidant molecules found in olive (Figure 2). Their efficiencies as antioxidant formulations were compared in emulsified systems with those of the corresponding antioxidant molecules without any vesicles carrier. As blank assays, unloaded vesicles (absence of antioxidant in their core) were also evaluated.

Antioxidant efficiency of loaded vesicles measured by the CAT assay

In order to evaluate the performance of the prepared vesicles as antioxidant systems in emulsion, we first used the CAT assay method. This method previously proved its efficiency to screen different types of antioxidants as natural phenolic compounds or extracts (Deyrieux et al., 2020; Phonsatta et al., 2019) or phenolipids (Laguerre et al., 2010; Laguna et al., 2020; Moltke Sørensen et al., 2014; Panya et al., 2012). In this method, the kinetics of oxidation of an oil-in-water emulsion made of tung oil are followed after initiation of lipid oxidation by a hydrophilic azo initiator (AAPH). Results are then expressed as CAT values using Trolox as reference antioxidant. The better the antioxidant efficiency the higher is the CAT value. All vesicles were evaluated at the same antioxidant molar quantities in the tung oil emulsion. Besides the loaded vesicles mentioned above, we also evaluated the efficacy of the antioxidants alone (OPC, HT, HT12, RC, and RC20) at the same antioxidant molar amounts in the emulsions. Moreover, empty artificial vesicles (unloaded in antioxidants) were also evaluated to estimate the potential effect alone and did not show any antioxidant activity. It is also worth noting that the total duration of the CAT assay does not exceed a few hours which meant that

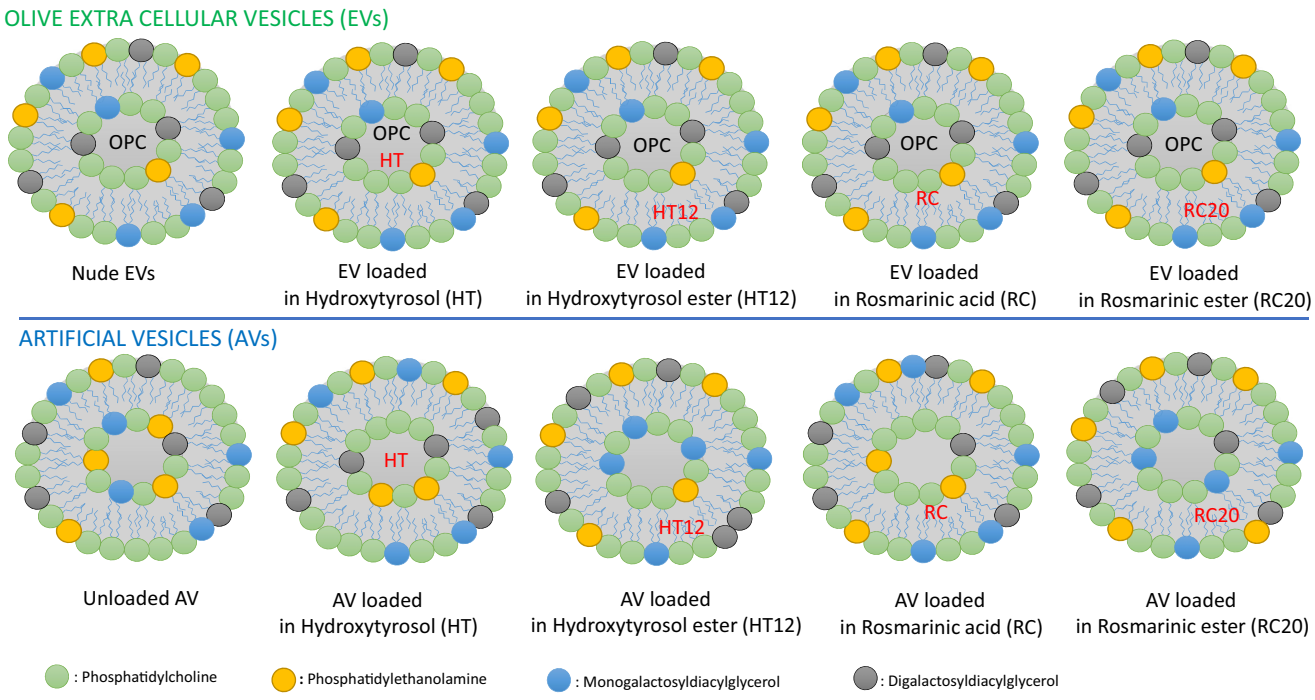

FIGURE 1 Type and loading of the studied olive extracellular and artificial vesicles (artificial vesicles were made from phospholipids and galactolipids mimicking the lipid membrane composition of extracellular vesicles).

all vesicles were physically stable throughout the assay (see Section 3.2). The obtained results (Figure 3) can be discussed through different scopes. Concerning the nature of the antioxidant itself, when added alone without vesicles carrier, the ranking of antioxidants according to their CAT values was the following one: RC (3.94 ± 0.30) > HT (2.39 ± 0.44) > HT12 (1.95 ± 0.03) > OPC (0.15 ± 0.01) > RC20 (0.11 ± 0.00). This ranking agrees with previous studies that showed for example that RC20 was a poor antioxidant in emulsions due to its tendency to form aggregate in the continuous phase of the emulsions (Laguerre et al., 2010). Such aggregate formation limits its mobility/diffusivity in the emulsion, and, consequently, strongly affects its efficiency as antioxidant for emulsified systems. The same authors also observed that rosmarinic acid was better than its RC20 ester as it is the case in the present work. Rosmarinic acid is also better than HT and showed a stronger radical scavenging activity which is not surprising considering that the former molecules has two catechol groups in its structure whereas HT has only one. Finally, although better than RC20, OPC did not

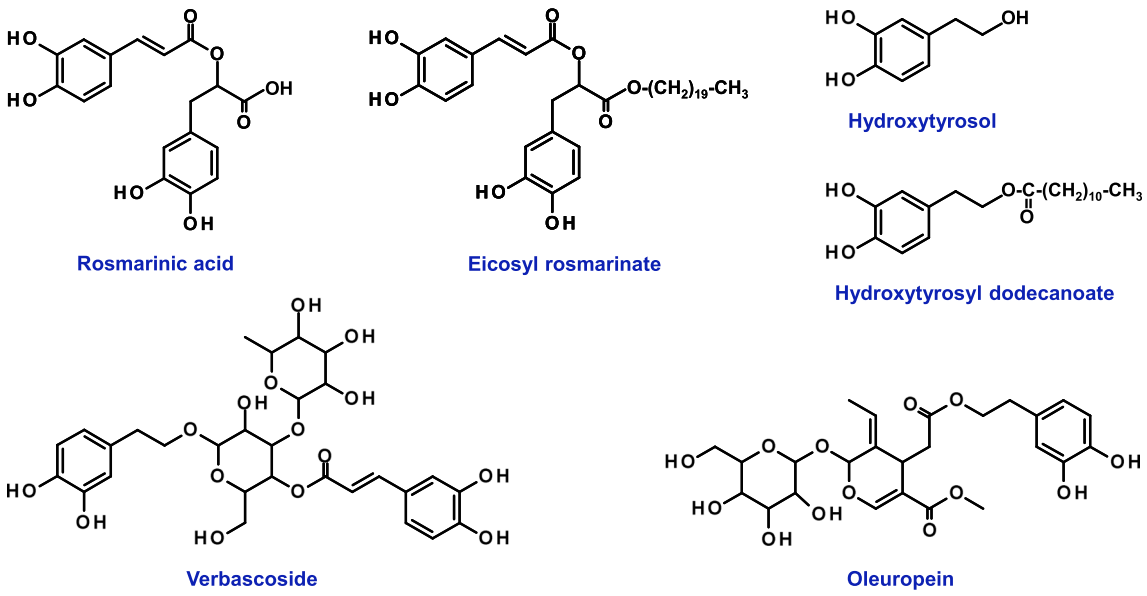

FIGURE 2 Chemical structures of tested antioxidants.

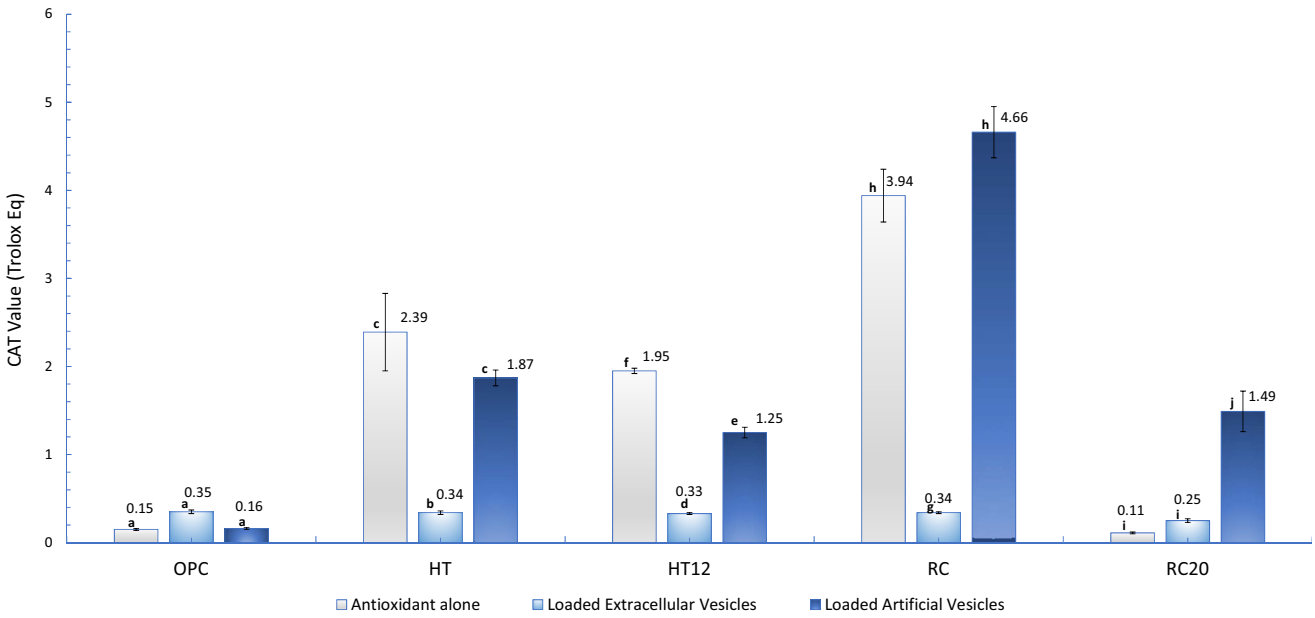

FIGURE 3 CAT assay values (Trolox Eq.) of all tested antioxidants and vesicles. (Values sharing the same superscript letter are not significantly different at $p < 0.05$ (ANOVA; $n = 3$) for each antioxidant group).

perform well in the CAT assay. This can be attributed to the hydrophilic nature of their most abundant phenolic compounds (verbascoside and oleuropein) as it is well known from the polar paradox theory that, in general, hydrophilic antioxidants are not satisfactory to protect lipids from oxidation in emulsions.

With loaded artificial vesicles the ranking of antioxidants was the following: RC > HT > HT12 > RC20 >> OPC. When loaded into artificial vesicles, RC20 showed an improved antioxidant effect in comparison with the antioxidant alone. 4.66 versus 3.84 for RC, 1.49 versus 0.11 for RC20 corresponding to a 1.84 folds improvement of antioxidant activity for RC and 13.54 for RC20. On the contrary, the antioxidant effect of HT and HT12 was lower when formulated in artificial membrane compared to the molecule alone while the one of OPC remained the same. Indeed, CAT value of HT and HT12 in artificial membranes were respectively 1.87 and 1.25 (2.39 and 1.95 for the same molecule alone). Finally, it was also interesting to note that extracellular vesicles exhibited a low antioxidant activity (CAT value = 0.35) and that their loading in HT, HT12, RC, or RC20 did not result in any improvement of that activity.

However, it is worth noting that the extracellular vesicles naturally containing OPC, despites their limited activity, had better performance than their extracted OPC loaded into artificial membranes. The reasons why loading of HT and HT12 in artificial membranes resulted in a loss of their activities compared to the molecules alone are still unclear. In emulsions, the efficiency of antioxidants is governed not only by their chemical reactivities but also by physico-chemical parameters concerning the capacity of antioxidants to be localized at the oil/water interphase and to limit mas transfer phenomena and the diffusion of lipid oxidation products. For RC and RC20, their encapsulation into the artificial vesicles improved their efficiency probably by favoring their location at the lipid droplet/water interface. Considering that the unloaded artificial vesicles did not express any antioxidant activity, these results suggest that artificial vesicles could act as carriers allowing the antioxidant molecule to express its activity. This could be due to the fact that antioxidant molecules encapsulated in these vesicles would be more available to exert their radical scavenging activity probably by an optimized encapsulation leading to a better orientation of the molecules into the particles. For HT and HT12, which showed a decreased activity when encapsulated in artificial vesicles, their availability as antioxidants within the artificial membrane would be hampered resulting in a loss of their chemical reactivity in comparison with the same molecules added alone in the emulsion.

Others studies have shown that the efficiency of some antioxidants could be improved in emulsions by their associations with vesicles or micelles. For example, Feng et al. (2017) prepared niosomes based on alkyl-O-glycosides and catechin, epigallocatechin and their dodecyl esters. They studied their antioxidants action to avoid liposomes oxidation induced by AAPH radicals. When not loaded into niosomes, both catechin and epigallocatechin expressed a better antioxidant activity than their dodecyl esters homologues. However, the antioxidant effect of these esters was significantly improved after niosomes encapsulation. The authors proposed that their cationic niosomal vesicles could bond and fuse with the positively charged liposomes allowing the encapsulated antioxidant to be incorporated into the liposomes for an optimized antioxidant activity. Interestingly, the authors mentioned that the incorporated dodecyl esters after vesicles fusion tended to reside in the liposome bilayer rather than diffuse into the aqueous phase because of their strong hydrophobicity. Such behavior could also explain our observations made with loaded artificial vesicles for which the increase of antioxidants efficiency was particularly strong for rosmarinic acid (intermediate polarity and negatively charged) and eicosyl rosmarinate (highly lipophilic). Another example was illustrated by Kiralan et al. (2014) who observed that the formation of surfactants-tocopherols co-micelles into the homogenous aqueous phase of emulsion improved the antioxidant effect of tocopherols. To explain this phenomenon, the authors suggested that such co-micelles would play the role of antioxidant reservoirs to maintain optimal level of antioxidants. They also envisaged that surfactant-tocopherols co-micelles could alternatively play a role in scavenging reactive oxygen species that could exist in the aqueous phase of the O/W emulsion. In this context, such antioxidant micelles would then participate in the limitation of lipid oxidation diffusion (mass transport) from droplets to droplets. Such a potential antioxidant mechanism is interesting when considering our results with RC20. As previously mentioned, Laguerre et al. (2010) showed that this phenolipid was weak in protecting lipids from oxidation in emulsions. This was attributed to the fact that this molecule tends to form aggregates in emulsions that strongly limit its availability as radical scavenger at the interface. The same observations were made in the present study as RC20 gave also a very low CAT value. However, when loaded into artificial membranes its activity was boosted. Using X-ray diffraction analysis, we previously showed Durand et al. (2017) that such long chains rosmarinic acid alkyl esters tends to deeply anchor into the lipid bilayer with their polar head expelled to the surface of the membrane vesicle. A similar location of RC20 in our vesicles is very probable and would favor its antioxidant activity as the polar head of RC20 (responsible of the radical scavenging reactivity) would be available at the surface for its antioxidant action. Therefore, as suggested by Kiralan et al. (2014), when loaded vesicles would locate at the O/W interface of the emulsions, RC20 could then express an optimized activity. Alternatively, one can

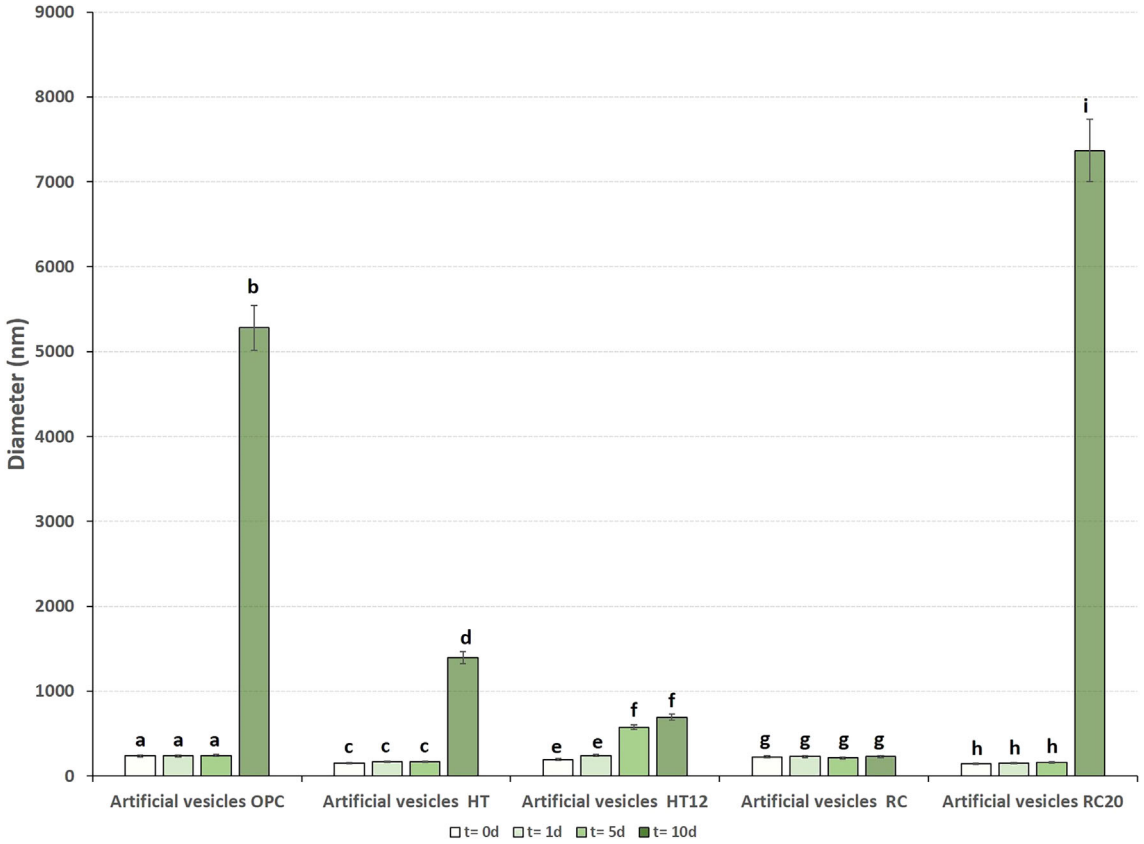

FIGURE 4 Size diameter during storage (4°C) of artificial vesicles loaded in antioxidants: OPC HT, HT12, RC, and RC20 (T0, T1, T5, T10 days). (Values sharing the same superscript letter are not significantly different at $p < 0.05$ (ANOVA; $n = 3$) within each vesicle group).

also envisaged that RC20 loaded in artificial membranes could constitute an antioxidant system capable of intercepting and scavenging reactive oxygen species present in the aqueous phase and therefore, contribute to an antioxidant action by limiting oxidative mass transfer phenomenon. Moreover, our results with RC20 loaded vesicles can be put in parallel with the ones from Panya et al. (2012) who showed that the activity of RC20 in emulsions could considerably be improved by co-micellization with excess surfactants present in the medium. In conclusion for the CAT assay, we could show that depending of their chemical structures, the loading in some antioxidants into artificial membranes mimicking the composition of extracellular membranes from olive-coproducts could either boost or decrease their antioxidant activity. In order to confirm these observations, we then evaluated the effect of such loaded vesicles in limiting the oxidation of rapeseed oil in emulsions and measuring primary and secondary oxidation products formation.

Physical stability of vesicles

Before the evaluation of their activity as antioxidant systems in rapeseed oil-in-water emulsions, the physical stability of all loaded artificial vesicles was first checked during storage at 4°C for 10 days (Figure 4). The vesicles loaded with OPC, HT, RC, and RC20 remained stable for the first 5 days as no significant increase of vesicles size could be observed. Whereas a diameter increase was observed at 10 days of storage for artificial membranes loaded in OPC, HT, HT12, and RC20, it is worth noting that the ones loaded in RC were particularly stable even at 10 days storage. In the case of HT12, artificial membrane vesicles showed an increase of their diameter (2 folds order of magnitude) after 5 days of storage. With RC20 loaded vesicles, while the stability at 5 days was good, a drastic increase of vesicles diameter was measured after 10 days resulting in particles size diameters above 7000 nm. If one excludes the potential chemical degradation (oxidation or hydrolysis) of the lipid bilayer of the vesicles, which is very improbable in the chosen storage conditions, one must consider that the observed increases in particle diameters are mainly due physical alterations such as the phenomena of fusion and aggregation, or leakage of entrapped antioxidant molecules. In terms of lipids composition, all artificial membranes do not differ. Their methods of formation were also similar. This suggests that the differences observed in terms in particles stability are most probably due to the nature of the

entrapped antioxidants themselves. It is well known that liposomes are able to encapsulate both hydrophilic or lipophilic bioactives, either in their internal aqueous core or their lipid bilayer, respectively. Therefore, the resulting stability of the obtained liposomes may be influenced by the polarity of the molecule they encapsulate.

For example, using fluorescein as model hydrophilic compound and rhodamine as hydrophobic one, (Khan et al., 2008) showed that fluorescein-encapsulated 1,2-dipalmitoyl phosphatidylcholine (DPPC) liposomes were more stable with respect to time and temperature. On the contrary, 1,2-distearoyl phosphatidylcholine (DSPC) liposomes exhibited no significant leakage differences most likely due to the higher transition temperature of DSPC compared with DPPC. Considering the polarity of both model compounds, the authors considered that with DPPC liposomes, fluorescein was more likely to be stored in the liposomal aqueous core while hydrophobic rhodamine would be located in the hydrophobic bilayer region of the liposomes and that this difference in location of both compounds would explain their leakage from the liposomal particles, with Rhodamine being more easily expelled from the vesicles. In a large computational study of more than 600 drug candidates for liposomal delivery systems, Cern et al. (2017) evaluated the influence of drugs structure on leakage and resulting liposomal stability and showed that the most important descriptors in the leakage models were those related to logP of the loaded molecules. Additionally, other authors showed that drug release from liposome was also linked to the charge of the drug, most likely due to electrostatic interactions with the liposome membrane depending of its charge (Aibani et al., 2020). In conclusion, beside the physical–chemical properties of the liposome itself, both logP and pka of loaded molecules are important parameters to explain drug leakage and eventually liposome stability. Various authors have studied the influence of such drug parameters on stability of vesicles (Gubernator et al., 2010; Soepenberg et al., 2004; Zhang et al., 2016; Zhigaltsev et al., 2005). In our specific case, artificial vesicles were loaded with antioxidants varying in polarity, either very hydrophilic ones such as verbascoside ($\log P = -0.707$) or oleuropein ($\log P = -0.072$) in the case of OPC extract, or intermediate polarity antioxidants such as HT ($\log P = 0.385$) or RC ($\mathrm{Log}P = 1.58$), or more lipophilic ones such as HT12 and RC20. From all the tested vesicles, the ones loaded in rosmarinic acid had the best stability as no increase of diameter of particles were observed. This could be attributed to the polarity of this molecules but more probably to its charge as under the condition of liposome formation, rosmarinic acid was predominantly deprotonated ($\mathrm{pKa} = 3.57$). In this form, it is probable that leakage from the liposomes is very limited due to electrostatic interactions of rosmarinic acid with lipid bilayer which is positively charged (Table 1). Vesicles loaded in HT presented a good stability at $t = 5$ days but an increase of vesicles size at $t = 10$. However, this increase was limited in comparison with the ones observed for lipophilic HT12 and RC20 and the ones of the vesicles loaded in OPC antioxidants which mainly contain hydrophilic verbascoside and oleuropein (Barouh et al., 2024). The case of HT12 and RC20 is of particular interest. Artificial vesicles loaded in HT12 started to show diameter increase as early as 5 days of storage. Vesicles loaded in RC20 drastically increased in size at 10 days. These two antioxidants, owing to the lipophilic chain they have in their structure are very probably located in the lipid bilayer of the liposome entities and not in their aqueous core. Indeed, with the example of C16 ester of rosmarinic acid, Durand et al. (2017) showed that this ester tail was deeply located within the liposome bilayer whereas its polar head was more expelled at its outer surface. Accordingly, the location of HT12 and RC20 at the lipid bilayer tends to favor their leakage and therefore, the instability of the corresponding vesicles. These results agree with the ones described by Cern et al. (2017) in their large computational study.

Antioxidant efficiency of loaded artificial vesicles in oil-in-water rapeseed oil emulsions

In order to confirm the previous results obtained with the CAT assay, we then evaluated the capacity of loaded artificial vesicles in limiting lipid oxidation in oil-in-water emulsions (1% rapeseed oil in PBS buffer and 5 mM SDS) and compared it with the effect of the corresponding antioxidants added alone in the same emulsion. For this, HT, HT12, RC, and RC20 loaded into artificial vesicles or the same antioxidants alone were added to the emulsions at 50 μM final concentration of antioxidants. The emulsions were then stored at 40°C for 21 days and their kinetics of lipid oxidation were followed by measuring primary oxidation compounds (Peroxide values) and secondary ones (TBARS) (Figure 5). When used alone (not incorporated into artificial vesicles), the efficiency of the tested antioxidants could be ranked as follows: RC > RC20 > HT ≈ HT12. Similarly, to the CAT assay, RC showed the best antioxidant effect among the four tested antioxidants. However, whereas RC20 was less efficient than HT and HT12 in the CAT assay, it performed better in the oil-in-water emulsion system. When comparing the effect of the various antioxidants alone with that observed with their vectorized forms (antioxidant loaded into artificial vesicles), we observed that the vectorized forms were less efficient for HT, HT12, and RC20 whereas no significant differences were observed for RC. With the exception of RC20, we therefore observed the same tendencies as those observed in the CAT assay when

TABLE 1 Artificial vesicle charges: Artificial membranes loaded in HT, HT12, RC, RC20, or OPC.

Vesicles	Artificial vesicles loaded in HT	Artificial vesicles loaded in HT12	Artificial vesicles loaded in RC	Artificial vesicles loaded in RC20	Artificial vesicles loaded in OPC
Charge (meV)	34.6 ± 1.2	33.2 ± 0.4	22.7 ± 3.4	20.8 ± 2.1	31.9 ± 0.9

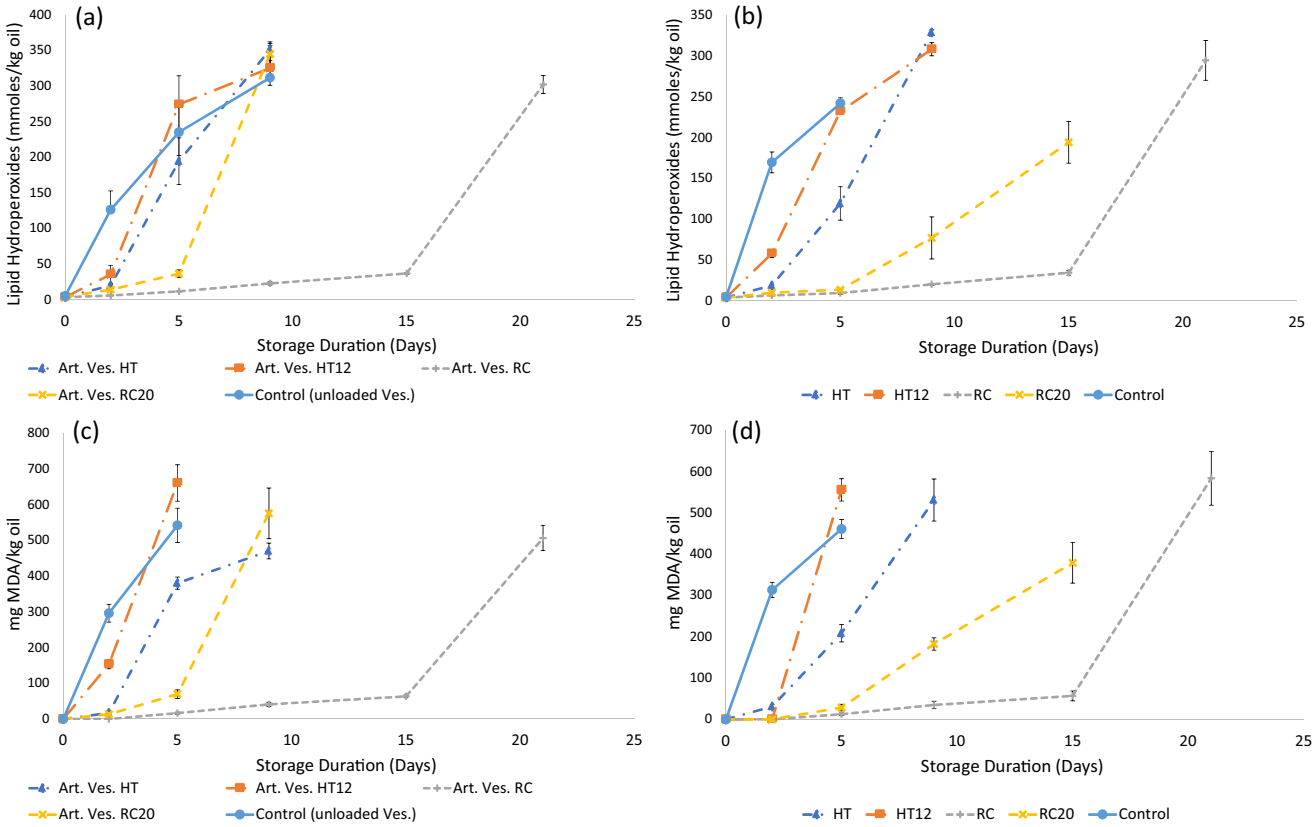

FIGURE 5 Oxidation kinetics (Peroxides Values and TBARS) of rapeseed oil-in-water emulsions (1% rapeseed oil in PBS (10 mM, pH 7.0) and SDS (5 mM)) in the presence of artificial loaded vesicles or antioxidants alone: (a) Peroxides Values with loaded Artificial Vesicles (b) Peroxides Values with antioxidants alone (c) TBARS with loaded Artificial Vesicles (d) TBARS with antioxidants alone.

using artificial vesicles as antioxidant carriers. The reasons why the results in oil-in-water emulsion did not confirm the significant improvement of the antioxidant activity of RC20 when vectorized in artificial vesicles as observed in the CAT assay is still unclear.

We previously showed (Figure 4) that the artificial vesicles loaded with HT, HT12, and RC20 where physically more instable than the ones loaded with RC. This physical instability was particularly pronounced for the RC20 loaded artificial vesicles. While the CAT assay is made with a few hours, the storage of these oil-in water emulsions was done for several days. Therefore, the aggregation of the artificial vesicles loaded in RC20 could have occurred resulting in a reduction of the efficiency of RC20 through the reduction of the antioxidant mobility into the system. The same explanation could be proposed for HT and HT12. However, when considering the first days of storage (Days <5) where the stability of the loaded vesicles is still acceptable, we still observed that RC20 alone was a better antioxidant than its vectorized counterpart. Therefore, the physical instability of the vesicles cannot explain alone the different antioxidant efficiencies observed. Panya et al. (2012) showed that the micellization of RC20 by surfactant can improve its antioxidant effect in emulsion. However, these authors used Tween 20 as emulsifier and above its critical micelle concentration (CMC). In our experimental protocol, SDS was used as surfactant at a concentration (5 mM) below its theoretical CMC (around 8 mM). Therefore, on our system, it is very unlikely that SDS micelles are present and that they may have modulated the antioxidant efficiency of RC20 alone. The emulsion used here and the one used in the CAT assay differ also by the nature of the used emulsifiers (Brij 35 vs. SDS), their lipid droplet size, the properties of the interfacial area or the way lipid oxidation is

induced. All these parameters may have contributed to the antioxidant efficiencies of the loaded vesicles and the antioxidants alone explaining the different observations made for RC20 depending of the used method.

CONCLUSION

The potential of olive extracellular vesicles or artificial liposomes mimicking their lipid composition was studied as potential carriers of antioxidants in emulsion to improve their efficiency. For this, a combination of various antioxidants with different polarities were evaluated. In terms of carrier stability, the ones loaded with the most polar antioxidants (hydroxytyrosol and rosmarinic acid) were the most stable whereas the ones loaded with lipophilic antioxidants (hydroxytyrosyl dodecanoate or eicosyl rosmarinate) were the most instable. This could be due to the localization of these lipophilic antioxidant into the lipid bilayer favoring the instability of the corresponding vesicles. When assessing the antioxidant efficiency of loaded vesicles in emulsified system using tung oil as oxidizable substrate and initiating the oxidation by the addition of a hydrophilic initiator (AAPH), the natural membrane vesicles loading in a specific antioxidant were more efficient than the corresponding antioxidant added alone in the system. This was particularly true when those vesicles were loaded with the most lipophilic tested antioxidant namely eicosyl rosmarinate. All extracellular vesicles exhibited a low antioxidant activity and loading any of the four tested antioxidants did not result in an improvement of that activity. The reason why extracellular vesicles showed a very weak activity whatever their loaded cargo is not clearly understood. Besides their lipid membrane components, extracellular vesicles are also made of proteins and genetic material (microRNA). Although found in lower quantities than in animal extracellular vesicles (Cao et al., 2023), these proteins and genetic material may alter the radical scavenging activities of the phenolic compounds found in these vesicles. This hypothesis may be supported by the fact that the use of natural or artificial liposomes mimicking the composition of membranes of extracellular membranes could boost the antioxidant activity of the loaded antioxidant. However, the boosting effect of natural and artificial carriers was not confirmed when using a 1% rapeseed oil-in-water emulsions and measuring its oxidation status during storage at 40°C. This could be due to a different type of emulsions compared to the one used with the tung oil assay as different surfactants and oxidation inducers were used.

AUTHOR CONTRIBUTIONS

Pierre Villeneuve, Claire Bourlieu-Lacanal: conceived and designed the study, and wrote the first draft of the manuscript. Bruno Baréa, Nathalie Barouh, Amal Fenaghra, Pascal Colosetti carried out the research. Jérôme Lecomte, Erwann Durand, Anne Mey, Fabienne Laugerette, Marie-Caroline Michalski analyzed the data. All authors contributed to and approved the final draft of the manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflict of interest.

ETHICS STATEMENT

No humans or animals were used in this research.

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