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"Exploration of plant derived extracellular vesicles as a new active ingredient for skin health"

Anita Coeurvolan [1,*], Laurianne Simon [1], Zhu Zhao [2], Noelia M Sanchez-Ballester [1], Frederic Zenhausern [2], Jerome Lacombe [2], Sylvie Begu [1]

[1]ICGM, Montpellier University, CNRS, ENSCM, Montpellier, France; [2]Center for applied NanoBioscience and Medecine, University of Arizona, Phoenix, United States

1. Introduction

Throughout life, our skin is subjected to external aggressions such as free radicals, pollution, ultraviolet radiation or pathologies. [1] To tackle those dilemmas and promote well-being by drawing inspiration from natural elements, our research focuses on plant-derived extracellular vesicles (PDEVs) as new active ingredients and nanocarriers of active compounds.

Extracellular vesicles (EVs) are lipid bilayer particles with a size from 50 nm to 5000 nm naturally produced by all types of cells (mammalian, bacterial and plant). Known for their role in cell communication, they contain a complex cargo composed of proteins, nucleic acids, lipids and bioactive compounds. [2] In the literature, mammalian-derived EVs have been extensively explored for therapeutics and biomarkers but are facing serious issues; quality of production at large-scale, loading and stability. For those reasons, PDEVs have been the subject of growing interest for the last decade. [3, 4] In fact, studies with multiple plants like blueberries or grapefruits showed that those PDEVs exerted interesting intrinsic biological properties such as antioxidant, regenerative and anti-inflammatory activities. Plus, the isolation of PDEVs is simple and can be done from several parts of the plant: peel, pulp, sap, roots or leaves, leading to a valorization of the plant as a whole. Furthermore, recent studies have also shown their capacity to vehicle active ingredients such as antioxidants in order to improve their efficiency.[4]

Considering all those benefits, PDEVs embody a potential agent in the cosmetic field both as an active ingredient and a vector. To emphasize the potential of PDEVs for cosmetics, this study will first focus on the isolation and physicochemical characterization of PDEVs from various sources. Then, antioxidant activity of black olives PDEVs and its stability under various storage conditions were investigated. Lastly, cell viability and internalization studies were conducted to assess their potential for cosmetic application.

2. Materials and Methods

2.1. Materials

Cans of black olives were purchased at the supermarket (Auchan, France). Plants were washed using ultrapure water (Milli-Q) from Merck (Germany). Filters with regenerative cellulose (RC) membrane (0.45 µm and 0.22 µm) were purchased from Sartorius (Germany). Hexane, sodium methylate and acetyl chloride solutions were purchased from HoneyWell (USA). 2, 2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), phenazine methosulfate (PMS), potassium persulfate, chloroform and methanol solutions were purchased from Sigma-Aldrich (Germany). 96-well microplates and 1,1'-Dioctadecyl-3,3,3',3'-Tetramethylindocarbocyanine Perchlorate (DiI) were brought from Thermofischer Scientific (USA). 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) was brought from Promega (USA). Ultrafiltration kits were purchased from Merck (Germany).

2.2. Isolation and purification of PDEVs

PDEVs were obtained by an ultracentrifugation-based method using fruits, pulps, juices or seeds from several sources. Plant materials were thoroughly washed and crushed into a paste using a blender. All samples were centrifuged at 4,800 x g for 25 minutes at 4 °C, then at 6,000 x g for 25 minutes at 4 °C in MegaStar 300 (VWR International, USA). The samples were centrifuged at 10,000 x g for 1 hour, two times at 4 °C. After each spin, pellets were discarded as supernatants were kept for the next round. After elimination of pellets, an ultracentrifugation at 100,000 x g for 2 hours at 4 °C was done with Optima XPN-80 (Beckman, Germany). The pellet obtained was resuspended in 1 mL of Milli-Q water and filtered on a 0.45 µm RC membrane. The solution was purified by size exclusion chromatography (SEC) with Sepharose CL-2B (Cytiva, USA) and Milli-Q water as the mobile phase. [5]

2.3. Nanoparticle tracking analysis

PDEVs size and concentration was determined by the nanoparticle tracking analysis (NTA) using the NanoSight NS300 (Malvern Panalytical, UK). PDEVs samples were diluted by 10,000 in Milli-Q water and injected in the capillary of the NTA. Measurements were performed with a 405 nm laser (temperature at 25°C, syringe pump at 40 AU, 5 videos of 60 s recorded). Data were analyzed using the NanoSight NTA 3.3 software. Videos were recorded with a camera level set to 14 and analyzed with a detection threshold of 5.

2.4. Transmission electron microscopy

Transmission electron microscopy (TEM) was performed on TEM Jeol 1200 EXII (Jeol. Ltbd, Tokyo, Japan) associated with a camera Jeol 2 K/2 K. The formulation was deposited on grids type Cu formvar carbon and negatively stained with an aqueous uranyl acetate solution. TEM images were taken at a 25 k magnification.

2.5. Fatty acids composition

Lipids were extracted by adding 600 µL of chloroform:methanol (2:1) and placed into a tube with 200 µL of PDEVs. After an additional 200 µL of chloroform, the tube was centrifuged at 1,000 rpm for 5 min. The bottom phase was kept and solvent was evaporated using a nitrogen evaporator N-EVAP (Organomation, USA). According to the fatty acid methyl esters (FAME) method, 1 mL of sodium methylate was added into the tube and heated at 65°C for 20 minutes. Then, 1 mL of acetyl chloride was added, and the tube was heated under the same conditions as previously described. Finally, 1 mL of hexane and 1 mL of Milli-Q water were added,

followed by centrifugation at 3,000 rpm for 5 minutes. The upper phase was collected and transferred into vials for analysis by gas chromatography (GC) using a Focus GC system (Thermo Fisher Scientific, USA). The blend of the plant is used as a control using the same protocol. [6]

2.6. Antioxidant activity

Antioxidant activity of PDEVs was determined using ABTS assay. 7mM of ABTS and 84 mM of potassium persulfate were mixed and placed in the dark overnight to generate ABTS radical cations. Then, 280 µL of ABTS radical cation solution ($ABTS^{\bullet+}$) and 10 µL of PDEVs at a constant concentration were added in a 96-well microplate for every sample. The absorbance was read at 734 nm with Infinite M1000 reader (TECAN, Switzerland). The standard curve was elaborated with different concentrations of $ABTS^{\bullet+}$ solution (from 0 to 100 %). Controls were made with $ABTS^{\bullet+}$ solution and solvent used for the isolation of PDEVs.

2.7. Stability of antioxidant activity

PDEVs samples were kept in solutions or freeze-dried (FD) using Heto PowerDry LL3000 (Thermo fischer, USA). For each condition, in solution or FD, a sample was placed at 4 °C and at 25 °C. At day 7 and 14, NTA measurements followed by ABTS assay were performed to study the influence of temperature and storage on antioxidant activity. For each time point, FD samples were reconstituted in Milli-Q water one day before measurements.

2.8. Cell culture

Mice fibroblasts cell line (NIH3T3) was purchased from American Type Cell Culture organization (USA). Cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) (Gibco) supplemented with 10 % fetal bovine serum (Life Technologies), 1 % L-glutamine and 1 % penicillin/streptomycin equivalent to final concentration of 2 nM for glutamine, 100 $U.mL^{-1}$ for penicillin and 100 $\mu g.mL^{-1}$ for streptomycin and incubated at 37 °C in humidified 5 % CO_2.

2.9. Cell viability assay

Cells were seeded at 20,000 cells/cm^2 in 96-well plate and incubated for 24 h at 37 °C, 5 % CO_2 to allow cell adhesion. The PDEVs concentration range was prepared in Milli-Q water from 1 x 10^{11} to 1 x 10^6 particles/mL. Cells were treated or not (control) with PDEVs at various concentrations. After 24 hours of exposure, CellTiter 96® Aqueous Non-Radioactive Cell Proliferation Assay (Promega, USA) was used to evaluate the cell viability following the manufacturer's instructions. The absorbances at 490 nm were recorded using Multiskan™ GO Microplate Spectrophotometer (Thermo Scientific, USA) and reduced from the background (wells free of cells). Each data corresponded to a mean of three measurements (mean of 3) was subtracted from each triplicate mean. Values of treated cells were normalized to the non-treated cells representing the 100 % cell viability.

2.10. PDEVs labelling with DiI

PDEVs at 1 x 10^{11} particles/mL were stained with 10 µM DiI by incubation at 37 °C for 1 hour under magnetic stirring. The solutions were purified by ultrafiltration with Amicon® filters (MWCO 100 kDa; regenerate cellulose; Merck MilliPore). 500 µL of the solution was loaded onto the upper compartment of the ultrafiltration device before centrifugation (2,000 x g, 4 °C for 30 minutes) using a centrifuge. Then, the volume was brought to 500 µL with DPBS added

in the upper compartment followed by a reverse spin at 1,000 x g at 4 °C for 2 minutes. Before addition to the cells, PDEVs were filtered through a 0.2 µm filter (RC membrane). The DiI control was prepared as previously described but without the PDEVs.

2.11. Cell internalization

NIH3T3 were seeded at 100,000 cells/cm^2 in a 24-well plate and incubated for 24 h at 37 °C with 5 % CO_2 to allow cells adhesion. The PDEVs labelled with DiI were added at a final concentration of 1 x 10^9 particles/mL to the cells corresponding to 2,500 PDEVs/cells. The incubation kinetics was carried at 4 hours and 24 hours. The solutions were removed and cells were rinsed twice with saline phosphate buffered before observation under EVOS microscope AMF4300 (Life Technologies, USA). Transmitted and RFP (for DiI fluorescence) channels were visualized using a magnification x40. DiI probe was used as a control to ensure the observance of PDEVs and distinguish the fluorescence from the lipophilic probe.

2.12. Statistical analysis

Results are expressed as mean ± standard deviation (SD) and with *n* number of replicates. The statistical analysis of the data resulting from the cell viability on cells was conducted with Origin Pro software 8.1 (OriginLab, USA). A two-sample t-test with equal variance was performed to compare cell viability of treated and untreated cells. The *p*-value reflects the significance with $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$.

3. Results

3.1. Isolation and characterization of PDEVs

First of all, plant materials are being thoroughly washed with distilled water and Milli-Q to take out any dirt and blended until the obtention of a smooth paste. Afterwards, several techniques can be used as the last step of isolation such as ultrafiltration (UF), polymer-based precipitation or electrophoresis coupled dialysis (ELD). However, they present disadvantages such as difficulty to purify samples for polymer-based precipitation or a low yield for UF and ELD. Therefore, ultracentrifugation (UC) -based method was selected as it is low cost, reduces the risk of contamination and supports large sample size. It relies on applying increasing centrifugation speeds at each round to sediment different materials at the bottom called pellets. The final speed results in a pellet containing the PDEVs. In fact, UC has been used for mammalian EVs but is spreading for the isolation of PDEVs. Then, to purify the sample of interest, a step of purification using size exclusion chromatography (SEC) is added. This chromatography allows the separation of molecules depending on their molecular weight. The bigger ones are eluted quickly while the small molecules such as PDEVs are eluted later due to the delay occurring when they are passing through the porous stationary phase. [4] (Figure 1)

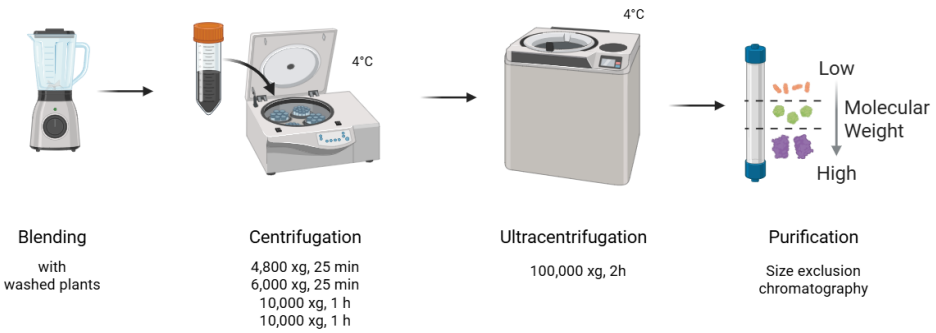


Figure 1. Isolation and purification process of PDEVs (created with BioRender)

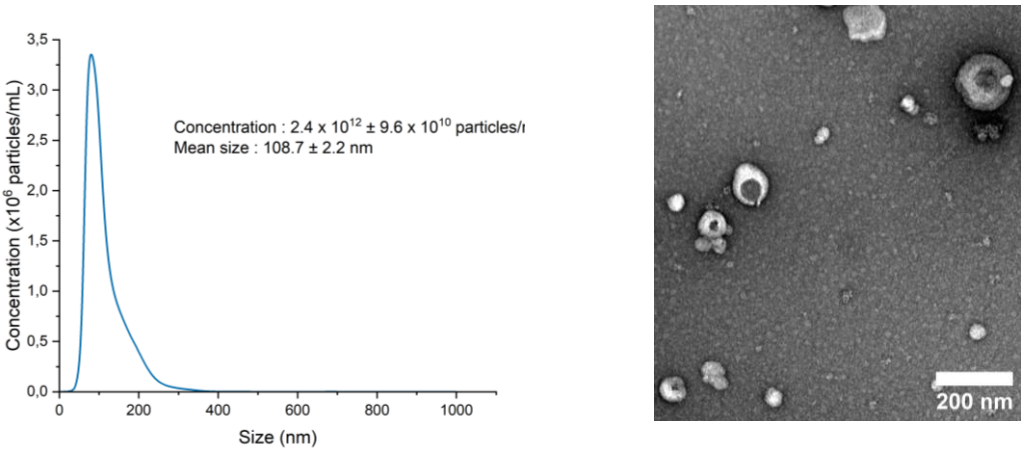
This process of isolation has been used to produce PDEVs from different types of plants such as fruits (black olives), leaves (basilic), roots (ginger) and spices (cloves). (Table 1)

Table 1. Characterization of PDEVs from multiple sources

Source of plant	Size range (nm)	Yield (PDEVs/100 g)	WF Size (mode) ± SD (nm)	PF size (mode) ± SD (nm)
Basil	50-650	$6.9 \times 10^{11} \pm 9.1 \times 10^{7}$	155.6 ± 31.6	102.1 ± 0.5
Black olives	50-600	$2.4 \times 10^{12} \pm 9.6 \times 10^{10}$	104.9 ± 6.0	110.2 ± 3.2
Cloves	50-650	$1.1 \times 10^{12} \pm 7.1 \times 10^{10}$	125.7 ± 7.6	96.5 ± 2.4
Ginger	50-400	$1.1 \times 10^{11} \pm 2.1 \times 10^{9}$	159.9 ± 16.2	87.2 ± 7.3
Lemon	50-450	$6.2 \times 10^{11} \pm 2.4 \times 10^{10}$	161.2 ± 9.2	150.4 ± 7.4

*WF : wide fraction; FD : purified fraction

Overall, with 100 g of each plant, a concentration from 10^{11} to 10^{12} particles/mL was obtained with a size range from 50 to 650 nm. As an example, the NTA profile of black olives PDEVs (BOEVs) showed a narrow size distribution with a concentration of $2.4 \times 10^{12} \pm 9.6 \times 10^{10}$ particles/mL and a mean size of 108.7 ± 2.2 nm. TEM image confirms the presence of PDEVs, indicated by visible bilayer and spherical shape, as well as the overall homogeneity of the sample. (Figure 2)



(**a**) (**b**)

Figure 2. (a) Nanoparticle tracking analysis profile of BOEVs with concentration and mean size (at 25 °C); (b) TEM image of BOEVs with negative staining (scale bar of 200 nm).

3.2. Fatty acids composition

PDEVs are made of a lipid bilayer membrane with a rich cargo composed of proteins, nucleic acids and lipids. It has been proven that lipids play an important role in the stabilisation of PDEVs. [4] For this reason, we investigated the fatty acid composition of BOEVs using a chloroform:methanol (2:1) lipid extraction method and FAME analysis with black olives as a reference. (Figure 3)

For black olives, a total of 15 fatty acids was detected including caproic acid (C6:0), caprylic acid (C8:0), capric acid (C10:0), pentadecanoic acid (C15:0), palmitic acid (C16:0), palmitoleic acid (C16:1), stearic acid (C18:0), oleic acid (C18:1), linoleic acid (C18:2) and linolenic acid

(C18:3), arachidic acid (C20:0), eicosenoic acid (C20:1), docosanoic acid (C22:0), tricosanoic acid (C23:0) and tetracosanoic acid (C24:0). BOEVs shared a similar fatty acid composition except for missing C16:1, C20:0, C20:1, C23:0 and C24:0 fatty acids. The concentration of these fatty acids may be below the GC's detection limit due to the low concentration of lipids extracted from BOEVs.

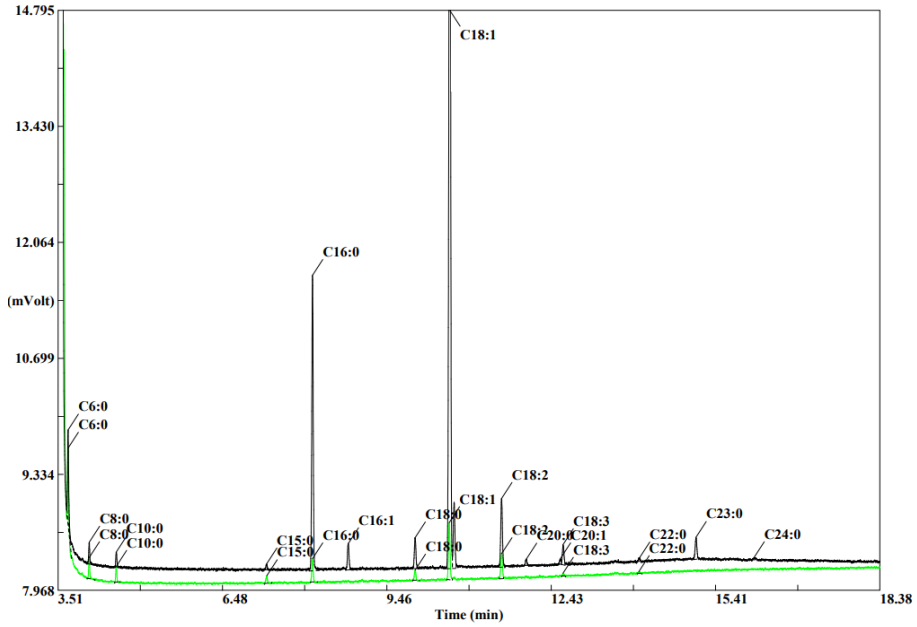


Figure 3. Overlay of chromatogram from black olives (black) and BOEVs (green) by gas chromatography.

3.3. Antioxidant activity and stability upon storage conditions

After physicochemical characterization of BOEVs, the antioxidant activity upon different conditions (4 °C, 25 °C, freeze-dried (FD) stored at 4 °C and 25 °C) at multiple time points was evaluated. The fresh BOEVs were used as a control for this study. Results were expressed by percentage of $ABTS^{\bullet+}$ reduction (%). $ABTS^{\bullet+}$ reduction of the samples stored at 4 °C were represented in blue while those from samples stored at 25 °C were shown in orange. Hatched bars represented results from FD samples. (Figure 4)

BOEVs displayed an $ABTS^{\bullet+}$ reduction of 46.4 ± 11.4 % at day 0. After being kept at 4 °C and 25 °C in solution for 7 and 14 days, the $ABTS^{\bullet+}$ reduction remained between 30% and 40%. Meanwhile, FD samples stored at 4 °C and 25 °C showed a clear lowering of $ABTS^{\bullet+}$ reduction compared to the control, as shown by the values in the range of 10-20 % at day 7 and 14.

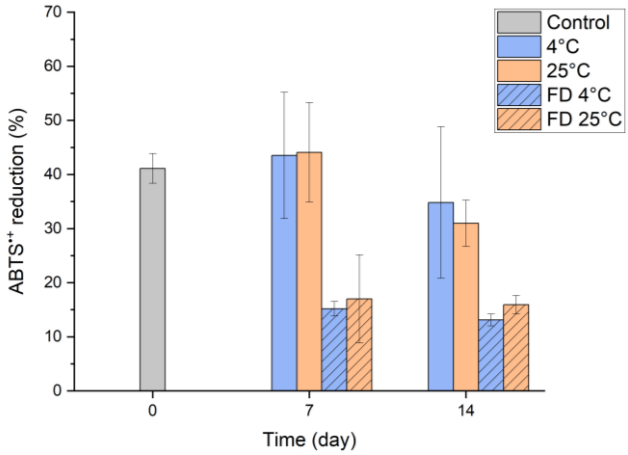


Figure 4. Antioxidant activity of BOEVs solutions and FD samples at day 0 (control), 7 and 14. (*n*=2)

These observations suggest that the antioxidant activity of BOEVs in solution remained equivalent at 4 °C and 25 °C for a period of 14 days. However, a loss of antioxidant activity is noticeable for the FD samples whether they were kept at 4 °C or 25 °C.

3.4. Cell viability and internalization

In order to investigate the cytotoxicity of PDEVs on cells, fibroblasts have been exposed to BOEVs with a concentration range from 10^5 to 10^{11} particles/mL. From 10^5 to 10^9 particles/mL, the cell viability of fibroblasts was not impacted. However, a significant difference with untreated cells, was observed at 10^{10} and 10^{11} particles/mL with respective cell viability of 89.8 ± 7.5 % and 80.4 ± 12.6 %. Thus, the selected working concentration for cell internalization was set on the highest concentration with no significant effects (10^9 particles/mL). (Figure 5)

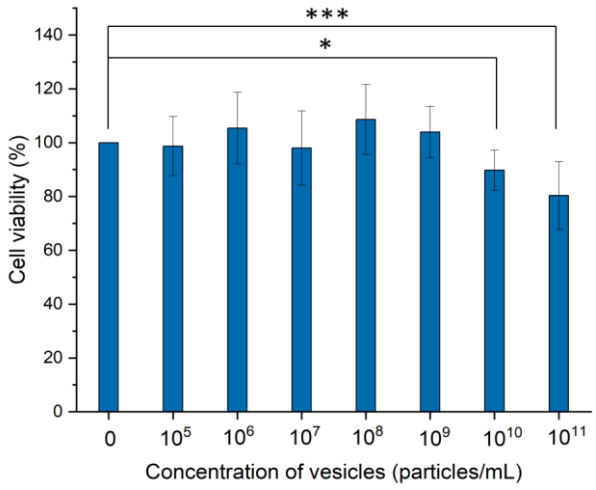
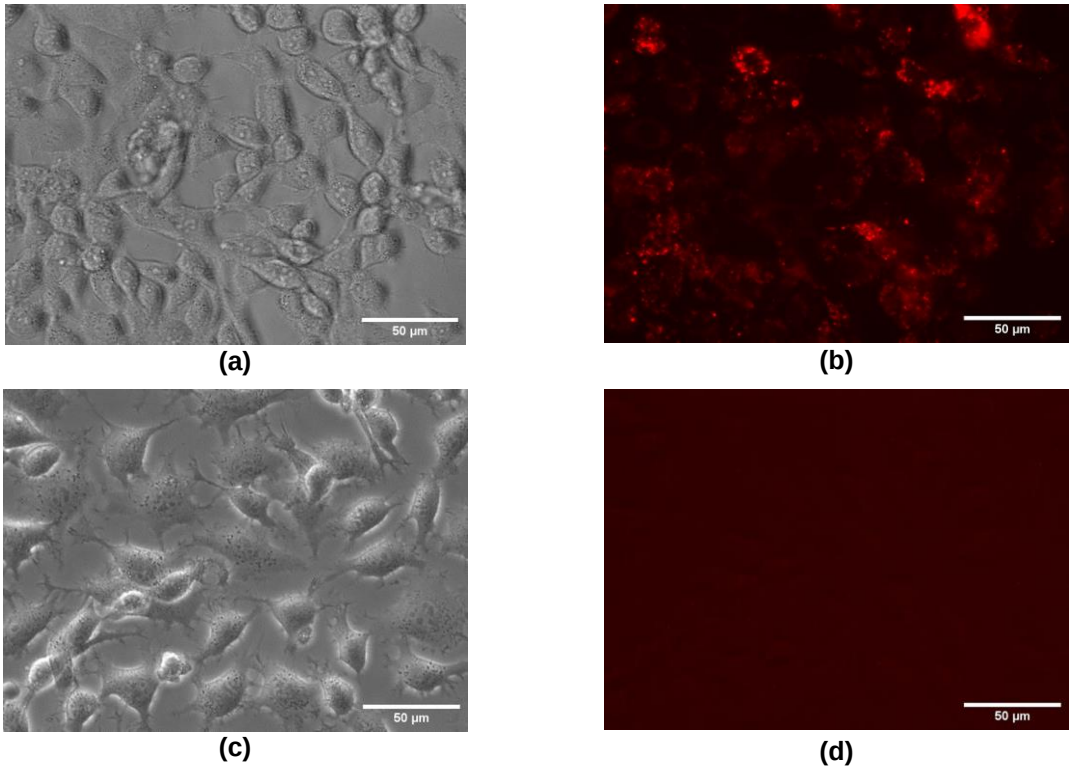


Figure 5. Cell viability of fibroblasts treated with varying concentration of BOEVs from 0 to 10^{11} particles/mL for 24 h (*n*=3, * $p < 0.05$ and *** $p < 0.001$)

To improve the understanding of how the BOEVs can be used for the skin, DiI-labelled PDEVs have been used to perform internalization test on fibroblasts to check the uptake by targeted cells.

Comparing the cells incubated for 24 hours with labelled BOEVs and the control group, a striking difference can be noticed between the two of them. In fact, the control group presented no fluorescence after 24 hours. This indicates that DiI was not internalized by our cells at that

time point. However, after 24 hours of incubation with labelled BOEVs, a clear appearance of fluorescence can be noted by the presence of red dots inside the cells which confirmed the intracellular uptake of BOEVs in the cytosol of fibroblasts. (Figure 6)



(a) **(b)**

(c) **(d)**

Figure 6. Representative images (x40) ; (a) of fibroblasts culture with BOEVs labelled with DiI and (b) corresponding fluorescence image after 24 hours of incubation ; (c) of fibroblasts culture with DiI and (d) corresponding fluorescence image after 24 hours of incubation.

4. Discussion

PDEVs represent the next class of potential natural ingredients for cosmetics due to their intrinsic properties such as antioxidant and anti-inflammatory activities. To highlight their potential, this article focuses on the isolation process of PDEVs, antioxidant activity and stability followed by cellular tests. To illustrate these characterizations, this article looks into black olives as a plant material, which are widely used in cosmetics due to their bioactive compounds such as flavonoids and phenols. [7]

The combination of UC and SEC allowed the isolation and purification of PDEVs from different sources such as black olives, ginger or cloves. Using 100 g of black olives as the source material, a concentrated sample of 2.4 x 10^{12} particles/mL with a mean diameter around 110 nm was obtained. This result lies with previous results in the literature, where an ultracentrifugation method followed by a sucrose gradient was used to obtain a concentration of 3.95 x 10^{12} particles/mL from 100 g of canned black olives. Both BOEVs and these PDEVs share comparable average size, spherical shape and concentration range. Thus, it can be concluded that this process yields outcomes similar to those previously reported while differing by its purification step. [8] These PDEVs are by definition cell communication vehicles composed of a lipid bilayer whose composition depends on the plant nature itself. As far as BOEVs, the fatty acid composition identified presents all the major fatty acids reported in the literature such as palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1), linoleic acid

(C18:2) and linolenic acid (C18:3). These characteristics are beneficial for human skin as it is composed of lipids such as fatty acids which play an important role in the permeability barrier, acidification of the stratum corneum and regulation of keratinocytes homeostasis. *In fine*, fatty acids ensure skin hydration, softness and elasticity. Therefore, fatty acids found in BOEVs allow for a better absorption in the epidermis and can improve the overall appearance of the skin. [7,9]

However, the skin is subject to oxidative stress by multiple factors like pollution, ultraviolet radiation and aging resulting in the overproduction of reactive oxygen species (ROS) leading to cell apoptosis. [9] To combat this phenomena, antioxidants are used to regulate ROS levels. Here, ABTS assay allowed to confirm the antioxidant activity of BOEVs which can scavenge 46.4 ± 11.4 % of $ABTS^{\bullet+}$. However, in the literature, antioxidant activity test is often evaluated via *in vitro* test to mimick biological complexity. Testing BOEVs on cells which have been exposed to H_2O_2 would be helpful to ensure that BOEVs can protect cells against oxydative stress. [10] Plus, black olives exert other properties such as anti-inflammatory activity [7].Thus, it would be interesting to measure the effect of BOEVs on cytokines pro-inflammatory expression with inflammation-induced cells treated with lipopolysaccharides. [11]

Regarding the storage of PDEVs, the stability of antioxidant activity was investigated using the ABTS assay at different time points (day 0, 7 and 14). It showed that the antioxidant activity of BOEVs in solution remains comparable for both conditions at 4 °C and 25 °C but shown a reduction to 30 % after 14 days for this last condition. This can be explained by the fact that EVs storage can lead to instability and rapid structural damages of the cargo at 25 °C after several days thus including antioxidant compounds of EVs. However, while testing FD samples at both conditions after 7 and 14 days, an important reduction of antioxidant activity can be noted as the value falls near 20 %. Several factors come into play to explain these results. First of all, the freezing done at -80 °C before freeze-drying can disrupt the PDEVs membrane to a certain extent and result in low recovery yield. Also, the freeze-drying of black olives PDEVs in water showed a low recovery yield under 40% in previous study. [8,12] In fact, lyophilisation without a cryoprotectant can severely damage PDEVs and its bioactive cargo leading to a weaker $ABTS^{\bullet+}$ reduction capacity. The addition of a cryoprotectant during lyophilisation such as trehalose has shown to enhance the stability of PDEVs and could allow for a long term antioxidant activity of FD BOEVs. [8] Additionally, cellular tests showed no toxicity for cells. Using the concentration of 10^9 particles/mL, the internalization of BOEVs within fibroblasts after 24 hours of incubation has been demonstrated. This observation shows that BOEVs can be uptaken by cells and therefore, deliver they payload into cells to exert their antioxidant effect.

All together, these results highlight the potential of BOEVs for cosmetics, as it contains fatty acids and antioxidant properties, useful for improving skin appearance, without initiating cytotoxicity.

5. Conclusion

PDEVs are originally communication vehicles produced by all types of cells including plants. They are timed with a panel of bioactive compounds presenting biological intrinsic properties such as antioxidant and anti-inflammatory properties valuable for skin applications. To shed light on these PDEVs, this article focuses on the process to isolate PDEVs from several plants and parts. By using, an ultracentrifugation-based method combined with SEC, samples containing 10^{12} particles/mL of black olives PDEVs, characterized by a spherical structure and

a size of approximately 110 nm, were obtained. These PDEVs showed no cytotoxicity and were able to be internalized by fibroblasts. They are composed of fatty acids which play an important role in the skin health by providing elasticity and hydration. Plus, the antioxidant activity of BOEVs has been proven and can be preserved up to 14 days at 4 °C and 25 °C in solutions. Ultimately, BOEVs has been brought to light as a potential new active ingredient for cosmetics by possessing fatty acids and antioxidant activity, which are useful for improving skin health.

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