Cytotoxicity assessment of microalgae-derived extracellular vesicle-enriched populations in Jurkat cells

Amira Batrisyia Shariman[1], Puteri Hanis Nafisah Megat Kamil[2], Fatin Syahirah Othman[3], Shaza Eva Mohamad[3], Nadiah Abu[2]

1 Department of Chemical Engineering & Sustainability, Kulliyyah of Engineering, International Islamic University Malaysia, 53100, Kuala Lumpur, MALAYSIA.
2 UKM Medical Molecular Biology Institute, Universiti Kebangsaan Malaysia, 56000, Cheras, MALAYSIA.
3 Malaysia-Japan International Institute of Technology, Universiti Teknologi Malaysia, 54100, Kuala Lumpur, MALAYSIA.
Corresponding author. E-mail: nadiah.abu@hctm.ukm.edu.my; Tel: +603 9145 9068

ABSTRACT Extracellular vesicles (EVs) are nano-sized particles that are naturally released by cells. Recently, increasing attention has been directed toward EVs derived from natural sources such as plants and fruits. This is due to their sustainability and cost-effectiveness compared to mammalian-derived EVs. Microalgae are photosynthetic unicellular organisms that have also been reported to secrete EVs. In this study, we aimed to isolate EVs from the well-known microalga *Chlorella vulgaris* and evaluate their cytotoxicity in Jurkat T cells. EVs were isolated using a combination of ultracentrifugation and size-exclusion chromatography (SEC). Characterization was conducted using MicroBCA protein assay, dynamic light scattering (DLS), and nanoparticle tracking analysis (NTA). Subsequently, the EV-enriched population was co-cultured with Jurkat T cells, and cell viability and apoptosis were assessed. Our results demonstrated that the EV-enriched population had a mean size below 200 nm. Moreover, the EVs did not cause any significant reduction in cell viability or induce apoptosis. Overall, our preliminary study has shown that microalgae-derived EVs are non-cytotoxic and are biocompatible in a mammalian system.

KEYWORDS: Microalgae; Nanocarriers; Jurkat; Extracellular Vesicles; *Chlorella vulgaris*.
Received 15 December 20205 **Revised** 25 December 2025 **Accepted** 26 December 2026 **In press** 26 December 2026 **Online** 27 December 2025

Original Article

INTRODUCTION

Extracellular vesicles (EVs) are nano-sized vesicles that are released into the extracellular space by cells. EVs are known to be involved in various cellular processes and can also carry cargo-specific markers. Apart from being endogenously involved in cellular processes, EVs also have the potential to carry drugs or external molecules to different sites (Du *et al.,* 2023). Apart from human sources, extracellular vesicles (EVs) can also be isolated from non-mammalian organisms such as plants and bacteria (Nemati *et al.,* 2022). Some EVs isolated from plants also possess the intrinsic anti-inflammatory properties. For example, a study by Cao *et al.,* has shown that plant-derived extracellular vesicles can be extracted from broccoli (Cao *et al.,* 2024). In fact, it has been shown that these entities can enhance the therapeutic effects of 5-flourouracil in colon cancer (Cao *et al.,* 2024).

Recently, there has been growing interest in exploring these alternative sources due to their sustainability and cost-effectiveness (Nemati *et al.,* 2022). Among them, microalgae have shown great potential as a source of biocompatible EVs. Earlier studies by Adamo *et al.,* have shown that they managed to isolate EVs from microalgae *Tetraselmis Chuii,* and termed them as nanoalgosomes (Adamo *et al.,* 2021). In fact, a study by Picciotto *et al.* (2021) managed to isolate EV-like particles from seven species of microalgae including *Cyanophora paradoxa*. On top of that, it has been shown that these EVs also possess anti-inflammatory properties as well (Adamo *et al.,* 2024). Among the different species of microalgae, *Chlorella vulgaris* is widely known for its therapeutic and nutritional values (Panahi *et al.,* 2016). This microalga has been reported to possess exceptional anti-inflammatory and antioxidant activities and is particularly beneficial for human use (Mendes *et al.,*

2024). Therefore, in our study we would like to isolate EVs from a well-known microalgae species, *Chlorella vulgaris* and evaluate its toxicity in an *in vitro* model.

METHODOLOGY

Preparation of Culture Medium- Bold's Basal Medium (BBM)

BBM was used as the culture medium for *Chlorella vulgaris* due to its physicochemical similarity to freshwater. The medium was prepared according to the standard recipe consisting of ten stock solutions. All required compounds were mixed and diluted with distilled water to prepare each stock solution. The appropriate volumes of each solution were then added into a 1 L Schott bottle and made up to volume with distilled water. The pH of the medium was adjusted to 6.6. The bottle was covered with aluminium foil and autoclaved to prevent contamination. After autoclaving, the medium was stored at room temperature. Table 1 presents the composition of BBM for 1 L preparation.

Table 1. Recipe for Bold's Basal Medium

Component	Amount in 1L	Stock solution concentration
$NaNO_3$	10 mL	10.0 g/400 mL
$CaCl_2.2H_2O$	10 mL	1.0 g/400 mL
$MgSO_4 \bullet 7H_2O$	10 mL	3.0 g/400 mL
K_2HPO_4	10 mL	3.0 g/400 mL
KH_2PO_4	10 mL	7.0 g/400 mL
NaCl	10 mL	1.0 g/400 mL
EDTA Stock	1 drop (0.05 mL)	
Iron Stock	1 drop (0.05 mL)	
Boron Stock	1 drop (0.05 mL)	
Bold Trace Stock	1 drop (0.05 mL)	

Microalgae Cultivation

The microalgae used in this study, *Chlorella vulgaris*, were obtained from the Research Laboratory of Algal Biomass, MJIIT, UTM Kuala Lumpur. For laboratory-scale cultivation, 200 mL of BBM was used to grow *C. vulgaris*. An inoculation volume of 10% of the total medium volume (20 mL) was taken from the stock culture provided by the laboratory. To prevent cross-contamination, all procedures were conducted under a laminar flow hood. The cultures were maintained at 25 °C, aeration with 2% of carbon dioxide and exposed to continuous illumination (24 h photoperiod) using a white, fluorescent lamp emitting 80 μmol photons m^{-2} s^{-1}. To maximize EV production, *Chlorella vulgaris* biomass was harvested at the exponential growth phase, corresponding to an OD_{750} nm in the range of 0.5–0.8.

Isolation of EV-enriched Population

Approximately 40-50 mLs of microalgae culture were subjected to differential centrifugation. The first step involved spinning the samples at 2000xg for 10 minutes, then the supernatant was transferred and centrifuged at 10,000xg for an additional 30 minutes. Subsequently, the supernatant was collected and subjected to ultracentrifugation for 100,000xg for 12 hours. Then, the pellet was resuspended in Phosphate Buffered Saline (PBS) and were further purified using the Exo-Spin Column (Cell Guidance Systems, USA) according to the manufacturer's instructions with slight modifications. The resulting EV-enriched population was used for downstream analysis.

BCA Assay

Protein content was determined using the microBCA Protein Assay Kit according to the manufacturer's instructions (Thermo Fisher, USA).

Dynamic Light Scattering

To assess the overall size distribution and dispersion of the EV-enriched preparation, we employed dynamic light scattering (DLS) using the Zetasizer Nano ZS instrument (Malvern Instruments, USA), following the manufacturer's instructions. Data acquisition and analysis were performed using the Zetasizer Software provided with the system.

Nanoparticle Tracking Analysis

Nanoparticle Tracking Analysis was conducted using the Nanosight NS300 machine and the corresponding software (Malvern Instruments, USA).

Cell culture

Jurkat cells were purchased from ATCC and maintained in UMBI's Biobank. The cells were cultured in RPMI1640 medium (Nacalai Tesque, Japan), supplemented with EV-depleted FBS and Penicillin-Streptomycin (Nacalai Tesque, Japan). The cells were maintained in a 37 °C humidified CO2 incubator.

Alamar Blue Assay

In brief, 5000 cells per well were seeded in a 96 well-plate before varying concentrations of EVs were added to the cells. The cultures were incubated for 48 and 72 hours. Afterwards, 10 ul of Alamar Blue (Biolegend, USA) was added and left to incubate for 30 minutes. Then, the relative fluorescence unit was obtained using a fluorescence microplate reader, Varioskan Lux (ThermoFisher, USA).

Apoptosis Annexin V Assay

Cells were seeded in a 24 well plate and were treated with EV-enriched population for 48 hours. Afterwards, the cells were collected and subjected to Annexin V staining using the Annexin V-FITC/PI Apoptosis Kit following the manufacturer's instructions (Elabscience, USA). The stained cells were then acquired using the BD FacsVerse Flow cytometer machine (BD, USA).

Statistical Analysis

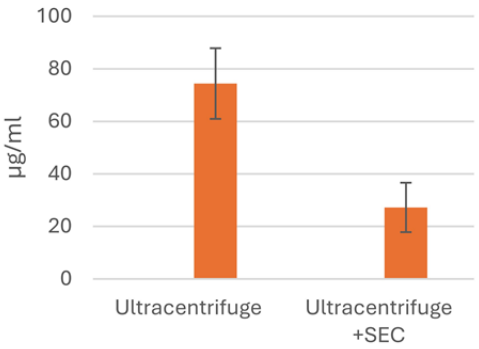
Statistical analysis for the Alamar Blue and Annexin V assays was performed based on the mean ± standard deviation (SD) using Student's t-test, with $p < 0.05$ considered statistically significant.

RESULT AND DISCUSSION

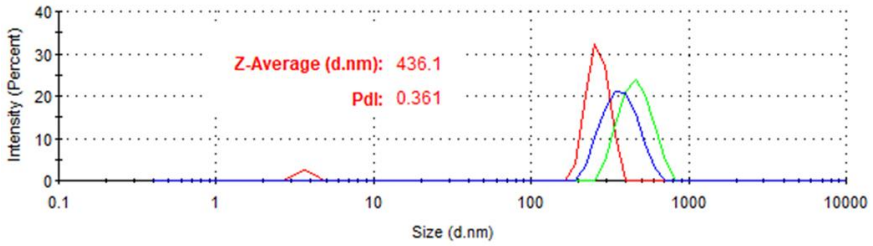
In this study, we utilized the microalga *Chlorella vulgaris* due to its well-established characteristics and ease of cultivation. For instance, a study by Savviddou *et al.*, has shown that extracts from *Chlorella vulgaris* is high with antioxidant activity (Savvidou *et al.*, 2023). As shown in Figure 1a, a visible pellet was obtained after ultracentrifugation, which was subsequently purified using size exclusion chromatography (SEC). The protein concentration of the EV-enriched populations was determined using the microBCA assay. We compared the protein content of EV samples isolated by ultracentrifugation alone with those subjected to an additional SEC purification step. As shown in Figure 1b, samples processed with SEC exhibited lower protein concentrations, indicating a higher level of purity in the EV population. Other studies have utilized other methods for EV isolation. For

instance, Adamo *et al.*, used a tangential flow filtration system (Adamo *et al.*, 2024). This may also result in different yields and composition of EVs.

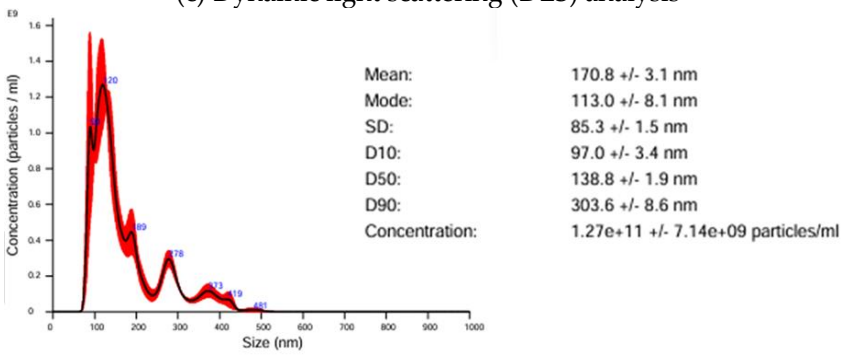
(a) Pellet obtained from the ultracentrifugation process.



(b) MicroBCA assay comparing EV yield from ultracentrifugation alone and ultracentrifugation followed by size-exclusion chromatography (SEC).



(c) Dynamic light scattering (DLS) analysis



(d) Nanoparticle tracking analysis (NTA) of the EV-enriched preparation.

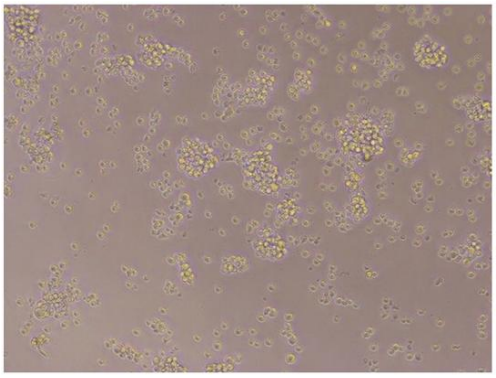
Figure 1. Figure shows the characteristics of the EV-enriched population.

Subsequently, we aimed to determine the overall size distribution of the isolated EV population. Two complementary methods were employed: dynamic light scattering (DLS) and nanoparticle tracking analysis (NTA). As shown in Figure 1c, the size distribution obtained from DLS was approximately 400 nm, which is relatively large for an EV-enriched population. It is known that DLS may overestimate the measurement as it is easily affected by outliers (Anderson *et al.*, 2013, Maas *et al.*, 2015). This measurement contrasts with the expected size range as an EV-enriched population often exhibits a mean size ranging at 50–200 nm, characteristic of small extracellular vesicles (sEVs) or exosomes (Welsh *et al.*, 2024). The optimum size range for a purified population of sEVs is below 200 nm, consistent with the guidelines established for biologically active vesicles (Welsh *et al.*, 2024). However, NTA measurements revealed a mean particle size of approximately 170 nm, consistent with the expected range for extracellular vesicles as shown in Figure 1d. A study by Garaeva *et al.*, observed that EVs isolated from *Parachlorella kessleri* and *Chlamydomonas reinhardtii* were sized approximately around 80nm and 90nm respectively based on NTA (Garaeva *et al.*, 2025). Different

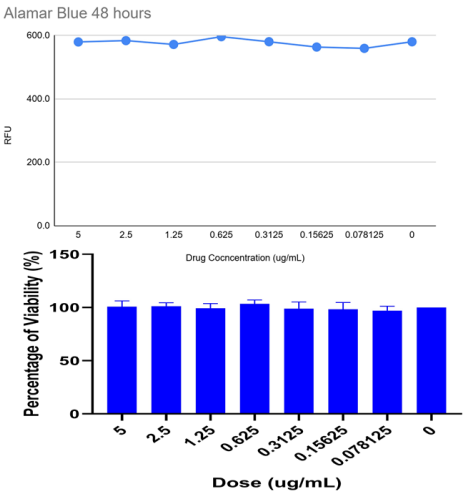
study by Adamo *et al.,* also showed that the EVs isolated from *Tetraselmis Chuii* were sized around ~100nm (Adamo *et al.,* 2024). The concentration of our preparation as shown by the NTA results was approximately ~1.2e11 particles/mL. Nevertheless, we are unable to compare this with current literature as we did not measure the dry biomass. Picciotto *et al.* (2021) reported varying concentrations of sEVs based on dry weight mass for different microalge species. For instance, the microalgae *Tetraselmis Chuii*, released sEVs ranging from ~2.6 e8 to 3e7 particle number per mg dry weight mass. Another limitation that we would like to highlight is the prolong period of ultracentrifugation, which may cause further aggregation and damage. This step was performed at 4 C and immediately proceeded with the SEC steps to minimize damage. While both NTA and DLS showed expected results of particle concentration and size, additional verification steps such as electron microscopy or determination of EV protein biomarkers could further strengthen the validation of the EV structure and integrity.

To further elucidate the cytotoxic effects of the isolated EV-enriched population in an in vitro cell culture, we utilized the Jurkat T cells as a model. As shown in Figure 2a, co-culture with EV-enriched population did not induce any noticeable morphological changes in the cells, indicating that EV-enriched population had minimal effects on them. On top of that, we also evaluated the cytotoxicity using an Alamar blue assay. After 48 hours of co-culture with EV-enriched population at concentrations up to 5 μg/mL, no significant changes were observed in cell viability, as shown in Figure 2b. This finding was consistent with the Annexin V assay results presented in Figure 2c, where the percentage of apoptotic cells following mEV treatment for 48 hours remained below 5%. These results indicate that EV-enriched population treatment did not induce significant cell death in Jurkat cells and may therefore be considered non-cytotoxic. Picciotto *et al.* (2022) reported that EVs isolated from *Tetraselmis Chuii* can be taken up by the *C.elegans* model as well as by MDA-MB231 cell line (Picciotto *et al.,* 2022). Although we did not confirm whether the Jurkat cells were able to take up the EV-enriched population we did show that there were indeed some minor physiological changes as shown in the annexin v results upon co-culturing. These findings suggest that the EVs may have been internalized by the cells. However, further experiments are required to confirm the uptake of EVs, such as using confocal or fluorescence microscopy, especially since it is a cross-kingdom setup.

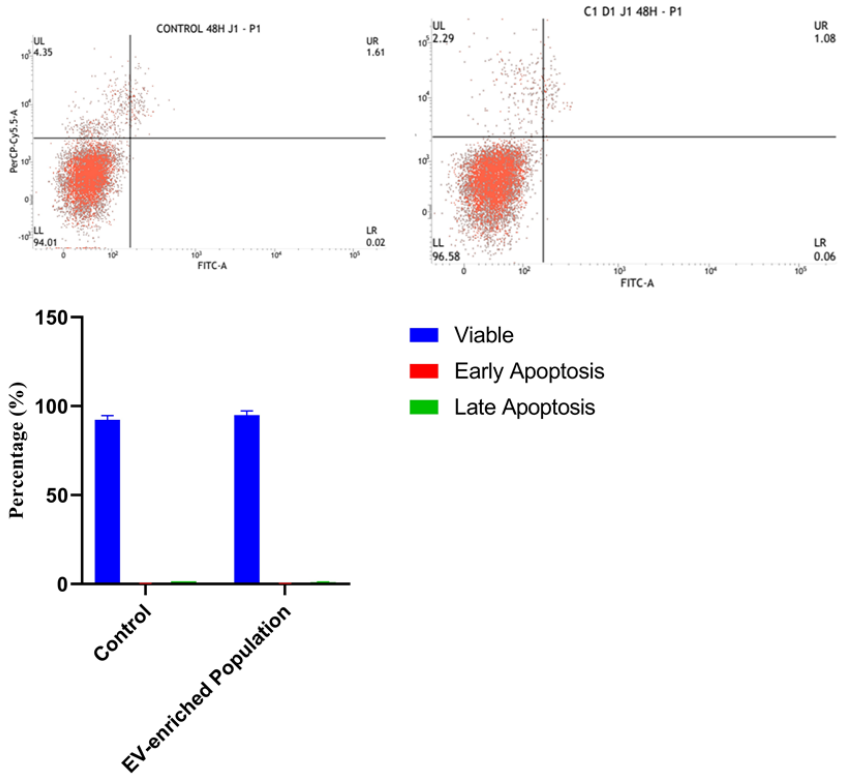
Despite the promising findings observed in this study, several limitations should be acknowledged. First, extracellular vesicle preparations derived from microalgae may contain potential contaminants, including residual proteins or endotoxin which could influence cellular responses. Although the current work focuses on *in vitro* functional assays, future studies should incorporate specific contaminant testing, such as endotoxin testing, to further validate biological safety. Second, this study was limited to EVs derived from a single microalgal species, and therefore the observed effects may not be the same across other microalgal sources. Comparative studies involving multiple species and standardized isolation protocols would strengthen translational relevance. In addition, all functional assessments were conducted *in vitro*; thus, in *vivo* validation using appropriate animal models will be essential to confirm safety and biodistribution,



a) Morphological assessment



b) Alamar blue assay



c) Annexin V assay (top: control; bottom: EV-enriched population -treated)

Figure 2. Figure showing functional assays of Jurkat cells incubated with the EV-enriched population for 48 hours.

CONCLUSION

Our preliminary results indicate that an EV-enriched population can be successfully isolated from *Chlorella vulgaris*, with a size distribution typically below 200 nm. Furthermore, these EVs were not cytotoxic to Jurkat cells, although more in-depth studies are required to validate this observation especially in terms of internalization. Overall, the EV-enriched population demonstrates potential as a biocompatible and naturally derived nanocarrier system for future therapeutic applications.

ACKNOWLEDGEMENTS

We would like to acknowledge Ms. Siti Hasanah Mohd Yusuff for her assistance in handling the flow cytometer machine and Ms. Farazela Mohd Radin and Ms. Lim Chai Ling for their assistance in cell culture techniques. We would also like to acknowledge the usage of an Open AI tool, ChatGPT that was used to increase the readability of the manuscript.

REFERENCES

[1] Adamo, G., Fierli, D., Romancino, D. P., Picciotto, S., Barone, M. E., Aranyos, A., Božič, D., Morsbach, S., Raccosta, S., Stanly, C., Paganini, C., Gai, M., Cusimano, A., Martorana, V., Noto, R., Carrotta, R., Librizzi, F., Randazzo, L., Parkes, R., Capasso Palmiero, U., Rao, E., Paterna, A., Santonicola, P., Iglič, A., Corcuera, L., Kisslinger, A., Schiavi, E. D., Liguori, G. L., Landfester, K., Kralj-Iglič, V., Arosio, P., Pocsfalvi, G., Touzet, N., Manno, M. & Bongiovanni, A. 2021. Nanoalgosomes: Introducing extracellular vesicles produced by microalgae. *Journal of Extracellular Vesicles*, 10(12), e12081.

[2] Adamo, G., Santonicola, P., Picciotto, S., Gargano, P., Nicosia, A., Longo, V., Aloi, N., Romancino, D. P., Paterna, A., Rao, E., Raccosta, S., Noto, R., Salamone, M., Deidda, I., Costa, S., Di Sano, C., Zampi, G., Morsbach, S., Landfester, K., Colombo, P., Wei, M., Bergese, P., Touzet, N., Manno, M., Di Schiavi, E. & Bongiovanni, A. 2024. Extracellular vesicles from the microalga *Tetraselmis chuii* are biocompatible and exhibit unique bone tropism along with antioxidant and anti-inflammatory properties. *Communications Biology*, 7, 941.

[3] Anderson, W., Kozak, D., Coleman, V. A., Jämting, Å. K. & Trau, M. 2013. A comparative study of submicron particle sizing platforms: Accuracy, precision and resolution analysis of polydisperse particle size distributions. *Journal of Colloid and Interface Science*, 405, 322–330.

[4] Cao, Y., Hou, L., Li, M., Zhang, J., Wang, L., Liu, C., Luo, T., Yan, L. & Zheng, L. 2024. Broccoli extracellular vesicles enhance the therapeutic effects and restore the chemosensitivity of 5-fluorouracil on colon cancer. *Food and Chemical Toxicology*, 186, 114563.

[5] Du, S., Guan, Y., Xie, A., Yan, Z., Gao, S., Li, W., Rao, L., Chen, X. & Chen, T. 2023. Extracellular vesicles: a rising star for therapeutics and drug delivery. *Journal of Nanobiotechnology*, 21(1), 231.

[6] Garaeva, L., Tolstyko, E., Putevich, E., Kil, Y., Spitsyna, A., Emelianova, S., Solianik, A., Yastremsky, E., Garmay, Y., Komarova, E., Varfolomeeva, E., Ershov, A., Sizova, I., Pichkur, E., Vinnikov, I. A., Kvanchiani, V., Kilasoniya Marfina, A., Konevega, A. L. & Shtam, T. 2025. Microalgae-Derived Vesicles: Natural Nanocarriers of Exogenous and Endogenous Proteins. *Plants*, 14(17), 2354.

[7] Maas, S. L. N., de Vrij, J., van der Vlist, E. J., Geragousian, B., van Bloois, L., Mastrobattista, E., Schiffelers, R. M., Wauben, M. H. M., Broekman, M. L. D. & Nolte-'t Hoen, E. N. M. 2015. Possibilities and limitations of current technologies for quantification of biological extracellular vesicles and synthetic mimics. *Journal of Controlled Release*, 200, 87–96.

[8] Mendes, A. R., Spínola, M. P., Lordelo, M. & Prates, J. A. M. 2024. Chemical Compounds, Bioactivities, and Applications of *Chlorella vulgaris* in Food, Feed and Medicine. *Applied Sciences*, 14(22), 10810.

[9] Nemati, M., Singh, B., Mir, R. A., Nemati, M., Babaei, A., Ahmadi, M., Rasmi, Y., Golezani, A. G. & Rezaie, J. 2022. Plant-derived extracellular vesicles: a novel nanomedicine approach with advantages and challenges. *Cell Communication and Signaling*, *20*(1), 69.

[10] Panahi, Y., Darvishi, B., Jowzi, N., Beiraghdar, F. & Sahebkar, A. 2016. *Chlorella vulgaris*: A Multifunctional Dietary Supplement with Diverse Medicinal Properties. *Current Pharmaceutical Design*, *22*(2), 164–173.

[11] Picciotto, S., Barone, M. E., Fierli, D., Aranyos, A., Adamo, G., Božič, D., Romancino, D. P., Stanly, C., Parkes, R., Morsbach, S., Raccosta, S., Paganini, C., Cusimano, A., Martorana, V.,

Noto, R., Carrotta, R., Librizzi, F., Capasso Palmiero, U., Santonicola, P., Iglič, A., Gai, M., Corcuera, L., Kisslinger, A., Di Schiavi, E., Landfester, K., Liguori, G. L., Kralj-Iglič, V., Arosio, P., Pocsfalvi, G., Manno, M., Touzet, N. & Bongiovanni, A. 2021. Isolation of extracellular vesicles from microalgae: towards the production of sustainable and natural nanocarriers of bioactive compounds. *Biomaterials Science*, *9*(9), 2917–2930.

[12] Picciotto, S., Santonicola, P., Paterna, A., Rao, E., Raccosta, S., Romancino, D. P., Noto, R., Touzet, N., Manno, M., Di Schiavi, E., Bongiovanni, A. & Adamo, G. 2022. Extracellular Vesicles From Microalgae: Uptake Studies in Human Cells and *Caenorhabditis elegans*. *Frontiers in Bioengineering and Biotechnology*, *10*, 830189.

[13] Savvidou, M. G., Georgiopoulou, I., Antoniou, N., Tzima, S., Kontou, M., Louli, V., Fatouros, C., Magoulas, K. & Kolisis, F. N. 2023. Extracts from *Chlorella vulgaris* Protect Mesenchymal Stromal Cells from Oxidative Stress Induced by Hydrogen Peroxide. *Plants (Basel)*, *12*(16), 2911.

[14] Welsh, J. A., Goberdhan, D. C. I., O'Driscoll, L., Buzas, E. I., Blenkiron, C., Bussolati, B., Cai, H., Di Vizio, D., Driedonks, T. A. P., Erdbrügger, U., Falcon-Perez, J. M., Fu, Q. L., Hill, A. F., Lenassi, M., Lim, S. K., Mahoney, M. G., Mohanty, S., Möller, A., Nieuwland, R., Ochiya, T., Sahoo, S., Torrecilhas, A. C., Zheng, L., Zijlstra, A., Abuelreich, S., Bagabas, R., Bergese, P., Bridges, E. M., Brucale, M., Burger, D., Carney, R. P., Cocucci, E., Crescitelli, R., Hanser, E., Harris, A. L., Haughey, N. J., Hendrix, A., Ivanov, A. R., Jovanovic-Talisman, T., Kruh-Garcia, N. A., Ku'ulei-Lyn Faustino, V., Kyburz, D., Lässer, C., Lennon, K. M., Lötvall, J., Maddox, A. L., Martens-Uzunova, E. S., Mizenko, R. R., Newman, L. A., Ridolfi, A., Rohde, E., Rojalin, T., Rowland, A., Saftics, A., Sandau, U. S., Saugstad, J. A., Shekari, F., Swift, S., Ter-Ovanesyan, D., Tosar, J. P., Useckaite, Z., Valle, F., Varga, Z., van der Pol, E., van Herwijnen, M. J. C., Wauben, M. H. M., Wehman, A. M., Williams, S., Zendrini, A., Zimmerman, A. J., Théry, C. & Witwer, K. 2024. Minimal information for studies of extracellular vesicles (MISEV2023): From basic to advanced approaches. *Journal of Extracellular Vesicles*, 13(2), e12404.