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SC-Nanophytosomes to address neurodegenerative diseases challenge: a novel concept of mitochondria-targeted therapy

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**SC-Nanophytosomes to address neurodegenerative
diseases challenge: a novel concept of mitochondria-
targeted therapy**

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and co-supervision of
Prof. Doctor Paula Cristina Branquinho de Andrade
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"Life is not easy for any of us.

But what of that?

We must have perseverance and, above all, confidence in ourselves.

We must believe that we are gifted for something, and that this thing,

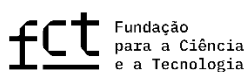
at whatever cost, must be attained."

– Marie Curie

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Publications

The data contained in the following works make up part of this thesis.

Scientific papers:

1. **Mendes D**, Valentão P, Oliveira MM, Andrade PB, Videira RA. A nanophytosomes formulation based on elderberry anthocyanins and Codium lipids to mitigate mitochondrial dysfunctions. Biomed Pharmacother 2021 Nov; 143: 112157.
2. **Mendes D**, Peixoto F, Oliveira MM, Andrade PB, Videira RA. Mitochondria research and neurodegenerative diseases: On the track to understanding the biological world of high complexity. Mitochondrion 2022 Jul; 65: 67-79.
3. **Mendes D**, Peixoto F, Oliveira MM, Andrade PB, Videira RA. Brain Effects of SC-Nanophytosomes on a Rotenone-Induced Rat Model of Parkinson's Disease—A Proof of Concept for a Mitochondria-Targeted Therapy. Int J Mol Sci 2022 Oct; 23: 12699.
4. **Mendes D**, Silva AM, Oliveira MM, Andrade PB, Videira RA. An Experimental Approach to Address the Functional Relationship between Antioxidant Enzymes and Mitochondrial Respiratory Complexes. Methods Protoc 2023 Mar; 6: 32.
5. **Mendes D**, Peixoto F, Oliveira MM, Andrade PB, Videira RA. Mitochondrial Dysfunction in Skeletal Muscle of Rotenone-Induced Rat Model of Parkinson's Disease: SC- Nanophytosomes as Therapeutic Approach. Int J Mol Sci 2023 Nov; 24:16787.

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Author statement

The author declares to have afforded a major contribution to the technical execution, interpretation of the results and manuscript preparation of all works included in this thesis, with the collaboration of other co-authors.

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Abstract

Abstract

The human brain, a functional system with a spatiotemporal multiscale architecture that raises the coordination skills, rational thinking, and memory to a unique and distinctive complexity level of the human species, is the most fascinating system produced by life during its long evolutionary journey on earth. However, the age-related neurodegenerative diseases that disturb progressively and in an irreversible way the brain process underlying coordination skills, rational thinking and memory are depriving millions of people of their very selves, creating a public health problem with global epidemic proportions. Thus, the development of new therapeutic technology to preserve the brain functioning over all human life is an urgent need – a challenge that is addressed by this Ph D thesis through a mitochondrion-focused investigation.

From the simple unicellular eukaryote to the highly complex multicellular organism like Humans, the mitochondrion emerges as a ubiquitous player to ensure the organism's functionality. It is popularly known as “the powerhouse of the cell” because of its key role in ATP generation. However, our understanding of the physiological relevance of mitochondrion is being challenged by data obtained in different fields. In the first chapter, a short history of the mitochondrion research field is presented, stressing the findings and questions that allowed the knowledge advances, and put mitochondrion as the main player in safeguarding multicellular organism life as well as a key to solving the puzzle of the neurodegenerative diseases, like Alzheimer's and Parkinson's diseases.

Many researchers consider mitochondrion a promising target to address the neurodegenerative issue; however, developing tools to modulate mitochondrial function effectively remains an unresolved scientific and technological challenge.

Although different neurodegenerative diseases exhibit disease-specific vicissitudes of mitochondrial dysfunction, a deep analysis reveals that all pathological roads start or converge to the inner mitochondrial membrane with a dysfunctional mitochondrial respiratory chain. Mitochondrial dysfunction associated with an anomalous activity of the respiratory chain complexes (mainly complex I) and an abnormal mitochondrial lipidome (mainly cardiolipins) are early pathological hallmarks of Alzheimer's and Parkinson's diseases. Thus, the present therapeutic strategy was rationalized to overcome mitochondrial dysfunction, by improving the respiratory chain functionally and tuning mitochondrial lipidome.

To overcome the dysfunctional complex I, it was selected an elderberry anthocyanin-enriched extract (*Sambucus nigra* L.) that preserves the anthocyanins in cationic form - redox-active compounds with the ability to oxidize NADH and deliver the electrons to

complex III of the mitochondrial redox chain, working as a membrane electron carrier. To modulate the mitochondrial lipidome a set of membrane polar lipids from *Codium tomentosum* Stackhouse was selected. The chloroplast-rich tissues of this green algae are rich in anionic phospholipids, mainly in phosphatidylglycerol that can be used by animal cells as cardiolipin precursor and in polyunsaturated fatty acid (mainly n-3 PUFA) that allow the formation of membranes with high curvature (typical of the inner membrane of mitochondrion) and enable the synthesis of mature cardiolipins. Additionally, the mitochondriotropic features of the elderberry anthocyanin-enriched extract (*Sambucus nigra*) were combined with the self-assembling properties of the membrane polar lipids from *Codium tomentosum* in water to develop a mitochondria-targeted nanocarrier, named SC-Nanophytosomes.

Although *Codium tomentosum* and berries of *Sambucus nigra* have been approved for food applications, it was developed new procedures to ensure that both SC-Nanophytosomes building blocks also meet this requirement. The membrane polar lipids were obtained as a chlorophyll-free extract, taking advantage of the differentiated solubilities of lipids in ethanol and cold acetone. The chemical characterization with several techniques confirms that the algae lipid extract has a distinctive composition, containing 26% non-phosphorous polar lipids and 74% phospholipids with a high degree of polyunsaturated fatty acids. Anthocyanin-enriched extract, obtained by taking advantage of the selective adsorption of anthocyanins to polyvinylpyrrolidone, is dominated by four cyanidin-glycosides (cyanidin-3,5-O-di-glucoside, cyanidin-3-O-sambubioside-5-O-glucoside, cyanidin-3-O-sambubioside and cyanidin-3-O-glucoside), representing about 86% of their phenolic content.

SC-Nanophytosomes engineered with algae membrane polar lipids (600 μ M in phospholipid), and 0.5 mg/L of the anthocyanin-enriched extract are nanosized vesicles (diameter = 108.74 ± 24.74 nm) with a negative surface charge (Zeta potential = -46.93 ± 6.63 mV). Transmission electron microscopy analysis confirmed the vesicles' hydrodynamic size distribution and showed their shape plasticity. It was also shown that this formulation is very stable, since its storage at 4 °C for 14 days, and the gastrointestinal tract-like pH changes do not change its chemical composition (e.g., polyphenols/lipid ratio), size and surface charge, nor its ability to preserve the anthocyanins in the flavylum cation form, with mitochondriotropic properties.

In vitro assays with neuronal SH-SY5Y cells (used as cellular models of neurons) revealed that SC-Nanophytosomes have the competence to overcome the cellular barriers and target mitochondrion, improving the activity of the mitochondrial respiratory chain complexes and preserving the mitochondrial membrane potential in the presence of

rotenone, a complex I inhibitor used to induce Parkinson's disease-like neuropathology. Additionally, SC-Nanophytosome formulation protects SH-SY5Y cells against rotenone-induced toxicity as well as against the excitatory toxicity promoted by glutamate in the millimolar range. Green-fluorescent labelled SC-Nanophytosomes were used to reveal that they are internalized by SH-SY5Y cells as well as by "normal" human fibroblast BJ cells via caveola-mediated endocytosis and reach mitochondrion and other cytosolic organelles. It was also shown that SC-Nanophytosomes are internalized by BV-2 cells (used as a cellular model of microglia). However, the cellular uptake does not involve micropinocytosis, clathrin-mediated endocytosis, or caveola-mediated endocytosis. Additionally, results show that SC-Nanophytosomes are not toxic for microglial cells in a resting state and under interferon- γ stimulation. However, they have a small impact on mitochondrial performance, detected by changes in mitochondrial membrane potential and activity of the respiratory complexes, without exhibiting anti-inflammatory activity.

Despite the minimal effects on BV-2 cells, the results with SH-SY5Y cells suggest that SC-Nanophytosomes have properties to support mitochondria-targeted therapy, and they were moved for *in vivo* assays. Thus, to obtain a proof-of-concept of the SC-Nanophytosomes as a mitochondria-targeted therapy, the oral administration route was rationalized to a rotenone-induced Parkinson's disease-like pathology animal model. The oral administration of SC-Nanophytosomes (delivery by drinking water for 3 weeks at 3 μ M in phospholipid and 2.5 mg/mL of elderberry anthocyanin-enriched extract) to a rotenone-induced Parkinson's disease-like pathology rat model showed significant positive outcomes detected during assay on disabling motor symptoms associated with the disease, and in *ex vivo* analysis on brain and skeletal muscle tissues.

Results obtained with two brain portions (BG-Cereb - with the basal ganglia with substantia nigra pars compacta and the cerebellum, and C-Cortex - with the cerebral cortex) showed that rotenone-induced neurodegeneration increases the α -synuclein levels in the BG-Cereb portion and compromises the mitochondrial respiratory chain functionality in both brain portions. These pathological hallmarks are accompanied by changes in the fatty acid profile of mitochondrial lipidome and the cytosolic activity of antioxidant enzymes like superoxide dismutase and catalase. However, the rotenone-induced Parkinson's disease-like pathology rat model does not show signals of dopaminergic neuron loss – a feature of human disease.

Treatment with SC-Nanophytosomes reverted the α -synuclein levels and the antioxidant enzyme activities to the values detected in control animals. Moreover, it mitigated mitochondrial dysfunction, with positive outcomes on respiratory control rate,

individual respiratory complexes' activity, and the membrane phospholipids' fatty acid profile.

Regarding the relevance of motor symptoms on Parkinson's disease progression, the effects of the SC-Nanophytosomes treatment on skeletal muscle tissues were also assessed. It is unveiled that the rotenone-induced Parkinson's disease rat model exhibits skeletal muscle tissues with deficient activity of mitochondrial complexes I and II along with small changes in antioxidant enzymes activity and skeletal muscle lipidome. Thus, skeletal muscle dysfunction can contribute to the motor disabilities of Parkinson's disease-like pathology, usually associated with brain dysfunction. SC-Nanophytosome treatment mitigates the impairment of complexes I and II activity, improving the mitochondrial respiratory chain performance at levels that surpass the control. Additionally, the skeletal muscle tissue's ability to deal with oxidative stress stimuli is improved by SC-Nanophytosomes treatment, providing a cellular environment with reduced redox potential. The small changes in skeletal muscle lipidome promoted by rotenone-induced Parkinson's disease-like pathology were also counteracted by SC-Nanophytosomes treatment.

The results obtained with brain and skeletal muscle tissues validate the concept of SC-Nanophytosomes as a mitochondria-targeted therapy for Parkinson's disease. This work paves the way to use nanomedicine-based phytochemicals in innovative oral formulations to address the challenge of neurodegenerative diseases by targeting the mitochondrion in multiple tissues.

Keywords: neurodegenerative diseases; mitochondria; anthocyanins; algae membrane lipids; nanomedicine; mitochondria-targeted therapy.

Resumo

Resumo

O cérebro humano é um sistema funcional com uma arquitetura que permite a coordenação no espaço e no tempo, o pensamento racional e a memória com um nível de complexidade único e distinto da espécie humana. Assim, o cérebro humano é o sistema mais fascinante desenvolvido pela vida durante a sua jornada evolutiva na Terra. No entanto, as doenças neurodegenerativas relacionadas com a idade, que perturbam progressivamente e de forma irreversível o processo cerebral subjacente às capacidades de coordenação, ao pensamento racional e à memória, estão a privar milhões de pessoas de si mesmas, criando um problema de saúde pública com proporções epidémicas globais. Portanto, o desenvolvimento de novas tecnologias terapêuticas para preservar o funcionamento do cérebro ao longo de toda a vida humana é uma necessidade urgente – o desafio que é abordado por esta tese de doutoramento através de uma investigação focada na mitocôndria.

Do mais simples organismo unicelular eucariota ao mais complexo dos organismos multicelulares (e.g., seres humanos), a mitocôndria é um ator ubíquo que garante a funcionalidade do organismo. Popularmente conhecida como o sistema gerador e fornecedor de energia celular, na designação anglo-saxónica “*the powerhouse of the cell*”, devido ao seu papel fundamental na produção de ATP. No entanto, a nossa compreensão da relevância fisiológica da mitocôndria está a ser desafiada por dados obtidos em diferentes áreas científicas. Assim, no primeiro capítulo é apresentada uma breve resenha histórica do campo de investigação sobre a mitocôndria, destacando-se as descobertas e questões que permitiram o avanço do conhecimento e colocaram a mitocôndria como o principal ator na salvaguarda da vida dos organismos multicelulares, bem como uma chave para resolver o puzzle das doenças neurodegenerativas, incluindo as patologias de Alzheimer e Parkinson.

Muitos investigadores consideram a mitocôndria um alvo promissor para resolver a questão neurodegenerativa, no entanto, o desenvolvimento de ferramentas para modular eficazmente a função mitocondrial continua a ser um desafio científico e tecnológico por resolver. Embora diferentes doenças neurodegenerativas exibam vicissitudes específicas da disfunção mitocondrial, uma análise profunda revela que todas as vias patológicas começam ou convergem para a membrana mitocondrial interna com a cadeia respiratória mitocondrial disfuncional. De facto, a disfunção mitocondrial associada a uma atividade anómala dos complexos da cadeia respiratória (principalmente do complexo I) e alterações do lipidoma mitocondrial (principalmente detetadas no perfil das cardiolipinas) são marcas patológicas precoces das doenças de Alzheimer e de Parkinson. Assim, a presente

estratégia terapêutica foi racionalizada para superar a disfunção mitocondrial, melhorando funcionalmente a cadeia respiratória e ajustando o lipidoma mitocondrial.

Para superar a disfunção do complexo I, foi selecionado um extrato de baga de sabugueiro (*Sambucus nigra* L.), enriquecido em antocianinas preservadas na forma catiónica. As antocianinas da baga de sabugueiro são compostos com capacidade de oxidar o NADH e entregar os eletrões ao complexo III da cadeia respiratória mitocondrial, funcionando como transportador de eletrões na membrana. Para modular o lipidoma mitocondrial, foi selecionado um conjunto de lípidos polares da membrana da alga *Codium tomentosum* Stackhouse. Os tecidos ricos em cloroplastos desta alga verde apresentam elevado teor de fosfolípidos aniónicos, principalmente fosfatidilgliceróis, e em ácidos gordos polinsaturados (principalmente PUFA n-3). As espécies de fosfatidilglicerol podem ser utilizados pelas células animais como precursor da cardiolipina e os ácidos gordos polinsaturados permitem a formação de membranas com uma alta curvatura (típico da membrana interna da mitocôndria), criando-se assim condições para a síntese de cardiolipinas maduras (com ácidos gordos polinsaturados) requeridas para assegurar a funcionalidade da membrana interna da mitocôndria. Adicionalmente, as características mitocondriótropas do extrato de baga de sabugueiro enriquecido com antocianinas foram combinadas com as propriedades de auto-organização em bicamada lipídica que os lípidos polares da membrana da *Codium tomentosum* exibem em meios aquosos, para desenvolver um nanossistema direcionado à mitocôndria, denominado SC-Nanofitosomas.

Embora a *Codium tomentosum* e as bagas de *Sambucus nigra* tenham aprovação para aplicações alimentares, foram desenvolvidos novos procedimentos para garantir que ambos os blocos de construção dos SC-Nanofitosomas também cumpram este requisito. Os lípidos polares da membrana foram obtidos na forma de extrato isento de clorofilas, usando um procedimento que tira vantagem da solubilidade diferenciada que as diferentes classes de lípidos apresentam em etanol e acetona a temperatura reduzida. A caracterização química com diversas técnicas confirma que o extrato lipídico da alga possui uma composição distintiva, contendo 26% de lípidos polares sem fósforo e 74% de fosfolípidos com elevado teor em ácidos gordos polinsaturados. O extrato enriquecido com antocianinas, obtido por adsorção seletiva de antocianinas à polivinilpolipirrolidona, é dominado por quatro cianinas (cianidina-3,5-O-di-glicosídeo, cianidina-3-O-sambubiosídeo-5-O-glicosídeo, cianidina-3-O-sambubiosídeo e cianidina-3-O-glicosídeo), as quais representam cerca de 86% do seu conteúdo fenólico.

SC-Nanofitosomas produzidos com os lípidos polares de membrana da alga (600 µM em fosfolípidos) e 0,5 mg/L de extrato de baga de sabugueiro enriquecido em

antocianinas são vesículas nanométricas (diâmetro = $108,74 \pm 24,74$ nm) com carga superficial negativa (potencial Zeta = $-46,93 \pm 6,63$ mV). A análise por microscopia eletrônica de transmissão confirmou a distribuição hidrodinâmica do tamanho das vesículas e mostrou a sua plasticidade quanto à forma. Mostrou-se também que esta formulação é muito estável, uma vez que o seu armazenamento durante 14 dias à temperatura de 4 °C, e as alterações de pH semelhantes às do trato gastrointestinal não alteram a composição química (*i.e.*, relação polifenóis/lípidos) das vesículas nem o seu tamanho e carga superficial. Adicionalmente, as antocianinas são preservadas na forma de catião flavínio, estrutura que apresenta propriedades mitocondriótropas.

Ensaio *in vitro* com células neuronais SH-SY5Y (utilizadas como modelo celular de neurónios) revelaram que os SC-Nanofitosomas têm a competência de superar as barreiras celulares e alvejar as mitocôndrias, melhorando a atividade dos complexos da cadeia respiratória e preservando o potencial de membrana mitocondrial na presença de rotenona, um inibidor do complexo I usado para induzir neuropatologia semelhante à doença de Parkinson. Além disso, a formulação de SC-Nanofitosomas protege as células SH-SY5Y da toxicidade induzida por rotenona, bem como da toxicidade excitatória promovida por glutamato quando em concentração milimolar. Usando SC-Nanofitosomas marcados com uma sonda que emite fluorescência na região do verde revelou-se que estes são internalizados por células SH-SY5Y, bem como por fibroblastos humanos “normais” (células BJ), através de um processo de endocitose mediado por cavéola e que tem competência para atingir as mitocôndrias e outros organelos citoplasmáticos. Também foi demonstrado que os SC-Nanofitosomas são internalizados pelas células BV-2 (usadas como modelo celular da micróglia), mas a entrada nestas células não envolve micropinocitose, nem processos de endocitose mediados por clatrina ou por cavéola. Todavia, o estudo com células BV-2 mostra que os SC-Nanofitosomas não são tóxicos para as células da micróglia, quer no estado de repouso quer sob estimulação com o interferão- γ . Adicionalmente, mostrou-se que os SC-Nanofitosomas não exibem atividade anti-inflamatória, apesar de terem um pequeno impacto no desempenho mitocondrial destas células, como detetado por alterações no potencial da membrana mitocondrial e na atividade dos complexos respiratórios.

Apesar dos efeitos menores sobre as células BV-2, os resultados obtidos com células SH-SY5Y conferem potencial terapêutico aos SC-Nanofitosomas, pelo que foram movidos para ensaios *in vivo*. Assim, para obter uma prova de conceito dos SC-Nanofitosomas como ferramenta terapêutica direcionada à mitocôndria efetuaram-se estudos com um modelo animal com patologia semelhante à doença de Parkinson, induzida pela rotenona, usando a via oral para administração da formulação. A

administração oral de SC-Nanofitossomas (3 μ M em fosfolípido e 2,5 mg/mL de extrato da baga de sabugueiro enriquecido em antocianinas na água potável) durante 3 semanas aos animais com patologia semelhante à doença de Parkinson mostrou resultados positivos significativos tanto nos sintomas motores associados à doença (avaliados *in vivo* durante o ensaio) como nos marcadores patológicos avaliados em tecidos do sistema nervoso central e do músculo esquelético (análise *ex vivo*).

Os resultados obtidos com duas porções do sistema nervoso central (BG-Cereb - contém os gânglios basais com substância nigra pars compacta e o cerebelo, e C-Cortex - contém o córtex cerebral) mostraram que a neurodegeneração induzida pela rotenona aumenta os níveis de α -sinucleína na porção BG-Cereb e compromete a funcionalidade da cadeia respiratória mitocondrial em ambas as porções do sistema nervoso central. Essas características patológicas são acompanhadas por alterações no perfil de ácidos gordos do lípido mitocondrial e na atividade citosólica de enzimas antioxidantes, tais como superóxido dismutase e catalase. No entanto, o modelo de rato com patologia semelhante à doença de Parkinson induzida pela rotenona não mostra sinais de perda de neurónios dopaminérgicos – uma característica da doença humana.

O tratamento com SC-Nanofitossomas reverteu os níveis de α -sinucleína e as atividades das enzimas antioxidantes para os valores detetados nos animais controlo. Além disso, mitigou a disfunção mitocondrial, com efeitos positivos no índice de controlo respiratório, na atividade dos complexos respiratórios individuais e no perfil de ácidos gordos dos fosfolípidos da membrana.

Considerando a relevância dos sintomas motores na progressão da doença de Parkinson, os efeitos do tratamento com SC-Nanofitossomas foram também avaliados em tecido de músculo esquelético. Revelou-se que as mitocôndrias do tecido muscular esquelético dos animais com patologia semelhante à doença de Parkinson induzida pela rotenona apresentam uma menor atividade dos complexos I e II, juntamente com pequenas alterações na atividade das enzimas antioxidantes e no perfil dos ácidos gordos do lipidoma do músculo esquelético. Portanto, conclui-se que uma disfunção específica ao nível do músculo esquelético pode contribuir para as deficiências motoras associadas à patologia, as quais são usualmente explicadas apenas pela disfunção cerebral ao nível dos neurónios dopaminérgicos.

O tratamento com SC-Nanofitossomas mitiga o comprometimento da atividade dos complexos mitocondriais I e II, melhorando o desempenho da cadeia respiratória mitocondrial para níveis que ultrapassam os detetados no controlo. Além disso, o tratamento com SC-Nanofitossomas melhora a capacidade do tecido muscular esquelético para lidar com estímulos de stress oxidativo, proporcionando um ambiente celular com

potencial de oxidação-redução mais reduzido. As pequenas alterações no perfil de ácidos gordos do lipidoma do músculo esquelético promovidas pela patologia também foram contrariadas pelo tratamento com SC-Nanofitossomas.

Os resultados obtidos com tecidos do sistema nervoso central e músculo esquelético validam o conceito dos SC-Nanofitossomas como uma ferramenta terapêutica direcionada à mitocôndria para tratamento da doença de Parkinson. Este trabalho abre caminho para o uso de fitoquímicos em nanoformulações orais inovadoras para enfrentar o desafio das doenças neurodegenerativas, visando a mitocôndria em múltiplos tecidos.

Palavras-chave: doenças neurodegenerativas; mitocôndria; antocianinas; lípidos de membrana de algas; nanomedicina; terapia direcionada às mitocôndrias.

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List of Abbreviations

$\Delta\Psi_m$ – mitochondrial membrane potential

16-AP – 16-(9-anthroyloxy)-palmitic acid

6-OHDOPA – 6-hydroxy-dopamine

A

AD – Alzheimer's disease

ADP – adenosine diphosphate

ALA – α -linolenic acid C18:3n-3

ATP – adenosine triphosphate

B

BG-Cereb – basal ganglia and cerebellum

BOLD – blood-oxygen-level-dependent

C

CAT - catalase

C-Cortex – cerebral cortex

CNS – central nervous system

CoASH – coenzyme A

Complex I – NADH-ubiquinone reductase

Complex II – succinate-ubiquinone reductase

Complex III – ubiquinol-cytochrome c reductase

Complex IV – cytochrome c oxidase

COX - cyclooxygenase

CS – citrate synthase

CTRL - control

Cu/Zn-SOD – superoxide dismutase

Cyt c – cytochrome c

D

DCF – 2',7'-dichlorofluorescein

DCFH-DA - 2',7'-

dichlorodihydrofluorescein diacetate

DCPIP – 2,6-dichlorophenolindophenol

DGCC - 1,2-diacylglycerol-3-O-carboxy-(hydroxymethyl)-choline

DGDG – digalactosyldiacylglycerol

DGTA - 1,2-diacylglycerol-3-O-2'-(hydroxymethyl)-(N,N,N-trimethyl)- β -alanine

DGTS – 1,2-diacylglycerol-3-O-4'-(N,N,N-trimethyl)-homoserine

DHA – docosahexanoic acid, C22:6n-3

DLS – dynamic light scattering

DMEM – dulbecco's modified eagle medium

DTNB – 5,5-dithio-bis-2-nitrobenzoic acid

E

EAE-extract – elderberry anthocyanins enriched extract

ECoG - electrocorticogram

EE – entrapment efficiency

ELISA – enzyme-linked immunosorbent assay

ELS – electrophoretic light scattering

EPA – eicosapentaenoic acid, C20:5n-3

F

FAD – flavin adenine dinucleotide

FADH₂ – reduce flavin adenine dinucleotide

FAME – fatty acid methyl esters

FBS – fetal bovine serum

FCCP – carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazine
 FMN – flavin mononucleotide

G

GC-FID – gas chromatography/flame ionization detector
 GC-MS - gas chromatography/mass spectrometer
 GPx – glutathione peroxidase
 GR – glutathione reductase
 GSH – reduced glutathione
 GSSG – oxidized glutathione
 GTP – guanosine triphosphate

H

HBSS – Hank's balanced salt solution
 HPLC-DAD – high-performance liquid chromatography with diode array detector

I

IFN- γ – interferon-gamma
 IMM – inner mitochondrial membrane
 IMS – intermembrane space
 iNOS – inducible nitric oxide synthase

J

JC-1 – 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolo-carbocyanine iodide

L

LA – linoleic acid, C18:2n-6
 LDH – lactate dehydrogenase
 L-DOPA – L-3,4-dihydroxyphenylalanine

LOX – lipoxygenase
 LPS - lipopolysaccharides

M

M β CD -methyl- β -cyclodextrin
 MEM – minimum essential medium
 MGDG - monogalactosyldiacylglycerol
 MitoQ - mitoquinone
 MPTP – 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
 mtDNA – mitochondrial DNA
 MTT – 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
 MUFA – monounsaturated fatty acids

N

NADH – reduced nicotinamide adenine dinucleotide
 NADPH - nicotinamide adenine dinucleotide phosphate
 NBT – nitrobluetetrazolium chloride
 NEM – *N*-ethylmaleimide
 NO $^{\bullet}$ – nitric oxide

O

O $_2^{\bullet -}$ - superoxide anion radical
 OMM – outer mitochondrial membrane
 OPT - orthophthalaldehyde

P

PA – phosphatidic acid
 PC - phosphatidylcholine
 PD – Parkinson's disease
 PDI -polydispersity index
 PE – phosphatidylethanolamine
 Pen-Strep – penicillin-streptomycin

List of Abbreviations

PI – phosphatidylinositol

PG – phosphatidylglycerol

PL – phospholipid

PLA2 – phospholipase A2

PS – phosphatidylserine

PUFA – polyunsaturated fatty acids

PVPP - polyvinylpyrrolidone

R

RCR – respiratory control rate

ROS – reactive oxygen species

ROT – rotenone

rRNA – ribosomal RNA

S

SFA – saturated fatty acids

SQDG - sulfoquinovosyldiacylglycerol

SS – Szeto-Schiller

T

TEM – transmission electron microscopy

TH – tyrosine hydroxylase

TLC – thin-layer chromatography

TNB – 2-nitro-5-thiobenzoic acid

TNF- α – tumour necrosis factor

TPP⁺ - triphenylphosphonium cation

tRNA – transfer RNA

U

U – enzyme's catalytic activity

UCP – uncoupling proteins

UQ - ubiquinone

Thesis outline

Chapter I – General Introduction

This chapter comprises a general introduction with a short history of the mitochondria research field, stressing the findings and questions that allowed the knowledge advances and putting mitochondrion as the leading player in safeguarding organism life and a key to solving the puzzle of neurodegenerative diseases. The second part of this introduction has presented the search for building blocks to support a mitochondria-targeted therapy using *Codium tomentosum* algae as a resource for structural and bioactive lipids and *Sambucus nigra* elderberry as a resource for anthocyanins, redox-active compounds with mitochondriotropic properties. Thus, is described a nanoplatform that can support nutraceutical and pharmacological approaches for neurodegeneration.

At the end of this chapter are presented the working hypothesis with open questions and the research objectives that arise from the presented background.

Chapter II – Experimental Section

This chapter describes all the methodologies used in this thesis.

Chapter III – Results and Discussion

This chapter presents a detailed description of all results obtained during this thesis, subdivided into four sub-chapters. The sub-chapters portray: i) the preparation, characterization, and *in vitro* effects of SC-Nanophytosomes formulation against toxicity induced by toxic stimuli with relevance for the neurodegenerative process; ii) *in vitro* effects of SC-Nanophytosomes on neuroinflammatory process; iii) brain effects of SC-Nanophytosomes oral administration using a rotenone-induced rat model of Parkinson's disease and a proof-of-concept for a mitochondria-targeted therapy, and iv) the therapeutic effects on skeletal muscle and associated motor symptoms.

Chapter IV – Integrated Discussion, Conclusion Remarks and Future Perspectives

This chapter integrates the key findings of the experimental work according to the available bibliography, followed by a summary of the conclusion's statements and the consequent future directions in this subject.

Chapter V – References

This chapter lists all the bibliographic references used in the writing of this thesis.

CHAPTER I

General Introduction

1. Mitochondria research and neurodegenerative diseases: On the track to understanding the biological world of high complexity

1.1. Outlining mitochondrion as a new research field – From the cytological studies to the enzymatic view of mitochondria

As a ubiquitous structure of eukaryotic cells, mitochondria emerged from the systematic observation of cells by optical microscopy performed by Richard Altmann at the end of the nineteenth century (1). Guided by the previous description of intracellular structures reported by others, this researcher detected within nearly all cells branched strings of granules that he designated “bioblasts”. He proposed that they are “elementary organisms” living inside the cells to ensure vital functions. Without any experimental evidence to support it, this idea was forgotten. However, the similarities between mitochondria and bacteria became evident in the first decades of the 20th century. The visionary idea of Altmann was recovered by the endosymbiotic theory, stating that mitochondria were once free-living aerobic (oxygen-using) bacteria that became an organelle of eukaryotic cells by an (endo)symbiotic process (2, 3). Whether this association involved two prokaryotic organisms of different life domains (one aerobic bacteria and one anaerobic archaeon as host cell) with the subsequent evolution for the eukaryotic cell, or if a eukaryotic host-cell is present from the beginning of the association remains a controversial subject (4, 5). Despite this, everyone agrees that the mitochondrion is an intracellular structure as defining and ubiquitous among eukaryotes as the nucleus itself (6). Additionally, we know that mitochondria contain their genome (mitochondrial DNA, mtDNA), characterized by multiple copies of a closed circular DNA molecule that is replicated and expressed within the organelle system (7). Human mtDNA only contains 37 genes coding for two rRNAs (ribosomal RNA), 22 tRNAs (transfer RNA), and 13 polypeptides, which are integral components of all subunits of enzyme complexes of the oxidative phosphorylation system (8, 9). Nevertheless, the human mitochondrial proteome contains approximately 1500 proteins. This fact indicates that the endosymbiotic process involved gene transfer from mitochondria to the host cell nucleus while developing a mitochondria-specific protein import mechanism to ensure organelle functionality (9-13).

Even so, the term “mitochondrion” (plural is “mitochondria”) was coined, in 1898, by Benda that aggregated two Greek words, “*mitos*” (thread) plus “*chondros*” (granules), to describe the intracellular “thread granules” detected by optical microscopy during spermatogenesis (14). In line with Altmann’s morphological description of the “bioblasts”, Benda was also the first to assign an intrinsic tubular structure to mitochondria. At that time,

knowing whether the intracellular structures named “bioblasts” or “mitochondria” were real intracellular structures or artefacts resulting from cell/tissue staining procedures fed the discussion among researchers. In 1899, Leonor Michaelis took the first step to resolve this issue, showing that the redox dye Janus Green B stains specifically the mitochondria within the cells (15). Although Michaelis had not associated the mitochondria with the cellular oxidations triggered by oxygen consumption, it was revealed that mitochondria staining with Janus Green B requires oxygen and is inhibited with cyanide (16). Therefore, Michaelis’s work is considered the starting point of a race to discover the mitochondrion’s role in cell metabolism, referred to by some authors as “enzymological” properties of mitochondria (17, 18). Thus, understanding how the dynamics of cytoplasmic granules known as mitochondria are related to cell metabolism raised the main research’s primary purpose.

In 1909, Regaud showed that mitochondria’s histological and staining behaviour resembled myelin, concluding they may be “corps lipoids”. A few years later, Kingsbury stated that the cytoplasmic granules known as mitochondria or chondriosomes, are the “structural expression of the reducing substances concerned in cellular respiration” (19). An idea reinforced by Warburg’s work, revealing that the cytoplasmic granules, named by him “grana”, enhance the activity of iron-containing respiratory enzymes (20). Using living cells obtained by tissue culture methods, Lewis and Lewis assigned structural plasticity and dynamics to mitochondria showing that mitochondria change in size, shape, and position inside the cell (21). Today, we know that these findings reflect the continuous processes of fusion and fission that ensure mitochondrial control quality. However, at that time, the lack of knowledge about the chemical composition, structure, and behaviour of the mitochondrial components did not allow for rationalizing the functional relevance of these observations.

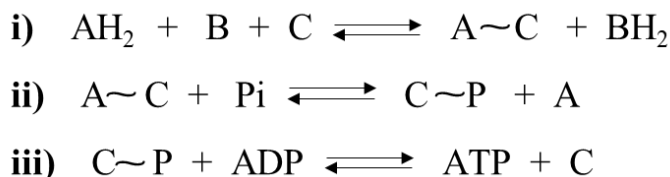
Bensley and Hoerr knew that to achieve mitochondria functions, it would be necessary to develop methodologies to fractionate the cells/tissues and isolate the mitochondria. Thus, in 1934, they reported a centrifugation-based procedure to obtain a fraction rich in globular or rod-shaped structures from liver homogenates in saline aqueous solutions (22). However, this fraction was not useful for studying mitochondria morphology and activity relationship since these globular or rod-shaped structures did not stain with Janus Green B dye. Claude and his collaborators developed a procedure to obtain a mitochondria-rich fraction with functional activity, using sucrose solutions to prepare tissue homogenates and combining the differential centrifugation fractionation technique with the enzymatic characterization of the different fractions obtained (23). Hogeboom used this strategy in rat liver to establish that the succinoxidase and cytochrome oxidase are localized exclusively in the mitochondria. It is also important to stress that the current standardized protocol resulted from upgrading of the above-mentioned procedure by reducing sucrose

concentration to overcome the inhibitory effect of a high sucrose concentration on several enzymes' activity (23, 24). Using this improved procedure, Kennedy and Lenhinger revealed that the reactions related to the citric acid cycle and fatty acids' oxidation are accomplished by mitochondria (25). Additionally, Lenhinger demonstrated the occurrence of ATP (adenosine triphosphate) synthesis (phosphorylation) coupled to the aerobic oxidation of NADH (nicotinamide adenine dinucleotide), concluding that oxidative phosphorylation occurs in mitochondria (26). In the same year, Schneider and Potter reported that mitochondria retain most rat liver homogenates' of the "oxalacetic" oxidase activity, highlighting their action in the Krebs citric acid cycle (27). In 1951, Green also showed that the so-called cyclophorase system, a particulate preparation obtained from the kidney and liver that catalyze the fatty acid and Krebs cycle oxidations, is a glimmer of mitochondrial activity (28). Another feature of intact mitochondria was recognized, in 1951, by Kielley and Kielley when they reported the "latency" of the ATPase activity, which is stimulated by agents that may compromise the mitochondrial structure integrity, such as dinitrophenol (29).

Thus, in the middle of the 20th century, it was well known that the enzymes required for the citric acid cycle, fatty acid oxidation, and oxidative phosphorylation were localized in mitochondria, allowing a complete description of the biochemical pathways that support the cell energy demand. These experimental data also indicated that the coupling of respiration to phosphorylation requires structural features of the mitochondrion and a set of functional enzymes. In this way, the mechanism used by mitochondria to couple the metabolites' oxidation to ATP synthesis became the main research challenge in the biochemistry field.

In 1953, Slater was the first to propose a scientific framework for mitochondrial oxidative phosphorylation, delineating a mechanism coupling oxidation to ATP synthesis (30). According to Slater's mechanism, known as the chemical-coupling hypothesis, the redox reactions between two successive members of the respiratory system (A and B) trap energy initially in a chemical complex (A~C). Then, C is transferred to phosphate to form a phosphorylated intermediate (C~P) which can donate its energy to the ultimate formation of ATP, recycling the unknown compound C (**Figure 1**). Slater also suggested that the high-energy complex could drive other reactions and be a general energy store within mitochondria.

Indeed, Slater's mechanism was based on the Efraim Racker mechanism for substrate-level phosphorylation by the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase. Despite the efforts developed by several researchers, including Lenhinger, the phosphorylated high-energy intermediates of the respiratory carriers have never been identified.



A and B are electron carriers.

Figure 1. The chemical couple hypothesis for mitochondrial oxidative phosphorylation proposed by Slater. AH_2 and B are adjacent components of the respiratory chain, C is required for the redox reaction and remains covalently linked to A in an energy-rich compound that can be used to synthesize ATP. A and C must be regenerated from the compound A~C to hydrogen-transfer proceed (adapted from reference (30)).

In the mid-1950s, Green's laboratory developed a procedure to obtain, in large-scale, preparations of beef heart mitochondria with a remarkably high degree of stability and purity which were used to resolve the puzzle of the respiratory chain and the phosphorylation system (31). It is important to stress that in the mammalian heart, mitochondria are more abundant than in any other tissue, accounting for approximately one-third of the cell volume (32). Working with this preparation, Singer and collaborators purified succinate dehydrogenase and demonstrated that the enzyme is a flavoprotein (33). Crane and collaborators (34) reported that ubiquinone is a redox carrier of the respiratory chain working between the NADH-dehydrogenase or succinate dehydrogenase and the cytochrome system. In 1960, Beinert and Sands discovered a new type of iron-containing redox catalysts that are nonheme iron proteins (35, 36). These catalysts, which were later found to contain iron-sulfur centres as their redox groups, were shown to be components of both succinate and NADH dehydrogenases.

Various groups in Green's laboratory made an effort to characterize the protein complexes that catalyze partial reactions of the respiratory chain allowed, in the early 1960s, the isolation of four different complexes, namely: NADH-ubiquinone reductase (complex I), containing FMN (flavin mononucleotide) and nonheme iron (37); succinate-ubiquinone reductase (complex II), containing FAD (flavin adenine dinucleotide) and nonheme iron (38); ubiquinol-cytochrome c reductase (complex III), which contains cytochromes b and c1, some bound ubiquinone (39); and cytochrome c oxidase (complex IV), containing cytochrome a and cooper (40). In 1962, Hatefi succeeded in reconstituting NADH oxidase and succinoxidase by combining complexes I, III, and IV, and complexes II, III, and IV, respectively, in the presence of cytochrome c (Cyt c) (41). These results gave rise to the concept that the respiratory chain components exist in mitochondria as a fixed assembly ("elementary particles"). Indeed, it was found that cyt c can form with complex III and complex IV supercomplexes, and the mitochondria contain the cytochromes in near-stoichiometric amounts (42).

At the functional level, the works of Chance and Williams have had a strong impact on the mitochondrial research field since they used an oxygen-selective electrode to record polarographically, with high sensibility, the oxygen consumption of mitochondria under different conditions, *i.e.*, substrates, inhibitors, ATP/ADP (adenosine diphosphate) and uncoupling agents. This methodology was decisive for studying the kinetics of the mitochondrial electron transport activity and its relationship with oxidative phosphorylation (43, 44) and is currently available in Hansateck and Oroboros devices. It made the first quantitative studies possible, characterizing the kinetics of electron-transport catalysts in intact mitochondria and integrated biological systems, including cells and tissue slices. The ATP produced per oxygen atom reduced by the respiratory chain (P/O ratios) activated by NADH-linked substrates or succinate were calculated. Usually, the experimental values were approximated for integers 3 and 2, respectively and rationalized under the chemical-coupling hypothesis framework proposed by Slater (43, 44).

The oxygen-selective electrode methodology also showed that isolated mitochondria can uptake Ca^{2+} from the suspending medium and, in the presence of phosphate, promotes the transient enhancement of the oxygen consumption rate like ADP. The energy-dependent accumulation of Ca^{2+} and many other divalent cations by respiring mitochondria in the presence of phosphate was quickly confirmed/revealed by other investigators (45). In 1963, Saris showed that Ca^{+2} uptake is accompanied by a release of protons (46). Moreover, Chance revealed that the mitochondrial Ca^{2+} uptake is driven by the energy of the respiratory chain independently of the phosphorylating system since it is sensitive to uncouplers (*e.g.*, dinitrophenol) and insensitive to oligomycin, an inhibitor of mitochondrial phosphorylation (47). These experiments highlighted the role of mitochondria in the regulation of intracellular fluxes of calcium. They also showed the failure of the chemical-coupling hypothesis to explain how mitochondria connect the metabolites' oxidation with ATP synthesis. Thus, a new vision is required to incorporate the available mitochondria architecture data and brings the membrane transport knowledge to the mitochondria research field to understand mitochondrial functionality.

1.2. Unveiling the interdependence between 3-D architecture and function: a scientific framework to describe the oxidative phosphorylation

With the application of electron microscopy to study cells and tissues during the 1950s, the first high-resolution micrographs revealing mitochondria as ellipsoid/spherical structures containing more than one membrane system emerged. Sjostrand described the mitochondria as a two membranes system, the outer and the inner membrane, and

postulated the existence of a third membrane that separates the matrix into multiple compartments (48). Palade also observed two mitochondrial membranes, proposing that the microscopy structures that separate the mitochondrial matrix into multiple compartments are invaginations of the inner membrane to form folded tubular structures, *i.e.*, cristae mitochondriales (49, 50). In agreement with Palade's model for mitochondria, Whittaker suggested that mitochondrial cristae are relatively narrow orifices; thereby, they can appear in many micrographs as structures without apparent connection to the inner membrane (51). Although cristae number was early recognized functional relevance, the technology to address the organization and opening size of these "relatively narrow orifices" just became available in the middle of the 1990s of the 20th century. Consequently, its putative functional relevance remained dormant for several decades. Therefore, mitochondria came to be represented as an ellipsoid/spherical structure that combines an outer mitochondrial membrane (OMM) and an inner mitochondrial membrane (IMM) as one continuous closed surface with multiples cristae, creating two distinct compartments: the intermembrane space (IMS), between two membranes, and the mitochondrial matrix, delimited by the IMM. The essential of Palade's model was preserved in the standard model for mitochondria structure, depicted in academic textbooks by cartoons showing the cristae with broad openings to the intermembrane space and relatively long projections to the matrix, like the bellows of an accordion (**Figure 2**).

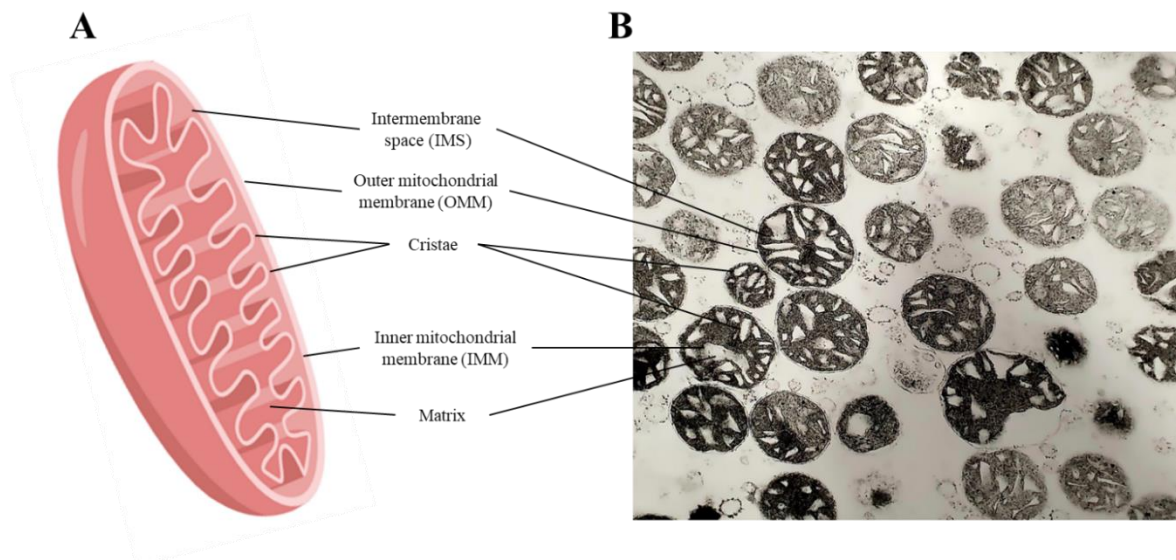


Figure 2. (A) Representation of Palade's mitochondria model, highlighting the outer mitochondrial membrane (OMM), inner mitochondrial membrane (IMM) with its cristae, and the intermembrane space (IMS). (B) Electron microscope micrograph of mitochondria isolated from rat liver with similar resolution to those allows building the model.

Focused on the emerging ideas about the structural organization and function of properties of cell membranes (52) that are also evidenced by the 3-D structures of mitochondria described above, Peter Mitchell advanced in 1961 with the chemiosmotic hypothesis to solve the puzzling problem of oxidative phosphorylation (53). With this hypothesis, Peter Mitchell intended to explain the coupling between oxidation-reduction and phosphorylation without assuming the existence of chemical intermediates common to the oxidation-reduction and phosphorylation pathways. Thus, he moves away from the shared vision proposed by Slater (explained above) to address the challenge of energy transduction in biological systems (*i.e.*, mitochondria, chloroplast, and plasma membrane of bacterial cells) with innovative ideas. The mature version of the chemiosmotic theory, published in 1966, is based upon four basic postulates: i) a membrane-located ATPase/synthase system which couples reversibly the translocation of protons across the membrane with ATP synthesis/hydrolysis. In mitochondria, the inwards protons translocation to the matrix is coupled with ATP synthesis, while a proton flows outwards (*i.e.*, from the matrix to intermembrane space) are generated by ATP hydrolysis; ii) the elements of the electron transport (respiratory) chain system exhibit an anisotropic spatial membrane organization that couples the redox reactions with a vectorial proton translocation from one side to another side of the membrane, creating a transmembrane electrochemical gradient of protons. In mitochondria, the elements of the electron transport chain use the energy of redox reactions to pump protons outwards, generating a transmembrane electrical potential (negative inside) and/or pH difference across the IMM; iii) the entry and exit of the essential metabolites are ensured by substrate-specific exchange/diffusion carriers that permit the reversible transmembrane exchange of the anionic metabolites against OH^- and the cationic against H^+ without the collapse of the membrane potential; iv) the specialized coupling membrane (*e.g.*, IMM) contains the systems postulated in i, ii, and iii, *i.e.*, ATPase/synthase system, elements of the oxidation-reduction chain system and the substrate-specific exchange/diffusion carriers, respectively, and has a low permeability to protons as well as to anions and cations in general (54). This set of postulates gives functional meaning to the available 3-D architecture of mitochondria. It explains the existing data about the oxidation-reduction reactions of the respiratory chain and phosphorylation system (ATPase/synthase) and the respiratory parameters obtained from different substrates, selective inhibitors, and uncoupling agents. They also allow the design of new experiments to test the validity of the hypothesis that energy conservation in the oxidation-reduction chain proceeds through a proton gradient across the coupling membrane.

The experimental data supporting the theory came successively, and they had contributions of many researchers, as reviewed elsewhere (55). However, the known “chloroplast acid bath experiment” pointed out in 1966 by Jagendorf and Uribe was particularly relevant for the general acceptance of chemiosmotic theory (56). These researchers obtained ATP synthesis in isolated chloroplasts inducing a transmembrane pH gradient. Thus, in 1978, Peter Mitchell was laureate of the Nobel Prize in chemistry for the formulating the chemiosmotic theory.

Despite the elegance and enormous application scope of Peter Mitchell’s proposal, the idea of an electrochemical gradient across a coupling membrane to drive ATP synthesis and other functions was far from the thought of many researchers of that time. For instance, Paul Boyer continued to work on the conformational hypothesis, considering that ATP synthesis was driven by the energy of conformational changes in the respiratory proteins energized by oxidation-reduction reactions. Using ^{32}P as a probe, he even announced the discovery of a catalytic intermediate in ATP formation with a phosphoryl group attached to a histidine residue. However, Paul Boyer’s research team quickly concludes that this enzyme is an intermediate in the substrate-level phosphorylation of the citric acid cycle rather than a key to oxidative phosphorylation (57).

Considering Peter Mitchell’s framework, understanding how energy stored in membrane potential and/or proton gradient generated by the respiratory chain could be used to drive ATP synthesis turns into a challenge. Racker and collaborators ascertained that the synthesis of ATP occurred on the so-called “spheres” referred to as F1 subunits of the enzyme “FoF1-ATPase” (58). Considering the available biochemical data, Boyer proposes the “binding change mechanism” for ATP synthesis, suggesting that the energy of the proton gradient is coupled to the rotational movement of catalytic subunits relative to a core of noncatalytic subunits. Consequently, the energy is required to bind ADP and the phosphate to the F1 subunits enzyme and release ATP from the enzyme (59, 60). Walker and his co-workers determined the protein subunits’ primary structure (amino acid sequences). They established the 3-D structure of the enzyme by crystallography (61), which allowed the validation of the mechanism proposed by Boyer. The scientific relevance of these findings was fully recognized in 1997 when Paul Boyer and John Walker were laureated with the Nobel Prize in Chemistry.

The chemiosmotic hypothesis complemented by the binding change mechanism for ATP synthesis has contributed significantly to our understanding of how biological systems convert and store energy. Additionally, it strongly influenced molecular biology development, especially protein technology. The principal IMM organizational vicissitudes exhibited by the mitochondrial redox chain complexes and FoF1-ATP synthase, unveiled

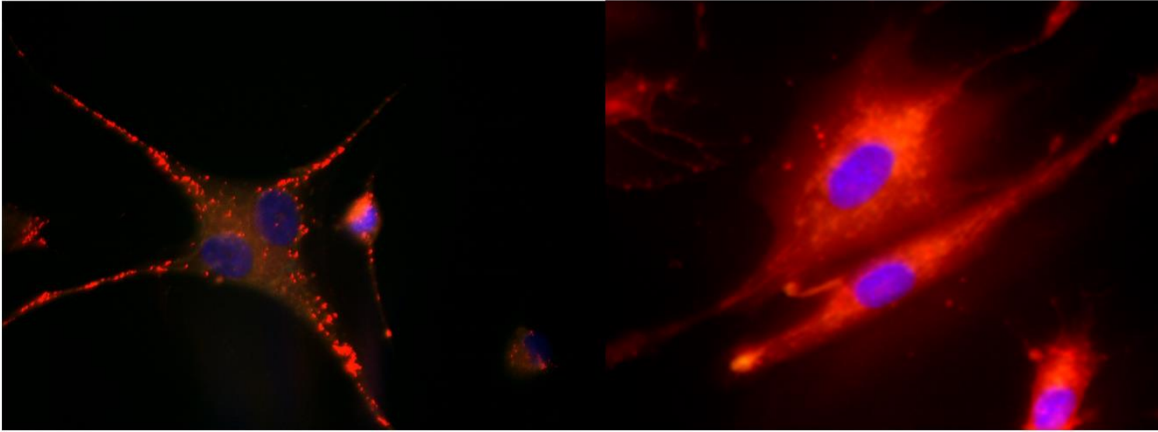
during the last four decades (61-70), support Peter Mitchell's vision for mitochondria functionality.

1.3. Current challenges in mitochondria research – How 3-D architecture fits with function

Mitochondrial bioenergetic functions are essential to cell life and death. Combining high-resolution fluorescent microscopy (including confocal microscopy) with mitochondria-targeted fluorescent dyes allows showing that, in living cells, mitochondria form a branched and extended network with a dynamic dependence of the metabolic state and cell-cycle phase (71, 72), as illustrated in **Figure 3A**. These mitochondrial networks maintain themselves in a dynamic morphology, mainly around the nucleus, by undergoing frequent cycles of division (fission) and merging (fusion) (73-77). Indeed, the unveiling of mitochondrial fusion and fission processes is the recognition of the functional relevance of the structural plasticity attributed to mitochondria by Lewis and Lewis in 1915 (21).

The application of electron microscopic tomography to study mitochondria provides important new insights into organelle 3-D architecture (78-80) that emphasize the need to develop a new model to represent the functional organization of the organelle and eventually update Peter Mitchell's vision. Electron microscopic cryo-tomography data show that cristae can vary from simple tubular structures to more complex lamellar structures merging with the inner boundary membrane through tubular structures with about 28 nm in diameter (79-81). Electron Cryo-Tomography studies of cells and tissues stained with protein-specific fluorescent dyes also revealed that "ATP synthase is arranged in rows of dimers along highly curved apices of the inner membrane cristae with complexes of the respiratory redox chain located in the membrane areas either side of the rows" (81-84) as represented in the **Figure 3B**. While the physiological relevance of this oxidative phosphorylation system membrane organization is not fully understood, it is known that FoF1-ATP synthase dimerization is a crucial player in 3-D inner membrane cristae organization (83, 84). Indeed, severe human diseases, including Leigh syndrome, are characterized by significant changes in the 3-D architecture of the mitochondria resulting from genetic alterations that hamper the establishment of FoF1 ATP synthase dimers (85, 86). Experimental evidence also showed that the disruption of FoF1-ATP synthase dimerization strongly impacts 3-D mitochondrial cristae organization with minor effects on the catalytic activity of the monomeric FoF1-ATP synthase form (87, 88).

A



B

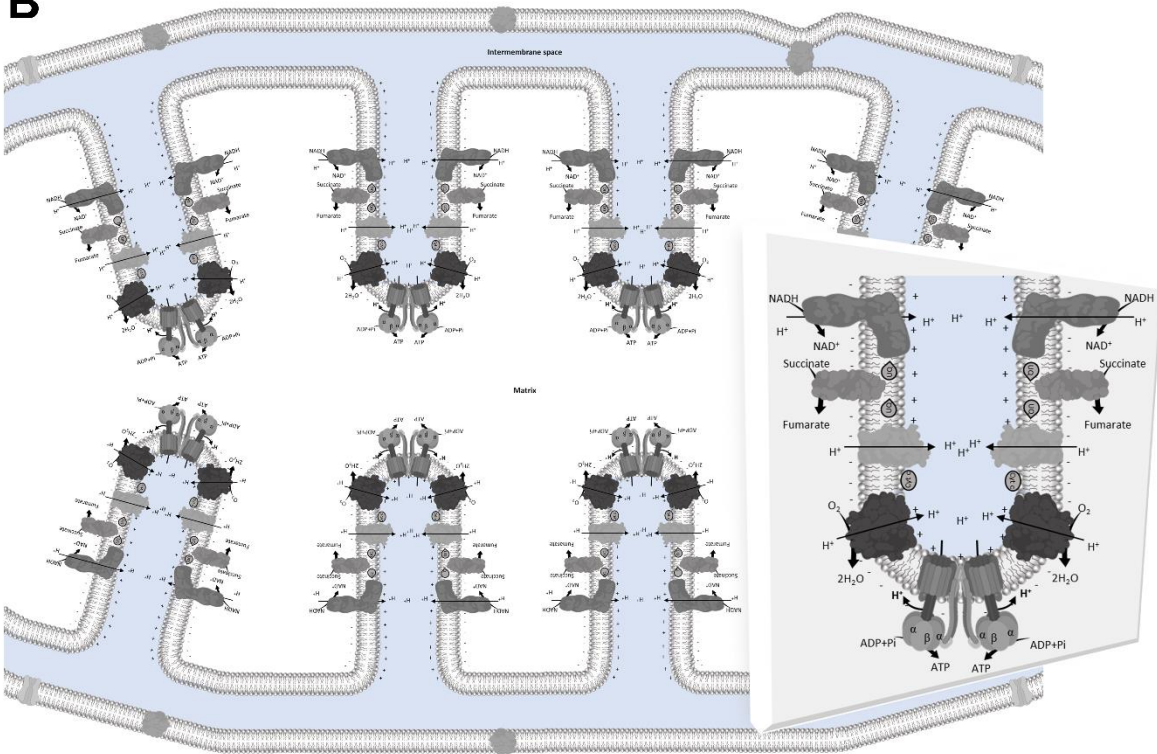


Figure 3. (A) Sertoli cells stained with the mitochondrial probe JC-1 and with Hoechst (colour blue nuclei) to show the mitochondrial network in the cytosol. The images were constructed by merging three channels – blue nuclei, red mitochondria polarized, and green mitochondria depolarized. (B) Schematic representations of the organization of the oxidative phosphorylation system in the IMM cristae according to the Cryo-Tomography studies, with one mitochondrial cristae magnified to highlight the schematic representation of the dimeric organization of the FoF1-ATP synthase and the respiratory chain complexes in the IMM invaginations that form the cristae. Abbreviations: cyt c, cytochrome c; IMM, inner mitochondrial membrane; JC-1, 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolo-carbocyanine iodide; UQ, ubiquinone.

Although Cryo-Tomography allows obtaining images of the 3-D architecture of mitochondria with a very high-spatial resolution, it cannot give information about the temporal scale of morphological changes since the dynamics of biological processes are stopped by cryo-preservation. Super-resolution nanoscopy techniques, including stimulated emission depletion microscopy, address this constraint by using fluorescent mitochondria

tags with improved photostability to detect the mitochondria dynamics for several minutes, recording sequential images with spatial resolution less than 60 nm (89-91). Thus, the mitochondrial fusion and fission processes and the dynamic reorganization of mitochondrial cristae (e.g., number, shape, matrix volume versus inner membrane volume) are being revealed in cell culture systems under different metabolic and external stimuli (89). The organization of redox complexes of the respiratory system and phosphorylation system, revealed by Cryo-Tomography studies, and cristae dynamics, revealed by super-resolution nanoscopy techniques, are also dependent on a specific pool of IMM lipids, mainly of the cardiolipin content and diversity (92-96). Cardiolipin is an anionic tetra-acyl phospholipid selected by evolution to optimize functional membrane assembly of redox complexes of the respiratory system, being considered the lipid fingerprint of mitochondria. The mitochondrial dysfunction associated with many human chronic diseases (e.g., Alzheimer's (AD) and Parkinson's (PD) diseases) is characterized not only by the deficit of the respiratory activity system and/or phosphorylation system but also by anomalous cardiolipin profile (93, 94, 97-101). These data suggest that the 3-D architecture of mitochondria with a particular oxidative phosphorylation system organization in IMM and a particular lipid composition was preserved by evolution not only to allow the coupling between oxidation-reduction and phosphorylation (ATP production) but also to support other fundamental requirements of multicellular life, as it will be exemplified below.

Mammals and other homeothermic organisms control their body temperature at values that generally, are higher than the environmental temperature, which requires a controlled process of continuous heat generation (thermogenesis) to counteract heat loss to the environment by thermic difference. Heat generation is related to mitochondrial functionality, and the shivering and non-shivering mechanisms were described as thermogenesis. Shivering thermogenesis is associated with the skeletal muscle tissue that releases significant amounts of heat during contractile activity. The contractile activity of the skeletal muscle is mainly supported by the ATP generated by the intermyofibrillar mitochondria population at the z-bands of the muscle sarcomere, it depends on the mitochondrial functionality (102-105). Non-shivering thermogenesis is a generic designation for the heat generated associated with the metabolic activity unrelated to movement (102, 103, 106). The heat released by the activity of mitochondria under uncoupled conditions, well documented in brown and beige adipose tissues, is particularly relevant. In this mechanism, uncoupling proteins (UCP) play a crucial role since they promote the diffusion of protons through the IMM, allowing that all energy of the oxidation of the NADH and FADH₂ (reduce flavin adenine dinucleotide) along the respiratory chain can be dissipated as heat rather than to ATP synthesis (102, 106, 107). Thus, mitochondrial architecture seemingly

tuned for ATP synthesis serves efficiently another purpose by including a new protein-type (e.g., UCPs) in its IMM. Recent studies also revealed that the temperature around the IMM rises 10 °C above the temperature of the cytosol (37 °C for human cells) when the respiratory chain is fully functional and coupled with ATP synthesis, as detected by temperature-sensitive fluorescent targeted to mitochondria (108). How mitochondria can dissipate such a tremendous amount of heat under coupled conditions and the role of this thermogenesis type for body temperature regulation remains open. In our understanding, the formulating any convincing explanatory hypothesis for these questions should be driven by the 3-D architecture of mitochondria with its oxidative phosphorylation system organization in the IMM.

A cornerstone of the highly complex eukaryotic organisms (as humans) is the homeostatic control of the balance between cell proliferation and cell death in tissues, which is required to ensure the organism's good development and physiological performance. Mitochondria are a key player in either cell proliferation or cell death. Cell proliferation requires continuous biosynthetic processes, which depend highly on ATP, reduced NADH and FADH₂ cofactors, heme prosthetic group, and intermediary metabolites produced in mitochondria (109-112). Cell death by apoptosis (113) is also associated with mitochondrial organization and functionality (114, 115). Two general mechanisms were used to describe apoptosis, one triggered by external stimuli that activate the cell death receptors on the plasma membrane (extrinsic) and the other by an intrinsic mechanism associated with mitochondrial dysfunction. In both mechanisms, the release of cyt c (an electron membrane carrier of the respiratory chain) from the IMM to the cytoplasm is a key event that precedes the activation of the apoptotic caspases cascade. Thus, apoptosis is associated with a profound change in the functional 3-D architecture of mitochondria required for cyt c release (5, 114-116).

1.4. Mitochondria and degenerative brain diseases - the case of Alzheimer's and Parkinson's diseases

Degenerative brain diseases that cause progressive and irreversibly brain functions, including memory, cognitive, behavioural, and motor coordination skills, are devastating the lives of millions of people. Currently, 45 million people worldwide suffer from AD and 10 million other neurodegenerative diseases, including PD, without any reliable therapeutic tool to stop or slow their progression (117). Thus, solving the problem of progressive brain degeneration is a scientific challenge with high social and economic relevance.

It is well-known that the loss of functional connectivity between different brain regions, resulting from distorted/disrupted neural networks, is the main macroscopic feature of brain pathologies. At the same time, mitochondrial dysfunction is a cellular hallmark of neurodegeneration, including AD and PD (118, 119). However, the relationship between mitochondria functionality and the brain processes used to generate and coordinate, in space and time, the information flows underlying the cellular networks connectivity remains to be determined. Consequently, some investigators consider mitochondrial dysfunction the first cause of the pathological cascade and others as a consequence of the degenerative process, as highlighted in many reviews (120-123).

In terms of therapeutic-oriented research, the problem of age-related neurodegeneration has been addressed by a disease-specific pathophysiological vision focused on protein misfolding and genetic vicissitudes connected specifically to each disease (*e.g.*, amyloid- β peptides and tau for AD and α -synuclein for PD) (124, 125). Hence, the canonical vision of neurodegenerative diseases recognizes mitochondrial dysfunction due to the degenerative process. However, the research developed for more than two decades under the canonical vision produced disappointing outcomes since the therapeutics developed to target amyloid- β peptides production/aggregation have failed in clinical trials (126), and α -synuclein-targeted therapies have not been successful either (127, 128). Thus, the arsenal of available drugs to fight AD and PD remains limited to the first generation of pharmacological molecules approved by their positive impact on specific neural networks. In AD, the pharmacological drugs were rationalized to modulate the cholinergic (acetylcholinesterase inhibitors such as donepezil, galantamine, and rivastigmine) or glutamatergic (memantine) neuronal networks (129, 130). In PD, they were designed to improve dopaminergic connectivity, increasing dopamine levels working as dopamine precursors (*e.g.*, carbidopa-levodopa) or as inhibitors of dopamine catabolism enzymes (*e.g.*, selegiline, rasagiline, and safinamide that are monoamine oxidase-B inhibitors) (131, 132). However, we all know that the therapeutic benefits of these drugs are not sustained in time, and their continuous use has been associated with significant toxic side effects (117).

In recent years the strategies to fight neurodegenerative diseases have been directed towards a vision considering mitochondrial dysfunction a primary cause of the neurodegenerative process. In defense of this vision, we can assert that: i) mitochondrial dysfunction is an early pathological mark of the neurodegenerative process, and the dysfunctional mitochondria generate large amounts of reactive oxygen species (ROS, mainly at levels of complexes I and III) and drive the cell to the release of pro-inflammatory mediators, leading to oxidative stress and chronic inflammation, other pathological

hallmarks of the neurodegeneration (133-135); ii) intoxications by drugs that work as specific mitochondrial complex I inhibitors (e.g., rotenone, MPTP 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) leads to a PD-like neurodegenerative process (136, 137); iii) in animal models for neurodegeneration, including AD and PD, the mitochondrial dysfunction is an upstream cellular signal from the aggregation of disease-specific misfolded proteins, preceding the clinical symptoms of each disease (138-141); iv) massive synapse loss precedes neurons death, and the mitochondrial dysfunction is initially detected in the synaptic brain mitochondria population (decline of complex I activity and abnormal cardiolipins profile) as reported for AD-like pathology (98); v) an imbalance between the dynamic mitochondrial fission and fusion process, mainly resulting from an enhancement of the fission with consequent excessive fragmentation of the mitochondrial network and dysfunctional intracellular distribution, is early detected in animal models of both AD and PD (142-145). Accordingly, mitochondria emerge as a promising target to address the neurodegeneration issue, with the putative benefit that a single therapeutic approach can be suitable for multiple common disorders. However, the functional organization of the mitochondrial supports a hub of cell functions impaired by neurodegeneration. The selection of the specific mitochondrial targets that need to be modulated to interrupt (or avoid) the degenerative process remains an unsolved issue.

In the context of neurodegeneration, the first attempts to modulate mitochondrial functionality relied on antioxidant molecules. The main goal of this approach is to prevent the oxidative stress resulting from the overproduction of ROS connected with mitochondrial dysfunction, and the main outcomes obtained in animal models for AD and PD with coenzyme Q10, vitamin E, and lipoic acid are highlighted in **Table 1**. Overall, positive outcomes in AD and/or PD animal models were reported for all antioxidant compounds indicated in **Table 1** as well as for many other antioxidants, including the plant polyphenols in formulations prepared with pure compounds or with complex mixtures like polyphenol-rich extracts (146-149).

The benefits obtained in animal models sustained that Coenzyme Q10 and lipoic acid were also examined in clinical trials for PD and AD, respectively (**Table 2**). Formulations of Coenzyme Q10, in a nanodispersion or combined with vitamin E, did not promote any detectable positive outcomes in randomized, double-blind, placebo-controlled phase III trials for PD (**Table 2**). The benefits of lipoic acid in animal models of neurodegenerative diseases were also not confirmed in clinical trials for AD (**Table 2**). It is important to stress that the cell uptake of the antioxidant molecules mentioned in **Table 1** is mainly driven by passive diffusion. Their lipophilic features lead to the accumulation of the lipid component of all biomembranes without selectivity for the mitochondrial

membranes. It was indicated that the significant drawback of these antioxidant molecules to support mitochondria therapy to fight neurodegeneration is related to their low availability for the mitochondria of brain cells (150, 151).

Table 1. Therapeutic approaches for AD and PD supported by antioxidants (coenzyme Q10, vitamin E, and lipoic acid) or antioxidant molecules engineered to target mitochondria (mitoQ, Mito-Apocynin, SkQ1, and SS-31), highlighting the main outcomes in the indicated animal model used under a specified administration route. Abbreviations: AD, Alzheimer's disease; PD, Parkinson's disease; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; 6-OHDOPA, 6-hydroxy-dopamine; ATP, Adenosine triphosphate; TPP⁺, triphenylphosphonium cation; iNOS, inducible nitric oxide synthase; mtDNA, mitochondrial DNA.

Antioxidant molecules	Disease	Animal model/ Administration route	Main outcomes
Coenzyme Q10	AD and diabetes	Diabetic Goto–Kakizaki aged rat model (20-months-old) – Treatment for 7 weeks (injected each 48h)/ Intraperitoneal.	Improved the mitochondrial respiratory control ratio and oxidative phosphorylation efficiency compromised in this animal model (152).
	AD	Transgenic Tg19959 mouse model (1-month-old) – Treatment from 1 to 4 months of age or 1 to 6 months of age/ Oral, chow supplementation.	Decreased brain oxidative stress, Aβ42 levels, plaque burden, and improved cognitive performance of the animal model (153).
	PD	MPTP-induced PD C57BLr6 mouse model (1-year-old) – Treatment for 5 weeks/ Oral, chow supplementation.	Attenuated the MPTP-induced loss of dopamine levels and dopaminergic neurons in the striatal brain region (154).
Vitamin E or γ-Cyclodextrin-Vitamin E complex	PD	6-OHDOPA-induced PD in adult Sprague-Dawley rat model – Treatment for 4 weeks (1h before the PD-like toxic stimulus and continued at 2-day intervals)/ Intramuscular.	Protected the brain dopaminergic neurons from 6-OHDOPA toxicity (155).
	Brain ageing	C57BL/6J mice (17-months-old) – Treatment for 24 weeks/ Oral, chow supplementation.	Increased the brain levels of ATP and enhanced the mitochondrial membrane potential of aged mice, suggesting benefits for healthy ageing (156).
Lipoic acid	PD	Rotenone-induced PD albino rat model (≥ 22-months-old) – Treatment for 60 days (start 1h before rotenone administration and continuous daily dose until de 60 days)/ Oral gavage.	Attenuated the rotenone-induced brain toxicity, preserving the striatal ultrastructure integrity (157).
	AD	Transgenic SAMP8 mouse model (11-months-old) – Treatment starting at 11 months of age until they were deceased/ Subcutaneous.	Improved memory and reversed indices of oxidative stress in extremely old mice (158).
Mitoquinone (MitoQ) – Antioxidant coenzyme Q ₁₀ analogue conjugated with TPP ⁺	AD	Transgenic 3xTg-AD mouse model (2-months-old)- Treatment for 5 months/ Oral, drinking water.	Prevented the onset of cognitive deficits and decreased oxidative stress, synaptic loss, astrogliosis, and caspase activation (159).
	PD	MPTP-induced PD C57BL/6 mouse model (8-weeks-old) – Treatment for 13 days (one day before PD-like toxic stimulus, 5 days co-administered with toxic and more 7 days)/ Oral gavage.	Improved motor functions and protected the nigrostriatal axis against MPTP-induced toxicity, decreasing the TH-positive neuronal loss (160).

Mito-Apocynin - Apocynin conjugated with TPP ⁺	PD	Transgenic MitoPark mouse model - Treatment thrice weekly from age 13 to 24 weeks/ Oral gavage.	Improved motor activity and coordination and neuroprotective effects, were detected at the level of mitochondrial aconitase and oxidative damage biomarkers (161).
	PD	MPTP-induced PD C57BL/6 mouse model (8- to 10-weeks-old) – Acute and sub-acute treatments designed under a preventive paradigm. Treatment starts one day before PD-like toxic stimulus followed by: i) 3 days of co-administration with the toxic stimulus (acute); ii) 5 days of co-administration with toxic stimulus and 6 additional days (sub-acute)/ Oral gavage.	Improved motor performance and nigrostriatal dopaminergic neuron survival and attenuated the inflammatory response, decreasing the expression of pro-inflammatory proteins, including iNOS (162).
Plastoquinone (SkQ1) – TPP ⁺ derivative conjugated with plastoquinone that is delivered for mitochondria	Brain ageing and AD	Senescence-accelerated OXYS rat model (1.5-months-old) – Treatment from 1.5 to 3 months of age/ Oral, chow supplementation.	Reduced the level of mtDNA deletions in the hippocampus and slowed the accelerated ageing of the brain exhibited by this animal model (163).
	AD	Senescence-accelerated OXYS rat model (4-months-old and 12-months-old) – Animals with 4 months old: treatment for 7 or 14 days; Animals with 12-month-old: treatment for 6 months/ Oral, chow supplementation.	Prevented neuronal loss and synaptic damage, attenuated spatial learning and memory impairments, and decreased the brain levels of both amyloid- β 1-42 polypeptide and hyperphosphorylated tau protein (164).
Szeto-Schiller tetrapeptides 31 (SS-31) – a water-soluble peptide with antioxidant and mitochondriotropic properties	AD	Transgenic APP Tg2576 AD mouse model (12-months-old) – Treatment twice a week for 6 weeks/ Intraperitoneal.	Decreased amyloid- β brain levels and mitochondrial dysfunction, maintained mitochondrial dynamics and enhanced mitochondrial biogenesis and synaptic activity (165).
	PD	MPTP-induced PD C57 black mouse model (3-months-old) Treatment 30 min. before PD-like toxic stimulus administration (a triple dose in 2h interval), with reinforcement at 1 and 12 h after the last MPTP dose/ Intraperitoneal.	Attenuated MPTP-induced depletion of dopamine levels in the striatum brain region and protected dopaminergic neurons against MPTP damage (166).

To overcome the constraints mentioned above several lipophilic cationic compounds were engineered to target mitochondria taking advantage of the mitochondrial electrical potential (negative inside) (150, 167-169). The antioxidant molecules conjugated with the monocharged lipophilic triphenylphosphonium (TPP⁺) cation (e.g., MitoQ, Mito-Apocynin, Plastoquinones SkQ1) and the Szeto-Schiller (SS) cell-permeable synthetic tetrapeptides with a triple-positive charge at physiological pH (e.g., SS31) are representative compounds of this mitochondria-targeted strategy that were tested in animal models for AD and PD. As indicated in **Table 1**, these studies revealed that the TPP-based antioxidants and the SS synthetic tetrapeptides promote benefits in several biochemical and physiological parameters impaired by AD- and/or PD-like degenerative processes.

Considering the Clinical Trials database of the U.S. National Library of Medicine, only MitoQ was tested in neurodegenerative disease patients. The data obtained with animal models pointing MitoQ as a promising tool to support a mitochondria-targeted therapy to treat age-related neurodegenerative diseases. However, the results obtained in clinical trials for PD were disappointing (**Table 2**). Despite this lack of therapeutic effectiveness against PD, the oral formulations of MitoQ showed the ability to improve vascular endothelial function in healthy older adults, which has relevance to healthy ageing management (170).

Table 2. Clinical trials performed with the antioxidant's coenzyme Q10 and lipoic acid and with the mitochondria-targeted antioxidant MitoQ to assess their therapeutic effectiveness against AD and/or PD, highlighting the main outcomes and the administration route. Data were obtained from the ClinicalTrials.gov database provided by the U.S. National Library of Medicine (www.clinicaltrials.gov).

Antioxidant molecules	Disease - Clinical trial level (Gov identifier; year)	Drug formulation/ Administration route	Main outcomes
Coenzyme Q10	PD - Phase III (NCT00740714; 2013)	Coenzyme Q10 plus vitamin E or vitamin E as placebo/ Oral, chewable wafers	Administered to patients with early PD; no therapeutic efficacy was demonstrated thereby it was not recommended for the treatment of early PD (171).
	PD - Phase III (NCT00180037; 2005)	Coenzyme Q10 Nanodispersion or placebo/ Oral.	Well tolerated and safe but did not display symptomatic benefits in midstage PD, thereby it was not recommended for PD treatment (172).
Lipoic acid	AD - Phase I and II (NCT00090402; 2017)	Lipoic acid plus fish oil/ Oral.	Slowed both cognitive and functional decline in mild to moderately impaired AD participants. Inconclusive therapeutic benefits require additional studies (173).
Mitoquinone (MitoQ)	PD - PROTECT phase II (NCT00329056; 2007)	MitoQ or placebo/ Oral.	Showed no difference between MitoQ and placebo on all measured parameters related to the PD progression. MitoQ did not slow down PD progression, thereby it was not recommended (174).

The data set presented in **Tables 1** and **2** indicates that the antioxidants, including those engineered to selectively target the mitochondria (e.g., MitoQ), are unsuitable as a therapeutic tool for AD and PD, despite their promising in pre-clinical data. The lack of therapeutic effectiveness of these antioxidant-based therapies indicates that the antioxidant does not fulfil the purpose for which it was engineered/selected, or the interruption of the mitochondrial oxidative stress-dependent pathological cascade is not enough to promote positive outcomes on the neurodegenerative process. It is also important to stress that these therapies were not rationalized to overcome any known structural mitochondrial parameters affected by the neurodegenerative process but only to avoid/attenuate the

oxidative damage resulting from the overproduction of ROS by dysfunctional mitochondria. Thus, we need a new vision to rationalize the fundamental assumptions required to develop an effective mitochondria-targeted therapy to fight AD and PD, which will be discussed in light of the functional 3-D architecture of the mitochondria in the next section.

1.5. Outlooking a mitochondria-targeted therapy for AD and PD

Despite more than a century of active research on the mitochondrion, from its discovery as the ubiquitous intracellular structure of the eukaryotic cell, mitochondria still hold many secrets to be unravelled. As highlighted above, we know that mitochondrion currently supports a hub of functionalities required to ensure cell survival and many of the physiological requirements of multicellular life. The mitochondrial-dependent biochemical pathways connected with carbon fluxes that occur in the matrix (*e.g.*, citric acid cycle) follow a similar strategy to those driven by cytosolic enzymes (*e.g.*, glycolysis), including GTP (guanosine triphosphate, ATP equivalent) synthesis by the substrate-level phosphorylation mechanism. As rationalized by Peter Mitchell, it is the dynamic 3-D architecture of mitochondria that enables a more efficient membrane energy conservation and transduction process, which can be used to supply more than 90% of the ATP required by cells (*e.g.*, as occurs in brain cells) as well as for many other purposes, such as thermogenesis and calcium homeostasis regulation. Additionally, the subtle way how the functional assembling and organization of respiratory chain complexes provided by a specific pool of lipids in the IMM uses the NADH and FADH₂ redox power to reduce the oxygen to water and generate an electrochemical proton gradient also leads to the release of ROS. Mitochondrial ROS play a key role in cell signalling under physiological conditions, but mitochondrial dysfunction amplifies ROS generation to pathological levels. Thus, mitochondrial dysfunction changes the redox-dependent cell signalling cascades and can lead to the oxidative damage of cellular biomolecules (oxidative stress), including the mitochondrial ones, with consequent mitochondrial dysfunction worsening. The interlink between mitochondrial dysfunction and oxidative stress under a vicious pathological cycle is highly relevant in neurodegeneration. However, several antioxidant-driven approaches, including those supported by engineered mitochondria-targeted molecules, have failed when tested in clinical trials for AD and PD, suggesting that oxidative stress should be a minor branch of the pathological cascade underlying neurodegeneration.

Within this framework, developing effective mitochondria-targeted therapies for AD and PD may require at least two challenging approaches. First, we need a set of compounds with the ability to overcome the anomalous activity of the respiratory chain complexes

(mainly complex I) and repair the abnormal mitochondrial lipidome (mainly cardiolipins), hallmarks of AD and PD mitochondrial dysfunction. These parameters are interlinked and directly related to the 3-D architecture of the mitochondria. Second, we need a capable delivery system to overcome the different physiological barriers and to supply the mitochondria of brain cells with the selected compounds. To overcome the dysfunctional complex I, we propose redox-active compounds that can oxidize NADH in the water phase and deliver the electrons to complex III of the mitochondrial redox chain, working as a membrane electron carrier. It was reported that methylene blue, a synthetic compound with several toxicological concerns, has the competence to oxidize NADH and deliver electrons to complex III (175). In the present thesis, an integrated approach supported by elderberry anthocyanins and algae membrane polar lipids, natural products obtained from edible sources, will address the challenges mentioned above.

2. On the track of an effective nanoplatform to support a mitochondria-targeted therapy for neurodegenerative diseases.

2.1. *Sambucus nigra* – resources of anthocyanins with redox-active mitochondriotropic properties

Anthocyanins, a Greek word that aggregates *anthos* (flower) with *kyanos* (blue), are vacuolar pigments responsible for the characteristic red to blue or purple colour exhibited by many flowers, vegetables, and fruits. Plants produce, via the phenylpropanoid pathway, anthocyanins aiming: i) to regulate metabolism and protect cells against abiotic (e.g., solar radiation) and biotic stress stimuli (e.g., pathogen infections), delaying the senescence process and increasing plants life expectancy; ii) to improve the flowers and mature fruits attractiveness for pollinating insects and animals that promote seed dispersal - evolutionary advantage to plants (176-179).

From a chemical point of view, anthocyanins are glucosides of the anthocyanidins, a flavonoids sub-group with the typical C6-C3-C6 carbon skeleton, *i.e.*, two phenyl rings (A and B rings) and one heterocycle pyran (ring C) fused with ring A (**Figure 4**). However, anthocyanidins are structurally distinguished from the other flavonoids due to their capacity to form a flavylium cation at acidic pH conditions – a key structural feature for their bioactivity (176,180,181). The first line of structural diversity of anthocyanidins emerges from hydroxylation at different carbons of the flavylium cation backbone and subsequent O-methylation, mainly on the B ring, as illustrated in **Figure 4** for the six major types (or families) of anthocyanidins synthesized by plants. The biodiversity within each family results

from enzyme-catalyzed O-glycosylation reactions, which can involve several hydroxyl groups. In general, the hydroxyl group at carbon 3 (C3-OH) is the most abundant glycosylation site in nature. Additional glycosylation at C5-OH is also very common in plants, while glycosylation at the C7-, C3'-, or C5'-OH groups is considered less usual despite being detected in some plant species (182). Different types of sugars can be used for glycosylation, including monosaccharides (e.g., glucose, galactose and rhamnose) and disaccharides like sophorose (β -1,2-glucose dimer), sambubiose (β -D-xylose-(1 $\rightarrow$ 2)- β -D-glucose) and rutinose (rhamnose α (1 $\rightarrow$ 6) glucose). The identity, number, and positions of sugars attached to the aglycone backbone expand the molecular species diversity of anthocyanins by one or two orders of magnitude. As the hydroxyl groups, the glycosylation also increases the water solubility of the anthocyanins and improves their stability by decreasing the electron-deficient properties of the flavylium cation (177,183).

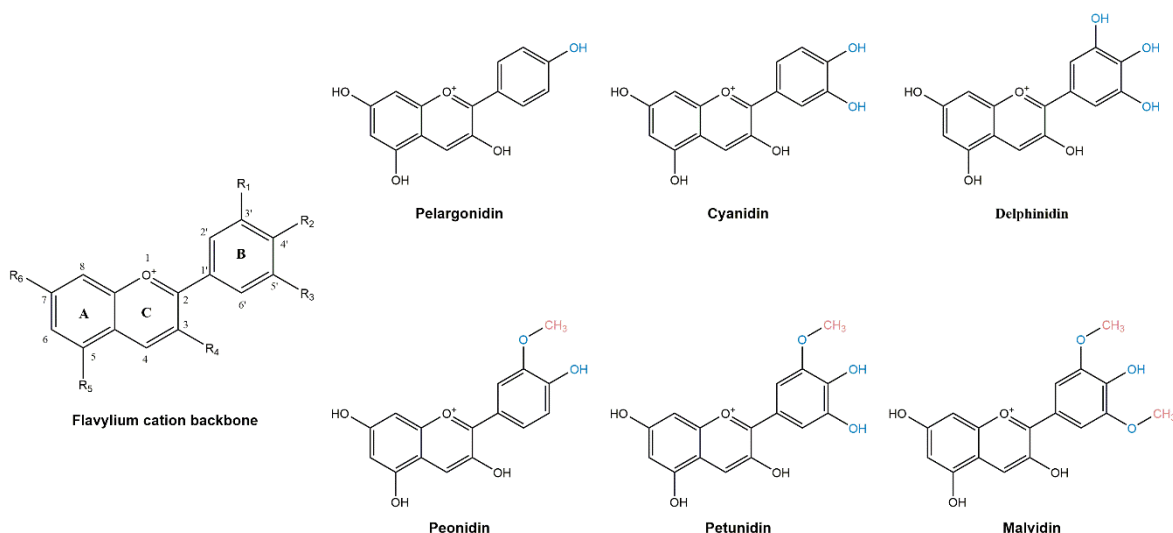


Figure 4. Flavylium cation backbone and the six major anthocyanidins families produced by plants (pelargonidin, cyanidin, delphinidin, peonidin, petunidin, and malvidin) by changing the number of hydroxyl groups and subsequent methylation of hydroxyl groups at B ring.

Despite that, the acid-base properties of the flavylium ion (a weak diacid) dominate the reactivity of anthocyanins in aqueous media, modulating many other chemicals (e.g., antioxidant activity) and physical (e.g., absorption bands and colour) properties. The first acid-base reaction ($\text{pK}_{\text{a}1} \leq 4$), mainly assigned to a proton release from the C7-OH group (A ring), leads to the formation of the neutral quinonoid base. In contrast, the second acid-base reaction ($\text{pK}_{\text{a}2} = 6\text{--}7$) is associated with a proton loss from the C4'-OH group (B ring) and yields an anionic base that amplifies the electron delocalization over the three rings (184-186). Consequently, the anthocyanins absorption band in the visible region undergoes a progressive blueshift with pH increasing. Thus, in aqueous solutions with a pH value below $\text{pK}_{\text{a}1}$, the dominant species is the flavylium cation which exhibits a red or orange colour,

depending on the number of hydroxyl and methoxyl groups. For pH values between pK_{a1} and pK_{a2} , a neutral quinonoid base with a violet colour is privileged, while the pH increasing for values above pK_{a2} leads to the anionic base formation, turning the solution blue (176,178,184-186). It is also important to highlight that the acidic form of anthocyanins (flavylium cation form) is also susceptible to water addition at C2 (an electrophilic carbon), through a reversible reaction that generates the colourless hemiketal. This structure occurs in balance with chalcones. On the other hand, the neutral and anionic quinonoid bases of anthocyanins, the main structures found at basic pH, are highly susceptible to nonenzymatic oxidation triggered by O_2 , an irreversible process that leads to their degradation and consequent loss of colour (176,178). Thus, the anthocyanins are more stable under acidic conditions favouring the flavylium cation form (176,187,188). Additionally, planar structures and extended electron delocalization over the three rings allow anthocyanins to develop π -stacking interactions, supporting their self-association and the recruitment of other phenols. Taking advantage of this anthocyanin's ability, the plants develop a co-pigmentation process that improves the anthocyanin's stability and colour by preventing the free access of the water molecules to the flavylium cation backbone. The co-pigmentation process can be further boosted by the recruitment of ions (e.g., Mg^{+2}) that leads to anthocyanins-metallic complexes formation and/or by the acylation of their glycosidic moieties with organic acids (e.g., *p*-hydroxycinnamic, *p*-hydroxybenzoic, acetic, malonic acids) (189,190).

The structural particularities of anthocyanins also allow antioxidant properties, well-evidenced by their high reactivity towards ROS (180,191-193). Thus, the health promoting benefits of anthocyanins are explained by their ability to protect the biomolecules (lipids, proteins, and nucleic acids) and cells from the oxidation promoted by ROS and other oxidant toxic stimuli (181,183). Additionally, the anti-inflammatory properties of anthocyanins are a well-recognized feature, being considered promising compounds for the prevention and treatment of many diseases (e.g., cardiovascular diseases, diabetes, obesity, and neurodegenerative diseases) characterized by oxidative stress and chronic inflammation (178,191,192,194,195). For example, it was reported that anthocyanins could ameliorate chronic brain inflammation by targeting microglial cells since they can prevent the interferon- or lipopolysaccharides (LPS)-induced activation of signalling cascades in BV-2 microglial cells by reducing the production of pro-inflammatory mediators (nitric oxide, tumour necrosis factor, interleukins) (196,197). Regarding effects on AD, anthocyanins consumption was associated with memory and cognitive function improvement simultaneous with reduced of brain levels of amyloid-beta peptides aggregates and attenuation of the inflammation-related parameters (198,199). In PD, the higher intake of anthocyanins has been associated with a lower risk of developing this type of brain pathology since anthocyanins lead to an

improvement of motor performance and increased hippocampal neurogenesis, positive outcomes that were associated with their anti-inflammatory properties (200,201).

While anthocyanins have shown great promise in supporting strategies to treat many types of disease, it is essential to highlight that the *in vivo* bioavailability of anthocyanins is relatively low. Additionally, in standard food formulations, they are easily oxidized and rapidly metabolized in gut microbiota and/or in liver enzymes producing compounds without or with less bioactivity (202,203). For this reason, encapsulating anthocyanins in suitable delivery systems is considered a good approach to ensure and/or enhance their therapeutic efficacy, as explained below.

Sambucus nigra L. is the most common species of the *Sambucus* L. genus of the Adoxaceae family (204). It is known in Portugal as “sabugueiro” while the international common names include elder, elderberry, black elder, European elderberry, and European black elderberry. There are three subspecies of *Sambucus nigra* L.: *S. nigra* L. ssp. *nigra*, a subspecies native to Europe and North Africa, and *S. nigra* L. ssp. *canadensis* and *S. nigra* L. ssp. *cerulea*, both naturally occur in North America. However, the cultivation of *S. nigra* L. ssp. *nigra* has been successively extended to other regions of the world, and currently, this subspecies can also be found in Asia, North America, New Zealand, and Australia (205,206). *S. nigra* produces white and small hermaphrodite flowers in spring, whereby the development and ripening of the berries occur during summer. Thus, at the end of August or the beginning of September, the berries exhibit a dark-violet colour indicating an optimum maturity state for harvest (**Figure 5**) (205,206). The development of the dark-violet colour is associated with increased of anthocyanins content that occurs during fruit ripeness.



Figure 5. Elderberry *S. nigra* L. flowers and fruits.

Elderberries are a rich source of phenolic compounds, including cinnamic acids, flavonols and anthocyanins, representing around 5%, 13% and 80%, respectively (204,207-209). The major cinnamic acid in elderberries is chlorogenic, followed by neochlorogenic

and cryptochlorogenic acids. Quercetin-3-rutinoside and quercetin-3-glucoside are the most reported flavonols in the elderberries. Regarding anthocyanins, cyanidin-3-O-sambubioside, cyanidin-3-O-glucoside, cyanidin-3-O-sambubioside-5-O-glucoside, and cyanidin-3,5-O-diglucoside dominate the phenolic composition of elderberries (**Figure 6**) (204,205,210). Therefore, the anthocyanins of *S. nigra* fruits are cyanidins, catechol-containing compounds.

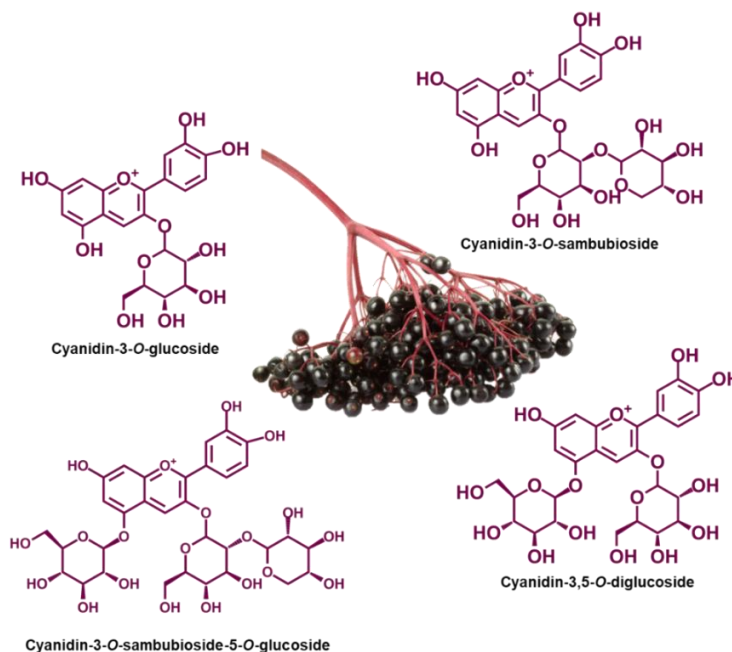


Figure 6. Elderberry *S. nigra* L. fruits and the structure of the main anthocyanins present: cyanidin-3-O-glucoside, cyanidin-3-O-sambubioside, cyanidin-3-O-sambubioside-5-O-glucoside, and cyanidin-3,5-O-diglucoside.

Elderberries have been used in folk medicine since ancient times to treat many diseases, and the reported health promotion is always associated with their phenolic composition, particularly with anthocyanins content (210-214). It was shown that an *S. nigra* anthocyanins-enriched extract, a polyphenols mixture dominated by four anthocyanins (cyanidin-3-O-glucoside, cyanidin-3-O-sambubioside, cyanidin-3-O-sambubioside-5-O-glucoside, and cyanidin-3,5-O-diglucoside) containing significant amounts of quercetin-3-glucoside, has remarkable antioxidant activity, working as broad spectrum free radical scavenger (210). It was also unveiled that this anthocyanins-enriched extract exhibits a reversible redox behaviour within the range of mitochondrial redox chain potentials – a redox feature that can be assigned to elderberry anthocyanins properties. Interestingly, these elderberry anthocyanins' reversible redox behaviour emerges from one electron reaction giving elderberry anthocyanins the potential to be active in the first segment of the mitochondrial redox chain, *i.e.*, NADH/complex I – ubiquinone/complex III. Moreover, in their acidic cationic form, elderberry anthocyanins exhibit a strong affinity for the

mitochondrial membranes, accumulating in both the polar region of the membrane and the hydrophobic core of the mitochondrial lipid bilayer (210). It was also unveiled that the *S. nigra* anthocyanins-enriched extract can overcome complex I inhibition promoted by rotenone, working as a membrane electron carrier that oxidizes NADH and carries the electrons for complex III (210). Thus, elderberries are an excellent renewable resource to obtain extracts rich in mitochondriotropic active compounds, which have a high potential to support a new mitochondria-targeted therapy for degenerative brain diseases, like AD and PD – pathologies with well-documented mitochondrial dysfunction associated with the anomalous activity of mitochondrial complex I.

2.2. *Codium tomentosum* – a resource of membrane polar lipids with self-assemble properties and suitable bioactivity to improve mitochondrial lipidome

Macroalgae are eukaryotic photoautotrophic organisms that use light and water to produce ATP and the reduced coenzymes, the power required to convert the carbon dioxide in biomass, generating oxygen as a by-product of an integrated metabolic process, generically known as photosynthesis (215,216). Currently, algae classification is based on six main criteria: i) nuclear organization; ii) nature of cell wall components; iii) pigmentation and photosynthetic apparatus; iv) nature of reserve food; v) flagellation; and vi) type of life cycle and reproduction. However, the traditional macroalgae classification, supported by their main pigmentation, as green algae (Phylum Chlorophyta), brown algae (Phylum Ochrophyta, Phaeophyceae) and red algae (Phylum Rhodophyta), still in use (215,217). The colour of green algae results from a high chlorophyll content, in general, with equal amounts of chlorophylls *a* and *b*. Concerning the other two types of macroalgae, the colour of brown algae is due to xanthophyll and fucoxanthin pigments. In contrast, the red algae result from phycoerythrin and phycocyanin, pigments that mask the colour effect of chlorophylls (217,218).

Regarding the macroalgae lipid composition, the major phospholipids found are phosphatidylglycerol (PG), phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylinositol (PI) and phosphatidic acid (PA) classes. The anionic PG is the dominant phospholipid class in thylakoid membranes (accounting for 20-40% of phospholipids), where it plays a crucial role in the functional organization of photosynthetic apparatus (219,220). The typical algae glycolipids are the glyceroglycolipids, including monogalactosyldiacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG) and sulfoquinovosyldiacylglycerol (SQDG). Glycolipids are predominantly located in photosynthetic membranes with MGDG and SQDG restricted to the thylakoid membranes

of the chloroplast while DGDG is also found in extraplastidial membranes (219,220). At least three different betaine lipids were reported in algae, namely, 1,2-diacylglycerol-3-O-4'-(*N,N,N*-trimethyl)-homoserine (DGTS), 1,2-diacylglycerol-3-O-2'-(hydroxymethyl)-(*N,N,N*-trimethyl)- β -alanine (DGTA), and 1,2-diacylglycerol-3-O-carboxy-(hydroxymethyl)-choline (DGCC). DGTS is particularly abundant in green algae membranes (from 5.2 to 56.5% of polar lipids), working as a substitute for PC (219-221).

Codium tomentosum (Stackhouse, 1797, **Figure 7**), known in Portugal as “chorão-do-mar”, is an edible green macroalgae of the family Codiaceae included in the phylum Chlorophyta. It is native to the northeast Atlantic Ocean from the British Isles southwards to the Azores and Cape Verde. *C. tomentosum* is continually recorded in the intertidal zone of the Portugal coast (MACOI – Portuguese Seaweeds Website (MARE, University of Coimbra, <http://macoi.ci.uc.pt/>)).



Figure 7. *C. tomentosum* green algae.

These edible green macroalgae are known as reservoirs of bioactive substances that can be used to ensure human health. *C. tomentosum* characterization showed high content of membrane polar lipids with a high relative abundance of polyunsaturated fatty acids (PUFA), including phospholipids, glycolipids, and betaine lipids (**Figure 8**) (222). These green algae have a high lipid content, reporting the presence of glycolipids (MGDG, DGDG, SQDG), phospholipids (mainly PG), and betaine lipids (DGTS, MGTS). The fatty acid profile is composed of saturated species, mainly 14:0, 16:0, 18:0, 22:0, and 24:0 and unsaturated species, namely 18:1, 16:1, 16:2, linoleic acid C18:2n-6 (LA), 18:3n-6, C18:3n-3 (ALA), 20:3n-3, eicosapentaenoic acid 20:5n-3 (EPA) species (222). Regarding the richness of the *C. tomentosum* membranes in betaine lipids and anionic phospholipids,

mainly PG, both with high levels of PUFA, these macroalgae showcase some interesting polar lipids that can be associated with health benefits.

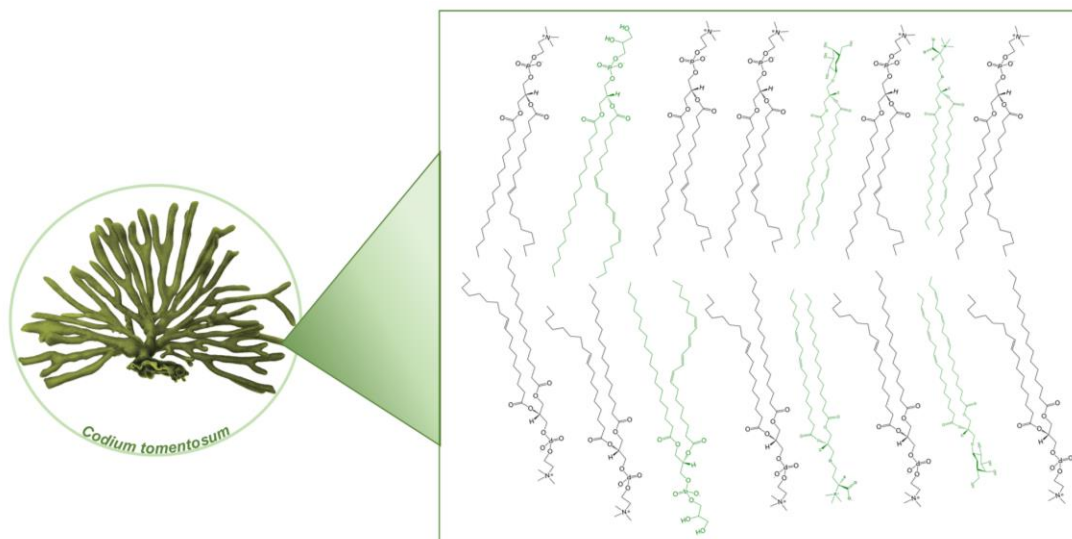


Figure 8. Structure of the common phospholipids, glycolipids and betaine lipids found in thylakoid membranes of *C. tomentosum* algae organized in a lipid bilayer. 1,2-diacylglycerol-3-O-4'(N,N,N-trimethyl)-homoserine (DGTS), monogalactosyldiacylglycerol (MGDG) and phosphatidylglycerol (PG).

The available scientific reports indicate that *C. tomentosum* has a high content of PUFA, mainly n-3 PUFA (222). In the diet is important to maintain an appropriate balance of n-3 and n-6 PUFA as these fatty acids work together to promote health. The n-3 PUFA are recognized by their anti-inflammatory and antioxidant activities, while the n-6 PUFA tend to promote inflammation and tumour growth (223,224). In addition, some n-3 PUFA, especially EPA and docosahexanoic acid C22:6n-3 (DHA), are major components of brain cells and crucial for the development and functioning of the nervous system (224,225). Currently, the diet n-6:n-3 ratio recommended by the WHO should be no higher than 10 to avoid inflammatory, cardiovascular, and nervous system disorders (226,227). Consequently, including this macroalgae in the diet is an excellent way to reduce the n-6:n-3 ratio to values of about 1.0. PG, the primary phospholipid of *C. tomentosum*, is a key phospholipid for mammalian since it is an intermediate in the biosynthesis of several other lipids, including the mitochondrial cardiolipins – an essential lipid for the proper functioning of the enzymes involved in oxidative phosphorylation (228,229). Betaine lipids accumulation depends on transporters, and primarily distributes to the kidneys, liver, and brain (229,230). Betaine's primary physiological function is to act as a methyl group donor in liver metabolism. However, it is utilized in most tissues such as the kidneys and brain as an osmolyte to protect cells under stress (231,232). Thus, betaine lipids are also associated with anti-inflammatory activity with putative benefits in numerous diseases.

Therefore, *C. tomentosum* is a practical resource of membrane polar lipids with self-assembly properties and bioactivity to improve the mitochondrial lipidome. Thus, *C. tomentosum* lipids can address a new mitochondria-targeted therapeutic strategy for degenerative brain diseases. This pool of selected lipids combined with the above-mentioned redox-active molecules has the potential to support a nanoplatform with properties to overcome the different physiological barriers and target brain mitochondria, as will be addressed below.

2.3. Conceptual framework to combine algae lipids and elderberry anthocyanins in a nanoplatform able to support nutraceutical and pharmacological approaches for neurodegeneration

Anthocyanins' antioxidant and anti-inflammatory properties are well-recognized. However, anthocyanins' pharmacological benefits are hindered by their low bioavailability. It is reported that only about 1-2% of the ingested anthocyanins can reach the target tissues/organs (180,233). Several factors explain the apparent low bioavailability, including their active metabolization by gastrointestinal microbiota, the chemical reaction associated with pH fluctuations and liver metabolism (180,233). The gastrointestinal tract has compartments with different pH values and specific enzymes; thereby, anthocyanins from the oral cavity to the small intestine are subjected to transformations by chemical and/or enzyme-catalysing reactions (180,234). In the stomach, the acidic environment favours the stability of anthocyanins in cationic form, reducing anthocyanins' degradation (180,233,235,236). Thus, some of the ingested anthocyanins reach the large intestine, where the alkaline pH favours their metabolization with consequent excretion in the faeces. Additionally, anthocyanins undergo phase 2 enzymatic metabolism in the liver, transforming into other metabolites (180,233,237,238). Another reason sometimes associated with the low bioavailability of anthocyanins is the positive or negative synergistic interactions between them and food-coexisting compounds. Compounds in the same food matrix of the anthocyanins can compete for specialized transporters or directly affect their stability with an impact on absorption and bioavailability (180,233,239,240).

Encapsulation has been recognized as a valuable approach to overcoming the "Achilles' heel" of anthocyanins (*i.e.*, low bioavailability) (180,241). Several formulation strategies have been reported to improve anthocyanins' bioavailability and therapeutic properties by increasing stability in the gastrointestinal tract and enhancing the competence to cross the blood-brain barrier. The lipid-based nanocarriers are promising tools to protect anthocyanins from degradation in the gastrointestinal tract, prevent their interaction with

other food constituents and enhance their potential to cross physiological barriers, including the blood-brain barrier (241-243). The health-related benefits of the encapsulation of anthocyanins in lipid-based nanocarriers and the stability and behaviour of the different types of nanoformulation produced are reported in many works (241,244-247). However, in all these works, lipids are selected to support the vehicle with the ability to carry the active compounds – anthocyanins.

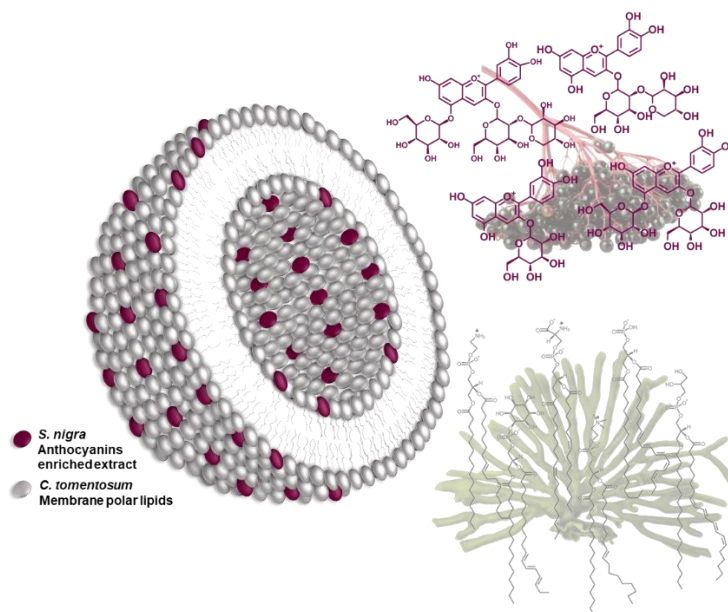


Figure 9. Possible design of a lipid-based nanocarrier built with *C. tomentosum* membrane polar lipids and *S. nigra* elderberry anthocyanins enriched extract.

A novel lipid-based nanocarrier (nanophytosomes) has been developed by taking advantage of this knowledge, utilizing the synergistic effects of lipids and anthocyanins to modulate mitochondrial functionality. Thus, *C. tomentosum* membrane lipids were selected since they have a high relative abundance of PUFA, n-3/n-6 PUFA ratio higher than 1, and anionic phospholipids, mainly PG. The self-assemble bilayer properties of this set of membrane polar lipids, mainly from chloroplast-rich tissues of green algae, may allow preparing nanocarriers with competence to preserve elderberry anthocyanins in their mitochondriotropic cationic form, mainly as a result of interactions with anionic phospholipids (**Figure 9**). In target tissues (e.g., brain), these nanocarriers may provide mitochondria not only with anthocyanins but also with lipids with the competence to improve the functional organization of the inner membrane. The elevated levels of PG in the nanocarrier could promote the synthesis of mitochondrial cardiolipin, while the abundant presence of PUFA may enhance cardiolipin remodeling, resulting in the production of mature cardiolipin species that predominate the lipid profile of brain mitochondria. (228,248).

3. The challenge of neurodegenerative diseases, working hypothesis and objectives

Degenerative brain diseases are devastating the lives of millions of people, perturbing progressive and irreversibly the brain functions, including memory, cognitive, behavioural, and motor coordination skills. Currently, 45 million people worldwide suffer from AD and more than 10 million from PD without hope since the available medicines cannot break the progressive brain degeneration. Thus, developing effective medicine to break or delay these neurodegenerative diseases is one greatest challenge of our time. The neurodegeneration is a very complex subject since many diseases-related symptoms emerge from a dysfunctional organization of the human brain that remains beyond our current understanding. Despite this, there are some questions that can be used to drive the research towards an effective therapy, some which are rationalized below.

What is the relationship among the common subcellular pathological hallmarks of degenerative brain diseases (e.g., mitochondrial dysfunction, oxidative stress, accumulation of misfolded proteins, increased levels of pro-inflammatory mediators) and the deficient brain connectivity detected by disease-specific pattern of clinical symptoms?

Are the well-known subcellular pathological events cause or consequence of the dysfunctional brain connectivity?

Can the same interlinked subcellular pathological events in different brain regions trigger the main degenerative brain diseases?

Different visions on the questions above can lead to a several hypotheses for neurodegenerative diseases, consequently different therapeutic approaches can be proposed. Thus, this work was developed considering that:

- i) the neuronal networks functionality (brain connectivity) is highly dependent on the mitochondria performance of the brain cells;
- ii) the dysfunctional organization of the inner mitochondrial membrane, characterized by an impairment of the mitochondrial redox chain, leads to many other subcellular pathological hallmarks, which together trigger the neurodegenerative cascade underlying neurodegenerative diseases, including AD and PD; and
- iii) the inner mitochondrial membrane emerges as the suitable therapeutic target to address the neurodegeneration.

3.1. Research objectives

The main goal of the present project is to develop an affordable therapeutic strategy to interrupt and/or delay the pathological cascade underlying the degenerative brain diseases by targeting the inner mitochondrial membrane, *i.e.*, a mitochondria-targeted therapy. The therapeutic approach is based on a cutting-edge nanophytosomes formulation designed: i) to overcome the mitochondrial dysfunction, by improving the respiratory chain functionally and tuning mitochondrial lipidome; ii) to avoid oxidative stress and to adjust the redox-dependent signalling pathways, by modulating of cytosolic redox status of cells.

Thus, the specific objectives are:

1. To develop scalable methodologies to obtain from *C. tomentosum* a specific pool of membrane polar lipids and from *S. nigra* fruits an anthocyanin-rich extract, fulfilling the requirements for biomedical and food applications;

2. To develop and characterize the physical-chemical properties of SC-Nanophytosomes formulation (*e.g.*, size, shape, surface charge, stability) with competence to overcome cell barriers, and to target mitochondria without cellular toxic effects, using different cellular models;

3. To evaluate the ability of the SC-Nanophytosomes formulation to overcome complex I-related mitochondrial dysfunction, repair mitochondrial lipidome, adjust cell redox state, and protects cells against toxic stimuli with relevance in neurodegeneration context;

4. To test *in vivo* the ability of the SC-Nanophytosomes formulation, delivered by drinking water, to disrupt the pathophysiological cascade that underlie the brain degenerative processes, using a rotenone-induced rat model of PD. The therapeutic efficacy will be evaluated during the treatment by the benefits on the motor symptoms, and in final of treatment by the effects on the main sub-cellular pathological hallmarks of PD assessed at level of brain and skeletal muscles tissues.

CHAPTER II

Experimental section

1. Elderberry anthocyanins-enriched extract preparation

Fresh mature fruits (ca. 1 kg) were obtained from five plants of the European elderberry (*S. nigra* L.) in September 2019 in northern Portugal (40°46'34"N, 7°3'46"W), and stored in plastic bags, at -20 °C. Elderberries (ca. 100 g) were extracted with water (300 mL) in a light maceration process for 4 h, under magnetic stirring, 200 rpm, at 40 °C. Then the seeds were separated from the fruit pomace by gravimetric filtration. After centrifugation at 2900 x *g* for 10 min., the resulting aqueous extract was filtered under reduced pressure and freeze-dried (210). Then, the dried extract was dispensed by screw flasks, sealed under a nitrogen atmosphere, protected from light, and stored in a desiccator. The elderberry anthocyanin-enriched extract (EAE-extract) was obtained by the selective adsorption of the polyphenols from elderberry aqueous extract to polyvinylpolypyrrolidone (PVPP), as previously reported (148). The polyphenols (mainly anthocyanins) adsorbed to PVPP were extracted with 250 mL of alkaline ethanol solution (ethanol: 100 mM of aqueous ammonia solution, 90:10, v/v) by shaking for 30 min. The extract was filtrated in a Buchner filter (Whatman no. 1 filter paper). The remaining PVPP in the Buchner filter was reextracted with 250 mL of fresh extraction solvent. The combined extracts were neutralized with an aqueous formic acid solution and evaporated at room temperature by rotary evaporation to remove and recover the ethanol. The resulting aqueous solution was freeze-dried under vacuum at -50 °C to remove both formic acid and ammonia, as volatile ammonium formate, and to obtain a final dried EAE-extract, which was dispensed by screw flasks, sealed under nitrogen atmosphere, protected from the light, and stored in a desiccator until to be used.

2. EAE-extract characterization by high-performance liquid chromatography with a diode array detector

The phenolic profile of the EAE-extract was assessed by high-performance liquid chromatography with a diode array detector (HPLC-DAD, Gilson Medical Electronics, Villiers le Bel, France) according to a previously validated method (249). For analytical purposes, the extract was re-dissolved in HPLC-grade water acidified with formic acid (pH 2.0) and filtered by a 0.22 µm pore membrane. 20 µL of the extract was injected into a Spherisorb ODS2 column, with a C18 ODS guard column, at a flow rate of 0.8 mL/min. The mobile phase consisted of water:formic acid:acetonitrile (87:10:3, v/v/v; eluent A) and water:formic acid: acetonitrile (40:10:50, v/v/v; eluent B), using a gradient that increases the eluent B from 10% to 25% after 10 min., 31% B at 15 min., 40% B at 20 min., and 50% B at 25 min. Chromatograms were recorded at 280, 320, 350, and 525 nm. Phenolic compounds

were identified by comparing their retention times and UV spectra (200 – 600 nm) with reference compounds running in the same conditions. Quantification in μg of compound/mg of dry weight EAE-extract was achieved by using the external calibration curves obtained with appropriated reference compounds, namely: *p*-hydroxybenzoic acid (peak 1), pelargonidin-3,5-*O*-diglucoside (peak 2), cyanidin-3-*O*-sambubioside (peak 3), cyanidin-3-*O*-glucoside (peak 4), and quercetin-3-*O*-glucoside (peak 5).

3. Extraction of the membrane polar lipids from *C. tomentosum*

Individuals of *C. tomentosum* were harvested in October 2019 at the beach of Aguda, Vila Nova de Gaia (41°02'58.9"N, 8°39'20.4"W) and transported to the laboratory in seawater. The material was thoroughly rinsed with water to remove traces of epiphytes visible, frozen, and freeze-dried (VirTis SP Scientific Sentry 2.0 lyophilizer). The dried material was stored at room temperature in a desiccator, protected from light. The lyophilized algae were sprayed on a sliding mill (Retsch, Grindomix 200) before being subjected to the extraction process. Algae membrane polar lipids were obtained following the flowchart depicted in **Figure 10**, using ethanol and acetone, EU approval solvents for food applications, combining ultrasound-assisted extraction with a lipid extraction procedure that takes advantage of the effect caused by the temperature drop to 4 °C or -18 °C on the solubility of different lipid classes in ethanol and acetone, respectively (250). Briefly, 10 g of dried algae were extracted with 200 mL of ethanol under ultrasound agitation for 30 min. followed by shaking for 60 min. The extract was filtrated in a Buchner filter (Whatman no. 1 filter paper). The mixture was centrifugated at 2500 x *g* for 5 min., the green supernatant containing extractable lipids and chlorophylls was collected, and the pellet was re-extracted with the abovementioned conditions. Both supernatants were combined in a glass flask, and the ethanol was evaporated under reduced pressure in the rotary evaporator (Rotavapor R-215 Buchi) until to obtain a solution of extractable lipids of 1 g algae/3 mL ethanol. This concentrated solution was incubated overnight at 4 °C to promote the crystallization/precipitation of the non-polar lipids (e.g., triacylglycerols) and then centrifugated (2500 x *g*, 5 min.) to obtain a clean solution of polar lipids in the supernatant. Afterwards, cooled acetone at -18 °C was added to supernatant (4 mL of acetone /1 mL of ethanol solution) to precipitate the polar membrane lipids (mainly phospholipids and betaine lipids), free of chlorophylls that remain soluble in cooled acetone. Membrane polar lipids, collected as a pellet by centrifugation, were dried by nitrogen pulse and stored at -18 °C.

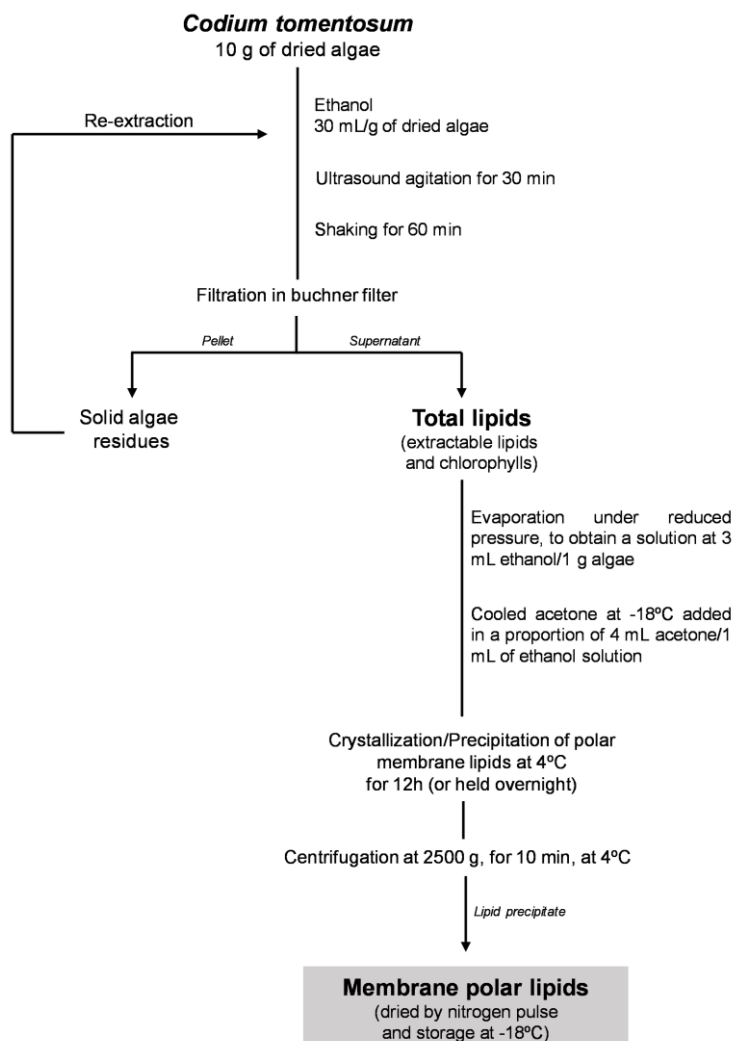


Figure 10 - Flow diagram to obtain a membrane polar lipid fraction from *C. tomentosum*, using ethanol and acetone as extractants.

4. Characterization of the membrane polar lipids from *C. tomentosum*

4.1. Phosphate quantification by the phosphorus method

Total phospholipid content in membrane polar lipid extract was quantified by the inorganic phosphate released after acid hydrolysis of the phospholipids. According to this method, the inorganic phosphate is converted into a blue-coloured phosphomolybdate complex (**Figure 11**) and quantified by spectrophotometry in a microplate reader (Synergy H1 Biotek) (98). Samples of membrane polar lipid extracts (300 μ L) dissolved in ethanol were transferred to glass tubes, and the solvent was evaporated under a stream of nitrogen. Simultaneously, standards from KH_2PO_4 aqueous solution (0, 5, 10, 25, 50, 100, and 250 nmol of phosphate) were transferred from glass tubes, and the solvent was evaporated in a Bunsen burner flame. The hydrolysis of the phospholipids was carried out with the addition

of 0.65 mL of 70 % perchloric acid and then the mixtures were incubated for 2 h at 180 °C. After cooling to room temperature, 3.3 mL of water, 0.5 mL of ascorbic acid (10 g/100 mL), and 0.5 mL of ammonium molybdate (2.5 g/100 mL) were added to each sample. The mixture was shaken and incubated for 5 min in a 100 °C water bath. The tubes were cooled to room temperature and samples' absorbance and standards were read at 800 nm on a microplate reader. The concentration of inorganic phosphate in the samples was calculated from the standard curve of KH_2PO_4 and the results were expressed in nmol/mg protein.

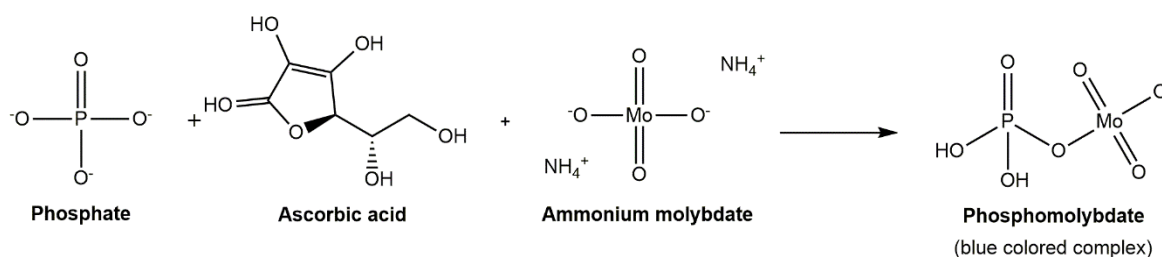


Figure 11 – Phosphorus assay to phosphate quantification.

4.2. Preparation of fatty acid methyl esters

The lipid extracts were submitted to transmethylation of fatty acids for further analysis by gas chromatography/flame ionization detector (GC-FID) and gas chromatography/mass spectrometer (CG-MS) (251). The extract volume containing 500 nmol of phospholipid and 30 nmol of saturated fatty acid C17:0, was inserted into screw tubes, and the solvent was evaporated to dryness with a stream of nitrogen. 5 mL of methylation solution (5 % (w/v) hydrochloric acid in methanol) was added to each tube containing the lipid residue. The solution in each tube was capped and incubated for 2 h in a 70 °C bath. After cooling to room temperature, the methyl esters were extracted by adding 5 mL of hexane, vortexed for 3 min, and centrifuged at 200 x g for 5 min. The upper phase (hexane) was collected, and the lower phase was re-extracted with 5 mL of hexane to increase the extraction yield. After shaking, it was centrifuged, and the upper phase was removed again. After the two hexane phases were brought together, approximately 1 g of anhydrous sodium sulfate was added to remove the water. The hexane extracts were filtered through Whatman no. 1 filter paper into a flask, and the solvent evaporated under reduced pressure on a rotary evaporator under a nitrogen atmosphere. The methyl esters were resuspended in about 200 µL of hexane and preserved at -20 °C for further use.

4.3. Profiling of the methyl esters of fatty acyl chains by GC-FID and GC-MS

The fatty acid methyl esters (FAME) profiling of the membrane polar lipids was analyzed by GC-MS (Thermo-Finningan-Trace Gas Chromatograph connected to the mass spectrometer Polaris Q MSn equipped with an Ion Trap analyzer and the column was a Supelcowax 10 M (30 m, 0.32 mm I.D., 0.50 mm film thickness, 0.45 mm O.D.), Supelco, Bellefonte, Pennsylvania, USA) for identification (252) and analyzed by GC-FID (Finnigan Focus GC apparatus, Thermo Fisher Scientific, Waltham, MA, USA equipped with a flame ionization detector and a VF-5 ms column 30 m×0.25 mm×0.25 µm, Varian BV, Middelburg, TheNetherlands) for quantification (250).

GC-MS injections were made in splitless mode with an injection volume of 1 µL. The column oven temperature was settled at an initial value of 140 °C (1 min hold), increased to 240 °C with a ramp of 2.0 °C/minutes (9 minutes hold), and finally increased to 280 °C with a ramp of 4.0 °C/minute. The total runtime was 70 minutes using Helium as the carrier gas, at a constant 1 mL/minute flow. The injector temperature was set at 250 °C. Data acquisition and results treatment were performed using an Xcalibur data system (V2.0, ThermoFinnigan, San Jose, CA, USA). Fatty acids were identified by comparison with retention time and fragmentation profile of standard reference mixtures FAME 37 (Supelco 37 Component FAME Mix). For GC-FID the injection volume was 1µL (derivatized lipid extract containing the IS, and FAME mixture). The carrier was high-purity helium C-60 (Gasin, Portugal) at a constant 1mL/min flow rate. Temperatures of the injector and detector were maintained at 250 °C. The oven temperature was set at 40 °C for 1min, then increased to 5 °C/min to 250 °C, 3 °C/min to reach 300 °C and held for 15 min. The compounds were identified by comparing their retention times with the FAME mixture, and their contents were calculated as a relative % of the internal standard C17:0. The relative abundance of betaine lipids was estimated by considering the total fatty acids amount and the predicted amount released from the phospholipids quantified as indicated above.

4.4. Separation of phospholipid classes by thin-layer chromatography

Membrane polar lipids were separated in thin-layer chromatography (TLC) on a silica plate to assess the relative abundance of each phospholipid class (252). Before separation, plates were washed with chloroform/methanol (1:1, v/v) and treated with boric acid 2.3% in ethanol. The plates with the spots were developed in the solvent mixture chloroform: ethanol: water: triethylamine (35:30:7:35, v/v/v/v). Lipid spots on TLC were detected at 365 nm, after revelation with primulin (50 µg/100 mL in acetone: water (80:20,

v/v)). The spots were scraped individually to extract and quantify the phospholipids using the phosphorus assay.

5. SC-Nanophytosomes preparation

SC-Nanophytosomes were prepared with EAE-extract and *C. tomentosum* membrane polar lipids, using a procedure to preserve anthocyanins in their flavylium cation form. *C. tomentosum* membrane polar lipids (600 μM in phospholipids) and 0.5 mg/mL of EAE-extract were dissolved in 5 mL of ethanol: methanol (1:1, v/v) in a round-bottom flask. Then the solvent was evaporated to dryness in a rotatory evaporator. The resulting thin lipid film in the inner wall of the flask was hydrated with a 5 mL volume of buffer solution (50 mM KCl, 10 mM HEPES, 2 mM citric acid, pH 6.4), previously heated at 37 °C. Then, the mixture was subjected to sonication cycles (2 min.) alternate with handshaking (2 min.) for 20 min., using a water bath sonicator (Bandelin Sonorex Digitec DT 100 H, Berlin, Germany, 80/320 W, 35 kHz), at 37 °C, to obtain SC-Nanophytosomes formulation. (**Figure 12**).

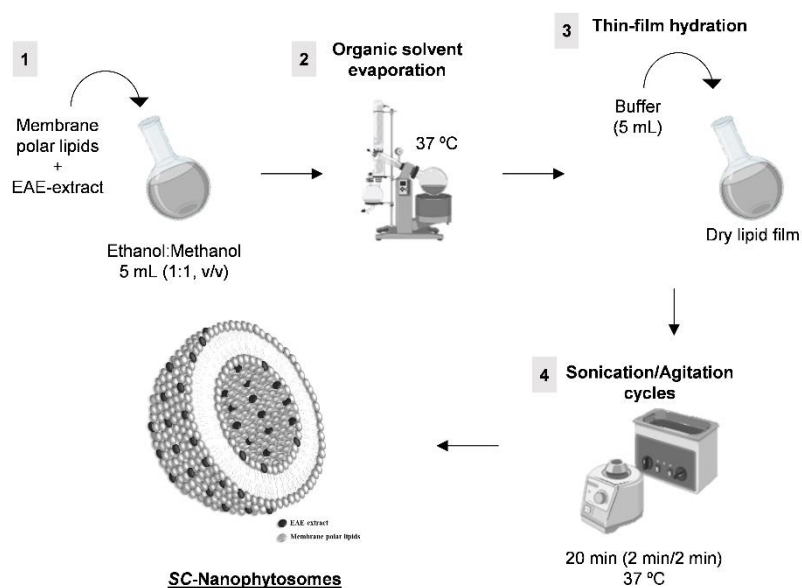


Figure 12 - Outline of the protocol for preparation of SC-Nanophytosomes with the membrane polar lipids and the EAE-extract.

6. SC-Nanophytosomes characterization

6.1. Assessment of the EAE-extract entrapment efficiency

1 mL of the formulation was collected to assess elderberry anthocyanins' entrapment efficiency (EE) into the SC-Nanophytosomes. The other 4 mL was submitted to

dialysis through a cellulose membrane (cut-off of 14 kDa Sigma-Aldrich) against 100 mL of aqueous buffer solution (50 mM NaCl, 10 mM HEPES, 2 mM citric acid, pH 6.4) to remove the non-encapsulated polyphenols. For sterilization purposes, dialysed SC-Nanophytosomes formulation was filtered through a sterile filter with 0.22 µm pore size, an aliquot was collected for anthocyanins EE assessment, and the remaining suspension was distributed by three sterilized vials that were stored (4 °C) until used. UV-Visible spectra of non-dialysed and dialysed SC-Nanophytosomes were recorded in the range of 250 - 700 nm with 1 nm of resolution using a microplate reader (Thermo Spectronic Unicam Helios Alpha UV-Visible spectrophotometer). Nanoliposomes prepared under the same conditions with the same algae lipid concentration without EAE-extract were used for background correction. The values of the absorption at 520 nm (typical of anthocyanins in cationic form) of the corrected spectra were used to determine the EE, according to the equation: EE (%) = $(\text{Abs}_{\text{dialysed SC-Nanophytosomes}} / \text{Abs}_{\text{non-dialysed SC-Nanophytosomes}}) \times 100$.

6.2. Vesicle's shape, size, and Zeta potential

All samples were diluted in HEPES buffer (1:4) to yield a suitable scattering intensity (count rate between 300 and 500 kcps). Vesicle's size, size distribution, and polydispersity index (PDI) were evaluated by dynamic light scattering (DLS) using a helium-neon laser wavelength of 658 nm and a detector angle of 90° (Brookhaven Instruments, NY, USA). Individual running time was 120 s, and 6 runs per measurement were performed.

Surface charge, assessed in terms of Zeta potential, was evaluated by electrophoretic light scattering (ELS) using the ZetaPALS instrument (Brookhaven Instruments, NY, USA). Data were recorded from six runs (10 cycles per run) with a relative residual value (a measure of data fit quality) of 0.03.

SC-Nanophytosomes formulations were also morphologically characterized by transmission electron microscopy (TEM) after negative staining with uranyl acetate (253). For this purpose, 1 µL of 2 % uranyl acetate solution was added to 9 µL of SC-Nanophytosomes aqueous suspension, and the mixture was gently agitated for 1 min. Then, the stained SC-Nanophytosomes suspension was loaded into Formvar-carbon coated grids (TAAB Laboratories, Aldermaston, England) using a 0-2 µL micropipette under binocular control. The grids were air-dried, and the adsorbed vesicles were examined in a Tecnai G2 Spirit Biotwin transmission electron microscope (FEI, Hillsboro, NE, USA) equipped with MegaView III-SIS CCD camera operated at 100 kV.

7. In vitro assays

7.1. Cell culture conditions and treatments

Neuroblastoma SH-SY5Y cells (ATCC, Barcelona, Spain) were cultured in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F12) supplemented with fetal bovine serum (FBS, 10 %) and penicillin-streptomycin (Pen-Strep, 1 %), at 37 °C, in a humidified incubator with 5 % carbon dioxide (Toreuse model 2428; Saint Louis, Missouri, USA). Normal human fibroblast BJ cells were cultured in Minimum Essential Medium (MEM) supplemented with FBS (10 %) and Pen-Strep (1 %) in the same conditions as SH-SY5Y cells. Microglial mouse BV-2 cells were cultured in DMEM supplemented with FBS (10 %) and Pen-Strep (1 %) in the same conditions described above.

7.2. Cell viability

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was chosen to assess SH-SY5Y, BJ, and BV-2 cells viability (254). SH-SY5Y cells were seeded in 96-well microplates at 30.000 cells/well, BJ cells at 6.000 cells/well and BV-2 at 40.000 cells/well. After 24 h, the cells were treated with serial dilutions of the SC-Nanophytosomes formulations (0-30 μ M PL/0-25 μ g mL⁻¹ EAE-extract) or vehicle for 22 h and then co-incubated with 3 μ M of rotenone or with 25 mM of glutamate for an additional 2 h. The assays were performed 24 h after SC-Nanophytosomes addition, preserving the total cell incubation time after seeding. The culture medium was then removed, and SH-SY5Y and BJ cells were incubated for 2 h, at 37 °C, with 0.5 mg/mL of MTT solution (in culture medium), while BV-2 cells were incubated for 1 h, at 37 °C with the MTT solution.

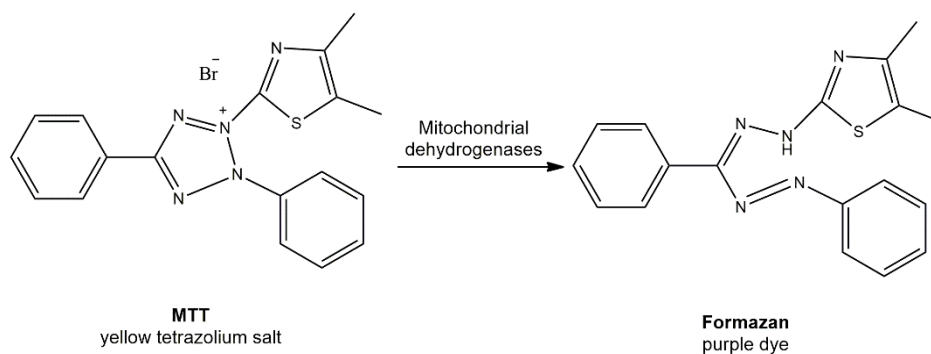


Figure 13 – Reduction of MTT to formazan, by mitochondrial dehydrogenases of metabolically active cells.

Mitochondrial dehydrogenases of metabolically active cells reduce MTT to form an insoluble purple formazan (**Figure 13**). The formazan crystals were dissolved in 200 μ L of

a dimethyl sulfoxide: isopropanol (3:1, v/v) mixture and the extent of MTT reduction by the cells was quantified at 560 nm on a Multiskan GO plate reader (Thermo Fisher Scientific; Waltham, MA, USA). Results were expressed as a percentage of MTT reduction of treated cells relative to control. Three independent assays were performed in triplicate.

7.3. Cell membrane integrity

SH-SY5Y cell membrane integrity was assessed by the lactate dehydrogenase (LDH) release assay (254). SH-SY5Y cells were seeded in 96-well microplates at a density of 30.000 cells/well and treated with serial dilutions of the SC-Nanophytosomes formulations (0-30 μM PL/0-25 $\mu\text{g mL}^{-1}$ EAE-extract) for 24h. The reaction used using 50 μL of cell-free extracellular medium, supplemented with 10 μM pyruvate and 300 μM of NADH. LDH activity was evaluated spectrophotometrically following NADH oxidation (**Figure 14**) at 340 nm for 3 min, using a Multiskan GO plate reader (Thermo Fisher Scientific; Waltham, MA, USA). LDH release in each well was determined by the ratio between LDH activity in the extracellular medium and LDH activity of controls treated with lysis solution (1% Triton X-100). Results were expressed as a percentage of LDH release.

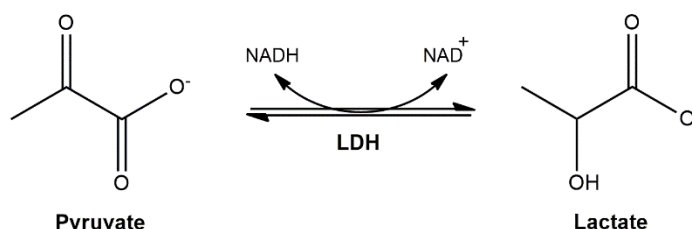


Figure 14 – Reaction catalyzed by lactate dehydrogenase (LDH) following NADH oxidation.

7.4. Nitric oxide determination

The capacity of SC-Nanophytosomes to modulate anti-inflammatory activity-dependent on the iNOS cascade was evaluated by measuring the levels of nitric oxide (NO) produced by BV-2 cells without and with interferon-gamma (IFN- γ) stimulation. The amount of NO released by cells was assessed according to the Griess assay (**Figure 15**) (255). Cells were seeded in 24-well plates (160.000 cells/well) for 24 h (37 $^{\circ}\text{C}$, 5 % CO_2) and then pre-treated with SC-Nanophytosomes (0-30 μM PL/0-25 $\mu\text{g mL}^{-1}$ EAE-extract) or with EAE-extract (12.5-50 $\mu\text{g mL}^{-1}$) for 2h. Then, cells were stimulated with IFN- γ (10 ng mL^{-1}), followed by an incubation period of 22 h (37 $^{\circ}\text{C}$, 5 % CO_2). Afterwards, 85 μL of cell culture medium was collected from each well and mixed with an equal volume of Griess reagent, the mixture was incubated for 10 min in the dark, and the absorbance was measured at 540

nm. The effect of both SC-Nanophytosomes and EAE-extract on NO levels produced by non- and stimulated cells are expressed as % of control, which correspond to NO levels produced by the cell in the absence of IFN- γ stimulation (*i.e.*, basal levels).

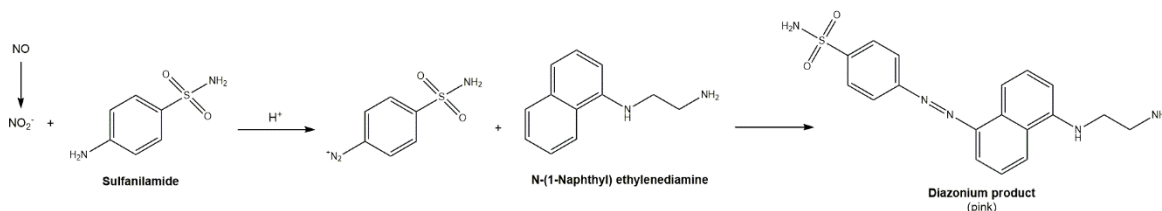


Figure 15 – Schematic representation of the reactions underlying NO assay by using Griess reagent.

7.5. Mitochondrial membrane potential by using JC-1 fluorescent probe

$\Delta\Psi_m$ was evaluated, in intact cells, by using JC-1 fluorescent probe (254). SH-SY5Y, BJ and BV-2 cells were seeded in 96-well black plates at a density of 30.000 cells/well, 6.000 cells/well, and 40.000 cells/well, respectively. Cells in the standard conditions (control) and pre-treated with SC-Nanophytosomes (concentrations indicated in figures) for 24 h were incubated with JC-1 (7 μ M) for 30 min. Afterwards, the medium was discarded, and cells were washed three times with Hanks' Balanced Salt Solution (HBSS). The microplate, with cells in HBSS, was transferred to a fluorescence microplate reader (Synergy H1 Biotek) and used to assess the time-dependent effects of different mitochondria active compounds (25 μ M rotenone, 25 μ M antimycin, 2 mM potassium cyanide (KCN), 50 μ M carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone (FCCP)) on the $\Delta\Psi_m$ of both control and SC-Nanophytosomes pre-treated cells. For $\Delta\Psi_m$ quantification, the plate was read every five minutes for 30 min. with excitation/emission settings at 485/535 and 560/595 nm to assess probe fluorescence as monomer and aggregates, respectively. The ratio of JC-1 aggregates to monomer (the 695:535 ratio) was used as a measure of the $\Delta\Psi_m$, and a decrease in this ratio was interpreted as $\Delta\Psi_m$ depolarization.

7.6. Reactive oxygen species generation

SH-SY5Y and BV-2 cells were seeded in black 96-well plates for fluorescence-based assays at 30.000 cells/well and 40.000 cells/well, respectively. The cells were treated with serial dilutions of the SC-Nanophytosomes formulations (0-30 μ M PL/0-25 μ g mL⁻¹ EAE-extract) or vehicle for 22 h and then co-incubated with 3 μ M of rotenone or with 25 mM of glutamate for an additional 2 h. In this way, assays were performed 24 h after SC-

Nanophytosomes addition, preserving the total cell incubation time after seeding. 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) passively diffuses into cells, where its acetate groups are cleaved by intracellular esterase enzymes, and its subsequent oxidation yields a fluorescent adduct 2',7'-dichlorofluorescein (DCF) (**Figure 16**). The medium was removed for ROS quantification, and cells were washed with HBSS before adding DCFH-DA (10 μ M) in HBSS. Afterwards, cells were incubated for 30 min at 37 °C, and the fluorescence was recorded at 520 nm (under excitation at 490 nm) in a microplate reader Synergy H1 Biotek. The results were expressed as % of control (210).

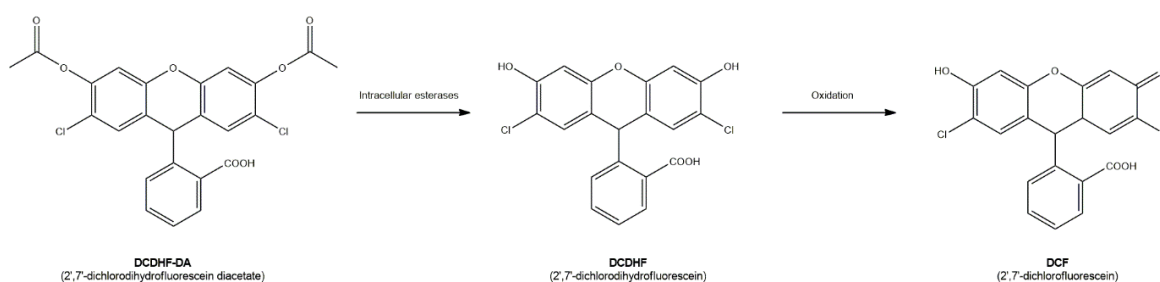


Figure 16 - 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) passively diffuses into cells, where its acetate groups are cleaved by intracellular esterases and its thiol-reactive chloromethyl group reacts with intracellular glutathione and other thiols. Subsequent oxidation yields a fluorescent adduct 2',7'-dichlorofluorescein (DCF).

7.7. Cellular uptake determination and evaluation of the underlying mechanism using fluorescence microscopy

To assess the cytoplasmic internalization of the SC-Nanophytosomes were used the SH-SY5Y, BJ and BV-2 cells. SC-Nanophytosomes were labelled with the fluorescent lipophilic membrane probe 16-(9-anthroyloxy)-palmitic acid (16-AP), which exhibits green fluorescence under ultra-violet light excitation when incorporated in the hydrophobic core of lipid bilayer (256). 2 μ L of the 16-AP probe, 3 mM in dimethylformamide, were injected into the 2 mL of SC-Nanophytosomes suspensions (300 μ M in phospholipids) to give a phospholipid/probe molar ratio of about 300/1. The mixture was incubated, in the dark, under magnetic stirring, at 37 °C, for 60 min., a procedure that ensures the random and complete incorporation of the fluorescent probe into lipid bilayers. Then we assessed the fluorescence emission spectra of the labelled SC-Nanophytosomes as a function of lipid concentrations, considering the concentrations used in cell assays. The fluorescence emission spectra of green-fluorescent labelled SC-Nanophytosomes treated cells were recorded in the range 395 - 650 nm, setting the excitation wavelength at 365 nm, using a microplate reader Synergy H1 Biotek. The spectra of non-labelled SC-Nanophytosomes (without 16-AP probe) with similar concentrations were recorded and used for background correction. Cells seeded in black 6-well plates were cultivated for 24 h and then treated with

increasing concentrations of the green-fluorescent labelled SC-Nanophytosomes for 2 h. Afterwards, the culture medium was removed, and cells were washed three times with HBSS before being used to assess the fluorescence retained by cells. The untreated (control) and treated cells' fluorescence emission spectra were recorded in the 395 - 650 nm range, setting the excitation wavelength at 365 nm, using a microplate reader (Synergy H1 Biotek). The maxima of the fluorescence emission spectra were used to calculate the % of cellular uptake. To evaluate the mechanism responsible for cellular uptake, cells were pre-incubated with amiloride (50 μ M), methyl- β -cyclodextrin (M β CD, 50 μ M) or sucrose (0.45 M), inhibitors of the main endocytosis pathways, for 30 min., and then co-incubated with the green-fluorescent labelled SC-Nanophytosomes for an additional 2 h before used to assess the % of cellular uptake.

Cellular uptake of the green-fluorescent labelled SC-Nanophytosomes was also assessed by fluorescence microscopy. For these assays, SH-SY5Y and BJ cells were seeded in glass coverslips with 30.000 cells/well and 60.000 cells/well, respectively, and treated with SC-Nanophytosomes. Before fluorescence microscopy examination, SH-SY5Y cells were incubated with Hoechst 33342 labelling solution for 15 min. at 37 °C to stain the cell nuclei. The Hoechst solution was removed, cells were washed three times with HBSS and then analyzed by fluorescence microscopy.

BJ cells treated with SC-Nanophytosomes were also incubated with Mito Red labelling solution for 30 min. at 37 °C to stain the mitochondria, washed with HBSS and incubated, for 15 min., with Hoechst dye to stain the nuclei. Then, cells were washed three times with HBSS and analyzed by fluorescence microscopy.

7.8. Cell fractionation – mitochondria-free cytosolic fraction and mitochondria-rich fraction

SH-SY5Y and BV-2 cells were seeded in T-25 flasks to obtain, 48 h after incubation, a cell number suitable for experiments (about 1.5×10^6 cells/flask). After 24 h after incubation, cells were treated with 7.5 μ M PL/6.25 μ g mL⁻¹ EAE-extract of SC-Nanophytosomes or vehicle (buffer used for dialysis) and re-incubated for 24 h. To stop cell metabolism, the cell culture medium was removed, washed with HBSS and the cells in the flask are frozen in liquid nitrogen and stored at -80 °C until to be used. To stop cell metabolism, the cell culture medium is removed, washed with HBSS, and the cells in the flask are frozen in liquid nitrogen and stored at -80 °C until used.

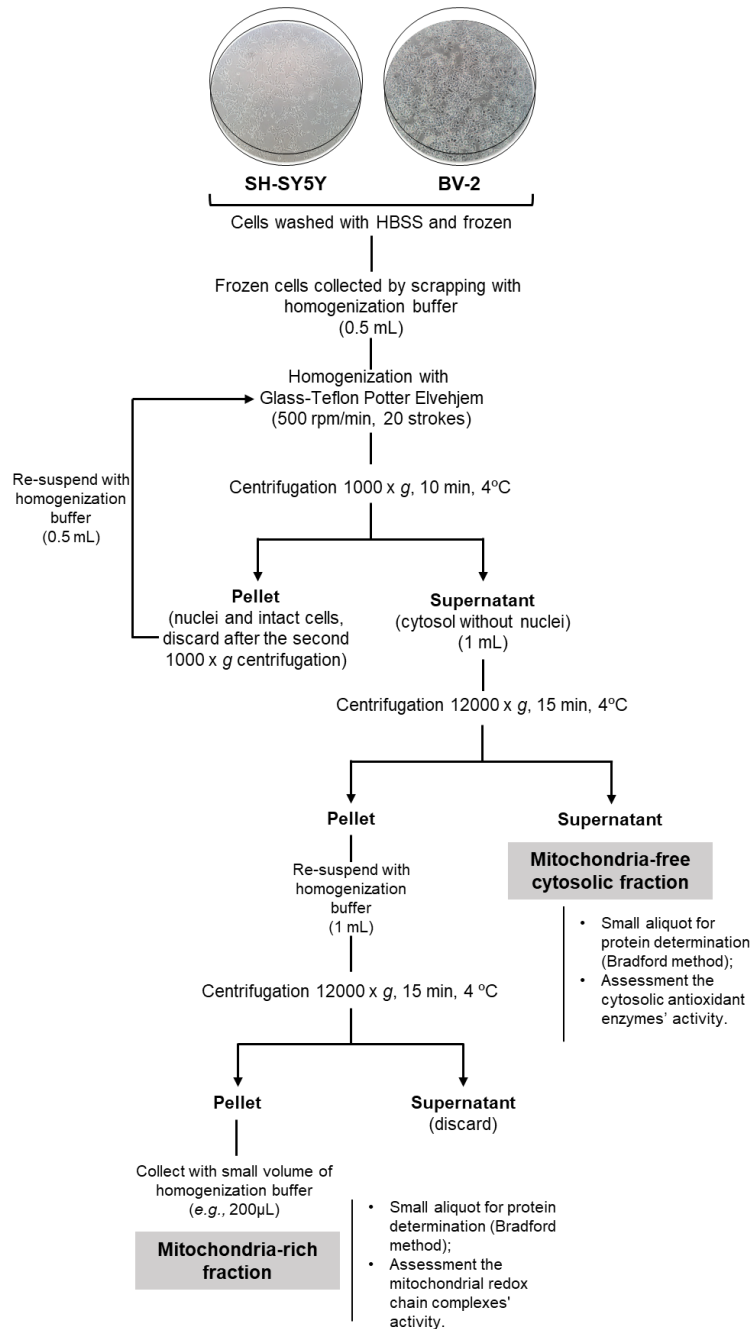


Figure 17 - Flow diagram representing the fractionation of cell pellet in mitochondria-free cytosolic and mitochondria-rich fractions on cell cultures (SH-SY5Y and BV-2 cells) by differential centrifugation.

The frozen adherent cells are collected from the flasks by scraping after adding 0.5 mL of homogenization buffer (Sucrose 130 mM, KCl 50 mM, MgCl₂ 5 mM, KH₂PO₄ 5 mM, HEPES 5 mM, pH 7.4) at 4 °C, and homogenized by using a Glass-Teflon Potter Elvehjem using 20 up- and downstrokes at 500 rpm. The mitochondria-free cytosolic and mitochondria-rich fractions are obtained by sequential differential centrifugation steps, as displayed in **Figure 17**, using standard procedures (210). Typically, about 1 mL of mitochondria-free cytosolic fraction with a protein concentration of 1.0 mg/mL was obtained.

In comparison, the mitochondria-rich fraction was collected in 200 μL with a protein concentration of about 0.5 mg/mL.

7.9. Assessment of the activity of the individual mitochondrial redox complexes in mitochondria-rich fraction

The activity of mitochondrial complex I, complex II, complex IV, and CS (citrate synthase), were assessed by spectrophotometric or spectrofluorimetric assays, at 37 $^{\circ}\text{C}$, in 250 μL of reaction medium supplemented with a volume of the mitochondria-rich fraction containing 10 μg of protein, using a microplate reader (Synergy H1 Biotek). The activity of mitochondrial redox chain complexes was normalized with CS activity.

Complex I was evaluated spectrofluorimetrically following NADH oxidation, detected by the blue/cyan fluorescence decay, as illustrated in **Figure 18** (98,257).

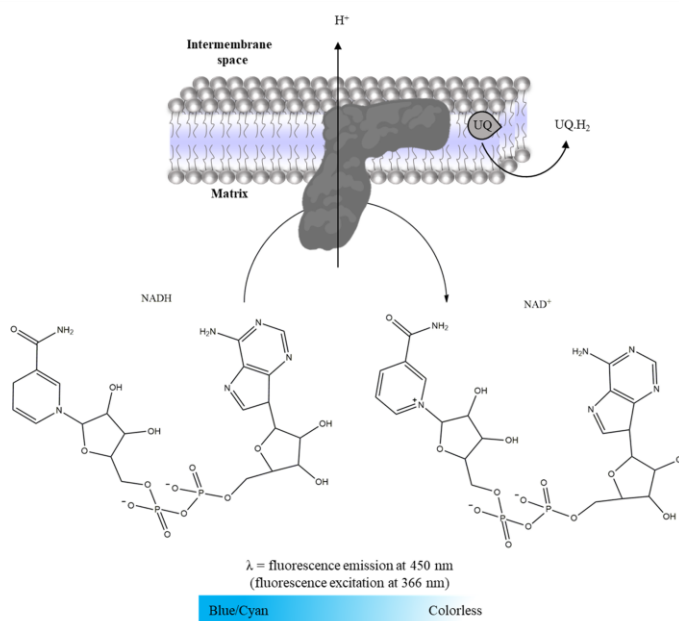


Figure 18 - Oxidation of NADH to NAD⁺, measured at 450 nm (setting excitation at 366 nm).

Thus, the time-dependent decrease of NADH (5 mM) fluorescence intensity was followed for 10 minutes at 450 nm (setting excitation at 366 nm) in the absence and presence of rotenone (600 μM , complex I inhibitor), with phosphate buffer (KH_2PO_4 25 mM, MgCl_2 5 mM, pH 7.5) supplemented with KCN (100 mM), antimycin A (2 mM) and decylubiquinone (38.75 mM). Complex I activity was determined by the difference between the slopes in the absence and presence of rotenone. A calibration curve of the NADH fluorescence intensity as a function of concentration, prepared in the same assay

conditions, was used to convert the rate of NADH fluorescence decay promoted by complex I activity in mol. Then, complex I activity is expressed as nmol NADH oxidized per minute per mg of protein.

Complex II activity was evaluated spectrophotometrically following the reduction of 2,6-dichlorophenolindophenol (DCPIP, color change from blue to colorless), which was used as an exogenous acceptor of the electrons (**Figure 19**) (98,258). In assay conditions (*i.e.*, complex I is inhibited by rotenone, complex III by antimycin A and complex IV by KCN), succinate (100 mM) oxidation promoted by complex II delivers the electrons for ubiquinone that promotes DCPIP (25 mM) reduction. Thus, the time-dependent decrease of absorbance intensity at 600 nm ($\epsilon_{\text{DCPIP}} = 20700 \text{ M}^{-1} \text{ cm}^{-1}$) in the absence and presence of oxaloacetate (500 mM, complex II inhibitor) is recorded in a microplate reader, with phosphate buffer (KH_2PO_4 25 mM, pH 7.5) supplemented with decylubiquinone (38.75 mM), rotenone (600 μM), antimycin (2 mM) and KCN (100 mM). Complex II activity was determined by the difference between the slopes in the absence and presence of oxaloacetate, and it is expressed as nmol DCPIP reduced per minute per milligram of protein.

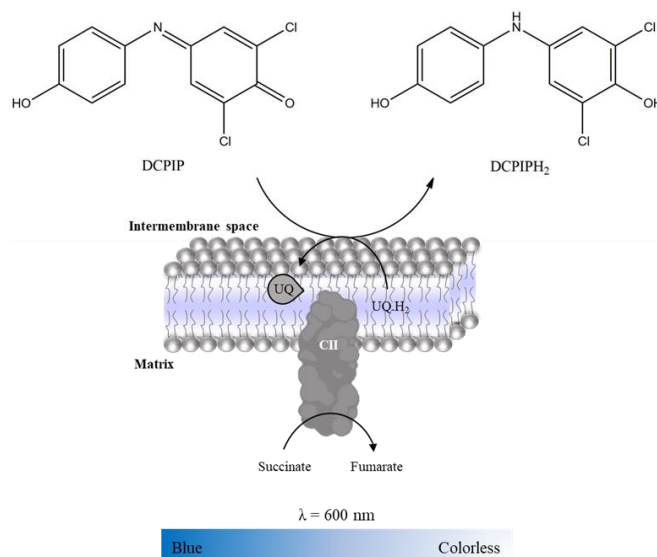


Figure 19 - Reduction of DCPIP with color change from blue to colorless, used as an exogenous acceptor of electrons resulting from succinate oxidation to fumarate promoted by Complex II.

Complex IV activity was evaluated spectrophotometrically following the oxidation of reduced Cyt c (200 μM , $\epsilon_{\text{Cyt c}} = 29500 \text{ M}^{-1} \text{ cm}^{-1}$) at 550 nm (color change from light red to dark red, **Figure 20**) (259). Thus, the time-dependent decrease in absorbance intensity at 550 nm in the absence and presence of KCN (100 mM, complex IV inhibitor) is recorded

for 10 minutes using a microplate reader, with phosphate buffer (KH_2PO_4 25 mM, pH 7.5) supplemented with antimycin A (2 mM) and rotenone (600 μM). The stock solution of reduced Cyt c is freshly prepared (in a spectrophotometer cuvette, Helios Alpha, UNICAM) by adding a volume of dithionite saturated solution required to increase the absorbance of Cyt c solution in 0.6 units, at 550 nm, after its saturation of nitrogen gas. In general, 10 μL of dithionite solution is enough to reduce 3 mL of a Cyt c solution at 200 μM . Complex IV activity was determined by the difference between the slopes in the absence and presence of KCN, and it is expressed as nmol oxidized Cyt c per minute per milligram of protein.

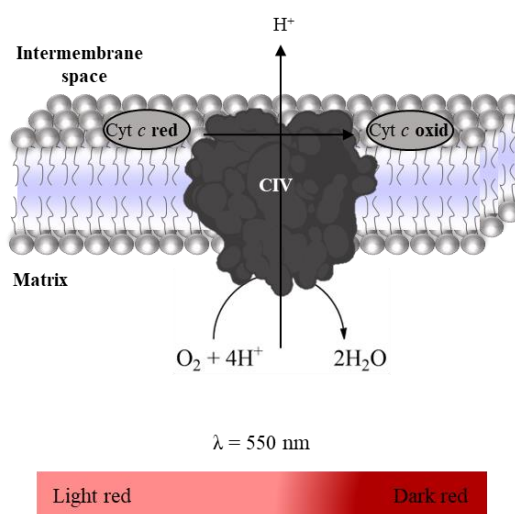


Figure 20 - Oxidation of the reduced Cyt c with water production.

CS activity was determined spectrophotometrically, monitoring the color change associated with the reduction of 5,5-dithio-bis-2-nitrobenzoic acid (DTNB) by the coenzyme A (CoASH), a product of the condensation reaction catalyzed by CS enzyme (acetyl-CoA plus oxaloacetate generate citrate and CoASH), as displayed in **Figure 21** (98,260).

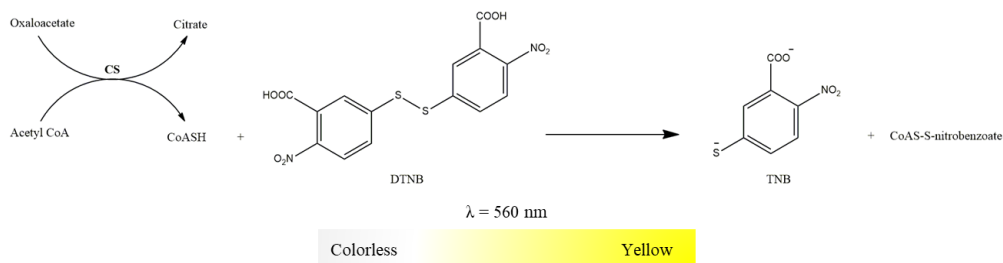


Figure 21 - Reduction of DTNB with color change from colorless to yellow by the CoASH resulting from the condensation reaction of acetyl-CoA and oxaloacetate to form citrate promoted by CS.

The time-dependent increase in absorbance at 412 nm was recorded in a microplate reader, with buffer (Tris-HCl 20 mM, pH 8, Triton X-100 0.02%) supplemented with DTNB (10 mM), oxaloacetate (100 mM) and acetyl-CoA (6.1 mM). Succinyl-CoA (60 mM), an

inhibitor of CS, is used to confirm the reduction of DTNB in couple to CoASH released by the CS activity. CS activity is calculated by the difference between the slopes of DTNB reduction ($\epsilon_{\text{TNB}} = 13600 \text{ M}^{-1} \text{ cm}^{-1}$) in the absence and presence of CS inhibitor, and it is expressed as nmol of 2-nitro-5-thiobenzoic acid (TNB) per minute per milligram of protein.

7.10. Fatty acid profile of cell lipidome: membrane lipids extraction, FAMES preparation and profiling by GC-FID and GC-MS

Membrane lipids extraction from BV-2 cells homogenates was carried out by a double extraction procedure using a combination of methanol/ chloroform/ water (2:1:0.8, v/v/v) (98). A volume of suspension corresponding to 2 mg of protein was reconstituted in 0.8 mL water. The samples were mixed with 2 mL methanol for 5 min. and then 1 mL chloroform was added and mixed for 5 min. The mixture was centrifuged at 3500 rpm for 5 min. at 4 °C. The supernatant was collected and an additional volume of 1 mL chloroform and 1.2 mL of water with potassium chloride was added, followed by 5 min mixing. Finally, the samples were centrifuged at 3500 rpm for 5 min. at 4 °C, to obtain a two-phase system: an aqueous top phase and an organic bottom phase from which lipids were collected. The pellet obtained in the first centrifugation was used to perform lipid re-extraction. The obtained lipid extracts were used for quantification of the total phospholipids and to assess the fatty acid profile by GC-FID and CG-MS, as described above.

8. *In vivo* assays

8.1. Preparation of SC-Nanophytosomes formulation for oral administration – evaluation of the SC-Nanophytosomes formulation stability under conditions that mimic the gastrointestinal tract

SC-Nanophytosomes were prepared with EAE-extract (0.5 mg/mL) and *C. tomentosum* membrane polar lipids (600 μM in phospholipids), as described above. The oral route will be used to evaluate the therapeutic competence of SC-Nanophytosomes formulation in a rotenone-induced rat model of PD. Thus, the effects of pH changes on the properties of SC-Nanophytosomes were evaluated by a successive dialysis process through a cellulose membrane (cut-off of 14 kDa Sigma-Aldrich, St. Louis, MO, USA) against buffer solutions that mimic the pH conditions throughout the gastrointestinal tract. The SC-Nanophytosomes formulation (5 mL), prepared in aqueous buffer solution (50 mM KCl, 10 mM HEPES, 2 mM citric acid, pH 6.4), was, in a first step, dialysed against 100 mL

of aqueous buffer solution (50 mM NaCl, 10 mM HEPES, 2 mM citric acid, pH 6.4) for 1h. Then, an aliquot was collected for analysis, and the remaining volume was submitted to dialysis against 100 mL of aqueous buffer solution at pH 2.0 for 1 h. An aliquot was collected, and the dialysed formulation was submitted to new dialysis against 100 mL of aqueous buffer solution at pH 7.4 for 1 h. Aliquots of SC-Nanophytosomes formulation collected in the different dialysis steps were used to determine the vesicle size, surface charge and EE of the anthocyanins. The values of the absorption at 520 nm (typical of anthocyanins in cationic form) obtained by UV-Visible spectra of non-dialysed and dialysed SC-Nanophytosomes, were used to determine the EE of elderberry anthocyanins, according to the procedure described above. The size, size distribution and Zeta potential of the dialysed SC-Nanophytosomes were evaluated, as described above.

8.2. Rotenone-induced rat model of PD and treatment with SC-Nanophytosomes

Animal experiments were conducted according to Portuguese (Decreto-Lei 113/2013) and European guidelines for Animal Care (EU Directives 2010/63/EU) and were approved by UTAD-ORBEA ethical committee. Animals were housed with food and water available *ad libitum* and maintained on a 12 h light/dark cycle in a temperature-controlled (22 °C±2 °C) room in the animal facilities of the University of Trás-os-Montes and Alto Douro. All efforts were made to minimize the number of animals used and their suffering. Fifteen *Wistar* rats (male) with 3-month-old were randomly assigned into two different experimental groups, one group (control) with five animals and the other group with ten animals that will be used to induce a PD-like pathology with rotenone and then subdivided into two groups according to the experimental design displayed in (**Figure 46A**). Considering the low water solubility of the rotenone, 3 mg/mL of rotenone was solubilized in a lipid-based carrier containing 15 mM of egg yolk phospholipids. Then, rats were injected intraperitoneally (*i.p.*) with either rotenone (3.0 mg/kg of animal weight) formulation or vehicle (water suspension of the lipid-based carrier without rotenone) three times a week for three weeks. Then, the ten rats with rotenone-induced PD-like pathology were randomly divided into two groups with five animals each. One week later, the experimental plan followed with three groups: control (CTRL), rotenone (ROT), and rotenone treated with SC-Nanophytosomes delivered by drinking water at a final concentration of 3 µM in phospholipids plus 2.5 mg/L in EAE-extract (ROT+SC-Nanophyt). SC-Nanophytosomes were prepared with 600 µM membrane polar lipids from *C. tomentosum* and 0.5 mg/mL of EAE-extract from *S. nigra* in the buffer as described above and diluted with commercially available water with acidic pH (total mineralization 26 mg/L, silica 10 mg/L, bicarbonate <0.3

mg/L, chloride 7.2 mg/L, nitrate 1.3 mg/L, calcium 0.4 mg/L, sodium 4.2 mg/L and magnesium 0.7 mg/L, pH 4.7). During three weeks of the treatment, animals had free access to the liquid (water for CTRL and ROT groups, and water supplemented with SC-Nanophytosomes for ROT+SC-Nanophyt group) and standard rodent chow composed of pellets of AIN-76A balanced diet (20.3% protein, 65% carbohydrates and 5% fat, <http://www.researchdiets.com>). Drinking liquid (water or water supplemented with SC-Nanophytosomes) was changed every 3 days. Animal body weight was monitored weekly throughout the study (**Figure 47**).

Considering the average animals' body weight (375.12 ± 20.69 g) and the daily liquid intake (41.10 ± 3.18 mL) for the ROT+SC-Nanophyt group, we have estimated an SC-Nanophytosomes oral dose of 300 nmol of phospholipid and 0.25 mg of EAE-extract/Kg/day, which is equivalent to a human dose of 56.20 μ mol of phospholipid and 46.80 mg of EAE-extract/Kg/day using Food and Drug Administration criteria for converting equivalent drug dosages across species (261). Humans' recommended total polyphenol intake is about 20 mg/Kg/day.

8.3. Beam walking test

The beam walking test assesses animals' motor coordination and balance disturbance. In this test, the animal must walk across a high beam with a narrow diameter that challenges its ability to maintain balance (262). The beam apparatus consists of a 100 cm beam with a flat surface of 6 mm (width) suspended 50 cm from the ground by wooden supports at either end. The wooden supports were put at the beginning of the beam and the end, next to which was placed a black box, serving as a home cage for the animal being tested (**Figure 22**). At the start of the beam, a line was drawn 20 cm that was used as starting zone. The test starts by placing the animal in the starting zone and finishes when the animal reaches the home cage. At the same time, a video camera records the performance and allows the measurement of the time the animal takes to cross the beam (80 cm).

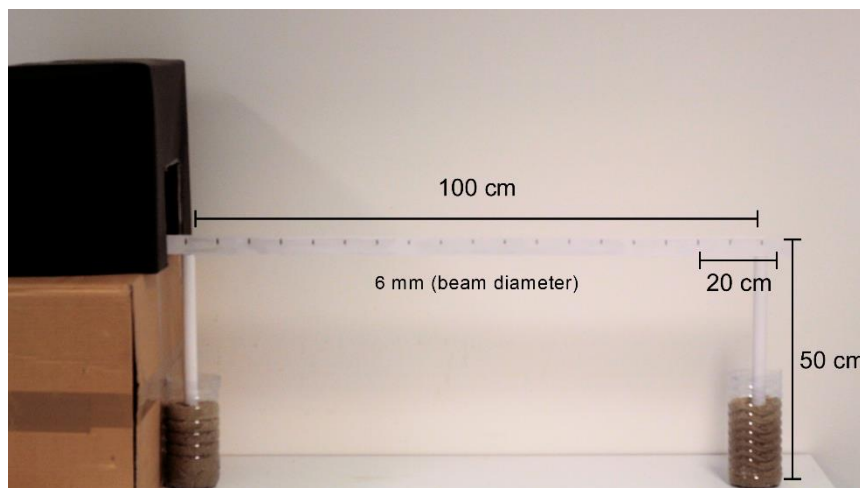


Figure 22 - Image of the apparatus used for beam walking test.

9. *Ex vivo* assays

9.1. Samples collection

After three weeks of SC-Nanophytosomes treatment, the animals were euthanized by cervical displacement followed by decapitation. Their brains were sectioned into two different portions: one comprising the basal ganglia, a brain macro-region that includes the basal ganglia and cerebellum (BG-Cereb), and the other with the cerebral cortex (C-Cortex) regions of the brain. The brain portions obtained were homogenized in buffer (sucrose 130 mM, KCl 50 mM, MgCl_2 5 mM, KH_2PO_4 5 mM, and HEPES 5 mM, pH 7.4, supplemented with a protease inhibitors cocktail) using a Glass-Teflon Potter Elvehjem (20 strokes) to promote the rupture of cells. The homogenates were centrifugated at $1000 \times g$ for 10 min, at 4 °C, to discard cell nuclei as a pellet. The supernatants containing cytosolic organelles, including mitochondria, were collected, and their total protein content was determined by the Biuret method (98). One sample of each brain supernatant ($1000 \times g$) was collected, quickly frozen in liquid nitrogen, and stored at -80 °C until to be used to evaluate the brain levels of α -synuclein. Another sample of the supernatant obtained with the BG-Cereb portion was immediately used to evaluate the bioenergetic performance of mitochondria with an OROBOROS Oxygraph-2k system, (as described below). As described above, a differential centrifugation procedure fractionated the remaining supernatants to obtain mitochondria-free cytosolic and mitochondria-rich fractions containing both synaptic and non-synaptic brain mitochondria. The protein content of these sub-cellular fractions was determined by the Biuret method and then kept at -80 °C until the time to be used. The heart, liver and kidneys were also collected, weighed (**Table 4**), and immediately frozen in liquid nitrogen and kept at -80°C for further studies.

Samples of skeletal-muscle tissues were collected from the thighs of both hind limbs, immediately frozen in liquid nitrogen, and kept at -80 °C until to be used. Lately, skeletal-muscle samples with a known mass, were cut into small pieces and homogenized in ice-cold buffer (sucrose 130 mM, KCl 50 mM, MgCl₂ 5 mM, KH₂PO₄ 5 mM, and HEPES 5 mM, pH 7.4, supplemented with a protease inhibitors cocktail) (1:10 p/v) using a Glass-Teflon Potter Elvehjem. The homogenate was filtered through standard surgical gauze to remove the solid debris, and the filtered homogenates were collected. One part of the filtered homogenate was quickly frozen in liquid nitrogen and stored at -80 °C until used for evaluation of the fatty acid profile. The remaining filtered homogenate was fractioned by differential centrifugation to obtain the mitochondria-free cytosolic and the mitochondria-rich fractions, as described above. The Biuret method determined the protein content of these sub-cellular fractions and the filtered homogenates.

9.2. Rat α -synuclein Enzyme-Linked Immunosorbent Assay

α -synuclein levels were quantified in the brain supernatants in the BG-Cereb and C-Cortex brain portions by using a rat α -synuclein Enzyme-Linked Immunosorbent Assay (ELISA) kit (Cusabio) according to the manufacturer's protocol. The absorbance was read at 450 and 570 nm, and the concentrations were calculated from standard curves. Results are expressed as ng per mg of protein.

9.3. Tyrosine hydroxylase activity

Tyrosine hydroxylase (TH) activity was assessed in mitochondria-free cytosolic fraction obtained from BG-Cereb and C-Cortex portions (263). A volume of the mitochondria-free cytosolic fraction containing 0.2 mg of protein was added to a 125 μ L of HEPES buffer (100 mM, pH=7.0) supplemented with 0.5 mM tetrahydrobiopterin and 5 μ M iron (II) sulphate and the mixture incubated for 5 min. The reaction started with the addition of 125 μ L of HEPES buffer supplemented with 100 μ M of tyrosine (enzyme substrate) and 200 μ M of sodium periodate (used to transform L-3,4-dihydroxyphenylalanine (L-DOPA) into the detectable dopachrome, **Figure 23**). The enzyme activity was followed spectrophotometrically at 475 nm, recording points every 20 seconds for 10 min., at 37 °C. Production of L-DOPA was determined using a molar extinction coefficient for dopachrome of 3700 M⁻¹ cm⁻¹. Assays conducted in the presence of 100 μ M of 3-iodo-tyrosine, a known competitive inhibitor of tyrosine hydroxylase, were used to confirm that the detected dopachrome is related to tyrosine hydroxylase activity.

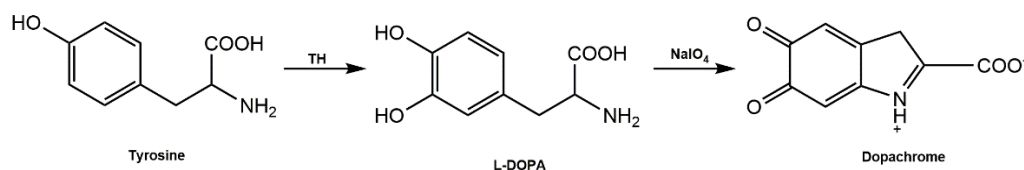


Figure 23 – Tyrosine hydroxylase transforms tyrosine in L-DOPA. Sodium periodate (NaIO₄) is used to transform L-DOPA into the detectable dopachrome.

9.4. Assessment of the mitochondrial respiratory function

Brain mitochondria oxygen consumption was monitored with a Clark-type electrode, at 37°C, in a 2 mL thermostatic chamber with continuous agitation using an OROBOROS Oxygraph-2k system (Oroboros Instruments, Innsbruck Austria). This high-resolution respirometry apparatus allows the detection of respiratory flux from 1 pmol.s⁻¹.mL (264). Brain supernatants (0.5-0.6 mg), containing the non-synaptic brain mitochondria in suspension and the synaptic population of brain mitochondria within synaptosomes, were added to 2 mL of reaction medium (sucrose 130 mM, KCl 50 mM, MgCl₂ 5 mM, KH₂PO₄ 5 mM, and HEPES 5 mM, pH 7.4) supplemented with digitonin to a final concentration of 0.02%. Digitonin is used to permeabilize the synaptosomes membrane, allowing the assessment of oxygen consumption by both non-synaptic and synaptic brain mitochondria populations (98). Simultaneously, oxygen concentration and flux were recorded and analyzed by Dat lab software Version 7.0 (Oroboros Instruments). Fifteen minutes after starting the record, a cocktail of mitochondrial respiratory substrates (pyruvate 2.5 mM and malate 1.25 mM, and succinate 5 mM) was added to allow the activation of the mitochondrial respiratory chain at the level of complex I (pyruvate and malate are NADH generators) in simultaneous with complex II activation (succinate is the substrate of this enzyme), as occurs in the physiological conditions. ADP (50 µM) was added to induce state 3 respiration (respiration is coupled ATP synthesis). Oxygen consumption decreases when all ADP is phosphorylated, and the system enters state 4. Then, sodium azide 20 µM was added to inhibit complex IV, which allows the assessment of the oxygen consumption not connected with mitochondrial respiration. Thus, the mitochondrial oxygen consumption rate values in states 3 and 4, expressed by pmol O₂/min./mg of protein, can be calculated. The respiratory control rate (RCR) is calculated as the ratio between the oxygen consumption rates in states 3 and 4. The concentrations mentioned earlier for respiratory substrates, ADP, and inhibitors refer to their concentrations in the chamber.

9.5. Mitochondrial respiratory chain complexes

The mitochondria-rich fraction (mg of protein) obtained from two brain portions (BG-Cereb and C-Cortex) and skeletal-muscle tissue were used to evaluate the activity of mitochondrial redox chain complexes (complex I, complex II and complex IV), and CS, at 37 °C, in 250 µL of the reaction medium, using a microplate reader (Synergy H1 Biotek). The activity of mitochondrial redox chain complexes was normalized by CS activity. The complex I, complex II, complex IV and CS activities was described above.

9.6. Determination of brain enzymatic and nonenzymatic antioxidant defenses

The activities of cytosolic antioxidant enzymes: superoxide dismutase (Cu/Zn-SOD), glutathione reductase (GR), and glutathione peroxidase (GPx) were evaluated in 250 µL of reaction medium by using a microplate reader (Synergy H1 Biotek) while catalase (CAT) activity was assessed polarographically following the oxygen production in 1 mL reaction medium by using a Clark-type electrode (Hansatech, Norfolk, UK). All assays are conducted at 37 °C using a volume of the mitochondria-free cytosolic fraction containing 50 µg of protein.

Cu/Zn-SOD activity was assessed indirectly based on competition for $O_2^{\cdot-}$, generated by the xanthine/xanthine oxidase system, between the enzyme and the yellow nitrobluetetrazolium chloride (NBT) that reacts with the $O_2^{\cdot-}$ to produce purple formazan, detectable by changes in absorption at 560 nm as illustrated in **Figure 24** (210,265). Thus, the time-dependent absorbance increased at 560 nm in the absence and presence of mitochondria-free cytosolic fraction was recorded in a microplate reader, with phosphate buffer (KH_2PO_4 100 mM, EDTA 5 mM, pH 7.4) supplemented with NBT (2 mM), xanthine oxidase (12.5 U/mL) and xanthine (2 mM). Cu/Zn-SOD activity is expressed as a unit of the enzyme's catalytic activity (U) per milligram of protein. When the $O_2^{\cdot-}$ is generated by 1 U of xanthine oxidase, one catalytic unit of Cu/Zn-SOD is defined by the amount of biological sample required to decrease the slope of NBT reduction by 50%.

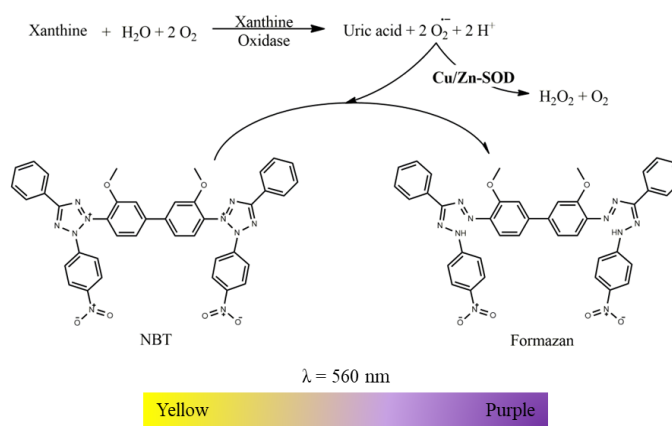


Figure 24 - The xanthine/xanthine oxidase system generates O₂^{•-} disputed between Cu/Zn-SOD enzyme and the yellow NBT. Cu/Zn-SOD is responsible for the dismutation of O₂^{•-} into H₂O₂ and molecular oxygen, and the NBT reacts with the O₂^{•-} producing the formazan, detectable at 560 nm.

GR activity was evaluated spectrofluorimetrically following reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidation associated with the reduction of oxidized glutathione (GSSG) to reduced glutathione (GSH), detected by the fluorescence decay, as illustrated in **Figure 25**. The time-dependent decrease of NADPH (10 mM) fluorescence intensity at 450 nm (setting excitation at 366 nm) promoted by the mitochondria-free cytosolic fraction with phosphate buffer (KH₂PO₄ 100 mM, EDTA 5 mM, pH 7.4) in the absence and presence of GSSG (10 mM, enzyme-substrate) is recorded in a microplate reader. GR activity was determined by the difference between the slopes of NADPH oxidation in the presence and absence of GSSG and expressed in nmol NADPH per minute per milligram of protein. To convert the slopes of NADPH fluorescence decay promoted by GR activity in moles, a calibration curve of the NADPH fluorescence intensity as a function of concentration prepared in the same assay conditions is required.

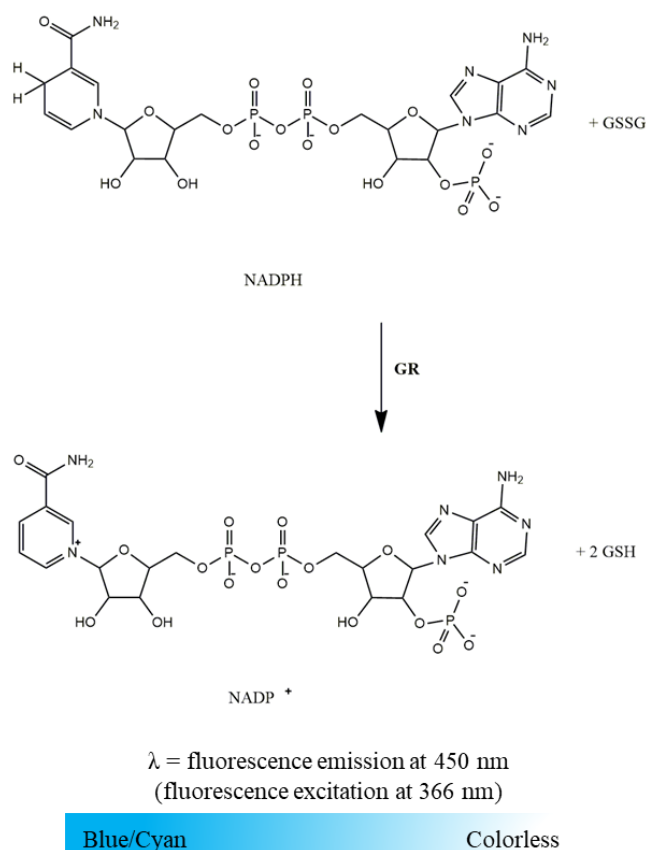


Figure 25 - Oxidation of NADPH to NADP⁺ with reduction of GSSG to GSH by GR. The NADPH oxidation intensity is measured at 450 nm (setting excitation at 366 nm).

GPx activity in the mitochondria-free cytosolic fraction was evaluated through a coupled reaction with the GR enzyme. GPx reduces hydrogen peroxide (H₂O₂) to water and oxidizes GSH to GSSG (210,254). The generated GSSG is reduced to GSH with NADPH consumption by GR, which can be followed by fluorescence decay, as described above in **Figure 26**. Under GR excess conditions, the NADPH oxidation is limited by GPx activity and can be easily measured spectrofluorimetrically. Thus, the time-dependent decrease of NADPH (10 mM) fluorescence intensity at 450 nm (setting excitation at 366 nm) promoted by the mitochondria-free cytosolic fraction in the absence and presence of GSH (10 mM, substrate of GPx enzyme) is recorded in a microplate reader with phosphate buffer (KH₂PO₄ 50 mM, EDTA 5 mM, pH 7.4) supplemented with GR (116.48 U/mL) and H₂O₂ (88 mM). GPx activity is determined, in the first step, by the difference between the slopes of NADPH oxidation in the presence and absence of GSH, expressed in nmol NADPH per minute per milligram of protein. To convert the slopes of NADPH fluorescence decay promoted by the GR/GPx coupled system in mol, a calibration curve of the NADPH fluorescence intensity as a function of concentration is prepared in the same assay conditions.

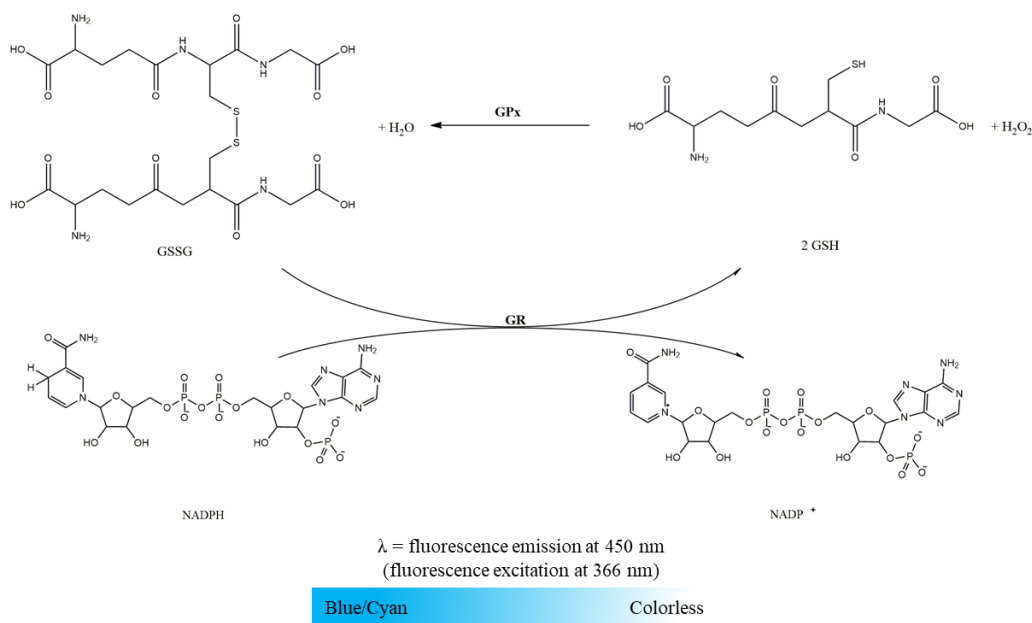


Figure 26 - GPx activity is evaluated by a coupled reaction with GR enzyme. GPx reduces hydrogen peroxide to water and oxidizes GSH to GSSG. The generated GSSG is reduced to GSH with NADPH consumption by GR.

CAT reduces H_2O_2 to water and O_2 (colorless reaction, **Figure 27**). CAT activity was determined polarographically following O_2 production for 2-3 minutes, using a Clark-type oxygen electrode, resulting from H_2O_2 (88 mM) decomposition, with phosphate buffer (KH_2PO_4 100 mM, EDTA 5 mM, pH 7.4) (98,266). CAT activity is determined by the difference between the slopes in the presence and absence of mitochondria-free cytosolic fraction, and it is expressed in terms of nmol O_2 produced per minute per milligram of protein.

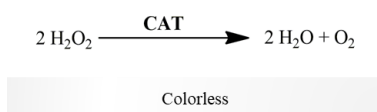


Figure 27 - CAT reduces H_2O_2 to water and O_2 , detected using a Clark-type oxygen electrode.

GSH and GSSG levels were determined in the mitochondria-free cytosolic fraction obtained from two brain portions (BG-Cereb and C-Cortex) and skeletal-muscle tissue by a spectrofluorimetric assay using a microplate reader (Synergy H1 Biotek) setting excitation at 339 nm and emission at 426 nm (98,148). To determine GSH content, the mitochondria-free cytosolic fraction was incubated at room temperature in 2.2 ml of phosphate buffer (100 mM KH_2PO_4 , 5 mM EDTA, pH 8.0) supplemented with 200 μL of fluorochrome orthophthalaldehyde (OPT, 2000 $\mu\text{g}/\text{mL}$) for 15 min, in the dark. To determine GSSG content, the mitochondria-free cytosolic fraction was first incubated at room temperature with 40 μL of N-ethylmaleimide (NEM, 12 mM) for 30 min. After that, 1.66 ml of NaOH (0.1M)

and 0.6 ml of phosphate buffer supplemented with 200 μ L of OPT (2000 μ g/mL) were added to the reaction, followed by 15 min incubation at room temperature in the dark. Concentrations were calculated using standard curves prepared with different concentrations of GSH and GSSG, which underwent the same sample treatment. The results were expressed as GSH/GSSG ratio.

9.7. Membrane lipids extraction, FAMES preparation and profiling by GC-FID and CG-MS

Phospholipids extraction from mitochondria-rich fraction obtained from two brain fractions (BG-Cereb and C-Cortex) and skeletal-muscle tissue homogenates, was carried out by a double extraction procedure using a combination of methanol/ chloroform/ water (2:1:0,8, v/v/v) (98), as described above. The lipid extracts were submitted to hydrolysis and methylation of fatty acid and analyzed by GC-FID and CG-MS, as described above.

CHAPTER III

Results and Discussion

CHAPTER III

Results and Discussion

In vitro assays

1. A nanophytosomes formulation based on elderberry anthocyanins and *Codium* sp. lipids to mitigate mitochondrial dysfunctions

Mitochondria are a hub of cell metabolism and signalling, ensuring the bioenergetic requirements for cell function and many other processes that support cell ability to sense and respond to changes in intra and extracellular environments (109,267). Acquire mitochondrial dysfunction has been implicated in disparate degenerative diseases currently without effective therapeutics and increasing incidence, including AD and PD (268, 269). Thus, mitochondria emerged as a promising therapeutic target to support a common therapeutic strategy to fight different age-related neurodegenerative diseases (270–272).

Several mitochondria-targeted approaches, supported by compounds engineered/selected to address a particular parameter of the organelle dysfunction (e.g., ROS overproduction and oxidative damage, defects in mitochondrial biogenesis and quality control mechanisms, redox changes, and/or metabolic-dependent signalling cascades) have been developed and tested (148,271,272). Despite the promising pre-clinical data obtained with some compounds/strategies pairs, all yielded disappointing or inconclusive results when moved to clinical trials (271). Two different explanations can be advanced for these poor results: i) the concentration of the bioactive compounds in the mitochondria of target cells remains below what is required to modulate mitochondrial functionality, reflecting a deficient delivery strategy; ii) the selected parameter of mitochondrial dysfunction was modulated, but the mitochondrial functionality was not fully restored, thereby the pair compound/strategy is not suitable to alter the course of human disease. The anomalous mitochondrial respiratory chain functionality, mainly characterized by a deficient complex I activity and abnormal cardiolipin profile, is a pathological hallmark of both AD and PD (98,273), which has never been the target of the previous attempts to develop a mitochondrial therapy for neurodegenerative diseases. Moreover, impaired activity of the mitochondrial redox chain: i) generates a high rate of ROS at the level of complex I, with consequent oxidative stress and changes in cell signalling (274,275); ii) disrupts the usual pattern of $\Delta\Psi_m$ fluctuations, with impact on ATP synthesis, calcium homeostasis, and cell death mechanisms (109,267).

Within this scientific framework, the development of a well-succeed mitochondria therapy for AD and PD requires a redox-active compound to overcome complex I-related mitochondrial redox chain dysfunction integrated into a delivery lipid-based system with competence not only to overcome the physiological barriers and target the mitochondria of brain cells but also to modulate the mitochondrial membranes lipidome.

Elderberry anthocyanins, obtained from *Sambucus nigra* L. as an EAE-extract, are mitochondriotropic compounds that work in the mitochondrial inner membrane as electron carriers that oxidize NADH and deliver electrons for complex III, bypassing mitochondrial redox chain abnormalities associated with impaired complex I activity (210). Elderberry anthocyanins can also protect neuronal cells against rotenone-induced toxicity and oxidative stress (210), expanding the well-known preventive health benefits of polyphenols for neurodegeneration (276). Thus, elderberry anthocyanins' mitochondriotropic properties were capitalized within an innovative lipid-based nanocarrier built with lipids obtained from *C. tomentosum*, an edible green seaweed. *C. tomentosum* membranes are a source of polar lipids characterized by a high richness of anionic phospholipids (mainly PG) that is a cardiolipin precursor), and a fatty acid profile with high levels of PUFA (222). Thus, the polar lipids obtained from *C. tomentosum* membranes may allow the design of a lipid-based nanocarrier with a high potential to accommodate and stabilize the elderberry anthocyanins in their cationic form as well as the potential to modulate the mitochondria lipidome.

In this chapter, a lipid-based nanoplatform built with *C. tomentosum* lipids and *S. nigra* anthocyanins, called SC-Nanophytosomes, will be prepared and characterized. Then, the competence of these SC-Nanophytosomes to support the development of an effective mitochondria-targeted therapy for both AD and PD will be addressed by *in vitro* approaches. Since the standard methodologies to extract the membrane lipids use solvents non-compatible with the regulations required for pharmaceutical and food applications, a new sustainable procedure to obtain a chlorophyll-free membrane polar lipid extract from *C. tomentosum* was developed and validated here.

1.1. Characterization of the building blocks for the assembly of the SC-Nanophytosomes

Both building blocks, EAE-extract, and *C. tomentosum* membrane polar lipids, used for assembling the SC-Nanophytosomes, were obtained by novel procedures. Thereby, the detailed chemical composition of both building blocks will be described below. As described in the experimental section, *C. tomentosum* membrane polar lipids were extracted from the freeze-dried algae with ethanol by an ultrasound-assisted procedure and purified by precipitation with cooled acetone, avoiding the toxic organic solvents used in traditional lipid extraction methodologies (277). This procedure allows for obtaining a lipid extract that represents 0.05% of the dry weight of the algae, containing only trace amounts of chlorophylls, as shown in **Figure 28** (277).

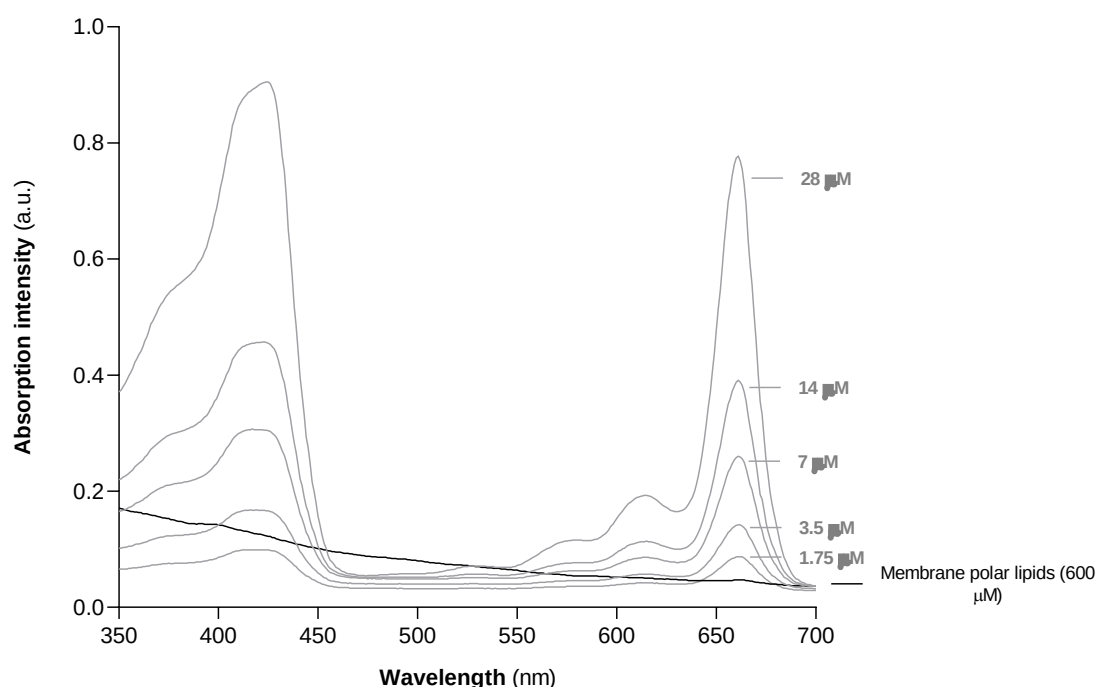


Figure 28. Typical UV-Visible absorption spectra (350-700 nm) of an ethanolic solution of *C. tomentosum* membrane polar lipids, at 600 μM in PL, (black line) and of chlorophyll *a* ethanolic solutions at different concentrations (grey lines).

As shown in **Figure 29A**, the phospholipids content, assessed by phosphorus assay after acid hydrolysis, represents 74% of the dry weight of the whole lipid extract, considering 777 g/mol as the average molecular weight of phospholipids. In comparison, the remaining 26% may result from the other families of polar lipids without the phosphate group that composes the membranes of algae cells. The fatty acid profile of *C. tomentosum* membrane polar lipids fraction was revealed by analysing their methyl esters by GC-FID and GC-MS. The fatty acid methyl esters were quantified by the area of each peak normalized by the standard internal peak ($\text{C}_{17:0}$), which was added to the lipid extract before *trans*-methylation. Quantitative GC-FID analysis of the fatty acid methyl esters prepared with samples of polar lipid extract containing a well-known number of phospholipids also indicates that *C. tomentosum* membrane polar lipids extract contain 26% of polar lipids with a diacylglycerol backbone, but without phosphorus in their polar group (**Figure 29A** and **29B**). According to the previously reported liquid chromatography with tandem mass spectrometry *C. tomentosum* lipid profile (222), betaine lipids are the most abundant among the non-phosphorus polar membrane lipids. Betaine lipids are characterized by a non-phosphorous zwitterionic polar head group connected to the diacylglycerol backbone by an ether bond. Thus, betaine lipids should also be the dominant non-phosphorus lipids in the extract obtained by our extraction/purification procedures. All non-phosphorus lipids will be considered as “betaine lipids” for structural rationalization of the membrane organization.

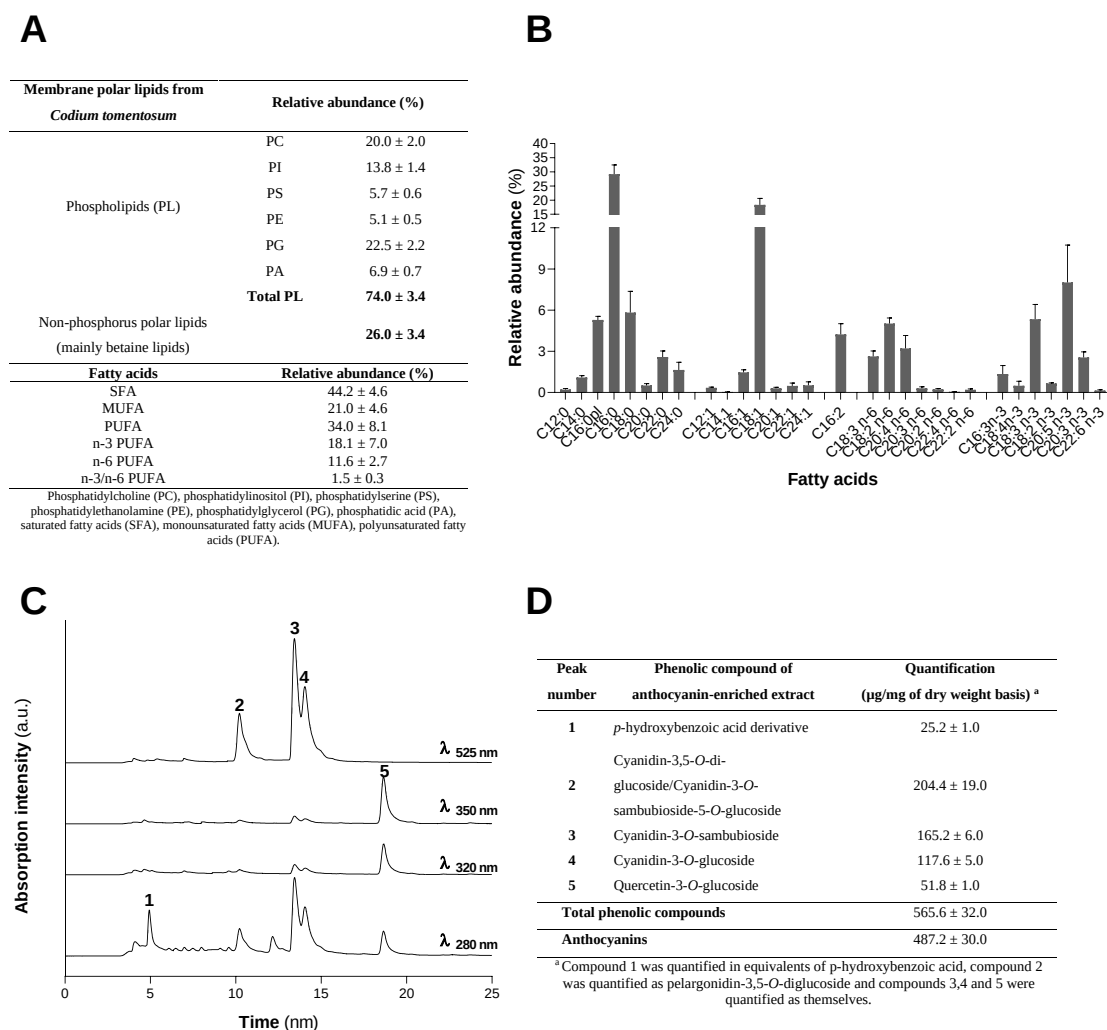


Figure 29. Characterization of *C. tomentosum* membrane polar lipids (**A**, **B**) and elderberry anthocyanins-enriched extract (**C**, **D**) used as building blocks to prepare SC-Nanophytosomes. (**A**) Relative abundance (%) of non-phosphorus polar lipids and phospholipids (PL), discriminated by phospholipid classes: phosphatidylcholine (PC), phosphatidylinositol (PI), phosphatidylserine (PS), phosphatidylethanolamine (PE), phosphatidylglycerol (PG), phosphatidic acid (PA). (**B**) Fatty acid profile of *C. tomentosum* membrane polar lipids: C12:0=lauric acid, C14:0=myristic acid, C16:0pl=hexadecyl dimethyl acetal, C16:0=palmitic acid, C18:0=stearic acid, C20:0=arachidonic acid, C22:0=docosanoic acid, C24:0= lignoceric acid, C12:1=lauroleic acid, C14:1=myristoleic acid, C16:1=palmitoleic acid, C18:1=oleic acid, C20:1=eicosenoic acid, C22:1=docosenoic acid, C24:1=nervonic acid, C16:2=hexadecadienoic acid, C18:3n-6=γ- linolenic acid, C18:2n-6=linoleic acid, C20:4n-6=arachidonic acid, C20:3n-6=dihomo-γ-linolenic acid (DGLA), C20:2n-6=eicosadienoic acid, C22:4n-6=docosatetraenoate, C22:2n-6=decosadienoic acid, C16:3n-3=hexadecatrienoate, C18:4n-3=octadecatetraenoate, C18:3n-3=α-linolenic acid, C18:2n-3=linoleic acid, C20:5n-3=eicosapentaenoic acid (EPA), C20:3n-3=eicosatrienoate acid, C22:6n-3=docosahexanoic acid (DHA). (**C**) HPLC-DAD chromatogram of anthocyanin-enriched extract recorded at 280, 320, 350, and 525 nm. (**D**) Content of the identified phenolic compounds. Peak assignment: 1. *p*-hydroxybenzoic acid derivative, 2. cyanidin-3,5-*O*-diglucoside/cyanidin-3-*O*-sambubioside-5-*O*-glucoside, 3. cyanidin-3-*O*-sambubioside, 4. cyanidin-3-*O*-glucoside, and 5. quercetin-3-*O*-glucoside.

TLC separated the obtained membrane polar lipids. The plate was revealed by primulin under ultraviolet light to detect the separated phospholipid classes in independent

"spots", which were identified by comparison with standards run in the same conditions. Each "spot" was scraped, and the phospholipids were quantified after acid hydrolysis. TLC analysis allowed identifying and quantifying six phospholipid classes as independent TLC "spots". Thus, the anionic PG and the zwitterionic PC, with an abundance of 22.5% and 20.0%, respectively, are the most abundant phospholipid classes. PI contributes 13.8%, while PA, PS, and PE, with relative abundances of 6.9%, 5.7%, and 5.1%, respectively, can be considered minority classes. Thus, the anionic phospholipids (PG, PI, PA, and PS) contribute 48.9%, and the other lipids, mainly zwitterionic lipids (PC, PE, and betaine lipids) maximum of 51.1% for the *C. tomentosum* membrane polar lipids.

Regarding the fatty acid profile of *C. tomentosum* membrane polar lipids obtained by GC-FID and GC-MS analyses (**Figure 29B**), thirty fatty acids species were detected and quantified, comprising eight saturated (SFA), seven monounsaturated (MUFA), and fifteen PUFA. SFA represents 44.2%, MUFA 21.0%, and PUFA 34.0% of the total content. The relative abundance of n-3 PUFA (18.1%) is higher than the n-6 PUFA (11.6%) (**Figure 29A**), which follows data previously reported (278). Considering the molecular species, palmitic acid (C16:0) is the most abundant SFA followed by stearic acid (C18:0), docosanoic acid (C22:0), and lignoceric acid (C24:0). *C. tomentosum* also has very reduced amounts of SFA with an odd number of carbons, namely, with 15, 21, and 23 carbons, supporting the use of heptadecanoic acid (C17:0) as an internal standard. Oleic acid (C18:1) is the dominant species of MUFA, while palmitoleic acid (C16:1) contributes about 2%, and the other four MUFA species have low relative abundance. Regarding PUFA, eicosapentaenoic acid (C20:5n-3) is the most abundant molecular species in the n-3 series, while linoleic (C18:2n-6) is the most abundant in the n-6 series. Arachidonic (C20:4n-6) and linolenic (C18:3n-3) acids also have high relative abundances, while eicosatrienoate (C20:3n-3) and hexadecatrienoate (C16:3n-3) acids (a fingerprint of the *C. tomentosum* algae (279)) were detected in low concentration. Therefore, a set of lipids rich in anionic phospholipids and betaine lipids containing a high level of PUFA will be used to assemble the SC-Nanophytosomes. This set of lipids was chosen not only to support the architecture of the nanocarrier but also to provide lipids with physiological relevance in the context of mitochondrial dysfunction and neurodegeneration. Thus, PG is a cardiolipins precursor required for mitochondrial functionality, the high n-3/n-6 PUFA ratio is associated with anti-inflammatory outcomes, and betaine is a key factor in improving the lipid metabolism compromised by neurodegeneration and mitochondrial dysfunctions (280-283).

Figure 29C shows the HPLC-DAD profile of the phenolic of the EAE-extract obtained by selective adsorption to PVPP with the chromatogram recorded at 280, 320, 350, and 525 nm. The phenolic profile was established according to the retention time and

UV spectra features, while the concentration of each phenolic compound was estimated by using standard curves prepared with appropriate reference compounds. As displayed in **Figure 29D**, it was possibly identified five phenolic compounds, namely *p*-hydroxybenzoic acid derivative (peak 1), cyanidin-3,5-*O*-di-glucoside/cyanidin-3-*O*-sambubioside-5-*O*-glucoside (peak 2), cyanidin-3-*O*-sambubioside (peak 3), cyanidin-3-*O*-glucoside (peak 4), and quercetin-3-*O*-glucoside (peak 5). The total phenolic compounds quantified were 565.6 ± 32.0 $\mu\text{g}/\text{mg}$ of dry weight extract, and the total of anthocyanins was 487.2 ± 30.0 $\mu\text{g}/\text{mg}$ of dry weight extract. Thus, anthocyanins represent 86% of the total phenolic content of the extract, in agreement with what was reported by others (282). This phenolic profile is very similar to the anthocyanin-enriched extract obtained by a solid-phase extraction clean-up procedure with Chroma bond C18 non-end-capped column (210), suggesting that PVPP is a suitable and sustainable tool to recover/purify elderberry anthocyanins. Therefore, an EAE-extract dominated by four anthocyanins will assemble the SC-Nanophytosomes.

1.2. SC-Nanophytosomes preparation and characterization

SC-Nanophytosomes were prepared with *C. tomentosum* membrane polar lipids, rich in anionic phospholipids and EAE-extract, using a two-step procedure rationalized to preserve elderberry anthocyanins in flavylum cation into the bilayer of the final vesicles. Additionally, they were prepared in an aqueous buffer containing potassium chloride (KCl) 50 mM and dialysed through a cellulose membrane (cut-off of 14 kDa) against a large volume aqueous buffer where the KCl was replaced by sodium chloride (NaCl) 50 mM to remove non-encapsulated phenolics and to obtain SC-Nanophytosomes with potassium in the intern aqueous compartment (intracellular environment-like) and sodium in the extern aqueous environment.

The characterization of the SC-Nanophytosomes formulations, in terms of EE of the EAE-extract, vesicle size, and surface charge during 14 days of storage, at 4 °C, and vesicles shape analysis at 7 days of storage, are summarized in **Figure 30**. The SC-Nanophytosomes formulations were prepared with a bulk concentration of 600 μM phospholipids (PL) and 0.5 mg/mL of EAE-extract. The EE of the elderberry phenolic compounds (mostly anthocyanins) was assessed by UV-vis spectrophotometry analysis (**Figure 30B**), recording the absorption spectra (between 250 and 600 nm) of the non-dialysed SC-Nanophytosomes (continuous line) and dialysed SC-Nanophytosomes (dashed line). Nanoliposomes prepared under the same conditions with the same algae lipid concentration without EAE-extract were used for background correction. As shown in **Figure 30B**, the absorption spectrum of the SC-Nanophytosomes exhibits the absorption

bands of elderberry phenolics with a maximum of 280 (as shoulder), 350, and 520 nm. The absorbance values at 520 nm, characteristic of anthocyanins in flavylium cation form, enable the determination of an EE value of 90.71 ± 3.04 %. These data suggest that the cationic anthocyanins may interact with the anionic phospholipids of *C. tomentosum*. This interaction prevents the protons transfer from anthocyanins to the water molecules (acid-base reactions); thereby acidic cationic form anthocyanins are stabilized in the lipid bilayer. Moreover, no changes in the spectral features and EE value were detected during the 14 days of storage, suggesting that the methodology used for SC-Nanophytosomes formulation preparation is an efficient way to encapsulate and preserve elderberry anthocyanins in the cationic form into vesicles.

The size and surface charge of the dialysed SC-Nanophytosomes suspensions, evaluated by DLS and ELS, respectively, at 1, 7, and 14 days of storage at 4 °C are displayed in **Figures 30A** and **30C**. The size and surface charge of a nanostructured system has a decisive influence on its stability and the ability to overcome various biological barriers (285). DLS data indicate that SC-Nanophytosomes have, on the first day, an average diameter of 108.74 ± 24.74 nm (for $n=6$, values ranged from 107.30 to 168.90 nm) and that their average size does not undergo significant changes during 14 days of storage at 4 °C. **Figure 30C** shows the hydrodynamic size distribution of a typical SC-Nanophytosomes formulation, expressed in terms of the relative abundance (by number) for each detected particle diameter. These data indicate that the vesicles exhibit size homogeneity (diameters ranged from 107 to 169 nm) and that this size distribution is preserved during storage. Therefore, the slight size differences that SC-Nanophytosomes underwent during storage at 4 °C may emerge from alterations in the lipid bilayer's fluidity triggered by temperature changes, not vesicle fusion. Zeta potential measurements show that dialysed SC-Nanophytosomes have a strongly negative surface charge, with a mean Zeta potential value of -46.93 ± 6.63 mV, at 1 day of storage (**Figure 30A**). The storage at 4 °C has no significant impact on the vesicle surface charge since Zeta potential values only increase up to -41.62 ± 6.26 mV during the 14 days of storage. The high density of negative surface charge of the SC-Nanophytosomes hinders vesicle fusion processes and explains the stability of the formulations during storage. From a pharmacological point of view, nanoparticles with zeta potential values greater than 30 mV (positive or negative) are considered stable (284), and those with negative Zeta potential are considered less toxic and a more efficient vehicle to transport drugs for the brain (285).

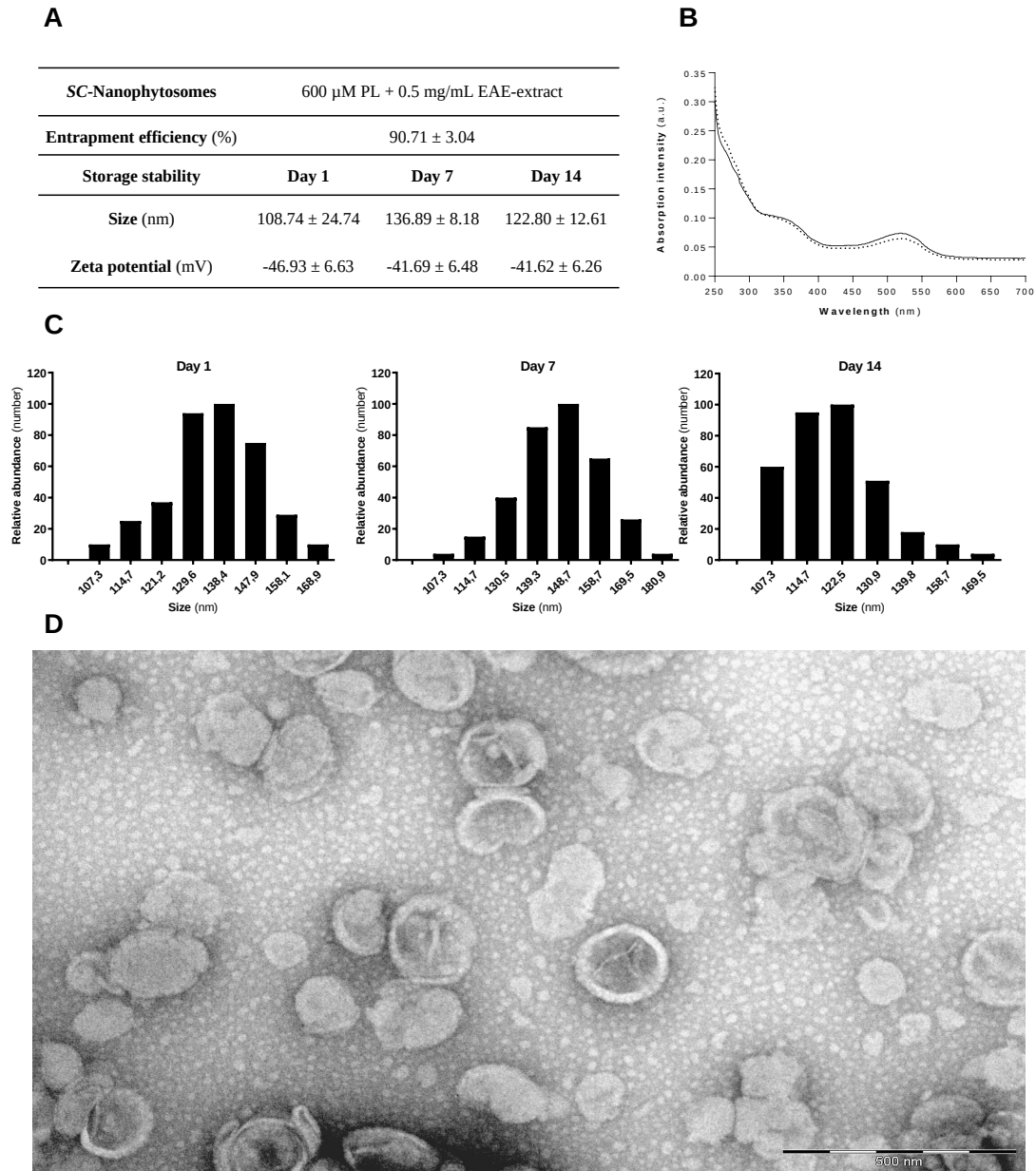


Figure 30. Characterization of the SC-Nanophytosomes. **(A)** Entrapment efficiency (%), vesicle size (nm), and zeta potential (mV) of dialysed SC-Nanophytosomes at 1, 7, and 14 days of storage. **(B)** Typical UV-Visible absorption spectra (250-700 nm) of the anthocyanins-enriched extract non-dialysed (continuous line) and dialysed (dashed line) SC-Nanophytosomes. **(C)** Typical plots of the size distribution of SC-Nanophytosomes at 1, 7, and 14 days of storage expressed in terms of relative abundance (by number). **(D)** Representative TEM image of SC-Nanophytosomes formulation at 7 days of storage.

TEM analysis (**Figure 30D**) showed that the SC-Nanophytosomes formulations are a mixture of nanovesicles with different shapes, ranging from spherical to discoid structure. It confirmed the hydrodynamic size distribution determined by DLS. The shape vesicle heterogeneity captured in the TEM image reflects the bilayer flexibility, and its ability to change the shape without losing integrity. This property is a consequence of noncovalent interactions among the amphiphilic lipids, lipids, and elderberry polyphenols, as well as among lipids, elderberry polyphenols, and water molecules in the bilayer structure. Thus,

SC-Nanophytosomes exhibit good stability and suitable size and surface charge for biomedical applications.

1.3. Effects of SC-Nanophytosomes on SH-SY5Y cells

As a first approach, SH-SY5Y cells were exposed to increasing concentrations of SC-Nanophytosomes for 24 h to assess the effects on cell viability by MTT reduction assay (**Figure 31A**) and membrane integrity by LDH release assay (**Figure 31B**). In the range of tested concentrations (up to 30 μM PL/25 $\mu\text{g mL}^{-1}$ EAE-extract), SC-Nanophytosomes do not alter the viability or membrane integrity of SH-SY5Y cells, suggesting no cytotoxicity and a good safety profile.

Previously, it was shown that elderberry anthocyanins could modulate the mitochondrial respiratory chain activity of mitochondria isolated from the rat brain and promote mitochondria-related beneficial effects on neuronal cells for concentration equal to or greater than 12.5 $\mu\text{g/mL}$ (210). However, the availability of dietary anthocyanins for brain mitochondria should be minimal since they are water-soluble, do not have a dedicated membrane transporter, and undergo significant transformation in the gastrointestinal tract and the liver (205). The entrapment of the flavonoids in lipid-based nanocarriers minimizes these drawbacks since their cell bioavailability is improved, and the threshold required to promote positive outcomes on intracellular targets is shifted for lower concentrations, as previously reported (286). Thus, the next step was to investigate if the SC-Nanophytosomes could target elderberry anthocyanins for the intracellular compartments of the SH-SY5Y cells. In the first set of assays, SH-SY5Y cells, grown in 96-well black plates, were treated with SC-Nanophytosomes (7.5 μM PL/6.25 $\mu\text{g mL}^{-1}$ EAE-extract) or vehicle (control) for 24 h and then incubated with mitochondrial potential-sensitive JC-1 fluorescent probe, for 30 min, to assess the effects on the $\Delta\Psi\text{m}$ in living cells. The $\Delta\Psi\text{m}$, generated by the mitochondrial electron transport chain enzymes with proton pump activity (Complexes I, II, and IV), is an essential component in the process of energy storage during oxidative phosphorylation, playing a key role in cell functioning and survival (287). Then, the sensitivity of these cells to rotenone (25 μM), antimycin A (50 μM), KCN (2 mM), or FCCP (0.2 mM) was assessed after 30 min. exposure, considering effects on $\Delta\Psi\text{m}$ (**Figure 31C**). Cell metabolism was supported by the 5 mM of glucose present in HBSS, and the ratio of the fluorescent probe JC-1 aggregate to monomer was used as a measurement of $\Delta\Psi\text{m}$. As shown in **Figure 31C**, SH-SY5Y cells exhibit polarized mitochondria, with $\Delta\Psi\text{m}$ values assessed by JC-1 aggregate/monomer ratio close to 3, and the treatment of SH-SY5Y cells with SC-Nanophytosomes does not promote significant effects on $\Delta\Psi\text{m}$. As expected, the

addition of FCCP, an uncoupler of mitochondrial oxidative phosphorylation, promotes the complete dissipation of the mitochondrial membrane potential, and the values of the JC-1 aggregate/monomer ratio decrease to close to 1 in both control and SC-Nanophytosomes treated cells (288). On the other hand, the treatment of the cells with SC-Nanophytosomes formulation modulated the response of the mitochondrial membrane potential to rotenone (complex I inhibitor) but not to antimycin A (complex III inhibitor) and KCN (complex IV inhibitor). Thus, rotenone decreased by 49% the value of the $\Delta\Psi_m$ of control cells, and only 30% of SC-Nanophytosomes treated cells, suggesting that anthocyanins were delivered for mitochondria by SC-Nanophytosomes and overcome the rotenone inhibition, as previously described for non-encapsulated anthocyanins (210). Regarding the effects of the mitochondrial complexes III and IV inhibitors, antimycin A only promoted small mitochondrial depolarization. In contrast, KCN promoted a full collapse of $\Delta\Psi_m$, suggesting uncoupler properties in addition to mitochondrial respiration inhibition. The second set of assays was performed with cells grown in T-25 flasks to assess the treatment effects with SC-Nanophytosomes (7.5 μ M PL/6.25 μ g mL⁻¹ EAE-extract) on the activities of mitochondrial complex I, II, and IV, using isolated cell mitochondria. The activities of the mitochondrial redox chain enzymes were normalized by the activity of the CS, expressed by nmol citrate per min per mg of protein, to discard the influence of putative differences in the mitochondria content of sample preparations. The values of the CS activity in mitochondria obtained from control cells (130.66 $\pm$ 42.43) are not different from those obtained with mitochondria isolated from SC-Nanophytosomes treated cells (103.34 $\pm$ 37.63). **Figures 31D, 31E and 31F** display CS normalized complex I, complex II, and complex IV activities of mitochondria-rich fraction obtained from cells treated with SC-Nanophytosomes and from control cells. Complex I and complex II activities of the mitochondria obtained from SC-Nanophytosomes treated cells are significantly higher than the activity of these complexes in control cell mitochondria. Regarding the mitochondrial complex IV activity, no differences were detected between control and SC-Nanophytosomes treated cells. The increased activity of the mitochondrial complex I and complex II promoted by SC-Nanophytosomes suggests an enhancement of the coupling efficiency between carbon flux that generates NADH and FADH₂ and electron flux through the respiratory chain, which increases cell ability to generate ATP by oxidative phosphorylation. These effects may positively impact diseases associated with bioenergetic declines, like neurodegenerative pathologies (252).

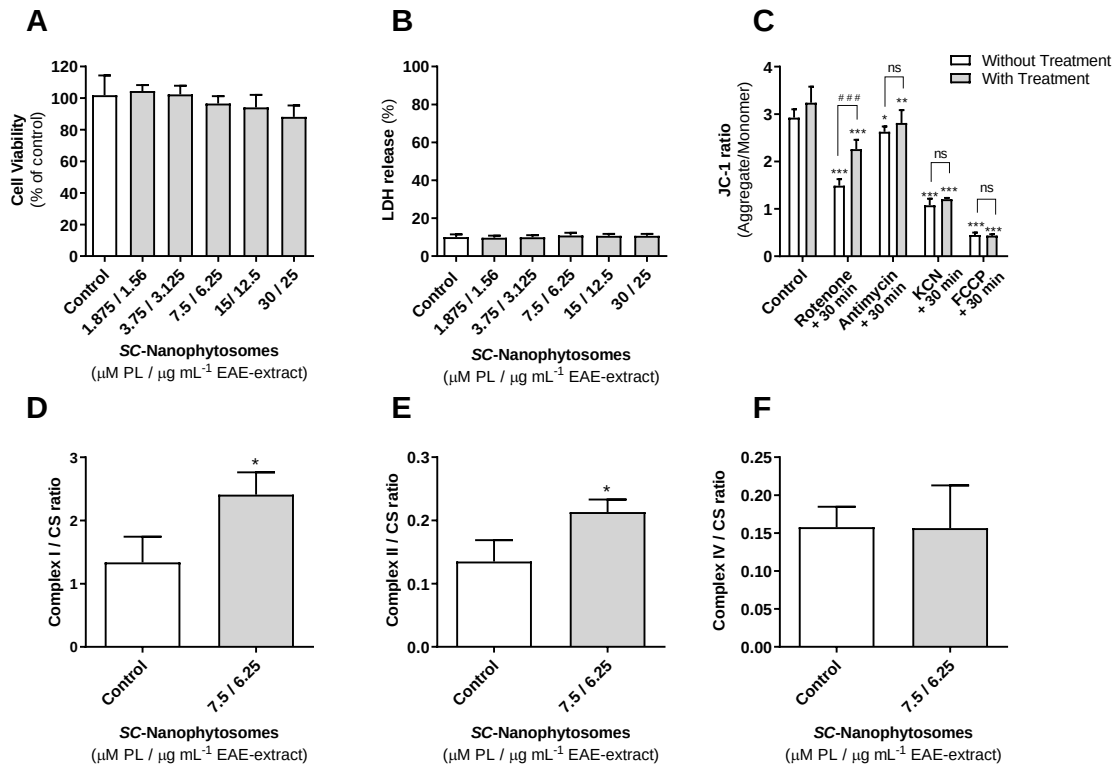


Figure 31. Effects of SC-Nanophytosomes on SH-SY5Y cell viability, membrane integrity, and mitochondrial functionality. Effects of increasing concentrations of SC-Nanophytosomes on the viability of SH-SY5Y cells, assessed by the MTT reduction assay (A) and on the activity of LDH released into the culture medium assay (B) after 24 h of incubation. Total LDH activity was assessed after cell lysis with Triton X-100. (C) Effects of the treatment of SH-SY5Y cells with SC-Nanophytosomes (7.5 μM PL/6.25 μg. mL⁻¹ EAE-extract) on the mitochondrial membrane potential sensitivity to rotenone, antimycin A, KCN, and FCCP, assessed by JC-1 probe. Results are expressed by the ratio of red (aggregate) to green (monomer) fluorescence JC-1. (D, E, F) Effects of the treatment of SH-SY5Y cells with SC-Nanophytosomes (7.5 μM PL/6.25 μg. mL⁻¹ EAE-extract) on the activity of mitochondrial complex I (D), complex II (E), and complex IV (F) normalized by citrate synthase. *, **, *** Significantly different from control condition, with $p \leq 0.05$, and $p < 0.01$, $p < 0.001$, respectively. ### Significantly different from the matched untreated cells, with $p < 0.001$, respectively; n.s without significant differences.

1.4. SC-Nanophytosomes' cellular protective effects

The ability of SC-Nanophytosomes (up to 15 μM PL/12.5 μg mL⁻¹ EAE-extract) to protect SH-SY5Y cells against the harmful effects promoted by toxic concentrations of glutamate (25 mM) or rotenone (3 μM) was assessed considering effects on cell viability and ROS generation. Glutamate is the major excitatory neurotransmitter, and its extracellular accumulation can lead to neuronal cell death and glutamate excitotoxicity is often associated with AD (289). Rotenone, a mitochondrial complex I inhibitor with insecticide properties, can cross the blood-brain barrier and accumulates within the mitochondria of brain cells leading to a PD-like neurodegenerative process (290).

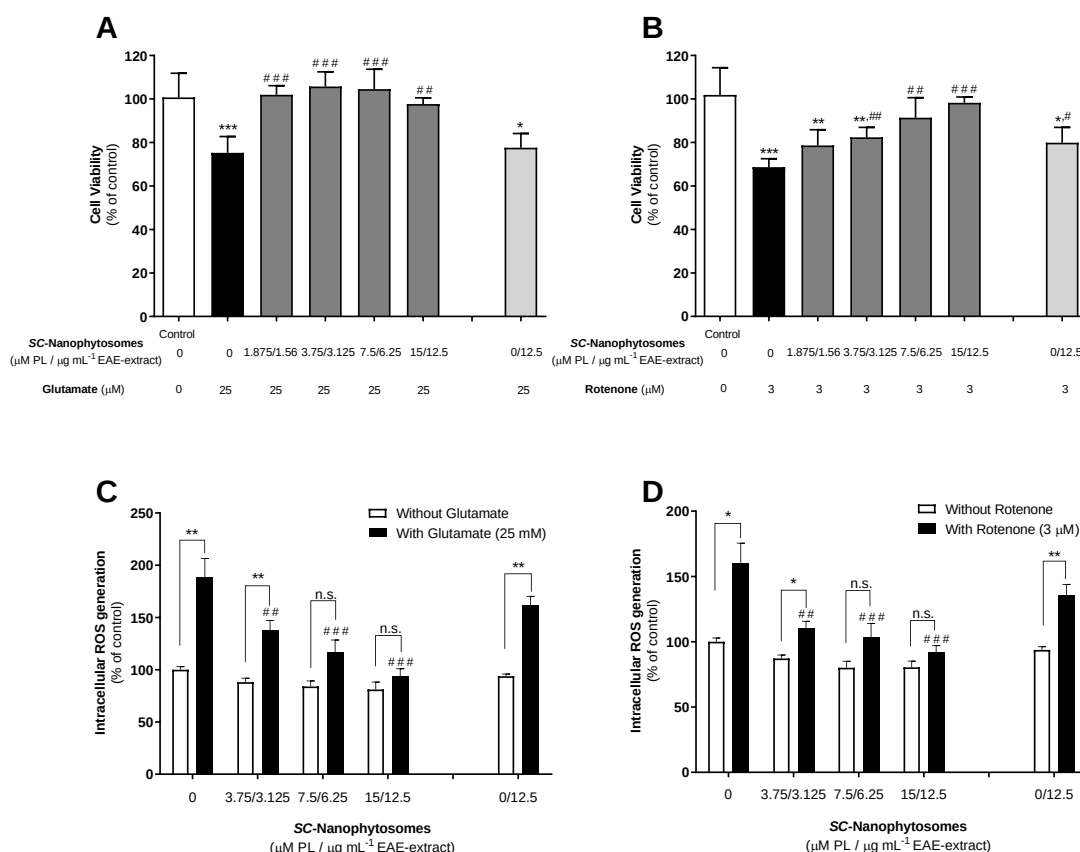


Figure 32. SC-Nanophytosomes protect SH-SY5Y cells against the toxicity and oxidative damage promoted by glutamate or rotenone. Effects of the pre-treatment, for 22 h, of SH-SY5Y cells with increasing concentrations of SC-Nanophytosomes on the cell viability (**A**, **B**) and ROS generation (**C**, **D**) induced by 25 mM glutamate (**A**, **C**) or 3 μM rotenone (**B**, **D**) after 2 h of exposure. *, **, *** Significantly different from control (untreated) cells, with $p \leq 0.05$, $p < 0.01$ and $p < 0.001$, respectively. #, ##, ### Significantly different from glutamate- or rotenone-treated cells. $p \leq 0.05$, $p < 0.01$ and $p < 0.001$, respectively; n.s. without significant differences.

In the control condition, SH-SY5Y cells exposure to glutamate or rotenone for 2 h promoted a significant decrease in cell viability (**Figure 32A** and **32B**, respectively) and a significant increase in the ROS generation (**Figure 32C** and **32D**, respectively). The pre-treatment of SH-SY5Y cells with SC-Nanophytosomes for 22 h protected the cells from glutamate- or rotenone-induced toxicity. As shown in **Figure 32**, all tested SC-Nanophytosomes concentrations prevented the glutamate-induced changes in cell viability (**Figure 32A**). At the same time, the increased generation of ROS promoted by the glutamate was progressively attenuated by the increasing concentrations of the SC-Nanophytosomes (**Figure 32C**). Regarding the effects of rotenone on SH-SY5Y cells pre-treated with the nanoformulation, the SC-Nanophytosomes were able to mitigate rotenone-induced cell toxicity in a concentration-dependent manner, and the two higher tested concentrations entirely prevented the rotenone-induced changes on both cell viability and ROS generation (**Figure 32B** and **32D**). For both toxic stimuli, the protective effects of the non-encapsulated EAE-extract at 12.5 μg mL⁻¹ were also evaluated for comparing

purposes. As shown in **Figure 32**, the magnitude of the beneficial effects promoted by this concentration of EAE-extract is similar to that obtained with SC-Nanophytosomes formulation at 3.75 μM PL/3.125 $\mu\text{g mL}^{-1}$ EAE-extract. Therefore, SC-Nanophytosomes improve at least four times the cellular bioactivity of elderberry polyphenols, suggesting that they are internalized by an endocytic pathway that allows elderberry anthocyanins to deliver in intracellular targets.

1.5. SC-Nanophytosomes cellular uptake and endocytic pathways on SH-SY5Y cells

SC-Nanophytosomes labelled with the lipophilic 16-AP fluorescent probe were used to quantify the uptake of SC-Nanophytosomes by SH-SY5Y cells and to investigate the main steps of the delivery process at the cell level: the passage through the cell membrane, the escape from the endosome, and the delivery in intracellular therapeutic targets (e.g., mitochondria). The aqueous suspensions of 16-AP-labeled SC-Nanophytosomes preserve the typical fluorescence features of the 16-AP probe in lipid bilayers (256). The intensity of the fluorescence in the green region of the spectrum increases with increasing SC-Nanophytosomes concentration, as shown in **Figure 33A**.

Cells incubated for 2 h with increasing concentrations of 16-AP-labeled SC-Nanophytosomes also exhibit the same fluorescence features, indicating that the SC-Nanophytosomes were internalized by SH-SY5Y cells (**Figure 33B**). Moreover, the cellular uptake increases with increasing SC-Nanophytosomes concentration in the incubation medium, thereby an enhancement of cellular internalization efficiency of about five times (from 5.95% to 25.20%) was detected when the SC-Nanophytosomes concentration ranged from 7.5 μM PL/6.25 $\mu\text{g mL}^{-1}$ EAE-extract to 60 μM PL/50 $\mu\text{g mL}^{-1}$ EAE-extract (**Figure 33B**). To investigate the mechanism of cellular uptake of the SC-Nanophytosomes, cells were exposed, for 30 minutes, to specific inhibitors of the main endocytosis pathways, such as amiloride, M β CD, and sucrose (inhibitors of macropinocytosis, caveola-mediated endocytosis, and clathrin-mediated endocytosis, respectively (291,292)) before to be incubated with the SC-Nanophytosomes formulation (30 μM PL/25 $\mu\text{g mL}^{-1}$ EAE-extract) for 2 h. As shown in **Figure 33C**, in the absence of inhibitors pre-treatment, SC-Nanophytosomes internalization efficiency by the cells in cultures is $17.63 \pm 3.30\%$. The pre-treatment of the cells with amiloride or sucrose does not affect the cells' capacity to internalize the SC-Nanophytosomes, suggesting that the lipid-based vesicles' cellular uptake does not involve macropinocytosis nor the clathrin-mediated endocytosis process. In opposition, the internalization efficiency of the SC-Nanophytosomes by the cells pre-treated with M β CD decreased to $5.06 \pm 1.94\%$, about four times lower than the value

obtained in the control condition, suggesting that SC-Nanophytosomes uptake occurs mainly via caveola-mediated endocytosis mechanism.

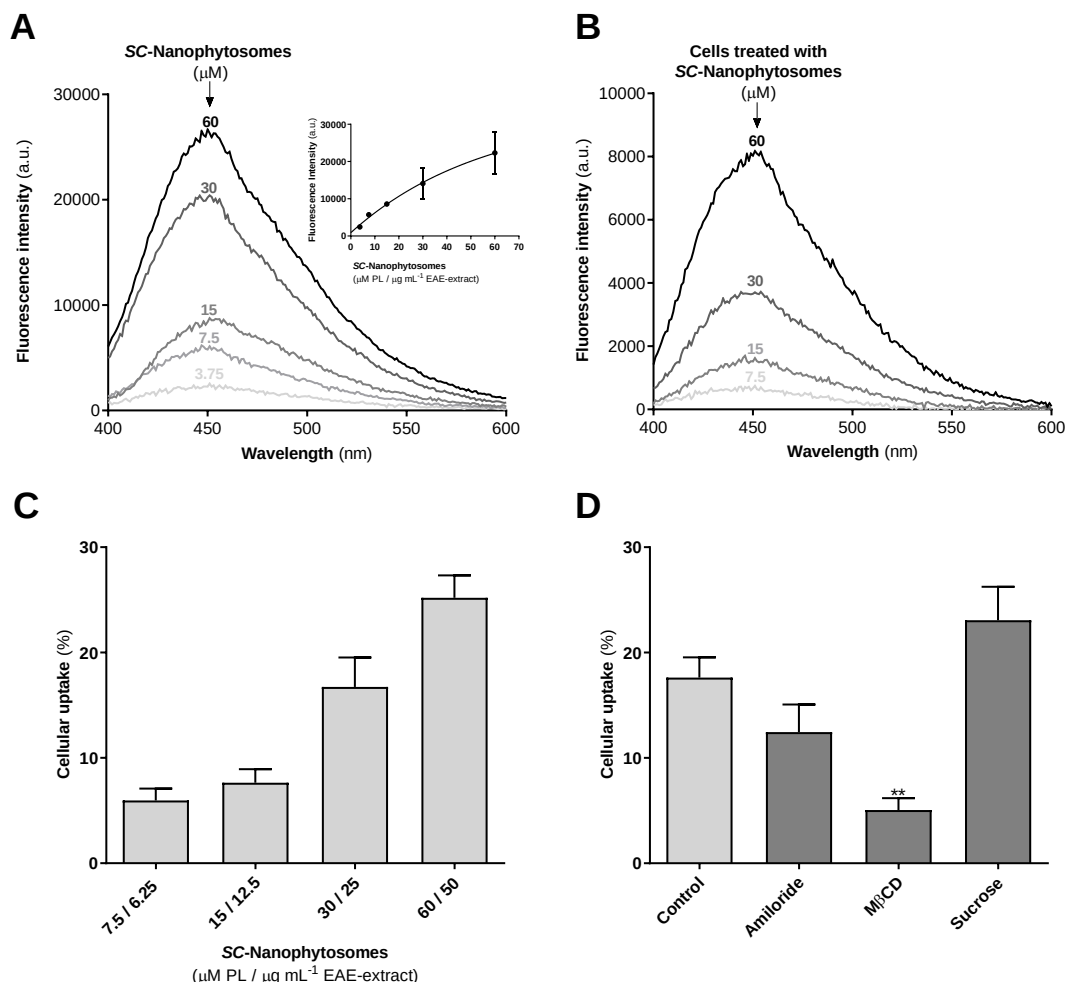


Figure 33. Cellular uptake of SC-Nanophytosomes labelled with the lipophilic fluorescent 16-AP probe, using a probe: phospholipid ratio of 1:300. **(A)** Typical fluorescence emission spectra of aqueous suspensions of 16-AP-labelled SC-Nanophytosomes with different concentrations (until 60 μM PL/50 μg. mL⁻¹ EAE-extract), the peak intensity (green fluorescence) as a function of the SC-Nanophytosomes concentration is represented in the insert considering mean ± std of three independent assays. **(B)** Typical fluorescence emission spectra of SH-SY5Y cells exposed at different concentrations of 16-AP-labelled SC-Nanophytosomes for 2 h. All emission spectra were obtained, setting the excitation wavelength at 365 nm. **(C)** Quantification of cellular uptake of green-fluorescent labelled SC-Nanophytosomes in SH-SY5Y cells exposed at different concentrations for 2 h. **(D)** Effects of the SH-SY5Y cells pre-treatment with amiloride, methyl-β-cyclodextrin (MβCD) or sucrose (endocytosis inhibitors) for 30 minutes on the cellular uptake of SC-Nanophytosomes (30 μM PL/25 μg. mL⁻¹ EAE-extract). ** Significantly different from control (i.e., in the absence of endocytosis inhibitors), with $p < 0.01$.

The internalization of the SC-Nanophytosomes was also evaluated by fluorescence microscopy, using SH-SY5Y cells treated, for 2h, with green-fluorescent labelled SC-Nanophytosomes at 30 μM PL/25 μg mL⁻¹ EAE-extract, and untreated cell as control. For both conditions, Hoechst dye was used to stain cell nuclei (**Figure 34**). In opposition to control cells, treated cells exhibit a strong green fluorescence in addition to the blue fluorescence of nuclei stained by the Hoechst dye. Plus, merging the images obtained

with blue and green filters shows the green fluorescence around the cell nuclei, indicating that SC-Nanophytosomes were internalized by cells. Their intrinsic properties should allow them to escape from the endosome and reach the cytoplasm organelles, including mitochondria (**Figure 34B**).

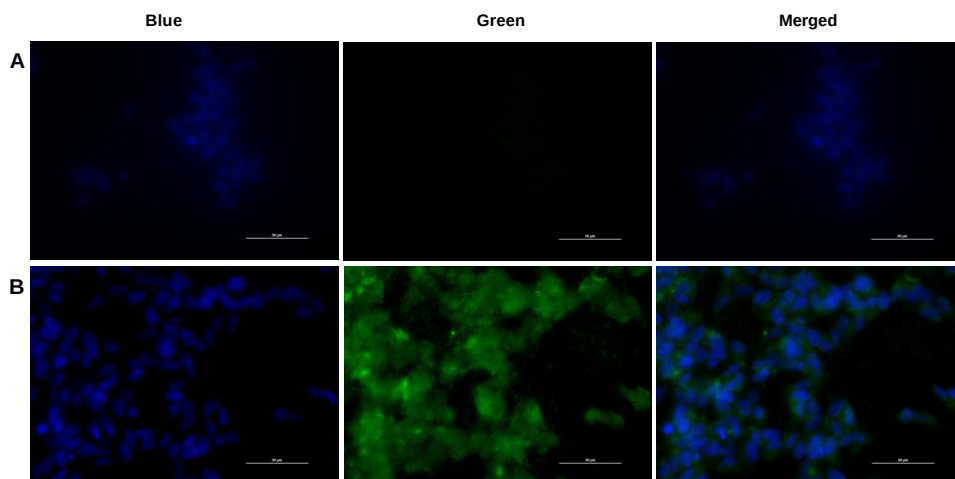


Figure 34. Cellular uptake of SC-Nanophytosomes detected by fluorescence microscopy. Fluorescence microscope images of control (**A**) and green-fluorescent labelled SC-Nanophytosomes treated (**B**) SH-SY5Y cells. Hoechst was used to stain the nuclei of the cells of both conditions.

Normal human fibroblast BJ cells were also used to assess the competence of SC-Nanophytosomes to enter cells and reach mitochondria. This cell type was chosen since it exhibits an extensive mitochondrial network within a large cytosol, and it has been used to explore the mechanisms involved in the pathophysiology of PD and AD (293,294). Fluorescence imaging of cells treated with green-fluorescent labelled SC-Nanophytosomes (at 15 μ M PL/25 μ g mL⁻¹ EAE-extract) and mitochondria stained with red fluorescence (by using Mito Red probe) and nuclei with blue (by using Hoechst dye) are shown in **Figure 35**. Merging the images obtained with blue, green, and red filters suggests that SC-Nanophytosomes reach not only mitochondria (orange) but also other organelles localized close to the cell nucleus. Moreover, SC-Nanophytosomes are not toxic for BJ cells and modulated the response of the mitochondrial membrane potential to rotenone, as described above for neuronal cells (**Figure 35**).

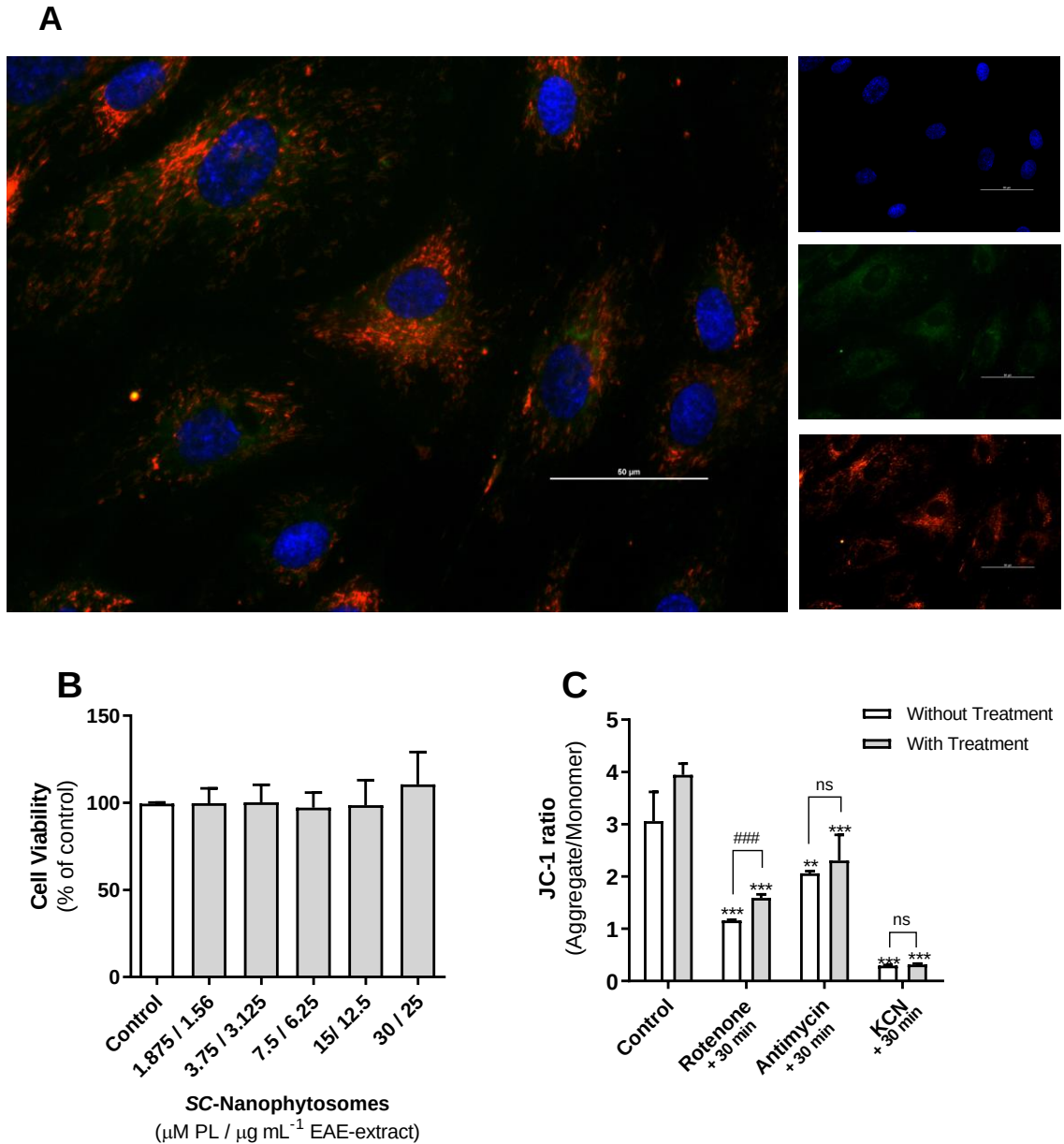


Figure 35. (A) Fluorescence microscope images of normal human fibroblasts BJ cells treated, for 1 h, with green-fluorescent labelled SC-Nanophytosomes (15 µM PL /12.5 µg. mL⁻¹ EAE-extract). Mitochondria were stained with Mito Red probe and nuclei with the blue Hoechst dye. (B) Effects of increasing concentrations of SC-Nanophytosomes on the viability of SH-SY5Y cells, assessed by the MTT reduction assay after 24 h of incubation. (C) Effects of the treatment of SH-SY5Y cells with SC-Nanophytosomes (7.5 µM PL/6.25 µg. mL⁻¹ EAE-extract) on the mitochondrial membrane potential sensitivity to rotenone, antimycin A and KCN, assessed by JC-1 probe. Results are expressed by the ratio of red (aggregate) to green (monomer) fluorescence JC-1. **, *** Significantly different from control condition, with $p < 0.01$ and $p < 0.001$, respectively. ### Significantly different from the matched untreated cells, with $p < 0.001$, respectively; n.s without significant differences.

1.6. Main conclusions

- i) SC-Nanophytosomes are a mixture of sphere- and discoid-shaped vesicles with nanometric hydrodynamic size and very high negative surface charge that preserve the mitochondriotropic properties of the elderberry anthocyanins;
- ii) SC-Nanophytosomes have a negative surface charge with Zeta potential values close to those reported for extracellular vesicles (e.g., exosomes) released by cells with intercellular communication purposes, which may explain its good stability during storage;
- iii) Cellular assays demonstrated the non-cytotoxicity of the SC-Nanophytosomes and confirmed their competence to target mitochondria using the caveola-mediated endocytosis pathway;
- iv) SC-Nanophytosomes enhance the elderberry anthocyanins' ability to modulate mitochondria functionality, improving the activity of mitochondrial complexes I and II and ensuring the $\Delta\Psi_m$ in the presence of a complex I inhibitor;
- v) SC-Nanophytosomes protect neuronal cells against the toxicity induced by rotenone or glutamate, which are toxics with relevance in the context of neurodegeneration;
- vi) SC-Nanophytosomes have excellent properties to support mitochondria-targeted therapies, particularly for pathologies associated with deficient activity of the mitochondrial redox chain activity like AD and PD.

2. Unveiling the bioactivity of the SC-Nanophytosomes on microglia cells

Microglia are central nervous system (CNS) resident immune-like cells that play a crucial role in brain development and brain remodelling functionality. The microglia's phagocytic and scavenging activities include removing dead cells, synapse pruning, and injury repair (295,296). Microglia cells are characterized by remarkable phenotypic plasticity, which can be detected by changes in cellular morphology, patterns of gene expression and released molecules, as well as by changes in proliferative capacity and phagocytic ability. Thus, in response to specific stimuli (e.g., IFN- γ), microglia switch from a resting state to an activated phenotype with the competence to orchestrate an inflammatory response by the production and release of high levels of inflammatory mediators, including NO, tumour necrosis factor (TNF- α) and interleukins (e.g., IL-1 β and IL-6) (296-298). In the healthy homeostatic CNS, the pro-inflammatory activated state of microglia is time-limited, since cells undergo a new phenotype remodelling that triggers inflammatory process resolution, avoiding brain damage (299). Experimental evidence shows that losing homeostatic control over microglial phenotypic plasticity leads to chronic inflammation with consequent brain damage and dysfunction (300). Although chronic inflammation is not considered the main initial event of the neurogenerative cascade, a positive correlation between chronic microglial activation and the progressive neurodegenerative pattern of many neurodegenerative diseases, such as AD and PD, is well established (301-304). Thus, microglia (particularly, the microglia cells with a pro-inflammatory phenotype) represent a promising cellular target for developing therapeutics to tackle neurodegenerative diseases. Unfortunately, the mechanisms underlying the phenotypic plasticity of the microglial remain poorly understood, hindering the design of effective therapeutic approaches.

Scientific evidence shows that the pro-inflammatory mediators produced by microglia cells with a pro-inflammatory phenotype can potentially compromise the functionality of their mitochondria. Additionally, cellular phenotype remodelling requires high metabolic energy levels and biosynthetic precursors dependent on mitochondrial performance (305). Thus, mitochondrial dysfunction of microglia cells with a pro-inflammatory phenotype can also be associated with the chronic inflammation underlying neurodegenerative progression. It is also important to highlight that synaptic mitochondrion (neurons) dysfunction is an early pathological hallmark of AD and PD (98,306-308).

Working within this scientific framework, the present sub-chapter describes the research designed to evaluate if the SC-Nanophytosomes can potentially mitigate chronic inflammation, by targeting the mitochondria of microglia cells. Murine microglial cell line BV-

2 was chosen as a model of the microglia since it has been used for many years to study neuroinflammation, including in the context of degenerative brain disorders. As microglial brain cells, BV-2 cells exhibit phenotypic plasticity in response to external stimuli, with a suitable pattern of morphological and functional changes (309-311). It was also needed to characterize the bioactivity of EAE-extract to understand the effects of SC-Nanophytosomes on BV-2 cells since elderberry anthocyanins are the mitochondriotropic component of the lipid-based nanocarrier. Thus, the effects of both SC-Nanophytosomes and EAE-extract on BV-2 cells will be presented and discussed simultaneously.

It was rationalized as an experimental design with two steps. The first step aims to reveal the effects on BV-2 cells in the resting state, and the second step the effects on BV-2 cells exposed to IFN- γ - a cytokine that induces a pro-inflammatory phenotype. The bioactivity of SC-Nanophytosomes and EAE-extract on BV-2 cells is characterized by considering effects on cell viability, cellular production of NO and ROS, and the $\Delta\Psi_m$.

2.1. Effects on resting state of BV-2 cells

BV-2 cells were exposed to increasing concentrations of EAE-extract (0 - 50 $\mu\text{g}\cdot\text{mL}^{-1}$) or SC-Nanophytosomes (0/0 - 30/25 $\mu\text{M PL}/\mu\text{g}\cdot\text{mL}^{-1}$ EAE-extract) for 24h to assess the effects on cell viability and generation NO. Then cells were used for MTT reduction assay and the culture medium to assess the NO levels by Griess assay. In the range of tested concentrations, EAE-extract and the SC-Nanophytosomes do not affect the viability of BV-2 cells (**Figures 36A** and **36E**) or their ability to produce NO (**Figures 36B** and **36F**), suggesting that they do not have cytotoxicity or competence to drive cells towards a pro-inflammatory phenotype.

To evaluate the effects on ROS generation and $\Delta\Psi_m$, BV-2 cells in 96-well black plates were treated with increasing concentrations of EAE-extract or SC-Nanophytosomes for 24h, and then incubated with DCFH-DA fluorescent probe to assess ROS, or with the mitochondrial-sensitive JC-1 fluorescent probe to assess $\Delta\Psi_m$. As shown in **Figures 36C** and **36D**, EAE-Extract does not produce detectable effects on the rate of ROS production, or the mitochondrial membrane potential of the BV-2 cell in the resting state. Regarding the effects of SC-Nanophytosomes, it is detected a decrease in ROS generation by BV-2 cells with an increase of SC-Nanophytosomes concentrations (**Figure 36G**), and the differences reach statistical significance for the higher tested concentration, *i.e.*, 30/25 $\mu\text{M PL}/\mu\text{g}\cdot\text{mL}^{-1}$ EAE-extract. SC-Nanophytosomes also promote a decrease of $\Delta\Psi_m$ of BV-2 cells, which is well-evidenced ($p<0.001$) for the two higher tested concentrations (**Figure 36G**). It is important to highlight that mitochondria of BV-2 cells in resting state exhibit a low value of

$\Delta\Psi_m$ (JC-1 aggregate/monomer ratio close to 1.5), as compared with the neuron-like SH-SY5Y cells (JC-1 aggregate/monomer ratio close to 3). Therefore, by decreasing the $\Delta\Psi_m$ to JC-1 aggregate/monomer ratio values close to 1.0, SC-Nanophytosomes promote a depolarization of the mitochondrial inner membrane potential that compromises the cell's ability to synthesize ATP through oxidative phosphorylation. In general, depolarization of mitochondrial membrane potential is strategy cells use to attenuate mitochondrial ROS generation (312,313). Comparing these results with those reported in the previous sub-chapter for SH-SY5Y cells, we can conclude that SC-Nanophytosomes affect the activity of microglial cells differently than neurons; thereby, they exhibit bioactivity dependent on cell type.

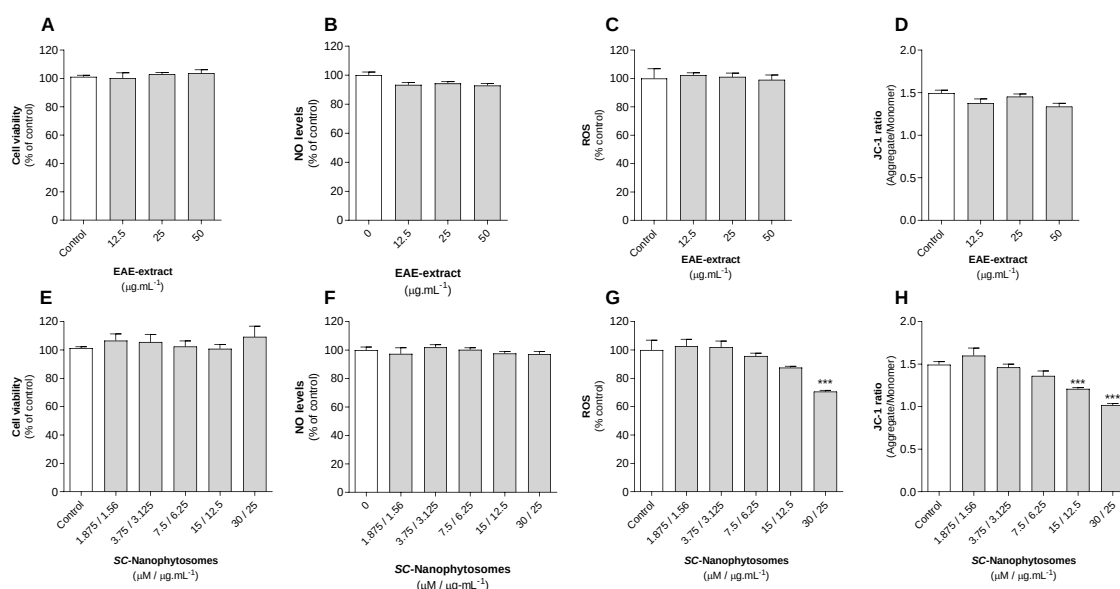


Figure 36. Effects of EAE-extract and SC-Nanophytosomes on BV-2 cell viability, NO levels, ROS levels, and mitochondrial functionality. Effects of increasing concentrations of EAE-extract (**A,B,C,D**) and SC-Nanophytosomes (**E,F,G,H**) on the viability of BV-2 cells, assessed by MTT reduction assay after 24h of incubation (**A,E**), NO levels (**B,F**), ROS levels (**C,G**) and JC-1 ratio (**D,H**). **,*** Significantly different from control condition, with $p < 0.01$ and $p < 0.001$, respectively.

2.2. Effects on BV-2 cells in the presence of IFN- γ – a pro-inflammatory stimuli

In the next step, BV-2 cells were incubated with EAE-extract (0 - 50 $\mu\text{g. mL}^{-1}$) or SC-Nanophytosomes (0/0 - 30/25 $\mu\text{M PL}/\mu\text{g. mL}^{-1}$ EAE-extract) for 2 h and then co-incubated with IFN- γ (10 ng. mL^{-1}) for 22 h. Regarding the effects of IFN- γ , the cell viability of BV-2 cells is not affected by the presence of IFN- γ . Additionally, EAE-extract or SC-Nanophytosomes together with IFN- γ do not show any detectable effects on the BV-2 cell viability (**Figures 37A and 37E**), suggesting the mixtures have no cytotoxicity.

Regarding NO generation, IFN- γ drives BV-2 cells towards a pro-inflammatory phenotype, as indicated by the significant increase in cellular NO production (**Figures 37B** and **37F**). Data in this figure also suggest that EAE-extract, as well as SC-Nanophytosomes, are unable to prevent the phenotypic remodelling of BV-2 cells promoted by IFN- γ since the NO levels detected in the cell culture medium of cells exposed to the mixtures (EAE-extract + IFN- γ ; SC-Nanophytosomes + IFN- γ) remains close to values detected for cells exposed only to IFN- γ . Concerning ROS production, cells stimulated with IFN- γ show a significant decrease in ROS generation compared to control cells in a resting state. The presence of EAE-extract or SC-Nanophytosomes does not mitigate the ROS generation decrease induced by IFN- γ (**Figures 37C** and **37G**). In fact, the experimental data even support the idea that SC-Nanophytosomes potentiate the IFN- γ effects since a cellular ROS generation decrease is detected with increasing SC-Nanophytosomes concentration used in the pre-treatment of the cell before the addition of IFN- γ .

Figures 37D and **37H** show that BV-2 cells stimulated with IFN- γ exhibit lower $\Delta\Psi_m$ than cells in the rest state. The pre-treatment of the cells with EAE-extract or SC-Nanophytosomes did not overcome the effects promoted by IFN- γ on $\Delta\Psi_m$. Additionally, SC-Nanophytosomes at the two slightest concentrations as well as at the higher concentration (30/25 μM PL/ μg . mL^{-1} EAE-extract) potentiate the effects promoted by IFN- γ on $\Delta\Psi_m$. Therefore, the decrease of $\Delta\Psi_m$ seems to run parallel with a decrease in cellular ROS generation.

Although the bioactivity of elderberry anthocyanins and algae polar lipids on microglia cells have not been studied, several studies report the effects of elderberry anthocyanins on macrophages. Thus, the ability of anthocyanins to attenuate NO production by macrophages stimulated by LPS and/or IFN- γ was previously reported (314,315). Another study reports that the bioactivity of elderberry extracts on the NO production by IFN- γ -stimulated macrophages is characterized by a biphasic effect (316). This work also showed that cyanidin 3-O-glucoside is more effective in attenuating ROS production than NO production by IFN- γ -stimulated macrophages (315). However, it is important to highlight that the stimulation of macrophages with IFN- γ triggers an increase in both NO and ROS generation while in BV-2 cells only an increase in NO generation is detected.

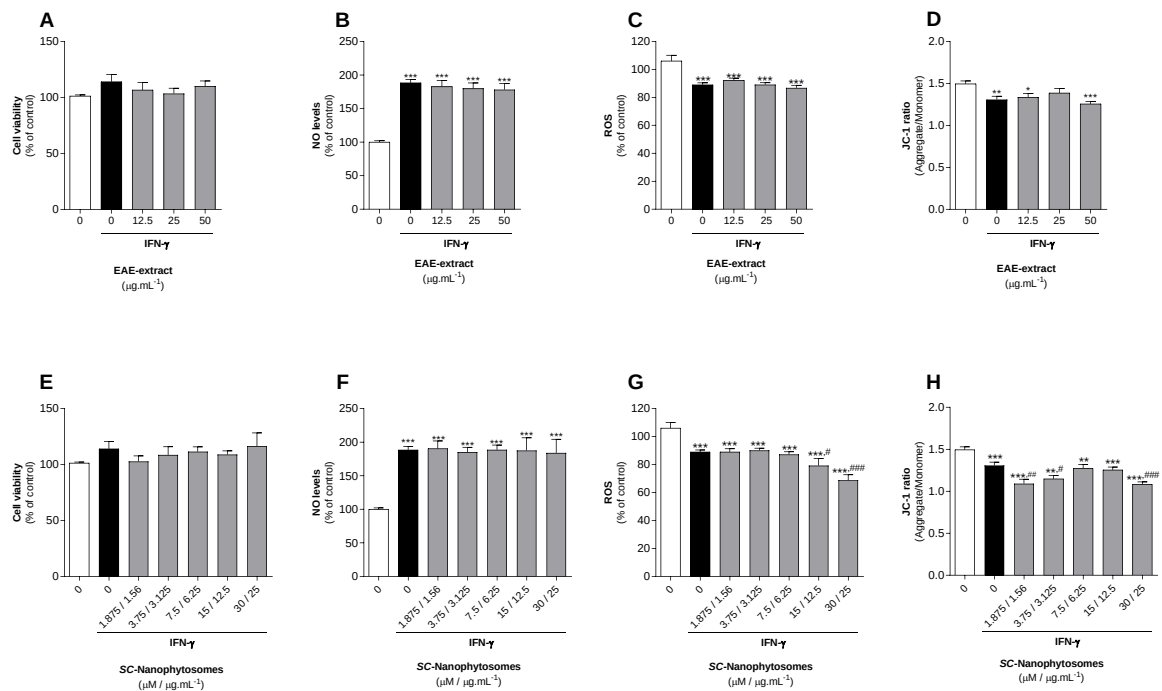


Figure 37. Effects of the treatment of BV-2 cells with increasing concentrations of EAE-extract (**A,B,C,D**) or SC-Nanophytosomes (**E,F,G,H**) on the cell viability by MTT reduction assay (**A, E**, respectively), on NO levels in culture supernatants (**B,F**, respectively), ROS levels (**C,G**, respectively), and JC-1 ratio (**D,H**, respectively) obtained with cells treated with IFN- γ (10 ng. mL⁻¹), and with cells without treatment or IFN- γ . *, **, *** Significantly different from control (without treatment or IFN- γ) with $p \leq 0.05$, and $p < 0.01$, $p < 0.001$, respectively. #, ##, ### Significantly different from the control (without treatment and with IFN- γ), with $p \leq 0.05$, and $p < 0.01$, $p < 0.001$, respectively.

Together, these results suggest IFN- γ promotes a phenotype remodelling of BV-2 cells, characterized by an increase of NO generation simultaneous with a decrease of ROS generation and $\Delta\Psi_m$. EAE-extract and SC-Nanophytosomes have no competence to reduce the cellular generation of NO, suggesting that the ability of IFN- γ to drive BV-2 cells towards a pro-inflammatory phenotype is not affected by the presence of EAE-extract or SC-Nanophytosomes. However, the present data also suggest that SC-Nanophytosomes modulate the metabolic pathways underlying ROS generation and $\Delta\Psi_m$ accompanying the BV-2 cells remodelling towards pro-inflammatory phenotype.

2.3. Effects on mitochondria of BV-2 cells

The metabolic reprogramming underpinning microglial cells' reactive phenotypes remains unclear, making it difficult to rationalise the effects of EAE-extract and SC-Nanophytosomes. Thus, the mitochondrial complexes activity, the cell redox state and the fatty acid profile of membrane lipids were analysed to shed light on metabolic reprogramming that sustains BV-2 cell phenotype remodelling by IFN- γ in the absence and presence of SC-Nanophytosomes or EAE-extract.

Figure 38 shows the activity of mitochondrial respiratory complexes of mitochondria obtained from BV-2 cells incubated without and with EAE-extract at $12.5 \mu\text{g mL}^{-1}$ or SC-Nanophytosomes at $7.5 \mu\text{M}$ PL/6.25 $\mu\text{g mL}^{-1}$ EAE-extract for 24 h, as well from cells stimulated with IFN- γ without and with pre-incubation with EAE-extract or SC-Nanophytosomes. The activities of complexes I, II, and IV were normalized by the activity of the CS, expressed by nmol citrate per min per mg of protein, to discard putative differences in the mitochondria content of the samples. The values of CS activity do not have significant differences among the different groups (CS values: 10.87 ± 0.42 and 11.69 ± 0.17 for mitochondria from control cells in the absence and presence of IFN- γ , respectively; 11.39 ± 0.24 and 10.68 ± 0.59 for mitochondria of cells treated with EAE-extract in the absence and presence IFN- γ , respectively; 10.79 ± 1.88 and 10.20 ± 0.28 for mitochondria of cells treated with SC-Nanophytosomes in absence and presence IFN- γ , respectively).

As shown in **Figure 38**, the mitochondria from BV-2 cells stimulated with IFN- γ (black bars) exhibit the lower activity of the complexes I, II, and IV as compared with mitochondria obtained from cells in resting state (white bars), despite the differences only reach statistical significance for complex II activity. Newsworthy, the exposition of BV-2 cells in resting state to EAE-extract or SC-Nanophytosomes also promotes a decrease in the activity of mitochondrial complexes I and II, but the differences did not reach statistical significance.

Interestingly, data from **Figure 38** also show that the activity of respiratory chain complexes from cells pre-treated with EAE-extract or SC-Nanophytosomes followed by co-incubation with IFN- γ is closer to the activity of mitochondrial complexes from BV-2 cells in resting state, as well evidenced for complex II activity (**Figures 38B** and **38E**). Therefore, the combining effects of EAE-extract or SC-Nanophytosomes with IFN- γ are no additives since EAE-extract and SC-Nanophytosomes decrease the activity of the mitochondrial respiratory complexes (mainly of complex II). However, they also prevent any additional decrease resulting from IFN- γ .

Regarding the understanding of the metabolic changes that support the BV-2 cells in an activated state promoted by IFN- γ , the present data indicate that the activity of mitochondrial redox complexes decreases simultaneously with depolarization of the inner mitochondrial membrane to a level that may compromise the ATP synthesis but avoid the ROS generation at the level of mitochondria. These data suggest that stimulating BV-2 cells with IFN- γ can potentially switch the bioenergetic metabolism from oxidative phosphorylation to glycolysis, as previously reported for macrophages (317-319). SC-Nanophytosomes modulate the cellular effects of the IFN- γ , enhancing the depolarization

of the inner mitochondrial membrane and pulling the activity of the respiratory complexes to values close to the values of BV-2 cells in the resting state. Therefore, combining EAE-extract or SC-Nanophytosomes with IFN- γ enables the transformation of bioenergetics from oxidative phosphorylation to glycolysis. This transition offers enhanced protection to the functionality of the mitochondrial respiratory complex, which is expected to positively impact the cells' capacity to undergo new phenotype remodelling.

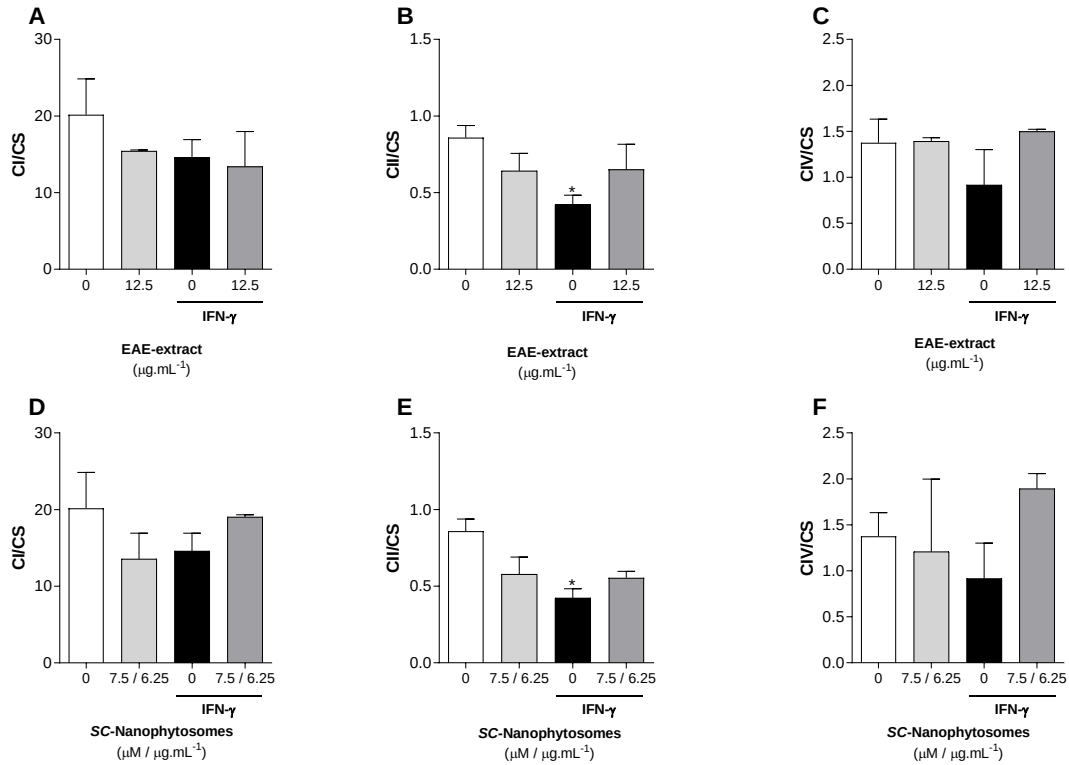


Figure 38. Effects of the treatment of BV-2 cells with EAE-extract (12.5 μ g. mL⁻¹) and SC-Nanophytosomes (7.5 μ M PL/6.25 μ g.mL⁻¹ EAE-extract) on the activity of mitochondrial complexes I (A,D, respectively), II (B,E, respectively) and IV (C,F, respectively), normalized by citrate synthase, obtained in control cells with and without IFN- γ (10 ng.mL⁻¹). * Significantly different from control (without treatment or IFN- γ) with $p \leq 0.05$.

2.4. Effects on the cellular redox state of BV-2 cells

Microglia activation undergoes a transformation that involves increased production of various proinflammatory cytokines and acquisition of cellular profile able to generate oxidative bursts (320,321). In this way, cell survival is highly dependent on the endogenous antioxidant defence system to adjust the cellular redox state, which includes the modulation of the activity of antioxidant enzymes like SOD, CAT, GR, and GPx (322). Thus, the activities of the SOD, CAT, GR, and GPx enzymes were assessed in mitochondria-free cytosolic fraction obtained from BV-2 cells without and with incubation of EAE-extract at 12.5 μ g mL⁻¹ or SC-Nanophytosomes at 7.5 μ M PL/6.25 μ g mL⁻¹ EAE-extract for 24 h, as

well from cells stimulated with IFN- γ without and with pre-incubation with EAE-extract or SC-Nanophytosomes (**Figure 39**).

Regarding the BV-2 cells stimulated with IFN- γ , it is observed that lower CAT and GR activities and higher GPx activity when compared with the cells in resting state, however, differences did not reach statistical significance. Additionally, the activity of antioxidant enzymes in the cytosol of the cells in the resting state exposed to EAE-extract or SC-Nanophytosomes did not reveal any statistical difference when compared with the control cells.

Concerning the cells pre-treated with EAE-extract or SC-Nanophytosomes followed by co-incubation with IFN- γ , SC-Nanophytosomes plus IFN- γ did not affect the activity of the antioxidant enzymes, while EAE-extract plus IFN- γ promote a significant increase of SOD activity when compared both with cells in resting or activated states, without affecting the activity of the other enzymes (**Figure 39**). Therefore, SC-Nanophytosomes do not affect the activity of antioxidant enzymes of BV-2 cells, while the cells pre-treatment with EAE-extract followed with IFN- γ only modulate the activity of SOD. These data suggest that the phenotype remodelling induced by IFN- γ has minor effects on the enzymatic antioxidant defence system of BV-2 cells.

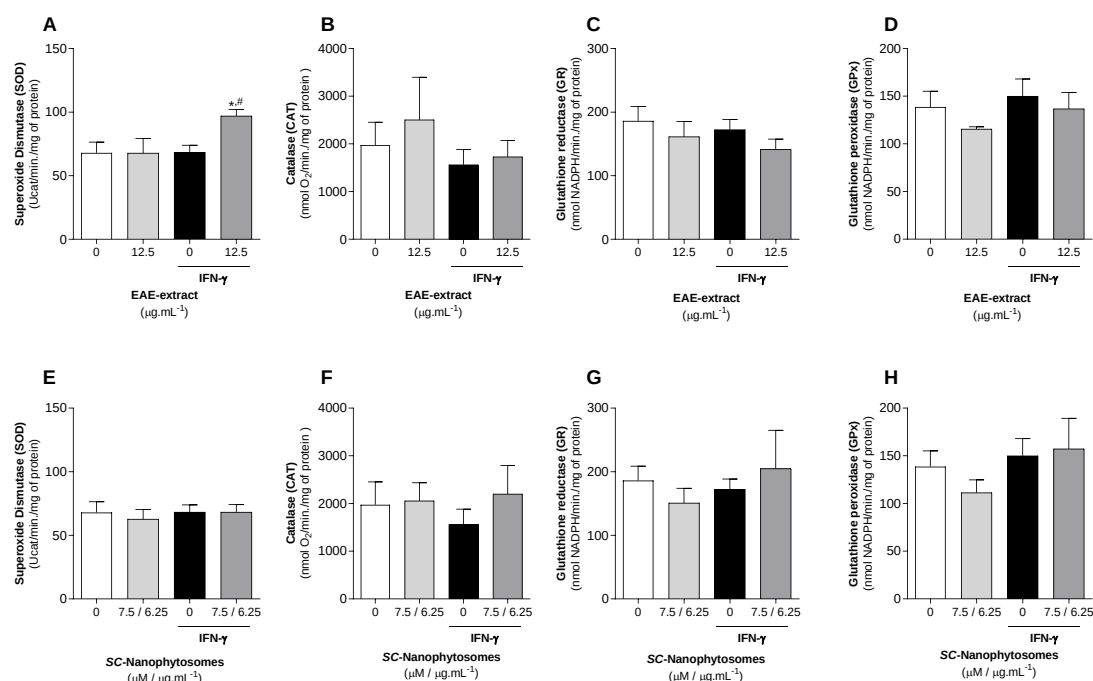


Figure 39. Effects of the treatment of BV-2 cells with EAE-extract (12.5 $\mu\text{g.mL}^{-1}$) and SC-Nanophytosomes (7.5 μM PL/6.25 $\mu\text{g.mL}^{-1}$ EAE-extract) on the activity of antioxidant enzymes SOD (**A,E**, respectively), CAT (**B,F**, respectively), GR (**C,G**, respectively) and GPx (**D,H**, respectively), obtained in control cells with and without IFN- γ (10 ng.mL^{-1}). * Significantly different from control (without treatment or IFN- γ) with $p \leq 0.05$. # Significantly different from the control (without treatment and with IFN- γ), with $p \leq 0.05$.

2.5. Effects on the fatty acid profile of BV-2 cells

The changes in the fatty acid profile of cell lipidome were also analysed to characterise how EAE-extract or SC-Nanophytosomes modulate the phenotype remodelling of BV-2 cells induced by IFN- γ . The obtained results are displayed in **Figure 40A** (effects of EAE-extract) and **Figure 40B** (effects of SC-Nanophytosomes). Thus, the fatty acid profile was evaluated using the total lipids extracted from BV-2 cells in resting state (CTRL group), BV-2 cells stimulated with IFN- γ (IFN- γ group) without and with pre-incubation of EAE-extract at 12.5 $\mu\text{g mL}^{-1}$ (EAE-extract + IFN- γ group) or SC-Nanophytosomes at 7.5 μM PL/6.25 $\mu\text{g mL}^{-1}$ EAE-extract (SC-Nanophyt + IFN- γ group).

Data from **Figure 40** show that BV-2 cells in a resting state exhibit a fatty acid profile with thirty-one fatty acids species, comprising eleven SFA, seven MUFA, and thirteen PUFA. Regarding SFA, C16:0 is the most abundant, followed by C18:0. Lauroleic (C12:1) and oleic acid (C18:1) acids are the most abundant species of MUFA. Regarding PUFA, C18:3n-3 is the most abundant molecular species in the n-3 series, while C18:2n-6 is the most abundant in the n-6 series. AA (C20:4n-6) and γ -linolenic (C18:3n-6) acid also have relative abundances higher than 3%. IFN- γ stimulation promotes small changes in the fatty acid profile of BV-2 cells, with differences detected by a significant decrease in the levels of C16:0 and C12:1 and by a significant increase in the levels of C18:0 and C14:1. The cells pre-treated with EAE-extract seems to prevent the effects promoted by stimulation with IFN- γ since cells of the EAE-extract + IFN- γ group have levels of C16:0 and C14:1 species equal to the cells of the CTRL group, but different from cells of IFN- γ group (**Figure 40A**).

Regarding the effects of cells pre-incubation with SC-Nanophytosomes on the stimulation promoted by IFN- γ (**Figure 40B**), we can assert that SC-Nanophytosomes effects follow the same trend described for EAE-extract. However, the significant differences are only detected at the level of C14:1. Additionally, the relative abundance of C18:2n-6 in BV-2 cells pre-treated with SC-Nanophytosomes or EAE-extract surpasses the levels detected in cells of both CTRL and IFN- γ groups (**Figures 40A and 40B**). Regarding the increase in the levels of C18:0 induced by IFN- γ stimulation, the EAE-extract does not counteract this increase. On the other hand, the SC-Nanophytosomes can reduce the C18:0 levels to values near the CTRL.

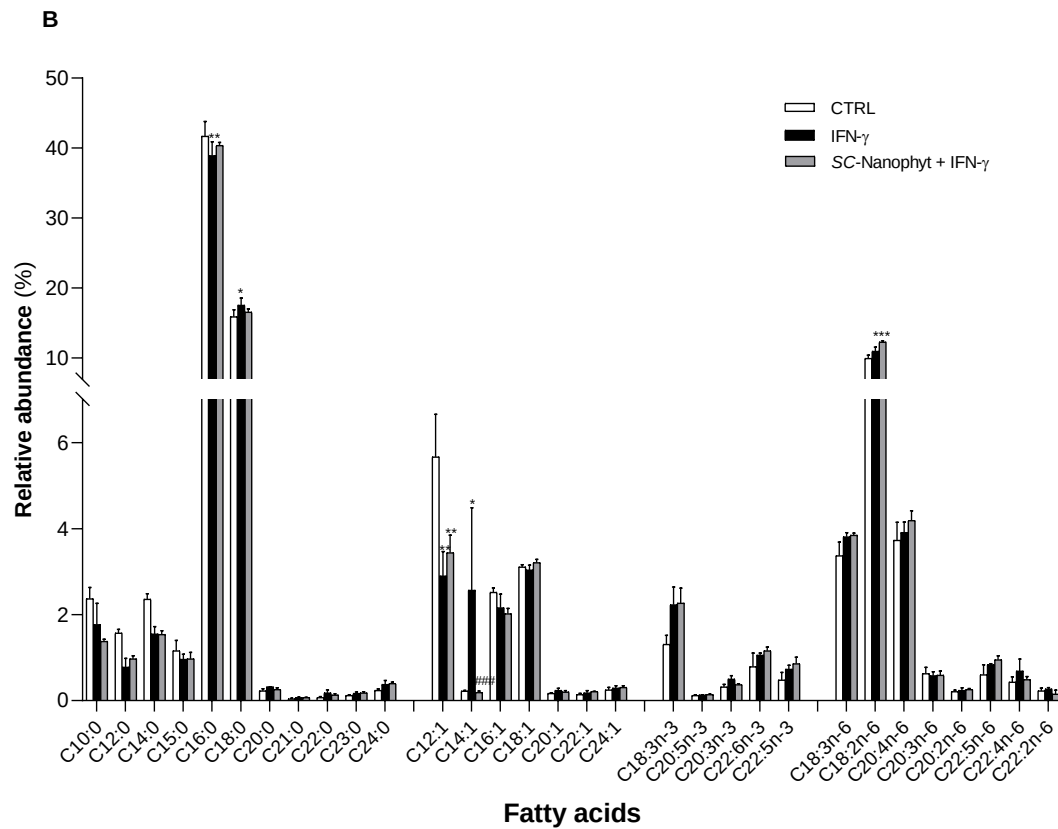
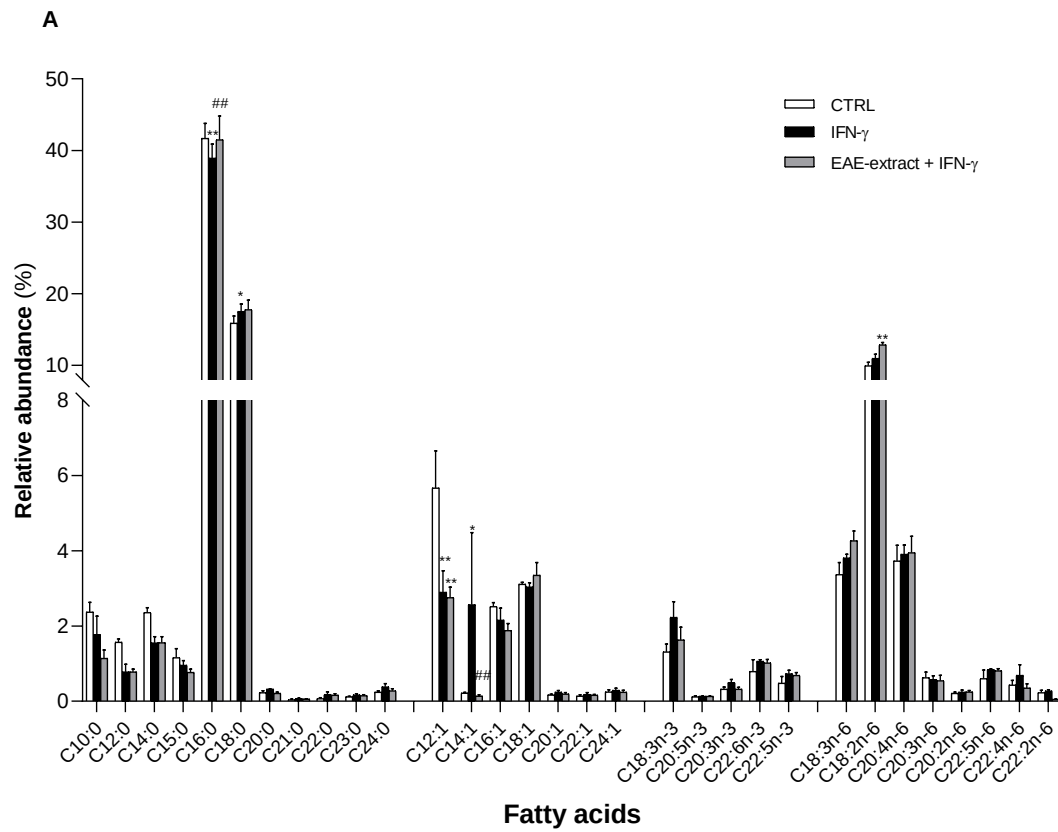


Figure 40. Fatty acid profile of total lipid extract obtained from BV-2 cells from control (CTRL), treated IFN- γ (10 ng.mL⁻¹) and pre-treated with EAE-extract 12.5 μ g.mL⁻¹) (A) or with SC-Nanophytosomes (7.5 μ M PL/6.25 μ g.mL⁻¹ EAE-extract) (B) followed with stimulation with IFN- γ . C10:0= decanoic acid, C12:0= lauric acid, C14:0= myristic acid, C15:0= pentadecanoic acid, C16:0= palmitic acid, C18:0= stearic acid, C20:0= arachidonic acid, C21:0= heneicosanoic acid, C22:0= docosanoic acid, C23:0= tricosanoic acid, C24:0= lignoceric acid, C12:1= lauroleic acid, C14:1= myristovaccenic acid, C16:1= palmitoleic acid, C18:1= oleic acid, C20:1= eicosenoic acid, C22:1= docosenoic acid, C24:1= nervonic acid, C18:3n-3= α -linolenic acid, C20:5n-3= EPA, C20:3n-3= eicosatrienoate acid, C22:6n-3= DHA, C22:5n-3= docosapentaenoic acid (DPA), C18:3n-6= γ -linolenic acid, C18:2n-6= linoleic acid, C20:4n-6= arachidonic acid (AA), C20:3n-6= DGLA, C20:2n-6= dihomo-linoleic acid, C22:5n-6= Osbond acid, C22:4n-6= docosatetraenoate, C22:2n-6= docosadienoic acid. Error bars represent SEM for n = 3 independent experiments. .*,**,*** Significantly different from control (without treatment or IFN- γ) with p $\leq$ 0.05, p < 0.01 and p < 0.001, respectively. #,### Significantly different from the control (without treatment and with IFN- γ), with p $\leq$ 0.05 and p < 0.001, respectively.

The total levels of SFA, MUFA and PUFA, with n-3 and n-6 PUFA levels discriminated, as well as the PUFA/SFA, MUFA/SFA, n-6/n-3 PUFA and DHA/AA ratios and unsaturation index, are displayed in **Table 3**. These parameters were calculated by using the quantitative data in **Figure 40**.

Compared with BV-2 cells in resting state (CTRL), IFN- γ stimulation promoted a slight decrease in the total levels of SFA and MUFA with a simultaneous increase in total PUFA. However, these changes did not reach statistical significance. Additionally, the pre-treatment with EAE-extract or SC-Nanophytosomes enhances the effects promoted by IFN- γ stimulation, and the increased levels of PUFA reach statistical significance in both EAE-extract + IFN- γ and SC-Nanophyt + IFN- γ groups when compared to the CTRL group (**Table 3**). The slight increase in PUFA levels of the IFN- γ group is accompanied by a decrease in the n-6/n-3 PUFA ratio and an increase in PUFA/SFA and DHA/AA ratios, but compared to the CTRL group, these changes did not reach statistical significance. However, the changes in SFA, MUFA, and PUFA levels promoted by cells' stimulation with IFN- γ lead to a significant increase in the unsaturation index (**Table 3**). Therefore, the increased membrane fluidity can be considered a lipid marker of IFN- γ -induced BV-2 cell phenotype remodelling.

Regarding the effects of EAE-extract plus IFN- γ on the fatty acid profile of whole lipidome of BV-2 cells, the significant increase in the unsaturation index induced by IFN- γ stimulation is prevented by pre-incubation of cells with EAE-extract. Despite the PUFA level in EAE-extract + IFN- γ group being higher than in the IFN- γ group, the levels of MUFA in this group decrease about IFN- γ , preserving the unsaturation index similar to the CTRL group. Additionally, the n-3 PUFA level in EAE-extract + IFN- γ group did not increase as much as the n-6 PUFA, preserving the n-6/n-3 ratio of the CTRL group, supporting the idea that the EAE-extract counteract the changes induced by IFN- γ stimulation (**Table 3**).

Regarding SC-Nanophytosomes effects, the significant increase in the unsaturation index and the decrease in the n-6/n-3 ratio promoted by IFN- γ stimulation are not avoided by cells pre-incubation with SC-Nanophytosomes (**Table 3**).

Table 3. General parameters of the fatty acid profile of phospholipid extracts obtained from BV-2 cells from control (CTRL), IFN- γ and pre-treated with EAE-extract (12.5 $\mu\text{g.mL}^{-1}$) or with SC-Nanophytosomes (7.5 μM PL/6.25 $\mu\text{g.mL}^{-1}$ EAE-extract) together with IFN- γ . AA - arachidonic acid, DHA – docosahexaenoic acid, SFA – saturated fatty acids, MUFA – monounsaturated fatty acids, PUFA – polyunsaturated fatty acids, UI – unsaturation index. *,** Significantly different from control (without treatment or IFN- γ stimulation) with $p \leq 0.05$ and $p < 0.01$ respectively.

	CTRL	IFN- γ	EAE-extract + IFN- γ	SC-Nanophyt + IFN- γ
SFA	65.74 $\pm$ 2.78	62.66 $\pm$ 1.78	64.38 $\pm$ 3.78	62.84 $\pm$ 1.16
MUFA	12.07 $\pm$ 1.34	11.37 $\pm$ 2.35	8.72 $\pm$ 1.19	9.58 $\pm$ 0.39
PUFA	22.19 $\pm$ 4.03	25.97 $\pm$ 0.93	26.89 $\pm$ 2.69 *	27.58 $\pm$ 1.42 **
n-6 PUFA	19.16 $\pm$ 3.02	21.32 $\pm$ 1.56	23.10 $\pm$ 1.94	22.78 $\pm$ 1.02
n-3 PUFA	3.03 $\pm$ 1.08	4.66 $\pm$ 0.91	3.79 $\pm$ 0.82	4.80 $\pm$ 0.74
PUFA/SFA	0.34 $\pm$ 0.07	0.41 $\pm$ 0.01	0.42 $\pm$ 0.06	0.44 $\pm$ 0.03
MUFA/SFA	0.18 $\pm$ 0.01	0.18 $\pm$ 0.04	0.14 $\pm$ 0.03	0.15 $\pm$ 0.01
n-6/n-3	6.94 $\pm$ 1.77	4.84 $\pm$ 1.37	6.30 $\pm$ 0.93	4.84 $\pm$ 0.66
DHA/AA	0.19 $\pm$ 0.13	0.27 $\pm$ 0.01	0.26 $\pm$ 0.03	0.28 $\pm$ 0.02
UI	34.26 $\pm$ 2.78	37.35 $\pm$ 1.77*	35.61 $\pm$ 3.79	37.16 $\pm$ 1.16*

Thus, the competence of SC-Nanophytosomes to modulate the effects promoted by IFN- γ stimulation on fatty acid profile and concomitant membrane fluidity is significantly lower than EAE-extract. However, it is important to highlight that the pro-inflammatory activated state of microglia is time limited. Therefore, conducting assays at only one time point, such as IFN- γ incubation for 22 hours, does not provide a comprehensive understanding of the complete phenotype remodelling process. In general, cells with a pro-inflammatory phenotype release pro-inflammatory lipid mediators, which result from the sequential activity of phospholipase A2 (PLA2) and cyclooxygenase (COX) or lipoxygenase (LOX) enzymes on n-6 PUFA (323). Thus, a decrease in the n-6/n-3 PUFA ratio of membrane phospholipids is expected during the activated stage of cells. The n-6/n-3 PUFA ratio IFN- γ group is about 30% lower than in the CTRL group, supporting this general idea of cells with pro-inflammatory phenotype.

However, microglia with pro-inflammatory phenotypes can undergo a new phenotype remodelling to trigger inflammatory process resolution. This phenotype is associated with the release of lipids mediator (resolvins), resulting from the mobilisation of n-3 PUFA of membrane phospholipids by PLA2, COX and LOX enzymes that leads to an increase of n-6/n-3 PUFA ratio (324). Therefore, the differential effects SC-Nanophytosomes and EAE-extract may emerge from the modulation of the effects promoted by IFN- γ stimulation for pro-inflammatory phenotype and the time required for cells to undergo a new phenotype remodelling to resolve the inflammation.

2.6. SC-Nanophytosomes cellular uptake and endocytic pathway on BV-2 cell line

The previous sub-chapter unveiled that SC-Nanophytosomes reach the mitochondria of neurons after cell uptake by a caveola-mediated endocytosis pathway. It is also known that the bioactivity of any lipid-based nanomedical platform depends on the cell internalization pathway and the nanocarrier's ability to escape from the endosome (325,326). Thus, the cellular uptake of SC-Nanophytosomes by BV-2 cells is characterized to assess their bioavailability for microglia and try to understand the specific bioactivity of this type of cells.

SC-Nanophytosomes labelled with the lipophilic 16-AP fluorescent probe were used to quantify the uptake by BV-2 cells. BV-2 cells incubated with increasing concentrations of aqueous suspensions of 16-AP-labelled SC-Nanophytosomes exhibit an increase of the fluorescence intensity in the spectrum's green region, indicating that the SC-Nanophytosomes are internalized by BV-2 cells (**Figure 41A**). As shown in **Figure 41B**, the cellular uptake increases with increasing concentrations of SC-Nanophytosomes in the incubation medium. In BV-2 cells, the internalization efficiency increases from 2.84% to 8.42% when the SC-Nanophytosomes ranged from 7.5 μM PL/6.25 $\mu\text{g}\cdot\text{mL}^{-1}$ EAE-extract to 60 μM PL/50 $\mu\text{g}\cdot\text{mL}^{-1}$ EAE-extract. Besides, the cellular uptake does not go beyond 10%, while in SH-SY5Y, it reaches around 25% in similar assay conditions.

BV-2 cells were submitted to treatments with specific inhibitors of the main endocytic pathways, such as amiloride, sucrose, and M β CD to investigate the routes of liposomes internalization before their incubation with SC-Nanophytosomes at 30 μM PL/25 $\mu\text{g}\cdot\text{mL}^{-1}$ EAE-extract for 2h. Without cell pre-treatment with inhibitors, SC-Nanophytosomes internalization efficiency by the BV-2 cells is 5.88%. The pre-treatment of the cells with amiloride, sucrose or M β CD (inhibitors of micropinocytosis, clathrin-mediated endocytosis, or caveola-mediated endocytosis, respectively) does not affect the internalization efficiency of SC-Nanophytosomes by BV-2 cells, suggesting that the cellular uptake does not involve any of these endocytic processes.

Despite microglia cells possessing a broad range of endocytic mechanisms, including the tested here, phagocytosis is their main mark, since they are in the first line of defence against invading pathogens (327-329). Thus, phagocytosis inhibitors should be considered in future studies to unveil the SC-Nanophytosomes internalization pathway by microglia.

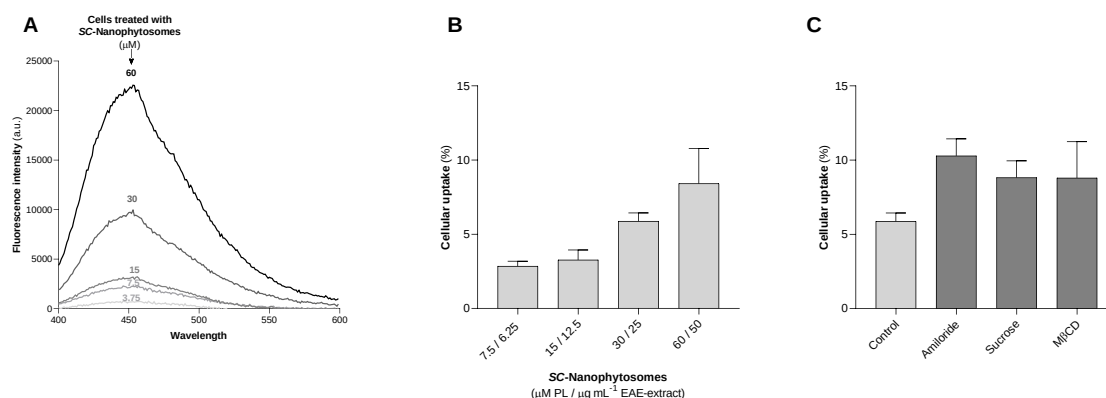


Figure 41. Cellular uptake of SC-Nanophytosomes labelled with the lipophilic fluorescent 16-AP probe, using a probe: phospholipid ratio of 1:300. Typical fluorescence emission spectra of BV-2 cells exposed at different concentrations of 16-AP-labelled SC-Nanophytosomes for 2 h (until 60 μM PL/50 $\mu\text{g. mL}^{-1}$ EAE-extract), the peak intensity (green fluorescence) as a function of the SC-Nanophytosomes concentration is represented in the insert considering mean $\pm$ std of three independent assays (**A**). All emission spectra were obtained, setting the excitation wavelength at 365 nm. Quantification of cellular uptake of green-fluorescent labelled SC-Nanophytosomes in BV-2 cells exposed at different concentrations for 2 h (**B**). Effects of the BV-2 cells pre-treatment with amiloride, methyl- β -cyclodextrin (M β CD), or sucrose (endocytosis inhibitors) for 30 min on the cellular uptake of SC-Nanophytosomes (30 μM PL/25 $\mu\text{g. mL}^{-1}$ EAE-extract) (**C**). *Significantly different from the control condition, with $p \leq 0.05$.

2.7. Main conclusions

- i) Cellular assays with BV-2 cells show that EAE-extract and SC-Nanophytosomes do not alter the viability or the NO production of BV-2 cells, suggesting no cytotoxicity;
- ii) EAE-extract and SC-Nanophytosomes did not show competence to overcome the alterations promoted by IFN- γ , such as the NO production, the decrease in ROS generation and the decrease in JC-1 ratio;
- iii) EAE-extract or SC-Nanophytosomes plus IFN- γ allow the bioenergetic remodelling from oxidative phosphorylation to glycolysis, but with the functionality of the mitochondrial respiratory complex is more protected, with a predicted positive impact on cells' ability to undergo new phenotype remodelling;
- iv) SC-Nanophytosomes do not have effects on the activity of antioxidant enzymes, while the EAE-extract only modulate the activity of SOD, suggesting that the phenotype remodelling induced by IFN- γ has minor effects on the enzymatic antioxidant defence system of BV-2 cells;
- v) The alterations promoted by IFN- γ in the fatty acid profile of BV-2 cell homogenates were ameliorated when treated both with EAE-extract or SC-Nanophytosomes;
- vi) The competence of SC-Nanophytosomes to modulate the effects promoted by IFN- γ stimulation on fatty acid profile and concomitant membrane fluidity is significantly lower than EAE-extract;
- vii) SC-Nanophytosomes have the competence to internalize the BV-2 cells, however, the cellular uptake seems not to involve any endocytic pathway tested;
- viii) These results, together with the results with SH-SY5Y cells shown in the previous chapter are a good prelude to the use SC-Nanophytosomes for animal studies, aiming to test this mitochondria-targeted therapy for neurodegenerative diseases, including AD and PD.

CHAPTER III

Results and Discussion

In vivo assays

3. Brain effects of SC-Nanophytosomes on a rotenone-induced rat model of Parkinson's disease - a proof of concept for a mitochondria-targeted therapy

Developing effective therapies to fight neurodegeneration is a major scientific challenge and an imperative need to ensure healthy lives and promote well-being for all ages. At least 50 million people suffer from a neurodegenerative pathology, with AD and PD being the most common diseases, without any reliable treatment to stop or slow the progression of the degenerative process (117,330).

Despite the fog that still surrounds the molecular mechanisms that trigger the neurodegenerative process, it is known that a successful therapy should be able to restore the functional connectivity among brain regions lost during the neurodegenerative process as a consequence of distorted and/or disrupted neural networks with progressive severity. The functional connectivity of brain regions is highly dependent on mitochondrial performance, as indicated by the solid spatiotemporal correlation between the synchronised neuronal activity in the frequency band (electrocorticogram, ECoG connectivity) and the high oxygen and glucose consumption patterns (blood-oxygen-level-dependent, BOLD connectivity) (118,330). It is well-known that mitochondrial dysfunction, resulting from abnormal respiratory chain activity, is a cellular hallmark of many neurodegenerative diseases (119,331-334). In AD and PD, mitochondrial dysfunction is mainly detected by a deficient complex I activity and by an abnormal membrane lipid profile (98,135). Additionally, a dysfunctional mitochondrial redox chain affects the bioenergetic performance of the cell and the ATP-dependent metabolic pathways, increasing the generation of ROS that trigger profound changes in the cellular signalling cascades, promoting oxidative stress, chronic inflammation, and the accumulation of misfolded proteins - other pathological marks of diseases (134,140,141,335). Mitochondrial dysfunction may have a negative impact on cell-to-cell communication processes, compromising brain connectivity before promoting the death of individual cells. There is scientific evidence for many neurodegenerative diseases that a massive synapse loss precedes the neurons' death (98). Thus, recognising the importance of mitochondria in brain intercellular networks is shifting the paradigm of neurodegeneration therapies toward mitochondria.

As explained in the general introduction, the first generation of mitochondria-targeted therapy for AD and PD, supported by antioxidant molecules, was designed to combat the oxidative damage resulting from the overproduction of ROS by a dysfunctional mitochondrial redox chain. Despite their positive outcomes in animal models, the results obtained in clinical trials were disappointing as mentioned in the chapter Introduction.

This chapter addresses the mitochondria-targeted therapy challenge from a different perspective, rationalising the structural and functional mitochondrial parameters affected by neurodegeneration that are impaired complex I activity and the changes in mitochondrial lipidome in PD and AD. The therapeutic tool is a SC-Nanophytosomes formulation that revealed, in vitro assays, competence to target mitochondria and to improve the activity of the mitochondrial redox chain, protecting the cells against the toxicity induced by rotenone or glutamate - toxins with relevance in the context of neurodegeneration. Thus, the SC-Nanophytosomes are moved to in vivo studies to investigate their ability to work as a mitochondria-targeted therapy when delivered orally. A rotenone-induced rat model of PD that mimic many behavioural and neuropathological features of the human disease, including mitochondrial complex I impairment (335,336), was used to obtain a proof-of-concept.

3.1. SC-Nanophytosomes formulation stability under pH changes that mimic the gastrointestinal tract environment

Developing of lipid-based nanoformulation to target the brain by oral administration requires nanoplatforms with the competence to overcome several physiological barriers. The nanoformulation should preserve its structural and functional properties throughout the digestive tract to maximize its uptake at different compartments (e.g., buccal cavity, small intestine). From the mouth to the small intestine, the SC-Nanophytosomes are subjected to different external conditions, including pH changes from 6.2–7.4 values (pH of saliva) to the high acidic conditions in the stomach (pH 1.0–2.0) and then for quasi-neutral pH environment along the small intestine (pH 6.0–7.5) (337,338). Thus, to evaluate the impact of the pH changes throughout the gastrointestinal tract on the SC-Nanophytosomes stability, the formulation was submitted to successive dialysis against an aqueous buffer solution with pH values representative of the above-mentioned gastrointestinal tract compartments. The effects on SC-Nanophytosomes properties were assessed regarding the EE of the EAE-extract, vesicle size, and surface charge (**Figure 42**).

The SC-Nanophytosomes, prepared with a concentration of 600 μ M of algae membrane polar lipids and 0.5 mg/mL of EAE-extract, exhibit the typical absorption bands of elderberry phenolics with a maximum at 280 (as the shoulder), 350, and 520 nm that is preserved during the successive dialysis against buffers with pH of 6.4, 2.0 and 7.4 (**Figure 42B**). The absorption band with a maximum at 520 nm is typical of the anthocyanins in flavylium cation form (acidic form) since its blue quinoidal bases, the dominant form in aqueous solution with pH > 3, exhibited a maximum at 430 nm (339). Therefore, the pH

changes in the external environment do not affect anthocyanins in the SC-Nanophytosomes, suggesting that they form a stable complex with algae membrane polar lipids. Moreover, at a quantitative level, the anthocyanins are preserved in the vesicles during the three steps of dialysis, as indicated by the EE value of $81.39 \pm 0.43\%$ after the dialysis against buffer with pH 6.4, $76.01 \pm 1.77\%$ after the dialysis against buffer with pH 2.0, and $68.33 \pm 4.69\%$ after the dialysis against buffer with pH 7.4 (**Figure 42A**).

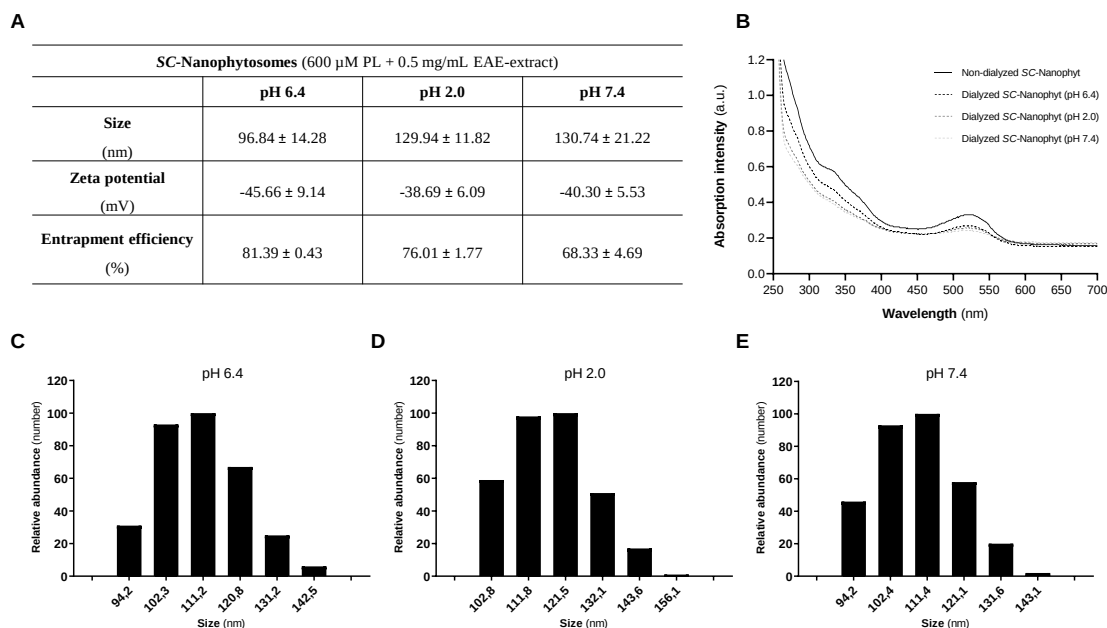


Figure 42. (A) Characterization of the dialysed SC-Nanophytosomes (600 μ M in phospholipid and 0.5 mg/mL EAE-extract) against buffer with different pH (6.4, 2.0, and 7.4) in terms of vesicle size (nm), Zeta potential (mV), and the entrapment efficiency (%). (B) Typical UV-Visible absorption spectra (250–700 nm) of the EAE-extract of non-dialysed and dialysed SC-Nanophytosomes at pH 6.4, 2.0, and 7.4. (C–E) Typical plots of the size distribution of dialysed SC-Nanophytosomes at pH 6.4 (C), 2.0 (D), and 7.4 (E).

The size and surface charge of the dialysed SC-Nanophytosomes suspensions, evaluated by DLS and ELS, respectively, at pH 6.4, 2.0, and 7.4, are also displayed in **Figure 42A**. DLS data indicate that SC-Nanophytosomes dialysed at pH 6.4 have an average diameter of 96.84 ± 14.28 nm and that size increases to 129.94 ± 11.82 nm after dialysis at pH 2.0 and for 130.74 ± 21.22 nm by the additional dialysis step against an aqueous solution with pH 7.4. Despite the detected fluctuations in the average diameter exhibited by vesicles in response to the external pH changes, the differences did not reach statistical significance. The typical plots of the size distribution, expressed by number in relative abundance, of SC-Nanophytosomes dialysed against pH 6.4, 2.0, and 7.4 buffer solutions also reveal vesicle size stability (**Figures 42C, 42D and 42E**). Zeta potential measurements show that dialysed SC-Nanophytosomes have a strongly negative surface charge, with a Zeta potential value of -45.66 ± 9.14 mV after dialysed at pH 6.4, $-38.69 \pm$

6.09 mV after dialysed at pH 2.0 and -40.30 ± 5.53 mV after dialysed at pH 7.4 (**Figure 42A**). Following the results in previous chapters, the SC-Nanophytosomes exhibit a strongly negative surface charge but preserve the anthocyanins in flavylum cation form. Data in **Figure 42** shows that SC-Nanophytosomes formulation is stable under the pH changes that mimic the gastrointestinal tract environments.

3.2. Effects of SC-Nanophytosomes on rotenone-induced PD animal model - behaviour benefits and α -synuclein outcomes

An experimental design was rationalised considering a suitable animal model, the route of SC-Nanophytosomes administration, and the concentration (dose) used to obtain a proof-of-concept for the mitochondria-targeted therapy for neurodegenerative diseases. A rotenone-induced PD animal model was chosen since it is highly reproducible and mimics PD-like motor deficits, including resting tremor and postural instability, as well as the progressive degeneration of the nigrostriatal dopamine system. Additionally, at a cellular level, it displays many pathological marks of PD, including impairment of mitochondrial complex I activity, accumulation of α -synuclein-containing Lewy bodies, and oxidative stress (336,337). Thus, it exhibits a pattern of changes that reflect impairment in functional connectivity involving different brain regions and specific peripheral tissues and the bioenergetic performance of the cells that support functional networks. SC-Nanophytosomes formulation delivered by drinking water at 3 μ M in PL and 2.5 mg EAE-extract/L was chosen since the aqueous suspensions SC-Nanophytosomes are stable for 14 days. The formulation is unaffected by pH changes throughout the gastrointestinal tract, and membrane phospholipids-based vesicles at 3 μ M in PL are highly compatible with the physiological conditions (340). Additionally, SC-Nanophytosomes delivered by drinking water allows SC-Nanophytosomes uptake in different gastrointestinal tract compartments, including in the buccal cavity, with privileged access to the brain (341). *Wistar* albino rats injected with rotenone (ROT group, $n = 10$) three times a week for three weeks developed a PD-like phenotype with postural instability and bradykinesia (**Figure 43**), that compromised their performance in beam walking test, as detected by an increased in the time required to complete the task ($t = 7.25 \pm 2.22$ s) when compared with control animals (CTRL group, $n = 5$, $t = 3.13 \pm 0.33$ s) (**Figure 44B**).

Then, animals with PD-like phenotype were randomly divided into two groups with five animals per group, and the experimental plan followed with CTRL, ROT, and ROT+ SC-Nanophyt groups.

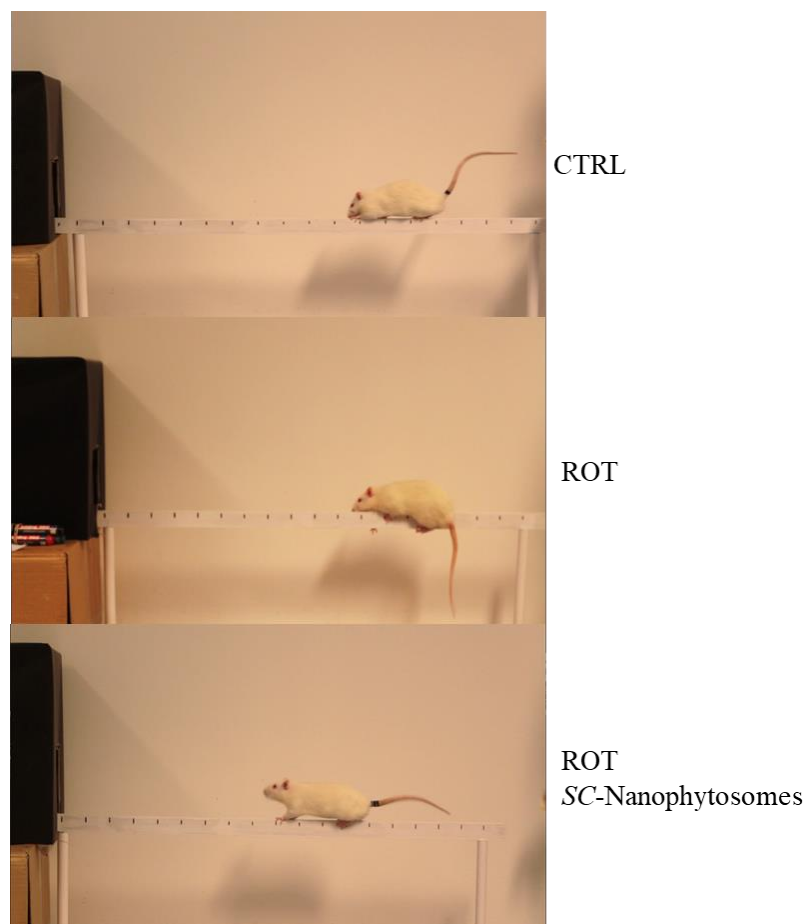
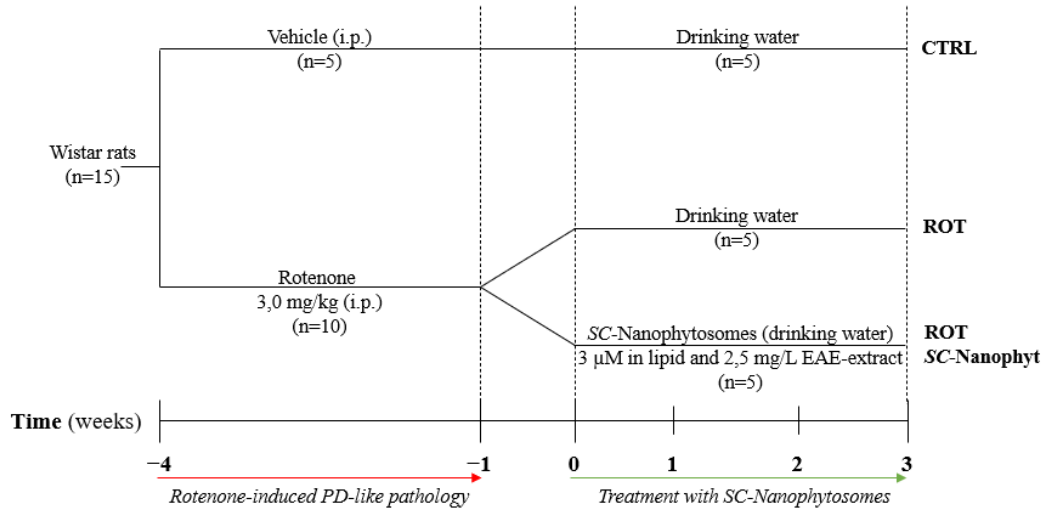


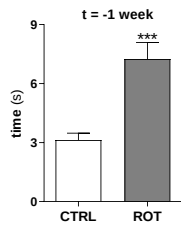
Figure 43. Captions obtained from the video camera records during the beam walking test for the three groups of the experimental plan: CTRL, ROT and ROT+SC-Nanophytosomes.

During the three weeks of treatment with SC-Nanophytosomes, the time required by the animals of the three groups to complete the beam walking test was measured once a week (**Figures 44C, 44D and 44E**). Data show that the animals treated with SC-Nanophytosomes (ROT+SC-Nanophyt group) exhibit better performance in the beam walking test compared with animals of the ROT group, and the differences concerning the CTRL group are also attenuated. Moreover, the SC-Nanophytosomes benefits are detected at the final of the first week and sustained during the three weeks of treatment. It is essential to highlight that the ROT group improved test performance two weeks after the induction of PD-like pathology (**Figures 44B and 44C**), suggesting that part of the motor deficits detected in the first measurement can be attributed to acute rotenone toxicity. One week of a break between the last rotenone injection and the beginning of the SC-Nanophytosomes treatment was rationalized to allow the rotenone clearance from the organism and to test the effects on the rotenone-induced neurodegenerative process.

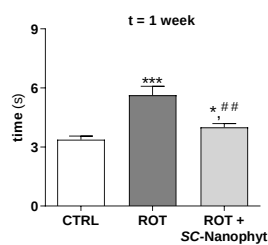
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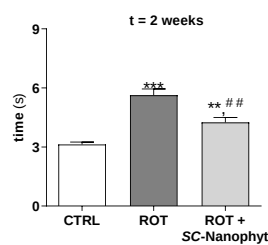
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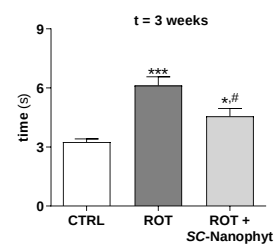


Figure 44. (A) Schematic representation of the experimental procedure. Rotenone-induced PD-like pathology for three weeks, one week break, and then three weeks of SC-Nanophytosomes treatment (3 µM in phospholipid and 2.5 mg/L in EAE-extract). (B–E) Average times from assessment of motor balance and coordination in the rats using the beam walking test. (B) Average times for rats crossing the 6 mm beam after three weeks of pre-treatment with rotenone (3.0 mg/kg, *i.p.*) and control. Effect of treatment with SC-Nanophytosomes on the first week (C), after two weeks (D), and three weeks (E) in average times for crossing the 6 mm beam in CTRL, ROT, and ROT+SC-Nanophyt groups. Error bars represent SEM for $n = 5$ independent experiments using five animals in each one. *, **, *** Significantly different from CTRL group, with $p \leq 0.05$, $p < 0.01$ and $p < 0.001$, respectively. #, ## Significantly different from ROT group with $p \leq 0.05$ and $p < 0.01$.

The obtained results indicated that the rotenone-induced PD animal model exhibits significant motor deficit, at least for four weeks, and that SC-Nanophytosomes formulation, delivered by oral route, promotes significant benefits on motor coordination and balance disturbance. It is also important to highlight that, after three weeks of treatment, the animal's body weight of the ROT+SC-Nanophyt group, as well as the weight of their peripheral organs (e.g., heart, kidneys, and liver), did not show significant differences compared with the CTRL group (Figure 45 and Table 4). Additionally, these animals no longer display visual signs of postural instability and bradykinesia or any other health problems (Figure 43).

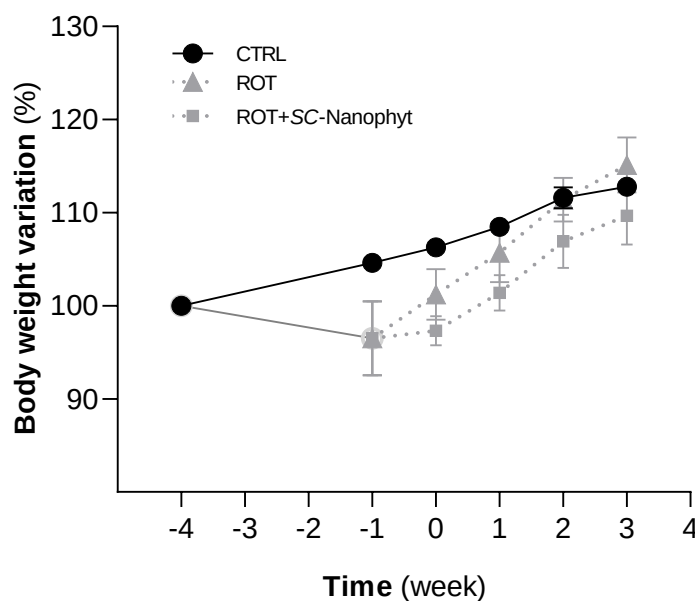


Figure 45. Body weight variation (%) of the rats during the experimental procedure for the CTRL, ROT and ROT+SC-Nanophyt groups. The average animals' body weight when the study started was: CTRL 397.5±21.4 g and ROT 362.1±32.5 g, and at the end of the study, the average body weight was: CTRL 448.2±22.5 g, ROT 417.5±37.4 g and ROT+SC-Nanophyt 405.2±19.8 g.

Regarding the classical PD model, the dopaminergic degeneration at substantia nigra pars compacta triggers the disease. However, experimental evidence supports the idea that the cerebellum is also involved in PD pathology, while reciprocal connections between the basal ganglia and cerebellum were revealed by anatomical studies (342). Additionally, the degeneration of dopaminergic nigrostriatal pathways in PD also disrupts the connectivity between the motor cortex and substantia nigra pars compacta (343,344). Thus, the biochemical studies described below were performed in two brain portions with similar weight (**Table 4**): one comprising the basal ganglia and cerebellum (BG-Cereb) and the other with the cerebral cortex (C-Cortex) regions.

Table 4. Average of the organ weights of Wistar rats from CTRL, ROT and ROT+SC-Nanophyt groups

Organ weights (g)	CTRL	ROT	ROT+SC-Nanophyt
BG-Cereb	0.868±0.086	0.813±0.030	0.827±0.089
C-Cortex	1.0328±0.136	1.101±0.120	1.018±0.059
Liver	12.638±0.927	12.163±0.907	11.931±1.152
Kidneys	2.648±0.100	2.615±0.283	2.419±0.097
Heart	1.175±0.124	1.210±0.356	1.048±0.080

α -synuclein is a cytosolic protein expressed in brain cells, mainly in the hippocampus, substantia nigra, thalamus, cerebellum, and neocortex. In physiological conditions, it occurs in soluble monomeric form playing multiple functions, including synaptic vesicle trafficking and regulation of dopamine metabolism. However, the abnormal intracellular aggregation of misfolded α -synuclein leads to the formation of intracellular inclusions named Lewy bodies, which is a pathological hallmark in PD (332,345). Thus, the levels of α -synuclein in rat brain BG-Cereb and C-Cortex portions were evaluated, and the results are displayed in **Figures 46A** and **46B**. Data show that the brain levels of α -synuclein in the BG-Cereb portion are significantly higher than in the C-Cortex portion. Moreover, only the BG-Cereb brain portion of the ROT group exhibits a higher level of α -synuclein than the CTRL group. Therefore, enhancing this pathological mark in the rotenone-induced PD animal model seems selective for the brain regions where the α -synuclein is highly expressed under physiological conditions. The SC-Nanophytosomes treatment (ROT+SC-Nanophyt group) promoted a significant decrease in α -synuclein levels in the BG-Cereb, re-establishing the levels of the CTRL group (**Figure 46A**). These results support the use of the rotenone toxic stimuli to induce a PD-like phenotype in rats and highlight the beneficial effect of the SC-Nanophytosomes treatment on this brain pathological mark of PD.

Tyrosine hydroxylase is a rate-limiting enzyme in dopamine biosynthesis, and a decrease in the tyrosine hydroxylase-positive neurons is considered a marker of nigrostriatal dopamine system degeneration. Thus, the activity of the tyrosine hydroxylase in rat brain BG-Cereb and C-Cortex portions was evaluated, and the results are displayed in **Figures 46C** and **46D**. In both brain portions, tyrosine hydroxylase activity did not show significant differences among the three groups. The absence of changes in the brain tyrosine hydroxylase activity between CTRL and ROT groups suggests that the rotenone-induced PD rat model (3.0 mg of rotenone/kg of animal weight, three times a week for three weeks) does not exhibit substantial loss of dopaminergic neurons, despite their deterioration in small brain regions cannot be excluded. The loss of tyrosine hydroxylase-positive neurons in substantia nigra resulting from rat treatment with rotenone (2.75–3.0 mg/kg/day, up to 60 days) was previously reported (346). However, it was also reported that the treatment of rodents with rotenone (2.0–4.0 mg/kg/day, for 45 days) does not promote significant changes in the density of the tyrosine hydroxylase-positive neurons, despite the well-evidenced debilitating PD-phenotype exhibited by animals (347). Therefore, the rotenone-induced PD-like phenotype can be more consistently related to changes in brain connectivity and motor dysfunction than to the loss of dopaminergic neurons in the substantia nigra.

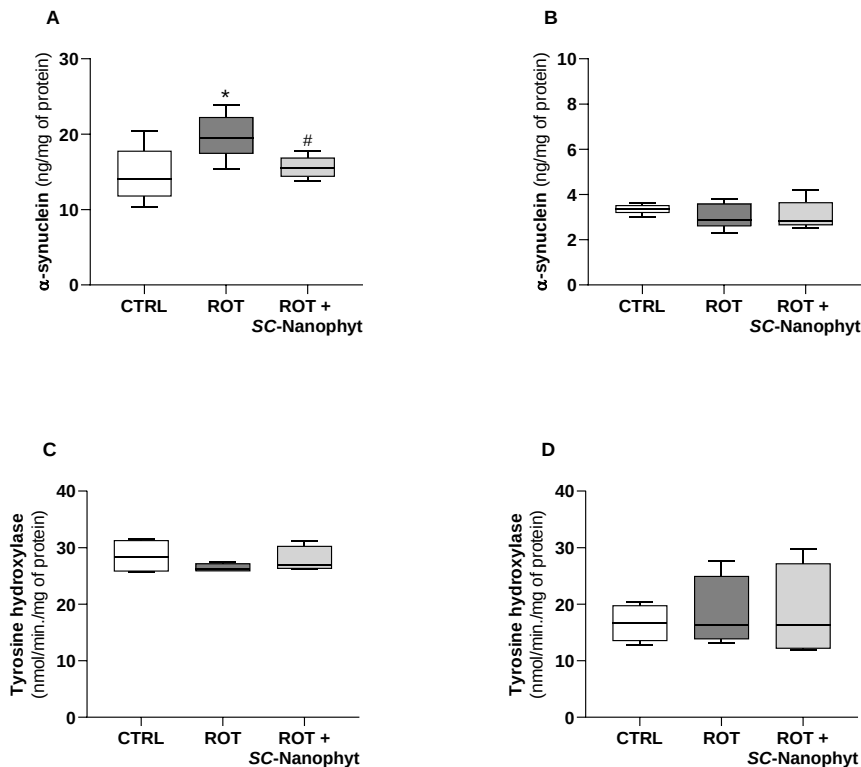


Figure 46. Brain α -synuclein levels assessed in brain homogenates from BG-Cereb (A) and the C-Cortex (B) portions of the CTRL, ROT, and ROT+SC-Nanophyt groups. The results are expressed as ng/mL/mg of protein. Tyrosine hydroxylase activity was assessed in the mitochondria-free cytosolic fraction from BG-Cereb (C) and the C-Cortex (D) portions of the CTRL, ROT, and ROT+SC-Nanophyt groups. The results are expressed as nmol/min/mg of protein. Error bars represent SEM for $n = 5$ independent experiments using five animals in each one. * Significantly different from the CTRL group, with $p \leq 0.05$. # Significantly different from the ROT group, with $p \leq 0.05$.

3.3. Effects on brain mitochondria

Rotenone-induced PD animal model exhibits impaired mitochondrial respiratory function, detected by deficient oxidative phosphorylation and by decreased complex I activity (273), which are also pathological hallmarks of PD (273,348). Thus, the competence of the oral administration of SC-Nanophytosomes to work as a brain mitochondria-targeted therapy was assessed considering effects: (i) on the mitochondrial respiratory function under conditions that ensure the coupling between mitochondrial respiratory chain and ATP synthesis (**Figure 47**), (ii) on the activity of individual respiratory complexes normalized by the activity of CS (**Figure 48**), and (iii) on the fatty acid profile of mitochondrial membrane polar lipids (**Figures 49 and 50**). **Figure 47** shows the results of the brain mitochondrial respiration parameters of the BG-Cereb portion for the three animal groups obtained with fresh preparations. Mitochondrial respiration was supported by pyruvate/malate plus

succinate to simulate the physiological conditions, and adenosine diphosphate (ADP) was used to induce state 3, characterized by a high oxygen consumption rate associated with ATP synthesis. After total ADP phosphorylation, the oxygen consumption rate decreased, reaching a new plateau used to assess respiration in state 4. The oxygen consumption not connected with mitochondrial respiration was assessed after adding sodium azide to inhibit complex IV. The RCR used to measure of the mitochondrial phosphorylation efficiency, is calculated as the ratio between the oxygen consumption rate in states 3 and 4. Although the oxygen consumption rates in states 3 and 4 do not exhibit significant differences among the three animal groups, the mitochondria of the ROT group exhibit a significant decrease in RCR compared with the CTRL group. On the other hand, the RCR value of ROT+SC-Nanophyt group mitochondria is comparable to those found for the CTRL group and significantly higher than that of ROT group mitochondria. Therefore, the phosphorylation efficiency of the brain mitochondria of the rotenone-induced PD animal model is compromised, and SC-Nanophytosomes treatment revealed competence to recover it for the values of control animals.

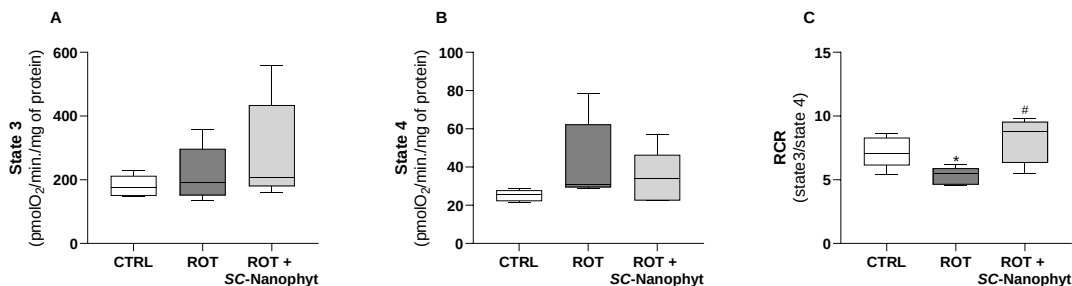


Figure 47. Mitochondrial respiration parameters were obtained with fresh preparations of BG-Cereb portion for CTRL, ROT, and ROT+SC-Nanophyt groups, supported by pyruvate/malate and succinate. The respiratory state 3 (**A**) was obtained by adding ADP to oxidize succinate, and state 4 (**B**) was reached after total phosphorylation of ADP. The RCR (**C**) was calculated as the ratio between the oxygen consumption rate in states 3 and 4. Results are expressed as pmolO₂/min/mg of protein. Error bars represent SEM for n = 5 independent experiments using five animals in each one. * Significantly different from the CTRL group, with p ≤ 0.05. # Significantly different from the ROT group, with p ≤ 0.05.

Mitochondrial electron transport chain complexes I, II, and IV activities were evaluated in the mitochondria-rich fraction obtained from BG-Cereb and C-Cortex brain portions (**Figure 48**). The activities of these complexes were normalized by the activity of CS, expressed by nmol citrate per min per mg of protein, to discard the influence of putative differences in the mitochondria content of sample preparations. In both brain portions, the values of CS activity do not have significant differences among the tested groups (BG-Cereb: CS values of 368.39±43.89, 323.31±40.57, 413.74±112.77 for CTRL, ROT and ROT+SC-Nanophyt groups, respectively; C-Cortex: CS values of 444.14±99.03,

452.57±63.50, 469.23±65.60 for CTRL, ROT, and ROT+SC-Nanophyt groups, respectively). Considering that the CS activity values are tightly correlated with morphometric data (349), are also conclude that the mitochondrial content is similar in both brain portions.

A general view of the results of **Figure 48** indicates that, in both brain portions, mitochondrial complex I, II, and IV activities are decreased in the ROT group compared to the CTRL group and that rats' treatment with SC-Nanophytosomes recovered the activities of these respiratory complexes. Additionally, the impaired activity of the complex I associated with the deterioration of other complexes' activity, detected in both brain portions of the ROT group, suggests that the rotenone promotes a neurodegenerative process with a mitochondrial dysfunction extendable to the entire brain that goes beyond the expectable inhibitory effects on complex I. Despite this, the brain effects of mitochondrial dysfunction promoted by rotenone and SC-Nanophytosomes treatment can also be dependent on the brain region since, under physiological conditions (CTRL group), the BG-Cereb mitochondria exhibit a respiratory redox chain with a 40% greater capacity to process the NADH produced in the citric acid cycle than mitochondria of C-Cortex portion. A detailed analysis of **Figure 48** shows that the results depend on the mitochondrial complex and the brain portion. Regarding complex I activity (**Figures 48A** and **48D**), the ROT group significantly decreases both brain portions, suggesting that the impairment of complex I promoted by rotenone is not restricted to any particular brain region. On the other hand, the SC-Nanophytosomes treatment recovered the complex I activity for values similar to the CTRL group. However, the differences between ROT+SC-Nanophyt and ROT groups only reached statistical significance in the BG-Cereb brain portion. Complex II has lower activity in the ROT group, but the significant differences are only detected in comparison to the CTRL group in the BG-Cereb portion (**Figures 48B** and **48E**). On the other hand, the complex IV activity in the ROT group is significantly lower than in the CTRL group in the C-Cortex (**Figures 48C** and **48F**). In this brain portion, the complex IV activity in the ROT+SC-Nanophyt group is also significantly higher than in the ROT group. These results indicate that SC-Nanophytosomes formulation has the competence to restore/attenuate the impairment of mitochondrial respiratory complexes activity exhibited by the rotenone-induced PD animal model. Using *in vitro* models was shown that elderberry anthocyanins are redox-active mitochondriotropic compounds with the ability to overcome the complex I inhibition promoted by rotenone (210) and that, as observed for SH-SY5Y cells in this work, SC-Nanophytosomes use the caveola-mediated endocytosis pathway to target mitochondrial, protecting the neuronal cells against the glutamate- and rotenone-induced toxicity. Therefore, the present *in vivo* results indicates that SC-Nanophytosomes, delivered

by drinking water at 3 M in PL and 2.5 mg EAE-extract/L for three weeks, ensure the targeting of the elderberry anthocyanins to the mitochondria of brain cells, allowing the redox-active anthocyanins to reach brain concentrations with therapeutic efficacy.

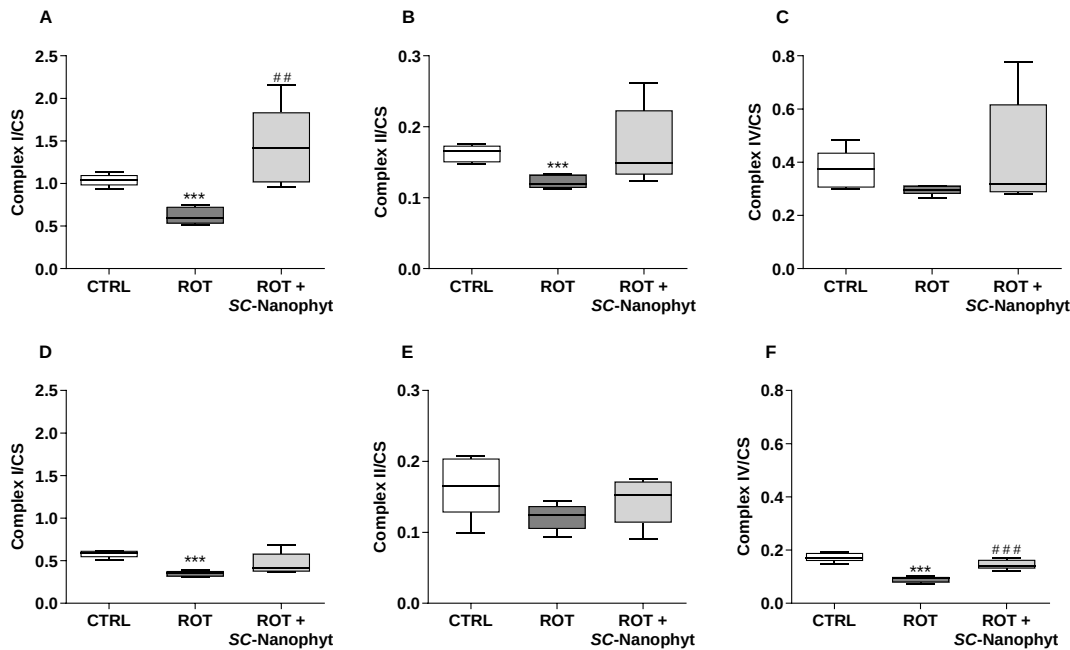


Figure 48. Mitochondrial complexes I (A,D), II (B,E), and IV (C,F) activity in the mitochondria-rich fraction from BG-Cereb (A–C) and C-Cortex (D–F) brain portions of CTRL, ROT, and ROT+SC-Nanophyt groups normalized by CS activity. Error bars represent SEM for $n = 5$ independent experiments using five animals in each one. *** Significantly different from CTRL group, with $p < 0.001$. ##, ### Significantly different from ROT group, with $p < 0.01$ and $p < 0.001$, respectively.

Considering that SC-Nanophytosomes were built with elderberry anthocyanins and *C. tomentosum* membrane polar lipids, the next step was to investigate if the algae lipids, in addition to the nanoplatform physical support, also have therapeutic benefits by modulating the mitochondrial lipidome. Thus, the fatty acid profile of the brain mitochondria phospholipids was revealed for three animal groups (CTRL, ROT, and ROT+SC-Nanophyt), with results discriminated for both BG-Cereb and C-Cortex brain portions as displayed in **Figures 49** and **50**. *C. tomentosum* membrane polar lipids are characterized by a fatty acid profile with high levels of PUFA (34%) and an n-3/n-6 PUFA ratio of 1.5 ± 0.3 , as previously shown. Therefore, an enrichment of the mitochondrial lipidome in PUFA leads to an increase in unsaturation index and to the enhancement of the n-3/n-6 PUFA ratio, which can be used as indicators of the SC-Nanophytosomes' ability to deliver algae lipids to brain mitochondria. The functional organization of the mitochondrial redox chain complexes requires phospholipid bilayers with fatty acids with high unsaturation index, mainly ensured by mature cardiolipin species with three/four PUFA (98,350). The n-3/n-6 PUFA and

DHA/AA ratios balance are essential players in the many physiological processes, and the changes in those ratios are associated with neuroinflammation and neurodegenerative diseases, including PD (351-353). It was also described that diets enriched in n-3 PUFAs and/or in DHA/AA ratios improve cognitive functions and exert neuroprotective effects in PD animal models (354-357).

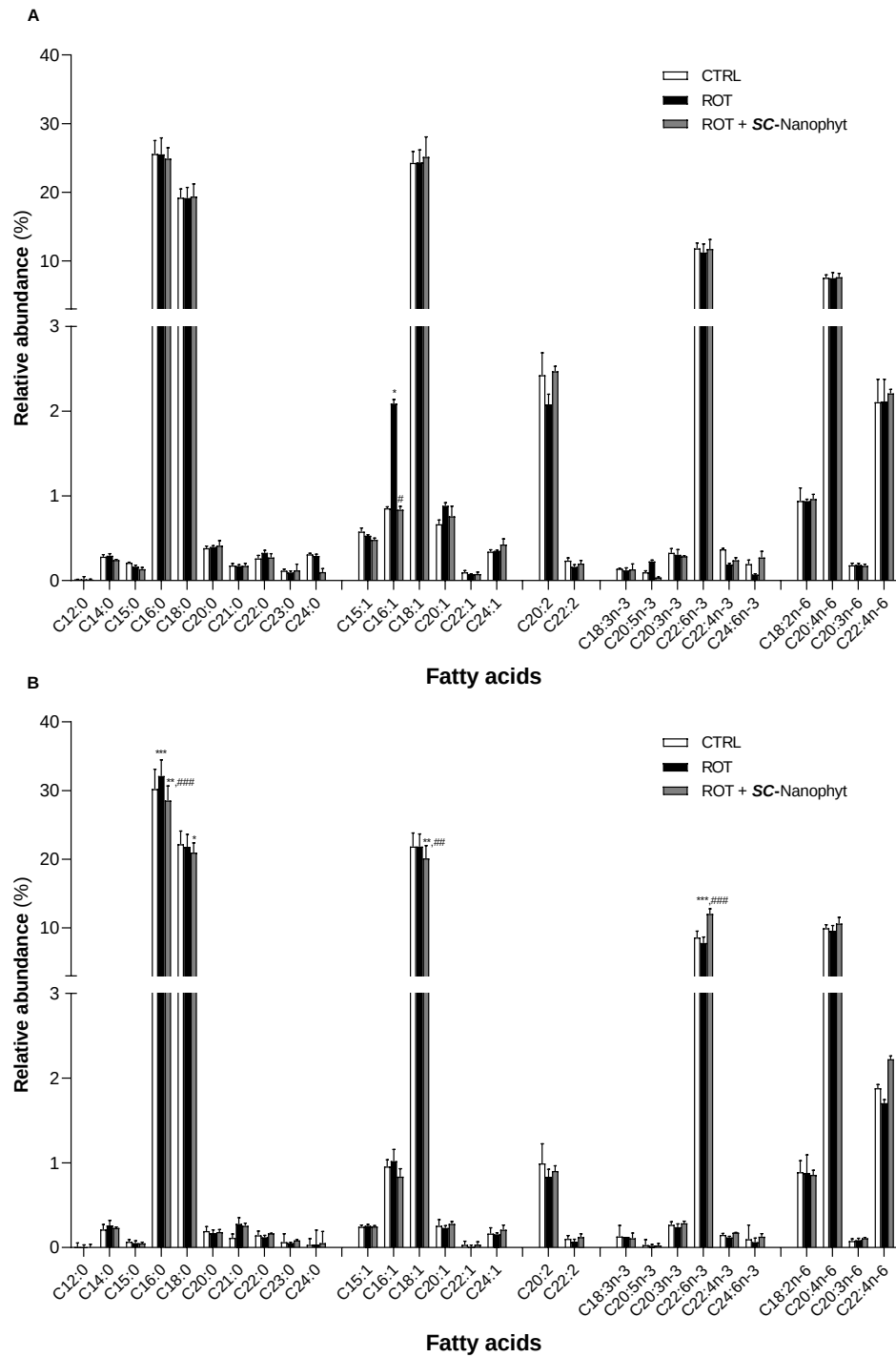


Figure 49. Fatty acid profile of phospholipid extracts obtained from mitochondria-rich fraction from BG-Cereb (A) and C-Cortex (B) brain portions of CTRL, ROT and ROT+SC-Nanophyt groups (CTRL:white bars; ROT:black bars; ROT+SC-Nanophyt: grey bars): C12:0 = lauric acid, C14:0 = myristic acid, C15:0 = pentadecanoic acid, C16:0 = palmitic acid, C18:0 = stearic acid, C20:0 = arachidonic acid, C21:0 = heneicosanoic acid, C22:0 = docosanoic acid, C23:0 = tricosanoic acid, C24:0 = lignoceric acid, C15:1 = pentadecenoic acid, C16:1 = palmitoleic acid, C18:1 = oleic acid, C20:1 = eicosenoic acid, C22:1 = docosenoic acid, C24:1 = nervonic acid, C20:2 = eicosadienoic acid, C22:2 = docosadienoic acid, C18:3n-3 = α -linolenic acid, C20:5n-3 = EPA, C20:3n-3 = eicosatrienoate acid, C22:6n-3 = DHA, C22:5n-3 = DPA, C22:4n-6 = docosatetraenoate, C24:6n-3 = tetracosahexaenoic acid, C18:2n-6 = linoleic acid, C20:4n-6 = AA, C20:3n-6 = DGLA. Error bars represent SEM for n = 5 independent experiments using five animals in each one. *, **, *** Significantly different from CTRL group, with $p \leq 0.05$, $p < 0.01$ and $p < 0.001$, respectively. #, ##, ### Significantly different from ROT group, with $p \leq 0.05$, $p < 0.01$ and $p < 0.001$, respectively.

Data from **Figure 49** shows that mitochondria from both brain portions of the CTRL group exhibit a fatty acid profile with twenty-eight fatty acids species, comprising ten SFA, six MUFA, and twelve PUFA. Regarding SFA, C16:0 is the most abundant SFA, followed by C18:0, while C18:1 is the dominant specie of MUFA. Regarding PUFA, DHA is the most abundant molecular species in the n-3 series, while AA is the most abundant in the series n-6. Eicosadienoic (C20:2), C18:2n-6 and docosatetraenoic (C22:4n-6) acids have relative abundances higher than 1%. The rotenone-induced PD-like pathology promotes minor changes in the mitochondrial fatty acid profile. Compared to CTRL, the ROT group significantly increases C16:1 content in the BG-Cereb portion. In contrast, in the C-Cortex portion, the major differences occur at C16:0 (increase) and C22:6n-3 (decrease). The treatment with SC-Nanophytosomes (ROT+SC-Nanophyt group) reverts the rotenone effects by decreasing C16:1 and C16:0 levels in BG-Cereb and C-Cortex portions, respectively. Additionally, the relative abundance of C22:6n-3 in the C-Cortex portion is increased, surpassing the CTRL level. The quantitative data in **Figure 49** were used to determine the total levels of SFA, MUFA, n-3, and n-6 PUFA, as well as to calculate the unsaturation index and the n-3/n-6 PUFA and DHA/AA ratios. The obtained results are displayed in **Figure 50**.

In the BG-Cereb portion, the small changes in the levels of individual fatty acid species detected among the three experimental groups did not significantly change the unsaturation index nor in the n-3/n-6 PUFA and DHA/AA ratios. However, in the C-Cortex portion, the mitochondria of the ROT group exhibit a fatty acid profile with a lower unsaturation index and lower n-3/n-6 PUFA and DHA/AA ratios, as compared with the CTRL group. Thus, the functional heterogeneity of the different brain portions is evidenced, associated with different lipid profiles and responses according to the function. The PD phenotype presented notorious lipid changes, mainly on the C-Cortex fraction. When treated with SC-Nanophytosomes (ROT+SC-Nanophyt group), the fatty acid profile was reverted to CTRL levels or even improved, as observed for the unsaturation index, n-3/n-6 PUFA, and DHA/AA ratios (**Figure 50**). These results indicate that the SC-Nanophytosomes

are competent in modulating the lipidome of the brain mitochondria, promoting positive outcomes in rotenone-induced PD-like pathology.

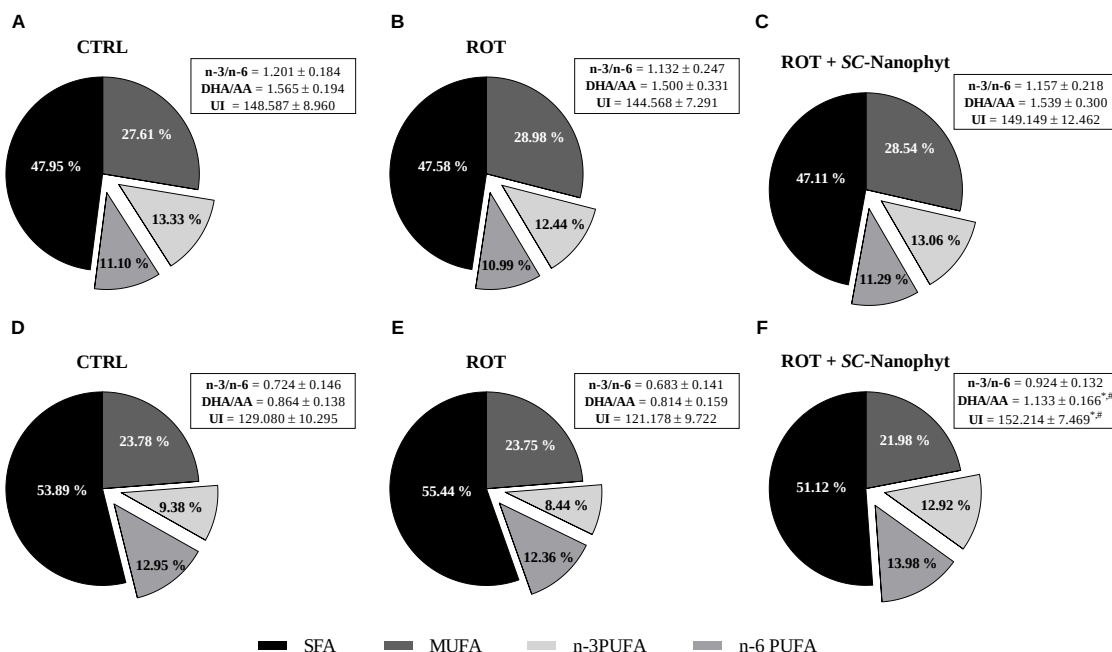


Figure 50. General parameters of the fatty acid profile of phospholipid extracts obtained from mitochondria-rich fraction from BG-Cereb (A–C) and C-Cortex (D–F) brain portions of CTRL, ROT and ROT+SC-Nanophyt groups. AA—arachidonic acid, DHA- docosahexanoic acid, SFA- saturated fatty acids, MUFA- monounsaturated fatty acids, PUFA- polyunsaturated fatty acids. * Significantly different from the CTRL group, with $p \leq 0.05$. # Significantly different from the ROT group, with $p \leq 0.05$.

3.4. Effects on the brain redox state

The brain redox state was evaluated in mitochondria-free cytosolic fraction obtained from rat BG-Cereb and C-Cortex brain portions regarding the levels of GSH and GSSG (Figure 51) and the activity of antioxidant enzymes, namely SOD, CAT, GR, and GPx (Figure 52). The concerted activity of these antioxidant enzymes promotes the management of the ROS simultaneous with the dynamic regulation of the GSH/GSSG ratio, used as an indicator of cell redox state (358-360). Therefore, the results of Figures 51 and 52 are discussed in an integrated fashion to provide an overview of the tissue's ability to respond to oxidative stress stimuli.

Data in Figure 51 indicate that the cells of the C-Cortex have a cytosolic environment significantly more reduced (a redox potential more negative) than the cells of the BG-Cereb portion since the values of the GSH/GSSG ratio in the C-Cortex portion are about three times higher than in BG-Cereb. Additionally, the total cytosolic content of glutathione (GSH + 2GSSG) is also significantly greater in C-Cortex cells, suggesting a high ability to handle ROS. In both brain portions, no significant differences were detected

for GSH/GSSG ratio between ROT and CTRL animal groups (**Figures 51C** and **51F**), suggesting that four weeks after the development of rotenone-induced PD-like phenotype (ROT group), the oxidative damage is not yet a pathological hallmark spread throughout the brain. Consequently, treatment with SC-Nanophytosomes of rats with a PD-like phenotype (ROT+SC-Nanophyt group) did not promote detectable effects on the cytosolic redox state of brain cells.

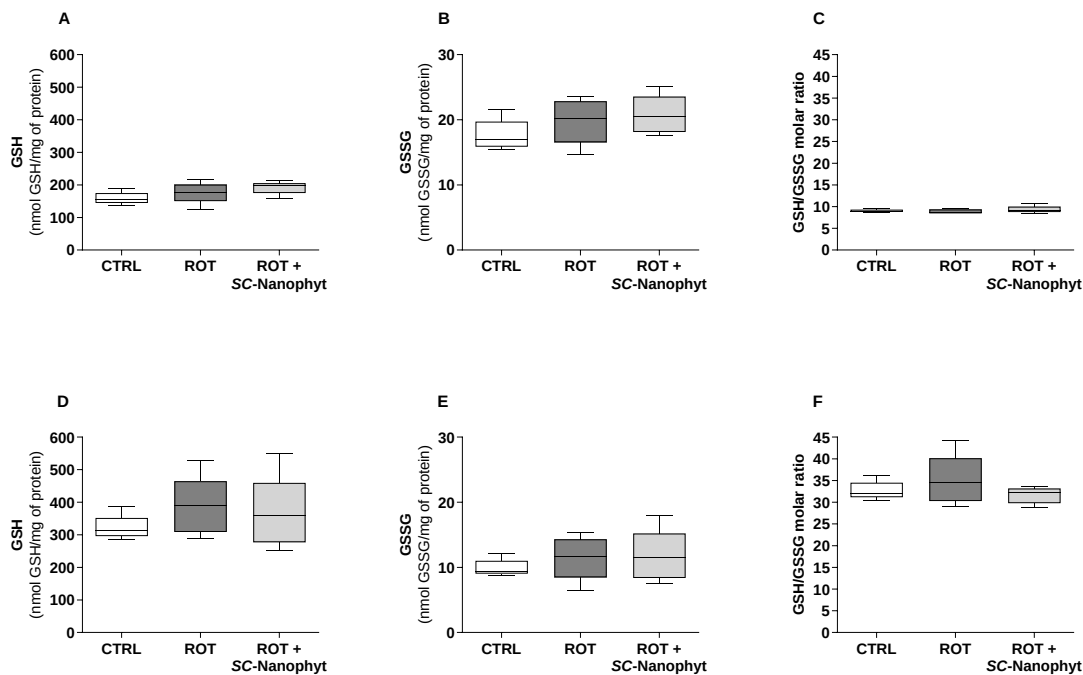


Figure 51. GSH (**A,D**) and GSSG (**B,E**) levels and GSH/GSSG molar ratio (**C,F**) in the mitochondria-free cytosolic fraction from BG-Cereb (**A–C**), and C-Cortex (**D–F**) brain portions of CTRL, ROT and ROT+SC-Nanophyt groups. Error bars represent SEM for n = 5 independent experiments using five animals in each one.

Despite this, the comparison of the activity of the antioxidant enzymes in three animal groups (**Figure 52**) suggests that the brains of the animals with rotenone-induced PD-like pathology regulate the activity of these enzymes in response to oxidative stress stimuli. In the BG-Cereb brain portion (**Figures 52A, 52B, 52C** and **52D**), SOD, CAT, and GR activity decreased in the ROT group compared to the CTRL group (significant differences were detected for CAT and GR). While for GPx activity, no significant differences were detected among the three groups, the values of the activity of SOD, CAT, and GR enzymes in the ROT+SC-Nanophyt group are not significantly different from those detected in the CTRL group. However, they are higher than those observed in the ROT group, with statistical significance for SOD and GR. Therefore, the rise of the vulnerability to oxidative stress exhibited by the ROT group is attenuated by treatment with SC-Nanophytosomes.

In the C-Cortex brain portion (**Figures 52E, 52F, 52G and 52H**), the effects of the rotenone-induced PD-like pathology on the activity of SOD and CAT follow a pattern with an opposite behaviour to the observed in the BG-Cereb brain portion. Thus, the ROT group exhibits significantly higher activity of SOD and CAT enzymes than the CTRL group. Consistently, in the ROT+SC-Nanophyt group, the values of the activity of SOD and CAT are not significantly different from those detected in the CTRL group. However, they are lower than those observed in the ROT group despite the differences not reaching statistical significance. The activity of GR and GPx enzymes did not show significant differences among the three groups. From the data in **Figures 51 and 52**, we can conclude that the treatment with SC-Nanophytosomes has the competence to attenuate and/or counteract the effects of the rotenone-induced PD-like pathology on the antioxidant enzymes of the cytosolic fraction of cells obtained from both brain portions. Additionally, the rotenone-induced PD rats have a BG-Cereb brain portion more vulnerable to oxidative damage than the C-Cortex brain.

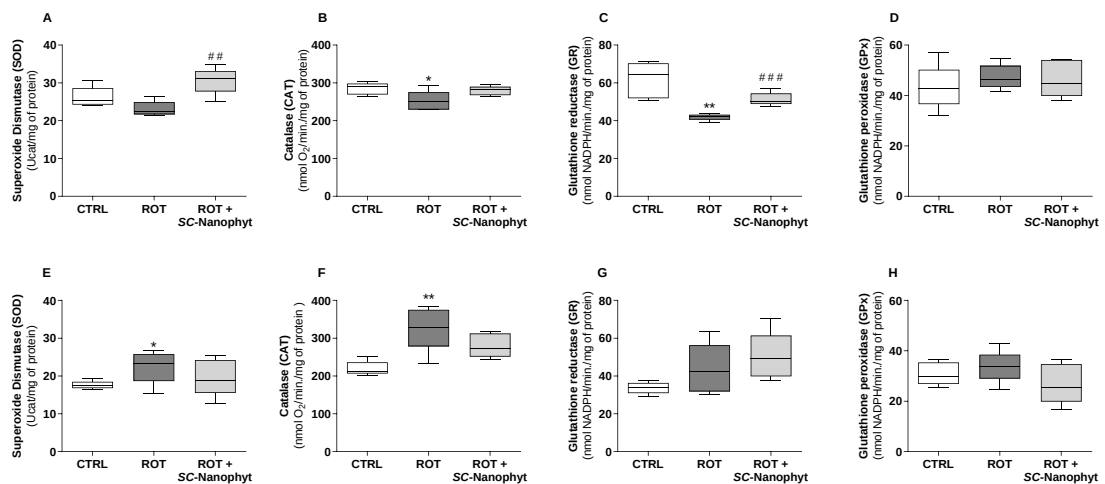


Figure 52. SOD (**A,E**), CAT (**B,F**), GR (**C,G**) and GPx (**D,H**) activity in the mitochondria-free cytosolic fraction from BG-Cereb (**A–D**) and C-Cortex (**E–H**) brain portions of CTRL, ROT and ROT+SC-Nanophyt groups. Error bars represent SEM for n = 5 independent experiments using five animals in each one. *, ** Significantly different from CTRL group, with p ≤ 0.05 and p < 0.01, respectively. ##, ### Significantly different from ROT group, with p < 0.01 and p < 0.001, respectively.

3.5. Main conclusions

- i) SC-Nanophytosomes formulation is suitable for oral administration since under pH conditions that mimic the gastrointestinal tract environments, the SC-Nanophytosomes preserve therapeutic-relevant properties, including size and surface charge, and high entrapment efficiency of anthocyanins in flavylium cation form - chemical structures with mitochondriotropic properties;
- ii) The rotenone-induced PD-like pathology animal model exhibits a set of phenotypic markers related to PD, comprising a significant motor deficit, higher brain levels of α -synuclein, compromised mitochondrial phosphorylation efficiency, impairment of the mitochondrial complex I, and an altered mitochondrial membrane lipidome, as well as changes in the cytosolic activity of the antioxidant enzymes;
- iii) Oral administration of SC-Nanophytosomes in a rotenone-induced rat model of PD is able: i) to promote significant benefits on motor coordination and balance disturbance; ii) to decrease the brain levels of α -synuclein to the values detected in control animals; iii) to mitigate the dysfunction of brain mitochondria, assessed in terms of phosphorylation efficiency, activities of mitochondrial respiratory complexes and fatty acid profile; iv) to modulate the activity of the antioxidant enzymes, improving the brain cells' ability to lead with toxic oxidant stimuli;
- iv) The positive outcomes of SC-Nanophytosomes treatment suggest that they have the competence to target the mitochondria of brain cells, whereas elderberry anthocyanins improve the functionality of mitochondrial redox chain and algae lipids contribute to changes in mitochondrial lipidome;
- v) SC-Nanophytosomes have excellent properties to support mitochondria-targeted therapy for neurodegenerative diseases like PD.

4. SC-Nanophytosomes effects on skeletal muscle of a rotenone-induced rat model of Parkinson's disease – From motor symptoms benefits to mitochondrial dysfunction mitigation

PD is a degenerative brain disease with a clinical pattern of progressive motor impairment characterized by bradykinesia, tremor, rigidity, and imbalance. PD patients exhibit many non-motor symptoms during disease progression, including sleep disturbances, depression, cognitive dysfunction, and dementia (331,333). Clinical symptoms are usually attributed to a gradual loss of functional connectivity among basal ganglia-cortex-cerebellum, brain regions dominated by dopamine-producing neurons that play a key role in turning movement thought into action (361).

However, skeletal muscle atrophy plays an important role in the progressive severity of the motor symptoms that characterize PD (362). The skeletal muscle of PD patients also exhibited several brain pathological hallmarks of the disease, including mitochondrial dysfunction related to deficient activity of the mitochondrial redox chain (363,364). Furthermore, exercise programs that improve mitochondrial plasticity, biogenesis, and respiration promote benefits in patients with PD (365), highlighting the role of the skeletal muscle-brain axis on health and diseases of the brain (366). Therefore, for therapy to be successful in the treatment of Parkinson's disease, it needs not only to improve the functionality and connectivity of brain cells but also of to mitigate skeletal muscle mitochondrial dysfunctionality to ensure the functionality of this tissue heavily affected by the disease. Thus, the present sub-chapter aims to characterize the effects SC-Nanophytosomes on the skeletal muscle of the rotenone-induced rat model of PD to consolidate the concept of mitochondria-targeted therapy for PD, established in the previous chapter considering brain benefits.

4.1. Benefits of SC-Nanophytosomes formulation on motor symptoms

As described in the previous sub-chapter, the animals of the three experimental groups were submitted to a beam walking test at the beginning and once a week during the three weeks of treatment with SC-Nanophytosomes to assess PD-related motor symptoms. The videos recorded during the beam walking test show that ROT group animals exhibit tremors, postural instability, balance disturbance, and a visible dragging of rear legs, symptoms were not detected in the animals of the CTRL group (<https://drive.google.com/file/d/1wPgRoKEjGj6veokvkOD0d2d5zipsV1ZY/view?usp=sharing>). The benefits of SC-Nanophytosomes treatment on PD-related motor symptoms are well-

evidenced in the videos recorded during the beam walking test by the absence of tremors and by reducing the imbalance. Therefore, the present video and quantitative results of animals' performance in beam walking presented in the previous subchapter confirm that the rotenone-induced PD animal model exhibits significant motor impairment, SC-Nanophytosomes treatment promotes benefits in motor coordination and balance.

Despite the PD motor impairments, major disease progression concerns have been attributed to neuronal degeneration issues, not the muscles' contractile capacity. The pathological hallmarks of PD, including mitochondrial dysfunction and oxidative stress, are also detected in human patients' skeletal muscle tissues and animal models for PD (367). Mitochondrial dysfunctions and oxidative stress are interlinked factors that compromise bioenergetic metabolism and lipid metabolism, which can be detected by changes in the membrane lipid profile (368). Thus, the metabolic changes in skeletal muscle tissues triggered by oxidative stress and mitochondrial dysfunction should also contribute to motor impairment with a pattern of progressive severity exhibited by PD patients. Considering this framework, the therapeutic potential of SC-Nanophytosomes as mitochondria-targeted therapy for PD was assessed in skeletal muscle tissues, considering effects on cell redox state, mitochondria functionality, and fatty acid profile.

4.2. Effects on the activity of mitochondrial redox chain complexes

Mitochondrial respiratory failure in the skeletal muscle of patients with PD has been reported (364), emphasizing their role in motor symptoms. Thus, the benefits of the oral administration of SC-Nanophytosomes on the skeletal muscle mitochondria were assessed considering the effects on the activity of the individual respiratory complexes I, II, and IV in the mitochondria-rich fraction (**Figure 53**). The activity of these mitochondrial complexes was normalized by the activity of CS to discard putative differences in the mitochondria content of the samples. Skeletal muscle mitochondria obtained from three experimental groups exhibit similar values of CS activity, namely 134.95 ± 16.05 , 137.19 ± 16.45 , and 106.30 ± 12.78 for CTRL, ROT, and ROT+SC-Nanophyt groups, respectively.

The results in **Figure 53** show that ROT group's mitochondrial complex I and II activities are decreased compared to the CTRL group. The results showed no difference between CTRL and ROT groups regarding the mitochondrial complex IV activity. The impairment of both complexes I and II in the skeletal muscle of ROT group animals suggests that the neurodegenerative process underlying PD is associated with a mitochondrial dysfunction extendable beyond the brain, which is detected in peripheral organs like skeletal muscle as reported here for rotenone-induced PD rat model (**Figure 53**), and previously for

other models (369,370). The brain and skeletal muscle are among the most metabolically active tissues. Consequently, their functional performance is particularly vulnerable to mitochondrial impairment (371,372), and the bidirectional connectivity between brain and skeletal muscle is also well-documented (373). Therefore, skeletal muscle mitochondrial dysfunction detected in the ROT group can emerge either by the direct effects of rotenone on skeletal muscles or by its degenerative brain effects.

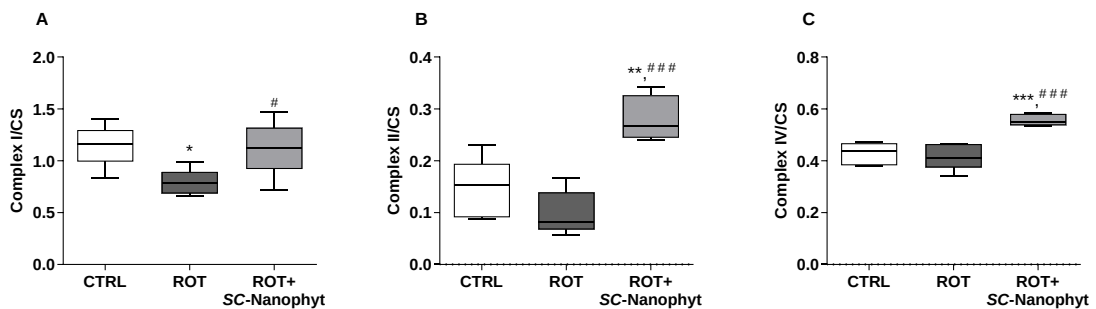


Figure 53. Mitochondrial complexes I (A), II (B) and IV (C) activity in the mitochondria-rich fraction from skeletal muscle of CTRL, ROT and ROT+SC-Nanophyt groups normalized by CS activity. Error bars represent SEM for n=5 independent experiments using five animals in each one. *, **, *** Significantly different from the CTRL group, with $p \leq 0.05$, $p < 0.01$ and $p < 0.001$, respectively. #, ### Significantly different from the ROT group, with $p \leq 0.05$ and $p < 0.001$, respectively.

Regarding the effects of SC-Nanophytosomes treatment (**Figure 53**), the activities of complexes I, II, and IV in the ROT+SC-Nanophyt group are significantly increased when compared with the ROT group, indicating that SC-Nanophytosomes have the competence to restore the impairment of mitochondrial respiratory complexes activities promoted by rotenone. Moreover, the mitochondria of skeletal muscle tissues of the ROT+SC-Nanophyt group exhibit activities of complexes II and IV significantly higher than mitochondria of the CTRL group, suggesting that the treatment could improve the mitochondrial respiration performance at a level that surpasses the control animals. These effects align with the mitochondrial bioactivity of elderberry anthocyanins revealed in isolated mitochondria, cells, and the brain of this set of animals (210).

4.3. Effects on the cell redox state

In general, oxidative stress results from an excessive generation of ROS and/or from deficient activity of the antioxidant enzymes. Oxidative stress biomarkers mirror the unbalance of cell redox status, mainly characterized by a decreased GSH/GSSG ratio. Therefore, the capacity of SC-Nanophytosomes treatment to modulate the redox status of skeletal muscle tissues of the rotenone-induced rat model of PD was evaluated in

mitochondria-free cytosolic fractions, measuring the levels of GSH and GSSG (**Figure 54**) and the activity of antioxidant enzymes, namely SOD, CAT, GR, and GPx (**Figure 55**). The integrated assessment of these enzymatic and nonenzymatic antioxidant parameters allows an overview of the response of skeletal muscle tissues to oxidative stress stimuli, such as rotenone used to induce PD-like pathology. While GSH/GSSG ratio is used as an indicator of the redox state of cells, the antioxidant enzymes are the key players in the management of the ROS generated in the cells, thereby the activity of antioxidant enzymes is interlinked with intracellular GSH/GSSG ratio (358-360).

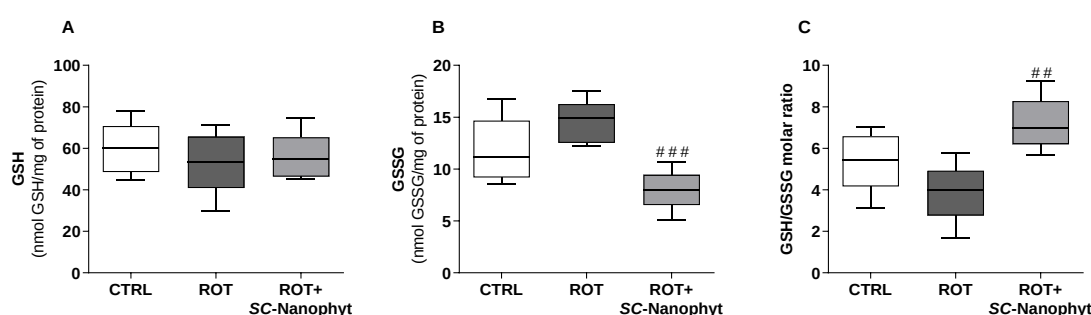


Figure 54. GSH (A) and GSSG (B) levels and GSH/GSSG molar ratio (C) in the mitochondria-free cytosolic fraction from skeletal muscle of CTRL, ROT and ROT+SC-Nanophyt groups. Error bars represent SEM for n=5 independent experiments using five animals in each one. ##, ### Significantly different from the ROT group, with $p < 0.01$ and $p < 0.001$, respectively.

Figure 54 shows that the cytosolic levels of GSH are similar in the three experimental groups and that GSSG levels in the ROT+SC-Nanophyt group are similar to the CTRL group but significantly lower than in the ROT group. The GSSG levels in the ROT group slightly increased as compared to the CTRL group, but the differences did not reach statistical significance. Consequently, GSH/GSSG ratio in the ROT+SC-Nanophyt group is significantly higher than in the ROT group ($p < 0.01$), but it is not statistically different from the CTRL group. These results suggest that the PD-like pathology induced by rotenone has a minor impact on the redox status of skeletal muscle tissues and that treatment with SC-Nanophytosomes promotes a cytosolic environment with more reduced redox potential (high GSH/GSSG ratio), enhancing the skeletal muscle cells competence to lead with oxidative stress stimuli.

Regarding the activity of SOD, CAT, GR, and GPx enzymes in the cytosolic fraction of the skeletal muscle tissues, data from **Figure 55** shows that the rotenone-induced rat model of PD only affects the activity of CAT, promoting a significant increase in its activity when compared to the CTRL group. The increased CAT activity in the ROT group suggests that the skeletal muscle cells reply to a cytosolic increase of hydrogen peroxide levels,

enhancing the activity of this enzyme. Additionally, treating with SC-Nanophytosomes in rats with PD-like pathology strongly impacts the activity of all evaluated antioxidant enzymes. Thus, it is detected that in the ROT+SC-Nanophyt group, there was a significant decrease in SOD, CAT, GR, and GPx enzyme activity compared with the CTRL group or the ROT group. The previous sub-chapter showed that the SC-Nanophytosomes have positive brain impacts on the rotenone-induced PD rat model by modulating the activity of antioxidant enzymes in brain cells.

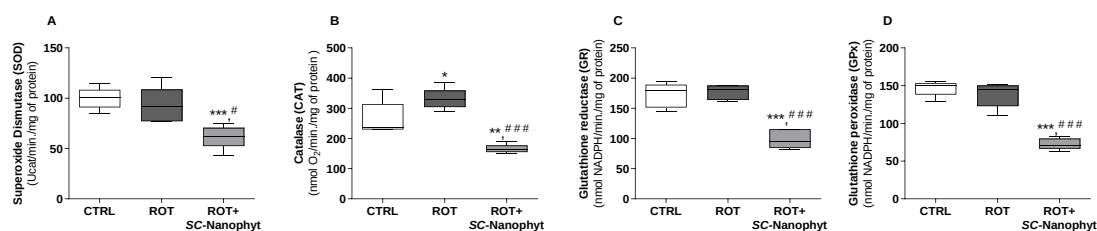


Figure 55. SOD (A), CAT (B), GR (C) and GPx (D) activity in the mitochondria-free cytosolic fraction from skeletal muscle of CTRL, ROT and ROT+SC-Nanophyt groups. Error bars represent SEM for n=5 independent experiments using five animals in each one. *, **, *** Significantly different from the CTRL group, with $p \leq 0.05$, $p < 0.01$ and $p < 0.001$, respectively. #, ### Significantly different from the ROT group, with $p \leq 0.05$ and $p < 0.001$, respectively.

From the data in **Figures 54** and **55**, we can conclude that the treatment with SC-Nanophytosomes has the competence to increase the GSH/GSSG ratio simultaneously with a decrease in the antioxidant enzyme activities. Therefore, the SC-Nanophytosomes treatment mitigates the effects of the rotenone-induced PD-like pathology, mainly detected by CAT activity, providing a cellular environment with a more reduced redox potential, which has high relevance in the context of degenerative diseases and the ageing process.

4.4. Effects on the fatty acid profile of the skeletal-muscle tissue

The next step is to investigate if SC-Nanophytosomes have therapeutic benefits by modulating the skeletal muscle lipidome. Lipid studies have high relevance in the PD context since the mitochondria functionality, synaptic signalling, and endosome–lysosome system functioning, all affected by the degenerative process, rely heavily on the membrane lipid composition of the cells and tissues affected by the disease (374-376). Thus, the characterization of the fatty acid profile of the three experimental groups' skeletal muscle tissues lipids changes connected with the rotenone-induced PD-like pathology and reveals the putative effects of SC-Nanophytosomes treatment. **Figures 56** and **57** show the fatty acid profile of the total membrane lipid extracted from the skeletal muscle tissues of the CTRL, ROT, and ROT+SC-Nanophyt groups.

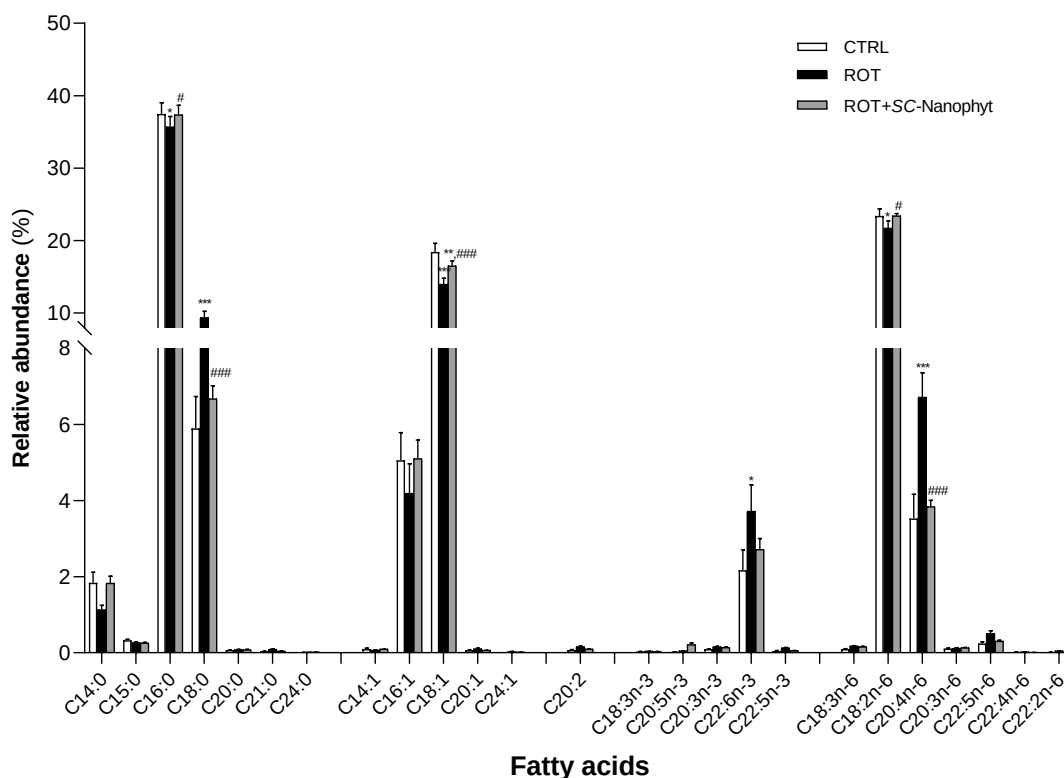


Figure 56. Fatty acid profile of phospholipid extracts obtained from skeletal muscle homogenates of CTRL, ROT and ROT+SC-Nanophyt groups (CTRL: white bars; ROT: black bars; ROT+SC-Nanophyt: grey bars): C14:0= myristic acid, C15:0= pentadecanoic acid, C16:0= palmitic acid, C18:0= stearic acid, C20:0= arachidonic acid, C21:0= heneicosanoic acid, C24:0= lignoceric acid, C14:1= myristoleic acid, C16:1= palmitoleic acid, C18:1= oleic acid, C20:1= eicosenoic acid, C24:1= nervonic acid, C20:2= eicosadienoic acid, C18:3n-3= α -linolenic acid, C20:5n-3= EPA, C20:3n-3= eicosatrienoic acid, C22:6n-3= DHA, C22:5n-3= DPA, C18:3n-6= γ -linolenic acid, C18:2n-6= linoleic acid, C20:4n-6= AA, C20:3n-6= DGLA, C22:5n-6= docosapentaenoic acid, C22:4n-6= docosatetraenoic acid, C22:2n-6= docosadienoic acid. Error bars represent SEM for n=5 independent experiments using five animals in each one. *, **, *** Significantly different from the CTRL group, with $p \leq 0.05$, $p < 0.01$ and $p < 0.001$, respectively. #, ### Significantly different from the ROT group, with $p \leq 0.05$ and $p < 0.001$, respectively.

As shown in **Figure 56**, the skeletal muscle of the CTRL group exhibits a fatty acid profile with twenty-five detectable fatty acids species, comprising seven SFA, five MUFA, and thirteen PUFA. Regarding SFA, C16:0 is the most abundant species followed by C18:0. In contrast, C18:1 is the most abundant MUFA, followed by C16:1. The C18:2n-6 is the dominant specie of PUFA and the most abundant molecular specie in the n-6 series followed by the AA, while DHA is the most abundant in the n-3 series. According to these results, Abbott and collaborators (377) showed that DHA accounted for most of the n-3 series in the rats' muscle lipidome. At the same time, C18:2n-6 and AA provided the highest relative abundance in the total n-6 series. Regarding the fatty acid profile of the ROT group, a significant increase in C18:0, DHA, and AA content is detected, and a significant decrease in the levels of C16:0, C18:1 and C18:2n-6 when compared with the CTRL group. The treatment with SC-Nanophytosomes, ROT+SC-Nanophyt group, promotes a reversion of

the effects promoted by rotenone, bringing the levels of the affected species to values close to the control. Thus, it is detected in the ROT+ SC-Nanophyt group, with a significant increase in C16:0, C18:1 and C18:2n-6 content and a significant decrease in the levels of C18:0 and AA when compared with the ROT group.

The quantitative data in **Figure 56** were used to determine the total levels of SFA, MUFA, n-3, and n-6 PUFA, as well as to calculate the unsaturation index and the n-3/n-6 PUFA and DHA/AA ratios (**Figure 57**). Thus, the ROT group has a fatty acid profile with higher PUFA and SFA richness and lower levels of MUFA when compared with the CTRL group. However, the differences only reach statistical significance for MUFA. No significant differences between CTRL and ROT groups are detected in the n-3/n-6 PUFA and DHA/AA ratios and unsaturation index parameters. Despite the SC-Nanophytosomes treatment promoting quantitative changes in fatty acid profile, the impact on the abovementioned parameters is minor. The differences did not reach statistical significance when compared with CTRL or ROT groups. However, the DHA/AA and n-3/n-6 PUFA ratios in the ROT+SC-Nanophyt group are closer to the CTRL group, which suggest that the treatment counteracts the small changes promoted by rotenone on skeletal muscle lipidome.

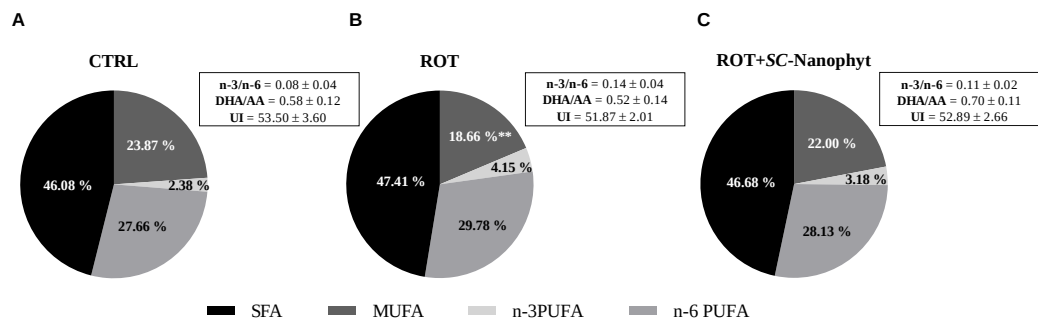


Figure 57. General parameters of the fatty acid profile of phospholipid extracts obtained from skeletal muscle homogenates of CTRL (**A**), ROT (**B**) and ROT+SC-Nanophyt (**C**) groups. AA - arachidonic acid, DHA – docosahexaenoic acid, SFA – saturated fatty acids, MUFA – monounsaturated fatty acids, PUFA – polyunsaturated fatty acids, UI – unsaturation index. ** Significantly different from the CTRL group, with $p < 0.01$.

4.5. Main conclusions

- i) The rotenone-induced PD-like pathology animal model exhibits significant motor deficit, characterized by tremors, postural instability, balance disturbance, and dragging of rear legs. These motor symptoms are accompanied by skeletal muscle sub-cellular pathological hallmarks, including mitochondrial dysfunction (strong deficiency of complex I activity), changes in cellular redox state (lower GSH/GSSG ratio) that compromise the tissue's ability to lead with toxic oxidant stimuli and small vicissitudes in the fatty acid profile of membrane lipids;
- ii) Oral administration of SC-Nanophytosomes in a rotenone-induced rat model of PD is able: i) to promote significant benefits on motor coordination and balance disturbance; ii) to mitigate the mitochondria dysfunction in skeletal muscles, overcoming the deficient complex I activity and improving the activities of complexes II and IV to values significantly higher than those detected in skeletal muscle mitochondria of CTRL group; iii) to modulate cell redox state, leading to the redox potential of the skeletal muscle cell towards a more negative value (higher GSH/GSSG ratio), with a concomitant modulation of the activity of the main antioxidant enzymes; iv) to modulate the fatty acid profile of membrane lipids, countering the lipids changes associated with PD like pathology;
- iii) SC-Nanophytosomes' effects on skeletal muscle consolidate the concept of mitochondria-targeted therapy for PD.

CHAPTER IV

*Integrated Discussion, Conclusions Remarks
and Future Perspectives*

In the past 30 years, the number of people suffering from neurodegenerative diseases has risen substantially in parallel with population growth and ageing. Currently, more than 50 million people are suffering from a neurodegenerative pathology, with AD and PD being the most common diseases (117,329). Insufficient advances in prevention and the absence of therapies capable of interrupting or reversing the clinical symptoms with a pattern of progressive severity allow us to predict that the number of people affected by these diseases will continue to increase in the coming years. In terms of the economy, this would require a high financial effort from the healthcare systems. In Europe Union, the annual cost estimated for brain disorders has about 800 billion euros in 2010 (378,379). Therefore, the development of effective medicine for degenerative brain diseases is an urgent and unquestionable emergency to keep not only our ageing societies healthy, but also our economy.

In the present Ph.D. thesis, the challenge of degenerative brain diseases was addressed by a novel therapeutic strategy, focused on mitochondria. This mitochondria-targeted therapy has the inner mitochondrial membrane as a main target, and it was rationalized to overcome the mitochondrial dysfunction, by improving the respiratory chain functionally and tuning mitochondrial lipidome, and adjusting the redox-dependent signalling pathways, by modulating the cytosolic redox status of cells.

As justified in the first chapter, the mitochondrion is the main player in safeguarding multicellular organisms' life and a promising key to solving the puzzle of neurodegenerative diseases. It is well-known that mitochondrial dysfunction associated with an anomalous activity of the respiratory chain complexes (mainly complex I) and an abnormal mitochondrial lipidome (mainly cardiolipins) are early pathological hallmarks of both AD and PD. Thus, to overcome the dysfunctional complex I, it was selected elderberry anthocyanins in cationic form - redox-active compounds with the ability to oxidize NADH and deliver the electrons to complex III of the mitochondrial redox chain, working as a membrane electron carrier. To modulate the mitochondrial lipidome, it was selected a set of membrane polar lipids from *C. tomentosum*. The chloroplast-rich tissues of this green algae are rich in anionic phospholipids (mainly in PGs used by animal mitochondria as cardiolipin precursor) and in PUFA (mainly n-3 PUFA) that allow the formation of membranes with high curvature (typical of the inner membrane of mitochondria) and enable the synthesis of mature cardiolipins. Taking advantage of the self-assembling properties of *C. tomentosum* membrane polar lipids and the great affinity of elderberry anthocyanins in cationic form by anionic phospholipids, it was developed the SC-Nanophytosomes, a nanoplatform with the potential to be a delivery system with competence to overcome the different physiological

barriers and supply the mitochondria of brain cells with elderberry anthocyanins and algae lipids.

To test the above-mentioned therapeutic strategy, it was developed sustainable procedures to obtain the building blocks of the SC-Nanophytosomes, fulfilling the requirements of the food and medical applications. The obtained EAE-extract and algae membrane polar lipids were characterized and used to prepare the SC-Nanophytosomes. Characterization of the SC-Nanophytosomes revealed that they are a mixture of sphere- and discoid-shaped vesicles with nanometric hydrodynamic size and very high negative surface charge that preserve the mitochondriotropic properties of the elderberry anthocyanins. The aqueous suspensions of SC-Nanophytosomes exhibit stability, at least, for three weeks when stored at 4 °C. In fact, SC-Nanophytosomes have a negative surface charge with Zeta potential values close to those reported for extracellular vesicles (e.g., exosomes) released by cells with intercellular communication purposes, which may explain their good stability during storage. Thus, we can conclude that the anionic phospholipids-rich *C. tomentosum* lipid extract has the competence to form complexes with the elderberry anthocyanins in cationic form, allowing the production of SC-Nanophytosomes with an improved negative surface charge and stability.

In vitro assays with the SH-SY5Y cells demonstrated the non-cytotoxicity of the SC-Nanophytosomes and revealed their competence to target mitochondria using the caveola-mediated endocytosis pathway. Additionally, the SC-Nanophytosomes enhance the elderberry anthocyanins' ability to modulate mitochondria functionality, improving the activity of mitochondrial complexes I and II and ensuring the $\Delta\Psi_m$ in the presence of a complex I inhibitor (e.g., rotenone). Moreover, they protect neuronal cells against the toxicity induced by rotenone or glutamate, which are toxins with relevance in the context of neurodegeneration.

In vitro assays with BJ cells revealed that SC-Nanophytosomes have no detectable toxicity for human fibroblasts and showed competence to enter in cell and target the mitochondria, modulating the $\Delta\Psi_m$ in the presence of rotenone. Therefore, in addition to neurons, SC-Nanophytosomes have ability to target mitochondria of other cell types, which can be considered positive for the treatment of degenerative brain diseases that affect not only the brain but also peripheral tissues.

Chronic brain inflammation is another pathological mark of neurodegenerative diseases, suggesting that the fundamental role of microglia cells in the maintenance of nervous system cells is affected. Therefore, it was evaluated the capacity of SC-Nanophytosomes to modulate the phenotype and activity of a cellular model of microglia. In response to specific stimuli, microglia cells switch from a resting state to an activated

phenotype which leads to an inflammatory response with the consequent production of inflammatory mediators (296-298). Thus, it was used BV-2 cells as a cellular model of microglia and IFN- γ was selected as a chemical stimulus to lead BV-2 cells towards a pro-inflammatory phenotype (380,381). It is important to highlight that some studies use a combination of IFN- γ and LPS as pro-inflammatory stimuli (382,383). However, IFN- γ is produced by brain cells, thereby it can be considered a more realistic stimulus to promote the activation of microglia cells in the context of neurodegenerative diseases, such as PD and AD. In fact, BV-2 cells exposure to IFN- γ , for 22 h, leads to an increase in cellular generation of NO, indicating an increase of iNOS expression/activity (used as a biomarker of pro-inflammatory phenotype), accompanied by a decrease in ROS generation and mitochondrial $\Delta\Psi_m$. Therefore, IFN- γ has the competence to promote a phenotype remodelling of BV-2 cells with detectable changes at levels of NO generation, mitochondrial functionality, and fatty acid profile of whole cell lipidome. Assays with BV-2 cells also showed that SC-Nanophytosomes, as well as elderberry anthocyanin-enriched extract, do not alter the viability nor the NO production of BV-2 cells in resting state, suggesting no cytotoxicity or competence trigger a cellular remodelling towards a pro-inflammatory phenotype. Despite this, the data reveal that SC-Nanophytosomes decrease both $\Delta\Psi_m$ and ROS generation of BV-2 cells in a resting state with minor effects on the activities of mitochondrial respiratory complexes and antioxidant enzymes. The pre-incubation of BV-2 cells with SC-Nanophytosomes or EAE-extract followed by co-incubation with IFN- γ did not prevent the phenotype remodelling towards a pro-inflammatory phenotype, characterized by an increase of cellular NO production in simultaneous with a decrease of ROS generation and mitochondrial $\Delta\Psi_m$. These results suggest that SC-Nanophytosomes, as well as EAE-extract, did not show anti-inflammatory activity on BV-2 cells under IFN- γ stimulation. However, data suggest that SC-Nanophytosomes modulate the cellular effects of the IFN- γ on mitochondria, enhancing the depolarization of the inner mitochondrial membrane and pulling the activity of the respiratory complexes to values close to the values of BV-2 cells in the resting state. Additionally, SC-Nanophytosomes do not have effects on the activity of antioxidant enzymes, despite the EAE-extract alone increases the activity of cytosolic SOD enzyme in cells stimulated with IFN- γ . Furthermore, SC-Nanophytosomes have a small impact on alterations promoted by IFN- γ in the fatty acid profile of whole BV-2 cell lipidome, which is detectable by an increase of PUFA reflected in the higher unsaturation index value.

The use of green-fluorescent labelled SC-Nanophytosomes with the lipophilic 16-AP fluorescent probe allows the confirmation of BV-2 cells' competence to internalize the SC-Nanophytosomes. However, the cellular uptake seems do not involve micropinocytosis, clathrin-mediated endocytosis, or caveola-mediated endocytosis. Thus, the main entry

route of SC-Nanophytosomes into microglial cells is not the same as the neurons (*i.e.*, caveola-mediated endocytosis). Regarding the high phagocytic activity of microglial cells (384), to understand the specific activity SC-Nanophytosomes on microglial cells it is important to unveil, in a future work, if they are internalized by a phagocytic pathway. In fact, the activation of a phagocytosis pathway can lead to the destruction of the integrity of SC-Nanophytosomes vesicles with total or partial metabolization of its building blocks. Thus, SC-Nanophytosomes' ability to reach and modulate the mitochondrial functionality of BV-2 cells can be compromised. Additionally, the microglia cells have a very low mitochondrial membrane potential when compared to neurons, which may bias the effectiveness of SC-Nanophytosomes to modulate the mitochondria. Regarding the microglia cells phenotype remodelling induced by IFN- γ , a study to assess the effects of dose and exposure time on the phenotype markers is also a relevant issue that is not addressed in the present work.

Comparing the results on BV-2 cells with those obtained with SH-SY5Y cells, we can conclude that SC-Nanophytosomes affect the activity of microglial cells differently than neurons; thereby, they exhibit bioactivity dependent on cell type. Thus, the effects on microglia cells suggest that SC-Nanophytosomes did not have anti-inflammatory activity. However, the results obtained with the SH-SY5Y cells suggest that SC-Nanophytosomes have excellent properties to support a mitochondria-targeted therapy to address the challenge of neuronal mitochondria dysfunction underlying neurodegenerative diseases, including AD and PD. Thus, SC-Nanophytosomes were moved for *in vivo* assays.

The oral administration route has many advantages over the various other administration routes of drug delivery, including safety, good patient compliance, ease of ingestion and pain avoidance (385). To evaluate if aqueous SC-Nanophytosomes formulation is suitable for oral administration, its properties under pH conditions that mimic the gastrointestinal tract environments were assessed. Results revealed that gastrointestinal tract-like pH changes do not affect the stability of the formulation nor its ability to preserve the anthocyanins in the flavylum cation form, with mitochondriotropic properties. Thus, to obtain a proof-of-concept of the SC-Nanophytosomes as a mitochondria-targeted therapy, the oral administration route was rationalized to a rotenone-induced PD-like pathology animal model.

To induce a PD-like pathology, animals were injected with rotenone (3.0 mg/kg of animal weight) three times a week for three weeks, then the animals were randomly divided into two groups (ROT and ROT+SC-Nanophyt groups). Simultaneously, animals of the control (CTRL) group were injected with the vehicle. One week later, the experimental plan followed with CTRL, ROT, and ROT+SC-Nanophyt groups. SC-Nanophytosomes were delivered to the animals of the ROT+SC-Nanophyt group by drinking water for three weeks,

at a final concentration of 3 μ M in phospholipids plus 2.5 mg/L in EAE-extract. *Ex vivo* assays were performed with two brain portions, the BG-Cereb and the C-Cortex.

Results show that with this experimental design, the PD-like pathology model exhibits a set of phenotypic markers related to PD, namely: i) a significant motor deficit; ii) higher brain levels of α -synuclein in BG-Cereb portion; iii) compromised mitochondrial respiratory chain functionality in both brain portions, well-evidenced by a 50% decrease in respiratory control rate and up to 40% in mitochondrial complex I; iv) an altered mitochondrial membrane lipidome in both brain portions and v) changes in the SOD and CAT activities that are dependent on the brain portion. The treatment with SC-Nanophytosomes promoted significant benefits on motor coordination and balance disturbance, decreased brain levels of α -synuclein levels to the values detected in control animals and recovered the mitochondrial phosphorylation efficiency and complex I activity for values similar to control animals. Furthermore, the fatty acid profile of the mitochondrial membrane phospholipids and the cytosolic activity of the antioxidant enzymes, affected by rotenone-induced PD-like neurodegeneration, were ameliorated to control levels. The positive outcomes on the brain mitochondrial respiratory chain can be attributed to elderberry anthocyanins, while the modulation of mitochondrial lipidome to the algae lipids. Although these results do not allow rationalize the nanoplatforms' ability to cross the blood-brain barrier, they support the idea that SC-Nanophytosomes is an effective tool to provide elderberry anthocyanins and algae lipids for the brain cells.

Regarding the relevance of motor symptoms on PD progression, the effects of the SC-Nanophytosomes treatment on skeletal muscle tissues were also assessed. Although the skeletal muscle tissues of the rats with PD-like pathology only exhibit small changes in the cellular redox state, the higher activity of the CAT enzyme, compared with control animals, suggests that they are under oxidative stress stimuli. SC-Nanophytosomes treatment modulates the redox potential of the skeletal muscle tissues leading cell cytosol towards more negative values (with a higher GSH/GSSG ratio), with a concomitant changing of the activity of the main antioxidant enzymes. In fact, results support the idea that SC-Nanophytosomes treatment improves the competence of the skeletal muscle tissues in counteracting oxidative stress damage. Furthermore, the SC-Nanophytosomes treatment mitigates the impairment promoted by PD-like pathology on the mitochondrial redox chain complexes activity and counteracts the changes in the fatty acid profile presented by this animal model of PD. These benefits suggest that SC-Nanophytosomes, delivered by oral route, have the competence to target mitochondria not only in the brain but also in skeletal muscle cells.

If the brain effects of the SC-Nanophytosomes restore the dysfunctional connection between the brain and skeletal muscle tissues underlying PD-like pathology, then the skeletal muscle positive outcomes can also emerge from the brain benefits. However, experimental data show that beneficial effects on skeletal muscle functionality positively impact brain disorders, including PD, a functional feature assigned to myokines and metabolites produced by skeletal muscle tissues (386,387). Given that oral administration has the potential to deliver SC-Nanophytosomes for brain and peripheral tissues, the positive SC-Nanophytosomes' outcomes on motor and non-motor symptoms of the PD-like pathology can emerge from effects on brain and skeletal muscle tissues simultaneously. In fact, the positive outcomes of SC-Nanophytosomes on cells and animals with PD-like pathology can be endorsed to the bioactivity of its building blocks organized in nanostructured vesicles with competence to overcome the different physiological/cellular barriers and deliver algae phospholipids and elderberry anthocyanins for subcellular targets (e.g., mitochondria) at concentrations with therapeutic efficiency. Thus, the functional organization of the inner membrane of mitochondria may be improved as a consequence of the lipid modulation promoted by membrane polar lipids from *C. tomentosum* and by the presence of exogenous elderberry anthocyanins that overcome putative deficiencies of complex I deficiencies working as membrane electron carrier that oxidizes NADH and deliver the electrons for complex III. In this way, the results in the brain and skeletal muscle tissues prove the concept of SC-Nanophytosomes as a mitochondria-targeted therapy.

The above-mentioned explanation is indirectly supported by the positive outcomes on mitochondrial functionality obtained in clinical trials designed to test the effects of membrane phospholipids along with antioxidants as functional oral supplements on aged subjects and patients with fatigue (388,389). Authors have named this strategy as lipid replacement therapy, highlighting that it differs from other lipid-based approaches since the membrane phospholipids, protected by antioxidant molecules, have the competence to target the mitochondria of different types of cells/ tissues (e.g., skeletal muscle), where they naturally replace and stimulate the removal of damaged membrane lipids (389,390). However, it is also important to claim that the lipid replacement therapy did not show how the membrane phospholipids, delivered as solid-like formulations, are able to escape from hydrolyses promoted by gut enzymes nor reveal its competence to surpass the physiological and cellular barriers required to reach mitochondria of target cells. On the other hand, our results support the idea that the therapeutic success of SC-Nanophytosomes, as a mitochondria-targeted therapy, emerges from the specific properties and bioactivities of the membrane polar lipids from *C. tomentosum* and elderberry anthocyanins self-organized in nanostructured vesicles within an aqueous

environment - a process driven by the entropy of the water molecules (hydrophobic effect) and that take advantage from strong interaction between anionic phospholipids and the anthocyanins in its positively charged flavylium form.

Considering that the motor deficit, mitochondrial dysfunction and α -synuclein accumulation are pathological features of PD in humans, it is expectable that SC-Nanophytosomes will be able to promote positive outcomes in human patients. However, the SC-Nanophytosomes formulation must be tested in other PD-animal models that recapitulate the above-mentioned pathological biomarkers (e.g., MPTP-induced PD-like pathology, MitoPark mice) to obtain a detailed pre-clinical overview, including their safety profile and pharmacokinetic parameters in blood, brain, and skeletal muscle tissues. These studies are a key to open the door for i) clinical trials, aiming the assessment of the effectiveness of SC-Nanophytosomes as a mitochondria-targeted therapy for neurodegenerative diseases, like PD; ii) development of innovative functional food supplements containing redox-active elderberry anthocyanins and membrane polar lipids rich in anionic phospholipids and essential omega-3 fatty acids assembled in lipid-based nanovesicles.

Before being used in the clinic, SC-Nanophytosomes need to go a long way to prove its effectiveness and non-toxicity in multiple steps. However, current knowledge allows to ensure, right now, the health benefits of SC-Nanophytosomes through their incorporation in food supplements. The development of foods with gel-like properties seems to be the more suitable way to deliver the SC-Nanophytosomes for elderly people with or at risk of developing degenerative brain diseases, like PD and AD. In fact, gel-type foods exhibit aqueous interstices with the size and amount of water needed to house and preserve the SC-Nanophytosomes with their functional properties, and taste and texture aligned with older people's appreciation. Thus, the next step of the research will be focused on the development of formulations containing the SC-Nanophytosomes and gel-like properties, *i.e.*, dietary supplements directed to older people, including those living with degenerative brain diseases.

CHAPTER V

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