

MESTRADO ESPECIALIDADE EM TOXICOLOGIA ANALÍTICA CLÍNICA E FORENSE

Development of lipid-based nanosystems (SLN and NLC) for nose-to-brain delivery: a potential strategy for the treatment of neurodegenerative diseases

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neurodegenerative diseases

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Abstract

Currently, research on the development of alternatives for the management of neurodegenerative diseases have become necessary due to the global increase in life expectancy. Herein, the use of active molecules from natural products has been extensively investigated. In particular, as oxidative stress is related with the progress of several neurodegenerative diseases, antioxidants seem to play an important role in their treatment and prevention. However, these compounds show difficulties in reaching the brain, as they need to enter the brain through the blood-brain barrier (BBB). Therefore, to circumvent this limitation, the use of the nose-to-brain route is being extensively investigated.

The objective of this work was to develop and characterize (particle size, polydispersity index - PDI, zeta potential - ZP, encapsulation efficiency – EE, and shape by CryoSEM) two lipid-based formulations, containing solid lipid nanoparticles (SLN) and nanostructured lipid carriers (NLC) loaded with astaxanthin (ASTX) for nose-to-brain delivery. Then, the biocompatibility of the formulations was evaluated in differentiated SH-SY5Y neuronal cells and in RPMI 2650 nasal cells. Finally, the neuroprotective effects of the formulations against the cytotoxicity induced by Iron (III), MPP⁺ and *t*-BHP were also assessed. In addition, an HPLC-UV method for ASTX detection and quantification was developed and validated.

The developed ASXT-loaded SLN and ASXT-loaded NLC formulations showed adequate particle sizes for intranasal administration (50% of the particles had sizes equal or lower than 63.400±0.001 nm and 59.200±0.000 nm, for SLN and NLC, respectively), PDI (0.349 ± 0.056 and 0.294 ± 0.035, for SLN and NLC, respectively), ZP (-24.333 ± 1.234 mV and -23.300 ± 1.058 mV, for SLN and NLC, respectively) and EE (98.75 ± 0.88 % and 98.55 ± 0.39 %, for SLN and NLC, respectively); and were stable over 30 days of storage at room temperature. These formulations demonstrated to be safe for differentiated SH-SY5Y cells and for RPMI 2650 cells, in concentrations up to 100 µg/mL.

Outstandingly, ASTX-loaded NLC demonstrated capacity to counteract the harmful effects of Iron (III), *t*-BHP and MPP⁺, while ASTX-loaded SLN only provided significant protective effects against *t*-BHP and MPP⁺-induced cytotoxicity. Noteworthy, the improved neuroprotective effect observed for ASTX-loaded NLC against *t*-BHP, when compared to free ASTX, can be justified by the synergistic antioxidant effect of ASTX and vitamin E, which are both present in the formulation.

Regarding the developed HPLC-UV method, a mobile phase containing methanol: water (97:3 v/v) was used, with an injection volume of 20 µL, at a flow rate of 1 mL/min. The validated method showed good linearity, as proven for the high value of r^2 (0,9911), good

accuracy and precision and low limit of detection (LOD) and limit of quantification (LOQ) values (2.2 and 6.9 ng/mL, respectively).

Given the significant neuroprotective effects observed for the developed formulations, in particular their antioxidant effect against oxidant aggressors, it is possible to conclude that ASTX-loaded NLC may have therapeutic potential in counteracting the oxidative stress observed in several neurodegenerative diseases. However, more research is needed to confirm this results, and to fully elucidate their therapeutic potential.

Keywords: neurodegenerative diseases; solid lipid nanoparticles (SLN); nanostructured lipid carriers (NLC); nose-to-brain; astaxanthin.

Resumo

Atualmente, investigação para o desenvolvimento de alternativas para o tratamento de doenças neurodegenerativas ganhou preponderância com o aumento da esperança média de vida. Para este efeito, o uso de princípios ativos provenientes de compostos naturais tem sido extensivamente estudado. Particularmente, os antioxidantes, dado o stress oxidativo estar presente em várias doenças neurodegenerativas, parecem ter um papel importante no tratamento e prevenção destas patologias. No entanto, este tipo de compostos apresenta dificuldades em chegar ao cérebro, pois têm de atravessar a barreira hematoencefálica, BBB (do inglês, *blood-brain barrier*). Assim, para ultrapassar esta limitação, o uso da via nariz-cérebro (do inglês *nose-to-brain*) está a ser extensivamente investigado.

O objetivo deste trabalho foi desenvolver e caracterizar (tamanho de partículas, índice de polidispersão [PDI], potencial zeta [ZP], eficácia de encapsulação [EE] e forma) duas formulações à base de nanopartículas lipídicas, SLN (do inglês, *solid lipid nanoparticles*) e NLC (do inglês, *nanostuctured lipid carriers*) contendo astaxantina (ASTX) encapsulada, para administração intranasal. Após a produção e caracterização, a biocompatibilidade das formulações foi avaliada em células neuronais diferenciadas (SH-SY5Y) e em células nasais (RPMI 2650). Por fim, o potencial de neuroproteção das formulações foi avaliado contra a citotoxicidade induzida pelo Ferro (III), MPP⁺, e *t*-BHP. Adicionalmente, um método em HPLC-UV para a deteção e quantificação de ASTX foi desenvolvido e validado.

Ambas as formulações desenvolvidas, SLN e NLC, demonstraram tamanho de partículas adequado à administração intranasal (50% dos valores de tamanho de partícula foram iguais ou inferiores a 63.400 ± 0.001 nm e 59.200 ± 0.000 nm, respetivamente), PDI (0.349 ± 0.056 e 0.294 ± 0.035 , respetivamente), ZP (-24.333 ± 1.234 mV e -23.300 ± 1.058 mV, respetivamente) e EE (98.75 ± 0.88 % e 98.55 ± 0.39 %, respetivamente), e estabilidade após 30 dias de armazenamento, à temperatura ambiente. As formulações revelaram-se não citotóxicas para células SH-SY5Y diferenciadas e células RPMI 2650 em concentrações até 100 µg/mL. É de salientar que as NLC com ASXT encapsulada demonstraram ser capazes de contrariar o efeito negativo do Ferro (III), *t*-BHP e MPP⁺, enquanto as SLN com ASXT encapsulada apenas apresentaram proteção significativa contra a citotoxicidade provocada pelo *t*-BHP e MPP⁺. É de reforçar que, o desempenho das NLC com ASXT encapsulada, quando comparado com o da ASTX livre, pode ser justificado pelo efeito antioxidante conjunto com a vitamina E presente na formulação dos NLC.

No que diz respeito ao método de HPLC-UV, uma fase móvel composta por metanol: água (97:3 v/v) foi utilizada, com um volume de injeção de 20 µL, a um ritmo de 1 mL/min. O método validado demonstrou boa linearidade, como se pode verificar pelo valor de r^2 (0.9911), boa exatidão e precisão, e um baixo limite de detecção (LOD) e de quantificação (LOQ) (2.2 e 6.9 ng/mL, respetivamente).

Concluindo, e tendo em conta os efeitos neuroprotetores observados nas formulações desenvolvidas, em particular o seu efeito contra oxidantes, é possível afirmar que estas formulações à base de SLN e NLC com ASTX encapsulada podem ter potencial terapêutico reduzindo o stress oxidativo observado em diversas doenças neurodegenerativas. Não obstante, mais investigação é necessária para confirmar estes resultados, e elucidar por completo o seu potencial terapêutico.

Palavras-chave: doenças neurodegenerativas; *solid lipid nanoparticles* (SLN); *nanostructured lipid carriers* (NLC); *nose-to-brain*; astaxantina.

Index

Acknowledgments.....	V
Abstract.....	VI
Resumo	VIII
Index	X
Figures Index	XII
Tables Index.....	XIII
Equations Index.....	XIV
Abbreviation List	XVI
1. Neurodegenerative Diseases – An Overview	1
2. Natural Compounds As A Promising Source Of New Biologically Active Drugs For The Treatment Of Neurodegenerative Diseases	4
2.1. Natural Antioxidants	13
3. Lipid Based Nanosystems As Smart Delivery Systems To The Brain.....	17
3.1. Solid Lipid Nanoparticles (Sln) And Nanostructured Lipid Carriers (Nlc)	18
3.2. Nose-To-Brain Delivery: An Alternative Route For Sln And Nlc To Reach The Brain	21
3.3. Relevant Studies With Lipid-Based Nanosystems For Brain Delivery Of Drugs And Natural Compounds	24
4. Objectives.....	27
5. Materials And Methods.....	28
5.1. Materials	28
5.2. Preparation Of The Lipid Nanoparticles Formulations	28
5.3. Characterization Of The Lipid Nanoparticles Formulations	29
5.3.1. Particle Size, Polydispersity Index And Zeta Potential.....	29
5.3.2. Morphology: Cryosem	30
5.3.2. Evaluation Of The Encapsulation Efficacy	31
5.4. <i>In Vitro</i> Studies With Differentiated Sh-Sy5y Cells	31
5.4.1. <i>SH-SY5Y Cells As An In Vitro Neuronal Model</i>	31
5.4.2. <i>SH-SY5Y Cells: Cell Culture, Seeding And Differentiation</i>	32
5.4.3. <i>Evaluation Of The Cytotoxicity Of The Developed Formulations</i>	32
5.4.3.1. <i>MTT Reduction Assay</i>	32
5.4.3.2. <i>Neutral Red Uptake Assay</i>	33
5.4.4. <i>Cellular Uptake Of Astx</i>	34
5.4.5. <i>Evaluation Of Potential Neuroprotective Effects Of The Developed Formulations</i>	34
5.4.5.1. <i>Protective Effects Against Fenta-Induced Cytotoxicity</i>	35
5.4.5.2. <i>Protective Effects Against T-Bhp-Induced Cytotoxicity</i>	35
5.4.5.3. <i>Protective Effects Against Mpp⁺-Induced Cytotoxicity</i>	36
5.5. <i>IN VITRO STUDIES WITH RPMI 2650 CELLS</i>	37
5.5.1. <i>RPMI 2650 As An In Vitro Model For Studies In Nose-To-Brain Delivery</i>	37
5.5.2. <i>RPMI 2650 Cells: Cell Culture And Seeding</i>	37
5.5.3. <i>Evaluation Of The Cytotoxicity Of The Developed Formulations</i>	37
5.6. DEVELOPMENT OF AN HPLC-UV METHOD FOR THE QUANTIFICATION OF ASTX.....	38

5.6.1.	<i>Instrumental And Chromatographic Conditions</i>	38
5.6.2.	<i>Preparation Of Astx Standards</i>	38
5.6.3.	<i>Method Validation</i>	38
5.6.3.1.	Specificity	39
5.6.3.2.	Linearity	39
5.6.3.3.	Accuracy	39
5.6.3.4.	Precision	40
5.6.3.5.	Sensitivity Of The Method- LOD And LOQ.....	42
5.6.3.6.	Stability	42
5.6.3.7.	Proof Of Applicability.....	43
5.7.	STATISTICAL ANALYSIS	43
6.	Results And Discussion.....	44
6.1.	Characterization Of Astaxanthin-Loaded Lipid Nanoparticles	44
6.1.1.	Particle Size, Polydispersity Index And Zeta Potential.....	44
6.1.2.	Morphology: Cryosem	45
6.1.3.	Evaluation Of The Encapsulation Efficiency	45
6.2.	Evaluation Of The Cytotoxicity Of The Developed Formulations	46
6.3.	Cellular Uptake Of Astx.....	52
6.4.	Evaluation Of Potential Neuroprotective Effects Of The Developed Formulations ...	53
6.4.1.	Protective Effects Against FeNTA-Induced Cytotoxicity.....	53
6.4.2.	Protective Effects Against <i>t</i> -BHP-Induced Cytotoxicity	56
6.4.3.	Protective Effects Against MPP ⁺ -Induced Cytotoxicity	59
6.5.	Method Validation.....	61
6.5.1.	Chromatographic Conditions.....	61
6.5.2.	Specificity	62
6.5.3.	Linearity	64
6.5.4.	Accuracy Of The Method	65
6.5.5.	Precision	65
6.5.6.	Sensitivity Of The Method: LOQ And LOD.....	66
6.5.7.	Stability	66
7.	Conclusions And Future Perspectives	68
8.	References.....	69

Figures Index

FIGURE 1. CHEMICAL STRUCTURE OF ASTX.	14
FIGURE 2. SCHEMATIC REPRESENTATION OF THE DIFFERENCES BETWEEN SLN AND NLC (ADAPTED FROM (133))	20
FIGURE 3. ADVANTAGES AND LIMITATIONS OF THE NASAL/INTRANASAL DRUG ADMINISTRATION (ADAPTED FROM (1))	22
FIGURE 4. CRYOSEM IMAGES OF THE PREPARED SLN AND NLC FORMULATIONS: (A) EMPTY NLC (MAGNIFICATION 35000X); (B) ASTAXANTHIN-LOADED NLC (MAGNIFICATION 10000X); (C) EMPTY SLN (MAGNIFICATION 20000X); AND (D) ASTAXANTHIN-LOADED SLN.	45
FIGURE 5. CYTOTOXICITY OF BOTH EMPTY AND ASTX-LOADED SLN (A) AND NLC (B) FORMULATIONS TOWARDS DIFFERENTIATED SH-SY5Y CELLS, EVALUATED BY THE NR UPTAKE ASSAY, 24 H AFTER EXPOSURE. THE CYTOTOXICITY OF FREE ASTX WAS ALSO EVALUATED	47
FIGURE 6. CYTOTOXICITY OF BOTH EMPTY AND ASTX-LOADED SLN (A) AND NLC (B) FORMULATIONS TOWARDS DIFFERENTIATED SH-SY5Y CELLS, EVALUATED BY THE MTT REDUCTION ASSAY, 24 H AFTER EXPOSURE.	48
FIGURE 7. CYTOTOXICITY OF BOTH EMPTY AND ASTX-LOADED SLN (A) AND NLC (B) FORMULATIONS TOWARDS RPMI 2650 CELLS, EVALUATED BY THE NR UPTAKE ASSAY, 24 H AFTER EXPOSURE.	50
FIGURE 8. CYTOTOXICITY OF BOTH EMPTY AND ASTX-LOADED SLN (A) AND NLC (B) FORMULATIONS TOWARDS RPMI 2650 CELLS, EVALUATED BY THE MTT REDUCTION ASSAY, 24 H AFTER EXPOSURE.	51
FIGURE 9. CELLULAR UPTAKE OF ASTX, EVALUATED IN DIFFERENTIATED SH-SY5Y 24 HOURS AFTER EXPOSURE TO 100 µG/ML ASTX-LOADED SLN (A) AND NLC (B) FORMULATIONS, AS WELL AS TO THE CORRESPONDING FREE ASTX CONCENTRATIONS.	53
FIGURE 10. POTENTIAL NEUROPROTECTIVE EFFECTS OF THE DEVELOPED SLN (A) AND NLC (B) FORMULATIONS (BOTH EMPTY AND ASTX-LOADED), AND FREE ASTX, AGAINST FENTA-INDUCED CYTOTOXICITY, EVALUATED IN DIFFERENTIATED SH-SY5Y CELLS, BY THE NEUTRAL RED UPTAKE ASSAY, 24 H AFTER EXPOSURE TO THE AGGRESSOR.	55
FIGURE 11. POTENTIAL NEUROPROTECTIVE EFFECTS OF THE DEVELOPED SLN (A) AND NLC (B) FORMULATIONS (BOTH EMPTY AND ASTX-LOADED), AND FREE ASTX, AGAINST T-BHP-INDUCED CYTOTOXICITY, EVALUATED IN DIFFERENTIATED SH-SY5Y CELLS, BY THE NEUTRAL RED UPTAKE ASSAY, 24 H AFTER EXPOSURE TO THE AGGRESSOR.	58
FIGURE 12. POTENTIAL NEUROPROTECTIVE EFFECTS OF THE DEVELOPED SLN (A) AND NLC (B) FORMULATIONS (BOTH EMPTY AND ASTX-LOADED), AND FREE ASTX, AGAINST MPP⁺-INDUCED CYTOTOXICITY, EVALUATED IN DIFFERENTIATED SH-SY5Y CELLS, BY THE NEUTRAL RED UPTAKE ASSAY, 24 H AFTER EXPOSURE TO THE AGGRESSOR.	60
FIGURE 13. CHROMATOGRAM OBTAINED AFTER INJECTION (20 µL) OF A 5 UG/ML ASTX STANDARD SPIKED WITH 1 UG/ML OF IS.	61
FIGURE 14. CHROMATOGRAM OBTAINED AFTER INJECTION (20 µL) OF A BLANK METHANOL SAMPLE SPIKED WITH 1 UG/ML OF IS.	62
FIGURE 15. CHROMATOGRAM OBTAINED AFTER INJECTION (20 µL) OF THE SUPERNATANT OF EMPTY SLN (A) AND EMPTY NLC (B) FORMULATIONS, PREVIOUS SUBMITTED TO THE EXTRACTION PROTOCOL USED FOR THE EVALUATION OF THE FORMULATIONS EE, AND FURTHER SPIKED WITH 1 UG/ML OF IS.	63
FIGURE 16. CHROMATOGRAM OBTAINED AFTER INJECTION (20 µL) OF METHANOLIC EXTRACTS OF DIFFERENTIATED SH-SY5Y CELL EXPOSED TO BOTH EMPTY SLN (A) AND NLC (B) FORMULATIONS, FOR 24 HOURS, AND FURTHER SPIKED WITH 1 UG/ML OF IS.	63
FIGURE 17. CALIBRATION CURVES OBTAINED FROM THE ANALYSIS OF SEVEN ASTX CALIBRATORS	64

Tables Index

Table 1. Natural Compounds Used In Management Of Ad, And Their Effects And Type Of Study (In Vitro/In Vivo) Performed To Evaluate Their Properties.....	8
Table 2. Natural Compounds Used As Potential Treatment In Als, As Well As Their Effect And The Type Of Study (In Vitro/In Vivo) Performed To Evaluate Their Properties.....	13
Table 3. Composition Of The SIn And Nlc Formulations, Empty And Loaded With Asxt.	29
Table 4. Particle Size Results (D10, D50, D90, Z-Ave), Pdi And Zp Of The SIn And Nlc Formulations (Loaded With Astaxanthin – Astx And Empty) Prepared On Day 0 And After Storage At $20.0 \pm 0.5^{\circ}\text{C}$, For 30 Days. All Results Are Presented As Mean Values $\pm$ Sd.	44
Table 5. Calibration Curves Obtained After Preparation And Injection (20 μl) Of Seven Astx Standards (0 - 7.5 $\mu\text{g}/\text{ml}$) Spiked With 1 $\mu\text{g}/\text{ml}$ Is. Five Astx Calibration Curves Were Prepared And Analyzed In Five Different Days.....	64
Table 6. Accuracy Of The Developed Hplc-Uv Method Evaluated At 3 Levels Of Astx Concentration (Low, Medium, High) Within The Linear Concentration Range.....	65
Table 7. Inter-Day And Intra-Day Precision Of The Developed Hplc-Uv Method Evaluated At 3 Levels Of Astx Concentration (Low, Medium, High) Within The Linear Concentration Range.	66
Table 8. Astx Stability Evaluated After Samples Storage At Different Temperature For 7 Days.	67

Equations Index

Equation 1. Equation Used To Calculate The Encapsulation Efficacy Percentage	31
Equation 2. Equation Used To Calculate Accuracy Percentage	40
Equation 3. Equation Used To Calculate Lod	42
Equation 4. Equation Used To Calculate Loq	42

Abbreviation List

AChE - acetylcholinesterase enzyme
AD - Alzheimer's disease
ALS - Amyotrophic lateral sclerosis
APP - amyloid precursor protein
ASTX - astaxanthin
A β - β -amyloid peptide
BBB - blood brain barrier
BDNF - brain derived neurotrophic factor
BIM - BCL2L11 protein
CAT - catalase
CNS - central nervous system
COMT - catechol-O-methyl transferase
CSF - cerebrospinal fluid
DLS - dynamic light scattering technique
DMEM - Dulbecco's modified eagle medium
DMSO - Dimethyl sulfoxide
EDTA - ethylenediamine tetraacetic acid
EE - encapsulation efficiency
EGCG - epigallocatechin gallate
ERK1 - extracellular regulated kinase-1
ER α - estrogen receptor α
FBS - fetal bovine serum
FDA - Food and Drug Administration
FeCl₃ - iron (III) chloride
FeNTA - iron (III)- nitrilotriacetic acid complex
GP1B - G- protein coupled receptor 1
GPX - glutathione peroxidase
GSH - glutathione
GSK-3 β - glycogen synthase kinase-3 beta
HBSS - Hanks' balanced salt solution
HPLC - High performance liquid chromatography
IFN- γ - interferon γ
IGF - insulin-like growth factor
iNOS - inducible nitric oxide synthase
IS - internal standard

JNK - c-Jun amino-terminal kinase

LD - laser diffraction

L-DOPA - L-3,4-dihydroxyphenylalanine

LOD - limit of detection

LOQ - limit of quantification

MAO-B - monoamine oxidase type B

MAPK - mitogen-activated protein kinases

MDA - malonaldehyde

MPP⁺ - 1-methyl-4-phenylpyridinium

MPTP - 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine

MTT - 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide

Na₂NTA - nitrilotriacetic acid disodium salt

Na₃NTA - nitrilotriacetic acid trisodium salt

NF-κB - inflammatory mediator nuclear factor kappa-light-chain-enhancer of activated B cells

NLC - nanostructured lipid carriers

NO - nitric oxide

NR – Neutral Red

Nrf2 - nuclear factor erythroid 2-related factor

NTA - nitrilotriacetic acid

PD - Parkinson's disease

PDI - polydispersity index

RA - retinoic acid

REZ - resazurin

ROS - reactive oxygen species

SD - standard deviation

SEM - scanning electron microscopy

SLN - solid lipid nanoparticles

SOD - superoxide dismutase

T-AOC - total antioxidant capacity

t-BHP - tert-Butyl hydroperoxide

TPA - phorbol 12-myristate 13-acetate

UV – ultraviolet radiation

ZP – zeta potential

1. Neurodegenerative diseases – an overview

With the aging of the world's population, neurodegenerative diseases appear to be a growing problem for public health. It is estimated that neurodegenerative diseases affect 50 million people around the world and, by the year of 2050, this number can rise up to 115 million people (2). Alzheimer's disease (AD), Parkinson's disease (PD), and amyotrophic lateral sclerosis (ALS) are three of the most prevalent neurodegenerative diseases. The incidence of these diseases upsurge severely with age and, therefore, the number of cases will probably rise in the near future, as life spans in several countries is increasing (3).

Despite the continuous efforts made to obtain a therapeutically effective solution for the treatment of neurodegenerative diseases, results have not been promising, and AD and dementia remain to be an unmet medical problem in elderly people (4, 5).

The blood–brain barrier (BBB) remains to be a real obstacle concerning the effective management of neurodegenerative diseases, as it constitutes a barrier to the passage of several drugs to the central nervous system (CNS). Regardless the accomplishments that have been with surgeries and other extremely invasive techniques, their clinical relevance is reduced due to many concerns about their long-term benefit, owed to the possible injury to the BBB (6).

Alzheimer's disease (AD) is the most common form of dementia, as approximately 35 million people worldwide live with AD (7). AD usually appears in late stages of life, but it can arise as early as at the third decade of life. Often, it starts with the loss of short memory, followed by slowly progressive dementia. Contrasting with other forms of dementia, AD doesn't disturb patients' motor function until late stages of the disease (8).

Although AD's pathogenesis is yet to be fully elucidated, the aggregation of misfolded tau proteins that leads to the formation of intraneuronal neurofibrillary tangles, the formation of extracellular senile plaques that consist predominantly of insoluble deposits of β -amyloid peptide ($A\beta$), the oxidative stress, the neuronal loss, ferroptosis and the activation of microglia are considered the main pathophysiological hallmarks of the disease (9).

Many environmental factors, including the exposure to aluminum, mercury, and viruses, have also been suggested as causes of AD, but several studies were unsuccessful to validate a significant role for any of these exposures. Equally, several studies imply that the use of nonsteroidal anti-inflammatory agents is associated with a reduced risk of AD, but this correlation has not been fully established (10).

Nowadays, there is no cure or an effective pharmacologic treatment for AD capable of stopping or delaying disease progression. The primordial focus is on the long-term improving of behavioral and neurologic associated problems, as well as offering caregiver support. Currently, there are three drugs being used in clinical practice to manage AD,

namely, galantamine, rivastigmine, and donepezil, which are cholinesterase inhibitors. However, these drugs, unfortunately, only help in reducing or controlling some behavioral and cognitive symptoms and do not stop or delay the disease progression (11). To counteract the limitation of the currently used drugs, some potential therapies are being tested, such as neurotransmitter-based therapies and therapies targeting the A β burden (9).

Parkinson Disease (PD) is a neurologic and progressive degenerative disease manifested by motor and nonmotor symptoms (12). It is projected that the number of people diagnosed with PD will be reaching approximately 10 million persons by 2030, based on the aging of the world population (13).

The chance of developing PD increases with age, as the mean age of PD onset is 60 years. Nonetheless, it can be diagnosed, in some cases, in patients in their twenties and even in earlier ages, exceptionally when it is correlated with a gene mutation (mutations in the *LRRK2*, *PARK7*, *PINK1*, *PRKN* and *SNCA* genes were already correlated with PD) (14).

PD is characterized by rest tremor, bradykinesia, stiffness and gait dysfunction with postural instability. Additional medical features can involve freezing of gait, trouble speaking, swallowing problems, mood disorders, trouble sleeping and dementia (9).

Pathologically, the main hallmarks of PD include the degeneration of the dopaminergic neurons of the *substantia nigra pars compacta*, the low striatal levels of dopamine and the formation of Lewy bodies, which are intraneuronal inclusions containing α -synuclein aggregates and ubiquitin that are present in the neocortex, brainstem, forebrain and other areas of the nervous system. The latter are also known as Lewy pathology can affect cholinergic, noradrenergic, and serotonergic neurons, as well as the neurons of the olfactory system, spinal cord, cerebral hemispheres and peripheral nervous system, with this “nondopaminergic” disease being responsible for the nonmotor symptoms (13).

Even existing numerous therapeutic drugs available for the treatment of PD, many patients still develop an intolerable disability (9), thus making the search for a new disease-modifying drug an emerging line of research. Nowadays, several therapeutic approaches are being used in the treatment of PD, such as the administration of levodopa, dopamine agonists, catechol-O-methyl transferase (COMT) enzyme inhibitors, monoamine oxidase type B (MAO-B) inhibitors, among others. Nevertheless, this existing approaches merely reduce symptoms and unluckily do not stop disease progression (9).

Amyotrophic lateral sclerosis (ALS) is the most usual form of progressive motor neuron disease. It is a major standard of a neurodegenerative disease and is possibly the most disturbing among the neurodegenerative disorders (15). However, the primary cell death pathway(s) involved in motor neuron death remain unclear. Motor neuron death was initially suggested to be apoptotic, caused by the activation of death receptors or by

activating the intrinsic mitochondrial pathway leading to caspase activation (16). Furthermore, it was demonstrated, in a mouse model of ALS, that the inflammatory form of regulated cell death called necroptosis was not involved in motor neuron death. Noteworthy, several pieces of evidence suggest that ferroptosis, an iron-dependent way of regulated cell death, is indeed present in ALS (16). Although ALS pathophysiology indeed remains largely unknown, several pathophysiological mechanisms were already linked to this disease, namely oxidative stress with increased levels of reactive oxygen species (ROS), iron metabolic dysregulation and ferroptosis, protein aggregation, altered astrocyte function, neuroinflammation and glutamate-induced excitotoxicity (17).

Currently, there is no treatment capable of stopping or delaying the pathologic process of ALS. Riluzole was approved by the Food and Drug Administration (FDA) for the treatment of ALS because it seems to extend the survival of the patients, although for a short period of time. The mechanism underlying this effect is not clearly known, but it seems that riluzole has the ability to decrease glutamate-mediated excitotoxicity by reducing glutamate release (18). The efficiency of riluzole was recognized in two clinical trials. Patients involved in these clinical trials had ALS for less than 5 years and possessed a baseline forced vital capacity $\geq 60\%$. In the first trial, the survival time was around 17.7 months in the riluzole group, while it was 14.9 months in the placebo group. On the other hand, in the second clinical trial, the survival time was about 16.5 months in the riluzole group versus 13.5 months in the placebo group (19, 20).

Another FDA-approved drug, edaravone, has also been approved by the FDA based on a study that verified a significant reduction in the worsening of ALS in patients with ALS (21). This drug is believed to be a scavenger capable of scavenging reactive oxygen species (ROS) and free radicals, thus acting as an antioxidant protecting neurons from oxidative stress (18, 22). For edaravone, one Phase II open-label trial and two Phase III placebo-control trials have been already performed. In all three clinical trials, the main sign assessed was the patients motor function. These trials suggested that edaravone is harmless and effective in ALS. It decreased 3-nitrotyrosine levels, a marker of oxidative stress, and also showed to slow the disease progression (18, 23). In May of 2022, an oral formulation of edaravone was approved in the USA and there is research undergoing to establish if a higher dose of oral formulations may be beneficial. A Phase 1 study has revealed a bioequivalence of a 105 mg oral suspension, and a Phase 3 clinical trial in Europe is assessing the efficacy of a separate oral formulation (24).

Neurons play a critical role in the normal function of the human brain, as they are essential in communication and are present all around the human body, although most are originated in the brain (25, 26). Neural stem cells produce, during childhood, the majority of neurons, which significantly decrease in number during adulthood (27). Neurons are not

immortal, and the neurodegeneration (i.e. the progressive loss of neuron structure, loss of neurons and/or their functions) is the principal pathophysiologic symptom of many brain disorders (28). Neurodegeneration is related with the deposition of physiochemical altered variants of proteins in the brain and with the dysfunction of neural network and synapse (29-32).

Age is the most contributive risk factor to the development of all neurodegenerative diseases, but recent findings disclose that the combination of environmental factors with genetic predisposition can similarly contribute to increasing the risk for neurodegenerative diseases (6).

As the brain regulates several features of the body's function, neurodegenerative diseases subsequently affect numerous aspects of the human performance and limit the capability to complete basic tasks (6). Therefore, and knowing that no effective drug exists able to cure or capable of stopping or delaying the progression of the most prevalent neurodegenerative diseases previously mentioned, there is a huge demand for the development of new disease-modifying drugs or therapeutic strategies to circumvent the BBB and increase the therapeutic efficacy of existing drugs. Additionally, and noteworthy, it is also currently recognized that, in contrast to the use of a cocktail of drugs, the development of a multitarget drug, acting on more than one of the disease pathophysiological pathways, should be faced as the foremost therapeutic approach, significantly reducing off-target side effects and drug-drug interaction, while increasing patients compliance and therapeutic effectiveness.

2. Natural compounds as a promising source of new biologically active drugs for the treatment of neurodegenerative diseases

Natural sources, such as plants, provide a huge variety of compounds that have been widely used by humans as medicines. The therapeutic use of plants can be followed back to the Sumerian and Akkadians civilization (33). Natural products have been important in primordial medicine systems, as in Unani, Chinese and Ayurveda, which have been used by humans until now. According to the World Health Organization, around 75% of the world population still trusts in plant-based ancient medicines for primary health care (34). Fruits, plant derived beverages, vegetables, nuts and herbs are great sources of bioactives for the treatment of neurological disorders (33).

As previously mentioned, the main neuropathological hallmark of AD is the development of A β fibrils composed of A β peptides. The exact mechanism underlying the formation of amyloid plaques remains unestablished. However, it is believed that A β is usually formed and aggregated within the extracellular matrix to create plaques. These A β

plaques are known as toxic for the contiguous neurons. Additionally, A β peptides are shown to be accumulated inside the neurons, consequently leading to synaptic malfunction, cognitive impairment and A β plaques development (35). Some naturally occurring therapeutic compounds, such as curcumin and berberine (36, 37), have shown to effectively suppress the formation of A β plaques and, therefore, have potential for the treatment of AD (38, 39). Furthermore, given the high cost associated with AD treatment and the limitations related with the conventional preventive techniques in use, natural products gained relevance in this therapeutic field (40). For instance, curcumin is a recognized element in traditional Indian cuisine, and which is obtained from the turmeric plant. This polyphenol successfully inhibited the formation of A β oligomers, bonded to the previously existing plaques and reduced A β burden *in vivo*, as shown by Yang *et al.* (41)

Multiple natural products proved to reinstate memory and cognitive shortages in the brain(42). The therapeutic properties of these products are essentially due to the action of phytonutrients on specific signaling pathways connected with protein folding and neuroinflammation (42). Furthermore, and knowing that several studies performed on AD patients demonstrated an increased oxidative stress in the affected areas of the brain (43), the use of antioxidants from natural origin may represent a potential therapeutic strategy in AD treatment. In agreement, reports from Heo and Lee (44) disclose that the compounds present in strawberry fenolic extracts demonstrated a very solid antioxidant potential against the neurotoxicity induced by hydrogen peroxide in PC12 cells. Indeed, the viability of PC12 cells was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) reduction assay upon exposure to hydrogen peroxide and the obtained results demonstrated that strawberry phenolics significantly decreased the neurotoxicity induced by the oxidative stress stimulus. Among all the three fruits tested in this study, the overall cell protection activity against oxidative stress followed the decreasing order strawberry > banana > orange, being the higher neuroprotective effects of strawberries probably explained their higher phenolic content, including anthocyanins, and anthocyanins (44).

The antioxidant potential of many natural compounds can be attributed to numerous phytoconstituents, such as gallic acid, anthocyanins, tannins, vitamin E, vitamin C and carotenoids. In particular, anthocyanins from *Vaccinium angustifolium* showed protective effects against oxidative and inflammatory stress in rats experimentally demyelinated with ethidium bromide (45). The rodents were divided in six groups: control, 30 mg/kg anthocyanins, 100 mg/kg anthocyanins, 0.1% ethidium bromide, ethidium bromide + 30 mg/kg anthocyanins and ethidium bromide + 100 mg/kg anthocyanins. The animals were treated by gavage for 7 days, once a day, with vehicle or anthocyanins, being the seventh day the peak in the demyelination. Accordingly to the obtained data, ethidium bromide-mediated demyelination increased biomarkers of oxidative stress, including the levels of 4-

hydroxynonenal, malondialdehyde, as well as increased protein carbonylation, decreased glutathione intracellular levels and decreased superoxide dismutase (SOD) activity. The concentration of 100 mg/kg anthocyanins ended up showing better protective effects against oxidative stress. Luxol fast blue staining was used to assess demyelination, demonstrating myelin loss in ethidium bromide-treated animals and confirming anthocyanins (100 mg/kg)-mediated neuroprotective effects, namely the significant decrease in ethidium bromide-mediated demyelination, restoration of the SOD levels, a decrease in the lipid peroxidation markers and it was achieved an increase in glutathione (GSH) levels (45).

Similarly, resveratrol obtained from *Vitis vinifera* has revealed alleviation of inflammation and oxidative stress (46). Jang *et al.*, conducted an *in vitro* study where PC12 cells were exposed to A β (20 μ M) for 24 hours. The treatment of those cells with resveratrol oligomers, vitisin A or heyneanol A (5- 50 μ M), significantly decreased A β -induced cell death, as evaluated by the MTT reduction assay. Additionally, A β - induced oxidative stress was significantly alleviated by the treatment with both oligomers (10, 25, 50 μ M), as demonstrated by the quantification of the intracellular ROS production (47, 48).

AD may be, as mentioned, the outcome of mutations in the gene that codes for the amyloid precursor protein (APP), originating a modified production of this peptide, which impacts the loss of neurons and disrupts synaptic connections. *Juglans regia* is recognized to assist in the inhibition of A β fibril development, in the defibrillation of the preformed A β and amyloidogenic action. Indeed, fatty acids, polyphenols, especially ellagic acid and α -tocopherol, are the main compounds responsible for this effect (49). In the study performed by Chauhan *et al.*, they assessed the potential of walnut extract on reducing A β fibrillization. Experimental samples containing 10 μ L of soluble A β (40 μ g) were incubated with different quantities of walnut extract (0,25- 5 μ L). The technique used to assess A β aggregation/fibrillization was Thioflavin T fluorescence spectroscopy and it was performed 2 and 3 days after incubation. Walnut extract showed to be effective against A β fibrillization and also demonstrated to defibrillize preformed A β fibrils, effects that occurred in a concentration and time- dependent manner. The best results were obtained for 10 μ L of the extract after 2 days of incubation (49).

A comparable type of activity was described by Frydman, *et al.*, in *Cinnamomum verum* where cinnamaldehyde, cinnamyl acetate, cinnamyl alcohol and eugenol are the mainly constituents responsible for its anti-AD activity, by stopping the oligomer and amyloid fibril formation (50). Frydman, *et al.*, evaluated the potential of cinnamon extract, orally administered to fly and mice models of AD, to reduce A β oligomerization and to correct cognitive impairment. Fly models were fed with the cinnamon extract (0,75 mg/mL) since larval stage. They were monitored daily for survival and locomotion ability. The extract

improved locomotion ability, as well as it increased the life span of the flies. The *in vivo* effect of the extract was also evaluated on an aggressive AD mouse model. Cinnamon extract was daily administered in the drinking water (100 µg/mL) from the age of 2 months, for 120 days. Then, the rodents were tested to assess cognitive abilities and presence of Aβ oligomers and plaques. In fact, they revealed an improvement of memory, when compared to the untreated group. A reduction in the quantity of oligomers was also achieved, as well as a reduction in Aβ plaques on the treated group. *In vitro*, it was also demonstrated that Cinnamon extract was capable of full restoring the viability of PC12 cells at very low concentrations, thus inhibiting Aβ-mediated cytotoxicity in this rat neuronal cell line (50).

Bromelain is a proteolytic enzyme abundantly found in fruit and stem of pineapple. It has been described that bromelain interacts and destroys synthetic amyloid-β42 monomers and oligomers. Cerebro-spinal fluid taken from AD patients, when incubated with bromelain, resulted in reduced levels of amyloid-β42 monomers and oligomers, showing that bromelain has a high potential for the treatment of AD (51).

Another important neuropathological hallmark of AD is the development of neurofibrillary tangles as a consequence of the accumulation of intracellular hyperphosphorylated neuronal microtubule-associated proteins, named “tau proteins” (52). It was shown by Liu *et al.* that berberine, an alkaloid found in many plants, significantly decreased the tau hyperphosphorylation and cytotoxicity induced by Calyculin A, a potent phosphatase inhibitor that reduced the axonal transport in neuroblastoma-2a cells (53). Additionally, it was also established by Yu *et al.*, in HEK293 cells, that berberine caused a substantial decrease of Calyculin A-induced tau hyperphosphorylation at Ser198/199/202, Ser396, Ser404, Thr205, and Thr231, and also recuperated the 2A activity of protein phosphatases and restricted glycogen synthase kinase-3 beta (GSK-3β) activation and GSK-3β phosphorylation at Tyr216 and Ser9 of tau (54).

Caffeoylquinic acid, citric acids and flavonoids from *Catharanthus roseus* have also shown to be advantageous in AD patients given their ability to inhibit acetylcholinesterase enzyme (AChE) (55). Indeed, the impairment of the cholinergic system was already reported in the basal forebrain in early AD patients, as well as in individuals at high risk of developing the disease. Moreover, AD pathology it is not only associated with a decreased number of cholinergic neurons but also with the increased levels of the enzyme required for the synthesis of acetylcholine in the basal forebrain, named AChE. As a consequence, decreased levels of this critical modulator of cognitive functions, acetylcholine, are expected (56, 57). Furthermore, research has suggested that AChE is responsible for many non-catalytic actions that include Aβ pro- aggregating activity. In AD patients, it was also demonstrated that the activity of AChE is correlated with the formation of amyloid plaques

and neurofibrillary tangles. Thus, AChE inhibition has a critical role in the AD management (58). Additionally, AChE inhibitory and memory boosting ability of *Magnolia officinalis* has been associated to the presence of honokiol, magnolol, obovatol and 4-O-methylhonokiol (59).

In table 1, are presented examples of natural compounds proposed for the management of AD, as well as their effects and type of study (*in vitro/in vivo*) used to evaluate their properties.

Table 1. Natural compounds used in management of AD, and their effects and type of study (*in vitro/in vivo*) performed to evaluate their properties.

Species designation	Common name	Natural compound	Action	Type of study	Ref.
<i>Arbutus unedo</i>	Strawberry tree	Anthocyanins, gallic acid, tannins, vitamin C, vitamin E and carotenoids	Antioxidant potential versus hydrogen peroxide induced neurotoxicity in PC12 cells, enhancement of memory and increase of spatial learning	<i>In vitro</i> (PC12 cells)	(44)
<i>Catharanthus roseus</i>	Madagascar periwinkle	Caffeoylquinic acid, flavonoids, citric acids	Inhibition of AChE	<i>In vitro</i> (AChE activity by Ellman's method)	(55)
<i>Cinnamomum verum</i>	Cinnamon	Cinnamaldehyde, Eugenol, Cinnamyl acetate, and cinnamyl alcohol	Stoppage of oligomer and amyloid fibril formation	<i>In vivo/ in vitro</i> (PC12 cell; <i>Drosophila Melanogaster</i>)	(50)
<i>Juglans regia</i>	Persian walnut	Fatty acids, alpha tocopherol, vitamin and polyphenols, especially ellagic acid	Inhibition of A β fibril formation, defibrillation of the preformed A β and amyloidogenic action	<i>In vitro</i> (Tioflavin T Fluorescence Spectroscopy)	(49)
<i>Magnolia officinalis</i>	Houpo	Magnolol, honokiol, obovatol and 4-O-methylhonokiol	AChE inhibitory and memory improving capacity	<i>In vivo</i> (Male ICR mice)	(59)
<i>Vaccinium angustifolium</i>	Lowbush blueberry	Anthocyanins	Decreasing of oxidative harm and drop in the expression of age-linked protein	<i>In vivo</i> (Male Balb-c mice)	(60)

Species designation	Common name	Natural compound	Action	Type of study	Ref.
<i>Vitis vinifera</i>	Grape vine	Resveratrol	Mitigation of inflammation and oxidative stress by stimulation of sirtuin 1 and consequently weakening of NF-kB activity and apoptotic activity of FOXO proteins	<i>In vivo/ in vitro</i> (C57B1/6 mice; HEK 293 cells, Na2 cells)	(61)
<i>Ananas comosus</i>	Pineapple	Bromelain	Destroys amyloid- β 42 monomers and oligomers in cerebrospinal fluid (CSF) of AD patients and damages synthetic forms.	<i>In vitro</i> (Cerebrospinal fluid of AD patients)	(51)
<i>Piper nigrum</i>	Pepper	Piperine	Reduces lipid peroxidation and AChE	<i>In vivo</i> (Adult male Wistar rats)	(62)

In what concerns to PD, the main pathological hallmark is, as mentioned, associated with a protein named as α -synuclein, which binds to ubiquitin and generates cytoplasmatic inclusions named as “Lewy bodies” that are round eosinophilic intraneuronal filamentous inclusions that subsequently cause deterioration and eventually the death of the dopaminergic neurons (34). The disease is also known to be linked to many other biochemical irregularities in the brain, such as defect of mitochondrial complex I, depletion of intracellular thiols and augmented nigral iron content (63). PD has no cure, and the currently available treatments are, as mentioned, only symptomatic. The typical treatment for PD uses the dopamine precursor L-3,4-dihydroxyphenylalanine (levodopa or L-DOPA), which reduces the motor symptoms. However, there are several obstacles involving L-DOPA-induced dyskinesias, among others (64).

To overcome the side effects linked with the synthetic compounds, researchers have currently changed to the use of diverse natural products. Estrogenic compounds such as 17 β -estradiol and progesterone (15 mg/kg for 10 days) have shown to have neuroprotective properties in C57BL/6 mice (65). Indeed, 17 β -estradiol and naturally appearing plant-derived compounds, as phytoestrogens, demonstrated to have neuroprotective effects through the activation of receptors like estrogen receptor- α (ER α), ER β , or G-protein coupled receptor-1 (GPER1) and through the stimulation of the signaling cascades involving extracellular regulated kinase-1 (ERK1)/ERK2, PI3K/Akt, and c-Jun amino-terminal kinase (JNK), that control numerous downstream transcription factors involved in the subsistence of neurons (66, 67). ER α , in the active form, may bind to insulin-like growth factor (IGF)-1 receptor and, therefore, control the signaling pathway involved in the neuroprotective action

of estradiol, which is the PI3K/Akt–glycogen synthase kinase-3 β (GSK3 β)– β -catenin (66, 68). The presence of antiapoptotic proteins, for example Bcl-2, Bcl-xL and Bcl-W, was increased in rat hippocampus while the expression of the pro-apoptotic proteins, as BCL2L4 and BAD, was decreased by the activated ERK1/ERK2 and PI3K/Akt signaling pathways (69, 70). Also, 17 β -estradiol, by triggering the JNK pathway, lead to a higher expression of anti-apoptotic proteins as Bcl-W and BCL2L11 (BIM) (71). The usual downstream molecule of ERK1/ERK2 and PI3K/Akt signaling pathways is GSK3 β that gets triggered and produces neuronal cell death. Consequently, the inhibition of GSK3 β leads to the survival of neurons and thus represents an interesting target for PD treatment (72).

Diverse spices, such as turmeric, ginger, pepper and cloves, have been shown to produce neuroprotective effects against PD (73, 74). Rajeswari and Sabesan tested the potential of curcumin and its metabolite, tetrahydrocurcumin, for MAO- B inhibition, using adult Swiss male albino mice where PD was induced using 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine (MPTP). Curcumin and its metabolite were intraperitoneally administered at a concentration of 80 and 60 mg/kg, respectively. In this PD model, dopamine and 3,4-dihydroxy phenyl acetic acid levels are usually augmented, mainly because of the increased levels of MAO-B. During this study, dopamine and 3,4- dihydroxy phenyl acetic acid were quantified using a high-performance liquid chromatograph (HPLC) coupled to an electrochemical detector and MAO-B levels were assessed by spectrofluorimetry. The depletion of dopamine and 3,4- dihydroxy phenyl acetic acid induced by MPTP was significantly reversed by curcumin and its metabolite, as well as MAO-B activity was significantly inhibited by the natural compounds. The results allowed the authors to conclude that both compounds, curcumin and tetrahydrocurcumin, have a neuroprotective action against MPTP-induced neurotoxicity (75). In another study, Luo *et al.*, tested the potential of *Astragalus membranaceus*, a medicinal herb used for decades in stroke patients in China, as a potential protector against ischemic brain injury. The *Astragaloside IV* is the main active constituent of the medicinal herb in study. Therefore, its protection potential was assessed in murine models of focal cerebral ischemia/ reperfusion that was produced by the occlusion of the middle cerebral artery for 1.5 hours. After the produced ischemia, the rodents were treated with *Astragaloside IV*, intraperitoneally, at a concentration of 20 or 40 mg/ kg and, 72 hours after, it was observed a reduction in infarct volume. The treatment with *Astragaloside IV* reduced the levels of malondialdehyde, indicating a reduction in lipid peroxidation, and increased the levels of antioxidant enzymes, glutathione peroxidase (GPX) and SOD, in ischemic tissues. These results provided the researchers an evidence of the neuroprotective effects of the tested compounds in the ischemic brain injury model (76). Additionally, Chonpathompikunlert *et al.*, assessed the potential of piperine, a constituent of pepper, against neurodegeneration and cognitive impairment in Wistar rats

AD models. Rodents were orally administered with piperine (5, 10 and 20 mg/kg/ body weight) for 3 weeks. The results showed that piperine afforded a significant protection against memory impairment and neurodegeneration at the hippocampus, which can be correlated with a diminished lipid peroxidation and decreased AChE activity (62).

Additionally, the ingestion of green tea also demonstrated to be advantageous against age-related neurological conditions and late onset of PD (77). In fact, tea ingestion was assessed as a potential delayer of PD onset. Additionally, smoking and coffee drinking were also assessed, retrospectively, to measure their actions in the onset age of PD. All factors were studied in a population of 278 PD patients. The results showed that smoking and drinking tea can delay PD onset age, being tea consumption the one that retarded the most PD onset (7.7 years) (77).

Securinine, a main natural alkaloid found in the root of the plant *Securinega suffruticosa*, also revealed to be a probable candidate for the treatment of neurodegenerative diseases associated with neuroinflammation, including PD (34). The anti-inflammatory effects of securinine were the responsible for the neuroprotection observed in glial cells. Securinine caused the inhibition of the inflammatory mediator nuclear factor kappa-light-chain-enhancer of activated B cells (NFkB), of mitogen-activated protein kinases (MAPK) and also caused the inhibition of interferon- γ (IFN- γ), apart from decreasing the mRNA expression of inducible nitric oxide synthase (iNOS) and reducing the concentration of nitric oxide (NO).

Apart from compounds from natural origin, probiotics may also be hypothesized in the treatment of PD. Probiotics are live microorganisms that, once administered in suitable quantities, confer health advantages to the host (78). Probiotics demonstrated to rise the concentration of dopamine through the production of L-DOPA, potentially allowing to restore dopamine levels (34). Padhi *et al.* genetically engineered *E. coli* Nissle 1917 to be capable of continuously producing L- Dopa. The *in vitro* outcomes showed that the probiotic can generate L- Dopa in a dose and time- dependent manner. To assess, *in vivo*, the properties of *E. coli* Nissle 1917, MitoPark mouse models of PD were used (an animal model of PD with impaired respiratory chain function in dopamine neurons) (79). The results in the rodents showed and improved response in motor, cognitive and mood- related tasks (80).

ALS is, as mentioned, a neurological disease which cause is yet unknown and that is characterized by the gradual deterioration of upper and lower motor neurons (81). ALS is fatal since it leads to respiratory muscle weakness. Riluzole is one of the FDA-approved drugs existing for ALS treatment, being recommended for symptoms relief in ALS patients. It can slightly improve respiratory weakness and increase the survival time up to only 3 months. Nevertheless, it displays no advantageous action on muscle wasting, atrophy, spasticity, weakness, dysarthria, dysphagia and life quality of ALS patients (82). Ferrante

et al., studied the gender-specific neuroprotective outcome of an oral ingestion of *Ginkgo biloba* extract against mitochondrial harm and oxidative stress in a transgenic mice model of ALS. The extract of the *Ginkgo biloba* (200 and 400 mg/kg/day) displayed substantial enhancement in motor performance and survival and reduced the loss of anterior motor neurons in male mice (81). Ginseng has about 20 ginsenosides, as protopanaxatriol and protopanaxadiol, that are recognized as the pharmacologically active compounds of ginseng. Jiang *et al.*, researched the advantageous effects of ginseng in a transgenic mice model of ALS in what concerns to the motor damage and survival correlated with ALS. The animals administered with ginseng (40 and 80 mg/ kg, orally administered) exhibited a prolongation in the beginning of symptoms and an increased survival, further confirming the potential helpful effects of ginseng in ALS (83).

Additional studies performed by Trieu *et al.*, certificated the properties of genistein treatment concerning its potential protection versus pathological settings like ALS and stroke. Genistein (16 mg/kg twice a day) showed protection against cerebral damage produced by singlet-oxygen, *in vivo*, by postponing the onset and decreasing the mortality rate in G93A mice (84). In ALS mice models, disease onset and mortality happened first in males than in females. This sexual dimorphism was attributed to estrogen as treatment with the phytoestrogen genistein eliminated the spotted sexual dimorphism observed in rodents. These results suggest that genistein has both dependent and independent estrogen neuroprotective actions and it must be explored as a preventive agent against disorders such as ALS (84).

Epigallocatechin gallate (EGCG) obtained from green tea is a bioactive natural component that has several pharmacological effects as anticancer, anti-inflammatory and antioxidant. Koh *et al.*, studied the function of EGCG in oxidative stress-injured motor neurons via PI3K/Akt and GSK-3 pathways. Pretreatment of VSC 4.1 cells with EGCG (100 μ M for 2 hours) preceding oxidative exposure rised cell viability by increasing the survival signals. They demonstrated that EGCG stimulated the activation of PI3K/Akt and inhibited GSK-3, thus representing a possible therapeutic strategy for ALS (85).

An extra pharmacologically active compound, resveratrol, encountered in red wine, peanuts, berries, grapes, can also exert antioxidant, anti-aging, anti-AD, anti-inflammatory, and neuroprotective effects. Yanez *et al.*, explained the protective actions of resveratrol versus neurotoxic effects induced by cerebrospinal fluid obtained from ALS patients in primary cultures of rat cortical motor neurons. The cerebrospinal fluid from ALS patients exerted significant cytotoxicity against primary neuronal cells. However, resveratrol significantly decreased Ca^{2+} concentration induced by ALS cerebrospinal fluid and behaved as a neuroprotective compound with probable therapeutic application in ALS patients (86).

In table 2, are described examples of natural products uses in ALS management as well as their effects and the type of study (*in vitro/in vivo*) performed to test their properties.

Table 2. Natural compounds used as potential treatment in ALS, as well as their effect and the type of study (*in vitro/in vivo*) performed to evaluate their properties.

Species designation	Common name	Natural compound	Action	Type of Study	Ref.
<i>Ginkgo biloba</i>	Ginkgo	Terpenestrilactones, Sesquiterpene trilactones, flavonol glycosides	Cognitive / memory improvement	<i>In vivo</i> (Male Sprague-Dawley rats)	(87)
<i>Panax ginseng and other species</i>	Ginseng	Ginsenosides (protopanaxadiol and protopanaxatriol)	Antioxidant, anti-inflammatory	<i>In vivo/ In vitro</i> (SOD1-G93A transgenic mice; Hep 2 cells; mouse fibroblast NIH3T3)	(83, 88)
<i>Genistein</i>	Genistein	4,5,7-trihydroxyisoflavone	Anti-viral, anti-angiogenic	<i>In vivo</i> (Murine FALS model)	(84, 89)
<i>Epigallocatechin Gallate (EGCG)</i>	Catechin	Flavan-3-ol	Antioxidant, anti-inflammatory	<i>In vitro</i> (VSC4.1 cells)	(85, 90)
<i>Resveratrol</i>	Resveratrol	3,5,4-trihydroxy trans stilbene	Anti-aging, anti-ischemic	<i>In vivo</i> (SOD1-G93A transgenic mice)	(86, 91, 92)
<i>Thunbergia laurifolia</i>	Rang Jued	Epicatechin, apigenin-7-O-glucoside, 7-hydroxycoumarin, apiin, betaine	Antioxidant	<i>In vitro</i> (HT-22 cells)	(93)

2.1. Natural Antioxidants

Oxidative stress is a consequence of an unbalance between ROS and antioxidant defenses. Free radicals are extremely reactive species that have unpaired electrons in their “shell” and react quickly with the cell membranes, eventually triggering cellular degeneration and, consequently, cell death. In fact, they react with polyunsaturated lipids of cell membranes and with the RNA and DNA. To manage with these radicals, the living systems produce several antioxidants or get them through the diet (94).

Noteworthy, oxidative stress interferes with several cellular functions and leads to many pathological conditions like ageing, asthma, arthritis, autoimmune diseases, cardiovascular dysfunction, carcinogenesis, cataract, diabetes and neurodegenerative diseases, including AD, PD and ALS (95). For this reason, among naturally occurring compounds, antioxidants deserve a special attention.

An antioxidant is every substance that, in a small concentration, protects proteins, carbohydrates, lipids and DNA from oxidation. They are divided into three main classes: the front-line defense antioxidants, which involve SOD, catalase, glutathione reductase and minerals as Se, Cu, Zn; the second line defense antioxidants, which involve GSH, vitamin C, albumin, vitamin E, carotenoids, and flavonoids; and the third line defense antioxidants, which involve a group of enzymes that repair damaged DNA, damaged proteins, oxidized lipids and peroxides, such as lipase, protease, DNA repair enzymes, transferases, methionine sulphoxide reductase and GPX (96).

Numerous studies tested the diverse health benefits of antioxidant supplementation in processes as ageing, apoptosis and neurological diseases. The brain is exceptionally susceptible to oxidative stress, given the elevated metabolic rate and high quantities of polyunsaturated lipids, the target of lipid peroxidation. Therefore, antioxidants are usually used as medicines to treat numerous forms of brain damage. Indeed, antioxidants are deeply being researched as potential treatments for neurodegenerative diseases such as AD, PD and ALS (94).

Astaxanthin (ASTX) or 3,3'-dihydroxy β - β '-carotene-4,4'-dione (Figure 1) is an oxygenated carotenoid with potent antioxidant activity, which can be 10 times greater than other carotenoids and above 500 times more effective than α -tocopherol (97).

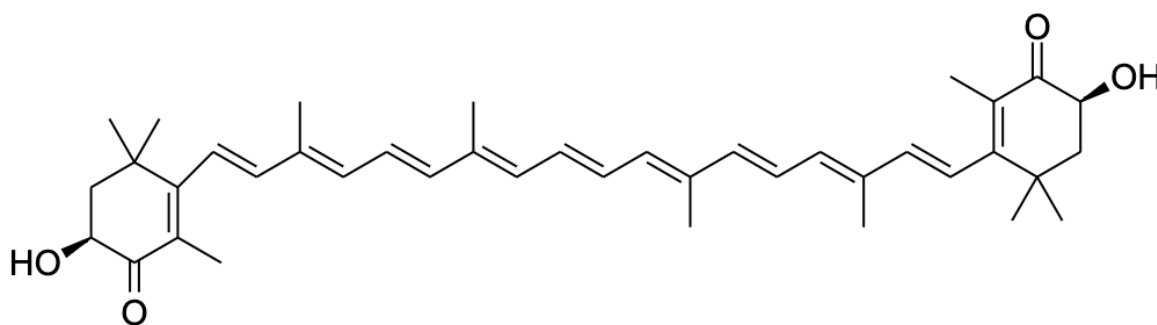


Figure 1. Chemical structure of ASTX.

ASTX is a product of the metabolism of several organisms. The most common sources of ASTX are the *algae Haematococcus pluvialis*, which is considered the most capable microorganism of producing ASTX (98); the waste of crustaceans, such as shrimps; the yeast *Phaffia rhodozyma*/*Xanthophyllomyces dendrorhous*; the bacteria *Agrobacterium aurantiacum*, and *Bacillus circulans*; and some plants, such as *Adonis* sp. (99).

Numerous *in vitro* studies shown that ASTX is a potent eliminator of ROS and nitrogen species, such as single- and two-electron oxidants and singlet oxygen (100). This dominant antioxidant activity led to the extensive application of ASTX in foods, cosmetics, and dietary supplements. In 1987, the FDA approved the use of ASTX as a food additive for the aquaculture industry and, in 1999, it was also approved as a dietary supplement (101).

As ASTX doesn't occur naturally in the human organism, its uptake is reliant on the dietary intake of foods, such as lobster, crab, shrimp and salmon (102). After ingestion, it is expected that the intestinal absorption of carotenoids, such as ASTX, are comparable to those of dietary lipids (102). Passive diffusion into the lining of the intestinal mucosa is allowed by a process resulting, for example, from the association of carotenoids, following consumption, with lipid micelles in the intestinal lumen. After this, carotenoids subsequently associate with chylomicrons and are then discharged into the lymphatic system. Chylomicrons are digested by lipoprotein lipase, at the liver, a process that permits carotenoids to relate themselves with other lipoproteins, as low-density lipoproteins, to be afterwards transported to other tissues in the body (102). Some research has been made, allowing the access to bioavailability data. The uptake and elimination kinetics of ASTX in human plasma was quantified using high-performance liquid chromatography (103-106). This method was used by Rüfer *et al.* (106) during the investigation where there were used 28 healthy males for 4 weeks. Before the start of the research, ASTX was quantified, and the concentrations found were classified as nondetectable. After that, two randomized groups ($n = 14$ each group) consumed both 250 g of wild or aquacultured salmon on a daily base, to obtain approximately 1.25 mg/day ASTX. After 6 days of consumption, ASTX concentrations reached a level of 52.4 ± 16.2 nmol/L (aquacultured salmon) and 33.7 ± 16.2 nmol/L (wild salmon), respectively. The concentrations of ASTX remained stable for the next 4 weeks (106). More research should, then, aim to elucidate the bioavailability of ASTX from a diversity of different sources, with data concerning the elimination kinetics that is also important and helps to clarify how the availability of ASTX may reduce in humans over time.

It is also important to deep elucidate ASTX-mediated antioxidant effect. In particular, ASTX apparently has an affinity for peroxy radical and singlet oxygen intermediates (102, 107-109). ASTX is able to quench singlet oxygen, for example, through the process of energy transfer, demanding ground state oxygen alongside ASTX in a triplet-excited state (102). As a carotenoid, ASTX is later capable to dissipate this energy reacting with the involving solvent, then returning to the ground state structurally unbroken, disposed to contribute to further quenching cycles (102, 110).

Although a specific mechanism of action is not clarified yet, research made in animal models reported increases in the expression of nuclear factor erythroid 2-related factor

(Nrf2) , a transcription factor that appears to induce the upregulation of endogenous antioxidant enzymes, including catalase (CAT), GPX and SOD, after ASTX administration (111-113).

Al-Amin *et al.* conducted a study where ASTX antioxidant potential was assessed. Swiss albino male mice were the *in vivo* model chosen to perform the work. A concentration of 2 mg/kg was orally administered in single daily doses for 4 weeks. To measure oxidative stress, the researchers quantified the levels of lipid peroxidation (measuring thiobarbituric acid reactive substances), the levels of nitric acid oxide, the activity of CAT, the level of advanced protein oxidation products, the activity of SOD and the levels of GSH. Lipid peroxidation was lowered by ASTX in the striatum, parietal and frontal cortex, hippocampus and cerebellum. All brain regions, except hippocampus, had the levels of nitric oxide reduced. ASTX had the capability to augment the activity of CAT in all researched groups. A noteworthy reduction of advanced protein oxidation product levels was also observed. ASTX seemed to raise the activity of SOD in all brain regions studied. GSH levels were raised by ASTX in the parietal cortex and hippocampus, while in striatum of aged animals, the levels were reduced. Therefore, the antioxidant markers evaluated showed that the treatment with ASTX improves the antioxidant activity (114).

Wu *et al.* tested the potential of ASTX to attenuate oxidative stress and increase brain derived neurotrophic factor (BDNF) to alleviate brain aging. The models used were male Sprague-Dawley rats. The animals were treated with ASTX emulsifier by intragastric administration with a dose of 0.02% during their daily diet, 3 times a week, for 8 weeks. The levels of total antioxidant capacity (T-AOC), malonaldehyde (MDA) and GSH, and the activities of GPX and SOD were evaluated using kits. The reduction of T-AOC was ameliorated with the ASTX treatment, the levels of malondialdehyde were reduced and the levels of GSH restored. In their work, ASTX proved to alleviate brain aging in the tested animals, as the oxidative capacity was improved. The upregulation of BDNF levels seemed to improve the hippocampal neurodegeneration (115).

Lee *et al.* assessed the potential of ASTX against mitochondrial dysfunction induced by MPTP/ MPP⁺ *in vivo* and *in vitro*. The models used were C57BL/6 mice (*in vivo*) and SH-SY5Y (*in vitro*). *In vivo*, the concentration of ASTX tested was 30 mg/kg/ day, intraperitoneally for 28 days and which was administered 2 hours before the treatment with MPTP. *In vitro*, the ASTX was used at a concentration of 50 μ M. The cell viability was assessed by the MTT reduction assay and, afterwards, the antioxidant enzyme activity (SOD and CAT) was evaluated. After the animals sacrifice, their *substantia nigra* was stored to further analysis by Western Blot, while the hemi-mesencephalon was prepared for the immunohistochemistry assay. The data obtained from cytometric assay showed that ASTX inhibited the apoptotic process induced by MPP⁺ in SH-SY5Y cells, evidencing the

antioxidant activity of the compound. *In vivo*, the neuronal loss induced by the exposure to MPTP was significantly reduced after the treatment with ASTX. Hence, this research work pointed that ASTX is capable of preventing cell death caused by ROS generation (116).

Liu *et al.* tested the use of ASTX as an inhibitor of ROS toxicity in SH-SY5Y cells. The ROS inducers chosen were DHA hydroperoxide and 6- hydroxydopamine, which originated a significant cell death assessed by the MTT reduction assay. Outstandingly, the pre-treatment with ASTX (100 nM) seemed to prevent the aggressors-mediated cell death. Besides that, the treatment with ASTX (100 nM) significantly inhibited apoptosis, ROS generation, and mitochondrial abnormalities caused by the two neurotoxins. Therefore, the results obtained in this work suggested that the neuroprotective effects of ASTX relies on its mitochondria protection and antioxidant potential (117).

Al-Amin *et al.* assessed the potential of ASTX against spatial memory impairment and neuronal oxidative stress, in Swiss albino male mice, induced by aluminum chloride. The ASTX was orally administered, once a day, for 6 weeks, at a concentration of 20 mg/kg body weight. Two behavioral tests were performed, and the oxidative stress markers were quantified (lipid peroxidation, nitric oxide, GSH and advanced oxidation of protein products). The rodents exposed to the aluminum presented a decrease in spatial memory performance and exhibited anxiety- like behaviors. However, when treated with ASTX it was observed an improvement in both behavioral tests. A reduction of neuronal oxidative stress was also observed. These results prove the antioxidant potential of ASTX (118).

3. Lipid based nanosystems as smart delivery systems to the brain

The creation of innovative approaches to treat brain disorders is very challenging for pharmaceutical companies nowadays. Presently, emergent developments in the field of nanomedicine have created several platforms that increase drug transport across the BBB (119). Currently, inorganic, and organic nanosystems have been produced to act as drug nanocarriers, aiming to improve drug delivery into the brain. Up to now, about 30 combinations concerning nanomaterials and therapeutic drugs have been accepted by the FDA for clinical use, plus some others that are under clinical trials (120).

Among the different nanomedicines investigated to improve drug delivery, lipid-based nanosystems, such as solid lipid nanoparticles (SLN) and nanostructured lipid carriers (NLC), have been extensively explored due to their advantages.

3.1. Solid lipid nanoparticles (SLN) and nanostructured lipid carriers (NLC)

SLN combines the benefits of conventional colloidal drug carrier systems, such as liposomes and polymeric nanoparticles, while prevent or minimize the drawbacks associated with them. Extra worthy features of SLN are their particulate nature, capability of incorporating hydrophobic compounds, manufacture processes with no organic solvents and the opportunity to make extremely concentrated lipid suspensions having lower cytotoxicity (121-124). Indeed, the principal advantage presented by lipid carriers for drug delivery is the use of physiological lipids or lipid molecules that are safe for human medicines, reducing the chance of acute and chronic toxicity (121-123). In addition, SLN do not bring the elevated costs typically associated to large scale manufacture and have fewer storage and drug leakage complications, when compared to other carrier systems as, for example, emulsions and liposomes. The toxicity related with biodegradable polymeric materials is not observed in SLN as well (125, 126).

SLN are formed by one solid lipid stabilized with one or two emulsifiers in water. The core of the SLN contains the drug dissolved or dispersed in the solid matrix. These nanoparticles can be made using an extensive diversity of lipids involving fatty acids, mono-, di-, or triglycerides, glyceride combinations or waxes (127). Drug release from SLN can be made in two different ways, by diffusion or by degradation of the lipid matrix that usually is made by enzymes (128).

Handicaps of SLN involve low drug encapsulation capacities, stability problems during storage and which are related to the occurrence of lipid polymorphic transitions that can lead to drug expulsion from the nanoparticles (122, 127).

The main aspects disturbing the loading capacity of compounds in the SLN lipid matrix include the solubility of the compound in the lipid melt and the physical and chemical structure of the solid matrix (121).

Three distinct theoretical models can explain the loading of molecules in SLN. One is the homogenous matrix model that is when the compound is either molecularly dispersed or presents as amorphous clusters in the lipid matrix. Another model is the drug-enriched shell model, when exists an outer lipid shell holding the molecules inside the lipid core. The last one is termed the drug-enriched core model, where a lipid layer or reservoir encloses the compound core. The morphological dissimilarities concerning these models depend on the properties of the molecules, on the composition of the matrix, as well as on the method chosen for their manufacture. On several occasions, a combination of the three factors is achieved and, depending on the partition coefficient of the compound between the lipid and

the aqueous phase, the molecules can also be adsorbed on the surface of the nanoparticles, becoming unprotected by the solid matrix (121, 129).

The homogenous matrix model is typical of the entrapped compounds that can display extended release from SLN. The drug-enriched shell model is achieved when phase separation happens during the cooling course of the liquid lipid droplet to the formation of SLN. The lipid is the first to precipitate, creating a nearly compound-free lipid core. Simultaneously, the concentration of compound in the remaining liquid lipid rises constantly. Lastly, the compound-enriched shell crystallizes. The drug-enriched core model is obtained when the contrary mechanism of the previous model occurs. In this situation, the compound precipitates first and the lipid shell created around this core will have particularly less compound. This takes to a membrane-controlled release ruled by Fick's law of diffusion. This model is originated when the compound concentration is almost the same as his saturation solubility in the melted lipid (121, 129).

SLN is the first generation of lipid nanoparticles. These systems have shown exciting applications, including the fast and effective production process that includes the possibility of production in large-scale, the possibility of not using organic solvents and the possibility to produce high concentrated lipid suspensions. Nonetheless, there was still some opportunity for improvements, which were made in the following generation of lipid nanoparticles, the NLC (130, 131). To surpass some limitations of the SLN, the NLC were developed. These second type of nanoparticles present a higher loading capacity for numerous active compounds and the potential of expulsion of the encapsulated compounds during storage is minimized, when compared to SLN (132).

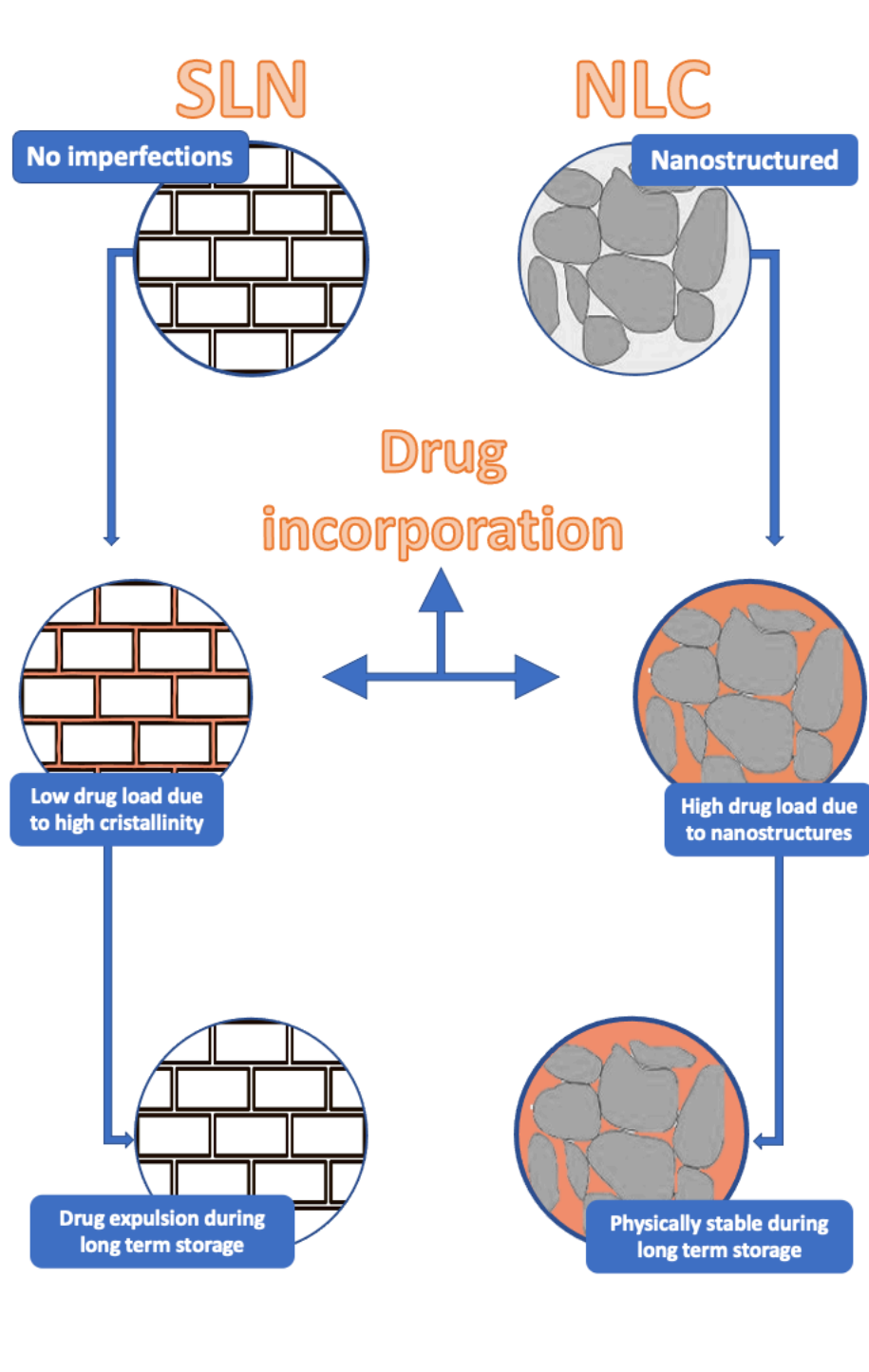


Figure 2. Schematic representation of the differences between SLN and NLC (adapted from (133))

The main distinction between SLN and NLC is that in the NLC is formed by nanostructuring the lipid matrix with the objective of increasing molecules loading and avoiding expulsion, which offers extra flexibility in the modulation of the release of the loaded molecules (Figure 2). As NLC have liquid lipids on its constitution, the degree of organization of the lipid matrix is reduced, which originates more imperfections where the

drugs can be placed (132). Thereby, NLC are made by mixing one solid lipid with one liquid lipid, in contrast with SLN that are made by only one solid lipid. Several imperfections make possible the accommodation of the compounds within the NLC, which are called the "imperfect crystal type" (131).

To minimize crystallization, which affects the maintenance of the molecules within the NLC, solid particles of amorphous lipid structure can be created, by adding particular lipids that do not recrystallize after homogenization and cooling down. This is called the "amorphous type" of NLC (131).

For some compounds, the drug solubility in the liquid lipid is greater than in the solid lipid (134). Then, the loading capacity for these compounds was increased in the NLC "multiple type" of NLC. Therefore, the NLC offer supplementary advantages over SLN, such as higher drug loading capacity and minimization of drug leakage during storage. This is owed to the non-ordered structure of these carrier systems (131).

3.2. Nose-to-brain delivery: an alternative route for SLN and NLC to reach the brain

The nasal route involves drug absorption in the nasal mucosa for local or systemic action, or brain targeting. Therefore, the term "intranasal" is used to mention drug absorption through the nasal mucosa with action in different locations, while "nasal" is used to refer a local action (135).

Nasal/intranasal administration offers numerous benefits over other routes, as non-invasiveness, easiness of application and pain free, in comparison with parenteral and rectal routes. In addition, through the nose, drugs can pass directly to the blood circulation, escaping gastrointestinal tract inactivation and hepatic metabolism, and showing a fast beginning of action with increased drug bioavailability, when compared to orally administered drugs. Intranasal route also offers the chance of drug delivery directly to the brain, escaping the need of crossing the BBB (136-139).

However, some disadvantages compromise the nasal/intranasal drug administration, such as (Figure 3): 1) short time available for absorption, between 15 and 30 minutes, due to the fast mucociliary elimination mechanism; 2) difficult permeation of the mucus barrier; 3) possibility of enzymatic degradation; 4) restricted volume accessible for administration (maximum of 150 μ l per nostril); 5) low permeability for hydrophilic and high molecular weight molecules. To surpass these difficulties, the use of mucoadhesive agents and permeation enhancers has been recommended, extending the permanence of the

formulation in the nasal cavity (140). Furthermore, injuries in the nasal cavity and nasal congestion may also contribute to reduce the nasal drug absorption (136, 141).

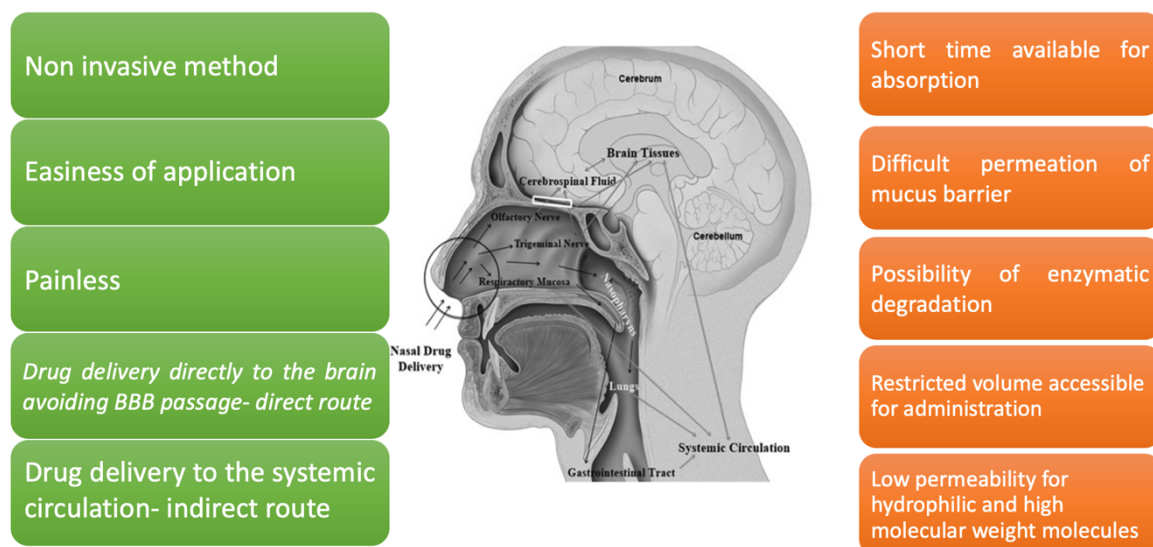


Figure 3. Advantages and limitations of the nasal/intranasal drug administration (adapted from (1))

The BBB and the CSF are physiological barriers that isolate the brain from its blood supply, controlling the internal environment. Among all protective structures, the BBB is the most wide and exclusive. It is mainly constituted of tightly linked brain endothelial cells and a discontinuous layer of pericytes. The cerebral endothelium has exclusive characteristics that preserve the BBB integrity, trans endothelial transport of cells and angiogenic ability to tolerate revascularization when needed. The passage between endothelial cells is made through a mechanism named paracellular, which is used for ions and solutes that do their passage based on a gradient of concentration. In contrast, the passage of compounds can occur through the endothelial cells by passive diffusion, which occurs for small lipophilic molecules given the lipophilic nature of the endothelium membrane. Alternatively, transcellular transport may also occur, which comprises carrier-mediated transport (involving, for example, transport proteins for glucose, amino acids, purine bases, among others) and receptor-mediated transport (e.g., to perform the transport of transferrin and insulin). The equilibrium between paracellular and transcellular transport is crucial to define the degree of permeability in a healthy BBB. Other ways of transport occur through the establishment of cellular invaginations identified as *caveolae*. These structures create vesicles around the molecules allowing their transport to or from the brain (142).

Tight junctions that exist between the BBB capillary endothelial cells mediate the drug passage into the brain. Low molecular weight and lipophilic molecules are the only ones

that can easily cross the BBB by passive diffusion. Macromolecules, as peptides and proteins, cannot directly enter into the CNS through the BBB by passive diffusion. In this situation, the nasal route surges as a promising alternative, offering direct access to the brain without the need of crossing the BBB (140).

Recently, the chance of directly accessing the brain from the nose has been widely investigated, as the brain is connected to the nasal cavity through the cribriform plate of the ethmoid bone. More specifically, the nasal cavity and the CNS are connected by the olfactory nerves, in the olfactory area, and through the trigeminal nerves, in the respiratory area. The olfactory area is the key region for the nose-to-brain transport and offers a direct course that implicates mechanisms of extracellular and paracellular diffusion and intracellular absorption in the olfactory neurons. Once they reach the olfactory mucosa, drugs can be transported to the brain by the olfactory nerve. Drugs that interact with the ciliated receptors of the olfactory neurons can be rapidly transported to the CNS, through the cribriform plate. In addition, drugs can be transported across the axons to the olfactory bulb and further to the CSF, where they are distributed into the brain. Therefore, the olfactory nerve pathway is usually separated into intra and extraneuronal pathways, which explain the transport of drugs to the brain via different mechanisms (143). The brain is also connected with the nasal cavity via the trigeminal nerve, which allows drugs to be directly delivered into the brainstem through the branches that innervate the respiratory mucosa. The drug is transported along the trigeminal nerve branches that innervate the nasal mucosa (143). The drug transport also occurs in an indirect way in the respiratory area and involves a countercurrent exchange of drugs in the blood circulation, which need to cross the BBB to reach the brain. The olfactory and respiratory epithelia have a high quantity of blood vessels that facilitate drug absorption into the systemic circulation. However, drugs may not reach the brain in therapeutic concentrations and can suffer elimination during their passage throughout the systemic circulation (143).

Numerous studies described that the drugs administered intranasally can reach the brain across the diverse pathways mentioned (135, 144, 145). Nonetheless, drug absorption in the nasal mucosa is affected by several factors such as, molecular lipophilic and hydrophilic characteristics and weight of the drug, capacity for mucus solubilization, ionization, mucociliary clearance, enzymatic degradation and pharmaceutical dosage form (146). Moreover, nasal formulations' efficiency is affected by the pH, osmolality, particle size, viscosity and physical state. Thus, an ideal drug for nasal delivery should include the following characteristics: acceptable mucus solubility; dose administration of an appropriate volume; non-irritant; fast onset of action; odorless and tasteless; good stability (147). Additionally, the administration device and the position of the patient head during administration also interfere with drug deposition in the nasal cavity (140).

For intranasal administration, SLN and NLC offer several benefits over other nanosystems, as they can be produced from biocompatible and biodegradable excipients. Additionally, these nanoparticles protect drugs from enzymatic degradation, improve the residence time in the nasal cavity, and increase drug bioavailability (148). However, the low viscosity of SLN and NLC aqueous dispersions reduces the interaction time of the formulation with the nasal cavity, disturbing drug transport and bioavailability in the brain. To avoid this obstacle, it has been suggested to include mucoadhesive materials in the NLC formulations (149).

3.3. Relevant studies with lipid-based nanosystems for brain delivery of drugs and natural compounds

Some research has been made to assess the potential of the intranasal administration as an alternative route for the drugs and natural compounds to reach the brain. For example, M. Igartua and R.M. Hernandez *et al.* produced NLC constituted of polyunsaturated fatty acids and assessed their protective and anti-inflammatory properties. In their research, they observed that the produced NLC were biocompatible towards dopaminergic and microglia cells and afforded neuroprotective effects to dopaminergic neuronal cells after exposition to the neurotoxin 6-hydroxydopamine hydrochloride. Also, the NLC formulations reduced the proinflammatory cytokine concentration in microglia cells. From these results, the researchers established that the developed polyunsaturated fatty acids NLC are promising for the treatment of neurodegenerative diseases (150).

Agrawal *et al.* tested the potential of curcumin for the treatment of AD. The researchers developed and optimized a curcumin-loaded NLC formulation with high entrapment efficiency (92.74 %) and improved drug release profile, when compared with a curcumin solution. Although further research will be needed, the developed curcumin-loaded NLC showed good results as a nanocarrier for brain delivery (151).

Skamula *et al.* developed an alpha-mangostin-loaded NLC formulation and assessed its potential in the treatment of PD. Mice with rotenone-induced PD were used for the *in vivo* study. The alpha-mangostin-loaded NLC was administered orally in a dose of 25 mg/Kg/48h for 4 consecutive weeks. The results showed that the tested NLC formulation of alpha-mangostin was able to protect against the neurons reduction caused by the oxidative stress induced by rotenone. Furthermore, it showed a better therapeutic efficiency against neurodegeneration than the alpha-mangostin alone, emphasizing the benefit of using alpha-mangostin-loaded NLC (152). Cunha *et al.* tested rivastigmine-loaded NLC for nose-to-brain delivery. In AD, AChE inhibitors are the main drugs used to manage it. Rivastigmine belongs to the AChE inhibitors groups and, for that reason, has potential as possible

manager of AD was tested. The biocompatibility of the formulation was performed using RPMI 2650 and Calu-3 cells, with the Alamar Blue assay being performed to determine the cells' viability. The results obtained in the biocompatibility studies demonstrated a reduction of cell viability only at the highest concentration of the formulation (814 $\mu\text{g/mL}$). Further tests are needed to confirm the potential of this formulation, but, overall, it appears to be a good candidate to manage AD by nose- to- brain administration (153).

Jojo *et al.* optimized the production of NLC-loaded with pioglitazone to manage AD. Furthermore, they tested the formulations *in vitro*, *in vivo*, and *ex vivo*. They performed a drug release study and tested the safety of the formulation in sheep nasal mucosa and in the SH-SY5Y cell line. To assess the effect *in vivo*, male Wistar rats were used. After confirming the safety of the NLC, the rodents were divided in three groups and each received a concentration of pioglitazone of 1 mg/kg. One group received the NLC-loaded with pioglitazone intranasally, while the other two groups received a pioglitazone solution, one intranasally and the other intravenously. After the different administrations of pioglitazone, the concentration of the drug that reached the brain was quantified. The results showed a higher concentration in the brain when administered intranasally in the NLC form (1.64 $\mu\text{g/mL}$ for NLC- intranasally; 0.88 $\mu\text{g/mL}$ for pioglitazone solution intranasally; and 0.38 for pioglitazone solution intravenously). From these results, it was concluded that using pioglitazone-loaded NLC intranasal provides better outcomes for the drug to reach the brain, thus increasing brain bioavailability (154).

Costa *et al.* optimized and tested a diazepam-loaded NLC for nose-to-brain delivery as a possible new formulation for the treatment of epilepsy. The biocompatibility assays were performed in SH-SY5Y cells and the used cytotoxicity tests were neutral red uptake, resazurin reduction and sulforhodamine B assays. The results obtained proved that the optimized formulations were safe at concentrations up to 100 $\mu\text{g/mL}$. These formulations seem to have potential to improve the treatment of epilepsy, although the authors reinforced the idea of being essential further studies in nasal cells and in animal models, to confirm this evidence (155). In another study, Alam *et al.*, optimized and assessed the potential an NLC-loaded with lamotrigine to manage epilepsy of. The study was carried in male Wistar rats, where an electroshock seizure was induced. After that, the animals were divided in four groups, with one being the control group (no treatment) and the other three receiving treatment, one orally administered with the drug solution, other intranasally administered with the drug solution and the last one receiving intranasally the NLC formulation. After the induced seizures, malondialdehyde and GSH levels increased and decreased, respectively, and for that reason, an evaluation of their levels was performed after treatment. The results permitted to conclude that the intranasal administration of the NLC formulation can improve the activity of lamotrigine, as it allowed for a much higher brain concentration, when

compared with the other two administration routes. Indeed, the neuroprotective effect of lamotrigine was observed with lower doses when the intranasal route is compared with oral route (156).

Additionally, Singh *et al.* developed alprazolam-loaded SLN for nose-to-brain delivery. The *in vivo* tests were conducted in male Wistar rats and gamma scintigraphy imaging was made in New Zealand rabbits, where the formulation was tagged with a radioactive substance. The rats were divided in different groups and received the drug by different routes of administration (alprazolam solution intranasally and alprazolam-loaded SLN, one group intravenously, and other intranasally), at a concentration of 0.08 mg/kg body weight. The results obtained suggested that alprazolam rapidly reached the brain by direct nose-to-brain transport. The high bioavailability of the drug in the brain obtained in the intranasal administration can help reducing the usually administered dose of alprazolam, which can increase the therapeutic index, as well as the clinical acceptance from patients (157).

4. Objectives

Oxidative stress is one of the main hallmarks underlying the pathogenesis of several neurodegenerative diseases. Accordingly, is important to study how to prevent, slow or even stop this deleterious process. Considering ASTX as one of the most powerful natural antioxidants, creating ways to efficiently deliver this compound to the brain can significantly improve the management of the neurodegenerative diseases that have in common this pathophysiological mechanism.

Hence, in this experimental work, the main objectives were:

- To develop and characterize two lipid-based nanoformulations (SLN and NLC) loaded with ASTX;
- To validate a HPLC- ultraviolet (UV) method to be used for the quantification of ASTX;
- To evaluate, *in vitro*, the biocompatibility of the developed formulations towards differentiated SH-SY5Y cells and RPMI 2650 cells;
- To assess the potential neuroprotective effects of the developed formulations against the cytotoxicity induced by Iron (III), MPP⁺ and *t*-BHP.

5. Materials and Methods

5.1. Materials

Dulbecco's modified eagle medium (DMEM)- high glucose and Trypsin-ethylenediamine tetraacetic acid (EDTA) solution (0.25% trypsin/1 mM EDTA) were obtained from Thermo Fisher Scientific (USA). Eagle's minimum essential medium (EMEM) with L-Glutamine was acquired from the American Type Culture Collection (ATCC, USA). Vitamin E, benzalkonium chloride, and Tween® 80 (polysorbate 80) were acquired from Acofarma (Spain). Glycerin was obtained from Acofarma (Portugal). Precirol®5ATO (glyceryl distearate) was kindly donated from GATTEFOSSÉ (France). Glacial acetic acid was acquired from Fischer Scientific (Portugal). Neutral Red (NR) solution, resazurin (REZ), 1-methyl-4-phenylpyridinium (MPP⁺), sodium deoxycholate, MTT, iron (III) chloride (FeCl₃), nitrilotriacetic acid disodium salt (Na₂NTA), nitrilotriacetic acid trisodium salt (Na₃NTA), tert-Butyl hydroperoxide (*t*-BHP), retinoic acid (RA), phorbol 12-myristate 13-acetate (TPA), trans-β-apo-8'-carotenal and astaxanthin (ASTX) were obtained from Sigma-Aldrich (Germany). Hanks' balanced salt solution (HBSS) with or without calcium and magnesium [HBSS (+/+) or HBSS (-/-), respectively], heat inactivated fetal bovine serum (FBS) and non-essential amino acids were obtained from PAN-Biotech (Germany). Methanol and ethanol were acquired from Honeywell (USA). Dimethyl sulfoxide (DMSO) was obtained from Merck (Germany). Antibiotic mixture (10,000 U/mL penicillin, 10,000 µg/mL streptomycin) was obtained from Biochrom (Germany). All sterile plastic material used was acquired from Corning Costar (USA). The purified water used in all experiments was obtained from a Direct- Q® Ultrapure Water Systems, Merck Millipore (Germany). All the reagents used were of analytical grade or of the highest grade available.

5.2. Preparation of the lipid nanoparticles formulations

SLN and NLC were developed according to other works previously conducted in the Laboratory of Pharmaceutical Technology and prepared using the ultrasound technique (155, 158). First, all the constituents were separately weighed, and then, the lipid(s) and ASTX were heated at a temperature 5 to 10°C above the melting point of the solid lipid (Precirol®5ATO). Simultaneously, the water phase containing benzalkonium chloride, Tween® 80 and glycerin was heated at the same temperature. When the two phases were at the same temperature, they were mixed and emulsified at 13400 rpm for 16 minutes with an Ultra-Turrax® T25 (Janke and Kunkel GmbH, Germany). Subsequently, the oil-in-water (O/W) emulsion formed was subjected to the ultrasound energy (VCX 130 ultrasonic processor, Sonics, Switzerland) at an amplitude of 50 % for 20 minutes (155). Finally, the

obtained O/W nanoemulsion was packed in glass vials and drastically cooled to form the lipid nanoparticles. In parallel, both empty NLC and SLN formulations (without ASTX) were also prepared as described above. The composition of all the prepared formulations is presented in table 3.

The prepared formulations were protected from the light and stored at room temperature (20.0±0.5 °C).

Table 3. Composition of the SLN and NLC formulations, empty and loaded with ASXT.

	SLN		NLC	
	ASXT-loaded	Empty	ASXT-loaded	Empty
Composition	% (w/w)		% (w/w)	
<i>Precirol®5ATO</i>	8.600	8.700	6.300	6.400
<i>Vitamin E</i>	-	-	2.700	2.700
<i>ASTX</i>	0.1	-	0.1	-
<i>Tween® 80</i>	4.200	4.200	4.200	4.200
<i>Sodium deoxycholate</i>	0.300	0.300	0.300	0.300
<i>Benzalkonium chloride</i>	0.020	0.020	0.020	0.020
<i>Glycerin</i>	0.003	0.003	0.003	0.003
<i>Ultrapure water</i>	q.s. 100	q.s. 100	q.s. 100	q.s. 100

5.3. Characterization of the lipid nanoparticles formulations

5.3.1. Particle size, polydispersity index and zeta potential

The developed NLC and SLN formulations, empty and ASTX-loaded, were characterized for particle size, polydispersity index (PDI) and zeta potential (ZP).

The particle size of the SLN and NLC formulations were evaluated by the laser diffraction (LD) technique and the Mastersizer 3000 equipment (Malvern, UK), using a particle absorption index of 0.001, a particle refractive index of 1.6, and water as a dispersing medium. For each sample, were performed 5 measurements (n=5) and the result was presented as the mean diameter ± standard deviation (SD). These are presented in the form of percentiles (D10, D50 and D90), which indicates that 10%, 50% or 90% of the particles have sizes equal or smaller than the value of D10, D50 and D90, respectively (155).

The particle size was also evaluated by the dynamic light scattering technique (DLS) using the Nanozetasizer equipment (Malvern, UK). The PDI and ZP were also evaluated using the same equipment. Before starting, the samples were diluted in ultrapure water (1:100) and a particle absorption index of 0.001, a particle refractive index of 1.6, an angle of 0.001 and a 173° backscattering were selected as ideal conditions for the determination of the parameters under study. All measurements were performed in triplicate (n=3) and the results were presented as mean values $\pm$ SD, and the ZP values were calculated by the software of the system, through the Helmholtz-Smoluchowski equation (155).

After 30 days of storage at room temperature (20.0 ± 0.5 °C), all formulations were characterized and evaluated in terms of particle size (D10, D50, D90, Z-Ave), PDI and ZP.

1.3.2. Morphology: CryoSEM

The analysis of the morphology of lipid nanoparticles, namely, the shape and appearance of their surface, can be performed using different electron microscopy techniques, namely, through the technique of cryoscanning electron microscopy. This technique is an extension of the differential scanning electron microscopy (SEM) technique, as it allows characterization of samples in the liquid state, unlike the SEM technique, which only allows the determination of the structure of solid preparations (159). Therefore, the morphology of empty SLN and NLC and loaded with astaxanthin was analyzed by cryoSEM, using a high-resolution Scanning Electron Microscope, with X-ray microanalysis and a system for observation of samples at low temperature, JEOL JSM 6301F/ Oxford INCA Energy 350/ Gatan Alto 2500. Before starting the analysis, each sample under study was quickly cooled by dipping into sub-cooled liquid nitrogen and then transferred, under vacuum, to the sample preparation chamber with the cooled platinum. Subsequently, each sample was fractured, sublimed for 120 seconds at -90°C, and coated with gold and palladium (Au/Pd) by ion spraying for 45 seconds at a current of 12mA. Finally, each sample was transferred to the SEM chamber and observed at a temperature of -150°C. It is important to point out that in the sample preparation process, namely in the cooling and vacuum processes, these may change the shape of the nanoparticles, which generally have a spherical shape (160). It is for this reason that the cooling process must be carried out quickly in order to reduce sample structure distortion; and its transfer to the preparation chamber must be done under vacuum in order to avoid its contact with the atmosphere, and consequently its sample moisture (159).

5.3.2. Evaluation of the encapsulation efficacy

To assess the encapsulation efficiency (EE) of the ASTX in the NLC and SLN formulations, liquid-liquid extractions on the developed formulations were made aiming the removal of the unloaded ASTX. First, 700 µL of ethanol absolute were added to 300 µL of each formulation, and vortexed. Then, the mixture was filtered using an Amicon® Ultra-4 Centrifugal Filter Unit. To facilitate filtration, the samples were centrifuged for 1 h, at 3500 rpm, at room temperature (20.0 ± 0.5 °C). After centrifugation, the supernatant was collected and filtered using a 0.22 µm cellulose acetate filter (Frlabo, Portugal). This first extraction was repeated performing two similar extractions where 1 mL of ethanol was added to the formulation remaining in the filter unit, followed by supernatants collection after centrifugation for 1 h, at 3500 rpm, and at room temperature. Afterwards, the supernatants were diluted 20x in methanol containing 1 µg/mL of IS and further injected in the HPLC-UV apparatus to quantify the amount of unloaded ASTX. The EE was calculated as the difference in the ASTX used in the preparation of the NLC and SLN formulations and the unload ASTX quantified in this study, expressed as percentage (equation 1) (161). At least 3 independent (n=3) NLC and SLN extractions of each formulation were performed.

$$EE\% = \frac{\text{Total amount of ASTX} - \text{Free ASTX}}{\text{Total amount of ASTX}} \times 100$$

Equation 1. equation used to calculate the encapsulation efficacy percentage

5.4. *In vitro* studies with differentiated SH-SY5Y cells

5.4.1. SH-SY5Y cells as an *in vitro* neuronal model

The SH-SY5Y cell line is a neuroblastoma cell line cloned from SK-N-SH cells. This cell line has been extensively used as a neuronal model back from the early 1980s as these cells hold numerous biochemical and functional properties of neurons (162). Among these properties is the ability to transport dopamine and noradrenaline, the expression of enzymes involved in the synthesis and metabolism of dopamine and acetylcholine and the expression of dopamine and acetylcholine receptors (163).

Although SH-SY5Y cells can be used in an undifferentiated form, different protocols can be used to differentiate these cells obtaining, for example, SH-SY5Y cells with a dopaminergic phenotype. Indeed, the use of undifferentiated SH-SY5Y cells as an *in vitro* model can present some limitations, such as low expression of enzymes involved in the synthesis of dopamine and the fact that undifferentiated cells have a continuous mitosis, which can affect the response to drugs and toxins. It was then demonstrated that

differentiating SH-SY5Y cells with RA followed by the treatment with TPA significantly improves these aspects (162). Therefore, this cell line represents an important *in vitro* tool in the research of pharmacologic and toxicologic properties of several drugs.

5.4.2. SH-SY5Y cells: cell culture, seeding and differentiation

The human neuroblastoma SH-SY5Y cell line was obtained from the American Type Culture Collection (ATCC, United States of America), and was routinely cultured in 25 cm² flasks using DMEM - high glucose supplemented with 10% heat inactivated FBS, 1% MEM non-essential amino acids, 1% antibiotic mixture (100 U/mL of penicillin and 100 µg/mL of streptomycin). The cells were kept in a 5% CO₂- 95% air atmosphere, at 37° C, the medium was changed every 2–3 days, and cultures were passaged weekly by trypsinization using 0.25% trypsin- 1mM EDTA solution. For each experiment, cells were seeded at a density of 25,000 cells/cm² and used between the 20th and 25th passages. The cells were always differentiated into a dopaminergic phenotype. Succinctly, the cells were seeded in complete DMEM medium containing RA (final concentration of 10 µM) and, 3 days after seeding, TPA (final concentration of 80 nM) was added for another 3 days. The cells were used in the experiments 6 days after seeding.

5.4.3. Evaluation of the cytotoxicity of the developed formulations

The cytotoxicity of the developed NLC and SLN formulations, both empty and ASTX-loaded, was assessed in differentiated SH-SY5Y cells, by the NR uptake and MTT reduction assays, 24 h after exposure. SH-SY5Y cells were seeded in 96-well plates (25,000 cells/cm²) and submitted to neuronal differentiation, as previously described. Six days after seeding, the cells were exposed to the NLC and SLN formulations (empty or loaded with ASTX, 0 - 500 µg/mL), as well as exposed to ASTX alone, in the same concentration range that is found in the ASXT-loaded formulations (0 - 3.8 µg/mL), for 24 h. For the *in vitro* studies, a 25 g/L ASTX stock solution was prepared in DMSO and stored at -20 °C. In the day of the experiment, the NLC and SLN formulations (both empty and ASTX-loaded), as well as ASTX, were serial diluted in fresh cell culture medium to obtain the desired final concentrations.

5.4.3.1. MTT reduction assay

The MTT reduction assay is a fast colorimetric method to determine cellular cytotoxicity and viability based on the viable cell mitochondrial activity (164). MTT is a water-soluble dye and a yellow tetrazolium salt. Viable cells cleave the tetrazolium ring by

mitochondrial dehydrogenases, turning the tetrazolium dye into purple water-insoluble formazan crystals, which can be solubilized in an organic solvent. Therefore, it is expected that formation of formazan is directly proportional to the quantity of metabolically viable cells, turning this method an effective and easy way of evaluating cells cytotoxicity (165, 166).

In this work, the cell culture medium was removed, at the chosen time point (24 h), and the cells were incubated with 150 μ L of fresh cell culture medium with 0.5 mg/mL MTT, for 1.5 h, at 37 °C, in a humidified 5% CO₂-95% air atmosphere. After this incubation period, the cell culture medium was removed, and the formed formazan crystals solubilized with 150 μ L of DMSO. Then, plates were softly shaken in a microtiter-plate shaker until a homogeneous solution was obtained and the absorbance measured at 550 nm in a multiwell plate reader (PowerWaveX BioTek Instruments, Vermont, US). Evaluation of cytotoxicity was made by comparing the percentage of MTT reduction comparatively to control cells (0 μ M). At least four independent experiments (n=4) were performed, in triplicate.

5.4.3.2. Neutral red uptake assay

The NR uptake assay is a colorimetric simple method used to assess cellular viability or cytotoxicity and is based on the aptitude of viable cells to incorporate and bind into the lysosomes the supravital NR dye (167). NR is as weak cationic dye that accumulates in the lysosomes. After penetrating the cells membrane by non-ionic passive diffusion, it binds through electrostatic hydrophobic interactions to phosphate groups and/or anionic sites. Because the characteristic lysosome proton gradient permits the dye to become charged and kept in the lysosomal matrix, the cells ability to maintain pH gradients directly affects the NR uptake, as there is no dye retention in case of a pH gradient reduction or cell death. Consequently, the amount of dye incorporated in the lysosomes is directly proportional to the quantity of viable cells in culture. Then, the dye is removed from the viable cells using an acidified ethanol solution and quantified using a spectrophotometer (168). Additionally, damage in the lysosomal membrane or differences in cell surface can alter the NR uptake as well, permitting the distinction between damaged, dead, and viable cells, based on their ability to incorporate the dye that is lysosomal specific. Therefore, the lysosome integrity and the binding of the neutral red dye that is related to it, brand this method as a sensitive indicator for cell viability (168).

In this work, the cell culture medium was removed, at the chosen time point (24h), and the cells were incubated with 150 μ L of fresh cell culture medium with 50 μ g/mL NR, for 1,5 h at 37 °C, in a humidified 5% CO₂-95% air atmosphere. Ended the incubation time, the cell culture medium was removed, and the dye was extracted with 150 μ L of absolute

ethanol/distilled water (1:1) with 5% glacial acetic acid. Then, plates were softly shaken in a microtiter-plate shaker until a homogeneous solution was produced and the absorbance measured at 540 nm in a multiwell plate reader (PowerWaveX BioTek Instruments, Vermont, US). Evaluation of cytotoxicity was made by comparing the percentage of NR uptake comparatively to control cells (0 μ M). At least four independent experiments (n=4) were performed, in triplicate.

5.4.4. Cellular uptake of ASTX

Uptake of ASTX was assessed in SH-SY5Y cells. To assess this uptake SH-SY5Y cells were seeded in 6-well plates at a density and 25,000 cells/cm², following the RA and TPA differentiation protocol mentioned above. Six days after seeding, SH-SY5Y cells were exposed to the prepared formulations (100 μ g/mL) containing the respective ASTX concentration (0.76 μ g/mL for SLN and 0.73 μ g/mL for NLC). The culture medium was removed 24 hours after exposition and 250 μ L of methanol containing 1 μ g/mL of internal standard (IS) was added to each well to extract the ASTX for 30 minutes. After the period of incubation, the plates were centrifuged at 250g's for 5 minutes, and the methanol with IS was collected and injected in the HPLC-UV to quantify the ASTX present in the samples. Finally, 250 μ L of NaOH were added to each well of the plate to solubilize the existent protein and it was left protected from the light at 4°C for 48h. After that, the protein samples were stored at -20°C until analysis. Protein analysis was made using the DC Protein Assay Kit from BioRad®. At least four independent experiments (n=4) were performed, in triplicate.

5.4.5. Evaluation of potential neuroprotective effects of the developed formulations

The potential neuroprotective effects of the developed NLC and SLN formulations, as well as the neuroprotective effects of the ASTX alone, were assessed in differentiated SH-SY5Y cells, which were exposed to different chemical aggressors, namely Iron (III) [in the form of iron (III)- nitrilotriacetic acid (NTA) complex (FeNTA) to simulate iron overload], MPP⁺ [the active metabolite of MPTP, used *in vitro* to mimic PD], and *t*-BHP (to induce oxidative stress).

5.4.5.1. Protective effects against FeNTA-induced cytotoxicity

The protective effects of the ASTX-loaded NLC and SLN formulations, and ASTX alone, were evaluated against an iron overload, a pathophysiological hallmark observed in several neurodegenerative diseases. To assess these neuroprotective effects against the cytotoxicity induced by ferric iron, FeNTA was the chosen iron aggressor. Indeed, for investigational purposes, FeNTA stops the hydrolysis of iron (III) at physiological pH and has been extensively used as an iron source for many biological studies, as NTA reasonable affinity for iron makes FeNTA an admirable model for imitating small ligands involved in metal-chaperone proteins (169). The FeNTA concentration tested (1000 μ M) was selected based on previous research works (170-172).

Firstly, the NTA (250 mM, pH 7.4) solution was set by mixing a nitrilotriacetic acid disodium salt (Na₂NTA) solution (100 mM) and a nitrilotriacetic acid trisodium salt (Na₃NTA) solution (100 mM) until pH = 7. Iron (III) chloride was then combined with NTA to obtain the FeNTA solution. For each experiment, a fresh FeNTA solution (100 mM) was prepared (protected from light) and incubated for 15 minutes, to assure the complete development of the ferric oxidation form before adding the solution to the cells (170, 173). The stock FeNTA solution was further diluted in fresh cell culture medium to obtain the desired final working concentration.

For this assay, SH-SY5Y cells were seeded at a density of 25,000 cells/cm² into 96-well plates, and submitted to the RA and TPA differentiation protocol as mentioned above. Six days after seeding, the cells were exposed to the ASTX-loaded NLC and SLN formulations (50 and 100 μ g/mL) or to the respective concentrations of ASTX alone ((0.38 and 0.76 μ g/mL for SLN and 0.37 and 0.73 μ g/mL for NLC, respectively) prepared in fresh cell culture medium. Empty NLC and SLN formulations were also tested. After 30 min of pre-incubation, FeNTA (final concentration of 1000 μ M) was added, and the cells were further incubated, at 37 °C, in a humidified 5% CO₂-95% air atmosphere, for 24 h. After 24 h of incubation, FeNTA cytotoxicity was assessed by the NR uptake assay, as described before. At least four independent experiments (n=4) were performed, in triplicate.

5.4.5.2. Protective effects against t-BHP-induced cytotoxicity

The imbalance between the ROS formation and the antioxidant defense is the definition of oxidative stress. Consequently, the vital constituents of cells, as lipids, proteins and nucleic acids, are oxidized by ROS, which can induce cell damage and apoptosis or necrosis and, consequently, cell death (174). As oxidative stress is one of the main causes underlying several neurodegenerative diseases, t-BHP is a ROS inducer that can be used to find drugs with potential neuroprotective effects (174).

In this assay, SH-SY5Y cells were seeded at a density of 25,000 cells/cm² into 96-well plates, and further submitted to a RA and TPA differentiation protocol as mentioned before. Six days after seeding, the cells were exposed to the ASTX-loaded SLN and NLC formulations (50 and 100 µg/mL), as well as to the respective concentrations of ASTX alone (0.38 and 0.76 µg/mL for SLN and 0.37 and 0.73 µg/mL for NLC, respectively) prepared in fresh cell culture medium. Empty NLC and SLN formulations were also tested. After 30 min of pre-incubation, *t*-BHP (final concentration of 75 µM) was added and the cells were further incubated at 37 °C in a humidified 5% CO₂-95% air atmosphere, for 24 h. After 24 h of incubation, *t*-BHP cytotoxicity was evaluated by the NR uptake assay, as described before. For each experiment, a fresh stock solution of *t*-BHP was prepared (protected from light) in fresh cell culture medium. At least four independent experiments (n=4) were performed, in triplicate.

5.4.5.3. Protective effects against MPP⁺-induced cytotoxicity

One of the pathological hallmarks of PD is the loss of dopaminergic neurons in the *substantia nigra*. To replicate this disease empirically, *in vivo* or *in vitro*, the MPP⁺ neurotoxin, the active metabolite of MPTP is extensively used, as it capable of severely damage neuronal cells, inducing cell death, thus leading to a condition that closely mimics PD (170, 175, 176). MPP⁺ is responsible for the inhibition of complex I of the mitochondrial electron transport chain, leading to ROS formation and ATP depletion, changing the mitochondrial membrane potential, which leads to apoptosis and death of dopaminergic neurons (177, 178). Hence, the cytotoxicity induced by MPP⁺ has been frequently used to research agents that may have potential neuroprotective effects, *in vitro* and *in vivo* (170).

In this assay, SH-SY5Y cells were seeded, at a density of 25,000 cells/cm² into 96-well plates, and submitted to the RA and TPA differentiation protocol, as mentioned above. Six days after seeding, the cells were exposed to the ASTX-loaded SLN and NLC formulations (50 and 100 µg/mL) or to the respective concentrations of ASTX alone ((0.38 and 0.76 µg/mL for SLN and 0.37 and 0.73 µg/mL for NLC, respectively), prepared in fresh cell culture medium. Empty NLC and SLN formulations were also tested. After 30 min of pre-incubation, MPP⁺ (final concentration of 1000 µM) was added and the cells were further incubated at 37 °C in a humidified 5% CO₂-95% air atmosphere, for 24 h. After 24 h of incubation, MPP⁺ cytotoxicity was evaluated by the NR uptake assay, as described before. In each experiment, a fresh stock solution of MPP⁺ (10 mM) was prepared (protected from light) in HBSS (+/+), diluted in fresh cell culture medium and immediately used. At least four independent experiments (n=4) were performed, in triplicate.

5.5. *In vitro* studies with RPMI 2650 cells

5.5.1. RPMI 2650 as an *in vitro* model for studies in nose-to-brain delivery

RPMI 2650 is a human nasal cell line that was isolated from a squamous cell carcinoma of the nasal septum (179). In experiments with nasal formulations the RPMI 2650 is the primary human nasal cell line used, as this is a non-invasive method to predict nasal drug delivery and shows an easy reproducibility of results and maintenance (179).

RPMI 2650 cells can reach confluence in a short time period, as they have a consistent growth rate. They are frequently selected over excised nasal tissue, as they are less costly and easier to maintain. It was shown that the cells create tight junctions, with transepithelial electrical resistance mimicking the nasal mucosa and can secrete a consistent coating of mucus on the cells surface (180). However, RPMI 2650 cells are unable to perform the mucociliary clearance system, as they do not contain cilia (181). There are some discussion on how valid RPMI 2650 cells are as an *in vitro* model. This discussion is based on the effects caused from different cell culturing conditions. Despite these concerns, a lot of research have been made to further improve the RPMI 2650 cells as a nasal model (179-181).

5.5.2. RPMI 2650 cells: cell culture and seeding

The human nasal septum carcinoma RPMI 2650 cell line was acquired from the American Type Culture Collection (ATCC, USA), and was routinely cultured in 75cm² flasks using EMEM with L-Glutamine supplemented with 10% heat inactivated FBS, 100 U/mL penicillin and 100 µg/mL streptomycin. The cells were kept in a 5% CO₂ -95% air atmosphere, at 37 °C, the medium was changed every 1-2 days, and cultures were passaged weekly by trypsinization using 0.25% trypsin-1 mM EDTA solution. For each experiment, the cells were always seeded at a density of 160,000 cells/cm² and used between the 5th and 10th passages. In all experiments, the cells were used 24 h after seeding.

5.5.3. Evaluation of the cytotoxicity of the developed formulations

The cytotoxicity of the developed NLC and SLN formulations, both empty and ASTX-loaded, was also assessed towards RPMI 2650 cells, by the NR uptake and MTT reduction assays, and 24 h after exposure. RPMI 2650 cells were seeded in 96-well plates (160,000 cells/cm²) and, 24 h after seeding, the cells were exposed to the NLC and SLN formulations (empty or loaded with ASTX, 0 - 500 µg/mL), or alternatively exposed to ASTX alone, in the same concentration range that is found in ASXT-loaded formulations (0 - 3.8 µg/mL), for 24

h. After the incubation time, cytotoxicity was assessed by the NR uptake and MTT reduction assays as described for the differentiated SH-SY5Y cells. Results were expressed as percentage of NR uptake or MTT reduction relative to control cells (0 μ M). Four independent experiments (n=4) were performed, in triplicate.

5.6. Development of an HPLC-UV method for the quantification of ASTX

5.6.1. Instrumental and chromatographic conditions

The quantification of ASTX was performed using a high-performance liquid chromatograph Chromaster, equipped with a Hitachi Chromaster 5160 pump, with a Hitachi Chromaster 5260 autosampler and with a Hitachi Chromaster 5310 column oven, and coupled to a Hitachi Chromaster 5410 UV detector. A C18 column from ACE with a 5 μ m diameter and 250 x 4.6 mm dimensions was used. The analysis was performed in an isocratic elution mode, with a mobile phase composed of 97:3 (v/v) methanol/water, at a flow rate of 1 mL/minute and at room temperature (20,0 $\pm$ 0,5 $^{\circ}$ C). The mobile phase was previously filtered using a 0.45 μ m membrane (Millipore, Madrid, Spain). The sample injection volume was 20 μ L and the UV detection was performed at 474 nm. Clarity® software was used for data analysis.

5.6.2. Preparation of ASTX standards

A stock solution of ASTX ([ASTX]= 25g/L) was prepared in DMSO and stored at - 20 $^{\circ}$ C. This stock solution was then diluted in methanol, by serial dilutions, resulting in 7 ASTX standards- 0.01, 0.05, 0.1, 0.5, 1.0, 5.0 and 7.5 μ g/mL. The ASTX standards were then spiked with 50 μ L of a prepared solution of trans- β - apo- 8'- carotenal, used as IS.

5.6.3. Method validation

The validation of the analytical procedure was completed according to the FDA and the International Conference on Harmonization (ICH) guidelines (182, 183).

The parameters evaluated were specificity, linearity, accuracy, precision (inter- and intra-day), limit of detection (LOD) and limit of quantification (LOQ). Assessment of analytes' stability throughout the processing time of samples was also executed. All calibrators, blank samples and samples were prepared in methanol. All data were analyzed using the GraphPad Prism Software 8.0 and Microsoft Excel 2016.

5.6.3.1. Specificity

The method's specificity is defined as the ability to unequivocally detect and quantify the analyte in the presence of other components, such as degradants, impurities and matrix components (182, 183). Consequently, to distinguish and quantify the response of the target compound, ASTX, from the response of other compounds, the specificity was evaluated. To accomplish this goal, blank samples (methanol spiked with IS) were injected (20 μ L), in duplicate, into the HPLC-UV system to assess and detect possible co-eluting peaks that could interfere with ASTX peak. An analysis of empty SLN and NLC, submitted to the same extractive procedure used for determination of EE was also performed to detect possible interferents. Lastly, methanolic extracts of differentiated SH-SY5Y cells previously exposed to empty SLN and NLC formulations for 24 h was also analyzed to detect the possible existence of interferent peaks resulting from cellular constituents.

5.6.3.2. Linearity

The linearity of an analytical procedure corresponds to the ability, within a certain range of concentrations, to obtain analytical results that are directly proportional to the analyte concentration in the samples (182, 183). To estimate the linearity of the method, the signal obtained from the peak area (Y) and the nominal standard concentration (X) are required (182, 183). In this particular case, in which an IS was used, the ratio of analyte peak area/ IS peak area (Y) and analyte concentration (X) were assessed by linear regression analysis studying the coefficient of determination (r^2) obtained with seven ASTX calibrators. In five distinct days, ASTX calibrators with concentrations ranging from 0 to 7.5 μ g/mL of ASTX were prepared each day (0, 0.01, 0.05, 0.1, 0.5, 1.0, 5.0, 7.5) and injected, in duplicate, into the HPLC-UV system. The linearity was then evaluated by the regression curves (ratio ASTX peak area/IS peak area versus ASTX concentration) and by the coefficient of determination (r^2). Five independent calibration curves ($Y = aX + b$) were obtained, and the regression analysis was performed using the GraphPad Prism 8.0 Software.

5.6.3.3. Accuracy

The accuracy of a method expresses the proximity of agreement between the measured quantity value (experimental results, in other words, a calculated value obtained from a calibration curve) and a true quantity value (nominal value) (182, 183). Therefore, accuracy was determined by spiking blank matrix (methanol) with three different ASTX

concentrations within the ASTX linear concentration range (low, medium and high: 0.5, 1.0 and 5.0 µg/mL, respectively). For each ASTX concentration, five independent replicates were prepared, spiked with IS, and injected (20 µL), in triplicate, in the HPLC-UV system. To calculate the experimental concentration of each calibrator, an ASTX calibration curve was also prepared and injected, in duplicate, to calculate the experimental concentration of each calibrator and the percent deviation between the calculated value and the nominal value through the equation 2:

$$\text{Accuracy (\%)} = (\text{mean experimental concentration} / \text{nominal concentration}) \times 100$$

Equation 2. equation used to calculate accuracy percentage

5.6.3.4. Precision

The precision of an analytical method expresses the closeness of agreement between independent results obtained from homogenous samples under stipulated conditions (182, 183). In other words, this parameter shows how similar are a sequence of measurements acquired from several injections of distinct calibrators, all under similar operating conditions. The precision of a given analytical procedure can be approached at three distinct levels: repeatability, intermediate precision and reproducibility. In the present study the method's repeatability (also termed as intra-day precision) was evaluated, which expresses the precision under the same operating conditions and over a short period of time. The method's intermediate precision (also termed as inter-day precision), which expresses variations within-laboratories, such as different days, was also determined. Reproducibility, which expresses the precision between laboratories, was not evaluated (182, 183). Precision was always expressed as the coefficient of variation (%CV) of a series of measurements.

5.6.3.4.1. Repeatability - Intra-day precision of the equipment and method

The intra-day precision expresses the degree of repeatability of a given analytical procedure, in the preparation of the same samples when performed by the same analyst, in the same laboratory, using the same instruments and during a short period of time. Thus, this parameter reflects the degree of agreement of the results when the experimental conditions are consistently maintained (182, 183). This parameter was assessed both for the HPLC-UV equipment and for the entire analytical method, since sample preparation until its injection in the HPLC-UV equipment.

The intra-day precision of the equipment was obtained by analyzing the results acquired for three different ASTX calibrators, with ASTX concentrations within the ASTX linear concentration range (low, medium and high concentrations: 0.5, 1.0 and 5.0 µg/mL,

respectively), which were prepared, spiked with IS and injected (20 μ L) five times in the HPLC-UV equipment, on the same day. Intra-day precision was then assessed by calculating the coefficient of variation (CV %) of the obtained values (ratio between ASTX peak area and IS peak area obtained for each ASTX calibrator). A CV % value $\leq$ 15 % was considered satisfactory.

The intra-day precision of the analytical method was calculated by analyzing three different ASTX calibrators with ASTX concentrations within the ASTX linear concentration range (low, medium and high concentrations: 0.5, 1.0 and 5.0 μ g/mL, respectively), which were independently prepared five times in the same day (5 replicates of each calibrator), spiked with IS and injected (20 μ L), in triplicate, in the HPLC-UV apparatus, also on the same day. Intra-day precision of the analytical method was then assessed by calculating the coefficient of variation (%CV) of the observed values (ratio between ASTX peak area and IS peak area for each ASTX calibrator). A %CV value of $\leq$ 15% was considered satisfactory.

5.6.3.4.2. Intermediate precision - Inter-day precision of the equipment and method

The inter-day precision of an analytical procedure expresses the variations within-laboratories such as, for example, different days. This parameter was also assessed both for the HPLC-UV equipment and for the entire analytical method, from the sample preparation until its injection in the HPLC-UV equipment.

The inter-day precision of the equipment was evaluated by analyzing the results of three representative ASTX calibrators with ASTX concentrations within the ASTX linear concentration range (low, medium and high concentrations: 0.5, 1.0 and 5.0 μ g/mL, respectively), which were prepared, spiked with IS, and injected (20 μ L), in triplicate, in the HPLC-UV equipment, over five consecutive days. The coefficient of variation (CV %) of the replicated values (ratio between ASTX peak area and IS peak area obtained for each ASTX calibrator) were then calculated. A CV% value of $\leq$ 15% was considered satisfactory.

The determination of inter-day precision of the analytical method was obtained by individually preparing and analyzing, in five consecutive days, three representative ASTX calibrators with ASTX concentrations within the ASTX linear concentration range (low, medium and high concentrations: 0.5, 1.0 and 5.0 μ g/mL, respectively), which were daily prepared, spiked with IS and injected (20 μ L), in triplicate, in the HPLC-UV equipment. The coefficient of variation (CV %) of the obtained values (ratio between ASTX peak area and

IS peak area obtained for each ASTX calibrator) were then calculated. A CV% value of $\leq 15\%$ was considered satisfactory.

5.6.3.5. Sensitivity of the method- LOD and LOQ

The LOD of a given method is defined as the smallest measured concentration of an analyte from which is possible to deduce its presence in a sample but which is not necessarily quantified as an exact value (i.e. it can be distinguished from the blank or noise of the system). The LOQ is defined as the smallest measured concentration of an analyte in a sample, which can be quantitatively detected with a specified degree of accuracy and precision, under the experimental conditions adopted (182, 183).

LOD and LOQ were calculated with the standard deviation (σ) of the analytical signal of 20 blank samples (analyte peak area/ IS area for each blank sample) injected (20 μL), in duplicate, into the HPLC-UV system and with the slope (S) of the ASTX calibration curve, which was prepared and analyzed in the same day. The following equations 3 and 4 were used to calculate the LOD and LOQ:

$$LOD = \frac{3.3 \times \sigma}{S}$$

Equation 3. equation used to calculate LOD

$$LOQ = \frac{10 \times \sigma}{S}$$

Equation 4. equation used to calculate LOQ

5.6.3.6. Stability

A remarkably important assay that should be performed during method validation consists in the evaluation of the analyte stability, once it allows to infer if the samples are subjected or not to chemical degradation. Accordingly, the amount of substance present in a given sample is analyzed to assess if it remains unchanged once subjected to different conditions, during a certain period of time (182, 183). The analyte stability can be evaluated by changing different factors, including the exposure to the light, different storage temperatures (-80°C , -20°C , 4°C or 20°C) and storage time.

In the present study, the temperature and time of storage were the stability parameters assessed. This assessment was performed by preparing a calibration curve with seven ASTX calibrators with concentration ranging from 0.01 and 7.5 $\mu\text{g/mL}$. All calibrators were spiked with IS and injected (20 μL), in duplicate, in the HPLC-UV apparatus immediately after preparation. After injecting each one of the calibrators, the remaining volume was stored in independent vials at different temperatures (room temperature, 4°C ,

–20 °C and – 80 °C) for 7 days. After this storage time, each calibrator was re-injected, in duplicate, and the obtained calibration curve compared with the calibration curve obtained in the day of the calibrators preparation.

5.6.3.7. Proof of applicability

As mentioned above, the developed and validated HPLC-UV method was applied in the quantification of the intracellular ASTX levels in samples obtained in ASTX uptake assays, as well as in the assessment of ASTX EE into both NLC and SLC-loaded ASTX formulations, thus representing a proof of applicability of the validated method.

5.7. Statistical analysis

The statistical calculations were executed using GraphPad Prism 8 for Windows (GraphPad Software, USA). The normality of the data distribution was measured using the Kolmogorov-Smirnov, D'Agostino & Pearson omnibus, and Shapiro-Wilk normality tests. Means of different groups were compared using a two-way ANOVA, followed by the Tukey's multiple comparisons test. In every cases, p values under 0.05 were pondered as significant.

6. Results and Discussion

6.1. Characterization of astaxanthin-loaded lipid nanoparticles

6.1.1. Particle size, polydispersity index and zeta potential

The results of the particle size, PDI and ZP of the astaxanthin-loaded SLN and NLC formulations showed that 50% of the particles had sizes equal or lower than 63.400 ± 0.001 nm and 59.200 ± 0.000 nm, respectively. After 30 days of storage, at 20.0 ± 0.5 °C, these formulations appeared to have no significant changes in the particle sizes, when compared to the values obtained in the production day (65.100 ± 0.000 nm for SLN, and 60.300 ± 0.000 nm for NLC) (Table 4). Regarding the results of the PDI, the closeness of the values of PDI to 0.25 suggests that the majority of the particles have similar sizes. Regarding the ZP, since the results are close to $|\pm 30|$ mV, it is possible to predict that the produced formulations will show good storage stability (184, 185). In addition, it was observed that ASXT encapsulation in the SLN and NLC did not affected size, PDI and ZP. Similar results were obtained in other works with ASTX-loaded lipid nanoparticles were produced (186, 187).

Table 4. Particle size results (D10, D50, D90, Z-Ave), PDI and ZP of the SLN and NLC formulations (loaded with astaxanthin – ASTX and empty) prepared on day 0 and after storage at 20.0 ± 0.5 °C, for 30 days. All results are presented as mean values $\pm$ SD.

	Time (days)	D10 (nm)	D50 (nm)	D90 (nm)	Z-Ave (nm)	PDI	ZP (mV)
SLN-ASXT	0	20.200 ± 0.000	63.400 ± 0.001	3520.000 ± 0.621	110.200 ± 6.482	0.349 ± 0.056	-24.333 ± 1.234
	30	20.400 ± 0.000	65.100 ± 0.000	3450.000 ± 0.038	106.547 ± 9.360	0.457 ± 0.067	-29.800 ± 1.058
Empty SLN	0	43.200 ± 0.000	69.800 ± 0.000	105.000 ± 0.000	98.043 ± 1.200	0.212 ± 0.006	-20.033 ± 0.643
	30	18.000 ± 0.001	41.300 ± 0.001	109.000 ± 0.003	98.107 ± 0.641	0.230 ± 0.008	-22.700 ± 0.361
NLC-ASTX	0	19.900 ± 0.000	59.200 ± 0.000	244.000 ± 0.002	97.610 ± 0.394	0.294 ± 0.035	-23.300 ± 1.058
	30	20.000 ± 0.000	60.300 ± 0.000	265.000 ± 0.002	109.000 ± 1.000	0.330 ± 0.021	-28.400 ± 0.954
Empty NLC	0	14.300 ± 0.001	30.800 ± 0.003	91.900 ± 0.011	109.033 ± 0.404	0.228 ± 0.006	-23.467 ± 1.079
	30	20.400 ± 0.000	52.200 ± 0.000	129.000 ± 0.000	116.033 ± 1.795	0.215 ± 0.004	-23.833 ± 0.462

6.1.2. Morphology: CryoSEM

Figure 4 shows that both NLC and SLN (empty and loaded with astaxanthin) had the typical spherical shape and smooth external surface of the lipid nanoparticles. In addition, it is possible to confirm the obtained nanometric values for particle size in the LD and DLS techniques (Table 4). These observations were already observed in other works with the same type of formulations (184, 188).

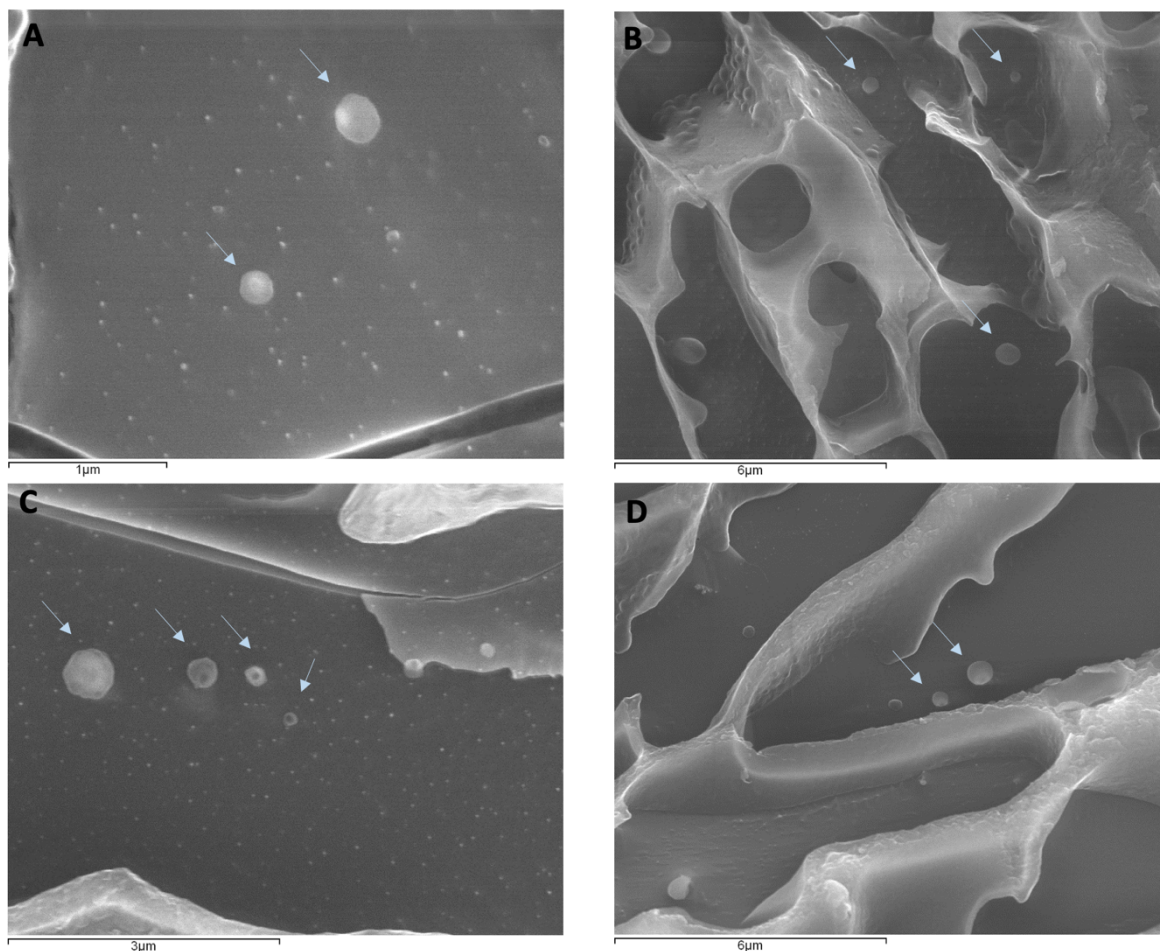


Figure 4. CryoSEM images of the prepared SLN and NLC formulations: (A) Empty NLC (Magnification 35000x); (B) Astaxanthin-loaded NLC (Magnification 10000x); (C) Empty SLN (Magnification 20000x); and (D) Astaxanthin-loaded SLN.

6.1.3. Evaluation of the encapsulation efficiency

The EE of ASTX in both SLN and NLC was very high (98.75 ± 0.88 and 98.55 ± 0.39 , respectively), which were expected results, as the ASTX is a lipophilic compound with affinity for the lipid matrix. These results suggest that the developed ASTX-loaded lipid nanoparticles can be effective carriers for lipophilic natural compounds. For SLN and NLC formulations these EE values are usually obtained when lipophilic drugs are encapsulated (189).

6.2. Evaluation of the cytotoxicity of the developed formulations

Cytotoxicity assays were performed to assess the safety of SLN and NLC formulations in differentiated SH-SY5Y and RPMI 2650 cells. In both cell lines, the cytotoxicity of the empty formulations, ASTX-loaded SLN and NLC formulations, and ASTX alone was evaluated by the NR uptake and MTT reduction assays. As shown in **figure 5**, the developed SLN and NLC formulations (both empty and ASTX-loaded) significantly decreased the viability of differentiated SH-SY5Y cells, when tested at concentrations equal or above 250 $\mu\text{g/mL}$, as evaluated by the significant decrease in NR uptake, 24 hours after exposure to 250 and 500 $\mu\text{g/mL}$ of each formulation. For free ASTX, no significant cytotoxic effects were detected at the tested concentration range. Furthermore, when tested at the highest concentrations, significant differences were detected between 250 and 500 $\mu\text{g/mL}$ of the SLN and NLC formulations (both empty and AST-loaded), when compared to the corresponding concentration of free ASTX. Moreover, and noteworthy, no significant differences were observed between empty and ASTX-loaded SLN and NLC formulations.

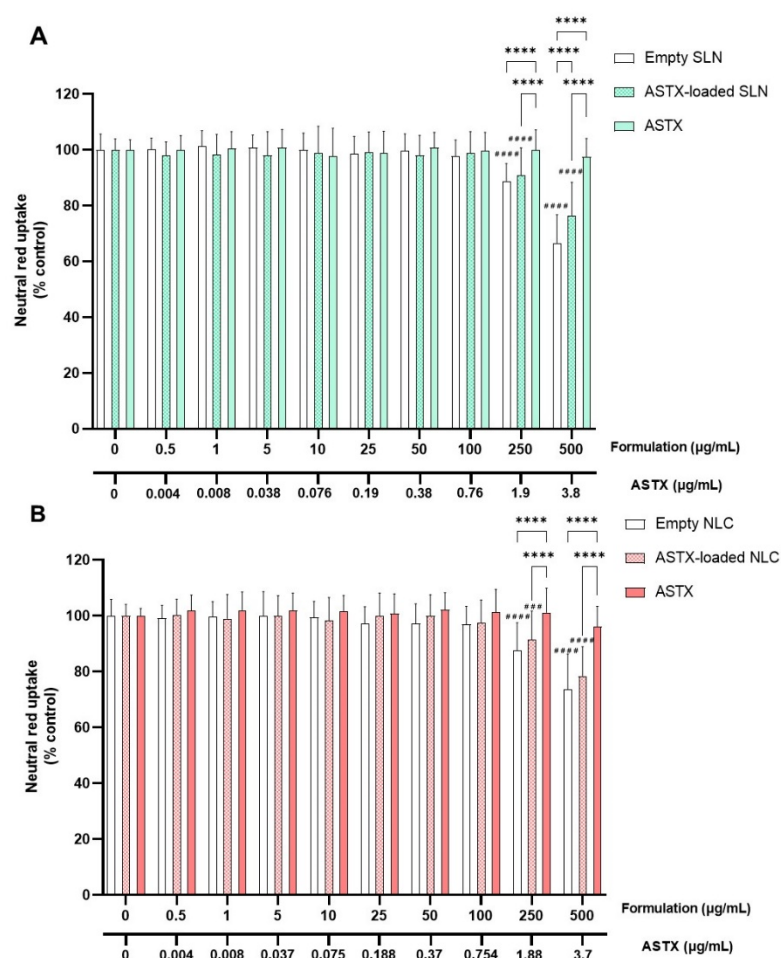


Figure 5. Cytotoxicity of both empty and ASTX-loaded SLN (A) and NLC (B) formulations towards differentiated SH-SY5Y cells, evaluated by the NR uptake assay, 24 h after exposure. The cytotoxicity of free ASTX was also evaluated. Results are presented as mean + SD from at least 4 independent experiments, performed in triplicate. Statistical comparisons were made using two-way ANOVA followed by the Tukey's multiple comparisons test (## $p < 0.01$, ### $p < 0.001$, #### $p < 0.0001$ for each formulation or ASTX vs. 0 µg/mL; **** $p < 0.0001$, for comparisons between formulations or between formulations and ASTX alone, at each concentration). In all cases, p values < 0.05 were considered significant.

In what concerns to the MTT reduction assay, and as shown in [figure 6](#), similar results were obtained for the NLC formulations (empty and ASXT-loaded). In contrast, for SLN formulations a small, but significant, decrease in cell metabolic activity was observed (MTT reduction significantly decreased to 87.6% and 89.7%, 24 hours after exposure to 100 µg/mL empty SLN and ASTX-loaded SLN, respectively). Again, a significant decrease in MTT reduction was also observed when the formulations were tested at 250 and 500 µg/mL. Additionally, and although no significant differences were observed between empty and ASTX-loaded formulations, significant differences were detected between 100, 250 and 500 µg/mL of SLN or 250 µg/mL of NLC formulations (both empty and AST-loaded) and the corresponding concentrations of free ASTX. The slightly difference found between both methods used to assess cell viability (with MTT reduction assay detecting significant

differences for the 100 $\mu\text{g/mL}$ SLN formulations) can be explained by the fact that these methods use different principals to assess cytotoxicity. Even though, accordingly to the obtained results, the developed SLN and NLC formulations (empty and ASTX-loaded) are safe towards differentiated SH-SY5Y cells for concentration equal or below 100 $\mu\text{g/mL}$ (% of cell viability always above the 85% threshold). Previous studies have reported similar results with this type of formulations, with the drug encapsulated being less toxic towards differentiated SH-SY5Y cells, when compared to the formulations themselves (154, 184, 190, 191). Accordingly, the 100 $\mu\text{g/mL}$ was the concentration selected as the highest safe concentration of SLN and NLC formulations to be used in the subsequent studies in differentiated SH-SY5Y cells, namely studies evaluating the potential neuroprotective effects of the developed formulations and their capacity to be incorporated intracellularly.

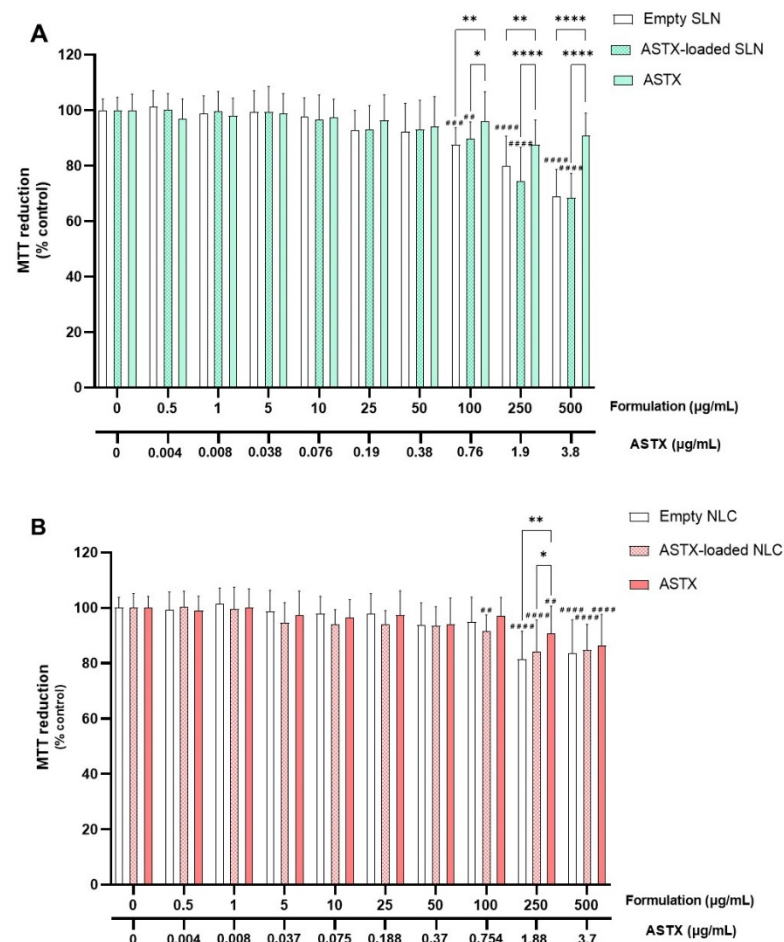


Figure 6. Cytotoxicity of both empty and ASTX-loaded SLN (A) and NLC (B) formulations towards differentiated SH-SY5Y cells, evaluated by the MTT reduction assay, 24 h after exposure. The cytotoxicity of free ASTX was also evaluated. Results are presented as mean + SD from at least 4 independent experiments, performed in triplicate. Statistical comparisons were made using two-way ANOVA followed by the Tukey's multiple comparisons test ($^{##}p < 0.01$, $^{###}p < 0.001$, $^{####}p < 0.0001$ for each formulation or ASTX vs. 0 $\mu\text{g/mL}$; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{****}p < 0.0001$, for comparisons between formulations or between formulations and ASTX alone, at each concentration). In all cases, p values < 0.05 were considered significant.

In what concerns to the RPMI 2650 cell, and as shown in **figure 7**, cell viability was reduced when cells were exposed to a concentration of 250 and 500 $\mu\text{g/mL}$ of SLN formulations. NR uptake significantly decreased to 83.1% and 61% 24 hours after exposure to 250 and 500 $\mu\text{g/mL}$ of empty SLN, respectively; and to 88.9% and 57.4% 24 hours after exposure to 250 and 500 $\mu\text{g/mL}$ ASTX-SLN, respectively. For the NLC formulations, a significant cytotoxicity was also observed with NR uptake significantly decreasing to 89.1% and 51.2% 24 hours after exposure to 250 and 500 $\mu\text{g/mL}$ of empty NLC, respectively, and to 88.9% and 67.3% 24 hours after exposure to 250 and 500 $\mu\text{g/mL}$ of ASTX-NLC, respectively. For free ASTX, no significant cytotoxic effects were detected at the tested concentration range. As a consequence, although no significant differences were observed between empty and ASTX-loaded formulations (for both SLN and NLC), significant differences were detected between free ASTX and SLN or NLC formulations, at the highest tested concentration.

In figure 8 are presented the results of the evaluation of the formulations cytotoxicity towards RPMI 2650 assessed by the MTT reduction assay. In agreement with the NR uptake assay, a significant decrease in cell metabolic activity was observed for all the tested formulations at a concentration of 250 and 500 $\mu\text{g/mL}$ (both empty and ASTX-loaded SLN and NLC formulations). Again, no significant cytotoxic effects were detected for free ASTX, at the tested concentration range.

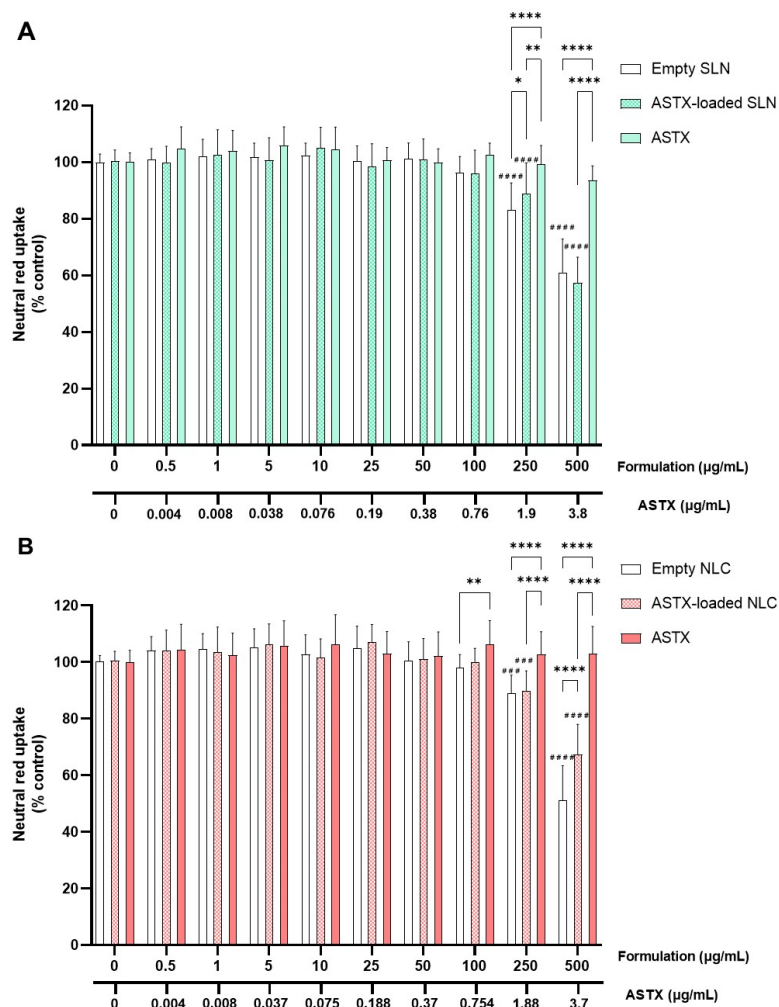


Figure 7. Cytotoxicity of both empty and ASTX-loaded SLN (A) and NLC (B) formulations towards RPMI 2650 cells, evaluated by the NR uptake assay, 24 h after exposure. The cytotoxicity of free ASTX was also evaluated. Results are presented as mean + SD from at least 4 independent experiments, performed in triplicate. Statistical comparisons were made using two-way ANOVA followed by the Tukey's multiple comparisons test (#### $p < 0.0001$, ##### $p < 0.00001$ for each formulation or ASTX vs. 0 µg/mL; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.0001$, for comparisons between formulations or between formulations and ASTX alone, at each concentration). In all cases, p values < 0.05 were considered significant.

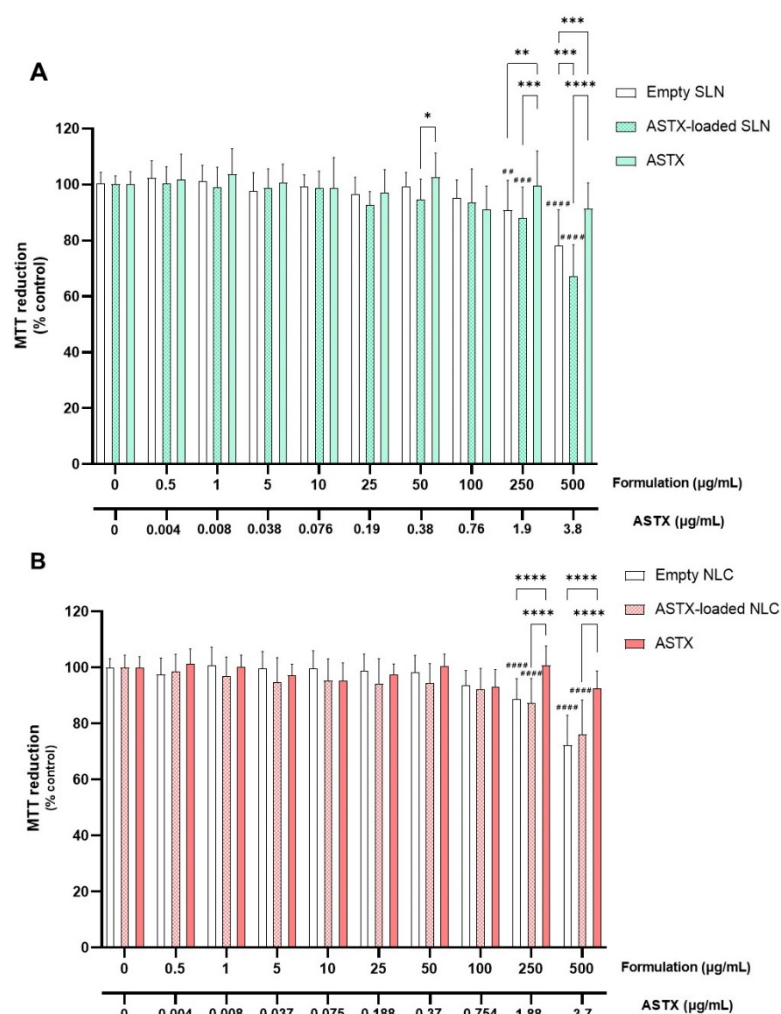


Figure 8. Cytotoxicity of both empty and ASTX-loaded SLN (A) and NLC (B) formulations towards RPMI 2650 cells, evaluated by the MTT reduction assay, 24 h after exposure. The cytotoxicity of free ASTX was also evaluated. Results are presented as mean + SD from at least 4 independent experiments, performed in triplicate. Statistical comparisons were made using two-way ANOVA followed by the Tukey's multiple comparisons test ($^{##}p < 0.01$, $^{###}p < 0.001$, $^{####}p < 0.0001$ for each formulation or ASTX vs. 0 µg/mL; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$, for comparisons between formulations or between formulations and ASTX alone, at each concentration). In all cases, p values < 0.05 were considered significant.

Overall, the obtained results, clearly demonstrated that the developed SLN and NLC formulations (both empty and ASTX-loaded) can be safely tested in RPMI 2650 cells, at concentrations equal or below 100 µg/mL (% of cell viability always above the 85% threshold).

6.3. Cellular uptake of ASTX

The ASTX intracellular uptake was evaluated in differentiated SH-SY5Y cells, 24 hours after exposure to 100 µg/mL of ASTX-loaded SLN or NLC formulations. The cellular uptake of free ASTX at the corresponding concentrations was also evaluated. Methanolic extract of the exposed differentiated SH-SY5Y cells were injected into the HPLC-UV and the obtained ASTX intracellular content normalized to the cell's protein content.

As observed in **Figure 9**, the exposure of differentiated SH-SY5Y cells to the ASTX-loaded SLN formulation (100 µg/mL) lead to an intracellular accumulation of ASTX of 139.4 ng ASTX/ mg protein, similar to the amount incorporated by the cells upon exposure to the corresponding concentration of free ASTX (0.76 µg/mL) (128.3 ng ASTX/ mg protein). On the other side, the exposure of differentiated SH-SY5Y cells to the ASTX-loaded NLC formulation (100 µg/mL) for 24 hours resulted in the cellular uptake of 38.7ng ASTX/ mg protein, an amount significantly smaller than the observed upon exposure to the corresponding concentration of free ASTX (0.73 µg/mL) (147.3 ng ASTX/ mg protein).

These results suggest that, contrary to ASTX-loaded SLN, ASTX-loaded NLC tend to stay more on the cell surface rather than penetrating. To support this conclusion, a greater neuroprotective effect was observed with ASTX-loaded NLC (see section 6.5.), which may be related to the formation of a protective barrier on the cell surface that prevented the entry of the aggressor agent.

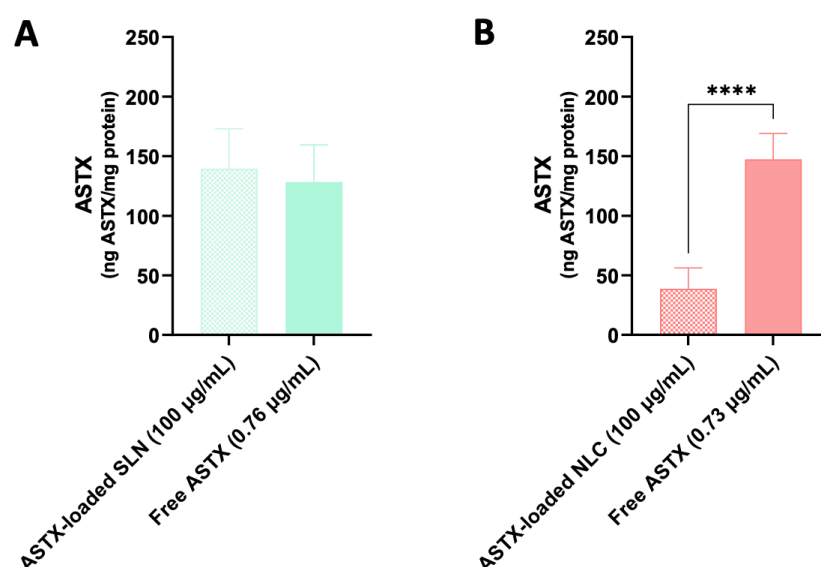


Figure 9. Cellular uptake of ASTX, evaluated in differentiated SH-SY5Y 24 hours after exposure to 100 µg/mL ASTX-loaded SLN (A) and NLC (B) formulations, as well as to the corresponding free ASTX concentrations. Results are presented as mean $\pm$ SD from 4 independent experiments, performed in triplicate. Statistical comparisons were made using Unpaired t test test (**** $p < 0.0001$ for comparisons between free ASTX and ASTX-loaded formulations). In all cases, p values < 0.05 were considered significant.

6.4. Evaluation of potential neuroprotective effects of the developed formulations

6.4.1. Protective effects against FeNTA-induced cytotoxicity

In brain functions, iron plays many important roles to turn them healthy. Nevertheless, when iron levels augment due to a disruption in its homeostasis, it can trigger oxidative stress and, consequently, cell death. Furthermore, iron deregulation was already associated with several neurodegenerative diseases (192). Iron has a significant role in ROS production and, as such, it can induce oxidative stress. Additionally, it is able to oxidize dopamine to a quinone, a highly reactive compound that leads, eventually, to cellular dysfunction or even cell death (170, 193). Moreover, ferric iron is an example of a pro-oxidant, which means that it induces ROS production, originating oxidative stress, as already reported in neuroblastoma cells (172, 194). The neuroprotective effects showed by some iron chelators further highlights the impact of iron-induced toxicity in neurodegeneration. This protection was observed in in vitro models used to investigate neurodegenerative diseases, such as the SH-SY5Y cells (170, 172, 195-197). Outstandingly, and as mentioned, iron has already been associated with the pathophysiology of numerous neurodegenerative diseases, namely with AD and PD (195,

198, 199). Therefore, metal ions offer an encouraging pharmacological target for the management of these impacting diseases (170, 173, 200). For experimental studies, the iron (III)-NTA complex avoids the hydrolysis of iron (III) at a functional pH, and has been extensively used as an iron source in many biological investigations, because as NTA has a moderate affinity for iron it makes FeNTA an outstanding model for simulating small ligands concerned in metal-chaperone proteins (169, 170). Thus, to assess the protective effect of ASTX- loaded SLN and ASTX- loaded NLC formulations against ferric iron-induced cytotoxicity, FeNTA was the chosen agent. Iron's cytotoxic outcome was, then, assessed in differentiated SH-SY5Y cells by the NR uptake assay, after 24 hours of exposure to FeNTA (1000 μ M). Previous works, where FeNTA exhibited cytotoxic effect from 250 up to 2500 μ M in SH-SY5Y cells, were reviewed to select the tested FeNTA concentration (170-172, 201).

In figure 10 are shown the results obtained in the assessment of the neuroprotective effects of both SLN and NLC, ASTX-loaded and empty, and the respective concentration of ASTX alone, against FeNTA-induced cytotoxicity. Accordingly, when tested alone, the iron overload promoted by FeNTA (1000 μ M) significantly reduced the viability of differentiated SH-SY5Y cells to ~70% ,24 hours after exposure, and when compared to control cells (0 μ M). However, when SLN formulations were tested, no significant protective effects against FeNTA-induced cytotoxicity were observed. When exposed to 1000 μ M FeNTA in the presence of empty SLN formulations (50 and 100 μ g/mL), the cells viability significantly decreased to 70.2% and 67.6%, respectively. Concerning the exposure to 1000 μ M FeNTA in the presence of ASTX- loaded SLN (50 and 100 μ g/mL), cell viability significantly decreased to 71.7% and 70.7%, respectively. However, significant protective effects against FeNTA-induced cytotoxicity were observed when the exposure to the aggressor occurred in the presence of ASTX alone (0.38 and 0.76 μ g/mL), with cell viability significantly increasing up to 77% for both ASTX concentrations.

Outstandingly, when FeNTA-induced cytotoxicity was assessed in the presence of NLC formulations (50 and 100 μ g/mL), remarkable protective effects were observed for all the tested conditions (empty NLC, ASTX-loaded NLC and free ASTX). Indeed, when the exposure to the aggressor occurred in the presence of 50 and 100 μ g/mL of empty NLC, cell viability significantly increased to 78.2% and 81.3%, respectively. On the other side, upon simultaneous exposure to FeNTA and 50 and 100 μ g/mL of ASTX-loaded NLC, cell viability significantly ascended to 88.6% and 90.2%, respectively. When ASTX alone was tested (0.37 and 0.73 μ g/mL), cell viability significantly increased to 77.3% and 75.6%, respectively. It is important to highlight that significant differences were observed between empty and ASTX-loaded NLC formulations, with the latter being significantly more effective than the empty NLC. Outstandingly, ASTX-loaded NLC was significantly more protective

against FeNTA-induced cytotoxicity, at both tested concentrations, when compared to ASTX alone. The protective effects afforded by the empty NLC may be justified by the antioxidant effect of vitamin E, that is also present in the composition of the NLC formulations, and which was already reported to protect against iron induced toxicity (202)

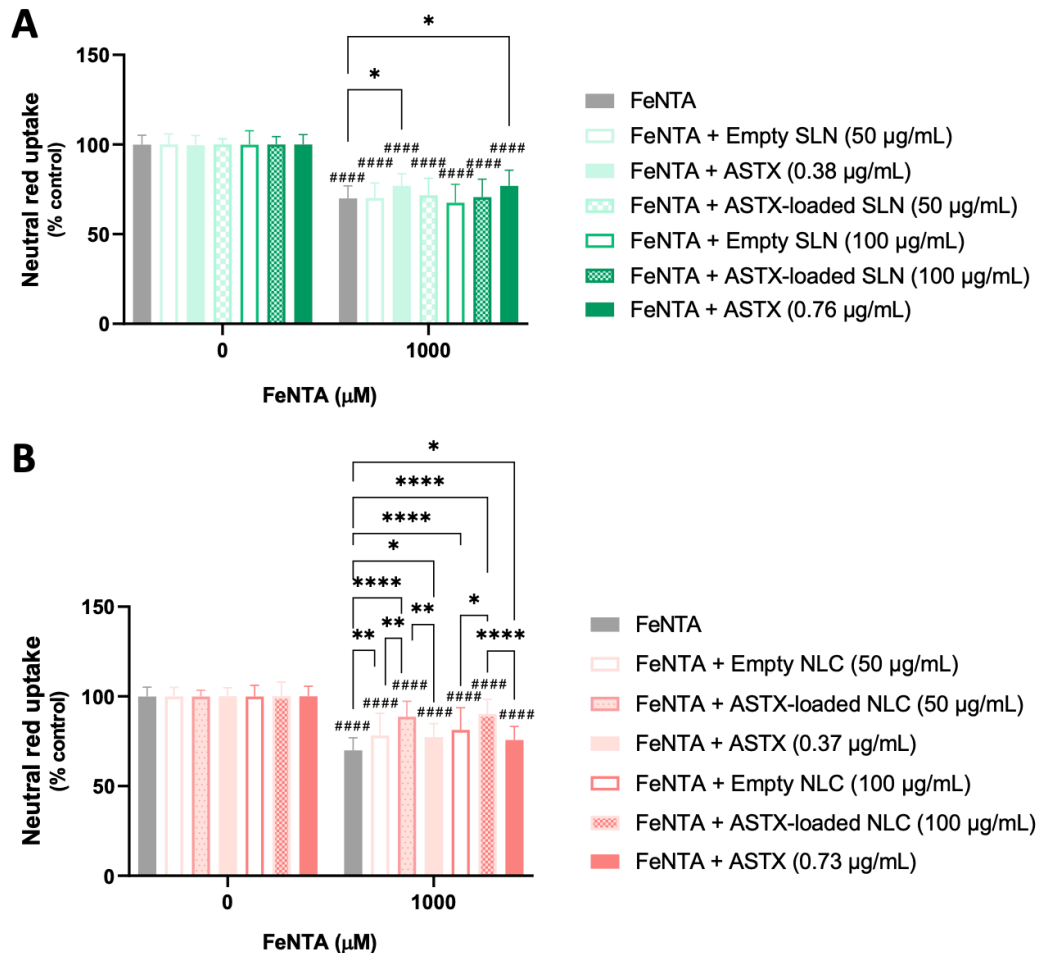


Figure 10. Potential neuroprotective effects of the developed SLN (A) and NLC (B) formulations (both empty and ASTX-loaded), and free ASTX, against FeNTA-induced cytotoxicity, evaluated in differentiated SH-SY5Y cells, by the neutral red uptake assay, 24 h after exposure to the aggressor. Results are expressed as mean + SD from 4 independent experiences, performed in triplicate. Statistical comparisons were made using two-way ANOVA followed by the Tukey's multiple comparisons test [#### $p < 0.0001$ versus control cells (0 µM FeNTA); * $p < 0.05$; ** $p < 0.01$; * $p < 0.001$; **** $p < 0.0001$, for comparison between formulations, ASTX and FeNTA alone, at each FeNTA concentration). In all cases, p values < 0.05 were considered significant.**

Overall analysis of the data obtained in the present study (figure 10), demonstrated that empty SLN (50 and 100 µg/mL) and ASTX-loaded SLN (50 and 100 µg/mL) formulations did not show significant protection against iron (III)-induced cytotoxicity. On the other side, ASTX alone (0.38 and 0.76 µg/mL) achieved a slight, but significant protection against the aggressor. In what concerns to the NLC formulations, all conditions tested (empty NLC [50 and 100 µg/mL]; ASTX- loaded NLC [50 and 100 µg/mL]; and ASTX alone

[0.37 and 0.73 µg/mL]) presented a significant protective effect towards iron (III)-induced cytotoxicity. In both cases, the neuroprotective effects of ASTX alone may be due to its antioxidant properties. However, the remarkably improved protective effects observed when the natural antioxidant was incorporated in the NLC should be highlighted, and putatively attributed to the synergistic antioxidant effect of ASTX and vitamin E. As mentioned, the latter is present in the composition of the NLC formulations (see table 3).

6.4.2. Protective effects against *t*-BHP-induced cytotoxicity

The imbalance between antioxidant defense and the ROS production causes oxidative stress. The fundamental components of cells, such as proteins, nucleic acids, and lipids, are oxidized and can trigger cell damage with consequent apoptosis (203). One of the main pathophysiological mechanisms underlying several neurodegenerative diseases is the oxidative stress. ROS increases with age, and brain function is altered. Abundant release of ROS is induced by mitochondrial dysfunction and declines the activity of antioxidant enzymes. In AD, it was observed in patients dopaminergic neurons an excess of oxidative stress (204). For this reason, in this work, *t*-BHP was the chosen aggressor, as it is a producer of oxidative stress by stimulating an excessive production of ROS (205-208). Indeed, *t*-BHP, when metabolized, leads to the appearance of peroxy and alkoxy radicals that are toxic and originate lipid peroxidation, disturb cell integrity, and create covalent bonds with cellular molecules, taking to cell death (209, 210). A model of oxidative stress-inducing cell damage in this work was acquired treating differentiated SH SY5Y cells with 75 µM *t*-BHP for 24 h [this working concentration was selected based on previous works where *t*-BHP was used as a ROS producer (174, 205-208)].

In figure 11 are depicted the results of the potential neuroprotective effects of both SLN and NLC, loaded and empty, and the respective concentration of ASTX alone, against *t*-BHP- induced cytotoxicity. According to the obtained results, when tested alone, *t*-BHP (75 µM) significantly reduced the viability of differentiated SH-SY5Y cells to ~67%, 24 hours after exposure, and when compared to control cells (0 µM). When differentiated SH-SY5Y cells were exposed to 75 µM *t*-BHP in the presence of empty SLN formulations (50 and 100 µg/mL), the cells viability were 68.3% and 67.2%, respectively, evidencing no significant protective effects, when compared to the exposure to the chemical aggressor alone. Concerning the simultaneous exposure to 75 µM *t*-BHP and 50 or 100 µg/mL ASTX-loaded SLN, cell viability increased to 79% and 72.3%, respectively, thus demonstrating a significant protection of differentiated SH-SY5Y cells against *t*-BHP-induced cytotoxicity. When ASTX alone was tested (0.38 and 0.76 µg/mL), a significantly higher cell viability was

observed, highlighting the ASTX potential in protecting cells against *t*-BHP-induced oxidative stress. NR uptake significantly increased to 82.1% and 74.1% when differentiated SH-SY5Y cells were exposed to the aggressor in the presence of 0.38 and 0.76 µg/mL ASTX, respectively. When cell viability was assessed after exposure to 75 µM *t*-BHP in the presence of 50 or 100 µg/mL empty NLC, a cell viability of 66.6% and 67.7% was obtained. On the other hand, when the exposure to the chemical aggressor occurred in the presence of 50 or 100 µg/mL ASTX-loaded NLC, cell viability ascended to 79.3% and 73.4%, respectively. However, and in agreement with results obtained for the ASTX-loaded SLN formulation, a significant protective effect was only observed for the lowest concentration of ASTX-loaded NLC (50 µg/mL). When the exposure to *t*-BHP occurred in the presence of 0.37 or 0.73 µg/mL ASTX alone, and in agreement with the studies performed with the SLN formulations, cell viability significantly increased to 82.2% and 73.8%, respectively, highlighting again the ASTX potential in protecting neuronal cells against *t*-BHP-induced oxidative stress.

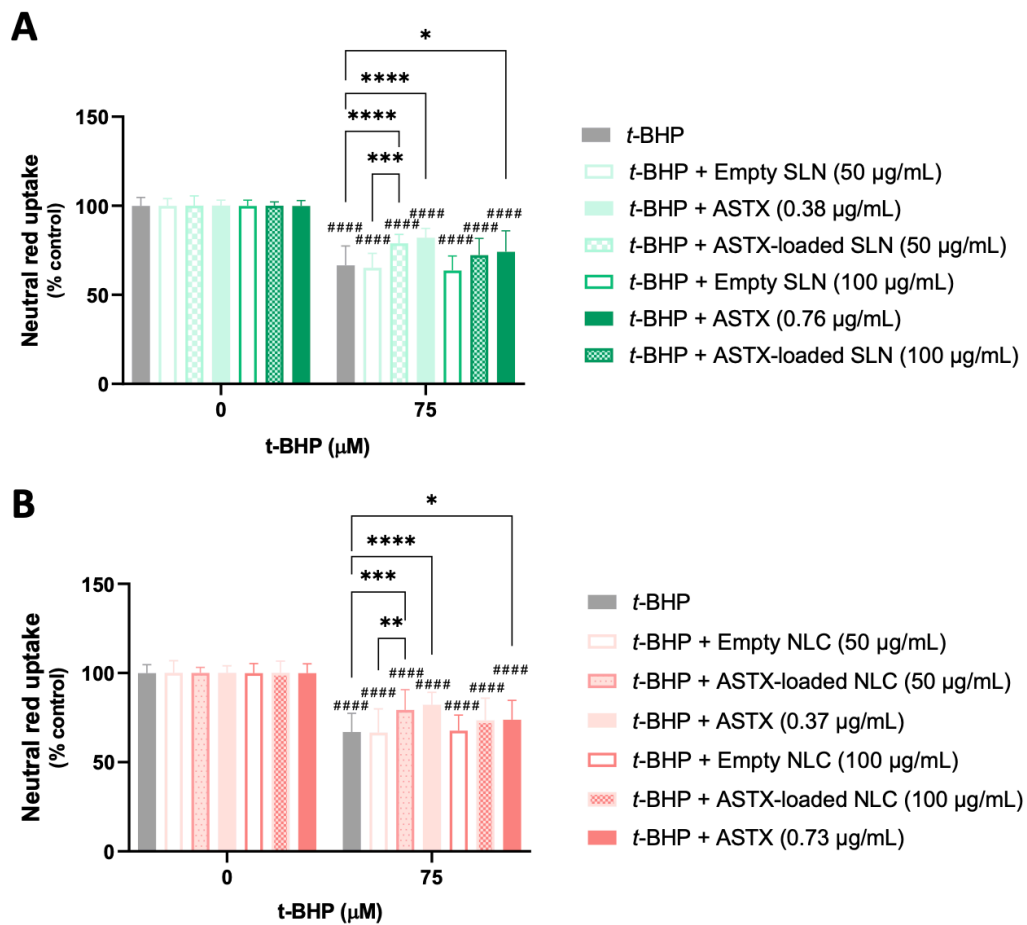


Figure 11. Potential neuroprotective effects of the developed SLN (A) and NLC (B) formulations (both empty and ASTX-loaded), and free ASTX, against *t*-BHP-induced cytotoxicity, evaluated in differentiated SH-SY5Y cells, by the neutral red uptake assay, 24 h after exposure to the aggressor. Results are expressed as mean + SD from 4 independent experiences, performed in triplicate. Statistical comparisons were made using two-way ANOVA followed by the Tukey's multiple comparisons test [##### $p < 0.0001$ versus control cells (0 μM *t*-BHP); * $p < 0.05$; ** $p < 0.01$; * $p < 0.001$; **** $p < 0.0001$, for comparison between formulations, ASTX and *t*-BHP alone, at each *t*-BHP concentration). In all cases, p values < 0.05 were considered significant.**

The overall analysis of the results obtained in this study (figure 11), clearly showed that ASTX-loaded SLN and ASTX-loaded NLC formulations, when tested at the smallest concentration of 50 μg/mL significantly protected differentiated SH-SY5Y cells against *t*-BHP induced cell death. Furthermore, free ASTX was capable to counteract *t*-BHP-mediated oxidative stress, significantly increasing cell viability at all concentrations tested. Given the lack of significant protection by the empty SLN and NLC formulations, the obtained results can be exclusively attributed to the ASTX antioxidant activity, which has the capability of neutralizing the deleterious effect of ROS.

Therefore, the developed ASTX-loaded SLN and ASTX-loaded NLC formulations showed encouraging antioxidant effects, with a protective effect against ROS-induced neurotoxicity and, for that reason, deserve to be further tested for their therapeutic potential in the management neurodegenerative diseases.

6.4.3. Protective effects against MPP⁺-induced cytotoxicity

One of the main hallmarks of PD is the loss of dopaminergic neurons at the *substantia nigra*. It is possible to experimentally replicate this neurodegenerative disease, *in vivo*, and *in vitro* models, using MPP⁺, the active metabolite of MPTP. This toxin is capable of brutally harming neuronal cells, provoking cell death, leading to a condition that closely mimics PD (175-178). MPP⁺ inhibits the complex I of the mitochondrial electron transport chain, inducing to ROS production and ATP depletion, this way changing the mitochondrial membrane potential and, consequently inducing apoptosis and dopaminergic neuron death (170, 176-178, 211, 212). Moreover, MPP⁺-induced dopaminergic cytotoxicity has been frequently used to explore the potential of drugs as neuroprotective agents, *in vitro* and *in vivo* (170, 211, 213-216). Several natural compounds, including natural antioxidants, have shown encouraging neuroprotective capabilities against MPP⁺-induced cytotoxicity being potential therapeutic strategies for neurodegenerative diseases, particularly for PD (170, 176, 213, 216, 217). Indeed, natural antioxidants such as anthocyanins, melatonin, tectorigenin, or ASTX itself have already shown protective effects against the cytotoxicity induced by the MPP⁺ neurotoxin in SH-SY5Y cells (116, 214-216, 218).

In figure 12 are illustrated the results obtained in the assessment of potential neuroprotective effects of both SLN and NLC, ASTX-loaded and empty, and the respective concentrations of ASTX alone, against MPP⁺-induced cytotoxicity. According to the obtained results, when tested alone, MPP⁺ (1000 μ M) significantly reduced the viability of differentiated SH-SY5Y cells to 78.9%, 24 hours after exposure, and when compared to control cells (0 μ M). When the exposure to the chemical aggressor occurred in the presence of 50 or 100 μ g/mL empty SLN, no significant protective effects were observed (cell viability of 82.1% and 80.1%, respectively). However, for ASTX- loaded SLN, only when tested at the lowest concentration (50 μ g/mL), was capable of significantly counteracting MPP⁺-induced cell death, significantly increasing cell viability to 82.5%. When ASTX alone was tested (0.38 and 0.76 μ g/mL), significant protective effects were also observed for both tested concentrations, with NR uptake significantly increasing to 85.7% and 85.4%, respectively.

When cell viability was assessed upon exposure to MPP⁺ in the presence of 50 or 100 μ g/mL empty NLC, no significant protective effects were observed (cell viability of 82.0% and 80.7% was obtained, respectively). However, when the exposure to the chemical aggressor occurred in the presence of the ASTX-loaded NLC (50 and 100 μ g/mL) the viability significantly ascended to 85.8% and 85.9%, respectively, thus demonstrating the ASTX-loaded NLC-mediated protection against MPP⁺-induced cell damage. When free

ASTX was tested (0.37 and 0.73 $\mu\text{g/mL}$), and in agreement with the results obtained for the SLN formulations, ASTX was capable of counteract MPP⁺-induced cytotoxicity, significantly increasing cell viability to 85.5% and 84.6%, respectively.

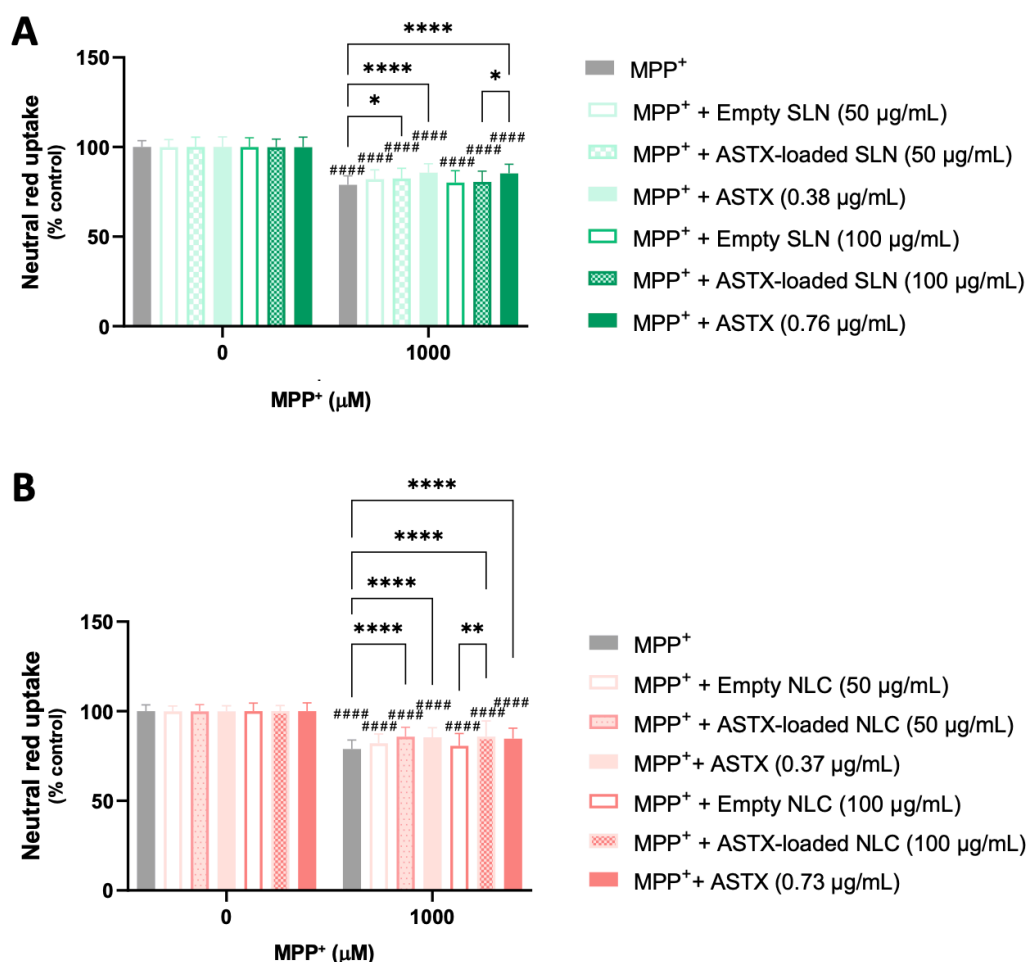


Figure 12. Potential neuroprotective effects of the developed SLN (A) and NLC (B) formulations (both empty and ASTX-loaded), and free ASTX, against MPP⁺-induced cytotoxicity, evaluated in differentiated SH-SY5Y cells, by the neutral red uptake assay, 24 h after exposure to the aggressor. Results are expressed as mean + SD from 6 independent experiences, performed in triplicate. Statistical comparisons were made using two-way ANOVA followed by the Tukey's multiple comparisons test [#### $p < 0.0001$ versus control cells (0 μM MPP⁺); * $p < 0.05$; ** $p < 0.01$; **** $p < 0.0001$, for comparison between formulations, ASTX and MPP⁺ alone, at each t -BHP concentration). In all cases, p values < 0.05 were considered significant.

The overall analysis of the results obtained in the present study (figure 12), clearly demonstrated that ASTX-loaded SLN, only when tested at the smallest concentration (50 $\mu\text{g/mL}$) was capable of slightly, but significantly reduce the cytotoxicity induced by the MPP⁺ toxin, 24 h after exposure. In what concerns to the ASTX- loaded NLC (50 and 100 $\mu\text{g/mL}$), remarkable protective effects were observed for both tested concentrations, clearly demonstrating the capacity of ASTX- loaded NLC to counteract MPP⁺-mediated cell death. Similar results were obtained for the free ASTX, with significant protective effects being

observed at all tested concentrations. As no protective effects were observed upon exposure to empty SLN or NLC, the obtained positive outcomes for ASTX- loaded NLC and ASTX-loaded SLN in counteracting the deleterious effects induced by this harmful neurotoxin can be exclusively attributed to the antioxidant potential of ASTX. Therefore, given the encouraging antioxidant effects of the developed ASTX-loaded SLN and ASTX-loaded NLC formulations, with remarkably protective effect against MPP⁺-induced neurotoxicity, these nanosystems deserve to be further tested for their therapeutic potential in the management neurodegenerative diseases, in particular for PD.

6.5. Method validation

6.5.1. Chromatographic conditions

A preliminary literature research was made to find HPLC methods currently available for ASTX quantification, in order to select the ideal conditions to perform the analysis (188, 219-222). During preliminary studies, the simplest mobile phase and IS found in the literature were selected. According to the results obtained during these preliminary studies, it was possible to obtain chromatograms with peaks with high resolution for both compounds, and a good chromatographic separation between ASTX and IS was also obtained in a 20-minute run. Therefore, the chromatographic conditions selected for the present work were a mobile phase composed of methanol: water (97:3 v/v); a volume of injection of 20 μ L; a flow rate of 1 mL/minute; and a run time of 20 minutes. Under these chromatographic conditions, a retention time of 5.31 and 16.57 minutes was obtained for ASTX and IS, respectively (Figure 13).

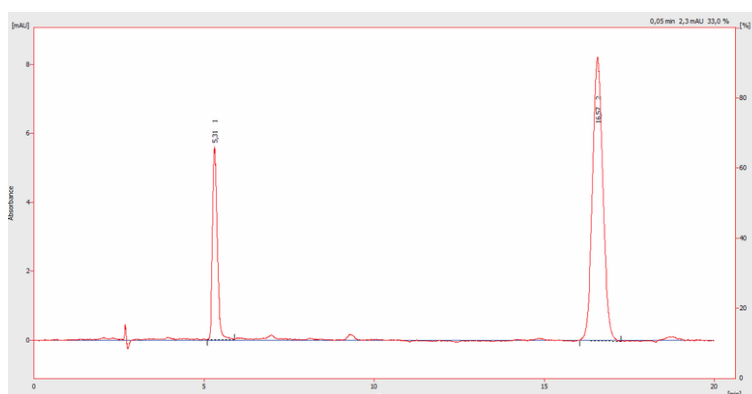


Figure 13. Chromatogram obtained after injection (20 μ L) of a 5 ug/mL ASTX standard spiked with 1 ug/mL of IS.

Afterwards, the method was validated according to the FDA (182) and ICH guidelines (183) for the following parameters: specificity, linearity, accuracy, precision (inter and intra-

day), LOD and LOQ, and stability. A proof of applicability of the validated method was performed through the quantification of the unloaded ASTX after NLC and SLN preparation (to assess EE indirectly), and in the evaluation of the ASTX intracellular uptake in differentiated SH-SY5Y cells.

6.5.2. Specificity

The specificity is assessed to confirm the ability of the developed method to detect and quantify the analyte in study in the presence of interferents, as degradants, impurities or matrix components, and other compounds (223). Therefore, to initially evaluate potential chromatographic interferences, blank samples (which correspond to methanol with IS, and without ASTX) were analyzed. No interference peaks were detected in the retention time of the analyte under study (Figure 14).

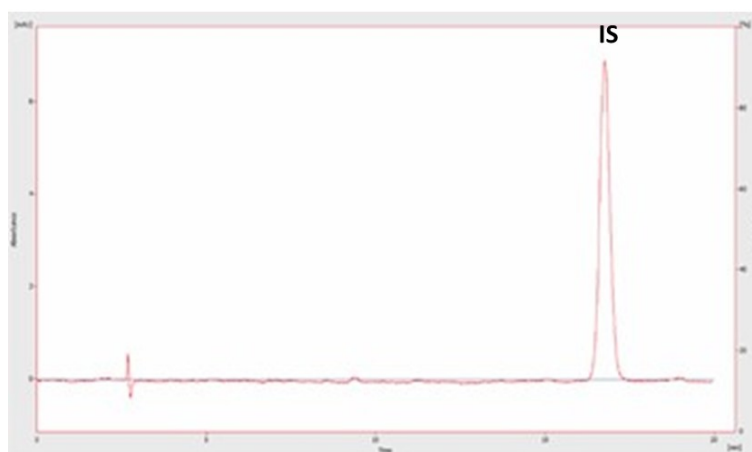


Figure 14. Chromatogram obtained after injection (20 μ L) of a blank methanol sample spiked with 1 μ g/mL of IS.

Aware that the chosen solvent was methanol and no interferent peaks were expected, specificity was further evaluated through the analysis of empty SLN and NLC formulations, submitted to the same extraction procedure used for the determination of EE. As depicted in Figure 15, after injecting the supernatant of both empty SLN and NLC formulations, no interference peaks were detected in the ASTX retention time, confirming that the method is specific for ASTX analysis and quantification, in the present experimental conditions.

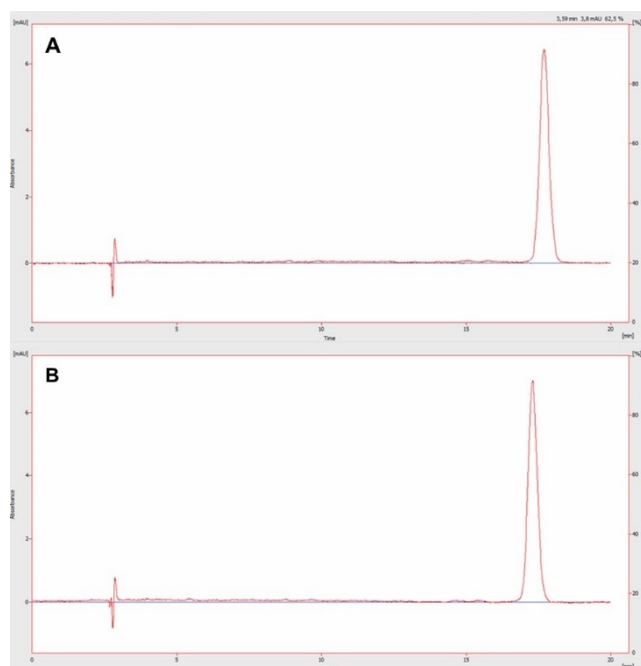


Figure 15. Chromatogram obtained after injection (20 μ L) of the supernatant of empty SLN (A) and empty NLC (B) formulations, previous submitted to the extraction protocol used for the evaluation of the formulations EE, and further spiked with 1 μ g/mL of IS.

Additionally, as the method was also applied to the assessment of the cellular uptake of ASTX, methanolic extracts of differentiated SH-SY5Y cells exposed to both empty SLN and NLC formulations, for 24 hours, were also analyzed in the search for potential interference peaks resulting from cellular constituents (Figure 16).

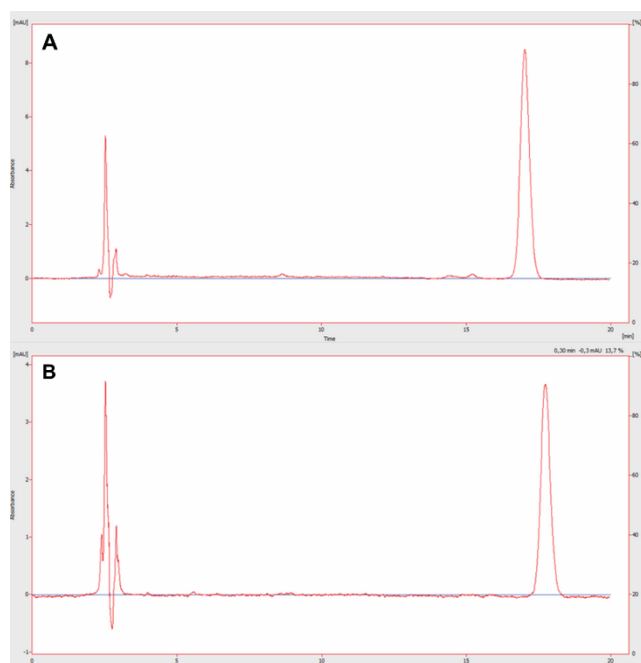


Figure 16. Chromatogram obtained after injection (20 μ L) of methanolic extracts of differentiated SH-SY5Y cell exposed to both empty SLN (A) and NLC (B) formulations, for 24 hours, and further spiked with 1 μ g/mL of IS.

Overall, all the obtained results clearly demonstrated that, in the present experimental conditions, the developed HPLC-UV method is specific for ASTX detection and quantification.

6.5.3. Linearity

The ability to obtain analytical results directly proportional to the concentration of the analyte in study in a given sample, within a certain concentration range, is designed as the linearity of the analytical method. The linearity is assessed by the linear regression between the signal obtained from the peak area (Y) and the nominal standard concentration (X) (223). In this work, as an IS was used, the ratio of ASTX peak area/ IS peak area (Y) and the nominal standard concentration (X) of seven ASTX calibrators were used to estimate the linear regression and the coefficient of determination (r^2).

Five ASTX calibration curves were independently prepared in five different days, and the regression analysis of the calibration data shows satisfactory linearity over the tested concentration range (0.01 to 7.5 $\mu\text{g/mL}$), further confirmed by the high r^2 coefficient (higher than 0.99). The linearity data for ASTX is presented in Table 5 and the equations of the five calibration curves along with the curves obtained are presented in Figure 17.

Table 5. Calibration curves obtained after preparation and injection (20 μL) of seven ASTX standards (0 - 7.5 $\mu\text{g/mL}$) spiked with 1 $\mu\text{g/mL}$ IS. Five ASTX calibration curves were prepared and analyzed in five different days.

COMPOUND	EQUATION	R^2	95% CONFIDENCE INTERVALS		
			Slope	Y-intercept	X-intercept
ASTX	$Y = 0.8988 \cdot X + 0.02842$	0.9911	0.8007 to 0.9970	-0.3085 to 0.3653	-0.4363 to 0.3235
N=5					

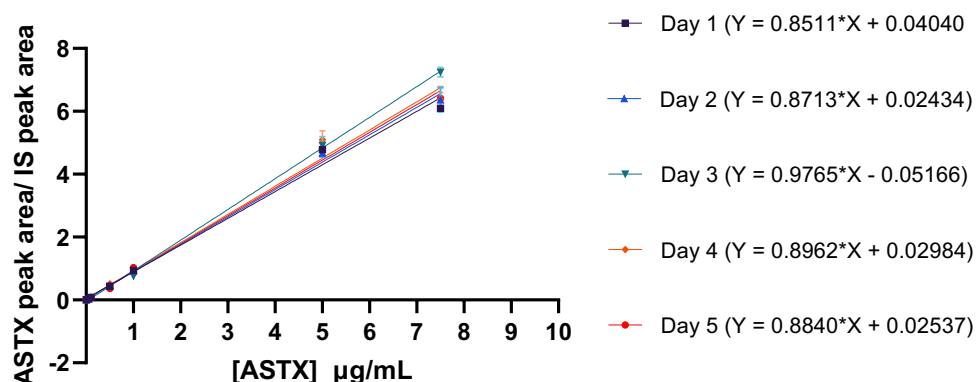


Figure 17. Calibration curves obtained from the analysis of seven ASTX calibrators

6.5.4. Accuracy of the method

The proximity between the nominal concentration and the experimental concentration expresses the accuracy of the method (223). The accuracy was assessed using ASTX calibrator with three different concentrations of the analyte in study (low, medium, high: 0.5, 1.0, 5.0 µg/mL, for this work). For each concentration, five independent replicates were prepared, and injected in the HPLC-UV apparatus. The accuracy % was calculated using equation 2.

The obtained results allowed to obtain accuracy values ranging between 95.604 and 108.783 % (Table 6). These values are within the proposed acceptance limits for this parameter ($100 \pm 15\%$), thus demonstrating that the developed method is accurate for ASTX quantification (182, 183).

Table 6. Accuracy of the developed HPLC-UV method evaluated at 3 levels of ASTX concentration (low, medium, high) within the linear concentration range.

Compound	[nominal] µg/mL	[experimental] µg/mL	Accuracy (%)
ASTX	0.5	0.496 ± 0.0195	99.14 ± 3.89
	1.0	1.088 ± 0.0834	108.78 ± 8.34
	5.0	4.780 ± 0.3912	95.60 ± 7.82

N=5

6.5.5. Precision

The closeness of agreement between a series of measurements is termed precision, evaluated in terms of CV%, which needs to be equal or less than 15% for the method to be accepted as precise (182, 183). The precision of a method can be evaluated at three distinct levels: repeatability, intermediate precision and reproducibility. In this work, only repeatability and intermediate precision were evaluated. Repeatability represents the precision under the same operating conditions and during a short period of time and is also called intra-day precision. Intermediate precision represents variations inside laboratories, such as different days and is also termed inter-day precision. The precision between different laboratories (reproducibility) was not evaluated in this work.

When the analysis is performed by the same analyst, with the same instrument, in the same laboratory, during a short period of time, it is possible to assess intra-day precision for both equipment and the complete method. Moreover, and in a given laboratory, it is also possible to assess inter-day precision, and this parameter was also evaluated for both the equipment and the entire method.

The obtained CV% values showed an acceptable intra-day precision for both equipment and method, which was always below 1.71% and 8.32%, respectively (Table 7). The CV% values obtained for the inter-day precision of the equipment and method were below 5.04% and 10.38% respectively, which further demonstrates that the developed method complies with the CV value considered as acceptable for a method to have a good intra-day precision (Table 7). Overall, the obtained results clearly demonstrated that the developed HPLC-UV method is precise for the quantification of ASTX.

Table 7. Inter-day and intra-day precision of the developed HPLC-UV method evaluated at 3 levels of ASTX concentration (low, medium, high) within the linear concentration range.

Compound	Concentration (µg/mL)	Inter-day Precision (CV%)		Intra-day Precision (CV%)		LOD (ng/mL)	LOQ (ng/mL)
		Equipment	Method	Equipment	Method		
ASTX	0.5	2.01	10.38	1.71	4.28	2.2	6.9
	1.0	5.04	10.23	0.97	8.32		
	5.0	1.56	8.75	1.65	8.24		

N=5 for all parameters except LOD and LOQ (N=20)

6.5.6. Sensitivity of the method: LOQ and LOD

The smallest concentration detected by an equipment defines the LOD. However, in this case, it is only possible to deduce the presence of the analyte, but not to quantify it as an exact value. The smallest concentration measured by an equipment that can be quantitatively detected with precision and accuracy defines the LOQ (223).

The LOQ and LOD values were calculated through the analysis of twenty blank samples and are presented on Table 7 (LOD of 2.2 ng/mL and LOQ of 6.9 ng/mL). This values show that the developed HPLC-UV method was sensitive for detection and quantification of ASTX, allowing to the detection/quantification of the analyte in the low ng/mL concentration range.

6.5.7. Stability

Some alterations can happen to the analyte in study, so it is important to assess his stability during storage periods and to be sure that the samples remain usable. For that, different conditions of storage can be evaluated, as an example, different storage temperatures. In this work, four different temperatures of storage were tested (20°C, 4 °C, -20 °C, -80 °C). The comparison was made between the results (ratio of ASTX peak area/ IS peak area) obtained in day 1 (preparation day) and the results obtaining after sample storage, at different temperatures, for seven days.

The % variation between the initial ASTX peak area/ IS peak area (day 1) and the ratio obtained after sample injection after storage under different temperatures (day 7) was assessed and is shown in **Table 8**. The obtained results suggest that the difference between values obtained in first day and in the last day of storage is low (under 10%), apart from room temperature storage, where it ASTX demonstrated a lower stability, with the analytical signal decreasing nearly 15%, when compared to day 1. Given the stability results obtained for ASTX, it is recommendable that, if needed, the samples should be kept at low temperature storage conditions.

Table 8. ASTX stability evaluated after samples storage at different temperature for 7 days.

Compound	Concentration (µg/mL)	Storage temperature ^a			
		20°C	4 °C	-20 °C	-80 °C
ASTX	0.5	-11.59	-2.29	-0.10	2.42
	1.0	-14.54	-4.47	0.16	0.71
	5.0	-14.79	1.35	4.16	7.50
^a Data presented as % ASTX peak area/ IS peak area relative to day 1					

7. Conclusions and future perspectives

In this work, it was possible to develop two ASXT-loaded SLN and ASXT-loaded NLC formulations with adequate size, PDI, ZP and EE for nose-to-brain administration.

A HPLC-UV method was developed and validated for the detection and quantification of ASTX. According to the validation parameters, the method was linear within the 0 – 7.5 µg/mL ASTX concentration range, demonstrated an excellent accuracy and precision and a remarkable sensitivity, with very low LOQ and LOD values being obtained. Additionally, the validated method was successfully applied to assess the EE and to determine the uptake of ASTX in differentiated SH-SY5Y cells.

In vitro studies in differentiated SH-SY5Y cells and in RPMI 2650 cells demonstrated that the developed formulations were safe for concentrations up to 100 µg/mL.

In what concern to the neuroprotection studies, the results obtained in this work showed that the developed ASTX-loaded SLN and ASTX-loaded NLC formulations were capable of counteracting some of the pathophysiological mechanisms underlying neurodegeneration, including iron overload and oxidative stress. However, when compared to the ASTX-loaded SLN, the ASTX-loaded NLC formulation showed more efficient neuroprotective effects against the cytotoxicity induced by the tested aggressors. Nevertheless, it is mandatory to perform more studies to confirm this potential of neuroprotection, such as the determination of GSH intracellular levels, evaluation of protective effects against lipid peroxidation, quantification of intracellular ROS levels, or the evaluation of the activity of antioxidant enzymes, such as SOD, AChE, and catalase, and to further elucidate the mechanism(s) underlying the observed neuroprotection. Moreover, and considering the intranasal route proposed for the tested formulations, permeability studies in RPMI 2650 cells should be performed to confirm the suitability of the developed formulations for nose-to-brain administration.

In conclusion, knowing that oxidative stress is related to several neurodegenerative diseases, and considering the results of the antioxidant potential observed for ASTX-loaded NLC formulations, the use of this innovative formulation may improve the treatment of these diseases, although *in vivo* experiments, in animals' models, and clinical studies should be performed to further confirm this application.

8. References

1. Costa CP, Moreira JN, Sousa Lobo JM, Silva AC. Intranasal delivery of nanostructured lipid carriers, solid lipid nanoparticles and nanoemulsions: A current overview of in vivo studies. *Acta Pharmaceutica Sinica B*. 2021;11(4):925-40.
2. Livingston G, Huntley J, Sommerlad A, Ames D, Ballard C, Banerjee S, et al. Dementia prevention, intervention, and care: 2020 report of the Lancet Commission. *Lancet*. 2020;396(10248):413-46.
3. Checkoway H, Lundin JI, Kelada SN. Neurodegenerative diseases. *IARC Sci Publ*. 2011(163):407-19.
4. Choonara YE, Pillay V, Du Toit LC, Modi G, Naidoo D, Ndesendo VMK, et al. Trends in the Molecular Pathogenesis and Clinical Therapeutics of Common Neurodegenerative Disorders. *International Journal of Molecular Sciences*. 2009;10(6):2510-57.
5. Rapp T, Chauvin P, Costa N, Molinier L. 42Health economic considerations in neurodegenerative disorders. In: Saba L, editor. *Imaging in Neurodegenerative Disorders*: Oxford University Press; 2015. p. 0.
6. Lamprey RNL, Chaulagain B, Trivedi R, Gothwal A, Layek B, Singh J. A Review of the Common Neurodegenerative Disorders: Current Therapeutic Approaches and the Potential Role of Nanotherapeutics. *International Journal of Molecular Sciences*. 2022;23(3):1851.
7. Oboudiyat C, Glazer H, Seifan A, Greer C, Isaacson RS. Alzheimer's disease. *Semin Neurol*. 2013;33(4):313-29.
8. Rabinovici GD. Late-onset Alzheimer Disease. *Continuum (Minneapolis)*. 2019;25(1):14-33.
9. Fauci AS. *Harrison's principles of internal medicine*. 21 ed. New York: McGraw-Hill, Health Professions Division; 2022.
10. Eratne D, Loi SM, Farrand S, Kelso W, Velakoulis D, Looi JC. Alzheimer's disease: clinical update on epidemiology, pathophysiology and diagnosis. *Australas Psychiatry*. 2018;26(4):347-57.
11. Cummings JL, Tong G, Ballard C. Treatment Combinations for Alzheimer's Disease: Current and Future Pharmacotherapy Options. *Journal of Alzheimer's disease : JAD*. 2019;67(3):779-94.
12. Beitz JM. Parkinson's disease: a review. *Front Biosci (Schol Ed)*. 2014;6(1):65-74.
13. Dorsey ER, Constantinescu R, Thompson JP, Biglan KM, Holloway RG, Kieburtz K, et al. Projected number of people with Parkinson disease in the most populous nations, 2005 through 2030. *Neurology*. 2007;68(5):384.
14. Dorsey ER, Constantinescu R, Thompson JP, Biglan KM, Holloway RG, Kieburtz K, et al. Projected number of people with Parkinson disease in the most populous nations, 2005 through 2030. *Neurology*. 2007;68(5):384-6.
15. Brown RH, Al-Chalabi A. Amyotrophic Lateral Sclerosis. *N Engl J Med*. 2017;377(2):162-72.
16. Wang T, Tomas D, Perera ND, Cuic B, Luikinga S, Viden A, et al. Ferroptosis mediates selective motor neuron death in amyotrophic lateral sclerosis. *Cell Death Differ*. 2022;29(6):1187-98.
17. Meyer T. [Amyotrophic lateral sclerosis (ALS) - diagnosis, course of disease and treatment options]. *Dtsch Med Wochenschr*. 2021;146(24-25):1613-8.

18. Jaiswal MK. Riluzole and edaravone: A tale of two amyotrophic lateral sclerosis drugs. *Med Res Rev.* 2019;39(2):733-48.
19. Bensimon G, Lacomblez L, Meininger V. A controlled trial of riluzole in amyotrophic lateral sclerosis. ALS/Riluzole Study Group. *N Engl J Med.* 1994;330(9):585-91.
20. Lacomblez L, Bensimon G, Leigh PN, Guillet P, Powe L, Durrleman S, et al. A confirmatory dose-ranging study of riluzole in ALS. ALS/Riluzole Study Group-II. *Neurology.* 1996;47(6 Suppl 4):S242-50.
21. Abe K, Aoki M, Tsuji S, Itoyama Y, Sobue G, Togo M, et al. Safety and efficacy of edaravone in well defined patients with amyotrophic lateral sclerosis: a randomised, double-blind, placebo-controlled trial. *The Lancet Neurology.* 2017;16(7):505-12.
22. Watanabe K, Tanaka M, Yuki S, Hirai M, Yamamoto Y. How is edaravone effective against acute ischemic stroke and amyotrophic lateral sclerosis? *J Clin Biochem Nutr.* 2018;62(1):20-38.
23. Sawada H. Clinical efficacy of edaravone for the treatment of amyotrophic lateral sclerosis. *Expert Opinion on Pharmacotherapy.* 2017;18(7):735-8.
24. Johnson SA, Fang T, De Marchi F, Neel D, Van Weehaeghe D, Berry JD, et al. Pharmacotherapy for Amyotrophic Lateral Sclerosis: A Review of Approved and Upcoming Agents. *Drugs.* 2022;82(13):1367-88.
25. van den Heuvel MP, Sporns O. Network hubs in the human brain. *Trends Cogn Sci.* 2013;17(12):683-96.
26. Pino A, Fumagalli G, Bifari F, Decimo I. New neurons in adult brain: distribution, molecular mechanisms and therapies. *Biochem Pharmacol.* 2017;141:4-22.
27. Ganat YM, Silbereis J, Cave C, Ngu H, Anderson GM, Ohkubo Y, et al. Early postnatal astroglial cells produce multilineage precursors and neural stem cells in vivo. *J Neurosci.* 2006;26(33):8609-21.
28. Przedborski S, Vila M, Jackson-Lewis V. Neurodegeneration: what is it and where are we? *J Clin Invest.* 2003;111(1):3-10.
29. Hoover BR, Reed MN, Su J, Penrod RD, Kotilinek LA, Grant MK, et al. Tau mislocalization to dendritic spines mediates synaptic dysfunction independently of neurodegeneration. *Neuron.* 2010;68(6):1067-81.
30. Milnerwood AJ, Raymond LA. Early synaptic pathophysiology in neurodegeneration: insights from Huntington's disease. *Trends Neurosci.* 2010;33(11):513-23.
31. Scott DA, Tabarean I, Tang Y, Cartier A, Masliah E, Roy S. A pathologic cascade leading to synaptic dysfunction in alpha-synuclein-induced neurodegeneration. *J Neurosci.* 2010;30(24):8083-95.
32. Kovacs GG. Molecular pathology of neurodegenerative diseases: principles and practice. *Journal of Clinical Pathology.* 2019;72(11):725-35.
33. Borchardt JK. The Beginnings of Drug Therapy: Ancient Mesopotamian Medicine. *Drug News Perspect.* 2002;15(3):187-92.
34. Rehman MU, Wali AF, Ahmad A, Shakeel S, Rasool S, Ali R, et al. Neuroprotective Strategies for Neurological Disorders by Natural Products: An update. *Curr Neuropharmacol.* 2019;17(3):247-67.
35. Takahashi RH, Nagao T, Gouras GK. Plaque formation and the intraneuronal accumulation of β -amyloid in Alzheimer's disease. *Pathol Int.* 2017;67(4):185-93.

36. Williams P, Sorribas A, Howes MJ. Natural products as a source of Alzheimer's drug leads. *Nat Prod Rep*. 2011;28(1):48-77.
37. Cai Z, Wang C, Yang W. Role of berberine in Alzheimer's disease. *Neuropsychiatr Dis Treat*. 2016;12:2509-20.
38. Ahmad Fazili N, Naeem A, Md. Ashraf G, Hua Gan S, A. Kamal M. Therapeutic Interventions for the Suppression of Alzheimer's Disease: Quest for a Remedy. *Current Drug Metabolism*. 2015;16(5):346-53.
39. Szeto JY, Lewis SJ. Current Treatment Options for Alzheimer's Disease and Parkinson's Disease Dementia. *Curr Neuropharmacol*. 2016;14(4):326-38.
40. Kumar GP, Khanum F. Neuroprotective potential of phytochemicals. *Pharmacogn Rev*. 2012;6(12):81-90.
41. Yang F, Lim GP, Begum AN, Ubeda OJ, Simmons MR, Ambegaokar SS, et al. Curcumin inhibits formation of amyloid beta oligomers and fibrils, binds plaques, and reduces amyloid in vivo. *J Biol Chem*. 2005;280(7):5892-901.
42. Noori T, Dehpour AR, Sureda A, Sobarzo-Sanchez E, Shirooie S. Role of natural products for the treatment of Alzheimer's disease. *European Journal of Pharmacology*. 2021;898:173974.
43. Behl C, Moosmann B. Antioxidant neuroprotection in Alzheimer's disease as preventive and therapeutic approach. *Free Radic Biol Med*. 2002;33(2):182-91.
44. Heo HJ, Lee CY. Strawberry and its anthocyanins reduce oxidative stress-induced apoptosis in PC12 cells. *J Agric Food Chem*. 2005;53(6):1984-9.
45. Carvalho FB, Gutierrez JM, Bohnert C, Zago AM, Abdalla FH, Vieira JM, et al. Anthocyanins suppress the secretion of proinflammatory mediators and oxidative stress, and restore ion pump activities in demyelination. *The Journal of Nutritional Biochemistry*. 2015;26(4):378-90.
46. Ma T, Tan M-S, Yu J-T, Tan L. Resveratrol as a therapeutic agent for Alzheimer's disease. *BioMed research international*. 2014;2014:350516-.
47. Jang MH, Piao XL, Kim HY, Cho EJ, Baek SH, Kwon SW, et al. Resveratrol oligomers from *Vitis amurensis* attenuate beta-amyloid-induced oxidative stress in PC12 cells. *Biol Pharm Bull*. 2007;30(6):1130-4.
48. Wang H, Joseph JA. Quantifying cellular oxidative stress by dichlorofluorescein assay using microplate reader. *Free Radic Biol Med*. 1999;27(5-6):612-6.
49. Chauhan N, Wang KC, Wegiel J, Malik MN. Walnut extract inhibits the fibrillization of amyloid beta-protein, and also defibrillizes its preformed fibrils. *Curr Alzheimer Res*. 2004;1(3):183-8.
50. Frydman-Marom A, Levin A, Farfara D, Benromano T, Scherzer-Attali R, Peled S, et al. Orally administered cinnamon extract reduces β -amyloid oligomerization and corrects cognitive impairment in Alzheimer's disease animal models. *PLoS One*. 2011;6(1):e16564.
51. Sancesario GM, Nuccetelli M, Cerri A, Zegeer J, Severini C, Ciotti MT, et al. Bromelain Degrades A β 1-42 Monomers and Soluble Aggregates: An In Vitro Study in Cerebrospinal Fluid of Alzheimer's Disease Patients. *Curr Alzheimer Res*. 2018;15(7):628-36.
52. Chauhan V, Chauhan A. Oxidative stress in Alzheimer's disease. *Pathophysiology*. 2006;13(3):195-208.
53. Liu X, Zhou J, Abid MDN, Yan H, Huang H, Wan L, et al. Correction: Berberine Attenuates Axonal Transport Impairment and Axonopathy Induced by Calyculin A in N2a Cells. *PLOS ONE*. 2016;11(3):e0152609.

54. Yu G, Li Y, Tian Q, Liu R, Wang Q, Wang JZ, et al. Berberine attenuates calyculin A-induced cytotoxicity and Tau hyperphosphorylation in HEK293 cells. *J Alzheimers Dis*. 2011;24(3):525-35.
55. Pereira DM, Ferreres F, Oliveira J, Valentão P, Andrade PB, Sottomayor M. Targeted metabolite analysis of *Catharanthus roseus* and its biological potential. *Food Chem Toxicol*. 2009;47(6):1349-54.
56. Grothe MJ, Schuster C, Bauer F, Heinsen H, Prudlo J, Teipel SJ. Atrophy of the cholinergic basal forebrain in dementia with Lewy bodies and Alzheimer's disease dementia. *J Neurol*. 2014;261(10):1939-48.
57. Teipel S, Heinsen H, Amaro E, Jr., Grinberg LT, Krause B, Grothe M. Cholinergic basal forebrain atrophy predicts amyloid burden in Alzheimer's disease. *Neurobiol Aging*. 2014;35(3):482-91.
58. Singh M, Kaur M, Kukreja H, Chugh R, Silakari O, Singh D. Acetylcholinesterase inhibitors as Alzheimer therapy: From nerve toxins to neuroprotection. *European Journal of Medicinal Chemistry*. 2013;70:165-88.
59. Lee YK, Yuk DY, Kim TI, Kim YH, Kim KT, Kim KH, et al. Protective effect of the ethanol extract of *Magnolia officinalis* and 4-O-methylhonokiol on scopolamine-induced memory impairment and the inhibition of acetylcholinesterase activity. *J Nat Med*. 2009;63(3):274-82.
60. Papandreou MA, Dimakopoulou A, Linardaki ZI, Cordopatis P, Klimis-Zacas D, Margarity M, et al. Effect of a polyphenol-rich wild blueberry extract on cognitive performance of mice, brain antioxidant markers and acetylcholinesterase activity. *Behav Brain Res*. 2009;198(2):352-8.
61. Vingtdeux V, Dreses-Werringloer U, Zhao H, Davies P, Marambaud P. Therapeutic potential of resveratrol in Alzheimer's disease. *BMC Neurosci*. 2008;9 Suppl 2(Suppl 2):S6-S.
62. Chonpathompikunlert P, Wattanathorn J, Muchimapura S. Piperine, the main alkaloid of Thai black pepper, protects against neurodegeneration and cognitive impairment in animal model of cognitive deficit like condition of Alzheimer's disease. *Food Chem Toxicol*. 2010;48(3):798-802.
63. Sayre LM, Smith MA, Perry G. Chemistry and biochemistry of oxidative stress in neurodegenerative disease. *Curr Med Chem*. 2001;8(7):721-38.
64. Mercuri NB, Bernardi G. The 'magic' of L-dopa: why is it the gold standard Parkinson's disease therapy? *Trends Pharmacol Sci*. 2005;26(7):341-4.
65. Callier S, Morissette M, Grandbois M, Pélaprat D, Di Paolo T. Neuroprotective properties of 17beta-estradiol, progesterone, and raloxifene in MPTP C57Bl/6 mice. *Synapse*. 2001;41(2):131-8.
66. Arevalo MA, Azcoitia I, Garcia-Segura LM. The neuroprotective actions of oestradiol and oestrogen receptors. *Nat Rev Neurosci*. 2015;16(1):17-29.
67. Bourque M, Dluzen DE, Di Paolo T. Neuroprotective actions of sex steroids in Parkinson's disease. *Front Neuroendocrinol*. 2009;30(2):142-57.
68. Cardona-Gomez P, Perez M, Avila J, Garcia-Segura LM, Wandosell F. Estradiol inhibits GSK3 and regulates interaction of estrogen receptors, GSK3, and beta-catenin in the hippocampus. *Mol Cell Neurosci*. 2004;25(3):363-73.
69. Garcia-Segura LM, Azcoitia I, DonCarlos LL. Neuroprotection by estradiol. *Prog Neurobiol*. 2001;63(1):29-60.

70. Mattson MP. Apoptosis in neurodegenerative disorders. *Nat Rev Mol Cell Biol.* 2000;1(2):120-9.
71. Yao M, Nguyen T-V, Pike C. Estrogen Regulates Bcl-w and Bim Expression: Role in Protection against -Amyloid Peptide-Induced Neuronal Death. *The Journal of neuroscience : the official journal of the Society for Neuroscience.* 2007;27:1422-33.
72. Morissette M, Al Sweidi S, Callier S, Di Paolo T. Estrogen and SERM neuroprotection in animal models of Parkinson's disease. *Mol Cell Endocrinol.* 2008;290(1-2):60-9.
73. Mythri RB, Bharath MM. Curcumin: a potential neuroprotective agent in Parkinson's disease. *Curr Pharm Des.* 2012;18(1):91-9.
74. Mythri RB, Harish G, Bharath MM. Therapeutic potential of natural products in Parkinson's disease. *Recent Pat Endocr Metab Immune Drug Discov.* 2012;6(3):181-200.
75. Rajeswari A, Sabesan M. Inhibition of monoamine oxidase-B by the polyphenolic compound, curcumin and its metabolite tetrahydrocurcumin, in a model of Parkinson's disease induced by MPTP neurodegeneration in mice. *Inflammopharmacology.* 2008;16(2):96-9.
76. Luo Y, Qin Z, Hong Z, Zhang X, Ding D, Fu JH, et al. Astragaloside IV protects against ischemic brain injury in a murine model of transient focal ischemia. *Neurosci Lett.* 2004;363(3):218-23.
77. Kandinov B, Giladi N, Korczyn AD. Smoking and tea consumption delay onset of Parkinson's disease. *Parkinsonism Relat Disord.* 2009;15(1):41-6.
78. Fijan S. Microorganisms with claimed probiotic properties: an overview of recent literature. *Int J Environ Res Public Health.* 2014;11(5):4745-67.
79. Ekstrand MI, Galter D. The MitoPark Mouse – An animal model of Parkinson's disease with impaired respiratory chain function in dopamine neurons. *Parkinsonism & Related Disorders.* 2009;15:S185-S8.
80. Padhi P, Abdalla A, Backes N, Zenitsky G, Jin H, Anantharam V, et al. Emerging Microbiome Genetic Engineering Technology for Stable Levodopa Delivery in Parkinson's Disease. *The FASEB Journal.* 2022;36(S1).
81. Ferrante RJ, Klein AM, Dedeoglu A, Beal MF. Therapeutic efficacy of EGb761 (Gingko biloba extract) in a transgenic mouse model of amyotrophic lateral sclerosis. *J Mol Neurosci.* 2001;17(1):89-96.
82. Nabavi SF, Daglia M, D'Antona G, Sobarzo-Sánchez E, Talas ZS, Nabavi SM. Natural compounds used as therapies targeting to amyotrophic lateral sclerosis. *Curr Pharm Biotechnol.* 2015;16(3):211-8.
83. Jiang F, DeSilva S, Turnbull J. Beneficial effect of ginseng root in SOD-1 (G93A) transgenic mice. *J Neurol Sci.* 2000;180(1-2):52-4.
84. Trieu VN, Uckun FM. Genistein is neuroprotective in murine models of familial amyotrophic lateral sclerosis and stroke. *Biochem Biophys Res Commun.* 1999;258(3):685-8.
85. Koh S-H, Kwon H, Kim KS, Kim J, Kim M-H, Yu H-J, et al. Epigallocatechin gallate prevents oxidative-stress-induced death of mutant Cu/Zn-superoxide dismutase (G93A) motoneuron cells by alteration of cell survival and death signals. *Toxicology.* 2004;202(3):213-25.
86. Yáñez M, Galán L, Matías-Guiu J, Vela A, Guerrero A, García AG. CSF from amyotrophic lateral sclerosis patients produces glutamate independent death of rat

motor brain cortical neurons: protection by resveratrol but not riluzole. *Brain Res.* 2011;1423:77-86.

87. Zhang Z, Peng D, Zhu H, Wang X. Experimental evidence of Ginkgo biloba extract EGB as a neuroprotective agent in ischemia stroke rats. *Brain Res Bull.* 2012;87(2-3):193-8.

88. Nakaya TA, Kita M, Kuriyama H, Iwakura Y, Imanishi J. Panax ginseng induces production of proinflammatory cytokines via toll-like receptor. *J Interferon Cytokine Res.* 2004;24(2):93-100.

89. Polkowski K, Mazurek AP. Biological properties of genistein. A review of in vitro and in vivo data. *Acta Pol Pharm.* 2000;57(2):135-55.

90. Khan N, Afaq F, Saleem M, Ahmad N, Mukhtar H. Targeting multiple signaling pathways by green tea polyphenol (-)-epigallocatechin-3-gallate. *Cancer Res.* 2006;66(5):2500-5.

91. Langcake P, Pryce RJ. The production of resveratrol by *Vitis vinifera* and other members of the Vitaceae as a response to infection or injury. *Physiological Plant Pathology.* 1976;9(1):77-86.

92. Mancuso R, del Valle J, Modol L, Martinez A, Granado-Serrano AB, Ramirez-Núñez O, et al. Resveratrol improves motoneuron function and extends survival in SOD1(G93A) ALS mice. *Neurotherapeutics.* 2014;11(2):419-32.

93. Vongthip W, Sillapachaiyaporn C, Kim K-W, Sukprasansap M, Tencomnao T. *Thunbergia laurifolia* Leaf Extract Inhibits Glutamate-Induced Neurotoxicity and Cell Death through Mitophagy Signaling. *Antioxidants.* 2021;10(11).

94. Warner DS, Sheng H, Batinić-Haberle I. Oxidants, antioxidants and the ischemic brain. *J Exp Biol.* 2004;207(Pt 18):3221-31.

95. Sindhi V, Gupta V, Sharma K, Bhatnagar S, Kumari R, Dhaka N. Potential applications of antioxidants – A review. *Journal of Pharmacy Research.* 2013;7(9):828-35.

96. Irshad M, Chaudhuri PS. Oxidant-antioxidant system: role and significance in human body. *Indian J Exp Biol.* 2002;40(11):1233-9.

97. Zhao X, Zhang X, Liu H, Zhu H, Zhu Y. Enzyme-assisted extraction of astaxanthin from *Haematococcus pluvialis* and its stability and antioxidant activity. *Food Science and Biotechnology.* 2019;28(6):1637-47.

98. Powtongsook S, Kaewpintong K, Shotipruk A, Pavasant P. Effect of superficial gas velocity on growth of the green microalga *Haematococcus pluvialis* in airlift photobioreactor. In: Rhee H-K, Nam I-S, Park JM, editors. *Studies in Surface Science and Catalysis.* 159: Elsevier; 2006. p. 481-4.

99. Martínez-Delgado AA, Khandual S, Villanueva-Rodríguez SJ. Chemical stability of astaxanthin integrated into a food matrix: Effects of food processing and methods for preservation. *Food Chemistry.* 2017;225:23-30.

100. Fassett RG, Coombes JS. Astaxanthin: A Potential Therapeutic Agent in Cardiovascular Disease. *Marine Drugs.* 2011;9(3):447-65.

101. Guerin M, Huntley ME, Olaizola M. *Haematococcus* astaxanthin: applications for human health and nutrition. *Trends in Biotechnology.* 2003;21(5):210-6.

102. Visioli F, Artaria C. Astaxanthin in cardiovascular health and disease: mechanisms of action, therapeutic merits, and knowledge gaps. *Food Funct.* 2017;8(1):39-63.

103. Østerlie M, Bjerkeng B, Liaaen-Jensen S. Plasma appearance and distribution of astaxanthin E/Z and R/S isomers in plasma lipoproteins of men after single dose administration of astaxanthin. *J Nutr Biochem.* 2000;11(10):482-90.

104. Mercke Odeberg J, Lignell A, Pettersson A, Höglund P. Oral bioavailability of the antioxidant astaxanthin in humans is enhanced by incorporation of lipid based formulations. *Eur J Pharm Sci.* 2003;19(4):299-304.
105. Coral-Hinostroza GN, Ytrestøyl T, Ruyter B, Bjerkeng B. Plasma appearance of unesterified astaxanthin geometrical E/Z and optical R/S isomers in men given single doses of a mixture of optical 3 and 3'R/S isomers of astaxanthin fatty acyl diesters. *Comp Biochem Physiol C Toxicol Pharmacol.* 2004;139(1-3):99-110.
106. Rüfer CE, Moeseneder J, Briviba K, Rechkemmer G, Bub A. Bioavailability of astaxanthin stereoisomers from wild (*Oncorhynchus* spp.) and aquacultured (*Salmo salar*) salmon in healthy men: a randomised, double-blind study. *British Journal of Nutrition.* 2008;99(5):1048-54.
107. Miki W. Biological functions and activities of animal carotenoids. *Pure and Applied Chemistry.* 1991;63(1):141-6.
108. Shimidzu N, Goto M, Miki W. Carotenoids as Singlet Oxygen Quenchers in Marine Organisms. *Fisheries science.* 1996;62(1):134-7.
109. Goto S, Kogure K, Abe K, Kimata Y, Kitahama K, Yamashita E, et al. Efficient radical trapping at the surface and inside the phospholipid membrane is responsible for highly potent antiperoxidative activity of the carotenoid astaxanthin. *Biochim Biophys Acta.* 2001;1512(2):251-8.
110. Stahl W, Sies H. Antioxidant activity of carotenoids. *Mol Aspects Med.* 2003;24(6):345-51.
111. Yang Y, Pham TX, Wegner CJ, Kim B, Ku CS, Park YK, et al. Astaxanthin lowers plasma TAG concentrations and increases hepatic antioxidant gene expression in diet-induced obesity mice. *Br J Nutr.* 2014;112(11):1797-804.
112. Yang Y, Seo JM, Nguyen A, Pham TX, Park HJ, Park Y, et al. Astaxanthin-rich extract from the green alga *Haematococcus pluvialis* lowers plasma lipid concentrations and enhances antioxidant defense in apolipoprotein E knockout mice. *J Nutr.* 2011;141(9):1611-7.
113. Xu J, Rong S, Gao H, Chen C, Yang W, Deng Q, et al. A Combination of Flaxseed Oil and Astaxanthin Improves Hepatic Lipid Accumulation and Reduces Oxidative Stress in High Fat-Diet Fed Rats. *Nutrients.* 2017;9(3):271.
114. Al-Amin MM, Akhter S, Hasan AT, Alam T, Nageeb Hasan SM, Saifullah ARM, et al. The antioxidant effect of astaxanthin is higher in young mice than aged: a region specific study on brain. *Metabolic Brain Disease.* 2015;30(5):1237-46.
115. Wu W, Wang X, Xiang Q, Meng X, Peng Y, Du N, et al. Astaxanthin alleviates brain aging in rats by attenuating oxidative stress and increasing BDNF levels. *Food Funct.* 2014;5(1):158-66.
116. Lee DH, Kim CS, Lee YJ. Astaxanthin protects against MPTP/MPP+-induced mitochondrial dysfunction and ROS production in vivo and in vitro. *Food Chem Toxicol.* 2011;49(1):271-80.
117. Liu X, Shibata T, Hisaka S, Osawa T. Astaxanthin inhibits reactive oxygen species-mediated cellular toxicity in dopaminergic SH-SY5Y cells via mitochondria-targeted protective mechanism. *Brain Res.* 2009;1254:18-27.
118. Al-Amin MM, Reza HM, Saadi HM, Mahmud W, Ibrahim AA, Alam MM, et al. Astaxanthin ameliorates aluminum chloride-induced spatial memory impairment and neuronal oxidative stress in mice. *European Journal of Pharmacology.* 2016;777:60-9.

119. Achar A, Myers R, Ghosh C. Drug Delivery Challenges in Brain Disorders across the Blood–Brain Barrier: Novel Methods and Future Considerations for Improved Therapy. *Biomedicines*. 2021;9(12):1834.
120. Anselmo AC, Mitragotri S. Nanoparticles in the clinic: An update. *Bioeng Transl Med*. 2019;4(3):e10143-e.
121. Müller RH, Mäder K, Gohla S. Solid lipid nanoparticles (SLN) for controlled drug delivery - a review of the state of the art. *European journal of pharmaceutics and biopharmaceutics : official journal of Arbeitsgemeinschaft fur Pharmazeutische Verfahrenstechnik eV*. 2000;50(1):161-77.
122. Wissing SA, Kayser O, Müller RH. Solid lipid nanoparticles for parenteral drug delivery. *Adv Drug Deliv Rev*. 2004;56(9):1257-72.
123. Joshi MD, Müller RH. Lipid nanoparticles for parenteral delivery of actives. *Eur J Pharm Biopharm*. 2009;71(2):161-72.
124. Dingler A, Gohla S. Production of solid lipid nanoparticles (SLN): scaling up feasibilities. *J Microencapsul*. 2002;19(1):11-6.
125. Müller RH, Rühl D, Runge S, Schulze-Forster K, Mehnert W. Cytotoxicity of solid lipid nanoparticles as a function of the lipid matrix and the surfactant. *Pharm Res*. 1997;14(4):458-62.
126. Nassimi M, Schleh C, Lauenstein HD, Hussein R, Lübbers K, Pohlmann G, et al. Low cytotoxicity of solid lipid nanoparticles in in vitro and ex vivo lung models. *Inhal Toxicol*. 2009;21 Suppl 1:104-9.
127. Mehnert W, Mäder K. Solid lipid nanoparticles: production, characterization and applications. *Adv Drug Deliv Rev*. 2001;47(2-3):165-96.
128. Olbrich C, Kayser O, Müller RH. Enzymatic Degradation of Dynasan 114 SLN – Effect of Surfactants and Particle Size. *Journal of Nanoparticle Research*. 2002;4(1):121-9.
129. Müller RH, Radtke M, Wissing SA. Solid lipid nanoparticles (SLN) and nanostructured lipid carriers (NLC) in cosmetic and dermatological preparations. *Adv Drug Deliv Rev*. 2002;54 Suppl 1:S131-55.
130. Müller RH, Radtke M, Wissing SA. Solid lipid nanoparticles (SLN) and nanostructured lipid carriers (NLC) in cosmetic and dermatological preparations. *Advanced Drug Delivery Reviews*. 2002;54:S131-S55.
131. Müller RH, Radtke M, Wissing SA. Nanostructured lipid matrices for improved microencapsulation of drugs. *Int J Pharm*. 2002;242(1-2):121-8.
132. Pardeike J, Hommoss A, Müller RH. Lipid nanoparticles (SLN, NLC) in cosmetic and pharmaceutical dermal products. *Int J Pharm*. 2009;366(1-2):170-84.
133. Patel D. DEVELOPMENT & SCREENING APPROACH FOR LIPID NANOPARTICLE: A REVIEW. *Int J Innovations Pharm Sci*. 2013;2:27-32.
134. Jennings V, Schäfer-Korting M, Gohla S. Vitamin A-loaded solid lipid nanoparticles for topical use: drug release properties. *J Control Release*. 2000;66(2-3):115-26.
135. Cunha S, Almeida H, Amaral MH, Lobo JMS, Silva AC. Intranasal lipid nanoparticles for the treatment of neurodegenerative diseases. *Current pharmaceutical design*. 2017.
136. Kapoor M, Cloyd JC, Siegel RA. A review of intranasal formulations for the treatment of seizure emergencies. *J Control Release*. 2016;237:147-59.
137. Kälviäinen R. Intranasal therapies for acute seizures. *Epilepsy Behav*. 2015;49:303-6.

138. Ugwoke MI, Agu RU, Verbeke N, Kinget R. Nasal mucoadhesive drug delivery: background, applications, trends and future perspectives. *Adv Drug Deliv Rev.* 2005;57(11):1640-65.
139. Dimova S, Brewster ME, Noppe M, Jorissen M, Augustijns P. The use of human nasal in vitro cell systems during drug discovery and development. *Toxicol In Vitro.* 2005;19(1):107-22.
140. Costa C, Moreira JN, Amaral MH, Sousa Lobo JM, Silva AC. Nose-to-brain delivery of lipid-based nanosystems for epileptic seizures and anxiety crisis. *Journal of controlled release : official journal of the Controlled Release Society.* 2019;295:187-200.
141. Shah B, Khunt D, Misra M, Padh H. Non-invasive intranasal delivery of quetiapine fumarate loaded microemulsion for brain targeting: Formulation, physicochemical and pharmacokinetic consideration. *Eur J Pharm Sci.* 2016;91:196-207.
142. Saraiva C, Praça C, Ferreira R, Santos T, Ferreira L, Bernardino L. Nanoparticle-mediated brain drug delivery: Overcoming blood-brain barrier to treat neurodegenerative diseases. *J Control Release.* 2016;235:34-47.
143. Cunha S, Forbes B, Sousa Lobo JM, Silva AC. Improving Drug Delivery for Alzheimer's Disease Through Nose-to-Brain Delivery Using Nanoemulsions, Nanostructured Lipid Carriers (NLC) and in situ Hydrogels. *Int J Nanomedicine.* 2021;16:4373-90.
144. Ahmad E, Lu Y, Qi J, Fan W, Ma Y, He H, et al. Evidence of nose-to-brain delivery of nanoemulsions: Cargoes but not vehicles. *Nanoscale.* 2016;9:1174-83.
145. Cunha S, Amaral MH, Lobo JMS, Silva AC. Lipid Nanoparticles for Nasal/Intranasal Drug Delivery. *Crit Rev Ther Drug Carrier Syst.* 2017;34(3):257-82.
146. Grassin-Delyle S, Buenestado A, Naline E, Faisy C, Blouquit-Laye S, Couderc LJ, et al. Intranasal drug delivery: an efficient and non-invasive route for systemic administration: focus on opioids. *Pharmacol Ther.* 2012;134(3):366-79.
147. Kumar A, Pandey AN, Jain SK. Nasal-nanotechnology: revolution for efficient therapeutics delivery. *Drug Deliv.* 2016;23(3):681-93.
148. Khan S, Baboota S, Ali J, Khan S, Narang RS, Narang JK. Nanostructured lipid carriers: An emerging platform for improving oral bioavailability of lipophilic drugs. *Int J Pharm Investig.* 2015;5(4):182-91.
149. Costa CP, Barreiro S, Moreira JN, Silva R, Almeida H, Sousa Lobo JM, et al. In Vitro Studies on Nasal Formulations of Nanostructured Lipid Carriers (NLC) and Solid Lipid Nanoparticles (SLN). *Pharmaceuticals (Basel).* 2021;14(8):711.
150. Hernando S, Herran E, Hernandez RM, Igartua M. Nanostructured Lipid Carriers Made of Ω -3 Polyunsaturated Fatty Acids: In Vitro Evaluation of Emerging Nanocarriers to Treat Neurodegenerative Diseases. *Pharmaceutics.* 2020;12(10).
151. Agrawal M, Saraf S, Pradhan M, Patel RJ, Singhvi G, Ajazuddin, et al. Design and optimization of curcumin loaded nano lipid carrier system using Box-Behnken design. *Biomed Pharmacother.* 2021;141:111919.
152. Sakamula R, Yata T, Thong-Asa W. Nanostructure lipid carriers enhance alpha-mangostin neuroprotective efficacy in mice with rotenone-induced neurodegeneration. *Metab Brain Dis.* 2022;37(5):1465-76.
153. Cunha S, Swedrowska M, Bellahnid Y, Xu Z, Sousa Lobo JM, Forbes B, et al. Thermosensitive in situ hydrogels of rivastigmine-loaded lipid-based nanosystems for nose-to-brain delivery: characterisation, biocompatibility, and drug deposition studies. *International Journal of Pharmaceutics.* 2022;620:121720.

154. Jojo GM, Kuppusamy G, De A, Karri V. Formulation and optimization of intranasal nanolipid carriers of pioglitazone for the repurposing in Alzheimer's disease using Box-Behnken design. *Drug Dev Ind Pharm*. 2019;45(7):1061-72.
155. Costa CP, Cunha S, Moreira JN, Silva R, Gil-Martins E, Silva V, et al. Quality by design (QbD) optimization of diazepam-loaded nanostructured lipid carriers (NLC) for nose-to-brain delivery: Toxicological effect of surface charge on human neuronal cells. *Int J Pharm*. 2021;607:120933.
156. Alam T, Pandit J, Vohora D, Aqil M, Ali A, Sultana Y. Optimization of nanostructured lipid carriers of lamotrigine for brain delivery: in vitro characterization and in vivo efficacy in epilepsy. *Expert Opinion on Drug Delivery*. 2015;12(2):181-94.
157. Singh AP, Saraf SK, Saraf SA. SLN approach for nose-to-brain delivery of alprazolam. *Drug Delivery and Translational Research*. 2012;2(6):498-507.
158. Vaz S, Silva R, Amaral MH, Martins E, Sousa Lobo JM, Silva AC. Evaluation of the biocompatibility and skin hydration potential of vitamin E-loaded lipid nanosystems formulations: In vitro and human in vivo studies. *Colloids and Surfaces B: Biointerfaces*. 2019;179:242-9.
159. Wightman R. An Overview of Cryo-Scanning Electron Microscopy Techniques for Plant Imaging. *Plants*. 2022;11(9):1113.
160. Iyer R, Arunagirinathan MA, Prabhu CS, Bellare J. An improved specimen loader for cryo-scanning electron microscopy. *Scanning*. 2005;27(3):141-6.
161. Silva A, Lopes C, Fonseca J, Soares M, Santos D, Souto EB, et al. Risperidone Release from Solid Lipid Nanoparticles (SLN): Validated HPLC Method and Modelling Kinetic Profile. *Current Pharmaceutical Analysis*. 2012;8:307-16.
162. Barbosa DJ, Capela JP, Silva R, Vilas-Boas V, Ferreira LM, Branco PS, et al. The mixture of "ecstasy" and its metabolites is toxic to human SH-SY5Y differentiated cells at in vivo relevant concentrations. *Archives of Toxicology*. 2014;88(2):455-73.
163. Presgraves SP, Ahmed T, Borwege S, Joyce JN. Terminally differentiated SH-SY5Y cells provide a model system for studying neuroprotective effects of dopamine agonists. *Neurotox Res*. 2004;5(8):579-98.
164. Mosmann T. Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. *Journal of Immunological Methods*. 1983;65(1):55-63.
165. Morgan DM. Tetrazolium (MTT) assay for cellular viability and activity. *Methods Mol Biol*. 1998;79:179-83.
166. van Meerloo J, Kaspers GJ, Cloos J. Cell sensitivity assays: the MTT assay. *Methods Mol Biol*. 2011;731:237-45.
167. Lowik CWGM, Alblas MJ, Vanderuit M, Papapoulos SE, Vanderpluijm G. Quantification of Adherent and Nonadherent Cells Cultured in 96-Well Plates Using the Supravital Stain Neutral Red. *Analytical Biochemistry*. 1993;213(2):426-33.
168. Repetto G, del Peso A, Zurita JL. Neutral red uptake assay for the estimation of cell viability/cytotoxicity. *Nature Protocols*. 2008;3(7):1125-31.
169. Jiang D, Li X, Williams R, Patel S, Men L, Wang Y, et al. Ternary complexes of iron, amyloid-beta, and nitrotriacetic acid: binding affinities, redox properties, and relevance to iron-induced oxidative stress in Alzheimer's disease. *Biochemistry*. 2009;48(33):7939-47.
170. Barreiro S, Silva B, Long S, Pinto M, Remião F, Sousa E, et al. Fiscalin Derivatives as Potential Neuroprotective Agents. *Pharmaceutics*. 2022;14(7):1456.

171. Chavarria D, Fernandes C, Aguiar B, Silva T, Garrido J, Remião F, et al. Insights into the Discovery of Novel Neuroprotective Agents: A Comparative Study between Sulfanylcinnamic Acid Derivatives and Related Phenolic Analogues. *Molecules*. 2019;24(23):4405.
172. Fernandes C, Pinto M, Martins C, Gomes MJ, Sarmento B, Oliveira PJ, et al. Development of a PEGylated-Based Platform for Efficient Delivery of Dietary Antioxidants Across the Blood–Brain Barrier. *Bioconjugate Chemistry*. 2018;29(5):1677-89.
173. Molina-Holgado F, Gaeta A, Francis PT, Williams RJ, Hider RC. Neuroprotective actions of deferiprone in cultured cortical neurones and SHSY-5Y cells. *Journal of Neurochemistry*. 2008;105(6):2466-76.
174. Cai L, Wang L-F, Pan J-P, Mi X-N, Zhang Z, Geng H-J, et al. Neuroprotective Effects of Methyl 3,4-Dihydroxybenzoate against TBHP-Induced Oxidative Damage in SH-SY5Y Cells. *Molecules*. 2016;21(8):1071.
175. Jantas D, Greda A, Golda S, Korostynski M, Grygier B, Roman A, et al. Neuroprotective effects of metabotropic glutamate receptor group II and III activators against MPP(+)-induced cell death in human neuroblastoma SH-SY5Y cells: The impact of cell differentiation state. *Neuropharmacology*. 2014;83:36-53.
176. Zhao Q, Ye J, Wei N, Fong C, Dong X. Protection against MPP+-induced neurotoxicity in SH-SY5Y cells by tormentic acid via the activation of PI3-K/Akt/GSK3 β pathway. *Neurochemistry International*. 2016;97:117-23.
177. Wang Y, Gao J, Miao Y, Cui Q, Zhao W, Zhang J, et al. Pinocembrin Protects SH-SY5Y Cells Against MPP+-Induced Neurotoxicity Through the Mitochondrial Apoptotic Pathway. *Journal of Molecular Neuroscience*. 2014;53(4):537-45.
178. Kim S-Y, Kim M-Y, Mo J-S, Park J-W, Park H-S. SAG protects human neuroblastoma SH-SY5Y cells against 1-methyl-4-phenylpyridinium ion (MPP+)-induced cytotoxicity via the downregulation of ROS generation and JNK signaling. *Neuroscience Letters*. 2007;413(2):132-6.
179. Kreft ME, Jerman UD, Lasič E, Lanišnik Rižner T, Hevir-Kene N, Peternel L, et al. The Characterization of the Human Nasal Epithelial Cell Line RPMI 2650 Under Different Culture Conditions and Their Optimization for an Appropriate in vitro Nasal Model. *Pharmaceutical Research*. 2015;32(2):665-79.
180. Pozzoli M, Ong HX, Morgan L, Sukkar M, Traini D, Young PM, et al. Application of RPMI 2650 nasal cell model to a 3D printed apparatus for the testing of drug deposition and permeation of nasal products. *Eur J Pharm Biopharm*. 2016;107:223-33.
181. Gonçalves VSS, Matias AA, Poejo J, Serra AT, Duarte CMM. Application of RPMI 2650 as a cell model to evaluate solid formulations for intranasal delivery of drugs. *International Journal of Pharmaceutics*. 2016;515(1):1-10.
182. Food, Drug Administration Department of H, Human S. Guidance for Industry : Bioanalytical Method Validation. <http://www.fda.gov/cder/Guidance/4252fnl.pdf>. 2001.
183. Harmonization I. Topic Q2 (R1) Validation of Analytical Procedures: Text and Methodology. 2005.
184. Costa CP, Cunha S, Moreira JN, Silva R, Gil-Martins E, Silva V, et al. Quality by design (QbD) optimization of diazepam-loaded nanostructured lipid carriers (NLC) for nose-to-brain delivery: Toxicological effect of surface charge on human neuronal cells. *International Journal of Pharmaceutics*. 2021;607:120933.
185. Cunha S, Costa CP, Loureiro JA, Alves J, Peixoto AF, Forbes B, et al. Double Optimization of Rivastigmine-Loaded Nanostructured Lipid Carriers (NLC) for Nose-to-

- Brain Delivery Using the Quality by Design (QbD) Approach: Formulation Variables and Instrumental Parameters. *Pharmaceutics*. 2020;12(7):599.
186. Geng Q, Zhao Y, Wang L, Xu L, Chen X, Han J. Development and Evaluation of Astaxanthin as Nanostructure Lipid Carriers in Topical Delivery. *AAPS PharmSciTech*. 2020;21(8):318.
 187. Khongkaew P, Chaemsawang W. Development and Characterization of Stingless Bee Propolis Properties for the Development of Solid Lipid Nanoparticles for Loading Lipophilic Substances. *Int J Biomater*. 2021;2021:6662867.
 188. Pereira A, Ramalho MJ, Silva R, Silva V, Marques-Oliveira R, Silva AC, et al. Vine Cane Compounds to Prevent Skin Cells Aging through Solid Lipid Nanoparticles. *Pharmaceutics*. 2022;14(2):240.
 189. Barichello JM, Morishita M, Takayama K, Nagai T. Encapsulation of hydrophilic and lipophilic drugs in PLGA nanoparticles by the nanoprecipitation method. *Drug Dev Ind Pharm*. 1999;25(4):471-6.
 190. Silva S, Marto J, Gonçalves L, Almeida AJ, Vale N. Formulation, Characterization and Evaluation against SH-SY5Y Cells of New Tacrine and Tacrine-MAP Loaded with Lipid Nanoparticles. *Nanomaterials (Basel)*. 2020;10(10).
 191. Li H, Chen Y, Deng Y, Wang Y, Ke X, Ci T. Effects of surface charge of low molecular weight heparin-modified cationic liposomes on drug efficacy and toxicity. *Drug Development and Industrial Pharmacy*. 2017;43(7):1163-72.
 192. Reichert CO, de Freitas FA, Sampaio-Silva J, Rokita-Rosa L, Barros PL, Levy D, et al. Ferroptosis Mechanisms Involved in Neurodegenerative Diseases. *Int J Mol Sci*. 2020;21(22).
 193. Remião F, Carmo H, Carvalho FD, Bastos ML. Inhibition of Glutathione Reductase by Isoproterenol Oxidation Products. *Journal of Enzyme Inhibition*. 1999;15(1):47-61.
 194. Mura CV, Delgado R, Aguirre P, Bacigalupo J, Núñez MT. Quiescence induced by iron challenge protects neuroblastoma cells from oxidative stress. *Journal of Neurochemistry*. 2006;98(1):11-9.
 195. Eid R, Arab NTT, Greenwood MT. Iron mediated toxicity and programmed cell death: A review and a re-examination of existing paradigms. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research*. 2017;1864(2):399-430.
 196. Zucca FA, Segura-Aguilar J, Ferrari E, Muñoz P, Paris I, Sulzer D, et al. Interactions of iron, dopamine and neuromelanin pathways in brain aging and Parkinson's disease. *Progress in Neurobiology*. 2017;155:96-119.
 197. Abdelsayed S, Ha Duong NT, Bureau C, Michel PP, Hirsch EC, El Hage Chahine JM, et al. Piperazine derivatives as iron chelators: a potential application in neurobiology. *BioMetals*. 2015;28(6):1043-61.
 198. Salvador GA, Uranga RM, Giusto NM. Iron and Mechanisms of Neurotoxicity. *International Journal of Alzheimer's Disease*. 2011;2011:720658.
 199. Cheignon C, Tomas M, Bonnefont-Rousselot D, Faller P, Hureau C, Collin F. Oxidative stress and the amyloid beta peptide in Alzheimer's disease. *Redox Biol*. 2018;14:450-64.
 200. Whitnall M, Richardson DR. Iron: a new target for pharmacological intervention in neurodegenerative diseases. *Semin Pediatr Neurol*. 2006;13(3):186-97.
 201. Knöbel Y, Glei M, Osswald K, Pool-Zobel BL. Ferric iron increases ROS formation, modulates cell growth and enhances genotoxic damage by 4-hydroxynonenal in human colon tumor cells. *Toxicol In Vitro*. 2006;20(6):793-800.

202. Villalón-García I, Álvarez-Córdoba M, Povea-Cabello S, Talaverón-Rey M, Villanueva-Paz M, Luzón-Hidalgo R, et al. Vitamin E prevents lipid peroxidation and iron accumulation in PLA2G6-Associated Neurodegeneration. *Neurobiol Dis.* 2022;165:105649.
203. Abarikwu SO, Pant AB, Farombi EO. 4-Hydroxynonenal induces mitochondrial-mediated apoptosis and oxidative stress in SH-SY5Y human neuronal cells. *Basic Clin Pharmacol Toxicol.* 2012;110(5):441-8.
204. Hashimoto M, Rockenstein E, Crews L, Masliah E. Role of protein aggregation in mitochondrial dysfunction and neurodegeneration in Alzheimer's and Parkinson's diseases. *NeuroMolecular Medicine.* 2003;4(1):21-35.
205. Cai L, Wang LF, Pan JP, Mi XN, Zhang Z, Geng HJ, et al. Neuroprotective Effects of Methyl 3,4-Dihydroxybenzoate against TBHP-Induced Oxidative Damage in SH-SY5Y Cells. *Molecules.* 2016;21(8).
206. Caprioli G, Maggi F, Bendif H, Miara MD, Cinque B, Lizzi AR, et al. Thymus lanceolatus ethanolic extract protects human cells from t-BHP induced oxidative damage. *Food Funct.* 2018;9(7):3665-72.
207. Bhattacharya S, Gachhui R, Sil PC. Hepatoprotective properties of kombucha tea against TBHP-induced oxidative stress via suppression of mitochondria dependent apoptosis. *Pathophysiology.* 2011;18(3):221-34.
208. Amoroso S, D'Alessio A, Sirabella R, Di Renzo G, Annunziato L. Ca(2+)-independent caspase-3 but not Ca(2+)-dependent caspase-2 activation induced by oxidative stress leads to SH-SY5Y human neuroblastoma cell apoptosis. *J Neurosci Res.* 2002;68(4):454-62.
209. Aust SD, Chignell CF, Bray TM, Kalyanaraman B, Mason RP. Free radicals in toxicology. *Toxicol Appl Pharmacol.* 1993;120(2):168-78.
210. Valentão P, Carvalho M, Fernandes E, Carvalho F, Andrade PB, Seabra RM, et al. Protective activity of Hypericum androsaemum infusion against tert-butyl hydroperoxide-induced oxidative damage in isolated rat hepatocytes. *J Ethnopharmacol.* 2004;92(1):79-84.
211. Chong C-M, Shen M, Zhou Z-Y, Pan P, Hoi P-M, Li S, et al. Discovery of a benzofuran derivative (MBPTA) as a novel ROCK inhibitor that protects against MPP+-induced oxidative stress and cell death in SH-SY5Y cells. *Free Radical Biology and Medicine.* 2014;74:283-93.
212. Mathiasen JR, McKenna BAW, Saporito MS, Ghadge GD, Roos RP, Holskin BP, et al. Inhibition of mixed lineage kinase 3 attenuates MPP+-induced neurotoxicity in SH-SY5Y cells. *Brain Research.* 2004;1003(1):86-97.
213. Zeissler ML, Eastwood J, McCorry K, Hanemann CO, Zajicek JP, Carroll CB. Delta-9-tetrahydrocannabinol protects against MPP+ toxicity in SH-SY5Y cells by restoring proteins involved in mitochondrial biogenesis. *Oncotarget.* 2016;7(29):46603-14.
214. Gong P, Deng F, Zhang W, Ji J, Liu J, Sun Y, et al. Tectorigenin attenuates the MPP(+)-induced SH-SY5Y cell damage, indicating a potential beneficial role in Parkinson's disease by oxidative stress inhibition. *Exp Ther Med.* 2017;14(5):4431-7.
215. Song Q, Peng S, Zhu X. Baicalein protects against MPP(+)/MPTP-induced neurotoxicity by ameliorating oxidative stress in SH-SY5Y cells and mouse model of Parkinson's disease. *Neurotoxicology.* 2021;87:188-94.
216. Zaim M, Kara I, Muduroglu A. Black carrot anthocyanins exhibit neuroprotective effects against MPP+ induced cell death and cytotoxicity via inhibition of oxidative stress mediated apoptosis. *Cytotechnology.* 2021;73(6):827-40.

217. Shen DF, Qi HP, Ma C, Chang MX, Zhang WN, Song RR. Astaxanthin suppresses endoplasmic reticulum stress and protects against neuron damage in Parkinson's disease by regulating miR-7/SNCA axis. *Neurosci Res.* 2021;165:51-60.
218. Jung YJ, Choi H, Oh E. Melatonin attenuates MPP(+)-induced apoptosis via heat shock protein in a Parkinson's disease model. *Biochem Biophys Res Commun.* 2022;621:59-66.
219. Lu M, Zhang Y, Zhao C, Zhou P, Yu L. Analysis and identification of astaxanthin and its carotenoid precursors from *Xanthophyllomyces dendrorhous* by high-performance liquid chromatography. *Z Naturforsch C J Biosci.* 2010;65(7-8):489-94.
220. Hu J, Lu W, Lv M, Wang Y, Ding R, Wang L. Extraction and purification of astaxanthin from shrimp shells and the effects of different treatments on its content. *Revista Brasileira de Farmacognosia.* 2019;29(1):24-9.
221. Tzanova M, Argirova M, Atanasov V. HPLC Quantification of astaxanthin and canthaxanthin in Salmonidae eggs. *Biomed Chromatogr.* 2017;31(4).
222. López-Cervantes J, Sánchez-Machado DI, Gutiérrez-Coronado MA, Ríos-Vázquez NJ. Quantification of astaxanthin in shrimp waste hydrolysate by HPLC. *Biomed Chromatogr.* 2006;20(10):981-4.
223. Silva B, Silva R, Fernandes C, Guedes de Pinho P, Remião F. Enantioselectivity on the absorption of methylone and pentedrone using Caco-2 cell line: Development and validation of an UHPLC method for cathinones quantification. *Toxicol Appl Pharmacol.* 2020;395:114970.