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Testing the application of new chemical formulations for neurological disease therapies

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Dissertação para obtenção do Grau de Mestre em
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(Emanuel Silva David)

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A eles dedico este trabalho

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Resumo

A doença de Parkinson é uma doença neurodegenerativa multifatorial, caracterizada pela diminuição dos níveis de dopamina libertados pelos neurónios dopaminérgicos, pela formação dos corpos de Lewis, constituídos maioritariamente por α-sinucleína e pela morte dos neurónios dopaminérgicos da *substantia nigra*. Diferentes mecanismos estão desregulados na Doença de Parkinson tais como o processo inflamatório que resulta na microgliose e astrogliose, disfunção da mitocôndria, síntese e degradação da dopamina e ativação da NADPH oxidase, que culima na produção de espécies reativas de oxigénio em grande quantidade criando um ambiente oxidativo nos neurónios dopaminérgicos. Dado a grande importância do stress oxidativo nesta patologia, é do maior interesse a utilização de antioxidantes no seu tratamento, como por exemplo o ácido vanílico, o ácido siríngico e o ácido salicílico. Contudo estes compostos apresentam baixa solubilidade e baixa biodisponibilidade o que dificulta a sua utilização no tratamento da Doença de Parkinson. Desta forma, na presente tese foram utilizadas formulações de Líquidos Iónicos derivados de colina que aumentam a solubilidade e biodisponibilidade dos antioxidantes. Para cada uma das formulações e respetivo precursor foram realizados ensaios em neurónios dopaminérgicos (linha celular N27) para determinar a sua citotoxicidade e o potencial neuroprotector. Dos resultados obtidos foi possível concluir que o ácido vanílico, o ácido siríngico, o ácido salicílico, a colina vanilato, a colina siringato e a colina salicilato não apresentam citotoxicidade para os neurónios dopaminérgicos. Foi ainda concluído que nenhum dos compostos testados (precursores e líquidos iónicos) apresentam potencial de neuroprotecção nos três modelos in vitro de doença de Parkinson.

Palavras-chave

Doença de Parkinson, stress oxidativo, antioxidantes, líquidos iónicos, neurónios dopaminérgicos

Resumo Alargado

A doença de Parkinson é uma doença neurodegenerativa multifatorial, que resulta da ação combinada de diversos fatores de ordem genética e ambiental. Esta doença é caracterizada pela diminuição dos níveis de dopamina libertados pelos neurónios dopaminérgicos no corpo estriado o que dá origem a distúrbios nos movimentos voluntários. A doença de Parkinson tem como características distintivas a formação dos corpos de Lewis nos neurónios dopaminérgicos, constituídos maioritariamente por α-sinucleína, e pela morte dos neurónios dopaminérgicos da *substantia nigra*. Esta é uma doença crónica sem cura e cujos tratamentos disponíveis apenas atenuam os sintomas. Nesta patologia existem vários mecanismos celulares e moleculares que se encontram desregulados, como por exemplo, um aumento da neuroinflamação, disfunção mitocondrial, aumento dos níveis de NADPH oxidases com consequente aumento do stress oxidativo, entre outros. O aumento de espécies reativas de oxigénio tem impacto no complexo da membrana mitocondrial interrompendo o fluxo de eletrões contribuindo ainda mais para o aumento da produção de espécies reativas de oxigénio e uma diminuição da síntese de ATP. Este ambiente altamente oxidante promove a oxidação do ADN mitocondrial o que impede a biogenética mitocondrial. A maior vulnerabilidade dos neurónios dopaminérgicos ao de stress oxidativo resultante da oxidação da dopamina e da presença das enzimas NAPDH oxidases nos neurónios dopaminérgicos reforçam o papel do stress oxidativo na neurodegeneração dopaminergica na Doença de Parkinson. Dado a grande importância do stress oxidativo na doença de Parkinson é do maior interesse a utilização de antioxidantes no tratamento desta patologia, tais como os compostos flavonoides, como por exemplo o ácido vanílico, o ácido siríngico e o ácido salicílico. No entanto, estes compostos apresentam baixa solubilidade e baixa biodisponibilidade o que torna impraticável a utilização destes compostos no tratamento da doença de Parkinson. De forma a aumentar a sua solubilidade e consequentemente a sua disponibilidade, novas formulações destes compostos necessitam ser desenvolvidas. Neste sentido, na presente tese, foram utilizadas novas formulações destes compostos com líquidos iónicos à base de colina.

Para determinar a citotoxicidade dos compostos e dos respetivos líquidos iónicos e mais tarde avaliar o potencial neuroprotector foi utlizada a linha celular N27 de neurónios dopaminérgicos para ambos os testes e, nos ensaios de avaliação do potencial neuroprotector, foram utilizadas as neurotoxinas 6-hidroxidopamina (6-OHDA), MPP+ e Paraquat (PQ).

Os resultados obtidos nos ensaios de citotoxicidade demostraram que a viabilidade nos vários tratamentos era semelhante ao respetivo controlo pelo que se concluiu que o ácido vanílico, o ácido siríngico, o ácido salicílico, a colina vanilato, a colina siringato e colina salicilato não apresentam citotoxicidade para os neurónios dopaminérgicos. Por outro lado, após administração das várias toxinas (6-OHDA, MPP+ e Paraquat) os diferentes compostos testados

(ácido vanílico, o ácido siríngico, o ácido salicílico, a colina vanilato, a colina siringato e colina salicilato) não demostraram ter nenhum potencial neuroprotetor.

Abstract

Parkinson's Disease (PD) is a multifactorial neurodegenerative disease, characterised by a decrease in the dopamine levels released by the dopaminergic neurons, the formation of Lewis bodies, formed mainly by α-synuclein and by the death of dopaminergic neurons of the *substantia nigra*. Several different mechanisms are dysregulated in Parkinson's disease such as the inflammatory process; the dysfunction of mitochondria; the synthesis and degradation of dopamine and the activation of NADPH oxidases, all resulting in the production of reactive oxygen species (ROS) in large quantity which originates a highly oxidative environment in the dopaminergic neurons. Given the high importance of oxidative stress in Parkinson's Disease, it is of particular interest the use of antioxidants in the treatment of this disease, such as vanillic acid, syringic acid and salicylic acid. However, these compounds present low solubility and low bioavailability which makes impracticable their use in the treatment of Parkinson's Disease. Thus, in this thesis, new formulations of cholinium-based ionic liquids were used in order to increase the solubility and therefore the bioavailability od these compounds. For each ILs based antioxidant and its respective precursor, cytotoxicity and neuroprotection tests were performed in the dopaminergic neurons cell line (N27). The obtained results disclosed that vanillic acid, syringic acid, salicylic acid, cholinium vanillate, cholinium syringate and cholinium salicylate do not present cytotoxicity to dopaminergic neurons. Furthermore, it was also possible to conclude that none of the tested compounds (precursors and respective ionic liquids) exhibited neuroprotective characteristics in the three different *in vitro* models of PD tested.

Keywords

Parkinson's disease, oxidative stress, antioxidants, ionic liquids, dopaminergic neurons.

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List of Abbreviations

[Chol][Sal]	Cholinium salicylate
[Chol][Syn]	Cholinium syringate
[Chol][Van]	Cholinium vallinate
6-OHDA	6-hidroxidopamine
ARE	Antioxidant response element
ATP	Adenosine triphosphate
ATP13A2	Probable cation-transporting ATPase 13A2
BBB	Brain-blood barrier
BDNF	Brain-derived neurotrophic factor
CAT	Catalase
CO_2	Carbon dioxide
COX-2	Cyclooxygenase-2
CR3	Complement receptors 3
DA	Dopamine
DAT	Dopamine transporter
DMSO	Dimethyl sulfoxide
DT	Dopamine transporter
EDTA	Ethylenediaminetetraacetic acid
FBS	Foetal bovine serum
GBA	B-glucocerebrosidase
GSH	Glutathione
H_2O_2	Hydrogen peroxide
HBSS	Hank's Balanced Salt Solution
HO-1	heme oxygenase-1
IFN-γ	Interferon-γ
IGF-1	Insulin-like growth factor
IL	Ionic liquids
IL-10	Interleukin-10
IL-1B	Interleukin-1B
IL-6	Interleukin-6
iNOS	Inducible nitric oxide
LPS	Lipopolysaccharides
LRRK2	Leucine-rich repeat kinase 2
MAO-B	Monoamine oxidase B
MPP^+	1-methyl-4-phenylpyridinium
MPTP	1-methyl-4-phenyl-1,2,4,6-tetrahydropyridine
mtDNA	Mitochondrial DNA
MTT	3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium
MV	Monoaminergic vesicles
NO	Nitric oxide
NOX	NADPH-oxidase
NRF-1	Nuclear respiratory factor-1
Nrf2	Nuclear factor (erythroid-derived 2)-like 2
NSAIDs	Nonsteroidal anti-inflammatory drugs

$O2^{-\bullet}$	Superoxide
$ONOO^{-}$	Peroxynitrite
PARK7	Parkinson disease protein 7
PBS	Phosphate-buffered saline
PD	Parkinson's Disease
PET	Positron emission tomography
PGE2	Prostaglandin-2
PINK1	PTEN-induced kinase 1
PRKN	parkin
PTEN	Phosphatase and tensin homolog
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Medium
SA	Syringic acid
SN	*Substantia nigra*
SNCA	α-synuclein
SNpc	*Substantia nigra pars compacta*
SOD	Superoxide dismutase
SS	Salicylic acid
TGF-B	Transforming growth factor-B
TLR	Toll-like receptor
TNF-α	Tumour necrosis factor- α
VA	Vanillic acid
VMAT-2	Vesicular monoaminergic-transporter 2

Chapter 1

1. Introduction

1.1. Parkinson's Disease – Pathology

Parkinson's disease (PD) is a multisystemic neurodegenerative disease that affects approximately 1% of world population over 60 years old (1). The hallmarks of PD are the loss of dopaminergic neurons from the *substantia nigra* (SN), with greater incidence in the pars compacta sub-region of *substantia nigra* (SNpc), the accumulation of abnormal spherical and eosinophilic inclusions known as Lewy bodies that are mainly composed of α-synuclein aggregates and ubiquitin; and the depletion in the content of dopamine (DA) in the SN. The neurodegeneration follows the nigrostriatal pathway, leading to a depletion in the amount of DA released into the striatum that translates in the observable motor defects observable in PD (2). In PD, neurodegeneration and formation of Lewy bodies do not exclusively affect dopaminergic neurons, it also affects acetylcholinergic, serotonergic and noradrenergic neurons, and thus contributing to the observable manifestations in non-motor symptoms.

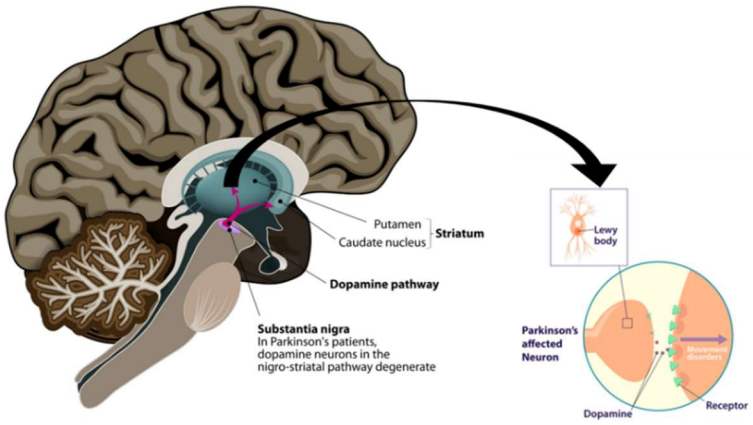

Figure 1.1 – Neuropathological hallmarks of Parkinson's Disease. Parkinson's disease is characterized by the loss of dopaminergic neurons from the *substantia nigra* region and for the formation of inclusion bodies called Lewy bodies that are mainly formed by α-synuclein. The dopaminergic neurons are responsible for the production and release of dopamine which follows the dopamine nigrostriatal ending in the striatum and is responsible for the coordination of voluntary movements. In the case of Parkinson's disease, due to the neurodegeneration of dopaminergic neurons and the inhibition of dopamine synthesis the dopamine levels in the synaptic cleft between the dopaminergic neurons and of *substantia nigra* and the cholinergic neurons from the striatum are diminished, which leads to the development of movement disorders such as bradykinesia and/or tremor. Adapted from: https://healthjade.com/what-is-parkinsons-disease/

PD symptoms are divided into motor symptoms and non-motor symptoms. The motor symptoms were first described by James Parkinson in 1817 in a medical assay in which he described what he called as “shaking palsy,” and include four main symptoms: tremor at rest, rigidity, bradykinesia and postural instability ((3), (4) and (2)). The non-motor symptoms are mainly involved with disturbances of autonomic functions, dementia, alterations in humour and sensory abnormalities ((5) and (4)).

Currently, there is no cure for PD. The most successful treatment to date, is the administration of L-dopa that considerably improves the motor symptoms of PD patients by increasing the content of DA precursor in the SN.

1.2. Causes

PD is a multifactorial disease, in which the interplay between genetic and environmental factors seems to play an important role, being considered as the principal cause of PD development and progression.

Genetic risks involve mutations and epigenetics in seven main genes. These genes are SNCA (α-synuclein), LRRK2, PARK7 (DJ-1), PINK1 (PTEN), PRKN (parkin), GBA, ATP13A2.

α-synuclein and LRRK2 inheritance follows an autosomal dominant pattern. LKKR2 is an enzyme with kinase activity, it's widely distributed within multiply organelles, and participates in the regulation of autophagosome and lysosome, and mitochondrial function ((6) and (7)). α-synuclein is the protein mainly associated with the development and progression of PD, it is involved in the process of synaptic vesicle formation. Mutations, epigenetics and post-translational modifications of α-synuclein promotes the α-synuclein aggregation. The accumulation of α-synuclein dysregulates mitochondrial function, autophagy and endoplasmic reticulum-Golgi complex transport and inhibits the exocytosis of synaptic vesicle content ((8), (9) and (10)).

PARK7, PINK1, PRKN, GBA and ATP13A2 inheritance follows an autosomal recessive pattern. DJ-1 is a peroxidase involved in the elimination of ROS ((11) and (12)). Parkin is an E3 ligase protein involved in the degradation of proteins by the proteasome and mitophagy. Phosphatase and tensin homologue (PTEN) and PTEN induced kinase 1 (PINK1)is an autosomal recessive gene involved in mitophagy ((13) and (11)). The GBA and ATP13A2 are involved in lysosome activity, whereas GBA localization is inside the lysosome and is responsible for the degradation of glucosylceramide into glucose, ATP13A2 is associated with lysosomes membrane ((14), (15) and (16)).

Environmental risks include exposure to pesticides/herbicides like paraquat, rotenone, organochlorine and organophosphates that promote inhibition of the complex I of mitochondria, increase in the production of reactive oxygen species (ROS) and activation of microglia (17). Chronic exposure to metals such as Cu, Al, Hg, Fe and Mn and consequent accumulation throughout life in the brain are a risk factor for developing PD, by promoting production of ROS, α-synuclein activation of microglia (17).

1.3. Neuroinflammation

In neurodegenerative diseases, such as PD, neuronal loss is accompanied by neuroinflammation. Neuroinflammation is a process that occurs as a response to injury, infection, toxins or protein aggregates involving the activation of microglia and astrocytes, and the release of pro- or anti-inflammatory cytokines and chemokines in an effort to remove the harmful stimulus and protect the brain ((18) and (19)). The occurrence of neuroinflammation in PD is demonstrated by the observation of microgliosis and astrogliosis in PD post-mortem brains, by *in vivo* positron emission tomography (PET) study of activated microglia in SN, and by the presence of activated microglia after the administration of toxins and by the decrease in neurodegeneration after the administration of nonsteroidal anti-inflammatory drugs (NSAIDs) ((20) and (21)).

Neuroinflammation has a dual character. A neuroinflammatory response can promote neuroprotection by promoting neuronal remodelling with the secretion of neurotrophic factors like the transforming growth factor-ß (TGF-B) and anti-inflammatory cytokines like IL-10 and removal of cell debris, protein aggregates and toxins ((22) and (23)). At the same time, neuroinflammation can promote neurodegeneration by the production of pro-inflammatory cytokines such as tumour necrosis factor-α (TNF-α) and interleukin-1ß (IL-1ß), and generation of ROS and reactive nitrogen species (RNS) (23).

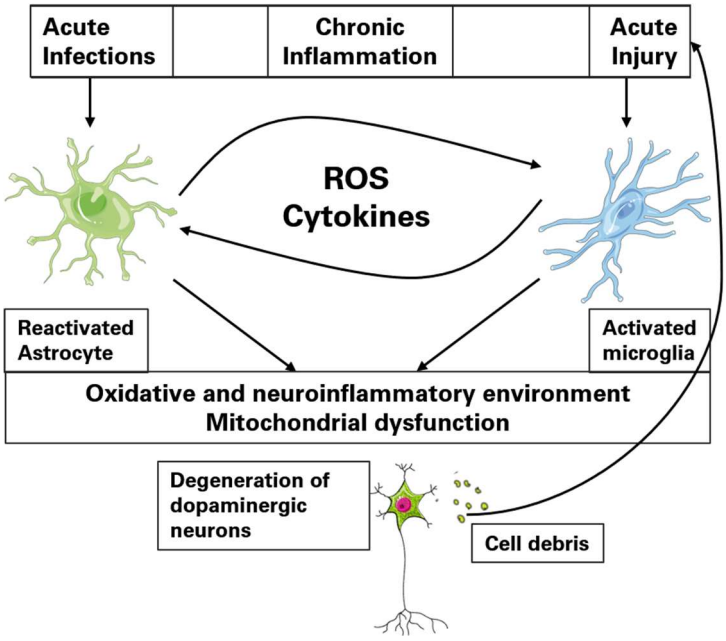

Figure 1.2 – Schematic representation of the neuroinflammatory response in PD. Neuroinflammation can be triggered by multiples factors such as acute infections mediated by bacteria and viruses, chronic inflammation caused by constant extracellular presence of inclusion bodies like α-synuclein or neuron injury. In the presence of these factors occurs the activation of microglia culminating with microgliosis, with preferential differentiation into M1 morphology, and reactivation of astrocytes which culminates with astrogliosis. The M1 microglia and reactive astrocytes are responsible for the production and release of pro-inflammatory cytokines and ROS which activate and reactivate even more microglia and astrocytes, respectively. These cytokines and ROS also act on neurons promoting the development of the inflammatory process which induces the neuronal degeneration. Adapted from: **Adapted: (Taylor, Main et al. 2013)**

Under physiological conditions, microglia are “inactivated” with a ramified morphology, but it is constantly monitoring the extracellular space and neuronal homeostasis, and constantly releasing trophic factors such as Brain-Derived Neurotrophic Factor (BDNF), Insulin-like Growth Factor-1 (IGF-1) and anti-inflammatory cytokines (IL-10) (24). In cases of PD, microglia from the SN are highly activated by externalized SNCA At3T aggregates in the form of oligomers and fibrils. After being phagocyted or bound to receptors expressed in the surface of microglia cells, (toll-like receptors (TLR), P2X7 receptor, Galectin-3 receptor and Complement Receptors 3 (CR3)) microglia acquire pro-inflammatory activity and produce high amounts of ROS and RNS ((25), (26), (27) and (28)). After activation of microglia, several morphological alterations occur and the ramified morphology changes to an amoeboid morphology. Furthermore, the levels of mRNA of TNF-α, IL-1b, Il-6, cyclooxygenase-2 (COX-2), inducible nitric oxide synthase (iNOS) and interferon-γ (IFN-γ) are increased. The levels of expression of NADPH oxidase (Nox) after the activation of microglia in PD’s patients are increased which is accompanied with an increased in the expression and translocation of p67phox and increased phosphorylation of p47phox and formation of p47phox-Nox2 complexes which increases the activity of NADPH oxidase 2 (Nox2) and the production of $O2^{-\bullet}$ and H_2O_2. ((29), (20), (30), (25), (31) and (32)). Microglial pro-inflammatory cytokines promote neurodegeneration by activating inflammation pathways in neurons and inducting apoptosis (33).

In PD reactivation of astrocytes accompanies the activation of microglia. Astrocytes are activated α-synuclein (phagocytosis or binding to receptors), or by the cytokines released by the microglia and ROS. By its turn, astrocytes when activated release complement components, IL-1B, IL-6, and chemokines that promotes further neurodegeneration and activation of more microglia cells (34).

1.4. Mitochondrial Dysfunction

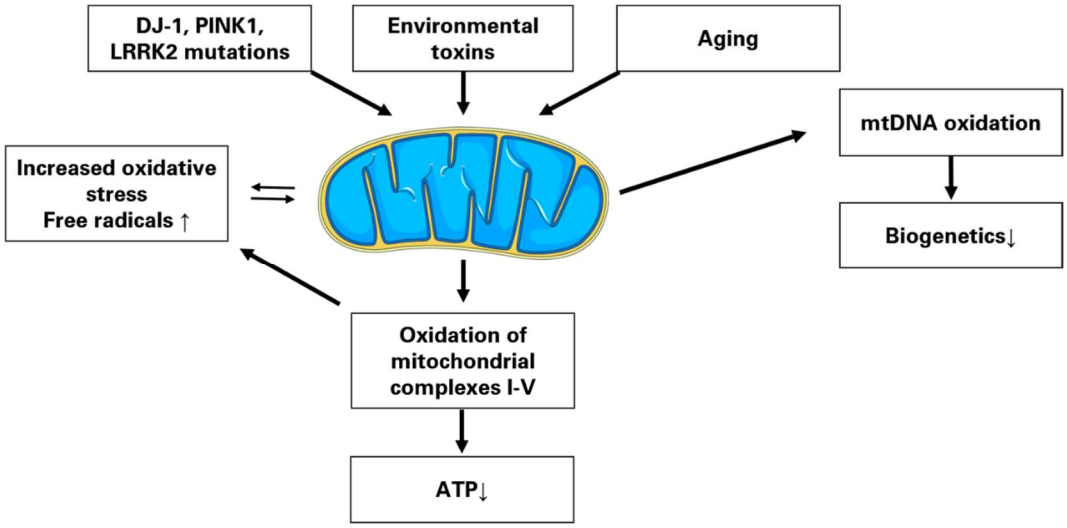

Figure 1.3 - Mitochondrial dysfunction. In mitochondria due to mutation of genes responsible for the transcription of proteins related with the mitochondrial biogenetics (PINK1 and LRRK2) and oxidative stress control (DJ-1), due to environmental toxins inhibitory of the mitochondrial respiratory chain and as a consequence to the production of ROS as a result of dopamine oxidation and neuroinflammation promote increased oxidative stress in mitochondria. The increased oxidative environment results in the oxidation of proteins of the mitochondrial complex I-V which causes the interruption of the transport of electrons through the mitochondrial respiratory chain promoting the production of more ROS and the inhibition of ATP synthesis. Other macromolecules are also oxidized such as mtDNA, which prevents the synthesis e substitution of proteins of the mitochondrial complex proteins and the lipids of the double lipidic layer which compromises the mitochondrial integrity. As a result, is promoted the release of the cytochrome C and the formation of the mTP which culminates in the apoptosis process.

There are plenty of evidences supporting the notion that the development of PD is accompanied by mitochondrial dysfunction. Mitochondria is composed of one external mitochondrial membrane and one internal mitochondrial membrane where complexes I-IV are coupled. A series of redox reactions through the mitochondria electron transporter chain provides energy to form proton gradient (mitochondrial membrane potential) that drives the synthesis of ATP. In PD, the SN neurons exhibit a reduction in the mitochondrial complex I activity which not only promotes a decrease in ATP synthesis but also increases the production of ROS. Inhibition of mitochondrial complex I can be in consequence of oxidation of cysteine residues in the catalytic subunits of mitochondrial complex I or due to accumulation of α-synuclein aggregates in the mitochondria ((35), (36) and (37)).

Because of this production of ROS, macromolecules are oxidized. The oxidation of proteins culminates with further inhibition of complex I enzymes with consequential production of even more ROS. Oxidation of mitochondrial DNA (mtDNA) may promote mutations that compromises the activity of the enzymatic subunits of mitochondrial complex I leading to the failure of the biogenetic process which culminates with the production more oxidative stress. Peroxidation of the mitochondrial lipids leads to the release of cytochrome c to the cytosol, which initiates the apoptotic pathway ((36), (37) and (38)).

1.5. Oxidative Stress

The cause and development of PD is not completely elucidated, but evidences, like high levels of lipid peroxidation, protein oxidation and DNA oxidation (8-hydroxyguanine) found in studies on post-mortem PD brains and the presence of high amounts of ROS in studies with animal models of PD and assays using toxins that inhibit the mitochondrial complex I such as MPTP strengthens the hypothesis that oxidative stress maybe the main mechanism of development in PD (39).

Neurons are more vulnerable to oxidative stress because they are exclusively aerobic and have low levels of antioxidants and high content in polyunsaturated fatty acids, dopaminergic neurons are even more susceptible to ROS due to high amounts of highly oxidizable DA, accumulation of Fe2+ in neuromelanin, low levels of mitochondria and a large resident microglia population ((40) and (41)). In a disease as complex as PD is, ROS and RNS production have multiple origins. Mitochondria is the main source of ROS and RNS production in PD, followed with age, oxidation of DA, neuroinflammation, α-synuclein.

DA is a highly reactive catecholamine; it is synthesized in the cytosol but is rapidly actively uptaked into monoaminergic vesicles (MV) with an acidic environment by the vesicular monoaminergic transporter-2 (VMAT-2) to prevent the oxidation of DA in the cytosol. In PD, due to mitochondria dysfunction and a decrease in ATP levels, the interaction of SNCA A53T MV culminates with the release of DA to the cytosol (42). In the cytosol, DA can have three different destinations: it can be degraded by monoamine oxidase-B (MAO-B) forming H_2O_2 as by-product; or due to a higher pH in the cytosol can suffer autoxidation and form dopamine-quinones; or can even be oxidized by COX in the presence of O_2 and forming $\bullet O_2$-. The resulting dopamine-quinones and ROS promotes mitochondrial dysfunction and further α-synuclein aggregation ((42), (43) and (44)).

As previously mentioned, in PD extracellular α-synuclein aggregated promotes activation of microglia. After activation, the three different types of NOX expressed in microglia, NOX-1, NOX-2, that catalyses the formation of O2-• from O_2 in the presence of NADPH, and NOX-4, that catalyses the formation of H2O2 and O_2, are activated along with the activation of iNOS that produces nitric oxide (NOS). The O2-• is a radical with low membrane permeability, therefore to pass through cell membranes O2-• needs to be converted into species that can pass through cell membrane such as H_2O_2 (spontaneously or by the action of SOD) or •OH through Fenton reaction in the presence of free Fe^{2+}. NOS is a molecule that can pass freely through the cell membranes and in the presence of O2-• can form peroxynitrite ($ONOO^-$). ROS and RNS promotes oxidation and nitration of α-synuclein which leads to protein aggregation initiating a vicious circle of neuroinflammation, oxidative stress and protein aggregation (45).

Under physiological conditions, ROS are constantly produced in small amounts by the mitochondria, mitochondrial complex I and II are responsible for producing small amounts of O_2-• that are quickly converted into H_2O_2 by SOD that are consequently converted into H_2O and O_2. In PD, as demonstrated by mitochondrial complex I inhibitors 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and rotenone in animal models, the inhibition of complex I enhances the production of ROS, and in result it disrupts the electron flow along the mitochondria electron transporter chain and decreases ATP production. This abnormal production of $\bullet O^{2-}$ oxidize proteins of the complex I or reacts with NO (nitric oxide) forming $ONOO^-$ that promotes the nitration of mitochondrial proteins in tyrosine residues, which further inhibits complex I and promotes production of more ROS ((41), (46) and (47)).

1.6. Neuroprotector potential of antioxidant compounds

Vanillic acid (VA), syringic acid (SA) and salicylic acid (SS), belong to a sub-family of phenolic compounds, in particular flavonoids. They are found in fruits, seeds and vegetables as a by-product of the plant metabolism, being involved in the plant defence against ultraviolet radiation and pathogens and are responsible for the vivid colouration of fruits and vegetables. The compounds from the flavonoid family are well-documented throughout the literature, in particular for their antioxidant and anti-inflammatory proprieties (48).

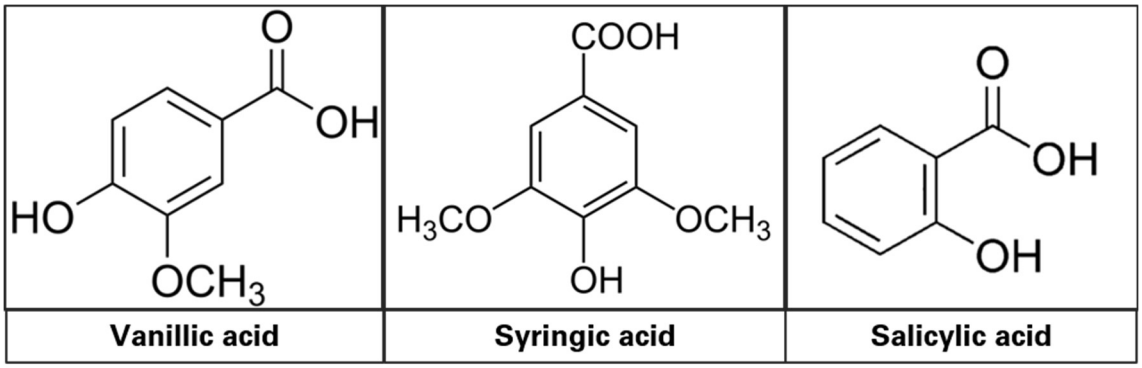

Figure 1.4 – Chemical structure of the antioxidant compounds under study: Vanillic acid (VA), syringic acid (SA) and salicylic acid (SS)

The antioxidant proprieties of VA, SA and SS are due to the radical scavenging activity and by increasing the levels and in the expression of superoxide dismutase (SOD), catalase (CAT), glutathione (GSH), the cofactors responsible for re-reduction of the antioxidant enzymes and inducible nitric oxide synthase (iNOS) expression and activation ((49), (50), (51), (52), (53), (54), (55) and (56)).

Another mechanism by which VA has an antioxidant effect is through the regulation of Nrf2, a transcription factor that binds to a specific DNA sequence antioxidant response element (ARE) which regulates the transcription of HO-1, an enzyme that regulates $Fe2^{+}$ homeostasis (57). When analysing the action of VA in neurons and macrophages. Kim at el, demonstrated the anti-inflammatory proprieties of VA when stimulated with lipopolysaccharide (LPS) by decreasing the synthesis of pro-inflammatory cytokines TNF-α and IL-6, by decreasing the expression levels of COX-2, and therefore, by decreasing the synthesis of prostaglandin E2 (PGE2). VA also down-regulates the activation of NF-κB pathway by inhibiting the degradation of IkB, the inhibitory proteins of NF-κB (58).

SA decreases oxidative stress through the regulation of mitochondria biogenesis and indirectly in the respiratory function by increasing the levels of Nuclear Respiratory Factor 1 (NRF-1) (56).

VA and SA administration also increases cell survival by lowering the number of cells entering in apoptosis process. This effect is accomplished by an increase in the expression of the anti-apoptotic BCL-2 proteins and a decrease in the levels of Bax (pro-apoptotic protein) with the administration of VA in hippocamp neurons (57). SA has an anti-apoptotic action by turning down the levels of expression of caspase-3 (56).

SS anti-inflammatory action is well known through the inhibition of COX-2, but another mechanism by which it exert anti-inflammatory action is through the inhibition of the NF-κB pathway (59).

These naturally occurring flavonoids, exhibit antioxidant and anti-inflammatory properties which make them molecules of interest in the treatment of neurodegenerative diseases. Notwithstanding, these flavonoids display low solubility, which results in lower bioavailability and the necessity of administrate higher doses of antioxidant molecules to reach therapeutic concentrations. Supplementary complications arise with a low permeability through the brain-blood barrier (BBB), which further decreases the bioavailability (60).

1.7. Ionic Liquids

To overcome the limited solubility and consequent decreased bioavailability of VA, SA and SS, we based on the works of (61) in which they converted phenolic acids into cholinium-based salts to find new formulations that increase the solubility of antioxidant and anti-inflammatory species. The IL that were used during the execution of this work are: cholinium vanillate [Chol][Van], cholinium syringate [Chol][Syn] and cholinium salicylate [Chol][Sal]

Cholinium-based salts are ionic liquids (IL). ILs are salts with a melting point below 100ºC, and the majority of (IL) are liquid at room temperature. IL are liquid at room temperature because of the interaction between a large and asymmetrical organic cation and an inorganic weakly coordinating anion ((62) and (63)). Because they are not volatile, they are considered as "green" solvents. Furthermore, they are highly tuneable, being considered as designer solvents. These characteristics alongside with their remarkable thermophysical properties makes ILs as remarkable solvents with applications in different scientific areas, including pharmaceutical industry ((64) and (63)).

Cholinium-based salts are very promising formulations due to their high solubility in water and reduced toxicity, while keeping or even increasing the antioxidant and anti-inflammatory proprieties. Furthermore, due the existence of cholinium receptors in the BBB that permits the passage of molecules with a cholinium group in their structure, these new formulations based on cholinium based salts increase the permeability of flavonoids through the BBB.

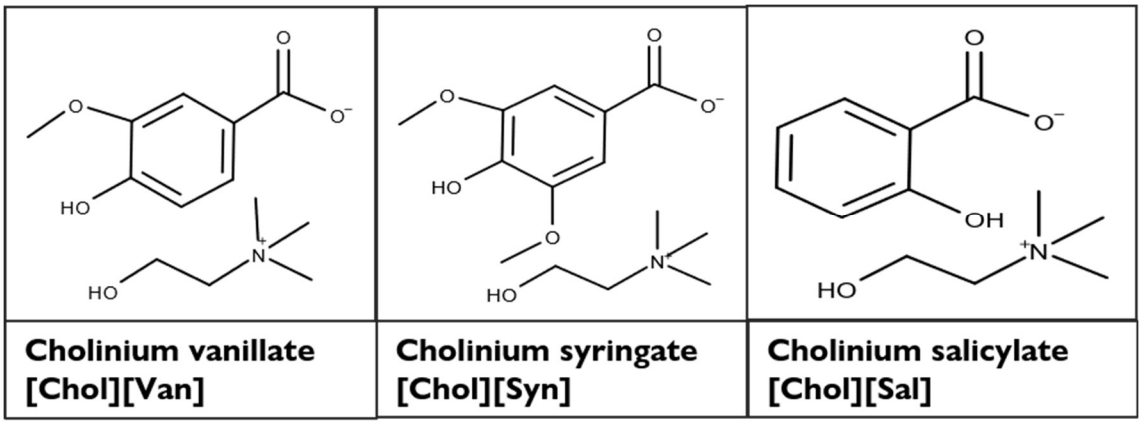

Figure 1.5 – Chemical structures of the ionic liquids under study: Cholinium vanillate ([Chol][Van]), Cholinium syringate [Chol][Syn] and Cholinium salicylate [Chol][Sal].

Chapter 2

2. Objectives

PD is neurodegenerative disease in which oxidative stress plays a major role. Given the high importance of oxidative stress in PD, the use of antioxidants in the treatment of this disease such as VA, SA and SS is of particular interest. However, these compounds present low solubility and low bioavailability which makes their use impracticable. Thus, in this thesis, new formulations of cholinium-based ionic liquids were used in order to increase the solubility and therefore the bioavailability.

The objectives of this thesis can be defined as follows: To evaluate the cytotoxic effect of the ILs formulations and their respective precursors into N27 (dopaminergic neurons) cell line; and to evaluate the neuroprotective potential of the Ils and precursors to the same cell line before the administrations of different neurotoxins.

Chapter 3

3.Methods

3.1 N27 Cell Line

The cell line of immortalized dopaminergic neurons from E12 primary rat mesencephalic tissue after being transfected with virus gene SV40 large T antigen was used. This N27 cell line does not express tyrosine hydroxylase and is transfected with a plasmid to express high levels of DA transporter (DAT).

3.2 Cell Culture of N27 Cell Line

N27 cells stored at -80ºC were quickly thawed. With a P1 000 micropipette they were transferred to a falcon tube containing 10 mL of Roswell Park Memorial Institute (RPMI) 1640 medium [(Sigma-Aldrich®; Darmstadt, Germany) (GibcoTM, Dreieich, Germany)] with pH 7.3, L-glutamine, phenol red, 0,20mL/mL of heat-inactivated foetal bovine serum (FBS) (Millipore, Germany), 100U/ml penicillin plus 100µg/ml streptomycin (Sigma-Aldrich®; Darmstadt, Germany).

The cell suspension was centrifuged for 3 minutes at 1 500 r.p.m. The supernatant obtained after centrifugation was discarded and the pellet containing the cells was resuspended with 2mL of medium. From the cell suspension, 1mL was transferred to a 35mm TC-treated culture dish (Corning®, Wiesbaden, Germany).

The cell culture was then incubated at 37ºC under 5% CO_2 until achieving 80% of confluence, after which they were dissociated with trypsin, giving rise to a new passage. The dissociation was performed by adding of 1mL of 0,05%/0,02% trypsin/EDTA (GibcoTM; Dreieich, Germany), prepared from 1mL 0,5%/0.02% trypsin PBS/EDTA plus 9mL 0,02% PBS/EDTA. After 2min, the cells were detached from the 35mm culture dish surface. Afterwards trypsin was removed and 5mL of new medium was added to the 35mm culture dish to obtain a cell suspension, of which 2 mL was transferred to a 100mm TC-tread culture dish (Corning®; Wiesbaden, Germany) with 15mL of medium. The culture dish was then incubated at 37ºC under 5% CO_2 until 80% of confluence was achieved. Upon achievement of 80% of confluence a new cell passage to a new 100mm culture dish was performed.

3.3 Test solutions

The solutions of VA, SA, SS, [Chol][Van], [Chol][Syn] and [Chol][Sal] were prepared by weighing on an Eppendorf the masses of VA, SA, SS, [Chol][Van], [Chol][Syn] and [Chol][Sal] represented on Table 1 and by adding 4mL of 0,9g/100mL saline solution (NaCl) to obtain stock solution 1.

From the stock solution 1 of VA, SS, [Chol][Van] and [Chol][Sal], 400µL of stock solutions 2 and 3 were prepared by two consecutive dilutions by a factor of 1:100.

For SA and [Chol][Syn], the 400µL stock solution 2 was obtained from a dilution by a factor of 1:80 and the stock solution 3 was obtained with a dilution by a factor of 1:100.

The rest of the solutions (4 out of 7 different concentrations prepared) were obtained from dilutions of the three stock solutions by a factor of 1:10, except the solution with lowest concentration, that was prepared from by a dilution factor of 1:100 from stock solution 3.

Table 3.1 - Mass and concentrations of VA, SA, SS, [Chol][Van], [Chol][Syn] and [Chol][Sal] of the different solutions prepared. The mass represented in this table corresponds to the mass required for the preparation of Stock1 solution. The concentrations shown correspond to the concentrations of the three Stock solutions used and the concentrations of the four intermediate solutions prepared from the 3 different Stock solutions.

Solutions	Mass (mg)	Concentrations µM						
		Stock1		Stock2		Stock3		
VA	7,2	10 000	1 000	100	10	1	0,1	0,01
SA	4,0	5 000	1 000	100	10	1	0,1	0,01
SS	5,5	10 000	1 000	100	10	1	0,1	0,01
[Chol][Van]	10,8	10 000	1 000	100	10	1	0,1	0,01
[Chol][Syn]	6,0	5 000	1 000	100	10	1	0,1	0,01
[Chol][Sal]	9,7	10 000	1 000	100	10	1	0,1	0,01

3.4 Cytotoxicity Tests

Cell Viability Tests were performed in quadruplicate in a 96 multi-well plate. The cells were used only when 80% of confluence was achieved in the 100mm culture dish (see section 3.2). The cells were detached from the 100mm cell dish surface with the addition of 2mL of trypsin/EDTA for 2 minutes. Then trypsin was removed, and cells were suspended in 5ml of medium and poured into a falcon tube. To obtain a cell suspension with the cell concentration $1x10^4$cells/well (62 500cell/cm^2), cells were counted in a haemocytometer.

The cell counting was performed after mixing up 25µL of cell suspension with 25µL of Trypan Blue in an Eppendorf, and with a P100 micropipette the Trypan Blue/cell suspension was applied to the haemocytometer. Under the microscope, and using the 10x objective, the unstained cells were counted with a hand tally counter in four sets of sixteen squares of the haemocytometer. Only unstained cells were considered because Trypan Blue only internalizes when the cell membrane is compromised and thus is a marker for dead cells. After counting, and through the calculation of the mean of the four sets, the number of cells in 1mL of cell suspension was determined.

From the obtained concentration in the cell suspension and the number of wells to be used, a new solution with the required volume of cell suspension to have a final cell concentration of $1x10^4$cells/well with 200µL medium per well was prepared.

The plating of 96 multi-well plate was performed with a P200 multichannel and after the plating was completed, the 96 multi-well plate was incubated at 37ºC under 5% CO_2 for 24 hours.

After 24 hours, the stimulation of cells with VA, SA, SS and [Chol][Van], [Chol][Syn] and [Chol][Sal] was carried out (Figure 3.1). First, intermediate solutions were prepared from the original solutions by adding, on an Eppendorf tube, 100µL of solution with 900µL of medium, these solutions have a final concentration represented in Table 2. After the preparation of working solutions, the medium was removed from the 96 multi-well plate by aspiration through a vacuum system with posterior addition of 200µL of each working solution except in the control where was only 200µL of RPMI-1640 medium was added. Then, the 96 multi-well plate was incubated at 37ºC under 5% CO_2 for 24 hours.

Table 3.2 - Final concentrations of VA, SA, SS, [Chol][Van], [Chol][Syn] and [Chol][Sal] to which the cells were exposed.

Solutions	Concentrations µM						
	Stock1		Stock2		Stock3		
VA	1 000	100	10	1	0,1	0,01	0,001
SA	500	100	10	1	0,1	0,01	0,001
SS	1 000	100	10	1	0,1	0,01	0,001
[Chol][Van]	1 000	100	10	1	0,1	0,01	0,001
[Chol][Syn]	500	100	10	1	0,1	0,01	0,001
[Chol][Sal]	1 000	100	10	1	0,1	0,01	0,001

After 24 hours of exposure to the stimulus the cell viability assay was performed (Figure 3.1). The initial assay used was MTT assay, but later the cell viability assay was changed for CCK-8. This change was made, due to limitations and disadvantages of using MTT assay, such as the necessity to prepare, freeze and thaw the reagent and an extra step to externalize and dissolve the formazan formed, which makes a very time-consuming assay. In contrast, the CCK-8 assay is much less time-consuming assay as it uses a ready-to-use solution, it is conserved at 4ºC (no freezing and thawing steps necessary) and is not necessary an extra step to externalize and dissolve the formazan formed. Other advantages of CCK-8 in comparison with MTT assay consist in the lower cytotoxicity and higher sensitivity ((65), (66) and (67)).

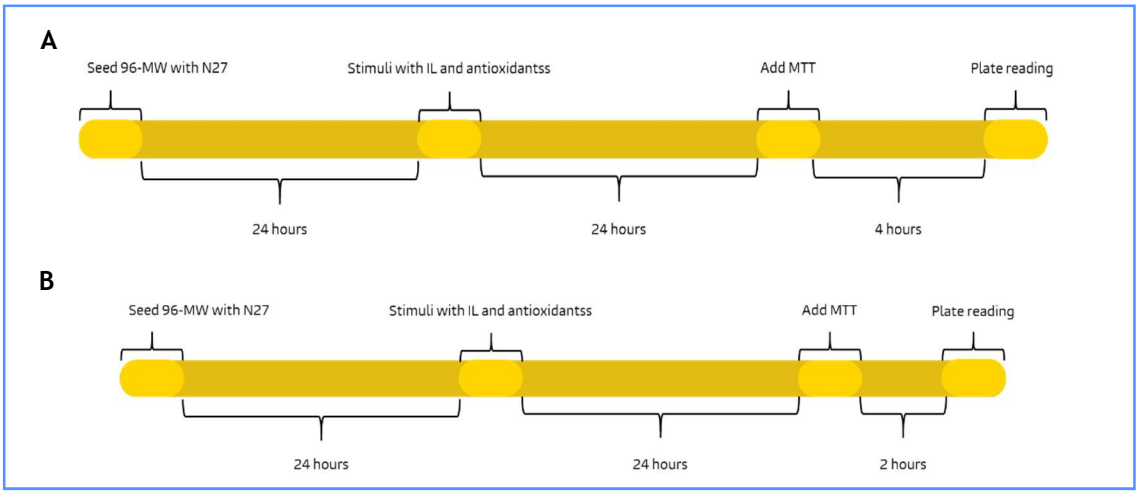

Figure 3.1 - Cytotoxicity Test. Cytotoxic tests were performed on cell line N27 and the number of viable cells was evaluated using Cell Viability Assays. In A we have description of procedure to Cytotoxic Test using the MTT Assay. N27 were seeded with a confluence of $1x10^4$ cells/well. After 24 hours were stimulated with precursors and respectively IL in a quintuplicate assay. Pass the follow 24 hours was remove the medium and added MTT solution after which was waited 4 hours before the addition of SDS. The plate reading was performed 15 minutes after the addition of SDS. In B we have description of the procedure to Cytotoxic Test using the CCK-8 Assay. N27 were seeded with a confluence of $1x10^4$ cells/well. After 24 hours were stimulated with precursors and respectively IL in a quintuplicate assay. Pass the follow 24 hours was added directly to each well CCK-8 solution. The plate reading was performed 2 hours after the addition of CCK-8.

3.5 Neuroprotection Potential Tests

The neurotoxins 6-OHDA (Sigma-Aldrich®; Darmstadt, Germany), MPP+ (Sigma-Aldrich®; Darmstadt, Germany) and Paraquat (Sigma-Aldrich®; Darmstadt, Germany) were prepared by weighing on an Eppendorf the respectively masses represented on Table 2 and by adding 2mL of 0,9g/100mL saline solution (NaCl) to MPP+ and Paraquat, and 2 mL of 0,1% (w/v) ascorbic acid to 6-OHDA. The three solutions were conserved at -4ºC.

Table 3.3 - Mass and concentrations initial prepared of the neurotoxins 6-OHDA, MPP^+ and Paraquat, and the concentrations of VA, SA, SS, [Chol][Van], [Chol][Syn] and [Chol][Sal] used on the neuroprotection tests based on the results obtained from the Cytotoxic tests.

Solutions	Mass (mg)	Concentrations µM
6-OHDA	10,3	25 000
MPP^+	5,9	10 000
Paraquat	5,12	10 000
VA	7,2	1 000
SA	4,0	1 000
SS	5,5	1
[Chol][Van]	10,8	1 000
[Chol][Syn]	6,0	1 000
[Chol][Sal]	9,7	1

Neuroprotection Potential Tests were performed in quadruplicate in a 96 multi-well plate and with two controls: a negative control in which only medium was present; and a positive control in which the medium with the neurotoxins was used. To each one of the target compounds, i.e., VA, SA, SS, [Chol][Van], [Chol][Syn] and [Chol][Sal] a concentration from the Cytotoxic Tests was selected and its neuroprotective potential against all three neurotoxins (6-OHDA, MPP+ and Paraquat) was tested.

Similarly, to cytotoxicity tests, a cell concentration of $2x10^4$ cells/well was used. The cell counting, and seeding followed the same protocol as described in for the cytotoxicity tests (section 3.4). The plating of 96 multi-well plate was performed with a P200 multichannel micropipette and after the plating was completed, the 96 multi-well plate was incubated at 37ºC under 5% ^{CO2} for 24 hours.

Table 3.4 - Final concentrations of neurotoxins 6-OHDA, MPP^+ and Paraquat present in the wells of the 96 multi-well plate.

Solutions	Concentrations µM
6-OHDA	50
MPP^+	1 000
Paraquat	1 000

After the 24h incubation period, the stimulation of cells with VA, SA, SS and [Chol][Van], [Chol][Syn] and [Chol][Sal] was performed. First, intermediate solutions were prepared from the original solutions by adding, on an Eppendorf tube, 100µL of solution with 900µL of medium.

After the preparation of working solutions, the medium was removed from the 96 multi-well plate by aspiration through a vacuum system with subsequent addition of 180µL of each working solution except in the negative control where only 200µL medium was added. Afterwards, the 96 multi-well plate was incubated at 37ºC under 5% CO_2 for 1 hour.

During that one-hour incubation period, the neurotoxins were thawed. Except 6-OHDA for which was prepared an intermediate solution by a dilution factor of 1:50 with medium RPMI-1640, the working toxin solutions PQ and MPP+ were used directly.

After the incubation period, 20µL of 6-OHDA, MPP+ and Paraquat was added to the respective wells and then the 96 multi-well plate was incubated at 37ºC under 5% CO_2 for 24 hours. The next day, the CCK-8 assay was performed (Figure 3.2).

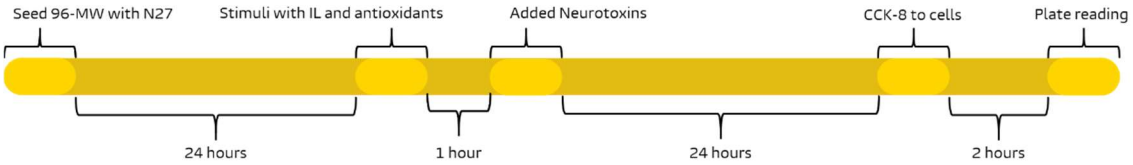

Figure 3.2 - Neuroprotection Potential Tests. The experimental procedure was carried out in the cell line N27 and in order to evaluate the neuroprotective potential of the antioxidants the number of viable cells using Cell Viability Assays after toxin exposure was compared with the conditions with no toxin exposure. N27 were seeded with a confluence of $1x10^4$ cells/well. After 24 hours were stimulated with precursors and respectively IL in a quintuplicate assay. The addition of neurotoxins was realized 1 hour after the stimuli with the precursors and IL. Pass the follow 24 hours was added directly to each well CCK-8 solution. The plate reading was performed 2 hours after the addition of CCK-8.

3.6 Cell Viability Assays

3.6.1 MTT

MTT assay is a colorimetric assay that quantifies *in vitro* cell viability. It uses the reduction of a pale yellow, water soluble and cell permeable substrate 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) by the NAD(P)H-dependent oxidoreductase enzymes of viable cells into formazan, an insoluble crystalline purple product that accumulates inside the cells and in the medium. The formazan crystals must be dissolved in an additional step with SDS to homogenize the solution for quantification of formazan generated by evaluating the absorbance at 570nm with microplate absorbance spectrophotometer. The amount of formazan generated is directly proportional to the number of viable cells (68).

MTT (Acros Organics; Geel, Belgium) stock solution with a concentration of 5mg/mL was prepared in dark conditions by dissolving 100 mg MTT in 20 mL of filtered phosphate-buffered saline. The solution was conserved in aliquots of 1mL frozen.

The assay was performed under dark conditions on a 96 multi-well plate. First, the MTT stock solution was diluted to a concentration of 0.5mg/mL with filtered HBSS/PBS followed by the removal of medium from the 96 multi-well by aspiration through a vacuum system and the addition of 100µL of MTT (0,5mg/mL) to each well. After the addition of the MTT, the 96 multi-well plate was incubated for 24 hours at 37º under 5% CO_2. After 24 hours, the MTT solution was removed and the formazan formed was dissolved by adding SDS 10% for 2 hours at 37º. The last step was measuring the absorbance at 570nm with a microplate absorbance scanning ((69) and (70)).

3.6.2 CCK-8

Cell Counting Kit–8 (Dojindo; Munich, Germany) is a colorimetric assay that permits the quantification of cell viability in proliferation and cytotoxicity assays. It is based on the reaction of reduction of a lightly yellow water-soluble 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt (WST-8) by the cell dehydrogenases in the presence of an electron carrier, 1-Methoxy PMS, into an orange soluble WST-8 formazan. To quantification of WST-8 formazan generated, the absorbance is measured at 450nm with a microplate absorbance scanning. The amount of WST-8 generated is directly proportional to the number of viable cells ((65) and (66)).

Cell viability evaluated with CCK-8 was performed following the manufacturer's protocol. CCK-8 comes already prepared in a bottle-solution, this way no initial steps of preparation were needed. Initially, under dark conditions, a solution containing the total volume of CCK-8 was prepared with the new medium necessary to the total number of wells to be used. The removal of medium from the 96 multi-well was the performed by aspiration through a vacuum system.

From the CCK-8 solution initially prepared 200µL to each well was added with a P200 multichannel. The 96 multi-well plate was then incubated on an incubator for 2 hours at 37º under 5% CO_2. At the end of the 2 hours the absorbance at 450nm was measured with a microplate absorbance scanning.

Chapter 4

4. Results

Cytotoxic and neuroprotection potential of the ionic liquids ([Chol][Van], [Chol][Syn], [Chol][Sal]) and respective precursors (VA, SA, SS) were evaluated in a cell line of mouse dopaminergic neurons (N27) and in cell models of PD, namely 6-hidroxidopamine (6-OHDA), 1-methyl-4-phenylpyridinium (MPP^+) and Paraquat (PQ). Based in the literature seven different concentrations of the compounds (Table 3.2) were selected to be tested in the cytotoxicity assays ((61), (71), (72)). The results obtained in the cytotoxicity tests allowed to select the target concentration of the antioxidants to be used in the neuroprotection assays (Table 3.3).

4.1 Cytotoxicity Tests

To study the potential cytotoxic effects of VA, SA, SS, [Chol][Van], [Chol][Syn] and [Chol][Sal] in dopaminergic neurons, cell viability assays using the cell line N27 were performed. Cells were stimulated for 24 hours with the different concentrations of the compounds of interest, in order to evaluate the number of viable cells. In the end of the experiments, the cell viability in % of control against the different the different concentrations of compounds tested were plotted, and the EC_{50}, (i.e., concentration of the different compounds that promotes a 50% decrease in cell viability) was calculated.

From the cytotoxic assays with VA and [Chol][Van], we can observe a cell viability close to 100% in all range of concentrations of VA and [Chol][Van] tested, meaning that neither VA or [Chol][Van] present any cytotoxic effect in any range of concentrations tested in N27 cells. As an outcome of such results is not possible to obtain the dose response curve and to determine the EC_{50} value to either VA and [Chol][Van] (Figure 4.1). Comparing the results obtained of cell viability between the VA and [Chol][Van] is it observable that both compounds leads to a cell viability with values very similar to each other, meaning that both compounds hare similar to each other in terms of cytotoxicity.

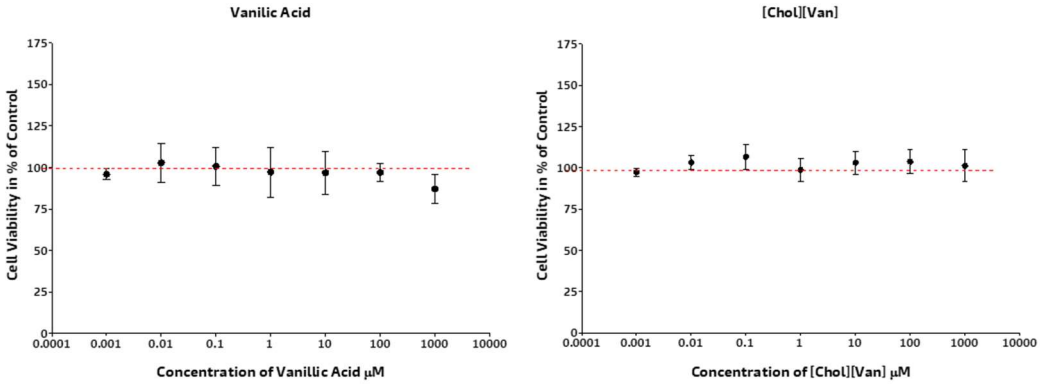

Figure 4.1 - Cytotoxicity assays of VA and [Chol][Van]. Cells were stimulated with VA and [Chol][Van] for 24h. Cell viability was assessed by MTT and CCK-8 assays. Results are expressed in % of control and represent the mean±SEM (n=4-5)

The results obtained for SA and [Chol][Syn] were very similar to the ones for VA and [Chol][Van]. In fact, a cell viability close to 100% in all range of concentrations of SA and [Chol][Syn] is noticeable, which means that neither SA or [Chol][Syn] present any cytotoxic effect to N27 cells the concentrations tested, and, like in the case of VA and [Chol][Van], such results do not permit to obtain the dose response curve and to calculate the EC_{50} value to either VA and [Chol][Van] (Figure 4.2).

When comparing the cell viability between SA and [Chol][Syn], is it observable that the stimulation with both formulations leads to values of cell viability very similar, meaning that both formulations have a similar cytotoxic effect in N27 cells.

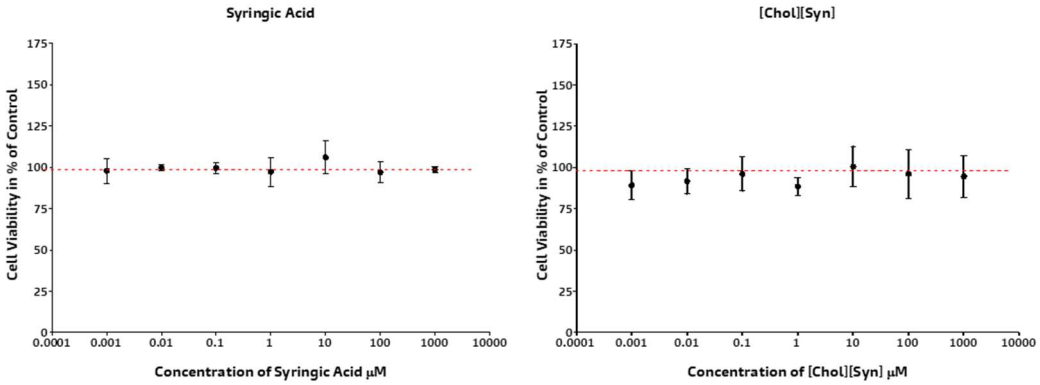

Figure 4.2 - Cytotoxicity of SA and [Chol][Syn]. Cells were stimulated with SA and [Chol][Syn] for 24h. Cell viability was assessed by MTT and CCK-8 assays. Results are expressed in % of control and represent the mean±SEM (n=3-4)

In the case of SS and [Chol][Sal], the results obtained are again very similar to the ones obtained with VA, SA, [Chol][Van] and [Chol][Syn]. That is, we can observe a cell viability close to 100% in all range of concentrations of VA and [Chol][Van], notwithstanding a slightly decrease in cell viability when using concentrations of SS superior to 1µM. Despite the slight decrease in SS, we

can determine that neither VA or [Chol][Van] present any cytotoxic effect to all range of concentrations tested in N27, and as consequence from these results is not possible to obtain the EC_{50} curve or determine the EC_{50} value to either SS and [Chol][Sal] (Figure 4.3). In this case comparing the cell viability between SS and [Chol][Sal] it is observable that, in concentrations superior to 1µM, [Chol][Sal] presents a slightly higher cell viability when compared with cell viability of SS, however none the less this slight difference, both compounds present a very similar cell viability.

When comparing the cell viability between SA and [Chol][Syn], is observable that the stimulation of both compounds leads to values of cell viability very similar, meaning that both compounds have an alike cytotoxic effect in N27 cells.

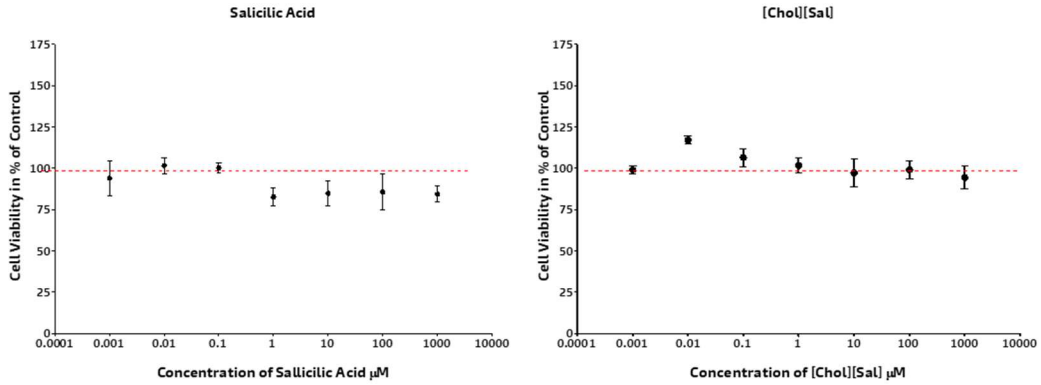

Figure 4.3 - Cytotoxicity of SS and [Chol][Sal]. Cells were stimulated with SS and [Chol][Sal] for 24h. Cell viability was assessed by MTT and CCK-8 assays. Results are expressed in % of control and represent the mean±SEM (n=3)

4.2 Neuroprotection

To study the neuroprotective potential of VA, SA, SS, [Chol][Van], [Chol][Syn] and [Chol][Sal] in dopaminergic neurons, the PD cell models 6-OHDA, MPP^+ and PQ were used. These models are known for promoting neurodegeneration of dopaminergic neurons (in this case was used dopaminergic cell line N27) by promoting an increase in the oxidative stress in dopaminergic neurons that leads to cell loss. To achieve this aim, one concentration for each precursor and it´s correspondent IL was chosen from the cytotoxic assays to test they neuroprotective potential in the three different PD cell models. The neuroprotection potential was assessed by performing cell viability assays to determine if the cell viability, after 24 hours, was increased in the PD cell models through the action of VA, SA, SS, [Chol][Van], [Chol][Syn] and [Chol][Sal] in comparison with assays in the presence of only 6-OHDA, MPP+ and PQ (positive control). All results obtained are presented in % of control, this control corresponds to the test performed only the presence of RPMI-1640 medium (negative control).

4.2.1. 6-OHDA PD cell model

In the tests 6-OHDA model two positive controls were performed separately, one for the assays with VA, SA and SS and the other to the assays with their ionic liquid formulations ([Chol][Van], [Chol][Syn] and [Chol][Sal]). As was initial expected, in both cases a decrease in the cell viability to 52% of control and 55% of control was observed, respectively.

To determine the neuroprotective potential of VA, SA and SS in the PD 6-OHDA cell model the cell viability obtained in each of these assays was compared with the cell viability obtained in the positive control experiments.

The obtained results disclosed that VA, SA and SS promoted a statistically significant decrease in cell viability in comparison with the negative control to 44% of control, 45% of control and 50% of control, respectively. When comparing, the cell viability of the positive control with the cell viability of VA, SA and SS, it was observed that these compounds promote a decrease in the cell viability, although not statistically significant. Such results suggest that in the 6-OHDA cell model VA, SA and SS are not neuroprotective neuroprotection (Figure 4.4).

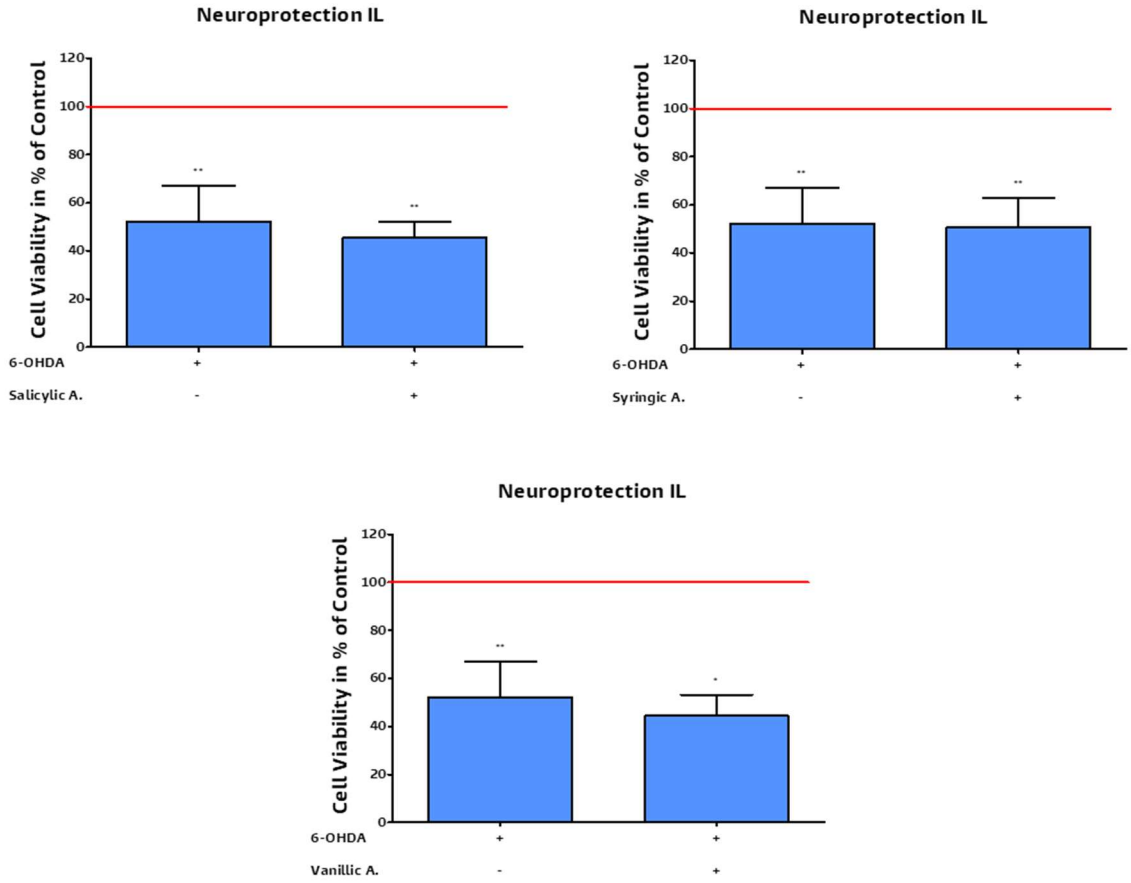

Figure 4.4 - Neuroprotection of VA, SA and SS against 6-OHDA. Cells were stimulated with 100µM of VA, 100µM of SA, 1µM of SS and 50µM of 6-OHDA for 24h. Cell viability was assessed with the CCK-8 assay. Results are expressed in % of control and represent the mean±SEM (n=3). Statistical analysis was performed using one-way ANOVA followed by Student Newan-Keuls Multiple Comparison Test. **P<0.001, *P<0.05 compared to control.

Using the same approach to [Chol][Van], [Chol][Syn] and [Chol][Sal] as to VA, SA and SS it was found that [Chol][Van], [Chol][Syn] and [Chol][Sal] promoted a significant decrease in cell viability in comparison with the negative control to 67% of control, 63% of control and 41% of control. However, when comparing the cell viability of the positive control with the cell viability of VA, SA and SS no significant difference was found, meaning that [Chol][Van], [Chol][Syn] and [Chol][Sal] doesn't increase the cell viability in the PD 6-OHDA cell model, thus they don't exert any neuroprotection (Figure 4.5).

When comparing VA, SA and SS with their respectively IL, it is observable that [Chol][Van] and [Chol][Syn] increases the cell viability in comparison with VA and SA, which can be view as higher neuroprotective potential to [Chol][Van] and [Chol][Syn], and in the opposite way [Chol][Sal] decreases the cell viability in comparison with SS, which can be view as reduced neuroprotective potential of [Chol][Sal].

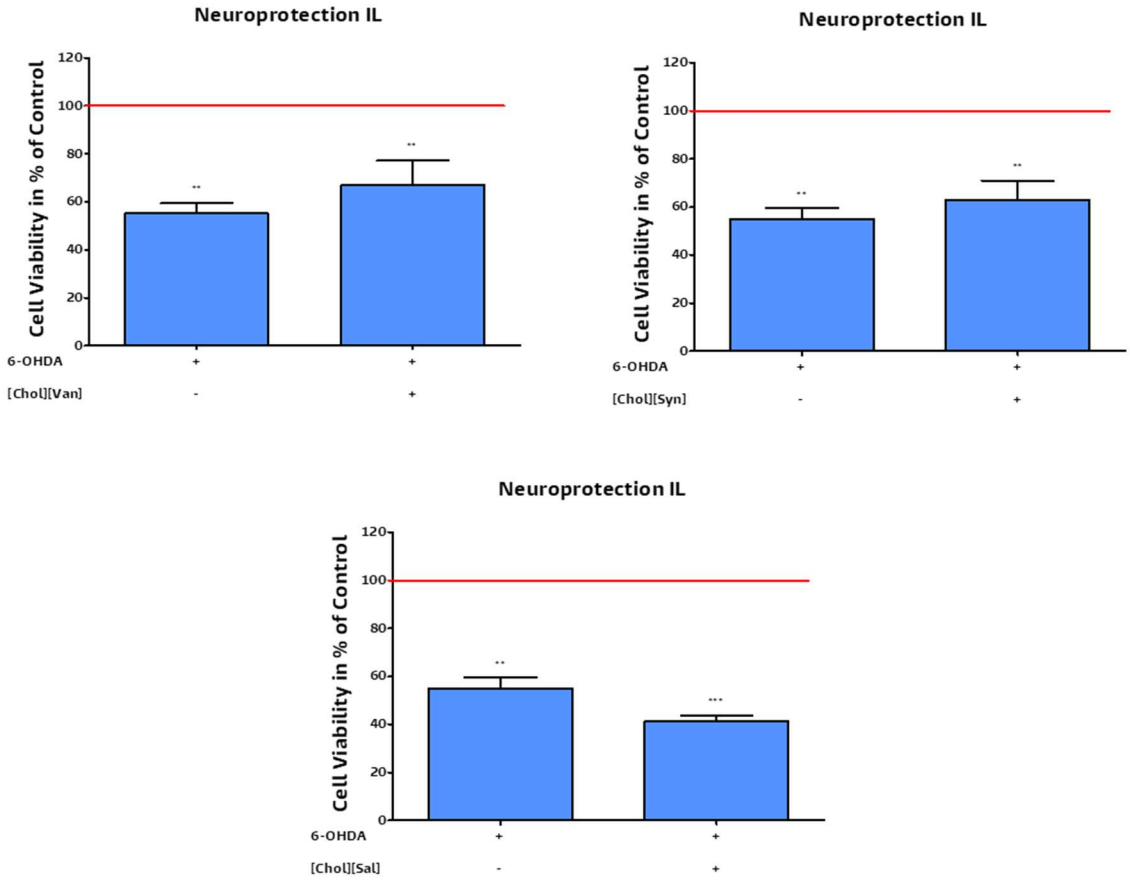

Figure 4.5 - Neuroprotection of [Chol][Van], [Chol][Syn] and [Chol][Sal] against 6-OHDA. Cells were stimulated with 100µM of [Chol][Van], 100µM of [Chol][Syn], 1µM of [Chol][Sal] and 50µM of 6-OHDA for 24h. Cell viability was assessed with the CCK-8 assay. Results are expressed in % of control and represent the mean±SEM (n=3). Statistical analysis was performed using one-way ANOVA followed by student Newan-Keuls Multiple Comparison Test.***P<0.0001, **P<0.001 compared to control.

4.2.2. MPP^+ PD cell model

The tests with the MPP^+ cell model was performed in a similar way to the ones for 6-OHDA model. In brief, two positive controls were performed separately, one for the assays with the precursors (VA, SA and SS) and the other for the assays with the Ionic Liquids ([Chol][Van], [Chol][Syn] and [Chol][Sal]). As expected, in both cases a decrease in the cell viability in relation to the control was observed (62% of control and 70% of control, respectively).

To determine the neuroprotective potential of VA, SA and SS in the MPP^+ model, the cell viability obtained in each of these assays was compared with the cell viability obtained in the positive control. The obtained results disclosed that VA, SA and SS promoted a statistically significant decrease in cell viability when compared with the negative control to 68% of control, 72% of control and 78% of control. However, when comparing the cell viability of VA, SA and SS with the cell viability of the positive control an increase in the cell viability was found, although it was not statistically significant. Such results suggest that VA, SA and SS don't exert any neuroprotection (Figure 4.6).

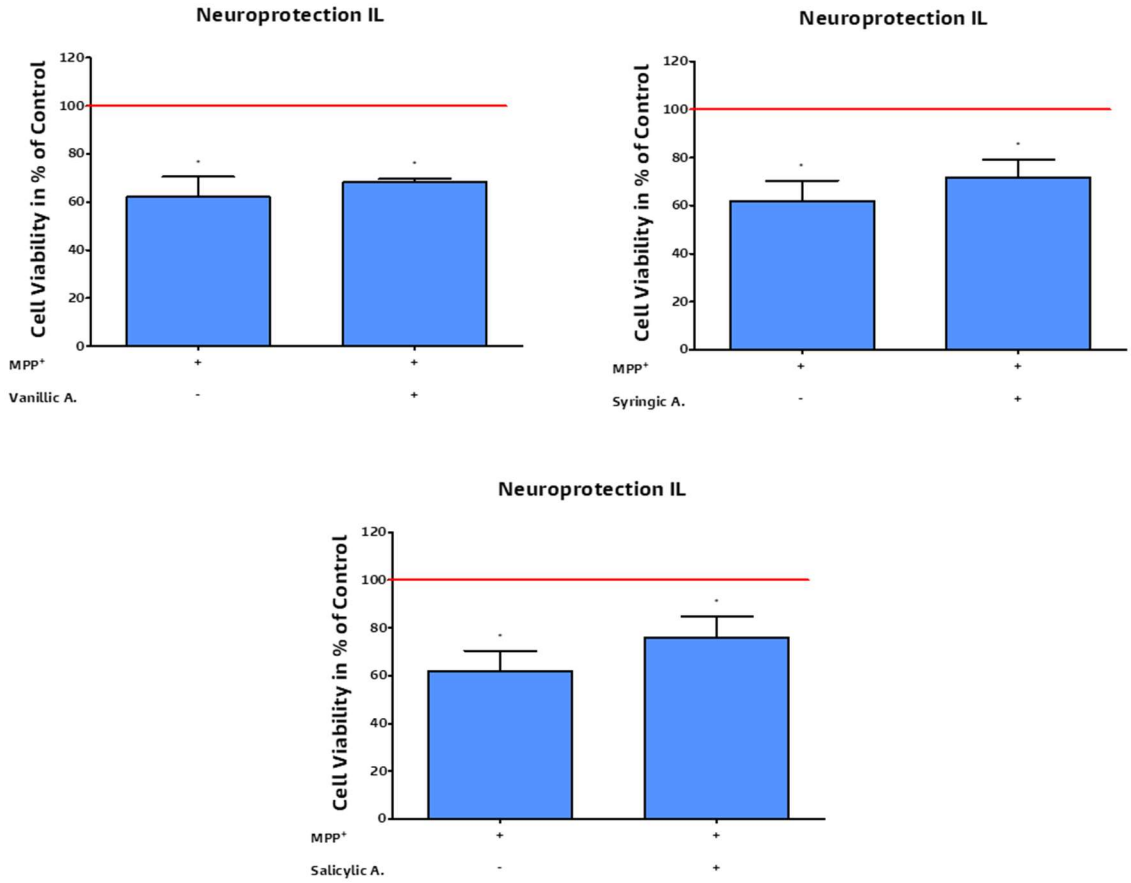

Figure 4.6 - Neuroprotection of VA, SA and SS against MPP⁺. Cells were stimulated with 100µM of VA, 100µM of SA, 1µM of SS and 1mM of MPP+ for 24h. Cell viability was assessed with the CCK-8 assay. Results are expressed in % of control and represent the mean±SEM (n=3). Statistical analysis was performed using one-way ANOVA followed by Student Newan-Keuls Multiple Comparison Test. **P<0.001, *P<0.05 compared to control.

Using the same approach to [Chol][Van], [Chol][Syn] and [Chol][Sal] it was found out that the ionic liquids promoted a decrease in cell viability in comparison with the negative control to 78% of control, 85% of control and 76% of control, yet this difference was not statistically significant. When comparing the cell viability of the positive control with the cell viability of VA, SA and SS an increase in the cell viability was found, again this increase was also not statistically significant. Such results suggest that [Chol][Van], [Chol][Syn] and [Chol][Sal] don't exert any statistically significant neuroprotection in the PD MPP⁺ cell model (Figure 4.7).

When comparing VA, SA and SS with their respectively IL, is observable that [Chol][Van] and [Chol][Syn] increases the cell viability in comparison with VA and SA, which can be view as higher neuroprotective potential of [Chol][Van] and [Chol][Syn], and in the opposite way [Chol][Sal] has the same cell viability as SS, which can be view as neuroprotective potential of both compounds in equal.

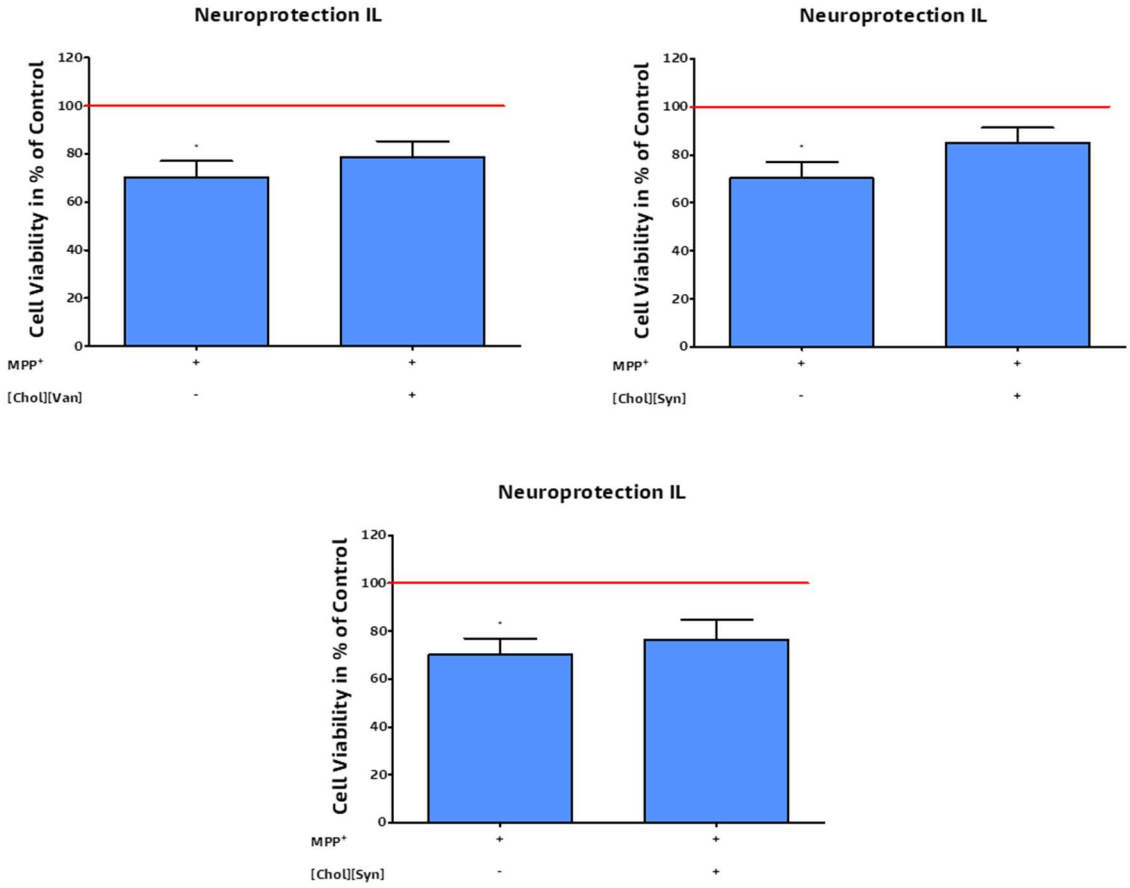

Figure 4.7 - Neuroprotection of [Chol][Van], [Chol][Syn] and [Chol][Sal] against MPP+. Cells were stimulated with 100µM of [Chol][Van], 100µM of [Chol][Syn], 1µM of [Chol][Sal] and 1mM of MPP+ for 24h. Cell viability was assessed with the CCK-8 assay. Results are expressed in % of control and represent the mean±SEM (n=3). Statistical analysis was performed using one-way ANOVA followed by Student Newan-Keuls Multiple Comparison Test.*P<0.05 compared to control.

4.2.3. Paraquat PD cell model

The tests performed with the paraquat (PQ) model follow the same procedure as the previous ones. Two positive controls were performed separately, one for the assays with VA, SA and SS and the other for the assays with [Chol][Van], [Chol][Syn] and [Chol][Sal]. As expected, in both cases a decrease in the cell viability in relation to the control was observed (61% of control and 58% of control, respectively).

To determine the neuroprotective potential of VA, SA and SS in the PQ model, the cell viability obtained was compared with the cell viability obtained in the positive control. The obtained results disclosed that SA promoted a statistically significant decrease in cell viability when compared with the negative control (61% of control), but VA and SS didn't promote any statistically significant decrease in the cell viability (with 67% of control and 63% of control, respectively). When comparing the cell viability of the positive control with the cell viability of VA, SA and SS no significant differences were found, which means that VA, SA and SS in presence of PQ don't exert any neuroprotection (Figure 4.8).

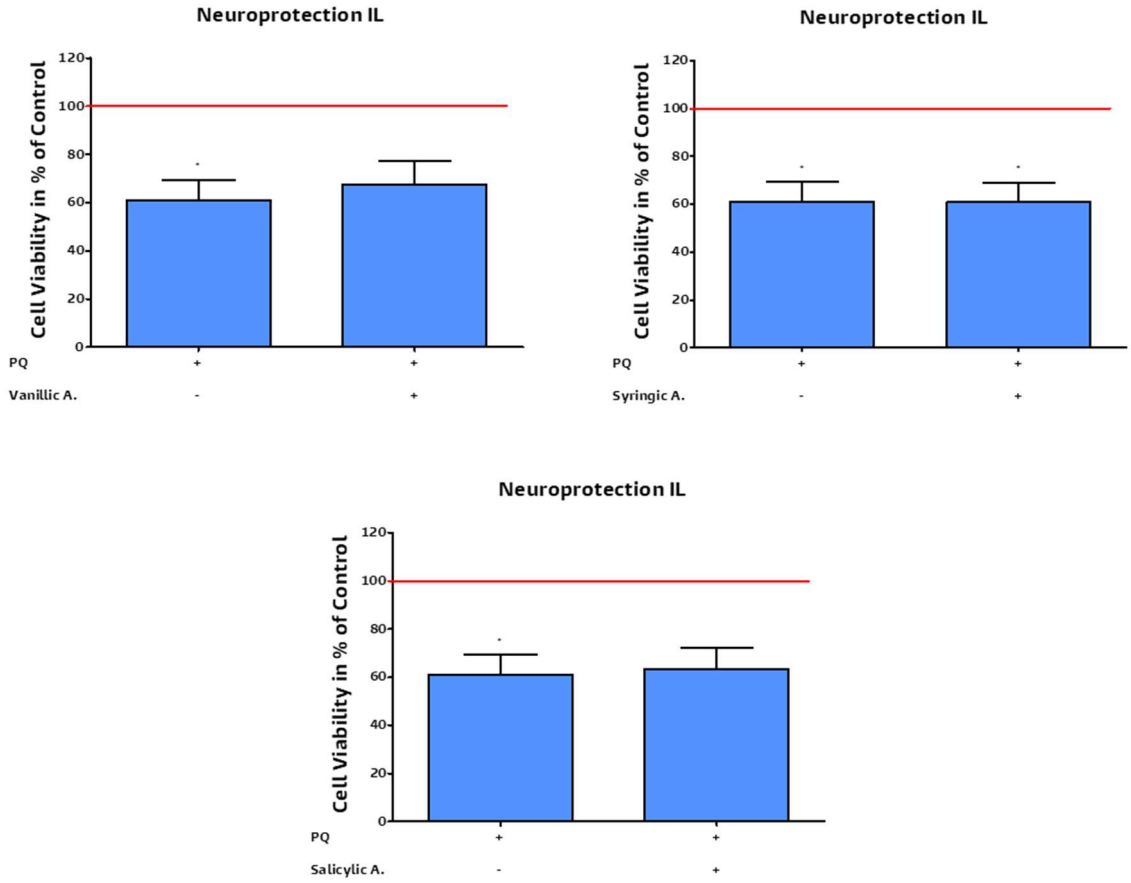

Figure 4.8 - Neuroprotection of VA, SA and SS against PQ. Cells were stimulated with 100µM of VA, 100µM of SA, 1µM of SS and 1mM of PQ for 24. Cell viability were assessed CCK-8 assay. Results are expressed in % of control and represent the mean±SEM (n=2-3). Statistical analysis was performed using one-way ANOVA followed by Student Newan-Keuls Multiple Comparison Test.*P<0.05 compared to control.

Using the same approach to [Chol][Van], [Chol][Syn] and [Chol][Sal] it was found out that [Chol][Van], [Chol][Syn] and [Chol][Sal] promoted a statistically significant decrease in cell viability in comparison with the negative control to 45% of control, 69% of control and 53% of control, respectively. However, when comparing the cell viability of the positive control with the cell viability of VA, SA and SS an increase in the cell viability was found, but this increase is also not statistically significant, meaning that [Chol][Van], [Chol][Syn] and [Chol][Sal] in the PQ cell model don't exert any neuroprotection (Figure 4.9).

When comparing VA, SA and SS with their respectively ILs, it is observable that [Chol][Syn] increases the cell viability in comparison with SA, which can be view as higher neuroprotective potential to [Chol][Syn], and in the opposite way [Chol][Van] and [Chol][Sal] decreases the cell viability in comparison with VA and SS, which can be view as a reduced neuroprotective potential of the ionic liquids [Chol][Van] and [Chol][Sal].

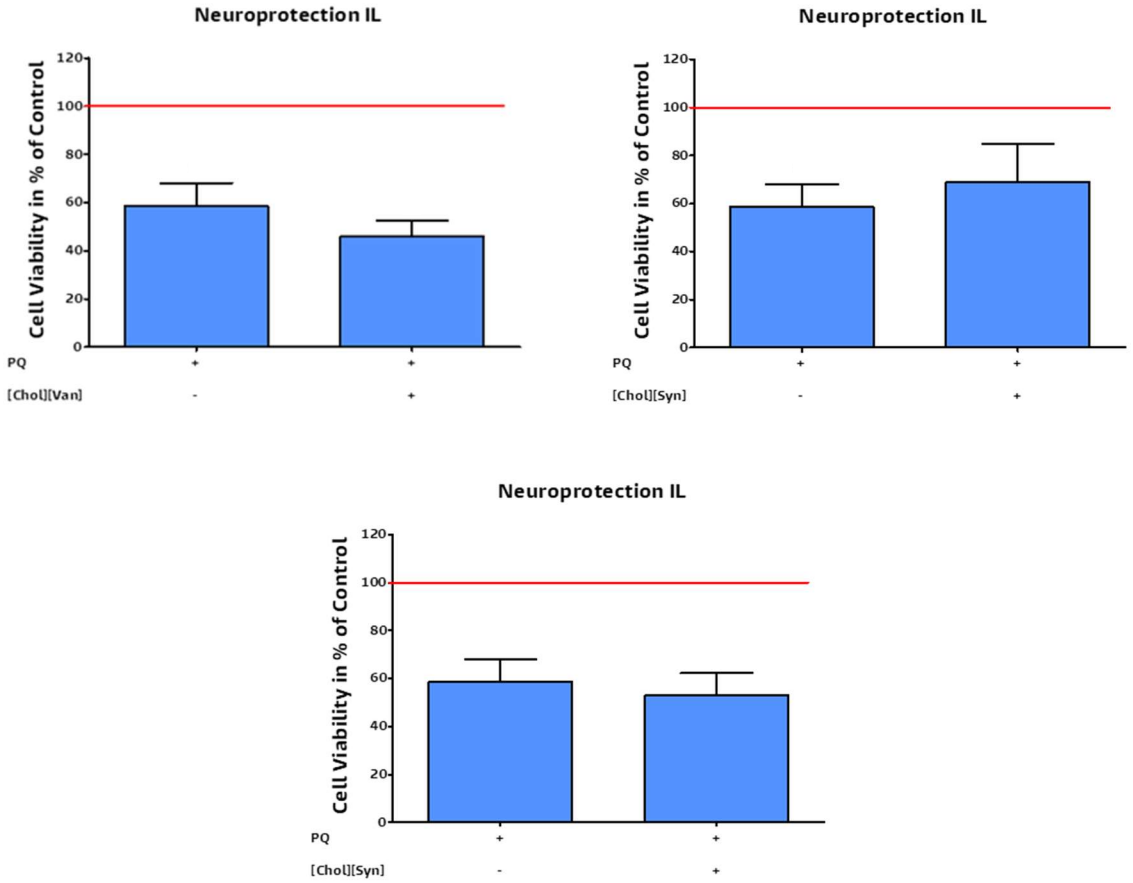

Figure 4.9 - Neuroprotection of [Chol][Van], [Chol][Syn] and [Chol][Sal] against PQ. Cells were stimulated with 100µM of [Chol][Van], 100µM of [Chol][Syn], 1µM of [Chol][Sal] and 1mM of PQ for 24h. Cell viability was assessed with the CCK-8 assay. Results are expressed in % of control and represent the mean±SEM (n=3). Statistical analysis was performed using one-way ANOVA followed by Newan-Keuls Multiple Comparison Test.

Chapter 5

5. Discussion

The main aim of this thesis was to find out a new non-neurotoxic formulation to deliver antioxidant molecules for the treatment of neurodegenerative diseases, particularly PD. Generally, neurotoxic organic solvents, like DMSO or ethanol, are used to solubilize water insoluble molecules, and increasing the permeability through the BBB. In order to avoid the use of such toxic solvents, in this thesis new formulations of ionic liquids were tested. For that, we based our approach on the already published works by (61) that used ILs formulations to improve the solubility and bioavailability of antioxidant compounds, namely VA, SA, SS. The cholinium based ILs formulations ([Chol][Van], [Chol][Syn] and [Chol][Sal]) presented higher water solubility.

The concentrations of the precursors and the ILs tested were chosen based on their solubility values and the concentrations already described in the literature ((61), (71), (72)). The tested concentration range varied between 10 000µM to 0,01µM (Table 3.1). However, given the low solubility of SA (Table 5.1) it was not possible to prepare a solution of SA with a concentration of 10 000µM, thus the most concentrated solution of SA was 5.000µM ((61) and (57)).

Table 5.5 - Solubility of VA, SA, SS. [Chol][Van], [Chol][Syn] and [Chol][Sal].

	Solubility (mmol/L)
Vanillic Acid	10,43
Syringic Acid	7,4
Salicylic Acid	15,66
Cholinium Vanillate	3 181,40
Cholinium Syringate	2 793,71
Cholinium Salicylate	-

The first objective of this thesis was to evaluate the cytotoxic potential of VA, SA, SS and their respectively IL formulation to establish whether the IL formulation presents a higher cytotoxicity than its respective precursors. For that, we exposed N27 dopaminergic neurons to seven different concentrations (Table 3.2) of VA, SA, SS and their respectively IL formulation and evaluated the cell viability. From the results obtained (Figures 4.1, 4.2 and 4.3) we concluded that the VA, SA, SS exhibited the same level of cytotoxicity as their IL formulations and that all the compounds do not show any cytotoxicity in all range of concentrations tested.

After establishing that the IL formulation do not show any cytotoxicity in the DA neurons, we moved to the second objective of this thesis that was to evaluate the neuroprotective potential

of VA, SA, SS and their respective IL formulations. PD is a neurodegenerative disease characterized by the death of DA neurons from the SNpc. The causes to this neurodegeneration are also related to a general increase in the oxidative stress in neurons, from different sources, such as mitochondria dysfunction, inversed NADPH oxidases and neuroinflammation. This in turn, contributes to the dysfunction of lysosomal pathway and protein accumulation. Oxidized DA is other important source of oxidative stress by promoting directly the formation of ROS or through the formation of dopamine-quinones ((73) and (74)). To reproduce the PD pathological hallmarks, we used 6-OHDA, MPP^+ and Paraquat in vitro models of PD. 6-OHDA is transported into the dopaminergic neurons through the DT and accumulates in the cytosol and since is readily oxidized promotes directly to the formation of ROS. MPP^+ is transported into the dopaminergic neurons through the DA transporters (DT) where it interacts and inhibits the complex I of mitochondria and impairs the production of ATP. Paraquat is converted into PQ and enters into the dopaminergic neurons through the DT where generates ROS (17).

We tested the neuroprotective potential of VA, SA, SS and their respectively IL formulations against the three aforementioned PD cell models: 6-OHDA, MPP^+ and Paraquat. From the results obtained we concluded that neither VA, SA, SS or their respectively IL formulations protected the neurons against the three neurotoxins used. The results obtained are not in line with the ones previously reported by other researchers for the neuroprotective potential of the VA, SA and SS, even using concentrations lower than the ones used by us (((49), (50), (51), (52), (53), (54), (55) and (56)). In fact, in such studies, the treatment with VA, SA or SS promoted neuroprotection followed by a decrease in the levels of oxidative stress, an increase in the levels of antioxidant enzymes SOD, CAT and GSH and inhibition of the activation of NF-κB factor ((49), (50), (51), (52), (53), (54), (55), (56), (58) and (59)).

One possible reason for the absence of neuroprotection in our assays could be related with the concentrations used that are not enough high to promote neuroprotection in the PD cell models. We can overcome this issue by increasing the concentrations used, which is not a problem for IL formulations, but due to solubility limitations, is not possible in the cases of VA, SA and SS (Table 5.1). The time between the stimulation with VA, SA and SS and their respectively IL formulations, and the addition of neurotoxins 6-OHDA, MPP^+ and Paraquat of 1hr could not be longer enough to allow the emergence of the neuroprotective effects, which is easily tested by changing the duration of time between the stimulation with VA, SA and SS and their respectively IL formulations and the addition of neurotoxins 6-OHDA, MPP^+, Paraquat.

Another explanation could be related with the absence of microglia. In fact, in the previously published research related with the neuroprotection potential of VA, SA and SS, these compounds were tested in the presence of microglia. VA, SA and SS showed anti-inflammatory proprieties by inhibiting the activation of NF-κB factor, which is an important activator of microglia and promotes the differentiation of microglia into the phenotype M1, which promotes the neuroinflammation process. When the inhibition of NF-κB factor occurs, it is probable that

microglia will preferably differentiate into the phenotype M2, which promotes neuroprotection and tissue remodulation ((57), (58), (72), (56), (75), (76)).

Chapter 6

6. Conclusion and Future Perspectives

In this thesis, we found that VA, SA and SS, are not cytotoxic to the dopaminergic neurons and that their IL formulation [Chol][Van], [Chol][Syn] and [Chol][Van] is also not cytotoxic to the dopaminergic neurons. In terms of neuroprotection, we found out that VA, SA and SS didn't protect the neurons against the neurotoxins 6-OHDA, MPP^+ or Paraquat and that neither the IL formulations [Chol][Van], [Chol][Syn] and [Chol][Van] did.

In order to obtain neuroprotection in the PD cell models, it would be important to better understand how these formulations may act as neuroprotective in the dopaminergic neurons. For instance, tuning the concentrations [Chol][Van], [Chol][Syn] and [Chol][Sal] in order to find the best one that prevents DA neurodegeneration in PD.

It would also be very interesting to test the neuroprotection potential of VA, SA, SS, [Chol][Van], [Chol][Syn] and [Chol][Sal] in the presence of microglia. The same principle is applied to the cytotoxic assays, that is, to evaluate the putative cytotoxicity of these formulation in the presence of microglia, to evaluate the response upon stimulation with VA, SA, SS and their respectively IL formulations and how that response affects the dopaminergic neurons viability.

Chapter 7

7. Bibliography

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