Universidade de Lisboa

Faculdade de Farmácia

The Effect of Natural Compounds on PC12 Cell Viability in the Absence and Presence of Reactive Oxygen Species

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Mestrado Integrado em Ciências Farmacêuticas

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Resumo

Ao longo de milénios, a contribuição de produtos naturais para a medicina e saúde tem sido de extrema importância. Ainda hoje, os compostos naturais são considerados "uma fonte contínua de identificação de novos fármacos" pelo que, medicamentos ou potenciais fármacos, derivados de fontes naturais, são estudados no seguimento de uma necessidade urgente de novas terapêuticas para patologias tão nefastas como o cancro ou as doenças neurológicas.

De acordo com a Organização Mundial de Saúde, devido ao prolongamento da esperança média de vida e ao envelhecimento da população a nível mundial, os distúrbios neurológicos são uma das maiores atuais ameaças à saúde pública. Assim, torna-se essencial que tratamentos ou intervenções profiláticas eficazes sejam descobertos num futuro próximo, ou os custos sociais, financeiros e emocionais das doenças neurológicas tornar-se-ão avassaladores. No entanto, no caso das doenças neurodegenerativas, a complexidade dos mecanismos patológicos que promovem a neurodegeneração tem dificultado a descoberta de fármacos eficazes no tratamento destas patologias. Deste modo, umas das principais linhas de investigação nesta área tem como objetivo identificar as principais causas de desencadeamento dos processos neurodegenerativos e desenvolver formas de os modelar de forma específica. Ainda assim, foi recentemente sugerido que a ação contra um só mecanismo patológico, mesmo com elevada potência e seletividade, pode não ser suficiente para fazer face ao carácter multifatorial das doenças neurodegenerativas, e que uma abordagem múltipla deve ser desenvolvida. Consequentemente, o stress oxidativo, um mecanismo patológico comum a vários distúrbios neurodegenerativos, foi selecionado como potencial alvo-terapêutico no presente estudo.

Foi investigado o efeito de vários compostos naturais na viabilidade de células PC12, na ausência e na presença de espécies reativas de oxigénio (*reactive oxygen species* - ROS), bem como a influência destes mesmos compostos na expressão da anexina A2 (AnxA2), uma nova proteína reguladora dos mecanismos de oxidação-redução a nível celular. A AnxA2 tem sido envolvida numa quantidade crescente de patologias prevalentes, desde doenças neurológicas e autoimunes, a diversos tipos de cancro. Apesar das suas já conhecidas numerosas funções, rigorosamente reguladas por modificações pós-tradução, foi ainda recentemente proposto um papel na proteção de células e tecidos contra o dano oxidativo. A AnxA2 interage diretamente com o peróxido de hidrogénio (H_2O_2) de forma reversível, pelo que uma única molécula de anexina A2 é capaz de inativar várias moléculas de H_2O_2. Foi ainda publicado um estudo que demonstra o seu papel na proteção do DNA contra as espécies reativas de oxigénio e tem sido

sugerido que um aumento na expressão de AnxA2 pode constituir uma adaptação ao stress oxidativo. Em função destes dados, deduz-se que um composto capaz de aumentar de forma transiente os níveis de expressão de AnxA2, seria um potencial antioxidante exógeno, apto a reduzir o dano oxidativo celular.

Foram então fornecidos vinte compostos naturais, cujo efeito na viabilidade celular, tanto na ausência como na presença de espécies reativas de oxigénio, tinha sido previamente testado dentro de uma gama de concentrações mais baixa (2 nM a 20 µM). Como nenhum dos compostos demonstrou influenciar de forma clara a viabilidade celular, foram selecionados quatro compostos (esciadina, dimetil-esciadinonato, naringenina(3→6'')luteolina e ácido mefenâmico) que foram estudados com maior detalhe numa gama de concentrações mais ampla (2 nM a 1 mM). Foi estudado o efeito destes compostos na viabilidade celular, tanto na ausência de tóxico, de modo a avaliar a sua possível citotoxicidade, como na prevenção do dano oxidativo e na recuperação após dano oxidativo. As alterações na viabilidade celular foram detetadas através do método fluorométrico CellTiter-Blue® (CTB).

A esciadina foi o composto natural que apresentou resultados mais promissores, dado que não demonstrou citotoxicidade e potenciou de forma dose-dependente a viabilidade celular após exposição a ROS, contribuindo deste modo para a recuperação celular após dano oxidativo. Para além disso, a exposição a este composto aumentou os níveis de expressão de AnxA2. No entanto, uma vez que um aumento continuado na expressão desta proteína está geralmente associado a um fenótipo invasivo e metastático de uma grande variedade de cancros, torna-se necessário investigar mais detalhadamente o caráter deste aumento de expressão. Caso se revele transiente e fisiologicamente seguro, a esciadina poderá eventualmente vir a ser um fármaco antioxidante com potencial aplicação no stress oxidativo excessivo que é verificado nos processos neurodegenerativos.

O dimetil-esciadinonato apresentou um efeito na viabilidade celular muito semelhante, embora mais fraco, de forma geral, comparativamente à esciadina, o que pode resultar da sua estrutura molecular estreitamente relacionada. Curiosamente, ao contrário da esciadina, a exposição a este composto não parece afetar os níveis de expressão de AnxA2, o que pode significar que os grupos funcionais específicos da esciadina poderão ser responsáveis por desencadear o mecanismo pelo qual este composto aumenta a expressão de AnxA2. Para além disso, o dimetil-esciadinonato pode ainda ser uma alternativa, caso a exposição à esciadina se venha a demonstrar prejudicial ao organismo devido um aumento sustentado na expressão de anexina A2.

O potencial antioxidante da naringenina(3→6'')luteolina é mais questionável, dado que este composto manifestou citotoxicidade generalizada, principalmente em concentrações mais elevadas. Concentrações inferiores demonstraram influenciar de uma forma positiva a viabilidade celular na presença de ROS, em particular na prevenção de dano oxidativo. No entanto, torna-se necessária uma posterior verificação destes resultados, dada a variabilidade encontrada nos resultados obtidos com baixas concentrações, devida eventualmente à elevada viscosidade e difícil solubilidade deste composto. Devem ainda ser realizados estudos toxicológicos que permitam determinar a segurança da naringenina(3→6'')luteolina, tanto *in vitro* como *in vivo*.

O ácido mefenâmico manifestou um duplo efeito na viabilidade celular: concentrações mais baixas potenciaram a viabilidade celular, enquanto que concentrações mais elevadas provocaram citotoxicidade significativa. No entanto, as concentrações inferiores não apresentaram os efeitos positivos que seria de esperar relativamente à viabilidade celular na presença de ROS, considerando o potencial antioxidante e neuroprotetor sugerido para este composto. Deste modo, devem ser testadas diferentes abordagens, como a exposição a níveis menos acentuados de H_2O_2 (que se traduzem em danos oxidativos mais moderados, o que acontece em estados patológicos mais precoces), ou o prolongamento do tempo prévio de incubação com ácido mefenâmico. Curiosamente, ambas as concentrações testadas aumentaram proporcionalmente os níveis de expressão de anexina A2.

Contudo, apesar de ter sido determinado o efeito dos compostos selecionados na viabilidade celular de células PC12, considerado o seu efeito tanto na ausência como na presença de ROS e ter sido identificada uma correlação entre a exposição a estes compostos naturais e níveis de expressão de AnxA2 alterados, os mecanismos bioquímicos subjacentes a estes resultados não foram ainda determinados. Para além disso, o efeito na viabilidade celular dos restantes compostos naturais fornecidos, bem como a sua influência na expressão de AnxA2, está ainda por estudar. Assim, é necessário um volume considerável de investigação adicional para averiguar se algum destes compostos poderá vir a ser um potencial fármaco antioxidante, capaz de fazer face ao stress oxidativo que desempenha um papel determinante na progressão das doenças neurodegenerativas.

Palavras-chave: Compostos naturais; viabilidade celular; stress oxidativo; anexina A2; neurodegeneração

Abstract

To this day, natural compounds have been regarded as “a continuing source of novel drug leads”, with naturally inspired drugs and potential drug candidates being studied in the urgent need for new medicines and therapies for major health disorders, such as neurological diseases - one of today’s greatest threats to public health. However, the complexity of the neurodegenerative processes has made it difficult for an effective drug to be discovered. Recently, it has been suggested that acting against a single pathogenic mechanism might prove insufficient to face the multifactorial nature of neurodegenerative disorders and that multiple approaches must be pursued. Therefore, oxidative stress, a common mechanism of neurodegeneration, is addressed in this study as a potential therapeutic option.

The effect of several natural compounds on PC12 cell viability in the absence and presence of H_2O_2-induced cell death was investigated, together with their influence in the expression of Annexin A2, which was recently proposed to protect cells and tissues from oxidative damage.

Beforehand, the effect of a library of twenty natural compounds (2 nM to 20 μM) on cell viability was investigated in PC12 cells, both in the absence and presence of H_2O_2. As none of the compounds exhibited a clear influence on cell viability at these concentrations, four compounds (sciadin, dimethyl sciadinonate, naringenin (3→6’’) luteolin and mefenamic acid) were selected and tested in further detail on a wider concentration range (2 nM to 1 mM).

Sciadin presented the most promising results, exhibiting no cytotoxicity and contributing for cellular recovery upon oxidative damage. If a transient increase in the expression of AnxA2 is confirmed, sciadin could be a potential antioxidant candidate to address the pathological oxidative stress observed in neurodegeneration. Although the other compounds induced a slight but significant increase in cell viability before and after H_2O_2 administration, they did not affect AnxA2 (dimethyl sciadinonate, naringenin (3→6’’) luteolin), or led to a significant increase in cell death in the absence of the toxic stimuli (naringenin (3→6’’) luteolin and mefenamic acid).

Nevertheless, a considerable amount of additional work is required before we can learn which (if any) of the given twenty compounds are strong antioxidant drug candidates to treat neurological diseases.

Keywords: Natural compounds; cell viability; oxidative stress; annexin A2; neurodegeneration

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Abbreviations

Aβ	Amyloid β-protein
AChE	Acetylcholinesterase
AD	Alzheimer Disease
AD-TNDCI	AD-type neurodegeneration with cognitive impairment
AIDS	Acquired immunodeficiency syndrome
ALS	Amyotrophic Lateral Sclerosis
ANOVA	Analysis of variance
AnxA2	Annexin A2
API	Active Pharmaceutical Ingredient
BCE	Before Common Era
Bcl-XL	B-cell lymphoma-extra large
CE	Common Era
COMT	Catechol-O-methyltransferase
CTB	CellTiter-Blue®
Cys	Cysteine
DALY	Disability-adjusted life year
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
ECL	Enhanced Chemiluminescence
EGTA	Egtazic acid
FBS	Fetal bovine serum
FTIR	Fourier Transform Infrared
GPx	Glutathione Peroxidase
HD	Huntington's disease
HIV	Human Immunodeficiency Virus

HO	Heme Oxygenase
HRP	Horse Radish Peroxidase
HS	Horse serum
HSD	Honest Significant Difference
IC50	Half maximal Inhibitory Concentration
IgG	Immunoglobulin G
IL	Interleukin
IR	Infrared
MAO-B	Monoamine Oxidase B
MIR	Mid-Infrared
Mr	Relative Molecular mass
MVB	Multivesicular body
NGF	Nerve Growth Factor
NSAID	Nonsteroidal Anti-inflammatory Drug
OHDA	Hydroxydopamine
PBS	Phosphate Buffered Saline
PC12	Pheochromocytoma 12
PD	Parkinson Disease
P/S	Penicillin-streptomycin
PTM	Post-translation Modification
qRT-PCR	Quantitative Reverse Transcription - Polymerase Chain Reaction
R&D	Research and Development
RIPA	Radioimmunoprecipitation assay
ROS	Reactive Oxygen Species
RPMI	Roswell Park Memorial Institute
SCA	Spinocerebellar Ataxia

SDS	Sodium Dodecyl Sulphate
SDS-PAGE	SDS-Polyacrylamide Gel Electrophoresis
SEM	Standard Error of the Mean
Ser	Serine
SOD	Superoxide Dismutase
SUMO	Small Ubiquitin-like Modifier
TBS/T	Tris-buffered saline – tween
TNF	Tumour Necrosis Factor
Tyr	Tyrosine
Ub	Ubiquitin
UV	Ultraviolet
WHO	World Health Organization

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1 Introduction

1.1 Natural compounds as drugs

Over the ages, humans have relied on nature to fulfil their basic needs and the medicinal use of natural products may even extensively precede recorded human history (1,2). Natural sources have been used by civilizations since the ancient times, to provide treatment for a broad range of ailments and diseases (1,3).

Paleoanthropological studies suggest that Neanderthals, more than 60 000 years ago, might have been aware of several plants' medicinal properties (2). First records, however, date from 2600 BCE, reporting the medical use of plant-derived compounds in Mesopotamia, some of which are still currently applied (1,4). Since then, documented records have multiplied, e.g.: the Egyptian "Ebers Papyrus" from 1500 BCE, a pharmaceutical record describing plant-based gargles, pills, infusions and ointments; the notorious Chinese Materia Medica, from 1100 BCE; and the Indian Ayurvedic medicine from before 1000 BCE, one of the world's oldest medical systems (1,4,5).

In the Western world, the Greeks and Romans played an important role in the rational use of herbal drugs, through personalities such as the Roman pharmacy and medicine teacher as well as practitioner, Galen (130–200 CE), and the Greek physician Dioscorides (100 CE) (1), who laid the foundations of pharmacology in Europe (2). Over the Dark and Middle Ages (500-1200 CE), this knowledge was preserved by the Arabs, who combined it with their own resources, together with their knowledge on Chinese and Indian herbs, (1) being the first to privately own pharmacies (4,6).

Despite the broad use of medicinal plants worldwide, it was not until the 18th and 19th centuries that their active components were identified, opening a new era for natural products' research and usage. Analytical and structural chemistry allowed for compound purification and molecular structure determination. Thus, chemical synthesis replaced isolation from natural sources, considerably reducing drug production costs. Furthermore, chemists could now modify their structures, in order to suppress or enhance specific properties (2). Due to this scientific breakthrough, relevant drugs were discovered and optimized, such as: penicillin (*Penicillium notatum*) (2); aspirin (*Salix alba*) (7); digitoxin (*Digitalis purpurea* L.) (4); morphine and verapamil (*Papaver somniferum* L.); quinine (*Cinchona* spp.); and even anticancer drugs, like paclitaxel (Taxol®) derived from the leaves of various Taxus species (1).

Remarkably, over millennia, the importance of natural products for medicine and health has been tremendous and an extensive range of natural compounds has been discovered and used (2). Even so, from the early 1980s, the boost in molecular biology and combinatorial chemistry allowed for the rational design of chemical compounds which target specific molecules and, thus, natural products have taken a secondary role in drug discovery and development. The belief that high-throughput screening and combinatorial chemistry would be the future source of new chemical entities and drug leads, was responsible for the discontinuation of most natural-based R&D programs from the pharmaceutical industry (1,2,8). However, although these techniques revolutionized the development of active chemical leads (8), they did not bring the expected outcomes in terms of new drug candidates (2). Hence, in the last years, attention has been turning back to natural compounds as a "continuing source of novel drug leads" (1,2), with naturally inspired drugs and potential drug candidates being studied in major therapeutic areas such as neurological (9–14), immunological and inflammatory (9,11), cardiovascular (1,11), infectious (9,11,15) and oncological diseases (9,11,13,15,16).

The cumulative experience of thousands of years of ancient medical knowledge, boosted by modern pharmaceutical research have, therefore, opened the doors to new and powerful drug combinations, on a renewed interest in natural products in drug discovery (2,17). Their potential must be addressed in the urgent need for new pharmaceuticals and therapies for health disorders as devastating as cancer or neurological diseases (1,2).

1.2 Neurological diseases and therapeutics

Due to prolonged life expectancy and the global ageing of populations, neurological disorders are one of today's greatest threats to public health. According to the World Health Organization (WHO), they constitute nearly 12% of total deaths and their burden is already higher than HIV/AIDS, malignant neoplasms and ischaemic heart disease, on a global scale.

Neurodegenerative diseases as Alzheimer disease (AD) and other dementias are estimated to increase by 66% from 2005 to 2030, referring to disability-adjusted life years (DALYs) (18). With such a drastic prevalence increase, the WHO launched a Comprehensive Mental Health Action Plan 2013-2020, which states the importance of strengthening evidence and research on this matter (19). In fact, a much deeper knowledge about the brain is necessary (18) and, although extensive research has been conducted (20–24), therapeutic options remain limited (25). It is, therefore, of the utmost importance that effective treatments or prophylactic

interventions for neurodegenerative disorders are discovered in a near future, or the societal, financial and emotional costs of these neurological diseases will be staggering (26).

1.2.1 Neurodegenerative disorders

Neurodegenerative disorders represent a set of pathological conditions resulting from a progressive and irreversible dysfunction and loss of neurons and synapses in specific areas of the nervous system, which determine clinical presentation and development (27). Examples are Alzheimer's disease (AD), Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS), Huntington's disease (HD) and spinocerebellar ataxias (SCAs), among others (28).

Basic processes leading to neurodegeneration are multifactorial, influenced by genetic, environmental and endogenous factors related to ageing, although their exact molecular and pathogenic mechanisms are still to be fully understood (27). Nonetheless, a common mechanism underlying several neurodegenerative disorders is extensive evidence of oxidative stress (27,29). Other mechanisms include: abnormal protein dynamics, often with actions and mutations of molecular chaperones; impaired bioenergetics, mitochondrial dysfunctions and DNA damage; fragmentation of neuronal Golgi complexes; disruption of the cellular/axonal transport; dysfunction of neurotrophins; and neuroinflammatory/neuro-immune processes. A comprehensive analysis of all these processes, which collectively lead to cell dysfunction and death, was reviewed by Jellinger K (27).

The complexity of neurodegeneration leading processes and its multifactorial nature have made it difficult for an effective drug to be discovered (30,31). In fact, current clinically relevant medicines and therapies are scarce and offer only limited and temporary symptomatic relief to patients, being unable to significantly slow or cease the underlying pathology progression of these diseases (28,30,31). This is the case of the approved acetylcholinesterase (AChE) inhibitors and memantine for managing AD (32), or the dopamine agonists, Monoamine oxidase B (MAO-B) and Catechol-O-methyltransferase (COMT) inhibitors, levodopa and other adjuvant therapies for managing PD (33,34). Therefore, most leading scientific research aims to identify the major causes of neurodegenerative disorders and develop ways to target them (28).

Different targets have been identified in sporadic and genetic neurodegenerative disorders, nonetheless, no efficient therapies have been found. For instance, in AD, the amyloid β-protein (Aβ) hypothesis was regarded as "the dominant model of AD pathogenesis", leading a global

effort to develop potential treatments targeting proteins involved in Aβ generation (35). On the other hand, in genetic disorders such as HD, caused by the expression of a mutant protein, effort has been directed to develop molecules targeting the mutated proteins, such as huntingtin, as the main therapeutic option (28). However, these approaches take nothing away from the need for alternative new molecules, able to target other early processes of these complex and devastating diseases. Instead of "one hypothesis against another", multiple approaches must be pursued to find a range of therapeutics which may work together as a solution (35). In fact, regarding the extensively studied AD, for example, strategies as polypharmacology have been addressed as "one of the most promising and innovative paradigms in drug research" and it has been suggested that acting against a single pathogenic mechanism, even with high potency and selectivity, might prove insufficient to face the multifactorial nature of neurodegenerative disorders (31). Thus, for being a common mechanism of neurodegeneration, oxidative stress has been selected as a subject of our work.

1.2.2 Neurodegeneration and oxidative stress

Reactive oxygen species (ROS) are highly reactive compounds with the ability to damage multiple biological molecules, including proteins, lipids, carbohydrates and DNA (36). Following molecular damage, a cascade of events is initiated, including dysfunction of mitochondrial respiration, excitotoxicity, and a lethal rise in cytosolic calcium, resulting in cellular dysfunction and a positive feedback loop of ROS (27,37,38). Examples of ROS are the superoxide anion radical ($O_2^{-\bullet}$), the hydroxyl radical ($^{\bullet}OH$), and hydrogen peroxide (H_2O_2), which readily forms hydroxyl free radicals by the Fenton or Haber-Weiss reaction (38,39). Although they can also be formed in response to external stimuli (40), oxygen free radicals and ROS are common products of aerobic cellular metabolism (38). When their production exceeds the cells' antioxidant defence mechanisms, oxidative stress takes place (27). Thus, oxidative stress can be defined as a balance disturbance between the production of ROS and antioxidant defences, which may lead to tissue injury (40).

Oxidative stress plays indeed a crucial role in the pathogenesis of various diseases, including neurodegenerative disorders, cancer and ischemia (37,41). The brain, however, is particularly vulnerable to oxidative damage due to high oxygen consumption, increased levels of polyunsaturated fatty acid (which are readily attacked by free radicals), relatively high levels of redox transition metal ions, reduced levels of physiological antioxidants (e.g. glutathione,

catalase, and superoxide dismutase) and low regenerative capacity (29,37,38). Thus, ageing is followed by an increase in oxidative stress (38).

Under complex disease conditions, oxidative stress is linked with major pathological processes in AD, such as Aβ-induced neurotoxicity, tau pathology, mitochondrial dysfunction, as well as metal dyshomeostasis, and it may promote the initiation and progression of AD (38). Extensive exposure to ROS can also indirectly lead to protein misfolding (e.g. α-synuclein in PD), as well as to the dysfunction of protein degradation systems (like the ubiquitin proteasome system or autophagy), which play a key role in the appearance of the deleterious events implicated in the neurodegenerative process (42). Therefore, besides genetic and environmental elements, oxidative stress is one of the leading factors in most neurodegenerative diseases like AD and PD (38), and thus proteins involved in the cellular antioxidant protection mechanism are potential therapeutic targets (29).

As previously mentioned, cells present robust defence systems against ROS in order to avoid cellular damage. These include: antioxidant compounds (vitamins E, A and C, uric acid, glutathione), antioxidant scavenging enzymes (superoxide dismutase, catalase, peroxidases) and secondary defences like lipolytic enzymes, DNA repair enzymes, endonucleases, proteases, among others. However, the complexity of the intracellular antioxidant network complicates the full understanding of the overall protective efficacy of these defence systems (43). Recently, a potential new defence mechanism against oxidative stress has been hypothesised, in which Annexin A2 (AnxA2) plays a critical role (44).

1.2.3 Oxidative stress and Annexin A2

Annexins are a large family of structurally related, calcium-dependent anionic phospholipid-binding proteins, present in all eukaryotic cells (44,45), including neuronal and glial brain cells (46,47). They participate in numerous processes, from regulation of membrane and cytoskeleton dynamics, to cell migration, proliferation and apoptosis (45).

Within this family, AnxA2 (Figure 1.1) is the most extensively studied member (48). This 39 kDa (36 kDa by SDS-PAGE) multifunctional protein consists of two principal domains: a 33 kDa C-terminal core structure folded in a tightly packed α-helical conformation; and a 3-4 kDa unique N-terminal region. AnxA2 also undergoes post-translation modifications (PTMs), believed to discriminate between its different functions. Tyr23 phosphorylation, for example, occurs in the N-terminal region and is required for stable binding of AnxA2 to endosomes.

Subsequently to this modification, the protein is transported from early to late endosomes, associated with lipid rafts and multivesicular bodies (MVB), and finally localized in the lumen of exosomes (36).

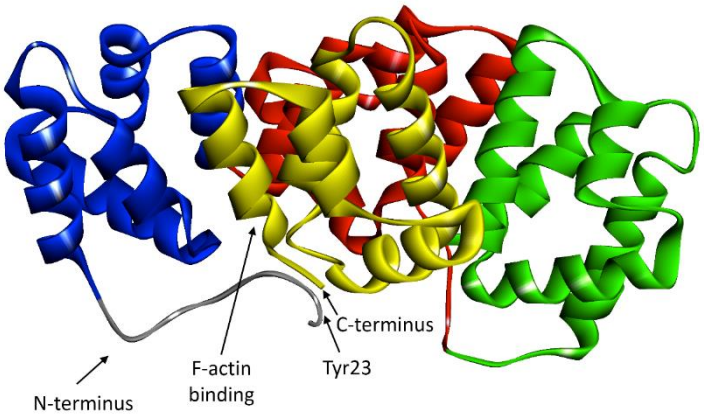


Figure 1.1 Annexin A2 structure. Crystal structure of bovine AnxA2. The first visible amino acid is Ser21. Blue, red, green and yellow represent the four annexin domains. F-actin-binding and Tyr23 phosphorylation sites, as well as the N- and C-terminal regions are also displayed. Protein Databank Code: 4X9P (49). Adapted from Grindheim AK, et al. (50).

The role of AnxA2 has been shown in a growing list of human diseases. It has been reported that dysregulation and abnormal expression of AnxA2 are correlated with prevalent pathologies, ranging from sepsis (48) and autoimmune diseases to a large number of cancers (36,51), where AnxA2 plays an essential role on tumour cell adhesion, proliferation, apoptosis, invasion and metastasis along with neovascularization, through diverse modes of action (52). AnxA2 upregulation is generally associated with an aggressive and metastatic cancer phenotype, being directly related with advanced clinical stages of several cancer types (50,52,53). On the other hand, the inverse correlation was identified for oesophageal carcinomas and head and neck squamous cell carcinoma, where AnxA2 downregulation is closely related with advanced clinical stage, more frequent recurrence and regional lymph node and distant metastasis (52). Moreover, regarding the immune system, the upregulation of AnxA2 stimulated the production of TNF-α, IL-1β, IL-6 as well as other chemokines, this way contributing to the recruitment and activation of macrophages, suggesting a positive role for AnxA2 in the establishment of inflammation and an immune response (54). In fact, total amounts of AnxA2 vary between different cells and tissues (53), suggesting distinct dominant

functions according to cell type, which are tightly regulated by complex PTMs, ligand binding and subcellular localisation (50,52,53).

Its expression and function in the brain have also been studied, although there is still much to learn on this topic when compared with the information obtained from peripheral tissues. However, it is clear that AnxA2 overexpression is largely associated with brain pathological conditions, such as tumour, inflammation and neurodegeneration (36,47,55,56).

In recent years, AnxA2 has been identified as "a novel cellular redox regulatory protein" (57), as it directly interacts with H_2O_2 in a reversible manner. Its unique Cys8 residue is readily oxidised by H_2O_2 and posteriorly reduced by the thioredoxin system, allowing AnxA2 to participate in multiple redox cycles. Therefore, a single molecule of AnxA2 is able to inactivate several molecules of H_2O_2 (58). In fact, its upregulation in response to H_2O_2-induced oxidative stress, as well as its increased phosphorylation, have also been reported for different cell types (36). Moreover, apart from being localised in the cytoplasm and plasma membrane, its presence in the nucleus is also significant, where it contributes to the protection of DNA against H_2O_2 (36,58). Recent findings show that, when rat adrenal pheochromocytoma (PC12) cells are exposed to oxidative stress, two separate events take place: nuclear pTyr32AnxA2 is dephosphorylated and cytoplasmic AnxA2 is Tyr23 phosphorylated (Figure 1.2A) (36). This phosphorylation step allows for AnxA2 to bind to the cytoplasmic surface of endosomes, which develop into MVBs due to the invagination of the endosomal membrane. Consequently, pTyr23AnxA2 is localized to the lumen of these vesicles. As the MVBs fuse with the plasma membrane, the vesicles are released to the extracellular space as exosomes (Figure 1.2B). In addition, it has been shown that cells exposed to these extracellular vesicles (Figure 1.2C) develop a higher tolerance for a subsequent exposure to H_2O_2, thus increasing their viability. Their level of AnxA2 and pTyr23AnxA2 are also increased during subsequent ROS exposure (Figure 1.2C and D). Therefore, it has been hypothesized that this increment in the expression of AnxA2 may represent an adaptation to oxidative stress (44). Thus, although the exact mechanisms are yet to be unveiled, it is clear that AnxA2 plays a key role in cellular redox regulation, particularly during oxidative stress (58). According to this, one could deduce that a compound able to increase the expression of AnxA2 would be a potential exogenous antioxidant, reducing cellular oxidative damage.

These findings indicate that sustained high levels of AnxA2 are largely associated with adverse effects, while a short-term transient upregulation may be beneficial regarding immune stimulation and protection against oxidative stress.

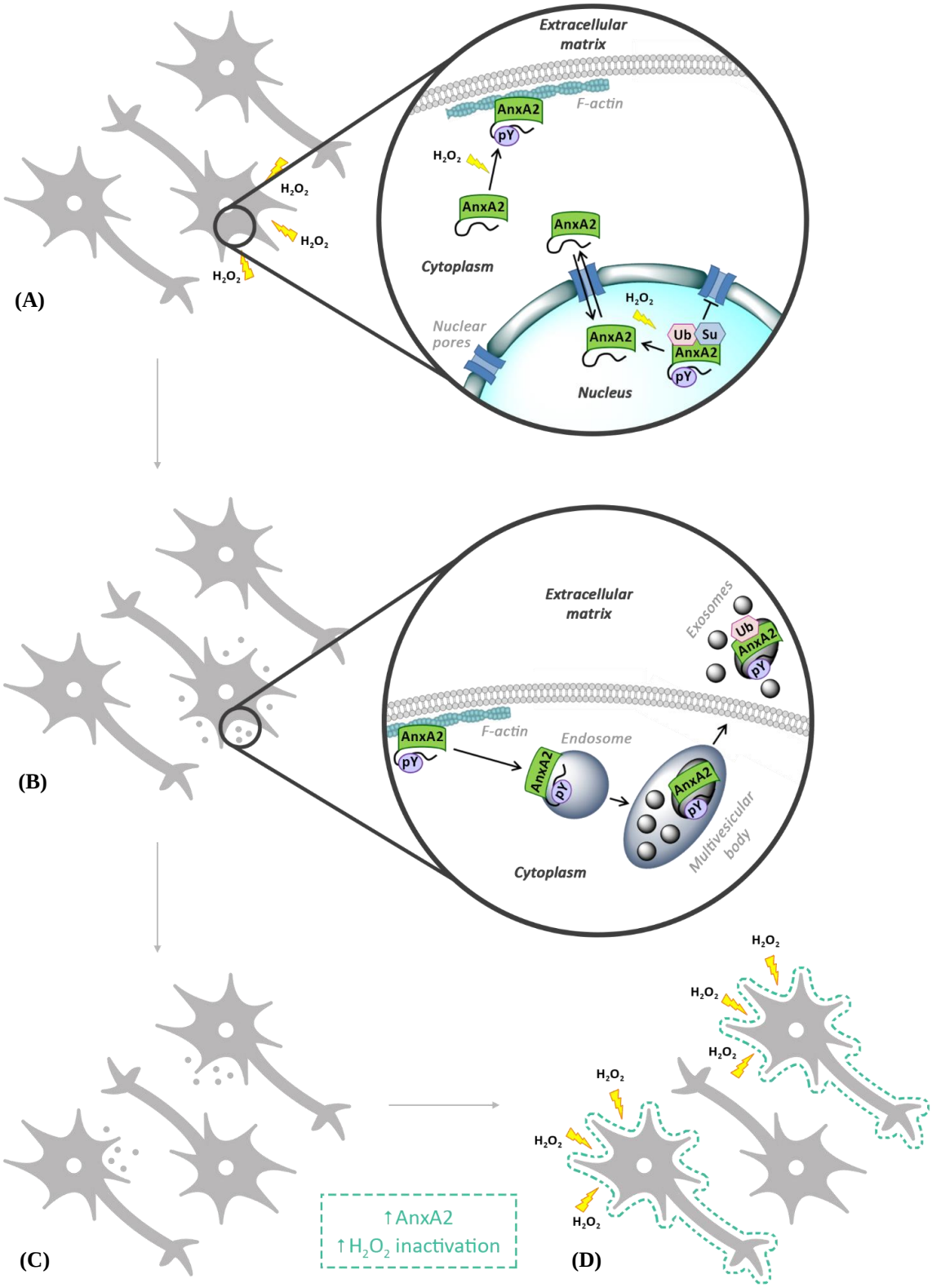


Figure 1.2 Schematic representation of the presumed AnxA2-based adaptation to oxidative stress. A: Due to oxidative stress (H_2O_2), nuclear pTyr23AnxA2 undergoes rapid and transient dephosphorylation, while cytoplasmic AnxA2 is phosphorylated at Tyr23 (pY). Ub and Su represent ubiquitin and SUMO, respectively. B: Cytoplasmic pTyr23AnxA2 binds to F-actin and associates with early endosomes, residing in MVBs and being finally released in the lumen of exosomes. C: Surrounding cells are exposed to these exosomes and appear to increase their level of AnxA2 and pTyr23AnxA2. D: During a subsequent exposure to ROS, higher H_2O_2 inactivation may be responsible for an increase in cell viability. Figure adapted from Grindheim AK, et al. (36).

2 Aims

Considering the urgent need for new effective drugs and therapies for neurodegenerative disorders, and given its complex multifactorial character, a multiple approach, targeting different pathological mechanisms, may be the best solution. Therefore, oxidative stress, a common mechanism of neurodegeneration, is addressed in this study. The aim of this project is to investigate the effect of several natural compounds on PC12 cell viability in the absence and presence of ROS, along with their influence on AnxA2 expression. As mentioned before, AnxA2 is a cellular redox regulatory protein, recently proposed to protect cells and tissues from oxidative damage.

To accomplish this aim, the following steps were established:

- Investigate the cytotoxic effect of a library of natural compounds;
- Investigate the effect of selected natural compounds in oxidative damage prevention;
- Investigate the effect of selected natural compounds in the recovery from oxidative damage;
- Determine the effect of selected natural compounds on AnxA2 expression.

Therefore, a library of twenty natural compounds was provided, of which four were arbitrarily selected and tested in further detail. PC12 cells were incubated with H_2O_2, and various concentrations of the selected natural compounds were used to determine which concentrations revert ROS-induced cell death. Finally, AnxA2 expression levels were determined by immunoblotting.

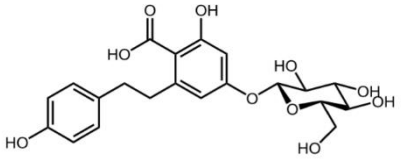
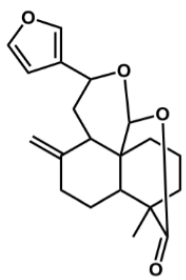
3 Materials and Methods

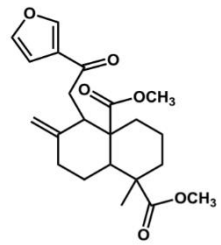
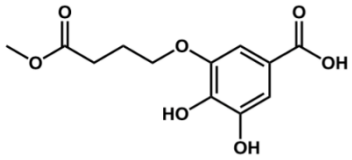
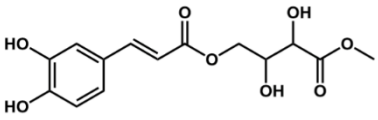
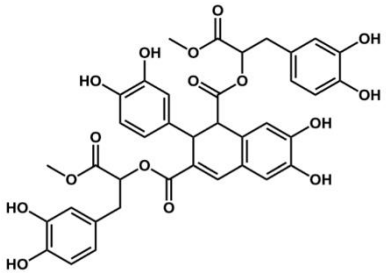
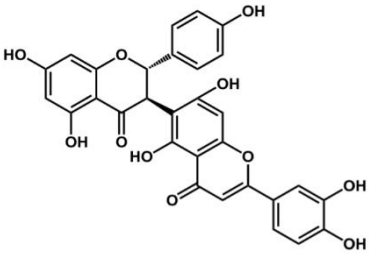
3.1 Preparation of stock solutions from natural compounds

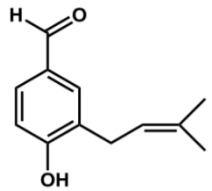
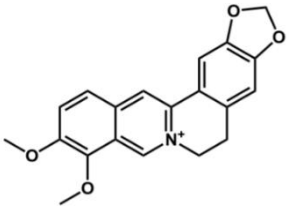
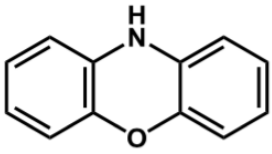
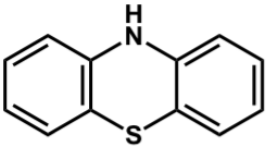
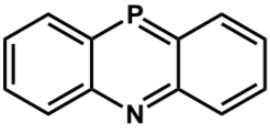
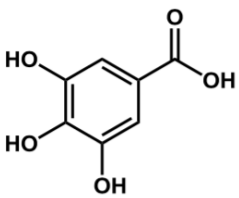
A library of 20 natural compounds was kindly provided by Prof. Torgils Fossen (UiB). In order to reduce or eliminate bias until final results were analysed, all compounds were numbered, and will hereafter be referred as so. Although compounds 8-16 and 20 are commercially available, compounds 1-7, 17 and 19 were isolated by Prof. Torgils Fossen's research group. Compound 18 was extracted from *Aglaia Meliaceae* and modified with Br by Prof. Laurent Désaubry (59).

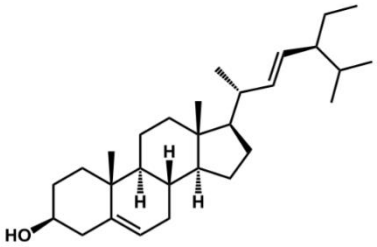
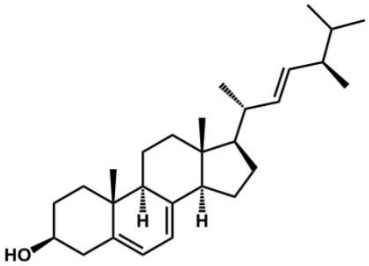
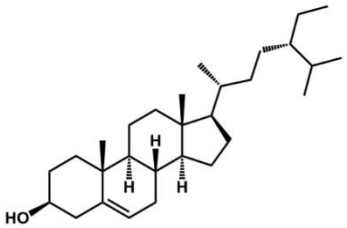
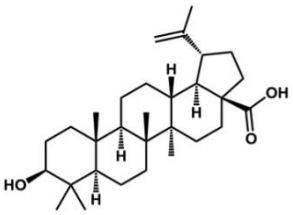
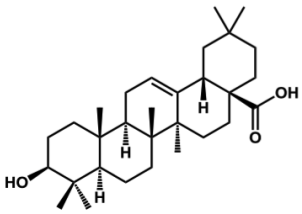
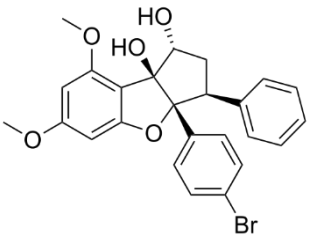
Purified samples from the natural compounds are listed in Table 3.1 and were provided as solids. For further testing on cell viability and effect on AnxA2 expression levels, the samples were solubilised with the amount of DMSO (Sigma®, St. Louis, USA) needed to obtain a final concentration of 150 mM (stock solution). Several 100x dilutions were made in separate vials using RPMI 1640 (Sigma®) as a solvent, allowing for practical pipetting. All solutions were stored at -20ºC.

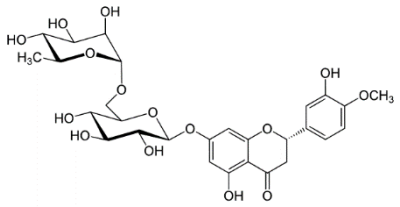
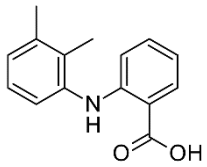
Table 3.1 Library of natural compounds tested.

No	Compound (Natural Source, Copyright supplier)	Chemical Formula	Mr	Molecular Structure
1	6-Carboxydihydroresveratrol 3-O-β-glucopyranoside (*Metasequoia glyptostroboides* (60), Torgils Fossen's group)	$C_{21}H_{24}O_{10}$	436.41	
2	Sciadin (*Sciadopitys verticillata* (61), Torgils Fossen's group)	$C_{20}H_{24}O_4$	328.41	

3	Dimethyl sciadinonate (*Sciadopitys verticillata* (61), Torgils Fossen's group)	$C_{22}H_{28}O_6$	388.46	
4	GHB-2 (*Viscum album* (62), Torgils Fossen's group)	$C_{12}H_{14}O_7$	270.23	
5	4-Oxy-(E)-caffeoyl-2,3--dihydroxybutanoic acid methyl ester (*Echium amoenum*, Torgils Fossen's group)	$C_{14}H_{16}O_8$	312.27	
6	Bis (3-(3,4--dihydroxyphenyl)--1-methoxy-1-oxopropan--2-yl) 2-(3,4--dihydroxyphenyl)-6,7--dihydroxy-1,2--dihydronaphthalene-1,3--dicarboxylate (*Echium amoenum*, Torgils Fossen's group)	$C_{38}H_{34}O_{16}$	746.67	
7	Naringenin(3→6'')luteolin (*Narthecium ossifragum* (63), Torgils Fossen's group)	$C_{30}H_{20}O_{11}$	556.48	

8	4-Hydroxy-3-(3-methylbut-2-enyl) benzaldehyde (*Narthecium ossifragum* / *Heterobasidion occidentale* (63,64), Aldlab Chemicals)	$C_{12}H_{14}O_2$	190.24	
9	Berberine (*Berberis* L. (65,66), Sigma)	$C_{20}H_{18}NO^+$	336.37	
10	Phenoxazine (*Streptomyces* spp. – modified actinomycin derivate (67), Sigma)	$C_{12}H_9NO$	183.21	
11	Phenothiazine (*Streptomyces* spp. – sulphur modified actinomycin derivate (67), Sigma)	$C_{12}H_9NS$	199.27	
12	Phenophosphazine (*Streptomyces* spp. – phosphor modified actinomycin derivate (67), Sigma)	$C_{12}H_8NP$	197.18	
13	Gallic acid (Several natural sources ex: *Viscum album* (62), Sigma)	$C_7H_6O_5$	170.12	

14	Stigmasterol (Several natural sources ex: *Ageratum conyzoides* (68,69), Carl Roth)	$C_{29}H_{48}O$	412.70	
15	Ergosterol (Several natural sources ex: *Aspergillus fischeri* (70), Carl Roth)	$C_{28}H_{44}O$	396.66	
16	β-sitosterol (Several natural sources ex: *Ageratum conyzoides* (69), Sigma)	$C_{29}H_{50}O$	414.72	
17	European Mistletoe Triterpene acids: - Betulinic acid (20%) - Oleanolic acid (80%) (*Viscum album* (71), Torgils Fossen's group)	$C_{30}H_{48}O_3$	456.71	 
18	Flavagline (FL3) (*Aglaia Meliaceae* modified Br derivate (59), Laurent Désaubry)	$C_{25}H_{23}BrO_5$	483.36	

19	Hesperidin (*Citrus* L. (72), Torgils Fossen's group)	$C_{28}H_{34}O_{15}$	610.56	
20	Mefenamic acid (*Viscum album* alkaloids' modified derivate (73), Sigma)	$C_{15}H_{15}NO_2$	241.29	

3.2 Cell culture

3.2.1 Working conditions

In order to prevent cell contamination and ensure reliability of results related to cell culture work, an aseptic working area was established: all cell handling experiments took place in a laminar vertical flow hood; the work surface and materials placed inside the hood were disinfected with 70% (v/v) ethanol and wiped clean; sterile disposable materials were used and personal protective equipment was accounted for. Whenever contamination was suspected, the material was discarded.

When not in use, all chemicals, reagents, substances, buffers and solutions were stored as recommended by the supplier. Cell cultures and plates were maintained inside a humid CO_2 incubator (T=37.0ºC; CO_2=5.0%).

3.2.2 Cultivation of PC12 cells

PC12 cells derive from a noradrenergic clonal cell line established in 1976 as a "useful model system for the study of numerous problems in neurobiology and neurochemistry" (74). PC12 cells can synthetize, store and release dopamine and norepinephrine, resembling noradrenergic adrenal chromaffin cells, and the phenotype of sympathetic ganglion neurons upon differentiation with nerve growth factor (NGF) (74,75). The fact that they can be subcultured indefinitely, together with their enormous versatility for pharmacological manipulation, ease of culture and the large background knowledge on their proliferation and differentiation, make

them an extremely useful widely used model for neuronal differentiation and neuropharmacological and neurotoxicological studies (75).

An adherent subclone of the original neuroendocrine PC12 cell line (74) was used in the experiments. It was generously provided by Prof. Eyvind Rødahl, Haukeland University Hospital, UiB. Cells were grown in complete RPMI medium, prepared as indicated in Appendix A1. Horse serum (HS) and fetal bovine serum (FBS) were added to allow cell growth in the absence of NGF; penicillin-streptomycin (P/S) and plasmocin were present to avoid biological contamination; extra L-glutamine prevented its depletion in the growth medium; and NaOH addition allowed for a final pH of ±7.4. Complete RPMI medium was stored at 2-8ºC.

3.2.3 Subculturing of PC12 cells

In order to enable further propagation of the cell line, while ensuring reproducibility and allowing cell culture health status monitoring, cells were split and subcultured on a regular basis. This occurred every two (1:3 split ratio) or three (1:5 split ratio) days, according to the dilution factor and use in experiments. At subculturing time, cells were approximately 80% confluent.

Firstly, the 25 cm^2 flask (T25), was knocked against the palm so that the cells would detach from the bottom. It was then examined under the microscope to ensure 90% detachment. Secondly, the content was resuspended 3-4 times before pipetting the necessary volume of cell suspension and complete RPMI medium into new cell culture flasks. Finally, the new flasks were gently shaken to provide homogenous cell dispersion and contact with the bottom, and were then incubated for further growth.

3.3 Cell viability testing

3.3.1 Plating of PC12 cells

When cultures reached 80% confluency, cells were detached (see Section 3.2.3) and transferred to a 50 mL tube. Cells were counted using a haemocytometer. PC12 cells were plated in 96-well plates (BD Falcon™, BD Biosciences, Erembodegem, Belgium). The volume containing 35 000 cells (76) was calculated and pipetted to each well, followed by the corresponding amount of complete RPMI to obtain 100 µL/well. Subsequently, the plate was gently shaken to

ensure contact with the bottom and incubated overnight. Cell growth and attachment was then examined under the microscope.

3.3.2 Cell viability assay

Cell viability was measured using Promega's CellTiter-Blue® (CTB) fluorometric method. 20 µL of CTB reagent (indicator dye resazurin) were pipetted into each well. Unlike dead cells, which have lost their metabolic capacity, viable cells retain the ability of metabolically reducing resazurin (dark blue, limited intrinsic fluorescence) into resorufin (pink, highly fluorescent, $560_{Ex}/590_{Em}$). After 1 hour of incubation (76), fluorescence was measured at λ=590 nm with a microplate reader (Victor^3, 1420 Multilabel Counter, Perkin Elmer, Norway). Since the reagent is light sensitive, experiments were conducted in the absence of direct light and, when not in use, the reagent was stored protected from light at -20ºC. On the day before usage, the reagent was stored in the dark at 2-8ºC, allowing for prompt application.

3.4 Preparation of cell lysates

PC12 cells were plated in 6-well plates (Nunc™, Roskilde, Denmark). Cells were incubated with the selected natural compounds and, 24 hours later, the plate was tapped and cells scraped off the wells' base. Cells were then transferred to 15 mL tubes and 8 mL of phosphate buffered saline (PBS) (Medicago, Uppsala, Sweden) was added. Cells were pelleted by centrifugation (2000 rpm, 5 min), rinsed with PBS and centrifuged once more (2000 rpm, 5 min). PBS was removed, the pellets were resuspended in 100 µL of lysis buffer (see Appendix A1), transferred to microcentrifuge tubes and incubated on ice for 15 min. Subsequently, the lysate was centrifuged (12000 rpm, 30 min, 4ºC) and the supernatant transferred to a new microcentrifuge tube. Samples were stored at -20ºC.

3.5 Protein quantitation

Total protein was determined by Mid-Infrared (MIR)-based protein quantitation. Infrared Spectroscopy analyses the interaction between molecules and IR light. Considering that proteins, lipids, carbohydrates and other biomolecular components show different IR spectra, they can be analysed separately by this technique, eliminating signal interferences from other components. Another advantage of MIR-based protein quantitation is minimal dependence

upon amino acid composition, when compared with e.g. 280 nm UV absorbance (77). The method required the addition of 2 µL lysis buffer (as blank) and 2 µL of each total protein sample (see Section 3.4) to IR sample reader cards. When the card spots were dry, total protein was quantified with a Fourier Transform Infrared (FTIR) spectrometer (Direct Detect®, Merck Millipore, Massachusetts, USA).

3.6 Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The sample volume corresponding to 30 µg of total protein was calculated and added to a microcentrifuge tube, together with acetone (4×sample volume). The new samples were homogenized and incubated overnight at -20ºC for protein precipitation. On the following day, they were centrifuged (16000 rpm, 20 min, 4ºC) and acetone was removed. After the pellet dried, 21 µL ddH_2O and 7 µL super4× denaturing buffer (see Appendix A1) were added to each tube. Samples were heated for 15 min at 56ºC and briefly centrifuged, immediately before loading. They were then loaded onto 10% (w/v) sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (PAGE) gels (10% acrylamide/bisacrylamide 37.5:1 crosslinker ratio) together with a prestained protein standard (PageRuler™, 10-180 kDa, Thermo Fisher Scientific, Vilnius, Lithuania). SDS-PAGE was performed at 25 mA (250 V limiting) in electrophoresis buffer (see Appendix A1), until the front nearly reached the gel bottom. Gels were removed from the electrophoresis equipment and analysed on a molecular imager (ChemiDoc™ XRS+, Bio-Rad, Oslo, Norway).

3.7 Western blot analysis

To allow for AnxA2 detection, proteins separated by SDS-PAGE were transferred to a nitrocellulose membrane (Amersham™Protran™ 0.2µm NC, GE Healthcare Life Sciences) by electrotransfer. A blotting cassette, two porous mats, two blotting papers and a nitrocellulose membrane were submerged in blotting buffer (see Appendix A1) and placed so that SDS-PAGE gel proteins could migrate onto the membrane. Any air bubbles were removed, cassettes were closed and transferred to the blotting chamber, filled with blotting buffer. A magnetic stirrer was added to the bottom and the transfer ran at 50 V/h (25 V, 2 hours).

The membrane was then incubated with tris-buffered saline – tween buffer (TBS/T) (see Appendix A1) containing 5% (w/v) dried defatted milk and 1% (w/v) glycine, for 2 hours, on

a platform shaker, to block unspecific binding. Primary monoclonal antibodies against AnxA2 (purified mouse monoclonal anti-AnxA2, BD Biosciences) (1:1000) were added together with TBS/T buffer containing 3% (w/v) dried defatted milk and 1% (w/v) glycine, and incubated overnight at 4ºC with gentle agitation. On the following day, the membrane was rinsed three times with TBS/T buffer and washed for another 15 min, allowing for any antibody excess to be removed. Washing was repeated four times before secondary antibodies (goat anti-mouse IgG, light chain, HRP-conjugated, Jackson ImmunoResearch Inc., Pennsylvania, USA) (1:2000) were added together with TBS/T buffer containing 3% (w/v) dried defatted milk and 1% (w/v) glycine. After 2 hours of incubation on a platform shaker, four washing cycles with TBS/T buffer were repeated every 15 min.

Finally, visualisation of luminescent substrates was done using enhanced chemiluminescence (ECL). In the presence of H_2O_2, horse radish peroxidase (HRP)-conjugated secondary antibodies oxidize luminol and produce light. ECL components 1 and 2 (WesternBright™ ECL HRP substrate, Advansta Inc., California, USA) were added 1:1 and used to cover the membrane. Light emission was then detected by a molecular imager (Bio-Rad).

3.8 Other materials

An extensive list of all chemicals, reagents, buffers, solutions, consumables, technical equipment and data processing software used in the experiments can be found in Appendix A1.

3.9 Statistical analysis

Statistical analysis was performed by the one-way Analysis of Variance (ANOVA) test, followed by Dunnett or Tukey HSD multiple comparisons tests. Data was analysed using STATISTICA version 8.0 for Windows, StatSoft Inc, Tulsa, Oklahoma, USA.

4 Results

4.1 Effect of natural compounds on cell viability

4.1.1 Determination of the cytotoxic effect of low doses of DMSO

It has been reported that DMSO is responsible for significant apoptotic cell death, even in small doses. However, minimal toxic concentrations of DMSO vary widely (78–80). Since DMSO was used to dissolve the original natural samples, the effect of low-doses of this compound on PC12 cell viability was determined.

Cells were plated as previously described and treated with increasing concentrations of DMSO (0%, 0.2%, 0.3%, 0.4%, 0.5%, 0.6%, 0.7%, 0.8%, 0.9%, 1%, 2%, 3% final concentration) on the following day. After 24 hours of incubation, cell viability was determined (Figure 4.1).

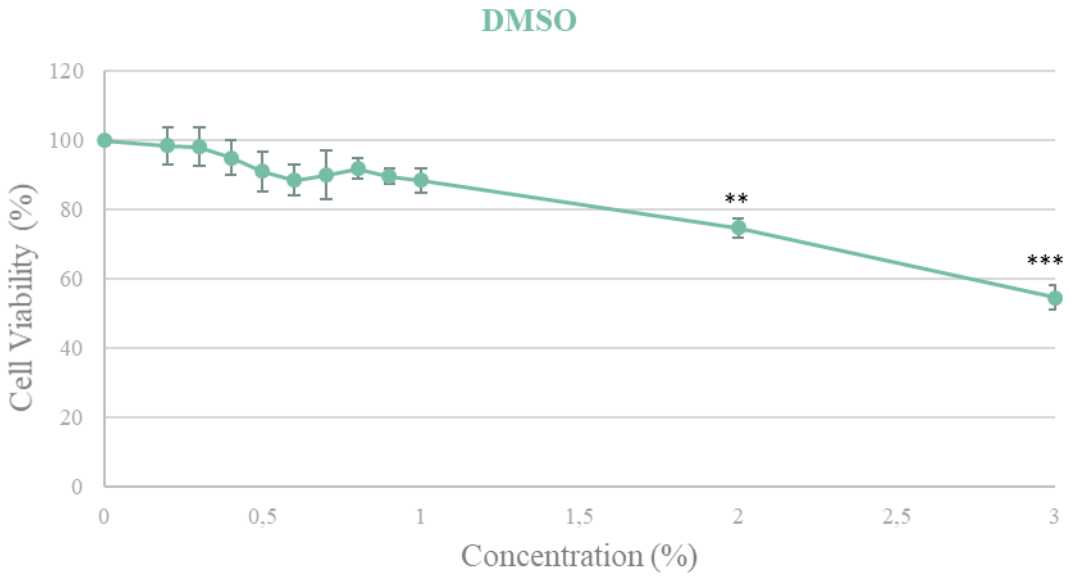


Figure 4.1 Effect of DMSO on PC12 cell viability. Cells were plated at a final density of 3,5×10^4 cells/well and incubated with increasing concentrations of DMSO on the following day. Cell viability was determined 24 hours later by the CTB assay, as described in Section 3.3.2. For each DMSO concentration, 6 replicates were made. Results are presented as mean values ± SEM and expressed in percentage of cell viability. **p<0.01; ***p<0.001 | n=4 (0.7%); n=5 (0.4%, 0.9%); n=6 (0-0.3%, 0.5%, 0.6%, 0.8%, 1-3%).

Results indicate a decrease in cell viability with increasing concentrations of DMSO. A deviation from linearity is observed for lower concentrations, but a significant decrease on cell viability of about 25% can only be observed in cells treated with 2% DMSO (one-way ANOVA $p<0.01$, $F=8.44$, Dunnett post-hoc test). Moreover, cells treated with 3% DMSO show a

significant decrease in cell viability of about 45% (one-way ANOVA $p<0.001$, $F=8.44$, Dunnett post-hoc test).

Due to the observed effect of DMSO in cell viability, we decided to use the lowest possible amount on subsequent experiments.

4.1.2 Determination of natural compounds' cytotoxic effect

Before investigating the protective effect of the natural compounds in the presence of ROS, it was crucial to ensure that these compounds were not cytotoxic by themselves. With this purpose, PC12 cells were treated with 2 and 20 nM of each natural compound for 24 hours (Figure 4.2). Moreover, for selected compounds (compounds 2, 3, 7 and 20), a dose-response curve was obtained by treating cells with 1, 10, 20, 50, 125, 250, 500, 750, 1000 µM and determining cell viability (Figure 4.3-4.6), since the cytotoxic effect of these molecules had only been tested in the nM range (76).

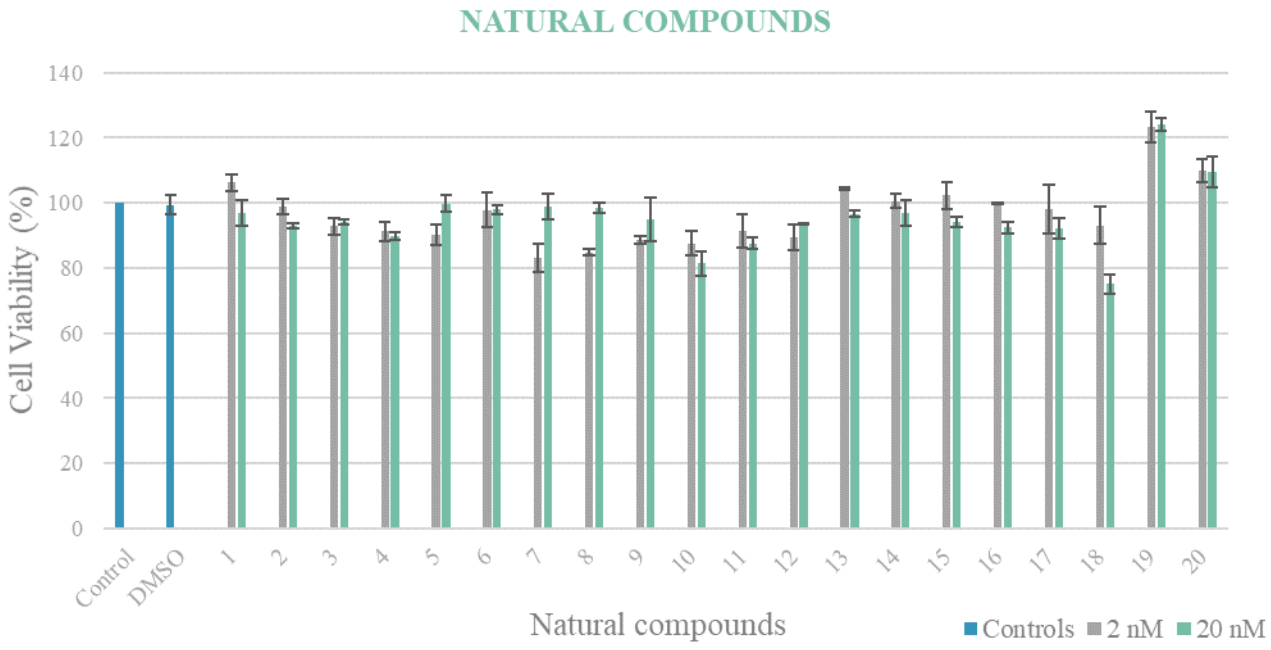


Figure 4.2 Effect of natural compounds on PC12 cell viability. Cells were plated at a final density of $3,5\times10^4$ cells/well and incubated on the following day with 2 and 20 nM of each compound. Cell viability was determined 24 hours later by the CTB assay, as described in Section 3.3.2. For each concentration, 3-6 replicates were made. Data is represented as mean values ± SEM and expressed in percentage of cell viability relative to Control. Control: untreated cells | DMSO: cells treated with the maximum concentration of DMSO (0.2 %)

Our results show mild cytotoxicity for compounds 10-12 and 18. On the contrary, it appears that compounds 19-20 increase cell viability.

For a more detailed analysis, four compounds (compounds 2, 3, 7 and 20) were arbitrarily chosen and tested in a wider concentration range (2 nM – 1 mM).

Our results show that compound 2 has indeed some effect on PC12 cell viability (Figure 4.3). For lower concentrations (2 nM – 250 µM) no significant fluctuations were obtained, except for 20 nM and 50 µM, where a mild cytotoxic effect can be observed. Interestingly, for concentrations above 500 µM, we observed a significant increase in cell viability. Indeed, in cells treated with 750 µM and 1 mM of compound 2, an increase of about 22% on cell viability was obtained, when compared with untreated cells (one-way ANOVA $p<0.001$, $F=34.35$, Dunnett post-hoc test).

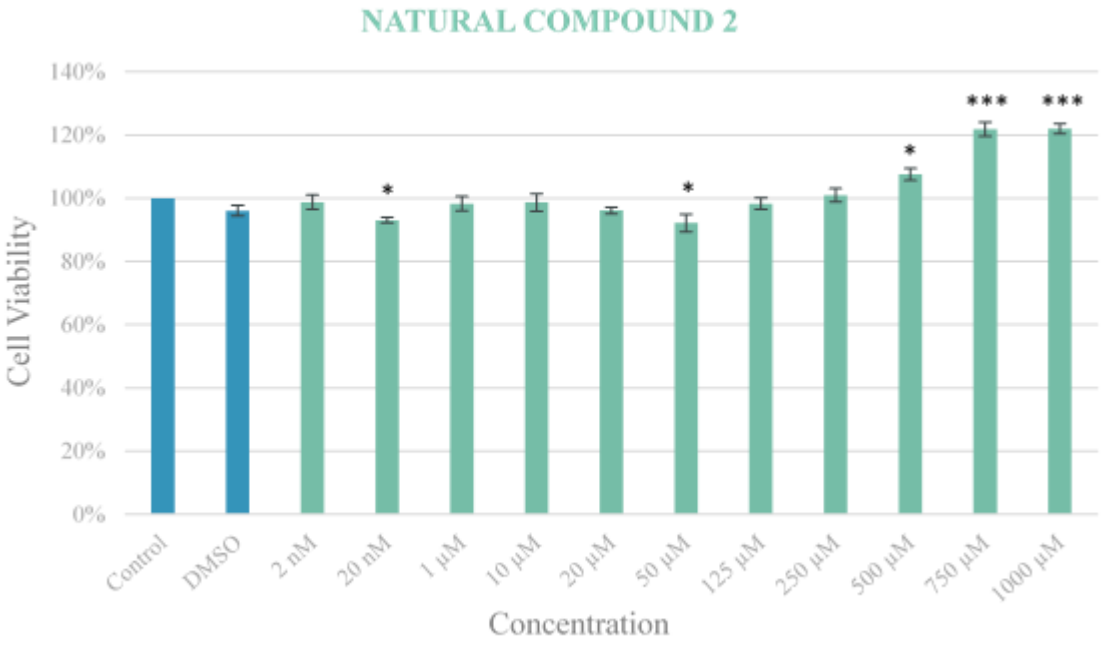


Figure 4.3 Effect of natural compound 2 on PC12 cell viability. Cells were plated at a final density of $3,5\times10^4$ cells/well and incubated on the following day with increasing concentrations of compound 2. Cell viability was determined 24 hours later by the CTB assay, as described in Section 3.3.2. For each concentration, 3-4 replicates were made. Data is represented as mean values ± SEM and expressed in percentage of cell viability relative to Control. Control: untreated cells | DMSO: cells treated with the maximum concentration of DMSO (0.7 %). *p<0.05; ***p<0.001 | n=3 (2 nM – 20 µM); n=4 (50 µM – 1 mM)

As far as compound 3 is concerned, it induced a mild cytotoxic effect at lower concentrations and it has a proliferative effect at higher concentrations (Figure 4.4). Indeed, at lower

concentrations (10 µM – 50 µM), a mild cytotoxic effect was observed, since treatment with this compound led to a decrease on cell viability of about 10%. On the other hand, higher concentrations of compound 3 (500 µM – 1 mM) revealed an increase on cell viability up to 15%, when compared with untreated cells (one-way ANOVA $p<0.001$, $F=25.13$, Dunnett post-hoc test).

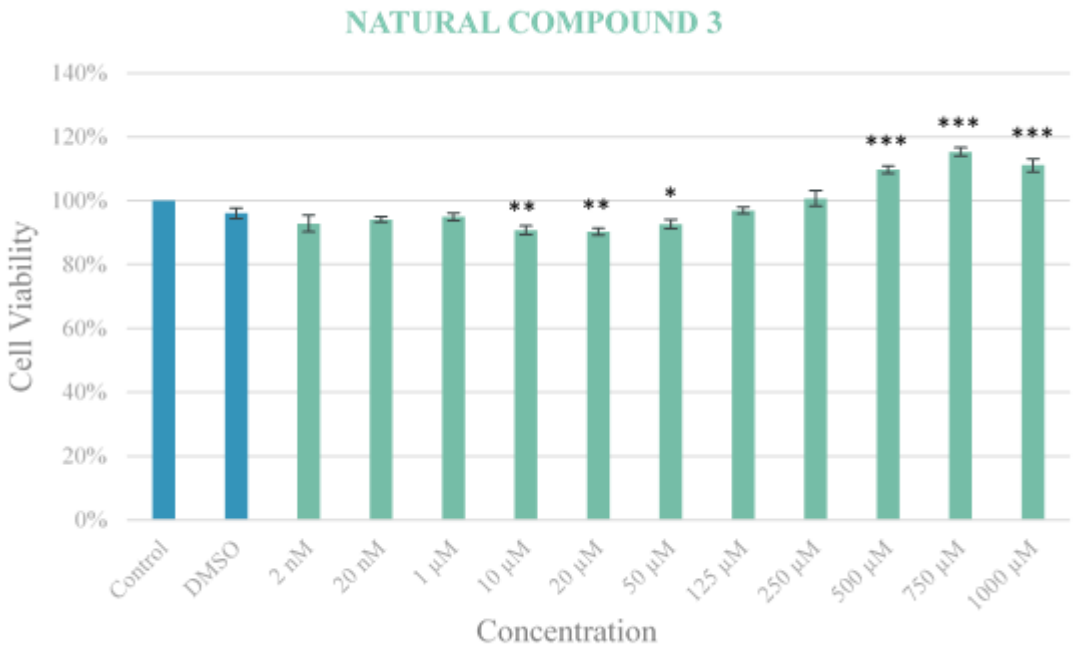


Figure 4.4 Effect of natural compound 3 on PC12 cell viability. Cells were plated at a final density of $3,5\times10^4$ cells/well and incubated on the following day with increasing concentrations of compound 3. Cell viability was determined 24 hours later by the CTB assay, as described in Section 3.3.2. For each concentration, 3-4 replicates were made. Data is represented as mean values ± SEM and expressed in percentage of cell viability relative to Control. Control: untreated cells | DMSO: cells treated with the maximum concentration of DMSO (0.7 %). *p<0.05; **p<0.01; ***p<0.001 | n=2 (20 nM); n=3 (2 nM, 1-20 µM); n=4 (50 µM – 1 mM)

Due to limited sample availability of natural compound 7, we decided to test fewer concentrations while maintaining the concentration range (Figure 4.5). Our results show that compound 7 induced significant cell death at higher concentrations. For lower concentrations (2 nM – 250 µM), no significant alterations in cell viability were obtained, except for cells treated with 1 µM, where a decrease in cell viability of about 10% was observed (one-way ANOVA $p<0.05$, $F=11.3$, Dunnett post-hoc test). Regarding cells treated with higher concentrations of compound 7 (500 µM – 1 mM), a significant decrease on cell viability was observed, when compared with untreated cells. This cytotoxic effect seems to be heightened

with higher concentrations, reaching a 73% drop in cell viability (1 mM final concentration) (one-way ANOVA p<0.001, F=64.75, Dunnett post-hoc test).

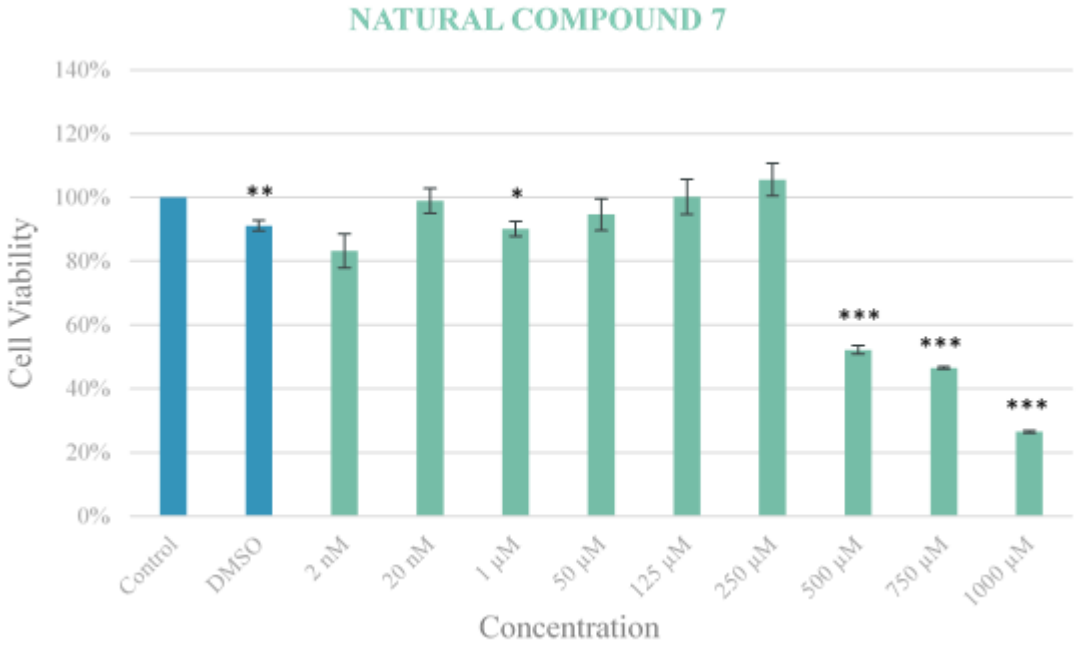


Figure 4.5 Effect of natural compound 7 on PC12 cell viability. Cells were plated at a final density of $3{,}5\times10^4$ cells/well and incubated on the following day with increasing concentrations of compound 7. Cell viability was determined 24 hours later by the CTB assay, as described in Section 3.3.2. For each concentration, 3-4 replicates were made. Data is represented as mean values ± SEM and expressed in percentage of cell viability relative to Control. Control: untreated cells | DMSO: cells treated with the maximum concentration of DMSO (0.7 %). *p<0.05; **p<0.01; ***p<0.001 | n=2 (2 nM); n=3 (20 nM, 1 µM, 750 µM, 1 mM); n=4 (50 – 500 µM)

Results show that compound 20 has indeed an effect on PC12 cell viability (Figure 4.6). Notably, unlike previous compounds, it demonstrated a dual effect depending on the concentration range. For lower concentrations (2 – 20 nM) a mild 10% increase on cell viability was observed (one-way ANOVA p<0.05, F=5.194, Dunnett post-hoc test). On the other hand, for higher concentrations (500 µM – 1 mM), there was a significant decrease on cell viability, which goes down to 52% (750 µM) (one-way ANOVA p<0.001, F=73.76, Dunnett post-hoc test).

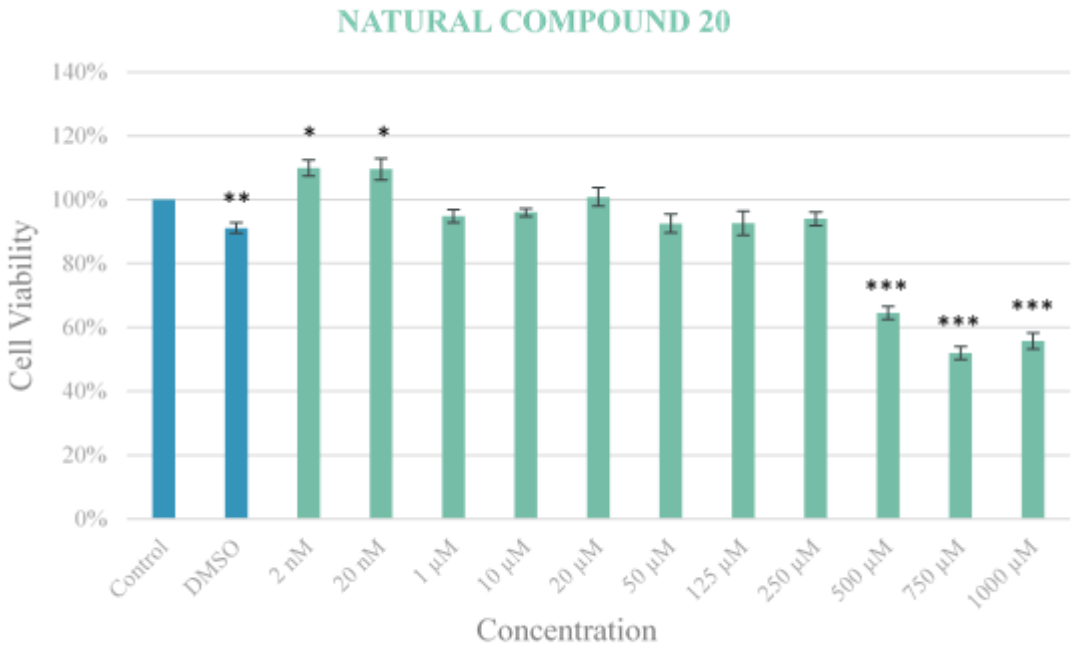


Figure 4.6 Effect of natural compound 20 on PC12 cell viability. Cells were plated at a final density of $3{,}5\times10^4$ cells/well and incubated on the following day with increasing concentrations of compound 20. Cell viability was determined 24 hours later by the CTB assay, as described in Section 3.3.2. For each concentration, 3-6 replicates were made. Data is represented as mean values ± SEM and expressed in percentage of cell viability relative to Control. Control: untreated cells | DMSO: cells treated with the maximum concentration of DMSO (0.7 %). *p<0.05; **p<0.01; ***p<0.001 | n=3 (1-20 µM); n=4 (50 – 750 µM); n=6 (2-20 nM; 1 mM)

4.1.3 Exposure to natural compounds in the presence of ROS

Hydrogen peroxide-induced oxidative stress was selected to replicate physiological apoptotic cell death induced by ROS. To study its effect both in prevention and recovery from oxidative damage, natural substances where added before and after treatment with H_2O_2, respectively.

4.1.3.1 Prevention of oxidative damage

PC12 cells were treated with natural compounds at different concentrations. As in the previous Section, an initial experiment had been done by pre-treating cells with all compounds at the final concentrations of 1, 10, 20 µM, 2 hours before the addition of H_2O_2. 24 hours later, cell viability was determined (Figure 4.7).

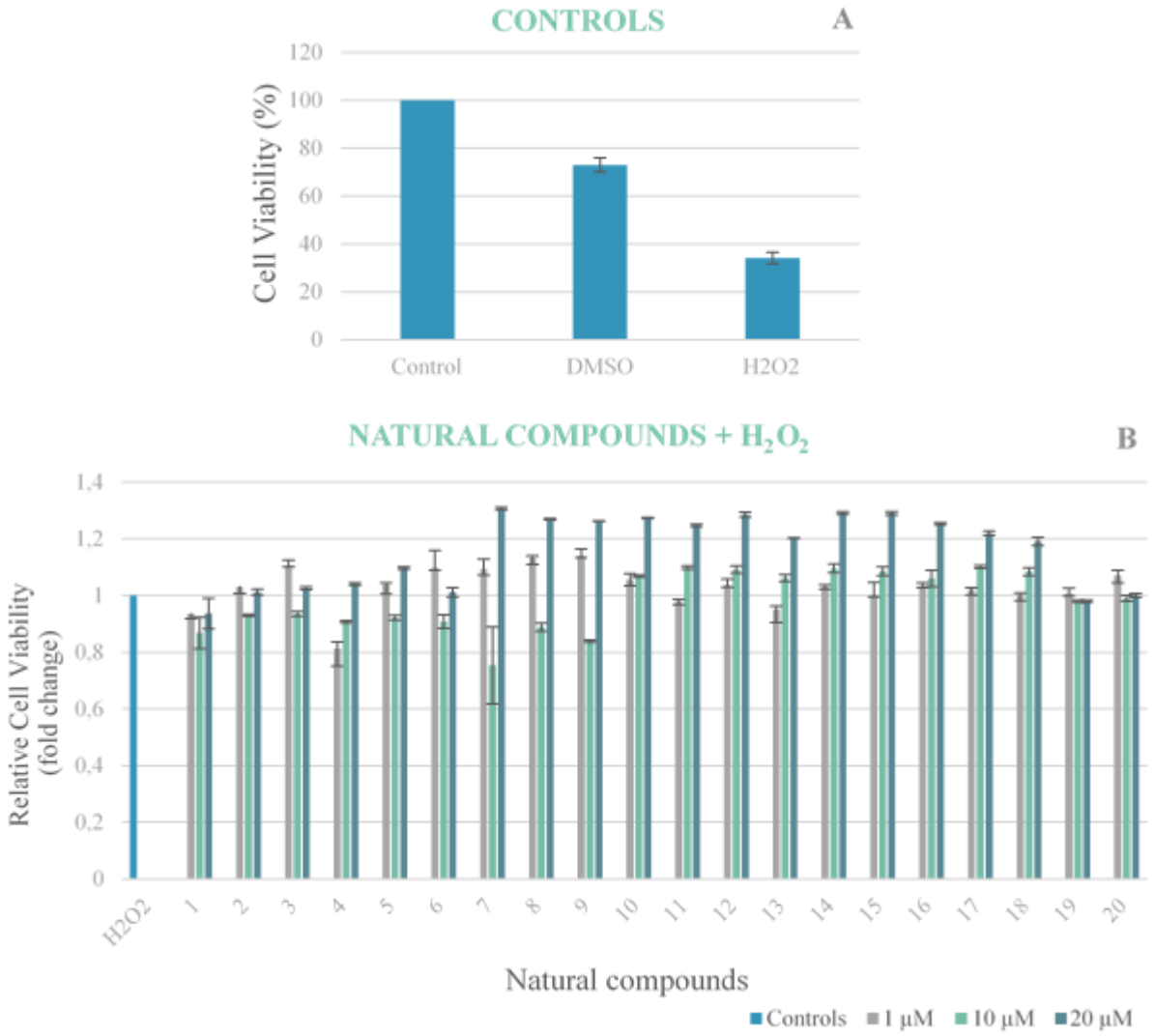


Figure 4.7 Effect of natural compounds in the prevention of H_2O_2 induced cell death. Cells were plated at a final density of $3{,}5\times10^4$ cells/well and incubated with the natural compounds 2 hours before the addition of 1 mM H_2O_2. Cell viability was determined 22 hours later by the CTB assay. For each concentration, 3-5 replicates were made. A- Data is represented as mean values ± SEM and expressed as percentage of cell viability relative to Control. Control: untreated cells | DMSO: cells treated with the maximum concentration of DMSO used to dissolve the compounds (1.4%) | H_2O_2: cells treated with 1 mM H_2O_2 for 22 hours. B- Data is represented as mean values ± SEM and expressed as fold change relative to H_2O_2 treated cells (cell viability of 34%).

Treatment with 1 mM H_2O_2 led to a significant increase in cell death up to approximately 50% when compared to DMSO-treated cells. Although none of the compounds could completely prevent the induced damage, a potential improvement in cell viability was clearly observed for compounds 7-18.

For a more detailed analysis, the same four compounds (compounds 2, 3, 7 and 20) were tested in a wider concentration range (1 µM – 1 mM final concentration). The obtained results were then added to the previous ones, allowing for a complete analysis.

Results show that compound 2 was unable to completely prevent H_2O_2 induced damage (Figure 4.8). However, an improvement in relative cell viability was observed in cells treated with compound 2 at concentrations above 125 µM (one-way ANOVA $p<0.001$, $F=82.00$, Dunnett post-hoc test). This increase corresponds to a maximum 3.04% enhancement on cell viability in cells treated with 1 mM of compound 2. Regarding lower concentrations, the effect on relative cell viability was minor or not significant.

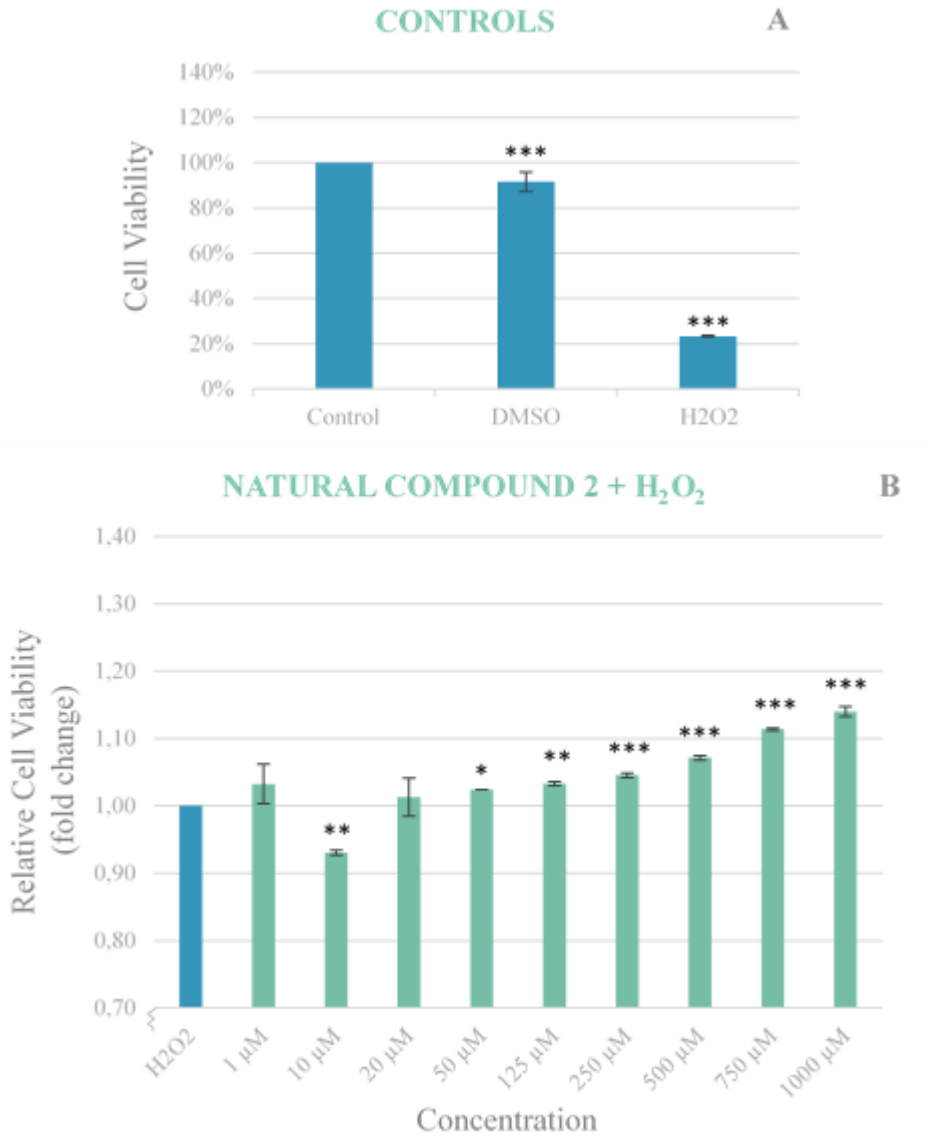


Figure 4.8 Effect of natural compound 2 in the prevention of H_2O_2 induced cell death. Cells were plated at a final density of $3{,}5\times10^4$ cells/well and incubated with increasing concentrations of natural compound 2, 2 hours before the addition of 1 mM H_2O_2. Cell

viability was determined 22 hours later by the CTB assay. For each concentration, 3-4 replicates were made. A- Data is represented as mean values ± SEM and expressed as percentage of cell viability relative to Control. Control: untreated cells | DMSO: cells treated with the maximum concentration of DMSO used to dissolve the compounds (0.7%) | H_2O_2: cells treated with 1 mM H_2O_2 for 22 hours. B- Data is represented as mean values ± SEM and expressed as fold change relative to H_2O_2 treated cells (average cell viability of about 23%). **p<0.01; ***p<0.001 | n=2 (1 µM); n=3 (10, 20, 250 µM); n=4 (50, 125, 500 µM – 1 mM)

As far as compound 3 is concerned, our results show that, once again, pre-treatment with this molecule was unable to completely prevent ROS-induced cell damage (Figure 4.9). However, similarly to the results obtained with compound 2, a significant improvement in relative cell viability can be observed on cells treated with compound 3 at concentrations above 125 µM (one-way ANOVA p<0.001, F=65.11, Dunnett post-hoc test). This corresponds to a maximum enhancement of approximately 4% on cell viability for cells treated with 1 mM of compound 3. Regarding lower concentrations, the effect on cell viability was minor or not significant.

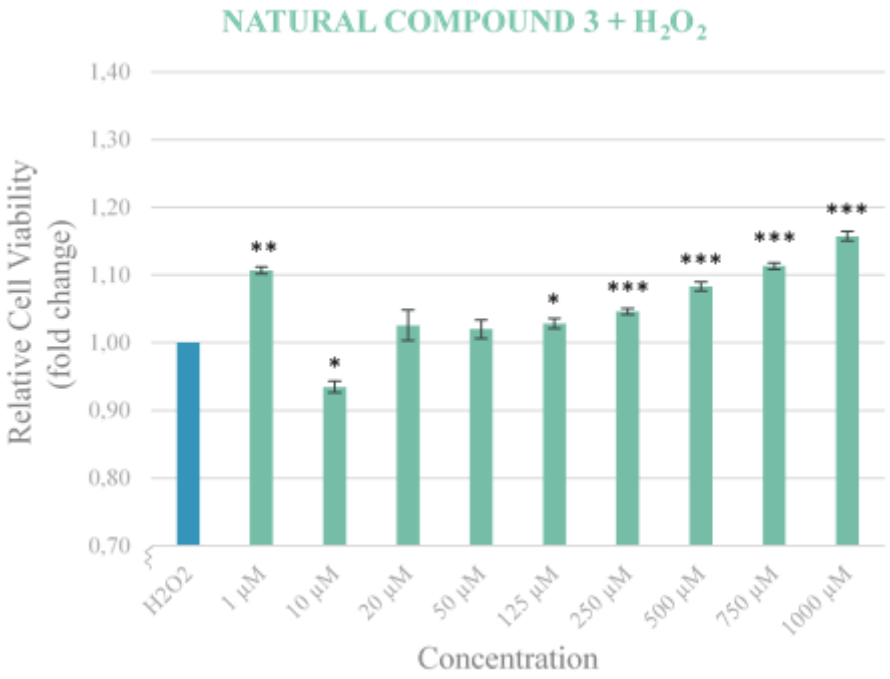


Figure 4.9 Effect of natural compound 3 in the prevention of H_2O_2 induced cell death. Cells were plated at a final density of $3{,}5\times10^4$ cells/well and incubated with increasing concentrations of natural compound 3, 2 hours before the addition of 1 mM H_2O_2. Cell viability was determined 22 hours later by the CTB assay. For each concentration, 3-4 replicates were made. Data is represented as mean values ± SEM and expressed as fold change relative to H_2O_2 treated cells (average cell viability of about 23%). *p<0.05; **p<0.01; ***p<0.001 | n=3 (1-20 µM); n=4 (50 µM – 1 mM)

Our results show that compound 7 was able to slightly prevent the induced damage in almost all tested concentrations (Figure 4.10). However, these experiments need to be repeated since some of the results are difficult to interpret. Indeed, a significant increase in relative cell viability was observed for all concentrations, except for 10 µM and 50 µM. A maximum protection was achieved when PC12 cells were treated with 20 µM of compound 20, with a 0.31-fold increase in relative cell viability when compared to vehicle-treated cells (one-way ANOVA $p<0.05$, $F=11.69$, Dunnett post-hoc test), corresponding to an increase of about 7.7% on cell viability.

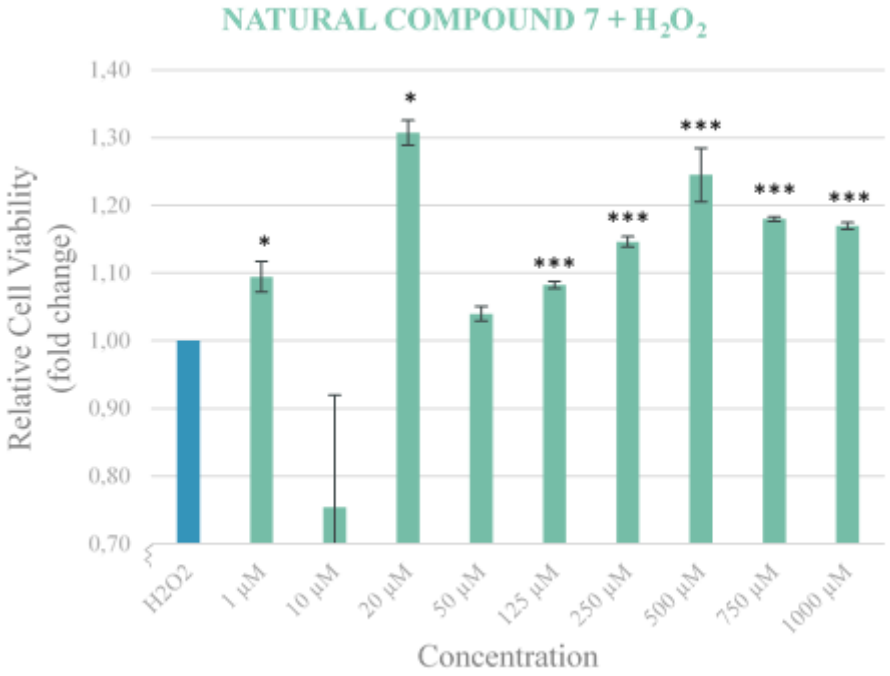


Figure 4.10 Effect of natural compound 7 in the prevention of H_2O_2 induced cell death. Cells were plated at a final density of $3,5\times10^4$ cells/well and incubated with increasing concentrations of natural compound 7, 2 hours before the addition of 1 mM H_2O_2. Cell viability was determined 22 hours later by the CTB assay. For each concentration, 3-4 replicates were made. Data is represented as mean values ± SEM and expressed as fold change relative to H_2O_2 treated cells (average cell viability of about 23%). *p<0.05; ***p<0.001 | n=2 (10-20 µM); n=3 (1 µM); n=4 (50 µM – 1 mM)

Finally, the results obtained for compound 20 reveal that, once again, there was some protection against ROS-induced cell death for concentrations above 125 µM (Figure 4.11). A maximum protection of 1.14-fold in relative cell viability was observed following cell treatment with 750 µM of compound 20 (one-way ANOVA $p<0.001$, $F=61.36$, Dunnett post-hoc test). This corresponds to a slight 3.03% enhancement on cell viability. Regarding lower concentrations, the effect on cell viability was minor or not significant.

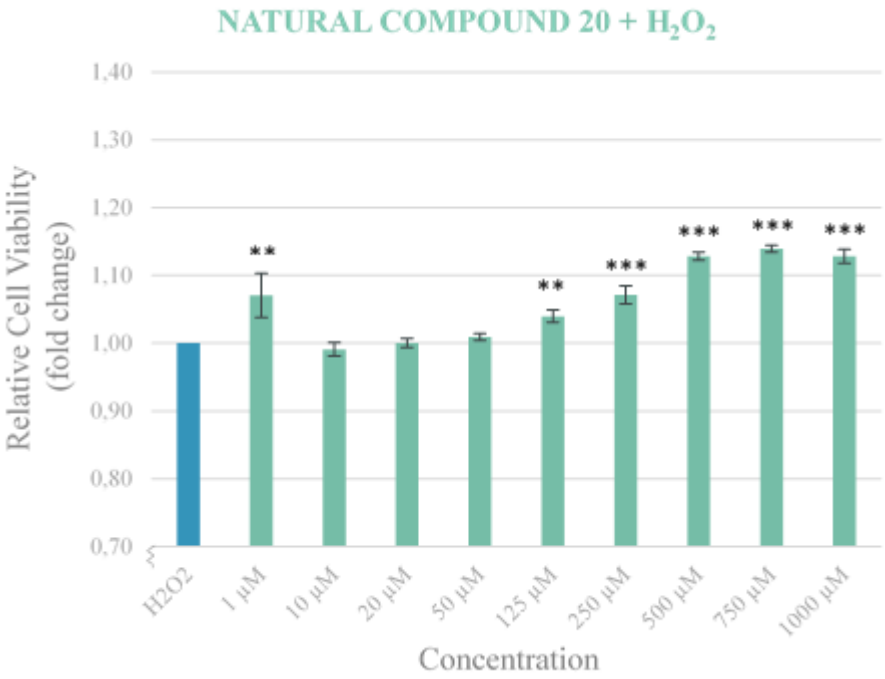


Figure 4.11 Effect of natural compound 20 in the prevention of H_2O_2 induced cell death. Cells were plated at a final density of $3{,}5\times10^4$ cells/well and incubated with increasing concentrations of natural compound 20, 2 hours before the addition of 1 mM H_2O_2. Cell viability was determined 22 hours later by the CTB assay. For each concentration, 4-5 replicates were made. Data is represented as mean values ± SEM and expressed as fold change relative to H_2O_2 treated cells (average cell viability of about 23%). **p<0.01; ***p<0.001 | n=3 (1, 250 µM); n=4 (50-125 µM, 500 µM – 1 mM); n=5 (10-20 µM)

Our results suggest that natural compounds 2, 3, 7 and 20 are able to slightly prevent cell death by exposure to H_2O_2-induced oxidative stress at mM concentrations.

4.1.3.2 Recovery from oxidative damage

As previously mentioned, we aimed to study the effect of natural compounds both in prevention and recovery from cell damage. Therefore, the previous experiments were repeated by adding the natural compounds after treatment with H_2O_2. PC12 cells were exposed to 1 mM H_2O_2 for 2 hours (76) before the natural compounds were added (all compounds: 20 nM; selected compounds: 1, 10, 20, 50, 125, 250, 500, 750, 1000 µM). After 24 hours of exposure to H_2O_2, cell viability was determined as formerly described. Since positive results were obtained in the prevention of oxidative damage for µM concentrations of several compounds, while compound 20 demonstrated a mild increase on cell viability at the nM range, it was decided to reintroduce the 20 nM final concentration in the following experiments.

As mentioned above, all natural compounds had been previously tested on the lowest concentration (20 nM) (76). This first screen showed little or no effect on relative cell viability (Figure 4.12). Nevertheless, compounds 2, 3, 7 and 20 demonstrated an apparent dose-dependent protective effect against oxidative stress, when tested in higher concentrations (Figure 4.13 – 4.16).

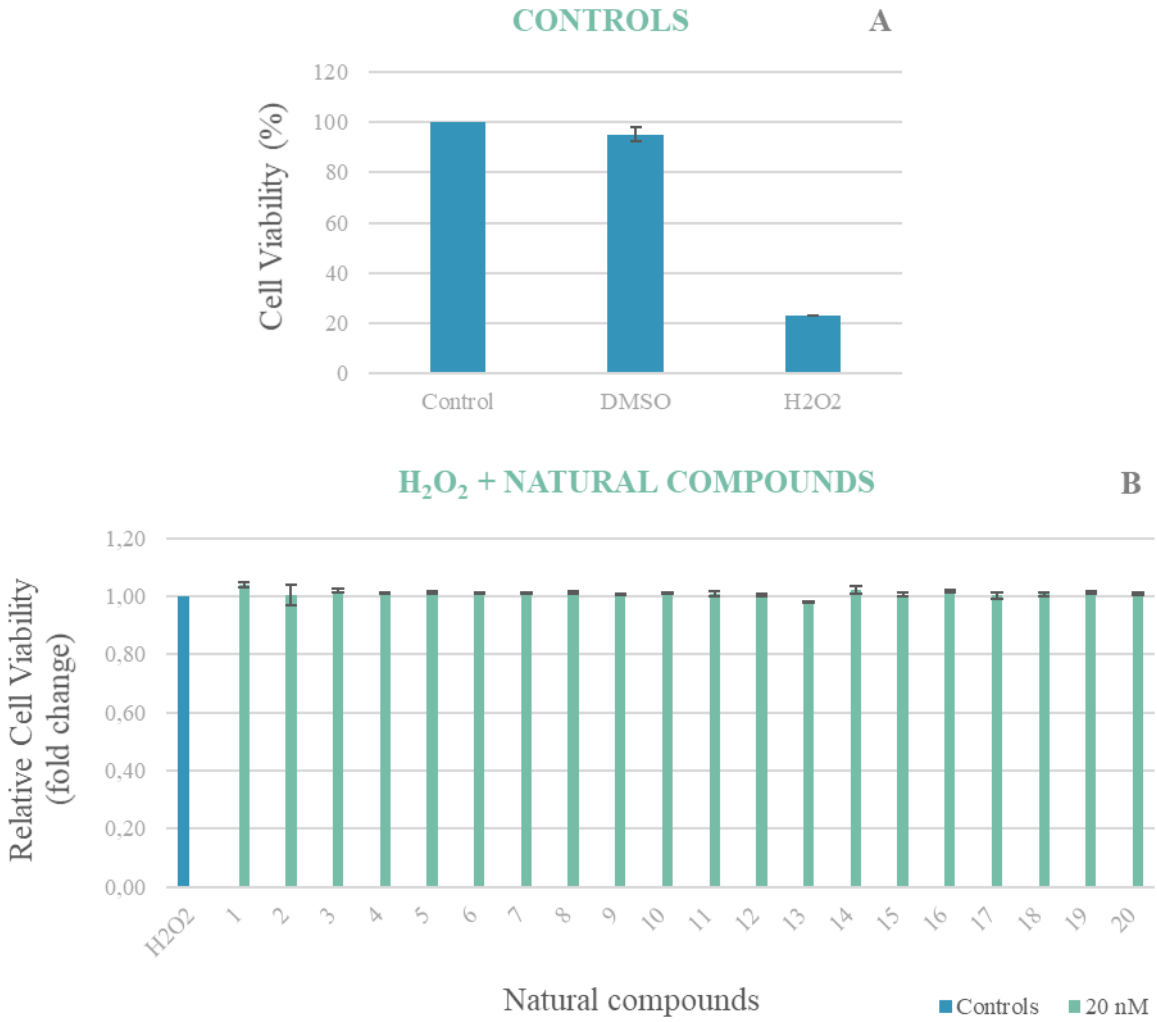


Figure 4.12 Effect of natural compounds in the protection against H_2O_2 induced cell death. Cells were plated at a final density of $3{,}5\times10^4$ cells/well and incubated with 1 mM H_2O_2, followed by the administration of 20 nM of natural compounds, 2 hours later. Cell viability was determined 24 hours after exposure to H_2O_2 by the CTB assay. For each concentration, 3-5 replicates were made. A- Data is represented as mean values ± SEM and expressed as percentage of cell viability relative to Control. Control: untreated cells | DMSO: cells treated with the maximum concentration of DMSO used to dissolve the compounds (0.2%) | H_2O_2: cells treated with 1 mM H_2O_2 for 24 hours. B- Data is represented as mean values ± SEM and expressed as fold change relative to H_2O_2 treated cells (cell viability of 23%).

Our results show that treatment with compound 2 leads to a significant dose-dependent protection against cell death (Figure 4.13). An increase up to 0.24-fold on relative cell viability was observed for the highest tested concentration (1 mM) (one-way ANOVA $p<0.001$, $F=125.9$, Tukey HSD post-hoc test). This corresponds to an enhancement of approximately 5.9% on cell viability.

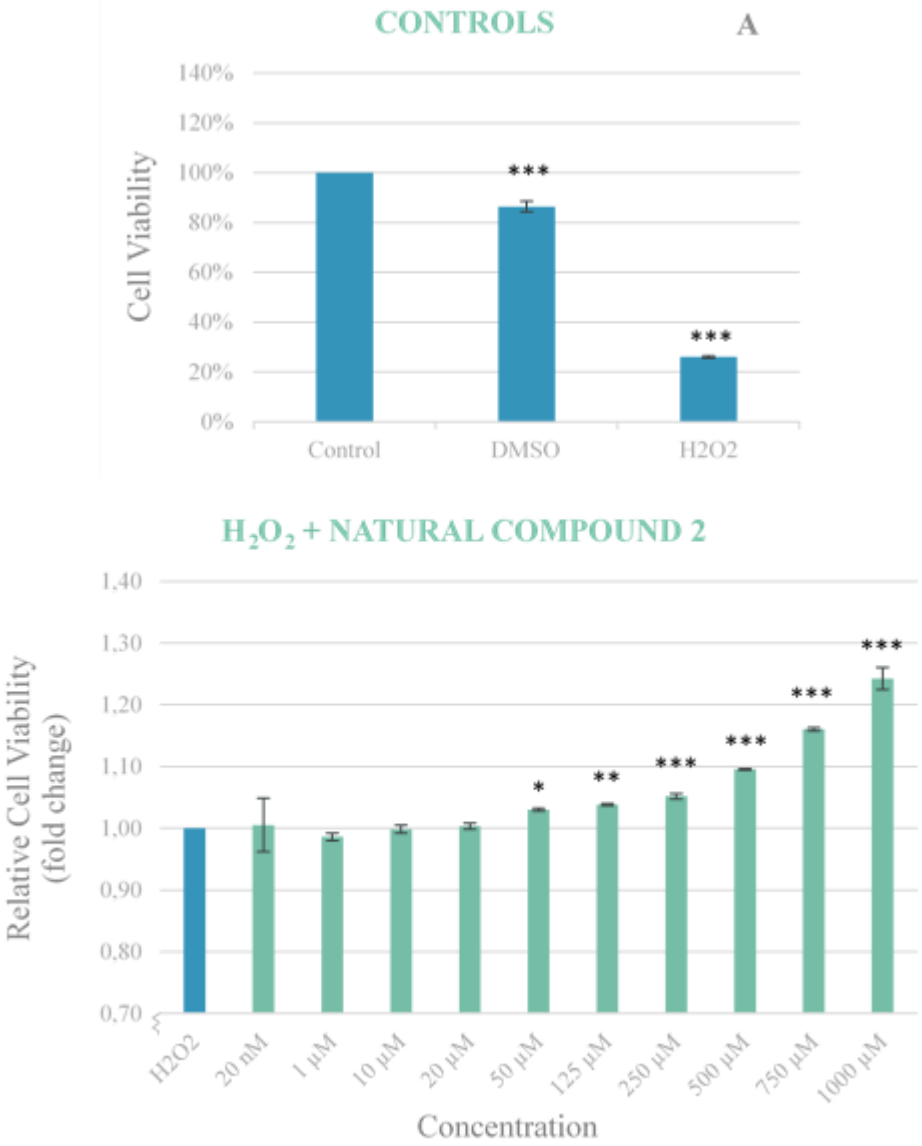


Figure 4.13 Effect of natural compound 2 in the protection against H_2O_2 induced cell death. Cells were plated at a final density of $3,5\times10^4$ cells/well and incubated with 1 mM H_2O_2, followed by the administration of 20 nM of natural compound 2, 2 hours later. Cell viability was determined 24 hours after exposure to H_2O_2 by the CTB assay. For each concentration, 3-4 replicates were made. A- Data is represented as mean values ± SEM and expressed as percentage of cell viability relative to Control. Control: untreated cells | DMSO: cells treated with the maximum concentration of DMSO used to dissolve the compounds (0.7%) | H2O2: cells treated with 1 mM H_2O_2 for 24 hours. B- Data is

represented as mean values ± SEM and expressed as fold change relative to H_2O_2 treated cells (cell viability of 26%).

Similar results were obtained for compound 3, with a significant dose-dependent protection against cell death (Figure 4.14). An increase up to 0.21-fold on relative cell viability was observed for the highest tested concentration (1 mM) (one-way ANOVA $p<0.001$, $F=135.0$, Dunnett post-hoc test). This corresponds to an increase of about 5.8% on cell viability.

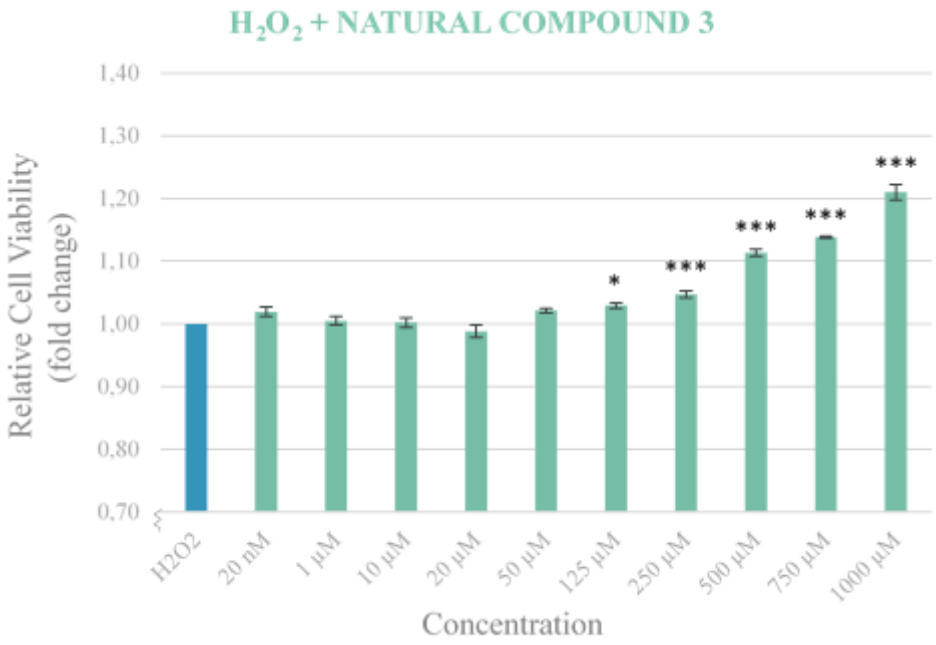


Figure 4.14 Effect of natural compound 3 in the protection against H_2O_2 induced cell death. Cells were plated at a final density of $3,5\times10^4$ cells/well and incubated with 1 mM H_2O_2, followed by the administration of 20 nM of natural compound 3, 2 hours later. Cell viability was determined 24 hours after exposure to H_2O_2 by the CTB assay. For each concentration, 3-4 replicates were made. Data is represented as mean values ± SEM and expressed as fold change relative to H_2O_2 treated cells (cell viability of 26%). *p<0.05; ***p<0.001 | n=3 (20 nM – 20 µM); n=4 (50 µM – 1 mM)

Once again, due to limited sample availability of natural compound 7, we decided to test it in fewer concentrations, while maintaining the concentration range. Results show that treatment with compound 7 led to a significant dose-dependent protection against cell death (Figure 4.15). An increase of 0.19-fold on relative cell viability was observed for 500 µM, reaching a plateau at this concentration (one-way ANOVA $p<0.001$, $F=126.2$, Dunnett post-hoc test). This corresponds to an enhancement of about 5.2% on cell viability.

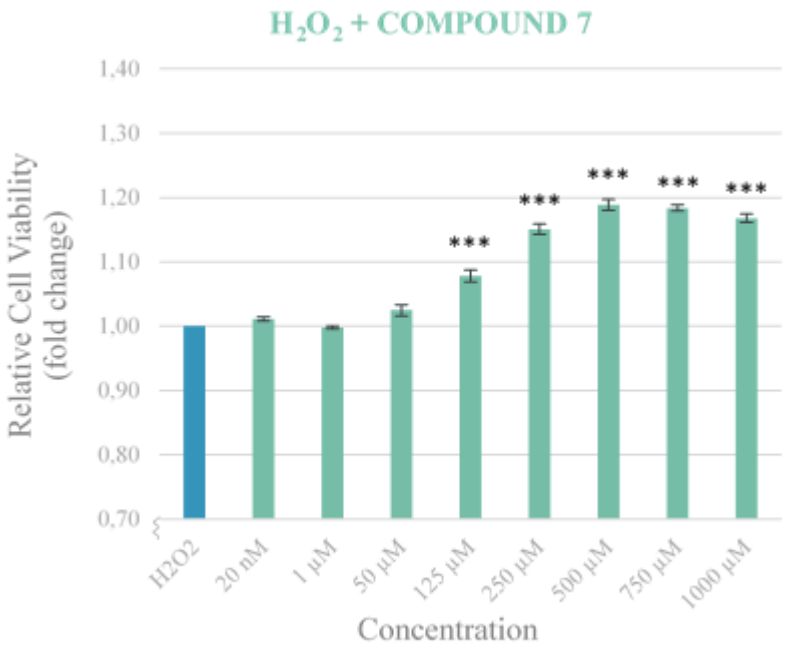


Figure 4.15 Effect of natural compound 7 in the protection against H_2O_2 induced cell death. Cells were plated at a final density of $3{,}5\times10^4$ cells/well and incubated with 1 mM H_2O_2, followed by the administration of 20 nM of natural compound 7, 2 hours later. Cell viability was determined 24 hours after exposure to H_2O_2 by the CTB assay. For each concentration, 3-4 replicates were made. Data is represented as mean values ± SEM and expressed as fold change relative to H_2O_2 treated cells (cell viability of 26%). ***p<0.001 | n=3 (20 nM – 1 µM); n=4 (50 µM – 1 mM)

Finally, administration of compound 20 showed a significant dose-dependent protection against cell death (Figure 4.16). An increase up to 0.18-fold on relative cell viability was observed for the highest tested concentration (1 mM) (one-way ANOVA $p<0.001$, $F=76.97$, Dunnett post-hoc test). This represents an increase of approximately 4.4% on cell viability.

Our results indicate that natural compounds 2, 3, 7 and 20 are slightly able to protect PC12 cells from cell death induced by exposure to H_2O_2 at mM concentrations. Moreover, the apparent dose-dependent protective effect suggests that higher concentrations should be tested.

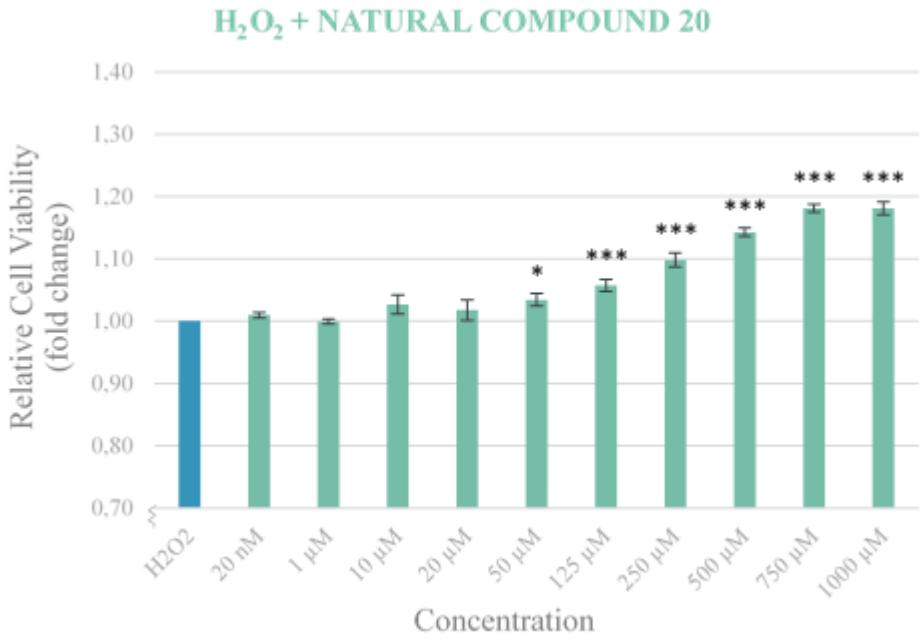


Figure 4.16 Effect of natural compound 20 in the protection against H_2O_2 induced cell death. Cells were plated at a final density of $3{,}5\times10^4$ cells/well and incubated with 1 mM H_2O_2, followed by the administration of 20 nM of natural compound 20, 2 hours later. Cell viability was determined 24 hours after exposure to H_2O_2 by the CTB assay. For each concentration, 3-5 replicates were made. Data is represented as mean values ± SEM and expressed as fold change relative to H_2O_2 treated cells (cell viability of 26%). *p<0.05; ***p<0.001 | n=2 (20 µM); n=3 (1-10 µM); n=4 (50 µM – 1 mM); n=5 (20 nM)

4.2 Effect of natural compounds on Annexin A2 expression levels

AnxA2 has been identified as a novel cellular redox regulatory protein (57), thus we aimed to uncover if this protein was involved in the natural compounds' observed prevention/ protection against H_2O_2-induced oxidative stress. Therefore, we performed a preliminary experiment in order to determine if AnxA2 expression levels were modulated in the presence of compounds 2, 3, 7 and 20 (Figure 4.17).

PC12 cells were plated on 6-well plates and treated with 750 µM of compounds 2 and 3, 1 mM of compound 7, or 2 nM and 750 µM of compound 20 for 24h. Cell lysates were prepared and AnxA2 protein levels were determined by Western Blot.

Interestingly, our results clearly show that treatment with 750 µM of compound 2 and 20 led to an increase in the expression of AnxA2 of approximately 5.0- and 2.6-fold, respectively.

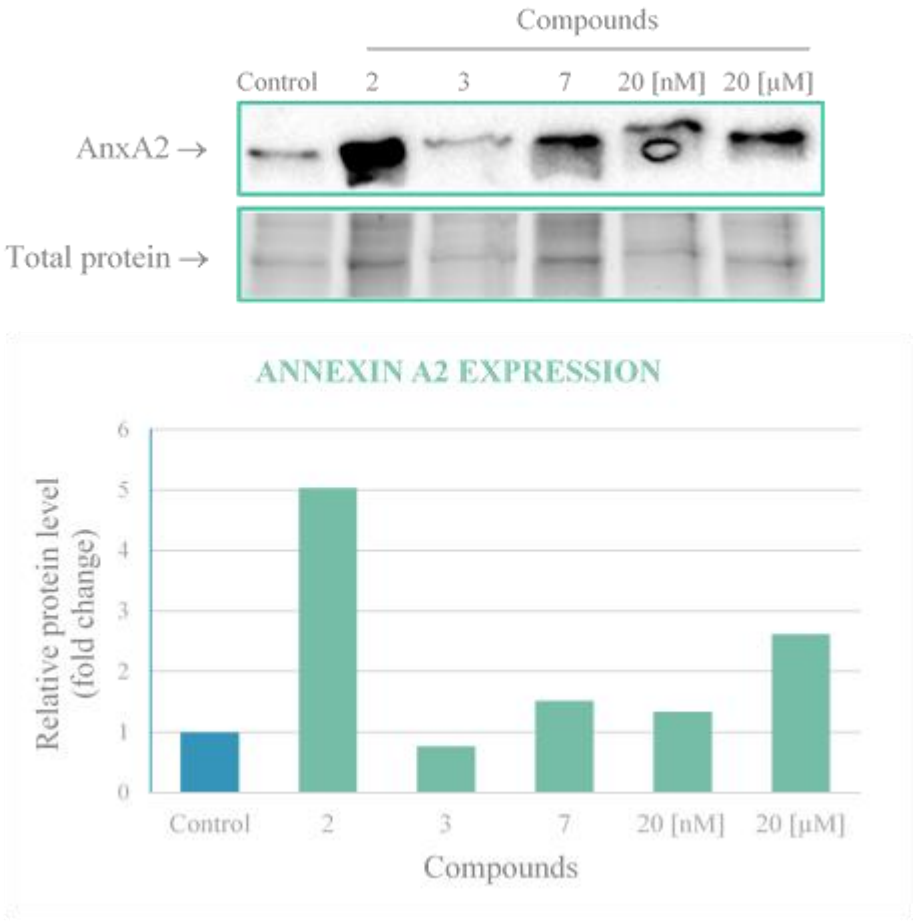


Figure 4.17 Effect of natural compounds on AnxA2 expression levels. Western blot analysis of AnxA2 protein levels in PC12 cells treated with selected natural compounds for 24 hours: compound 2 (750 µM), compound 3 (750 µM), compound 7 (1 mM) and compound 20 (2 nM and 750 µM). Coomassie blue staining was used as loading control. Data is expressed as fold change relative to vehicle-treated cells.

Although preliminary, our results suggest that an increase in AnxA2 could eventually underlie the protective effect against H_2O_2-induced oxidative stress observed for compounds 2 and 20.

5 Discussion

In the present study, the effect of selected natural compounds on PC12 cell viability in the absence and presence of ROS was investigated, together with their influence in the expression of AnxA2.

5.1 Natural compound 2: Sciadin

Sciadopitys verticillata Siebold et Zucc. (commonly known as umbrella pine) (81) is regarded to be amongst the most relictual of all plants, as the last living member of an ancient conifer lineage: the Sciadopityaceae. In fact, this species is morphologically and genetically diverged from all other conifers and considerably older than other renowned 'living fossil' gymnosperms such as *Metasequoia* or *Wollemia*, having diverged around 220 million years ago. It thereby represents one of the most early diverging plant lineages survived by a single species in the world, together with other ancient plants like *Amborella* and *Ginkgo*. How the species withstood millions of years being exposed to extreme climate conditions (including glacial–interglacial cycles) and deep variations in fauna, flora and microorganisms, persisting until today, is not well known (82). However, it is certainly a species of great interest, whose composition should be addressed.

Sciadin is a diterpenoid originally isolated from the heartwood of *Sciadopitys verticillata*, currently an endemic sole species to Japan (83,84). This terpenoid was later extracted from the plant's seeds and it was found to have a growth inhibitory effect on seed germination (61,84). However, little else is known about its biological activity and the compound was never pharmacologically addressed. Nonetheless, several other diterpenoids are known for their medical use e.g ginkgolide–B, colforsin and paclitaxel. The lack of information on sciadin makes it impossible to predict its effect on PC12 cells.

The effect of sciadin on PC12 cell viability was addressed in three stages: preliminary exposure (Figure 4.3), prevention of oxidative damage (Figure 4.8) and recovery from oxidative damage (Figure 4.13). This compound presented the most promising results, exhibiting no cytotoxicity and enhancing cell viability in a dose-dependent manner after exposure to ROS, this way contributing for cellular recovery upon oxidative damage. The dose-dependent result suggests that higher concentrations may cause an even stronger improvement. Regarding exposure to H_2O_2, it must also be noted that in our work, we used a concentration of 1 mM, which was

responsible for a 77% drop on cell viability alone. Considering that this high concentration leads to severe cell damage, these experiments should be repeated with lower concentrations of H_2O_2, and with other oxidative stress inducers. Moreover, the effect of these compounds should be compared with known antioxidants such as N-acetylcysteine.

To address sciadin's influence on AnxA2, the protein's expression level in PC12 cells was determined (Figure 4.17). Exposure to sciadin upregulated the expression of AnxA2. As previously mentioned, AnxA2 was found to increase cell proliferation and inhibit cell apoptosis via different pathways in a wide range of cell types (52,85). Therefore, in agreement with previous reports, the observed increase in the expression of AnxA2 may explain the obtained increase in PC12 cell viability. However, because the sustained overexpression of AnxA2 is mostly associated with malignant tumour progression (53), additional work is required to investigate whether this upregulation is transient or sustained. Moreover, it would also be interesting to determine if these compounds affect AnxA2 PTMs and its cellular localization, to ascertain whether or not this enhancement can be physiologically harmful. If not, sciadin might be a potential antioxidant candidate to address the pathological oxidative stress observed in neurodegeneration.

5.2 Natural compound 3: Dimethyl sciadinonate

Dimethyl sciadinonate is another diterpenoid, structurally related to sciadin, and extracted from the leaves and seeds of the native conifer *Sciadopitys verticillata* Sieb. et Zucc. (61,86). This terpenoid was later isolated from *Osmunda asiatica* (87) and the leaves of avocado (*Persea americana*) where it inhibits the growth of silkworm larvae (88,89). However, little else is known about its biological activity and the compound was never pharmacologically addressed. The lack of information on dimethyl sciadinonate makes it impossible to predict its effect on PC12 cells.

The effect of dimethyl sciadinonate on PC12 cell viability was addressed in three stages: preliminary exposure (Figure 4.4), prevention of oxidative damage (Figure 4.9) and recovery from oxidative damage (Figure 4.14). Dimethyl sciadinonate presented a milder but similar effect on PC12 cell viability when compared with sciadin. The compounds' closely related structure might be responsible for these similar results. It can additionally be hypothesised that these different functional groups might play an important role in the mechanism leading to increased cell viability.

To address dimethyl sciadinonate's influence on AnxA2, the protein's expression level in PC12 cells was determined (Figure 4.17). Interestingly, in contrast to sciadin, exposure to dimethyl sciadinonate did not affect the expression of AnxA2, meaning that sciadin's specific functional groups could be responsible for triggering the mechanism by which this molecule upregulates AnxA2. Also, dimethyl sciadinonate might be an alternative, in case sciadin exposure proves to be physiologically harmful following AnxA2 upregulation.

5.3 Natural compound 7: Naringenin(3→6'')luteolin

Narthecium ossifragum L. Huds (commonly known as bog asphodel) is mostly known for causing poisoning in cattle, goats and sheep. Its harmful effects were reported for the first time in 1667 (63) and currently include nephrotoxicity and hepatotoxicity (90). The plant is also believed to cause *alveld*, a photodynamic disease in lambs. Although this species has been known and studied for centuries, little is known about its chemical constituents and their biological activity. In fact, it wasn't until recently that the first aromatic compounds were identified, together with a fungal metabolite suggested to play a significant role in the plant's pathogenicity (63).

Naringenin(3→6'')luteolin is a new flavonoid isolated from the fruits of *Narthecium ossifragum* and hitherto unique to this plant source. The first reported *in vitro* tests showed significant cytotoxicity for this natural compound: IC_{50}=230 µM (primary rat kidney epithelial cells) and IC_{50}=115 µM (MOLM13 acute myeloid leukaemia cells) (63). So far, nothing else has been published regarding the compound's biological activity. However, a cytotoxic effect on PC12 cell viability would be expected from this information.

Nonetheless, if we look at naringenin or luteolin alone, they present very interesting outcomes. Naringenin was reported to have a protective effect on Aβ-induced *in vitro* neuronal cytotoxicity (91), to ameliorate AD-type neurodegeneration with cognitive impairment (AD-TNDCI) in rats (92), to improve learning and memory in an intrahippocampal Aβ-injected rat model of AD (93) and to have a neuroprotective role on rotenone induced PD rat model (94). Luteolin promotes a unique anti-inflammatory, antioxidative and neuroprotective phenotype (95) and it was reported to protect PC12 cells from 6-OHDA-induced apoptosis (96) and to have a neuroprotective and neurotrophic role against the MPTP-induced parkinsonian mouse model (97).

As naringenin(3→6'')luteolin was not tested in neuronal cells thus far, these contrasting results make it difficult to predict its effect on PC12 cells.

The effect of naringenin(3→6'')luteolin on PC12 cell viability was addressed in three stages: preliminary exposure (Figure 4.5), prevention of oxidative damage (Figure 4.10) and recovery from oxidative damage (Figure 4.15). This compound's antioxidant potential was more doubtful, as it exhibited a general cytotoxic effect on PC12 cell viability, especially in higher compound concentrations, matching earlier *in vitro* tests which showed significant cytotoxicity for this compound (63). Surprisingly, lower concentrations showed a positive influence on cell viability in the presence of ROS, especially in the prevention of oxidative damage. However, further verification of these results is crucial, given the obtained broad SEM values derived from the compound's viscosity and poor solubility, which sometimes led to uncertainty when handling small volumes. Nonetheless, our results suggest that naringenin(3→6'')luteolin could have a positive influence on PC12 cell viability in the presence of ROS, especially in the prevention of oxidative damage. This is a very interesting outcome, considering that both naringenin and luteolin were reported to have a neuroprotective effect against neurodegeneration (91–97). However, thorough toxicological studies must be performed to investigate the compound's safety.

To address naringenin(3→6'')luteolin's influence on AnxA2, the protein's expression level in PC12 cells was determined (Figure 4.17). Exposure to this compound did not affect the expression of AnxA2. To ensure that the protein is not involved in the observed decline in PC12 cell viability, it would be important to determine how this compound influences AnxA2 PTMs and their cellular localization.

5.4 Natural compound 20: Mefenamic acid

Viscum album L. (commonly known as European white-berry mistletoe) has been acknowledged as an important medicinal plant for millennia (73). Mistletoe extracts' medical uses date back to the 5th century BCE, when it was used to treat diseases of the spleen and complaints associated with menstruation. In the following centuries, it was used to cure illnesses and disorders as diverse as epilepsy, infertility, ulcers, labour pains, oedema or the so called "weakness of the heart". In the 20th century, its uses in Europe as a traditional remedy included hypertension, diabetes, arthrosis and cancer (73,98). The anticancer activity has been attributed to the presence of lectins, viscotoxins and alkaloids, the latter being unidentified until

recently. The novel group of aminoalkaloids was suggested as a potential source of future anti-cancer drugs (73). Interestingly, the structure of these newly isolated alkaloids resembles the one of a synthetic widely used nonsteroidal anti-inflammatory drug (NSAID): mefenamic acid.

Mefenamic acid is produced and commercialised worldwide on an industrial scale, being synthesised from e.g. 2-chlorobenzoic acid and 2,3-dimethylaniline (99). In Portugal, it is the active pharmaceutical ingredient (API) of Ponstan®, whose marketing authorization dates from 1965. This anthranilic acid derivative is an anti-inflammatory, antipyretic and analgesic drug used for the symptomatic treatment of rheumatoid arthritis, osteoarthrosis, headache, flu states, primary dysmenorrhoea, fever and postoperative, postpartum or muscular pain, to name a few applications (100). Nevertheless, recent studies suggest that its pharmacological activity may be extended to neuroprotection against neurodegeneration (101–104). NSAIDs have been implied on neurodegenerative disorders such as AD, not only for the association of this disease with inflammation (105) and their typical role as cyclooxygenase inhibitors (104), but also for NSAIDs' newly identified potential antioxidant properties (101,106). They appear to minimize the risk of AD, delay the onset of dementia, decelerate its progression and reduce the severity of cognitive symptoms (105). Particularly, mefenamic acid demonstrated an *in vitro* neuroprotective effect in differentiated PC12 cells, by inhibiting cytochrome c release from mitochondria and caspase-3 activation, possibly derived from its inhibitory effects on ROS and nitric acid accumulation. Mefenamic acid could also promote cell survival by up-regulating the expression of the antiapoptotic protein Bcl-X_L. In the same study, mefenamic acid improved learning and memory impairment in an $A\beta_{1-42}$-infused AD rat model (102).

These remarkable outcomes, together with mefenamic acid's recently identified radical scavenging potential (indicating an antioxidant activity) (101,106), make this compound a promising candidate to address neurodegeneration. Given the aforementioned results, this compound was expected to have an overall positive effect on PC12 cell viability.

The effect of mefenamic acid on PC12 cell viability was addressed in three stages: preliminary exposure (Figure 4.6), prevention of oxidative damage (Figure 4.11) and recovery from oxidative damage (Figure 4.16). Mefenamic acid enhanced PC12 cell viability in lower concentrations and exhibited significant cytotoxicity in higher concentrations. This could be the adverse result of exceeding the maximum safe concentration on the therapeutic window for this compound (107). Unexpectedly, the cytotoxic effect was not observed in the presence of ROS, where the compound had a positive influence in the prevention and recovery from oxidative stress. Because preliminary exposure to higher concentrations proved to be toxic for

PC12 cells and did not present a strong positive effect in the presence of ROS, they should be disregarded in future studies. However, according to previous research, it would be expected for lower concentrations to demonstrate an effect on PC12 cell viability in the presence of ROS. For example, it has been reported that exposure to 5 μM of mefenamic acid for 24 hours exhibited a neuroprotective effect on PC12 cells (102). Hence, in the present work, lower concentrations did not bring the expected positive outcomes regarding cell viability in the presence of ROS. Considering the reported antioxidant and neuroprotective potential of this compound, different approaches must be tested, including the exposure to milder ROS damage or the extension of the preceding incubation time with mefenamic acid. Once again, it would be interesting to test this compound with lower concentrations of the toxic stimuli.

To address mefenamic acid's influence on AnxA2, the protein's expression level in PC12 cells was determined (Figure 4.17). Despite the opposite effect of lower and higher concentrations on cell viability, they seem to influence AnxA2 in a similar way. Moreover, AnxA2 was proportionally upregulated by both tested concentrations. As previously mentioned, AnxA2 was found to increase cell proliferation and inhibit cell apoptosis in a wide range of cell types (52,85). Therefore, in agreement with previous reports, the observed upregulation of AnxA2 would produce an increase in cell viability for both concentrations, which did not occur. These results suggest that AnxA2 may not be directly involved in the effect of mefenamic acid on PC12 cell viability. In any case, although a correlation between exposure to mefenamic acid and increased AnxA2 expression levels was observed, the underlying mechanism behind this outcome is yet to be unveiled. Furthermore, because the sustained overexpression of AnxA2 is mostly associated with malignant tumour progression (53), additional work is required to investigate whether this upregulation is transient or sustained, as well as which PTMs are overexpressed and their cellular localization, to ascertain whether or not this enhancement can be physiologically harmful.

6 Conclusions

From the originally provided twenty natural compounds, sciadin, dimethyl sciadinonate, naringenin(3→6'')luteolin and mefenamic acid were selected and tested in detail. Sciadin presented the most promising results, exhibiting no cytotoxicity and contributing for cellular recovery upon oxidative damage. If a transient increase in the expression of AnxA2 is confirmed, sciadin could be a potential antioxidant candidate to address the pathological oxidative stress observed in neurodegeneration.

Nonetheless, the underlying mechanism behind the obtained results is yet to be unveiled. In further studies, in order to identify which pathways are involved in the natural compounds' antioxidant effect, the expression of other antioxidant enzymes, superoxide dismutase (SOD1), manganese SOD (SOD2), catalase, glutathione peroxidase (GPx1) and heme oxygenase 1 (HO1), could be determined by qRT-PCR. Moreover, ROS levels and total, reduced and oxidised glutathione could also be measured. Regarding the compounds' influence in AnxA2, the protein's expression levels in the presence of ROS-inducer must be determined and an experiment investigating which PTMs (especially Tyr23 phosphorylation) are involved, as well as AnxA2 subcellular localisation would be of great interest. Also, the natural compounds' effect in the expression of AnxA2 should be analysed on a time-dependent manner, to learn whether the observed upregulation is transient or sustained and, hence, physiologically safe or harmful.

Furthermore, PC12 cells should be exposed to lower concentrations of H_2O_2, as well as to other oxidative stress inducers, to investigate how this affects the natural compounds' ability to protect cells against oxidative damage. Moreover, a positive control, with a known antioxidant such as N-acetylcysteine, should be included. The concentration range must also be expanded to ascertain potential antioxidant effects outside the tested span. In addition, besides the applied dose-response approach, a time-response analysis could also be performed.

For now, the effect of the remaining natural compounds on PC12 cell viability, together with their influence in the expression of AnxA2 is still waiting to be revealed. In fact, a considerable amount of additional work is required before we can learn which (if any) of the given compounds are strong antioxidant drug candidates to address neurological diseases.

Nonetheless, it was the first time that the effect of these four compounds on PC12 cell viability was addressed with a particular focus on AnxA2, as a potential defence mechanism against the excessive oxidative damage observed in neurodegenerative diseases.

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Appendix

A1. Detailed materials

a. Chemicals and reagents

Table A.1 Chemicals and reagents

Chemical	Abbreviation, Chemical Formula	Mr	Supplier
Ethanol	EtOH, C_2H_5OH	46.07	Sigma
Double-distilled water	ddH_2O, H_2O	18.02	UiB
Dimethyl sulfoxide	DMSO, C_2H_6OS	78.13	Sigma
Resazurin	$C_{12}H_7NO_4$	229.19	Promega
Hydrogen peroxide	H_2O_2	34.01	Sigma
Acetone	C_3H_6O	58.08	Sigma

b. Buffers and solutions

b.1 Culture growth medium

Table A.2 – Culture growth medium

Medium	Supplier	Substance added	Volume	Final Concentration
RPMI 1640 *complete for cell culture, with:*	Sigma	-	500 mL	-
		Horse serum (HS)	*50 mL*	*9.8% (v/v)*
		Fetal bovine serum (FBS)	*25 mL*	*4.9% (v/v)*
		Penicillin-Streptomycin (P/S)	*5 mL*	*1.0% (v/v)*
		L-Glutamine	*5 mL*	*1.0% (v/v)*
		Plasmocin (prophylaxis)	*100 µL*	*0.02% (v/v)*
		NaOH 1M (pH adjustment)	*± 1 mL*	*± 0.2% (v/v)*

b.2 Buffers and solutions for Annexin A2 determination

Table A.3 – Buffers and solutions for Annexin A2 determination

Buffer/solution	Supplier	Substance	Final Concentration
PBS	Medicago	NaCl	140 mM
		KCl	2.7 mM
		Phosphate buffer (pH=7.4)	10 mM
1× RIPA buffer	Sigma/Merck	Tris-HCl (pH=7.6)	25 mM
	Merck	NaCl	150 mM
	Sigma	NP-40	1.0% (v/v)
	Sigma	Sodium deoxycholate	1.0% (w/v)
	Sigma	SDS	0.1% (w/v)
Lysis buffer	-	1× RIPA buffer	-
	Sigma	EGTA	2 mM
	Roche	Protease inhibitor cocktail	2% (v/v)
	Sigma	Sodium orthovanadate	200 mM
	Sigma	N-Ethylmaleimide	20 mM
Super 4× Denaturing buffer	Sigma/Merck	Tris-HCl (pH=6.8)	250 mM
	Sigma	SDS	20% (w/v)
	Merck	2-Mercaptoethanol	20% (v/v)
	Sigma	87% Glycerol	20% (v/v)
	Sigma	3% Bromophenol Blue	0.02% (w/v)
Electrophoresis buffer	Sigma	Tris (pH=8.3)	25 mM
	Merck	Glycine	190 mM
	Sigma	SDS	0.1% (v/v)
Blotting buffer	Sigma	Methanol	10% (v/v)
	Merck	Glycine	0.19 M
	Sigma	Tris	0.02 M
TBS	Sigma	Tris (pH=7.4)	30 mM
	Merck	NaCl	140 mM
TBS/Tween buffer	Sigma	Tris (pH=8.0)	20 mM
	Merck	NaCl	150 mM
	Sigma	Tween 20	0.05% (v/v)

c. Consumables

Table A.4 – Consumable materials

Consumable	Product name	Supplier
Vials	2 mL Screw Top Vials	Agilent
Tips	-	VWR; Gilson; Sarstedt
Tubes	Centrifuge Tubes with CentriStar™ Cap	Corning
Cell culture flasks (25 cm^3)	-	Thermo scientific; Sarstedt
Serological pipettes	-	Sarstedt; VWR
Microcentrifuge tubes	-	Eppendorf
Multiwell plates (96 wells)	-	BD Falcon
Multiwell plates (6 wells)	Cell-Culture Treated Multidishes	Nunc

d. Technical equipment

Table A.5 – Technical equipment

Equipment	Product name	Supplier
Laminar-flow hood	Holten LaminAir S2010 0.9	Holten
Incubator	CO_2, water jacketed incubator, Series II	Forma Scientific,Inc.
Water bath	Water Bath Swbd 1D	Stuart scientific
Inverted microscope	Olympus CKX31 SF	Olympus
Vacuum aspiration pump	Vacusafe comfort	Integra Biosciences
Microplate Reader	Victor^3 Multilabel Counter 1420-012	PerkinElmer
Automatic pipettor	Easypet Pipette Controller	Eppendorf
Hemocytometer	Reichert Bright-Line	Hausser Scientific

Micropipettes	-	Eppendorf, Thermo Scientific, Gilson
Cell scraper	-	Sarstedt
Centrifuges	5804 R; 5415 D; Galaxy Mini	Eppendorf; VWR
FTIR spectrometer	Direct Detect® Spectrometer	Merck Millipore
IR sample reader cards	Direct Detect® assay-free cards	Merck Millipore
Heater	Techne Dri-Block® DB2A	Nerliens
10% SDS gels	Mini-PROTEAN® TGX Stain-Free™ Precast Gels	Bio-Rad
Electrophoresis equipment	Mini PROTEAN II	Bio-Rad
Electrophoresis power supply	EPS 3500	Pharmacia Biotech
Western blot equipment	-	Bio-Rad
Power supply	PS500XT DC Power Supply	Hoefer Scientific
Nitrocellulose blotting membrane	Amersham™ Protran™ 0.2µm NC	GE Healthcare Life Sciences
Blotting paper	Whatman® 3MM Chr	GE Healthcare
Porous mats	-	GE Healthcare
Magnetic stirrer	-	IKA
Molecular Imager	ChemiDoc™ XRS+	Bio-Rad
Platform shaker	-	Heto Lab Equipment

e. Data processing software

Table A.6 – Data processing software

Programme	Supplier	Purpose
Wallac 1420 Workstation	Perkin Elmer	Fluorescence measurement
Excel 2016	Microsoft	Data processing and analysis
Direct Detect® Spectrometer Software	Merck Millipore	Infrared protein quantitation
Image Lab™ Software	Bio-Rad	Gel and blot imaging and analysis