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NEUROPROTECTIVE ROLE OF NUTRACEUTICAL AND PHYTOCHEMICAL COMPONENTS

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ABSTRACT

The central nervous system (CNS) consists of around 100 billion neurons and as many glial cells such as astrocytes, oligodentrocytes and microglial cells. Alterations in normal CNS function can lead to detrimental structural and functional consequences. Neurodegenerative diseases (NDs), a heterogeneous group of diseases that affect the CNS of which the main ones are Parkinson's, Alzheimer's and Huntington's disease, are all characterized by progressive damage and death of neuronal cells and represent a health problem mainly affecting the elderly. The increase in the average age of the population is inevitably linked to an increase in the incidence of chronic degenerative diseases. These pathologies are characterized by a multifactorial etiology, in which oxidative stress and inflammation are the main causative factors. For this reason, the scientific world pays increasing attention to the characterization and the identification of nutraceuticals and phytochemicals with intrinsic pleiotropic activity. Moreover, in a Circular Economy perspective, these natural compounds can be obtained, as well as from natural sources, also from renewable resources derived from the food industry by-products and can be used for both preventive and therapeutic purposes.

The aim of this PhD program was to identify nutraceuticals and phytochemicals, both as extracts and pure compounds and obtained from both plant and renewable sources, which due to their antioxidant and anti-inflammatory properties, were able to counteract cellular and molecular alterations that characterize neurodegenerative diseases. Their neuroprotective potential (antioxidant and / or anti-inflammatory) has been evaluated in an in vitro model of neuroinflammation (the BV-2 microglial cell line activated with LPS), and / or in an in vitro model of neuronal oxidative stress (the neuron-like SH-SY5Y cell line differentiated with retinoic acid and exposed to H_2O_2).

In particular, 4 projects, distinct from each other but all deeply linked by the aforementioned common goal, have been discussed in this thesis. Following the data obtained in the 4 projects developed during this PhD project, it is therefore possible to conclude for each of them:

Study 1_ Impact of phenolic profile of different cherry cultivars on the potential neuroprotective effect in SH-SY5Y cells: new sweet cherries from Unibo breeding program can be considered a new functional food with a high antioxidant and neuroprotective activity. Furthermore, their protective activity seems to vary according to the specific phenolic pattern of the different cherry extracts.

Study 2_Anti-inflammatory activities of Spilanthol-rich essential oil from Acmella oleracea (L.) R.K. Jansen and its nanoemulsion: Acmella oleracea essential oil (OA), having shown both anti-inflammatory and antioxidant properties, can be considered as a valid strategy to counteract

neurodegenerative disorders. Moreover, the potential of this essential oil could be further enhanced if included it in an OA nanoemulsion, as a new technological formulation.

Study 3_Study of the anti-inflammatory activity of novel tacrine derivatives with lipids extracted from cashew nutshell liquid: in the pattern of studied compounds (hybrids 5-17), molecules 5 and 6 were identified as the most effective anti-inflammatory ones. Indeed, 5 and 6 reduced the expression levels of the main mediators of inflammation (such as TNF-a, IL-1β, COX2 and iNOS), the levels of the pro-inflammatory cytokine IL-1β and the nuclear translocation of the transcription factor NF-kB, becoming candidates as potential novel drugs for the treatment of neurodegenerative diseases.

Study 4_Coffee Silverskin (CSS) and Spent Coffee Grounds (SCG): coffee industry by-products as a promising source of neuroprotective agents: coffee by-products have proven to be a valuable source of bioactive compounds with high health-beneficial potential. Both extracts obtained from Coffee Silverskin (CSSs), for their antioxidant activities, and extracts obtained from Spent Coffee grounds (SCGs), for their antioxidant and anti-inflammatory activities, which are both coffee industry by-products, can be considered as a valid strategy to prevent and/or counteract neurodegeneration.

In general, it can be concluded that the natural compounds studied in this thesis have been proven, due to their antioxidant and/or anti-inflammatory properties, to be valid preventive and therapeutic strategies for the treatment of neurodegenerative diseases, to improve the life quality of these patients and of the general population by preventing and combating the onset of these deleterious diseases.

INDICE

1 NEURODEGENERATIVE DISEASES

The central nervous system (CNS) consists of around 100 billion neurons and as many glial cells such as astrocytes, oligodentrocytes and microglial cells. Therefore, its articulate structure makes it one of the most complex systems in our body [1]. Alterations in normal CNS function can lead to detrimental structural and functional consequences. Neurodegeneration is a common feature of CNS disorders and involves processes through which normal functions, such as mobility, memory, learning, judgement and coordination, are progressively lost [2].

Neurodegenerative diseases (NDs) are a heterogeneous group of diseases that affect the CNS and are all characterized by progressive damage and death of neuronal cells [3]. Neuronal deterioration can lead to cognitive deficits, dementia, motor impairment, behavioral and psychological disorders that vary in different diseases. The main NDs include Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease, and many others.

The causes and mechanisms underlying the onset and progression of these diseases are still unclear. However, it is clear that the presence of a single trigger is not sufficient for the development of these pathologies, but rather the involvement of several factors, which contribute to each other in the onset of neurodegeneration, is fundamental.

Although NDs are characterized by a multifactorial etiology, it is possible to identify some common features:

- a predictable pathological evolution, progressively involving larger and larger regions of the brain [2];
- the presence of modified proteins with a pathological tendency to self-aggregate and depositate at neuronal and extra-neuronal levels. The resulted self-aggregation protein products are toxic to the cells and the toxicity spreads to different areas of the brain [4];
- compromised axonal transport, mitochondrial dysfunction and induction of cell death pathways [3];
- perturbed Ca^{2+} homeostasis and excitotoxicity processes [5];
- all NDs show a strong involvement of inflammatory processes and oxidative stress conditions.

Normally, proteins assume specific three-dimensional conformations (folding) that ensure their proper functionality [6]. Altered protein folding causes the formation of sticky protein surfaces, which can abnormally “re-aggregate” to form non-functional and toxic filiform protein aggregates (called amyloid fibrils). Thus, misfolding is the inability of polypeptides to remain in their functional conformational state [7]. Protein misfolding insidiously contributes to a wide variety of diseases [8].

In neurodegenerative diseases, such protein accumulations are thought to contribute to neurodegeneration. As mentioned above, one of the most characteristic pathophysiological features of NDs is the presence of unfolded proteins, so that NDs are also called “proteinopathies” [9]. The generation of unfolded proteins leads to an increased ability to interact, aggregate and accumulate both within neurons and in the spaces between them (amyloid plaques and Lewy bodies) [10].

Among the well-known proteins involved in neurodegeneration are [11]:

- amyloid-β (Aβ) which is the primary constituent of senile plaques in Alzheimer's disease;
- α-synuclein which, under pathological conditions (e.g. Parkinson's disease), aggregates to form insoluble fibrils characterized by Lewy bodies;
- superoxide dismutase 1 (SOD1) or TAR DNA-protein 43 (TDP-43) that are mutated in amyotrophic lateral sclerosis (ALS) [3], [12];
- huntingtin (Htt) which undergoes a DNA mutation known as the CAG trinucleotide repeat typical of Huntington disease. In fact, this disease is also known as "trinucleotide repeat disorders" [3], [6], [9];
- tau which, when phosphorylated, forms the neurofibrillary tangles that characterize Alzheimer's disease.

When proteins are not correctly folded, they are normally degraded by the cell, which reuses their essential constituents, the amino acids. If this degradation does not take place or is insufficient, unfolded protein aggregates accumulate in the cells. Under physiological conditions, misfolded proteins deposited in the various cellular compartments (cytoplasm, nucleus and endoplasmic reticulum (ER)) are normally and efficiently removed by the cell control systems, such as the ubiquitin (Ub)-proteasome system (UPS), macroautophagy and chaperone-mediated autophagy (CMA) [13]. In addition to these intracellular systems, in the CNS there are also several extracellular mechanisms involved in the clearance of neurotoxic proteins, such as:

- the blood-brain barrier (BBB) and the glymphatic system responsible for removing toxic proteins from extracellular spaces;
- the cerebrospinal fluid (CSF) and the interstitial fluid (ISF) where the altered proteins can be phagocytosed by microglia and astrocytes or degraded by proteases [12].

Thus, in parallel to the formation of these insoluble protein aggregates, another factor causing the accumulation and spread of these altered proteins is the impairment of normal intra- and extra-cellular control and elimination systems [6], [12], [14]–[18] (Figure 1).

Therefore, these pathways of altered protein accumulation represent an important molecular mechanism underlying the pathogenesis of NDs [17].

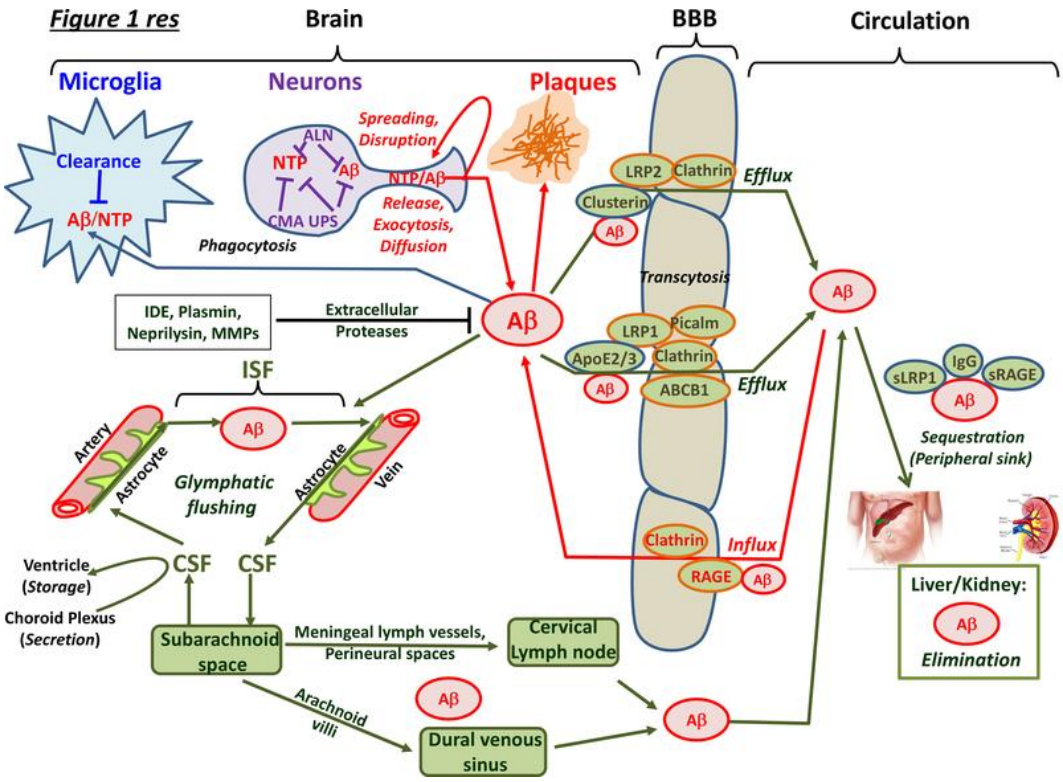

Figure 1. Overview of intracellular and extracellular mechanisms for the clearance of neurotoxic proteins from the brain [12].

Regardless of the specific protein involved, an individual risk of developing one of these diseases increases markedly with ageing [12]. Even under normal physiological conditions, ageing leads to a loss/alteration of the efficiency of the normal processes of protein synthesis, folding and degradation, resulting in an accumulation of misfolded proteins [18]. This is one of the reasons for the close correlation between age and neurodegeneration.

In light of these, neurodegenerative diseases are a health problem that mainly affects the elderly population. In fact, they arise mainly in the later stages of life, making time an essential cofactor in the pathogenesis of these disorders. Over the years, the scientific community has made great efforts to reduce premature mortality due to the onset of cancer, infectious diseases and cardiovascular disorders, making it possible to increase life expectancy. On the other hand, unfortunately, the direct consequence of this increase in the average age of the population corresponded to an increase in the incidence of neurodegenerative diseases [19]. For example, in the coming decades, the number of PD patients is expected to grow to about 1.24 million [20], while reaching 13.8 million people (aged 65+) with AD only in the United States [21].

In addition, NDs have a progressive course that is clinically evident when the anatomical brain damage is already at an advanced stage. In this regard, it has been reported that at the time of diagnosis the patients have already lost up to 70% of neurons [22], thus reducing the possibility of effective

therapeutic intervention. Unfortunately, to date there are no effective therapies available to halt or reverse the disease. Treatments currently in clinical use are only able to alleviate symptoms, without slowing down the progression of neurodegeneration [5], [12].

In conclusion, the increasing diffusion of these diseases due to their multifactorial nature, lack of effective therapy, late diagnosis and increased life expectancy, is a health problem that affects the entire world population, with implications that have a strong economic impact on public health costs and a social impact on the quality of life of patients and their families [6]. Therefore, it is fundamental to find new and effective therapeutic strategies and, in this effort, a clear and deep understanding of the multiple mechanisms involved in the onset of these deleterious diseases is crucial and necessary. Recently, increasing attention is being paid to the multifactorial nature of NDs for potential new therapeutic approaches. In particular, there is a growing body of evidence supporting the key role of oxidative stress and inflammation in the initiation and progression of the neurodegenerative process. Indeed, epidemiological, animal and clinical studies confirm the role of oxidative stress and the inflammatory cascade in the initiation and progression of neurodegenerative disorders [23]–[25]. For this reason, multi-target therapeutic strategies capable of simultaneously counteracting these two conditions represent a promising opportunity to contrast these deleterious diseases.

1.1 ALZHEIMER DISEASE (AD)

Alzheimer's disease (AD) is a degenerative process that slowly and progressively destroys brain cells, leading to an irreversible deterioration of higher cognitive functions such as memory, reasoning and language up to a total impairment of functional status and ability to perform normal basic daily activities [26]–[28]. Alzheimer's disease has been known as a form of dementia since the early 1900s, when the physician and neurologist Alois Alzheimer, after whom the disease is named, discovered, through a brain autopsy of a 51-year-old man who had been suffering from memory loss, disorientation, inability to speak properly and hallucinations for at least five years, the presence of senile extraneuronal plaques and intraneuronal neurofibrillary tangles (NFTs) in the brain, together with a severe form of brain atrophy [27], [29].

AD is characterized by two distinctive histopathological features, both of which can be identified by the formation and deposition of insoluble protein clusters: extracellular plaques of β-amyloid (Aβ) and intracellular neurofibrillary tangles (NFTs) of hyperphosphorylated tau protein. Extracellular β-amyloid (Aβ) plaques are generated by the cutting of Amyloid Precursor Protein (APP) by specific proteases, β-secretases and γ-secretases, and are extremely toxic at the synaptic level. Aβ peptides consist of 36 to 43 amino acids and are products of the progressive proteolytic degradation of APP

by the amyloid β-site precursor protein 1 (BACE-1) and enzyme complexes consisting of the proteases β-secretase and γ-secretase with presenilin in the catalytic core. When Aβ plaques are accumulated in the brain, due to an imbalance between production, clearance and aggregation of Aβ peptides, we speak of the "amyloid hypothesis" [10], [26], [27].

Following the amyloidogenic pathway, APP undergoes progressive cuts leading to the formation of Aβ (Aβ1-42), which are released into the extraneuronal space where they are deposited [10], [26], [27]. The main constituents of these extracellular deposits are Aβ40 and Aβ42, of which Aβ42 is considered the most toxic [30], [31] and generally associated with neuronal degeneration and synapse disruption [27]. In contrast, in healthy patients, APP follows the non-amyloidogenic pathway, mediated by γ- and α-secretases and from which residues not including Aβ are generated [26] (Figure 2).

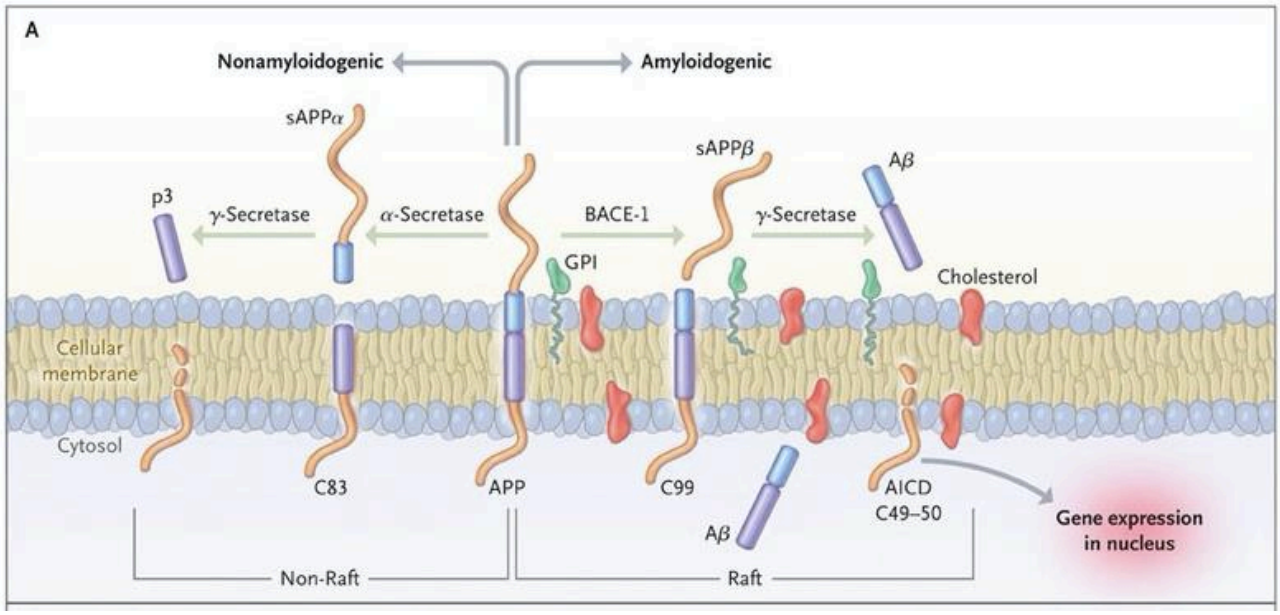

Figure 2. Processing of Amyloid Precursor Protein (APP). Modified from [26].

On the other hand, intracellular neurofibrillary tangles (NFTs) are intracellular protein aggregates consisting predominantly of hyperphosphorylated and misfolded tau protein (Figure 3). Being important for microtubule stability, tau disruption leads to impaired axonal transport [1], [10], [26], [27], [32]. When hyperphosphorylated, tau becomes insoluble by losing its affinity for binding to microtubules and self-aggregates into paired helical filamentous structures [26]. Phosphorylation of this protein, in addition to altering microtubule structure, interferes with normal synaptic communication between neurons, leading to loss of synapses, neuronal branching and neuronal death [31].

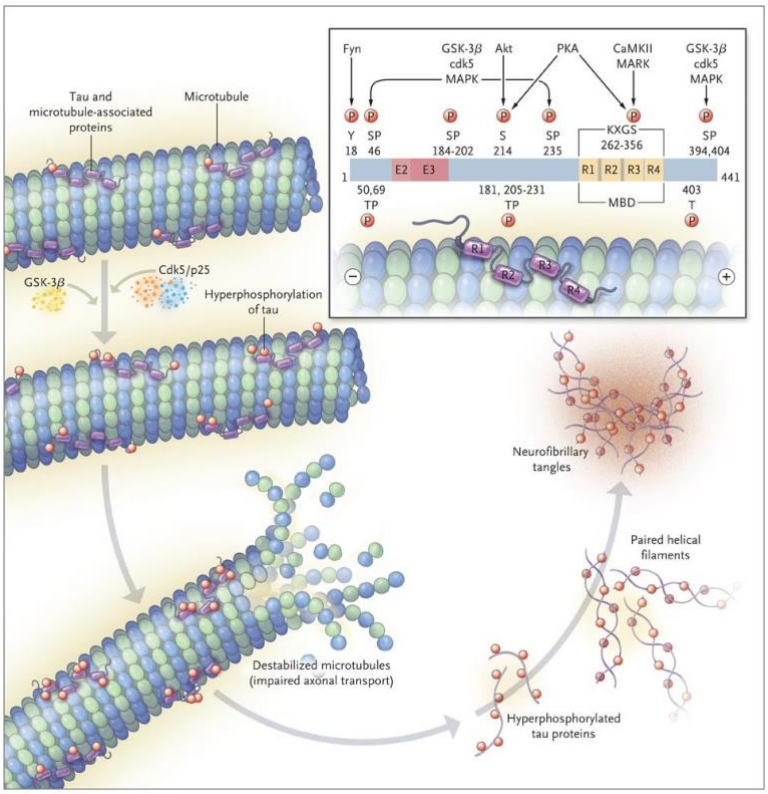

Figure 3. Tau Structure and Function [26].

In addition to this, AD is also characterized by the loss of neurons and white matter, congophilic (amyloid) angiopathy, oxidative damage and inflammation [26]. The chronicity of the originally protective inflammatory response leads to alterations in the morphology and distribution of microglial cells resulting in increased release of cytokines and inflammatory mediators, that contribute to a neurotoxic oxidative stress condition that is correlated with the neuronal death observed in AD patients [27], [33], [34]. Over time, the incidence of this disease has grown exponentially and accounts for about 75% of the causes of dementia [35]. Today, around 50 million people worldwide are affected by AD and this is expected to increase to over 100 million by 2050 [27], [36], [37]. Except for rare cases of early onset (3%), AD is a dementia that mainly affects the elderly population over 65 years of age [27], [32], [38]–[40].

Therefore, its increasing incidence is closely related to the increased life expectancy of the world population that has been witnessed in the last century [27]. Today, the availability of therapies that can only alleviate the severity of symptoms and the late diagnosis (the appearance of the first clinical symptoms occurs about 10-12 years after the onset of the first neurodegenerative processes) are other factors contributing to the increased incidence and severity of this disease [10], [27].

The precise mechanisms underlying the onset of AD are still unknown. In particular, AD is considered a disease with a multifactorial etiology that cannot be traced back to a single cause but

rather to multiple factors that cooperate and interact with each other to promote its course [1], [5], [26], [27], [32].

Two types of Alzheimer's disease have been determined: the early-onset hereditary familial form and the late-onset sporadic form. Familial AD accounts for only 3% of AD cases and is genetically based. This form of AD is characterized by an early development of the disease, usually before the age of 50 (about 10-12 years earlier than the late forms), and is associated with mutations in the genes coding for APP and for Presenilin1 (PS1) and Presenilin2 (PS2). On the other hand, the second form of AD, known as sporadic or late (over 65 years), affects the highest percentage of the population [27], [41]. As anticipated, although Aβ plaques and NFTs are the main signs of AD, mitochondrial dysfunction, loss of calcium regulation, oxidative damage, inflammation, diabetes mellitus or hyperinsulinemia, overweight and hypercholesterolemia, hypertension, aging and sedentary lifestyle play an important role in the onset and progression of the disease [1], [26], [27].

1.2 PARKINSON DISEASE (PD)

Parkinson's disease (PD) is part of a group of pathologies defined as "movement disorders" [42]. Parkinson's disease is a slowly progressive disease characterized by some distinctive clinical features including a movement disorder consisting of bradykinesia, tremor at rest and rigidity, with postural instability occurring at a later stage [43]. Parkinson's disease, the second most common neurodegenerative disease after Alzheimer's disease (AD), has an incidence of about 0.5-1% among people over 65 years of age and grows to 3- 4% over the 80-year-olds [43]–[47] with a higher prevalence in men (2%) than women (1.3%) [45]. Age is the most impactful risk factor for PD and, in parallel with the ageing population, the prevalence and incidence of this disease are expected to increase by more than 30% by 2030 [43], [45] with both direct and indirect economic consequences for society.

Parkinson's disease was first described by James Parkinson, an English physician after whom the disease is named, in 1817, who defined it as “agitating paralysis”. His description of PD is still applicable today [43], [48].

" Involuntary tremulous motion, with lessened muscular power, in parts not in action and even when supported; with a propensity to bend the trunk forwards, and to pass from a walking to a running pace." [49]

Although the disease is defined by the described motor manifestations, PD is also characterized by a whole range of non-motor symptoms that often precede the motor symptoms by years or even decades

[43], [48]. The “premotor or prodromal” phase of the disease often occurs as early as 12-14 years before diagnosis [50] and it is characterized by non-motor symptoms, that could be classified into neuropsychiatric, sensory, sleep and autonomic symptoms [48]. These symptoms can be particularly difficult to treat as, having mostly a non-dopaminergic basis, they do not respond to treatments used for motor symptoms [43], [48]. Non-motor symptoms contribute significantly to the patient's disability and poor quality of life, and include impaired ability to smell, autonomic dysfunction, pain, fatigue, dream enactment (a behavioral disorder of REM sleep), orthostatic hypotension, cognitive and psychiatric disorders, hallucinations, depression, hyposmia, constipation, urinary incontinence and rapid eye movement (RDB) [43], [44], [46], [47].

PD occurs when the production of the neurotransmitter dopamine, which is essential for the control of body movements, declines considerably in the brain as a result of neuronal degeneration in the substantia nigra [51]. In particular, at the subcellular level Parkinson's disease (PD) is characterized by the progressive loss of dopaminergic neurons in the region of the substantia nigra pars compacta, and by the presence of cytoplasmic protein aggregates called Lewy bodies and Lewy neurites, i.e., protein aggregates consisting mainly of α-synuclein, in the remaining neurons [52], [53]. The cause of PD is still generally unknown. Although PD is in most cases an idiopathic or sporadic disorder (having a not known specific cause and showing the involvement of multiple causes), there is a minority of cases (10-15%) with a familiar history (Familial Parkinson's) and about 5% of these show a Mendelian inheritance [43], [44], [46]. Genetically based PD generally has an early onset (between 20 and 50 years of age) and it is usually mainly linked to genetic mutations in the α-synuclein protein (SNCA) and parkin (PARK) [48], [53], [54]. The genes considered responsible for these forms of genetic PD have been given the name “PARK” and, to date, around twenty have been identified. Mutations in some of these are associated with autosomal dominant inheritance (e.g., SCNA, LRRK2 and VPS32), others with autosomal recessive inheritance (e.g. PRKN, PINK1 and DJ-1) [43].
In contrast, to date about 90% of diagnosed PD cases are idiopathic [45] and have been associated with several causes, including mitochondrial dysfunction, oxidative stress, glutathione loss, neuroinflammation, altered signaling of neurotrophic factors, accumulation of abnormal proteins and environmental toxins (Figure 4) [43], [46], [47], [52], [53], [55].

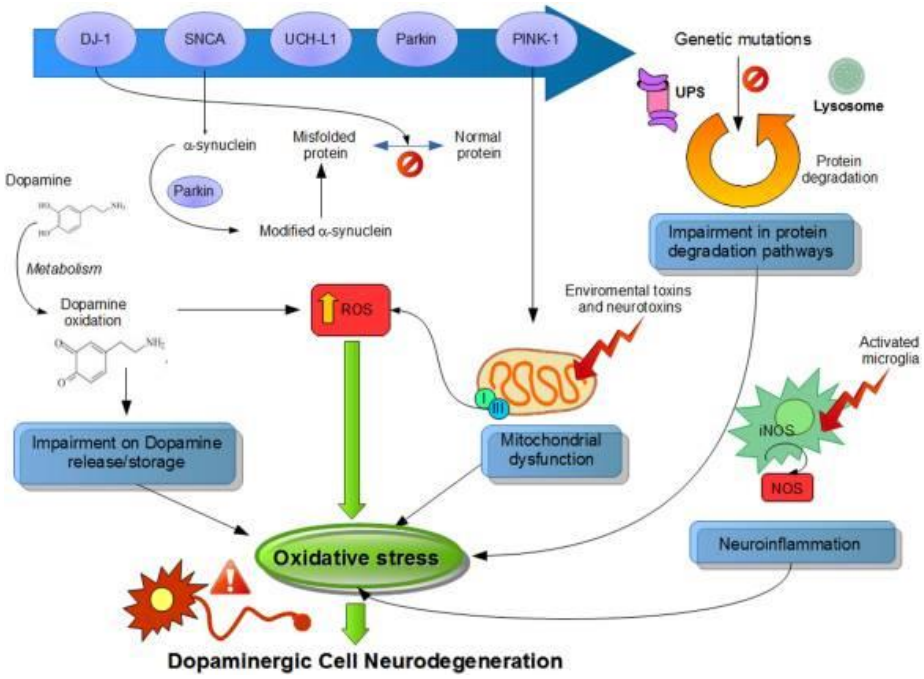

Figure 4. Suggested different pathways involved in PD [55].

It has been documented that dopaminergic neurons are highly vulnerable to oxidative stress. Dopamine (DA) can spontaneously undergo autoxidation producing toxic molecules such as hydrogen peroxide, superoxide radicals and toxic dopamine quinones [53], [55]–[57], leading to what is called "dopamine-dependent oxidative stress" [53]. Since cytoplasmic DA can rapidly lead to ROS formation, it must be rapidly inactivated/vehiculated from the cell by internalizing it into synaptic vesicles (Figure 5) [53].

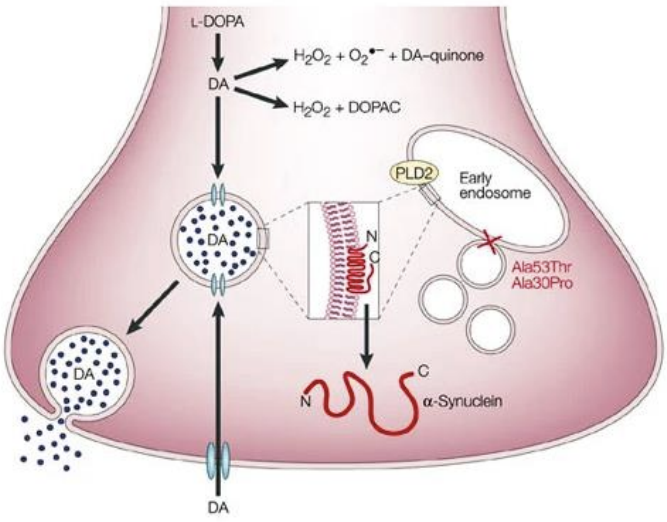

Figure 5. α-synuclein and intracellular dopamine storage [53].

In turn, oxidized dopamine can react with sulfhydryl groups of protein cysteines and generate additional ROS through the redox cycle [58]–[60]. Furthermore, the functional impact of oxidative stress in PD was also confirmed by the reduction of reduced GSH and the main antioxidant enzyme systems (superoxide dismutase, catalase and glutathione peroxidase) in the brain [61].

Another fundamental factor in the evolution of this disease is mitochondrial dysfunction, which plays a key role in the pathogenesis of both idiopathic and genetic forms [43], [61]. It is primarily linked to the deficiency of complex I of the mitochondrial respiratory chain mainly at the level of the substantia nigra [43], [61]. At the genetic level, mutations with consequent loss of function have been found in the PARK2 and PARK6 genes (coding for PINK1 and parkin respectively), both of which are fundamental in the process of mitophagy, i.e. the correct removal of dysfunctional mitochondria from the cellular environment [43], [61]. Finally, α-synuclein can accumulate within mitochondria and damage complex I, further supporting mitochondrial dysfunction and increasing oxidative stress.

Although it is still unclear whether it should be considered more cause or consequence, the strong involvement of neuroinflammation in the pathogenesis of PD [43], [47], [55], [61], [62] is certain. Post-mortem studies have found high levels of several cytokines, mainly TNF-α, and iNOS, in the substantia nigra and cerebrospinal fluid of brains of PD patients [43].
Initially, neuroinflammation was considered a secondary detrimental effect triggered by chemical or genetic stressors related to PD. For example, microglia can be activated by neuromelanin, a pigment present in neurons of the substantia nigra pars compacta [63], further increasing the susceptibility of this area of the brain to the inflammatory process. In addition, microglia can also detect the presence of misfolded α-synuclein, which promotes the production of ROS and proinflammatory cytokines [64], [65]. All this leads to an increase in neurotoxicity and a promotion of neuroinflammation [47]. On the other hand, over the years increasing evidence has shown that the neuroinflammatory process is also a fundamental causal factor in the onset of PD [47], [61], [62]. In particular, a strong interrelationship has been demonstrated between neuroinflammation and other pathogenetic processes, the main ones being mitochondrial dysfunction and oxidative stress [1], [62]. In addition to an immunoregulatory action of DA during the inflammatory process [66], it has been found that the inflammatory process may contribute to the intrinsic vulnerability of dopaminergic neurons and may interfere with the expression of genes coding for proteins such as α-synuclein, Parkin and DJ-1 [62].
In addition, there are many concomitant causes underlying the development of PD and, above all, there is a strong interrelationship between them, creating a self-sustaining and self-promoting circle

of pathology. Together with the already difficult treatment of motor and non-motor symptoms, the complex multifactorial nature of this disease makes it difficult to find an effective treatment and promotes the identification of multitarget molecules that can act simultaneously on the multiple involved pathways.

1.3 HUNTINGTON DISEASE (HD)

Huntington's disease (HD) is an autosomal dominant hereditary neurodegenerative disorder with a prevalent onset in young adulthood and leading to death within 15-20 years [67]–[69].

Huntington's disease affects approximately 5-10 individuals per 100.000 between the ages of 35 and 40 years [67]–[70]. However, its incidence range may vary from childhood to the ninth decade of life [70]. Clinically it is characterized by choreic movements, alterations in the emotional sphere and loss of cognitive abilities, probably caused by neuronal damage and death [68]. It was initially named "Huntington's chorea" in order to emphasize the choreic movement as the most characteristic trait, i.e. a particular type of abnormal movement characterized by sudden, involuntary, non-rhythmic jerks involving different parts of the body resulting in a particular gait, defined as "dancing".

Although choreic movements remain one of the most characteristic elements of this disorder, the heterogeneity of its manifestations makes it clinically much more complex. Even if chorea is usually the predominant symptom in the first phase of the disease, further motor disturbances, in some cases even more disabling, arise later, such as incoordination, progressive bradykinesia and rigidity (so-called motor impairment) [68], [71]. Moreover, many patients show substantial cognitive or behavioral disturbances before the onset of the diagnostic motor signs [72] such as depression, loss of self and spatial awareness, anxiety and dementia [68].

At the molecular level, HD is a hereditary disorder caused by mutations in the Htt gene that encodes for an expanded polyglutamine (polyQ) tract in the huntingtin (Htt) protein. The mutation involves a segment of DNA known as the CAG trinucleotide repeat. This segment consists of a series of three DNA building blocks (cytosine, adenine, and guanine) that appear several times in a row. Normally, the CAG segment is repeated between 10 and 35 times within the gene. In people with Huntington's disease, the CAG segment is repeated 36 to more than 120 times. People with 36-39 CAG repeats may or may not develop signs and symptoms of Huntington's disease, while people with 40 or more repeats almost always develop the disorder [67].

Although there is a strong influence of both genetic and environmental factors, ageing plays a key role in the onset of HD. Firstly, in most cases, there seems to be an inverse correlation between the age of onset of the disease and the number of CAG repeats (i.e. higher is the expansion of repeats, earlier is the onset of the disease [69]).

Given the breadth of molecular interactions in which Htt is involved, unsurprisingly its mutated form results in large-scale destabilization of the proteome and subsequent disruption of multiple cellular processes [67].

Indeed, mutated Htt (mHtt) is responsible for the disruption of multiple cellular processes, including protein-protein interaction, protein clearance, mitochondrial function, DNA maintenance, cell cycle regulation, N-methyl-D-aspartate (NMDA) receptor activation, energy metabolism, axonal trafficking, post-translational modification and gene transcription [70]. In addition, the toxic effect of mHtt also promotes detrimental conditions such as excitotoxicity, oxidative stress and inflammation [73]–[76], as well as being involved in the loss of beneficial functions of wild-type huntingtin (Htt) protein, including BDNF translation, vesicle transport and the scaffold for autophagic machinery [75] (Figure 6).

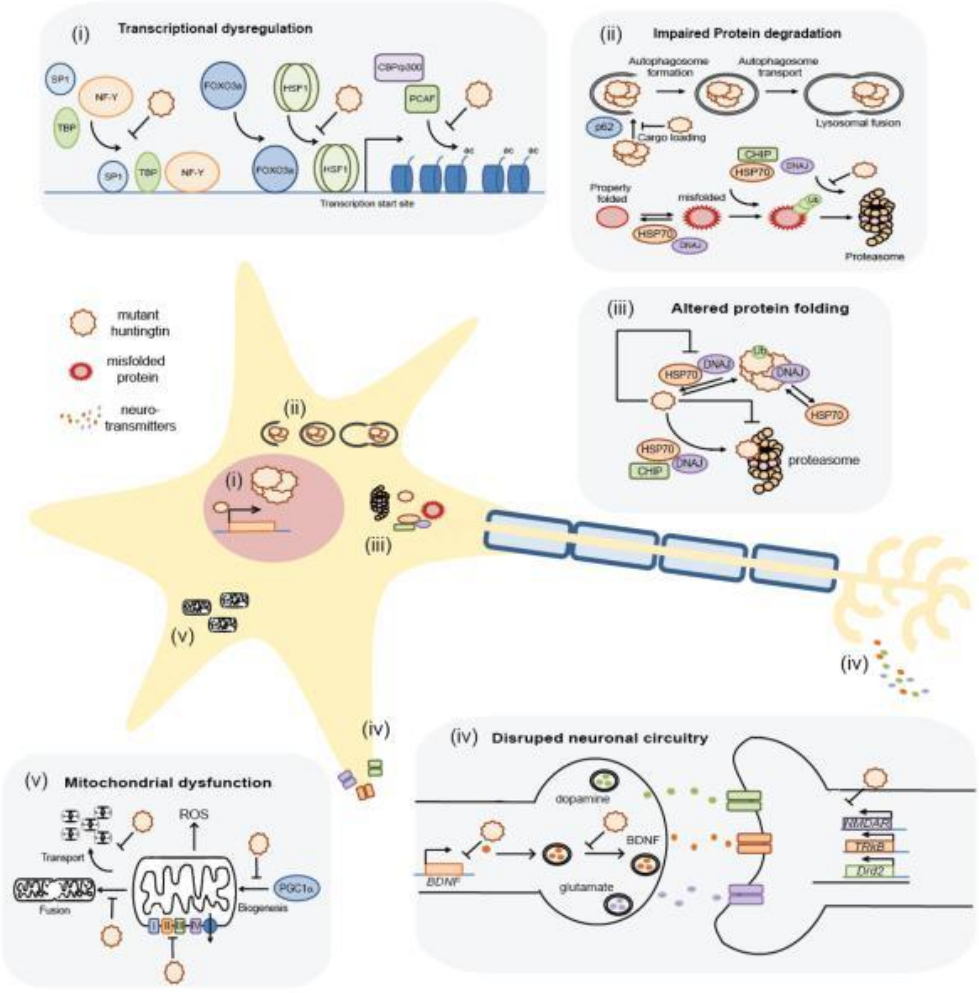

Figure 6. Major cellular pathways disrupted in Huntington's disease [67].

Despite progress in understanding the disease, the exact molecular mechanisms involved in the relationship between mHtt aggregates and cellular dysfunction/pathological symptoms remain unclear. Certainly mitochondrial dysfunction, transcriptional dysregulation, oxidative stress, neuroinflammation and neuronal excitotoxicity are some of the key pathways most impactful in the pathogenesis of HD [67], [70], [74]–[76] (Figure 7).

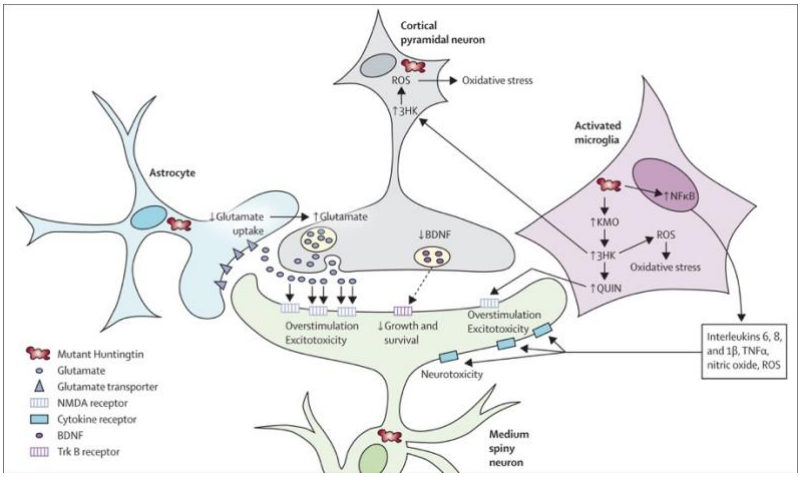

Figure 7. Intracellular pathways involved in pathogenesis of HD [68].

2 OXIDATIVE STRESS AND NEURODEGENERATION

2.1 OXIDATIVE STRESS

The normal functioning of the Central Nervous System (CNS) is ensured by maintaining the biochemical integrity of the brain, which can be altered by the development of an oxidative stress condition [77].

Oxidative stress occurs when there is an imbalance between the production of oxidizing substances, mainly reactive oxygen species (ROS), and the body's endogenous antioxidant defenses. This condition expresses a series of alterations that occur in cells, biological macromolecules and tissues when exposed to an excess of oxidant agents. In particular, an excess of ROS can cause damage within the cell (at the level of proteins, lipids and nucleic acids [78]–[80]), leading to cell apoptosis [81].

In all organisms, there is a fine balance between oxidant species and antioxidant defense systems, the purpose of which is to prevent and/or repair any damage generated. Under physiological conditions, oxidant species resulting from metabolism are adequate to maintain tissue homeostasis and are promptly neutralized by antioxidant systems that ensure efficient use of cellular oxygen and the maintenance of essential redox reactions [77]. The relationship between reactive species and antioxidant defenses could be represented as a balance that loses its physiological equilibrium when oxidative stress occurs. This imbalance is due to an excessive presence of oxidizing species or a depletion of antioxidant defenses [82] (Figure 8).

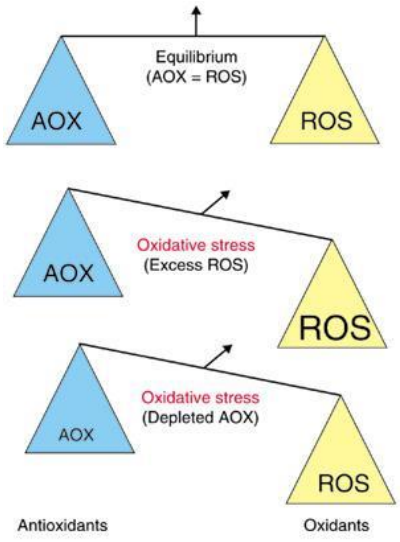

Figure 8. Balance between oxidizing species (ROS) and endogenous antioxidant defenses (AOX) [81].

Thus, ROS and other reactive species, being physiologically crucial in adequate quantities for the maintenance of cellular homeostasis and protection from pathogens, can be considered essential components in innumerable biological processes in which they act as second messengers [80], [83].
In general, according to the "redox window" hypothesis, depending on their quantity in the body, ROS show two faces and act in opposite ways: redox signaling and oxidative stress. An appropriate production of ROS (low levels) is required for the maintenance of normal cell signaling and cellular physiological functions, whereas an excess of ROS (high levels) establishes an oxidative state and may contribute to the development of pathological conditions [79], [84].
Alteration of the normal redox state can lead to significant toxic effects on different cellular components [85] and may accelerate normal ageing processes and be involved in various acute, chronic and degenerative pathological conditions, such as atherosclerosis, cardiac damage, ageing, diabetes and cancer [80], [84]. As anticipated, excessive production of ROS, correlated with the presence of an oxidative state, causes deleterious cellular damage and this condition is further enhanced by the damaged macromolecules themselves, which can behave as and/or become ROS. Indeed, on this basis, oxidative stress can be defined as a self-propagating phenomenon [77]. The body is continuously exposed to these reactive molecules either because they are physiologically produced by the body or because they come from exogenous sources such as air pollution, exposure to sunlight, ultraviolet (UV), ionizing radiation, stress, drugs, smoking and unbalanced diets that induce their production [82], [86] (Figure 9).
Therefore, a perfect balance between pro-oxidants and antioxidant systems is crucial to maintain cellular homeostasis and well-being.

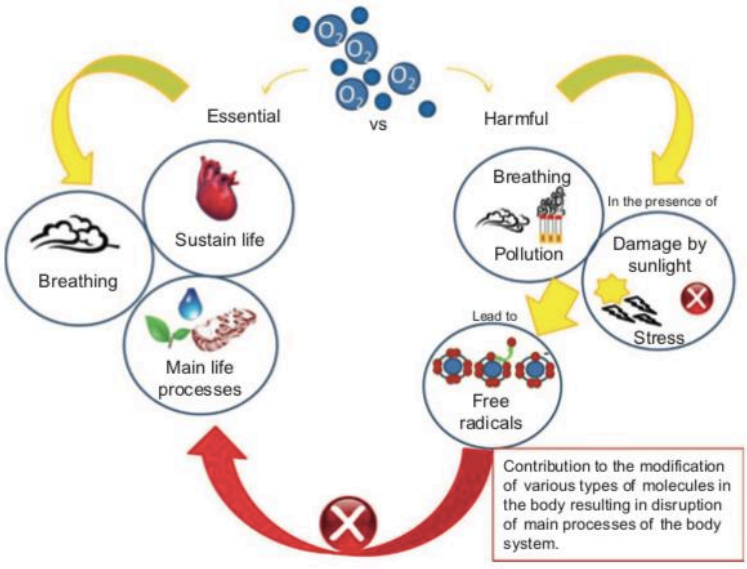

Figure 9. Double effect of O_2 in the body: essential and harmful [87].

2.2 FREE RADICALS AND REACTIVE OXYGEN SPECIES (ROS)

A free radical is defined as:

"a chemical species with an existence of its own containing one or more unpaired electrons on an atomic or molecular orbital" [82], [87].

Free radicals are very high reactive and unstable chemical species, which tend to steal an electron from other molecules to become stable. Depending on the atom responsible for their reactivity, they can be classified into 3 main categories:

- reactive oxygen species (ROS)
- reactive nitrogen species (RNS)
- reactive sulphur species (RSS).

All these molecules act as pro-oxidant chemical species and, consequently, if present at too high levels, lead to oxidative damage. Among these, ROS are the best known and most involved in cellular processes.

The mitochondria are the intracellular organelle designated to use molecular oxygen to generate energy in the form of ATP. In fact, they produce around 95% of intracellular ATP and are the destination of most of the O_2 breathed in. O_2 molecules that are not properly metabolised during this process are transformed into highly reactive molecules [87]. Under physiological conditions, approximately 2% of the total O_2 consumed by mitochondria can be related to the formation of ROS [82]. Specifically, ROS are produced in an aerobic environment in the mitochondria during the cellular respiration process. In this process, each oxygen molecule (O_2) is reduced to water (H_2O), producing energy. This requires the transfer of four electrons, not simultaneously, but by progressive steps with the generation of highly reactive intermediate species, ROS [87], [88] (Figure 10).

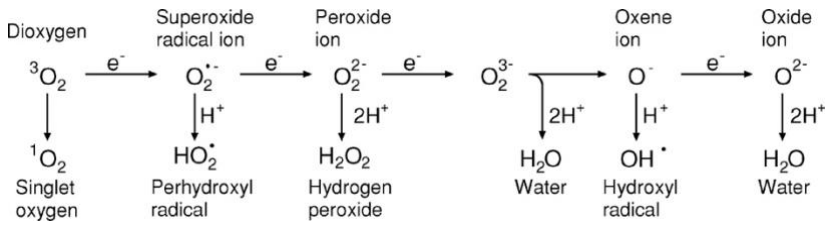

Figure 10. Generation of different ROS [88].

In the mitochondria, the respiration process takes place through the so-called mitochondrial electron transport chain (ETC), which is a transmembrane multimeric protein complex consisting of five multi-enzyme complexes: NADH-coenzyme Q (CoQ) reductase (NADH dehydrogenase, complex I),

succinate dehydrogenase (complex II), coenzyme Q-cytochrome c reductase (complex III), cytochrome C oxidase (complex IV) and ATP synthase (complex V) [82] (Figure 11).

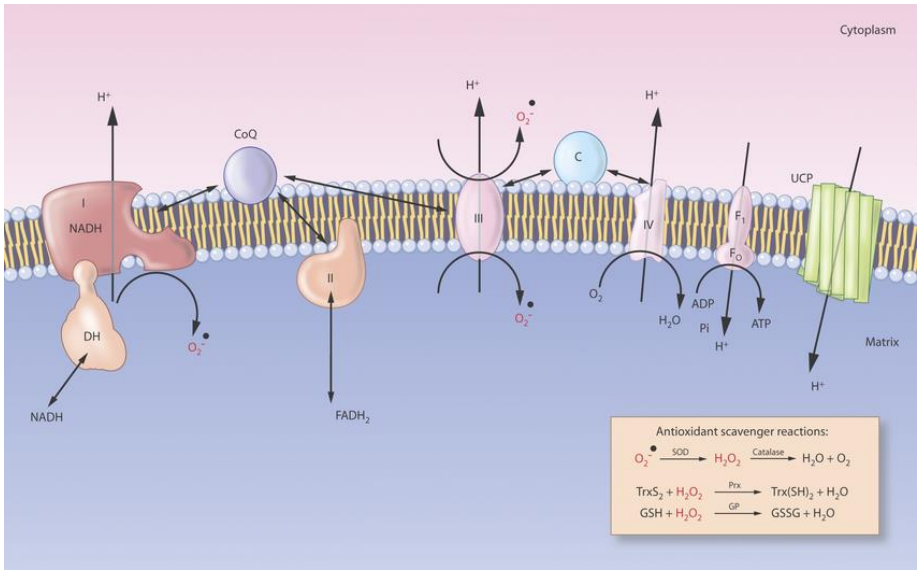

Figure 11. ROS production by mitochondrial electron transport chain (ETC) [89].

Interestingly, the production of ROS by these five enzymatic complexes can change between organs or during disease conditions [90]. In particular, Complex I seems to be the most related to oxidative damage in the CNS. Indeed, it appears to be the main generator of $O_2^{\bullet-}$ in the brain and the one most involved in ROS formation during pathological conditions such as accelerated ageing and neurodegenerative processes [82], [90].

In addition to the mitochondrial process of cellular respiration, which is the main source of ROS, other endogenous sources of this kind of reactive molecules include several non-mitochondrial enzymes such as nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX), xanthine oxidase (XO), cytochrome P450 from endoplasmic reticulum (ER) and flavin oxidases from peroxisomes [82]. Among them, one of the most involved in ROS production is the enzyme NADPH oxidase (NOX), a transmembrane glycoprotein that consumes NADPH to generate $O_2^{\bullet-}$ and subsequently H_2O_2 [82], [91]. Specifically, NOXs are multimeric enzyme complexes classified according to their catalytic subunit. NOX exists in several isoforms and shows a specific subcellular localization, which is required to localize H_2O_2 production and to activate specific redox signaling pathways, that are important in the regulation of several functions, such as cellular signaling processes and tissue homeostasis [84], [84], [92], [93]. In the CNS, among the various isoforms of this enzyme, NOX2 is the predominant isoform mainly in microglia and astrocytes [82], [94]. NOX-generated

ROS can act as molecules with antimicrobial properties and they are involved in growth factors signaling cascades. [95].

As mentioned above, free radicals come from both endogenous and exogenous sources. Endogenously, in addition to the main described mechanisms, there are several conditions or systems that can further contribute to the production of ROS such as inflammation, activation of immune system cells, cancer, infection, ischaemia, excessive exercise, ageing and mental stress [79]. In addition, exposure to radiation or particular chemicals such as heavy metals (Cd, Hg, Pb, Fe and As), environmental pollutants, chemical solvents, certain drugs, cooking (smoked meat, consumed oil and fat) and alcohol represents an external stimulus that promotes the body to produce free radicals. In particular, free radicals are formed as a by-product of the body's metabolism of these substances [79].

ROS refer to a variety of reactive molecules of which the main ones are [79], [80], [82], [96] (Figure 12):

- superoxide anion ($O_2^{\bullet-}$), which is formed both by the process of cellular respiration and through some enzymatic reactions catalysed by xanthine oxidase, NADPH oxidase (NOX), cytochrome P450 and nitric oxide synthase [80], [82]. As it is produced directly from O_2, O_2 concentration and/or a hypoxic condition appear to have a great impact on superoxide anion levels [84]. In addition to being a radical, it is also an anionic species and is characterized by very high reactivity. $O_2^{\bullet-}$ can be partially converted to H_2O_2 and O_2 by the enzyme superoxide dismutase (SOD) [82], [97].
- hydroxyl radical (·OH), which can be generated by various mechanisms such as ionizing radiation on the H_2O molecule, the Fenton reaction and the Haber-Weiss reaction [79], [80], [82], [98]. Among ROS, ·OH represents the most damaging species for cells, as it is responsible for triggering most of the oxidative processes in the biological environment [80], [82], [86].
- hydrogen peroxide (H_2O_2), which is obtained both from the dismutation of $O_2^{\bullet-}$, and through enzymatic reactions catalysed by specific peroxidases in peroxisomes [82]. H_2O_2 can be rapidly converted to water (H_2O) by various enzymatic systems, such as catalase [99] and glutathione peroxidase (GPx) [100] (Figure 12). H_2O_2 can also be detoxified and converted to H_2O by another enzyme complex, the thioredoxin-peroxiredoxin system [101]. Unlike other ROS, it is not a radical species, but still has strong oxidizing properties [86].

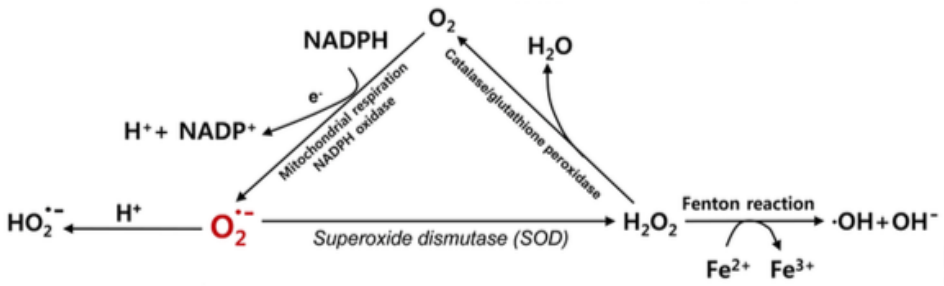

Figure 12. ROS generation mechanisms [82].

Under physiological conditions, the H_2O_2 redox cycle is a key cellular defense mechanism to reduce lipid oxidation and preserve membranes from oxidative damage [77]. This molecule can be considered one of the main ROS involved in important pathways of cell growth, proliferation and survival [79].

Among ROS, H_2O_2 has the longest half-life and is the only one that can readily diffuse across membranes [102].

Therefore, this allows it, by integrating environmental stimuli and activating a downstream signal transduction cascade, to act as a second messenger in numerous cellular signaling pathways [84]. On the other hand, the ability of this molecule to cross cell membranes and spread to other districts is also at the basis of the risk of activating degenerative peroxidation processes [103].

2.3 ANTIOXIDANT DEFENSES

ROS are extremely reactive molecules that can react with a myriad of biological macromolecules to induce damage in all cellular districts. They can inactivate proteins and alter the composition of the plasmatic membrane by oxidizing membrane phospholipids (lipid peroxidation). They can inactivate proteins and alter the composition of the plasma membrane by oxidizing membrane phospholipids (lipid peroxidation). Some of these species can cross the nuclear membrane (especially H_2O_2) and modify the structure of DNA, causing mutations at the genetic level [80].

However, the organism is equipped with efficient endogenous defense systems. In addition to endogenous defenses, there are several exogenous antioxidant molecules, mostly of plant origin, which can be taken mainly through diet or nutritional supplementation [80].

These defense systems are both endogenous and exogenous, enzymatic and non-enzymatic, intracellular and extracellular, and can be classified as follows [87], [88] (Figure 13):

- endogenous:
    - enzymatic antioxidants: SOD, CAT, GPx, GR, NQO1, HO-1 and TR

- intracellular non-enzymatic antioxidants: coenzyme Q and glutathione (GSH)
- extracellular non-enzymatic antioxidants (metal-binding molecules): transferrin, ceruloplasmin, albumin, uric acid and metallothionenins

- exogenous non-enzymatic scavengers: ascorbic acid (Vitamin C), tocopherols (Vitamin E), carotenoids (β-carotene and lycopene), polyphenols (mainly flavonoids) and many others [80].

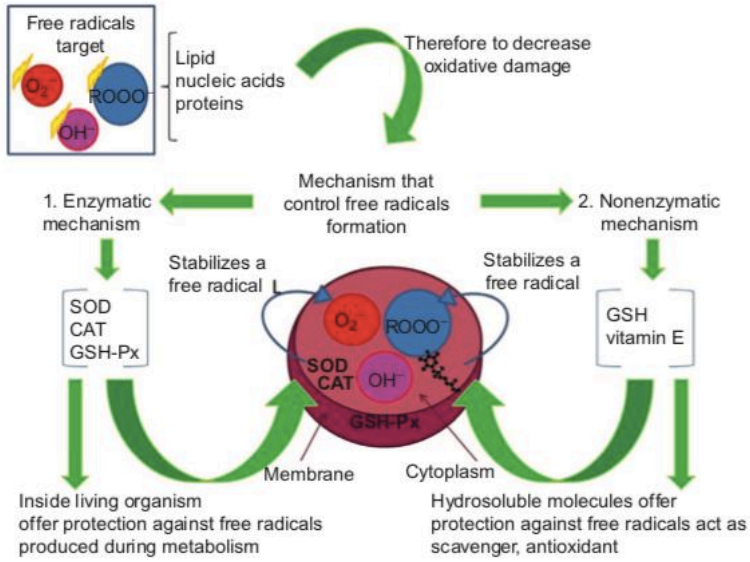

Figure 13.Main enzymatic and non-enzymatic control mechanisms of the fate of free radicals [87].

2.3.1 ENDOGENOUS ANTIOXIDANT DEFENSES

Our body produces a wide range of antioxidant molecules that physiologically balance the production of ROS. In general, their function is to oxidize themselves instead of more sensitive and vital structures for the cell, first and foremost DNA. The most important of these are the enzymatic antioxidant system and low molecular weight antioxidants (mainly GSH) [77], [82].

2.3.1.1 ENZIMATIC ANTIOXIDANTS

Superoxide dismutase (SOD) is an enzyme belonging to the class of oxidoreductases and catalyses the conversion of $O_2^{\bullet-}$ into H_2O_2 (Figure 14) [82].

There are several isoforms of this enzyme, which are distinguished according to their different localization and the metal that acts as a cofactor. There is a cytoplasmic SOD1 with Cu and Zn as cofactors (Cu/ZnSOD), a mitochondrial SOD2 with Mn as its associated metal (MnSOD) and an extracellular SOD3 [104], [105].

This enzyme represents the first form of defense against $O_2^{\bullet-}$.

Catalase (CAT) is an oxidoreductase with H_2O_2 as a substrate. CAT catalyses the dismutation of H_2O_2 to H_2O and O_2[82] (Figure 14). It can be found in peroxisomes, cytoplasm and mitochondria. CAT activity increases or decreases as a function of H_2O_2 levels [82], [106]. Overexpression of this enzyme at the cytosolic or mitochondrial level has shown a protective effect against oxidative damage [107].

Glutathione peroxidase (GPx) is a selenium-dependent enzyme located both in the cytosol and the mitochondria that, together with CAT, is essential in the detoxification of H_2O_2 [82]. GPx exists in several isoforms, of which GPx1, being expressed predominantly in microglia but not in neurons, is considered to have the greatest antioxidant action in the brain. Indeed, numerous studies have demonstrated its protective action against neuronal injury [82], [108]. This enzyme utilizes glutathione (GSH) as an electron donor for the reduction of H_2O_2 and lipid hydroperoxides [106], [109]. GSH acts as a co-substrate of GPx, which during the redox reaction is oxidised to GSSG through the formation of a disulphide bridge between two GSH molecules.

GSSG can return to its reduced form thanks to **glutathione reductase (GR)**. GR is a NADPH-dependent enzyme that converts GSSG to GSH through the oxidation of NADPH to NADP+, restoring endogenous GSH stores (Figure 14).

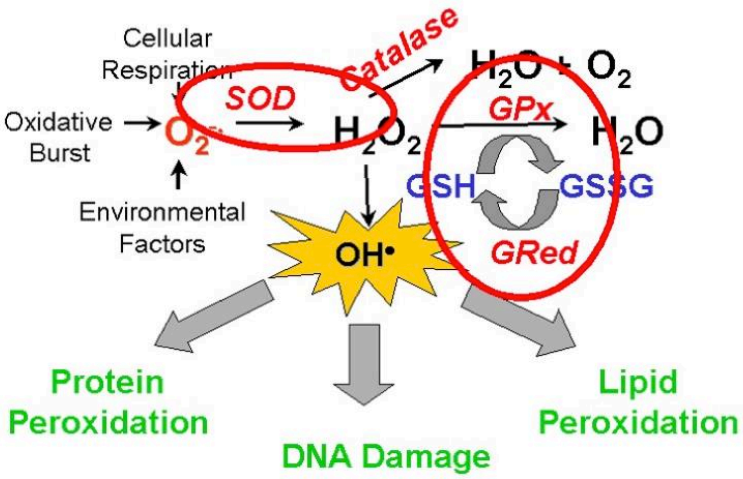

Figure 14. Mechanisms of action of SOD, CAT, GR, GPx.

The **Thioredoxin (Trx)/Thioredoxin reductase (TrxR) system** is another important enzymatic system involved in the reduction of H_2O_2. TrxR uses NADPH to catalyse the conversion of oxidised Trx into reduced Trx. Reduced Trx, in turn, is the reducing equivalent required by Thioredoxin peroxidase to convert H_2O_2 into H_2O [110].

In addition, the Trx / TrxR system has also been shown to play an important role in the de-nitrosylation of proteins containing the S-NO group or disulphide bridges [111].
The activity of this enzyme is implicated in several cellular processes, including transcriptional regulation, apoptosis and exocytosis [112], inflammatory processes, synthesis and repair of DNA. The Trx/TrxR system exists in all living cells and represents the main defense against RNS-mediated oxidative damage.

NAD(P)H quinone oxidoreductase is a flavoprotein that catalyses the reduction of quinones and other xenobiotics to less reactive compounds.
The oxidation-reduction reaction catalysed by this enzyme uses, without distinction, NADH or NADPH as cofactors and takes place through a mechanism defined as 'ping-pong' [113] (Figure 15). NQO1 reduces quinones to hydroquinones by a two-step reduction, avoiding the formation of the reactive semiquinone intermediate [113]. This avoids the generation of ROS from the interaction of the semiquinone intermediate with molecular oxygen [114]. Thus, NQO1 has been reported to protect cells against oxidative stress, cyclic redox reactions, neoplastic lesions [114] and Alzheimer's disease [115]. Indeed, NQO1 shows a protective effect against mutagenesis, carcinogenesis and other toxic conditions due to the generation of toxic compounds with quinones as their precursors [116].

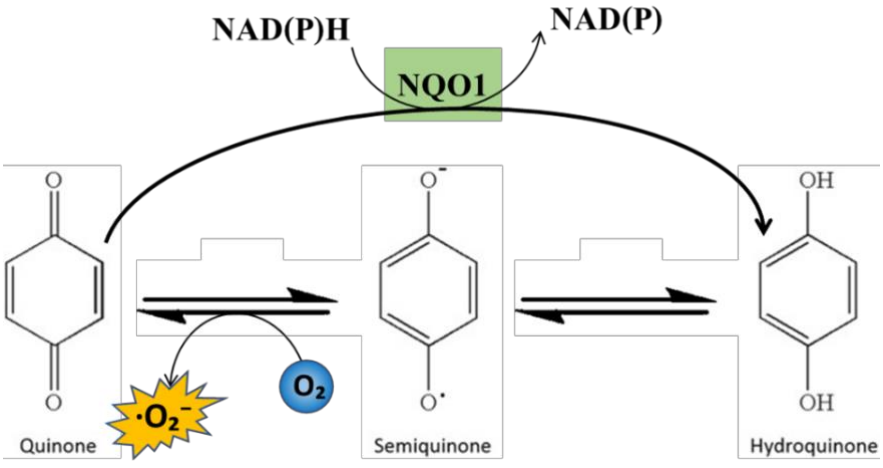

Figure 15. NQO1 mechanism of action.

The **heme oxygenase (HO) system** consists of several isoforms, of which HO-1 is the one that is induced by oxidative stress [117]. This enzymatic system is crucial for heme catabolism. In particular, HO-1 catalyses the conversion of heme to biliverdin using NADH or NADPH. Biliverdin is then turned into bilirubin through the action of biliverdin reductase. Bilirubin, the end-product of heme catabolism, exhibits antioxidant properties.

2.3.1.2 INTRA-CELLULAR NON ENZIMATIC ANTIOXIDANTS: GLUTATHIONE (GSH)

Glutathione (GSH) is an important non-enzymatic antioxidant ubiquitously present in cells and is one of the most abundant antioxidants synthesized by cells [91]. GSH is a hepatically synthesized tripeptide consisting of glutamic acid, L-lysine and L-glycine and represents the largest non-protein thiol component in the body. Glutathione is synthesized by the consecutive action of two enzymes: γ-glutamylcysteine synthetase and γ-glutathione synthetase [82]. Glutathione can be present in cells in either a reduced form (GSH) or an oxidized form (GSSG), and an adequate ratio between the two forms contributes to the maintenance of an optimal redox state.

GSH, GPx and GR together form a system that detoxifies ROS and oxidized substrates by continuous consumption of GSH by GPx and restoration of GSH by GR [77]. The ability of cells to regenerate GSH, either by reducing GSSG or by synthesizing new GSH, is fundamental for the body to counteract oxidative stress [82], [118]. Therefore, GSH plays a crucial role in cellular defense against oxidative stress, both as a non-enzymatic antioxidant (acting as free radical scavenger [77], [87]) and as a co-substrate of specific antioxidant enzymes such as glyoxalase and peroxidase [77]. Thus, there is a close relationship between high levels of intracellular GSH and the cell's ability to counteract ROS [119]. Indeed, the GSH/GSSG ratio is considered an indicator of cellular redox homeostasis [77].

2.3.2 ESOGENOUS ANTIOXIDANT DEFENSES

In nature, there are a several compounds with antioxidant properties, which are able to combat reactive chemical species such as ROS. They are particularly abundant in vegetables and are consumed by humans through the diet. These include mainly compounds such as vitamin C, tocopherols, carotenoids, glucosinolates and polyphenols.

Many studies have been carried out on these compounds to assess their protective effect on health, and there are numerous indications that natural antioxidants, as exogenous sources, can boost the body's endogenous antioxidant defenses, helping to combat the damage induced by an excess of free radicals.

More specifically, exogenous antioxidants can be classified into two broad categories according to their mechanism of action:

- direct antioxidants, i.e. molecules that react directly with radicals by reducing oxidative stress and exerting their protective effects against cellular damage [120];
- indirect antioxidants, i.e. compounds that counteract oxidative damage by modulating/enhancing the endogenous antioxidant defenses.

Among the various naturally occurring compounds, polyphenols and flavonoids have been shown to be particularly effective in counteracting oxidative stress and, in some cases, even more effective than the most historically used antioxidant molecules (vitamins C and E, and β-carotene) [121].
Polyphenols comprise a wide range of compounds, divided into several classes, including anthocyanins, flavonols, flavones, flavanols, isoflavones, stilbenes and lignans. They are mainly found in fruit, vegetables, wine, tea, coffee and cocoa products [122]. Epidemiological studies have shown that an increased intake of polyphenols is associated with a reduced risk of cardiovascular disease, cancer and neurodegenerative disorders [123]. The beneficial effects of polyphenols are mainly attributable to their ability to counteract oxidative stress that is associated with these diseases. Several polyphenols have been shown to have clear antioxidant properties in vitro, as they can act, depending on their chemical structures, by blocking radical chain reactions, as radical scavengers or by inducing endogenous antioxidant defenses, thus improving cell survival [124].

2.4 PHYSIOLOGICAL FUNCTIONS OF ROS

When referring to ROS, there is a tendency to emphasise the harmful role of these molecules on the body, but, as mentioned above, adequate levels of ROS are produced by the body itself as it needs them to function properly. As anticipated, low to moderate levels of ROS are crucial in numerous cellular processes, primarily in cell signaling and pro-survival pathways [82]. For example, ROS can activate survival pathways, such as mitogen-activated protein kinase (MAPK) pathways. MAPKs are serine/threonine-specific kinases and their action is finely regulated by the redox state. Their importance is given by the numerous signaling cascades in which they are involved such as mitosis, proliferation, cell survival, migration, cardiovascular systems expression and apoptosis [82]. NOX-produced ROS are critical at the cardiovascular level in cell signaling pathways [125] and NOX-2-generated ROS in phagocytes play a key role in the defense against pathogens by cells of the immune system [126].

In addition to this, ROS can modulate the activation of important transcription factors, such as Nrf2 and NF-kB.
Nrf2, the NF-E2-related factor 2 (Nrf2), is one of the most important redox-sensitive transcription factors and modulates the expression of the major endogenous antioxidant enzymes such as GPx, SOD, HO-1 and PRX [82].
This transcription factor is normally found in the cytoplasm bound to a suppressor protein, Kelch-like ECH-associated protein 1 (Keap1), which prevents its translocation into the nucleus and keeps it inactive [127]. When ROS levels increase, the binding between Keap1 and Nrf2 is broken, Nrf2 is

free to translocate into the nucleus where it binds to the antioxidant response element (ARE) in the promoter regions of many genes coding for antioxidant enzymes and activates their transcription [77], [82].

Over the years, Nrf2 has been extensively studied for its protective properties. In particular, it has been considered as a possible therapeutic target for the treatment of diseases characterised by oxidative and inflammatory events such as neurodegenerative diseases [77].

Another transcription factor modulated by ROS is nuclear factor-kappa B (NF-κB). NF-κB is a transcription factor that mediates pro-survival cellular responses. It is also normally found in the cytoplasm in an inactive form due to its binding to an inhibitory protein (IkB). NF-kB activity is finely regulated and varies according to the levels of ROS present at cellular level. Indeed, moderate levels of ROS lead to the release and activation of NF-kB which, in turn, promotes the transcription of anti-apoptotic proteins and inhibits caspase-mediated cell death pathways [82]. Conversely, if ROS levels increase excessively, NF-kB binding to DNA is prevented with inhibition of survival pathways and subsequent apoptotic cell death [128].

2.5 OXIDATIVE STRESS AND NEURODEGENERATION

The onset of a central oxidative condition leads to the impairment of neuronal function and even to micro- and macroscopic morphological changes in the structure of the brain itself. The exact molecular mechanisms by which ROS cause damage to the brain tissue are not yet fully known, nor are their temporal sequence, the reciprocal impact of the various involved mechanisms and the threshold that determines the transition from a physiological to a pathological state. The specificity of neurodegeneration mechanisms is still unclear: whether each disease exhibits unique mechanisms that are different from the others, or whether several diseases may share the same mechanisms. A widely accepted hypothesis, which is common to several pathological conditions and also takes into consideration the consequences of the physiological ageing process, associates the accumulation of oxidative damage with the loss of function at various levels [129]. Different tissues have different susceptibility to oxidative stress and the central nervous system (CNS) is extremely sensitive to this type of damage. The low level of antioxidant enzymes, the high content of oxidisable substrates (mainly unsaturated lipids), the high O_2 consumption, the high metal content (especially iron), the high metabolic activity and the large amount of ROS produced during neurochemical reactions are at the basis of the CNS vulnerability to oxidative damage [73], [77], [82], [87]. Extensive protein oxidation and lipid peroxidation develop when an oxidative state is established in the brain, promoting cell degeneration and functional decline. For example, it has been shown that high amounts of ROS in the brain can become particularly dangerous for normal brain function triggering several

molecular cascades, that reduce LTP (long-term potentiation), decrease synaptic signaling and brain plasticity mechanisms, alter brain morphology and increase permeability of the blood-brain barrier leading to neuroinflammation and neuronal death [77].

In particular, highly reactive lipids are formed through the process of lipid peroxidation, which, when reacting with proteins, can easily generate secondary oxidation products [130]. Due to the high quantity of polyunsaturated fatty acids, the CNS is particularly susceptible to this type of oxidative damage.

In addition, mitochondrial DNA is much more vulnerable to oxidative damage than nuclear DNA, which favours mitochondrial dysfunction and ROS production.

It is widely known that the CNS is an extremely metabolically active system (to support normal axonal transport and neuronal conduction, it requires about 20% of the total body energy consumption) and is consequently rich in particularly active mitochondria that are responsible for high ROS production [130]. The impact is even greater considering that many mitochondrial enzymes require iron to function, so the content of this metal is high in CNS cells. This facilitates the Fenton reaction and the subsequent generation of ROS in the mitochondrial matrix, highlighting the sensitivity of mitochondria to oxidative stress.

Another important aspect is the ROS-mediated protein oxidation that leads to the aggregation and accumulation of proteins, compromising their degradation by the proteasome. The proteasome is physiologically responsible for the rapid degradation of damaged proteins and organelles, thus preventing their accumulation and subsequent cell death [1]. In neurons, due to limited neurogenesis, the removal of non-functional proteins is particularly important. This aspect becomes increasingly important during ageing, since with increasing age physiological clearance slows down, leading to the accumulation of damaged molecules and organelles [131]. Therefore, it is not surprising that ageing is one of the main risk factors in the development of neurodegenerative diseases.

There is a growing body of evidence supporting the involvement of oxidative stress in the development of neurodegeneration and a growing interest in understanding the exact role played by oxidative stress in the development of neurodegenerative disorders. There is strong evidence that free radicals are implicated in the development of ischaemic-reperfusion disease, Parkinson's disease, Huntington’s disease, Amyotrophic lateral sclerosis (ALS) and Alzheimer's disease [73], [77], [82], [87], [132] (Figure 16).

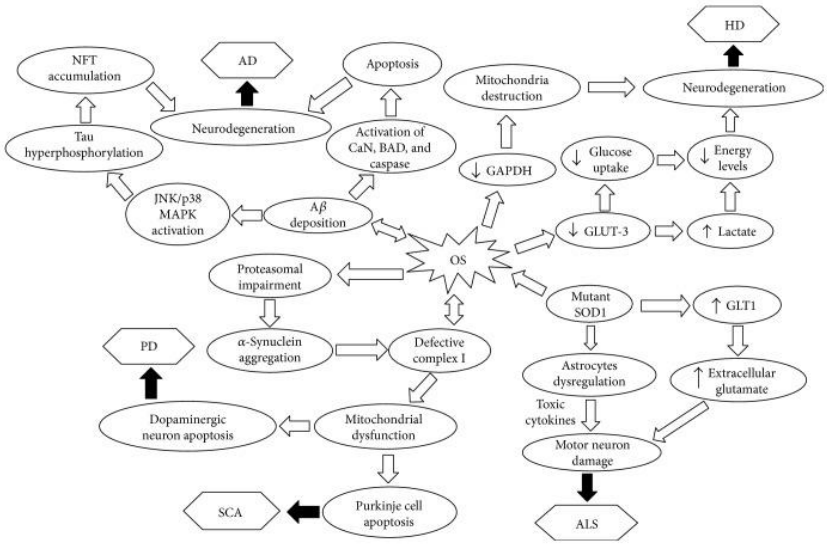

Figure 16. Schematic overview of the involvement of oxidative stress in the development of the main neurodegenerative disorders [73].

Due to their multifactorial aetiology, at the base of their development, neurodegenerative diseases, in addition to oxidative stress, are characterised by the establishment of numerous other mechanisms that often interact with each other to further exacerbate the pathological condition.

For example, in the CNS, the redox balance can be negatively affected by the establishment of inflammation or mitochondrial dysfunction and vice versa. Two of the most impactful hallmarks common to most neurodegenerative disorders are brain atrophy and accumulation of misfolded proteins (plaques, neurofibrillary tangles and other protein aggregates [73], [77]). Indeed, the main neurodegenerative diseases include Alzheimer's disease (AD), Parkinson's disease (PD) and Huntington's disease (HD), which are all characterised by the neurotoxic aggregation of specific proteins in the brain. Specifically, in AD there is an accumulation of misfolded β-amyloid (Aβ) and tau proteins, in PD of misfolded α-synuclein and in HD of misfolded huntingtin (mHtt). These abnormally folded proteins are prone to association resulting in the formation of insoluble aggregates that are deposited and accumulated in various areas of the brain (intra- and extra-cellular) causing neurotoxic processes. The close relationship between oxidative stress and these protein aggregates in neurodegeneration has been widely documented [1], [73], [77]. It is now clear that ROS are involved in the formation of all kinds of protein aggregates, depending on the respective pathology, and this is often enhanced by the ageing process [77]. Moreover, in most cases, a self-propagating process is generated between the two phenomena: ROS promote the formation of protein aggregates, which, once formed, induce further ROS production [77]. For example, in PD, the aggregation of α-synuclein in dopaminergic neurons is stimulated by ROS and, in turn, the misfolded α-synuclein

further promotes the intracellular formation of ROS [133]. ROS are also involved in mHtt-induced neurotoxicity and, in turn, the accumulation of this protein has a negative influence on the expression of the antioxidant enzyme Prx (Peroxiredoxin) indirectly improving ROS levels in PC12 cells [134]. In addition, the formation of these misfolded protein aggregates may also trigger an inflammatory response in the CNS resulting in ROS production [73].

Concomitantly with the above, an influence of ROS-stimulated Aβ on lipid peroxidation mechanisms has also been documented. Lipid peroxidation leads to the formation of aldehydes [135] that impair the normal functioning of mitochondrial enzymes [136], [137] and the activation of excitotoxicity mechanisms, alter the normal membrane permeability and increase calcium (Ca^{2+}) influx: all of these mechanisms strongly impair neurotransmission and normal cognitive functions [77]. In support of these findings, numerous studies have identified a correlation between ROS and impaired learning and memory processes due to ROS-mediated improvement of Aβ in LTP [77] as well as damage to neuronal, cerebrovascular and microglia cells resulting from the pro-oxidant action of Aβ mediated by the receptor for glycation end products (RAGE) [138].

High levels of free bivalent ions, such as iron, copper and zinc (Fe^{2+}, Cu^{2+} and Zn^{2+}) along with aluminium (Al^{3+}), are related to ROS and mediate neurodegenerative damage in several ways. These ions also promote aggregation of the tau protein, causing its conformational changes and phosphorylation [139]–[145].

Another fundamental aspect common to many neurodegenerative disorders is the existence of mitochondrial dysfunction. Oxidative stress and mitochondrial dysfunction are two mutually supportive mechanisms [73]. For example, in HD, an mHtt-mediated alteration in normal energy production processes in mitochondria has been reported [68]. As the mitochondrial respiratory chain is the primary endogenous source of ROS, an alteration at this level generates high levels of ROS [1]. Indeed, it has been widely observed that in chronic neurodegenerative diseases, oxidative stress can also be induced by impairment of mitochondrial functions [1]: lipid peroxidation, DNA damage and protein oxidation can cause fragmentation of the mitochondria and their abnormal distribution, altering their metabolic activity and leading to the formation of further reactive species [146] (Figure 17).

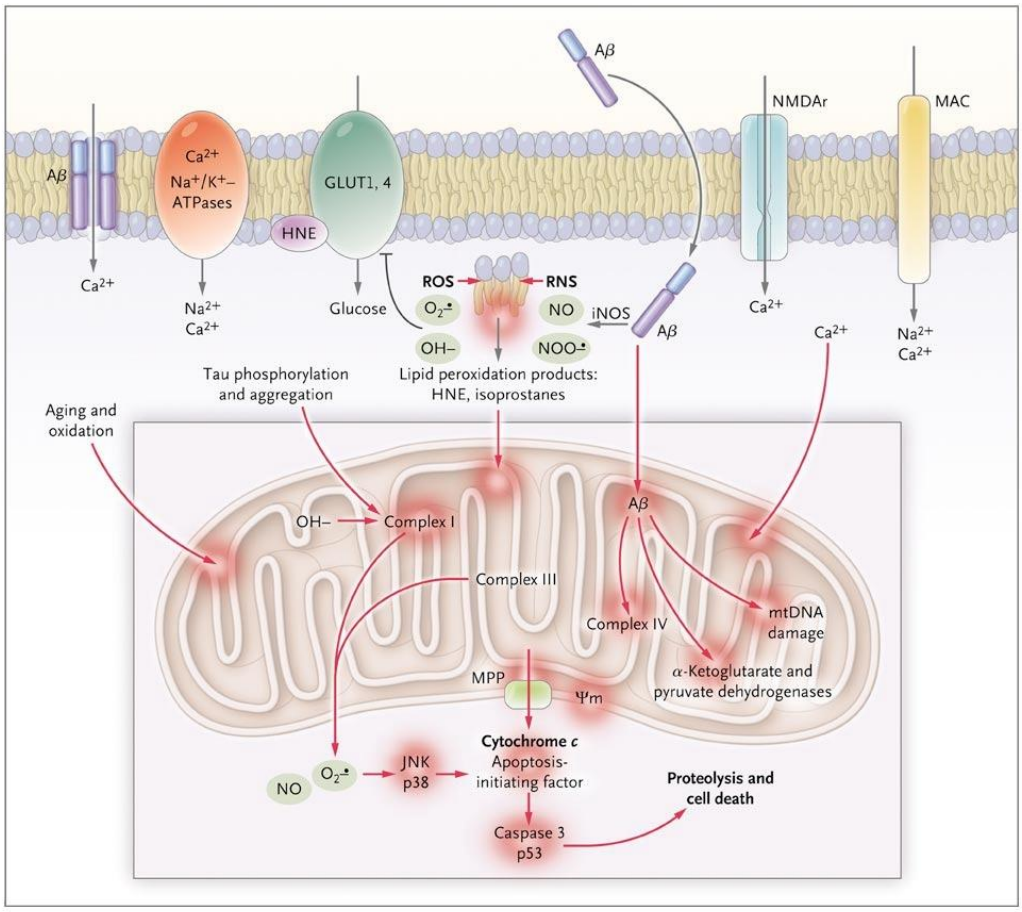

Figure 17. Relationship between mitochondrial dysfunction and ROS in neurodegeneration [26].

In Table 1 the specific involvement of neurodegeneration in Alzheimer's, Parkinson's, and Huntington's disease is reported.

Table 1. Focus on oxidative stress in neurodegenerative diseases.

FOCUS ON OXIDATIVE STRESS IN NEURODEGENERATIVE DISEASES	
Alzheimer's disease	*Over the years, oxidative stress is increasingly emerging as a highly relevant factor in the pathogenesis of AD, as overproduction of these reactive species is thought to play a critical role in the accumulation and deposition of extracellular Aβ plaques.* *Recent clinical evidence has shown that β-amyloid aggregates can directly initiate free radical formation through the activation of NADPH oxidase. In particular, NOX2 stimulates microglia to release ROS. Therefore, the resulting oxidative stress status contributes to neurodegeneration and the pathogenesis of AD [147].* *In addition, Aβ has been shown to play an important role in mediating cellular apoptotic cascades [148].* *Under oxidative stress conditions, mitochondrial impairment triggers chain reactions responsible for the hyperphosphorylation of tau protein, which accumulates in abnormal quantities and forms highly neurotoxic intracellular tangles [149].* *The severe oxidative stress observed in AD patients could be the result of over-activation of N-methyl-D-aspartate glutamate receptors (NMDARs), leading to an alteration in cell permeability and neurotoxic levels of ROS and RNS in the brain [150].*

Parkinson's disease	*A preventive approach to this disease, i.e. the use of compounds with neuroprotective and antioxidant properties that can act on ROS-mediated signaling cascades, is becoming increasingly promising.* *The pathological mechanisms underlying the degeneration of dopaminergic neurons have often been linked to the excessive accumulation of ROS and/or other free radicals.* *ROS accumulation in PD may worsen when individuals are exposed to environmental risk factors such as pesticides. This is demonstrated by the significant association between increased risk of PD and exposure to pesticides [151].* *Other studies have suggested that the loss of dopaminergic neurons, a feature of PD, may also be associated with the presence of neuromelanin in the neuron. Highly pigmented neurons may be more susceptible to damage. The formation of neuromelanin appears to be related to the autoxidation of dopamine, a process induced by the overproduction of ROS.* *In addition, dopamine is an excellent metal chelator and electron donor, so it has a high tendency to link with Fe^{3+}. As in the Fenton reaction, this bond with Fe^{3+} leads to the reduction of the metal and consequently to the generation of H_2O_2 [152].* *Similarly to Alzheimer's disease, mitochondrial disfunction promotes free radical production in the respiratory chain. In particular, it has been documented that PD neuronal apoptosis can be attributed to a defect in complex I of the respiratory chain. This defect is related to a mutation in the kinase PINK1. PINK1, which is ubiquitously expressed in the body, is crucial in the defense against oxidative stress and in the maintenance of mitochondrial membrane potential [153]. In addition to PINK1, α-synuclein aggregation also interferes with the functions of mitochondrial complex I, leading to impaired ATP synthesis and mitochondrial dysfunction [154].* *The process of autoxidation to which Dopamine (DA) is subjected results in the overproduction of ROS and the subsequent inefficiency of the proteasome in the degradation of proteins. The accumulation of α-synuclein may also be caused by this mechanism [56].* *Although no effective therapies currently exist to treat Parkinson's disease, understanding the mechanisms underlying ROS production in the brain provides important insights for possible treatments to alleviate the symptoms of this disease [73].*
Huntington's disease	*Huntington's disease is a progressive neurodegenerative disorder related to an unstable expansion of the cytosine/adenine/guanine (CAG) repeats in the Htt gene, which codes for the protein huntingtin (Htt). This mutation leads to the elongation of the Htt protein, which becomes mHtt and is extremely susceptible to aggregation. These alterations are responsible for the motor and cognitive disorders characteristic of HD [67]. Initial studies supported the theory that in HD patients there was a reduction in energy levels in the brain due to reduced glucose uptake and increased lactate levels.* *Recent studies have shown that oxidative damage causes a reduction in the expression of the glucose transporter GLUT-3, resulting in decreased glucose uptake and lactate accumulation [76]. In addition, it has been shown that the mHTT protein plays a key role in mitochondrial dysfunction (mitochondria are the main site of ATP synthesis), as it can interact with mitochondrial membranes causing inhibition of electron transport, increased levels of ROS and reduced ATP production [75]. In 2015, a new mechanism for mitochondrial dysfunction in HD was proposed. This suggests that oxidative stress may inactivates glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a key enzyme in selective degradation by lysosomes, in compromised mitochondria. However, in the presence of mHtt, normal GAPDH activity is impaired and damaged mitochondria cannot be digested by lysosomes. This leads to an accumulation of mHtt in the cells and neuronal cell death [73].* *Although the involvement of mitochondrial alterations and ROS overproduction in the progression of HD was well established, it is unclear which event triggers the disease.*

3 NEUROINFLAMMATION IN NEURODEGENERATION

Neuroinflammation is a fundamental process within the neuroimmunological mechanisms that occurs in the central nervous system (CNS). A large body of research demonstrates the crucial role of neuroinflammation in the development of various neurological disorders, such as neurodegenerative diseases and CNS lesions.

Neuroinflammation is a complex and dynamic process that is finely regulated by the interaction of different cell types in the CNS such as glial cells and immune system cells [155].

The CNS is considered an immunologically quiescent organ, whose homeostasis is highly dependent on the maintenance of a fine balance of the immune system. In fact, numerous studies have shown that a disruption of innate immunity in the CNS is at the basis of many neurological disorders [155].

Neuroinflammation is a finely regulated self-defense mechanism and constitutes the articulated innate immune response of the CNS against injury or pathogenic stimuli. It aims to protect neural tissue and, in case of injury, to promote the healing process.

The immune system can be classified as innate or adaptive.

The innate immune system, the first line of defense against insults, is responsible for generating a rapid, but short-term response. Mononuclear phagocytes (dendritic cells, macrophages, microglia and monocytes), natural killer cells and neutrophils are responsible for the activation of this response.

On the other hand, the adaptive immune system generates a pathogen-specific, non-rapid and long-lasting response through T and B lymphocytes [156].

In general, inflammation develops when the body's immune cells become activated and trigger a series of reactions to prevent tissue damage. When effective, the inflammatory response counteracts pathogens, initiates the processes of angiogenesis and then subsides itself to complete exhaustion [157]. Under normal conditions, this process is a homeostatic protective action that the body performs. Conversely, when the inflammatory response is abnormally prolonged, it leads to the uncontrolled release of inflammatory mediators such as pro-inflammatory cytokines, chemokines, ROS and secondary messengers [158].

Of note, neuroinflammation and peripheral inflammation differ from each other due to the cell types involved and how they communicate with each other [159]. The neuroinflammatory process is the result of the communication and collective action of all kinds of glial cells.

When neuroinflammation becomes persistent, damage to CNS tissues and cells occurs and a chronic condition underlies many neurological disorders.

In particular, a failure in regulating the production and release of these pro-inflammatory molecules leads to the generation of a chronic condition, which is implicated in most neurological disorders, including AD, PD and HD, and it is a major cause of neurodegeneration [160].

3.1 CELL TYPES INVOLVED IN THE INFLAMMATORY PROCESS

Neurons and neuroglial cells are the two broad categories of cells that populate the CNS. The term 'neuroglial cells' refers to oligodendrocytes, astrocytes, microglia and ependymal cells. Among these, microglia represent the first form of defense of the CNS against insults and constitute about 5-10 % of the total number of cells in the brain and 20% of glial cells [62], [155], [161], [162]. The continuous and complex communication of all these cell types, together with the cells of the immune system (IS), ensures the fine regulation of the signaling cascades and the maintenance of the brain tissue homeostasis (Figure 18).

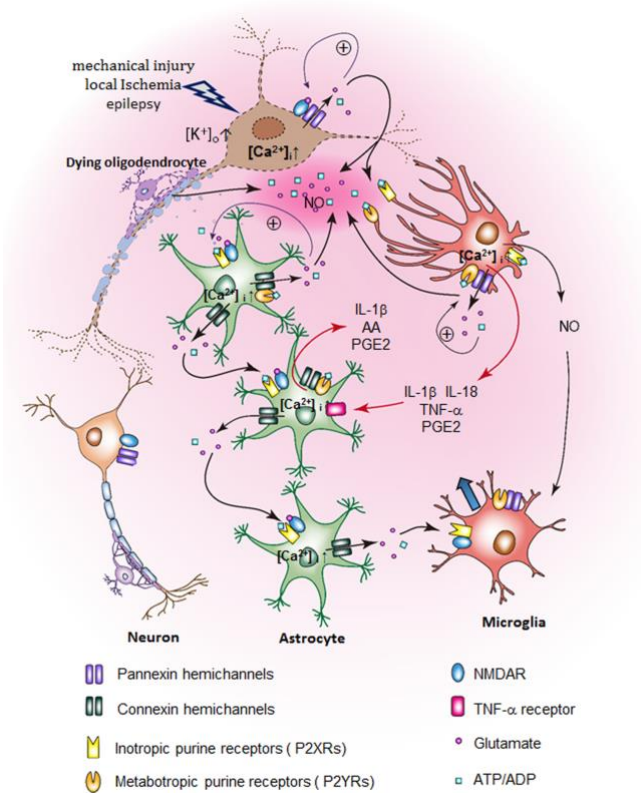

Figure 18. Fine communication of CNS cell types [155].

In particular, in case of damage, all glial cells become activated and secrete pro-inflammatory mediators triggering the opening of the blood-brain barrier (BBB) and attracting immune cells from

the peripheral blood to the CNS. Therefore, after IS cells infiltration into the brain parenchyma, a coordinated inflammatory response between glial and immune cells is activated to resolve the damage. At the same time, a similar coordination exists between the responses mediated by these two populations of cells in order to attenuate the defense response and protect cerebral tissues from the secondary damage caused by the hyperactivation of inflammation [155]. Indeed, the activation of the signaling cascade can promote both pro- and anti-inflammatory responses, on the one hand to combat the damage, and, on the other hand, to stop the process when the situation is restored and activate the repair of the nervous tissue. This "bilateral" character of neuroinflammation is finely regulated by the various cellular phenotypes and the interactions between them [155]. Thus, it is crucial to understand the mutual involvement of all different cell types and how they mediate the neuroinflammatory process (Figure 19).

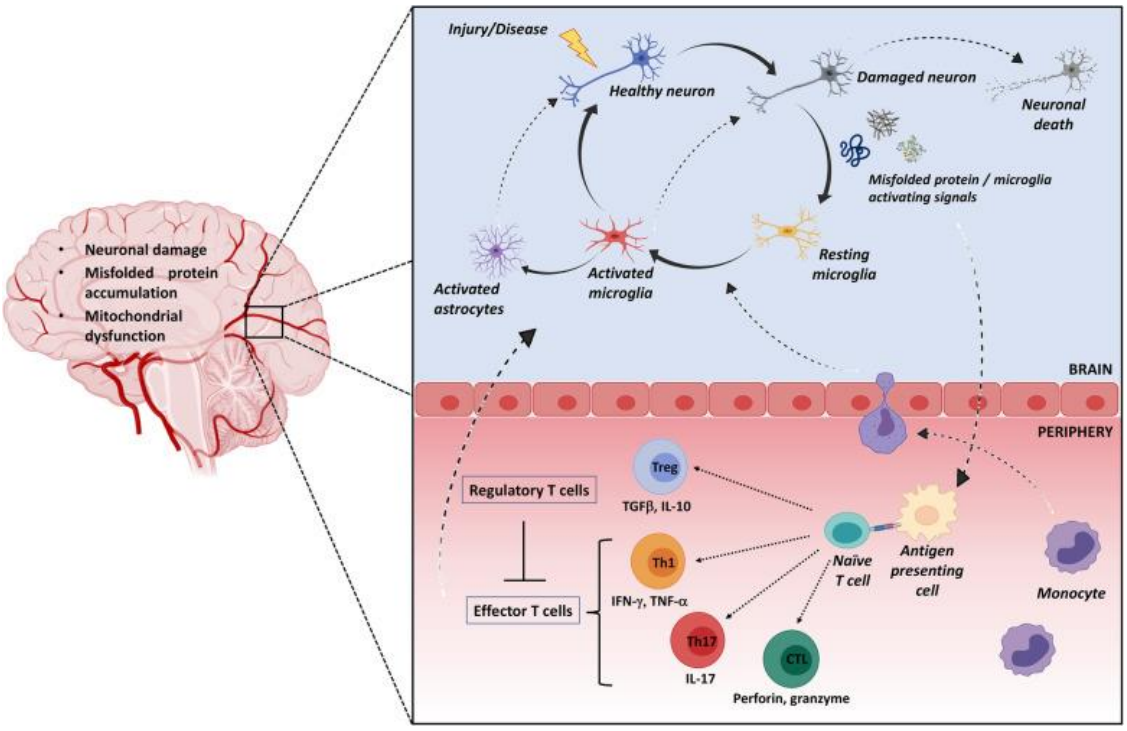

Figure 19. Central and peripheral inflammatory/immune response in neurodegeneration [163].

3.1.1 MICROGLIA

Microglia are immune cells, permanently resident in the brain and spinal cord and are the primary mediators of neuroinflammation. They share many features with macrophages, which, outside the CNS, are essential in defense against exogenous pathogens and endogenous harmful stimuli. Microglial cells are mostly uniformly distributed in the adult brain, both in the white and grey matter, but with variable density. In particular, the highest concentrations are found in the brainstem (substantia nigra), basal ganglia and hippocampus. Microglia consist of cells with a long half-life and

a particularly slow turnover (approximately 28% per year) [164]. In particular, the ageing process is one of the main predisposing factors of neurodegeneration, so the peculiar longevity of this type of cells could be an important starting point for the treatment of neurodegenerative disorders. [165].
Under physiological conditions, microglia exist as so-called “resting” or “surveillance” microglia. Each cell consists of a small body and numerous branches that extend through the surrounding tissue without overlapping with the apophyses of an adjacent microglial cell and exhibits minimal expression of surface antigens [74], [155], [162].
On their surface there is an enormous variety of receptors that allow them to recognize pathogens, toxic and bacterial agents, viral and fungal xenobiotics, as well as substances released from dead or dying cells. Indeed, thanks to their antigen presentation and phagocytic function, microglial cells are the main effector of the innate immune response in the brain [62].
Microglia play a key role in maintaining the homeostasis of brain tissue and in the development of the CNS. Indeed, microglia support neuronal survival and synaptogenesis, also through the release of neurotrophic factors (NFs), promote cell death of damaged neurons, stimulate neuronal tissue repair, removal of toxic substances, synaptic pruning, and synaptic remodelling [62], [161]. These cells constantly monitor the surrounding environment through their branches, which in real time explore and sample the local microenvironment looking for possible stimuli to promptly activate a related response [161], [165]. Thanks to this “surveillance” action, they are often the first to intervene in case of injury or disease. In addition, they play an active role in the proper development and maintenance of the CNS because [161]:
- they secrete NFs such as basic fibroblast growth factor (bFGF) and nerve growth factor (NGF).
- they promote the programmed cell death of defective or improperly developed neurons and the removal of cellular debris by phagocytosis (this process is further supported by the dying neurons themselves, which, in turn, release factors that stimulate phagocytosis and chemotaxis by microglia, accelerating debris removal).
- they are involved in the process of synaptic stripping/pruning, i.e. removing damaged/unused synapses and re-establishing functioning neural circuits.

All these physiological functions of microglia contribute to the correct development of the CNS in its complexity and to the generation of an efficient and mature neuronal circuit [161].
In addition, microglial cells have an enormous variety of receptors on their surface, such as toll-like receptors (TLRs), which allow the recognition of a wide variety of exogenous agents such as pathogens, toxic and bacterial agents, viral and fungal xenobiotics, as well as substances released from dead or dying cells [62], [71].

Microglia remain in a dormant/resting state until mobilised by a potential stressor event. These cells can undergo a transformation called 'activation' as a result of various stimuli including infection, trauma and/or brain injury, stroke, oxidative damage and neurodegeneration [1], [62]. As a result of this process, the cell soma enlarges, the complexity of cellular processes is reduced and the microglia take on an unbranched amoeboid appearance, acquiring the ability to move actively towards the lesion and activate phagocytosis [2], [74], [161], [165] (Figure 20).

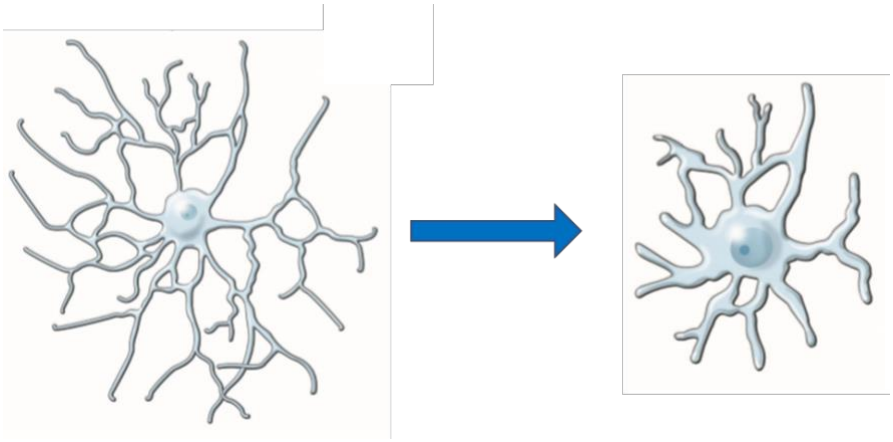

Figure 20. Morphology of quiescent/ramified (healthy CNS) and activated/ameboid (in case of injury) microglia. Modified from [2].

Thus, under stress conditions, microglia react promptly to damage by activating, through the release of pro-inflammatory signal molecules (e.g. cytokines/chemokines and nitric oxide), other cell types involved in the immune defense of the CNS and, therefore, stimulating tissue repair or inducing cell death [62], [165], [166].

However, recently the concept of “resting” microglia has been overturned by a series of scientific evidence. The researchers observed that the healthy state of the tissue is characterised by microglia with relatively stationary bodies and highly and spontaneously active branches. For the first time, these studies have indicated that microglia do not exclusively exhibit a “reactive” immune function, i.e. they are not only mobilized following infection or injury, but also play an essential role in monitoring normal synaptic activity [167].

In addition, under stressful condition, the response of activated microglia is more complex as it is driven by several microglial phenotypes (M1 and M2) that mediate both damaging and protective responses. In addition, activated microglia exert its effect on other cell populations in the CNS, namely neurons and macroglia (astrocytes and oligodendrocytes) [74]. Moreover, the activity of microglia is, in turn, modulated by neurons and astrocytes in an intricate network of signaling and

communication among the various cell populations of the CNS that collectively modulates a finely regulated process known as neuroinflammation [74] (Figure 21).

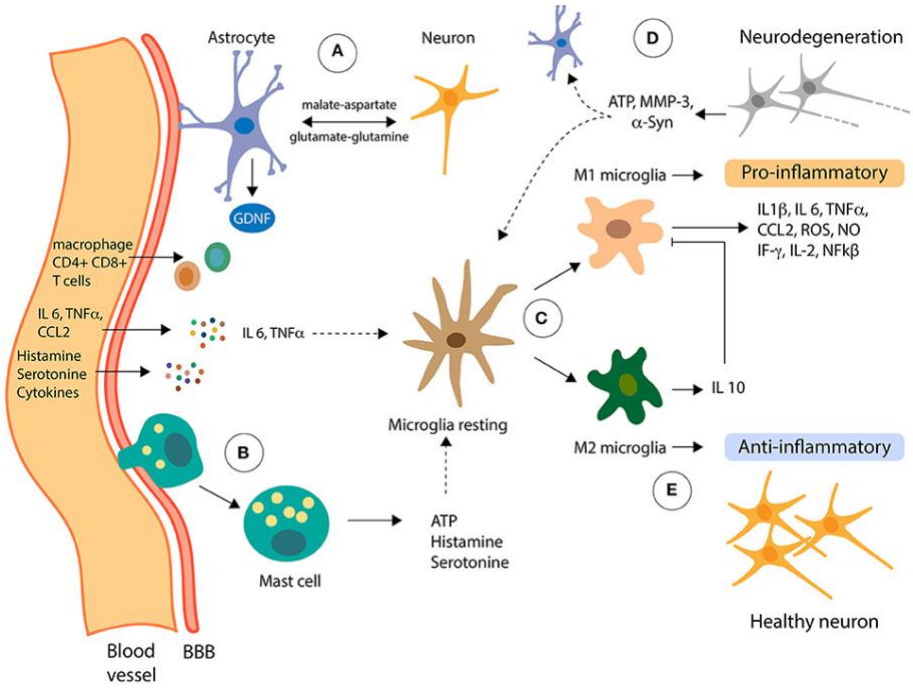

Figure 21. Fine regulated interrelationship between non-neuronal cells in neuroinflammation [62].

Generally, it can be assumed that when activation is short-term, neuroprotective responses are modulated, whereas chronicization of such activation is often implicated in the onset of neurodegenerative diseases [62].

3.1.1.1 ACTIVATED MICROGLIA

As previously mentioned, the biological function of microglia in the CNS is extremely complex, since they can mediate both pro-inflammatory and anti-inflammatory events, as well as neuroprotective and neurotoxic responses. Microglia can be activated by a wide range of substances and events and, in turn, can produce and release an equally wide range of mediators and signaling molecules. It can conventionally be assumed that, depending on the type of triggering event, microglia can transform into two different phenotypes [62], [161], [168], [169]:

-M1 phenotype (pro-inflammatory), generally activated by a pro-inflammatory environment (e.g. cytokines) and cellular or bacterial debris,

-M2 (anti-inflammatory) phenotype, generally induced by parasites and interleukins such as IL-4 and IL-13.

Moreover, always depending on the type of stimulus, the activation process may be of different types and, depending on the followed mechanism, microglia will assume one phenotype rather than another. More specifically, the activation pathways that microglia can follow are [62], [161], [168], [169]:

- classical microglial activation,
- alternative microglial activation,
- acquired deactivation,
- toll-like receptor (TLR)-mediated activation.

Classical microglial activation

Both in vitro and in vivo studies have shown that several stimuli can induce classical microglial activation, such as lipopolysaccharide (LPS), interferon (IFN)-γ, β-amyloid (Aβ), and α-synuclein. This kind of activated microglia is considered as the M1 phenotype [161], [168]. It represents the first line of defense, as it can eliminate pathogens by secreting pro-inflammatory factors/cytotoxic mediators including Interleukin-1β (IL-1β), Interleukin-6 (IL6) and TNF-α. However, chronic activation of microglia results in excessive accumulation of these factors, which, in association with increased NO and ROS production, causes damage to surrounding neuronal cells [161]. This has been confirmed by post-mortem examination of subjects with brain disorders such as AD and PD [165] (Figure 22).

Alternative microglial activation

Microglia can also follow an alternative activation pathway, classically referred to as the M2 phenotype, which is mainly activated by two interleukins: Interleukin-4 (IL-4) and Interleukin-13 (IL-13). Cells belonging to the M2 phenotype predominantly mediate anti-inflammatory responses through the secretion of anti-inflammatory cytokines such as Interleukin-10 (IL-10) and factors promoting extracellular matrix (ECM) reconstruction and tissue repair such as Quinitase-3 (Chil3) and Arginase (Arg-1) [161]. This type of microglia intervenes, after the M1 phenotype has eliminated foreign organisms, to restore tissue homeostasis. It is essential that cells can switch from M1 to the M2 phenotype to avoid uncontrolled production of pro-inflammatory cytokines, NO and ROS, which would continue to induce cell death and tissue damage [161], [170] (Figure 22).

Acquired deactivation

This microglial activation pathway is mainly promoted by IL-10, Transforming Growth Factor-β (TGF-β) or apoptotic cells [161], [168]. Similar to alternative activation, this mechanism also mediates anti-inflammatory responses. Although microglia also assume an M2 phenotype here, cells

derived from this mechanism secrete a different pattern of cytokines and chemokines as they are in a more typically immunosuppressive rather than anti-inflammatory state [171]. Indeed, the molecules that mediate this type of activation, IL-10 and TGF-β, by their association with STAT3- and Smad-mediated signaling pathways respectively, promote immunosuppressive events [161] (Figure 22).

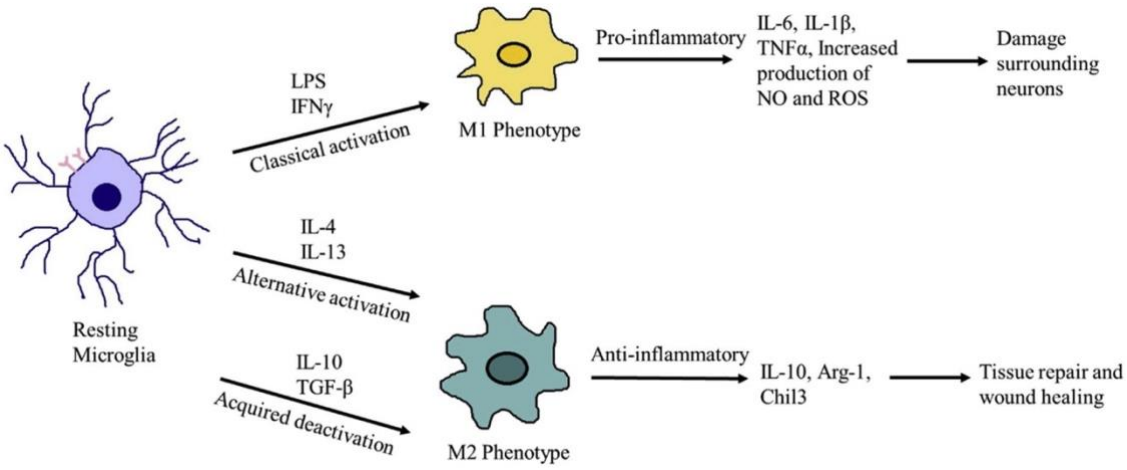

Figure 22. Classical activation, alternative activation and acquired deactivation of microglia [161].

Toll-like receptor-mediated activation

Toll-like receptors (TLRs) play a key role in innate immunity. They can be triggered by the presence of PAMPs (Pathogen Associated Molecular Patterns) such as LPS, an important component of the outer membrane of Gram-negative bacteria. After interaction with these receptors, pro-inflammatory cytokines are secreted and maturation processes of antigen-presenting cells (APCs) are triggered [161]. There are several isoforms of TLRs, among which TLR4 is the most widely expressed in microglia. This activation pathway is often involved in neurodegenerative processes. Between the several ligands of this receptor, LPS is one of the most studied to understand the relationship between microglia activation and the onset of neurodegeneration. Recognition of this molecule by microglia, activates, through the release of pro-inflammatory mediators such as IL-6, TNF-α and NO, a pro-inflammatory response that leads to neuronal death. In fact, in parallel to mediating chronic inflammatory processes due to strokes and spinal cord injury resulting in brain damage, TLR4 is often associated with various neurodegenerative diseases such as AD and PD [161], [172].

Another activator of this pathway is IFN-γ and its activation is particularly characteristic in multiple sclerosis [161].

Of note, in neurodegenerative diseases the M1/M2 activation ratio is generally altered. A modulation of the ratio between the two cellular phenotypes could be an aspect to be taken into account for therapeutic approaches in this kind of diseases [169].

3.1.2 ASTROCYTES

Astrocytes are the most abundant glial cells in the central nervous system (CNS), about five times the number of neurons [62]. As these cells account for almost half of the cells in the human brain, there is no CNS pathology that does not involve them [173]. Initially it was thought that microglia were the main actor in the neuroinflammatory process and astrocytes intervened secondarily only to amplify and propagate the immunological signals that are sent to them by microglia. However, it has since been shown that astrocytes, although more passively than microglia, play an important role in the defense of the CNS. In fact, these cells are responsible for establishing a functional network, called "neurovascular unit" (NVU), with neurons and blood vessels of the BBB with which they are physically and directly connected [174] (Figure 23). Indeed, they provide energy and trophic support to neurons, regulate synaptic neurotransmission, synaptogenesis, cerebral blood flow, brain ion and neurotransmitter levels and maintain the integrity of the blood-brain barrier [155], [175]. Furthermore, due to their close communication with immune cells, they are crucial regulators of innate and adaptive immune responses in the damaged CNS, thus playing an active role in the development of neuroinflammation [155].

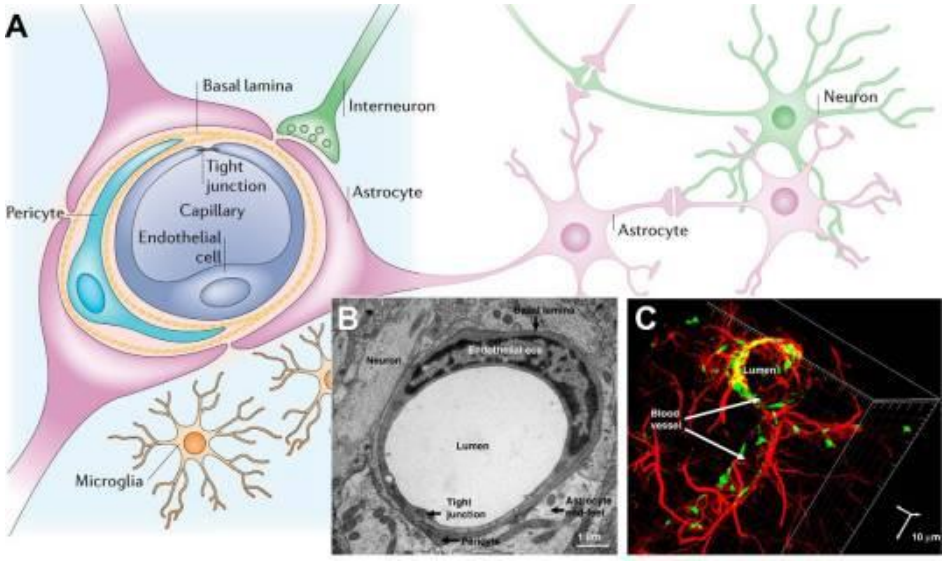

Figure 23. Neurovascular unit (NVU) [176].

Like other glial cells, astrocytes respond to various inflammatory stimuli, such as IL-1β, LPS and TNF-α, by activating the production of pro-inflammatory cytokines [62]. Moreover, under

neuroinflammatory conditions, astrocytes upregulate the receptor for IL-17, an important inflammatory cytokine released by T-lymphocytes, and trigger the production of pro-inflammatory cytokines, chemokines and metalloproteinases. This category of cells also plays a fundamental role in the formation and maintenance of the BBB, which, if its integrity is altered, induces an inflammatory state and can facilitate the entrance of molecules and immune cells into the CNS [177]. Astrocytes, following damaging events and/or lesions such as infection, trauma, ischaemia and neurodegeneration, may react to the stimulus becoming reactive, a process known as reactive astrogliosis. When in the reactive state, these cells undergo/activate changes in their morphology [178] and gene expression [173] resulting in the formation of a glial scar at the site of injury.

Indeed, prolonged activation of astrocytes can induce the release of extracellular matrix proteins that cause scar formation along the edges of tissues damaged by necrosis, infection or autoimmune inflammatory infiltration. For a long time, it was unclear whether reactive astrogliosis was beneficial or detrimental to the CNS. Recent experimental evidence suggests that reactive astrocytes may perform both functions. Indeed, these scars, on the one hand, act as neuroprotective barriers against inflammatory cells and infectious agents and, on the other hand, limit tissue regeneration [62], [174]. This dual function of reactive astrocytes has suggested the possibility of the existence of different astrocyte populations associated with different functions. Indeed, it has been demonstrated that there are two phenotypes of reactive astrocytes:

- A1 reactive astrocytes, activated by LPS;
- A2 reactive astrocytes, induced by ischaemic processes [62].

Of note, LPS is a strong activator of microglia, which, when activated, secrete IL-1alpha and TNF that promote astrocyte activation to the A1 phenotype [62]. This underlines the strong interrelationship between the various cell populations in the brain.

In addition, the A1 phenotype has been associated with a loss of normal astrocyte functions and an increase in toxic functions. In fact, A1 reactive astrocytes are no longer able to form functional synapses and completely lose their phagocytic function [62]. In some studies it has been seen that co-cultures of neurons and A1-reactive astrocytes are associated with a higher percentage of cell death (about 25% more), generally by apoptosis [62], [179]. This, in parallel to confirming the loss of the protective role of A1 astrocytes on neuronal survival, underlines the strong interrelationship between astrocytes and microglia in integrating responses at the basis of neurodegeneration.

3.2 INFLAMMATORY MEDIATORS

NF-kB is one of the most important regulatory factors in the regulation of proinflammatory and immune gene expression as well as playing a controlling role in the processes of cell proliferation

and protection from apoptosis [180]. It is ubiquitously present in all cells and is the link between early signal transduction events at the membrane and changes in gene expression [181]. The NF-kB family consists of 5 members: RelA (p65), RelB, cRel (Rel), NF-κB1 (p50 and its precursor p105) and NF-κB2 (p52 and its precursor p100) [180], [182]. They are all characterised by the presence in their structure of a Rel domain that is critical for DNA binding. More precisely, RelA (p65), RelB, cRel (Rel) activate the transcription of NF-kB target genes by the presence of a transactivation domain in their structure, while the other two members are under form of precursor proteins (p100 and p105) and are then respectively transformed into p52 and p50 following the action of the proteasome [1].

Physiologically, the NF-kB family forms a variety of homo- and hetero-dimers that are maintained in an inactive form, complexed with an inhibitory protein called IkB that prevents their translocation to the nucleus. Following cellular stimulation, NF-kB can follow two main activation pathways [1], [180]:

- Classic: activation of various receptors, such as TNFRs, causes phosphorylation of the IκB inhibitory proteins by the IKK complex. This causes degradation by the 26S proteasome of IkB through phosphorylation of two specific serine residues. Released from their inhibitory interaction with the IκB protein, the RelA and p50 dimers translocate into the nucleus, where they bind to the kB consensus sequence (most commonly GGGACTTTCC), generally resulting in increased expression of the target gene [183]. Generally, activation of this pathway occurs following stressor signals, among which the most common are: interleukin-1 (IL-1), inflammatory cytokine tumour necrosis factor α (TNFα), toll-like receptors (TLRs) and lipopolysaccharide (LPS). This pathway is crucial in the regulation of innate immunity and inflammation and mediates a strong and rapid NF-kB activation signal [180].
- Alternative: This pathway is slower and has specific long-term effects. In this case it is activated by a more restricted set of TNF family members, such as B-cell activating factor (BAFF), lymphotoxin-β (LTβ) and CD40 ligand [180]. It generally results in the activation of RelB-containing dimers and involves the NF-κB inducing kinase (NIK), which activates IKKα. This leads to the release of RelB/p50 and RelB/p52 dimers following phosphorylation and proteasome-dependent processing of p100 [180]. Lymphotoxin-β is a pro-inflammatory cytokine that activates the alternative NF-κB pathway, resulting in several crucial changes in gene expression of both genes involved in inflammatory processes, and genes related to lymph nodal development and gastrointestinal immune homeostasis [180] (Figure 24).

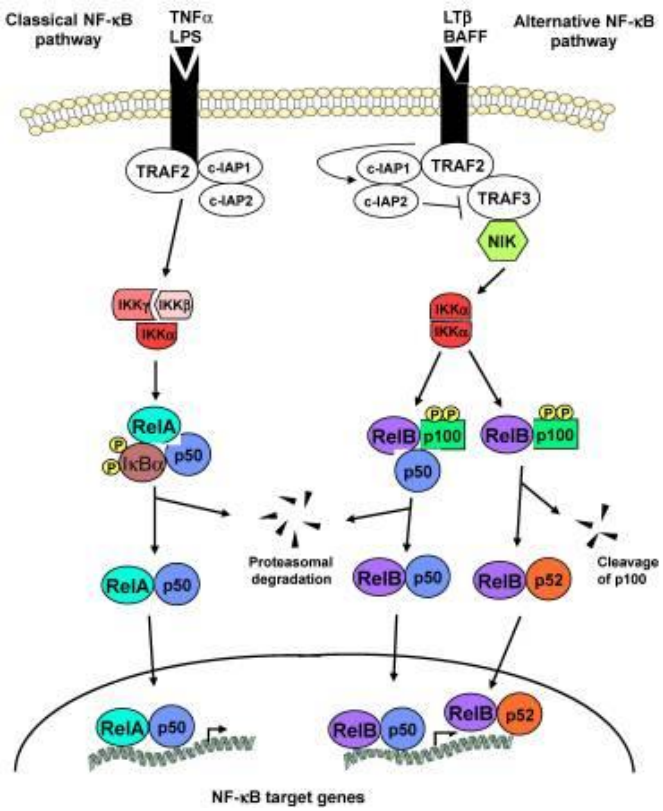

Figure 24. NF-kB activation pathways: Classical and alternative [180].

When cytokines, PAMPs (Pathogen Associated Molecular Patterns) and DAMPs (Damage Associated Molecular Patterns) stimulate receptors such as TLRs to activate NF-κB, genes coding for pro-inflammatory cytokines are expressed and the CNS immune response is enhanced. In addition, NF-kB plays a key role in cellular responses to oxidative stress [1]. NF*κ*B promotes ROS/RNS production under proinflammatory conditions, as it is a potent inducer of NOX2 and iNOS [184]. This transcription factor can also further recruit inflammatory cells through the induction of COX-2 /cPLA2-mediated prostaglandin production. This mechanism further promotes a condition of oxidative stress, as superoxide is also generated during prostaglandin production [184]. This demonstrates the strong involvement of NF-kB in the interrelationship between ROS/RNS generation and the induction of proinflammatory cytokines, resulting in increased cellular damage and improved neurodegeneration [1].

The NF-kB-mediated inflammatory state is maintained until the brain environment reaches the right conditions to trigger deactivation of the immune response.

Among the pro-inflammatory cytokines induced by NF-κB, TNF-α, IL-1β and IL-6 are the main ones.

Tumour necrosis factor alpha (TNF-α)

TNF-α is a cytokine that exists both as a membrane-bound protein and in a soluble form generated by the activity of a metalloprotease known as "TNF-α-converting enzyme" (TACE). Although microglia are primarily responsible for TNF-α production in neurodegenerative disorders and CNS insults, numerous studies have shown that astrocytes and neurons may also be a source of this pro-inflammatory cytokine [185]. TNF-α signaling occurs through two distinct receptors called TNFR1, which is expressed by almost all cell types, and TNFR2, which is less expressed in glial cells. TNFR1 appears to have important roles in promoting neuronal death through the activation of pro-apoptotic proteins [186].

Interleukin 1 (IL-1)

The IL-1 family consists of several proteins including IL-1α and IL-1β.

IL-1α is mainly found bound to the membrane, while IL-1β is typically in a soluble form. Both are synthesised in an inactive form which, following the action of specific proteases, are converted into mature proteins. IL-1 induces transcription of inflammation-associated genes, in particular iNOS and cyclooxygenase type 2 (COX-2) whose products, NO, prostaglandins and leukotrienes, are potent pro-inflammatory mediators. As gene expression and synthesis of these enzymes is increased, the effects of IL-1 persist for a long time after activation of the cell [187].

Interleukin 6 (IL-6)

IL-6 is a glycoprotein that binds to and activates a receptor complex consisting of a non-membrane-associated α subunit and two other subunits responsible for signal transduction. IL-6 is involved in the regulation of apoptosis, stimulates the differentiation of monocytes into macrophages, and promotes the infiltration of all components of the immune system (IS) by stimulating chemotaxis.

IL6 is a myokine, so in addition to being secreted by immune cells, it is also produced by muscles, especially during aerobic exercise. In this capacity it has extensive anti-inflammatory functions as an antagonist of the IL-1 receptor [188].

3.3 NEUROINFLAMMATION IN NEURODEGENERATION

Many neurodegenerative diseases, such as Parkinson's disease (PD), Alzheimer's disease (AD) and Huntington's disease (HD) show several common features. Among these, neuroinflammation is one of the most impactful and characterizing events at the basis of these diseases [1], [155], [161], [168]. In fact, the presence of damaged neurons in these pathologies implies a continuous and constant

activation of microglia and astrocytes, promoting a neuroinflammatory environment that inevitably initiates and supports neurodegeneration processes [1].

In recent decades, the role of neuroinflammation as a crucial factor underlying the onset and progression of neurodegeneration and neuronal loss in neurodegenerative diseases has been extensively established [155], [169], although it is still unclear whether neuroinflammation is more a cause or a consequence.

As already widely discussed, neuroinflammation is a complex and finely regulated mechanism in which several cell types are involved and which can have a dual (beneficial/damaging) effect on the CNS. When there is a failure in the regulation of the balance between pro- and anti-inflammatory mechanisms, this leads to a chronic inflammatory condition characterized by the loss of CNS homeostasis and the develop of neurodegeneration.

Epidemiological studies have shown that lesions in brain tissue may represent a risk factor for the development of pathologies such as AD and PD [155]. Furthermore, it has been shown that, even after the lesion has been repaired, microglia remain in an active state without promptly returning to a quiescent state. This prolonged microglia activation generates a chronic inflammatory response that could contribute to neurodegeneration [155]. However, in most cases neurodegenerative diseases are not triggered by lesions in the CNS, but rather their initiation is more attributable to intrinsic changes in CNS tissues and functions as occurs, for example, during the ageing process.

As neurodegenerative diseases are typical of the elderly, ageing is one of the key predisposing factors of these diseases [155], [168], [189]. Glia and neurons are not exempt from the consequences of the normal ageing process and are particularly susceptible to it. In fact, the microglia of the aged brain, through molecular and morphological changes typical of cellular senescence, lose their protective function in the CNS becoming what is known as "microglia dysfunction or dystrophic" [168]. Indeed, aged microglia are associated with dystrophic phenotypes, characterized by reduced branching and mobility of cellular processes, replicative senescence and altered response to insults. In addition, among the hallmarks of aged microglia there is an altered inflammatory profile. In fact, the aged brain shows increased levels of pro-inflammatory molecules such as L-1β and IL-6 and reduced levels of anti-inflammatory molecules such as IL-10 [168], as well as increased expression of NF-kB [190]. Studies performed on astrocytes isolated from aged brains have shown an increase of pro-inflammatory processes through the release of IL-1β, IL-6, IL-18, TNF-α, and COX-2, oxidative/nitrosative stress, mitochondrial dysfunction, impaired antioxidant defenses and release of trophic factors such as GDNF, BDNF and TGF-β [155], [191], [192].

What has been observed is in agreement with the change in the M1/M2 microglial ratio with prevalence of the M1 phenotype in neurodegenerative diseases. Indeed, a promotion of the classical (pro-inflammatory) activation pathway has been found in parallel with a reduction in the alternative (anti-inflammatory) one, making the ageing brain more vulnerable to neurodegeneration [168].

Another distinctive aspect of neurodegenerative diseases is the aggregation and accumulation of misfolded proteins such as β-amyloid (Aβ), α-synuclein, tau and huntingtin (Htt). The interaction of glial cells with these toxic proteins is one of the most relevant mechanisms for the development of chronic inflammation at the basis of the pathogenesis of these diseases [155], [165] and generally a mechanism of mutual self-propagation between the two events is established: the formation of toxic protein aggregates promotes the development of a chronic inflammatory condition, which, in turn, supports and stimulates further formation of such aggregates.
Aggregates of misfolded proteins, such as Aβ and α-synuclein, can bind and activate TLR receptors (mainly TLR2 and TLR4) promoting a dual response [1], [155]: the phagocytosis of toxic proteins by microglia and the release of chemokines and cytokines (TNF, IL, CCL2) contributing to disease progression [1], [193]. In particular, persistent activation of TLR2 has been found to be strongly involved in the development of neuroinflammation and subsequent neurodegeneration [193].

Moreover, as anticipated above, microglia and astrocytes can also promote the production and release of highly reactive molecules such as ROS and RNS [1], [155]. In particular, it has been shown that excessive NOX-mediated production of superoxide may be deeply implicated in neurodegenerative processes [147]. The profound involvement of oxidative stress in neurodegenerative diseases has already been widely discussed, but it is interesting to note the strong involvement and interrelation between oxidative damage and neuroinflammation in these diseases. Indeed, it should be observed that astrocytes and microglia, when activated, can induce the expression of NADPH oxidase and NO synthase (especially NOX2 and iNOS), which lead to an increase in superoxide and NO levels with consequent neuronal damage [1], [169]. Moreover, in neurodegenerative diseases, Aβ and α-synuclein can directly activate NOX [1], [169], further supporting oxidative damage, neuroinflammation and neurodegeneration in a vicious cycle of self-propagation. In addition to this, excessive levels of ROS and RNS can activate pro-inflammatory signaling cascades through the induction of stress-responsive protein kinases such as JNK and p38 MAP kinase and the activation of transcription factors such as AP-1, HIF-1 and NFκB [98], [194].

In conclusion, neuroinflammation, through the involvement of different cell types and biological systems, is strongly implicated in the onset and progression of neurodegenerative disorders. It probably plays the role of both cause and consequence, establishing a vicious circle that increasingly promotes neurodegeneration. In Table 2 the specific involvement of neurodegeneration in Alzheimer's, Parkinson's, and Huntington's disease is reported.

Table 2.Focus on neuroinflammation in neurodegenerative diseases.

Alzheimer's disease	*Neuroinflammation is one of the most important neuropathological features in AD. It is mediated by activated astrocytes and microglia, which are responsible for the inflammatory reaction against the progressive accumulation of Aβ plaques and neurofibrillary tangles in the brain [32]. However, due to inefficient phagocytosis of these protein aggregates, activation becomes chronic and uncontrolled, producing large amounts of pro-inflammatory mediators resulting in neuronal damage or death [195].* *In AD, activated microglia interact with Aβ oligomers and several receptors, such as G-protein-coupled receptors and Toll-like receptors (TLR4), and initiate the NF-κB-mediated pathway. Furthermore, in astrogliosis, Aβ-mediated activation of astrocytes enhances neuroinflammation through activation of the NF-κB pathway and microglia phagocytosis is promoted [196]. Neuroinflammation in AD patients is characterised by the expression of several pro-inflammatory cytokines including:* - *TNF-α, responsible for the generation of Aβ through β- and γ-secretase;* - *IL-1 which increases tau protein phosphorylation through the p38-MAPK pathway [197].* - *IL-6 which enhances the phosphorylation of tau protein via the cdk5 / p35 pathway [188].* *Another typical feature of AD is the loss of synapses. This phenomenon can occur years before clinical symptoms and is responsible for cognitive decline. The mechanisms underlying synapse loss and dysfunction are still poorly understood. It is believed that complement system proteins, abundant in the hippocampus, send signals to microglia to degrade damaged synapses even before amyloid plaque deposition [198].*
Parkinson's disease	*Several mechanisms have been described as causative factors for dopaminergic neurodegeneration in PD. Some of these are common among neurodegenerative diseases, such as protein aggregation due to misfolding and/or reduced protein degradation, ROS/RNS-induced oxidative stress, mitochondrial dysfunction and chronic neuroinflammation [199]. Studies in experimental animal models of PD have shown that neuroinflammation plays a key role in the onset and progression of this disease [200]. Indeed, like Aβ plaques, α-synuclein plaques induce IL-1β production by microglia through a TLR-dependent mechanism [201].* *In addition, the integrity of the blood-brain barrier is compromised and components of the innate immune system are activated [62].* *Several human studies have shown that the cytokines IL-1, IL-6, TNF-α and IFN-γ are increased in the serum of PD patients [202] compared to healthy subjects at the same age [203].* *Post-mortem studies on PD brain sections have shown that Lewy bodies contain proteins of the complement system (a serum-mediated mechanism designed to eliminate immune targets).* *Taken together, these observations indicate that in PD patients there is an inflammatory process with the involvement of the innate immune system in the CNS [204].*
Huntington's disease	*The first evidence of microglial abnormalities in Huntington's disease was described in a study by Singhrao et al. in 1999 [205], which showed a considerable increase in both number of microglial cells and expression of complement factors. Recent advances in imaging technology have made it possible to identify the brain areas with the greatest increase in glial activation, confirming the correlation between microglial activation and the subclinical progression of HD [74]. In several autopsy examinations of HD patients, a specific profile of inflammatory mediators was observed: IL-1 and TNF- α were increased only in the striatum, while IL-6, IL-8 and MMP-9 were also upregulated in the cortex. This is markedly different from the*

neuroinflammatory profile of other neurodegenerative diseases, such as AD or PD, which show a more general upregulation of a wide range of inflammatory mediators [206]. Of note, in HD, in contrast to multiple sclerosis and AD, peripheral immune cells, such as lymphocytes or neutrophils, have not been found to be involved. Thus, in this disease, the neuroinflammation appears to be sustained exclusively by the interactions among microglia, neurons and astrocytes [74].

4 AIM

In the 20th century there was a strong demographic event such as an ageing population. Life expectancy increased by about 40 years only in Europe [207]. Initially, this phenomenon affected only the most developed countries, but in recent years it has also emerged in developing countries. Ageing is a complex demographic phenomenon which, on a global scale, is linked to social, economic and cultural aspects. Over the next few decades, there will be an extraordinary increase in the number of elderly people worldwide: recent ISTAT data show that by 2065 the average life expectancy will increase by more than five years for both genders. In fact, it is reported that there will presumably be a shift in average survival from 80.6 to 86.1 in men and from 85 to 90.2 in women [208]. In this scenario, any intervention focused on improving life quality in old age can be considered fundamental for the well-being and socio-economic growth of any country.

Along with this, the coming decades will inevitably be marked by an increase in the incidence of chronic degenerative pathologies, being typical of the elderly part of the population, such as cardiovascular pathologies, neurodegenerative diseases and cancer [209]. Among these, neurodegenerative diseases, the most common of which are Alzheimer's, Parkinson's and Huntington's disease, occurring at a particularly advanced age (except sporadic genetic forms, the average onset age is over 60-65 years), will become an increasingly pressing global health problem. This was confirmed by the analysis of a series of epidemiological studies carried out by García-Morales et all., which, beyond highlighting a prevalent incidence of AD in women (5.1%) compared to men (3.8%), underlined how the incidence of this pathology grows proportionally and exponentially with age [27] (Figure 25).

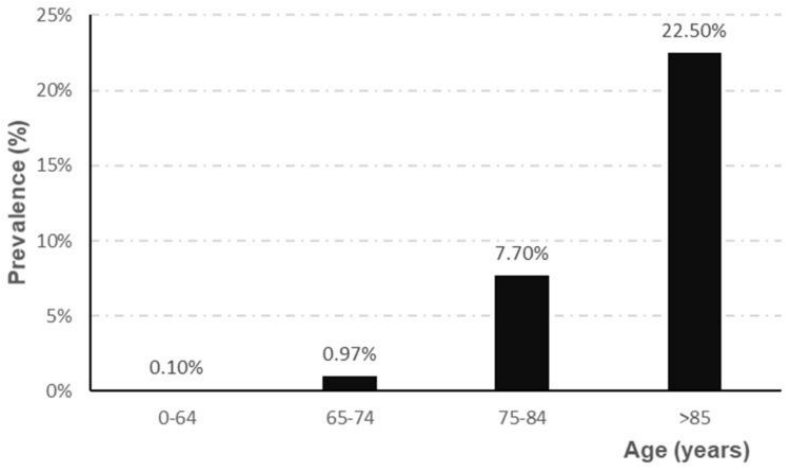

Figure 25. Evolution of the prevalence of AD according to age range [27].

Moreover, as mentioned above, this increased incidence of neurodegenerative diseases will have a significant socio-economic impact. The economic cost for the treatment and care of these patients, in addition to proportionally increasing with the average incidence of the disease, grows with the evolution of the pathology due to the increasing patients request for assistance. Of note, in 2010 the cost for just treating AD was estimated to be around $600 billion worldwide and, in just 5 years, it has grown by 5% reaching around $800 billion in 2015 [27], [210]. On the other hand, no less important is the enormous social impact of these diseases that compromise not only the life quality of patients but also of their families. As the disease progresses and the patient needs increase, the costs and social commitment for the healthcare system and families also increase.

Despite the efforts and resources invested by researchers to find an effective therapy for these forms of dementia, unfortunately, to date only a few drugs are available and only able to mitigate the symptoms without slowing down the progression of the disease [1]. Among the reasons for this lack of therapy, in addition to the recruitment of patients too far ahead in the progression of the disease, the main reason is the difficulty in identifying the molecular target on which to act.

The main neurodegenerative diseases can also be defined as 'proteinopathies', as they are based on the formation and accumulation of aggregates of specific misfolded proteins [9]. However, considering them only as 'proteinopathies' is an oversimplification, since their real hallmark is their multifactorial nature. If this aspect is not taken into account, it will be difficult to develop an effective therapy. Infact, every day there is more and more evidence supporting the necessity of a multitarget treatment, rather than a single target, in order to effectively counteract the various concomitant causal factors of the diseases [211].

Therefore, neurodegenerative diseases are characterized by a multifactorial etiology and share common pathological features such as oxidative stress, inflammation, glycation, abnormal protein deposition, microglia dysfunction and apoptosis [212], [213]. Among these, oxidative stress and neuroinflammation, besides being the most impactful, are highly interconnected events that constitute two causal factors that are mutually self-sustaining and exponentially promote neurodegeneration [1].

The central nervous system is extremely sensitive to oxidative stress for several reasons including: a low level of antioxidant enzymes, a high content of oxidable substrates and a large amount of ROS produced during metabolic reactions due to high brain oxygen consumption [214]–[216]. Oxidative damage, which in turn leads to a further increase in ROS levels, generates a self-propagation cycle in

which oxidative stress and ROS mutually enhance each other. ROS also promote protein misfolding and aggregation, which are key features of neurodegenerative diseases such as Parkinson's and Alzheimer's [157].

Although the brain is considered an immunologically privileged organ, it is actually the site of several immune responses. Among the CNS cells, microglia represent the resident immune cells of the brain and have the physiological function of detecting, transducing, integrating and responding to extracellular signals and participating in brain development and maintaining CNS homeostasis [195]. Microglia cells represent the first defense system of the CNS [27]. Indeed, it is known that in case of infectious agents or neuropathological conditions, microglia rapidly switch from a quiescent to a reactive form, triggering inflammatory and phagocytosis mechanisms through the release of cytotoxic molecules such as free radicals like nitric oxide (NO) and superoxide anion, proteases, several pro-inflammatory cytokines including tumour necrosis factor (TNF-α) and interleukin-1β (IL-1β) [217]. Thus, while an acute neuroinflammatory response is beneficial to the CNS, a prolonged microglia activation generates a chronic inflammatory condition through the continuous release of these toxic molecules and the promotion of oxidative stress; both events that perpetuate the inflammatory cascade by further protracting inflammation and sustaining neurodegeneration [218].Therefore, the regulation of redox balance and microglia-mediated inflammatory processes may lead to the development of effective strategies for the treatment of neurodegenerative diseases.

As mentioned above, due to the multifactorial nature of neurodegenerative diseases, it is difficult to counteract them by using molecules with a single 'molecular target'. For this reason, in recent years, the scientific community has focused on the development and characterization of molecules with pleiotropic activity. Moreover, given that the clinical symptoms of these disorders appear when the damage to the patient is already at an advanced stage, in addition to pharmacological therapy, prevention is now another valid intervention strategy to counteract the neurodegenerative process. Nutritional research, which in recent years has paid increasing attention to the identification and characterization of phytochemicals and nutraceuticals as potential neuroprotective agents, is part of this context.

To date, several studies have demonstrated the health benefits of natural compounds (mainly plant extracts and their bioactive compounds) to counteract neurodegeneration through their proven antioxidant, anti-inflammatory, neuroprotective, antithrombotic, anti-monoamine oxidase (MAO) and anti-acetylcholinesterase (AChE) properties [219], [220].

Traditionally, the main sources of bioactive compounds have always been plants and vegetal food. In addition to these, in recent decades the food industry processing by-products are proving to be valuable sources of bioactive natural compounds.

The post-industrial society is facing alarming global problems caused by the impact of human activities on the environment. Every year, the food industry produces huge amounts of waste, the disposal of which is becoming an increasingly urgent global problem, as it exceeds the planet's capacity to regenerate resources and process waste and pollutants [221].

As reported by the Food and Agriculture Organization of the United Nations (FAO), about one third of the world's food production for human consumption (about 1.3 billion tons per year) is lost or wasted [222]. In fact, since 2015, the United Nations has proposed and outlined Sustainable Development Goals (SDGs) in order to "halve the per capita global food waste at the retail and consumer levels and reduce food losses along production and supply chains, including post-harvest losses" by 2030 [223]. In this context, the Circular Economy is based on the reuse of materials normally considered as waste and moves more towards the concept of "upcycling" [224], i.e. any process that can transform waste into higher value products by making it a raw material for new products [221]. The potential for recycling food processing by-products is extremely broad, ranging from their reuse as fertilizers and animal feed to their potential application as sources of bioactive compounds with high potential for human health benefits in the nutraceutical, cosmetic and pharmaceutical fields [223].

In this context, nutritional research further broadens its horizons, as phytochemicals and nutraceuticals can be obtained both traditionally from plant and from renewable sources derived from the food industry by-products. Moreover, compounds of natural origin, i. e. extracts or pure compounds, can be used for both preventive and therapeutic purposes. Thus, nutritional research can assume several connotations:

-Nutraceuticals and phytochemicals obtained directly from natural sources or from processing byproducts (extracts or pure compounds) and intended as normal dietary components and/or dietary supplements

-Nutraceuticals and phytochemicals isolated directly from natural sources or from processing by-products and considered as isolated biologically active compounds that can be exploited in chemical synthesis to obtain molecular hybrids that can act as "multitarget" drugs.

The aim of this PhD program was to identify nutraceuticals and phytochemicals, both as extracts and pure compounds and obtained from both plant and renewable sources, which due

to their antioxidant and anti-inflammatory properties, were able to counteract cellular and molecular alterations that characterize neurodegenerative diseases. Their neuroprotective potential (antioxidant and / or anti-inflammatory) has been evaluated in an in vitro model of neuroinflammation (the BV-2 microglial cell line activated with LPS), and / or in an in vitro model of neuronal oxidative stress (the neuron-like SH-SY5Y cell line differentiated with retinoic acid and exposed to H_2O_2).

In particular, in this thesis I will discuss 4 projects, distinct from each other but all deeply linked by the common goal of identifying phytochemicals and nutraceuticals as potential neuroprotective agents in order to suggest preventive and therapeutic strategies for the treatment of neurodegenerative diseases and to improve the life quality of these patients.

Study 1_ Impact of phenolic profile of different cherry cultivars on the potential neuroprotective effect in SH-SY5Y cells

Study 2_Anti-inflammatory activities of Spilanthol-rich essential oil from Acmella oleracea (L.) R.K. Jansen and its nanoemulsion.

Study 3_Study of the anti-inflammatory activity of novel tacrine derivatives with lipids extracted from cashew nutshell liquid

Study 4_Coffee Silverskin (CSS) and Spent Coffee Grounds (SCG): coffee industry by-products as a promising source of neuroprotective agents

5 Study 1_Impact of phenolic profile of different cherry cultivars on the potential neuroprotective effect in SH-SY5Y cells

The results of this part have been published on:

Fruit Quality Characterization of New Sweet Cherry Cultivars as a Good Source of Bioactive Phenolic Compounds with Antioxidant and Neuroprotective Potential

Antognoni, F.; Potente, G.; Mandrioli, R.; Angeloni, C.; **Freschi, M**.; Malaguti, M.; Hrelia, S.; Lugli, S.; Gennari, F.; Muzzi, E.; Tartarini, S.

Antioxidant 2020 (2020): E677
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5.1 INTRODUCTION

Many chronic diseases (cardiovascular diseases, cancer, diabetes and neurodegenerative disorders) can be largely prevented, and dietary habits play a crucial role in this context [225]. The intake of fruit and vegetables, key components of the human diet, is related to the maintenance of good health and the prevention of a wide range of diseases [226]. In temperate areas, sweet cherry (Prunus avium L.) fruits are widely consumed, and their market is steadily growing with a production of about 2.2 million tons per year [227]. Major producers include Turkey, USA and Iran, and Italy was the sixth largest producer in the world and the largest in Europe in 2017. In particular, in the spectrum of the Italian market, Emilia-Romagna is among the 5 regions with the highest production of sweet cherries [227]. There are some quality traits that make one cultivar more agronomically interesting than another and these are constantly sought such as large fruit size, taste, sweetness, extremely early or late ripening time, firmness, light or dark red color [228]. In addition to these, there is great interest in trying to develop cultivars that have a longer harvest season. For this purpose, breeding programs are continuously being developed to obtain new cultivars with improved characteristics. The University of Bologna developed a sweet cherry breeding program allowing the determination of 7 new cultivars. In this breeding program, European genotypes selected for quality were crossed with American self-fertile counterparts [229]. Sweet cherries are fruits rich in a wide range of food constituents such as simple sugars (glucose, sorbitol and fructose), dietary fiber, organic acids (succinic and malic acid), fat-soluble (A, E and K) and water-soluble (B and C) vitamins [230].

In addition to the proven protective properties of fiber and vitamins, numerous studies have demonstrated the bioactivity of numerous secondary metabolites in cherry plants [231]. This refers to a wide range of secondary compounds, among which the phenolic compounds stand out. These compounds, which are responsible for the taste, color, bitterness and astringency of the fruit [232], have increasingly attracted the attention of the scientific world for their health benefits [233]. Among the superfamily of phenolic compounds, sweet cherries are rich in anthocyanins, which are mainly responsible for the intense color of the fruit, together with flavonols, hydroxycinnamic acid derivatives and flavan-3-ols [234]. These compounds possess antioxidant properties [235], [236] and their concentration and composition within the plant can vary depending on genetic and environmental factors [237].

The antioxidant activity of these phenolic compounds has been shown to be related to the shelf life and quality of the fruit [238] and developing cultivars rich in these compounds could delay ageing, as well as better preserve their nutritional and nutraceutical value. In addition, the antioxidant activity of phenolic compounds in counteracting oxidative stress has been widely documented in literature [239], and to date there is a large body of pre-clinical and clinical research supporting the health

benefits related to their intake [240]–[242]. Oxidative stress is an imbalance between levels of reactive oxygen species (ROS) and the body's endogenous antioxidant defenses [243] and causes severe damage to essential biological macromolecules [244].

Chronic degenerative diseases are characterized by a multifactorial etiology and oxidative stress plays a key role in their onset and progression. In particular, oxidative stress plays a key role in neurodegenerative disorders, due to the brain high oxygen consumption, low levels of antioxidants and high polyunsaturated fatty acid content [77]. To date, there are numerous studies supporting the strong involvement of oxidative stress in the onset of Parkinson's [151] and Alzheimer's diseases [245]. Several neurotrophic factors are involved in neurodegenerative processes and among these, brain-derived neurotrophic factor (BDNF) is among the most studied [246]. Indeed, there is extensive evidence that both Parkinson's and Alzheimer's diseases are related to low levels of this neurotrophin [247]. In particular, in Parkinson's patients, low BDNF levels are associated with an increase in the degeneration of dopaminergic neurons [248], while in Alzheimer's patients with memory impairment [249].

Therefore, modulation of this neurotrophin could be considered as a valid preventive/therapeutic strategy to counteract neurodegeneration. Interestingly, anthocyanins have been proposed as potential agents able in modulate BDNF expression [250].

The aim of this project was to evaluate the potential neuroprotective effect of extracts of different cherry cultivars on the differentiated neuron-like SH-SY5Y cell line. This study is part of the "Sweet Unibo" project, developed by the University of Bologna, with the aim of obtaining new sweet cherry cultivars through a new breeding program. In this project 7 new sweet cherry cultivars (Sweet Valina, Sweet Saretta, Sweet Aryana, Sweet Stephany, Marysa, Sweet Lorenz and Sweet Gabriel) were compared to 3 reference cultivars (Burlat, Grace Star and Lapins).

Based on the analysis of the phytochemical profile (carried out by the research group of Prof. Fabiana Antognoni of the University of Bologna), 3 "Sweet Unibo" cultivars (Sweet Lorenz, Sweet Gabriel and Marysa) were chosen as the most promising to continue the studies and to evaluate their neuroprotective potential focusing on their ability to counteract oxidative stress and to modulate BDNF expression (Figure 26).

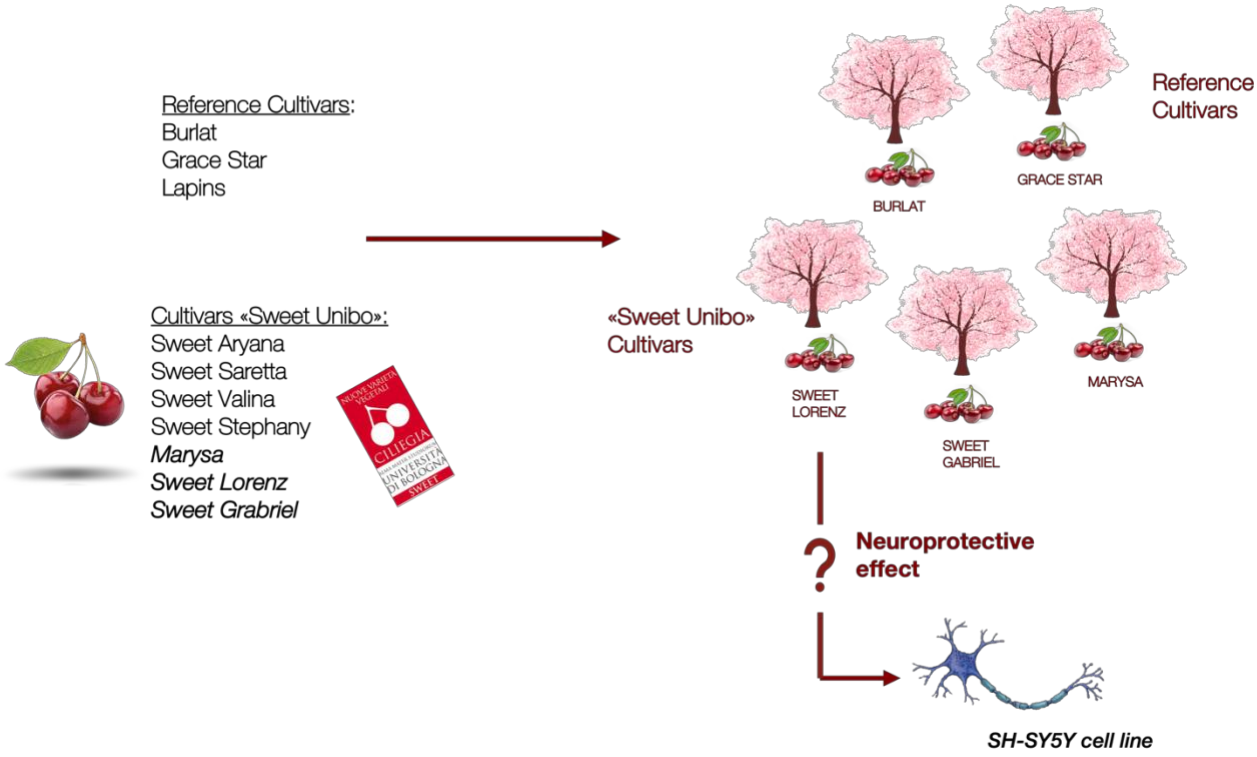

Figure 26. Aim of the study.

5.2 MATERIALS AND METHODS

5.2.1 Chemicals

Dulbecco's modified Eagle's medium (DMEM), penicillin/streptomycin, L-glutamine solution, trypsin-EDTA solution, phosphate buffered saline (PBS), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA), all-trans-retinoic acid (RA), H_2O_2, monochlorobimane (MCB), primers for real-time polymerase chain reaction (RT-PCR), dimethyl sulfoxide (DMSO) and all other chemicals of the highest analytical grade were produced by Merck Italia (Milan, Italy). Fetal bovine serum (FBS) was purchased from Euroclone (Milan, Italy). RNeasy Mini Kit was from Qiagen (Hilden, Germany). iScript cDNA Synthesis Kit, SsoAdvanced Universal SYBR Green Supermix Kit were purchased from Bio-Rad (Hercules, USA).

5.2.2 Plant materials

Fruits of sweet cherry (Prunus avium L.) cultivars were collected at the full ripeness stage in 2016 from an orchard located in Vignola (Emilia-Romagna, Italy). This work comprised three reference cultivars (Burlat, Grace Star*, Lapins) and seven new cultivars obtained from the UNIBO sweet cherry breeding program (Sweet Aryana® PA1UNIBO*, Sweet Lorenz® PA2UNIBO*, Sweet Gabriel® PA3UNIBO*, Sweet Valina® PA4UNIBO*, Sweet Saretta® PA5UNIBO*, and Marysa® PA6UNIBO* grafted on Colt, and Sweet Stephany® PA7UNIBO* grafted on CAB11E). The abbreviated names, Aryana, Gabriel, Lorenz, Saretta, Stephany and Valina will be used subsequently. Similar agronomic conditions (fertilization, irrigation, and pest control) were used for the growth of all cherry trees. Fruits samples were collected randomly from four different trees of each cultivar. The collected samples were used for both quality qnalysis/phytochemical determinations and extraction.

5.2.3 Extraction procedures

After freeze-drying, the pulp of cherries harvested from the same tree was finely ground in a knife mill for 4 × 30 s. Two technical replicates from the pulp powder were obtained using the "coning and quartering" sampling procedure.

The protocol of Ballistreri et al. [251], with modifications, was used to extract flavonoids and anthocyanins from the samples. 1 g of freeze-dried powder was mixed with 5 mL of methanol/HCl mixture (95.5/0.5, v/v) before homogenizing for 1 min (speed 5.5), and the resulting suspension was sonicated for 20 min at 35 ◦C. Subsequently, a centrifugation for 10 min at 1400× g was performed,

the supernatant was collected in a new vial and the sediment was extracted twice more by the same procedure. The resulting supernatants were pooled together and filtered through grade 44 (3 μm) ash-free filter paper. Further filtration was done using a syringe filter (nylon, pore diameter 0.22 μm) from Thermo Fisher Scientific.

The protocol of Milinovic´ et al. [252], with modifications, was employed to extract phenolic acids from the samples. 1 g of powder was mixed with 20 mL of methanol/water mixture (80/20, v/v), homogenized for 1 min (speed 5.5), and after centrifugation for 15 min at 1400× g, the supernatant was filtered through grade 44 (3 μm) ash-free filter paper. The clear liquid was diluted with 85% methanol in a volumetric flask until a final volume of 25 mL was obtained.

5.2.4 Fruit quality analysis and GC determination of sugar and acids in cherry extracts.

A sample of 30 fruits/plant were used to evaluate the fruit quality profile. The following parameters were evaluated: fruit color (L*, a*, b* measured with a CR-400 Konica Minolta colorimeter, Tokyo, Japan), delta absorbance (DA) index (using a Cherry DA-meter, Sinteleia, Bologna, Italy), fruit weight, fruit diameter, fruit firmness (using a Güss Fruit Texture Analyzer equipped with a 4-mm tip, Strand, South Africa), fruit pH and acidity (using titration with a Crison Titromatic 1S, Barcelona, Spain), soluble solids (using an Atago PAL-1 pocket refractometer, Tokyo, Japan), and kernel weight. Chroma and Hue angle was calculated using color coordinates.

Analysis of sugar and simple acid content was carried out employing a GC approach following the protocol of Bartolozzi et al. [253]. Precisely, 450 μL of imidazole buffer (pH 7.0) was added to 500 μL aliquots of each sample. Aliquots of 500 μL of each diluted juice were dried under continuous air flow before derivatization. 1 μL of the obtained solution was injected into a VARIAN GC 3900 equipped with electronic flow control (EFC), flame ionization detector (FID), CP-Sil 5 CB capillary column (30 m long), and a CP8410 autosampler (helium as carrier gas and nitrogen as make-up gas).

5.2.5 HPLC determination of phenolic compounds

A Jasco (Tokyo, Japan) HPLC-DAD system consisting of a PU-4180 pump, an MD-4015 PDA detector, and an AS-4050 autosampler was used for the determination of phenolic compounds in the extracts. The stationary phase consisted of an Agilent (Santa Clara, CA, USA) Zorbax Eclipse Plus C18 reverse phase column (100 mm × 3 mm I.D., 3.5 μm) and the injection volume used for all determinations was 20 μL.

The method of Milinovic´ et al. [252] was adapted for flavonoid analysis. A mixture of solvent A (water/formic acid 95/5, v/v) and solvent B (acetonitrile), with a composition gradient ranging from 97 to 36% of solvent A and flowing at 0.5 mL/min was used for the elution. A signal at 370 nm was

used for quantitative purposes. Flavonoid recovery values in spiked samples ranged from 79.2 to 91.4% (RSD < 9.4%, n = 6) (Figure 27).

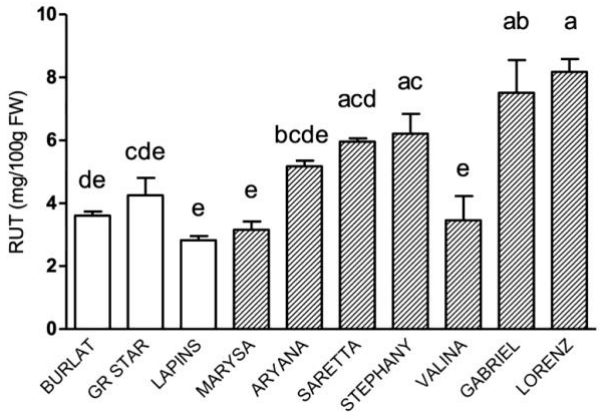

Figure 27. Rutin levels (mg/100 g FW) in sweet cherry cultivars. Data are the mean ± SE of four biological replicates. Different letters indicate statistical significance ($p < 0.05$) [254].

Analysis of phenolic acids was conducted following the method of Antognoni F, et al. [255]. A mixture of solvent A (50 mM phosphate buffer, pH 2.5) and solvent B (acetonitrile) flowing at 0.7 mL/min, increasing from 97 to 50% (v/v) of solvent A was used to carried out the gradient elution. Quantification of the analyte was performed at 254, 280, and 329 nm. Phenolic acid recovery values in the spiked samples ranged from 80.2 to 91.3% (RSD < 10.7%, n = 6) (Figure 28).

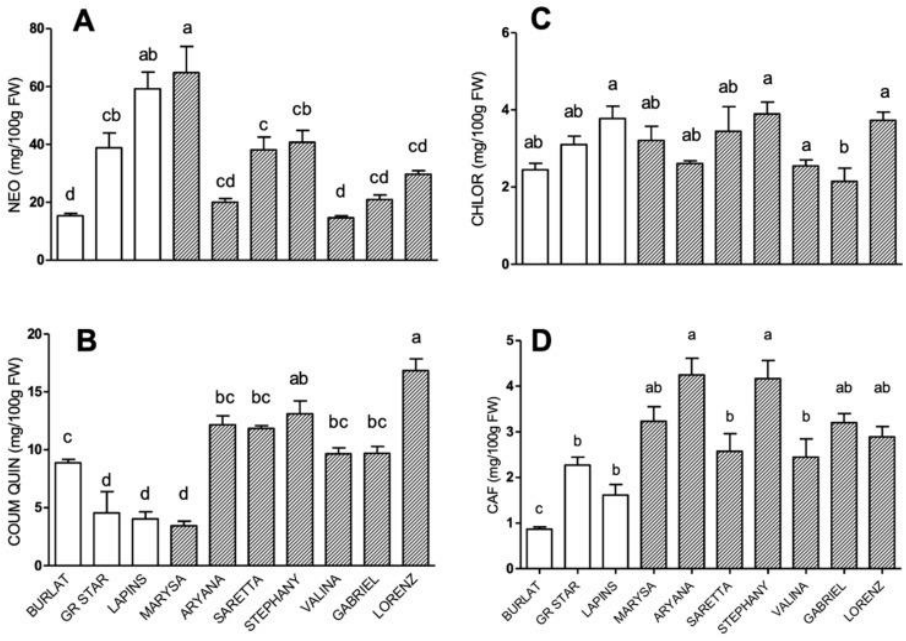

Figure 28. Phenolic acid levels (mg/100 g FW) in sweet cherry cultivars: (A) neochlorogenic acid, (B) coumaroylquinic acid, (C) chlorogenic acid, and (D) caffeic acid. Data are the mean ± SEM of four biological replicates. Different letters indicate statistical significance $p<0.05$ [254].

Anthocyanins were analyzed following the method of Ballistreri et al. [251] with modifications. A mixture of solvent A (water/formic acid/acetonitrile 87/10/3, v/v/v) and solvent B (water/formic acid/acetonitrile 40/10/50, v/v/v), with a composition gradient ranging from 94 to 40% solvent A and flowing at 1.0 mL/min was used for elution. Quantification was carried out at 520 nm. Anthocyanin recovery values in spiked samples ranged from 78.6 to 89.3% (RSD < 9.7%, n = 6) (Figure 29).

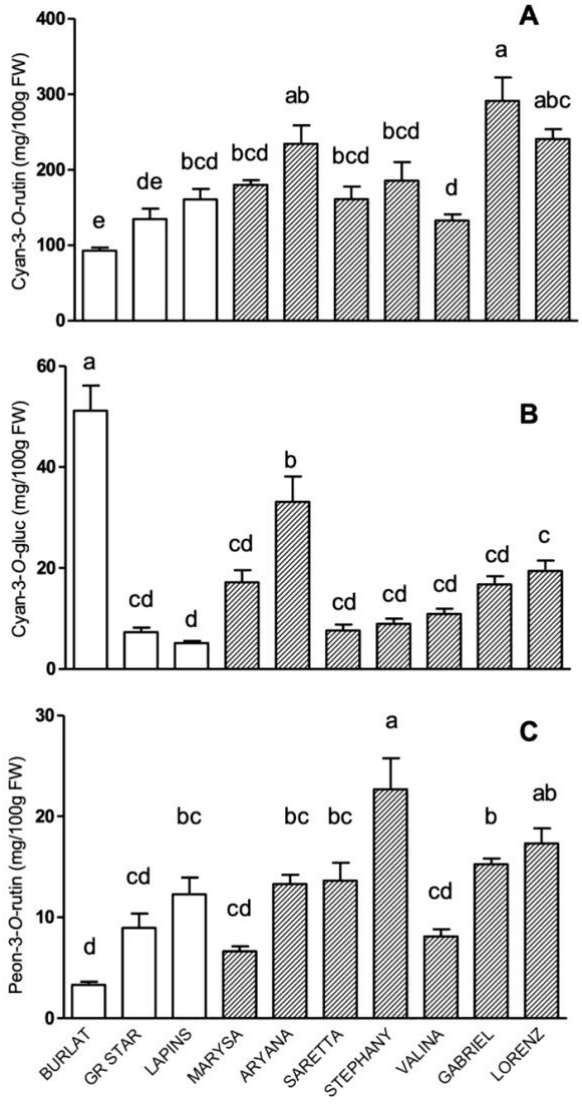

Figure 29. Anthocyanins levels (mg/100 g FW) in sweet cherry cultivars: (A) cyanidin-3-O-rutinoside, (B) cyanidin-3-O-glucoside, and (C) peonidin-3-O-rutinoside. Data are the mean ± SEM of four biological replicates. Different letters indicate statistical significance p<0.05 [254].

5.2.6 Cell Culture

The SH-SY5Y human neuroblastoma cell line (ECACC 94030304) was purchased from Merck (Italy). Cells were grown in DMEM supplemented with 10% (*v*/*v*) of FBS, 2 mM of L-glutamine, 50 U/mL of penicillin, and 50 µg/mL of streptomycin and maintained at 37 °C in a humidified incubator

with 5% CO_2 as previously reported [256]. Differentiated SH-SY5Y cells were used for experiments. Before experiments cell differentiation was induced by treatment with RA (10 μM) for 7 days (1% FBS).

5.2.7 Viability Assay

Cells were treated with different concentrations (0.1–100 μg/mL) of five cherry extracts (Burlat, Grace Star, Gabriel, Lorenz, Marysa) for 24 h, then exposed to H_2O_2 700 μM for 1 h to induce oxidative stress. At the end of the treatments cell viability was evaluated by MTT assay as previously reported [257]. At the end of the experiments, cells were incubated with MTT work solution (0.5 mg/mL) for 90 min at 37 °C. After this period, MTT solutions were replaced with 100 μL of DMSO to dissolve the formed formazan crystals and the absorbance was measured at a wavelength of 595 nm using a multilabel plate spectrophotometer (VICTOR3 V Multilabel Counter; Perkin-Elmer, Wellesley, MA, USA).

5.2.8 Determination of Intracellular ROS levels

Intracellular ROS levels were evaluated using the fluorescent DCFH-DA probe as previously reported [258]. SH-SY5Y cells were treated with 50 μg/mL of each cherry extract for 24 h, then incubated with 10 μM DCFH-DA (DMEM 1% FBS w/o phenol red) for 30 min. After probe removal, cells were exposed to 400 μM H_2O_2 (DMEM 1% FBS w/o phenol red) for 15 min. Then, H_2O_2 was replaced by PBS. Cell fluorescence was measured at 485 nm (excitation) and 535 nm (emission) with a multilabel plate reader (VICTOR3 V Multilabel Counter; Perkin-Elmer).

5.2.9 Determination of Reduced Glutathione (GSH) levels

The monochlorobimane (MCB) fluorometric assay was used to determine reduced glutathione (GSH) levels as previously reported [259]. Briefly, SH-SY5Y cells were treated with 50 μg/mL of each cherry extract for 24 h and then exposed to 700 μM H_2O_2 for 1 h to induce oxidative stress. At the end of experiments, cells were incubated with 50 μM MCB (1% FBS w/o phenol red) for 30 min at 37 °C. Subsequently, fluorescence was measured at 355 nm (excitation) and 460 nm (emission) with a multilabel plate reader (VICTOR3 V Multilabel Counter; Perkin-Elmer).

5.2.10 Real-Time polymerase chain reaction (PCR)

SH-SY5Y cells were treated with 50 μg/mL of each cherry extract for 24 h and, after treatment, total RNA was extracted using a RNeasy Mini Kit (Qiagen, Hilden, Germany), following the

manufacturer's protocol. The yield and purity of RNA were measured using a NanoVue spectrophotometer (GE Healthcare, Milan, Italy).

1 μg of total RNA was reverse-transcribed into cDNA using an iScript cDNA Synthesis Kit (BIO-RAD, Hercules, CA, USA), following the manufacturer's protocol. The subsequent PCR was carried out in a total volume of 10 μL constituted as follows: 2.5 μL (12.5 ng) of cDNA, 5 μL SsoAdvanced Universal SYBR Green Supermix (BIO-RAD), and 0.5 μL (500 nM) of each primer.

Primers used are below (Table 3):

PRIMER	SEQUENCE (5'-3')
BDNF_H_F	CAAAAGTGGAGAACATTTGC
BDNF_H_R	AACTCCAGTCAATAGGTCAG
GR_H_F	GACCTATTCAACGAGCTTTAC
GR_H_R	CAACCACCTTTTCTTCCTTG
NQO1_H_F	AGTATCCACAATAGCTGACG
NQO1_H_R	TTTGTGGGTCTGTAGAAATG
RPS18_H_F	CAGAAGGATGT AAAGGATGG
RPS18_H_R	TATTTCTTCTTGGACACACC

Table 3. Primers for RT-PCR.

RPS18 was used as a reference gene. Amplification of cDNA was initiated by activating the polymerase for 30 s at 95 °C, followed by 40 cycles of 5 s at 95 °C and 30 s at 60 °C. A melt curve was run to ensure quality control and generation of a single product. Normalized expression levels were calculated with respect to control cells according to the $2^{-\Delta\Delta CT}$ method [260].

5.2.11 Statistical Analysis

Each experiment was performed at least three times, and all values are represented as mean ± SEM. One-way analysis of variance (ANOVA) was used to compare differences among groups, followed by Tukey's test or Dunnett's test (Prism 6; GraphPad Prism Software, San Diego, CA, USA). Values of $p < 0.05$ were considered statistically significant. Correlation analysis was carried out with the same software.

5.3 RESULTS

5.3.1 Neuroprotective effect of cherry extracts against oxidative stress

First aim of this project was to evaluate the cytotoxicity profile of the different cherry extracts (0.1–100 µg/mL, for 24 h) on differentiated SH-SY5Y cells using the MTT viability assay.

The exposure to extracts up to 100 µg/mL did not influence the cell viability, so no cytotoxicity was observed as shown in Figure 30.

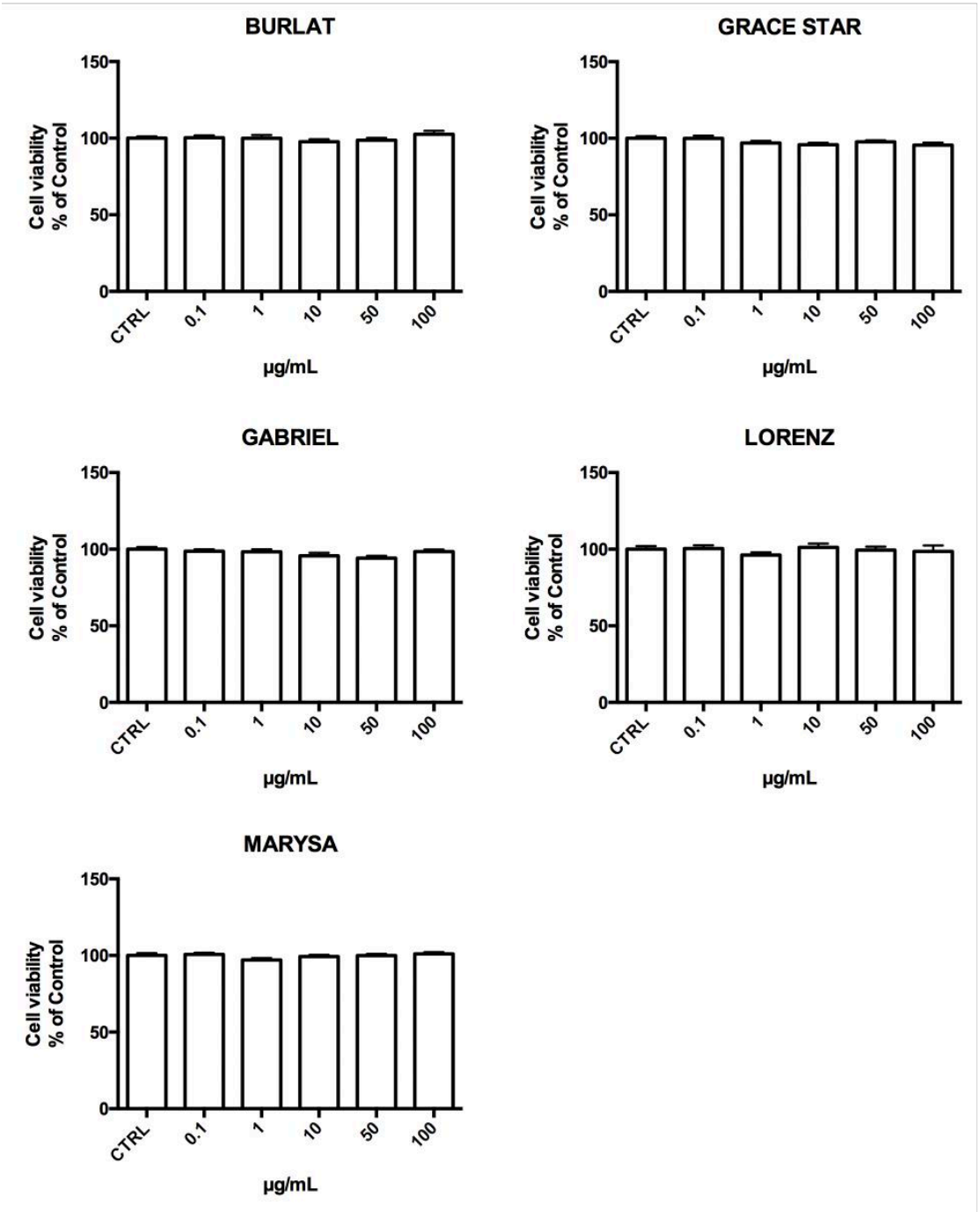

*Figure 30. Effect of different concentration of cherry extracts on SH-SY5Y cell viability. Cell viability was measured by MTT assay. Each bar represents the mean ± SEM of at least three independent experiments. Data were analyzed by one-way ANOVA followed by Dunnett's test. *p<0.05 compared to control (CTRL).*

To evaluate the potential protective effect of the five extracts, SH-SY5Y cells were treated with 0.1–100 µg/mL of each extract for 24 h and then exposed to H_2O_2 700 µM for 1 h to induce oxidative stress [256]. Lorenz, Gabriel, Grace star and Marysa cultivars showed a protective effect against H_2O_2 significantly increasing cell viability compared to H_2O_2-treated cells. Of note, Gabriel showed the greatest effect in fact it significantly increased cell viability at all concentrations higher than 1 µg/mL; Lorenz showed a protective effect only at 10 and 50 µg/mL, while Grace star and Marysa only at the concentration of 50 µg/mL. Burlat had no effect (Figure 31).

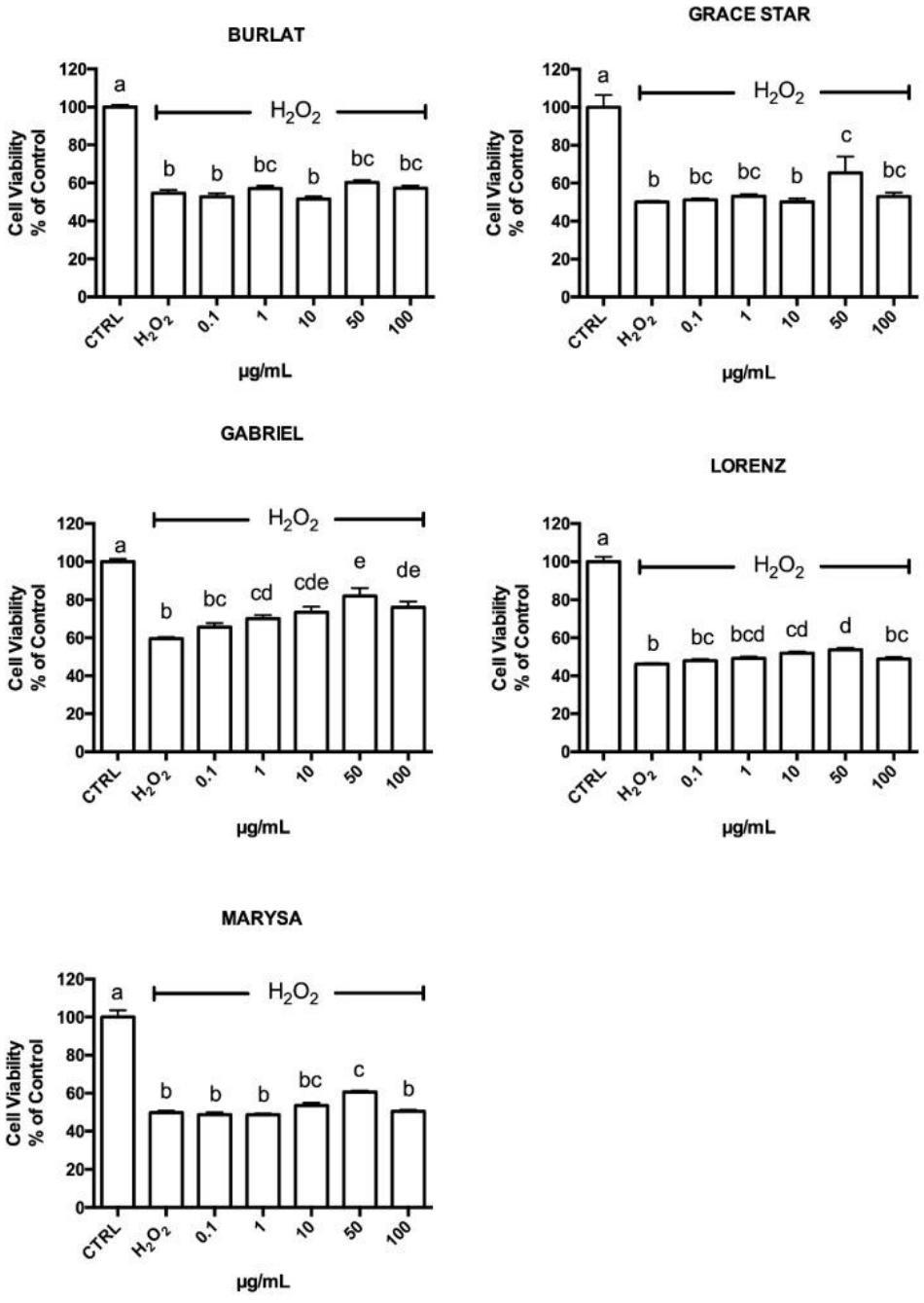

Figure 31. Protective effect of cherry extracts against H_2O_2-induced damage to SH-SY5Y cells. Cell viability was measured by MTT assay. Each bar represents the mean ± SEM of at least three independent experiments. Different letters indicate statistical significance ($p<0.05$)

As 50 µg/mL concentration showed a protective effect in all extracts except Burlat, it was selected to continue the study. To better investigate the mechanisms behind this protective effect, the ability of the tested extracts to counteract intracellular ROS production was investigated by DCFH-DA assay. In agreement with the viability data, Gabriel, Grace Star, Lorenz, and Marysa significantly reduced intracellular ROS compared H_2O_2 treated cells. As expected, Burlat did not show any effect (Figure 32A).

GSH is the most abundant intracellular antioxidant and therefore the effect of cherry extracts on this parameter was also evaluated using the MCB assay. SH-SY5Y cells were treated with 50 µg/mL of each cherry extract for 24 h and then exposed to 700 µM H_2O_2 for 1 h to induce oxidative stress. As expected, exposure to H_2O_2 significantly reduced GSH levels compared to control cells. As shown in Figure 32B, GSH levels were significantly increased only by Gabriel and Grace Star to values comparable to control cells, whereas the other extracts did not induce any increase in respect to H_2O_2 treated cells.

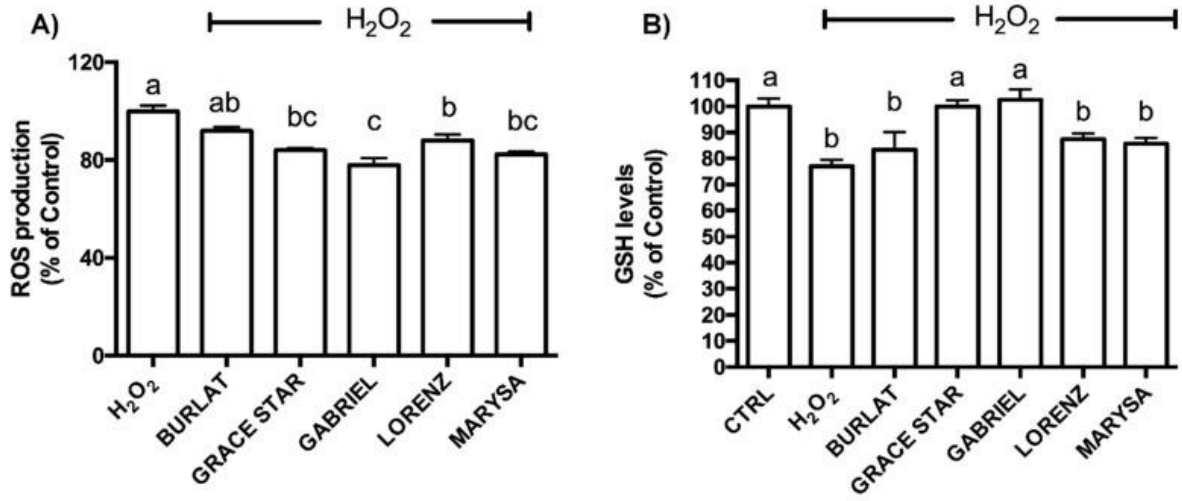

Figure 32. Effect of cherry extracts on intracellular (A) reactive oxygen species (ROS) and (B) reduced glutathione (GSH) levels in SH-SY5Y cells. ROS data are expressed as % compared to H_2O_2-treated cells, and GSH levels are expressed as % compared to control (CTRL) cells. Each bar represents the mean ± SEM of at least three independent experiments. Different letters indicate statistical significance ($p<0.05$)

5.3.2 Effect of cherry extracts on antioxidant enzymes and BDNF expression

It is well known that the endogenous antioxidant system is involved in the formation of GSH and hydroquinones in cells, so cherry extracts could act modulating it. To investigate this aspect, the modulation of two endogenous antioxidant enzymes, GR and NQO1, was evaluated. Cells were treated with 50 µg/mL of the five extracts and then the modulation of mRNA levels of these two enzymes was assessed by RT-PCR (Figure 33). Consistent with previous data, the extracts Gabriel,

Grace Star, Lorenz, and Marysa significantly up-regulated the expression of GR and NQO1, whereas Burlat showed no effect. Notably, Gabriel was the most effective in up-regulating GR expression.

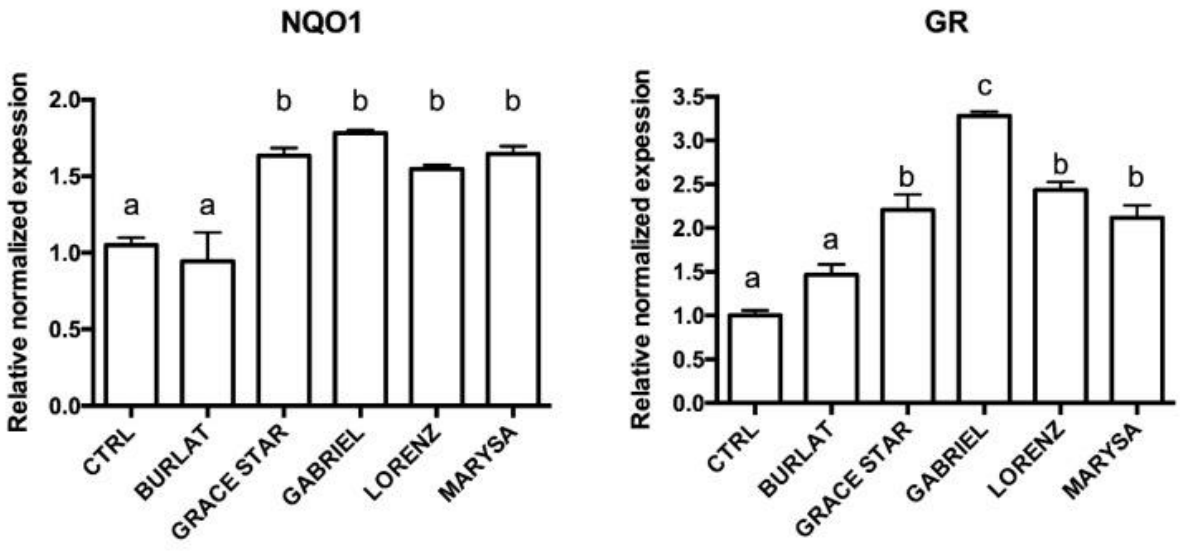

Figure 33. Effect of cherry extracts on the expression of NQO1 and GR in SH-SY5Y cells. Each bar represents the mean ± SEM of at least three independent experiments. Different letters indicate statistical significance ($p<0.05$)

Neurodegenerative diseases are characterized by reduced levels of brain-derived neurotrophic factor (BDNF) [249]. Therefore, the effect of the different extracts on BDNF expression levels was studied. Cells were treated with 50 µg/mL of the extracts before measuring changes in BDNF mRNA levels. As shown in Figure 34 all the extracts were able to significantly up-regulate BDNF in respect to control cells. This suggests a further protective role of cherries in neurodegeneration in addition to the demonstrated antioxidant one.

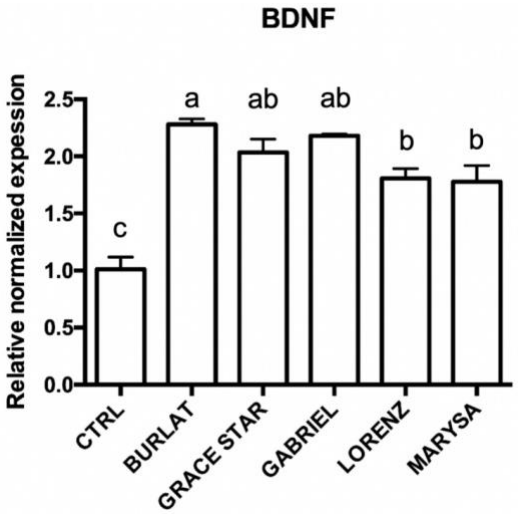

Figure 34.Effect of cherry extracts on the expression of brain-derived neurotrophic factor (BDNF) in SH-SY5Y cells. Each bar represents the mean ± SEM of at least three independent experiments. Different letters indicate statistical significance ($p<0.05$)

5.4 DISCUSSION

Oxidative stress is a harmful condition characterized by the intracellular accumulation of reactive oxygen species (ROS) due to an imbalance between ROS production and endogenous antioxidant defense mechanisms [243], [244], [261]. The onset of an oxidative condition has serious consequences for important cellular biomolecules (lipids, proteins and DNA) and compromises their functionality [244]. Thus, oxidative stress is a mechanism closely related to the onset and progression of a wide range of chronic degenerative diseases such as cardiovascular diseases, diabetes, cancer, chronic intestinal inflammation and neurodegenerative disorders [262]. It is clear how important is the adequate modulation of oxidative damage to prevent/counteract these diseases [261], [263]–[265]. Numerous studies highlight the impact of ROS in the development and progression of the neurodegenerative process. Specifically, a strong involvement of mitochondrial pathways, a reduction in endogenous antioxidant defenses and a stimulation of ROS production due to iron accumulation in the brain have been described [266]. Antioxidant defenses are both endogenous (antioxidant enzymes and glutathione system), constitutively present in the body and finely regulated, and exogenous, i.e. antioxidant compounds obtained from the diet such as (poly)phenols [262]. These molecules can act both as direct antioxidants, i.e. by directly neutralizing ROS, and as indirect antioxidants, i.e. as metal chelators and/or modulators of the endogenous antioxidant systems [243], [244], [261], [267]. Cherries, and in particular sweet cherries, are a nutrient-rich food with a low-calorie content and containing a large variety and quantity of bioactive compounds [268]. In particular, cherries are fruit rich in polyphenolic and other functional compounds such as hydroxycinnamates, potassium, fiber, vitamin C, carotenoids and melatonin [262]. Growing, harvesting, and processing conditions (UV concentration, degree of ripeness, post-harvest storage and processing) can significantly influence the content of nutrients and bioactive compounds in these fruits. These functional molecules present in cherries are responsible for the documented health benefits of these fruits. Several biological activities related to cherry consumption have been demonstrated, such as reduction of oxidative stress and/or inflammation, tumor suppression, regulation of glucose levels and inhibition of uric acid production [268]. Thus, cherry consumption is related to a beneficial preventive effect against a wide range of chronic degenerative diseases such as cancer, cardiovascular disease, diabetes, inflammatory diseases and Alzheimer's disease [262].

In this contest, aim of this study was to investigate the antioxidant activity of different sweet cherries (*Prunus avium* L.) cultivars, obtained through a natural breeding program, in differentiated neuron-like SH-SY5Y cells line.

The results obtained in this study show that different cherry cultivars vary in terms of fruit quality and content of bioactive compounds, showing significant quantitative inter-cultivar differences in the pattern of polyphenolic compounds.

Based on the analysis of the phytochemical profile, 3 “Sweet Unibo” cultivars (Sweet Lorenz, Sweet Gabriel and Marysa) and 2 reference cultivars (Burlat and Grace Star) were selected to evaluate their neuroprotective potential focusing on their ability to counteract oxidative stress and to modulate BDNF expression on the differentiated neuron-like cell line SH-SY5Y. The SH-SY5Y cell line is widely used in scientific research as an in vitro model of neuronal function [269]. Furthermore, their exposure to H_2O_2 triggers biochemical pathways that mimic neurodegenerative processes and lead to neuronal death [270]. Interestingly, treatment with the extracts obtained from the new cultivars resulted in a significant reduction in intracellular ROS and a significant increase in GSH levels, showing a protective effect against oxidative damage. Among the tested extracts, the one richest in anthocyanins, Gabriel, was associated with the greatest antioxidant effect. Of fundamental importance is the ability of anthocyanins and their metabolites, due to their chemical characteristics, to cross the blood-brain barrier and reach the brain in vivo [271], [272]. In agreement with the results of the present study, Leong et al. [273] observed greater efficacy in counteracting H_2O_2-induced damage in Caco-2 cells from cultivars with higher anthocyanin content. In contrast, another study evaluated the ability of three different Saco cherry fractions (uncolored fraction, colored fraction and total extract) to counteract tert-butylhydroperoxide-induced oxidative stress in Caco-2 cells, reporting a lower efficacy of the colored extract [274].

Several studies have also evaluated the antioxidant effect of cherry extracts in neurons. In particular, the ability of an anthocyanins-rich extract from Saco cherries to protect SK-N-MC cells from oxidative stress has been reported [262]. Gabriel and Lorenz cultivars, i.e. those with the highest content of peonidin-3-O-rutinoside, cyanidin-3-O-rutinoside, rutin and some minor phenolic acids (e.g. caffeic acid), were associated with the highest neuroprotective potential. Furthermore, the results showed that the rutinoside forms of anthocyanins were more effective than the glucoside forms. In accordance with the data of the present study, a strong influence of caffeic acid on the brain has recently been documented, demonstrating a protective effect in Alzheimer's disease [275].

Modulation of the expression of the antioxidant enzymes GR and NQO1 was evaluated to further investigate the intracellular antioxidant mechanisms of the extracts. GR is critical in regulating the intracellular ratio of reduced to oxidized glutathione (GSH/GSSG) [276], while NQO1, through a catalytic mechanism, promotes the two-electron reduction of quinones to their corresponding hydroquinones, inhibiting the generation of semiquinones and ROS [277]. Of note, all new cherry cultivars strongly up-regulated both antioxidant enzymes compared to controls. In particular, Gabriel

resulted in a significant up-regulation of GR compared to the other extracts. The antioxidant activity of Gabriel is probably attributable to the strong modulation of this enzyme. Although the ability of anthocyanins to positively modulate GR and NQO1 expression has been documented in other studies [278], this was the first time that such modulation was observed with cherry extracts.
CNS depletion of the neurotrophic factor BDNF, which is essential in neuronal survival, maintenance and regeneration [279], is closely related to neurodegenerative diseases such as Parkinson's and Alzheimer's [248], [249]. Therefore, any therapeutic strategy that increases levels of this neurotrophic factor should be strongly considered. Although there is no direct correlation with the anthocyanin content found in the various cultivars, BDNF expression was significantly increased by all extracts. In a study evaluating the effect of blueberry extract in mice, an increase in BDNF levels in the hippocampus was observed, attributing this effect not only to anthocyanins but also to flavonols [280].

In conclusion, the present study supports the idea that these new sweet cherries can be considered a new functional food with a high antioxidant and neuroprotective activity and these effects seem to be related to the specific phenolic pattern of the different cherry extracts.

6 Study 2_Anti-inflammatory activities of Spilanthol-rich essential oil from Acmella oleracea (L.) R.K. Jansen and its nanoemulsion.

The results of this part have been published on:

Spilanthol-rich essential oil obtained by microwave-assisted extraction from Acmella oleracea (L.) R.K. Jansen and its nanoemulsion: insecticidal, cytotoxic and anti-inflammatory activities.

Spinozzi E.; Pavela R.; Bonacucina G.; Perinelli D.R.; Cespi M.; Petrelli R.; Cappellacci L.; Fiorini D.; Scortichini s.; Garzoli S.; Angeloni C.; **Freschi M**.; Hrelia S.; Quassinti L.; Bramucci M.; Lupidi G.; Sut S.; Dall'Acqua S.; Benelli G.; Drenaggi E.; Maggi F.

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6.1 INTRODUCTION

Acmella oleracea (L.) R.K. Jansen (syn. Spilanthes acmella (L.) L.) is an aromatic and medicinal plant native to subtropical areas such as Peru and Brazil, which belongs to the family of Compositae (Asteraceae). To date, it is spread worldwide and is easily founded in areas such as Central America (Mexico), Asia (China, India and Taiwan) and Africa [281].

In Brazil, the leaves of A. oleracea, also known "jambù," are widely used as a spice in the local cuisine for their sialagogue property, i.e., responsible for increased salivation and tingling in the mouth, and for its acrid, pungent flavor [282]–[284].

In many parts of the world, this plant has a thousand-year history as a remedy in traditional folk medicine [283]. Traditionally, in subtropical regions, the various parts of this plant (flowers, leaves and stem), for their analgesic effect, have been chewed to treat toothache, gingivitis, sore throat and stomatitis [282]. In addition to “jambù”, it is also known as “paracress” or “toothache plant”, precisely in association with its well-known use in the treatment of tooth pain [285]. In fact, in traditional medicine, the flowers of this plant are chewed to promote sensations of numbness, tingling, and local anesthesia in the mouth [281]. The alkylamides present in the plant, among which spilanthol is the principal, are primarily responsible for these chemesthetic sensations in the oral mucosa [284]. In addition, A. oleracea has been widely used as an antiviral, analgesic, anesthetic, aphrodisiac, antiparasitic, antiseptic, fungicide, diuretic, and for wound healing [281], [286]–[289]. Therefore, this plant can be used for many applications in horticulture and in the fight against arthropods, but also in the pharmaceutical and cosmetic fields. EFSA (European Food Safety Authority) [290] has classified it as safe. As it has also been included in the BELFRIT list [291], it is a plant that has been validated by the Italian Ministry of Health as a component of dietary supplements [292]. A wide range of chemical compounds such as essential oils, phytosterols and lipophilic alkylamides are present in A. oleracea [283]. However, it has been shown that the major contributor to the main biological properties of A. oleracea is (2E,6Z,8E)-N-isobutyl-2,6,8-decatrienamide, the main secondary metabolite of this plant also known as spilanthol [286]. Spilanthol, a compound chemically belonging to the class of alkylamides, has been associated with several biological activities such as antimicrobial, antioxidant, antinociceptive, anti-inflammatory and insecticidal [282], [287], [293]–[297]. Therefore, this molecule can be widely used in industry. From 2005 to 2007, the use of spilanthol for various purposes grew exponentially, until 2014 when a large number of patents related to its use in the food, cosmetic and pharmaceutical industries were drafted [298]. As stated by EFSA in 2015, spilanthol can be taken as a flavoring agent in the amount of 24 mg/capita/day [292]. From leaves, inflorescences, and stems, an essential oil (EO) can be obtained through hydrodistillation (HD). Although this extraction technique is widely used, it has some disadvantages including very

long times, large consumption of energy and water, and frequently loss of thermosensitive compounds [299]. Therefore, researchers tried to develop novel extraction techniques that could be green, solvent-free, and most importantly, capable of increasing the yield of volatile organic compounds (VOCs) from different matrices [300]. Therefore, an extraction procedure, known as microwave-assisted extraction (MAE), was developed, which relies on the vibration of water and polar molecules within matrix cells using microwave radiation. This technique allows to obtain higher temperatures resulting in the breakdown of cell membranes through the vaporization of water and finally the release of VOCs with higher yields and lower costs [299]. Selecting the optimal extraction conditions allows to influence and modulate the chemical profile of the resulting EO [301]. EO from A. oleracea is receiving increasing attention for the variety of biological properties associated with it, and recently, its chemical composition and biological activities have been evaluated [302]. Unfortunately, the poor stability of the EO active compounds, make the development of new technological formulations necessary, such as micro and nano formulations that can increase efficacy and stability [303]–[306].

In this context, the aim of this project was to evaluate the potential anti-inflammatory effect of Acmella oleracea essential oil (EO), pure spilanthol and a nanoemulsion of EO (NE-EO), developed to increase its bioavailability, in an in vitro model of neuroinflammation, the LPS-activated microglial BV-2 cell line.
The essential oil was obtained by microwave-assisted extraction (MAE) and was characterized by the research group of Prof. Filippo Maggi of the University of Camerino (Figure 35).

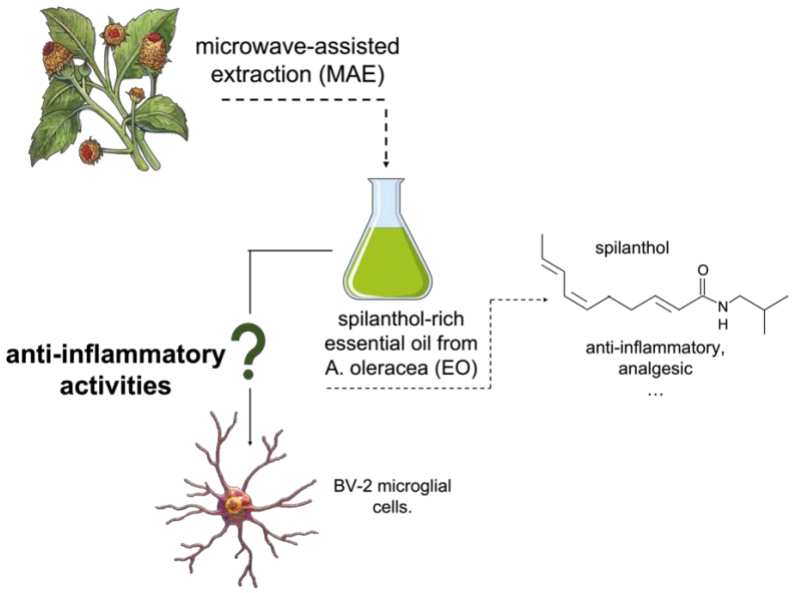

Figure 35. Aim of the study.

6.2 MATERIALS AND METHODS

6.2.1 Chemicals

Dulbecco's modified Eagle's medium (DMEM), penicillin/streptomycin, L-glutamine solution, trypsin-EDTA solution, phosphate buffered saline (PBS), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA), LPS from Escherichia coli serotype O127:B8, primers for real-time polymerase chain reaction (RT-PCR), dimethyl sulfoxide (DMSO) and all other chemicals of the highest analytical grade were produced by Merck Italia (Milan, Italy). Low-endotoxin Fetal bovine serum (low-endotoxin FBS) was purchased from Euroclone (Milan, Italy). RNeasy Mini Kit was from Qiagen (Hilden, Germany). Synthesis Kit, SsoAdvanced Universal SYBR Green Supermix Kit were purchased from Bio-Rad (Hercules, USA).

6.2.2 Plant materials

Plants of Acmella oleracea var. oleracea were transplanted in the 2nd decade of May 2019 in the flat soil of the medium-fine section of the Musone River bed, in the Municipality of Castelfidardo (AN) at 38 m above sea level. The plantation was south-facing, in a sandy-loamy soil and characterized by a submediterranean-low mesotemperate climate. Organic, enzymatic and microbiological fertilizers, as well as water-soluble organic NPK fertilizers were used for fertilization. Irrigation was carried out with a periodic cadence every 1-2 days in the presence of water stress. Planting was protected by removal of floral biomass. Harvesting was done at 70% of flowering (when flower color turned red) and in a manual manner by cutting flower stems and collecting limited leaf biomass. Harvesting had occurred every 10-14 days during September through October 2019. The harvested plant material was dried for approximately 5-6 days in an open environment (hangar) with protection from solar irradiation and in an open environment without solar protection (greenhouse with plastic covering) [286].

6.2.3 Microwave-assisted extraction (MAE) and hydrodistillation (HD)

A Milestone ETHOS X microwave extraction system (Milestone, Italy) was used to carried out MAE. Extraction was done at 1800 W for 160 min using the "Fragrances set-up" on the plant material (1.750 kg) previously allowed to thaw in the 12 L glass reactor (Pyrex) for 30 min according to the manufacturer's instructions. Subsequently, the OE was stored at 4°C after being properly separated from water and collected in a vial with a PTFE-silicon septum. The yield had been 0.47% (w/w dw) and the percentage of moisture in the biomass was calculated by placing the plant in an oven at 110 ◦C for 24 hours [286].

On the other hand, 2 kg of fresh plant material distilled with 12 L of distilled water was used for the isolation of OE through hydrodistillation (HD). The hydrodistillation was carried out with a Clevenger-type apparatus for 4 h. A Falc MA mantle system (Falc Instruments, Treviglio, Italy) was used to heat the distillation system. After extraction, Na_2SO_4 was used to dehydrate the EO. After that, the dehydrated EO was stored in a PTFE/silicon cap vial at 4 ◦C. The yield of isolated EO was 0.22% (w/w dw). The moisture content was calculated by placing the plant in an oven at 110 ◦C for 24 hours [286].

6.2.4 Preparation of A. oleracea hexane extract (HE)

Dried and crushed flowers, stems, and leaves of A. oleracea were extracted with n-hexane (1:10 w/v) employing an ultrasonic bath (Analogic Ultrasonic Bath Mod. AU-220, ARGOLAB, Carpi, Italy) for 3 hours at 40 ◦C. The extract was then filtered first on cotton and then under vacuum using a Buchner funnel fitted with filter paper. Next, a rotary evaporator (Buchi Rotavapor R- 200, Büchi Labortechnik AG, Flawil, Switzerland) was used to dryness the solution while checking that the bath temperature was maintained below 40 ◦C. The resulting residue was dissolved in n-hexane (1:4 w/v). To this was added 50% (w/w) activated carbon and then placed in a hot bath at 30 ◦ C for one hour maintaining constant stirring. Finally, the mixture was filtered over Celite®, evaporated to dryness, and stored at 4 ◦ C until use [286].

6.2.5 Spilanthol isolation and characterization

1 g of the extract obtained from the dried leaves of A. oleracea was purified to obtain pure spilanthol through chromatography with a 90:10-80:20 hexane/ethyl acetate gradient solvent system on a silica gel flash column (230-400 mesh, Merck).

The purity of the obtained spilanthol was performed by HPLC-DAD analysis using different wavelengths (210, 220, 230, 240, 250, 270 nm). The registered purity was 100% purity at 270 nm and 96.60% purity at 210 nm [286].

6.2.6 Gas chromatography-mass spectrometry (GC-MS) analysis of A. oleracea EO

A gas chromatograph coupled to a mass spectrometer detector (GC-MS, Agilent 6890 N equipped with a single quadrupole detector 5973 N) was employed to perform chemical analysis of the two EOs obtained from A (Table 4). oleracea by MAE and HD. The analysis conditions and the identification of the chemical constituents of the EO were in accordance with the work of Benelli et al. [302].

No.	Component[a]	RI[b]	RI Lit.[c]	Relative peak area (%)		ID[d]
				HD	MAE	
1	(2*E*)-hexenal	847	846	–	0.1	RI,MS
2	*n*-hexanol	864	863	–	tr[e]	RI,MS
3	*α*-thujene	921	924	0.1	tr	RI,MS
4	*α*–pinene	926	932	2.1	1.7	Std,RI,MS
5	Camphene	939	946	Tr	tr	Std,RI,MS
6	sabinene	965	969	0.7	1.5	Std,RI,MS
7	*β*-pinene	967	974	14.7	10.8	Std,RI,MS
8	myrcene	989	988	17.4	12.3	Std,RI,MS
9	*p*-mentha-1(7),8-diene	1001	1004	0.3	0.2	RI,MS
10	*α*-terpinene	1013	1014	0.2	tr	Std,RI,MS
11	*p*-cymene	1021	1020	0.2	tr	Std,RI,MS
12	*β*-phellandrene	1024	1025	6.5	4.9	RI,MS
13	(*Z*)-*β*-ocimene	1036	1032	6.0	3.8	Std,RI,MS
14	(*E*)-*β*-ocimene	1046	1044	0.1	tr	Std,RI,MS
15	*γ*-terpinene	1054	1054	0.3	tr	Std,RI,MS
16	terpinolene	1084	1086	Tr	0.1	Std,RI,MS
17	allo-ocimene	1128	1128	0.3	0.1	RI,MS
18	terpinen-4-ol	1171	1174	0.5	tr	Std, RI,MS
19	*δ*-elemene	1330	1335	0.3	0.8	RI,MS
20	*α*-copaene	1366	1374	0.1	0.2	RI,MS
21	*β*-elemene	1384	1389	0.3	0.2	RI,MS
22	cyperene	1386	1398	Tr	0.2	RI,MS
23	(*Z*)-caryophyllene	1396	1408	0.4	–	RI,MS
24	(*E*)-caryophyllene	1408	1417	19.4	19.4	Std,RI,MS
25	*β*-copaene	1418	1430	0.1	0.1	RI,MS
26	*γ*-elemene	1426	1434	0.3	0.1	RI,MS
27	*α*-humulene	1441	1452	1.3	1.1	Std, RI,MS
28	germacrene D	1470	1484	11.8	15.2	RI,MS
29	bicyclogermacrene	1486	1500	–	0.3	RI,MS
30	1-pentadecene	1492	1493	–	5.5	RI,MS
31	(*Z*,*E*)-*α*-farnesene	1493	1491	2.1	2.7	RI,MS
32	*δ*-amorphene	1498	1511	0.1	–	RI,MS
33	*γ*-cadinene	1503	1513	0.3	tr	RI,MS
34	(*E*,*E*)-*α*-farnesene	1505	1505	0.6	0.4	RI,MS
35	*δ*-cadinene	1515	1522	1.1	0.2	RI,MS
36	kessane	1520	1529	1.4	0.5	RI,MS
37	germacrene B	1543	1559	0.4	0.2	RI,MS

38	(*E*)-nerolidol	1560	1561	0.7	0.2	Std,RI,MS
39	caryophyllene oxide	1569	1583	0.9	0.3	Std,RI,MS
40	(*Z*,*Z*)-1,8,11-heptadecatriene	1660	1664	–	0.2	RIMS
41	spilanthol	1887	1888[f]	2.3	11.7	MS[g]
42	acmellonate	1997	–	2.1	2.5	MS[h]
43	*n*-tricosane	2297	2300	Tr	0.2	RI,MS
44	*n*-pentacosane	2497	2500	Tr	0.1	RI,MS
	Oil yield (%, w/w)			0.22	0.47	
	Total identified (%)			95.3	97.8	
	Grouped compounds (%)					
	Monoterpene hydrocarbons			48.8	35.5	
	Oxygenated monoterpenes			0.5	tr	
	Sesquiterpene hydrocarbons			38.6	41.2	
	Oxygenated sesquiterpenes			2.9	0.9	
	Alkylamides			2.3	11.7	
	Others			2.2	8.6	

Table 4. Chemical composition of the Acmella oleracea EOs obtained by HD and MAE [286].

6.2.7 Quantification of the Acmella oleracea EO marker compounds by GC-FID analysis

A GC coupled to flame ionization detector (FID) was used to quantify α-pinene, β-pinene, myrcene, 1,8-cineole, (E)-β-ocimene, terpinolene, (E)-caryophyllene, α-humulene, germacrene D, caryophyllene oxide and spilanthol in OEs obtained from HD and MAE along with the extract. An Agilent 6850 GC series GC-FID was used for the analysis (Figure 36). A solution (0.5 µL) of EO (50 µL) in analytical grade n-hexane (5mL) was injected into the GC. Instead, for the analysis of the extract, preliminary trans-methylation was performed. The extract (15 mg) was dissolved in 1 mL of analytical-grade n-hexane, then a 2 N KOH solution in methanol (0.1 mL) was added and the resulting solution was stirred vigorously for 2 min using a vortex. 1.5 mL of a saturated brine was used to quench the reaction. The resulting mixture was stirred for 2 min and then centrifuged for 5 min (5000 rpm). Each sample was analyzed in duplicate by recovering the organic phase and injecting 0.5 µL of it. Carrier gas (hydrogen), flowing at 3.7 mL/min, was produced by a PGH2-250 generator (DBS Analytical Instruments, Vigonza, Italy). The injector was set at 300 ◦ C in split mode (1:30). The analytical conditions for the separation and quantification of the main constituents of EO are in line with previously reported by Fiorini et al. [301], with the addition of spilanthol analysis.

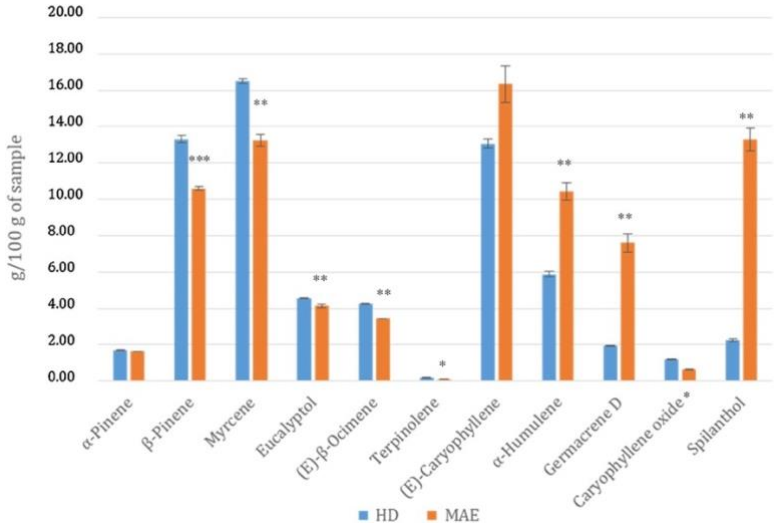

Figure 36. Quantification by GC-FID analysis of the marker compounds of Acmella oleracea EO obtained by HD (blue) and MAE (orange). Data were analyzed by one-way ANOVA, highlighting various degrees of significance ($p < 0.05$, ** $p < 0.01$, *** $p < 0.001$) between HD and MAE [286].*

6.2.8 High performance liquid chromatography-mass spectrometry (HPLC-MS) analysis of A. oleracea HE.

Analysis of alkylamides by LC-MS (Table 5) was performed by dissolving the extract in methanol (10 mg/10 mL) with 10 min of sonication, centrifuging at 13,000 g for 10 min and the resulting supernatant was used for the analysis. The stationary phase consisted of a Phenomenex RP-MAX 150 × 3.0 mm column (particle size 4 μm), while the mobile phases was made up of acetonitrile (A) and 0.1% formic acid water (B), with the following gradient: min0 (15%A,85%B); min33 (85%A,15%B); min33.5 (15%A,85% B) with 4 min of re-equilibration time. The injection flow was 0.4 mL/min and the injection volume was 10 μL. The MS 500 ion Trap was used in positive ion mode to identify the alkylamides in a range of 100-700 Da in TDDS (Turbo Detection Data Scanning) mode allowing acquisition of the fragmentation path of each ion. Fragments of ion were compared to those reported by Boonen et al. [307] and each peak was assigned. The relative area of the peak was used to calculate the amount of each compound [286].

$[M+H]^+$	RT	Identification	%
204	21	(2*Z*)-*N*-isobutyl-2-nonene-6,8-diynamide	0.18
232	23.9	(2*E*)-*N*-isobutyl-2-undecene-8,10-diynamide	0.43
222	25	(2*E*,6*Z*,8*E*)-*N*-isobutyl-2,6,8-decatrienamide (spilanthol)	42.67
246	25.8	(2*E*)-*N*-(2-methylbutyl)-2-undecene-8,10-diynamide	0.11
258	26.1	(2*E*,7*Z*)-*N*-isobutyl-2,7-tridecadiene-10,12-diynamide	0.43
224	27.1	(2*E*,7*Z*)-*N*-isobutyl-2,7-decadienamide	0.89
236	27.1	(2*E*,6*Z*,8*E*)-*N*-(2-methylbutyl)-2,6,8-decatrienamide	6.10
248	28	(2*E*,4*E*,8*Z*,10*Z*)-*N*-isobutyl-dodeca-2,4,8,10-tetraenamide	0.09

Table 5. Identification and quantification of alkylamide derivatives in Acmella oleracea HE. Retention time (RT) and [M+H]+ for each compound were reported [286].

6.2.9 Encapsulation of the A. oleracea EO in nanoemulsion (NE-EO)

A high-energy method was used to obtain A. oleracea EO NE. To an aqueous solution of polysorbate 80 (P80, Sigma-Aldrich), ethyl oleate and EO (in a ratio of 0.5) were added drop by drop under high-speed stirring (Ultraturrax T25 basic, IKAfi Werke GmbH & Co.KG, Staufen, Germany) for 5 min at 9500 rpm. The resulting emulsion was homogenized using a French pressure cell press (American Instrument Company, AMINCO, MY, USA) with a pressure of 130 MPa four times. An MT9000 polarizing optical microscope (Meiji Techno Co Ltd., Japan) equipped with a CMOS camera (Invenio 3S, DeltaPix, Denmark) at 10X magnification was used for visual inspection of the formulation and assessment of NE formation, while a Zetasizer nanoS (Malvern Instruments, Worcestershire, UK) coupled with a backscattered light detector operating at 173 was exploited for measurement of internal phase droplet size. 1 mL of the formulation was analyzed 3 times at 25 °C using an equilibration time of 180 s [308], [309](Benelli et al., 2020b; Kavallieratos et al., 2021). To assess the room temperature physical stability of the formulations, an optical microscope observation was performed and DLS analysis were carried out at different intervals (i.e. 0, 15, 30, 90 and 360 days, T0, T15, T30, T90, T360, respectively).

6.2.10 Cell Culture

Murine microglial cells (BV-2) were provided by Prof. Elisabetta Blasi (University of Modena and Reggio Emilia, Italy) and cultured in DMEM supplemented with 10% (*v*/*v*) of low-endotoxin FBS, 2 mM of L-glutamine, 50 U/mL of penicillin, and 50 µg/mL of streptomycin and maintained at 37 °C in a humidified incubator with 5% CO_2 as previously reported [211].

6.2.11 Viability Assay

Cells were treated for 24 h with different concentrations of *A. oleracea* EO, spilanthol and *A. oleracea* EO-NE in DMEM supplemented with 10% (*v*/*v*) of low-endotoxin FBS, subsequently activated with 100 ng/mL LPS for another 24 h in DMEM supplemented with 1% (*v*/*v*) of low-endotoxin FBS and then cell viability was evaluated by MTT assay as previously reported [257]. At the end of the experiments, cells were incubated with MTT work solution (0.5 mg/mL) for 30 min at 37 °C. After this period, MTT solutions were replaced with 100 µL of DMSO to dissolve the formed formazan crystals and the absorbance was measured at a wavelength of 595 nm using a multilabel plate spectrophotometer (VICTOR3 V Multilabel Counter; Perkin-Elmer,). Cell viability was expressed as % of control cells.

6.2.12 Determination of Intracellular ROS levels

Intracellular ROS levels were evaluated using the fluorescent DCFH-DA probe [258] in BV-2 cells. Cells were treated with 50 μg/mL of EO or 10 μg/mL of spilanthol for 24 h (DMEM 10% low-endotoxin FBS w/o phenol red), then exposed to LPS 100 ng/mL for 24 h (DMEM 1% low-endotoxin FBS w/o phenol red).

After all treatments BV-2 cells were incubated with 10 μM DCFH-DA (DMEM 1% low-endotoxin FBS without phenol red) for 30 min. After incubation period DCFH-DA solution was replaced with PBS. Cell fluorescence was measured at 485 nm (excitation) and 535 nm (emission) with a microplate spectrofluorometer (VICTOR3 V Multilabel Counter; Perkin-Elmer). Intracellular ROS levels were expressed as % of LPS treated cells.

6.2.13 Real-Time polymerase chain reaction (PCR)

Cells were treated with 50 μg/mL of EO or 10 μg/mL of spilanthol for 24 h (DMEM 10% low-endotoxin FBS w/o phenol red), then exposed to LPS 100 ng/mL for 24 h (DMEM 1% low-endotoxin FBS w/o phenol red). After treatment, total RNA was extracted using a RNeasy Mini Kit (Qiagen), following the manufacturer's protocol. The yield and purity of RNA were measured using a NanoVue spectrophotometer (GE Healthcare).

1 μg of total RNA was reverse-transcribed into cDNA using an iScript cDNA Synthesis Kit (BIO-RAD, Hercules, CA, USA), following the manufacturer's protocol. The subsequent PCR was carried out in a total volume of 10 μL constituted as follows: 2.5 μL (12.5 ng) of cDNA, 5 μL SsoAdvanced Universal SYBR Green Supermix (BIO-RAD), and 0.5 μL (500 nM) of each primer.

Primers used are below (Table 6):

PRIMER	SEQUENCE (5'-3')
GAPDH_M_F	ACCACAGTCCATGCCATCAC
GAPDH_M_R	TCCACCACCCTGTTGCTGTA
IL-1β_M_F	GTTCCCATTAGACAACTGCACTACAG
IL-1β_M_R	GTCGTTGCTTGGTTCTCCTTGTA
TNFα_M_F	CCCCAAAGGGATGAGAAGTTC
TNFα_M_R	CCTCCACTTGGTGGTTTGCT
iNOS_M_F	CCTCCTCCACCCTACCAAGT
iNOS_M_R	CACCCAAAGTGCTTCAGTCA
COX2_M_F	TGGGGTGATGAGCAACTATT
COX2_M_R	AAGGAGCTCTGGGTCAAACT

Table 6. Primers for RT-PCR.

GAPDH was used as a reference gene. Amplification of cDNA was initiated by activating the polymerase for 30 s at 95 ˚C, followed by 40 cycles of 5 s at 95 ˚C and 30 s at 60 ˚C. A melt curve

was run to ensure quality control and generation of a single product. Normalized expression levels were calculated with respect to control cells according to the $2^{-\Delta\Delta CT}$ method [260].

6.2.14 Statistical Analysis

Each experiment was performed at least three times, and all values are represented as mean ± SEM. One-way analysis of variance (ANOVA) was used to compare differences among groups, followed by Bonferroni's test (Prism 7; GraphPad Prism Software, San Diego, CA, USA). Values of $p < 0.05$ were considered statistically significant.

6.3 RESULTS

6.3.1 Cytotoxic activity of Acmella oleracea (L.) EO and spilanthol on BV-2 murine microglial cells.

First aim of this study was to investigate the cytotoxicity of EO and spilanthol on BV-2 murine microglial cells. Cells were treated for 24 h with different concentrations of *A. oleracea* EO (0.5− 500 µg/mL) and spilanthol (10− 200 µM), subsequently cell viability was evaluated by MTT assay as reported in section 6.2.11.

EO showed no cytotoxicity up to 50 µg/mL (Figure 37A), whereas spilanthol was not toxic at any of the tested concentrations (Figure 37B).

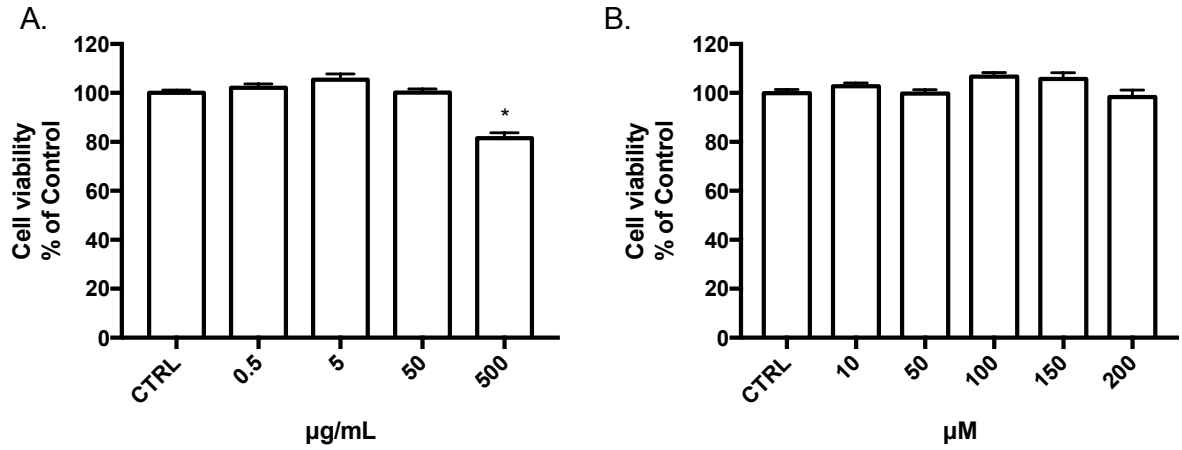

Figure 37. Cytotoxicity of Acmella oleracea EO and spilanthol in BV-2 cells. Cells were treated with (A) 0.5–500 µg/mL essential oil and (B) 10-200 µM spilanthol for 24 h. Cell viability was measured by MTT assay. Each bar represents the mean ± SEM of at least four independent experiments. Data were analyzed by one-way ANOVA followed by Dunnett's test.

**p<0.05 compared to control (CTRL).*

6.3.2 Anti-inflammatory effect of Acmella oleracea (L.) EO and spilanthol on cell viability and intracellular levels of ROS.

BV-2 cells were treated with different concentration of EO (0.005− 50 µg/mL) and spilanthol (10− 150 µM) for 24 h before activation for further 24 h with LPS (100 ng/mL) to evaluate their ability to counteract inflammation. LPS is an inflammatory agent that is commonly used, in vitro, to trigger the pro-inflammatory pathway in microglial cells [310], [311]. As expected, LPS significantly reduced cell viability in respect to control cells. The treatment with EO at 0.5-50 µg/mL was able to significantly improve cell viability compared to LPS-treated cells. In particular, the concentration 50 µg/mL of EO was associated with the highest capacity in counteracting LPS-induced damage (Figure 38A). Likewise, spilanthol countered LPS-induced inflammation in BV-2 cells. The concentration

10, 50 and 100 µM had led to an increase in cell viability in respect with LPS-treated cells (Figure 38B). 50 µg/mL EO and 10 µM spilanthol were chosen to continue the study.

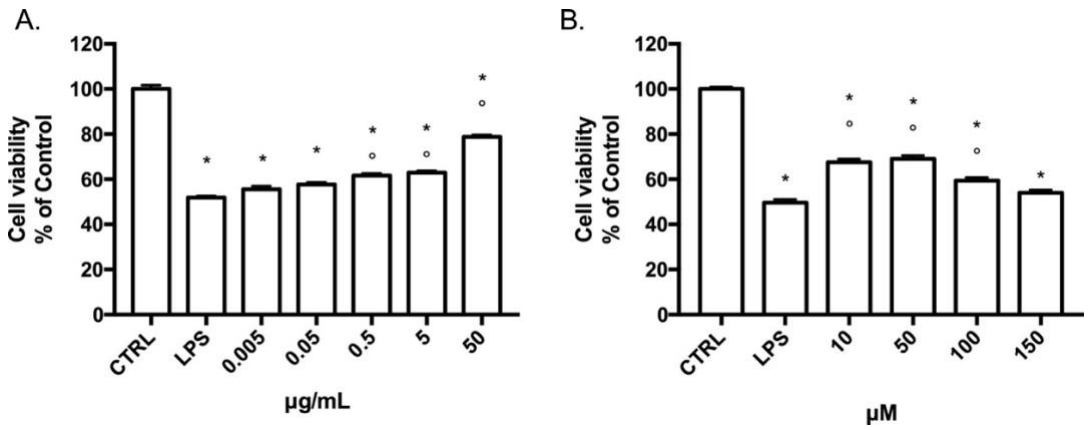

Figure 38. Anti-inflammatory activity of Acmella oleracea OE and spilanthol against LPS in BV-2 cells. Cells were treated with (A) 0.005–50 µg/mL EO and (B) 10-150 µM spilanthol for 24 h before inducing inflammation with LPS 100ng.mL for further 24 h. At the end of experiments, cell viability was measured by MTT assay. Each bar represents the mean ± SEM of at least three independent experiments. Data were analyzed by one-way ANOVA followed by Bonferroni's test.

**p<0.05 compared to control (CTRL), °p<0.05 compared to LPS.*

As reported in several studies, LPS, in addition to stimulating the inflammatory pathway, also causes an increase in ROS production leading to a condition of oxidative stress [312], [313]. To also investigate this aspect, intracellular ROS levels were evaluated through the DCFH-DA assay. Cells were pre-treated with 50 µg/mL of EO or 10 µg/mL of spilanthol for 24 h before activation with 100 ng/mL LPS for 24 h. As shown in Figure 39, interestingly, only EO significantly reduced ROS levels compared to LPS-activated cells. On the other hand, spilanthol had no effect on this parameter.

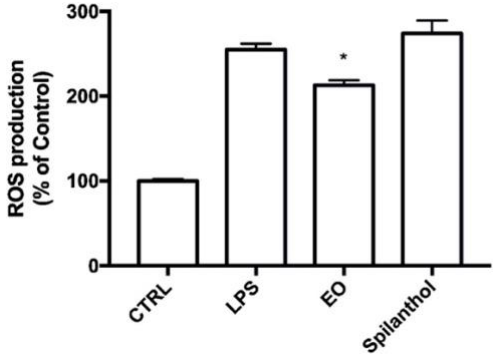

Figure 39. Effect of EO and spilanthol on intracellular ROS levels in LPS-stimulated BV-2 cells. Cells were treated with 50 µg/mL EO and 10 µM spilanthol for 24 h before inducing inflammation with LPS 100 ng/mL for further 24 h. At the end of the experiments, intracellular ROS were measured using the fluorescent probe DCFH-DA. Each bar represents the mean ± SEM of at least three independent experiments. Data were analyzed by one-way ANOVA followed by Bonferroni's test.

**p<0.05 compared to LPS.*

6.3.3 Effect of Acmella oleracea (L.) EO and spilanthol in modulating gene expression of IL-1β, TNF-α, iNOS and COX2.

To better clarify the mechanisms underlying the anti-inflammatory effect of EO and spilanthol, the gene expression of some of the main pro-inflammatory mediators (IL-1β, TNF-α, iNOS and COX2) was evaluated by RT-PCR as reported in section 6.2.13. Cells were pre-treated with 50 µg/mL of EO or 10 µg/mL of spilanthol for 24 h before activation with 100 ng/mL LPS for 24 h.

As shown in Figure 40 the exposure to LPS led to a significant increase of the expression of IL-1β, TNF-α, iNOS and COX2 in BV-2 cells. EO significantly down-regulated the expression of all tested genes. In particular, EO treatment showed a very strong effect on iNOS and COX2. Otherwise, spilanthol determined a significant down-regulation of only IL-1β expression, while had no effect in modulating the expression of other genes.

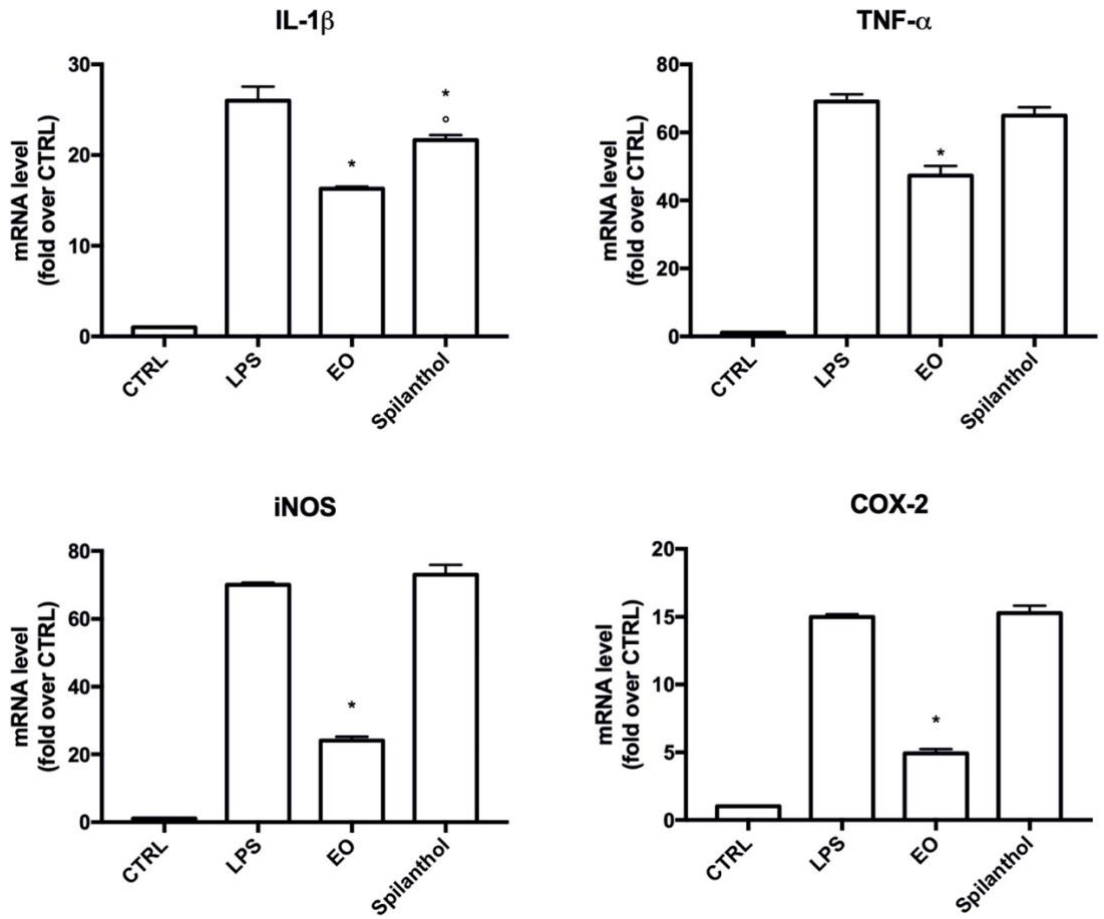

*Figure 40. Anti-inflammatory effect of Acmella oleracea (L.) EO and spilanthol in modulating gene expression of IL-1β, TNF-α, iNOS and COX2. Cells were treated with 50 µg/mL EO and 10 µM spilanthol for 24 h before inducing inflammation with LPS 100ng.mL for further 24 h. At the end of experiments, RT-PCR was carried out. Data are expressed as relative abundance compared to control (CTRL) cells. Each bar represents the mean ± SEM of at least three independent experiments. Data were analyzed by one-way ANOVA followed by Bonferroni's test. °p<0.05 compared to CTR, *p<0.05 compared to LPS.*

6.3.4 Effect of Acmella oleracea (L.) nanoemulsion EO-NE on cell viability of BV-2 microglial cells.

To further investigate the possible uses of Acmella oleracea, the effect of its nanoemulsion (EO-NE) was also studied. The first aspect evaluated was the cytotoxicity profile of EO-NE on BV-2 cells. Cells were treated with 0.001-10 mg/mL EO-NE for 24 h and then cell viability was assessed by MTT assay. EO-NE showed no cytotoxic effect on BV-2 cells up to the concentration 0.1 mg/mL, whereas concentrations 1 and 10 mg/mL significantly reduced cell viability compared to control cells (Figure 41A).

Subsequently, the anti-inflammatory activity of this nanoemulsion was investigated pre-treating cells with different concentration of EO-NE (0.0625-0.5 mg/mL) before LPS exposure. Treatment with EO-NE showed a protective effect against LPS-induced damage, as it increased cell viability by almost 30% at 0.25 mg/mL and 40% at 0.5 mg/mL compared with LPS-activated cells (Figure 41B).

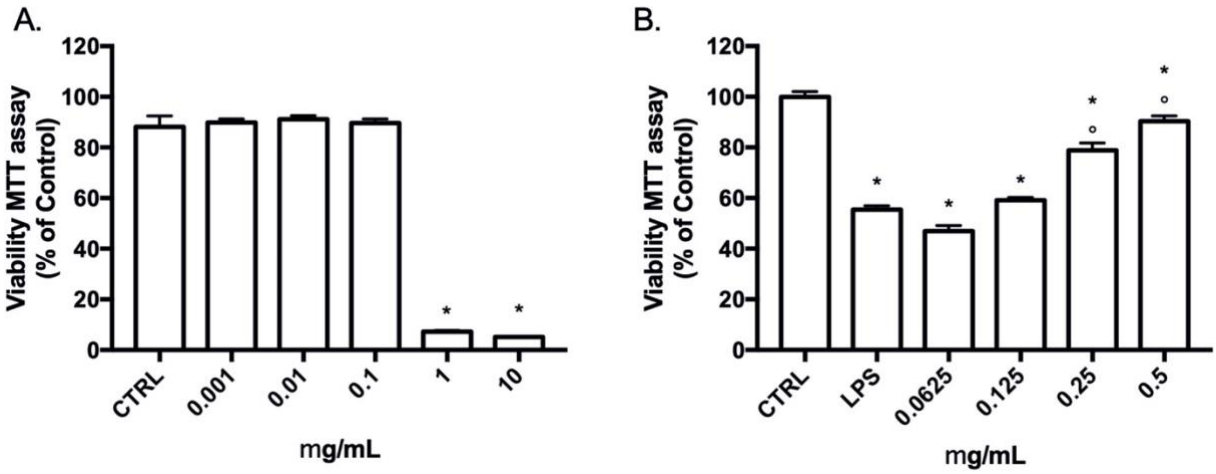

*Figure 41. Effect of Acmella oleracea nanoemulsion (EO-NE) on BV-2 cells. Cells were treated with (A) 0.001-10mg/mL of EO-NE for 24 h, (B) 0.0625-0.5 mg/mL EO-NE then activated with LPS (100ng/mL for 24 h). At the end of each treatment cell viability was measured by MTT assay. Each bar represents the mean ± SEM of at least four independent experiments. Data were analyzed by one-way ANOVA followed by (A) Dunnett's test, (B) Bonferroni's test. °p<0.05 compared to CTR, *p<0.05 compared to LPS.*

6.4 DISCUSSION

Neuroinflammation plays a key role in the development of several diseases, including neurodegenerative diseases [169], [314], brain trauma [315], strokes [316], infections [317] and even mental disorders [155]. A growing body of scientific evidence suggests that microglia, i.e. the cells of the innate immune system that make up 10% of the cells in the central nervous system (CNS), play an active role in the pathophysiological processes associated with these diseases [195]. Under physiological conditions BV-2 cells, brain macrophages, are crucial in protecting the CNS from various exogenous insults [318]. Under pathological conditions, there is an over activation of these cells resulting in increased release of pro-inflammatory mediators such as IL-1β, TNF-α, IL-6, nitric oxide (NO) and ROS [319]. Recently, the involvement of microglia in the onset and progression of neurodegenerative disorders such as Parkinson's disease and Alzheimer's has been widely documented [320]–[322].

In recent years, epidemiological studies have highlighted the role of several plant-derived compounds in preventing neurodegenerative disorders and cognitive decline [323], [324].

Among medicinal plants, Acmella oleracea is a species of flowering plant belonging to the Asteraceae or Compositae family. It is cultivated as an ornamental or medicinal plant and attracts fireflies when in flower. When chewed, the leaves and flowers of Acmella produce a tingling sensation on the lips and tongue. This sensation is caused by the action of spilanthol, an isobutylamide compound that is responsible for the local anaesthetic action. Because of this characteristic, Acmella oleracea is used in traditional medicine for its power against toothache [307]. Several studies, in vitro and in vivo, have shown that spilanthol also has other biological activities including inhibition of prostaglandin synthesis [325], activation of the opioidergic [284], serotonergic and GABAergic systems [326]. In particular, the anti-inflammatory activity of Acmella dried flowers was demonstrated in murine macrophages RAW 264.7 activated with lipopolysaccharide (LPS) [282]. These results suggest that Acmella, thanks also to the presence of spilanthol, could be considered an effective source of bioactive components as part of a preventive strategy to counteract neuroinflammation.

In this context, the aim of this project was to evaluate the potential anti-inflammatory effect of Acmella oleracea essential oil (EO), pure spilanthol and a nanoemulsion of EO (NE-EO), developed to increase its bioavailability, in an in vitro model of neuroinflammation, the LPS-activated microglial BV-2 cells.

First aim of this study was to investigate the cytotoxicity of EO and spilanthol on BV-2 murine microglial cells by MTT assay. EO showed no cytotoxicity up to the concentration 50 μg/mL, whereas spilanthol was not toxic at any of the tested concentrations. Once assessed the cytotoxic profile, the anti-inflammatory potential of EO and spilanthol was evaluated by exposing BV-2 cells to LPS. LPS

is a pro-inflammatory agent widely exploited in research to induce an inflammatory state in vitro, as it triggers the inflammatory cascade in microglia [310], [311]. The treatment with both EO and spilanthol was able to significantly improve cell viability compared to LPS-treated cells. In particular, the concentrations 50 μg/mL of EO and 10 μM of spilanthol were associated with the highest capacity in counteracting LPS-induced damage, so were chosen for the subsequent experiments. As reported in several studies, LPS, in addition to stimulating the inflammatory pathway, also causes an increase in ROS production leading to a condition of oxidative stress [312], [313]. Therefore, this parameter was also evaluated by DCFH-DA assay. Interestingly, only EO significantly reduced ROS levels compared to LPS-activated cells. To better clarify the mechanisms underliying the anti-inflammatory effect of EO and spilanthol, the gene expression of some of the main pro-inflammatory mediators (IL-1β, TNF-α, iNOS and COX2) was evaluated by RT-PCR. EO significantly down-regulated the expression of all tested genes, while spilanthol only reduced the expression of IL-1β. In particular, EO treatment showed a very strong effect on iNOS and COX2. Spilanthol represents the main N-alkylamide present in the aerial parts of A. oleracea and seems to be the main responsible for the anti-inflammatory activity of this plant [282], [325], [327]–[330]. The results obtained in the present study suggest a greater effect of EO than spilanthol in counteracting LPS-induced damage. This could suggest an involvement of other compounds present in the extract in the observed protective effect. Furthermore, as there are no other studies in the literature reporting the effect of A. oleracea on microglia, this suggests the potential action of EO as an anti-neuroinflammatory agent. In a study conducted by Bakondi et al. [282], the ability of spilanthol to inhibit NO production and iNOS expression in RAW macrophages was demonstrated. In the same cell model, spilanthol was also able to downregulate the expression of TNF-α, IL-1, IL-6, iNOS and COX-2 by inhibiting NF-kB activation [330]. The action of spilanthol was also evaluated in other cell models including vascular smooth muscle cells (VSMC) in which an inflammatory state was induced by high glucose levels. In this cellular model of inflammation, spilanthol triggered the upregulation of the antioxidant enzyme catalase and the reduction of NO [329]. In HaCaT keratinocytes, spilanthol counteracted the release of pro-inflammatory molecules (IL-6, IL-8, MCP-1, ICAM-1 and COX-2), inhibited the pJNK-MAPK signaling pathway and up-regulated HO-1 expression [327].

In contrast to what has been documented, in the present study spilanthol was not able to reduce the pro-inflammatory mediators. These discrepancies could be attributed to both the different experimental model employed and the significantly lower concentrations of spilanthol used in the present study (approximately 10-fold less than in the other studies). Furthermore, as described above, the greater effect of EO compared to spilanthol could be associated with the action of other compounds contained in the extract. Indeed, EO is extremely rich in bioactive compounds, in addition

to spilanthol, such as myrcene, β-pinene, germacrene D and (E)-caryophyllene, whose relative anti-inflammatory activities have already been documented. Indeed, Basholli-Salihu et al. [331], demonstrated a reduction in IL-6 secretion in LPS-activated macrophages by three different Pinus EO-rich β-pinene, (E)-caryophyllene and germacrene D. A reduction of inflammation was also observed in guinea pigs treated with different β-pinene-rich EO obtained from leaves and fruits of Schinus polygama (Cav.) Cabrera [332]. Another plant rich in β-pinene, (E)-caryophyllene, myrcene and germacrene D is Eremanso erythropappus (DC.) MacLeish, whose leaf extracts reduced inflammation in rats through the inhibition of carrageenan-induced oedema [333].
Mouse macrophages showed a reduction in the LPS-induced inflammatory state following treatment with EO of Liquidambar formosana Hance, which is rich in β-pinene [334]. (E)-Caryophyllene is also associated with an anti-inflammatory effect as it inhibited cytokines by modulating the arachidonic acid and histamine pathways in a mouse model [335].
To further investigate the possible uses of Acmella oleracea, the effect of its nanoemulsion (EO-NE) was also studied. The first aspect evaluated was the cytotoxicity profile of EO-NE on BV-2 cells. EO-NE showed no cytotoxic effect on BV-2 cells up to the concentration 0.1 mg/mL, whereas concentrations 1 and 10 mg/mL significantly reduced cell viability compared to control cells. Subsequently, the anti-inflammatory activity of this nanoemulsion was investigated exposing cells to LPS. EO-NE showed a protective effect against LPS-induced damage, as it increased cell viability by almost 30% at 0.25 mg/mL and 40% at 0.5 mg/mL. Of note, these data suggest an increase in the bioactivity of EO in the form of nanoemulsion, as the effective concentrations of EO-NE contain 15 and 30 μg/mL of EO, respectively. The observed increase in bioactivity of the nanoemulsion is likely due to an increase in cellular availability of EO when included in this type of technological formulation. In support of this, 1 mg/mL of EO-NE containing 60 μg mL of EO was found to be cytotoxic, whereas the corresponding concentration of EO had shown no toxicity effects.

In conclusion, the data obtained from this study allow to suggest the essential oil of Acmella oleracea, for its anti-inflammatory and antioxidant properties, as a valid strategy to counteract neurodegeneration. Moreover, the nanoemulsion of OA, as a new technological formulation, could be exploited to further increase the potential of this essential oil.

7 Study 3_Study of the anti-inflammatory activity of novel tacrine derivatives with lipids extracted from cashew nutshell liquid

The results of this part have been published on:

Sustainable Drug Discovery of Multi-Target-Directed Ligands for Alzheimer's Disease.

Rossi, M.[§]; **Freschi, M**.[§]; de Camargo Nascente, L.[§]; Salerno, S.; de Melo Viana Teixeira, S.; Nachon, F.; Chantegreil, F.; Soukup, O.; Prchal, L.; Malaguti, M.; Bergamini, C.; Bartolini, M.; Angeloni, C.; Hrelia, S.; Romeiro, LAS.; Bolognesi, ML.

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64(8): 4972–4990.

[§]These authors contributed equally the paper

7.1 INTRODUCTION

Nowadays, dementia is a health problem that mainly affects elderly population and its increasing incidence is closely related to the rising average age of the world population. Research has estimated that the number of people suffering from this type of disorder, of which Alzheimer's disease (AD) is the most common, will grow exponentially. To date, it is estimated that every 3 seconds someone in the world develops dementia and that the incidence of these disorders will increase from 35 million to 135 million by 2050 [336]. Today, 60% of those affected live in low- and middle-income countries and by 2050 this percentage will rise to 71% in these countries [336].

The problem of AD is made even greater by the absence of an effective treatment. Currently the drugs in clinical use (3 acetylcholinesterase inhibitors (AChEI) and memantine) are only able to alleviate symptoms, but not to halt the disease progression.

Unfortunately, very few drug candidates from basic research became novel therapies with a success rate of only 0.4 % (more than 200 drug candidates have not passed the clinical phase) [337]. In the last two decades, only one novel drug out of more than 400 clinical trials has been approved, namely GV-971 which was approved in China in 2019 and is currently only available in China [338].

The increase in the incidence of dementia is a problem that has become even more impactful, especially in more populous countries such as Brazil, India and Indonesia, because it is closely related to the consequent difficulty in fair distribution of the treatments across the affected population. The development of new therapies that are not only effective but also easily accessible is becoming an issue increasingly important and urgent.

Among the causes contributing to the difficulty of finding an effective treatment is the multifactorial nature of neurodegenerative diseases [339] and multi-target treatments, in respect to single target treatments, appear to be associated with a higher success rate [340]–[342]. For this reason, multifunctional drugs are increasingly considered a viable alternative for the treatment of AD [343]–[345] and other neurodegenerative diseases [343].

Currently, it is crucial to develop molecules that are not only effective but also obtained in a sustainable way to protect the environment and to be cheap and accessible for the population and the healthcare systems.

In this context, the aim of this project was to develop novel pharmacological strategies for AD from cashew nutshell liquid (CNSL), an inexpensive and inedible food waste [346], [347]. In particular, this study concerns the evaluation of the anti-inflammatory activity of novel sustainable MTDLs derived from CNSL in an in vitro model of neuroinflammation, the BV-2 microglial cell line (Figure 42).

The compounds investigated in this study were synthesized by the research group of Prof. Bolognesi (University of Bologna) using a rational framework combination approach.

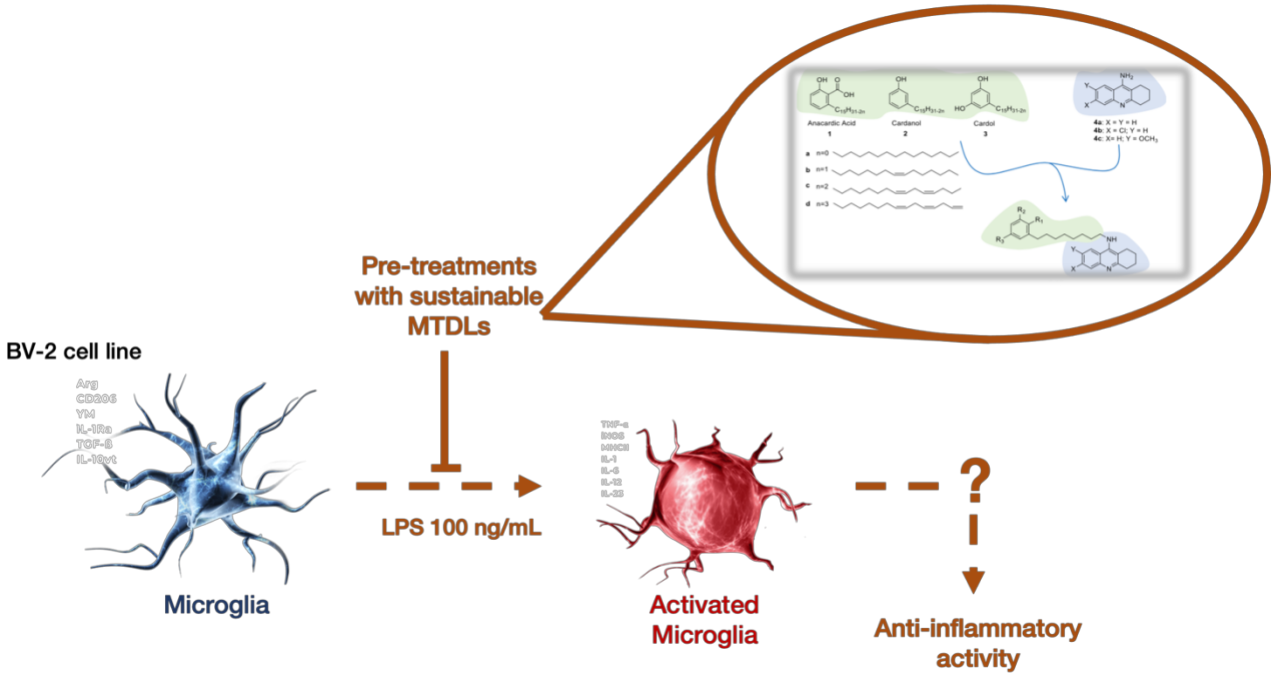


Figure 42. Aim of the study

7.2 MATERIALS AND METHODS

7.2.1 Chemicals

Dulbecco's modified Eagle's medium (DMEM), penicillin/streptomycin, L-glutamine solution, trypsin-EDTA solution, phosphate buffered saline (PBS), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA), all-trans-retinoic acid (RA), LPS from Escherichia coli serotype O127:B8, paraformaldehyde 2%, Triton X-100, 4′-6-diamidino-2- phenylindole (DAPI), primers for real-time polymerase chain reaction (RT-PCR), dimethyl sulfoxide (DMSO) and all other chemicals of the highest analytical grade were produced by Merck Italia (Milan, Italy). Fetal bovine serum (FBS) and Low-endotoxin Fetal bovine serum (low-endotoxin FBS) were purchased from Euroclone (Milan, Italy). RNeasy Mini Kit was from Qiagen (Hilden, Germany). Synthesis Kit, SsoAdvanced Universal SYBR Green Supermix Kit were purchased from Bio-Rad (Hercules, USA).

7.2.2 Extraction of Anacardic acid mixtures (1) from natural CNSL and of mixtures of Cardanols (2) and Cardol (3) from technical CNSL.

To obtain anacardic acid mixtures (1) 30 g of natural CNSL was mixed with a solution of 15 g calcium hydroxide in methanol/water (6:1, 210 mL) and placed under stirring at 60 °C for 3 hours. The resulting mixture was concentrated under vacuum and filtered, and the resulting solid was transferred to a 1 L Erlenmeyer flask into which ethyl acetate (150 mL), distilled water (50 mL), and a 50% HCl solution were added to achieve pH = 1.0. The resulting solution was washed with a saturated solution of sodium chloride (50 mL) and dried over anhydrous Na2SO4. The mixture, after evaporation of the solvent under reduced pressure, was purified by silica gel column chromatography (hexane/ethyl acetate: 0-30%). 16.5 g of the anacardic acid mixture (1), corresponding to approximately 55% of the mass of natural CNSL used, was obtained.

To obtain mixtures of Cardanols (2) and Cardol (3) 20 g of technical CNSL was purified by silica gel column chromatography (hexane/ethyl acetate: 5−35%) to provide 14 g of the mixture of cardanols (2, 70% of the applied mass) and 4.8 g of the mixture of cardols (3, 24% of the applied mass). The technical CNSL was donated from the company Resibras.

7.2.3 Chemical synthesis of compounds 5-17

CNSL constituents 1-3 and tacrine templates 4a-4c were used to obtain the final hybrid compounds 5-17 according to the following design strategy scheme (Figure 43).

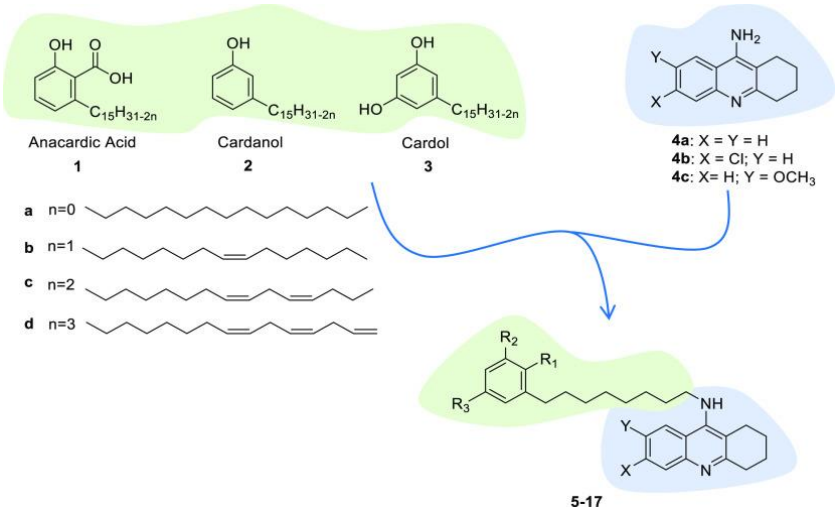


Figure 43. Design strategy toward hybrids 5–17, starting from CNSL constituents 1–3 and tacrine templates 4a–4c [211].

In particular, the chemical synthesis of the compounds 5-17 was carried out obtaining first the CNSL mesylate intermediates 21a-21d[a] and then synthetizing the final hybrids 5-17 as showed in Figure 44 and Figure 45 and described by Rossi M et al. [211].

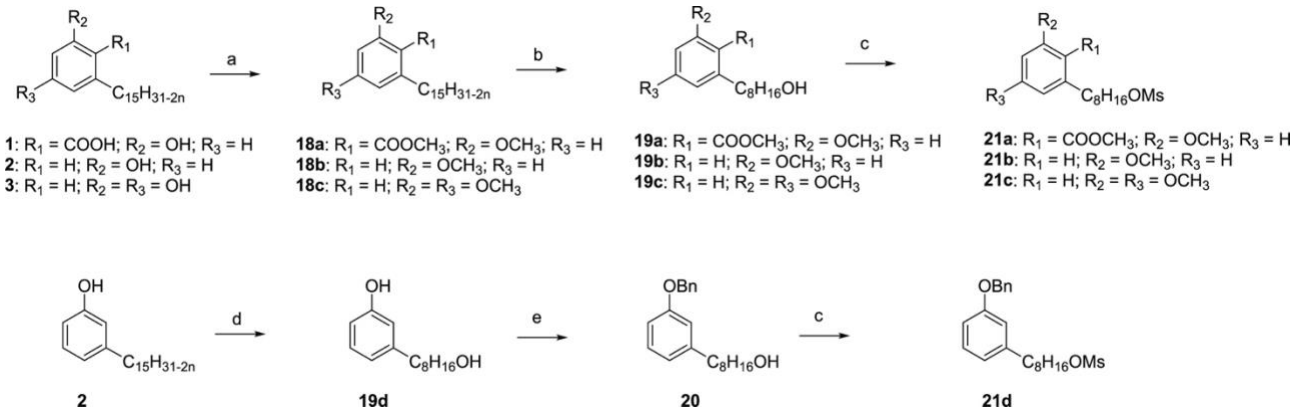


Figure 44. Synthesis of CNSL mesylate intermediates 21a-21d[a]. Reagents and conditions: (a) K_2CO_3 and acetone; MeI, 24h, and 110°C (66-80%); (b) 0_3, DCM/MeOH, and 0°C; $NaBH_4$, 24h, and rt (60-70%); (c) methanesulfonyl chloride, TEA, DCM, 12h, and rt (60-85%); (d) acetic anhydride, MW:450W, and 3 min; O_3, DCM/MeOH, and 0°C; $NaBH_4$, 16h, and rt; HCL conc. (67%); and (e) benzyl bromide, K_2CO_3, acetone, 12h, and reflux (92%).

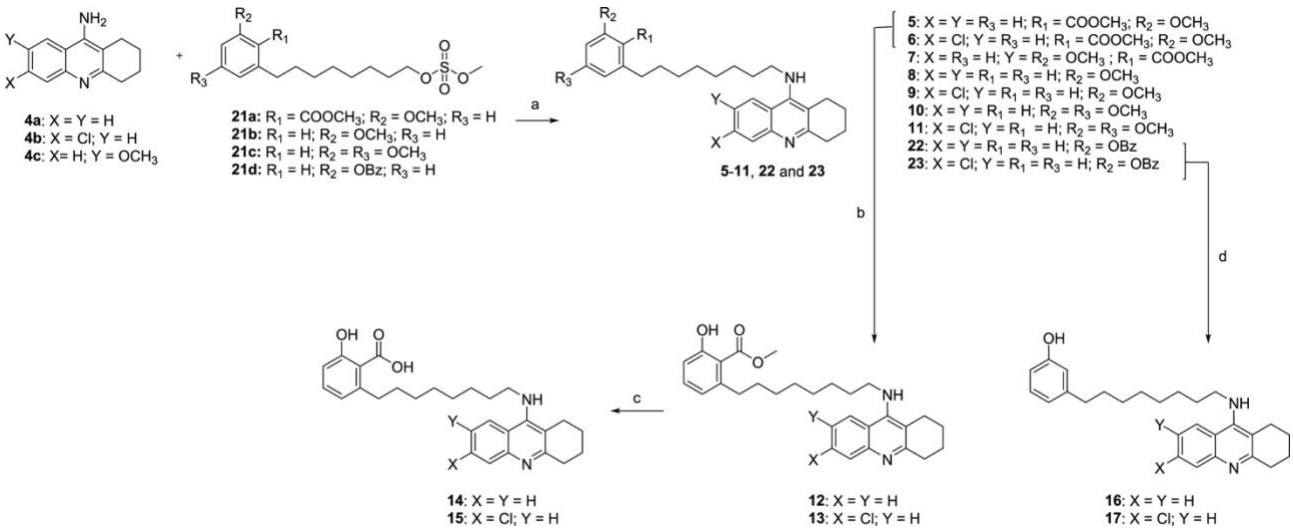
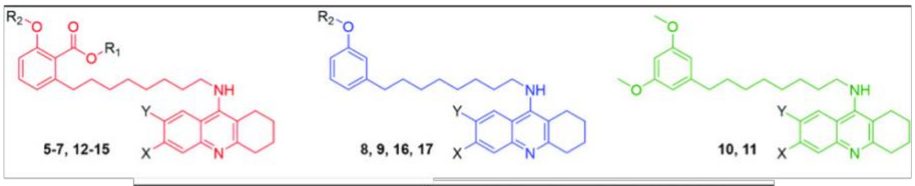


Figure 45. Synthesis of final compounds 5-17. Reagents and conditions: (a) KOH, DMSO, MW:120°C, and 12 min (20-34%); (b) BBr_3, 0°C to rt, DCM, and 40 min (25%); (c) KOH, MeOH/H_2O, MW: 100 °C, and 10 min (54-84%); and (d) H-Cube H_2, 5 bar, and Pd/C 10% (41-45%).

7.2.4 Determination of epatotoxicity of 5-17 on HepG2 cells

HepG2 cells (human hepatocytes from liver cancer, American Type Culture Collection, ATCC) were cultured in DMEM supplemented with 10% FBS and 50 units/mL penicillin/ streptomycin (Life Technologies Italia, Monza, MB, Italy) at 37 °C in a humidified atmosphere containing 5% CO2. HepG2 cells (0.3 × 105 cells/well) were seeded in a 96-well plate in complete medium for the experiments. After 24 h, cells were treated with increasing concentrations (0.1, 1, and 10 μM) of hybrids 5-17, reference compounds, or vehicle for 24 h. MTT assay was used to evaluate cell viability (Figure 46) [211].



Compound					Cytotoxicity in HepG2 Cells *% of viable cells*[a]		
no.	**R_1**	**R_2**	**X**	**Y**	**0.1 µM**	**1 µM**	**10 µM**
5	CH_3	CH_3	H	H	93.4±4.0	95.7±6.8	93.8±2.9
6	CH_3	CH_3	Cl	H	95.4±2.4	95.1±1.8	81.5±2.4**
7	CH_3	CH_3	H	OCH_3	94.7±3.9	92.9±9.2	93.2±1.3
8		CH_3	H	H	89.7±7.2	90.2±1.4	89.6±3.6
9		CH_3	Cl	H	100.0±2.5	93.4±3.6	99.4±5.3
10			H	H	94.4±6.0	85.5±0.5	61.4±3.5***
11			Cl	H	94.9±7.7	92.5±4.9	78.5±2.3***
12	CH_3	H	H	H	95.6±5.6	81.0±1.3	84.2±10.3*
13	CH_3	H	Cl	H	90.3±4.2	86.6±2.4	88.1±5.2
14	H	H	H	H	105.5±0.5	108.2±5.7	100.8±2.5
15	H	H	Cl	H	100.0±5.0	100.0±2.9	92.7±7.6
16		H	H	H	98.8±5.0	86.0±15.9	59.1±3.1***
17		H	Cl	H	91.3±3.6	95.5±3.7	90.0±3.3
1a					95.4±2.2	92.2±2.6	110±18
4a					97.79±6.5	95.0±3.6	82.3±2.8*
4b							
4c							

*Figure 46. Hepatotoxicity in HepG2 of 5-17 and reference compounds 4a-4c and 1a[b]. [a]Results are expressed as the mean of at least three experiments. [b]*Significance was determined by ANOVA, Dunnett's multiple comparison test $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$. Modified by [211].*

7.2.5 Cell Culture

The SH-SY5Y human neuroblastoma cell line (ECACC 94030304) was purchased from Merck (Italy). Cells were grown in DMEM supplemented with 10% (*v*/*v*) of FBS, 2 mM of L-glutamine, 50 U/mL of penicillin, and 50 µg/mL of streptomycin and maintained at 37 °C in a humidified incubator with 5% CO_2 as previously reported [256]. Differentiated SH-SY5Y cells were used for experiments. Cell differentiation was induced by treatment with RA (10 µM) for 7 days (1% FBS). Differentiated SH-SY5Y cells were treated for 24 h with different concentrations of the tested compounds.

Murine microglial cells (BV-2) were provided by Prof. Elisabetta Blasi (University of Modena and Reggio Emilia, Italy) and cultured in DMEM supplemented with 10% (*v*/*v*) of low-endotoxin FBS, 2 mM of L-glutamine, 50 U/mL of penicillin, and 50 µg/mL of streptomycin and maintained at 37 °C

in a humidified incubator with 5% CO_2. BV-2 microglial cells were treated for 24 h with different concentrations of the tested compounds and then activated with LPS (100ng/mL) for another 24 h.

7.2.6 Viability Assay

Cell viability was measured by MTT assay as reported in sections 5.2.7 and 6.2.11. Cell viability was expressed as % of control cells.

7.2.7 Real-Time polymerase chain reaction (PCR)

Total RNA was extracted using a RNeasy Mini Kit (Qiagen), following the manufacturer's protocol. The yield and purity of RNA were measured using a NanoVue spectrophotometer (GE Healthcare, Milan, Italy).

The Real-Time polymerase chain reaction (PCR) was carried out as described in section 6.2.13.

7.2.8 Immunofluorescence confocal microscopy

BV-2 cells were seeded directly on glass coverslips in 6-well plates. After treatments, cells were incubated first with paraformaldehyde 2% 15 min at room temperature to be fixed and then with Triton X-100 0.1% for 10 min to be permeabilized. Once fixed and permeabilized, BV-2 cells were incubated overnight with a polyclonal antibody (1:500) against NF-κB p65 (Sigma-Aldrich− Merck). After PBS extensive washing, cells were exposed to a secondary Alexa Fluor 488-conjugated antirabbit IgG antibody (1:1000) (Life Technologies Italia, Monza, MB, Italy) for 1 h at room temperature. 1 μg/mL 4′-6-diamidino-2- phenylindole (DAPI) was used to stain nuclei. Slides were analyzed with a C2 Plus confocal laser scanning microscope (Nikon Instruments, Firenze, Italy). Images were processed using NIS-Elements imaging software (Nikon Instruments, Firenze, Italy).

7.2.9 IL-1β quantification

At the end of experiments, the culture media were collected to detect IL-1β concentration. IL-1β quantification was carried out using an IL-1β ELISA Kit following the manufacturer's instructions (Sigma-Aldrich− Merck). Absorbance (450 nm) was measured using a microplate spectrophotometer (VICTOR3 V Multilabel Counter).

7.2.10 Statistical Analysis

Each experiment was performed at least three times, and all values are represented as mean ± SEM. One-way analysis of variance (ANOVA) was used to compare differences among groups, followed

by Dunnett's or Tukey's test (Prism 7; GraphPad Prism Software, San Diego, CA, USA). Values of $p < 0.05$ were considered statistically significant.

7.3 RESULTS

7.3.1 Cytotoxicity of 5,6,9,12-15 and 17 on neuronal SH-SY5Y and microglial BV-2 cells.

Preliminary analysis carried out by Cristian Bergamini's research group (University of Bologna) using the HEPG2 cell line demonstrated that only compounds **5**,**6**,**9**,**12-15** and **17** were not hepatotoxic. For this reason, only these compounds were investigated in the next experiments. Neurotoxicity of **5**,**6**,**9**,**12-15** and **17** was evaluated in both differentiated SH-SY5Y and BV-2 cells by MTT assay. Differentiated SH-SY5Y cells were treated with increasing concentration (0.1-1 μM) of the selected compounds for 24 h. Interestingly, all of the compounds did not reduce cell viability in respect to control cells at both the concentrations tested (Figure 47).

Similarly to the neuronal cell line, BV-2 cells were treated with 0.1-1 μM of **5**,**6**,**9**,**12-15** and **17** for 24 h, and at the end of the treatments MTT assay was carried out. At the lowest concentration (0.1 μM) none of the tested compounds reduced cell viability compared with control cells, whereas at concentration 1 μM only compounds **5**, **6** and **9** resulted in slight, although significant, cytotoxicity (Figure 48). Based on the cytotoxicity results both on SH-SY5Y and BV-2 cells, 0.01 and 0.1 μM were chosen to continue the study.

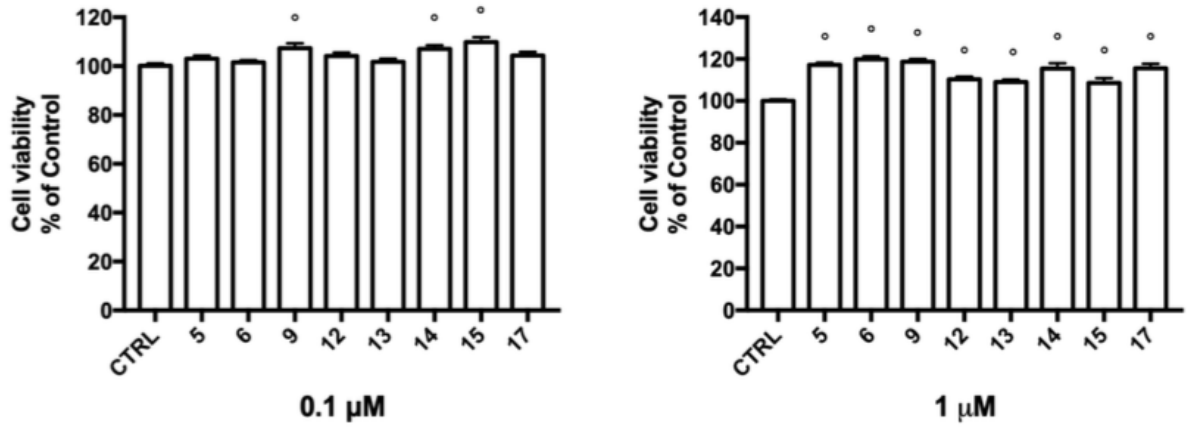


Figure 47. Neurotoxicity of 5,6,9,12-15 and 17 on SH-SY5Y cells. Differentiated SH-SY5Y cells were treated with increasing concentration (0.1-1 μM) of the selected compounds for 24 h. Cell viability was measured by MTT assay. Each bar represents the mean ± SEM of at least four independent experiments. Data were analyzed by one-way ANOVA followed by Dunnett's test.

°p<0.05 compared to control (CTRL).

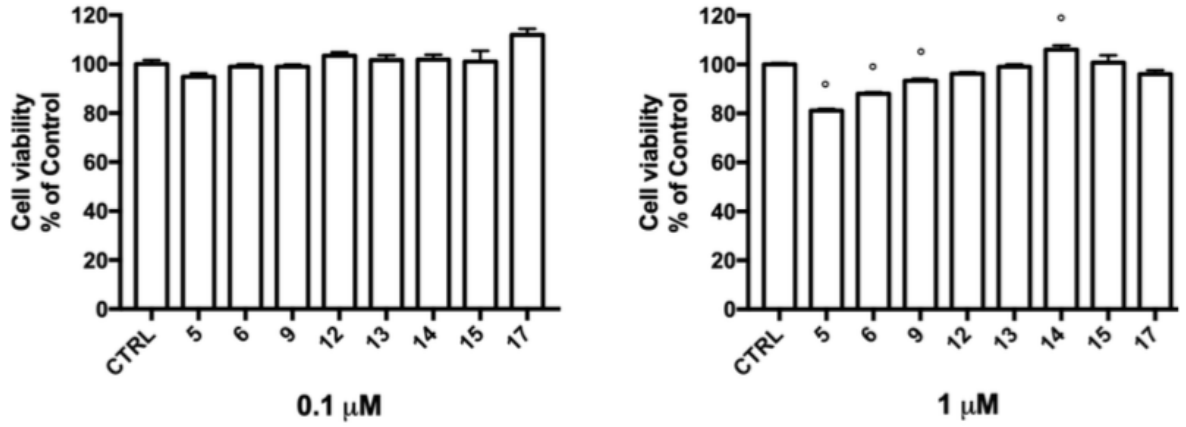


Figure 48.Citotoxicity of 5,6,9,12-15 and 17 on BV-2 murine microglial cells. Cells were treated with increasing concentration (0.1-1 μM) of the selected compounds for 24 h. Cell viability was measured by MTT assay. Cell viability was measured by MTT assay. Each bar represents the mean ± SEM of at least four independent experiments. Data were analyzed by one-way ANOVA followed by Dunnett's test. °p<0.05 compared to control (CTRL).

7.3.2 Effect on cell viability of 5,6,9,12-15 and 17 in microglial BV-2 cells against LPS-induced inflammation.

To evaluate the potential anti-inflammatory activity of the selected compounds, BV-2 cells were pre-treated with **5**,**6**,**9**,**12-15** and **17 (**0.01 and 0.1 μM**)** for 24 h before LPS activation (100 ng/mL) and cell viability was measured by MTT assay. As expected, LPS exposure significantly reduced cell viability in respect to non-treated cells. **13-15** and **17** hybrids had no effect in counteracting LPS-induced damage as cell viability of **13-15** and **17**-treated cells was comparable to cells exposed only to LPS. On the contrary, hybrids **5**, **6**, **9** and **12** significantly counteracted LPS-induced cell death. As shown in Figure 49, **12** exhibited a slightly significant protective effect against LPS already at 0.01 μM, while **9** showed a total protection only at the highest concentration (0.1 μM). Of note, compounds **5** and **6** restored cell viability to values comparable to control cells already at the lowest concentration (0.01 μM).

Compounds **5** and **6** proved to be the most effective ones against LPS-induced damage. For this reason, they were chosen for next experiments.

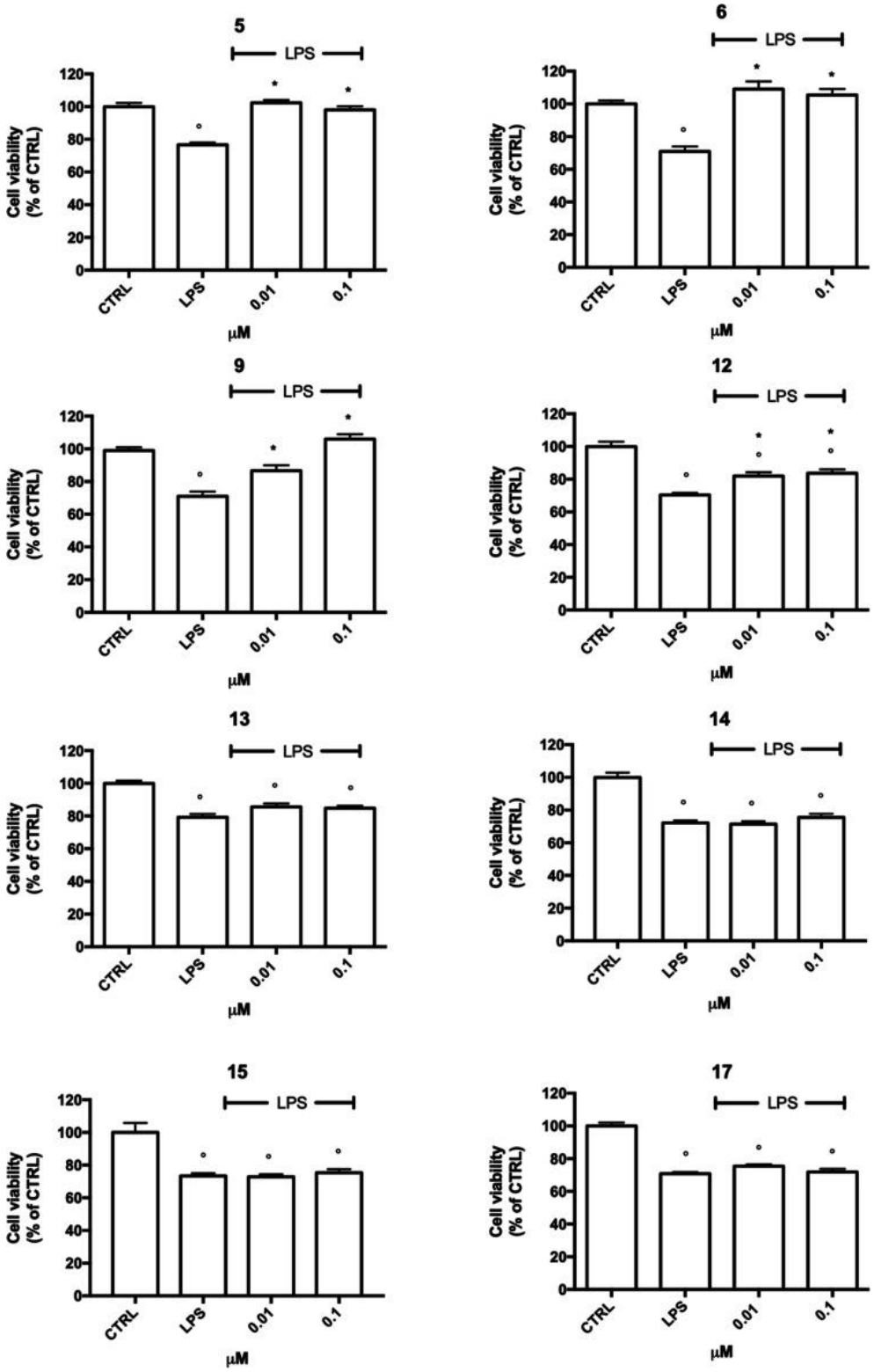


*Figure 49. Anti-inflammatory effects of 5, 6, 9, 12−15, and 17 against LPS in BV-2 cells. Cells were treated with increasing concentration (0.01-0.1 μM) of the selected compounds for 24 h and exposed to LPS 100 ng/mL for further 24 h. Cell viability was measured by MTT assay. Each bar represents the mean ± SEM of at least four independent experiments. Data were analyzed by one-way ANOVA followed by Tukey's test. °p<0.05 compared to control (CTRL), *p<0.05 compared to LPS.*

7.3.3 Anti-inflammatory effect of compounds 5 and 6 in modulating expression of pro-inflammatory mediators.

To study the mechanisms underlining the anti-inflammatory effect of hybrids **5** and **6**, their ability in modulating the expression of some of the main pro-inflammatory mediators (IL-1β, TNF-α, iNOS and COX2) was evaluated. BV-2 cells were treated with 0.1 μM of **5** and **6** for 24 h before LPS activation (100 ng/mL) for further 24 h and then RT-PCR was carried out as described in section 7.2.7. Parent compounds **1a** (chemically resembles salicylic acid, but with an higher anti-inflammatory activity than acetylsalicylic acid in vitro as reported by de Souza et al. [348]) and **4a** (Tacrine) were included in the experimental settings as, respectively, positive and negative controls. As expected, the stimulation with LPS significantly increased the expression of all the tested genes in respect to control cells. Predictably, **4a** did not show any effect, whereas **1a**, **5** and **6** successfully suppressed the transcription of all considered pro-inflammatory cytokines and enzymes. Interestingly, compounds **5** and **6** showed a higher anti-inflammatory activity than **1a**. In particular, **6** significantly downregulated all the tested genes, **5** significantly reduced the expression of IL-1β, iNOS and COX2, while **1a** was able to reduce only the expression of IL-1β and COX2 (Figure 50).

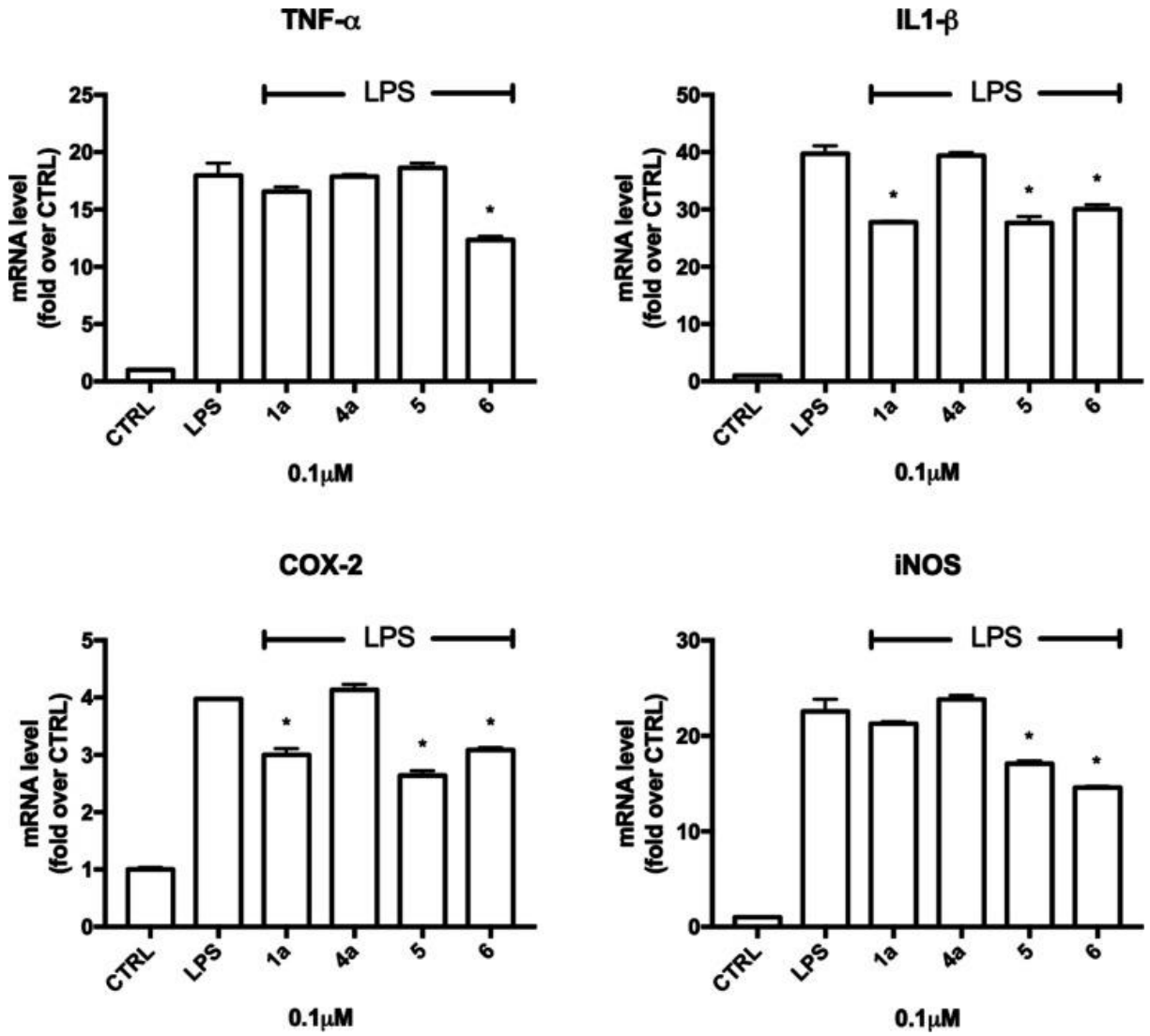


Figure 50. Expression of TNF-α, IL-1β, iNOS, and COX-2 in LPS-activated BV-2 cells treated with 1a, 4a, 5, and 6. Cells were treated with 5, and 6 (0.1 μM) and 1a and 4a as reference compounds (0.1 μM) for 24 h and then exposed to 100 ng/mL LPS for further 24 h. At the end of the experiments, RT- PCR was performed. Data are expressed as relative abundance compared to control (CTRL) cells. Each bar represents the mean ± SEM of at least three independent experiments. Data were analyzed by one-way ANOVA followed by Tukey's test.

**p<0.05 compared to LPS.*

The anti-inflammatory activity of compounds **5** and **6** was also confirmed through the evaluation of the pro-inflammatory cytokines IL-1β secretion by ELISA assay. BV-2 cells were treated with 0.1 μM of **5** and **6** for 24 h before LPS activation (100 ng/mL) for further 24 h and then ELISA assay was performed to detect IL-1β concentration in the culture medium. The pre-treatment with hybrids **5** and **6** significantly reduced the IL-1β release in the culture medium in respect to cells only exposed to LPS (Figure 51).

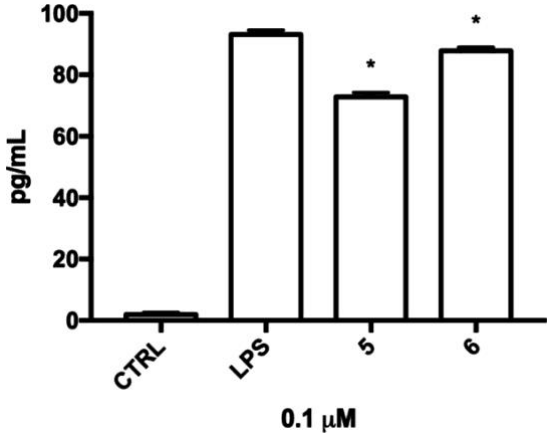


Figure 51. Release of IL-1β in activated BV-2 cells treated with compounds 5 and 6. BV-2 cells were treated with 0.1 μM of 5 and 6 for 24 h before LPS activation (100 ng/mL) for further 24 h and then ELISA assay was performed. Each bar represents the mean ± SEM of at least three independent experiments. Data were analyzed by one-way ANOVA followed by Tukey's test.

**p<0.05 compared to LPS.*

7.3.4 Anti-inflammatory effect of compounds 5 and 6 on nuclear translocation of transcription factor NF-kB in BV-2 cells.

The translocation to the nucleus of NF-kB is another factor that underlines the instauration of the inflammatory process, so the effect of the pre-treatment with compounds **5** and **6** on this parameter was also studied. BV-2 cells were pre-treated with 0.1 μM of **5** and **6** for 24 h, then exposed to 100 ng/mL LPS for further 24 h and, at the end of the treatment, the localization of NF-kB was evaluated by confocal immunofluorescence as described in section 7.2.8. Similarly to RT-PCR experiments, reference compound **1a** and **4a** were included in the experimental settings. As shown in the images, the LPS exposure induced a strong increase in NF- kB levels both in the cytoplasm and in the nucleus. In line with the RT-PCR data, LPS-induced nuclear translocation of NF-kB was markedly reduced by the pre-treatment with **5** and **6** and, to a lesser extent, with **1a** (Figure 52).

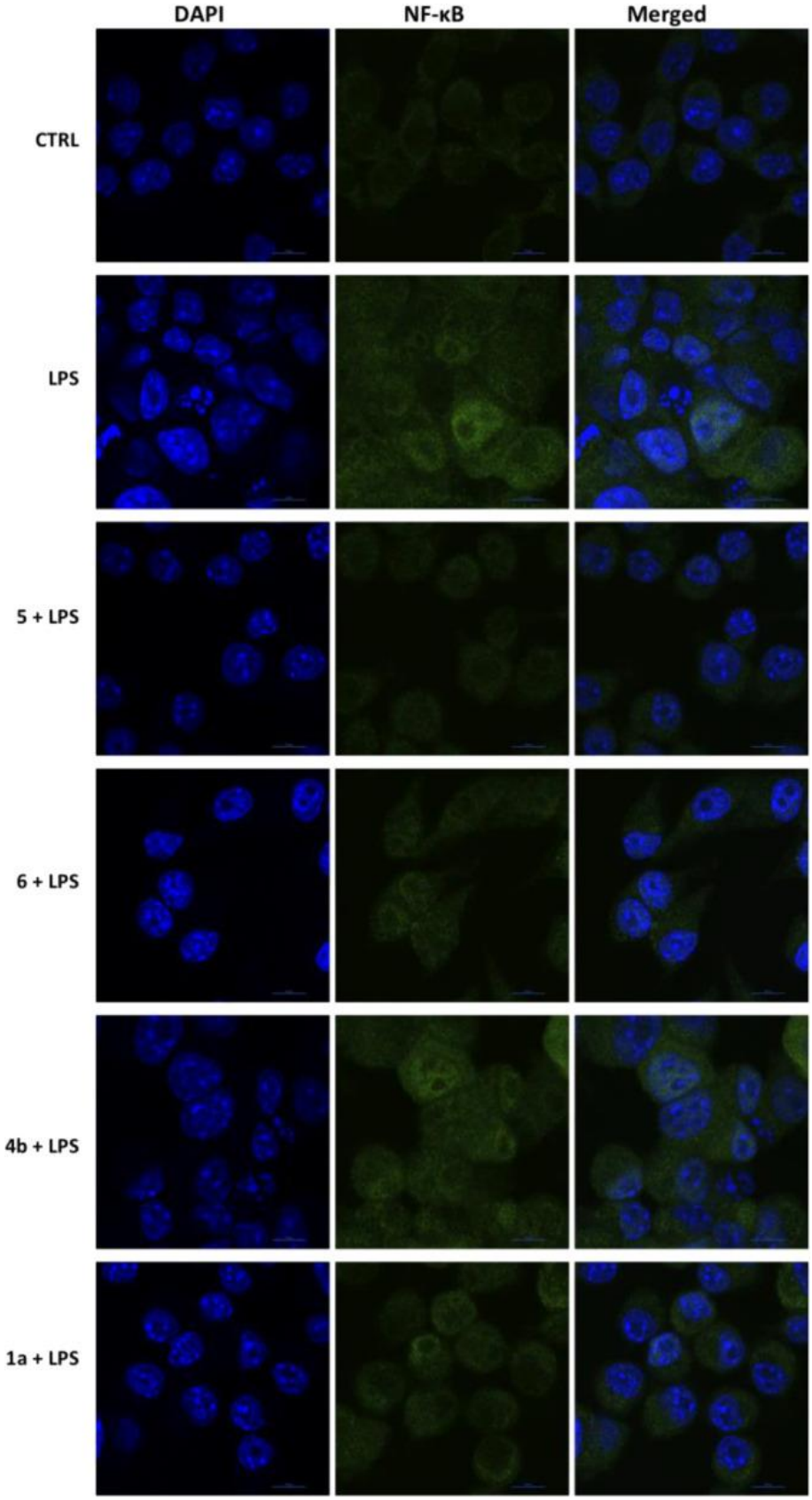


Figure 52. Nuclear translocation of NF-κB in LPS-activated BV-2 cells treated with 1a, 4a, 5, and 6. Cells were treated with 5, and 6 (0.1 μM) and 1a and 4a as reference compounds (0.1 μM) for 24 h and then exposed to 100 ng/mL LPS for further 24 h. BV-2 cells were immunostained with a primary antibody against NF-κB p65 followed by secondary Alexa Fluor 488-conjugated antirabbit IgG antibody (green), and cell nuclei (blue) were visualized with DAPI. Scale bars: 10 μm.

7.4 DISCUSSION

Neurodegenerative diseases are characterized by a multifactorial etiology [349] and, of the various causal factors, the process of neuroinflammation is one of the most impactful [169]. Neurodegenerative diseases, including Alzheimer's disease (AD), are associated with neuroinflammation which initiates the degeneration of neurons by over-activating microglia in the brain. It is now widely accepted that clustered populations of reactive microglia are hallmarks of AD, and these brain cells are likely to contribute to the mechanisms of neuronal damage and cognitive loss [161]. Therefore, the prevention and/or modulation of key pro-inflammatory molecules from microglia is a promising strategy for the prevention and possible treatment of AD. To date, available therapies are only able to reduce the symptoms of the disease without slowing or halting its course [169]. One of the main reasons for the absence of effective drugs is the multifactorial nature that characterizes these diseases and lays the basis for the development of multi-target-directed ligands (MTDL) [211]. Indeed, multi-target therapy has been suggested as a valuable strategy to improve the efficacy of drugs and to treat concomitant neurodegenerative disease conditions [169], [350]–[352]. Moreover, given the considerable increase in the incidence of these diseases in developing countries, as well as the urgent and growing demand for new effective drugs, it is necessary that these drugs are also accessible to patients.

Anacardium occidentale L phenolic lipid (LDT11) is used in traditional medicine as an anti-inflammatory, astringent, anti-diarrheal, anti-asthmatic and depurative. Recently, phenolic derivatives extracted from cashew nut (Anacardium occidentale L.) shell fluid (CNSL) have been shown to have biological and pharmacological properties as promising new anti-inflammatory agents [348].

Tacrine was approved in 1993 as the first AChEI for the treatment of AD and was withdrawn shortly thereafter due to liver toxicity, but its structure is still important for new drug design [353].

In this context, the aim of this project was to develop novel compounds to potential counteract AD from cashew nutshell liquid (CNSL), an inexpensive and inedible food waste [346], [347]. In particular, this study concerns the evaluation of the anti-inflammatory activity of novel sustainable MTDLs derived from CNSL in an in vitro model of neuroinflammation, the BV-2 microglial cell line.

During the initial phase of developing of new drug candidates for AD, a basic prerequisite is that the novel molecules possess an adequate safety and efficacy profile [354]. In fact, to date the lack of an effective therapy for the treatment of this pathology is also linked to the development of drug toxicity during chronic administration [354]. Moreover, in the elderly there are numerous concomitant causes that facilitate the onset of a toxic reaction. This is due to the concomitant presence of comorbidity,

ageing and, in most cases, polytherapy; all these factors predispose the affected subjects to the development of hepatic toxicity and to the increased risk of drug interactions. Furthermore, as mentioned above, tacrine (**4a**) has been withdrawn from the market due to its strong hepatotoxicity and, in general, the serious side effects associated with its clinical use. However, in the literature there are studies supporting the possibility of developing hepatotoxicity-free hybrids of this molecule [355], [356] . Therefore, the first aspect investigated in this study was the evaluation of the cytotoxic effect of the tested compounds on several cell lines: the hepatic cell line HepG2, the human neuron-like SH-SY5Y cell line and the murine microglial line BV-2.

Given the high risk of these classes of compounds to cause hepatotoxic damage, the first extensive screening was conducted on HepG2 liver cells and the results allowed the selection of compounds **5**, **6**, **9**, **12-15** and **17** as the most promising to continue the study and assess their anti-inflammatory/neuroprotective potential against neurodegeneration.

Concerning the SH-SY5Y cell line, no toxicity was observed for all the compounds at 0.1 and 1 μM, meanwhile a slight reduction in cell viability was observed in microglial BV-2 cells when treated with 1 μM of compounds **5**, **6** and **9**. In light of these results, the next experiments were carried out at concentrations lower than 1 μM.

The strong involvement of the neuroinflammatory process in the pathogenesis of AD has been widely documented. This is due to an increase in pro-inflammatory mediators in AD patients and a close correlation between innate immune functions and AD risk genes [357]. In the CNS, the neuroinflammatory cascade is mainly carried out by microglia which constitute the main form of defense of the CNS against various damaging events [358]. Under normal physiological conditions microglia play a key role in the normal development, structural formation and functional regulation of the CNS [217], [359], [360]. Under pathological conditions (changes in the microenvironment in the brain and/or exogenous stimulation) resting microglia switch to an activated phenotype with subsequent expression of pro-inflammatory genes and neuronal damage [361], [362]. When microglia are in their activated form it triggers several signaling pathways such as Toll-like receptor 4 (TLR4), nuclear factor kappa B (NF-κB), and mitogen-activated protein kinase (MAPK) pathways that stimulate the release of a wide range of pro-inflammatory molecules such as TNF-α and IL-1β [363], [364]. In addition, under activated microglia conditions there is also an increase in the expression of enzymes involved in the inflammatory process such as COX-2 [365] and inducible nitric oxide synthase (iNOS) [361]. Therefore, neuroinflammation in AD could be counteracted by inhibiting the excessive activation of microglia and reducing the production of proinflammatory molecules.

On this basis, the anti-inflammatory potential of compounds **5**, **6**, **9**, **12-15** and **17**was carried out LPS-activated microglial BV-2 cells. The ability of LPS to promote a pro-inflammatory phenotype

in both primary microglia and BV-2 cells has been widely documented [366], [367]. Moreover, BV-2 cells can be considered a valid simulator of primary microglia as they show the same reaction patterns and modulation by LPS [368].

Compounds **5** and **6** resulted as the most effective against LPS-induced damage. For this reason, their anti-inflammatory activity was further investigated.

The ability of **5** and **6** in modulating the expression of some of the main pro-inflammatory mediators (IL-1β, TNF-α, iNOS and COX2) was studied. It is well known that IL-1β and TNF-α, pro-inflammatory neurotoxic cytokines, play a key role in neuronal dysfunction and loss [369]. The production of nitric oxide (NO), a crucial cytotoxic mediator involved in the innate immune response, is upstream regulated by iNOS. Although iNOS is not commonly expressed in the brain, activated microglia constitute a primary source of this enzyme and a subsequent excessive release of NO by these cells strongly promotes the progression of neurodegeneration.

Similarly, the COX-2 enzyme promotes the establishment of neurotoxic processes and, in fact, its inhibition is linked to a reduction in cerebral lesions and a slowdown in neurodegenerative processes [370].

The evaluation of these pro-inflammatory mediators by RT-PCR showed that **6** significantly downregulated all the tested genes, **5** significantly reduced the expression of IL-1β, iNOS and COX2. Of note, supporting the efficacy of the combined approach of the applied framework was a greater anti-inflammatory effect of compounds **5** and **6** compared to the positive control (**1a**).

Partially in agreement with the results of the present study, de Souza et al. [348] reported an effect of 1a (LDT11) in significantly reducing the expression of IL-1β and IL-6, TNF-α, COX-2, iNOS and NF-κB. In addition to the different cell model used by de Souza et al. (RAW264.7 murine macrophage cell line) [348], the different concentrations used (50 μM vs 0.1 μM) in the two studies could justify the discrepancy observed.

A key factor in modulating the expression of pro-inflammatory cytokines and enzymes is the migration of the transcription factor NF-κB from the cytoplasm, where it is normally bound to IκBα, to the nucleus. Phosphorylation and degradation of IκBα results in the release of NF-κB, which is then free to translocate into the nucleus [371], [372]. The rate of this translocation was assessed in LPS-activated BV-2 cells by confocal immunofluorescence. In line with the RT-PCR data, LPS-induced nuclear translocation of NF-kB was markedly reduced by the pre-treatment with **5** and **6** and, to a lesser extent, with **1a**. This result, in addition to confirming the greater efficacy of **5** and **6** compared to **1a**, proposes the inhibition of NF-κB transcription as a possible anti-inflammatory mechanism by which the two compounds act.

The data obtained in this study identified compounds **5** and **6** as the most effective anti-inflammatory compounds, as they reduce the expression levels of the main mediators of inflammation (such as TNF-a, IL-1β, COX2 and iNOS), the levels of the pro-inflammatory cytokine IL-1β and the nuclear translocation of the transcription factor NF-kB. This makes them valuable candidates as potential novel drugs for the treatment of neurodegenerative diseases.

8 Study 4_Coffee Silverskin (CSS) and Spent Coffee Grounds (SCG): coffee industry by-products as a promising source of neuroprotective agents

The results of this part have been published on:

Coffee silverskin extracts: quantification of 30 bioactive compounds by a new HPLC-MS/MS method and evaluation of their antioxidant and antibacterial activities.

Nzekoue, F.K.; Angeloni, S.; Navarini, L.; Angeloni, C.; **Freschi, M**.; Hrelia, S.; Vitali, L.A.; Sagratini, G.; Vittori, S.; Caprioli, G.

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And

Antioxidant and Anti-inflammatory Profiles of Spent Coffee Ground Extracts for the Treatment of Neurodegeneration

Angeloni, S.[§]; **Freschi, M**.[§]; Marrazzo, P.; Hrelia, S.; Beghelli, D.; Juan-García, A.; Juan, C.; Caprioli, G.; Sagratini, G.; Angeloni, C.

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8.1 INTRODUCTION

Significant amounts of waste products are generated every day by the food industry. It is critical that these by-products are properly disposed in an environmentally friendly way. Moreover, a proper waste management is also economically fundamental, as it reduces both the production of new products, often non-renewable, and the energy required to produce them [373]. Coffee industry by-products are among the most exploited food wastes for recycling [374]–[378]. Coffee is one of the most widely consumed beverages worldwide and it is generated by extraction with hot water or brewing the powder obtained by grinding roasted coffee beans. Over the past 150 years its commercial importance has grown exponentially. In 2019, the International Coffee Organization (ICO) estimates that 9.5 billion kg were produced, most of which were consumed in the EU, the US, Brazil and Japan [379]. Together with the large amount of coffee produced every year, many tones of by-products are generated such as spent ground coffee (SCG) and coffee silverskin (CSS).

The most abundant waste is spent coffee grounds (SCG), a solid residue that constitutes 55-67% of the total waste from the coffee industry [380]. For example, 1000 kg of green coffee beans yields about 650 kg of SCG, and the preparation of 1 kg of soluble coffee generates almost 2 kg of wet SCG [381]. The SCG disposal into the environment is critical because it is considered an inedible toxic residue due to its caffeine, tannin, and polyphenol content [380], [381].

Another coffee by-products is represented by coffee silverskin that is the thin silvery tegument that coats the coffee bean [382] (Figure 53) and is a by-product of the roasting stage with a generation of approximately 60 kg of CSS for every 8 tones of roasted coffee [383].

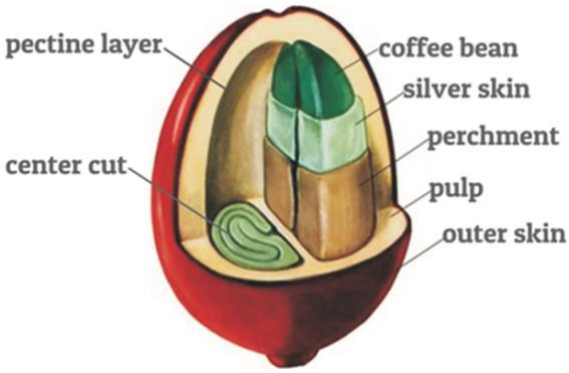

Figure 53. Constitution of a coffee drupe [384].

Due to the high coffee production and the consequent generation of waste, it is essential to find new ways to reduce and dispose coffee by-products in order not to burden the environment [385] and, to develop new applications to re-use them [386]–[388] in a circular economy perspective.

From this point of view, to date, several solutions have been proposed. In particular, CSS and SGC can:

- be used as a source of oil to produce biofuels (biodiesel, renewable diesel, bioethanol, bio-ethers, bio-oil, biochars and biogas) production [389]–[392],
- be used to produce adsorbent material for the removal of toxic metals in water [393], [394].
- be converted into galactomannans [395], fertilizers (rich in tannins, polyphenols and caffeine), bio-absorbents, activated carbons, polyols, polyhydroxyalkanoates and bio-film [392], [395]–[397].
- be used as a source of recoverable sugars either for bioethanol production or used as a food additive [391], [398]–[400].

Increasing attention has been paid to CSSs as a potential source of functional compounds for food-products enrichment, such as, for example, soluble (15%) and insoluble (50%) dietary fibers of which CSSs are rich [401].

In addition to these, SCGs and CSSs, being extremely rich in many other bioactive compounds, can find a wide range of applications in the food, nutraceutical and cosmetic industry [402] [403] [375], [404]–[406]. Caffeine, polysaccharides, phenols, proteins, lipids, minerals, and melanoidins are among the major components [407]. Phenols present in these coffee wastes are phenolic acids such as chlorogenic, ellagic, caffeic, gallic, trans-ferulic, p-coumaric, p-hydroxybenzoic, tannic, and protocatechuic acids; flavonoids such as rutin, catechin, epicatechin, and quercetin [375], [408], [409].

Polyphenols are known to be a class of molecules, commonly found in plants, with well-established antioxidant, antiproliferative, anti-allergic, anticarcinogenic, antimicrobial, anticancer, anti-inflammatory, and neuroprotective properties [401], [410].

Although traditionally the beneficial effects of coffee have been mainly attributed to its most known and studied component, caffeine, it is now widely accepted that other bioactive compounds contribute to the precious properties of this beverage [382].

Neurodegenerative diseases, among which Parkinson's and Alzheimer's disease, represent a health problem that primarily affects the elderly population. These diseases are a growing health globally problem, also due to the increase in the average lifespan of the population. Neurodegenerative diseases are characterized by a multifactorial etiology and, although they show distinct symptoms and clinical features, they share common mechanisms such as neuroinflammation, oxidative stress, induction of apoptosis, abnormal protein deposition, impaired mitochondrial function, and impaired proteostasis [77] [5].

Among these, oxidative stress and neuroinflammation are the most impactful [1]. Neurons are particularly susceptible to oxidative stress because the brain is characterized by a high oxygen consumption, high polyunsaturated fatty acid content in membranes, and weak antioxidant defenses [73]. Oxidative stress, besides damaging the main cellular components, can contribute to the abnormal protein aggregation, one of the main hallmarks of neurodegenerative diseases, by ROS-induced protein misfolding [157].

Another factor that plays a key role in the onset and progression of neurodegeneration is the immune system [411], [412], which can trigger immune cell proliferation and migration, altered cytokine signaling, reactive gliosis and altered phagocytosis [413].

Microglia and astrocytes, immune cells of the CNS, are crucial to maintain tissue homeostasis and physiological brain development and are an essential defense system to protect neurons from microorganisms and toxic substances. In case of various injuries or insults, these cells are stimulated to produce both pro-inflammatory factors, that can counteract the damage, and anti-inflammatory factors that promote tissue repair and recovery of a normal condition. Under physiological conditions, this mechanism is fundamental for preserving the integrity of the CNS by eliminating insults and re-establishing homeostasis [414]. However, excessive activation of these cells damages the surrounding tissue and, in turn, the factors released by the dead cells trigger a series of events that cause progressive neuronal loss [161], [415].

To date, there are no drugs available that can block, or at least slow down, these deleterious pathologies. Due to the multifactorial etiology of neurodegenerative diseases, it is difficult to counteract them using molecules with a single molecular target.

More and more studies are focusing on the identification and characterization of molecules with pleiotropic activity, such as natural compounds that can act as potential preventive/protective agents against neurodegeneration. Numerous epidemiological studies have reported that caffeine consumption is related to a reduction of the incidence of various neurological and neurodegenerative diseases [416]. Caffeine consumption has been associated with a decrease in cognitive decline in both healthy older adults and Alzheimer's patients [417]–[420]. Most studies on the beneficial effects of coffee have focused mainly on caffeine, but coffee contains more than 1000 components that may have neuroprotective effects [421]–[424].

In this context, the identification and characterization of bioactive compounds in CSSs and SCGs able to counteract neurodegeneration could be of great importance.

The aim of this study was to evaluate the potential neuroprotective effect (antioxidant and anti-inflammatory) of 4 different extracts obtained from spent coffee grounds (Spent Coffee) and coffee Silverskin (CSS), in a circular economy perspective.

In particular, their potential neuroprotective activity against H_2O_2-induced damage was assessed in an in vitro model of oxidative stress (differentiated neuron-like SH-SY5Y cell line exposed to H_2O_2). Following promising data in SCG extracts, their neuroprotective potential has also been assessed in an in vitro model of neuroinflammation (BV-2 microglial cell line activated with LPS) (Figure 54).

CSS and SCG extracts were obtained by 4 different extraction methods (E1 _MeOH, E2_H_2O, E3_MeOH/ H_2O (50:50), E4_EtOH/ H_2O (70:30)) and were characterized by the research group of Prof. Sagratini of the School of Pharmacy of the University of Camerino using an HPLC-MS/MS system.

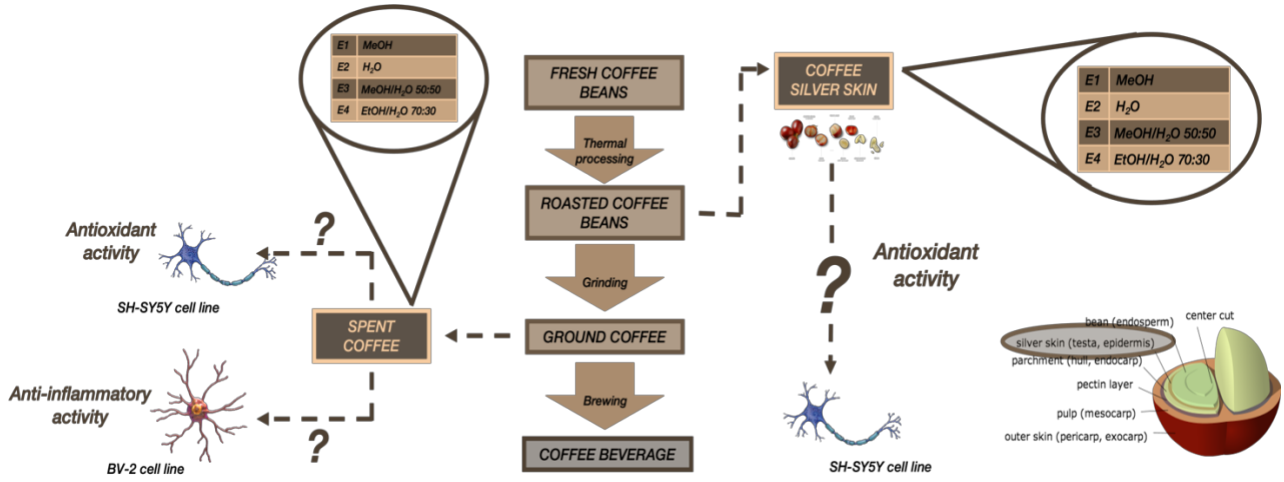

Figure 54. Aim of the study.

8.2 MATERIALS AND METHODS

8.2.1 Chemicals

Dulbecco's modified Eagle's medium (DMEM), penicillin/streptomycin, L-glutamine solution, trypsin-EDTA solution, accutase solution, phosphate buffered saline (PBS), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA), all-trans-retinoic acid (RA), H_2O_2, LPS from Escherichia coli serotype O127:B8, primers for real-time polymerase chain reaction (RT-PCR), RIPA buffer, sodium pyrophosphate, phenylmethylsulfonyl fluoride, Triton X-100, paraformaldehyde, 4′-6-diamidino-2- phenylindole (DAPI), dimethyl sulfoxide (DMSO) and all other chemicals of the highest analytical grade were produced by Merck Italia (Milan, Italy). Fetal bovine serum (FBS) and Low-endotoxin Fetal bovine serum (low-endotoxin FBS) were purchased from Euroclone (Milan, Italy). RNeasy Mini Kit was from Qiagen (Hilden, Germany). Synthesis Kit, SsoAdvanced Universal SYBR Green Supermix Kit were purchased from Bio-Rad (Hercules, USA).

8.2.2 Coffee silverskin (CSS) and Spent coffee (SCG) extract preparation.

Coffee silverskin was obtained by roasting green C. arabica beans of Ethiopian origin (supplied by Gardelli specialty coffees, Forli, Italy). An exposure at a maximum temperature of 195 °C for 9 minutes using an Ikawa coffee roaster (IKAWA Ltd, London, UK) was done to carry out the roasting process. The discarded silverskin was finally grounded under nitrogen condition and was stored at 4 °C until extraction.

To obtain Spent coffee grounds, Simonelli Group S.p.A. (Belforte del Chienti, Italy) supplied the roasted beans of 100% Coffea arabica L., Ethiopian origin. The Mythos 1 grinder (Simonelli Group S.p.A.) was used to grind the roasted beans. The resulting grounded coffee was used to obtain spent coffee ground (SCG) through a series of replications of espresso coffee preparations with a VA833 Black Eagle espresso machine (Victoria Arduino, Simonelli Group S.p.A., Belforte del Chienti, Italy).

Espresso coffee extraction was performed as follows: 7 ± 0:05 g roasted and ground coffee (R&G) per cup, 25 ± 1 s extraction time, water pressure and temperature 9 bar and 92.0°C, respectively, and 25 ± 2 g per cup. Subsequently, the obtained SCG samples from the coffee espresso extraction were dried in an oven at 50°C for approximately 48 h or otherwise until constant weight and then stored at 4°C.

The preparation of the extracts was carried out as described by Caprioli, et al. [425] with some modifications.

10 g of each SCG and CSS was extracted with 50 mL of solvent using a FALC ultrasonic bath (FALC, Treviglio, Italy) at a frequency of 40 kHz at 20°C for 120 min. The following 4 different solvents were selected: H_2O, MeOH, a mixture of MeOH:H_2O (50 : 50, v/v), and a mixture of EtOH:H_2O (30:70, v/v). Subsequently, SCG extracts, after appropriate filtration with filter paper, were lyophilized using a LyovaporTM L-200 (Buchi, Cornaredo, Italy) and stored in the dark at -20°C until use.

8.2.3 HPLC-MS/MS Triple Quadrupole.

HPLC-MS/MS analysis were performed as previously described [374]. For HPLC analysis, 5 mg of lyophilized extract were solubilized in 5 mL of MeOH (1 mg/mL), sonicated for 10 minutes, filtered with 0.2 μm pore size filter and then aliquots of the obtained solutions were injected in HPLC-MS/MS [373].

The HPLC-MS/MS analysis was carried out employing an Agilent 1290 Infinity series coupled to a Triple Quadrupole 6420 from Agilent Technology (Santa Clara, CA, USA) equipped with an electrospray (ESI) source operating in negative and positive ionization modes. A Kinetex PFP analytical column (100 mm × 2.1 mm i.d., particle size 2.6 μm) from Phenomenex (Torrance, CA, USA) was used to perform the separation. The mobile phase consisted of a mix of water (A) and methanol (B) both with formic acid 0.1% in gradient elution mode at a flow rate of 0.2 mL/min. The mobile phase composition chanced as follows: 0–2 min, 20% B; 2–15 min, 80% B; 15–18 min, 80% B; 18–23 min, 100% B, 23–35 min, 20% B. The injection volume was 2 μL. The column was at 30 °C whereas the drying gas in the ionization source at 350 °C. The nebulizer pressure was 25 psi, the gas flow was 10 L/min, and the capillary voltage was 4000 V. The dynamic "multiple reaction monitoring" (Dynamic-MRM) mode was employed for the detection. The characterization of the four selected CSS extracts was as reported in Table 7 [374], while in Table 8 is reported the content of bioactive compounds (μg/g of dry weight extract) of each SCG [373].

n.	Compounds	E1 (MeOH)	E2 (H_2O)	E3 (MeOH: H_2O)	E4 (EtOH: H_2O)
	ALKALOIDS				
1	Caffeine	10010.22 ± 389.40	19599.04 ± 1842.31	25176.74 ± 1072.53	35879.16 ± 3236.30
2	Quinine	0.23 ± 0.01	0.49 ± 0.02	0.43 ± 0.04	0.61 ± 0.03
	POLYPHENOLS				
	Phenolic acids				
3	3,5-diCQA	43.79 ± 2.04	47.82 ± 3.93	145.9 ± 4.00	201.08 ± 2.03
4	3-CQA	3390.85 ± 221.08	2748.70 ± 83.56	4014.6 ± 307.52	2725.61 ± 57.24
5	5-CQA	2009.35 ± 153.92	319.08 ± 6.70	396.51 ± 14.87	388.39 ± 0.66
6	Caffeic Acid	n.d	79.00 ± 0.13	112.65 ± 16.36	212.38 ± 27.86
7	Ferulic Acid	n.d	66.52 ± 3.09	104.64 ± 8.60	226.23 ± 6.20
8	Gallic Acid	16.59 ± 1.41	24.36 ± 1.59	31.07 ± 0.94	15.76 ± 1.21
9	Loganic acid	n.d	n.d	n.d	n.d
10	p-Coumaric Acid	2.96 ± 0.39	7.75 ± 0.87	9.91 ± 0.46	18.18 ± 1.49
11	Shikimic Acid	n.d	n.d	n.d1	n.d
12	Syringic Acid	n.d	39.00 ± 5.12	52.5 ± 5.86	77.5 ± 3.60
13	Trans-cynnamic acid	1.10 ± 0.05	2.98 ± 0.27	3.55 ± 0.18	4.20 ± 0.27
14	Vanillic Acid	n.d	138.27 ± 5.19	184.73 ± 0.31	345.13 ± 50.11
	Flavonoids				
15	Catechin	n.d	n.d	n.d	n.d
16	Cyanidin 3-glucoside	n.d	n.d	n.d	n.d
17	Delphinidin 3,5-diglucoside	n.d	n.d	n.d	n.d
18	Epicatechin	151.07 ± 0.26	n.d	n.d	n.d
19	Hyperoside	0.39 ± 0.00	0.28 ± 0.00	0.37 ± 0.02	n.d
20	Kaempferol	0.96 ± 0.08	0.76 ± 0.06	1.4 ± 0.01	1.66 ± 0.03
21	Naringin	n.d	0.32 ± 0.01	n.d	n.d
22	Quercetin Dihydrate	2.13 ± 0.17	2.19 ± 0.01	2.46 ± 0.04	1.53 ± 0.26
23	Quercitrin	0.38 ± 0.04	0.15 ± 0.01	0.33 ± 0.03	0.51 ± 0.00
24	Resveratrol	n.d	n.d	n.d	n.d
25	Rutin Hydrate	1.63 ± 0.05	1.74 ± 0.02	8.7 ± 0.12	3.37 ± 0.19
	Xanthone				
26	Isogentisin	0.032 ± 0.00	0.40 ± 0.01	0.5 ± 0.08	0.31 ± 0.06
	Secoiridoids				
27	Swertiamarin	n.d	n.d	n.d	n.d
28	Sweroside	n.d	n.d	n.d	n.d
29	Gentiopicroside	n.d	n.d	n.d	n.d
30	Amarogentin	n.d	n.d	n.d	n.d
	Total level of bioactive compounds	15631.69 ± 768.88	23078.85 ± 1952.89	30247.00 ± 1431.96	40101.63 ± 3387.54
	% of bioactive compounds (%w/w)	1.56 ± 0.08	2.31 ± 0.20	3.02 ± 0.14	4.01 ± 0.34
	Total level of polyphenols	5621.23 ± 379.48	3479.32 ± 110.59	5069.83 ± 359.40	4221.85 ± 151.21

n.d : not detectable. the signal was lower than LOQ; CQA: caffeoylquinic acid.

Table 7. Quantification of 30 bioactive compounds (expressed as μg/g of dry weight extract) in CSS extracts by HPLC-MS/MS analysis [374].

No.	Analytes[a]	E1 (MeOH)	E2 (H_2O)	E3 (MeOH : H_2O)	E4 (EtOH : H_2O)
1	Shikimic acid	38.52 ± 1.84	23.11 ± 1.23	86.70 ± 3.26	71.15 ± 3.12
2	Gallic acid	87.65 ± 3.33	57.62 ± 2.65	112.29 ± 4.26	75.91 ± 2.81
3	Loganic acid	n.d.[c]	n.d.	n.d.	n.d.
4	3-CQA[b]	3637.65 ± 157.21	2324.33 ± 100.89	3587.15 ± 163.24	4317.31 ± 185.42
5	Swertiamarin	n.d.	n.d.	n.d.	n.d.
6	Gentiopicroside	n.d.	n.d.	n.d.	n.d.
7	(+)-Catechin	0.95 ± 0.04	n.d.	1.25 ± 0.05	1.02 ± 0.04
8	Del 3,5-diglu[b]	n.d.	n.d.	n.d.	n.d.
9	Sweroside	n.d.	n.d.	n.d.	n.d.
10	5-CQA[b]	12699.32 ± 483.26	7569.25 ± 305.21	13256.35 ± 499.74	12868.75 ± 401.68
11	Caffeine	41047.71 ± 1896.25	45568.32 ± 2121.56	51236.74 ± 2036.15	52346.41 ± 2536.98
12	Cya 3-glu[b]	1.56 ± 0.07	1.02 ± 0.05	1.85 ± 0.08	2.03 ± 0.09
13	Vanillic acid	65.23 ± 2.36	82.65 ± 3.33	122.36 ± 5.14	105.41 ± 4.21
14	Caffeic acid	81.58 ± 1.65	103.28 ± 4.78	170.83 ± 5.98	220.71 ± 10.36
15	(-)-Epicatechin	87.23 ± 2.98	n.d.	85.11 ± 2.22	n.d.
16	Syringic acid	23.56 ± 1.01	44.15 ± 1.87	43.65 ± 2.10	78.63 ± 3.88
17	*p*-Coumaric acid	8.36 ± 0.32	9.45 ± 0.29	15.23 ± 1.12	28.12 ± 1.15
18	Ferulic acid	82.47 ± 3.45	87.54 ± 2.65	118.96 ± 4.13	155.32 ± 5.89
19	3,5-diCQA[b]	915.43 ± 55.32	902.34 ± 58.12	1025.84 ± 64.32	1325.98 ± 88.23
20	Quinine	1.44 ± 0.07	1.69 ± 0.06	2.75 ± 0.10	3.23 ± 0.12
21	Naringin	n.d.	0.62 ± 0.03	0.40 ± 0.02	0.47 ± 0.02
22	Rutin	3.33 ± 0.15	5.36 ± 0.33	8.75 ± 0.52	10.11 ± 0.61
23	Hyperoside	0.98 ± 0.04	0.86 ± 0.03	0.75 ± 0.03	1.23 ± 0.06
24	*trans*-Cin acid[b]	6.27 ± 0.24	5.44 ± 0.30	6.49 ± 0.32	8.11 ± 0.35
25	Resveratrol	n.d.	n.d.	n.d.	n.d.
26	Amarogentin	n.d.	n.d.	n.d.	n.d.
27	Kae 3-glu[b]	1.54 ± 0.06	1.03 ± 0.05	1.97 ± 0.08	2.84 ± 0.11
28	Quercitrin	0.47 ± 0.02	0.28 ± 0.01	0.74 ± 0.03	1.12 ± 0.05
29	Quercetin	3.42 ± 0.12	3.15 ± 0.13	3.96 ± 0.15	3.87 ± 0.11
30	Isogentisin	1.65 ± 0.06	1.12 ± 0.04	1.23 ± 0.05	1.45 ± 0.05
Total compounds		58796.31 ± 2756.32	56792.60 ± 2521.98	69891.35 ± 3102.12	71629.19 ± 3025.85

[a]Each sample was analyzed in triplicate ($n = 3$); [b]3-CQA: 3-caffeoylquinic acid; 3,5-diCQA: 3,5-dicaffeoylquinic acid; 5-CQA: 5-caffeoylquinic acid; Del 3,5-diglu: delphinidin 3,5-diglucosiede; Cya 3-glu: cyanidin 3-glucoside; *trans*-Cin acid: *trans*-cinnamic acid; Kae 3-glu: kaempferol 3-glucoside; [c]n.d.: not detectable.

Table 8. Bioactive compounds content (μg/g of dry weight extract) in spent coffee ground (SCG) extracts [373].

8.2.4 Spectrophotometric analysis: total phenolic content (TPC), total flavonoid content (TFC) and DPPH radical scavenging activity

The method of Siatka and Kašparová [426] with some modifications was used to determine TPC. To 0.5 mL of extract solution (1 mg/mL in MeOH) were added 2.5 mL of Folin-Denis reagent solution and 7 mL of Na2CO3 solution (7.5% w/w in water) and this mixture was incubated for 2 h in the dark at room temperature. After the incubation period, the absorbance was measured at 765 nm. The ∑ TPC quantification of the extracts was carried out using the gallic acid calibration curve and was expressed as mg gallic acid equivalents (GAE) per g of dry weight of the CSS extracts (Figure 55A) [374] and SCG extracts (Table 9) [373].

The method of Chen, Chen, Xiao and Fu [427] with slight variations was used to evaluate the TFC of the 4 CSS. 0.5 mL of extract solution (1 mg/mL) was mixed with 0.15 mL of $NaNO_2$ (0.5 M), 3.2 mL of methanol (30% V/V) and 0.15 mL of $AlCl_3$-$6H_2O$ (0.3 M) and after 5 min, 1 mL of NaOH (1 M) was added. The absorbance was measured at 506 nm against the white reagent. A standard

solution of rutin (0 to 100 mg/L) was used to make the standard calibration curve for TFC. The TFC of CSS (Figure 55B) [374] and SGC (Table 9) [373] was expressed as mg rutin equivalent (RE) per g dry extract.

The method of Venditti et al. [428] with modifications was used to determine the ability of the extracts to scavenge the 2,2-diphenyl-1- picrydrazyl (DPPH) radical. 0.5 mL of extract solution (1 mg/mL in MeOH) was mixed with 4.5 mL of DPPH ethanolic solution (0.1 mM) and incubated in the dark at room temperature for 30 min. The disappearance of DPPH was measured spectrophotometrically at 517 nm. The percentage of DPPH scavenging was calculated as follows: % I = [($A_{control}$ - A_{sample})/ $A_{control}$] × 100. $A_{control}$ and A_{sample} represent the absorbance obtained without and with antioxidants, respectively. The scavenging activity was expressed as the IC_{50} value (µg/mL), which is the concentration of the extract required to cause 50% of DPPH inhibition. The interpolation from linear regression analysis was performed to obtain the IC_{50} value. Trolox® was used as a reference antioxidant (1-50 µg/mL). DPPH activity of CSS (Figure 55C) [374] and of SCG extracts was (Table 9) [373] as shown below.

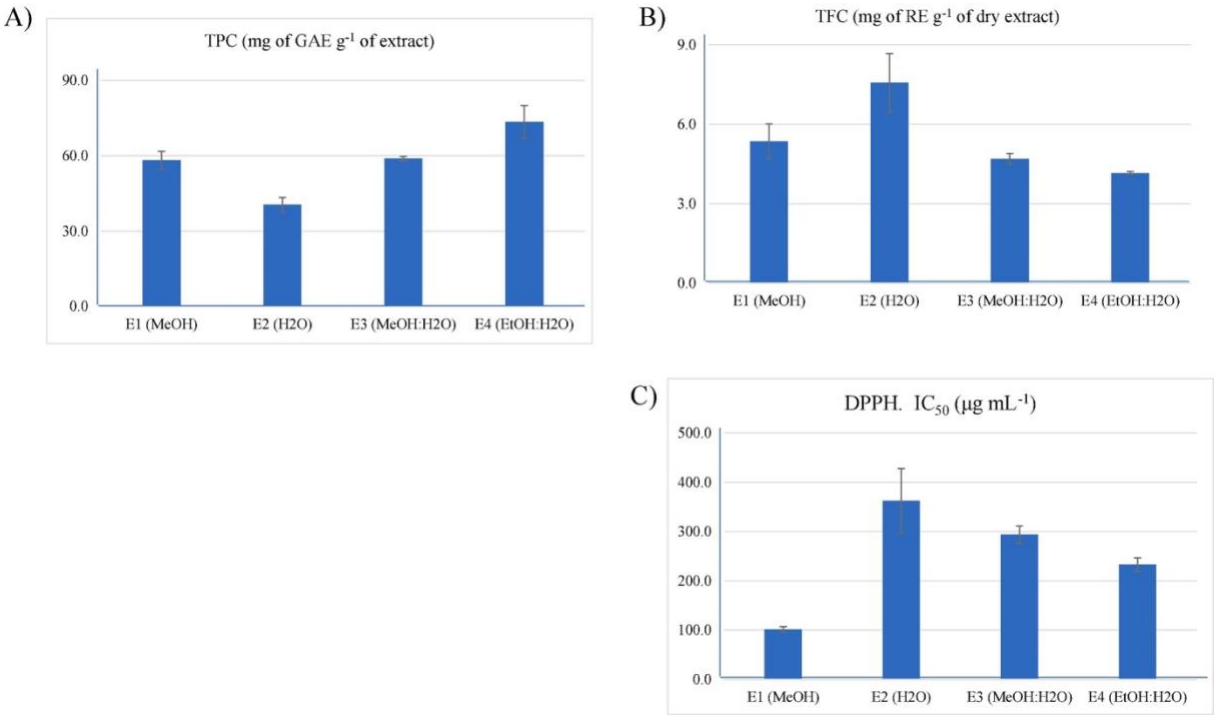

Figure 55. Spectrophotometric analysis of coffee silver skin (CSS) extracts (n = 3). (A) Total phenolic contents (TPC) of CSS extracts, expressed as mg of gallic acid equivalents per g of dry weight of extract (mg of GAE/g of dry extract). (B) Total flavonoid contents (TFC) of CSS extracts, expressed as mg of rutin equivalents per g of dry weight of extract (mg of RE/g of dry extract). (C) DPPH radical scavenging activity of the 4 CSS extracts, expressed as IC_{50} value (µg/mL). DPPH: 2,2-diphenyl-1-picrydrazyl; IC_{50} which is the concentration of the extract necessary to cause 50% of DPPH inhibition [374].

Extracts	Total phenolic content (mg GAE/g)	Total flavonoid content (mg RE/g)	DPPH IC50 (μg/mL)
E1 (MeOH)	88.75 ± 2.13	6.17 ± 0.16	215.35 ± 7.42
E2 (H_2O)	69.32 ± 2.11	3.15 ± 0.14	585.32 ± 25.32
E3 (MeOH : H_2O)	95.12 ± 3.56	6.29 ± 0.23	298.44 ± 13.12
E4 (EtOH : H_2O)	112.65 ± 4.53	5.56 ± 0.12	196.25 ± 6.87

Table 9. Total phenolic content (TPC), total flavonoid content (TFC) and DPPH radical scavenging activity of the 4 SCG extracts [373].

8.2.5 Cell Culture and treatments

The SH-SY5Y human neuroblastoma cell line (ECACC 94030304) was purchased from Merck (Italy). Cells were grown in DMEM supplemented with 10% (*v*/*v*) of FBS, 2 mM of L-glutamine, 50 U/mL of penicillin, and 50 µg/mL of streptomycin and maintained at 37 °C in a humidified incubator with 5% CO_2 as previously reported [256]. Differentiated SH-SY5Y cells were used for experiments. Before experiments cell differentiation was induced by treatment with RA (10 µM) for 7 days (1% FBS). Differentiated SH-SY5Y cells were treated for 24 h with different concentrations of the CSS and SCG extracts, subsequently exposed to 700 µM H_2O_2 (1 h) to induce oxidative stress.
Controls were set adding the highest volume of the respective vehicle. Vehicles, at this concentration, did not influence cell viability (data not shown).

Murine microglial cells (BV-2) were provided by Prof. Elisabetta Blasi (University of Modena and Reggio Emilia, Italy) and cultured in DMEM supplemented with 10% (*v*/*v*) of low-endotoxin FBS, 2 mM of L-glutamine, 50 U/mL of penicillin, and 50 µg/mL of streptomycin and maintained at 37 °C in a humidified incubator with 5% CO_2 as reported in [211]. BV-2 microglial cells were treated for 24 h with different concentrations of the SCG extracts and then activated with LPS (100 ng/mL) for further 24 h.

8.2.6 Viability Assay

Cell viability was evaluated by MTT assay as previously reported [257]. At the end of each experiment, cells were incubated with MTT work solution (0.5 mg/mL) for 90 min (SH-SY5Y cells) or 30 min (BV-2 cells) at 37 °C. After this period, MTT solutions were replaced with 100 µL of DMSO to dissolve the formed formazan crystals and the absorbance was measured at a wavelength

of 595 nm using a multilabel plate spectrophotometer (VICTOR3 V Multilabel Counter; Perkin-Elmer, Wellesley, MA, USA). Cell viability was expressed as % of control cells.

8.2.7 Trypan Blue Assay

Differentiated SH-SY5Y cells were treated with the 4 SCG extracts (50 µg/mL) and after 24 h cells were stained with 0.4% trypan blue. Countess™ Cell Counting Chamber Slides (Invitrogen, Carlsbad, CA, USA) using the Countess® Automated Cell Counter (Invitrogen) was employed to evaluate cell viability. For each sample, the number of living cells was determined by discriminating them from dead cells through the incorporation of trypan blue. Percent of viability was calculated as follows: Viability (%) = Live cell number/Total cell number x 100.

8.2.8 Determination of Intracellular ROS levels

Intracellular ROS levels were evaluated using the fluorescent DCFH-DA probe as previously reported [258]. SH-SY5Y cells were incubated with 10 µM DCFH-DA (DMEM 1% FBS w/o phenol red) for 30 min, the probe was removed and cells were exposed to 400 µM H_2O_2 (DMEM 1% FBS w/o phenol red) and after 15 min H_2O_2 was replaced by PBS.

BV-2 cells were exposed to LPS (100 ng/mL) for 24 h, then incubated with 10 µM DCFH-DA (DMEM 1% FBS w/o phenol red) and after 30 min the probe was replaced with PBS.

Cell fluorescence was measured at 485 nm (excitation) and 535 nm (emission) with a multilabel plate reader (VICTOR3 V Multilabel Counter; Perkin-Elmer, Wellesley, MA, USA). Intracellular ROS levels were expressed as % of peroxide treated cells.

8.2.9 Real-Time polymerase chain reaction (PCR)

Total RNA was extracted using a RNeasy Mini Kit (Qiagen, GmbH, Hilden, Germany), following the manufacturer's protocol. The yield and purity of RNA were measured using a NanoVue spectrophotometer (GE Healthcare, Milan, Italy).

1 µg of total RNA was reverse-transcribed into cDNA using an iScript cDNA Synthesis Kit (BIO-RAD, Hercules, CA, USA), following the manufacturer's protocol. The subsequent PCR was carried out in a total volume of 10 µL constituted as follows: 2.5 µL (12.5 ng) of cDNA, 5 µL SsoAdvanced Universal SYBR Green Supermix (BIO-RAD, Hercules, CA, USA), and 0.5 µL (500 nM) of each primer.

Primers used are below (Table 10 and Table 11):

PRIMER	SEQUENCE (5'-3')
GAPDH_M_F	ACCACAGTCCATGCCATCAC
GAPDH_M_R	TCCACCACCCTGTTGCTGTA
IL-1β_M_F	GTTCCCATTAGACAACTGCACTACAG
IL-1β_M_R	GTCGTTGCTTGGTTCTCCTTGTA
TNFα_M_F	CCCCAAAGGGATGAGAAGTTC
TNFα_M_R	CCTCCACTTGGTGGTTTGCT
iNOS_M_F	CCTCCTCCACCCTACCAAGT
iNOS_M_R	CACCCAAAGTGCTTCAGTCA
COX2_M_F	TGGGGTGATGAGCAACTATT
COX2_M_R	AAGGAGCTCTGGGTCAAACT

Table 10. Primers for RT-PCR in BV-2 cells.

PRIMER	SEQUENCE (5'-3')
RPS18_H_F	CAGAAGGATGT AAAGGATGG
RPS18_H_R	TATTTCTTCTTGGACACACC
GR_H_F	GACCTATTCAACGAGCTTTAC
GR_H_R	CAACCACCTTTTCTTCCTTG
NQO1_H_F	AGTATCCACAATAGCTGACG
NQO1_H_R	TTTGTGGGTCTGTAGAAATG
HO1_H_F	CAACAAAGTGCAAGATTCTG
HO1_H_R	TGCATTCACATGGCATAAAG
TRX_H_F	AGACAGTTAAGCATGATTGG
TRX_H_R	AATTGCCCATAAGCATTCTC

Table 11. Primers for RT-PCR in SH-SY5Y cells.

RPS18 was used as a reference gene for SH-SY5Y cells, while GAPDH for BV-2 cells. Amplification of cDNA was initiated by activating the polymerase for 30 s at 95 °C, followed by 40 cycles of 5 s at 95 °C and 30 s at 60 °C. Melt curves were run to ensure quality control and generation of a single product. Normalized expression levels were calculated with respect to control cells according to the $2^{-\Delta\Delta CT}$ method [260].

8.2.10 Western Immunoblotting

Cells were washed with ice-cold PBS and lysed on ice using RIPA buffer containing mammalian protease inhibitor mixture (1:100 dilution), 1 mM sodium pyrophosphate, 10 mg/mL phenylmethylsulfonyl fluoride, PhosSTOP 1X (Roche, Mannheim, Germany). Before separation on 4-20% SDS-polyacrylamide gels (20 µg/lane) (BIO-RAD, Hercules, CA, USA), samples were boiled for 5 minutes. Protein transfer (Hybond-C; GE Healthcare, Buckinghamshire, UK) was carried out in Tris-glycine buffer at 110 V for 90 min using a nitrocellulose membrane. The membranes were subsequently incubated first in blocking buffer (5% (w/v) bovine serum albumin (BSA)) and then with anti-HO1 (Cell Signaling Technology, Beverly, MA) (1:1000 dilution) and anti-β- actin (Sigma Aldrich–Merck) (1:5000 dilution) as internal loading control, overnight at 4°C on a 3D rocking table. Marked proteins were visualized using ClarityTM Western ECL Substrate (BIO-RAD, Hercules, CA,

USA). Densitometric analysis of specific immunolabeled bands was performed using ImageJ software.

8.2.11 Flow cytometry

To evaluate the membrane expression of TLR4 receptor on BV-2 cells, 1×10^5 cells were seeded in 12-well tissue culture plates. At the end of each treatment, cells were detached with accutase solution after PBS washing. Cells were centrifuged at 300 g for 5 min, then the obtained cell pellet was washed twice through centrifugation and resuspension in washing buffer (0.2% BSA-PBS), in 1.5mL tubes. After removing the supernatant, the cells were resuspended and incubated in FITC-conjugated rabbit anti-TLR4 antibody (Stressmarq, cat. no. SPC-200), 1:100 dilution in 0.2% BSA-PBS, for 30 min in the dark at 37°C according to the manufacturer's instructions. After antibody incubation, the cells were washed twice as above. The supernatant was discarded by aspiration and the samples were appropriately diluted to 5×10^5 cells/mL and finally resuspended in BSA 0.1% PBS for flow cytometry reading. Guava® easyCyteTM 5 HT instrument was used to collect all raw data. FlowJo software was used to analyze the mean fluorescence intensity (MFI). Unstained samples were used as negative controls.

8.2.12 Immunofluorescence confocal microscopy

BV-2 cells were seeded directly on glass coverslips in 6-well plates. After treatments, cells were incubated first with paraformaldehyde 2% 15 min at room temperature to be fixed and then with Triton X-100 0.1% for 10 min to be permeabilized. Once fixed and permeabilized, BV-2 cells were incubated overnight with a polyclonal antibody (1:500) against NF-κB p65 (Sigma-Aldrich− Merck). After PBS extensive washing, cells were exposed to a secondary Alexa Fluor 488-conjugated antirabbit IgG antibody (1:1000) (Life Technologies Italia, Monza, MB, Italy) for 1 h at room temperature. 1 μg/mL 4′-6-diamidino-2- phenylindole (DAPI) was used to stain nuclei. Slides were analyzed with a C2 Plus confocal laser scanning microscope (Nikon Instruments, Firenze, Italy). Images were processed using NIS-Elements imaging software (Nikon Instruments, Firenze, Italy).

8.2.13 Statistical Analysis

Each experiment was performed at least three times, and all values are represented as mean ± SEM. One-way analysis of variance (ANOVA) was used to compare differences among groups, followed by Dunnett's or Bonferroni's test (Prism 7; GraphPad Prism Software, San Diego, CA, USA). Values of $p < 0.05$ were considered statistically significant.

8.3 RESULTS

8.3.1 Antioxidant activity of CSS

8.3.1.1 Neurotoxicity of CSS extracts in SH-SY5Y cells.

First aim of this study was to investigate the cytotoxicity of E1 (MeOH), E2 (H_2O), E3 (MeOH/H_2O) and E4 (EtOH/H_2O) obtained by CSS in SH-SY5Y cells. Cells were treated for 24 h with increasing concentrations (1-200 µg/mL) of the 4 extracts, subsequently cell viability was evaluated by MTT assay. Until 100 µg/mL all the extracts did not show any neurotoxic effect maintaining cell viability at values comparable, and in most cases even higher, to controls.

Only 200 µg/mL of E4 significantly reduced cell viability. For this reason, the concentration 200 µg/mL in E4 was excluded from subsequent experiments (Figure 56).

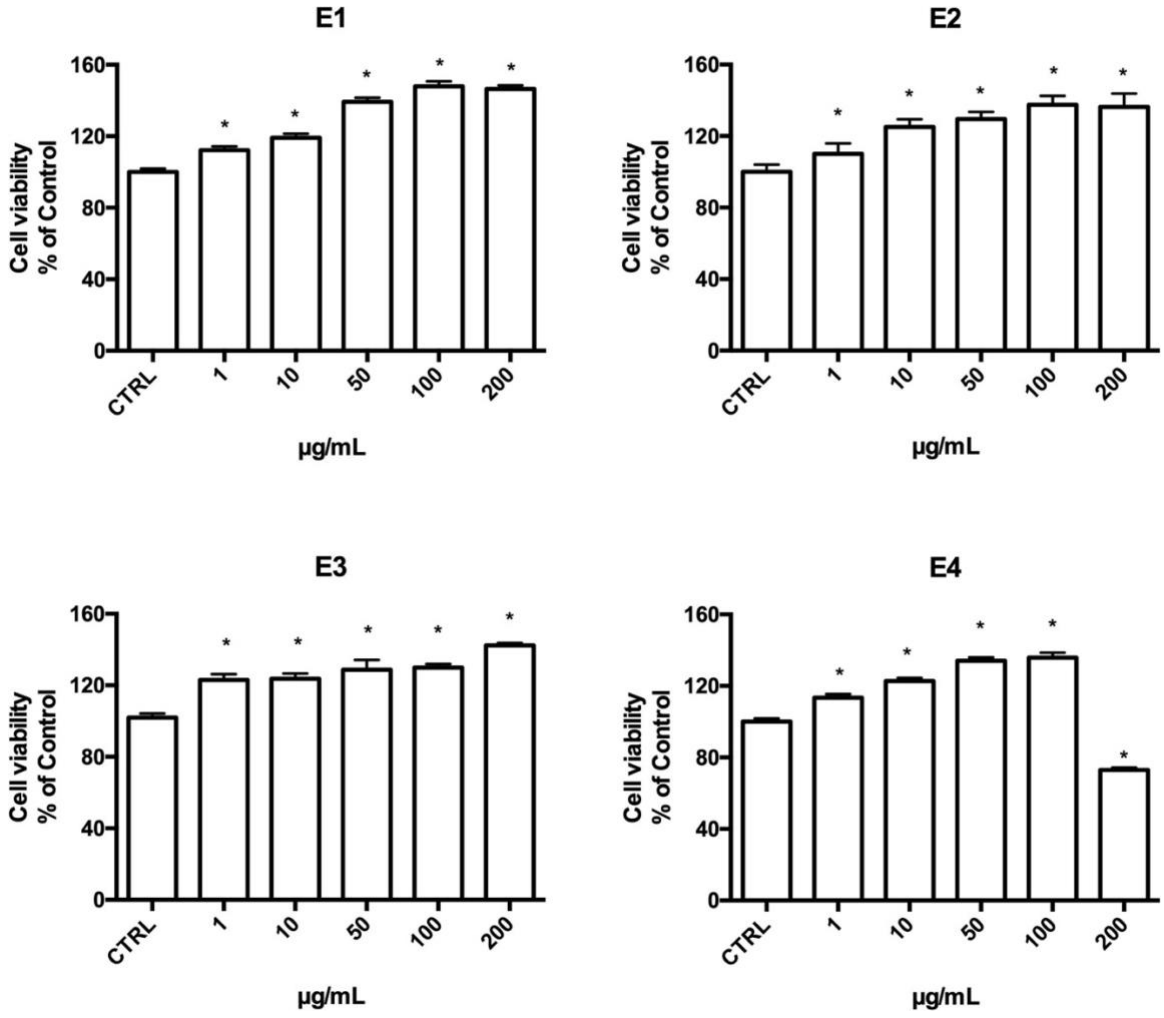

*Figure 56. Cell viability of differentiated SH-SY5Y treated with the four silverskin extracts. Cells were treated with increasing concentration (1–200 µg/mL) of E1, E2, E3, and E4 and then 24 h cell viability was evaluated by MTT assay. Each bar represents the mean ± SEM of at least four independent experiments. Data were analyzed by one-way ANOVA followed by Dunnett's test. *p<0.05 compared to control (CTRL).*

8.3.1.2 Effect of CSS extracts against H_2O_2-induced damage in SH-SY5Y cells.

To evaluate the potential protective effect of the 4 Silverskin extracts against H_2O_2-induced damage, cells were pre-treated with different concentration (1-200 µg/mL) of E1, E2, E3 and E4 and then exposed to H_2O_2 700 µg/mL for 1 h. At the end of the treatments cell viability was measured by MTT assay. H_2O_2 700 µg/mL for 1 h was the selected stress condition because able to reduce cell viability by 50%. As data shown, at the lower concentration (1-10 µg/mL) only E1 and E4 was able to significantly increase cell viability in respect with H_2O_2-treated cells displaying a protective effect. On the other hand, E2 and E3 showed a protective effect against oxidative stress only from 50 µg/mL (Figure 57).

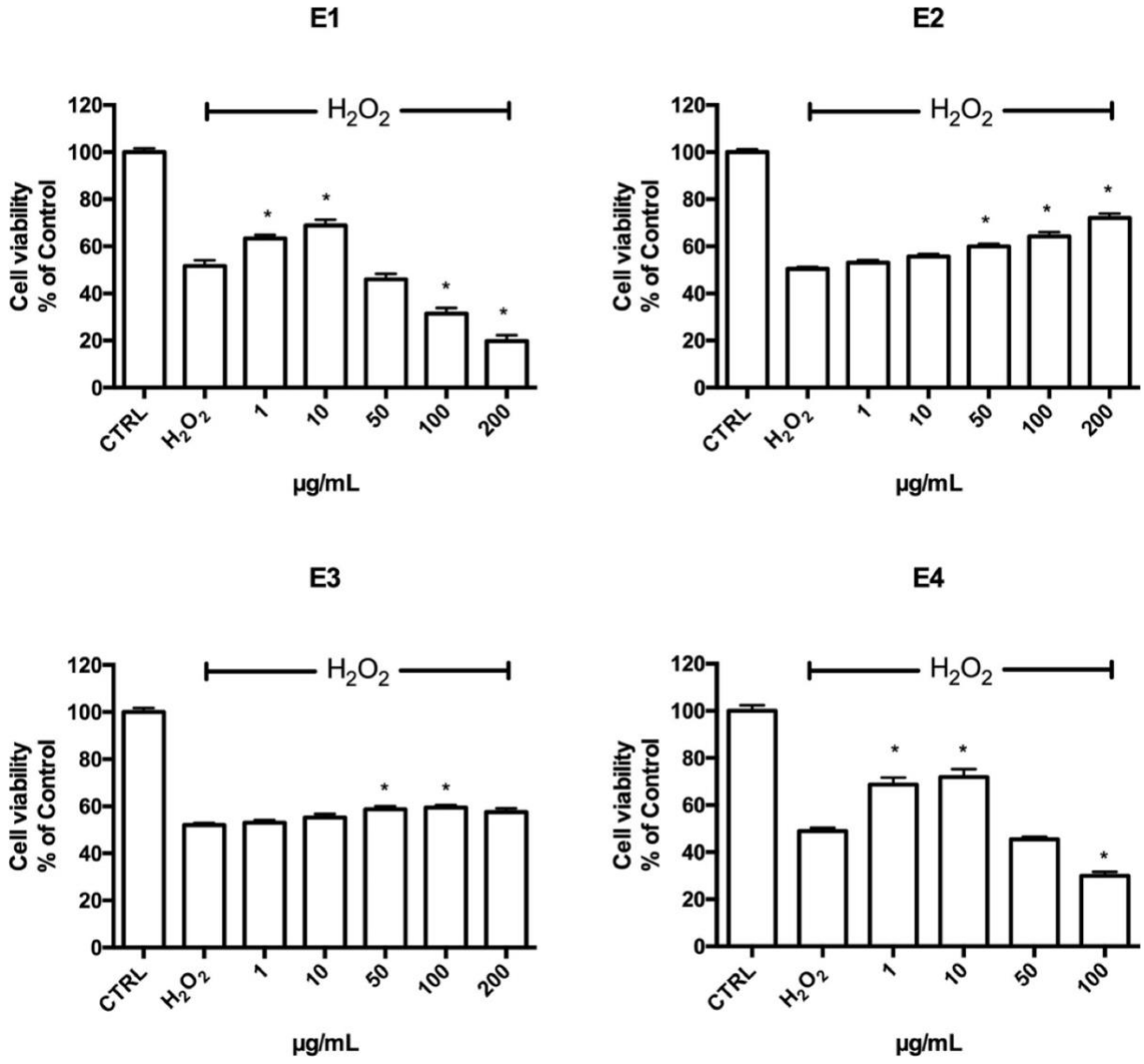

*Figure 57. Effect of E1, E2, E3 and E4 against H_2O_2-induced damage on cell viability in differentiated SH-SY5Y cells. Cells were pre-treated with different concentration (1-200 µg/mL) of E1, E2, E3 and E4 and then exposed to H_2O_2 700 µg/mL for 1 h. cell viability was evaluated by MTT assay. Each bar represents the mean ± SEM of at least four independent experiments. Data were analyzed by one-way ANOVA followed by Bonferroni's test. *p<0.05 compared to H_2O_2.*

Subsequently, to study more in depth the observed protective effect cells were pre-treated with 1-200 µg/mL of the 4 extracts before H_2O_2 (400 µM, 15 min) exposure and intracellular ROS levels were measured by DCFH-DA.

Consistently with the viability results, E1 and E4 were the most effective in counteract ROS production. Notably, 10 and 50 µg/mL of E1 significantly decreased intracellular ROS, while 50 and 100 µg/mL of E4 significantly reduced ROS levels. E2 and E3 had an effect in reducing intracellular ROS levels only at the highest concentration (Figure 58).

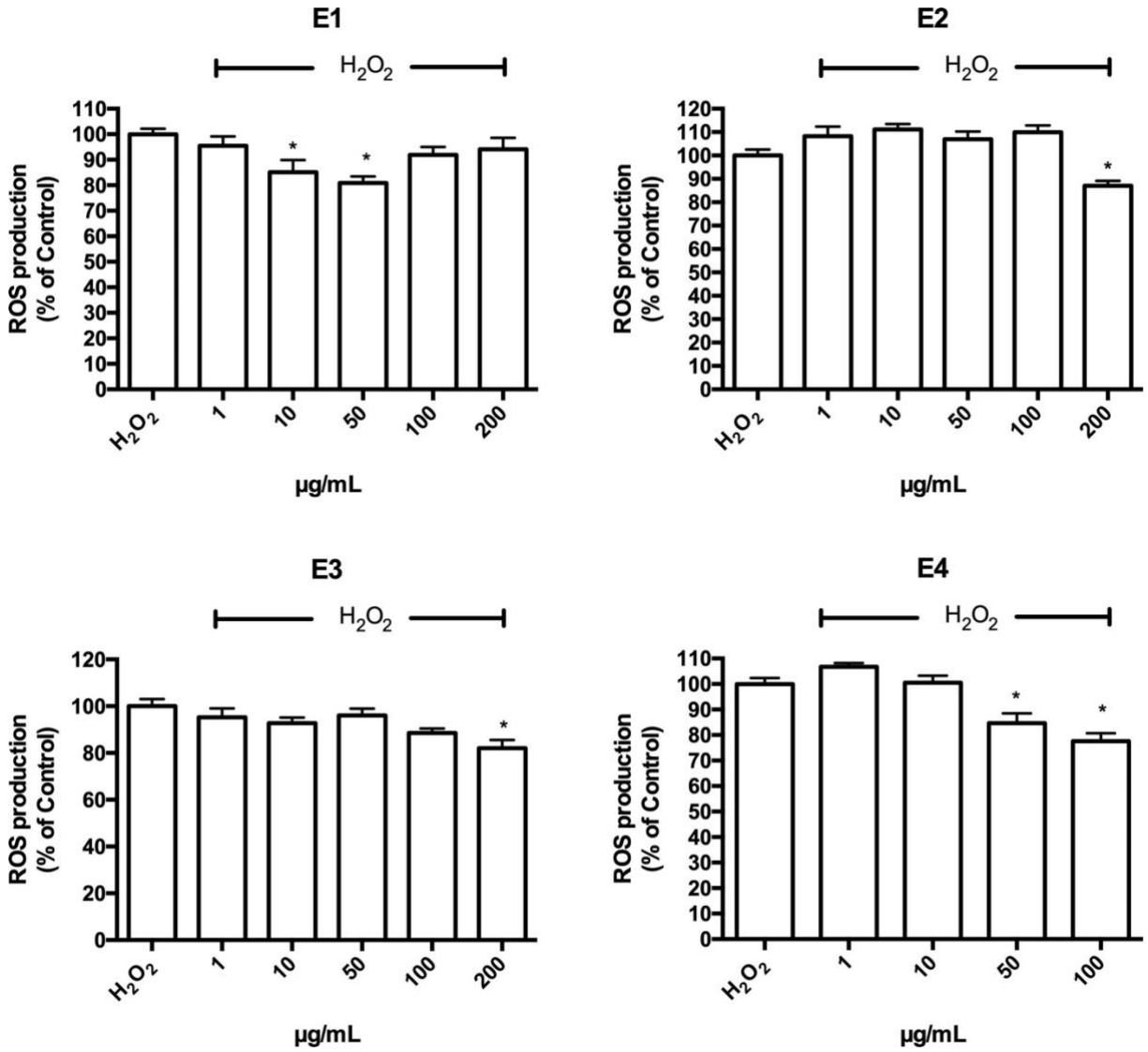

*Figure 58. Effect of E1, E2, E3 and E4 against H_2O_2-induced ROS production in differentiated SH-SY5Y cells. Cells were pre-treated with different concentration (1-200 µg/mL) of E1, E2, E3 and E4 and then exposed to H_2O_2 400 µg/mL for 15 min. Intracellular ROS levels were measured by DFDH-DA fluorescent assay. Data are expressed as % compared to H_2O_2-treated cells. Each bar represents the mean ± SEM of at least four independent experiments. Data were analyzed by one-way ANOVA followed by Bonferroni's test. *p<0.05 compared to H_2O_2.*

8.3.2 Antioxidant activity of SCG

8.3.2.1 Neurotoxicity of SCG extracts in neuronal differentiated SH-SY5Y cells.

Cells were treated with increasing concentrations (1-200 μg/mL) of E1, E2, E3 and E4 obtained by SCG for 24 h and cell viability was measured by MTT assay. Only 200 μg/mL of E1 significantly reduced cell viability in respect to untreated cells, while in all other cases no cytotoxic effect was detected and in some cases the treatment with the extracts determined a significant increase of cell viability in respect to control cells (Figure 59).

To clarify whether the observed increase was related only to increased mitochondrial activity, cell viability was evaluated with different assays. Cells were treated with 50 μg/mL for 24 h of each SCG extracts and Trypan Blue was used to assess both cell viability and total cell number. Interestingly, in both assays no treatment rose cell viability compared to the controls (Figure 60).

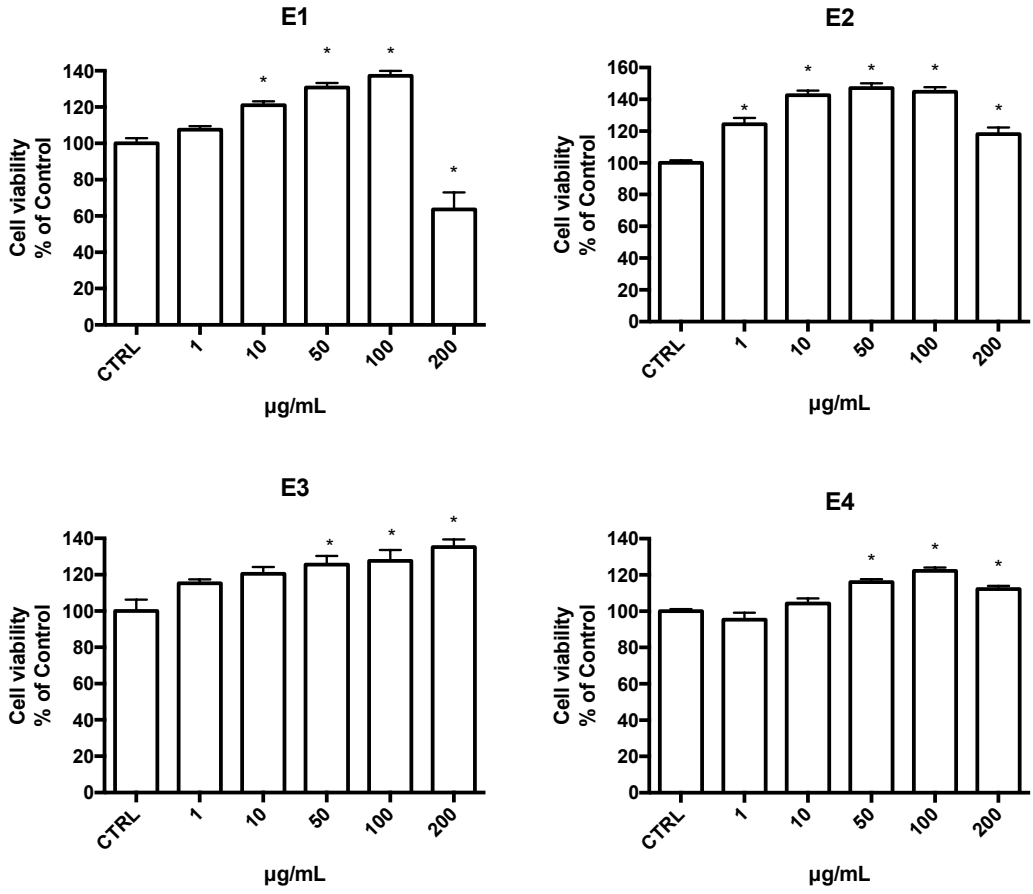

*Figure 59. Neurotoxicity of SCG extracts in neuronal differentiated SH-SY5Y. Cells were treated with increasing concentration (1–200 μg/mL) of E1, E2, E3, and E4 for 24 h and then viability was evaluated by MTT assay. Each bar represents the mean ± SEM of at least four independent experiments. Data were analyzed by one-way ANOVA followed by Dunnett's test. *p<0.05 compared to control (CTRL).*

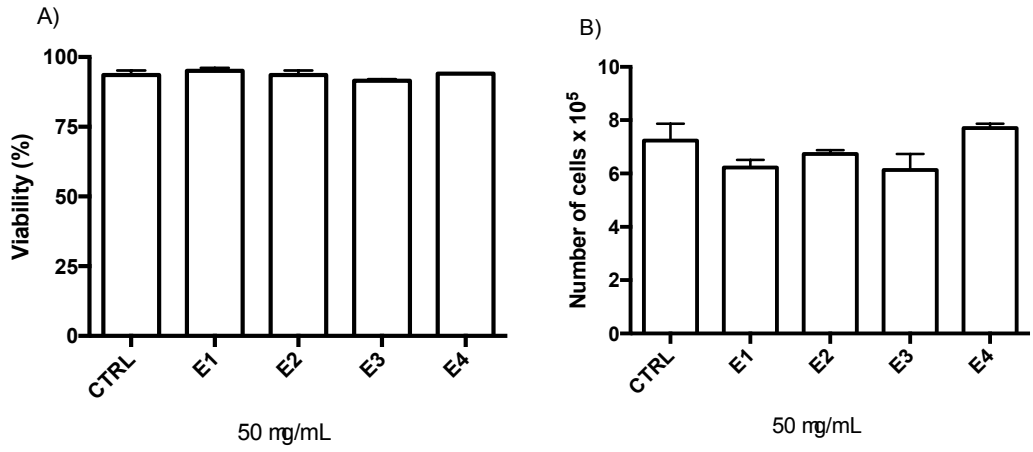

*Figure 60. Neurotoxicity of SCG extracts in neuronal differentiated SH-SY5Y. Cells were treated with 50 μg/mL of E1, E2, E3, and E4 for 24 h and then Trypan Blue was used to assess A) cell viability and B) total cell number. Each bar represents the mean ± SEM of at least four independent experiments. Data were analyzed by one-way ANOVA followed by Dunnett's test. *p<0.05 compared to control (CTRL).*

8.3.2.2 Effect of SCG extracts in neuronal differentiated SH-SY5Y against oxidative stress.

To evaluate the antioxidant activity of the SCG extracts differentiated SH-SY5Y cells were pre-treated with 1-100 μg/mL of each extract for 24 h and then exposed to 700 μM H_2O_2 for 1 h to create an oxidative stress condition. Only E4 significantly increased cell viability compared to H_2O_2-treated cells at the lowest concentration, while E1 showed a protective effect starting from 10 μg/mL. On the other hand, E2 and E3 counteracted oxidative stress at higher concentrations: E3 significantly increased cell viability in respect to H_2O_2-treated cells at 50 and 100 μg/mL, meanwhile E2 only at the highest concentration (Figure 61).

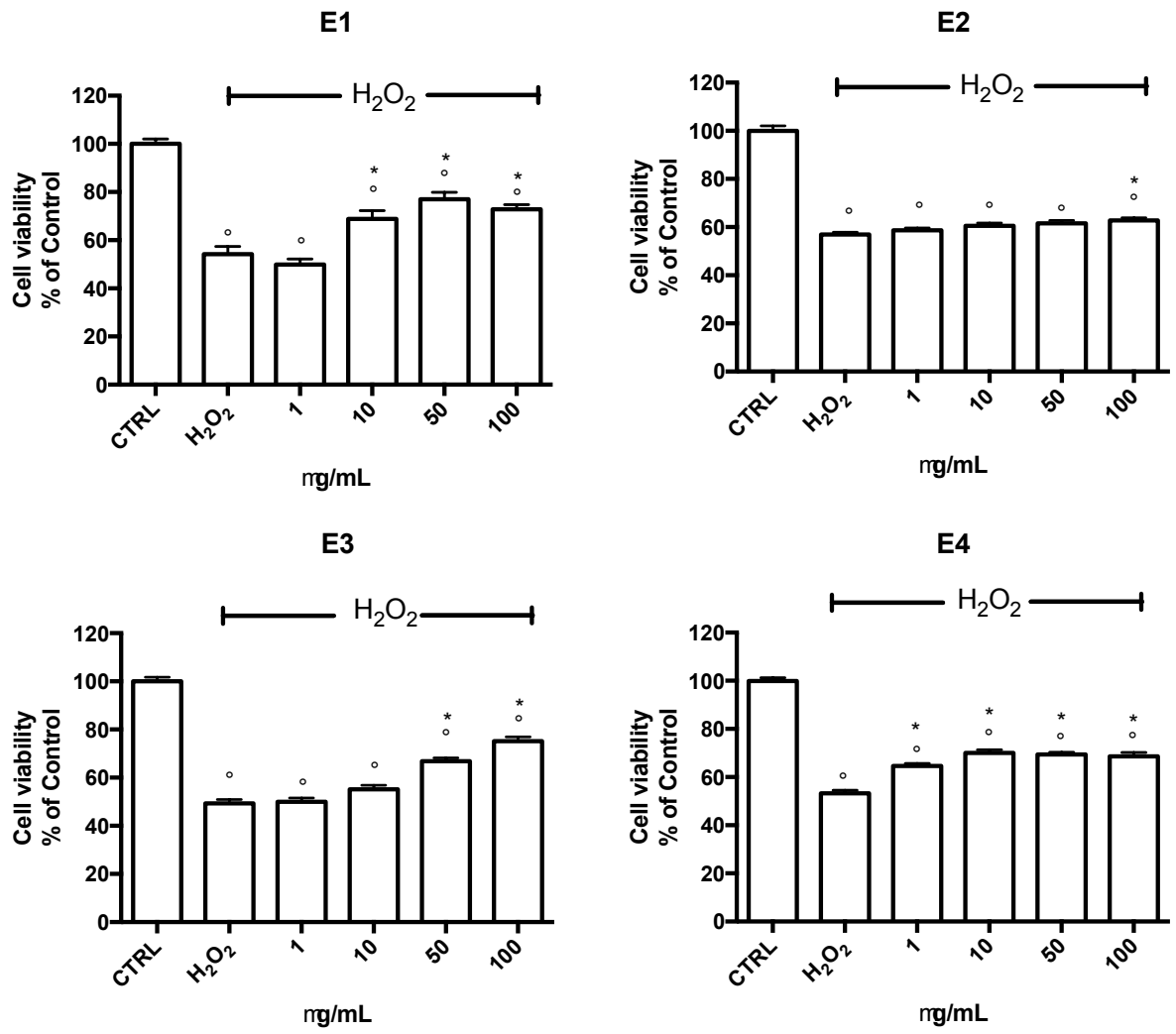

Figure 61. Cytoprotective effect of SCG extracts in neuronal differentiated SH-SY5Y against oxidative stress. Cells were treated with increasing concentration (1–100 μg/mL) of E1, E2, E3, and E4 for 24 h, then exposed to H_2O_2 700 μg/mL for 1 h and cell viability was evaluated by MTT assay. Each bar represents the mean ± SEM of at least four independent experiments. Data were analyzed by one-way ANOVA followed by Bonferroni's test. °p<0.05 compared to control (CTRL),

**p<0.05 compared to H_2O_2.*

To deeper investigate the antioxidant profile of the tested extracts, differentiated SH-SY5Y cells were pre-treated with increasing concentration (1-100 μg/mL) of E1, E2 E3 and E4 before inducing oxidative stress (H_2O_2 400 μM for 15 minutes) and intracellular ROS levels were measured by DCFH-DA fluorescent assay. E1 and E4 were the extracts associated with the highest effect, whereas E3 significantly reduced intracellular ROS only at 100 μg/mL and E2 had no effect on this parameter (Figure 62).

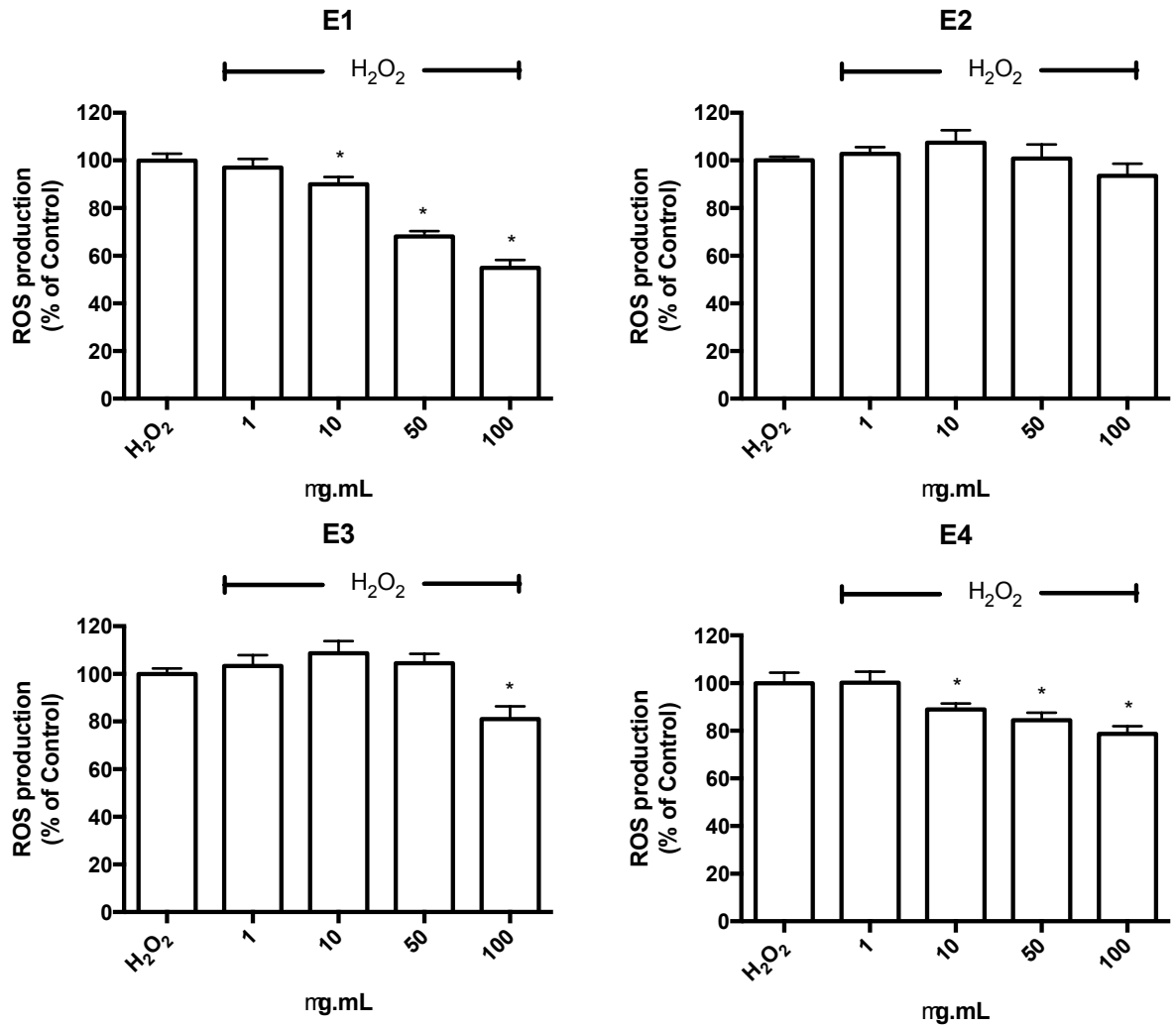

*Figure 62. Effect of SCG extracts against H_2O_2-induced ROS production in differentiated SH-SY5Y cells. Cells were pre-treated with different concentration (1-100 µg/mL) of E1, E2, E3 and E4 and then exposed to H_2O_2 400 µg/mL for 15 min. Intracellular ROS levels were measured by DFDH-DA fluorescent assay. Data are expressed as % compared to H_2O_2-treated cells. Each bar represents the mean ± SEM of at least four independent experiments. Data were analyzed by one-way ANOVA followed by Dunnett's test. *$p<0.05$ compared to H_2O_2.*

8.3.2.3 Effect of SCG extracts on the modulation of the endogenous antioxidant system in differentiated SH-SY5Y cells.

To better study the observed antioxidant activity of the four SCG extracts, the gene expression of the main antioxidant enzymes, glutathione peroxidase (GR), heme oxygenase 1 (HO1), NADP(H) oxidoreductase 1 (NQO1) and thioredoxin reductase (TRX) was evaluated by RT-PCR. This assay was carried out both in basal and stress conditions. First, differentiated SH-SY5Y cells were treated with 50 µg/mL of each SCG extracts for 5 h before RNA extraction. All the extracts were able to induce a significant up-regulation of HO1, NQO1 and TRX, while the expression of GR was significantly up-regulated only by E1, E3 and E4 (Figure 63).

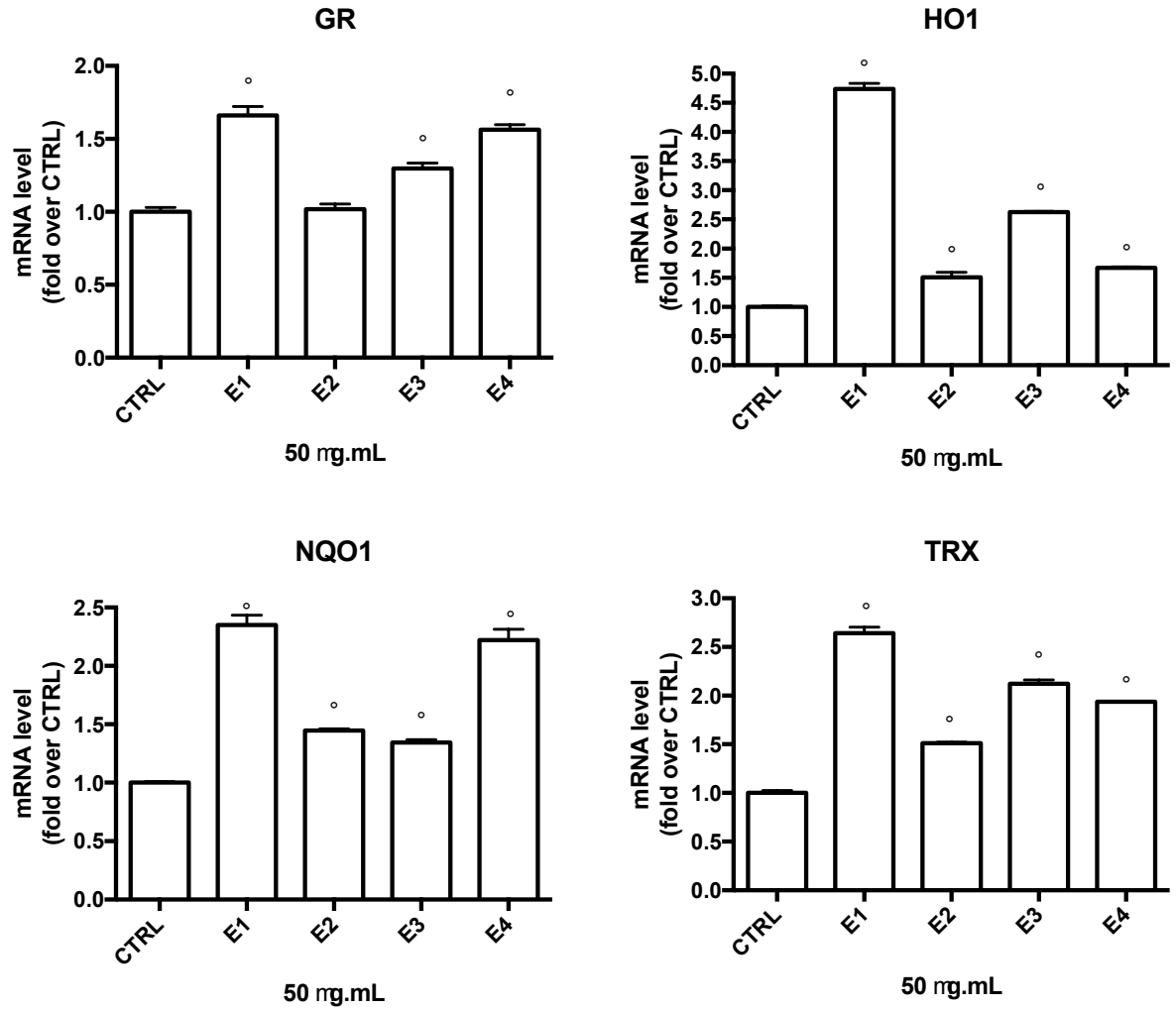

Figure 63. Effect of SCG extracts on the gene expression of antioxidant enzymes in differentiated SH-SY5Y cells. Cells were treated with 50 µg/mL of E1, E2, E3, and E4 for 5 h and then RT-PCR was carried out to measure GR, HO1, NQO1 and TRX mRNA levels. Data are reported as relative abundance in respect with control cells (CTRL). Each bar represents the mean ± SEM of at least three independent experiments. Data were analyzed by one-way ANOVA followed by Dunnett's test. °p<0.05 compared to CTRL.

Subsequently, the same evaluation was done in stress condition. Differentiated SH-SY5Y cells were treated with 50 µg/mL of each SCG extracts for 5 h and the exposed to 700 µM H_2O_2 for 1 h to induce oxidative stress. At the end of the experiments RT-PCR was carried out. As expected, the exposure to H_2O_2 significantly downregulated the expression of all tested genes compared to controls. Interestingly, E1 significantly and strongly up-regulated all the tested genes in respect to controls and H_2O_2-treated cells. E2 slightly but significantly increased HO1 and NQO1 expression with respect to only stressed cells, whereas had no effect on the modulation of GR an TRX expression. E3 treatment was able to up-regulate the expression of NQO1 compared to H_2O_2-treated cells and of HO1 and TRX with respect to both control and H_2O_2-exposed cells. E4 significantly increased the mRNA

levels of GR with respect to H_2O_2, and up-regulated HO1, NQO1 and TRX in respect to both control and stressed cells (Figure 64).

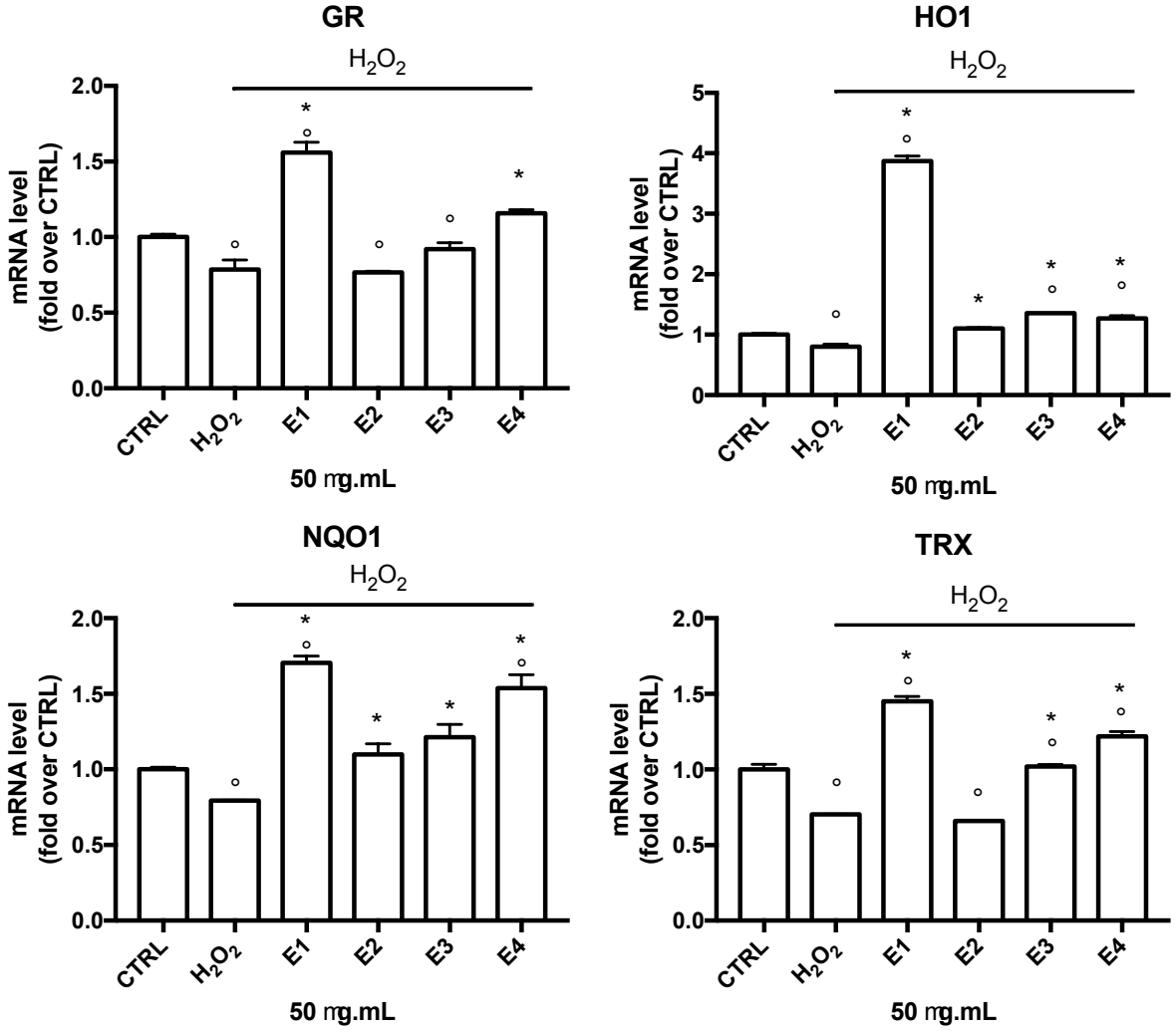

Figure 64. Effect of SCG extracts on the gene expression of antioxidant enzymes in differentiated SH-SY5Y cells in presence of H_2O_2. Differentiated SH-SY5Y cells were treated with 50 μg/mL of each SCG extracts for 5 h and the exposed to 700 μM H_2O_2 for 1 h to induce oxidative stress. At the end of the experiments RT-PCR was carried out measure GR, HO1, NQO1 and TRX mRNA levels. Data are reported as relative abundance in respect with control cells (CTRL). Each bar represents the mean ± SEM of at least three independent experiments. Data were analyzed by one-way ANOVA followed by Bonferroni's test.

*°$p<0.05$ compared to CTRL, *$p<0.05$ compared to H_2O_2.*

8.3.2.4 Effect of SCG extracts on the protein expression of HO1 in differentiated SH-SY5Y cells.

Taking into consideration the strong modulation of HO1 compared to the other tested genes, an immunoblotting analysis was performed to study HO1 induction also at a protein level. Cells were treated with 50 μg/mL of E1, E2, E3 and E4 for 24 h and the exposed to 700 μM H_2O_2 for 1 h. At the

end of the experiments, the protein expression of HO1 was measured by western blot analysis. H_2O_2 exposure led to a decrease, although not significant, of HO1 level in respect to control cells. In agreement with the RT-PCR results, E1 treatment was able to strongly and significantly induced HO1 protein expression (Figure 65).

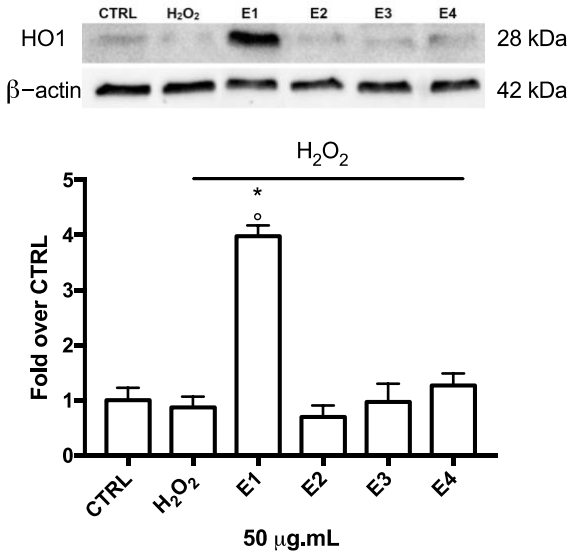

Figure 65. Effect of SCG extracts on protein expression of HO1 in SH-SY5Y differentiated cells. Cells were treated with 50 μg/mL of E1, E2, E3 and E4 for 24 h and the exposed to 700 μM H_2O_2 for 1 h. Immunoblotting was carried out using anti-HO1. Data are expressed as fold over control (CTRL) and normalized by -actin. Each bar represents the mean ± SEM of at least three independent experiments. Data were analyzed by one-way ANOVA followed by Bonferroni's test.

*°p<0.05 compared to CTRL, *p<0.05 compared to H_2O_2.*

8.3.3 Anti-inflammatory activity of SCG extracts

8.3.3.1 Cytotoxicity of SCG extracts in microglial BV-2 cells.

The potential anti-inflammatory activity of SCG extracts was evaluated in microglial BV-2 cells. First aspect studied was the cytotoxicity of the four extracts (E1, E2, E3 and E4) in BV-2 cells using MTT assay. Cells were treated with increasing concentrations (1-200 μg/mL) of E1, E2, E3 and E4 for 24 h and cell viability was measured. All extracts significantly reduced cell viability compared to control cells only at 200 μg/mL, whereas at all other concentrations no cytotoxicity was detected (Figure 66). On this basis, the concentration 200 μg/mL was excluded from the subsequent experiments.

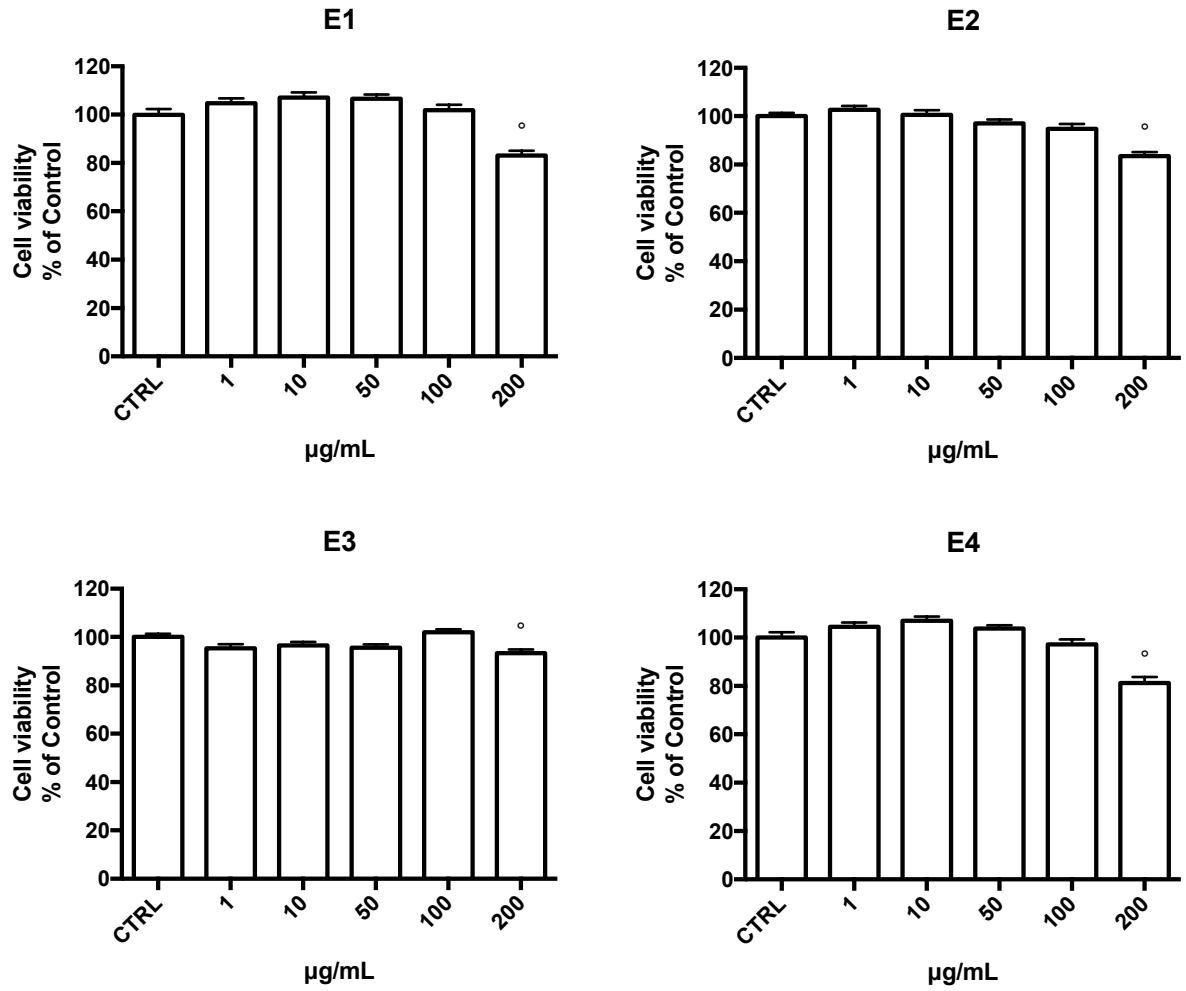

*Figure 66. Cytotoxicity of SCG extracts in microglial BV-2 cells. Cells were treated with increasing concentration (1–200 µg/mL) of E1, E2, E3, and E4 for 24 h and then viability was evaluated by MTT assay. Each bar represents the mean ± SEM of at least four independent experiments. Data were analyzed by one-way ANOVA followed by Dunnett's test. *p<0.05 compared to control (CTRL).*

8.3.3.2 Effect of SCG extracts in microglial BV-2 cells against LPS-induced inflammation.

Prior, to investigate the anti-inflammatory activity of SCG extracts the ability of the four extracts to counteract the LPS-induced damage was evaluated in term of cell viability. BV-2 cells were pre-treated with different concentration (1-100 µg/mL) of E1, E2, E3 and E4 for 24 h and then cells were exposed to LPS (100 ng/mL) for further 24 h to induce an inflammatory condition. At the end of the experiments cell viability was measured by MTT assay.

As expected, the exposure to LPS caused a reduction in cell viability by ≈ 40% compared to the controls. E1, E2 and E4 significantly increased cell viability in respect to LPS-exposed cells, meanwhile E3 did not show any protective effect against the inflammatory process (Figure 67).

Of note, at 50 µg/mL E1, E2 and E4 showed a strongly protective activity maintaining cell viability at values comparable to the controls, so 50 µg/mL was chosen as the best concentration to continue the study.

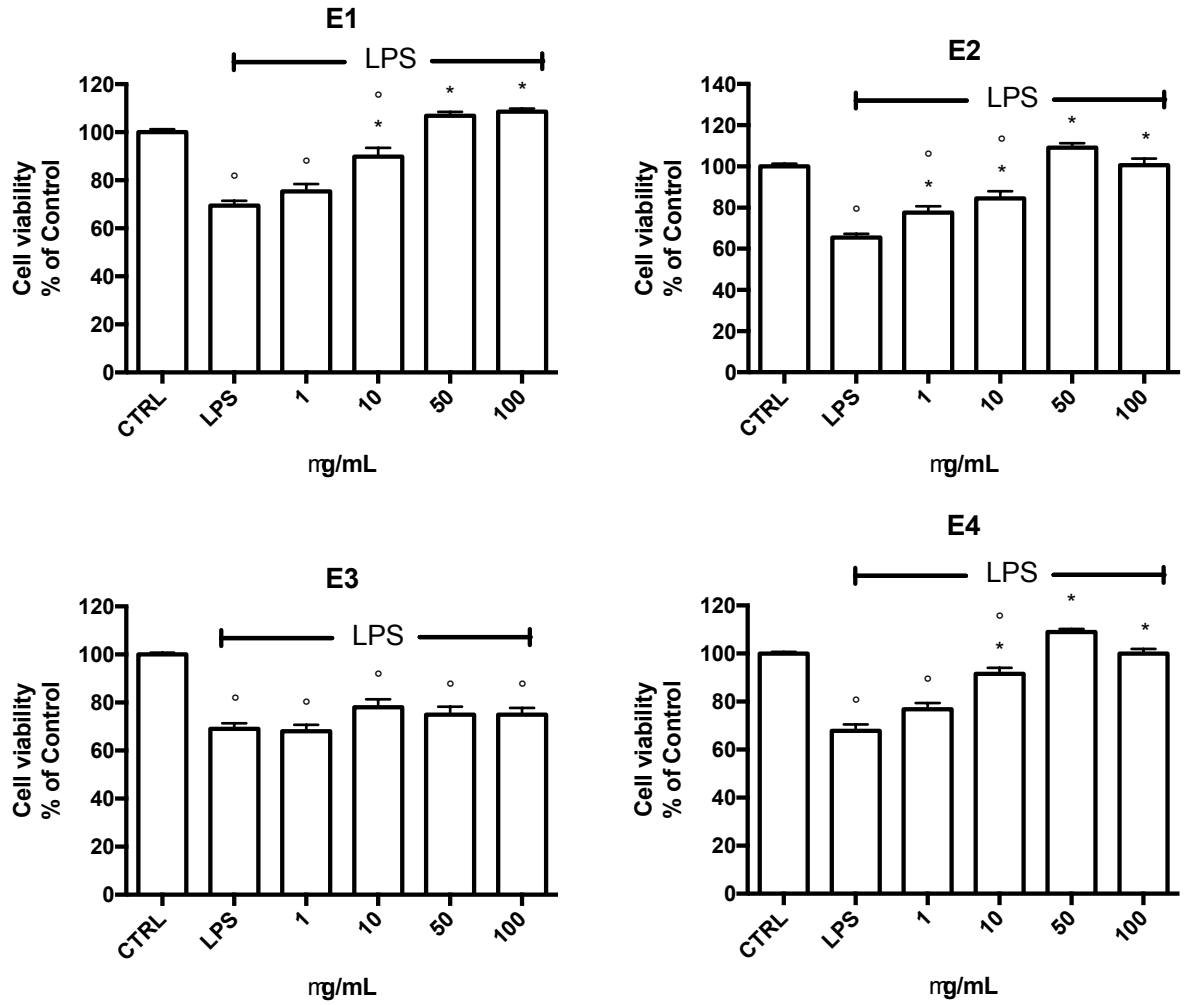

*Figure 67. Effect of SCG extracts on cell viability of BV-2 against LPS-induced damage. Cells were treated with increasing concentration (1–100 μg/mL) of E1, E2, E3, and E4 for 24 h before LPS exposure (100ng/mL for further 24h) and then viability was evaluated by MTT assay. Each bar represents the mean ± SEM of at least four independent experiments. Data were analyzed by one-way ANOVA followed by Dunnett's test. °p<0.05 compared to control (CTRL), *p<0.05 compared to LPS.*

It is widely known that exposure to LPS also promotes ROS production establishing an oxidative stress condition [312], [313]. To this end, cells were pre-treated with 50 μg/mL of each extract before LPS exposure (100 ng/mL for further 24 h) and then intracellular ROS production was evaluated by the fluorescent probe DCFDH-DA. Predictably, LPS exposure induced a significant increase of intracellular ROS levels compared to control cells. In agreement with the data obtained in SH-SY5Y cells, E1, E3 and E4 were able to counteract the LPS-mediated oxidative stress significantly reducing ROS levels in respect with LPS-treated cells, while E2 had no effect on this parameter (Figure 68).

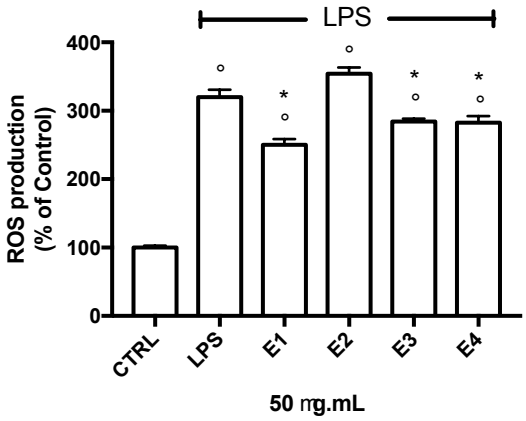

*Figure 68. Effect of SCG extracts against LPS-mediated ROS production in BV-2 cells. Cells were pre-treated with different concentration (1-100 μg/mL) of E1, E2, E3 and E4 and then exposed to LPS 100ng/mL for further 24 h. Intracellular ROS levels were measured by DFDH-DA fluorescent assay. Data are expressed as % compared to control cells. Each bar represents the mean ± SEM of at least four independent experiments. Data were analyzed by one-way ANOVA followed by Bonferroni's test. °p<0.05 compared to control (CTRL), *p<0.05 compared to LPS.*

8.3.3.3 Effect of SCG extracts on the modulation of pro-inflammatory mediator gene expression in LPS-stimulated BV-2 cells.

To further investigate the anti-inflammatory potential, the ability of SCG extracts to modulate the gene expression of some of the main pro-inflammatory mediators was evaluated. For this purpose, the mRNA levels of tumor necrosis factor α (TNF-α), interleukin 1β (IL-1β), cyclooxygenase 2 (COX-2), and inducible nitric oxide synthase (iNOS) were measured by RT-PCR. BV-2 cells were pre-treated with 50 μg/mL of each extract and then exposed to 100 ng/mL LPS for further 24 h. LPS increased the expression of all tested gene compared to controls. In agreement with viability results, E3 significantly increased only the expression of IL-1β, while it had no effect on TNF-α and COX-2 expression and slightly, although significantly, reduced the mRNA levels of iNOS. On the other hand, E1 and E4 were able to induce a significant reduction of iNOS and COX-2 expression with respect to LPS-exposed cells, whereas they were not able to modulate the expression of IL-1β. Regarding TNF-α expression, E1 and E4 had opposite behaviours: E1 significantly reduced the LPS-mediated expression of this cytokine, meanwhile E4 significantly increased it compared to LPS-treated cells. Interestingly, E2 was able to strongly and significantly inhibit the LPS-induced expression of all tested genes (Figure 69).

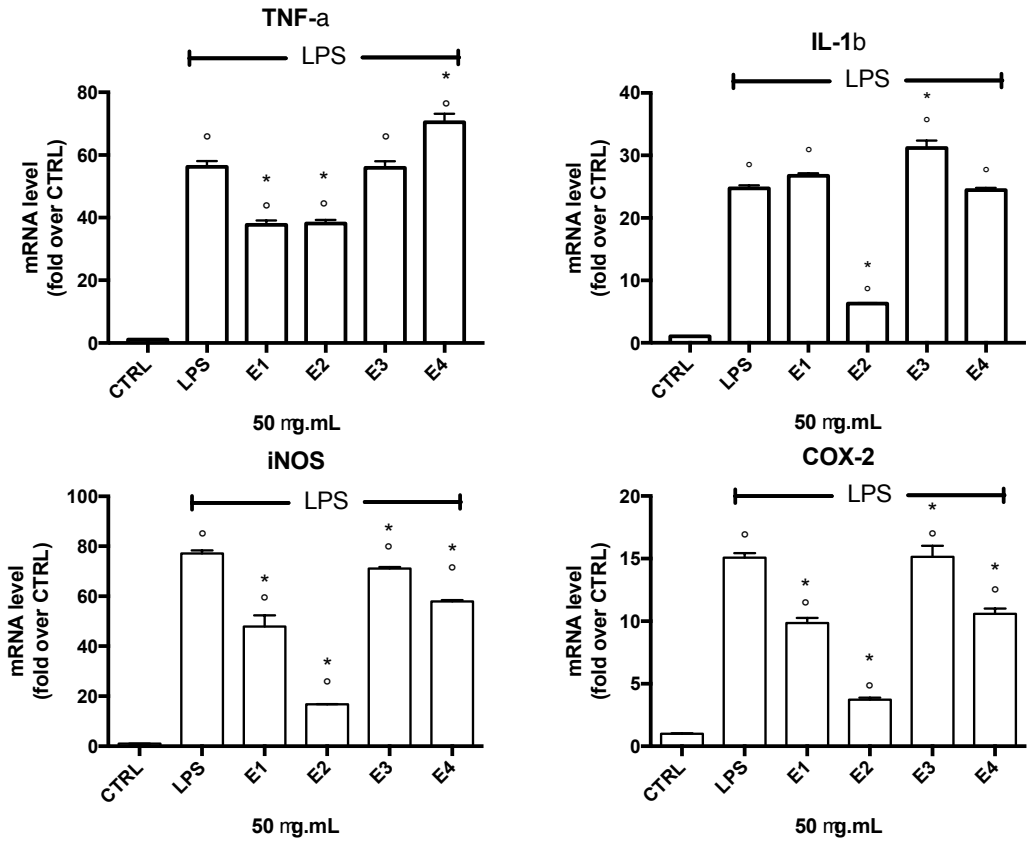

Figure 69. Effect of SCG extracts on the gene expression of pro-inflammatory cytokines and enzymes in LPS-stimulated BV-2 cells. Cells were pre-treated with different concentration (1-100 μg/mL) of E1, E2, E3 and E4 and then exposed to LPS 100ng/mL for further 24 h. At the end of the experiments RT-PCR was carried out measure IL-1β, TNF-α, iNOS and COX-2 mRNA levels. Data are reported as relative abundance in respect with control cells (CTRL). Each bar represents the mean ± SEM of at least three independent experiments. Data were analyzed by one-way ANOVA followed by Bonferroni's test.

*°p<0.05 compared to CTRL, *p<0.05 compared to LPS.*

8.3.3.4 Anti-inflammatory effect of SCG extracts on NF-kB/TLR4 signaling pathway in LPS-stimulated BV-2 cells.

To better clarify the mechanisms by which the extracts exert their anti-inflammatory activity, the modulation of the NF-kB/TLR4 signaling pathway was studied. First, cells were pre-treated with 50 μg/mL of each extract before LPS exposure (100 ng/mL for further 24 h) and then NF-kB activation was studied through confocal microscopy. At the end of the treatments, cells were immunostained with a primary antibody against NF-kB p65, followed by Alexa Fluor 488-conjugated secondary antibody. As expected, LPS exposure strongly stimulated the levels of NF-kB and led to a clear translocation of this pro-inflammatory transcription factor to the nucleus compared to control cells. In accordance with the previous results, E1, E2 and, to a lesser extent, E4 decreased protein levels of NF-kB compared to LPS-exposed cells. On the contrary, E3 was not able to modulate the expression of this factor (Figure 70).

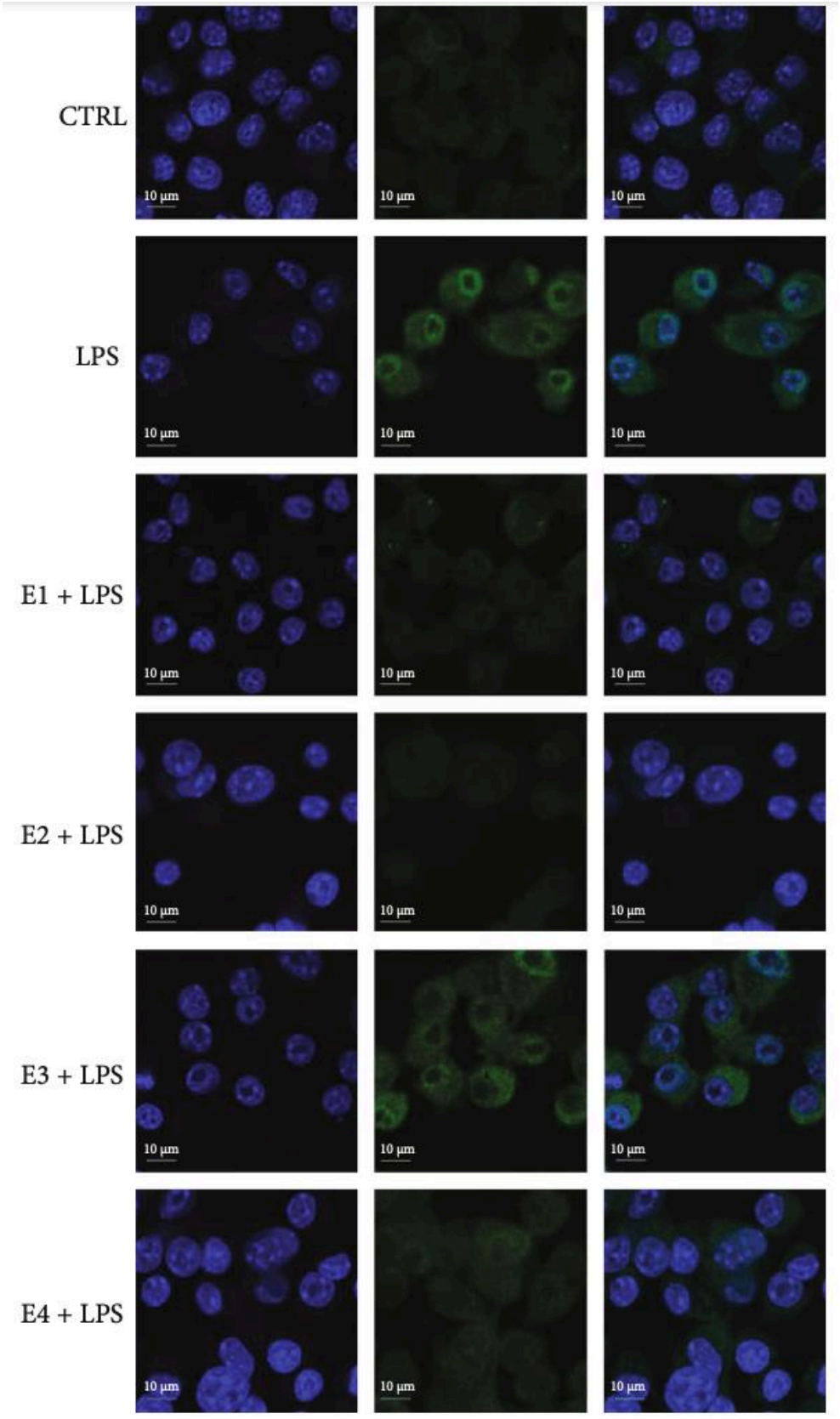

Figure 70. Modulation of NF-κB expression by SCG extracts in LPS-stimulated BV-2 cells. Cells were pre-treated with different concentration (1-100 µg/mL) of E1, E2, E3 and E4 and then exposed to LPS 100ng/mL for further 24 h. BV-2 cells were immunostained with a primary antibody against NF-κB p65 followed by secondary Alexa Fluor 488-conjugated antirabbit IgG antibody (green), and cell nuclei (blue) were visualized with DAPI. Scale bars: 10 µm.

Considering that the induction of NF-kB is closely correlated with the dimerization of TLR4, the levels of this receptor have also been investigated by cytofluorimetric analysis. Predictably, LPS strongly and significantly triggered an increase of TLR4 membrane expression compared to the controls. Perfectly in agreement with what has been seen above, E2 significantly reduced TLR4 surface expression in respect with both LPS-treated cells and control cells. E1, E4 and, to a lesser extent, E3 significantly decreased TLR4 surface expression compared to LPS-exposed cells (Figure 71).

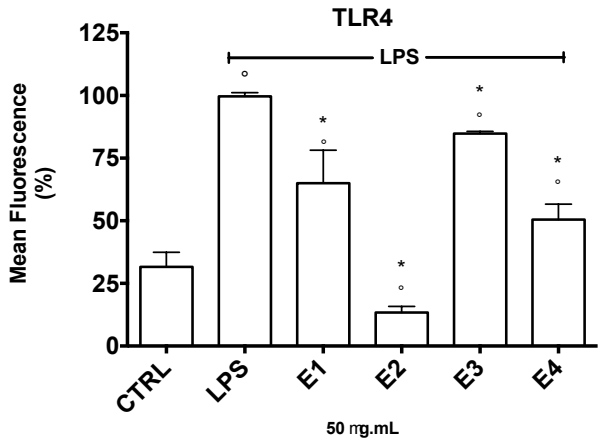

*Figure 71. Effect of SCG extracts on cell surface expression of TLR4 in LPS- stimulated BV-2 cells. Cells were pre-treated with 50 µg/mL of E1, E2, E3 and E4 before LPS activation (100ng/mL for further 24 h) and then TLR4 surface expression was evaluated by flow cytometry. Data are expressed as % compared to LPS-activated cells. Each bar represents the mean ± SEM of at least three independent experiments. Data were analyzed by one-way ANOVA followed by Bonferroni's test. °p<0.05 compared to control (CTRL), *p<0.05 compared to LPS.*

8.4 DISCUSSION

Neurodegenerative diseases, among which Parkinson's disease (PD), Alzheimer's disease (AD), amyotrophic lateral sclerosis (ALS), Huntington's disease (HD) and spinocerebellar ataxia (SCA) [38], [429]–[431], are characterized by an impairment of cognitive and motor functions due to progressive neuronal damage [73].

In this context, identifying molecules that can prevent/counteract neurodegeneration through their antioxidant and anti-inflammatory activities may be a valid strategy. Nutritional research is paying increasing attention to the characterization of phytochemicals and nutraceuticals that may act as potential neuroprotective agents.

Furthermore, in a circular economy perspective, in recent decades it has been found that by-products/waste materials from the food industry could be a rich source of biologically active compounds with health-promoting properties.

More and more attention has recently been paid to the valorization of by-products from coffee industry. It is well known that coffee is one of the most widely consumed beverages in the world, and huge amount of waste material are inevitably obtained from its processing.

In recent decades, coffee has been widely associated with positive health effects and, in particular, with a preventive/beneficial effect on several neurological and neurodegenerative disorders [416], [421]–[424]. Tons of by-products of the coffee industry are produced every year worldwide [383].

In particular, the potential protective effect of coffee and its by-products, such as SCG and CSS, is related to the presence of a wide range of substances with proven biological properties such as caffeine, chlorogenic acid, polyphenols, carotenoids, melanoidins and many other antioxidant compounds [392], [396], [432],[403].

Considering this, in this study we evaluate the potential neuroprotective effect (antioxidant and anti-inflammatory) of 4 different extracts obtained from SSC and SCG.

Both Silverskin and spent coffee extracts were obtained by 4 different extraction methods (E1 _MeOH, E2_H_2O, E3_MeOH/ H_2O (50:50), E4_EtOH/ H_2O (70:30)) and characterized in terms of bioactive compounds.

Interestingly, the treatment of SH-SY5Y with all the extracts led to a significant increase of cell viability in respect to control cells and we suggest that this increase can be attributed to an improvement in mitochondrial respiration. In fact, the MTT assay measures cell viability in terms of the ability of living cells to convert tetrazolium salts into water-insoluble formazan crystals by mitochondrial dehydrogenases. On this basis, the MTT assay is correlated with mitochondrial respiration [433], [434], so it can be assumed that this increase in cell viability could be due to an enhancement of mitochondrial respiration. To confirm this observation, we used other methods to

measure cell viability of SH-SY5Y treated cells. The results demonstrated that the extracts did not influence the ratio between live and dead cells and the number of total cells, so the increase of cell viability observed by MTT assay is probably associate to an enhancement of the mithocondrial respiration.

This promotion of mitochondrial respiration could be attributable to the high caffeine content of the extracts. Several studies have shown that caffeine, by stimulating the expression of the peroxisome proliferation-activated receptor gamma coactivator 1-alpha (PGC-1α) [435]–[437], a key factor in the regulation of nuclear respiratory factors 1 and 2 (NRF1/2), induces a positive stimulation of mitochondrial content [438]–[440]. Furthermore, mitochondrial transcription factor A (TFAM), which is crucial in modulating the respiratory components of the electron transport chain, is upstream regulated by PGC-1α and NRF1/2 [438]–[442]. Human muscle fibers treated with caffeine also reported an increase in mitochondrial respiration rates and a simultaneous decrease in mitochondrial membrane potential, highlighting a direct effect of caffeine on mitochondrial respiration [443].

Based on the quantification of the bioactive compounds present in the extracts, there is no direct correlation between the observed viability increase and caffeine content. This suggests a synergistic effect of other compounds contained in the extracts that could influence caffeine metabolism and absorption. To better clarify this aspect, in the future, it might be interesting to evaluate the effect of pure caffeine on neuron-like SH-SY5Y cells.

The CSS and SCG extracts were then tested for their ability to counteract oxidative stress induced by hydrogen peroxide in SH-SY5Y cells.

Interestingly the methanolic (E1) and EtOH/H2O (E4) extracts of both CSS and SCG were the most effective in counteracting oxidative stress.

Regarding CSS extracts, the observed antioxidant activity of E1 and E4 in SH-SY5Y was in agreement with the results obtained by DPPH assay. Interestingly, E1 in respect to the other extracts present a higher total polyphenolic content and the higher concentration of epicatechin and 5-CQA

Caffeoylquinic acid (CQA), an ester consisting of one quinic and one caffeic acid molecule, has several isomers [444]. Among these, 5-CQA has been shown to have the greatest antioxidant activity in vitro [444]. Moreover, its effect was also studied on TNF-α-exposed EA.hy926 endothelial cells, in which it resulted in a reduction of ROS levels and a recovery of depleted GSH [445]. Also, in PMA + IFNγ-stimulated Caco2 cells, pre-treatment with 5-CQA showed similar effects [446], as it resulted in increased reduced GSH, reduced intracellular ROS and activated the Nrf2 signaling pathway.

Epicatechin, due to its biological functions, can also be considered a good antioxidant. Indeed, it is able to eliminate free radicals, reduce lipid peroxidation and produce protein complexes [447]. The deterioration of spatial memory induced by Aβ25-35, was reduced in rats pre-treated with

epicatechin, probably through an antioxidant and ant-inflammatory mechanisms in the hippocampus [448]. Recently, a study in neuronal cells showed that methamphetamine-induced neuronal death via oxidative and ER stress was reduced by epicatechin. Following methamphetamine-induced apoptosis, treatment with epicatechin inhibited ROS production, DR4 and CHOP expression and MAPK activity.

Furthermore, it was seen that mouse cortical neurons damaged by oxygen/glucose deprivation through upregulation of the antioxidant enzyme heme oxygenase 1 and activation of the Nrf2-mediated pathway were protected by epicatechin [449]. This molecule was also able to increase HO1 activity and modulate the Nrf2 pathway in Nrf2 and HO1 knockout mice [450]. It has been shown by the same authors that epicatechin is able to counteract H_2O_2 /tert-butyl hydroperoxide-induced oxidative stress in embryonic cortical neuronal cells. On this basis, it can be assumed that the higher 5-CQA and epicatechin content of E1 compared with the other extracts justifies its protective effect.

The antioxidant effect of E4 could be associated with its higher content of quercitrin, p-coumaric acid, 3,5-diCQA and ferulic acid, as all these phenolic compounds counteracted oxidative damage in various cell lines. P-coumaric acid is a hydroxy derivative of cinnamic acid and counteracted Aβ toxicity and reduced ROS production in PC12 neuronal cells [451]. Treatment with ferulic acid, a phenolic acid derivate, in neuronal cells protected, through several mechanisms including induction of the cellular stress response, neurons from nitrosative/oxidative stress caused by exposure to Aβ or other radical initiators [452]. 3,5-diCQA, another caffeoylquinic acid derivative, counteracted H_2O_2-induced activation of caspase-3 and cell death in SH-SY5Y cells [453], as well as restoring H_2O_2-induced reduction of intracellular GSH. Quercitrin, a glycosylated form of quercetin, shows better absorption than quercetin due to the binding of the sugar to the aglycone portion, which increases its solubility in polar solvents [454]. The antioxidant and anti-inflammatory effect of quercitrin is well documented [455]. Quercitrin counteracted the production of ROS and malondialdehyde (MDA), enhanced antioxidant enzyme activities,

reduced tissue plasminogen activator (t-PA) activity and inhibited the induction of cytochrome P450 2E1 (CYP2E1) in ICR mice treated with carbon tetrachloride [456] demonstrating its antioxidant effect.

On the other side, results obtained with SCG extracts revealed that, according to the data obtained from the DPPH assay on this group of extracts, E1 and E4 were the extracts associated with the highest antioxidant effect, while extract E2 was associated with the lowest effect. Inefficient extraction of low-polarity compounds, of which coffee is rich and which are known for their high antioxidant activity [457], [458], could be the cause of the low antioxidant effect of E2. There is

increasing evidence to support the indirect mechanism by which some phytochemicals perform their antioxidant actions, ie, they act by increasing the expression of cytoprotective proteins and antioxidant enzymes [459]–[462].

Although both groups of tested coffee extracts (CSS and SCG extracts) showed an extremely promising antioxidant profile, comparing the significance of their respective antioxidant effects, a higher efficacy of extracts derived from SCGs than those from CSSs can be observed. In addition, taking into consideration that SCGs are the most abundant waste from the coffee industry (55-67% of the total waste produced [380] every day) and the consequent urgency of proper disposal and, possibly, re-use as renewable sources of them, the 4 SCG extracts were selected for further investigation of both the antioxidant and inflammatory profiles.

Therefore, to further study the antioxidant profile of the 4 SCG extracts, the effect of their pre-treatment on the modulation of gene expression of the main antioxidant enzymes (GR, HO-1, NQO1, and TRX) was evaluated by RT-PCR in both basal and stressful conditions. In basal conditions, all the extracts were able to induce a significantly up-regulation of HO1, NQO1 and TRX, while the expression of GR was significantly up-regulated only by E1, E3 and E4. The same evaluation was done in the presence of H_2O_2 and, as expected, the exposure to H_2O_2 significantly down-regulated the expression of all tested genes compared to controls and, given the short exposure to H_2O_2 (1 h), this downregulation is probably associated with H_2O_2-induced mRNA oxidation. Interestingly, E1 was the most effective extract in increasing the expression of the tested genes. In particular E1 treatment strongly up-regulated HO1 and these results were also confirmed immunoblotting analysis.

Chemical characterization of the extracts showed that E1 is associated with the highest content of (-)-epicatechin and isogentisin. Although there is no correlation between the tested biological parameters and the content of (-)-epicatechin, instead there is both a positive correlation between the content of isogentisin with the protection against H_2O_2 ($r = 0{:}9745$, $p < 0{:}05$) / GR expression ($r = 0{:}9575$, $p < 0{:}05$), and an inverse correlation with levels of ROS ($r = -0{:}9604$, $p < 0{:}05$).

Isogentisin is a xanthone typical of plants belonging to the Gentianaceae family [463]. Although few research have investigated its biological properties, it has been shown to inhibit monoamine oxidases type A and B in rat brain [464], [465] and counteract smoke damage in human umbilical vein endothelial cells (HUVECs) [466].

The involvement of monoamine oxidases in disorders such as anxiety, depression, and neurodegenerative diseases, including Alzheimer's and Parkinson's diseases, is widely recognized [467], [468]. In the future, it will be interesting to continue the study to evaluate both the ability of E1 to inhibit monoamine oxidases and the antioxidant effect of pure isogentisin in neurons.

Following the promising data on the antioxidant profile of the extracts under study, it was investigated another fundamental factor involved in neurodegenerative diseases, inflammation. For this purpose, it was evaluated the potential protective effect of the 4 SCG extracts on the microglial cell line BV-2. Microglia constitute the macrophages of the brain and represent the main defense system of the brain against various types of insults to which the CNS may be exposed. Microglia, through phagocytosis of dead cells, protein aggregates, cellular debris and pathogens, and through the production of neurotrophic factors, are essential for the protection of the brain and the regulation of neuronal survival under physiological conditions [318].

However, excessive activation of these cells can trigger and sustain neurotoxic processes through the release of proinflammatory molecules such as nitric oxide (NO), tumor necrosis factor-α (TNF-α), interleukins (IL-1β and IL-6), and ROS [319]. Therefore, as shown in several studies, the development and course of neurodegenerative diseases, such as Alzheimer's and Parkinson's, is strongly influenced by the action of microglia [320]–[322].

The potential anti-inflammatory effect of the extracts was, therefore, evaluated following exposure of BV-2 microglial cells to LPS. LPS is the most widely used and accredited pro-inflammatory mediator in literature to simulate inflammation in vitro, as it activates microglial cells and triggers the inflammatory signaling cascade [310], [311]. Exposure of BV-2 cells to LPS resulted in a ~40% reduction of cell viability compared to controls. E1, E2 and E4 significantly increased cell viability in respect to LPS-exposed cells, meanwhile E3 did not show any protective effect against the inflammatory process.

Of note, at 50 μg/mL E1, E2 and E4 showed a strongly protective effect maintaining cell viability at values comparable to the controls, so 50 μg/mL was chosen as the best concentration to continue the study. It is widely known that exposure to LPS also promotes ROS production establishing an oxidative stress condition [312], [313], therefore, the intracellular levels of ROS in BV-2 cells were also evaluated. Similarly to what was found in SH-SY5Y cells, all extracts, except E2, determined significant decreases in intracellular ROS levels compared to LPS-treated cells.

To better elucidate the mechanisms underlying the observed anti-inflammatory effect, the influence of the extracts on the gene-level modulation of the main pro-inflammatory cytokines and enzymes (TNF-α, IL-1β, COX-2 and iNOS) was assessed by RT-PCR. In agreement with viability results, E3 was the less effective extract in reducing these inflammatory mediators, on the other hand E2 was able to strongly inhibit the LPS-induced expression of all tested genes proving to be the most effective. Data obtained from RT-PCR analysis are partially in agreement with the viability results which showed similar behaviour of E1, E2 and E4 against LPS-induced damage, and full protection against inflammation for all of them at the concentration 50 μg/mL. This inconsistency could be

clarified by assuming different mechanisms by which these different extracts counteract inflammation. For example, E2 exerts its anti-inflammatory effect by strongly reducing the expression of all pro-inflammatory mediators evaluated. Otherwise, the anti-inflammatory action of E1 and E4 must be explained also by their antioxidant activity as there is a strict connection between inflammation and oxidative stress. The production and release of pro-inflammatory cytokines and chemokines is also modulated by another key inflammatory mediator, NF-kB, which in turn is activated by Toll-like receptors (TLRs) [469]. Several studies have shown that the development of neurodegenerative diseases is strongly favoured by the activation of NF-kB and the release of its subunits [470], [471]. In addition, this transcription factor is also involved in the transcriptional regulation of TNF-α, IL-1β, iNOS and COX-2. To further clarify the mechanisms by which the extracts exert their anti-inflammatory action, the ability of the extracts to modulate NF-kB activation was also assessed by confocal microscopy. In agreement with the previous results, E1, E2 and, to a lesser extent, E4 decreased protein levels of NF-kB compared to LPS-exposed cells. On the contrary, E3 was not able to modulate the expression of this factor. As mentioned above, TRL receptors play a key role in the activation of the inflammatory cascade. In particular, Toll-like receptor 4 (TLR4), of which LPS is an accredited ligand [472], is one of the main receptors in modulating microglia activation and the release of pro-inflammatory mediators. TLR4 dimerization triggers an NF-kB-mediated signaling cascade that leads to microglia activation [473]. In this context, the modulatory capacity of extracts on TLR4 expression on the cell surface was also assessed by cytofluorometry. Perfectly in agreement with what has been seen above, E2, was the most effective in reducing TLR4 membrane expression in respect to LPS-treated cells and control cells. Interestingly, there is no specific correlation between the chemical composition (in terms of the presence of specific compounds) of the extracts and the observed anti-inflammatory effect. In particular, E2, which is the one with the greatest anti-inflammatory effect, contains the same compounds of the other extracts at concentrations comparable or even lower. Thus, this suggests the presence in E2 of yet unidentified bioactive compounds and future studies should be carried out to better characterize this extract.

In conclusion, it can be stated that the use of different solvents resulted in a different composition of the extracts. In particular, the extracts that proved to be the richest in total bioactive compounds were those in MeOH:H_2O (E3) and EtOH:H_2O (E4). On the other hand, biological data showed that these two extracts were not those associated with the greatest antioxidant or anti-inflammatory effect. In particular, E2 (MeOH) was the one associated with the greatest antioxidant effect on neuron-like SH-SY5Y cells, as it reduces intracellular ROS levels and up-regulates the expression of NQO1, GR, TRX and HO1. As mentioned above, this greater antioxidant effect of E1 could be associated with its higher isogentisin content compared to the other extracts.

On the other hand, regarding anti-inflammatory activity, it was E2 (H_2O) that was associated with the greatest effect, as it is able to down-regulate the expression of pro-inflammatory mediators through modulation of the TLR4/NF-kB pathway. As mentioned above, the chemical composition of E2 does not support its major effect therefore it will be interesting to further characterize the extracts in the future.

In conclusion, both CSSs, for their antioxidant activities, and SCGs, due to their antioxidant and anti-inflammatory activities, can be considered as a valid strategy to prevent and/or counteract neurodegeneration.

9 CONCLUSION

Neurodegenerative disorders are a kind of chronic degenerative disease manifested by a progressive loss of the normal structure and function of the central nervous system. The pathophysiology of these diseases, of which the main ones are Parkinson's, Alzheimer's and Huntington's disease, derives from the complex interaction of both genetic and environmental factors. While the genetic forms of these disorders are sporadic and characterized by an earlier onset, the higher percentage of cases of these diseases is mainly due to the combined effect of environmental factors and lifestyle alterations [474] and represent a health problem mainly affecting the elderly.

Ageing is a process characterized by the accumulation of biological alterations that inevitably lead, over time, to the functional decline of the organism and to the risk of developing pathologies such as cancer, diabetes, cardiovascular, musculoskeletal and neurodegenerative diseases [475]. The increase in the average age of the population, which has occurred and will continue to occur in the coming decades, is inevitably linked to an increase in the incidence of chronic degenerative diseases.

Although neurodegenerative diseases present distinct symptoms and clinical features, they share common mechanisms. In particular, the etiology of this degeneration involves a number of concomitant factors that lead to the generation of a multifactorial toxic reaction that is manifested by dysfunction of the ubiquitin-proteasome system, mitochondrial dysfunction, an activated inflammatory cascade, oxidative stress, impairment of the normal neuronal survival pathways, leading to apoptosis/neuronal necrosis and cell death [474], [476]. The proven multifactorial etiology of these pathologies, in which oxidative stress and inflammation are the main causative factors [1], has prompted the scientific world to pay increasing attention to the characterization and identification of molecules that do not have a "single molecular target", but have an intrinsic pleiotropic activity.

Taking into account the complex multifactorial etiology of these disorders and the scarceness and low effectiveness of currently available therapies, the role of natural agents and plant extracts as valid therapeutic (alone or in combination with synthetic drugs) [474] and preventive solutions is increasingly emerging.

Moreover, in a circular economy perspective, in the last decades it has been found that processing by-products/waste materials of the food industry can represent a rich source of biologically active compounds with beneficial properties for health. The researchers have not only stopped at the evaluation and study of primary products of the agro-food industry as a source of nutraceutical

compounds, but, pursuing the basic principles of circular economy, have shown increasing interest in the valorization of by-products normally considered as waste [475].

In light of these observations, nutritional research, both from a preventive and therapeutic perspective, includes these features and qualities. In fact, more and more attention has been paid to nutraceuticals and phytochemicals as possible tools, for their antioxidant and anti-inflammatory properties, to contrast the onset of these deleterious diseases.

Therefore, the aim of this PhD program was to identify nutraceuticals and phytochemicals, both as extracts and pure compounds and obtained from both plant and renewable sources, which due to their antioxidant and anti-inflammatory properties, were able to counteract cellular and molecular alterations that characterize neurodegenerative diseases. Their neuroprotective potential (antioxidant and / or anti-inflammatory) has been evaluated in an in vitro model of neuroinflammation (the BV-2 microglial cell line activated with LPS), and / or in an in vitro model of neuronal oxidative stress (the neuron-like SH-SY5Y cell line differentiated with retinoic acid and exposed to H_2O_2).

Following the data obtained in the 4 projects developed during this PhD project, it is therefore possible to conclude for each of them:

Study 1_ Impact of phenolic profile of different cherry cultivars on the potential neuroprotective effect in SH-SY5Y cells

New sweet cherries from Unibo breeding program can be considered a new functional food with a high antioxidant and neuroprotective activity. Furthermore, their protective activity seems to vary according to the specific phenolic pattern of the different cherry extracts.

Study 2_Anti-inflammatory activities of Spilanthol-rich essential oil from Acmella oleracea (L.) R.K. Jansen and its nanoemulsion.

Acmella oleracea essential oil (OA), having shown both anti-inflammatory and antioxidant properties, can be considered as a valid strategy to counteract neurodegenerative disorders. Moreover, the potential of this essential oil could be further enhanced if included it in an OA nanoemulsion, as a new technological formulation.

Study 3_Study of the anti-inflammatory activity of novel tacrine derivatives with lipids extracted from cashew nutshell liquid

In the pattern of studied compounds (hybrids 5-17), molecules 5 and 6 were identified as the most effective anti-inflammatory compounds. Indeed, 5 and 6 reduced the expression levels of the main mediators of inflammation (such as TNF-a, IL-1β, COX2 and iNOS), the levels of the pro-inflammatory cytokine IL-1β and the nuclear translocation of the transcription factor NF-kB, becoming candidates as potential novel drugs for the treatment of neurodegenerative diseases.

Study 4_Coffee Silverskin (CSS) and Spent Coffee Grounds (SCG): coffee industry by-products as a promising source of neuroprotective agents

Coffee by-products have proven to be a valuable source of biologically active compounds with high health-beneficial potential. Both extracts obtained from Coffee Silverskin (CSSs), for their antioxidant activities, and extracts obtained from Spent Coffee Grounds (SCGs), for their antioxidant and anti-inflammatory activities, which are both coffee industry by-products, can be considered as a valid strategy to prevent and/or counteract neurodegeneration.

In general, it can be concluded that the natural compounds studied in this thesis have been proven, due to their antioxidant and/or anti-inflammatory properties, to be valid preventive and therapeutic strategies for the treatment of neurodegenerative diseases, to improve the life quality of these patients and of the general population by preventing and combating the onset of these deleterious diseases.

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