

# Evaluation of neuroprotective potential of polyphenols derived from Portuguese native plants: *Juniperus* sp. and *Rubus* sp.

Lucélia Rodrigues Tavares



Dissertation presented to obtain the Ph.D degree in Biochemistry  
Instituto de Tecnologia Química e Biológica | Universidade Nova de Lisboa

Oeiras,  
November, 2012



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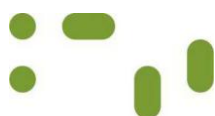
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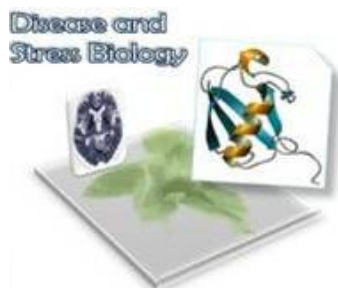


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## Abbreviation list

Abbreviation	Term
$\Delta\Psi_m$	Mitochondrial transmembrane potential
4CL	4-coumarate:CoA ligase
6-OHDA	6-hydroxydopamine
8-OHdG	8-hydroxydeoxyguanosine
A $\beta$	$\beta$ -amyloid
ABC	ATP-binding cassette
AC	Antioxidant capacity
ACC	Acetyl-CoA carboxylase
ACh	Acetylcholine
AChE	Acetylcholinesterase
ACT	Actin
AD	Alzheimer's disease
Aha1	ATPase homolog 1
Akt	Protein kinase B
ALP	Autophagy-lysosome pathway
ALS	Amyotrophic lateral sclerosis
ANR	Anthocyanidin reductase
ANS	Anthocyanidin synthase
AP-1	Activating protein-1
APC/CDC20	Anaphase-promoting complex/cell division cycle 20 homolog
APP	Amyloid precursor protein
Arc	Activity-regulated cytoskeleton-associated protein
ARE	Antioxidant response element
Bcl-2	B-cell lymphoma 2
BDNF	Brain-derived neurotrophic factor
bp	Base pair
Brca	Breast and ovarian cancer susceptibility protein
BuChE	Butyrylcholinesterase
C3H	p-coumarate 3 hydroxylase
C4H	Cinnamate 4-hydroxylase
cAMP	Cyclic adenosine monophosphate
CAT	Catalase
CBF	Cerebral blood flow
CE	Catechin equivalents
CFTR	Cystic fibrosis transmembrane conductance regulator
CHI	Chalcone isomerase
CHS	Chalcone synthase
CNS	Central nervous system
COP	Coat protein complex
COX	Cyclooxygenase
CREB	cAMP response element binding
CyDAGlc	Cyanidin-3-O-dioxyglucoside
CyGlc	Cyanidin-3-O-glucoside
CyHMGG	Cyanidin-3-O-hydroxymethyl-glutaroyl glucoside
CyMalG	Cyanidin-3-O-malonylglucoside
CyXyl	Cyanidin-3-O-xyloside
CyXyl	Cyanidin-3-O-xyloside
DAPI	4',6-diamidino-2-phenylindole
DAVID	Database for Annotation, Visualization and Integrated Discovery
DCF	2',7'-dichlorofluorescein
DFR	Dihydroflavonol reductase
DG	<i>Dentate gyrus</i>
DiOC <sub>2</sub> (3)	3,3'-dihexyloxacarbocyanine iodide
DSB	Double strand break
DW	Dry weight
EA	Ellagic acid
ECAC	European Collection of Cell Cultures

EF1A	Elongation factor 1 $\alpha$
EGCG	Epigallocatechin gallate
eIF2	Eukaryotic translation initiation factor 2
EMEM	Eagle Minimum Essential Medium
ENaC	Epithelium sodium channel
ER	Endoplasmic reticulum
ERK	Extracellular-signal-regulated kinase
ET	Ellagitannin
F3'5'H	Flavonoid 3'5'-hydroxylase
F3'H	Flavonoid 3'-hydroxylase
F3GT	Flavonoid-3-glucosyltransferase
F3H	Favanone 3-hydroxylase
FA	Formic acid
FBS	Foetal bovine serum
FLS	Flavonol synthase
FS	Flavone synthase
GA	Golgi apparatus
GABA <sub>x</sub>	Gamma-aminobutyric acid
GAE	Gallic acid equivalents
GAPDH	Glyceraldeide 3-phosphate dehydrogenase
GCN2	General control nonrepressed 2
GI	Gastrointestinal
GITC	Guanidine Isothiocyanate
GPx	Glutathione peroxidase
GR	Glutathione reductase
GRAS	Generally Recognized As Safe
Grx	Glutaredoxin
GSH	Glutathione (reduced form)
GSK 3	Glycogen synthase kinase 3
GSSG	Glutathione disulphide
H <sub>2</sub> DCFDA	2',7'-dichlorofluorescein diacetate
HD	Huntington's disease
HNE	4-hydroxynonenal
HO-1	Heme oxygenase
HSD	Honest Significant Difference
HSP	Heat shock protein
IAPs	Inhibitors of apoptosis
IL-1 $\beta$	Interleukin-1 $\beta$
iNOS	Inducible nitric oxide synthase
IVD	<i>In vitro</i> digestion
JNK	c-Jun N-terminal kinase
KV	Voltage-gated K <sup>+</sup>
Lamb	Lambertianin C
LAR	Leucoanthocyanidin reductase
LC-MS	Liquid chromatography-mass spectrometry
LPO	Lipid peroxidation
m/z	Mass-to-charge ratio
MAPK	Mitogen-activated protein kinase
MEAD	Methyl ellagic acid derivative
MeEAGlcA	Methyl ellagic acid glucuronide
MeEApent	Methyl ellagic acid pentose
MeJa	Methyl jasmonate
MEK	Mitogen-activated protein kinase kinase
MMR	Mismatch repair
MPP <sup>+</sup>	1-methyl-4-phenylpyridinium
Chk2	Cell cycle checkpoint kinase 2
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MRE11	Meiotic recombination element 11
MRN	MRE11/Rad50/NBS1 complex
mTOR	Mammalian target of rapamycin

NBT	Nitro blue tetrazolium
NDDs	Neurodegenerative diseases
NER	Nucleotide excision repair
Nf- $\kappa$ B	Nuclear factor- $\kappa$ B
NFT	Neutrophin
NHEJ	Non-homologous end joining
NMDA	N-Methyl-D-aspartic acid
NP	Natural product
NQO1	NAD(P)H dehydrogenase, quinone 1
Nrf2	Nuclear factor erythroid 2-related factor 2
OMT	o-methyltransferase
OPA	Orthophthalaldehyde
ORAC	Oxygen radical absorbance capacity
OST	Oligosaccharyltransferase
PAL	Phenylalanine ammonia-lyase
PBS	Phosphate buffer saline
PCD	Programmed cell death
PCNA	Proliferating cell nuclear antigen
PCR	Polymerase chain reaction
PD	Parkinson's disease
PDA	Photo diode array
PEF	Phenolic-enriched fraction
PG	Post gastric (digest)
PI	Propidium iodide
PI3K	Phosphoinositide 3-kinase
PKA	cAMP-dependent protein kinase
PLA2	Group II phospholipase A2
POLR2A	DNA-directed RNA polymerase II subunit A
PP	Polyphenol
PP1-cat	Protein phosphatase catalytic subunit
PP2A	Protein phosphatase 2A catalytic subunit
PP2C	Protein phosphatase 2C
QGlc	Quercetin-glucoside
QGlcA	Quercetin-glucuronide
QHMGG	Quercetin-HMG-glucoside
QHMGGlc	Quercetin-HMG-glucoside
Qpent	Quercetin pentose
Qrut	Quercetin-rutinoside
QXyl	Quercetin-xyloside
RAN	Ras-related nuclear protein
RMA	Robust Multi-array average
RNS	Reactive nitrogen species
RONS	Reactive oxygen and nitrogen species
ROS	Radical oxygen species
RT	Retention time
RT-PCR	Reverse transcription-polymerase chain reaction
Sang	Sanguin
SD	Standard deviation
SIRT1	Sirtuin 1
SN	<i>Substantia nigra</i>
SOD	Superoxide dismutase
SPE	Solid phase extraction
STS	Stilbene synthase
TE	Trolox equivalents
TFC	Total flavonoid content
TFIIH	Transcription factor II H
TOP	Topoisomerase
TPC	Total phenolic content
TrkB	Tyrosin-related kinase B
UFGT	UDP glucose-flavonoid 3-glucosyltransferase

UPS	Ubiquitin-proteasome system
UPW	Ultra pure water
XPC	Xeroderma pigmentosum complementation group G
XRE	Xenobiotic responsive element

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## Abstract

Neurodegenerative diseases have become a challenge to modern societies in industrialized countries due to the high social and economic impacts and therefore demand intensive research. These diseases present diverse common features, in which oxidative stress plays a very important role. As result of oxidative stress, some molecular mechanisms become dysfunctional and ultimately lead to cell death. Amongst the compounds displaying neuroprotective activity, phenolics have been identified amongst the most active substances from natural sources.

The purpose of the work presented in this thesis was to address the neuroprotective capacity of phenolic compounds from juniper leaves (*Juniperus* sp.) and blackberry fruits (*Rubus* sp.) from Portuguese native plants that display high phenolic content. Two different approaches in the prevention of neurodegenerative diseases could be foreseen: nutritional and pharmacological approaches.

Concerning the pharmacological approach, leaves from four junipers were evaluated and all displayed characteristics for promising neuroprotective products. Among these species, *Juniperus oxycedrus badia* was distinguished by the capacity of its phenolic compounds to protect neuronal viability and functionality. Junipers are evergreen plants with leaves available all the year, although their phenolic composition could be affected by environmental factors. Therefore, it is important to understand the seasonal dynamics of their phytochemical content and variation due to stress. In periods when active growth is retarded, particularly in winter, junipers had higher phenolic content. Juniper species displayed different susceptibilities to stresses, and salt stress enhanced the phenolic content of *J. oxycedrus badia*. The increase in total content was due to a general rise in all classes of compounds with a few alterations in the relative content of specific compounds. This increase was concomitant with the enhancement of transcripts encoding phenylalanine ammonia lyase, a key enzyme of the pathway responsible

by the production of polyphenols. Therefore, junipers phytochemicals yield can be modulated/maximized.

Related to the nutritional perspective, the impact of gastrointestinal digestion on blackberry polyphenols was assessed to ensure that the physiological conditions affecting foods were taken into account. This approach was then used to evaluate the neuroprotective potential of polyphenols from Portuguese wild blackberries compared to commercial blackberries. Although they presented few quantitative chemical differences, differential cytoprotective activities were discovered. Wild blackberry species were revealed as more effective in neuroprotection possibly through the mediation of molecular adaptive responses. Therefore, a transcriptomic approach was used to compare the differential molecular mechanisms mediating neuroprotection between commercial species and *R. vagabundus*. Blackberry metabolites were able to differentially prevent transcript alterations induced by oxidative stress, with wild blackberry more effective in reducing the number of genes altered. The main pathways differentially affected between stressed and blackberry pre-treated cells were related to transport, cell cycle and DNA repair. A common regulation by small GTP-binding proteins and/or associated pathways could be foreseen, although confirmatory biochemical assays are required. In conclusion, metabolites from *J. oxycedrus badia* and *R. vagabundus* show neuroprotection potential that could be explored in future studies.

## Resumo

As doenças neurodegenerativas têm-se tornado um desafio das sociedades modernas nos países industrializados, devido ao elevado impacto económico e social, requerendo por isso de uma investigação intensiva. Estas doenças apresentam diversas características em comum das quais se destaca o stress oxidativo. Como resultado deste, alguns mecanismos moleculares tornam-se disfuncionais podendo conduzir à morte celular. Entre os compostos apresentando atividade neuroprotetora, os compostos fenólicos têm sido identificados como entre as substâncias mais ativas obtidas da Natureza.

O objetivo do trabalho apresentado na presente tese foi de determinar a capacidade neuroprotetora de compostos fenólicos de folhas de zimbro (*Juniperus* sp.) e amoras de silva (*Rubus* sp.), obtidos de plantas nativas portuguesas com um elevado conteúdo em compostos fenólicos. Duas abordagens diferentes podem ser antecipadas na prevenção das doenças neurodegenerativas: farmacológica e nutricional.

Relativamente à abordagem farmacológica, folhas de quatro espécies de zimbro foram avaliadas e todas demonstraram características importantes para a obtenção de produtos neuroprotetores promissores. *J. oxycedrus badia* foi distinguido pela capacidade dos seus compostos fenólicos protegerem a viabilidade e funcionalidade neuronal. Sendo os zimbros plantas perenes, cuja composição fenólica das folhas pode ser afetada pelos fatores ambientais, revelou-se importante perceber a dinâmica sazonal dos fitoquímicos bem como as suas variações devido a stresses. Em períodos em que o crescimento ativo é retardado, particularmente no Inverno, os zimbros apresentaram um elevado conteúdo em compostos fenólicos. As diferentes espécies de zimbro apresentaram diferentes suscetibilidades aos stresses, tendo o stresse salino aumentado o conteúdo em polifenóis de *J. oxycedrus badia*. O aumento do conteúdo total deveu-se ao aumento geral de compostos das várias classes, apresentando poucas alterações no conteúdo relativo de compostos em

particular. Este aumento foi concomitante com o aumento de transcritos que codificam fenilalanina amónia liase, um enzima chave da via responsável pela produção de polifenóis. Verificou-se portanto que a produção de fitoquímicos nos zimbros pode ser modulada/maximizada. Quanto à perspectiva nutricional, de forma a garantir que as condições fisiológicas que afetam os alimentos eram contempladas, foi determinado o impacto da digestão gastrointestinal sobre os polifenóis de amoras. Esta abordagem foi posteriormente usada na avaliação do potencial neuroprotetor dos polifenóis de amoras silvestres portuguesas comparativamente com amoras comerciais. Apesar da composição química das amoras apresentar poucas diferenças quantitativas entre espécies, as atividades citoprotetoras foram diferenciadas. As espécies de amora silvestres mostraram-se mais efetivas na neuroprotecção, possivelmente devido à mediação de respostas adaptativas moleculares. Consequentemente, foi usada uma abordagem transcritómica para comparar os mecanismos moleculares que intervêm na neuroprotecção e que são diferentes entre as amoras comerciais e selvagens, particularmente *R. vagabundus*. Os metabolitos de ambas as amoras conseguiram prevenir as alterações nos transcritos induzidas pelo stresse oxidativo, apresentando-se a amora silvestre mais efetiva na redução do número de genes alterados. As principais vias diferencialmente alteradas entre células stressadas e pré-tratadas com amoras encontram-se relacionadas com funções de transporte, ciclo celular e reparação de DNA. Uma regulação comum pelas pequenas proteínas de ligação ao GTP e/ou vias associadas pode ser antecipada, apesar de ser necessário a realização de ensaios bioquímicos confirmatórios. Em conclusão, os metabolitos de *J. oxycedrus badia* e *R. vagabundus* mostram um potencial neuroprotetor que poderá ser explorado em estudos futuros.

## Thesis outline

This thesis is organized in seven chapters.

The first chapter (General introduction) brings in the main topics related to the work developed and necessary to its comprehension, namely neurodegenerative diseases, polyphenols and the possible role of polyphenols activities in protecting human body against neurodegeneration.

Presentation of experimental work is divided in two parts: *Juniperus* sp. (A) and *Rubus* sp. (B), where is presented work developed applied to species of these two genus.

In the second and third chapters are presented the results concerning the first type of strategy, the role of phenolic extracts from *Juniperus* sp. leaves as natural products. The second chapter rely on the potential neuroprotective activity of the extracts and the third chapter on understanding polyphenols dynamics in plants due to seasonality and stress induction.

The fourth, fifth and sixth chapters rely on a nutritional approach, where the neuroprotective potential of blackberries polyphenols are determined. In the fourth chapter it is highlighted the importance of using a more physiological model to unravel the real potential of polyphenols that are submitted to gastrointestinal digestion, by using a commercial variety of blackberry. In the fifth chapter it is evaluated the neuroprotective potential of two Portuguese wild blackberries. In the sixth chapter the potential molecular mechanisms underlying the protective effect previously verified by blackberries are studied using transcriptomics approach.

In the seventh chapter (General discussion) it is presented a general discussion of findings obtained and the main conclusions as well as future perspectives of the work.



## Chapter 1: Introduction

## **1. Neurodegenerative diseases (NDDs)**

NDDs are a heterogeneous debilitating and until now an incurable group of degenerative disorders. They are characterized by a slow and progressive loss of neuronal cells, leading to gradual and progressive impairments of selective functions of central nervous system (CNS), depending upon the involved type of neuronal cells <sup>1</sup>. The increase in life expectancy observed over the last century has led to the emergence of these age-related disorders that pose novel challenges to modern societies in industrialized countries and demand intensive research <sup>2</sup>.

### **1.1. Social and Economic impact**

These diseases cause devastating effects on the patient and are currently only treated palliatively. Despite the profound impact on patients and on their families, the combination of expensive formal care services, increasing number of affected people and major reliance on informal care, remains a great challenge for society. It was estimated in 2004 by WHO that NDDs (Alzheimer's disease (AD), other dementias and Parkinson's disease (PD)) reached 29.4 million people all around the world <sup>3</sup>.

Focusing just on dementia, a more recent survey estimated that there were about 10.11 million people affected in Europe <sup>4</sup>. The total costs of illness for these disorders in the whole Europe were €177 billion, being the cost per demented person about €22,000 per year <sup>4</sup>.

### **1.2. The main NDDs**

#### **Alzheimer's disease**

AD is the most common NDD, where patients experience progressive cognitive deterioration, behavioral changes, and neuropsychiatric changes <sup>5</sup>. AD is characterized by the loss of neurons, synapses, and neurotransmitters throughout the brain, especially in the hippocampus and cerebral cortex <sup>6</sup>. The pathological hallmarks of AD are neuronal loss, extracellular senile plaques containing the peptide  $\beta$ -amyloid ( $A\beta$ ) and neurofibrillary tangles, composed of a hyperphosphorylated form of the microtubular protein tau <sup>7</sup>.

**Parkinson's disease**

PD is a slowly progressive NDD, the second most common, characterized by loss of catecholaminergic neurons from the *substantia nigra* (SN) that eventually leads to bradykinesia, muscle rigidity, resting tremor, and postural instability<sup>8</sup>. The motor symptoms of PD are directly related to the loss of pigmented cells in the SN and to the reduction of the neurotransmitter dopamine in the striatum. The characteristic hallmark is also the presence of the Lewy body, aggregates of proteins containing ubiquitin and alpha-synuclein, within the cytoplasm of dying nerve cells.

**Huntington's disease (HD)**

HD is characterized by personality changes as well as motor and cognitive symptoms, including the hallmark feature, chorea, psychiatric disturbances and dementia<sup>9</sup>. Pathologically it is characterized by loss of long projection neurons in the cortex and striatum and premature death<sup>10,11</sup>. HD is an autosomal dominant disease that causes degeneration of medium spiny GABAergic neurons in the striatum. This neurodegeneration causes a progressive atrophy of the *caudate nucleus*, *putamen*, and *globus pallidus*<sup>5</sup>. HD is a genetic disease caused by a CAG repeat expansion of exon 1 in the gene that encodes huntingtin protein.

**Amyotrophic lateral sclerosis (ALS)**

ALS is a devastating disease resulting in progressive skeletal muscle weakness, muscle atrophy, paralysis, and ultimately death within 2–5 years of onset. ALS results from a progressive degeneration of motor neurons within the ventral horn of the spinal cord, brainstem, and motor cortex. Degeneration also occurs within the corticospinal tract. Approximately 20 % of patients have a mutation in the superoxide dismutase 1 (SOD1) gene, however, the majority of patients have normal SOD1 activity<sup>12</sup>.

**1.3. Common features of NDDs**

The various NDDs have different symptoms, affect different parts of the brain and have different physiological causes<sup>13</sup>. NDDs constitute a heterogeneous group of pathologies with a complex multifactorial

pathogenesis. Cellular features that have been thought to contribute to neuronal loss include oxidative stress, abnormal protein dynamics, molecular chaperones, fragmentation of neuronal Golgi apparatus (GA), disruption of cellular/axonal transport, ion channel changes, neuroinflammatory process, dysfunction of neurotrophins, choline deficit and neuronal death (Fig. 1) <sup>14</sup>.

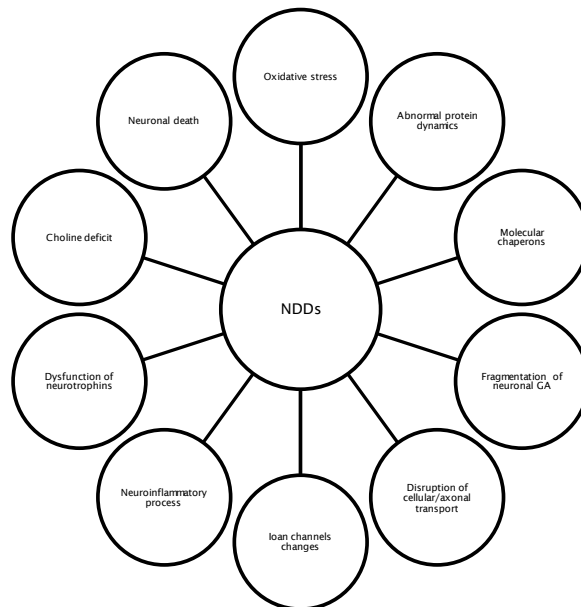


Fig. 1- Cellular features that contribute to neuronal loss.

### 1.3.1. Oxidative stress

Oxidative stress has been implicated in the pathogenesis of several disease processes, including, ischemia/reperfusion injury, AD, PD, among others <sup>15</sup>. It is basically a pathologic metabolic condition arising upon imbalance between the production of potentially toxic reactive oxygen species (ROS; *e.g.* hydrogen peroxide, superoxide and hydroxyl radical) and the antioxidants, in favor of the former <sup>16</sup>. ROS include oxygen free radicals and non-radical derivatives of O<sub>2</sub> that participate in free radical production.

ROS have been shown to play both beneficial and deleterious roles. At very low concentration, in non-pathological conditions, it may act as a

second messenger in some signal transduction pathways <sup>17</sup>. However, overproduction of ROS can damage cells through free radicals generation that leads to a progressive decline in physiological function <sup>18</sup>. ROS can attack proteins <sup>19</sup>, oxidize lipids and damage DNA <sup>20</sup>.

### **Protein oxidation**

ROS may damage the proteins via nitration or oxidation <sup>21,22</sup>. Protein oxidation contributes to the inhibition or impairment of multiple enzymes, thus affecting multiple cellular functions, ranging from protein synthesis, energy production, and cytoskeleton dynamics to signal transduction <sup>23</sup>. Additionally, protein oxidative modification could contribute to the formation of intracellular protein aggregates, which may have additional effects on intracellular homeostasis <sup>24</sup>. Once protein aggregation begins, other proteins may be sequestered into the protein aggregates, resulting in their loss of cellular function <sup>22,25</sup>.

### **Lipid peroxidation**

All cellular membranes are especially vulnerable to oxidation due to their high concentrations of unsaturated fatty acid <sup>21</sup>. Thus lipid peroxidation (LPO) is the consequence of ROS attack. Their role is well established in the pathogenesis of a wide range of NDDs <sup>26</sup>. LPO causes changes in fluidity and permeability of the cell membranes leading to the production of conjugated dienic hydroperoxides <sup>27,28</sup>. These unstable substances decompose either into various aldehydes, such as malondialdehyde and 4-hydroxynonenal (HNE) <sup>28</sup>. HNE in primary neurons and tissues leads to the activation of cell repair or cell death programs, being its adducts used as biomarkers of oxidative damage within cells <sup>29</sup>. HNE accumulation has been observed in NDDs, including AD, PD, and ALS <sup>30</sup>.

### **DNA oxidation**

Although DNA is a stable and well-protected molecule, ROS can promote extensive damages. The damage of nuclear DNA can be caused by two different mechanisms: oxidative modification and endonuclease-mediated DNA fragmentation <sup>31</sup>. Several types of DNA damages could be caused by ROS: modification of DNA bases, single- and double-DNA breaks, loss of

purines, damage to the deoxyribose sugar, DNA–protein cross-linkage and damage of the DNA repair system <sup>21</sup>. All these chemical damages impair function and leads to cellular debilitation and possibly premature death <sup>32</sup>. The 8-hydroxydeoxyguanosine (8-OHdG), an oxidized form of guanine, is a major oxidative DNA-damage product that can produce mutations.

The consequences of oxidative stress increase may result in imbalance calcium homeostasis, alterations in cellular signaling cascades and changes in gene expression <sup>33,34</sup>. These effects are detected as more elevated in the aging population <sup>35,36</sup>, AD <sup>37-39</sup> and PD <sup>40,41</sup>.

Cells are also equipped to counteract these oxidative attacks with numerous cellular antioxidant defenses, such as glutathione (GSH), glutathione peroxidase (GPx), SOD, and catalase (CAT) <sup>42</sup>. However, during aging and various disease states, these antioxidant defense systems can be altered leading to progressive oxidative damage and subsequent cell death and/or significant loss of function <sup>43</sup>.

#### **Brain sensitivity to oxidative stress**

The brain is particularly sensitive to oxidative stress since it presents: high content of peroxidizable unsaturated fatty acids, high oxygen consumption per unit of weight, high content in iron and ascorbate (LPO key ingredients) and a scarcity of antioxidant defense systems(*e.g.* GSH, GPx, CAT and vitamin E) <sup>44-49</sup>.

In fact, neurons have high metabolic demand. In humans, the brain accounts for only a few percent of the body weight, but it processes 20 % of basal oxygen consumption. A neuron uses much of oxygen via mitochondrial respiratory chain to make ATP for maintaining low gradients (high intracellular K<sup>+</sup>, low Na<sup>+</sup>, very low and free Ca<sup>2+</sup>) <sup>50,51</sup>.

#### **Mitochondria role**

Mitochondria are the “powerhouses” of the cells. The main function is the production of ATP <sup>52</sup>. It has been estimated that about 90 % of mammalian oxygen consumption is mitochondrial, which primarily serves to synthesize ATP. This makes mitochondria particularly important in

neurons, owing to their high demands for energy because of their specialized functions, complex morphology, and synaptic activity<sup>53</sup>. It has been increasing attention for the recognition of NDDs as mitochondrial diseases<sup>54</sup>. Although each of the NDDs has its distinct etiological processes and different affected brain regions, neurodegenerative disorders as a whole, share similar mitochondrial dysfunctions<sup>54</sup>. Since neurons in the brain strongly depend on mitochondrial driven aerobic respiration, when mitochondria become dysfunctional, it starts a vicious and detrimental cycle in which neurons become much more susceptible to oxidative stress. Mitochondria have already a high level of oxidative stress and, therefore, any increase in internal or external ROS leads to dysfunctional mitochondria, which in turn produces more ROS. Additionally, mitochondrial DNA is also a target of oxidation which can mediate impairment of mitochondrial functions, leading to more oxidative stress and eventual cell death (Fig. 2).

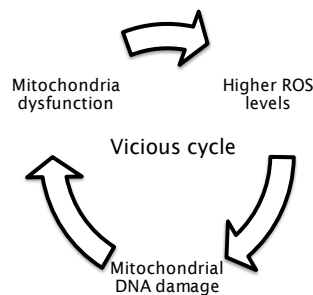


Fig. 2- Vicious cycle of mitochondria promoted by oxidative stress.

### Role of oxidative stress on the main NDDs

Oxidative stress seems to be involved in the neuronal loss that occurs in AD although its cause is unclear<sup>55</sup>. Oxidative damage occurs early in the AD brain, before the onset of significant plaque pathology, and seems also to precede the A $\beta$  deposition in animal models<sup>56</sup>. Increased protein oxidation, protein nitration, and LPO occur in neurofibrillary tangles and neuritic plaques of AD patients and also levels of oxidation products are increased in cerebrospinal fluid of AD patients<sup>57</sup>. Increased amounts of

glutathione reductase (GR) glutaredoxin (Grx) and GPx were found in *postmortem* samples from AD patients, suggesting the activation of an antioxidant defense response <sup>58,59</sup>.

Oxidative stress is also involved in the pathology of PD. Although the disease could be attributed to genetic or environmental factors, both lead to oxidative stress, mitochondrial dysfunction and protein aggregation <sup>60</sup>. The pathogenesis of PD has been reported to include increased dopamine turnover, diminished GSH content, and increased iron levels in the SN <sup>61</sup>. Dopaminergic neurons of the SN from brains of PD patients also exhibit hallmarks of oxidative stress, including LPO, nucleic acid and protein oxidation, and changes in some antioxidant molecules <sup>62</sup>. Mitochondrial defects are observed in PD <sup>63</sup>; specifically, reduced activity of the mitochondrial respiratory complex I (NAD(P)H: quinone oxidoreductase (NQO)) in the SN <sup>64</sup> as well as the frontal cortex <sup>65,66</sup>. It is well known that PD pathology includes the selective loss of dopamine neurons <sup>67</sup>, and it is hypothesized that the main cause of this loss is the high levels of ROS in dopamine neurons due to dopamine metabolism and their high iron content <sup>68</sup>.

Oxidative stress is also present in HD as represented by increased DNA oxidative products and elevated DNA strand breaks <sup>5</sup>. Oxidative challenges present with HD are thought to arise from poor mitochondrial membrane integrity. Impaired function of complex II and complex III of the electron transport chain <sup>69</sup>, should contribute to the decreased ATP levels detected in HD patients.

In ALS like disease, onset and progression is also associated with enhanced production of ROS in the spinal cords <sup>70</sup>. Oxidative stress has been suggested as favoring neuronal damage <sup>71</sup>. Protein carbonyls and protein nitration have been found to be elevated in human ALS patients <sup>72,73</sup>. Markers for LPO and higher levels of 8-OHdG were detected in spinal cord from ALS patients <sup>74,75</sup>. Other cellular characteristics of ALS include a loss of mitochondrial membrane potential and increased cytosolic calcium <sup>12</sup>.

### 1.3.2. Abnormal protein dynamics

Aberrant self-aggregating misfolded proteins with formation of intra- or extra-cellular high-ordered insoluble fibrils deposits are common pathological hallmarks of many NDDs <sup>76,77</sup>. A causative link between the formation of protein aggregates and NDD has been established, which may occur as a result of the toxic action of substances produced during early phases, where soluble oligomers and protofibrillar derivatives of misfolded proteins may play a pathogenic role <sup>78-86</sup>.

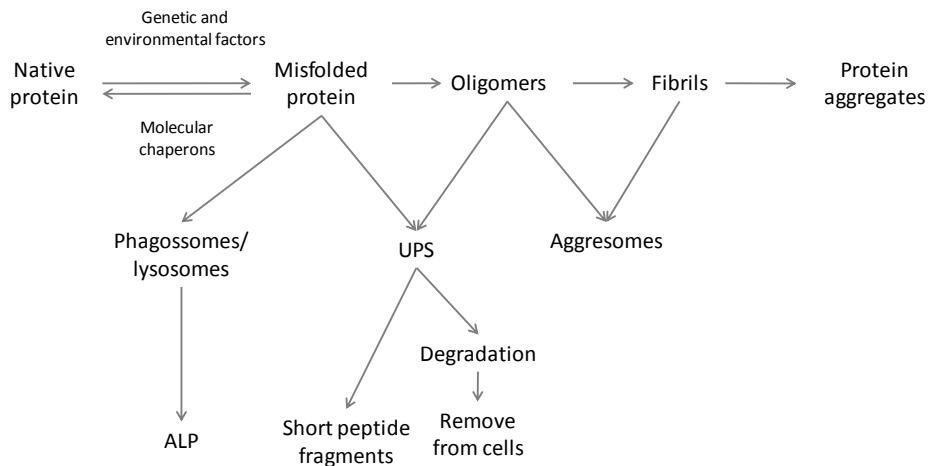


Fig. 3- Abnormal protein model of protein misfolding and fibrillation leading to the deposition of aggregated proteins via misfolded proteins, formation of aggresomes or to degradation via autophagy-lysosome pathway (ALP) or ubiquitin-proteasome system (UPS) (adapted from Jellinger <sup>14</sup>).

Progressive intracellular protein accumulation can result from various pathological processes: abnormal synthesis and folding, abnormal interaction with other proteins, overproduction of protein constituents, impaired degradation and turnover, altered post-translational modifications of newly synthesized proteins, abnormal proteolytic cleavage, improper expression or altered gene splicing, insufficient molecular chaperone activity, impaired intracellular transport of proteins and protein folding/misfolding modified on surfaces such as lipid membranes <sup>87</sup>.

Aberrant proteins often cannot fold correctly and will be trapped in misfolded conformations. To get rid of the misfolded proteins, the living

cell contains a large number of intracellular proteases, which, together with the chaperones, comprise the cellular protein quality-control systems in the endoplasmic reticulum (ER) <sup>88</sup>. The two principal routes of intracellular protein catabolism are the UPS and the ALP (autophagy). Both were implicated to play important roles in the pathogenesis of NDDs <sup>89</sup>, but also to collaborate in neuroprotection <sup>90</sup>. When the capacity of UPS to degrade misfolded proteins is overwhelmed, aggregation occurs and proteins are moved to ubiquitin-rich structures termed aggresome <sup>91</sup>. The formation of these structures has been associated with activation of caspases and apoptosis <sup>92</sup>. However, is not clear if the formation of aggresomes is causative or protective, although data suggest that they facilitate the degradation of toxic proteins <sup>93</sup>. Not all aberrant proteins can be eliminated and the misfolded protein may accumulate and form toxic oligomeric and/or aggregated inclusions. By this way, the loss of protein function may be accompanied by a gain of pathogenic function, culminating in death of affected cells <sup>94,95</sup>.

### **1.3.3. Molecular chaperones defective function**

Molecular chaperones have essential roles in many cellular processes, including protein folding, targeting, transport, degradation, and signal transduction. Chaperones have been found to be effective in preventing misfolding of different disease-causing proteins, reducing the severity of several NDDs and many other protein-misfolding diseases. They are ubiquitous in cells and are stress-induced or administered pharmacologically. Mutations on chaperones, such as heat shock proteins (HSP) have been increasingly associated to diseases <sup>96</sup>. It is likely that molecular chaperones facilitate neuroprotection by functioning at various levels. They could prevent protein aggregation, interfere with oxidative stress (*eg.* HSP27 through GSH uphold) and block apoptotic signaling pathways, thus promoting survival. Thus, molecular chaperones may increasingly become new targets for the therapy of NDDs <sup>96</sup>.

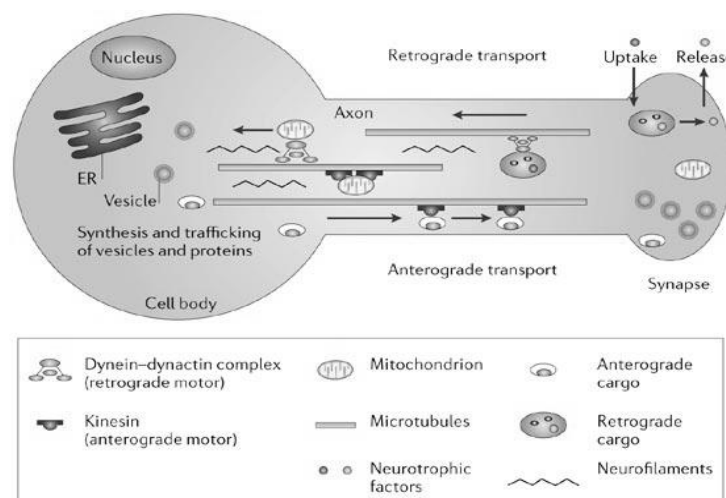
#### 1.3.4. Fragmentation of neuronal GA

Fragmentation of the neuronal GA was reported in ALS, AD, PD, Creutzfeldt-Jakob disease and in spinocerebellar ataxia type 2<sup>97</sup>. Since the GA is involved in numerous important functions, such as the transport, processing, and targeting of proteins synthesized in the ER, fragmentation of Golgi might have detrimental effects and lead to dysfunction of the cytoplasmic machinery in neurons. In ALS cell model expressing mutant SOD1, fragmentation of the GA is associated with dysfunction of the secretory pathway<sup>98</sup>. Several types of neuronal insult induce Golgi fragmentation, including excitotoxicity (nerve cells are damaged and killed by excessive stimulation by neurotransmitters), ROS, reactive nitrogen species (RNS) and ER stresses. GA has been found as a sensor for controlling entry into apoptosis<sup>99</sup>. Evidences from Nakagomi *et al.* work have suggested that GA fragmentation/dispersal is a process downstream mitochondria and ER cell death pathway in response to apoptotic effectors<sup>99</sup>. Moreover, this process seems to be an early event in neuronal cell death, initiated prior to tubulin degeneration, which is observed in the middle stages of apoptosis and not a consequence of cytoskeletal degradation during cell death. Finally, it has been suggested that GA fragmentation aberrant entry into the cell cycle can trigger neuronal apoptosis. Since mature neurons cannot undergo mitosis, the initiation of cell cycle events, such as GA fragmentation, may contribute to an apoptotic pathway<sup>99,100</sup>.

#### 1.3.5. Disruption of cellular/axonal transport

Defective neuronal and axonal transport also plays a mechanistic role in several NDDs<sup>101-103</sup>. Most of the transport uses the microtubule system, which is proposed to form a network of trafficking highways, and also active proteins (Fig. 4). All axonal components are synthesized in the cell body and transported from there into the axonal processes. Axonal transport is essential for the movement of vital proteins, vesicles, organelles, signaling molecules, and other materials to the axon, and between cell body and synapse. A complementary mechanism functions in

the opposite direction, i.e., away from the axon into the cell body. Similar mechanisms exist in the dendrites, regulated by synaptic plasticity, but little is known about these mechanisms. It has been postulated that defects in axonal transport may be responsible for NDDs <sup>104</sup>. Three developments have highlighted the significance of disrupted cellular/axonal transport in human NDDs: discovery of human motor protein mutations in these disorders; axonal transport defects in animal and *in vitro* cellular models harboring human mutations; and newly discovered roles for pathogenic proteins like amyloid precursor protein (APP), tau, presenilin, and synuclein, in the regulation of axonal transport <sup>102</sup>.



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Fig. 4- Axonal transport. The motors for anterograde and retrograde fast axonal transport are the kinesins and dynein complex proteins, respectively; microtubules provide the tracks for these motors. Vesicles for transport are sorted and loaded onto transport motors both in the cell body and the distal nerve terminal. The former are transported not only into the axon but also into dendrites. Those in the distal nerve terminal permit uptake and axosomatic movement of substances such as trophic proteins. Figure from Pasinelli *et al.* <sup>105</sup>

### 1.3.6. Ion channels changes

Ion channels have a critical role in maintaining proper CNS function, and have long been suspected as a critical factor in NDDs <sup>106</sup>. Perturbations in

Ca<sup>2+</sup> homeostasis can promote alterations of Ca<sup>2+</sup> buffering capacities, deregulation of Ca<sup>2+</sup> channel activities, or excitotoxicity and were observed in several neurodegenerative disorders including AD, PD, HD and ALS<sup>107-110</sup>. In AD, accumulation of A $\beta$  peptides in neurons has been shown to activate ion channels, causing an influx of Ca<sup>2+</sup> that disrupts homeostasis, leading to mitochondria dysfunction, oxidative stress and apoptosis of neurons<sup>111</sup>. Moreover, reduction of K<sup>+</sup> in cells can cause neuronal apoptosis<sup>112</sup>. Voltage-gated K<sup>+</sup> (KV) channels are responsible for the electrical activity in neurons. The drop in cytosolic K<sup>+</sup> relieves inhibition of an array of pro-apoptotic enzymes such as caspases and nucleases<sup>113,114</sup>. Blocking KV channels has been known to prevent neuronal apoptosis by preventing K<sup>+</sup> efflux.

### 1.3.7. Neuroinflammatory process

Chronic inflammatory reactions and signs of immune activation in the CNS, involving major histocompatibility complex class II expression, glial reaction, T-cell infiltration and blood-brain-barrier (BBB) dysfunction are well-known features in the pathogenesis and progression of NDDs<sup>115-120</sup>. Microglial cells are the primary immune cells in the CNS. Their primary functions are to promote defense by destroying pathogens, removing deleterious debris, promoting tissue repair and facilitating tissue homeostasis, through their influence on surrounding astrocytes and neurons<sup>121</sup>. In most cases, neuroinflammatory process promoted by microglial activation stops once homeostasis has been restored. However, continued, uncontrolled activation of microglia can lead to an excess production of various factors that contribute to neuronal injury<sup>122-124</sup>. The insufficient clearance by microglia is also present in several NDDs and in aging, associated with an inadequate regenerative response<sup>125</sup> (Fig. 5).

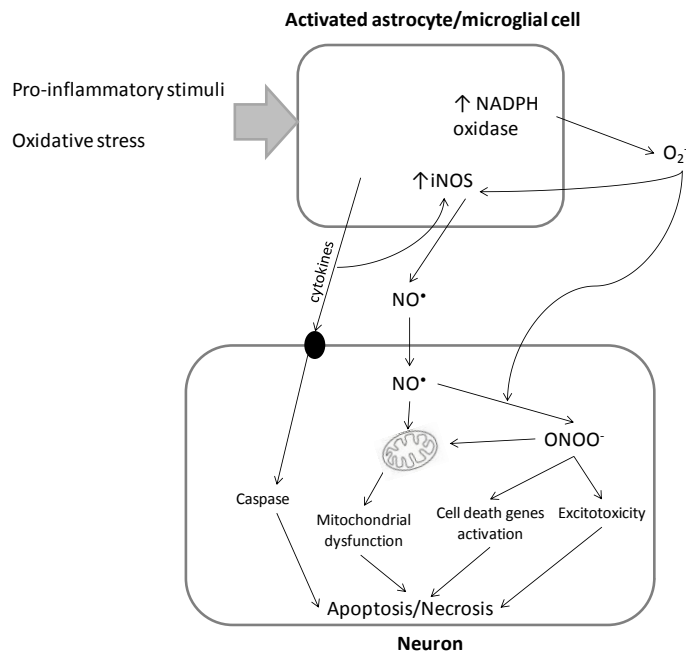


Fig. 5- Neuroinflammatory process. Microglia activation induce increased expression of inducible nitric oxide synthase (iNOS)<sup>126</sup> that promotes excessive nitric oxide production that can lead to protein S-nitrosylation and nitration<sup>127</sup> and disruption of neuronal mitochondrial electron transport chain function<sup>128</sup>. The activation of NADPH oxidase<sup>129</sup> also promoted by microglia activation mediates superoxide production and the release of pro-inflammatory molecules<sup>130</sup>. Superoxide itself can increased iNOS expression<sup>131</sup> and react with nitric oxide generating peroxynitrite<sup>132</sup>. This highly reactive oxygen and nitrogen species (RONS) can inhibit mitochondrial respiration, induce caspase-dependent neuronal apoptosis, and to induce glutamate release resulting in excitotoxicity and neuronal death<sup>132,133</sup>. Additionally, longer lived cytokines are produced which enhance the expression of iNOS, increasing nitric oxide production and stimulating the release of additional cytokines that activate neuronal death signaling cascades.<sup>134,135</sup>. Glial cytokine may also bind to specific cell surface receptors in neurons that activate pro-apoptotic pathways<sup>136,137</sup>.

### 1.3.8. Dysfunctions of neurotrophins (NTFs)

Reduced neurotrophic support is a significant factor in the pathogenesis of NDDs<sup>138,139</sup>. NTFs regulate development and maintenance of the CNS. They affect neuronal survival, influence synaptic function and plasticity and are central to many aspects of the CNS. Since NTFs in neurons are subject to transport from and to targeting neurons, their effects may be related to synthesis or to changes in axonal transport. NTFs activate signaling pathways through binding of tropomyosin-receptor-kinase (Trk)

receptors, receptors dimerization and subsequent phosphorylation of the intracellular kinase domain <sup>139</sup>. Neurotrophic support is inversely correlated with oxidative stress <sup>140</sup>. In NDDs, such as AD, PD and HD, oxidative stress appears linked to the loss of neurotrophic support, causing down-regulation of NTFs which, in turn, can up-regulate antioxidant enzymes and promote the expression of antioxidant proteins.

### 1.3.9. Choline deficit

Acetylcholine (ACh) is a neurotransmitter widely distributed in the nervous system and has been implicated in cerebral cortical development, cortical activity, controlling of cerebral blood flow, sleep-wake cycle as well as in modulating cognitive performances and learning and memory processes <sup>141</sup>. It plays an essential role in structural and functional remodelling of cortical circuits by establishing synaptic contacts in networks of cells that will support complex cognitive functions in adulthood <sup>142</sup>. However, in NDDs it has been registered a cholinergic deficit <sup>143</sup>. To the cholinergic dysfunction may contribute imbalances in the expression of neurotrophins, their precursors or in neurotrophin receptors, changes in ACh release, high-affinity choline uptake, as well as alterations in muscarinic and nicotinic ACh receptor expression. As a consequence of cholinergic hypofunction, cell atrophy may be mediated by decrements in gene expression, impairments in intracellular signaling and cytoskeletal transport that lead to functional decline in the brain. Malfunction of the cholinergic system may be tackled pharmacologically by intervening in cholinergic as well as neurotrophic signaling cascades that have been shown to ameliorate the cholinergic deficit at early stages of the disease, and slow-down its progression. Acetylcholinesterase (AChE), catalyzes the hydrolysis of the neurotransmitter ACh to choline (Fig. 6). This process is necessary to return an activated cholinergic neuron to a resting state. AChE inhibition has been reported to ameliorate the symptoms of NDDs <sup>144-146</sup>.

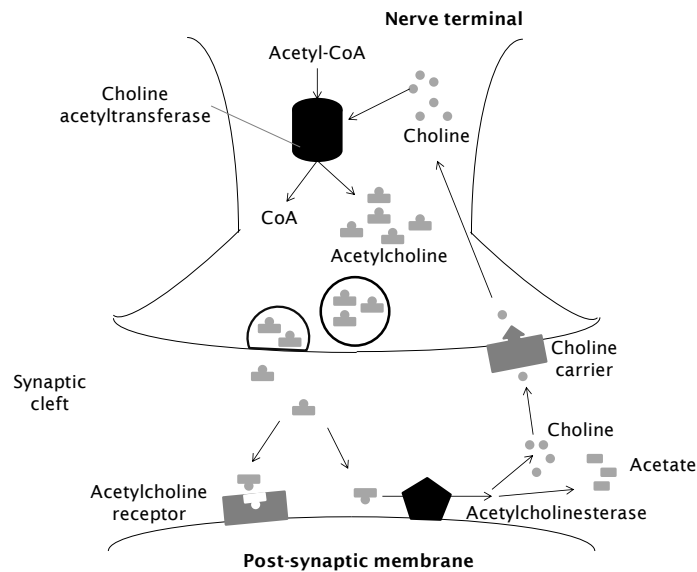


Fig. 6- Acetylcholine cycle. Cholinergic nerve transmission is terminated by the enzyme AChE, that is found both on the post-synaptic membrane of cholinergic synapses and in other tissues. ACh binds to AChE and is hydrolysed to acetate and choline. This inactivates the ACh and the nerve impulse is halted. AChE inhibitors prevent the hydrolysis of ACh, increasing its concentration of in the synaptic cleft.

### 1.3.10. Neuronal death

Neuronal death is the most critical hallmark of many NDDs <sup>14</sup>. The nature, time course, molecular causes of cell death in NDDs and their relations to basic processes are still a matter of discussion. Based on distinct morphologic criteria and biochemical features, three major mechanisms of neuronal failure could be found: apoptosis, a specific form of gene-directed programmed cell death (PCD); necrosis, a passive killing of the cell; autophagic degeneration <sup>147,148</sup>.

Morphologically, apoptotic cell death is characterized by chromatin condensation (pyknosis), nuclear fragmentation, cell shrinkage, and plasma membrane blabbing. Eventually, the cell breaks into small membrane surrounded fragments (apoptotic bodies), which are cleared by phagocytosis without inciting an inflammatory response <sup>149</sup>. Apoptosis can occur locally, without damaging healthy adjacent cells <sup>150</sup>.

In contrast, necrotic cell death, an accidental and uncontrolled mode of cell death, exhibits rapid cell swelling and subsequent rupture of the

plasma membrane that due to an inflammatory response, usually induces substantial secondary cell damage in the surrounding tissue <sup>151</sup>.

Cell death with autophagy is a normal physiological process active in both homeostasis and atrophy, probably representing a failure of neuroprotective mechanisms <sup>152</sup>. It is characterized by the formation of numerous autophagic vacuoles, endocytosis, enlargement of the GA for the vacuolization of the ER and moderate condensation of nuclear chromatin that may ultimately leave the pyknotic nuclei and to be destroyed by autophagosomes <sup>153</sup>.

Increasing evidences suggests that regulation of neuronal cell death is complex, utilizing multiple pathways that are dependent on the damaging insult <sup>154</sup>.

## 2. Polyphenols

### 2.1. General aspects

#### 2.1.1. Occurrence and function

Polyphenols (PPs) are among the most widespread class of metabolites in nature, and their distribution is almost ubiquitous. It is estimated that exist 100,000 to 200,000 secondary metabolites in plants <sup>155</sup> and 20 % of the carbon fixed by photosynthesis is channeled into the phenylpropanoid pathway, thus generating the majority of the natural-occurring phenolics <sup>156</sup>. PPs are plant secondary metabolites, *i.e.* substances that in plants have little or no role in primary metabolism: photosynthesis, respiration, growth and development, but which may accumulate in surprisingly high concentrations <sup>157</sup>.

PPs appear to have many diverse functions in plants, *e.g.* colour of leaves, flowers and fruit, anti-microbial function, anti-fungal function, insect feeding deterrence, screening from damage by solar UV radiation, chelation of toxic heavy metals and antioxidant protection from free radicals generated during the photosynthetic process <sup>158</sup>.

One of the most obvious roles for colored phenolics is in making flowers attractive to pollinating animals. Also certain soil bacteria, including

*Rhizobium* spp., are positively chemotactic towards plant phenolics<sup>159</sup>, which is advantageous to the plant in the development of a favourable rhizosphere environment and in the establishment of symbiotic relationships, such as with nitrogen-fixing bacteria. Phenolics also protect plants against damage produced by UV light, by enhancing their accumulation or shifting towards more hydroxylated PPs<sup>160</sup>. Flavonoids, a particular group of phenolic compounds, are major components of this screen, that occur in highest levels in plant tissues exposed to strong light, such as flowers and leaves.<sup>161</sup>

One of the most important roles of phenolics is the defense from pathogens and herbivorous predators. This protective role results from PPs activities such as antibacterial, antiviral, damage tissues seal and insect feeding deterrents. Moreover, the higher-molecular-weight PPs (also known as tannins) precipitate proteins in the mammalian digestive system and impair digestion.

### 2.1.2. Chemistry

PPs are characterized structurally by the presence of one or more six-carbon aromatic rings and two or more phenolic (i.e. linked directly to the aromatic ring) hydroxyl groups. Some compounds are usually integrated in the group of PPs as "honorary" PPs, however not sharing the characteristic chemical properties, such as phenolic acids, stilbenes, etc. Phenolic compounds display diverse chemical aspects relevant to their biological actions, such as: (a) interaction of the hydroxyl groups with the p-electrons of the benzene ring make them able to scavenging reactive radicals and generate more stable radicals; (b) chelation of pro-oxidants metal ions, that requires vicinal hydroxyl groups; (c) readily ionized, acting as weak acids and thus influencing their chemical reactivity; (d) good hydrogen donors, forming complexes with other molecules extremely stable and with tendency to precipitate; (e) *cis-trans* isomerism. PPs are a diverse class of plant secondary metabolites (Fig. 7). They possess a vast range of precise chemical structures, ranging from simple

molecules such as catechol, through to complicated macromolecular polymers <sup>162</sup>.

#### **2.1.2.1. Biosynthesis**

Although a large variety of plant phenols exists, PPs are mostly synthesized from phenylalanine, from shikimic acid pathway. Biosynthesis is then conducted via phenylpropanoids pathway, producing the large variety of plant phenols (Fig. 8).

The flux into phenolic biosynthesis in plants is known to be highly sensitive to environmental conditions and also biotic and abiotic stresses <sup>158</sup>. Detailed examination by molecular biological approaches has indicated that the phenomenon is largely due to enhanced transcription of the phenolic biosynthetic genes following exposure to the inducing stimulus <sup>163</sup>. Genes for the initial enzymes in the phenylpropanoid pathway appear to be induced very early on, typically in a co-ordinated, “block-wise” manner <sup>158</sup>. Mineral nutrition, such as a limited nitrogen supply and boron deficiency, lower temperatures, water availability and light are environmental factors able to influence phenolic metabolism<sup>164</sup>.

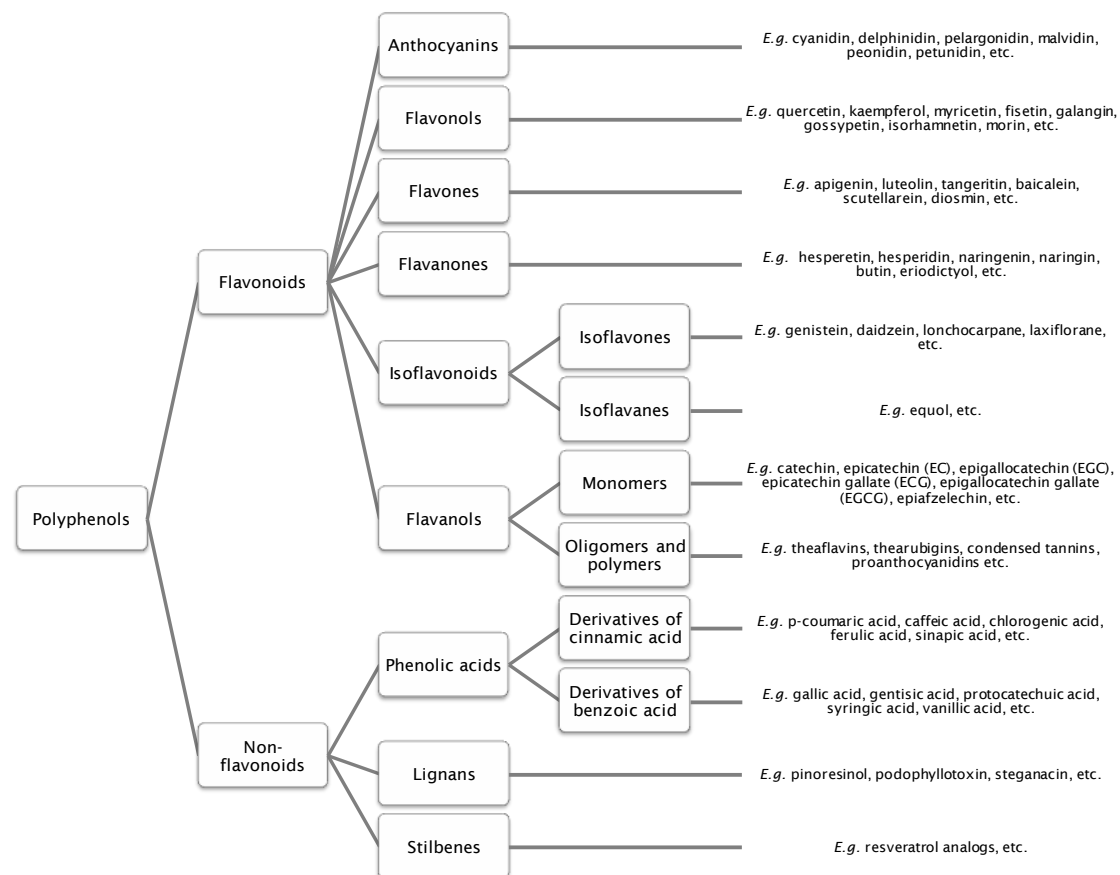


Fig. 7- Polyphenols classification. Schematic representation of polyphenols groups, including main examples of molecules.

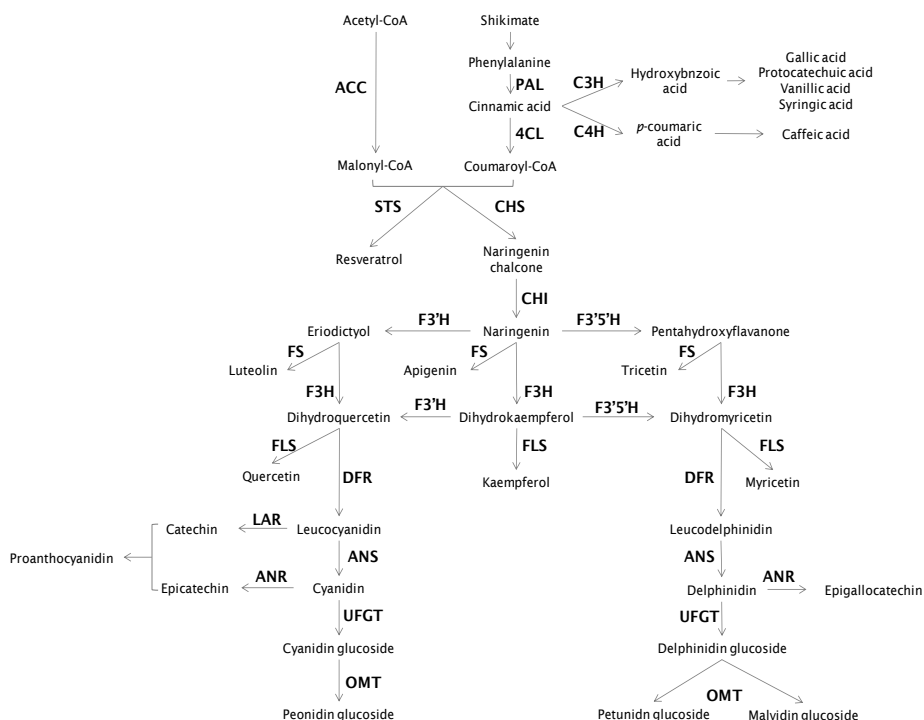


Fig. 8- Biosynthetic pathway of phenylpropanoids. Adapted from Berli *et al.* <sup>165</sup>. Legend: 4CL- 4-coumarate:CoA ligase (EC 6.2.1.12); ACC- acetyl-CoA carboxylase (EC 6.4.1.2); ANR- anthocyanidin reductase (EC 1.3.1.77); ANS- anthocyanidin synthase (EC 1.14.11.19); C3H- p-coumarate 3 hydroxylase (EC 1.14.1.1); C4H- cinnamate 4-hydroxylase (EC 1.14.13.11); CHI- chalcone isomerase (EC 5.5.1.6); CHS- chalcone synthase (EC 2.3.1.74); DFR- dihydroflavonol reductase (EC 1.1.1.219); F3H- flavanone 3-hydroxylase (EC 1.14.11.9); F3'H- flavonoid 3'-hydroxylase (EC 1.14.13.21); F3'5'H- flavonoid 3'5'-hydroxylase (EC 1.14.13.88); FLS- flavonol synthase (EC 1.14.11.23); FS- flavone synthase (EC 1.14.11.22); LAR- leucoanthocyanidin reductase (EC 1.17.1.3); OMT- o-methyltransferase (EC 2.1.1.1); PAL- phenylalanine ammonia-lyase (EC 4.3.1.5); STS- stilbene synthase (EC 2.3.1.95); UFGT- UDP glucose-flavonoid 3-glucosyltransferase (EC 2.4.1.115). The pathway starts with PAL that catalyzes the conversion of phenylalanine to cinnamate. The C4H catalyzes the synthesis of p-hydroxycinnamate from cinnamate and 4CL converts p-coumarate to its coenzyme-A ester, activating it for reaction with malonyl CoA. The flavonoid biosynthetic pathway starts with the condensation of one molecule of 4-coumaroyl-CoA and three molecules of malonyl-CoA, yielding naringenin chalcone. This reaction is carried out by the CHS. Chalcone is isomerised to a flavanone by the CHI. From these central intermediates, the pathway diverges into several side branches, each resulting in a different class of flavonoids. F3H catalyzes the stereospecific 3 $\beta$ -hydroxylation of (2S)-flavanones to dihydroflavonols. For the biosynthesis of anthocyanins, DFR catalyzes the reduction of dihydroflavonols to flavan-3,4-diols (leucoanthocyanins), which are converted to anthocyanidins by ANS. Leucoanthocyanins can also be catalyzed by LAR to flavan-3-ol. ANR catalyzes the reduction of anthocyanidins to epi-flavan-3-ol. The formation of glucosides is catalyzed by UFGT, which stabilize the anthocyanidins by 3-O-glucosylation.

## 2.1. Neuroprotective potential

It has been suggested that phenolics are among the most active substances from natural sources, displaying a variety of health-promoting properties<sup>166,167</sup>. PPs and PP-rich extracts have been implicated as beneficial agents in a multitude of disease states<sup>168,169</sup>, most commonly cancer, cardiovascular disease, and neurodegenerative disorders.

It is now believed that the activity of these low-molecular-weight, non nutrient components and their metabolites on neurological processes can be defined through a number of distinct biological processes: (a) neuronal signaling pathways, leading to inhibition of apoptosis, neuronal survival and differentiation<sup>170,171</sup>; (b) inhibition of neuropathological processes in specific brain regions<sup>172,173</sup>; (c) changes in brain blood flow<sup>174-176</sup>.

### 2.1.1. Interactions with neuronal signaling pathways

There is extensive evidence indicating that some native flavonoids<sup>171,177</sup>, their small intestinal metabolites<sup>178,179</sup> and also large intestinal derivatives<sup>180-182</sup> are capable of exerting beneficial effects on neurological processes and that this is linked to their interactions with neuronal signaling pathways. Receptor binding by flavonoids and their metabolites may underlie changes in the activation status of various downstream kinases, including various members of both the mitogen-activated protein kinase (MAPK) and the phosphoinositide 3-kinase (PI3K) pathways<sup>170,171,183,184</sup> and the nuclear factor- $\kappa$ B (Nf- $\kappa$ B) pathway<sup>185</sup>. Various flavonoid-binding sites on neurons have now been described, whose include adenosine<sup>186</sup>, gamma-aminobutyric acid (GABA<sub>A</sub>)<sup>187,188</sup>, delta-opioid<sup>189,190</sup>, nicotinic<sup>191,192</sup>, TrkB<sup>193</sup>, estrogen<sup>194</sup>, and testosterone receptors<sup>195</sup>. Also a specific brain plasma membrane binding site for PPs has been proposed<sup>196</sup>.

### Neuronal survival and differentiation

Modulation of protein kinases and lipid kinases signaling cascades by PPs allows changes in caspase activity and/or alterations on gene expression, thus affecting neuronal survival<sup>171</sup>.

Signaling pathways changes promoted by PPs are highly likely to underpin enhancements in spatial memory through the facilitation of changes in synaptic strength and the induction of morphological changes <sup>197</sup>, in particular changes in neuronal spine density and morphology that are considered vital for learning and memory <sup>198</sup>. Compounds or drugs that are capable of enhancing the activity of cAMP response element binding (CREB) may be capable of facilitating memory consolidation by promoting gene expression related with synaptic morphology and associated changes that are critical for long-term memory <sup>199,200</sup>. Moreover, molecules that are capable of entering the brain and activate upstream regulators of CREB (*e.g.*, extracellular-signal-regulated kinase (ERK) and protein kinase B (Akt)) may also be considered excellent candidates for memory-enhancing drugs. Flavonoids, due to their ability to localize in the brain <sup>201</sup> and their capacity to activate ERK- CREB and Akt-CREB circuitry <sup>170,202</sup> may be regarded as promising candidates for the enhancement of memory and aid patients suffering from AD, PD, stroke, or even normal age-associated memory deficits.

### **2.1.2. Inhibition of neuropathological processes**

Flavonoids are also able to counteract the neuronal injury underlying these disorders and thus slowing the disease progression <sup>203,204</sup>. Recent evidences suggests that nonsteroidal anti-inflammatory drugs are effective at delaying the onset of neurodegenerative disorders, particularly PD <sup>205</sup>. As such, there has been an interest in the development of new compounds with an ability to counteract neuroinflammatory injury to the brain. Likewise, flavonoids have been shown to be effective at blocking oxidant-induced neuronal injury <sup>206</sup>, although not via direct radical or oxidant-scavenging activity <sup>170,178,179,207-209</sup>.

#### **'Direct' versus 'indirect' antioxidant activity**

Until recently the conventional wisdom was that the direct antioxidant activity of phytochemicals was the responsible to confer their health benefits <sup>210</sup>. Phytochemicals have been considered able to modulate cellular responses to various stimuli interacting with RONS-mediated

intracellular signaling by scavenging RONS or suppressing their generation, either by inhibiting/activating enzymes or by chelating trace elements involved in free radical production <sup>211,212</sup>. More recently, the real impact of flavonoids and in general of PPs, as antioxidants has been reconsidered and questioned, opening up to the evidence that the molecular basis of their activity is much larger than originally considered <sup>213</sup>. Diverse mechanisms have been proposed to explain the biological activity of PPs, including the capacity to bind protein and eventually affect enzyme activity by competitive or allosteric interactions <sup>174,214</sup>; regulation of signal transduction; modulation of redox-sensitive transcription factors, including nuclear factor erythroid 2-related factor 2 (Nrf2), NF-κB, and activating protein-1 (AP-1) <sup>215,216</sup>; GSH biosynthesis <sup>217</sup>; and gene expression in general <sup>218-220</sup>. Regarding phytochemicals concentrations, they are only effective as radical scavengers when they are present in supra-micromolar concentrations. Thus, it has been speculated that this antioxidant activity cannot account for the bioactivity of flavonoids *in vivo*, particularly in the brain, where they are found at only very low concentrations <sup>174</sup>. Indeed, when examined, there is a biphasic dose-response relationship for many phytochemicals (low dose beneficial effects and high dose toxic effects), arguing against an exclusive antioxidant mechanism of action. Moreover, data from epidemiological studies and clinical trials have failed to demonstrate benefits of dietary supplementation with antioxidants such as vitamins C and E <sup>221,222</sup>.

It is becoming clear that many of the beneficial phytochemicals evolved as toxins (in which PPs are included), at subtoxic doses, activate adaptive cellular stress-response pathways, under control of genes defined as vitagenes, in a variety of cells including neurons. This mechanism defined as preconditioning, hormesis or neurohormesis, when applied to neurons, is an adaptive response of cells and organisms to a moderate stress <sup>223,224</sup>. It is suggested that hormetic effects represent an adaptive responses to environmentally induced disruptions in homeostasis, such as ischemic

preconditioning, exercise, dietary energy restriction and exposures to low doses of certain phytochemicals (*e.g.* resveratrol and curcumin). Recent findings have elucidated the cellular signaling pathways and molecular mechanisms that mediate hormetic responses which typically involve enzymes such as kinases and deacetylases, and transcription factors such as Nrf2 and NF- $\kappa$ B. As a result, cells increase their production of cytoprotective and restorative proteins including growth factors, phase II and antioxidant enzymes, and protein chaperones<sup>223,224</sup>. By this way PPs can provide significant protection from oxidative stress at concentrations much lower than would be required for “chemical” antioxidant protection and by mechanisms other than radical scavenging. It appears that PPs act by maintaining upregulated levels of our endogenous antioxidant and other defenses, thereby minimizing the response time for the defense against stresses. Actions exerted through signaling cascades are likely to proceed at intracellular concentrations lower than those required for antioxidant actions. This is important as the accumulation of flavonoids in the brain and their potential bioactivity may be restricted by processes at the BBB in that it may aid or prevent the entry of such compounds to the brain<sup>225</sup>. Results have demonstrated that flavonoids and their metabolites are able to cross the BBB but that its potential for permeation is dependent on compound lipophilicity and/or transporters<sup>226,227</sup>.

### 2.1.3. Angiogenesis

There is also strong evidence to suggest that flavonoids, in particular flavanols, are capable of promoting significant improvements in cardiovascular health after oral intake<sup>228-230</sup>, facilitating more efficient cerebral blood flow (CBF). CBF is known to deteriorate with age, to be vital for optimal brain function and to be decreased in patients with dementia<sup>231,232</sup>. Modulation of the cardiovascular risk factors by flavonoids may then result in lessening reductions in cognitive function. Epidemiological data suggest that this may be the case of flavonoids that could be capable of preventing many forms of cerebrovascular disease<sup>233,234</sup>. As increased cerebrovascular function is known to facilitate adult

neurogenesis in the hippocampus <sup>235</sup>, appearing new hippocampal cells clustered near blood vessels and areas of angiogenesis and proliferate in response to vascular growth factors <sup>236</sup>. The vascular effects of some flavonoids are partly mediated by their ability to induce nitric oxide production, a vasodilator, in the endothelium <sup>237,238</sup>.

#### **2.1.4. Plants and phytochemicals with recognized neuroprotective effects**

Growing body of evidences have surged in the literature, showing pure PPs or PPs-enriched extracts as displaying neuroprotective activity (Table 1). Those evidences are coming from several types of studies: *in vitro*, *in vivo*, epidemiological and clinical. Each type of study presents different advantages and disadvantages, being the clinical ones more expensive but more reliable on the effects of compounds in the human organism and the *in vitro* the cheapest and the less physiological, however very important to unravel the molecular mechanisms underlying the effects observed on other models and useful for screenings. However, most of the *in vitro* studies rely on pure compounds, usually in the form as they occur in plants, being forgotten the possible synergisms between different compounds and their physicochemical alterations when they are orally administered.

Table 1- Neuroprotective effects of foods presenting high content of PPs, phenolic-enriched extracts and pure PPs.

Compound	Type of study	Effect
<i>Ginkgo biloba</i> (EGb 761)	<i>In vitro</i>	Scavenger of RONS <sup>239-241</sup> Protection from A $\beta$ peptide neurotoxicity <sup>242-245</sup> Protection mitochondria from H <sub>2</sub> O <sub>2</sub> , A $\beta$ toxicity <sup>246</sup> and APP overexpression <sup>247</sup>
	<i>In vivo</i>	Improvement of spatial learning and memory in AD mice model <sup>248</sup> Decreases APP levels in the cortex by 50 % in long-term treatment <sup>249</sup> Decreases iNOS expression <sup>250</sup> , up-regulates genes encoding antioxidant enzymes <sup>251</sup> and activates sirtuin 1 (SIRT1), which in turn suppresses A $\beta$ peptide-mediated NF- $\kappa$ B activation <sup>242</sup>
	Clinical	Meta-analysis over 50 clinical studies indicates pharmacological effects controversy <sup>252</sup> More recent meta-analysis indicate it more effective than the placebo in cognition scores, but no consistent results were obtained concerning the quality of life and neuropsychiatric symptoms <sup>253</sup> Ongoing GuidAge study results, a 5-year double blind randomized trial for the prevention of AD will be available soon <sup>254</sup>
Green tea	<i>In vitro</i>	Direct scavenging of RONS <sup>255</sup> and induction of endogenous antioxidant systems, preventing oxidative DNA modifications <sup>255</sup> and inhibiting LPO <sup>256,257</sup> Catechins contribute for neuronal viability through modulation of signal transduction pathways, cell survival/death genes and mitochondrial function <sup>258,259</sup> also reduce neurotoxins damages and A $\beta$ -induced toxicity either in established cell lines and primary cell cultures <sup>257,260-263</sup> Epigallocatechin gallate (EGCG) prevents A $\beta$ fibril formation <sup>264</sup> and protects against A $\beta$ -induced apoptosis by inhibiting caspase activation <sup>257</sup>
	<i>In vivo</i>	Protection of proteins and lipids against oxidation, prevents the increase of lipofuscin deposition in hippocampal neurons of elderly rats <sup>265</sup> and improves the spatial learning abilities <sup>265</sup> Improvement of cognitive and behavioural abilities during aging and neurodegenerative conditions <sup>259</sup> . Catechins expand the life span in transgenic mouse and in <i>Coenorhabditis elegans</i> and prevent the spatial learning and memory decline in old rats <sup>266-270</sup> EGCG improves age-related cognitive decline and protects against cerebral ischemia/reperfusion <sup>271,272</sup> , brain inflammation <sup>273</sup> , rescues memory impairments induced by A $\beta$ peptide <sup>264</sup> through inhibition of NF- $\kappa$ B pathway and mitigation of induced oxidative stress in

Green tea	<i>In vivo</i>	the brains of rats <sup>274</sup> Up-regulates genes and enhances activities of antioxidant protective enzymes (SOD, CAT and heme oxygenase (HO-1) <sup>259,275,276</sup> ), increases the activity and nuclear binding of Nrf1 and Nrf2 to the antioxidant regulatory element (ARE) <sup>259,277-279</sup> EGCG treatment restores mitochondrial functions 50–85 % detected in mitochondria isolated from the hippocampus, cortex, and striatum <sup>280</sup> EGCG supplemented diet rescues transgenic mice presenting phenotypes of brain morphogenesis defects, low brain-derived neurotrophic factor (BDNF) levels and mnemonic deficits <sup>281</sup>
	Epidemiological	Tea consumption inversely correlates with incidence of dementia, AD and PD <sup>282</sup> Higher consumption is associated with lower prevalence of cognitive impairment in Japanese elderly people <sup>283,284</sup> In Chinese elderly people the cognitive impairments were correlated with the lower prevalence of tea habits consumption <sup>285</sup> In American, Finnish and Singaporean people, PD incidence was inversely correlated with the consumption of tea <sup>286-288</sup>
	Clinical	No obvious disease-modifying results were obtained <sup>289,290</sup>
Curcumin	<i>In vitro</i>	Exhibits intracellular direct and indirect antioxidant activities <sup>291-294</sup> anti-amyloid activities <sup>295</sup> and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) protective activity <sup>296,297</sup> Anti-inflammatory through inhibition of lipoxygenase and cyclooxygenase-2 (COX-2) <sup>298,299</sup> , suppression of iNOS and inhibition of NF- $\kappa$ B and AP-1 activation <sup>300-302</sup> Inhibits the formation and extension of A $\beta$ fibrils and destabilizes preformed A $\beta$ fibrils <sup>303</sup>
	<i>In vivo</i>	Reduces A $\beta$ levels and plaques, oxidized proteins and interleukin-1 $\beta$ (IL-1 $\beta$ ) in aged transgenic mice with advanced A $\beta$ accumulation <sup>304</sup> Able to cross the BBB, targeting senile plaques and disrupting existing plaques <sup>305</sup> Protects rat brain from MPTP-induced neurotoxicity <sup>296</sup>
	Epidemiological	Prevalence of AD in people aged 70-79 years in India is 4.4-fold less than in the United States <sup>306</sup>
	Clinical	Phase I clinical studies have demonstrated low systemic bioavailability following oral intake <sup>307,308</sup>
Rosemary phytochemicals	<i>In vitro</i>	Rosmarinic acid scavenges RNS, peroxynitrite, and various ROS <sup>309,310</sup> Rosmarinic and carnosic acid protects human neurons from oxidative stress <sup>311,312</sup>

Rosemary phytochemicals	<i>In vivo</i>	Rosmarinic acid alleviates memory impairment associated with A $\beta$ neurotoxicity, delays disease onset and prolongs lifespan in mouse models of AD and ALS <sup>313,314</sup> Carnosic acid crosses the BBB and preserves GSH levels in the brain, protecting it against injury induced by middle cerebral artery ischemia/reperfusion <sup>312</sup>
Grape seed polyphenolic extract	<i>In vitro</i>	Inhibits A $\beta$ -protein aggregation, suppressing self-assembly of A $\beta$ peptide and cytotoxicity <sup>315</sup>
	<i>In vivo</i>	Rescues AD animal models from cognitive deteriorations with reduced high molecular weight soluble oligomeric A $\beta$ in the brain <sup>173</sup> and in serum <sup>316</sup> , reduces formation of amyloid plaques and microglial activation <sup>316</sup> Selectively decreases a 56 kDa A $\beta$ oligomer previously shown to induce memory impairment in rats, without alterations in the levels of transgenic APP, monomeric A $\beta$ peptide, or other A $\beta$ oligomers <sup>317</sup> Attenuates of ERK 1/2 signalling in the brain of a mouse model of AD, suppressing the development of AD type tau neuropathology <sup>318</sup>
Red wine	<i>In vivo</i>	Attenuates deterioration of spatial memory performance and A $\beta$ neuropathology and A $\beta$ -related cognitive deterioration in AD transgenic mice <sup>319,320</sup> Able to cross BBB <sup>321</sup>
Resveratrol	<i>In vitro</i>	Reduces A $\beta$ -induced intracellular accumulation of ROS and apoptotic death <sup>322</sup>
	<i>In vivo</i>	Reduces formation of amyloid plaques in the medial cortex, striatum and hypothalamus without affecting APP level in mice overexpressing APP <sup>323</sup> Neuroprotective through modulation of the NF- $\kappa$ B activity or NF- $\kappa$ B/SIRT1 pathways <sup>322,324,325</sup> Reduces neurodegeneration in the hippocampus and prevents learning impairment in a model of AD and tauopathies by intracerebroventricular injection <sup>326</sup>
Berries	<i>In vitro</i>	Blueberry extracts decreased production of proinflammatory cytokines and enzymes in murine microglial cells activated by LPS <sup>327</sup> Blueberry extracts induced increase in A $\beta$ clearance, inhibition of its aggregation in fibres and suppression of microglia activation in murine cell culture <sup>328</sup> Biotransformed blueberry juice activated molecular pathways promoting cell survival (p38 and c-Jun N-terminal kinase (JNK) pathways) and the blocking of cell death mediated by extracellular signal-regulated kinases in neuronal cultures submitted to H <sub>2</sub> O <sub>2</sub> <sup>329</sup>

Berries	<i>In vivo</i>	<p>Pomegranate, strawberry, and blueberry ameliorate several aspects of memory and learning: rapid and slow memory acquisition<sup>330-332</sup>, short-term working memory<sup>201,333</sup>, long-term reference memory<sup>334</sup> and memory retention/retrieval<sup>335</sup></p> <p>Strawberry, blueberry, and blackberry retard functional age-related CNS and cognitive behavioural deficits in rodent models<sup>331,332,336</sup></p> <p>Blueberries reverse age related deficits in spatial working memory<sup>201,333,334,336-339</sup> through: increases in CREB activity and BDNF<sup>201</sup>; phosphorylation of hippocampal Akt, the activation downstream of mammalian target of rapamycin (mTOR), and the increased expression of activity-regulated cytoskeletal-associated protein (Arc/Arg3.1)<sup>201</sup>; by acting indirectly on the <i>dentate gyrus</i> (DG), the hippocampal subregion most sensitive to the effects of aging<sup>340-342</sup></p> <p>Blueberry and blackberry effects are pronounced in terms of short-term memory<sup>336,343</sup></p> <p>Blueberries anthocyanins can cross BBB<sup>337,344</sup></p> <p>Pomegranate juice improves in cued and spatial learning tasks and has a significantly reduced burden of plaque load and soluble A<math>\beta</math> in the hippocampus in transgenic mice expressing the APP<sup>330</sup></p>
Quercetin	<i>In vitro</i>	<p>Increases cell survival in the presence of H<sub>2</sub>O<sub>2</sub><sup>345,346</sup>, linoleic acid hydroperoxide<sup>347</sup>, tert-butyl hydroperoxide<sup>348</sup> and IL-1<math>\beta</math><sup>349,350</sup></p> <p>Protects cells against the PD toxins, such as 6-hydroxydopamine (6-OHDA) and 1-methyl-4-phenylpyridinium (MPP<sup>+</sup>)<sup>261</sup></p>
	<i>In vivo</i>	<p>Improves memory and hippocampal synaptic plasticity after impairment induced by chronic lead exposure<sup>351</sup></p> <p>Neuroprotective against colchicine that induces cognitive impairments<sup>352</sup></p> <p>Decreases ischemic lesion<sup>353</sup> and suppresses hippocampal neuronal death in a rat ischemia model<sup>354</sup></p> <p>Improves motor function in a model of acute spinal cord injury<sup>355</sup></p>

### 3. Strategy to deal with NDDs

As already described NDDs are multifactorial diseases for which currently there are no cures. Licensed drugs for dementia are still few: cholinesterase inhibitors (four drugs developed to inhibit AChE), butyrylcholinesterase (BuChE) and memantine (a glutamate *N*-Methyl-D-aspartic acid (NMDA) receptor antagonist). A range of other drugs often prescribed incidentally include antipsychotics, antidepressants, tranquillizers, and hypnotics <sup>356</sup>. Afterwards, the development of new therapies aimed at halting or reversing the progression of these ailments is urgently needed. Moreover, most of PPs exhibit an immense natural diversity and appear to have a number of different molecular targets, participating on several signaling pathways, and showing pleiotropic activity on cells <sup>357</sup>. In addition, their occurrence in plants in complex mixtures make it possible to take advantages of additive or synergistic activities <sup>358,359</sup>. Thus, PPs appear as promising candidate compounds to be explored in the treatment of NDDs. The uses of natural products (NPs) in pharmacological or nutritional approaches are two different strategies to supply PPs to humans that are described below.

#### 3.1. Natural products

##### 3.1.1. The current interest

NPs are promising candidates for drug discovery and they still playing an important role in the development programs of future small organic compound drug <sup>360</sup>. In the years 2000-2006, NPs produced or were involved in the production of 50 % of all small molecules <sup>360</sup>. NPs play a dominant role in the discovery of leads for the development of drugs to be used in the treatment of human diseases. From 2005 to 2007, 13 NPs related drugs were approved <sup>361</sup>. They include compounds from plants (including elliptinium, galantamine and huperzine), microbes (daptomycin) and animals (exenatide and ziconotide), as well as synthetic or semi-synthetic compounds based on NPs (*e.g.* tigecycline, everolimus, telithromycin, micafungin and caspofungin). Over 100 NPs-derived compounds are currently undergoing clinical trials and a similar number

of projects are in preclinical development. Most are derived from leads obtained from plants and microbial sources <sup>361</sup>. With the growing realization that the chemical diversity of NPs is a better match to that of successful drugs than the diversity of collections of synthetic compounds <sup>362</sup>, the interest in applying natural chemical diversity to drug discovery appears to be increasing once again <sup>363</sup>. NPs have inspired many developments in organic chemistry <sup>364,365</sup>, leading to advances in synthetic methodologies and to the possibility of making analogues of the original lead compound with improved pharmacological or pharmaceutical properties <sup>366</sup>. There are several examples of libraries based on alkaloids, polyketides, terpenoids <sup>367</sup> and flavonoids <sup>368</sup>. Despite a period in which pharmaceutical companies cut back on their use of NPs in drug discovery, there are many promising drug candidates in the current development pipeline that are of natural origin.

### 3.1.2. Lead *versus* multidrug approach

Most NP drug discovery work is focused on single compounds with a particular biological activity (single target–single compound paradigm) tested at the molecular level (*e.g.* receptor binding inhibition or enzyme screening) <sup>369</sup>. This approach is usual in Western medicine and has been responsible for the successful delivery of many potent drugs in the past, such as the huperzine A for AD <sup>370</sup>. It is considered for some authors not the best approach for NP studies since the presence of synergism and pro-drugs in an extract can be misleading <sup>369</sup>. Recently, new approaches might help to get a better view of the mode of action of herbal or NPs and lead to the possibility of obtaining proof and guarantee of their pharmacological activity over different batches. These approaches are based on considering the NP holistically, *i.e.* on the bulk of chemical constituents rather than focusing on certain individual components or groups of compounds. Genomics, proteomics and metabolomics platforms, nowadays available, are crucial since they allow the identification of detectable protein/genes or compounds which might relate to the biological activity of complex extract <sup>371</sup>. This may shift the

paradigm in the development and application of complex plant/phytochemical mixtures in modern medicine <sup>372</sup>. Besides, new or unsuspected, mode(s) of action and targets might also be revealed in this approach <sup>369</sup>. St John's Wort extract is an example of holistic approach, since no single compound or even group of compounds has been found to be responsible for its activity, the possibility of synergism and pro-drugs was evident <sup>369,371</sup>.

### 3.1.3. NPs challenges

NP-based drug discovery projects also poses some challenges <sup>373</sup>, such as: (a) dereplication, which is the rapid identification of known and unknown compounds from partially purified mixtures; (b) interaction between metabolites present in extracts, which could promote antagonism or synergism; (c) structure determination by spectroscopic methods; (d) sourcing of authenticated plants, a very important issue for quality control; (e) large scale production of the active component (as the identified NP lead might be a minor compound of the extract, or the supply of the material for extraction might be limited).

### 3.1.4. NPs as neuroprotectants leads

NPs are anticipated to play a significant role in the development of new therapeutic leads for NDDs, as exemplified by galanthamine and huperzine A, which have both proven to be potent inhibitors of AChE and are currently in clinical use for the treatment of AD <sup>374,375</sup>. Some important properties for a CNS-active drug are present in NPs within the desired ranges, presenting chirality and structure rigidity and displaying privileged structures that make them able to interact with biological targets <sup>376,377</sup>. Therefore, secondary metabolite leads should be regarded as generally compatible with the BBB penetration needs of CNS-active agents. Moreover, the chemical properties of the small-molecule NPs that have been recently developed into drugs, have been analyzed and it was found that half of them are more readily absorbed than synthetic drugs <sup>378</sup>. As already described, a substantial number of cellular dysfunctions are shared among neurons experiencing NDDs. Accordingly, many research

groups have examined these non-specific disease targets in the hope of finding compounds that have broad applications to more than one neurodegenerative disorder. Several assays have been described for identifying generalized neuroprotective agents, including preventing glutamate-induced neurotoxicity<sup>374,379,380</sup>, modulating neurotrophic activity<sup>381-383</sup>, providing protection against inflammation<sup>384,385</sup>, and inhibiting monoamine oxidases<sup>386</sup>. Numerous flavonoids compounds (*e.g.* gossypin, quercetin, gnapthalin) have been associated with antiinflammatory activity<sup>387</sup> and may have potential in the management of inflammatory disorders. Among species with a range of relevant neurobiological activities, that have shown some promising effects on cognition in dementia patients it can be found *Salvia officinalis* L. (Lamiaceae), *S. lavandulifolia* Vahl. (Lamiaceae), *Melissa officinalis* L. (Lamiaceae), *Crocus sativus* L. (Iridaceae) and the extensively studied *Ginkgo biloba* L. (Ginkgoaceae)<sup>356</sup>.

The combination of the attractive chemical properties of NPs leads with the variety of druggable targets that have been identified for neurodegeneration, makes it clear that many opportunities remain for new discoveries in the area of NPs relevant to NDDs<sup>388</sup>.

### 3.2. Nutrition

Compounds within plant foods may be considered as being more safe and healthy, compared to the ones isolated and presented in high doses, such as those in supplements. Two main factors contribute to the trust on beneficial activities of plant foods: (a) their general low concentration of nutrients and non-nutrients; (b) the additive or synergistic actions of complex mixture profiles of phytochemicals and nutrients.

PPs are phytochemicals widespread in fruits, vegetables, cereals, dry legumes, chocolate, and plant-derived beverages (*e.g.* such as tea, coffee, and wine) and represent the most abundant “minor components” in the diet<sup>389</sup>. Daily intake is estimated at 100–150 mg per day in Western populations<sup>389</sup> and their concentration in fruits and vegetables are around up to several 100 mg/100 g<sup>390</sup>.

### 3.2.1. Epidemiological/clinical evidences

There is increasing evidence that plant-derived flavonoid-rich foods or supplements might delay the initiation/slow the progression of neurodegenerative disorders. Regular consumption of flavonoid-rich foods such as tea and wine has been associated with better performance on cognition and decreased risk of cognitive decline in elderly populations in Asia and Europe <sup>391,392</sup>. The adherence to the Mediterranean diet has been associated to the lower risk of developing AD, mild cognitive impairment, the conversion of such impairment to AD <sup>393-395</sup> and to lower mortality in AD <sup>396</sup>. Moreover, the consumption of polyphenolic-rich vegetables, fruit juices, and red wine has been shown to delay the onset of AD disease <sup>233,397,398</sup>. One study, at a large-scale population level, negatively correlated intake of flavonoids with the years of life lost prematurely due to dementia and costs in disability <sup>399</sup> and evidencing flavonols as negatively correlated with dementia incidence. There are a number of studies that have indicated that the consumption of these foods and extracts may also be capable of inducing improvements in cognitive performance, human vascular function and on improving memory and learning, including in elderly people <sup>170,174,183,204,215,234,392,400-417</sup>. Collectively these reports lend some support to the hypothesis that dietary intervention with plant-derived flavonoid-rich foods or supplements has an impact on the development of NDDs.

### 3.2.2. The fate of phytochemicals supplied by nutrition

Phytochemicals supplied by food ingredients are subjected to extensive alterations due to the gastrointestinal digestion, absorption and liver detoxification. The first step upon ingestion is the release of PPs from their matrix. Deglycosylation of flavonoid glycosides, the cleavage of polymeric proanthocyanidins, as well as the hydrolysis of esterified phenolic acids, are for most compounds a prerequisite for absorption through the intestinal barrier <sup>389</sup>. These hydrolyses can be performed from the small intestine onward by either brush border or microbial enzymes. Aglycones, monomers to trimers of flavonols, and some intact glycosides

(anthocyanins) can rapidly be absorbed by enterocytes, most likely by passive diffusion. During intestinal absorption and passage in the liver, PPs are extensively glucuronidated and/or sulphated (phase II metabolism), whereas phase I metabolism (oxidation/reduction reactions) appears to be minor<sup>418</sup>. As a result, circulating PPs are mainly glucuronides. Polyphenol suffer metabolization by colonic microflora, being posteriorly reabsorbed to the enterohepatic circulation. Although faecal excretion is low, urinary recoveries of the parent PPs account for only a small fraction of the ingested doses, suggesting a high degree of metabolization of phenolic compounds during digestion.

#### 4. Plants

The chemical diversity of plants constitutes an immense and relatively untapped reservoir of molecules with potential pharmacological/nutraceutical value. The diversity of Portuguese plants represents a pool of phytochemicals as yet poorly characterized and explored.

##### 4.1. Juniper (*Juniperus* sp.)

*Juniperus* sp. belong to Cupressaceae family and they are known a total of 70 species and 27 varieties all around the world<sup>419</sup>. The major part of these species are spread in Laurasia region, except *J. procera* that grows from the mountains of East Africa until the south hemisphere and some mediterranean species such as *J. oxycedrus*, *J. phoenicea*, and *J. thurifera* that also grow in mountain regions of North Africa<sup>419</sup>.

Plants from the *Juniperus* genus have found application in different European cuisines as a spice, flavouring alcoholic drinks as well as in cosmetics<sup>420</sup>. Furthermore, these plants have an extensively history of use in global folk medicine for various disorders, such as common colds, urinary and kidney infections and dermatological disorders<sup>421</sup>. Many biological activities have been more recently reported for *Juniperus* sp.. In this work the species evaluated were *J. navicularis* Gand., *J. oxycedrus badia* H. Gay, *J. phoenicea* L. (= *J. phoenicea phoenicea* L.) e *J. turbinata*

*Guss.* (= *J. phoenicea turbinata* Guss.), all of them native from Portugal, being *J. navicularis* a Portuguese endemism. *J. navicularis* are usually found in sandy soils in the Portuguese coast, pinewoods and deciduous woods in Baixo Alentejo, Estremadura and Ribatejo regions <sup>422,423</sup>. *J. oxycedrus badia* can be found in xerophytic regions, plains, open hills or woods up to 1000 m high, especially in Portuguese inner regions: Beira Alta, Beira Baixa, Trás-os-Montes and also in Ribatejo <sup>422,423</sup>. *J. phoenicea* and *J. turbinata* usually inhabit sandy areas such as dunes and xerophytic woods in central and southern parts of coastal area of the country, with *J. phoenicea* also colonizing cliffs and rocky areas <sup>422,423</sup>.

#### 4.2. Blackberry (*Rubus* sp.)

Fruits from *Rubus* sp. have long been collected and consumed worldwide, regardless of whether they were recognized for their possible health benefits from their natural phytochemicals <sup>424,425</sup> or simply because they have a good taste. The worldwide popularity of *Rubus* fruit has increased in part due to the repeated published accounts of highly colored berries/fruit and their potential health benefits <sup>336,426-429</sup>. The worldwide commercial production of blackberry is estimated to be approximately 154,578 tons annually <sup>430</sup>, being wild blackberries also cultivated in considerable amounts in some regions, reaching 13,460 tons worldwide <sup>430</sup>.

Among the huge diversity of blackberries found in Europe, it is tough that the areas which were not covered with ice during the last two glaciations constituted refuges for the pre-glacial *Rubus* sp. <sup>431</sup>. These areas found in Portugal, Pyrenees, Switzerland, Syria, Carpathian and Caucasus mountains should be the habitat of ancestral species, different from those found in the North countries of Europe <sup>431</sup>. In Portugal it was already identified diverse species whose occurrence is relatively limited to some Iberian areas <sup>422</sup>. Examples of these species are *R. brigantinus* Samp., *R. caesius* L., *R. castellarnau* Pau, *R. genevieri* Boreau, *R. henriquesii* Samp., *R. lainzii* H. E. Weber, *R. praecox* Bertol., *R. radula* Weihe in Boenn., *R. sampaioanus* Sudre ex Samp., *R. vagabundus* Samp., and *R. vigo* R. Roselló, Peris & Stübing. These different species are potentially

constituted by a different combination of bioactive phytochemicals. Blackberries wild species are then a source of genetic variability to be explored.

## 5. Aim

This work envisages the study of plants as sources of phytochemicals with neuroprotective potential. Two different approaches will be described: the first utilizes juniper leaves for a potential pharmacological application; the second uses blackberries for a nutritional strategy. The two groups of plants chosen to be studied (junipers and blackberries) included species/cultivars with broader occurrences and others presenting a more restricted distribution, being in some cases endemic from some Portuguese regions.

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Part A:  
*Juniperus* sp.





## Chapter 2: The neuroprotective potential of phenolic-enriched fractions from four *Juniperus* species found in Portugal

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LT performed the majority of the experiments presented in this chapter (except AChE inhibitory assay, performed in collaboration with SF) and wrote the manuscript cited above.

### **Abstract**

The increase in population lifespan has enhanced the incidence of NDDs, for which there is, as yet, no cure.

We aimed to chemically characterize phenolic-enriched fractions (PEFs) from four wild *Juniperus* sp. found in Portugal (*Juniperus navicularis*, *Juniperus oxycedrus badia*, *Juniperus phoenicea* and *Juniperus turbinata*) and address their potential as sources of natural products for treatment of NDDs.

Leaves from the four *Juniperus* sp. evaluated contained a range of phenolic components which differed quantitatively between the species. The PEFs obtained were rich sources of phenolic compounds, exhibited AChE inhibitory activity and also displayed effective intracellular radical scavenging properties in neurons submitted to oxidative injury but showed a different order of effectiveness compared to AChE inhibition. These properties made them good candidates for testing in a neurodegeneration cell model. Pre-incubation with *J. oxycedrus badia* PEF for 24 h protected neurons from injury in the neurodegeneration cell model.

### **Keywords**

Acetylcholinesterase inhibitor; intracellular antioxidant capacity; *Juniperus* sp.; natural products; neurodegeneration cell model; phenolic enriched fraction.

## 1. Introduction

In the developed world, the increase in population lifespan has been accompanied by an increase in the incidence of many age-related diseases such as neurodegenerative disorders<sup>4</sup>. These diseases are associated with enormous economic and social costs<sup>5,6</sup>: it has been estimated that the European healthcare costs of for AD in 2007 were €117 billion and that the number of cases are predicted to rise to 81.1 million by 2040<sup>6</sup>. This, in conjunction with the lack of any cure, has made the development of new therapies for halting, retarding or reversing such disorders imperative. Research in recent years has provided substantial evidence supporting the theory that oxidative stress plays a major role in the pathogenesis of neurodegenerative disease<sup>8-10</sup>. Oxidative stress is primarily caused by deviation of the cells redox balance from the norm and generally this is associated with an excessive accumulation of ROS in cells; a process previously implicated in the development of many NDDs including PD, HD, ALS and AD<sup>8,11,12</sup>. Furthermore, a consistent neuropathological occurrence associated with memory loss is a cholinergic deficit, which has been correlated with the severity of AD. AChE inhibition has been reported to ameliorate the symptoms of some NDDs and has been used as a rationale to develop drugs to treat AD<sup>14</sup>.

NPs have made significant contributions towards the treatment of degenerative diseases<sup>15</sup>. Since plants constitute a rich and diverse source of secondary metabolites they have long contributed to the development of small-molecule based therapeutics; the class of compounds called phenolics, which display a significantly diverse combination of chemical moieties, structures and associated potent bioactivities, are a good example of this. Indeed, before the advent of high-throughput screening and the post-genomic era around 80% of drugs were natural product derived and more recently natural product derivatives still accounts for more than half of the drugs approved since 1994<sup>16</sup>. Several small molecules, including (poly)phenolics, have been reported to exhibit

neuroprotective properties <sup>14,17,18</sup>. In addition, reports suggest that some phenolic compounds may target numerous pathways and protein kinases that underlie these conditions and that strengthen their utility in multifactorial diseases, such as neurodegeneration <sup>17</sup>.

Plants from the *Juniperus* genus have found application in different European cuisines as a spice, flavouring for alcoholic drinks as well as in cosmetics <sup>19</sup>. Furthermore, these plants have an extensively history of use in global folk medicine for various disorders, such as common colds, urinary and kidney infections and dermatological disorders <sup>20</sup>. Many biological activities have been reported for *Juniperus* sp. These include anti-inflammatory <sup>3,21</sup>, diuretic, antiseptic (bacterial and fungal) <sup>2,22-25</sup>, anthelmintic <sup>26</sup>, hypoglycaemic <sup>27</sup>, hypotensive, abortifacient, antinociceptive <sup>21</sup>, antiviral <sup>28</sup>, anticancer <sup>29</sup>, antioxidant <sup>2,3,30-32</sup>, anticholinesterase <sup>31,32</sup> and analgesic properties <sup>33</sup>. They have also been found to be useful in the treatment of psoriasis <sup>34</sup> and neurasthenic neurosis <sup>35</sup>. Although the chemical composition of the essential oil from diverse *Juniperus* sp. has attracted significant interest <sup>36-42</sup> much less attention has been paid to other components and there is paucity of detail on the phenolic characterization of *Juniperus* sp.. Previous reports have highlighted the presence of phenolic compounds including flavonoids, neolignans and phenylpropanoids <sup>7,43-46</sup>, but also terpenoids <sup>47-49</sup>. Studies on *Juniperus* species occurring in Portugal have been even more limited and the focus again was on essential oils <sup>40-42</sup>. The aim of this work is to chemically characterize wild Portuguese *Juniperus* sp., in terms of phenolic compounds and address their potential as sources of NPs for treatment of NDDs. The rationale of this work is the search for AChE inhibitors as well as intracellular antioxidants as candidates to be tested in a neurodegeneration cell model.

## 2. Material and methods

### 2.1. Plant material and extract preparation

Leaves of *Juniperus navicularis* Gand., *Juniperus oxycedrus subsp. badia* (H. Gay) Debeaux, *Juniperus phoenicea* L. and *Juniperus turbinata* Guss. were collected, frozen and then freeze-dried. *Juniperus navicularis* is a Portuguese endemic and threatened plant and was sampled under a license for capture (11/2008/CAPT and 12/2008/CAPT). For all species, voucher samples were authenticated and deposited at the herbarium "João de Carvalho e Vasconcelos", Instituto Superior de Agronomia, Lisbon, Portugal. The herbarium numbers are: *Juniperus navicularis* (LISI 1064/2007), *J. oxycedrus subsp. badia* (LISI 1/2008), *J. phoenicea* (LISI 3/2008) and *J. turbinata* (1067/2007). Freeze-dried leaves were ground in a IKA M20 mill to pass a 0.5 mm sieve and stored at -80 °C prior to extraction. Leaf extracts were prepared using a hydroethanolic solution (ethanol 50 % (v/v)) as previously described<sup>50</sup>. Samples were dried, by centrifugal evaporation.

### 2.2. Fractionation by solid phase extraction

PEFs were obtained by fractionation by Solid Phase Extraction (SPE) using a Giga tubes 2 g 12 mL<sup>-1</sup>, C18-E units (Phenomenex®) as described before<sup>50</sup>. PEFs were dried under vacuum, to suitable phenol contents for subsequent assays.

### 2.3. Chemical characterization

#### 2.3.1. Total phenolic quantification

Determination of total phenolic content (TPC) was performed by the Folin-Ciocalteu method adapted to microplate reader<sup>51</sup>. Gallic acid was used as the standard and the results were expressed as mg of gallic acid equivalents (mg GAE) per g of dry weight (DW).

### 2.3.2. Liquid chromatography-mass spectrometry (LC-MS) phenolic profile determination

Dried extracts were redissolved and then applied to a C-18 column (Synergi Hydro C18 with polar end capping, 4.6 mm x 150 mm, Phenomenex Ltd.) and eluted over a gradient of 95:5 solvent A:B at time=0 minutes to 60:40 A:B at time=60 minutes at a flow rate of 400  $\mu\text{L min}^{-1}$ . Solvent A was 0.1% (v/v) formic acid in ultra pure water and solvent B 0.1% (v/v) formic acid in acetonitrile. Samples were analysed on a LCQ-DECA system controlled by the XCALIBUR software (2.0, ThermoFinnigan), as reported by Tavares *et al.* <sup>52</sup>. The LCQ-DECA system comprised a Surveyor autosampler, pump and photo diode array (PDA) detector and a Thermo Finnigan mass spectrometer iontrap.

### 2.4. AChE inhibitory assay

AChE inhibition was determined in 96-microtiter well plate, based on Ellman's reaction, as described previously <sup>53</sup>. Different concentrations of PEFs of the four species were tested (50, 100, 200, 400 and 800  $\mu\text{g GAE mL}^{-1}$ ). The effect on AChE activity was calculated as percentage inhibition (%) of the control activity without inhibitor.

### 2.5. Cell culture

Human neuroblastoma SK-N-MC cells were obtained from the European Collection of Cell Cultures (ECACC) and cultured in Eagle Minimum Essential Medium (EMEM, Sigma) supplemented with 2 mM L-glutamine (Sigma), 10 % (v/v) heat inactivated foetal bovine serum (FBS, Gibco), 1 % (v/v) non-essential amino acids (Sigma), 1 mM sodium pyruvate (Sigma) and containing 50 U  $\text{mL}^{-1}$  penicillin and 50  $\mu\text{g mL}^{-1}$  streptomycin. The cells were maintained at 37 °C in 5 %  $\text{CO}_2$  and harvested at sub-confluence of 70-80 % using 0.05 % trypsin/EDTA (Gibco).

### 2.6. Cytotoxicity profile

PEFs were dissolved in cell medium for measuring cell viability as previously described <sup>52</sup>. Briefly, SK-N-MC neuroblastoma cells were seeded

in a 96-well plate using  $1.25 \times 10^5$  cells  $\text{mL}^{-1}$  and grown for 48 h prior to addition of PEFs. Cytotoxicity was assessed using 24 h incubation with PEFs in the range 0-500  $\mu\text{g}$  GAE  $\text{mL}^{-1}$  medium. Cell viability was assessed using the CellTiter-Blue® Cell Viability Assay (Promega), according to the manufacturer's instructions.

### 2.7. Intracellular ROS production

To evaluate the ability of PEFs to reduce cellular ROS levels, the conversion of 2',7'-dichlorofluorescein diacetate ( $\text{H}_2\text{DCFDA}$ , Invitrogen) to fluorescent 2',7'-dichlorofluorescein (DCF) was monitored, as already described<sup>52</sup>. Briefly, SK-N-MC neuroblastoma cells were seeded in a 96-well plate ( $1.25 \times 10^5$  cells  $\text{mL}^{-1}$ ), grown for 24 h prior to treatment then pre-incubated with PEFs prepared in medium (0.5% (v/v) FBS) for 2 h or 24 h. Cells were then incubated with 25  $\mu\text{M}$   $\text{H}_2\text{DCFDA}$  for 30 min at 37 °C, followed by addition of  $\text{H}_2\text{O}_2$  (200  $\mu\text{M}$ ) and the fluorescence determined ( $\lambda_{\text{ex}}$ : 485 nm,  $\lambda_{\text{em}}$ : 530 nm) over 1 h at 37 °C. ROS generation was calculated as an increase in fluorescent signal between control and  $\text{H}_2\text{O}_2$ -treated cells.

### 2.8. Determination of antioxidant enzyme activity

Cells were seeded in 6-well plates and grown for 24 h before treatment with PEFs. After 24 h, cells were treated with  $\text{H}_2\text{O}_2$  (200  $\mu\text{M}$ , 1 h), which has been previously reported as inducing oxidative stress without cell death<sup>52</sup>. Cells were washed with PBS and lysed with 200  $\mu\text{L}$  of lysis buffer, containing 50 mM Tris-HCl pH 8.0, 120 mM NaCl, 0.5 mM EGTA and 0.05 % (v/v) Triton X-100. Cells were incubated for 20 min on ice with vortexing every 5 min, then centrifuged 15 min at 9,000g. The supernatant was used to determine the activities of superoxide dismutase (EC 1.15.1.1) and catalase (EC 1.11.1.6). The activity of both enzymes was normalized for protein content determined by the Lowry method<sup>54</sup>.

### 2.8.1. SOD assay

SOD activity was measured based on the extent of inhibition of amino blue tetrazolium formazan formation and was adapted from Mockett *et al.*<sup>55</sup>. Briefly, cell extracts were incubated in 100 mM PBS (pH 7.8) containing 0.1 mM EDTA, 0.1 mM xanthine, 100  $\mu$ M nitro blue tetrazolium (NBT) and 2.5 nM xanthine oxidase (EC 1.1.3.22, CAS No: 9002-17-9, purified from bovine milk, Grade I, Sigma). The reduction of NBT was measured at 560 nm. One unit of SOD activity was determined as the rate of absorbance change per min.

### 2.8.2. CAT assay

CAT activity was measured by the method of Aebi<sup>56</sup> in which the rate of  $H_2O_2$  decomposition was determined spectrophotometrically at 240 nm. The reaction mixture contained 30 mM of  $H_2O_2$  in 50 mM potassium phosphate buffer, at pH 7.0. The reaction was initiated by adding 50  $\mu$ L of cell lysate and the decrease in absorbance per min was calculated from the initial linear portion of the curve, using as molar extinction coefficient 0.0394  $cm^{-1} mM^{-1}$ . The enzyme activity was expressed as U  $mg^{-1}$  protein, with 1 U being the amount of enzyme consuming 1  $\mu$ mol of  $H_2O_2$  per min.

## 2.9. Neuroprotective effect against oxidative stress

Fractions were evaluated for their neuroprotective effect in a neurodegeneration cell model previously described<sup>52</sup>. The model involved the treatment of SK-N-MC neuroblastoma cells with  $H_2O_2$  to induce cell death. Briefly, cells were seeded at  $7.4 \times 10^4$  cells  $mL^{-1}$  and grown for 24 h. After 24 h of pre-incubation with medium supplemented with non-toxic concentrations of PEFs, cells were treated with medium containing  $H_2O_2$  (300  $\mu$ M). After 24 h, the medium was removed and cells were washed with PBS and collected by trypsinisation. Cells were then incubated with two fluorescent probes for 30 min at 37 °C. Mitochondrial transmembrane potential ( $\Delta\Psi_m$ ) was assessed using 3,3'-dihexyloxycarbocyanine iodide (DiOC<sub>6</sub>(3), 20 nM, Invitrogen) and cell viability was determined by assessing plasma membrane integrity using propidium iodide (PI, 1  $\mu$ g

mL<sup>-1</sup>, Invitrogen) <sup>57</sup>. These parameters were analysed by flow cytometry using a blue solid state laser (488 nm) with FL1 green fluorescence channel for DiOC<sub>6</sub>(3) at 530 nm and a FL3 red fluorescence channel for PI detection at 650 nm. The acquisition and analysis of the results were performed with FlowMax<sup>®</sup> (Partec) software.

#### 2.10. Statistical analysis

The results reported are the averages of at least three independent experiments and are represented as the mean  $\pm$  SD. Differences among treatments were detected by analysis of variance with Tukey HSD (Honest Significant Difference) multiple comparison test ( $\alpha = 0.05$ ) using SigmaStat 3.10 (Systat).

### 3. Results

#### 3.1. Chemical characterization

Hydroethanolic extracts were obtained from the four Juniper species and chemically characterized (Table 1). The phenolic content was highest for the extract obtained from *J. oxycedrus badia*, followed by *J. turbinata*, *J. navicularis* and *J. phoenicea*, respectively. Following SPE fractionation, PEFs were obtained for all species with similar phenolic content. They were used at an equivalent phenolic load for all subsequent comparative bioactivity assessments.

All four *Juniperus* sp. gave LC-MS-PDA profiles, in which some shared peaks presented quantitative differences (Fig. 1 and Table 2). Their phenolic composition is also in agreement with the phenolic identifications found in the literature for *Juniperus* sp.. Until now the identifications of *Juniperus* sp. compounds are spread across different reports, in which different species were used and for each only a few number of compounds were identified <sup>2,3,7,13,31,44,45,58-61</sup>.

Table 1- Total phenolic content of crude extracts and PEFs obtained from the four *Juniperus* sp.. Values are expressed as mg GAE g<sup>-1</sup> of dried powder. Statistically significant differences at p<0.05 are denoted with different letters (a-c). All values are mean ± SD, n = 3.

Species	Crude extract	PEF
<i>J. navicularis</i>	247±28 <sup>bc</sup>	310±40
<i>J. oxycedrus badia</i>	354±10 <sup>a</sup>	414±90
<i>J. phoenicea</i>	221±5 <sup>c</sup>	330±24
<i>J. turbinata</i>	274±3 <sup>b</sup>	401±55

Catechins, procyanidins, flavonol derivatives, flavones and biflavones (Fig. 1 and Table 2) were putatively identified in the *Juniperus* sp.. Two major compounds that have been previously reported in *J. communis* as unknown<sup>13</sup> were present and identified as psydrin (peak 1) and procyanidin trimer (peak 9). Psydrin was the major peak of *J. oxycedrus badia*, *J. phoenicea* and *J. turbinata* but significantly less prevalent in the *J. navicularis* LC-MS-PDA profile. Amongst the major compounds of *Juniperus* sp. were catechins, a range of flavonol derivatives (such as quercetin rutinoside and quercetin rhamnoside), flavones (such as isoscutellarein and luteolin glycosides) and biflavones. Procyanidins were present in all PEFs but in variable amounts. Some procyanidins components eluted as discrete peaks (*e.g.* peaks 2, 4, 5 and 7) but they were also presented as a poorly resolved smear across retention times (RTs) 17-25 mins (Fig. 1, annotated by bar). All four species contained flavonol derivatives mainly as quercetin, but also as myricetin, glycosides but they varied in content and composition between the species. For example, quercetin rutinoside (peak 13) was a major component of *J. navicularis* but it was present in much lower amounts in the other species. Another major difference between *J. navicularis* and the other species was the abundance of procyanidin trimer (peak 9, the unidentified phenolic component reported by Martz *et al.*<sup>13</sup>). Putative flavone derivatives were detected but whether these were isoscutellarein and/or luteolin glycosides could not be completely confirmed by LC-MS. However,

these flavone derivatives (especially peaks 20 and 21) were more abundant in *J. oxycedrus badia*, *J. phoenicea* and *J. turbinata*. Such flavone derivatives have already been identified in *J. communis* var. *depressa*<sup>44,45</sup>. Biflavone derivatives were putatively identified in all four species (peak 22, RT ~35 min). However, these apolar compounds eluted late in the gradient and were not sufficiently separated to allow us to distinguish between the three biflavones already identified in *J. drupacea*, amentoflavone, cupressuflavone and hinokiflavone<sup>7</sup>.

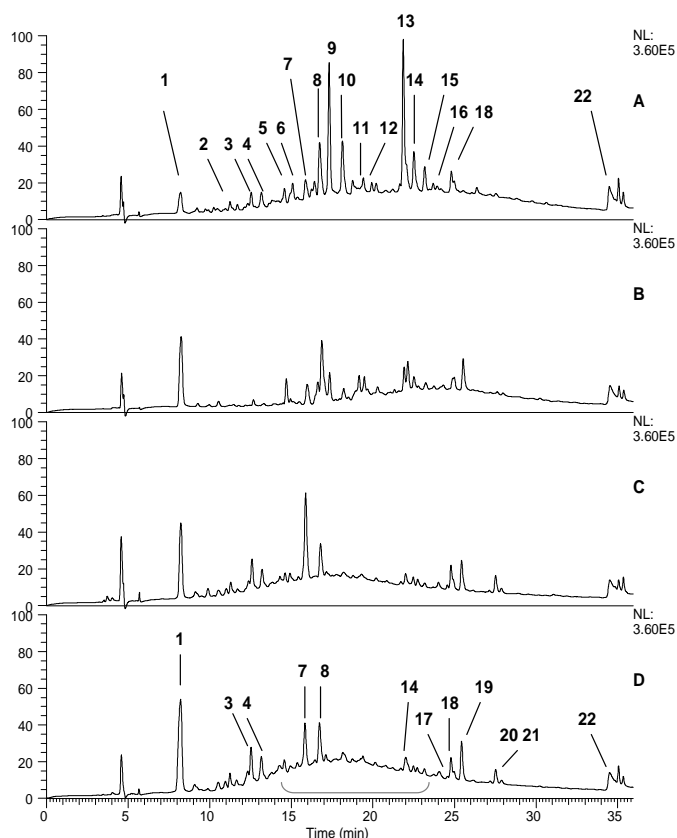


Fig. 1- Representative PDA profiles for *J. navicularis* (A), *J. oxycedrus badia* (B), *J. phoenicea* (C) and *J. turbinata* (D). Peak numbers correspond to their putative identifications based on MS fragmentation data presented in Table 2. The full scale deflection for each trace is shown in the top right corner. The area denoted by the bar is discussed in the text.

Table 2- Putative identities of major peaks in Juniper fractions

Peak no.	RT	PDA	m/z [M-H]	MS <sup>2</sup>	Putative Identity
1*	8.22	271	<b>275.1</b> [321.1] *277.1, 115.1	<b>161.1</b> , 113.1 *115.1, 87.1	Psydrin, a furanone glycoside <sup>1</sup>
2	11.23-11.41	280-310	<b>593.2</b> , 425.4, <b>289.8</b>	549.1, <b>289.1</b>	Epicatechin-epigallocatechin (EC-EGC)
3	12.54	285	<b>305.1</b>	179.1	Gallocatechin
4	13.17	269	<b>896.7</b> , <b>593.0</b> , 425.3, <b>289.1</b>	710.9, <b>593.1</b> , 425.0	Procyanidin (EC-EGC-EGC)
5	14.58	277	<b>577.1</b> , 289.2	451.0, 407.1, <b>289.1</b>	Procyanidin dimer (EC2)
6	15.07	283-314	<b>305.2</b>	219.1, <b>179.0</b>	Epigallocatechin
7	15.87	275	<b>577.0</b> , 289.1	<b>289.1</b>	Procyanidin dimer (EC2)
8	16.74	279	<b>289.2</b>	245.1, 179.2	Epicatechin
9	17.32	275	<b>865.1</b> , 577.0	695.1, <b>577.1</b>	Procyanidin trimer (EC3)
10	18.14	275	<b>881.2</b>	593.1, 695.1	Procyanidin trimer (EC-EGC-EC)
11	19.42	275, 378	<b>479.2</b> , 317.1	317.1	Myricetin hexose <sup>2</sup>
12	19.93	270, 357	<b>625.3</b> , 317.0	317.1	Myricetin rutinoside
13	21.86	255, 354	<b>609.2</b> , 301.1	301.2,	Quercetin rutinoside <sup>3</sup>
14	22.06	270-365	<b>449.2</b> , 317.1	317.1	Myricetin pentose
15	22.51	221, 269	<b>755.0</b>	<b>569.0</b> , 289.1	Proanthocyanidin derivative
16	23.17	280-365	<b>181.5</b> <b>463.7</b> , 301.2	None 301.2	Unknown compound Quercetin hexose
17	24.56	280-340	<b>447.1</b> , 285.0	285.1	Isoscutellarein hexoside
18	24.81	276, 342	<b>433.2</b> , 301.2	301.2	Quercetin pentoside
19	25.43	280-340	<b>447.1</b> , 301.1	301.1	Quercetin rhamnoside
20	27.57	280-340	<b>417.0</b> , 285.2	285.2	Luteolin/isoscutellarein pentoside
21	27.88	280-340	<b>431.1</b> , 285.1	285.1	Luteolin/isoscutellarein rhamnoside
22	35.06, 35.34	272, 329	<b>537.9</b>	375.2	Amentoflavone or Cupressuflavone or Hinokiflavone <sup>7</sup>

Figures in bold are the main m/z or MS<sup>2</sup> signals and were those used for MS<sup>2</sup> fragmentation. Compound 1 is equivalent to the unknown compound as detected previously by Martz et al. <sup>13</sup>. Figures in parentheses are formate adducts. \* denotes [M + H] signals.

Other m/z signals for larger proanthocyanidins (e.g. 1153 for epicatechins tetramers) are present but do not elute as discrete peaks.

\* - Exact mass data obtained on the Orbitrap system supports this identification; molecular formula C<sub>11</sub>H<sub>17</sub>O<sub>8</sub> in negative mode (RDB 4.5, delta amu 0.001). The difference in MS<sup>2</sup> fragmentation pattern between positive and negative mode is also noted by Comte *et al.* <sup>1</sup>.

### 3.2. AChE inhibitory assay

AChE inhibition by the PEFs was tested at the following concentrations: 50, 100, 200, 400 and 800 µg mL<sup>-1</sup> (Table 3). *J. turbinata* was the most potent species. Using 800 µg mL<sup>-1</sup> of *J. turbinata* PEF the inhibition was 72.65 %. This species was followed by *J. phoenicea*, *J. navicularis* and *J. oxycedrus badia*. The last species was the only not able to inhibit more than 50 % at the concentrations tested.

Table 3- AChE inhibitory capacity by *Juniperus* species. Results are reported as % inhibition compared to the control activity. Values are the average of at least three replicates  $\pm$  SD.

Fraction concentration ( $\mu\text{g mL}^{-1}$ )	<i>J. navicularis</i>	<i>J. oxycedrus badia</i>	<i>J. phoenicea</i>	<i>J. turbinata</i>
50	6.44 $\pm$ 3.28	14.97 $\pm$ 8.71	14.36 $\pm$ 4.84	16.38 $\pm$ 5.76
100	14.31 $\pm$ 6.46	14.26 $\pm$ 3.60	15.93 $\pm$ 4.71	29.33 $\pm$ 3.90
200	25.76 $\pm$ 3.48	24.02 $\pm$ 5.03	33.74 $\pm$ 3.32	47.07 $\pm$ 4.88
400	30.69 $\pm$ 7.60	27.03 $\pm$ 8.47	53.44 $\pm$ 3.02	83.84 $\pm$ 7.90
800	55.60 $\pm$ 5.23	38.78 $\pm$ 4.07	65.48 $\pm$ 3.03	72.65 $\pm$ 17.06

### 3.3. Cytotoxicity profile

Cell viability was determined for SK-N-MC neuroblastoma cells submitted to increasing concentrations of PEFs (0 to 500  $\mu\text{g GAE mL}^{-1}$ ; Fig. 2). The four species presented different toxicity profiles. The less toxic species were *J. navicularis* and *J. oxycedrus badia*, which required more than 100  $\mu\text{g GAE mL}^{-1}$  to cause complete cell death. *J. turbinata* and *J. phoenicea* PEFs were more toxic, as they required less than 100  $\mu\text{g GAE mL}^{-1}$  to drop cell viability to 0%. The different toxicities were also reflected in the amount of PEFs required to attain 50% of cell viability (around 20  $\mu\text{g GAE mL}^{-1}$  for *J. phoenicea* and 60-70  $\mu\text{g GAE mL}^{-1}$  for *J. navicularis*).

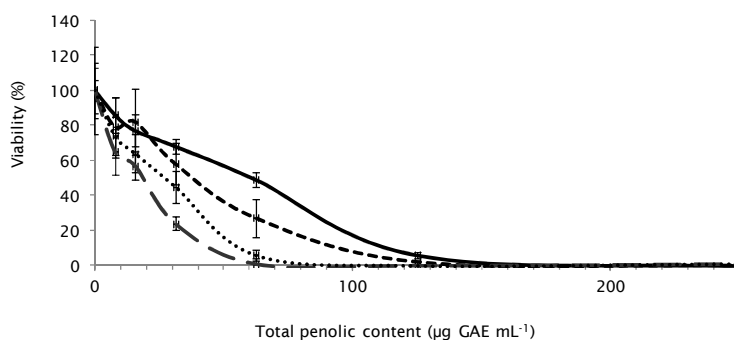


Fig. 2- Cytotoxicity profiles of *Juniperus* sp. PEFs. Cell viability was determined for SK-N-MC neuroblastoma incubated with *Juniperus* sp. PEFs (0 to 500  $\mu\text{g GAE mL}^{-1}$ ) for 24 h. *J. navicularis* (—), *J. oxycedrus badia* (----), *J. phoenicea* (— —) and *J. turbinata* (·····). All values are mean  $\pm$  SD, n = 3.

### 3.4. Intracellular ROS production

The effect of pre-incubation with *Juniperus* PEFs on the production of ROS by neuroblastoma cells in the presence or absence of oxidative stress was evaluated (Fig. 3). PEFs were applied at concentrations demonstrated to be non-toxic for cells. In the absence of stress, all four *Juniperus* sp. PEFs reduced the basal production of ROS in cells exposed for 2 h or 24 h (Figs. 3A and B, respectively), and this reduction was more significant after 24 h incubation (Fig. 3B). When cells treated with PEFs for 2 h were exposed to a non-lethal stress (200  $\mu\text{M}$   $\text{H}_2\text{O}_2$  for 1 h)<sup>52</sup>, a significant decrease in ROS levels was recorded compared to the stress in the absence of PEFs but not for all PEF concentrations used (Fig. 3C). For example, the highest concentration of PEFs from *J. phoenicea* and *J. turbinata* (5  $\mu\text{g}$  GAE  $\text{mL}^{-1}$ ) did not significantly reduce ROS production although lower concentrations did. Indeed, when cells were pre-incubated with PEFs for 24h, only *J. navicularis* and *J. oxycedrus badia* PEFs significantly reduced ROS production, for all three concentrations used (Fig. 3D).

The 24 h pre-incubation with PEFs gives sufficient time that ROS levels may be affected through alterations in indirect antioxidant defences. Therefore we decided to evaluate the activities of two important endogenous antioxidant enzymes (SOD and CAT). Their activities were determined in neuroblastoma cells pre-incubated with PEFs for 24 h in the absence or presence of the non-lethal stress (200  $\mu\text{M}$   $\text{H}_2\text{O}_2$  for 1 h). No differences were detected in SOD or CAT activities, when cells were stressed with  $\text{H}_2\text{O}_2$  (results not shown). The lack of changes in SOD and CAT activities were also verified in cells pre-incubated with PEFs or pre-incubated with PEFs followed by the oxidative challenge.

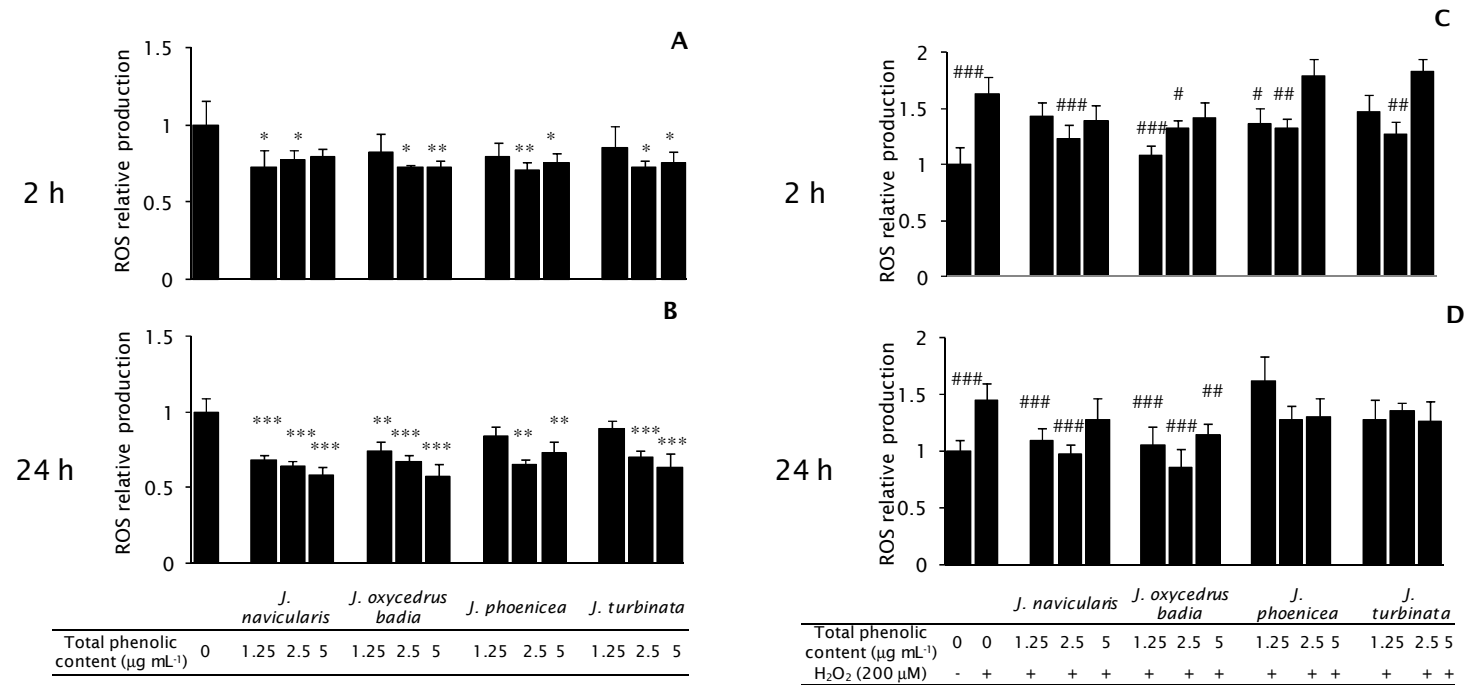


Fig. 3- Relative intracellular ROS production by SK-N-MC neuroblastoma cells pre-incubated with *Juniperus* PEFs. Neuroblastoma were pre-incubated with PEFs for 2 h (A) or 24 h (B) in the absence of stress or for 2 h (C) or 24 h (D) in the presence of stress ( $\text{H}_2\text{O}_2$  200  $\mu\text{M}$ , 1h). ROS were detected by fluorimetry using  $\text{H}_2\text{DCFDA}$  as probe. Statistical differences in relation with untreated cells are denoted as \*  $p < 0.05$  \*\*  $p < 0.01$  \*\*\*  $p < 0.001$ ; statistical differences in relation with stressed cells are denoted as #  $p < 0.05$  ##  $p < 0.01$  ###  $p < 0.001$ . All values are mean  $\pm$  SD,  $n = 3$ .

### 3.5. Neuroprotective effect against oxidative stress

PEFs were also tested in a neurodegeneration cell model for their protective effects. This model consists of neuroblastoma cells injured with 300  $\mu\text{M}$   $\text{H}_2\text{O}_2$  for 24 h, a condition that routinely reduces viability to 50 %. Two parameters were used to assess the protective effect of the PEFs: cell viability through the measurement of membrane integrity (Fig. 4A) and measurement of  $\Delta\Psi\text{m}$  (Fig. 4B).

All *Juniperus* sp. PEFs were tested but only the *J. oxycedrus badia* PEF was able to protect cell viability and promote an increase in  $\Delta\Psi\text{m}$  (Fig. 4) at the concentrations used. The others *Juniperus* sp. PEFs did not improve these parameters over the stressed conditions (results not shown). The highest concentration of *J. oxycedrus badia* PEF (5  $\mu\text{g}$  GAE  $\text{mL}^{-1}$ ) did not change viability, but decreased  $\Delta\Psi\text{m}$  in the absence of  $\text{H}_2\text{O}_2$ , suggesting that it may influence cellular metabolism. Under  $\text{H}_2\text{O}_2$  stress, 2.5  $\mu\text{g}$  GAE  $\text{mL}^{-1}$  PEF was the most effective cytoprotective concentration, in both parameters (viability and  $\Delta\Psi\text{m}$ ). The use of 5  $\mu\text{g}$  GAE  $\text{mL}^{-1}$  of PEF was not able to produce a better protection than 2.5  $\mu\text{g}$  GAE  $\text{mL}^{-1}$  PEF.

## 4. Discussion

Previous work has attributed antioxidant activity and AChE inhibitory activity to extracts of some *Juniperus* sp. These properties along with their multiple aforementioned bioactivities (see section 1. Introduction) make *Juniperus* sp. interesting plants in the search for new NPs to treat NDDs. Despite this, diverse species of *Juniperus*, and in particular those growing in Portugal have not been characterized with respect to their phenolic composition; the proposed source of some of their bioactivities. Botanically, *J. navicularis* and *J. oxycedrus badia* belong to section *Juniperus* and *J. phoenicea* and *J. turbinata* belong to section *Sabina* and these inter-relationships might also underlie chemical diversity<sup>62</sup>.

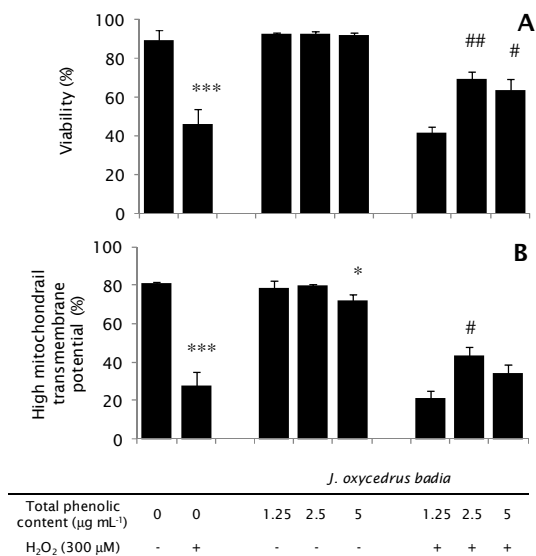


Fig. 4- Cell viability and mitochondrial transmembrane potential ( $\Delta\Psi_m$ ) for cells pre-incubated with *J. oxycedrus badia* PEF. (A) Cell viability, expressed as percentage of cells containing intact membrane, using PI as fluorochrome. (B) Percentage of cells presenting high  $\Delta\Psi_m$ , using DiOC<sub>6</sub>(3) as probe. Neuroblastoma cells were pre-incubated with PEFs for 24 h and then injured by 300 µM H<sub>2</sub>O<sub>2</sub> for 24 h. Statistical differences comparatively with untreated cells are denoted as \* p<0.05 \*\*\* p<0.001; statistical differences comparatively with injured cells are denoted as # p<0.05 ## p<0.01. All values are mean  $\pm$  SD, n = 3.

The four species yielded extracts with high TPCs when compared with extracts obtained in other works<sup>31,32</sup>. After enrichment by SPE fractionation the fractions exhibited a higher phenolic content without organic acids, sugars and minerals which could confound any subsequent bioactivity analyses.

This is the first time that the phenolic composition of these four *Juniperus* sp. has been described. Previous work on these species has mainly focused on essential oils composition<sup>23,40,42</sup> or have described biological properties without attempting to define or chemically characterise the derivation of the bioactivity<sup>26,33,63</sup>. This work showed that the main polyphenolic compounds were common among the different species and some have been identified previously in various studies in different

*Juniperus* sp.<sup>2,3,7,13,44,58-60</sup>. The main differences noted between the four species under study were in the relative amounts of certain compounds. For example, in *J. oxycedrus badia*, *J. phoenicea* and *J. turbinata*, psydin was a major peak. However, in *J. navicularis*, this compound was not so dominant and other components, including the procyanidin trimer (peak 9, RT= 17.32) and quercetin rutoside (peak 13, RT = 21.86), were more abundant. On an equivalent phenolic basis, *J. oxycedrus badia*, *J. phoenicea* and *J. turbinata* were characterised by having greater levels of psydin but also enhanced levels of procyanidins and certain individual peaks such as peak 7 (a procyanidin dimer) as well as the flavone glycosides (peaks 20, 21). Indeed, on further examination, small differences in the flavonol and flavone composition of the species could be discerned which would not explain their different bioactivities, but could be used to distinguish them. However this would require more extensive examination.

Among the phenolic compounds identified, biflavones such as amentoflavone, have already been described as useful in NDDs. It should be noted that the levels of these components were similar between the four PEFs. Amentoflavone has been reported as possessing inhibitory effect on the group II phospholipase A2 (PLA2) activity and also in inhibiting COX<sup>64</sup>. Collective evidence from many recent studies suggests that increased PLA2 activity and PLA2-generated mediators play a central role not only in acute inflammatory responses in the brain, but also in oxidative stress associated with neurological disorders<sup>65</sup>. PLA2 reportedly contributes to the pathogenesis of these disorders by attacking neural membrane phospholipids and releasing proinflammatory lipid mediators such as prostaglandins among others and also by generating 4-hydroxynonenal, a well reported toxic product of lipid oxidation<sup>66</sup>. Thus, inhibition of PLA2 activity provides an attractive approach for the treatment of inflammation and oxidative stress associated with acute neural trauma and some neurodegenerative disorders such as AD<sup>67</sup>.

Among the possible strategies for the treatment of AD, PD, senile dementia, ataxia and myasthenia gravis is the enhancement of brain cholinergic activity by AChE inhibition<sup>68-70</sup>. The principal role of AChE is the termination of nerve impulse transmission at the cholinergic synapses by rapid hydrolysis of ACh. A variety of plants have been reported to show AChE inhibitory activity and constitute sources of potential new NPs in the treatment of neurodegenerative disorders<sup>70</sup> with diverse phytochemistries including alkaloids such as physostigmine and galantamine<sup>71</sup>, ursolic acid<sup>71</sup>, lignans<sup>72</sup>, flavonoids, terpenoids and coumarins<sup>14</sup>. Leaves of the *Juniperus* sp. used here caused similar levels of AChE inhibition as reported for other plants<sup>70</sup> and comparable to extracts from other *Juniperus* sp. leaves<sup>31</sup>. In that work, *J. excelsa* extracts caused 45 % inhibition using 200 µg mL<sup>-1</sup>, whilst here *J. turbinata* extracts inhibited 47 % of AChE activity at the same concentration of PEF. The remaining three species were less effective (causing 20 to 30 % inhibition at this concentration). Among the four species, the most effective AChE inhibition was caused by *J. phoenicea* and *J. turbinata*, which reached 50 % of inhibition at ~400 and 200 µg mL<sup>-1</sup> respectively. Since the PEFs, by definition, are enriched in phenolic components but free of sugars, organic acids and minerals, it is likely that AChE inhibitory activity was derived from phenolic compounds either singly or in combination. Psydrin and as well as quercetin derivatives have been identified as a good inhibitors of AChE<sup>73,74</sup>. It has been demonstrated that quercetin inhibits AChE by a competitive mechanism<sup>75,76</sup>. However, other components must contribute to AChE inhibition since *J. navicularis* exhibited the highest amount of quercetin rutinoside (peak 13) but was the least effective PEF. The most effective PEFs for AChE inhibition (*J. phoenicea* and *J. turbinata*) had higher relative levels of procyanidins and lower levels of flavonols. Their higher effectiveness as AChE inhibitors could be related to their different composition. Due to the adverse side-effects and bioavailability problems of some AChE inhibitors already in use, there still is great

interest in finding better molecules<sup>77,78</sup>. However, as the bioavailabilities of the juniper phenolics, are unknown, especially the unidentified components, their potential as effective agents remains to be discovered. Since many of the NPs exhibit an hormetic effect<sup>79</sup> it was necessary to confirm that the concentrations used were not cytotoxic before testing PEFs in neuroblastoma cells. Once again, there was a clear difference between the *Juniperus* sp., with *J. navicularis* and *J. oxycedrus badia* being the least toxic and *J. turbinata* and *J. phoenicea* more toxic. This difference in cytotoxicity must be related to differences in the polyphenol composition.

Non-toxic concentrations of PEFs (0 and 5  $\mu\text{g GAE mL}^{-1}$ ) from the four *Juniperus* sp. reduced basal ROS production (without  $\text{H}_2\text{O}_2$  stress) at 2 and 24 h incubation. When a non-lethal stress was imposed (200  $\mu\text{M H}_2\text{O}_2$  for 1 h), all PEFs retained the ability to reduce intracellular ROS following pre-incubation for 2 h. However, at 24 h pre-incubation, only PEFs from *J. navicularis* and *J. oxycedrus badia* caused diminished ROS levels. Antioxidant activity in biological systems could be due to direct or indirect effects of compounds and the *Juniperus* PEFs could be acting by both direct as well as indirect antioxidant capacities. The direct antioxidant capacity could be achieved through a direct radical scavenging activity and the indirect antioxidant capacity through the induction of cellular defenses generally through the Nrf2 system, such as antioxidant enzymes<sup>80</sup>. Therefore, at 2 h pre-incubation with PEFs, we assumed that changes in ROS levels were the consequence of a direct radical scavenging and at 24 h pre-incubation the changes were the consequence of the indirect antioxidant defenses. Thus, the direct scavenging that the four PEFs were able to mediate did not last for a longer time (Fig. 3 C and D). At 24 h pre-incubation other molecular events should take place, but they only should occur in cells pre-incubated with *J. navicularis* and *J. oxycedrus badia* PEFs. Since the amount of phenolic compounds used to determine the intracellular ROS production was equivalent for the four

species, the differences obtained must be the result of the differential composition/relative amount of each compound within species. Concerning the indirect antioxidant defences, the activities of SOD and CAT, considered primary antioxidant enzymes<sup>81</sup>, were not altered as ROS production was decreased. Although both enzymes are responsible for protecting cells against ROS produced during normal metabolism and after an oxidative insult, these results suggest that alternative pathways could be regulated to cope with the imposed increase of ROS. These alternative pathways could be acting through the ARE and/or the xenobiotic response element (XRE) systems<sup>82</sup> with associated induction of induce genes for antioxidant and detoxification enzymes, such as NQO1<sup>83</sup>. Only the PEF from *J. oxycedrus badia* could protect neurons against the injury caused by H<sub>2</sub>O<sub>2</sub>. This PEF enhanced values of viability and  $\Delta\Psi_m$ , compared with cells treated with H<sub>2</sub>O<sub>2</sub>. A PEF concentration of 2.5  $\mu\text{g GAE mL}^{-1}$  produced effective protection, enhancing viability by 51 % and  $\Delta\Psi_m$  by 57 % compared with cells only treated with H<sub>2</sub>O<sub>2</sub>. If considering at the same time the number of cells presenting adequate membrane integrity and a high  $\Delta\Psi_m$ , the augmentation caused by 2.5  $\mu\text{g GAE mL}^{-1}$  of *J. oxycedrus badia* PEF was about 70 %.

These results, together with the data for SOD and CAT activities, suggest that reduction of intracellular ROS levels verified for 24 h pre-incubation with PEFs should be due to the induction of other intracellular antioxidant mechanisms. The involvement of ARE and XRE systems may be valid systems for investigation.

## 5. Conclusions

All four *Juniperus* sp. PEFs, and in particular *J. oxycedrus badia*, constitute potential sources of neuroprotective NPs. The four species are rich sources of phenolic compounds and contain molecules previously described with neuroprotective potentialities, such as quercetin derivatives (AChE inhibitor) and amentoflavone (effect on the group II

PLA2 inhibitor and cyclooxygenase inhibitor). Phenolic compounds from these species caused effective intracellular radical scavenging in neurons submitted to oxidative injury. Moreover, *J. navicularis* and *J. oxycedrus badia* PEFs induced mechanisms that reduced ROS formation induced by H<sub>2</sub>O<sub>2</sub> treatment. Finally, *J. oxycedrus badia* PEF had a protective effect in the neurodegeneration cell model. The lack of impact of the components on the standard antioxidant enzymes CAT and SOD suggest alternative mechanisms underpin this bioactivity. Further research is merited to elucidate the source and mechanisms of these bioactivities, particularly if these are to be used in dietary-based neuroprotection strategies such as functional foods and/or functional food ingredients.

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### Chapter 3: Elucidating phytochemical production in *Juniperus* sp.: seasonality and response to stress situations

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LT performed the majority of the experiments presented in this chapter in collaboration with RP and wrote the manuscript cited above.

### **Abstract**

Phenolic enriched extracts from endemic Portuguese junipers were described as potential neuroprotective NPs. However, evaluation of plants for pharma/nutraceutical industry is based on secondary metabolites content, which is influenced by the environmental conditions. Therefore, it became important to elucidate the metabolic response of the junipers to seasonality and to stress conditions, concerning PPs production.

Seasonal conditions modulated total phenolic and flavonoids contents of the four juniper species, being higher levels of phytochemicals obtained when plants were not actively growing. However, it was only register few differences in the relative contents of psydrin and a procyanidin dimer, accordingly to species. Salt stress and methyl jasmonate (MeJa) elicitation presented different susceptibilities within the species. They were effective in enhancing phenolic accumulation, with just few alterations in the relative contents of procyanidins, epicatechin and quercetin derivatives. These differences were also reflected in gene expression of important enzymes from biosynthetic pathway.

### **Keywords**

Methyl jasmonate; phenolic content; polyphenols biosynthesis; salinity; season variability.

## 1. Introduction

NPs are important sources of novel leads for therapeutic drugs <sup>2</sup>. Between 1981 and 2002, of the 877 novel medicines developed, 49 % were NPs, their derivatives or synthetic products developed with the natural product as a basis <sup>3</sup>.

Among the diverse phytochemicals produced by plants, PPs are an important class. These compounds have many diverse functions in plants, such as colour of leaves, flowers and fruits, anti-microbial, anti-fungal, insect feeding deterrence, screening from damage by solar UV radiation, chelation of toxic heavy metals and anti-oxidant protection from free radicals generated during the photosynthetic process <sup>4</sup>. Some have also been recognized as beneficial for human health since they exhibit activities such as anti-inflammatory, antioxidant, insulin-potentiating, anti-carcinogenic, anti-viral, anti-ulcer, anti-apoptotic, cardiovascular and neurodegenerative protectant among others <sup>5</sup>.

Junipers (*Juniperus* sp.) are plants rich in secondary compounds, particularly terpenoids and phenolics <sup>6-11</sup>. Common juniper extract is an important natural product used widely in many pharmaceutical and technical preparations, in cosmetic products and as a food additive. Some studies have emerged reporting on the diverse biological activities, such as: anti-inflammatory <sup>12</sup>, diuretic, antiseptic <sup>13,14</sup>, hypoglycaemic <sup>15</sup>, hypotensive, analgesic, abortifacient, antinociceptive <sup>16</sup>, antiviral <sup>17</sup>, anticancer <sup>18</sup>, antioxidant <sup>19,20</sup> and anticholinesterase <sup>19,20</sup>. Recently phenolic enriched extracts from endemic Portuguese *Juniperus* sp. were described as potential NPs in the treatment of NDDs <sup>1</sup>.

The quality of plants intended for processing by the pharmaceutical industry is usually judged on the basis of secondary metabolites content <sup>21</sup>. However, environmental conditions have an important effect on the secondary metabolisms <sup>22</sup>. It has been reported that PPs levels can change in response to biotic (pest and pathogens) and abiotic (drought, hypoxia, high light intensity, low or high temperature) stresses <sup>22,23</sup>. Therefore, it is critical to understand the metabolic response of the *Juniperus* sp. to

seasonality as well as to stress conditions, in terms of PPs production, if the species is to realise its full potential.

## 2. Materials and methods

### 2.1. Plant material and growth conditions

#### 2.1.1. Seasonal evaluation

Five years old plants of *J. navicularis* Gand., *J. oxycedrus badia* (H. Gay) Debeaux, *J. phoenicea* L. and *J. turbinata* Guss were used for seasonal evaluation. As *J. navicularis* is a Portuguese endemic and threatened plant, a license for capture (11/2008/CAPT and 12/2008/CAPT) was required for sampling. For all species, voucher samples were authenticated and deposited at the herbarium "João de Carvalho e Vasconcelos", Instituto Superior de Agronomia, Lisbon, Portugal. The herbarium numbers are: *Juniperus navicularis* (LISI 1064/2007), *J. oxycedrus* subsp. *badia* (LISI 1/2008), *J. phoenicea* (LISI 3/2008) and *J. turbinata* (1067/2007). All plants were grown in pots, exposed to the same local (Lisbon area) environmental conditions of light, temperature and humidity. Plants were watered when necessary. Leaf samples were collected monthly through the year 2008, freeze-dried, ground to fine powder and stored at -80 °C.

#### 2.1.2. Stress conditions

For stress evaluation, two year old plants of *J. oxycedrus badia*, *J. phoenicea* and *J. turbinata* were used. Plants were maintained in 1 L pots, inside a growth chamber (Fitoclima 700 EDTU, Aralab, Lisbon, Portugal) with standard conditions: 30 °C temperature, 60 % relative humidity, 800  $\mu\text{mol m}^{-2} \text{s}^{-1}$  and 16 h/8 h (light/dark) photoperiod. All the plants, except the ones submitted to drought stress, were watered frequently with 300 mL of water. Plants were acclimatized for one week prior to stress initiation. For each condition tested five plants were used. **Drought stress** Plants were maintained in standard conditions but without watering for 20 days. Control plants were maintained under standard conditions for 20 days.

*Salt stress* Plants were kept in standard conditions but watered with 300 mL of 250 mM NaCl for 15 days. Control plants were maintained in standard conditions for 15 days.

*Methyl jasmonate (MeJa) treatment* Plants were sprayed with an aqueous solution containing 5 mM MeJa (Sigma-Aldrich) and 0.1 % (v/v) Tween 20 (Sigma-Aldrich) at day 0 and effects followed for 120 h. Control plants were sprayed with aqueous solution of 0.1 % (v/v) Tween 20.

### 2.1.3. Phenolic extracts preparation

Phenolic compounds were extracted from leaves using a 50 % ethanol solution (v/v) in a ratio of 12 mL g<sup>-1</sup> of freeze dried leaf powder, as previously described <sup>24</sup>.

## 2.2. Chemical characterization

### 2.2.1. Total phenolic content (TPC)

Determination of total phenolic compounds was performed by the Folin-Ciocalteu method adapted to microplate reader <sup>25</sup>. Gallic acid was used as the standard and the results were expressed as mg of GAE.

### 2.2.2. Total flavonoid content (TFC)

Measurement of total flavonoids was performed by a modification of the AlCl<sub>3</sub> complexation method as described before <sup>25</sup> and expressed as mg catechin equivalents (CE).

### 2.2.3. Phenolic composition

Samples containing 20 µg GAE were applied to a C18 column (Synergi Hydro C18 column with polar end capping, 4.6 mm x 150 mm, Phenomenex Ltd.) and analysed on a LCQ-DECA system controlled by the XCALIBUR software (2.0, ThermoFinnigan), as reported by Tavares *et al.* <sup>26</sup>. The LCQ-Deca system comprised a Surveyor autosampler, pump and PDA detector and a Thermo Finnigan iontrap mass spectrometer. Samples were analysed in triplicate and in positive and negative mode to aid identification. Peak areas were assigned and quantified using the XCALIBUR software.

### 2.3. Relative accumulation of enzyme transcripts from phenylpropanoid biosynthesis pathway

#### 2.3.1. Achievement of specific RNA sequences from *Juniperus* sp.

##### 2.3.1.1. RNA isolation and cDNA synthesis

Total RNA was isolated using an adaptation of the method described by Chang *et al.* <sup>27</sup>. The extraction buffer contained 2 % (w/v) CTAB, 2 % (v/v) 2-mercaptoethanol, 3 M NaCl, 25 mM EDTA, 100 mM Tris-HCl (pH 8.0), 2% (w/v) PVP-40 and 0.04 % (w/v) of spermidine. Briefly, leaf were ground with liquid nitrogen and each 1 g of leaf powder was incubated with 20 mL of pre-heated extraction buffer for 30 min at 65 °C with periodic shaking. Protein extraction was carried out using with chloroform:isoamyl alcohol (24:1) then RNA was precipitated with LiCl overnight at 4 °C <sup>27</sup>. The RNA obtained was dissolved in SSTE (1 M NaCl, 0.5% (w/v) SDS, 10 mM Tris-HCl pH 8 and 1 mM EDTA pH 8) and a second protein removal was performed followed by nucleic acid precipitation with absolute ethanol at -20 °C. The pellet was dried and resuspended in sterile water. RNA quantity and quality were measured spectrophotometrically (ND 1000, ThermoScientific) and checked by agarose gel electrophoresis. Afterwards, RNA was treated with Turbo DNA-free™ kit from Applied Biosystems. Reverse transcription (RT)-PCR used 2 µg of total RNA in a 20 µL volume reaction, 25 µg mL<sup>-1</sup> anchored oligo(dT)12-18 primer (Invitrogen) and SuperScript™ II first-strand synthesis system for reverse transcription (RT)-PCR (Invitrogen).

##### 2.3.1.2. Gene Amplification, cloning and sequencing

To determine the accumulation of transcripts that encode some of the most important phenylpropanoyd biosynthesis pathway enzymes, specific sequences of PAL, F3H, ANS, LAR and ANR for *J. oxycedrus badia* and *J. phoenicea* were amplified. For Real-time RT-PCR normalization purposes, the sequences of actin (ACT), elongation factor 1 $\alpha$  (EF1A) and glyceraldeide 3-phosphate dehydrogenase (GAPDH) were also determined. For each gene of interest, amino acid and nucleic acid sequences from diverse species, giving special attention to gymnosperm species, were

aligned and primers were designed for the most conserved regions. Primers were chosen in order to obtain PCR products with approximately 200 to 500 bp size.

Genes of interest were amplified in PCR reactions containing 0.1 to 2  $\mu$ L of cDNA, 0.625 U *Taq* DNA Polymerase (Fermentas), 0.5  $\mu$ M of each primer, 2.5 mM  $MgCl_2$ , 0.2 mM of each dNTP, 75 mM Tris-HCl (pH 8.8) and 20 mM  $(NH_4)_2SO_4$ . The quality of PCR products was visually inspected by electrophoresis, and the generation of only one single band of the expected size was the criterion for specificity. The PCR products were then directly cloned using TA Cloning® Kit Dual Promoter (pCR®II) from Invitrogen, accordingly to the manufacturer's instructions. One Shot® INVαF' Chemically Competent *E. coli* (Invitogen) were transformed using the vector previously prepared. Transformed colonies grown in selective medium and the plasmids were purified using illustra plasmidPrep Mimi Spin Kit (GE Healthcare).

The fragments were sequenced using the plasmid internal primers and their identity confirmed by comparison with sequences in database. Obtained sequences were also deposited in database with GenBank Accession numbers GQ389669 to GQ389686 (Table 1).

#### 2.3.2. Relative quantification of *Juniperus* sp. transcripts by quantitative Real Time RT-PCR

Primers for Real Time RT-PCR were designed using the conserved regions of sequences previously obtained for both species. Beacon Designer™ 7.5 (PREMIER Biosoft International) software was used and primers were designed to obtain products with size between 80 and 200 bp (Table 1). Total RNA isolation, DNase treatment and cDNA synthesis were performed as described above.

Table 1- Enzymes transcripts from the phenylpropanoid biosynthetic pathway evaluated in *J. phoenicea* and *J. oxycedrus badia* by Real Time RT-PCR. For each are shown the geneGenBank accession numbers, gene sequence of primers used in Real Time RT-PCR and respective product length.

Gene	Species	GenBank accession number	Primers	PCR product length (bp)
PAL	<i>J. phoenicea</i>	GQ389685	Fwd: ATGGCTTCTTACACCTCTGAG	188
	<i>J. oxycedrus badia</i>	GQ389686	Rev: TCCACCGCTTGACAAATCC	
F3H	<i>J. phoenicea</i>	GQ389679	Fwd: GGATTGGAAGCCGACGCGGT	168
	<i>J. oxycedrus badia</i>	GQ389680	Rev: TGGAGGAGCAGAGTGATG	
LAR	<i>J. phoenicea</i>	GQ389683	Fwd: CCAGTTGAACCTGCTTTGAG	190
	<i>J. oxycedrus badia</i>	GQ389684	Rev: TGACAGTGCCATCACCATAG	
ANS	<i>J. phoenicea</i>	GQ389673	Fwd: TCAGTGCTCTCACATCC	193
	<i>J. oxycedrus badia</i>	GQ389674	Rev: ATCCTTACTTTCTCCTTGTTTC	
ANR	<i>J. phoenicea</i>	GQ389671	Fwd: ACTTGCCCTTAGCCTACTTACAG	117
	<i>J. oxycedrus badia</i>	GQ389672	Rev: AGTTGAGCACTACAAACATCATCC	
ACT	<i>J. phoenicea</i>	GQ389669	Fwd: CCGCGATGTATGTTGCTATTC	157
	<i>J. oxycedrus badia</i>	GQ389670	Rev: CCAGCGAGATCCAACCTAAG	
EF1A	<i>J. phoenicea</i>	GQ389677	Fwd: CTCTCAGGTTGCCACTTCAG	131
	<i>J. oxycedrus badia</i>	GQ389678	Rev: TTCAGTTGTCAGCCCAAGTTG	
GAPDH	<i>J. phoenicea</i>	GQ389681	Fwd: AACTAATTGTCTTGCTCCTCTTG	200
	<i>J. oxycedrus badia</i>	GQ389682	Rev: CCTTTCCAACCTGCCTTTGC	

Real-time RT-PCR was performed using an iCycler iQ system (Bio-Rad). Each reaction contained 10  $\mu$ L of 2X iQ<sup>TM</sup> SYBR<sup>®</sup> Green Supermix (Bio-Rad), 1.0  $\mu$ L of diluted cDNA sample, and 0.5 mM of each gene-specific primer in a final volume of 20  $\mu$ L. The following program was applied: 95  $^{\circ}$ C, 3 min; then 45 cycles at 94  $^{\circ}$ C, 10 s; 60  $^{\circ}$ C, 20 s; 72  $^{\circ}$ C, 30 s. All reactions were performed in triplicate. To check the specificity of the PCR reaction, melting curves were analyzed for each data point. Efficiency was determined by comparison of experimentally determined and theoretically expected threshold values, in cDNA mixtures using serial dilutions. ACT, EF1A and GAPDH were used for internal normalization. The expression rates were determined using Gene Expression Analysis for iCycler iQ<sup>®</sup> Real-Time PCR Detection System v1.10-©2004 (Bio-Rad). The calculation method took into account the different amplification efficiencies determined for each gene and also calculated a normalization factor which is the geometric mean of the relative quantities for all reference genes.

## 2.4. Statistical analysis

The results reported in this work are the averages of at least three independent experiments and are represented as the mean  $\pm$  SD. Differences among treatments were detected by analysis of variance with Tukey HSD multiple comparison test ( $\alpha=0.05$ ) using SigmaStat 3.10 (Systat).

## 3. Results

### 3.1. Seasonal evaluation of phytochemicals

To understand the seasonal dynamics of phytochemical compounds in the four different juniper species, plants were grown in the same environmental conditions to avoid differences in key growth drivers such as soil, moisture, temperature, solar exposure amongst others parameters. The highest value of TPC was obtained for *J. turbinata* and the lowest for *J. phoenicea* (Table 2). These were also the two species where the highest differences between the minimum and the maximum value attained through the year were observed. These differences were ~ 235 %, 118 %, 82 % and 78% for *J. phoenicea*, *J. turbinata*, *J. oxycedrus badia* and *J. navicularis*, respectively. The highest TPC were usually registered in the winter (months of October to January) and for *J. navicularis* and *J. turbinata* also in August and September. The lowest values were noted at beginning of spring (Feb/Mar to Apr/May) depending on the species and also in July, except for *J. turbinata*. A similar pattern was observed across the species for the TFC (Table 2). However, the highest TFC was exhibited by *J. oxycedrus badia* and the lowest in *J. phoenicea*. Selected samples were analysed for phytochemical composition using LC-MS. The months chosen were April, May, July and December. The rationale was to analyze junipers at the stage of active growth (April), the stage when they stop growing (May), the months when most of junipers register low levels of TPC and TFC (July) and high TPC and TFC (December). The main phytochemicals were then identified (Fig. S1 on Supplementary material) and their levels quantified relative to each other (Fig. 1).

Table 2- Total phenol content and total flavonoid content for the four juniper species (*J. navicularis*, *J. oxycedrus badia*, *J. turbinata* and *J. phoenicea*) across different months. Different letters (a-f) denote significantly different samples at  $p < 0.05$ .

Species	Months	Total phenolic content (mg GAE g <sup>-1</sup> DW)		Total flavonoid content (mg CE g <sup>-1</sup> DW)	
<i>J. navicularis</i>	Jan	80.84 ± 1.32	a	37.50 ± 0.98	a
	Feb	45.66 ± 1.30	f	20.52 ± 0.38	g
	Mar	57.91 ± 0.23	de	24.80 ± 0.47	f
	Apr	56.75 ± 1.67	d	24.92 ± 0.65	ef
	May	69.48 ± 2.41	bc	33.14 ± 0.89	b
	Jun	63.07 ± 2.56	cd	29.83 ± 1.06	d
	Jul	54.26 ± 0.97	e	27.20 ± 0.33	e
	Aug	65.52 ± 4.05	b	30.81 ± 0.87	cd
	Set	65.30 ± 5.59	bcd	30.24 ± 1.25	d
	Oct	68.43 ± 1.35	b	31.88 ± 0.68	bcd
	Nov	71.61 ± 0.36	b	32.68 ± 0.66	bc
	Dec	81.24 ± 2.77	a	36.10 ± 0.67	a
<i>J. oxycedrus badia</i>	Jan	94.37 ± 7.68	ab	54.18 ± 1.02	b
	Feb	72.91 ± 2.73	d	43.96 ± 0.99	f
	Mar	57.06 ± 2.20	f	35.71 ± 0.51	g
	Apr	55.86 ± 0.89	e	37.71 ± 0.38	g
	May	56.30 ± 2.81	f	38.32 ± 0.58	g
	Jun	80.61 ± 2.35	cd	50.91 ± 0.79	cd
	Jul	60.89 ± 2.94	f	37.60 ± 2.01	g
	Aug	77.68 ± 3.80	d	48.44 ± 1.55	de
	Set	74.65 ± 5.78	d	47.23 ± 1.21	ef
	Oct	83.52 ± 2.22	bcd	52.07 ± 0.73	bc
	Nov	90.86 ± 0.62	bc	54.70 ± 0.49	b
	Dec	102.55 ± 6.29	a	62.01 ± 2.26	a
<i>J. phoenicea</i>	Jan	61.42 ± 0.69	d	25.94 ± 1.57	bc
	Feb	41.30 ± 0.43	e	16.90 ± 1.32	e
	Mar	26.56 ± 1.34	f	9.95 ± 0.46	f
	Apr	44.68 ± 1.75	e	16.95 ± 0.09	e
	May	31.32 ± 0.67	f	10.67 ± 0.18	f
	Jun	60.18 ± 0.61	d	23.89 ± 0.74	cd
	Jul	43.52 ± 0.81	e	15.30 ± 0.92	e
	Aug	69.75 ± 1.14	c	25.75 ± 0.83	bc
	Set	71.65 ± 3.69	bc	23.17 ± 0.15	d
	Oct	77.65 ± 2.81	b	27.27 ± 1.10	ab
	Nov	74.61 ± 2.64	b	26.92 ± 0.47	b
	Dec	89.07 ± 3.85	a	29.73 ± 0.67	a
<i>J. turbinata</i>	Jan	90.90 ± 1.84	b	34.50 ± 1.05	b
	Feb	62.89 ± 2.15	de	23.20 ± 0.84	d
	Mar	51.94 ± 1.57	e	19.88 ± 0.11	e
	Apr	91.66 ± 4.03	b	34.25 ± 0.70	b
	May	75.67 ± 0.23	cd	29.83 ± 0.80	c
	Jun	73.05 ± 1.27	d	28.60 ± 0.73	c
	Jul	86.67 ± 2.25	bc	33.40 ± 0.71	b
	Aug	94.11 ± 2.55	b	33.88 ± 1.98	b
	Set	95.07 ± 3.73	b	34.63 ± 1.47	b
	Oct	70.31 ± 2.39	d	25.00 ± 0.50	d
	Nov	113.52 ± 12.66	a	38.44 ± 0.90	a
	Dec	95.74 ± 10.15	b	33.69 ± 0.76	b

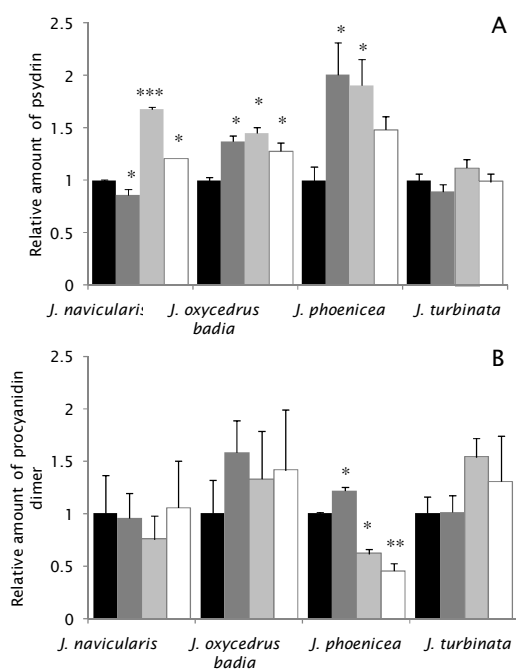


Fig. 1- Relative quantification of psydrin (A) and procyanidin dimer (B) for the four *Juniperus* sp. in April (■), May (■), July (■) and December (□). Phytochemicals were quantified by LC-MS and normalized against the April values, which was the month generally presenting the lowest TPC contents. Statistical differences relative to April values are denoted by \* p<0.05, \*\* p<0.01 and \*\*\* p<0.001.

Analysed on the basis of equivalent phenol content, LC-MS identified that only two compounds showed differentially significant quantitative changes across the seasons; psydrin (5-methyl-4-*O*-( $\beta$ -D-glucopyranosyl)-3(2H)-furanone; peak 1, RT 8.22 in Fig. S1; Fig. 1A) and a procyanidin dimer (peak 7, RT 15.87 in Fig. S1; Fig. 1B). Psydrin exhibited significant changes in *J. navicularis*, *J. oxycedrus badia* and *J. phoenicea* but not in *J. turbinata*. Levels of psydrin increased in May, July and December in comparison with the levels in April. This increase was ~50 % for *J. navicularis* (July) and *J. phoenicea* (May and July). The exception was in May for *J. navicularis*, when psydrin levels slightly decreased. Only *J. phoenicea* showed statistically significant alterations in the levels of the procyanidin dimer, being its levels increased in May and decreased in July and December.

### 3.2. Changes in polyphenol composition in response to stress

Young plants from *J. oxycedrus badia*, *J. phoenicea* and *J. turbinata* were submitted to drought and salinity stresses as well as MeJa elicitation. These stresses are reported to induce changes in phenolic content in plants<sup>28-32</sup>. We selected young plants because these are normally more responsive to stress treatment. However, drought stress did not significantly alter TPC and TFC (results not shown). Different *Juniperus* species responded differently to salt stress and MeJa treatment. *J. oxycedrus badia* was the only species that responded to salt stress (Fig. 2) and *J. phoenicea* the only species that responded to MeJa treatment (Fig. 3). In response to salt stress, *J. oxycedrus badia* showed increased TPC and TFC (Fig. 2A). These increases were noted 2 days after stress application and persisted until day 7. Then, levels of TPC and TFC returned to initial levels, with only a slight increase in TPC on day 15. To assess if changes in total phenol content were accompanied by qualitative changes in composition, the samples were analysed by LC-MS. Based on an equivalent amount of phenols, the profiles were very similar and only the levels of epicatechin (Fig. 2B) and a procyanidin trimer were altered significantly by the salt treatment (Fig. 2C). In comparison with control plants, both epicatechin and the procyanidin dimer decreased between the 2<sup>nd</sup> and the 7<sup>th</sup> day of salt stress.

Following MeJa application, TPC and TFC changed significantly (Fig. 3A). The response was biphasic, with an initial increase at 12 h after treatment, which was accompanied by a non-significant increase in TFC, followed by a decrease. At 72 h, both parameters (TPC and TFC) showed significant increases which persisted until 96 h. LC-MS analysis revealed that the only compounds that were differentially altered in response to the treatment were a procyanidin dimer, epicatechin and quercetin-rhamnoside (Fig. 3B, C and D, respectively). In comparison with control plants that were only sprayed with Tween 20, MeJa induced increases in a procyanidin dimer 72 h after treatment (Fig. 3B). Epicatechin also clearly increased from 12 to 72 h after treatment (Fig. 3C).

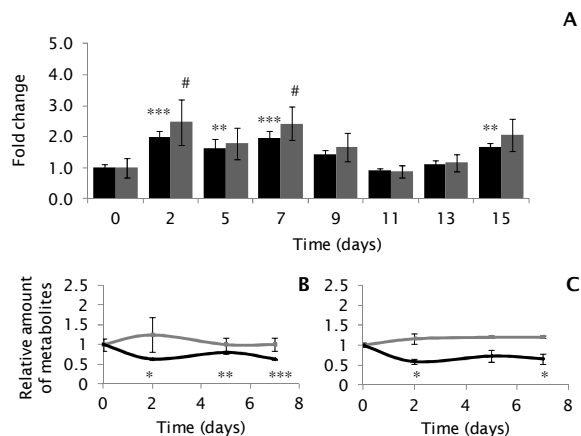


Fig. 3- Changes in total phenol content and total flavonoid content in *J. oxycedrus badia* subjected to salt stress. TPC (■) and TFC (▒) were evaluated (A). Significant statistical changes in total phenolic content in comparison with plants at 0 days are denoted by \* p<0.05, \*\* p<0.01 and \*\*\* p<0.001; significant statistical changes in total flavonoid content in comparison with control plants are denoted by # p<0.05, ## p<0.01 and ### p<0.001. LC-MS also noted that the contents of epicatechin (B) and procyanidin trimer (C) were the only two compounds that changed due to salt stress. Values of control plants are represented by the grey line and at black values for the salt stressed plants. Significant statistical changes in metabolites levels in comparison with control plants are denoted by \* p<0.05, \*\* p<0.01 and \*\*\* p<0.001

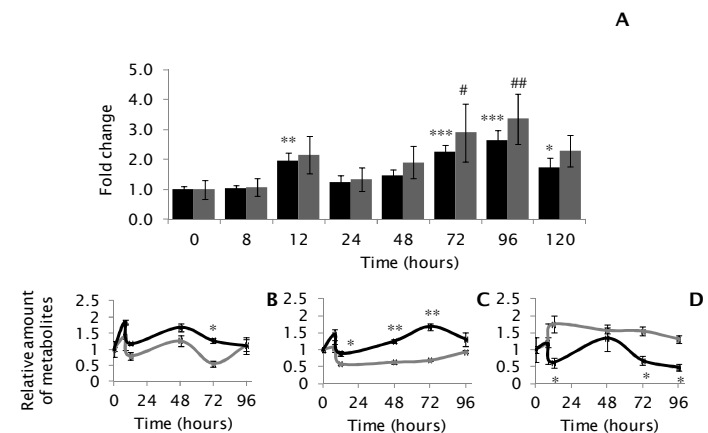


Fig. 2- Changes in total phenol content and total flavonoid content in *J. phoenicea* subjected to Meja treatment. TPC (■) and TFC (▒) were evaluated (A). Significant statistical changes in total phenolic content in comparison with plants at 0 h are denoted as \* p<0.05, \*\* p<0.01 and \*\*\* p<0.001; significant statistical changes in total flavonoid content in comparison with control plants are denoted by # p<0.05, ## p<0.01 and ### p<0.001. LC-MS also noted that the contents of procyanidin dimer (B), epicatechin (C) and quercetin-rhamnoside (D) were the only three compounds that changed due to Meja treatment. Values of control plants are represented by the grey line and at black values for the elicited plants. Significant statistical changes in metabolites levels in comparison with control plants are denoted by \* p<0.05, \*\* p<0.01 and \*\*\* p<0.001

Quercetin-rhamnoside levels decreased compared to control at 12h after methyl jasmonate application, recovered to control levels at 48h then were reduced at 72 to 96 h (Fig. 3C).

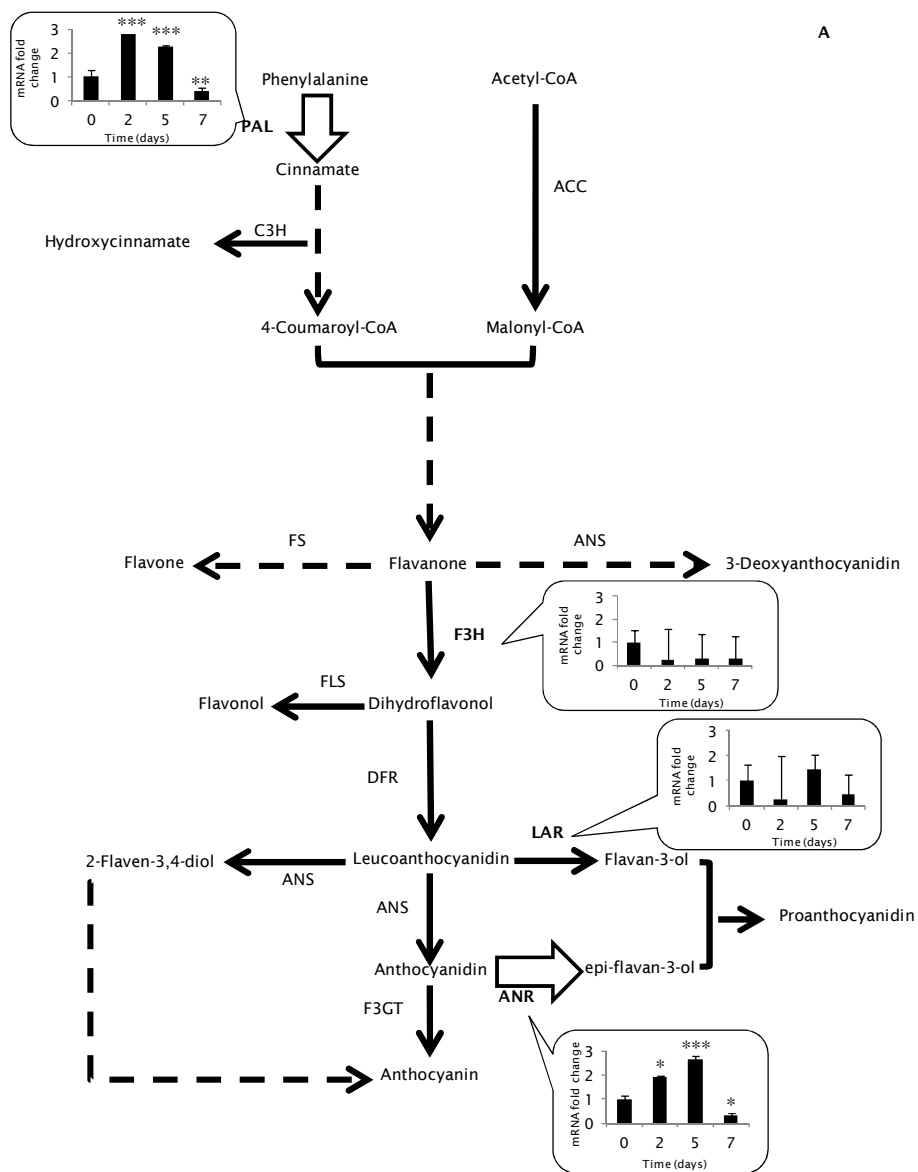
### 3.3. Transcription regulation of enzymes from phenylpropanoid biosynthetic pathway as response to stresses

To verify the transcriptional regulation of the synthesis of metabolites affected by stresses, the mRNA levels of some crucial enzymes of the phenylpropanoid biosynthetic pathway were determined by Real Time RT-PCR. For that, specific nucleotide sequences of *J. oxycedrus badia* and *J. phoenicea* were determined. Relative quantification of transcripts was determined for each enzyme using a normalization factor calculated using the relative quantities of the reference genes ACT, EF1A and GAPDH. Transcript levels of reference genes were monitored for all conditions tested and it was verified that their levels did not vary significantly.

In response to salt stress, the levels of PAL and ANR transcripts increased in *J. oxycedrus badia* (Fig. 4A). For both enzymes, their transcript accumulation was enhanced two days after stress initiation and persisted until day 5. The relative accumulation of F3H and LAR transcripts were not significantly changed in response to salt stress.

On the other hand, MeJa treatment induced changes in the accumulation of PAL, ANS, F3H and ANR transcripts in *J. phoenicea* (Fig. 4B) but with no change in transcript level for LAR. The level of PAL transcripts increased 8 h after treatment and stayed high until 96 h. ANS and F3H also showed a biphasic response with increases within 12h and a second peak at 96h. The expression of ANR was slightly different as it was increased at 8h compared to control, reduced at 12h, increased at 48h then was maintained to 96h.

Chapter 3: Elucidating phytochemical production in *Juniperus* sp.: seasonality and response to stress situations



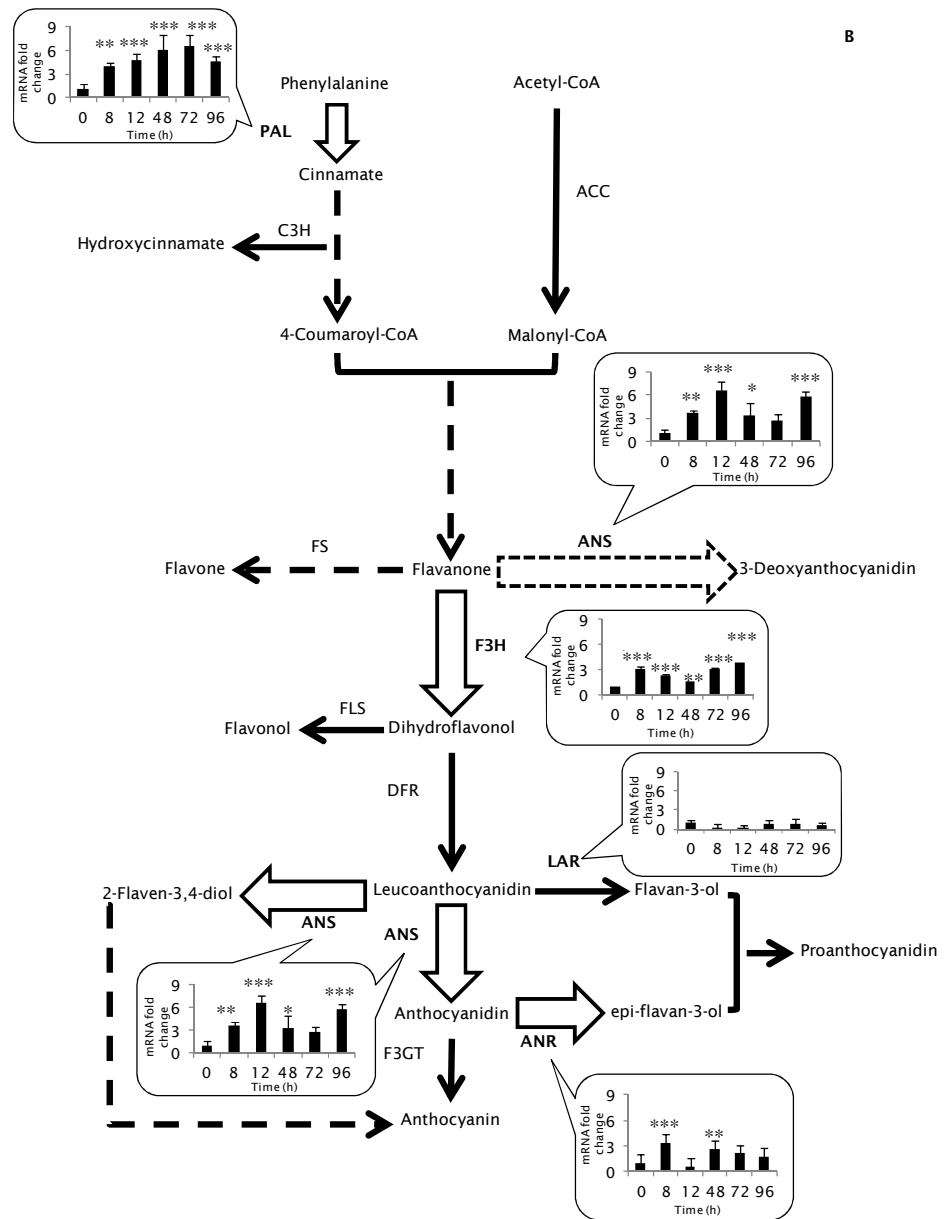


Fig. 4- Schematic representation of the main alteration in expression of enzymes of phenylpropanoid biosynthetic pathway under stress conditions. *J. oxycedrus badia* subjected to salt stress (A) and *J. phoenicea* subjected to MeJa treatment (B). Direct reactions are denoted with solid arrows and indirect with dashed arrows. Enzymes whose mRNA levels were analyzed are written in bold. Reactions mediated by enzymes whose mRNA showed significant differences due to stress are highlighted with block arrows. Graphical representations of mRNA fold change are shown in the respective balloon. Results were obtained from three independent replicates and significant statistical changes are denoted as \*  $p < 0.05$ , \*\*  $p < 0.01$  and \*\*\*  $p < 0.001$ . Legend: ACC- Acetyl-CoA carboxylase (EC 6.4.1.2), ANR- anthocyanidin reductase (EC 1.3.1.77), ANS- anthocyanidin synthase or leucocyanidin oxygenase (EC 1.14.11.19), C3H- *p*-coumarate 3 hydroxylase (EC 1.14.1.1), DFR- dihydroflavonol-4-reductase (EC 1.1.1.219), F3GT- flavonoid-3-glucosyltransferase or anthocyanidin/flavonol 3-O-glucosyltransferase (EC 2.4.1.115), F3H- flavanone 3-hydroxylase or flavanone 3-dioxygenase (EC 1.14.11.9), FLS- flavonol synthase (EC 1.14.11.23), FS- flavone synthase (EC 1.14.11.22), LAR- leucoanthocyanidin reductase (EC 1.17.1.3), PAL- phenylalanine ammonia-lyase (EC 4.3.1.5).

## 4. Discussion

### 4.1. Seasonal evaluation of phytochemicals

*Juniperus* sp. have been widely used in the cosmetic industry, gastronomy, and many uses and biological properties have been attributed to these plants<sup>12,16,18</sup>. The Juniper species are rich in secondary metabolites, compounds well described as greatly affected by environmental conditions<sup>33</sup>. Since the industrial quality (and relevance) of plants to the pharma/nutraceutical industries are valorised based on their content in secondary metabolites, it is imperative to understand secondary metabolite content, diversity and dynamics. Phytochemicals from some Portuguese *Juniperus* sp. have recently been described as exhibiting potential in the development of therapies for the prevention of NDDs<sup>1</sup>. Therefore, to translate this to practical application it is important to understand the seasonal dynamics of phytochemical compound production. Here, four Portuguese juniper species were evaluated and grown under the same environmental conditions to eliminate any growth condition differences. Although some differences in TPC and TFC amongst the different species were found (Table 2), some general trends were found. The highest TPC were obtained in the winter months of October to January and the lowest values in the beginning of spring (February/March

to April/May) and in July. A similar tendency was verified for TFC. In the literature, the seasonal dynamics of polyphenolic compounds have been reported. For example, in *Salix* species<sup>34</sup>, phenolic glycoside levels decreased over the growing season, with the highest contents detected during plant dormancy (winter). A similar trend was also seen for *Apocynum venetum* and *Poacynum species*<sup>35</sup>, where the influence of environment (geolocation and climate) was also shown to be significant with respect to polyphenol content.

To identify and quantify phytochemicals that may be preferentially affected due to environmental factors varying during the seasons, extracts were evaluated by LC-MS based on the same amount of phenol content. The phytochemical profile of *Juniperus* sp. gives a characteristic LC-MS profile and is relatively similar within species<sup>1</sup>. Consequently, our focus was in the relative quantification of compounds that changed significantly with respect to the different conditions. The chemical profile of junipers species only presented small differences. The compounds that were significantly altered between species (Fig. 1) were psydrin (peak 1, RT 8.22 in Fig. S1) and a procyanidin dimer (peak 7, RT 15.87 in Fig. S1). Psydrin exhibited seasonal changes in *J. navicularis*, *J. oxycedrus badia* and *J. phoenicea*. For all these species psydrin levels are higher after the phase of active growth (April). The content of the procyanidin dimer only changed in *J. phoenicea* in May, the month after active growth, when its levels were highest.

Resource allocation hypotheses such as the carbon-nutrient balance<sup>36</sup> and growth differentiation balance<sup>37</sup> have been proposed that changes in carbon source-sink relationships may be a consequence of carbon availability. That availability determines variations in the relative partitioning of carbon to growth and production of carbon based secondary metabolites. Secondary metabolite production has been found in diverse species [such as *Zingiber officinale*<sup>38</sup>, *Digitalis lanata*<sup>39</sup>, *Brassica oleracea*<sup>40</sup> and *Hypericum perforatum*<sup>41</sup>] to increase as a result of primary metabolism enhancement, for instance, through CO<sub>2</sub>

modulation. The shikimic acid pathway is able to convert simple carbohydrate precursors derived from glycolysis and the pentose phosphate pathway into aromatic amino acids<sup>38</sup>, such as phenylalanine, the precursor compound in phenylpropanoid biosynthetic pathway.

In Mediterranean conditions, temperatures and water availability in winter and summer are more extreme for plants, which may decrease photosynthetic efficiency<sup>42,43</sup>. In spring and autumn, conditions that allow a high photosynthetic yield are exploited and active growth occurs. Thus, it is reasonable to hypothesise that during periods of growth inactivity (winter and summer), available carbon may be reallocated to secondary metabolites. Under conditions favourable to growth, the opposite may occur. Our results fit this hypothesis as maximum TPC and TFC were found in winter and minimum values in spring. The production of secondary metabolites was modulated across the seasons but the main differences were in the total levels of phenolics, not in their composition. Any enrichment in a few metabolites (psydin and a procyanidin dimer) was mostly observed after the period of active growth.

#### 4.2. Influence of stress on PPs production

To assess the influence of stresses on secondary metabolite production, young plants from *J. oxycedrus badia*, *J. phoenicea* and *J. turbinata* were submitted to drought, salinity and MeJa elicitation. However, only *J. oxycedrus badia* changed its phenolic content as response to salt stress and *J. phoenicea* as consequence of MeJa elicitation. The fact that *J. oxycedrus badia* was the only species that exhibited enhanced TPC and TFC contents in response to salt stress (Fig. 2A) is likely to be associated with the adaptation of this species to mountainous environments rather than the saline coastal regions, favoured on the other species. In the literature, contradictory effects of salinity on phenolic contents can be found, depending on the plant and salinity levels applied<sup>28,44,45</sup>. Although there was a general trend of increasing TPC and TFC with salt treatment, there were also changes in the relative amounts of specific compounds (Fig. 2B and 2C) as determined by LC-MS. However, changes in the relative

amounts were only significant for epicatechin (peak 8, RT 16.74 in Fig. S1) and a procyanidin trimer (peak 9, RT 17.32 in Fig. S1). The relative contents of these two particular metabolites were decreased by salt treatment. Other phenolics showed increases in their relative amounts (without reaching significance) were quercetin rutinoside (peak 13, RT 21.86 in Fig. S1) and luteolin/isoscutellarein rhamnoside (peak 21, RT 27.88 in Fig. S1). It appears that the salt stress produced a general increase in polyphenol content *per se* and that a differential response in specific polyphenolics is minor. These alterations in metabolites were accompanied by increase in transcripts of PAL and ANR enzymes (Fig. 4A). The significant enhancement of PAL transcripts corroborates the hypothesis of a general enrichment in phenolic compounds, without specific enhancement/reduction of any particular class of compounds. Induction of PAL transcripts and activity by salt stress is well reported in the literature for other plants <sup>46,47</sup>. However, enhancements in ANR transcript levels were accompanied by decreases in epicatechin and procyanidin trimer levels. These results may suggest that post-transcriptional/translational regulation is evident, limiting the translation of those transcripts and/or the activity of those enzymes. Alternatively, reductions in epicatechin or the procyanidin dimers may have associated with enhanced levels of higher molecular weight oligomers and therefore unextractable procyanidins <sup>48</sup>. That regulation should be theme of future studies to elucidate the specific mechanisms of stress responses in junipers.

Endogenous MeJa is a signaling compound that modulates various physiological processes in plants <sup>49</sup>. Under stresses such as wounding and pathogen attack, volatile MeJa can be released into the air from the wounded plants <sup>50</sup> and can induce plants to increase secondary (defense) metabolite production and consequentially enhance their defensive systems. Therefore, exogenous MeJa has been used to usefully induce secondary metabolites such as alkaloids, terpenoids, and phenolics in some plants and plant cell cultures <sup>51,52</sup>. In the present work *J. phoenicea*

was the only species that responded to MeJa by increasing TPC and TFC levels (Fig. 3A). The TPC response showed a biphasic pattern which was reflected in the increase of the relative amounts of the procyanidin dimer (peak 7, RT 15.87 in Fig. S1) and epicatechin (peak 8, RT 16.74 in Fig. S1) (Figs. 3B and C). Conversely, the relative amount of quercetin-rhamnoside (peak 19, RT 25.43 in Fig. S1) was reduced, also in a biphasic manner (Fig. 3D). These results are corroborated by the transcriptomic analysis (Fig. 4B). MeJa induced the gene expression of PAL from 8 h to 96 h, which would drive a generalized augmentation of phenolic compounds. Induction of PAL transcripts by MeJa is well reported in literature<sup>52,53</sup>. Increases in F3H, ANS and ANR transcripts were also registered and this was reflected in the levels of some of their associated metabolic end points (procyanidin dimer, epicatechin). The increase in transcripts of this branch could lead to metabolic flux deviation to the synthesis of proanthocyanidin and epi-flavan-3-ol, thus disfavoring flavonol biosynthesis.

## 5. Conclusion

Overall, the four juniper species presented a common pattern of phytochemicals production with higher levels in periods when plants were not actively growing and lower levels in the remaining periods. Therefore, seasonal conditions modulated the total content of phenolics and flavonoids. However, qualitatively, phytochemical composition was not greatly changed with only the relative contents of psydriin and a procyanidin dimer enhanced in some months and this varied accordingly to species. Salt stress and MeJa elicitation were effective in enhancing phenolic accumulation, however different susceptibilities were exhibited by the different species. In addition to the increase of total phenolic and flavonoids content, both stresses caused alterations in the relative phytochemical composition. Procyanidins, epicatechin and quercetin derivatives were the most responsive to stresses, being altered their relative amounts in both stresses. Salt stress reduced procyanidin and

epicatechin contents in *J. oxycedrus badia* extracts and enrichment in quercetin derivatives, while the opposite effect was noted after MeJa elicitation in *J. phoenicea*. This suggests differential metabolic regulation in response to those stresses between species. The mechanisms underlying these responses are important considering juniper a viable and sustainable source of compounds/extracts for end-user industries such as pharmaceuticals/nutraceuticals.

## 6. Acknowledgements

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## 8. Supplementary material

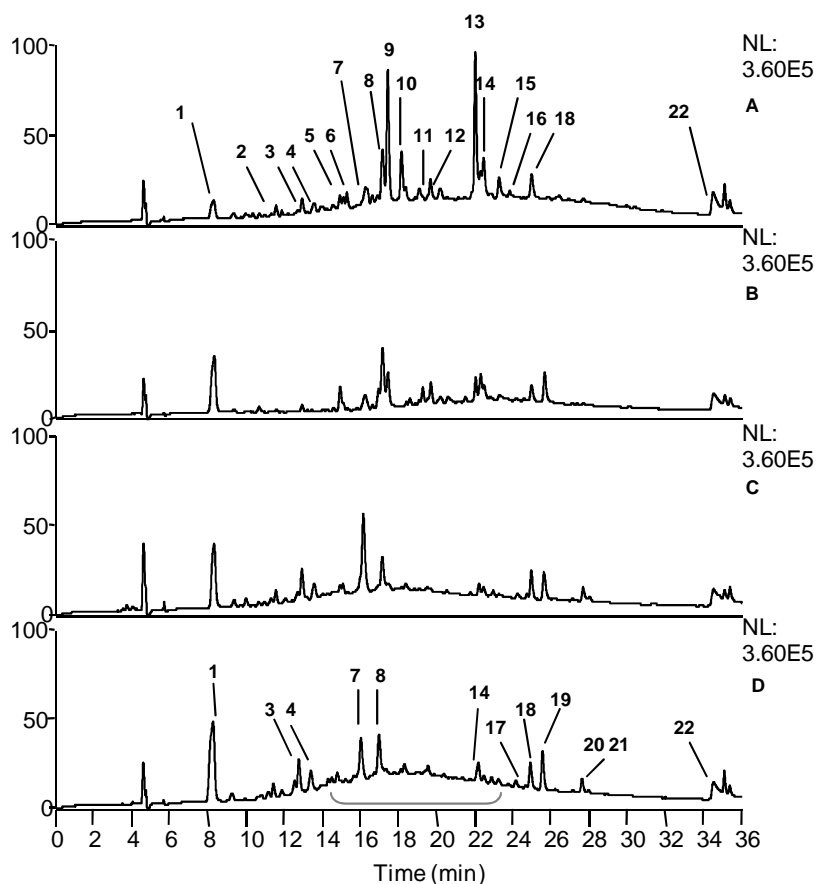


Figure S1- Representative PDA profiles for *J. navicularis* (A), *J. oxycedrus badia* (B), *J. phoenicea* (C) and *J. turbinata* (D). Peak numbers correspond to their putative identifications based on MS fragmentation, already published<sup>1</sup>. The full scale deflection for each trace is shown in the top right corner. The area denoted by the bar contain some procyanidins components that are poorly resolved across RTs 17-25 mins. Legend: 1- Psydrin; 2- Procyanidin dimer; 3- Gallocatechin; 4- Procyanidin trimer; 5- Procyanidin dimer; 6- Epigallocatechin; 7- Procyanidin trimer; 8- Epicatechin; 9- Procyanidin trimer; 10- Procyanidin trimer; 11- Myricetin hexose; 12- Myricetin rutinoside; 13- Quercetin rutinoside; 14- Myricetin pentose; 15- Proanthocyanidin derivative; 16- Unknown compound + Quercetin hexose; 17- Isoscutellarein hexoside; 18- Quercetin pentoside; 19- Quercetin rhamnoside; 20- Luteolin/isoscutellarein pentoside; 21- Luteolin/isoscutellarein rhamnoside; 22- Amentoflavone or Cupressuflavone or Hinokiflavone.

Part B:  
*Rubus* sp.





## Chapter 4 : Neuroprotective effect of blackberry (*Rubus* sp.) polyphenols is potentiated after simulated gastrointestinal digestion

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Author contribution:

LT performed the majority of the experiments presented in this chapter (except *in vitro* digestion and GSH and GSSG quantification, performed in collaboration) and wrote the manuscript cited above.

### **Abstract**

Blackberry ingestion has been demonstrated to attenuate brain degenerative processes in rodents with the benefits ascribed to the (poly)phenolic components. The aim of this work was to assess the efficacy of blackberry polyphenolics in a neurodegeneration cell model before and after simulated gastrointestinal digestion.

Digested blackberry metabolites protected neuroblastoma cells from H<sub>2</sub>O<sub>2</sub>-induced death at low, non-toxic levels that approaches physiologically-relevant serum concentrations. However, the undigested extracts were not protective even at five-fold higher concentrations. This potentiation may reflect alterations in the polyphenolic composition caused by the digestion procedure as detected by LC-MS analysis. This protection was not caused by modulation of the intracellular antioxidant capacity or through alteration of glutathione levels, although the undigested extract influenced both these parameters. This work reinforces the importance of evaluating digested metabolites in disease cell models and highlights the possible involvement of other mechanisms beyond antioxidant systems.

### **Keywords**

Blackberry; *In vitro* digestion model; Neurodegenerative diseases; Phenolic compounds.

## 1. Introduction

Epidemiological studies have shown that dietary habits can influence the incidence of AD and PD<sup>1-4</sup>. Foods and food ingredients, in particular, components chemically classified as antioxidants have been described to exert a beneficial effect in neurodegeneration<sup>5-8</sup>.

In the developed world, the population lifespan is increasing with a concomitant increased incidence of many age-related diseases such as cancer, cardiovascular and neurodegeneration<sup>9</sup>. The impact of this at the financial and social level is immense with the health care costs in 2008 for Alzheimer's disease and other forms of dementia recently estimated at €160 billion for the EU27 and €177 billion for the whole of Europe<sup>10</sup>. Clearly it is paramount that preventative, amelioration and/or, ideally, inhibition strategies are developed to retard or reverse neuronal and behavioral deficits that occur in aging<sup>9</sup>. Indeed, these foci are areas of intense research effort but the delivery of (pharma) products<sup>11,12</sup> and therapeutic strategies<sup>13</sup> have been limited. Furthermore the mechanisms involved in the behavioral deficits during aging remain to be discerned<sup>14,15</sup>. Substantial evidence supports the hypothesis that oxidative stress plays a major role in neurodegenerative disease pathogenesis<sup>16-18</sup>. Oxidative stress is generally caused by the excessive accumulation of ROS in cells and has been implicated in the development of many NDDs including PD, HD, ALS and AD<sup>16,19,20</sup>. In tissues from patients with neurodegenerative disorders, an increase in markers of ROS damage has been found<sup>5,21</sup>. In the affected regions of brain, these markers are elevated levels of lipid<sup>22</sup>, protein<sup>23,24</sup> and DNA<sup>24,25</sup> oxidation products.

Behavioral studies in rodents have revealed an attenuation of brain aging when strawberries, blueberries or blackberries are ingested<sup>26-29</sup> and the authors propose that the benefits are due to the presence of (poly)phenolic compounds. These phytochemicals, and by association the foods, are accruing a significant evidence base for beneficial effects on human health and the reduction of risk of cardiovascular disease<sup>30,31</sup>, cancer<sup>32,33</sup> and type II diabetes<sup>34</sup>. Driving these beneficial endpoints are

the numerous pathways and protein kinases that have been reported as being targets for phenolic compounds, thereby demonstrating the broad spectrum of targets and strengthen their usefulness in addressing multifactorial diseases <sup>5</sup>.

In many of the *in vitro* studies focused on PP-derived health benefits, the doses used are significantly higher than those to which humans are exposed through the diet or that could be found in the blood <sup>35</sup>. On average, and depending on the PP class, plasma bioavailabilities in healthy humans are in the range of 0.5-1.6  $\mu\text{M}$  <sup>36</sup>. Furthermore, it was concluded from an analysis of 97 bioavailability studies that total PP-derived metabolite concentration in plasma after an intake of 50 mg of aglycone equivalents ranges from 0 to 4  $\mu\text{M}$  <sup>37</sup>. However, the majority of *in vitro* studies use PP concentrations ranging from 10 to 100  $\mu\text{M}$  <sup>38</sup>: around 2-25 fold difference. Additionally, most of *in vitro* cell-based studies evaluate metabolites “as they are in food”, ignoring the chemical alterations occurring during digestion, absorption and metabolism with the consequential impact on bioavailability and bioefficacy. Moreover, many studies have evaluated the effect of single purified phenolic compounds, thereby losing possible synergic/cooperative or competitive activities between phenolic compounds <sup>38</sup>. Thus, in order to evaluate the potential role of fruit phytochemicals in human body, we should take into account the physiochemical changes occurring in the gastrointestinal tract <sup>38</sup>.

The aim of this work is to compare the neuroprotective effect of non-digested blackberry extract against digested blackberry metabolites. This work encompasses a more physiological approach which takes into account: i) the chemical changes occurring during digestion; ii) the effects of phytochemical mixtures found in authentic foods; iii) treatment of cell models with relevant *in vivo* concentrations of phytochemical metabolites.

## 2. Materials and methods

### 2.1. Plant material and extract preparation

Blackberry (*Rubus* L. subgenus *Rubus* Watson) cv. Apache was grown in Fataca experimental field (Odemira, Portugal) and berries were harvested at full ripeness. Berries were harvested (yield approx. 500 g), frozen and then freeze-dried. After, fruits were ground without seed separation in an IKA M20 mill to pass a 0.5 mm sieve and stored at -80 °C prior to extraction. Fruit extracts were prepared as previously described<sup>39</sup>. Briefly, to each 1 g of lyophilized powder, 12 mL of hydroethanolic solvent (50 % (v/v) ethanol/water) was added and the mixture was shaken for 30 min at room temperature in the dark. The mixture was then centrifuged at 12,400 *g* for 10 min at room temperature. The supernatant was filtered through paper filter and then through 0.2 µm cellulose acetate membrane filters. The resulting extracts were stored frozen at -80 °C.

### 2.2. *In vitro* digestion (IVD)

Phytochemical alterations during digestion were mimicked using the IVD model previously described by McDougall *et al.*<sup>40</sup>. Briefly, the undigested extract (final volume 20 mL) was adjusted to pH 1.7 with 5 M HCl, then pepsin (Sigma Product number P6887) was added at 315 units mL<sup>-1</sup> and incubated at 37 °C in a heated water bath for two hours with shaking at 100 rpm. Aliquots (2 mL) of the post-gastric digestion were removed and frozen. The remainder was placed in a 250 mL glass beaker and 4.5 mL of 4 mg mL<sup>-1</sup> pancreatin, 25 mg mL<sup>-1</sup> bile salts mixture added. A segment of cellulose dialysis tubing (molecular mass cut-off 12 kDa) containing sufficient 0.1 M NaHCO<sub>3</sub> to neutralise the sample's titratable acidity was added and the beaker sealed with parafilm. The NaHCO<sub>3</sub> diffused out of the dialysis tubing and the pH reached neutrality within 45 min. After two hours incubation at 37 °C, the solution inside the dialysis tubing (fraction IN) and the solution outside the dialysis tubing (fraction OUT) were taken. Small samples (2 mL) were immediately frozen. The digested fractions IN and OUT were acidified until 0.5 % (v/v) by slow addition of 10 % formic acid.

After centrifugation (2,500 *g*, 10 min, 5 °C), the soluble material from fractions IN and OUT were applied to C18 solid phase extraction columns (GIGA tubes, 1000 mg capacity, Phenomenex Ltd) which had been pre-equilibrated in ultra pure water (UPW) containing 0.25 % (v/v) formic acid (FA). After a wash with 2 volumes of FA/ultra pure water, the bound material was eluted by the addition of 0.25 % (v/v) FA in 25 % (v/v) acetonitrile. This afforded complete separation of total phenolics from the bile salts present in samples <sup>41</sup>. The fractions were then concentrated in a Speed-Vac to suitable phenol concentrations.

### 2.3. Chemical characterization

#### 2.3.1. Total phenolic quantification

Determination of total phenolic compounds was performed by the Folin-Ciocalteu method adapted to microplate reader <sup>42</sup>. Gallic acid was used as the standard and the results were expressed as mg GAE.

#### 2.3.2. Peroxyl radical scavenging capacity determination

Peroxyl radical scavenging capacity was determined by the ORAC (Oxygen Radical Absorbance Capacity) method as described by Tavares *et al.* <sup>39</sup>. The final results were calculated using the differences in area under the fluorescence decay curves between the blank and the sample, and were expressed as  $\mu\text{M}$  Trolox equivalents ( $\mu\text{M}$  TE).

#### 2.3.3. Phenolic Profile Determination by LC-MS

Extracts and digested fractions were applied to a C-18 column (Synergi Hydro C18 column with polar end capping, 4.6 mm x 150 mm, Phenomenex Ltd.) and analysed on a LCQ-DECA system controlled by the XCALIBUR software (2.0, ThermoFinnigan), as reported by Tavares *et al.* <sup>39</sup>. The LCQ-Deca system comprised a Surveyor autosampler, pump and PDA detector and a Thermo Finnigan mass spectrometer iontrap.

### 2.4. Cell culture

Human neuroblastoma SK-N-MC cells were obtained from the ECACC and cultured in EMEM (Sigma) supplemented with 2 mM L-glutamine (Sigma), 10 % (v/v) heat inactivated FBS (Gibco), 1 % (v/v) non-essential amino acids (Sigma), 1 mM sodium pyruvate (Sigma) and containing 50 U/mL penicillin

and 50 µg/mL streptomycin. The cells were maintained at 37 °C in 5 % CO<sub>2</sub> and split at sub-confluence of 70-80 % using 0.05 % trypsin/EDTA (Gibco).

### 2.5. Cytotoxicity profile

The undigested blackberry extracts and fraction IN were concentrated under vacuum and dissolved in cell medium for the cytotoxicity tests<sup>43</sup>. The cell viability assay was performed in a 96-well plate cell and employed the neuroblastoma human cell line SK-N-MC to identify the non-toxic range of extract concentrations. Cells were seeded at 1.25 x 10<sup>5</sup> cells mL<sup>-1</sup> and grown for 48 h prior to incubation with extracts. Toxicity tests involved 24 h incubation in the range of 0 to 500 µg GAE mL<sup>-1</sup> medium. Cell viability was assessed using the CellTiter-Blue® Cell Viability Assay (Promega), according to the manufacturer's instructions. Non-viable cells rapidly lose their metabolic capacity and thus do not generate the fluorescent signal.

### 2.6. Neuroprotective effect against oxidative stress

To evaluate the neuroprotective effect of extracts, SK-N-MC neuroblastoma cells were incubated in the presence of H<sub>2</sub>O<sub>2</sub>. Cells were seeded at 7.4 x 10<sup>4</sup> cells mL<sup>-1</sup> and 24 h after seeding, growth medium was removed and wells washed with PBS. Cells were pre-incubated with medium containing 0.5 % (v/v) FBS supplemented with non-toxic concentrations of blackberry extracts. After 24 h pre-incubation, cells were washed again with PBS and medium replaced by medium containing 0.5 % (v/v) FBS and H<sub>2</sub>O<sub>2</sub> at a final concentration of 300 µM. After 24 h, medium was removed, cells were washed with PBS, collected by trypsinisation and incubated with two fluorescent probes for 30 min at 37 °C. DiOC<sub>6</sub>(3) (20 nM, Invitrogen) was used to evaluate the ΔΨ<sub>m</sub> and PI (1 µg mL<sup>-1</sup>, Invitrogen) was used to determine cell viability, based on plasma membrane integrity<sup>44</sup>. Cells were then analyzed by flow cytometry. A flow cytometer (Partec) containing a blue solid state laser (488 nm) with FL1 green fluorescence channel for DiOC<sub>6</sub>(3) at 530 nm and a FL3 red fluorescence channel for PI detection at 650 nm was used. The acquisition

and analysis of the results were performed with FlowMax® (Partec) software.

### 2.7. Intracellular ROS production

To evaluate the ability of extracts to reduce ROS levels produced by cells, the conversion of H<sub>2</sub>DCFDA (Invitrogen) to fluorescent DCF was monitored<sup>45,46</sup>. SK-N-MC neuroblastoma cells were seeded in a 96-well plate at 1.25 x 10<sup>5</sup> cells mL<sup>-1</sup>. Cells were grown for 24 h and then they were washed with PBS and then pre-incubated with extracts prepared in medium (0.5 % (v/v) FBS) for 2 h or 24 h. After pre-incubation, cells were washed with PBS and incubated with 25 μM H<sub>2</sub>DCFDA in PBS for 30 min at 37 °C. Cells were washed and H<sub>2</sub>O<sub>2</sub> (200 μM) in PBS, added. Fluorescence was measured (λ<sub>ex</sub>: 485 nm, λ<sub>em</sub>: 530 nm) using a FLx800 Fluorescence Microplate Reader (Biotek) during 1 h at 37 °C. ROS generation was calculated as an increase in fluorescent signal between control and H<sub>2</sub>O<sub>2</sub>-treated cells.

### 2.8. Glutathione and glutathione disulphide (GSSG) quantification

To quantify GSH and GSSG, cold 10 % (v/v) metaphosphoric acid was carefully added to samples or standards. After incubation (4 °C, 10 min) and centrifugation (16,000 *g*, 20 min, 4 °C) supernatants were transferred into 1.5 mL propylene tubes (50 μL for determination of GSH and 200 μL for determination of GSSG).

Derivatization was performed accordingly to Kand'ar *et al.*<sup>47</sup>, adapted from Hissin and Hilf<sup>48</sup>. Briefly, for GSH analysis 1 mL of 0.1 % (w/v) EDTA in 0.1 M sodium hydrogen phosphate, pH 8.0, was added to 50 μL of supernatant. To 20 μL portion of this mixture, 300 μL of 0.1 % (w/v) EDTA in 0.1 M sodium hydrogen phosphate, and 20 μL of 0.1 % orthophthalaldehyde (OPA) (w/v) in methanol, was added. Tubes were incubated at 25 °C for 15 min in the dark. The reaction mixture was then stored at 4 °C until analysis. For GSSG analysis, a 200 μL of supernatant was incubated at 25 °C with 200 μL of 40 mM N-ethylmaleimide for 25 min in dark. To this mixture, 750 μL of 0.1 M NaOH was added. A 20 μL portion was taken and mixed with 300 μL of 0.1 M NaOH and 20 μL of

0.1 % OPA. Tubes were incubated at 25 °C for 15 min in dark and stored at 4 °C until analysis.

Chromatographic analysis was accomplished using isocratic elution on C18 analytical column (Supelcosil™ ABZ+Plus HPLC Column 15 cm x 4.6 mm, 3 µm (Supelco)) at 40 °C on an Acquity™ Ultra Performance LC system (Waters). The mobile phase consisted of 15 % methanol in 25 mM sodium hydrogen phosphate (v/v), pH 6.0. The flow rate was kept constant at 0.7 mL min<sup>-1</sup>. The excitation and emission wavelengths were set at 350 and 420 nm, respectively. The amount of GSH and GSSG was quantified from the corresponding peak area using Empower® Pro 2.0 software. The concentration of GSH and GSSG in the samples was determined from standard curves with ranges 0-100 µM for GSH and 0-5 µM for GSSG. Values were normalized for total protein content, determined by Lowry method <sup>49</sup>.

### 2.9. Statistical analysis

The results reported in this work are the averages of at least three independent experiments and are represented as the mean ± SD. Differences among treatments were detected by analysis of variance with Tukey HSD multiple comparison test ( $\alpha=0.05$ ) using SigmaStat 3.10 (Systat).

## 3. Results

### 3.1. Chemical characterization

Blackberry fruits are well known to be a rich source of PPs and to exhibit high antioxidant capacity. However, after ingestion blackberry phytochemicals undergo many modifications or even degradation by the processes of gastrointestinal (GI) digestion. In the present work, an IVD model was used to mimic the effect of GI digestion on blackberry phenolic extract. IVD produces different digested fractions: post gastric digest (PG) and two pancreatic digested fractions (IN and OUT). There was a large

reduction in both TPC and antioxidant capacity (AC) following digestion, in particular for the pancreatic digest fractions IN and OUT (Table 1).

Table 1- Total phenolic content (TPC) antioxidant capacity (AC) and ratio antioxidant capacity per total phenolic content (AC/TPC) of undigested extract of blackberry and IVD fractions. Values are reported as means  $\pm$  SD (n=3) and percentage in comparison to the undigested extract. Statistical differences compared to the undigested extract are denoted as \* p<0.05 \*\* p<0.01 \*\*\* p<0.001. PG - Post gastric; IN - post pancreatic; OUT - post pancreatic.

		Undigested extract	PG	IN	OUT
Total phenolic content (TPC)	mg GAE	16.7 $\pm$ 0.2	15.5 $\pm$ 0.4	0.9 $\pm$ 0.2	4.6 $\pm$ 0.2
	%	100.0	93.0	5.5	27.5
	significance		*	***	***
Antioxidant capacity (AC)	$\mu$ mol TE	544.2 $\pm$ 26.9	268.6 $\pm$ 71.1	91.7 $\pm$ 6.2	294.3 $\pm$ 23.5
	%	100.0	49.4	16.9	54.1
	significance		**	***	**
AC/TPC		32.6	17.3	101.9	64.0

Interestingly, IVD increased the relative antioxidant capacity (expressed as a ratio of total phenolic content) of fraction IN by three-fold and by two-fold in fraction OUT (AC/TPC in Table 1). There were substantial alterations in phytochemical composition caused by IVD as assessed by LC-MS (Fig. 1; Table 2). The major anthocyanins, cyanidin-3-O-glucoside (CyGlc), cyanidin-3-O-xyloside (CyXyl) and cyanidin-3-O-dioxyl-glucoside (CyDAGlc) were greatly reduced by pancreatic digestion with recoveries in fractions IN and OUT at around 10 % of the undigested extract (Fig. 2). Quercetin derivatives were more highly recovered after digestion with recoveries ranging from 40 to >80 %. The recovery of the major ellagitannin components was substantial in the PG fraction but much lower in the pancreatic digests, around 30-40 % for fraction OUT and essentially zero for IN. It was also notable that new peaks were identified after IVD. For example, after gastric digestion, a major new peak was present (G1, Fig. 1B) but the MS properties did not permit identification. After pancreatic digestion, a number of new peaks appeared in fractions IN and OUT (Fig. 1 C and D, peaks P1-P3). Peak P3 gave PDA and MS

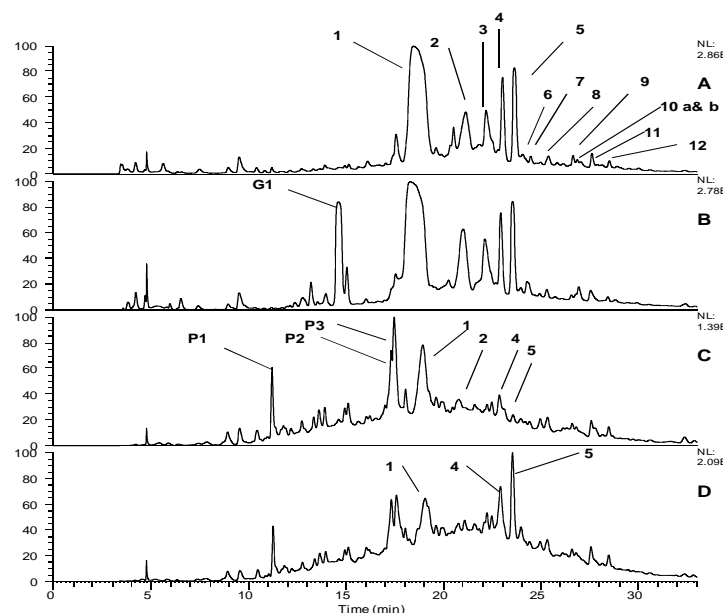


Fig. 1- Analysis of blackberry samples by liquid chromatography-mass spectrometry (LC-MS). A- Undigested extract; B- Post gastric; C- IN post pancreatic; D- OUT post pancreatic. All figures are representative traces recorded at 280 nm. The full scan deflection is shown in the upper right corner of each panel. Peaks are labelled as described in Table 2 with components appearing in the PG or the pancreatic digests, IN and OUT, labelled, *e.g.* G1 and P1, respectively.

Table 2- Identification of indicated phenolic components from blackberries.

Peak No.	RT	PDA	<i>m/z</i>	MS <sup>2</sup>	Putative ID
1	18.46	515, 280	<b>+449</b> , 287	287	Cyanidin-3- <i>O</i> -glucoside <sup>50,51</sup>
2	21.14	515, 208	<b>+419</b> , 287	287	Cyanidin-3- <i>O</i> -xyloside <sup>50,51</sup>
3	22.18	520, 280	<b>+593</b> , 287	287	Cyanidin-3- <i>O</i> -dioxayl-glucoside <sup>50</sup>
4	23.03	240-300	<b>1401</b> , 1250, 934, 301	<b>1869</b> , 1567, <b>1250</b> , 1235, 934, 633	Lambertianin C <sup>51,53</sup>
5	23.63	240-300	<b>1869</b> , 935, 301	<b>1567</b> , 1265, 1235, 1103, 933, etc.	Sanguin H6 <sup>51-54</sup>
6	24.06	240-300	<b>1103</b>	multiple	Unidentified ellagitannin
7	24.47	355	<b>433</b> , 301	301	Quercetin-xyloside <sup>55</sup>
8	25.39	355	<b>433</b> , 301	301	Quercetin-xyloside <sup>55</sup>
9	25.83	355	<b>609</b> , 301	301	Quercetin-rutinoside <sup>50,53,56</sup>
10a	26.64	355	<b>463</b> , 301	301	Quercetin-glucoside <sup>50,51,53,56</sup>
10b	26.86	355	<b>477</b> , 301	301	Quercetin-glucuronide <sup>51,56</sup>
11	27.61	355	<b>607</b> , 505, 463, 301	545, 505, <b>463</b> , 301	Quercetin-HMG-glucoside <sup>56</sup>
12	28.50	365	447	315	Methyl ellagic acid derivative <sup>51,53</sup>
G1	14.61	250-300, 270 max	none	-	Unknown
P1	11.21	220-300, 260 max	282, 150	150, 133	Unknown
P2	17.32	290	none	-	Unknown
P3	17.47	265	153	109	Dihydroxy benzoic acid

There was also evidence for cyanidin-3-*O*-rutinoside which co-eluted with the main anthocyanin peak. Assignments are supported by previous work. Peak numbers refer to Fig. 1. Compounds 1-12 were identified in the undigested extract; compound G1 was more apparent in PG and compounds P1-P3 were more apparent in the IN and OUT. + denote detection in the positive mode MS only. In bold are denote the most abundant ions.

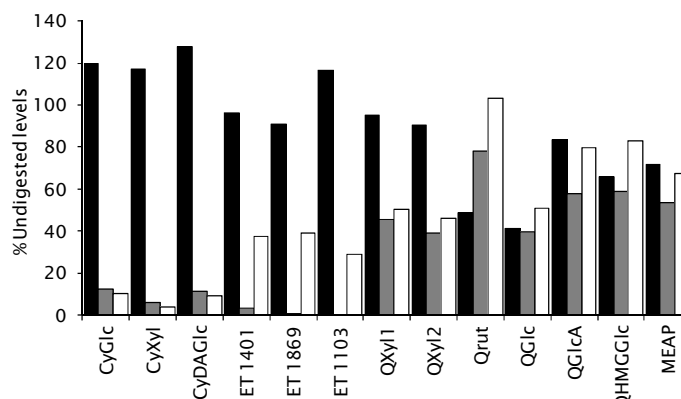


Fig. 2- Recovery of selected blackberry phenolics after in vitro digestion. The main metabolites identified in undigested blackberry extract were relatively quantified and values reported as percentage of recovery based on the peak area of the mass spectrometer response for each m/z. ■- PG (Post gastric); ■- IN post pancreatic; □- OUT post pancreatic. Metabolites analyzed: CyGlc- Cyanidin-3-O-glucoside; CyXyl- Cyanidin-3-O-xyloside; CyDAGlc- Cyanidin-3-O-dioxyl-glucoside; ET 1401- Lambertianin C; ET 1869- Sanguin H6; ET 1103- Unidentified ellagitannin; QXyl1- Quercetin-xyloside 1; QXyl2- Quercetin-xyloside 2; Qrut- Quercetin-rutinoside; QGlc- Quercetin-glucoside; QGlcA- Quercetin-glucuronide; QHMGGlc- Quercetin-HMG-glucoside; MEAD- Methyl ellagic acid derivative.

properties consistent with assignment as dihydroxybenzoic acid, perhaps derived from the breakdown of cyanidin anthocyanins at pH > 7<sup>40,50</sup>.

### 3.2. Cytotoxicity profile determination

Prior to the assessment of the neuroprotective potential, cytotoxicity assays were performed using the assessment of cell metabolism of SK-N-MC neuroblastoma cells. Fraction IN which presented a higher relative antioxidant capacity, was more toxic than the undigested extract (Fig. 3). Non-toxic concentrations of the

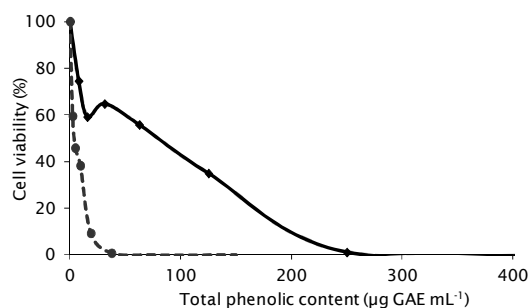


Fig. 3- Cytotoxicity profile obtained for undigested extract (solid line) and IN (dashed line). Cell viability was determined by CellTiter-Blue® Cell Viability Assay (Promega) after cells being incubated with phenolic compounds for 24 h.

undigested extract and fraction IN were selected for further assays.

To evaluate if the effect of the PPs was due to interactions with extracellular receptors or to uptake, we took advantage of the intrinsic autofluorescence properties of polyphenolic compounds<sup>51</sup>.

Cells incubated for 1 h with increasing concentrations of blackberry extract showed a concentration-dependent autofluorescent signal within the cytoplasm (see column A in Fig. S1 in Supplementary material). This suggests cellular uptake of, at least, part of the PPs.

### 3.3. Evaluation of neuroprotective effect

Concentrations of blackberry extract and IN fraction, which were non-toxic as assessed by cellular metabolism, were tested in an H<sub>2</sub>O<sub>2</sub>-stress neurodegeneration cell model. Neuroblastoma cells were treated with 300 µM H<sub>2</sub>O<sub>2</sub> for 24 h, which reduced cell viability to around 50 % (based on cells with an intact cell membrane, negative for PI in flow cytometry assay; Fig. 4A). Only fraction IN was able to significantly protect neuroblastoma cells from H<sub>2</sub>O<sub>2</sub> injury (Fig. 4A). The greatest protection was achieved when higher metabolite concentrations were used (0.5 and 1 µg GAE mL<sup>-1</sup>) and pre-incubation with IN fraction (0.5 µg GAE mL<sup>-1</sup>) resulted in an increase in viability to 78 % (Fig. 4A). However, in this neurodegeneration cell model, neither the undigested extract nor fraction IN could prevent the dissipation of the  $\Delta\Psi_m$  (Fig. 4B).

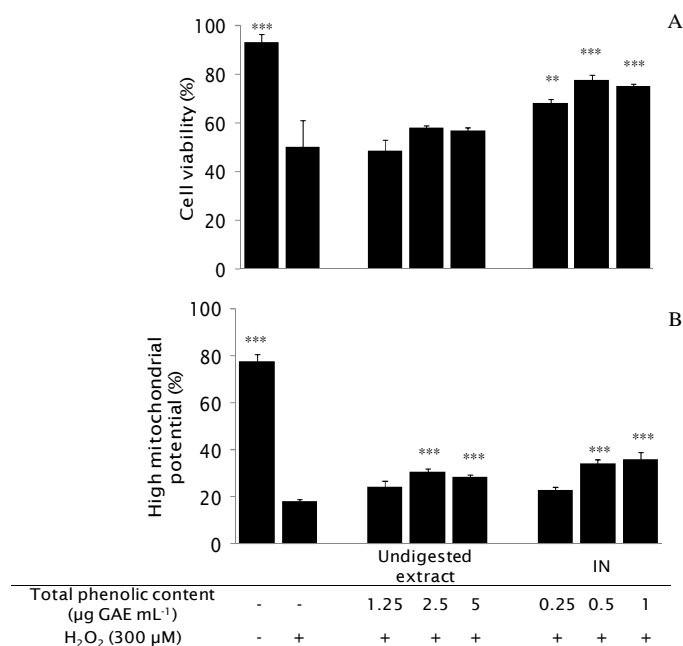


Fig. 4- Cell viability and mitochondrial transmembrane potential ( $\Delta\Psi_m$ ) assessed by flow cytometry. A) Cell viability, expressed as percentage of cells containing intact membrane, using PI as fluorochrome. B) Percentage of cells presenting high mitochondrial transmembrane potential, using DiOC<sub>6</sub>(3) as probe. Neuroblastoma cells were pre-incubated with blackberry undigested extract or IN fraction for 24 h and then injured by 300  $\mu$ M H<sub>2</sub>O<sub>2</sub> for 24 h. Statistical differences between treatments are denoted as \*  $p < 0.05$  \*\*  $p < 0.01$  \*\*\*  $p < 0.001$ . All values are mean  $\pm$  SD, n = 3.

### 3.4. Intracellular ROS production determination

The same concentrations were also tested for intracellular antioxidant capacity (Fig. 5). Two different pre-incubation times were tested, at 2 h and 24 h (Fig. 5A and 5B, respectively).

At both time points and in the absence of an imposed oxidative stress, both undigested extract and IN fraction significantly reduced the basal ROS production. H<sub>2</sub>O<sub>2</sub> was used to promote an oxidative stress in cells and doubled ROS production compared with the control (Fig. 6) without promoting cell death. With a 2 h pre-incubation, neither the undigested extract nor IN fraction reduced intracellular ROS caused by H<sub>2</sub>O<sub>2</sub> stress. With a 24 h pre-incubation followed by H<sub>2</sub>O<sub>2</sub> stress, the undigested extract but not IN fraction reduced intracellular ROS production. Conversely, the

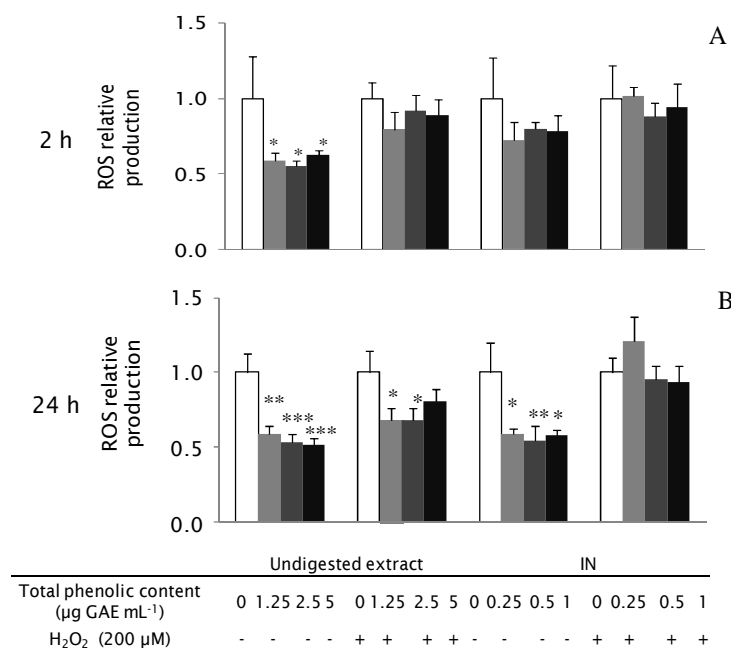


Fig. 5- Relative intracellular ROS production by SK-N-MC neuroblastoma cells in presence/absence of oxidative stress. Oxidative stress (200 µM H<sub>2</sub>O<sub>2</sub>) was applied for 1 h. Cells were pre-incubated with undigested blackberry extract or fraction IN for A) 2 h; B) 24h. ROS were detected by fluorimetry using DCF as probe. Statistical differences between treatments are denoted as \* p<0.05 \*\* p<0.01 \*\*\* p<0.001. All values are mean ± SD, n ≥ 3.

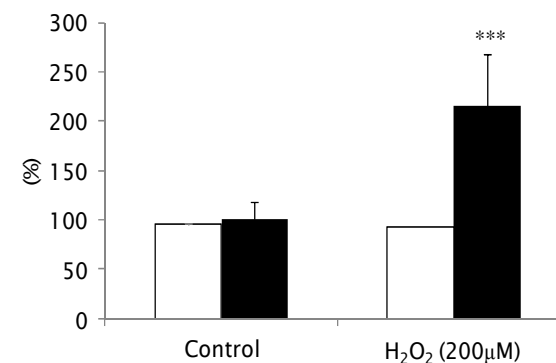


Fig. 6- Characterization of stress conditions used to determine intracellular ROS production. □- Cell viability; ■-ROS production. Values are reported as percentage relative to the control condition. Cells were incubated in presence/absence of an oxidative stress (200 µM H<sub>2</sub>O<sub>2</sub> for 1 h), cell viability determined by FACS with PI as probe and ROS production determined by fluorimetry with DCF as probe. Statistical differences between treatments are denoted as \* p<0.05 \*\* p<0.01 \*\*\* p<0.001. All values are mean ± SD, n = 3.

undigested extract was able to promote ROS scavenging, although not enough to protect cells from death.

### 3.5. GSH and GSSG quantification

To verify the alterations induced by metabolites pre-treatment and post application of H<sub>2</sub>O<sub>2</sub> stress, the redox pair GSH/GSSG was quantified. GSH and total thiols (GSH+2xGSSG) are presented in Fig. 7. Cells subjected to H<sub>2</sub>O<sub>2</sub> (300 μM, 24 h) exhibited a reduction in the level of total thiols as a result of GSH depletion. Conversely, cells subjected to a 24 h pre-treatment with blackberry extract at 1.25 and 2.5 μg GAE mL<sup>-1</sup> showed an increase in GSH and total thiols. Pre-treatment with IN fraction did not prevent the H<sub>2</sub>O<sub>2</sub>-dependent depletion of GSH levels.

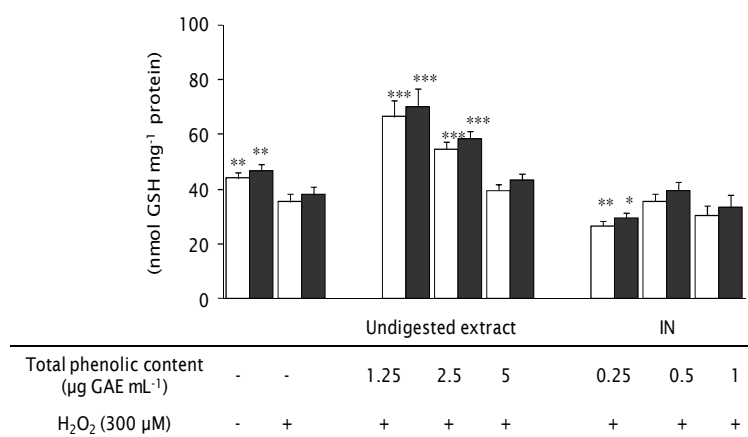


Fig. 7- Quantification of GSH and total thiols. □- GSH and ■- total thiols levels in SK-N-MC neuroblastoma cells pre-treated with undigested blackberry extract and fraction IN. After pre-treatment for 24 h, cells were subjected to 300 μM H<sub>2</sub>O<sub>2</sub> for 24h. Cells were harvested and analyzed for their content in GSH and GSSG by UPLC. Total thiol content was calculated as GSH+2xGSSG and values are expressed in nmol GSH mg<sup>-1</sup> protein. Statistical differences between treatments are denoted as \* p<0.05 \*\* p<0.01 \*\*\* p<0.001. All values are mean + SD. n > 3.

## 4. Discussion

Blackberry fruits are well known to be a rich source of PPs and to exhibit high antioxidant capacity<sup>52,53</sup>. Besides that, like many fruits of the *Rubus* genus they are increasingly being attributed with a significant potential for

human health benefits<sup>54-56</sup> and, in particular, blackberries have been reported to enhance short-term memory performance in animal models<sup>27</sup>. After ingestion, blackberry phytochemicals undergo many modifications or even degradation by the processes of GI digestion. Although transport and metabolic mechanisms cannot be effectively reproduced, *in vitro* studies can provide a simple predictive instrument to investigate the potential bioavailability under conditions mimicking the GI tract. In the present work, an IVD model was used to mimic the effect of GI digestion on blackberry phenolic extract. This model was first described by Miller *et al.*<sup>57</sup> and then adopted to evaluate secondary metabolites<sup>50,58</sup>. In the present study, following digestion, blackberries presented a large reduction in both TPC and AC, in particular for IN and OUT (Table 1). Gastric digestion reduced the antioxidant capacity by 50.6 %, although the total phenolic content was reduced by no more than 7 %. The results for total phenolic content of PG digest are in accordance with other studies performed with pomegranate, red cabbage and chokeberry<sup>50,59,60</sup>. The TPC of IN fraction was reduced to 5.5 % and the AC to 16.9 % compared to the undigested blackberry extract. In OUT, these values were 27.5 % and 54.1 % respectively. Both TPC and AC values are in the same range as those obtained for grapes by Tagliazucchi *et al.*<sup>61</sup> (55.5 % for TPC and 62.4 % for AC).

There were substantial alterations in phytochemical composition caused by IVD as assessed by LC-MS (Fig. 1; Table 2).

The major anthocyanins were greatly reduced by pancreatic digestion (recoveries at around 10 % of the undigested extract; Fig. 2). These low recoveries are concordant with the values reported after IVD of pomegranate juice<sup>59</sup> and raspberry<sup>58</sup>. Anthocyanins are generally stable in the acidic conditions of the stomach, but less stable at the higher pH of the small intestine<sup>40,59,62</sup>. Quercetin derivatives were more highly recovered after digestion (40 to >80 %). In some cases, the total recovery in fractions IN plus OUT was greater than 100 %, which may be due to interconversion of quercetin components during digestion or enhanced detection of

quercetin derivatives due to reductions in other components. Increased relative recovery of flavonols was also noted after IVD of green tea <sup>63</sup>. The recovery of the major ellagitannin components was around 30-40 % for fraction OUT and essentially zero for IN. Ellagitannins are more stable to pH changes than anthocyanins but degrade to smaller components <sup>64</sup>. Additionally, after IVD some new peaks were identified (Fig. 1). As mentioned in section 3.1, fraction IN had approximately three-fold higher antioxidant capacity than the undigested extract when expressed as a ratio of phenolic content. This increased antioxidant capacity may result from its greatly altered phenolic composition (lower levels of anthocyanins and ellagitannins but relatively enhanced levels of quercetin derivatives and evidence of accumulation of breakdown products) (Fig. 2) and made it the obvious choice for further studies on neuroprotective effects.

Fraction IN also revealed more toxic than the undigested extract (Fig. 3), presumably as a result of digestion-induced chemical modifications. Interestingly, the non-toxic range of fraction IN (maximum 1  $\mu\text{g GAE mL}^{-1}$  corresponding to 6  $\mu\text{M}$ ) was similar to that reported for dietary polyphenolic-derived metabolites in plasma at 0 to 4  $\mu\text{M}$  <sup>37</sup>, which is physiologically relevant with respect to polyphenolic-load. The maximum non-toxic concentration for the undigested extract was higher, 5  $\mu\text{g GAE mL}^{-1}$  (equivalent to 30  $\mu\text{M}$ ). It should be noted that these concentrations of total PPs do not refer to specific chemical moieties, rather it is a value for the total mix of PPs obtained from fruits or their simulated digestion. Non-toxic concentrations of blackberry extract and IN fraction were then tested in an  $\text{H}_2\text{O}_2$ -induced neurodegeneration cell model. Production of  $\text{H}_2\text{O}_2$  is related with age-related diseases and more particularly with neurodegeneration <sup>65,66</sup>. Only fraction IN was able to significantly protect neuroblastoma cells from  $\text{H}_2\text{O}_2$  injury (Fig. 4A) even though the undigested extract was applied at five-fold higher concentration. The lowest concentration of IN fraction (0.25  $\mu\text{g GAE mL}^{-1}$  equivalent to 1.5  $\mu\text{M}$ ) was able to exert a protective effect and this concentration is in the same range as the serum bioavailability reported

for individual PPs (maximum 4  $\mu\text{M}$ <sup>37</sup>) which reinforces the biological significance of this neuronal protection. Furthermore, higher protection was achieved for pre-incubation with higher metabolite concentrations of IN fraction (0.5  $\mu\text{g GAE mL}^{-1}$ ) resulting in an increase in viability to 78 % (Fig. 4A). However, in this neurodegeneration cell model, blackberry metabolites before and after digestion were not able to prevent the dissipation of  $\Delta\Psi\text{m}$  (Fig. 4B). This dissipation reflects the earlier stage  $\text{H}_2\text{O}_2$ -induced cytotoxicity associated with its diffusion into the mitochondrial matrix and with subsequent loss of integrity, ability to generate ATP and finally cell death<sup>67,68</sup>. Therefore, although not able to completely protect neuroblastoma cells, digested metabolites in fraction IN were able to modulate molecular mechanisms of survivability in response to the imposed oxidative stress. The same concentrations were tested for intracellular antioxidant capacity (Fig. 5), using two different pre-incubation times (2 h and 24 h; Fig. 5A and 5B, respectively), to cover different timescale events. We presumed that the 2 h pre-incubation would evaluate the direct scavenging events caused by the phenolic compounds. As it turned out, as mentioned above (section 3.2), we verified that 1 h should be enough for compounds presented in blackberry extract to be taken up by cells (column A in Fig. S1 in Supplementary material). We presumed that 24 h pre-incubation with PP-derived metabolites could influence ROS levels via indirect effects on endogenous antioxidant systems. At both time points and in the absence of an imposed oxidative stress, both undigested extract and IN fraction significantly reduced the basal ROS production. This suggests that the undigested extract and IN fraction could alter the oxidative environment of cells. Contrary happened with a 2 h pre-incubation, neither the undigested extract nor IN extract reduced intracellular ROS caused by  $\text{H}_2\text{O}_2$  stress. With a 24 h pre-incubation followed by  $\text{H}_2\text{O}_2$  stress, the undigested extract but not IN fraction reduced intracellular ROS production. Comparison of this data with that derived from the neuroprotection assay suggests that the neuroprotection exhibited by

fraction IN was not mediated by ROS scavenging. Indeed, similar results were obtained by Cilla *et al.*<sup>69</sup> who reported that intracellular ROS production was not diminished by pre-incubating cells with an *in vitro* digested fruit beverage. Conversely, the undigested extract was able to promote ROS scavenging, although not enough to protect cells from death. Protection from ROS over a 24 h time scale may be indicative of priming of the endogenous antioxidant systems via events like nuclear receptor modulation, gene expression and enzyme activity regulation, subcellular signaling pathways modulation and involvement in mechanisms of DNA protection/repair among others<sup>70-72</sup>. Phenolic compounds present in the undigested blackberry extract could be modulating some endogenous antioxidant defenses and consequently reducing the intracellular ROS, as suggested for grape derived PPs<sup>73,74</sup>. Indeed, recent work by Xiao<sup>75</sup> showed that fruit PPs stimulated the expression and production of mammalian detoxification/antioxidant enzymes via Nrf2 transcription factor. Compounds responsible for decreasing ROS levels may have been removed during digestion, since the same response was not observed with IN. These results suggest that neuronal protection caused by IN fraction is produced by other mechanisms than modulating ROS levels. GSH is the major antioxidant within cells and is involved in maintaining a tight control of redox status<sup>76</sup>. To verify the alterations in GSH/GSSG homeostasis induced by metabolites pre-treatment and post application of H<sub>2</sub>O<sub>2</sub> stress, the redox pair GSH/GSSG was quantified. Contrarily to cells subjected pre-treated with blackberry extract that exhibited an increase in GSH and total thiols, pre-treatment with IN fraction did not prevent the H<sub>2</sub>O<sub>2</sub>-dependent depletion of GSH levels. These changes in GSH levels are in accordance with ROS levels observed after 24 h pre-treatment followed by oxidative stress (Fig. 6). The augmentation in GSH following pre-treatment with blackberry extract and induction of oxidative stress could contribute to the reduction in ROS levels detected. Although IN fraction did not prevent GSH depletion, it promoted cell protection, unlike the undigested blackberry extract. Again, these results reinforce the

differences noted between cell model studies performed with metabolites obtained from food digestion rather than the direct food components.

## 5. Conclusion

The potential of blackberry to contribute to dietary strategies to prevent or retard neurodegeneration was evaluated. The present work clearly compares and distinguishes the neuroprotective effect of non-digested blackberry extract (undigested extract) and digested blackberry metabolites (IN fraction) at concentrations approaching physiological levels.

The undigested blackberry extract, although exhibiting a significant antioxidant capacity *in vitro*, was not able to protect neurons in a neurodegeneration cell model, but enhanced GSH levels and reduced ROS production. However, enhancing intracellular antioxidant capacity *per se* was not enough to effectively protect neurons.

Conversely, digested PP metabolites were able to maintain cell membrane integrity, protecting neurons from death. Interestingly, this protection was not related to enhanced intracellular antioxidant capacity, since the IN fraction did not directly/indirectly reduce ROS levels. In addition, there were no alterations in GSH redox status after IN pre-incubation. This highlights the involvement of other mechanisms beyond antioxidant systems and the complexity of how, what may be thought of as a simple food, fruit, can interact at the fundamental level with our cells. Overall, this work illustrates the importance of evaluating the effect of digested metabolites in disease cell models.

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## 8. Supplementary material

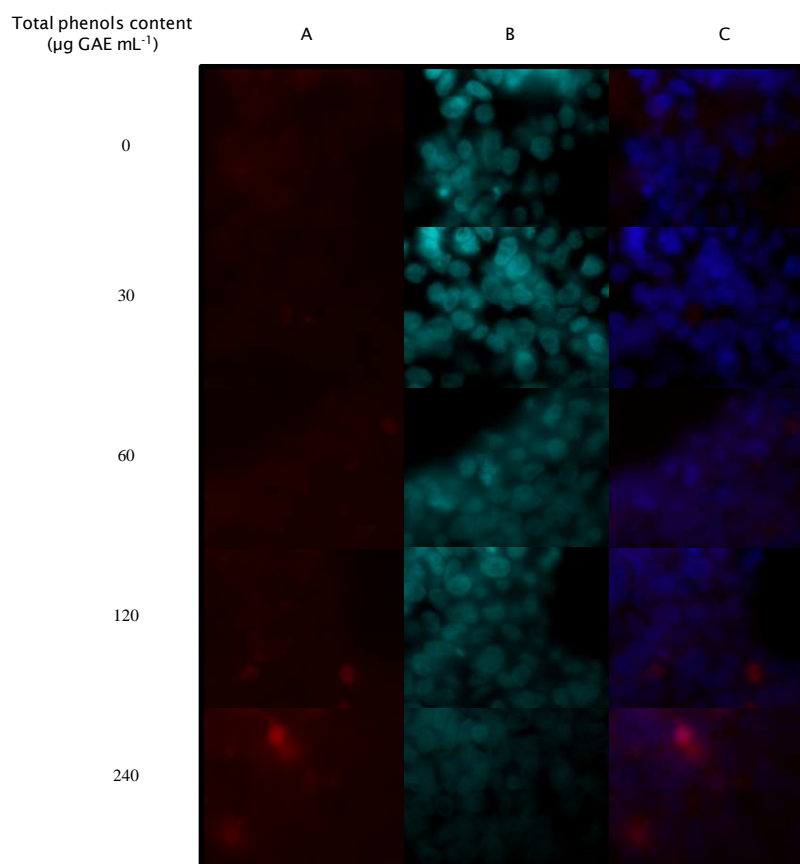


Fig. S1- Polyphenol uptake by SK-N-MC neuroblastoma cell line.

Cells were treated with increasing concentrations of blackberry undigested extract for 1 h. Slides were prepared and nuclei were stained with DAPI. Cells were visualized using a fluorescence microscope (Leica DM RB) with two different filters. Column A: Images were obtained using a filter with excitation range on UV  $\lambda_{\text{ex}}$  515-560 nm, LP 590 nm to detect the intrinsic autofluorescence of the PPs. Column B: Images were obtained using a filter with excitation range on green  $\lambda_{\text{ex}}$  340-380 nm, LP 425 nm to detect DAPI-stained nuclei. Column C: Merged images of A and B columns using ImageJ software.

## Chapter 5: Neuroprotective effects of digested polyphenols from wild blackberry species

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Author contribution:

LT performed the majority of the experiments presented in this chapter (except *in vitro* digestion and GSH and GSSG quantification, performed in collaboration) and wrote the manuscript cited above.

### **Abstract**

Blackberry ingestion has been demonstrated to attenuate brain degenerative processes with the benefits ascribed to the (poly)phenolic components. The aim of this work was to evaluate the neuroprotective potential of two wild blackberry species in a neurodegeneration cell model and compare them with a commercial variety.

This work encompasses chemical characterization before and after an IVD and the assessment of neuroprotection by digested metabolites. Some studies targeting redox/cell death systems were also performed to assess possible neuroprotective molecular mechanisms.

The three blackberry extracts presented some quantitative differences in PP composition that could be responsible for the different responses in the neurodegeneration cell model. Commercial blackberry extracts were ineffective but both wild blackberries, *R. brigantinus* and *R. vagabundus*, presented neuroprotective effects. It was verified that a diminishment of intracellular ROS levels, modulation of glutathione levels and activation of caspases occurred during treatment. The last effect suggests a preconditioning effect since caspase activation was not accompanied by diminution in cell death and loss of functionality.

This is the first time that metabolites obtained from an *in vitro* digested food matrix, and tested at levels approaching the concentrations found in human plasma, have been described as inducing an adaptative response.

### **Keywords**

Caspase activity; Glutathione balance; *In vitro* digestion; Neurodegenerative diseases; Wild blackberries.

## 1. Introduction

In the developed world, the population lifespan is increasing with a concomitant increase in the incidence of age-related diseases such as neurodegeneration<sup>1</sup>. Due to the high impact at both financial and social levels<sup>2,3</sup>, strategies to retard or reverse neuronal and behavioral deficits that occur in aging are urgently required. Indeed, these foci are areas of intense research effort but the therapeutic strategies<sup>4</sup> and delivery of (pharma) products<sup>5,6</sup> have been limited. Epidemiological evidence indicates that antioxidant supplementation may provide neuroprotection against age-related neurodegenerative disorders, including PD, ALS and AD<sup>7-9</sup>. Increased dietary intake of antioxidant fruits, in particular berry fruits, may cause positive and profound impacts on human health, performance, and disease<sup>10</sup>. Their biological properties are attributed to the wide diversity and high levels of phenolic compounds, frequently associated with a high antioxidant capacity. Due to the multitude of phytochemicals found in these fruits, instead of a single compound, they can promote complementary, additive, and/or synergistic effects<sup>10,11</sup>.

*In vitro* studies have revealed blackberries as possessing potent antioxidant, antiproliferative and anti-inflammatory activities<sup>12,13</sup>. Moreover, in aged rats these fruits were capable of improving performance on motor tests, which relied on balance and fine motor coordination, and on measures of spatial working memory<sup>14</sup>. However, these effects were not accompanied by an improvement in dopamine release<sup>14</sup>, and consequently by an improvement of receptor sensitivity, events usually related with the observed effects<sup>15,16</sup>. A large number of pathways and protein kinase cascades, such as protein kinase C, Nrf2/ARE antioxidant pathway, pro-survival mitogen-activated protein kinase kinase (MEK)/ERK and PI3K/AKT pathways among others, have been reported as targets for phenolic compounds; nevertheless the target pathways affected remain unknown<sup>17</sup>.

The chemical diversity of plants constitutes an immense and relatively untapped reservoir of molecules with potential

pharmacological/nutraceutical value. The diversity of Portuguese plants represents a reservoir of phytochemicals as yet poorly characterized and explored. In particular, in the north of Iberian Peninsula there are endemic *Rubus* species, such as *Rubus brigantinus* Samp. and *Rubus vagabundus* Samp.<sup>18</sup>, of which chemical diversity could be further explored.

The aim of this work is to evaluate the neuroprotective potential of two endemic blackberry species in a neurodegeneration cell model. This work encompasses an IVD to mimic some alterations in metabolites that fruits are submitted to when ingested. Some studies targeting redox and cell death systems were performed to illustrate molecular mechanisms by which blackberry metabolites could exert beneficial effects.

## 2. Methods and Materials

### 2.1. Plant material

Fruits of wild blackberry species (*Rubus brigantinus* Samp. and *Rubus vagabundus* Samp.) were collected in September 2009 in Bragança region (northeast region of Portugal) and frozen. Fruits were collected from several populations, growing in different locations in order to be representative of species. For both species, voucher samples were authenticated and deposited at the herbarium "João de Carvalho e Vasconcelos", Instituto Superior de Agronomia, Lisbon, Portugal (voucher number 716/2010 and 722/2010). For comparison purposes, the commercial blackberry cv. Apache (*Rubus* L. subgenus *Rubus* Watson) produced in Fataca experimental field (Odemira, Portugal) was also used. The samples were freeze-dried, ground without separation of seeds in an IKA M20 mill to pass a 0.5 mm sieve and stored at -80 °C prior to extraction.

### 2.2. Extract preparation

Fruit extracts were prepared using an hydroethanolic solution (ethanol 50 % (v/v)) as previously described<sup>19</sup>. Briefly, 12 mL of ethanol 50 % were added for each gram of blackberry freeze-dried powder. Homogenate was

shaken for 30 min and filtered. Extracts obtained were dried under vacuum.

### 2.3. *In vitro* digestion

Phytochemical alterations during digestion were mimicked using the IVD model already described<sup>20</sup>. Briefly, the undigested extract was submitted to conditions that mimic the gastric digestion such as adjusted to pH 1.7, the addition of pepsin and incubation at 37 °C with shaking at 100 rpm for 2 h. After, small intestine conditions were mimicked by the addition of pancreatin and bile salts, followed by dialysis with a cellulose tube containing NaHCO<sub>3</sub> to neutralize titratable acidity. After 2 h incubation at 37 °C, the solution inside the dialysis tubing (IN) and the solution outside the dialysis tubing (OUT) were taken.

### 2.4. Chemical characterization

#### 2.4.1. Total phenolic quantification

Determination of total phenolic compounds was performed by the Folin-Ciocalteu method adapted to microplate reader<sup>21</sup>. Gallic acid was used as the standard and the results were expressed as mg GAE.

#### 2.4.2. Peroxyl radical scavenging capacity determination

Peroxyl radical scavenging capacity was determined by the ORAC method as described by Tavares *et al.*<sup>19</sup>. The final results were calculated using the area differences under the fluorescence decay curves between the blank and the sample, and were expressed as μM TE.

#### 2.4.3. Determination of Phenolic Profile by LC-MS

Extracts and digested fractions, containing 500 mg GAE mL<sup>-1</sup> were applied to a C18 column (Synergi Hydro C18 column with polar end capping, 4.6 mm x 150 mm, Phenomenex Ltd.) and analyzed on a LCQ-DECA system controlled by the XCALIBUR software (2.0, ThermoFinnigan), as reported by Tavares *et al.*<sup>19</sup>. The LCQ-Deca system comprised a Surveyor autosampler, pump and PDA detector and a Thermo Finnigan iontrap mass spectrometer.

### 2.5. Animal cell culture

Human neuroblastoma SK-N-MC cells were obtained from the ECACC and cultured in EMEM (Sigma) supplemented with 2 mM L-glutamine (Sigma), 10 % (v/v) heat-inactivated FBS (Gibco), 1 % (v/v) non-essential amino acids (Sigma), 1 mM sodium pyruvate (Sigma) and containing 50 U penicillin and 50 µg streptomycin per mL of medium. The cells were maintained at 37 °C in 5 % CO<sub>2</sub> and split at sub-confluence of 70-80 % using 0.05 % trypsin/EDTA (Gibco).

### 2.6. Cytotoxicity evaluation

Digested fractions were dried under vacuum and dissolved in cell medium for the cytotoxicity tests by measuring cell viability as previously described<sup>20</sup>. Briefly, SK-N-MC neuroblastoma cells were seeded in a 96-well plate using 1.25 x 10<sup>5</sup> cells mL<sup>-1</sup> and grown for 48 h prior to incubation with the IN fractions. Toxicity tests involved 24 h fractions incubation in the range 0-100 µg GAE mL<sup>-1</sup> medium. Cell viability was assessed using the CellTiter-Blue® Cell Viability Assay (Promega), according to the manufacture instructions.

### 2.7. Neuroprotective evaluation

To evaluate the neuroprotective effect of fractions, a neurodegeneration cell model already described was used<sup>20</sup>. The model described the treatment of SK-N-MC neuroblastoma cells with H<sub>2</sub>O<sub>2</sub> to induce cell death. Briefly, cells were seeded at 7.4 x 10<sup>4</sup> cells mL<sup>-1</sup>, grown for 24 h, then after 24 h of pre-incubation with medium supplemented with non-toxic concentrations of blackberry fractions, the cells were treated with medium containing H<sub>2</sub>O<sub>2</sub> (300 µM). After 24 h, the medium was removed and cells were washed with PBS and collected by trypsinisation. Cells were then incubated with two fluorescent probes for 30 min at 37 °C. 3,3'-Dihexyloxacarbocyanine iodide (DiOC<sub>6</sub>(3), 20 nM, Invitrogen) was used to evaluate the ΔΨ<sub>m</sub> and PI (1 µg mL<sup>-1</sup>, Invitrogen) was used to determine cell viability, based on plasma membrane integrity<sup>22</sup>. A flow cytometer (Partec) was used to analyze parameters. This cytometer contains a blue solid state laser (488 nm) with FL1 green fluorescence

channel for DiOC<sub>6</sub>(3) at 530 nm and a FL3 red fluorescence channel for PI detection at 650 nm. The acquisition and analysis of the results were performed with FlowMax<sup>®</sup> (Partec) software.

### 2.8. Intracellular ROS production determination

To evaluate the ability of fractions to reduce ROS levels produced by cells, the conversion of H<sub>2</sub>DCFDA (Invitrogen) to fluorescent DCF was monitored<sup>23,24</sup>. SK-N-MC neuroblastoma cells were seeded in a 96-well plate (1.25 × 10<sup>5</sup> cells mL<sup>-1</sup>), grown for 24 h, then washed with PBS and pre-incubated with fractions prepared in medium (0.5 % (v/v) FBS) for 2 h or 24 h. After pre-incubation, cells were washed with PBS and incubated for 30 min at 37 °C with 25 μM H<sub>2</sub>DCFDA prepared in PBS. Cells were washed with PBS then H<sub>2</sub>O<sub>2</sub> (200 or 300 μM) was added. Fluorescence was measured (λ<sub>ex</sub>: 485 nm, λ<sub>em</sub>: 530 nm) using a FLx800 Fluorescence Microplate Reader (Biotek) over 1 h at 37 °C. ROS generation was calculated as an increase in fluorescent signal compared with cells not treated with H<sub>2</sub>O<sub>2</sub>.

### 2.9. GSH and GSSG quantification

GSH and GSSG were quantified by HPLC after derivatization with orthophthalaldehyde, performed accordingly to Kand'ar *et al.*<sup>25</sup> as already described in Tavares *et al.*<sup>20</sup>.

Chromatographic analysis was accomplished using isocratic elution on a C<sub>18</sub> analytical column (Supelcosil<sup>™</sup> ABZ+Plus HPLC Column 15 cmx4.6 mm, 3 μm (Supelco)) at 40 °C on an Acquity<sup>™</sup> Ultra Performance LC system (Waters). The mobile phase was 15 % (v/v) methanol in 25 mM sodium hydrogen phosphate, pH 6.0. The flow rate was kept constant at 0.7 mL min<sup>-1</sup>. The excitation and emission wavelengths were set at 350 and 420 nm, respectively. The amount of GSH and GSSG was quantified from the corresponding peak areas using Empower<sup>®</sup> Pro 2.0 software. The concentration of GSH and GSSG in the samples was determined from standard curves with ranges 0-100 μM for GSH and 0-5 μM for GSSG. Values were normalized for total protein content, determined by Lowry method<sup>26</sup>.

### 2.10. Caspase 3/7 activity determination

Caspase activity was determined using the Caspase-Glo™ 3/7 assay (Promega). SK-N-MC neuroblastoma cells were seeded in a 96-well plate ( $1 \times 10^4$  cells mL<sup>-1</sup>). Cells were grown for 24 h, then washed with PBS and pre-incubated with fractions prepared in medium (0.5% (v/v) FBS) for 24 h. After this period, cells were washed again with PBS and medium containing 300  $\mu$ M H<sub>2</sub>O<sub>2</sub> was added. Cells were incubated for 24 h and then 100  $\mu$ L of proluminescent caspase 3/7 substrate was added to each well. Cells were incubated at room temperature for 3 h and luminescent signal was recorded. Values were normalized for cell viability, determined by flow cytometry, as described above.

### 2.11. Statistical analysis

The results reported in this work are the averages of at least three independent experiments and are represented as the mean  $\pm$  SD. Differences among treatments were detected by analysis of variance with Tukey HSD multiple comparison test ( $\alpha=0.05$ ) using SigmaStat 3.10 (Systat).

## 3. Results

### 3.1. Characterization of the blackberry extracts

Characterization of the three blackberry extracts was performed and, although the TPC of the three blackberries was very similar, their AC was different (Table 1). The wild blackberries had a higher AC compared to the commercial variety, especially *R. brigantinus* which had 60 % higher antioxidant capacity than the commercial blackberry.

The IVD model provided two fractions (IN and OUT) after pancreatic digestion. The fraction that passes through the dialysis membrane constitutes the IN fraction and contains metabolites that equate to those that should be able to reach serum by paracellular transport. The material that remains outside the dialysis tubing constitutes the OUT fraction and contains metabolites that equate to those that reach colon after digestion.

After IVD, the TPC and AC were greatly changed (Table 1) and the TPC of the IN fractions as reduced to less than 10 % of the original content. The AC values were also reduced in all samples, but since the reduction was lower than in the values of TPC, the ratio AC/TPC became higher in the IN fractions comparatively to the undigested extracts (Table 1). Concerning these ratios for the IN fraction, *R. brigantinus* was the most potent (222  $\mu\text{mol TE mg}^{-1}$  GAE) followed by the commercial blackberry (100  $\mu\text{mol TE mg}^{-1}$  GAE) then *R. vagabundus* (44  $\mu\text{mol TE mg}^{-1}$  GAE; Table 1). Moreover, IN fractions had a higher ratio of AC/TPC than the respective OUT fractions (results not shown). Since IN fraction equates to the compounds that could potentially reach the serum through paracellular transport and at the same time, this fraction seems to be the most chemically reactive (higher AC/TPC), these factors led us to choose the IN fraction to be tested in neuroprotective studies.

LC-MS analysis (Fig. S1 and Table S1 on Supplementary material) showed that the PP profiles of the wild blackberries before digestion were similar to that of the commercial blackberry (Fig. S1A on Supplementary material and Fig. 1A).

Table 1- Chemical characterization of commercial blackberry and two wild blackberry species (*R. brigantinus* and *R. vagabundus*) extracts before and after *in vitro* digestion. For undigested extracts and IN fractions the total phenolic content (TPC) and antioxidant capacity for peroxy radical (AC) were determinate and the ratio AC/TPC was calculated. Values of TPC, AC and AC/TPC in the undigested extract were expressed as  $\text{mg GAE g}^{-1}$  dw,  $\mu\text{mol TE g}^{-1}$  dw and  $\mu\text{mol TE mg}^{-1}$  GAE, respectively. Values in the IN fraction were expressed as % of the values determined for the undigested extract. Statistical significant differences for  $p < 0.05$  are denote with different letters (a-c). All values are mean  $\pm$  SD,  $n = 3$ .

	Undigested extract			IN fraction		
	TPC ( $\text{mg GAE g}^{-1}$ dw)	AC ( $\mu\text{mol TE g}^{-1}$ dw)	AC/TPC ( $\mu\text{mol TE mg}^{-1}$ GAE)	TPC (% of undigested extract)	AC	AC/TPC
Commercial blackberry	27.51 $\pm$ 0.98 <sup>a</sup>	221 $\pm$ 22 <sup>c</sup>	33 $\pm$ 2 <sup>a</sup>	5.5	17	307
<i>R. brigantinus</i>	28.17 $\pm$ 3.65 <sup>a</sup>	357 $\pm$ 10 <sup>a</sup>	21 $\pm$ 3 <sup>b</sup>	0.5	5	1040
<i>R. vagabundus</i>	31.07 $\pm$ 1.47 <sup>a</sup>	274 $\pm$ 57 <sup>b</sup>	15 $\pm$ 3 <sup>b</sup>	1.7	5	292

The main differences were in the amount of anthocyanins (cyanidin-3-*O*-glucoside, -xyloside and -hydroxymethyl-glutaroyl glucoside), which were lower for the endemic species and in the quantity of ellagitannins

(sanguins H-2, C, H-6 and H-10), which were particularly high in *R. vagabundus* (approx. 3.5-fold the amount detected in commercial blackberry). In addition, the endemic species had higher levels of certain flavonols (quercetin pentoside) and ellagic acid or ellagic acid derivatives (methyl ellagic acid glucuronide). After IVD (Fig. 1B), the amounts of anthocyanins and ellagitannins detected in the IN fractions were considerably reduced while, conversely, quercetin derivatives were present in higher levels in fractions from the wild species than those from the commercial blackberry (especially quercetin pentoside; Fig. 1B). The most obvious difference between the *R. brigantinus*, *R. vagabundus* and the commercial blackberry IN samples was the presence of a component with properties that suggest a methyl ellagic glucuronide structure (compound 10 in Fig. S1A on Supplementary material).

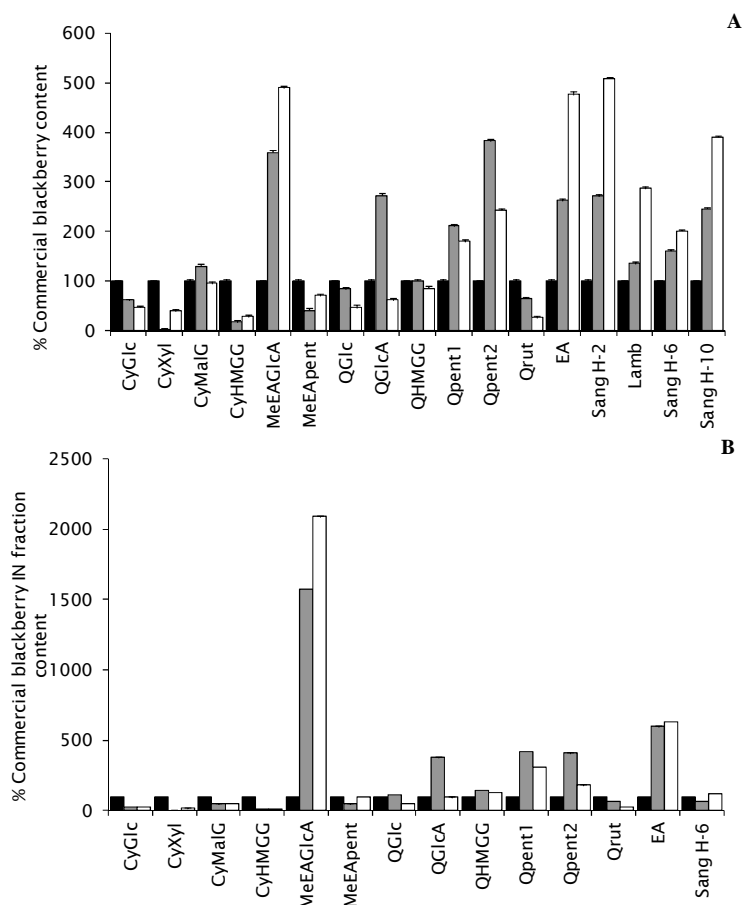


Fig. 1- Relative quantification of PPs in blackberries. A) Undigested extract before in vitro digestion, B) IN samples. All values are expressed as relative to the commercial blackberry variety (■), *R. brigantinus* (■) and *R. vagabundus* (□). Values were calculated as peak area of the mass spectrometer response for the m/z characteristic of each component. Quantified metabolites: cyanidin-3-O-glucoside (CyGlc), cyanidin-3-O-xyloside (CyXyl), cyanidin-3-O-malonylglucoside (CyMalG), cyanidin-3-O-hydroxymethyl-glutaroyl glucoside (CyHMGG), methyl ellagic acid glucuronide (MeEAGlcA), methyl ellagic acid pentose (MeEApent), quercetin-glucoside (QGlc), quercetin-glucuronide (QGlcA), quercetin-HMG-glucoside (QHMGG), quercetin pentose 1 (Qpent1), quercetin pentose 2 (Qpent2), quercetin-rutinoside (Qrut), ellagic acid (EA), sanguin H-2 (Sang H-2), lambertianin C (Lamb), sanguin H-6 (Sang H-6), sanguin H-10 (Sang H-10). Lambertianin C, sanguin H-2 and sanguin H-10 were not identified in IN samples.

### 3.2. Cytotoxicity evaluation

Cytotoxicity of digested blackberry metabolites was evaluated by conversion of resazurin into resorufin by viable cells (Fig. 2).

*R. brigantinus* metabolites were more toxic than commercial blackberry and *R. vagabundus* (Fig. 2) which had similar toxicity profiles. Based on these profiles, ranges of non-toxic concentrations were selected as 0 to 1  $\mu\text{g mL}^{-1}$  GAE for commercial blackberry and *R. vagabundus* and 0 to 0.4  $\mu\text{g mL}^{-1}$  GAE for *R. brigantinus*.

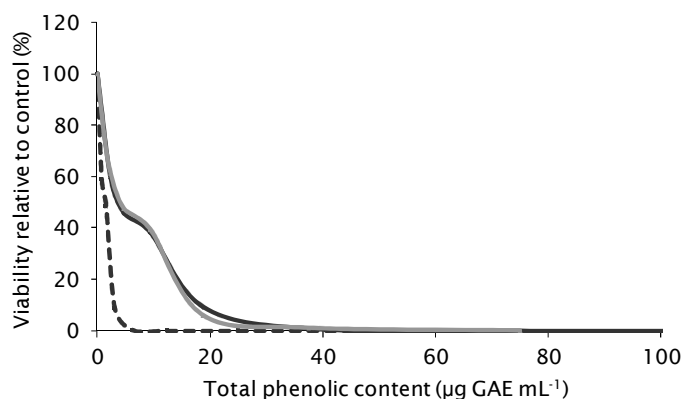


Fig. 2- Cytotoxicity evaluation of digested fractions of the three *Rubus* sp. SK-N-MC neuroblastoma cells were incubated for 24 h with digested fractions (0-100  $\mu\text{g GAE mL}^{-1}$ ) and the cytotoxicity was determined evaluating cell viability by CellTiter-Blue® Cell Viability Assay (Promega). Values are expressed as percentage relatively to control (without extract): Commercial blackberry (—), *R. brigantinus* (---■), *R. vagabundus* (—●)

### 3.3. Neuroprotective evaluation

SK-N-MC neuroblastoma cells were pre-incubated for 24 h in the presence or absence of IN fractions and then challenged with  $\text{H}_2\text{O}_2$  (300  $\mu\text{M}$ , 24 h). Neuroprotection was then evaluated by monitoring cells that maintained both high  $\Delta\Psi\text{m}$  and cell membrane integrity (Q4 positive cells; Fig. 3B). Incubation with all IN fractions in the absence of  $\text{H}_2\text{O}_2$  injury did not alter cellular responses compared to the controls (Fig. 3A), which confirmed the absence of toxicity previously detected on neuroblastoma cells. When cells were challenged with  $\text{H}_2\text{O}_2$ , pretreatment with the IN fraction from commercial blackberry did not cause a protective effect (Figs. 3B and 3C). However, IN fractions from wild species simultaneously increased  $\Delta\Psi\text{m}$  and cell membrane integrity (Figs. 3B and 3C).

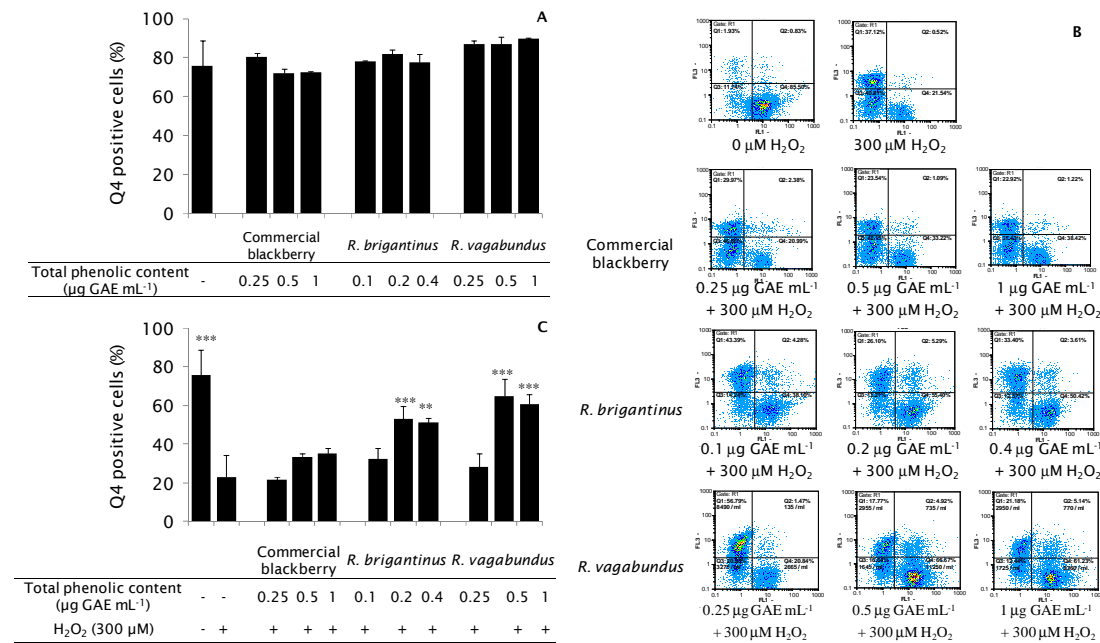


Fig. 3- Cell viability assessed by flow cytometry. Cell viability was assessed in Q4, showing cells presenting membrane integrity (negative for PI) and high mitochondrial potential ( $\Delta\psi_m$ ; positive for DiOC<sub>6</sub>(3)) and expressed as percentage. A) Cell viability for cells incubated with IN digested fractions without oxidative stress (0  $\mu\text{M}$  H<sub>2</sub>O<sub>2</sub>). Statistical differences of pre-incubated cells in comparison with cells not treated with H<sub>2</sub>O<sub>2</sub> are denote as \* p<0.05 \*\* p<0.01 \*\*\* p<0.001. All values are mean  $\pm$  SD, n  $\geq$  3. B) Flow cytometry outputs obtained with H<sub>2</sub>PI and DiOC<sub>6</sub>(3) allow the assessment of the percentage of viable cells, represented in quadrant Q4 (cells presenting membrane integrity and high mitochondrial potential). C) Cell viability for cells incubated with IN digested fractions with oxidative stress (300  $\mu\text{M}$  H<sub>2</sub>O<sub>2</sub>). Neuroblastoma cells were pre-incubated with digested fractions for 24 h and then injured by 300  $\mu\text{M}$  H<sub>2</sub>O<sub>2</sub> for 24 h. Graphic representation of flow cytometry panels and respective statistical evaluation. Statistical differences of pre-incubated cells in comparison with cells only treated with H<sub>2</sub>O<sub>2</sub> are denote as \* p<0.05 \*\* p<0.01 \*\*\* p<0.001. All values are mean  $\pm$  SD, n  $\geq$  3

### 3.4. Intracellular ROS production

Intracellular ROS levels were monitored (Fig. 4) at two different times of pre-incubation, 2 h and 24 h, to cover different time events. All blackberry digested extracts at 2 h and/or 24 h pre-incubation (Fig. 4A) reduced basal ROS production.

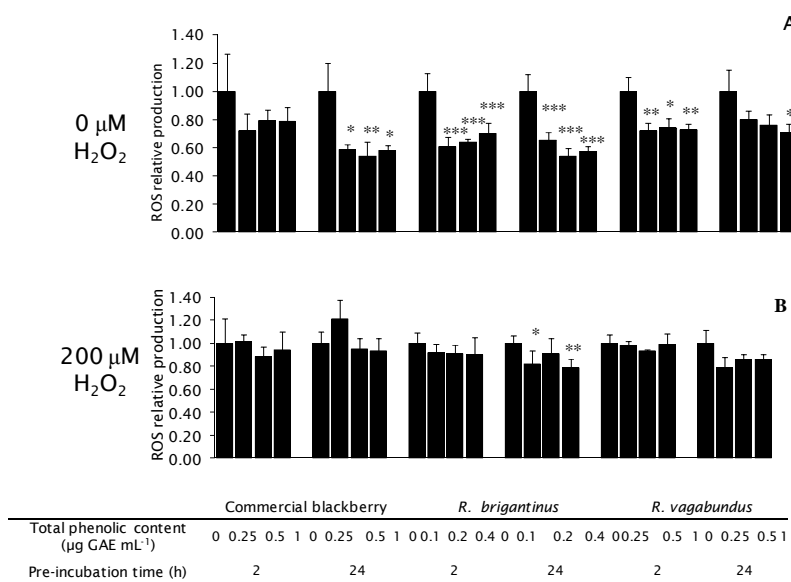


Fig. 4- Relative intracellular ROS production by SK-N-MC neuroblastoma cells pre-incubated with digested fractions for 2 h or 24 h. A) In absence of an oxidative stress B) In the presence of an oxidative stress (200 μM H<sub>2</sub>O<sub>2</sub> for 1 h). ROS were detected by fluorimetry using H<sub>2</sub>DCFDA as probe. Statistical differences in relation to cells not treated with blackberries metabolites (0 μg GAE mL<sup>-1</sup>) are denote as \* p<0.05 \*\* p<0.01 \*\*\* p<0.001. All values are mean ± SD, n = 3

### 3.5. GSH and GSSG quantification

GSH and GSSG were measured after pre-incubation with digested metabolites and after application of H<sub>2</sub>O<sub>2</sub> stress (Fig. 5). The commercial blackberry fraction caused a decrease in GSH content (Fig. 5A), contrarily to *R. brigantinus* and *R. vagabundus* fractions that augmented GSH with *R. vagabundus* being more effective (Fig. 5A).

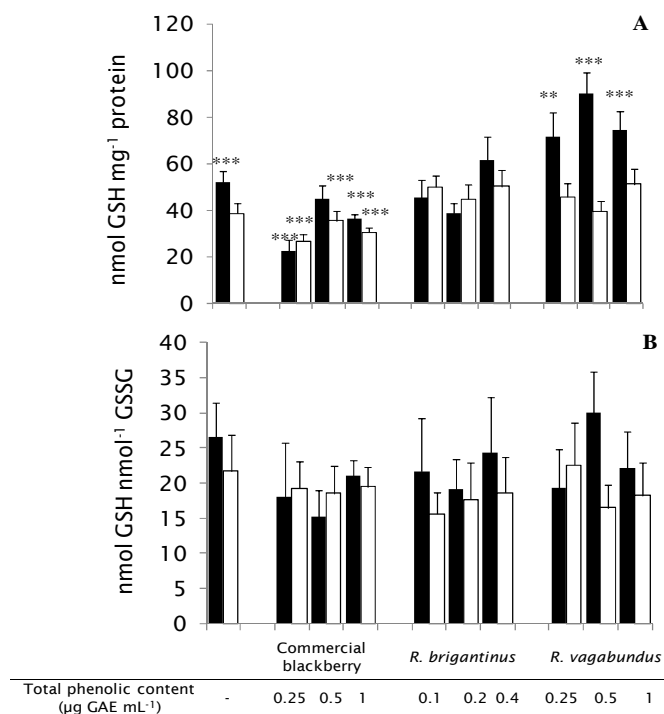


Fig. 5- Quantification of GSH and ratio GSH/GSSG. SK-N-MC neuroblastoma cells pretreated with digested blackberries extract for 24 h, in the absence of stress (■) and in cells subjected to stress (300 µM H<sub>2</sub>O<sub>2</sub> for 24h) (□) A) GSH; B) ratio GSH/GSSG. Cells were harvested and analyzed for their content in GSH and GSSG by HPLC. Statistical differences between treatments in relation to control were determined for p<0.01 and are denoted as \*\* p<0.01 \*\*\* p<0.001. All values are mean ± SD, n ≥ 3

H<sub>2</sub>O<sub>2</sub> insult (300 µM for 24 h) induced a GSH depletion (Fig. 5A). Pre-treatment with commercial blackberry metabolites could not maintain GSH levels under H<sub>2</sub>O<sub>2</sub> insult. Conversely, pre-incubation with the IN fractions from the wild blackberries prevented GSH depletion, maintaining GSH levels at around control levels. However, analysis of the GSH/GSSG did not highlight any detectable any significant alteration due to H<sub>2</sub>O<sub>2</sub> stress or pre-incubation with digested metabolites (Fig. 5B).

### 3.6. Caspase 3/ 7 activity determination

Pre-incubation with IN metabolites in the absence of H<sub>2</sub>O<sub>2</sub> injury enhanced caspase activity up to twofold (Fig. 6A). When cells were challenged with 300 μM H<sub>2</sub>O<sub>2</sub>, caspase activation was augmented by around fourfold compared to the control (Fig. 6B). Indeed, the same augmentation in caspase activity was verified when cells were pre-incubated with any of the blackberry IN samples and submitted to stress. Curiously, lower levels of endemic metabolites (0.1-0.25 μg GAE mL<sup>-1</sup>) strongly promote caspase activation.

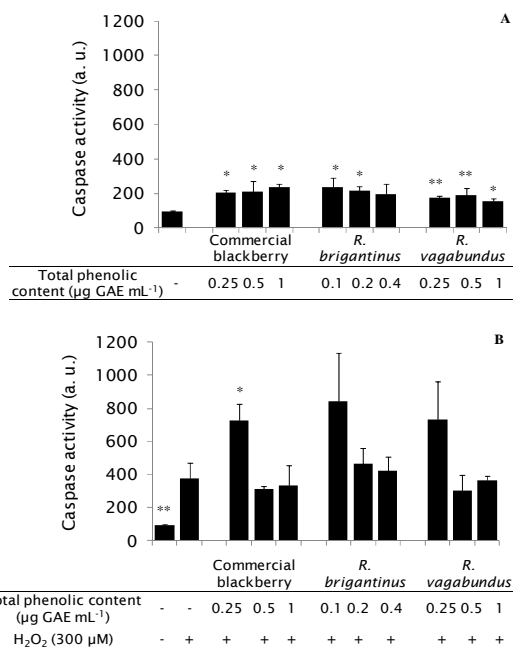


Fig. 6- Caspase 3/7 relative activity in SK-N-MC cells pre-treated with digested fractions for 24 h. A) In the absence of H<sub>2</sub>O<sub>2</sub> injury. B) In the presence of H<sub>2</sub>O<sub>2</sub> (300 μM, 24 h) injury. Values of caspase 3/7 activity were normalized by cell viability determined by flow cytometry. Statistical differences are denote as \* p<0.05 \*\* p<0.01 \*\*\* p<0.001. All values are mean ± SD, n = 3

### 4. Discussion

Wild species represent a rich source of phytochemicals that can be explored with nutritional, nutraceutical or pharmaceutical purposes. *R. brigantinus* and *R. vagabundus* are two *Rubus* sp. endemic from the North of Iberia Peninsula with a very restrict occurrence<sup>18</sup>. These two species have, until now, not been chemically characterized. The occurrence of different (quantities of) phenolic compounds could promote different bioactivities, thereby identifying this germplasm as sources of compounds to be further explored and exploited. Blackberries are berries

with a recognized high phenolic content <sup>27</sup>. However, although the TPC of the three blackberry extracts was very similar, the wild blackberries exhibited higher ACs compared to the commercial variety, especially *R. brigantinus* (Table 1). LC-MS analysis of blackberry extracts before IVD showed some quantitative differences among species concerning anthocyanins, ellagitannins, flavonols and ellagic acid (Fig. 1A and Fig. S1 on Supplementary material). However, the physical-chemical alterations during GI digestion have a major impact on the quantity and diversity of the phenolic compounds present in fruits <sup>20</sup>. After IVD, the TPC of the IN fractions was reduced to less than 10 % of the original content (Table 1). These values are in accordance with recoveries obtained in previous work on red wine and red cabbage <sup>28,29</sup>. The AC was also greatly reduced (Table 1). Nevertheless, the IN fractions presented relatively higher AC/TPC ratio than the undigested extracts (Table 1). IN fractions from *R. brigantinus* were the most potent followed by the commercial blackberry and then by *R. vagabundus*. These differences, reflecting the different chemistries, could be reflected in different biological potencies and/or effects. After IVD, the amounts of anthocyanins and ellagitannins detected in the IN fractions were considerably reduced (Fig. 1B). Total anthocyanin content was reduced to 12.8 % in commercial blackberry and to around 5 % in the endemic species; these recoveries are in agreement with other work on red cabbage, red wine and pomegranate juice <sup>28-30</sup>. Anthocyanins are stable in acidic conditions of the stomach, but less stable at elevated pH of the small intestine <sup>28,30,31</sup>. Also ellagitannins, a characteristic group of metabolites in the Rosaceae, were extensively reduced in all species. A proportion of the “missing ellagitannins” was recovered in the OUT sample having been unable to pass through the dialysis tubing presumably as a result of their larger size or binding to proteins and/or bile acids <sup>32</sup>. The increased levels of ellagic acid in the IN fractions is indicative of ellagitannin breakdown. After IVD, quercetin derivatives were present in higher levels in extracts from the wild species than those from the commercial blackberry. These higher recoveries reflect the greater

stability of flavonols to IVD<sup>29</sup> as other components were degraded during IVD but also reflect higher levels in the undigested extracts. The most obvious difference between the *R. brigantinus*, *R. vagabundus* and the blackberry IN samples was the presence of a component with properties indicating a methyl ellagic glucuronide structure. Methyl ellagic glycosides have been identified in blackberry and raspberry<sup>33,34</sup>, and methyl ellagic glucuronides have been identified in other plants<sup>35</sup>. However, further work is required to confirm this putative identification. Although this component appeared greatly enriched in the IN samples after digestion compared to the commercial blackberry IN sample, it still only comprised a small proportion of the total phenolic content. The IN samples contained many peaks, presumably breakdown products, that could not be identified, and these metabolites may also have important biological properties.

Based on the cytotoxicity profiles, ranges of non-toxic concentrations of IN fractions were selected as 0- 1  $\mu\text{g mL}^{-1}$  GAE for commercial blackberry and *R. vagabundus* and 0-0.4  $\mu\text{g mL}^{-1}$  GAE for *R. brigantinus*. These ranges correspond to 0-6  $\mu\text{M}$  GAE and 0-2.4  $\mu\text{M}$  GAE, respectively. These levels are near to the physiological concentrations of certain phenolic components noted in human plasma (0-4  $\mu\text{M}$ )<sup>36</sup>. Then, these non-toxic physiological concentrations were tested on a neurodegeneration cell model.

SK-N-MC neuroblastoma cells were pre-incubated for 24 h in the presence or absence of IN fractions and then challenged with  $\text{H}_2\text{O}_2$ . Neuroprotection was then evaluated by monitoring cells that maintained both high  $\Delta\Psi\text{m}$  and cell membrane integrity (Q4 positive cells; Fig. 3B). Unlike the commercial blackberry variety, the wild species were able to recover both parameters, ensuring cell viability and functionality (high cell membrane integrity and high  $\Delta\Psi\text{m}$ ). Therefore, *R. brigantinus* and *R. vagabundus*, but not the commercial blackberry, extracts contain phytochemicals with a potential to be explored in a neuroprotective perspective. However, the mechanism(s) by which those metabolites are exerting a beneficial effect

are unknown. In order to unravel possible mechanisms behind the neuroprotection detected, some cellular redox/death mechanisms were tested.

Intracellular ROS levels were monitored (Fig. 4) at two different times of pre-incubation, 2 and 24 h, to cover different time events. Within 2 h, direct scavenging effects from metabolites can be determined, and at 24 h, indirect scavenging resulting from activation/modulation of other molecular mechanisms can be assessed. Diverse phytochemicals can act directly through scavenging properties or indirectly through modulation of thiol-containing molecules (such as glutathione), ROS-inactivating enzymes (*e.g.* SOD, CAT) or phase II detoxifying enzymes (*e.g.* glutathione S-transferases, HO-1) <sup>37</sup>. All blackberry extracts at 2 h and/or 24 h pre-incubation (Fig. 4A) reduced basal ROS production. This effect suggests a possible preventive role in oxidative stress-associated diseases, such as neurodegeneration. These results agree with previous work on various phenolic components (*e.g.* naringin, nobiletin, luteolin, quercetin, gingerol) <sup>38-40</sup>. Following the application of a stress of 200  $\mu\text{M}$   $\text{H}_2\text{O}_2$  for 1 h (Fig. 4B), *R. brigantinus* was the only species that maintained reduced ROS levels after 24 h pre-incubation. The different composition of *R. brigantinus* digested metabolites could activate indirect antioxidant systems more effectively than the other species. Digested metabolites from commercial blackberry, as already described <sup>20</sup>, and from *R. vagabundus* lost part of their intracellular antioxidant capacity especially against  $\text{H}_2\text{O}_2$ -induced stress.

GSH and GSSG were determined after pre-incubation with blackberry fractions and after application of  $\text{H}_2\text{O}_2$  stress (Fig. 5). Different IN fractions caused different responses. The commercial blackberry fraction caused a GSH depletion (Fig. 5A), while conversely, *R. brigantinus* and *R. vagabundus* fractions augmented GSH levels with *R. vagabundus* being more significant (Fig. 5A). Intracellular GSH depletion is an early hallmark in the progression of cell death in response to different apoptotic stimuli <sup>41</sup>.  $\text{H}_2\text{O}_2$  insult induced a GSH depletion (Fig. 5A). Pretreatment with

commercial blackberry metabolites could not maintain GSH levels under  $H_2O_2$  insult, which corresponds to the lack of neuroprotection by commercial blackberry digests (Fig. 3B). However, no significant changes were detected in GSH/GSSG ratio, induced by  $H_2O_2$  stress or IN fraction pre-incubation (Fig. 5B). This redox homeostasis is due to changes in GSH that are accompanied by changes in GSSG. Pre-incubation with the IN fractions from the wild blackberries, although not changing GSH/GSSG ratio, increased GSH levels (Fig. 5A) which could occur via *de novo* synthesis of GSH by  $\gamma$ -glutamylcysteine ligase. This increase should contribute to the cellular protection, since after imposition of oxidative stress, levels of GSH were maintained around control levels (Fig. 5B) and cell death was prevented (Fig. 3C). The decrease in GSH and GSSG levels after pre-incubation with wild blackberries fractions followed by the oxidative stress could be due to their mobilization by other molecules, such as proteins. This mechanism denominated S-glutathionylation is an important post-translational modification that provides protection of proteins against irreversible modifications and protein damage in response to higher levels of oxidative stress<sup>42</sup>.

Acute and chronic neurodegenerative conditions have been associated with both apoptotic and necrotic cell death, and caspases can play a role in both mechanisms<sup>43</sup>. A range of pure phenolics have been shown to cause caspase inhibition and reduce cell death<sup>38,39,44</sup>. However, pre-incubation with IN metabolites in the absence of  $H_2O_2$  injury enhanced caspase activity up to twofold (Fig. 6A) without reducing cell viability (Fig. 3A). When cells were challenged with  $H_2O_2$ , caspase activation was augmented by comparison with the control (Fig. 6B). The same augment in caspase activity was verified when cells were pre-incubated with any of the blackberry IN samples and submitted to stress. However, for the fractions from wild blackberries, this caspase activation was not accompanied by a decrease in cell viability, in contrast to cells only challenged with  $H_2O_2$  (Fig. 3C). This protective effect was not verified for the commercial blackberry fraction. Again, differences in PP composition

must be responsible for these observed differences. Curiously, although lower levels of endemic metabolites ( $0.1\text{-}0.25\ \mu\text{g GAE mL}^{-1}$ ) strongly promote caspase activation, they did not diminish cell death in stressed cells (Fig. 3C). Results suggest that the existing mechanism of death in the presence of metabolites is not caspase-dependent. Different levels of caspases-3 and -7 activation by metabolites pre-incubation (Fig. 6A) did not lead to different levels of death (Fig. 3A). Also, similar caspases-3 and -7 activation by metabolites (Fig. 6B) led to reduced levels of death after oxidative injury (Fig. 3C). Exposure to components in the digests from wild blackberries could be an initiating event that leads to protection against subsequent, potentially lethal stimuli. This mechanism, also known as preconditioning or hormetic effect, has been reported as an effective neuroprotective mechanism, where activation of caspase-3 could be involved<sup>45-47</sup>. In those cases where caspase-3 is activated, arrest of cell death can be triggered by downstream mechanisms like defense against apoptosome assembly and cleaved caspases. This protection can be mediated by factors such as calbindin, inhibitors of apoptosis (IAPs), B-cell lymphoma 2 (Bcl-2) family and HSPs, induced by sub-lethal insults<sup>46,47</sup>.

The evaluation of (*in vitro*) digested metabolites, as opposed to the common approach employing undigested/pure compounds, revealed a differential metabolic response indicating that considering dietary PPs metabolism is crucial to effectively assess and determine physiological bioefficacy<sup>20</sup>. In fact, the metabolism of these compounds is quite complex. PPs can be absorbed in the stomach and small intestine, by diffusion or transport<sup>36</sup>. Once absorbed, they are biotransformed through phase II enzymatic conjugation in both the small intestine and liver. Flavonoids not absorbed in the upper GI tract reach the colon and are subjected to colonic microflora action and their catabolites absorbed into the circulatory system.

Once we aim to study digestion-derived metabolites in neurodegenerative disorders, we should also take into account that circulating metabolites must cross and/or interact with BBB. There are numerous studies

reporting PP-mediated neuroprotection. However, there is paucity of information regarding the interaction of PPs metabolites with the brain endothelial cells forming the BBB, which has complicated identification of PPs compounds entering the central nervous system. Among PPs found in berries, there are some reports indicating the presence of anthocyanins and flavanols in brain tissues <sup>48-52</sup>.

## 5. Conclusions

In summary *R. brigitinus* and *R. vagabundus* were revealed to be promising sources of metabolites with neuroprotection capabilities. Digested metabolites from these blackberries, at levels that could be found in human plasma, activated adaptative cellular stress response pathways such as caspase activation, GSH modulation and also ROS diminishment. These effects protected neuronal cells against oxidative injury, one of the most important features of neurodegeneration. This is the first report highlighting a neuroprotective effect by a digested food matrix involving activation of caspases, suggesting a preconditioning effect. It has been already described for some isolated phytochemicals that beneficial effects could be achieved through neurohormesis pathways, such as stimulating the vitagene system, the production of antioxidant enzymes, protein chaperons and other proteins that help cells to withstand stress <sup>53</sup>. Therefore, these molecular targets should be a goal for future studies into the neuroprotection mediated by digested metabolites from wild blackberries.

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## 8. Supplementary material

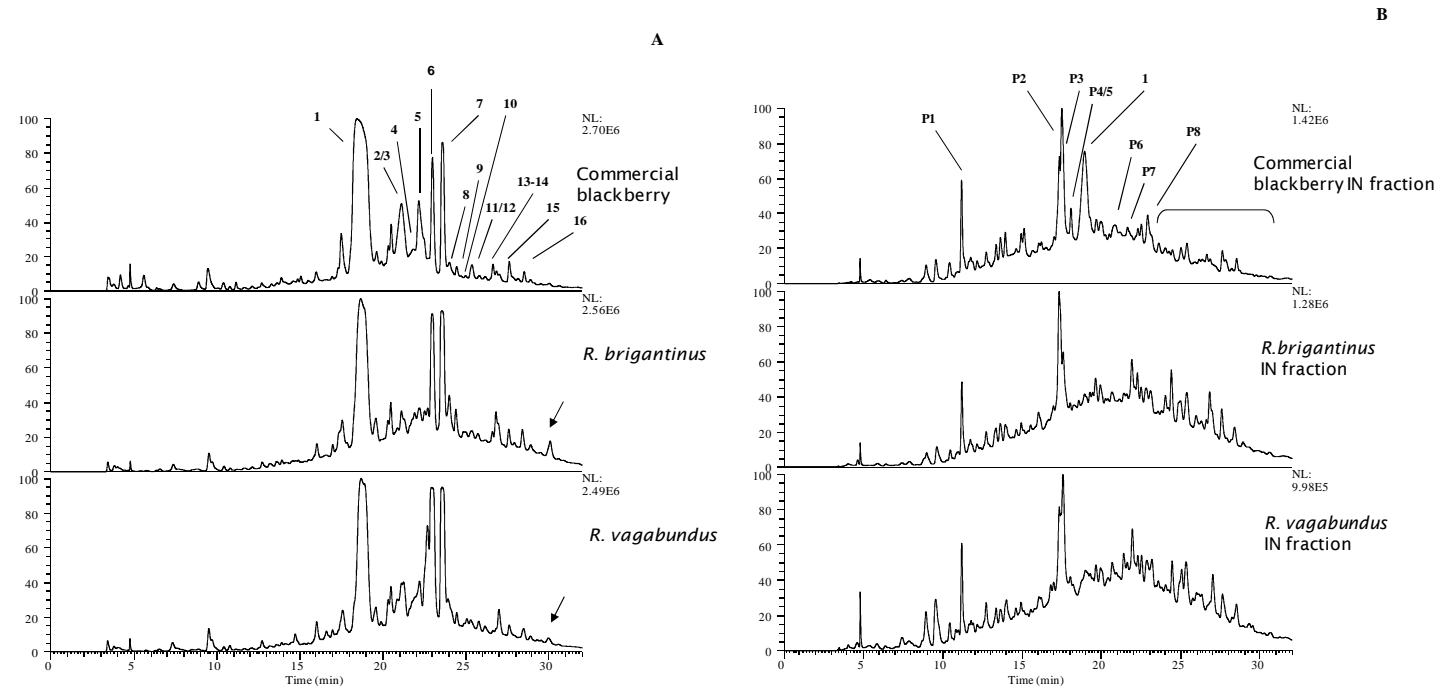


Figure S1- HPLC profile of the three blackberries recorded at absorption maximum wavelength for each peak A) Undigested extracts and B) IN samples. The full scan deflection is shown in the upper right corner of each panel. Numbers in some peaks correspond to their putative identifications based on MS fragmentation pattern presented in Table S1. Peaks under the bracket are covered by peaks 9-16 (mainly quercetin and ellagic acid derivatives) which are enriched in the wild species and survive digestion. The arrows denote unknown components which are enriched in only the wild species.

Table S1- Identification of phenolic components from blackberry extracts

Peak No.	RT	PDA	<i>m/z</i>	MS <sup>2</sup>	Putative ID
<b>Blackberry extracts</b>					
1	18.46	515, 280	<b>+449</b> , 287	287	Cyanidin-3- <i>O</i> -glucoside <sup>33,54</sup>
2	21.11	515, 280	<b>+419</b> , 287	287	Cyanidin-3- <i>O</i> -xyloside <sup>33,54</sup>
3	21.77	240-300	<b>1567</b> , 783, 301	1265, 1103, <b>933</b>	Sanguin H-10 <sup>33,54,55</sup>
4	21.85	520, 285	<b>+535</b> , 287	287	Cyanidin-3- <i>O</i> -malonyl glucoside <sup>54</sup>
5	22.16	520, 280	<b>+593</b> , 287	287	Cyanidin-3- <i>O</i> -hydroxymethyl-glutaroyl glucoside <sup>55</sup>
6	22.98	240-300	<b>1401</b> , 1250, 934, 301	1869, 1567, <b>1250</b> , 1235, 934, 633	Lambertianin C <sup>33,34,56</sup>
7	23.58	240-300	<b>1869</b> , 935, 301	<b>1567</b> , 1265, 1235, 1103, 933, etc.	Sanguin H-6 <sup>34,56</sup>
8	24.00	240-300	<b>1103</b>	multiple	Sanguin H-2 <sup>57</sup>
9	24.43	355	<b>433</b> , 301	301	Quercetin pentose 1 <sup>58</sup>
10	24.83	365	<b>491</b> , 315, 301	<b>315</b> , 301	Methyl ellagic acid glucuronide <sup>35</sup>
11	25.25	355	<b>433</b> , 301	301	Quercetin-pentose 2 <sup>58</sup>
12	25.40	355	<b>609</b> , 301	301	Quercetin-rutinoside <sup>34,54,58</sup>
13a	26.63	355	<b>463</b> , 301	301	Quercetin-glucoside <sup>33,34,54,58</sup>
13b	26.83	355	<b>477</b> , 301	301	Quercetin-glucuronide <sup>33,58</sup>
13c	27.00	355	<b>477</b> , 301	301	Quercetin-glucuronide <sup>33,58</sup>
14	27.57	370	301	301, 275	Ellagic acid <sup>59</sup>
15	27.95	355	<b>607</b> , 505, 463, 301	545, 505, <b>463</b> , 301	Quercetin-HMG-glucoside <sup>58</sup>
16	28.50	365	447, 315	315	Methyl ellagic acid pentose <sup>33,34</sup>
<b>IN fractions</b>					
P1	11.22	230-300, 250 max	<b>779</b> , 667, 281, 267	737, 547, 397, 265	Unknown
P2	17.32	260, 290	611	575, 465, 285	Unknown
P3	17.47	270	443	237, 219, 163	Unknown
P4	17.85	260, 290	<b>1057</b> , 471	869	Unknown
P5	18.06	275	<b>855</b> , 715, 552, 519	757, 739, 713, 695	Unknown
P6	20.48	335, 285	465	303, <b>285</b>	Unknown
P7	20.74	280	<b>767</b> , 565, 463, 445	731, 587, 551	Unknown
P8	22.24	235	881	ND	Unknown

Assignments are supported by previous work. Peak numbers refer to Fig. S1. Compounds 1-14 were identified in the original extract; compounds P1-P8 were more apparent in the IN samples. + denote detection in the positive mode MS only. In bold are denote the most abundant ions. ND- not detected.

## Chapter 6: Differential expression of genes involved in neuroprotection conferred by blackberry polyphenols after simulated gastrointestinal digestion

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Work on this chapter needs further experiments to be submitted for publication.

Author contribution:

LT performed the majority of the experiments presented in this chapter (except RNA quality control and labeling and hybridization on microarrays).

### **Abstract**

Clinical and preclinical research has identified neurological benefits associated with the consumption of berry fruits in relation to reducing the risk of incidence of age-related neurodegeneration. Signaling mechanisms and protein kinases cascades have been reported as affected by phenolic compounds; however the impact upon pathways affected remain unknown. The aim of this study is to unravel the neuroprotective pathways mediated by extracts of *Rubus vagabundus*, a wild blackberry species, in comparison with a commercial variety, which have already been proven to promote different neuroprotective actions. In model systems To facilitate the achievement of this aim a transcriptomic approach will be exploited. Blackberry extracts subjected to IVD were able to differentially impact upon gene expression alterations induced by oxidative stress. However, *R. vagabundus* was more effective in reducing the number of genes altered. Potential pathways differentially affected by stress and blackberry metabolites were mainly related with functions of transport, cell cycle and DNA repair. Complementary biochemical studies are necessary to evaluate possible further post-translational regulation and validate the differential protective role of blackberry metabolites on those pathways. Pathways involving small GTP-binding proteins were also highlighted as differentially regulated. This impact upon several cellular pathways with the effect on those pathways could be interesting to follow. Moreover, study of small GTP-binding proteins could reveal them as important regulators.

### **Keywords**

Cell cycle; DNA repair mechanism; Microarrays; Neurodegeneration cell model; *Rubus* sp.; Small GTP-binding proteins; Transport.

## 1. Introduction

The increase in life expectancy in populations from developed countries has increased the incidence of aging related diseases, such as NDDs <sup>1</sup>. These diseases are becoming a major problem in terms of health and well-being with an associated high impact in health and welfare care costs. Dietary supplementation with berry fruits is reported to have direct and beneficial effects on the brain <sup>2</sup>. There is a growing body of preclinical and clinical research identifying neurological benefits associated with the consumption of berry fruits, preventing age-related neurodegeneration, by mediating cognitive and motor functions <sup>3</sup>. Recent clinical trials have extended the findings of the model systems identifying antioxidant, anti-inflammatory, and cognition sparing effects to humans <sup>3</sup>. In cell and animal models, berry fruits have been shown to mediate signaling pathways involved in inflammation and cell survival in addition to neuroplasticity and neurotransmission enhancement, leading to attenuation of age- and pathology-related deficits in behavior <sup>2,4</sup>. A large number of signaling pathways and protein kinase cascades, such as protein kinase C, Nrf2/ARE antioxidant pathway, pro-survival MEK/ERK and PI3K/AKT pathways among others, have been reported to be affected by phenolic compounds; nevertheless the specific details of the mode and site of action of these phenolic compounds remains unclear <sup>5</sup>. Recently, wild blackberries, with a slightly different chemical composition from a commercial blackberry cultivar, were shown to exhibit a better neuroprotective capacity than the commercial cultivar <sup>6</sup>. Therefore, as well as understanding the target pathways affected it become also important to understand the differential regulation mediated by the different blackberries.

Many reports on the effects of PPs are based on *in vitro* cell assays that have studied single compounds at relatively high doses and in the form as they occur in food <sup>7</sup>. Although these studies are import to unravel the potential mechanisms involved, the experimental conditions are largely non-physiological. PPs are normally ingested as mixtures of different

compounds immersed in a complex food matrix that undergoes a digestion process and then must pass several barriers before reaching the target organ.

Dietary interventions generally results in small effects on a large number of genes compared to a pharmaceutical intervention which may targets a specific biomarker <sup>8</sup>. Transcriptional profiling using DNA microarrays has become an essential technology to conduct a broad survey of the changes in gene expression that accompany specific cellular responses such as those triggered by food or food components on cells. It can also highlight the combined effect of several genes belonging to a similar biological pathway and to identify gene products of potential significance that can be examined further thereby giving a feel for the global cell response to a specific treatment. Moreover, it can identify modulations of multiple gene networks, in the form of the expression of thousands of genes, within a single experiment.

The aim of this work is to use a transcriptomic approach to unravel the mechanism/pathways mediated, at the gene expression level, by *Rubus vagabundus*, a wild blackberry species, in comparison with a commercial variety, which have already been shown to promote different neuroprotective actions.

## 2. Material and Methods

### 2.1. Preparation of *in vitro* digested fractions

Fruits of wild blackberry (*Rubus vagabundus* Samp.) were collected in September 2009 in Bragança region (northeast region of Portugal) and frozen. Fruits were collected from several populations, growing in different locations in order to be representative of the species. Voucher samples were authenticated and deposited at the herbarium "João de Carvalho e Vasconcelos", Instituto Superior de Agronomia, Lisbon, Portugal (voucher number 722/2010). The commercial blackberry cv. Apache (*Rubus* L. subgenus *Rubus* Watson) was produced in Fataca experimental field (Odemira, Portugal). The samples were freeze-dried,

ground without separation of seeds in an IKA M20 mill to pass a 0.5 mm sieve and stored at -80 °C prior to extraction. *In vitro* digested fractions were obtained as previously described <sup>6</sup>. Fruit extracts were prepared using a hydroethanolic solution (ethanol 50 % (v/v)). Briefly, 12 mL of ethanol 50 % were added for each gram of blackberry freeze-dried powder. Homogenate was shaken for 30 min and filtered. Extracts obtained were dried under vacuum. Phytochemical alterations during digestion were mimicked using the IVD model and a fraction which contains metabolites that equate to those that should be able to reach serum by paracellular transport were evaluated.

## 2.2. Animal cell culture

Human neuroblastoma SK-N-MC cells were obtained from the ECACC and cultured in EMEM (Sigma) supplemented with 2 mM L-glutamine (Sigma), 10 % (v/v) heat inactivated FBS (Gibco), 1 % (v/v) non-essential amino acids (Sigma), 1 mM sodium pyruvate (Sigma) and containing 50 U penicillin and 50 µg streptomycin per mL of medium. The cells were maintained at 37 °C in 5 % CO<sub>2</sub> and split at sub-confluence of 70-80 % using 0.05 % trypsin/EDTA (Gibco). SK-N-MC cells were seeded at 5 x 10<sup>4</sup> cells cm<sup>2</sup> and grown for 24 h. Afterwards, medium was replaced and *in vitro* digested fractions that contains metabolites that could reach serum by paracellular transport supplied to cells (0.5 µg GAE mL<sup>-1</sup>) dissolved in medium containing 0.5 % (v/v) FBS. Cells were incubated for 24 h, then medium was removed, replaced by medium (0.5 % (v/v) FBS) containing 300 µM H<sub>2</sub>O<sub>2</sub> and cells incubated for 3 h.

## 2.3. Cell sorting

After incubations cells were washed with PBS, trypsinized, collected to a tube and 0.05 volumes of FBS added, to inactivate trypsin. Two fluorescent probes (DiOC<sub>6</sub>(3), Invitrogen and PI, Invitrogen) were then added, in PBS, to final concentrations of 20 nM and 1 µg mL<sup>-1</sup>, respectively. Cells were then incubated with the probes for 30 min at

37 °C. DiOC<sub>6</sub>(3) was used to evaluate the  $\Delta\Psi_m$  and PI to determine cell viability, based on plasma membrane integrity, as previously described <sup>6</sup>.

Cells were then sorted in a FACSAria High Speed Cell Sorter (Becton Dickinson), using a 100  $\mu$ m nozzle with 206.8 kPa (30 psi) sheath pressure. A 488 nm laser was used for DiOC<sub>6</sub>(3) and PI excitation and detection was performed using a 530/30 nm and a 695/40 nm HQ band pass filter, respectively. Only cells that presented cellular membrane integrity and high mitochondrial transmembrane potential were collected in tubes containing PBS.

#### 2.4. RNA isolation, quality control and labeling and hybridization

Followed centrifugation 10 min at 500 *g*, total RNA extraction of sorted cells was performed using AxyPrep Multisource Total RNA Miniprep (Axygen), accordingly to manufacturers and as previously described <sup>9</sup>. Total RNA was treated with Turbo™ DNase I (Ambion), accordingly to the manufacturer's instructions. RNA quantity was assessed through Nano-Drop® ND-1000 spectrophotometer (NanoDrop Technologies) and quality using an Agilent 2100 Bioanalyser with an RNA 6000 Nano Assay (Agilent Technologies). The RNA, in biological triplicates, was processed for use on Affymetrix (Santa Clara, CA, USA) GeneChip HuGene 1.0 ST Arrays, according to the manufacturer's Whole Transcript Sense Target Labeling Assay. Briefly, 100 ng of total RNA containing spiked in Poly-A RNA controls (GeneChip Expression GeneChip Eukaryotic Poly-A RNA Control Kit; Affymetrix) was used in a reverse transcription reaction (GeneChip® WT cDNA Synthesis Kit; Affymetrix) to generate first-strand cDNA. After second-strand synthesis, double-stranded cDNA was used in an *in vitro* transcription (IVT) reaction to generate cRNA (GeneChip® WT cDNA Amplification Kit; Affymetrix). Fifteen  $\mu$ g of this cRNA was used for a second cycle of first-strand cDNA synthesis (GeneChip® WT cDNA Synthesis Kit; Affymetrix). From the single stranded cDNA 5.5  $\mu$ g was fragmented and end-labeled (GeneChip® WT Terminal Labeling Kit; Affymetrix). Size distribution of the fragmented and end-labeled cDNA,

respectively, was assessed using an Agilent 2100 Bioanalyzer with a RNA 6000 Nano Assay.

Five  $\mu\text{g}$  of end-labeled, fragmented cDNA was used in a 100- $\mu\text{L}$  hybridization cocktail containing added hybridization controls. 80  $\mu\text{L}$  of mixture was hybridized on arrays for 17 h at 45°C. Standard post hybridization wash and double-stain protocols (FS450\_0007; GeneChip HWS kit, Affymetrix) were used on an Affymetrix GeneChip Fluidics Station 450. Arrays were scanned on an Affymetrix GeneChip scanner 3000 7G. Scanned arrays were analyzed first with Affymetrix Expression Console software for quality control. GeneChip datasets for the eighteen arrays used in this study will be available in a MIAME-compliant format through Gene Expression Omnibus.

### 2.5. Data Analysis and Gene Functional Classification

Absent and present calls were generated using Chipster 2.0 platform as of 24 of January 2012 (<http://chipster.csc.fi/><sup>10</sup>). Array data were normalized using Robust Multi-array average (RMA) and statistical differences evaluated by a two groups test (empirical Bayes), for  $p < 0.01$ .

The DAVID gene functional classification tool<sup>11,12</sup> was used to condense the list of genes detected in our sample set into functionally-related groups. We used an agglomeration method (Functional Annotation Clustering) to cluster the three main gene ontology charts (Biological Process, Molecular Function and Cellular Component) in a meaningful network context. The following parameters were used: classification stringency (medium), similarity term overlap (3), similarity threshold (0.50), initial group membership (3), final group membership (3), and multiple linkage thresholds (0.50). Only clusters presenting an enrichment score higher than 1.3 (equivalent to  $p < 0.05$ ) were considered. To identify the canonical pathways involved in the protection by berry metabolites, differentially-expressed genes were analyzed using a manually curated proprietary database (MetaCore™, GeneGo, by Thomson Reuters, St. Joseph, MI). Analysis involves in the enrichment of matching gene IDs of

possible targets for the sets with gene IDs in functional ontologies in MetaCore. The probability of a random intersection between a set of IDs the size of target list with ontology entities is estimated in p-value of hypergeometric intersection. The lower p-value means higher relevance of the entity to the dataset, which shows in higher rating for the entity. Pathways identified were sorted using the criterion “Statistically significant Maps”.

### 3. Results

Transcript levels were determined in neuroblastoma cells pre-incubated with blackberry metabolites and/or subjected to oxidative insult, a model for neurodegeneration. Cells were subjected to control (C) conditions, oxidative stress [subjected to 3 h H<sub>2</sub>O<sub>2</sub> 300 μM (S)], incubated with blackberry metabolites for 24 h (BB and RV for commercial blackberry and *R. vagabundus*, respectively) and pre-incubated with blackberry metabolites for 24 h followed by oxidative stress (BB+S and RV+S). All the results were obtained by comparison with C conditions.

#### 3.1. General characterization

Oxidative stress (S treatment; Table 1) resulted in the differential expression 967 genes compared with control treatment, which corresponds to 4.8 % of the total genes covered by the array. Of those, 495 were up-regulated and 472 down-regulated. BB and RV treatments induced the differential expression of 524 and 85 genes, respectively (Table 1). In the BB+S treatment, 844 (4.2 % of total) genes were differentially-expressed. Fewer genes were differentially-expressed by the RV+S treatment, 441 (2.2 % of total) genes, compared with the C cells.

Within the differentially-expressed genes, some were commonly differentially regulated as result of H<sub>2</sub>O<sub>2</sub> treatment and pre-incubation with blackberry metabolites (78 and 19 genes for BB and RV, respectively; Fig. 1). When cells were pre-incubated with metabolites followed by oxidative stress, some genes that were differentially-expressed by the

stress itself (S) remained changed (42 % and 29 % of the genes altered by S remain altered in BB+S and RV+S, respectively).

Table 1- Comparison of numbers of genes differentially-expressed against controls. Legend: C- control; S- oxidative stress (300  $\mu\text{M}$   $\text{H}_2\text{O}_2$ , 3h); BB- incubation with commercial blackberry (0.5  $\mu\text{g}$  GAE  $\text{mL}^{-1}$ , 24 h); BB+S- pre-incubation with commercial blackberry followed by stress imposition; RV- incubation with *R. vagabundus* (0.5  $\mu\text{g}$  GAE  $\text{mL}^{-1}$ , 24 h); RV+S- pre-incubation with *R. vagabundus* followed by stress imposition.

	Up-regulated	Down-regulated	Total
S	495	472	967
BB	316	208	524
BB + S	487	357	844
RV	48	37	85
RV + S	258	183	441

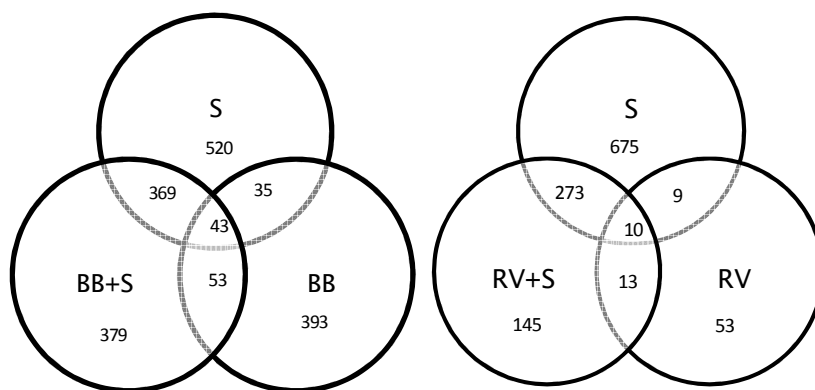


Fig. 1- Venn diagram illustrating differential gene expression. Numbers correspond to differentially-expressed genes in comparison with the control. S- oxidative stress (300  $\mu\text{M}$   $\text{H}_2\text{O}_2$ , 3h); BB- incubation with commercial blackberry (0.5  $\mu\text{g}$  GAE  $\text{mL}^{-1}$ , 24 h); BB+S- pre-incubation with commercial blackberry pre-incubation followed by stress imposition; RV- incubation with *R. vagabundus* incubation (0.5  $\mu\text{g}$  GAE  $\text{mL}^{-1}$ , 24 h); RV+S- pre-incubation with *R. vagabundus* followed by stress imposition.

### 3.2. Gene enrichment

Using term enrichment algorithms it was possible to associate biological relevance and consequences to some sample comparisons (Table 2). After S treatment, differentially-expressed genes were mostly grouped by terms

associated to “endosome- and vesicle-mediated transport”. BB treatment promoted the alteration in transcription of genes mainly associated with terms related to “response to stimulus”, “carbohydrate metabolic process” and “regulation of cell migration” pathways. When pre-incubation was followed by H<sub>2</sub>O<sub>2</sub> treatment (BB+S), the differentially-expressed genes were mostly associated with changes in components of the “Ras GTPases” and “ATP-grasp” (enzymes, catalyzing ATP-dependent ligation of a carboxylate containing molecule to an amino or thiol group-containing molecule) superfamilies. In case of RV and RV+S, the differentially-expressed genes could not be grouped in any significant term, and presented widespread locations, functions and properties.

Using gene enrichment algorithms, it was possible to associate alterations with pathways that showed genes differentially expressed as result of treatments applied (Table 3). For each comparison of treatments, the top ten pathways were determined using MetaCore™<sup>13</sup>. The ranked pathways are those that displayed higher number of differentially-expressed genes in response to the treatments. Differentially-expressed genes in these pathways and their respective fold changes are listed in supplementary material and are grouped by pathways (Table S1). Among the pathways highlighted, were those related to basic/general cell functions, such as nucleotide metabolism (“ATP/ITP”, “CTP/UTP” and “GTP-XTP” metabolism). Their importance is supported by their presence in the top 10 of almost all treatments tested. Other representative pathways were related with transport, cell cycle, and DNA repair, and 14 pathways were identified amongst all treatments that play important roles on these events.

S treatment mainly induced changes in genes associated with nucleotide metabolism, cell cycle and DNA repair functions.

Table 2- Functional categories significantly over-represented in differentially expressed genes. Enrichment term analysis was performed using Functional Annotation Clustering on DAVID. Only clusters presenting an enrichment score higher than 1.3 (equivalent to  $p < 0.05$ ) were considered. All comparisons were performed against the control. Legend: C- control; S-stress imposition (300  $\mu\text{M}$   $\text{H}_2\text{O}_2$ , 3h); BB- commercial blackberry incubation (0.5  $\mu\text{g}$  GAE  $\text{mL}^{-1}$ , 24 h); BB+S- commercial blackberry pre-incubation followed by stress imposition; RV- *R. vagabundus* incubation (0.5  $\mu\text{g}$  GAE  $\text{mL}^{-1}$ , 24 h); RV+S- *R. vagabundus* pre-incubation followed by stress imposition.

	S		BB		BB+S		RV		RV+S	
	Term	Enrichment score	Term	Enrichment score	Term	Enrichment score	Term	Enrichment score	Term	Enrichment score
1	Endosome	1.671	Response to stimulus	1.737	Ras GTPases	1.766	-	-	-	-
2	Vesicle-mediated transport	1.403	Carbohydrate metabolic process	1.515	ATP-grasp	1.340				
3			Regulation of cell migration	1.329						

Table 3- Pathways identified by gene enrichment in top 10 of each comparison, sorted using the criterion 'Statistically significant Maps'. Genes differentially expressed genes were analyzed using a manually curated proprietary database (MetaCore™, GeneGo, by Thomson Reuters, St. Joseph, MI).

	Pathway
Nucleotide metabolism	ATP/ITP metabolism CTP/UTP metabolism GTP-XTP metabolism
Transport	Clathrin-coated vesicle cycle Aldosterone-mediated regulation of ENaC sodium transport CFTR folding and maturation (norm and CF) Normal wtCFTR traffic/ER-to-Golgi Delta508-CFTR traffic/ER-to-Golgi in CF Ran regulation pathway
Cell cycle	Role of Nek in cell cycle regulation Transition and termination of DNA replication Chromosome condensation in prometaphase Spindle assembly and chromosome separation
DNA repair	Role of Brca1 and Brca2 in DNA repair NHEJ mechanisms of DSBs repair Mismatch repair Nucleotide excision repair
Translation	Regulation of eIF2 activity
Amino acid biosynthetic process	Glycine, serine, cysteine and threonine metabolism
Peptide hormone processing	POMC processing

ENaC, epithelium sodium channel; CFTR, cystic fibrosis transmembrane conductance regulator; ER, endoplasmic reticulum; Nek, NimA related kinase; Brca, breast and ovarian cancer susceptibility protein; NHEJ, non-homologous end joining; DSBs, double strain breaks; eIF2, eukaryotic translation initiation factor 2.

However, when cells were incubated with berry metabolites, the enrichment pattern was altered. The group composed of “ATP/ITP”, “CTP/UTP” and “GTP-XTP” metabolism was affected in all conditions.

Pathways related to transport function were more prevalent amongst those affected when cells were (pre-)incubated with berry extracts, especially in *R. vagabundus* treatments (RV and RV+S). Pathways related with the cell cycle were less impacted upon by pre-incubation with berry ingested metabolites, most clearly in *R. vagabundus* treatments. The remaining highlighted pathways were related with DNA repair, translation, amino acid biosynthetic process and peptide hormone processing. Those pathways appear to be affected by blackberry incubation per se irrespective of the source species (BB and RV).

For the more prevalently representative pathways, genes that showed differential expression following pre-incubation with blackberry

metabolites reversed the changes induced by S treatment were considered for detailed analysis (Figs. 2 to 4). For pathways related to transport function, the “clathrin-coated vesicle cycle” was affected by S treatment and some of the genes implicated in this pathway were differentially-expressed in response to blackberry pre-incubation (Fig. 2). These genes included those encoding clathrin and other genes that encode Rab proteins or proteins that regulate their activity, such as Rab-5A, Rab-7, RABGDIA, RAB9P40, Rab-8 and Rab-11A. For Rab-11A expression, pre-incubation with *R. vagabundus*, and for Rab-8 expression pre-incubations with *R. vagabundus* and commercial blackberry maintained transcript levels at the same level as control conditions. In addition, the pathways associated with “Cystic Fibrosis Transmembrane conductance Regulator (CFTR) folding and maturation” and “CFTR traffic from ER to Golgi” were affected by S treatment as well as by blackberry metabolite treatments (Fig. 2). Moreover, there were differences between the responses to BB+S and RV+S treatments. In response to S treatment, levels of important genes that regulate CFTR function were enhanced but this enhancement was inhibited or repressed by BB+S treatment for oligosaccharyltransferase (OST) complex, HSP70 and HSP90beta genes, which are related with folding and maturation of CFTR. Treatment with RV+S modulated the levels of diverse transcripts, related with “CFTR folding and maturation” and also “CFTR traffic” (including OST complex, HSP40, HSP70, HSP90beta, ATPase homolog 1 (Aha1), coat protein complex-II (COPII), syntaxin 6 and coatomer). “Aldosterone-mediated regulation of epithelium sodium channel (ENaC)”, a pathway related and regulated by CFTR pathway<sup>14</sup>, was also affected by the treatments (Tables 3 and S1 on Supplementary material). Some genes of the “aldosterone-mediated regulation of ENaC” pathway were significantly differentially-expressed by S treatment and by blackberry (pre-)incubation (results not shown). The neuronal precursor cell expressed developmentally downregulated 4 (NEDD4) gene, an important player in “ENaC regulatory” pathway had its transcript levels altered by S treatment but they were

restored to C levels in response to pre-incubation with *R. vagabundus* (RV+S) (Table S1 on Supplementary material). The “Ras-related nuclear protein (RAN) regulation” pathway is also related with transport function. However, due to its crucial role in cell cycle and DNA replication<sup>15</sup>, results concerning the changes in their transcript levels are presented associated to cell cycle (Fig. 3).

Concerning the cell cycle, four pathways appear as differentially-regulated at transcriptomic level by blackberry metabolites (Fig. 3): “transition and termination of DNA replication”, “chromosome condensation in prometaphase”, “spindle assembly and chromosome separation” and “Ran regulation”. The first three have a role in specific phases of the cell cycle, while the Ran regulation pathway is important throughout the entire cycle (Fig. 3). In the “transition and termination of DNA replication” pathway, S treatment changed levels of DNA ligase I, DNA polymerase alpha/primase, DNA polymerase delta, DNA polymerase epsilon, topoisomerase I and II (TOPI and TOPII) and ribonuclease H1 transcripts. BB+S treatment only reverted transcript levels of ribonuclease H1, to levels below C. Conversely, RV+S treatment diminished levels of DNA ligase I, DNA polymerase alpha/primase, DNA polymerase delta, DNA polymerase epsilon, TOP2 and ribonuclease H1 transcripts in relation to stress, although in the last case to levels lower than C. In the “chromosome condensation in prometaphase” pathway, the levels of condensin transcripts were enhanced by S treatment and similar levels were noted in response to BB+S. However, RV+S treatment was accompanied by a smaller increase in transcription but not to the level exhibited in the control. This pattern was also followed for the “spindle assembly and chromosome separation” pathway (anaphase-promoting complex/cell division cycle 20 homolog (APC/CDC20 complex)). In the “Ran regulation” pathway, importin (karyopherin)-alpha transcripts were also up-regulated by S treatment but these levels were not reversed by pre-incubation with either blackberry.

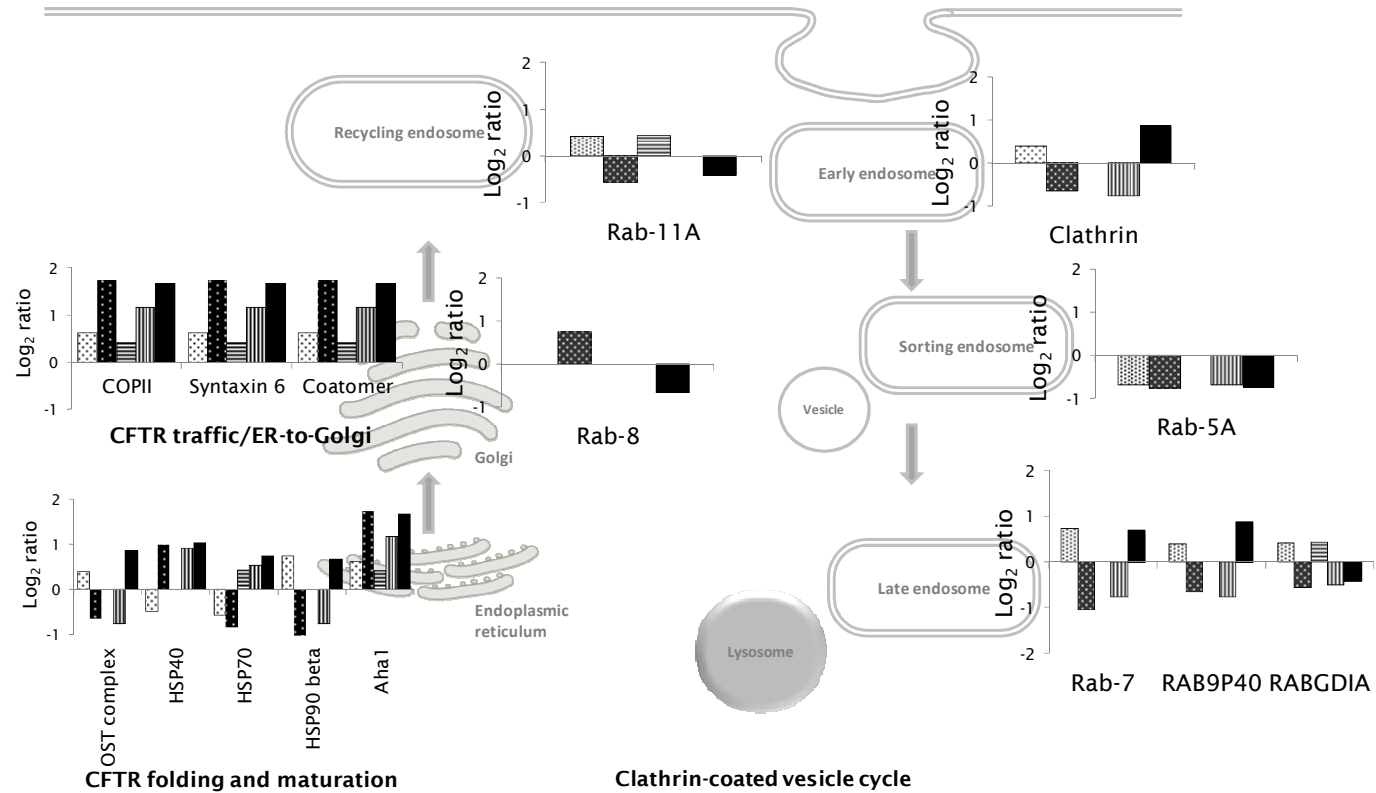


Fig. 2- Transcript levels of some important genes that regulate CFTR folding and maturation, CFTR traffic/ER-to-Golgi and clathrin-coated vesicle cycle, pathways related with transport process. Transcript levels correspond to fold change in comparison to control cells. Legend: BB; BB+S; RV; RV+S; S.

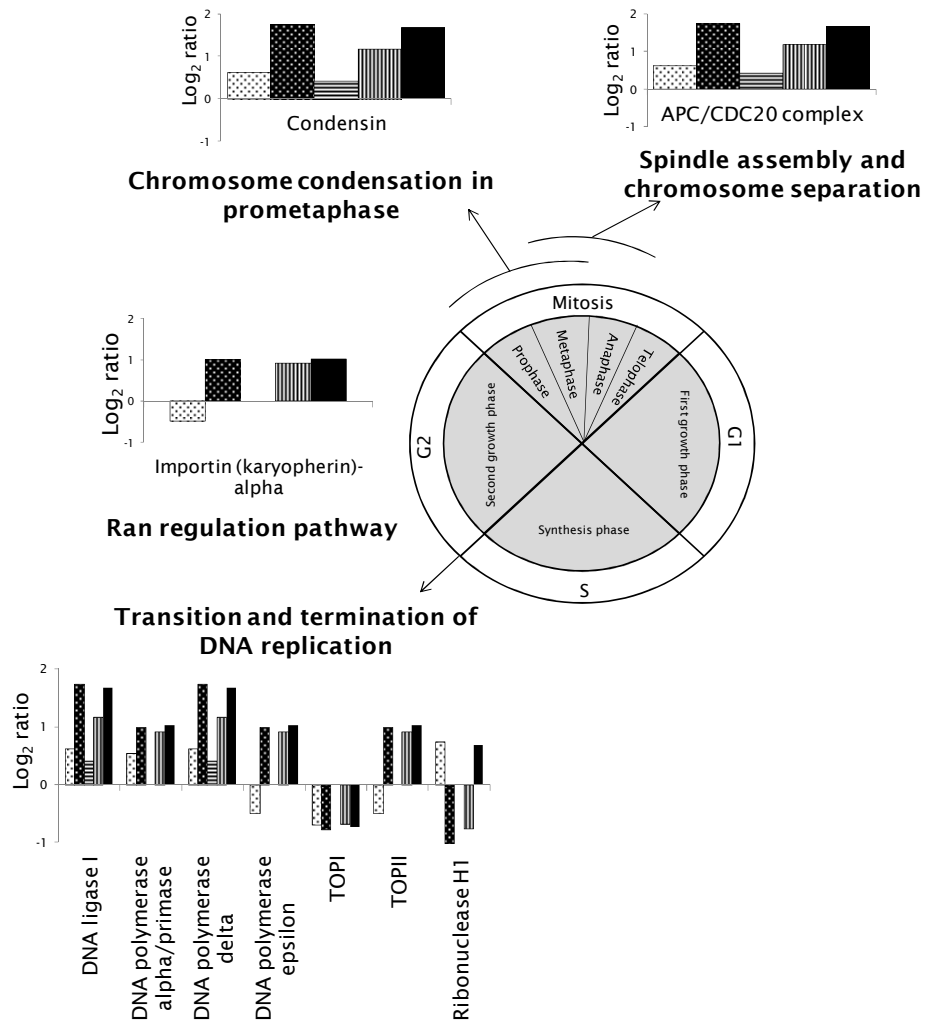


Fig. 3- Transcript levels of some important genes that regulate Ran regulation, transition and termination of DNA replication, chromosome condensation in prometaphase and spindle assembly and chromosome separation pathways, related with cell cycle. Transcript levels correspond to fold change in comparison to control cells. Legend: BB; BB+S; RV; RV+S; S.

Concerning DNA repair, diverse pathways were differentially-regulated at the transcriptomic level, these included: the “role of breast and ovarian cancer susceptibility protein 1 and 2 (Brca1 and Brca2) in DNA repair”, “non-homologous end joining (NHEJ) mechanism of double strain breaks (DSBs) repair”, “mismatch repair (MMR)” and “nucleotide excision repair

(NER)" (Fig. 4). In the "Brca1/Brca2 role in DNA repair" pathway, the transcript levels of histone H2AX were enhanced by S treatment (Fig. 4) and these levels were decreased by RV+S but not to C levels. The transcript levels of Brca1, a protein indirectly controlled by histone H2AX, were not changed by stress conditions, in the presence or absence of blackberry metabolites. In the MMR pathway, four genes were highlighted as differentially expressed: MutSalpha complex, MutLalpha complex, MutLbeta complex and proliferating cell nuclear antigen (PCNA). The MutSalpha complex, which recognizes single base mismatches and small insertion/deletion in DNA, showed enhanced levels of transcripts in response to S treatment, whereas RV+S treatment reduced expression, without reaching C values. MutLalpha and MutLbeta complex also presented different expression patterns in response to S treatment. BB+S treatment reversed the effects of S treatment, whereas RV+S only affected MutLbeta transcript levels. The transcript levels of PCNA, a processivity factor, was also enhanced under S treatment. However, pre-incubation with commercial blackberry metabolites, decreased PCNA transcript levels to below C values and pre-incubation with *R. vagabundus* returned PCNA transcript levels to C values. This gene is also involved in the "NER" pathway, where various other genes were differentially-expressed from the "lesion type recognition to the repair process", these included DNA-directed RNA polymerase II subunit A (POLR2A), RNA polymerase II, Xeroderma pigmentosum complementation group G (XPG), DNA polymerase delta, DNA polymerase epsilon, transcription factor II H (TFIIH) and DNA ligase I. The transcript levels of POLR2A and RNA polymerase II, which are involved in recognition steps, were enhanced by S but were significantly reduced in response to pre-incubation with *R. vagabundus* (RV+S), although they remained higher than C. The same pattern was noted for DNA polymerase delta, TFIIH and DNA ligase I. For XPG, transcript levels in response to both BB+S and RV+S treatments decreased to values below C, reversing the effect of S treatment.

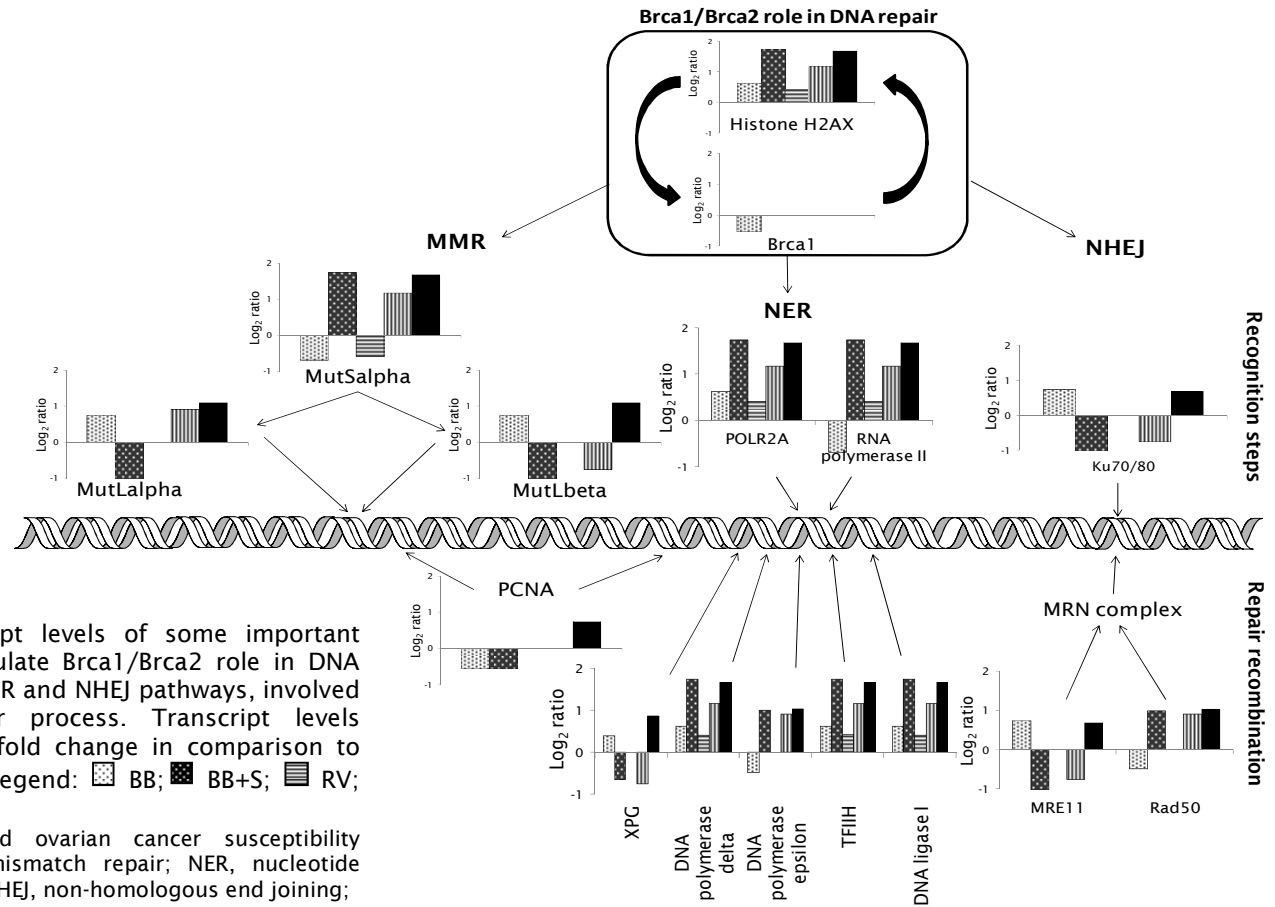


Fig. 4- Transcript levels of some important genes that regulate Brca1/Brca2 role in DNA repair, MMR, NER and NHEJ pathways, involved in DNA repair process. Transcript levels correspond to fold change in comparison to control cells. Legend: BB; BB+S; RV; RV+S; S. Brca, breast and ovarian cancer susceptibility protein; MMR, mismatch repair; NER, nucleotide excision repair; NHEJ, non-homologous end joining;

In the “NHEJ mechanism of DSBs repair” pathway, among diverse genes differentially expressed, three genes, Ku70/80, MRE11 and Rad50 were highlighted (Fig. 4). The first two genes showed increased levels of transcripts in response to S treatment but BB+S and RV+S treatments diminished these levels to values below C. MRE11 together with nibrin and Rad50 constitute the MRN complex. However, Rad50 did not show the same expression pattern as MRE11. Although S treatment enhanced Rad50 transcript levels, BB+S and RV+S treatments did not alter these transcript levels. Nibrin expression was not affected by any treatment.

In the pathway “regulation of eukaryotic translation initiation factor 2 (eIF2) activity”, various genes presented altered expression levels in response to S treatment and some of these alterations were reversed by pre-incubation with berry metabolites (Table S1 on Supplementary material). However, once again, the effects of blackberry pre-incubation were different between the commercial and wild blackberries, with *R. vagabundus* more efficacious.

### 3.3. Examination of biochemical pathways previously implicated in neuroprotection

Two important biochemical targets previously suggested to be regulated by pre-incubation of neuroblastoma cells with blackberry metabolites were caspases and glutathione-related pathways, namely the apoptosis and glutathione metabolism pathways<sup>6</sup>. Indeed, in the “Bad phosphorylation” pathway, which is related with cell apoptosis and survival, various genes were differentially regulated, mainly those involved in the direct phosphorylation/dephosphorylation of Bad, such as protein phosphatase catalytic subunit (PP1-cat) alpha, protein phosphatase 2A catalytic subunit (PP2A), protein phosphatase 2B (calcineurin A), protein phosphatase 2C (PP2C) and cAMP-dependent protein kinase (PKA) (Table S1 in Supplementary material). Also proteins presenting a broader occurrence, such as ERK and MEKs, are differentially expressed and may participate in Bad phosphorylation.

Concerning glutathione metabolism, two genes were highlighted as differentially-expressed (phospholipid hydroperoxide glutathione metabolizing peroxidase (GPx4) and glutathione peroxidase (GPx7); Table S1 in Supplementary material). Both genes encode glutathione peroxidases with different specificities and metabolites from both blackberries contradicted the alterations promoted by the stress treatment.

#### 4. Discussion

Neurological benefits have been associated with the consumption of berry fruits with the implication being that of reducing the risk of incidence of age-related neurodegeneration. Our previous work has shown that small quantitative chemical differences could mediate different cell responses concerning neuroprotection by blackberry metabolites <sup>6</sup>. Consequently, it is important to understand which molecular pathways that underpin the differential protection if these fruit are to be successfully exploited for human health benefits. Therefore, the gene expression alterations mediated by the metabolites of a commercial variety and a wild species of blackberry were evaluated. The commercial variety was reported as able to protect cell viability but not mitochondria functionality in model systems, while *R. vagabundus*, the wild blackberry, was able to protect both viability and mitochondria functionality as well as activating adaptive cellular stress response pathways such as caspase activation and GSH modulation <sup>6</sup>. Transcript levels of neuroblastoma cells subjected to different conditions were determined. Treatment of SK-N-MC neuroblastoma cells with H<sub>2</sub>O<sub>2</sub> 300 μM for 24 h was already reported to promote cell death <sup>6</sup>. In the present work, H<sub>2</sub>O<sub>2</sub> treatment was conducted for a shorter period to allow the evaluation of early events at transcriptomic level. To elucidate the possible role of blackberry digested metabolites on cells, neuroblastoma cells were also incubated with digested metabolites of commercial and wild blackberries for 24 h, followed or not by H<sub>2</sub>O<sub>2</sub> treatment and their transcript levels evaluated.

Oxidative stress (S treatment) induced the differential-expression of 967 genes (Table 1 and Fig. 1). However, when cells were pre-incubated with blackberry metabolites prior to H<sub>2</sub>O<sub>2</sub> treatment, the number of genes changed was reduced, especially for *R. vagabundus* treatment (to around half this number). *R. vagabundus* metabolites were more efficacious to contradict the effect of oxidative stress, since only 29 % of genes altered by S treatment were changed in response to RV+S and 42 % of genes were changed by BB+S. These results suggest that the reduction in total number of altered genes due to blackberries pre-incubation (BB+S and RV+ S) in comparison with stress alone could be associated to their previously described neuroprotective ability<sup>6</sup>. In addition, after incubation with blackberry metabolite alone, the number of differentially-expressed genes was considerably higher for commercial blackberry than for *R. vagabundus* (524 and 85, respectively). It appears that the commercial blackberry extract induced more changes in gene expression than *R. vagabundus*, however, without mediating such efficacious protection. Since RV treatment was considerably more effective in this neuroprotection model<sup>5</sup>, it is tempting to assume that the lower number of genes affected by RV treatment are those most crucial to the process. These considerations are reflected in the absence of terms associated to RV and RV+S in gene enrichment analysis (Table 2). Terms associated with stress treatment alone highlighted those terms associated with transport mechanisms (endosome- and vesicle-mediated transport). In fact, during neurodegeneration, the fragmentation of neuronal GA and disruption of cellular/axonal transport are important features<sup>16-19</sup>. Indeed, GA fragmentation is thought that may contribute to the initiation of apoptotic pathway<sup>20,21</sup>. Pre-treatment with blackberry metabolites, commercial blackberry was the only one whose treatments (BB and BB+S) were associated with enrichment terms. These results show the difference in impact that the two blackberries have in cells at transcriptomic level. Although *R. vagabundus* also induce alteration in gene expression of SK-N-MC neuroblastoma cells, those changes were affecting different

targets, with different functions and locations, reason why it was not associated with any term with statistical significance.

To assess which specific pathways were significantly impacted upon with respect to transcriptional changes due to digested metabolites of the two blackberries, an approach based on the use of enrichment analysis of canonical pathways was followed. With respect to gene enrichment terms analysis, “transport” was a term highly associated to the transcriptomic alterations induced by S treatment. This importance was corroborated using Metacore™ analysis that applies a different algorithm of enrichment analysis. Some genes that encode proteins from “clathrin-coated vesicle cycle”, “CFTR folding and maturation” and “CFTR traffic” pathways had their levels of expression altered due to S treatment (Fig. 2). However, pre-incubation with *R. vagabundus* digested metabolites made the expression levels of Rab-8, Rab-11A return to C levels and decrease the levels of COPII, syntaxin 6, coatomer, Aha1, HSP70 and HSP40 in comparison with stress. CFTR are ATP-binding cassette (ABC) transporters and are important in regulation of membrane vesicles trafficking/fusion, acidification of organelles, normal anion transport such as chloride and neuropeptide transport<sup>22</sup>. They are widespread in the human brain and involved in the transport of GSH, which could act in brain as neurotransmitter and neuromodulator. Furthermore CFTR also controls ENaC activity<sup>14,22,23</sup>. The CNS could be affected by CFTR (dis)function, for instance, by the maintenance of the steady state of cellular electrolytes<sup>22</sup>. Other phenolic compounds, as curcumin, have already been identified as interfering in CFTR genes<sup>24</sup>. ENaC transporters are non-voltage-gated channels associated with sodium and water transport<sup>25</sup> and have been identified in sensory neurons as putative mechanosensors wherein they may participate in neuronal regeneration after injury or degenerative diseases<sup>25</sup>. Their activity is dependent on an increase in CFTR activity<sup>14</sup>. Therefore, it is not surprising that the “aldosterone-mediated regulation of ENaC activity” pathway was highlighted as differentially-expressed.

Another important pathway differentially regulated was the “Ran regulation” pathway. Ran is a protein that plays a central role in the nucleocytoplasmic transport and organizes the microtubules during the M phase of cell cycle. Consequently, this pathway is important in transport function as well as in the cell cycle, being essential through phases G1, S, G2 and M<sup>15</sup>.

Other pathways that play a role in the cell cycle were differentially-expressed (Fig. 3) *e.g.* the “transition and termination of DNA replication”, the “chromosome condensation in prometaphase” and “spindle assembly and chromosome separation” pathways all play a role in specific phases of the cell cycle. The expression of genes associated with these pathways was altered in response to S treatment and treatment with blackberry metabolites tended to reverse this alteration, bringing gene expression level closer to the control values. As in the previous pathways, the changes in transcript levels mediated by commercial blackberry was different from those mediated by wild blackberry, and in most cases, the latter was more effective in minimizing the alterations promoted by stress. Overall, these results point to the cell cycle as an important target of oxidative stress and that blackberry metabolites tend to ameliorate or reduce the impact of that stress. However, further biochemical studies are required to better understand the specific effects of each treatment on cell cycle, such as the cell cycle analysis by flow cytometry.

Beyond their participation in “transition and termination of DNA replication” pathway, DNA polymerase delta and DNA polymerase epsilon, as well as DNA ligase, are also shared by DNA repair mechanisms. Among the DNA repair pathways, genes associated with “NER”, “MMR”, “NHEJ” and “Brca1/Brca2 roles” were modulated following the stress treatment (Fig. 4). The “Brca1” pathway assumes a central role in DNA repair, facilitating the assessment of DNA repair machinery. Brca1 activation by phosphorylation is mediated indirectly by participation of histone H2AX in the activation of the cell cycle checkpoint kinase 2 (Chk2). On the other hand, Brca1 directly regulates histone H2AX activity by ubiquitination. Histone H2AX

transcript levels were affected by stress treatment and RV+S treatment attenuated these stress-induced transcript alterations. However, Brca1 transcripts were not regulated according to this pattern. It is possible that regulation of Brca1 could be mediated by Histone H2AX but not at transcriptomic level and thus not reflected in the transcriptomic data pattern seen here. This is an example of one case where activity/levels must be verified by complementary methodologies, to better understand the general regulation of pathways. Among the DNA repair systems, genes differentially regulated in recognition steps and in repair steps were identified. Concerning “NER”, which combines subpathways of global genomic NER and transcription-coupled NER, the latter seemed to be more affected by treatments. In terminally differentiated neurons and neuroblastoma cells, where synthesis of new DNA molecules is not occurring, global genomic NER is described as poorly active<sup>26,27</sup>. However, the transcription process is active and transcription-coupled NER ensures that errors in DNA during transcription are repaired to maintain the quality of genetic information for future transcription. Oxidative stress is well known to promote DNA damage, therefore transcription of genes involved in DNA repair mechanisms are activated. Results suggest that pre-incubation with digested metabolites could protect DNA damage, directly or indirectly, reducing the requirement of further increase in transcription.

Another point of regulation at transcriptional level is the regulation of eIF2 activity pathway. This pathway regulates mRNA translation, transferring Met-tRNA to the 40S ribosomal subunit, which also involves GTP/GDP conversion<sup>28</sup>. The initiator factor eIF2 controls gene expression under conditions of stress, such as viral infection and apoptosis<sup>28</sup>. The regulation of this pathway also appears associated to protein unfolded diseases, an important hallmark in most NDDs. Protein unfolding causes endoplasmic reticulum stress, activating the apoptosis pathway and the control of eIF2 attenuates the unfolded protein synthesis<sup>29</sup>. The fact that blackberry metabolites regulated the activation at transcriptional level of

the eIF2 pathway is an important clue unraveling a potential protective mechanism.

Previous work has also shown that the differences on cell protection mediated by commercial and wild blackberry species was accompanied by caspases-3 and -7 activation and a differential modulation of GSH levels <sup>6</sup>. Therefore, caspases and glutathione related pathways, namely apoptosis and glutathione metabolism pathways were relevant to be analyzed in the present work. In the Bad phosphorylation pathway, genes involved in the direct phosphorylation/dephosphorylation of Bad were differentially affected by S treatment and by blackberry metabolites. However, some genes linked to signaling functions (MEK, ERK, etc) are ubiquitous thereby making it difficult to understand the specific events/alteration occurring due to the treatments imposed. Concerning glutathione metabolism, two genes that encode glutathione peroxidases were differentially expressed (GPX4 and GPX7). Both enzymes mediate the conversion of GSH (reduced form) to GSSG (oxidized form) but use different electron acceptors. While GPX7 exhibits a strong capacity to neutralize H<sub>2</sub>O<sub>2</sub> independent of glutathione, GPX4 can use a wide range of reducing substrates in addition to glutathione and act directly to reduce lipid peroxides and other membrane-bound complex hydroperoxides in addition to H<sub>2</sub>O<sub>2</sub> <sup>30,31</sup>. Lipid hydroperoxides have important metabolic roles, since they can (i) activate of lipoxygenase and cyclooxygenase, (ii) act as signal molecules, in apoptotic cell death and receptor-mediated signal transduction, and (iv) participate in inflammation <sup>30</sup>. Therefore, GPX4 plays a key role in mammalian cells. The up-regulation of GPX4 transcripts by the stress here indicates its need to deal with the damage stimulus promoted by oxidative condition. The fact that blackberry metabolites contradicted that up-regulation, returning transcription to levels of C (in the case of RV) or even below it (in the case of BB), indicates an interesting shift in oxidative metabolism.

Among the highlighted pathways, seven exhibited a common regulation by small GTP-binding proteins (Fig. 5). Small GTP-binding proteins are

monomeric G proteins with molecular masses of 20 to 40 kDa that have both GDP/GTP-binding and GTPase activities. More than 100 members of this superfamily have been identified <sup>32</sup> and they are classified in five families: Ras, mainly regulators of gene expression; Rho regulators of cytoskeletal reorganization and gene expression; Rab regulators of intracellular vesicle trafficking; Sar1/Arf also regulators of intracellular vesicle trafficking; Ran regulators of nucleocytoplasmic transport during G1, S and G2 phases of the cell cycle and microtubule organization during the M phase <sup>15</sup>. The families more represented in the presented work as differentially-expressed were Ras, Rab, Sar1/Arf and Ran (Fig. 5). Functions associated to these proteins were previously identified in term enrichment analysis (BB and BB+S; Table 2) as “regulation of cell migration”, “Ras GTPases” and “response to stimulus”. In mammalian cells, Ras proteins directly bind to and activate Raf protein kinase, which then induces gene expression through the MAPK cascade in response to various extracellular signaling stimuli <sup>33</sup>. They regulate cell proliferation, differentiation, morphology and apoptosis. The plasma membrane localization is essential for their functions and their association to ER and GA assumes an important role in this function. In this work, three pathways were highlighted as regulated by Ras family proteins: the “aldosterone-mediated regulation of ENaC sodium transport”, the “regulation of eIF2 activity” and the “Bad phosphorylation” pathways. Rho proteins are involved in diverse cellular events, such as cell growth, membrane trafficking, development, axon guidance and extension. Rho family proteins regulate actin polymerization, required for reorganization of actin cytoskeleton and by this way, regulating cell shape changes, cell motility, cell adhesion and cytokinesis <sup>34</sup>. However, among the highlighted pathways none were directly regulated by Rho proteins but their regulation is not negligible considering a significant term outline in Table 2 (Regulation of cell migration). Rab proteins have been identified as regulating intracellular vesicle trafficking from ER to GA and from this to the plasma membrane. Exocytosis, endocytosis and recycling are

performed by intracellular vesicle trafficking. Sar1/Arf proteins are also associated to vesicles formation and their trafficking. Vesicle budding requires the assembly of specific proteins coating the cytoplasmic face of a donor membrane to provide the mechanical force to pull the membrane into a bud and to capture specific receptors and their bound cargo molecules<sup>35</sup>. Coated vesicles could be classified as clathrin-, COPI- and COPII-coated vesicles. Sar1 and Arf proteins play crucial roles in membrane recruitment of COPI and COPII components. In the present work, two pathways highlighted in top 10 (Table 3) are regulated by proteins belonging to Rab and Sar1/Arf families (Fig. 5). In the “clathrin-coated vesicle cycle”, Rab proteins play an important role, with some transcripts that encode Rab proteins or proteins that regulate them being differentially expressed (Fig. 3). In “normal wtCFTR traffic/ER-to-Golgi” pathway both families, Rab and Sar1/Arf, are involved (Fig. 2). Ran proteins play a central role in nucleocytoplasmic transport, chromosome localization as well as in microtubule organization in M phase of cell cycle. Therefore, these proteins are vital in the cell cycle ensuring the transport from nucleus to cytoplasm and in the opposite direction, through the nuclear pore complexes. In this work, the Ran regulation pathway and the “spindle assembly and chromosome separation” pathway are both differentially regulated (Figs. 3 and 5).

In general, small GTP-binding proteins regulate a wide and diverse range of cellular functions such as transport, cell cycle, translation and cell death/survival<sup>15</sup>. Those proteins are affected by S treatment as well as by blackberry metabolites pre-incubation. Therefore these pathways associated to small GTP-binding proteins are interesting targets for further investigation as is the involvement of these proteins as possible common upstream regulators modulated by blackberry metabolites. Nevertheless, additional biochemical experiments must be conducted to connect this regulation with the neuroprotection afforded by blackberry metabolites. Different regulation at transcriptomic level was mediated by digested metabolites from the commercial and wild blackberry species,

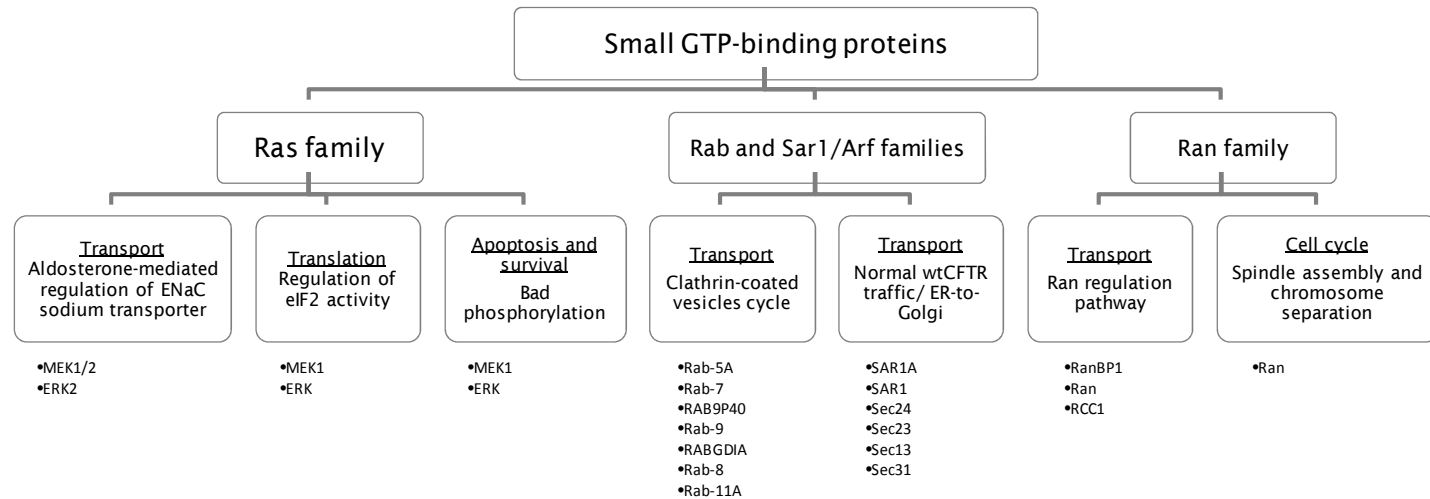
which only had differences in the relative amounts of phytochemicals. However, to provide a clear distinction between mechanisms mediated by commercial and wild blackberries, further biochemical assays targeting molecules on pathways highlighted by microarrays analysis are required.

## 5. Conclusion

Overall, blackberry metabolites were able to prevent some of the gene expression alterations caused by oxidative stress. *R. vagabundus* was more efficient, since its pre-incubation promotes a higher reduction in the number of genes altered by stress in comparison with commercial blackberry. Transcripts changed in response to *R. vagabundus* pre-incubation were spread among different cell locations and mechanisms and produced a different effect from commercial blackberry. Thus, different regulation at transcriptomic level seems to be mediated by metabolites from the commercial and wild blackberry species.

Potential pathways differentially affected by stress and blackberry metabolites were identified, they were mainly related with functions of transport, cell cycle and DNA damaging. Also pathways related with apoptosis and GSH metabolism displayed some transcripts differentially expressed. The possibility that small GTP-binding proteins play a role on diverse pathways differentially regulated between blackberries and stress suggests a possible common regulation by blackberry metabolites. However, supplementary biochemical studies are necessary to understand possibly additional post-translational regulation and validate the differential protective role of blackberry metabolites on those pathways.

Fig. 5- Pathways highlighted as differentially regulated in the present work and regulation by the respective small GTP-binding protein families. Small GTP-binding proteins as well as proteins directly involved in their regulation (up- and down-stream are indicated), such as guanine nucleotide exchange factors and GTPase activating proteins. Transcript fold changes of proteins indicated can be found in Table S1 in supplementary material.



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## 8. Supplementary material

Table S1- List of gene products codified by genes differential expressed, belonging to pathways of top 10 (Table 3), Bad phosphorylation and glutathione metabolism pathways. Legend: C- control; S- stress imposition (300  $\mu\text{M}$   $\text{H}_2\text{O}_2$ , 3h); BB- incubation with commercial blackberry (0.5  $\mu\text{g}$  GAE  $\text{mL}^{-1}$ , 24 h); BB+S- pre-incubation with commercial blackberry followed by stress imposition; RV- incubation with *R. vagabundus* (0.5  $\mu\text{g}$  GAE  $\text{mL}^{-1}$ , 24 h); RV+S- pre-incubation with *R. vagabundus* followed by stress imposition.

Gene product	Type	Input IDs	BB+S		BB		RV		RV+S		S	
			Log <sub>2</sub> ratio	p-value	Log <sub>2</sub> ratio	p-value	Log <sub>2</sub> ratio	p-value	Log <sub>2</sub> ratio	p-value	Log <sub>2</sub> ratio	p-value
<b>ATP/ITP metabolism</b>												
KAD6	Generic kinase	9212_at; 9263_at; 9232_at; 2824_at; 2825_at; 9235_at; 2760_at; 9236_at; 9213_at	-1.04	0.00002	0.74	0.00478			-0.76	0.00028	0.68	0.00028
Ribonucleotide reductase	Generic enzyme	10849_at; 11041_at; 10788_at; 3052_at; 4015_at; 4026_at; 5476_at; 2274_at; 5438_at; 4048_at; 10847_at; 5458_at; 3081_at; 3006_at	-0.84	0.00376	0.48	0.00142	0.43	0.00653	-0.36	0.00695	-0.51	0.00146
Exosome multienzyme ribonuclease complex	Generic enzyme	4288_at; 2633_at; 2107_at; 2138_at; 2354_at; 2353_at; 8767_at; 8744_at; 8739_at; 2730_at; 4300_at; 4299_at; 2137_at; 4684_at; 4677_at; 4627_at; 4616_at; 2192_at; 2178_at; 5876_at; 5868_at; 5837_at; 5802_at; 5792_at; 2355_at; 2317_at; 2316_at; 2314_at; 8798_at; 8731_at; 2744_at; 2737_at; 2736_at; 2729_at; 5795_at; 8745_at; 8706_at; 8078_at; 4690_at; 4685_at; 2146_at; 5831_at; 8754_at; 4640_at	1.74	0.00023	-0.68	0.00443	0.42	0.00862	1.17	0.00824	1.68	0.00005
AMP deaminase 2	Generic enzyme	9212_at; 9232_at; 9263_at; 2760_at; 2825_at; 9235_at; 2824_at; 9236_at; 9213_at	-1.04	0.00002	0.74	0.00478			-0.76	0.00028	0.68	0.00028
AMP deaminase	Generic enzyme	9212_at; 9232_at; 9263_at; 2760_at; 2825_at; 9235_at; 2824_at; 9236_at; 9213_at	-1.04	0.00002	0.74	0.00478			-0.76	0.00028	0.68	0.00028
NDPK complex	Generic enzyme	10440_at; 10460_at; 1521_at; 2963_at; 254958_at; 10432_at; 10456_at; 10457_at	-0.97	0.00007	0.55	0.00562	-0.53	0.00044	-0.55	0.00114	-0.63	0.00040
PNPH	Generic enzyme	9824_at; 9814_at; 2959_at; 9787_at; 9821_at; 9828_at; 9788_at; 9780_at; 2901_at; 2899_at; 2843_at; 2882_at; 2957_at	-0.97	0.00006	0.49	0.00497			-0.77	0.00003	-0.85	0.00001

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ADA	Generic enzyme	8767_at; 8744_at; 8739_at; 2730_at; 2744_at; 2729_at; 2736_at; 2737_at; 8731_at; 8798_at; 8706_at; 8745_at; 8754_at	1.00	0.00013	-0.49	0.00215			0.92	0.00006	1.03	0.00004
APRT	Generic enzyme	9212_at; 9263_at; 9232_at; 2824_at; 2825_at; 9235_at; 2760_at; 9236_at; 9213_at	-1.04	0.00002	0.74	0.00478			-0.76	0.00028	0.68	0.00028
AK2	Generic kinase	2354_at; 2353_at; 2355_at; 2314_at; 2316_at; 2317_at; 5876_at; 5868_at; 5837_at; 5802_at; 5792_at; 5795_at; 5831_at	1.74	0.00023	0.62	0.00158	0.42	0.00862	1.17	0.00824	1.68	0.00005
ADSSL1	Generic enzyme	8767_at; 8744_at; 8739_at; 2730_at; 2744_at; 2729_at; 2736_at; 2737_at; 8731_at; 8798_at; 8706_at; 8745_at; 8754_at	1.00	0.00013	-0.49	0.00215			0.92	0.00006	1.03	0.00004
ADSL	Generic enzyme	9787_at; 9814_at; 9824_at; 2959_at; 9828_at; 9821_at; 9788_at; 9780_at; 2901_at; 2899_at; 2882_at; 2843_at; 2957_at	-0.97	0.00006	0.49	0.00497			-0.77	0.00003	-0.85	0.00001
RNA polymerase II	Generic enzyme	2617_at; 2633_at; 2354_at; 2353_at; 4026_at; 4015_at; 1594_at; 7030_at; 6999_at; 2527_at; 5868_at; 5837_at; 5802_at; 5792_at; 2355_at; 2317_at; 2316_at; 2314_at; 5876_at; 5795_at; 7468_at; 7005_at; 8398_at; 8078_at; 5831_at; 1486_at; 4048_at; 7025_at; 7020_at; 8336_at; 7026_at; 7023_at; 2542_at	1.74	0.00023	-0.68	0.00443	0.42	0.00862	1.17	0.00824	1.68	0.00005
RNA polymerase I	Generic enzyme	2633_at; 2617_at; 8767_at; 8744_at; 8739_at; 2730_at; 2527_at; 7030_at; 6999_at; 8798_at; 8731_at; 2744_at; 2737_at; 2736_at; 2729_at; 8745_at; 7468_at; 8706_at; 8078_at; 7025_at; 7020_at; 7005_at; 7023_at; 7026_at; 8336_at; 2542_at; 8754_at	1.00	0.00013	-0.68	0.00443			0.92	0.00006	1.03	0.00004
CTP/UTP metabolism												
KCY	Generic kinase	10440_at; 10460_at; 2963_at; 10456_at; 10432_at; 10457_at	-0.97	0.00007	0.55	0.00562			-0.55	0.00114	-0.63	0.00040
Exosome multienzyme ribonuclease complex	Generic enzyme	2633_at; 2138_at; 8767_at; 8744_at; 8739_at; 2730_at; 4288_at; 2107_at; 2354_at; 2353_at; 2317_at; 2316_at; 2314_at; 5876_at; 5868_at; 5837_at; 5802_at; 5792_at; 2355_at; 8798_at; 8731_at; 2744_at; 2737_at; 2736_at; 2729_at; 4300_at; 4299_at; 2137_at; 4684_at; 4677_at; 4627_at; 4616_at; 2192_at; 2178_at; 5795_at; 4690_at; 2146_at; 4685_at; 8745_at; 8706_at; 8078_at; 5831_at; 4640_at; 8754_at	1.74	0.00023	-0.68	0.00443	0.42	0.00862	1.17	0.00824	1.68	0.00005



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		GTP-XTP metabolism										
KGUA	Generic kinase	2354_at; 2353_at; 5802_at; 5792_at; 2355_at; 2317_at; 2316_at; 2314_at; 5837_at; 5876_at; 5868_at; 5795_at; 5831_at	1.74	0.00023	0.62	0.00158	0.42	0.00862	1.17	0.00824	1.68	0.00005
Exosome multienzyme ribonuclease complex	Generic enzyme	2354_at; 2138_at; 2353_at; 2633_at; 8767_at; 8744_at; 8739_at; 2730_at; 4288_at; 2107_at; 2317_at; 2316_at; 2314_at; 5876_at; 5868_at; 5837_at; 5802_at; 5792_at; 2355_at; 4684_at; 4677_at; 4627_at; 4616_at; 2192_at; 2178_at; 4300_at; 4299_at; 2137_at; 8798_at; 8731_at; 2744_at; 2737_at; 2736_at; 2729_at; 5795_at; 5831_at; 8745_at; 8706_at; 8078_at; 4690_at; 4685_at; 2146_at; 8754_at; 4640_at	1.74	0.00023	-0.68	0.00443	0.42	0.00862	1.17	0.00824	1.68	0.00005
NDPK complex	Generic enzyme	10460_at; 10440_at; 1521_at; 2963_at; 254958_at; 10432_at; 10456_at; 10457_at	-0.97	0.00007	0.55	0.00562	-0.53	0.00044	-0.55	0.00114	-0.63	0.00040
PNPH	Generic enzyme	9824_at; 9814_at; 9787_at; 2959_at; 2901_at; 2899_at; 9828_at; 9821_at; 9780_at; 9788_at; 2843_at; 2882_at; 2957_at	-0.97	0.00006	0.49	0.00497			-0.77	0.00003	-0.85	0.00001
IMD1	Generic enzyme	2107_at; 2959_at; 4288_at; 9212_at; 9824_at; 9263_at; 9700_at; 9787_at; 9814_at; 9232_at; 2137_at; 2899_at; 2901_at; 4299_at; 4300_at; 9699_at; 9780_at; 9788_at; 9821_at; 9828_at; 2760_at; 9235_at; 2824_at; 2882_at; 2843_at; 2825_at; 9213_at; 9236_at; 2957_at	-1.16	0.00010	0.74	0.00478			-0.77	0.00003	-0.85	0.00001
RNA polymerase II	Generic enzyme	2617_at; 2633_at; 2354_at; 2353_at; 2317_at; 2316_at; 2314_at; 5876_at; 5868_at; 5837_at; 5802_at; 5792_at; 2355_at; 4026_at; 4015_at; 1594_at; 7030_at; 6999_at; 2527_at; 5795_at; 7025_at; 7020_at; 7005_at; 8078_at; 4048_at; 5831_at; 7468_at; 8398_at; 1486_at; 7026_at; 7023_at; 8336_at; 2542_at	1.74	0.00023	-0.68	0.00443	0.42	0.00862	1.17	0.00824	1.68	0.00005
RNA polymerase I	Generic enzyme	2617_at; 2633_at; 8767_at; 8744_at; 8739_at; 2730_at; 7030_at; 6999_at; 2527_at; 8798_at; 8731_at; 2744_at; 2737_at; 2736_at; 2729_at; 8745_at; 7005_at; 8706_at; 7468_at; 8078_at; 7025_at; 7020_at; 8754_at; 2542_at; 8336_at; 7026_at; 7023_at	1.00	0.00013	-0.68	0.00443			0.92	0.00006	1.03	0.00004

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RNA polymerase III	Generic enzyme	2633_at; 2730_at; 8767_at; 8744_at; 8739_at; 2617_at; 2354_at; 2353_at; 10460_at; 10440_at; 2744_at; 2729_at; 5876_at; 5868_at; 5837_at; 5802_at; 5792_at; 2355_at; 2317_at; 2316_at; 2314_at; 7030_at; 6999_at; 2527_at; 8798_at; 8731_at; 2737_at; 2736_at; 2963_at; 5795_at; 7468_at; 8745_at; 8706_at; 5831_at; 10456_at; 10432_at; 8078_at; 7025_at; 7020_at; 7005_at; 2542_at; 8754_at; 10457_at; 7026_at; 7023_at; 8336_at; 8364_at	1.74	0.00023	-0.68	0.00443	0.42	0.00862	1.17	0.00824	1.68	0.00005
Guanine deaminase	Generic enzyme	2354_at; 2353_at; 5802_at; 5792_at; 2355_at; 2317_at; 2316_at; 2314_at; 155054_at; 5837_at; 5876_at; 5868_at; 5795_at; 5831_at	1.74	0.00023	0.62	0.00158	0.42	0.00862	1.17	0.00824	1.68	0.00005
Clathrin-coated vesicle cycle												
Eps15	Generic binding protein	2527_at; 6999_at; 7030_at; 7005_at; 7020_at; 7025_at; 7026_at; 7023_at	-0.57	0.00066	-0.57	0.00334					0.75	0.00111
Clathrin heavy chain	Generic binding protein	2138_at; 4677_at; 4684_at; 4627_at; 4616_at; 2192_at; 2178_at; 2146_at; 4685_at; 4690_at; 4640_at	-0.64	0.00042	0.39	0.00914			-0.75	0.00138	0.87	0.00098
Rab-7	RAS - superfamily	9212_at; 9263_at; 9232_at; 2825_at; 2760_at; 2824_at; 9235_at; 9213_at; 9236_at	-1.04	0.00002	0.74	0.00478			-0.76	0.00028	0.68	0.00028
Syntaxin 5	Transporter	9787_at; 9814_at; 9824_at; 2959_at; 9780_at; 2901_at; 2899_at; 9788_at; 9821_at; 9828_at; 2882_at; 2843_at; 2957_at	-0.97	0.00006	0.49	0.00497			-0.77	0.00003	-0.85	0.00001
Rab-5A	RAS - superfamily	2633_at; 8078_at	-0.76	0.00268	-0.68	0.00443			-0.68	0.00161	-0.74	0.00036
GOS-28	Transporter	4288_at; 2107_at; 2137_at; 4299_at; 4300_at	-1.16	0.00010	-0.58	0.00064			-0.61	0.00020	-0.85	0.00006
YKT6	Generic enzyme	10440_at; 10460_at; 2963_at; 10432_at; 10456_at; 10457_at	-0.97	0.00007	0.55	0.00562			-0.55	0.00114	-0.63	0.00040
PI3K cat class III (Vps34)	Lipid kinase	2959_at; 9787_at; 9814_at; 9824_at; 9821_at; 9828_at; 9788_at; 9780_at; 2901_at; 2899_at; 2843_at; 2882_at; 2957_at	-0.97	0.00006	0.49	0.00497			-0.77	0.00003	-0.85	0.00001
Syntaxin 12	Transporter	2633_at; 8078_at	-0.76	0.00268	-0.68	0.00443			-0.68	0.00161	-0.74	0.00036

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AP complex 2	Transporter	10849_at; 10788_at; 3052_at; 10987_at; 10847_at; 3006_at; 3081_at	-0.84	0.00376	-0.41	0.00308					-0.36	0.00695	0.57	0.00316
NSF	Generic binding protein	2527_at; 6999_at; 7030_at; 7005_at; 7020_at; 7025_at; 7026_at; 7023_at	-0.57	0.00066	-0.57	0.00334							0.75	0.00111
RAB9P40	Protein	2138_at; 4684_at; 4616_at; 2192_at; 2178_at; 4677_at; 4627_at; 2146_at; 4685_at; 4690_at; 4640_at	-0.64	0.00042	0.39	0.00914					-0.75	0.00138	0.87	0.00098
HIP1	Generic binding protein	8739_at; 8744_at; 8767_at; 2730_at; 8731_at; 2744_at; 2737_at; 2736_at; 2729_at; 8798_at; 8745_at; 8706_at; 8754_at	1.00	0.00013	-0.49	0.00215					0.92	0.00006	1.03	0.00004
Rab-11A	RAS - superfamily	5476_at; 2274_at; 5438_at; 5458_at	-0.56	0.00285	0.42	0.00366	0.43	0.00653					-0.41	0.00560
Myosin I	Generic binding protein	10460_at; 9212_at; 9232_at; 9263_at; 10440_at; 2963_at; 10432_at; 10456_at; 2760_at; 2824_at; 2825_at; 9235_at; 9236_at; 9213_at; 10457_at	-1.04	0.00002	0.74	0.00478					-0.76	0.00028	0.68	0.00028
RABGDIA	Regulators (GDI, GAP, GEF)	5577_at; 5476_at; 2274_at; 5438_at; 5458_at	-0.56	0.00285	0.42	0.00366	0.43	0.00653	-0.49	0.00090	-0.41	0.00560		
Rab-8	RAS - superfamily	5291_at; 5289_at; 5287_at; 5290_at	0.76	0.00050									-0.66	0.00018
PICALM	Generic binding protein	2138_at; 4288_at; 2107_at; 4300_at; 4299_at; 2192_at; 2178_at; 2137_at; 4616_at; 4627_at; 4684_at; 4677_at; 4685_at; 4690_at; 2146_at; 4640_at	-1.16	0.00010	-0.58	0.00064					-0.75	0.00138	0.87	0.00098
Eps15	Generic binding protein	2527_at; 6999_at; 7030_at; 7005_at; 7020_at; 7025_at; 7026_at; 7023_at	-0.57	0.00066	-0.57	0.00334							0.75	0.00111
PI3K reg class III (p150)	Generic binding protein	2527_at; 6999_at; 7030_at; 7020_at; 7005_at; 7025_at; 7026_at; 7023_at	-0.57	0.00066	-0.57	0.00334							0.75	0.00111
Syntaxin 16	Transporter	2633_at; 8078_at	-0.76	0.00268	-0.68	0.00443					-0.68	0.00161	-0.74	0.00036

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VT11A	Transporter	9212_at; 9263_at; 9232_at; 2760_at; 9235_at; 2825_at; 2824_at; 9213_at; 9236_at	-1.04	0.00002	0.74	0.00478								-0.76	0.00028	0.68	0.00028
Syntaxin 6	Transporter	2353_at; 2354_at; 2316_at; 2314_at; 2317_at; 2355_at; 5876_at; 5802_at; 5792_at; 5868_at; 5837_at; 85458_at; 5795_at; 5831_at	1.74	0.00023	0.62	0.00158	0.42	0.00862	1.17	0.00824	1.68	0.00005					
ARF1	RAS - superfamily		1.74	0.00023	0.62	0.00158	0.42	0.00862	1.17	0.00824	1.68	0.00005					
VPS45A	Generic binding protein	2138_at; 2192_at; 4616_at; 4684_at; 4677_at; 4627_at; 2178_at; 4690_at; 4685_at; 2146_at; 4640_at	-0.64	0.00042	0.39	0.00914								-0.75	0.00138	0.87	0.00098
Aldosterone-mediated regulation of ENaC sodium transport																	
MEK1/2	Protein kinase	2354_at; 2353_at; 2316_at; 2314_at; 2317_at; 2355_at; 5792_at; 5802_at; 5837_at; 5876_at; 5868_at; 5795_at; 5831_at	1.74	0.00023	0.62	0.00158	0.42	0.00862	1.17	0.00824	1.68	0.00005					
GCR-alpha	Transcription factor	706_at				0.56	0.00187										
NEDD4L	Generic enzyme	5476_at; 2274_at; 5438_at; 5458_at	-0.56	0.00285	0.42	0.00366	0.43	0.00653								-0.41	0.00560
ERK2 (MAPK1)	Protein kinase	2138_at; 2178_at; 2192_at; 4616_at; 4627_at; 4677_at; 4684_at; 4690_at; 4685_at; 2146_at; 4640_at	-0.64	0.00042	0.39	0.00914								-0.75	0.00138	0.87	0.00098
SGK1	Protein kinase	973_at	-0.43	0.00202	-0.33	0.00922											
ICMT	Generic enzyme	2354_at; 2353_at; 5837_at; 5876_at; 5792_at; 2355_at; 2317_at; 2316_at; 2314_at; 5802_at; 5868_at; 5795_at; 5831_at	1.74	0.00023	0.62	0.00158	0.42	0.00862	1.17	0.00824	1.68	0.00005					
SAHH	Generic enzyme	5476_at; 2274_at; 5438_at; 5458_at	-0.56	0.00285	0.42	0.00366	0.43	0.00653								-0.41	0.00560
CFTR folding and maturation (norm and CF)																	
MA1B1	Generic enzyme	10849_at; 3052_at; 10788_at; 10847_at; 3006_at; 3081_at	-0.84	0.00376	-0.41	0.00308								-0.36	0.00695	-0.41	0.00400

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Aha1	Generic binding protein	2353_at; 2354_at; 5876_at; 5868_at; 5837_at; 5802_at; 5792_at; 2355_at; 2317_at; 2316_at; 2314_at; 5795_at; 5831_at	1.74	0.00023	0.62	0.00158	0.42	0.00862	1.17	0.00824	1.68	0.00005
HSP40	Generic binding protein	8739_at; 8744_at; 8767_at; 2730_at; 2744_at; 2737_at; 2736_at; 2729_at; 8731_at; 8798_at; 8745_at; 8706_at; 8754_at	1.00	0.00013	-0.49	0.00215			0.92	0.00006	1.03	0.00004
OST complex	Generic enzyme	2138_at; 4627_at; 4616_at; 2192_at; 2178_at; 4684_at; 4677_at; 4690_at; 4685_at; 2146_at; 4640_at	-0.64	0.00042	0.39	0.00914			-0.75	0.00138	0.87	0.00098
HSP90 beta	Generic binding protein	2617_at; 9263_at; 9232_at; 9212_at; 2760_at; 2824_at; 2825_at; 7468_at; 9235_at; 9236_at; 9213_at; 2542_at	-1.04	0.00002	0.74	0.00478			-0.76	0.00028	0.68	0.00028
Calnexin	Generic binding protein	3157_at; 3156_at; 3097_at; 11238_at; 11272_at	0.50	0.00079			0.47	0.00901	0.59	0.00093	0.46	0.00147
HSP70	Generic binding protein	2617_at; 10849_at; 7030_at; 6999_at; 2527_at; 3052_at; 10788_at; 5476_at; 2274_at; 10847_at; 7025_at; 7020_at; 7005_at; 7468_at; 5438_at; 5458_at; 3081_at; 3006_at; 7026_at; 7023_at; 2542_at	-0.84	0.00376	-0.57	0.00334	0.43	0.00653	0.53	0.00156	0.75	0.00111
Normal wtCFTR traffic/ER-to-Golgi and Delta 508-CFTR traffic/ER-to-Golgi in CF												
Coatomer	Generic binding protein	2353_at; 2354_at; 2138_at; 2730_at; 8739_at; 8744_at; 8767_at; 2617_at; 115361_at; 8798_at; 8731_at; 2744_at; 2737_at; 2736_at; 2729_at; 5876_at; 5868_at; 5837_at; 5802_at; 5792_at; 2355_at; 2317_at; 2316_at; 2314_at; 4684_at; 4677_at; 4627_at; 4616_at; 2192_at; 2178_at; 5795_at; 2146_at; 5831_at; 8745_at; 8706_at; 7468_at; 4690_at; 4685_at; 8754_at; 2542_at; 4640_at	1.74	0.00023	0.62	0.00158	0.42	0.00862	1.17	0.00824	1.68	0.00005
COPII	Generic binding protein	9824_at; 9814_at; 9787_at; 2959_at; 2617_at; 2354_at; 2353_at; 4288_at; 2107_at; 9828_at; 9821_at; 9788_at; 9780_at; 2901_at; 2899_at; 5876_at; 5868_at; 5837_at; 5802_at; 5792_at; 2355_at; 2317_at; 2316_at; 2314_at; 2137_at; 4026_at; 4015_at; 4300_at; 4299_at; 5795_at; 2843_at; 2882_at; 7468_at; 4048_at; 5831_at; 2542_at; 2957_at	1.74	0.00023	0.62	0.00158	0.42	0.00862	1.17	0.00824	1.68	0.00005

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Syntaxin 6	Transporter	2354_at; 2353_at; 5837_at; 5802_at; 5792_at; 2355_at; 2317_at; 2316_at; 2314_at; 5868_at; 85458_at; 5876_at; 5795_at; 5831_at	1.74	0.00023	0.62	0.00158	0.42	0.00862	1.17	0.00824	1.68	0.00005
Ran regulation pathway												
NTF2	Transporter	5476_at; 2274_at; 5438_at; 5458_at	-0.56	0.00285	0.42	0.00366	0.43	0.00653			-0.41	0.00560
Ran	RAS - superfamily	9824_at; 2633_at; 9787_at; 2959_at; 9814_at; 9780_at; 2901_at; 2899_at; 1839_at; 9828_at; 9821_at; 9788_at; 8078_at; 2882_at; 2843_at; 2957_at	-0.97	0.00006	-0.68	0.00443			-0.77	0.00003	-0.85	0.00001
RCC1	Regulators (GDI, GAP, GEF)	2138_at; 2178_at; 2192_at; 4616_at; 4627_at; 4677_at; 4684_at; 1196_at; 2146_at; 4685_at; 4690_at; 4640_at	-0.64	0.00042	0.39	0.00914			-0.75	0.00138	0.87	0.00098
E2I	Generic enzyme	2354_at; 2353_at; 2317_at; 2316_at; 2314_at; 2355_at; 5792_at; 5802_at; 5837_at; 5876_at; 5868_at; 5795_at; 5831_at	1.74	0.00023	0.62	0.00158	0.42	0.00862	1.17	0.00824	1.68	0.00005
RanBP1	Regulators (GDI, GAP, GEF)	2354_at; 2353_at; 5802_at; 5792_at; 2355_at; 2317_at; 2316_at; 2314_at; 5837_at; 5876_at; 5868_at; 5795_at; 5831_at	1.74	0.00023	0.62	0.00158	0.42	0.00862	1.17	0.00824	1.68	0.00005
Importin (karyopherin)-beta	Transporter	9212_at; 9787_at; 9814_at; 9824_at; 9232_at; 9263_at; 2959_at; 9828_at; 9821_at; 9788_at; 9780_at; 2901_at; 2899_at; 2760_at; 2825_at; 9235_at; 2882_at; 2843_at; 2824_at; 2957_at; 9236_at; 9213_at	-1.04	0.00002	0.74	0.00478			-0.77	0.00003	-0.85	0.00001
Role of Nek in cell cycle regulation												
RCC1	Regulators (GDI, GAP, GEF)	2138_at; 4616_at; 2192_at; 2178_at; 4627_at; 4684_at; 4677_at; 1196_at; 4690_at; 4685_at; 2146_at; 4640_at	0.39	0.00914	-0.64	0.00042			-0.75	0.00138	0.87	0.00098
Cyclin B1	Generic binding protein	2617_at; 7468_at; 2542_at	-0.40	0.00411	0.73	0.00019			0.53	0.00156	0.53	0.00132
Insulin receptor	Receptor with enzyme activity	65110_at									-0.45	0.00356
MAD1 (mitotic checkpoint)	Generic binding protein	2138_at; 2192_at; 2178_at; 4616_at; 4627_at; 4684_at; 4677_at; 2146_at; 4690_at; 4685_at; 4640_at	0.39	0.00914	-0.64	0.00042			-0.75	0.00138	0.87	0.00098

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Ran	RAS - superfamily	9824_at; 2633_at; 9787_at; 2959_at; 9814_at; 9828_at; 9821_at; 9788_at; 9780_at; 2901_at; 2899_at; 1839_at; 2843_at; 8078_at; 2882_at; 2957_at	-0.68	0.00443	-0.97	0.00006					-0.77	0.00003	-0.85	0.00001
HEC	Generic binding protein	4026_at; 4015_at; 4048_at	0.48	0.00142									-0.51	0.00146
Transition and termination of DNA replication														
PCNA	Generic binding protein	6999_at; 2527_at; 7030_at; 7020_at; 7005_at; 7025_at; 7023_at; 7026_at	-0.57	0.00066	-0.57	0.00334							0.75	0.00111
TOP2	Generic enzyme	8767_at; 2730_at; 8739_at; 8744_at; 2737_at; 2736_at; 2729_at; 8798_at; 8731_at; 2744_at; 8706_at; 8745_at; 8754_at	1.00	0.00013	-0.49	0.00215					0.92	0.00006	1.03	0.00004
TOP1	Generic enzyme	2633_at; 8078_at	-0.76	0.00268	-0.68	0.00443					-0.68	0.00161	-0.74	0.00036
PCNA	Generic binding protein	6999_at; 2527_at; 7030_at; 7020_at; 7005_at; 7025_at; 7023_at; 7026_at	-0.57	0.00066	-0.57	0.00334							0.75	0.00111
DNA ligase I	Generic enzyme	2354_at; 2353_at; 2355_at; 5792_at; 2317_at; 2316_at; 2314_at; 5876_at; 5837_at; 5868_at; 5802_at; 5795_at; 5831_at	1.74	0.00023	0.62	0.00158	0.42	0.00862	1.17	0.00824	1.68	0.00005		
RFC complex	Generic binding protein	2633_at; 10849_at; 1054_at; 8767_at; 8744_at; 2730_at; 8739_at; 7030_at; 6999_at; 2527_at; 8798_at; 8731_at; 2744_at; 2737_at; 2736_at; 2729_at; 3052_at; 10788_at; 8078_at; 10847_at; 7020_at; 8706_at; 8745_at; 7025_at; 7005_at; 8754_at; 3081_at; 3006_at; 7023_at; 7026_at	1.00	0.00013	-0.68	0.00443					0.92	0.00006	1.03	0.00004
MCM2	Generic binding protein	2527_at; 7030_at; 6999_at; 7020_at; 7025_at; 7005_at; 7023_at; 7026_at	-0.57	0.00066	-0.57	0.00334							0.75	0.00111
FEN1	Generic enzyme	10460_at; 10440_at; 2963_at; 10456_at; 10432_at; 10457_at	-0.97	0.00007	0.55	0.00562					-0.55	0.00114	-0.63	0.00040
RFC1	Generic binding protein		-0.76	0.00268	-0.68	0.00443					-0.68	0.00161	-0.74	0.00036

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DNA polymerase alpha/primase	Generic enzyme	10460_at; 2730_at; 10440_at; 8767_at; 8744_at; 8739_at; 4015_at; 4026_at; 8798_at; 8731_at; 2744_at; 2737_at; 2736_at; 2963_at; 2729_at; 4048_at; 8706_at; 8745_at; 10432_at; 10456_at; 8754_at; 10457_at	1.00	0.00013	0.55	0.00562			0.92	0.00006	1.03	0.00004
CDK2	Protein kinase	7030_at; 6999_at; 2527_at; 7005_at; 7020_at; 7025_at; 7023_at; 7026_at	-0.57	0.00066	-0.57	0.00334					0.75	0.00111
POLD cat (p125)	Generic enzyme	2354_at; 2353_at; 2317_at; 2316_at; 2314_at; 5876_at; 5868_at; 5837_at; 5802_at; 5792_at; 2355_at; 5795_at; 5831_at	1.74	0.00023	0.62	0.00158	0.42	0.00862	1.17	0.00824	1.68	0.00005
DNA polymerase epsilon	Generic enzyme	8767_at; 8744_at; 8739_at; 2730_at; 2729_at; 2736_at; 2737_at; 8798_at; 2744_at; 8731_at; 8706_at; 8745_at; 8754_at	1.00	0.00013	-0.49	0.00215			0.92	0.00006	1.03	0.00004
Brcal/Bard1	Generic enzyme	604_at				-0.53	0.00054					
Ubiquitin	Generic binding protein	9787_at; 9814_at; 9824_at; 2959_at; 9780_at; 2901_at; 2899_at; 9788_at; 9821_at; 9828_at; 2882_at; 2843_at; 2957_at	-0.97	0.00006	0.49	0.00497			-0.77	0.00003	-0.85	0.00001
Ribonuclease H1	Generic enzyme	9263_at; 9232_at; 9212_at; 9373_at; 2824_at; 2760_at; 2825_at; 9235_at; 9213_at; 9236_at	-1.04	0.00002	0.74	0.00478			-0.76	0.00028	0.68	0.00028
Chromosome condensation in prometaphase												
Cyclin B	Generic binding protein	2617_at; 7468_at; 2542_at	0.73	0.00019	-0.40	0.00411			0.53	0.00156	0.53	0.00132
TOP2	Generic enzyme	2730_at; 8739_at; 8744_at; 8767_at; 8798_at; 8731_at; 2744_at; 2737_at; 2736_at; 2729_at; 8745_at; 8706_at; 8754_at	1.00	0.00013	-0.49	0.00215			0.92	0.00006	1.03	0.00004
TOP1	Generic enzyme	2633_at; 8078_at	-0.76	0.00268	-0.68	0.00443			-0.68	0.00161	-0.74	0.00036
Aurora-A	Protein kinase	2617_at; 7468_at; 2542_at	0.73	0.00019	-0.40	0.00411			0.53	0.00156	0.53	0.00132
BRRN1	Protein	6999_at; 2527_at; 7030_at; 7005_at; 7020_at; 7025_at; 7023_at; 7026_at	-0.57	0.00066	-0.57	0.00334					0.75	0.00111
Histone H1	Generic binding protein	2617_at; 7468_at; 2542_at	0.73	0.00019	-0.40	0.00411			0.53	0.00156	0.53	0.00132

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Histone H3	Generic binding protein	8767_at; 2730_at; 8739_at; 8744_at; 2729_at; 2527_at; 8798_at; 8731_at; 7030_at; 6999_at; 2744_at; 2737_at; 2736_at; 8745_at; 7005_at; 7025_at; 7020_at; 8706_at; 8754_at; 7026_at; 7023_at	1.00	0.00013	-0.57	0.00334			0.92	0.00006	1.03	0.00004
Condensin	Protein	2353_at; 2354_at; 9824_at; 9814_at; 9787_at; 2959_at; 5802_at; 5792_at; 2355_at; 2317_at; 2316_at; 2314_at; 5876_at; 5868_at; 5837_at; 2901_at; 2899_at; 5291_at; 5289_at; 5287_at; 9828_at; 9821_at; 9788_at; 9780_at; 7030_at; 6999_at; 2527_at; 5795_at; 2843_at; 2882_at; 7025_at; 7020_at; 7005_at; 5831_at; 2957_at; 7026_at; 7023_at; 5290_at	1.74	0.00023	0.62	0.00158	0.42	0.00862	1.17	0.00824	1.68	0.00005
Spindle assembly and chromosome separation												
Tubulin (in microtubules)	Generic binding protein	9212_at; 9232_at; 9263_at; 5287_at; 5289_at; 5291_at; 2824_at; 2760_at; 9235_at; 2825_at; 5290_at; 9213_at; 9236_at	-1.04	0.00002	0.74	0.00478			-0.76	0.00028	0.68	0.00028
Ran	RAS - superfamily	9824_at; 2633_at; 9787_at; 2959_at; 9814_at; 9780_at; 2901_at; 2899_at; 1839_at; 9828_at; 9821_at; 9788_at; 2843_at; 2882_at; 8078_at; 2957_at	-0.97	0.00006	-0.68	0.00443			-0.77	0.00003	-0.85	0.00001
APC/CDC20 complex	Generic enzyme	10460_at; 2353_at; 10440_at; 2138_at; 2354_at; 4288_at; 2107_at; 2963_at; 90637_at; 4684_at; 4677_at; 4627_at; 4616_at; 2192_at; 2178_at; 2355_at; 2317_at; 2316_at; 2314_at; 4300_at; 4299_at; 2137_at; 5876_at; 5868_at; 5837_at; 5802_at; 5792_at; 7030_at; 6999_at; 2527_at; 5795_at; 162466_at; 5831_at; 7025_at; 7020_at; 7005_at; 10456_at; 10432_at; 4690_at; 4685_at; 2146_at; 7026_at; 7023_at; 10457_at; 4640_at	1.74	0.00023	0.62	0.00158	0.42	0.00862	1.17	0.00824	1.68	0.00005
Ubiquitin	Generic binding protein	9787_at; 9814_at; 9824_at; 2959_at; 9780_at; 2901_at; 2899_at; 9788_at; 9821_at; 9828_at; 2882_at; 2843_at; 2957_at	-0.97	0.00006	0.49	0.00497			-0.77	0.00003	-0.85	0.00001
Mitotic cohesin complex	Protein	10849_at; 5287_at; 5289_at; 3052_at; 10788_at; 5291_at; 10847_at; 3081_at; 3006_at; 5290_at	-0.84	0.00376	-0.41	0.00308			-0.36	0.00695	-0.66	0.00018





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RFC complex	Generic binding protein	8767_at; 10849_at; 1054_at; 2730_at; 8739_at; 8744_at; 2633_at; 3052_at; 7030_at; 6999_at; 2527_at; 10788_at; 2729_at; 2736_at; 2737_at; 2744_at; 8731_at; 8798_at; 7020_at; 8078_at; 7025_at; 10847_at; 8706_at; 8745_at; 7005_at; 7026_at; 7023_at; 8754_at; 3081_at; 3006_at	1.00	0.00013	-0.68	0.00443		0.92	0.00006	1.03	0.00004	
RPA1	Generic binding protein	11238_at; 3097_at; 3157_at; 3156_at; 11272_at	0.50	0.00079			0.47	0.00901	0.59	0.00093	0.46	0.00147
MutSbeta complex	Generic binding protein	860_at; 2633_at; 8078_at	-0.76	0.00268	-0.68	0.00443	-0.59	0.00049	-0.68	0.00161	-0.74	0.00036
MutSalpha complex	Generic binding protein	860_at; 2353_at; 2633_at; 2354_at; 5876_at; 5868_at; 5837_at; 5802_at; 5792_at; 2355_at; 2317_at; 2316_at; 2314_at; 5795_at; 8078_at; 5831_at	1.74	0.00023	-0.68	0.00443	-0.59	0.00049	1.17	0.00824	1.68	0.00005
RPA2	Generic binding protein	8739_at; 2730_at; 8767_at; 8744_at; 8798_at; 2729_at; 8731_at; 2744_at; 2737_at; 2736_at; 8706_at; 8745_at; 9172_at; 1119_at; 8754_at	1.00	0.00013	-0.71	0.00008		0.92	0.00006	1.03	0.00004	
PCNA	Generic binding protein	7030_at; 2527_at; 6999_at; 7020_at; 7005_at; 7025_at; 7026_at; 7023_at	-0.57	0.00066	-0.57	0.00334					0.75	0.00111
Nucleotide excision repair												
RPA1	Generic binding protein	11238_at; 3157_at; 3156_at; 3097_at; 11272_at	0.50	0.00079			0.47	0.00901	0.59	0.00093	0.46	0.00147
TFIIH	Generic enzyme	9787_at; 2959_at; 2617_at; 2354_at; 2353_at; 9824_at; 9814_at; 2899_at; 5876_at; 5868_at; 5837_at; 5802_at; 5792_at; 2355_at; 2317_at; 2316_at; 2314_at; 2901_at; 9780_at; 4026_at; 4015_at; 5476_at; 9828_at; 9821_at; 9788_at; 5795_at; 2274_at; 7468_at; 5831_at; 4048_at; 5438_at; 2882_at; 2843_at; 2542_at; 5458_at; 2957_at	1.74	0.00023	0.62	0.00158	0.43	0.00653	1.17	0.00824	1.68	0.00005
XPF	Generic enzyme	2617_at; 7468_at; 2542_at	0.73	0.00019	-0.40	0.00411		0.53	0.00156	0.53	0.00132	

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DNA polymerase epsilon	Generic enzyme	8767_at; 8744_at; 8739_at; 2730_at; 2729_at; 2736_at; 2737_at; 8798_at; 2744_at; 8731_at; 8706_at; 8745_at; 8754_at	1.00	0.00013	-0.49	0.00215			0.92	0.00006	1.03	0.00004
XPG	Generic binding protein	2138_at; 2192_at; 2178_at; 4616_at; 4627_at; 4684_at; 4677_at; 2146_at; 4685_at; 4690_at; 4640_at; 4734_at	-0.64	0.00042	0.39	0.00914			-0.75	0.00138	0.87	0.00098
Ubiquitin	Generic binding protein	9814_at; 9787_at; 2959_at; 9824_at; 9828_at; 2901_at; 9780_at; 9788_at; 9821_at; 2899_at; 2882_at; 2843_at; 2957_at	-0.97	0.00006	0.49	0.00497			-0.77	0.00003	-0.85	0.00001
ERCC6	Generic enzyme	10849_at; 10788_at; 3052_at; 10847_at; 3006_at; 3081_at	-0.84	0.00376	-0.41	0.00308			-0.36	0.00695	-0.41	0.00400
POLD cat (p125)	Generic enzyme	2354_at; 2353_at; 2316_at; 2314_at; 5876_at; 5868_at; 5837_at; 5802_at; 5792_at; 2355_at; 2317_at; 5795_at; 5831_at	1.74	0.00023	0.62	0.00158	0.42	0.00862	1.17	0.00824	1.68	0.00005
COP9 signalosome	Generic enzyme	1789_at	-0.46	0.00426								
RAD23B	Generic binding protein	5476_at; 2274_at; 5438_at; 5458_at	-0.56	0.00285	0.42	0.00366	0.43	0.00653			-0.41	0.00560
DNA ligase I	Generic enzyme	2354_at; 2353_at; 5802_at; 5792_at; 2355_at; 2317_at; 2316_at; 2314_at; 5837_at; 5876_at; 5868_at; 5795_at; 5831_at	1.74	0.00023	0.62	0.00158	0.42	0.00862	1.17	0.00824	1.68	0.00005
RAD23B	Generic binding protein	5476_at; 2274_at; 5438_at; 5458_at	-0.56	0.00285	0.42	0.00366	0.43	0.00653			-0.41	0.00560
RPA2	Generic binding protein	8739_at; 2730_at; 8767_at; 8744_at; 8798_at; 2729_at; 8731_at; 2744_at; 2737_at; 2736_at; 1119_at; 8706_at; 8745_at; 9172_at; 8754_at	1.00	0.00013	-0.71	0.00008			0.92	0.00006	1.03	0.00004
RNA polymerase II	Generic enzyme	2353_at; 2354_at; 2633_at; 2617_at; 5802_at; 5792_at; 2355_at; 2317_at; 2316_at; 2314_at; 5876_at; 5868_at; 5837_at; 2527_at; 6999_at; 7030_at; 1594_at; 4015_at; 4026_at; 5795_at; 8078_at; 4048_at; 7025_at; 7020_at; 7005_at; 5831_at; 1486_at; 7468_at; 8398_at; 8336_at; 2542_at; 7023_at; 7026_at	1.74	0.00023	-0.68	0.00443	0.42	0.00862	1.17	0.00824	1.68	0.00005

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RFC complex	Generic binding protein	8767_at; 2633_at; 10849_at; 1054_at; 2730_at; 8739_at; 8744_at; 2736_at; 2729_at; 2527_at; 6999_at; 7030_at; 8798_at; 2737_at; 2744_at; 8731_at; 10788_at; 3052_at; 8745_at; 8706_at; 8078_at; 7005_at; 7020_at; 7025_at; 10847_at; 8754_at; 7026_at; 7023_at; 3006_at; 3081_at	1.00	0.00013	-0.68	0.00443			0.92	0.00006	1.03	0.00004
PCNA	Generic binding protein	2527_at; 7030_at; 6999_at; 7005_at; 7020_at; 7025_at; 7023_at; 7026_at	-0.57	0.00066	-0.57	0.00334					0.75	0.00111
Regulation of eIF2 activity												
MEK1(MAP2K1)	Protein kinase	2353_at; 2354_at; 5792_at; 2355_at; 2317_at; 2316_at; 2314_at; 5802_at; 5837_at; 5868_at; 5876_at; 5795_at; 5831_at	1.74	0.00023	0.62	0.00158	0.42	0.00862	1.17	0.00824	1.68	0.00005
GSK3 alpha/beta	Protein kinase	9212_at; 9493_at; 9263_at; 9232_at; 2760_at; 2824_at; 2825_at; 9235_at; 9236_at; 9213_at	-1.04	0.00002	0.74	0.00478			-0.76	0.00028	0.68	0.00028
Insulin receptor	Receptor with enzyme activity	65110_at									-0.45	0.00356
GCN2	Protein kinase	2354_at; 2353_at; 5802_at; 5792_at; 2355_at; 2317_at; 2316_at; 2314_at; 5837_at; 5876_at; 5868_at; 5795_at; 5831_at	1.74	0.00023	0.62	0.00158	0.42	0.00862	1.17	0.00824	1.68	0.00005
PP1-cat	Protein phosphatase	5476_at; 2274_at; 5438_at; 5458_at	-0.56	0.00285	0.42	0.00366	0.43	0.00653			-0.41	0.00560
GAB1	Generic binding protein	60488_at			0.53	0.00338						
eIF2B	Regulators (GDI, GAP, GEF)	10460_at; 2353_at; 2354_at; 9814_at; 10440_at; 2959_at; 9787_at; 2138_at; 9824_at; 4677_at; 4627_at; 4616_at; 2192_at; 2178_at; 5876_at; 5868_at; 5837_at; 5802_at; 5792_at; 2355_at; 2317_at; 2316_at; 2314_at; 4684_at; 4919_at; 2899_at; 2901_at; 9780_at; 9788_at; 9821_at; 9828_at; 2963_at; 5795_at; 10456_at; 10432_at; 2882_at; 2843_at; 4690_at; 4685_at; 2146_at; 5831_at; 10457_at; 2957_at; 4640_at	1.74	0.00023	0.62	0.00158	0.42	0.00862	1.17	0.00824	1.68	0.00005

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eIF2B5	Regulators (GDI, GAP, GEF)	2354_at; 2353_at; 5837_at; 5802_at; 5792_at; 2355_at; 2317_at; 2316_at; 2314_at; 5876_at; 5868_at; 5795_at; 5831_at	1.74	0.00023	0.62	0.00158	0.42	0.00862	1.17	0.00824	1.68	0.00005
eIF2	Generic binding protein	2617_at; 10849_at; 10788_at; 3052_at; 8013_at; 10847_at; 7468_at; 3081_at; 3006_at; 2542_at	-0.84	0.00376	-0.41	0.00308			0.53	0.00156	0.53	0.00132
Casein kinase I	Protein kinase	8767_at; 8744_at; 8739_at; 4288_at; 2730_at; 2107_at; 8798_at; 2137_at; 2729_at; 2736_at; 2737_at; 2744_at; 4299_at; 4300_at; 8731_at; 8706_at; 8745_at; 8754_at	-1.16	0.00010	-0.58	0.00064			0.92	0.00006	1.03	0.00004
Casein kinase II, alpha chains	Protein kinase	2633_at; 4015_at; 4026_at; 8078_at; 4048_at	-0.76	0.00268	-0.68	0.00443			-0.68	0.00161	-0.74	0.00036
PP1-cat alpha	Protein phosphatase	5476_at; 2274_at; 5438_at; 5458_at	-0.56	0.00285	0.42	0.00366	0.43	0.00653			-0.41	0.00560
ERK1/2	Protein kinase	2138_at; 6999_at; 2527_at; 7030_at; 2178_at; 2192_at; 4616_at; 4627_at; 4677_at; 4684_at; 7025_at; 7020_at; 2146_at; 4685_at; 4690_at; 7005_at; 4640_at; 7023_at; 7026_at	-0.64	0.00042	-0.57	0.00334			-0.75	0.00138	0.87	0.00098
Casein kinase II, beta chain (Phosvitin)	Generic binding protein	2633_at; 8078_at	-0.76	0.00268	-0.68	0.00443			-0.68	0.00161	-0.74	0.00036
Glycine, serine, cystein and threonine metabolism												
SerRS	Generic enzyme	2354_at; 2353_at; 5802_at; 5792_at; 2355_at; 2317_at; 2316_at; 2314_at; 5837_at; 5876_at; 5868_at; 5795_at; 5831_at	1.74	0.00023	0.62	0.00158	0.42	0.00862	1.17	0.00824	1.68	0.00005
CBS	Generic enzyme	2138_at; 2178_at; 2192_at; 4616_at; 4627_at; 4677_at; 4684_at; 2146_at; 4685_at; 4690_at; 4640_at	-0.64	0.00042	0.39	0.00914			-0.75	0.00138	0.87	0.00098
SHMT1	Generic enzyme	9787_at; 9814_at; 9824_at; 2959_at; 9821_at; 9788_at; 9780_at; 2901_at; 2899_at; 9828_at; 2843_at; 2882_at; 2957_at	-0.97	0.00006	0.49	0.00497			-0.77	0.00003	-0.85	0.00001
GlyRS	Generic enzyme	6999_at; 2527_at; 7030_at; 7005_at; 7020_at; 7025_at; 7026_at; 7023_at	-0.57	0.00066	-0.57	0.00334					0.75	0.00111
AGXT	Generic enzyme	5291_at; 5289_at; 5287_at; 5290_at	0.76	0.00050							-0.66	0.00018



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MEK1(MAP2K1)	Protein kinase	2353_at; 2354_at; 5792_at; 2355_at; 2317_at; 2316_at; 2314_at; 5802_at; 5837_at; 5868_at; 5876_at; 5795_at; 5831_at	1.74	0.00023	0.62	0.00158	0.42	0.00862	1.17	0.00824	1.68	0.00005
PKA-reg (cAMP-dependent)	Generic binding protein	4026_at; 4015_at; 4048_at			0.48	0.00142					-0.51	0.00146
ERK1/2	Protein kinase	2138_at; 4684_at; 4677_at; 4627_at; 4616_at; 2192_at; 2178_at; 7030_at; 6999_at; 2527_at; 7020_at; 2146_at; 4685_at; 4690_at; 7005_at; 7025_at; 7023_at; 7026_at; 4640_at	-0.64	0.00042	-0.57	0.00334			-0.75	0.00138	0.87	0.00098
Calcineurin A (catalytic)	Protein phosphatase	2959_at; 9787_at; 9212_at; 9232_at; 9824_at; 9814_at; 9263_at; 2899_at; 9780_at; 9788_at; 9821_at; 9828_at; 2901_at; 2882_at; 2843_at; 2824_at; 2825_at; 9235_at; 2760_at; 9213_at; 9236_at; 2957_at	-1.04	0.00002	0.74	0.00478			-0.77	0.00003	-0.85	0.00001
PP1-cat alpha	Protein phosphatase	5476_at; 2274_at; 5438_at; 5458_at	-0.56	0.00285	0.42	0.00366	0.43	0.00653			-0.41	0.00560
Bcl-2	Generic binding protein	2060_at; 1909_at	-0.47	0.00846	0.40	0.00678						
PP2A catalytic	Protein phosphatase	2354_at; 2353_at; 5802_at; 5792_at; 2355_at; 2317_at; 2316_at; 2314_at; 5837_at; 5876_at; 5868_at; 5795_at; 5831_at	1.74	0.00023	0.62	0.00158	0.42	0.00862	1.17	0.00824	1.68	0.00005
p70 S6 kinase1	Protein kinase	238_at			-0.63	0.00208						
PP2C	Protein phosphatase	2633_at; 1870_at; 8078_at	-0.76	0.00268	-0.68	0.00443			-0.68	0.00161	-0.74	0.00036
Bcl-XL	Generic binding protein	1615_at			0.37	0.00897						
GSH metabolism												
GGT1	Generic enzyme	9814_at; 9787_at; 2959_at; 9824_at; 9821_at; 9828_at; 2899_at; 2901_at; 9780_at; 9788_at; 2843_at; 2882_at; 2957_at	0.49	0.00497	-0.97	0.00006			-0.77	0.00003	-0.85	0.00001
GSHR	Generic enzyme	2617_at; 7468_at; 2542_at	-0.40	0.00411	0.73	0.00019			0.53	0.00156	0.53	0.00132

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5-oxoprolinase	Generic enzyme	10797_at; 10460_at; 10440_at; 2963_at; 10456_at; 10432_at; 10457_at	0.55	0.00562	-0.97	0.00007	-0.55	0.00114	-0.63	0.00040
GPX4 (PHGPx)	Generic enzyme	7030_at; 2527_at; 6999_at; 7020_at; 7025_at; 7005_at; 7026_at; 7023_at	-0.57	0.00334	-0.57	0.00066			0.75	0.00111
GCL cat	Generic enzyme	10849_at; 10788_at; 3052_at; 10847_at; 3081_at; 3006_at	-0.41	0.00308	-0.84	0.00376	-0.36	0.00695	-0.41	0.00400
GSHB	Generic enzyme	2633_at; 8078_at	-0.68	0.00443	-0.76	0.00268	-0.68	0.00161	-0.74	0.00036
GPX7	Generic enzyme	4026_at; 4015_at; 4048_at	0.48	0.00142					-0.51	0.00146

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## Chapter 7: General discussion

The increase in population lifespan has enhanced the incidence of neurodegenerative diseases, for which there is no cure, yet. A common feature of this diseases group is the occurrence of oxidative stress, on which there are still some uncertainties about its role on the onset and/or propagation of disease <sup>1</sup>. Among the natural compounds exhibiting potential on neuroprotective activity, polyphenols are important molecules, due to their chemical properties <sup>2,3</sup>. Therefore, plants displaying high content in polyphenols constitute interesting sources of those compounds to be evaluated for neuroprotective purposes. Portugal display a great diversity of plants, several of them endemic and chemically uncharacterized. Therefore, they represents a huge reservoir of phytochemicals with potential to be explored.

The present work aimed to study the protective potential of polyphenols from juniper leaves and blackberry fruits on a neurodegenerative cell model.

In the literature it can be found several reports about potential cellular bioactivities that rely on the antioxidant capacities of several compounds, however they are usually evaluated by chemical assays, using pure compounds and at very high concentrations. With that approach cell environment is not taken into account; possible existence of synergisms and/or antagonism within compounds that occur in plants could be missing; high concentrations of compounds tested could mediate different mechanisms of action even conducting to cell toxicity. These issues were taken into consideration in this thesis: to maximize protective effects derived from compounds interactions, phenolic extracts were obtained from plants; to avoid toxicity derived from solvents, Generally Recognized As Safe (GRAS) solvents were used for polyphenols extraction; to pursue a physiological approach, non-toxic concentrations of extracts were used on the treatment of cell models.

Concerning juniper leaves evaluation, it was considered four species occurring in different areas of Portugal, one of them an endemism (*J. navicularis*). Junipers leaves are already described as used in

traditional medicine and they are also used as seasonings or as drink flavourings. A possible pharmacological application was studied for juniper leaves polyphenols. Non-cytotoxic concentrations of phenolic-enriched fractions of the four junipers were evaluated on a neurodegenerative cell model, constituted by neuroblastoma cells subjected to an oxidative insult. From the four species evaluated only *J. oxycedrus badia* was able to protect cell viability and ensure mitochondrial functionality, being considered as potentially neuroprotective. ROS scavenging are among the possible mechanisms involved in that protection. This species exhibited intracellular antioxidant capacity prior and after oxidative insult, mediated by direct scavenging and indirect antioxidant mechanisms. However, SOD and CAT antioxidant activities were excluded as involved in the indirect protection verified. Other enzymes activated by Nrf2/ARE transcription factor pathway, other than SOD and CAT, could be involved, such as gamma-glutamylcysteine synthetase, GPx, GR, thioredoxin reductase, peroxiredoxin and HO-1. Several polyphenols have been identified as hormetic effectors and activating Nrf2 (kaempferol, resveratrol, quercetin, EGCG, hesperidin, cyaniding-3-rutinoside, etc) <sup>4</sup>. Thus, further work is necessary to elucidate molecular mechanisms underpin the observed neuroprotection. Another complementary bioactivity used as rationale on the search of new neuroprotective compounds is AChE inhibitory activity that was also identified in junipers leaves, especially in *J. phoenicea* and *J. turbinata*. The use of juniper phenolic-enriched extracts in this exploratory stage does not exclude the possibilities of future bio-guided fractionation in order to produce a refined NP or the isolation of a lead for future drug development. Actually, this should constitute a future step on this research in attempt to associate particular phytochemicals to those bioactivities. Many phytochemicals are characterized by a biphasic dose-response relationship <sup>5</sup>. Although the concentrations tested in this work were not toxic for cells, in possible future applications toxicity studies on animal models are an important issue to be considered.

Having the perspective of a pharmaceutical application of juniper polyphenols, their content in plant material is an important issue for industrial processing. Since junipers are evergreen plant, plant material are available all year. However, phytochemicals content can vary, since they are greatly influenced by the environmental conditions <sup>6</sup>. Then, understand polyphenols dynamics and their possible modulation through control of external conditions is an important subject to be addressed. In fact, all four species registered seasonal variations in their phenolics and flavonoids contents, being winter the best period for plant harvesting, when plants are not actively growing. Although polyphenols content is higher in winter, the relative amount of individual compounds is not greatly affected, only being registered a decrease on procyanidin dimer relative content for *J. phoenicea*. Polyphenols content modulation was studied and it was verified that the different species have differential susceptibility to stresses, probably reflecting their adaptation to the environment where they are usually found. Polyphenols from *J. oxycedrus badia* that previously showed to protected neuroblastoma cells from oxidative stress, can have their content enhanced through salinity treatment. Polyphenols from *J. phoenicea* that presented AChE inhibitory activity could also be modulated through MeJa treatment. In both treatments only epicatechin, procyanidins and quercetin derivatives had their relative contents altered. Although those alterations did not privilege any particular class of compounds, the maintenance of the cell bioactivities detected must be confirmed. The enrichment in epicatechin, procyanidin or quercetin can potentiate activities related with the contest of NNDs such as modulation of pro-inflammatory signals <sup>7,8</sup>. Total phenolics contents variation was supported by PAL transcripts quantification. Transcripts profiles anticipated the changes verified on the phytochemicals contents. Transcripts levels of *J. phoenicea* treated with MeJa are better related with alterations in the content of specific compounds than transcript levels of *J. oxycedrus badia* subjected to

salinity. Those results suggest a differential post-transcriptional and/or post-translational regulation specific of treatments/species.

Regarding blackberries evaluation it was considered a nutritional perspective. In literature the majority of works that evaluate food phytochemicals does not take into account alterations occurring in gastrointestinal track as well as the concentrations that metabolites are usually found in serum. Then, following the purpose of a physiological approach, an IVD model already adopted on phytochemicals evaluation<sup>9</sup> was used. In order to show the impact that gastrointestinal digestion produces on phytochemicals alterations and consequently on their bioactivities, it was conducted the comparison of effects produced on cells by blackberry polyphenols before and after IVD. In addition to the different chemical profile presented by phenolic extracts before and after IVD, cell protection, ROS scavenging and GSH/GSSG balance were also differentially affected. IVD potentiated protection of cell membrane integrity, however not mediated by ROS scavenging neither by GSH modulation. Contrarily, undigested extract did not protect cells although it was able to scavenge ROS and to modulate GSH redox status. This work really stated the necessity of considering gastrointestinal digestion on the study of phytochemicals effects from foods. Additionally, the protection promoted by the digested extract, not associated to antioxidant mechanisms, highlighted the occurrence of other protective mechanism mediated by polyphenols. Actually as consequence of IVD the antioxidant capacity of metabolites is greatly lost, deterring a possible antioxidant effect on cells. Moreover, the effective and non-cytotoxic concentrations of digested extract were on the range of physiologic concentrations of polyphenols found in plasma<sup>10</sup>.

Once clarified the importance of using physiological conditions on bioactivities study of food phytochemicals, that approach was used to evaluate the neuroprotective potential of two Portuguese wild blackberry species (*R. brigantinus* and *R. vagabundus*). *R. brigantinus* is actually a Portuguese endemic species. In comparison with a commercial blackberry

variety, the two wild species presented different relative contents of some metabolites, inducing different cellular effect. While the digested phenolic extracts of the two wild species protected cell membrane integrity and mitochondria functionality, being considered neuroprotective, the digested extract of the commercial variety only was able to protect cell membrane integrity. Then wild blackberries are more protective since they ensure the functionality of mitochondria, essential for energy production and crucial on neurodegenerative diseases. The neuroprotection conferred by wild blackberries should be mediated by decrease of ROS levels, GSH content augmentation and caspases activation prior to stress imposition. These effects indicate that digested metabolites should constitute a stimulus that activate cells defenses that make them able to withstand to stress. This effect has been already described for pure undigested polyphenols <sup>4</sup>, however, as far as we know, not for a digested phenolic extracts.

It was verified that few relative differences in the chemical composition of blackberries were responsible by the different cell protection produced. Then, it is crucial to introduce the wild species in culture conditions and confirm the stability of their chemical profiles as well as their biological activities. The recognition of wild Portuguese blackberry species as potential neuroprotective fruits is important in diverse perspectives: i) for consumers have access to fruits with nutritional add value; ii) for farmers produce plants well adapted to Portuguese environment and to growing conditions with lower inputs, eventually more adapted to a sustainable agriculture; iii) for species conservation; iv) for public health policies, making possible to reduce future cost associated to neurodegenerative diseases through a nutritional prevention.

Another important issue to be addressed is the attribution of bioactivities to specific classes of polyphenols. A possible approach to be followed is the comparison of polyphenols bioactivities derived from isogenic fruits. The use of varieties that present markedly different chemical composition and then considered quasi-isogenic fruits could constitutes a good tool.

The advantage of using these fruits on studies with a similar approach to the one used in this thesis, is that fruits present all the same matrix and are enriched in specific polyphenols classes. Then, factors that could determine the extractability of compounds or possible interferences on some methods are similar within fruits, working as true matched matrix control.

Until now, and mostly due to animal experiments, it is known that flavonoids should act on neurodegeneration due to modulation of signaling cascades and activating intracellular defenses, however no specific molecular pathways are known <sup>11</sup>. In order to have some clues about the pathways differentially affected by wild and commercial blackberries that make cells able to cope with oxidative stress, a transcriptomic approach was followed. Although further experiments are needed for pathways/targets confirmation, interesting results were already found. The wild blackberry tested (*R. vagabundus*) revealed to better contradict the number of transcripts altered due to stress imposition, than the commercial blackberry. Alterations contradicted by wild blackberry pre-incubation prior to stress could be responsible by the protective effect demonstrated. Among blackberries pre-incubation, differential levels of transcripts were verified in some pathways. Products encoded by those transcripts are considered important targets to be followed by complementary biochemical experiments. Among pathways differentially expressed by blackberries and stressed cells were highlighted some related to transport (clathrin-coated vesicles cycle, CFTR folding/maturation/traffic, aldosterone-mediated regulation of ENaC transport), DNA repair mechanism (role of Brca1/Brac2 on DNA repair, NHEJ mechanism of DSBs repair and nucleotide excision repair) and cell cycle (Ran regulation pathway, transition and termination of DNA replication, chromosome condensation in prometaphase and spindle assembly and chromosome separation). Transport assume high importance in NDDs, since fragmentation of neuronal GA and disruption of cellular/axonal transport are common features of those diseases <sup>12-15</sup>.

Actually, GA fragmentation is thought that may contribute to the initiation of apoptotic pathway<sup>16,17</sup>. If blackberries treatment could revert that process, it could protect cells to enter into apoptosis. Moreover, DNA repair mechanisms are fundamental to counteract the deleterious effects of oxidative stress. Oxidative stress promotes DNA damage, therefore enhancing transcription of genes involved in DNA repair. All these differential responses induced by blackberries pre-incubation must help cells to counteract stress effects. In addition, small GTP-binding proteins also emerged as possible regulators to ensure the maintenance of cell homeostasis. Although those proteins not always showed changes in their transcript levels, other proteins/enzymes that belong to highlighted pathways and are described as down-stream regulated by small GTP-binding proteins (Bad phosphorylation, regulation of eIF2 activity, spindle assembly and chromosome separation, RAN regulation, clathrin-coated vesicles cycle, CFTR traffic and aldosterone-mediated regulation of ENaC transport) were differentially regulated. However, all these indications must be confirmed by adequate biochemical experiments.

Overall, among plants from the two groups in study, there are potential sources of neuroprotective compounds to be further investigated. Cell models relying on other features of neurodegeneration beyond oxidative stress could constitute interesting and complementary tools to further investigated juniper and blackberry beneficial effects. Moreover, more representative cells, such as primary neurons can be explored. Furthermore, the question of BBB permeability should be also taken into account and then appropriate models should be used to verify the concentrations and metabolites that reach brain tissues.

Juniper phenolic-enriched fractions constitute a good source of a NP or a lead for pharmaceutical purposes. However, fractions should be refined by bioguided purification and after *in vivo* activity confirmation, toxicity tests and future animal trials must be conducted.

From the nutritional perspective, the use of digested metabolites was an improvement. However, IVD model used does not mimic true bioavailable

metabolites since active transport in gut barrier, colon microflora metabolism and metabolites conjugation are missing. Nevertheless, there is no model accomplishing all these aspects and also delivering metabolites to be further tested. The fruits could be also investigated in long-term intervention studies on animals and/or humans to really access the benefits ascribed.

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## **Appendix: Comparison of different methods for DNA-free RNA isolation from SK-N-MC neuroblastoma**

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Author contribution:

LT performed all the experiments presented in this chapter and written the manuscript cited above

### **Abstract**

**Background:** RNA quality and quantity are important factors for ensuring the accuracy of gene expression analysis and other RNA-based downstream applications. Extraction of high quality nucleic acids is difficult from neuronal cells and brain tissues as they are particularly rich in lipids. In addition, most common RNA extraction methods are phenol-based, resulting in RNA that may be incompatible with downstream applications such as gene expression.

**Findings:** In this work, a comparative analysis of the RNA quality obtained from SK-N-MC cells was performed using six commonly used RNA isolation kits: two phenol-based kits and four non-phenol based kits. The non-phenol based kits tested AxyPrep Multisource Total RNA Miniprep, RNeasy® Mini, EasySpin and Illustra RNAspin Mini RNA Isolation, all performed well and resulted in the isolation of high quality RNA, as evaluated by  $A_{260}/A_{280}$ . The RNA extracted with AxyPrep Multisource Total RNA Miniprep, RNeasy® Mini and EasySpin provided the highest RNA yields. In particular, the RNA isolated by AxyPrep Multisource Total RNA Miniprep Kit did not show any detectable genomic DNA contamination even without previous DNase treatment or after RNA direct PCR amplification using universal 18S primers.

**Conclusions:** The RNA extracted from SK-N-MC cells with AxyPrep Multisource Total RNA Miniprep Kit was superior with respect to the RNA quality and concentration. This kit does not use aggressive organic solvents and RNA free of genomic DNA was isolated without the need for DNase treatment.

## 1. Introduction

The accuracy of gene expression evaluation is influenced by the concentration and quality of input RNA. The purity and integrity of RNA are critical elements for the overall success of RNA-based analyses <sup>1</sup>. Starting with a low quality RNA may compromise the results of downstream applications which are often labour-intensive, time-consuming and very expensive <sup>2,3</sup>. The integrity of the total RNA used should be examined prior to its use in quantitative RT-PCR, microarrays and any array-based applications.

To ensure acceptable total RNA quality, the RNA extraction procedure must fulfill a number of requirements: including, the final preparation must be free from protein, genomic DNA or enzyme inhibitors and must not include any phenol or alcohol carryover which may compromise downstream reactions <sup>4</sup>. Also, the purified RNA should also be free of nucleases to maintain integrity under appropriate storage conditions. Reverse transcriptase and PCR reactions are strongly dependent on the purification and clean-up methods, as well as on the presence of exogenous contaminants. For example, the presence of hemoglobin, fat, glycogen, Ca<sup>2+</sup>, high genomic DNA concentrations, DNA binding proteins or other cell constituents are critical contaminants <sup>5,6</sup>.

There are three major techniques extensively used for RNA extraction: organic extraction, such as phenol-Guanidine Isothiocyanate (GITC)-based solutions, silica-membrane based spin column technology, and paramagnetic particle technology. One of the most commonly used methods is the phenol-GITC-based organic extraction. However, RNA samples isolated by this method are frequently contaminated with proteins and other cellular materials, organic solvents such as phenol-chloroform, salts and ethanol. Additionally, these methods require safety precautions (i.e., the use of fume hoods) which lengthen the procedure and employ liquid-liquid extraction leading to incomplete phase separation and increased carryover contamination with genomic DNA. Silica column and paramagnetic particle based RNA isolation systems do

not require the use of toxic organic solvents, are relatively simple, efficient, low cost, and yield total intact RNA with low levels of contamination from proteins and other cellular materials<sup>7</sup>. However, these methods can often result in significant levels of genomic DNA contamination.

Digestion with DNase removes traces of DNA and is compulsory if the RNA samples are destined for use in RT-qPCR. DNase digestion after the final RNA precipitation step involves adding extra salts and proteins to the sample and since this can affect the efficiency of the cDNA synthesis, additional purification steps are required.

In this work, a comparative analysis of the RNA quality achieved from a neuroblastoma cell line (SK-N-MC) by six commonly used RNA isolation kits is presented; two phenol-based kits and four kits utilizing non-aggressive solvents. For the SK-N-MC cell line in particular, both types of extraction methods have previously been described, but RNA has been isolated mainly using phenol-GITC-based methods<sup>8-13</sup>.

## 2. Methods

### 2.1. Cell culture

Human neuroblastoma SK-N-MC cells were obtained from the European Collection of Cell Cultures (ECACC) and were cultured in EMEM supplemented with 2 mM glutamine, 10% (v/v) heat-inactivated fetal bovine serum (Gibco), 1% (v/v) of non-essential amino acids (Sigma) and 1 mM sodium pyruvate. Cells were cultivated at 37 °C in a humidified atmosphere containing 5% (v/v) CO<sub>2</sub>. For routine culture, cells were grown until reaching approximately 90% confluence. For RNA isolation, cells were harvested using trypsin and stored frozen at -80 °C.

### 2.2. RNA isolation

For SK-N-MC cell RNA isolation, six commercially available kits were tested: AxyPrep Multisource Total RNA Miniprep (Axygen), RNeasy® Mini (Qiagen), EasySpin (Citomed), Illustra RNAspin Mini RNA Isolation (GE), TRIzol® Plus RNA Purification System (Invitrogen) and E.Z.N.A.™ Total RNA kit II (Omega

Bio-Tek). The same amount of cells ( $6.92 \times 10^6$ ) and the manufacturer protocols were followed for each kit.

When appropriate, the isolated RNA was treated with Turbo™ DNase I (Ambion), accordingly to the manufacturer's instructions.

### 2.3. RNA quality and yield

For assessing RNA quality and yield,  $A_{260/A280}$  and  $A_{260/A230}$  ratios for RNA preparation samples were analysed with a Nano-Drop® ND-1000 spectrophotometer (NanoDrop Technologies). RNA integrity and DNA contamination were determined by 28S/18S rRNA visualization in agarose gel, stained with ethidium bromide.

PCR was used to detect potential DNA contamination, using primers specific for 18S rRNA as Quantum RNA Universal 18S Internal Standards primers (Ambion). PCR reaction contained 1  $\mu$ L of input (20-200 ng RNA), 0.6 U Taq DNA Polymerase (Fermentas), 2.5  $\mu$ L of 18S PCR Primer Pair (Ambion), 2 mM  $MgCl_2$ , 0.2 mM of each dNTP (Invitrogen), 75 mM Tris-HCl (pH 8.8 at 25 °C), 20 mM  $(NH_4)_2SO_4$  and 0.01% (v/v) Tween 20. The following program was applied: 1 cycle of 3 min. at 95 °C for denaturation, followed by 30 cycles (30 s at 95 °C for denaturation, 30 s at 57 °C for annealing, 30 s at 72 °C for extension) and a final 5 min extension at 72 °C.

PCR products were visualized in agarose gels, stained with ethidium bromide.

## 3. Results

RNA isolation methods such as acid phenol extraction, glass fibre filter purification, and single-step reagents can provide RNA with acceptable quality. However, all RNA isolation methods do not have the ability to completely remove genomic DNA contamination from RNA samples. To evaluate the differential efficiency in obtaining RNA with minimal DNA contamination, six commercial kits for RNA extraction were tested using SK-N-MC cells. Among these kits, two of them (TRIZOL® Plus RNA Purification System (Invitrogen) and E.Z.N.A.™ Total RNA kit II (Omega Bio-

Tek)) involve a more aggressive methodology which includes a monophasic solution of phenol and guanidine isothiocyanate. E.Z.N.A.<sup>™</sup> Total RNA Kit II was selected for this analysis because it is mainly designed for fatty tissues by combining the advantage of one step RNA isolation technology and silica-membrane technology.

Among the six kits tested, AxyPrep Multisource Total RNA Miniprep, RNeasy<sup>®</sup> Mini, EasySpin and Illustra RNAspin Mini RNA Isolation allow the isolation of higher quality RNA when compared to the other two kits (Table 1). The kits from Axygen, Qiagen, Citomed and GE, respectively, were qualitatively superior, providing a good  $A_{260}/A_{280}$  ratio (around 2.10). An  $A_{260}/A_{280}$  ratio greater than 1.8 is usually considered an acceptable indicator of good quality RNA with a low level of protein contamination<sup>14,15</sup>. An  $A_{260}/A_{230}$  ratio higher than 1.8 is used as an indicator of extracted RNA with a low level of polysaccharides contamination. Quantitatively, the highest RNA concentration and yield was obtained by the AxyPrep Multisource Total RNA Miniprep kit while RNeasy<sup>®</sup> Mini kit, EasySpin kit and TRIzol<sup>®</sup> Plus RNA Purification System presented intermediate values, and Illustra RNAspin Mini RNA Isolation kit and E.Z.N.A.<sup>™</sup> Total RNA kit II provided the lowest recovery values (Table 1). However, based on the standard deviation illustrated in Table 1, the TRIzol<sup>®</sup> Plus RNA Purification System -(GITC based procedure) demonstrates low RNA recovery reproducibility. In terms of yield, among Axygen, Qiagen and Citomed kits, clearly the highest and most reproducible is the AxyPrep Multisource Total RNA Miniprep Kit, where RNA yield is at least four fold higher than the other methods. E.Z.N.A.<sup>™</sup> Total RNA Kit II, a GITC based kit, and Illustra RNAspin Mini RNA Isolation Kit revealed rather poor efficiencies of RNA extraction from this neuroblastoma cell line (Table 1).

Table 1- Evaluation of quality and quantity parameters of RNA samples extracted from SK-N-MC neuroblastoma.

Kit	A <sub>230</sub>	A <sub>260</sub>	A <sub>280</sub>	A <sub>260</sub> /A <sub>280</sub>	A <sub>260</sub> /A <sub>230</sub>	Concentration (ng/μL)	Elution volume (μL)	Yield (μg RNA/1 × 10 <sup>6</sup> cells)
AxyPrep Multisource Total RNA Miniprep Kit (Axygen)	5.38	6.82	3.29	2.07	1.26	272.80±28.55	100	3.94±0.41
RNeasy® Mini Kit (Qiagen)	1.32	2.14	1.03	2.07	1.63	85.74±72.26	50	0.62±0.52
EasySpin (Citomed)	1.79	3.54	1.73	2.06	1.92	141.5±74.58	50	1.02±0.54
Illustra RNAspin Mini RNA Isolation Kit (GE)	0.3	0.15	0.07	2.13	0.37	5.98±7.44	100	0.09±0.11
TRizol® Plus RNA Purification System (Invitrogen)	1.13	2.31	1.15	1.86	1.65	92.4±111.50	50	0.67±0.80
E.Z.N.A.™ Total RNA Kit II (Omega bio-tek)	0.30	0.55	0.29	1.89	1.52	21.87±4.14	50	0.16±0.03

Most gene expression experiments require RNA samples free of DNA contamination; therefore it is imperative to minimize this contamination. Removal of DNA is especially critical for RT-PCR applications, since DNA can be amplified during the PCR portion of the experiment, resulting in false positive results and high background “noise” levels.

DNase I digestion has consistently proven to be the most effective method for removing DNA contamination from RNA samples. DNase I treatment efficacy test was evaluated for the two best performing kits; RNA extracted with AxyPrep Multisource, Total RNA Miniprep and RNeasy® Mini. Kits, with or without DNase treatment, were visualized in agarose gel stained with ethidium bromide (Fig. 1). RNA isolated with AxyPrep Multisource Total RNA Miniprep kit did not show

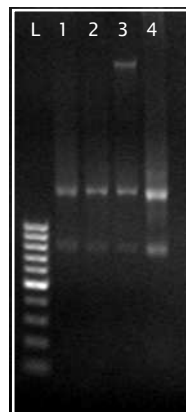


Fig. 1- Electrophoresis of RNA samples in 2% (w/v) agarose gel, stained with ethidium bromide. L- 100 bp ladder; 1- RNA (untreated sample) isolated by AxyPrep Multisource Total RNA Miniprep kit (Axygen); 2- RNA (treated with Turbo™ DNase, Ambion) isolated by AxyPrep Multisource Total RNA Miniprep kit (Axygen); 3- RNA (DNase I untreated sample) isolated by RNeasy® Mini kit (Qiagen); 4- RNA (treated with Turbo™ DNase, Ambion) isolated by RNeasy® Mini kit (Qiagen).

visible genomic DNA contamination even without DNase treatment (Fig. 1, lanes 1 and 2). On the contrary, RNA extracted using the RNeasy® Mini Kit clearly contained DNA contamination, which disappeared promptly after DNase treatment. Very low levels of DNA contamination, albeit not detectable by agarose gel electrophoresis, may be amplified and then corrupt the results obtained by highly sensitive techniques such as Real-Time PCR. In an attempt to address and evaluate this hypothesis, RNA samples (with and without DNase treatment) isolated by Axygen and Qiagen kits were directly amplified by PCR using universal 18S primers. The corresponding reaction products were visualized by agarose gel electrophoresis (Fig. 2). Although not visualized by direct RNA electrophoresis in Figure 1, RNA isolated using the RNeasy® Mini kit and

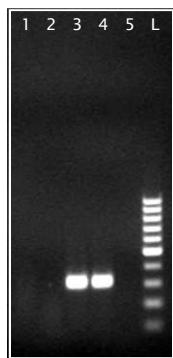


Fig. 2- Electrophoresis of 18S PCR amplification products of RNA samples without reverse transcription, in 2% (w/v) agarose gel, stained with ethidium bromide. 1- 18S PCR amplification products from RNA isolated by AxyPrep Multisource Total RNA Miniprep Kit (Axygen); 2- 18S PCR amplification products from RNA isolated by AxyPrep Multisource Total RNA Miniprep Kit (Axygen) and treated with Turbo™ DNase (Ambion); 3- 18S PCR amplification products from RNA isolated by RNeasy® Mini Kit (Qiagen); 4- 18S PCR amplification products from RNA isolated by RNeasy® Mini Kit (Qiagen) and treated with Turbo™ DNase (Ambion); 5- 18S PCR amplification products from water (negative control); L- 100 bp ladder.

treated with DNase was shown to contain DNA contamination after PCR amplification (Fig. 2, lanes 3 and 4). This result was obtained after confirming experimentally that the PCR product amplification was a result of the RNA concentration in the sample (and therefore the DNA contaminant amount) and was not due to extensive amplification (results not shown). After direct PCR amplification, the RNA isolated by AxyPrep Multisource Total RNA Miniprep kit (with or without previous DNase treatment) did not reveal the presence of any band on the agarose gel (Fig. 2, lanes 1 and

2). Therefore, the Axygen kit provides reliable and good quality RNA isolation from SK-N-MC neuroblastoma cells, thus suitable for a successful RNA amplification without the need of any DNase treatment. DNase treatment is considered disadvantageous by some investigators, as it adds extra salts and protein to the sample and can affect the efficiency of the subsequent cDNA synthesis.

#### **4. Conclusions**

Quality control is an extremely important issue when isolating RNA, especially when the quantity is small and the amount required is large, for example microarray experiments (15 µg). In regards to quality and yield the AxyPrep Multisource Total RNA Miniprep kit was determined to be the best kit tested for the isolation of RNA from SK-N-MC cells. This kit uses no aggressive organic solvents and delivers RNA devoid of genomic DNA, without the need for DNase treatment. This is an important finding, especially in large scale gene-expression studies, since DNase treatment is time consuming and adds a substantial cost to the overall cost for such experiments. Furthermore, DNase treatment may lead to a loss in both RNA amount and mRNA integrity due to the exposure of the RNA samples to high temperatures during the heat inactivation step required for many commercial DNases. This effect extends further to include any downstream applications demanding compulsory genomic DNA removal. AxyPrep Multisource Total RNA Miniprep Kit allows the use of mild treatments minimizing the introduction of further contaminants to the extracted RNA.

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