Rita João Rosado Serranito Ramos

Degree in Biology

Evaluation of Neuroprotective Effects of Natural Extracts obtained from Portuguese Agro-food Residues

Dissertation to obtain a Master Degree in Biotechnology

Supervisor: Ana Matias, Ph.D, IBET/ITQB-UNL

Co- Supervisor: Cláudia Santos, Ph.D, IBET/ITQB-UNL

Júri:

Presidente: Prof. Doutor Pedro Miguel Calado Simões

Arguente: Prof. Doutora Margarida Castro Caldas Braga

FACULDADE DE CIÊNCIAS E TECNOLOGIA
UNIVERSIDADE NOVA DE LISBOA

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Abstract

Countries are currently faced with problems derived from changes in lifespan and an increase in lifestyle-related diseases. Neurodegenerative disorders such Parkinson's (PD) and Alzheimer's (AD) diseases are an increasing problem in aged societies. Data from World Alzheimer Report 2011 indicate that 36 million people worldwide are living with dementia. Oxidative stress has been associated with the development of AD and PD. Therefore there is interest to search for effective compounds or therapies to combat the oxidative damage in these diseases. Current evidence strongly supports a contribution of phenolic compounds present in fruits and vegetables to the prevention of neurodegenerative diseases such AD and PD.

The industrial processing of a wide variety of fruits results in the accumulation of by-products without commercial value. *Opuntia ficus-indica* (cactus pear) is consumed fresh and processed like in juice. *Prunnus avium* (sweet cherry) is consumed fresh but the organoleptics characteristics of the fruits leads to the smaller and ragged fruits have no commercial value. Fruit extracts of both species has described to be rich in phenolic compounds and to have high antioxidant activities due to its composition. The aim of this work was assessing the efficacy of *O. ficus-indica* and *P. avium* by-products extracts obtained with conventional solvent extraction and pressurized liquid extraction in a neurodegeneration cell model. All extracts have protected neuroblastoma cells from H_2O_2-induced death at low, non-toxic levels, which approach to physiologically-relevant serum concentration. However, cherry extract has a slighter neuroprotective activity. The protective effect of *Opuntia* extracts are not conducted by a direct antioxidant activity since there are not decreases in intracellular ROS levels in cell treated with extracts and challenged with H_2O_2, while cherry extract neuroprotection seems to be due to a direct scavenging activity. Extracts from different biological matrixes seems to protect neuronal cells trough different cellular mechanisms.

Keywords: Neuroprotection, Agro-food residues, *Opuntia ficus-indica*, *Prunus avium*, Natural extracts.

Resumo

Em países desenvolvidos, o envelhecimento da população está a aumentar assim como a incidência de doenças relacionadas com a idade e estilo de vida como as doenças neurodegenerativas. O stress oxidativo tem sido associado ao desenvolvimento de doenças neurodegenerativas como a doença de Alzheimer (AD) e a doença de Parkinson (PD). Assim sendo existe um interesse crescente na busca por novos compostos ou terapias eficazes no combate aos danos provocados pelo stress oxidativo nestas doenças. Evidências apoiam fortemente um contributo de compostos fenólicos na prevenção de doenças neurodegenerativas. O processamento industrial de uma grande variedade de frutos resulta na acumulação de subprodutos sem qualquer valor comercial. *Opuntia ficus-indica* (figo da índia) é consumido fresco ou por exemplo processado em sumo. *Prunnus avium* (cereja) é consumido preferencialmente fresco, mas o tamanho e as características visuais necessárias leva a que frutos pequenos e de forma irregular não tenham valor comercial. Extratos de ambos os frutos têm sido descritos como ricos em compostos fenólicos e com elevada capacidade antioxidante, devido à sua composição. O objetivo principal desta dissertação é avaliar os efeitos neuroprotectores de extratos naturais obtidos a partir de resíduos agro-industriais destas duas espécies com recurso à extração convencional ou com fluidos pressurizados. A avaliação da capacidade neuroprotectora é realizada recorrendo à linha celular neuronal, proveniente de um neuroblastoma, SK-N-MC, submetida a um stress oxidativo induzido por uma solução de H_2O_2. Todos os extratos demonstraram um efeito neuroprotector em concentrações baixas, não tóxicas, fisiologicamente relevantes por se encontrarem perto de concentrações séricas. O efeito protetor dos extratos de *Opuntia* parece não se dever a uma atividade antioxidante direta, enquanto a neuroproteção exercida pelo extrato de cereja parece ser devida a uma ação antioxidante direta. Pode-se concluir que diferentes extratos de matrizes biológicas distintas parecem proteger as células neuronais por diferentes mecanismos celulares.

Palavras-chave: Neuroprotecção, Resíduos agroindustriais, *Opuntia ficus-indica*, *Prunus avium*, Extratos naturais;

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List of abbreviations, acronyms and symbols

Abbreviation	Full Form
AAPH	2”,2”-Azobis (2-amidinopropane) dihydrochloride
AD	Alzheimer’s Disease
ADT	Adsorption Technology
ATP	Adenosine tri-phosphate
BBB	Blood Brain Barrier
CAE	Caffeic Acid Equivalents
CCE	Crude Cherry Extract
CE	Catechin Equivalents
CNS	Central Nervous System
CO_2	Carbon Dioxide
CSE	Conventional Solvent Extraction
CVD	Cardiovascular Diseases
DAD	Diode Array Detector
DCFH – DA	2”,7”-Dichlorofluorescin Diacetate
ECACC	European Collection of Cell Cultures
ED	Electrochemical Detector
EGCG	Epigallochatequine -3- gallate
EMEM	Eagle Minimum Essential Medium
EtOH	Ethanol
ETC	Electron Transport Chain
EU	European Union
FBS	Fetal Bovine Serum
FDA	Food and Drug Administration
FL	Disodium Fluoresceín
GAE	Gallic Acid Equivalents
GPx	Glutathione Peroxidase
GR	Glutathione Reductase
GSH	Glutathione
GSSG	Glutathione disulphide
H_2O	Water
H_2O_2	Hydrogen Peroxide
HORAC	Hydroxyl Radical Adverting Capacity
HPLC	High Performance Liquid Chromatography
KA	kainate
MeOH	Methanol
NMDA	N-methyl-D-aspartate

OBS – CSE	Opuntia Beja/Serpa – Conventional Solvent Extract
O_2^-	Superoxide anion
OGD	Oxygen-Glucose Deprivation
$OH^·$	Hydroxyl Radical
ORAC	Oxygen Radical Absorbance Capacity
PBS	Phosphate Buffer Saline
PD	Parkinson's Disease
PGI	Protected Geographical Indication
PLE	Pressurized Liquid Extraction
PLE – A	Pressurized Liquid Extract – A
PLE – B	Pressurized Liquid Extract – B
PLE – C	Pressurized Liquid Extract – C
ROS	Reactive Oxygen Species
SOD	Superoxide Dismutase
SPE	Solid Phase Extraction
TE	Trolox Equivalents
TFC	Total Flavonoid Content
TPC	Total Phenolic Content

1. Introduction

1.1. Neurodegenerative diseases

Countries are currently faced with problems derived from changes in population demography and an increase in lifestyle-related diseases. Neurodegenerative disorders such Parkinson's (PD) and Alzheimer's (AD) diseases are an increasing problem in aged societies like the Europe. There is an increased prevalence of both diseases with age *(1)*. Neurodegenerative diseases are a heterogeneous debilitating group of degenerative disorders characterized by a slow and progressive loss of neuronal cells. Such phenomena lead to gradual and progressive impairments of selective functions of central nervous system (CNS), depending upon the involved type of neuronal cells, and so far are incurable *(2)*. Data from World Alzheimer Report 2011 indicate that 36 million people worldwide are living with dementia, with numbers doubling every 20 years to 66 million by 2030, and 115 million by 2050. Alzheimer's disease has becoming the most common neurodisorder of today *(3, 4)*. And at financial and social level, AD represents an impact of €160 billion for the UE27 and €177 billion for whole Europe.

1.2. Neurodegeneration and Oxidative Stress

Neurodegenerative disorders appear to be triggered by multi-factorial events including neuroinflammation, depletion of endogenous antioxidants and increases in oxidative stress.*(1, 5, 6)*. The high metabolic rate and the low concentration of endogenous antioxidants as well as the proportion of polyunsaturated fatty acids make the brain tissue particularly susceptible to oxidative damage *(7)*. The most effective way to produce adenosine tri-phosphate (ATP) at cellular level is through oxidative phosphorylation within the mitochondria via the electron transport chain (ETC). The ETC is not completely efficient showing a basal level of electron leak. The reaction of leaked electrons with molecular oxygen produce a short-lived free radicals such superoxide anion ($O_2^{\cdot-}$), hydroxyl radical ($OH^{\cdot}$), and hydrogen peroxide (H_2O_2) called reactive oxygen species (ROS)*(8, 9)*. Under normal conditions, mitochondria have an efficient biochemical defense mechanism to neutralize the effect mediated by these free radicals. Endogenous defenses are composed of glutathione (GSH), glutathione peroxidise (GPx), glutathione reductase (GR), superoxide dismutase (SOD), NADPH, vitamins E and C *(10, 11)*. Generally, intracellular ROS are maintained at low but measurable level and are regulated by the balance between the rate of production and scavenging by the components of this biochemical defense mechanism. Oxidative stress is generally caused by an imbalance between ROS regeneration and antioxidant defenses and can lead to direct cellular organelles damage such proteins and nucleic acids and this fact can cause mitochondrial damage and eventual cell death *(12)*.

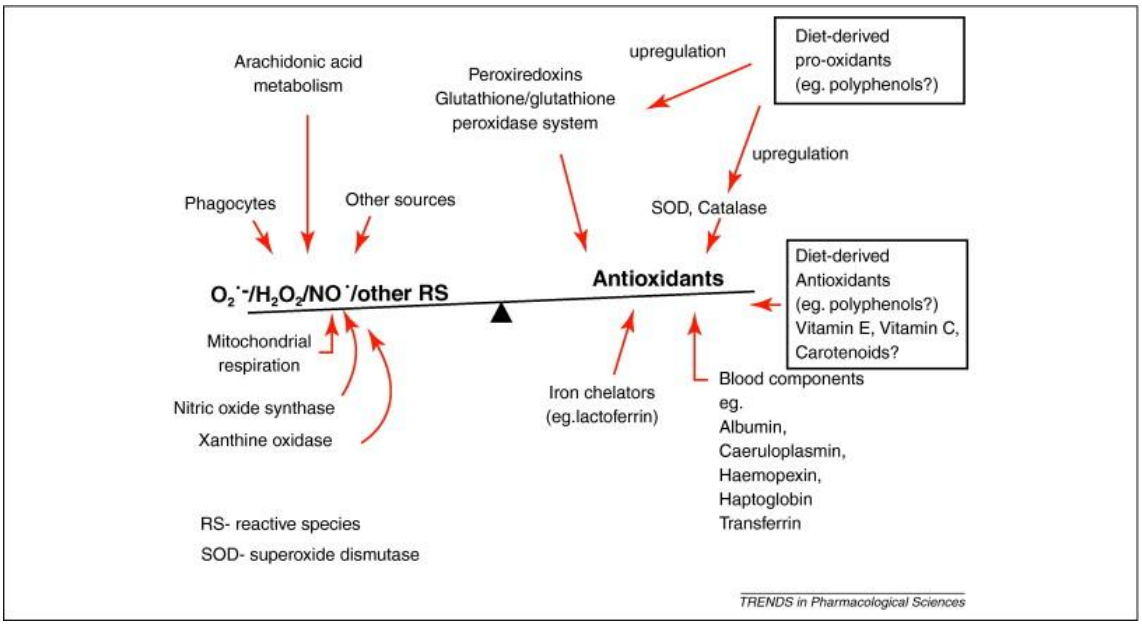


Figure 1.1. The approximate balance of antioxidants and reactive species *in vivo*.*(13)*

That is a large body of evidences demonstrating that the accumulation of iron ion species in the brain, the involvement of mitochondria, the decreased levels of endogenous antioxidants, and other ROS-mediated pathways are the major pathological factors that contributes to neurodegenerative diseases like PD and AD *(14, 15)*.

Biological systems have several mechanisms to protect themselves from the ROS consequences. These antioxidant defenses mechanisms include scavenging of ROS species and their precursors, binding catalytic metal ions needed for ROS formation, generating and up-regulating endogenous antioxidant defenses *(11)*. Central nervous system dysfunction has been observed in all diseases, related to errors in GSH metabolism, suggesting that the brain is particularly susceptible to alterations in GSH homeostasis. The reason for the sensitivity is unknown, although may be consider the possibility of the high susceptibility of the brain to oxidative stress, due to its high oxygen consumption*(10)*.

Glutathione (GSH) plays a central role in the cellular protection mechanism against oxidative and other forms of stress. Glutathione is present in both reduced (GSH) and oxidized (GSSG) state and the redox state of the GSH/GSSG couple can serve as an important indicator of cell redox environment. It has been described that a decrease in neuronal GSH levels leads to mitochondrial dysfunction and apoptosis.

1.2.1.Neurodegeneration Cell Model

Studies to determine biological effect of specific phytochemicals and plant-based foods involve experimentation with *in vitro* and *in vivo* systems. *In vitro* systems present advantages as a first approach, testing their potential effects within cells. They can provide an indication of biological activity for the phytochemical in question, which can be used to design animal experiments in the future. The major benefits of the use of in vitro cell models is practical convenience, such as

ease of culturing, their relatively low cost, and moderate throughput capabilities *(16)*.

The creation of a cellular model based in the application of oxidative damage in neurons may be helpful to understand the molecular mechanisms underlying in the development of neurodegenerative diseases. Therefore, exposure of cultured neurons to relatively low concentrations of H_2O_2 induces changes in cell metabolism and lead to a moderate neuronal death, reproducing what may occur during neurodegeneration process *(17)*.

In the Disease and Stress Biology (DSB) lab, SK-N-MC cell line is used as neuronal cell model and is a continuous cell line, obtained from human metastic neuroblastoma tissue *(17-19)*.

1.3. Nutraceuticals and Functional Foods

It is well known that consumption of plant-based foods such fruits, vegetables, grains and cereals have key role in health promotion and disease prevention *(20)*. There has been an increased concern for consuming health-promoting food products and two new concepts appear in the market: Nutraceuticals and Functional Foods. The short-term goal of nutraceuticals and dietary supplements is to improve the quality of life and enhance health status while its long-term goal is to increase lifespan while maintaining health *(20)*.

The term nutraceutical was coined from nutrition and pharmaceutical (Fig. 1.2) in 1989 by Stephen DeFelice, founder and chairman of foundation for innovation in medicine, an American organization which encourages medical health *(21)*. Nowadays the term Nutraceutical is defined as “a product isolated or purified from the food, generally sold in medicinal form not associated with food and demonstrated to have a physiological benefit .It also provides benefit against chronic disease” *(21)*. Such products may range from isolated nutrients, dietary supplements to genetically engineered designer foods and herbal products and can be found in presentations similar to drug such pills, extracts or tablets *(22)*.

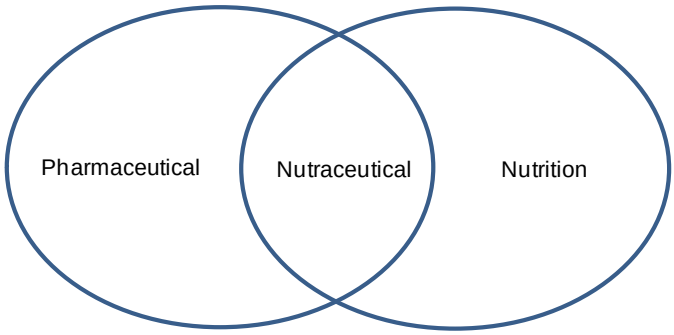


Figure 1.2. The term "nutraceutical" was coined from "nutrition" and "pharmaceutical" in 1989 by Stephen DeFelice, MD, founder and chairman of the Foundation for Innovation in Medicine (FIM), Cranford, NJ. Adapted from Pandey *et al*. 2010 .

Functional foods represents a type of food that when consumed regularly exert a specific health-beneficial effect (i. e., a healthier status or a lower risk of disease) beyond their nutritional properties, and this effect must be scientifically proven *(22, 23)*. From consumers point of view,

functional foods and nutraceuticals may offer many benefits such as an increasing of health value of our diet, help people to live longer, help to avoid particular medicinal conditions, be perceived to be more "natural" than traditional medicine and less likely to produce unpleasant side-effects and present food for population with special needs. Some of the most common bioactive ingredients found in nutraceutical and functional food market are bioactive non-nutrient plant compounds. These compounds called phytochemicals, have raised interest in human nutrition because of their potential effects as antioxidants, antiestrogenics, anti-inflammatory, immunomodulatory and anticarcinogenics *(22, 23)*. Nutraceuticals are able to exert neuroprotection via a wide range of proposed mechanisms, such as scavenging of free radicals and ROS, metal chelation, modulation of cell-signalling pathways, and inhibition of inflammation *(24)*. Most of phytochemical compounds with biological activities present in nutraceuticals and functional foods are isolated from natural sources using several extraction techniques.

1.4. Phytochemicals

The composition of foods cannot be reduced to the macronutrients and essential micronutrients. Foods also contain a large number of other compounds that, although not essential, also have influence in human health. Several hundreds of phytochemicals such as polyphenols, carotenoids, glucosinolate, saponins or alkaloids have been identified in foods of plant origin. Some of these compounds may contribute to explain the beneficial health effects of consuming fruits and vegetables *(25)*.

Polyphenols constitute a large group of phytochemicals with more than 8000 indentified compounds *(26)* and generally, involved in defence against ultraviolet radiation or aggression by phatogens *(27)*. Polyphenols may be classified in several ways such as a classification based on structure and function. Simple phenols are substances containing only one aromatic ring and having at least one phenolic hydroxyl group and have the possibility of have other substituent. Phenols and polyphenols may occur as unconjugated aglycones or, as conjugates, frequently with sugar or organic acids. Flavonoids is the most extensively studied polyphenols, all characterized by a C_6-C_3-C_6 structure, subdivides by the nature of C_3 element into anthocyanins, flavanols, flavanones, flavones, flavonols, isoflavones as shown in figure 1.3 *(28)*.

Epidemiological studies suggest that high dietary intake of polyphenols is associated with decreased risk of a range of diseases including cardiovascular disease (CVD), specific forms of cancer and neurodegenerative diseases such AD *(29)* and PD *(30-31)*. A regular dietary intake of flavonoid-rich foods and/or beverages has been associated with 50% reduction in the risk of dementia, a preservation of cognitive performance with ageing, a delay in the onset of Alzheimer's disease and a reduction in the risk of developing Parkinson's disease *(1)*. Many studies have reported the bioavailability of polyphenolic compounds in the systemic circulation *(32-34)*. However, less is known about their degree of brain bioavailability, flavanones such as hesperetin, naringenin and their *in vivo* metabolites, have been shown to traverse the blood brain barrier (BBB) in relevant *in vitro* and *in situ* models *(35).* Moreover, several anthocyanins

have also been identified in the cortex and cerebellum of rat and pig following feeding with blueberries.

Together, these results suggest that polyphenols are able to transverse the BBB, in different degrees and depending on their structure. Thus, such compounds are likely to be candidates for direct neuroprotective and neuromodulatory actions *(1)*. The contribution of phenolic compounds to the protection against degenerative diseases as well as their effects on health has been attributed to their antioxidant activities *(36, 37)*, but also to their cellular modulatory actions through a direct interaction with receptors or enzymes involved in signal transductions, such as protein and lipid kinases signaling pathways. Therefore, a wide range of mechanisms have been described to explain polyphenol's health benefits such an antioxidant action by scavenging radicals, a induction of endogenous antioxidants (glutathione peroxidase, glutathione reductase or superoxide dismutase); iron chelating properties; modulation of genes related to cell survival/death modulation, gene/protein and cell signaling pathway regulatory activity as well as regulation of mitochondrial function *(38-40)*.

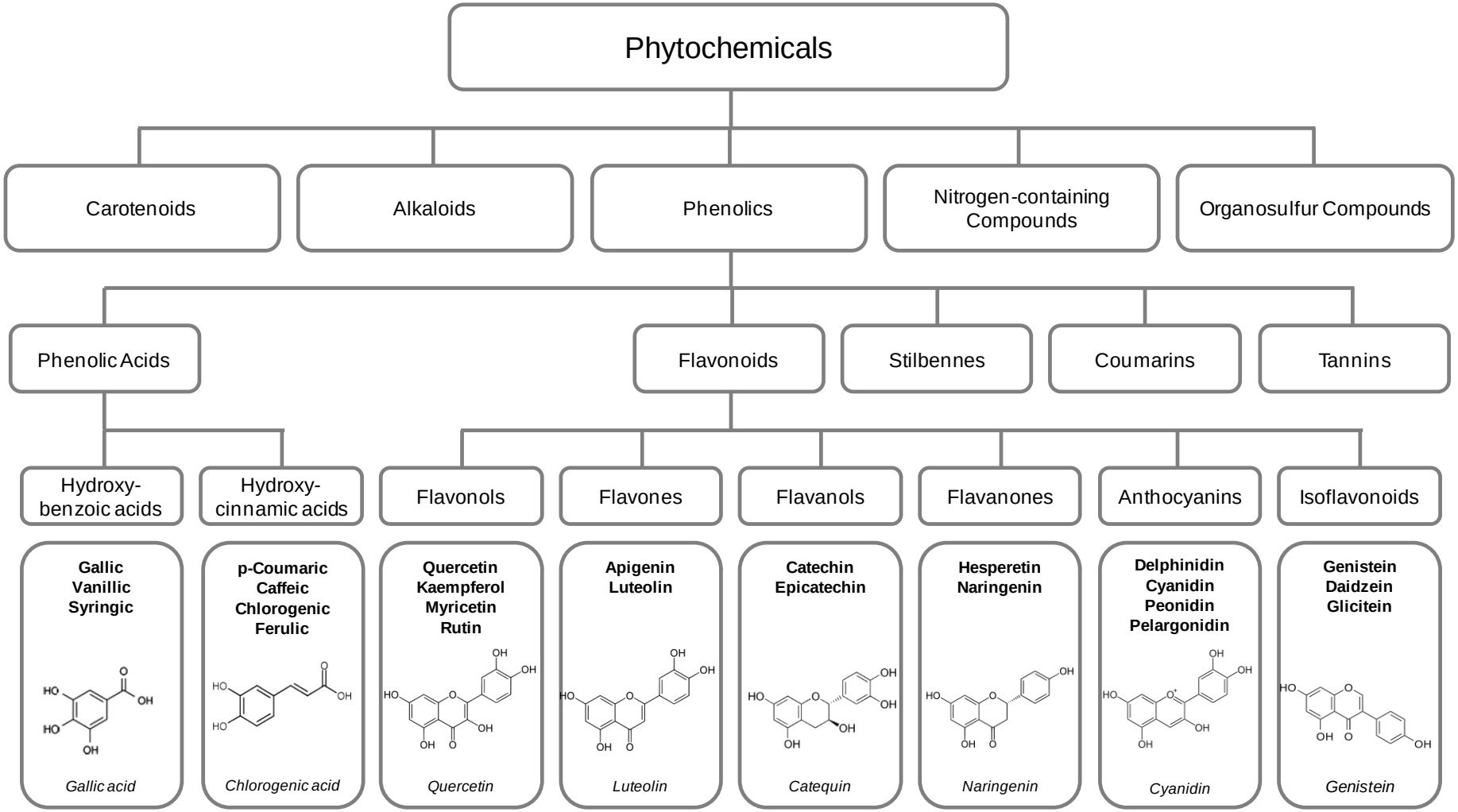


Figure 1.3. Classification of dietary phitochemicals (Adapted from Liu, 2004) . *(41)*

Table 1.1. Polyphenols effects in *in vivo* and *in vitro* models of neurotoxicity and neurodegeneration (Adapted from Ebrahimi *et al*. (2012). *(14)*

Substance	Cell line/ Animal model	Effect	
Aloe-emodin	N-methyl-d-aspartate (NMDA)-induced toxicity in retinal ganglion cells (RGCs)	– Elevates levels of RNA and protein expression of superoxide dismutase (SOD); Attenuates NMDA-induced apoptosis of RGCs	*(42)*
Curcumin	N27 dopaminergic neurons	– Protects against mitochondrial complex I inhibition (leading to mitochondrial dysfunction) and NS	*(43)*
Curcumin	Homocysteine-induced neurotoxicity in rats	– Reduces Malondialdehyde (MDA)a and Superoxide anion levels; Reduces lipid peroxidation; Improves learning and memory in rats	*(44)*
Curcumin	N27 dopaminergic neuronal cell line	– Increases glutathioneb (GSH) levels	*(45)*
Curcumin	3-Nitropropionic acid (3-NP)-induced neurotoxicity in rats	– Improves the 3-NP-induced motor and cognitive impairment; Attenuates 3-NP-induced OS (including lipid peroxidation, reduced GSH and nitrite activity); Restores the decreased succinate dehydrogenasec activity	*(46)*
Epigallocatechingallat (EGCG)	Glucose oxidase-induced neurotoxicity in H 19-7 (a rat neuronal cell line)	– Enhances cellular resistance to glucose oxidase-mediated oxidative damage; Elevates heme oxygenase-1d (HO-1) mRNA and protein expression; Activates transcription factor Nrf2e	*(47)*
EGCG	Glutamate-induced toxicity in HT22 mouse hippocampus neuronal cells, Kainic acid-induced neurotoxicity in Rats	– Reduces glutamate-induced oxidative cytotoxicity; Inactivates the NF-_B signaling pathway; Reduces ROS accumulation and NF-_B transcriptional activity	*(48)*
EGCG	Transient global cerebral ischemia C57BL/6 in mice	– Reduces the development of delayed neuronal death after transient global cerebral ischemia in mouse brain	*(49)*
EGCG	Age-associated oxidative damage in rat brain	– Amplifies the activities of enzymic antioxidants like SOD, catalase, glutathione peroxidase, glutathione reductase and glucose-6-phosphate dehydrogenase; Improves the activity of non-enzymic antioxidants like tocopherol, ascorbic acid and glutathione;Ameliorates the MDA and protein carbonyl levels	*(50)*
EGCG	Progressive neurotoxic model of long-term serum deprivation in human SH-SY5Y neuroblastoma cells	– Decreases protein levels and mRNA expression of the beta subunit of the enzyme prolyl 4-hydroxylase; Decreases protein levels of two molecular chaperones that are associated with HIF regulation, the immunoglobulin-heavy-chain binding protein and the heat shock protein 90 beta	*(51)*
EGCG	SOD1-G93A transgenic mice (a model of ALS)	– Maintains the normal expression of p85a PI3-K, pAkt, and pGSK-3 (molecular signals of survival); Reduces activation of NF-kB and the cleaved form of caspase-3; Reduces microglial activation; Prolongs the life span; Delays the onset of symptoms	*(52)*
Mangiferin	Glutamate-induced neurotoxicity in rat cerebral cortex neurons	– Prevents neuronal death, oxidative stress and mitochondrial depolarization	*(53)*
Mangiferin	1-Methyl-4-phenyl pyridinium (MPP(+))-induced oxidative stress in the murine neuroblastoma cell	– Restores the GSH content (to 60% of control levels), and down-regulates both SOD and catalase mRNA expression	*(54)*

	line N2A	–	Quenches reactive oxygen intermediates	
Mangiferin Morin	Glutamate-induced neurotoxicity in rat primary culture of neurons	–	Reduces ROS formation; Activates enzymatic antioxidant system; Restores mitochondrial membrane potential	*(55)*
Polyphenol-rich *Hedeoma multiflorum* extract	Biochemical assay on rat brain Homogenates	–	Inhibits lipid peroxidation; Scavenges 2,2_-diphenyl-1-picrylhydrazyl (DPPH) radicals	*(56)*
Polyphenol-rich osmanthus fragrans extract	Glutamate, arachidonic acid, and 6 hydroxydopamine-induced neurotoxicity in rat primary cortical neurons	–	Scavenges DPPH and hydroxyl anions Inhibits lipid peroxidation	*(57)*
Red wine polyphenol compound	Rat model of ischemic cerebral stroke	–	Prevent the burst of excitatory amino acids in response to ischemia; Reduce brain infarct volumes; Enhance the residual cerebral blood flow during occlusion and reperfusion; Modulate expression of proteins involved in the maintenance of neuronal caliber and axon formation	*(58)*
Resveratrol	Lipopolysaccaride (LPS)-induced dopaminergic neurodegeneration in rat	–	Reduces NADPHg oxidase-mediated generation of ROS; Inhibits microglia activation; Attenuates the activation of MAPK and NF-_B signaling pathways; Implies neuroprotection against LPS-induced dopaminergic neurodegeneration	*(59)*
Resveratrol	Glutamate-induced toxicity in mice primary culture of neurons; Optimized ischemic-reperfusion stroke model in mice	–	Induces heme oxygenase 1d (HO-1) in dose- and time-dependent manner; Protects mouse neurons, subjected to an optimized ischemic-reperfusion stroke model; Protects neurons against ecitotoxicity	*(60)*
Resveratrol	1-methyl-4-phenyl-1,2,3,6 tetrahydropyridine (MPTP)- induced Parkinson in mice	–	Protects from MPTP-induced motor coordination impairment, hydroxyl radical overloading, and neuronal loss; Scavenges free radicals	*(61)*
Resveratrol	MPP(+)-induced neurotoxicity in dopaminergic neurons of midbrain slice culture	–	Prevents accumulation of ROS, depletion of cellular glutathione, and cellular oxidative damage induced by MPP(+); Activates sirtuin family of NAD-dependent histone deacetylases	*(62)*
Resveratrol	A-beta induced toxicity in neurons from a mouse model (Tg2576 line) and mouse neuroblastoma (N2a) cells	–	Maintains normal expression of peroxiredoxins and mitochondrial structural genes; Maintains normal mitochondrial function	*(63)*
Tea polyphenols	NMDA-induced neurotoxicity in mice	–	Attenuates the increased production of synaptosomal ROS; Reduces the deteriorative ROS-sensitive $Na^{(+)}$, K(+)-ATPase and $Mg^{(2+)}$-ATPase activity	*(64)*

1.5. Extraction Technologies

Amongst fruits, vegetables and herbs, agricultural and industrial by-products are attractive sources for extraction of compounds with nutraceutical interest such pigments or natural antioxidants. Special attention has been focused on the extraction from inexpensive or residual sources from agricultural industries. Some studies have already been done on by-products, which could be potential sources of antioxidants *(65)*. Once the industrial processing of a wide variety of fruits results in the accumulation of large quantities of residues, the recovery of these food industry by-products could reduce waste disposal problems and serve as a potential new source of phytochemicals that can be used in nutraceutical and functional food market creating value-added applications. In order to obtain such value compounds, extraction techniques have been investigated. Conventional Solvent Extraction (CSE) is a traditional extraction method characterized by a solid-liquid principle. Solid-liquid extraction can be defined as a mass transport phenomenon in which solids contained in a solid matrix migrate into a solvent brought into contact with the matrix. The single-stage system represents the complete operation of contacting the solid matrix with the fresh solvent. However, these techniques have, generally, negative environmental impact and some drawbacks such as long extraction times, large amounts of initial matrix and organic solvents and lower extraction yields *(65)*.

An attractive and alternative methodology to conventional extraction, to produce extracts for pharmaceutical or nutraceutical application is Pressurized Liquid Extraction (PLE). The technique involves the utilization of water and/or organic solvents at considerable elevated temperatures (313 – 473 K) and pressures (3.3 – 20.3 MPa). Liquid carbon dioxide is found to be a good extraction solvent in food industry due to its physicochemical properties, low toxicity and price. Since carbon dioxide is non-polar specie it is not suitable solvent to extract polar polyphenols. The addition of solvents like ethanol increases the solvating power of carbon dioxide and the extraction yield of phenolic compounds. Ethanol is a permitted co-solvent in food industry.The advantages of PLE technique are usually related with the usage of carbon dioxide *(66)* that has been described as a “green solvent” and gives to the method improved characteristics in terms of mass transfer and of solvation properties*(66, 67)*. Extraction time can be decreased using high temperature and high pressure: there is enhanced diffusivity of the solvent and, at the same time, there is the possibility of working under an inert atmosphere and with protection from light *(68)*.

Adsorption technology (ADT) is a commonly applied process to recover bioactive compounds from plant materials and consists on a solid phase extraction (SPE). SPE consists in a partitioning of compounds between two phases. The compounds presents in the liquid phase and these analytes must have a greater affinity for the solid phase than for the sample matrix. Compounds retained on the solid phase can be removed at a later stage by eluting with a solvent with a higher affinity for the analytes (elution or desorption step). The different retention and elution mechanisms are due to intermolecular forces between the analyte, the active sites on the surface of the solid phase and the liquid phase or matrix *(69)*.

The adsorption capacity of an adsorbent for a solute may vary with processing conditions such as temperature and pH value of solution. The elution step is usually performed with alcohols, in particular ethanol as it is acceptable for food and pharmaceutical applications.

The adsorption processes can be performed in a batch model *(70)* or in continuous, where the adsorbent is packet into a column *(71)*. The use of resins for food production proposes is regulated by the US Food and Drug Administration (FDA) and the Council of Europe.

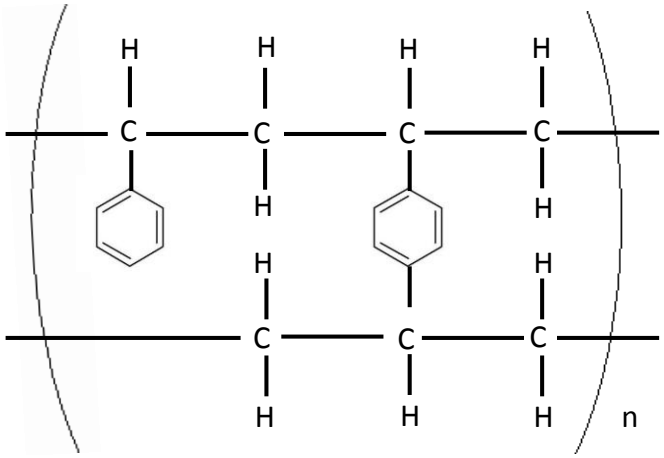


Figure 1.4. Chemical structure of Amberlite® XAD 16.

The selection of adsorbents is critical for the development of an adsorption process. Adsorbent characteristics, such as particle size, surface, area and porosity should be taken in consideration in each case study. For the purification of polyphenols various adsorbents have been used *(72)* One of the most commonly applied is a polymeric resin Amberlite® XAD16 (Figure 1.7) *(73)*.

1.6. *Opuntia ficus-indica*

Opuntia ficus-indica (cactus pear) is the larger member of *Cactaceae* family that mainly grows in arid and semi-arid regions and is largely distributed in Mexico, much Latin America, South Africa and Mediterranean area*(74)*. *Opuntia* fruits are oval and elongated berries of 67 to 216 g weigh. Cactus pear fruits have a thick pericarp (peel) and a juicy pulp with many seeds. The pulp is the edible part of the fruit and is mainly composed of water (84-90%) and reducing sugars (10-15%). Fruit seeds represent about 10-15% of the edible pulp and are usually discarded as waste after pulp extraction *(75)*. The high season for harvesting *Opuntia* cactus fruits is between the end of July and November in the Mediterranean regions *(76)*. Fruit and stems of *Opuntia* species have been used in folk medicine for burns, wounds, edema, bronchial asthma, hypertension, indigestion, and type II diabetes *(77)*. They nutritional properties have long been known and it is also used in traditional medicine. In the industrialized countries of Mediterranean area, cladodes are not consumed but the fruits are largely used as nutritional sources *(76)*. The plant is used mainly for fruit production and the fruit are consumed as fresh fruit or juice. Although in some countries it is used as a vegetable for fodder *(78, 79)*.

Figure 1.5. Images of *Opuntia ficus-indica* cladodes and fruits.

Cactus pear was largely ignored by the scientific community until the beginning of the 1980's when several studies were published on their biological functions. In recent years, investigations on the chemical and nutritional value of *Opuntia spp*. have attracted attention in the food, nutritional and pharmacological sciences *(80, 81)* . Based on various studies, fruit pulp is considered a good source of minerals, especially calcium, potassium and magnesium and phosphorous*(74)*. The concentration in bioactive compounds is dependent on the cultivar, cultivation site, maturation stage and environmental conditions.

Fruit is commonly consumed fresh but there is increased interest in the transformation into different products such as juice, jam and jelly among others. After being consumed or transformed, fruit peels and seeds are discarded and taking in account they composition in phytochemicals make them very attractive as sources for extraction. After extraction these by-products can be used as addictive in food preparation or in the pharmaceutical and nutraceutical market *(82)*. The nutraceutical benefits of *Opuntia* spp. fruits are believed to be related to their content in ascorbic acid and phenolic compounds, including flavonoids, and a mixture of yellow betaxanthin and red betacyanin pigments. Betaxanthins and betacyanins belong to betalains group that are water soluble compounds described as excellent radical scavengers.*(79)*

In vitro studies have demonstrated that a variety of compounds present in *Opuntia* sp. fruits are able to exert neuroprotective activity. The methanolic extract of *O. Ficus-indica* produced dose-dependent neuroprotective effects on hydroxyl- and superoxide radical-mediated neuronal damage to mouse primary cultures *(83)* and the pre-treatment of cultured neurons and glia cells with methanolic extract of *Opuntia* fruits inhibited N-methyl-D-aspartate (NMDA)-, kainate (KA)-

and oxygen-glucose deprivation (OGD)-induced neurotoxicity dose-dependent.*(84)* Kim and collaborators (2006) also have demonstrated that when gerbils receive different doses of the Opuntia extract, the neuronal damage in the hippocampus was reduced. Moreover *Opuntia* polysaccharides as described to exert a neuroprotective activity against a H_2O_2-induced oxidative damage in PC12 cells by maintain cell viability, reduce apoptosis and decrease intracellular ROS levels.*(77)*

1.7. *Prunus avium*

Prunus avium (sweet cherries) are very attractive fruits for consumers due to their taste, colour, sweetness and wealth of nutrients being easily adapted to a regular diet. Portugal produces more than 15 000 tons of cherries every year. It is in the northeast of the country, in the region of Beira Interior, where the fruit is more cultivated and the cherries has protected geographical indication (PGI) registration according to the European Union (EU) regulations.

Figure 1.6. Cherries (*Prunus avium*) from *Saco* cultivar collected in “Cova da Beira” region, Portugal.

Saco cultivar is an old traditional cherry. It is a very promising functional fruit due to its powerful antioxidant attributed to the phenolic composition *(85)*. Serra *et al.* evaluated nine “Cova da Beira” cherry varieties in terms of polyphenolic content and bioactivity (antioxidant properties) and it was shown that *Saco* cherry extract has a strong antioxidant activity. However there is little information about cherries biological activities. However, due to its small size and weight the acceptance of *Saco* cherry by consumers could be compromised since larger fruits are preferred.

It has been described that sweet cherries cultivars such as *Saco* contain various anthocyanins with the total anthocyanins in a range of 19.4 to 95.7 mg.100 g^{-1} of fresh cherries. Serra and

collaborators described that sweet cherries contain cyaniding -3- glucoside and cyaniding-3-rutinuside being that 77.3 to 86.6 % of the total anthocyanins content. Among hydroxycynamic acid derivates, cherries had neochlorogenic acid and *p*-coumaroylquinic acid as the predominant compounds and the glycosides of peonidin and pelargonidin as minor compounds *(86)*. Cyaniding -3- glucoside is described as able to transpose the BBB. *(87)*

Nevertheless, there is little information available on the possible health benefits of phenolic compounds present in fresh cherries on animal cells exposed to cell-damaging oxidative stress.

Taking this in account the major aim of this work is to obtain and characterize natural extracts obtained from *Opuntia ficus-indica* and *Prunus avium*, residues using different extraction technologies, evaluate their neuroprotective effect in a H_2O_2-induced neurodegeneration cellular model and understand the cellular mechanisms behind their potential bioactivity.

2. Materials and Methods

2.1. Materials

For phytochemical characterization: Amberlite® XAD 16 and sodium carbonate (Na_2CO_3) were purchased from Sigma- Aldrich (St Quentin Fallavier, France), Folin Ciocalteau reagent was acquired from Panreac (Barcelona, Spain) and gallic acid from Fluka (Germany). Sodium nitrite ($NaNO_2$) was purchased from Riedel-de-Haën (Seelze, Germany), aluminium chloride *(*$AlCl_3$) and sodium hydroxide (NaOH) was obtained from Sigma-Aldrich, in Germany. (+)-Catechin hydrate was purchased from Sigma (Japan).

For antioxidant activity assays chemicals used were: 2‟,2‟-Azobis (2- amidinopropane) dihydrochloride (AAPH), 6-hydroxy-2,5,7,8- tetramethylchroman-2-carboxylic acid (Trolox), caffeic acid ($C_9H_8O_4$), cobalt floride tetrahydrate (CoF_2), hydrogen peroxide (H_2O_2) and picolinic acid ($C_6H_5NO_2$) were purchased from Sigma-Aldrich (St Quentin Fallavier, France). Disodium fluorescein (FL) was from TCI Europe (Antwerp, Belgium). Sodium chloride (NaCl), potassium chloride (KCl) and monopotassium phosphate (KH_2PO_4) were from Sigma-Aldrich (St Quentin Fallavier, France) and sodium phosphate dibasic dehydrate ($Na_2HPO_4{\cdot}2H_2O$) from Riedel-de Haën (Seelze, Germany) were used for phosphate buffer solution preparation (PBS).

Cell based assays were performed using: 2',7'-dichlorofluorescein diacetate (DCFH-DA), hydrogen peroxide (H_2O_2), sodium dodecyl sulphate (SDS), GSH and GSSG standards, orthophthalaldehyde (OPA), Eagle Minimum Essential Medium (EMEM), glutamine, non-essential amino acids, sodium pyruvate, were purchased from Sigma-Aldrich (St. Quentin Fallavier, France), Disodium fluorescein was obtained from TCI Europe (Antwerp, Belgium), CellTiter – Blue Reagent® Cell Viability Assay was obtained from Promega (San Luis Obispo, CA, USA). Fetal bovine serum (FBS), Trypsin-EDTA and penicillin-streptomycin (PenStrep) were obtained from Invitrogen (Gibco, Invitrogen Corporation, Paisley, UK). Phenolic standards used were: chlorogenic acid from Sigma-Aldrich (St. Louis, MO, USA), and cyanidin-3-glucoside, cyanidin-3-rutinoside were all from Extrasynthèse (Genay, France). Ultra-pure water (18.2 MΩ.cm) was obtained from a Millipore Direct Q3 UV system (Millipore, USA).

2.2. Raw Materials

Cactus pear (*Opuntia ficus-indica)* fruits were collected at Beja/Serpa, Portugal, in September 2011. After remove the spikes, fruits were processed using a kitchen robot (UFESA, LC5005, China) and the peels and seeds were collected. The waste (peels and seeds) was kept at -20^{o}C.

Sweet cherries (*Prunus avium*), from *Saco* cultivar, grown in Cova da Beira region, Portugal, were harvested by hand between May and June of 2011. The whole fruits were processed with seeds and stalks, milled and frieze dried. Milled cherries were kept at – 20 ºC.

2.3. Preparation of *Prunus avium* and *Opuntia ficus-indica* Extracts

2.3.1.*Opuntia ficus-indica* Extracts

2.3.1.1. Conventional Solvent Extraction

Opuntia ficus-indica fruits were processed into juice and the peels and seeds were collected and submited to a conventional solid-liquid extraction with a biocompatible mixture: ethanol (EtOH): water (H_2O) (1:1 v/v) solution (1:20, w/v). The extraction were processed in the dark for 4 h under constant agitation (200 rpm), at room temperature (IKA® dual-speed mixer RW 20.n, Aldrich, St. Luis, USA) and protected from light. The homogenates were centrifuged at 9000 rpm for 10 min. The supernatants were filtered under vacuum and the solvent was evaporated in a rotary evaporator (Buchi, Switzerland) at 40 ºC. The extract was freeze dried at and kept at – 20 ºC until analyses.

2.3.1.2. Pressurized Liquid Extraction

Extracts obtained during pressurized liquid extraction from *Opuntia ficus-indica* fruits residues were kindly provided by Dr. Hermínio de Sousa and his laboratory team group (Laboratory of Polymer Processing and Supercritical Technology, Universidade de Coimbra, Portugal). Three different extracts were obtained at 313 K and 200 MPa using three different EtOH/CO_2/H_2O mixtures (table 2.1). The extract was designated as PLE-A for the 0.4/0.3/0.3 mixture, PLE-B and PLE-C for 0.5/0.4/0.1 and 0.5/0.1/0.4 mixtures, respectively.

Table 2.1. Molar fraction of Pressurized Liquid Extraction (PLE) solution.

Extracts	Molar Fractions		
	EtOH	**CO_2**	**H_2O**
PLE – A	**0.4**	**0.3**	**0.3**
PLE – B	**0.5**	**0.4**	**0.1**
PLE – C	**0.5**	**0.1**	**0.4**

2.3.2.*Prunus avium* Extract

2.3.2.1. Conventional Solvent Extractio (CSE)

After dehydratation by freeze dryer, the cherries were homogenized in a grinder (UFESA, LC5005, China). Powdered cherries were extracted in the dark with EtOH:H_2O (1:1, v/v) solution (1:20, w/v), for 2 h at room temperature, under constant agitation (200 rpm) (IKA® dual-speed mixer RW 20.n, Aldrich, St. Luis, USA). The homogenates were filtered under vacuum and the solvent was evaporated in a rotary evaporator (Buchi, Switzerland) at 40 ^{o}C. The remaining extracts were diluted in bi-distilled water. The extract was kept at – 20 º C.

2.3.2.2. Adsorption Technology (AD)

To concentrate in anthocyanins of *Prunus avium* extract obtained on 2.3.2.1. section, food grade macroporous resin Amberlite® XAD-16 was used as adsorbent. This resin is allowed for food applications by the U.S. Food and Drug Administration Code of Federal Regulation Title 21 *(88)*.

The preconditioning of the adsorbent was done by an extensive wash with abundant distilled water to remove salts and impurities, and was then dried at 70 ^{o}C for 24 h. The dried resin was immersed in EtOH (96%) for 12 h. EtOH was then replaced by bi-distilled water through washing.

The production of cherry polyphenols rich extract was performed in batch mode. Briefly, aqueous crude cherry extract (CCE) obtained previously (section 2.3.2.1) were maintained in contact with the resin in a ratio of 80 mg GAE.g^{-1} of resin, protected from light and submitted to 200 rpm of agitation, during 4 h. After 4 h of contact, supernatant was removed and resin washed three times with bi-distilled water. Anthocyanins were then eluted two times with EtOH (96 %). The ethanolic fraction were concentrated by evaporation, freeze dried and kept at – 20 ºC.

2.4. Extracts Characterization

The extracts were analyzed for their chemical composition and antioxidant capacity against peroxyl and hydroxyl radicals. Composition in polyphenols was determined using Folin-Ciocalteau colorimetric method and flavonoids content was performed by the $AlCl_3$ complexation method *(89)* modified by Tavares et al, 2010. Antioxidant activity was assessed using two different in vitro chemical assays, ORAC and HORAC adapted by Feliciano *et al*, 2009 and Serra *et al*, 2011, respectively. Extracts phenolic profile were obtained by High Performance Liquid Chromatography with Diode Array Detector and Electrochemical Detector (HPLC – DAD – ED).

2.4.1. Total Phenolic Content (TPC)

Total phenolic content present in extracts was determined according to the Folin- Ciocalteau method. To perform the colorimetric method, 20 µL of the appropriate dilutions of extracts were added to 1580 µL of distilled water. Than 100 µL of the Folin Ciocalteau reagent was added. The reaction was neutralized with 300 µL of sodium carbonate. The absorbance, at 765 nm of each sample was measured after 40 min at 40 ºC of incubation, in a Genesys10uv spectrometer (Thermo Spectronic, New York, USA). The results were expressed as means of independent triplicates as mg of Gallic Acid Equivalents (GAE) per gram of dry weight extract (mg GAE.g^{-1} *dw*). A calibration curve was made using Gallic Acid as standard.

2.4.2. Total Flavonoid Content (TFC)

The total flavonoid content was determined using a spectrophotometric method adapted to 96-well microplate by Tavares *et al,* 2010. Briefly, 25 µL of each sample, 125 µL of distilled water and 7.5 µL of $NaNO_2$ 5% (v/v) was added to each well of a 96-well plate. The plate was incubated for 6 min at room temperature. Than 15 µL of a 10% $AlCl_3$ (v/v) solution was added. After 5 min of incubation, 100 µL of NaOH 1 M was added and the solution of each well mixed. The absorbance was measured at 510 nm in a microplate reader (BioTek™ Power Wave XS). (+)-Catechin hydrate, minimum 98% (w/w) was used as standard, and the results are expressed in means of triplicates as mg catechin equivalents (CE) per mg of dry weight extract (mg CE.mg^{-1} *dw*).

2.4.3.HPLC – DAD – ED analysis

HPLC analysis of *Opuntia ficus-indica* and *Prunus avium* fruit residues extracts were performed by Analytical Group of IBET (Oeiras, Portugal) coordinated by Dr. Rosário Bronze. Briefly the analysis were performed on a Waters® Alliance 2695 (Waters) equipped with a quaternary pump, solvent degasser, auto sampler and column oven, coupled to a Photodiode Array Detector Waters 996 PDA (Waters). A pre-column (RP-18, 5 µm, Lichrocart) and reverse phase column (RP-18, 2.5 µm, from Manu-cart) with oven at 35 ºC were used for separation. The gradient mobile phase consisted of 0.1 % phosphoric acid p.a. in ultra pure water (A): 0.1 % phosphoric acid p.a. in LC-MS grade acetonitrile (B) and ultra pure water at a flow rate of 0.30 mL.min^{-1}. The injection volume was 5 µL.Photodiode Array Detector was used to scan wavelength absorption from 210 to 600 nm.

For HPLC quantification of compounds in cherry extract, the mobile phase used consisted of a gradient mixture of eluent A water:formic acid (90:10 v/v) and eluent B acetonitrile:water:formic acid (40:50:10 v/v/v). The following gradient of eluents was used: 0- 15min from 0 until 20% of eluent B; 10 min with 20% eluent B; 25–70 min, from 20 until 70% eluent B; 70–75 min, with 70% of eluent B; 75–85 min from 70 until 100% eluent B; 85–90 min, with 100% eluent B; 90-95 min from 100 to 0% of eluent B; and 95-100 min 100% of eluent A. The solvent flow rate was 0.7 mL.min^{-1}. The injection volume was 20 µL. Acquisition range was set between 190 and 700 nm and chromatogram was monitored at 280, 360, and 527 nm. Chlorogenic acid, Cyanidin glucosides, rutin and quercetin were quantified using standard compounds. Coefficients of variation on the HPLC quantifications were <5% and final concentrations were expressed as mg.g^{-1} dry extract.

2.4.4.TLC analysis

Presence of terpenoids compounds was assessed in the three PLE extracts by thin layer chromatography (TLC) using Lupeol as standard. 10 µL of extracts and standard were carefully placed in a Plate Silica gel 60 F_{254}. The mobile phase was an acetate:methanol:water (77:15:8) solution. In order to reveal the terpenoid compounds present Liebermann – Burchard reagent were prepared adding carefully 5 mL of acetic anhydride and 5 mL concentrated sulphuric acid to 50 mL absolute ethanol, while cooling on ice. The TLC plate is sprayed with freshly prepared solution, heated for 10 min at 100 ºC and inspected in UV-365nm.

2.5. Extracts Chemical Antioxidant Activity

2.5.1.Oxygen Radical Absorbance Capacity (ORAC)

The oxygen radical absorbance capacity was assessed by ORAC modified by Ou. *et al*. 2001*(90)*. The oxidation of disodium flourescein (FL) is catalyzed by peroxyl radicals generated by 2, 2'-azobis(2-amidopropane)dihydrochloride (AAPH) at 37 ^{o}C. ORAC assay measure the ability of the antioxidant compounds present in the sample to inhibit that oxidation.

The protective effect of an antioxidant specie present in the sample is obtained by the difference between the area under the fluorescence decay curve (AUC) of the sample and the blank, in which no antioxidant is present. Briefly, sodium fluorescein (150 µL; $2{,}0x10^{-8}$ M in PBS 75 mM pH 7,4) and samples or standards (25 µL) were added to a 96-well microplate and incubated during 10 min at 37 ºC. AAPH (25 µL,$1.28x10^{-2}$ prepared in PBS 75mM pH 7,4) were added to each well. FL800 (Bio-Tek Instruments) microplate fluorescence reader was used (λex: 485 nm, λem: 530 nm). The blank contained PBS (pH 7.4, 75 mM) instead of sample and 5 to 50 µM of Trolox was used to the calibration curve. The results were expresses as means of independent triplicades as µmol of Trolox Equivalents (TE) *per* gram of dry weight extract (µmol TE.g^{-1} *dw*).

2.5.2. Hydroxyl Radical Adverting Capacity (HORAC)

HORAC assay was based on a method reported by Ou *et al* 2002 *(91)* modified for the FL800 microplate fluorescence reader FL800 (Bio- Tek Instruments). HORAC assay evaluates the hydroxyl radical prevention capacity by monitoring the fluorescence decay of fluoresceín (FL), which is used as a probe. The hydroxyl radical was generated by a Co(II)-mediated Fenton like reaction and the fluorescence decay curve of FL was used to quantify the HORAC value.

Briefly, 10 µL of each sample were added to 180 µL of FL (4×10^{-3} µM) and then was added 10 µL of a 0.55 M H_2O_2 solution. The reaction starts when it is added 10 µL of CoF_2 to each well of a 96-well microplate at 37 ºC. The FL800 (Bio-Tek Instruments) microplate fluorescence reader was used (λex: 485 nm, λem: 530 nm). Caffeic acid was used as a standard and the data were

expressed as means of independent triplicates as µmol of Caffeic Acid Equivalents (CAE) per gram of dry weight extract (µmol CAE.g^{-1} *dw*).

2.6. Cell Based Assays

2.6.1.Cell Culture

Human Neuroepithelioma from Supra-Orbital Metastasis SK-N-MC cells, obtained from the European Collection of Cell Cultures (ECACC), were routinely grown in EMEM (Eagle Minimum Essential Medium, Sigma) supplemented with 2mM L-Glutamine (Sigma), 10% (v/v) heat-inactivated fetal bovine serum (FBS) (Sigma), 1% (v/v) non-essential amino acids (NEM)(Sigma), 1 mM sodium pyruvate and 50 U.mL penicillin and 50 µg.mL streptomycin. According to ECACC instructions, stock cells were maintained in 25 cm^2 T-flask, incubated at 37 ºC and 5% CO_2. Cells are split to sub-confluence of 70 – 80 % confluence using 0.05 % (v/v) trypsin-EDTA (Gibco).

2.6.2.Cytotoxicity Profile determination

Extracts toxicity assays were performed using CellTiter-Blue® Reagent. Briefly, SK-N-MC cells were seeded at a density of $2.5x10^4$ cells/well in 96 well plate. After 24 hours, SK-N-M cells were pre-incubated with the extracts in a range 0-250 µg GAE.mL^{-1} medium, for 24 h. A control was performed with cells incubated only with cell culture medium.

After 24 of incubation with the extracts, 20 µL of CellTiter-Blue® Reagent was added to each well for 3 h. Resazurin present in CellTiter-Blue® Reagent is a dark blue dye that has little intrinsic fluorescence, is reduced by metabolically active cells into a fluorescent product, resorfurin. Therefore, fluorescent signal is proportional to the number of viable cells. The product was quantified by measurement of the fluorescence (λex: 560 nm, λem: 590 nm) on microplate fluorescence reader FL800 (Bio-Tek Instruments).

2.6.3.Evaluation of Neuroprotective effect

To evaluate the neuroprotective effect of each extract, a neurodegeneration cell model was used. The model describes the treatment of SK-N-MC neuroblastoma cells with H_2O_2 to induce 50% of cell death. Cells were seeded at 2.5×10^4 cells per well. After 24 hours of growth the medium was removed and the cells were washed with PBS. Cells were pre-incubated with non-toxic concentrations of each extract and after 24 hours, cells were washed again with PBS and medium was replaced by medium containing 0,5% (v/v) FBS and 300 µM H_2O_2 for 24h. Cell viability was monitorized with CellTiter-Blue® Reagent as previously described (Section 2.6.1).

2.6.4.Intracellular reactive oxygen species (ROS) production determination

In order to evaluate the ability of extracts o reduce ROS levels produced by cells, the conversion of 2',7' – dichlorofluorescein diacetate (H_2DCFDA) to fluorescent 2',7' – dichlorofluorescein (DCF) was monitored. SK-N-MC cells were seeded at 1.25 x 10^4 cells per well in a 96-well plate, grown for 24 h, then washed with PBS and pre-incubated with the different extracts diluted in medium (0.5 % (v/v) FBS) for 2 or 24h. After this pre-incubation, cells were washed with PBS and incubated for 10 min at 37 ºC with 25 µM H_2DCFDA prepared in PBS. Cells were washed with PBS and then 300 µM H_2O_2 was added for 30 min. The FL800 (Bio-Tek Instruments) fluorescence microplate reader was used, with flouorescence filters for an excitation wavelength of 485 ± 20 nm and an emission wavelength of 530 ± 25 nm, over 1h at 37 ºC. ROS generation was calculated as an increase in fluorescent signal compared with cells not treated with H_2O_2.

2.6.5. Glutathione (GSH) and glutathione disulphide (GSSG) quantification

In order to quantify GSH and GSSG, cold 10% (w/v) metaphosphoric acid was carefully added to samples or standards. After incubation (4 ºC for 10 min) and centrifugation (16,000 g during 20 min at 4 ºC) supernatants were transferred into 1.5 ml tubes (50 µL for GSH determination and 200 µL for determination of GSSG). GSH and GSSG were quantified by HPLC after derivatization with orthophthalaldehyde, performed accordingly to Kand'ar *(83)* and already described in Tavares *et al*. *(84)*. Briefly, for GSH quantification, 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 a 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% (w/v) orthophthalaldehyde (OPA) in methanol, were added. Tubes were incubated at 25 ºC for 15 min in the dark. The reaction mixture was then stored at 4 ºC until analysis. For GSSG analysis, 200 µL of supernatant were incubated at 25 ºC with 200 µL of 40 mM N-ethylmaleimide for 25 min in the dark. To this mixture, 750 µL of 0.1 M NaOH were added. A 20 µL portion was taken and mixed with 300 µL of 0.1 M NaOH and 20 µL of 0.1% (w/v) OPA. Tubes were incubated for 15 min in the dark at 25 ºC and stored at 4 ºC until analysis. Chromatographic analysis was accomplished using isocratic elution on a C18 analytical column (Kinetex Column 15 cm x 4.6 mm, 2.6 µm (Phenomenex)) at 40 ºC on an Acquity^{TM} 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 0.3 mL.min^{-1}. The excitation and emission wavelengths were set at 350 and 420 nm, respectively. The amounts of GSH and GSSG were quantified from the corresponding peak areas, using Empower® Pro 2.0 software. The concentrations of GSH and GSSG in the samples were determined from standard curves with ranges 0–100 µM for GSH and 0–10 µM for GSSG.

2.7. Statistical analysis

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

3. Results and Discussion

3.1. *Opuntia ficus-indica*

Opuntia ficus-indica fruits were harvested in Beja/Serpa region, Portugal, and were processed with peel and seeds and separated into juice and residue.

In this work two different extraction methods of *Opuntia ficus-indica* fruit residues were compared, Conventional Solvent Extraction (CSE) and Pressurized Liquid Extraction (PLE). CSE were performed with a EtOH:H_2O (1:1 v/v) solution and PLE were performed using different CO_2, H_2O and EtOH molar fractions. The three extracts obtained with PLE (PLE – A, PLE – B and PLE – C) and the extract obtained with CSE (OBS – CSE) was analyzed for their content and composition of their phytochemical content, namely polyphenolic composition and presence of terpenes. For the compositional analysis High Performance Liquid Chromatography with Diode Array Detection and Electrochemical Detection (HPLC–DAD–ED) was used and Folin-Ciocalteau method was apllied to quantify the total content. The total content in flavonoids was assessed by a colorimetric assay. All samples were characterized for their chemical antioxidant activity using two different *in vitro* assays: ORAC, HORAC. The extracts obtained from both extraction technologies were analyzed for their neuroprotective effect in SK-N-MC neuroblastoma cell line.

3.1.1. Chemical Characterization

The product obtained from CSE is a light yellow colored extract and the products of the PLE are a brown-yellow and very viscous extracts. The total phenolic content was assessed by Folin-Ciocalteau colorimetric assay (table 3.1).and total flavonoids content was assessed by $AlCl_3$ complexation colorimetric assay (table 3.2).

Table 3.1. Total phenolic content of *Opuntia* extracts, assessed by Folin-Ciocalteau method, expressed as mg GAE.g^{-1} of extract, in a dry weight basis. Significant statistical differences are denoted with different letters. All values are means of three independent experiments ± SD.

***Opuntia ficus-indica* Extracts**	**Total Phenolic Content TPC± SD (mg GAE.g^{-1} *dw*)**
OBS	23.75 ^{a} ± 0.47
PLE - A	22.43^{b} ± 0.46
PLE – B	9.12 ^{c} ± 0.21
PLE – C	13.29 ^{d} ± 0.34

Table 3.2. Total flavonoid content of *Opuntia* extracts, assessed by a colorimetric method, expressed as mg CE.g^{-1} of extract, in a dry weight basis. Significant statistical differences are denoted with different letters. All values are means of three independent experiments ± SD.

***Opuntia ficus-indica* Extracts**	**Total Flavonoid Content TFC± SD (mg CE.g^{-1} *dw*)**
OBS – CSE	1.88 ± 0.20
PLE - A	1.51 ± 0.38
PLE – B	1.21 ± 0.44
PLE – C	1.46 ± 0.23

As shown in table 3.1., the three *Opuntia* extracts obtained with PLE technology present different phenolic contents. TPC ranged from 9.12 to 22.43 mg GAE.g^{-1} *dw*, demonstrating that the phenolic composition are dependent of the extraction conditions. OBS – CSE is the extract with higher water content in the solution and the one with higher phenolic content. PLE extract that shows a higher phenolic content is PLE – A while PLE – B shows the lowest value. The latter was extracted with a solution with less water resulting in an extract depleted in phenolic acids.

Total flavonoid content (TFC) ranged between 1.21 and 1.88 mg CE.g^{-1} *dw*. Among all *Opuntia* extracts, the one obtained with CSE shows the highest content in phenolic compounds and in flavonoids with 23.75 mg GAE.g^{-1} *dw and* 1.88 mg CE.g^{-1} *dw*, respectively.

However it's interesting to note that flavonoids concentrations varied within extraction conditions of the same matrix. The extract that shows a higher amount of flavonoids per total polyphenols is PLE – B, that shows 0.133 mg CE.mg^{-1} GAE (approximately 13 %), being the extract with lower TPC but with a higher percentage of flavonoids. These percentages of flavonoids content in *Opuntia* by-products are in accordance with the values described by Cardador – Martinez. The study reveal that flavonoid content of *Opuntia ficus-indica* residues extracts range from 14 to 23.5 % of the fruit peel and is approximately 12 % of phenolic compounds for seeds *(82)*.

In order to analyze and compare the phenolic composition of samples HPLC-DAD-ED was performed at different UV absorption, namely 280 nm, 360 nm and 480 nm which are the characteristic wavelength of phenolic compounds, flavonoids and betaxanthins (yellow-orange pigments), respectively.

When comparing HPLC profiles of *O. ficus indica* extracts at 280 nm (Figure 3.1. and 3.2.) some differences were observed between CSE and PLE derived products. OBS – CSE have higher amount of compounds with short retention time, reflecting compounds with higher

polarity and lower molecular weight. This is related with the water content of the extraction solution, since conventional extract is the one with higher water content (50%).

However, as shown, the phenolic profiles of the three PLE extracts are similar between the three samples (PLE – A - PLE – C) at 280 (Fig 3.2.) and 360 nm (Fig 3.3.). However PLE – B seems to be less concentrate in phenolics compounds with lower molecular weight. The most significant differences between PLE extracts are in the betaxanthins profile at 480 nm (Fig 3.4.). Furthermore, betaxanthin compounds were identified in both PLE – A and PLE – C, whereas no betaxanthins pigments were identified in PLE – B. PLE-A and PLE-C extracts presents two and three different betaxanthins, respectively. The absence of betaxanthins in PLE-B is consistent with the expected since that betaxanthins are water-soluble pigments and PLE-B extract is the one that have lower content of water in the extraction solution. The absence of betaxanthins in OBS – CSE (Fig. 3.1.) could be explained with the viscosity of the matrix. Sugars and fibers present in high amounts in original matrix could difficult the extraction of bexanthins pigments.

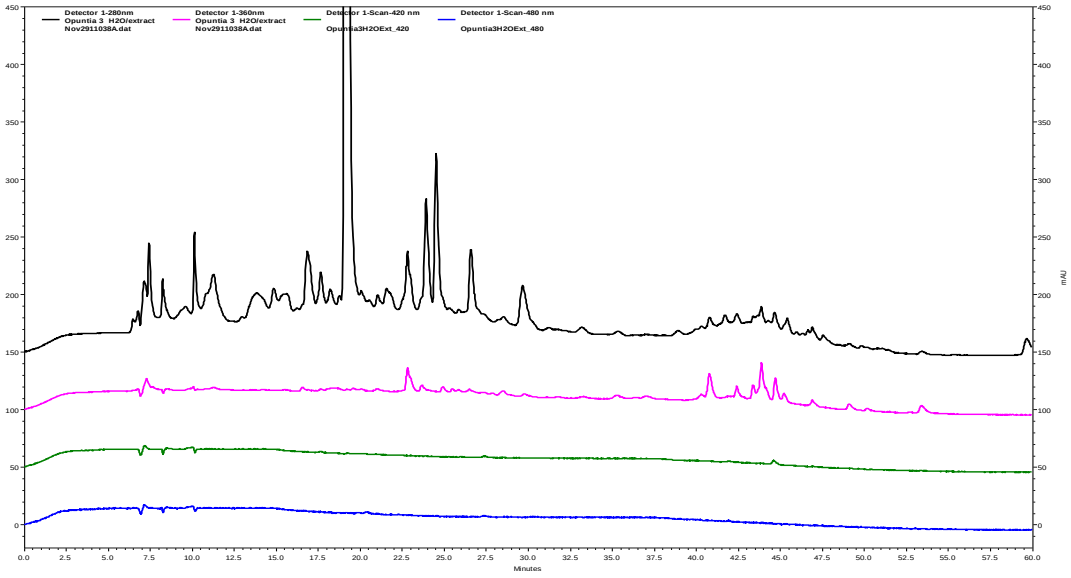


Figure 3.1. Chromatographic profile of *O. ficus indica* from Beja/Serpa fruit by-products extract (OBS-CSE) obtained using Conventional Solvent Extraction (CSE) at 280, 360, 420, AND 480 nm.

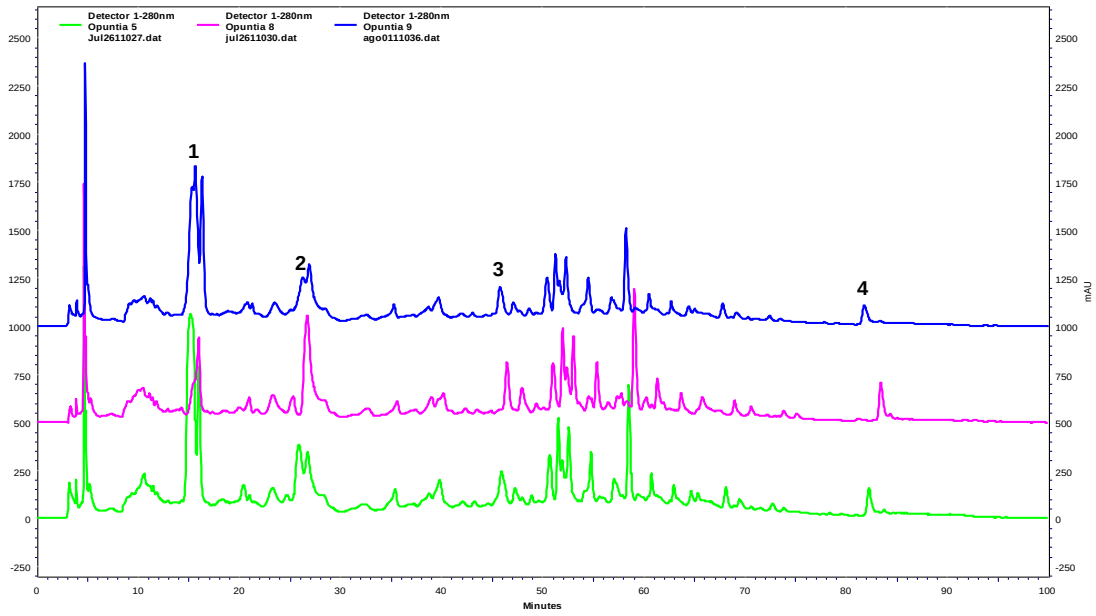


Figure 3.2. Chromatographic profile of *O. ficus indica* from Beja/Serpa fruit by-products extracts obtained using Pressurized Liquid Extraction (PLE), at 280 nm. **PLE – A**, **PLE – B**, and **PLE – C**.Piscidic acid (1), Eucomic acid (2), Ferulic acid (3) and Isorhamnetin (4) were identified.

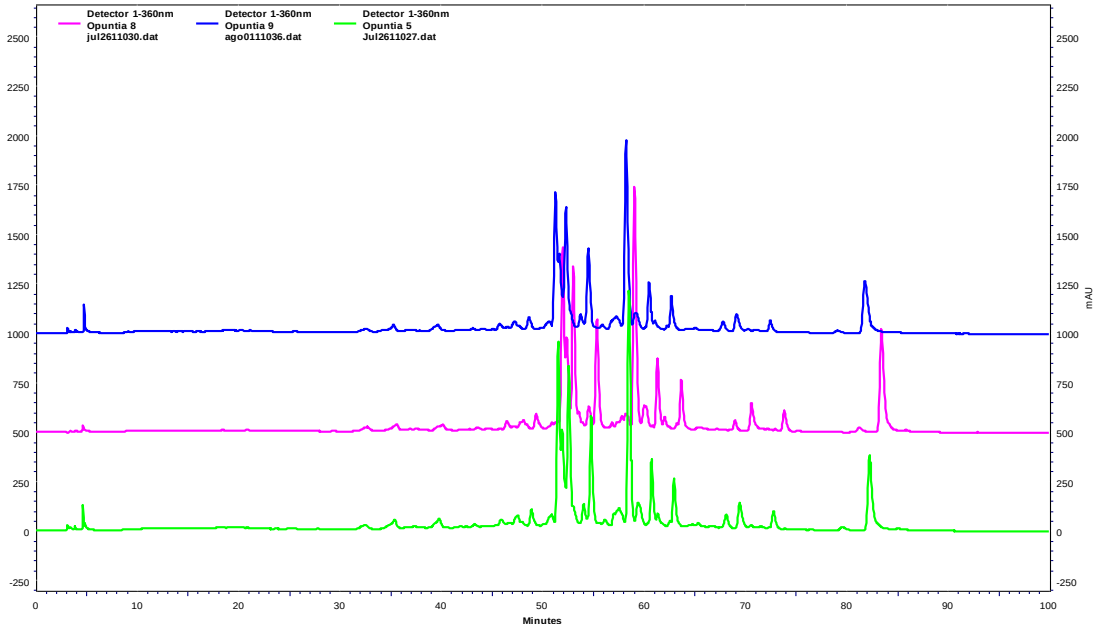


Figure 3.3. Chromatographic profile of *O. ficus indica* from Beja/Serpa fruit by-products extracts obtained using Pressurized Liquid Extraction (PLE), at 360 nm. **PLE – A**, **PLE – B**, and **PLE – C**.

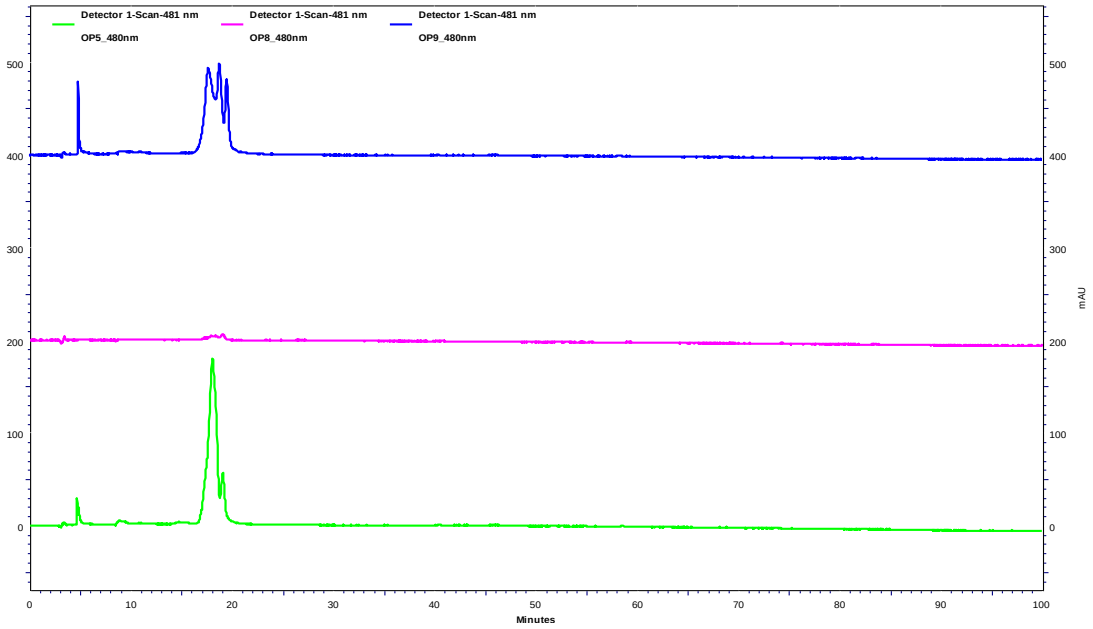


Figure 3.4. Chromatographic profile of *O. ficus indica* from Beja/Serpa fruit by-products extracts obtained using Pressurized Liquid Extraction (PLE), at 480 nm. **PLE – A**, **PLE – B**, and **PLE – C**.

In PLE extracts, piscidic acid (1), eucomic acid (2), ferulic acid (3) and Isorhamnetin (4) were identified by HPLC. Closer to piscidic acid retention time was identified another compound with similar UV-Spectra. This compound may be a piscidic acid derivative or another compound from hydroxycinnamic acids class. All the compounds present between 50' and 70' are quercetin derivatives such as isorhamnetins, once they show an absorbance maximum at 360 nm. Quercetin, kaempferol and isorhamnetin and their derivatives has been reported as the main compounds found in *O. ficus-indica*. Cai and collaborators shows that the main flavonoids found in *Opuntia* spp. were isorhamnetin 3-*O*-(2,6-dirhamnosyl)glucoside and isorhamnetin 3-*O*-*D*-rutinuside *(94)*.

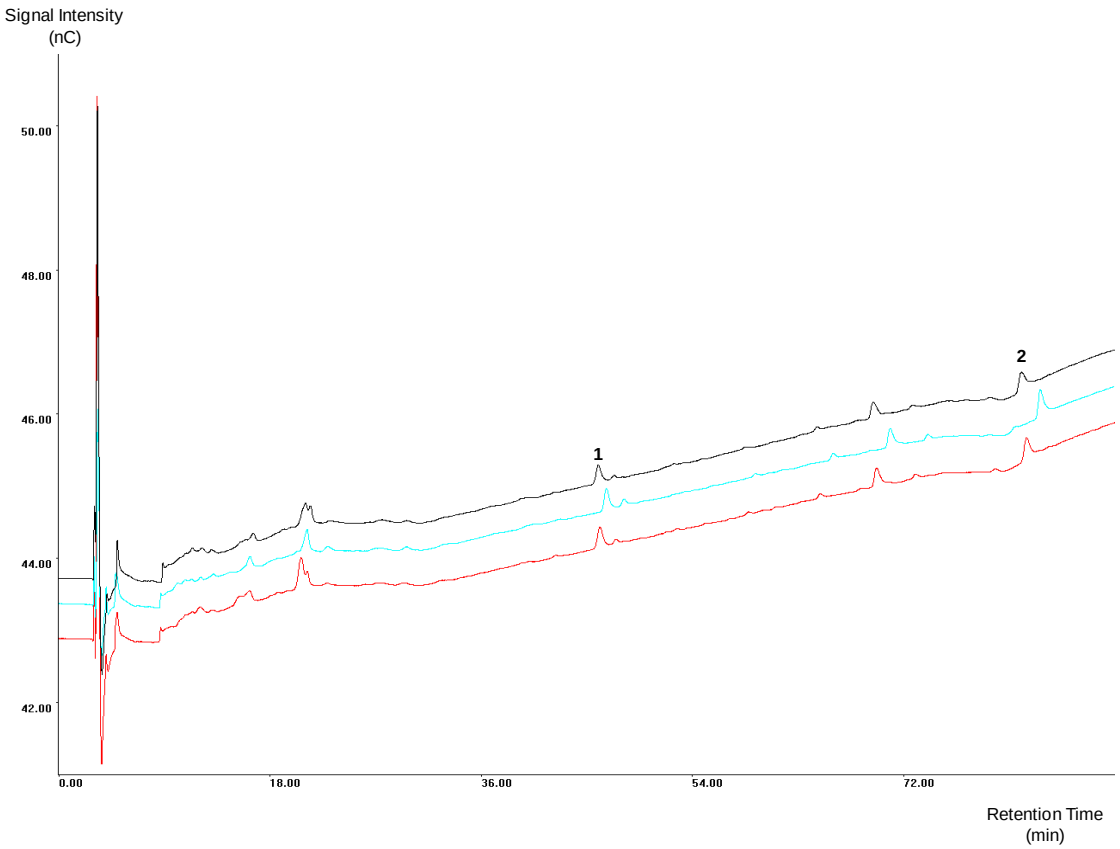


Figure 3.5. Chromatographic profile of *O. ficus indica* fruit residues extracts obtained with PLE using ED. **PLE – A, PLE – B,** and PLE – C. Ferulic acid (1) and Isorhamnetin (2).

Moreover it was also performed the HPLC with the electrochemical detection mode (Figure 3.5) since it is an important tool to detect substances with antioxidant activity. Peaks detected by the electrochemical detector correspond to reactive species with strong capacity to donate electrons. Therefore, a positive correlation between the total area of peaks detected in the electrochemical chromatogram and the antioxidant properties of the extracts is likely to be found. All of the compounds identified have been reported as potent antioxidant compounds*(38)*.

Through the analyze of ED chromatogram we be concluded that the compounds that give to PLE extracts their antioxidant activities are, two phenolic acid present around 18' and a querecetin derivate around 72' not identified, and three identified compounds, ferulic acid at 45' and isorhamnetin at 82'.

Beyond the content in polyphenols, cactus pear has been described to have other compounds with biological activities such terpenes. In stems and fruits were identified two terpenoids: (6S,9S)-3-oxo-α-ionol-β-D-glucopyranoside and corchoionoside C.*(95)* In order to investigate the presence of terpenes in fruit by-products extracts Thin Layer Chromatography (TLC) were performed, using lupeol as standard, a mobile phase and a solution to reveal terpenes. TLC revealed the presence of terpenes in all PLE extracts (Fig. 3.6).

Sample	Lupeol	PLE – A	PLE – B	PLE – C
Concentration ($mg.mL^{-1}$)	10	50	50	50
Volume (µL)	10	10	10	10

Figure 3.6. TLC plate for assess the presence of terpenoids in *O. ficus-indica* fruit residues extract obtained with PLE (PLE – A, PLE – B, and PLE – C in a concentration of 50 $mg.mL^{-1}$ *dw* extract).

The presence of a fluorescent band in the same molecular weight of the standard is common to all PLE extracts. This may reveal that the PLE extracts have lupeol in their composition or a similar compound with the same molecular weight. TLC reveals also the presence of other terpenoids compounds with higher molecular weights which identification is under evaluation.

3.1.2.Chemical Antioxidant Capacity

In order to evaluate the chemical antioxidant activities of *Opuntia ficus-indica* extracts, two different assays were performed: ORAC and HORAC.

ORAC assay measures the potential to scavenge harmful oxygen species such peroxyl and HORAC assay analyze the efficacy of the extract to prevent hydroxyl radical formation.

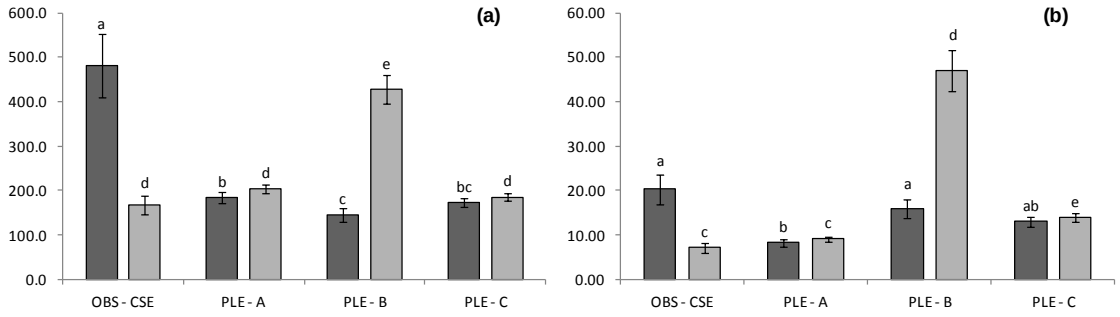


Figure 3.7. Chemical antioxidant capacity of *Opuntia ficus-indica* extracts measured by ORAC and HORAC assays. **(a)** ORAC (■) expressed as µmol TE.g^{-1} *dw*, and HORAC (■) expressed as µmol CAE.g^{-1} *dw*. (**b**) Antioxidant capacity per unit of phenol ORAC (■) expressed as µmol TE.g^{-1} GAE, and HORAC (■) expressed as µmol CAE.g^{-1} GAE. All values are means of three independent experiments ± SD.

ORAC and HORAC values obtained for *O. ficus-indica* extracts range from 145 to 481 µmol TE.g^{-1} *dw*, and 167 to 427 µmol CAE.g^{-1} *dw*, respectively (Fig. 3.7). As verified for TPC and for TFC, OBS – CSE had the highest ORAC value. Between *Opuntia* extracts PLE – B have higher ORAC and HORAC/TPC ratio, being ORAC/TPC ratio similar to OBS – CSE.

These results suggest that PLE and CSE technologies have the ability to extract different phenolic compounds from *Opuntia* fruit residues. While conventional solvent extraction have the mostly radical chain breaking antioxidants since it presents a higher ORAC/TPC value, pressurized liquid extracts are mainly constitute for preventive antioxidants showing a higher HORAC/TPC values. That could be traduced in different *in vivo* potencies or bioactivities.

3.1.3.Cytotoxicity Profile Determination

Prior to the evaluation of neuroprotective potential of different *Opuntia* extracts, cytotoxicity assays were performed using CellTiter-Blue® Reagent (Promega). This reagent is a dark blue dye that has little intrinsic fluorescence. When present in the cytoplasm of metabolically active cells is reduced into a fluorescent product, resorfurin. Therefore, fluorescent signal of resorfurin is proportional to the number of viable cells.

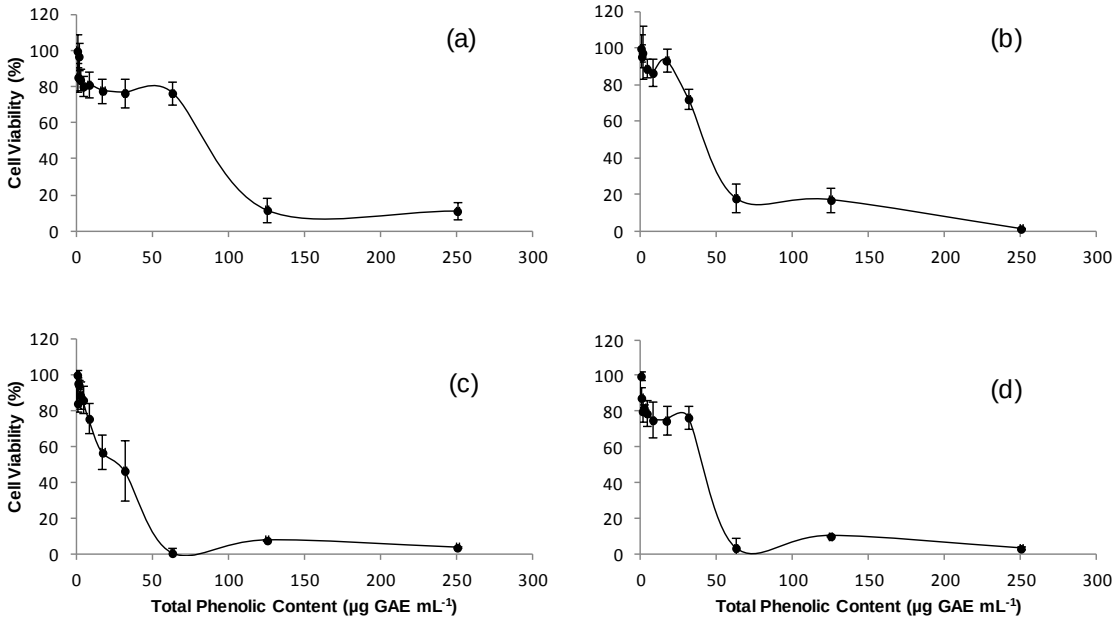


Figure 3.8. Cytotoxicity profile of *Opuntia ficus-indica* extracts, assessed by CellTiter-Blue Reagent Cell Viability Assay (Promega). OBS – CSE (a),PLE – A (b), PLE – B (c), and PLE – C (d). SK-N-MC neuroblastoma cells were incubated for 24 h with the extract (0-250 µg GAE mL^{-1}). Cell viability is expressed as percentage of viable cells. Values are expressed as percentage relatively to control (without extract). All values are means of three independent experiments ± SD.

PLE – B (fig 3.8. (c)), seems to be more toxic extract, while it shows a more pronounced reduction of cell viability for lower concentrations than the other *Opuntia* extracts. The extract that shows a lower toxicity is OBS-CSE (fig 3.8. (a)). All extracts are completely toxic to concentrations higher than 125 µg GAE.mL^{-1}. Cytotoxicity profiles are under the expected since most polyphenols show a bell-shape dose-response curve, presenting cellular toxicity at high concentrations *(96)*.

Non-toxic concentrations of the extracts selected for neuroprotection assays were 0.25, 0.5, 1, 2.5 and 5 µg GAE.mL^{-1}. Curiously, the non-toxic range of *Opuntia* extracts (maximum 5 µg GAE.mL^{-1} for all *Opuntia* extracts) is closer to the values reported for dietary polyphenolic-derived metabolites in plasma at 4 µM *(97)*. The maximum plasma level of flavonoids in humans is usually reached between 0.06 and 7.6 µM *(33)*. For quercetin the concentration in human plasma ranges from 0.1 to 10 µM *(34)*.

3.1.4. Evaluation of Neuroprotective Effect

Today, cell models can provide an indication of biological activity for the phytochemicals in question. The main advantage of the use of *in vitro* cell models is the practical convenience, such as easy culturing, relatively low cost and moderate throughput capabilities *(16)*. Neuronal

cell death caused by oxidative stress has been implicated in a variety of brain pathologies including neurodegenerative diseases such as AD and PD *(7)*.

SK-N-MC cells were pre-incubated for 24 h in the presence or absence of *Opuntia* extracts and then challenged with 300 µM H_2O_2 for 24 h. Neuroprotection was then evaluated by monitoring cell viability using Cell Titer Blue – Reagent® (Promega). Figure 3.9. shows the different responses of SK-N-MC cells to stress after pre-incubation with the *Opuntia* extracts.

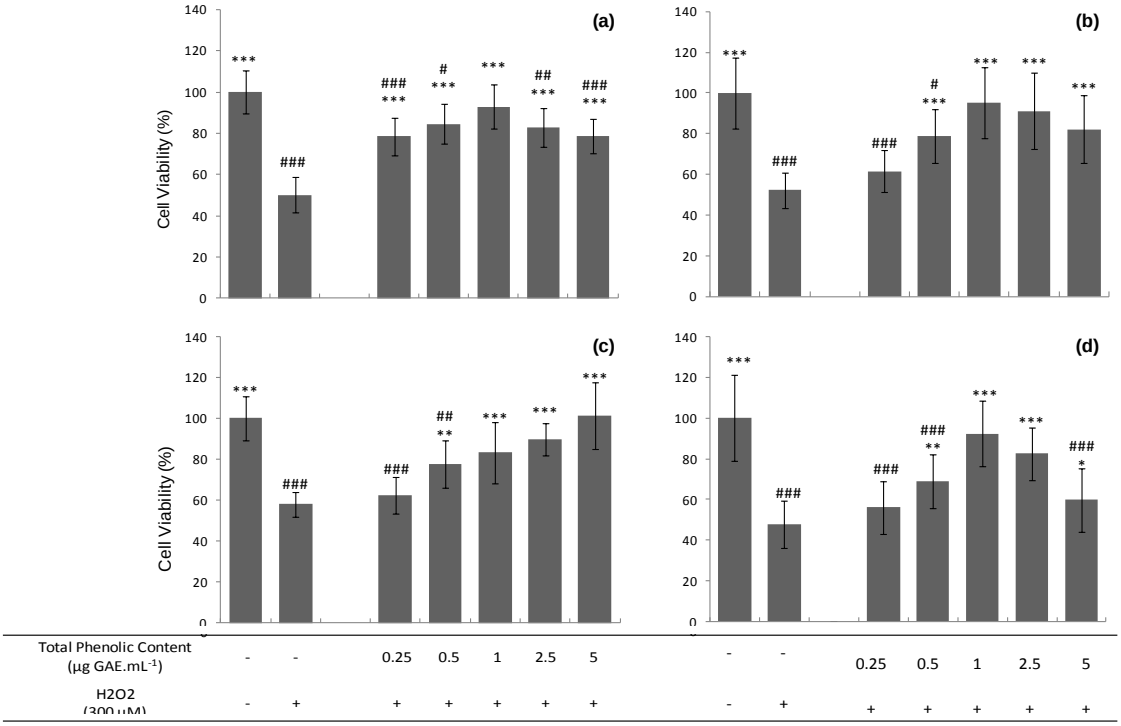


Figure 3.9. Neuroprotective effect of *Opuntia ficus-indica* extracts, assessed by CellTiter-Blue Reagent Cell Viability Assay (Promega). OBS – CSE (**a**),PLE – A (**b**), PLE – B (**c**), and PLE – C (**d**). Cell viability is expressed as percentage of viable cells. SK-N-MC cells were pre-incubated with 0.25, 0.5, 1, 2.5, and 5 µg GAE.mL^{-1} of all extracts for 24 h and then injured by 300 µM H_2O_2 for 24 h. Statistical differences between treatments and stressed cells are denoted as *p < 0.05, **p < 0.01, ***p < 0.001 and statistical differences between treatments and not-treated cells are denoted as $^{\#}$p < 0.05, $^{\#\#}$p < 0.01, $^{\#\#\#}$p < 0.001. All values are means of three independent experiments ± SD.

Only OBS – CSE (Figure 3.9. (a)) is able to increase significantly cell viability for 0.25 µg GAE.mL^{-1}, while no extract from PLE technology can exert any protective effect. However, all the extracts in the range of 0.5 to 2.5 µg GAE.mL^{-1} have the same behavior, exerting a significant maintenance of cell viability, with no statistical differences between the treatments with different extracts.

The similarities observed in the intermediary concentrations are not observed in the higher concentration 5 µg GAE.mL^{-1}, when PLE – C are not able to protect neuronal cells at the same level of the remain *Opuntia* extracts (Figure 3.9. (d)). Comparing the results of neuroprotective activity with the phenolic profile of *Opuntia* extracts, it is suggested to exist a negative correlation of neuroprotection and the presence of betaxhantin pigments, since PLE – C is the one with possess three compounds from this family.

Kim and co-authors (2006) have already report the neuroprotective effect of a methanolic extract of *O.ficus-indica* fruits against NMDA-, KA-, and OGD- induced neuronal injury. The treatment of neuronal cells with 30, 300 and 1000 µg.mL^{-1} of *Opuntia* extract significantly reduced the neurotoxicity in a dose-dependent way. While interesting results it is important to note that the concentrations tested by the authors are not dietary relevant and the content in phenolic compounds of the extracts was not assessed.

3.1.5. Intracelular ROS production determination

The concentration that shows a higher neuroprotective effect was also tested for intracellular antioxidant capacity. Two different pre-incubation times were tested, at 2 and 24 h, to cover different timescale events. It was expect that the 2 h pre-incubation would cover the time period when direct scavenging events caused by the phenolic compounds present in the extracts while 24 h of pre-incubation with extracts could reflect ROS levels influence by indirect effects on endogenous antioxidant systems.

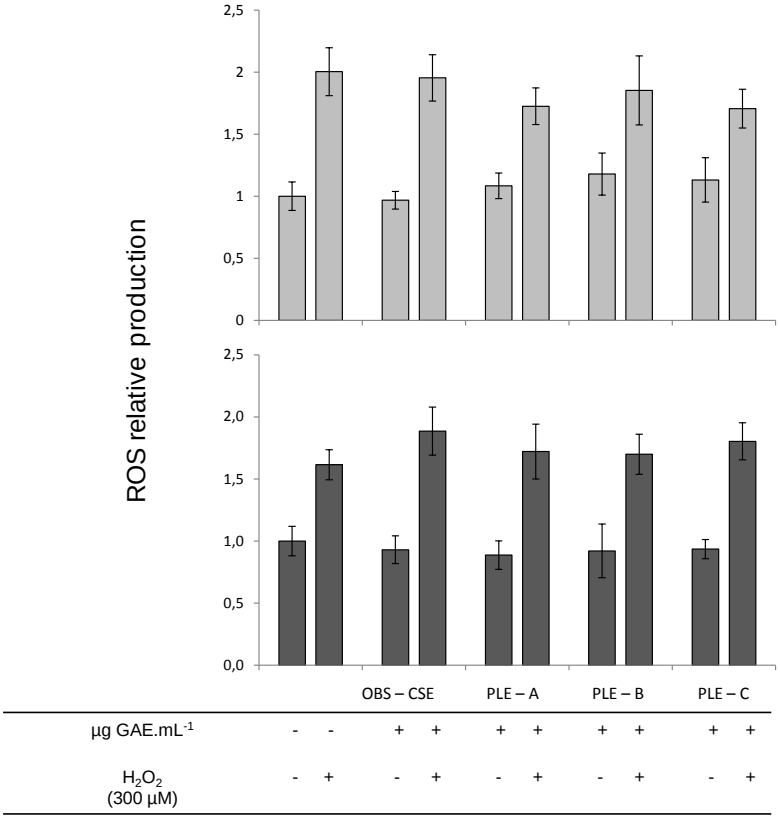


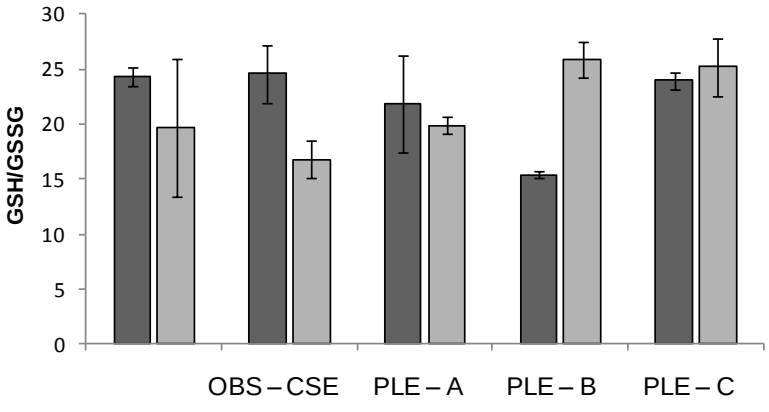
Figure 3.10. Relative ROS production by SK-N-MC neuroblastoma cells pre-incubated with *O. ficus-indica* extracts for 2h (■) and 24 h(■) and submitted to an oxidative stress (300 µM H_2O_2 for 30 min). ROS were detected by fluorimetry using H_2DCFDA as probe. Statistical differences in relation with cells not treated are denoted as *p < 0.05, **p < 0.01, ***p < 0.001. All values are means of three independent experiments ± SD.

At both time points, 2 and 24 hours, and in the absence of an imposed oxidative stress all *Opuntia* extracts maintain the basal ROS production. H_2O_2 was used to promote an oxidative

stress in cells and in fact, ROS production duplicates after 30 min compared with the control, however without promoting cell death. With a 2 h pre-incubation, none of the extract reduced intracellular ROS caused by H_2O_2-induced stress. With a 24 h pre-incubation, followed by H_2O_2 stress also none extract reduced intracellular ROS production either. Comparing these results with those obtained for neuroprotection assay, it is suggested that the neuroprotection carried out by *Opuntia* extracts was not mediated by a direct ROS scavenging activity or even by activating endogenous antioxidant systems. Antioxidant compounds may be characterized into two types, the direct antioxidants, which are a short-lived redox-active molecules, and indirect antioxidants, which may or may not show redox activity and are effective by interfering in other molecular mechanisms *(98)*.

3.1.6. Glutathione (GSH) and Glutathione disulphide (GSSG) Quantification

GSH is the major antioxidant within cells and is involved in maintaining a tight control of redox status *(10)* to verify the modifications induced by metabolites pre-treatment and post application of H_2O_2 stress, the redox pair GSH/GSSG was quantified. Preliminary results that need to be confirmed are presented for GSH and GSSG quantifications after a 24h pre-incubation with *Opuntia* extracts and an oxidative stress applied with H_2O_2 (Fig 3.11).



Total Phenolic Content (µg GAE.mL^{-1})	-	-	1	1	1	1	1	1	1	1		
H_2O_2 (300 µM)	-	+	-	+	-	+	-	+	-	+		

Figure 3.11. Quantifications of GSH and ratio GSH/GSSG after pre-treated for 24 h with *O. ficus-indica* extracts, SK-N-MC neuroblastoma cells were subjected to 300 uM H2O2 for 24 h. cells were collected and analyzed for their content in GSH and GSSG by HPLC. The values of GSH are expressed in nmol GSH.mg $^{-1}$ protein. All values are means ± SD.

The cells challenged with H_2O_2 presented slightly a decrease in the GSH/GSSG ratio as expected, although not significant in the assay.

Cells subjected to a pre-incubation with PLE – A and PLE – C extracts, when challenged with an oxidative stress, did not show any modifications on GSH/GSSG levels. However, OBS – CSE seems to promote a decrease in the ratio levels. This decrease after with pre-incubation with a polyphenolic extract and followed by an oxidative stress was also observed by Tavares and collaborators *(93)* and could be due to the mobilization of GSH molecules conducted by other identities such as proteins. On the contrary, PLE – B extracts promote a slightly increase in GSH/GSSG ratio. The increment may be related with an effect of the extract in promoting activities related with restore of GSH. In fact PLE-B extract is the only that after 24 h incubation reduces the GSH/GSSG ratio in cells, and if followed by stress the cells seems able to recover to levels higher than the control.

Taking these results in account seems that the neuroprotective effect showed by PLE – B extract in neuroprotection assay can be due to their capacity of increase GSH/GSSG ratio levels.

Moreover interesting is the fact that results suggest that the same levels of neuroprotection capacity at 1 µg GAE.mL-1 observed in all extracts may have different causes. Overall these preliminary results are very interesting for further confirmations.

It has been shown that curcumin and flavonoids enhance the *in vivo* synthesis of GSH *(99)*. The increase in GSH levels in various cell types subjected to polyphenol treatment results from an increased transcription of the γ-GSS (glutathione synthase) gene *(100)*. GSH may has multifunctional roles, including being a reducing agent and a cofactor in enzyme function, as well as playing a role in the chemical antioxidant defense and the detoxification of xenobiotics.

3.2. *Prunus avium*

Fruits were selected from the ones with no commercial value and were consider waste. Fruits were processed with peel, seeds and stalks to obtain the extract from the residue. Cherry extract were obtain trough a conventional solvent extraction (in batch mode) with a H2O:EtOH (1:1) solution as solvent. To recover and concentrate polyphenols from *Saco* cherry crude extract macroporous adsorption resin Amberlite® XAD16 was used as described before (material and methods, 2.3). Resin Amberlite® XAD16 was chosen due to its low operation cost, simple handling and high absorption capacity for a large number of compounds, namely compounds with aromatic rings such as phenolics *(72, 88)*.

3.2.1. Chemical Characterization

The extract obtained from cherry residue is a red/pink powder. TPC obtained by Folin Ciocalteau method increases after resin concentration (Fig 3.12), it was 10 mg GAE.g^{-1} *dw* for crude extract (CCE) and 375.5 mg GAE.g^{-1} *dw* for polyphenol rich cherry extract (PRCE).

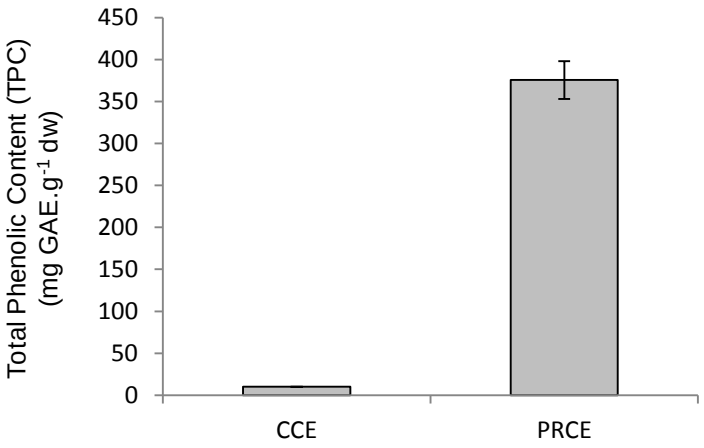


Figure 3.1 Total phenolic content of crude cherry extract (CCE) and polyphenol-rich cherry extract (PRCE), expressed as mg GAE.g^{-1} of extract in a dry weight basis. All values are means of three independent experiments ± SD.

A very important fact is that was a high enrichement in TPC after the submission to AD technology, proving that Amberlite® XAD16 resin is a good approach to enhance the concentration in phenolic compounds present in a biological sample. Figure 3.13 shows the phenolic profile of PRCE at 280, 360 and 527 nm.

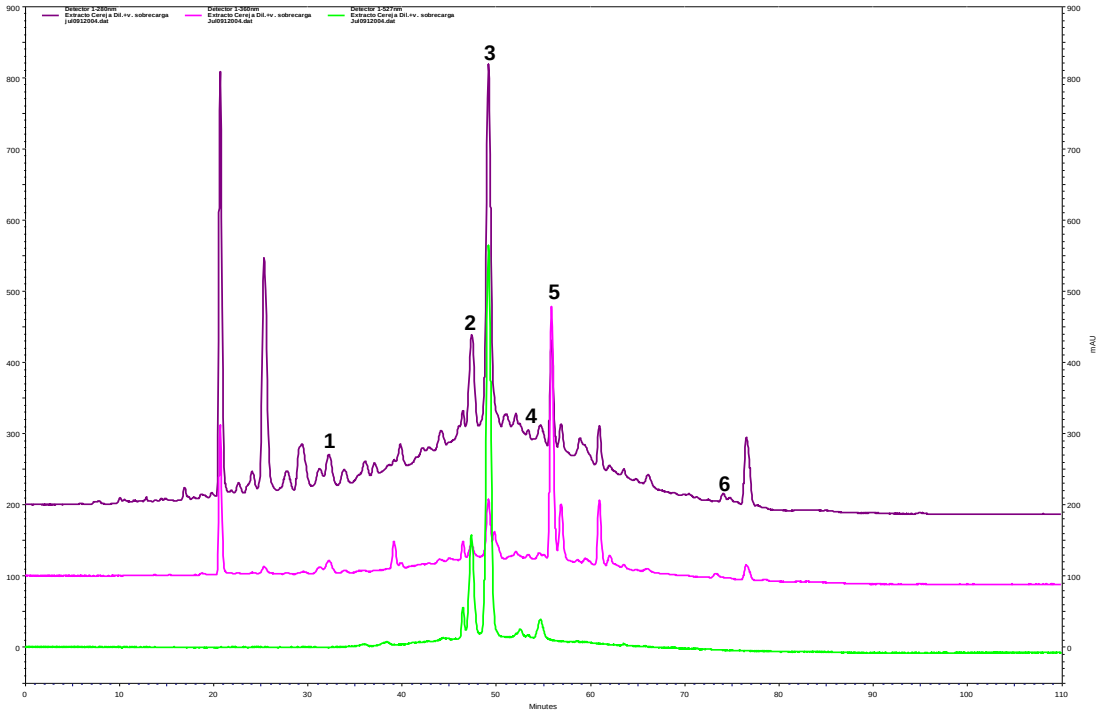


Figure 3.13. Chromatographic profile of Polyphenol-Rich Cherry Extract (PRCE) at 280, 360 e 527nm. Chlorogenic Acid (1), Cyanidin-3-*O*-glucoside (2), Cyanidin-3-*O*-rutinuside (3), Peonidin-3-glucoside (4), Rutin (5), and Quercetin (6) were indentified.

The compounds identified in PRCE by HPLC were a phenolic acid, chlorogenic acid (1), three anthocyanins namely cyanidin-3-*O*-glucoside (2), cyanidin-3-*O*-rutinuside (3), peonidin-3-glucoside (4), and two flavonoids, rutin (5) and quercetin (6). Anthocyanins are flavonoids recognized for their promoting health benefits mainly because their strong antioxidant activity *(101, 102)*. Due to their singular structure, anthocyanins can scavenge ROS and also chelate metals preventing these way the formation of free radicals by Fenton or Fenton-like reactions*(103)*.

Through the analyses of ED chromatogram was identified also a flavonol namely Quercetin (Fig 3.14 (5)).

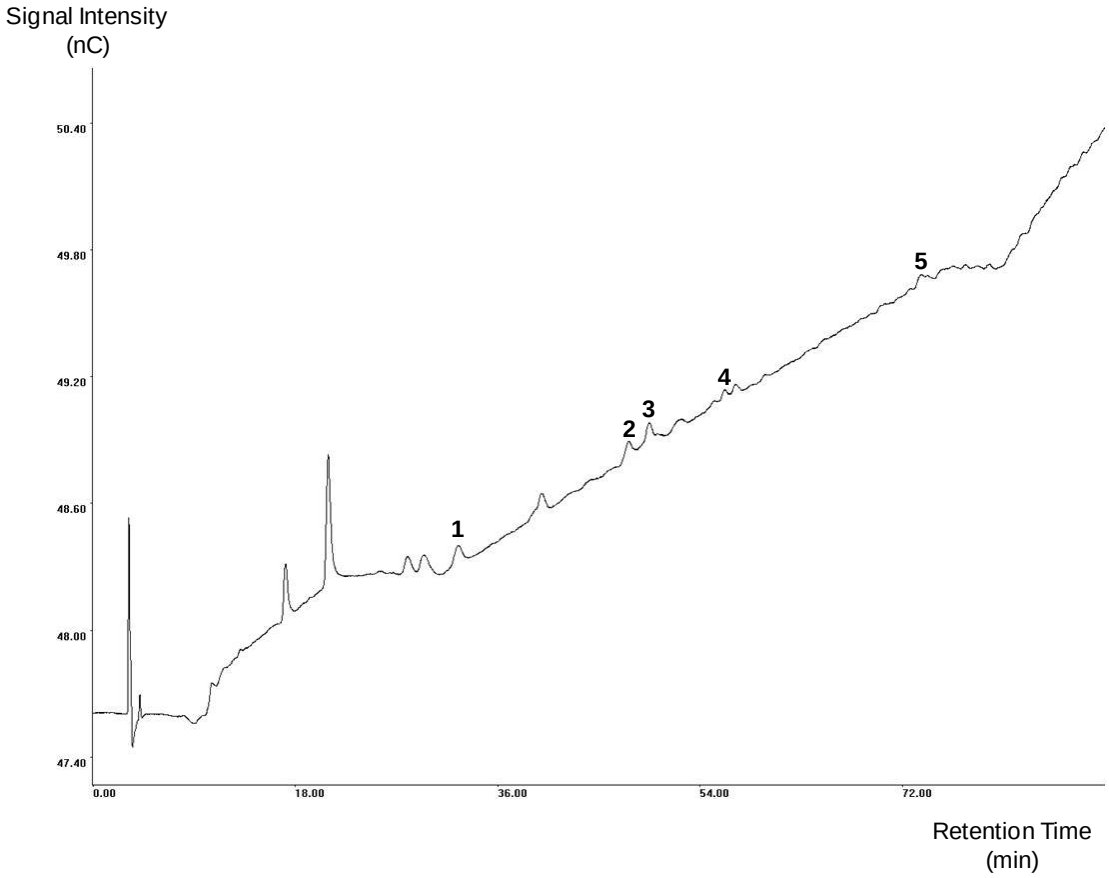


Figure 3.14. Chromatographic profile of PRCE using ED. **Ch**lorogenic acid (1), Cyanidin-3-glucoside (2), Cyanidin-3-rutinoside (3), Rutin (4) , Quercetin (5).

As showed before, the identification of compounds detected by HPLC with the electrochemical detection mode is an important tool to detect substances with antioxidant activity. All of the compounds identified (Figure 3.14.) have been reported as potent antioxidant compounds*(38)*. Moreover phenolic compounds identified by HPLC – DAD – ED have already been described by other authors to be the active antioxidant species in cherries. For example, Piccolella and collaborators (2008) found that flavonoids like epicatechin and anthocyanins, and hydroxyl-

cinnamic acids such chlorogenic and neochlorogenic acid are the most active compounds in scavenging free radicals *(104)*. Previous study with apples and wines have reported good correlations between the total area of electrochemical chromatogram and chemical antioxidant activity *(105)*.

3.2.1.Chemical antioxidant activity

Results of antioxidant activity of cherry extract measured by ORAC (µmol TE.g^{-1} *dw* extract) and HORAC (µmol CAE.g^{-1} *dw* extract) assays are presented in figure 3.15.

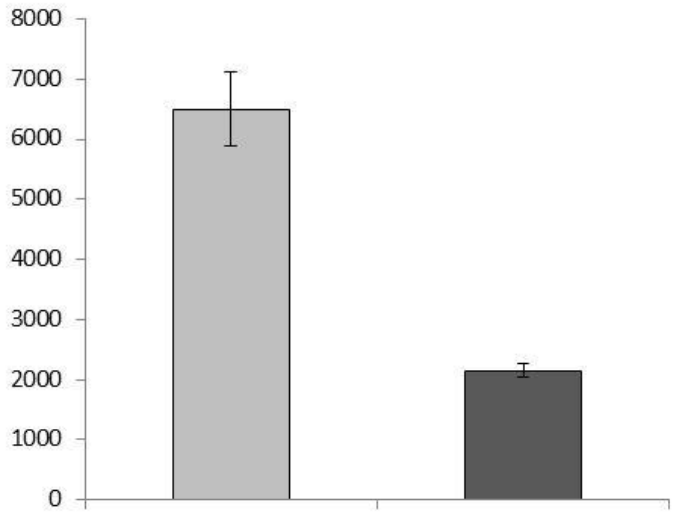


Figure3.15. Antioxidant activity of polyphenol-rich cherry extract (PRCE) measured by ORAC (■) (µmol TE.g^{-1} *dw* extract) and HORAC (■) (µmol CAE.g^{-1} *dw* extract) assays. Statistical differences are denoted with different letters. All values are mean ± SD, n=3.

For ORAC assay, Blando and co-authors (2004)*(106)* showed that the antioxidant values of cherries are related with their total anthocyanin content whereas for HORAC assay, no study has been published for cherry extracts to date. A previous study with apple cultivars good correlations were obtained between antioxidant values of ORAC and HORAC with catechin, epicatechin and chlorogenic acid, suggesting that these compounds are the major contributors of antioxidant capacity in several fruit matrices.

3.2.2.Cytotoxicity profile determination

SK-N-MC cells were incubated with PRCE with concentrations from 0 to 250 µg GAE.mL^{-1} and cell viability assayed using CellTiter-Blue® Reagent (Promega) (Fig 3.16). .

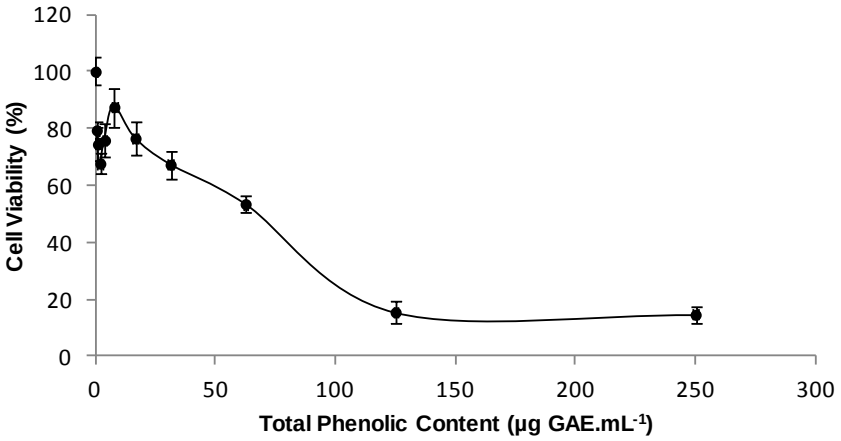


Figure 3.16. Cytotoxicity profile of *Prunus avium* extract (PRCE), assessed by CellTiter-Blue Reagent® Cell Viability Assay (Promega). SK-N-MC neuroblastoma cells were incubated for 24 h with the extract (0-250 µg GAE mL^{-1}). Cell viability is expressed as percentage of viable cells. Values are expressed as percentage relatively to control (without extract). All values are mean ± SD.

Non-toxic concentrations of PRCE selected for neuroprotection assay were 0.25, 0.5, 1, 2.5 and 5 µg GAE.mL^{-1}. As verified for *Opuntia* extracts, the higher non-toxic concentration of PRCE (5 µg GAE.mL^{-1}) is closer to the values reported for anthocyanins present in human plasma, that is less than 0.15 µM (59).

3.2.3.Neuroprotective effect evaluation

PRCE neuroprotective ability was assayed in the human cell model previously described. SK-N-MC cells were pre-incubated for 24 h in the presence or absence of PRCE and then challenged with 300 µM H_2O_2 for 24 h. Neuroprotection was then evaluated by monitoring cell viability using Cell Titer Blue – Reagent® (Promega). Figure 3.17. shows the different responses of SK-N-MC cells to stress.

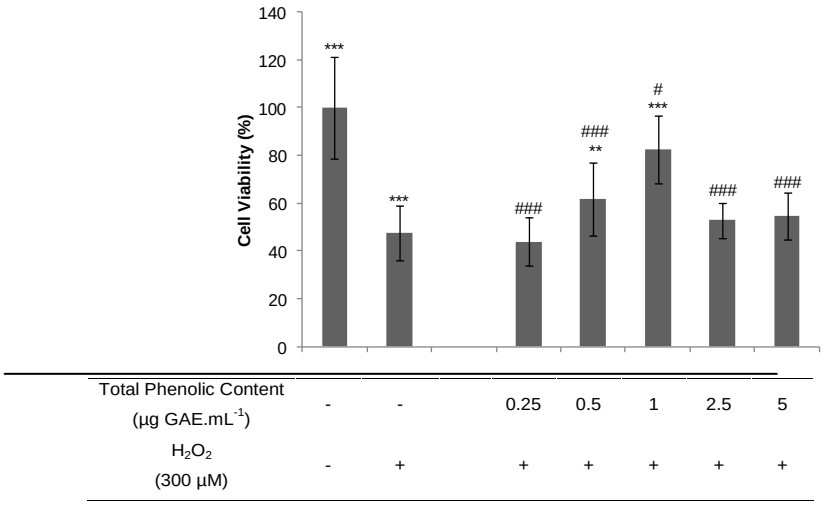


Figure 3.17. Neuroprotective effect of *Prunus avium* extract (PRCE), assessed by CellTiter-Blue Reagent ® Cell Viability Assay (Promega). Cell viability is expressed as percentage of viable cells. SK-N-MC cells were pre-incubated with 0.25, 0.5, 1, 2.5, and 5 µg GAE.mL^{-1} of all extracts for 24 h and then injured by 300 µM H_2O_2 for 24 h. Statistical differences between treatments and stressed cells are denoted as *p < 0.05, **p < 0.01, ***p < 0.001 and statistical differences between treatments and not-treated cells are denoted as $^{\#}$p < 0.05, $^{\#\#}$p < 0.01, $^{\#\#\#}$p < 0.001. All values are means of three independent experiments ± SD.

As verified for all *O. ficus-indica* extracts obtained with PLE technology, PRCE can not exert neuroprotection at the lower tested concentration (0.25 µg GAE.mL^{-1}). At 0.5 and 1 µg GAE.mL^{-1} PRCE are able to increase cell viability higher than cells submitted to an oxidative stress, however the extract can not promote a cell survival at the same scale that the control cells. In two higher concentrations (2.5 and 5 µg GAE.mL^{-1}), PRCE are unable to promote any increase in cell viability.

3.2.4.Intracellular ROS production determination

After measure the chemical antioxidant capacity of PRCE, the cellular antioxidant capacity was assessed in SK-N-MC cells. H_2O_2 can generate hydroxyl radicals by Fenton or Fenton-like reactions*(107)* and are a stress inductor capable to generate oxidative stress in SK-N-MC cells with an increase in ROS production *(17, 93)*. After a pre-incubation of cells with the extract during 2 and 24 h, cell were challenged with H_2O_2 and cellular levels of ROS were measured against cells not submitted to H_2O_2 – induced stress.

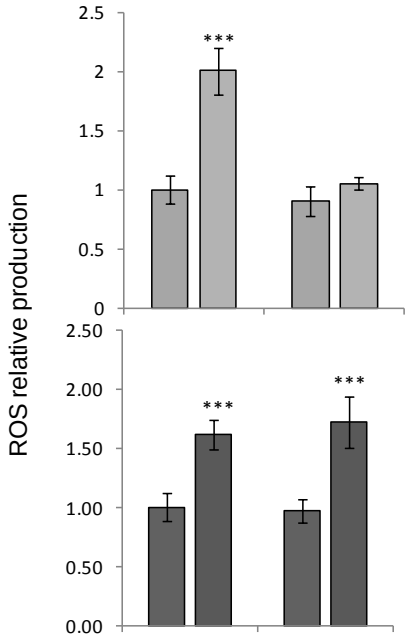
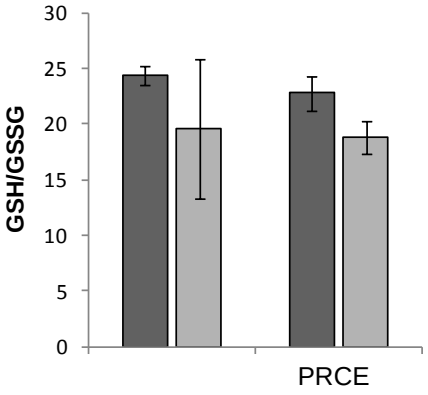


Figure 3.18. Relative ROS production by SK-N-MC neuroblastoma cells pre-incubated with *Prunus avium* extract (PRCE) for 2h (■) and 24 h(■) and submitted to an oxidative stress (300 µM H_2O_2 for 30 min). ROS were detected by fluorimetry using H_2DCFDA as probe. Statistical differences in relation with cells not treated are denoted as $p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. All values are means of three independent experiments ± SD.

In the absence of H_2O_2, treatment of SK-N-MC cells with PRCE at 1 µg GAE.mL^{-1} for 2 and 24 h did not affect endogenous ROS levels (Figure 3.18.). The pre-treatment of neuronal cells with PRCE during 2 h followed by a H_2O_2 – induced stress shows a significant decrease of ROS levels (Figure 3.18. (a)). This effect is not detected at 24 h of PRCE pre-treatment (Figure 3.18. (b)). As shown by Serra and co-authors (2011) cyaniding 3-glucoside and cyaniding 3-rutinoside, that are the main phenolics present in PRCE, are compounds with high chemical antioxidant capacities. Therefore the neuroprotective effect exerted by this extract could be related with their capacity of exert a direct scavenge of free radicals in neuronal cells, which are associated with the decrease detected with 2 h incubation.

3.2.6.Glutathione (GSH) and Glutathione disulphide (GSSG) Quantification



Total Phenolic Content (µg GAE.mL^{-1})	-	-	1	1
H_2O_2 (300 µM)	-	+	-	+

Figure 3.19. Quantification of GSH and GSH/GSSG ratio. After pre-treated for 24 h with *Prunus avium* extract (PRCE), SK-N-MC neuroblastoma cells were subjected to 300 µM H_2O_2 for 24 h. cells were collected and analyzed for their content in GSH and GSSG by HPLC. The values of GSH are expressed in nmol GSH.mg $^{-1}$ protein. All values are means ± SD.

Regarding the ability of PRCE extract to modulate the GSH/GSSG ratio, also a preliminary assay was performed. Cells pre-incubated with PRCE do not seem to react by altering the GSH/GSSG balance.suggesting that the neuroprotective effect showed by PRCE is not mediated by a regulation of GSH/GSSG redox pair.

4. Final Conclusions

The aim of this work was to assess the efficacy of *O. ficus-indica* and *P. avium* fruit by-products extracts in a neurodegeneration cell model with SK-N-MC neuroblastoma cell line submitted to an oxidative injury. All the *O. ficus-indica* and *Prunus avium* extracts have protected neuroblastoma cells from H_2O_2-induced death at low, non-toxic levels, revealing both species as promising sources for neuroprotective compounds. This reinforces the importance of the approach since the concentrations used are found able to protect neuronal cells at physiologically-relevant serum concentration.

Based on cellular events evaluated the extracts from different biological matrixes seems to protect neuronal cells trough different cellular mechanisms. While cherry extract are able to reduce ROS levels, cactus pear extracts are able to modulate the GSH/GSSG homeostasis.

On the other hand the different responses revealed by neuronal cells to extracts obtained with different extraction technology from the same matrix highlights the influence of chemical composition. The results suggest that different extraction methods are able to isolate compounds with differential biological activities and even using the same technology and the same biological matrix, the conditions of the extraction could influence the phytochemical content and consequently, the extracts biological activities.

It is now thought more likely that some phytochemicals, including polyphenols, are processed by the body metabolism as xenobiotics. They stimulate stress- related cell signalling pathways that result in increased expression of genes that encode cytoprotective genes. This provides grounds for the hormesis theory, i.e. when mild stress triggers defense mechanisms. In the case of polyphenols could explain how they could have an indirect antioxidant activity. However, other phytomolecules could act as antioxidant defences against ROS. Ascorbic acid (Vitamin C), Vitamin E, glutathione (GSH) and carotenoids are examples of nonenzymatic antioxidant defenses.

Many in vitro studies are focused on polyphenol-derived health benefits, but using doses significantly higher than those to which humans are exposed through the diet or that could be found in the blood (Collins, 2005). This work encompasses a more physiological approach which takes into account treatment of cell models with relevant *in vivo* concentrations of phytochemical metabolites. However and since is food based extract that may be consider to constitute a supplement/nutraceutical for oral consumption other concerns should be consider for the more promising extracts. In particular consider the physiochemical changes occurring in the gastrointestinal tract, absorption and metabolism, these are important factors with a consequential impact on bioavailability and bioefficacy.

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