Elena Valeria Girlus

Degree in Biochemistry

Protective activity of phytochemicals from natural extracts

Dissertation to obtain a Master Degree in Biochemistry for Health

Supervisor: Regina Menezes Echaniz, Ph.D, IBET/ITQB-UNL
Co-Supervisor: Ana Alexandra Figueiredo Matias, Ph.D, IBET/ITQB-UNL

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Acknowledgments

Quero agradecer a todas as pessoas do laboratório de *Disease and Stress Biology* e do laboratório *Nutraceuticals and Delivery*, em especial à Dra. Regina Menezes, Dra. Cláudia Santos, Dra. Ana Matias e Dra. Catarina Duarte, por me terem dado a oportunidade de trabalhar nos dois grupos, e pelo apoio e disponibilidade ao longo destes meses. Obrigada por me incentivarem em continuar pois sem isso não teria conseguido chegar até aqui, e obrigada por todas as vezes em que me disseram que vai correr bem quando estava desanimada, foi muito importante ouvir isso!

Muito obrigada à Carolina Jardim, Inês Costa e Gonçalo Garcia que me acompanharam mais de perto e que me ajudaram a conseguir acabar este trabalho, obrigada mesmo. Carolina obrigada por teres puxado por mim e por todos os conselhos! Muito obrigada pelo apoio e amizade Inês Figueira, Andreia Gomes, Diana Macedo, Joel Guerra, Joana Pereira, Ana Margarida Nunes, Sónia Moita.

Muito obrigada à Ana Nunes por me ter acompanhado e por me ter introduzido aos conhecimentos e métodos para eu avançar com o meu trabalho, e a todas as pessoas do laboratório, Joana Poejo, Inês Silva, Agostinho Alexandre, Liliana Rodrigues, Daniel Lopes, Vanessa Gonçalves, Cátia Carmo, Catarina Maia pela disponibilidade em me esclarecerem as dúvidas que foram surgindo ao longo do trabalho. Obrigada também à Dra. Rosário Bronze, à Elsa e Andreia pela disponibilidade em analisar as minhas amostras.

Tive um enorme prazer em conhecer-vos e poder trabalhar com vocês. Obrigada por tudo!

Obrigada Tiago Matos, Francisco Oliveira e André Preto pela vossa amizade, por me darem força para continuar e por todos os momentos de descontração. Obrigada por estares ao meu lado e por todo o apoio que me tens dado Luís Batalha!

Muito obrigada aos meus pais, sem vocês não teria sido possível ter conseguido tudo o que sou hoje, obrigada pelo esforço, apoio e confiança durante estes anos todos! Obrigada especialmente à minha mãe pela tua força!

Abstract

The rise in the incidence of neurodegenerative diseases leads to an increased demand for the development of alternative therapeutic strategies. Phytochemicals have proved beneficial effects for human health, exerting protective roles towards several diseases including neurodegenerative disorders. Thus, the search for new bioactivities is a field in constant growth. The beet and fruits belonging to *Opuntia spp.* are rich in betalains and phenolic compounds, which confer several benefits to human health such as regulators of antioxidant and anti-inflammatory responses as well as potential protective effect for chronic diseases.

The objectives of this study were the optimization of solid-liquid extractions of beets, purple and yellow fruits of *Opuntia spp*. in order to obtain extracts enriched in betalains (betacyanins and betaxanthinas) and (poly)phenols. The beetroot purified fraction was selected as the matrix displaying higher contents of betalains and (poly)phenols, and bioactivities for key processes associated with neurodegenerative diseases (Parkinson's disease, Amyotrophic Lateral Sclerosis, Huntington's and Alzheimer's) were assessed using humanized yeast models. As chronic inflammation is a common pathological process associated with neurodegeneration, the potential anti-inflammatory properties of the beetroot purified fraction were also evaluated using a yeast model Crz1/NFAT activation. Although the beetroot phytochemicals did not reveal significant protective activities for amyloidogenic processes associated with these diseases, they showed a potential anti-inflammatory activity as revealed by the great potential to attenuate Crz1/NFAT activation. The most abundant compound present in beet extract, betanin (pure standard), was used to infer whether the potential of the beetroot purified fraction to modulate Crz1/NFAT activation was associated with betalains compounds, revealing that was not the case. Possibly, the phenolic compounds present in this fraction exert the potential anti-inflammatory effects observed or maybe the synergistic action between betalains and (poly)phenols are in the ground of the bioactivity displayed by the beetroot phytochemicals.

Keywords: Natural extracts, Betalains, (Poly)phenols, Neuroprotection, Anti-inflammatory activity

Resumo

O aumento na incidência de doenças neurodegenerativas tem levado a um aumento da demanda para o desenvolvimento de estratégias terapêuticas alternativas. Os fitoquímicos têm revelado efeitos benéficos para a saúde humana, exercendo funções de proteção para várias doenças, incluindo doenças neurodegenerativas. Assim, a procura de novas bioatividades é um campo em crescimento constante. A beterraba e frutos pertencentes a *Opuntia spp.* são ricos em compostos fenólicos e betalaínas, os quais conferem vários benefícios para a saúde humana, tais como a regulação de respostas antioxidantes e anti-inflamatórias, assim como efeitos protetores potenciais para doenças crónicas.

Os objetivos deste estudo foram a otimização da extração sólido-líquido de beterraba, frutos roxos e amarelos de *Opuntia spp.* de modo a obter extratos enriquecidos em betalaínas (betacianinas e betaxantinas) e (poli)fenóis. A fração purificada de beterraba foi selecionada como a matriz exibindo teores de betalaínas e (poli)fenóis mais elevados. As bioatividades desta matriz para processos-chave associadas a doenças neurodegenerativas (doença de Parkinson, Esclerose Lateral Amiotrófica, Huntington e Alzheimer) foram avaliadas utilizando modelos humanizados de levedura. Como a inflamação crónica é um processo patológico comum associado à neurodegeneração, as potenciais propriedades anti-inflamatórias da fração de beterraba purificada foram também avaliadas usando um modelo de levedura de ativação do fator de transcrição Crz1/NFAT. Embora os fitoquímicos da beterraba não tenham revelado atividades de proteção significativas para processos amiloidogénicos associados a estas doenças, eles mostraram uma potencial atividade anti-inflamatória conforme revelado pelo grande potencial para atenuar a ativação Crz1/NFAT. O composto mais abundante presente no extrato de beterraba, betanina (padrão puro), foi usado para inferir se o potencial da fração de beterraba purificada para modular a ativação Crz1/NFAT estava associado às betalaínas, revelando que este não era o caso. Possivelmente, os compostos fenólicos presentes nesta fração exercem os efeitos protetores observados ou talvez a ação sinérgica entre betalaínas e (poli)fenóis estejam na base das propriedades protetoras exibidas pelos fitoquímicos da beterraba.

Palavras-chave: Extratos naturais, Betalaínas, (Poli)fenóis, Neuroprotecção, Atividade anti-inflamatória

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Figure **3.15** Page 36 Modulation of Crz1 activity by beetroot phytochemicals. Pre-cultures grown in glucose were diluted in fresh media supplemented or not with beetroot purified fraction (0.06 % to 6.25%, v/v). Crz1 activity was induced with 3 mM $MnCl_2$ and FK506, at a final concentration of 10 $\mu g.mL^{-1}$, was used as a positive control. Quantitative β-galactosidase assays were performed as described and values are given in Miller units. Values are presented as mean ± SD of at least three replicates.

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List of abbreviations, acronyms and symbols

Aβ42	Beta amyloid protein
AD	Alzheimer´s Disease
ALS	Amyotrophic Lateral Sclerosis Disease
APPH	2`, 2`-Azobis (2-amidinopropane) dihydrochloride
BC	Betacyanins
BX	Betaxanthins
CDRE	Calcineurin-dependent response element
C_2H_3N	Acetonitrile
CH_3COOH	Acetic acid
CH_2O_2	Formic acid
CH_4O	Methanol
Crz1	*C*alcineurin-responsive zinc finger
CSM	Complete Synthetic Mixture
Cyclo-DOPA	Cyclo-3, 4-dihydroxyphenylalanine
EGCG	Epigallocatechine-gallate
FDA	Food and Drug Administration
FL	Disodium fluorescein
FUS	Fused in sarcoma DNA-binding protein
FK506	Tacrolimus
GAE	Gallic Acid Equivalents
h	Hours
HCl	Hydrochloric acid
HD	Huntington´s Disease
H_2O	Water
H_2O_2	Hydrogen peroxide
Htt	Huntingtin protein
KCl	Potassium chloride
KH_2PO_4	Monopotassium phosphate
min	Minutes
$MgSO_4.7H_2O$	Magnesium Sulfate Heptahydrate
$MnCl_2$	Manganese chloride
Na_2CO_3	Sodium carbonate
NaCl	Sodium chloride
$NaH_2PO_4.H_2O$	Sodium phosphate monobasic monohydrate
$Na_2HPO_4.2H_2O$	Sodium phosphate dibasic dehydrate

NFAT	Nuclear Factor of Activated T-cells
OD	Optical Density
ONPG	Ortho-Nitrophenyl-β-galactoside
ORAC	Oxygen Radical Absorbance Capacity
PBS	Phosphate Buffer Solution
PD	Parkinson´s Disease
spp.	Species
SPE	Solid Phase Extraction
TE	Trolox Equivalents
TPC	Total Phenolic Content
Trolox	6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid
YNB	Yeast Nitrogen Base
Y-Per	Yeast Protein Extraction Reagent

1) Introduction

1.1) Phytochemicals

Phytochemicals are natural compounds found in plants, and may present bioactivity. They can be found in fruits, vegetables and also grains. Although a large number of these chemical compounds have been identified so far, many are still unknown and need to be identified (Angel Nivya et al. 2012). The main phytochemicals occurring in plants (figure **1.1**) consumed by humans as foods, are phenolics, carotenoids, alkaloids, organosulfur compounds, as well as nitrogen containing compounds. Among these, carotenoids and anthocyanins have been more extensively studied, however, in the last years there has been also a growing interest in studying nitrogen-containing compounds such as betalains (Iriti et al. 2010).

Bioactive compound family	**Primary source in fruits and vegetables**
Terpenoids	
Carotenoids	Leafy vegetables, red and yellow fruits and vegetables
Monoterpenes	Citrus, cherries, mint and herbs
Saponins	Alliaceae, asparagus
Apocarotenoids	Fruits
Polyphenols	
Phenolic acids	Small fruits, apples, fruit and vegetables
Hydrolysable tannins	Fruit and vegetables, pomegrenate, raspberry
Stylbenes	Grapes, small fruits
Proanthocyanidins	Fruit and vegetables, cacao, small fruits, cranberry, blueberry
Monophenolic alcohols tyrosol	Olive oil, wine
Organo sulphur compounds	
Glucosinolates	Brassicaceae
γ-Glutamyl cysteine sulphoxides	Alliaceae
Alkaloids	
Capsaicin	Chili Pepper
Betalain	Red Beet, Prickly pear, Pittaya

Figure 1.1 – Major phytochemicals present in fruits and vegetables (Dixon & Aldous 2014).

The growing interest in plant sources rich in phytochemicals, which are natural products with potential health benefits for humans, made necessary a clear description and classification of these compounds. In 1989, the Foundation for Innovation in Medicine (New York, US) created the term ´nutraceutical´. Although the definition of the term is in some way questioned, the idea behind the concept is to define substances from foods that have been identified as displaying a medicinal effect on human health (Andlauer & Fürst 2002).

Traditional medicine, in other words, the use of plants (leaves, stems, roots, flowers, fruits and seeds), has been used to treat the symptoms associated with various disorders for thousands of years. They mostly contain a mixture of compounds, and although their evident biological activity, is often difficult to know which compound/s confers the plant its beneficial effect on human health. The reported health-promoting benefits of phytochemicals include protective action against cardiovascular diseases, type 2 diabetes, cancer, neurological decline and metabolic diseases (Chaalal et al. 2013; Tiwari & Cummins 2013; Rao 2003). These protective effects of phytochemicals are mainly related to their antioxidant properties, reducing the oxidative damage associated with these diseases (Shebis et al. 2013), as well as their capacity to modulate the immune system (Rao 2003). However, the precise mechanism(s) of action of these compounds has not yet been fully elucidated due to difficulties in isolating the protective compounds. Moreover, this task becomes even more challenging considering that the potential protective activities may result from the synergistic or additive action of several compounds.

Betalains

Many phytochemicals are pigments absorbing light in the visible region, thus humans have used them since earliest times for food and cloth coloring (Jiménez 2014). Nowadays, these products are mostly colored with synthetic colorants, consequently, there has been a growing concern about their safety and possible adverse effects on human health. So, attending to the consumer´s request, the attention of the scientific research has been directed toward the discovery and isolation of natural pigments. Nevertheless, the use of natural pigments as food additives is very limited because of the strict regulations that need approval by the European Union and the U.S. Food and Drug Administration (FDA) (Delgado-Vargas et al. 2000). Since natural pigments became the focus, also because of their use as part of foods with potential health effects, the research area focusing these compounds has been growing in the last decades, being betalains one relevant class of natural pigments.

In 1968, Mabry and Dreiding introduced the term betalains. This class of compounds currently includes more than 50 molecules, which has aroused a great interest in the last decade (Delgado-Vargas et al. 2000). Betalains are water-soluble nitrogen-containing pigments, which accumulate in the vacuoles of cells, where they are synthesized. They are predominant in plants of the order Caryophyllales, they can be found in leaves, stems, seeds, bracts and flowers, and also in some higher fungi (Gandía-Herrero et al. 2014). A few edible sources containing these compounds are red and yellow beetroot (*Beta vulgaris ssp. vulgaris*), Swiss chard (*Beta vulgaris ssp. cicla*), amaranth (*Amaranthus sp*.) and cactus fruits of *Opuntia* and *Hylocereus* genera (Agrawal 2013). Betalains present a broad spectrum of colors (yellow-violet), and are often difficult to distinguish from the red-purple pigments anthocyanins. The two pigments are structurally distinct and mutually exclusive in the same plant. Taking into account the applicability of these compounds as natural colorants for food industry, betalains are more suitable than anthocyanins because of their higher stability between pH 3 to 7 (Gandía-Herrero et al. 2014; Strack et al. 2003; Dabas et al. 2014).

Betalains are divided into two groups: the violet betacyanins (λ_{max} = 535-540 nm, and a peak in the UV-range between 270 and 280 nm due to the cyclo-Dopa structure) and the yellow betaxanthins (λ_{max} = 480 nm), being their color attributable to the resonating double bonds. The maximum wavelength absorption is dependent on the solvent used for their extraction (Gandía-Herrero et al. 2014; Stintzing & Carle 2004; Jiménez 2014). The common structural unit, *i.e.*, the chromophore of both molecules, is betalamic acid (a protonated 1,2,4,7,7-pentasubstitued 1,7-diazaheptamethin system). This, in condensation with cyclo-DOPA, gives rise to betacyanins, and with amines and amino acids to betaxanthins (Delgado-Vargas et al. 2000). The three general structures are illustrated in figure **1.2**.

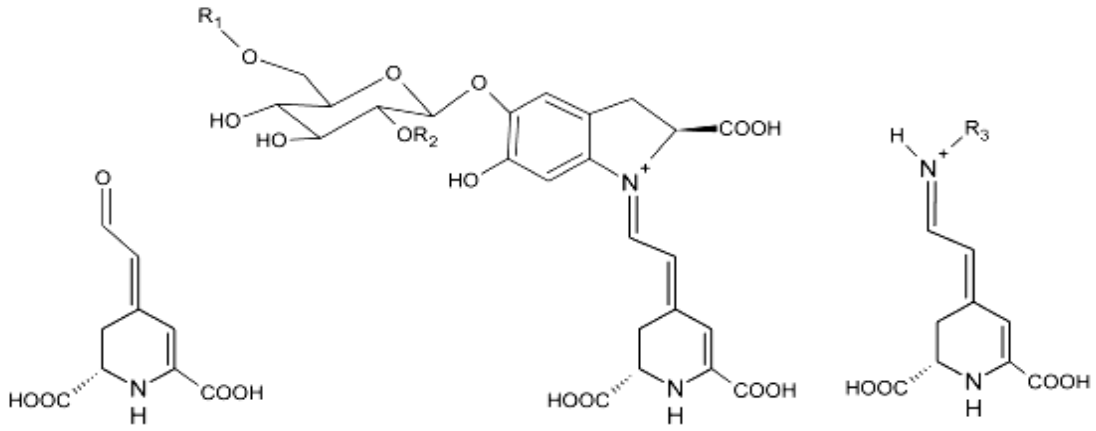

Figure 1.2 - General structures of betalamic acid, betacyanins and betaxanthins, from left to right. R_1= R_2 = H; R_3 = amine or amino acid group (Azeredo 2009).

Betalains are affected by multiple factors such as pH, water activity, exposure to light, oxygen, metal ions, temperature and enzymatic activities. UV or visible light absorption excites π electrons of the pigment chromophore to a more energetic state (π*), which leads to an increase of reactivity. Light-induced betalains then react with oxygen. Thus, to maintain their stability, they should be stored in the dark, under low water activity and oxygen levels, since low oxygen levels allow the pigment to be partially recovered after degradation (Agrawal 2013).

In the last years the study of these pigments has witnessed an increase, also because of their bioactive potential. The health benefits of betalains accounted so far include antiviral, antimicrobial, antioxidant activity, chemoprevention against lung and skin cancers (Slavov et al. 2013).

(Poly)phenols

(Poly)phenols, another class of phytochemicals, are structurally characterized by hydroxyl groups that are linked to aromatic rings. Most are water-soluble, being stored in cellular vacuoles (Sant'Anna et al. 2013; Pace et al. 2014; Yordi et al. 2012). They are divided in sub-groups according to the number of phenol rings and to the structural elements between the phenol rings (Ignat et al. 2011). These features contribute to the great structural diversity and physicochemical properties of (poly)phenols (Tsao 2010).

Indeed, more than 8000 (poly)phenol compounds are currently known, flavonoids being considered as the most relevant compounds belonging to this class (Kujala et al. 2001). Major (poly)phenols found in various sources such as plant and plant derived products are presented in table **1.1**.

Table 1.1 – Major phenolic compounds and their sources (Prakash et al. 2012).

(Poly)phenols	Sources
Flavonoids (Anthocyanidins): Cyanidin 3-glycosides, Malvidin, Delphinidin, Pelargonidin	Blue berries, black berries, cranberry, raspberry, black currant, black grape, straw-berries, cherries, plums, pomegranate, juice, red wine
Flavonoid glycosides: Rutin, Hesperidin, Naringin	Orange, orange juice, lemon, grapefruit, tangerine juice
Flavones: Apigenin, Luteolin	Celery hearts, celery, olives, peppers, fresh parsley, dry parsley, oregano, rosemary, thyme
Flavanones: Naringenin, Eriodictyol, Hesperetin	Grapes, citrus fruits and their juices, tangerine juice, peppermint
Flavanols: Morin, Procyanidins Prodelphinidins, Catechin, Epicatechin and their gallates	Apricots, apples, grapes, peaches, pears, plums, raisins, berries, cherries, red wine, tea, chocolate
Anthoxanthins (Flavonols): Myricetin, Fisetin, Quercetin, Kaempferol, Isorhamnetin	Cherry, tomatoes, spinach, celery, onions, peppers, sweet potato, lettuce, broccoli, kale, buckwheat, beans, apples, apricots, grapes, plums, berries, currants, cherries, juices, *ginkgo biloba*, red wine, tea, cocoa
Isoflavones: Genistein, Daidzein, Equol	Soybean, soy products, soy cheese and sauces, grape seeds/skin,
Phenolic acids: Caffeic acid, Chlorogenic acid, Ferulic acid, p-coumaric acid, Sinapic acid, Ellagic acid,Gallic acid	Lemon, peach, lettuce, coffee beans, tea, coffee, cider, Strawberry, raspberry grape juice, pomegranate juice bluberry, cranberry, pear, cherry, cherry juice, apple, apple juice, orange, grapefruit
Tannins: Catechin, Epicatechin polymers, Ellagitannins, Proanthocyanidins, Tannic acids	Pomegranate, walnuts, peach, olive, plum, chick pea, peas, grape seeds and skin, apple juice, strawberries, raspberries, blackberry, lentils, haricot bean, red wine, cocoa, chocolate, tea, coffee, immature fruits
Diferuloylmethane: Curcuminoids	Turmeric

Recently, phenolics from plants have received interest due to its potential use as food additives, and health-promoting compounds to improve memory, learning and general cognitive ability (Kujala et al. 2001). On the other hand, it is has been claimed that continuous, long-term consumption of (poly)phenols can have helpful effects against cancer and chronic diseases such as cardiovascular diseases and type 2 diabetes, chronic inflammation and neurodegenerative diseases (Del Rio et al. 2012).

1.2) Beetroot (*Beta vulgaris*)

Beet belongs to the *Chenopodiaceae* family and has been used as a common vegetable. The cultivated group of the genus *Beta* includes sugar beets (*Beta vulgaris saccharifera*), fodder beets (*Beta vulgaris crassa*), leaf beets (*Beta vulgaris cicla*) and garden beets (*Beta vulgaris rubra*) (Ninfali & Angelino

2013). Red beetroot (figure **1.3**) is a good source of water (~87%), carbohydrates, fibers, proteins, minerals (sodium, potassium, calcium and iron). It contains mostly betalain pigments such as betaxanthins and betacyanins, and a large number of phenolic compounds (phenolic acids, flavonoids). The phenolic concentration is dependent on the maturity stage of the plant, being maximized when the crop is fully mature (Ninfali & Angelino 2013; Stintzing & Carle 2004). It also contains high levels of nitrates and has an earthy flavor due to geosmin (a bicyclic alcohol) and various pyrazines (heterocyclic aromatic organic compound) (Clifford et al. 2015). Beet is considered a potent antioxidant vegetable due to the betalain pigments that it contains. On the other hand, it is a low caloric vegetable (Straus et al. 2012). Consumption of beetroot appears to help protecting against hypertension and cardiovascular diseases due to its high content in nitrate, and in turn to the produced nitric oxide, the last acting as vasoprotective due to its vasodilatation effect (Hobbs et al. 2012; Coles & Clifton 2012). It can also be chemopreventive, as reported in a study showing that commercial red beetroot inhibits tumorigenesis in a rat esophageal cell carcinoma model (Lechner et al. 2010), in addition to its protective effect on liver injury (Váli et al. 2007) and potential kidney protection (El Gamal et al. 2014).

1.3) *Opuntia spp.* fruits

Cactus pears plants belong to the *Cactaceae* family. They are originated from Central America (Mexico), but they have been well adapted to the arid areas in Europe. The genus *Opuntia* includes around 1500 species, and many of the species produce edible and highly flavored fruits, with different shapes, sizes, and colors (Moßhammer et al. 2006). Cactus fruits (figure **1.3**) are a rich source of water (about 80-95%), carbohydrates, ascorbic acid, vitamin E, carotenoids, fibers, amino acids, phenolic compounds and betalains (Osuna-martínez et al. 2014). *Opuntia ficus-indica*, an edible species of the genus *Opuntia*, has been used in folk medicine to treat arteriosclerosis, diabetes, hypertension, asthma, burns, edema, indigestion and gastritis (El-Mostafa et al. 2014). On the other hand, due to its high phenolic content, it exhibits antioxidant and anti-inflammatory properties (M. Abou Elella 2014; Kaur et al. 2012). It was reported that oral administration of *Opuntia ficus-indica* extracts ameliorates neuronal damage after ischemic insult in cultured mouse cortical cells (Kim et al. 2006).

Figure 1.3 – Beetroot and *Opuntia spp.* fruits. The left image shows red beetroot and the two images on the right show *Opuntia spp,* fruits, purple and yellow, respectively.

1.4) Neurodegenerative diseases

Neurodegenerative diseases are a heterogeneous group of pathologies, defined as hereditary or sporadic conditions. They are characterized by a slow and progressive degeneration of neurons in specific locations of the central nervous system, including the brain, spinal cord and peripheral nerves (Hirsch 2007; Ullah & Khan 2008). The cases of neurodegenerative disorders have increased significantly worldwide, and it is estimated to rise from 13.5 million in 2000 to 36.7 million in 2050 (Chen & Zheng 2012).

Neurodegenerative diseases such as Parkinson's (PD), Amyotrophic Lateral Sclerosis (ALS), Huntington's (HD) and Alzheimer's (AD) are pathologies characterized by the loss or dysfunction of particular groups of neurons (Cacciatore et al. 2012). The pathological hallmarks of these diseases involve accumulation and aggregation of misfolded proteins, which are thought to lead to neurodegeneration. The accumulation of misfolded proteins results from the disruption of protein homeostasis, that is, the equilibrium between protein synthesis and protein degradation (Narayan et al. 2014; Gorman 2008; Takalo et al. 2013; Ross & Poirier 2004; Nieoullon 2011). On the other hand, due to the high oxygen consumption rate, the brain is a vulnerable organ to oxidative damage. Therefore, the generation of reactive oxygen species (ROS) and oxidative damage is directly associated to the pathogenesis of neurodegenerative disorders (Melo et al. 2011; Cacciatore et al. 2012).

Parkinson´s Disease (PD)

PD is the second most common neurodegenerative disease. The clinical symptoms of the disease are due to the degeneration of dopaminergic neurons, which leads to the slow movement, resting tremor and muscle rigidity (Gorman 2008). The major pathological hallmark of the disease is the formation of α-synuclein insoluble aggregates that constitutes cytoplasmic inclusions called Lewy bodies (Braun et al. 2010). The potential protective effect of (poly)phenols for PD has already been stated (Caruana & Vassallo 2011). For example, (poly)phenolic molecules such as resveratrol, cathechin or epigallocatechine-gallate (EGCG) originated from grape, blueberry and green tea extracts show a potential protective effect against this disease (Masuda et al. 2006). In addition, (poly)phenol from *Corema album* leaves extracts were able to inhibit α-synuclein aggregation and reduced its cytotoxicity (Macedo et al. 2015).

Amyotrophic Lateral Sclerosis Disease (ALS)

ALS is a neurodegenerative disorder that results in the progressive death of motor neurons in the central nervous system. It is characterized by progressive muscle disorder, muscle atrophy and paralysis being among the symptoms (Kiernan et al. 2011). Of the identified genes, mutations in *SOD1* (encoding the copper/zinc ion-binding superoxide dismutase), *TDP-43 (*encoding the TAR DNA binding protein), and *FUS* (encoding the fused in sarcoma protein) are mostly responsible for the development of the disease (Kiernan et al. 2011; Poppe et al. 2014). There is evidence of the potential neuroprotective effect

targeted against ALS for a number of (poly)phenolic compounds (Nabavi et al. 2015). The oral administration of EGCG in a transgenic ALS mouse model has shown that this compound is able to increase life span by delaying the disease´s onset (Xu et al. 2006). Also, oral administration of a chemical mixture composed of flavonols, terpenes and other compounds from *Gingko biloba* extract revealed a neuroprotective effect in transgenic ALS mice models (Ferrante et al. 2001).

Huntington´s Disease (HD)

HD is characterized by neuronal cell death due to the accumulation of toxic mutated Huntingtin protein fragments. The symptoms are progressive motor abnormality and intellectual deterioration, and manifest typically between 30 and 45 years of age (Li & Li 2006). The main hallmark of the disease are intranuclear and cytoplasmic inclusions of Huntingtin (Htt), which leads to brain lesions (Outeiro & Muchowski 2004). It has been shown that oral administration of grape seed (poly)phenolic extract inhibited the abnormal aggregation of the Huntingtin protein (Wang et al. 2010). On the other hand, antioxidants such as lycopene have shown their efficacy in slowing disease progression in transgenic HD mouse models (Johri & Beal 2012).

Alzheimer's Disease (AD)

AD is the most common neurodegenerative disease leading to progressive memory loss and cognitive dysfunction (Gorman 2008; Quinn et al. 2004). The disease is characterized by the accumulation in the brain tissue of beta amyloid (Aβ42) proteins, resulting in synaptic degeneration, neuronal loss and decline in cognitive function (Axelsen et al. 2011; Zhao & Zhao 2013). Compounds from *Opuntia ficus-indica* appear to protect primary cultured rat cortical cells against neuronal injury induced by oxidative stress, excytotoxins, and amyloid β (Aβ) (Cho et al. 2007). In addition, the flavonoids quercetin and rutin showed an inhibitory action on Aβ formation in AD case studies, suggesting their potential protection against Aβ toxicity (Jiménez-Aliaga et al. 2011).

1.5) Inflammation

Inflammation is a protective response of the organism to infection and tissue injury in order to reestablish the homeostasis, but it can cause undesirable effects if the response persists more than it is needed to restore normal immune function of the organism, leading to disorders characterized by chronic inflammation. Neurodegenerative disorders are associated with a variety of inflammatory responses, which may be activated by the accumulation of proteins with abnormal conformations or by signals originated from injured neurons (Wyss-coray & Mucke 2002). However, it is known that systemic inflammation links with the brain through signals, which lead to modifications in the behavior, and recent indications suggest that this fact may lead to the development of neurodegenerative diseases (Perry 2010). Although the inflammatory response may occur in a certain location of the body, the inflammatory mechanisms can communicate. One example is the possible use of conventional

nonsteroidal anti-inflammatory drugs, which could delay the progression of some neurodegenerative diseases (Akiyama et al. 2000).

The mechanism by which cells respond to stimuli is the contribution of the immune cells such as macrophages, dendritic cells, mast cells, neutrophils and leucocytes, and other non-immune cells. Mediators called cytokines and chemokines regulate the arrival of these cells at the damaged tissue to begin the anti-inflammatory process. On the other hand, the inflammatory response is regulated by genes encoding for these mediators through the activation of transcription factors. In normal conditions they remain inactive in the cytoplasm, and enter the nucleus in response to a stimulus to induce expression of specific genes (Ahmed 2011). This is the case of the transcription factor NFAT (Nuclear Factor of Activated T cell), which regulate T cells immune response. NFAT is regulated by the calmodulin/calcineurin complex, leading to its dephosphorylation and localization to the nucleus. Therefore, NFAT transport between cell compartments is dependent on the level of calcium intracellular and calcineurin activity (Pan et al. 2013).

Calcineurin is a eukaryotic serine/threonine protein phosphatase containing a catalytic and a regulatory subunit, and functions along with the calmodulin/Ca^{2+}-complex, leading to dephosphorylation of NFAT. As the intracellular calcium level rises, the calcineurin is activated. FK506 (Tacrolimus) inhibits the NFAT dephosphorylation, blocking its translocation to the nucleus and the activation of pro-inflammatory genes (figure **1.4**) (Rusnak & Mertz 2000).

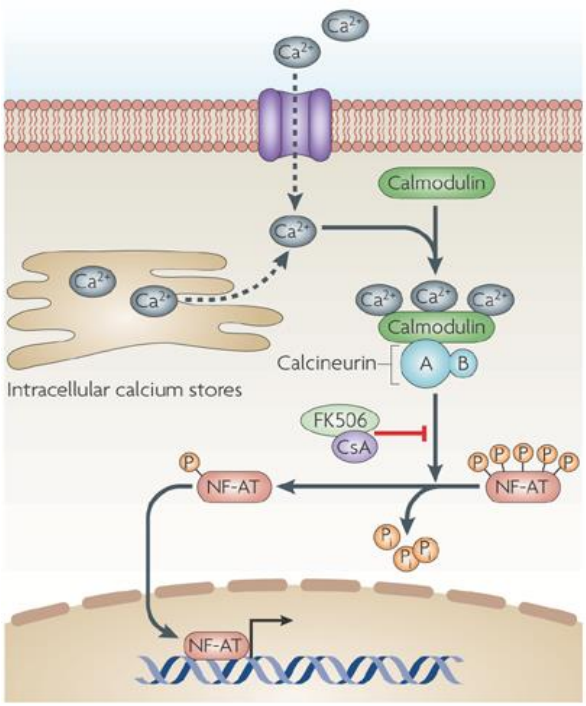

Figure 1.4 – Schematic representation of the cellular pathway leading to NFAT activation in mammalian cells (Steinbach et al. 2007).

1.6) Yeast as a discovery platform for therapeutic compounds

The knowledge of disease mechanisms comes from models, either *in vivo* or *in vitro*, which uses a toxin and/or genetic mutations to recapitulate specific disease features. The yeast *Saccharomyces cerevisiae* is the simplest eukaryotic model organism with proven utility in the study of cellular pathologies associated to neurodegenerative diseases (Khurana & Lindquist 2010). Yeast cells are unicellular and contain a nucleus and organelles bounded by membranes like mammalian cells (Duina et al. 2014).

The advantages of using yeast models for biological studies include easy manipulation and short doubling time. Additionally, yeast encodes various genes with human orthologues (Khurana & Lindquist 2010). Most importantly, it shares many of the fundamental biological features of the eukaryotic cells, including basic cellular pathways (figure **1.5**) involved in neurodegeneration, such as protein homeostasis, mitochondria biogenesis, intracellular transport, cell division and vesicular trafficking (Ocampo & Barrientos 2008; Pereira et al. 2012). On the other hand, the triggering of mammalian Ca^{2+}-signaling pathways and NFAT activation is highly conserved in yeast, which in turn culminates with the induction of the calcineurin-responsive zinc finger – Crz1 – transcription factor, a NFAT orthologue (Thewes 2014).

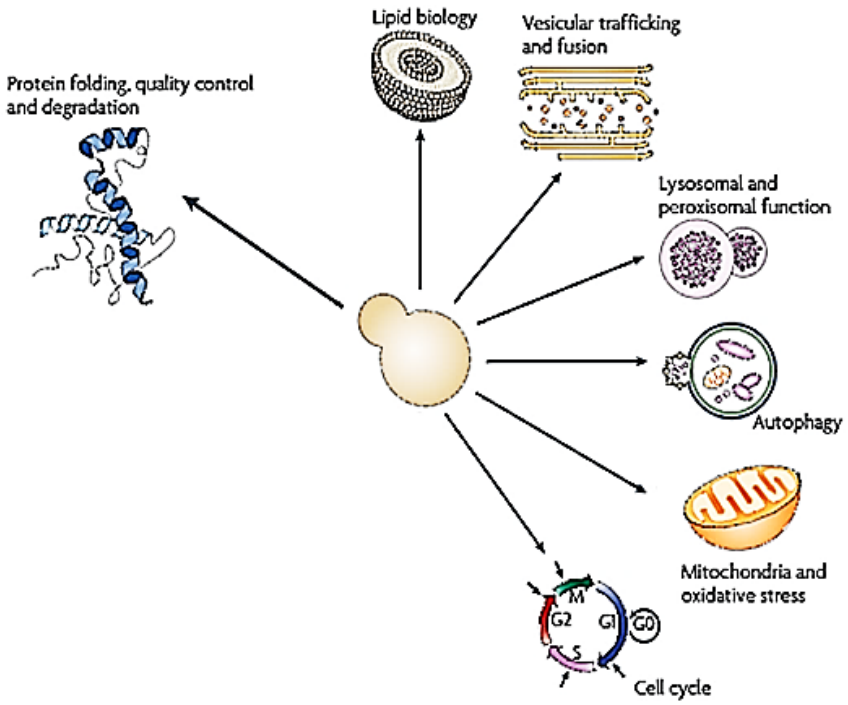

Figure 1.5 – Illustration of the main cellular pathways relevant for neurodegeneration, which are conserved between yeast and human (adapted from Khurana & Lindquist 2010).

Yeast models of neurodegenerative diseases

The humanized yeast models are based on the premise that disease protein aggregation is a relevant feature of neurodegenerative processes associated to PD, ALS, HD and AD. In the yeast models, recombinant versions of α-synuclein (PD), FUS (ALS), Htt (HD) and Aβ42 (AD) fused to GFP are regulated by the inducible *GAL1* promoter and shift of cells to galactose-supplemented media triggers high fusion protein expression, leading to protein mislocalization and cell death. All the humanized yeast models mimic fundamental aspects of disease-protein pathobiology, including aggregation, proteostasis unbalance and cytotoxicity (Braun et al. 2010; Khurana & Lindquist 2010; Outeiro & Lindquist 2003; Kryndushkin & Shewmaker 2011; Mason & Giorgini 2011), offering a great opportunity to screen for compounds with protective functions for these pathological processes.

Yeast model of Crz1/NFAT activation

Crz1 regulation is highly conserved with the NFAT human orthologue. The yeast model encodes an integrated copy of the bacterial *lacZ* reporter gene under the control of a calcineurin-dependent response element promoter (CDRE). At low cytosolic Ca^{2+} levels, phosphorylated Crz1 is kept in the cytosol; cellular stimuli increasing cytosolic Ca^{2+} levels activate the calmodulin/calcineurin complex, leading to Crz1 dephosphorylation and translocation to the nucleus, where it binds the CDRE and activates *lacZ* expression. Therefore, it is possible to monitor the degree of Crz1 activation by evaluating the β-galactosidase activity (Schumacher et al. 2008; Garcia et al. 2015, in press). Compounds able to interfere with this process are good candidates to modulate NFAT activation, since both pathways are highly conserved.

1.7) Objectives

The major goal of this thesis was to identify potential therapeutic compounds for neurodegenerative disorders and chronic inflammatory processes in beetroot and *Opuntia* fruit matrixes. This objective was accomplished through the following intermediate goals:

(a) Isolation of betalain- and (poly)phenol- rich extracts from beetroot and *Opuntia* fruits (purple and yellow), using biocompatible solvents (water), and selection of the matrix containing the highest contents of both classes of compounds;

(b) Evaluation of the protective potential of the selected matrix using humanized yeast models of the major neurodegenerative disorders (Parkinson's, Amyotrophic Lateral Sclerosis, Huntington's and Alzheimer's) and Crz1/NFAT activation.

2) Materials and Methods

2.1) Samples and extracts preparation

The red beetroots (*Beta Vulgaris*) were purchased, in early September 2014, from a local supermarket (Pingo Doce, Oeiras, Portugal). After being washed, they were cut into small pieces and freeze-dried during 72 h (the whole vegetable, including the peel). Afterwards, the pieces were freeze-dried in a FreeZone Plus 4,5 L Cascade Freezer Dry System (Labconco), milled, sieved and stored at -20 ˚C. The purple and yellow *Opuntia spp.* fruits were collected in Quarteira (Algarve, N37º 04´ 24´´ W8º 06´ 06´´) and Ribeira dos Moinhos (Sines, N37º 58´ 41´´ W8º 50´ 27´´), Portugal, respectively, at the end of September 2014. Firstly, the spikes on the surface of the fruits were removed, and the fruits were cut into pieces and freeze-dried during 72 h to obtain the dry matrix. After being freeze-dried using the same equipment cited above, the fruits were milled and stored at -20 ˚C.

The parameters of the solid-liquid extraction applied to the three matrixes were selected taking into account two optimized extraction procedures already published for beetroot (Swamy et al. 2014) and for *Opuntia spp.* fruits (Prakash Maran et al. 2013), with modifications. For beetroot, the optimum extraction parameters were defined as 1.5 g fresh weight extracted with 50 mL water, during 84 min at 60 ˚C (Swamy et al. 2014). For *Opuntia spp*. fruits, the optimum extraction parameters were 1.2 g of fresh fruits extracted with 50 mL water at pH 6.9, during 115 min at 42 ˚C (Prakash Maran et al. 2013). Nevertheless, since for beetroot the pH of the water was not mentioned, it was performed an optimization of this parameter. Additionally, the procedure was carried out using stirring at 405 rpm, to promote a better diffusion of the pigments into the solvent, and all the extractions (in triplicate) were made in a thermostatic bath, in the absence of light, to prevent degradation of phytochemicals. When necessary, the acidification of the extraction solvent (distilled water) was done with 1% citric acid (Sigma-Aldrich) to enhance betalain stability (Strack et al. 2003). The obtained extracts from beetroot and *Opuntia spp.* fruits were kept at -20 ºC, in the dark.

Therefore, two procedures were carried out for beetroot phytochemicals extraction (figure **2.1**):

<u>Procedure 1</u>:

Condition (a): As the water pH of the extraction solvent was not well established in the previous studies, the pH optimization was conducted using three different pH conditions: 3, 5 and 7. Since the matrix was lyophilized, the ratio of dry matrix/solvent used was 0.165 g/51.335 mL (1:311). The calculation of this ratio took into consideration the mass of fresh fruit/solvent ratio from the original reference (1.5 g/50 mL) and the water content of beetroot determined in the lyophilization process.

Condition (b): To replicate the conditions used in the original reference, it was used a 1.5 g/50 mL ratio (1:33) of fresh matrix/water. As the water pH (extraction solvent) was not mentioned, the water was not acidified considering that the authors used water without acidification. So, the water pH was 7.1.

Procedure 2: This procedure was established according to the best condition resulting from procedure 1 with the aim to maximize betalains and (poly)phenols contents. Extractions were performed using dry matrix, a 1:10 (10 g/100 mL) ratio of dry matrix/water and pH 5.

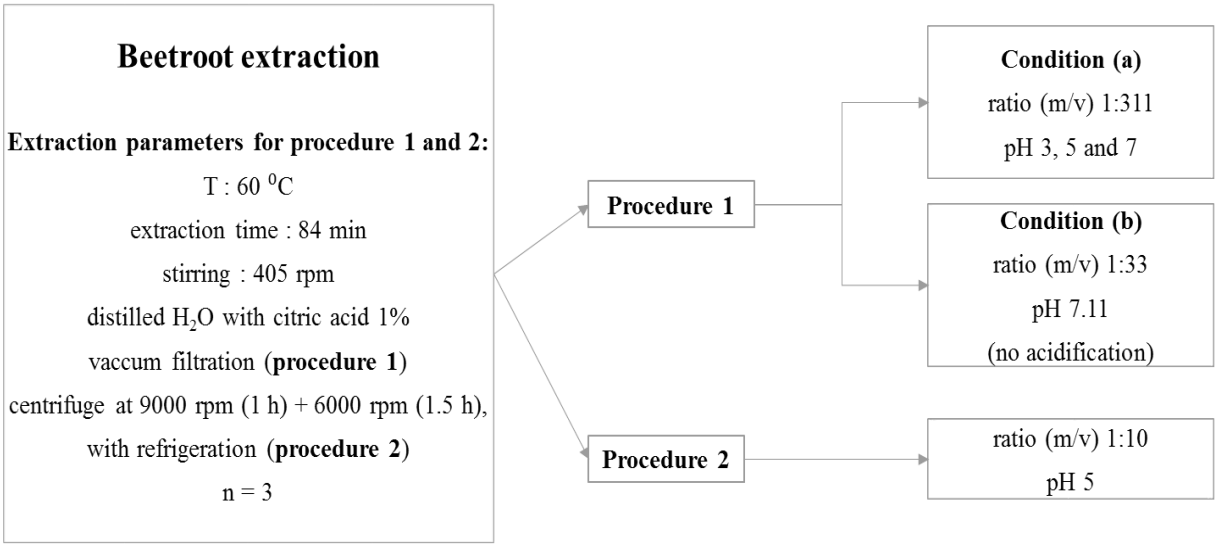

Figure 2.1 – Schematic representation of the procedures used for beetroot solid-liquid extractions.

Two procedures were also carried out for purple *Opuntia spp.* fruits phytochemicals extraction (figure **2.2**):

The ratio dry matrix/solvent for the purple fruits was calculated according to the ratio described in the literature (1.2 g/50 mL) (Prakash Maran et al. 2013) and the water percentage of the fruits obtained from the lyophilization process, obtaining a final ratio of 0.264 g/50.936 mL (1:193).

Procedure 1: It was performed according to the parameters described by Prakash Maran and co-workers (2013).

Procedure 2: Taking into account the parameters established for the beetroot, extractions were carried out at pH 5 for 84 min to allow yielding an increase on phytochemical contents and to evaluate the impact of a lower residence time, which could avoid degradation due to prolonged exposure to high temperature.

For yellow *Opuntia spp.* fruits, phytochemical extractions were performed using a 1:10 ratio of dry matrix/water (1 g/10 mL) and different temperatures (40 ºC, 50 ºC and 60 ºC) to evaluate their influence in the final contents of betalains and (poly)phenols. The extracts were centrifuged instead of filtrated because of their high viscosity (figure **2.2**).

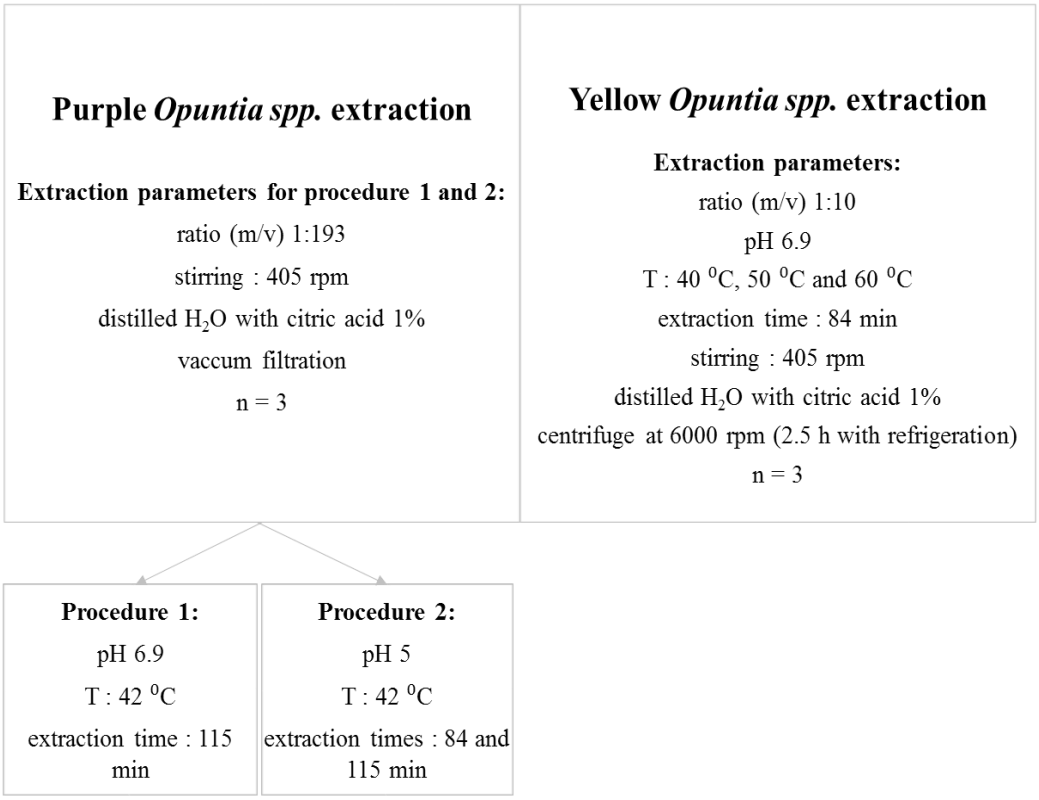

Figure 2.2 – Schematic representation of the procedures used for purple and yellow *Opuntia spp.* fruits solid-liquid extraction.

2.2) Solid Phase Extraction (SPE)

Purification of selected beetroot extracts was performed by solid phase extraction, at room temperature (~25 ^{0}C), using a reversed phase C-18-E (2 g/12 mL, Strata, Phenomenex). The reagents used for the procedure were acetic acid (CH_3COOH), formic acid (CH_2O_2) and acetonitrile (C_2H_3N), purchased from Panreac, and methanol (CH_4O) from Fischer Scientific. Briefly, the procedure consisted in five steps. First the column was washed with 15 mL of 0.5% CH_3COOH/C_2H_3N (5 mL each time) to activate stationary phase. It was then equilibrated with 15 mL of 0.5% CH_3COOH/H_2O (5 mL each time) to prepare sorbent for optimized interaction with the analyte, and the extract was loaded (5 mL each time) for analyte selective retention. Fractions 1 and 2 were retrieved. The column was washed with 15 mL of 0.5% CH_3COOH/H_2O (5 mL each time) to remove impurities from the sample, providing fractions 3 and 4. Finally, analytes were eluted 6 mL of 2% CH_2O_2/CH_4O, giving rise to fractions 5 and 6.

2.3) Extracts characterization

2.3.1) Betalain determination

Spectrophotometric determination of betalains was performed using an UV-Vis spectrometer (Thermo Spectronic, Genesys 10uv). Measurements were performed by diluting the samples in 0.05 mM citrate-phosphate buffer pH 6.5 (Na_2HPO_4/KH_2PO, acidified with 0.1 M hydrochloric acid (HCl), reagents purchased from Sigma-Aldrich). Betacyanins (BC) and betaxanthins (BX) were determined in

triplicates according to Nilsson equations (1970), by means of three wave lengths as reference: 480 nm ($\lambda_{max.}$ indicaxanthin), 538 nm (λ_{max} betanin) and 600 nm (correction of the absorbance due to impurities of the solutions), using the following equations (Guzmán-Maldonado et al. 2010; Slavov et al. 2013):

% **Betacyanins** (BC) = (a/1129)*FD (Eq. 1)

and

% **Betaxanthins** (BX) = (y/750)*FD (Eq. 2)

where:

a = 1,095*($A_{538\ nm}$ - $A_{600\ nm}$);

y = A_{476nm} - ($A_{538\ nm}$ - a) - (a/3.1);

FD = dilution factor.

The results were expressed as a mean of triplicates and were presented as mg of betacyanins or betaxanthins per liter (L) and per 100 g of dry extract (d.e.).

2.3.2) Total phenolic content (TPC) determination

Total phenolics concentration of extracts was determined according to the Folin-Ciocalteau colorimetric method (Singleton et al. 1999) as previously described by Serra (2010). The reagents used were Folin-Ciocalteau reagent (Panreac), saturated sodium carbonate solution (Na_2CO_3, Sigma- Aldrich) and 1000 $mg.L^{-1}$ gallic acid ($C_7H_6O_5$, Fluka).

The procedure was carried out on a 96-well plate. Briefly, 3 µL of appropriately diluted samples were added to 273 µL of distilled water, and 15 µL of Folin-Ciocalteau reagent was added. 45 µL of saturated Na_2CO_3 were added to neutralize the reaction, and it was incubated at 37-40 ˚C for 30 min. The absorbance of samples and blank (distilled water) was recorded at 765 nm using the Genesys10uv spectrometer (Thermo Spectronic). To quantify the total phenolic content, it was used a calibration curve with gallic acid as standard, with the following concentrations: 0, 50, 100, 200, 400, 600 and 800 $mg.L^{-1}$, prepared in distilled water. Samples were analyzed as triplicates. The total phenolic content of the samples were expressed in gallic acid equivalents (GAE) per liter and per 100 g of dry extract (d.e.).

2.3.3) Oxygen radical absorbance capacity (ORAC)

The ORAC assay was carried out as previously described (Huang et al. 2002), with adaptations for the FL800 microplate fluorescent reader (Serra 2010). The reagents used for the procedure were 0.3 µM disodium fluorescein (FL, TCI Europe), 153 mM 2, 2'- Azobis (2-amidinopropane) dihydrochloride (AAPH, Sigma-Aldrich), 5 mM 6-hydroxy-2, 5, 7, 8- tetramethylchroman-2-carboxylic acid (Trolox, Sigma-Aldrich). Sodium chloride (NaCl), potassium chloride (KCl) and monopotassium phosphate (KH_2PO_4) were purchased from Sigma-Aldrich and sodium phosphate dibasic dehydrate ($Na_2HPO_4{\cdot}2H_2O$) from Riedel-de-Haën to prepare 75 mM phosphate buffer solution (PBS) at pH 7.4. All samples, the calibration curve (Trolox), AAPH and FL were prepared in PBS, the former was used as blank.

The procedure was performed in a 96-well microplate using 25 μL of properly diluted sample, and 150 μL of 0.3 μM FL. The microplate was incubated in a fluorescent reader (Bio-Tek Instruments) at 37 ºC, for 10 min. 25 μL of 153 mM AAPH were added through an injector to start the reaction. Trolox, at the concentrations of 5, 10, 20, 40 and 50 μmol.L^{-1} was used as a standard. Samples were analyzed as triplicates. Fluorescence was recorded every 1 min, during 40 min, at the emission wavelength of 530±25 nm, and excitation wavelength of 485±20 nm. ORAC values were calculated by a regression equation between the Trolox concentration and the net area under the FL decay curve, and were expressed as Trolox Equivalents (TE) per liter per 100 g of dry extract (d.e.).

2.4) Sucrose, D-fructose and D-glucose assay procedure

The assays were performed using the Megazyme kit (K-SUFRG 06/14, Megazyme International Ireland) according to manufacturer´s instructions. The reagents included in the kit were sodium phosphate buffer plus sodium azide (0.02% w/v), NADP+ plus ATP, hexokinase plus glucose-6-phosphate dehydrogenase suspension, phosphoglucose isomerase suspension and β-Fructosidase. Spectrophotometric measurements were carried out at 340 nm using the Shimadzu UV-1603 equipment (Model CPS-240A). Sucrose, D-fructose and D-glucose contents were quantified in the beetroot extract and in the SPE purified fractions, samples being appropriately diluted in distilled water.

2.5) Strains and plasmids

The yeast strains used in this study are listed bellow:

- PD yeast model: W303 *MATα can1-100 his3-11 15 leu2-3 112 ade2-1 GAL1pr-syn WT::TRP1 GAL1pr-syn WT::URA3* (Outeiro and Lindquist 2003), which encodes two integrated copies of *SNCA* (α-synuclein) gene fused to GFP, under the control of the yeast *GAL1* promoter;
- ALS yeast model: W303 *MATα can1-100 his3-11 15 leu2-3 112 ade2-1 GAL1pr-FUS WT::TRP1 GAL1pr-syn WT::HIS3* (Ju et al 2011), encoding two integrated copies of the *FUS* gene fused to GFP, under the control of the yeast *GAL1* promoter;
- HD yeast model: BY4741 *MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 YML008c*::*kanMX4 <p425_GAL1pr_HTTpolyQ103_GFP> <p426_GAL1pr_HTTpolyQ103_GFP> (*Menezes et al, unpublished), which encodes two episomal copies of *HTTpolyQ103* cDNA fragment fused to GFP, under the control of the yeast *GAL1* promoter. The recombinant multi-copy plasmid *p425_GAL1pr_HTTpolyQ103*_GFP (Krobitsch and Lindquist 2000) was kindly provided by Prof Dr Tiago Outeiro;
- AD yeast model: BY4741 *MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 YML008c*::*kanMX4 <p425_GAL1pr_GFP_Aβ-42> <p426_GAL1pr_GFP_Aβ-42>*, encoding two episomal copies of *Aβ-42* cDNA fused to GFP, under the control of the yeast *GAL1* promoter (Menezes et al, unpublished);

- Yeast model of Crz1/NFAT: BY4741 *MATa his3 leu2 lys2 ura3 aur1::AUR1-C-4xCDRE-lacZ* (Araki et al 2009), encoding an integrated copy of the *lacZ* reporter gene under the control of a promoter containing four copies of the Crz1 binding sequence CDRE.

2.6) Yeast media and reagents

Humanized yeast strains expressing PD, ALS, HD and AD disease-proteins were grown in synthetic media containing 6.7 $g.L^{-1}$ yeast nitrogen based (YNB, Difco), 0.79 $g.L^{-1}$ complete supplement mixture (CSM-6AA w/o adenine, histidine, leucine, lysine, tryptophan and uracil; MP Biomedicals), and the required amino acids. Glucose ($C_6H_{12}O_6$, Sigma-Aldrich), galactose ($C_6H_{12}O_6$, Fluka Analytical, Sigma-Aldrich) or raffinose ($C_{18}H_{32}O_{16}.5H_2O$; Fluka Analytical, Sigma-Aldrich) at a final concentration of 2.0% (w/v) were used as carbon sources.

The yeast strain encoding the Crz1-reporter system was grown in synthetic complete media containing 6.7 $g.L^{-1}$ YNB (Difco), 0.79 $g.L^{-1}$ of CSM (QBiogene) and 2.0% (w/v) glucose. 100 mM manganese chloride ($MnCl_2$, Sigma–Aldrich) and 1$mg.mL^{-1}$ FK506 (Tacrolimus, Sigma–Aldrich) were used as a Crz1 inducer and as an immunosuppressant, respectively. Yeast Protein Extraction Reagent (Y-PER, Thermo Fisher Scientific Inc.–Life Technologies), LacZ buffer (prepared with 8.5 $g.L^{-1}$ Na_2HPO_4, 5.5 $g.L^{-1}$ $NaH_2PO_4.H_2O$, 0.75 $g.L^{-1}$ KCl, 0.246 $g.L^{-1}$ $MgSO_4.7H_2O$), and 2 $mg.mL^{-1}$ Ortho-Nitrophenyl-β-galactoside (ONPG, Sigma–Aldrich) were used to monitor β-galactosidade activity.

2.7) Growth conditions

The evaluation of the protective activity of phytochemicals using the humanized yeast models was performed by means of growth curve assays. For that, stationary phase pre-cultures grown overnight at 30 ºC in raffinose-supplemented synthetic media, with orbital agitation at 200 rpm, were diluted in fresh media and incubated for 6-8 h under the same conditions. The optical density of the cultures was monitored at 600 nm (OD_{600}), using the BioTek, PowerWaveXS spectrophotometer, and cultures were diluted to obtain a final OD_{600} 0.4 ± 0.04 after 16 h incubation, according to the equation:

$ODi \times Vi = (ODf/(2^{(t/gt)}) \times Vf$ (Eq. 3)

where:

ODi = initial optical density of the culture;

Vi = initial volume of culture;

ODf = final optical density of the culture;

t = time of incubation;

gt = generation time of the strain;

Vf = final volume of culture according to the number of conditions tested.

Monitoring of phytochemical bioactivities towards Crz1/NFAT regulation was carried out through the measurement of Crz1-driven β-galactosidase activity. For that, stationary phase pre-cultures grown overnight at 30 ºC in glucose-containing synthetic media, with orbital agitation at 200 rpm, were diluted in fresh media and incubated for 6-8 h under the same conditions. The OD_{600} was monitored and cultures were diluted according to same equation described above, to obtain a final OD_{600} 1 ± 0.1 after 16 h.

2.8) Growth curves

The assay to evaluate the protective activity of the extracts was performed using sterile 96-well microplates. The lyophilized extracts were first resuspended in sterilized water and were then diluted in glucose or galactose media. Exponential-phase control and humanized cell cultures (obtained from **2.7**) were diluted in glucose (disease protein expression repressed) and galactose (disease protein expression induced) media to obtain a final OD_{600} of 0.08 ± 0.008. The microplates were incubated at 30 ^{0}C, with continuum agitation, in the spectrometer (BioTek, PowerWaveXS) during 24 h. The OD_{600} was monitored every hour for 24 h. Control and humanized cultures, without the addition of phytochemicals, were diluted in fresh glucose and galactose media, and were used for comparison purposes. Each tested concentration of extracts/fractions had a corresponding blank.

2.9) β-galactosidase assays

To 300 µL of exponential-phase cultures obtained from section **2.7**, it was added 20 µL of extracts/fractions to be tested, sterilized water (negative control) or the positive control FK506 (to a final concentration of 10 $\mu g.mL^{-1}$), and cultures were incubated at 30 ^{0}C for 90 min with orbital agitation at 200 rpm. The tubes were then centrifuged for 3 min at 1000 g, and the pellet was ressuspended in 300 µL of fresh media. After homogenizing the cells, 150 µL corresponding to each condition was transferred into a new tube containing 7.5 µL of $MnCl_2$ (to a final concentration of 3 mM). These tubes correspond to the induced condition. To the remaining 150 µL of each culture, 7.5 µL of water were added to mimic the uninduced condition. The tubes were incubated for further 90 min under the same conditions. 10 µL of these cultures were retrieved for OD_{600} measurements. For the monitoring of β-galactosidase activity (in triplicate), 10 µL of each sample were transferred to a 96-well microplate containing 20 µL Yeast Protein Extraction Reagent (Y-PER) per well. The microplate was incubated at 37 ºC for 20 min and 240 µL of LacZ/ONPG buffer were added to each well. The 96-well microplates were incubated at 30 ºC in a spectrophotometer (BioTek, Synergy HT) for 2 h and OD_{420}/OD_{550} were monitored every 10 min.

The β-galactosidase activity values were given in Miller units, calculated according the equation:

$1000 \times ((OD_{420} - 1.75 \times OD_{550})/(t \times V \times OD_{600})$ (Eq. 4)

where:

t = reaction time in min

V = volume of culture assayed in mL

The OD_{420} values used in the equation were chosen taking into account the best differences between the β-galactosidase activities from the positive and negative controls.

2.10) Statistical analyses

Statistical analyses were performed using SigmaPlot software and results were presented as mean ± standard deviations. Differences were tested using one-way ANOVA, performing the Tukey test for multiple comparison, and were considered significant when $p<0.05$.

3) Results and Discussion

3.1) Extracts chemical characterization:

3.1.1) Betalain (betacyanins and betaxanthins) determination

The betalain contents of extracts/fractions were determined spectrophotometrically using Nilsson equations (Eq. 1 and 2 section **2.3.1**) and are presented as $mg.L^{-1}$ or $mg.100\ g^{-1}$ dry extract (d.e.).

3.1.1.1) Beetroot extracts

Based on literature data, two extraction procedures were carried out to improve the extraction of betalains. Along with betalains, (poly)phenols are co-extracted, improving the composition complexity. Beetroot matrix was extracted with water, during 84 min at 60 ^{0}C and matrix/water ratio was calculated as previously described (Swamy et al. 2014). For procedure 1, and once the pH was not specified, in this work an optimization of extraction solvent pH was studied. Three different pH (3, 5 and 7) were evaluated using a dry matrix/volume (m/v) ratio of 1:311. At pH 7.1 a comparison was made using a different m/v ratio (1:33) and in this case fresh matrix was used (based on reference Swamy et al. 2014). For the best pH attained in this first approach (procedure 1), pH 5, other comparison was performed (procedure 2) in order to verify the effect of increasing m/v ratio (dried matrix) and with this reduce the experimental error. For this m/v of 1:10 was applied.

The extracts were evaluated in terms of betacyanin and betaxanthin content, and are presented in $mg.L^{-1}$ and $mg.100\ g^{-1}$ dry extract in table **3.1**.

Table 3.1 – Betalain determination of beetroot extracts, at different pH (3, 5, 7 and 7.1), matrix/solvent ratios (1:311, 1.5:50 and 1:10), extraction at 60 ºC for 84 min. Values are presented as mean ± standard deviation (SD) from three replicates.

		Freeze-dried matrix			Fresh matrix	Freeze-dried matrix
Beetroot extracts		pH 3 **(1:311)**	pH 5 **(1:311)**	pH 7 **(1:311)**	pH 7.1 **(1:33)**	pH 5 **(1:10)**
BC	$mg.L^{-1}$	5±0.2	10±0.4	9±0.1	16±4.5	282±1
	$mg.100\ g^{-1}$ d.e.	9 ±5	**289±13**	160±11	375±104	390±1
BX	$mg.L^{-1}$	4±0.1	9±0.4	9±0.3	11±2.3	217±0.3
	$mg.100\ g^{-1}$ d.e.	76±3.5	**250±15**	152±9	274±47	301±0.4

BC: Betacyanins, BX: Betaxanthins, d.e.: dry extract

The optimization of solid-liquid extraction procedure for beetroot indicated that pH 5 was the best condition to extract betacyanins and betaxanthins in any of the freeze-dried matrix/solvent ratios. On the other hand, the extraction made with fresh matrix (1:33 ratio) allowed an efficient betalain extraction, but less reproducibility (as inferred by the high deviation among triplicates), compared to

the dry matrix. According to Kujala and co-workers (2000), pigments distribution in fresh beetroot surface (peel, flesh and crown) varies, and so the pigment extraction using fresh matrix may present higher variability depending on the beetroot portion and its size. Since the condition at pH 5 was optimum, it was prepared an extract using the same conditions but with a higher m/v ratio (1:10). The extract presented a much higher content in betalains in terms of $mg.L^{-1}$ compared to the obtained for 1:311 ratio, although per dry extract the content was very close. Since the condition at pH 5 demonstrated to be optimum, it was prepared an extract using the same conditions but with a higher m/v ratio (1:10) in order to reduce the error associated to mass losses. The extract prepared with this higher m/v ratio presents, as expected, a much higher betalains concentration ($mg.L^{-1}$) and high amount of betalains (both BX and BC) per mass of dry extract. The betalains extraction was successfully attained in both conditions, but as less solvent was available per gram of raw material, the extraction was more selective (regarding betalains). This fact contributes to the reducing of co-extracted compounds in the extract. As expected, the experimental error also decreased.

3.1.1.2) *Opuntia spp.* purple and yellow extracts

The purple and yellow *Opuntia spp.* fruit extracts were obtained using three different solid-liquid extraction conditions. Betalain extraction from purple fruits were performed using a dry matrix/water ratio of 1:193 (calculated according to Prakash Maran and co-workers (2013)), using pH 5 and 84 min as pH and extraction times parameters, respectively, since these conditions were found to be optimum for beetroot betalains extraction (section **3.1.1.1**). As the optimum parameters for *Opuntia spp.* fruits betalain extraction according to Prakash Maran and co-workers (2013), pH 6.9, 42 ºC and 115 min, the combination of parameters pH 5 and 115 min was also studied.

The extracts were evaluated in terms of betacyanin and betaxanthin content and presented in $mg.L^{-1}$ and $mg.100\ g^{-1}$ dry extract in table **3.2**. However, it was not possible to detect betaxanthins in the extracts.

Table 3.2 – Betalain determination of purple *Opuntia spp.* extracts at different pH (5 and 6.9), matrix/solvent ratio of 1:193, extraction at 42 ºC for 84 and 115 min. Values are presented as mean ± standard deviation (SD) from three replicates.

.

***Opuntia spp.* purple extracts**		**pH 6.9** (1:193) (115 min)	**pH 5** (1:193) (84 min)	**pH 5** (1:193) (115 min)
BC	$mg.L^{-1}$	9±0.7	7±0.4	7±0.3
	$mg.100\ g^{-1}$ d.e.	332±4	314±5	302±4

BC: Betacyanins, d.e.: dry extract

The betacyanins content of purple fruits was similar to those determined on the beetroot extract in terms of $mg.100\ g^{-1}$ d.e. (see tables **3.1** and **3.2**). It can also be concluded that the extraction time (84 min

versus 115 min) did not influence betalain extraction, contrarily to higher pH, which favors betacyanins extraction. Among the three conditions, extraction performed at 42 ºC and pH 6.9 for 115 min was selected as the more efficient for betacyanins extraction.

The yellow *Opuntia spp.* extracts were obtained using three different solid-liquid extraction conditions at pH 6.9 (established as the optimum for the purple fruits), using a 1:10 dry matrix/water ratio and extraction time of 84 min. The influence of extraction temperature was evaluated (40 ^{0}C, 50 ^{0}C and 60 ^{0}C) since increasing the temperature in an extraction process can promote the diffusion of the pigments into the solvent (Swamy et al. 2014). The betalain contents in mg.L^{-1} and mg.100 g^{-1} dry extract for each condition are presented in table **3.3**.

Table 3.3 – Betalain determination of yellow *Opuntia spp.* extracts at pH 6.9, 1:10 matrix/solvent ratio, extraction at 40 ºC, 50 ºC and 60 ºC for 84 min. Values are presented as mean ± standard deviation (SD) from three replicates.

Opuntia spp. **yellow extracts**		**pH 6.9** (1:10) (40 ºC,)	**pH 6.9** (1:10) (50 ºC)	**pH 6.9** (1:10) (60 ºC)
BC	mg.L^{-1}	3±0.5	3±0.5	4±0.5
	mg.100 g^{-1} de	4±1	5±1	4±1
BX	mg.L^{-1}	9±1.2	9±1.3	9±0.5
	mg.100 g^{-1} de	16±1.5	17±3.5	16±0.5

BC: Betacyanins, BX: Betaxanthins, d.e.: dry extract

The betalain content of yellow *Opuntia spp*. extracts was shown to be very low, therefore it was not possible to obtain an extract rich in both compounds (betacyanins and betaxanthins). The variation from 40 to 60 ^{0}C didn´t allow a significant improvement in the betalain extraction, the content being very similar for the three conditions. This was possibly caused by the fact that the fruits were mostly not ripen enough because of the weather conditions at the time that they were collected. The ripeness of the fruits is determined by the betalain biosynthesis, which is complex, pigments being accumulated only in certain tissues and at specific stages of development (Delgado-Vargas et al. 2000). On the other hand, the maturity of the fruits/vegetables determines the composition in betacyanins/betaxanthins, total phenolic content and antioxidant activity (Herrera-Hernández et al. 2011).

The results from beetroot extraction were according to literature since it was reported that betalains are more efficiently extracted at pH between 4 and 6 (Delgado-Vargas et al. 2000). The beetroot betalain content were also within the values found in the literature, considering that they vary according to the cultivar, maturation etc. Furthermore, the vegetable was used with the peel to maximize betalains extraction; the peel has higher contents of betalain and total phenolic content than the crown and flesh (Kujala et al. 2000). The betalain contents were also found to be within the values reported in literature

for the extraction of betalains from beets (5.41±0.02 mg BC.g^{-1} dry pulp and 3.21±0.01 mg BX.g^{-1} dry pulp) using water as solvent (Castellanos-Santiago & Yahia 2008).

The betalain content of purple *Opuntia spp.* fruits (332±4 mg.100 g^{-1} d.e.) was very similar to those obtained by other authors (Saldanha et al. 2015) (332.9±0.1 mg.100 g^{-1} d.e.), using the same type of fruits, ripen in the same location in 2012.

For the yellow fruits, the betacyanin and betaxanthin contents were around 4±1 mg.100 g^{-1} d.e. and 16±0.5 mg.100 g^{-1} d.e., respectively. These values were similar to those reported in the literature (5-7.2 mg.100 g^{-1} dry pulp and 12-41 mg.100 g^{-1} dry pulp for betacyanins and betaxanthins, respectively) (Castellanos-Santiago & Yahia 2008), even considering that the pigment contents for the yellow fruits vary greatly according to the species, cultivar and ripeness.

3.1.2) Total Phenolic Content (TPC)

Total concentration of phenolic compounds present in the selected extracts of each matrix was determined according to the Folin-Ciocalteau colorimetric method (Singleton et al. 1999). For beetroot, the extract selected was the one obtained using a 1:10 matrix/water ratio, at pH 5. For the purple *Opuntia spp.* fruits, TPC was determined for the extract obtained at pH 6.9, ratio of 1:193, 42 ºC and 115 min. For the yellow fruits, the extract characterized was obtained at pH 6.9, using a 1:10 ratio, 60 ºC and 84 min. The results in mg GAE.100 g^{-1} dry extract (d.e.) are presented in figure **3.1**.

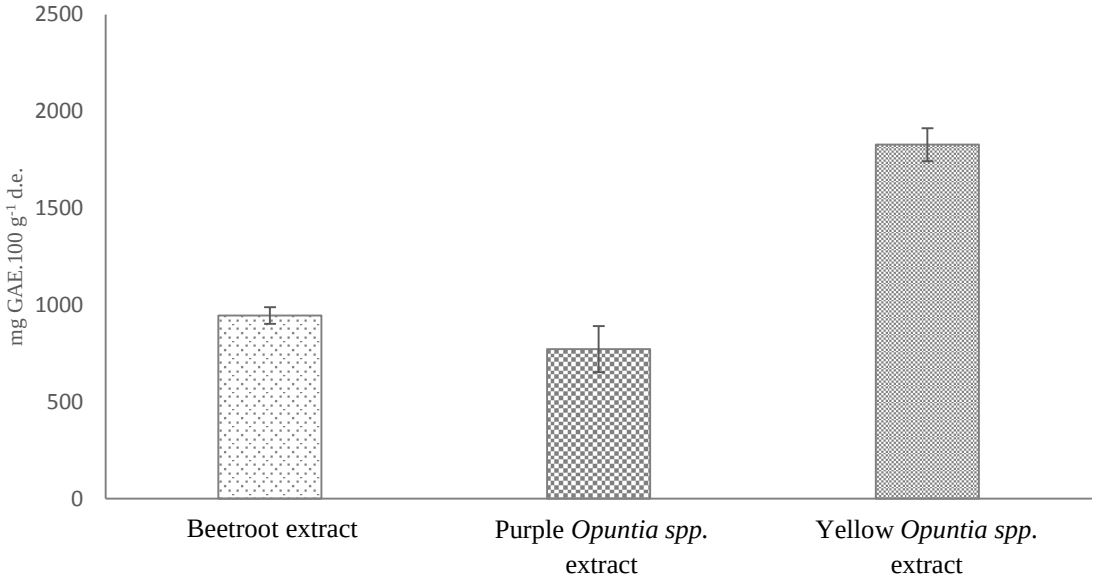

Figure 3.1 – Total phenolic content, assessed by Folin-Ciocalteu method, for beetroot extract (pH 5, ratio 1:10, 60 ºC, 84 min) and Opuntia spp. extracts using purple fruits (pH 6.9, ratio 1:193, 42 ºC, 115 min) and yellow (pH 6.9, ratio 1:10, 60 ºC, 84 min). Values are presented as mean ± standard deviation (SD) from three replicates.

The content in (poly)phenols determined for beetroot, purple and yellow *Opuntia spp.* fruits were 945±43 mg GAE.100 g^{-1} d.e., 772±119 mg GAE.100 g^{-1} d.e. and 1828±85 mg GAE.100 g^{-1} d.e., respectively. Beetroot and purple *Opuntia spp.* fruits extracts presented similar content in

(poly)phenols. Between both *Opuntia* fruits, the extract from the yellow fruits presented more (poly)phenols than the purple ones. One reason may be that the yellow, since they were not ripe enough, may contain more ascorbic acid, which could interfere with the quantification method (Singleton et al. 1999). Therefore, the TPC value may not represent an accurate estimation of the phenolic contents of this matrix. The total phenolic content for the purple *Opuntia* fruits was lower than the two other matrixes, and although richer in betacyanins, it was poor in betaxanthins and phenolics. Thus, it was decided not to proceed with it to further studies. Total phenolic content obtained for beetroot (freeze-dried with peel) is similar to the content determined for a lyophilized beetroot extract (with water) mentioned by Kujala and co-workers (2000), although determined separately for peel (15.5 mg/g dry weight), crown (11.4 mg/g dry weight) and flesh (4.2 mg/g dry weight). For the *Opuntia spp.* fruits, according to Poejo (2011), using fruits collected in Sines (dark-yellow) and Quarteira (purple) in the year 2010, the total phenolic content of yellow juice fruits collected from Sines was higher comparing to the purple ones. However, the TPC contents obtained for the juices of both fruits (3.84 and 0.36 mg GAE.g^{-1} dry weight, respectively) are much lower than the obtained in this study, revealing that the use of the whole fruit valorizes the extracts and allows the extraction of more (poly)phenols.

3.1.3) Oxygen Radical Absorbance Capacity (ORAC)

The ORAC assay was carried out as previously described (Huang et al. 2002). The antioxidant activity measured in the ORAC assay results from the ability of the antioxidant species present in the sample to inhibit the oxidation of disodium fluorescein by peroxyl radicals generated from AAPH (Prior et al. 2005). ORAC content for beetroot and yellow *Opuntia spp.* fruits (the same extracts as referred in section **3.1.2** above), in µM TE. 100 g^{-1} dry extract (d.e.), are presented in figure **3.2.**

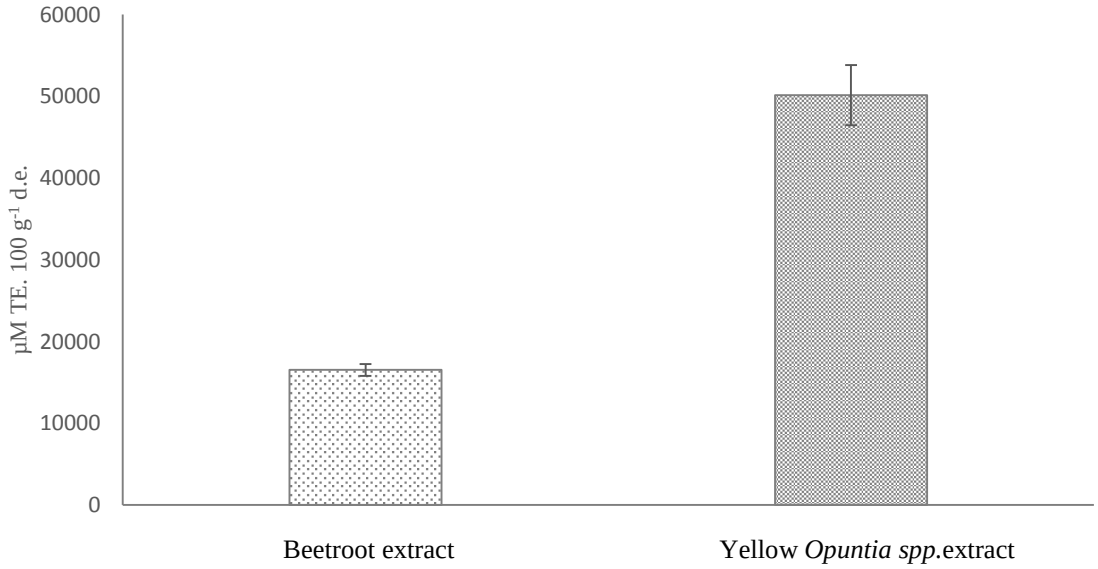

Figure 3.2 – Antioxidant activity, assessed by Oxygen Radical Absorbance Capacity (ORAC) method, for beetroot extract (pH 5, ratio 1:10, 60 ºC, 84 min) and yellow *Opuntia spp.* extracts (pH 6.9, ratio 1:10, 60 ºC, 84 min).Values are presented as mean ± standard deviation (SD) from three replicates.

The ORAC values determined for beetroot and yellow *Opuntia spp.* fruits extracts were 16519±740 µM TE.100g^{-1} d.e. and 50157±3 680 µM TE.100g^{-1} d.e., respectively, indicating that yellow *Opuntia spp.* fruit extracts had a higher *in vitro* antioxidant activity. ORAC content for beetroot is within the content determined by Ou et al. (2002), 115±36 µM TE.g^{-1} freeze-dried weight, using acetone/water beetroot extracts. For the yellow *Opuntia spp.* fruits, the content obtained in this work is approximately 2 fold higher from the content determined from the juice´s fruits by Poejo (2011), 257±16.5 µM TE.g^{-1} dry weight.

(Poly)phenol content, mostly flavonoids which are the main contributor compounds of fruits/vegetables antioxidant activity, depends on the cultivar, harvest period, weather conditions, and ultimately to variability in the total phenolic content and antioxidant activity (Ou et al. 2002). Still, as is referred in literature, a high total phenolic content contributes to a higher antioxidant activity (Číž et al. 2010), as it may be shown in the case of beetroot and yellow *Opuntia spp.* fruits matrixes.

Since the goal was to study the effect of natural compounds - betalains and (poly)phenols - on neuroprotection and inflammation, and considering the betalain content obtained for beetroot and *Opuntia* extracts, is clear that beetroot extract is richer in betalains, and it also contains a high content in (poly)phenols. This fact is due to the type of the matrix, which is richer in both pigments - betacyanins and betaxanthins, comparing to both purple and yellow *Opuntia* fruits. The purple *Opuntia* lacks in betaxanthins, and for the yellow ones, although their promising antioxidant activity, their content in betalains was very low. Consequently, the beetroot extract was the extract selected to be used for further cell assays. The summarized content in betalains, (poly)phenols and antioxidant activity for the beetroot extract are presented in table **3.5**.

Table 3.4 – Summary of the content in betacyanins, betaxanthins, (poly)phenols (TPC) and antioxidant activity (ORAC) obtained for the beetroot extract.

Beetroot extract	
Betacyanins	282±1 mg.L^{-1} 390±1 mg.100 g^{-1} d.e.
Betaxanthins	217±0.3 mg.L^{-1} 301±0.4 mg.100 g^{-1} d.e.
TPC	709±48 mg GAE.L^{-1} 945±43 mg GAE.100 g^{-1} d.e
ORAC	12808±1 289 µM TE.L^{-1} 16519±740 µM TE.100g^{-1} d.e

3.2) Solid Phase Extraction (SPE) – Purification of beetroot extract

Beetroot is a vegetable presenting high sugar contents of which approximately 90% is sucrose (Wootton-Beard et al. 2014). Given that sugars interfere with the yeast bioactivity assays, the beetroot

extracts were subjected to a solid-phase extraction to remove sugars and other contaminants. Since the compounds of interest have different polarities, their affinity with the stationary phase (silica C-18) is different. Thus, purification was performed according to the steps described in the Material and Methods section (**2.2**), and various fractions, containing a mixture of betacyanins, betaxanthins and (poly)phenols, were collected. To monitor the sugar contents of each fraction, sucrose, D-glucose, D-fructose concentrations, which are the main sugars found in fruits and vegetables, were determined. The results are presented in the figure **3.3**, in $g.L^{-1}$.

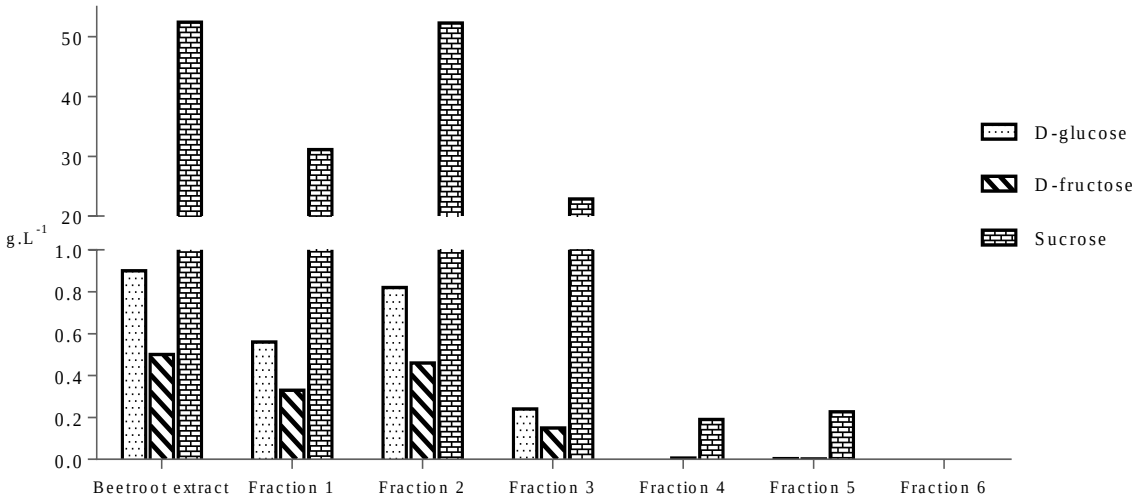

Figure 3.3 – D-glucose, D-fructose and Sucrose content of the beetroot extract and the SPE fractions of the extract.

As expected, sucrose is the main sugar present in beetroot extract. The results for the sugar quantification for the beetroot extract are within the values found in literature, for different beetroot variants (Wruss et al. 2015). High sugar levels are still observed in fractions 1, 2 and 3, derived from the sample application and column washing, respectively. The fractions 4 and 5 display only residual sucrose levels, whereas sugar levels are undetectable in fraction 6. Thus, the SPE procedure efficiently removed sugars from fractions 4 to 6.

Regarding the phytochemical (betacyanins, betaxanthins, total phenolic contents) profile of fractions, fractions 1 and 2 were shown to contain moderate levels of betaxanthins and (poly)phenols, especially fraction 2. Fraction 5 was found to include the higher amounts of phytochemicals, with the exception of betaxanthins. As shown in figure **3.4**, the fractions collected from the SPE procedure present different phytochemical contents. The betacyanin ($mg.L^{-1}$), betaxanthin ($mg.L^{-1}$), and total phenolic content (TPC) (mg $GAE.L^{-1}$) for each fraction are presented in figure **3.4**.

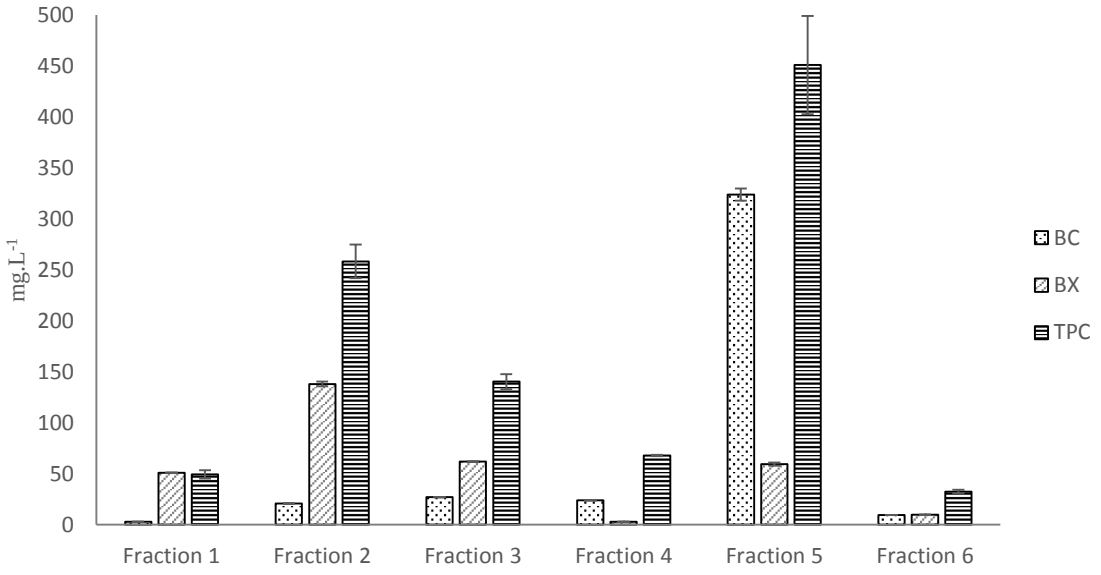

Figure 3.4 – Betalain - betacyanins (BX) and betaxanthins (BX) - and total phenolic content (TPC) of beetroot extract fractions 1 to 6 obtained by using solid phase extraction (C-18 resin). Values are presented as mean ± standard deviation (SD) from two replicates.

The majority of betaxanthins (MW ~374 $g.mol^{-1}$) and smaller (poly)phenols, pass more easily through the silica, being collected in the first fractions (1 and 2). As larger molecules (MW ~550 $g.mol^{-1}$), betacyanins remain attached to the silica. Column washing with a polar mixture of solvents (0.5% CH_3COOH/H_2O) enabled the collection of two fractions (3 and 4) with different colors, indicating different phytochemical composition. In this step, it seems that there was not a great loss of compounds, although some betaxanthins (which are more polar than betacyanins) and some (poly)phenols may be solubilized into the mixture of polar solvents, being wash out from the column. The mixture of formic acid and methanol (2% CH_2O_2/CH_3OH) was ultimately used in order to disrupt the hydrophobic interactions between the analytes and the silica, given that it is slightly polar and allows the removal of all polar compounds still attached to the column, enabling the collection of two fractions (5 and 6) with different colors.

Based on the chemical characterization of phytochemicals (betacyanins, betaxanthins, total phenolic content) and sugar quantification, fraction 5 was selected for further analysis given its high phytochemical contents and low sugar contamination. Although fraction 6 also showed low sugars levels, it contains low phytochemical concentrations (figure **3.4)**.

3.3) Bioactivity of beetroot phytochemicals

The potential protective activity of beetroot phytochemicals against key pathological aspects of the neurodegenerative processes associated to PD, ALS, HD and AD was assessed using humanized yeast models of the respective diseases. The models are based on the premise that heterologous expression of aggregation-prone disease proteins in yeast leads to proteotoxicity and cellular death. In these models, protein expression is controlled by the *GAL1* promoter in such way that, in the presence of glucose,

protein expression is repressed and, in the presence of galactose, it is strongly activated. An uninduced intermediate condition is obtained when cells are incubated in raffinose-containing media, as the condition of the pre-cultures (see section **2.8**), in which protein expression is readily activated when galactose is added to the media. Therefore, the cellular growth of yeast disease models was compromised in galactose media compared to control cells (figure **3.5**), bearing empty vectors. These controls are necessary to ensure that growth impairment is mediated by the expression of disease proteins. Thus, it is expected that compound(s) interfering with disease-protein aggregation and subcellular dynamics may improve cellular growth in cells grown in galactose media.

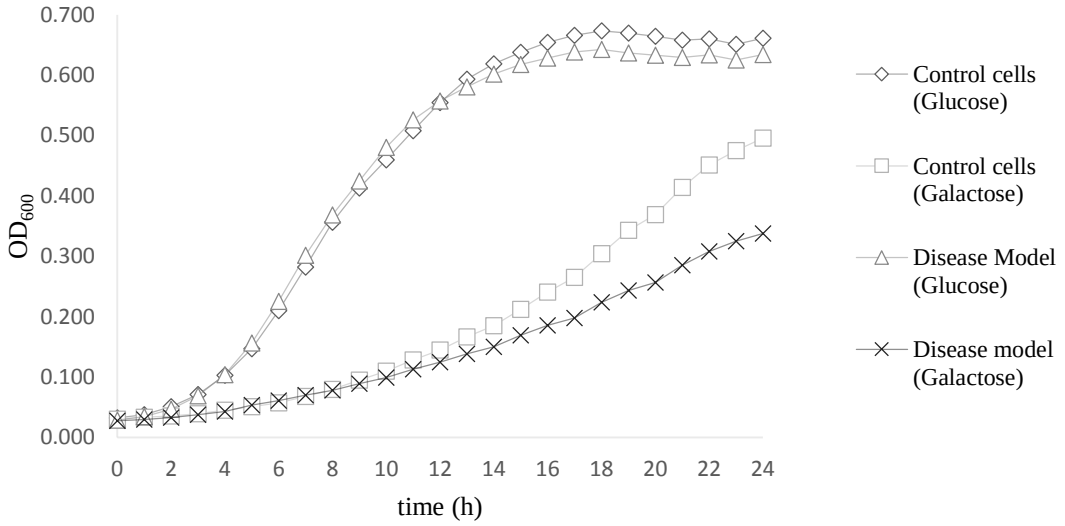

Figure 3.5 – Representative growth curves of a yeast disease model and cells in the presence of glucose or galactose. Pre-cultures grown in raffinose were diluted in glucose or galactose media and growth was monitored at OD_{600} each 1 h for up to 24 h.

3.3.1) Toxicity assays

Before bioactivity evaluation, cytotoxicity of total extracts/fractions was performed for each model to establish the highest non-toxic concentration to be further used in the protection assays. The percentage of extract was calculated according to the volume used in relation to the volume of cultures. The percentages were converted to $\mu g.mL^{-1}$ of betacyanins, betaxanthins and (poly)phenols, according to table **3.5.**

Table 3.5 - Concentration of betacyanins, betaxanthins and (poly)phenols (TPC) for each percentage (0.4 to 25%, v/v) of beetroot extract.

% (v/v) beetroot extract	Phytochemicals		
	TPC (µg GAE.mL^{-1})	**Betacyanins** (µg.mL^{-1})	**Betaxanthins** (µg.mL^{-1})
0.4	3.92	1.56	1.17
0.8	7.81	3.13	2.35
1.6	15.63	6.25	4.69
3.1	31.25	12.5	9.38
6.25	62.5	25	18.75
12.5	125	50	37.5
25	250	100	75

Figure **3.6** shows the growth curves of cytotoxicity assays using control cells incubated in galactose media supplemented with several concentrations of beetroot extract. As anticipated, the results indicate that sugars from beetroot extract improve cell growth, even at the lowest concentration.

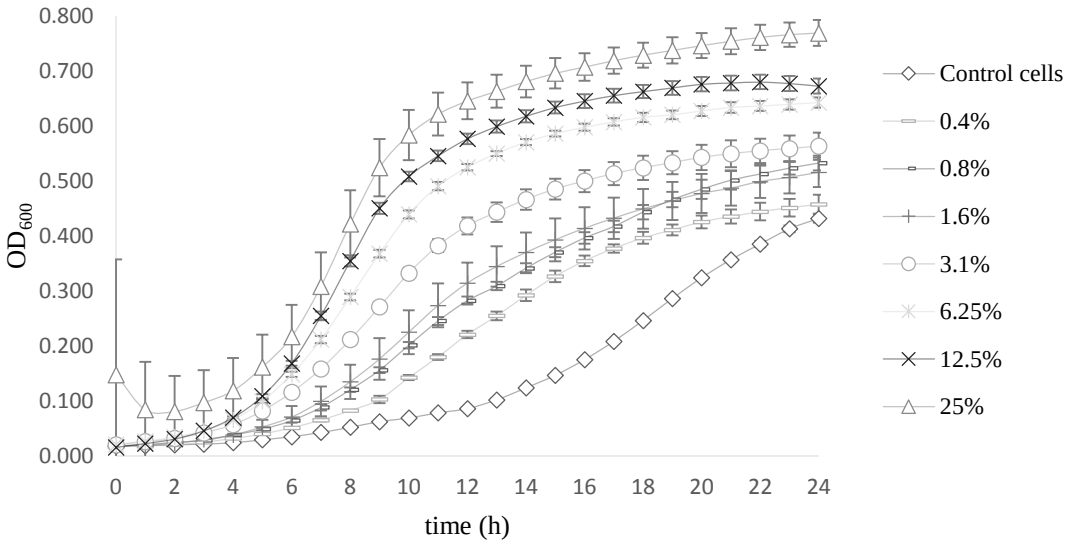

Figure 3.6 – Representative growth curves of control yeast cells grown in galactose media supplemented with different concentrations of beetroot total extracts (0.4 to 25%, v/v). Pre-cultures grown in raffinose were diluted in galactose media and growth was monitored at OD_{600} each 1 h for up to 24 h. Values are presented as mean ± SD of at least three replicates.

The same rationale was used to denote the concentration of the beetroot purified fraction used in the cytotoxicity and bioactivity assays. It was calculated according to the volume of fraction used relative

to the volume of cell cultures, being the percentages subsequently converted to $\mu g.mL^{-1}$ of betacyanins, betaxanthins and (poly)phenols (table **3.6**). The cytotoxicity of the fraction was then determined for control cells corresponding to each yeast disease model, to establish the higher non-toxic concentration to be used in the bioactivity assays.

Table 3.6 – Concentration of betacyanins, betaxanthins and (poly)phenols (TPC) for each percentage (0.8 to 50%, v/v) of beetroot purified fraction.

% (v/v) purified fraction	**Phytochemicals**		
	TPC ($\mu g\ GAE.mL^{-1}$)	**Betacyanins** ($\mu g.mL^{-1}$)	**Betaxanthins** ($\mu g.mL^{-1}$)
0.8	1.96	1.41	0.26
1.6	3.91	2.81	0.51
3.1	7.81	5.63	1.03
6.25	15.63	11.25	2.06
12.5	31.25	22.5	4.11
25	62.5	45	8.22
50	125	90	16.44

The highest percentage of beetroot purified fraction (50%) was shown to be very toxic to PD control cells (figure **3.7**). As the concentration of purified fraction decreases, increases cellular growth. The growth of cells incubated in media supplemented with 6.25% of fraction or less was almost identical to that of untreated cells. Therefore, this concentration was chosen to evaluate the potential of beetroot phytochemicals to circumvent α-synuclein proteotoxicity in the yeast PD model.

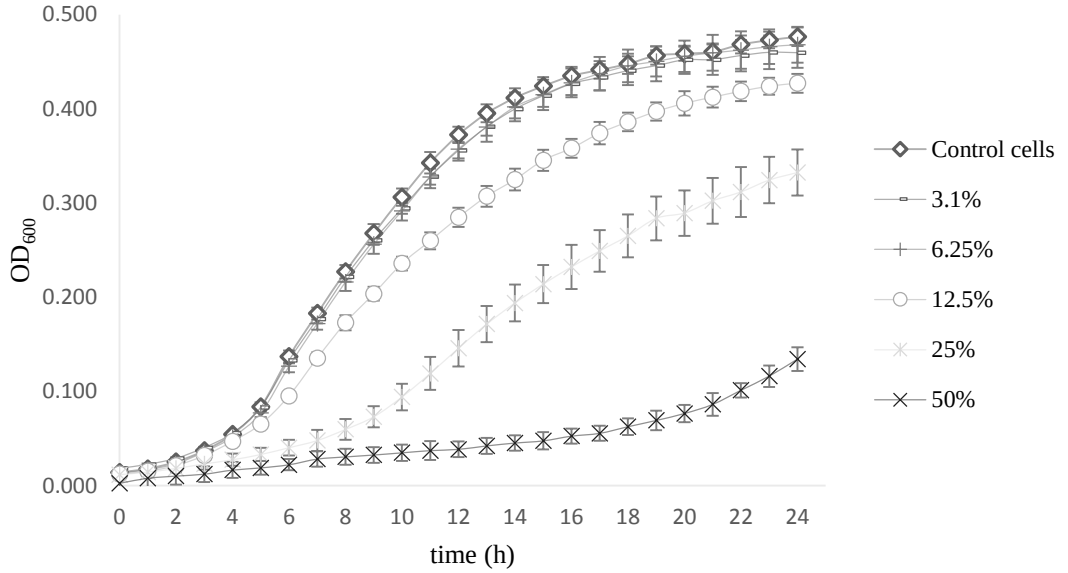

Figure 3.7 – Growth curves of PD control cells grown in galactose media supplemented with different concentrations of beetroot purified fraction (3.1 to 50%, v/v). Pre-cultures grown in raffinose were diluted in galactose media and growth was monitored at OD_{600} each 1 h for up to 24 h. Values are presented as mean ± SD of at least three replicates.

All beetroot purified fraction concentrations were shown to be cytotoxic to ALS control cells. The fraction concentration of 6.25% slightly impair cellular growth being therefore selected for the bioactivity assays (figure **3.8**).

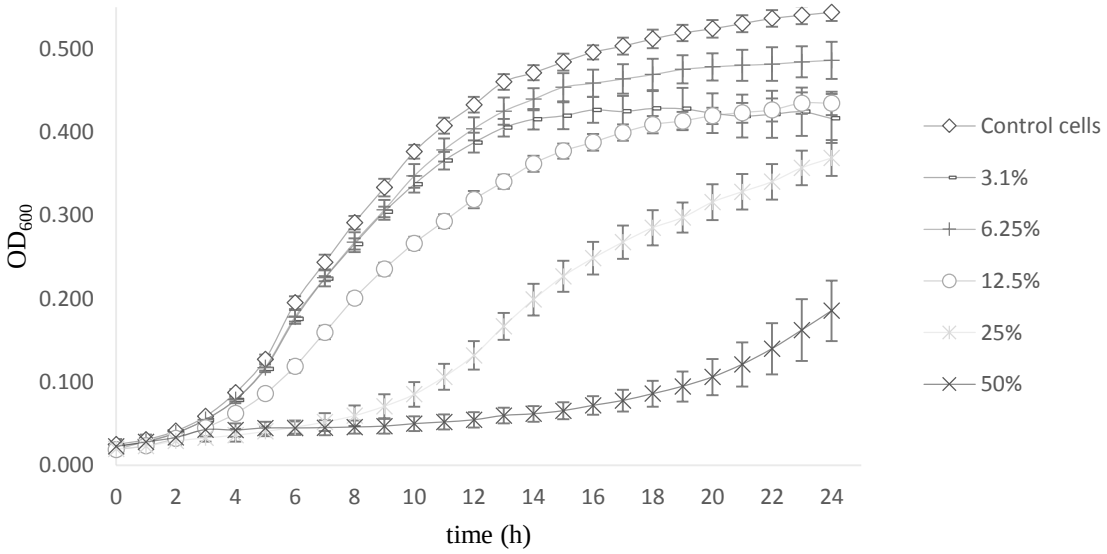

Figure 3.8 – Growth curves of ALS control cells grown in galactose media supplemented with different concentrations of beetroot purified fraction (3.1 to 50%, v/v). Pre-cultures grown in raffinose were diluted in galactose media and growth was monitored at OD_{600} each 1 h for up to 24 h. Values are presented as mean ± SD of at least three replicates.

The beetroot purified fraction concentrations used to evaluate toxicity in the HD/AD control cells were lower than those used for the other control cells (figure **3.9**), due to the auxotrophic requirements of this

strain (synthetic media devoid of leucine and uracil to allow plasmid selection). Fraction concentrations higher than 25% were shown to be toxic (data not shown), so cytotoxicity of lower fraction concentrations (12.5% to 0.8%) was re-tested. Curiously, it can be verified that at this concentration range, the lowest fraction concentrations seem to be more toxic than the higher ones. This may be caused by the auxotrophic requirements of this strain and/or different strain background.

Although fraction concentrations of 3.1% and 1.6% were slightly cytotoxicity for these cells, these concentrations were chosen to evaluate the potential protective activity in the HD and AD disease models.

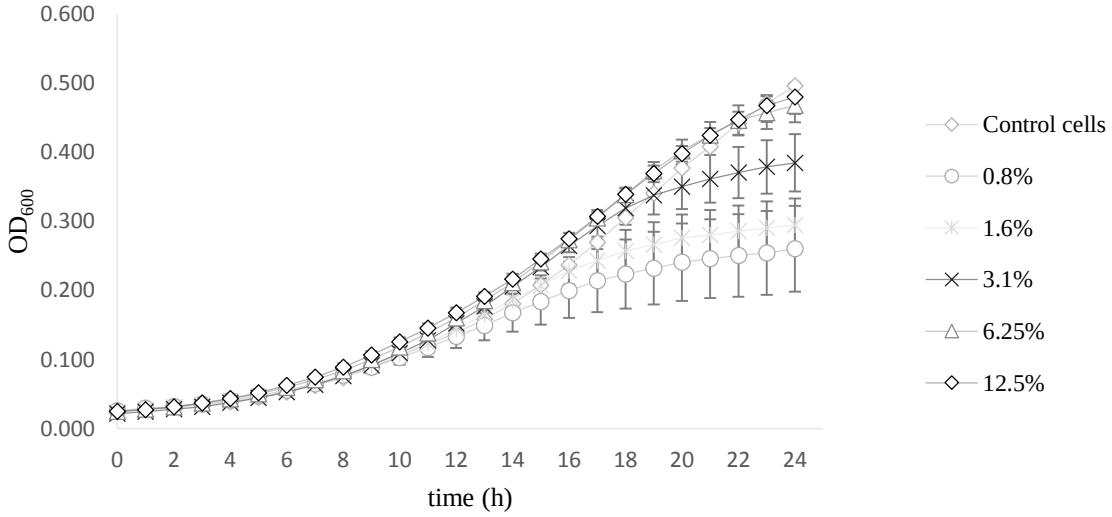

Figure 3.9 – Growth curves of HD/AD control cells grown in galactose media supplemented with different concentrations of beetroot purified fraction (0.8 to 12.5%, v/v). Pre-cultures grown in raffinose were diluted in galactose media and growth was monitored at OD_{600} each 1 h for up to 24 h. Values are presented as mean ± SD of at least three replicates.

3.3.2) Evaluation of the protective potential of beetroot purified fraction

Once established the cytotoxicity of fractions, the next step was to evaluate whether media supplementation with beetroot phytochemicals is translated into an improved growth and survival upon expression of the disease proteins α-synuclein, FUS, Huntingtin and Aβ42, thus revealing potential therapeutic potential for PD, ALS, HD and AD, respectively.

PD yeast cells displayed reduced growth compared to control cells, which indicates α-synuclein proteotoxicity. Media supplementation with both 3.1% or 6.25% beetroot purified fraction did not improve cell growth implying that the phytochemicals present in this fraction were not able to revert the toxic effects of α-synuclein expression in yeast (figure **3.10**).

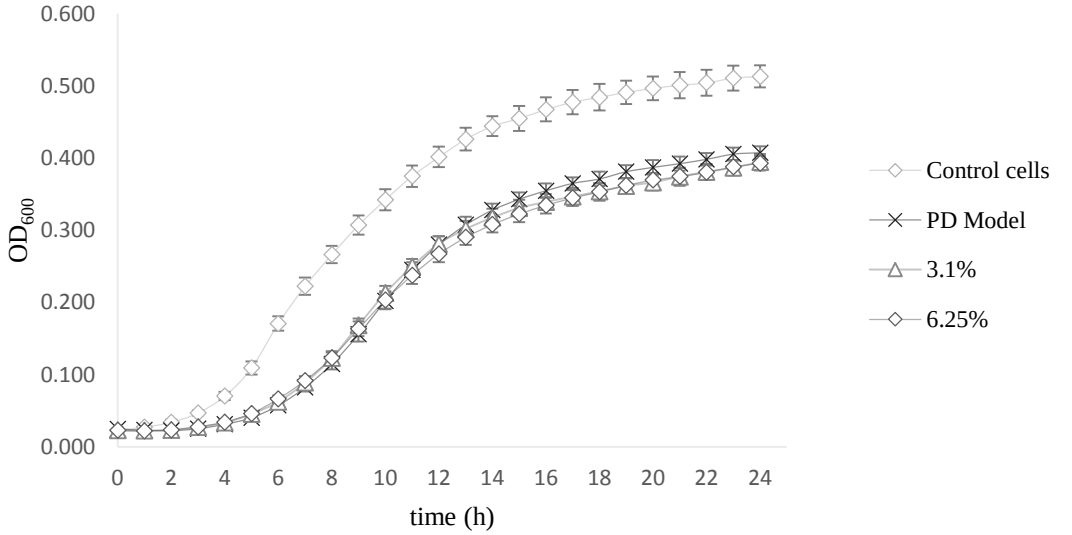

Figure 3.10 – Growth curves of PD model and control cells grown in galactose media supplemented with 3.1% and 6.25% of beetroot purified fraction. Pre-cultures grown in raffinose were diluted in galactose media and growth was monitored at OD_{600} each 1 h for up to 24 h. Values are presented as mean ± SD of at least three replicates.

Similar results were obtained for the ALS, HD, and AD yeast models, in which the selected concentrations of beetroot purified fraction were not able to revert the toxic effect of the FUS (figure **3.11**), Huntingtin (figure **3.12**), and Aβ42 (figure **3.13**) expression, respectively. Altogether, these results indicate that phytochemicals from beetroot purified fraction do not have protective activity towards the pathological processes of these diseases, at least as regard to disease-protein toxicity.

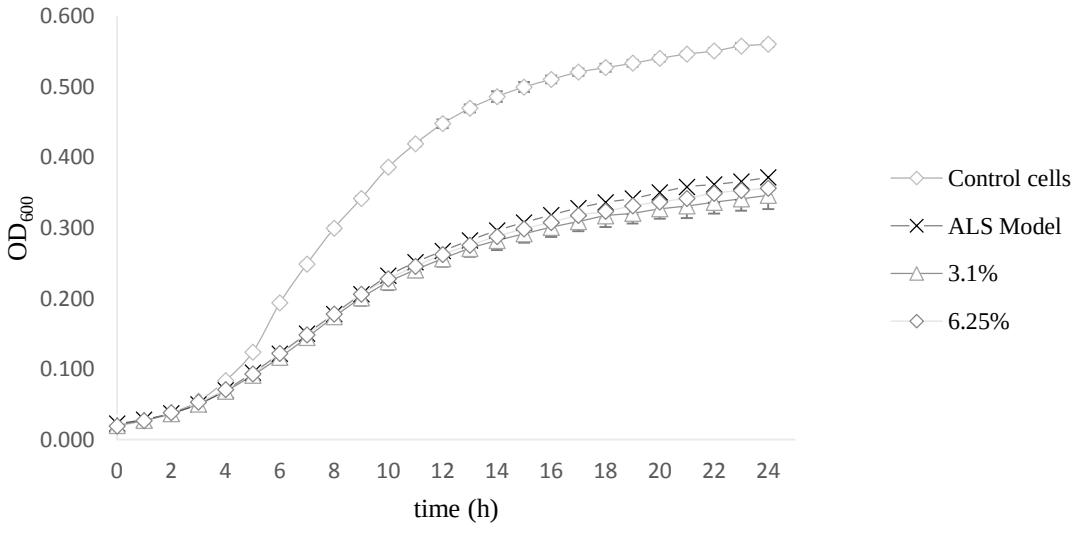

Figure 3.11 – Growth curves of ALS model and control cells grown in galactose media supplemented with 3.1% and 6.25% of beetroot purified fraction. Pre-cultures grown in raffinose were diluted in galactose media and growth was monitored at OD_{600} each 1 h for up to 24 h. Values are presented as mean ± SD of at least three replicates.

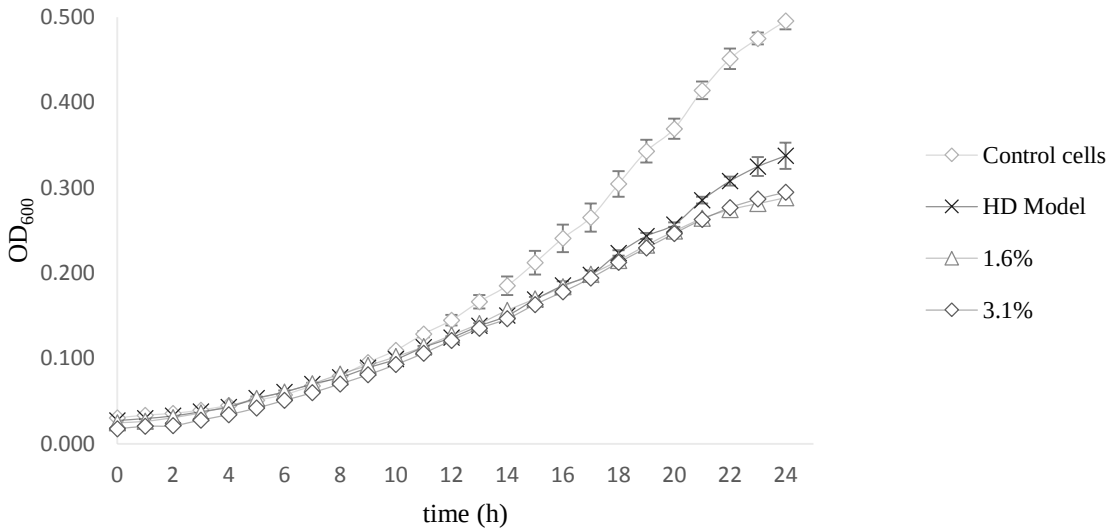

Figure 3.12 – Growth curves of HD model and control cells grown in galactose media supplemented with 1.6% and 3.1% of beetroot purified fraction. Pre-cultures grown in raffinose were diluted in galactose media and growth was monitored at OD_{600} each 1 h for up to 24 h. Values are presented as mean ± SD of at least three replicates.

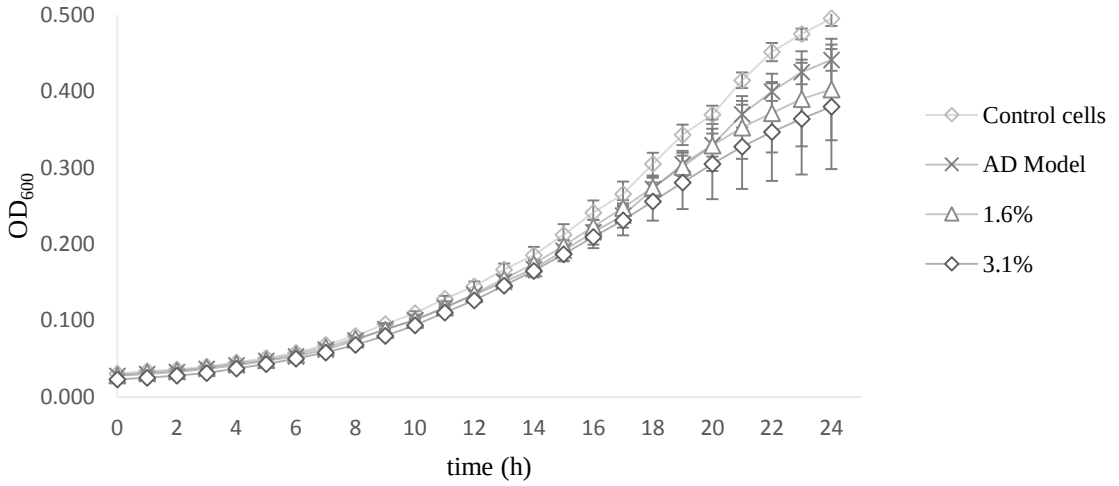

Figure 3.13 – Growth curves of AD model and control cells grown in galactose media supplemented with 1.6% and 3.1% of beetroot purified fraction. Pre-cultures grown in raffinose were diluted in galactose media and growth was monitored at OD_{600} each 1 h for up to 24 h. Values are presented as mean ± SD of at least three replicates.

The use of yeasts as simple models organisms brings many methodological advantages, including the investigation of particular aspects of complex diseases such as the neurodegenerative disorders. The beetroot phytochemicals did not have potential to circumvent the deleterious effects of disease-proteins toxicity but they can affect other cellular process associated to neurodegeneration, for example chronic inflammation.

3.4) Yeast model of Crz1/NFAT activation

Chronic inflammation is a process closely associated to neurodegeneration. To investigate the potential bioactivity of beetroot phytochemicals for the prevention of inflammatory processes, it was used a yeast model of Crz1/NFAT activation. In these assays, the incubation time in the presence of fraction was reduced, compared to the previous assays (2 h versus 24 h), and were not long enough to significantly interfere with cell viability. Therefore, several concentrations of beetroot purified fraction were used as indicated in table **3.7**.

Table 3.7 – Concentration of betacyanins, betaxanthins and (poly)phenols (TPC) for each percentage (0.06 to 6.25%, v/v) of beetroot purified fraction.

% (v/v) purified fraction	**Phytochemicals**		
	TPC (μg GAE.mL^{-1})	**Betacyanins** (μg.mL^{-1})	**Betaxanthins** (μg.mL^{-1})
0.06	10	7.2	1.3
0.31	25	18	3.3
0.63	50	36	6.6
1.25	100	72	13.2
3.13	250	180	33
6.25	500	360	66

To mimic a condition of inflammation, yeast cells were subjected to a treatment with $MnCl_2$, which triggers a robust activation of Crz1, as assessed by the high levels of β-galactosidase activity observed in these cells (figure **3.14**). The rationale of the assay is based on the assumption that compounds interfering with Crz1 activity, and putatively with NFAT, will reduce the β-galactosidase activity as a result of impaired Crz1 activation.

Thus, cells were first treated with the indicated concentrations of beetroot purified fraction and, after phytochemicals removal, cells were challenged with $MnCl_2$ to induce Crz1 activity. The immunosuppressant drug FK506 was used as a positive control.

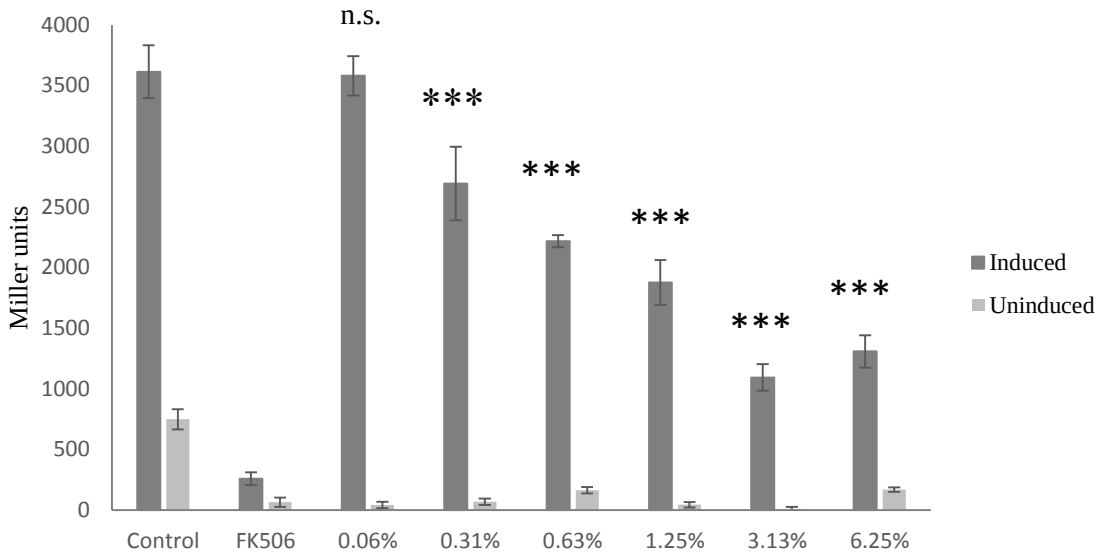

Figure 3.14 – Modulation of Crz1 activity by beetroot phytochemicals. Pre-cultures grown in glucose were diluted in fresh media supplemented or not with beetroot purified fraction (0.06 % to 6.25%, v/v). Crz1 activity was induced with 3 mM $MnCl_2$ and FK506, at a final concentration of 10 µg.mL^{-1}, was used as a positive control. Quantitative β-galactosidase assays were performed as described and values are given in Miller units. Values are presented as mean ± SD of at least three replicates. p *** < 0.001, p ≥ 0.05, n.s. – not statistically significant.

The β-galactosidase activity of cells exposed for the lowest concentration of purified fraction (0.06%) was not statistically different from the induced control cells, indicating that at this concentration the beetroot purified fraction did not reveal protective properties (figure **3.14**). However, beetroot phytochemicals concentrations above 0.31% showed a dose-dependent protective activity as indicated by the reduced levels of Crz1-driven β-galactosidase activity compared to the control condition. Additionally, uninduced cells showed a basal β-galactosidase activity that is also reduced in all fraction concentrations tested as well as in the presence of the immunosuppressant FK506.

As the purified fraction is composed by a complex mixture of compounds, (poly)phenols and betalains, it was not clear which class of compounds was responsible for the protective effect, or whether there was a synergy between the compounds present in the fraction. Therefore, purified betacyanin, the major compound in beetroot extracts, was used as a betanin standard (at the concentrations indicated in table **3.8**) to evaluate if the protective activity could be derived from this class of compounds.

Table 3.8 – Concentration of betanin standard (in terms of µg.mL^{-1} betacyanins - BC) for each percentage of standard tested (0.06 to 6.25%, v/v).

% (v/v) Betanin Standard (BC)	0.06%	0.16%	0.31%	0.62%	1.56%	3.13%	6.25%
Betacyanins (BC) (µg.mL^{-1})	10	25	50	100	250	500	1000

None of the betanin standard concentrations tested were able to reduce $MnCl_2$-induced Crz1 activation suggesting that betanin was not the compound conferring the bioactivity of this fraction (figure **3.15**). Since this fraction contains a mix of phytochemicals, its protective effect may be due to other compounds or even a consequence of the synergy among different compounds.

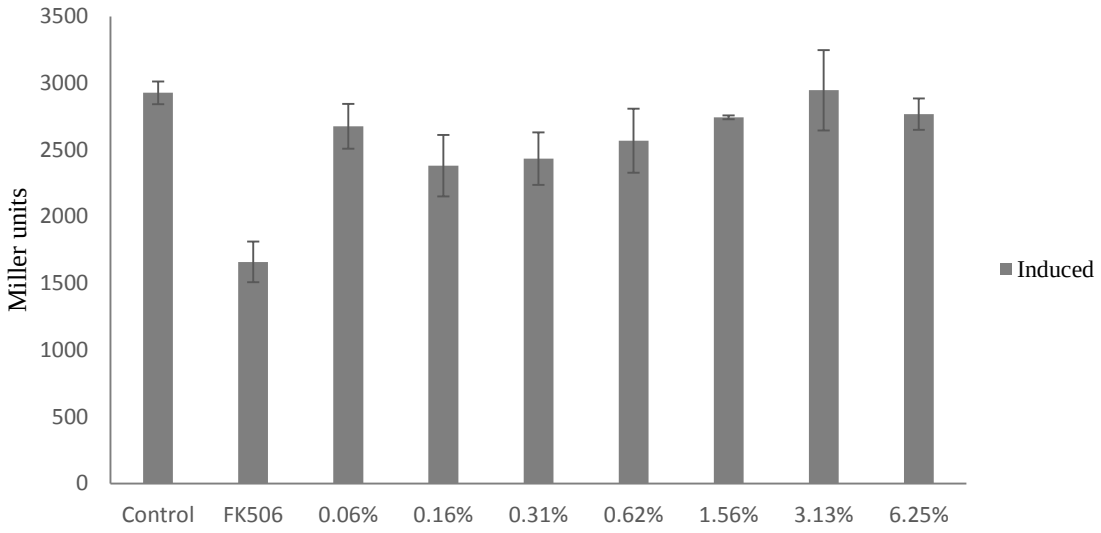

Figure 3.15 – Modulation of Crz1 activity by beetroot phytochemicals. Pre-cultures grown in glucose were diluted in fresh media supplemented or not with beetroot purified fraction (0.06 % to 6.25%, v/v). Crz1 activity was induced with 3 mM $MnCl_2$ and FK506, at a final concentration of 10 $\mu g.mL^{-1}$, was used as a positive control. Quantitative β-galactosidase assays were performed as described and values are given in Miller units. Values are presented as mean ± SD of at least three replicates.

Although betanin has been referred in the literature as an attenuator of NF-κB activation in the kidney (Tan et al. 2015), it does not seem to interfere with Crz1/NFAT activation in the conditions tested in this study. Another explanation for the results obtained is that other compound(s) from the class of betaxanthins and/or (poly)phenols, including phenolic acids and flavonoids (Kim et al. 2004; Yoon & Baek 2005), could exert the protective activity. Indeed, beetroot extracts were rich in (poly)phenols (Wootton-Beard & Ryan 2011; Ninfali & Angelino 2013).

4) Conclusion

Medicine alone cannot respond in an effective way to all the conditions affecting human health, so people are directing their attention to natural alternatives such as traditional medicine. The search of bioactive natural compounds begins with the extraction, isolation of the compounds and their characterization, followed by studies that can evaluate their bioactivity.

The objective of this study was to evaluate the potential protective activity of phytochemicals, namely betalains (betacyanins and betaxanthins) and (poly)phenols extracted from beetroot and *Opuntia spp.* fruits, to pathological processes associated to the neurodegenerative disorders Parkinson's disease, Amyotrophic Lateral Sclerosis, Huntington's disease and Alzheimer's disease. Bioactivities were evaluated using an innovative approach grounded on the use of humanized yeasts recapitulating fundamental disease cellular aspects.

The protocol to extract these compounds were optimized, the extract content in betalains and (poly)phenols was monitored, and their *in vitro* antioxidant capacity was evaluated. This study revealed that beetroot was the matrix presenting higher betalain contents (betacyanins and betaxanthins) compared to purple *Opuntia spp.* fruits, which contain only betacyanins, and to yellow fruits, which exhibit very low contents of both pigments. Although beetroot does not present the highest phenolic content and antioxidant activity, it contains both classes of pigments, and so the beetroot matrix was selected for further bioactivity assays. After removing interferences, such as from sugars, and enriching fractions with the compounds of interest – betacyanins, betaxanthins and (poly)phenols – the bioactivity of purified fraction was tested using yeast models for the diseases indicated above. The beetroot purified fraction was not able to revert the proteotoxicity associated to disease-proteins expression, however, it potentially attenuates inflammation as revealed by the use of a yeast model of Crz1/NFAT activation. Betanin, the major beetroot compound, did not seem to be the phytochemical class conferring bioactivity, at least as an isolated compound, as the betanin standard did not interfere with Crz1 activation as it does the beetroot purified fraction.

In conclusion, the results indicate that beetroot purified fraction has a potential to attenuate inflammation through the modulation Crz1/NFAT activation. On the other hand, it is reported in literature that beetroot exert a positive effect on the attenuation of NF-κB transcription factor related to the immune responses (El Gamal et al. 2014). In line with the notion that neuroinflammation, triggered by the overproduction of inflammatory mediators, can lead to neurodegeneration (Frank-Cannon et al. 2009), the discovery of compounds that can modulate the inflammatory response through different pathways is a valuable asset in therapeutic.

As future work, the beetroot extracts and fractions require a better chemical characterization in order to identify the putative protective compounds. These efforts can also contribute to build a library of compounds that would be a useful tool for actual and future research. Also, the results must be validated in more complex models such as mammalian cells lines and primary cell cultures, which are models

physiologically more similar to human cells. To be able to evaluate the potential of betacyanins, betaxanthins and (poly)phenol compounds, they need to be isolated and purified, and their potential evaluated in separate or as mixtures so to understand the sources of bioactive compounds. Additionally, since compounds are altered due to the digestion process, so extracts/compounds should be submitted to *in vitro* digestion and then tested in models systems with various degrees of complexity.

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