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Hypericum perforatum phenolic compounds: Protective role in the toxicity induced by heterologous expression of α-synuclein and huntingtin in yeast cells

Declaração

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Abstract

Parkinson's (PD) and Huntington's (HD) diseases are neurodegenerative disorders with high prevalence. Several studies implicate abnormal protein accumulation, protein phosphorylation, mitochondrial dysfunction and oxidative stress as common pathways implicated in PD and HD pathogenesis. Polyphenolic compounds are commonly found in both edible and medicinal plants, and they have been reported to have multiple biological effects, including antioxidant activity. The budding yeast *Saccharomyces cerevisiae* has been used as a model to study the toxicity and the biological functions of α-synuclein in PD and huntingtin in HD. The heterologous expression of wild-type and A53T mutant form of α-synuclein and huntingtin 103Q mutant form in yeast model to PD and HD, respectively, causes toxicity and death of yeast cells. Therefore, the aim of this study was to evaluate the possible protective effect of *Hypericum perforatum* phenolic compounds (quercetin, kaempferol and biapigenin) in the toxicity induced by the heterologous expression of α-synuclein and huntingtin, in *S. cerevisiae*. Preliminary results indicate that the presence of these phenolic compounds decreased the α-synuclein toxicity. Our results showed a possible synergistic effect between the phenolic compounds, and biapigenin was the compound with higher protective effects in the α-synuclein-induced toxicity. Mixtures of these compounds inhibited the foci formation, and kaempferol increased the aggresome formation. Relatively to the expression of huntingtin 103Q mutant form, our results showed that the presence of phenolic compounds did not interfere in the huntingtin-induced toxicity. We concluded that these phenolic compounds apparently show beneficial biological properties that consequently could have a potential use in preventing Parkinson's disease.

Key words: Parkinson's disease, α-synuclein, Huntington's disease, huntingtin, quercetin, kaempferol, biapigenin, ROS, foci and yeast.

Resumo

A doença de Parkinson (DP) e a doença de Huntington (DH) são desordens neurodegenerativas com bastante prevalência. Diversos estudos associam a acumulação de proteínas disfuncionais, fosforilação de proteínas, disfunção mitocondrial e stress oxidativo à patogénese da DP e da DH. Os compostos fenólicos são normalmente encontrados em plantas comestíveis e em plantas medicinais e têm sido estudados devido aos seus múltiplos efeitos biológicos, incluindo a actividade antioxidante. A levedura *Saccharomyces cerevisiae* tem sido usada como modelo de estudo de toxicidade e das funções biológicas das proteínas α-sinucleína e huntingtina inplicadas na DP e na DH, respectivamente. A expressão heteróloga das formas tóxicas da proteína α-sinucleína (normal e da forma mutante A53T) e da forma patogénica (103Q) da huntingtina, em leveduras, causa toxicidade e morte celular. Assim, o objectivo deste trabalho foi a avaliação de possíveis efeitos protetores de compostos fenólicos (quercetina, kaempferol e biapigenina), presentes na planta *Hypericum perforatum*, na toxicidade induzida pela expressão heteróloga das proteínas, α-sinucleína e huntingtina, usando para tal a levedura *S. cerevisiae* como modelo. Resultados preliminares demonstraram que a presença dos compostos fenólicos diminuiu a toxicidade da α-sinucleína. Os resultados mostraram ainda a possibilidade de efeitos sinergéticos entre os compostos fenólicos e que a biapigenina foi o composto com maior efeito protetor na toxicidade induzida pela α-sinucleína. Misturas desses compostos inibiram a formação de "foci" e o kaempferol aumentou a formação de agressomas. Os resultados mostraram ainda que a presença dos compostos fenólicos não interferiu na toxicidade induzida pela expressão da forma patogénica da huntingtina. Conclui-se assim que os compostos fenólicos utilizados aparentam ter propriedades biológicas benéficas e consequentemente poderão ter um potencial uso na prevenção da DP.

Palavras-chave: Doença de Parkinson, α-sinucleína, Doença de Huntington, huntingtina, quercetina, kaempferol, biapigenina, espécies reactivas de oxigénio, "foci" e levedura.

Index

Abreviations

Aβ	Amyloid β-protein
AD	Alzheimer's disease
ALS	Amyotrophic Lateral Sclerosis
ATP	Adenosine triphosphate
ATP7B	Wilson's disease protein
CFUs	Conting colony-forming units
CLN3	Ceroid lipofuscinosis neuronal 3
DHE	Dihydroethidium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
GFP	Green fluorescent protein
GSH	Glutathione
GSSH	Oxidized glutathione
HD	Huntington's disease
HSP	Hereditary Spastic Paraplegia
Htt	Huntingtin
mPTP	Mitochondrial permeability transition pore
NFT	Neurofibrillary tangles
OS	Oxidative stress

PD	Parkinson´s disease
PolyQ	Polyglutamine
RNA	Ribonucleic acid
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SNcp	Substantia nigra pars compacta
UPS	Ubiquitin proteasome system
WT	Wild-type
YEPD	Yeast extract, peptone and dextrose

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I.   Introduction

1. Neurodegenerative disorders

Neurodegenerative diseases are progressive disorders of the central nervous system. They have clinical heterogeneity and are characterized by motor, cognitive, and/or behavioural dysfunction (Woulfe, 2008).

Age is directly linked with these diseases and not only makes patients more bent to neurodegenerative diseases, but also impairs their abilities of self-repairing. During ageing, DNA, RNA and proteins become more susceptible to suffer damage, such as oxidation, and ubiquitin proteasome system (UPS) activity decreases (Hung et al., 2010; Woulfe, 2008). Another factor associated with many neurodegenerative diseases (table 1) is the accumulation of disease-specific misfolded proteins in the central nervous system – the conversion of a protein from normal, functional, and benign conformations to aberrant and toxic conformations (Shastry, 2003). The proteins α-synuclein in Parkinson's disease (PD), amyloid β-protein (Aβ) and tau protein in Alzheimer's disease (AD) and polyglutamine (polyQ) – expansion in huntingtin (htt) protein in Huntington's disease (HD) are good examples of misfolded proteins in neurodegenerative disorders (Wood-Kaczmar et al., 2006; Shastry, 2003). The aggregation of misfolded proteins leads to a selective loss of neurons and affect a diverse neuronal population but the exact causes of misfolding and accumulation of proteins are still not totally understood (Hung et al., 2010; Wood-Kaczmar et al., 2006 and Shastry, 2003).

Table 1 – Three neurodegenerative diseases, their inclusions and protein (Shastry, 2003)

Disease	Inclusions	Protein
Alzheimer's	Plaques and neurofibrillary tangles (NFT)	Presenilins 1 and 2; Apolipoprotein E and Amyloid precursor protein
Parkinson's	Lewy bodies	α-Synuclein; Parkin and Ubiquitin carboxy-terminal hydrolase L1
Huntington's	Huntingtin inclusions	Huntingtin

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Parkinson's disease and α- synuclein

Parkinson's disease is one of the most common neurodegenerative diseases and was first described by James Parkinson in 1817. Most patients are over 55 years old and men seem to be more affected than women (Wood-Kaczmar et al., 2006). PD is a chronic, progressive neurodegenerative disorder and its typical clinical symptoms are resting tremor, slowness of movement, cogwheel rigidity, bradykinesia, and postural instability (Ziemssen & Reichmann, 2007). Patients with PD also exhibit nom-motor symptoms that consist of disturbances of sleep, cognition, autonomy and olfaction (Hou & Lai, 2007).

A study on the prevalence of PD in Portugal shows that age-standardized rates were 1.4/1000 for males and 1.3/1000 for females and sex-specific rates increase gradually with age for males and females, covering a level of 9/1000 for ages over 75 years (Dias et al., 1994). It is believed that most cases are sporadic. However in some cases, PD is thought to have a genetic component in recessive and dominant modes of inheritance (Wood-Kaczmar et al., 2006). Some of the key factors associated with this disease process, both familiar and sporadic forms, are protein phosphorylation, oxidative stress, mitochondrial dysfunction, protein misfolding and impairment of the UPS (Dauer & Przedborski, 2003).

From the pathological point of view, PD is characterized principally by the loss of dopaminergic neurons in the *substantia nigra* resulting in a deficiency of dopamine, an important neurotransmitter for the basal ganglia circuit and the presence of cytoplasmic inclusions known as Lewy bodies (Fig.1) (Hou & Lai, 2007 and Ruipérez, Darios, & Davletov, 2010)

α- Synuclein is an abundant presynaptic protein and is expressed in a number of neuronal and non-neuronal cell types. Aggregations of this protein have been implicated in the pathogenesis of PD (Outeiro et al., 2006; Brighina et al., 2009 and Dev et al., 2003). The α-synuclein protein has a highly conserved N-terminal domain that consists of two α-helical regions interrupted by a short break and a C-terminal region. The protein is small and the normal function is not well understood. However, α-synuclein is known to interact with membranes and to bind to fatty acids. It is also implicated in the regulation of dopamine neurotransmission and certain enzymes and involved in neuronal survival (Wood-Kaczmar et al., 2006 and Dev et al., 2003).
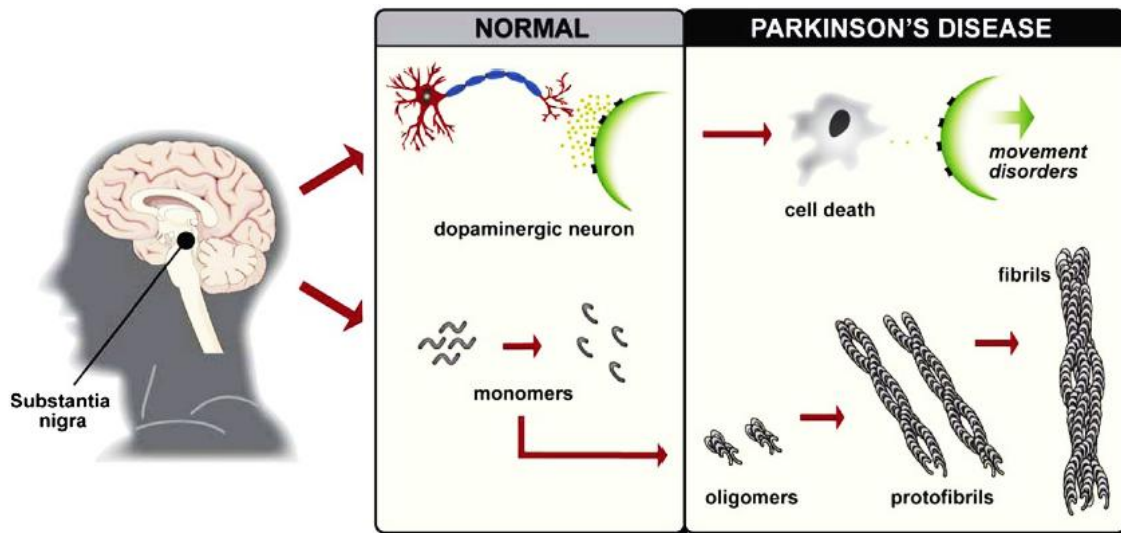
Figure 1 - Loss of dopaminergic neurons and formation of α-synuclein fibrils (Ruipérez et al., 2010).

Studies show that α-synuclein is the main component of Lewy bodies, having an important role in PD (Dauer & Przedborski, 2003). Three mutations of this protein, A30P, A53T and E46K, lead to the development of familial PD. Many studies show that triplications and duplications of the α-synuclein gene cause an early onset of PD. Mutations in this protein have not been found in sporadic PD (Dauer & Przedborski, 2003; Ruipérez et al., 2010).

Overexpression of α-synuclein in several cellular models (animal, yeast and mammalian cells) leads to cytotoxicity and inclusion body formation, facilitates cell death, promotes mitochondrial deficit, improves vulnerability to oxidative stress, and can impair cellular organelles including lysosomes. The pathological role of inclusion bodies is still a debated topic, but the fibrillation and aggregation of α-synuclein have been implicated in the pathogenicity of PD (Outeiro et al., 2006 and Brighina et al., 2009). Aggregation of α-synuclein in disease (Fig.2), is caused by increased oxidative stress and decreased protein degradation of abnormal proteins by UPS (Raichur et al., 2006 and Branco et al., 2010).

Several studies show differences in fibrils formation between α-synuclein mutant forms and wildtype (WT) α-synuclein. The A53T mutant fibrilizes more rapidly than WT α-synuclein while the A30P mutant fibrilizes more slowly (Dauer & Przedborski, 2003). Apart from their divergent rates in fibril formation, the A30P mutant contrasting the A53T mutant is also defective in binding to vesicles and membranes. The mutant forms, seem to sensitize cells to proteasome inhibition and thus reduce the

neuroprotective effects as compared to WT α-synuclein (Dev et al., 2003; Dauer & Przedborski, 2003).
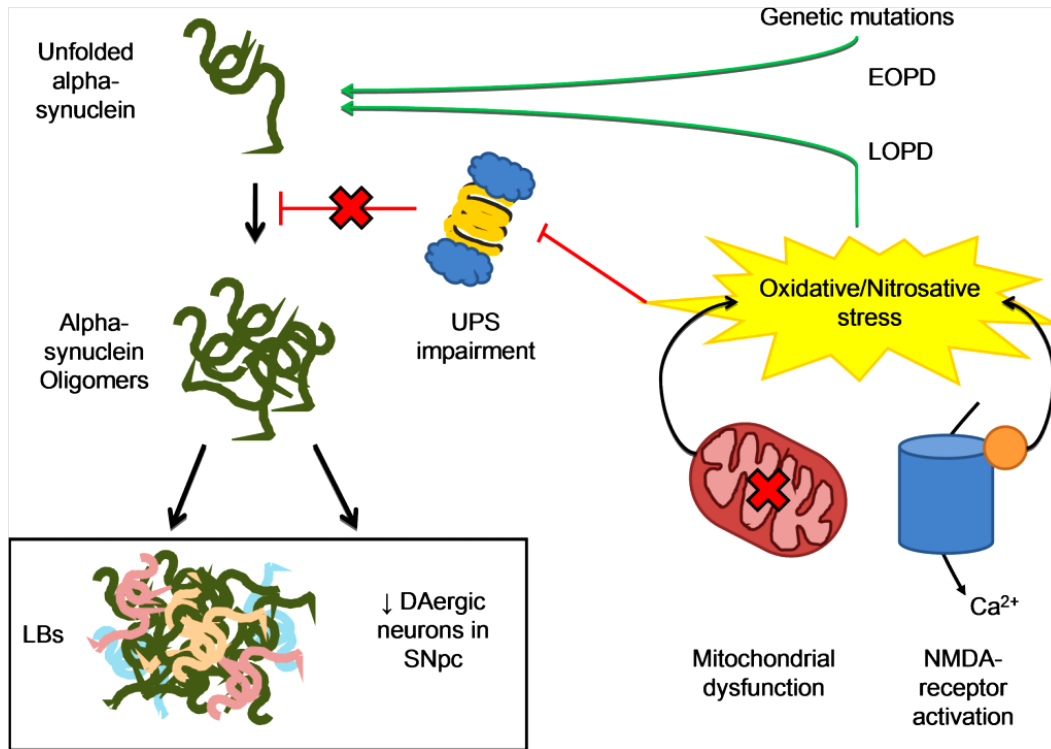

Figure 2 – Overview of the α-synuclein aggregation process (Branco et al., 2010).

Huntington's disease

HD belongs to a family of dominantly inherited neurodegenerative disorders characterized by degeneration of neurons in the striatum and, to a lesser extent, in the cerebral cortex (Sorolla et al., 2011). This disease begins in middle age and is caused by an abnormal expansion of a CAG repeat, located in the exon 1 of the IT15 gene that encodes a stretch of polyglutamines (polyQ) at the N-terminal of the htt protein (Damiano et al., 2010). Htt is a 350-kDa multidomain protein that contains a polymorphic glutamine/proline-rich domain at its amino-terminus (Sorolla et al., 2008; Qin & Gu, 2004). The normal function of this protein has been complicated to establish because it encloses very little sequence homology to other known proteins, is ubiquitously expressed and is localized in many subcellular compartments. Htt is present in the nucleus, cell body, dendrites and nerve terminals of neurons, and is also associated with a number of organelles including the

endoplasmic reticulum, mitochondria and Golgi apparatus (Cattaneo et al., 2005; Landles & Bates, 2004).

Clinical symptoms include psychiatric and cognitive abnormalities, chorea, and dementia. Degenerative events begin years before the occurrence of clinical symptoms. With the progression of the disease, motor rigidity and dementia dominate and death normally occurs after 15–20 years of progression (Damiano et al., 2010).

Age of onset and severity of this disease, correlates with the length of CAG expansion and HD appears when the number of glutamines expands beyond 40. A polyQ extension in proteins is implicated in some autosomal dominant neurological diseases and HD is the most prevalent of these diseases, affecting 1 in 10^5 persons (Sorolla et al., 2011).

The major hallmark of cytopathology of HD and other polyglutamine disorders is the accumulation of misfolded polyglutamine proteins as aggregates or neuronal intranuclear inclusions (Duennwald et al., 2006). In fact, mutant proteins with extended polyQ tracts gain an unusual conformation that simplifies their oligomerization and aggregation, generating cell toxicity. In addition to the toxicity induced by aggregates, polyQ proteins can also be toxic in the absence of a detectable formation of aggregates and misfolded htt monomers and oligomers are believed to be toxic before development of macromolecular inclusions (Sorolla et al., 2011; Damiano et al., 2010).

Several pathways are affected by the existence of pathological polyQ such as: vesicular and organelle trafficking; UPS; kinurenine pathway; transcription and mitochondrial function (Sorolla et al., 2011).

In HD, the combination of function failure of the wild type protein with a gain-of-function mutation, probably leads to neuronal degeneration. The mutant htt polypeptides get an uncommon conformation that produced cell toxicity, and facilitates the aggregation the aggregation into intracellular inclusion bodies (Landles & Bates, 2004).

Different models have been used to study this disease, and most of them express exon 1 or it's fragment, which promotes protein misfolding and aggregation, and causes toxicity (Sorolla et al., 2011; Damiano et al., 2010). Examples of these models are mouse, worm, fly, cell cultures and yeast (Sorolla et al., 2011).

2. Oxidative stress, mitochondrial dysfunction and ubiquitin proteasomal system in Parkinson's and Huntington's diseases

Oxidative stress (OS) happens when oxygen free radicals are formed in excess through the reduction of oxygen. Many of these radicals are generated during normal physiological conditions at low levels and are scavenged by endogenous antioxidant systems (Gilgun-sherki et al., 2001). The production of reactive oxygen species (ROS) leads to oxidative stress when endogenous antioxidant system is not capable to remove the high amount of ROS. This process has been suggested to concur in the onset of several neurodegenerative diseases, including PD and HD (Gilgun-sherki et al., 2001; Floyd & Hensley, 2002).

In fact, the brain is inclined to ROS production and oxidative stress due to its elevated metabolic rate, combined with the content of oxidizable molecules, such as dopamine and neuromelanin, whose metabolism generates ROS. In addition, the elevated concentration of transition metals and the low content of antioxidants, can contribute to exacerbate oxidative challenge (Raichur et al., 2006; Branco et al., 2010; Tsang & Chung, 2009). Indeed, many studies have showed a reduction in glutathione (GSH) and an increase in oxidized glutathione (GSSH) levels (Gilgun-sherki et al., 2001).

The oxidative stress in dopaminergic neurons of the *substantia nigra* pars compacta (SNpc) is connected with increased ROS levels, decreased levels of cellular antioxidants and increased levels of iron. Oxidative stress may contribute to SNpc neuronal cell death (Fig. 3) and plays an important role in neuronal degeneration of pigmented dopaminergic neurons in the SNpc (Raichur et al., 2006; Branco et al., 2010 and Tsang & Chung, 2009).

Various studies in HD brains have demonstrated the existence of oxidative damage, and that antioxidants slow the disease development. Therefore, the role of oxidative stress in the pathogenesis of HD has been growing attention (Sorolla et al., 2011).

Oxidative damage in mitochondria has also been implicated in the pathogenesis of PD and HD. Mitochondria are organelles with a vital role in the cells such as fatty acid oxidation, steroid metabolism, amino acid biosynthesis and principally, mitochondria maintain the cellular energy reserves with ATP production (Chong et al., 2005 and Hald & Lotharius, 2005).

Post-mortem studies show a complex I deficiency in the *substantia nigra* of patients with PD (Hald & Lotharius, 2005). In fact the inhibition of complex I activity leads to: an energy crisis; a discrepancy

of mitochondrial membrane potential; an increase on ROS levels; and, eventually, to neuronal death (Chong et al., 2005 and Hald & Lotharius, 2005). The active generation of ROS can lead to a subsequent irreversible inhibition of complex I in mitochondria and enhance ROS production (Tsang & Chung, 2009).

HD is characterized by degeneration of neurons in the striatum and it is known that this brain region is very sensitive to deficiency in energy metabolism (Cattaneo et al., 2005). In fact, some studies suggested a association between mitochondrial defects and striatum susceptibility to ROS, which presents the initial and most striking neuropathological changes in patients (Damiano et al., 2010). Mutant htt possibly may destabilize mitochondrial membrane, and increase the sensitivity of the mitochondrial permeability transition pore (mPTP) to calcium or other apoptotic stimuli through interactions with the external mitochondrial membrane (Damiano et al., 2010).
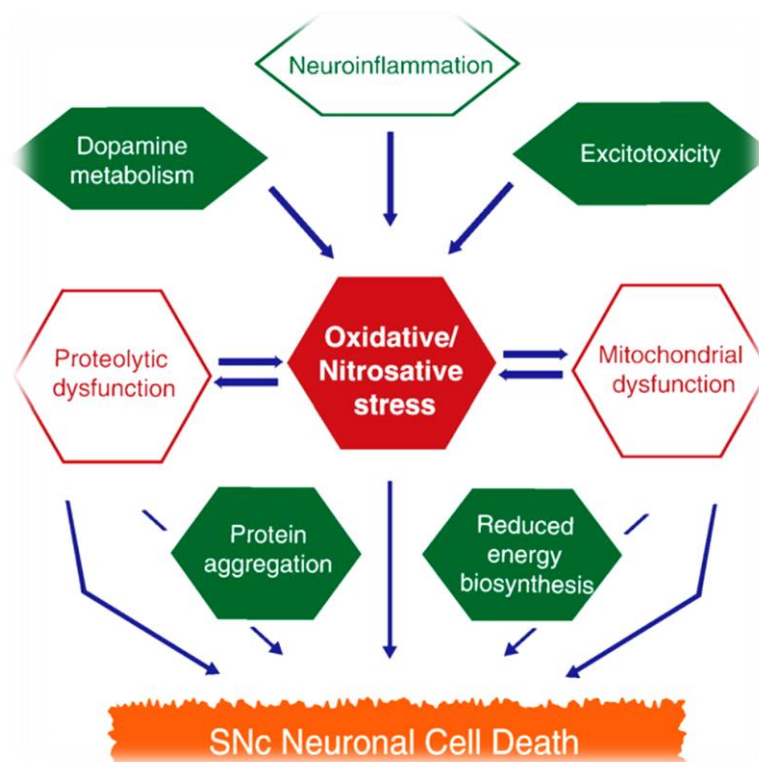

Figure 3 – Pathways that contribute to the SNcp neuronal cell death (Tsang & Chung, 2009)

Post mortem studies shows that defects in respiratory chain, diminution in the activity of complex II/III and milder reduction of complex IV, were observed in HD patients (Damiano et al., 2010).

The regulation of protein levels involved in fundamental cellular processes is made through mechanisms of protein degradation (Betarbet et al., 2005). The UPS is one of the pathways involved in targeting and detoxification of short-lived damaged proteins for degradation and provides the specificity and control of several regulatory proteins (Nedelsky et al., 2008). The UPS also contribute to maintain amino acid pools in acute starvation and contribute considerably to the degradation of defective proteins (Salomons et al, 2010).

The UPS involves the coordinated actions of a large number of proteins that consecutively recognize the substrates, tags substrates with poly-ubiquitin chains, and promote the destruction of poly-ubiquitylated substrates (Nedelsky et al., 2008). The recognition and tagging are mediated by a large group of ubiquitylation enzymes that conjugate poly-ubiquitin chains to substrates, while the final destruction is executed by the proteasome,a large multi-subunit proteolytic complex (Salomons et al., 2010 and Esser et al., 2004).

The accumulation of toxic proteins can arise due to a directly or indirectly impairment of UPS, under conditions of stress or other insults and lead to neuronal dysfunction or death (Betarbet et al., 2005). Anomalous accumulation of protein and aggregation affect the normal function of degradative pathways to degrade mutated or oxidized proteins (Nedelsky et al., 2008 and Ross & Pickart, 2004).

The aggregation of mutated α-synuclein can inhibit the UPS and it is thought that an impairment of the UPS and protein aggregation may affect each other (Betarbet et al., 2005). In fact, besides α-synuclein, proteasomal subunits are found in Lewy bodies suggesting the involvement of UPS in PD pathogenesis (Ross & Pickart, 2004).

Post mortem studies, show a deficit of proteasomal activity in nigral tissue from sporadic PD patients, suggesting that a dysfunctional UPS may cause susceptibility and deterioration of nigral dopaminergic neurons in PD (Betarbet et al., 2005). Other studies show the possibility of α-synuclein to block the polybiquitin recognition by binding to a subunit of 19S complex – UPS (Ross & Pickart, 2004).

In what concerns HD, the formation of both intranuclear and cytoplasmic ubiquitinated aggregates result from de existence of pathological polyQ and these aggregates containing the protease-resistant mutated N-terminal htt fragment in neurons of affected areas. This proposes that abnormal htt is targeted for proteolysis but is resistant to its removal (Bennett et al., 2007 and Sorolla et al., 2008).

3. Yeast model

Yeast models have been developed to study several neurodegenerative disorders (Scherzer & Feany, 2004; Summers & Cyr, 2011 and Winderickx et al., 2008). *Saccharomyces cerevisiae*, also known as budding yeast, is extensively used for studying the molecular events and cell composition relevant for human diseases and is one of the most recognised model organisms (Summers & Cyr, 2011 and Zabrocki et al., 2008). *S. cerevisiae* was the first eukaryote organism to be fully sequenced and there is a well-functioning research infrastructure, such as the Saccharomyces Genome Database (Zabrocki et al., 2008 and Petranovic & Nielsen, 2008).

S. cerevisiae is a unicellular microorganism and several cellular processes such, cell division, protein turnover, DNA replication and recombination, are well conserved between yeast and higher eukaryotes, including humans. In fact around 31% of yeast genes have a mammalian homolog and an additional 30% of yeast genes have domain similarity with mammalian (Scherzer & Feany, 2004 and Winderickx et al., 2008). There are some factors that make yeast an exceptional model: easy to cultivate in large populations, quickly and inexpensive; exists in haploid and diploid forms that makes possible sexual and budding reproduction, allowing genetic manipulations and screenings; can express heterologous genes, being rather simple to mutate, insert or delete any genomic sequence due to the presence of homologous recombination pathway (Winderickx et al., 2008; Zabrocki et al., 2008 and Miller-Fleming et al., 2008).

S. cerevisiae offers an experimental model system to explore the cell biological effects of several proteins involved in neurodegenerative diseases (table 2) (Scherzer & Feany, 2004). Although it is impossible to study the neuronal effects of this proteins in yeast, some mechanisms and pathways correlated to neurodegenerative disorders such as proteasomal dysfunction and mitochondrial dysfunction, are well conserved between yeast and humans, enabling the modelling of neurodegenerative diseases in these cells (Winderickx et al., 2008 and Miller-Fleming et al., 2008).

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Table 2 – Proteins involved in neurodegenerative diseases modeled in yeast

Protein involved	Disease
Superoxide dismutase 1	Amyotrophic Lateral Sclerosis (ALS) (Scherzer & Feany, 2004)
Frataxin	Friedreich's Ataxia (Scherzer & Feany, 2004)
Huntingtin	HD and PoyQ disorders (Winderickx et al., 2008)
α-synuclein	PD and synucleinopathies (Winderickx et al., 2008)
Tau and Amyloid precursor protein	AD and tauopathies (Winderickx et al., 2008)
ATP- dependent proteases	Hereditary Spastic Paraplegia (HSP) (Winderickx et al., 2008)
Wilson's disease protein (ATP7B)	Wilson's disease (Scherzer & Feany, 2004)
CLN3	Ceroid lipofuscinosis (Scherzer & Feany, 2004)

Yeast model for α-synuclein aggregation and toxicity

The yeast model has been used in the study of the pathogenic basis of PD (Fig.4), especially the biochemistry and toxicity of α-synuclein and its mutant forms (Scherzer & Feany, 2004). Through this model it is possible to comprehend the molecular pathways underlying the cytotoxic effects of α-synuclein malfunction, the cell response to an accumulation of toxic aggregates and the intracellular location of α-synuclein aggregates (Petranovic & Nielsen, 2008; Braun et al., 2010 and Outeiro & Giorgini, 2006).

When α-synuclein is expressed in yeast, the formation of inclusions (Fig.5) seems to be a process that involves the aggregation of monomers at the plasma membrane, which are then delivered and continue to grow in the cytoplasm (Braun et al., 2010 and Wang et al., 2009).
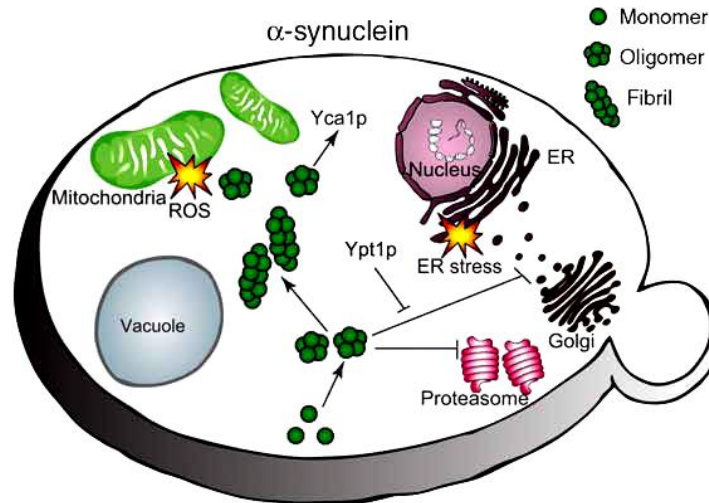
Figure 4 – Yeast model for α-synucleinopathies (Braun et al., 2010)

The heterologous expression of WT α-synuclein and A53T mutant form inhibited the yeast growth, which is not the case of A30P mutant that merely produced partial growth inhibition when expressed from high-copy plasmid (Winderickx et al., 2008).

Unlike the WT α-synuclein and the A53T, the A30P mutant does not form cytoplasmatic inclusions, due to its low membrane-binding capability. The WT α-synuclein and the A53T inclusions are involved in impaired proteasomal degradation, inhibition of phospholipase D, blockage of endoplasmic reticulum to Golgi transport and delayed endocytosis (Zabrocki et al., 2008 and Winderickx et al., 2008).

Several studies show that the heterologous expression of WT α-synuclein or mutant forms, leave the yeast cell more vulnerable to oxidative stress induced by peroxide, and this is because the expression of the protein induced the ROS production and release of cytochrome c from mitochondria, proposing that α-synuclein activates the apoptotic cell death program (Braun et al., 2010 and Winderickx et al., 2008).
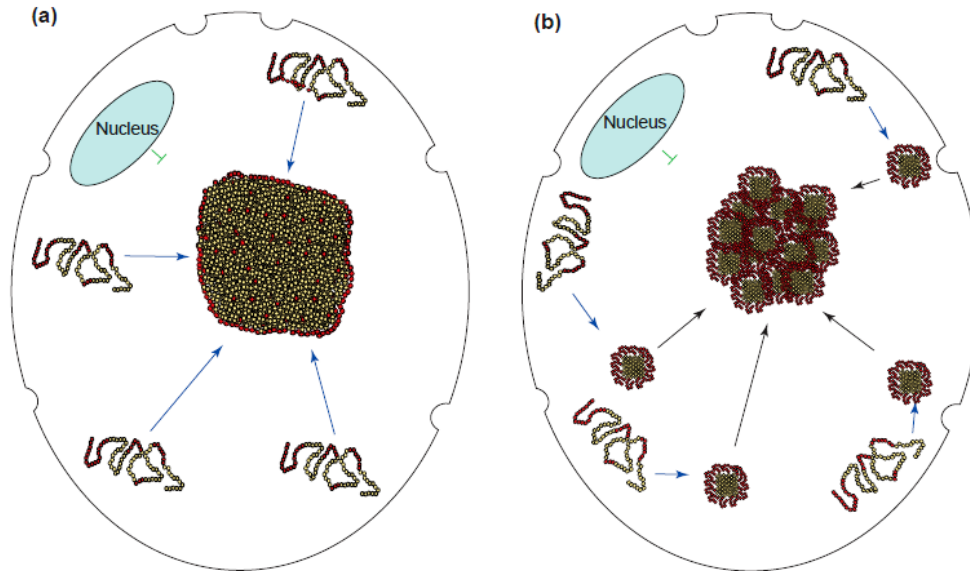
Figure 5 – Two models to cytoplasmic inclusion formation. (a) Monomers directly diffuse to the deposition site. (b) Monomers aggregate in the cell periphery and are transported to the inclusion body (Kopito, 2000).

Yeast model of Huntington's disease

Studies have been developed in order to understand the folding and behaviour of mutant htt, as well as to determine the basic conserved mechanism of mutant htt-dependent toxicity and yeast models of HD have been developed as valuable tools in these studies (Miller-Fleming et al., 2008).

Yeast models express a fragment of the human polyQ protein huntingtin (htt), htt exon I. The first studies using yeast as a model demonstrated that heterologous expression of htt results in aggregation dependent of polyQ length (Duennwald et al., 2006). One of these models shows toxicity dependent of polyQ length, such that expression of a htt fragment with higher polyQ length (htt103Q) produces cellular toxicity, whereas the expression of a non-expanded polyQ tract (htt25Q) shows no effects upon growth (Duennwald, 2011).

These models offer a useful tool to discover modulators of polyQ toxicity in a defined and consistent cellular environment and provide a powerful instrument to explore the intramolecular and intermolecular factors that are connected with polyQ toxicity (Duennwald, 2011 and Duennwald et al., 2006).

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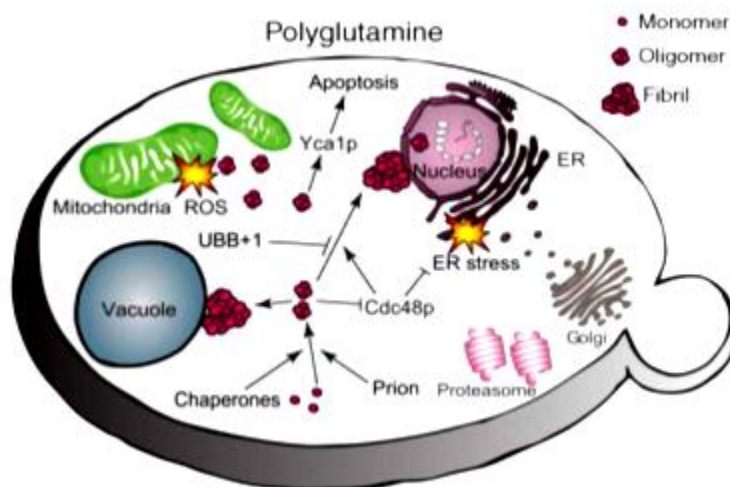

Figure 6 - Yeast model for polyglutamine disorders (Braun et al., 2010)**.**

Mitochondrial dysfunction, increased levels of ROS, defects in endocytosis, apoptotic-like events and perturbations in the kynurenine pathway are examples of factors that many studies related to toxicity in yeast model of HD (Solans et al., 2006 and Miller-Fleming et al., 2008).

4. *Hypericum perforatum* and phenolic compounds

Medicinal plants have been used for long time, due to their significant biological activities, as well as many natural compounds from these plants (Maciel et al., 2002).

Hypericum, a genus of the family *Clusiaceae*, has been commonly used in traditional medicine for a long time. *H. perforatum* is common in Europe, North Africa, Asia and North America (Langosch et al., 2002). The plant spreads quickly and can emerge in different locations such as plains and sides of roads. Its popular name St John's wort raises to its flowering period around St John's day, the 24th of June (Langosch et al., 2002 and Saddiqe et al., 2010). The medicinal plant *H. perforatum* has been used as an antidepressant with scarce side effects, and it is very interesting to observe that several *Hypericum* species showed good antioxidant activity (Saddiqe et al., 2010). Evidences from the literature show that the quercetin, kaempferol and biapigenin are present in *H. perforatum* extracts (Table 3), in particular biapigenin, which is only produced by this plant (Silva et al., 2008 and Schulte-Löbbert et al., 2003).

Hypericum perforatum phenolic compounds: Protective role in the toxicity induced by heterologous expression of α-synuclein and huntingtin in yeast cells

Table 3 – Phenolic composition of *Hypericum perforatum* total ethanolic extract (Silva et al., 2008)

Compounds	µg/ g dwb	Compounds	µg/ g dwb
Phloroglucinols		*Naphthodianthrones*	
Hyperforin	6880	Hypericin	570
Adhyperforin	1207	Pseudohypericin	699
Total	8087	Protopseudohypericin	48
		Protohypericin	27
		Total	1334
Flavonol glycosides		*Flavonoid aglycones*	
Hyperoside	7861	Quercetin	5128
Rutin	3949	Kaempferol	156
Isoquercitrin	1942	Biapigenin	2135
Querct 3-glucuronic + querct 3-galacturonic	352	Amentoflavone	108
Hyperoside-acetyl	758	Total	7527
Rutin-acetil	233		
Kampferol 3-rutinoside	283		
Quercetin 3-rhamnoside	3760		
Total	19,138		
Phenolic acids			
Neochlorogenic acid	2880		
Chlorogenic acid	1181		
Unidentified	189		
Total	4250		
		Total phenolics	40,336

Phenolic compounds are abundant in plant foods, and have been associated with the sensory and nutritional value of fresh and processed plant foods (Gonzalez et al., 1999). They are secondary metabolites that are derivatives of the pentose phosphate, shikimate, and phenylpropanoid pathways in plants (Balasundram et al., 2006). These compounds, ubiquitous in plants, have physiological and morphological relevance in these organisms. Phenolic compounds show an important role in growth and reproduction, providing defense against pathogens and predators, also contributing to the color and sensory characteristics of fruits and vegetables (Balasundram et al., 2006 and Martins et al., 2011). These compounds are considered relatively non-toxic bioactive substances and have various biological activities, such as antioxidants, chelation of metals, scavengers of active oxygen species, blockers of nitration, anti-allergenic, anti-artherogenic, anti-inflammatory, anti-microbial, anti-thrombotic, cardioprotective and vasodilatory effects. (Martins et al., 2011; Cai et al., 2004;

Balasundram et al., 2006 and Saddiqe et al., 2010) They can suffer autoxidation to produce hydrogen peroxide in the presence of metals and are capable of modulating certain cellular enzyme activities (Saddiqe et al., 2010). Flavonoids have a great antioxidant potential due to structural characteristics such as the arrangement of hydroxyl groups on the benzene ring (García-Mediavilla et al., 2007). These compounds have gained much attention, since their consumption has been associated with a decrease in age-related cognitive declines and risk of developing neurodegeneration (Filomeni et al., 2010). In fact, studies with rat brain have demonstrated that biapigenin, kaempferol and quercetin, may play a neuroprotective role against neuronal excitotoxicity and mitochondrial dysfunction (Silva et al., 2008).

Quercetin

Quercetin is a major dietary flavonoid (Fig. 7) present in fruits and vegetables and several others foods, and has gained attention as a good antioxidant (Cornish et al., 2002).
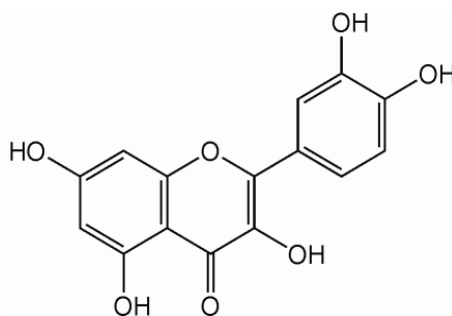

Figure 7 – Structural formula of quercetin (Silva et al., 2008)

Within the flavonoids, quercetin is the most active scavenger of ROS and reactive nitrogen species (RNS). This can be explained by the structure of the molecule that has the optimum configuration for free radical scavenging (García-Mediavilla et al., 2007). Several studies using human and rat cells, show that quercetin exhibits anti-inflammatory effects, inhibiting tumor necrosis factor receptor family signaling in bone cells and can display a neuroprotective role (García-Mediavilla et al., 2007; Pang et al., 2006 and Arredondo et al., 2010).

Kaempferol

Kaempferol is a natural flavonoid (Fig. 8) which has been isolated from tea, broccoli, witch-hazel, propolis, grapefruit, and other plant sources (Yoshida et al., 2008). Some studies with neuronal cells have shown that this compound, even at the dose of 30 µM, may provide neuroprotection by autophagy and protect against mitochondrial dysfunction (Filomeni et al., 2010). This compound such as quercetin, have been reported as antioxidant, as illustrated by a study with cerebellar granule cells that shows that kaempferol decreased oxidative stress (Samhan-Arias et al., 2004).
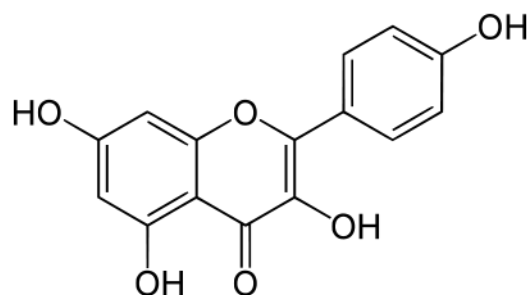

Figure 8 – Structural formula of Kaempferol (Silva et al., 2008)**.**

Biapigenin

Biapigenin is the most abundant biflavone (Fig. 9) in *Hypericum perforatum* and some studies suggest that biapigenin may contribute to some of the pharmacological actions of this herb (Colovic & Caccia, 2008). It is mainly localized in the buds and flowers of this plant, reaching its maximum amount just before blooming of the buds (Schulte-Löbbert et al., 2003).

This compound has a variety of biological effects. Biapigenin was remarkably active in an established screening model for antidepressants (Colovic & Caccia, 2008). A study using macrophage cells show that biapigenin may inhibit the activation of nuclear factor-κB and thus contribute to blocking effects in inducible nitric oxide synthase and ciclooxygenase-2 (Woo et al., 2006). Another study with rat hippocampal neurons shows that this compound confers neuroprotection against excitotoxic insults by protection from calcium deregulation (Silva et al., 2008).

Hypericum perforatum phenolic compounds: Protective role in the toxicity induced by heterologous expression of α-synuclein and huntingtin in yeast cells
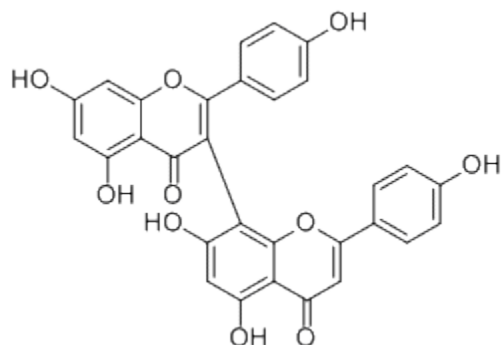

Figure 9 - Structural formula of Biapigenin (Silva et al., 2008)**.**

5. Goals

The aims this study were to evaluate the possible effects of *Hypericum perforatum* phenolic compounds (quercetin, kaempferol and biapigenine) in the toxicity induced by the heterologous expression of α-synuclein and huntingtin. For these studies the yeast *Saccharomyces cerevisiae* was used as a model.

II.   Material and Methods

1. Phenolic compounds

Quercetin (≥ 98%), kaempferol (≥ 98%) and biapigenin (≥ 98%) were purchased from Sigma. All compounds were dissolved in dimethyl sulfoxide (DMSO) (Sigma).

The compounds were used at concentration 15 μM and were made two different mixtures of the 3 compounds (Mix a: 10μM quercetin, 10μM kaempferol and 10μM biapigenin; Mix b: 15μM quecetin, 15μM kaempferol and 15μM biapigenin).

1. Strains and growth conditions

The yeast strains and plasmids used in this study are listed in Table 3.

Table 3 – Yeast strains and plasmids used in this study

Strain	Genotype	Plasmids		Source
W303-1A	MAT**α** ade2-1 can1-100 his3-11,14 leu2-3 112 trp1-1 ura3-1	**WT**	pRS304Gal-αSynWT-GFP pRS306Gal-αSynWT-GFP	Outeiro, T.F.
		A53T	pRS304Gal-αSynA53T-GFP pRS306Gal-αSynA53T-GFP	
		A30P	pRS304Gal-αSynA30P-GFP pRS306Gal-αSynA30P-GFP	
W303-1A	MAT**a** ura3-52 trp1Δ 2 leu2-3,112 his3-11 ade2-1	**25Q**	pRS303Gal-htt25Q-GFP	Outeiro, T.F.
		103Q	pRS303Gal-htt103Q-GFP	

To perform the assays (Fig. 10) with the yeast Parkinson's disease (PD) models, the cells were inoculated in YEPD medium with 0.5 % (w/v) yeast extract (Difco Laboratories), 1% (w/v) peptone (Difco Laboratories) and 2% (w/v) glucose at 26°C and 150 rpm, until the stationary phase. In the assays (Fig. 11) with the yeast Huntington's disease (HD) models, the cells were inoculated in

selective YNB medium, containing 0.67 % (w/v) Yeast Nitrogen Base (Difco Laboratories), 2% (w/v) glucose as carbon source, supplemented with the appropriate amino acids: 100 mg/L uracil, 300 mg/L leucine, 100 mg/L tryptophan and 100 mg/L adenine. Growth was monitored (each 2 hours) by measuring the turbidity of the culture at 640 nm in a Spectronic Genesys 20 spectrophotometer (Thermo Spectronic).

To study the effects of the phenolic compounds, cells were collected at exponential phase (OD 640 nm ≈ 0.4), washed twice with distilled water and inoculated in YEPDGal medium with 0.5 % (w/v) yeast extract (Difco Laboratories), 1% (w/v) peptone (Difco Laboratories) and 2% (w/v) galactose or YNBGal medium with 0.5 % (w/v) yeast extract (Difco Laboratories), 1% (w/v) peptone (Difco Laboratories) and 2% (w/v) galactose at optical density ≈ 0.24 at 640 nm. Then, the yeast cells were transferred to 12-well culture plates and Falcon tubes to PD and HD yeast model, respectively. Next the phenolic compounds were added.

For determination of yeast cell survival, cellular samples were serially diluted and platted on YEPD agar plates, consisting in 0.5 % (w/v) yeast extract (Difco Laboratories), 1% (w/v) peptone (Difco Laboratories), 2% (w/v) glucose and 2% (w/v) agar. The viability was determined by counting colony-forming units (CFUs) after 2 days of incubation at 30°C. The CFUs on time 0 (when the phenolic compounds were added to the culture medium) were considered as 100% survival.

Hypericum perforatum phenolic compounds: Protective role in the toxicity induced by heterologous expression of α-synuclein and huntingtin in yeast cells
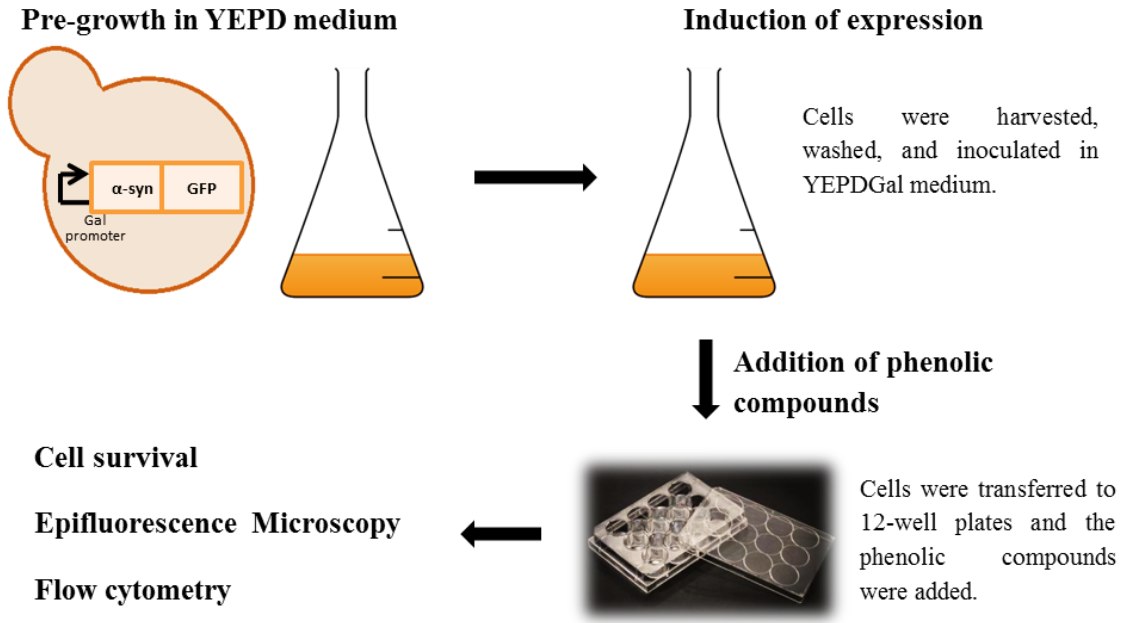

Figure 10 - Schematic representation of the methods used to study the toxicity induced by heterologous expression of α-synuclein.
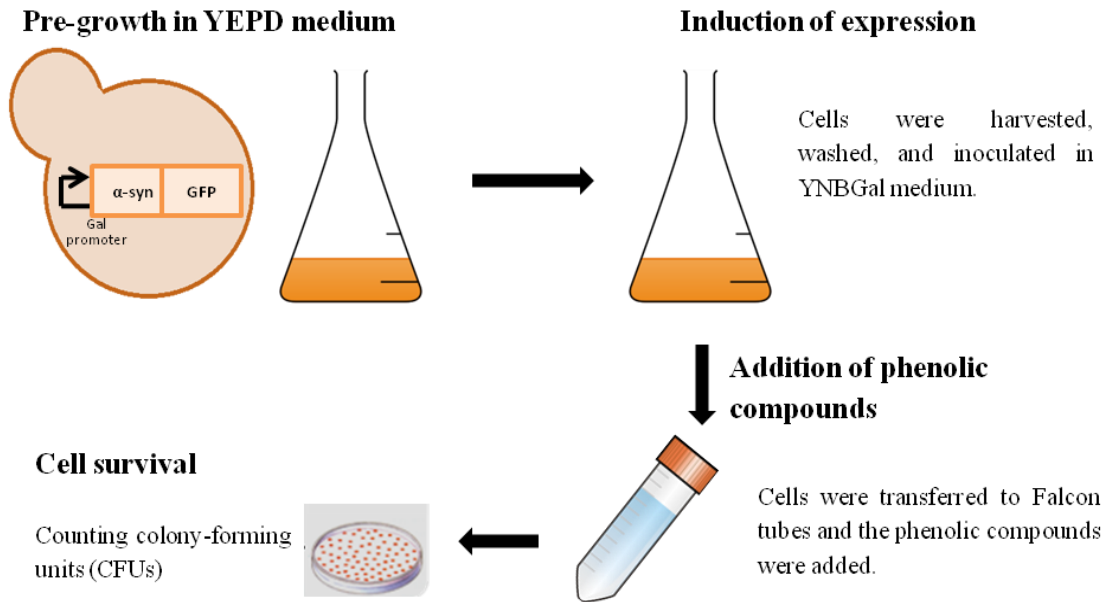

Figure 11 - Schematic representation of the methods used to study the toxicity induced by expression of htt.

2. Epifluorescence microscopy

All the samples were collected at specific time points, 20h and 40h, and then they were washed and suspended in PBS and Vectashield® (Vector Laboratories, Burlingame, Canada).

Images were acquired with an Olympus BX61 (Olympus, United States) microscope with filter wheels, to control excitation and emission wavelengths, equipped with a high-resolution DP70 digital camera and using an Olympus UPlanSApo 100X/oil objective, with a numerical aperture of 1.40.

3. Measurements of cellular reactive oxygen species levels

Cellular reactive oxigen species (ROS) production was kinetically monitored by flow cytometry with dihydroethidium (DHE) staining. This assessment was obtained by measuring the ROS production of cell culture samples harvested at different time points of α-syn expression. For DHE staining, cells were harvested by centrifugation, resuspended in PBS and incubated with 10 µg/ml DHE in the dark for 10 minutes at 30°C. Afterwards, the fluorescence was detected by flow cytometry. Cells presenting high red fluorescence were considered to have high intracellular ROS levels.

Flow cytometry assays were performed on an EPICS XL-MCL (Beckman-Coulter Corporation, Hialeah, FL) flow cytometer, equipped with an argon-ion laser emitting a 488-nm beam at 15mW. DHE was collected through a 488-nm blocking filter, a 590 nm long-pass dichroic and a 620-nm bandpass. Twenty thousand cells per sample were analyzed. The data were evaluated with the MULTIGRAPH software included in the system II acquisition software for the EPICS XL/XL-MCL version 1.0.

4. Statistical analysis

The results shown are mean values and standard deviations of at least three independent assays. Statistical analyses were determined using *t*-test. A *p*-value less than 0.05 was assumed to denote a significant difference.

III.   Results and Discussion

1. Effect of phenolic compounds in toxicity induced by heterologous expression of α-synuclein

The protein α-synuclein is the main component of Lewy bodies, one of the hallmarks of Parkinson's disease (PD) (Corti et al., 2005). Several studies in different cellular models showed that overexpression of the α-synuclein resulted in cell toxicity and culminates in cell death (Raichur et al., 2006). *Saccharomyces cerevisiae*, genome does not encode an orthologue of α-synuclein and thus could be used to study the effects of heterologous expression of α-synuclein. In fact, yeast cells have already proved to be an excellent model to study the cellular processes linked to PD (Outeiro & Lindquist, 2003). To study the cellular toxic effects of α-synuclein a model in which the yeast cells carrying human wild-type (WT) or mutant α-synuclein fused with GFP (green fluorescent protein) was developed. The α-synuclein-GFP fusion construct was integrated into the yeast genome under the control of a galactose-inducible promoter (Outeiro & Lindquist, 2003), meaning that the presence of galactose in the yeast culture medium allows the α-synuclein-GFP heterologous expression.

In yeast, the toxicity of neurotoxic proteins is tested by clonogenic assays on agar plates or in liquid cultures, and cell survival is calculated by the number of colony-forming units (CFUs) (Braun et al., 2010). Therefore, clonogenic assays were performed with the addition of phenolic compounds (quercetin, kaempferol and biapigenin) in the medium.

The phenolic compounds have various biological activities (Silva et al., 2008), including quercetin and kaempferol which have been used in several studies due to their antioxidant properties (Pang et al., 2006). The use of polyphenols has been associated with a decrease in age-related cognitive declines and risk of developing neurodegeneration (Filomeni et al., 2010). In addition, studies in isolated rat brain mitochondria have been developed and showed that biapigenin confers protection against excitotoxic insults (Silva et al., 2010).

To gain further insight into the bioactivities of these compounds, yeast clonogenic assays were performed on cells expressing an vector control (Empty), WT α-synuclein and mutant forms, A30P

and A53T in the presence of quercetin, kaempferol and biapigenin at concentration of 15μM each, to verify the occurrence of possible effects of the phenolic compounds on the toxicity caused by heterologous expression of the proteins. The data showed that biapigenin seems to be more effective in reducing the toxicity of heterologous expression of WT α-synuclein (Fig. 12B) when compared to cells without the addition of compounds, or even when compared with the quercetin and kaempferol.
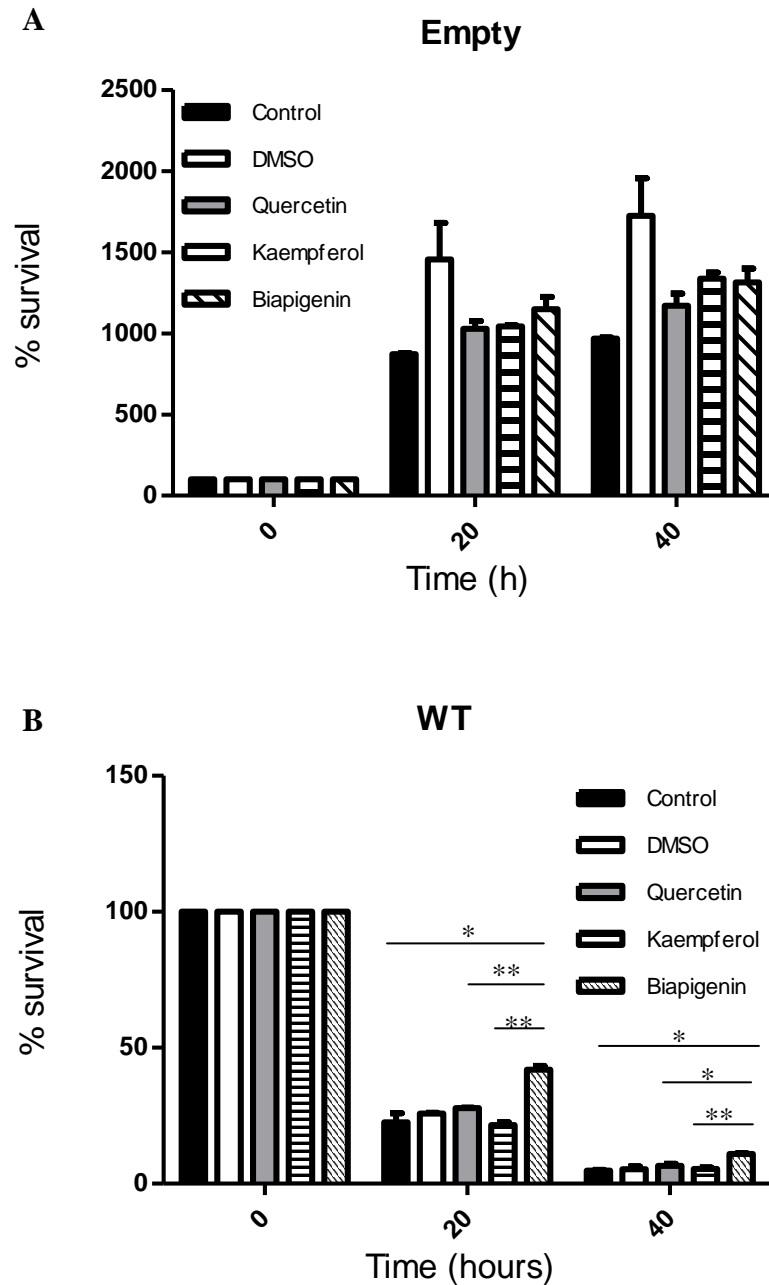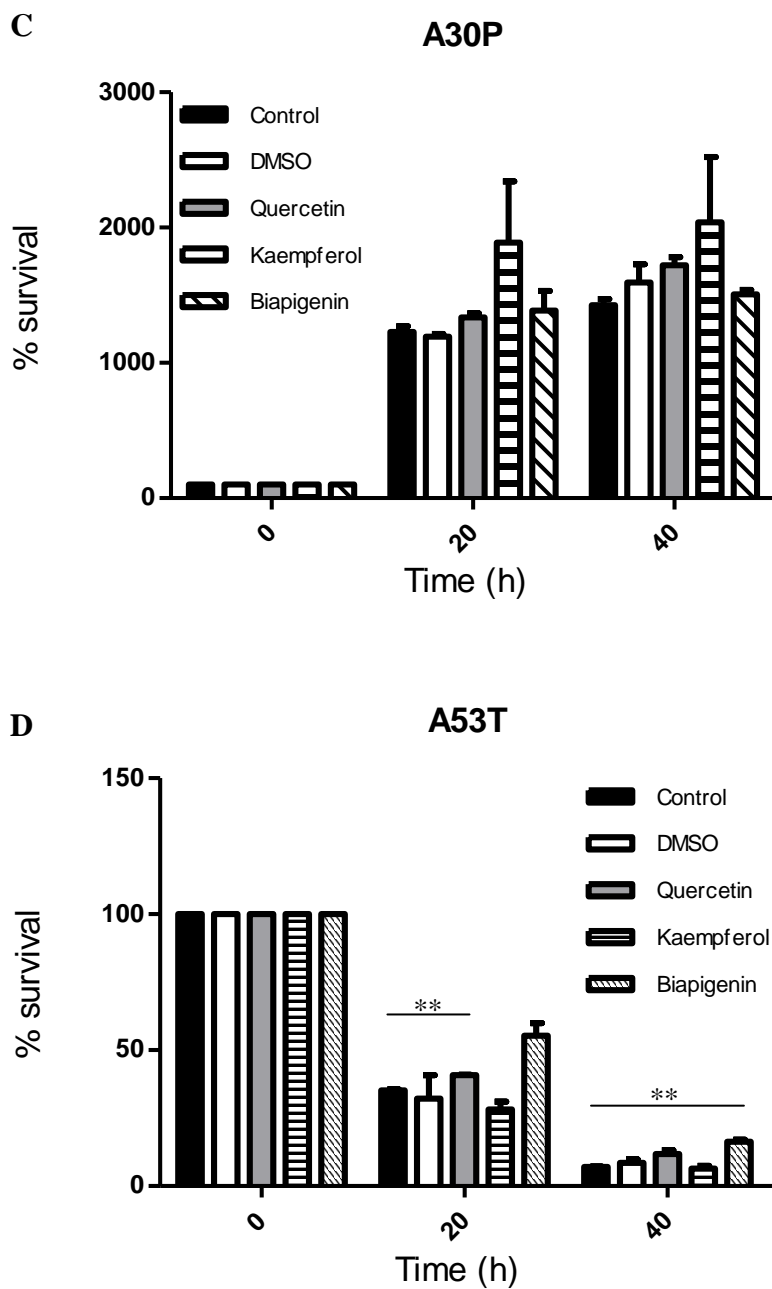
Figure 12 - Survival determined by colony-forming units of cell expressing WT (B) and point mutants A53T (D) and A30P (C) forms of α-synuclein or harboring the empty vector (A) after induction of expression by changing to 2% galactose medium in presence of quercetin, kaempferol and biapigenin. Data represent mean ± S.E. of 3 independent experiments. Statistical significance (*p <0.05, **p <0.01) were determined by t-test.

As expected there was a continued growth in the case of the cells harboring the empty vector (Fig. 12A) functioning as a control. Also in the case of the A30P mutant form (Fig. 12C) there was a

continuous growth, similarly to the control conditions (empty). The non-toxic phenotype of α-synuclein A30P mutant form was also expected, since the A30P mutation reduces the affinity of the protein to interact with phospholipids, a pre-requisite to aggregation (Auluck et al., 2010). The A30P mutant form only produced partial growth inhibition when expressed in a high-copy plasmid (Scherzer & Feany, 2004). In the case of WT α-synuclein (Fig. 12B), the heterologous expression of the protein reduced the cell survival to values of 22.57% and 4.82% after 20h and 40h of induction of protein expression, respectively. While the A53T mutant form (Fig. 12D) the heterologous expression reduces the cell survival to values of 35.24% and 7.1% to 20h and 40h, respectively. The treatment with biapigenin appears to reduce the toxicity induced by heterologous expression of α-synuclein, since the values of cell survival in the presence of this compound were about 41.95% and 10.82% after 20h and 40h of induction of WT α-synuclein expression, respectively (Fig. 12B). In the case of A53T mutant form the values of cell survival (Fig. 12D) in the presence of biapigenin were 55.27% and 16.35% to 20h and 40h, respectively. The research about biapigenin and its actions is scarce, but some studies using rat brain mitochondria, suggest that biapigenin contributes for neuroprotection against excitotoxicity, since this compound can modulate mitochondrial permeability transition pore opening (mPTP), probably through modulating adenosine nucleotide translocator function, which contributes to improve the mitochondrial calcium efflux, reducing in this manner the calcium burden (Silva et al., 2008 and Silva et al, 2010).

Additionally, the presence of quercetin, also demonstrated a reduction of the toxicity induced by heterologous expression of α-synuclein, since the values of cell survival were about 40.75% and 11.28% to 20h and 40h respectively (Fig. 12D). Nevertheless, relatively to the cells expressing the A53T mutant form in the presence of quercetin, the toxicity reduction was only observed at 20h. The effects observed with quercetin may be due to its role as an antioxidant, catalysing electron transport, chelating transition metal and scavenging free radicals, since several studies using human and rat cells show that quercetin decreases oxidative stress (Dias et al., 2005 and Jamshidzadeh & Mehrabadi, 2010).

The use of dimethyl sulfoxide (DMSO) does not affect the cells, as observed in figure 12.

2. Effect of the mix compounds in toxicity induced by heterologous expression of α-synuclein

An effect in reducing the toxicity induced by heterologous expression of α-synuclein under phenolic compounds treatment was observed, therefore, we wanted to understand if the presence of the three compounds together could induce a synergistic effect. Hence, to perform this assay two mixtures with different concentrations (Mix a: 10µM quercetin, 10µM kaempferol and 10µM biapigenin and Mix b: 15µM quecetin, 15µM kaempferol and 15µM biapigenin) were used. We used two different compounds concentrations, for as little or nothing is known about the use of the compounds together, we decided to try the two concentrations mentioned above. The data (Fig. 13) showed that the mix a, has significant effects in protecting against the toxicity of the protein expression in the case of α-synuclein A53T mutant form. Although the mix b had no significant effects, however there was observed a strong tendency to reduce the toxicity induced by heterologous expression of α-synuclein.
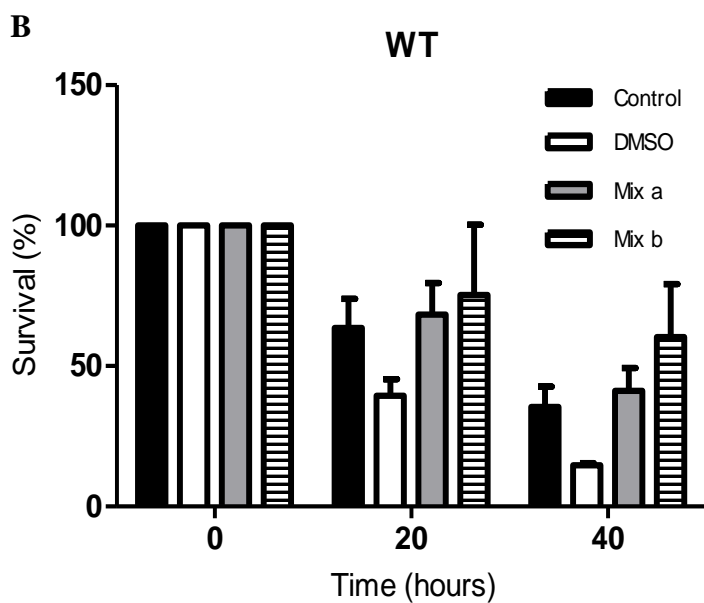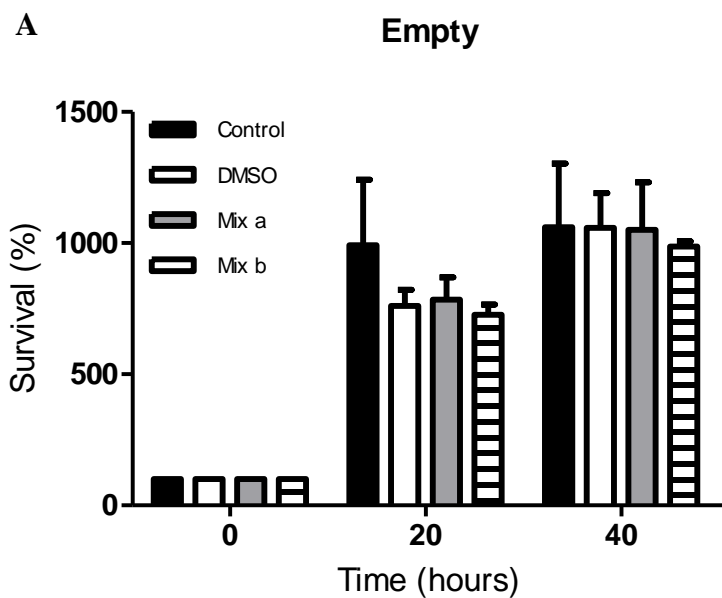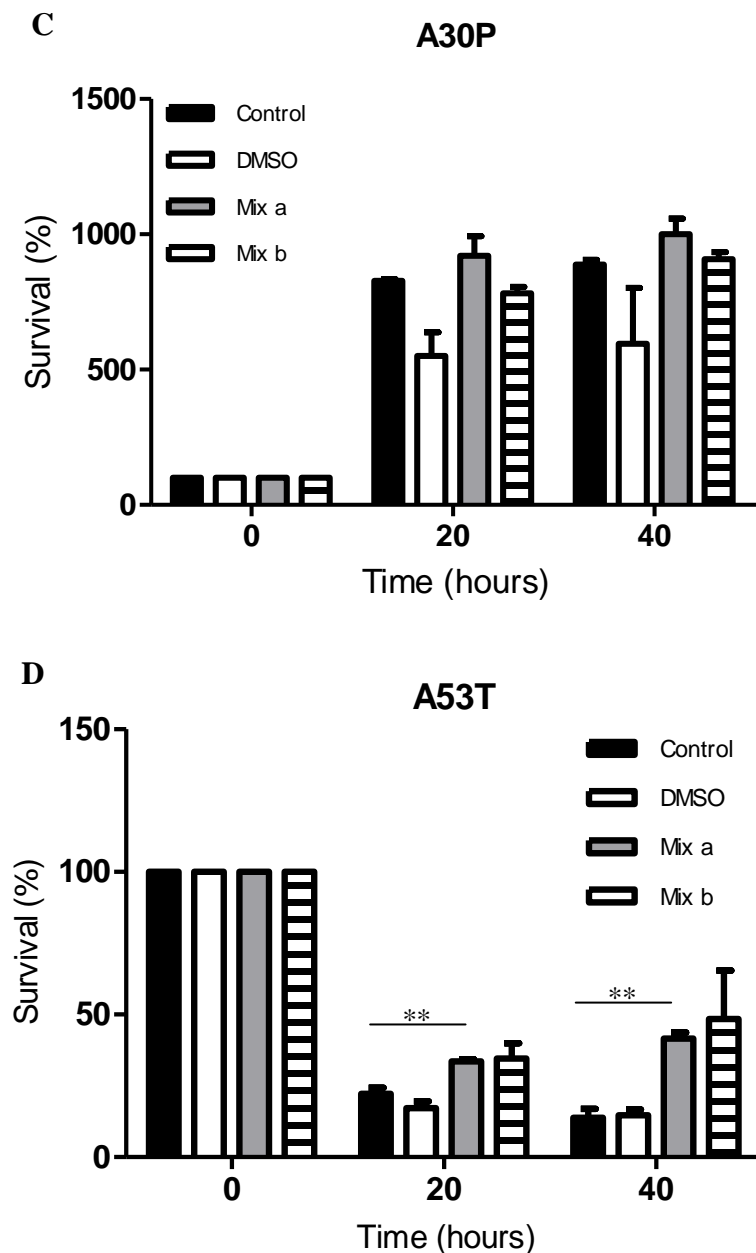
Figure 13 - Survival determined by colony-forming units of cell expressing WT (B) and point mutants A53T (D) and A30P (C) forms of α-synuclein or harboring the empty vector (A) after induction of expression by changing to 2% galactose medium in presence of compounds mix. Data represent mean ± S.E. of 3 independent experiments. Statistical significance ($*p<0.05$, $**p<0.01$) were determined by t-test.

Once again, as expected, the cells harboring the empty vector and the A30P mutant form (Fig. 13A and 13C) showed a non-toxic phenotype, being possible to observe cell growth. In the case of expression of α-synuclein A53T mutant form (Fig. 13D), the values of cell survival at time 20h and

40h without compounds were about 22.13% and 13.84%, respectively. In the presence of mix a, we could observe an higher percentage of cell survival, 33.51% and 42.53% after 20h and 40h of induction of protein expression, respectively. Relatively to the mix b, that has the higher concentration of compounds, no significant effect has been detected, however there was a strong tendency of this mixture to reduce the α-synuclein-induced toxicity, also in the case of α-synuclein A53T mutant form (Fig. 13D). Relatively to the expression of WT α-synuclein, again, no significant effects were shown (Fig. 13B), but there was a strong tendency of the both mixtures to reduce the toxicity induced by heterologous expression of WT α-synuclein. Therefore, it is necessary to repeat the experiments with mix b, to validate the observations and to confirm their statistical significance.

The data showed that, contrary to previous results, the presence of DMSO reduces the cell survival, since the amount of DMSO used (1.3%) is toxic to yeast cells. Thus, we can assume that, in this case, the effects of the compounds are more significant than those observed.

Although there are some studies about quercetin, kaempferol and biapigenin, little or nothing is known about their use as a mixture. The data showed possible synergistic effects of these compounds, that may have been originated by the combined action of quercetin and kaempferol, due to their antioxidant properties (Dias et al., 2005 and Samhan-Arias et al., 2004) and/or ability of biapigenin to regulate the calcium homeostasis (Silva et al., 2010). A study with neuronal cells showed that kaempferol can interfere with mitochondrial homeostasis (Filomeni et al., 2010), a target of the α-synuclein induced toxicity. Therefore, we can assume that there may be a combined effect of the actions of kaempferol and biapigenin by impinging on cellular energetics.

3. Effect of phenolic compounds in huntingtin expression

The formation of aggregates and misfolded huntingtin (htt) monomers and oligomers are the major hallmark of cytopathology of Huntington's disease (HD) and several pathways are affected by their presence (Qin & Gu, 2004). The disease is caused by an abnormal expansion of a CAG repeat located in the exon 1 of the IT15 gene of htt protein. The longer polyQ domain seems to induce conformational changes in the protein, which causes it to form intracellular aggregates that, in most cases, manifest as nuclear inclusions (Sorolla et al., 2011 and Damiano et al., 2010). The increase

in the length of polyglutamine tract alters biochemical and biophysical properties of proteins, and consequently, these proteins accumulate and forms aggregates (Dehay & Bertolotti, 2006).

The yeast genome does not have a homologous htt gene, however it is possible to induce its heterologous expression. The yeast model of HD used, employs the W303-1a strain harboring a plasmid containing the first exon of htt gene, with a polyQ tract of 25 glutamines (25Q) or 103 glutamines (103Q), under the control of a galactose-inducible promoter (Solans et al., 2006 and Hughes et al., 2001), furthermore, the exon 1 of htt gene is fused with the GFP, allowing its visualization.

Like what was developed with the PD yeast model, with the HD model, we studied the effect of phenolic compounds on the survival of yeast cells expressing the normal or extended htt polyQ tract (Fig. 14), to access the possible effects of the phenolic compounds on the toxicity levels caused by an abnormal expansion of a CAG repeat.
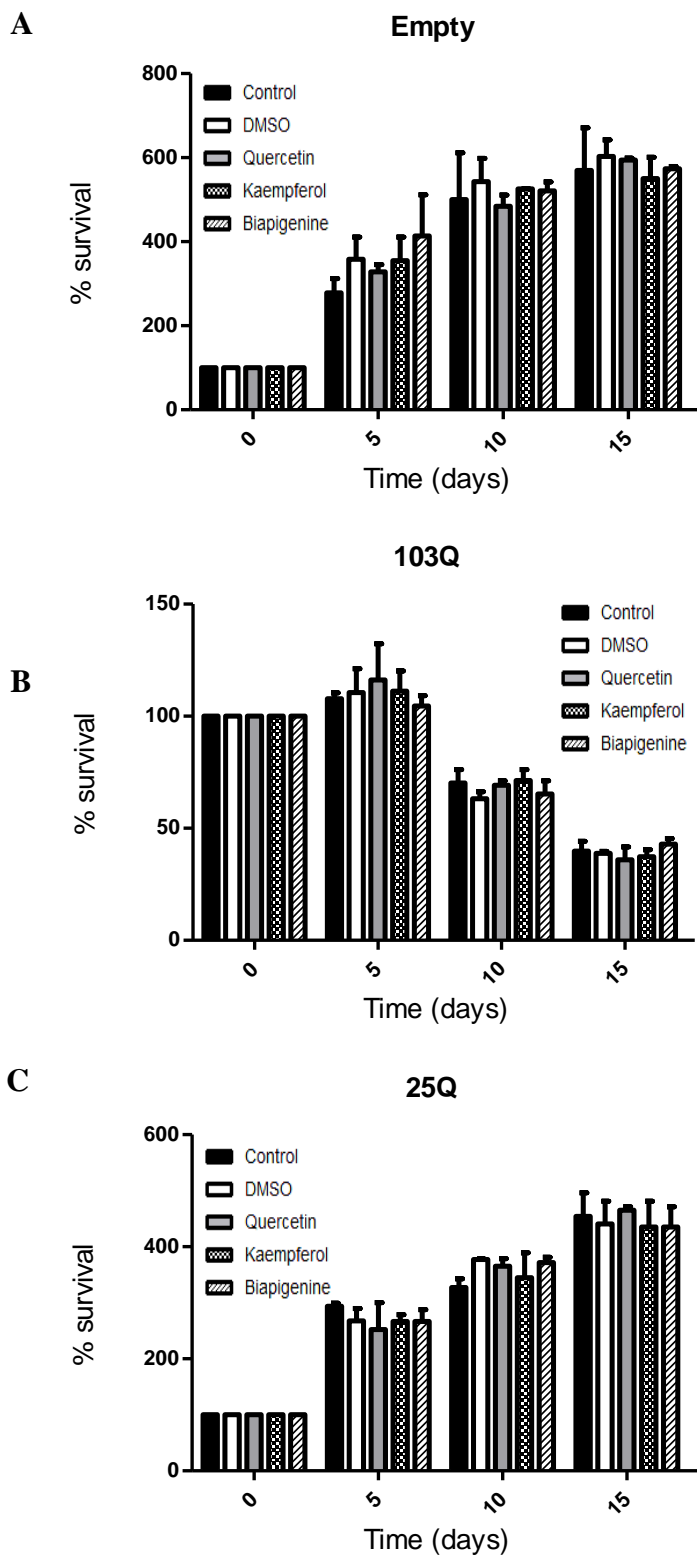
Hypericum perforatum phenolic compounds: Protective role in the toxicity induced by heterologous expression of α-synuclein and huntingtin in yeast cells

Figure 14 - Survival determined by colony-forming units of cell expressing point mutants 25Q (C) and 103Q (B) forms of htt or harboring the empty vector (A) after induction of expression by changing to 2% galactose medium in presence of quercetin, kaempferol and biapigenin. Data represent mean ± S.E. of 3 independent experiments.

The yeast model used to study the effect of phenolic compounds in htt expression has lower toxicity than the yeast model used to α-synuclein expression. Thus, for this study the cell survival was monitored for 15 days.

In the case of cells harboring the empty vector (Fig. 14A), it was possible to observe a continued growth, as expected, since protein expression does not occur. The same happened in case of normal htt form 25Q (Fig. 14C) since the severity of the disease is correlated with the length of CAG expansion and the toxicity appears when the number of glutamines expands beyond 40 (Sorolla et al., 2011). The data showed a reduction in cell survival in the case of htt 103Q mutant form (Fig. 14B). These results are not surprising because the length of CAG expansion is higher and consequently the toxicity occurs (Dehay & Bertolotti, 2006). In fact, other studies using yeast and other cell models showed that the expression of htt 103Q form has a greater toxic effect when compared to normal htt 25Q form (Truant, 2003). Several investigations using various models show that unusually long polyQ tracts induce disease because they interfere with the normal function of cellular proteins (Sorolla et al., 2008 and Sorolla et al., 2011).

The presence of quercetin, kaempferol or biapigenin seems to have no effect in the toxicity induced by expression of toxic protein, htt 103Q (Fig. 14B), since the values of cell survival were 70.2% and 35.8% after 10 and 15 days of induction of protein expression, respectively, while in the presence of the phenolic compounds, the values of cell survival ranged between 65.3% and 71.3% after 10 days of induction of htt expression and 35.2% and 42.9% after 15 days of induction of htt expression. Probably, the concentrations of compounds used were not high enough to cause effects in htt expression, therefore it is necessary to repeat the experiments with other concentrations.

4. Effect of the mix compounds in huntingtin expression

Although there have been no effects of phenolic compounds in cells expressing htt toxic form, it was performed an assay in which two different mixtures of the phenolic compounds, quercetin, kaempferol and biapigenin, were used (Fig. 15) to understand if there was the possible synergistic effects occurring between the compounds, that consequently could rescue the cells expressing the toxic form of htt.
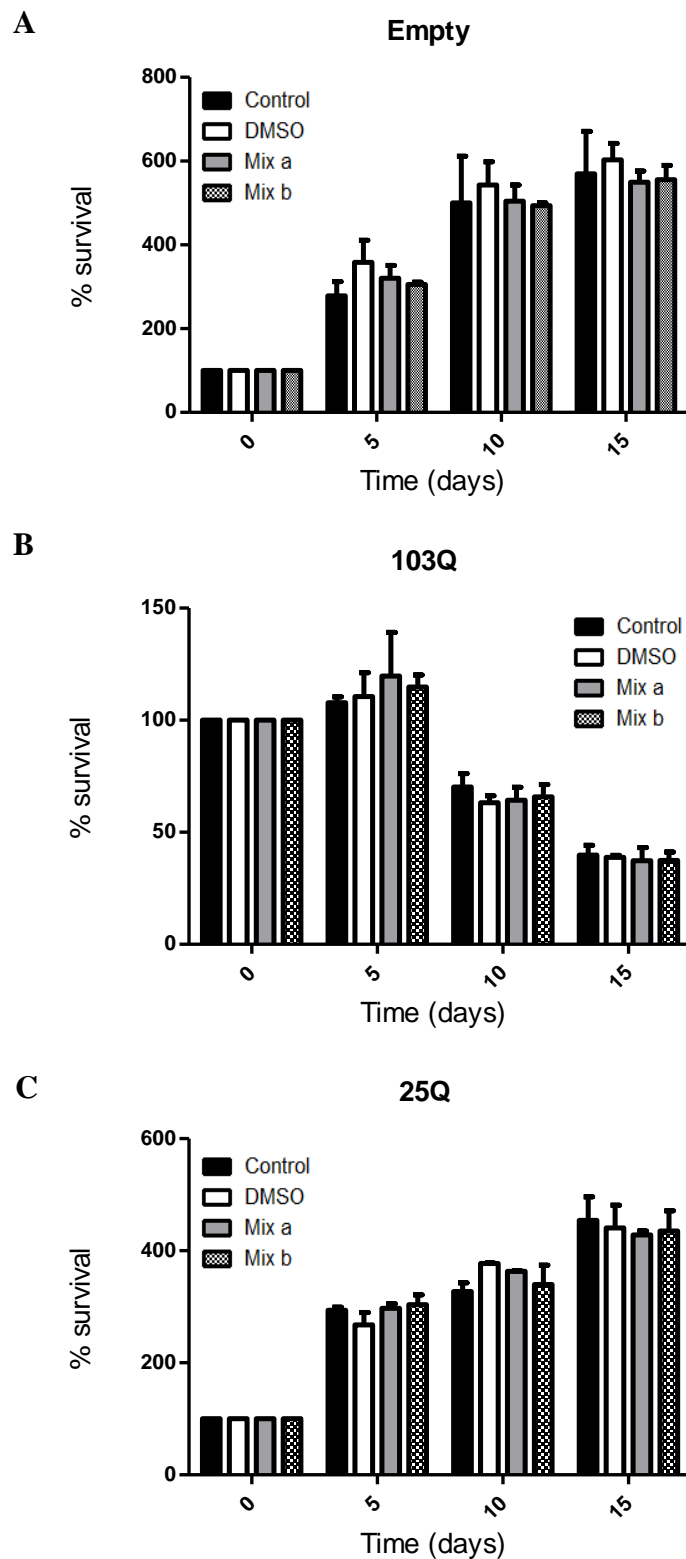
Hypericum perforatum phenolic compounds: Protective role in the toxicity induced by heterologous expression of α-synuclein and huntingtin in yeast cells

Figure 15 - Survival determined by colony-forming units of cell expressing point mutants 25Q (C) and 103Q (B) forms of htt or harboring the empty vector (A) after induction of expression by changing to 2% galactose medium in presence of compounds mix. Data represent mean ± S.E. of 3 independent experiments.

Once again, and as expected, the cells expressing the empty vector and the normal htt 25Q form (Fig. 15A and 15C), revealed a continuous growth and presented a non-toxic phenotype, since it was not observed cellular death during the time that the assay was performed. On other hand, the cells expressing the 103Q mutant form (Fig. 15B), presented a reduction in cell survival, of about 30% and 60% at time 10 days and 15 days, respectively, as expected, and the use of mixtures apparently, did not demonstrate any interference with htt induced toxicity, since it was not observed any difference between the survival of untreated and treated cells with the phenolic compounds.

Previous results seemed to demonstrate that the effects of the phenolic compounds were essentially on the α-synuclein-induced toxicity. Therefore, other studies about α-synuclein aggregation and ROS levels were performed.

5. Epifluorescent microscopy

The heterologous expression of α-synuclein can induce the foci formation (Griffioen et al., 2006). The protein aggregation results from a tendency of unfolded polypeptides to associate with each other (Kopito, 2000). In fact, in certain aggregation pathways, small foci may converge, by microtubule-based transport, forming a large inclusion of protein foci, called an aggresome (Wang et al., 2009). In order to understand whether phenolic compounds interfere with the aggregation of α-synuclein and since that in the models used in this work the α-synuclein protein is fused with GFP, it was performed the microscopic visualization of the cells in the presence of quercetin, kaempferol and biapigenin.

Examination of cells, using epifluorescent microscopy analysis in the studied conditions, allowed the visualization of GFP fused with the α-synuclein forms. The cells expressing the WT α-synuclein and the A53T mutant form (Fig. 16) revealed the presence of several foci, being more pronounced after 20h of expression induction, while in the case of cells expressing the α-synuclein A30P mutant form, there are no cytoplasmatic inclusions, due to its low membrane-binding capacity (Dauer & Przedborski, 2003).

The analyses of the images of epifluorescent microscopy led to the counts observed in results described below. The images (Fig. 16), made possible the distinction between small foci and foci with large size (aggresomes).
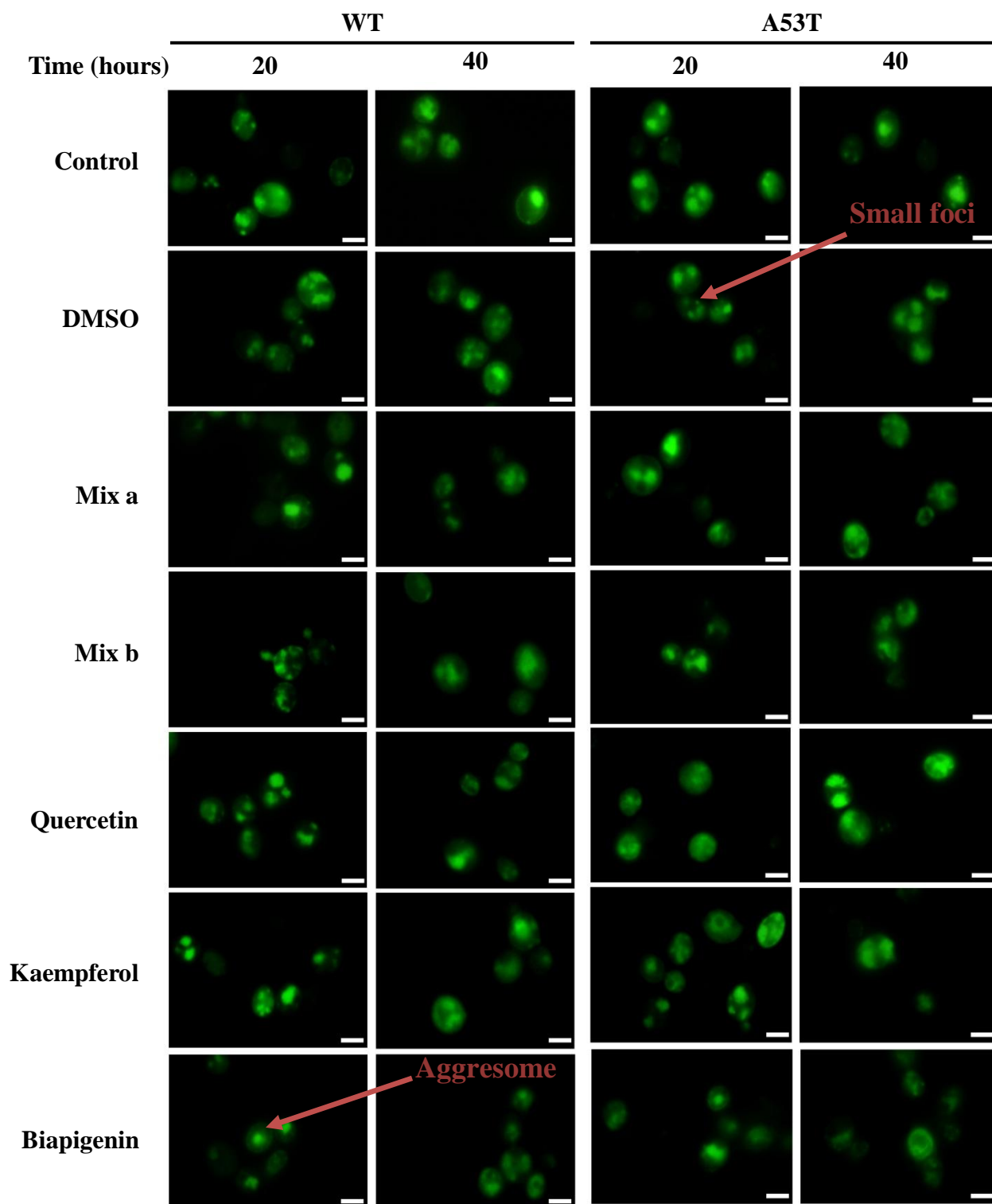
Figure 16 - Foci visualization of the cells expressing WT and point mutant A53T form of α-synuclein, after induction of expression by changing to 2% galactose medium in presence of phenolic compounds and compounds mix. 20 μm.

It was performed a count of 300 cells in triplicate, and it was estimated the amount of the cells containing foci, to study the possibility of the compounds interfering in the aggregation of α-synuclein. Thus if during the treatment of cells with the phenolic compounds occurs a decrease in the cell number with foci, it is possible to admit that the compounds have effects in the protein aggregation.
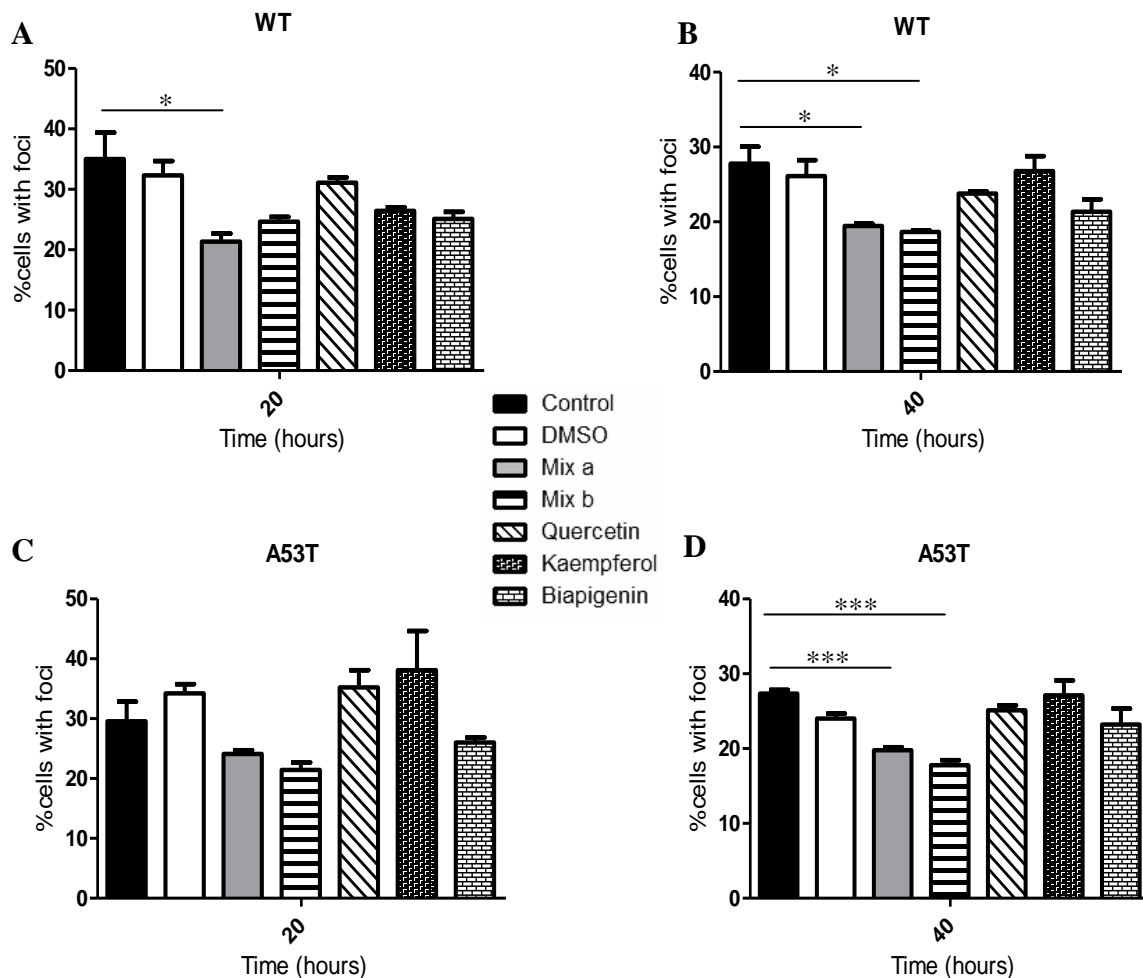

Figure 17 - % of the cells expressing WT (A and B) and point mutant A53T form of α-synuclein (C and D), after induction of expression by changing to 2% galactose medium in presence of phenolic compounds. Data represent mean ± S.E. of 3 independent experiments. Statistical significance (*$p<0.05$, **$p<0.01$ and ***$p<0.001$) were determined by *t*-test.

The data showed that the compounds mix have effects (Fig. 17) on protein aggregation, since the number of cells with foci is significantly reduced, during the time considered. For WT α-synuclein, mix a reduced the cell number with foci approximately 13.67 % (Fig. 17A) when compared with control, after 20h of protein induction. After 40h of α-synuclein induction, the mix a and mix b reduced the

percentage of cells with foci around 8.33% and 9.1% respectively. In the case of α-synuclein A53T mutant form, significant effects of mix a and b, after 40h of protein induction, were observed that reduced the cell number with foci approximately 7.56% and 9.56% respectively.

Once more, the results showed the synergistic effects between different compounds. These suggest that compounds mixtures may have a potential use for preventing foci formation. In fact, some natural compounds can depolymerize microtubules (Wilson & Jordan, 1995) and thus inhibit the aggregation. The aggregation inhibitors that act early on the aggregation pathway can prevent toxic species from forming, and allow cells to clear the mutant protein before it can do much damage (Bodner et al., 2006).

Next and using a similar approach, we determined the number of foci per cells. For this count were admitted only small foci.
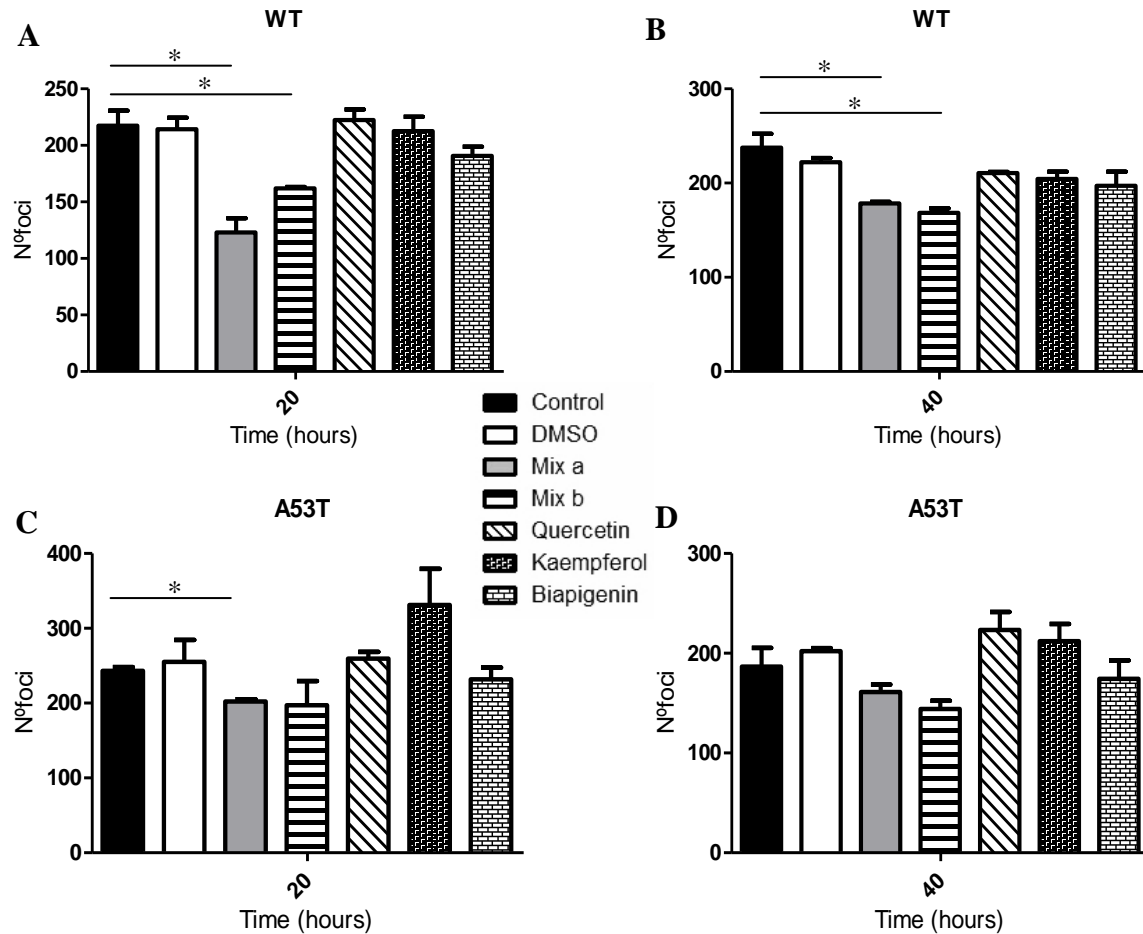
Hypericum perforatum phenolic compounds: Protective role in the toxicity induced by heterologous expression of α-synuclein and huntingtin in yeast cells

Figure 18 – Number of foci present in the cells expressing WT (A and B) and point mutant A53T form of α-synuclein (C and D), after induction of expression by changing to 2% galactose medium in presence of phenolic compounds. Data represent mean ± S.E. of 3 independent experiments. Statistical significance (*p <0.05, **p <0.01 and ***p <0.001) were determined by t-test.

The number of foci in the presence of the phenolic compounds and compounds mixture was analyzed. The data (Fig. 18) showed that mix a and mix b, have significant effects in reducing the number of foci mainly for WT α-synuclein (Fig. 18A and 18B) After 20h of induction of WT α-synuclein expression (Fig. 18A), the number of foci was reduced from 217 to 123 and 162 in the case of mix a and b, respectively. Also after 40h both mix a and b, reduced the foci number from 217 to 178 and 168, in that order (Fig. 18B). In the case of α-synuclein A53T mutant form expression, the foci number was reduced significantly only at time 20 and in the presence of the mix a, from 243 to 202 (Fig. 18C). Thus, these results accentuate once again the idea of synergistic effects between different compounds.

Next we determined the number of aggresomes per cells, since several investigations into the mechanisms of intracellular protein aggregation show that the aggresomes facilitate the degradation by autophagy (Wang et al., 2009) and we also observe the presence of higher foci. This suggests that aggresome formation represents a protective response of cells to an accumulation of foci under conditions in which chaperones and ubiquitin proteasomal system (UPS) machineries fail (Wang et al., 2009).

Investigations with mammalian cells showed that therapeutic intervention for PD and HD may involve the discover of compounds that decrease the size or number of inclusions and showed that the aggresome formation can be blocked by drugs that depolymerize microtubules (García-Mata et al., 1999). On the other hand, studies showed that there are compounds that enhance the formation of aggresome. These compounds can contribute for the treatment of neurodegenerative disorders (Olzmann et al., 2008).
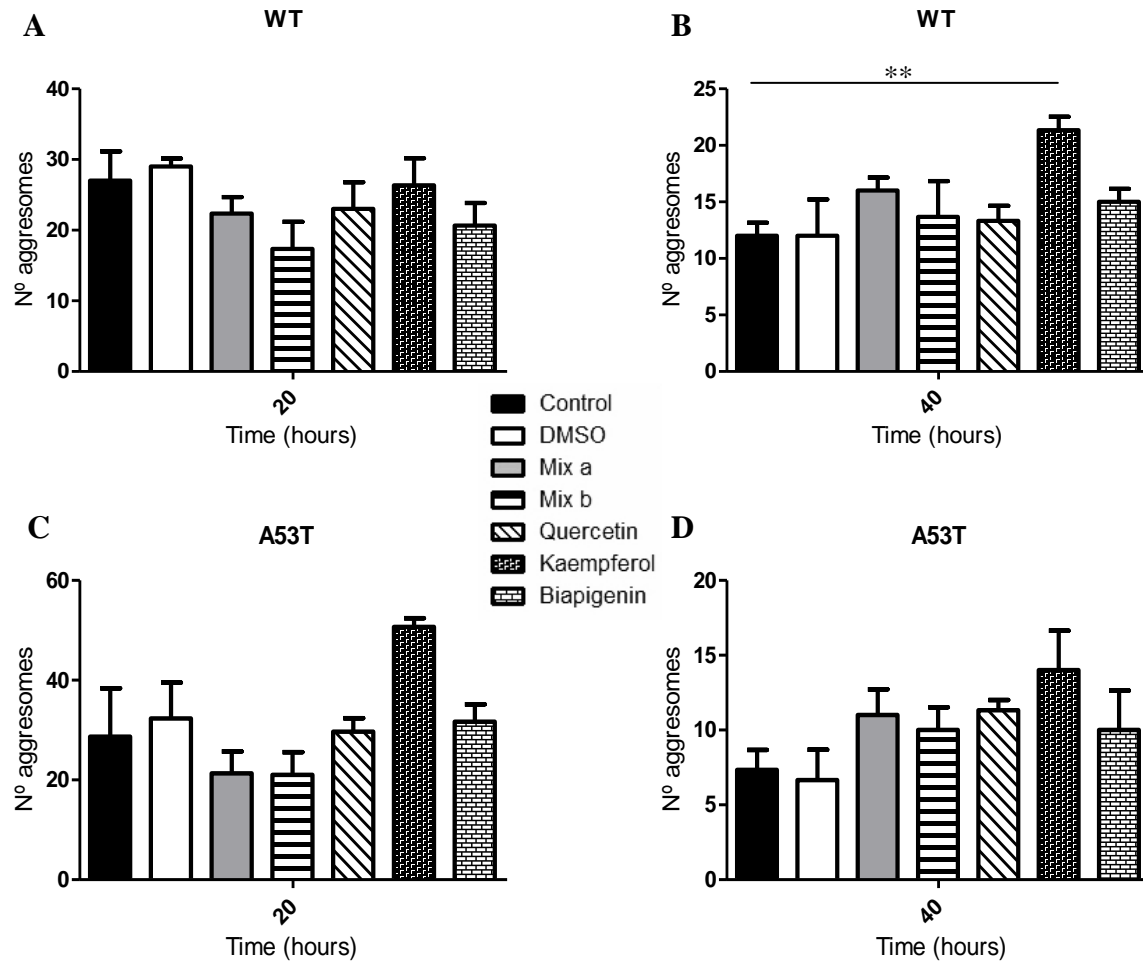
Figure 19 - Number of aggresomes present in the cells expressing WT (A and B) and point mutant A53T form of α-synuclein (C and D), after induction of expression by changing to 2% galactose medium in presence of phenolic compounds. Data represent mean ± S.E. of 3 independent experiments. Statistical significance ($*p<0.05$, $**p<0.01$ and $***p<0.001$) were determined by t-test.

Phenolic compounds and their mixtures do not seem to have much influence in the formation of aggresomes, (Fig. 19). However, in the presence of kaempferol there was an increase of the aggresomes number after 40h induction of heterologous expression of WT α-synuclein (Fig. 19B) from 12 to 21. Relatively to other situations (Fig. 19A, 19C and 19D) there were no significant effects, but apparently a tendency to increase the number of aggresomes in the presence of kaempferol was observed.

A study with neuronal cells treated with rotenone show that kaempferol at concentration of 30µM, is able to induce autophagy (Filomeni et al., 2010). Therefore, we suggest that kaempferol enhances

the formation of aggresomes, that way facilitating their degradation by autophagy.This aspect will be investigated in the future.

6. Measurements of cellular ROS levels

Oxidative stress (OS) plays an important role in the pathogenesis of several neurodegenerative disorders, including, PD and HD (Chong et al., 2005). Indeed, several postmortem studies from patients with PD, showed that OS is involved in the degeneration of dopaminergic neurons. Other studies about HD in animals, as well as humans, support the theory of association of OS with metabolic dysfunction (Gilgun-sherki et al., 2001).
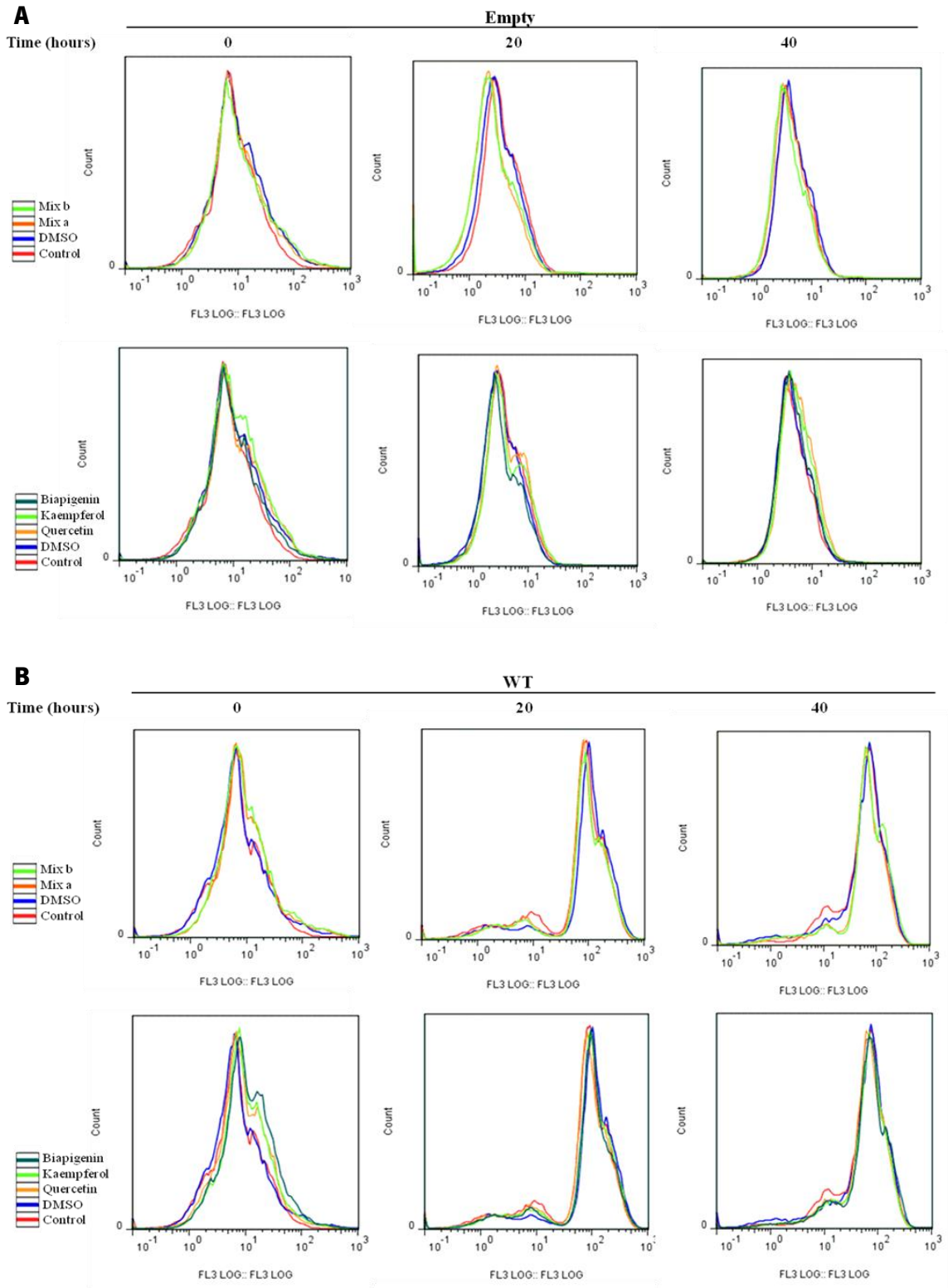
Reactive oxygen species (ROS) are normally produced in physiological conditions, at low levels. However, when the ROS levels exceed the capacity of the endogenous antioxidant system to eliminate surplus ROS, cellular damage occurs (Chong et al., 2005) Thus, compounds that have antioxidant properties and assist the endogenous antioxidant system may be potential targets for the therapy of neurodegenerative disorders (Floyd & Hensley, 2002)

Phenolic compounds have been linked with health benefits derived from fruit and vegetables consuming and one of the most frequently reported activities of phenolic compounds is the antioxidant activity (Balasundram et al., 2006).

Flow cytometry is a tool that has been developed for making fast measurements on individual particles or cells as they flow in a fluid flow and has been used in the evaluation of ROS level. Thus, to access the effects of quercetin, kaempferol and biapigenin in ROS levels, we analyzed the yeast cells in the presence of these compounds by flow cytometry with dihydroethidium (DHE) staining for superoxide.

The data (Fig. 20) showed that quercetin, kaempferol, biapigenin and their mixtures, did not demonstrate any interference with the intracellular ROS accumulation. These results were not expected, especially in the case of quercetin, since this compound is known to have elevated antioxidant properties (Cornish et al., 2002). Therefore this aspect will be investigated in the future to explore the known antioxidant properties of quercetin, as well as, try to understand if kaempferol and biapigenin can interfere with intracellular ROS.

Figure 20- Measurements of intracellular ROS levels in cells expressing WT (B) and point mutants A53T (D) and A30P (C) forms of α-synuclein or harboring the empty vector (A) after induction of expression by changing to 2% galactose medium in presence of phenolic compounds and their mixtures.

IV.   Conclusion

Conclusion and future perspectives

Neurodegenerative diseases are caused by the specific failure of neuronal activity, which habitually involves the definitive demise of neurons (Shastry, 2003). The brain is exposed throughout life to oxidative stress, and Parkinson's (PD) and Huntington's (HD) diseases are thought to involve free radical processes and oxidative damage (Gilgun-Sherki et al., 2001). Another factor associated with PD and HD is the presence of excessive protein misfolding. The accumulation of these aberrant proteins leads to a selective loss of neurons in an age-dependent mode (Miller-Fleming et al., 2008). Therefore, substances that can protect the brain to oxidative damage or reduce the accumulation of misfolded proteins or foci formation are potential targets to therapy of neurodegenerative disorders and yeast has been used as a potential target for identification of novel therapeutic compounds in PD and HD (Outeiro & Giorgini, 2006).

The natural compounds of several medicinal plants have been used in different studies , in special the phenolic compounds that are considered relatively non-toxic bioactive substances and have various biological activities, such as antioxidants, chelation of metals, scavengers of active oxygen species, blockers of nitration. In addition, anti-inflammatory, anti-microbial, cardioprotective and vasodilatory effects have been attributed to these secondary plant metabolites (Martins et al., 2011). Silva et al (2008) relate that quercetin, kaempferol and biapigenine have neuroprotective effects against synaptic dysfunction processes.

Our results raise the possibility of synergistic effects between quercetin, kaempferol and biapigenin in reducing the toxicity induced by heterologous expression of α-synuclein and shows that biapigenin is the compound with higher effects in the reduction of α-synuclein-induced toxicity. However, in the case of heterologous expression of huntingtin (htt), the phenolic compounds and their mixtures have no effects in the htt-induced toxicity. Nevertheless, further studies using the HD yeast model with the compounds previously used but ranging their concentrations are crucial to verify the possible existence of effects of quercetin, kaempferol and biapigenin in the toxicity induced by heterologous expression of htt.

With regard to foci formation, the mixtures of phenolic compounds reduce the number of cells with foci and reduce the number of foci presents in yeast cells. Kaempferol increased the number of aggresome, so we can admit that kaempferol may contribute to the defense response of cells to an accumulation of foci under conditions in which chaperones and ubiquitin proteasomal system (UPS) machineries fail (Wang et al., 2009) and thus facilitate autophagy. Therefore, it may be interesting to investigate the connection among misfolded proteins and autophagy.

It would also be interesting to repeat some of the studies conducted to validate and confirm the statistical significance, in particular, studies with the mixture more concentrate. Another situation that is attractive to note is the measurements of cellular reactive oxygen species (ROS) levels.

In the scope of this work, there may be performed several studies, such as: evaluation of protein levels, analysis of the role of autophagy in the presence of quercetin, kaempferol and biapigenin, employment other phenolic compounds and use different concentrations of the compounds which were used in this work.

We concluded that these phenolic compounds apparently have beneficial biological properties that consequently could have a potential use in preventing Parkinson's disease.

V.   References

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