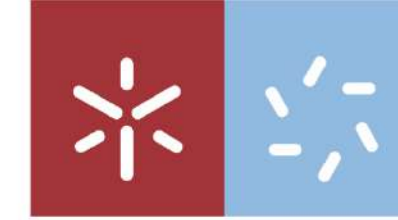




Study of the neuroprotective activity of medicinal plants in
Caenorhabditis elegans models of Neurodegenerative Diseases

Jorge Fernandes

UMinho | 2021

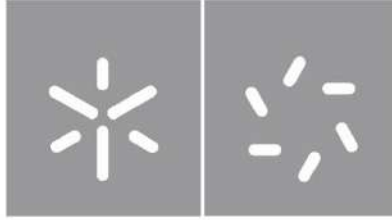


Universidade do Minho
Escola de Ciências

Jorge Humberto Esperança Fernandes

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fevereiro de 2021



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Master's Dissertation

Master in Applied Biochemistry

Specialization in Biomedicine

Work developed under supervision of

Professor Doctor Alberto Carlos Pires Dias

and

Professor Doctor Patrícia Espinheira de Sá Maciel

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Statement of Integrity

I hereby declare having conducted this academic work with integrity. I confirm that I have not used plagiarism or any form of undue use of information or falsification of results along the process leading to its elaboration.

I further declare that I have fully acknowledged the Code of Ethical Conduct of the University of Minho.

Resumo

Estudo da atividade neuroprotetora de plantas medicinais em modelos de *Caenorhabditis elegans* de Doenças Neurodegenerativas

A Demência Frontotemporal com Parkinson associada ao cromossoma 17 (FTDP-17) e a doença Machado-Joseph (DMJ) são doenças neurodegenerativas causadas por mutações nos genes que codificam para as proteínas tau e a ataxina-3, respectivamente, resultando na expressão de formas proteicas propensas a agregação e levando à perda neuronal. Actualmente não existe tratamento eficaz para nenhuma destas doenças e são necessárias novas estratégias terapêuticas.

Os produtos naturais têm sido explorados como fonte de terapias inovadoras. Neste estudo, descrevemos o estudo de um conjunto de 5 extractos naturais e 5 compostos purificados provenientes de plantas medicinais chinesas, nos modelos *C. elegans* da DMJ e de FTDP-17.

Um extracto etanólico (etanol a 30%) da planta asiática *Hemerocallis citrina* (HCE) foi identificado como tendo a capacidade de melhorar significativamente os défices locomotores de ambos os modelos. Os resultados sugerem também que o referido efeito é totalmente depende da sinalização serotoninérgica, nomeadamente da actividade dos receptores SER-1, SER-4, SER-5 e SER-7 e do transportador de serotonina (MOD-5). O tratamento com HCE também induziu diferentes respostas adaptativas, em estirpes repórter, causando uma activação moderada da expressão dos genes *gcs-1* e *hsp-6*, envolvidos respectivamente na síntese de glutatona (um potente antioxidante celular) e na resposta da mitocôndria ao stress proteico. De forma mais evidente, o tratamento dos animais com HCE induziu a translocação nuclear do factor de transcrição HLH-30, ortólogo do factor de transcrição TFEB em mamíferos, que regula a expressão de vários genes relacionados com a autofagia, sugerindo um papel deste extracto na activação deste mecanismo proteolítico, cujos efeitos neuroprotectores e anti-envelhecimento são já conhecidos. No entanto, mais estudos serão necessários para identificar quais os mecanismos chave para a acção terapêutica deste extracto. .

Estes resultados reforçam a importância de compostos bioactivos derivados de plantas como novas estratégias terapêuticas a serem exploradas para doenças neurodegenerativas que permanecem sem cura.

Palavras Chave: Plantas Medicinais, Doenças Neurodegenerativas, *Caenorhabditis elegans*

Abstract

Study of the neuroprotective activity of medicinal plants in *Caenorhabditis elegans* models of Neurodegenerative Diseases

Frontotemporal Dementia with Parkinsonism associated to chromosome 17 (FTDP-17) and Machado-Joseph Disease (MJD) are two genetic neurodegenerative diseases caused by mutations in genes encoding for tau and ataxin-3, respectively, resulting in the expression of aggregation-prone forms of these proteins and leading to neuronal loss. Currently there is no effective treatment for neither of these diseases and novel therapeutic strategies are needed.

Natural products have been explored as a source for innovative therapies. In this study, we describe the study of 5 natural extracts and 5 purified compounds from Chinese medicinal plants, in the *C. elegans* models of MJD and FTDP-17.

We demonstrate the neuroprotective potential of an ethanolic (30% ethanol) extract from the Asian plant *Hemerocallis citrina* (HCE), which was able to improve significantly the locomotor deficits that characterize both disease models. Our results also revealed a complete dependency of the beneficial effects of HCE on specific components of the serotonergic signalling, namely the activity of the serotonin receptors SER-1, SER-4, SER-5 and SER-7 and transporter MOD-5. In addition, HCE treatment also activated distinct adaptative responses, in reporter strains, causing a moderate induction of the *gcs-1* and *hsp-6* genes, associated with the synthesis of glutathione (a potent cellular antioxidant) and with the mitochondrial unfolded protein stress response, respectively. The same treatment induced an evident nuclear translocation of the transcription factor HLH-30, an orthologue of the TFEB factor in mammals, a regulator of several autophagy genes, suggesting an impact of this extract in the activation of this proteolytic mechanism, known to have neuroprotective and anti-aging effects. However, additional studies are still needed to identify which mechanisms are key for the therapeutic action of HCE.

These results strengthen the relevance of bioactive compounds derived from plants as new therapeutic options to be explored for neurodegenerative disorders that remain without cure.

Key Words: Medicinal Plants, Neurodegenerative Diseases, *Caenorhabditis elegans*

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Abbreviation list

AChE – Acetylcholinesterase
AD – Alzheimer’s Disease
APP – Amyloid Precursor Protein
ATXN3 – Ataxin-3
A β – Amyloid Beta
CREB – cAMP response element binding protein
DMSO - Dimethyl sulfoxide
dsRNA – Double stranded RNA
FOXO – Forkhead box protein O transcription factor(s)
FTD – Frontotemporal Dementia
FTDP-17 - Frontotemporal Dementia with Parkinsonism Associated to Chromosome 17
GFP – Green Fluorescent Protein
HCE – *Hemerocallis citrina* 30% ethanol extract
HD – Huntington’s Disease
HSF-1 – Heat-Shock Factor 1
HSP – Heat Shock Proteins
IPTG - Isopropyl β - d-1-thiogalactopyranoside
LB – Luria Broth
MAPT – Microtubule Associated Protein tau
MJD – Machado-Joseph disease
MTB – Microtubule Binding Domain
MTBR – Microtubule Binding Region
ND – Neurodegenerative disease
NGM – Nematode Growth Medium
PD – Parkinson’s disease
PN – Proteostasis Network
PolyQ – Polyglutamine
PPD – Protopanaxadiol
PPT – Protopanaxatriol
RNAi – Interference RNA
SCA – Spinocerebellar Ataxia
UIM – Ubiquitin Interaction Motifs
UPR – Unfolded Protein Response
UPR^{ER} - Endoplasmatic Reticulum Unfolded Protein Response
UPR^{mt} – Mitochondrial Unfolded Protein Response
UPS – Ubiquitin Proteasome System
WMT – WmicroTracker
WT – Wild-type
YFP – Yellow Fluorescent Protein

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Figure 30 - Activation of *hsp-6* transcription after treatment with HCE. (A-F) Representative images of a strain expressing GFP under the regulation of *hsp-6* promoter. Brightfield and GFP fluorescence images were acquired in animals **(A and B)** treated with vehicle (DMSO 1%), **(C and D)** grown in vehicle and treated with Antimycin A 50 μ M for 24 hours before imaging, **(E and F)** treated with HCE 1 mg/mL over 4 days. **G** Representation of GFP fluorescence intensity divided by the total area of each animal and normalized to the vehicle (DMSO) control. HCE treatment induced a mild GFP expression. Antimycin A, an oxidative phosphorylation inhibitor, was used as positive control, inducing a 4-fold change in GFP signal intensity. Graphic represented with Mean $\pm$ SEM. One-way ANOVA; * $p < 0.05$, ** $p < 0.01$, *** $P < 0.0001$ 68

Figure 31 – Translocation of HLH-30 to the nucleus and activation of autophagy by HCE. (A-F)

Representative microphotographs of a strain expressing HLH-30 fused to GFP. Brightfield and GFP fluorescence images were acquired in animals **(A and B)** treated with vehicle (DMSO 1%) for 4 days, **(C and D)** treated with vehicle and restricted of food for 30 minutes prior to imaging, inducing autophagy, **(E and F)** treated with HCE 1 mg/mL. Arrows indicate the localized pattern observed upon HLH-30 nuclear translocation. **G** Quantification of the number of animals displaying diffuse or localized GFP fluorescence. HCE treatment resulted in a majority of worms displaying localized GFP fluorescence, indicating HLH-30 nuclear translocation..... 71

Figure 32 – Neuronal silencing of autophagy did not impact HCE treatment effect. A. Representative schematic of the molecular pathway of autophagy. Underline in red is represented the genes silenced by RNAi. Adapted from (Wong et al., 2020) **B** Locomotor activity of AT3q130;LC108 animals after RNAi-mediated silencing of *hlh-30*, *lgg-1* and *atg-9* genes, involved in the autophagic flux. AT3q130 animals, treated with vehicle and HCE were used as controls. Treatment remained effective in all conditions assessed, reducing the number of worms scored as locomotion defective. **C** Locomotor activity of CK10;LC108 animals after RNAi silencing of the same genes. CK10 animals treated with vehicle and HCE were used as controls. Treatment remained effective after silencing of all genes. In both cases, RNAi-mediated silencing control was done through silencing of *unc-70*, that resulted in increased motor deficits. Graphics represented with Mean $\pm$ SEM. One-way ANOVA; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.0001$, (n = 3 biological replicates, total of 150 animals per condition). 72

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1. Introduction

1.1. Neurodegenerative diseases

In the last 100 years, due to advances in healthcare and quality of life, we have been witnessing an increase of life expectancy in developed countries. While globally positive, this demographic increase of the elderly population also represents a challenge for healthcare systems and society, since with ageing there is an increased risk of chronic and severe disease. Especially, ageing is one of the major risk factors for neurodegenerative diseases (ND) (Hou et al., 2019). NDs are a group of devastating diseases characterized by a progressive loss of nervous system function and integrity. Degradation of specific neuronal circuitry by synaptic dysfunction and/or neuronal death, results in specific behavioural symptoms, originating diverse clinical manifestations. Different motor, cognitive and mood symptomatology might be observed for different NDs (Hou et al., 2019; Chen et al., 2015). Despite the diverse clinical presentation, these diseases might share some common features such as the presence of aberrant post-translational modifications in specific proteins, protein misfolding and overall loss of protein homeostasis (also referred to as proteostasis), resulting in protein aggregation and deposition and eventually leading to mitochondrial dysfunction, axonal transport impairments, , among others (Chen et al., 2015), culminating in neuronal dysfunction, neuroinflammation (reflected in astrocytic reactivity and microglial activation) and ultimately neuronal death.

In the brain of patients with neurodegenerative diseases it is possible to observe abnormal aggregates of cytosolic or nuclear proteins, namely amyloid β peptide ($A\beta$) plaques in Alzheimer's Disease (AD); fibrils of Tau protein, in AD and in tauopathies such as Frontotemporal Dementia (diseases characterized by toxicity induced by tau protein); α -synuclein in Parkinson's Disease (PD) or proteins with abnormally expanded polyglutamine tracts in polyglutamine diseases [huntingtin in Huntington's Disease (HD) or ataxin-3 in Machado-Joseph Disease (MJD)] (reviewd in Da Silva et al., 2019; Taylor et al., 2002). The fact that the proteins involved in the diseases do not share neither functional nor structural homology led to the hypothesis that protein misfolding and accumulation is the main mechanism behind the pathologies. (Jalles and Maciel, 2015).

1.2. Protein misfolding in neurodegenerative diseases

Since protein misfolding is a key pathogenic mechanism of several NDs, it is important to understand protein folding and quality control mechanisms responsible for maintaining a balanced proteome.

After translation is completed, polypeptides must fold into the correct three-dimensional conformation in order to become completely functional. A polypeptide chain can adopt a very large number of conformations, making folding a highly complex phenomenon. Hydrophobic forces seem to be particularly important in driving the conformational changes needed to reach a final tertiary structure, especially in soluble proteins (Hartl et al., 2011). Currently, the most accepted model postulates that hydrophobic forces drive a “chain collapse”, reducing the contact of polar amino-acid residues with the solvent, burying them in the interior of the protein. This leads the protein to adopt progressively constrained conformations, named intermediate states. Electrostatic forces drive the intermediate states’ exposed hydrophobic residues to interact with their counterparts in other polypeptide chains, reducing the contacts with the solvent, making these intermediate states prone to aggregation (Hartl et al., 2011). A similar mechanism is observed with misfolded proteins. Moreover, ordered and thermodynamically stable structures, such as fibrillar aggregates named amyloid fibrils, are also observed. These ordered aggregates consist of β -strands organized along the fibril axis and are highly stable but can lead to protein loss of function (if fibrils are not the functional form of that protein) and may generate important cellular stress and dysfunction (Hartl et al., 2011).

Hence, the folding process needs to be tightly controlled i. e. monitored and assisted. Moreover, misfolded proteins and aggregates need to be refolded, disaggregated and, if irreversibly misfolded, targeted for degradation before damaging the cells. The machinery responsible for maintaining a dynamic but balanced proteome is named proteostasis network.

This machinery helps to maintain a functional proteome by: controlling the translational machinery and consequently the rate of protein synthesis; promoting the action of molecular chaperones and co-chaperones, such as Heat Shock Proteins (HSP) 70, HSP 40, HSP 90 and small Heat Shock Proteins, which are a group of proteins that identify non-native conformations and act as proteins folding modulators, to assist in folding and assembly of other proteins; controlling the membrane trafficking patterns, influencing the stability of different proteins due to compartmentalization in different cell environments; and by mediating protein turnover, controlling

the flow of proteins towards the Ubiquitin Proteasome System (UPS) and the autophagy machinery. The UPS is responsible mainly for degrading soluble proteins, tagged with polyubiquitin, while the autophagy system is responsible for degrading large aggregates and for the turnover of deficient/damaged organelles. These referred mechanisms are known as the “core pathways of the Proteostasis Network (PN)” (Labbadia and Morimoto, 2015; Powers et al., 2009; Hartl et al., 2011). However, assisting the “core PN” there are other complementary pathways, namely stress responsive pathways:

- The Heat Shock Response is a concerted mechanism in which, under stress conditions, the transcription factors Heat-Shock Factor 1 (HSF-1) and the Forkhead box protein O (FOXO) transcription factors can trigger the expression of chaperone proteins such as Heat Shock Proteins. Molecular chaperones bind to other proteins, inhibiting the interaction between the hydrophobic amino-acid residues, avoiding aggregation and assisting in the folding process (Vabulas et al., 2010);
- The Unfolded Protein Response (UPR) of the Endoplasmic Reticulum (UPR^{ER}) is prompted in response to the accumulation of damaged proteins of the secretory pathway in the ER, which trigger the inhibition of protein synthesis, to reduce the protein load, and also recruit chaperones to the ER lumen, to increase the refolding ability (Buchberger et al., 2010). Conformational stress in the mitochondria triggers a similar response in this organelle (UPR^m), adapting the protein-folding capacity in the matrix and protecting the mitochondrial function (Haynes and Ron, 2010);

Despite the existence of such mechanisms for protein control, when this machinery is damaged or overloaded, misfolded proteins tend to accumulate and cluster in cells, affecting their normal function and viability. As stated above, misfolded proteins tend to aggregate and become toxic. Several factors might induce proteostasis disruption, namely ageing, genetic mutations, oxidative stress, among others (McClellan et al., 2005). Moreover, when protein aggregation begins, it tends to create further impairments in protein quality control mechanisms, creating a “snowball effect”. Protein misfolding can result in a toxic gain-of-function of proteins due to their aberrant conformation, or it can also lead to a loss of native function of the proteins. These different

mechanisms can create severe constraints for normal cellular function and can ultimately result in cell death. Hence, protein misfolding, aggregation and loss of cell quality control mechanisms are important hallmarks in the context of several diseases, including the majority of NDs (McClellan et al., 2005).

In this study we focused on two genetic neurodegenerative diseases: Frontotemporal Dementia with Parkinsonism associated to chromosome 17 and Machado-Joseph Disease. These two genetically determined NDs, that are part of larger groups of diseases, are caused by mutations that result in the expression of aggregation-prone protein forms and ultimately lead to protein homeostasis disruption. Therefore, we considered them to be interesting models to explore possible neuroprotective effects of different drugs and test the hypothesis of translation to other NDs.

1.3. Frontotemporal dementia

Frontotemporal dementia (FTD) is a syndrome characterized by progressive changes in behaviour, personality and language, while memory is relatively preserved. It accounts for 5 to 15% of all the dementia cases. A large number of the patients reveal familial precedents of a similar disease, which indicate a strong genetic influence (Neumann et al., 2009). It is an heterogenous group of diseases and can be classified in different sub-classes, depending on several criteria: **clinically**, FTD can be divided in behavioural variant FTD, progressive non-fluent aphasia and semantic dementia (Rademakers et al., 2012). In all types of FTD described, it is possible to observe shrinkage of the frontotemporal cortex, with loss of neurons and gliosis of the most superficial layers (van Swieten and Spillantini, 2007). **Histologically**, FTD can be divided according to the presence of protein inclusions in the brain: tau-positive inclusions; ubiquitin-positive but tau-negative inclusions; and those lacking a distinctive histopathology (van Swieten and Spillantini, 2007). Approximately 30% of the familial FTD cases present tau aggregation and tau pathology, related with mutations in the MAPT gene (van Swieten and Spillantini, 2007).

1.3.1. Frontotemporal dementia and Parkinsonism linked to chromosome 17 (FTDP-17)

Frontotemporal Dementia with Parkinsonism associated to chromosome 17 (FTDP-17) is one of the familial forms of FTD with autosomal dominant inheritance, characterized by frontotemporal atrophy, neuronal loss, degeneration of *substantia nigra* and basal ganglia and

abundant tau inclusions (van Swieten and Spillantini, 2007). Tau is a microtubule associated protein important for microtubule assembly and stabilization (van Swieten and Spillantini, 2007). It is an intrinsically disordered protein and can be divided in four domains: N-terminal, proline-rich domain, microtubule binding domain (MTB) and C-terminal. The MTB binds to the carboxy-terminal of tubulin. In healthy conditions, tau is mainly located in the axonal microtubules of neurons, while in pathological conditions it is hyperphosphorylated and is mainly located in the somatodendritic compartment (Bodea et al., 2016). In AD, tau is reported to aggregate in paired-helical filaments and subsequently forming neurofibrillary tangles that deposit intracellularly. This tau aggregation is reported to occur downstream of A β accumulation in amyloid plaques. In FTD however, it is possible to observe tau neurofibrillary tangles and tau pathology without A β accumulation (van Swieten and Spillantini, 2007).

The microtubule associated protein tau (MAPT) gene, that encodes for the tau protein, is located in chromosome 17 and comprises 16 exons (Strang et al., 2019). Alternative splicing of its mRNA might lead to different tau isoforms. In the human brain there are 6 different isoforms, created by alternative splicing of exons 2, 3 and 10, as represented in **figure 1** (Strang et al., 2019). The exons 1, 4, 5, 7, 9, 11 and 12 are constitutively expressed in all tau isoforms, while exons 4A, 6 and 8 were not identified in any human brain mRNA (Bodea et al., 2016). Exons 2 and 3 are involved in the formation of tau isoforms with 0, 1 or 2 N-terminal repeats and exon 10 is involved in forming tau isoforms with 3 or 4 MBT repeats (Strang et al., 2019). In normal brains, the balance between 3 or 4 MBT repeats tau isoforms is roughly equal. More than 50 different mutations, both on exons and introns of MAPT have been associated with FTDP-17, with different outcomes, such as enhanced tau aggregation, altered tau interaction with microtubules and alternative splicing of the MAPT gene (Mutreja et al., 2019). In particular, mutations near the exon 10 affect the ratio of 4 and 3 MTB repeat forms (Mutreja et al., 2019), while mutations in the Microtubule Binding Region (MTBR) might affect tau affinity for Microtubule and trafficking. Mutant forms of tau may also present enhanced propensity to form filaments (van Swieten and Spillantini, 2007).

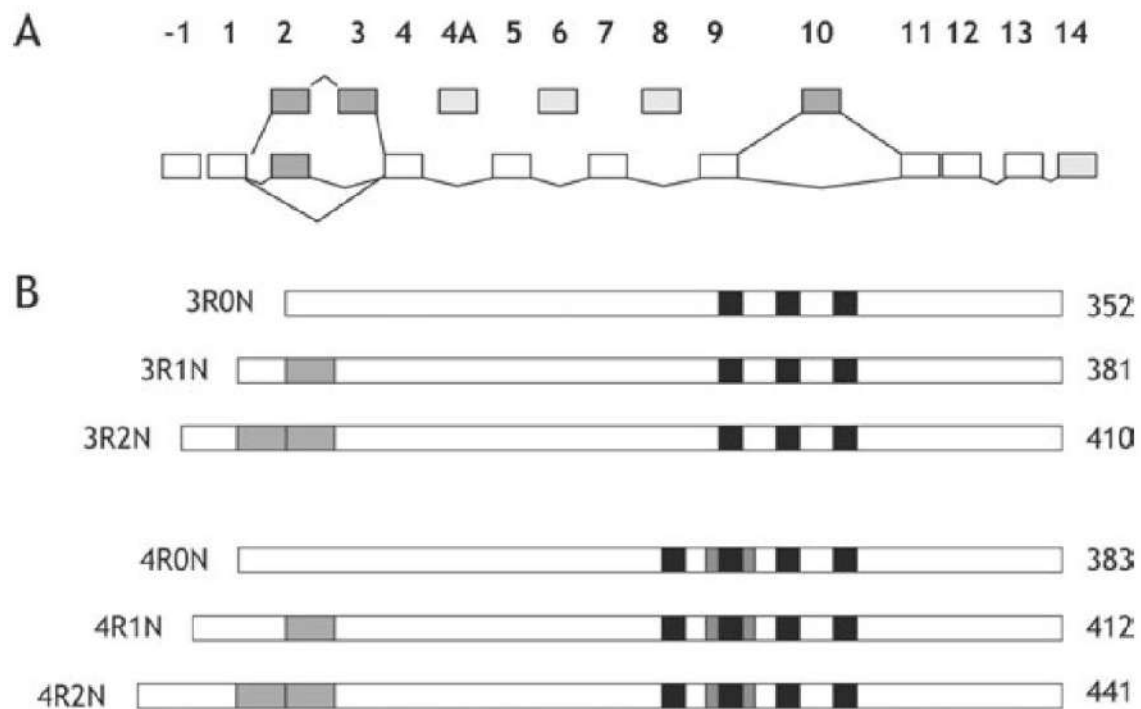


Figure 1 - Schematic representation of tau gene and protein. **A** tau gene with the respective introns and exons. **B** Alternative mRNA splicing of exons 2, 3 and 10, represented in grey boxes, originates 6 different tau protein isoforms. The microtubule binding repeats are represented by the black boxes. The different tau isoforms have 0, 1 or 2 N-terminal repeats and 3 or 4 microtubule binding repeats. (Adapted from van Swieten and Spillantini, 2007)

Figure 2 - Schematic representation of Tau toxicity process. Hyperphosphorylation of tau drives microtubule destabilization and disassembly. Deficits in cell transport processes result in synapses depleted of mitochondria and cause mitochondrial, ER and oxidative stress. Hyperphosphorylated tau leads to overactivation of Fyn and consequent constant NMDA receptor activation, provoking excitotoxicity by increasing Ca^{2+} influx. As a result, tau is capable of inducing synapse dysfunction and neuronal loss.

Figure 3 - Schematic representation of tau gene and protein. **A** tau gene with the respective introns and exons. **B** Alternative mRNA splicing of exons 2, 3 and 10, represented in grey boxes, originates 6 different tau protein isoforms. The microtubule binding repeats are represented by the black boxes. The different tau isoforms have 0, 1 or 2 N-terminal repeats and 3 or 4 microtubule binding repeats. (Adapted from van Swieten and Spillantini, 2007)

Mutant tau can promote neurotoxic effects (**figure 2**). It directs the protein Fyn, a kinase,

to the post-synaptic space, where it is able to phosphorylate the NMDA receptors and induce the formation of an excitotoxic complex (Bodea et al., 2016). Studies suggested that activation of Fyn and sequential inactivation by a phosphatase are necessary for regulation of NMDA receptors. However, pseudophosphorylated tau was shown to interact more tightly with Fyn than wild-type tau and that 3MTBR-tau appears to have increased affinity when compared with the 4MTBR-tau isoform. Moreover, the P301L mutation, commonly found in FTDP-17, was shown to increase the affinity of 4MTBR-tau to Fyn, therefore increasing Fyn activity and consequently synaptic damage and further neurodegeneration (Bodea et al., 2016). The overactivation of NMDA receptors results in neuronal loss due to increased Ca^{2+} influx and NO activation (Palop and Mucke, 2010). Microtubule dysfunction, caused by hyperphosphorylated tau, leads to improper transport process that can result in synapses depleted of mitochondria, causing synaptic loss (Kopeikina et al., 2012). Moreover, impairments in mitochondrial transportation might result in mitochondrial dysfunction including ROS formation and activation of cell death pathways (Kopeikina et al., 2012). Tau accumulation also causes dephosphorylation of cAMP response element binding protein (CREB) in the nucleus. CREB functions are vital to synapse and cognitive function, thus tau interferes with the normal function of synapses. (Ying Yang 2017). Tau accumulation is also capable of inducing ER stress, with increased phosphorylation of several enzymes, leading to cell death (Yang and Wang, 2018; Abisambra et al., 2013).

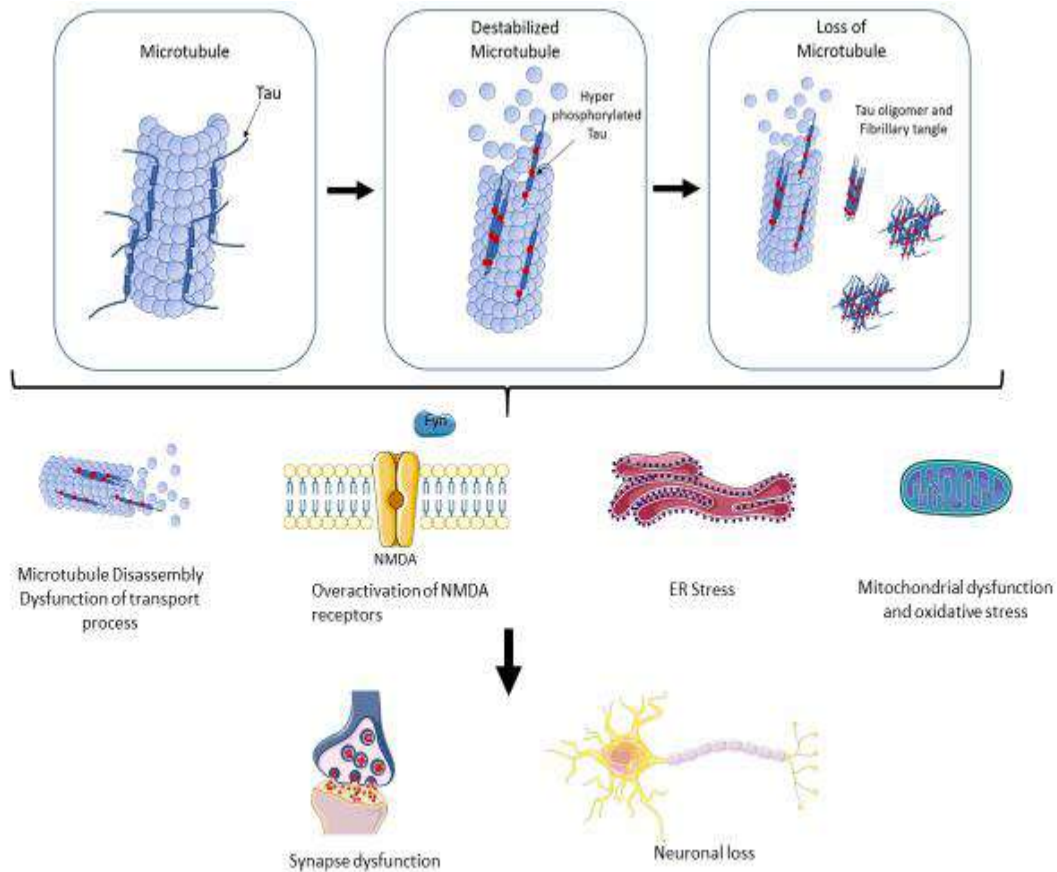


Figure 4 - Schematic representation of Tau toxicity process.

Hyperphosphorylation of tau drives microtubule destabilization and disassembly. Deficits in cell transport processes result in synapses depleted of mitochondria and cause mitochondrial, ER and oxidative stress. Hyperphosphorylated tau leads to overactivation of Fyn and consequent constant NMDA receptor activation, provoking excitotoxicity by increasing Ca^{2+} influx. As a result, tau is capable of inducing synapse dysfunction and neuronal loss.

1.4. Polyglutamine (PolyQ) Diseases

Polyglutamine expansion diseases are a group of disorders that result from the abnormal expansion of a CAG repeat that is translated as a longer polyglutamine (polyQ) tract, in different

proteins. The expansion of the polyQ tract is associated with an increased propensity of these proteins to aggregate, involving both gain and loss of function effects (reviewd in Zoghbi and Orr, 2000; Esteves et al., 2017).

This group of diseases includes Huntington's Disease, Spinobulbar Muscular Atrophy, Dentatorubral-pallidoluysian Atrophy and Spinocerebellar Ataxias (SCA), which include Spinocerebellar ataxias 1, 2, 3, 6, 7 and 17 (Cohen-Carmon and Meshorer, 2012). Notably, although expansion of the CAG repeat is neurotoxic, each disease has a specific polyQ protein associated and different patterns of neurodegeneration, which might indicate that the remaining aminoacidic sequence of each protein is also important in the context of the disease (Zoghbi and Orr, 2000).

1.4.1. Machado-Joseph Disease

Spinocerebellar ataxia 3 (SCA3), also known as Machado-Joseph Disease (MJD), is the most common dominant spinocerebellar ataxia worldwide and it is caused by an expansion of the CAG trinucleotide in the *atxn-3* gene (**figure 3**), mapped to chromosome 14q32.1. In normal individuals, the CAG repeat size of ataxin-3 varies between 12 and 44 repeats, while in MJD patients it ranges from 61 to 87 (Maciel et al., 2001). This alteration is translated as an abnormal tract of polyglutamines (polyQ) in the corresponding protein ATXN-3. This protein, expressed throughout the body, has a Josephin Domain with a papain-like fold and an unstructured C-terminal, containing the polyQ tract and two to three ubiquitin interaction motifs (UIM) (**figure 3**) (Da Silva et al., 2019; Nicastro et al., 2010, 2005). The Josephin domain, together with the UIMs has been shown to present deubiquitylase activity, trimming polyubiquitin chains (Kieling et al., 2007). This means that ataxin-3 might be involved in the rescue of proteins from degradation by the Ubiquitin-Proteasome System (UPS) or in some cases it might be responsible for removing inhibitory poly-ubiquitin chains and therefore stimulating the breakdown of proteins. The deubiquitination of proteins is vital for cellular homeostasis, regulating protein breakdown by the UPS and recycling ubiquitin. Moreover, poly-ubiquitination of lysine 63 or linear poly-ubiquitin chains have non-proteasome-related functions, being involved in phenomena such as activation of kinases or directing protein for degradation through autophagy. While the Josephin domain of ataxin-3 is responsible for the isopeptidase activity of the protein, the UIMs are responsible for mediating the binding of ataxin-3 to poly-ubiquitin chains of four or more ubiquitin with linkages to lysin 48 and

lysine 63. The UIMs also allow the binding of ataxin-3 to mixed linkage ubiquitin chains, with preference for lysine 63-tagged ubiquitin (Kieling et al., 2007; Nicastro et al., 2010).

The most relevant symptoms of MJD are related to the progressive ataxia, meaning dysfunction of motor coordination, and the spasticity and hyperreflexia that result from impairments on the pyramidal tract. This symptomatology is a result of the MJD affecting mainly the cerebellum, brainstem and some cranial nerves. The spinal cord is also affected. In contrast to FTD and other dementias, and even to HD, cognitive impairment is not relevant in the context of this disease (Fahl et al., 2015; Saute and Jardim, 2015). After onset, the median survival time has been estimated to be around 20 years (Sequeiros and Coutinho, 1993).

When mutated, ATXN-3 becomes highly aggregation prone, resulting in impaired proteostasis (reviewed in Da Silva et al., 2019; Teixeira-Castro et al., 2015). It is believed that the basis of the toxicity is related to an aberrant association and deposition of critical components of the proteostasis network with the ATXN-3 aggregates, namely molecular chaperones, proteasome subunits and other metastable proteins, such as the transcription coactivator cAMP response element-binding protein (CREB)-binding protein (CBP) and the ATPase valosin-containing protein (VCP/p97) (**figure 3**) (Cohen-Carmon and Meshorer, 2012; Da Silva et al., 2019; Perez et al., 1998; Yang et al., 2014a). This sequestration of critical components of the proteostasis network, as well as loss of natural de-ubiquitylating activity leads to impairment of folding and clearance that may trigger neuronal dysfunction (Cohen-Carmon and Meshorer, 2012; Da Silva et al., 2019). Ataxin-3 aggregation might also affect DNA repair, transcription and translation mechanisms. It is known to interact with proteins involved in DNA repair mechanism such as homologous of Rad32 (HHR23A and B), RNA polymerase II (RNAP II) or classical nonhomologous end-joining proteins (C-NHEJ), such as the polynucleotide kinase 3'-phosphatase (PNKP) (Da Silva et al., 2019; Chatterjee et al., 2015; Chakraborty et al., 2020). The nucleus also has specific protein quality control mechanisms, especially design to detect and repair damaged protein after nuclear import (Enam et al., 2018). Deposition of ATXN3 in the nucleus might lead to impairment in this organelle's quality control and further protein deposition, abnormal interactions with transcription factors/cofactors and histone deacetylases, among others, affecting the normal function of the cell nucleus (Perez et al., 1998; reviewed in Da Silva et al., 2019). Furthermore, ATXN3 has also been found in mitochondrial fractions of cells and to interact with other known mitochondrial proteins (Kristensen et al., 2018). Ataxin-3 protein dysfunction might therefore explain increased the oxidative stress found in patients by provoking abnormalities in mitochondrial function (Da Silva

et al., 2019). Ataxin-3 was also found to have a role in the activation of transcription of the manganese superoxide dismutase (SOD2) gene by interacting with forkhead box O transcription factor FOXO4. However, mutant ataxin-3 has impaired capacity to interact with FOXO4 and MJD patients present downregulation of SOD-2 in pontine brain tissue (Araujo et al., 2011).

As stated before, aggregates of ataxin-3 can contain other proteins such as VCP/p97. Thus, ER degradation pathways, autophagy and homeostasis can be affected (Hill et al., 2021; reviewed in Da Silva et al., 2019).

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1.5. *Caenorhabditis elegans* as a model organism

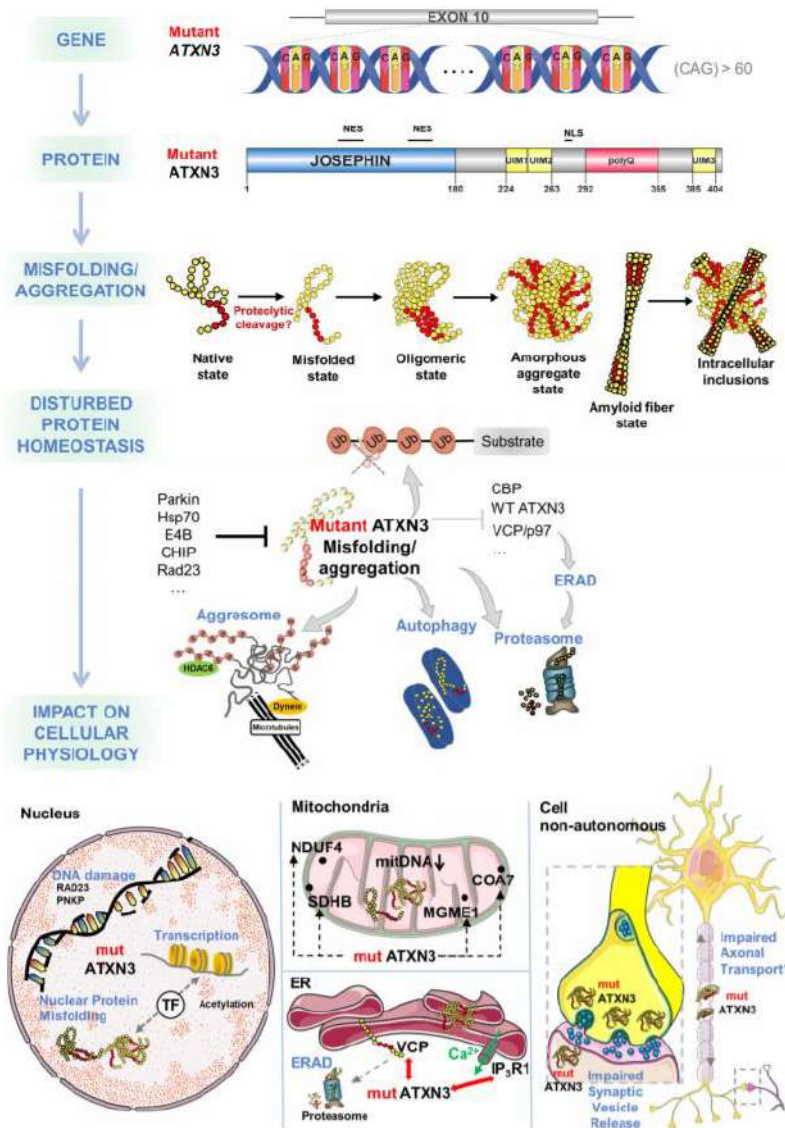


Figure 3 - Representation of the MJD-causing CAG expansion in ataxin-3 gene and the current view on the toxicity process associated with mutant ataxin-3. CAG expansion is translated into a polyglutamine tract that promotes aggregation of mutant ataxin-3. It is believed that mutant ataxin-3 sequesters critical components of the proteostasis network, such as molecular chaperones, proteasome subunits and other metastable proteins, such as CBP and theUCP/p97, leading to impaired proteostasis. This may impact cellular physiology at several levels, from DNA damage and repair, mitochondria function or normal axonal transport in neurons. Adapted from (Da Silva et al., 2019)

Driven by the need to find a model in which it was possible to determine the complete structure of the nervous system and that was suitable for genetic studies, Brenner presented the small free-living soil nematode *Caenorhabditis elegans* as a powerful tool for biological studies. *C. elegans* is a self-reproducing hermaphrodite, capable of producing both eggs and sperm (Brenner, 1974). Hermaphrodites present 5 autosomal chromosomes and 2 sexual chromosomes (5 AA + XX). Occasionally, a few males, bearing 5 autosomal chromosomes and only 1 sexual chromosome (5AA + X0) may be found in cultures (Brenner, 1974). The *C. elegans* genome was completely sequenced in 1998 (Genome sequence of the nematode *C. elegans*: a platform for investigating Biology, 1998), and shown to contain approximately 20.000 genes. At least 41,7% of the *C. elegans* predicted proteome presents human orthologs, as assayed by 4 different automated programs for orthology-prediction, conferring increased value to the model (Kim et al., 2018). An adult hermaphrodite has exactly 959 somatic cells, and this complete cell lineage, invariable between animals, enabled researchers to follow each single cell (Sulston and Horvitz, 1977). The nervous system of the *C. elegans* provides a compromise between simplicity, when compared to other animal models, with the possibility of addressing relatively complex behaviours, including response and adaptation to environmental stimuli and associative learning. The hermaphrodite *C. elegans* has 302 neurons, that can be categorized into 39 classes of sensory neurons, 27 of motor neurons and 52 different classes of interneurons. Worms present around 7,000 chemical synapses and gap junction connections that occur mainly *en passant*, using classical neurotransmitters such as serotonin or dopamine, as well as a few aminoacids (Bessa et al., 2013). For example, serotonin is thought to be involved in *C. elegans* adaptation to the surrounding environment by controlling different behaviours such as locomotion (slowing in response to the presence of food) and egg-laying. Serotonin is synthesised in the neurosecretory motor neurons, which have projections in the pharynx, thus being able to detect the presence of food and regulate the motor behaviour accordingly. Serotonin is also produced in the hermaphrodite specific neurons and stimulates egg-laying. At least four different serotonin receptors have been identified in *C. elegans*: The G-protein-coupled receptors SER-1, SER-4, SER-5 and SER-7, which regulate different behaviours. Also, a serotonin-gated pre-synaptic channel has been identified, MOD-5, responsible for the re-uptake of serotonin from the synaptic cleft (Chase and Koelle, 2007; Hapiak et al., 2009). Dopamine is also one of the neurotransmitters involved in adaptation to the surrounding environment. Dopamine is thought to play a role in the modulation of the motor behaviour in response to food, to maintain animals nearby the food source. Dopamine is synthesized in eight neurons in the hermaphrodite

and in six more neurons in the male. At least four dopamine receptors have been identified (DOP-1, DOP-2, DOP-3 and DOP-4). DOP-1 and DOP-3 have been shown to act antagonistically, resembling D1 and D2 dopamine receptors in mammals (Chase and Koelle, 2007). Importantly, some of the basic neuronal signalling pathways are also conserved from *C. elegans* to humans (Bessa et al., 2013).

Moreover, *C. elegans* is relatively easy to maintain and manipulate in the laboratory. It is small (1mm length) and transparent and feeds mainly on bacteria (Brenner, 1974). It can be maintained in Petri dishes filled with agar and with a bacterial lawn. The nematodes also present great resistance to low temperatures, allowing cryopreservation of different strains for long periods of time, up to several years, in -80 °C.

C. elegans lifecycle is well characterized. At the standard temperature of 25 °C, the duration of the *C. elegans* life cycle is 3 days, from egg to a fertile adult (**figure 4**) (Brenner, 1974). It comprises 3 major stages: embryogenesis; larval development and adult stage. Embryogenesis is the period from fertilization until hatching and occurs in 14 hours. The larval development comprises 4 different stages (L1 to L4) and lasts for 35 hours in optimal conditions. However, if starved, the L2 larvae enters a stage called Dauer. These larvae, which do not feed and limit their metabolic activity, display structural and behavioural adaptations that increase resistance to multiple stresses and ultimately their lifespan. If food becomes available, the worms exit the Dauer larvae stage and proceed to the adult stage. The average lifespan of *C. elegans* is 2-3 weeks (**Figure 4**). An adult self-fertilized hermaphrodite produces about 300 offspring, whilst male-fertilized hermaphrodites can produce more than 1000 (Strange, 2006).

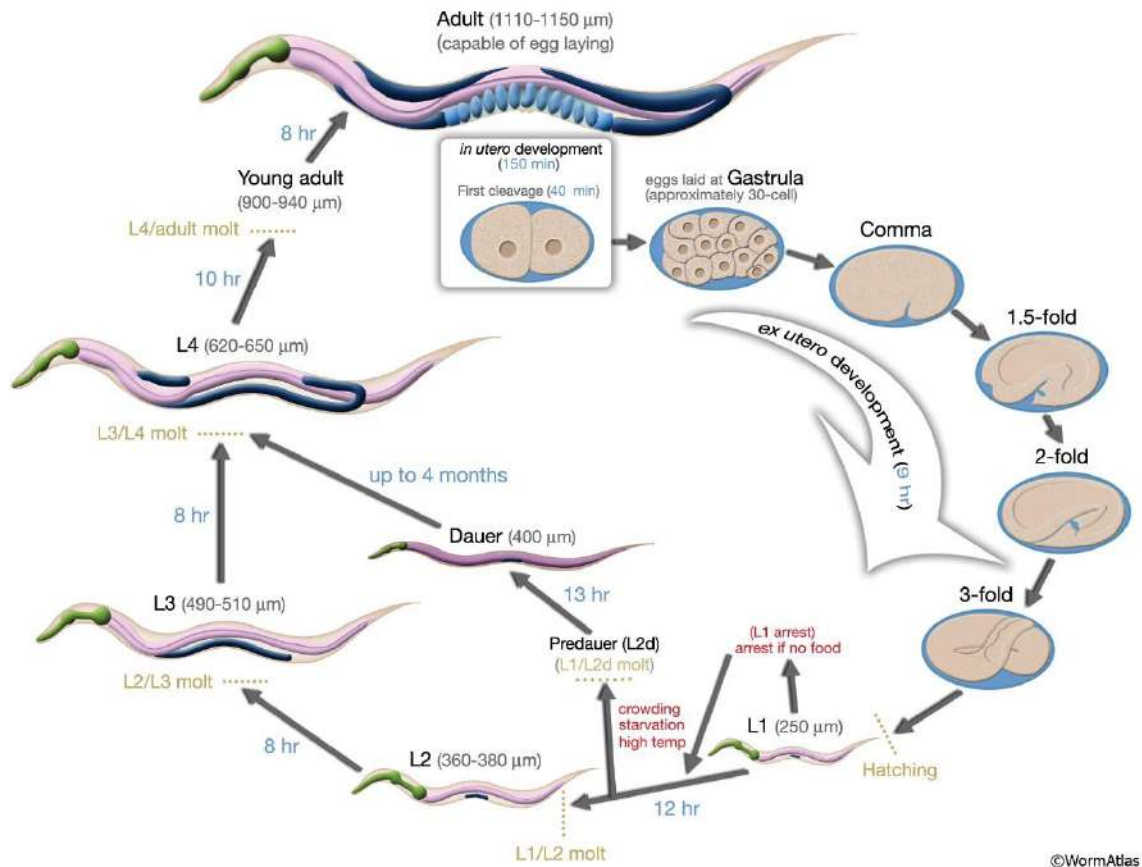


Figure 4 - The complete *C. elegans* life cycle, from egg to reproductive adult stage, extends for about three days at 25°C and comprises 3 major stages: embryogenesis; larval development and adult stage. In adverse conditions, such as starvation, animals can enter the Dauer stage, becoming resistant to environmental stresses, expanding *C. elegans* life span some to months. Figure adapted from WormAtlas (<https://www.wormatlas.org/>).

One of the main advantages of *C. elegans* as a biological model is its amenability to genetic manipulations, either by classic genetic methods or using new technologies (Antoshechkin and Sternberg, 2007). Promoters driving cell-specific expression of proteins allow the generation of transgenic strains displaying phenotypes that replicate key aspects of some human diseases (Pir et al., 2017b). This is of particular interest regarding studies of age-related neurodegenerative diseases, where mutant protein expression and accumulation/aggregation is a common hallmark. The transparent body of *C. elegans* also allows the *in vivo* study of the dynamics of some proteins, using fluorescent tags. Moreover, interference RNA (RNAi), first described in animals for this nematode, may easily be used to specifically knock down a gene. This may be useful in several ways. First, it can be used to detect new disease-causing genes. Second, it may be used to identify

modulators of the disease. There are several methods to use RNAi in *C. elegans*. Double stranded RNA (dsRNA) can be injected into the worms using a capillary needle. The dsRNA will spread throughout the worm, including the germline, assuring the transference of the gene silencing to the next generation. An alternative method, much simpler, use bacteria expressing dsRNA as food source to induce RNAi in the worms by feeding. This method may be adapted to perform genome-wide RNAi screenings (Conte et al., 2015). In *C. elegans*, the study of some genes and their related pathways may also culminate in the identification of drug targets for several diseases. In this case, RNAi may also be used to validate the target of a given drug – by showing dependency on a specific gene for the beneficial effect of the drug.

Altogether, the ability to replicate human diseases that unicellular organisms cannot, while maintaining some model simplicity in a multi-organ system; the complete knowledge of genome; the simply assessable phenotypes; the genetic tools, including gene expression, tagged protein dynamic evaluation and RNAi knock-down (O'Reilly et al., 2014) make *C. elegans* a potent model for disease understanding and drug screening.

There is a great need for novel treatments for NDs that either relieve the symptoms or act on some level of the disease, preventing the toxicity and neurodegeneration. Cell cultures most often do not offer the complexity needed, and for some studies it is not suitable to use mammals, such as rodents, since the time and number of animals required for high-throughput drug screening would be tremendous, with increased issues at the ethical level. To overcome these challenges, *C. elegans* reveals itself as very powerful model.

1.5.1. *C. elegans* as a model of tauopathies

C. elegans is able to replicate several essential aspects of neurodegenerative diseases characterized by the aggregation of mutant proteins. In particular, diseases characterized by tau-mediated neurotoxicity have been studied in this model organism. In *C. elegans*, the only gene encoding for a protein with similarity to tau is *ptl-1*, a member of the microtubule-associated protein family. This gene encodes for a protein with tau-like repeats, predominantly similar to the C-terminal repeat region of tau, that as the mammalian tau, displays several phosphorylation sites (Pir et al., 2017a). Moreover, this gene can also undergo alternative splicing, originating two isoforms, with different number of repeats. Studies have associated PTL-1 in *C. elegans* with several functions such as egg hatching, responsiveness to touch, neuronal integrity and lifespan regulation. Moreover, similarly to mammalian tau, PTL-1 binds and stabilizes microtubules, but appears to

have a broader role in microtubule cytoskeleton repair after mechanical induced damage (Pir et al., 2017a).

In the last years, researchers have tried to replicate several aspects of tau-mediated neurotoxicity by expressing mutant forms of human tau, using neuronal promoters. In 2003, Kraemer et al proposed a transgenic model for FTDP-17 to identify critical steps of neurodegeneration induced by tau (Kraemer et al., 2003). They produced two strains expressing a human 4R1N-tau (4 MTB repeats and 1 N-terminal repeat) with the V337M and P301L mutations, commonly found in FTDP-17 cases, under the regulation of the *aex-2* (pan)neuronal promoter. The strains also expressed GFP under the regulation of the *myo-2* promoter, meaning that they express a fluorescent marker in the pharynx of the nematode, which makes them easily identifiable in the dissecting scope, without the need for genotyping. In humans, the P301L mutation increased the expression of the 4R tau isoform, whilst V337M promoted tau aggregation. As a result, worms present a decreased lifespan, uncoordinated locomotion, reduced egg laying and dumpiness, that worsens with age. The progressive accumulation of mutant tau led to neurodegeneration, with gaps being found in axonal bundles of the dorsal and ventral cords. It was observed that the impairment in locomotion also occurred before major insoluble tau accumulation, and that this accumulation also occurred previous to gross neurodegeneration. Therefore, tau aggregation may not be necessary for neuronal deficiencies but may contribute to neurodegeneration with further neuronal loss (Kraemer et al., 2003).

Miyasaka and colleagues reported in 2005 to have generated transgenic strains expressing either WT tau or a form of human tau related to FTDP-17, bearing the P301L and R406W mutations or only the R406W mutation in 6 motor neurons and in the touch neurons. The strain expressing WT tau presented a progressive age-dependent impairment in the touch neurons. On the other hand, strains expressing tau with the P301L and R406W mutations displayed a stronger impairment on the touch response, as well as a stronger accumulation of tau in the PLM neuron, causing neuritic abnormalities (Miyasaka et al., 2005).

Fatouros et. al. also reported a *C. elegans* model of FTDP-17. They used the *rab-3* promoter to express pan-neuronally a fragment of human tau bearing the K280 deletion, a mutation commonly found in this disease. In addition, they crossed these new strains with the CK10, generating animals with a strong toxicity phenotype. This strain had severe deficits in locomotion at day one of adulthood and accelerated aggregation of insoluble tau. Moreover, impairment of pre-synaptic transmission and deficits on the development of the nervous system were detected.

Importantly, when these strains also expressed the proline substitutions I277P and I308P, that act as β -sheet breakers and consequently prevent aggregation, the locomotion defects were lost, and the nervous system developed with minimal perturbations. This indicates that the toxicity displayed in the model is related with the aggregation of tau, providing an interesting model to assess the toxic mechanisms of aggregation (Fatouros et al., 2012).

1.5.2. *C. elegans* as a model of PolyQ disorders

In the same manner as for tauopathies, polyQ disorders have also been studied using the nematode *C. elegans*, with researchers trying to replicate several aspects of the neurotoxicity associated with PolyQ expansions. Brignull *et al.* expressed in *C. elegans* several pure polyQ protein fragments of different length (Q0, 19, 35, 40, 67 and 86) tagged with CFP or YFP in neurons, using the F25B3.3 gene promoter (the gene encodes for a *C. elegans* ortholog of the Ca²⁺-regulated Ras nucleotide exchange factor, and is expressed throughout the nervous system), resulting in expression throughout the nervous system (Brignull et al., 2006). This study suggested that *C. elegans* neurons have a pathogenic threshold of 35-40 glutamine repeats, with larger constructs being associated with discrete fluorescent foci in some neurons instead of diffuse fluorescence. These animals showed motor dysfunction, reduction of the pharyngeal pumping rate and erratic defecation cycles. (Brignull et al., 2006).

Although the polyQ models provide useful insight into polyglutamine diseases as a group, it is important to remember that, as discussed above, in each of polyglutamine diseases the affected proteins are different, which may impact on the specificity of each disorder. In MJD, for example, the polyQ expansion occurs in the ATXN3 protein. A first model of MJD was constructed by expressing full-length and truncated human ataxin-3, with the wild-type length or expanded polyQ tract in the nervous system of the animals. Expression of the expanded polyQ tracts provoked deficiencies on the survival of embryos, and the surviving animals were egg-laying deficient and uncoordinated in locomotion. Aggregation of the fragments also lead to interruption of pre-synaptic transmission, as well as aberrant branching in neuronal processes. It also resulted in impaired Ubiquitin-Proteasome System function in these animals (Khan et al., 2006).

In 2011, Teixeira-Castro *et al.* described another *C. elegans* model capable of replicating key aspects of this disease. Making use of the *C. elegans* promoter of the F25B3.3 gene they expressed mutant full-length and truncated forms of a human ATXN3 in the nervous system of the

nematode: with 14, 75 and 130 polyQs.(Teixeira-Castro et al., 2011a). AT3q14 and AT3q75 animals presented diffuse YFP fluorescence while AT3q130 animals showed discrete foci in some neurons of the nerve cords and in the nerve ring, corresponding to immobilized aggregates (Teixeira-Castro et al., 2011a). AT3q130 animals displayed defective motility, reduced lifespan and age dependent ATXN3 aggregation in some neurons. The AT3q130 animals were still viable and able to develop to adulthood with similar brood sizes (Teixeira-Castro et al., 2011a).

Christie *et al.* also proposed a model of MJD in which polyQ-containing fragments of ataxin-3, either with 45 or 63 glutamines, were expressed in *C. elegans* body-wall muscles. This model showed defects in the thrashing (swimming) behaviour and aggregation dependent on the polyQ length. However, these defects, unlike those seen in other models of MJD, were not influenced by aging (Christie et al., 2014).

In 2017, Fardghassemi and colleagues published yet another transgenic *C. elegans* model of MJD that expressed human ATXN3 cDNA in GABAergic neurons, using the *unc-47* gene promoter, with wild-type length and with 10 and 89 CAG repeats. Worms expressing the ATXN3-CAG89 form presented decreased lifespan, progressive motoneuron degeneration and age-dependent movement impairment and paralysis (Fardghassemi et al., 2017).

1.6. Natural compounds as a source for novel drugs

In spite of the significant advances in the scientific understanding of neurodegeneration witnessed in the last decades, the majority of ND therapeutics are only palliative, focusing on the symptomatic relief. The currently approved treatments are not capable of preventing or reversing the progression of the disease and the decline of patients' function (Cummings and Zhong, 2006).

For PolyQ diseases and specifically MJD, potential therapeutic targets might address directly the polyQ proteins, namely enhancing mutant protein degradation or preventing its aggregation, or even induce gene silencing (Duarte-Silva and Maciel, 2018). Other therapeutic targets might be related to the downstream toxic effects of polyQ protein aggregation and toxicity, namely addressing the UPS impairment or mitochondrial dysfunction, oxidative stress and cell death pathways (Duarte-Silva and Maciel, 2018; Neves-Carvalho et al., 2020).

In tauopathies, the therapeutic options are also focussed on symptomatic effects. Some interesting targets for the development of disease-modifying drugs include the inhibition of tau phosphorylation or aggregation, anti-tau immunotherapies, activating tau clearance and anti-sense MAPT-targeted oligonucleotides (Panza et al., 2020).

Compounds with potential for therapeutic use in NDs might come from different sources: neurotherapeutics approved for other disorders might be repurposed for NDs; high-throughput screenings using commercial compound libraries have also identified some possible therapeutic compounds; *in silico* directed modelling of compounds for specific targets is an emerging field due to technological advances; lastly, but not less importantly, natural products may also play an important part in the drug discovery process for ND-targeting disease-modifying drugs (Cummings and Zhong, 2006).

Traditional medicines worldwide have long been using natural products for disease treatments. Dating back from 1100 BCE it is possible to find records of prescriptions based on natural products from several advanced civilizations such as Chinese; Indian Ayurvedic; Mesopotamian; Egyptian; Greek and Roman (Cragg and Newman, 2013). Particularly, plants have been extensively used for disease treatments due to their diverse biological activities. Plant-produced compounds may be divided into two major groups: Primary and secondary metabolites. Primary metabolites play a role in basic life functions, namely growth, cell division, reproduction and energy production (Bourgaud et al., 2001) and are mainly carbohydrates, lipids and amino acids (Oksman-Caldentey and Inze, 2004). Higher plants, however, are also capable of producing low molecular weight compounds that play an important role in the interaction between plant and its environment. Mainly, secondary metabolites present roles in anti-microbial and anti-insect activities, attraction of symbionts and pollinators, anti-germinative effects on other plants, providing competitive advantages and protection against UV damage, with UV absorbing compounds (Bourgaud et al., 2001). Secondary metabolites can be divided in three major groups: phenolics; terpenes/steroids and alkaloids (Bourgaud et al., 2001). Besides their natural role in plants, secondary metabolites can provide activities for human health, partly because of their structural diversity and complexity, with a variety of chemical and steric properties, resulting in selectivity and efficiency towards some targets (Yuan et al., 2016). They may present a direct activity, or they can be used as lead compounds, i. e. as a starting point for the rational design and synthesis of new active molecules.

1.6.1. *Panax ginseng* and derived molecules

Panax ginseng (**Figure 5**), is one of the most used plants in East Asian traditional medicine. Other plants of the *Panax* genus present similar properties to *Panax ginseng* and have also been referred to as Ginseng. This herb has been used in Traditional Chinese Medicine for the treatment of “*fatigue, poor appetite, diarrhoea, shortness of breath, spontaneous perspiration, diabetes, febrile diseases, amnesia, insomnia and impotence*” (Chen et al., 2008).

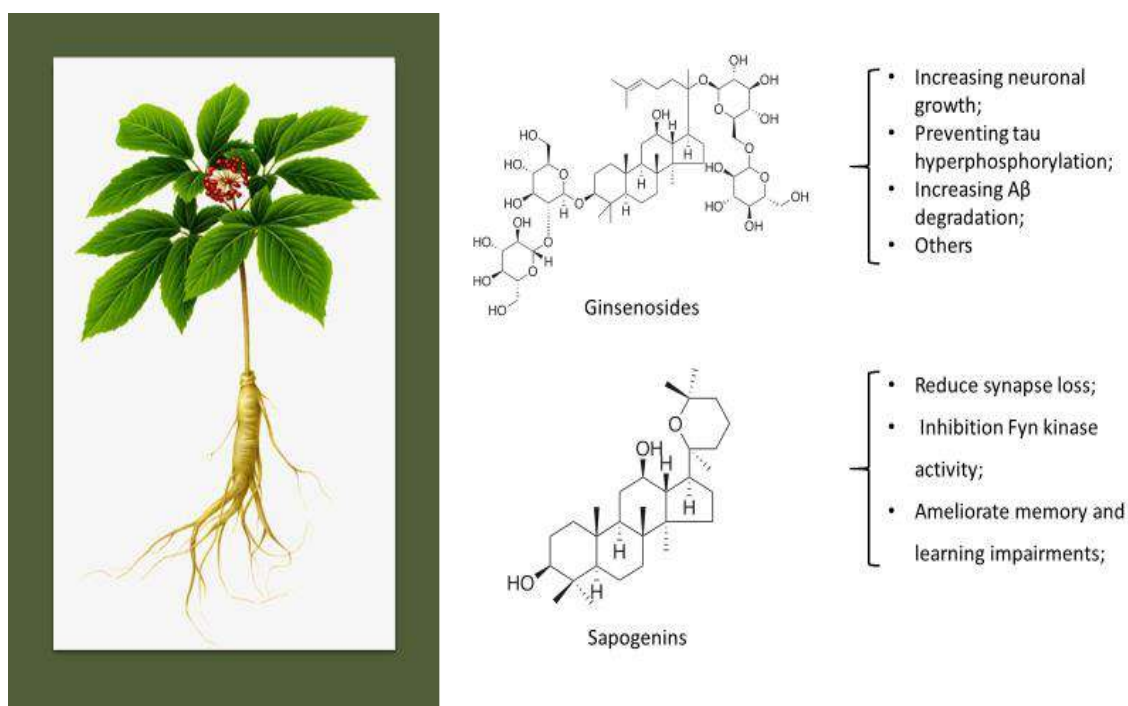


Figure 5 - *Panax ginseng* and some of its bioactive molecules. The main effects of *P. ginseng* are attributed to ginseng saponins, also known as ginsenosides, which are metabolized by the intestinal bacteria to ginseng sapogenins by glycosylation. Some of the main effects of these compounds are indicated in the figure.

Experimental data in studies with AD patients treated with powder of *Panax ginseng* roots suggested that it is capable of improving cognitive performance of the individuals (Lee et al., 2008). Previous to these studies, extracts from *Panax ginseng* were found to reduce the levels of Aβ₁₋₄₂ in mammal cell-based systems in a dose-dependent manner (Chen et al., 2006). It also promoted the phagocytosis of Aβ peptides by microglial activation (Joo and Lee, 2005) and protected the cholinergic function in the hippocampus of rats (Wang et al., 2006).

Most of the effects of *Panax ginseng* have been attributed to ginseng saponins, hereby referred to as ginsenosides (**figure 5**) (Shin et al., 2015). Saponins consist of an aglycone unit, that can also be named sapogenin, and a carbohydrate side chain, usually linked to carbon 3 of the sapogenin. It is believed that most of the ginsenosides are synthesized from 2,3-oxidosqualene, a common precursor in plants. Dammarenydiol-II synthase transforms 2,3-oxidosqualene in dammarenydiol-II, which is then hydroxylated to protopanaxadiol (PPD). PPD can suffer O-glycosylation in C-3 or C-20 to originate PPD-derived ginsenosides, namely Rb1, Rb2 and others (Shin et al., 2015). Other pathway for ginsenoside synthesis involves further hydroxylation of PPD to protopanaxatriol (PPT) and O-glycosylation of C-6 or C-20. The most common ginsenosides originated by this pathway are ginsenoside Rg1 and Re. Previous studies showed that ginsenoside Rb1 showed anti-neurodegenerative effects by increasing neuronal growth and activating the transcription of kinases such as AKT, that are involved in promoting growth in mouse hippocampal cultured cells exposed to glucose-induced neurotoxicity; inhibiting apoptosis induced by A β in mouse hippocampal cultured cells; preventing tau hyperphosphorylation induced by aluminium in mice; increased A β degradation by activating the expression of endopeptidases that degrade A β (Ahmed et al., 2016; Akao et al., 1998). Ginsenoside Rg1 was also capable of modulating Amyloid Precursor Protein (APP) processing by inhibiting γ -secretase activity and lowering A β levels. Moreover, it was capable of activating the PKA/CREB signalling pathway, increasing the levels of phosphorylated CREB, that was previously mentioned to be affected by tau in AD (Fang et al., 2012).

However, these ginsenosides are poorly absorbed. Instead, intestine bacteria metabolize them to deglycosylated metabolites (Akao et al., 1998). When orally administered, ginsenoside Rb1 is thought to be metabolized by the gut microbiota to compound K and to PPD. On the other hand, ginsenoside Rg1 is proposed to be metabolized to PPT and other dammarane sapogenins (Kim, 2018).

1.6.2. *Dendrobium nobile* Lindl. Polysaccharides and Alkaloids

Dendrobium nobile Lindl. is another plant extensively used in Traditional Chinese Medicine (Figure 6). Various compounds produced by this plant, such as alkaloids and polysaccharides, were described as having interesting bioactivities.

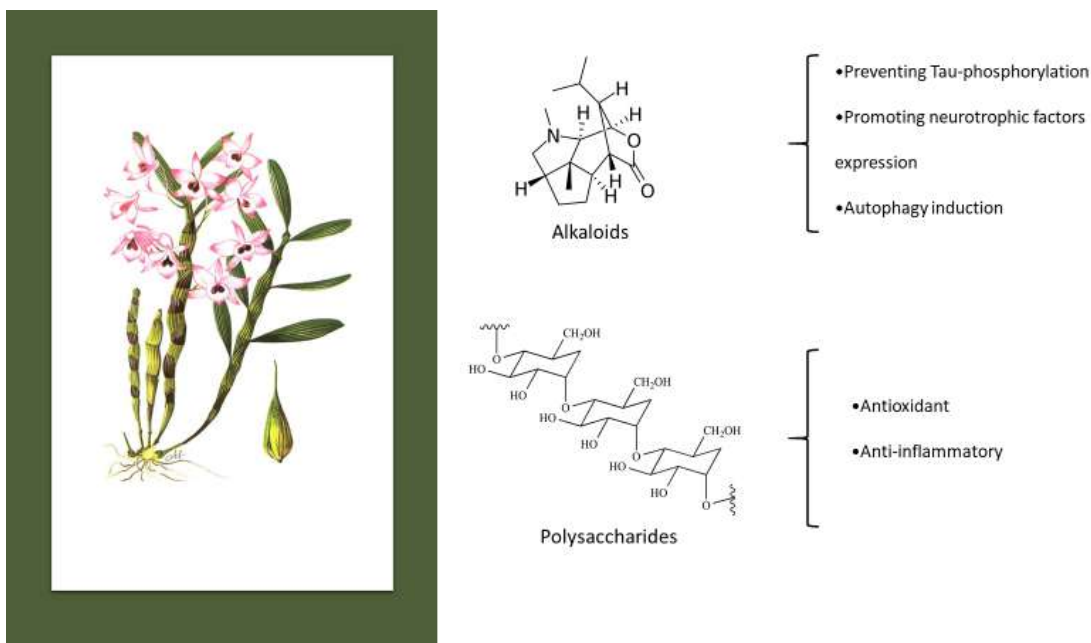


Figure 6 – *Dendrobium nobile* and some of its bioactive molecules. The main effects of *D. nobile* are attributed to alkaloids and polysaccharides, and include as antioxidant and anti-inflammatory activities, prevention of Tau phosphorylation, induction of neurotrophic factors expression and autophagy activation.

Dendrobium polysaccharides present antioxidant activity, are capable of modulating the immune response and present neuroprotective effects, namely on focal cerebral ischemia/reperfusion in rats (Xu et al., 2017). *Dendrobium* alkaloids also present several activities as antitumor agents, anti-cataract agents and improving memory and learning functions in APP/PS1 transgenic mice. They also reduced the damage caused by A β 25-35 accumulation and synaptic dysfunction in cultured cortical rat neurons (Zhang et al., 2017). This probably occurred through the induction of neurotrophic factor expression (Nie et al., 2016) and/or by inducing autophagy, seen in cultured hippocampal rat cells exposed to A β 25-35 (Zeng et al., 2019). In a mouse model with tau phosphorylation induced by lipopolysaccharide, an extract enriched in alkaloids attenuated tau hyperphosphorylation and inhibited apoptosis in neurons of (Yang et al., 2014b), strengthening the

neuroprotective activities of these compounds. Among the alkaloids present in this plant, dendrobine is thought to be the main active molecule (Xu et al., 2017).

1.6.3. *Nelumbo nucifera* (Lotus) leaf alkaloids

One other plant used in traditional medicines is *Nelumbo nucifera* (**figure 7**), commonly known as Sacred Lotus. It is an aquatic perennial plant, widely found in East Asia and often used in Asian cuisine.

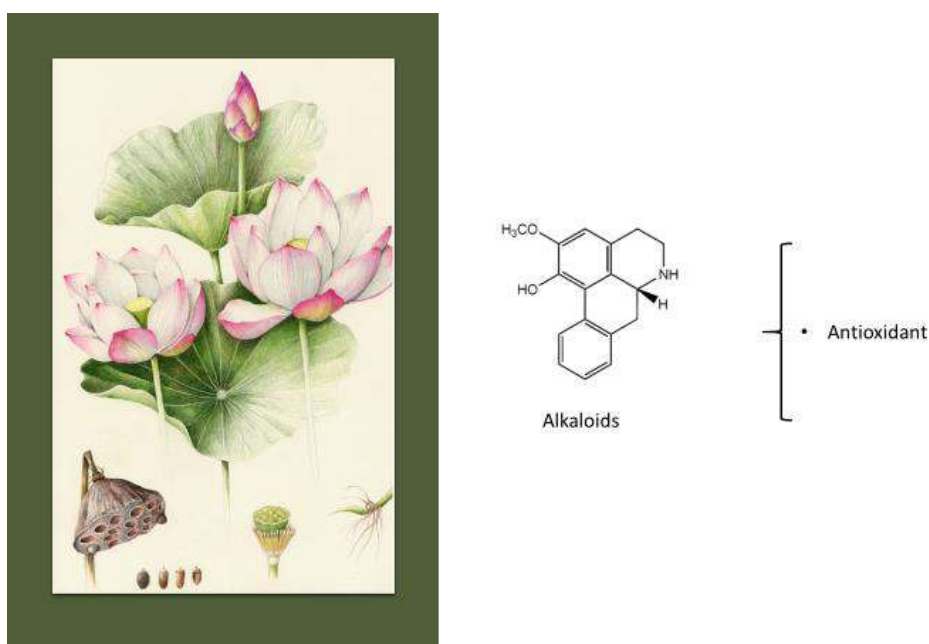


Figure 7 - *Nelumbo nucifera* and its bioactive molecules. The leaves of *N. nucifera* are rich in alkaloids, a chemical class with antioxidant capacity and potential anti-AD effects.

Besides the recreational use of *N. nucifera*, it has also been used for its bioactive effects in traditional medicines, such as the Chinese and Ayurvedic. In particular, lotus has been used as a diuretic, to improve cardiac tonus, as a demulcent for haemorrhoids, for dysentery and others. The leaves of this plant are particularly rich in alkaloids which, as seen before, is a class of molecules with potential to be used as ND therapeutics. Alkaloids from the leaves were shown to increase the levels of GABA in mice brain, producing a sedative hypnotic effect (Yan et al., 2015). Aporphine and derivatives, a class of molecules found in the leaves of *N. nucifera*, were shown to

present antioxidant and antiproliferation effects in some types of cancer cells, namely human melanoma cell lines (Liu et al., 2014a).

1.6.4. *Hemerocallis citrina* extracts

Hemerocallis citrina (**figure 8**) is a common plant in East Asia, from the Liliaceae family, whose flower is commonly called “daylily flower” or also “yellow flower vegetable”, since it is widely used in Asian cuisine. In addition to this, *H. citrina* is also recorded in the “*Compendium of Materia Medica*” for its bioactive effects and it has been used as a sedative, sleep promoting agent and febrifuge.

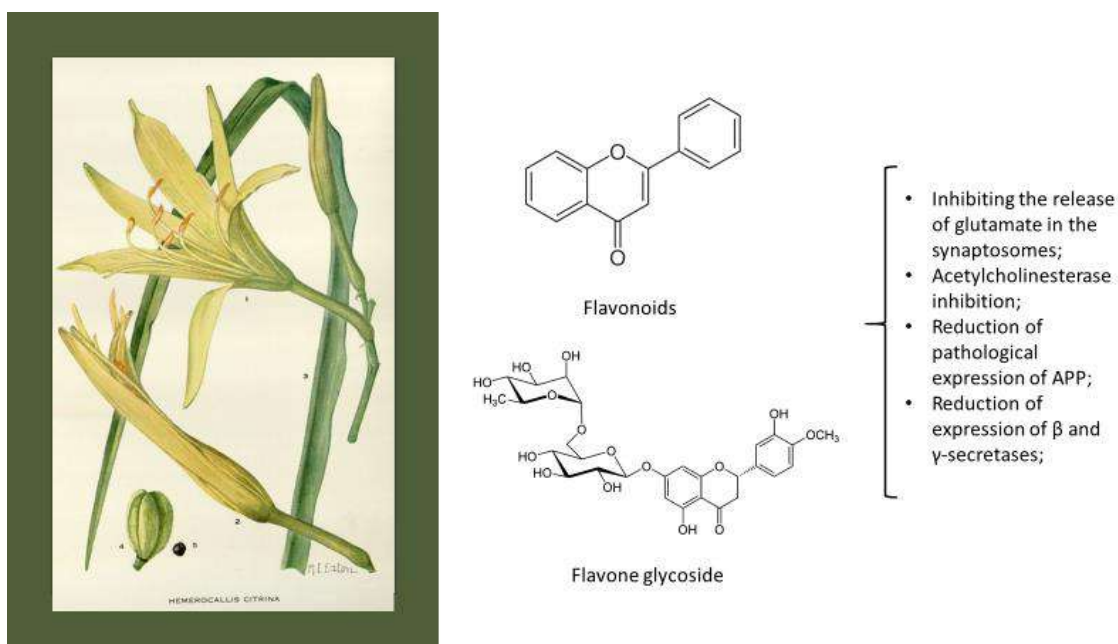


Figure 8 - *Hemerocallis citrina* and some of its bioactive molecules. The main effects of *H. citrina* effects have been attributed to flavonoids and their flavone glycosides, namely hesperidine. The effects range from inhibition of glutamate release in synaptosomes to reduction of APP expression and amyloidogenic processing.

In 2012, Lan Gu and colleagues demonstrated that *H. citrina* ethanolic extracts elevated the levels of serotonin, noradrenaline and dopamine in the mouse brain, creating an antidepressant-like effect (Gu et al., 2012). Du et al. later suggested that these antidepressant-like effects were mainly related with flavonoids, in particular Rutin and Hesperidin (Du et al., 2014). In 2017, a group of researchers described that a pre-treatment of PC12 cells with the phenolic and

flavonoid fraction of an ethanolic extract of *H. citrina* reversed the corticosterone and glutamate induced neurotoxicity. The phenolic acid fraction increased the level of dopamine released to the cell culture medium whilst flavonoids increased the level of acetylcholine and serotonin (Tian et al., 2017). In a model of depression, where rats were exposed to chronic unpredictable mild stress, chronic administration of *H. citrina* ethanolic extract reversed the decreased sucrose preference (a readout for anhedonia) displayed by this model. Moreover, ethanolic extracts inhibited the expression of interleukins-1 β (IL-1 β) and -6 (IL-6) and of the tumour necrosis factor- α (TNF- α) and increased the activity of indoleamine 2,3-dioxygenase in the model (Liu et al., 2014b).

Hesperidin is a flavone glycoside, particularly abundant in citrus fruits such as oranges. Anti-carcinogenic, anti-oxidant, anti-inflammatory and neuroprotective effects have been attributed to hesperidin in different models (Hajjalyani et al., 2019). Hesperidin was shown to be able to protect neurons from oxidative stress, either by its radical scavenging activities or by enhancing endogenous antioxidant mechanisms. In mouse models of AD, treatment with hesperidin decreased A β -aggregation and APP expression (Li et al., 2015). In a rat model of AD induced by aluminium, hesperidine showed several neuroprotective effects such as metal chelating activity, AChE inhibition, activation of signalling pathways that reduce pathological expression of APP and β and γ -secretases, inhibiting the amyloidogenic processing and resulting in the reduction of A β 1-42 levels (Justin Thenmozhi et al., 2015). In a PD mouse model, it prevented memory impairment and depressive-like behaviour by increasing the resistance to oxidative stress and the levels of dopamine in the brain. It was also demonstrated to increase the levels of neuronal growth factors, such as BDNF (Antunes et al., 2014).

Rutin is also a flavonoid glycoside found in several plants. It has been suggested that rutin is a strong antioxidant molecule, with potential to be used in the treatment of NDs. For example, Rutin was shown to protect dopaminergic neurons in a 6-hydroxy-dopamine (6-OHDA)-induced Parkinson's disease rat model (Li et al., 2015). It was also suggested to display an anti-inflammatory action in a rat model of Subarachnoid Hemorrhage, possibly by suppressing the RAGE–NF- κ B Inflammatory Pathway (Li et al., 2015).

1.7. Phytochemicals and the hormetic effect

In the past few years, the beneficial effects of molecules originated from plants and herbal formulas has been suggested to be a result of an hormesis-like effect. Hormesis is a process that describes a biphasic dose response of organisms to stress. Studies have shown that mild biological stresses, such as caloric restriction, intermittent fasting and physical exercise, were able to generate beneficial effects in several models, by inducing stress resistance mechanisms, whilst more severe stress generated prejudicial effects (Martel et al., 2019). For example, caloric restriction was able to extend lifespan in yeast, *Drosophila melanogaster*, *C. elegans*, mice and non-human primates (Mattison et al., 2017; Pifferi et al., 2018). Moreover, it was able to improve organ function and reduced the development of common chronic diseases (Martel et al., 2019).

These beneficial effects are thought to be promoted by the induction of defensive cellular responses. For instance, caloric restriction was able to activate autophagy, while the activation of sirtuin-1, a histone deacetylase (which in fact deacetylates many non-histone substrates, modifying their activity), triggered different events, such as the induction of DNA repair mechanisms and the activation of forkhead box protein O (FOXO) transcription factors, leading to the expression of antioxidant enzymes. Energy depletion in muscles and other organs, as a result of exercise, activates similar pathways: activation of SIRT-1 and FOXO transcription factors; production of small amounts of ROS that consequently activate endogenous antioxidant pathways via the nuclear (erythroid-derived 2) factor (Nrf2) (Martel et al., 2019; Mattson and Magnus, 2006). A congruous response is observed in the nervous system. A moderate level of stress in neurons leads to an enhancement of the response to more severe stress. This effect is promoted by protective pathways that include up-regulation of expression of neurotrophic factors, heat-shock proteins, sirtuins and mitochondrial uncoupling proteins (Mattson and Cheng, 2006; Mattson and Magnus, 2006).

In this sense, many of the neuroprotective effects of phytochemicals share common features to the hormetic effect promoted by mild biological stress. For example, epigallocatechin activates the Nrf2 pathway in a rodent model of cerebral ischemia. Other examples comprise the sulforaphane, present in broccoli, that was able to activate the Nrf2 pathway in astrocytes, producing antioxidant molecules capable of protecting neurons from oxidative stress (Mattson and Cheng, 2006; Martel et al., 2019).

Autophagy is also one of the defensive cellular responses that can be activated in response to phytochemicals. Several plant-based compounds have been found to activate autophagy with promising effects in both *in vitro* and *in vivo* models of neurodegenerative diseases. For example, resveratrol, a polyphenol, was able to activate the first step of autophagy and to ameliorate the cognitive impairment observed in a mouse model of AD. On the other hand, flavonoids such as baicalein were also shown to promote the autophagic flux in both a cell culture and a mouse model of Parkinson Disease, preventing mitochondrial damage (Stacchiotti and Corsetti, 2020).

1.7.1. *C. elegans* stress responses

Resembling other organisms, *C. elegans* has its own stress response pathways, many of which are conserved between nematodes and mammals. Up-regulation of Heat-Shock Proteins has been faced as key for protecting cells against several kinds of stress. The heat-shock transcription factor 1 (HSF-1) is one of the main regulators of HSP transcription. Under normal conditions, HSF-1 is localized in the cytosol and it is inhibited by a weak association with HSPs. Upon stress, such as heat-shock or accumulation of misfolded proteins, the interaction of HSF-1 with HSPs is lost and it can be translocated to the nucleus, exerting its activity by promoting the expression of various molecular chaperones (Rodriguez et al., 2013).

Other transcription factors can bind to HSP genes cis-regulatory elements, inducing their expression. In *C. elegans* these transcription factors are DAF-16, a FOXO homologue, and SKN1, an nrf-2 homologue (Prahlad and Morimoto, 2009).

The transcription factor DAF-16 is negatively regulated by the Insulin Signalling pathway (IIS). In normal conditions, with abundance of food source, the IIS pathways is activated, DAF-2 (the insulin/IGF receptor) initiating a phosphorylation cascade that results in inactivation of DAF-16 and sequestration in the cytosol. However, under stress conditions, DAF-16 is not phosphorylated and it is translocated to the nucleus where it induces the expression of HSP genes (Prahlad and Morimoto, 2009; Rodriguez et al., 2013). Similarly, upon oxidative stress, SKN-1 is translocated to the nucleus, where it activates the expression of genes involved in the response to this type of stress, such as catalases, superoxide dismutase and glutathione-S-transferases (Park et al., 2009; Prahlad and Morimoto, 2009).

It was recently discovered that many of these stress response pathways of *C. elegans* can be regulated in a non-cell autonomous way, meaning that an insult on a specific tissue might activate stress response pathways in other tissues. For example, the heat-shock response is

regulated at the whole-animal level, in a non-cell-autonomous fashion, since thermosensory neurons are capable of modulating this response in other somatic tissues (Nussbaum-Krammer and Morimoto, 2014). Similarly, it has been shown that ER or mitochondrial stress in neurons are capable of activating the same pathways in other tissues (Nussbaum-Krammer and Morimoto, 2014). Moreover, regulation of the insulin-like growth factor pathway has been described to be similar, with expression of *daf-16* in muscle cells influencing the expression of the target genes in other tissues, such as in the intestine (Taylor et al., 2014). Interestingly, it has been shown that serotonin plays an important role in modulating some of these cell non-autonomous stress responses. Neuronal release of serotonin in response to different sensory stimuli was able to activate HSF-1 and other chaperones in non-neuronal tissues of *C. elegans* (Tatum et al., 2015). Likewise, serotonin also mediates the activation of the mitochondrial unfolded protein response in the intestine of nematodes in response to proteotoxic stress in neuronal mitochondria (Berendzen et al., 2016).

1.7.2. Hormesis-like effects of phytochemicals in *C. elegans*

The neuroprotective effects of phytochemicals in worms might be mediated by stress-response pathways that are conserved from *C. elegans* to higher animals' context. For example, tomatidine, a compound found in tomatoes, improved several aspects of worm healthspan, expanding the lifespan of the animals. This compound generated a hormetic effect by inducing a mild oxidative stress, which in turn activated the SKN-1/Nrf2 pathway, resulting in increased antioxidant capacity and activation of mitophagy (Fang et al., 2017).

The beneficial effects of hormesis-like effects promoted by phytochemicals were also shown in *C. elegans* models of disease. In 2018, Maulik and co-workers, using a strain expressing a human form of α -synuclein, showed a positive effect of a *Vacuum uliginosa* extract in reducing the expression of this protein. Moreover, the extract improved the motor function of the animals and restored lipid content (lipid droplet accumulation being a readout for impaired autophagy in this model). The group suggested that these effects were due to the xenohormetic properties of the extract, that resulted in increased levels of *sir-2.1* expression; this gene encodes for SIR2, a sirtuin, homologue to Sirt1, that modulates DAF-16 activity by deacetylation of this transcription factor. As a result, DAF-16 translocates to the nucleus and activates expression of its target genes, with an overall neuroprotective effect (Maulik et al., 2018).

In strains expressing A β in different tissues, the benefits of some phytochemicals have also been shown to involve stress response pathways. For example, in a strain that expresses A β peptide in the muscle, treatment with epigallocatechin gallate (EGCG), found in the leaves of *Camellia sinensis*, inhibited the formation of deposits of this peptide and increased the nuclear localisation of DAF-16 (Abbas and Wink, 2010). Otophyllaside B, originated from *Cynanchum otophyllum*, extended lifespan, reduced A β deposition, delayed paralysis, improved chemotaxis and increased resistance to heat stress when used to treat neuronal and muscle *C. elegans* models of AD. These effects were mediated by the up-regulation of *hsf-1* expression and consequently of its target genes, such as *hsp-12.6*, *hsp-16.2* and *hsp-70* and by the activation of DAF-16, resulting in increased expression of antioxidant enzymes (Yang et al., 2017).

In the same line of evidence, rutin reduced neuronal death of ASH neurons and polyQ aggregation in a *C. elegans* model of HD. Additionally, it increased the lifespan of strains expressing polyQ fragments or human huntingtin (Htn-Q150) in the worm neurons. The treatment with rutin resulted in increased nuclear localization of DAF-16 and activation of both antioxidant enzymes and autophagy (Cordeiro et al., 2020).

2. The Aim

In this study, we proposed to assess the potential neuroprotective effects of a set of extracts and purified natural compounds, originating from plants used in traditional Asian medicines, using *C. elegans* models of neurodegenerative diseases.

The natural products under study comprise ginsenosides Rb1 and Rg1 and the sapogenins protopanaxadiol (PPD), protopanaxatriol (PPT), all originated from *Panax ginseng*. *Dendrobium nobile* extract and its specific alkaloid dendrobine, *Nelumbo nucifera* leaf alkaloids and *Hemerocallis citrina* extracts were also evaluated, since most of them have been used through the ages in traditional medicines as treatment for several diseases.

To this end, *C. elegans* models capable of replicating key aspects of the pathophysiology of FTDP-17 and MJD were used. These models, previously described, are a result of the overexpression of human 4R1N-tau (V337M) and of a mutant ataxin-3 protein (130 polyQ) in nematodes' nervous system. The motor impairment of the models was the main readout evaluated after animals' treatment. The evaluation of motor function was made using an automated detection strategy, proposed for high throughput screenings, and the results were validated using an additional motility assay.

Taking advantage of distinct genetic tools, this study also aimed to understand which targets and pathways are key for the compounds' or extracts' effect. This knowledge can culminate in the identification of additional therapeutic targets for these currently untreatable diseases.

3. Methods

3.1. Compounds and extract preparation

All the compounds and extracts used in this study were kindly provided by Professor Qiong Wang from the School of Pharmacy, Southwest Medical University, Sichuan province, China. The compounds, listed in **table 1**, were provided by Professor Qiong Wang, with a purity > 98% confirmed by HPLC, and transported to Portugal at room temperature conditions.

The compounds to be used in the assays were resuspended in DMSO 100% to a stock concentration of 1 mM. The stock solutions were stored at -20°C and for each experiment, fresh dilutions were made to obtain final concentrations of 100; 50; 10; 1; 0,1; 0,01 and 0,001 µM in DMSO 1%.

Table 1 – Compounds used in this study

Name	Species	Chemical formula	Molecular weight (g/mol)	CAS number
Protopanaxatriol (PPT)	<i>P. ginseng</i>	C ₃₀ H ₅₂ O ₄	476.73	34080-08-5
Protopanaxadiol (PPD)	<i>P. ginseng</i>	C ₃₀ H ₅₂ O ₃	460.73	7755-01-3
Ginsenoside Rb1	<i>P. ginseng</i>	C ₅₄ H ₉₂ O ₂₃	1109.3	41753-43-9
Ginsenoside Rg1	<i>P. ginseng</i>	C ₄₂ H ₇₂ O ₁₄	801.01	22427-39-0
Dendrobine	<i>D. nobile</i>	C ₁₆ H ₂₅ NO ₂	263.381	2115-91-5

Three different extracts from *Hemerocallis citrina* were used. Flowers were collected in Hunan, China and were extracted for three times with 20 volumes of 80% ethanol, for 1 hour each. The resulting extracts were combined and evaporated in a rotary vacuum evaporator. After drying, the combined extract was resuspended in water and re-extracted three times with different solvents: petroleum ether, ethyl acetate and n-butanol. Some of the n-butanol part was stored and the remaining was run by a HP-20 macroporous resin and eluted with water, ethanol 30%, ethanol

50%, ethanol 70% and ethanol 95%. In this study we assessed both the 30% ethanol and 50% ethanol parts, as well as the previously obtained n-butanol extract.

For the *Dendrobium nobile* extract, stems of the plant were collected in the Sichuan province, China. After dried, stems were crushed and extracted with 80% ethanol overnight. The resulting extract was re-extracted three times using 80% ethanol at 80°C. After being filtrated, the extraction solution was evaporated using a rotary vacuum evaporator and freeze-dried for 12H.

Nelumbo nucifera alkaloids were obtained from the lotus leaves. After air-dried, the leaves were crushed and extracted three times with 0,1% HCl (10L). The resulting solution was filtered and passed through a column filled with D001 resin and 95% ethanol. After intense washing, the adsorbed compounds were eluted with a mixture of ethanol 95% and ammonia 1%. The solution was then dried in a rotary vacuum evaporator.

All the extracts were resuspended in DMSO 100% to a stock concentration of 100 mg/mL and stored at -20°C. For the tests, each stock solution was diluted to obtain final concentrations of 1; 0,75; 0,50; 0,25; 0,125 mg/mL in DMSO 1%.

Table 2 – Plant derived extracts used in this study

Name	Species
<i>H. citrina</i> 30% ethanolic extract	<i>H. citrina</i>
<i>H. citrina</i> 50% ethanolic extract	<i>H. citrina</i>
<i>H. citrina</i> n-butanol extract	<i>H. citrina</i>
<i>D. nobile</i> extract	<i>D. nobile</i>
<i>N. nucifera</i> alkaloids	<i>N. nucifera</i>

3.2. *C. elegans* strains and maintenance

Nematodes were grown on NGM (Nematode Growth Medium) plates and seeded with *Escherichia coli* OP50 strain at 20° C, using standard methodologies (Brenner, 1974). A list of all the strains used in this work is available in **Supplementary table 1**. All media and buffers used in this work are listed in **Supplementary table 2**.

3.3. Freezing *C. elegans* strains

C. elegans can be conserved at -80°C for extended periods of time. For this purpose, and in order to keep stocks of the strains for the different experiments, seven hermaphrodites at L4 stage of development were transferred to NGM plates freshly seeded with *E. coli* OP50 and maintained at 20°C for 5 days. At day 5, before starvation was reached and when a large number of L1 animals were observed, the different strains were frozen. To this end, worms were transferred to a 15 mL falcon using M9 medium and the falcons were centrifuged for 1 minute at 2000 g using a Thermo Electron Heraeus Multifuge 3L-R (Thermo Electron Corporation, Waltham, Massachusetts, USA). The supernatant was discarded, and animals were resuspended in M9 again. This step was repeated twice to wash the animals. After the final wash, animals were resuspended in a 1:1 solution of M9 and freezing solution (**Supplementary Table 2**) and the volume was distributed by cryovials and stored at -80°C using a Coolcell® LX 1°C/min (Corning Incorporated, Corning, Nova York, USA), that assured a progressive temperature decrease, allowing the preservation of the animals.

3.4. Animal synchronization

To obtain synchronised populations of worms, animals were collected from the NGM plates to 15 mL conical centrifuge tubes using M9 medium and adult animals were allowed to deposit. The M9 was discarded and animals were treated with a solution of 20% alkaline hypochlorite for 5 minutes with vigorous shaking, until all animals were lysed, and only eggs remained, for a maximum period of 5 minutes. The Falcon tubes were then centrifuged for 1 minute at 2000 g using a Thermo Electron Heraeus Multifuge 3L-R (Thermo Electron Corporation, Waltham, Massachusetts, USA). The supernatant was discarded, and eggs were washed twice using M9 followed by centrifugation in the same conditions as stated before. For experiments requiring the use of eggs, they were immediately resuspended in S-Medium whilst for other experiments that used L1 stage animal, eggs were resuspended in M9 and allowed to hatch overnight.

3.5. Inactivation of *Escherichia coli* OP50 for drug assays

To minimize the drug metabolization by bacteria during drug assays, *E. coli* OP50 was inactivated by cycles of freeze/thaw. Bacteria were grown in LB medium for 16 hours at 37° C with shaking at 200 rpm. It was then distributed by 50 mL falcons and centrifuged at 4° C and 4000 g for 30 minutes. Supernatants were discarded and pellets submitted to 3 consecutive cycles of flash-freezing in liquid nitrogen and water bath defrosting at 37° C. After the last cycle, the pellets were frosted one last time in liquid nitrogen and stored at -80° C.

Whenever a drug assay was performed, *E. coli* pellets were thawed and resuspended in S-medium complete (Supplementary table 2) to the desired optical density at 595 nm (OD₅₉₅).

3.6. Food clearance assay for *in vivo* toxicity evaluation

The effect of the compounds on the nematodes' development, growth and survival was assessed to check for possible toxic effects. This was done by monitoring the consumption rate of *E. coli* OP50 suspension food solution in liquid medium, along time.

Synchronized N2 Bristol strain eggs were resuspended in S-Medium to a density of 20-25 animals per 15 µL of volume. Using a 96-well microtiter plate, 15 µL of egg suspension solution was added to 20 µL of inactivated *E. coli* OP50 with OD₅₉₅ 0,7 and 25 µL of compound or extract solution to final concentrations between 100 µM - 0,001 µM and 1 mg/mL – 0,125 mg/mL, respectively, in 1% DMSO. A toxic concentration of 5% DMSO was used as negative control, and 1% DMSO was used as positive control. Four replicates were used per condition. Microtiter plates were incubated at 20° C for 7 days, involved in humidified paper and aluminium foil to protect animals and drugs from drought and light. The absorbance was measured daily at 595 nm, using a Tecan Infinite M200 Pro plate reader (Männedorf, Switzerland).

3.7. WMicroTracker Motility Assessment

The WMicroTracker from Nemametrix is an automated device that allows the simultaneous measurement of overall locomotor activity of nematode populations in liquid media in microtiter plates. This device uses infrared microbeams crossing each well of conventional microtiter plates to quantify the locomotor activity of the animals. When the infrared microbeams are scattered by

moving worms, interruptions are scored as movement of animals. Therefore, the device is able to determine the overall locomotor function of the worm population in each well. (Simonetta and Golombek, 2007).

Synchronized eggs were allowed to hatch overnight in M9 medium, the resultant L1 animals were diluted to a final concentration of 40-50 L1 worms per 40 μL . This volume of worm solution was added to 50 μL of *E. coli* OP50 suspension with OD₅₉₅ 0,6 and 10 μL of compound/extract solution to the final concentrations previously described in 1% DMSO. Four replicates were used per condition. Microtiter plates were incubated at 20° C for 4 days, involved in humidified paper and aluminium foil to protect from drought and light.

At day 3, 1 μL of Fluorodeoxyuridine (FudR) (120 μM) was added to the final 100 μL volume, to block the hatching of eggs laid by adult animals (this would affect the WMicroTracker reading). At day 4, the plates were read for 150 minutes in the WMicroTracker device (**Figure 9**). Three independent biological replicates were used.

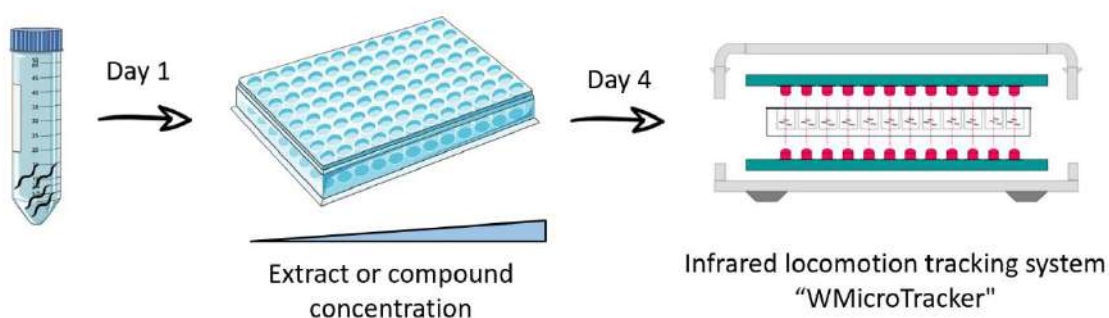


Figure 9 – WMicroTracker assessment experimental design. Experimental procedure design to assess the impact of chronic treatment in *C. elegans* strains' motor phenotype in liquid medium using the infrared locomotion tracking system "WMicroTracker".

3.8. Motility Assay

The motor phenotype of worms was also evaluated using a motility assay. After 4 days of growth (after hatching) in the same conditions referred above, worms were transferred from the 96-well microtiter plate to unseeded NGM plates and left to dry at room-temperature of 20° C for 45 minutes. Five to ten worms were then picked and placed in the centre of a 30 mm freshly seeded NGM plate. Animals remaining inside a circle with 1 cm of diameter were scored as “locomotion defective”, until 50 animals per condition were scored. The experiment was repeated 3 independent times, reaching a total of 150 animals scored (**Figure 10**).

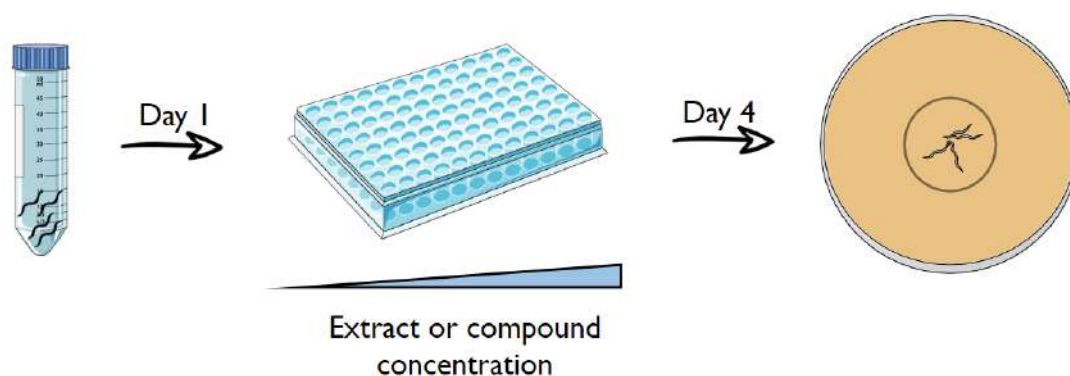


Figure 10 – Motility Assay experimental design. Experimental procedure design to assess the impact of chronic treatment in *C. elegans* strains' motor phenotype in solid medium using motility assay.

3.9. Thrashing Assay

To assess the swimming behaviour of the animals we used a thrashing assay (Bravo et al., 2018). Animals grown for 4 days in liquid media were transferred from the 96-well microtiter plate to unseeded NGM plates and left to dry at room-temperature of 20° C for 45 minutes. We picked one single worm to a drop of M9 buffer in a glass slide and allowed the worm to stabilize for 30 seconds. Animals were then filmed for a period of 1 minute at a rate of 15 frames-per-second, in a total of 600 frames, using an Olympus SZX16 stereomicroscope. The number of body bends was scored manually by analysis of the video. At least ten animals per condition were assessed in each experiment.

3.10. Evaluation of ATXN-3 aggregation

In vivo confocal imaging was used to quantify mutant ATXN-3 aggregation. Live AT3q130 animals grown in liquid media were picked and mounted on slides with 3% agarose pads and immobilized with a solution of 3 mM levamisole. Images of the animal heads were captured using an Olympus FV1000 (Japan) confocal microscope, under a x60 oil objective. The confocal pinhole was adjusted to 1.0 Airy unit of optical slice and Z-series images were acquired every 0,5 μm along the axis, using the 515 nm excitation laser for yellow fluorescent protein (YFP).

Quantification of aggregates was performed in an automated manner, as described by Teixeira-Castro and colleagues using the software MeVisLab (Teixeira-Castro et al., 2011b). Three different parameters were examined in this study: total number of aggregates present in the head of the animal; number of aggregates/total area; and total area occupied by the aggregates/ total area. Three independent assays were conducted, with at least six animals per condition assessed in each experiment.

3.11. Gene expression analysis using *C. elegans* fluorescence reporter strains

3.11.1. Antioxidant Response Pathways

The strains LD1171, CL2166 and CF1553 (**Supplementary table 1**), expressing GFP under the regulation of the *gcs-1*, *gst-4* and *sod-3* gene promoters respectively, were grown in liquid media and treated with 1mg/mL of HCE as described previously. At day 4, worms were picked to slides with a 3% agarose pad, anesthetised with sodium azide (NaN_3 , 20mM) and oriented using an eyelash. Excess sodium azide was removed, animals were covered with a cover slide and sealed using 3% agarose. In each assay, at least 10 worms were analysed per condition.

As positive control, animals grown in vehicle (1% DMSO) were incubated in 5-Hydroxy-1,4-naphthoquinone (or juglone) (Sigma-Aldrich, St. Louis, MO) at 150 μM for 1h and allowed to recover at 20° C for 3h for LD1171 and CL2166 strains. For CF1553 strain, animals grown in vehicle (DMSO 1%) were submitted to a heat-shock of 37°C for 1h and allowed to recover for 3h prior to use.

Brightfield and Fluorescence (GFP filter) images were obtained using an Olympus Widefield Upright Microscope BX61 at 4x magnification. Fluorescence exposure time was adjusted to control conditions and applied equally for treated animals in each experiment.

Fluorescence intensity of each individual worm was measured using ImageJ software, adjusted for background fluorescence and normalized to the mean intensity of the vehicle treated worms.

3.11.2. Autophagy

The JIN1679 strain (**Supplementary table 1**), expressing GFP fused to the HLH-30 transcription factor, a regulator of several autophagy genes, was grown in liquid medium and treated with 1 mg/mL HCE as previously described. At day 4, adult animals were mounted onto slides with 3% agarose pads, immobilized with sodium azide (20mM) and covered with a cover slide.

Starvation was used to induce autophagy and therefore used as a positive control. For this, worms treated with vehicle were washed 3 times with M9 buffer and allowed to starve for 30 minutes. The animals were mounted onto slides exactly as described before.

Brightfield and Fluorescence (FITC filter) images were acquired using an Olympus Widefield Upright Microscope BX61, at 4x magnification. Settings were adjusted to control conditions and used for the other conditions analysed in each experiment. Animals were considered to have nuclear translocation of HLH-30 if well-defined fluorescent nuclei were observed. At least 10 animals per condition were scored in each experiment.

3.11.3. Mitochondrial Unfolded protein response (UPR^{mt})

The SJ4100 strain (**Supplementary table 1**), expressing GFP fused to the hsp-6 promoter, was used to assess the ability of HCE treatment to activate the mitochondrial UPR. The nematodes were synchronized and grown in liquid media as described and treated either with 1 mg/mL of HCE or vehicle (DMSO 1%). At day 4, adult animals were mounted onto slides with 3% agarose pads, anesthetized with sodium azide (20 mM), oriented using an eyelash and covered with a cover slide.

Twenty-four hours prior to use, 1 µL of antimycin A was added to wells containing animals treated with vehicle only, to a final concentration of 5 µM. Antimycin A is an oxidative

phosphorylation inhibitor, capable of causing mitochondrial stress and activating the UPR^{mt} and therefore was used as a positive control.

An Olympus Widefield Upright Microscope BX61 was used to acquire brightfield and fluorescence (GFP filter) images, at 4x magnification. Imaging settings were adjusted to control conditions and maintained throughout each experiment.

Fluorescence intensity was measured for every animal, adjusted for background fluorescence and normalized to the mean intensity of the vehicle treated worms using ImageJ software. Two independent assays were performed with at least 10 animals per condition.

3.12. RNAi-mediated genetic silencing

3.12.1. LC108 x CK10 strain crossing

To generate a strain sensitive to RNAi mediated silencing in neurons, nematodes of the mutant-tau expressing line CK10 were crossed with animals of the LC108 strain (**Figure 11**).

Ten N2 hermaphrodite nematodes at the L4 stage were subjected to heat-shock in a water bath at 30°C for 4h, originating the presence of males among the progeny. After growing for three days at 20°C, ten male nematodes were transferred to a 60 mm NGM plate, along with three CK10 hermaphrodites in L4 stage, and maintained at 15°C for two days, allowing time for male mating to occur. The adult hermaphrodites were separated to different plates and maintained at 20°C. Three days later, plates containing male animals were selected. Ten male CK10 heterozygous males were transferred to a new 60 mm NGM plate, with three LC108 L4 stage hermaphrodites and placed at 15°C for two days. The hermaphrodites were isolated and maintained at 20°C, allowing progeny to develop. At least three L4 hermaphrodites, exhibiting both GFP and mCherry fluorescence, were isolated from plates containing male animals (indicating a successful crossing) and grew at 20°C for 4 days. From these plates, at least eighteen nematodes exhibiting both GFP and mCherry fluorescence were picked for new plates, in an attempt to isolate at least one homozygous CK10;LC108 animal. When these worms reached adulthood and originated progeny, they were considered to be homozygous for both genes if all the progeny exhibited GFP and mCherry fluorescence. Plates displaying mixed fluorescent phenotypes were discarded. The CK10 (WT LC108) and the LC108 (WT CK10) products of the crossing were also isolated.



Figure 11 – Schematic representation of the crossing CK10 × LC108.

3.12.2. RNAi by feeding

HT115 bacteria expressing double stranded (ds)RNA (from Ahringer library) (Kamath and Ahringer, 2003) were used as a food source, in order to induce specific gene silencing by RNAi. The identity of the clones was confirmed by Sanger sequencing. Bacterial clones were grown overnight in Luria broth (LB) medium containing ampicillin (100 µg/mL) at 37°C and 220 rpm. The OD600 nm of bacteria was then adjusted to 1.6 using LB+ampicillin.

RNAi-plates were prepared by supplementing NGM with ampicillin (100 µg/mL) and isopropyl β-D-1-thiogalactopyranoside (IPTG, 400 µM), here used to induce dsRNA expression and seeded with the respective bacterial clones after 24h. Worms were treated with HCE or vehicle. For this, HCE was diluted in medium with the HT115 bacteria to a final concentration of 1mg/mL.

For motility assessment after gene silencing, LC108;AT3q130 and LC108;CK10 worms were synchronized and grown in the RNAi-plates for 4 days at 20°C. At day 4, worms were picked and the motility assay was performed as described above.

4. Results

There is significant evidence that compounds of herbal origin used in traditional medicines might have potential neuroprotective effects in several NDs. Thus, the effect of the following plant extracts and derived compounds was studied in *C. elegans* models of FTDP-17 and MJD: Ginsenoside Rb1 and Rg1, Protopanaxadiol (PPD) and Protopanaxatriol (PPT) from *P. ginseng*; the extract of *D. nobile* and its alkaloid dendrobine; a mixture of alkaloids extracted from *N. nucifera* and a N-butanol, a 30% ethanolic extract and a 50% ethanolic extract of *H. citrina*.

4.1. Determination of safe concentrations of extracts and compounds for *C. elegans*

The first step consisted in the evaluation of a range of concentrations of extracts and compounds regarding their ability to cause any toxic effect in wild-type *C. elegans*. For this, we used the Food Clearance Assay described by Voisine and colleagues in 2007 (Voisine et al., 2007). This assay allowed to monitor the effect of the compound or the extract on animal survival, growth and fecundity by measuring, daily, the rate at which the food source (*E. coli* OP50) was consumed in liquid medium. In non-toxic conditions, as is the case of the control treatment with 1% DMSO (the compound/extract vehicle), the consumption of OP50 decreased along time, being particularly evident after the third day of the assay, when adult animals are capable of generating hundreds of descendants. Comparing the rate of bacteria consumption from animals treated with compound or extract with the rate of consumption from animals grown in 1% DMSO we can determine if the treatment induced any toxic effect in animal development. A concentration of 5% DMSO, which is toxic for the nematodes, was also used as control. Our results revealed that treatment with concentrations up to 100 μ M for each compound and 1 mg/mL for extracts did not produce any apparent toxic effect on the development and fertility of wild-type animals (**Table 3** and **4**, respectively). **Figure 12 and 13** shows the toxicity data for the compounds and extracts, respectively.

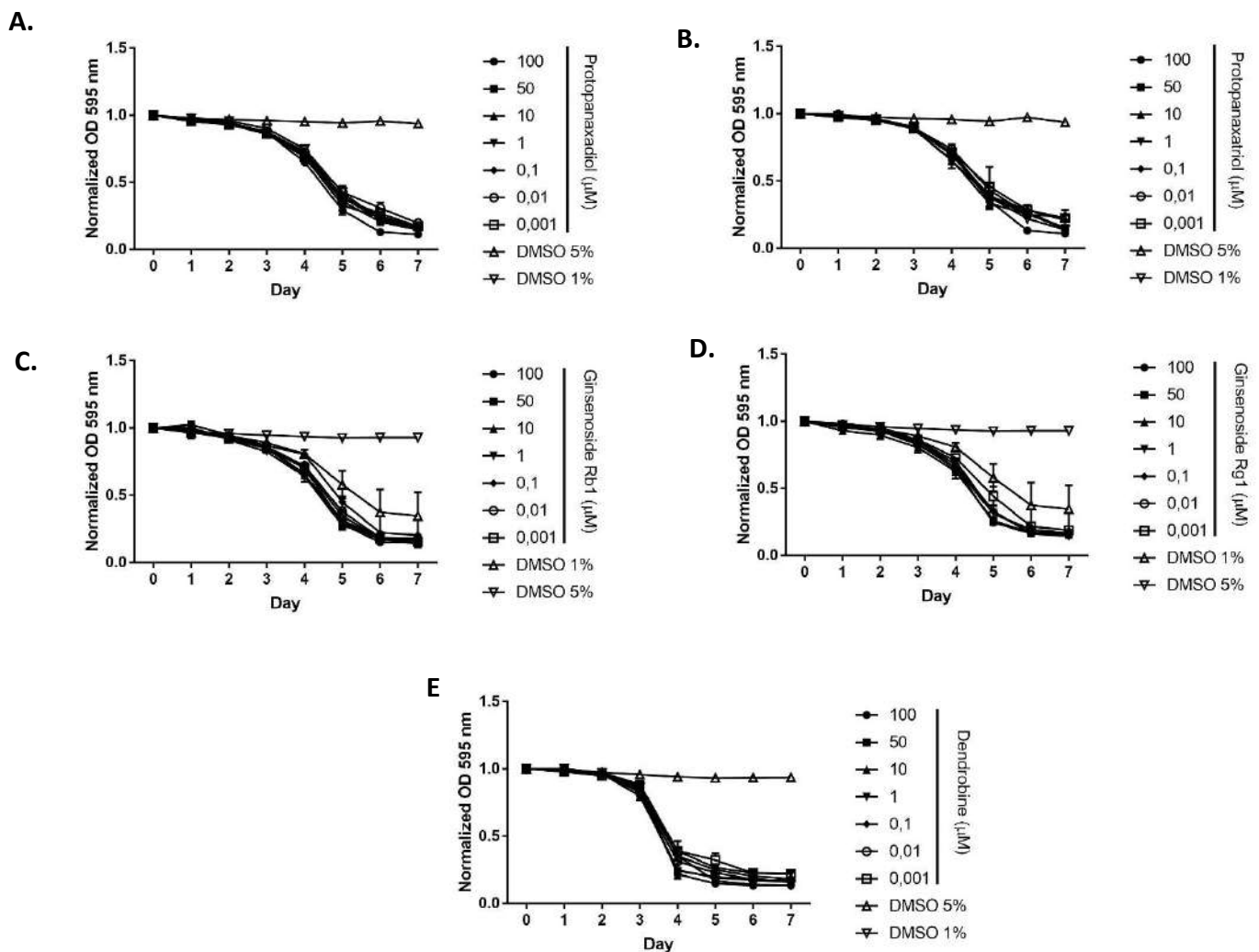


Figure 12 - Toxicity evaluation of compounds using the food clearance assay, in *C. elegans*.

Evaluation of the toxic effects of **A.** Protopanaxadiol; **B.** Protopanaxatriol; **C.** Ginsenoside Rb1, **D.** Ginsenoside Rg1 and **E.** Dendrobine treatment. The OD of *E. coli* was evaluated daily for each concentration and normalized for day 0 values. Statistical analysis revealed no significant differences between any treatment and DMSO 1% (drug vehicle, non-toxic). Graphic represented with Mean $\pm$ SEM. Non-linear regression, sigmoidal, 4PL, X is log(concentration), profile likelihood, $P \leq 0,05$.

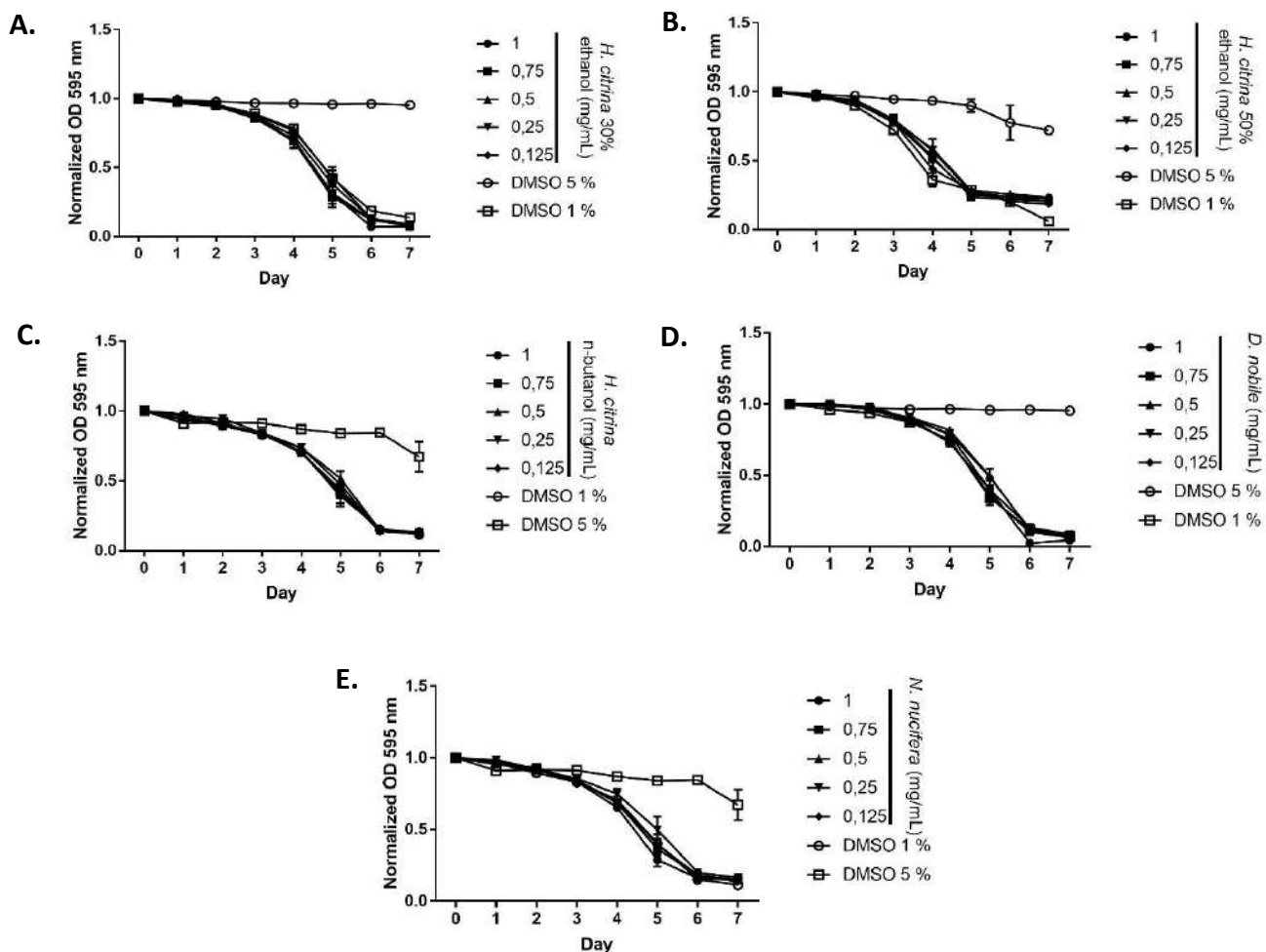


Figure 13 - Toxicity evaluation of plant extracts using the food clearance assay. Evaluation of the toxic effects of **A.** *H. citrina* 30% ethanol; **B.** *H. citrina* 50% ethanol; **C.** *H. citrina* n-butanol and **D.** *D. nobile* extract treatment. The OD of *E. coli* was evaluated daily for each concentration and normalized for day 0 values. Statistical analysis revealed no significant differences between the different extract concentrations and DMSO 1% (drug vehicle, non-toxic). Graphic represented with Mean $\pm$ SEM. Non-linear regression, sigmoidal, 4PL, X is log(concentration), profile likelihood, $P \leq 0,05$.

Table 3 – Summary of compound toxicity results

Group	Concentrations	Name	Toxicity
Compound	(μ M)	Protopanaxatriol	Safe for all concentrations
	100	Protopanaxadiol	Safe for all concentrations
	50	Dendrobine	Safe for all concentrations
	10	Ginsenoside Rb1	Safe for all concentrations
	1	Ginsenoside Rg1	Safe for all concentrations
	0,1		
	0,01		
	0,001		

Table 4 - Summary of extract toxicity results

Group	Concentrations	Name	Toxicity
Extract	(mg/mL)	<i>H. citrina</i> 30% ethanol	Safe for all concentrations
	1	<i>H. citrina</i> 50% ethanol	Safe for all concentrations
	0,750	<i>H. citrina</i> N-buthanol	Safe for all concentrations
	0,500	<i>D. nobile</i>	Safe for all concentrations
	0,250	<i>N. nucifera</i> alkaloids	Safe for all concentrations
	0,125		

4.2. Screening for neuroprotective activity of plant-derived compounds in a *C. elegans* model of FTDP-17 using an automated movement analysis in WMicroTracker

The impact of chronic treatment of the *C. elegans* model of FTDP-17 with each of the 5 compounds was evaluated using animal motor function as readout and taking advantage of an automated device for motility evaluation, the WMicroTracker (WMT).

At day 4 after hatching, *C. elegans* WT animals, that do not display any impairment in locomotor activity, presented activity counts, in the WMT, of around 1600-2000 after 150 minutes. On the other hand, mutant tau-expressing animals (CK10 model) presented the expected significant motor defects, scoring around 1000-1200 counts per 150 minutes (**Figure 14**). The chronic treatment of CK10 animals with the compounds did not result in statistically significant differences concerning the motility of the animals, except for the treatment with 0.1 μ M of Ginsenoside Rb1. Even so, the treatment of the model with different concentrations of Protopanaxatriol showed a tendency to increase the number of activity counts, suggesting a possible improvement of the motor function, when compared with the remaining compounds.

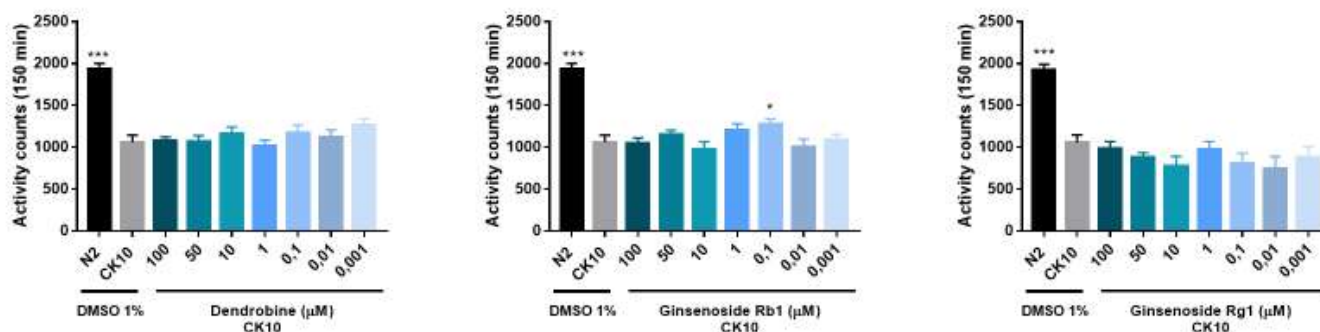


Figure 14 – Protopanaxatriol and Ginsenoside Rb1 treatment partially improved the mutant tau-mediated motor behaviour defects. Motor evaluation in WMicroTracker was performed using N2 (WT) and CK10 (FTDP-17 model) strains treated with DMSO 1% (drug vehicle) and 0.001-100 μ M/mL of: **A** Dendrobine; **B** Ginsenoside Rb1; **C** Ginsenoside Rg1; **D** Protopanaxadiol; **E** Protopanaxatriol. (n = 3 biological replicates, total of 600 animals per condition). Statistical analysis revealed statistically differences for Ginsenoside Rb1 (0.1 μ M) and no significant differences for the other compounds (although a slight effect is observed for Protopanaxatriol treatment (**E**)). Graphic represented with Mean $\pm$ SEM. One-way ANOVA planned contrasts; For Protopanaxatriol, planned contrasts with bootstrapping; *** P<0.001.

4.2.1. Effect of plant extracts on mutant tau mediated motor dysfunction of the *C. elegans* model of FTDP-17, evaluated using the Wmicrotracker

Concerning treatment with plant extracts, the same procedure was adopted using a range of concentrations from 1 to 0,125 mg/mL. In the majority of cases, the plant extract treatments did not improve the motor function of CK10 animals. However, upon treatment with the 30% ethanol extract of *H. citrina*, CK10 animals showed significant improvements in locomotor activity, at 1 mg/mL and 0.75 mg/mL, when compared to the model treated with drug vehicle. **(Figure 15)**. Higher concentrations (1 and 0,75 mg/mL) of *N. nucifera* alkaloids displayed toxic effects for the tau expressing model, with animals not surviving the 4-day treatment (despite not presenting any toxic effect on WT animals, suggesting a differential susceptibility) and were therefore excluded.

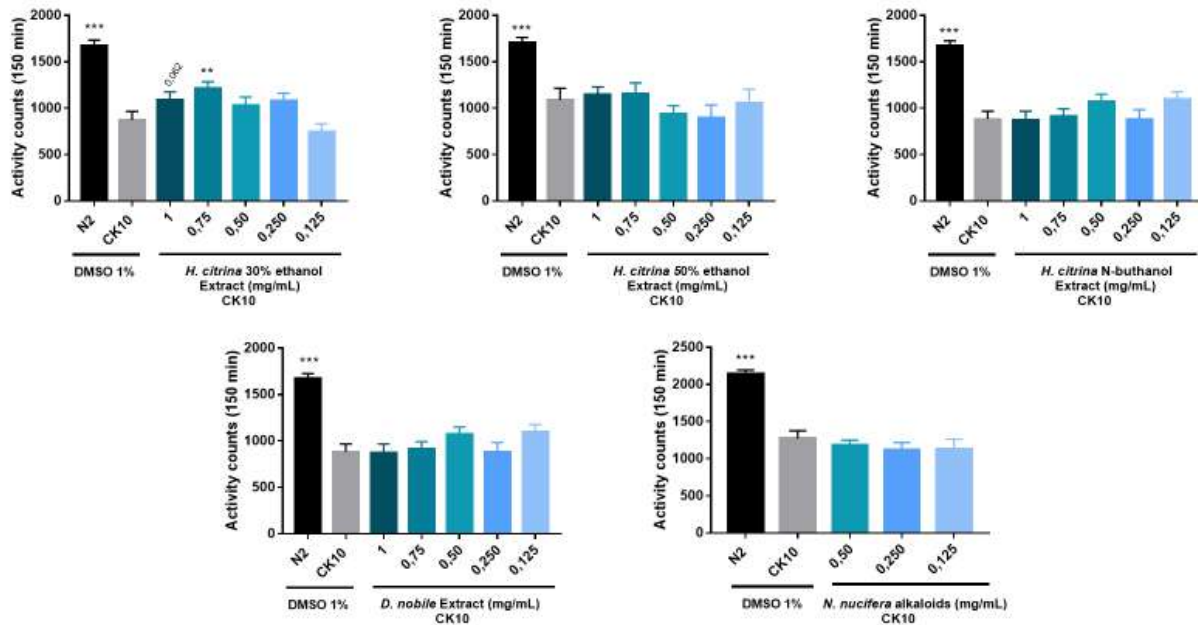


Figure 15 – *Hemerocallis citrina* 30% ethanol extract treatment partially suppressed the mutant tau-mediated motor behaviour defects. WMicroTracker Motility assays were performed using N2 (WT) and CK10 (FTDP-17 model) strains treated with DMSO 1% (drug vehicle) in comparison with CK10 treated with 1-0,125 mg/mL of **A** *H. citrina* 30% ethanol extract; **B** *H. citrina* 50% ethanol extract; **C** *H. citrina* N-buthanol extract ; **D**. *D. nobile* extract; **E** *N. nucifera* alkaloids. (n = 3 biological replicates, total of 600 animals per condition). Statistical analysis revealed significant differences between CK10 animals treated with 1% DMSO and treated with *H. citrina* 30% ethanol extract 1mg/mL. Graphic represented with Mean $\pm$ SEM. One-way ANOVA planned contrasts; **p<0.01, *** P<0.001.

4.2.2. Validation of the extracts/compounds effect on tau-mediated motor deficits using an additional motor behaviour assay

To validate the results obtained with the WMT, an independent assessment of motility was carried out, using the behaviour assay described in Teixeira-Castro (Teixeira-Castro et al., 2015) which assesses spontaneous motility of the worms in an agar plate. The *H. citrina* 30% ethanol extract, Protopanaxatriol and Protopanaxadiol treatments were selected to be independently tested using this method.

An improvement of the motor dysfunction of CK10 animals was observed (**figure 16**) upon treatment with *H. citrina* ethanolic extract, as suggested before by the WMT analysis, in a dose-dependent manner. Treatment with Protopanaxatriol ameliorated motor dysfunction at the highest concentration tested and it was confirmed that Protopanaxadiol treatment did not change the tau-mediated motor phenotype. Overall, these results confirmed the beneficial effect of both *H. citrina* 30% and Protopanaxatriol initially observed in the Wmicrotracker evaluation, suggesting that this automated device can be a reliable system for drug screenings in this model, in a high-throughput manner. The data also suggested that increments in the order of 20% in the activity counts of CK10 animals, when evaluated in the WMT, can be regarded as having potential biological relevance, even if no statistically significant differences are found. This value may be useful in future experiments as a “cut-off” value to select relevant conditions in screenings.

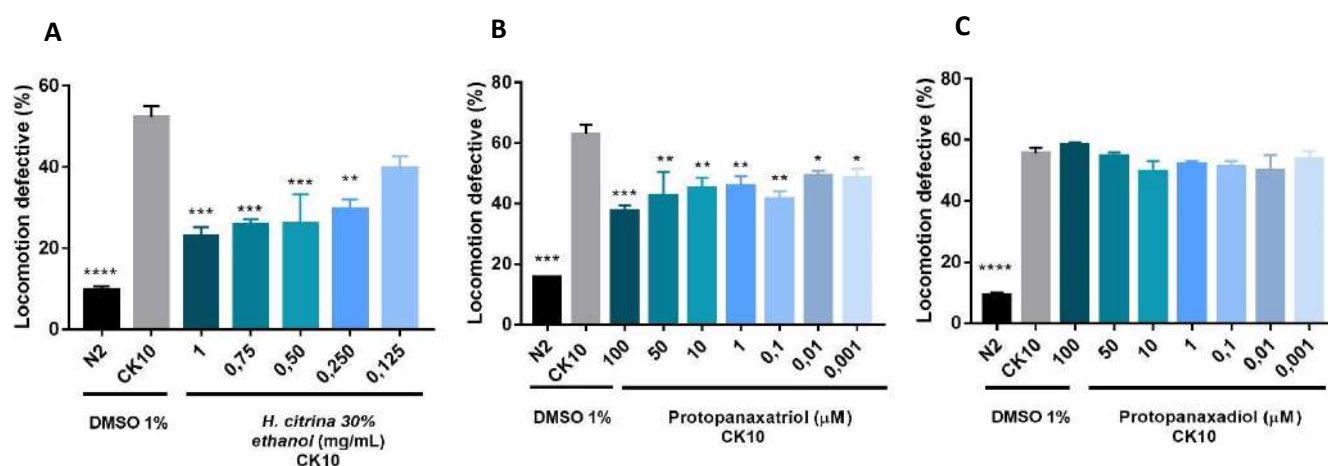


Figure 16 – *H. citrina* 30% ethanol extract and Protopanaxatriol ameliorated mutant tau-mediated motor behaviour defects. Motility assays in solid media were performed using N2 (WT) and CK10 (FTDP-17 model) strains treated with DMSO 1% (drug vehicle) in comparison with CK10 treated with 1-0,125 mg/mL of A *H. citrina* 30% ethanol extract; and with 100-0,001 μ M of B Protopanaxatriol; C Protopanaxadiol. (n = 3 biological replicates, total of 150 animals per condition). A significant improvement was observed upon treatment with all concentrations of *H. citrina* ethanolic extract in a dose dependent manner. Protopanaxatriol also caused a significant beneficial effect in motility. Protopanaxadiol did not improve the motor impairment present in the model. Graphic represented with Mean $\pm$ SEM. One-way ANOVA; *p<0.05, *** P<0.001.

4.2.3. Effect of protopanaxatriol and *H. citrina* 30% ethanol extract on mutant ataxin-3 mediated motor dysfunction in the *C. elegans* model of Machado-Joseph Disease, evaluated using the Wmicrotracker.

After having shown promising effects in the CK10 model, the impact of the treatment with Protopanaxatriol was also evaluated in another *C. elegans* model of a neurodegenerative disease, namely Machado-Joseph disease, in which motor deficits are also a key hallmark (Teixeira-Castro et al., 2011a). Worms were subjected to the same treatment as described before for CK10 model and were initially scored with the WMT system.

In accordance to the previous results, N2 WT strain did not display defects in motor function, resulting in readings of around 2000 activity counts over 150 minutes in the WMT assay. As expected, the activity measurements of the AT3q130 were significantly reduced in comparison

with the WT strain, scoring around 1000 counts per 150 minutes. For this model, no significant difference in motility was detected upon treatment with Protopanaxatriol (**Figure 17**).

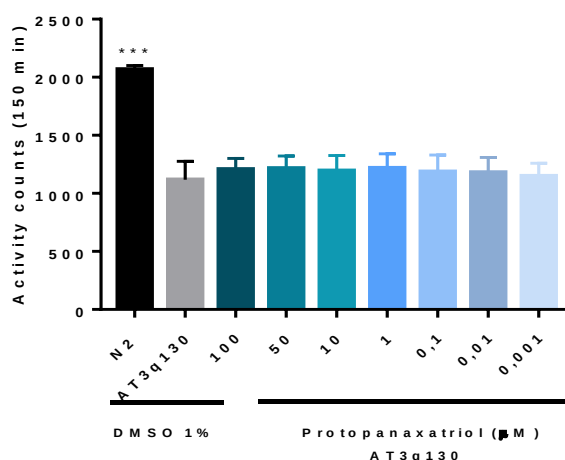


Figure 17 – WMicroTracker analysis showed no impact of Protopanaxatriol treatment in mutant ataxin-3-mediated motor deficits. *WMicroTracker* analysis was performed using N2 (WT) and AT3q130 (MJD model) strains treated with DMSO 1% (drug vehicle) in comparison with AT3q130 treated with 100-0.001 μ M of Protopanaxadiol, represented ($n = 3$ biological replicates, total of 600 animals per condition). Protopanaxatriol treatment did not caused improvement of the motor dysfunction. Graphic represented with Mean $\pm$ SEM. One-way ANOVA planned contrasts; *** $P < 0.001$.

The effect of treatment with *H. citrina* 30% ethanol extract in the mutant ataxin-3 mediated motor dysfunction of the MJD model was also assessed. Treatment with any concentration of this extract did not produce an effect detectable by the WMicroTracker device, (**figure 18**), since the overall activity for worm populations treated with *H. citrina* extract was around 1000 counts per 150 minutes, a similar value of that detected for the model treated only with drug vehicle (DMSO 1%).

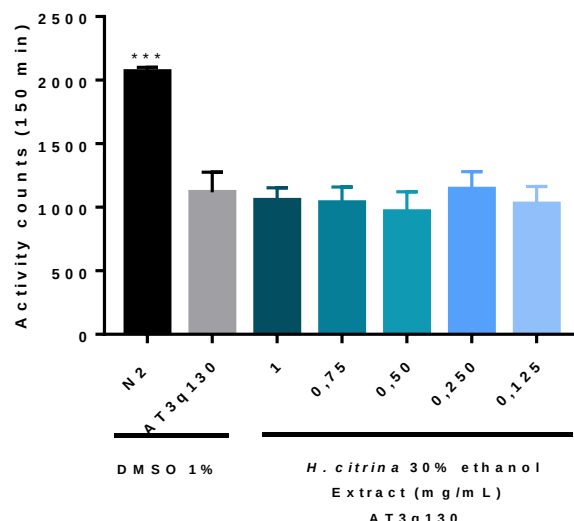


Figure 18 - WMicroTracker analysis did not reveal an impact of *H. citrina* 30% ethanol extract treatment on AT3q130 motor deficits. *WMicroTracker* was performed using N2 (WT) and AT3q130 (MJD model) strains treated with DMSO 1% (drug vehicle) in comparison with AT3q130 treated with 1-0,125 mg/mL of *H. citrina* 30% ethanol extract, represented (n = 3 biological replicates, total of 600 animals per condition). Graphic represented with Mean $\pm$ SEM. One-way ANOVA planned contrasts; *** P<0.001.

4.2.4. Validation of the compound/extract effect on mutant ataxin-3 mediated motor impairment using a distinct motility assay

Despite the lack of effect observed in the WMT assessment, we decided to ascertain the impact of Protopanaxatriol and *H. citrina* 30% ethanol treatments using the Motility Assay methodology. Using this method, around 50% of AT3q130 animals treated with drug vehicle (1% DMSO) were scored as locomotion defective, whilst only a few N2 animals (~10%) presented locomotion deficits. Upon treatment with 100 and 1 μ M of Protopanaxatriol, a significant improvement on the AT3q130 motor phenotype was observed (**figure 19**). An effective recovery of locomotor defects was also observed upon treatment with *H. citrina* 30% ethanol extract at different concentrations (**figure 20**).

Since *H. citrina* 30% treatment revealed to be the most effective among those tested in this model, its impact on mutant ATXN3 neuronal aggregation, another key aspect of MJD, was also investigated, making use of confocal dynamic imaging and analytical methods previously described by Teixeira-Castro and colleagues (**Figure 21 A;B**). Our data showed that treatment with 1 mg/mL of HCE 30% did not impact neither in the number of aggregates (**Figure 21 C**),

nor the area occupied by them in relation to the total area of the head of the animal (**Figure 21 D**). No significant changes were also observed in estimated mean area of each mATXN3 aggregate (**figure 21 E**). These results demonstrated an uncoupled effect of HCE 30% on motility and mATXN3 aggregation in this model.

Overall, these results suggested that both PPT and *H. citrina* 30% ethanol extract can ameliorate the locomotor function of animals expressing aggregation-prone proteins in the nervous system.

In the specific case of the *C. elegans* model of MJD, the motility evaluation by WMicroTracker device was not coincident with the subsequent scoring of the locomotion deficits in the motility assay. Therefore, additional experiments were performed to understand these puzzling results and to evaluate the applicability of Wmicrotracker analysis in this model.

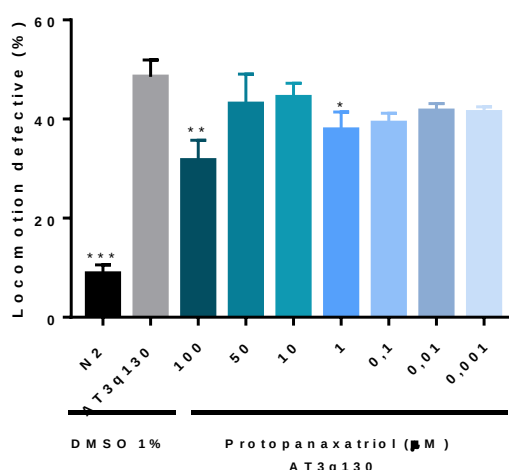


Figure 19 - Protopanaxatriol partially improved the motor behaviour defects in AT3q130 model. The motility assays were performed using N2 (WT) and AT3q130 (MJD model) strains treated with DMSO 1% (drug vehicle) in comparison with AT3q130 animals treated with 100-0,001 μ M of Protopanaxatriol ($n = 3$ biological replicates, total of 150 animals per condition). A significant improvement was observed upon treatment with 100 and 1 μ M of Protopanaxatriol. Graphic represented with Mean $\pm$ SEM. One-way ANOVA; * $p < 0.05$, *** $P < 0.001$.

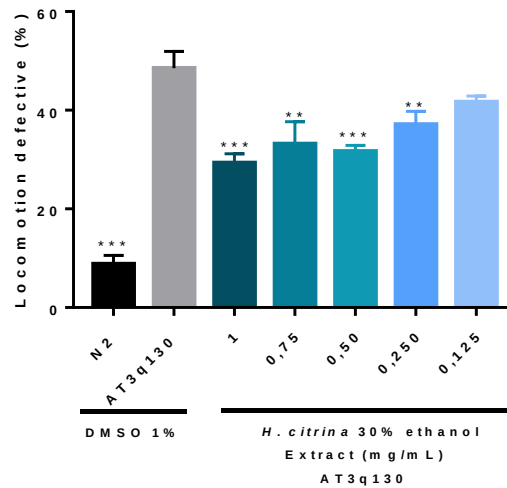


Figure 20 – *H. citrina* 30% ethanol extract improved motor behaviour impairments in AT3q130 animals. The motility assays were performed using N2 (WT) and AT3q130 (MJD model) strains treated with DMSO 1% (drug vehicle) in comparison with AT3q130 animals treated with 1-0,125 mg/mL μ M of extract (n = 3 biological replicates, total of 150 animals per condition). A significant improvement was observed upon treatment with 1, 0,75, 0,50 and 0,25 mg/mL. Graphic represented with Mean $\pm$ SEM. One-way ANOVA; *p<0.05, **p<0.01, ****P<0.0001.

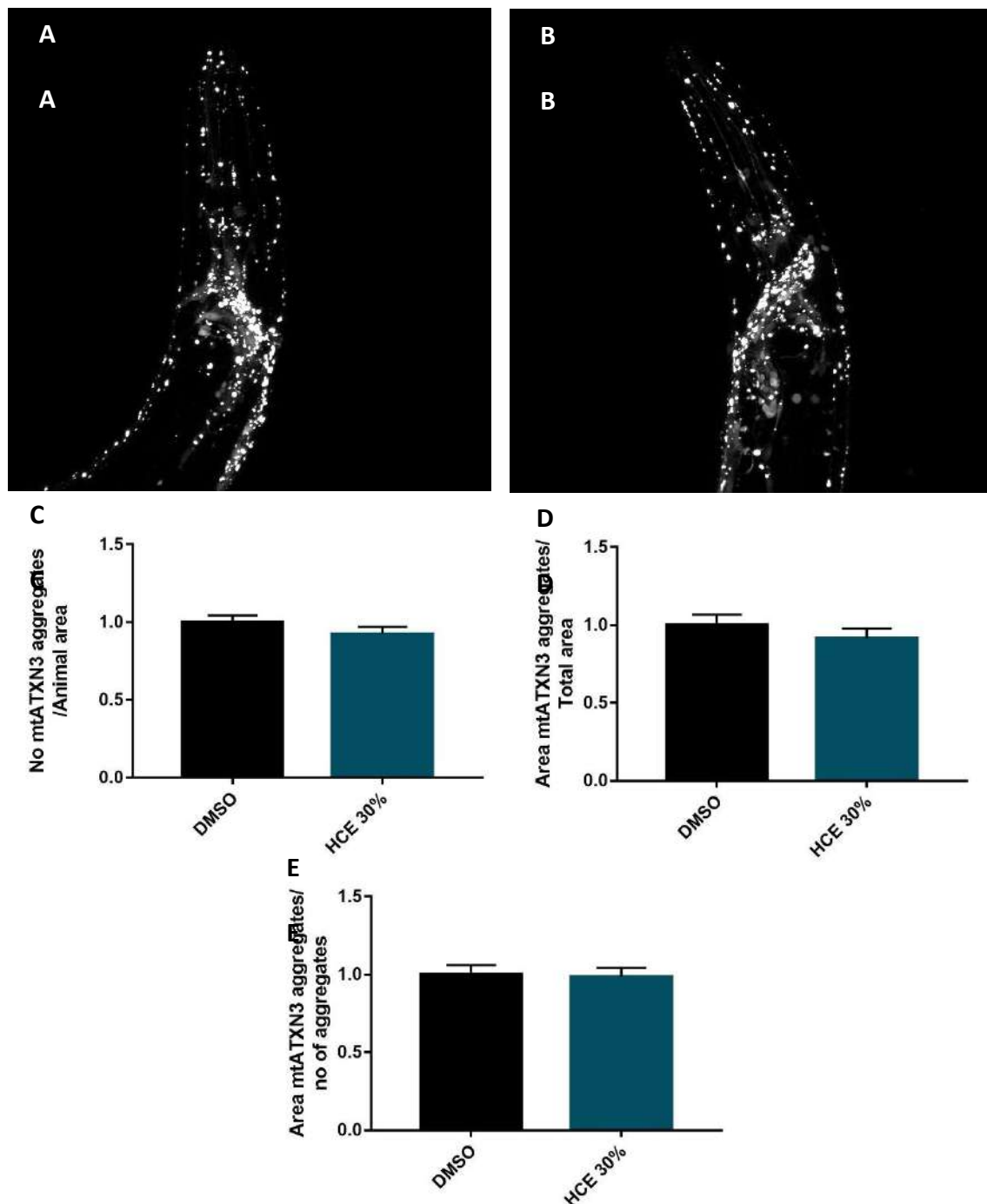


Figure 21 – Treatment of AT3q130 animals with HCE 30% extract was ineffective in changing mutant ATXN3 aggregation in neurons. Representative confocal microscopy images of animals grown in **A**. DMSO 1% and **B**. HCE 30% 1 mg/mL. Treatment with HCE 30% did not impact the three parameters assessed: **C**. the number of mutant ataxin-3 aggregates normalized for the area of the animals' head; **D**. the area occupied by aggregates in the area of the head; **E**. the mean area occupied per aggregate. Graphs show the values obtained normalized for DMSO control condition, represented with Mean $\pm$ SEM. Two-tailed t-test; * $p < 0.05$, ** $p < 0.01$, *** $P < 0.0001$, $n > 30$.

4.2.5. Effect of compound and extract treatments on crawling and swimming behaviours of the *C. elegans* model of MJD

We decided to further understand the discrepancies found concerning the effects of PPT and HCE30% upon evaluation in the automated device Wmicrotracker and in the manually performed motility assay, for the *C. elegans* model of MJD. It is important to refer that WMT evaluates the motor phenotype of a group of animals in liquid medium (swimming activity or “thrashing”) while in the motility assay the motor function of animals is evaluated in solid medium (“crawling”). In the case of the CK10 model, the effect of the compounds/extracts was first evaluated in the WMT, which, in a high-throughput manner, allowed us to quickly find the most effective compounds/extracts. Even so, their effects were mostly confirmed in the motility assay.

For the *C. elegans* model of MJD, and in order to try and understand the discrepant results obtained in the two motility assessment methods, we assessed the effect of two drugs, citalopram (25 μ M) and NLX-112, (5 μ M) previously shown to have a positive impact in the motor phenotype of this model evaluated through the motility assay in solid medium (Teixeira-Castro et al., 2015; Pereira-Sousa et al., 2021). The effect of these drugs was used in the thrashing activity of the model, scored visually by an observer by the estimation of animals’ body bends/30s in liquid medium and automatically using the WMT.

The two methods of analysis distinguished the motor phenotype of the AT3q130 animals, indicating that ataxin-3 expressing animals presented defects in crawling and swimming behaviours, when compared with the WT strain. However, while treatment with citalopram and NLX-112 reduced the motor deficits of the transgenic animals when tested in solid medium (**Figure 22 A**), these drugs were unable to improve their ability to swim in liquid medium, neither upon evaluation in WMT (**Figure 22 B**) nor when scored in the thrashing assay (**Figure 22 C**). These results suggested to us that some treatments can in fact differentially modulate different aspects of the motor function of these animals, improving the crawling capacity but not the swimming behaviour, therefore creating discrepancies between WMT and crawling evaluations.

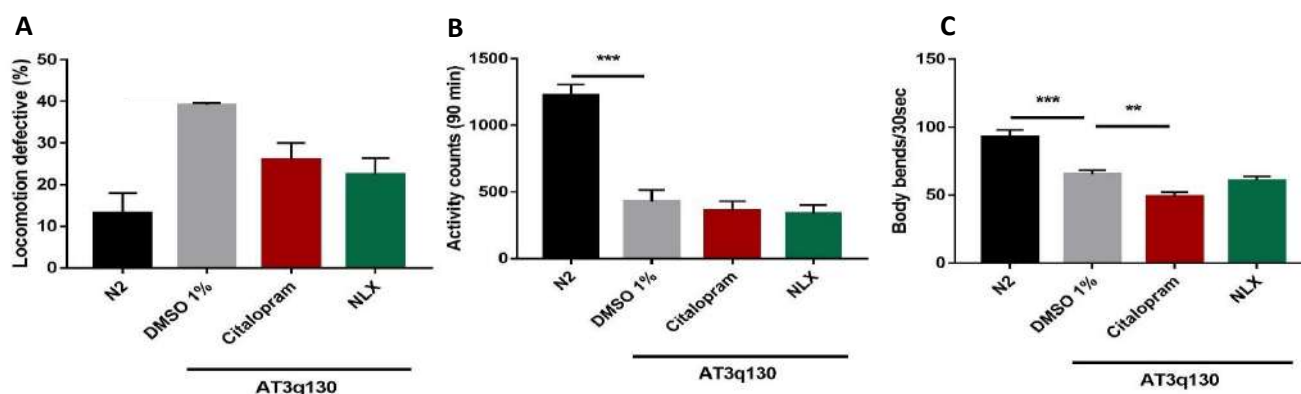


Figure 22 – Analysis of the motor function of AT3q130 animals after treatment with Citalopram (25 μ M) and NLX-112 (5 μ M) using different assays: **A.** Motility Assay (n = 2 biological replicates, total of 100 animals per condition). **B.** WMicroTracker analysis (n = 2 biological replicates, total of 20 animals per condition) **C.** Thrashing Assay (n = 2 biological replicates, total of 400 animals per condition). All graphics represented with Mean $\pm$ SEM. One-way ANOVA; *p<0.05, **p<0.01, **** P<0.0001.

4.3. Mechanism of action of *Hemerocallis citrina* 30% ethanolic extract

Given the promising therapeutic effect of *H. citrina* 30% extract (from now on designated HCE) in models of neurodegenerative diseases with distinct etiology, we attempted to investigate its mechanism of action. Previous studies revealed antidepressant-like effects of *H. citrina* extracts in mice, by increasing the levels of serotonin and dopamine in the brains of the animals. In the same line of evidence, the positive impact of serotonergic signalling modulation by specific serotonin reuptake inhibitors (SSRIs) of known antidepressant effects in humans was previously demonstrated in *C. elegans* and mouse models of MJD (Teixeira-Castro et al., 2015; Esteves et al., 2019; Ashraf et al., 2019). Taking this into consideration, we proceeded to address the question of whether the beneficial effect of HCE was dependent on serotonin and dopamine signalling in the *C. elegans* model of MJD, using double mutants already available in the lab (Pereira-Sousa et al., 2021).

4.3.1. Serotonergic signalling modulation

The relevance of three post-synaptic receptors, SER-1, SER-5 and SER-7 for the beneficial effect of HCE was next addressed in the *C. elegans* model of MJD. SER-1 is a 5-HT₂-like receptor, a G protein-coupled receptor involved in different processes from male-mating behaviour to oviposition. Animals in which SER-1 is lacking (AT3q130; *ser-1* (*ok345*)), kept a similar motor phenotype to the MJD model itself. However, treatment with HCE at a concentration of 1mg/mL did not ameliorate the locomotion impairments of this double mutant as it did in the AT3q130 model, suggesting that SER-1 receptors are essential for the HCE effect (**Figure 23 A**).

Similar results were obtained when the role of SER-5 receptor in the HCE effect was evaluated. SER-5 is also a G-coupled receptor involved in the regulation of oviposition. Ablation of the *ser-5* resulted in a strain that displayed similar locomotor deficits to the AT3q130 model. However, treatment with HCE 1 mg/mL was not effective in restoring the motor function in these animals. This result suggested that SER-5 is also necessary for the beneficial effect of the HCE treatment (**Figure 23 B**).

In *C. elegans* the SER-7 resembles the 5HT₇ receptors in mammals, being involved, among other things, in regulating pharyngeal pumping. Ablation of this receptor in the AT3q130 genetic background prevented the therapeutic effect of HCE treatment, also suggesting that this receptor is necessary for its action (**Figure 23 C**).

Taken together, these results indicated that these three post-synaptic serotonin receptors are completely required for the HCE effect in this model. Next, a similar approach was performed to evaluate the impact of the orthologs of SERT transporter and serotonin auto receptor.

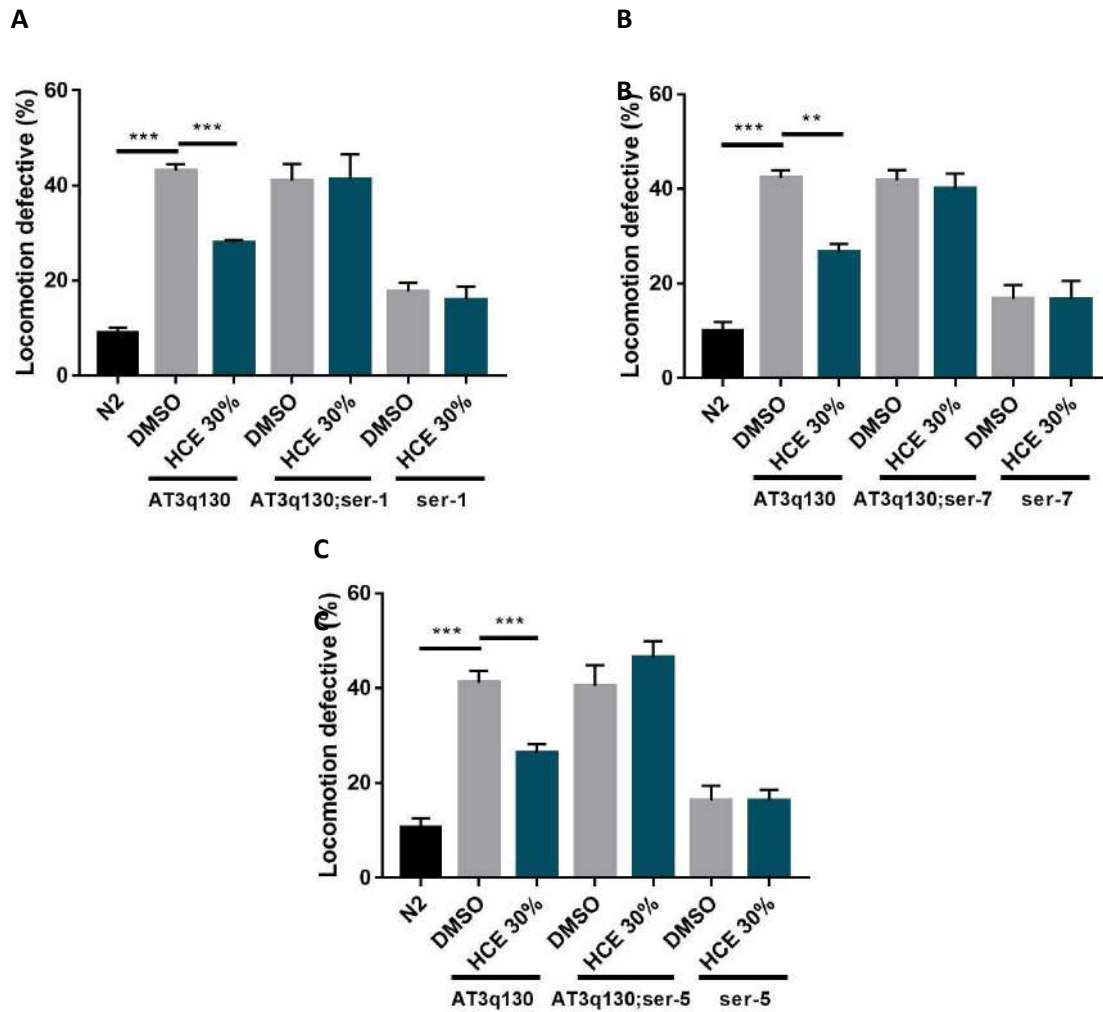


Figure 23 - SER-1, SER-5 and SER-7 receptor ablation blocked the beneficial effect of *H. citrina* 30% ethanol extract treatment in the *C. elegans* model of MJD .

Motility Assay was performed using N2 (WT), AT3q130 (MJD model), **A.** ser-1 and AT3q130;ser-1, **B.** ser-5 and AT3q130;ser-5, **C.** ser-7 and AT3q130;ser-7 strains treated with DMSO 1% (drug vehicle) and 1 mg/mL μ M of HCE (n = 4 biological replicates, total of 200 animals per condition). A significant improvement was observed in AT3q130 animals upon treatment with 1 mg/mL of extract, but not when the receptors were knock-down. Treatment did not improve motor phenotype of ser-1, ser-5 and ser-7 single-mutants. Graphic represented with Mean $\pm$ SEM. One-way ANOVA, planned contrasts; *p<0.05, **p<0.01, *** P<0.0001.

In *C. elegans* the orthologue of the vertebrate pre-synaptic serotonin transporter SERT, responsible for the re-uptake of the neurotransmitter from the synaptic cleft to the pre-synaptic neuron, is named MOD-5. The ablation of this transporter in the AT3q130 background was previously demonstrated to be sufficient, *per se*, to ameliorate the motor dysfunction and aggregation phenotypes (Teixeira-Castro et al., 2015). While treatment with citalopram was unable to further improve the phenotype of this double mutant strain, suggesting MOD-5 is the molecular target of this drug in the nematode as known for humans, the treatment with the steroid hormone estrone, was effective in improving the phenotype of this model, most likely by acting independently from pre-synaptic serotonergic receptors (Teixeira-Castro et al., 2015). This pharmacogenetics approach was also used here to help clarify the role of such receptors for HCE action.

Our data reveals that the treatment of AT3q130 animals lacking the MOD-5 transporter with HCE did not improve significantly the motor function of these double mutant animals, whereas the estrone treatment was still able to further reduce their motor deficits. These results suggested to us that HCE may be also acting through the modulation of the serotonergic signalling by MOD-5.(figure 24).

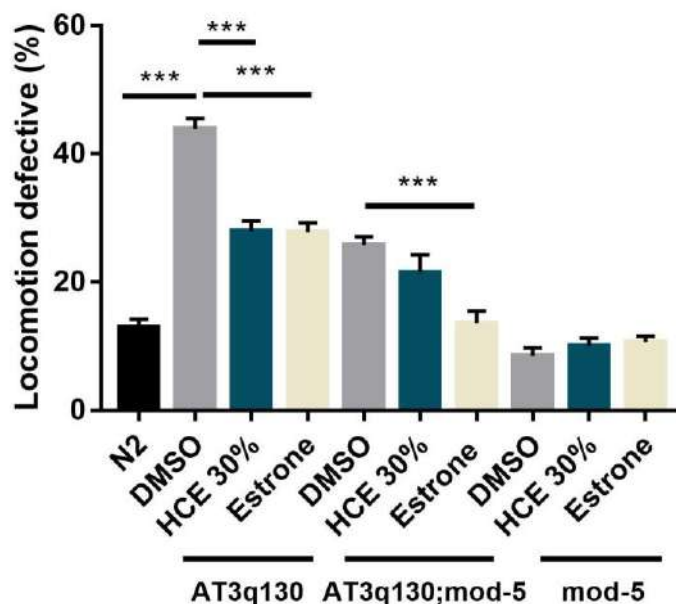


Figure 24 – MOD-5 transporter ablation inhibited the beneficial effect of *H. citrina* 30% ethanol extract treatment. Motility Assay was performed using N2 (WT), AT3q130 (MJD model), mod-5 and AT3q130;mod-5 strains treated with DMSO 1% (drug vehicle), 1 mg/mL μ M of HCE and Estrone (25 μ M) (n = 4 biological replicates, total of 200 animals per condition). A significant improvement was observed in AT3q130 animals upon treatment with 1 mg/mL of HCE and Estrone. Treatment with HCE was not able to reduce the number of AT3q130;mod-5 animals scored as locomotion defective, unlike Estrone which was effective in reducing the motor defects of the worms. Graphic represented with Mean $\pm$ SEM. One-way ANOVA; *p<0.05, **p<0.01, *** P<0.0001.

C. elegans also present a serotonin auto-receptor, SER-4, which is thought to be responsible for the negative feedback and to stop the release of serotonin. Ablation of SER-4 in the *C. elegans* likely leads to an increase of the availability of the neurotransmitter in the synaptic cleft (Teixeira-Castro et al., 2015). As result, AT3q130 animals lacking SER-4 display less locomotion

defects. Also in these experiments, estrone was used as a positive control, since it is thought to act independently of serotonergic signalling.

Treatment of AT3q130; ser-4 animals with HCE was not able to improve significantly the motor function beyond the effect of SER-4 ablation itself, unlike what was seen for the treatment with estrone (**figure 25**).

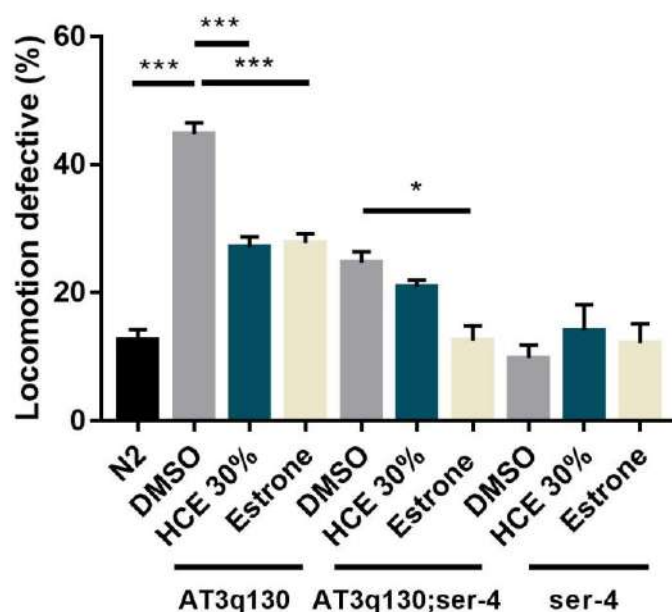


Figure 25 – SER-4 receptor ablation inhibited the beneficial effect of *H. citrina*

30% ethanol extract treatment. Motility Assay was performed using N2 (WT), AT3q130 (MJD model), ser-4 and AT3q130;ser-4 strains treated with DMSO 1% (drug vehicle), 1 mg/mL μ M of HCE and Estrone (25 μ M) (n = 4 biological replicates, total of 200 animals per condition). A significant improvement was observed in AT3q130 animals upon treatment with 1 mg/mL of HCE and Estrone. Treatment with estrone reduced the motor defects of the AT3q130;ser-4 animals, but HCE treatment was not capable to do so. Graphic represented with Mean $\pm$ SEM. One-way ANOVA; *p<0.05, **p<0.01, *** P<0.0001.

Taken together, these results suggested that HCE treatment impact is also dependent on the presence of pre-synaptic serotonergic receptors and transporter. These results strongly supported the hypothesis of HCE treatment exerting its impact on this model through modulation of the serotonergic signalling.

4.3.2. Dopaminergic signalling modulation

The impact of the deletion of dopaminergic receptors was also assessed in AT3q130 animals treated with *H. citrina* extract.

In the absence of the dop-1 receptor, treatment with 1 mg/mL of HCE was still able to reduce the percentage of locomotion defective animals in the *C. elegans* model of MJD suggesting that HCE effect is independent from dop-1 activity. (**figure 26 A**).

Likewise, treatment with 1 mg/mL of HCE was able to reduce the number of worms scored as locomotion defective in both the AT3q130 and AT3q130;dop-3 strains (**figure 26 B**).

Taken together, these results suggested that dopaminergic signalling is not necessary for the effect of HCE on improving the motor impairment mediated by the expression of mutant ataxin-3.

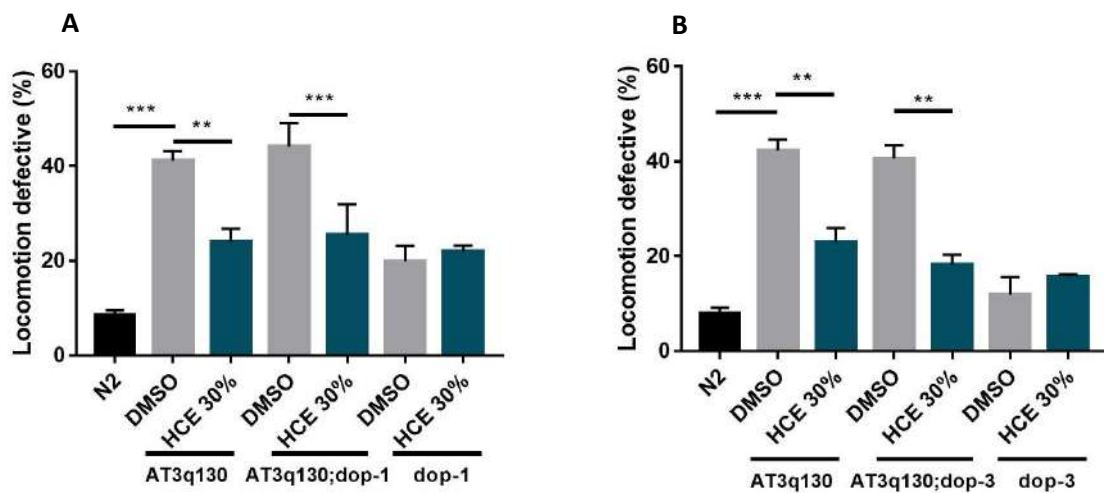


Figure 26 - DOP-1 and DOP-3 receptor ablation did not impact in the beneficial effect of *H. citrina* 30% ethanol extract treatment. Motility Assays were performed using N2 (WT), AT3q130 (MJD model), **A.** dop-1, AT3q130;dop-1, **B.** dop-3 and AT3q130;dop-3 strains treated with DMSO 1% (drug vehicle) and 1 mg/mL μ M of HCE (n = 4 biological replicates, total of 200 animals per condition). A significant improvement was observed in AT3q130 animals upon treatment with 1 mg/mL of extract and on AT3q130;dop-1. Treatment did not improve the motor phenotype of the Dop-1 single mutant either. Graphic represented with Mean $\pm$ SEM. One-way ANOVA, planned contrasts: *p<0.05, **p<0.01, *** P<0.0001.

4.4. Transcriptional and translational activation of cellular adaptative responses upon HCE treatment

To identify pathways potentially activated downstream to the serotonergic signalling modulation, which was shown to be required for the pharmacological effect of HCE 30% treatment, *C. elegans* transcriptional and translational reporter strains of distinct cellular adaptative responses were used. In these strains, fluorescent proteins, such as GFP, are expressed under the regulation of specific gene promoters, enabling to assess the levels of transcriptional induction of those genes under specific conditions. It is particularly informative when these genes are markers of diverse cellular adaptative responses. Alternatively, some of the strains expressed the fluorescent protein fused to a protein of interest, and since the function of the native protein is maintained, information regarding protein function and subcellular localization can be obtained.

4.4.1. Antioxidant Response

The impact of HCE treatment on the activation of endogenous antioxidant pathways in *C. elegans* was ascertained. Thus, *C. elegans* strains able to report the transcriptional activation of *gst-4*, *gcs-1* and *sod-3* genes were chronically treated with HCE and acutely treated with juglone (a ROS inducer and therefore a positive control for oxidative stress response induction).

The *gst-4* gene is a target of the SKN-1 transcription factor and encodes for the enzyme glutathione-S-transferase 4, involved in the metabolism of glutathione (Pohl et al., 2019). According to our results, HCE treatment was not able to induce the expression of *gst-4*, since GFP expression in *gst-4* reporter strain remained unchanged after HCE treatment, although it had increased significantly upon juglone acute treatment, as expected. (**figure 27**).

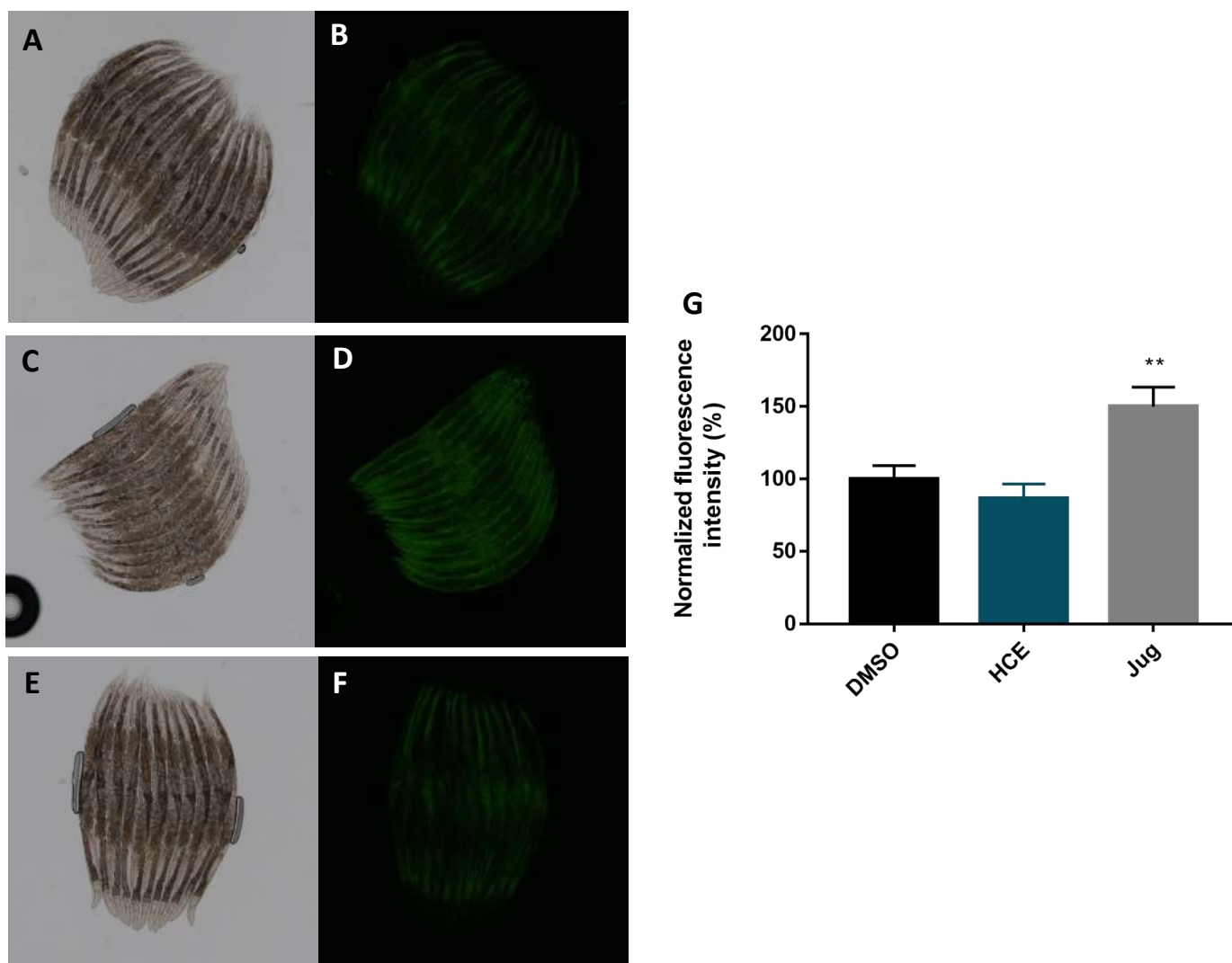


Figure 27 – Impact of HCE treatment in the activation of *gst-4* transcription. (A-F) Representative microphotographs of a strain expressing GFP under the regulation of *Pgst-4*. Brightfield and GFP fluorescence images were acquired in animals **(A and B)** treated with vehicle (DMSO 1%) for 4 days, **(C and D)** grown in vehicle, subjected to juglone for 1 hour and allowed to recover, **(E and F)** treated with HCE 1 mg/mL over 4 days. **G** Representation of GFP fluorescence intensity divided by the total area of each animal and normalized to the vehicle (DMSO) control. HCE treatment was not able to induce GFP expression. Juglone used as positive control. Graphic represented with Mean $\pm$ SEM. One-way ANOVA; * $p < 0.05$, ** $p < 0.01$, *** $P < 0.0001$; $n > 30$.

The activation of the *gcs-1* gene, which encodes for the gamma-glutamyl cysteine synthetase enzyme, involved in glutathione synthesis, was also analysed. This gene is also a target of the SKN-1 transcription factor (van der Hoeven et al., 2011). The *gcs-1*-reporter animals grown in HCE 1 mg/mL showed a 39% increase in GFP intensity, when compared to worms grown in vehicle only, suggesting a mild induction of GCS-1 expression upon HCE treatment (**figure 28**).

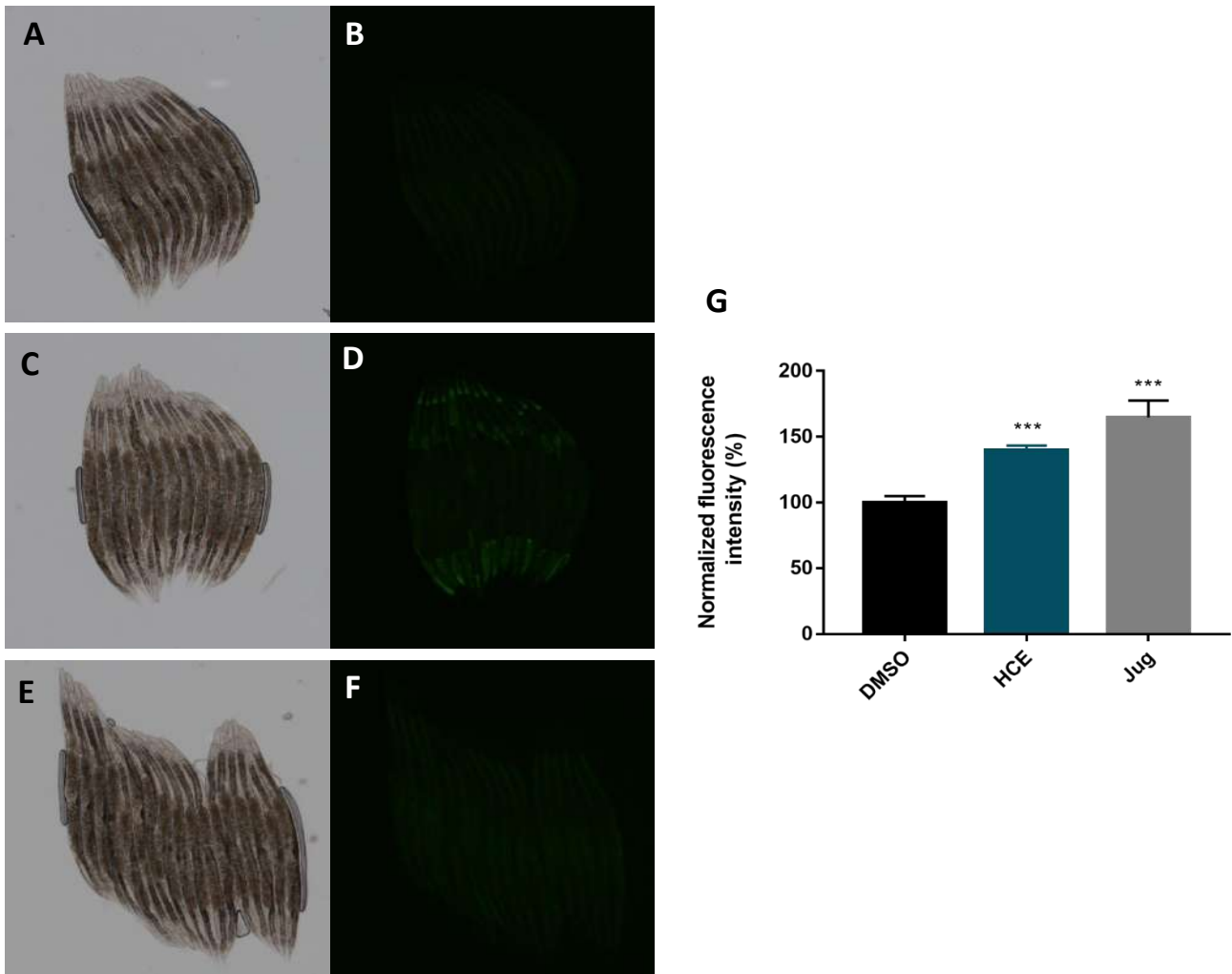


Figure 28 - Impact of HCE treatment in the activation of *gcs-1* transcription. (A-F)

Representative microphotographs of a strain expressing GFP under the regulation of *Pgcs-1*. Brightfield and GFP fluorescence images were acquired in animals (A and B) treated with vehicle (DMSO 1%) for 4 days, (C and D) grown in vehicle, subjected to juglone for 1 hour and allowed to recover, (E and F) treated with HCE 1 mg/mL over 4 days. G Representation of GFP fluorescence intensity divided by the total area of each animal and normalized to the vehicle (DMSO) control. HCE treatment significantly increased the intensity of GFP fluorescence. Juglone used as positive control. Graphic represented with Mean $\pm$ SEM. One-way ANOVA; * $p < 0.05$, ** $p < 0.01$, *** $P < 0.0001$; $n > 30$.

On the other hand, *sod-3* encodes for the mitochondrial manganese superoxide dismutase, involved in superoxide metabolism. The *sod-3* reporter animals treated with vehicle or HCE displayed similar levels of GFP fluorescence, indicating that HCE treatment did not induce the expression of SOD-3 (**figure 29**).

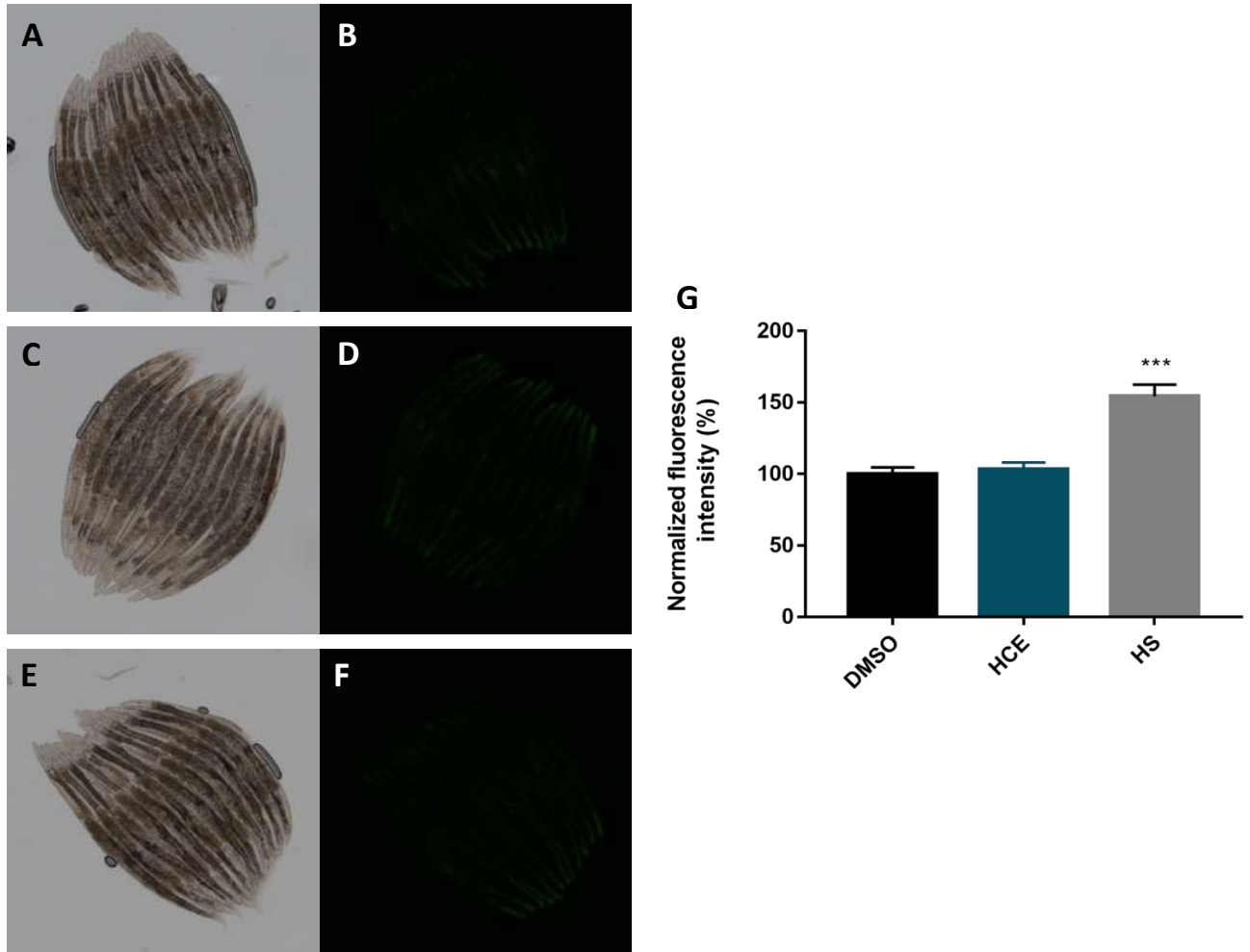


Figure 29 - Impact of HCE treatment in the activation of *sod-3* transcription. (A-F) Representative images of a strain expressing GFP under the regulation of *Psod-3*. Brightfield and GFP fluorescence images were acquired in animals (A and B) treated with vehicle (DMSO 1%) for 4 days, (C and D) grown in vehicle and subjected to heat shock prior to imaging, (E and F) treated with HCE 1 mg/mL over 4 days. G Representation of GFP fluorescence intensity divided by the total area of each animal and normalized to the vehicle (DMSO) control. HCE treatment was not able to induce GFP expression. Heat Shock was used as positive control. Graphic represented with Mean $\pm$ SEM. One-way ANOVA; * $p < 0.05$, ** $p < 0.01$, *** $P < 0.0001$.

Taken together, these results were indicative of a mild antioxidant induction capacity of this extract, only evidenced by the activation of GCS-1, possibly involved in the glutathione synthesis.

4.4.2. Mitochondrial Unfolded Protein Response (UPR^{mt})

The potential activation of the mitochondrial stress response UPR^{mt} by HCE treatment was also investigated. To evaluate the activation of this pathway, we resorted to the SJ4100 strain,

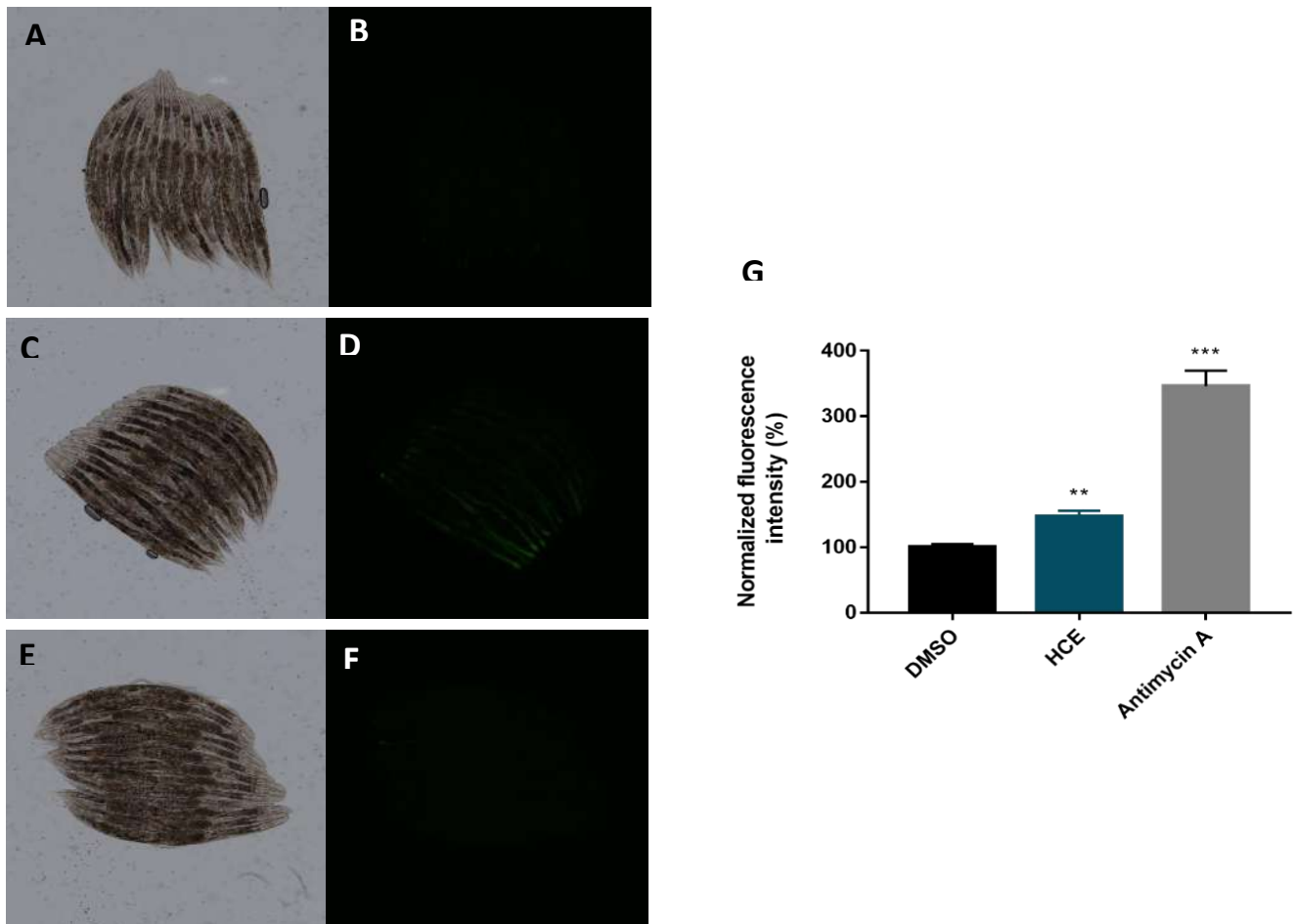


Figure 30 - Activation of hsp-6 transcription after treatment with HCE. (A-F) Representative images of a strain expressing GFP under the regulation of hsp-6 promoter. Brightfield and GFP fluorescence images were acquired in animals (A and B) treated with vehicle (DMSO 1%), (C and D) grown in vehicle and treated with Antimycin A 50 μ M for 24 hours before imaging, (E and F) treated with HCE 1 mg/mL over 4 days. G Representation of GFP fluorescence intensity divided by the total area of each animal and normalized to the vehicle (DMSO) control. HCE treatment induced a mild GFP expression. Antimycin A, an oxidative phosphorylation inhibitor, was used as positive control, inducing a 4-fold change in GFP signal intensity. Graphic represented with Mean $\pm$ SEM. One-way ANOVA; * p <0.05, ** p <0.01, *** P <0.0001.

expressing GFP under the regulation of the *hsp-6* gene promoter, that encodes for a mitochondrial stress-responsive chaperone.

Our data showed a statistically significant increase of GFP signal intensity after HCE treatment (in the order of 47%) (**figure 30**). Even so, the treatment with Antimycin A, an oxidative phosphorylation inhibitor, produced a much more intense increase (four-fold) in GFP signalling, which can suggest that HCE induces a moderate mitochondrial stress response in worms.

4.4.3. Activation of Autophagy

Animals expressing the transcription factor HLH-30 fused to GFP were used to monitor autophagy induction. HLH-30 is an ortholog of the mammalian TFEB, a regulator of several autophagy-related genes. In basal conditions HLH-30 is located in the cytoplasm, as demonstrated by the diffuse GFP fluorescence observed (**figure 31 A;B**). When animals were restricted of food for a period of 30 minutes (starvation), autophagy was activated (Lapierre et al., 2013) and as expected, HLH-30/TFEB nuclear translocation occurred, resulting in a localized GFP signal (**Figure 31 C;D**). Upon chronic treatment with HCE 1 mg/mL, the number of animals presenting a similar fluorescence pattern was much higher, indicating that HCE treatment induced significantly the HLH-30/TFEB nuclear translocation and, therefore, suggesting that autophagy may be activated in *C. elegans*, upon treatment.

Next, and in order to understand whether autophagy activation was the mechanism underlying the neuroprotection observed in the neurodegenerative disease models, autophagy related genes were silenced by RNAi in the treated *C. elegans* models of MJD and FTDP17, genetically mutated to be hypersensitive to RNAi-based silencing in neurons, by the overexpression of the SID-1 transporter in the neurons. The SID-1 transporter is a *C. elegans* transmembrane protein that allows a passive cellular uptake of dsRNA. This transporter is present in every cell of the worm except for neurons, the reason why there is a lack of neuronal response to systemic RNAi. Expression of this transporter in the neurons of the worms enabled dsRNA to be delivered to neurons by feeding and reduced the non-neuronal RNAi (Calixto et al., 2010). The strains LC108 expresses SID-1 transporter in neurons making use of the *unc-119* promoter. The CK10; LC108 double mutant was generated through crossing between CK10 animals with LC108, whereas the AT3q130; LC108 double mutant was already available in the lab. In both cases, the double mutants displayed slightly more severe motor defects when compared to the models *per se*.

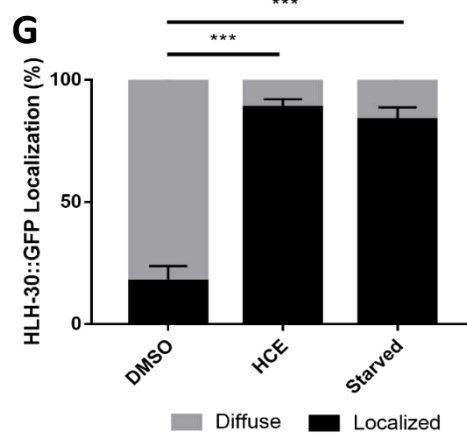
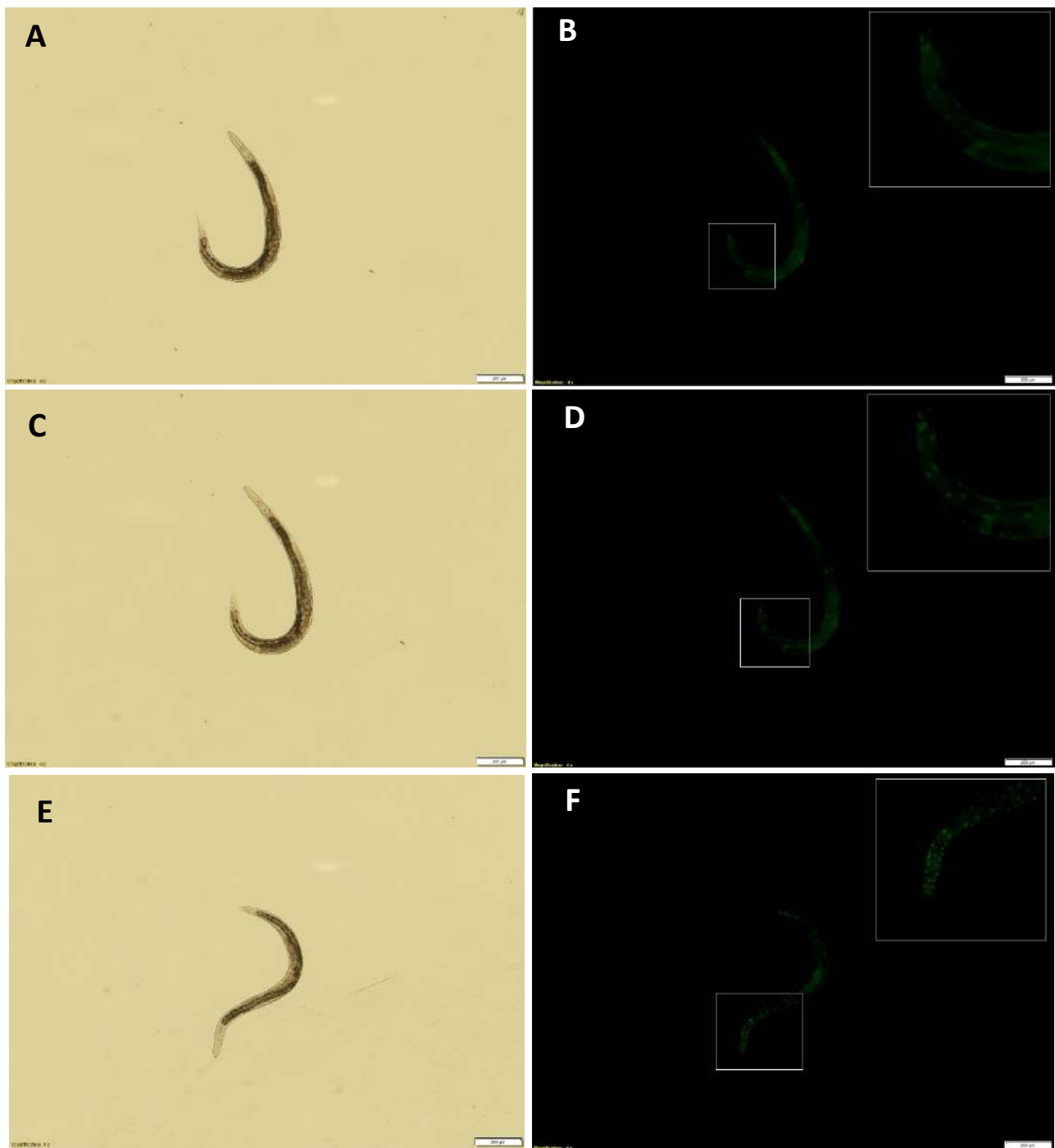


Figure 31 – Translocation of HLH-30 to the nucleus and activation of autophagy by HCE. (A-F) Representative microphotographs of a strain expressing HLH-30 fused to GFP. Brightfield and GFP fluorescence images were acquired in animals **(A and B)** treated with vehicle (DMSO 1%) for 4 days, **(C and D)** treated with vehicle and restricted of food for 30 minutes prior to imaging, inducing autophagy, **(E and F)** treated with HCE 1 mg/mL. Arrows indicate the localized pattern observed upon HLH-30 nuclear translocation. **G** Quantification of the number of animals displaying diffuse or localized GFP fluorescence. HCE treatment resulted in a majority of worms displaying localized GFP fluorescence, indicating HLH-30 nuclear translocation.

In contrast to our expectation, given the results obtained with the autophagy reporter strain, our preliminary data from RNAi this experiment indicates that HCE treatment remained effective in both strains when *hlh-30*, *lgg-1* or *atg-9* genes were silenced. These three genes are involved in different stages of the autophagic flux **(figure 32 A)**, and their silencing should block (partially or totally) this cellular process. In all of the situations, however, treatment with HCE still resulted in a significant reduction of the number of worms scored as locomotion defective in the motility assay **(figure 32 B;C)**. If the efficacy of silencing is confirmed (experiments ongoing), these results would suggest that, although HCE may be inducing autophagy in wild-type animals, neuronal autophagy is either not being induced or it is not required for the neuroprotective effect of HCE in the tested transgenic models of NDs.

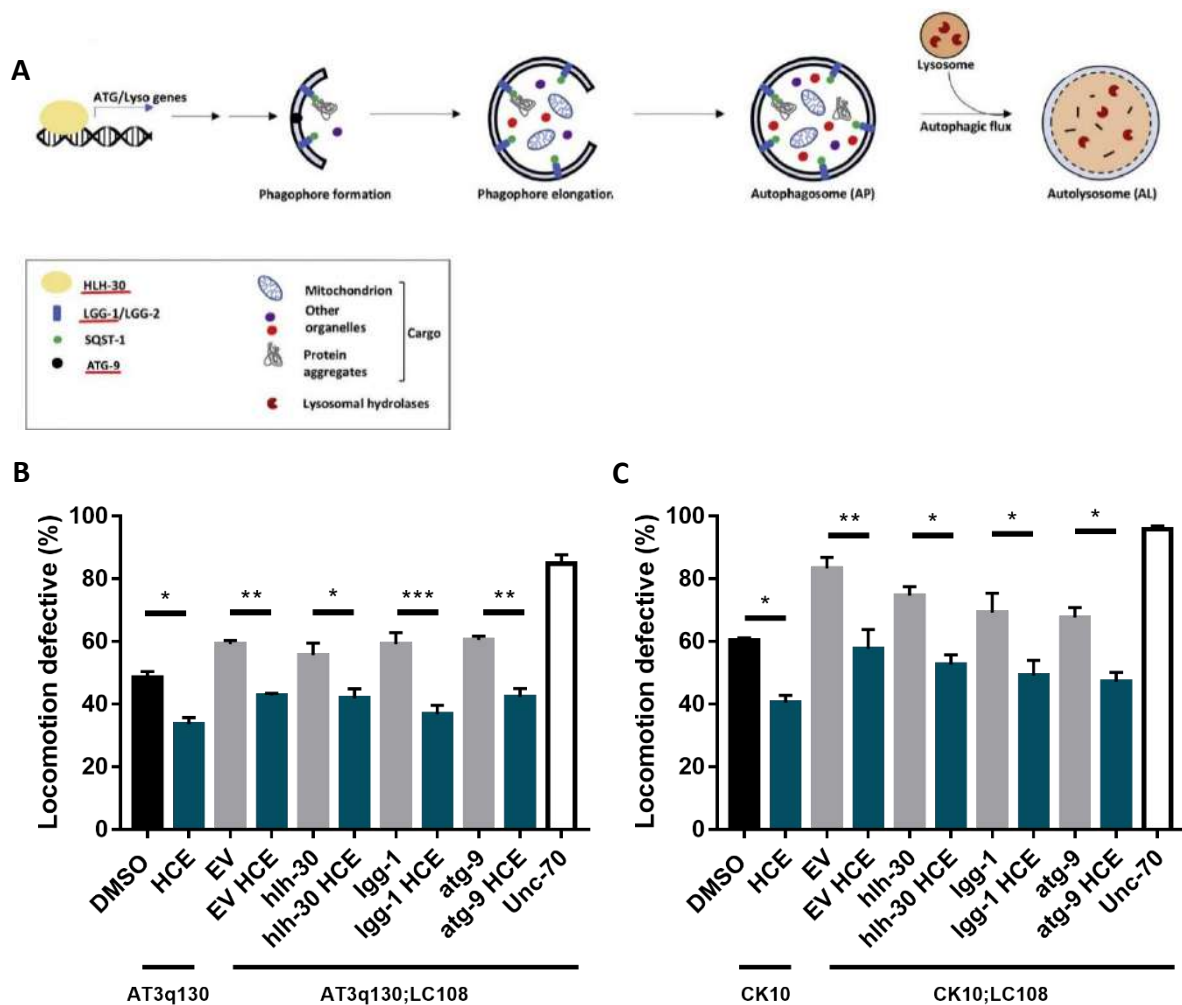


Figure 32 – Neuronal silencing of autophagy did not impact HCE treatment effect.

A. Representative schematic of the molecular pathway of autophagy. Underline in red is represented the genes silenced by RNAi. Adapted from (Wong et al., 2020) **B** Locomotor activity of AT3q130;LC108 animals after RNAi-mediated silencing of *hlh-30*, *lgg-1* and *atg-9* genes, involved in the autophagic flux. AT3q130 animals, treated with vehicle and HCE were used as controls. Treatment remained effective in all conditions assessed, reducing the number of worms scored as locomotion defective. **C** Locomotor activity of CK10;LC108 animals after RNAi silencing of the same genes. CK10 animals treated with vehicle and HCE were used as controls. Treatment remained effective after silencing of all genes. In both cases, RNAi-mediated silencing control was done through silencing of *unc-70*, that resulted in increased motor deficits. Graphics represented with Mean $\pm$ SEM. One-way ANOVA; * $p < 0.05$, ** $p < 0.01$, **** $P < 0.0001$, (n = 3 biological replicates, total of 150 animals per condition).

5. Discussion

In this study we performed an *in vivo* study of a set of extracts and compounds originated from medicinal plants, to assess their neuroprotective effects in transgenic *C. elegans* models of Machado-Joseph Disease (MJD) and Frontotemporal Dementia with Parkinsonism associated to Chromosome 17 (FTDP-17). Although MJD and FTDP-17 are quite different diseases from the clinical standpoint, they share common features, namely protein misfolding and aggregation, oxidative stress, ER stress, among others (reviewd in Strang et al., 2019; Da Silva et al., 2019). These common features of the diseases might indicate that similar pathways are affected in both conditions, thus suggesting that therapies addressing the shared aspects may have beneficial effects in both and perhaps also in other neurodegenerative diseases.

The transgenic *C. elegans* strains chosen for this work have previously been used in other drug screenings to search for potential treatments for these incurable disorders. In particular, the *C. elegans* model of MJD was used in a drug screening that revealed citalopram as an effective treatment, subsequently validated in vertebrate models of this disease (Teixeira-Castro et al., 2015; Esteves et al., 2019; Ashraf et al., 2019). In the same line, different natural products and plant-derived compounds have been also tested in these models, with promising outcomes (Vilasboas-Campos et al., 2020; Pohl et al., 2019). Since both models display motor deficits as a result of the expression of the disease-causing proteins in their nervous system, their motor function was used as a read-out for possible neuroprotective effects (Teixeira-Castro et al., 2015; McCormick et al., 2013). To this end, we started by using an automated device to facilitate the screening of compounds of interest. The WMicroTracker is a device able to evaluate the overall locomotion activity of a worm population in each well of a 96-well microplate and it has been used in several studies to assess the impact of acute treatments in the motility of the *C. elegans* (Simonetta and Golombek, 2007). We developed efforts to adapt the device to assess chronic treatments in these models, and to overcome some of the practical challenges of these chronic exposure studies. In parallel, we also evaluated the effect of some of the treatments on the locomotion of the animals using a method previously adopted in our laboratory to screen for therapeutic compounds for MJD (Teixeira-Castro et al., 2015). It is, however, important to refer that although both methods evaluate the motor function of the worms, they address different motor behaviours. While the WMicroTracker measured the movement of the animal in liquid medium, the Motility Assay surveyed the movement of the animal as they crawl in solid NGM medium.

Our study revealed that the treatment of CK10 model with the *H. citrina* 30% ethanol extract or with Protopanaxatriol displayed an interesting impact on the motor function of these animals, measured in the WMicroTracker and by the motility assay. Although WMicroTracker results failed to show robust and statistically significant improvements on the motor phenotype for some treatments, the subsequent validation steps using the additional motor assay, led us to conclude that increments in the range of 20% in the number of activity counts, when compared to the model, may be biologically relevant, even if not statistically significant. Since it is a new method of analysis that we are trying to implement, we may consider this value informative to select biologically significant conditions in future works.

HCE and Protopanaxatriol treatments also reduced the percentage of mutant ATXN3-expressing worms scored as locomotion defective in the Motility assay, not having demonstrated a modifying effect in the thrashing impairments of the model evaluated with the WMT. This distinct impact of the compounds/extracts towards distinct aspect of the motor function of the model was also observed for other drugs such as citalopram and NLX-112/befiradol, previously proven to be effective in this and other models of MJD, suggesting that such defects are probably more difficult to rescue and therefore are less suitable to be used as a readout in drug screenings. It is also important to refer that, besides its positive impact in motor function of the ATq130 animals, HCE treatment was unable to change the pattern of mATXN3 neuronal aggregation, presenting therefore a distinct effect towards two key aspects of the same disease. This uncoupled effect of a treatment between motility and protein aggregation is not new and has been reported in other studies, in this and other polyglutamine diseases (Teixeira-Castro et al., 2015; Pohl et al., 2019; Beam et al., 2012; Silva et al., 2011).

Plant secondary metabolism is able to synthesize compounds that play specific functions in the plant, namely involving plant defence. These compounds generally have particular chemical and sterical properties which might result in specificity to determined targets in the human body, and thus generating beneficial bioactivities. However, in the past few years, beneficial effects of phytochemicals have also been attributed to the ability to mimic a phenomenon called hormesis. Hormesis is a biphasic response to stress in which low doses of biological stress, just as caloric restriction, might result in the activation of stress response pathways, for instance, dependently from Skn-1/Nrf-2 or Daf-16/FOXO, leading to increased resistance to stronger insults (Martel et al., 2019), However the demonstration of a direct dependency of these pathways for the therapeutic outcome is always necessary (Vilasboas-Campos et al., 2020).

For the particular case of HCE, the literature also revealed that its ethanolic extracts of *H. citrina* originated an antidepressant-like effect in mice. The main effects were thought to be promoted by flavonoids, hesperidin and rutin, present in the extract (Du et al., 2014; Gu et al., 2012). Also, our study pointed to the higher neuroprotective effect of the ethanolic 30% fraction of HCE in comparison with other fractions of the HCE extraction. The thorough identification of the specific compounds in this extract, is therefore of extreme relevance to identify the bioactive components. An High Performance Liquid Chromatography (HPLC) analysis of the composition of the extracts might provide useful insights regarding this matter. Treatment with other extracts from this plant in mice also elevated the levels of dopamine and serotonin in the brain (Du et al., 2014; Gu et al., 2012). In the same line of evidence, our results indicated that serotonergic signalling, mediated by pre- and post-synaptic serotonin receptors, is completely required for the HCE beneficial effect in the *C. elegans* model of MJD, while the action of dopaminergic receptors is irrelevant for the therapeutic effect of this extract. The future evaluation of neurotransmitter levels upon treatment, by HPLC, namely to determine its impact on the levels of serotonin, will add further insights into the understanding of the mode(s) of action of the HCE extract in the nervous system.

The dysfunction of serotonergic signalling has been described in different neurodegenerative diseases, namely in Alzheimer's Disease and Amyotrophic lateral sclerosis (Dorszewska et al., 2017). In MJD, Teixeira-Castro and colleagues have shown that the modulation of serotonergic signalling, through the genetic and pharmacological modulation of the MOD-5 re-uptake channel (ortholog of mammalian SERT), and SER-4 (5-HT_{1A}R orthologue) ameliorated the motor dysfunction of the *C. elegans* MJD model and reduce the aggregation of mtATNX-3, by a still unknown mechanism. Importantly, treatment with the SSRI citalopram was also effective in mouse models of this disease, (Teixeira-Castro et al., 2015; Ashraf et al., 2019). Intriguingly, in *C. elegans*, serotonin was shown to mediate inter-tissue communication of the folding status of the organism, namely in response to the neuronal proteotoxicity caused by mutant PolyQ proteins; in this situation, serotonin was involved in an organismal response that culminated in the improvement of proteostasis in other tissues through the activation of mtUPR, even though the perturbation of proteostasis by polyQ was occurring only in neurons. (Berendzen et al., 2016; Tatum et al., 2015; Zhang et al., 2018).

Accordingly, the HCE treatment in *C. elegans* was also associated with the activation of distinct adaptative responses, albeit to different extents: a moderate transcriptional induction of components of the antioxidant response (namely of GCS-1, involved in the synthesis of glutathione,

a potent cellular antioxidant) (Pohl et al., 2019), a moderate induction of hsp-6 expression (and probably of the mtUPR), as well as a prominent activation of HLH-30, a transcription factor of autophagy-related genes known to initiate this cellular process. This is particularly relevant since oxidative stress, mitochondrial dysfunction and dysfunctional autophagy has been described as important aspects of the pathogenesis of numerous neurodegenerative pathologies, among which MJD and FTDP-17 (Menzies et al., 2015; Son et al., 2012). In agreement, the pharmacological modulation of these pathways has been proposed as a promising disease-modifying strategy (Stacchiotti and Corsetti, 2020). Although our study was strongly suggestive of a mechanistic action of HCE through the modulation of different domains of the proteostasis network with particular relevance to autophagy, the subsequent neuronal ablation of autophagy related genes in the genetic background of both disease models revealed irrelevant for the effect of HCE, failing to prove the dependency of this pathway for its effect, particularly in neurons. Additional studies and validation experiments (such as the confirmation of the gene silencing efficacy) are needed to clarify further this issue.

This study reveals the relevance of plant-derived compounds and natural products as effective therapeutic strategies for distinct neurodegenerative diseases. HCE ethanolic 30% extract treatment showed a potent modifying effect in MJD and FTDP-17 *C. elegans* models, in the first case, dependently from the modulation of the serotonergic signalling, which in conjugation with previous evidence from our team, strengthen the modulation of serotonergic signalling as a promising therapeutic approach for MJD. An effort to clarify further the HCE mechanistic action, revealed the ability of this treatment to also modulate important branches of the proteostasis network, which in the disease context, can help the organism to cope with the proteotoxic stress associated with the expression of an aggregation-prone mutant protein. However, further studies are still needed to identify which mechanisms are key for the HCE effect. In this sense, a broader-range approach, namely a transcriptomic or proteomic analysis of drug vs. vehicle treated animals, could give a more complete and non-hypothesis-based view about the pathways altered upon HCE treatment. The future dissection of the chemical composition of this extract together with further studies focused on the therapeutic effect of its isolated components will be a first step to help us identify the key bioactive molecules.

This study reinforced the relevance of plant extracts and natural products as source of novel therapeutic targets for neurodegenerative diseases.

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7. Supplementary material

Supplementary table 1 - List of every *C. elegans* strain used in this work

Strain	Abbreviation	Genotype
Bristol N2	N2	Wild-type
CK10	CK10	bkIs10 [aex-3p::hTau V337M + myo-2p::GFP]
AM685	AT3q130	rmls263[PF25B3.3::AT3v1-1q130::yfp]
MAC75	AT3q130;ser-1	rmls263[PF25B3.3::AT3v1-1q130::yfp]; ser-1(ok345) X
DA1814 (ser-1)	Ser-1	ser-1(ok345) X
MAC13	ser-5; AT3q130	ser-5 (tm2654) I; rmls263[PF25B3.3::AT3v1-1q130::yfp] II
tm2654 (ser-5)	Ser-5	ser-5 (tm2654) I
MAC38	AT3q130 (WT ser-5)	ser-5(+); rmls263[PF25B3.3::AT3v1-1q130::yfp] II
MAC17	AT3q130;ser-7	rmls263[PF25B3.3::AT3v1-1q130::yfp] II; ser-7(tm1325) X
DA2100 (ser-7)	ser-7	ser-7(tm1325) X
MAC39	AT3q130 (WT ser-7)	rmls263[PF25B3.3::AT3v1-1q130::yfp] II; ser-7 (+) X
MAC72	AT3q130;ser-4	ser-4(ok512) III;rmls263[PF25B3.3::AT3v1-1q130::yfp]
AQ866 (ser-4)	Ser-4	ser-4(ok512) III
MAC73	AT3q130;mod-5	mod-5(n822) I;rmls263[PF25B3.3::AT3v1-1q130::yfp]
MT8944 (mod-5)	mod-5	mod-5(n822) I
MAC171	AT3q130;dop-1	rmls263[PF25B3.3::AT3v1-1q130::yfp] II; dop-1(vs101) X

MAC170	Dop-1	dop-1(vs101) X
MAC168	AT3q130 (WT dop-1)	rmls263[PF25B3.3::AT3v1-1q130::yfp] II; dop-1 (+) X
MAC368	AT3q130;dop-3	rmls263[PF25B3.3::AT3v1-1q130::yfp] II; dop-3(vs106) X
LX703	Dop-3	dop-3(vs106) X
MAC367	AT3q130 (WT dop-3)	rmls263[PF25B3.3::AT3v1-1q130::yfp] II; dop-3 (+) X
JIN1679	Hlh-30	jinEx10 [hlh-30p::hlh-30::GFP + rol-6(su1006)]
LC108	LC108	uls69 [(pCFJ90) myo-2p::mCherry + unc-119p::sid-1]
MAC161	AT3q130; LC108	rmls263[PF25B3.3::AT3v1-1q130::yfp] II; uls69 [pCFJ90(myo-2p::mCherry) + unc-119p::sid-1]
	CK10;LC108	bkl510 [aex-3p::hTau V337M + myo-2p::GFP]; uls69 [pCFJ90(myo-2p::mCherry) + unc-119p::sid-1]
LD1171	gcs-1::GFP	ldls3 [gcs-1p::GFP + rol-6(su1006)]
CF1553	sod-3::GFP	mul84 [(pAD76) sod-3p::GFP + rol-6(su1006)]
CL2166	gst-4::GFP	dvls19 [(pAF15)gst-4p::GFP::NLS] III

Supplementary table 2 – List of Media and Buffers used in this work

Medium/Buffer	Composition
NGM (Nematode Growth Medium)	17 g/L agar, 2.5 g/L bacto peptone, 3 g/L NaCl, 1 mM CaCl ₂ , 25 mM Phosphate Buffer, 1 mM MgSO ₄ , and 5 mg/mL cholesterol
M9 Buffer	3 g/L KH ₂ PO ₄ , 6 g/L Na ₂ HPO ₄ , 5 g/L NaCl, 1 mM MgSO ₄
S-Basal	NaCl 5.85 g/L, K ₂ HPO ₄ 1 g/L, KH ₂ PO ₄ 6 g/L, cholesterol (5 mg/mL in ethanol 100%) 1 mL, dH ₂ O
Trace Metal	1.86 g/L disodium EDTA, 0.69 g/L FeSO ₄ • 7 H ₂ O, 0.2 g/L MnCl ₂ • 4 H ₂ O, 0.29 g/L ZnSO ₄ • 7 H ₂ O, 0.025 g/L CuSO ₄ • 5 H ₂ O
Potassium Citrate	20 g/L C ₆ H ₈ O ₇ • H ₂ O, 293.5 g/L C ₆ H ₅ K ₃ O ₇ • H ₂ O
S-Medium	S-Basal, 10ml/L trace metal, 10mL/L potassium citrate
S-Medium Complete	S-Medium, x100 Penicillin/Streptomycin, x100 Nystatin
Freezing Solution	NaCl 5.8 g/L, KH ₂ PO ₄ 1M (pH=6) 50 mL, glycerol 85% 240 mL, dH ₂ O 710 mL