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BIOACTIVE COMPOUNDS OF PLANTS AS MODULATORS OF HEAT STRESS AND PROTEIN AGGREGATION INDUCED TOXICITY IN THE NEMATODE *CAENORHABDITIS ELEGANS*

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ABSTRACT

The average global temperature increased by climate change represents a new environmental risk factor which influences people, animals and plants, in a complex interconnection that the One Health approach aims to address in order to promote global health. From the human health perspective, heat stress effects on neurotoxic protein aggregation, typical of several neurodegenerative diseases and, particularly, on a small family of abnormally long poly-glutamine (polyQ) repeats induced disorders, are still partially unknown. Simultaneously, plants respond to acute climate stressors enhancing their secondary metabolites production, known as beneficial in humans. This study investigates the protective and antioxidant potential against heat stress of several bioactive compounds of plants in *Caenorhabditis elegans*, thereafter focusing on erucin (ERN), an isothiocyanate naturally present in its oxidized form, sulforaphane, in broccoli, and in its precursor molecule, glucoerucin, in rocked salad leaves. Through the treatment of *C.elegans* strains expressing polyQ in different tissues, this research demonstrates that ERN protects from polyQ-induced toxicity both in standard and under heat stress conditions. Moreover, it evidences that ERN action is dependent on the catalytic subunit of AMP-activated protein kinase (*aak-2*/AMPKα2) and, downstream in this pathway, on the *daf-16*/FOXO transcription factor, since nematodes defective of *aak-2*/AMPKα2 and *daf-16* did not respond to the treatment, respectively.

Additionally, the treatment of *C.elegans* models of Parkinson’s Disease, affected by a different source of neurotoxicity than polyQ disorders, i.e by α-synuclein (α-syn) aggregates, evidences that ERN reduces α-syn-aggregates in standard conditions and improves the motility of worms even after heat stress.

Taken together, these results demonstrate ERN modulation of heat stress and protein aggregates induced toxicity and, at least partially, the pathway involved in its mechanism of action in *C. elegans*, justifying further pre-clinical studies in mice models of protein aggregation to test whether it could represent a potential neuroprotective compound even in humans.

Keywords: erucin; *Caenorhabditis elegans*; neuroprotection; heat stress; protein aggregation; polyQ toxicity; α-syn toxicity; AMPK; *daf-16*/FOXO; One Health

SUMMARY

1. INTRODUCTION

1.1 Climate change: actual impacts and future projections

According to the Intergovernmental Panel on Climate Change (IPCC) Sixth Assessment Report, climate change represents a multifactorial challenge of the 21st century, capable of impacting environments, societies and different aspects of human life and health through various events, such as the extreme weather events, droughts and floods, frequent wildfires, rising sea levels and temperatures (1,2). The IPCC asses that global surface temperature was 1.09 [0.95 to 1.20] °C higher in 2011-2020 than in 1850-1900, and that its rate of increase was faster since 1970 than in any other range of 50 years over the last 2000 years. Increasing greenhouse gases emissions, mainly coming from human activities, which have been estimated to be 12% higher in 2019 than 2010 and 54% higher than in 1990, are likely to have caused a warming of 1.0°C to 2.0°C from 1850-1900 to 2010-2019 (2). Moreover, the Global Annual to Decadal Climate Update issued annually by the World Meteorological Organization predicts that the global mean near-surface temperature for each year between 2024 and 2028 will be between 1.1 °C and 1.9 °C higher than the average over the pre-industrial baseline (years 1850-1900), and that there is 80% chance that it will be 1.5 C° higher than the 1850-1900 average for at least one year by the year 2028. Moreover, it is very likely (86% chance) that at least one year between 2024 and 2028 will be warmer than the warmest year on record (currently 2023) (3). Supporting this prediction, the last global climate summary for August 2024 by NOAA National Centers for Environmental Information confirms this trend, explaining that the August global surface temperature was 1.27°C above the 20th century average of 15.6°C, with Europe having its warmest August on record (4). Even sea level pressure and precipitations accumulated anomalies over the last five years in comparison to the most recent long period average (1991-2020) and the prediction is about low rainfalls over North-East Brazil and an increased chance of wet conditions in the African Sahel, probably due to the warmer North Atlantic (3).

1.2 **One Health: the approach to prevent and mitigate the impacts of climate change**

Considering its broad and multilevel impact, climate change becomes one of the biggest contributors to global change, as it affects a large number of sectors at the human – animal - environment interface, becoming a risk factor for species extinction, disruption of nutrient cycles, zoonosis, re-emergence of vector borne diseases, biodiversity loss, malnutrition, increase of cardiovascular diseases, emerging mental health problems and forced migrations (5). The Lancet Countdown on Health and Climate Change considers infectious diseases, heat stress impacts, the decrease in food quality and in safety index the most impacting risk factor for human health, underlining the interconnection of human, animal and environmental well-being. Therefore, addressing the effect of climate change on human health requires a more comprehensive approach based on the inextricable linkage of humans, animals and their environment and social context. The One Health approach, which aims to the simultaneous protection of humans, animals and ecosystems from climate change impacts, represents an integrated method to develop adaptation and preventive strategies much more efficient and cheaper than the human and animal health sectors working separately (6). More extensively, the term "One Health", firstly used in 2003 with the emergence of the severe acute respiratory disease (SARS), is defined by the US Centers for Disease Control and Prevention and the One Health Commission as "a collaborative, multisectoral, and transdisciplinary approach—working at the local, regional, national, and global levels—with the goal of achieving optimal health outcomes recognizing the interconnection between people, animals, plants, and their shared environment". This emerging concept embraces a wide category of disciplines including environmental and ecosystem health, social sciences, ecology, wildlife, land use, and biodiversity, finding in its interdisciplinarity the strength of an increasingly necessary strategy in the complex context of global change (7,8).

1.3 **Adverse effects on human health under the climate change scenario**

Climate change may negatively impact human health in different aspects, according to the direct and indirect environmental parameters that affects. Several factors, such as heavy rainfall and drought periods, together with temperature and humidity changes, can deeply influence the distribution, reproduction and incubation rates of vector-borne diseases, as demonstrated by the expansion of the African trypanosomiasis, Lyme disease, tick-borne encephalitis, yellow fever and the plague and Dengue (9,10). Even in the recent COVID-19 pandemic, the SARS-CoV-2 virus spread and the mortality rates were affected by air pollution, temperature, humidity and UV radiation (11–15). Changes in weather parameters and extreme weather events, represented by droughts, wildfires, hurricanes and floods, and especially heat waves, were proved to adversely affect humans (16,17). Particularly, high temperatures and heat waves were linked to an increased risk of respiratory and cardiovascular diseases (18,19) and mortality (20), especially for the aging population (21). Moreover, several environmental factors influenced by climate change, such as air pollution and radiations, were reported to be the causative risk for an increased rate of asthma, allergies (22–24) and even cancer (25,26) in children. Recent findings associated climate change with the onset and progression of neurodegenerative diseases (NDDs) (27), underlining a strict relation between the daily mean temperature and an increased death risk for both low and high temperatures (28). Even mental health was affected in 20% of individuals that experienced an extreme weather event, for the direct and indirect effects posed on stress levels by climate change (29), with an increased rate of suicide under high temperature conditions (30). Food security and production are projected to be adversely affected in many steps of the production, together with a higher risk of food borne diseases. Even aquatic systems will be impacted in their biology and ecology, with an accelerated proliferation of waterborne pathogens (31). In general, the interaction between climatic and environmental conditions posed by climate change produce many direct and indirect effects which enhance the risk of several health outcomes, summarized in figure 1.

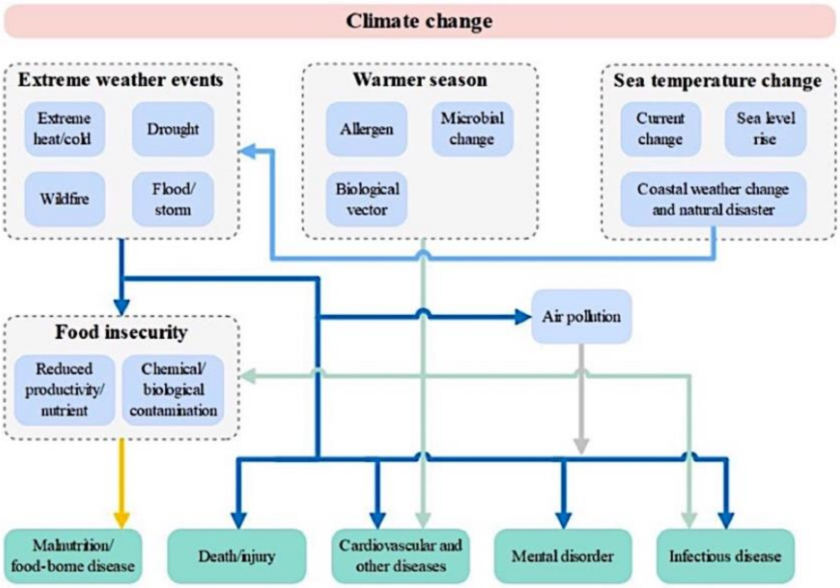


Figure 1. **Main pathways between climate change and health outcomes** (31)

1.4 **Heat stress: an emerging risk factor for several NDDs**

Among all the environmental factors related to climate change, increased temperatures and heat waves are the most dangerous for human health. According to a World Health Organization assessment climate change is projected to increase deaths by 250,000 between 2030 and 2050, 38,000 of which due to heat exposure in the aging population. Prolonged heath exposure may increase disease and death, worsening pre-existing chronic conditions of several cardiovascular (32) and neurological disorders (33), with Parkinson's diseases (PD) related deaths as the most impacted by high temperatures (28). Considering that neurological disorders have been recognized as the second cause of death globally in 2019 (34), an improved investigation on the pathways involved in the heat response under climate change may improve the knowledge about the preventive measures against their onset and progression.

According to epidemiological data, the incidence of neurologic disorders has been enhanced by climate change: both ischemic stroke and PD incidence was reported to be increased by high ambient temperature, even if this was not confirmed for dementia or motor neuron diseases (MNDs) (35,36). Moreover, even the severity of neurological diseases is impacted by climate change as mentioned for ischemic and hemorrhagic stroke and the neurodevelopmental regression in the autism spectrum disorder. Neuro-infectious diseases like tick-borne encephalitis and Lyme disease, coccidioidomycosis and meningitis increased their incidence in relation to elevated temperature and other climate factors (37).

Nevertheless, the neurological disorders impacted by heat stress which raise the highest interest are the NDDs (38). Bongioanni et al. highlighted the neuropathology of the most relevant NDDs, underlying the main processes activated by heat stress under the onset and progression of these disorders (27). When the external temperature is increased by even 1 °C by global warming, an acclimatization process begins activating those pathways that are connected to neurodegeneration, such as oxidative stress, excitotoxicity and neuroinflammation (27,39), which are potentially at the onset and development of NDDs under heat stress conditions (40,41). Excitotoxicity is a complex process induced by an excess in excitatory amino acids receptor activation. It involves the generation of free radicals, an imbalance of calcium buffering, the activation of the mitochondrial permeability transition pore, the degeneration of dendrites and cell death (42). Heat stress can also induce an imbalance between the antioxidant activity and the reactive oxygen species (ROS) production in neuronal cells, inducing oxidative stress and mitochondrial disfunction in several *in vitro* and *in vivo* studies. Moreover, high temperature, by increasing oxidative stress and inducing protein misfolding and aggregates formation, represents an input for the inflammatory and immune response, which play a crucial role in the onset and progression of NDDs. The proinflammatory responses in microglial brain cells imply the release of neurotoxic mediators and inflammatory cytokines which activate the process of brain disfunction, as represented in figure 2 (27).

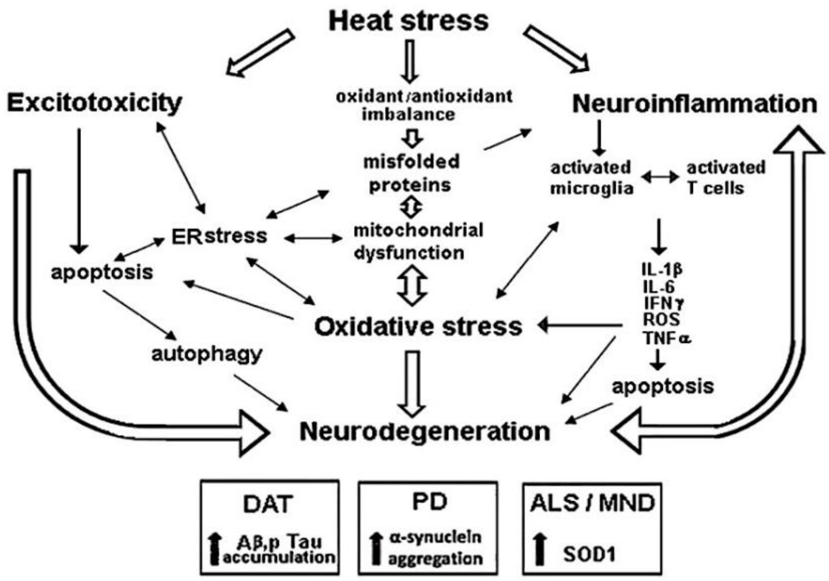


Figure 2. **Scheme of the main heat-affected pathways in brain cells** (27)

1.4.1 ***Protein aggregates: a common source of neurotoxicity in NDDs***

Even if of different nature and causative factors or genes, protein aggregates could be referred as the main source of neurotoxicity common to many NDDs. The term "protein aggregates" refers to a group of protein species that presents a higher molecular weight instead of the one desired for that species (e.g an oligomer instead of a monomer). Generally, they can be differentiated for the type of bond, reversibility, size and protein conformation (43). From the human health perspective, protein aggregates are associated to several disease conditions, both non-neurodegenerative, such as the preeclampsia, cataract and sickle cell anemia (44) and neurodegenerative (45). The aggregates usually consist of fibers containing misfolded proteins, which cause malfunctions in different neuronal types. Protein aggregates may be of different nature as demonstrated by amyloidoses, tauopathies and α- synucleinopathies which are caused by diverse pathways, including prion self-catalytic conformational conversion, mutations altering the protein stability, and uncontrolled pathological increase in the intracellular content of proteins. Alzheimer's disease (AD) with the amyloid-beta (Aβ), PD with the alpha-synuclein (α-syn), Huntington's disease (HD) with the huntingtin protein (Htt), together with the superoxide dismutase (SOD1) in the Amyotrophic Lateral Sclerosis (ALS)/MNDs, share the same hallmark feature and causative factor, represented by protein misfolding and aggregation into cells or into extracellular deposits (27,46–49).

1.4.1.1 *Poly-glutamine* (*polyQ) diseases*

Abnormal expansions of simple sequences in the genome represent a common risk factor for many human diseases, most of all concerning the nervous system. Microsatellites may fall in noncoding DNA regions, inducing both loss- and gain-of-function effects through different pathways, generating potentially aberrant proteins in the latter case. When microsatellite expansions fall within encoding tracts of DNA, they involve trinucleotide repeats, mostly regarding the CAG repeats encoding glutamine. These tracts imply the formation of neurotoxic proteins very prone to lose their folding and to form aggregates, becoming the causative factor of nine NDDs. In some cases, unfolded proteins become part of the inclusion bodies (IBs) , which are composed of the mutant proteins with unrelated proteins sequestered within (50). PolyQ expansions within endogenous proteins represent the same source of toxicity of various NDDs, probably due to the different expression pattern of the involved genes, which imply malfunctions in different neuronal types. Depending on the polyQ tracts length and the genes involved, the caused diseases present several natures and phenotypes: HD, six of the spino-cerebellar ataxias (SCA 1, 2, 3, 6, 7, and 17), the dentatorubral-pallido-luysian atrophy, and the spino-bulbar muscular atrophy (SBMA). Except for the SBMA, which occurs only in men, the other eight polyQ disorders are inherited in an autosomal dominant manner (51–53). Moreover, the length of polyglutamine repeats increases through transmission to subsequent generations and is related to a more recent disease onset and severe phenotype. Whatever the neuronal cell type which is involved, PolyQ diseases imply a progressive deterioration of neurons accumulating protein aggregates within (54). Figure 3 represents a scheme concerning the human diseases caused by abnormal repeat expansions and the region of the DNA involved in each of them.

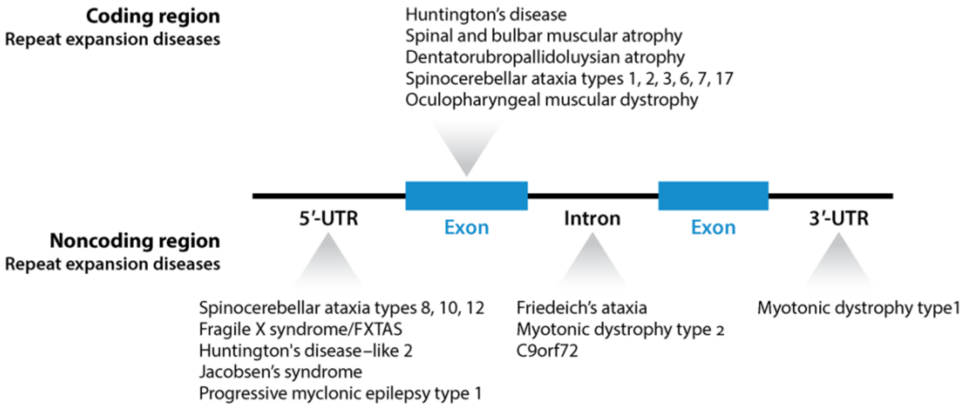


Figure 3. **Microsatellite repeat expansion diseases.** Microsatellite expansions of reiterated sequences of varying length occur in coding and noncoding regions of genes to cause human disease. Noncoding repeat expansions occur in 5′ or 3′ untranslated regions or introns and may encode thousands of repeated units. Coding region microsatellite expansions are restricted to trinucleotide repeats and tend to be more modest in length compared with noncoding region expansions. Abbreviation: FXTAS, fragile X–associated tremor/ataxia syndrome (53).

1.4.2 *The causative links between protein aggregation and heat stress*

The direct evidences of the causative factors for the neurotoxic protein aggregates formation are limited. Nevertheless, in today's scenario, in which humans are exposed to always increasing external risk stressors, understanding the impact of environmental factors, and their interlink with the physiological ones, becomes an increasingly crucial priority in human health science. Physiological parameters such as pH and mechanical stress (including shaking, stirring, pumping, and freeze-thawing) can cause alteration in protein conformation, together with environmental toxicants, such as heavy metals, pesticides and pollutants, that have been associated to a wide range of protein aggregation diseases (55). At the interface between internal (i.e physiological) and external (i.e environmental) parameters involved in protein conformational changes, temperature represents a relevant factor, especially in the global warming scenario, which can influence the physiological temperature of the human body threatening the human thermoregulatory capacity (56).

Generally, temperature is the most influencing factor of protein function, conformation and stability. Both high and low temperatures may cause the folding and unfolding of a given protein, in relation to its proper stability, which is related to its melting temperature, i.e the temperature at which 50% of protein molecules are unfolded during a thermal unfolding transition. In many cases, unfolding promotes aggregation (43,55). Figure 4 explains the cellular impact of environmental stress factors on protein conformation (55).

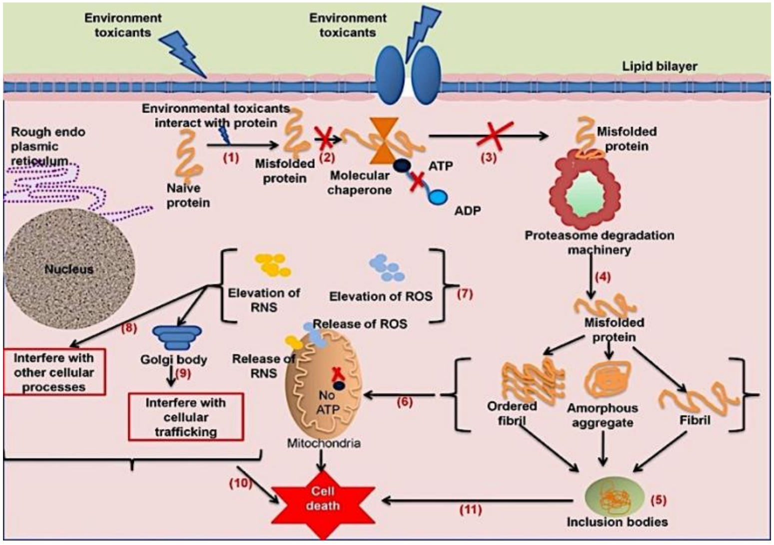


Figure 4. **Cellular impact of environmental stress factors and protein interactions.** Upon exposure to environmental toxicants, the native proteins may lead to misfolding [1], impaired folding machinery, including molecular chaperones and proteasome degradation machinery [2,3]. Collectively these malfunctions in protein homeostasis lead to the accumulation of partially folded, misfolded proteins in the cytosol leading to the formation of larger aggregates in the inclusion bodies [5]. The misfolded or aggregated protein can impair mitochondrial functions [6], generate the reactive oxygen species (ROS), and reactive nitrogen species (RNS) in the cytosol [7]. An increase in inclusion bodies, ROS, RNS further interfere with cellular trafficking [8] and other vital cellular processes (9), causing severe cellular stress and leading to cell death [10,11] (55).

Different types of stress, including heat stress, may cause protein misfolding, producing protein aggregates that the proteasomal and lysosomal system tries to degrade (57). During cell exposure to various stresses, among which warming, the unfolded protein response (UPR) triggers the upregulation of the heat shock proteins (HSPs) (such as the hsp-10, hsp-60 and hsp-70) that function as molecular chaperons and act to restore the conformation of those proteins denaturated by heat, trying to avoid the formation of neurotoxic protein aggregates (27,58).

Nevertheless, when the UPR clearance capacity is exceeded, misfolded proteins tend to form aggregates: under hyperthermic conditions, Aβ peptide and intracellular neurofibrillary tangles composed of hyper-phosphorylated Tau protein, which are the causative factors of AD, form extracellular amyloid plaques, exacerbating the risk and the course of dementia, especially in those which suffer from a circadian dysfunction in core body temperature (59). Similarly, misfolded forms of α-syn forms oligomers in PD patients, that suffer from a spectrum of thermoregulatory symptoms that can be affected by heat stress and heat waves.

Thermal dysregulation in PD patients' brain is based on mitochondrial dysfunction as the main causative source of oxidative stress and, downstream in the pathway, in cell death, lower metabolism and intraventricular temperature (60). In ALS/MND, the progressive death that interests motor neurons is caused even by protein misfolding, principally regarding mutations in the SOD1 gene (46) and by a defective thermoregulation and some mutated genes coding for thermoreceptors (27,61).

1.4.2.1 *Heat stress as a risk factor for polyQ-induced toxicity*

Nowadays, no direct correlation between heat stress exposition and polyQ disorders onset and progression has been reported in literature through epidemiological data, mainly due to the intrinsic rare frequency of these diseases, reported to be ∼1–10 cases per 100,000 people (62). Despite this, there are evidences that may support this hypothesis: considering that the oxidative stress-induced Htt aggregation may be triggered even by heat stress (27,63), the protection from polyQ aggregation cytotoxic effects exerted by hsp-40 and hsp-70 factors and, in general, by the heat shock response (HSR) pathway (27,58,64,65) is well documented. Moreover, HSR activation has been reported to increase the frequency of cell containing IBs in a HD cellular model, suggesting that this pathway does not prevent from the IBs-induced toxicity given by Htt accumulation (66) and both protein aggregation and IBs have been reported to be enhanced by heat-shock in cellular models of HD (67). These *in vitro* results are integrated by the evidence that the regulating mechanisms of proteostasis and particularly the HSR are impaired *in vitro* and *in vivo* models of HD (68–71), mainly for reduced levels of heat-shock factor-1 (HSF-1) (72–74), supporting the hypothesis that a dysfunction in the HSR system may be the causative factor of protein aggregates formation in HD, and extensively polyQ disorders, under heat stress conditions even in humans.

1.5 **Climate change benefits for human health: the greening trend**

Besides the adverse effects of climate change on animals, ecosystems and humans, some beneficial outcomes have been outlined. Extreme rainfall or droughts events can destroy the natural habitat and the eggs of several vector-borne pathogens; global warming can mitigate the negative impacts, and especially deaths, exerted by cold temperatures in certain countries. Some food sectors, as marine fisheries, are projected to be positively influenced in terms of productivity (31). However, the world of plants is the mainly positively impacted by global warming. The higher frequency and duration of warm days increases the period for plant growth and the more concentrated carbon dioxide (CO_2) promotes photosynthesis. One evidence of this effect is given by the greening trend showed by the 25-50% of the vegetated territories, with a prevalence of this phenomenon in high northern latitudes and northern temperate regions (75).

1.5.1 ***Plants secondary metabolites (PSMs) production it's enhanced by climate change***

Plants' endogenous circadian clock is influenced by daily and seasonal environmental fluctuations in temperature, light, humidity, and precipitation, which are strongly influenced by the global changing of climate. Consequently, the overall phytochemical composition, highly adaptive to the plants needs at any point in time, is affected by climate change (76). In particular, PSMs together with antioxidant enzymes are enhanced during stress conditions of various kind, both biotic and abiotic, which led to alterations in primary metabolism in plants and induce a defense response to protect plants from oxidative stress (77,78). They serve also as pollinator attraction. Their concentration in plants is dependent on many variables and even on the leaves dimension: younger leaves present a faster metabolites accumulation and a better response to stresses of various nature (79). Referring to climate change scenario, the environmental parameters which are affecting most PSMs production are elevated CO_2 concentration and high temperature (77,80,81), with a general positive effect in PSMs concentration and protection against short-term environmental stress, although such response is species – specific (82–84) and determined by the optimum temperature for plant's growth and survival, which is different among species. Nevertheless, the HSFA1s are the most activated under heat stress conditions in plants (85).

1.5.1.1 *PSMs impact on human health: the antioxidant role in NDDs*

PSMs provide a wide range of benefits for human health, as they act as valuable bioactive compounds in the human body (86). They are recognized as strong antifungal and antimicrobial; moreover, they are used for their antioxidant and anti-inflammatory activity, and, considering their cytotoxic potential, they are efficient anti-cancer substances. Particularly, antioxidant properties, anticancer and anti-inflammatory activity and cardiovascular protection are recognized to specific phenolic compounds, carotenoids and glucosinolates (GLs) (87–93). More extensively, bioactive compounds produced by plants to face oxidation triggered by biotic and abiotic stressors become the treatment and prevention for oxidative stress induced by genetic and environmental factors in humans, protecting cells and tissues from damages given by inflammatory states, and more generally from the onset and progression of a wide group of diseases, as explained by figure 5 (94).

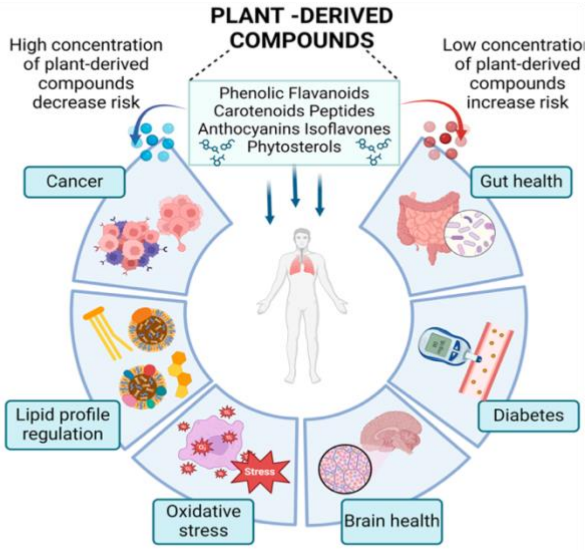


Figure 5. **Multifarious role of plant-based compounds associated with the prevalence of chronic diseases (**94).

Specifically, there is significant evidence that oxidative stress and damage may play a crucial role in the progression and possibly in the onset of several NDDs. This has been demonstrated by several studies for PD, AD, HD and ALS. In general, oxidative stress can lead to protein damage and to toxic aggregates, which in turn enhance the oxidative damage, altering the cellular redox potential and particularly causing mitochondrial dysfunction, in a vicious circle, represented in figure 6 (95,96). Therefore, nutrients and secondary metabolites able to prevent and mitigate oxidative stress, are now of great interest (97,98). The main chemical and biological characteristics of three classes of PSMs will be listed below, with a specific focus on the bioactive natural compounds from plants used in the present research.

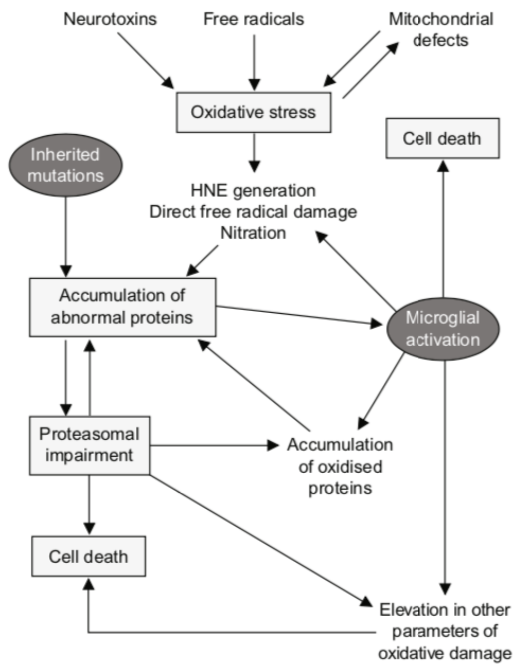


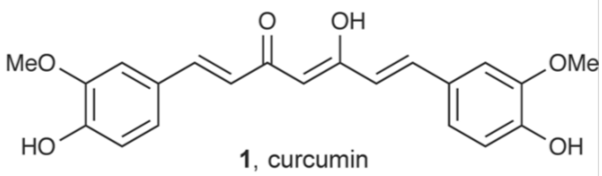
Figure 6. **The vicious cycle of proteasomal impairment, oxidative damage and cell death** (96).

1.5.1.1.1 Phenolic compounds

Phenolics contain a phenol group, an aromatic ring with a hydroxyl functional group. They display many functions from pathogen defense, pollinator attraction, stress tolerance to growth regulation, antioxidant activity and leaves protection. There are several subclasses of phenolics such as flavonoids, anthocyanins and tannins. Flavonoids are the most widespread PSMs with many defense properties in terms of ROS scavenging and membrane stabilization (79). Variable results have been collected about phenolics production under heat stress conditions, depending on pathway of biosynthesis. Indeed, in some species flavonoids are reduced (99–102), while phenolics and anthocyanin content is enhanced under heat stress in other plants (100,103). In any case, considering the ROS scavenging role of phenolic compounds, they act as preventive and protective molecules against the oxidative damage induced by heat stress (79).

1.5.1.1.1.1 *Curcumin*

Turmeric (*Curcuma longa* L.) is a member of the ginger family (Zingiberaceae). The crude extract prepared from it contains 1-6% of curcuminoids, of which 60-70% are represented by curcumin, 20−27% by demethoxycurcumin and 10−15% by bisdemethoxycurcumin. Chemically curcumin is a diferuloylmethane with a crystalline yellow-orange color (104)(figure 7).



1, curcumin
(60-70% of turmeric extract)

Figure 7. **Chemical structure of curcumin** (104).

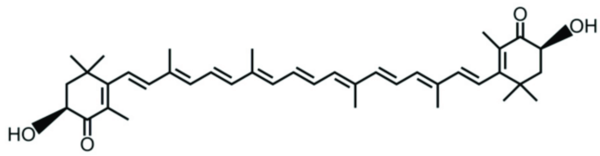
It is already known for its anti-inflammatory, antioxidant and anti-tumorigenesis properties via several pathways, mainly based on the nuclear factor erythroid 2–related factor 2 (Nrf2) and the nuclear factor kappa B (NFkB). These anti-inflammatory and antioxidant properties make the compound a successful neuroprotective treatment against various brain-related diseases, such as cerebral ischemia, intracerebral hemorrhage, AD and PD (105–107). Moreover, it attenuates heat stress-induced oxidative damage (108–110), characteristic that makes it suitable for the purposes of this study.

1.5.1.1.2 Carotenoids

Carotenoids are long combined form of carotenes, which are basically hydrocarbons differentiated into acyclic (lycopene and phytoene), cyclic (α- and β-)-carotene and xanthophylls such as lutein, bixin, capsanthin, zeaxanthin and β-cryptoxanthin. As PSMs they act in light harvestation and photoprotection of plants. Besides giving colors to the flowers and fruits, offering a tool in pollination and seed dispersal through vector attraction, they provide antioxidant protection both in plants and in humans, acting as ROS scavengers and suppressors of a wide range of human diseases (111). Under heat stress conditions of growth, carotenoids total content is reported to be increased (112–114), except in some species in which a decrease in the total content of carotenoids is reported (115).

1.5.1.1.2.1 *Astaxanthin*

Astaxanthin (3,3′-dihydroxy-β, β′-carotene-4,4′-dione) (figure 8) is a xanthophyll carotenoid, found in *Haematococcus pluvialis*, *Chlorella zofingiensis*, *Chlorococcum*, and *Phaffia rhodozyma*. It provides anticancer and antioxidant properties, for which it is considered one of the most powerful available antioxidants. For this reason, it is an efficient tool for the treatment of diabetes, cardiovascular diseases, and NDDs; it also stimulates the immune system (116–118). Even if the data do not accord with the temperature, it has been reported that astaxanthin content is increased under high temperatures of growth, reaching its maximum concentration at 28 °C (119) or 33 °C (120).



Astaxanthin

Figure 8. **Chemical structure of astaxanthin**

1.5.1.1.3 Glucosinolates

GLs are thioglucosides that contain a thiol group and a sugar that is always glucose. They are found in the Brassicaceae family. In plants they act as defensive compounds, especially in their bioactive form represented by isothiocyanates (ITCs), because they protect plants from pathogens and herbivores. In humans they modulate the xenobiotic metabolism, they protect against oxidative stress and they inhibit tumor growth, through the induction of apoptosis and the inhibition of angiogenesis. Moreover, they are protective against heart disease, inflammation and microbial infections (79,121,122). Their concentration is increased under heat stress conditions of growth (123–125).

1.5.1.1.3.1 *Erucin (ERN)*

Chemically ERN is an ITC derived from the enzymatic hydrolysis of glucoerucin, its precursor molecule, found in rocket salad leaves of different species, among which *Eruca sativa* Mill. (also known as salad rocket) and *Diplotaxis tenuifolia* L. (known as wild rocket) are the most representative. ERN is also the reduced molecule of sulforaphane (SFN), its oxidized analog, typical of broccoli (*Brassica oleracea* L. ssp. *italica*). The pharmacokinetic and bioavailability of ERN is represented in figure 9.

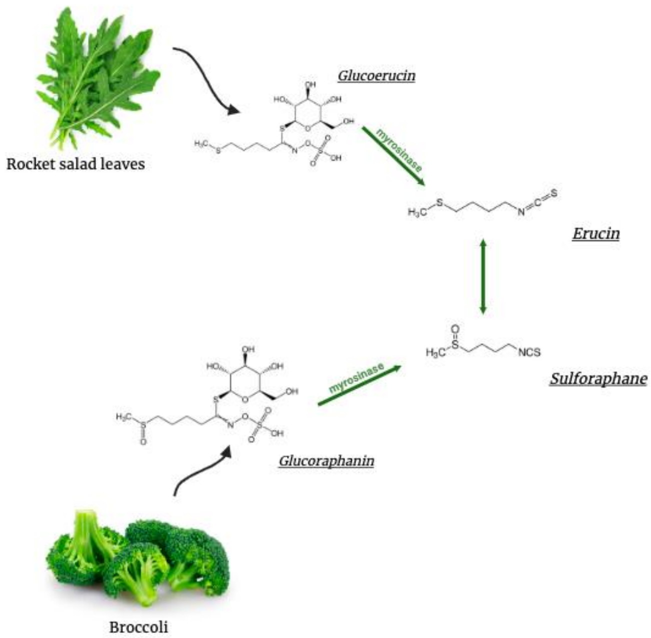


Figure 9. **Pharmacokinetics and bioavailability of ERN.** The isothiocyanate ERN is a reduced analog of the isothiocyanate SFN, and it is formed both from enzymatic hydrolysis of glucoerucin, a GL found in rocket salad leaves (*Eruca sativa* Mill., *Diplotaxis tenuifolia* L.) and from the *in vivo* reduction of SFN, derived from broccoli (*Brassica oleracea* L. ssp italica).

It's recognized for several health promoting properties, ranging from an antidiabetic (126), antioxidant (127) and anti-ulcer activity (128), to anti-nephrotoxic (129), antigenotoxic (130) and chemopreventive (131) actions (132). Moreover, different studies have assessed the neuroprotective role of GLs and ITCs against the progression of several NDDs (131). The choice of this natural compound was justified by the presence of *in vitro* and *in vivo* studies that had already demonstrated its anti-inflammatory, antioxidant and neuroprotective properties, via the c-Jun N-terminal kinases, the extracellular signal-regulated kinase 1/2, and the mitogen-activated protein kinases p38 signaling transduction pathways, able to enhance the nuclear Nrf2 levels (132,134,135). In lipopolysaccharide (LPS)-challenged umbilical vein endothelial cells ERN reduced the increase of ROS, tumor necrosis factor- α, and decreased cyclooxygenase 2, even increasing NFkB levels (136). Moreover, ERN, as a hydrogen sulphide-donor, has exerted neuroprotective effects in an LPS-induced microglia inflammation model and elicited a decrease in nitric oxide levels in AD LPS-stimulated cells (137). Finally, because of their molecular interchangeability, ERN has gained *in vitro* and *in vivo* effects similar to that of SFN in an SH-SY5Y cells simultaneous treatment with 6-hydroxydopamine (6-OHDA) and in a 6-OHDA-PD mouse model, respectively (138). Finally, it has been documented that, even if high growth temperatures (40°C) reduce considerably growth and productivity of rocket cultivars, they increase GLs accumulation, which biosynthesis is inherently tied to the stress responses of all Brassicales plants (139).

2. AIM OF THE STUDY

The impactive effect of climate change on human health and ecosystems and the projections about a progressively increasingly warmer word provide the justificatory basis for researching solutions to prevent and/or mitigate the negative health outcomes induced or worsened by those factors directly and indirectly linked to the changing climate (1–3). Precisely, high temperature and heat waves provoke mental health outcomes and a cascade of cellular events activating those pathways that are connected to neurodegeneration, such as oxidative stress, excitotoxicity and neuroinflammation, increasing the risk for the onset and progression of NDDs (27–30). Moreover, the common causative factor of several NDDs (AD, PD, ALS, polyQ diseases), recognized as the progressive deposition of protein aggregates of various kind and origin (48,49), is reported to be worsened by heat stress, phenomenon which is partly explained by an impairment in the antioxidant response system and in the HSR system (55–61). In parallel, plants, in response to high temperature, activating their defense response, produce PSMs (77), bioactive compounds which can be evaluated, applying the One Health approach, for their well-documented antioxidant capacity and their neuroprotective potential in humans (95–98).

In this regard, the research was articulated in subsequent experimental approaches with specific aims.

Firstly, three natural PSMs already recognized for their antioxidant capacity, curcumin (108–110), astaxanthin (116,117,119) and ERN (133–138), the concentration of two of them (astaxanthin and ERN) is even increased under high temperature conditions of growth (119,139), were selected and evaluated to verify their protective and antioxidant potential under heat stress conditions.

Secondly, ERN, the best-performing compound, was tested for its preventive capacity against protein aggregates induced toxicity (by polyQ and α-syn), both in standard and under heat stress conditions.

Finally, to deepen the mechanism of action of ERN, the signaling pathway of the compound was investigated.

The model organism selected for these purposes was the nematode *Caenorhabditis elegans*, for its intrinsic advantages that will be discussed below.

3. MATERIALS AND METHODS

3.1 **The nematode *C.elegans*: the model organism to study heat stress and protein aggregation induced toxicity**

C.elegans is a very tiny, free-living nematode that can be found worldwide, whose dimensions vary from 0.25 mm in the first larval stage to 1 mm in the adult stage of development. It has a very rapid life cycle of about 3 days at 25 °C and in normal growth conditions it’s a self-fertilizing hermaphrodite, although males arise at a frequency of < 0.2%. In laboratory it grows on agar plates seeded with a thin layer of *Escherichia coli.* Animals grow at temperatures ranging from 12 °C to 25 °C and can be even frozen for years. Temperatures above 25 °C make the worms sterile. Its life cycle, presented in figure 10, develops through four larval stages (L1-L4), before the adult stage. At 20°C, the embryogenesis takes about 16 hours, the L1 stage is about 16 hours long and the other stages are about 12 hours long. Adults produce progeny for 2-3 days; therefore, they live several more weeks before dying of senescence. When food is depleted, worms can enter in the L3 dauer stage, for which the development is arrested. These larvae can survive for months and restart growing when transferred in plates with food. *C.elegans* provides many advantages in the research practice, such as the small size, large brood size, ease of cultivation, low maintenance costs, long-term cryopreservation, quick generation time, transparency and invariant cell number. Moreover, its fully sequenced genome and its stable genetic made it a successful tool for genetics studies, especially because it presents over 60% of homologous genes to human disease genes (140,141). Moreover, as its neuroanatomy has been widely described, many studies have demonstrated that *C.elegans* provides many advantages for the study of NDDs; additionally it represents a model system to study protein misfolding-induced toxicity, the pathways involved and the treatment and/or prevention strategies that may be adopted against these invalidating diseases (142,143). Finally, the capacity to perceive changes in temperature of even 0.01 °C, through a very complex thermosensory molecules and pathways makes this organism a successful model to study heat stress effects on the redox balance and on NDDs. Even if literature is lacking of a standardized protocol for the heat-shock stress in *C.elegans*, reporting different developmental stages, temperatures and times of exposition to the stress, the heat-shock response is a very well-known pathway in *C.elegans*. It involves three main neuroendocrine signaling pathways (144): the nuclear hormone receptor pathway, the transforming growth factor-b pathway, and the IGF/insulin-like signaling (ILS) pathway, the most studied. In stress conditions, the ILS pathway promotes the dephosphorylation and drives the entry of DAF-16 and HSF-1 transcription factors into the nucleus, where they perform their functions (145). Particularly, HSF-1 regulates the heat-shock response inducing the expression of HSPs, which act to maintain cellular proteostasis in eukaryotes and prevent protein and cellular damage induced by stresses of various kind (65,146). Moreover, both DAF-16 and HSF-1, besides regulating protein homeostasis, regulate lifespan of worms (147–150), suggesting the link between the aging process and aggregation-mediated proteotoxicity. Finally, thermosensation in *C.elegans* is fundamental for organismal homeostasis and is regulated by 60 ciliated sensory neurons able to perceive external stimuli and govern ubiquitin-dependent protein turnover (151).

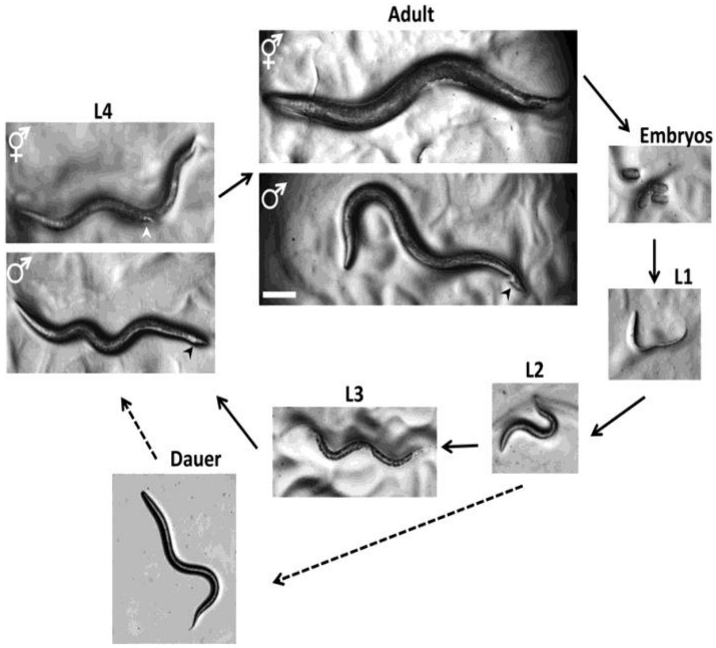


Figure 10. **Life Cycle of *C. elegans*.** Animals increase in size throughout four larval stages (L1-L4), but individual sexes are not easily distinguished until the L4 stage. At the L4 stage, hermaphrodites have a tapered tail, and the developing vulva (white arrowhead) can be seen as a clear half circle in the center of the ventral side. The males have a wider tail (black arrowhead) but no discernable fan at this stage. In adults, the two sexes can be distinguished by the wider girth and tapered tail of the hermaphrodite and slimmer girth and fan-shaped tail (black arrowhead) of the male. Oocytes can be fertilized by sperm from the hermaphrodite or sperm obtained from males through mating. The dauer larvae are skinnier than all of the other larval stages. Photographs were taken on Petri dishes (note the bacterial lawns in all but the dauer images). Bar 0.1 mm (140).

3.2 **Chemicals**

- Astaxanthin was furnished by Laura Pezzolesi, from the Department of Biological Sciences, Geological and Environmental BIGeA, University of Bologna. The astaxanthin stock solution was prepared in dimethyl sulfoxide (DMSO) at 10 mg/mL. The stock solutions were diluted in M9 1X Buffer as solvent to get the requested concentrations of astaxanthin in a maximum of 0.1% DMSO.

- Curcumin was purchased from Sigma Aldrich (Merck Spa) (Via Monte Rosa, 93 - 20149 Milano (MI), Italy). The curcumin stock solution was prepared in DMSO at 20 mM. The stock solutions were diluted in M9 1X Buffer as solvent to get the requested concentrations of curcumin in a maximum of 0.1% DMSO.
- ERN was purchased from LKT Laboratories (LKT Laboratories, St. Paul, MN, USA). The ERN stock solution was prepared in DMSO at 10 mM. The stock solutions were diluted in M9 1X Buffer as solvent to get the requested concentrations of ERN in a maximum of 0.1% DMSO.
- All the other substances and reagents utilized in this study were purchased from Sigma Aldrich (Merck Spa) (Via Monte Rosa, 93 - 20149 Milano (MI), Italy).

3.3 *C. elegans* strains

The strains N2 standard wild-type, AM141: rmIs133[unc-54p::40Q::YFP], AM101: rmIs110[F25B3.3p::Q40::YFP] were obtained from the Caenorhabditis Genetics Center (CGC Elegans) (University of Minnesota, MN, USA). The strains RVM455: rmIs133[unc-54p::Q40::YFP];aak-2(ok524), RVM131: vltEx131[mec-3p::112Q::TdTomato; myo-2p::GFP], RVM584: rmIs110[F25B3.3p::Q40::YFP]; daf-16(mu86), NL5901: pkIs2386 [unc-54p::alphasynuclein::YFP + unc-119(+)] were obtained from the Laboratory of Molecular, Cellular and Genomic Biomedicine, Instituto De Investigación Sanitaria La Fe, Valencia, Spain; Joint Unit for Rare Diseases IIS La Fe-CIPF, 46012 Valencia, Spain.

3.4 Culture and sampling of *C. elegans*

All strains were grown in NGM (0.3% NaCl, 1.7% agar, 0.3% peptone, 5 mg/ml choles-terol, 1 M KPO4, 1 M MgSO4) agar plates and fed with the bacterium *E.coli* OP50 strain (CGC Elegans but cultivated for a while both in the Laboratory of the Department for Life Quality Studies, University of Bologna, Corso d'Augusto, 237, 47921 Rimini (RN), and in the Laboratory of Molecular, Cellular and Genomic

Biomedicine, Instituto De Investigación Sanitaria La Fe, Valencia, Spain; Joint Unit for Rare Diseases IIS La Fe-CIPF, 46012 Valencia, Spain), distributed in a thin layer over the agar. All worm strains were maintained at 20 °C degrees, according to the standard protocol as described elsewhere (152).
For the sake of clarity, the methods described below are subdivided in protocols, according to the different and subsequent steps of this research, described in the aim (see chapter 2) and reported in the results (see chapter 4).

3.5 **Protocol A**

Protocol A is summarized and described in figure 11.

3.5.1 ***C. elegans* strains**

- N2: standard wild-type

3.5.2 **Treatment of *C. elegans* with curcumin, astaxanthin and ERN**

In order to perform the treatment of the N2 wild-type nematodes, two concentrations of curcumin and ERN and three of astaxanthin were selected. NGM agar plates (60 mm) were seeded with *E. coli* strain OP50 as a source of food and with

- 300 µL of 20 µM and 40 µM curcumin. Curcumin solutions were prepared diluting curcumin stock (20 mM) in M9 1X buffer.
- 300 µL of 100 µg/mL, 200 µg/mL and 400 µg/mL astaxanthin. Astaxanthin solutions were prepared diluting curcumin stock (10 mg/mL) in M9 1X buffer.
- 300 µL of 100 µM and 200 µM ERN. ERN solutions were prepared diluting ERN stock (10 mM) in M9 1X buffer.

All the solutions prepared for the treatment contained a maximum of 0.1% DMSO.

In order to perform the treatment of the strains, the concentrations of each substance described above were selected as considered not toxic by lifespan assays performed previously (data not shown).

To treat the worms in solid culture, ten 1-day-old adult worms four days after egg hatching were transferred to NGM agar plates, with or without each of the substances and maintained at 20 °C for 48 hours, until their third day of adulthood, before the heat stress.

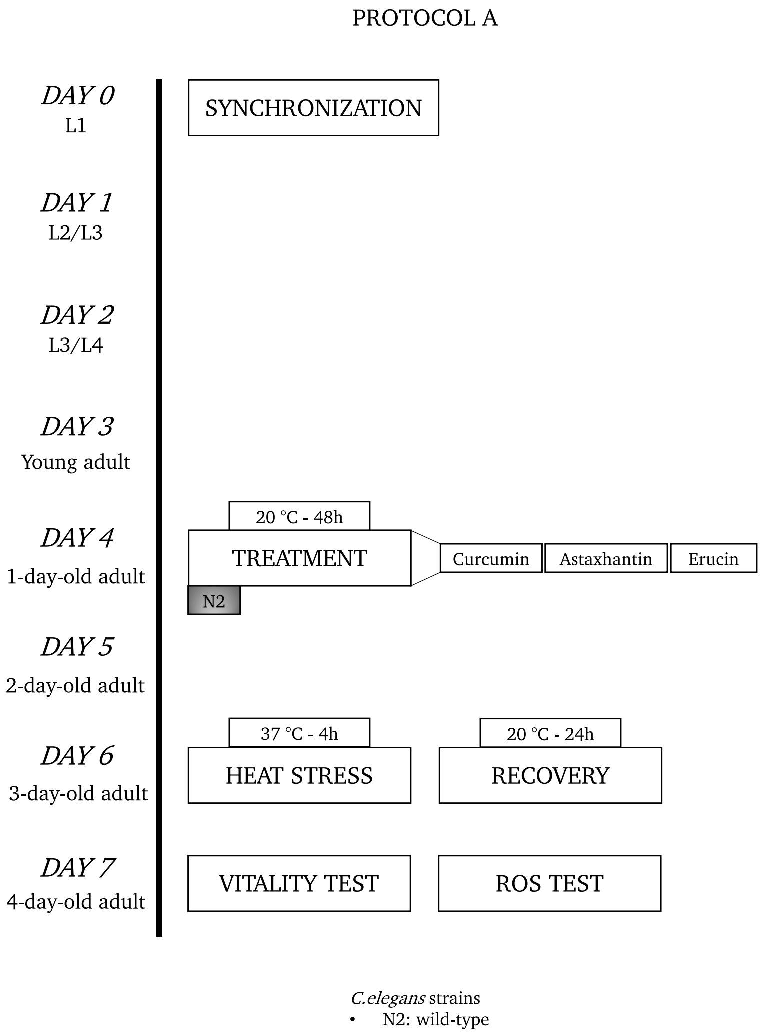


Figure 11. **Experimental protocol A.** In the first day of adulthood N2 wild-type worms are subjected to the treatment with curcumin, astaxanthin and ERN at 20 °C for 48 hours. Subsequently, the treatment is interrupted, and the 3-day old adult nematodes subjected to a 4 hours heat stress at 37 °C, followed by a 24 hours recovery at 20 °C. Finally, the counting of living nematodes and the evaluation of the redox status of worms are performed.

3.5.3 **Heat stress**

After the 48 hours treatment with each compound listed above, a total number of at least 40 3-day-old adult N2 wild-type nematodes, divided in two plates containing 20 worms each, were selected and transferred in NGM agar plates seeded with *E. coli* OP50 strain. Each plate was placed in an incubator and subjected to a 4 hours heat stress at 37 °C, according to one of the most frequent heat shock protocols, despite the many variations exposure temperatures and times reported in literature (153). Therefore, the nematodes were placed at 20 °C for 24 hours for recovery. At least 100 3-day-old adult nematodes per concentration of each compound were subjected to heat stress, subdivided in three independent experiments.

3.5.4 **Viability test**

The test relies on counting the still living nematodes in each considered plate. The nematodes that responded to the stimulus given by a slight touch with a platinum wire were considered alive. At least 40 4-day-old adult animals in total per condition were analyzed in three independent experiments, for a total number of at least 100 treated/untreated nematodes.

3.5.5 **Detection of intracellular ROS**

The nematodes of each plate were collected by adding 1-1.5 mL M9 1X buffer to each plate and gently shaking it to allow the buffer to distribute uniformly. Then, by tilting the plate, the worms were collected with a pipette and transferred in a 1,5 mL tube. Each tube was centrifuged at 6.6 rpm at ambient temperature for 10 minutes and the M9 buffer removed, without aspirating the nematodes on the bottom. The wash was repeated two more times for each tube. Under the yellow light, the working solution of 2′,7′- dichlorodihydrofluorescein diacetate (H_2DCFDA) 100 µM was prepared by diluting the stock solution (5mM) in M9 buffer, according to a solution concentration considered effective to enter the membrane of the nematodes, as described elsewhere (154). The nonpolar and non-ionic probe, H_2DCFDA, can easily penetrate the cellular membrane and is enzymatically deacetylated by esterases. This

biochemical reaction converts H_2DCFDA into the non-fluorescent compound 2′,7′-dichlorodihydrofluorescein (H_2DCF) which is then rapidly oxidized to highly fluorescent 2’,7’-dichlorofluorescein (DCF) in the presence of ROS.
After a vortexing of the working solution, 500 µL of H_2DCFDA solution was added in each tube and mixed with the pipette to let the solution distribute uniformly. After 1 hour of incubation in the dark at 20 °C, the working solution was removed from each tube per experimental point, taking care not to aspire the worms on the bottom. Therefore, at least two washes with 1 mL of M9 as described above were executed. Finally, ~5 µL of solution containing worms were mounted for microscopy on a glass slide, nematodes paralyzed with a little drop of 10 mM sodium azide and analyzed through fluorescence microscopy with Nikon Eclipse Ti-U Inverted Fluorescence Microscope at 20x magnification. The intensity of fluorescence was quantified for the region of interest (ROI) (an area within the image in which performing the quantification) selected and was dependent on intracellular oxidation of the deacetylated H_2DCFDA, the non-fluorescent compound H_2DCF, by superoxide/ROS to form highly fluorescent DCF in the presence of ROS. 10 untreated/treated 4-day-old adult animals in total per condition were analyzed and each experiment was executed three times, for a total number of at least 30 nematodes.

3.5.6 **Fluorescent microscopy imaging**

Images were collected using Nikon Eclipse Ti-U Inverted Fluorescence Microscope and the NIS-Elements Basic Research software. Live animals were selected and positioned above 2% agarose pads inside a drop of 10 mM sodium azide to anaesthetized them. The analysis and ROS quantification were performed in the selected ROI through the NIS-Elements Basic Research software and the fluorescence of the negative control was set as 1.

3.6 **Protocol B**

This protocol was performed both in standard conditions (figure 12), meaning with this term the experiments performed at 20 °C, and under heat stress (figure 13), i.e

applying a heat shock to worms as detailed later, with the strains listed below. For the sake of clarity, this chapter will be divided into two sections, in accordance with the application of the same protocol under two different temperature conditions to which the nematodes were subjected after the treatment with ERN.
The first section, at paragraph 3.6.1, is relative to the protocol performed in standard conditions, and the second one, at paragraph 3.6.2, is relative to the protocol which included heat stress, for a more detailed description of which please refer to the corresponding paragraphs.

3.6.1 **Standard conditions**

The protocol performed in standard conditions, i.e at 20°C, is summarized and described by figure 12.

3.6.1.1 ***C. elegans* strains**

- RVM131: vltEx131[mec-3p::112Q::TdTomato; myo-2p::GFP]
- AM141: rmIs133[unc-54p::40Q::YFP]
- AM101: rmIs110[F25B3.3p::Q40::YFP]
- NL5901: pkIs2386 [unc-54p::alphasynuclein::YFP + unc-119(+)]
- N2: standard wild-type

3.6.1.2 **Treatment of *C. elegans* with ERN**

In order to perform the treatment of the strains, two ERN concentrations (100 µM and 200 µM) were selected, as considered not toxic by lifespan assays performed previously (data not shown).
NGM agar plates (60 mm) were seeded with *E. coli* strain OP50 as a source of food and with 300 µL of 100 µM and 200 µM ERN. ERN solutions were prepared diluting ERN stock (10 mM) in M9 1X buffer.
To treat the worms in solid culture, ten 1-day-adult worms 4 days after egg hatching were transferred to NGM agar plates, with or without ERN at the two selected

concentrations, and allowed to lay eggs for 4 hours. Then, the adult nematodes were removed. Five hours later—with small variations depending on the worm strain—larvae at stage 1 (L1) hatched. After the development of young adults, approximately 3 days later, the number of polyQ aggregates on the 40Q::YFP worms was scored and the touch response on 112Q::TdTom nematodes assessed, as these worms start accumulating aggregates in this stage of development. Moreover, as worms expressing α-syn construct tend to form aggregates lately, the number of α-syn aggregates was scored and motility evaluated in 2-day-old adult animals, approximately 5 days after hatching of eggs. Worms were maintained at 20 °C until they reached young adulthood (40Q::YFP and 112Q::TdTom worms) or the 2-day-old adult stage (α-syn::YFP worms).

3.6.1.3 ***In vivo* scoring of polyQ aggregates in muscle cells**

The production of muscular polyQ aggregates induced by the 40Q::YFP transgene is age dependent and can be evaluated through a dissecting microscope equipped with fluorescence. At least 10 young adults were analyzed for each genotype and each independent experiment for each condition (untreated/treated worms) using an M165FC Leica dissecting microscope (Leica, Wetzlar, Germany). The analysis was repeated in three independent experiments for a total number of at least 30 worms.

3.6.1.4 ***In vivo* scoring of polyQ aggregates in the ventral nerve cord**

The production of neuronal polyQ aggregates induced by the 40Q::YFP transgene is age dependent but is not appreciable through a dissecting microscope. For this reason, the analysis was performed with THUNDER-equipped Leica DM6 B Upright Microscope with the Leica DMC5400 Camera and the LAS X Software. At least 10 young adults were analyzed for each genotype and each independent experiment for each condition (untreated/treated worms). The analysis was repeated in three independent experiments for a total number of at least 30 worms.

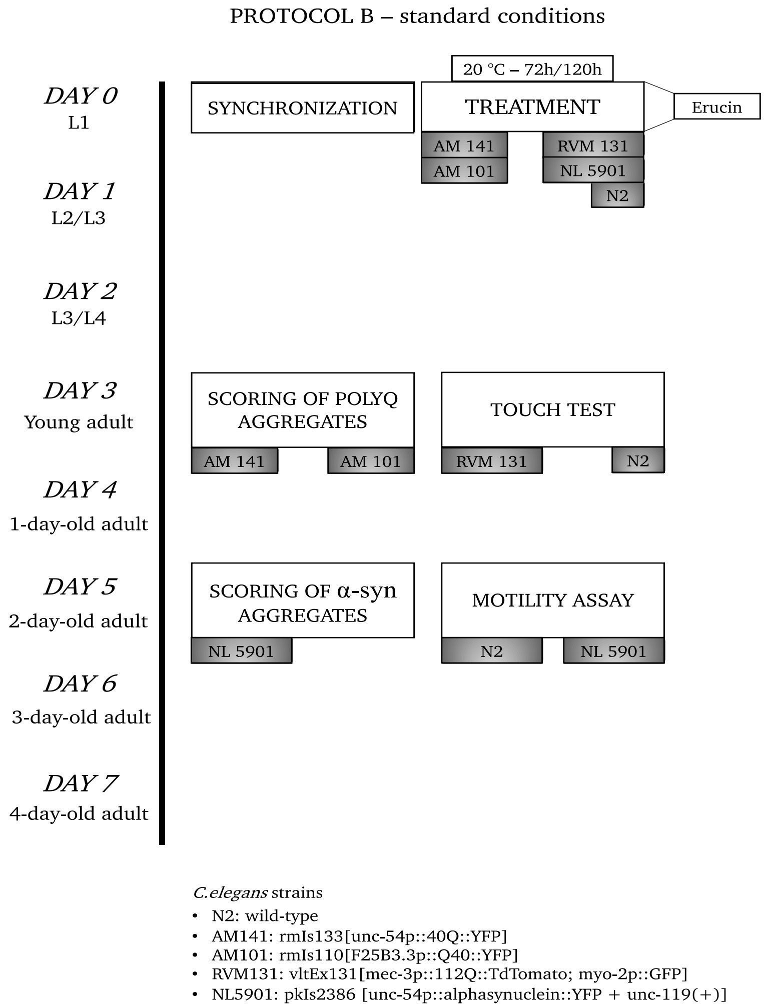


Figure 12. **Experimental protocol B – standard conditions.** The same day of synchronization, the treatment with ERN on L1 worms at 20 °C for 72 hours (N2, AM141, AM101, RVM131) and for 120 hours (N2, NL5901) is started. Subsequently, when the treatment is interrupted, the young adult nematodes are subjected to the scoring of polyQ aggregates both in muscle cells (AM141) and in the ventral nerve cord (AM101), and to the touch test (RVM131, N2), while the 2-day old worms are subjected to the scoring of α-syn aggregates (NL5901) and to the motility assay (N2, NL5901).

3.6.1.5 **Evaluation of the touch response**

The accumulation of polyQ aggregates in mechanosensory neurons produces a touch response dysfunction in the 112Q::TdTomato worms (155). Positive responses to a light touch in these neurons induce the worms to move forward. The touch response was recorded by gently touching the posterior part of the nematodes trough an eyelash fused on the top of a pipette tip. Each animal was touched ten times and the response counted as positive when each worm moved forward. Here the result is presented as the percentage of times each animal responded to the touch, as described elsewhere (156). Each test was repeated on 10 worms in three independent experiments both on the 112Q::TdTomato strain and on the N2 wild-type nematodes for each condition (untreated/treated worms), for a total number of at least 30 nematodes per strain. The touch response phenotype was scored using a Leica MS5 dissecting microscope.

3.6.1.6 ***In vivo* scoring of α-synuclein aggregates**

Differently from polyQ worms that express aggregates in muscular and neuronal cells already in the young adulthood, animals carrying the α-syn::YFP transgene, accumulate aggregation in old worms (2-day-old adult animals or older). Therefore, the treatment with 100 μM and 200 μM ERN was prolonged until the second day of adulthood of worms and the analysis performed, selecting the area between the two pharyngeal bulbs, since the counting of aggregates in the whole muscle tissue is not feasible, as described elsewhere (157). The assay was performed with the THUNDER-equipped Leica DM6 B Upright Microscope with the Leica DMC5400 Camera at 63x magnification. The test included 10 untreated/treated 2-day-old adult animals and each experiment was performed three times, for a total number of at least 30 nematodes per condition.

3.6.1.7 **Motility assay**

To evaluate the effect exerted by ERN on the motility capacity of worms expressing the α-syn::YFP gene, the behavior of nematodes was assessed by counting the number of thrashes per treated animal for 30 seconds and extrapolating the average data to represent thrashes per minute. Before scoring, each animal was immersed in M9 1X buffer solution for 30 seconds to let it acclimatize to the liquid medium, as described elsewhere (157). At least 10 untreated/treated 2-day-old adult animals were analyzed per each condition. To confirm the data, and to assess that ERN did not induce any toxic effect on the motility capacity of worms in any of the concentrations selected, the same analysis was executed even on the N2 wild-type strain at the same stage of development.

Each experiment was performed three times to obtain reproducible values, for a total number of at least 30 animals per each condition and strain.

3.6.1.8 **Fluorescent microscopy imaging**

Images were collected using THUNDER-equipped Leica DM6 B Upright Microscope with the Leica DMC5400 Camera and the LAS X Software. Live animals were selected and positioned above 2% agarose pads inside a drop of 0.25 M sodium azide to anaesthetized them.

3.6.2 **Heat stress conditions**

The protocol which included heat stress is summarized and described by figure 13.

3.6.2.1 ***C. elegans*** **strains**

- AM141: rmIs133[unc-54p::40Q::YFP]
- RVM131: vltEx131[mec-3p::112Q::TdTomato; myo-2p::GFP]
- NL5901: pkIs2386 [unc-54p::alphasynuclein::YFP + unc-119(+)]
- N2: standard wild-type

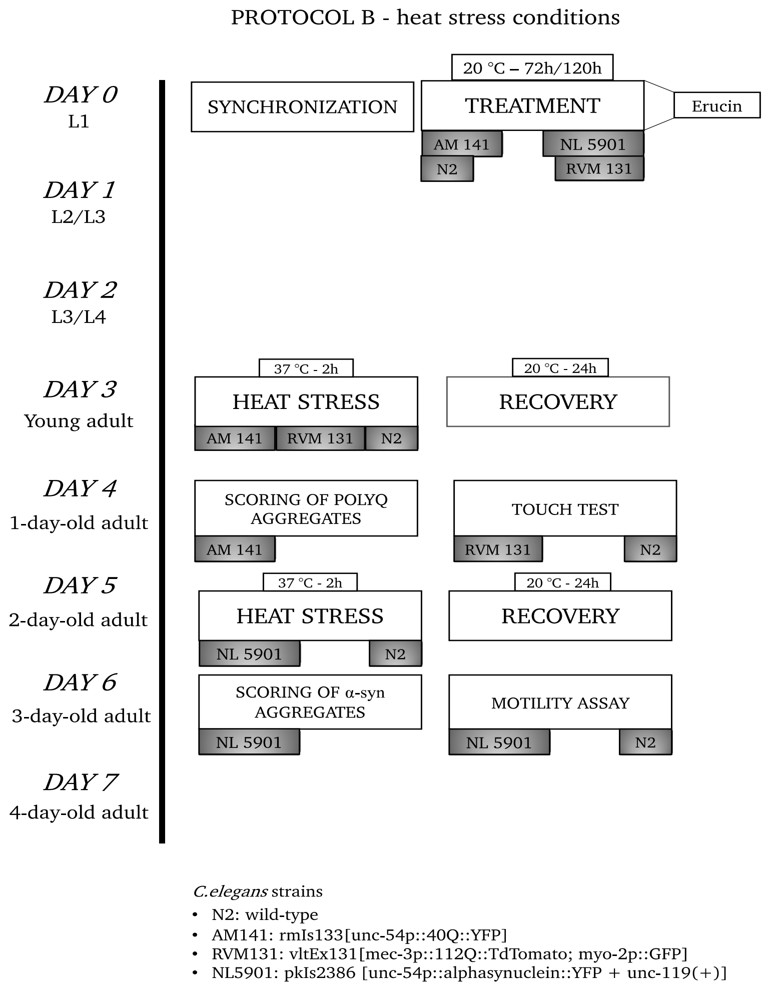


Figure 13. **Experimental protocol B – heat stress conditions.** The same day of synchronization, the treatment with ERN on L1 worms at 20 °C for 72 hours (N2, AM141, AM101,RVM131) and for 120 hours (N2, NL5901) is started. Subsequently, when the treatment is interrupted, the young adult nematodes are subjected to a 2 hours heat stress at 37 °C and, after a 24 hours recovery at 20 °C, to the scoring of polyQ aggregates in muscle cells (AM141) and to the touch test (RVM131, N2). Similarly, the 2-day-old adult worms are subjected to a 2 hours heat stress at 37 °C and, after a 24 hours of recovery at 20 °C, to a scoring of α-syn aggregates (NL5901) and to the motility assay (N2, NL5901).

3.6.2.2 **Treatment of *C. elegans* with ERN**

The treatment of the strains with ERN followed the same protocol described in paragraph 3.6.1.2.

3.6.2.3 **Heat stress**

In order to maintain the same length of the treatment described at paragraph 3.6.1.2, young adults 40Q::YFP and 112Q::TdTomato nematodes and 2-day-old α-syn::YFP worms were transferred in NGM agar plates seeded with *E. coli* OP50 strain. Each plate was placed in an incubator and subjected to a 2 hours heat stress at 37 °C. Therefore, the nematodes were placed at 20 °C for 24 hours for recovery. Each experiment was performed on at least 40 worms, divided in two plates with 20 nematodes each, in three independent experiments, to obtain reproducible values and perform the analysis described below.

3.6.2.4 ***In vivo* scoring of polyQ aggregates in muscle cells**

For the description of this analysis see paragraph 3.6.1.3. Even if the technique of this test was identical to the one described in the corresponding section 3.6.1.3, the difference consisted in the stage of development, in fact worms were analyzed in the first day of adulthood, one day after the young adult animals described before, after the recovery period that followed heat stress (paragraph 3.6.2.3). The analysis was repeated on 10 treated/untreated worms in three independent experiments, for a total number of at least 30 worms per condition.

3.6.2.5 **Evaluation of the touch response**

For the description of this analysis see paragraph 3.6.1.5. Even if the technique of this test was identical to the one described in the corresponding section 3.6.1.5, the difference consisted in the stage of development. Worms were analyzed in the first day of adulthood, one day after the young adult animals described before, after the recovery period that followed heat stress. The analysis was repeated on 10 treated/untreated worms, both on the 112Q::TdTomato strain and on the N2 wild-type nematodes, in three independent experiments, for a total number of at least 30 worms per condition and strain.

3.6.2.6 ***In vivo* scoring of α-synuclein aggregates**

For the description of this analysis see paragraph 3.6.1.6. Even if the technique of this test was identical to the one described in the corresponding section 3.6.1.6, the difference consisted in the stage of development. The test was performed on 3-day-old-adult worms, one day after the 2-day-old adult animals described before, after the recovery period that followed heat stress. The analysis was repeated on 10 treated/untreated nematodes in three independent experiments for a total number of at least 30 worms per condition.

3.6.2.7 **Motility assay**

For the description of this analysis see paragraph 3.6.1.7. Even if the technique of this test was identical to the one described in the corresponding section 3.6.1.7, the difference consisted in the stage of development. The test was performed on 3-day-old adults α-syn::YFP and wild-type worms, one day after the 2-day-old adult animals described before, after the recovery period that followed heat stress. The analysis was repeated in three independent experiments for a total number of at least 30 worms per condition and strain.

3.6.2.8 **Fluorescent microscopy imaging**

Images were collected using both Nikon Eclipse Ti-U Inverted Fluorescence Microscope and THUNDER-equipped Leica DM6 B Upright Microscope with the Leica DMC5400 Camera and the LAS X Software. Live animals were selected and positioned above 2% agarose pads inside a drop of 0.25 M sodium azide to anaesthetized them.

3.7 **<u>Protocol C</u>**

This protocol is summarized and described by figure 14.

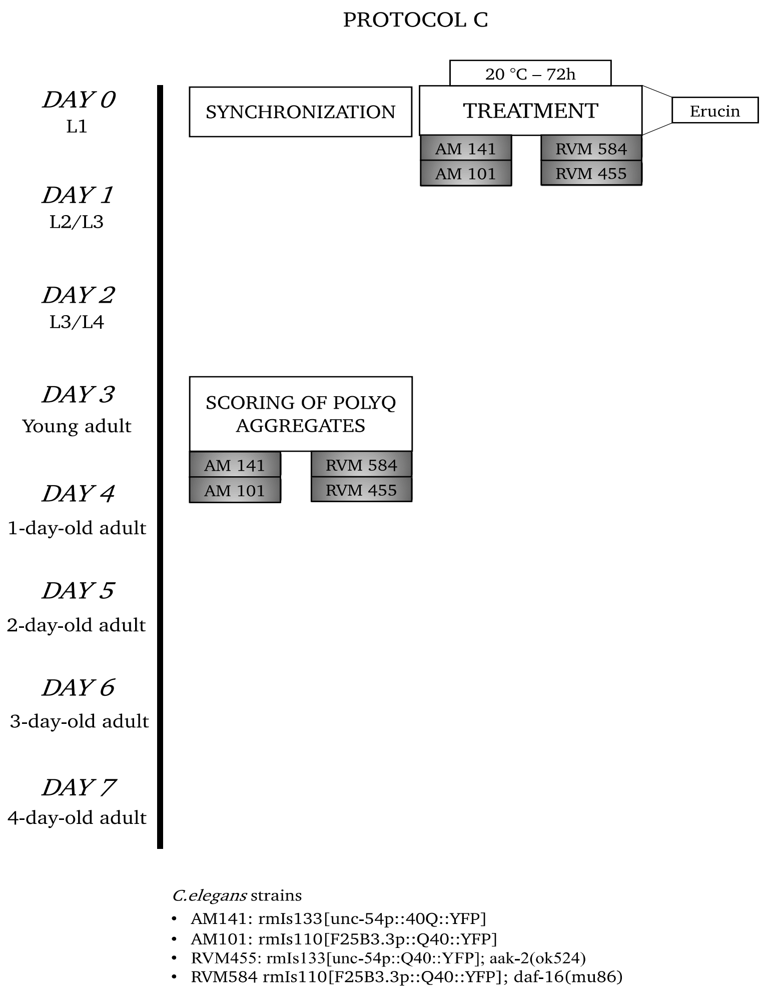


Figure 14. **Experimental protocol C.** The same day of synchronization, L1 worms are subjected to the treatment with ERN at 20 °C for 72 hours (AM141, RVM455, AM101, RVM584). Subsequently, when the treatment is interrupted, the young adult nematodes are subjected to the scoring of polyQ aggregates both in muscle cells (AM141, RVM455) and in the ventral nerve cord (AM101, RVM584).

3.7.1 ***C. elegans*** **strains**

- AM141: rmIs133[unc-54p::40Q::YFP]
- AM101: rmIs110[F25B3.3p::Q40::YFP]

- RVM455: rmIs133[unc-54p::Q40::YFP]; aak-2(ok524)
- RVM584: rmIs110[F25B3.3p::Q40::YFP]; daf-16(mu86)

3.7.2 **Treatment of *C. elegans* with ERN**

The treatment of the strains followed the same protocol described in paragraph 3.6.1.2.

3.7.3 ***In vivo* scoring of polyQ aggregates in muscle cells**

For the description of this analysis see paragraph 3.6.1.3. The analysis was repeated on 10 treated/untreated worms from the AM141 and RVM455 strains in three independent experiments for a total number of at least 30 worms per condition and strain.

3.7.4 ***In vivo* scoring of polyQ aggregates in ventral nerve cord**

For the description of this analysis see paragraph 3.6.1.4. This test was repeated on 10 treated/untreated worms from the AM101 and the RVM455 strains in three independent experiments for a total number of at least 30 worms per condition and strain.

3.7.5 **Fluorescent microscopy imaging**

Images were collected using THUNDER-equipped Leica DM6 B Upright Microscope with the Leica DMC5400 Camera and the LAS X Software. Live animals were selected and positioned above 2% agarose pads inside a drop of 0.25 M sodium azide to anaesthetized them.

3.8 **Statistical analysis**

One-way analysis of variance (one-way ANOVA) was applied to the data of every trial to study the effect of ERN treatment at the concentrations selected. In particular, Dunnett's method for multiple comparisons was applied to compare the mean of each treated group and the mean of the control group for each strain. GraphPad software (version 10.1.1) was used to perform the statistical analyses. The results are presented with the relative p-value to indicate the significance of the data.

4.RESULTS AND DISCUSSION

4.1 **Effects of bioactive compounds of plants against heat stress induced toxicity in N2 wild-type *C.elegans***

Initially, three bioactive compounds of plants were evaluated for their protective potential to induce heat stress resistance in terms of viability and oxidative stress in adult nematodes, as described in protocol A (chapter 3.5).

The 48 hours treatment of N2 wild-type 1-day-old adult nematodes with 20 µM and 40 µM curcumin at 20 °C did not provide any significant protection in terms of viability against a 4 hours heat stress at 37 °C, which induced a significant reduction of living worms respect to the negative control, as shown by figure 14A.

Nevertheless, the abovementioned antioxidant properties of curcumin (108–110), were confirmed by the quantification of ROS production in N2 wild type 4-day-old adult worms, here quantified as ROI, as described in section 3.5.5 of materials and methods, which was significantly enhanced by the 4 hours heat stress at 37 °C respect to the negative control and strongly suppressed by the highest concentration of curcumin tested down to levels similar to the negative control, as demonstrated by figure 14B.

This phenomenon suggests that there might be other mechanisms besides oxidation that induced the death of nematodes, even if the positive trend in viability provided in a dose-depended manner by curcumin suggests that ROS scavenging remains one of the main molecular mechanisms behind the protection from the negative effects induced by heat stress.

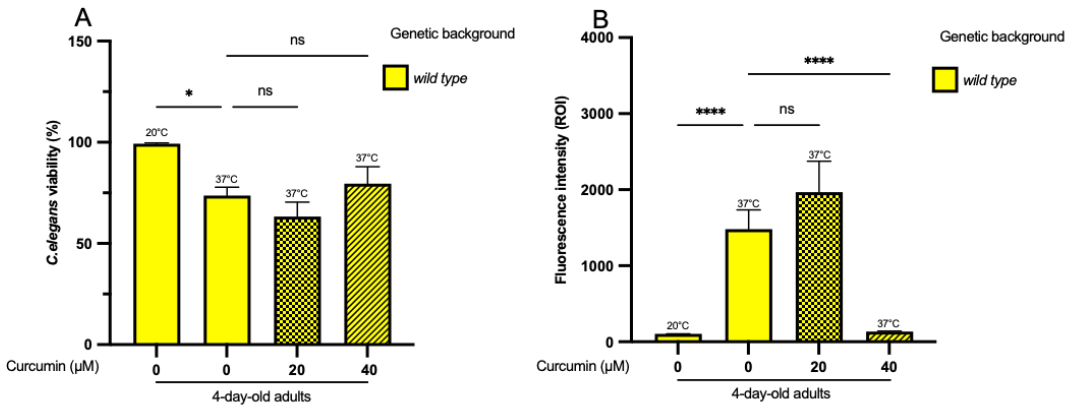


Figure 14. **Curcumin does not increase heat stress resistance.** A) N2 wild-type *C.elegans* 3-day-old adult nematodes, after a 48 hours treatment with 20 µM and 40 µM curcumin at 20 °C, are transferred to NGM agar plates seeded with *E.coli* OP50. These worms are placed at 37 °C for 4 hours and then recovered at 20 °C for 24 hours. Therefore, the viability is assessed through the counting of surviving 4-day-old adult worms, which value is set as 100 in the negative control (yellow column, 20° C). The percentage of surviving untreated and treated worms subjected to heat stress is calculated related to the negative control (% viability). Curcumin does not provide significant protection against heat stress induced mortality with any of the concentrations tested. B) N2 wild-type *C.elegans* 4-day-old adult nematodes, after a 48 hours treatment with 20 µM and 40 µM curcumin at 20 °C and a 4 hours heat stress at 37 °C, are incubated with 100 µM H_2DCFDA fluorescent probe. The total intensity of fluorescence of each worm (ROI) is analyzed by fluorescence microscopy and the fluorescence of the negative control (yellow column, 20 °C) is set as 1. Curcumin suppresses ROS production at the 40 µM concentration significantly. The data are expressed as the mean ± SEM. *P <0.05, ****P <0.0001.

The second natural compound tested for its protective potential against heat stress induced toxicity was astaxanthin.

The 48 hours treatment of N2 wild-type 1-day-old adult nematodes with 100 µg/mL, 200 µg/mL and 400 µg/mL astaxanthin at 20 °C did not provide any significant protection in terms of viability against a 4 hours heat stress at 37 °C, which induced a significant reduction of living worms respect to the negative control, as shown by figure 15A. In parallel, the quantification of ROS intracellular production confirmed the strong antioxidant properties of astaxanthin (116,117,119), for each of the concentrations tested and especially for the highest ones, for which the intensity of fluorescence was similar to the one of the negative control, as explained by figure 15B.

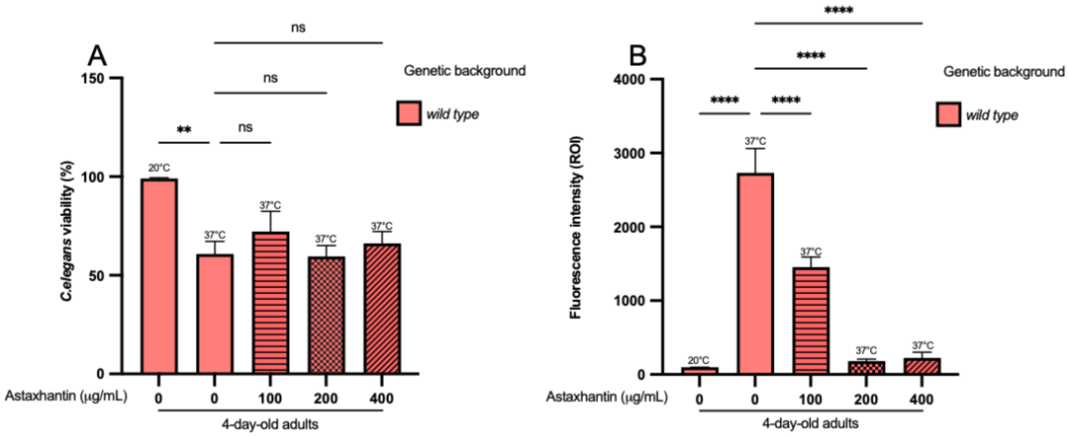


Figure 15. **Astaxanthin does not increase heat stress resistance.** A) N2 wild-type *C.elegans* 3-day-old adult nematodes, after a 48 hours treatment with 100 µg/mL, 200 µg/mL and 400 µg/mL astaxanthin at 20 °C, are transferred to NGM agar plates seeded with *E.coli* OP50. These worms are placed at 37 °C for 4 hours and then recovered at 20 °C for 24 hours. Therefore, the viability is assessed through the counting of surviving 4-day-old adult worms, which value is set as 100 in the negative control (red column, 20° C). The percentage of surviving untreated and treated worms subjected to heat stress is calculated related to the negative control (% viability). The compound does not provide significant protection against heat stress induced mortality with any of the concentrations tested. B) N2 wild-type *C.elegans* 4-day-old adult nematodes, after a 48 hours treatment with 100 µg/mL, 200 µg/mL and 400 µg/mL astaxanthin at 20 °C and a 4 hours heat stress at 37 °C, are incubated with 100 µM H_2DCFDA fluorescent probe. The total intensity of fluorescence of each worm (ROI) is analyzed by fluorescence microscopy and the fluorescence of the negative control (red column, 20 °C) is set as 1. Astaxanthin suppresses ROS production induced by heat stress significantly. The data are expressed as the mean ± SEM. $^{**}P < 0.01$, $^{****}P < 0.0001$.

This demonstrated that the antioxidant effect of the compound was not sufficient to provide a protection in terms of vitality in worms at the conditions tested and that other mechanisms that may be involved in the protection of worms from heat stress induced mortality might be investigated.

The third bioactive compound tested for its capacity to increase heat stress resistance in *C.elegans* was ERN.

The 48 hours treatment of N2 wild-type 1-day-old adult nematodes with 100 µM and 200 µM ERN at 20 °C provided a significant dose-dependent protection in terms of viability after a 4 hours heat stress at 37 °C, which induced a strong significant reduction of living worms respect to the negative control, as shown by figure 16A. Moreover, ERN exerted antioxidant protection, confirming the pre-existing

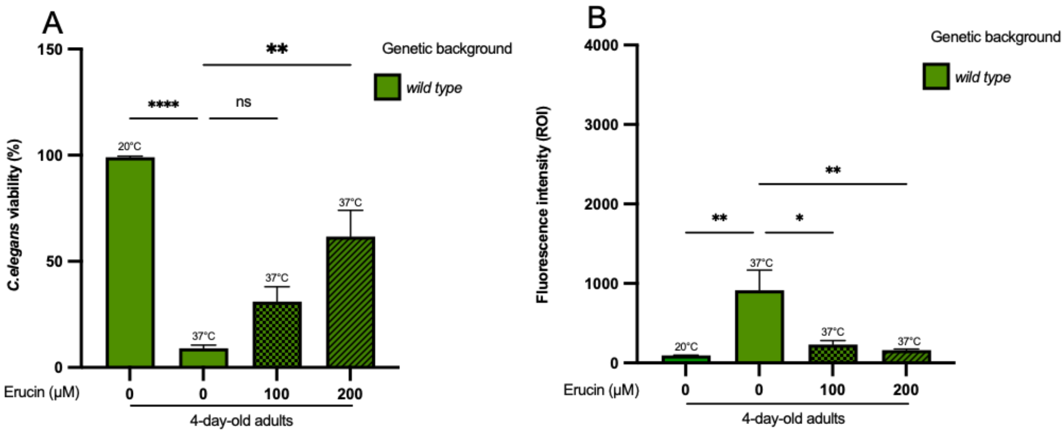


Figure 16. **Erucin increases heat stress resistance.** A) N2 wild-type *C.elegans* 3-day-old nematodes, after a 48 hours treatment with 100 µM and 200 µM ERN at 20 °C, are transferred to NGM agar plates seeded with *E.coli* OP50. These worms are placed at 37 °C for 4 hours and then recovered at 20 °C for 24 hours. Therefore, the viability is assessed through the counting of surviving 4-day-old adult worms, which value is set as 100 in the negative control (green column, 20° C). The percentage of surviving control and treated worms subjected to heat stress is calculated related to the negative control (% viability). ERN provides a significant dose-dependent protection against heat stress induced mortality. B) N2 wild-type *C.elegans* 4-day-old adult nematodes, after a 48 hours treatment with 100 µM and 200 µM ERN at 20 °C and a 4 hours heat stress at 37 °C, are incubated with 100 µM H_2DCFDA fluorescent probe. The total intensity of fluorescence of each worm (ROI) is analyzed by fluorescence microscopy and the fluorescence of the negative control (green column, 20 °C) is set as 1. ERN suppresses ROS production induced by heat stress significantly. The data are expressed as the mean ± SEM. **P* <0.05, ***P* <0.01, *****P* <0.0001.

evidences (133–138), against ROS production induced by heat stress, suppressing the fluorescence of worms down to basal levels similar to those of worms not subjected to heat stress, as shown by figure 16B.

These evidences confirmed that a significant contribution to the protective effect exerted by ERN against heat stress-induced mortality was provided by ROS scavenging, posing the justificatory basis to select ERN as the compound to be tested for its neuroprotective potential both under standard and heat stress conditions.

However, some critical points should be noticed. The fact that the mortality provided by a 4 hours heat stress at 37 °C in N2 wild-type 1-day-old adult worms was different between experiments executed for each compound (see viability in untreated worms at 37 °C in figure 14A, 15A and 16A) is a critical point that affects the subsequent significance of the effect provided by each compound after heat stress. As the applied protocol for heat stress was always the same and the strain came from the same source and unfreezing, some environmental factors might have affected the sensitivity of worms to temperature, as explained for its temperature-dependent longevity and thermotaxis (158,159). As an example, the treatment with curcumin was performed in summer, the one with astaxanthin in winter and the treatment with ERN was performed during autumn. Room temperature was not stable during months, for technical problems occurred in the building, therefore, even if worms were grown at 20 °C, since their handling and test set-up was executed in the laboratory, some adaptive mechanisms that affected thermosensation might have been induced. Nevertheless, the high number of nematodes subjected to heat stress in two consecutive independent experiments for each compound confirmed the data. Even fluorescence intensity of worms subjected to heat stress and untreated with any compound (i.e positive controls, see fluorescence intensity in figure 14B, 15B and 16B) showed great variability among experiments. Analyzing the trend among the compounds, the fluorescence was higher when the vitality was affected less by heat stress, as demonstrated by curcumin and astaxanthin respect to ERN (figure 14A, 15A and 16A), suggesting that this might be one of the first mechanism induced by stresses before death.

Moreover, another criticism was that worms were treated in an already adult stage. The results regarding a protective action of each substance studied would have been more complete and, potentially, pronounced, if treatment had also been performed from the first larval stage (L1) after eggs hatching in order to mimic a real preventive strategy against heat stress effects in nematodes. Supporting this, it has already been demonstrated that curcumin exerts a protection in terms of lifespan and oxidative stress induced even by heat stress in nematodes subjected to the treatment from the L1 and L4 stage of development (160,161) and that astaxanthin reduces heat stress induced oxidation in worms treated from the L4 stage (162), demonstrating a protective action exerted by these compounds when supplemented during the development. These results integrate those evidences demonstrating that the effects provided by curcumin and astaxanthin are maintained only in part in adult worms, suggesting different age-related effects exerted by the bioactive compounds evaluated.

Nevertheless, for the initial purpose of the study, which was a rapid screening among several bioactive compounds of plants to find the one which exhibited the best – protective properties in terms of viability and ROS production under heat stress conditions, these data were considered sufficient to select ERN. Considering all the results, it was evident that the alteration of the intracellular redox status connected to ROS production wasn't the primary mechanism induced by heat stress and that the antioxidant action alone exerted by all the bioactive compounds tested was not sufficient to guarantee an effective thermoprotective action, except for ERN.

In fact, ERN was the only compound that provided protection against both mortality and ROS production significantly, with a dose-dependent trend. Therefore, it's reasonable to assume that the protective ability of this ITC implies the involvement of other cellular damage defense mechanisms and heat-protective actions, justifying the subsequent evaluation of ERN's action in protein aggregation processes even in worms subjected to heat stress, which is known as a factor involved in several neurodegenerative processes.

4.2 **Effects of ERN against polyQ and α-syn induced toxicity in *C.elegans***

4.2.1 **Standard conditions**

The first part of the experimental protocol was focused on the evaluation of the neuroprotective potential of ERN in standard conditions, i.e with a treatment of worms performed and maintained ad 20 °C, as this was the usual temperature of growth of nematodes models of polyQ and α-syn-induced toxicity.

The research on the ability of ERN to exert a neuroprotective action started with a *C.elegans* model of neuronal toxicity, carrying a transgene coding for a 112Q fused to the dimeric fluorescent protein TdTomato (TdTom) in mechanosensory neurons, that provokes an impairment in their function. Indeed, these nematodes present an impaired sensitivity to a light touch given by an eyelash fused on the top of a pipette tip on the posterior part of the body, providing a model to test the neuronal functionality. These nematodes usually respond only 35 % of the time, while N2 wild-type worms do it 7 out of 10 times ($P < 0.0001$) (figure 17).

The treatment from the L1 to the young adult stage of development with 100 µM and 200 µM ERN separately at 20°C, both for the N2 wild-type and for the TdTom strain provided the evidence that ERN was able to reduce polyQ-induced toxicity, rescuing the neuronal functionality, compared to untreated nematodes (P <0.001 for 100 µM ERN and P <0.0001 for 200 µM ERN). Moreover, the same treatment in N2 wild-type worms did not affect the normally observed rate of response to the touch, for each concentration of ERN tested, confirming that the concentrations of the compound were not toxic (figure 17).

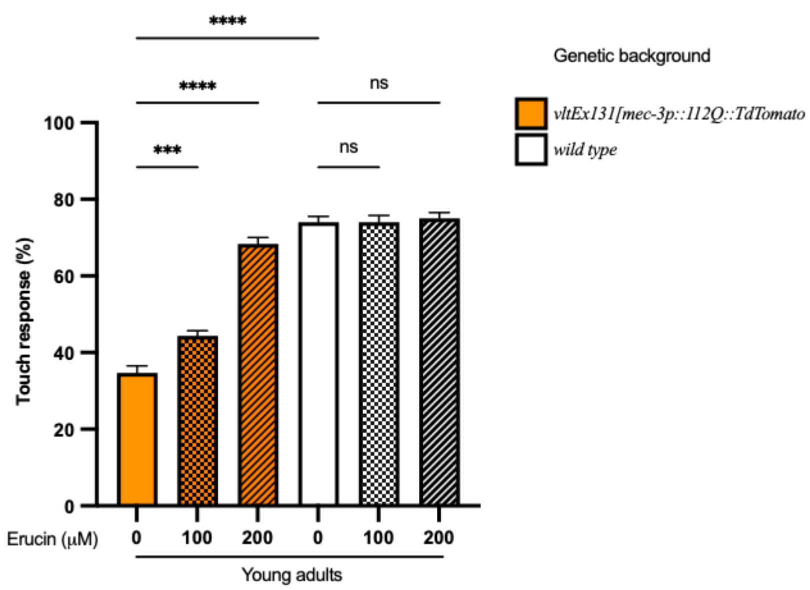


Figure 17. **Erucin restores neuronal function.** The expression of 112Q:TdTom in mechanosensory neurons induces an impairment of neuronal function compared to animals that do not have the transgene. Treatment in N2 wild-type worms does not affect the rate of response to the touch, for each concentration of ERN tested compared to untreated animals. Treatment with 100 µM and 200 µM ERN rescues the neuronal functionality of 112Q::TdTom young adult animals compared to untreated nematodes. The data are expressed as the mean ± SEM. ****P* <0.001, *****P* <0.0001.

Therefore, in order to understand if the neuroprotection provided by ERN was in some way connected to polyQ aggregates clearance mechanisms, two *C.elegans* strains expressing 40CAG repeats in frame with the yellow fluorescence protein (40Q::YFP) both in muscle cells and in the ventral nerve cord, that accumulate aggregates in an age-dependent manner, were treated with 100 µM and 200 µM ERN separately. The results of the treatment, shown in figure 18, demonstrated that both 100 µM and 200 µM ERN reduced the number of polyQ muscular aggregates significantly compared to untreated young adult animals (*P* <0.0001 for 100 µM and 200 µM ERN) (Figure 18A) and even the number of neuronal aggregates (*P* <0.001 for 100 µM ERN and *P* <0.01 for 200 µM ERN) (figure 18B).

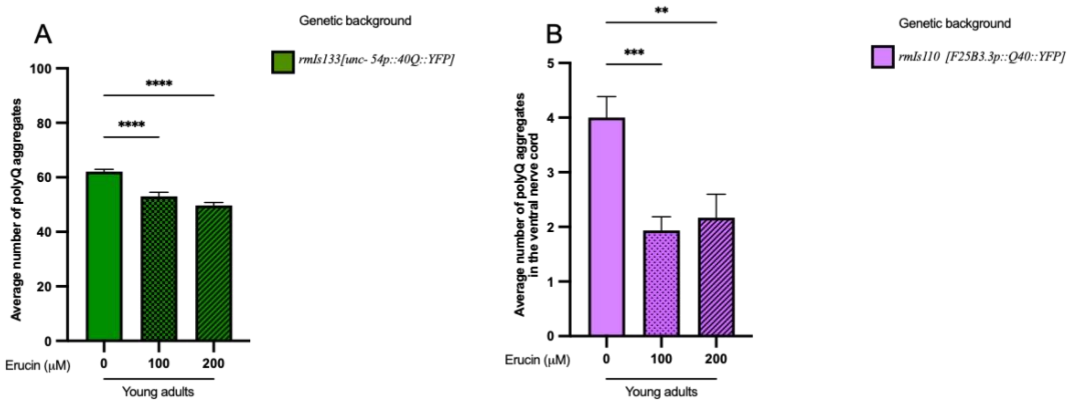


Figure 18. **Erucin reduces polyglutamine-induced muscular and neuronal toxicity.** The expression of 40CAG sequences in muscular and neuronal cells induces a progressive formation of neurotoxic protein aggregates. A) ERN treatment reduces polyQ-induced muscular aggregation in 40Q young adult animals significantly. B) ERN treatment reduces polyQ-induced aggregates formation in the ventral nerve cord in 40Q young adult animals significantly. The data are expressed as the mean ± SEM. $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

Representative images of polyQ muscular and neuronal aggregates are provided by figures 21B and 24B, respectively.

Considering that oxidative stress is one of the most relevant risk factors in NDDs (163), and that ERN is interconverted *in vivo* to its structurally related analog SFN (132), it is reasonable to assume that the neuroprotective role of ERN may be comparable to SFN's. SFN enhances antioxidant responses through Nrf2 pathway; it even exerts kelch like ECH associated protein 1/Nrf2-independent mechanisms stimulating anti-inflammatory responses through the inhibition of NF-kB and provides an epigenetic effect suppressing histone deacetylases and DNA methyltransferases. Moreover, it induces proteostasis, preventing neurodegeneration (164) and promotes longevity and health span via DAF-16/DAF-2 insulin/ insulin-like growth factor 1 (IGF-1) signaling (165), demonstrating the involvement even of these pathways in its mechanism of action. Till now, even though ERN activity has been related to the induction of Nrf2 both in vitro in HT-29 cells and in vivo in mice (134), its neuroprotective role and the molecular pathways involved in it are still largely unknown. These results showed that ERN reduces polyQ-induced toxicity at both muscular and neuronal level, providing further insights on the efficacy of this natural compound.

In order to broaden the range of NDDs included in the study, the experimental protocol aimed to verify even if ERN was able to interfere with the toxicity induced by α-syn through the treatment of the well-known PD model strain which expresses α-syn fused to a fluorescent protein in muscle cells (166). These nematodes tend to accumulate aggregates in 2-day-old adults, therefore the treatment with 100 µM and 200 µM ERN was prolonged until the second day of adulthood. The count of the aggregates was performed in the area included between the two bulbs of the pharynx through a fluorescence microscope, as described elsewhere (167), and represented in figure 19B. The test demonstrated a significant reduction of muscular α-syn aggregates with both concentrations of ERN tested (P <0.0001 for 100 µM and 200 µM ERN) (figure 19A). Moreover, in order to test whether ERN treatment improved the behavior of α-syn::YFP worms, the trashing assay was performed, both on α-syn::YFP worms and on N2 wild-type animals, because α-syn aggregates accumulation in muscle cells induces an impairment in motility respect to wild-type animals (168). This behavioral test relies on the counting of the frequency of complete lateral swimming movements of worms in a given window of time. When compared to wild-type nematodes, untreated α-syn::YFP showed a significant reduction in their motility capacity (P <0.0001), which was recovered significantly by the highest concentration of ERN tested (P <0.05 for 200 µM ERN). Moreover, ERN did not affect the natural movement of wild-type worms, confirming that it was not toxic for their natural movement capacity (figure 19C).

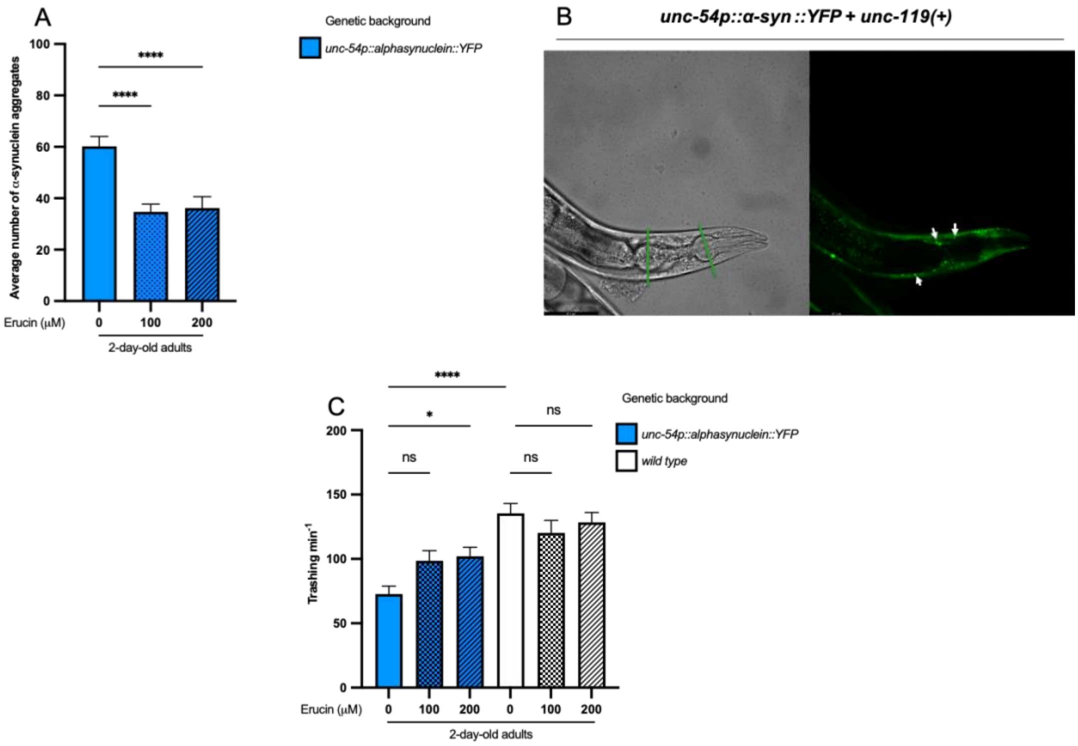


Figure 19. **Treatment with erucin reduces α-synuclein aggregation and slightly restores motor capacity.** The expression of α-syn in muscle cells induces the progressive formation of α-syn protein aggregates and motility impairment. A) Treated animals with 100 µM and 200 µM ERN show a reduction of α-syn aggregates compared to untreated α-syn::YFP 2-day-old adult animals. B) Representative images from fluorescence microscopy showing α-syn aggregates (white arrows) between the two bulbs of the pharynx (outlined by green lines in the left image) of α-syn ::YFP 2-day-old animals at 63x magnification. C) Treated N2 wild-type worms with both concentrations of ERN tested do not show motility impairment. The treatment of α-syn::YFP nematodes with 100 µM and 200 µM ERN rescue motility capacity of 2-day-old adult animals significantly after the treatment only with the higher dose of ERN tested. Motility capacity is analyzed as the total number of trashes per minute (trashing min^{-1} in the graph). The data are expressed as the mean ± SEM. *P <0.05, ****P <0.0001.

From these results it is possible to assume that a higher concentration of ERN could be more effective in rescuing the trashing impaired due to α-syn aggregates accumulation. Nevertheless, at the concentrations tested, ERN reduced the aggregation of the protein linked genetically and neuropathologically to PD. Several studies have already demonstrated that AMP-activated protein kinase (AMPK), as one of the key regulators of autophagy, which is a conservative intracellular degradation process, drives the lowering of α-syn aggregation, protecting cells from impairment and degeneration (169–171), together with the downstream action of *daf-*

16 signaling pathway (172), providing a hypothetical pathway common to polyQ-induced toxicity clearance mechanisms, as it will be described in the following paragraphs.

4.2.2 **Heat stress conditions**

In order to understand if the neuroprotective action of ERN against protein aggregates formation was maintained even under heat stress conditions, the experimental protocol developed on the abovementioned *C.elegans* models of polyQ and α-syn induced toxicity was applied even under heat stress conditions. In this regard, to make the data comparable, the treatment with ERN was maintained of the same length, i.e from the L1 to the young adult stage of development (or the second day of adulthood for the α-syn::YFP strain), moment in which worms were subjected to the 2 hours heat stress at 37°C and a recovery of 24 hours at 20°C. Therefore, each parameter described below was acquired one day after in respect of protocol B performed under standard conditions, i.e during the first day of adulthood for polyQ strains and on three-day-old adult α-syn nematodes.

The ability of ERN to provide a neuroprotective action under heat stress conditions was firstly tested through the RVM131 strain, carrying a transgene coding for a 112Q fused to the dimeric fluorescent protein TdTomato (TdTom) in mechanosensory neurons, that provokes an impaired response to a light touch given by an eyelash on the posterior part of the body, providing a model to test the neuronal functionality, as described in paragraph 3.6.1.5. In standard conditions, these nematodes usually respond only 35 % of the time, while N2 wild-type worms do it 7 out of 10 times (P <0.0001), as described in figures 17 and 20 (orange and white columns at 20 °C). Under heat stress conditions, the touch response of the untreated mutant worms wasn't affected respect to that collected at 20°C, while N2 wild-type nematodes showed a significant reduction in the sensitivity to the touch stimulus respect to those maintained at 20 °C (Figure 20). Nevertheless, the treatment with 100 µM and 200 µM ERN provided, in both strains, a recovery of the neuronal function even after heat stress, restoring the touch response in the N2 wild-type strain in a dose-dependent manner. Moreover, interestingly, it induced a strong improvement in the

112Q::TdTom strain touch response, up to levels similar to the wild-type nematodes in the same conditions of stress and stage of development.

These results demonstrated that ERN maintained its neuroprotective action even under heat stress, justifying the subsequent trials to understand if its ability in restoring neuronal function was connected to polyQ-induced aggregates clearance mechanisms even under hyperthermic conditions.

Moreover, from a first analysis, these data suggested that the heat stress applied didn't worsen the pattern of response in the mutant strain (orange columns at 20°C and 37°C, figure 20), even if it induced a significant impairment in the N2 wild-type touch response (white columns at 20 °C and 37 °C, figure 20). Therefore, an objection could be raised to the necessity of applying a stronger thermal stress to induce an effect in the response to the stimulus in the 112Q::TdTom strain. However, preliminary trials on this strain subjected to the same 4 hours stress at 37 °C described in protocol A (paragraph 4.1) demonstrated that these conditions were not compatible with viability (data not shown), as they recorded close to 100% mortality, making the touch test analysis, which is inherently performed on live nematodes, impossible.

This effect could be ascribed to a hypersensitivity to the temperature that might be constitutive of the mutated strain, as well as to the different developmental stage of the nematodes under stress. Indeed, being in the young adult stage, they may have been be more sensitive to thermal stimulus than adults at day 3 of adulthood described in protocol A (paragraph 4.1).

Moreover, another observation could be made among the effect of high temperature on the touch response, meaning the neuronal function. Indeed, the data collected for the N2 wild-type nematodes confirmed that even a 2 hours heat stress induced an impairment in the touch stimulus, while the absent worsening in neuronal functionality of the 112Q::TdTom strain, which was unexpected, seemed to contrast this evidence. The constitutive difference in the genetic background of the two strains makes the comparison difficult, nevertheless one explanation about the phenomenon observed in the RVM 131 strain could be, along with the hypothesis of a hypersensitivity to temperature, the activation of the HSF-1 and HSPs by the short heat stimulus, factors that might have counteracted the stress-induced cellular damages by preserving protein homeostasis (64,65), which have already been reported to be impaired in models of HD, and, extensively, in polyQ disorders (71–74).

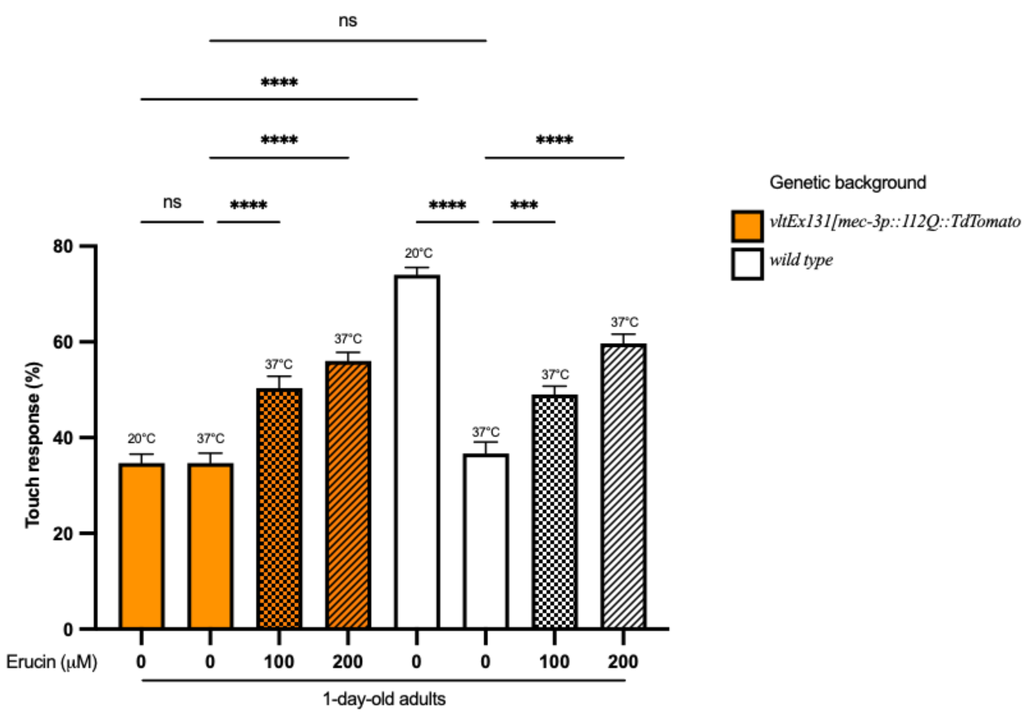


Figure 20. **Erucin restores neuronal function under heat stress conditions.** The expression of 112Q::TdTom in mechanosensory neurons (orange column) induces an impairment of neuronal function compared to N2 wild-type animals that do not have the transgene (white column) at 20°C. 112Q::TdTom and N2 wild-type *C.elegans* young adult nematodes, after a 72 hours treatment with 100 µM and 200 µM ERN at 20 °C, are transferred to NGM agar plates seeded with *E.coli* OP50. These worms are placed at 37 °C for 2 hours and then recovered at 20 °C for 24 hours, moment in which the touch response is performed. Heat stress at 37°C does not provoke any significant negative impact in the touch response in 112Q::TdTom untreated worms, while reduces the sensitivity to the stimulus in wild-type untreated nematodes down to levels similar to the mutant strain. The treatment with 100 µM and 200 µM ERN restores the touch response both in 112Q::TdTom 1-day-old adults worms and in 1-day-old adults wild-type nematodes significantly and in a dose-dependent trend. The data are expressed as the mean ± SEM. ***P< 0.001, ****P< 0.0001.

In any case, the results confirming that the neuroprotective action of ERN was maintained even under heat stress conditions, provided the justifying basis to test the compound under heat stress on the AM141 strain, which expresses 40CAG repeats in frame with a yellow fluorescent protein, mutation that imply an age-dependent accumulation of protein aggregates in the muscle cells of the worms. As mentioned for the RVM131 strain, even the 40Q::YFP nematodes died with a 4 hours stress at 37 °C (data not shown), leading to the decision of maintaining the 2 hours heat stress at 37 °C as the standardized protocol for heat stress on mutated strains involved in the present study. Figure 21 represents the average number of polyQ aggregates counted on ERN treated/untreated 1-day-old 40Q::YFP nematodes 24 hours after heat stress. The data are provided also with the negative control, represented by the polyQ aggregates in untreated, non-heat-stressed nematodes. Heat stress induced a significant increase (P <0.0001) in polyQ aggregates formation respect to the negative control, suggesting a proof for the role of oxidative stress in Htt aggregation (63) and for the involvement of an impaired HSR pathway both *in vitro* and *in vivo* models of HD, mainly due to reduced levels of HSF-1 (71–74). Moreover, ERN treatment provided a protection from thermal stress induced neurotoxicity at the highest concentration tested (P <0.0001), lowering the number of total polyQ aggregates to levels similar to the negative control. The molecular mechanisms by which ERN displayed this action, both in standard (see paragraph 4.2.1, figure 18A) and under heat stress conditions, remained unknown: they were therefore investigated and will be discussed and described in section C.

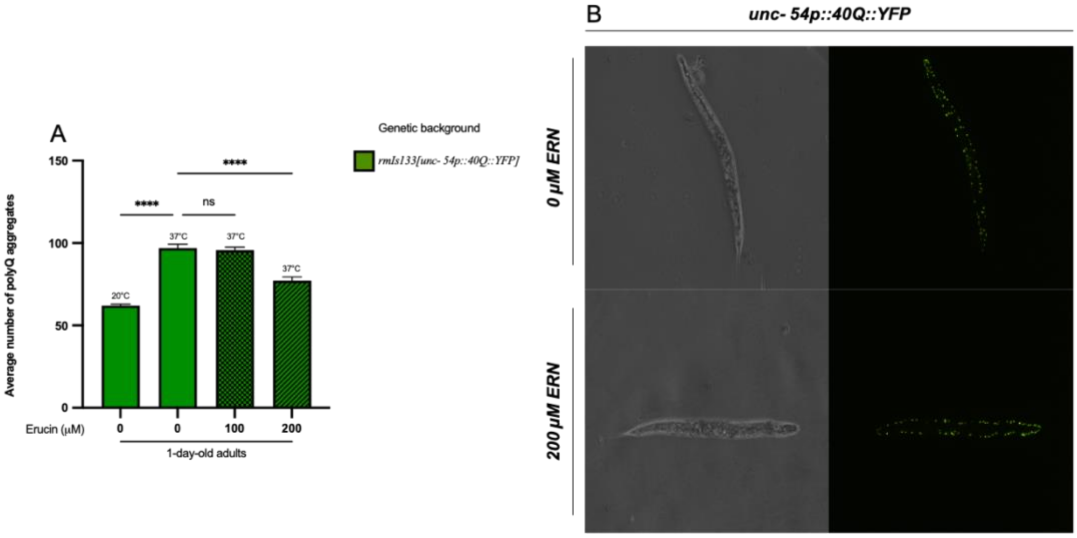


Figure 21. **Erucin reduces polyglutamine-induced muscular toxicity under heat stress conditions.** 40Q::YFP *C.elegans* young adult nematodes, after a 72 hours treatment with 100 µM and 200 µM ERN at 20 °C, are transferred to NGM agar plates seeded with *E.coli* OP50. These worms are placed at 37 °C for 2 hours and then recovered at 20 °C for 24 hours, moment in which the count of the aggregates is performed. A) The expression of 40CAG repeats in muscle cells implies an age-dependent accumulation of protein aggregates, which is significantly enhanced under heat stress conditions (green columns without any pattern, at 20°C and 37°C, respectively). ERN treatment reduces polyQ-induced muscular aggregation in 40Q 1-day-old adult animals subjected to a 2 hours heat stress at 37°C in the young adult stage significantly. B) Representative images from fluorescence microscopy showing polyQ muscular aggregates (fluorescence dots) of 40Q::YFP 1-day-old adult animals at 10x magnification, both for untreated (0 µM ERN) and treated (200 µM ERN) worms after heat stress. Data are expressed as mean ± SEM. ****P <0.0001.

Comparing the protocol developed under standard conditions at 20 °C with the one performed under thermal stress at 37 °C, this section lacks the stress performed on strain AM101 (figure 18B) for a technical reason: the strain, which presents 40CAG repeats in frame with a yellow fluorescent protein and develops polyQ aggregates in neuronal cells, can be analyzed through a confocal fluorescent microscope or with an upright microscope for fluorescence, transmitted light, and true color imaging equipped with the THUNDER imaging system, as described in section 3.6.1.4. As the thermal stress experiments were performed in a laboratory provided only with an Inverted Fluorescence Microscope (described in section 3.6.2.8), it was technically impossible to perform heat stress and the relative analysis on this strain. Nevertheless, considering the results collected under standard conditions both in AM141 and

AM101 strains (figure 18), which reflected the same mechanisms exerted by ERN, i.e a clearance of polyQ aggregates, the evidences collected on the only AM141 strain were sufficient to confirm that ERN maintained its neuroprotective action against polyQ induced toxicity even under heat stress conditions.

Finally, the heat stress experiment included even the PD *C.elegans* model strain, which expresses α-syn fused to a yellow fluorescent protein that enables the visualization and analysis of the α-syn aggregates in muscle cells of worms from the second day of adulthood on. Moreover, this progressive aggregation provokes a defect in motility capacity of worms, respect to wild-type nematodes, enabling behavioral assay on the NL5901 strain (168). The parameters evaluated 24 hours after the 2 hours heat stress at 37°C are presented in figure 22. The reduction of the average number of α-syn aggregates in the nematodes treated with 100 µM and 200 µM ERN was evident but not statistically significant (figure 22A). The analysis of the movement through the trashing assay showed that heat stress increased the impairment in the movement both in the untreated α-syn::YFP worms and in the untreated N2 wild-type nematodes (P <0.01 and P <0.0001, respectively). Nevertheless, ERN treatment did not induce any significant improvement in the trashing of N2 wild-type nematodes, while it ameliorated the movement in α-syn::YFP worms significantly (P < 0.05 for 100 µM and 200 µM ERN) (figure 22B). The results regarding α-syn protein aggregation, which seemed to be slightly reduced after heat stress both in untreated nematodes and even under the effect of ERN (figure 22A), even if not significantly, support the hypothesis that the short heat shock could have enhanced the ILS pathway, therefore activating the DAF-16 and/or HSF-1, that acted to maintain protein homeostasis in stressful conditions (145–150). Nevertheless, the short heat shock was sufficient to induce an impairment in motility, both in N2 wild-type nematodes and in α-syn::YFP worms. Interestingly, ERN treatment at both concentrations tested provided an improvement of motor capacity similar between strains, which became significant only for the α-syn::YFP strain, whose untreated nematodes suffered more for the heat shock stress (figure 22B). These results confirmed the protective role of ERN in terms of locomotion, as an indirect effect of reduction of α-syn aggregates in muscle cells, even under heat stress conditions.

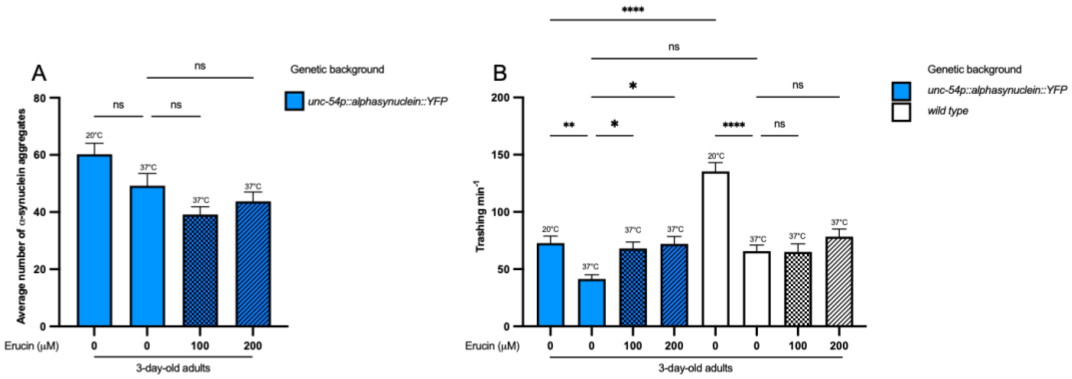


Figure 22. **Treatment with erucin reduces α-synuclein aggregation and restores motor capacity under heat stress conditions.** The expression of α-syn in muscle cells induces the progressive formation of α-syn protein aggregates and motility impairment. α-syn::YFP *C.elegans* 2-day-old adult nematodes, after the treatment with 100 μM and 200 μM ERN at 20 °C from the L1 stage of development are transferred to NGM agar plates seeded with *E.coli* OP50, placed at 37 °C for 2 hours and then recovered at 20 °C for 24 hours, moment in which the count of the aggregates and the trashing assay are performed. A) Untreated animals at 20 °C present more aggregates than those subjected to heat stress, even if this difference is not significant. Treated animals with 100 μM and 200 μM ERN do not show a significant reduction of α-syn aggregates compared to untreated α-syn::YFP 3-day-old adult animals. B) α-syn aggregates in worms subjected to heat stress induce an impairment of motility compared to wild-type animals at 20 °C. Heat stress reduces motility capacity of both α-syn::YFP and N2 wild-type worms significantly. Treated N2 wild-type worms with both concentrations of ERN tested do not show an improvement in motility respect to untreated ones. α-syn::YFP 3-day-old-adult nematodes treated with 100 μM and 200 μM ERN show a significant improvement in trashing. Motility capacity is analyzed as the total number of trashes per minute (trashing min^{-1} in the graph). Data are expressed as mean ± SEM. **P* <0.05, ***P* <0.01, *****P* <0.0001.

4.3 **ERN prevents polyQ-induced toxicity via *aak-2*/AMPK and *daf-16*/FOXO signaling**

The evidences collected about the neuroprotection exerted by ERN against protein aggregates induced toxicity sustained further tests to investigate its mechanism of action.

Supported by the chemical similarity between SFN and ERN, the first trial aimed to test weather AMPK, already reported to be involved in SFN anti-oxidative and autophagy promoting action both by *in vitro* (173) and *in vivo* studies (174,175), is activated by ERN. Moreover, AMPK protects neurons from dysfunction induced by the expression of human exon 1 Htt in *C.elegans*, preventing polyQ induced toxicity (176), in a *daf-16*/forkhead box O-dependent manner (177).

Therefore, the protocol included the treatment with ERN of worms defective of the only catalytic subunit of AMPK (aak-2/AMPKα2), which has been reported to affect the lifespan and health in *C. elegans* (178). The treatment of 40Q; *aak-2*(ok524) worms from L1 to young adult stage with 100 µM and 200 µM ERN did not induce any significant difference in the number of polyQ aggregates in nematodes treated in comparison to untreated ones. Considering that ERN treatment reduced polyQ muscular aggregation in 40Q young adult animals of the control strain without the mutation significantly (*P* <0.01 for 100 µM ERN and *P* <0.0001 for 200 µM ERN) (figure 23), the test demonstrated the AMPK involvement in the mechanism of action of ERN.

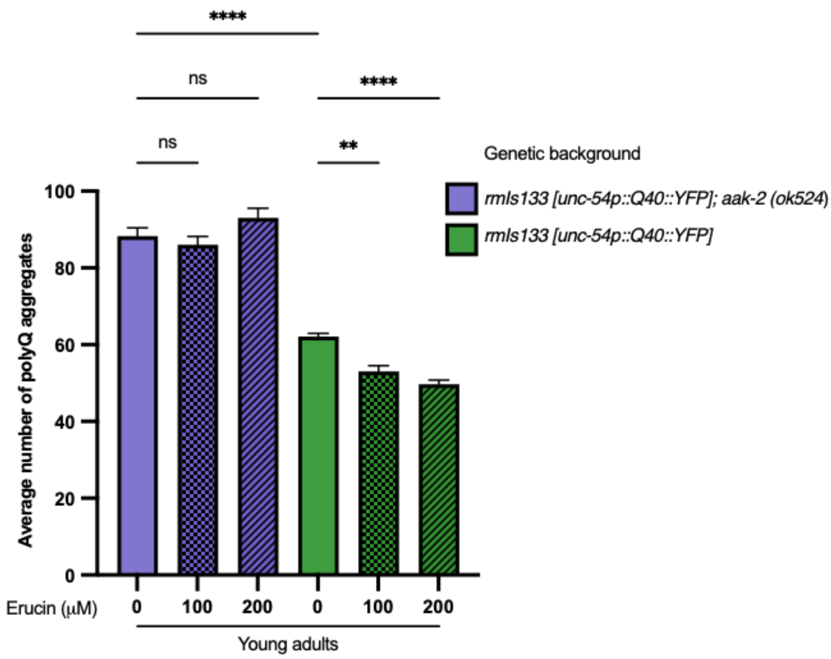


Figure 23. **Reduction of polyQ aggregation induced by erucin requires AMPK catalytic function.** The treatment with 100 µM and 200 µM ERN of 40Q::YFP worms defective of the catalytic subunit of AMPK (*aak-2*/AMPKα2) does not produce any significant difference in polyQ-induced muscular aggregates formation in comparison to untreated worms. In parallel, ERN treatment reduces polyQ-induced muscular aggregation in 40Q young adult animals of the control strain significantly. Data are expressed as mean ± SEM. ***P* <0.01, *****P* <0.0001.

Moreover, AMPK exerts its action activating, among several factors, even the FOXO factors (177,178). Considering that *daf-16*/ FOXO exerts a strong protective action in *C. elegans* models of HD (179), and plays a crucial role in lifespan and protein homeostasis regulation in response to abiotic stresses (145–150), the study investigated whether the neuroprotection exerted by ERN through AMPK might be dependent on *daf-16*/FOXO.

To verify this hypothesis, the 40Q::YFP nematodes defective of the *daf-16* gene were treated with 100 µM and 200 µM ERN from the L1 to the young adult stage. The treatment did not produce any significant variation in aggregates formation in the ventral nerve cord of the worms (represented in figure 24B). In parallel, the treatment of the relative control 40Q::YFP strain without mutation induced a significant reduction in the number of aggregates for both concentrations of ERN tested (P <0.0001 for 100 µM ERN and P <0.001 for 200 µM ERN) (figure 24A). The effect exerted by ERN confirmed the evidence that *daf-16*/FOXO is involved in SFN mechanism of action, promoting longevity and lifespan in *C. elegans* (165).

From these results it was evident that the aggregation in the rmls110;daf-16 strain was reduced in comparison to the rmls110 strain: this unexpected trend might have appeared because of a change in the genetic backgrounds during the crossing process or because worms had different growing times and the rmls110;daf-16 young animals were slightly younger than those of rmls110 strain, consequently with fewer aggregates. However, beyond the differences between the two strains, the important evidence was that the rescuing effect was lost in the strain carrying the mutation, which demonstrated that the *daf-16*/FOXO factor is involved in ERN neuroprotective action.

These evidences suggest a similar signaling pathway between that of polyQ-induced toxicity clearance and that of PD (169–172), supporting further studies to test this hypothesis.

Moreover, these results do not confirm that *aak-2* and *daf-16* operate in the same signaling pathway. Double mutants 40Q;*aak-2*;*daf-16* are not viable in the strain expressing 40CAG repeats in muscle cells, even if this hypothesis might be tested in 40Q;*aak-2*;*daf-16* double mutants in the strain expressing 40Q in neuronal cells, posing a future purpose to better delineate the mechanism of action of ERN.

Nevertheless, it has already been demonstrated in a 128Q strain expressing impairment in the touch response that the reaction to the stimulus in 128Q;*daf-16* nematodes is comparable to that of 128Q;*aak-2* worms and that double mutants 128Q;*aak-2*;*daf-16* do not experience an additive effect, demonstrating that AMPK and *daf-16*/FOXO operate in the same signaling (177). Therefore it is reasonable to assume that these factors are involved in the identical pathway even in the 40Q::YFP worms. Concluding, these evidences confirm the involvement of both *aak-2*/AMPK and *daf-16*/FOXO signaling in the mechanism of action of ERN, posing the justifying basis for testing whether other further factors and pathways are activated by this effective ITC.

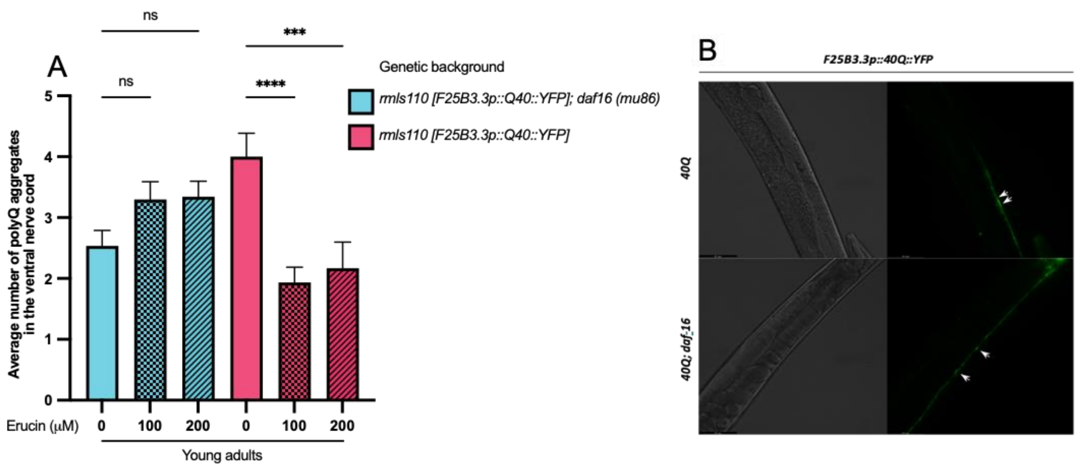


Figure 24. **Reduction of polyQ aggregation induced by erucin requires *daf-16*/FOXO transcription factor.** A) The treatment with 100 µM and 200 µM ERN of 40Q::YFP worms defective of the *daf-16* gene does not produce any significant difference in polyQ-induced neuronal aggregates formation in comparison to untreated worms (blue columns). In parallel, ERN treatment reduces polyQ-induced muscular aggregation in 40Q young adult animals of the control strain significantly (*P* <0.0001 for 100 µM ERN and *P* <0.001 for 200 µM). B) Representative images from fluorescence microscopy showing an equal number of polyQ neuronal aggregates (white arrows) in the ventral nerve cord of F25B3.3p::40Q::YFP and F25B3.3p::40Q::YFP; daf-16(mu86) young adult animals at 20x magnification.

5. CONCLUSIONS

In conclusion, the present research furnishes a One Health integrated approach at the human – animal – environment interface under the climate change scenario, investigating firstly heat stress induced toxicity in terms of loss of viability and of impairment of the redox balance in wild-type *C.elegans* nematodes. Thereafter, it considers the neurotoxic effects of protein aggregates formation in standard conditions and after heat stress, investigating the consequences of thermal stress on the balance of protein homeostasis and aggregation. Moreover, supporting the increasingly urgent necessity of finding preventing and mitigating solutions for humans against the negative health outcomes, and specifically NDDs, driven by climate change, this study focuses on the neuroprotective action of a natural ITC, ERN, both in standard and under heat stress conditions, in terms of protection of viability, reduction of ROS production and clearance of protein aggregates induced toxicity. Through different *C.elegans* models, the results presented in this research evidence that ERN protects from heat stress induced mortality and ROS formation, mechanism that, however, only partially explains its protective potential. Consequently, these data confirm that ERN prevents polyQ-induced aggregates formation both in muscular and neuronal tissues by activating AMPK catalytic subunit and, downstream in the pathway, inducing the expression of the *daf-16*/FOXO transcription factor. Moreover, they demonstrate the worsening of the polyQ-induced toxicity by heat stress, confirming ERN neuroprotective action even in thermal stress conditions. Moreover, they demonstrate that ERN reduces the aggregation of α-syn, the protein linked genetically and neuropathologically to PD, in standard, even though not under heat stress, conditions, and slightly rescues the motor capacity impaired by α-syn in both of the temperature conditions tested.

Finally, the results of this research partly explain the neuroprotective potential of ERN and pose the basis to further investigate whether other signaling pathways are involved in its protein aggregation induced toxicity clearance properties both in standard and under hyperthermic conditions, identifying in *C.elegans* an efficient experimental model to delineate the genes and molecular mechanisms involved in the action of a given compound. Nevertheless, besides its many advantages, the *in vivo* model represented by *C.elegans* possesses intrinsic limitations, for the simplicity of the body structure and lack of the complex physiology typical of humans. Therefore, further pre-clinical studies in mice models of protein aggregation are justified and could provide insights to test whether ERN could be a potential neuroprotective compound even in humans, highlighting the possibility of developing a nutraceutical product for people's use.

Concluding, considering that, as demonstrated in rocket salad leaves and even in many other plant species, the production of bioactive compound, and particularly PSMs, in plants is enhanced under high temperature of growth, and extensively under stressful weather parameters affected by climate change, this study represents one of the first evidences that, while the health risks posed by the environment to human beings rise, the solutions provided by nature also increase accordingly, in an eternal dialogue between man and nature that the One Health approach has the potential to investigate and develop.

6. ABBREVIATION'S INDEX

A

Aβ	Amyloid-beta
AD	Alzheimer's disease
ALS	Amyotrophic Lateral Sclerosis
AMPK	AMP-activated protein kinase
α-syn	Alpha-synuclein

C

CO_2	Carbon dioxide

D

DCF	2′,7′-dichlorofluorescein
DMSO	Dimethyl sulfoxide

E

ERN	Erucin

G

GLs	Glucosinolates

H

HD	Huntington's disease
H_2DCFDA	2′,7′-dichlorodihydrofluorescein diacetate
H_2DCF	2′,7′-dichlorodihydrofluorescein
HSF-1	Heat- shock factor-1
HSP	Heat shock protein
HSR	Heat shock response
Htt	Huntingtin protein

I

IBs	Inclusion bodies
IGF-1	Insulin-like growth factor 1
ILS	Insulin-like signaling
IPCC	Intergovernmental Panel on Climate Change
ITC	Isothiocyanate

L

LPS	Lipopolysaccharide

M

MND	Motor Neuron Disease

N

NDDs	Neurodegenerative diseases
NFkB	Nuclear factor kappa B
Nrf2	Nuclear factor erythroid 2–related factor 2

O

6-OHDA	6-hydroxydopamine

P

PD	Parkinson's diseases
PolyQ	Poly-glutamine
PSMs	Plant secondary metabolites

R

ROS	Reactive oxygen species

S

SARS	Severe acute respiratory disease
SBMA	Spino-bulbar muscular atrophy
SFN	Sulforaphane
SOD1	Superoxide dismutase

U

UPR	Unfolded protein response

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