Discovery of Fifteen New Aging-Delaying Plant Extracts That Extend the Longevity of Budding Yeast and Make Yeast Cells More Resistant to Long-Term Oxidative and Thermal Stresses

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ABSTRACT

Discovery of Fifteen New Aging-Delaying Plant Extracts That Extend the Longevity of Budding Yeast and Make Yeast Cells More Resistant to Long-Term Oxidative and Thermal Stresses

Monica Enith Lozano Rodriguez, M.Sc.

In a quest for previously unknown aging-delaying (geroprotective) natural chemicals, we used a robust cell viability assay to conduct a screen of a library of commercially available plant extracts. Our screen was aimed at the identification of those plant extracts in the library that can significantly prolong the chronological lifespan of budding yeast. Many of the plant extracts in the library have been used in traditional Chinese and other herbal medicines or the Mediterranean and other customary diets. The screen allowed us to discover fifteen plant extracts that considerably extend the longevity of chronologically aging budding yeast not limited in calorie supply. We demonstrated that each of the fifteen longevity-extending plant extracts is a geroprotector that decreases both the extrinsic and the intrinsic rates of aging in budding yeast. Our findings provided evidence that each of the fifteen longevity-extending plant extracts makes yeast more resistant to chronic (long-term) oxidative and thermal stresses. We also revealed that each of the fifteen geroprotective plant extracts mimics the longevity-extending and stress-protecting effects of a caloric restriction diet in yeast cells that are not limited in calorie supply and age chronologically.

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ATG, autophagy; CFU, colony-forming units; CLS, chronological lifespan; CR, caloric restriction; non-CR, non-caloric restriction; PKA, protein kinase A; RLS, replicative lifespan; PKH1/2, Pkb-activating kinase homolog; ROS, reactive oxygen species; Sch9, serine-threonine protein kinase Sch9; SNF1, sucrose non-fermenting; TORC1, the target of rapamycin complex 1; WT, wild-type strain.

1 Introduction

1.1 Studies in the budding yeast *Saccharomyces cerevisiae* discovered mechanisms of cellular processes that are common to all eukaryotes

The budding yeast *S. cerevisiae* is a useful model for elucidating mechanisms that underlie various cellular processes in eukaryotic organisms across species. *S. cerevisiae* is a single-cellular eukaryotic organism that people used in winemaking, brewing and baking over the last ten thousand years [1-3]. Since the discovery of alcoholic yeast fermentation and because of the significant improvement of the genetics approaches for studying *S. cerevisiae* [4, 5], this fast-growing under controllable culturing conditions unicellular eukaryote has also been used extensively as a model organism for elucidating the molecular mechanisms of many cellular processes [2, 6-8]. These cellular processes include the critical pathways of primary and secondary metabolism, DNA replication, DNA damage and repair, cell cycle regulation, vesicular protein traffic, transcription and translation mechanisms, cytoskeleton organization and function, mitochondrial respiration and oxidative phosphorylation, cellular signal transduction, cellular proteostasis maintenance, autophagy, lipid metabolism and transport, and many others [2, 6-10]. The rapid progress in using *S. cerevisiae* as a model organism in molecular biological studies of complex cellular processes was further speeded up after its genome was sequenced entirely, and the commercial libraries of gene-deletion, gene-overexpression and protein-tagging mutants of various genes became available [2, 7, 8, 10-13]. This progress was enhanced even more with the development and application of high-throughput methods for chemical biological, system biological and microfluidic dissection analyses of cellular processes in budding yeast [6-8, 10, 14-16]. The subsequent studies in multicellular eukaryotes have convincingly demonstrated that the molecular mechanisms underlying many cellular processes and initially discovered in budding yeast operate similarly in other eukaryotic organisms [6-8, 10, 17-19]. Such a great extent of evolutionary conservation further emphasizes the importance of using *S. cerevisiae* as a model organism for uncovering the mechanistic basis of essential cellular processes.

1.2 Two different methods of investigating *S. cerevisiae* aging in laboratory settings

Aging of *S. cerevisiae* under the controllable laboratory conditions can be investigated in two different ways, each corresponding to a different way the eukaryotic cells can become old. It

is a tradition to use robust laboratory assays for examining each of these two modes of cellular aging separately from each other [20-23]. These two different modes are called the replicative and chronological modes of yeast aging.

S. cerevisiae reproduces by asymmetric cell divisions called buddings [21, 23]. In the laboratory assay for yeast replicative aging, every new daughter cell formed by budding from a mother cell is removed with the help of a micromanipulator and the maximal number of daughter cells that each mother cell can form before irreversibly exiting mitosis is counted (Figure 1.1) [21, 23]. Thus, the maximal number of the mitotic divisions that a yeast cell can undergo before becoming mitotically senescent (i.e., incapable of dividing) defines the replicative mode of *S. cerevisiae* aging under laboratory conditions (Figure 1.1) [21, 23]. It is commonly believed that the replicative mode of yeast aging mimics the aging of mitotically active mammalian cells that can undergo only a limited number of divisions before becoming unable to divide [24-27]. Emerging evidence supports the notion that the replicative mode of yeast aging can be also be considered as a model for the aging of post-mitotic tissues in the nematode *Caenorhabditis elegans* and the organismal aging in humans [28-30]. Indeed, many genes that influence the replicative lifespan in *S. cerevisiae* also affect the reproductive lifespan in *C. elegans* [28, 29]. Furthermore, the hallmark events characteristic of replicatively old yeast cells are very similar to the ones observed in cells of elderly human individuals [30].

After *S. cerevisiae* cells cultured under laboratory conditions in a liquid medium with glucose consume this exogenous carbon source, they undergo a cell-cycle arrest in the G_1 phase and enter a reversible G_0 state of quiescence [20, 22]. Two characteristics of quiescent yeast cells distinguish them from non-quiescent cells. First, a quiescent yeast cell can form a colony after being transferred from a nutrient-depleted liquid medium to a surface of a nutrient-rich solid medium [20, 22]. Second, a population of quiescent yeast cells can synchronously re-enter the mitotic cell cycle after being transferred from a nutrient-depleted liquid medium to a nutrient-rich liquid medium [20, 22]. Under the laboratory conditions, a yeast cell in a nutrient-depleted liquid medium can maintain quiescence only for a limited number of days [20, 22]. Following this limited number of days, the *S. cerevisiae* cell exits quiescence and enters an irreversible state of mitotic senescence [20, 22]. The mitotically senescent yeast cells ultimately undergo an apoptotic or necrotic mode of regulated death [20, 22, 31, 32]. A laboratory clonogenic assay for yeast chronological aging measures the number of days required for such transition of the yeast cell from

being quiescent to becoming senescent (Figure 1.1) [20, 22, 31, 32]. Hence, the number of days during which a yeast cell undergoes a transition from quiescence to senescence defines the chronological mode of *S. cerevisiae* aging under laboratory conditions (Figure 1.1) [20, 22]. It is generally accepted that the chronological mode of yeast aging mimics the aging of mitotically inactive mammalian cells that lost the ability to proliferate by growth and division [25, 26, 33, 34].

The above two methods have been employed to investigate the replicative and chronological modes of yeast aging separately from each other and under controllable laboratory conditions. Such conditions may differ substantially from those existing within various natural ecosystems inhabited by budding yeast. Recent findings indicate that the replicatively old yeast cells are also chronologically old and, thus, that an aging process of yeast residing in the wild may integrate the replicative and chronological modes of aging [35-40]. A challenge for the future is to investigate the aging process of budding yeast within natural ecosystems and/or under field-like laboratory conditions.

1.3 *S. cerevisiae* is a valuable model organism for uncovering mechanisms of cellular aging and longevity regulation in other eucaryotes

As discussed above, the replicative and chronological lifespans of budding yeast are both relatively short and easy to measure under controllable laboratory conditions [20-23]. Besides (as was also discussed above), many advanced molecular and cell biological approaches for investigating mechanisms of aging and other complex cellular processes have been developed for *S. cerevisiae* [6-8, 10, 14-16]. These two factors facilitate the discoveries of the key molecular players in the replicative and chronological modes of aging in budding yeast. Because the research topic of my thesis concerns yeast chronological aging, I will mainly discuss this mode of yeast aging further in the text.

Research on chronological aging of *S. cerevisiae* led to the discovery of many genes and their protein products that control the rate of chronological aging and regulate longevity of budding yeast (Figure 1.2) [17, 25, 41, 42].

Studies in budding yeast also revealed a distinct set of cellular processes that are essential contributors to the pace of yeast chronological aging and define the longevity of chronologically aging *S. cerevisiae* [17, 25, 41, 42]. These processes include protein synthesis in the cytosol and mitochondria, stress protection, genome stability maintenance, mitochondrial respiration,

glyoxylate metabolic cycle, gluconeogenesis, glycogen synthesis and degradation, amino acid and fatty acid synthesis, autophagy, and peroxisome biogenesis (Figure 1.2) [17, 25, 41, 42].

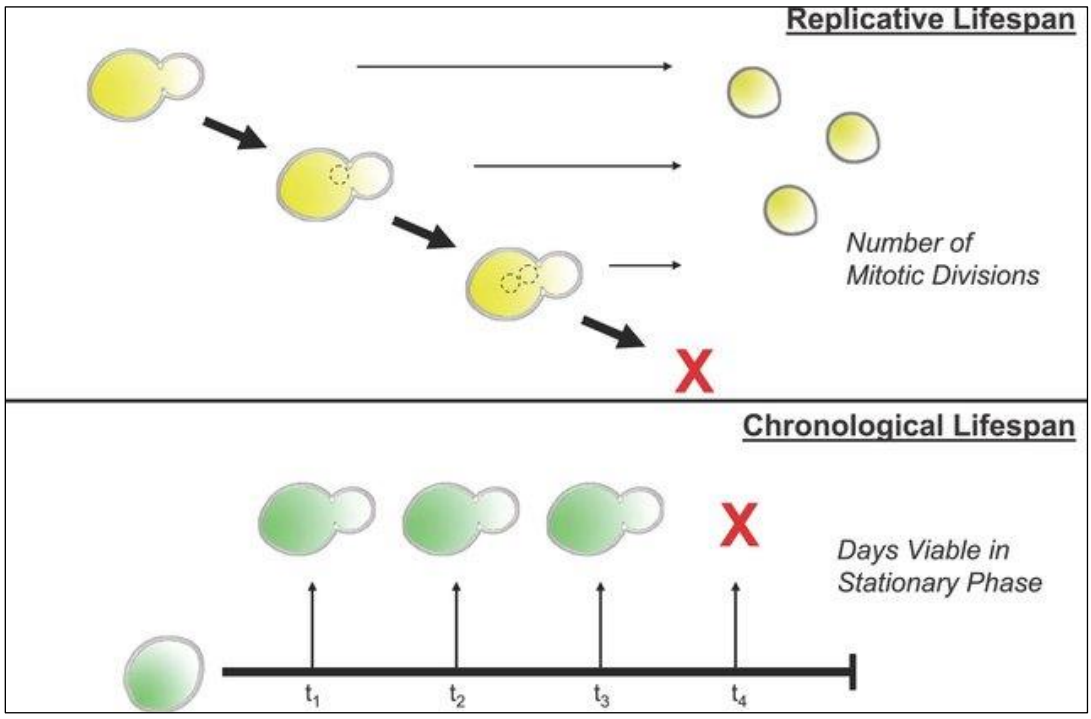

Figure 1.1. Laboratory assays for measuring the replicative and chronological lifespans under controllable conditions. A laboratory assay for replicative yeast aging measures the maximal number of mitotic divisions that a mother cell can undergo by budding a daughter cell before irreversibly exiting mitosis. A laboratory clonogenic assay for yeast chronological aging measures the number of days that are required for a quiescent cell to become senescent. See the text for more details.

Also, aging research in *S. cerevisiae* revealed specific signaling pathways and protein kinases that control the longevity-defining cellular processes [17, 25, 41, 42]. It was discovered that these signaling pathways and protein kinases are integrated into a hierarchical network (Figure 1.2) [17, 25, 41, 42]. The network assimilates the pro-aging (further referred to also as "aging-accelerating") TORC1 (target of rapamycin complex 1) pathway, pro-aging PKA (protein kinase A) pathway, pro-aging PKH1/2 (Pkb-activating kinase homolog) pathway, anti-aging (further referred to also as "aging-decelerating" or "aging-delaying") SNF1 (sucrose non-fermenting) pathway, anti-aging ATG (autophagy) pathway, pro-aging protein kinase Sch9 and anti-aging protein kinase Rim15 (Figure 1.2) [17, 25, 41, 42].

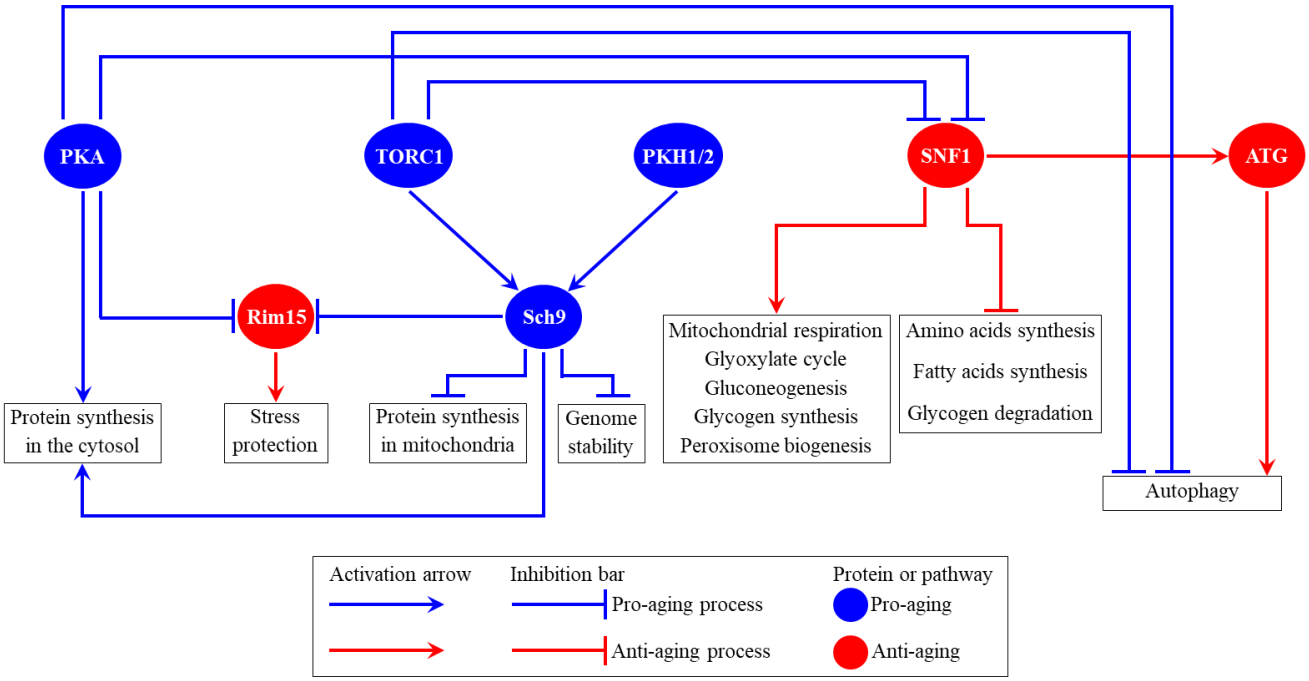

Figure 1.2. Several signaling pathways and protein kinases are integrated into a network that controls the rate of yeast chronological aging and defines yeast longevity. This network controls longevity-defining cellular processes shown in the boxes. Activation arrows and inhibition bars indicate pro-aging processes (presented in blue color) or anti-aging processes (shown in red color). Pro-aging or anti-aging signaling pathways and protein kinases are shown in blue or red color, respectively. Please see the text for additional details. Abbreviations: ATG, autophagy; PKA, protein kinase A; PKH1/2, Pkb-activating kinase homologs 1 and 2; Rim15, an anti-aging protein kinase; Sch9, a pro-aging protein kinase; SNF1, sucrose non-fermenting protein 1; TORC1, the target of rapamycin complex 1.

The pro-aging TORC1 pathway and its downstream target, the pro-aging protein kinase Sch9, are activated in response to the excessive amounts of nitrogen-rich nutrients (ammonium sulfate and amino acids) in a growth medium [17, 25, 41, 42]. Mutations that impair the functionalities of TORC1 or Sch9 delay yeast chronological aging and extend chronological lifespan in budding yeast [17, 25, 41, 42]. Furthermore, rapamycin-, caffeine- or cryptotanshinone-dependent inhibition of TORC1 has aging-delaying and longevity-extending effects in chronologically aging *S. cerevisiae* [17, 25, 41, 42]. After being activated by an excessive supply of nitrogen-rich nutrients, TORC1 and Sch9 accelerate chronological aging and shorten the chronological lifespan of *S. cerevisiae* because they phosphorylate and inhibit proteins involved in several anti-aging cellular processes [17, 25, 41, 42]. These anti-aging processes include the maintenance of nuclear genome stability and protein synthesis in mitochondria Rim15 (Figure 1.2)

[17, 25, 41, 42]. Besides, after being activated in response to excessive supply of nitrogen-containing nutrients, TORC1 and Sch9 phosphorylate and stimulate the pro-aging cellular process of protein synthesis in the cytosol (Figure 1.2) [17, 25, 41, 42]. Another downstream target of TORC1 and Sch9 is the anti-aging protein kinase Rim15. After being subjected to the Sch9-dependent phosphorylation and inhibition, the ability of Rim15 to stimulate the ant-aging process of cellular stress protection declines (Figure 1.2) [17, 25, 41, 42].

Sch9 is not the only phosphorylation target of activated TORC1 in budding yeast. The other TORC1 target is the anti-aging SNF1 pathway (Figure 1.2) [17, 25, 41, 42]. After being subjected to the TORC1-driven inhibitory phosphorylation, SNF1 less intensively promotes the anti-aging processes of mitochondrial respiration, glyoxylate cycle in mitochondria and the cytosol, gluconeogenesis in the cytosol, cytosolic glycogen synthesis, peroxisome biogenesis and autophagy in vacuoles (Figure 1.2) [17, 25, 41, 42]. The TORC1-driven phosphorylation of SNF1 also suppresses its ability to inhibit the pro-aging cellular processes of amino acids synthesis in mitochondria and the cytosol, fatty acid synthesis in the cytosol and cytosolic glycogen degradation (Figure 1.2) [17, 25, 41, 42].

Yet, another inhibitory phosphorylation target of activated TORC1 is the aging-delaying process of autophagy. After activated TORC1 phosphorylates several proteins implicated in the autophagic degradation of damaged proteins and organelles, the intensity of such degradation declines, cellular proteostasis is weakened and the process of yeast chronological aging is accelerated (Figure 1.2) [17, 25, 41, 42].

The essential role of TORC1 in the acceleration of cellular and organismal aging has been conserved in the course of evolution. Indeed, the mechanistic target of rapamycin TOR complexes 1 (mTORC1) and 2 (mTORC2) promote aging in nematodes, fruit flies, laboratory rodents and non-human primates because they phosphorylate and inhibit an evolutionarily conserved set of aging-decelerating cellular processes and phosphorylate and activate a set of aging-accelerating cellular processes similar to the ones subjected to activating phosphorylation in budding yeast (Figure 1.2) [17, 25].

The pro-aging protein kinase Sch9 is a downstream activating phosphorylation target not only for TORC1 but also for the sphingolipid long-chain base-activated PKH1/2 pathway in budding yeast (Figure 1.2) [17, 25, 41, 42]. In response to an increase in the concentration of complex sphingolipids within the plasma membrane, the PKH1/2 pathway promotes the activating

phosphorylation of Sch9, thus initiating the Sch9-dependent activation of pro-aging cellular processes and inhibition of anti-aging cellular processes in budding yeast (Figure 1.2) [17, 25, 41, 42]. Noteworthy, mutations that impair the functionality of the Pkh1 and Pkh2 protein components of the PKH1/2 pathway or cell treatment with myriocin (an indirect inhibitor of this pathway) slow down aging and prolong lifespan in chronologically aging *S. cerevisiae* [17, 25, 41, 42].

The longevity regulation network depicted in Figure 1.2 also integrates the pro-aging PKA pathway [17, 25, 41, 42]. This pathway is activated when yeast cells are cultured in a medium initially containing high concentrations of glucose [17, 25, 41, 42]. Mutations that impair the functionality of several protein components of the PKA pathway slow down yeast chronological aging and prolong chronological lifespan in budding yeast [17, 25, 41, 42]. The activated PKA pathway accelerates yeast chronological aging and shortens yeast chronological lifespan because it elicits the inhibitory phosphorylation of Rim15, thus weakening the aging-delaying process of cellular stress protection (Figure 1.2) [17, 25, 41, 42]. The activated PKA pathway also promotes aging and decreases the longevity of chronologically aging yeast by promoting activating phosphorylation of proteins involved in the aging-accelerating process of protein translation in the cytosol (Figure 1.2) [17, 25, 41, 42]. The PKA pathway is an essential contributor to longevity regulation not only in budding yeast but also in laboratory mice [17, 25]. Hence, the essential role of the PKA pathway in accelerating cellular and organismal aging has been conserved in the course of evolution.

Moreover, studies in budding yeast discovered several small chemical molecules that slow yeast chronological aging and prolong yeast longevity because they control the information flow through the signaling network of longevity regulation [17, 25, 41, 42]. These molecules include resveratrol, rapamycin, caffeine, myriocin, spermidine, cryptotanshinone, quercetin and lithocholic bile acid [17, 25, 41, 42]. Although resveratrol increases the replicative lifespan of budding yeast because it stimulates the NAD^+-dependent protein deacetylase Sir2, it extends yeast chronological lifespan in a Sir2-independent manner – perhaps by activating some other NAD^+-dependent protein deacetylases [17, 25, 41, 42]. The longevity-extending abilities of rapamycin, caffeine and cryptotanshinone in chronologically aging yeast are due to their direct (rapamycin and caffeine) or indirect (cryptotanshinone) inhibitory effects on TORC1 [17, 25, 41, 42]. Myriocin extends yeast chronological lifespan because it indirectly inhibits the Pkh1 and Pkh2 protein components of the PKH1/2 pathway [17, 25, 41, 42]. Spermidine increases the replicative

lifespan of budding yeast because it stimulates transcription of genes encoding proteins that involve in the aging-decelerating process of autophagy [17, 25, 41, 42]. Quercetin extends the chronological lifespan of budding yeast by causing a significant decline in the intracellular concentration of reactive oxygen species (ROS), thereby protecting cellular macromolecules from oxidative damage [17, 25, 41, 42]. Lithocholic bile acid increases yeast chronological lifespan because it alters the mitochondrial membrane lipidome, thereby improving mitochondrial functionality [17, 25, 41, 42].

After all these genes, proteins, cellular processes, signaling pathways, protein kinases and small chemical molecules were discovered in chronologically aging yeast, aging research in several multicellular eukaryotic organisms (including the nematode *Caenorhabditis elegans*, the fruit fly *Drosophila melanogaster*, laboratory rodents and non-human primates) revealed that similar genes, proteins, cellular processes, signaling pathways, protein kinases and small chemical molecules control the rates of cellular and organismal aging and regulate organismal lifespan and healthspan in these eukaryotes. These findings provided evidence that mechanisms of aging and aging delay have been conserved in the evolution [8, 10, 17-19, 25-27, 29, 30, 41, 42]. Thus, *S. cerevisiae* is a useful model organism for uncovering these evolutionarily conserved mechanisms.

1.4 Phytochemicals produced by plants slow aging and prolong the longevity of *S. cerevisiae*

Plants synthesize phytochemicals as the products of secondary metabolic pathways that, unlike the primary metabolic pathways, do not provide the host plants with significant amounts of energy or abundant quantities of biosynthetic products needed for their growth and reproduction [43-45]. Based on the chemical structure and functionality of phytochemicals, they belong to eleven different classes. These chemical classes of phytochemicals include phenolic compounds, terpenes, betalains, polysulfides, organosulfides, indoles, protease inhibitors, organic acids, modified purines, quinones and polyamines [46-48].

It has been proposed that plants evolved the secondary metabolic pathways for the synthesis of phytochemicals because these diverse chemical compounds can help the host plants to survive and reproduce [49-56]. The survival and reproduction benefits that phytochemicals provide to the host plants include protection from various environmental stresses and pollutants, a

defense from invading insects and many viral and microbial infections, and an attraction of pollinators and other symbiotes [49-56].

In addition to the above benefits that phytochemicals provide to the host plants, they are known for their abilities to slow cellular aging and prolong longevity in budding yeast [57-62].

Resveratrol is a polyphenolic phytochemical that increases the replicative lifespan of budding yeast because it stimulates the NAD+-dependent protein deacetylase called Sir2 [57]. The resveratrol-dependent stimulation of Sir2 suppresses rDNA recombination, thereby enhancing the stability of nuclear DNA and extending the longevity of replicatively aging *S. cerevisiae* [57].

Quercetin is a flavonoid phytochemical compound that extends the longevity of chronologically aging budding yeast because it elicits a significant decline in the intracellular level of ROS, thus lowering the extent of glutathione oxidation, lipid peroxidation and protein carbonylation [58]. These quercetin-driven changes increase cell resistance to oxidative stress, thus slowing down the chronological aging of *S. cerevisiae* [58].

Caffeine is a purine-like phytochemical compound that slows the chronological mode of yeast aging by inhibiting the protein kinase activity of TORC1, thus stimulating the aging-delaying cellular processes and suppressing the aging-accelerating cellular processes described in section 1.3. The aging-delaying cellular processes that are stimulated by the caffeine-dependent inhibition of TORC1 include the maintenance of nuclear genome stability, protein synthesis in mitochondria, cellular stress protection, mitochondrial respiration, glyoxylate cycle in mitochondria and the cytosol, gluconeogenesis in the cytosol, cytosolic glycogen synthesis, peroxisome biogenesis, autophagy and cellular proteostasis maintenance (Figure 1.2) [17, 25, 41, 42, 59]. The aging-accelerating cellular processes that are suppressed in response to the caffeine-dependent inhibition of TORC1 include protein synthesis in mitochondria, amino acids synthesis in mitochondria and the cytosol, fatty acid synthesis in the cytosol and cytosolic glycogen degradation (Figure 1.2) [17, 25, 41, 42].

Spermidine is a polyamine phytochemical compound that prolongs the chronological lifespan oh *S. cerevisiae* because it promotes the deacetylation of histone H3 by inactivating histone acetyltransferases [83]. The spermidine-dependent deacetylation of histone H3 promotes transcription of several autophagy-related genes, thus enhancing autophagic removal of dysfunctional organelles, promoting cellular proteostasis and delaying the onset of an age-related mode of regulated necrotic cell death [83].

Phloridzin is a polyphenolic phytochemical compound that increases the replicative lifespan of *S. cerevisiae* because it promotes transcription of the genes for the cytosolic and mitochondrial forms of superoxide dismutase (Sod1 and Sod2, respectively) as well as for the NAD+-dependent protein deacetylase Sir2 [272]. The phloridzin-driven increase in the enzymatic activities of Sod1 and Sod2 suppresses the aging-accelerating process of oxidative macromolecular damage by reducing the intracellular levels of ROS [272]. The phloridzin-driven rise in the enzymatic activity of Sir2 inhibits rDNA recombination, thereby stimulating the aging-delaying process of nuclear DNA maintenance [272]. All these effects of phloridzin extend the replicative lifespan of budding yeast.

Cryptotanshinone is a quinone phytochemical compound that extends the chronological lifespan of *S. cerevisiae* because it elicits the following two effects: 1) it inhibits the pro-aging protein kinases TORC1 and Sch9, thus lowering the inhibitory phosphorylation of their numerous aging-decelerating protein targets and promoting the activating phosphorylation of their many aging-accelerating protein targets (see section 1.3), and 2) it activates mitochondrial superoxide dismutase Sod2, thus reducing the intracellular levels of ROS and suppressing the aging-accelerating process of oxidative macromolecular damage [62].

1.5 The objectives of studies described in this thesis

The objective of studies presented in my thesis was to apply a robust clonogenic assay to search for previously unknown aging-delaying (geroprotective) plant extracts that can extend the longevity of chronologically budding yeast. Many of these commercially available plant extracts have been used in traditional Chinese and other herbal medicines or the Mediterranean and other customary diets. However, none of them has been known for their ability to slow aging and prolong the organismal lifespan. My search discovered fifteen plant extracts that significantly extend the longevity of chronologically aging yeast not limited in calorie supply. I found that each of the fifteen longevity-extending plant extracts makes yeast cells resistant to long-term oxidative and thermal stresses. All findings described in my thesis were published in Oncotarget. 2020; 11:2182-2203. Dr. Titorenko intellectually directed this project. He also corrected the first draft of my thesis and the entire manuscript of the above research paper.

2 Materials and methods

2.1 Yeast strains, media and growth conditions

The wild-type (WT) strain *Saccharomyces cerevisiae* BY4742 (*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*) and single-gene-deletion mutant strains in the BY4742 genetic background (all from Thermo Scientific/Open Biosystems) were grown in a synthetic minimal YNB medium (0.67% (w/v) Yeast Nitrogen Base without amino acids from Fisher Scientific; #DF0919-15-3) initially containing 2% (w/v) or 0.5% (w/v) glucose (#D16-10; Fisher Scientific), 20 mg/l *L*-histidine (# H8125; Sigma), 30 mg/l *L*-leucine (#L8912; Sigma), 30 mg/l *L*-lysine (#L5501; Sigma) and 20 mg/l uracil (#U0750; Sigma), with a plant extract (PE) or without it. A stock solution of each PE in ethanol was made on the day of adding this PE to cell cultures. For each PE, the stock solution was added to growth medium with 2% (w/v) or 0.5% (w/v) glucose immediately following cell inoculation into the medium. In a culture supplemented with a PE, ethanol was used as a vehicle at the final concentration of 2.5% (v/v). In the same experiment, yeast cells were also subjected to ethanol-mock treatment by being cultured in growth medium initially containing 2% (w/v) or 0.5% (w/v) glucose and 2.5% (v/v) ethanol. Cells were cultured at 30ºC with rotational shaking at 200 rpm in Erlenmeyer flasks at a "flask volume/medium volume" ratio of 5:1.

2.2 Chronological lifespan (CLS) assay

A sample of cells was taken from a culture at a certain day following cell inoculation and PE addition into the medium. A fraction of the sample was diluted to determine the total number of cells using a hemacytometer. Another fraction of the cell sample was diluted, and serial dilutions of cells were plated in duplicate onto YEP medium (1% (w/v) yeast extract, 2% (w/v) peptone; both from Fisher Scientific; #BP1422-2 and #BP1420-2, respectively) containing 2% (w/v) glucose (#D16-10; Fisher Scientific) as carbon source. After 2 d of incubation at 30ºC, the number of colony-forming units (CFU) per plate was counted. The number of CFU was defined as the number of viable cells in a sample. For each culture, the percentage of viable cells was calculated as follows: (number of viable cells per ml/total number of cells per ml) × 100. The percentage of viable cells in the mid-logarithmic growth phase was set at 100%.

2.3 Plating assays for the analysis of resistance to oxidative and thermal stresses

For the analysis of hydrogen peroxide (oxidative stress) resistance, serial dilutions (1:100 to $1:10^5$) of cells removed from each culture at various time-points were spotted onto two sets of plates. One set of plates contained a YP medium with 2% glucose alone, whereas the other set contained a YP medium with 2% glucose supplemented with 5 mM hydrogen peroxide. Pictures were taken after a 3-day incubation at 30ºC.

For the analysis of thermal stress resistance, serial dilutions (1:100 to $1:10^5$) of cells removed from each culture at various time-points were spotted onto two sets of plates containing the YP medium with 2% glucose. One set of plates was incubated at 30ºC. The other set of plates was initially incubated at 60ºC for 60 min and was then transferred to 30ºC. Pictures were taken after a 3-day incubation at 30ºC.

2.4 Statistical analysis

Statistical analysis was performed using Microsoft Excel's Analysis ToolPack-VBA. All data on cell survival are presented as mean ± SEM (n = 6). The *p* values for comparing the means of two groups using an unpaired two-tailed *t*-test were calculated with the help of the GraphPad Prism 7 statistics software. The logrank test for comparing each pair of survival curves was performed with GraphPad Prism 7. Two survival curves were considered statistically different if the *p* value was less than 0.05.

3 Results

3.1 Identification of new PEs that prolong the longevity of chronologically aging budding yeast

In search of new aging-delaying (geroprotective) PEs, we performed a screen of fifty-three commercially available PEs. The origin and properties of these PEs are shown in Table 3.1. These PEs are believed to have positive effects on human health, and many of them have been used in traditional Chinese and other herbal medicines or the Mediterranean and other long-established diets.

Table 3.1. Properties of plant extracts (PEs) used to conduct a new screen for PEs that can prolong the longevity of chronologically aging budding yeast.

Abbreviated name of a PE	The botanical name of a plant	Plant part used to make a PE	Properties of a PE	A commercial source of a PE
PE26	*Serenoa repens*	Berry	Extraction solvent: carbon dioxide. Extract ratio: 15:1. Composition: natural extract (oil) (45-55%), silica (45-55%).	Idunn Technologies
PE38	*Centella asiatica*	Herb	Extraction solvent: alcohol (50-70%), water (30-50%). Extract ratio: (8-12):1. Composition: 10% asiaticoside, 30% total triterpenes.	Idunn Technologies
PE39	*Hypericum perforatum*	Aerial parts	Extraction solvent: ethanol (60-80%), water (20-40%). Extract ratio: (5-10):1. Composition: 0.3% hypericin.	Idunn Technologies
PE40	*Boswellia serrata*	Resin	Extraction solvent: methanol (80%), water (20%). Extract ratio: 20:1. Composition: 65% boswellic acids.	Idunn Technologies
PE41	*Ruscus aculeatus*	Root	Extraction solvent: ethanol (70-80%), water (20-30%). Extract Ratio: 8:1. Composition: 10% ruscogenins.	Idunn Technologies
PE42	*Ilex paraguariensis*	Leaf	Extraction solvent: water. Extract ratio: (3-10):1. Composition: 2% caffeine.	Idunn Technologies
PE43	*Schisandra chinensis*	Berry	Extraction solvent: ethanol (30%), water (70%). Extract ratio: 4/1, 1% schizandrins.	Idunn Technologies

PE44	*Cynara scolymus L.*	Leaf	Extraction solvent: water. Extract ratio: 4:1. Composition: > 5% cynarin.	Idunn Technologies
PE45	*Allium cepa L.*	Bulb skin	Extraction solvent: ethanol (70%), water (30 %). Extract ratio: (20-25):1. Composition: > 5% quercetin glycoside derivates.	Idunn Technologies
PE46	*Matricaria recutita L.*	Flower	Extraction solvent: ethanol (80%), water (20%). Extract ratio: 5:1. Composition: 3% apigenins.	Idunn Technologies
PE47	*Ocimum tenuiflorum*	Leaf	Extraction solvent: ethanol (90%), water (10%). Extract ratio: 10:1. Composition: > 5% ursolic acid.	Idunn Technologies
PE48	*Rhaphanus sativus L. var. niger*	Root	Extraction solvent alcohol (60-80%), water (40-20%). Extract ratio: 4:1. Composition: unknown.	Idunn Technologies
PE49	*Rosmarinus officinalis L.*	Leaf	Extraction solvent: acetone. Extract ratio: (35-50):1. Composition: > 50% carnosic acid.	Idunn Technologies
PE50	*Angelica archangelica L.*	Root	Extraction solvent: ethanol (50%), water (50%). Extract ratio: 4:1. Composition: > 3% organic acids.	Idunn Technologies
PE51	*Epimedium grandiflorum*	Herb	Extraction solvent: ethanol (60%), water (40%). Extract ratio: 20:1. Composition: 20% icariin.	Idunn Technologies
PE52	*Bacopa monnieri*	Leaf	Extraction solvent: aqueous alcohol. Extract ratio: 10:1. Composition: 20% bacosides.	Idunn Technologies
PE53	*Phaseolus vulgaris*	Bean	Extraction solvent: aqueous alcohol. Extract ratio: 10:1. Composition: unknown.	Idunn Technologies
PE54	*Allium sativum L.*	Bulb	Extraction solvent: water. Extract ratio: 120:1. Composition: 4.5% alliin.	Idunn Technologies
PE55	*Morus alba*	Leaf	Extraction solvent: ethanol (70%), water (30%). Extract ratio: 4:1. Composition: 1% 1-deoxynojirimycin.	Idunn Technologies

PE56	*Saphora Japonica*	Flower	Extraction solvent: ethanol, water. Extract ratio: unknown. Composition: rutin (40%), quercetin (60%).	Idunn Technologies
PE57	*Morus nigra*	Fruit	Extraction solvent: ethanol, water. Extract ratio: 4:1. Composition: unknown.	Idunn Technologies
PE58	*Magnolia officinalis*	Bark	Extraction solvent: unknown. Extract ratio: (35-40):1. Composition: 40% honokiol.	Idunn Technologies
PE59	*Solidago virgaurea*	Herb	Extraction solvent: ethanol (30%), water (70%). Extract ratio: 4:1. Composition: > 2% flavonoid hyperosides.	Idunn Technologies
PE60	*Astragalus membranaceus*	Root	Extraction solvent: ethanol, water. Extract ratio: 8:1. Composition: 16% polysaccharides.	Idunn Technologies
PE61	*Lepidium meyenii*	Root	Extraction solvent: water, then ethanol (96%) and water (4%). Extract ratio: (22-27):1. Composition: 0.6% macamides and macaenes.	Idunn Technologies
PE62	*Taraxacum officinale*	Leaf	Extraction solvent: ethanol (70-80%), water (20-30%). Extract ratio: (4-7):1. Composition: 3% vitexin.	Idunn Technologies
PE63	*Taraxacum officinale*	Root	Extraction solvent: ethanol (60%), water (40%). Extract ratio: 15:1. Composition: 0.3-0.4% phenolic acids (chicoric, chlorogenic and caftaric acids).	Idunn Technologies
PE64	*Citrus sinensis*	Fruit	Extraction solvent: unknown. Extract ratio: unknown. Composition: ≥ 20% limonene.	Idunn Technologies
PE65	*Piper methysticum*	Root	Extraction solvent: ethanol (65%), water (35%,). Extract ratio: 8:1. Composition: > 30% kavalactones.	Idunn Technologies
PE66	*Handroanthus chrysotrichus*	Bark	Extraction solvent: ethanol (70%), water (30%). Extract ratio: (9-15):1. Composition: unknown.	Idunn Technologies
PE67	*Euterpe oleracea*	Fruit	Extraction solvent: water. Extract ratio: 20:1.	Idunn Technologies

			Composition: > 10% polyphenols.	
PE68	*Humulus lupulus*	Whole plant	Extraction solvent: unknown. Extract ratio: (5.5-6.5):1. Composition: unknown.	Idunn Technologies
PE69	*Vitis vinifera*	Grape skin	Extraction solvent: ethanol (30%), water (70%). Extract ratio: 450:1. Composition: ≥ 5% trans-resveratrol.	Idunn Technologies
PE70	*Vitis vinifera*	Grape	Extraction solvent: water (4%), ethanol (96%). Extract ratio: 200:1. Composition: ≥ 20% oligostilbenes.	Idunn Technologies
PE71	*Malus domestica* + *Vitis vinifera*	Grape + Fruit	Extraction solvent: water (5%), ethanol (95%). Extract ratio: (500-600):1. Composition: ≥ 95% polyphenols.	Idunn Technologies
PE72	*Andrographis paniculata*	Whole plant	Extraction solvent: unknown. Extract ratio: unknown. Composition: ≥ 20% andrographolids.	Idunn Technologies
PE73	*Oryza sativa* fermented with *Monascus purpureus* yeast	Fermented rice	Extraction solvent: unknown. Extract ratio: unknown. Composition: ≥ 20% monacolin K.	Idunn Technologies
PE74	*Melissa officinalis*	Leaf	Extraction solvent: unknown. Extract ratio: 4:1. Composition: ≥ 1% rosmarinic acid.	Idunn Technologies
PE75	*Hydrastis canadensis*	Root	Extraction solvent: ethanol (75%), water (25%). Extract ratio: (5-7):1. Composition: ≥ 5% berberine and other alkaloids.	Idunn Technologies
PE76	*Polygonum cuspidatum*	Root	Extraction solvent: unknown. Extract ratio: unknown. Composition: ≥ 20% resveratrol.	Idunn Technologies
PE77	*Trigonella foenum-graecum*	Seed	Extraction solvent: ethanol (60%), water (40%). Extract ratio: (5-8):1. Composition: 50% saponins.	Idunn Technologies
PE78	*Berberis vulgaris*	Root bark	Extraction solvent: ethanol (50%), water (50%). Extract ratio: (10-12):1. Composition: 6% berberine.	Idunn Technologies

PE79	*Crataegus monogyna*	Leaf, flower and stem	Extraction solvent: ethanol (80%), water (20%). Extract ratio: (3-6):1. Composition: 1.5% flavonoids.	Idunn Technologies
PE80	*Sophora japonica L.*	Flower bud	Extraction solvent: water. Extract ratio: (16-20):1. Composition: 95% quercetin.	Idunn Technologies
PE81	*Taraxacum erythrospermum*	Leaf	Extraction solvent: ethanol (70-80%), water (20-30%). Extract ratio: (4-7):1. Composition: 3% vitexin.	Idunn Technologies
PE82	*NA*	NA	Na-RALA Powder, Sodium R-lipoate (> 80 % Total R-lipoic Acid) from synthesis.	Idunn Technologies
PE83	*Ilex paraguariensis*	Whole plant	Extraction solvent: unknown. Extract ratio: unknown. Composition: unknown.	Idunn Technologies
PE84	*Vitis vinifera L.*	Seed	Extraction solvent: ethanol, water. Extract ratio: unknown. Composition: 95% polyphenols.	Idunn Technologies
PE85	*Ganoderma lucidum*	Mushroom body	Extraction solvent: unknown. Extract ratio: unknown. Composition: unknown.	Idunn Technologies
PE86	*Panax ginseng*	Root	Extraction solvent: unknown. Extract ratio: unknown. Composition: unknown.	Idunn Technologies
PE87	*Lycium barbarum*	Whole plant	Extraction solvent: unknown. Extract ratio: unknown. Composition: unknown.	Idunn Technologies
PE88	*Hemerocallis fulva*	Flower	Extraction solvent: unknown. Extract ratio: unknown. Composition: unknown.	Idunn Technologies
PE89	*Curcuma L.*	Root	Extraction solvent: unknown. Extract ratio: unknown. Composition: curcumin solid lipid microparticles to improve absorption.	Idunn Technologies

To conduct the screen, we exploited a robust clonogenic cell viability assay for measuring yeast chronological lifespan (CLS) [63]. In this assay, the wild-type (WT) strain BY4742 was cultured in the synthetic minimal YNB medium initially containing 2% (w/v) glucose, as described in Materials and Methods. Cells of budding yeast cultured under such non-caloric restriction (non-CR) conditions are known to age chronologically faster than the ones cultured under CR conditions on 0.2% (w/v) or 0.5% (w/v) glucose [25, 26, 42, 63].

At the time of cell inoculation into the culturing medium, we added each of the assessed PEs at a final concentration ranging from 0.02% (w/v) to 1.0% (w/v). We found that PE40, PE41, PE44, PE50, PE53, PE66, PE73, PE84, PE86 and PE87 do not affect the mean and maximum CLS of WT yeast if exogenously supplemented within this wide range of initial concentrations (Figures 3.1-3.7). In contrast, PE38, PE43, PE45, PE46, PE48, PE49, PE51, PE52, PE54-PE58, PE60-PE63, PE65, PE67, PE70, PE71, PE74, PE76, PE80, PE82, PE85, PE88 and PE89 were cytotoxic at certain concentrations; they decreased the mean and/or maximum CLS of WT yeast if used at the final concentrations in the 0.1 (w/v) to 1.0% (w/v) range (Figures 3.1-3.7).

Our screen revealed that fifteen of fifty-three tested PEs statistically significantly increase the mean and maximum CLS of WT yeast cultured under non-CR conditions on 2% (w/v) glucose (Figures 3.1-3.6; Figures 3.8-3.9). Each of these fifteen PEs extended the longevity of chronologically aging WT yeast if used within a specific concentration range and exhibited the highest longevity-extending effect at a certain concentration within this range (Figures 3.1-3.6). The following PEs exhibited the highest longevity-extending effect under non-CR conditions of cell culturing: 0.5% (w/v) PE26 from berries of *Serenoa repens* (Figures 3.1 and 3.8A), 0.5% (w/v) PE39 from aerial parts of *Hypericum perforatum* (Figures 2.1 and 2.8B), 0.5% (w/v) PE42 from leaves of *Ilex paraguariensis* (Figures 3.1 and 3.8C), 0.3% (w/v) PE47 from leaves of *Ocimum tenuiflorum* (Figures 3.1 and 3.8D), 0.3% (w/v) PE59 from the whole plant of *Solidago virgaurea* (Figures 3.1 and 3.8E), 0.1% (w/v) PE64 from fruits of *Citrus sinensis* (Figures 3.4 and 3.8F), 0.5% (w/v) PE68 from the whole plant of *Humulus lupulus* (Figures 3.4 and 3.8G), 1.0% (w/v) PE69 from grape skins of *Vitis vinifera* (Figures 3.5 and 3.8H), 0.1% (w/v) PE72 from the whole plant of *Andrographis paniculata* (Figures 3.5 and 3.9A), 0.3% (w/v) PE75 from roots of *Hydrastis canadensis* (Figures 3.5 and 3.9B), 0.5% (w/v) PE77 from seeds of *Trigonella foenum-graecum* (Figures 3.6 and 3.9C), 0.3% (w/v) PE78 from root barks of *Berberis vulgaris* (Figures 3.6 and 3.9D), 0.5% (w/v) PE79 from leaves, flowers and stems of *Crataegus monogyna* (Figures 3.6 and 3.9E), 0.3% (w/v) PE81 from leaves of *Taraxacum erythrospermum* (Figures 3.6 and 3.9F), and 0.5% (w/v) PE83 from the whole plant of *Ilex paraguariensis* (Figures 3.6 and 3.9G).

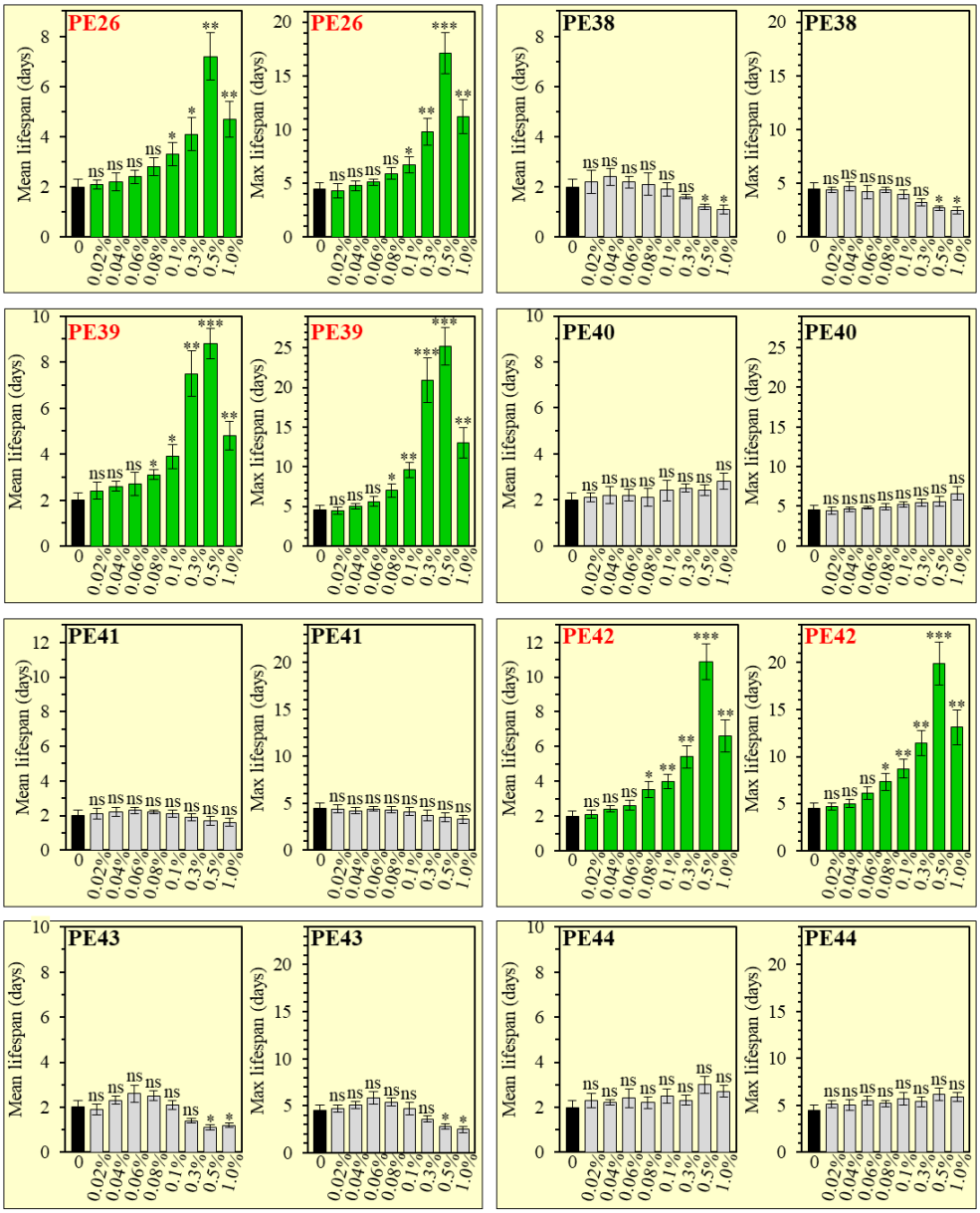

Figure 3.1. PE26, PE39 and PE42, but not PE38, PE40, PE41, PE43 or PE44, increase the mean and maximum CLS of WT yeast cultured under non-CR conditions on 2% (w/v) glucose. WT cells were cultured in the synthetic minimal YNB medium initially containing 2% (w/v) glucose, in the presence of a PE or its absence. The mean and maximum lifespans of chronologically aging WT strain cultured under non-CR conditions without a PE or with a PE added at various concentrations are shown; data are presented as means ± SEM (n = 6; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, *ns*, not significant; the *p* values for comparing the means of two groups were calculated using an unpaired two-tailed *t* test as described in Materials and Methods). Note that PE38 and PE43 can decrease the CLS of WT yeast under non-CR conditions if added at a final concentration of 0.5 (w/v) or 1.0% (w/v).

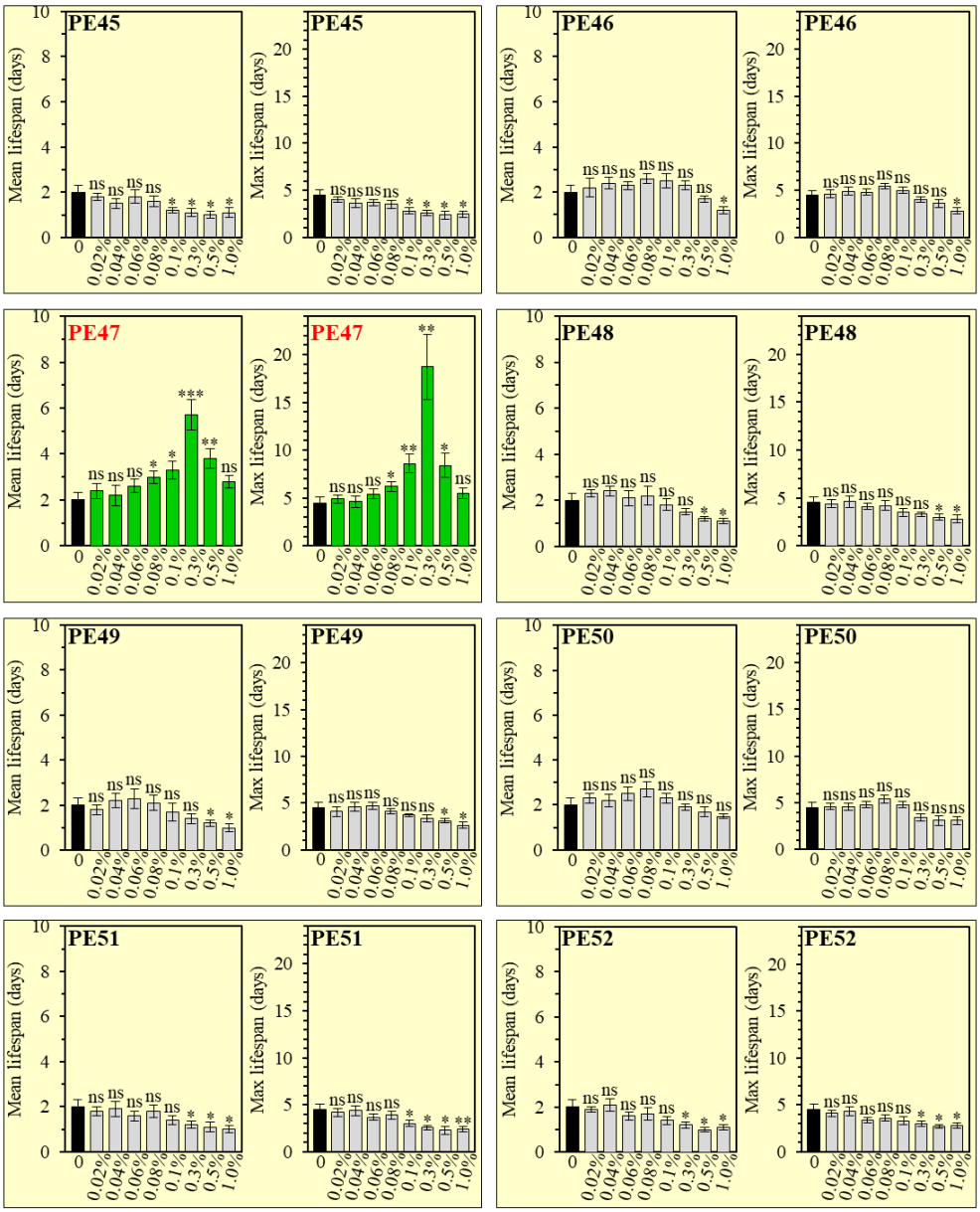

Figure 3.2. PE47, but not PE45, PE46, PE48, PE49, PE50, PE51 or PE52, increases the mean and maximum CLS of WT yeast cultured under non-CR conditions on 2% (w/v) glucose. WT cells were cultured as described in the legend to Figure 2.1. The mean and maximum lifespans of chronologically aging WT strain cultured under non-CR conditions without a PE or with a PE added at various concentrations are shown; data are presented as means ± SEM (n = 6; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, *ns*, not significant; the *p* values for comparing the means of two groups were calculated as described in the legend to Figure 2.1). Note that PE45, PE46, PE48, PE49, PE51 and PE52 can decrease the CLS of WT yeast under non-CR conditions if added at a final concentration ranging from 0.1% (w/v) to 1.0% (w/v).

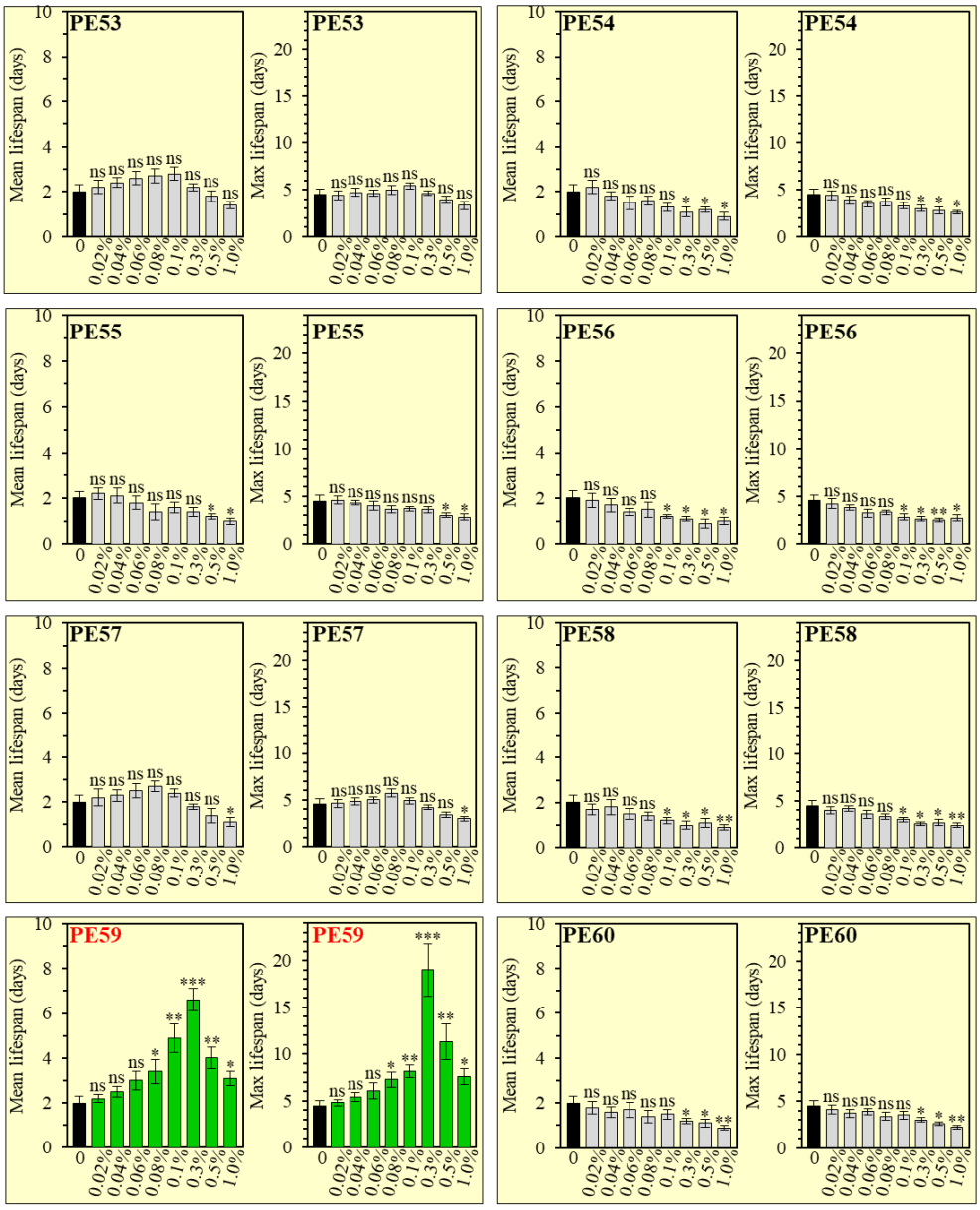

Figure 3.3. PE59, but not PE53, PE54, PE55, PE56, PE57, PE58 or PE60, increases the mean and maximum CLS of WT yeast cultured under non-CR conditions on 2% (w/v) glucose. WT cells were cultured as described in the legend to Figure 2.1. The mean and maximum lifespans of chronologically aging WT strain cultured under non-CR conditions without a PE or with a PE added at various concentrations are shown; data are presented as means ± SEM (n = 6; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, *ns*, not significant; the p values for comparing the means of two groups were calculated as described in the legend to Figure 2.1). Note that PE54, PE55, PE56, PE57, PE58 and PE60 can decrease the CLS of WT yeast under non-CR conditions if added at a final concentration ranging from 0.1% (w/v) to 1.0% (w/v).

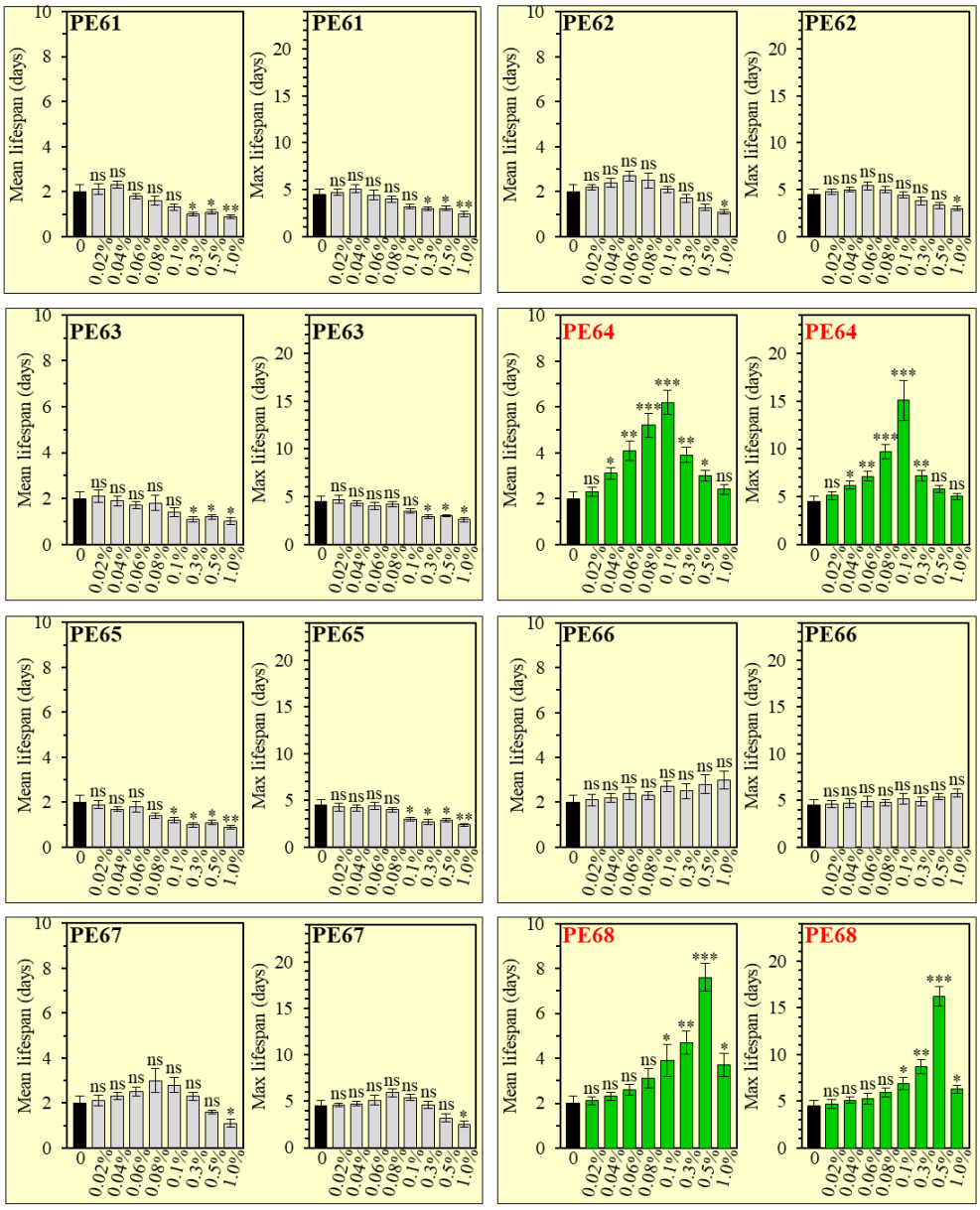

Figure 3.4. PE64 and PE68, but not PE61, PE62, PE63, PE65, PE66 or PE67, increase the mean and maximum CLS of WT yeast cultured under non-CR conditions on 2% (w/v) glucose. WT cells were cultured as described in the legend to Figure 2.1. The mean and maximum lifespans of chronologically aging WT strain cultured under non-CR conditions without a PE or with a PE added at various concentrations are shown; data are presented as means ± SEM (n = 6; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, *ns*, not significant; the *p* values for comparing the means of two groups were calculated as described in the legend to Figure 2.1). Note that PE61, PE62, PE63, PE65 and PE67 can decrease the CLS of WT yeast under non-CR conditions if added at a final concentration ranging from 0.1% (w/v) to 1.0% (w/v).

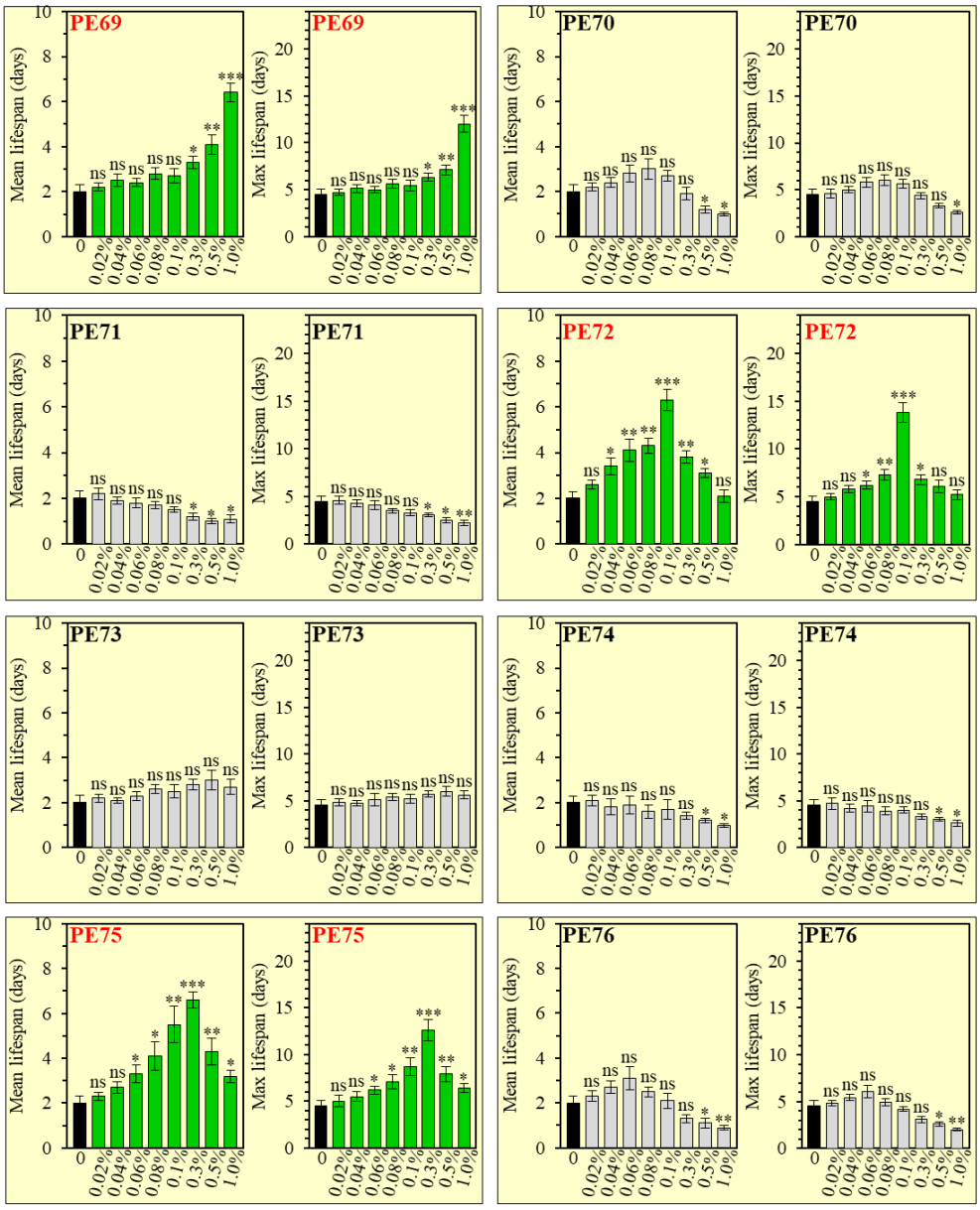

Figure 3.5. PE69, PE72 and PE75, but not PE70, PE71, PE73, PE74 or PE76, increase the mean and maximum CLS of WT yeast cultured under non-CR conditions on 2% (w/v) glucose. WT cells were cultured as described in the legend to Figure 2.1. The mean and maximum lifespans of chronologically aging WT strain cultured under non-CR conditions without a PE or with a PE added at various concentrations are shown; data are presented as means ± SEM (n = 6; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, *ns*, not significant; the p values for comparing the means of two groups were calculated as described in the legend to Figure 2.1). Note that PE70, PE71, PE74 and PE76 can decrease the CLS of WT yeast under non-CR conditions if added at a final concentration ranging from 0.3% (w/v) to 1.0% (w/v).

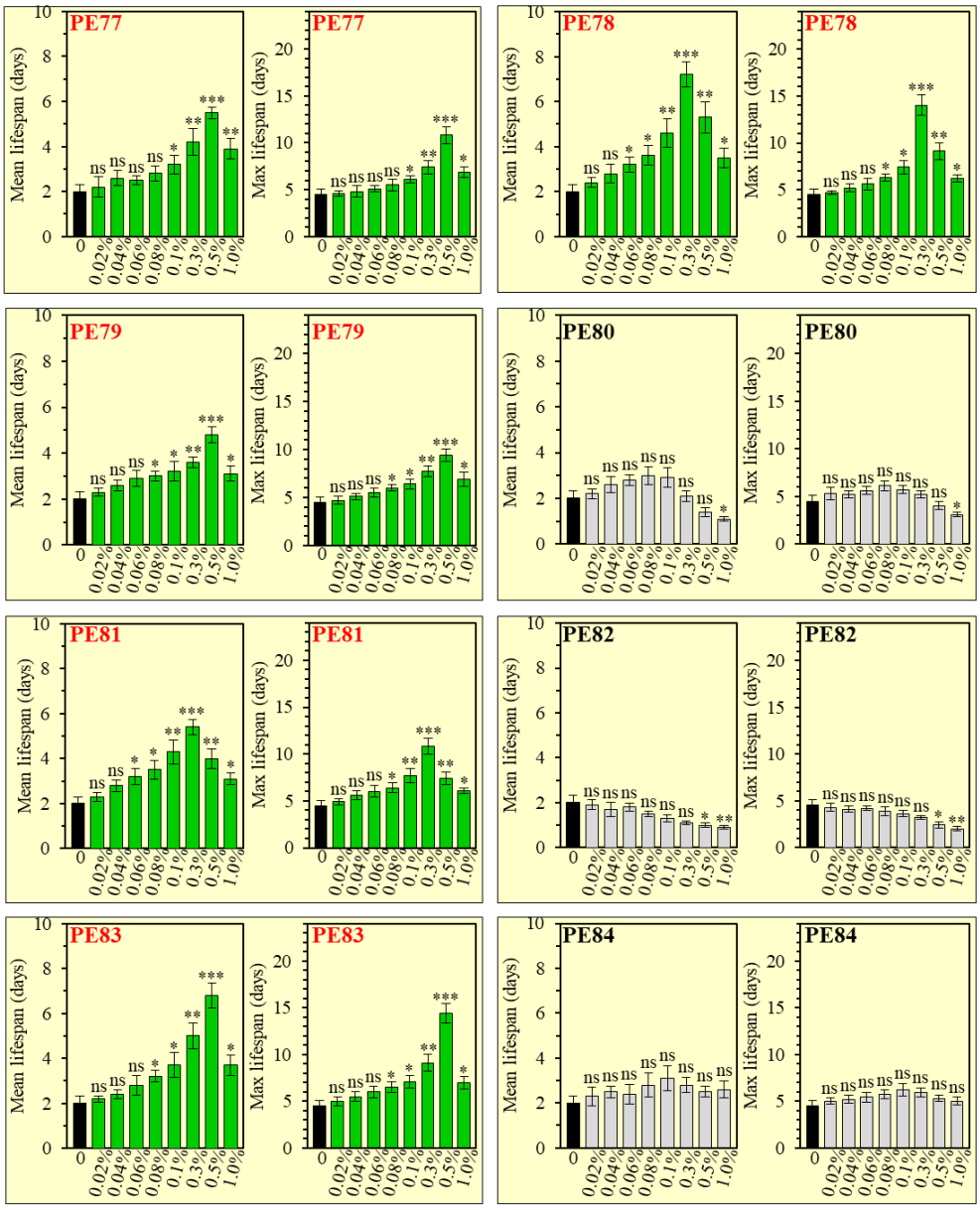

Figure 3.6. PE77, PE78, PE79, PE81 and PE83, but not PE80, PE82 or PE84, increase the mean and maximum CLS of WT yeast cultured under non-CR conditions on 2% (w/v) glucose. WT cells were cultured as described in the legend to Figure 2.1. The mean and maximum lifespans of chronologically aging WT strain cultured under non-CR conditions without a PE or with a PE added at various concentrations are shown; data are presented as means ± SEM (n = 6; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, *ns*, not significant; the p values for comparing the means of two groups were calculated as described in the legend to Figure 2.1). Note that PE80 and PE82 can decrease the CLS of WT yeast under non-CR conditions if added at a final concentration of 0.5% (w/v) or 1.0% (w/v).

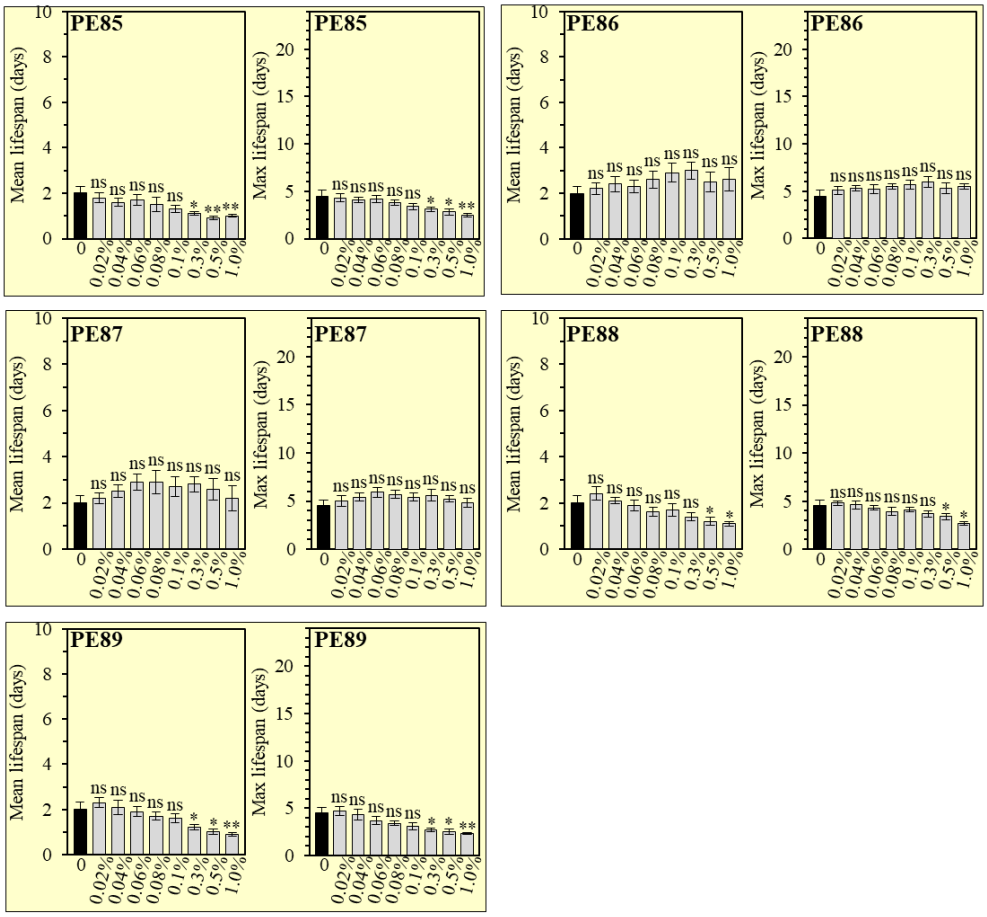

Figure 3.7. Neither PE85, PE86, PE87, PE88 nor PE89 can increase the mean or maximum CLS of WT yeast cultured under non-CR conditions on 2% (w/v) glucose. WT cells were cultured as described in the legend to Figure 2.1. The mean and maximum lifespans of chronologically aging WT strain cultured under non-CR conditions without a PE or with a PE added at various concentrations are shown; data are presented as means ± SEM (n = 6; * $p < 0.05$, ** $p < 0.01$, *ns*, not significant; the *p* values for comparing the means of two groups were calculated as described in the legend to Figure 2.1). Note that PE85, PE88 and PE89 can decrease the CLS of WT yeast under non-CR conditions if added at a final concentration ranging from 0.3% (w/v) to 1.0% (w/v).

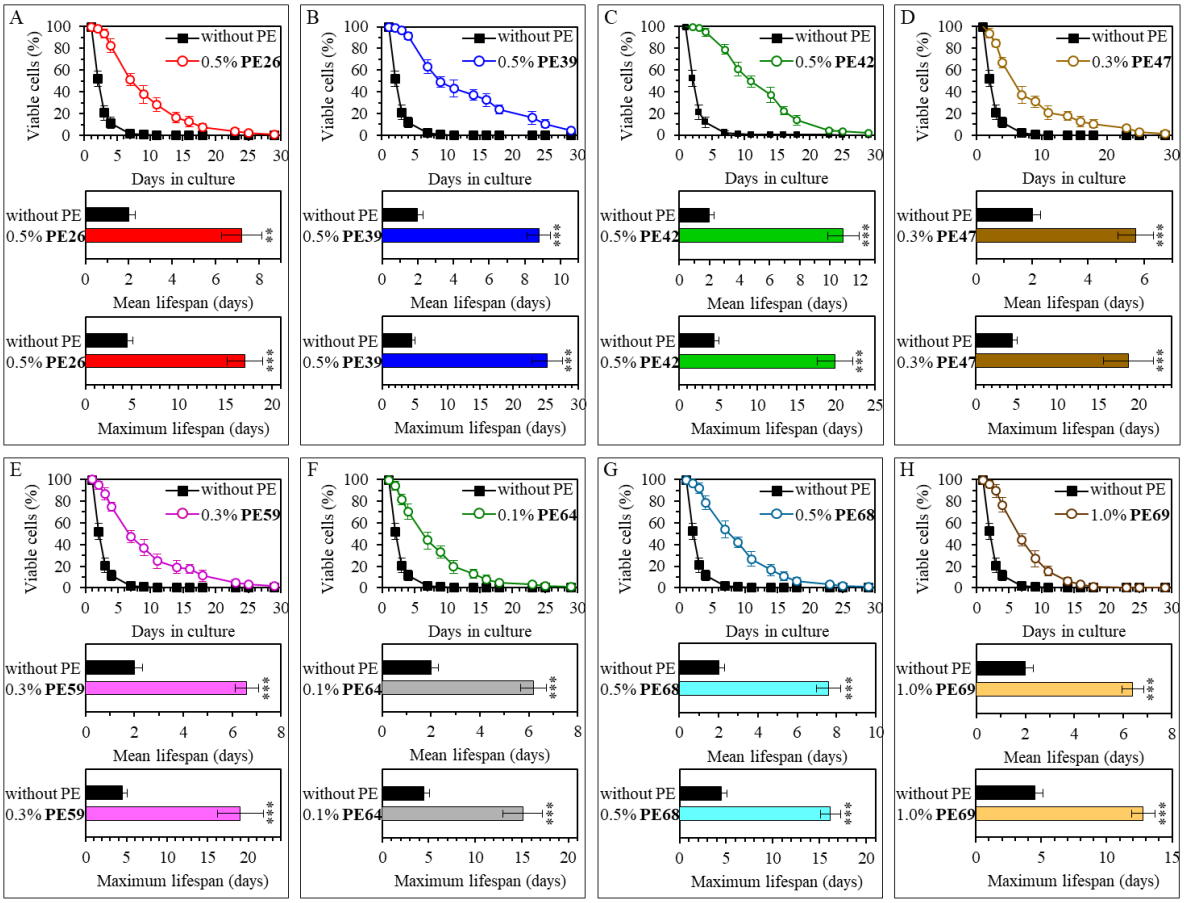

Figure 3.8. 0.5% (w/v) PE26, 0.5% (w/v) PE39, 0.5% (w/v) PE42, 0.3% (w/v) PE47, 0.3% (w/v) PE59, 0.1% (w/v) PE64, 0.5% (w/v) PE68 and 1.0% (w/v) PE69 exhibit the highest extending effects on the chronological lifespan (CLS) of wild-type (WT) yeast cultured under non-CR conditions on 2% (w/v) glucose. WT cells were cultured in the synthetic minimal YNB medium initially containing 2% (w/v) glucose, in the presence of a PE or its absence. In the cultures supplemented with a PE, ethanol was used as a vehicle at a final concentration of 2.5% (v/v). In the same experiment, WT cells were also subjected to ethanol-mock treatment by being cultured in the synthetic minimal YNB medium initially containing 2% (w/v) glucose and 2.5% (v/v) ethanol. Survival curves (the upper panels in A-H) and the mean and maximum lifespans (the lower two panels in A-H) of chronologically aging WT cells cultured without a PE (cells were subjected to ethanol-mock treatment) or with a PE (which was added at the concentration optimal for CLS extension) are shown. Data are presented as means ± SEM (n = 6). In the upper panels in A-H, CLS extension was significant for each of the PEs tested ($p < 0.05$; the p values for comparing each pair of survival curves were calculated using the logrank test as described in Materials and Methods). In the lower two panels in A-H, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$; the p values for comparing the means of two in groups were calculated using an unpaired two-tailed t test as described in Materials and Methods). Data for mock-treated WT cells are replicated in graphs A-H of this Figure. Data for WT cells cultured with a PE added at the concentration optimal for CLS extension are replicated in Figure 2.1 (for 0.5% (w/v) PE26, 0.5% (w/v) PE39 and 0.5% (w/v) PE42), Figure 2.2 (for 0.3% (w/v) PE47), Figure 2.3 (for 0.3% (w/v) PE59), Figure 2.4 (for 0.1% (w/v) PE64 and 0.5% (w/v) PE68) and Figure 2.5 (for 1.0% (w/v) PE69).

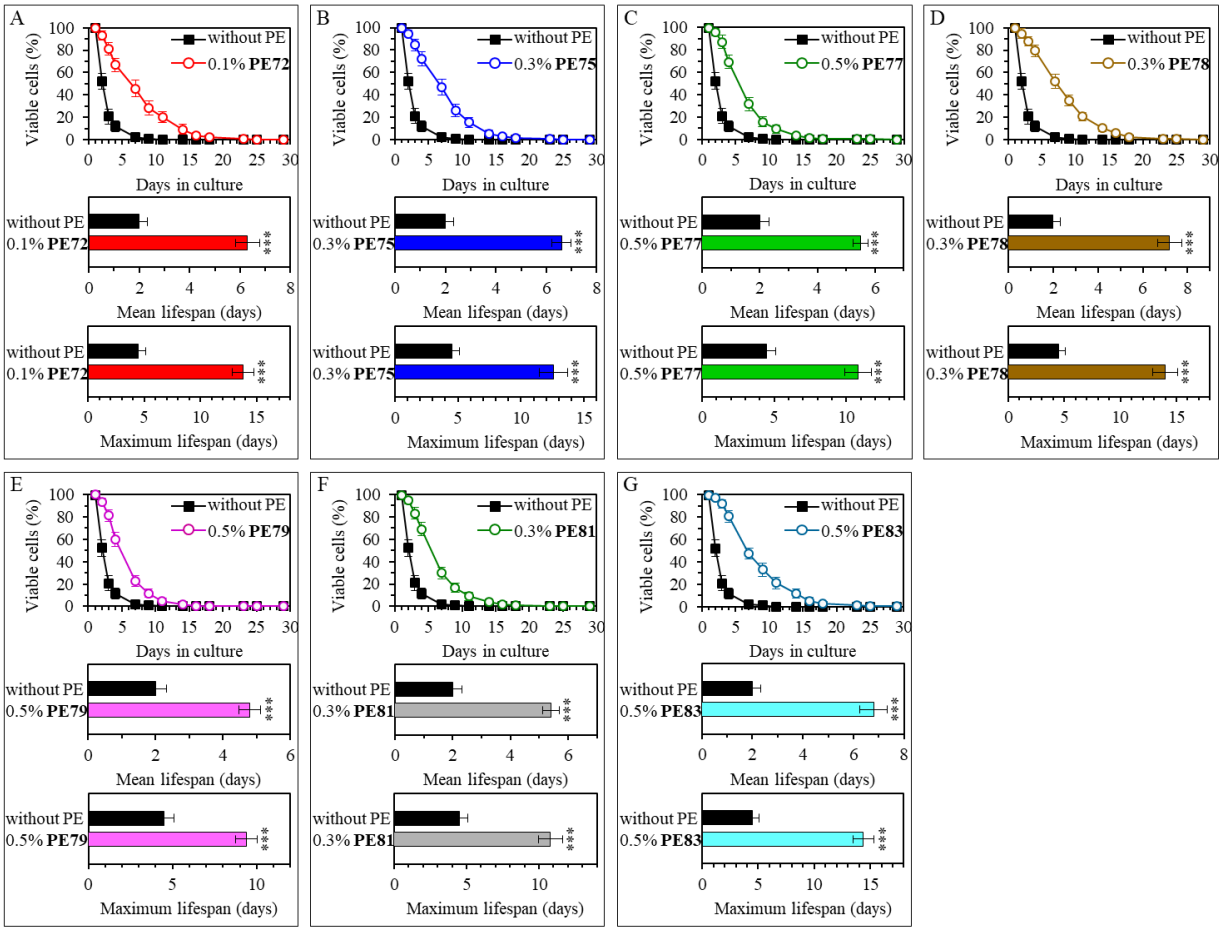

Figure 3.9. 0.1% (w/v) PE72, 0.3% (w/v) PE75, 0.5% (w/v) PE77, 0.3% (w/v) PE78, 0.5% (w/v) PE79, 0.3% (w/v) PE81 and 0.5% (w/v) PE83 exhibit the highest extending effects on the CLS of WT yeast cultured under non-CR conditions on 2% (w/v) glucose. WT cells were cultured in the synthetic minimal YNB medium initially containing 2% (w/v) glucose, in the presence of a PE or its absence. In the cultures supplemented with a PE, ethanol was used as a vehicle at a final concentration of 2.5% (v/v). In the same experiment, WT cells were also subjected to ethanol-mock treatment by being cultured in the synthetic minimal YNB medium initially containing 2% (w/v) glucose and 2.5% (v/v) ethanol. Survival curves (the upper panels in A-G) and the mean and maximum lifespans (the lower two panels in A-G) of chronologically aging WT cells cultured without a PE (cells were subjected to ethanol-mock treatment) or with a PE (which was added at the concentration optimal for CLS extension) are shown. Data are presented as means ± SEM (n = 6). In the upper panels in A-G, CLS extension was significant for each of the PEs tested ($p < 0.05$; the p values for comparing each pair of survival curves were calculated using the logrank test as described in Materials and Methods). In the lower two panels in A-G, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$; the p values for comparing the means of two in groups were calculated using an unpaired two-tailed t test as described in Materials and Methods). Data for mock-treated WT cells are replicated in graphs A-G of this Figure. Data for WT cells cultured with a PE added at the concentration optimal for CLS extension are replicated in Figure 1.4 (for 0.1% (w/v) PE72 and 0.3% (w/v) PE75) and Figure 2.6 (for 0.5% (w/v) PE77, 0.3% (w/v) PE78, 0.5% (w/v) PE79, 0.3% (w/v) PE81 and 0.5% (w/v) PE83).

3.2 Each of the fifteen longevity-prolonging PEs mimics longevity extension by CR

CR without malnutrition is a low-calorie dietary regimen that extends lifespan in many evolutionarily distant organisms and improves healthspan in laboratory rodents and rhesus monkeys [17, 26, 64-66]. Certain natural chemicals and synthetic drugs have been shown to elicit the CR-like lifespan-extending and healthspan-improving effects even under non-CR conditions (i.e., when calorie supply is not limited) [67-72]. These natural and synthetic chemical compounds are called CR mimetics (CRMs) if they not only extend longevity under non-CR conditions but also if they exhibit three other effects. First, CRMs do not impair food intake. Second, CRMs have CR-like effects on metabolism and physiology. Third, akin to CR, CRMs decrease the susceptibility to diverse stresses [73, 74]. In the present study, we found that each of the fifteen longevity-extending PEs increases yeast CLS under non-CR conditions on 2% (w/v) glucose (Figures 3.1-3.6, Figures 3.8 and 3.9). Besides, our recent unpublished data revealed that none of the fifteen longevity-extending PEs compromises glucose intake during culturing under these conditions. Thus, it seems that all these PEs are CRMs. This conclusion is further supported by our observations that each of the fifteen longevity-extending PEs exhibits a CR-like effect on stress resistance (see below).

Of note, we previously reported that if the CR diet is administered by culturing yeast in the YNB medium initially containing 0.5% (w/v) glucose, it significantly increases both the mean and maximum CLS of *S. cerevisiae* [63]. In the present study, we investigated how each of the fifteen PEs that extends longevity under non-CR conditions influences the longevity of yeast cultured under CR conditions on 0.5% (w/v) glucose. We found that eight of the fifteen PEs that prolong the longevity of chronologically aging yeast under non-CR conditions do not increase either the mean or the maximum CLS of *S. cerevisiae* under CR conditions (Figures 3.10 and 3.11). These PEs included 0.3% (w/v) PE47 (Figure 3.10D), 0.1% (w/v) PE64 (Figure 3.10F), 1.0% (w/v) PE69 (Figure 3.10H), 0.1% (w/v) PE72 (Figure 3.11A), 0.3% (w/v) PE75 (Figure 3.11B), 0.5% (w/v) PE77 (Figure 3.11C), 0.5% (w/v) PE79 (Figure 3.11E) and 0.3% (w/v) PE81 (Figure 3.11F). It seems conceivable, therefore, that each of these eight PEs increases yeast CLS because it modulates the same or highly overlapping sets of longevity-defining cellular processes under both CR and non-CR conditions.

We also revealed that seven of the fifteen PEs that extend yeast longevity under non-CR conditions also increase both the mean and maximum CLS of *S. cerevisiae* under CR conditions

(Figures 3.10 and 3.11). 0.5% (w/v) PE26 (Figure 3.10A), 0.5% (w/v) PE39 (Figure 3.10B), 0.5% (w/v) PE42 (Figure 3.10C), 0.3% (w/v) PE59 (Figure 3.10E), 0.5% (w/v) PE68 (Figure 3.10G), 0.3% (w/v) PE78 (Figure 3.11D) and 0.5% (w/v) PE83 (Figure 3.11G) were among these PEs. Therefore, we hypothesize that each of these seven PEs increases yeast CLS under CR conditions because it regulates the sets of longevity-defining cellular processes that differ from (or only partially overlap with) the ones it modulates under non-CR conditions.

We then compared the efficiency with which each of the fifteen PEs increases yeast CLS under non-CR conditions to that under CR conditions. Our comparison revealed that each of these PEs extends the longevity of chronologically aging yeast under non-CR conditions significantly more efficiently than it does under CR conditions (Figure 3.12). This finding shows that each of the fifteen PEs is a more effective longevity-prolonging intervention in chronologically aging yeast not-limited in calorie supply than it is in yeast placed on a CR diet.

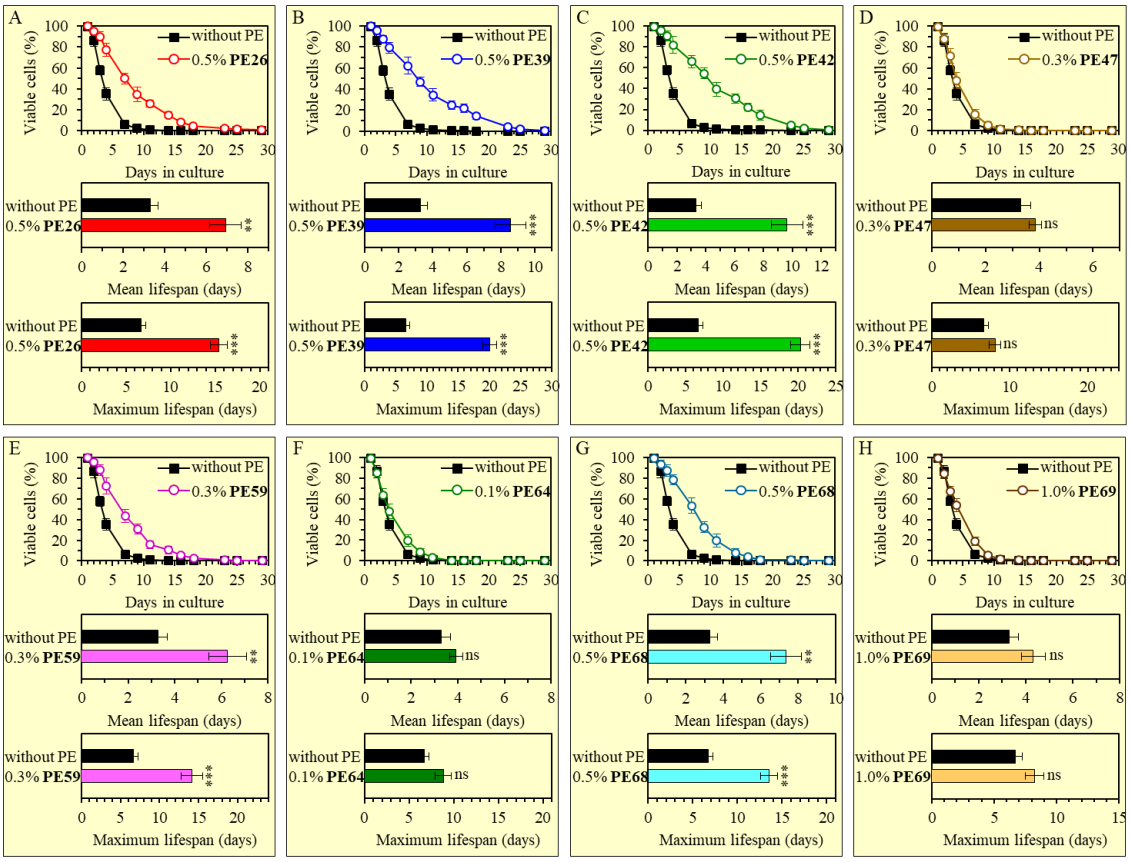

Figure 3.10. 0.5% (w/v) PE26, 0.5% (w/v) PE39, 0.5% (w/v) PE42, 0.3% (w/v) PE59 and 0.5% (w/v) PE68 (but not 0.3% (w/v) PE47, 0.1% (w/v) PE64 or 1.0% (w/v) PE69) extend the CLS of WT yeast cultured under CR conditions on 0.5% (w/v) glucose. WT cells were

cultured in the synthetic minimal YNB medium initially containing 0.5% (w/v) glucose, in the presence of a PE or its absence. In the cultures supplemented with a PE, ethanol was used as a vehicle at a final concentration of 2.5% (v/v). In the same experiment, WT cells were also subjected to ethanol-mock treatment by being cultured in the synthetic minimal YNB medium initially containing 0.5% (w/v) glucose and 2.5% (v/v) ethanol. Survival curves (the upper panels in A-H) and the mean and maximum lifespans (the lower two panels in A-H) of chronologically aging WT cells cultured without a PE (cells were subjected to ethanol-mock treatment) or with a PE (which was added at the concentration optimal for CLS extension under non-CR conditions) are shown. Data are presented as means ± SEM (n = 6). In the upper panels in A-C, E and F, CLS extension was significant for each of the PEs tested ($p < 0.05$; the p values for comparing each pair of survival curves were calculated using the logrank test as described in Materials and Methods). In the lower two panels in A-C, E and F, $^{**}p < 0.01$, $^{***}p < 0.001$; the p values for comparing the means of two in groups were calculated using an unpaired two-tailed t test as described in Materials and Methods). In the upper panels in D, F and H, CLS extension was statistically not significant for each of the PEs tested (the p values for comparing each pair of survival curves were calculated using the logrank test as described in Materials and Methods). In the lower two panels in D, F and H, *ns*, not significant; the p values for comparing the means of two in groups were calculated using an unpaired two-tailed t test as described in Materials and Methods). Data for mock-treated WT cells are replicated in graphs A-H of this Figure and graphs A-G of Figure 2.11.

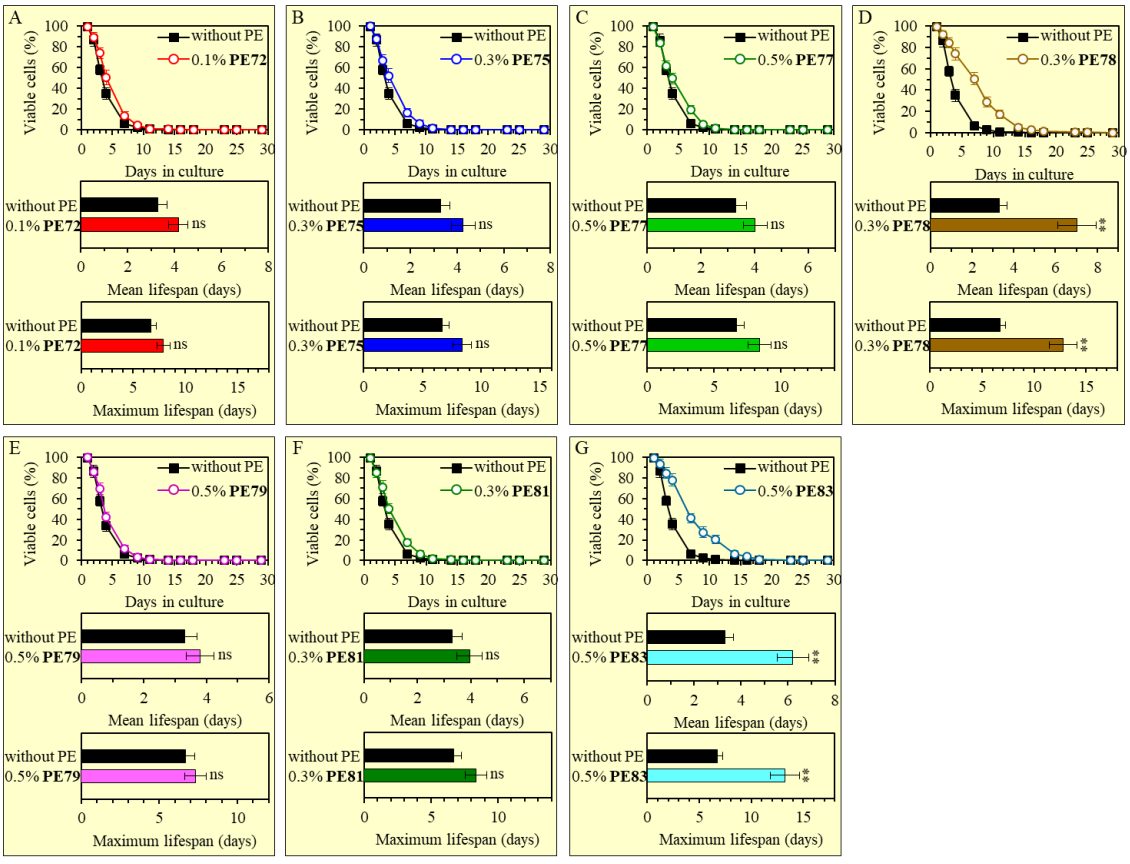

Figure 3.11. 0.3% (w/v) PE78 and 0.5% (w/v) PE83 (but not 0.1% (w/v) PE72, 0.3% (w/v) PE75, 0.5% (w/v) PE77, 0.5% (w/v) PE79 or 0.3% (w/v) PE81) extend the CLS of WT yeast

cultured under CR conditions on 0.5% (w/v) glucose. WT cells were cultured in the synthetic minimal YNB medium initially containing 0.5% (w/v) glucose, in the presence of a PE or its absence. In the cultures supplemented with a PE, ethanol was used as a vehicle at a final concentration of 2.5% (v/v). In the same experiment, WT cells were also subjected to ethanol-mock treatment by being cultured in the synthetic minimal YNB medium initially containing 0.5% (w/v) glucose and 2.5% (v/v) ethanol. Survival curves (the upper panels in A-G) and the mean and maximum lifespans (the lower two panels in A-G) of chronologically aging WT cells cultured without a PE (cells were subjected to ethanol-mock treatment) or with a PE (which was added at the concentration optimal for CLS extension under non-CR conditions) are shown. Data are presented as means ± SEM (n = 6). In the upper panels in D and G, CLS extension was significant for each of the PEs tested ($p < 0.05$; the p values for comparing each pair of survival curves were calculated using the logrank test as described in Materials and Methods). In the lower two panels in D and G, $^{**}p < 0.01$; the p values for comparing the means of two in groups were calculated using an unpaired two-tailed t test as described in Materials and Methods). In the upper panels in A-C, E and F, CLS extension was statistically not significant for each of the PEs tested (the p values for comparing each pair of survival curves were calculated using the logrank test as described in Materials and Methods). In the lower two panels in A-C, E and F, *ns*, not significant; the p values for comparing the means of two in groups were calculated using an unpaired two-tailed t test as described in Materials and Methods). Data for mock-treated WT cells are replicated in graphs A-G of this Figure and graphs A-H of Figure 2.10.

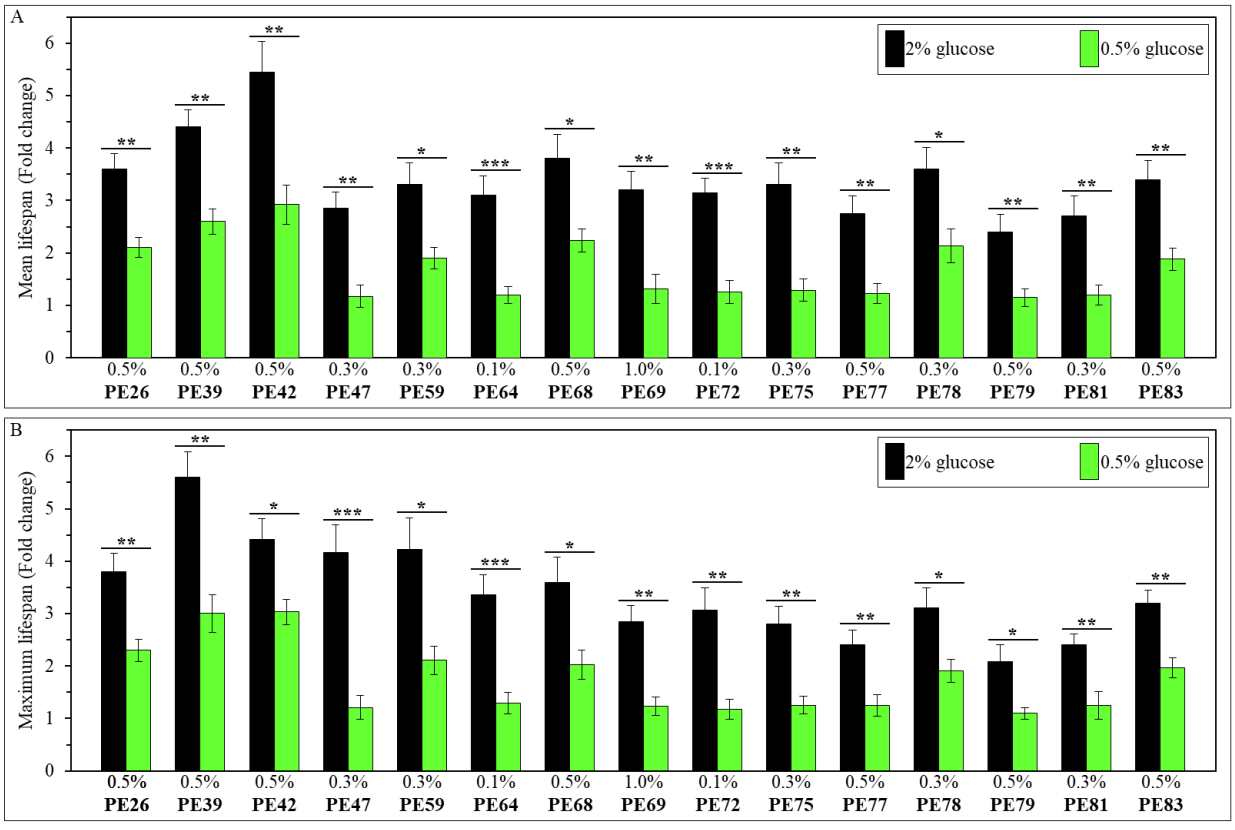

Figure 3.12. Each of the fifteen PEs extends the longevity of chronologically aging yeast under non-CR conditions on 2% (w/v) glucose significantly more efficiently than it does

under CR conditions on 0.5% (w/v) glucose. WT cells were cultured in the synthetic minimal YNB medium initially containing 2% (w/v) or 0.5% (w/v) glucose, in the presence of a PE or its absence. In the cultures supplemented with a PE, ethanol was used as a vehicle at a final concentration of 2.5% (v/v). In the same experiment, WT cells were also subjected to ethanol-mock treatment by being cultured in the synthetic minimal YNB medium initially containing 0.5% (w/v) or 2% (w/v) glucose and 2.5% (v/v) ethanol. The extent to which each of the PE tested increases the mean (A) and maximum (B) CLS under non-CR and CR conditions were calculated based on the data presented in Figures 2.8-2.11. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$; the p values for comparing the means of two in groups were calculated using an unpaired two-tailed t test as described in Materials and Methods.

3.3 Each of the fifteen longevity-prolonging PEs is a geroprotector that decreases both the extrinsic and the intrinsic rates of aging in budding yeast

Our data allow us to conclude that each of the fifteen longevity-prolonging PEs slows yeast chronological aging because it decreases both the extrinsic and the intrinsic rates of aging. This conclusion is based on our findings that each of these PEs extends both the mean and maximum CLS of yeast (Figures 3.10-3.12). The mean lifespans of evolutionarily distant organisms are thought to depend on specific environmental (extrinsic) factors to which cells are exposed before they enter the quiescent or senescent state [75, 76-78]. In contrast, the maximum lifespans of organisms across species are considered to rely on specific cellular and organismal longevity modifiers that operate after cells enter the quiescent or senescent state [75-77, 79, 80].

3.4 Each of the fifteen geroprotective PEs increases cell resistance to long-term oxidative and thermal stresses

Genetic, dietary and chemical interventions that decrease cell susceptibility to chronic (long-term) oxidative and/or thermal stresses have been shown to decelerate the aging process and extend longevity in yeast and other organisms across species [25, 26, 42]. Therefore, we investigated the effect of each of the fifteen geroprotective PEs on the susceptibility of chronologically aging yeast cells to these two types of chronic stresses.

To examine aging-associated changes in cell susceptibility to these long-term stresses, we recovered aliquots of yeast cells on days 1, 2, 3 and 4 of culturing under non-CR conditions in liquid YNB medium with 2% (w/v) glucose. To assess cell susceptibility to chronic oxidative stress, we spotted serial dilutions of these cell aliquots on solid YEP medium with 2% (w/v) glucose and 5 mM hydrogen peroxide and incubated them for 3 days. To assess cell susceptibility

to chronic thermal stress, we spotted serial dilutions of these cell aliquots on solid YEP medium with 2% (w/v) glucose, incubated at 60ºC for 60 min, transferred the plates to 30ºC and incubated at this temperature for 3 days.

We found that each of the fifteen geroprotective PEs makes yeast cells more resistant to chronic oxidative and thermal stresses, especially cells in ST-phase cultures recovered on days 3 and 4 (Figures 3.13B and 3.13C, respectively).

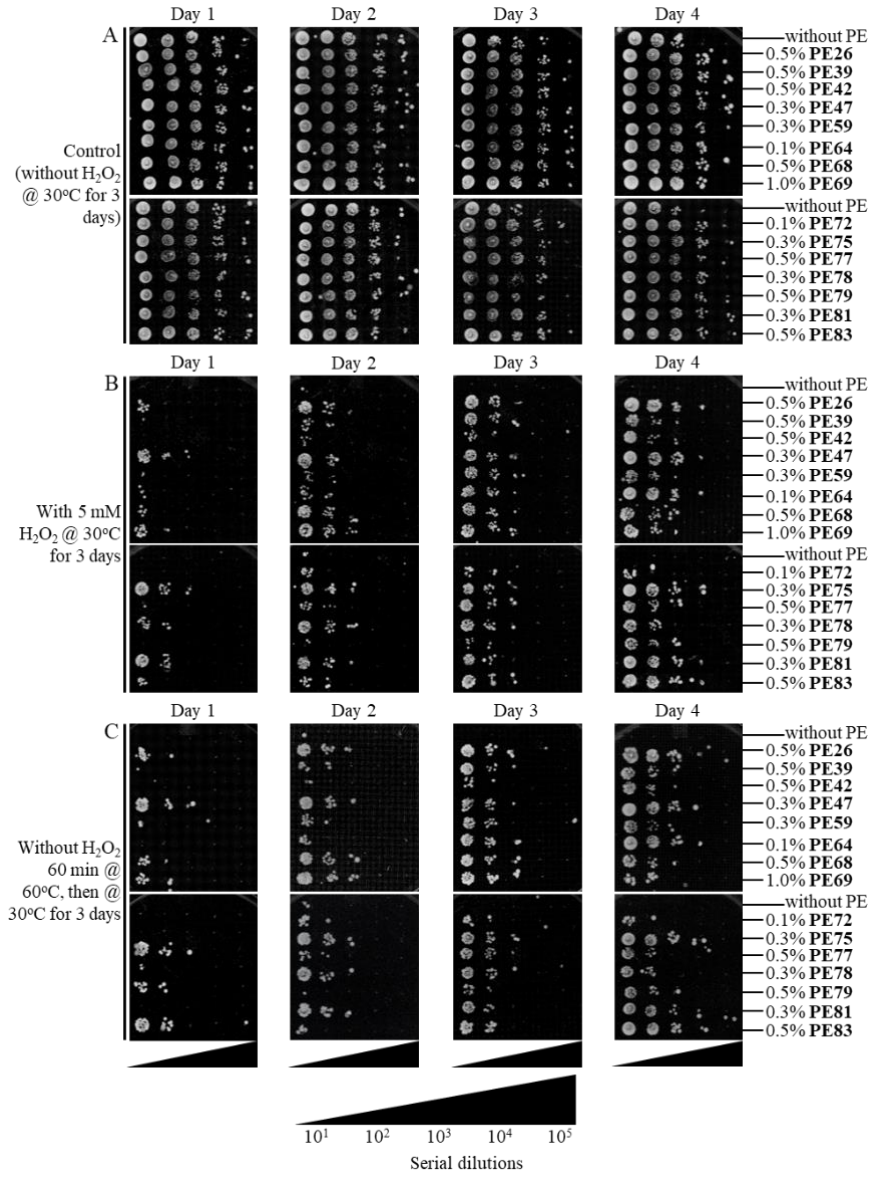

Figure 3.13. Each of the fifteen geroprotective PEs makes yeast more resistant to chronic (long-term) oxidative and thermal stresses. WT cells were cultured in the synthetic minimal YNB medium initially containing 2% (w/v) glucose, in the presence of a PE or its absence. In the

cultures supplemented with a PE, ethanol was used as a vehicle at a final concentration of 2.5% (v/v). In the same experiment, WT cells were also subjected to ethanol-mock treatment by being cultured in the synthetic minimal YNB medium initially containing 2% (w/v) glucose and 2.5% (v/v) ethanol. Spot assays for examining cell resistance to chronic oxidative (B) and thermal (C) stresses were performed as described in Materials and Methods. (A) In control samples, serial 10-fold dilutions of cells recovered on different days of culturing were spotted on plates with solid YEP medium containing 2% (w/v) glucose. All pictures were taken after a 3-d incubation at 30ºC. (B) In samples subjected to long-term oxidative stress, serial 10-fold dilutions of cells recovered on different days of culturing were spotted on plates with solid YEP medium containing 2% (w/v) glucose and 5 mM hydrogen peroxide. All pictures were taken after a 3-d incubation at 30ºC. (C) In samples subjected to long-term thermal stress, serial 10-fold dilutions of cells recovered on different days of culturing were spotted on plates with solid YEP medium containing 2% (w/v) glucose, incubated at 60ºC for 60 min and then transferred to 30ºC. All pictures were taken after a 3-d incubation at 30ºC.

4 Discussion

In search of new aging-delaying (geroprotective) PEs, we screened a library of PEs for extracts that can increase yeast CLS. This library includes 53 different PEs of known origin and properties. We discovered 15 PEs that extend the longevity of chronologically aging budding yeast. The high percentage (i.e., ~ 28%) of geroprotective PEs in this library was not surprising because our previous screen of a different set of 35 PEs from the same library led to the identification of 6 PEs exhibiting geroprotective effects [63]; thus, the percentage of geroprotective PEs discovered in this previous screen was ~ 17%. How to explain such high percentages of geroprotective PEs within the same library that was used for both screens? Two important aspects of these PEs origin and their geroprotective efficiencies need to be considered. First, it needs to be emphasized that the library includes mainly PEs known for their positive effects on human health because they have been for centuries used in traditional Chinese and other herbal medicines. Furthermore, some PEs from the library have been used in the Mediterranean and other long-established diets. Second, it is important to note that each of the 15 geroprotective PEs identified here extends yeast CLS much more efficiently than any of the 42 individually added geroprotective compounds (either synthetic drugs or natural chemicals) known to prolong lifespan in budding and fission yeast as well as in other organisms [63]. These other organisms include filamentous fungi, nematodes, fruit flies, daphnias, mosquitoes, honeybees, fishes, mammals and cultured human cells [63]. Indeed, we found that under non-CR conditions, the 15 geroprotective PEs increase the mean and maximum CLS of *S. cerevisiae* by 140%-445% and 109%–460%, respectively (Figure 3.12). Furthermore, the 6 geroprotective PEs that were discovered in the previous screen of the same library increase the mean and maximum CLS of yeast by 145%–475% and 80%–369%, respectively [63]. In contrast, any of the 42 presently known geroprotective compounds is known to prolong cellular and/or organismal lifespan in evolutionarily distant eukaryotes much less efficiently, within the 5% to 75% range, if it is applied individually [63].

Considering the above two aspects of the origin and longevity-extending efficiencies of the 15 geroprotective PEs that we discovered here (and of the 6 geroprotective PEs that were found earlier in a screen of the same library), we propose the following. It is possible that the high percentages of geroprotective PEs within the library used for both screens and the high longevity-extending efficiencies of these geroprotectors was due to the presence of several geroprotective chemical compounds within each of the discovered PEs. We also propose that each of these

geroprotective chemical compounds may extend yeast CLS by targeting different signaling pathways and cellular processes contributing to longevity assurance in *S. cerevisiae*. Hence, both the high percentages of geroprotective PEs within the same library and the high longevity-extending efficiencies of these PEs may be caused by the additive or synergistic longevity-extending effects of the individual geroprotective chemical compounds that are present within each of the discovered PEs.

A similar idea of the additive or synergistic longevity-extending effects for the mixtures of individual geroprotective chemicals was proposed by others [92-96]. The following three multi-component combinations of several chemical compounds were proposed in these studies as therapeutic geroprotective interventions. First, a three-component mixture was proposed. It contains epigallocatechin gallate (which is a known activator of cAMP synthesis), N-acetyl-L-cysteine (which suppresses cell proliferation) and myricetin (which is known for its ability to stimulate integrin signaling pathway, DNA repair, cAMP synthesis and hypoxia-responsive signaling pathways) [92]. Second, a seven-component mixture of rapalogs (i.e., rapamycin and its synthetic chemical derivates that directly inhibits the aging-accelerating TORC1 pathway), metformin (which stimulates AMP-activated protein kinase (AMPK), a master regulator of glucose and lipid metabolism), losartan or lisinopril (both of which are known inhibitors of angiotensin II signaling), a statin (atorvastatin, simvastatin or lovastatin, all of which are known for their abilities to lower blood cholesterol concentrations), propranolol (which is known as a non-cardioselective beta-adrenergic antagonist), aspirin (which inhibits cyclooxygenase) and a phosphodiesterase 5 inhibitor, in combination with physical exercise and CR diet or intermittent fasting [94, 96]. Third, another three-component mixture was proposed. It includes rapamycin, acarbose (which inhibits α-glucosidase) and a cardiolipin-binding peptide [95].

Considering the high longevity-extending efficiencies of all 21 discovered geroprotective PEs in budding yeast and the fact that the Health Canada government agency defines 19 of them [83] as the ones that are health-improving natural product safe for human consumption (see below for more details), it is not unreasonable to think that in the future some of them can be used to extend the lifespan and healthspan of humans. For each of them, Health Canada provides a detailed description of the source material, routes of administration, doses and dosage forms, uses or purposes, durations of use, risk information, cautions and warnings, contraindications, known adverse reactions, non-medicinal ingredients, specifications, references cited and reviewed,

examples of appropriate dosage preparations, and frequencies of use [83]. The ongoing collaboration between the Titorenko laboratory and Idunn Technologies Inc. addresses this challenge by investigating which of the discovered geroprotective PEs can increase the replicative lifespan of cultured human fibroblasts or improve the health of the *ob/ob* mice model of type II diabetes.

Certain natural chemicals and synthetic drugs have been shown to elicit the CR-like lifespan-extending and healthspan-improving effects even under non-CR conditions (i.e., when calorie supply is not limited) [67-72]. These natural and synthetic chemical compounds are called CR mimetics (CRMs) if they not only extend longevity under non-CR conditions but also if they exhibit three other effects. First, CRMs do not impair food intake [67-72]. Second, CRMs have CR-like effects on metabolism and physiology [97-102]. Third, akin to CR, CRMs decrease the susceptibility to diverse stresses [67-72]. Data presented in this Thesis and a recent article from the Titorenko laboratory [97] provide evidence that each of the 15 geroprotective PEs satisfies all the criteria previously proposed for a CRM. Indeed, each of these PEs increases yeast CLS under non-CR conditions on 2% (w/v) glucose and none of them compromises glucose intake during culturing under these conditions (this thesis and reference [97]). Furthermore, each of the 15 geroprotective PEs exhibits CR-like effects on specific aspects of the metabolism and physiology of budding yeast [97]. These effects include an increased rate of coupled mitochondrial respiration and an altered chronology of changes in intracellular ROS concentrations [97]. ROS are mildly toxic cellular molecules known to play essential pro-hormetic roles in regulating the longevity of many evolutionarily distant organisms) [97]. These effects also include a decline in the oxidative damage to cellular proteins, membrane lipids and mitochondrial DNA [97]. Moreover, each of the 15 geroprotective PEs makes yeast cells more resistant to long-term oxidative and thermal stresses (this thesis), likely because of the above effects of these PEs on intracellular ROS and oxidative macromolecular damage.

Of note, PE26, PE39, PE42, PE59, PE68, PE78 and PE83 can prolong yeast CLS even under CR conditions, when all cellular processes that limit longevity under non-CR conditions are likely to be suppressed. Therefore, it is possible that each of these 7 PEs may stimulate the longevity-extending cellular processes and/or may suppress the longevity-shortening cellular processes that operate only under CR conditions. Moreover, each of these 7 PEs may target both

CR-regulated and housekeeping (i.e., not regulated by CR) cellular processes (including cell susceptibility to long-term oxidative and thermal stresses).

Our goals for the future research of the 15 geroprotective PEs described here are outlined below.

First, we collaborate with Idunn Technologies Inc. in identifying chemical compounds that are responsible for the geroprotective effects of these PEs. Each of the 15 geroprotective PEs has already been fractionated with the help of the high-performance liquid chromatography on a C18 reversed-phase column. We identified individual chromatography fractions capable of a statistically significant extension of yeast CLS. Chemical compounds present in these fractions will be identified by Idunn Technologies Inc. with the help of tandem mass spectrometry and nuclear magnetic resonance spectroscopy. It is possible that some of these chemical compounds are present in some (or even all) of the 15 geroprotective PEs, whereas others are unique to a particular PE.

Second, we are interested in investigating and understanding the molecular and cellular mechanisms through which each of these PEs slows yeast chronological aging. We have recently described mechanisms underlying the aging-delaying action of PE21 [91], an extract from the white willow *Salix alba* we discovered in our previous screen for geroprotective PEs [63].

Third, we would like to explore how each of the 15 geroprotective PEs may coordinate the information flow through a longevity-defining network of signaling pathways and protein kinases operating in budding yeast and other organisms. This network incorporates the pro-aging TORC1, PKA and PKH1/2 pathways as well as the pro-aging serine/threonine-protein kinase Sch9 [17, 26, 42, 81]. This network also integrates the anti-aging SNF1 and ATG pathways as well as the anti-aging serine/threonine-protein kinase Rim15 [17, 26, 42, 81]. Our recent study has revealed that each of the 6 geroprotective PEs we discovered in the previous screen [63] slows yeast chronological aging through different functional modules of this longevity-defining signaling network [81]. Of note, pairwise mixes of these 6 geroprotective PEs slow the process of yeast chronological aging in a synergistic or additive manner only if they include the PEs that target different modules of this network [82]. Therefore, we are interested in investigating how different combinations of the 15 geroprotective PEs described here influence the extent of yeast chronological aging delay. We will be looking for the combinations of geroprotective PEs that exhibit synergistic or additive effects on the extent of yeast chronological aging delay.

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