The Effects of Six Potent Age-Delaying Plant Extracts on the Cellular Lipidome of *Saccharomyces cerevisiae*

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Abstract

The Effects of Six Potent Age-Delaying Plant Extracts on the Cellular Lipidome of *Saccharomyces cerevisiae*

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Concordia University, 2023

The objective of this study is to better understand the involvement of anti-aging plant extracts on the lipidome. My hypothesis was that some of the previously studied twenty-one age-delaying plant extracts extend aging by altering lipid and free fatty acid synthesis and/or metabolism. To test this hypothesis, my thesis analyzed the lipidome of *Saccharomyces cerevisiae* after treatment with Plant Extract 4, 6, 12, 21, 26, and 39, all of which have been previously shown to delay aging. The lipids were extracted from the treated and untreated *S. cerevisiae* and analyzed by a Mass Spectrometer. My results revealed that the six plant extracts enhanced the level of certain lipids and significantly decreased the level of free fatty acids. Similar results were obtained in a previous study done on caloric restriction, where caloric restriction enhanced the level of lipids and decreased the level of free fatty acids. Notably, while some lipids increased or decreased, during both caloric restriction and plant extract exposure, there was a general trend for free fatty acids to decrease, supporting that they may have a more consistent role in aging compared to lipids.

Acknowledgments

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Table of Contents

List of Figures

List of Tables

List of Abbreviations

AMP-activated protein kinase (AMPK)

Cardiolipin (CL)

Ceramide (Cer)

Diacylglycerol (DAG)

Free Fatty Acid (FFA)

Fourier transform mass spectrometry (FTMS)

Insulin/insulin-like growth factor signaling (IIS)

Lithocholic acid (LCA)

Monoacylglycerol (MAG)

Reactive oxygen species (ROS)

Organization for Economic Cooperation and Development (OECD)

Phosphatidic acid (PA)

Phosphatidylcholine (PC)

Phosphatidylethanolamine (PE)

Phosphatidylglycerol (PG)

Phosphatidylinositol (PI)

Phosphatidylserine (PS)

Triacylglycerol (TAG)

Mammalian Target of Rapamycin (mTOR)

Sirtuin pathway (Sir)

Unfolded protein response (UPR)

Ubiquitin-proteasome system (UPS)

Chapter 1: Introduction

1.1 Aging

Aging is a gradual, unavoidable outcome for all organisms. Aging is caused by an accumulation of cellular damage which leads to the gradual loss of physiological function and subsequent death [1] [2]. Aging is a complex multilevel process that involves alterations in molecular, cellular, and systematic processes [2]. Data shows that human lifespan expectancy has more than doubled since the 19th century, when it was estimated that humans lived 30 years on average [3]. Now the average human lifespan in industrialized countries is approximately 70 years [3]. With this colossal increase in lifespan comes diseases that occur later in life that were not as highly prevalent during the pre-modern era [3]. A study performed in 2015 by the Organization for Economic Cooperation and Development (OECD) found that before the age of 65, the likelihood of a person developing dementia is 0.002% whereas, after the age of 65 this percentage increases to 0.010%, and to 50% in Europeans of 95 and older [4]. This study shows that there is a correlation between the appearance of diseases and the aging of an individual. Given the inevitability of health decline with age, researchers are currently focused on increasing the health span rather than lifespan per se [3]. This goal has been difficult to achieve due to the complexity of the mechanisms responsible for aging [5]. The past two decades have led to many findings in this field [5], although more work is needed, particularly at the tissue and systems level before this knowledge can be applied to humans. Many studies of the molecular mechanisms regulating aging have been done in model organisms including *Saccharomyces cerevisiae*, *Caenorhabditis elegans*, and *Drosophila melanogaster*.

1.2 Aging in *S. cerevisiae*

S. cerevisiae has been an important model for studying aging mechanisms at the molecular level [5–9]. This unicellular eukaryote is inexpensive to use, has a short generation time, has a fully sequenced genome, and is amenable to manipulations in laboratories [10]. There are two models of aging: chronological and replicative aging (Figs 1, 2) [6]. Chronological aging is defined as the amount of time a cell is viable once it enters the stationary phase of growth [6]. Chronological lifespan mimics the aging seen in non-dividing cells like neurons [6]. Chronological lifespan is measured by counting the number of days the non-dividing cells are viable (Fig. 2) [11]. Replicative lifespan refers to the number of daughter cells a mother cell produces before it dies [12], as seen in Figure 2. Replicative lifespan mimics the aging seen in mitotically active dividing cells like lymphocytes [6]. This model of lifespan is measured in the laboratory by using a micromanipulator that removes the buds from the mother cells and then the number of mitotic divisions is counted [13,14]. In both types of aging, cells acquire damage that affects cellular processes including DNA damage, decrease in autophagy, mitochondrial dysfunction, and/or extrachromosomal rDNA circles (Fig. 1).

As indicated above, replicative lifespan in *S. cerevisiae* is measured by dissecting daughter cells away from the mother and counting them. Since this approach can be tedious, replicative lifespan can also be measured by the mother enrichment program [11]. This method is achieved by using a genetically modified strain where UBC9 and CDC20 mutations are introduced via Cre-*lox* recombination, and Cre recombinase found in daughter cells binds to the SCW11 promoter only when it is activated by the estrogen binding domain which is estradiol-dependent [11]. Thus, replication can be controlled via estradiol, and used to study replicative lifespan [11].

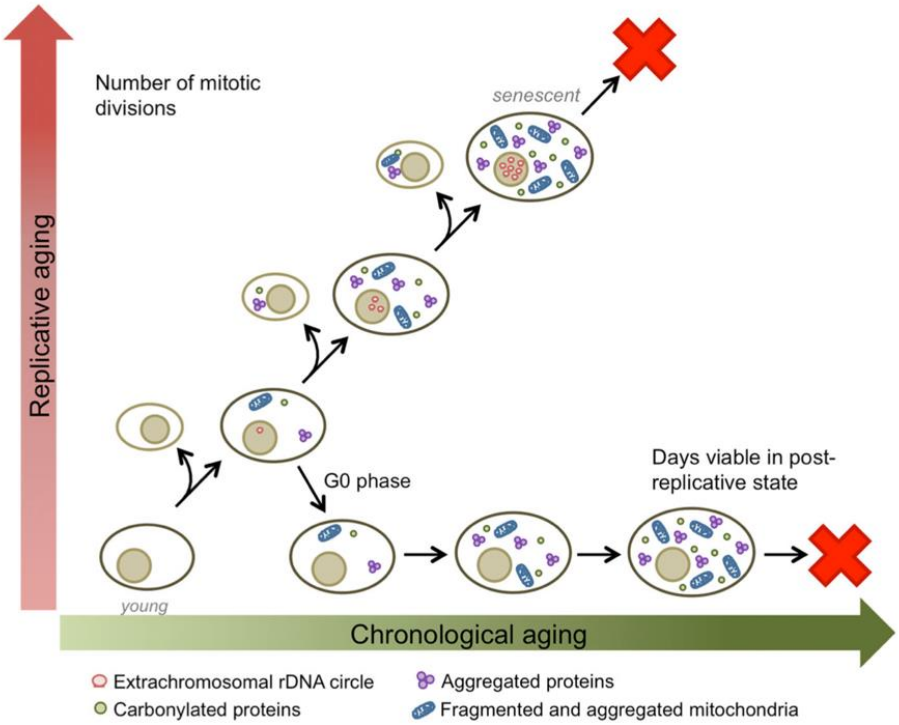

Figure 1. Two aging models in *S. cerevisiae* (modified from Oliveira et al., 2017) [15]**.** Chronological aging is shown on the X-axis as the amount of time a cell can live, vs. replicative aging on the Y-axis which refers to the number of times a cell can divide. Both types of aging are caused by the accumulation of damage and loss of physiological function of major cellular processes. The figure shows just a few examples of the many changes that typically occur.

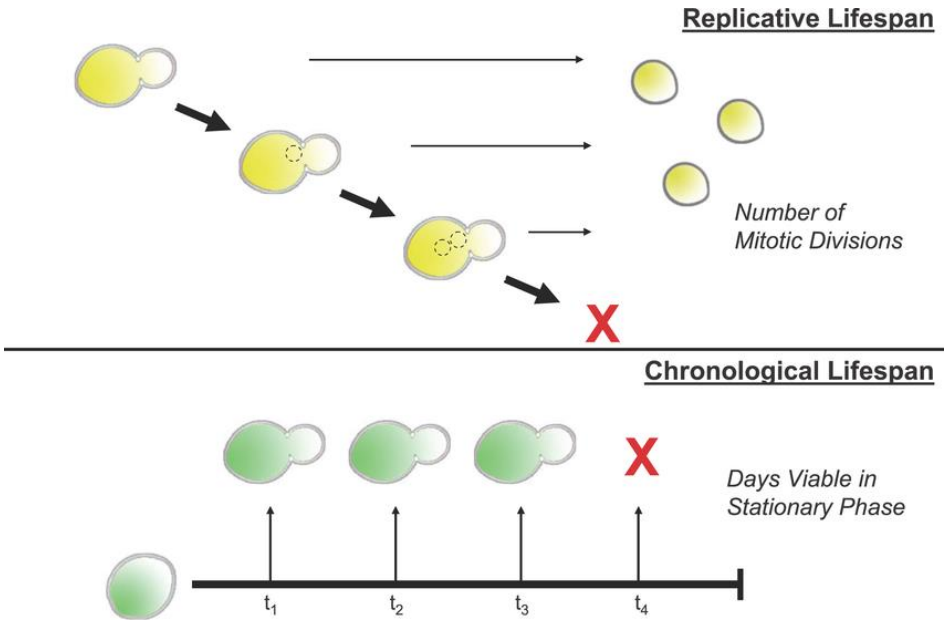

Figure 2. Measuring age in *S. cerevisiae* (modified from Kaeberlein et al., 2007) [16]**.** Chronological aging is measured by calculating the amount of time the *S. cerevisiae* cell is viable once it entered the stationary phase of growth. Replicative aging is measured by calculating the number of daughter cells one single mother cell produces before the mother cell dies.

Chronological lifespan can also be measured by different methods [11]. The first method is known as colony-forming unit assay [11,17]. This method is measured by plating dilutions of cells from media and counting the number of colonies that grow after incubation [11,17]. As cells age, the number of colony-forming decreases and are considered senescent when the number of colonies is below 1-10% [11]. Unfortunately, this method is hard to reproduce and relies on technical skills, which are not always precise [11]. Another method uses fluorescence to quantify chronological lifespan [11]. This method uses two fluorescent probes, FUN1 and propidium iodide (PI), with a flow cytometer and/or a fluorescence microscope to detect the cells [11,18,19]. These two florescent probes function differently, FUN1 passively enters cells and makes a cylindrical structure in the vacuole whereas, PI binds to exposed nucleic acids, which occurs when the membrane has permeabilized [18,19]. The main difference between these two probes is that FUN1 fluoresces red when the cell is metabolically active, whereas PI fluoresces red when the cell is dead or damaged [18,19]. Another method involves using an automated reader that calculates the size of the cells as they age [11,20,21]. This method involves the dilution of cells in a rich medium at each of the selected time points [11]. A sample of each time point is then taken and the optical density at 600nm is taken to create a growth curve [11]. Once this curve has been created it can be used to calculate the relative survival curve [11]. The relative survival curve is created by using the delay between the growth curve of the old and young *S. cerevisiae* and calculating the amount of time it takes for the *S. cerevisiae* colony to double in size [11].

1.3 Pathways that regulate aging in *S. cerevisiae*

Four important age-regulating pathways function in *S. cerevisiae*, some of which are conserved in other eukaryotes: proteostasis, the mTOR pathway (mTOR), the Sirtuin pathway

(Sir), and AMP-activated protein kinase (AMPK) [5,22], as seen in Figure 3. Proteostasis describes the turnover of proteins, including their synthesis and degradation, and the persistence of misfolded or damaged proteins can cause disease [23]. Thus, mechanisms of proteostasis that are associated with aging include protein folding, protein degradation, and unfolded protein response [23]. Proper protein folding is crucial for cells, and while chaperones or chaperonins can prevent misfolding, the accumulation of improperly folded proteins with low free energy could persist and form higher order structures [23]. For example, in humans, Parkinson's, Alzheimer's, and Huntington's disease arise due to improperly folded proteins with low free energy that give rise to irreversible higher ordered structures, such as fibrils [24–26]. Proteostasis is driven by insulin/insulin-like growth factor signaling (IIS) which uses insulin or insulin-like peptides to activate downstream effectors [27]. IIS is responsible for the synthesis, degradation, and modification of proteins although how insulin does this is still unclear [28]. Protein degradation controls the levels of endogenous proteins, and removes misfolded or damaged proteins via the ubiquitin-proteasome system (UPS) or autophagy [23]. While the UPS will degrade proteins that have been covalently tagged with ubiquitin, autophagy removes larger complexes and organelles [23]. Autophagy is always functional and occurs via a range of mechanisms, and increases or decreases via signaling caused by different pathways in response to stress and starvation, among others [29,30]. The third mechanism is unfolded protein response (UPR) [31,32]. UPR can be triggered in response to damaged proteins in specific conditions, and when active, proteins stop accumulating to permit misfolded proteins to be refolded with the help of chaperones [23]. When any of these mechanisms fails to function properly, this leads to unbalanced proteostasis which can promote aging and related diseases.

One of the most well-known pathways involved in aging involves signaling through mammalian target of rapamycin (mTOR) [22,33]. Most organisms have one or more isoforms of TOR, which is conserved through mammalian phyla, and is responsible for cell growth, metabolism, stress resistance, and inhibition of autophagy among other functions [34,35]. Several studies have shown that the mTOR pathway is responsible for cell proliferation and tumor growth in mammalian cells [33,36], and that TOR inhibition leads to an extension of lifespan was in *S. cerevisiae*, *C. elegans*, *M. musculus*, and *D. melanogaster* [37–41]. Studies performed using *S. cerevisiae*, *C. elegans*, *M. musculus*, and *D. melanogaster* also found that signaling through the TOR pathway was reduced under caloric restriction [39,42]. The TOR pathway was also found to regulate the replicative and chronological lifespan in *S. cerevisiae* [11]. *S. cerevisiae* contains two paralog genes: TOR1 and TOR2 [11]. When TOR1 was deleted in *S. cerevisiae*, both replicative and chronological lifespans were extended [37,43–45]. The extension of the replicative lifespan caused by the loss of TOR1 function was found to be Sir2-dependent [46]. This could be due to Sir2-mediated stabilization of extrachromosomal rDNA which was shown to decrease when TOR was inhibited [46,47]. However, Sir2 has epigenetic functions that could also contribute to the extended lifespan. Chronological lifespan also increased upon loss of TOR1 function, which could be due to changes in mitochondrial function [44]. There are likely differences in how carbon sources are used and metabolized, as well as the accumulation of specific byproducts [48].

Recently researchers have been interested in the Sirtuin pathway as it has been discovered to have a potential role in anti-aging in many different organisms as a type of conserved modulator [49]. *S. cerevisiae* have four sirtuins (Sir1-Sir4), which are NAD^+-dependent deacetylases [49], and each could play a role regulating lifespan [49–51]. The inactivation or depletion of Sir2 or its homolog in *S. cerevisiae*, *C. elegans,* and *D. melanogaster* caused a

decrease in lifespan, while its overexpression had the opposite effect [49,50,52]. Due to the complexity of metazoans, it is more challenging to distinguish between chronological aging and replicative aging as in *S. cerevisiae* [53]. Instead, their lifespan is measured by recording the time from birth until death [53]. While Sir2 could regulate extrachromosomal rDNA circle formation among other functions in *S. cerevisiae*, it functions differently in other organisms [6]. Therefore, different Sir2 orthologs may have evolved separately to regulate longevity through different pathways [6].

AMPK is another enzyme that regulates aging, and is involved in TOR signaling [54], as seen in Figure 4. AMPK functions as a metabolic sensor, based on the relative levels of AMP and ATP, and controls the production of fats and glucose [55]. Essentially, AMP kinase senses the level of available nutrients, and depending on this level, it activates catabolic pathways or represses anabolic pathways [34]. For example, researchers found that high levels of AMP/ATP lead to inactivation of the mTOR pathway [55]. Studies performed on *C. elegan* and *M. musculus* found that lifespan was extended in both these organisms when the AMPK pathway was overexpressed [39,56].

Figure 3 shows that each of the four pathways are important for different processes that have been found to affect anti-aging like autophagy, stress resistance, fatty acid synthesis and degradation and many more [27]. Although each of these pathways is important alone it was found that these pathways are interconnected, and they affect each other though inhibiting or promoting each other [27], as seen in Figure 4. The interconnection between these pathways reveals the complexity of aging [27].

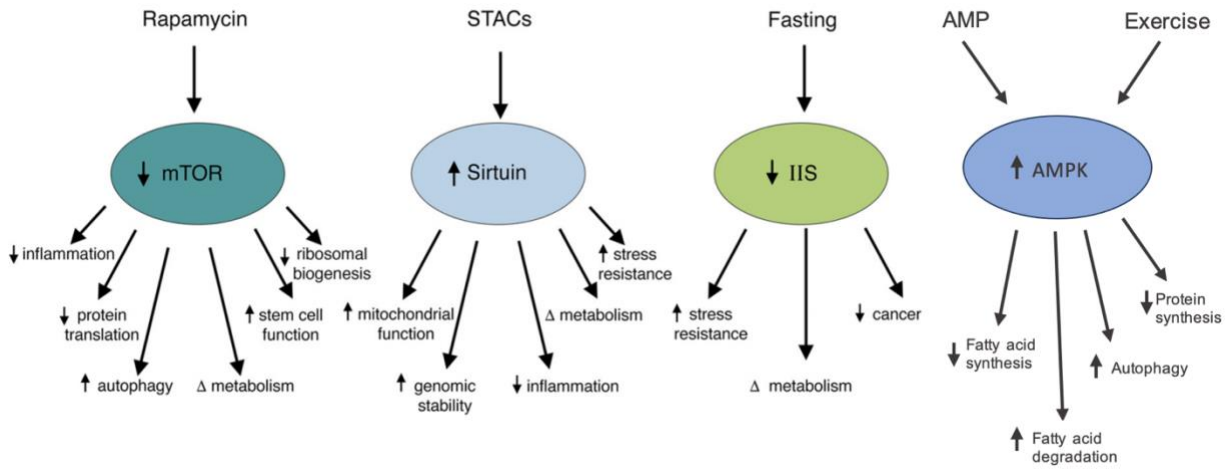

Figure 3. Overall outcomes of AMPK, IIS, Sir, and mTOR, when they are inhibited or activated (modified from Pan and Finkel, 2017) [27,57]**.** The core regulators of aging are shown along with drugs like rapamycin and STACs or environmental conditions like caloric restriction and exercising that extend lifespan and have anti-aging effects.

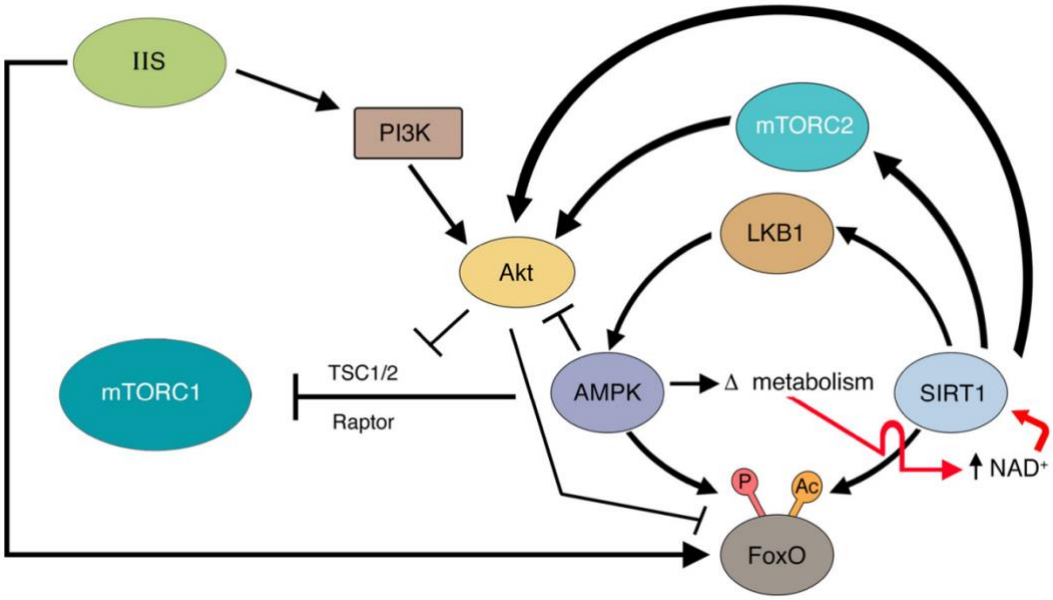

Figure 4. The interactions between four important pathways involved in anti-aging (modified from Pan and Finkel, 2017) [27]. The schematic shows the major pathways controlling aging; AMPK, IIS, Sir, and TOR, and how they are interconnected. For example, nutrients, energy and metabolism which influence IIS are key factors that impact TOR, AMPK, and Sir signaling.

1.4 The role of lipids in aging

Studies found that cellular lipid concentrations vary depending on the stage of the developmental stage of *S. cerevisiae* [58]. Phospholipids are amphipathic molecules that form the lipid bilayer of the plasma membrane and organelles [59,60]. Lipids also play a role in signaling, including the recruitment and regulation of proteins, and energy storage, among other functions [60,61]. Lipid turnover is important to generate the distinct pools that contribute to these various functions. Depending on the type of lipid, different mechanisms may be involved in their metabolism [58,62]. Different classes include triglycerides, phospholipids, sterols, fatty acids, triacylglycerol (TAG), sterols and sphingolipids (ceramide) [63] [62,64–68]. For the phospholipid class, those more commonly found in cell membranes of *S. cerevisiae* include phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), and phosphatidylserine (PS), which are enriched on the inner or outer leaflet [69,70]. Phosphatidylglycerol (PG) and cardiolipin are found in mitochondrial membranes, while phosphatidic acid (PA) and diacylglycerol (DAG) can be more variable in their concentrations in the different membranes [79,80, 81,82]. The most common fatty acids found in the glycerophosphate backbone are palmitic acid, palmitoleic acid, stearic acid, and oleic acid [69,73–75]. Multiple studies have shown that the relative amount of each lipid can vary depending on the growth conditions: carbon source, availability of nutrients, temperature, and growth phase [69,70,76]. Thus, depending on the growth conditions, the amount of each lipid class will vary depending on which pathways are activated during these conditions [58,77] as shown in Figure 5. Several studies have shown that the synthesis of lipids involves multiple organelles [78]. For example, some lipids can be synthesized in the mitochondria membrane and the ER, and could require different substrates and enzymes depending on the conditions [78]. Glycerol phospholipids like PS, PC, and PI are generally synthesized in the

endoplasmic reticulum [78]. Lipids like PE and cardiolipin, on the other hand, are synthesized in the mitochondria [78]. The pathways controlling their synthesis and metabolism change in response to nutrients and aging and are predicted to be the output of pathways controlling caloric restriction.

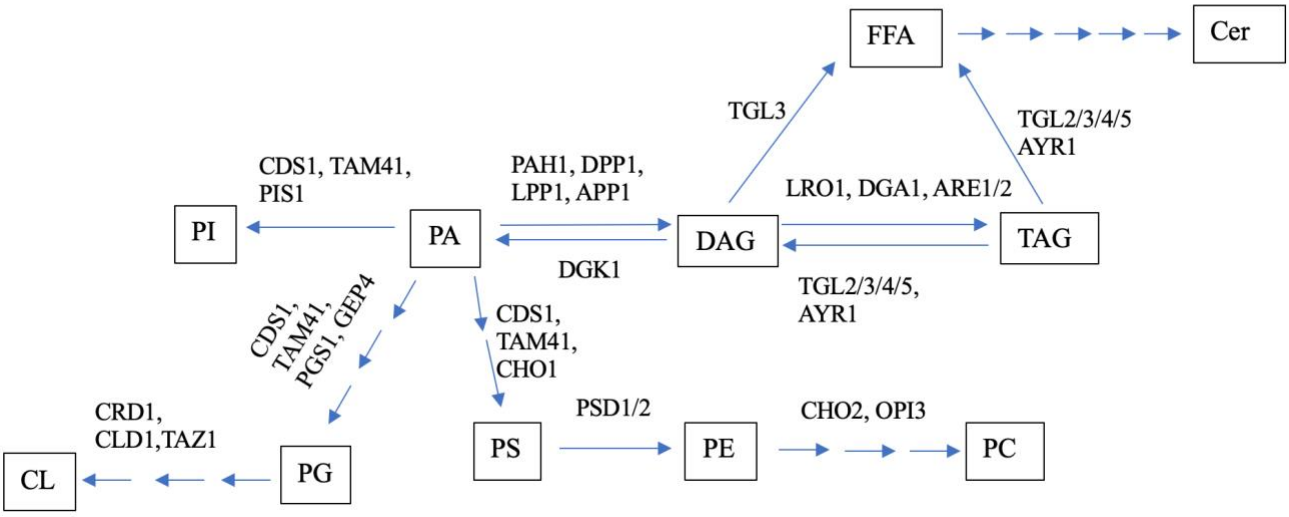

Figure 5. Lipid pathways found in *S. cerevisiae* (modified from Klug and Daum, 2014 and Mohammed et al, 2021) [77,79]**.** This figure shows how lipid metabolism is interconnected in *S. cerevisiae*. The different lipids, free fatty acids or ceramides are shown in the boxes, while the genes that regulate their turnover and/or conversion from one to the other are indicated with the relevant arrow.

1.5 Caloric restriction and intermittent fasting

Our understanding of aging has also been influenced by studies performed on different interventions that slow down aging [6]. The most well-studied intervention is caloric restriction and intermittent fasting. Caloric restriction is the decreased intake of calories without going below the threshold of amino acids, vitamins, and other nutrients that are needed for survival [8,55,80]. In *S. cerevisiae*, two mechanisms are affected by caloric restriction: ethanol metabolism and synthesis/degradation of lipids [10,81]. A study performed using *S. cerevisiae* found that when glucose was reduced (0.2-0.5% glucose) there was also a reduction in ethanol, unlike during non-caloric restriction (1-2% glucose) which led to an accumulation of ethanol [81]. The change in ethanol is due to glucose fermentation produced by *S. cerevisiae* [81]. Thus, the less glucose available the less ethanol is produced [81]. The study also showed that lifespan was prolonged in *S. cerevisiae* in the presence of 0.2% glucose (caloric restriction) than those in the presence of 2% glucose (non-caloric restriction) [81]. The results obtained in this study showed that *S. cerevisiae* under caloric restriction had a significant increase in lifespan, which correlated with a decrease in ethanol and other byproducts of metabolism [81].

Although it has not been shown, intermittent fasting could also impact lifespan, and would be a healthier option given that there could be extreme long-term consequences caused by caloric restriction in more complex organisms [55]. For intermittent fasting, there is less food provided only on some days, but not others [82]. While there was no change in lifespan, a study done in *M. musculus* found that there was a delay in the development of aging-associated diseases like Parkinson, Alzheimers, myocardial infarction, stroke, and diabetes [83,84]. In humans there have been quite a few studies with controlled nutritional intake which can lead to reduction in diseases typically associated with obesity (diabetes, cardiovascular disease), however, there is very

little knowledge of the impact of controlled nutrition at the molecular level, especially over the long-term. Regardless, it would be beneficial to find alternative solutions to extend lifespan, or in the case of humans, to improve the quality of life over a longer period of time rather than caloric restriction or intermittent fasting which could be problematic for many people.

1.6 The effects of geroprotective chemicals on aging

Geroprotective chemicals are chemicals that protect against aging [85]. These chemicals include resveratrol, rapamycin, curcumin, fistein, quercetin, caffeine, and spermidine [8,55,85–94]. Studies performed on *S. cerevisiae* found that these seven geroprotective chemicals help slow down the chronological and replicative aging, however they are not effective during caloric restriction [8,55,85–94]. The Titorenko laboratory also discovered a geroprotective chemical known as lithocholic acid (LCA), which is a bile acid in mammals [94]. The study found that LCA slows down chronological aging in *S. cerevisiae* under caloric restriction, suggesting it works through parallel pathways compared to the other geroprotective compounds [94]. A lipidomic study performed on *S. cerevisiae* after exposure to LCA under caloric restriction revealed that there was an increase in the level of PG, while there was a decrease in TAG and free fatty acids [94]. These results suggest that LCA could prevent aging by altering the metabolism of lipids and/or free fatty acids. Since LCA functions in parallel to caloric restriction, it may affect a distinct set of enzymatic regulators of lipid and/or free fatty acid metabolism. There are likely other chemicals that have anti-aging effects which are found in plants or extracts commonly used in Mediterranean diets or Chinese medicine to prolong healthy lifespans.

1.7 Using plant extracts to study aging

In many cultures, plants or plant extracts have been used to improve health. Our group chose to study a subset of extracts from plants used in Chinese medicine and Mediterranean diets to determine if they impact aging in *S. cerevisiae* [95]. These plant extracts were derived from stems, leaves, flowers, fruits, bark, roots, and other parts of plants, and of those tested, 92 showed anti-aging properties [95,96]. Further studies of these 92 plant extracts revealed that only 21 of them extended the lifespan of *S. cerevisiae* [95,96]. This study was conducted by culturing *S. cerevisiae* with or without plant extract and measuring changes in lifespan. As shown in Figure 6, while caloric restriction (0.5% glucose vs. 2% glucose) increased the proportion of viable *S. cerevisiae* after 2 days in culture (Figure 6A) and increased both the mean and maximum lifespan (Figures 6B, C), adding Plant Extract 4 had no impact on the viability of *S. cerevisiae* grown in caloric restriction (Figure 6D), but had a drastic impact on viability when grown in normal conditions (Figure 6E). Thus, these results support the hypothesis that some plant extracts can extend the lifespan of *S. cerevisiae* by affecting the same anti-aging pathways as caloric restriction [96]. The research done in the Titorenko laboratory found that for Plant Extract 4, Plant Extract 6, and Plant Extract 12 viability is extended only when the *S. cerevisiae* is under non-caloric restriction [95,96]. Unlike, Plant Extract 21, Plant Extract 26, and Plant Extract 39 which were found to extend lifespan under caloric restriction and under non-caloric restriction [95,96]. Based on this prediction, we proposed that caloric restriction and plant extract treatment would cause similar changes in the lipidome.

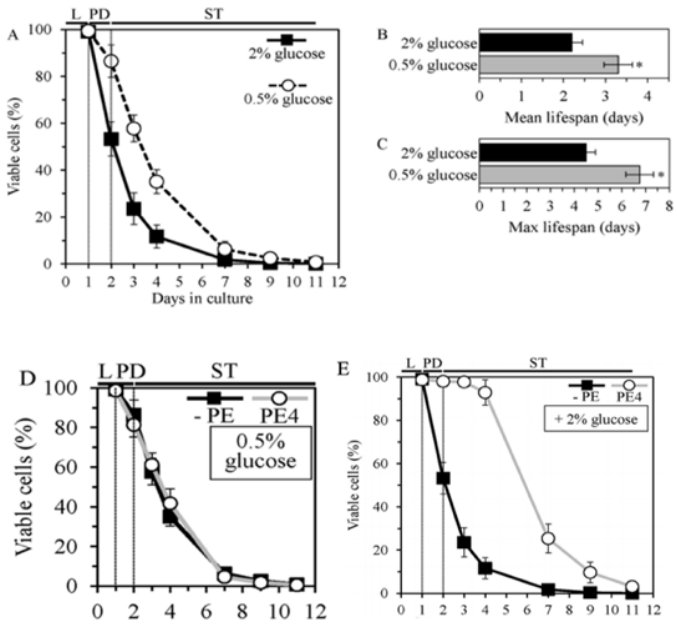

Figure 6. *S. cerevisiae* cells cultured with different glucose concentrations with or without Plant Extract 4 (modified from Lutchman et al., 2016) [96]**.** a) *S. cerevisiae* cells grown in YNB medium (0.67% Yeast Nitrogen Base without amino acids) with 2% glucose (non-caloric restriction) or 0.5% glucose (caloric restriction), b) mean lifespan of the *S. cerevisiae* grown in YNB medium under non-caloric and caloric restriction, c) maximum lifespan of *S. cerevisiae* grown in YNB medium under non-caloric and caloric restriction, d) *S. cerevisiae* cells were grown on YNB medium under caloric restriction with or without Plant Extract 4, and e) *S. cerevisiae* were grown on YNB medium under non-caloric restriction with or without Plant Extract 4.

Since Plant Extract 21 caused the most dramatic increase in lifespan, a previous study in the Titorenko lab performed studies to determine the genes involved in the anti-aging effects of Plant Extract 21 [97]. Four of these enzymes are *faa1, faa4, ale1,* and *slc1,* which help incorporate free fatty acids into PA [97]. As shown in Figure 7, when *faa1* (A), *faa4* (B), *ale1* (C), or *slc1* (D) were deleted, cell viability was similar or less than the non-mutant strain, while viability was increased after adding Plant Extract 21, but not to the same extent as adding Plant Extract 21 to the non-mutant. These results revealed that these four enzymes are at least partially needed for the full anti-aging effect of Plant Extract 21 [97]. A similar study explored the function of genes that convert TAG into free fatty acids; *tgl1,3,4,5* [97]. As shown in Figure 7 when *tgl1* (E), *3* (F), *4* (G), or *5* (H) were deleted, they had no effect on cell viability compared to control cells. However, when combined with Plant Extract 21, each mutant had increased viability above what was observed for Plant Extract 21 in control cells. This revealed that pathways controlling the conversion of TAG to free fatty acids counteract the effects of Plant Extract 21 [97].

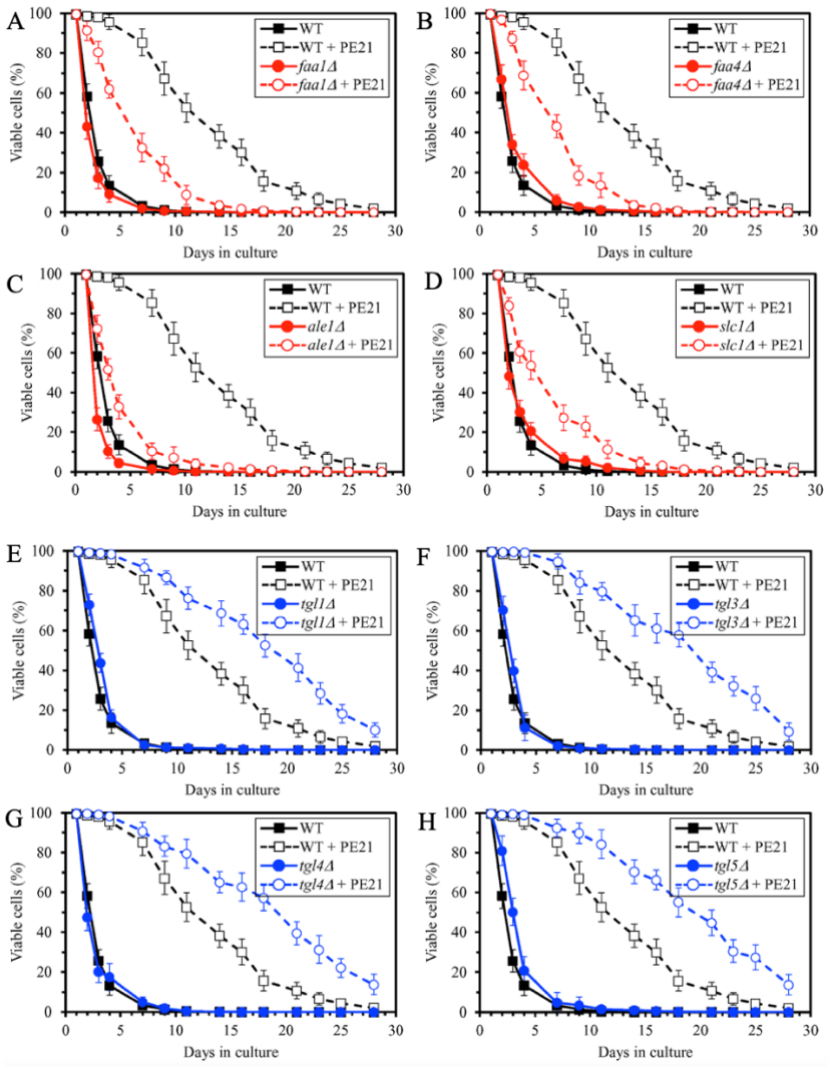

Figure 7. How mutations in genes required for fatty acid metabolism affect viability in combination with Plant Extract 21 (modified from Medkour et al., 2019) [97]**.** (A, B, C, D) shows the viability of cells containing deletions of *faa1, faa4, ale1,* or *slc1,* respectively, which incorporate free fatty acids into PA (closed red circles), and in combination with Plant Extract 21 (open red circles) compared to controls (black circles). (E, F, G, H) shows the viability of cells containing deletions of *tgl1,3,4,5*, respectively, which produce free fatty acids from TAG (closed blue circles), and in combination with Plant Extract 21 (open blue circles) compared to controls (black circles).

To further understand how Plant Extract 21 confers anti-aging, the Titorenko lab identified fifteen genes that are upregulated after Plant Extract 21 treatment and/or UPR, and determined if Plant Extract 21 still extends aging after their deletion. As shown in Figure 8 deleting genes *emc2* (A), *alg3* (B), *ctt1* (C), *fes1* (D), *nde1* (E), *cit1* (F), *ccp1* (G), *cox10* (H), *bsc1* (I), *hot13* (J), *dnm1* (K), *ifm1* (L), *bst1* (M), *fat1* (N) and *abp140* (O) all had no impact on cell viability [97]. With the exception of *dnm1*, *ifm1*, and *abp140*, the deletions decreased the extended viability caused by Plant Extract 21, suggesting that these genes are at least partially required for the mechanism by which Plant Extract 21 extends lifespan [97]. The even greater increase in viability caused by the combination of *dnm1*, *ifm1* and *abp140* deletions and Plant Extract 21, suggests that these genes counteracts the effect of Plant Extract 21 [97]. These results revealed that genes promoting the effects of anti-aging via Plant Extract 21 (*faa1, faa4, ale1, slc1, emc2*, *alg3*, *ctt1*, *fes1*, *nde1*, *cit1*, *bst1*, and *fat1*) include regulators of free fatty acid consumption by incorporating these free fatty acids into PA, chaperons need in protein folding, enzymes required in the ER for N-linkage of glycosylated proteins, stress response proteins that act against oxidative damage, components of the UPS pathway, while those promoting aging (*tgl1,3,4,5, dnm1*, *ifm1*, and *abp1*) include regulators responsible for the formation of free fatty acids from the lipolysis TAG [97]. These results led to the interest in lipids and how they are affected by these plant extracts.

In addition to these studies of Plant Extract 21, the Titorenko lab has also studied how the lipidome changes with caloric restriction. The lipidomes of *S. cerevisiae* grown under caloric and non-caloric restriction were compared, and it was found that caloric restriction enhances the level of PA, PI, PC, PS, PE, PC, and Cer [79]. Caloric restriction also decreases the level of TAG and does not alter the level of DAG [79]. Caloric restriction also decreases the level of free fatty acids [79]. These findings support that anti-aging effects of plant extracts could occur due to

changes in lipid and free fatty acid metabolism. To determine this, we performed lipidomic research on *S. cerevisiae* after exposure to plant extracts to compare the lipidome to the effects seen with caloric restriction.

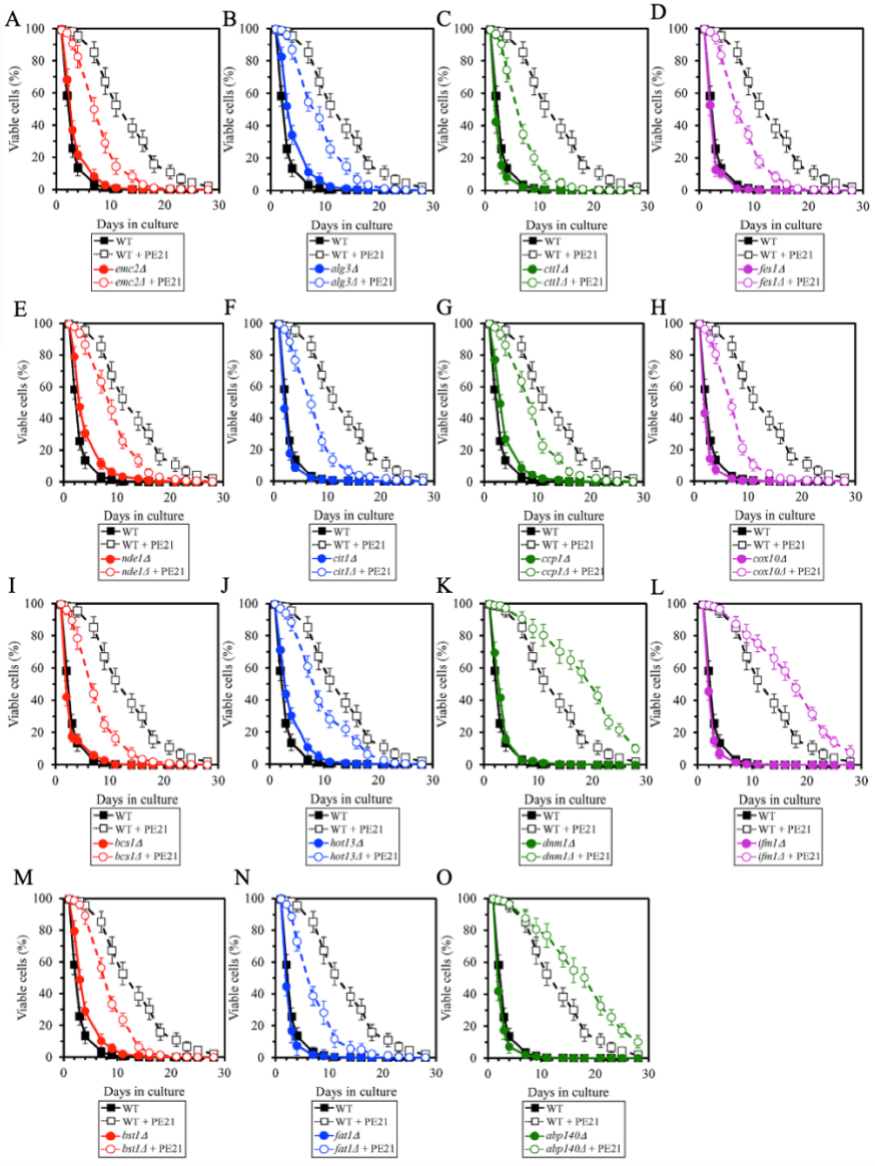

Figure 8. Single gene mutation of different genes that have been previously found to be involved in anti-aging pathways (modified from Medkour et al., 2019) [97]**.** (A-O) shows the viability of cells containing deletions of *emc2, alg3, ctt1, fes1, nde1, cit1, ccp1, cox10, bcs1, hot13, dnm1, ifm1, bst1, fat1,* or *abp140,* respectively, which has been previously found to be up-regulated (closed circles) when treated with Plant Extract 21 (open circles) compared to controls (black squares).

1.8 The Objectives of this Study

The Titorenko laboratory uses the model organism *S. cerevisiae* to study the mechanisms controlling aging. More recently, the laboratory found that 21 different plant extracts extend the lifespan of *S. cerevisiae* [95,96]. As shown in Figures 6-8, at least some of these plant extracts extend aging by mechanisms that alter lipid and free fatty acid metabolism similar to caloric restriction. We hypothesized that plant extracts cause changes in the lipidome like caloric restriction as an anti-aging mechanism. Out of the 21 plant extracts, 6 were chosen to determine their effect on the lipidome of *S. cerevisiae*: Plant Extracts 4, 6, 12, 21, 26, and 39. Quantitative mass spectrometry was used to calculate the level of each lipid in *S. cerevisiae* after treatment with the plant extracts compared to untreated *S. cerevisiae*. This thesis found that when *S. cerevisiae* was exposed to the plant extracts the lipid level changed when compared to the untreated. The most noteworthy revelation was the decrease of free fatty acids in all the plant extracts.

Chapter 2: Materials and Methods

2.1 *S. cerevisiae* strains and growth conditions

The wild-type *Saccharomyces cerevisiae* BY4742 (MATα *his3Δ1 leu 2Δ0 lys2Δ0 ura3Δ0*) obtained from Thermo Scientific/Open Biosystems was used in this research. The *S. cerevisiae* was grown on a synthetic minimal 0.67% Yeast Nitrogen Base medium (YNB) with no amino acids. The media was supplemented with 2% glucose, 20mg/l *L*-histidine, 30mg/l *L*-leucine, 30mg/l *L*-lysine, and 20mg/l uracil. The *S. cerevisiae* cells were cultured in 200ml Erlenmeyer flasks inside of a rotational shaker rotating at 200rpm with a stable temperature of 30 Celsius. The stock solution of ethanol/plant extract was produced on the same day as cell culturing began. The addition of the ethanol/plant extract solution was closely followed by cell inoculation. The control group received the sample volume of ethanol and the test group received the ethanol/plant extract solution. The six plant extracts that were studied were Plant Extract 4, Plant Extract 6, Plant Extract 12, Plant Extract 21, Plant Extract 26, and Plant Extract 39.

Table 1. Identification of Plant Extract 4, 6, 12, 21, 26 and 39 and their final concentrations

Plant Extract number	Scientific Name	Common Name	Concentration in Medium (%)	Batch Number	Supplier
4	*Actaea racemosa*	Black Cohosh	0.5	EC330312	Naturex
6	*Passiflora*	Passion Flower	1.0	132901	Nexira
12	*Apium graveolens*	Celery Seed	0.1	EC334426	Naturex
21	*Salix alba*	White Willow Bark	0.1	ED162368	Naturex
26	*Serenoa repens*	Saw Palmetto Fruit	0.5	ED842054	Naturex
39	*Hypericum perfortum* L	St John's Wort flower	0.5	N21397K0617	Pharmanager ingredients

2.2 Lipid extraction [98]

Two billion cells were collected for lipid extraction. These cells were collected 24 hours, 48 hours, 36 hours, and 72 hours after cell inoculation. The two billion cells were collected using a spectrophotometer at 600nm. The OD obtained was placed in a formula obtained using a hemocytometer. These measurements gave the precise volume needed to collect two billion *S. cerevisiae* cells. A cell pellet was then collected using a Centra CL2 clinical centrifuge at 3000 X g for 5 minutes at room temperature. The pellet was resuspended using 1ml of ice-cold ammonium bicarbonate (pH 8.0) was then used to resuspend the cells. A microfuge centrifuge at 16000 X g for 5 minutes at room temperature was used to obtain a pellet. The cell pellets were stored in a -80°C freezer until they were used for lipid extraction.

The *S. cerevisiae* cell pellets were taken from the -80°C freezer and thawed on ice for 20 minutes. The cell pellet was resuspended using 200µl of ice-cold LC/MS nano pure water and transferred to a 15ml glass screw top centrifuge tube. 100µl of acid-washed glass beads and 600µl of a 17:1 chloroform (HPLC grade) /methanol (LC/MS grade) solution (both chemicals were obtained from Fisher Scientific, see table 2) were added to the centrifuge tube. The centrifuge tubes were vortex for a minute at high speeds and then placed in an ice bath for a minute. This step was repeated five times to ensure the *S. cerevisiae* cells are properly broken apart. Once the *S. cerevisiae* cells have been broken the centrifuge tubes were vortex for an hour at a low speed to facilitate lipid extraction. The samples were incubated for 40 minutes at 4°C to promote protein precipitation and separate the organic phase from the aqueous phase. To further help this separation the samples were centrifuged at 3000 X g for 10 minutes at 4°C. 400µl of the lower organic phase (which contains TAG, PC, PE, and PG) is then transferred into Eppendorf tubes. These Eppendorf

tubes are centrifuged at 15000 X g for 40 minutes at 4°C. 300µl is taken from the Eppendorf tubes and placed into 5ml glass culture tubes. The sample in the glass tubes is then dried under nitrogen gas (AirLiquid) until there is no liquid left. 300µl of 2:1 chloroform (HPLC grade)/methanol (LC/MS grade) mixture (both chemicals were obtained from Fisher Scientific, see table 2) is added to the aqueous layer found in the 15ml centrifuge tubes. This step is done to extract the sphingolipids and PA, PI, PS, and CL. The centrifuge tubes are vortex at high speeds for 30 minutes at room temperature. After the vortex, they are incubated in an ice bath for 30 minutes. To further help with separation the centrifuge tubes are centrifuged at 3000 X g for 5 minutes at 4°C. 200µl of the organic phase is transferred from the 15ml centrifuge tubes to the Eppendorf tubes that previously contained the 400µl of the organic phase. The Eppendorf tubes are centrifuged at 15000 X g for 40 minutes at 4°C. 200µl is transferred from the Eppendorf tubes to the glass tubes containing the samples dried under nitrogen gas. 50µl of lipid standard (obtained by Adventi Lipids, see table 2.1). The samples in the glass tubes are dried under nitrogen gas until no liquid remains. 200µl of 65:35:5 of acetonitrile/isopropanol/nano pure water (all chemicals were LC/MS grade and from Fisher Scientific, see table 2.2) was added to the dried samples. The glass tubes were vortexed at high speed for a minute and a half. The samples were sonicated for 15 minutes to ensure that the sample was properly mixed. The samples were vortexed at high speeds for a minute and a half and then transferred to Eppendorf tubes for overnight storage at -20°C. The following day the samples were vortexed at high speeds for a minute and a half. The Eppendorf tubes were then centrifuged at 15000 X g for 30 minutes at 4°C and transferred to glass vials used in Mass Spectrometry.

Table 2. Lipid standard

Class of Lipid	Lipid chain composition(Number of carbon atoms: Number of double bonds on the fatty acid chain)	Exact Mass (g/mol)	Mass/Ion Charge (m/z)	Source	Product Number
PE	15:0-18:1-d7	710.559	728.593	Avanti Lipids	791638C

Table 3. Chemical solutions used to extract lipids

Chemical Solution	Chloroform (ml)	Methanol (ml)	Acetonitrile (ml)	Isopropanol (ml)	Nano pure water (ml)
Chloroform: Methanol 2:1	68	3	-	-	-
Chloroform: Methanol 17:1	50	25	-	-	-
Acetonitrile:Isopropanol: Nanopure water 65:35:5	-	-	52	28	4

2.3 Analysis of lipid species using Mass Spectrometry

The extracted lipids were run in the Thermo Orbitrap Velos Mass Spectrometer. Two mobile phases were used to run these samples mobile phase A and mobile phase B (see Table 2.3). Mobile phase A contains acetonitrile and nano pure water and mobile phase B contains acetonitrile and isopropanol. The polarity of the mobile phases was altered using two different salts. The positive mobile phases were obtained by the addition of ammonium formate (NH_4HCO_2, obtained from SIGMA-ALDRICH, BCCG7486). The negative mobile phases were obtained by the addition of ammonium acetate ($C_2H_7NO_2$, obtained from Fisher Scientific, 156070). Between each sample run, the mass spectrometry line was cleaned using the 2:1 chloroform/methanol mixture (see Table 2.2). The line was cleaned until the ion detection level returned to the baseline. The lipid standard was injected several times, during the data acquisition, to make sure that the instruments' sensitivity did not decrease.

Table 4. Chemical mixtures used to make the mobile phases

Mobile Phase	Acetonitrile (ml)	Isopropanol (ml)	Nanopure (ml)	Ammonium formate (g/500ml)	Ammonium Acetate (g/500ml)
A+	300	-	200	0.32	-
A-	300	-	200	-	0.39
B+	50	450	-	0.32	-
B-	50	450	-	-	0.39

Table 5. Thermo Orbitrap Velos Mass Spectrometer tuning setting

Polarity of Instrument	Positive	Negative
Voltage Source (kV)	3.9	4
Capillary temperature (Celsius)	275	275
Sheath gas flow	5	5
Aux gas flow	1	1
FTMS injection time (ms)	100	500
FTMS micro scans	3	1

Abbreviation: FTMS, Fourier transform mass spectrometry

Table 6. Thermo Orbitrap Velos Mass Spectrometer data acquisition instrument setup [98]

	Acquisition time	5 minutes (with a 0.25-minute delay)	
Segments	Instrument polarity	Positive	Negative
MS (segment I)	Analyzer	FTMS	FTMS
	Mass Range	Normal	Normal
	Resolution	100,000	100,000
	Data Type	Centroid	Centroid
	Scan Range	400-1,200	400-1,200
Data-dependent MS/MS (segments 2-10)	Analyzer	FTMS	FTMS
	Resolution	30,000	30,000
	Data Type	Centroid	Centroid
	Activation	HCD	HCD
	Activation Time (ms)	0.1	0.1
	Isolation Width	1	1
	Collision Energy	35	65
	Mass Range	Normal	Normal
	Data Type	Centriod	Centriod
	Scan Range	-	-

Chapter 3 RESULTS

The research was done in the Titorenko laboratory on age-delaying plant extracts and identified six plant extracts that extend chronological aging in *S. cerevisiae* [95,96]. PEs include Plant Extract 4 (Black Cohosh), Plant Extract 6 (Passion flower), Plant Extract 12 (Celery seed), Plant Extract 21 (White willow bark), Plant Extract 26 (Saw palmetto fruit), and Plant Eextract 39 (St John's wort flower). Changes in their lipidomes were identified using mass spectrometry as shown in each of the subsections below.

3.1 The effects of Plant Extract 4 on lipids and free fatty acids

3.1.1 The effects of Plant Extract 4 on nine different lipids

Figure 9 shows the level of the nine lipid classes measured by a mass spectrometer, and it revealed that when *S. cerevisiae* was treated with Plant Extract 4 the level of some lipids increased while others decreased, and some were not altered. Six of the nine lipid classes were increased when *S. cerevisiae* was treated with Plant Extract 4. These lipid classes are DAG, PC, PI, PE, PA, and Cer. A decrease in level is seen in TAG and PG when *S. cerevisiae* was treated with Plant Extract 4. The only lipid class that was not altered when *S. cerevisiae* was treated with Plant Extract 4 is PS. Apart from the overall increase or decrease seen in the treated *S. cerevisiae* when compared to the untreated *S. cerevisiae* there was also an observed trend when looking at the levels over the four days measured. The untreated *S. cerevisiae* is seen to decrease in level over the four days for all lipid classes. Unlike, the treated *S. cerevisiae* which exhibits increases in level during the four days for lipid DAG, PI, and Cer. A decrease in level of PE, PA, PC, and PS in the treated *S.*

cerevisiae is seen over the four days. Lastly, the level of TAG and PG remains stable over the four days in the *S. cerevisiae* treated with Plant Extract 4.

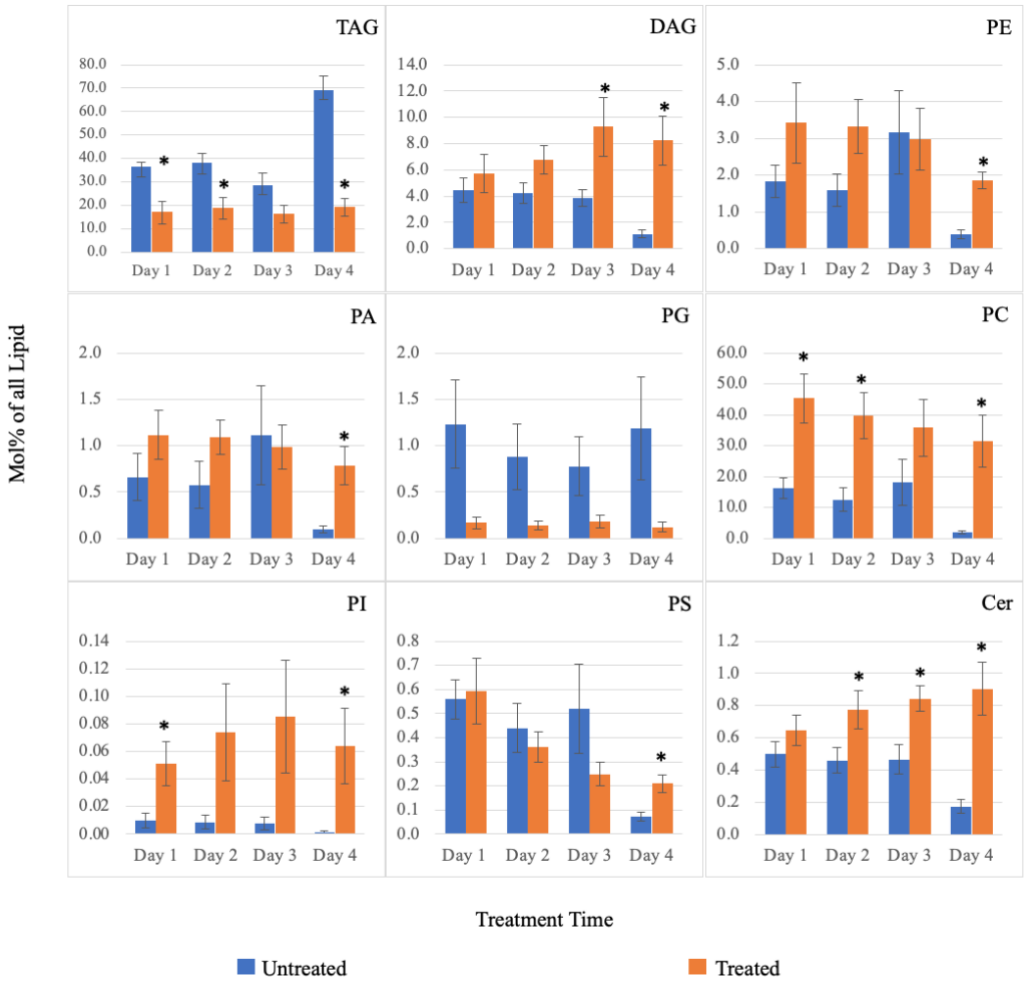

Figure 9. Average mol% of different lipid classes obtained from *S. cerevisiae* cells grown under 2% glucose with or without Plant Extract 4. A wild-type strain of *S. cerevisiae* was cultured in 2% glucose (w/v) with or without Plant Extract 4. The cells were then collected on days 1, 2, 3, and 4 of their culturing. The mol% was calculated using the sum of molar amounts of lipids in all classes for each technical repeat. Each lipid class had three different biological repeats and three technical repeats per biological repeat. The sum is then divided by the total lipid

concentration of all lipids. The error bars were calculated by SEM (n= 9). The asterisks indicate the significance between the treated and the untreated *S. cerevisiae* using a T-test ($p \leq 0.05$).

3.1.2 The effects of Plant Extract 4 on four free fatty acids

Figure 10 shows four free fatty acids studied in *S. cerevisiae* treated with Plant Extract 4 using a mass spectrometer. The results showed that the levels of oleic acid, palmitic acid, and stearic acid were not altered, while the level of palmitoleic acid in the treated *S. cerevisiae* decreased. However, there was a trend seen with the free fatty acids over the four days measured. An overall decreasing level is seen over the four days in the untreated *S. cerevisiae*. The treated *S. cerevisiae* on the other hand showed an increased level over the four days for oleic acid, palmitic acid, and stearic acid. Palmitoleic acid is the only free fatty acid that remains stable over the four days when treated with Plant Extract 4.

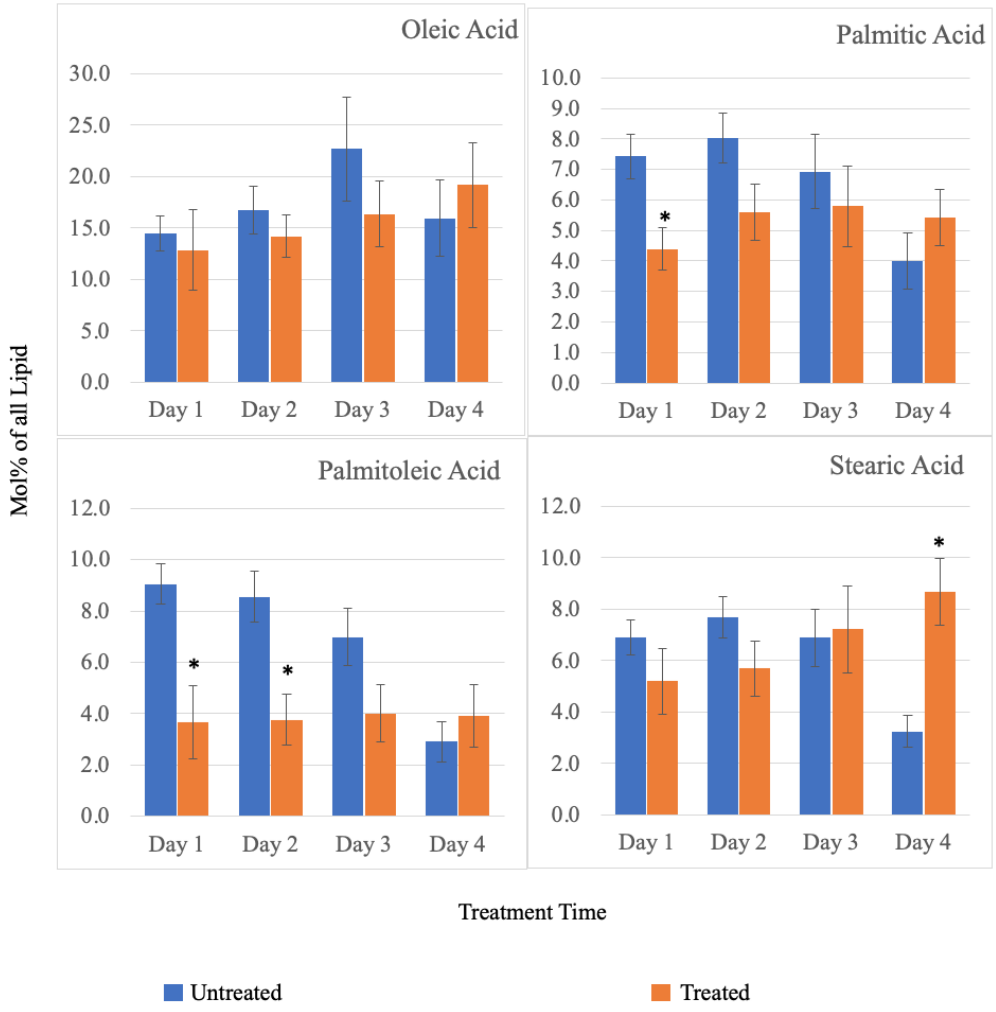

Figure 10. Average mol% of different free fatty acids obtained from *S. cerevisiae* cells grown with or without Plant Extract 4. A wild-type strain of *S. cerevisiae* was cultured in 2% glucose (w/v) with or without Plant Extract 4. The cells were then collected on Days 1, 2, 3, and 4 of their culturing. The mol% was calculated using the sum of molar amounts of lipids in all classes for each technical repeat. Each lipid class was analyzed in three different biological replicates and each with three technical replicates. The sum is then divided by the total lipid concentration of all lipids. The error bars were calculated by SEM (n= 9). The asterisks indicate the significance between the treated and the untreated *S. cerevisiae* using a T-test ($p \leq 0.05$).

3.2. Effects of Plant Extract 6 on lipids and free fatty acids

3.2.1 The effects of Plant Extract 6 on nine different lipids

Figure 11 shows the level of nine lipid classes measured by a mass spectrometer. Out of the nine lipid classes only two were seen to have an increased level when treated with Plant Extract 6: TAG and DAG. Plant Extract 6 did not alter PE, PA, PG, PC, PS, and Cer. When looking at the untreated *S. cerevisiae* it is seen that over the four days there is a decrease in level for all the lipids except TAG. The level of lipids over the four days in the treated *S. cerevisiae* changes depending on the lipid. An increase is observed over the four days in TAG. A decrease in level over the four days is observed in DAG, PA, PC, and PE. No visible trend is observed in PG, PS, and Cer. The level of lipid PI is too low to observe any trend or any fluctuations when *S. cerevisiae* is treated or untreated with Plant Extract 6.

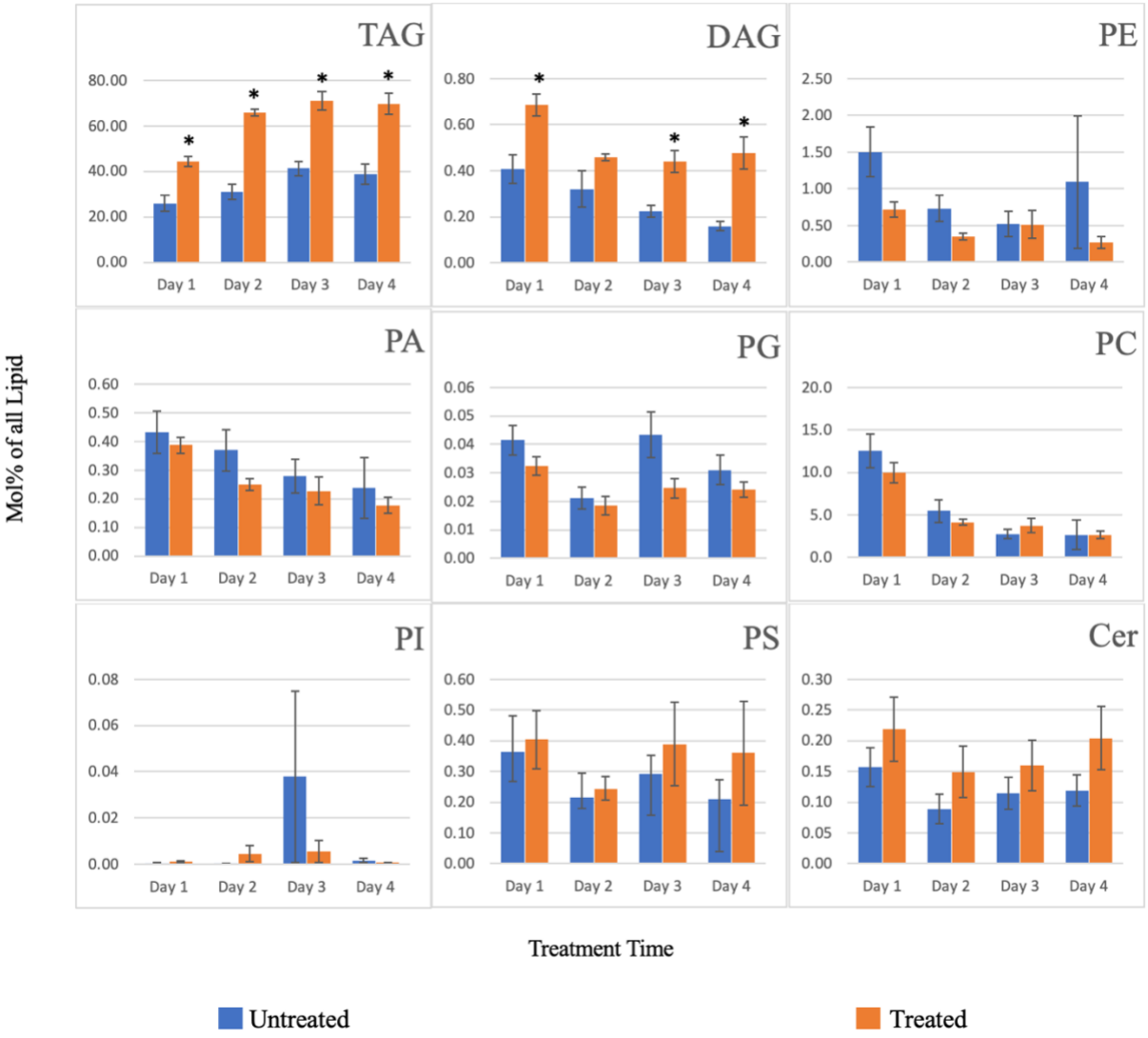

Figure 11. Average mol% of different lipid classes obtained from *S. cerevisiae* cells grown with or without Plant Extract 6. A wild-type strain of *S. cerevisiae* was cultured in 2% glucose (w/v) with or without Plant Extract 6. The cells were then collected on Days 1, 2, 3, and 4 of their culturing. The mol% was calculated using the sum of molar amounts of lipids in all classes for each technical repeat. Each lipid class was analyzed in three different biological replicates and each with three technical replicates. The sum is then divided by the total lipid concentration of all lipids. The error bars were calculated by SEM (n= 9). The asterisks indicate the significance between the treated and the untreated *S. cerevisiae* using a T-test ($p \leq 0.05$).

3.2.2 The effects of Plant Extract 6 on four free fatty acids

Figure 12 shows four free fatty acids studied in *S. cerevisiae* treated with Plant Extract 6 using a mass spectrometer. The results show that there was a decrease in all four free fatty acids when *S. cerevisiae* was treated with Plant Extract 6. A trend can be seen in some of the free fatty acids. In the untreated *S. cerevisiae,* a stable level of palmitic acid and palmitoleic acid is seen over the four days. An increasing trend over the four days is seen in both oleic and steric acid in the untreated *S. cerevisiae.* A decreasing trend is seen over the four days for palmitic and stearic acid when the *S. cerevisiae* is treated with Plant Extract 6. Lastly, a stable trend is seen in the level of oleic and palmitoleic acid over the four days in the treated *S. cerevisiae.*

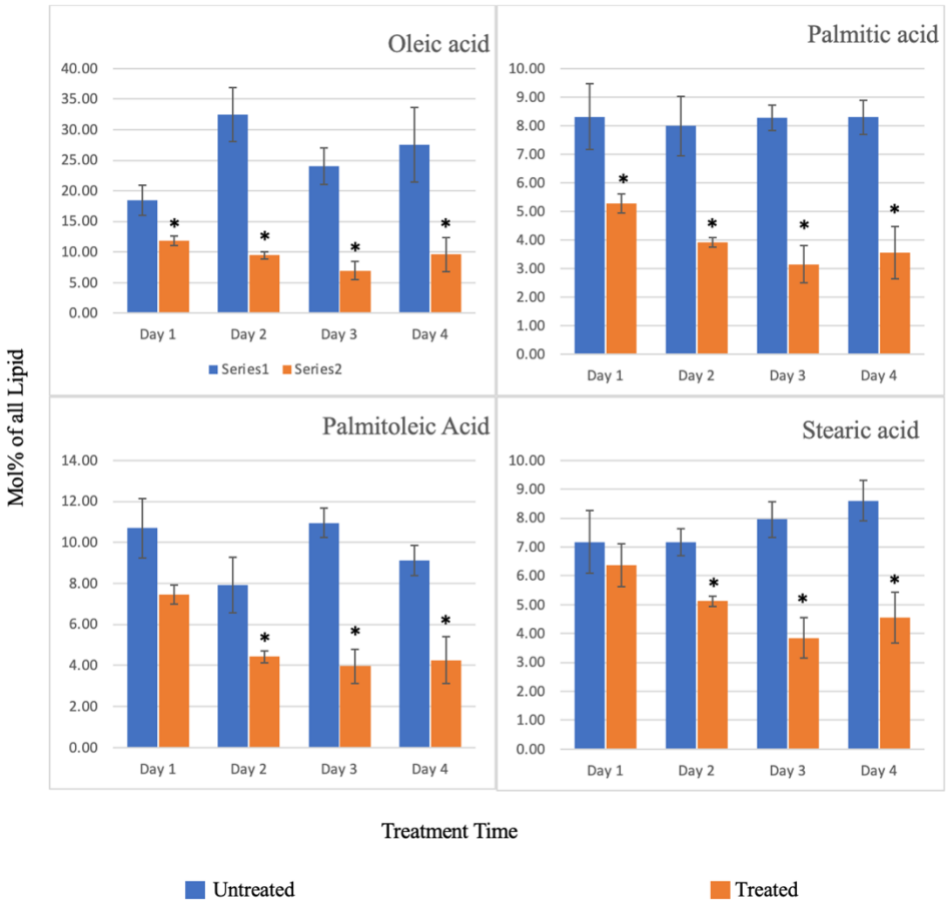

Figure 12. Average mol% of different free fatty acids obtained from *S. cerevisiae* cells grown with or without Plant Extract 6. A wild-type strain of *S. cerevisiae* was cultured in 2% glucose (w/v) with or without Plant Extract 6. The cells were then collected on Days 1, 2, 3, and 4 of their culturing. The mol% was calculated using the sum of molar amounts of lipids in all classes for each technical repeat. Each lipid class was analyzed in three different biological replicates and each with three technical replicates. The sum is then divided by the total lipid concentration of all lipids. The error bars were calculated by SEM (n= 9). The asterisks indicate the significance between the treated and the untreated *S. cerevisiae* using a T-test ($p \leq 0.05$).

3.3 Effects of Plant Extract 12 on lipids and free fatty acids

3.3.1 The effects of Plant Extract 12 on nine different lipids

Figure 13 shows the level of nine different lipid classes, measured by a mass spectrometer. Out of the nine lipid classes only TAG was seen to increase in the presence of Plant Extract 12. Four lipid classes, PE, PA, PG, PS, and PC were seen to decrease in the presence of Plant Extract 12. Both DAG and Cer were not altered in the presence of Plant Extract 12. There was no visible trend or change in the level of PI with or without Plant Extract 12. When looking at the trend of lipid levels over the four days for the untreated *S. cerevisiae* an increase is seen in PE, PA, and PC. A decrease in level over the four days in the untreated *S. cerevisiae* is seen in PG, PS, and Cer. No change in level is observed in TAG and DAG for the untreated *S. cerevisiae* over the four days. When looking at the treated *S. cerevisiae* it is seen that there is an increase in level over the four days in only Cer. The level of TAG, DAG, PE, PA, PG, PC and PS remain the same over the four days when *S. cerevisiae* is treated with Plant Extract 12. No lipid is observed to decrease over the four days when the *S. cerevisiae* is treated with Plant Extract 12.

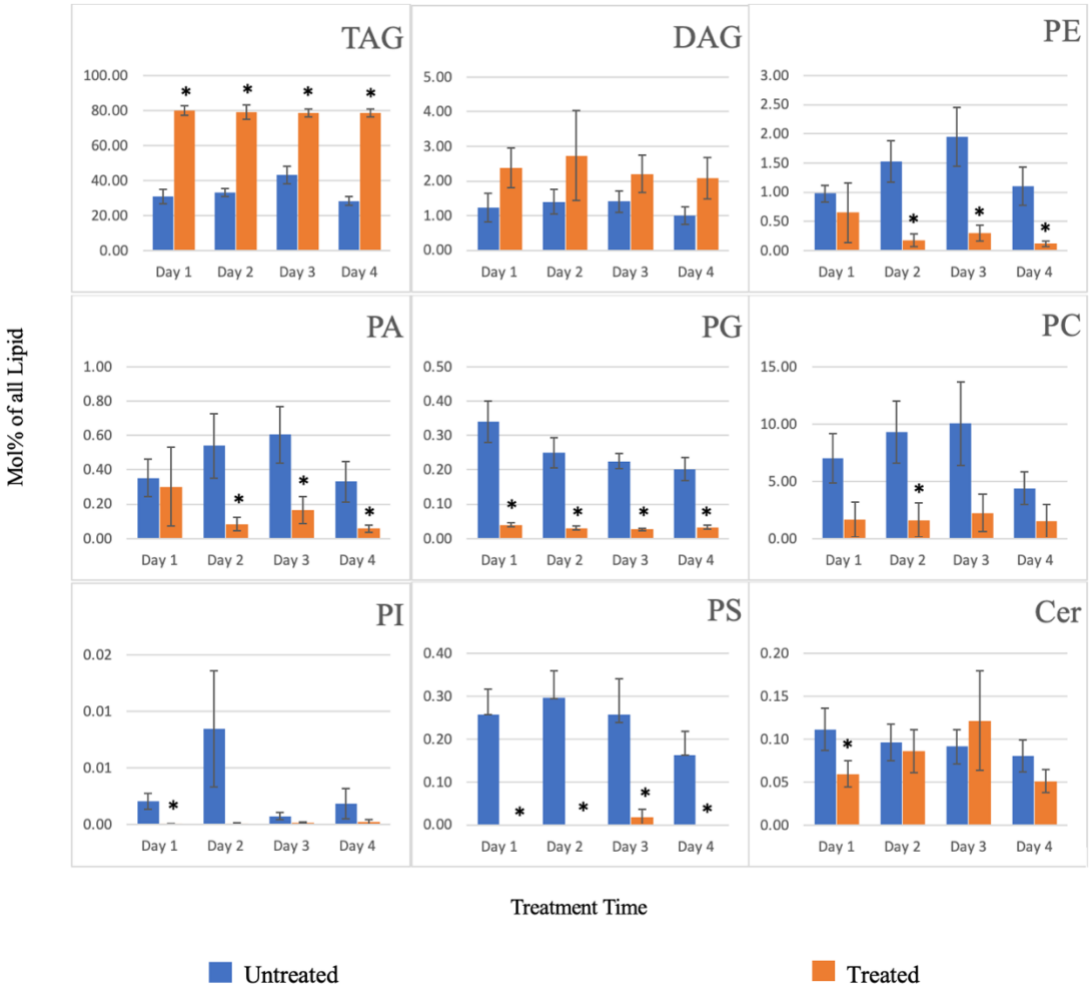

Figure 13. Average mol% of different lipid classes obtained from *S. cerevisiae* cells grown with or without Plant Extract 12. A wild-type strain of *S. cerevisiae* was cultured in 2% glucose (w/v) with or without Plant Extract 12. The cells were then collected on Days 1, 2, 3, and 4 of their culturing. The mol% was calculated using the sum of molar amounts of lipids in all classes for each technical repeat. Each lipid class was analyzed in three different biological replicates and each with three technical replicates. The sum is then divided by the total lipid concentration of all lipids. The error bars were calculated by SEM (n= 9). The asterisks indicate the significance between the treated and the untreated *S. cerevisiae* using a T-test ($p \leq 0.05$).

3.3.2 The effects of Plant Extract 12 on four free fatty acids

Figure 14 shows four free fatty acids studied in *S. cerevisiae* treated with Plant Extract 12 using a mass spectrometer. The results show a decrease in the level of all free fatty acids of *S. cerevisiae* treated with Plant Extract 12 when compared to the untreated *S. cerevisiae*. When looking at the trend of the level of the free fatty acids over the four days it is seen that palmitic and oleic acid in untreated *S. cerevisiae* does not change. Stearic acid is seen to increase unlike palmitoleic acid is seen to decrease in the untreated *S. cerevisiae*. The S. cerevisiae treated with Plant Extract 12 shows a stable level of all four free fatty acid over the four days of treatment.

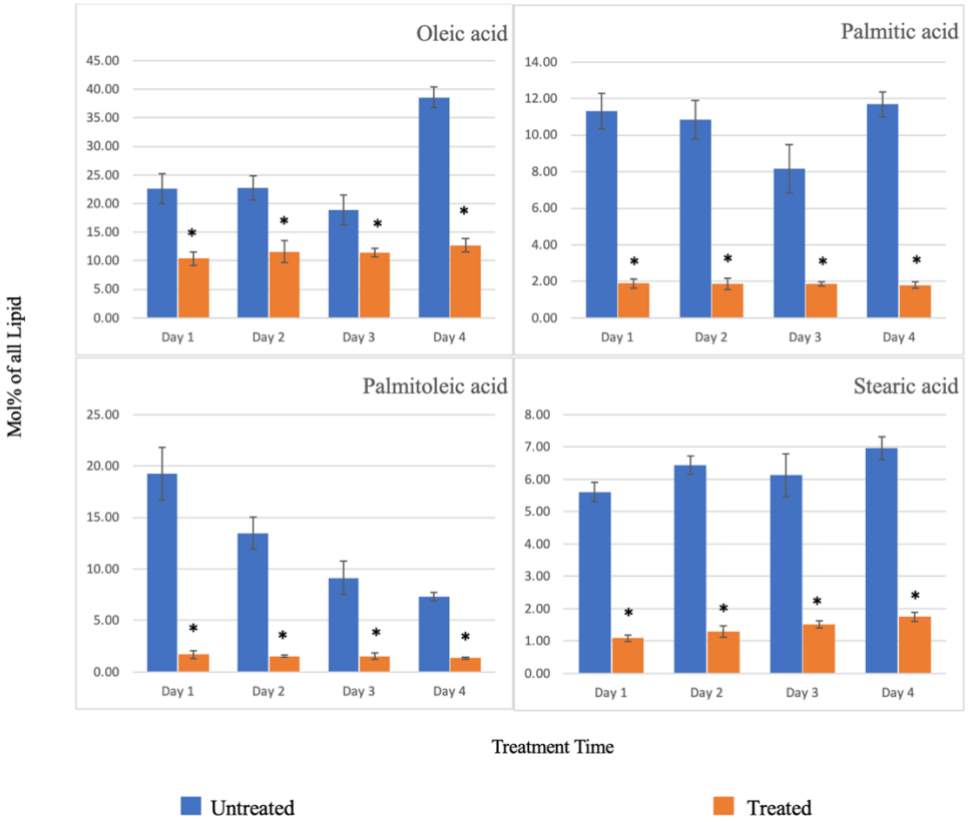

Figure 14. Average mol% of different free fatty acids obtained from *S. cerevisiae* cells grown with or without Plant Extract 12. A wild-type strain of *S. cerevisiae* was cultured in 2% glucose (w/v) with or without Plant Extract 12. The cells were then collected on Days 1, 2, 3, and 4 of their culturing. The mol% was calculated using the sum of molar amounts of lipids in all classes for each technical repeat. Each lipid class was analyzed in three different biological replicates and each with three technical replicates. The sum is then divided by the total lipid concentration of all lipids. The error bars were calculated by SEM (n= 9). The asterisks indicate the significance between the treated and the untreated *S. cerevisiae* using a T-test ($p \leq 0.05$).

3.4. The effects of Plant Extract 21 on lipids and free fatty acids

3.4.1 The effects of Plant Extract 21 on nine different lipids

Figure 15 shows the level of nine different lipid classes studied using mass spectrometry. An increase in the level of lipids TAG, DAG, PE, PI, PG, and Cer was observed in *S. cerevisiae* treated with Plant Extract 21. Plant Extract 21 did not alter the level of PA, PC, and PS. When looking at the untreated *S. cerevisiae* a decreasing level of lipid is seen over the four days in all the lipid classes. Unlike in the treated *S. cerevisiae* which shows an increasing level over the four days in PG. A decreasing level of TAG, DAG, PE, PA, PC, and PS is seen over the four days in *S. cerevisiae* treated with Plant Extract 21. The only lipid class that has no change in lipid level in the treated *S. cerevisiae* over the four days is Cer.

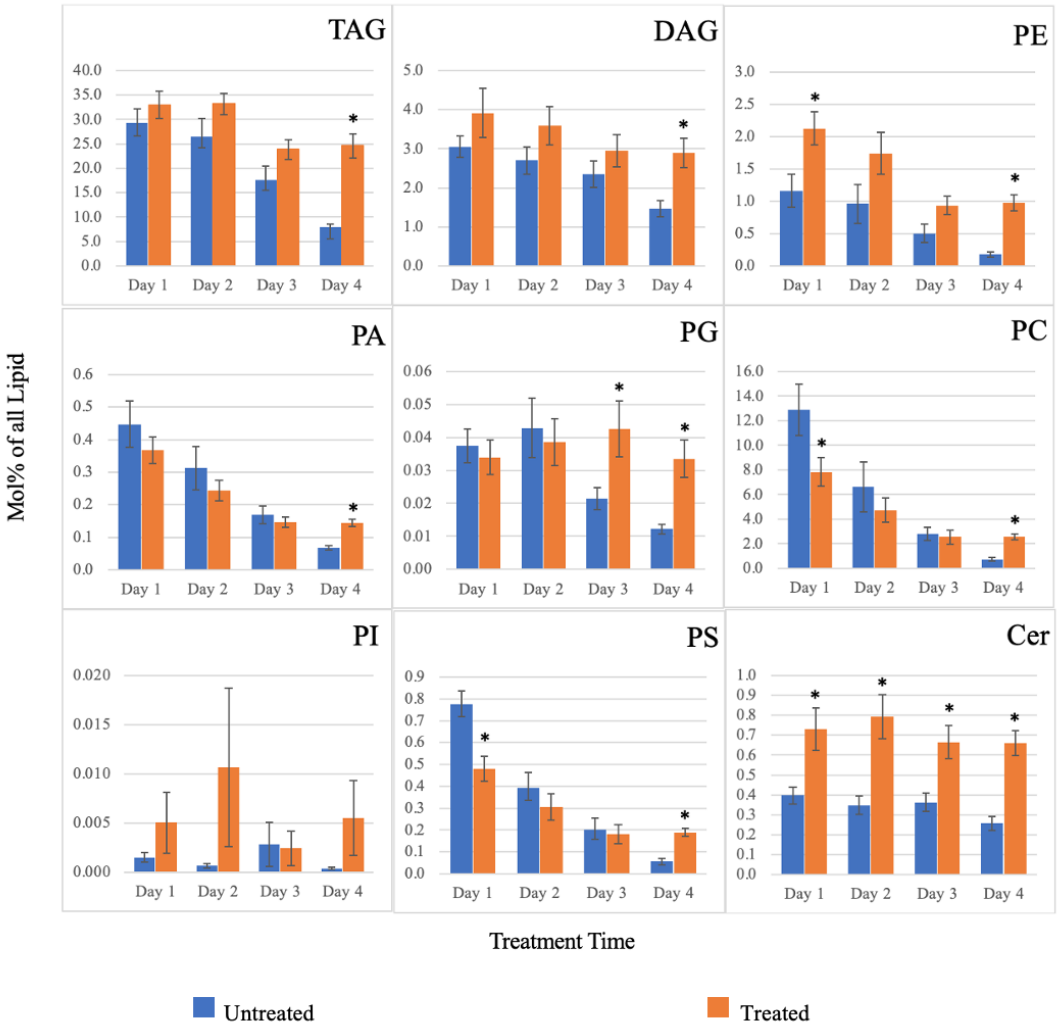

Figure 15. Average mol% of different lipid classes obtained from *S. cerevisiae* cells grown with or without Plant Extract 21. A wild-type strain of *S. cerevisiae* was cultured in 2% glucose (w/v) with or without Plant Extract 21. The cells were then collected on Days 1, 2, 3, and 4 of their culturing. The mol% was calculated using the sum of molar amounts of lipids in all classes for each technical repeat. Each lipid class was analyzed in three different biological replicates and each with three technical replicates. The sum is then divided by the total lipid concentration of all lipids. The error bars were calculated by SEM (n= 9). The asterisks indicate the significance between the treated and the untreated *S. cerevisiae* using a T-test ($p \leq 0.05$).

3.4.2 The effects of Plant Extract 21 on four free fatty acids

Figure 16 shows four free fatty acids studied in *S. cerevisiae* treated with Plant Extract 21 using a mass spectrometer. Plant Extract 21 was seen to cause a decrease in palmitic acid, unlike the other three free fatty acids which showed no change when compared to the untreated *S. cerevisiae*. When looking at the four-day trend of the level of free fatty acids it is seen that in the untreated *S. cerevisiae* there is an increase over the four days in oleic acid, palmitic acid and stearic acid. There is a decrease in level over the four days of palmitoleic acid of the untreated *S. cerevisiae*. In the treated *S. cerevisiae* there is an increase in oleic and stearic acid over the four days. The levels of palmitic and palmitoleic acid are stable over the four days in the treated *S. cerevisiae.*

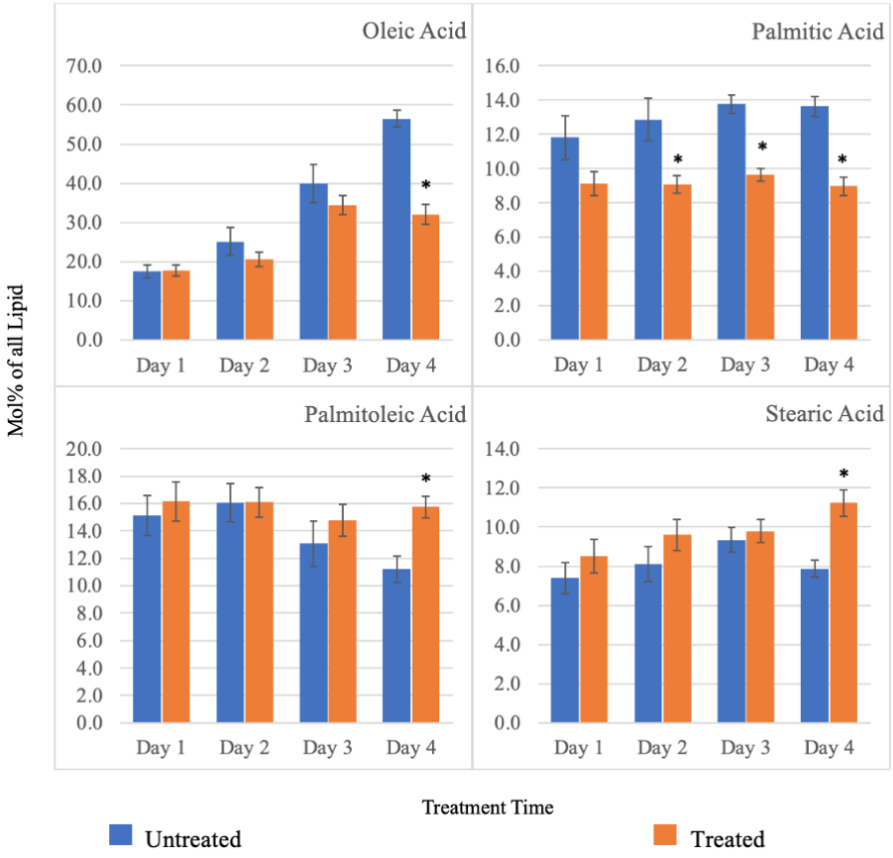

Figure 16. Average mol% of different free fatty acids obtained from *S. cerevisiae* cells grown with or without Plant Extract 21. A wild-type strain of *S. cerevisiae* was cultured in 2% glucose (w/v) with or without Plant Extract 21. The cells were then collected on Days 1, 2, 3, and 4 of their culturing. The mol% was calculated using the sum of molar amounts of lipids in all classes for each technical repeat. Each lipid class was analyzed in three different biological replicates and each with three technical replicates. The sum is then divided by the total lipid concentration of all lipids. The error bars were calculated by SEM (n= 9). The asterisks indicate the significance between the treated and the untreated *S. cerevisiae* using a T-test ($p \leq 0.05$).

3.5. The effects of Plant Extract 26 on lipids and free fatty acids

3.5.1 The effects of Plant Extract 26 on nine different lipids

Figure 17 shows the level of nine different lipid classes studied using mass spectrometry. Plant Extract 26 caused DAG, PC, and PI to increase in level. It also caused a decrease in the level of PE and PG. There was no observed change in level in TAG, PA, PS, and Cer, when they are compared to the untreated *S. cerevisiae*. When looking at the four-day trend of the lipids in the untreated *S. cerevisiae* it is seen that levels decrease in PC, PA, and Cer. The four-day trend in the untreated *S. cerevisiae* is seen to increase in level for lipids PE and PS. TAG, DAG, and PG is seen to have no change in level over the four days in the untreated *S. cerevisiae*. No trend can be observed over the four days for PI since there is not enough PI available to see the trend. When looking at the four-day trend of the lipid level of the treated S. cerevisiae it is seen that there is a decrease for PE and PI. All the other lipids have no change in lipid level over the four days when S. cerevisiae is treated with Plant Extract 26.

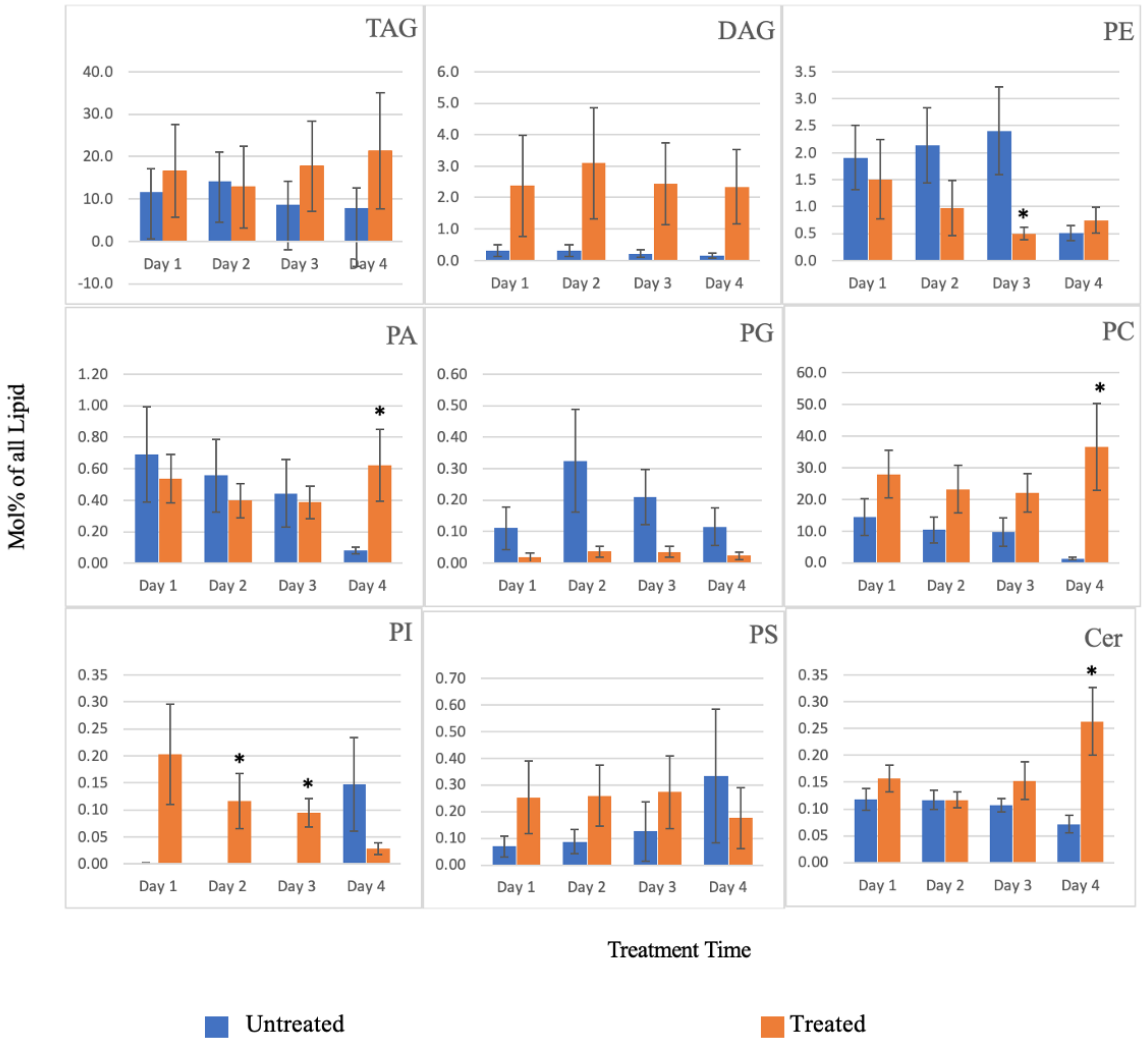

Figure 17. Average mol% of different lipid classes obtained from *S. cerevisiae* cells grown with or without Plant Extract 26. A wild-type strain of *S. cerevisiae* was cultured in 2% glucose (w/v) with or without Plant Extract 26. The cells were then collected on Days 1, 2, 3, and 4 of their culturing. The mol% was calculated using the sum of molar amounts of lipids in all classes for each technical repeat. Each lipid class was analyzed in three different biological replicates and each with three technical replicates. The sum is then divided by the total lipid concentration of all lipids. The error bars were calculated by SEM (n= 9). The asterisks indicate the significance between the treated and the untreated *S. cerevisiae* using a T-test ($p \leq 0.05$).

3.5.2 The effects of Plant Extract 26 on four free fatty acids

Figure 18 shows four free fatty acids studied in *S. cerevisiae* treated with Plant Extract 26 using a mass spectrometer. Palmitic acid, palmitoleic acid, and stearic acid were all seen to decrease in level when treated with Plant Extract 26. Unlike, oleic acid which had no change in level between the treated and untreated *S. cerevisiae*. When looking at the four-day trend in level in the untreated *S. cerevisiae* it is seen that oleic acid and stearic acid are increasing, whereas palmitoleic acid is decreasing and palmitic acid remains the same. When looking at the treated *S. cerevisiae* it is seen that the level of free fatty acids over the four days remains the same in all the four free fatty acids.

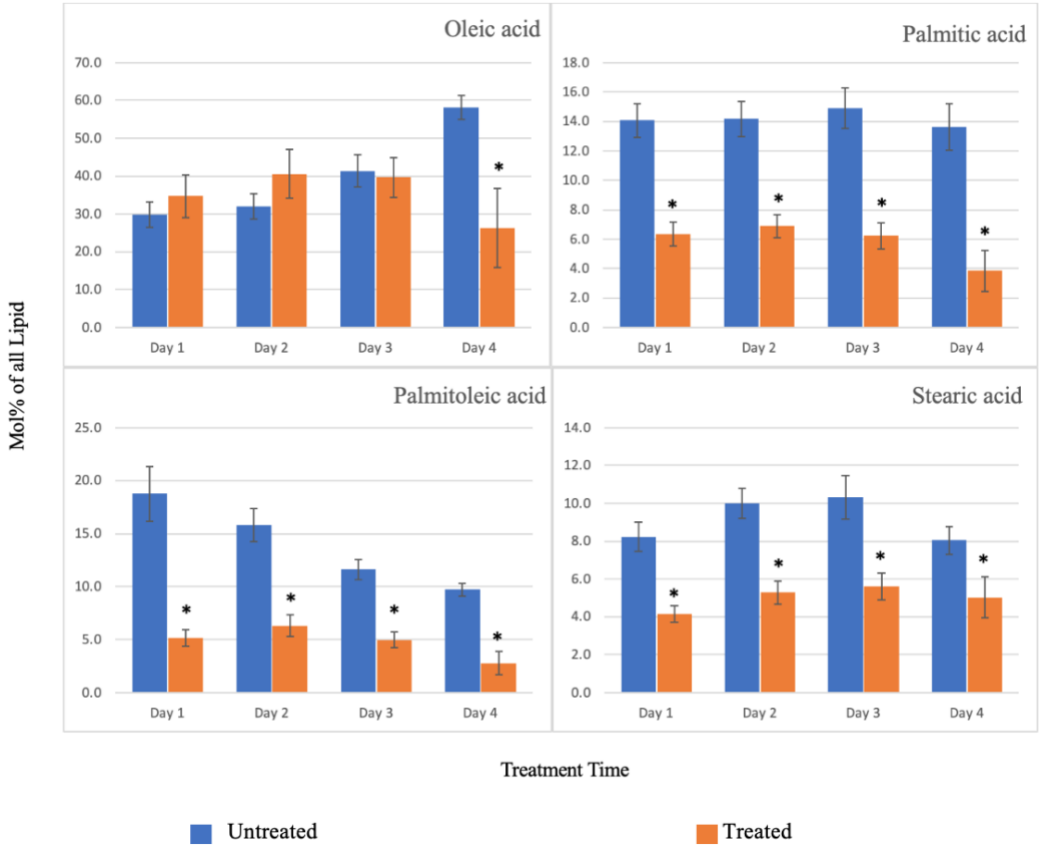

Figure 18. Average mol% of different free fatty acids obtained from *S. cerevisiae* cells grown with or without Plant Extract 26. A wild-type strain of *S. cerevisiae* was cultured in 2% glucose (w/v) with or without Plant Extract 26. The cells were then collected on Days 1, 2, 3, and 4 of their culturing. The mol% was calculated using the sum of molar amounts of lipids in all classes for each technical repeat. Each lipid class was analyzed in three different biological replicates and each with three technical replicates. The sum is then divided by the total lipid concentration of all lipids. The error bars were calculated by SEM (n= 9). The asterisks indicate the significance between the treated and the untreated *S. cerevisiae* using a T-test ($p \leq 0.05$).

3.6. The effects of Plant Extract 39 on lipids and free fatty acids

3.6.1 The effects of Plant Extract 39 on nine different lipids

Figure 19 shows the level of nine different lipid classes studied using mass spectrometry. In *S. cerevisiae* treated with Plant Extract 39 there was an increase in the levels of PC and Cer. This plant extract caused a decrease in TAG, PE and PA, and did not alter the levels of DAG, PG, PI, and PS. When looking at the four-day trend in the level of lipids in the untreated *S. cerevisiae* it is seen that the levels decrease for DAG, PE, PC, and PS. No change in level is seen in TAG, PA, PG, PI, and Cer, over the four days in the untreated *S. cerevisiae*. When looking at the treated *S. cerevisiae* it is seen that over the four days the level of all the lipid classes remains the same.

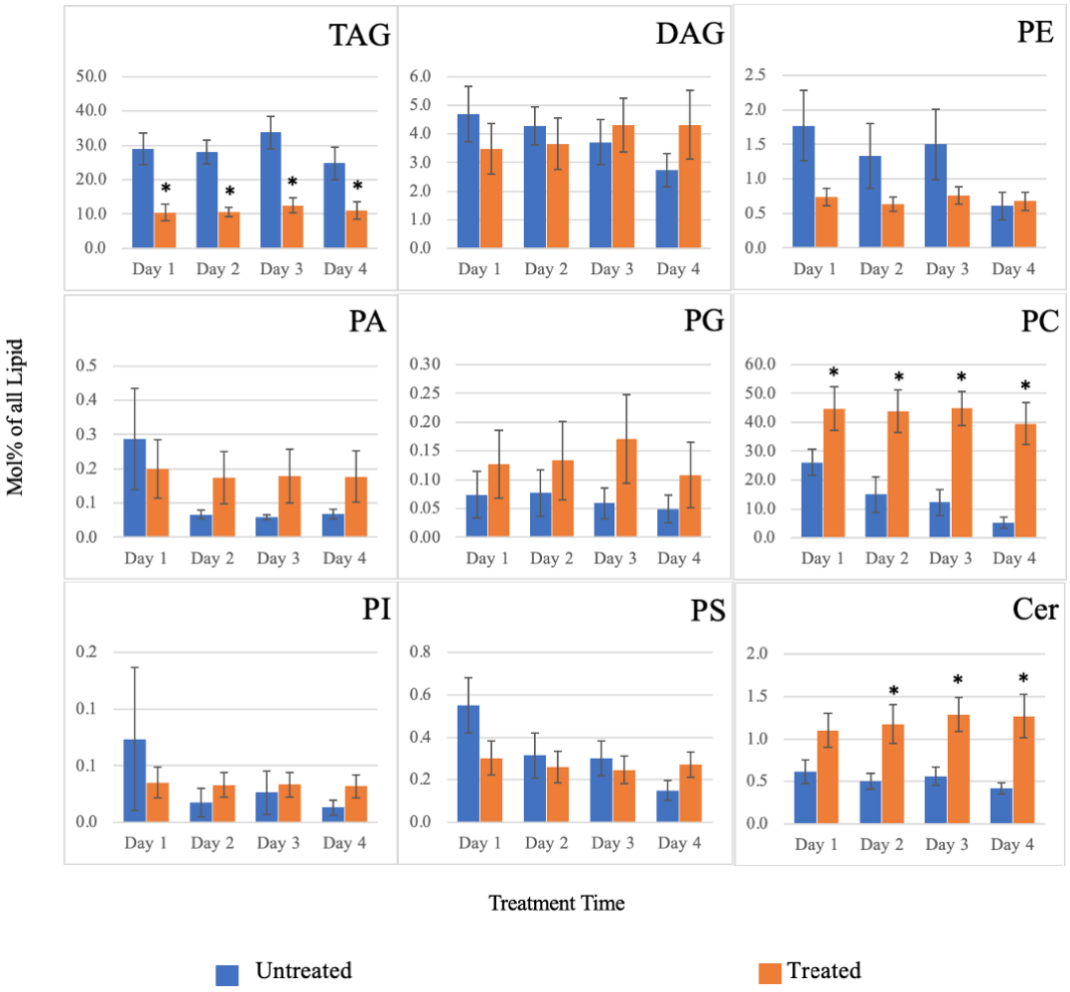

Figure 19. Average mol% of different lipid classes obtained from *S. cerevisiae* cells grown with or without Plant Extract 39. A wild-type strain of *S. cerevisiae* was cultured in 2% glucose (w/v) with or without Plant Extract 39. The cells were then collected on Days 1, 2, 3, and 4 of their culturing. The mol% was calculated using the sum of molar amounts of lipids in all classes for each technical repeat. Each lipid class was analyzed in three different biological replicates and each with three technical replicates. The sum is then divided by the total lipid concentration of all lipids. The error bars were calculated by SEM (n= 9). The asterisks indicate the significance between the treated and the untreated *S. cerevisiae* using a T-test ($p \leq 0.05$).

3.6.2 The effects of Plant Extract 39 on four free fatty acids

Figure 20 shows four free fatty acids studied in *S. cerevisiae* treated with Plant Extract 39 using a mass spectrometer. Three out of four free fatty acids in *S. cerevisiae* were not altered when treated with Plant Extract 39. Palmitoleic acid level was seen to decrease in the presence of Plant Extract 39. Even though the level was not altered a trend was observed over the four days in the free fatty acids. When looking at the untreated *S. cerevisiae* an increase in level is seen over the four days in oleic acid, palmitic acid, and stearic acid. In palmitoleic acid the untreated *S. cerevisiae* is seen to decease in level over the four days. When looking at the treated *S. cerevisiae* the level over the four days is seen to remain the same in all the four free fatty acids.

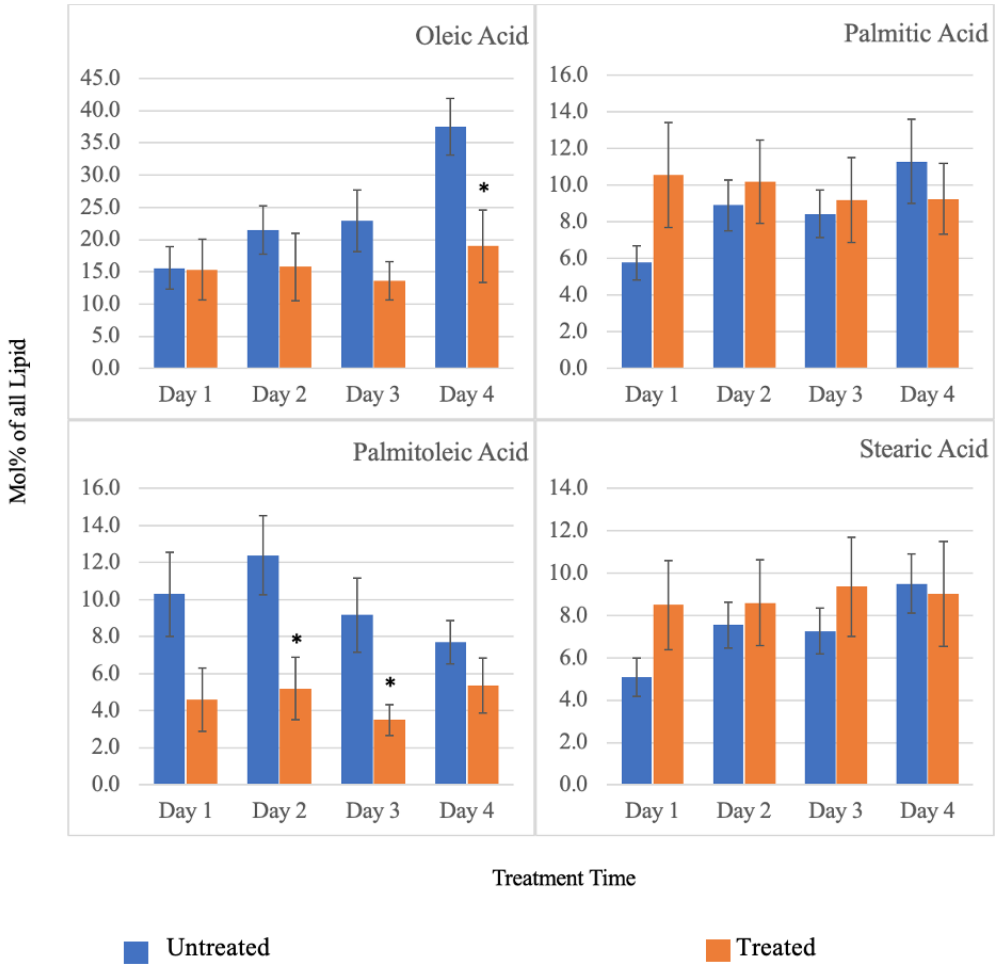

Figure 20. Average mol% of different free fatty acids obtained from *S. cerevisiae* cells grown with or without Plant Extract 39. A wild-type strain of *S. cerevisiae* was cultured in 2% glucose (w/v) with or without Plant Extract 39. The cells were then collected on Days 1, 2, 3, and 4 of their culturing. The mol% was calculated using the sum of molar amounts of lipids in all classes for each technical repeat. Each lipid class was analyzed in three different biological replicates and each with three technical replicates. The sum is then divided by the total lipid concentration of all lipids. The error bars were calculated by SEM (n= 9). The asterisks indicate the significance between the treated and the untreated *S. cerevisiae* using a T-test ($p \leq 0.05$).

Chapter 4 Discussion

My data show that all six plant extracts alter the lipidome of *S. cerevisiae* in different ways. It should be noted that there were large data variations in both treated and non-treated cells. A possible explanation for these fluctuations would be the change in colony for each biological repeat. During each of the biological repeats, a different colony was taken and grown in a rich media and then used for the different technical repeats. My data also show great fluctuations in the error bars depending on the lipids. A potential reason for the change in the size of the error bars would be that some of these lipid classes are less present than other lipids. For example, PI has a very low mol% compared to TAG, which is more abundant. PI values are more variable, as they are near the detection limit mass spectrometry quantifications.

Figure 21 summarizes the effect of Plant Extract 4 treatment on the cellular lipid profile. When *S. cerevisiae* was treated with Plant Extract 4, the free fatty acid and TAG decreased compared to the control cells. The reduction in the free fatty acid could be due to the production of Cer from free fatty acids, the inhibition of the free fatty acid production from DAG, or a combination of these two processes. On the other hand, Plant Extract 4 treatment causes increased levels of Cer, DAG, PC and PI. An increase in DAG may be due to more TAG changing into DAG or due to the inhibition of the enzyme responsible for the transformation of DAG into the free fatty acids. An increase in PC could be caused by its conversion from PE, which decreased over the four days. PS also decreased over the four days, and thus, this may further indicate the conversion of PC from PS and PE. An increase in PI level was observed and could be facilitated by increased production from PA, which showed a decreasing trend over the four days.

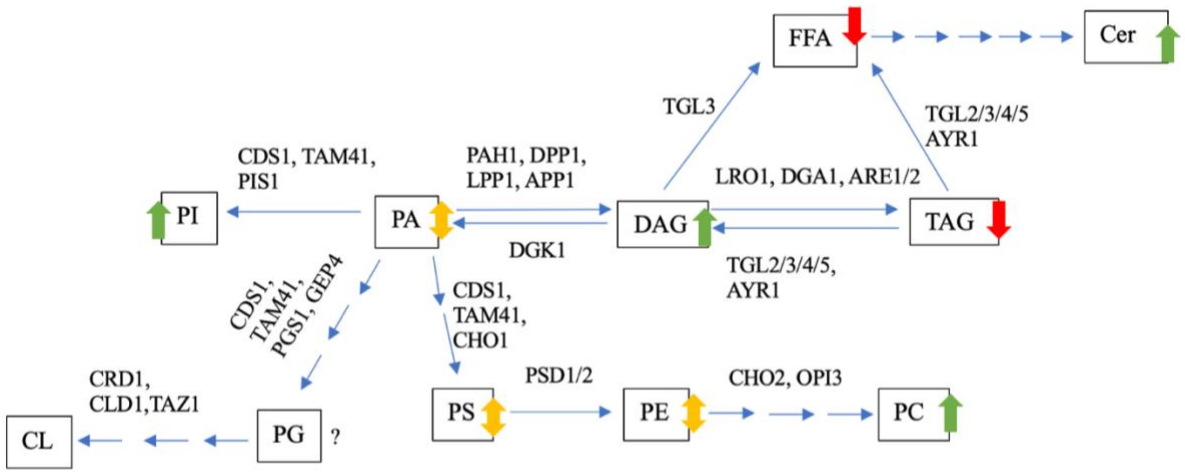

Figure 21. Changes in lipids when *S. cerevisiae* is treated with Plant Extract 4. This figure shows the change of lipids in the *S. cerevisiae* treated with Plant Extract 4 compared to the lipids in the *S. cerevisiae* that are untreated. The green and red arrows indicate an increase and decrease, respectively. The yellow arrow represents no change between the treated and untreated cells. The question mark means that it is impossible to determine the effect.

Figure 22 shows the effect of Plant Extract 6 on the cellular lipid profile. Plant Extract 6 treated cells showed an increase in TAG and DAG, and a decrease in the free fatty acids. These changes may be due to the inhibition of the production of free fatty acids from TAG and DAG which leads to an accumulation of TAG and DAG. Other lipids showed similar levels as the untreated *S. cerevisiae*. PA, PC, and PE decreased over the four days in both treated and non-treated cells (Figure 9). Thus, DAG does not become PA over the four days. Cer also fluctuates during the four days. Lastly, Plant Extract 6 caused a reduction in free fatty acids, suggesting that it promotes the breakdown of free fatty acids.

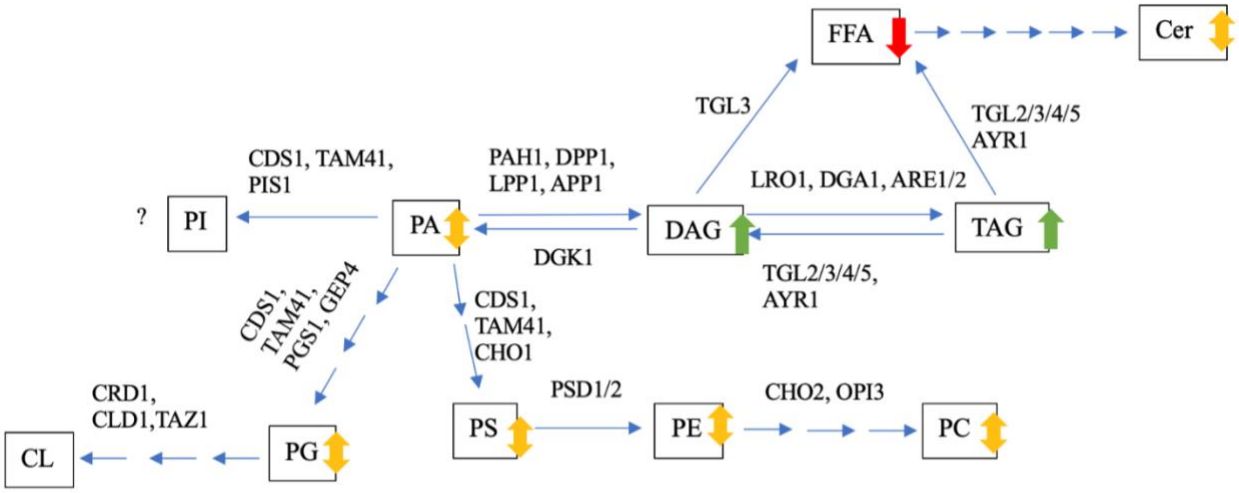

Figure 22. Changes in lipids when *S. cerevisiae* is treated with Plant Extract 6. This figure shows the change of lipids in the *S. cerevisiae* treated with Plant Extract 6 compared to the lipids in the *S. cerevisiae* that are untreated. The green and red arrows indicate an increase and decrease, respectively. The yellow arrow represents no change between the treated and untreated cells. The question mark means that it is impossible to determine the effect.

The Plant Extract 12 treatment caused an increase in TAG, which could be due to the decrease in free fatty acids or in DAG. PA, PG, PS, PE, and PC decreased when *S. cerevisiae* was treated with Plant Extract 12. Cer is the only lipid that is seen to follow the same trend as the untreated cells. Plant Extract 12 also greatly decreases the level of PS over the four days, which could be due to the conversion of PE and then PC.

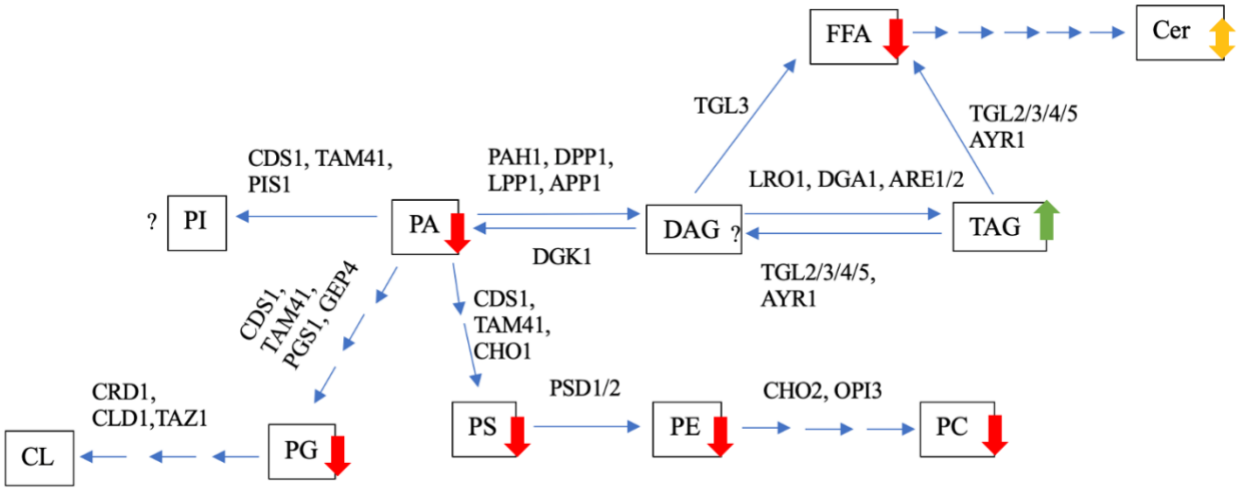

Figure 23. Changes in lipids when *S. cerevisiae* is treated with Plant Extract 12. This figure shows the change of lipids in the *S. cerevisiae* treated with Plant Extract 12 compared to the lipids in the *S. cerevisiae* that are untreated. The green and red arrows indicate an increase and decrease, respectively. The yellow arrow represents no change between the treated and untreated cells. The question mark means that it is impossible to determine the effect.

Figure 24 shows that the exposure of Plant Extract 21 causes an increase in Cer and free fatty acids. DAG and TAG decrease over the four days of treatment, which may be depleted due to production of other lipids such as Cer, PE and PG. PA is also decreasing over the four days, which may be used for the PE and PG production. Although the level of PE in the treated *S. cerevisiae* is increased compared to the untreated *S. cerevisiae*, a decreasing trend is seen over the four days in both the treated and untreated. A decrease in PA, PS, PE, and PC over the four days suggests that there must be something unknown after this pathway that depletes these lipids. Plant Extract 21 was seen to enhance the level of PG most likely by using PA which was seen to decrease over the four days. Lastly, Plant Extract 21 decreased the level of palmitic acid when treated with

plant extract, whereas Plant Extract 21 did not cause any detectable changes in oleic acid, palmitoleic acid, or stearic acid.

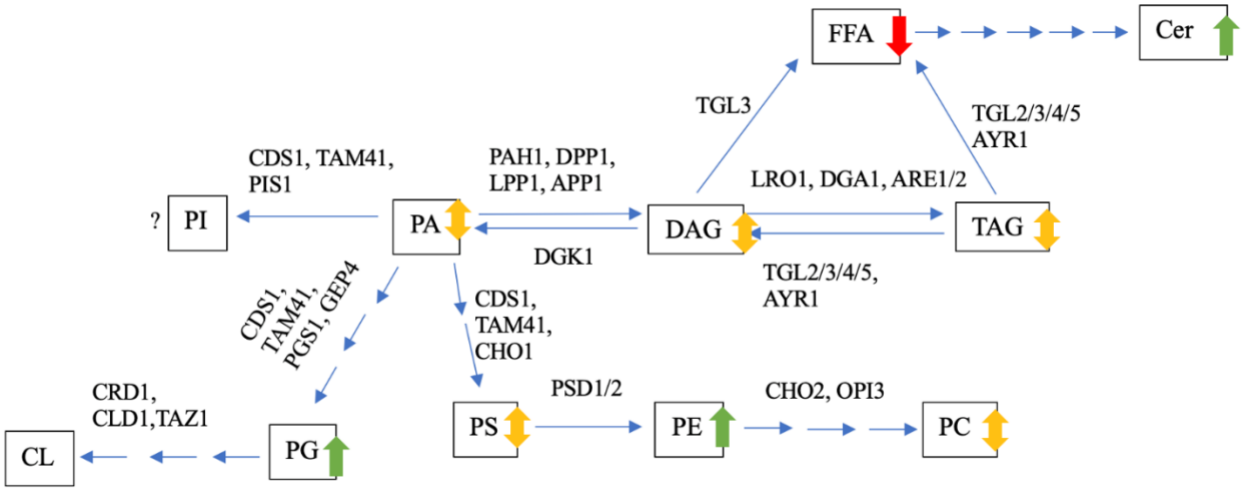

Figure 24. Changes in lipids when *S. cerevisiae* is treated with Plant Extract 21. This figure shows the change of lipids in the *S. cerevisiae* treated with Plant Extract 21 compared to the lipids in the *S. cerevisiae* that are untreated. The green and red arrows indicate an increase and decrease, respectively. The yellow arrow represents no change between the treated and untreated cells. The question mark means that it is impossible to determine the effect.

Figure 25 shows that Plant Extract 26 enhances the level of DAG and PI. The increased level of DAG could be due to the decreased level of free fatty acids which was observed as the enzyme used to produce the free fatty acids from DAG may be inhibited. My results show that Plant Extract 26 promotes the conversion of DAG from PA since an increase of DAG is observed, and a gradual decrease of PA over the four days is also seen. The results obtained also reveal a decrease in PA, PS, PE, and PC over the four days, which shows that there must be something unknown after this pathway that depletes these lipids. Plant Extract 26 enhances the level of PI by PA. The data also suggest that Plant Extract 26 promotes the depletion of certain free fatty

acids, as seen with the decrease of palmitic acid, palmitoleic acid, and stearic acid when *S. cerevisiae* was treated with the plant extract.

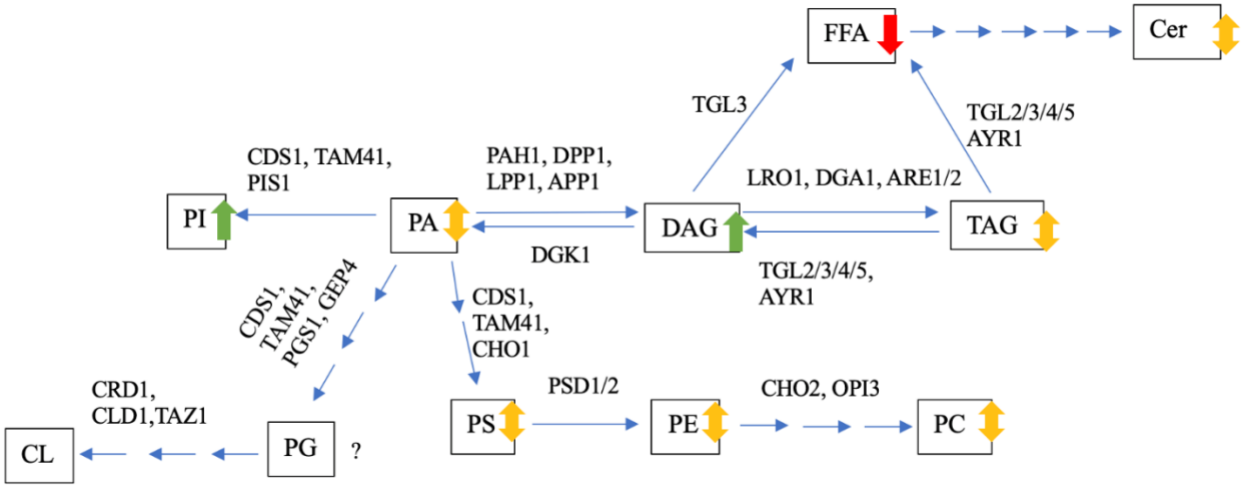

Figure 25. Changes in lipids when *S. cerevisiae* is treated with Plant Extract 26. This figure shows the change of lipids in the *S. cerevisiae* treated with Plant Extract 26 compared to the lipids in the *S. cerevisiae* that are untreated. The green and red arrows indicate an increase and decrease, respectively. The yellow arrow represents no change between the treated and untreated cells. The question mark means that it is impossible to determine the effect.

Figure 26 shows that Plant Extract 39 enhances the level of Cer and PC and decreases the level of free fatty acids and TAG. These results reveal that the increase in Cer may be caused by TAG changing into free fatty acids and the free fatty acids changing into Cer. The increase in PC is not clear since all the lipids preceding PC remain stable over the four days. Although the decrease in TAG may also be responsible for the increase in PC, for this to happen TAG would need to be changed into DAG, PA, PS, PE and finally PC. This would be a lot of changes. There may be an easier explanation for the increase in PC that is not seen in this diagram. Plant Extract

39 decreases the level of oleic acid and palmitoleic acid and does not alter the level of palmitic acid and stearic acid.

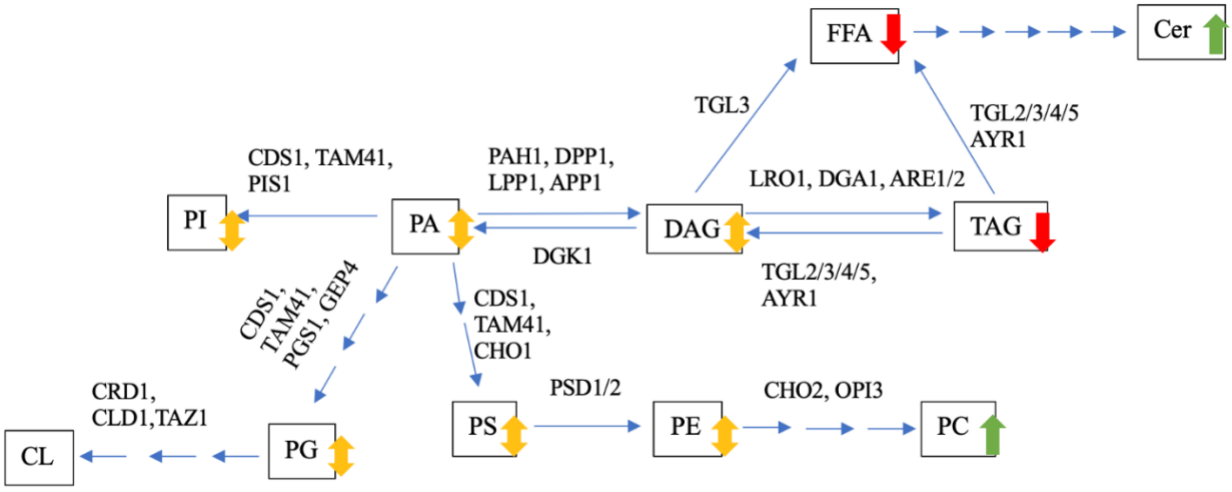

Figure 26. Changes in lipids when *S. cerevisiae* is treated with Plant Extract 39. This figure shows the change of lipids in the *S. cerevisiae* treated with Plant Extract 39 compared to the lipids in the *S. cerevisiae* that are untreated. The green and red arrows indicate an increase and decrease, respectively. The yellow arrow represents no change between the treated and untreated cells. The question mark means that it is impossible to determine the effect.

Table 7 shows the summary of all the lipid classes and the free fatty acids for every plant extract and previous research on caloric restriction and LCA. When analyzing my data, I required at least two bars in a graph to be significant for said lipid to be declared as increased or decreased, as seen in Table 7. This table shows that all these plant extracts work on their own; they do not affect the lipidome the same way. All six plant extracts had different lipids increasing and decreasing, although all the plant extracts decreased the level of free fatty acids. A decrease in free fatty acids was also seen in caloric restriction and LCA. Thus, these free fatty acids could be critical for anti-aging pathways.

A previous study by the Titorenko laboratory found that the aging-delaying effect of Plant Extract 21 is reduced in mutants showing defects in PA formation from free fatty acids *faa1*, *faa4*, *ale1*, and *slc1* [97]. Thus, these results signify that these genes are required for the Plant Extract 21 pathway. On the other hand, the aging-delaying effect of Plant Extract 21 is enhanced in mutants showing defects in free fatty acid formation.[97]. Thus, these results signify that *tgl1,3,4,5*, counteracts the anti-aging pathway by Plant Extract 21. A decrease in free fatty acids appears to be associated with an increase in longevity. For genes *faa1*, *faa4*, *ale1*, and *slc1*, this decrease in free fatty acids is seen when they are not mutated and exposed to Plant Extract 21 (caused the greatest longevity) since the free fatty acids are incorporated into PA [97], see Figure 7. In genes *tgl1,3,4,5,* this decrease of free fatty acids is seen when these genes are mutated and exposed to Plant Extract 21 which results in the greatest viability observed [77,97], see Figure 8. This genetic study further proves the importance of free fatty acids in anti-aging pathways. This thesis study has shown that all six anti-aging plant extracts caused a reduction in free fatty acids. This finding is consistent with other studies that reported the involvement of free fatty acids in necrosis and lipoapoptosis [99]. Free fatty acids have also been found to be needed in pathways that make reactive oxygen species (ROS) [99]. Excess ROS are harmful to the cell and are known to cause aging [99].

There are discrepancies between the data obtained in this study and a previous lipidome study using Plant Extract 21 [97]. A possible cause for these differences is that the colonies tested were different, and this could have affected the levels of the lipids.

	TAG	DAG	PE	PA	PG	PC	PI	PS	Cer	Palmitic Acid	Palmitoleic Acid	Stearic Acid	Oleic Acid
PE 4	↓	↑	↕	↕	-	↑	↑	↕	↑	↕	↓	↕	↕
PE 6	↑	↑	↕	↕	↕	↕	-	↕	↕	↓	↓	↓	↓
PE 12	↑	-	↓	↓	↓	↓	-	↓	↕	↓	↓	↓	↓
PE 21	↕	↕	↑	↕	↑	↕	-	↕	↑	↓	↕	↕	↕
PE 26	↕	↑	↕	↕	-	↕	↑	↕	↕	↓	↓	↓	↕
PE 39	↓	↕	↕	↕	↕	↑	↕	↕	↑	↕	↓	↕	↓
CR	↓	-	↑	↑	↑	↑	↑	↑	↑		↓		
LCA	↓	-	↕	↕	↑	↕	↕	↕	-		↓		

Table 7. Summary of effect of six plant extracts on *S. cerevisiae* lipid profiles. The green arrow represents an increase, the red arrow represents a decrease, and the yellow arrow represents no change between the treated and untreated S. *cerevisiae* cells. The hyphen means that it is impossible to determine the trends. The lipid profile data for caloric restriction (CR) and LCA are obtained from prior studies in Titorenko laboratory [79]. LCA is a geroprotective chemical that has been shown to increase longevity in *S. cerevisiae* [94].

My data shows that six plant extracts cause changes in the lipidome of *S. cerevisiae*, see Table 7, which supports the importance of lipid metabolism in aging. The data in Table 7 also shows that each plant extract causes unique changes in lipid metabolism. Yet, all six aging-delaying plant extracts caused a decrease in free fatty acids. A decrease in free fatty acids has also been reported for two other aging-delaying treatments, caloric restriction and LCA [79, 94]. Further research is still needed to better understand plant extracts and how the changes in the lipidome affect aging.

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