THE ANTIAGING ACTIVITY OF *Polyalthia longifolia* (Sonn.) Thwaites METHANOLIC EXTRACTS ON *Saccharomyces cerevisiae* BY611 YEAST CELLS

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by

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I take full responsibility for any remaining errors or shortcomings in this work.

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DCF	2′,7′-dichlorodihydrofluorescein diacetate
8-oxodG	8-hydroxy-deoxyguanosine
8-OHG	8-hydroxyguanosine
8-oxoG	8-oxo-7,8-dihydro guanosine
ATP	Adenosine triphosphate
AD	Alzheimer's disease
ALS	Amyotrophic lateral sclerosis
ANOVA	Analysis of variance
BSA	Bovine Serum Albumin
BS	Bud scars
B	Budding
CVD	Cardiovascular diseases
CAT	catalase
CM	Cell membrane
CW	Cell wall
CLS	Chronological lifespan
CER	Circular Endoplasmic Reticulum
DNA	Deoxyribonucleic acid
DEG	Differential Expression Genes
DMSO	Dimethyl Sulfoxide
DAD	Diode-Array Detection
ER	Endoplasmic reticulum

EtBr	ethidium bromide
FC	fold change
FDA	Food and Drug Administration
FOXO	Forkhead box
FRTA	Free radical theory of aging
GO	Gene Ontology
GSSG	Glutathione disulfide
GPx	glutathione peroxidase
HMDS	Hexamethyldisilane
HPLC	High-performance liquid chromatography
H_2O_2	Hydrogen peroxide
OH^-	Hydroxyl radical
IIS	Insulin/IGF-1 signalling
KEGG	Kyoto Encyclopedia of Genes and Genomes
LM	light microscope
$LOO^\bullet$	lipid peroxyl radical
mRNA	Messenger RNA
M	Mitochondria
NGS	Next-Generation Sequencing
N	Nucleus
OD	optical density
OsO4	Osmium tetroxide
GSSH	Oxidized glutathione
PD	Parkinson’s disease
P	Peroxisome

PBS	phosphate buffer solution
P. longifolia	*Polyalthia longifolia*
PLME	*Polyalthia longifolia* leaf methanolic extract
PCR	Polymerase chain reaction
PQC	Protein quality control
QC	Quality Control
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RSS	Reactive sulfur species
GSH	Reduced glutathione
RI	Regional invaginations
RLS	Replicative lifespan
Rpm	Revolutions per minute
RNA	Ribonucleic acid
R	Ribosome
RIN	RNA integrity number
RNA-seq	RNA sequencing
S. cerevisiae	*Saccharomyces cerevisiae*
SGD	Saccharomyces Genome Database
SEM	Scanning electron microscopy
SIR2	silent information regulator 2
SD	standard deviation
ST	stationary
O_2^-	Superoxide
SOD	superoxide dismutase

SR	Surface roughness
SD	synthetic dextrose
TERT	telomerase reverse transcriptase
TLS	total lifespan
TPM	Transcripts per Million
TEM	Transmission Electron Microscopy
T2DM	Type 2 diabetes patients
V	Vacuoles
v/v	Volume by volume
w/w	weight by weight
W	Wrinkled
YPD	yeast extract peptone dextrose

AKTIVITI ANTIPENUAAN OLEH EKSTRAK METHANOL *Polyalthia longifolia* (Sonn.) Thwaits KE ATAS SEL YIS *Saccharomyces cerevisiae* BY611

ABSTRAK

Usaha mencari agen anti-penuaan berasaskan bahan semulajadi telah mendapat momentum sejak kebelakangan ini. *Polyalthia longifolia*, tumbuhan perubatan tempatan yang berharga, banyak digunakan dalam perubatan tradisional disebabkan kandungan polifenolnya yang tinggi. Kajian ini menilai potensi anti-penuaan ekstrak metanol daun *P. longifolia* (PLME) menggunakan *Saccharomyces cerevisiae* strain BY611 sebagai model organisme. Dalam kajian ini, pendekatan yang terintegrasi, merangkumi ujian "*in vitro*'', "*in situ*'' dan teknik penjujukan RNA digunakan untuk meyiasat sifat anti-penuaan PLME. Ekstrak methanol daun *P. longifolia* telah disediakan, dan kuantifikasi rutin menghasilkan kandungan rutin sebanyak 0.013 µg/mL (1.30%) dalam 1000 µg PLME. Kepekatan optimum 1 mg/mL PLME telah ditetapkan untuk mencapai kesan anti-penuaan maksimal. Rawatan PLME didapati berkesan secara ketaranya ($p < 0.05$) dalam melanjutkan kedua-dua jangka hayat replikatif dan kronolog sel yis, serta memanjangkan jangka hayat keseluruhan sambil meningkatkan potensi proliferatif sel berbanding dengan kumpulan yang tidak dirawat. Seperti yang disokong hasil peninjauan mikroskop cahaya, PLME juga meningkatkan ketahanan hidup dalam sel yis BY611 dengan mengurangkan perkembangan vakuol besar (Jenis 3) seumpama apoptosis. Dapatan ini diperkukuh menerusi kajian mikroskop elektron imbasan (SEM) dan transmisi (TEM) yang menunjukkan penundaan manifestasi morfologi penuaan yang khas dalam sel yis yang dirawat dengan PLME. Manakala, sel yis BY611 yang tidak dirawat menunjukkan perubahan morfologi penuaan awal yang jelas, termasuk

pembengkakan vakuol dan mitokondrion, permukaan sel berkedut, kemunculan retikulum endoplasma sirkular (CER), pemecahan membran nuclear, fragmentasi nukleus, dan kerosakan sitoplasma yang meluas. Sementara itu, penyelidikan ini mengesahkan peranan aktiviti antioksida PLME dalam aktiviti anti-penuaannya. Ini dibuktikan oleh peningkatan rintangan tekanan oksidatif, pengurangan produksi spesies oksigen reaktif (ROS), dan peningkatan aktiviti pengurangan glutation (GSH) dalam sel yang dirawat dengan PLME. Analisa penjujukan RNA mendedahkan pembezaan ekpresi yang signifikan bagi gen berkaitan penuaan iaitu, *Sirtuin 2* (*SIR2*), *Superoxide dismutase* 1 (*SOD1*), dan *Superoxide dismutase* 2 (*SOD2*), dalam sel yang dirawat dengan PLME. Cerapan dari analisis Gene Ontology dan laluan KEGG menawarkan perspektif baru ke dalam peranan penting gen *SIR2* dan *SOD* dalam konteks kesan anti-penuaan PLME menerusi pengukuhan mekanisme pertahanan antioksidan yis, pengawalan metabolisma dan penyelenggaraan kestabilan genom. Eksperimen pengesahan menggunakan RT-qPCR dan ujian aktiviti enzim mengesahkan impak pengawalseliaan PLME terhadap gen *SIR2*, *SOD1,* dan *SOD2* dan menyebabkan peningkatan aktiviti enzim yang mejelaskan asas molekul kesan anti-penuaan PLME. Temuan kolektif ini memberikan bukti kukuh untuk kesan anti-penuaan PLME dalan sel yis BY611 melalui modulasi gen *SIR2*, *SOD1* dan *SOD2*, dengan demikian, mendedahkan potensi PLME sebagai agen anti-penuaan baru untuk menggalakkan penuaan yang sihat.

THE ANTIAGING ACTIVITY OF *Polyalthia longifolia* (Sonn.) Thwaites METHANOLIC EXTRACTS ON *Saccharomyces cerevisiae* BY611 YEAST CELLS

ABSTRACT

The pursuit of natural-based antiaging agents has gained momentum in recent years. *Polyalthia longifolia*, a valuable indigenous medicinal plant, is widely used in traditional medicines due to its high polyphenol concentration. This study assessed the anti-aging potential of *P. longifolia* leaf methanolic extracts (PLME) using *Saccharomyces cerevisiae* strain BY611 as a model organism. In this research, an integrated approach, encompassing *in vitro*, *in situ* assays and RNA sequencing techniques was employed to investigate the anti-aging properties of PLME. The methanolic extract was prepared, and rutin quantification yielded 0.013 µg/mL (1.30%) rutin content in 1000 µg of PLME. Furthermore, an optimal concentration of 1 mg/mL PLME was determined for achieving maximum anti-aging effects. Intriguingly, PLME treatment significantly ($p < 0.05$) extended both replicative and chronological lifespans and total lifespan while enhancing cellular proliferative potential compared to the vehicle control group. Corroborated by light microscopy, PLME also promoted longevity in BY611 yeast cells by mitigating the progression of large apoptotic-like Type 3 vacuoles. This was reinforced by scanning and transmission electron microscope findings, demonstrating delayed manifestation of typical aging morphologies in PLME-treated cells. Meanwhile, vehicle control BY611 yeast cells exhibited distinct early aging morphological alterations, including swollen vacuoles and mitochondria, wrinkled yeast cell surfaces, circular endoplasmic reticulum (CER) emergence, ruptured nuclear membrane, nucleus

fragmentation, and extensive cytoplasmic damage. Besides, this research confirmed the robust antioxidant function of PLME, substantiating its role in potential antiaging activity. This was evidenced by enhanced oxidative stress resistance, reduced reactive oxygen species (ROS) production, and heightened activity of reduced glutathione (GSH) in PLME-treated cells. RNA sequencing analysis unveiled significant differential expression of aging-related genes—Sirtuin 2 (*SIR2*), Superoxide dismutase 1 (*SOD1*), and Superoxide dismutase 2 (*SOD2*), in response to PLME treatment. Insights from Gene Ontology and KEGG pathway analysis offered a novel perspective into the significant impact of *SIR2* and *SOD* genes in the context of PLME-induced anti-aging effects by reinforcement of yeast's antioxidant defence mechanisms, metabolism regulation and genome stability maintenance. Validation experiments using RT-qPCR and enzymatic activity assays confirmed PLME's regulatory impact on *SIR2*, *SOD1*, and *SOD2* genes, leading to increased enzyme activities elucidating the molecular basis of PLME's antiaging effects. These collective findings provided compelling evidence for the anti-aging effects of PLME in BY611 yeast cells through the modulation of *SIR2* and *SOD* genes, thus, revealing its promising potential as a novel antiaging agent for promoting healthy aging.

CHAPTER 1

INTRODUCTION

1.1 Overview and Rationale of the Study

Aging is a predestined process that everyone undergoes in their phase of life at their own time and pace. Broadly, aging reflects the changes occurring over the journey of life, much beyond human control, usually resulting from the impact of the accumulation of various cellular and molecular damages over time. This complex and multifactorial process proceeds in a gradual and declining manner (Amarya *et al*., 2018). Though the idea of aging might be exciting to the young, the aging process is subjected to undesirable changes in physiology during old age like the development of wrinkles, weight gain, and greying of hair. More importantly, aging is linked to a variety of physiological changes that impede normal biological functions, making an individual more vulnerable to external stressors and increasing their risk of chronic diseases and death (Sangeetha *et al*., 2020).

According to the World Health Organization (WHO), the rate of global aging has increased considerably and is faster than in the past. People could expect to live a longer life beyond their sixties for the first time in history, with the majority of this surge resulting from better nutrition, livelihood, hygiene, and health care. Roughly 900 million people worldwide are 60 years and above, and these numbers are estimated to spike to 21.5% (approximately 2 billion) of the global population in 2050 (Liu *et al*., 2019). Malaysia currently has nearly 7% of the population (estimated 3.5 million individuals) above the age of 65, and these numbers are expected to quadruple to 14% by 2044 and 20% by 2056. If this current trend continues, Malaysia will be considered a “super-aged” nation (Wei, 2021). Unfortunately, the reality of living longer has increased the number of elderly

patients suffering from debilitating chronic diseases, usually with multiple co-morbidities. Despite the excitement about the prospect of living longer, this must be accompanied by an increase in healthy years rather than more years of impairment and disease (McAuley *et al*., 2017).

The irreversible loss of physiological function that occurs as people age is followed by an increase in the prevalence of chronic diseases, such as diabetes, heart disease, neurological or renal disease, and cancer, all of which have aged as strong risk factors (Niccoli and Partridge, 2012). Indeed, around 90% of people over 65 years of age have at least one chronic disease and more than 70% have two or more. Furthermore, the increase in the occurrence of several age-related diseases accounts for deaths in over 70% of Americans aged 65 years and above (Si and Liu, 2014). These disorders also necessitate long-term treatment, implying a link between a longer lifespan the emergence of chronic diseases and a higher social and economic burden (Hahad *et al*., 2021). According to a study of the Global Burden of Disease in 2017 (GBD, 2017), 92 out of 293 (31.4%) diseases were age-related diseases which include neurodegenerative disease, cancer, cardiovascular and metabolic diseases (Lublóy, 2020). Age-related illnesses as a whole are a considerable economic and healthcare challenge on a global scale. With that, the paramount importance is to improve the understanding of the underlying molecular mechanisms of aging. This is to facilitate the development of strategies for preventing or delaying the onset of these chronic diseases to satisfy the expanding healthcare needs of aging human populations while also promoting healthy aging.

The most popular and well-accepted theory is the free radical theory proposed by Denham Harman, which posits that aging affects the body's ability to cope with oxidative stress that occurs throughout the lifespan (Harman, 1956). This theory

proposes that collective oxidative stress produced by highly reactive oxygen free radicals causes oxidative damage to the cellular macromolecules (lipid membranes, proteins, nucleic acids) and cell death. The structural and functional damage in macromolecules will accumulate over time, inducing progressive cell senescence and organ failure, which will conveniently lead to aging and age-related diseases (Sangeetha *et al*., 2020). Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are produced from a variety of external sources in addition to within mitochondria during oxidative phosphorylation. Typically, the complex antioxidant defence system in the human body neutralizes the excess ROS/RNS to prevent the damaging effects on biomolecules. However, a decline in defence mechanisms' ability over time makes it increasingly tough to eliminate the cumulative damage due to ROS. Hence, this prompts oxidative stress conditions where free radicals' production overwhelms the antioxidant defence mechanism to regulate them, thus accelerating the aging process (Fusco *et al*., 2007). ROS at high quantities in the cell can damage macromolecules such as nucleic acids (DNA/RNA), proteins, lipids, membranes, and organelles (Salisbury and Bronas, 2015).

Defects induced by ROS, commonly during *in vitro* and *in vivo* oxidative stress could challenge normal cellular function, leading to uncontrolled cell proliferation or cell death. Indeed, emerging evidence strongly suggests that the inability of cells to clear oxidatively damaged RNA contributes to deleterious long-lasting effects on cells and organs, which are strongly implicated in developing age-related disorders (Simms and Zaher, 2016). Numerous studies have suggested that oxidized DNA and RNA are related to the pathogenesis of a range of diseases, such as Alzheimer's disease, Parkinson's disease, multiple system atrophies, dementia with Lewy bodies, myopathies, and atherosclerosis (Fimognari, 2015). The alarming

rate of aging populations over the age of 60 years around the world and the strong involvement of chronic diseases associated with the aging process have switched the focus of research towards aging biology and effective interventions that could slow the progression of aging or prevent age-related diseases. The antiaging molecular mechanisms, however, are still poorly understood (Tungmunnithum *et al*., 2022). Understanding the mechanism underlying the cellular aging process that occurs in humans could therefore provide significant insights that motivate the discovery of better anti-aging agents to target aging pathologies (Nunomura *et al*., 2012). Undoubtedly, the ultimate goal is to extend the healthy lifespan of individuals, thus improving their overall quality of life and promoting healthy aging. To date, no Food and Drug Administration (FDA)-approved drugs on the market are specifically designed to delay the aging process (Kennedy and Pennypacker, 2014; Rolland *et al*., 2023). Consequently, there is a considerable demand for research focused on identifying medicinal plants or active compounds that could enhance the quality of life, extend lifespan, and serve as a protective measure against age-related disorders. These studies aim to discover natural remedies that can contribute to a healthier and longer lifespan.

Among the approaches to combat cellular aging, the use of antioxidant agents is studied as one of the promising means to minimise the detrimental effect of ROS (Sarima *et al*., 2019). For thousands of years, traditional medicine systems have used plant-based products as a vital component in preserving human health. The problem of pharmaceutical product safety in the modern medical system has sparked a worldwide interest in medicinal plants, which have inspired leading pharmaceutical medications. In many model systems, studies have demonstrated that natural antioxidants may play a role in preventing aging and age-related illnesses by

scavenging free radicals created during aging (Mendes *et al*., 2015). Plant-derived antioxidants in particular polyphenols have been of immense interest which could delay the aging process as they are capable of scavenging ROS in cells thus defending the human body from the oxidation process and enabling them to react as an anti-aging agent (Tungmunnithum *et al*., 2020). Hence, delaying the aging processes and age-related diseases in humans by exploiting medicinal plants for the production of anti-aging agents has emerged as a novel and attractive approach (Sangeetha *et al*., 2020).

Polyalthia longifolia is one of the most important indigenous medicinal plants that is found throughout Malaysia where it is widely used traditionally as a febrifuge and tonic (Jothy *et al*., 2016). Fundamentally, the selected plant should be rich in antioxidants to minimize free radical generation, and a savour for macromolecules like DNA, should be able to enhance internal defence mechanisms and also be a good immune rejuvenator. Previous studies have indicated *P. longifolia* as a good anticancer agent, radioprotective agent, genoprotective agent, *in vitro* and *in vivo* antioxidant agent, and non-toxic towards animals which are all good attributes for an anti-aging agent (Jothy *et al*., 2012; Jothy *et al*., 2013a; Vijayarathna *et al*., 2017; Braganza and Sasidharan, 2023). Based on this rationale, *P. longifolia* can be employed as a remedy to delay aging progression and offer potential preventive or curative effects against aging-related diseases by scavenging free radicals, which largely contribute to the onset or development of the disease. Nevertheless, the antiaging effects of *P. longifolia* leaf extracts have not been determined.

Hence current research was designed to fill the important research gap by studying the antiaging activity of the polyphenolic-rich *P. longifolia* leaf methanolic extract (PLME) in the BY611 strain, *Saccharomyces cerevisiae*. This study aims to

validate the potential of *P. longifolia* as a novel natural antiaging agent. Since studying the aging process in mammalian model organisms has proven to be difficult, yeast has emerged as a popular and effective aging model. This is owing to its short lifespan, ease of handling, and significant conservation of essential cellular pathways with higher organisms (Fabrizio and Longo, 2003a; Lasserre *et al*., 2015; Liu *et al*., 2017). On that basis, BY611 yeast strains were cultured in the presence of PLME to assess potential effects on replicative and chronological lifespan as well as on aging-related extracellular and intracellular morphological features. Additionally, this study aims to investigate how PLME mitigates oxidative stress and ROS, thereby exerting its preventive and curative antiaging effects. Finally, the research endeavours to unravel the mode of action governing the anti-aging action of PLME in *S. cerevisiae* BY611 yeast cells, with a specific focus on the influence of PLME on the key aging-related genes, namely silent information regulator 2 (*SIR2*) and superoxide dismutase (*SOD1* and *SOD2*) genes.

Given the booming global aging population, it is more crucial to develop interventions that protect health in old age and postpone the onset of age-related diseases (Magalhães *et al*., 2017).

1.2 Research Objectives

The general objective of this study was as follows

- To explore the antiaging properties of *Polyalthia longifolia* leaf methanolic extracts (PLME) in *Saccharomyces cerevisiae* yeast strain, BY611.

The current study was undertaken with the following main objectives:

1. To determine the yeast replicative and chronological life span of vehicle control and *P. longifolia* leaf methanolic extract-treated *S. cerevisiae* BY611 yeast cells.
2. To evaluate the *in situ* morphological changes in vehicle control and *P. longifolia* leaf methanolic extract-treated *S. cerevisiae* BY611 yeast cells via microscopy techniques.
3. To establish the involvement of the antioxidative action in inhibiting cellular reactive oxygen species (ROS) and sensitivity to oxidative stress in *P. longifolia* leaf methanolic extract-treated and vehicle control *S. cerevisiae* BY611 yeast cells.
4. To examine the influence of *P. longifolia* leaf methanolic extract on the activation of key aging-related genes, silent information regulator 2 (*SIR2*) and superoxide dismutase (*SOD1* and *SOD2*) genes in *S. cerevisiae* BY611 yeast cells.

1.3 Workflow

The operational framework of this study is summarized as shown in Figure 1.1

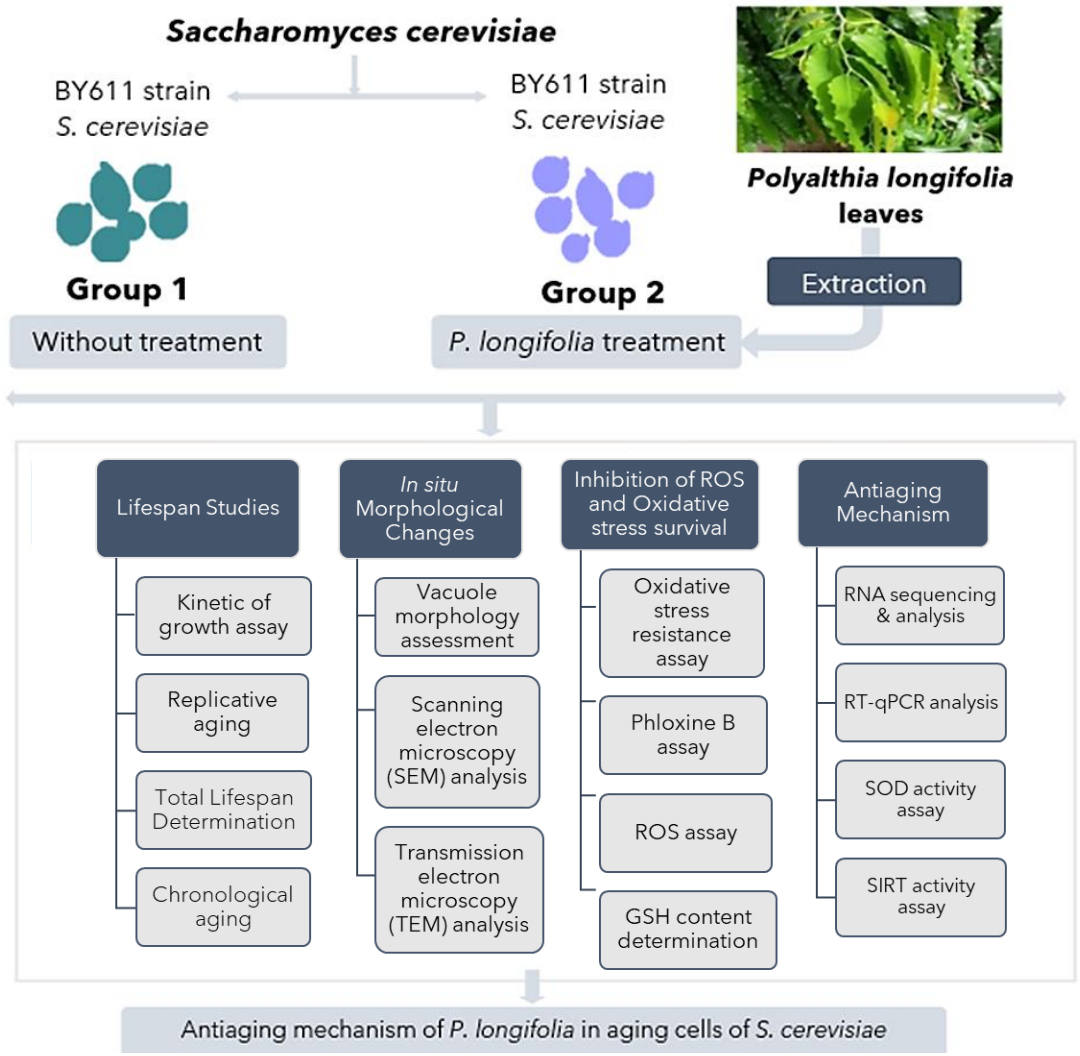

Figure 1.1: Workflow of the overall research

CHAPTER 2

LITERATURE REVIEW

2.1 Aging

2.1.1 Definition and Process of Aging

The definition of aging is very subjective as people's perspectives differ and the aging concept is defined based on what best fits their knowledge. Ideally, there are two ways to define aging that are both considered comparable. The first defines the aging process as an age-related deterioration of biological functions (Kirkwood and Austad, 2000) while the second way describes aging as an age-related increase in mortality (Lenart *et al*., 2018). Both defining ways are related, the only difference is that the mortality rate is a measurable parameter, whereas a precise definition is required for measurements of the decline of biological functions (Libertini, 2019).

Likewise, most evolutionary biologists classify aging as an age-reliant decline in intrinsic physiological function, that would eventually lead to an increase and decrease in age-specific mortality and reproductive rate respectively (Promislow and Bronikowski, 2006; Flatt and Schmidt, 2009; Bronikowski and Flatt, 2010; Fabian and Flatt, 2011). Ultimately, aging is commonly defined as the accumulation of progressive deterioration of practically every bodily function over time, resulting in an increased vulnerability to environmental challenges and a growing risk of chronic diseases that precedes death (Harman, 2003; Kirkwood, 2005).

The aging process has been traditionally seen as a result of multiple random mechanisms affecting cellular and molecular pathways (Kirkwood, 2011). Briefly, biological systems shield themselves against the constant disorder of randomness that preserves molecular fidelity. The physiological functional loss with age mirrors gradual loss in the ability to defend the chance. Additionally, the aging process is

recognized as heterogeneous, characterized by varying rates of aging among different organisms and asynchronous aging of various cells and tissues within a single organism (Carmona and Michan, 2016). Research from the past three decades portrays aging as a flexible process significantly influenced by factors such as genetics, epigenetics, diet, physical activity, and chance (McAuley *et al*., 2017).

The process of aging begins from birth and progresses gradually throughout the life cycle, entailing maturation and changes. Middle age is the point in time when many bodily functions start to steadily decline with noticeable age-related changes (Amarya *et al*., 2018). The dynamic process of aging encompasses biological, physiological, psychological, and social changes (Tully and Pawelec, 2021). The biological systems or physiological age, suggest aging as the stochastic changes in the body that usually occur as people age due to escalating loss of molecular and cellular fidelity. These losses in fidelity eventually surpass repair capacity and increase susceptibility to aging-related diseases (Hayflick, 2004). Conversely, social and psychological level changes refer to subjective age equivalent changes. Psychological aging involves the way people feel and act comprising the decline in mental functioning and personality. Meanwhile, social aging is the changes in a person's social roles and relationships (Charles and Carstensen, 2010).

Aging processes encompass two distinct categories either normal or pathological. Natural aging, an inevitable progression, entails changes like wrinkled skin, greying hair, modest physical decline, decreased stress tolerance, and heightened susceptibility to illnesses. In contrast, pathological aging stems from external factors like environmental shifts, genetic alterations, and unforeseen occurrences such as diseases (Ho *et al*., 2010).

2.1.2 Theories of Aging

Various theories attempt to explain aging, but none suffice individually due to their overlapping explanations within the complexity of the process (Weinert and Timiras, 2003). Whilst all theories possess some evidence most have equally strong flaws. Over time many theories have fallen out of favour while few broad theories have become more widely accepted. As aging is a complex process with complicated mechanisms, the initial theory of aging includes evolutionary, molecular, cellular, and system theories (Mehdi *et al*., 2021). These theories imply that a better understanding of homeostatic mechanisms and individual maintenance pathways can lead to a longer life (Bengtson *et al*., 2016).

The evolutionary theory includes mutation accumulation, disposable soma, and antagonistic pleiotropy and indicates aging as the consequence of a decline in the influence of natural selection (Tosato *et al*., 2007). The molecular theory discusses gene regulation, codon restriction, error catastrophe, and somatic mutation, while neuroendocrine and immunologic were highlighted under system theories. Finally, the cellular level theories include the telomere theory, free radical theory of aging, wear-and-tear theory, and apoptosis theory of aging (Weinert and Timiras, 2003). Interestingly, among all theories, the free radical theory of aging emerged as the most popular with the widest acceptance as a credible explanation of the primary chemical reactions at the basis of the aging process (Sangeetha *et al*., 2020).

2.1.2(a) Free Radical Theory of Aging

The free radical theory of aging (FRTA) is a structural damage-based theory proposed by Denham (Harman, 1956). FRTA has been termed an oxidative stress theory due to the contribution of oxygen species like peroxides and aldehyde in the

process of oxidative damage to cells (Pérez-Hernández *et al*., 2016). This theory suggests that age-related functional losses are from permanently damaged macromolecular cell components accumulated due to the prolonged and inevitable attack of free radicals. Free radicals are highly reactive, unstable, and unpaired molecules that attack macromolecules (nucleic acids, proteins, and lipids). The nucleic acid, in particular, picks up an additional base or sugar group, breaks into the single and double-stranded form in the backbone, and cross-links to other molecules during free radical attacks (Jin, 2010). The rise in accumulated cellular damage triggered by unrestrained oxidative stress will inevitably extend outwards towards tissues and organs, which may express itself as a degenerative disease that conveniently leads to aging (Harman, 1956). Therefore, leading to the assumption of cellular aging concerning oxidative stress. Hence, the deciding factor in determining lifespan would be the degree of oxidative damage to the cells and an organism's coping power.

Harman later revised this theory, centring more on the role of reactive oxygen species (ROS) generated by mitochondria during metabolism (Harman, 2009). ROS formation originates endogenously during the metabolism process and is genetically faulty and from exogenous environment sources. Hence, the oxidation of biomolecules by ROS precedes diseases that may be considered as varied forms of expression of the aging process (Harman, 2006). The free radical theory has subsequently inspired more research than other theories in aging. Findings validate Harman’s speculation that free radical damage is a major causative factor in the aging process and perhaps many other age-related diseases like cancer, neurodegenerative, arthritis, and atherosclerosis diseases (Clancy and Birdsall,

2013). Following the theory's acceptance, current research has switched focus towards exogenous antioxidants' role in restoring the redox balance.

2.1.3 Hallmarks of Aging

The cellular and molecular markers for aging must first be clarified to illuminate the mechanisms and effects of anti-aging treatments on aging-related disorders. Through geroprotective intervention experiments on many various types of model organisms, nine hallmarks that contribute to the process of aging have been well-defined, with indications that they are also conserved in humans (López-Otín *et al*., 2013). These hallmarks were characterized based on their fulfilment of precise aging-related criteria for an occurrence during normal aging, accelerated aging when experimentally induced and attenuation delays aging, resulting in increased lifespan. The interplay between the molecular homeostasis that leads to the cellular hallmarks of aging is considered the core underlying machinery of how our bodies age and contributes to frailty and diseases (Carmona and Michan, 2016).

The nine cellular and molecular hallmarks of aging are categorized into primary hallmarks, antagonistic hallmarks, and integrative hallmarks groups (López-Otín *et al*., 2013). Moreover, most of these hallmarks of aging are likely to be causative to accumulating cellular damage in their involvement in cascading degenerative pathways and being the most common associated phenomena with aging. Genomic instability, telomere attrition, epigenetic alterations, and loss of proteostasis are all included in the primary hallmarks. Antagonistic hallmarks include deregulated nutrient sensing, cellular senescence, and mitochondrial dysfunction contrarily and portray opposite impacts depending on their intensity: valuable at low levels but deleterious at higher levels. Finally, altered intracellular

communication and stem cell exhaustion are the integrative hallmarks that directly influence tissue homeostasis and function (van der Rijt *et al*., 2020). These hallmarks are usually in a networked fashion instead of being just independent of one another.

2.1.3(a) Genomic Instability

As we age, the stability and integrity of chromosomes are constantly challenged by exogenous and endogenous threats with the deteriorating capability of repair mechanisms (Hoeijimakers, 2009). This initiates the accumulation of mutations with age and genomic instability that disrupts cell homeostasis, leading towards accelerated aging and related diseases (Niedernhofer *et al*., 2018).

2.1.3(b) Telomere Attrition

Telomeres, repetitive sequences of DNA protect the ends of linear chromosomes. When telomere approaches critical length during cell division, cells sense it and the replication machinery is permanently turned off, entering a state of senescence (López-Otín *et al*., 2013). In aging cells, telomerase that prevents telomere shortening and restores telomere length appears silenced and rapid telomere shortening occurs. This subsequently results in diminished cell proliferation and cellular senescence. Hence, this indicates signs of premature aging, linking telomere attrition to aging (Li *et al*., 2021).

2.1.3(c) Epigenetic Alteration

As cells are constantly exposed to environmental stressors, genomes are susceptible to changes through epigenetic mechanisms (López-Otín *et al*., 2013). Epigenetic changes connected to cell aging include changes in DNA methylation, histone modifications, and chromatin remodelling (Kane and Sinclair, 2019). Over time,

epigenetic change accumulates in the cell and correlates with the deterioration observed in aging cells with compromised specific coordination of gene activity (Li *et al*., 2021). Unlike genomic mutations, epigenetic modifications are reversible, thus correcting epigenetic alterations is a treatment target for delaying cell aging (Freije and López-Otín, 2012).

2.1.3(d) Loss of Proteostasis

Intracellular protein homeostasis or proteostasis entails specialized molecular systems to repair and refold irrecoverably damaged proteins by degrading and replacing them (Powers *et al*., 2009). During aging, thermal, oxidative, and osmotic stressors disturb proteostasis mechanisms, resulting in protein instability, failure of autophagic processes, and accumulation of toxic misfold proteins all of which accelerate aging (Basisty *et al*., 2018).

2.1.3(e) Deregulation of Nutrient Sensing

As metabolism and its by-products damage cells through oxidative stress and mitochondrial dysfunction, organisms depend on several nutrient-sensing pathways to ensure only adequate nutrition is taken up (van der Rijt *et al*., 2020). Nevertheless, metabolic events add stress to cells by deregulating the nutrient-detecting molecules and downstream pathways. Therefore, with high metabolic activity and changes in nutrient availability, cells are prone to accelerated aging (Li *et al*., 2021).

2.1.3(f) Cellular Senescence

Cellular senescence is the irreversible cell cycle arrest where cells enter a permanent non-dividing state due to damages (Hernandez-Segura *et al*., 2018) including the generation of several molecules together known as the senescence-associated

secretory phenotype (SASP). The SASP is involved in the spread of senescence to neighbouring cells, inflammation, and tissue malfunction (Guerville *et al*., 2020). The apoptosis and immune system usually clear young senescent cells, but these cells persist and secrete damaging molecules in older tissues. Hence, aging is thought to occur due to the large accumulation of senescent cells in tissue (Hernandez-Segura *et al*., 2017).

2.1.3(g) Mitochondrial Dysfunction

Mitochondria lose their integrity as the cell ages, triggering a build-up of oxidative stress with the rise in ROS production and reduced adenosine triphosphate (ATP) levels hence compromising mitochondrial function (Harman, 1956). Ultimately, mitochondria dysfunction prompts various events for instance increased apoptosis induction and triggers inflammation, which correlates with aging and age-related diseases (Li *et al*., 2021).

2.1.3(h) Reduction of Somatic Stem Cell Activity

A prominent attribute of aging is the deterioration in tissues and organs' homeostatic and regenerative potential. The regenerative capability lies in healthy stem cells, the ultimate supplier of new cells (López-Otín *et al*., 2013). Although stem cells have qualities such as a rapid turnover rate and a specialized niche that protects them from aging insults, data suggests that there is a deterioration in stem cell functionality but not depletion in stem cells (Liu and Rando, 2011). With advancing age, stem cell fitness at all levels, particularly activation, repairing and replication ability gradually drops due to functional attrition in several cell compartments. Since the resident stem cells are responsible for the tissues' homeostatic and regenerative functions, these age-related changes reflect a decline in stem cell activity. Hence, the loss of stem cell

functionality influences an organism's health and viability. Aside from a lack of stem cell proliferation capability, excessive proliferation of stem and progenitor cells can also be detrimental by accelerating stem cell exhaustion (López-Otín *et al*., 2013). Unlike the amazing regenerative power found in lower animals, mammals, and especially humans have only limited potential to rejuvenate their injured tissues such as skin and bone marrow (Ho *et al*., 2005). With age, this regenerative potential, and other stem cell activities decline, lowering organ function and delaying tissue repair, contributing to age-related diseases that speed up the aging process (Goodell and Rando, 2015). Reduction in stem cell activity is considered an integrative consequence of the primary and antagonistic hallmarks and is most likely one of the primary culprits of tissue and organismal aging.

2.1.3(i) Altered Intercellular Communication

Apart from cell-autonomous changes, an essential factor in maintaining good health involves appropriate intracellular communication, be it neuroendocrine, endocrine, or neuronal (López-Otín *et al*., 2013). Aging is linked to alterations in cell communication, which are mostly caused by inflammaging, a chronic low-grade systemic inflammation in aged people. Similar to stem cell exhaustion, the age-reliant changes in intracellular communication are cohesive impacts of various hallmarks of aging. Cells exhibit an increase in self-preserving signals as they age, obstructing intracellular communication and resulting in damage elsewhere, for example, inflammaging (Ferrucci and Fabbri, 2018).

2.1.4 Aging-Related Diseases

Aging is associated with increased susceptibility to a range of diseases, posing significant threats to health and quality of life among older individuals (Liu *et al*.,

2019). As life expectancy rises, so does the prevalence of age-related chronic conditions, leading to a rise in morbidity and mortality rates. Among the most common age-related diseases worldwide are cancer, neurodegenerative disorders, cardiovascular diseases (CVD), and metabolic disorders (Li *et al*., 2021).

Aging is associated with the development of neurodegenerative disorders as the brain's ability to transmit signals and communicate during aging significantly declines (Amarya *et al*., 2018). Besides, oxidative stress is closely related to neurodegenerative diseases as the central nervous system is vulnerable to oxidants due to the presence of high lipid content and consumption of oxygen with low levels of antioxidant enzymes resulting in brain aging (Phaniendra *et al*., 2015). Neurodegenerative diseases associated with aging include Alzheimer's (Pan *et al*., 2011), Parkinson's (Sevcsik *et al*., 2011), multiple sclerosis (Witherick *et al*., 2011), and cognitive dysfunction.

Pilleron et al. (2021) reported that in 2018, an estimated 2.3 million cancer cases were diagnosed among aged people worldwide. Generally, the most common cancer sites diagnosed in older women were breast, lung, and colon cancer whereas prostate, lung, and colon cancers were prevalent in older males. In this current projectile, in 2050, we could expect over 6.9 million cancer cases in aged people globally with the leading cause of death (Pilleron *et al*., 2021). Epigenetic modification is a crucial link between aging and cancer. The increased risk of cancer during aging can be justified by Harman's (1993)'s theory that with advancing age and deficient antioxidant defences, there would be elevated levels of endogenous free radical reactions. This will consequently result in an increased rate of mutation in tumour-suppressing genes that suppress cell proliferation and proto-oncogenes that are involved in normal cell growth and development. Cancer is more prone in

aging people due to the increased rate of mutation with the gradual declining capacity of the immune system to eliminate transformed cells (Aunan *et al*., 2017).

Aging populations have a significant vulnerability to degenerative heart and blood vessels which are pathologies of CVD (Hahad *et al*., 2021). By 2030, CVD is predicted to result in 40% of deaths in elderly people globally, stressing it as a prominent cause of death in elderly people (Heidenreich *et al*., 2011). A steady deterioration in physiological processes at the molecular, cellular, and tissue stages is characterised as cardiovascular aging (cardiac and vascular aging). Aging cardiovascular tissues exhibit key hallmarks like endothelial dysfunction attended with increased arterial rigidity, pulse wave velocity, systolic blood pressure, and central venous pressure (Lakatta and Levy, 2003; Jani and Rajkumar, 2006). These aspects render aged vessels more prone to atherosclerosis and arteriosclerotic cardiovascular diseases such as coronary heart disease, hypertension, stroke, cognitive dysfunction, and peripheral vascular disease (Xu *et al*., 2021).

The shift in age distribution towards aged people and gradual aging over the last decades has been paralleled by a global epidemic of chronic metabolic disorders (Spinelli *et al*., 2020). The high prevalence of metabolic diseases such as diabetes and obesity are strongly linked with oxidative stress, inflammation, and decreased activity of antioxidant systems that precede aging (Guarner and Rubio-Ruiz, 2015). The malfunction of adipose tissue is a significant risk factor for aging, as it produces system-wide metabolic alterations such as the build-up of ectopic lipids, insulin resistance and chronic inflammation. Consequently, these adverse effects are responsible for the elevated risk of diabetes and obesity onset correlated to aging. Furthermore, chronic metabolic disorders may also embody the state of accelerated aging as these diseases have integrated physiological characteristics in the aging

process, for example, heightened cellular senescence and epigenetic alteration (Spinelli *et al*., 2020).

2.2 Free Radicals and Reactive Oxygen Species (ROS)

Free radicals are atoms, molecules, or ions that are produced during normal cellular metabolism consisting of one or more unpaired electrons in their valency shell. Free radicals are likely to participate in chemical reactions and draw off electrons from other molecules due to their odd electrons which are typically unstable, short-lived, and highly reactive. While this process provides free radicals stability, the attacked molecule in contrast develops into a free radical itself as it loses an electron of its own hence initiating a destructive chain reaction cascade that ultimately harms healthy cells (Phaniendra *et al*., 2015; Valko *et al*., 2006). However, despite regaining electrons from a balanced molecule, free radicals do not recover their original form and function, implying that damage is permanent (Halliwell *et al*., 1992). Free radicals are made up of the three most common elements, oxygen, nitrogen, and sulfur which become reactive oxygen species (ROS), reactive nitrogen species (RNS), and reactive sulfur species (RSS) (Carocho and Ferreira, 2013). ROS and RNS are free radicals or pro-oxidant groups with biological importance in the human body. Emphasis is given to ROS as they are largely produced during normal metabolic or physiological processes by cytosolic enzymes and membrane-bound ones (Sohal and Weindruch, 1996).

ROS are ubiquitous molecules produced as a product of singlet oxygen which reacts randomly by attacking electrons from other molecules. The most common ROS include superoxide (O_2^-), hydroxyl radical (OH^-), and hydrogen peroxide (H_2O_2) (Działo *et al*., 2016). The stimuli that activate the generation of ROS species

are either endogenous or exogenous sources. Endogenous sources for ROS production include various cellular organelles that have high oxygen consumption such as mitochondria via xanthine oxidase, peroxisomes, microsomes, and endoplasmic reticulum. Other endogenous sources involve inflammation processes, phagocytosis, arachidonate pathways, ischemia, mental stress, and extreme physical exercise, which play a crucial role in immune response and cell signalling (Phaniendra *et al*., 2015; Yan and Zaher, 2019). Apart from endogenous sources, ROS are also generated through different exogenous sources for instance X-rays and gamma-rays, UV radiation, and toxic compounds from air pollution, tobacco, and xenobiotics namely drugs, environmental agents, and natural compounds (Cadet and Wagner, 2013).

ROS species are known to be a two-edged sword as both beneficial and toxic compounds in the human body. At appropriate levels, ROS holds a beneficial role such as for inflammation, autophagy, stress response maintenance of the redox state, and signal transduction (Schieber and Chandel, 2014). Conversely, at higher concentrations, ROS become toxic and disrupt the antioxidant defence system of the body to regulate them, causing an imbalance between the generation and detoxification of ROS which may lead to oxidative stress (Pham-Huy *et al*., 2008). This is in relevance with the free radical theory that postulates a gradual mitochondrial dysfunction due to oxidative stress with aging to increased ROS production, which in turn results in oxidative damage to biomolecules (Harman, 1956; Srivastava, 2017). The exact mechanism behind the complex aging process remains widely ambiguous due to several cofounders. The onset and progression of aging have been largely associated with the delicate balance between many endogenous and exogenously produced ROS and their scavenging by an efficient

antioxidative system comprised of nonenzymatic along with enzymatic antioxidants. Although ROS and ROS-induced oxidative damage may not be the sole cause of the aging process, there is broad consensus that ROS plays a vital role in the molecular mechanisms that affect longevity. Hence, bridging the gap between the free radical theory and present aging knowledge could help us better understand how the interaction between ROS-induced oxidative damage and cellular metabolism affects aging and identify genetic and pharmaceutical therapies that might attenuate this interaction (Santos *et al*., 2018).

2.2.1 Oxidative Stress and Aging

Oxidative stress is often defined as the physiological condition where the damaging effect of free radicals occurs due to the overrun of ROS generation that overwhelms the cell's endogenous antioxidant defence system to regulate them bringing about potential biological damage (Liguori *et al*., 2018). In a healthy state of the organism, the regulation of ROS is well handled by broad monitory systems to ensure a redox balance state. Even so, the crucial balance can often be challenged by various endogenous and exogenous factors (Lushchak, 2014). This subsequently results in cumulative oxidative damage in macromolecules such as nucleic acids, proteins, and lipids that then leads to cell death (Scheibye-Knudson *et al*., 2015), and disrupts the health span of various major organ systems (Dai *et al*., 2014). Hence, a mechanism known as redox regulation is important in maintaining the critical balance between favourable and unfavourable effects of free radicals in an organism.

Oxidative stress has been proposed to play a crucial role in the process of aging, in fact, recently the free radical theory of aging has been modified and advanced into the "oxidative stress hypothesis" (Ghezzi *et al*., 2017). This modified

hypothesis highlights the involvement of antioxidant defence as an important determinant of the overall redox imbalance, apart from unrestricted ROS production (Tan *et al*., 2018). Moreover, both reactive oxygen species and oxidative stress have been associated with age-linked disorders such as neurological conditions (Alzheimer's, Parkinson's disease, muscular dystrophy), cancers, cardiovascular and metabolic diseases (diabetes and obesity) (Liu *et al*., 2017; Tan *et al*., 2015).

2.2.2 What Happens When Cells Cannot Handle Oxidative Stress?

Whether endogenous or exogenous sources, the production of ROS is unavoidable in living cells. During oxidative stress conditions, however, levels of ROS increase that if left unattended will result in oxidative modification of major cellular macromolecules such as nucleic acids (DNA and RNA), proteins, and lipids (Salisbury and Bronas, 2015) which are also biomarkers of oxidative stress.

2.2.2(a) Lipid Peroxidation

Due to its involvement in a variety of clinical diseases, lipid peroxidation, and lipid damage due to ROS is a vital metabolic process *in vivo*. This three-step process causes membrane function failure, for instance, reduced fluidity, and deactivation of membrane-bound enzymes and receptors (Engwa, 2018). In the initiation stage, ROS attacks lipids with carbon-carbon double bond(s), within cell membranes and abstracts hydrogen atoms to generate a carbon-centred lipid radical. Since cellular membranes are rich in unsaturated fatty acids, they are generally susceptible to oxidative damage, however, the most vulnerable lipid membrane to oxidation by ROS is the polyunsaturated fatty acid (PUFA), residues of phospholipids (Siems *et al*., 1995). The lipid radical with subsequently rearrange through a cyclization reaction to form endoperoxides, which ultimately produce malondialdehyde (MDA)

and 4-hydroxyl nonenal (4-HNA), the toxic end products of lipid peroxidation that cause damage to the DNA and proteins (Phaniendra *et al*., 2015). In the propagation step, the lipid radical reacts with molecular oxygen forming a lipid peroxyl radical (LOO•). The radicals are capable of further propagating down the peroxidation process by removing hydrogen atoms from other lipid molecules. The last step is chain termination occurs following the interaction of one LOO• with another radicals or antioxidants (Engwa, 2018).

2.2.2(b) Protein Oxidation

The expression status of each protein has a substantial impact on the longevity of the organism as well as the health of individual cells. Oxidative stress can also locally affect charged side chains or cleave the polypeptide backbone which leads to total unfolding, local conformational changes to protein, and directing for disposal (Hanson *et al*., 2000). Proteins are one of the primary targets of oxidative stress because of their cysteine and methionine build-ups that are commonly found at the catalytic and regulatory sites of proteins and enzymes (Stadtman and Levine, 2000). These sulfur-containing amino acids such as cysteine and methionine are naturally more vulnerable to oxidation by ROS. Therefore, upon oxidative stress, these residues are readily oxidized by ROS, causing protein-protein cross-links formation that disrupts the function and structure of a protein, enzyme activity, function of receptors, and transport proteins (Butterfield *et al*., 1998). When oxidized, the cysteine and methionine are converted to reversible disulphides and methionine sulphoxide that can only be switched back to native form by disulfide reductases and methionine sulfoxide reductases respectively. During such events, the protein quality control (PQC) mechanism present in the proteasome system will be activated to