ANTIAGING ACTIVITY OF POLYPHENOL RICH *CALOPHYLLUM INOPHYLLUM* FRUIT EXTRACT IN AGING MUTANTS OF *S. CEREVISIAE*

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LIST OF ABBREVIATIONS

μg/mL	Microgram per milliliter
ACD	Accidental Cell Death
ATP	Adenosine triphosphate
BCA	Bicinchoninic Acid
BOS	Basal Oxidative Stress
BSA	Bovine Serum Albumin
CIFE	Calophyllum inophyllum fruit extract
CFU	Colony Forming Unit
cm	Centimeter
cDNA	Complementary DNA
CLS	Chronological Life Span
ct	Cycle threshold
DNA	Deoxyribonucleic acid
DMSO	Dimethyl sulfoxide
ETC	Electron Transport Chain
ET	Electron Transfer
EBU-EA	Epstein Born Virus-early antigen
EC_{50}	Half maximal effective concentration
F-C	Follin Ciaocalteau
GAE	Gallic acid equivalent
g	Gram
HAT	Hydrogen Atom Transfer
HOS	High Intensity Oxidative stress
HIV	human immunodeficiency virus
HT-29	human colon adenocarcinoma
H_2O_2	Hydrogen peroxide
HOCl	Hypochlorous acid
HOBr	Hypobromous acid
HNO_2	Nitrous acid
IOS	Intermediate Oxidative stress

IC_{50}	half maximal inhibitory concentration
LOS	Low Intensity Oxidative stress
LD_{50}	median lethal dose
mg/mL	Milligram per milliliter
mM	Millimolar
MCF-7	Michigan Cancer Foundation-7
WHO	World Health organization
MOS	Mild Oxidative stress
mm	Millimeter
mg/kg	Milligrams per kilograms
mL	Milliliter
min	Minutes
mtDNA	Mithochondrial DNA
ng/mL	Nanograms per milliliter
nm	Nanometer
Na_2CO_3	Sodium carbonate
NO+	Nitrosyl cation
NO−	Nitrosyl anion
N_2O_3	Dinitrogen trioxide
N_2O_4),	dinitrogen tetraoxide
NAD	Nicotinamide adenine dinucleotide
NBT	Nobiletin
OD	Optical Density
OXPHOS	Oxidative Phosphorylation
O_3	Ozone
O_2	Singlet oxygen
ONOOH	Peroxynitrite
PUFA	Polyunsaturated fatty acids
PBS	Phosphate-buffered saline
ROS	Reactive Oxygen Species
RONS	Reactive Oxygen Nitrogen Species
RT-PCR	Real time PCR
RNA	Ribonucleic acid

RLS	Replicative life span
rpm	Revolutions per minute
RCD	Regulated cell death
SOD	Superoxide Dismutase
SIRT	Sirtuin
SOS	Severe Oxidative stress
SD	Synthethic Dextrose
sec	Seconds
TOS	Temperate Oxidative stress
TPC	Total phenolic content
USM	Universiti Sains Malaysia
U/mg	Enzyme units per milligram
YPD	Yeast peptone dextrose

AKTIVITI ANTI-PENUAAN MUTAN *S. CEREVISIAE* DENGAN EKSTRAK BUAH *CALOPHYLLUM INOPHYLLUM* KAYA DENGAN POLYPHENOL

ABSTRAK

Calophyllum inophyllum L. adalah tumbuhan perubatan yang penting dengan banyak nilai etnoperubatan dan menunjukkan aktiviti anti-oksidan yang ketara. Oleh itu, kajian ini telah dilakukan untuk menentukan sifat anti-penuaan *in vitro* dari ekstrak buah *C. inophyllum* (CIFE) terhadap Strain *Saccharomyces cerevisiae* BY611 dengan mekanisme molekul proses anti-penuaan. Kesan anti-penuaan CIFE terhadap sel yis dinilai melalui fasa pertumbuhan sel yang berbeza, ujian tekanan anti-oksidatif, tindak balas rantai polimerase kuantitatif masa nyata (RT-PCR) untuk mengkaji ekspresi gen Superoxide dismutase (*sod*) dan Sirtuin 1 (*sirt1*) dan ujian aktiviti enzim SOD dan protein SIRT1 dilakukan pada nilai kepekatan CIFE sebanyak 1 mg/mL. Setelah dirawat dengan CIFE, jangka hayat sel yis berpanjangan dengan ketara berbanding dengan kumpulan yang tidak dirawat ($P < 0.05$) dan juga meningkatkan daya hidup sel yis di bawah tekanan oksidatif sebanyak 4 mM H_2O_2 dengan ujian spot. Kaedah pewarnaan Phloxine B juga membuktikan daya tahan sel yis meningkat di bawah tekanan oksidatif dengan rawatan CIFE. Penelitian aktiviti spesies oksigen reaktif (ROS) menunjukkan bahawa rawatan CIFE telah mengurangkan tekanan oksidatif pada sel yis ($P < 0.05$). RT-PCR mengesahkan bahawa peningkatan ekspresi gen *sod* dan *sirt1 s*etelah dirawat dengan CIFE masing-masing dengan nilai ΔCt sebanyak 1.046 dan 1.063. Aktiviti protein SIRT1 dicatat sebagai 1702 ng/ml dalam sel yis yang dirawat sementara aktiviti SOD dalam sel yis yang dirawat dicatat sebagai 42,23 U/mgprot yang dua kali lebih tinggi daripada pada kumpulan yang tidak dirawat ($P<0.05$). Kesimpulannya, penemuan ini menunjukkan bahawa CIFE memperlihatkan

sifat anti-penuaan dengan mengawalatur pengekspresan gen *sod* dan *sirt1* dan mengurangkan tekanan oksidatif dalam sel *S. cerevisiae* BY611 yang dirawat dengan ekstrak CIFE. Oleh itu, buah *C. inophyllum* boleh menjadi calon makanan berfungsi anti-penuaan novel yang memberi harapan.

ANTIAGING ACTIVITY OF POLYPHENOL RICH *CALOPHYLLUM INOPHYLLUM* FRUIT EXTRACT IN AGING MUTANTS OF *S. CEREVISIAE*

ABSTRACT

Calophyllum inophyllum L. is an important medicinal plant with many ethnomedicinal values and exhibited significant anti-oxidant activity. Hence, this study was conducted to determine the *in vitro* anti-aging property of *C. inophyllum* fruit extract (CIFE) against *Saccharomyces cerevisiae* BY611 Strain with the molecular mechanism of the anti-aging process. The anti-aging effect of CIFE against the yeast was evaluated through chronological lifespan assay, anti-oxidative stress assays, real-time quantitative polymerase chain reaction (RT-PCR) to study the regulation of superoxide dismutase (*sod)* and sirtuin 1 (*sirt1)* genes and superoxide dismutase enzyme (SOD) and sirtuin 1(SIRT1) protein activity assays were conducted at 1 mg/mL of CIFE. After administrating CIFE, the lifespan of the yeast was significantly prolonged in comparison with the untreated group ($P<0.05$) and also increases the yeast cell viability under oxidative stress by 4 mM H_2O_2 by spot assay. The Phloxine B stain method also further proves yeast cells' viability increased under oxidative stress by CIFE treatment. The reactive oxygen species (ROS) assay revealed that CIFE notably reduces the oxidative stress in treated yeast cells ($P<0.05$) comparing to untreated yeast cells. RT-PCR confirms that the gene *sod* and *sirt1* expressions differ when compare to before and after the CIFE extract administration. The SIRT1 protein activity was recorded as 1702 ng/ml in the treated yeast cells while the SOD activity in the treated yeast cells was recorded as 42.23

U/mgprot which is twice higher than in the untreated group ($P<0.05$). Conclusively, these findings showed that CIFE exhibited the anti-aging properties by upregulating the *sod* and *sirt1* genes and reducing the oxidative stress in *S. cerevisiae* BY611 treated with CIFE extract. Therefore, *C. inophyllum* fruit could be a promising novel anti-aging functional food candidate.

CHAPTER 1

INTRODUCTION

1.1 Introduction

Aging is the accumulation of variety molecular and cellular damages such as damaged protein molecules and altered deoxyribonucleic acid (DNA) molecules over time, which results in senescence in an organism (Rose *et al.*, 2012).However, the damages are neither consistent nor linear, and they also do not correlate with a person's age in years. While some 70-year-olds enjoy extremely good health and functioning, other 70-year-olds are frail and require significant help from others. According to World Health Organization (WHO), by 2020, the number of people aged sixty years and older will outnumber children younger than five years. The pace of aging by age, among the population is much faster than in the past.

Reactive oxygen species (ROS) were believed to play a vital key role in instigating the damage of the lifespans of various organisms. ROS induced damage leads to altered structure of biological macromolecules, such as protein backbone fragmentation, loss of biological functions and modification of the amino acid chain (Reisz *et al.*, 2014). The scientist Harman was the first scientist to propose the idea of free radical theory of aging in 1950s (Harman, 1992).

According to his theory endogenous free radicals might cause macromolecular damage *in vivo* and leads to free radical-mediated damage such as aging, mutation and cancer. The most relevant source of reactive oxygen species (ROS) in *vivo* could be the superoxide radical produced as by-product of normal oxidative phosphorylation (OXPHOS) (Treberg *et al.*, 2010). Superoxide radicals are mainly responsible for the free radical reactions involved in mutations, disease and death (Pujari *et al.*, 2014). ROS are persistently generated in the human body and eliminated by antioxidant defenses. As the age proceeds, the effectiveness of antioxidant defense systems reduces, and the capacity to remove toxic ROS decreases. This leads to oxidative stress or also known as prevalent free radical states, initiates the oxidation of polyunsaturated fatty acids (PUFA), proteins, DNA, and sterols (Ashok and Ali, 1999).

The concern to reduce oxidative stress in human body was in demand and it was reported that the addition of potential antioxidants in the food matrix could balance the stress (Brewer, 2011). The usage of natural antioxidants has achieved phenomenal value due to the concerns on negative health effects developed by the use of synthetic antioxidants (Neiva *et al*., 2019). The natural antioxidants are primarily derived from medicinal plants and food, such as vegetables, fruits, mushrooms, flowers, spices and traditional medicinal herbs (Xu *et al*., 2017). These antioxidants are mainly polyphenols (phenolic acids, flavonoids, anthocyanin, lignans and stilbenes), carotenoids (xanthophyll and carotenes) and vitamins (vitamin E and C) (Manach *et al*., 2004).

Increment in intake of vegetables, herbs and fruits containing high amounts of anti-oxidative nutraceuticals has also been proven to be associated with the balance of the free radical status, as it helps to minimize the oxidative stress in the body and to reduce the risks of age-related diseases (Gupta and Prakash, 2009). Hence, anti-oxidative substances can be used as an antiaging agent. In recent years, evidence that phytochemicals such as resveratrol and quercetin extend the lifespan of yeast, nematodes, fruit flies, and mice has been studied and recorded (Liu *et al*., 2014). In another study, the free radical scavengers such as anti-oxidative substances are found to be one of the solutions toward Alzheimer’s Disease which is an age-related neurodegenerative disorder (Tönnies and Trushina, 2017). Hence, in this study

Calophyllum inophyllum fruit was evaluated as anti-aging agent in *Saccharomyces cerevisiae* BY611 strain yeast model.

C. inophyllum is universally known as Pannay Tree, Tamanu and Alexandrian Laurel in English (Dweck and Meadows, 2002). Local Malay names of this *C. inophyllum* include bintagor or penaga laut (Blust and Trussel, 2013). This medicinal plant has a long history of therapeutic value in which all the parts of this plant are widely used in traditional practices such as antiseptics, astringents, expectorants, diuretics and purgatives (Ali *et al*., 1999). A study conducted on the chemical constituents of the nut and barks of *C. inophyllum*, shows that this specific species contains antimicrobial and cytotoxic compounds especially xanthone derivatives (Yimdjo *et al*., 2004) which are claimed to be one of the natural anti-oxidant (de Almeida *et al*., 2019). Besides, scientific studies have also identified compounds such as, benzophenones, neo flavonoids, and coumarin derivatives in *C. inophyllum* and these compounds are reported to have anti-cancer, anti-tumor and lipid peroxidation properties (Patra and Thatoi, 2011).

In a recent anticancer study conducted by Shanmugapriya (Shanmugapriya *et al*., 2016), proves that the fruit induced the apoptosis in breast cancer MCF-7 cell line via the mitochondrial pathway in a dose dependent manner. However, the anti-aging activity of *C. inophyllum* fruit extract is least investigated and reported. Hence, detail studies on *C. inophyllum* fruits are required to have a better understanding on their role in anti-aging activity. Therefore, the present study was conducted in *S. cerevisiae* BY611 which commonly known as budding yeast, possesses 23% homologous genes to humans (Liu *et al*., 2017). Besides, *S. cerevisiae* BY611 has contributed to the identification of mammalian genes that affect aging comparing to any other model organism (Longo *et al*., 2012).

1.2 Objectives

The current study was undertaken with the following objectives:

1) To study the anti-aging activity of *C. inophyllum* fruit extract in *Saccharomyces cerevisiae* BY611.

2) To investigate the possible anti-aging mechanism of action of *C. inophyllum* fruit extract in *Saccharomyces cerevisiae* BY611.

CHAPTER 2

LITERATURE REVIEW

2.1 Free radicals

Oxygen is a vital element of life which cells utilize in the process of generating energy. It is during the production of adenosine triphosphate (ATP) by mitochondria which results in the formation of free radicals. Free radicals can be defined as extensively reactive molecules or molecular fragments containing one or more unpaired electron(s) in their external shell (Valko *et al*., 2007). Free radicals are said to be unstable and highly reactive because of the odd number of electron(s) in their atomic or molecular shell. The presence of the unpaired electron tends to donate or attract electrons from other compounds to attain its stability. This process results in a chain reaction cascade as the attacked molecule loses its electron and becomes a free radical itself which brings about the damage in living cells (Phaniendra *et al*., 2015). Since these radicals are produced by losing or accepting a single electron, therefore, they are also known as reductants and oxidants respectively. The existence of free radicals in biology was discovered less than 50 years ago (Commoner *et al*., 1954; Droge, 2002).

Free radicals were described as Pandora's Box of evil due to their possibility to account for cellular damage, cancer as well as the degenerative process of biological aging. In the later years, discovery of enzyme superoxide dismutase (SOD) brought about the evolution of free radicals in living organisms which convinced most researches regarding its importance in biology (McCord and Fridovich, 1969).Ever since, the mention of free radicals has gained an ever-increasing curiosity due to their pivotal role in various physiological conditions and a diverse range of diseases (Phaniendra *et al*., 2015)

Free radicals typically include both reactive oxygen and nitrogen species referred to as reactive oxygen-nitrogen species (RONS). Both the reactive oxygen and nitrogen species are categorized into two compound groups namely radicals and non-radicals. Radicals primarily consist of those species which contain at least one unpaired electron in the external shells and are capable of independent existence (Phaniendra *et al*., 2015).

One excellent example of a radical is the oxygen molecule. However, because of the presence of two unpaired electrons in the shell surrounding its atomic nucleus, it is referred to as bi-radical. Besides oxygen, superoxide, hydroxyl, alkoxyl, peroxyl radical, nitric oxide and nitrogen dioxide are some other examples of radicals. Non-radical species in contrary are not free radicals but are capable to bring about its reactions in living organisms. Some examples of non-radical species include hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl), hypobromous acid (HOBr), ozone (O_3), singlet oxygen (O_2), nitrous acid (HNO_2), nitrosyl cation (NO+), nitroxyl anion (NO−), dinitrogen trioxide (N_2O_3), dinitrogen tetraoxide (N_2O_4), and peroxynitrite (ONOOH) (Phaniendra *et al.*, 2015).

ROS and RONS in the human body are produced primarily by means of essential cellular metabolism and also external sources. In other words, these sources of free radicals are conveniently known as endogenous and exogenous sources. Endogenous sources simply refer to internally generated sources of free radicals like mental stress, excessive exercise, infection, inflammation, phagocytosis, reperfusion injury, mitochondria and peroxisomes (Lobo *et al.*, 2010). Continuous formation of free radical takes place in the cells as a consequence of both enzymatic and non-enzymatic reactions. Enzymatic reactions contributing to this process include those involved in the respiratory chain, prostaglandin synthesis, phagocytosis, and cytochrome P- 450 system. Non-enzymatic reactions include the reaction of oxygen with organic compounds and those initiated by ionization (Lobo *et al.*, 2010). Exogenous free radicals on the other hand result from cigarette smoke, air pollutants, industrial solvents, radiation, ozone, heavy and/or transition metals such as mercury, lead, and iron as well as some drugs like cyclosporine & gentamycin. Upon the absorption/penetration of these external compounds into the body, they undergo decomposition or metabolization which brings about the formation of free radicals.

There are various physiological and pathological conditions in which free radicals play its fundamental part. By targeting and attacking essential macromolecules, reactive oxygen-nitrogen species (RONS) leads to cell damage and disruption of homeostasis (Lobo *et al.*, 2010). Major essential molecules targeted in the body by RONS include nucleic acids (RNA & DNA), lipids, and proteins. Severe damage to these macromolecules occurs abundantly with the accumulation of free radicals as a consequence of antioxidants and oxidants imbalance. This leads to tissue damage in various disease conditions such as diabetes mellitus, neurodegenerative diseases, cancer, cardiovascular diseases, rheumatoid, arthritis, and asthma, therefore speeding the progression and growth of the disease (Phaniendra *et al*., 2015).

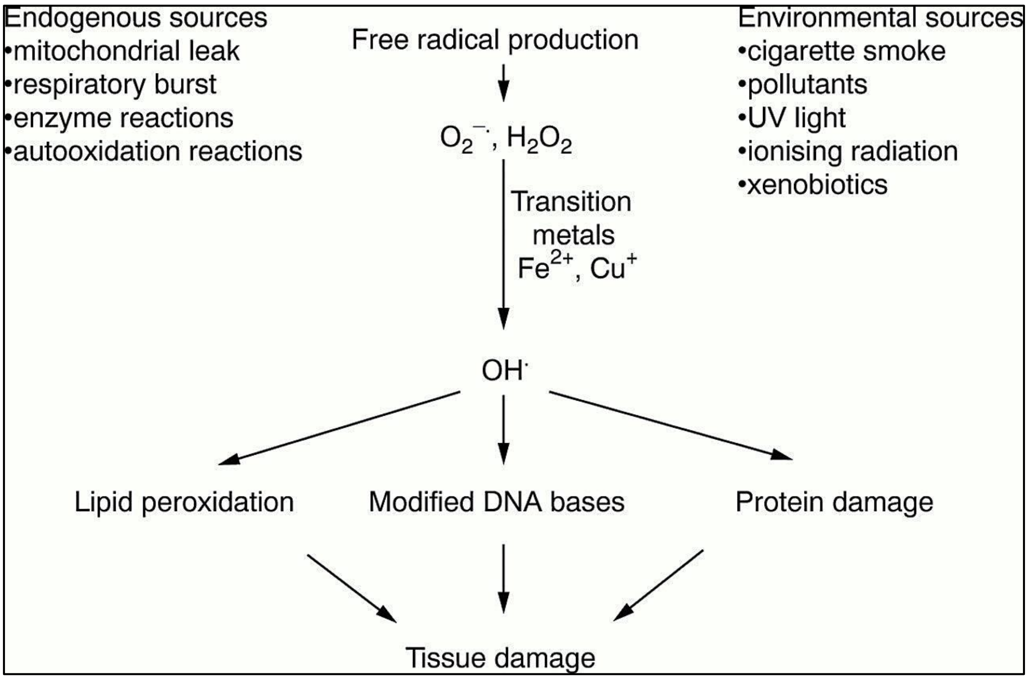

Figure 2.1: Schematic Diagram of Free Radical Production and The Effects.

Free radicals are formed by environmental and also through endogenous sources. Thus, the formation and accumulation of free radicals effects the tissue by damaging the proteins, DNA and lipids.

Source : Young and Woodside, 2001

Although both reactive oxygen and nitrogen species are frequently studied for their ability to cause extensive damage on some major molecules in the body, these free radicals too is undoubtedly recognized for their dual role as both deleterious and beneficial species (Valko *et al*., 2007). The harmonious balance between both antagonistic effects plays a significant part in the body. Reactive species, at low or moderate levels, portray beneficial effects in cellular signaling systems and immune function (Pham-Huy *et al*., 2008). In contrary, its overproduction leads to the exertion of undesirable effect by inducing oxidative stress that damages the cell.

2.2. Oxidative Stress

The term "stress" initially used in works of literature was described as the hyperactivity of the hormone system particularly concerning corticosteroids (Breitenbach and Eckl, 2015). After about two decades, the understanding of "stress" was brought to considerable attention once again regarding its importance in the study of diseases as well as general physiology. In other words, "stress" was primarily seen as a disease-causing factor. With the evolution of theories over the years, the term "oxidative stress" was proposed based on the profound knowledge of its mechanism and involvement in disease studies. Oxidative stress is basically the detrimental effect of free radicals which brings about possible biological damage. Oxidative stress occurs because of an imbalance between reactive oxygen species (ROS) formation and cell capacity to eliminate them (Liguori *et al*., 2018). This arises when the production of ROS overpowers the content level of intrinsic antioxidants, rendering the antioxidant defence mechanism unfavorable.

The generation and elimination of ROS are usually very well balanced by extensive regulatory systems to maintain an equilibrium state of ROS level (Lushchak, 2014). However, this critical balance can be interrupted by several sources/factors. The factors include depletion of low molecular mass antioxidant reserves, inactivation and/or decreased production of antioxidant enzymes as well as the combinations the stated factors (Lushchak, 2014). The degree of outcomes due to the imbalance depends on the location of ROS production, efficiency of antioxidant defence systems and the cellular targets in which the free radicals interact with (Lushchak, 2014).

As mentioned, oxidative stress is a destructive process which negatively affects cellular components like membranes, lipids, proteins, and nucleic acids. This process leads to the oxidation of the cellular components resulting in their structural and functional changes (Pizzino *et al.*, 2017).Thus, the critical balance between beneficial and deleterious effects of free radicals is vital and is achieved by a mechanism known as redox regulation (Droge, 2002). Maintenance and regulation of ROS homeostasis play a crucial role in cellular growth, survival, and metabolism. Presence of ROS at low level is important in maintaining cellular functions such as viability and apoptosis while extreme level, on the other hand, causes biological systems to incompletely detoxify the reactive intermediates and prevents the normal functions of biomolecules.

Both reactive oxygen species and oxidative stress have been proposed to play significant roles in various illnesses and health conditions. They contribute mainly in the process of aging and diseases such as cancer, inflammatory disorders (arthritis, vasculitis, & systemic lupus erythematosus), ischemic disorders (heart diseases, stroke), acquired immunodeficiency syndrome, hypertension, neurological conditions (Alzheimer's, Parkinson's disease, muscular dystrophy) and many more (Bajpai *et al.*, 2014). The metabolism of molecular oxygen by cells brings about the formation of reactive oxygen species which interacts with vital macromolecules. This interaction becomes the basis of most diseases and conditions stated. In the current time, it is a challenge to mention any illness for which the roles of oxidative stress and ROS have not been postulated (Ghezzi *et al*., 2017). Many researchers, with the help of strong evidence, suggest that oxidative stress can be associated with numerous diseases. Both reactive oxygen species and oxidative stress have been proposed to play significant roles in various illnesses and health conditions. They contribute mainly in the process of aging and diseases such as cancer, inflammatory disorders (arthritis, vasculitis, & systemic lupus erythematosus), ischemic disorders (heart diseases, stroke), acquired immunodeficiency syndrome, hypertension, neurological conditions (Alzheimer's, Parkinson's disease, muscular dystrophy) and many more (Bajpai *et al*., 2014). The metabolism of molecular oxygen by cells brings about the formation of reactive oxygen species which interacts with vital macromolecules. This interaction becomes the basis of most diseases and conditions stated. In the current time, it is a challenge to mention any illness for which the roles of oxidative stress and ROS have not been postulated (Ghezzi *et al.*, 2017). Many researchers, with the help of strong evidence, suggest that oxidative stress can be associated with numerous diseases.

With virtually three decades passed since the primary definition of oxidative stress was introduced, there still has been no accepted categorization to date. Consequently, in efforts to understanding the possible degrees of stress and effects, a basic classification based on intensity was presented. This intensity-based categorization includes basal oxidative stress (BOS), low-intensity oxidative stress (LOS), intermediate intensity oxidative stress (IOS) as well as high-intensity oxidative stress (HOS) (Lushchak, 2014). In addition, another classification of potential stress degree too was proposed consisting of three simple terms namely mild oxidative stress (MOS), temperate oxidative stress (TOS) and severe (strong) oxidative stress (SOS) (Lushchak, 2014). Normally, the level of ROS fluctuates to an extent outlined by their generation and elimination. However, because of various endogenous and exogenous factors, its usual level increases beyond the normal range. In circumstances at which the antioxidant defence systems are capable to deal with increased free radical amounts, they are known as “acute oxidative stress’’ (Lushchak, 2012). In contrary, “chronic oxidative stress” takes place when cells are unable to counteract the enhanced ROS content. At such state, even an improved expression of antioxidant and related enzymes would be unable to return ROS to its initial range. Stabilization at the new increased ROS level (quasi-stationary level) occurs and modification of cellular components is enhanced, significantly interrupting the redox homeostasis (Lushchak, 2012).

2.3 Aging

Humankind has come a long way through adaptation and evolution to the variety of changes that take place in the environment. Over the years, we have managed to overcome quite a number of limitations and succeeded in the areas once thought to be impossible. But there are, nonetheless, still certain frontiers we have yet to overcome in spite of our best efforts. One such matter is aging. Aging is a predestined process of life that as a living organism on this planet, we have to undergo it at any course. The outcome of aging is an inevitable part which brings about various effects such as the development of wrinkles, weight gain, greying of hair and many more. Though this matter is beyond the expertise of humankind to be prevented, experts/specialists have worked on possibilities to delay aging through advanced technology and modern governance.

Definition of aging is something which is very subjective as opinions vary among people and the concept of aging is defined on the basis of what suits their understanding best. Most evolutionary biologists describe aging as an age-dependent or age-progressive decrease in intrinsic physiological function leading to an increase and decrease in age-specific mortality rate and reproductive rate respectively (Flatt, 2012).

Aging can be defined as a time-related progressive impairment of physiological processes required for survival and reproduction (López-Otín *et al*., 2013). In addition to that, aging is also defined as "a persistent decline in the age-specific fitness components of an organism due to internal physiological degeneration." It is a process that is genetically determined and environmentally modulated (Rogina *et al*., 2000).

The aging process is associated with a wide range of physiological alteration which restrains an individual's normal biological processes rendering them to various diseases and also making them vulnerable to death. It involves changes in biological, physiological, psychological as well as social processes. Some of these transformations that take place such as the formation of wrinkles and greying of hair are considered harmless while there are also other alterations which result in biological function impairment as well as increased susceptibility to illnesses and disability. The degrees at which these changes occur are different in every individual. Example of some internal and external modifications of aging include wrinkled skin, weak bones, joints and muscles, mobility issues, hormonal changes, memory problems, weakened immune system, reduced sensitivity of hearing and vision impairment.

With aging, skin becomes less flexible and more prone to easy bruising. The decreased production of natural skin oil too causes the skin to become dry. Also, bones tend to lose its density making it susceptible to possible fractures which along with joint and muscle issues, become one of the major contributing factors to mobility and balance problems. Additionally, hormonal changes are commonly seen in aging individuals especially those related to glucose and carbohydrate metabolism and the immune system of the body also is weakened with aging. More frequent infections take place and with time may lead to some serious immunological disorders.

Many theories have been proposed by researches in order to explain the concept of aging. No single theory can be used to describe aging as none are fully sufficient (Davidovic *et al*., 2010) and also, all these theories are associated with each other in a complex system. Conventional theories stick to an understanding that aging process is not programmed genetically while most modern theories proposed are categorized into two main types, programmed and damage or error theories (Jin, 2010). The programmed theories state that aging occurs in accordance with a biological schedule structured to regulate growth and development. In contrary, the damage or error theories highlight that aging is caused by progressive damage resultant from external assaults (Jin, 2010). The programmed theory is further categorized into three sub-theories. One of which is the programmed longevity theory where aging is said to be the result of the "on-off" operation of specific genes in sequence. The second sub-theory which is endocrine theory states that the rhythm of aging is modulated by the body's innate mechanism via the help of hormones. Immunological theory, on the other hand, describes the occurrence of aging as a process that takes place with the programmed decline of the immune system which leads to an increased susceptibility to infectious disease. It is of no doubt that the efficacy of immune system reduces with age. As a person grows older, the antibodies in the body lose their ability to combat diseases effectively contributing to cellular stress (Cornelius, 1972).

Damage or error theory which focuses more on external factors consist of about three sub-theories namely wear-and-tear theory, cross-linking theory, and free radical theory. Wear-and-tear theory believes that cells and/or living organisms similar to instruments have essential parts which eventually wear out with time. However, this theory is not the best to refer because like machines, the worn-out parts can be repaired by the ability of a living organism to mend and restore. The second one which is the cross-linking theory assumes that the production of cross-links within molecules of collagen results in the loss of elasticity in tendons, skin, blood vessels and even enzymes. Accumulation of cross-links slows down essential bioprocesses by damaging cells and tissues, conveniently leading to aging (Jin, 2010).

Free radical theory suggests that free radicals cause oxidation of macromolecules (lipids, nucleic acids, and proteins) which results in their structural and functional damage and with the accumulated damage, induces the cells and organs to deteriorate (Harman, 2006; Jin, 2010). This demonstrates the possible counteract response towards oxidative stress that would have taken place. With further studies done on this matter, the mitochondrial theory of aging was introduced. Mitochondria are able to give rise to abundant reactive oxygen species resulting in extensive oxidative stress. According to the latter theory, accumulated mutation of mitochondrial DNA by oxidative stress causes the functional defect in mitochondria. In other words, they are no longer able to generate an adequate amount of energy for cellular use and with time leads to tissue impairment and degradation.

2.4 Oxidative Stress & Cellular Derived Mechanisms in Aging

There has always been a constant struggle to keep oneself from aging though it is an inevitable substance of the human body. For almost half a century various researches done have proposed oxidative stress to play a major role in aging and also pathophysiology generally associated with aging (Pole *et al*., 2016). Reactive oxygen species (ROS) are generally described as the key components contributing significantly to aging in mammalian cells. ROS such as superoxide anion (O^{-}_2), hydroxyl radical (·OH), and hydrogen peroxide (H_2O_2) are extremely reactive molecules that are capable of deteriorating essential macromolecules like nucleic acids, lipids, and proteins (Cui *et* *al*., 2012).This directly brings about oxidative cell damage leading to conditions associated with aging.

Researchers have suggested many theories explaining the event of aging. These include two of the most referred theories namely the free-radical theory and mitochondrial theory of aging. The free radical theory of aging is one of the most renowned theories suggested by American gerontologist, Denham Harman more than fifty years back. This theory proposes that aging occurs as a consequence of the build-up of detrimental effects caused by free radicals (Pole *et al*., 2016). The degree of oxidative damage to cells along with the coping capacity of an organism acts as a deciding factor in determining a lifespan (Harman, 1992).

However, Harman himself improved this theory in the later years where the latter theory focused more on the role of ROS generated by mitochondria (Harman, 2006). The revised version was brought about mainly because of the fact that mitochondrial energy-generating processes were seen as a major production pathway of ROS in mammalian cells. This latter theory was termed as the mitochondrial theory of aging.

Reactive oxygen species are basically extremely unstable molecules which are produced by multiple endogenous and exogenous sources where the majority come from mitochondria as a consequence of electron leakage in the electron transport chain (ETC). In an effort to combat these highly reactive molecules, the body has specific defence mechanisms where excess ROS are neutralized by enzymes like superoxide dismutase (SOD), catalase, thyroiodin, antioxidants like Vitamin C, E as well as glutathione (Gruber *et al*., 2008).Eventually, a decline in the ability of defence mechanisms becomes increasingly tough over time and as a result prompts the activation of the aging process.

The balance between the production of free radicals and counterbalance reactions of antioxidants are extremely crucial to manage and prevent any form of oxidative damage to macromolecules and cells. Nevertheless, as we age, we tend to observe a growing decrease in the capacity of internal antioxidants causing the obstruction in the balance. ROS accumulation exceeding the normal antioxidant quenching capacity, results in extensive oxidative stress. Cell damage which cannot be restored by internal defence molecules leads to organ mass loss and other features of overall organism aging. Numerous age-related diseases such as asthma, cancer, diabetes and cardiovascular disease have been associated with the state of cumulative oxidative stress and damage (Pole *et al.*, 2016).

Four main cellular effects occur with the excess production of ROS in the cell. These include cell apoptosis, necrosis, autophagy and senescence (Pole *et al.*, 2016). These cellular effects are parallel to the negative consequences to normal homeostasis of cells and tissues resulting in evolution of various age-related conditions and can adversely impact organism lifespan. Senescence could be said to be one of the hallmark and fundamental processes in the development of aging. Senescent cells increase in number in aging individuals. These cells, instead of undergoing normal cellular division and assisting tissue generation give rise to potentially dangerous chemical signals that influences neighbouring cells to enter the same state (Dodig *et al*., 2019). Usually, these cells are removed independently through self-programmed death (apoptosis) and/or via the immune system but with the decrease in immune functioning as one age, senescent cells tend to accumulate in tissues.

2.5 Medicinal Plants and Antioxidant Phytochemicals

Usage of medicinal plants as a fundamental source of commercial medicine can be traced back to the time of its use in folk treatment (Adeosun *et al*., 2016).

Traditional plants have been utilized to maintain human health for over a period of several thousand years throughout the world. Studies done, describe medicinal plants as a potential storage chamber of a large range of natural anti-oxidative compounds mainly plant secondary metabolites which include phenolic compounds and flavonoids (Tungmunnithum *et al*., 2018). These plant metabolites are categorized in accordance with their composition, physical and chemical properties as namely alkaloids, glycosides, corticosteroids and essential oils (Shejale and Yeligar, 2019).

Recent findings on adverse toxicology reports regarding synthetic antioxidant usage as well as emerging change in perception has brought growing attention by health practitioners and researchers towards natural antioxidants (Ramalakshmi *et al*., 2008). As such, medicinal plants are being studied and utilized more because of its availability and function as an excellent source of natural antioxidants. They contain abundant active composites that are able to exhibit their role individually or synergistically. A single plant may contain an assortment of phytochemicals ranging from compounds that prompt digestion system, phenolic compounds possessing antioxidant and various other pharmacological properties, antibacterial and antifungal compounds, tannins that work as natural antibiotics, alkaloids and more (Bhatt *et al*., 2013; Miguel, 2010).

Efforts made to possibly increase the usage of medicinal plants, is due to the main concern regarding the side effects of synthetic drugs which can be even more harmful than the diseases itself. Unlike artificial medicine, plant-derived medication contains natural substances which can promote better health besides relieving illness, has better patient tolerance and is comparatively economical (Bhatt *et al.*, 2013).
In the context of compatibility, medicinal plants have a better profile as the chemical constituents in plants; similar to that of humans are a part of physiological functions of living cells.

Therapeutic compounds/components derived from plants are obtained from their many different parts which include seeds, roots, fruits, leaves, barks, flowers and may also come from the entire plant as a whole. Different sections within a single plant may contain different active ingredients respectively which give rise to diverse medicinal properties (United States Department of Agriculture, 2018). Medicinal plants are traditionally used in the form of infusion, decoction, tincture, herbal crude extracts and sometimes may be used by combining two or more plants for therapeutic use.

Antioxidants are defined as substances capable of inhibiting a specific oxidizing enzyme and/or a substance that reacts with oxidizing agents prior to damaging other molecules or substances that sequesters metal ions or even a substance effective of repair (Bhatt *et al*., 2013; Brewer, 2011). Antioxidants reduce the risk of persistent and degenerative diseases by playing a significant role in neutralizing, disabling and scavenging free radicals and/or reactive oxygen species (ROS) (Guleria *et al*., 2013).

Antioxidants contribute to combating oxidative stress and free radical-related events through a variety of manner and level. Some of its major functions include reducing localized oxygen concentration, scavenging free radicals to prevent chain initiation, decomposing lipid peroxides to peroxyl and alkoxyl radicals as well as disintegrating and converting peroxides to non-radical molecules (Miguel, 2010; Bhatt *et al.*, 2013).

Some antioxidant phytochemicals present in medicinal plants include phenolic compounds, flavonoids, tannic acids, carotenoids, and vitamins. Phenolic compounds are biologically active secondary metabolites that contribute at the molecular level and are potent natural antioxidants (Martins *et al*., 2016). These components are the largest group of phytochemicals that exhibits anti-oxidative activity in plants (John *et al.*, 2014). Phenolic acids are present in natural products derived from several cereals and fruits where they are found at high concentrations at the outer layers of the kernel which set-up the bran (Shahinoor *et al.*, 2019).

Flavonoids are the main and biggest constituent of phenolic compounds which are observed to have many biological properties namely antimicrobial, anti-ulcer, anti-arthritic, anti-angiogenic, anticancer, mitochondrial and protein kinase inhibition and more (Sulaiman and Balachandran, 2012). Based on its composition and structural properties, flavonoids are further distributed into six classes known as flavones, flavanones, flavonols, isoflavones, anthocyanidins and flavanols (or catechins) (Shahinoor *et al.*, 2019). Carotenoids are yellow, orange or red pigments present in fruits and vegetables generally divided into two main classes called carotenes and xanthophylls (Majo *et al*., 2005). This effective antioxidant is proposed to carry out a specific task in the antioxidant mechanism due to their distinctive structure (Stahl and Sies, 2003). Certain vitamins like vitamins A, C, and E too are some major antioxidant phytochemicals in medicinal plants.

2.6 Antioxidant Activity of Medicinal Plants

Medicinal plants have been used since ages in numerous disease treatments and as excellent nutritional supplements. One of the main properties of medicinal plants which of special interest, is their role as vital sources of natural antioxidants (Tiwari *et al*., 2009). Recently, much emphasis is being made on obtaining and utilizing natural antioxidants derived from various aromatic plants. The increased use of organic antioxidants is due to the main concern regarding side effects of synthetic antioxidants which can be even more harmful than the diseases they ought to treat. Unlike artificial drugs, plant-derived medication contains organic substances capable of promoting better health besides relieving illness, has better patient tolerance and is comparatively economical (Bhatt *et al.*, 2013).

Antioxidants play an essential role in disease prevention and cure with its capability to scavenge reactive oxygen species present in the body. There are namely three main mechanisms by which they are able to counteract free radicals that is hydrogen atom transfer (HAT) mechanism, electron transfer (ET) mechanism and also a combined mechanism of both HAT and ET (Prior *et al*., 2005).

2.7 Defect in RNA Caused by Oxidative Stress

Imbalance between reactive oxygen species (ROS) production and the efficiency of antioxidant defence mechanism leads to what is known as oxidative stress. ROS such as superoxide radical, hydroxyl radical as well as hydrogen peroxide, because of their extremely reactive nature can significantly affect the cells by damaging DNA, RNA, proteins, and lipids (Droge, 2002). Free radicals are generated by various sources categorized into two main classes that are endogenous and exogenous sources. The major endogenous source of ROS is mitochondria where radical species are leaked through its electron transport chain (Finkel and Holbrook, 2000). Among various types of ROS produced in the biological system, hydroxyl radicals (OH·) are said to be one of the more significant molecules which are extremely reactive and capable of negatively affecting macromolecules mainly nucleic acids (Kong and Lin, 2010).

High concentration of ROS in living cells results in the extensive occurrence of oxidative damage to vital macromolecules especially DNA. When compared to nuclear DNA, mitochondrial DNA is exposed more to ROS attack because these nucleic acids are closely situated to the source of free radicals (mitochondria) (Phaniendra *et al.*, 2015). Though RNA is also to be affected by oxidative stress at a similar degree as DNA or any other macromolecules, its damage is least studied about and has not been given prime focus in relation to the context of oxidative stress (Kikuchi *et al*., 2002).

In was only till the past decade where more researchers were interested in understanding the consequences of oxidative insult to RNA. As a matter of fact, recent studies have demonstrated a higher exposure of RNA (messenger and ribosomal RNA) to oxidative injury compared to that of DNA. Based on a study conducted by (Fiala *et al*., 1989) where the degree of oxidation was measured by means of 8-hydroxyguanosine (8-oxo-G) content, the oxidative damage was observed to be higher in RNA especially with the presence of exogenous hydrogen peroxide (Hofer *et al*., 2005).

The possibility of RNA being more vulnerable to oxidative stress damage compared to DNA can be explained by a few factors. These include the single-stranded nature of RNA, deficiency of an active repair mechanism for oxidized RNA, lower concentration of protective proteins as well as the location of cytoplasmic RNA which is nearby the major endogenous source of ROS (Phaniendra *et al.*, 2015). The single-stranded structure of RNA renders its bases unguarded by hydrogen bonding and also specific proteins. Consequently, the higher oxidation level of RNA is definitely deleterious to cells and may play a significant role in oxidative stress-related conditions like neurodegenerative diseases and aging (Brégeon and Sarasin, 2005).

Hydroxyl radicals which are formed at the areas where RNA is present is able to alter the RNA structure mainly due to their highly reactive nature and also their slow diffusion rate from the site of production. A slow diffusion rate from its site of formation allows a high concentration of these molecules to remain close to cytoplasmic RNAs and thus reacting with them (Kong and Lin, 2010). Therefore, modifications prompted by hydroxyl radicals make up the most adverse category of RNA damage. A study carried out by (Barciszewski *et al*., 1999) was able to recognize more than 20 different types of base damage induced by hydroxyl radicals. Commonly, 8-hydroxyguanosine (8-OHG) is generated as a consequence of RNA oxidation by hydroxyl radicals. It is the most prevalent base formed when these reactive radicals react with guanine causing the loss of an electron and proton sequentially (Kong and Lin, 2010).

The primary role of RNA in protein synthesis is adversely affected by the occurrence of oxidative stress. Studies performed have shown increased evidence of RNA impairment as a result of oxidation (Liu *et al*., 2012).

Some of the adverse effects resulting from oxidation of messenger RNA include protein synthesis diminution, elevated production of aggregated protein products and ribosome stalling (Shan *et al*., 2003). The decline in normal protein concentration, as well as increased protein aggregates due to defective protein generation, is a common feature which can be observed in various neurodegenerative diseases and aging too (Shan *et al.*, 2003; Nunomura *et al.*, 2006). Recent findings obtained regarding deleterious effects of oxidative stress on RNA suggest possible detrimental outcomes on biological functions of living cells. RNA damage through oxidation leading to cell-cycle arrest and eventually cell death (Bellacosa and Moss, 2003) is a matter worthy of note. In spite of the rising appeal in understanding and studying theories of RNA oxidation and defence, the relatively low number of studies carried out results in the shallow knowledge on degree and variability in RNA oxidative damage.

Since the past decades, several illnesses such as Alzheimer's disease (Ding *et al*., 2006), Parkinson's disease (Kikuchi *et al*., 2002; Zhang *et al*., 1999), Down syndrome and dementia with Lewy bodies (Kikuchi *et al*., 2002) have been associated with the onset of oxidative insults to RNA molecules as a part of its pathological mechanism. Several reports have indicated increased rates of RNA oxidation in the growth of numerous degenerative disorders (Wurtmann and Wolin, 2009). ROS attack towards RNA is described also as a prime attribute in association with aging (Liu *et al*., 2012; Seo *et al*., 2008).

2.8 *Calophyllum inophyllum*

Kingdom:	Plantae
Subkingdom:	Tracheobionta
Phylum:	Trachephyta
Class:	Magnoliopsida
Subclass:	Dilleniidae
Order:	Theales
Family:	Clusiaceae-Guttiferae
Subfamily:	Kielmeyeroideae
Tribe:	Calophylleae
Genus:	*Calophyllum*
Species:	*C. inophyllum L.*

Table 2.1: Scientific Classification of *Callophylum inophyllum*.

Source: Barstow, 2019