Elucidating the Anti-Senescence Effects of *Calophyllum* Species and its Underlying Mechanism in Hydrogen Peroxide Induced Premature Senescence in Endothelial Cells

by

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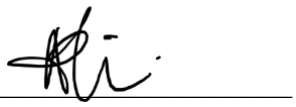
DECLARATION

I hereby declare that the work presented in this thesis was conducted in full compliance with the regulations of Universiti Malaysia Sarawak (UNIMAS). Except where proper acknowledgment is given, this work is solely the effort of the author. This thesis has not been accepted for the award of any other degree and is not being **concurrently** submitted for any other academic qualification.

Signature 

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Elucidating the Anti-Senescence Effects of Calophyllum Species and its Underlying Mechanism in Hydrogen Peroxide-Induced Premature Senescence in Endothelial Cells

ABSTRACT

Ageing, marked by endothelial cell cycle arrest and changes in metabolic pathways, is recognised as a crucial element in the onset and advancement of cardiovascular diseases (CVDs). Senescent cells play a role in oxidative stress are linked to endothelial dysfunction that ultimately leads to CVDs. Xanthone is a part of a large group of phenolic compounds, active ingredients that can be found in *Calophyllum* spp. proven to have valuable properties such as antioxidants, antimicrobial and anti-inflammation, inhibit the enzymes that lead to Reactive Oxygen Species (ROS) formation, which is one of the many factors leading to cellular senescence and the development of CVDs. However, the study on the anti-senescence effects of *Calophyllum* spp. extracts on stress-induced premature senescence (SIPS), which is accelerated cellular ageing that occurs when cells react to a variety of exogenous cellular stressors, including oxidative stress, remain underexplored. Thus, this research aims to explore the anti-senescence effects of *Calophyllum* spp. extracts on SIPS HUVECs. HUVECs were incubated with H_2O_2 (25 μM) to establish the SIPS model and treated with various concentrations of ethyl acetate (EA, 5 and 20 μg/ml), and the positive control Apocynin (20 μM) to evaluate the cell viability using phase contrast microscopy and MTT Assay. The senescence markers, senescence-associated β-galactosidase (SA-β-gal), were measured by the SA-β-gal assay. Additionally, the involvement of Nicotinamide Adenine Dinucleotide Phosphate (NADPH) and Cyclooxygenase-2 (COX-2) pathways was analysed using Colorimetric Assay and ELISA Assay. Results demonstrated that H_2O_2 (25 μM) induced senescence in HUVECs and was then reversed by the EA extract from *C. gracilentum* and *C. soulattri* and Apocynin (20 μM). Treatment with EA also upregulated the NADPH levels, indicating the involvement of EA in inhibiting oxidative stress effects in SIPS HUVECs. In contrast, a significant decrease in expression of COX-2 as an inflammatory response, after treatment with EA extract was not portrayed. Taken together, the findings underscore that *Calophyllum* spp. extracts potential in preventing CVDs by alleviating endothelial senescence and reducing endothelial dysfunctions of SIPS in HUVECs through the inhibition of SA-β-gal and regulation of NADPH but not COX-2.

Keywords: Anti-senescence, stress-induced premature senescence; NADPH, COX-2, cardiovascular diseases; *C. gracilentum; C. soulattri*

Kesan Anti-Penuaan Calophyllum Spesies dan Mekanisme dalam Penuaan Pramatang Diinduksi oleh Hidrogen Peroksida dalam Sel Endotelium

ABSTRAK

Penuaan yang dicirikan oleh pemberhentian kitaran sel endothelial dan perubahan laluan metabolik dikenali sebagai faktor utama menyumbang kepada pembentukan dan perkembangan penyakit kardiovakular. Penuaan sel yang dipengaruhi oleh tekanan oksidatif, menyumbang kepada disfungsi endotelium dan seterusnya membentuk penyakit kardiovaskular. Xantona iaitu sebahagian dari sebatan fenolik, bahan aktif yang ditemui dalam Calophyllum spesies terbukti mempunyai keupayaan antioksidatif, antimikrobial dan anti-keradangan menghalang pembentukan spesies oksigen reaktif (ROS) yang menyumbang kepada penuaan sel dan juga pembentukan penyakit kardiovaskular. Namun, kajian mengenai kesan anti-penuaan Calophyllum spesies terhadap penuaan pramatang sel yang didorong oleh tekanan (SIPS), iaitu penuaan sel yang dipercepat akibat tindak balas terhadap pelbagai stresor sel eksogen, termasuk stress oksidatif, masih kurang dikaji. Oleh yang demikian, kajian ini bertujuan untuk mengkaji kesan anti-penuaan C. gracilentum dan C. soulattri ekstraks terhadap SIPS "HUVECs". HUVECs diinkubasi dengan "H_2O_2" (25 µM) untuk membentuk model SIPS dan diinkubasi dengan pelbagai kepekatan ekstrak etil asetat (EA, 5 dan 20 µg/ml) serta kawalan positif "Apocynin" (20 µM) untuk menilai daya hidup sel menggunakan kaedah kontras fasa, ujian MTT dan ujian "SA-β-gal". Selain itu, penglibatan "Nicotinamide Adenine Dinucleotide Phosphate (NADPH)" dan "Cyclooxygenase-2 (COX-2)" dianalisis menggunakan Ujian Kolorimetrik dan ELISA. Keputusan menunjukkan bahawa "H_2O_2" (25 µM) mendorong kesan penuaan dalam sel "HUVECs", dan telah berjaya diterbalikkan oleh ekstrak EA dan "Apocynin" (20 µM). Rawatan dengan ekstrak EA turut meningkatkan tahap "NADPH", membuktikkan penglibatan EA dalam menghalang tekanan oksidatif dalam SIPS "HUVECs". Sebaliknya, penurunan signifikan dalam penghasilan enzim COX-2 sebagai tindak balas keradangan, hasil daripada rawatan oleh ekstrak EA tidak dapat diperhatikan. Secara keseluruhannya, hasil kajian mencadangkan bahawa ekstrak EA Calophyllum spesies berpotensi mencegah penyakit kardiovaskular dengan mengurangkan penuaan endotelium dan disfungsi endotelium dalam SIPS "HUVECs" melalui perencatan "SA-β-gal" serta pengawalan "NADPH" namun tidak "COX-2".

Kata Kunci: Anti-penuaan; penuaan pramatang didorong oleh tekanan; NADPH; COX-2; penyakit kardiovaskular; *C. gracilentum; C. soulattri*.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF ABBREVIATIONS

SIPS	Stress-Induced Premature Senescence
CVDs	Cardiovascular Diseases
NO	Nitric Oxide
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NOX	Nicotinamide Adenine Dinucleotide Phosphate Oxidase
COX-2	Cyclooxygenase-2
ROS	Reactive Oxygen Species
NRS	Nitrogen Reactive Species
RSS	Reactive Sulphur Species
H_2O_2	Hydrogen Peroxide
$O2^{-\bullet}$	Superoxide anion
(•OH)	Hydroxyl free radical
NOXs	NADPH Oxidases
SA-β-gal	Senescence Associated beta-Galactosidase
SASP	Senescence-Associated Secretory Phenotype
SOD	Superoxide dismutase
GSH	Glutathione
GSS	Glutathione Synthetase
PG12	Prostacyclin
MAOA	Monoamine Oxidase A
$ONOO^-$	Peroxynitrite
IL-1 α	Interleukin-1 α
IL-1β	Interleukin-1 β
IL-6	Interleukin-6
IL-8	Interleukin-8
VCAM-1	Vascular Cell-Adhesion Molecule-1
ICAM-1	Intercellular Adhesion Molecule-1
LDL	Low Density Lipoprotein
HIV	Human Immunodeficiency Virus
p53	Tumor protein p53
BH4	Tetrahydrobiopterin
AMPK	Adenosine Monophosphate-Activated Protein Kinase

eNOS	Endothelial Nitric Oxide Synthase
PI3K	Phosphoinositide 3-kinase
TNF-α	Tumor Necrosis Factor alpha
mTOR	Mechanistic Target of Rapamycin
NF-kB	Nuclear Factor kappa-light-chain-enhancer of activated B cells
AP-1	Activator protein-1
iNOS	Inducible Nitric Oxide Synthase
Akt	Protein kinase B
PPARγ	Peroxisome Proliferator-Activated Receptor gamma
Ser1177	Serine 1177
EGCG	Epigallocatechin Gallate
SIRT1	Sirtuin 1
NRF2	Nuclear Factor-erythroid 2-related factor 2
p-CA	p-coumaric acid
PAI-1	Plasminogen Activator Inhibitor-1

CHAPTER 1:

INTRODUCTION

1.1 Background of Study

Endothelial dysfunction is a condition where abnormal accumulation of senescent cells in the vessel wall leads to a compromised vascular function contributing to vascular ageing. Endothelial dysfunctions can be characterised by impaired endothelium-dependent vasorelaxation due to the specific endothelium-dependent agents like acetylcholine and bradykinin, or in response to stimuli that enhance shear stress that include flow-mediated dilation (Mah et al., 2015).

Endothelial dysfunction caused by senescent cells is one of the causes of cardiovascular diseases (CVDs). The premature senescence in the endothelial cell is associated with the upregulation of the Nicotinamide Adenine Dinucleotide Phosphate (NADPH) Oxidase (NOX) and cyclooxygenase-2 (COX-2) pathway (Panday et al., 2015). Due to the complexity that exists between accumulated oxidative stress and endothelial premature senescence which further increases risks of endothelial dysfunctions, there is a need to identify potential therapeutics to reduce the harmful effect of oxidative stress as well as to alleviate the presence of premature senescent cell morphology (Y. Higashi, 2022).

Cellular senescence, marked by cell cycle arrest and changes in metabolic pathways, is recognised as a crucial element in the onset and advancement of CVDs (Boccardi & Mecocci, 2020). During senescence, endothelial cells experience morphological changes whereas the normal spindled and cobblestone cells evolved into flattened and enlarged morphology (Bloom et al., 2023). Stress-Induced Premature Senescence (SIPS) is a type of cellular senescence that differs from replicative senescence, whereas SIPS is independent of the failure of cells to proliferate due to telomere shortening that can be demonstrated in replicative senescence. SIPS in cardiovascular cells possess a variety of morphological and molecular features and serve targeted markers such as flattened enlarged morphology,

increased senescence beta-galactosidase (SA-β-gal) activity and buildup of senescence-associated secretory phenotype (SASP) (Song et al., 2020).

In the context of ageing, senescent cells contribute to chronic inflammation and oxidative stress, which reduce nitric oxide (NO) availability. These processes promote endothelial dysfunction, which ultimately leads to CVDs. (Shakeri et al., 2018). Cellular senescence's impact on the vascular system's pathophysiology, especially in relation to oxidative stress during ageing has been highly highlighted in many researches (Bozaykut, 2019). The results highlight the possibility of focusing on senescent cells as a feasible treatment strategy for CVDs.

Oxidative stress can result from the accumulation of free reactive radical species including reactive oxygen species (ROS), nitrogen reactive species (NRS) and reactive sulphur species (RSS). Some of the by-products of cellular metabolic processes such as hydrogen peroxide (H_2O_2), also partake in the physiological signal transduction inside the cell (Shaito et al., 2022). The primary functions of these particles are in the balancing of the redox state of the cell, such as leading to activation/inactivation of the residue of redox reactions of cysteine proteins. Despite the importance of these free radical reactive species, their accumulation production may lead to many pathologies including CVDs (Incalza et al., 2018). Any imbalance or excessive accumulation of ROS is detrimental and may lead to oxidative stress, thus the roles of antioxidant enzymes are important to neutralise the excessive generated ROS (Sies, 2017).

In many CVDs, one of the main mechanisms that leads to endothelial dysfunction is the reduction in NO bioavailability. NO bioavailability is an important precursor for vascular health as it is endothelium-dependent dilatation and promotes immune cell rolling, adhesion and infiltration, platelet aggregation and endothelial permeability (Lau, 2022). Some evidence suggests that the onset of CVDs is by the excessive accumulation of ROS expressed by the activation of NOXs, induces endothelial dysfunction and inflammation, attenuates the levels of NO, increases vascular smooth muscle proliferation and modifies vascular response and vasotone (Saleh Al-Shehabi et al., 2016). A rodent animal model study reported that inhibiting ROS generation via inhibition of NOX successfully decreases blood pressure, proving that CVDs can be controlled by regulating the ROS generation with antioxidants (Zeng et al., 2009).

Traditional medicine and ethnomedicine, which involves studying the traditional remedies used by different ethnic groups, have existed since the beginning of human history. Historically, traditional medicine used natural resources as remedies. Throughout history, herbs, broadly categorised as any type of plant or plant-derived substance, were the primary components of the initial medications in traditional medical systems throughout various cultures and societies. Plants and herbs have long been a prevalent source of pharmaceuticals, whether in the form of traditional extracts or as pure active chemicals (Shaito et al., 2020). Nature is a crucial source for discovering novel medications that can be used to treat ailments. The use of plant-based medicines has attracted much interest because of the lower toxicity and cost benefits. Plant sources or herbs have yielded famous drugs, including aspirin from the *Salix alba* L. tree, ephedrine from *Ephedra sinica*, lovastatin from *Monascus purpureus* L., reserpine from *Rauvolfia serpentina* and many others (Weber et al., 2014). The discovery of antimalarial drugs which is quinine from the bark of *Cinchona* species is one of the notable examples of how ethnomedicine can steer towards drug discovery from natural sources (Cragg & Newman, 2013). Many different secondary metabolites have been isolated from plant bioactives and refined to produce a biological effect, but they have not yet undergone thorough examination for therapeutic applications. Recent scientific findings, technical advancements, and research trends indicate that naturally derived chemicals will be significant sources of novel medications (Davison & Brimble, 2019; Otvos et al., 2019). Therefore, our study uses extracts derived from *Calophyllum* species, including *C. gracilentum* and *C. soulattri,* that have been reported to possess many bioactive compounds as an alternative approach for the treatment of H_2O_2-induced senescence in endothelial cells (Zamakshshari et al., 2021).

1.2 Problem Statements

SIPS is one of the common factors that contribute to endothelial dysfunction and CVDss. Senescent cells are characterised by proliferation arrest, persistent accumulating at lesions of cardiovascular systems, and impaired cardiovascular functions that eventually lead to the onset of CVDs (Hu et al., 2022). Cellular senescence is triggered by many factors including oxidative stress, telomere and mitochondrial dysfunction, and SIPS is highly evident in CVDs.

Interventions to reduce the occurrence of premature senescence are vital to overcoming age-related CVDs. The prevalence of death due to CVDs is primarily developed from the onset of ageing, evident in an 8.4% chance among 74≥ years old whereas 1.3% chance among 55-64 years old (Hu et al., 2022). Evidently, urge of therapies that target senescence include serotherapeutic approaches that use senolytics, senomorphics, exogenous cell-based products and also non-pharmacological therapies (Huang et al., 2022). However, many of the harmful side effects of senotherapeutics may occur including tissue atrophy, thrombocytopenia and neutropenic disorders among many others (Park & Shin, 2022; Sharma et al., 2020). Therefore, there is an unmet demand for alternative novel mechanisms from natural products that can contribute to the development treatment of premature senescence that may lead to cardiovascular senescence.

Calophyllum species, is newly discovered to possess many phenolic compounds and act as a defence mechanism against injury in any biological system, has been traditionally used as skin treatments such as skin infections and ulcers (Cechinel Filho et al., 2009). It has been proposed that *Calophyllum* sp. possesses multiple biological properties including anti-inflammatory, anti-cancer and antioxidant (Nguyen et al., 2017; Ruangsuriya et al., 2023). *Calophyllum gracilentum* has been proven to possess high antioxidant activity due to its high phenolic and flavonoid compounds contents (Nurr et al., 2023). However, the anti-senescence effects and mechanisms involved of *Calophyllum* sp. have not been extensively investigated in the vascular system, particularly in the SIPS condition. Therefore, the purpose of this research is to investigate the anti-senescence effects and mechanisms undermine the protective effects of two new *Calophyllum* species, *Calophyllum gracilentum* and *Calophyllum soulattri* against SIPS models with a focus on its underlying mechanisms in treating premature senescence associated with CVDs.

1.3 Objectives of Study

1. To determine the anti-senescence effects of *C. gracilentum* and *C. soulattri* extracts in SIPS HUVECs *in vitro*.

2. To investigate the mechanism behind the anti-senescence effects of extracts of *C. gracilentum* and *C. soulattri* on SIPS HUVECs.

1.4 Scope of Study

This study focused on the anti-senescence effects of *Calophyllum* sp. extracts against SIPS Human Umbilical Vein Endothelial (HUVECs). HUVECs were incubated with H_2O_2 to establish SIPS model that could mimic normal physiological changes during ageing. The anti-senescence effects of *Calophyllum* sp. extract were assessed via cytotoxicity assay, phase contrast and SA-β-gal assay, whereas the anti-senescence pathways involved were determined by NOX and COX-2. This study was limited to laboratory-scale for determining the anti-senescence effects and the underlying mechanisms of *Calophyllum* sp. extracts.

CHAPTER 2:

LITERATURE REVIEW

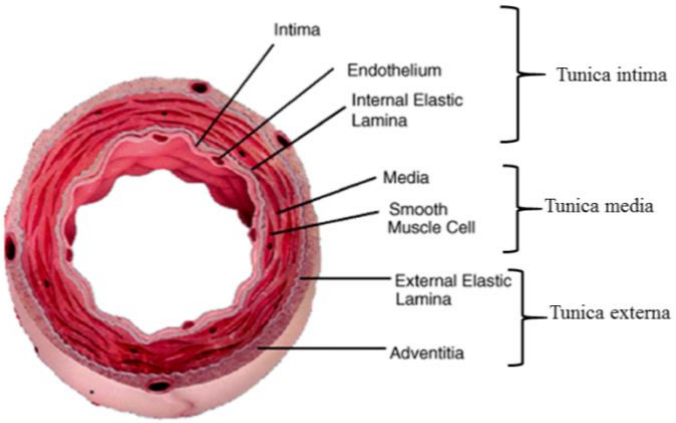
2.1 Vascular Endothelium and Blood Vessesls

2.1.1 Anatomy of endothelium and blood vessel

A blood vessel is composed of three layers: the tunica intima (inner layer), the tunica media (middle layer) and the tunica adventitia (outer layer) (Figure 2.1). The tunica intima acts as a barrier between the blood artery and blood flow (Yukihito Higashi, 2022). It consists of a layer of endothelial cells and an internal elastic lamina. Endothelial cells are orientated along the vessel axis and are highly elongated polygons, typically hexagons in the shape of "cobblestone" morphology (Suda et al., 2023). A single layer of endothelial cells, also called endothelium, is exceedingly thin and lines the wall, controlling the exchange of substances between the bloodstream and nearby tissues (Medina-Leyte et al., 2021).

Figure 2.1:

Layers of blood vessels.



Illustrations adapted from Di Nubila et al. (2024).

2.1.2 Physiology of endothelium and blood vessel

Endothelial cells are crucial key players in the cardiovascular network through their plethora of homeostatic, immune and inflammatory processes. Endothelial cells are responsible for metabolic and physiological functions such as redox balance, regulation of vascular tone, the interaction of platelets and leukocytes with the wall of vessels and the development of acute and chronic inflammation, among many others (Shaito et al., 2022). Regulation of vascular tone relies mostly on the endothelium-derived vasoconstrictions and vasodilators. Vasoconstrictions include endothelin-1 and thromboxane A-2 while vasodilations include NO and prostacyclin (PG12), give important effects on vascular smooth muscle regulations (Kant et al., 2022).

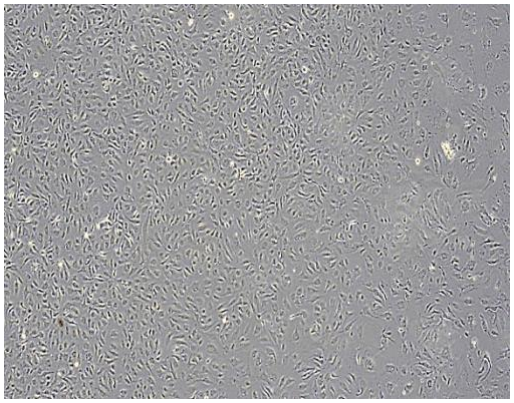
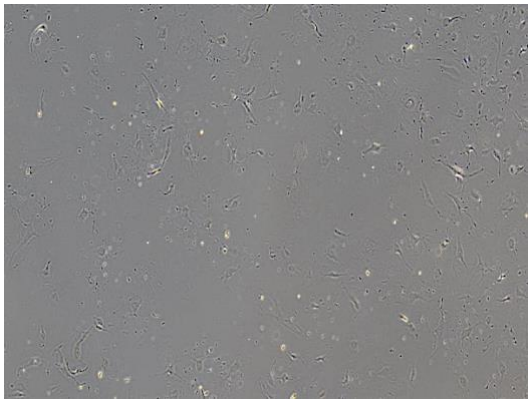
Endothelial cells also act as regulators that control the recruitment and movement of pro-inflammatory white blood cells in response to tissue damage and infection by expressing cell adhesion molecules and cytokines (Yau et al., 2015). The endothelial cells show a vital role in the healing process after inflammation or wounding by regulating the activation of platelets and the coagulation cascade which are then able to maintain the blood flow and prevent the formation of thrombus post vessels inflammation (van Hinsbergh, 2012). Thus, endothelial cells have become one of the key factors to regulate vascular health and disease. Endothelial cells are the primary source and also target of ROS that are involved in inflammation and vascular oxidative stress, which has become the main focus for therapeutic interventions (Glassman et al., 2020).

2.1.3 Endothelial Cell Senescent

Cell senescent occurs when the cells experience several physiological activities that change cell function, morphology and gene expression. Normal healthy endothelial cells exhibit “cobblestone” morphology (Figure 2.2a). In contrast, endothelial cell senescence is characterised by an irreversible cell cycle arrest with enlarged and flattened cell morphology (Figure 2.2b). Endothelial cell senescence also characterised by the release of growth factors, proteases and cytokines with potent autocrine and paracrine actions that contribute to the development of age-related diseases by affecting the function of neighbouring cells (Di Micco et al., 2021).

Figure 2.2:

Comparison of normal endothelial cells versus senescent endothelial cells.

(a)  (b) 

Cellular senescence is marked by an inflammatory phenotype known as senescence-associated secretory phenotype (SASP), the buildup of oxidative stress-induced damage, telomere shortening and mitochondrial dysfunction (Lo Curto et al., 2021). Thus, senescent cells with pro-inflammatory SASP are highly capable of producing pathological damage and ultimately leading to ageing (Sun et al., 2022). Chronic inflammation may be induced by SASP, through its continuous secretion of pro-inflammatory cytokines including interleukins consists of IL-1α, IL-1β, IL-6 and IL-8. Long-term inflammation may lead to immunosenescence, which is the functional deterioration of the immune system. If the inflammation persists over a long time, it can lead to an increase in the risk of premature mortality (Ratih et al., 2024).

One of the biomarkers to detect senescent cells is senescence associated-β-galactosidase (SA-β-gal). Cells with features of senescence expressed SA-β-gal activity as such that could be found accumulate at sites of ageing-associated diseases and in aged tissues (Di Micco et al., 2021). A study showed that endothelial cells from human atherosclerotic plaques expressed senescence markers, including SA-β-gal activity (Wang et al., 2015). Endothelial cells in human coronary arteries showed elevated SA-β-gal activity due to increasing senescence as individuals age. This suggests decreased endothelial cell regeneration and endothelial cell ageing, resulting in a decrease in endothelial cell-mediated arterial relaxation (Jia et al., 2019). The exposure of senescence endothelial cells to inhibitors of either senescence nicotinamide–adenine dinucleotide phosphate (NADPH) oxidase reduced the level of oxidative stress and the SA-ß-gal activity, indicating that NADPH oxidase contributes to the redox-sensitive induction of senescence (Cao et al., 2017).

2.1.4 Oxidative Stress

Oxidative stress is a situation where an imbalance occurs between the generation and accumulation of ROS within cells and tissues and the capabilities of biological systems such as the antioxidant defense system, to neutralise them (Jomova et al., 2023). The accumulation of ROS, free reactive radical species (Chang et al., 2016) and RSS are the precursors for the development of oxidative stress. Some of the by-products of cellular metabolic processes such as H_2O_2, also partake in the physiological signal transduction inside the cell (Shaito et al., 2020). The primary functions of these particles are in the balancing of the redox state of the cell, such as leading to activation/inactivation of the residue of redox reactions of cysteine proteins. However, the excessive accumulation of these free radical species may lead to pathologies including CVDs.

Antioxidant enzymes including catalase, superoxide dismutase (SOD) and glutathione synthase (GSH/GSS) are important enzymes that will be activated when ROS are at a high level (Bellezza et al., 2018). Small molecular weight antioxidants including flavonoids, carotenoids, vitamins C and E and others also possess the ability to ameliorate the harmful effects of oxidative stress. One of the prominent antioxidants that can boost the cellular antioxidant system by showing weak prooxidant properties is flavonoids (Jomova et al., 2023). Polyphenols of which half consist of flavonoids, found in almond skins were proven to provide protection against CVDs by enhancing the antioxidant enzyme and inhibiting the oxidation of low-density lipoprotein (Chen et al., 2019).

2.1.5 Endothelial Dysfunction

Increasing evidences suggest that endothelial dysfunctions share the same phenotypic features that occur in diseases, mainly in CVDs whereas the metabolic phenotypes experience changes (Kant et al., 2022). The altered metabolic phenotypes are changes that can be seen in oxidative stress where all lead to the impaired normal endothelial function and become the contributing factor to CVDs (Incalza et al., 2018).

One of the most prominent free radical species that highlighted is ROS, whereas ROS which also includes molecules such as H_2O_2, superoxide anion ($O2^{-\bullet}$) and hydroxyl free radical ($^{\bullet}OH$), are produced as byproducts of mitochondrial metabolism and by the activity of enzymes such as xanthine oxidase and NOXs (Zhang et al., 2022).

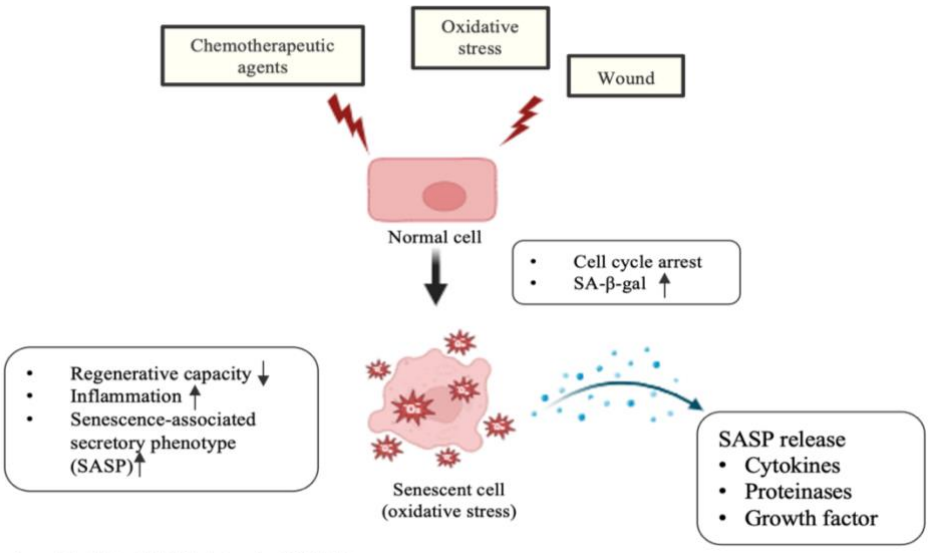
2.1.6 Mechanism of Senescence-Induced Premature Senescence (SIPS) and Cardiovascular Diseases (CVDs)

The connection between cellular senescence and CVDs is reinforced by the correlation between senescence and frailty, a prevalent illness in older individuals that is frequently linked with CVDs (Gorgoulis et al., 2019). Interventions to reduce the occurrence of premature senescence are vital to overcoming age-related CVDs. Understanding mechanisms driving SIPS is crucial in developing therapeutic interventions to mitigate age-related diseases and lifespan improvements. Recent research highlights a mechanistic pathway where oxidative stress lead to NOX overexpression, excessive ROS production, NO downregulation, endothelial senescence, dysfunction and ultimately CVDs.

Several studies have adopted SIPS (Figure 2.3), a form of accelerated cellular ageing occurs when cells react to variety of exogenous cellular stressors, including oxidative stress (Machado-Oliveira et al., 2020). This process is characterised by the expression of senescence markers, including cyclin-dependent kinase inhibitors including p16 and p21, mitochondrial dysfunction and the production of pro-inflammatory factors known as senescence-associated secretory phenotype (SASP).

Figure 2.3:

Mechanism of stress-induced premature senescence (SIPS).

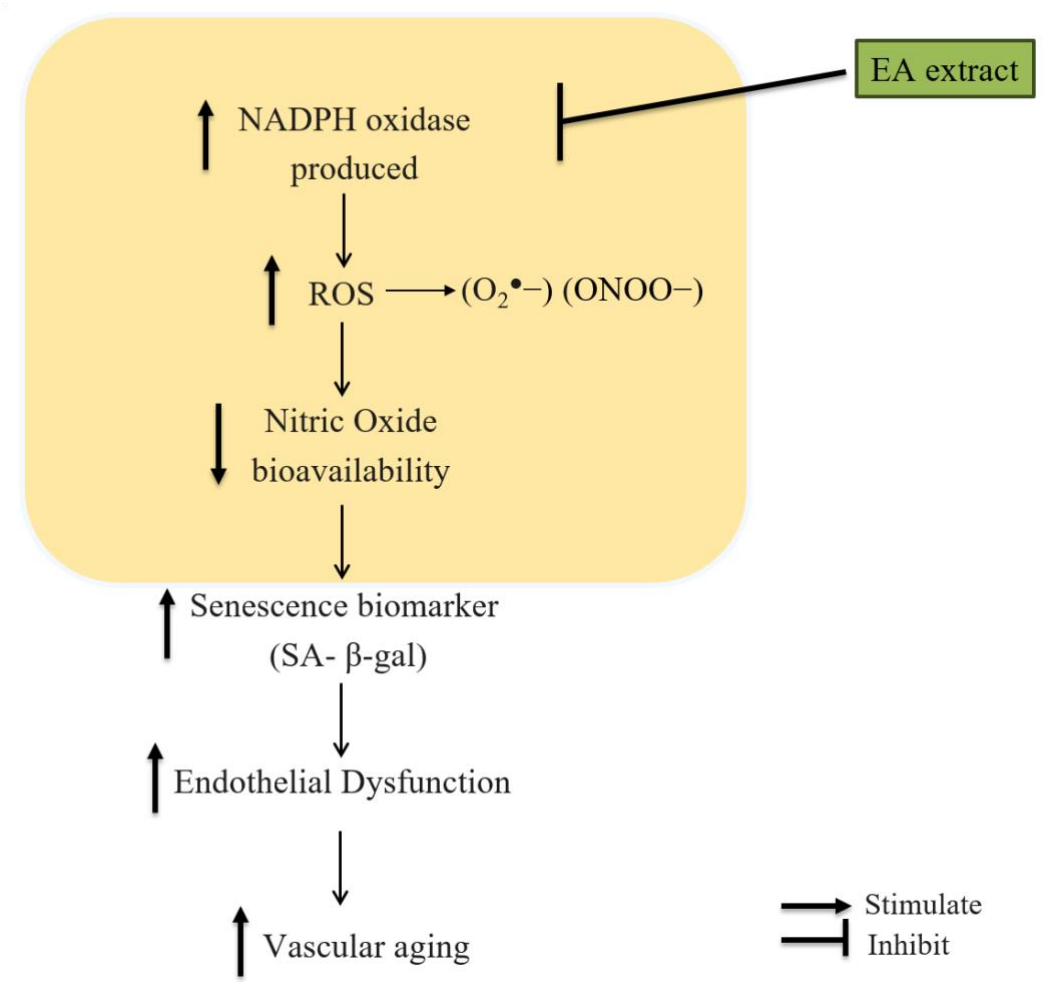


Oxidative stress upregulates NADPH oxidase (NOX) isoforms (notably NOX1, NOX2, NOX4, and NOX5) in vascular cells. This enzyme complex uses NADPH as an electron donor, and its overactivity often triggered by cytokines, hyperlipidemia, or high glucose leads to excessive ROS generation, particularly superoxide and hydrogen peroxide (Daiber & Chlopicki, 2020; Yukihito Higashi, 2022; Marqués et al., 2022; Myszko et al., 2025; Scioli et al., 2020). The overspending of NADPH relative to $NADP^+$ further amplifies this redox imbalance, fueling a vicious cycle of oxidative stress. (Daiber & Chlopicki, 2020; Marqués et al., 2022).

ROS produced by mitochondrial malfunction have the ability to harm lipids, proteins, and DNA, among other cellular constituents. For example, oxidative stress causes mitochondrial damage and telomere-associated foci, two important indicators of senescence (Martini et al., 2021). In cardiomyocytes, oxidative stress induced by monoamine oxidase A (MAOA) activity promotes premature senescence in cardiac stromal cells, contributing to cardiac dysfunction (Martini et al., 2021). Similarly, oxidative stress in skeletal muscle leads to muscle atrophy and fibrogenesis by the interaction between progerin and p53 (Xiang et al., 2022).

Figure 2.4:

The role of EA extract in pathophysiological of endothelial senescence and cardiovascular diseases.



As illustrated in Figure 2.4, elevated ROS from NOX overexpression reacts with NO, reducing its bioavailability and forming peroxynitrite (ONOO−) and superoxide (O2- −), types of cytotoxic species. This not only impairs vasodilation but also oxidises tetrahydrobiopterin (BH4), a critical endothelial Nitric Oxide Synthase (eNOS) cofactor, leading to eNOS uncoupling where eNOS produces superoxide instead of NO further exacerbating oxidative stress (Hernandez-Navarro et al., 2024; Janaszak-Jasiecka et al., 2021; Lee et al., 2020; Penna & Pagliaro, 2025). The resulting NO deficiency and oxidative stress promote endothelial cell senescence, marked by growth arrest, increased pro-inflammatory signaling, and altered cellular metabolism (Hernandez-Navarro et al., 2024; Lee et al., 2020). The same phenomena also observed in the improved arterial function in

old mice by fisetin via improved endothelial function mediated with increased NO bioavailability (Mahoney et al., 2024).

Senescent cells exhibit an increased in SA-β-gal activity, a marker of cellular senescence. This is associated with the activation of pathways including p53/p21 and p16INK4a, leads to cell cycle arrest and contributing to inflammations linked to CVDs (Ratih et al., 2024). In addition to SA-β-gal activity, senescent cells secrete pro-inflammatory cytokines as part of the SASP, which includes factors like IL-6 and IL-8. COX-2 is the predominant enzyme which induced by various stimuli including inflammation, growth factors and cytokines (Ziemlewska et al., 2024). This inflammatory response is linked to the development of many CVDs including hypertension and atherosclerosis (Qin et al., 2024). NADPH oxidase, one of the major sources of O_2- and H_2O_2 in which was especially observed in endothelial cells, is the main precursor for the increase in NOX activity leads to the adhesion of monocytes to the endothelium (Lee & Jose, 2021). Adhesion of monocytes to endothelium by signaling of Vascular cell-adhesion molecule-1 (VCAM-1) and Intercellular adhesion molecule-1 (ICAM-1) allow the transformation of monocytes into macrophages once inside the vessel wall. Then, the macrophages are able to take up oxidised low-density lipoprotein (LDL) and turn into foam cells, contributing to major part of plaque in arteries. The inflammatory molecule, IL-1β also released from macrophages worsens the damage and exacerbate plaque growth (Williams et al., 2020). Over time, as the increasing accumulation of apoptosis macrophages eventually coalesces into an acellular necrotic core within the plaque, disrupting normal form of vascular wall contribute to the formation of atherosclerotic lesion (Pickett et al., 2023).

Senescent endothelial cells exhibit reduced NO production, increased ROS, and upregulated adhesion molecules, fostering inflammation, leukocyte adhesion, and vascular remodeling keys of endothelial dysfunction. This dysfunction is a precursor to atherosclerosis, hypertension, and other CVDs (Yukihito Higashi, 2022; Marqués et al., 2022; Myszko et al., 2025; Scioli et al., 2020). Animal and human studies have confirmed that NOX overexpression and ROS excess are central to endothelial dysfunction and CVD pathogenesis, while interventions targeting NOX or enhancing antioxidant defenses can restore endothelial function (Yukihito Higashi, 2022; Lee et al., 2020; Vlad et al., 2025).

However, many of the therapies targeted at senescence are being focused rather than the onset of premature senescence, including senotherapeutics approaches that use senolytics, senomorphics, exogenous cell-based products and also non-pharmacological therapies (Huang et al., 2022). Conversely, emerging evidence of protection against age-associated endothelial senescence and oxidative stress was achieved by inhibition of promoter of p53-mediated SASP, Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) signaling in cells of rodent and human (Machado-Oliveira et al., 2020). Thus, this research focuses on the bioactive properties of plant-derived polyphenols to treat age-related ailments due to environmentally sustainable compared with conventional alternatives (Ziemlewska et al., 2024).

2.2 *Calophyllum* sp.

2.2.1 Calophyllum spp. and its phenolic compounds

Calophyllum species is a genus of the family Calophyllaceae family, a type of Sarawak's native shrubs or hardwood trees that can reach up to 30 meters in high (Figure 2.5). *Calophyllum* species, which is locally known as "Bintangor", is traditionally used as a folk remedy against ailments such as skin infections, peptic ulcers and inflammations (Cechinel Filho et al., 2009). *Calophyllum* spp. is famously known for its chemistry properties and possesses phenolic compounds such as xanthones, flavonoids, terpenes and coumarins. Xanthones are an important class of organic molecules abundant in living organisms. They have two benzene rings fused to a γ-pyrone ring, giving them a molecular formula of $C_{13}H_8O_2$ (Figure 2.6). These molecules have shown great potential in many biological applications, including as antioxidant agents (Cruz et al., 2017). This is due to its ability to donate or accept electrons to deactivate the free radicals, making it exhibit such biological activities (Liu et al., 2019).

Figure 2.5:

The stem bark of *Calophyllum* spp. is sourced from Sarawak.

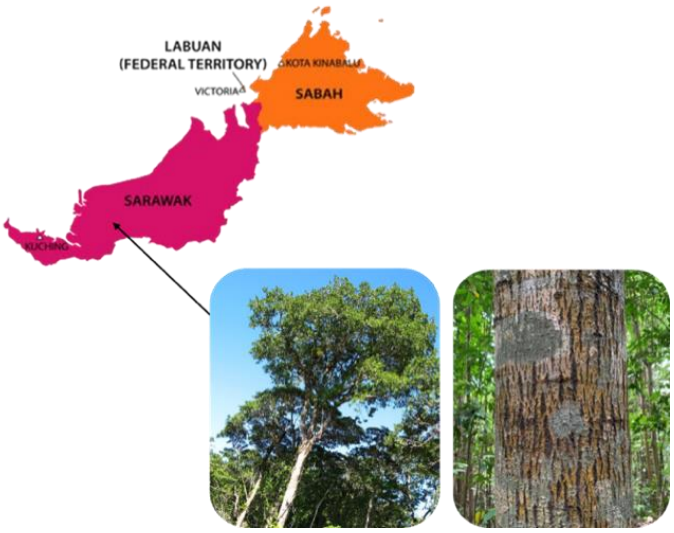
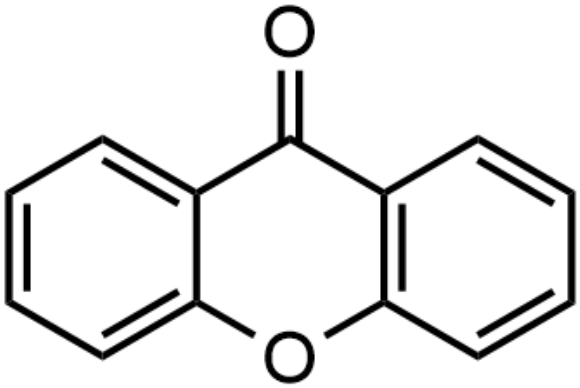


Figure 2.6:

Chemical structure of Xanthone.



2.2.2 Biological activities of *Calophyllum* Species

Phytochemicals are biologically active compounds that have been proven to be present in *Calophyllum* sp.. Phytochemicals represented as secondary metabolites assist plants in adaptation and protection against environmental stressors, including UV radiation, predators and pathogens (A. Kumar et al., 2023). Over the years, many studies focused on phytochemicals unveiled biologically active compounds from *Calophyllum* sp. and

xanthones are proven to be the most notable compounds exhibited biological activities (Marta et al., 2018).

Nguyen et al. (2017) isolated bioactive compounds from *Calophyllum inophyllum* Linn. has been reported to exhibit wound healing and anti-inflammatory activities proven with decreased fibrosis formation and wound closure in mouse models. Evidently, antioxidant activity also been reported through isolated compounds found in *Calophyllum gracilentum* and *Calophyllum ferrugineum* whereas both methanolic extract showed highest antioxidant activity respectively (Noh & Mian, 2020; Nurr et al., 2023). In addition, *Calophyllum lanigerum* and *Calophyllum brasiliense* chemotypes demonstrate selective defense activity against Human Immunodeficiency Virus (HIV) (H. Kumar et al., 2023) through reverse transcriptase inhibition and antimycobacterial effects (Ito et al., 2002; Nahar et al., 2020). It has also been reported that xanthones are most commonly found in *Calophyllum* spp. among other phenolic compounds that may contribute to the biological activities (Lizazman et al., 2022). Overall, these findings overview that biological activities and therapeutic potentials of *Calophyllum* spp. varied systematically by species and compounds class rather than being uniformly distributed across the genus.

CHAPTER 3:

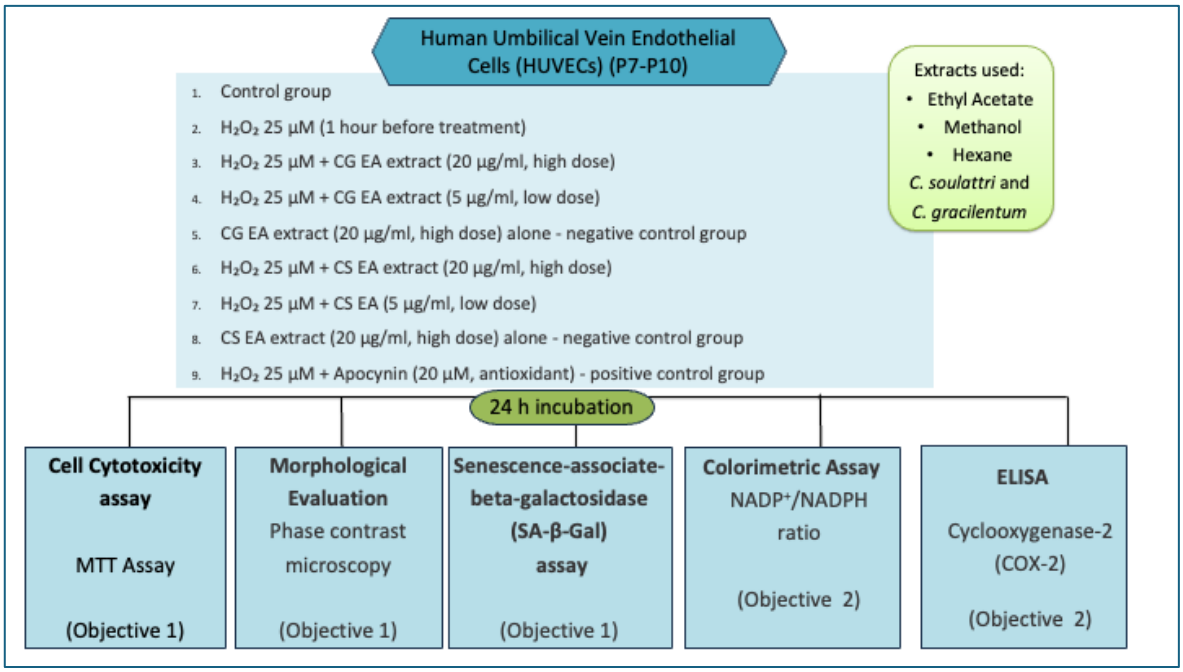
RESEARCH METHODOLOGY

3.1 Research Design

This study consists of an *in-vitro* experimental model of SIPS in HUVECs. Figure 3.1 illustrated methodology flowchart of the study. The anti-senescence effects of *Calophyllum gracilentum* (CG) and *Calophyllum soulattri* (CS) in H_2O_2-induced premature senescence were measured by assessing cell viability via phase contrast and by evaluating senescence-associated beta-galactosidase (SA-β-Gal) activity by SA-β-Gal assay. Additionally, the pathway involved in the anti-senescence effects of the *Calophyllum* spp. was investigated by colorimetric assay and ELISA for measuring NADPH and COX-2.

Figure 3.1:

Methodology flowchart of the study.



3.2 Chemicals and Reagents

Chemicals and reagents including methanol (MeOH), hexane (Hex), ethyl acetate (EA), dimethyl sulfoxide (DMSO), H_2O_2 and apocynin (Sigma-Aldrich, St. Louis. MO, USA) were used to extract from the plant samples, dissolve the extracts, induce SIPS and as treatment reagents respectively.

3.3 Methods

3.3.1 Plant Materials

Calophyllum soulattri (UiTM 3049) and *Calophyllum gracilentum* (UiTM 3048) stem barks were obtained from Sri Aman, Sarawak, Malaysia. The voucher specimens for each species were deposited in the Universiti Teknologi Mara Sarawak Herbarium and the samples were identified by the botanist. The stem bark of *Calophyllum soulattri* (2.9 kg) and C*alophyllum gracilentum* (2.1 kg) were dried in the oven and ground into fine powder by using a mill grinder. The powdered stem bark of both *Calophyllum* species was then extracted using EA, Hex and MeOH solvent for 72 h. This process was done three times, and the macerated samples were filtered to separate the filtrate and the residue. The filtrate was then combined and concentrated using a rotary evaporator to obtain the crude extracts.

3.3.2 Cell Culture

HUVECs (Lonza, Bassel, Switzerland; No. CC-2517) were cultured in an endothelial cell medium supplemented with 5% fetal bovine serum, 1% penicillin-streptomycin and 1% endothelial cell growth supplement (ScienCell Research Laboratories, USA). Cells were cultured in the CO_2 incubator maintained at 37°C, aerated with 5% CO_2 and 95% O_2. Cells from passages 7 to 10 were used. The experiments were performed once the cells reach 80% confluency.

HUVECs were treated with different concentration of extracts (625 to 5 µg/ml) from different extracts (EA, Hex and MeOH) of *C.gracilentum* and *C. soulattri* to determine the optimal concentration to evaluate the cell viability as the optimisation study. Afterwards, to elucidate the mechanism involved in the anti-senescence effects of *Calophyllum* spp., the

extract will be co-incubated with H_2O_2 as reported previously (Zheng et al., 2020). The preliminary study found that EA from CG and CS at 20 µg/ml significantly reduced cell viability and was used for subsequent experiments. Apocynin, a type of antioxidant, was used as positive control of the study. Then, the cells were cultured for 24 h in 9 experimental groups (n=3):

1. Control
2. H_2O_2 25 µM (1 h before treatment)
3. H_2O_2 25 µM + *C. gracilentum* ethyl acetate (20 µg/ml)
4. H_2O_2 25 µM + *C. gracilentum* ethyl acetate (5 µg/ml)
5. *C. gracilentum* ethyl acetate (20 µg/ml)
6. H_2O_2 25 µM + *C. soulattri* ethyl acetate (20 µg/ml)
7. H_2O_2 25 µM + *C. soulattri* ethyl acetate (5 µg/ml)
8. *C. soulattri* ethyl acetate (20 µg/ml)
9. H_2O_2 25 µM + Apocynin (antioxidant, 20 µM)

3.3.3 Induction of Stress Induced Premature Senescence (SIPS) in HUVECs

The induction of HUVECs premature senescence by hydrogen peroxide (H_2O_2) was performed as previously described (Zhu et al., 2021). HUVECs were incubated for 1 h in a growth medium containing 25 µM H_2O_2 to establish the SIPS model. Afterwards, to elucidate the mechanism involved behind the anti-senescence effect of *Calophyllum* spp. plant extract were co-incubated with H_2O_2 as reported previously (Zheng et al., 2020).

3.3.4 Cell Viability

Each extract was evaluated for its cytotoxic activities against HUVEC cell lines using the MTT assay. The HUVEC cells were seeded in a 96-well plate of 10,000 cells per well

until confluency was achieved. Once the cells reached 90% confluency, the cells were treated with extracts at volume (625 µg/ml to 5 µg/ml) in a two-fold dilution method. The plates were then incubated for 24 h at 37 ˚C in a 5% CO_2 humidified incubator. After 24 h, 10 µL of MTT solution (5mg/ml) was added to each well followed by incubation period of 3 h. Then, 80% of media was removed from each well and the same amount of DMSO was added to each well. The absorbance was measured at 550nm using a microplate reader (Multiskan FC, ThermoFisher). The percentage of cell viability relative to control were calculated using the following formula:

$$\text{Cell viability (\%)} = \frac{\text{mean absorbance in test wells}}{\text{mean absorbance in control}} \times 100\% \qquad \text{Equation 3.1}$$

3.3.5 Phase Contrast

The treated HUVECs according to the 9 experimental groups were cultured in 48 well plates with cell density of 30,000 cells per well. Morphological observations of HUVECs were analysed using phase contrast microscopy at 100x magnification via Leica DMi1 inverted microscope (Leica Microsystems, Mannheim, Germany) after 24 h.

3.3.6 Detection of senescence associated beta-galactosidase (SA- β-Gal) in HUVECs

The SA-β-Gal protocol was followed according to the manufacturer (Abcam, Senescence Detection Kit). Cells were seeded with cell density of 30,000 cells per well of the 48-well plates and treated for 24 h. After 24 h of incubation, the cells were washed once with 0.25 ml 1× PBS. 0.125 ml of fixation buffer was added and left for 10-15 min at room temperature. Then, the cells were washed using 0.25 ml PBS 1× twice. Next, 0.125ml of Staining Solution Mix was added to each well. The staining solution mix was prepared by mixing Staining Solution I/ Staining Solution 117.5 µl, 1.25 µl Staining Supplement and 40 mg/ml X-Gal in DMF 6.25 µl for each well. The cells were then incubated at 37 ˚C for the period of 1 h to overnight. Cells were then monitored after 24 h under a Leica DMi1 inverted microscope (Leica Microsystems, Mannheim, Germany). The appearance of the blue cell is the galactosidase-positive cells as a senescent cell representation. The percentage of SA-β-

gal-positive cells was calculated by the formula, counting the number of SA-β-gal-positive (blue) cells over the number of total cells:

$$\text{SA-β-gal positive cells (\%) (\%)} = \frac{\text{number of SA}-\beta-\text{gal}-\text{positive (blue) cells}}{\text{total number of cells}} \times 100\%$$

Equation 3.2

3.3.7 Measurement of COX-2 and NADPH levels in H_2O_2-induced SIPS HUVECs

3.3.7.1 Protein Extraction

Proteins were extracted after the treatment of 9 experimental groups HUVECs. HUVECs were washed in 1× PBS. Then, the cells were homogenised in extracting solution, blown and beaten gently, stand for 10 min to lyse cells. Then, the cells were centrifuged at 12000 ×g for 10 min at 4 ˚C to remove insoluble material. The supernatants were then collected and proceeded with protein determination using BCA Protein Colorimetric Assay Kit (E-BC-K318-M). The OD value of the concentration of the protein were measured by microplate reader. The protein concentrations were determined by plotting the standard curve of absoluted OD value of standard and correspondent concentrations.

3.3.7.2 Colorimetric assay: NADPH levels

The NADPH levels produced intracellularly in HUVECs after treatment according to 9 experimental groups were measured from the cell lysates by $NADP^+$/NADPH Colorimetric Assay Kit purchased from Elabscience (Houston, Texas, USA) according to the manufacturer's protocol [$NADP^+$/NADPH Colorimetric Assay Kit (WSR-8) (E-BC-K803-M)]. The Colorimetric Assay Kit has reagent WSR-8 that detects NADPH activity. The standard solution and samples were added in the appropriate 96 well plate as well as reaction working solution added into each well. The mix was then mixed fully with microplate reader for 5 sec and incubate at 37 ºC for 10 min. Next, chromogenic agent was added for each well immediately followed with mixed with microplate reader for 5 sec and incubated for another

37 ºC for 10 min. The OD value of each well was measured using microplate reader at 450 nm with 3 replicates (n=3).

3.3.7.3 Enzyme-linked immunosorbent assay (ELISA): COX-2

The intracellular senescence marker COX-2 levels in cell lysates from treated HUVECs were determined using enzyme-linked immunosorbent assay (ELISA) from Elabscience (Houston, Texas, USA). The ELISA assay was performed according to manufacturer's protocol [Human PTGS2/COX-2 (Prostaglandin Endeperoxide Synthase 2) ELISA Kit, E-EL-H1846]. The micro ELISA plate provided in this kit had been pre-coated with an antibody specific to Human PTGS2/COX-2. Samples and standards at a volume of 100 µl were then added to the micro ELISA plate wells and combined with the specific antibody, incubated for 37 ºC for 90 min. Next, Biotinylated Detection Ab working solutions were added to each well and incubated for 1 h at 37 ºC. Each well was then washed thrice using wash buffer and then incubated for 37 ºC at 30 min after added with HRP Conjugate working solution. Then, Substrate Reagent was added for each well and proceeded with incubation for 15 min at 37ºC followed by addition of stop solution. The optical density (OD value) was read with a microplate reader at 450nm with 3 replicates (n = 3).

3.3.8 Statistical analysis

The results are reported as mean ± SEM from n biological replicates. Statistical significance was determined using one-way analysis of variance (ANOVA) followed by post-hoc test Bonferroni's multiple comparison test by GraphPad Prism version 8 (GraphPad Software, Inc., Boston, MA, USA). p-value < 0.05 was considered as statistically significant.

CHAPTER 4:

FINDINGS AND DISCUSSION

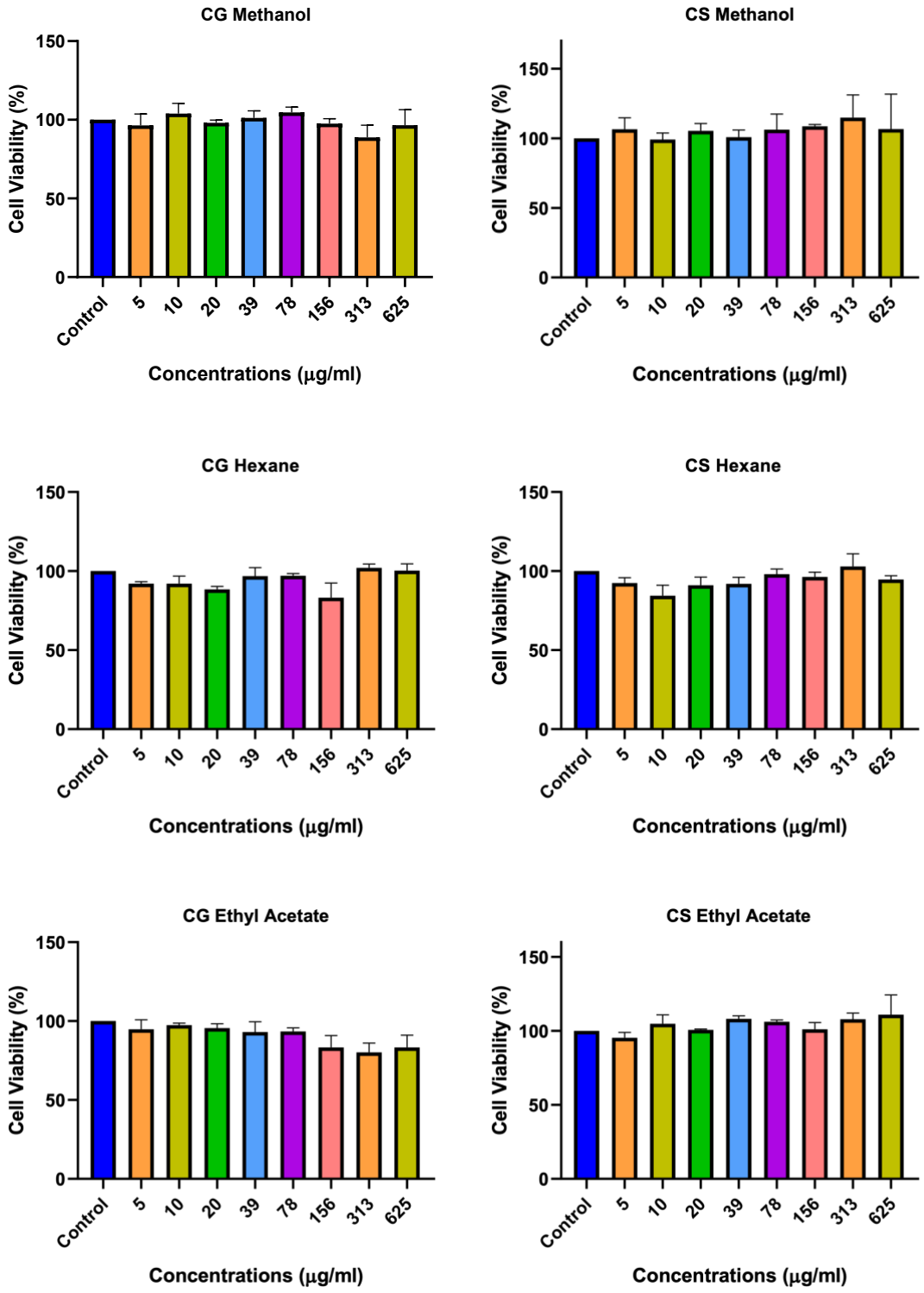
4.1 Cytotoxic activity of *Calophyllum* spp. extracts in HUVECs

To evaluate the cytotoxic activities of *Calophyllum gracilentum* (CG) and *Calophyllum soulattri* (CS) extracts against HUVECs, different concentrations of MeOH, Hex and EA in two-fold dilutions (625, 313, 156, 78, 39, 20, 10 and 5 µg/ml) were incubated for 24 h followed with MTT Assay.

Figure 4.1 shows MeOH, Hex and EA extracts have no significant of cell viability of HUVECs in a concentration-dependent manner from 625 to 5 µg/ml as compared to control. As shown in Figure 4.2, phase contrast of control HUVECs portrayed normal spindled cobblestone morphology, whereas HUVECs treated with MeOH and Hex show apoptotic morphology of shrinkage of cells and spherical (625 to 5 µg/ml) as indicated by the red arrow. Whereas HUVECs incubated with EA extract starting from 625 to 39 µg/ml, show apoptotic morphology outweigh the enlarged and flattened morphology of senescent cells in a concentration-dependent manner. However, Figure 4.2 shows most apoptotic morphology of HUVECs treated with MeOH and Hex extracts in 625 to 5 µg/ml. In contrast, HUVECs treated with CG EA extract in 39, 20, 10 and 5 µg/ml show cell viability of 93.042%, 95.549%, 97.386%, and 94.830%, whilst 108.149%, 100.787%, 104.875% and 95.374% in CS EA respectively (Figure 4.1). Taken together, 20 µg/ml EA extract of CG and CS indicating no toxicity against HUVECs, therefore chosen as the safety dose and used in subsequent experiments.

Figure 4.1:

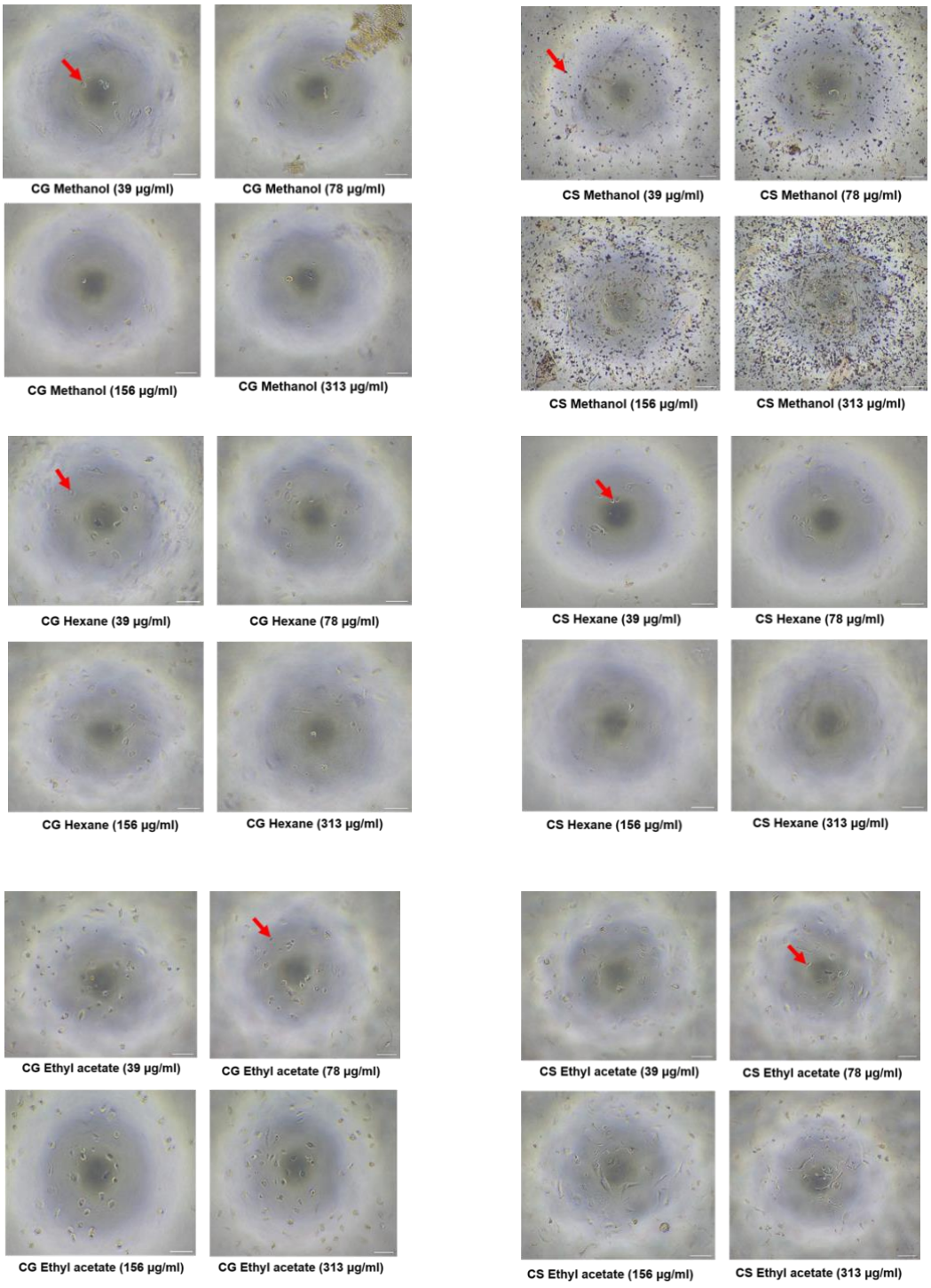
Cell viability test of HUVECs treated with methanol, hexane and ethyl acetate extracts in different concentrations (625, 313, 156, 78, 39, 20, 10 and 5 µg/ml).



Results are mean ± S.E.M of n=3 experiments. *p<0.05 compared with control.

Figure 4.2:

Phase contrast of HUVECs treated with methanol, hexane and ethyl acetate extracts in different concentrations (625, 313, 156, 78, 39, 20, 10 and 5 µg/ml).



Red arrow indicates apoptotic cells. The scale bar represents 100 µm.

The cytotoxic activity of each extract of *Calophyllum* sp. (MeOH, Hex and EA) was assessed through MTT Assay as previously reported (Wang et al., 2021). Screening extracts for cytotoxicity against HUVECs was vital as to help identify the safe concentration of extracts with potential therapeutic effect whilst minimizing harm to HUVECs such as apoptosis, cell cycle arrest and inhibition of cell proliferation (Batır et al., 2025). Past research found that the cell viability *Calophyllum inophyllum* seed oil, in which has been proven to possess many phenolic compounds including xanthone and tripertene, showed that in the range of 36 to 2 µg/ml have cell viability percentages above 70% in a dose-dependent manner (Ginigini et al., 2019; Saki et al., 2022). Arguably, CG and CS EA extract as portrayed in Figure 4.1 shows the highest cell viability at the range of 20 to 5 µg/ml, whereas MeOH and Hex show least cell viability in the dose-dependent manner. Hence, 20 and 5 µg/ml CG and CS EA extract were chosen as the optimal concentration to establish effective dosage therapeutical regimens for optimal clinical effectiveness in the absence of toxicity.

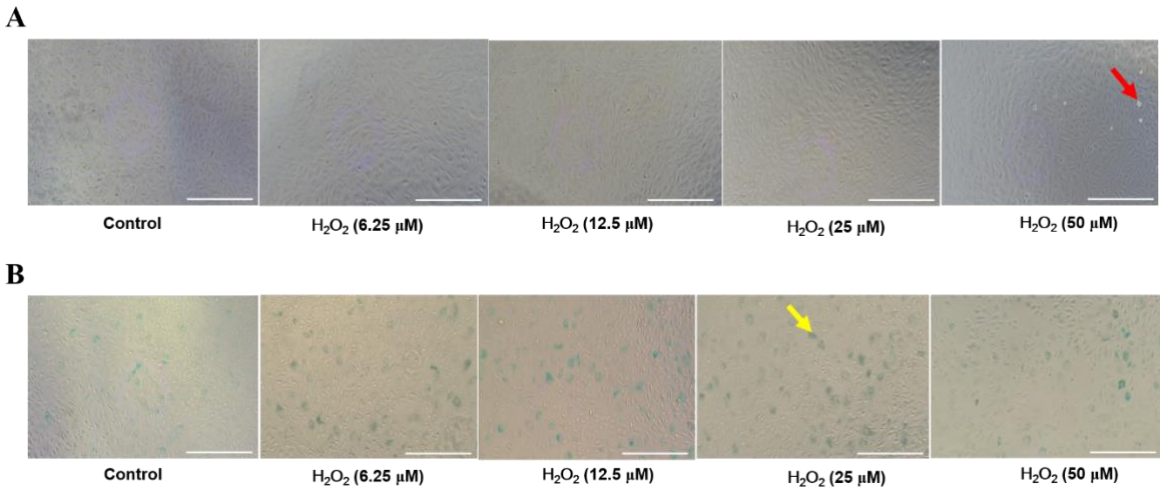
4.2 H_2O_2 induced SIPS in HUVECs

To determine the optimal concentration to induce SIPS by H_2O_2, HUVECs were incubated with different concentrations of H_2O_2 (50, 25, 12.5, 6.25 µM) for 1 h followed with senescence associated beta-galactosidase (SA- β-Gal) assay. Figure 4.3A shows control cells exhibited spindle cobblestone morphology, typical in normal HUVECs. In contrast, 50 µM H_2O_2 indicated toxic concentration portrayed by the shrinkage and spherical of cells including cell detachment as indicated by red arrow. HUVECs treated with H_2O_2 (25, 12.5 and 6.25 µM) show flattened and enlarged morphology with no spherical and detachment of cells.

Further SA-β-Gal assay confirmed the toxic dosage of 50 µM H_2O_2 with the significant SA-β-Gal positive cells compared with control and apoptotic cells indicated by red arrow (Figure 4.3B). HUVECs treated with 25, 12.5 and 6.25 µM H_2O_2 show significant SA-β-Gal positive cells indicated by yellow arrow compared to control. Collectively, 25 µM of H_2O_2 served as the optimal concentration to induce SIPS in HUVECs.

Figure 4.3:

Phase contrast of HUVECs treated with different concentrations of **(A)** H_2O_2 (50, 25, 12.5, 6.25 µM) and followed with **(B)** SA-β-Gal assay.



Red arrow indicates apoptotic cells and yellow arrow indicate positive-SA-β-Gal cells. The scale bar represents 100 µm. The total magnification was set at 100x.

H_2O_2 has been extensively validated as a robust inducer of stress-induced premature senescence in HUVECs, serving as a standard model for investigating endothelial ageing and senescence mechanisms. The H_2O_2-induced SIPS model recapitulates key features of replicative senescence, including increased expression of senescence markers (SA-β-gal, p16INK4a, p21WAF1, p53), elevated ROS production, DNA damage response activation, and telomere dysfunction (Katsuumi et al., 2018; Rippe et al., 2012; Shimizu & Minamino, 2020). The model's reliability stemmed from its ability to mimic physiologically relevant oxidative stress conditions encountered by endothelial cells *in-vivo*, particularly in the context of CVDs, hypertension, diabetes, and atherosclerosis (Bloom et al., 2023; Mudau et al., 2012). The H_2O_2-induced SIPS model continues to serve as an invaluable tool for elucidating anti-senescence mechanisms of natural products and developing therapeutic strategies for age-related vascular diseases. Treatment protocols typically employ H_2O_2 concentrations ranging from 20 to 200 µM, with exposure durations varying from 1 to 24 h depending on experimental objectives, whereas our preliminary experiment through MTT Assay employed 25 µM H_2O_2 as the optimum concentration to induce SIPS in HUVECs (Kiyoshima et al., 2012; Makpol et al., 2012).

4.3 Ethyl acetate (EA) extract restored the normal morphology in SIPS HUVECs

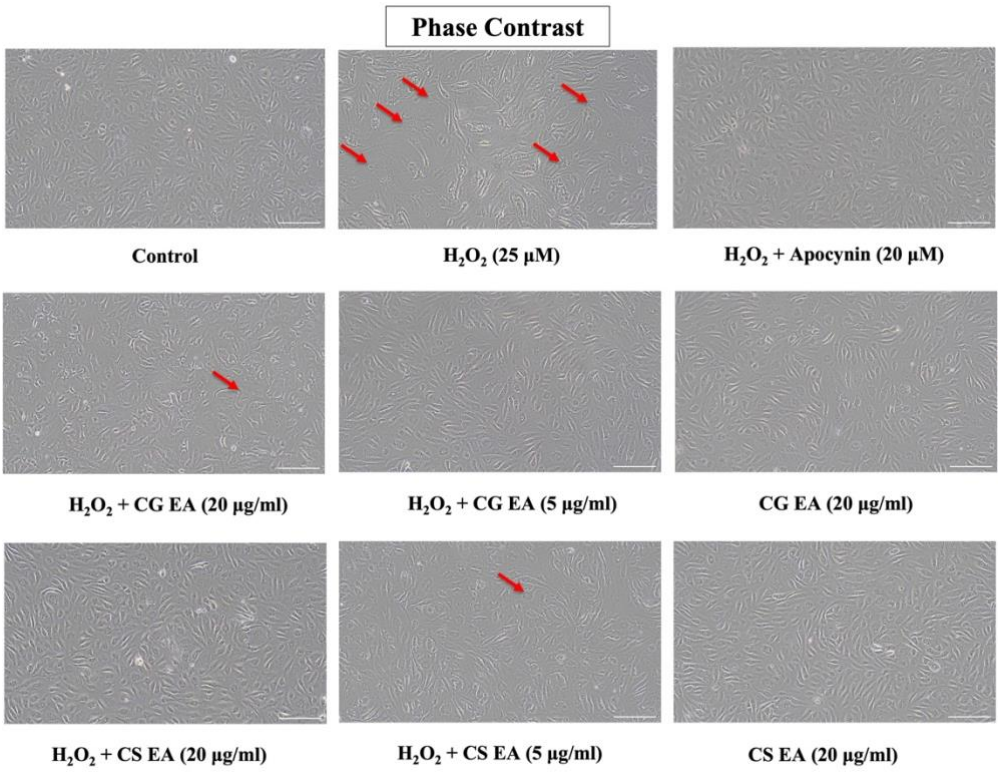
To evaluate the effect of EA in inhibiting senescence in SIPS of HUVECs induced by H_2O_2, phase contrast was examined. Figure 4.4 shows control group exhibited regular morphology of cobblestone spindled shape of HUVECs, significantly different than in HUVECs treated with H_2O_2 alone, which portrayed irregular enlarged and flattened morphology as indicated with the red arrow. Regular cobblestone spindled shape morphology also observed in HUVECs co-treated with 25 µM H_2O_2 and 20 µM Apocynin (positive control), similarly observed in control group.

HUVECs treated with 20 µg/ml CG and CS EA also shows normal cobblestone spindled morphology. Similarly, co-treated HUVECs with 25 µM H_2O_2 and 5 µg/ml CG EA and 20 µg/ml CS EA has cobblestone spindled shape morphology. Furthermore, HUVECs co-treated with 25 µM H_2O_2 and 20 µg/ml CG EA and 5 µg/ml CS EA show flattened and enlarged morphology however less significant as compared to HUVECs co-treated with H_2O_2 alone, suggesting the normalisation of regular morphology of HUVECs via co-treatment with CG and CS EA extracts.

Cellular senescence manifests distinct morphological alterations that can be readily observed under phase contrast microscopy, with senescent endothelial cells exhibiting characteristic enlarged and flattened morphology, which represents one of the most recognisable hallmarks of cellular ageing (Shimizu & Minamino, 2020). The structural alterations associated with endothelial senescence extend beyond simple morphological changes to include deviant phenotypic characteristics that contribute to endothelial dysfunction and subsequent cardiovascular pathology (Bloom et al., 2023). Studies on various polyphenolic compounds, including resveratrol, quercetin, and curcumin, have demonstrated their capacity to reverse the enlarged and irregular morphology characteristic of senescent cells, restoring a more youthful, spindle-shaped appearance typical of proliferative endothelial cells (Bloom et al., 2023; Shimizu & Minamino, 2020; Sun et al., 2015). Similarly, the treatment of H_2O_2-induced SIPS HUVECs with CG and CS EA extract normalised the flattened and enlarged morphology suggesting the protective effects of CG and CS EA extract against SIPS.

Figure 4.4:

Phase contrast of HUVECs treated with 25 µM H_2O_2 and different concentrations of CG and CS ethyl acetate extracts and 20 µM Apocynin.



Red arrow indicates senescent cells. The scale bar represents 100 µm. The total magnification was set at 100x.

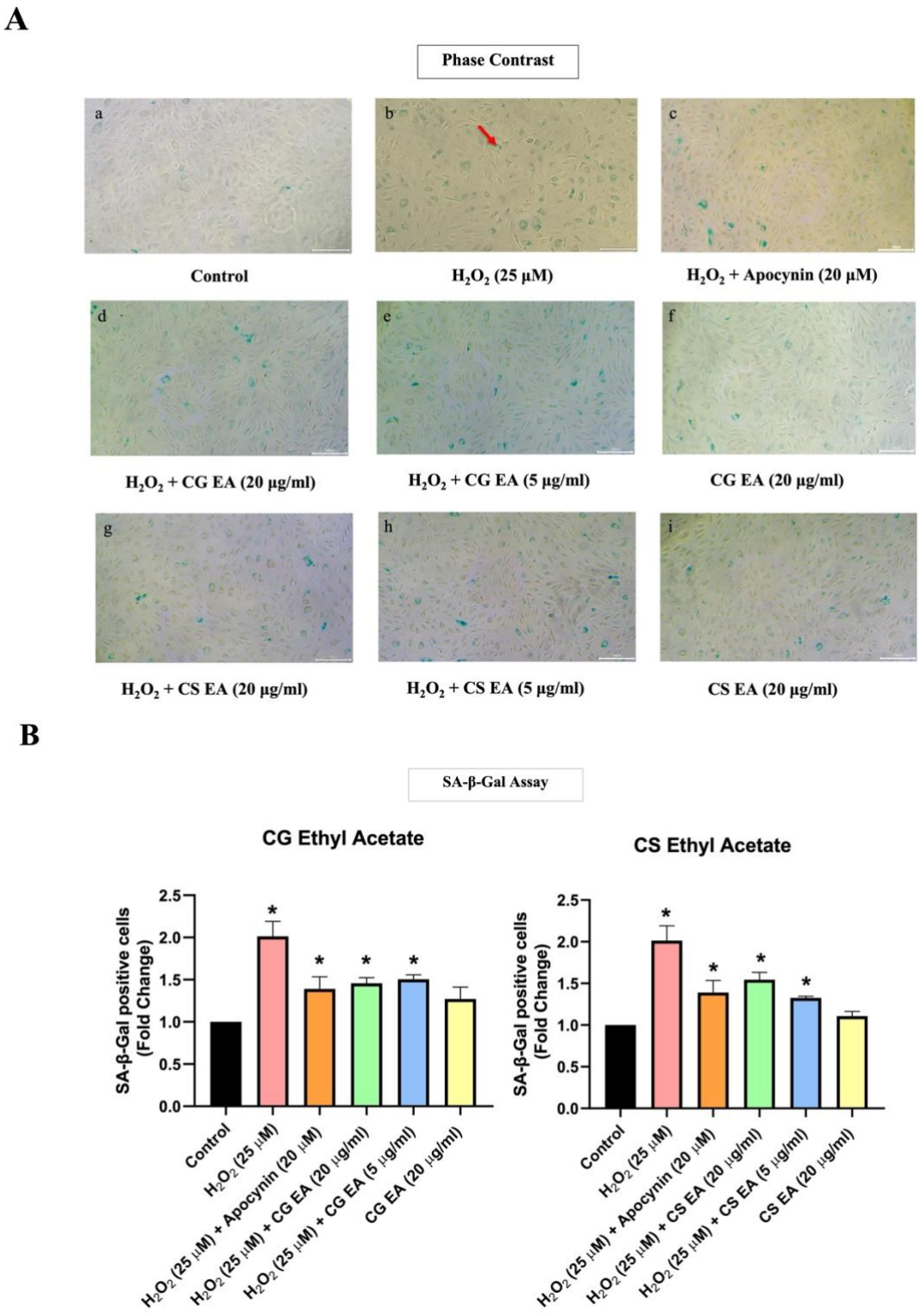
4.4 Ethyl acetate extract attenuates the production of SA- β-Gal in SIPS HUVECs

To evaluate the effect of EA extracts of *Calophyllum* spp. against SIPS HUVECs induced by H_2O_2, phase contrast and SA-β-Gal assay were performed. Figure 4.5A shows that exposure to H_2O_2 significantly increased the SA-β-Gal positive cells as indicated by red arrow as compared to control group. HUVECs co-treated with H_2O_2 and EA extracts (CG and CS) at 20 and 5 µg/ml show fewer SA-β-Gal positive cells, similarly seen in HUVECs co-treated with H_2O_2 and Apocynin, as compared with HUVECs treated with H_2O_2 only. Treatment of HUVECs with EA (CG and CS) at 20 µg/ml alone exhibited less SA-β-Gal positive cells, has no significant difference compared to control indicating that there is no toxicity of EA against HUVECs at this concentration.

Exposure of H_2O_2-treated HUVECs followed with treatment of EA extracts significantly attenuates the production of SA- β-Gal. Figure 4.5B shows HUVECs co-treated with 25 µM H_2O_2 induced 2.0145- fold increase expression of SA- β-Gal, whereas the simultaneous addition of Apocynin (20 µM, positive control group), significantly reduced the SA- β-Gal expression with 1.3900-fold increase. Similarly, HUVECs co-treated with H_2O_2 and EA extracts at 20 and 5 µg/ml shows a significant decreased expression of SA- β-Gal positive cells of 1.5802- and 1.5062-fold increase in CG extract, whereas 1.5454- and 1.3268-fold increase in CS extract respectively compared to HUVECs treated with H_2O_2 alone. Treating HUVECs alone with EA extract at 20 µg/ml (CG and CS) do not show any significant difference as compared to control, contrarily show significant difference as compared with treated with H_2O_2 alone group.

Figure 4.5:

(A) Phase contrast and **(B)** SA-β-Gal Assay of the effect of HUVECs treated with or without H_2O_2 (25 µM) and different concentrations of CG EA and CS EA (5 and 10 µg/ml).



Results are mean ± S.E.M of n=3 experiments. *p<0.05 compared with control #p<0.05 compared with H_2O_2. The scale bar represents 100 µm. Red arrow represents positive SA- β-Gal cells

Senescence-associated β-galactosidase (SA-β-Gal) activity represents the most reliable and widely utilised biomarker for identifying senescent cells, distinguishing them from cells merely arrested in the cell cycle (Bulbiankova et al., 2023). In H_2O_2-induced SIPS models, SA-β-Gal positive cells increased dramatically, serving as a definitive marker of the senescent phenotype, simultaneously decreased after co-treated with CG and CS EA extracts, shows the protective effects of CG and CS EA extracts against senescence (Figure 4.5). Correspondingly, quercetin, a flavonoid with potent senolytic properties, significantly decreases the number of SA-β-Gal positive cells in H_2O_2-induced senescence models while suppressing ROS production and inflammatory cytokine expression (Zoico et al., 2021), further postulated the reliability of SA-β-Gal positive cells as senescence biomarkers. These findings collectively underscore the therapeutic potential of *Calophyllum* spp. in mitigating SA-β-Gal in oxidative stress-challenged endothelial cells.

4.5 Ethyl acetate extract inhibited the upregulation of NADPH and no effects of COX-2 expression in SIPS HUVECs

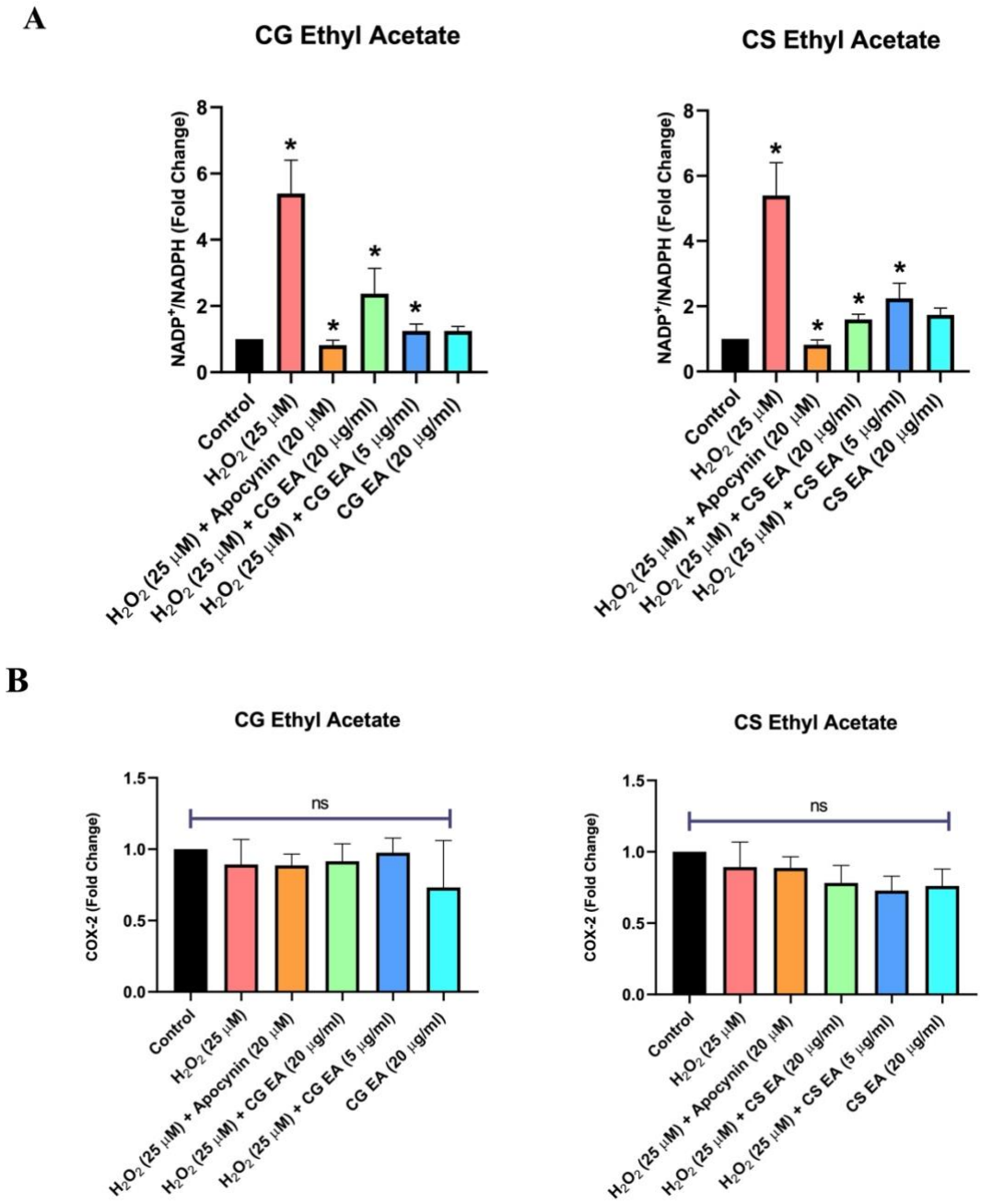
The involvement of endothelial stress (ES) pathways and the protective effect of EA extract against induced SIPS in HUVECs was measured by determining NADPH and COX-2, which served as SIPS biomarkers through Colorimetric Assay and ELISA respectively.

EA extracts (CG and CS) showed the ability to inhibit the protein expression of ES, NOX with the downregulated expression of the $NADP^+$/NADPH ratio. The $NADP^+$/NADPH ratio protein expression was significantly increased for HUVECs treated with H_2O_2 alone compared to control. HUVECs co-treated with 25 µM H_2O_2 and EA extract (20 and 5 µg/ml, CG and CS) show significant downregulated $NADP^+$/NADPH ratio (Figure 4.6A) as compared to H_2O_2 alone group, also similarly portrayed in HUVECs co-treated with Apocynin (20 µM, positive control group). Moreover, HUVECs treated with EA extract (20 µg/ml, CG and CS) alone did not show a significant difference expression of $NADP^+$/NADPH ratio compared to the control, contrary as compared with H_2O_2-treated alone group.

Nonetheless, the changes observed in $NADP^+$/NADPH ratio did not reflect to changes observed in COX-2 expression (Figure 4.6B). Incubation of H_2O_2 increased the expression of COX-2, remained the same in co-incubation of H_2O_2 and EA extracts, similarly seen in co-incubation with Apocynin. The results demonstrated no significant changes observed in co-incubation of H_2O_2 and EA extracts suggesting the anti-senescence effects of EA extracts did not involve in the COX-2 pathways of ES.

Figure 4.6:

(A) Fold change expression of $NADP^+$/NADPH ratio and **(B)** COX-2 expression of HUVECs co-treated with or without H_2O_2 (25 µM) and different concentrations of CG EA and CS EA (5 and 10 µg/ml).



Results are mean ± S.E.M of n=3 experiments. *p<0.05 compared with control #p<0.05 compared with H_2O_2.

NADPH oxidase (NOX) constitute major sources of ROS generation in endothelial cells, and their dysregulation significantly contributes to oxidative stress-induced cellular senescence (Chen et al., 2013; Fraga et al., 2023). NOX family enzymes, including NOX1, NOX2, and NOX4, are membrane-bound enzymes whose primary function is the transfer of electrons from NADPH to molecular oxygen, producing superoxide and other ROS as signaling molecules or stress agents (Cipriano et al., 2023). These ROS act as second messengers that can modulate various cellular processes, including inflammation, DNA damage, and cell cycle arrest, all of which are hallmarks of senescence (Kračun et al., 2025). Selective inhibition of NOX1 has been shown to rescue age-related impairments in blood flow and angiogenesis, reduce senescence markers, and restore vascular function, emphasizing the therapeutic potential of targeting NOX enzymes in ageing-related vascular dysfunctions (Li et al., 2021). NADPH oxidase also participates in a feedforward signaling loop involving inflammation, DNA damage, cell cycle arrest, and senescence-associated secretory phenotypes (SASP), intensifying tissue dysfunction and ageing phenotype. This has been particularly noted with NOX1 in both animal models and human tissues, where targeting NOX1 can effectively suppress senescence markers and inflammation, improving tissue repair and function (Li et al., 2021). According to our findings, HUVECs co-incubated with H_2O_2 and EA extracts downregulates the expression of NOX primarily induced by H_2O_2, simultaneously proved the protective effects of EA extract against senescence, mediated through decreasing NOX and inflammation caused by oxidative stress (Figure 4.6).

Next, cyclooxygenase-2 (COX-2) a type of inducible enzyme, is one of the many inflammatory stimuli specifically responsible for the conversion of arachidonic acid to pro-inflammatory prostaglandins. Under oxidative stress condition, COX-2 expression is elevated in response to ROS elevated mediated signaling and inflammatory signaling. Oxidative stress activated transcription factors including nuclear factor kappa B (NF- κB) and activator protein-1 (AP-1) further translocate to the nucleus and bind to promoter regions of inflammatory genes including COX-2 (Yamamoto & Gaynor, 2001). COX-2 was found to derive vasoconstrictor prostanoids and high amounts of NO from Inducible Nitric Oxide Synthase (iNOS) lead to vascular dysfunction including hypertension in angiotensin-II-infused mice (Lopes et al., 2022). Further studies support involvement of COX-2 in hypertensive rats, emphasised the upregulation of COX-2 in endothelial cells due to the sustained increase of oxidative stress (Wang et al., 2022; Wong et al., 2010). Arguably, COX-2 inflammatory pathway showed no significant difference of COX-2 values shows in H_2O_2-

induced SIPS HUVECs as compared to control (Figure 4.5B). This further postulate that the inflammation pathway of oxidative stress in H_2O_2-induced SIPS HUVECs may not be reflected in COX-2 inflammation pathway. On the other hand, further research may deem necessary to investigate other inflammation pathway including NF- κB which is a vital transcription factor mediating gene expression of inflammation and contributor to development of CVDs, in which when activated, possess elevated levels of pro-inflammatory cytokines including tumor necrosis factor-alpha, interleukin-1beta (IL-1 ß) and interleukin-6 (IL-6) (Anrather et al., 2006; Izzo et al., 2021).

4.6 *Calophyllum* sp. as potential natural senotherapeutics inhibiting endothelial senescence

The intricate relationship between oxidative stress, eNOS uncoupling, NO bioavailability, and endothelial dysfunction represents a central pathophysiological mechanism linking cellular senescence to CVDs. Under physiological conditions, eNOS catalyses NO production from L-arginine, generating a crucial vasodilatory and atheroprotective mediator (Raddino et al., 2007; Vallance & Hingorani, 1999). However, oxidative stress conditions, particularly those induced by H_2O_2 and other ROS, precipitate eNOS uncoupling which is a pathological state wherein eNOS generates superoxide (O2- −) rather than NO due to insufficient availability of the essential cofactor tetrahydrobiopterin (BH4) or L-arginine substrate depletion (Vallance & Hingorani, 1999; Verhaar et al., 2004). This uncoupling phenomenon transforms eNOS from a protective enzyme into a source of free radical production, generating peroxynitrite (ONOO−) through the reaction of superoxide with residual NO, thereby creating a vicious cycle of oxidative damage (Mudau et al., 2012; Verhaar et al., 2004). The resultant diminished NO bioavailability impairs endothelium-dependent vasodilation, promotes platelet aggregation, enhances leukocyte adhesion, and facilitates vascular smooth muscle proliferation (Little et al., 2021; Sukhovershin et al., 2015; Vallance & Hingorani, 1999). Natural product extracts intervene therapeutically at multiple nodes within this pathophysiological cascade. Anthocyanins from blueberries protect endothelial function against high-glucose injury via antioxidant and vasodilatory mechanisms, significantly ameliorating the vasodilatory effect by increasing NO and eNOS expression through the Phosphoinositide 3-kinase (PI3K)/protein kinase B

(Akt)/peroxisome proliferator-activated receptor gamma (PPARγ) signaling pathway (Huang et al., 2020). Olive-derived hydroxytyrosol increases NO bioavailability, thus favoring vasodilation, lowering blood pressure, and supporting vascular integrity (Milena & Maurizio, 2025; Vijakumaran et al., 2023; Zrelli et al., 2013). The PI3K/Akt/eNOS signaling axis represents a critical pathway through which natural products restore NO bioavailability. Alpha-lipoic acid activates eNOS through PI3K-Akt signaling pathway activation, inducing dose- and time-dependent phosphorylation of Akt and eNOS at serine 1177, the primary activating phosphorylation site (Fulton et al., 1999; Ying et al., 2015). Adenosine monophosphate-activated protein kinase **(**AMPK)-mediated eNOS phosphorylation at Serine 1177 (Ser1177) constitutes an alternative pathway exploited by natural compounds, with berberine and resveratrol demonstrating capacity to activate AMPK and subsequently enhance eNOS activity (McCarty, 2014; Reihill et al., 2007). These multifaceted interventions collectively restore the endothelial NO-dependent vasoprotective phenotype, counteracting senescence-associated endothelial dysfunction.

Next, endothelial dysfunction, characterised fundamentally by impaired NO bioavailability, represents an independent predictor of adverse cardiac events and serves as a pivotal early, reversible precursor of atherosclerosis (Mudau et al., 2012; Verhaar et al., 2004). The accumulation of senescent endothelial cells in the cardiovascular system drives vascular remodeling and promotes the pathogenesis of multiple cardiovascular and metabolic diseases, including atherosclerosis, heart failure, diabetes, hypertension, chronic kidney disease, and associated complications (Little et al., 2021; Wang & He, 2024). Cellular senescence in arterial tissues manifests through characteristic features including irreversible proliferation termination, flattened and enlarged morphology, pro-inflammatory secretory profile (senescence-associated secretory phenotype, SASP) and contribution to chronic sterile inflammation that drives cardiovascular pathology (Bu et al., 2023; Shimizu & Minamino, 2020).

The mechanistic links connecting endothelial senescence to CVDs encompass multiple converging pathways: telomere shortening accelerates endothelial senescence and promotes atherosclerotic plaque development (Chang & Harley, 1995; Katsuumi et al., 2018; Nazari-Shafti & Cooke, 2015), oxidative stress induces DNA damage and activates p53/p21/p16INK4a tumor suppressor pathways leading to growth arrest (Chen et al., 2022; Donato et al., 2015; Rippe et al., 2012), and mitochondrial dysfunction generates excessive ROS that perpetuates senescence and vascular inflammation (Chen et al., 2022; Katsuumi et

al., 2018; Torimoto & Eguchi, 2022). Studies examining telomere length in human vascular tissues have documented significantly greater rates of telomere loss in arterial endothelium compared to venous endothelium, with observations suggesting that chronic stress leading to cellular senescence is more pronounced in arterial intima than media, consistent with a role for focal replicative senescence in CVDs (Chang & Harley, 1995). The SASP associated with senescent endothelial cells secretes pro-inflammatory cytokines including IL-6, IL-1β, tumor necrosis factor alpha (TNF-α), matrix metalloproteinases and growth factors that disrupt vascular homeostasis, promote leukocyte recruitment, stimulate smooth muscle cell proliferation and migration, and destabilise atherosclerotic plaques (Bloom et al., 2023; Bu et al., 2023; Shimizu & Minamino, 2020). Due to many risk factors that contribute to

Natural product particularly *Calophyllum* sp. interventions targeting endothelial senescence offer promising therapeutic strategies for CVDs prevention and treatment. Polyphenols from diverse sources including resveratrol from grapes, (Epigallocatechin Gallate) EGCG from green tea, curcumin from turmeric, anthocyanins from berries, oleuropein and hydroxytyrosol from olive, and ellagic acid from pomegranate demonstrate capacity to reduce SASP-associated inflammation, restore tissue homeostasis, and attenuate cellular senescence across various ageing models (Bulotta et al., 2014; Della Vedova et al., 2025; Lockyer et al., 2017; Santos et al., 2024; Serino & Salazar, 2018; Woolf et al., 2023). These compounds improve anti-oxidant capacity, enhance mitochondrial function, promote autophagy, and reduce oxidative stress, inflammation, and cellular senescence in both vascular smooth muscle cells and endothelial cells (Serino & Salazar, 2018). The therapeutic potential extends to modulation of key longevity pathways including Sirtuin 1 (SIRT1) activation, AMPK stimulation, mechanistic target of rapamycin (mTOR) inhibition, and nuclear factor-erythroid 2-related factor 2 (Nrf2)-mediated antioxidant response, collectively promoting healthy vascular ageing and mitigating CVDs risk (Campagna et al., 2024; Dai et al., 2023; Fernandes & Demetriades, 2021; Law et al., 2024). Phenolic acids and flavonoids are organic compounds that can be found in many plants and have valuable properties such as antioxidants, inhibit the enzymes that lead to ROS formation which is one of the many factors leading to cellular senescence. p-coumaric acid (p-CA) is another antioxidant agent that can slow down the senescence process of cells by decreasing inflammatory response and chondrocyte activity, however, it is less proven to rectify the premature senescence of cells (Varesi et al., 2022).

Collectively, emerging evidence positions *Calophyllum* sp. as promising natural senotherapeutics for promoting healthy lifespan extension and reducing the burden of age-related CVDs (Della Vedova et al., 2025; Santos et al., 2024). This is evidently due to the Trapezifolixanthone compound, commonly found in *Calophyllum* sp. reportedly possess myriad of pharmacological bioactivities including anti-inflammatory, anti-bacterial and anti-viral effects (Lizazman et al., 2022). Hence, further support the presence of Trapezifolixanthone found in EA extract *Calophyllum* sp., mitigated H_2O_2-induced SIPS via decreasing the expression of senescence biomarkers including SA-β-Gal and NOX, which reduce the premature senescence and as a result, decreases ROS excess production and regulates the NO bioavailability. The present study provides new insights on understanding senescence pathways and therapeutic anti-senescence effects of *Calophyllum* sp. in preserving endothelial function in CVDs.

CHAPTER 5:

CONCLUSION

5.1 Conclusion

Senescence plays an important role in endothelial dysfunction. The high accumulation of senescent cells in vessel walls have been proven to contribute to risks of CVDs. The present study undermines the anti-senescence effects of *Calophyllum* sp. against SIPS-induced endothelial dysfunction.

First and foremost, the study focused on *Calophyllum* sp. EA extract against H_2O_2-induced SIPS in HUVECs, which normalised the enlarged and flattened morphology of senescent HUVECs to cobblestone-spindled shaped, which is typical in normal HUVECs. The application of EA extracts and Apocynin as NADPH inhibitor on H_2O_2-induced SIPS in HUVECs show no significant changes to control, with 25 µM H_2O_2 as the optimum concentration to induce SIPS. These results demonstrate that EA extract prevents H_2O_2-induced endothelial dysfunction by downregulating the effects of SIPS in endothelial cells.

Next, the study focused on the involvement of *Calophyllum* sp. EA extract in the expression of senescence biomarkers, SA-β-Gal in H_2O_2-induced SIPS HUVECs. Incubation of H_2O_2-induced SIPS HUVECs resulted in increased expression of positive SA-β-Gal, which was reversed by Apocynin and EA extracts. The findings further demonstrated the protective effects of *Calophyllum* sp. EA extracts against endothelial senescence induced by oxidative stress via inhibition of senescence markers, SA-β-Gal, among many others.

The third part of the study demonstrated the anti-senescence effects and involvement of EA extract in the endothelial stress pathway. Initially, incubation of H_2O_2-induced SIPS increased the expression of NADPH oxidase, which were then downregulated with the co-incubation with EA extracts. Similarly, the co-incubation of H_2O_2-induced SIPS with Apocynin, a type of NADPH inhibitor also downregulated the expression of NADPH oxidase. This further amplifies the protective effect of *Calophyllum*

sp. EA extract against senescence via regulation of NADPH oxidase pathway induced by oxidative stress.

Lastly, the study focused on the involvement of EA extract on COX-2 pathway, which is one of the vital pathways in regulating oxidative stress. This study undermined that incubation with H_2O_2-induced SIPS and EA extract as well as with Apocynin proved to have no significant differences. The incubation of H_2O_2-induced SIPS HUVECs increases the expression of COX-2, correspondingly seen in co-incubation H_2O_2-induced SIPS with EA extract. These results suggested that the no linkage of COX-2 pathways with the anti-senescence effect of *Calophyllum* sp. EA extracts and thus further investigation is crucial in understanding the pathways involved.

Overall, the current investigation demonstrated the therapeutic effects of *Calophyllum* sp. EA extract against senescence via normalisation of the senescence morphology, decreasing the expression of senescence biomarkers including SA-β-Gal, and inhibition of NADPH oxidase. These potent pharmacological effects of *Calophyllum* sp. not only mitigate senescence in endothelial cells but also improve the NO bioavailability in the vascular system, thus reduce the risk of CVDs. Taken together, *Calophyllum* sp. extracts offer remarkable therapeutic bioactive compounds with promising potential as a natural remedy in preventing endothelial dysfunction caused by endothelial senescence in the future.

5.2 Recommendations

HUVECs were induced by H_2O_2 to establish the SIPS model and produced significant differences as compared to the control, which were shown in the results of phase contrast, senescence biomarkers including SA-β-Gal, and senescence pathways involved, NOX, but not in the COX-2 inflammatory pathway. This further highlighted the need for a standardised SIPS induction method to improve efficacy. Furthermore, an *in silico* approach as a step to predict the binding and molecular interactions between the isolated compounds and targeted proteins in the senescent-related pathways as well as filtering prior to *in vitro* screening and *in vivo* testing, could also be executed to avoid unnecessary cost waste and sacrifice of animals. Moreover, additional method detection for different senescence pathways should be explored to produce insights on the protective effects of EA extracts against senescence, including IL-6, IL-8, Plasminogen activator inhibitor-1

(PAI-1) and TNF-α, among many others. Additionally reveals the invaluable insights of selectivity or broad activity of *Calophyllum* sp. EA extracts mitigating senescence and the pathways involved. Future studies should emphasise the anti-senescence effects of *Calophyllum* sp. extracts as well as isolated compounds to elucidate their pharmacological effects and further contribute to pharmaceutical applications due to their myriad phenolic compounds.

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