PROTECTIVE EFFECTS OF *Alternanthera sessilis* AGAINST SELECTED PRO-INFLAMMATORY MEDIATORS-INDUCED OXIDATIVE STRESS AND ENDOTHELIAL ACTIVATION IN HUMAN AORTIC ENDOTHELIAL CELLS

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By

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A dissertation submitted to the Department of Pre-Clinical Sciences,

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ABSTRACT

PROTECTIVE EFFECTS OF *Alternanthera sessilis* AGAINST SELECTED PRO-INFLAMMATORY MEDIATORS-INDUCED OXIDATIVE STRESS AND ENDOTHELIAL ACTIVATION IN HUMAN AORTIC ENDOTHELIAL CELLS

Nur Nadia binti Mohd Razali

Oxidative stress and activation of human aortic endothelial cells (HAEC) have been shown to contribute to the early stage of various pathological conditions such as atherosclerosis and hypertension. In these conditions, excess production of reactive oxygen species (ROS) and increased expression of cell adhesion molecules impair the endothelial barrier function, thereby increasing endothelial permeability. Tumor necrosis factor-α (TNF-α), a key player of atherogenesis, stimulates endothelial activation and oxidative stress in early atherosclerosis. Currently, statins which are widely used in the treatment of atherosclerosis by lowering cholesterol levels can cause adverse side effects and the treatment outcome is often unsatisfactory. Therefore, new therapeutic agents which target endothelial activation needs to be discovered for the prevention of early atherosclerosis. *Alternanthera sessilis,* popularly known as "sessile joyweed" or "*daun keremak*", is a traditional herbal plant previously shown to possess anti-oxidant and anti-inflammatory effects. This study aimed to investigate protective effects of *A. sessilis* ethanolic extract on HAEC hyperpermeability, vascular cell adhesion-1 (VCAM-1) expression, ROS and hydrogen peroxide (H_2O_2) productions. Activities of anti-oxidant enzymes including superoxide dismutase (SOD) and catalase (CAT) were also examined. To select a range of doses which were not cytotoxic, effects of 6.25 - 800 µg/ml of *A. sessilis* ethanolic extract on HAEC viability was first determined using 3-(4,5-

Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) assay. Effects of *A. sessilis* on endothelial hyperpermeability was then evaluated using fluorescein isothiocyanate (FITC)-dextran permeability assay. Soluble VCAM-1 levels were assessed using enzyme-linked immunosorbent assay (ELISA). ROS and H_2O_2 productions were studied using 2',7'-dichlorodihydrofluorescein diacetate (H_2-DCFDA) and Amplex Red fluorescent dyes, respectively. SOD and CAT activities were evaluated using commercial SOD and CAT assay kits. The MTT result showed that 25-200 µg/ml *A. sessilis* ethanolic extract did not cause significant HAEC death. *A. sessilis* ethanolic extract significantly inhibited TNF-α-induced hyperpermeability in HAEC at 200 µg/ml. The ROS assay also showed that 200 µg/ml of *A. sessilis* ethanolic extract significantly reduced TNF-α-induced increased ROS production in HAEC. However, *A. sessilis* failed to suppress increased H_2O_2 production induced by TNF-α. *A. sessilis* at all concentrations tested did not reduce increased VCAM-1 expression stimulated by TNF-α. Furthermore, *A. sessilis* ethanolic extract dramatically improved H_2O_2-induced decreased SOD and CAT activities. In conclusion, *A. sessilis* protects against selected pro-inflammatory mediator-induced endothelial activation and this is associated with inhibition of oxidative stress. This study reveals new knowledge on how *A. sessilis* suppresses endothelial activation and provides understanding on the mechanism by which *A. sessilis* protects HAEC against detrimental effects caused by TNF- α and H_2O_2.

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APPROVAL SHEET

This dissertation/thesis entitled "**PROTECTIVE EFFECTS OF *Alternanthera sessilis* AGAINST SELECTED PRO-INFLAMMATORY MEDIATORS-INDUCED OXIDATIVE STRESS AND ENDOTHELIAL ACTIVATION IN HUMAN AORTIC ENDOTHELIAL CELLS**" was prepared by NUR NADIA BINTI MOHD RAZALI and submitted as partial fulfilment of the requirements for the degree of Master of Medical Science at Universiti Tunku Abdul Rahman.

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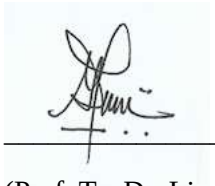

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SUBMISSION OF THESIS

It is hereby certified that **Nur Nadia binti Mohd Razali** (ID No: **17UMM07810**) has completed this thesis entitled "PROTECTIVE EFFECTS OF *Alternanthera sessilis* AGAINST SELECTED PRO-INFLAMMATORY MEDIATORS-INDUCED OXIDATIVE STRESS AND ENDOTHELIAL ACTIVATION IN HUMAN AORTIC ENDOTHELIAL CELLS" under the supervision of Assistant Professor Dr. Fong Lai Yen (Supervisor) from the Department of Pre-Clinical Sciences, Faculty of Medicine and Health Sciences, Professor Ts. Dr. Lim Yang Mooi (Co-Supervisor) from the Department of Pre-Clinical Sciences, Faculty of Medicine and Health Sciences, and Dr. Ng Chin Theng (External Co-Supervisor) from Physiology Unit, Faculty of Medicine, AIMST University.

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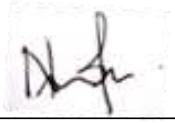

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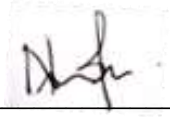
(Nur Nadia binti Mohd Razali)

DECLARATION

I Nur Nadia binti Mohd Razali hereby declare that the thesis is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UTAR or other institutions.



(NUR NADIA BINTI MOHD RAZALI)

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LIST OF ABBREVIATIONS

H_2-DCFDA	2′,7′-Dichlorodihydrofluorescein diacetate
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
ACE	angiotensin-converting enzyme
BSA	bovine serum albumin
CCl_4	carbon tetrachloride
CAT	catalase
CAM	cell adhesion molecules
JNK	c-Jun N-terminal kinase
COX-2	cyclooxygenase-2
CRD	cysteine-rich domain
DMSO	dimethyl sulphoxide
ERK1/2	extracellular signal-regulated kinase 1/2
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
GPX	glutathione peroxidase
HAEC	human aortic endothelial cell
HCAEC	human coronary aortic endothelial cell
H_2O_2	hydrogen peroxide
•OH	hydroxyl radical
ICAM-1	intercellular adhesion molecule-1
IL	interleukin
LPS	lipopolysaccharide
LC-MS	liquid chromatography mass spectrometry

LDL	low density lipoprotein
MAPK	mitogen-activated protein kinase
NAC	N-acetyl cysteine
NOX	NADPH oxidase
NADPH	nicotinamide adenine dinucleotide phosphate
NF-κB	nuclear factor kappa B
NSAID	non-steroidal anti-inflammatory drug
ONOO•	peroxynitrite
PBS	phosphate buffered saline
PECAM-1	platelet endothelial cell adhesion molecule
PES	polyether sulphones
ROS	reactive oxygen species
S.E.M	standard error of means
O_2^-	superoxide anion
SOD	superoxide dismutase
TNFR	TNF-α receptor
TNF-α	tumor necrosis factor-α
UHPLC	ultra high-performance liquid chromatography
VCAM-1	vascular cell adhesion molecule-1
VWF	von Willebrand factor
WPB	Weibel-Palade bodies

CHAPTER 1

INTRODUCTION

1.1 Background of Study

Natural products play an important role in the development of new drug entities as approximately 26% of new drugs that are currently being developed are derived from natural products. Interestingly, natural products also serve as the origin of about 50% of cardiovascular drugs developed since 1980 to present (Zhao et al., 2020). *Alternanthera sessilis*, popularly known as "sessile joyweed" worldwide or "*daun keremak*", is a traditional herb which is usually consumed as raw vegetables in Malaysia (Bhuyan et al., 2018). It is also traditionally used as a herbal remedy to treat some diseases such as asthma, bronchitis, wounds, diarrhea and diabetes (Gupta et al., 2012; Hosamani et al., 2004). In the context of scientific evidence, *A. sessilis* has previously been reported to exhibit hepatoprotective, anti-hyperglycemic, analgesic, anti-inflammatory and anti-oxidant effects (Bhuyan et al., 2018; Chai et al., 2016; Hazli et al., 2019; Hossain et al., 2014; Rayees et al., 2013).

A. sessilis whole plant ethanolic extract has been reported to protect liver tissues in carbon tetrachloride (CCl_4)-induced rats from hepatotoxicity by improving lipid profile and reducing oxidative stress (Bhuyan et al., 2018). Besides, methanolic extract of the aerial part of *A. sessilis* has also been reported to possess analgesic and antihyperglycemic activities in acetic acid-induced mice (Hossain et al., 2014). In RAW 264.7 cells, *A. sessilis* stem extract demonstrated anti-inflammatory activities through reducing the production of cytokines and pro-inflammatory mediators induced by lipopolysaccharide (Muniandy et al., 2018a). A previous study on the total phenolic content of *A. sessilis* showed that the plant contains flavonoids,

phenols, alkaloids, terpenoids and physterols (Kota et al., 2017). The authors also demonstrated that organic solvent extracts of *A. sessilis* possess both free radical scavenging activity and reducing power ability. Despite a number of studies which reported about pharmacological activities of *A. sessilis*, the effect of *A. sessilis* on endothelial activation and oxidative stress remains unknown.

Atherosclerosis is a chronic inflammatory disease where excessive accumulation of lipids, cholesterol, inflammatory cells and connective tissue in blood vessel walls causes the buildup of atherosclerotic plaques, which eventually leads to cardiovascular diseases (Funk et al., 2012). Endothelial activation is known to be one of the main causes of atherosclerosis. The endothelium, which is composed of endothelial cell monolayer, lines the wall of blood vessels and functions to maintain vascular homeostasis through the regulation of permeability, inflammatory responses, vascular tone and hemostasis. In physiological conditions where endothelial cells are not activated, the endothelium serves as a semi–permeable barrier that tightly regulates the basal permeability and exhibits anti-thrombotic and anti-inflammatory phenotypes. Upon stimulation with various types of pro-inflammatory mediators such as cytokines, endotoxin and oxidized-low density lipoprotein, the endothelium is activated where the endothelium switches its phenotypes to pro-thrombotic and pro-inflammatory (Medina-Leyte et al., 2021). As a result, disruption of the endothelial barrier occurs and this causes an increase in endothelial permeability and increased leukocyte extravasation.

Apart from endothelial activation, oxidative stress also plays a crucial role in the development of atherosclerosis, in which the interaction between reactive oxygen species (ROS) and free radicals with endothelial cells can cause endothelial activation (Nowak et al., 2017). Oxidative stress is characterized by an imbalance between ROS production and the anti-oxidant defense

mechanism in cells. At a basal level, ROS are important for cell signaling and many other physiological functions including angiogenesis and differentiation (Sena et al., 2012). However, excessive amount of ROS oxidizes protein molecules in cells and causes irreversible cellular damage. Accumulating studies from the past decade have also highlighted the role of vascular cell adhesion molecule-1 (VCAM-1), a cell surface receptor, in mediating increased endothelial permeability through ROS production. Endothelial cells increase the surface expression of VCAM-1 in response to cytokines, thereby promoting leukocyte adhesion and transducing intracellular signals to impair the endothelial barrier (Vockel et al., 2013).

The anti-oxidant defense system in cells is maintained by several anti-oxidant enzymes such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX). Reduced activity of anti-oxidant enzymes is closely associated with metabolic and coronary artery diseases (Lubrano et al., 2015). SOD converts the highly reactive radical superoxide anion (O_2^-) to the less reactive radical hydrogen peroxide (H_2O_2), which can be further destroyed by CAT and GPX. An overexpression of SOD has been shown to inhibit tumor necrosis factor-α (TNF-α)-induced both increased cell adhesion molecule expression and enhanced neutrophil adhesion in human aortic endothelial cells (HAEC), suggesting the importance of this enzyme in the prevention of early atherosclerosis (Fujimoto et al., 2007).

In addition to lifestyle modifications and supplementations, current approaches to treat atherosclerosis focus on medical treatment which includes lipid-lowering drug treatment by statins, as well as reduction of inflammation using non-steroidal anti-inflammatory drugs (NSAID), glucocorticoids or cyclooxygenase-2 inhibitors (Pedro-Botet et al., 2020; Ruiz-León et al., 2019). However, these pharmacological approaches are often associated with side effects, whereas bioactive components from natural products have potentials to reduce the progression

of atherosclerosis and are associated with minimal side effects (Scolaro et al., 2018). Studies on phytochemical constituents of *A. sessilis* indicated that the plant contains flavonoids, phenols and alkaloids which are associated with antioxidant, anti-inflammatory and cardioprotective properties. Besides that, *A. sessilis* has also been previously shown to suppress the release of pro-inflammatory cytokines and to possess antihyperglycemic and analgesic activities. Therefore, in this study, the effect of *A. sessilis* on the prevention of early atherogenic events such as endothelial activation and oxidative stress in HAEC was examined.

1.2 Problem Statement

Activation of the endothelium and oxidative stress initiate the formation of atherosclerotic lesions, which eventually lead to cardiovascular diseases. Currently, treatments for atherosclerosis focus on lowering plasma lipids. Statins are commonly used in clinical practice to treat atherosclerosis by lowering plasma cholesterol levels. Despite successful stories of statins in reducing risks of cardiovascular events, statins have been shown to cause adverse effects such as myopathy and hepatotoxicity (Bellosta et al., 2018; Broniarek et al., 2020). Therefore, new therapeutic agents which target endothelial activation needs to be discovered for the prevention and treatment of atherosclerosis. Previously, *A. sessilis* has been shown to suppress the release of pro-inflammatory cytokines and to possess antihyperglycemic and analgesic activities. However, no study has highlighted the protective effect of *A. sessilis* in activated HAEC, and the mechanism of action underlying the endothelial protective effect of *A. sessilis* has also yet to be elucidated. More evidence is needed to understand how *A. sessilis* can prevent endothelial activation and oxidative stress, which are key cellular events that occur in early atherosclerosis.

1.3 Research Questions

(i) Does treatment with *A. sessilis* ethanolic extract affect HAEC viability?

(ii) Does *A. sessilis* ethanolic extract protect against barrier impairment in TNF-induced HAEC?

(iii) What are the effects of *A. sessilis* ethanolic extract on TNF-α-induced increased ROS and H_2O_2 generation in HAEC?

(iv) What are the effects of *A. sessilis* ethanolic extract on TNF-α-induced increased soluble VCAM-1 expression?

(v) What are the effects of *A. sessilis* ethanolic extract on the activities of antioxidant enzymes (SOD and CAT) in H_2O_2 induced HAEC?

1.4 Objectives

General objective:

The aim of this study was to evaluate effects of *A. sessilis* ethanolic extract on TNF-α or H_2O_2-induced endothelial activation and oxidative stress in HAEC.

Specific objectives:

(i) To determine the viability of HAEC treated with *A. sessilis* ethanolic extract

(ii) To determine the endothelial barrier protective effect of *A. sessilis* ethanolic extract

(iii) To evaluate the effects of *A. sessilis* ethanolic extract on TNF-α-induced increased ROS and H_2O_2 generation

(iv) To evaluate the effects of *A. sessilis* ethanolic extract on TNF-α-induced increased soluble VCAM-1 expression

(v) To assess the effects of *A. sessilis* ethanolic extract on the activity of antioxidant enzymes (SOD and CAT) in H_2O_2-induced HAEC

1.5 Hypotheses

(i) *A. sessilis* ethanolic extract will not cause HAEC death

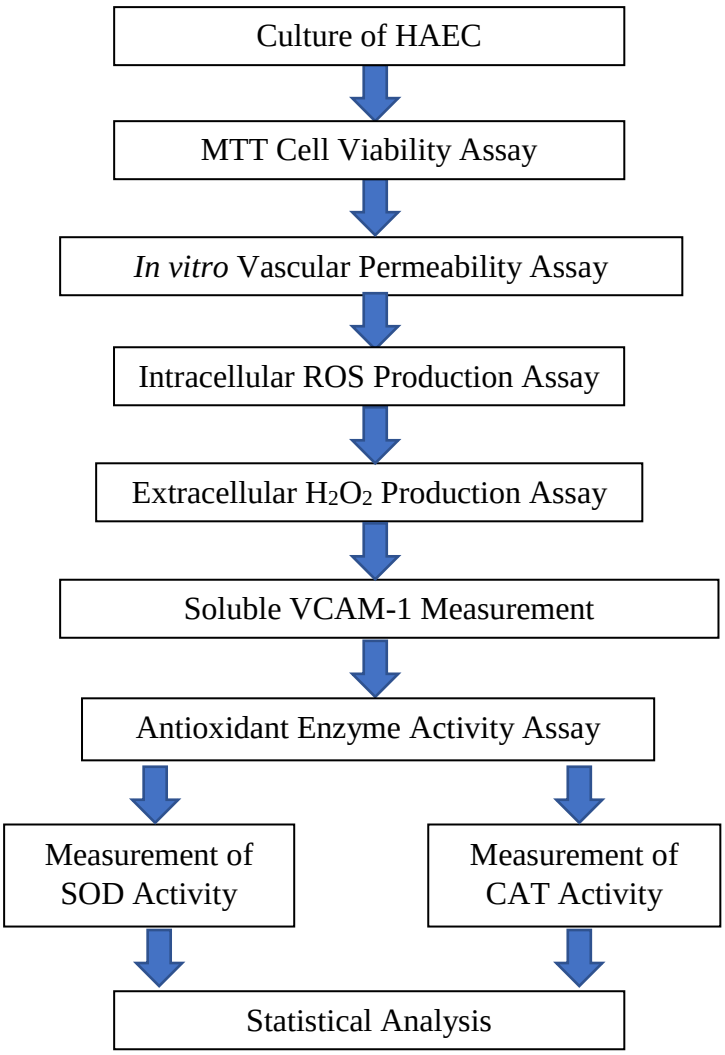
(ii) *A. sessilis* ethanolic extract will improve TNF-α-induced endothelial barrier impairment in HAEC

(iii) *A. sessilis* ethanolic extract will reduce increased ROS and H_2O_2 generation induced by TNF-α in HAEC

(iv) *A. sessilis* ethanolic extract will reduce increased soluble VCAM-1 expression induced by TNF-α in HAEC

(v) *A. sessilis* ethanolic extract will increase reduced SOD and CAT activities in H_2O_2-induced HAEC

1.6 Research Flow



CHAPTER 2

LITERATURE REVIEW

2.1. *Alternanthera sessilis*

Alternanthera sessilis **(Figure 2.1),** also known as '*keremak*', '*kermak putih*', '*daun tolod*', or '*bunga-bunga*' in Malaysia, is a traditional herbal remedy that belongs to the Amaranthaceae family. It is also popularly known as "sessile joyweed" or "dwarf copperleaf" worldwide. Other names for *A. sessilis* include "*Hongtyang wu*" (Malaysia, Singapore and Taiwan), "*Matikanduri*" (India), "*Dauntolod*" (Indonesia), "*Brede chevrette*" (France), "*Lian zi cao*" (China), "*Chanchii shak*" (Bangladesh), "*Gandal*" or "*Gudisak*" (Pakistan) (Bhuyan et al., 2018; Hossain et al., 2014; Tan et al., 2013). The plant is also found in other countries such as Indonesia, Sri Lanka and Nepal (Mondal et al., 2014).

This plant has been reported to grow in a variety of soil types. In India, *A. sessilis* can be found in the warmer regions, while in Bangladesh and Pakistan, it is reported to grow in more humid settings such as wetlands and swampy areas (Rayees et al., 2013). In India, its height was reported to be up to 50 cm, whereas in Malaysia it was reported to be up to 1.4 meters with 1.3 to 3 cm long and 0.5 to 1 cm wide leaves in linear-oblong shape (Kota et al., 2017). The leaves and stems are widely consumed by Indians especially in South India as leafy vegetables for multiple health benefits. The leaves are either cooked or consumed as raw salad (Saqib et al., 2016).

Figure 2.1: *Alternanthera sessilis* plant (Adapted from (Shehzad et al., 2018)).

2.1.1. Traditional Uses of *A. sessilis*

A. sessilis has been traditionally used as a herbal remedy by folk medicinal practitioners in India, Bangladesh and Ayurvedic medicine for hundreds of years to treat wound, vomiting, infertility, gonorrhea, leucorrhea, night blindness, malaria, pain, fever, diarrhea, cough, asthma, headache, inflammation, diabetes, blood dysentery and nausea, among others (Hossain et al., 2014; Saqib et al., 2016). Apart from being a popular traditional herb, *A. sessilis* is also a popular vegetable for general health wellbeing. It has been reported to be prepared as decoction and consumed orally to treat fever by communities in traditional communities in Pakistan (Ahmad et al., 2016). In Malaysia, *A. sessilis* is listed as one of the popular *ulam* (salad), commonly consumed raw or boiled and usually eaten with rice (Bachok et al., 2014).

2.1.2. Phytochemical Constituents and Isolated Compounds from *A. sessilis*

Pharmacological effects of *A. sessilis* are mainly attributed to the presence of phytochemical contents in the plant. Several studies have been done to identify active compounds and phytochemical constituents in different parts of the plant. Kota *et al.* in 2017 published a study on the investigation of phytochemicals in *A. sessilis* leaves, in which the plant was extracted using organic solvents of different polarities including petroleum ether, ethyl acetate, chloroform, acetone and methanol. These plant extracts were screened using various assays

and the results showed that all these organic solvent extracts contain flavonoids, alkaloids, phenols, terpenoids and phytosterols. In addition, methanolic extract of *A. sessilis* was also shown to contain other constituents such as tannins, steroids, reducing sugars and cardiac glycosides (Kota et al., 2017). The presence of these constituents, together with high 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity, total phenolic content and reducing power, suggests the antioxidant potential of *A. sessilis* extract. This finding is in agreement with another study previously done on an ethanolic extract derived from *A. sessilis* whole plant, where flavonoids, tannins, alkaloids, cardiac glycosides, saponins and phenols were identified (Saqib et al., 2016).

Flavonoids have been widely reported to be beneficial to cardiovascular health by showing antioxidant and anti-inflammatory effects in endothelial cells by inhibiting TNF-α-induced CAM expression (Lotito et al., 2006). Besides flavonoids, alkaloids are also important phytochemical constituents present in natural products that have been contributing to positive effects in various studies involving endothelial functions and atherosclerosis. For instance, previous studies have shown that rhynchophylla total alkaloid, which is derived from *Uncaria,* protects endothelial cells from autophagy by reducing oxidative stress and increasing vasodilation of endothelium (Li et al., 2018). Other *in vitro* and *in vivo* studies have reported the potential of phytochemicals including alkaloids, flavonoids and terpenoids in protecting endothelial cells by targeting inflammatory mediators such as TNF-α, ROS, CAM and interleukins (Bujor et al., 2021).

Identification of compounds in *A. sessilis* stem ethanolic extract using liquid chromatography mass spectrometry (LC-MS) showed the presence of 2”-O-rhammnosylvitexin, apigenin-6,8-di-C-β-D-glucopyraroside isomer, kaempferol monosulfate, p-hydroxybenoic acid, p-

hydroxycinnamoyl moiety, protocatehuic acid, gibberellin, daidzein and benzophenone-4 (Muniandy et al., 2018a). In addition, another study also demonstrated the presence of other phenolic compounds such as ferulic acid, apigenin, quercertin and rutin in *A. sessilis* (red) leaf and stem using ultra high performance liquid chromatography (UHPLC) (Hazli et al., 2019). This study also revealed that the total phenolic content present in *A. sessilis* leaf is higher compared to that in the stem, and the ethanolic extract has higher total phenolic content than water, ethyl acetate and hexane extracts. (Hazli et al., 2019).

2.1.3. Biological Activities of *A. sessilis*

A. sessilis has been reported to demonstrate a number of biological activities. The reported activities include anti-hyperglycemic, analgesic, hepatoprotective, anti-inflammatory, antioxidant, and wound healing effects (Bhuyan et al., 2018; Chai et al., 2016; Hazli et al., 2019; Hossain et al., 2014; Muniandy et al., 2018b; Rayees et al., 2013).

In vitro studies have been done to demonstrate biological activities of *A. sessilis*. Anti-inflammatory activity of an ethanolic extract derived from the stem of *A. sessilis* was shown in lipopolysaccharide (LPS)-stimulated RAW 264.7 cells. 50-200 µg/ml of *A. sessilis* stem extract suppressed nitric oxide production and inhibited the production of pro-inflammatory mediators such as prostaglandin E2, IL-6, IL-1β and TNF-α in a dose-dependent manner. In addition, *A. sessilis* also inhibited both nuclear factor kappa B (NF-κB) p65 subunit translocation and increased expression of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) dose-dependently. The authors postulated that anti-inflammatory effect of *A. sessilis* is associated with the inactivation of the NF-κB pathway (Muniandy et al., 2018a). Apart from anti-inflammatory activity, another *in vitro* study also showed wound healing activity of *A. sessilis*. *A. sessilis* was found to promote wound closure progression, which was indicated by

increased migration of keratinocytes and fibroblast cells in an *in vitro* wound scratch model (Muniandy et al., 2018b). Besides that, ethyl acetate fraction from the methanolic extract of *A. sessilis* leaves and callus has previously been shown to possess antiglucosidase and antihyperglycemic activities (Chai et al., 2016)

Several *in vivo* models have also been done to investigate biological activities of *A. sessilis*. 50 – 400 mg/kg body weight of *A. sessilis* methanolic extract was reported to reduce blood glucose concentration dose-dependently, and the inhibitory effect was comparable to a standard drug, glibenclamide, in glucose-fed albino mice. Using an acetic acid-induced pain model, the *A. sessilis* extract was also shown to reduce the number of abdominal constrictions in Swiss albino mice. The authors hypothesized that flavonoid and alkaloid present in *A. sessilis* might contribute to the anti-hyperglycemic activity, while the analgesic effect could be due to the ability of *A. sessilis* to inhibit lipooxygenase and cyclooxygenase activities, blocking prostaglandin synthesis that is responsible for pain sensation (Hossain et al., 2014). The hepatoprotective effect of *A. sessilis* was also demonstrated by Bhuyan *et al.* in 2017. In this study, methanolic extract of *A. sessilis* was shown to lower elevated concentrations of serum glutamic pyruvic transaminase, glutamic-oxaloacetic transaminase, alkaline phosphate, cholesterol and bilirubin in Wister albino rats with liver injury induced by CCl_4. In addition, the study also showed that *A. sessilis* inhibits oxidative stress in CCl_4-induced rats by suppressing lipid peroxidation and increasing the reduced activities of anti-oxidant enzymes such as glutathione reductase and CAT (Bhuyan et al., 2018).

2.2. The Endothelium

The endothelium is a continuous monolayer of cells that forms the wall of blood vessels, separating tissues and the blood. Vesicles and caveolae are present in the apical membrane of

endothelial cells and function to transport substances to the basal membrane via a transcellular route (Christiakov et al., 2015). Endothelial cells line blood vessels including arteries, veins and microvessels. The endothelium is supported by the basement membrane and smooth muscles cells at the basal surface. Endothelial cells bind to each other with the help of tight junctions and adherens junctions (Pierce et al., 2017).

One distinctive marker for endothelial cells is the Weibel-Palade bodies (WPB), which is an organelle that exclusively presents only in vascular endothelial cells. WPB are secretory granules that act as intracellular storage pools for a number of protein mediators and activate rapid responses upon vascular perturbation through the release of crucial protein components from endothelial cells to the circulation (Cookson et al., 2013; Rondaij et al., 2006). Among the components of WPB is the von Willebrand factor (VWF) which plays an important role in blood coagulation. Besides that, other markers also include angiotensin-converting enzyme (ACE), CAM and cobblestone morphology of the cell monolayer (Goncharov et al., 2017).

2.2.1. Functions of the Endothelium

The endothelium plays an active role to control the vascular integrity and permeability. It acts as a semipermeable barrier that regulates homeostasis between fluid and tissue by controlling macromolecule, solute and fluid exchange, and leukocyte migration (Christiakov et al., 2015; Di et al., 2016). Under normal circumstances, endothelial cells exhibit low permeability towards fluid and macromolecules and this results in the formation of the endothelial barrier, whereby only a basal level of permeability is noticed. The selective transport of fluid and macromolecules are tightly regulated by transcellular vesicles and intercellular junction proteins (Gimbrone et al., 2016).

Besides, endothelial cells also regulate vascular inflammation by mediating leukocyte recruitment upon infection or tissue damage. In physiological conditions, endothelial cells do not express adhesion molecules on their luminal surface and their interaction with leukocytes are therefore, prevented. Surface adhesion molecules are highly expressed on endothelial cells in response to inflammation and chemokines are produced to recruit leukocytes to the site of inflammation. (Pierce et al., 2017).

Furthermore, endothelial cells are also in charge of controlling the vascular tone by producing nitric oxide synthase that generates nitric oxide. As endothelial cells are continuously exposed to a laminar blood flow, nitric oxide is produced at basal levels based on the information received from shear force. The shear stress, which is detected by endothelial cells, arises from the friction between the vessel wall and the blood volume, as well as from the blood pressure. Nitric oxide diffuses from endothelial cells into smooth muscle cells and causes vasodilation, where smooth muscle cells relax and the lumen of blood vessels becomes wider (Fels et al., 2020).

2.2.2. Arterial versus Venous Endothelium

Arteries are blood vessel that carries oxygenated blood from the heart, while veins are the ones that carries deoxygenated blood towards the heart. Arteries are equipped with thick layers of tunica media and tunica adventitia since they are exposed to high blood pressure. On the other hand, veins have larger lumen diameter and thinner vessel wall to minimize blood flow resistance as it accommodates larger volume of blood (Niklason et al., 2018). Although arterial and venous endothelium generally serves the same purpose, some of their characteristics differ in terms of the cellular morphology, gene expression profile, extracellular matrix production and cell surface properties (Kruger-Genge et al., 2019). Morphologically, arterial endothelial

cells are thicker, narrower and longer compared to venous endothelial cells. The intercellular junctions in arterial endothelium are also much tighter compared to those in veins (dela Paz et al., 2009). In the arteries, platelet-rich thrombi are formed, while in veins, venous thrombosis are often initiated in the valves (Goncharov et al., 2017).

2.2.3. Atherosclerosis

Atherosclerosis is a chronic inflammatory disease with excessive accumulation of lipids, cholesterol, inflammatory cells and connective tissue in the arterial wall. Risk factors contribute to atherosclerosis include high non-high density lipoprotein (HDL) cholesterol levels, high low density lipoprotein (LDL) cholesterol levels, smoking, hypertension, obesity and diabetes mellitus (Funk et al., 2012). The American Heart Association (AHA) has classified atherosclerosis into several types based on the coronary thrombosis mechanisms which are AHA Type I lesion (intimal thickening), AHA Type II lesion (intima xanthoma/fatty streak), AHA Type III lesion (pathological intimal thickening) and AHA Type IV lesion (fibroatheroma) (Sakakura et al., 2013). Atherosclerosis is strongly associated with coronary heart disease and cerebrovascular disease, as the atherosclerotic plaque rupture or thrombosis is the common cause that leads to myocardial infarction and stroke. Atherogenesis starts with the increase of cholesterol levels in the plasma and altered permeability of endothelial cells, which in turn leads to lipid accumulation in the intima layer of the arterial wall (Libby et al., 2011).

Atherosclerosis formation is a continuous process, and is divided into six major stages. The first stage is the activation of endothelial cells induced by oxidized-LDL. This is followed by the second stage where there is dysfunction of endothelial cells, characterized by overexpression of CAM. This in turn initiates stage three, where inflammation occurs and

circulating immune cells are recruited into the inflammation site. In the fourth stage, smooth muscle cells migrate from the tunica media layer into the tunica intima layer, forming a fibrous cap and increasing extracellular matrix. Finally, in stage five, resident and immune cells secrete calcified fibro-lipid plaque, leading to unstable plaques and thrombus formation in stage six (Georgescu et al., 2021).

2.2.4. Endothelial Activation

Endothelial activation **(Figure 2.2**), occurs in lesion-prone regions in the arterial vasculature, is associated with cardiovascular diseases and is one of the key cellular events that happens in the early stage of atherosclerosis. It has been suggested that endothelial activation is manifested as a non-adaptive alteration of the endothelial function, which greatly affects the regulation of homeostasis and thrombosis, redox balance, vascular tone and inflammation (Gimbrone et al., 2016). Endothelial activation is characterized by an alteration of the phenotype of endothelial cells, from anti-inflammatory and anti-thrombotic to pro-inflammatory and pro-thrombotic, which results in enhanced permeability, increased adhesion molecule expression and increased leukocyte recruitment.

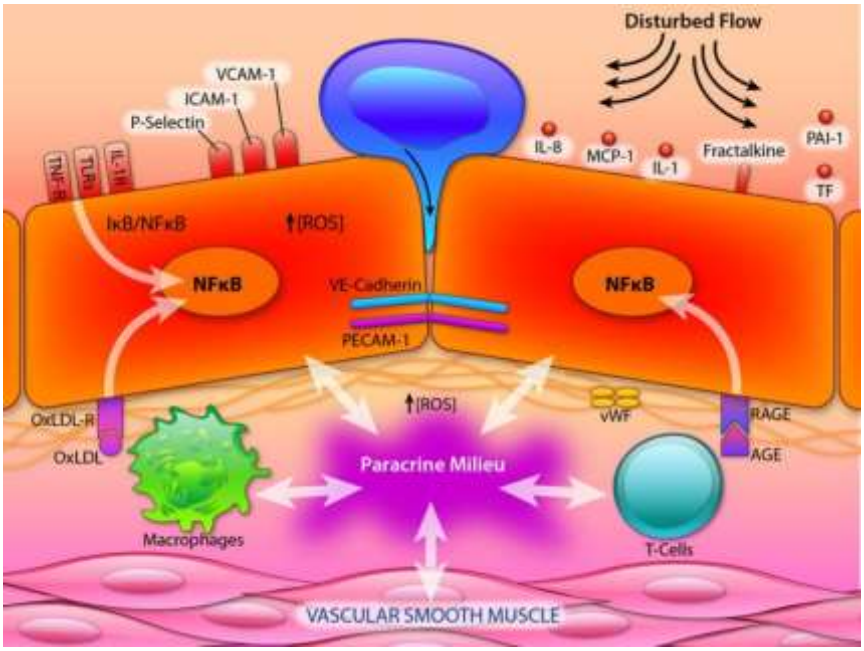


Figure 2.2: Endothelial activation. Inflammatory signaling mechanisms including CAM are upregulated through the release of inflammatory cytokines and chemokines. Circulating leukocytes are then recruited and transmigrate through the endothelium. This is accompanied by increased permeability and endothelial barrier dysfunction (Adapted from (Gimbrone et al., 2016)).

Activation of endothelial cells can be triggered by various types of stimuli include TNF-α, interleukins, lipopolysaccharides, oxidised low-density lipoprotein, vasoconstrictors, shear stress and extracellular matrix proteins (Funk et al., 2012). When endothelial cells are exposed to these stimuli, they increase the expression of CAM such as VCAM-1, intercellular adhesion molecule-1 and E-selectin on the luminal surface in order to attract and enhance the attachment of leukocytes (Pierce et al., 2017). In the early stage of atherosclerosis, recruited monocytes pass through the endothelial cell monolayer and move into the intima layer where they differentiate into macrophages and initiate cell-to-cell signaling, leading to inflammation. Besides that, activation of endothelial cells can also be caused by platelet activation, which is accompanied by the release of VWF, the production of platelet activating factor and chemokines, and the appearance of P-selectin on the plasma membrane (Kruger-Genge et al., 2019).

2.2.5. Endothelial Permeability

Leukocytes, plasma protein and solutes move across the endothelial barrier via transcellular or paracellular routes. In transcellular permeability, also known as transcytosis, the transport of solutes is mediated by caveolae that enriches the fission of plasma membrane microdomains. Albumin, albumin-bound ligands and hormones are transported from the luminal surface of endothelial cells to the basal surface by caveolar vesicles. This transport process is important to maintain homogenous oncotic pressure of interstitial fluid and that in the endothelium (Di et al., 2016).

On the other hand, the paracellular permeability is regulated by interendothelial junctions - tight junctions and adherens junctions which connect adjacent endothelial cells together. Tight junctions are composed of claudins, occludins and junctional adhesion molecules while adherens junctions are made up of catenins and cadherins. Together, these junctions maintain the endothelial barrier and restrict the transport of albumin and plasma proteins from the blood vessel to the stroma (Wettschureck et al., 2019). In endothelial activation, remodeling of endothelial cell-cell junctions takes place, which in turn increases endothelial permeability (Funk et al., 2012).

2.3. Oxidative Stress in Vascular Cells

Increased oxidative stress in vascular cells has been shown to contribute to the development of various vascular diseases including atherosclerosis and ischemic heart disease. Risk factors of atherosclerosis such as diabetes mellitus and hypertension are also associated with oxidative stress in the vascular wall (Margaritis et al., 2014). Oxidative stress occurs when there is an imbalance between oxidants that can cause cellular damage and antioxidants which counteract the damaging action of oxidants. When this happens in the vascular wall, a cascade of events

is then initiated, leading to endothelial dysfunction and other chronic inflammatory events (Castellon et al., 2016).

2.3.1. Reactive Oxygen Species (ROS)

In anaerobic organisms, oxygen is metabolized into ROS. ROS are molecules that have one or more unpaired electrons in their orbital. In order to stabilize themselves, unpaired electrons of ROS take electrons from other molecules, initiating a chain reaction. Besides that, some ROS molecules such as non-radical molecules which do not possess any unpaired electron are also considered as reactive. Examples of ROS include O_2^-, hydroxyl radical (•OH), H_2O_2 and peroxynitrite (ONOO•). Among all ROS, H_2O_2 and O_2^- are known to contribute the most as signaling molecules which initiate oxidative stress (Wang et al., 2018).

In most of the cells, ROS are produced endogenously by mitochondria. In endothelial cells, however, mitochondria are not the major source of ROS since the energy source of endothelial cells does not depend on mitochondrial oxidative phosphorylation. Instead, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX) including NOX1, NOX2, NOX4 and NOX5 isoforms, contribute vastly on ROS production in endothelial cells in both physiological and pathological conditions. Other sources of ROS in endothelial cells include perixosomes, lysosomes, cytochrome P450, endoplasmic reticulum and xanthine oxidase. (Pendyala et al., 2009).

In physiological conditions, ROS are constantly produced in small amount to initiate defense mechanism against microorganisms. However, under pathological conditions, overproduction of ROS can lead to oxidative stress. ROS are responsible to induce rearrangement of actin, resulting in the disruption of endothelial tight junctions (Moldovan et al., 2000). This allows

leukocytes to migrate from the vessel lumen into the intima via the paracellular route. Increased level of ROS in endothelial cells also activates NF-κB, which in turn induces the synthesis of inflammatory cytokines. The synergistic effect between ROS and these cytokines initiates an inflammatory cascade by upregulating the production of adhesion molecules and enhancing the recruitment of inflammatory cells across the vessel wall (Closa et al., 2004; Yuan et al., 2019).

ROS, particularly the free radicals, are also toxic to endothelial cells. This is due to the reaction between these molecules with molecular components in the cells which generates radical chain reaction, causing protein modifications and loss of functions. This reaction also results in DNA crosslinking and lipid peroxidation, which affects membrane protein activities and membrane fluidity (Closa et al., 2004).

Various methods have been developed to measure the production of ROS *in vitro*, *ex vitro* and *in vivo* with different sensitivities and specificities. To detect intracellular production of ROS within subcellular compartments and organelles, dichlorodihydrofluorescein diacetate (DCFH-DA), a cell-permeable probe is commonly used in cells or tissues culture. The DCFH, when reacts to oxidants, specifically O_2^- and H_2O_2, is oxidized and a fluorescent product called DCF is then produced. This fluorescent product can be observed or measured using a fluorescent microscope or a fluorescent microplate reader. To detect extracellular ROS, particularly H_2O_2, the most recommended assay is Amplex Red assay, where it measures H_2O_2 released from cells and in cell-free systems. Upon oxidization, Amplex Red, with the help of horseradish peroxidase, is converted to resorufin, which is a fluorescent product (Griendling et al., 2016).

2.3.2. Hydrogen Peroxide

H_2O_2 is a type of ROS that can cause damage to lipids, proteins and nucleic acids. It is a stable, uncharged molecule and is found to be more flexible compared to O_2^-. This is mainly due to its property where H_2O_2 can easily pass through cellular membranes via channel protein-dependent diffusion, and thus able to distribute redox signals to distant targets (Bienert et al., 2006). Therefore, a lot of studies focus on detecting H_2O_2 concentration intracellularly, in cell culture supernatant, as well as in serum samples. Different approaches have been developed with regards to the possibility of using H_2O_2 production as an indicator of oxidative stress. In human, H_2O_2 is produced as a by-product of aerobic metabolism in the mitochondria. Since O_2^- is highly unstable, it is spontaneously or enzymatically catalyzed by SOD into H_2O_2. Generation of H_2O_2 by SOD also causes H_2O_2 flux and activation of the redox-sensitive pathway such as mitogen-activated protein kinase (MAPK) (Breton-Romero et al., 2014).

Previous study have shown that H_2O_2 is able to alter different types of intracellular proteins such as phosphatases, antioxidants, transcription factors, protein kinases, metabolic enzymes, ion channels, and structural proteins that contain deprotonated cysteine residue by oxidization (Breton-Romero et al., 2014). In addition, accumulation of intracellular H_2O_2 was reported to inactivate MAPK phosphatases, leading to activation of c-Jun N-terminal kinase (JNK) and p38 MAPK signaling pathways (Kamata et al., 2005).

2.4. Cell Adhesion Molecules

CAM are transmembrane receptors that are expressed on the cell surface. They are classified into three groups, which are selectins, integrins and immunoglobulins. On vascular endothelial cells, CAM mediate the adhesion of circulating leukocytes on the endothelium and facilitate the migration of monocytes into the intima layer (Ling et al., 2012). During endothelial cell

activation, endothelial cells overexpress CAM, such as VCAM-1, PECAM-1, ICAM-1, and selectins (E-selectin and P-selectin) on their apical membrane surface (Khodabandehlou et al., 2017).

2.4.1. VCAM-1

VCAM-1, also known as CD106, is a 90 kDa type 1 transmembrane glycoprotein that resides on the endothelial membrane. VCAM-1 can be liberated from activated endothelium, resulting in soluble VCAM-1 that circulates in the blood plasma (Videm et al., 2008). Being a member of immunoglobulin gene superfamily, VCAM-1 is composed of an extracellular domain, which contains seven homologous immunoglobulin-like domains, a transmembrane domain and a cystosolic domain. VCAM-1 is mostly expressed in endothelial cells upon activation by extracellular stimuli including pro-inflammatory cytokines such as TNF-α and interleukins. Besides that, other stimuli such as ROS also elicit an increase in the expression of VCAM-1. (Elices et al., 1990; Kim et al., 2017)

Previous studies have shown that VCAM-1 is a key mediator of atherosclerosis, whereby the molecule is abundantly detected at atherosclerotic lesion sites of low-density lipoprotein receptor-deficient mice (Vogel et al., 2017). The expression of VCAM-1 is regulated by signaling molecules which include p38 kinase, JNK, extracellular signal-regulated kinase 1/2 (ERK1/2) and protein kinase B/Akt. Interestingly, decreased expression of ERK2 and protein kinase B/Akt have been reported to upregulate VCAM-1 expression (Pott et al., 2016).

VCAM-1 is one of the major regulators of leukocyte adhesion. VCAM-1 selectively binds to integrins (α4β1 and α4β7) expressed on monocytes and T- lymphocytes (Kong et al., 2018), adhering these leukocytes firmly to the endothelium. Leukocytes are then recruited into the

vascular intima with the help of other CAMs such as PECAM-1. During inflammation, TNF-α upregulates the expression of VCAM-1 on the luminal membrane of endothelial cells (**Figure 2.3**). VCAM-1 directly interacts with α4β1 integrin on leukocytes, which in turn activates downstream signaling molecules including ROS, allowing leukocytes to migrate through interendothelial junctions (Alon et al., 1995; Marchese et al., 2012). The cross-linking of VCAM-1 and the resulting signaling cascades cause intercellular gap formation and focal loss of VE-cadherin between adjacent cells, thus results in increased endothelial permeability (van Wetering et al., 2003).

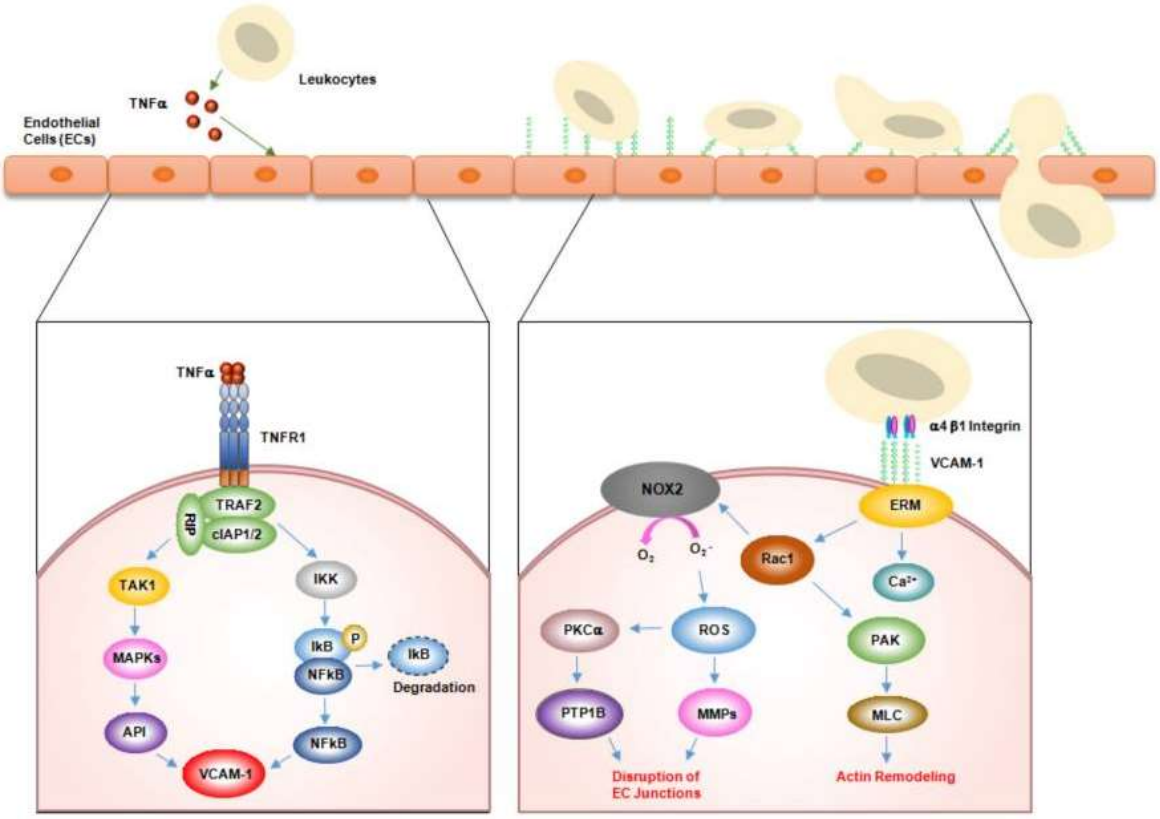


Figure 2.3: VCAM-1 induces ROS production, causing an increase in endothelial permeability. During inflammation, TNF-α is secreted from leukocytes causing upregulation of VCAM-1 expression. VCAM-1 expression then activates downstream signaling molecules including ROS, which eventually disturb interendothelial junctions, allowing leukocytes to migrate through the endothelial cells. (Adapted from (Kong et al., 2018)).

2.5. Antioxidants in Vascular Cells

Human body is capable of producing its own enzymatic and non-enzymatic antioxidants as defence system to balance the level of oxidants produced. Examples of antioxidant enzymes are SOD, CAT and GPX while non-enzymatic antioxidants include glutathione, albumin, bilirubin, vitamin A, C and E (Lubrano et al., 2015; Siti et al., 2015).

2.5.1. Superoxide Dismutase

SOD is expressed in all living organisms as the first line of defense against free radicals. There are three classes of SOD possessing three different catalytic metal ions as cofactors, which are Mn-SOD/Fe (SOD2), Cu/Zn-SOD (SOD1 and SOD3) and Ni-SOD. Although three classes of SOD exist, humans only possess SOD2 in the mitochondria, SOD1 in the cytoplasm and the intermembrane space of the mitochondria, whereas SOD3 is expressed in extracellular matrix and in extracellular fluids (Wang et al., 2018; Wassmann et al., 2004). SOD efficiently converts highly reactive O_2^- into H_2O_2 and oxygen which are more stable, thereby regulating the level of ROS (**Figure 2.4**). Studies have shown that SOD2 is essential in eliminating O_2^- produced in the mitochondria, whereas SOD1 and SOD3 are not essential for eliminating O_2^- although they act as the first line of defense to catalyze O_2^- dismutation (Lubrano et al., 2015).

The role of SOD in preventing atherosclerosis can be seen by the inhibition of O_2^--induced oxidative stress in the vascular endothelium (Forstermann et al., 2017). Although SOD is important in reducing the oxidative burden in endothelial cells, excessive SOD1 expression is, however, found to enhance oxidative stress due to the generation of large quantities of H_2O_2 as a byproduct (Fukai et al., 2011).

2.5.2. Catalase

CAT is mainly found in peroxisomes, but the enzyme can also be present in the cytosol. It is essential to tolerate oxidative stress, especially when GPX activity is reduced. CAT converts H_2O_2 into water and oxygen, which is an important step to protect the cells from the damaging effect caused by H_2O_2 (Poznyak et al., 2020) (**Figure 2.4**). Apart from that, CAT also catalyzes the conversion of hydrogen donors and organic peroxides into water and organic alcohols through its peroxidase activity (Wassmann et al., 2004). Previous study also indicated that CAT conjugated with PECAM-1 antibodies attenuates endothelial permeability caused by exogenous H_2O_2 (J. Han et al., 2011). Unlike SOD1, CAT overexpression reduces the formation of atherosclerosis, suggesting accumulation of peroxides in endothelial cells upon stimulation by atherogenic stimuli such as oxidised low-density lipoprotein (Forstermann et al., 2017).

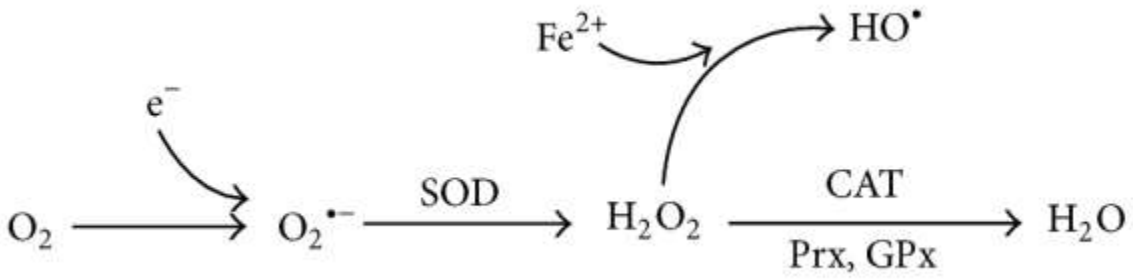


Figure 2.4: The relationship between O_2^-, SOD, H_2O_2 and CAT (Adapted from (Moreno-Arriola et al., 2014)).

2.6. Tumor Necrosis Factor-α

TNF-α is a 26 KDa transmembrane protein and is a member of the tumor necrosis factor ligand family. This cytokine was named tumor necrosis factor after its discovery in 1975. TNF-α was first isolated and identified from Meth A sarcoma cells and other transplanted tumors from mice sera infected with Bacillus Calmette-Guerin, where TNF was found to mediate tumor necrosis induced by endotoxins *in vitro* (Carswell et al., 1975). TNF-α is produced by immune

cells such as activated monocytes or macrophages, natural killer cells and T-lymphocytes. Besides immune cells, TNF-α is also expressed by some non-immune cells such as endothelial cells and fibroblasts. Over years, TNF-α was successfully purified biochemically and recombinant TNF was made available for *in vivo and in vitro* experiments (Sedger et al., 2014).

TNF-α works by binding to TNF receptor 1 (TNFR1) and TNFR2; where the former present in most cell types and the latter present in specific cells such as endothelial cells. TNFR1 is a type 1 transmembrane protein which contains intracellular and extracellular domains. Three cysteine-rich domains (CRD) which are CRD1, CRD2 and CRD3 are components of the extracellular domain, with TNF-α mostly binds to CRD2 and CRD3. On the other hand, intracellular domain of TNFR1 comprises a death domain which induces programmed cell death upon activation of TNFR1. In contrast, TNFR2 is lacked of the death domain. (Kalliolias et al., 2016; Kong et al., 2018).

TNF-α is originally synthesized as an insoluble protein expressed on the cell membrane. TNF-α can also be cleaved by a proteolytic enzyme, TNF-α-converting enzyme into a 17 kDa soluble TNF. Soluble TNF is present in the blood plasma and circulates in the body, therefore, can be found in sites distant from where it is synthesized. Both forms of TNF-α can bind to TNFR1 and TNFR2, although it has been shown that TNF-α is a more potent ligand for TNFR2. (Grell et al., 1995; Sedger et al., 2014).

Several signaling pathways are activated upon the binding of TNF and TNFR including NF-κB and MAPK pathways. JNK and p38 signal transduction pathways have also been shown to be activated upon induction by TNF-α (Zhou et al., 2017). These signaling pathways are known

to be important pathways initiating vascular injury, which eventually lead to endothelial barrier disruption as well as increased VCAM-1 expression and oxidative stress.

2.6.1. Disruption of Endothelial Barrier, Increased VCAM-1 Expression and Increased ROS Production Caused by TNF-α

TNF-α is known to be a key player of endothelial barrier dysfunction in view of its role in promoting chronic inflammatory responses. Disruption of the endothelial barrier can be due to a direct action of TNF-α upon endothelial cells, or an indirect effect resulting from leukocyte recruitment and adherence (Madge et al., 2001). TNF-α exposure has been reported to impair the endothelial barrier integrity by activating Rho GTPases, increasing formation of filamentous actin and remodeling the endothelial cell morphology (Marcos-Ramiro et al., 2014; Radeva et al., 2018). A number of studies have reported the effects of TNF-α on endothelial barrier disruption, which is mainly characterized by increased endothelial cell permeability. In microvascular endothelial cells, TNF-α was demonstrated to induce capillary leakage by disrupting tight junction via NF-κB-dependent pathways (Clark et al., 2015).

TNF-α is also a well-known positive regulator of VCAM-1 as demonstrated in many studies. VCAM-1 mRNA has been shown to be upregulated in the presence of TNF-α (Zapolska-Downar et al., 2012). Besides that, surface VCAM-1 expression has been shown to be significantly elevated in many types of endothelial cells, and this is associated with NF-κB activation (Scott et al., 2013). TNF-α and VCAM-1 interaction is interdependent. This means that TNF-α upregulates VCAM-1 expression, which in turn accelerates monocyte recruitment on the endothelial cells and facilitates their transmigration across the endothelium. Monocytes then mature into macrophages in the smooth muscle cell layer and express more TNF-α (Pott et al., 2016).

Besides upregulating VCAM-1 production, the binding of TNF-α to TNFR also leads to inflammation, ROS production and apoptosis which are associated with a number of diseases including atherosclerosis, heart failure, diabetes, cancer and autoimmune diseases (Kong et al., 2018). When TNF-α binds to TNFR, adaptor proteins are recruited to the intracellular domain of TNFR, causing generation of ROS. TNF-α-induced ROS has been demonstrated to sustain JNK and p38 MAPK activations, leading to programmed cell death (Kamata et al., 2005). Increased oxidative stress in endothelial cells also leads to disruption of cell-cell junctions, resulting in increased endothelial permeability (Craige et al., 2015).

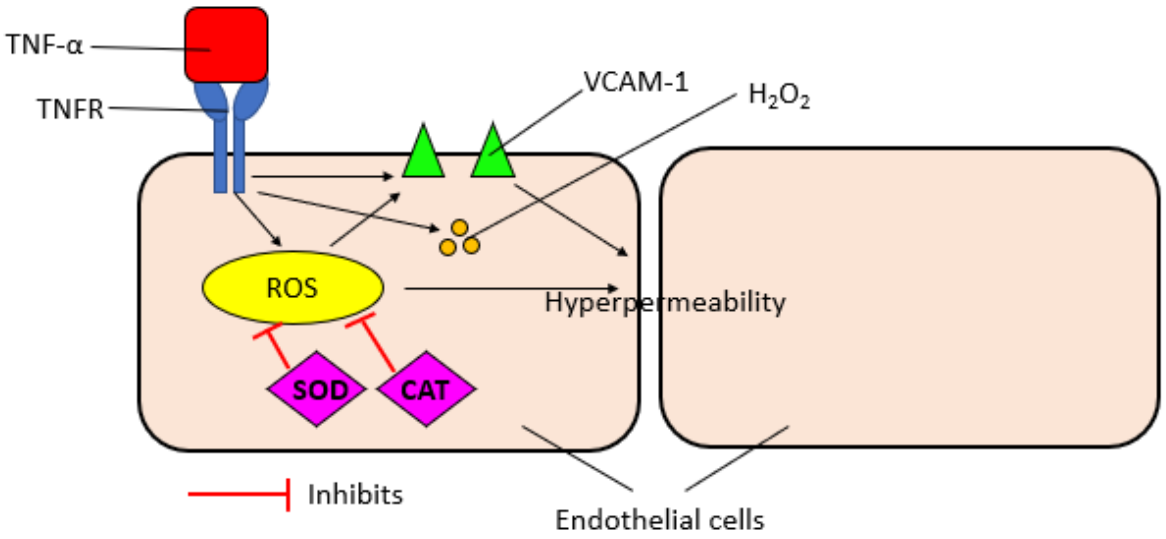


Figure 2.5: TNF-α impairs endothelial barrier function. Upon binding to TNFR, TNF-α induces increased ROS production, including the release of H_2O_2, and stimulates increased VCAM-1 expression on the luminal surface of endothelial cells. ROS causes an increase in endothelial permeability through VCAM-1 or without involving VCAM-1. TNF-α also directly causes overexpression of VCAM-1, which in turn triggers barrier disruption and endothelial hyperpermeability. Anti-oxidant enzymes such as SOD and CAT quickly eliminate excessive ROS formed in the cell.

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

Primary HAEC were purchased from American Type Culture Collection (ATCC) (VA, USA). Endothelial cell media and supplements including 5% fetal bovine serum (FBS), 100 U/mL penicillin,100 ug/mL streptomycin and endothelial cell growth factor were purchased from ScienCell (CA, USA). Human recombinant tumour necrosis factor-α (TNF-α) was purchased from PeproTech (NJ, USA). H_2O_2 was purchased from Merck. Simvastatin, 2′,7′-Dichlorodihydrofluorescein diacetate (H_2-DCFDA), Fluorescein isothiocyanate (FITC)-Dextran, dexamethasone and N-acetyl cysteine (NAC) were purchased from Sigma Aldrich (MO, USA). 10X Trypsin-EDTA was purchased from Biowest (Nuaille, France). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Dimethyl sulphoxide (DMSO), phosphate buffered saline (PBS) was purchased from Oxoid (UK).

3.2 Cell Culture

Primary HAEC were cultured in endothelial cell media supplemented with 5% FBS, 1% endothelial cell growth factors and 1% Penicillin/Streptomycin and were grown in T-25 culture flasks at a starting density of 2.5 x 10^3 cells/cm^2. The cells were incubated in CO_2 incubator at 37 ºC with 5 % CO_2. The culture media was changed the next day after thawing and subsequently every day until the cells reached about 70 % confluence. The cells were sub-cultured until desired passage. Cells at passages three to five were used for assays. To harvest the cells, media were removed and 1 ml of 10X Trypsin-EDTA was added into each flask. The cells were incubated at 37ºC for 2 min and the flask were then gently tapped to detach the cells.

The flask was observed under the microscope to confirm 90-100% cell detachment. Cells were cryopreserved in endothelial cell media with 5% DMSO and stored in liquid nitrogen. To recover the cells, the cells were thawed in 37ºC water bath for 1 – 2 min and were divided into four to six T-25 flasks.

3.3 Extraction of *A. sessilis*

A. sessilis whole plant was collected from herb garden of Persatuan Memperbaiki Akhlak Che Ru, Endau, Johor, Malaysia. A sample of the plant with voucher specimen number of RG5040 was deposited at Department of Biology, Faculty of Science, Universiti Putra Malaysia and the plant was verified by Dr. Rusea Go. *A. sessilis* ethanolic extract was prepared by the collaborators of this project, Dr. Mah Siau Hui from Taylor's University and Dr. Teh Seok Sin from Malaysia Palm Oil Board. Approximately 104.4 g of air-dried *A. sessilis* was ground to a fine powder and extracted in a Soxhlet apparatus with ethanol for 4 h (Mah et al., 2017). The extract was evaporated to dryness under vacuum to give 31.3 g of ethanolic extract.

3.3.1 Preparation of Plant Extract for Experiment

To prepare a stock solution of *A. sessilis* ethanolic extract, the extract was dissolved in endothelial basal media to a concentration of 5 mg/ml. The stock was filter-sterilised through a 0.2 µm polyether sulphones (PES) membrane filter before stored at 4°C for a maximum of 3 months. The working solutions were freshly prepared using endothelial cell media on the day of usage. Any leftover was discarded.

3.4 Cell Viability Assay

The cell viability was determined using MTT assay (Zhan et al., 2016). HAEC were seeded into 96-well plates at the density of 1 x 10^4 cells/well for overnight. The media were then

replaced with 25, 50, 100, 200, 400 and 800 µg/ml of *A. sessilis* ethanolic extract and further incubated for 24 h. Cells treated with endothelial cell media only served as a normal control group. Then, 10 µl of 5 mg/ml of MTT in PBS was added into each well, and the plate was incubated for another 4 h. Finally, all solution was removed from the well, and 100µl of DMSO was added into each well to dissolve the crystals formed. The absorbance was read using a microplate reader (Infinite M200pro, TECAN, Switzerland) at 450 nm.

3.*5 In vitro* Vascular Permeability Assay

This assay was performed according to the procedure previously described with some modifications (Fong et al., 2018). To study effects of *A. sessilis* on TNF-α-induced hyperpermeability, cell culture inserts with 1.0 µm pore size (Falcon, USA) flanked on 24-well companion plates were used. 2 x 10^5 cells were seeded into each cell culture insert pre-coated with 1.5 mg/ml of Type 1 rat tail collagen (BD Biosience, USA). The bottom well of each insert was filled with 500 µl of endothelial cell media to provide nutrients to the cells. The cells were grown for 3 to 4 days until a monolayer was formed. Media in the inserts, as well as in the bottom well were changed every 2 days. After a cell monolayer was formed, the cells were then treated with *A. sessilis* ethanolic extract (25 – 200 µg/ml) or simvastatin (2 µM) for 24 h. Then, the cells were induced with 10 ng/ml of TNF-α for 6 h. Following TNF-α incubation, the media in the insert and in the bottom well were removed. The bottom well was filled with 500 µl of endothelial cell basal media, while 150 µl of 0.04 mg/ml FITC-dextran (2000 kDa) was added to each insert and incubated at room temperature to allow permeation of FITC-dextran from the insert into the bottom well through the cell monolayer. After 20 min, the permeation of FITC-dextran was stopped by removing the culture insert from the well. The plate was shaken gently to mix the media and FITC-dextran homogenously. 100 µl of the media-FITC-dextran mixture was then transferred into 96-well black plates. The fluorescence

intensity was measured using a fluorescent microplate reader (Infinite M200pro, TECAN, Switzerland) at an excitation and emission wavelengths of 485 nm and 530 nm, respectively.

3.6 Intracellular ROS Production Assay

The protocol was adapted and improvised from Ganji et al. (2009). Cells were seeded onto 96-well plates at a density of 1 x 10^4 cells/well overnight. To optimize the duration required for TNF-α to induce maximal production of ROS levels in HAEC, the media in each well were removed and replaced with 100 µl of 10 µM H_2-DCFDA. After 30 min of incubation, the cells were then treated with 10 ng/ml of TNF-α for 30 min, 1 h, 2 h, 4 h, 6 h and 24 h. The fluorescence intensity was measured at excitation and emission wavelengths of 480 nm and 570 nm, respectively, using a fluorescent microplate reader (Infinite M200, TECAN, Switzerland).

To study the effect of *A. sessilis* on TNF-α-induced increased ROS production, the same seeding protocol was done. After overnight incubation, the cells were pre-treated with 25, 50, 100, and 200 µg/ml of *A. sessilis* ethanolic extract for 24 h. Then, the media with treatment were replaced with H_2-DCFDA (10 µM). H_2-DCFDA was then removed after 30 min of incubation, and this was followed by addition of TNF-α (10 ng/ml). The plate were then further incubated for 4 h. The fluorescence intensity was measured as described above.

3.7 Extracellular H_2O_2 Production Assay

This assay was performed using Amplex Red Hydrogen Peroxide/Peroxidase assay kit (Invitrogen, USA) according to manufacturer's protocols. HAEC were cultured overnight in 6-well plates at a density of 3 x 10^5 cells/well. To optimize the TNF-α concentration and induction

time required to induce maximal H_2O_2 levels, the cells were induced with TNF-α at concentrations of 10, 20, 100 and 200 ng/ml for 30 min, 1 h and 2 h, 4 h and 6 h.

The supernatant was collected and immediately assayed after indicated treatments using the assay kit and a 96-well plate. Briefly, 50 µl of samples were added to 50 µl of 0.1 mM Amplex Red and 0.2 U/ml horseradish peroxidase solution diluted in 1X reaction buffer. The plates were then incubated for 30 min at room temperature. The fluorescence intensity was measured using a microplate reader (Infinite M200, TECAN, Switzerland) at excitation/emission wavelengths of 540/590 nm.

To study the effect of *A. sessilis* on H_2O_2 production, the cells were treated with 25, 50, 100 and 200 µg/ml of *A. sessilis* ethanolic extract or 2 µM simvastatin for 24 h, followed by induction with 20 ng/ml TNF-α for 1 h. Cell culture supernatant was collected and the assay was performed as described above.

3.8 Soluble VCAM-1 Expression Assay

The assay was performed using DuoSet VCAM-1/CD106 enzyme linked-immunosorbent assay (ELISA) and ancillary reagent kit (R&D System, Minnesota, USA). HAEC were seeded onto 24-well plates at a density of $2x10^5$ cells/well overnight. The cells were then treated with *A. sessilis* ethanolic extract at 25, 50, 100 and 200 µg/ml, 2 µM of simvastatin or 10 mM of NAC for 24 h. NAC was prepared by dissolving NAC powder in water to a stock concentration of 612.8 mM. NAC stock solution was kept at 4°C, while working solution at a concentration of 10 mM was freshly prepared prior to each experiment. Next, the cells were induced with 10 ng/ml TNF-α for 6 h. The cell culture supernatant was collected and centrifuged at 1500 rpm

for 10 min at 4 ºC. Then, the supernatant was collected into new tubes and stored at – 80 ºC if not assayed on the same day. Repetitive freeze-thaw cycle was avoided.

A 96-well ELISA plate was coated with 2 µg/ml mouse anti-human VCAM-1 capture antibody diluted in PBS without carrier protein. After overnight incubation, the wells were washed with wash buffer containing 0.05% Tween 20 in PBS for three times. The plate was then incubated with reagent diluent made of 1% bovine serum albumin (BSA) in PBS for at least 1 h and the wells were washed again thrice. Then, recombinant human VCAM-1 standards and samples diluted with reagent diluent at a dilution factor of 2 were added into each well and incubated for 2 h. The wells were washed thrice with wash buffer and 100 µl of 200 ng/ml of biotinylated sheep anti-human VCAM-1 detection antibody was added into each well. Then, the plate was incubated for another 2 h. The washing step was repeated and 100 µl of streptavidin conjugated with horseradish-peroxidase (Steptavidin-HRP D) was then added to each well and incubated for 20 min. After washing the wells, substrate solution which consisted of 1:1 mixture of H_2O_2 and tetramethylbenzidine was then added into each well for 20 min. The reaction was stopped by adding a stop solution containing 2 N H_2SO_4 into each well. The absorbance was immediately read at 450 nm, and corrected at 540 nm using a microplate reader (Infinite M200, TECAN, Switzerland).

Standard curves were generated using Microsoft Excel by plotting the absorbance of each standard on Y-axis against the concentration of 15.6 – 1000 pg/ml of recombinant human VCAM-1 on X-axis. The best fit curve was obtained with $R^2 > 0.95$. The concentration of VCAM-1 in the samples was calculated by finding the x-value based on the absorbance reading of each sample in the standard curve.

3.9 Antioxidant Enzymes Production Assays

3.9.1 Measurement of SOD Activity

This assay was performed using the SOD Assay Kit (Cayman, USA) based on the instruction provided with the kit. HAECs were seeded on 12-well plates at a density of 2 x 10^5 cells/well. For this assay, H_2O_2 was used to induce decreased SOD activity in HAEC. To optimize the H_2O_2 concentration and induction time, the cells were induced with H_2O_2 at concentrations of 50, 100, 200 and 400 µM for 30 min, 1 h and 2 h.

Cell lysates of the samples were collected using rubber cell scrapers. Media were removed from each well, and iced-cold 20 mM N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES) buffer containing 1 mM ethylene glycol tetraacetic acid (EGTA), 210 mM mannitol and 70 mM sucrose (pH7.2) was then added. Cell lysates were then collected using a rubber cell scraper and transferred into centrifuge tubes. The cell lysate was centrifuged at 1500 x g for 5 min at 4ºC. The supernatant was kept at -80ºC if not assayed on same day.

To perform the SOD assay, 200 µl of radical detector containing tetrazolium salt solution was first added into a 96-well plate. Then, 10 µl of samples and bovine erythrocyte SOD (Cu/Zn) standards were added into the plate. To initiate the reaction, 20 µl xanthine oxidase was added into each well, and the plate was incubated for 30 min at room temperature. The absorbance was read using a microplate reader (Infinite M200, TECAN, Switzerland) at 440 nm.

To study the effects of *A. sessilis* on SOD activity, the cells were treated with 25, 50, 100 and 200 µg/ml of *A. sessilis* ethanolic extract or 2 µM of simvastatin or 10 mM of NAC or 10 µM of dexamethasone for 24 h, followed by induction with 100 µM of H_2O_2 for 2 h. Collection of cell lysates and the measurement of SOD activity were performed as described above.

3.9.2 Measurement of CAT Activity

This assay was performed using the CAT Assay Kit (Cayman, USA) based on the instruction provided with the kit. HAEC were seeded on 6-well plates at a density of 3 x 10^5 cells/well. H_2O_2 was used to induce reduced CAT activity in HAEC. To optimize the H_2O_2 concentration and induction time, the cells were induced with H_2O_2 at concentrations of 50, 100, 200 and 500 µM for 30 min, 1 h and 2 h, 4 h and 6 h.

The CAT activity was detected using cell lysate samples. Media were first removed from each well and iced-cold lysis buffer consisting of 50 mM potassium phosphate and 1 mM ethylenediaminetetraacetic acid (EDTA) at pH 7.0 was added. The cell lysates were then harvested using a rubber cell scraper and transferred into centrifuge tubes. The cell lysate was centrifuged at 10,000 x g for 15 min at 4ºC. The supernatant was then kept at -80ºC if not assayed on the same day.

To perform the CAT assay, samples with buffer and ethanol were added into each well of a 96-well plate. To initiate the reaction, 20 µl of hydrogen peroxide was added to the well and incubated for 20 min. The reaction was terminated by adding 30 µl potassium hydroxide into the well. 30 µl of chromogen and 10 µl of potassium periodate were then added. The absorbance was read using a microplate reader (Infinite M200, TECAN, Switzerland) at 540 nm.

In the subsequent experiment, effects of *A. sessilis* on CAT activity were evaluated by first treating the cells with 25, 50, 100 and 200 µg/ml of *A. sessilis* ethanolic extract or 2 µM of simvastatin for 24 h, and this was followed by induction with 100 µM of H_2O_2 for 2 h. Collection of cell lysates and the CAT assay were performed as described above.

3.10 Statistical Analysis

All experiments were performed in triplicates for at least three times. The results were expressed as mean of readings and standard error of means (S.E.M). Statistical analysis was performed using one-way Analysis of Variance (ANOVA) and Dunnett's test with GraphPad Prism 7 (CA, USA).

CHAPTER 4

RESULTS

4.1. Effect of *A. sessilis* Ethanolic Extract on Cell Viability

HAEC were incubated with various concentrations of *A. sessilis* ethanolic extract (6.25 – 800 µg/ml) for 24 h and the MTT assay was used to evaluate whether *A. sessilis* ethanolic extract would affect the cell viability. 400 and 800 µg/ml of *A. sessilis* ethanolic extract were found to cause significant cell death with mean cell viabilities of 23.74 ± 0.83 and 33.42 ± 0.81 % of control, respectively (**Figure 4.1**) ($P<0.05$). 6.25 – 200 µg/ml *A. sessilis* ethanolic extract did not affect the viability of HAEC (87.16 ± 7.13, 86.37 ± 5.71, 94.11 ± 3.05, 94.91 ± 5.00, 99.86 ± 6.22, and 100.60 ± 6.59 % of control respectively). Therefore, four highest concentrations (25, 50, 100 and 200 µg/ml) which did not cause significant cell death were used in subsequent experiments.

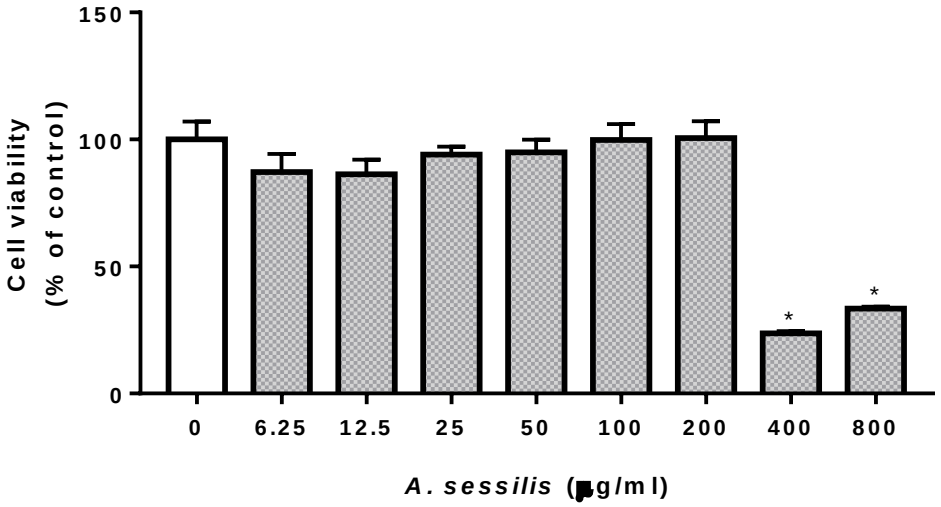


Figure 4.1: **The effect of *A. sessilis* ethanolic extract on HAEC viability.** HAEC were cultured in 96-well plates and treated with various concentrations of *A. sessilis* ethanolic extract (0–800 mg/ml) for 24 h. The percentage of cell viability was determined using MTT assay. Data are represented as the mean ± S.E.M of three independent experiments. **P*<0.05 compared to untreated group.

4.2. Effect of *A. sessilis* Ethanolic Extract on TNF-α-Induced Endothelial Hyperpermeability

In vitro vascular permeability assay is an assay used to measure the permeability of endothelial cells by quantifying the passage of fluorescent probe-labelled tracers across a cell monolayer grown on collagen-coated cell culture inserts. The effect of *A. sessilis* ethanolic extract on endothelial cell permeability was studied. As shown in **Figure 4.2**, 200 µg/ml of *A. sessilis* ethanolic extract alone without induction with TNF-α did not significantly affect the permeability of HAEC (95.99 ± 19.17 % of control). 10 ng/ml of TNF-α significantly increased the permeability of HAEC to 175.8 ± 15.03 % of control (P<0.05) at 6 h (**Figure 4.2**). Pre-treatment of HAEC with 200 µg/ml of *A. sessilis* ethanolic extract for 24 h significantly reduced the increased permeability to a level which is comparable to the unstimulated control group (101.0 ± 8.26 % of control) (P<0.05). A positive control drug, simvastatin, also significantly decreased TNF-α-induced hyperpermeability to 88.64 ± 13.32 % of control (P<0.05) at 2 µM. 25, 50 and 100 µg/ml of *A. sessilis* ethanolic extract, however, did not have significant effect on the increased permeability induced by TNF-α. These results show that *A. sessilis* ethanolic extract prevents impairment of the endothelial barrier induced by TNF-α, which was indicated by the suppression of FITC-dextran permeability.

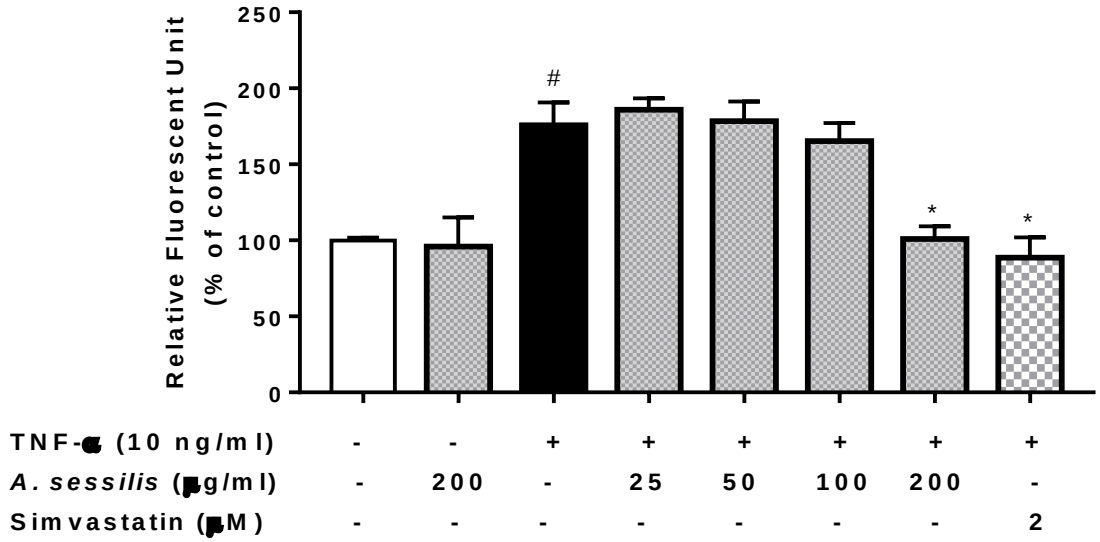


Figure 4.2: The effect of *A. sessilis* ethanolic extract on TNF-α-induced hyperpermeability of HAEC. HAEC cultured on collagen-coated transwell inserts were pre-treated with various concentrations of *A. sessilis* ethanolic extract (25 - 200 µg/ml) or simvastatin (2 µM) for 24 h, followed by stimulation with TNF-α (10 ng/ml) for 6 h. At the end of the experiment, the media in the bottom well were collected and the fluorescence intensity was measured. Data are presented as the mean ± S.E.M of three independent experiments, where each was performed in triplicates. #P<0.05 compared to unstimulated control, *P<0.05 compared to TNF-α treated group.

4.3 Effect of TNF-α on Intracellular ROS Production

Intracellular ROS production was measured using H_2-DCFDA dye. This non-fluorescent dye penetrates cell membranes and is converted to fluorescent DCF in the presence of ROS. HAEC were first stimulated with 10 ng/ml of TNF-α for 30 min, 1 h, 2 h, 4 h, 6 h and 24 h in order to identify the time point which shows the highest ROS accumulation in HAEC. The result shows that 10 ng/ml of TNF-α significantly increased intracellular ROS levels at 4 h (134 ± 2.27% of control), compared to unstimulated control **(Figure 4.3)**. Therefore, the cells were induced with TNF-α for 4 h in the subsequent experiment which measured the effect of *A. sessilis* ethanolic extract on increased intracellular ROS levels stimulated by TNF-α.

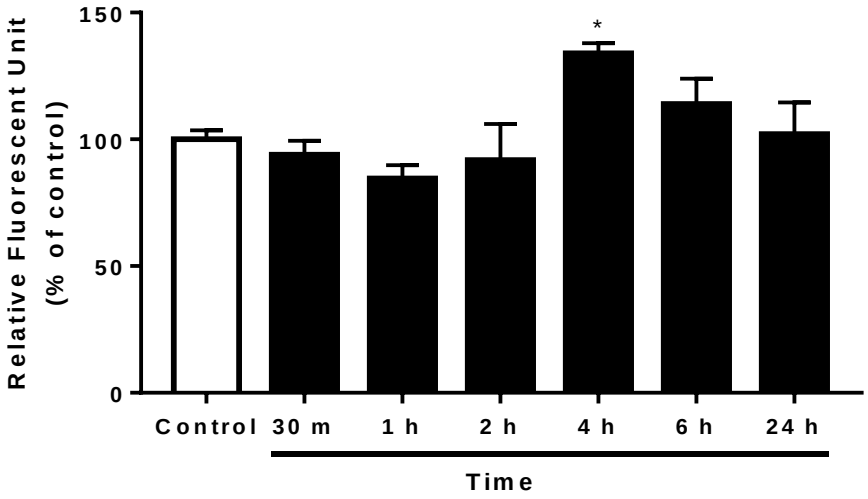


Figure 4.3: Time response effects of TNF-α on ROS levels. HAEC were stimulated with 10 ng/ml of TNF-α for different durations (30 min – 24 h). After staining the cells with H_2-DCFDA for 30 min, relative fluorescent unit at each well was measured using a microplate reader. Data are presented as the mean ± S.E.M of three independent experiments, each was performed in triplicates. *P<0.05 compared to unstimulated control.

4.4. Effect of *A. sessilis* Ethanolic Extract on TNF-α-Induced Increased Intracellular ROS Levels

The result shows that 200 µg/ml of *A. sessilis* ethanolic extract alone did not affect the basal levels of ROS in HAEC. 10 ng/ml of TNF-α significantly increased intracellular ROS levels in HAEC to 116.6 ± 1.67 of control (P<0.05) (**Figure 4.4**). 200 µg/ml of *A. sessilis* ethanolic extract significantly reduced increased intracellular ROS levels induced by TNF-α (98.1 ± 1.84 % of control) (P<0.05). Pre-treatment with 2 µM of simvastatin significantly reduced intracellular ROS production in HAEC (98.43 ± 9.19% of control) (P<0.05). However, pre-treatment with 25 – 100 µg/ml of *A. sessilis* ethanolic extact did not alter TNF-α-induced ROS release in HAEC. These data suggest that *A. sessilis* reduces increased intracellular ROS production induced by TNF-α.

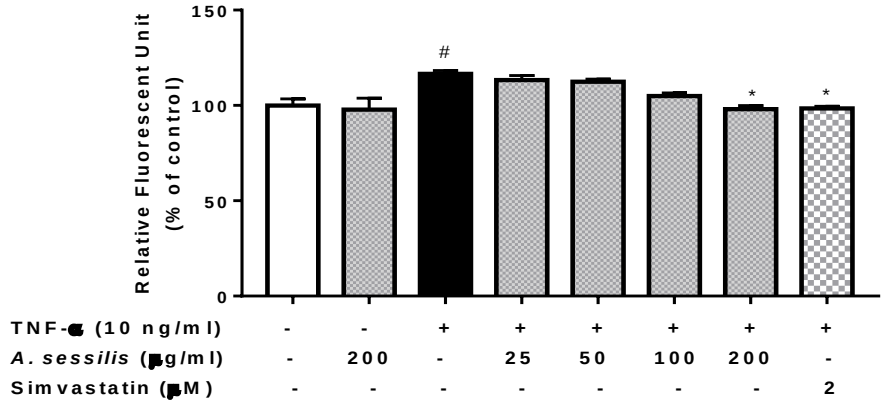
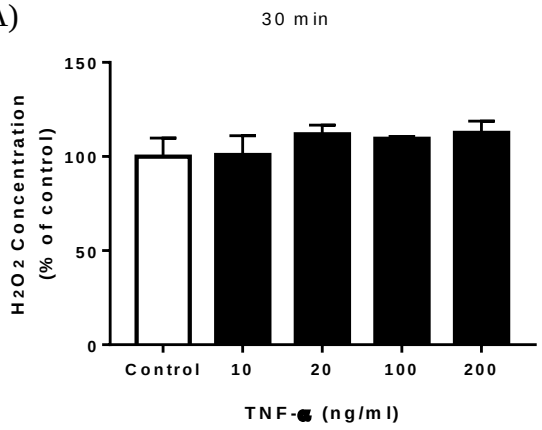


Figure 4.4: The effect of *A. sessilis* on TNF-α-induced increased ROS levels in HAEC. HAEC were pre-treated with various concentrations of *A. sessilis* ethanolic extract (25 - 200 µg/ml) or simvastatin (2 µM) for 24 h. After incubation with H_2-DCFDA for 30 min, the cells were stimulated with TNF-α (10 ng/ml) for 4 h.and the relative fluorescent unit of each well was measured using a microplate reader. Data are presented as the mean ± S.E.M of three independent experiments, each was performed in triplicates. #P<0.05 compared to unstimulated control. *P<0.05 compared to TNF-α treated group.

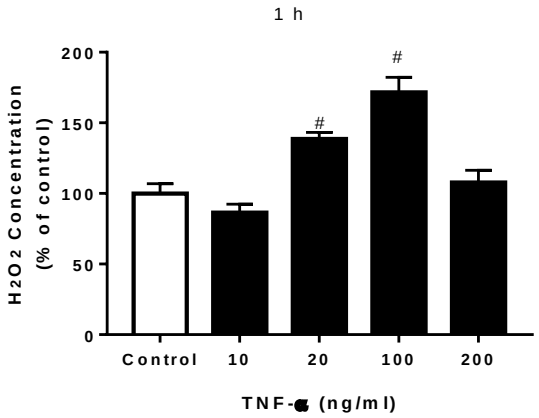
4.5. Effect of TNF-α on Extracellular H_2O_2 Production

The release of H_2O_2 in cell culture supernatant was measured using Amplex Red. Amplex Red, when combined with horseradish peroxidase, reacts with H_2O_2 to yield resorufin, which is a fluorescent molecule. To optimize the dose and induction period of TNF-α which can cause the maximal extracellular H_2O_2 production, HAEC were treated with 10 – 200 ng/ml TNF-α for various incubation periods ranging from 30 min to 6 h. The result shows that 20 ng/ml of TNF-α significantly increased extracellular H_2O_2 levels at 1 h (138.6 ± 4.69% of control), compared to unstimulated control (**Figure 4.5B**). On the other hand, 10 – 200 ng/ml TNF-α did not stimulate any increment in H_2O_2 production at 30 min, 2 h, 4 h and 6 h (**Figures 4.5A, C, D and E**). Therefore, 20 ng/ml of TNF-α with 1 h induction period was used in the subsequent experiment to measure the effect of *A. sessilis* ethanolic extract on extracellular production of H_2O_2 stimulated by TNF-α. Although 100 ng/ml of TNF-α also caused significant increment in H_2O_2 production at 1 h, but 20 ng/ml of TNF-α was chosen instead. This is because 100 ng/ml of TNF-α could be toxic to HAEC.

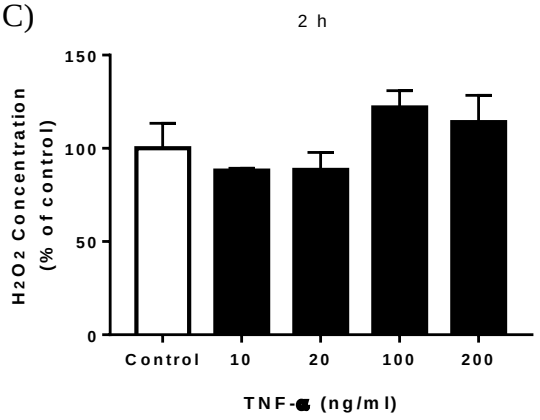
A)


30 min
H2O2 Concentration (% of control)
150
100
50
0
Control
10
20
100
200
TNF-α (ng/ml)

B)


1 h
H2O2 Concentration (% of control)
200
150
100
50
0
#
#
Control
10
20
100
200
TNF-α (ng/ml)

C)


2 h
H2O2 Concentration (% of control)
150
100
50
0
Control
10
20
100
200
TNF-α (ng/ml)

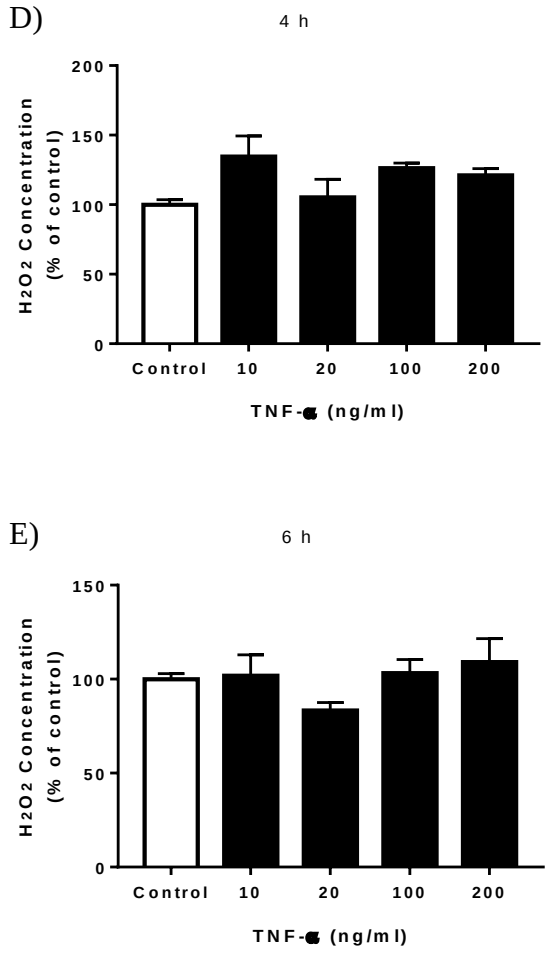


Figure 4.5: The effect of TNF-α on extracellular H_2O_2 production in HAEC. HAEC were treated with various concentrations of TNF-α (10, 20, 100 or 200 ng/ml) for **A) 30 min, B) 1 h, C) 2 h, D) 4 h and E) 6 h**. The cell culture supernatant was collected and the assay was performed immediately using H_2O_2 assay kits. Results are presented as the mean ± S.E.M. from three independent experiments, each was performed in triplicates. #P<0.05 compared to unstimulated control.

4.6. Effect of *A. sessilis* Ethanolic Extract on TNF-α-Induced Increased Extracellular H_2O_2 Production

As shown in **Figure 4.6**, treatment with 200 µg/ml of *A. sessilis* ethanolic extract alone caused a slight elevation of H_2O_2 production, but the elevation was not significant (158.8 ± 5.28 % of control). HAEC induced with 20 ng/ml TNF-α for 1 h showed an increase in H_2O_2 production (189 ±16.95%) compared to unstimulated control. However, pre-treatment of *A. sessilis* ethanolic extract at doses of 25, 50, and 100 µg/ml did not reduce the H_2O_2 production induced by TNF-α (203.4 ± 13.82, 203.8 ± 0.89 and 200.7 ± 6.69 % of control respectively). Although pre-treatment of 200 µg/ml of *A. sessilis* ethanolic extract and simvastatin also suppressed TNF-α-stimulated elevated H_2O_2 production to 171.85 ± 12.84 and 185.6 ± 6.37 % of control, respectively, but the suppression was not significant in terms of statistical analysis. In summary, *A. sessilis* ethanolic extract does not inhibit H_2O_2 production triggered by TNF-α.

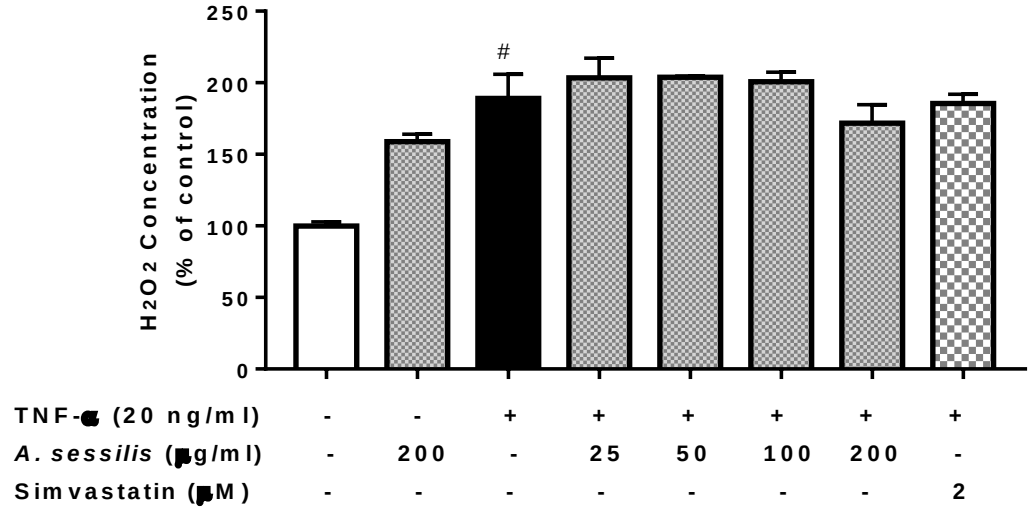


Figure 4.6: The effect of *A. sessilis* ethanolic extract on TNF-α-induced increased extracellular H_2O_2 production. HAEC were pre-treated with various concentrations of *A. sessilis* ethanolic extract (25 – 200 µg/ml) or simvastatin (2 µM) for 24 h, followed by stimulation with 20 ng/ml of TNF-α for 1 h. The assay was performed immediately using H_2O_2 assay kit after the collection of cell culture supernatant. Results are presented as the mean ± S.E.M. from three independent experiments, each was performed in triplicates. #P<0.05 compared to unstimulated control.

4.7. Effect of *A. sessilis* Ethanolic Extract on Increased Soluble VCAM-1 Expression Stimulated by TNF-α

The amount of soluble VCAM-1 released in cell culture supernatant was measured using ELISA method. sVCAM-1 expression was dramatically increased by 10 ng/ml of TNF-α to 890.2 ± 6.87% of control (P<0.05) compared to unstimulated control **(Figure 4.7)**. Pre-treatment of HAEC with *A. sessilis* ethanolic extract at all concentrations (25 – 200 µg/ml) did not inhibit TNF-α-induced increased soluble VCAM-1 expression (835.5 ± 40.97, 868.9 ± 17.95, 844.4 ± 20.98, 808.1 ± 27.97 % of control respectively). Interestingly, pre-treatment with 200 µg/ml *A. sessilis* ethanolic extract alone significantly increased VCAM-1 expression to 251.5 ± 31.01% (P<0.05), compared to unstimulated control. Simvastatin, however, was not able to decrease soluble VCAM-1 expression induced by TNF-α, with a result of 733.55 ± 22.41 % of control. Therefore, NAC was used as a control drug to validate this assay. 10 mM of NAC, which is a potent ROS inhibitor, dramatically reduced soluble VCAM-1 expression induced by TNF-α to 67.17 ± 4.61% of control (P<0.05). This result shows that VCAM-1 expression could be inhibited by a ROS inhibitor, suggesting that VCAM-1 is a downstream component of ROS in TNF-α signaling cascade. In summary, *A. sessilis* does not lower the increased release of soluble VCAM-1 induced by TNF-α.

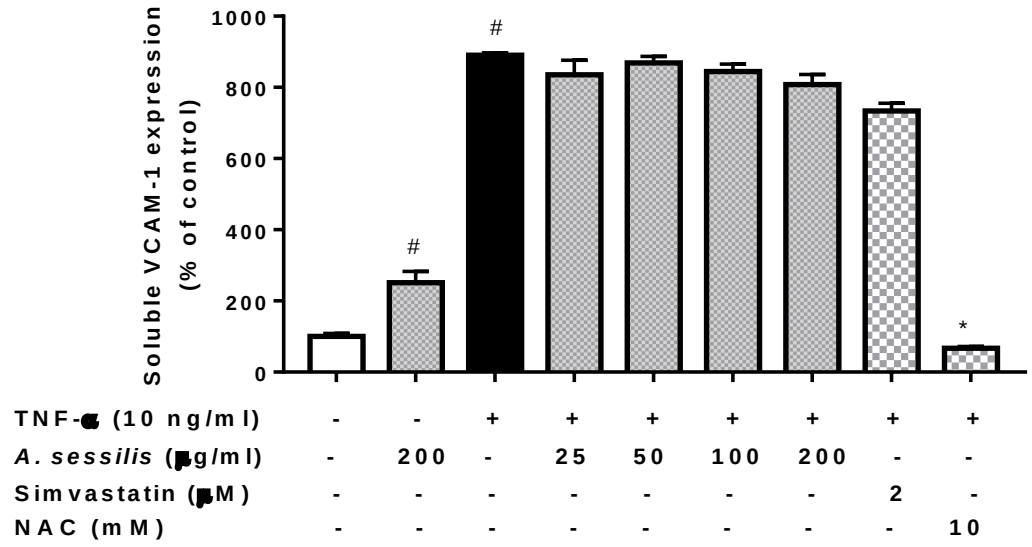


Figure 4.7: The effect of *A. sessilis* ethanolic extract on TNF-α-induced increased soluble VCAM-1 release in HAEC. HAEC cultured on 6-well plates were pre-treated with various concentrations of *A. sessilis* ethanolic extract (25 – 200 µg/ml), simvastatin (2 µM) or NAC (10 mM) for 24 h, followed by stimulation with TNF-α (10 ng/ml) for 6 h. The cell culture supernatant was collected and assayed using VCAM-1 ELISA kits. The absorbance was measured at 450 nm and corrected at 540 nm. Data are presented as mean ± S.E.M of three independent experiments, each was performed in triplicates. #P<0.05 compared to unstimulated control. *P<0.05 compared to TNF-α-treated group.

4.8. Effects of H_2O_2 on Antioxidant Enzymes

4.8.1. Effects of H_2O_2 on SOD Activity

Preliminary data showed that 10 and 20 ng/ml of TNF-α was not able to suppress SOD activity significantly at 1 h to 6 h (**Appendix J**). Hence, in this assay, H_2O_2 was used as an inducer to suppress SOD activity in HAEC instead of TNF-α. To identify the optimum induction time and concentration of H_2O_2 for the SOD assay, HAEC were treated with 50 – 400 µM of H_2O_2 for 30 min, 2 h and 4 h. The result shows that 100 µM of H_2O_2 significantly reduced SOD activity at 2 h (19.92 ± 7.1% of control), compared to unstimulated control (**Figure 4.8B**). On the other hand, H_2O_2 at all concentrations tested did not reduce SOD activity significantly at 30 min and 4 h (**Figures 4.8A and C**). Therefore, HAEC were induced with 100 µM of H_2O_2 for 2 h in the subsequent experiment which measured the effect of *A. sessilis* ethanolic extract on reduced SOD activity caused by H_2O_2.

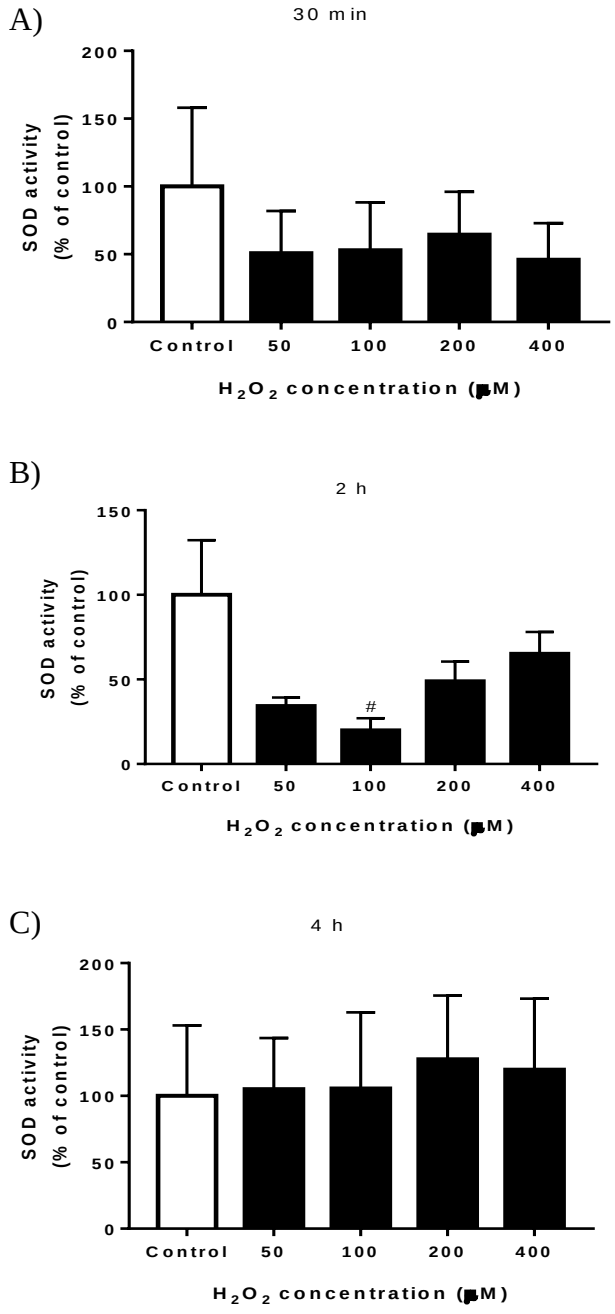
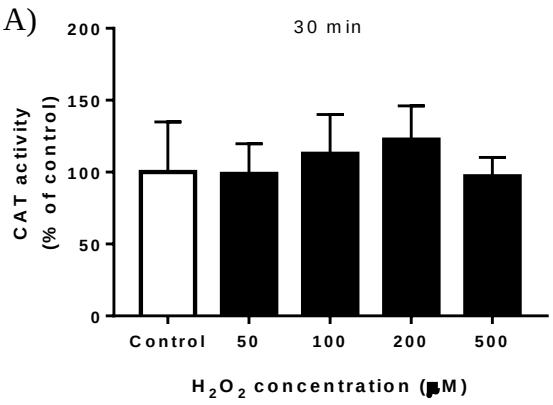


Figure 4.8: **The effect of H_2O_2 on SOD activity in HAEC**. HAEC were pre-treated with various concentrations of H_2O_2 (50, 100, 200 and 400 µM) for **A) 30 min, B) 2 h and C) 4 h**. Then, the cells were lysed in iced-cold buffer. Cell lysates were collected and the SOD activity assay was performed using SOD assay kits. Data are presented as the mean ± S.E.M of three independent experiments, each was performed in triplicates. #P<0.05 compared to unstimulated control.

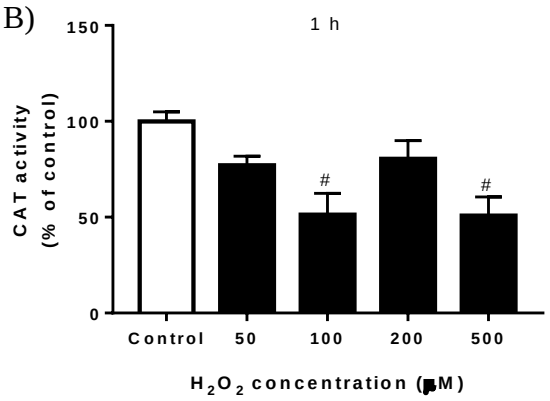
4.8.2. Effects of H_2O_2 on CAT Activity

The optimization result shows that 100 µM and 500 µM of H_2O_2 significantly reduced CAT activity at 1 h to 51.3 ± 11.11 and 50.8 ± 9.81 % compared to unstimulated control, respectively (**Figure 4.9B**). In addition, 100 µM and 500 µM of H_2O_2 also significantly reduced CAT activity to 44.6 ± 2.90 and 36.4 ± 10.90 % of control, respectively, at 2 h (**Figure 4.9C**). To standardise the H_2O_2 induction period between SOD and CAT assays, 2 h induction period was chosen and was used in the subsequent experiment. Given that 100 µM and 500 µM of H_2O_2 inhibited CAT activity to a similar extend at 2 h, the lower concentration (100 µM) was chosen for the subsequent experiment to avoid possible cell death caused by the relatively higher concentration of H_2O_2 (500 µM). Previous studies demonstrated that 500 µM of H_2O_2 was toxic to HAEC (Al-Ruzzeh et al., 2004). In summary, 100 µM of H_2O_2 with 2 h incubation period was chosen for the subsequent experiment which examined the effect of *A. sessilis* ethanolic extract on H_2O_2-induced reduced CAT activity.

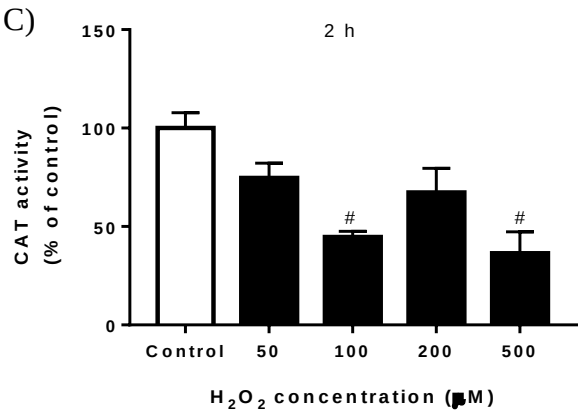
A)


30 min
CAT activity (% of control)
200
150
100
50
0
Control
50
100
200
500
H2O2 concentration (μM)

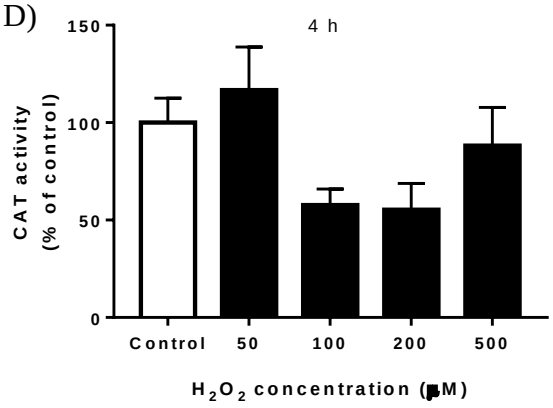
B)


1 h
CAT activity (% of control)
150
100
50
0
#
#
Control
50
100
200
500
H2O2 concentration (μM)

C)


2 h
CAT activity (% of control)
150
100
50
0
#
#
Control
50
100
200
500
H2O2 concentration (μM)

D)


4 h
CAT activity (% of control)
150
100
50
0
Control
50
100
200
500
H2O2 concentration (μM)

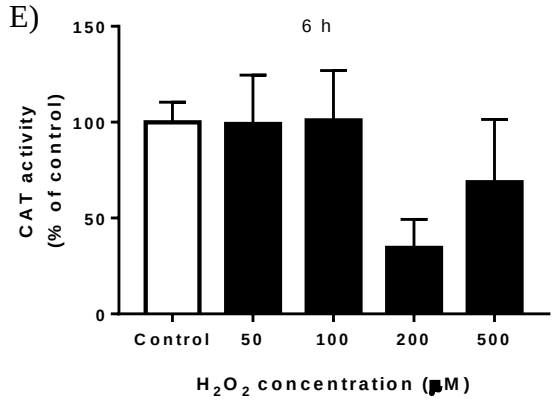


Figure 4.9: **The effect of H_2O_2 on CAT activity in HAEC**. HAEC were pre-treated with various concentrations of H_2O_2 (50, 100, 200 and 500 μM) for **A) 30 min, B) 1 h, C) 2 h, D) 4 h and E) 6 h**. Cell lysates were collected and the CAT activity was measured using CAT assay kits. Data are presented as mean ± S.E.M of three independent experiments, each was performed in triplicates. #P<0.05 compared to unstimulated control.

4.9. Effects *of A. sessilis* on Activities of Antioxidant Anzymes

4.9.1. Effect of *A. sessilis* Ethanolic Extract on H_2O_2-Induced Decreased SOD Activity

In **Figure 4.10**, it was demonstrated that *A. sessilis* alone significantly reduced SOD activity in HAEC to 49.9 ± 7.44 % of control. 100 µM of H_2O_2 significantly reduced SOD activity in HAEC to 66.8 ± 3.37 % of control, compared to unstimulated control (P<0.05). 50, 100 and 200 µg/ml of *A. sessilis* ethanolic extract significantly increased SOD activity reduced by H_2O_2 in a dose-dependent manner (120.7 ± 3.15%, 123.2 ± 6.67% and 136.1 ± 4.01% of control, respectively). HAEC that was pre-treated with 10 µM of dexamethasone which serves as a positive control drug showed significant increment of SOD activity (117.4 ± 5.80 % of control) (P<0.05). Dexamethasone was used as a control drug in this experiment as simvastatin and NAC were not able to significantly elevate SOD activities reduced by H_2O_2 (89.3 ± 6.93 and 67.8 ± 4.70 % of control respectively). These results indicate that *A. sessilis* ethanolic extract improves SOD activity in HAEC stimulated with H_2O_2.

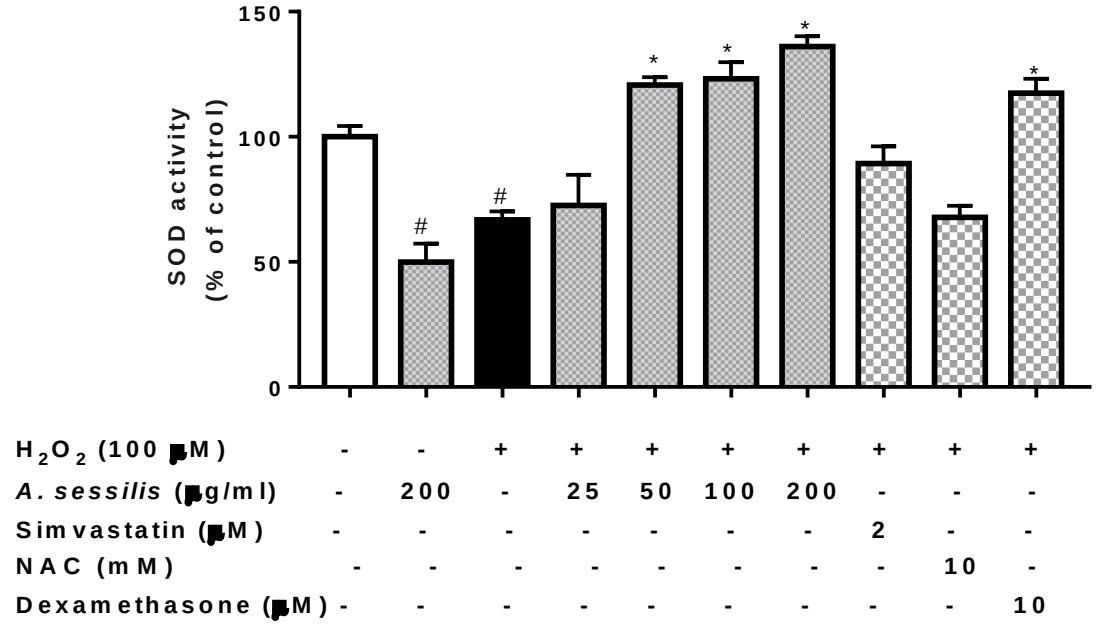


Figure 4.10: **The effect of *A. sessilis* ethanolic extract on H_2O_2-induced reduced SOD activity**. HAECs were pre-treated with various concentrations of *A. sessilis* (25 – 200 µg/ml), simvastatin (2 µM), NAC (10 mM) or dexamethasone (10 µM) for 24 h, followed by stimulation with 100 µM of H_2O_2 for 2 h. Cell lysates were collected and the assay was performed immediately using SOD assay kits. Results are presented as the mean ± S.E.M. from three independent experiments, each was performed in triplicates. #P<0.05 compared to unstimulated control. *P<0.05 compared to H_2O_2-induced group.

4.9.2. Effects of *A. sessilis* Ethanolic Extract on H_2O_2-Induced Decreased CAT Activity

As shown in **Figure 4.11**, treatment with *A. sessilis* alone did not affect the basal CAT activity in HAEC (110.4 ± 4.67 % of control). 100 µM of H_2O_2 significantly lowered CAT activity in HAEC to 74.1 ± 2.07 of control (P<0.05). Pre-treatment of *A. sessilis* ethanolic extract for 24 h, at 25, 50 and 200 µg/ml, significantly elevated the lowered CAT activity (112.5 ± 8.65%, 96.84 ± 8.46% and 110.1 ± 1.28% of control, respectively). A non-dose-dependent effect was observed as 100 µg/ml of *A. sessilis* ethanolic extract did not enhance the reduced CAT activity caused by H_2O_2. Simvastatin also significantly improved the lowered CAT activity to 105.6 ± 3.05 % of control (P<0.05). These results show that *A. sessilis* ethanolic extract protects against H_2O_2-induced decreased CAT activity.

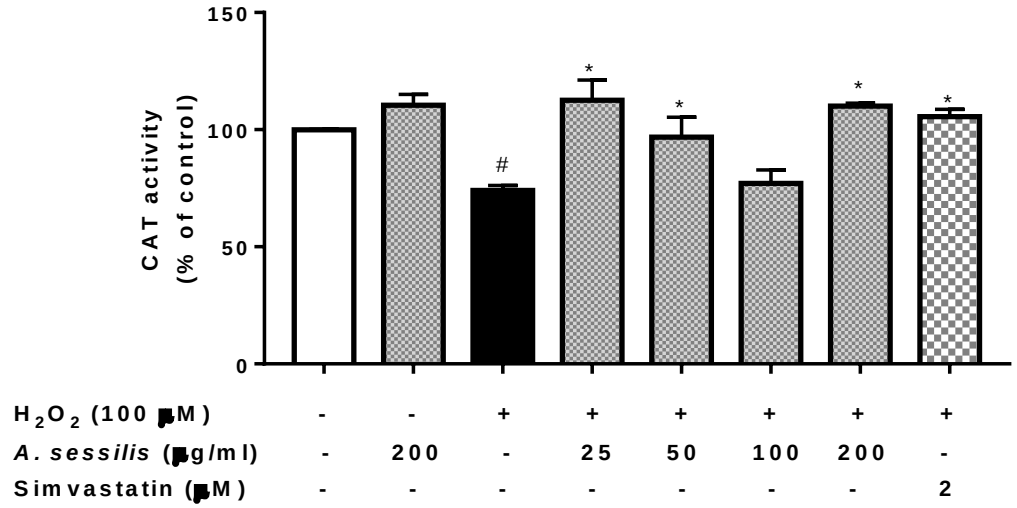


Figure 4.11: **The effect of *A. sessilis* ethanolic extract on H_2O_2-induced decreased CAT activity**. HAEC were pre-treated with various concentrations of *A. sessilis* ethanolic extract (25 – 200 μg/ml) or simvastatin (2 μM) for 24 h, and this is followed by stimulation with 100 μM H_2O_2 for 2 h. The assay was performed immediately using CAT assay kits after the collection of cell lysates. Results are presented as the mean ± S.E.M. from three independent experiments, each was performed in triplicates. #P<0.05 compared to unstimulated control. *P<0.05 compared to H_2O_2-induced group.

CHAPTER 5

DISCUSSION

A. sessilis Ethanolic Extract

The study of natural products, particularly medicinal and herbal plants as source of medicine has been rising. The main reason of this uprising popularity is due to their potential applications as alternative treatments with minimal side effects. Besides that, natural products are also cheaper, easily available and easy to administer. The preparation process of natural products such as herbal plants usually involves extraction of the dried, ground plant to separate active components of the plant from their inactive materials. Subsequently, fractionation and isolation can also be done in order to further separate the components into single active compound (Abubakar et al., 2020).

In this study, extraction using ethanol was done on *A. sessilis* whole plant to obtain *A. sessilis* ethanolic extract. Ethanol is a polar solvent and is considered as a universal solvent for phytochemical investigations of plant extract, as it has polarity value near to the polarity of solutes in most plants (Zhang et al., 2018). Moreover, ethanol has been extensively used to extract antioxidant compounds from various plants as it covers most of the metabolites present in the plant and yields more total phenolic contents and more potent antioxidant activities (Sultana et al., 2009). This current study is the first study that investigates the activity of *A. sessilis* on HAEC. Therefore, ethanolic extract was chosen as it captures most metabolites including a wide range of active compounds that can potentially show protective effects in pro-inflammatory stimuli-induced HAEC.

Biological activities of *A. sessilis* remain largely unexplored until the last decade. Among early findings about *A. sessilis* biological effect is an article published by Swemimo et. al. in 2009 where the cytotoxic effect of *A. sessilis* ethanolic extract against HeLa cell lines was studied (Swemimo et al., 2009). *A. sessilis* started to garner the attention of researchers after Bachok et al. listed the plant as one of the thirty-two popular *ulam* consumed by Malaysians (Bachok et al., 2014). To date, different types of extractions using various solvents have been done on *A. sessilis* to study the biological activities of *A. sessilis* whole plant or parts of the plant. Ethanolic extract has been a prevalent solvent for *A. sessilis* extraction in biological assays (Mondal et al., 2014; Rayees et al., 2013). Other than ethanol, methanol also is a popular solvent used to extract *A. sessilis* (Chalannavar et al., 2013; George et al., 2010; Hossain et al., 2014). On the other hand, a study also did further fractionization of the ethanolic extract of *A. sessilis* using different types of solvents; water, ethyl acetate and hexane (Tan et al., 2013).

There are a few methods that can be chosen to extract herbal plants depending on the suitability and stability of the plant, as well as the nature of the solvent used for extraction. Some of the commonly used methods include maceration and Soxhlet extraction. In this study, *A. sessilis* was extracted using Soxhlet extraction method. Since ethanol is a volatile solvent, Soxhlet extraction is considered as the best method to extract *A. sessilis* for this study. During Soxhlet extraction, the solvent in a flask is continuously heated and evaporates into a condenser. The vapour is then condensed, passes through finely powdered dried plant and flows down into the flask together with extracted active components of the plants. This extraction method produces more concentrated extracts in relatively small amount, compared to the maceration method (Abubakar et al., 2020).

HAEC

The commonly used vascular endothelial cell culture models are HAEC, human umbilical vein endothelial cells, human brain microvascular endothelial cells, human coronary endothelial cells and human pulmonary artery endothelial cells because all these cell lines are commercially available. In atherosclerosis-related studies, HAEC and human coronary artery endothelial cells (HCAEC) are most commonly used. Although atherogenesis occurs primarily in human coronary artery, but HAEC is still a popular choice of cell culture model for atherosclerosis studies due to the constant exposure of aorta to high hemodynamic changes, as well as its modulation on CAM expression. It has been shown that the basal levels of VCAM-1, ICAM-1 and E-selectin in HAEC were lower than that of HCAEC. When stimulated with TNF-α at concentrations higher than 0.1 ng/ml, the level of VCAM-1 expression in HAEC did not differ significantly from the expression in HCAEC (Mcdouall et al., 2001). Primary HAEC was used in the present study to investigate the effect of TNF-α on endothelial activation and oxidative stress, which are the key events in early progression of atherosclerosis. HAEC have a cobblestone appearance with large dark nuclei and during proliferation, the cells appear small and even size. Based on a previous study, it was mentioned that the aortic arch region is more prone to atherogenesis because of disturbed laminar blood flow (Gimbrone et al., 2012).

Cell Viability

Cell viability assay is a crucial preliminary assay in *in vitro* experiments to identify the dose range of any plant extract or active compound which does not affect the viability of cells. It is important to select non-toxic range of concentrations of a plant extract to ensure that all experiments performed are valid where the results are not being affected by cell death. In MTT cell viability assay, tetrazolium salt penetrates the membrane of viable cells. The cells metabolize the tetrazolium salt into insoluble purple formazan products. DMSO is then added

to solubilize the purple formazan, and a stabilized color product is formed, where the absorbance of the product can be measured.

In this study, the MTT cell viability assay was performed to examine effects of *A. sessilis* ethanolic extract on HAEC viability. Based on previous studies, various cell culture models were treated with *A. sessilis* extracts for a period of 24 h and therefore in this study, 24 h treatment time was chosen for *A. sessilis* ethanolic extract. In **Figure 4.1**, it was shown that 6.25 – 200 µg/ml of *A. sessilis* ethanolic extract did not have any effect on HAEC viability, whereas 400 and 800 µg/ml of the extract dramatically caused death in HAEC. While no study had reported on toxicity doses of *A. sessilis* on HAEC, some studies on other cell types showed a similar non-toxic dose range of *A. sessilis* extracts, ranging from 25 – 500 µg/ml. In a study which involves the use of human skin cells including human dermal fibroblast, keratinocytes, and diabetic human dermal fibroblast, 25 – 500 µg/ml of *A. sessilis* ethanolic extract of the plant stem did not show any effect on cell viability (Muniandy et al., 2018b). In another study using RAW 264.7 murine macrophage cell line, *A. sessilis* stem extract dose-dependently decreased the cell viability. As 400 and 500 µg/ml of *A. sessilis* stem extract slightly affect the viability of RAW 264.7 cells, only concentrations up to 200 µg/ml of *A. sessilis* extract were used (Muniandy et al., 2018a).

Endothelial Hyperpermeability

During endothelial activation, pro-inflammatory mediators are released, leading to increased endothelial permeability which has been strongly correlated with atherosclerosis. Therefore, targeting on inhibiting hyperpermeability is a crucial therapeutic strategy in order to prevent the initiation of atherosclerosis (Kumar et al., 2009). TNF-α is a potent cytokine that has previously been demonstrated to increase the permeability of endothelial cells. After non-toxic

concentrations of *A. sessilis* ethanolic extract were determined, the effect of *A. sessilis* ethanolic extract on HAEC hyperpermeability induced by TNF-α was evaluated. Previously, a dose and time-response study of TNF-α demonstrated that 10 and 15 ng/ml of TNF-α significantly induced increased endothelial permeability in HAEC at 6 h, which was sustained up to 24 h (Fong et al., 2015). Therefore, in this present study, TNF-α at a dose of 10 ng/ml and an induction period of 6 h was used to induce hyperpermeability in HAEC. The permeability data in **Figure 4.2** showed that induction with TNF-α as aforementioned caused barrier disruption by increasing the permeability of HAEC monolayer and allowing more FITC-dextran to pass through the cell monolayer and accumulate at the bottom well.

The permeability data in current study showed that 200 µg/ml of *A. sessilis* ethanolic extract, but not other lower concentrations, protects against TNF-α-induced increased endothelial permeability, as demonstrated by reduced FITC-dextran passage through the cell monolayer (**Figure 4.2)**. This suggests that *A. sessilis* ethanolic protects against TNF-α-induced endothelial barrier disruption. This inhibitory effect in endothelial hyperpermeability has also been demonstrated by other medicinal plants. One of the well-studied plants is *Centella asiatica*, in which the ethanolic extract has previously been shown to reduce peritoneal and ear vascular permeability in mice (Seo et al., 2021). In a clinical study, total triterpenic fraction of *C. asiatica* improved capillary permeability in patients with venous hypertension (Belcaro et al., 1990). In addition, active compounds isolated from *C. asiatica* such as asiatic acid and asiaticoside also suppressed endothelial hyperpermeability induced by TNF-α (Fong et al., 2016; Fong et al., 2015). Besides that, WS® 1442, a proven multi-component drug from hawthorn extract also protects against endothelial hyperpermeability by blocking FITC-dextran permeability induced by thrombin (Bubik et al., 2012)

Intracellular ROS and Extracellular H_2O_2 Productions

In vascular endothelial cells, increased oxidative stress contributes to the development of various vascular diseases including atherosclerosis and ischemic heart disease (Griendling et al., 2016). ROS are molecules that have one or more unpaired electrons in their orbital. Among all ROS, H_2O_2 and O_2^- are known to contribute the most as signaling molecules that initiate oxidative stress (Wang et al., 2018). The ROS and H_2O_2 assays showed that TNF-α increases the levels of ROS including H_2O_2 in HAEC (**Figures 4.3 and 4.5**). Previous studies reported that TNF-α triggers increased ROS production in vascular cells, leading to increased endothelial permeability (Craige et al., 2015; Kong et al., 2018). Besides that, a lot of studies focus on detecting H_2O_2 concentration, and different approaches have been developed with regards to the possibility of using H_2O_2 production as an indicator of oxidative stress (Breton-Romero et al., 2014). Our results showed that pre-treatment with *A. sessilis* significantly reduced ROS production, but not H_2O_2 production induced by TNF-α. These data indicate that the activity of *A. sessilis* on TNF-α-induced endothelial activation is associated with reduction of oxidative stress mechanism caused by ROS other than H_2O_2. Antioxidant activity of *A. sessilis* demonstrated in the present study is supported by other studies that previously reported antioxidant activity of *A. sessilis* using DPPH radical scavenging assay (Kota et al., 2017; Muniandy et al., 2018b).

Furthermore, the results generated from the H_2O_2 assay (**Figure 4.6**) are also in contrast with the previously reported H_2O_2 scavenging activity of *A. sessilis* published by Aryal et. al. In the previous study, it was demonstrated that *A. sessilis* methanolic extract had a high IC_{50} value for H_2O_2 scavenging activity (16.25 µg/ml), a value that was comparable to the IC_{50} of ascorbic acid, which is a well-known antioxidant (Aryal et al., 2019). The inconsistency between the findings of Aryal's study and the findings of this study could be due to different solvents used

for plant extraction and different experimental models performed. Aryal et al. used methanolic extract of *A. sessilis* and therefore, the active constituent that contributed to the positive effect of the extract might not be present in the ethanolic extract of *A. sessilis*. Besides that, the experimental method conducted by Aryal et al. (2019) is also different from the method used in this present study. The authors used H_2O_2 scavenging assay method which solely depends on the chemical reaction that occurs between a plant extract and H_2O_2, without involving intracellular production of ROS. On the other hand, cell culture supernatant was used in the present study to measure the effect of *A. sessilis* ethanolic extract on extracellular H_2O_2 release by HAEC. In comparison to the H_2O_2 scavenging activity assay, this *in vitro* cell culture method resembles the *in vivo* environment more closely where ROS including H_2O_2 are produced by endothelial cells in response to pro-inflammatory stimuli. As shown in **Figure 4.4** and **Figure 4.6**, simvastatin improved ROS level in HAEC, but not H_2O_2 production. In the future, H_2O_2 assay should be done with different positive control that has radical scavenging effect such as NAC.

Soluble VCAM-1 Production

VCAM-1 has been reported to be one of the major leukocyte adhesion regulators among other CAMs. The binding of integrins with VCAM-1 results in production of superoxide (O_2^-) and increases oxidative stress levels in endothelial cells. (Kim et al., 2017; Vogel et al., 2017). Previous studies demonstrated that VCAM-1 is a key mediator in atherosclerosis, where VCAM-1 expression was abundantly detected at atherosclerotic lesion sites of vascular endothelial cells (Vogel et al., 2017). TNF-α is a well-known positive regulator of VCAM-1 as the cytokine was demonstrated to upregulate VCAM-1 mRNA expression (Zapolska-Downar et al., 2012). It has been shown that 10 ng/ml of TNF-α significantly upregulated VCAM-1 expression in HAEC at 6 h (Fong, Ng, et al., 2016). Therefore, TNF-α at a dose of

10 ng/ml and an induction period of 6 h was used to induce soluble VCAM-1 expression in HAEC in this study.

Unexpectedly, *A. sessilis* ethanolic extract alone significantly increased soluble VCAM-1 release in HAEC (**Figure 4.7**). This might be the reason that *A. sessilis* also failed to reduce the increased secretion of soluble VCAM-1 induced by TNF-α in this present study (**Figure 4.7**). Increased VCAM-1 expression has been shown to cause disruption of the endothelial barrier, which in turn facilitates the transmigration of leukocytes across the endothelium (Kong et al., 2018) Further investigations are needed to clarify how *A. sessilis* increases the production of soluble VCAM-1 and to understand the indication of this enhancement effect. Besides that, the present study also showed that simvastatin failed to inhibit TNF-α-induced increased soluble VCAM-1 levels. Conflicting data are found among previous studies which report about the effect of simvastatin on the expression of CAM including VCAM-1. Both inhibitory and enhancement effects of simvastatin on CAM expression have previously been demonstrated (Meng et al., 2020; Sadeghi et al., 2000). These contradicting results between studies are probably due to different experimental conditions used for the measurement of CAM expression. A previous study showed that in static culture condition, simvastatin augmented VCAM-1 and ICAM-1 expressions, but the induction was prevented by laminar shear stress (Rossi et al., 2010). These previous data implies that the effect of simvastatin on the endothelium is greatly dependent on the shear stress levels applied to endothelial cells. On the other hand, NAC, which is a ROS inhibitor, was able to reduce TNF-α-induced VCAM-1 expression in HAEC. Based on this result, it is proposed that VCAM-1 expression is ROS-dependent.

SOD and CAT Activities

SOD and CAT are major antioxidant enzymes that act as first line of defence against ROS and free radicals. SOD converts O_2^- to H_2O_2 and CAT then converts H_2O_2 into water and oxygen. The optimization data showed that TNF-α failed to induce the reduction of SOD and CAT activities in HAEC (**Appendix J**). The inability of TNF-α to induce reduction of SOD and CAT activities found in this study is in contrast to a previous study reported in which TNF-α significantly decreased SOD and CAT activities (Zhou et al., 2017). Therefore, H_2O_2 was used as an inducer in SOD and CAT assays where the optimal dose and incubation time was determined.

Based on the H_2O_2 dose and time optimization, 100 µM of H_2O_2 significantly reduced SOD activity at 2 h, whereas 100 and 500 µM of H_2O_2 significantly reduced CAT activity at both 1 and 2 h (**Figure 4.8** and **Figure 4.9**). The use of H_2O_2 as an inducer to decrease SOD and CAT activities in cells is supported by many studies that have previously shown the inhibitory effect of H_2O_2 on SOD and CAT activities in HUVEC and human retinal endothelial cells (Yan et al., 2018; Zheng et al., 2020). The result in this present study confirmed that *A. sessilis* ethanolic extract improves both SOD and CAT activities in H_2O_2-induced HAEC (**Figure 4.9.1** and **Figure 4.9.2**). This result is consistent with previous findings where *A. sessilis* red ethyl acetate fraction dramatically increased pancreatic total SOD activity in diabetic rats (Tan & Kim, 2013). As shown in **Figure 4.9.1**, simvastatin and NAC failed to improve SOD activity and therefore, dexamethasone was used as a positive control. Dexamethasone was able to increase SOD activity reduced by H_2O_2 in this assay. On the other hand, simvastatin elevated CAT activity reduced by H_2O_2 **(Figure 4.9.2)**.

Taken together, these results show that *A. sessilis* alleviates oxidative stress by suppressing ROS production and upregulating the activities of anti-oxidant enzymes such as SOD and CAT. SOD and CAT has been known to act as defence system against oxidative stress, therefore reducing lipid peroxidation and low-density lipid modification that leads to atherosclerosis. Reduced SOD and CAT activities has been observed in patients with vascular disorders, particularly atherosclerosis (Khosravi et al., 2019). The mechanism of action of *A. sessilis* is illustrated in **Figure 5.1**. Apart from simvastatin, NAC and dexamethasone, some compounds with established antioxidant effects that can improve endothelial functions can also be used as positive controls to validate the assays.

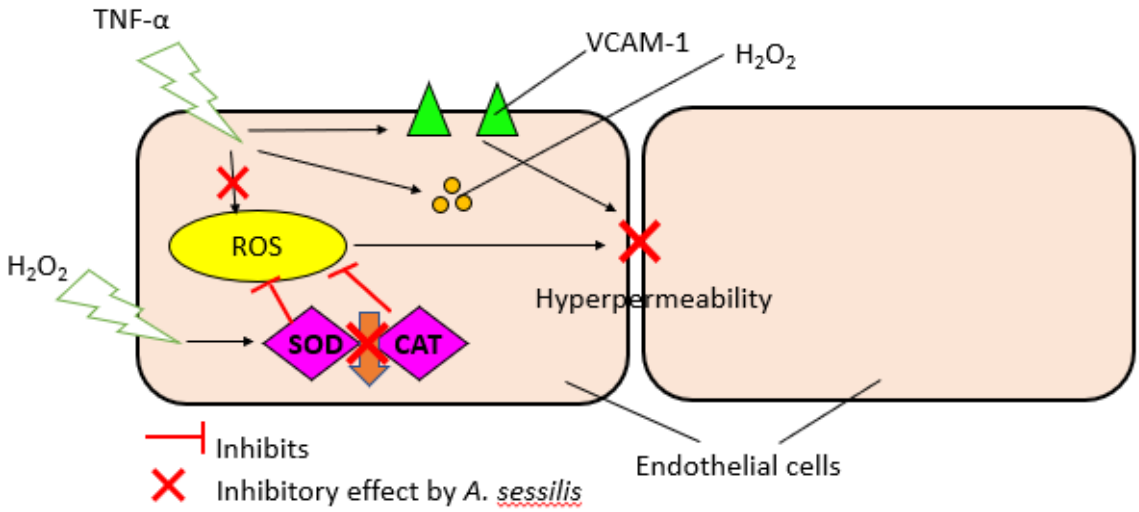


Figure 5.1: The mechanism of action of *A. sessilis*. *A. sessilis* suppresses TNF-α-induced endothelial hyperpermeability and reduces TNF-α-induced increased ROS production. Besides that, *A. sessilis* also prevents the reduction of SOD and CAT activities induced by H_2O_2. However, *A. sessilis* does not downregulate the increased expression of VCAM-1 and increased H_2O_2 production induced by TNF-α. The suppression of endothelial hyperpermeability by *A. sessilis* is associated with inhibition of oxidative stress.

Simvastatin

Simvastatin is a common drug widely used for treatment of cardiovascular diseases and risk factors associated to it by inhibiting 3-hydroxy-3-methylglutaryl-coenzyme A reductase which reduces cholesterol synthesis, thus lowering cholesterol (D. Han et al., 2020). In this study, simvastatin successfully reduced HAEC hyperpermeability and increased ROS level induced by TNF-α, as well as increased CAT activity lowered by H_2O_2. However, simvastatin failed to show positive effect on TNF-α-induced increased H_2O_2 production and sVCAM-1 expression and H_2O_2-induced reduced SOD activity.

Other drugs or compounds that can be used as positive control in this study are ROS inhibitor and NSAIDs. ROS inhibitor such as NAC has been used as positive control in other anti-atherosclerotic effect study by reducing ROS production in HUVEC induced with TNF-α (Ran et al., 2016). Apart from that, NSAIDs like dexamethasone, ibuprofen, PDTC and aspirin has also been used as positive control in previous study, showing reduced expression of ICAM-1 and VCAM-1 in TNF-α-induced HAEC (Chao et al., 2013; Zapolska-Downar et al., 2012).

Beside statins, ROS inhibitor and NSAIDs, some natural compounds are also well-known for their protective effects on cardiovascular diseases, particularly on endothelial function. They include resveratrol, quercetin, curcumin and epigallocatechin gallate, which are polyphenols that are commonly used to treat endothelial dysfunction in clinical, *in vivo* and *in vitro* studies (Khurana et al., 2013). These natural compounds, given their ability to reduce oxidative stress and inflammation by modulating various signaling pathways, can also be used as positive control in the present study.

CHAPTER 6

CONCLUSION

In summary, the findings suggest a possible mechanism of action which underlies the protective effect of *A. sessilis* ethanolic extract in pro-inflammatory mediators-stimulated oxidative stress and endothelial activation. The results demonstrated that *A. sessilis* ethanolic extract protects HAEC against TNF-α-induced endothelial hyperpermeability. Besides, *A. sessilis* lowers the increased ROS production, but not the release of H_2O_2 stimulated by TNF-α. However, *A. sessilis* extract fails to suppress the release of soluble VCAM-1, which is a molecule downstream of ROS. *A. sessilis* ethanolic extract also improves both H_2O_2-induced decreased SOD and CAT activities. These data suggest that the anti-hyperpermeability effect of *A. sessilis* is concomitant with abrogation of ROS release and enhancement of the antioxidant system in endothelial cells. *A. sessilis* protects against endothelial activation and this is associated with the inhibition of oxidative stress.

In conclusion, this study reveals new knowledge on how *A. sessilis* extract suppresses endothelial activation and provides a better understanding on the mechanism by which *A. sessilis* protects HAEC against detrimental effects caused by pro-inflammatory mediators such as TNF-α and H_2O_2. Importantly, this is the first study which reports the protective effect of *A. sessilis* in the endothelium. The findings, hence, suggest a new pharmacological use of *A. sessilis* in the prevention of early atherogenic events by inhibiting endothelial activation and oxidative stress.

For future research works, it is recommended to further explore the effect of *A. sessilis* in HAEC permeability in term of the signaling pathway involved and mechanisms. In addition,

different types of *A. sessilis* extracts, other than the ethanolic extract, should be assayed to determine the extract which possesses the most potent pharmacological activity. Bioassay guided extraction, fractionation and isolation of pure compound can also be done to identify the active compound which is responsible for pharmacological effects of *A. sessilis*. Besides that, phytochemical profile identification from *A. sessilis* extracts can also be performed. Apart from *in vitro* studies, *in vivo* studies also should be conducted for better understanding on the *in vivo* effect of *A. sessilis* in suppressing endothelial activation and vascular oxidative stress.

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APPENDICES

Appendix A

Preparation of *A. sessilis* ethanolic extract

Preparation of *Althernanthera sessilis* ethanolic extract stock solution (5 mg/ml)

1. Approximately 10 mg of plant extract was weighed.
2. Volume of basal media needed to dissolve the extract was calculated.
   e.g if 10 mg extract, 2 ml of basal media
   if 8.8 mg extract, 1.76 ml basal media
3. Extract was dissolved in basal media. Vortex / sonicator was used until it was fully dissolved.
4. The extract was filtered into a new sterile microcentrifuge tube using 0.22 µl syringe filter.
5. The tube was covered with aluminium foil and stored at 4ºC.
6. Stable for about 3 months.

Preparation of *Althernanthera sessilis* working solution (25, 50, 100, 200 µg/ml)

1. Working solution was freshly prepared.
2. The working volume needed was calculated.
3. From stock solution, working concentration of 200, 100, 50 and 25 µg/ml was prepared.

   (M1) (V1) = (M2) (V2)

   (5000 µg/ml) (V1) = (200 µg/ml) (2000 µl)
   V1 = 80 µl of stock extract + 1920 µl of media

   (5000 µg/ml) (V1) = (100 µg/ml) (2000 µl)
   V1 = 40 µl of stock extract + 1960 µl of media

   (5000 µg/ml) (V1) = (50 µg/ml) (2000 µl)
   V1 = 20 µl of stock extract + 1980 µl of media

   (5000 µg/ml) (V1) = (25 µg/ml) (2000 µl)
   V1 = 10 µl of stock extract + 1990 µl of media

Appendix B

Preparation of simvastatin

Preparation of simvastatin stock solution (65.8829 mM)

1. 50 mg of simvastatin powder
2. Dissolve with 1 ml EtOH + 812 µl NaOH

Preparation of simvastatin working solutions (2 µM)

Stock: 65.88 mM

1. Working solutions were freshly prepared
2. Volume of simvastatin working solution needed was calculated.
3. From stock solution, working concentration of 2 µM was prepared.

(M1) (V1) = (M2) (V2)

(65.88 mM) (V1) = (10 mM) (6.6 µl)

V1 = 1 µl of 65.88 mM simvastatin + 5.6 µl of media

(10 000 µM) (V1) = (100 µM) (100 µl)

V1 = 1 µl of 10 mM simvastatin + 99 µl of media

(100 µM) (V1) = (2 µM) (800 µl)

V1 = 16 µl of 100 µM simvastatin + 784 µl of media

Appendix C

Preparation of N-acetyl cysteine

Preparation of NAC stock solution (612.8 mM)

1. 0.1 g of NAC powder
2. Dissolve with 1 ml of water

Preparation of NAC working solutions (10 mM)

Stock: 612.8 mM

1. Working solutions were freshly prepared
2. Volume of NAC working solution needed was calculated.
3. From stock solution, working concentration of 10 mM was prepared.

(M1) (V1) = (M2) (V2)

(612.88 mM) (V1) = (100 mM) (1000 µl)

V1 = 163.19 µl of 612.8 mM NAC + 836.81 µl of media

(100 mM) (V1) = (10 mM) (1000 µl)

V1 = 100 µl of 100 mM NAC + 900 µl of media

Appendix D

Preparation of dexamethasone

Preparation of dexamethasone stock solution (10 mM)

1. 1.5 mg of dexamethasone powder
2. Dissolve with 382.2 µl methanol

Preparation of dexamethasone working solutions (10 µM)

Stock: 10 mM

1. Working solutions were freshly prepared
2. Volume of working dexamethasone needed was calculated.
3. From stock solution, working concentration of 10 µM was prepared.

(M1) (V1) = (M2) (V2)

(10 000 µM) (V1) = (100 µM) (1000 µl)

V1 = 10 µl of 10 mM dexamethasone + 990 µl of media

(100 µM) (V1) = (10 µM) (1000 µl)

V1 = 100 µl of 100 µM dexamethasone + 900 µl of media

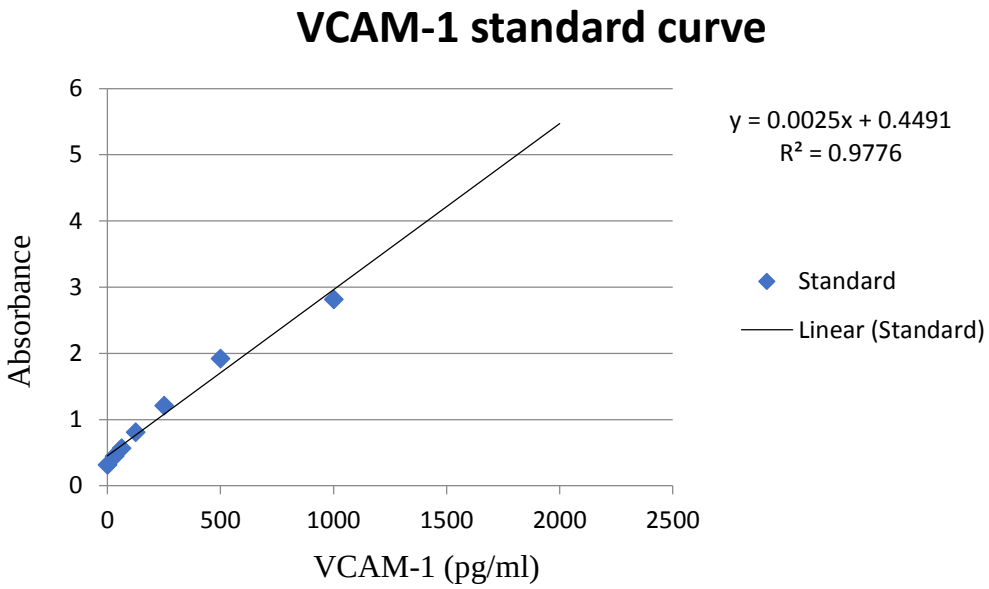
Appendix E

Volumes of media

Type of plate	6-well	12-well	24-well	96-well
Seeding volume (ml)	2000	1000	400	100
Treatment volume (ml)	1000	500	250	100

Appendix F

VCAM-1 Assay standard curve

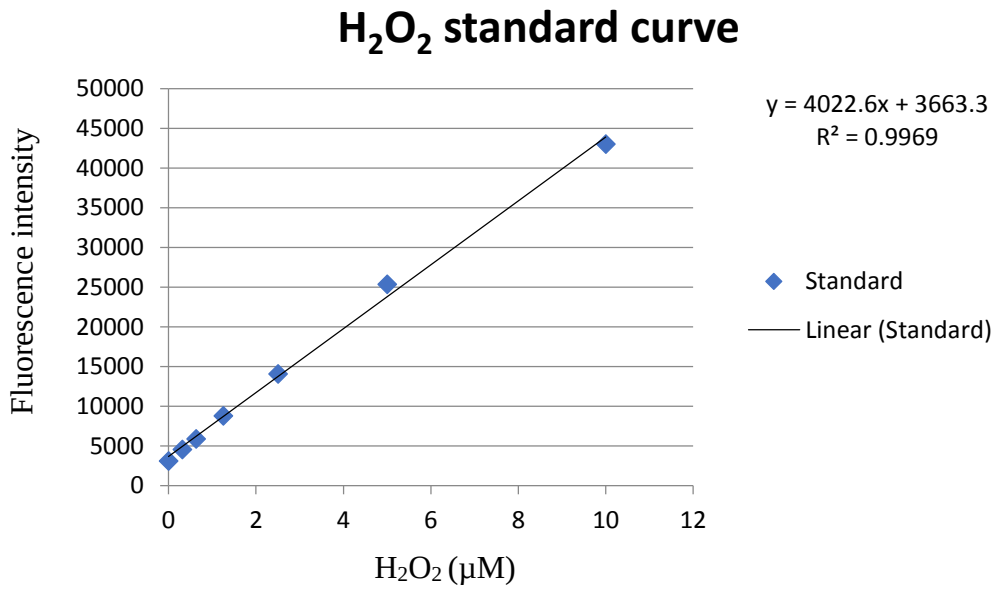


Calculation of VCAM-1 concentration

VCAM-1 concentration = (Mean absorbance – 0.4491) / 0.0025

Appendix G

H_2O_2 Assay standard curve

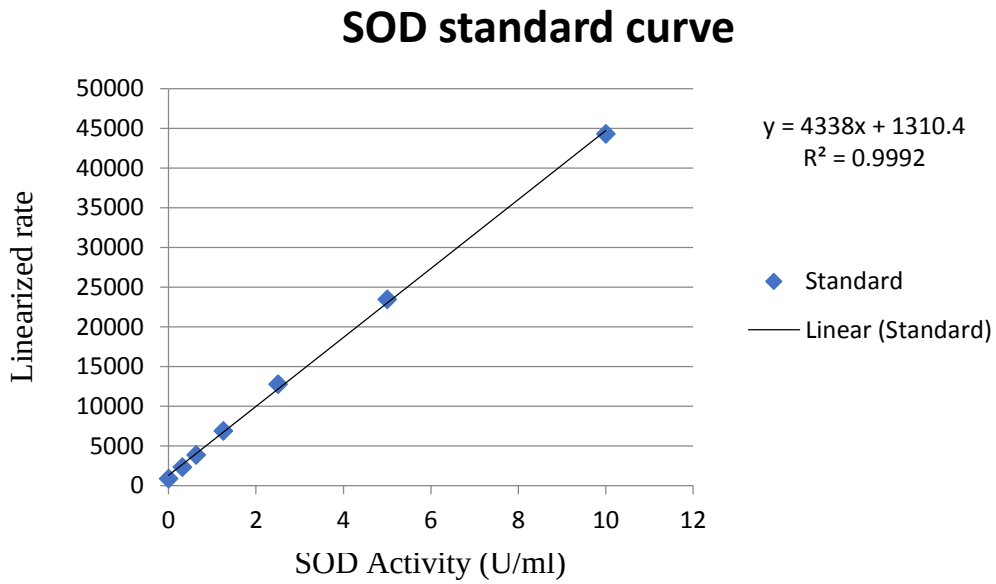


Calculation of H_2O_2 concentration

H_2O_2 concentration = (Mean fluorescence intensity – 3663.3) / 4022.6

Appendix H

SOD Assay standard curve



Linearized rate (LR)

LR for standard A = Absorbance of standard A / Absorbance of standard A

LR for standard B = Absorbance of standard A / Absorbance of standard B

LR for standard C = Absorbance of standard A / Absorbance of standard C

LR for standard D = Absorbance of standard A / Absorbance of standard D

LR for standard E = Absorbance of standard A / Absorbance of standard E

LR for standard F = Absorbance of standard A / Absorbance of standard F

LR for standard G = Absorbance of standard A / Absorbance of standard G

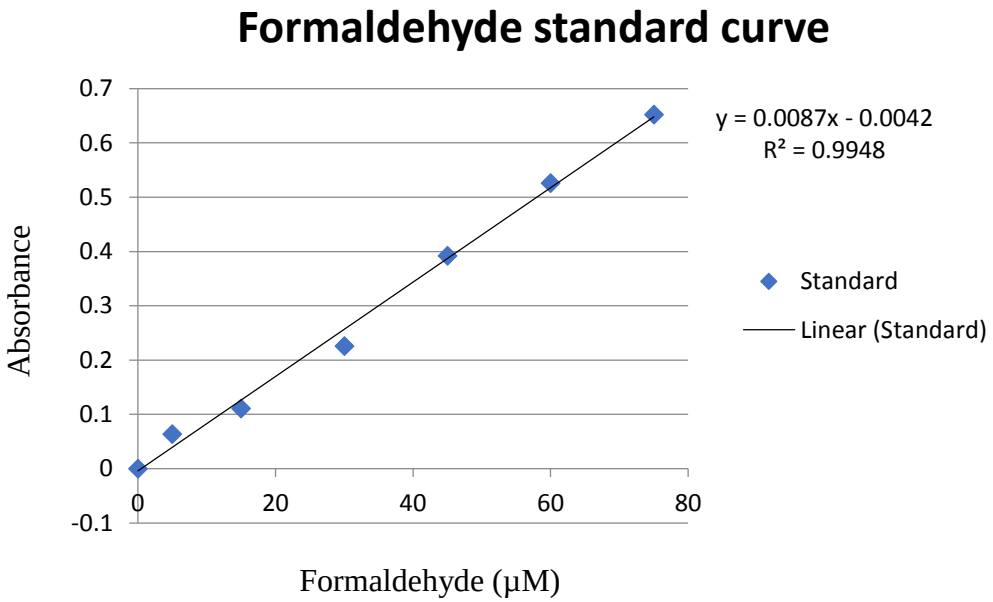
LR for sample = Absorbance of standard A / Absorbance of sample

Calculation of SOD activity

SOD (U/ml) = [(Sample LR – 1310.4) / 4338] x [0.23 ml / 0.01 ml]

Appendix I

CAT Assay standard curve



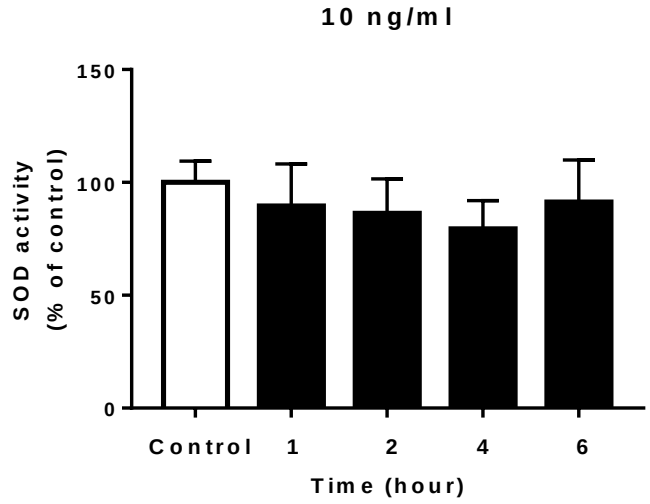
Calculation of CAT activity

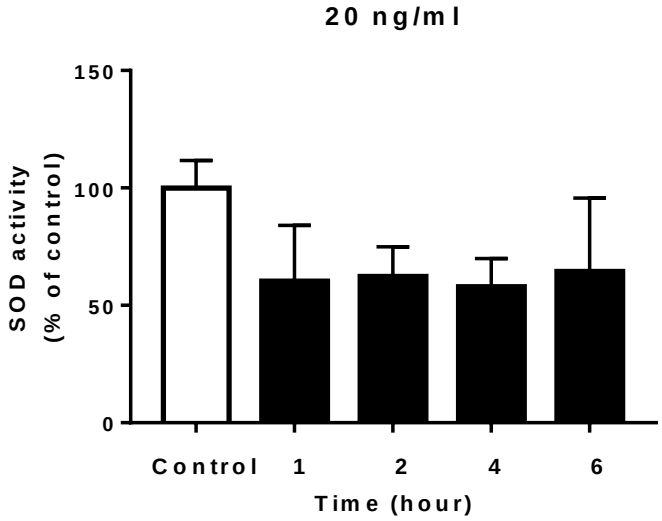
Formaldehyde (µM) = [(Sample absorbance – 0.0042) / 0.0087] x [0.17 ml / 0.02 ml]

CAT activity = (20 µM / 20 min) x sample dilution = nmol/min/ml

Appendix J

Effects of 10 and 20 ng/ml of TNF-α SOD activity





Appendix K

JOURNAL PUBLICATION

1. **Razali NNM**, Teh SS, Mah SH, Yong YK, Ng CT, Lim YM, Fong LY. (2022). Protective Effects of *Alternanthera sessilis* Ethanolic Extract on TNF-α-Induced Endothelial Barrier Activation and Oxidative Stress in Human Aortic Endothelial Cells. *Evidence-Based Complementary and Alternative Medicine, 2022,* 8728435.