



UNIVERSITI PUTRA MALAYSIA

***LAWSONE AS POTENTIAL ANTIOXIDANT: STUDIES ON TOTAL
ANTIOXIDANT ACTIVITY IN VITRO AND MOLECULAR DOCKING
ANALYSIS ON SUPEROXIDE DISMUTASE (SOD) ACTIVATION IN
SILICO***

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ABSTRACT

LAWSONE AS POTENTIAL ANTIOXIDANT: STUDIES ON TOTAL ANTIOXIDANT ACTIVITY *IN VITRO* AND MOLECULAR DOCKING ANALYSIS ON SUPEROXIDE DISMUTASE (SOD) ACTIVATION *IN SILICO*

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Introduction: Reactive oxygen species (ROS) are produced during normal cellular metabolism and play vital roles in the activation of signalling pathways. However, the imbalance between the ROS production and their elimination through protective mechanisms cause oxidative stress that can lead to chronic inflammation such as eczema and psoriasis on the skin. One of the most important preventive agents for reducing inflammation induced by oxidative stress are natural antioxidants. One of the natural sources with reported high antioxidant properties is *Lawsonia inermis* or henna, a plant that also scientifically proven for its anti-inflammatory characteristics. Lawsone as the most prominent active compound of henna, is expected to be responsible for the mentioned properties. Superoxide dismutase (SOD) is an anti-oxidative enzyme, where binding of phytochemicals to the domain of SOD receptor will increase its activity and reduce oxidative stress. **Objectives:** To evaluate the total antioxidant activity of lawsone *in vitro* and to predict the SOD activation property of lawsone via molecular docking study *in silico*. **Methodology:** Cytotoxicity (IC₅₀) of lawsone on human epidermoid carcinoma (A431) and normal mouse fibroblast (3T3) was obtained by performing MTT assay. Next, Ferric Reducing Antioxidant Power (FRAP) assay was carried out in two conditions: (i) in A431 and 3T3 cell lines; (ii) without cell line to assess the total antioxidant activity of lawsone. Next, molecular docking analysis was performed to investigate the activation mechanism of lawsone on SOD via AutoDock Tools (ADT). Prediction of the allosteric site of SOD was made by using AlloSite 2.0 webserver. The interaction between lawsone and SOD was analysed by using Pymol software, ProteinsPlus and PLIP webserver. **Results:** IC₅₀ of lawsone on A431 cell line was determined at 3650 µM while no IC₅₀ was detected on 3T3 cell line. Meanwhile, lawsone also exhibited the total antioxidant activities in the absence of cells, A431 and 3T3 cell lines at 200µM, with the highest FRAP values of 4.04 ± 0.18µM, 94.41 ± 1.21µM and 93.50 ± 8.48µM respectively. *In silico* results showed that lawsone binds to two allosteric regions of SOD1; namely Region A and B with binding affinities of -7.3 kcal/mol and -6.6 kcal/mol respectively. Results from PLIP webserver illustrated the hydrophobic interactions between lawsone and protein residues; - i.e. Asn53A, Val7F, Lys9F, Asn53F and Val148F from allosteric region A, and Ile113A, Ile151A and Ile113F from allosteric region B. Four hydrogen bondings with Gly108 (2.09Å & 2.94Å) and Val148 (1.91Å & 2.82Å) of chain A were also detected. Meanwhile, ProteinsPlus showed hydrophobic interaction of lawsone with

SOD1 pocket of Ile113A. **Conclusion:** Lawsone exhibited a low range increment in total antioxidant activity at the low concentration *in vitro* and have an excellent binding affinity at the allosteric sites of SOD *in silico* which is predicted to simultaneously trigger the activation of SOD enzyme.

Keywords: Lawsone; Anti-inflammatory; Antioxidant; FRAP; SOD; activation



ABSTRAK

LAWSON SEBAGAI ANTIOKSIDAN YANG BERPOTENSI: KAJIAN TERHADAP JUMLAH AKTIVITI ANTIOKSIDAN *IN VITRO* DAN ANALISIS MENGEDOK MOLEKUL TERHADAP PENGAKTIFAN SUPEROKSIDE DISMUTASE (SOD) *IN SILICO*

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Pengenalan: Spesies oksigen reaktif (ROS) terhasil apabila sel mengalami metabolisme yang normal dan ia mempunyai peranan yang penting dalam pengaktifan tapak jalan. Walau bagaimanapun, ketidakseimbangan yang berlaku di antara penghasilan dan penyingkiran ROS melalui mekanisme perlindungan menyebabkan berlakunya tekanan oksidatif yang boleh menyebabkan radang kronik kepada kulit seperti ekzema dan psoriasis. Salah satu agen pencegahan yang paling penting untuk mengurangkan radang yang disebabkan oleh tekanan oksidatif adalah antioksidan semula jadi. Salah satu sumber semula jadi yang dilaporkan dan dibuktikan secara saintifik mempunyai sifat antioksidan yang tinggi adalah tumbuhan yang dikenali sebagai *Lawsonia inermis* atau inai. Sebatian aktif inai yang paling utama, lawson dijangka bertanggungjawab di atas sifat yang dinyatakan. Superoksida dismutase (SOD) merupakan enzim anti-oksidatif, di mana pengikatan fitokimia di bahagian reseptor SOD akan meningkatkan aktiviti serta mengurangkan tekanan oksidatif.

Objektif: Untuk menilai jumlah aktiviti antioksidan lawson *in vitro* dan untuk meramalkan ciri pengaktifan lawson terhadap SOD melalui kajian mengedok molekul *in silico*. **Metodologi:** Kesitotoksikan (IC₅₀) lawson pada titisan sel karsinoma epidermoid manusia (A431) dan titisan sel normal fibroblas tikus (3T3) diperolehi dengan menjalankan asai MTT. Seterusnya, asai kuasa pengoksidaan pengurangan ferik (FRAP) dilaksanakan dalam dua kaedah: (i) titisan-titisan sel A431 dan 3T3; (ii) tanpa titisan sel untuk menilai jumlah aktiviti antioksidan lawson. Seterusnya, analisis mengedok molekul dijalankan untuk menyiasat mekanisme pengaktifan lawson terhadap SOD melalui perisian AutoDock Tools (ADT). Ramalan tapak alosterik SOD dilakukan dengan menggunakan pelayan web AlloSite 2.0. Saling tindak di antara lawson dan SOD dianalisis dengan menggunakan perisian Pymol, pelayan web ProteinsPlus dan PLIP. **Keputusan:** IC₅₀ lawson pada titisan sel A431 ditentukan pada 3650 µM manakala tiada IC₅₀ dikesan pada titisan sel 3T3. Sementara itu, dinyatakan juga jumlah aktiviti antioksidan lawson dan lawson terhadap titisan-titisan sel A431 dan 3T3, di mana nilai FRAP tertinggi pada 200 µM masing-masing adalah 4.04 ± 0.18 µM, 94.41 ± 1.21 µM dan 93.50 ± 8.48 µM. Keputusan *in silico* menunjukkan bahawa lawson ditambat pada dua tapak alosterik SOD1 yang dinamakan tapak A dan B dengan tambat afiniti sebanyak -7.3 kcal/mol dan -6.6 kcal/mol. Keputusan daripada pelayan web PLIP menggambarkan interaksi hidrofobik di antara lawson dan residu protein, iaitu Asn53A, Val7F, Lys9F, Asn53F dan Val148F dari tapak A dan Ile113A, Ile151A dan Ile113F dari tapak B. Empat pengikatan hidrogen dengan Gly108 (2.09Å

& 2.94Å) dan Val148 (1.91Å & 2.82Å) pada rantai A juga turut dikesan. Sementara itu, ProteinsPlus menunjukkan interaksi hidrofobik di antara lawson and poket Ile113A pada SOD1. **Kesimpulan:** Lawson mempamerkan kenaikan yang rendah dalam jumlah aktiviti antioksidan pada kepekatan rendah *in vitro* dan mempunyai tambat afiniti yang bagus di tapak alosterik SOD dan pada masa yang sama diramalkan mampu mencetuskan pengaktifan enzim SOD *in silico*.

Kata Kunci: Lawson, Anti-Radang, Antioksidan, FRAP, SOD, Pengaktifan



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LIST OF ABBREVIATIONS

3T3	Normal mouse fibroblast cell lines
A431	Human epidermoid carcinoma cell lines
ADT	AutoDock Tools
ANOVA	Analysis of Variance
FRAP	Ferric Reducing Antioxidant Power
IC ₅₀	Half Maximal Inhibitory Concentration
<i>L. inermis</i>	<i>Lawsonia inermis</i>
PDB	Protein Data Bank
ROS	Reactive oxygen species
SEM	Standard error mean
SOD	Superoxide dismutase
TPTZ	2,4,6-Tripyridyl-S-triazine

CHAPTER 1

INTRODUCTION

1.1 Background

Any molecular species that is capable of independent existence which contains an unpaired electron in their atomic orbital can be described as free radicals. The unpaired electron has resulted in certain properties that other radicals have in general. A lot of radicals are highly reactive and unstable. They can behave as oxidants or reductants, either by accepting an electron or donating an electron from other molecules (Lobo et al., 2010). Meanwhile, reactive oxygen species (ROS) is a term that is used to include the free radicals and non-radicals such as singlet oxygen, peroxynitrite and hydrogen peroxide. All aerobic organisms generate and eliminate reactive oxygen species, resulting in either the physiological amount needed for regular cell function or unsustainable amounts, a phenomenon called oxidative stress. Oxidative stress is the term that refers to the inequity between the production of ROS and the production of the protective antioxidant (Poljšak & Dahmane, 2012). Oxidative stress results in altered metabolism, disrupted signal transduction activities, and biomolecular injury, all of which lead to pathological changes in cell and tissue function (Trouba et al., 2002).

Oxidative stress can cause inflammation on the skin. According to Okayama (2005), the role of the skin as an interface between the body and the atmosphere continuously exposes the skin to both endogenous and environmental pro-oxidants,

contributing to the production of dangerous reactive oxygen species (ROS). Oxidative stress has already been shown as the primary mechanism of various diseases as the correlation between the oxidative stress, particularly in lipid peroxidation, and different inflammatory skin condition such as atopic dermatitis and psoriasis has been shown by compelling evidence (Santucci, Braconi, & Bernardini, 2010). Moreover, the formation of ROS activates the inflammatory process in the skin through environmental stimuli, such as sun rays, cigarette smoke and toxins as well as the natural ageing process (Pillai, Oresajo & Hayward, 2005).

Thus, some compounds can protect the skin from free radicals. They are known as antioxidants. According to Jaradat et al. (2018), antioxidants are chemical molecules that limit the propagation or initiation of oxidative chain reactions by inhibiting or delaying lipids or the oxidation of another compound. Antioxidants have an ingesting significant magnitude, which is the capability to reduce free radical activity that minimise the damage that leads to ailment and ageing (Biesalski, 2002). Hence, there are naturally occurring antioxidants in our skin that protect cells and tissue during normal metabolism from the continuous development of reactive oxygen metabolites, which consists of enzymatic and non-enzymatic antioxidants. Enzymatic antioxidants act as the first line of defence, for instance, glutathione peroxidase (GPx), superoxide dismutase (SOD) and catalase (CAT) while non-enzymatic antioxidants such as metal chelating agent, binds to the redox-active metals and suppress the production of metal-catalysed free radicals. However, they do not directly interact with reactive oxygen metabolites and decompose them (Conner & Grisham, 1996).

Superoxide dismutase (SOD) is the primary antioxidant defence system by catalysing the conversion of superoxide anion and hydrogen peroxide participating in cell signalling at each subcellular location. In addition, SOD also plays a crucial role in suppressing oxidative inactivation of nitric oxide, thereby preventing the production of peroxynitrite and endothelial and mitochondrial impairment (Fukai & Ushio-Fukai, 2011). Along with their classical role as detoxification enzymes against singlet oxygen, there is growing evidence showing that SOD is actively involved in the modulation of various cellular processes as they are progressively known to regulate multiple cellular processes, ranging from mitogenic to oxidative stress response (Che et al., 2016).

Nowadays, a lot of studies prove that compounds from natural products act as antioxidant, which can give protection from inflammation to the skin. The extracts of *Lawsonia inermis*, commonly known as henna, are among the most frequently used skin dyes. Henna has been widely used in traditional medicine to treat inflammation, cancer, bacterial and fungal infections and various skin ailments. Furthermore, the great Islamic scholar, Ibn Al-Qayyim wrote in his book “The Prophetic Medicine” about the medicinal benefit of henna such as to treat headache, migraine, nail infection and hot inflammation (Neni Kartini, Noorshilawati, & Rosita, 2016). The primary active component of *L. inermis* is lawsone or 2-hydroxy-1,4-naphthoquinone. A lot of studies have proven that lawsone possesses several remarkable biological functions, such as antioxidant, antibacterial and antifungal, anti-inflammatory, antipyretic and analgesic and anticancer (Oliveira et al., 2017).

The present study aims to investigate the total antioxidant activity of lawsone from *L. inermis* via *in vitro* and *silico* studies. The total antioxidant activity of lawsone on skin cells will be determined by ferric reducing antioxidant power (FRAP) assay. This study will also illustrate the activation mechanism of lawsone on superoxide dismutase (SOD) enzyme by using molecular docking.



1.2 Objective of the Study

1.2.1 General Objective

The objective of this study is to evaluate the total antioxidant activity of lawsone *in vitro* and SOD activation property of lawsone *in silico*.

1.2.2 Specific Objectives

The specific objectives of this study are:

- i. To determine the half maximal inhibitory concentration (IC_{50}) of lawsone by using MTT assay.
- ii. To evaluate the total antioxidant activity of lawsone by using the biochemical FRAP assay.
- iii. To evaluate the total antioxidant activity of lawsone on A431 and 3T3 cell line by using FRAP assay.
- iv. To investigate the activation mechanism of lawsone on superoxide dismutase (SOD) enzyme via molecular docking method.

1.3 Hypothesis

Lawsone will possess high antioxidant level through *in vitro* study and lawsone will also show a good activation effect towards superoxide dismutase (SOD) enzyme via molecular docking.

CHAPTER 2

LITERATURE REVIEW

2.1 Reactive Oxygen Species

Reactive oxygen species (ROS) is a term which covers all the highly reactive, oxygen-containing molecules, including free radicals. Hydroxyl radical, the superoxide anion radical, hydrogen peroxide, singlet oxygen, nitric oxide radical, hypochlorite radical and various lipid peroxides are the examples of ROS. They are capable of reacting with nucleic acids, membrane lipids, protein, enzymes and other small molecules, which can cause cellular damage (Krishnamurthy & Wadhwani, 2012).

As ROS are generated by the number of pathways, they produced by the cells because of normal aerobic metabolism which about 90% of the oxygen in the cell is used by the mitochondrial electron transport system. According to Sabharwal and Schumacker (2014), mitochondria produce ROS when electrons residing on groups of flavin, iron-sulphur centres or other "way-stations" electron transport are redirected to O₂, which produces superoxide. Various "antioxidant enzymes" scavenge ROS and reverse the effects of ROS on proteins, lipids and DNA while reducing the extent of oxidative damage or redox signals. ROS is also developed due to oxidative burst from phagocytes. In most condition, the white blood cells are destroyed as the part of the process by bacteria and viruses that denature the foreign substances such as antigens. Moreover, ROS production can be occurred by xenobiotic metabolism such as detoxification of toxic product (Krishnamurthy & Wadhwani, 2012).

In many biochemical processes, low levels of ROS are essential; however, oxidative stress results in overproduction or inadequate removal of ROS. Oxidative damage results from ROS interaction with cellular macromolecules such as protein, lipid, and DNA. These result in various pathological conditions including cardiovascular dysfunction, neurodegenerative diseases and cancer. Cells have developed enzymic and non-enzymic systems that convert oxidants into non-toxic molecules, thereby protecting the organism from the harmful effects of oxidative stress (Sener et al., 2006). ROS can be produced nonenzymatically or enzymatically, by ultraviolet (UV) and in the cells respectively. Depending on the availability of oxygen and the expression of ROS-producing enzymes, reactive oxygen species production varies greatly in different tissues (Shekh-Ahmad et al., 2019). The continuous growth of ROS facilitates cell proliferation and carcinogenesis. Amusingly, once the toxic threshold has been surpassed, overproduction of ROS can contribute to oxidative stress ultimately led to cell death (Xue et al., 2020).

Despite of all harmful effects of ROS to our body, it also offers some beneficial effect. The beneficial effect of ROS arises at low and moderate concentrations and it involves in physiological functions in cellular reactions, such as protection against infectious agents and the action of a variety of cellular signalling mechanisms. The initiation of a mitogenic reaction is another useful example of ROS at low and moderate concentrations (Valko et al., 2007). Furthermore, ROS also showed its beneficial role as it is also involved in redox balancing. It has been widely accepted that moderate concentrations of ROS in vivo act as regulatory mediators in signalling processes and as initiators in restoring "redox homeostasis" (Li, 2013).

2.2 Free Radicals

Free radicals are very unstable and reactive in body compounds which are produced during normal metabolism or exposed to exogenous factors (Hatami et al., 2014). The free radicals are molecules with one or more electrons unpaired. Thus, these highly reactive molecules attack the nearest stable molecule to get an electron. The targeted molecule then develops into a free radical and initiates events that can eventually lead to cellular damage. However, free radicals also help to maintain homeostasis at physiological levels by acting as signal transducers (Kothari et al., 2010). Free radicals play a significant role in several biological processes, such as phagocytic cells killing bacteria intracellularly, which also involved in cellular redox signalling. Excessive amounts of free radicals, however, can lead to cell damage and death because of their reactivity (Ahn et al., 2011).

According to Sen et al. (2010), free radicals are produced by the homolytic cleavage of a chemical bond and redox reactions, which can initiate a chain reaction when the highly reactive radicals are generated. The chain reactions require numerous stages, each of which constitutes a free radical that induces the next step (Wu, Kosten, & Zhang, 2013). According to Nimse and Pal (2015), the generation of free radicals starts with the rapid accumulation of oxygen, the activation of NADPH oxidase and the production of radical superoxide anions. Then, SOD (superoxide dismutase) rapidly converted the superoxide radicals to hydrogen peroxide, H_2O_2 . Such ROS can operate by one of the two oxygen-dependent mechanisms that result in a microorganism or other foreign matter being destroyed. Another, the myeloperoxidase–halide– H_2O_2 system will also produce the reactive species. The

enzyme myeloperoxidase (MPO) occurs in cytoplasmic granules of neutrophils. H_2O_2 is then converted to hypochlorous (HOCl), a potent oxidant and antimicrobial agent, in the presence of the chloride ion, which is ubiquitous.

The metabolism of oxygen produces hydroxyl radical, superoxide, and non-radical H_2O_2 . The hydroxyl radical is highly reactive and reacts at or near diffusion-controlled levels, causing chemical modifications with biological molecules such as DNA, proteins, lipids, except for superoxide radicals and H_2O_2 , which they do not react with most of the biological molecules. The reaction between the $\text{O}_2^{\bullet-}$ and H_2O_2 producing $\bullet\text{OH}$, which also known as Haber-White reaction, is catalysed by transition metal ions including iron and copper ion. Cellular water interaction with ionising radiation generates $\bullet\text{OH}$, $\text{O}_2^{\bullet-}$, H_2O_2 , hydrated electron, and H atom ($\text{H}^\bullet$), which is also a free radical (Dizdaroglu & Jaruga, 2012).

Intracellular superoxide ($\text{O}_2^{\bullet-}$) is mainly formed by NADPH oxidation by NAPH oxidase enzymes (NOXs) or through an electron leak in mitochondria from aerobic respiration. Compartment-specific superoxide dismutases (SODs) are said to rapidly converting superoxide into hydrogen peroxide (H_2O_2). To trigger the redox biology, H_2O_2 will oxidise cysteine residues on proteins. Alternatively, cellular antioxidant proteins including peroxiredoxins (PRx), glutathione peroxidase (GPx), and catalase (CAT) may convert H_2O_2 to H_2O . In the case of uncontrollably increase in levels of H_2O_2 , hydroxyl radicals ($\text{OH}^\bullet$) are produced with metal cation (Fe^{2+}) reactions and cause irreversible damage to cellular macromolecules (Schieber & Chandel, 2014).

Examples of endogenous sources from normal metabolism are mitochondria, peroxisomes, endoplasmic reticulum and phagocytic cells meanwhile free radicals which are derived from exogenous sources such as pollutions, alcohol, tobacco smoke, heavy metals, transition metals, industrial solvents, pesticides, drugs and radiation (Phaniendra, Jestadi, & Periyasamy, 2015). Enzymes from the mitochondrial respiratory chain, NADPH oxidase, xanthine oxidase and dysfunctional endothelium NOS (eNOS) are the primary endogenous examples of free radicals. Additionally, free redox-active metals such as iron and copper could even produce free radicals through catalytic hydrogen peroxide decomposition, known as Fenton reaction (Poprac et al., 2017).

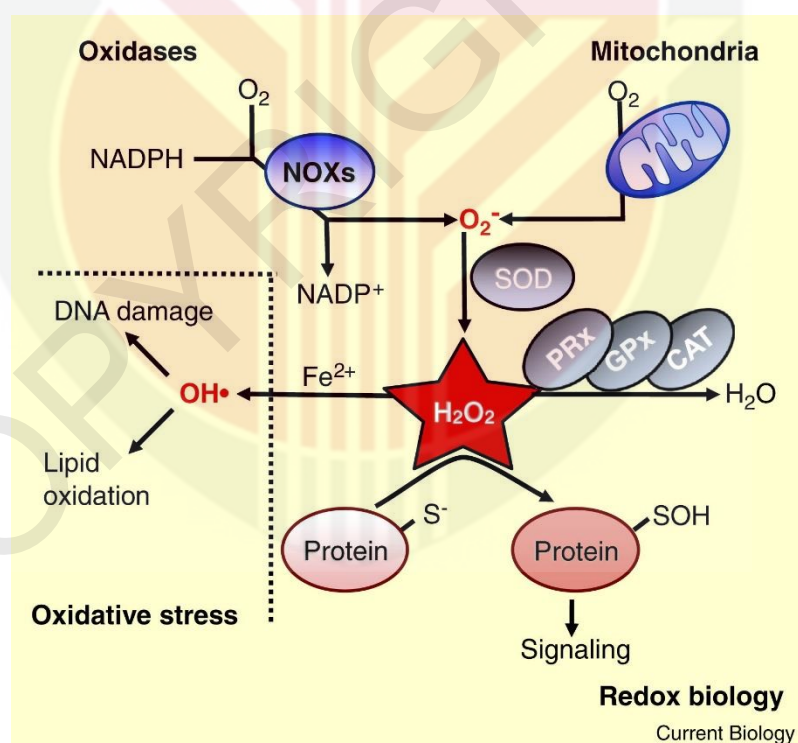


Figure 2.1 Formation of free radicals (Adapted from Schieber & Chandel, 2014)

2.3 Oxidative Stress

Oxidative stress is described as an imbalance between the development of free radicals and reactive metabolites, known as oxidants or reactive oxygen species (ROS), and their removal through protective mechanisms called antioxidants (Ďuračková, 2010). Oxidative stress arises when free radicals and active intermediates in a system are produced which surpass the capacity of the system to neutralise and remove them (Federico et al., 2007). Oxidative stress encompasses all the primary levels, from genome integrity and maintenance to redox metabolome, either as reversible oxidative eustress during redox regulation or as a cause of disruption to the biomolecules (Sies, Berndt & Jones, 2017).

According to Burton and Jauniaux (2011), the definition of pro-oxidant-antioxidant equilibrium is key to recognising oxidative stress. First, it points out that the disruption may be triggered by shifts on either side of the equilibrium, for example, an unusually large number of generations of ROS or deficiency in antioxidant defences. Next, it illustrates the homeostatic concentrations of ROS. Although ROS has first been recognised by the biologists as potentially dangerous aerobic metabolism by-products, they now play a crucial role in many intracellular pathways as secondary messengers. Finally, the theory of equilibrium refers to a degree of response to oxidative stress. Minor equilibrium disturbances are likely to lead in response to changes in the immediate environment to homeostatic adaptations, whereas more severe disturbances can cause permanent damage and cell death.

Oxidative stress can cause a detrimental effect on the skin. The skin has an enzymatic system to transform extremely hydrophobic and otherwise inert xenobiotics, such as polynuclear aromatic hydrocarbons, to reactive species through oxidative metabolism catalysed by a wide family of inducible heme-enzymes known as cytochrome P450s. Uncontrolled release of ROS is correlated with the pathogenesis of a variety of human skin diseases, including cutaneous neoplasia. Exposure of the skin to a variety of chemical and physical environmental factors causes oxidative stress contributing to activation of cutaneous lipid peroxidation with concomitant regulation of antioxidant and drug-metabolising enzymes (Bickers & Athar, 2006).

For instance, solar UV radiation is also one of the significant causes of oxidative stress in the skin as it is believed to play a key role in the cellular reaction due to UV exposure. UV-A and UV-B is the only solar UV radiation that will be exposed to the skin. Thus, UV-A photons are absorbed by endogenous UV-absorbing chromophores such as riboflavin, quinine, tryptophan, and porphyrin and eventually convert this energy to molecular oxygen, leading to the formation of $O_2^{\bullet-}$. In the case of redox-active transition metals, the superoxide anions are then transformed to H_2O_2 , which can be transformed into $HO\bullet$. UV-B radiation is mostly absorbed by the epidermis and is mainly responsible for sunburn, while UV-A penetrates extensively into the flesh of the skin. UV ultraviolet exposure has been found to induce oxidative stress in the skin leading to the degradation of endogenous antioxidant enzymes (Wagener et al., 2013).

2.4 Antioxidant

An antioxidant is a molecule which can prevent many particles from experiencing oxidation (Gülçin, 2012). Antioxidants are substances that delay, control or prevent oxidative processes which lead to food quality deterioration or initiation and propagation of degenerative diseases in the body when present in food or the body at deficient concentrations. As this concept implies, the physiological function of these compounds is to prevent damage to cellular components resulting from chemical reactions involving free radicals (Young & Woodside, 2001). Examples of antioxidants include free radical scavengers, singlet oxygen quenchers, peroxide inactivators and other reactive oxygen species (ROS), metal ion chelators, secondary oxidation products quenchers, and pro-oxidant enzyme inhibitors (Shahidi & Zhong, 2015).

The antioxidant also needed in all the organism to combat oxidative stress via intrinsic cellular defences (Kapoor et al., 2019). Cells contain sophisticated intracellular antioxidant defence systems for countering and preventing oxidative stress. These include an enzymatic antioxidant system as well as a non-enzymatic antioxidant system. Examples of enzymatic antioxidants are superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione reductase (GR) while non-enzymatic antioxidants include ascorbic acid, tocopherol and carotenes (Wang et al., 2016). Moreover, some antioxidant-active dietary nutraceutical products may contribute to the redox homeostasis of the human body cells. According to Shahidi and Zhong (2010), the best sources of natural antioxidants are higher plants and their constituents. The primary sources of plant-derived antioxidants are fruits,

vegetables, spices, herbs, cereals, grains, oilseeds, leguminous seeds, teas, coffee and cocoa.

Generally, there are two major classes of antioxidants based on the ROS inhibiting pathway. Firstly, so-called direct antioxidants that can react directly with ROS can break down the procession of the radical reactions in the chain such as quercetin and edaravone. Next, indirect antioxidants are compounds that do not react directly with ROS but that stimulate cellular endogenous antioxidant signalling pathways and facilitate the transcription of a wide range of cytoprotective genes to eliminate ROS, where KEAP1/NRF2/ARE is one of the significant antioxidants signalling pathways (Wang et al., 2019). The nuclear factor erythroid 2-related factor 2 (NRF2) signalling pathway acts to improve the intracellular antioxidant potential in response to excess oxidant stress. NRF2, triggered by KEAP1 dissociation, attaches to the antioxidant reaction and upregulates the transcription of numerous different gene forms (Zhang, 2006). Thus, KEAP1-NRF2 is an important factor for the treatment of cancer and neurodegenerative disorders, autoimmune diseases and inflammatory diseases. The inhibition of KEAP1 development and NRF2 activation is efficient in fostering antioxidant reactions in the combat against diseases (Yong & Ahn, 2018).

2.4.1 Role of Antioxidant Properties in Skin Inflammation

According to Sander et al. (2004), the skin is susceptible to ROS, which is considered as one of the challenges in treating skin inflammation induced by oxidative stress. Thus, antioxidant exists whether in enzymatic or non-enzymatic types to reduce oxidative stress. The plant-based natural product also can be one of the antioxidants

that can reduce oxidative stress in skin inflammation. According to Mueller, Hobiger and Jungbauer (2010), the acknowledged anti-inflammatory pathways include reduction of pro-inflammatory cytokines IL-6 and TNF- α , enhanced anti-inflammatory IL-10 secretion, and reduction of cyclooxygenase-2 (COX-2) and nitric oxide synthase production, which are based from 30 extracts from a wide range of plant families.

According to Benavente-García and Castillo (2008), a low molecular weight polyphenolic compounds known as flavonoids present in many plant-based foods are said to be the attributes to inhibit or modulate certain enzymes which lead to antioxidant properties such as free radical scavenging. Scavenging free radicals happens to play a significant role in the antioxidant function of flavonoids, which in turn is due to phenolic hydroxyl groups bonded to the flavonoid molecule (Yu et al., 2005). Example of flavonoid with antioxidant properties is naringenin or 4,5,7-trihydroxyflavanone, which acts as active metallic ion chelator and xanthine oxidase inhibitor that increase the antioxidant and antiradical properties (Cavia-Saiz et al., 2010).

On the other hand, nutrition is one of the factors needed to preserve optimal skin health. Deteriorated nutritional status changes the structural integrity and biological activity of the skin, resulting in an unstable skin barrier. In particular, the significance of micronutrients to the protection of the skin has been emphasised in cell culture, animal and clinical research. Such micronutrients are used not only as active substances in the treatment of some skin disorders, but also as components in beauty

products (Park, 2015). According to Masaki (2010), examples of micronutrients that play their role as an excellent antioxidant are ascorbic acid and tocopherols. Ascorbic acid destroys the majority of ROS due to oxidation of ascorbate to monodehydroascorbate and then to dehydroascorbate and has multiple roles in preserving normal physiological state in humans. It also enhances the blood flow through the generation of nitric oxide radicals via the increment of tetrahydrobiopterin stability, which is a cofactor of constitutive nitric oxide synthase. Meanwhile, α -tocopherol induces the production of GSH in HaCaT keratinocytes by the upregulation of γ -glutamylcysteine synthetase mRNA. The hydroxyl group in the chromanol ring of tocopherol that donates a hydrogen atom to reduce free radicals is said to be the antioxidative mechanism of tocopherols.

2.5 Skin Antioxidant Enzymes

The skin cells are built with an extensive network of redox modulation molecules. They defend against oxidative damage induced by excessive ROS/RNS production or remove potentially harmful molecules generated in reaction to environmental stress, such as exposure to sunlight. Redox regulation molecules contain enzymatic antioxidants such as superoxide dismutase (SOD), glutathione peroxidase (GPX), glutathione reductase (GSR), glutathione S-transferase (GST), thioredoxin reductase (TRX-R) and catalase (CAT). They are functioning in synergy to establish redox status (Svobodová & Vostálová, 2010). Intracellular enzyme processes generally are triggered by ROS molecules via transcription factors (FoxO) which stimulate antioxidant expression of the enzyme such as SOD. Increased FoxO levels reduce the proliferation of cells and cause apoptosis. Such factors lead to cell growth, proliferation, differentiation and longevity (Addor, 2017).

A study conducted by Nevin and Rajamohon (2010) described that increased antioxidant enzyme production in treated rats caused a decrease in lipid peroxides (MDA). The levels stayed lower even though the antioxidant enzymes returned to normal rates on the 14th day. It is also stated that mild oxidative stress could modulate cell growth and differentiation.

2.5.1 Superoxide Dismutase (SOD)

Superoxide dismutase (SOD) is the first detoxifying enzyme and most active antioxidant in the organism. It is an essential endogenous antioxidant enzyme which acts as an element of the first-line protection mechanism against the reactive oxygen

species (ROS). It catalyses the dismutation of two molecules of superoxide anion to hydrogen peroxide (H_2O_2) and molecular oxygen (O_2) and thus renders the potentially lethal superoxide anion less dangerous (Ighodaro & Akinloye, 2018). Among eukaryotic species, SOD is the only enzyme capable of detoxifying superoxide. SODs are found in all aerobic and some even anaerobic species. Anaerobics without SOD uses other mechanisms for detoxification of superoxides including superoxide reductase or increased levels of intracellular manganese, to enable them to withstand brief interactions with oxygen (Van Raamsdonk & Hekimi, 2012).

According to Ighodaro and Akinloye (2018), SOD is a metalloenzyme and thus needs a metal cofactor for its function. There are different types of the enzyme depending on the type of metal ion produced as a cofactor of SOD. Four isoenzymes of superoxide dismutase have been established which are SOD1, SOD2, SOD3 and SOD4. SOD1 is a zinc and copper-binding metalloprotein that can be found in the cytosol of the cell. Meanwhile, SOD2 is found in mitochondria and linked to the manganese or iron ions and SOD3 is a high molecular weight, therefore often correlated to copper and zinc with extracellular localisation. The sulphates of heparin and heparin have a strong affinity towards SOD3. SOD4, which has a high association with nickel, are found in various aerobic *Streptomyces* bacteria mainly in the soil (Aguilar, Navarro & Perez, 2016).

The intracellular degree of O_2 is governed by the functions of the two crucial scavenger enzymes, Cu/Zn-SOD and Mn-SOD in the eukaryotic cells. There are two stages for the proposed enzymatic mechanism for Cu/Zn-SOD. The first stage is the

release of dioxygen which is the product of the reduction of oxidised Cu(II) form of the enzyme by superoxide. In contrast, the second stage is the generation of H_2O_2 by the oxidation of reduced Cu(I) by another superoxide anion and two protons. For MnSOD, the cycling between oxidised (Mn^{3+}) and reduced (Mn^{2+}) ions are involved in MnSOD dismutation mechanism (Patlevič et al., 2016).



2.6 *Lawsonia inermis*

Lawsonia inermis is also known as henna, samphire and Cypress shrub in English while in Sanskrit, the common name given is Mendi, Mendika, Timir and Rakigarbha (Ponugoti, 2018). The name 'Inai' is commonly known in Sumatra and 'Panchar Kuku' in Java (Susi et al., 2002). "*Lawsonia*" is the Latin plant name, which is coined after Isaac Lawson, a Scottish army doctor in the 18th century by Carl Linnaeus, the father of the modern taxonomy (Singh, Luqman & Mathur, 2015). Under genus *Lawsonia*, *L. inermis* has different synonyms to other species which are *L. alba* and *L. spinosa* as they belong to the Lythraceae family (Chaudhary, Goyal & Poonia, 2010).

Australia, Asia and along the African Mediterranean coasts; these locations mentioned are the places where the henna is originated (Pradhan et al., 2012). Henna is cultivated at a tropical native place as well as subtropical areas such as India, Sri Lanka and the Middle East mainly for cosmetic and medicinal purposes (Jallad & Espada-Jallad, 2008). It is grown extensively in Afghanistan, Pakistan, Iran, Libya, Morocco, Somalia, Sudan and Yemen. The Pali district of Rajasthan state is currently the highest cultivated henna area in India (Kapadia et al., 2013). A dry and hot condition with iron-rich soil produces henna with high dyeing power produces high active constituent content. In contrast, fertile soil and moist conditions give the opposite outcome (Gallo et al., 2014).

L. inermis plant is about 2 to 6 metres of its height with the features of much branched glabrous shrub or small tree, which can be spiny. The branches turn red with age, but young branches are in quadrangular shape and green in colour. The

characteristics of *L. inermis* leaves are opposite, round, sub-sessile, elliptic to broadly lanceolate, 1.5-5x0.5-2 cm, glabrous, acuminate, while the upper surface veins are depressed. The flowers in the large pyramidal terminal are small, white, numerous, fragrant, 1cm across with four petals crumpled in the bud. The petals are orbicular to ovate, white or red. The pairs on the rim of the calyx tube are inserted with eight stamens. The calyx has a 2mm tube and three spread lobes. The ovary is four celled and the style up to 5mm long, erect. Henna fruits are small, brown, four to eight-millimetre diameter globosely capsules, many-seeded, irregularly opening, divided into four sections with persistent style. Their seeds are 3mm wide, angular, and it has a thick coat of seed (Zumrutdal & Ozaslan, 2012).

The usage of henna is already nearly five millenniums. It is stated that the ancient Egyptians use henna to dye the hair and nails. Significantly, Queen of Egypt uses Henna or Egyptian Privet to beautify their skin, besides dying their hair. Other, applying henna to the beard is also one of Prophet Mohammed's practices (Dweck, 2002). Arabic women design gorgeous temporary tattoos on their hands, arms and feet by using henna (Budair, 2019). Henna tattoo is used in a lot of rituals, social or aesthetic purposes at some ceremonies such as weddings in Jewish, Christian, Muslim and Buddhist cultures (Lamchahab et al., 2011). Also, henna is being used in ancient times by Arab doctors to treat dangerous maladies such as leprosy, smallpox, chickenpox and tumours (Mehkam, Nabati, & Kafshboran, 2014). In India, henna is popular in the hot Indian summers because of its cooling effect (Chaudhary et al., 2010). Moreover, in some events, special henna dance is introduced by the Malays during weddings and they also use the leaves of henna for medicinal intentions, such as to alleviate sore feet (Dweck, 2002).

L. inermis is used by all the dwellers around the world since primaeval times because of its effective ethnopharmacological effects. For example, henna is used for mummy embalmment in the form of oilmen, and the hair is coloured for rejuvenation purposes, which is proved by the blonde hair of Ramses III during his youth according to the study of Royal Mummies (Gallo et al., 2014). Furthermore, the Indians use henna to treat various maladies such as rheumatoid arthritis, headache, ulcers, diarrhoea, leprosy, fever, leucorrhoea, diabetes, cardiac disease, hepatoprotective and colouring agent (Chaudhary et al., 2010). They also use the whole henna plant to heal the wounds for external use (Wadankar, Malode, & Sarambekar, 2011). In Pakistan, the henna leaves, in the form of powder is used to treat hair and skin complications (Sultana & Zafar, 2013).

2.7 Lawsone

Lawsone, or 2-hydroxyl-1,4-naphthoquinone is an active agent which can be acquired from natural henna which produces greyish green colour (Chung et al., 2002). It is also the major component of henna, and it is renowned for its staining ability and many biological activities (Pradhan et al., 2012). Lawsone, a naphthoquinone compound produces red-orange dye molecules from the leaves of *L. inermis*. The dye molecules combines with the natural colour of hair and turn into red-orange (Jain et al., 2010).

Lawsone interacts chemically to protein keratin in the skin and hair via Michael addition, resulting in a strong permanent stain that persists until the skin or hair is sloughed off (Hijji, Barare, & Zhang, 2012). Whole, unbroken leaves of henna will not stain the skin. Henna will not dye the skin until the lawsone molecules are extracted from the henna plant. Fresh henna leaves dye the skin if they are mixed with a mildly acidic liquid. Gradually, lawsone will migrate from the henna paste to the outer layer of the skin and attach to the proteins in it, producing a fast stain (Ashnagar & Shiri, 2011).

Lawsone derives from its precursor called the hennosides during henna preparation and it is not present as free molecules in the leaves. The tautomeric form of the keto-enol interconversion of the naphthoquinone structure forms the three isomers of hennosides. This gives rise to three possible hydroxyl group and changes into diketonic form consequently when the second ring is thrice oxygenated. The three isomers are rise from the glucosidation of each hydroxyl groups. Lawsone, the dying active compound is produced due to the oxidation of aglycone, which is derived from

the hydrolysis of the three isomers of hennosides (Gallo et al., 2014). Lawsone also found in three tautomeric models; the 1,4-naphthoquinone configuration is the most stable form preceded by 1,2-naphthoquinone and 1,2,4-naphthotriene. The triene type is the least stable yet likely to be in equilibrium with the other two tautomeric forms. Such stabilisation was attributed to the cancellation of dipolar moments in the carbonyl groups in conjunction with intramolecular hydrogen bonds in the 1,4 isomer (López López et al., 2014).

2.7.1 Antioxidant Activities of Lawsone

Lawsone has been proven as one of the natural products which are rich with antioxidant properties. Lawsone and other 1,4-naphthoquinones can serve as natural and reversible redox system in the plant and act as autoxidising hydrogen acceptors in cell respiration and photosynthesis. Their electron affinity can uncouple oxidative phosphorylation and cause both normal and tumour cell deaths. This has been proven as there have been reports of a modulative impact in mice of 80% ethanolic extracts from fresh henna leaves in the metabolism of phase I and phase II enzymes, antioxidant enzymes, lipid peroxidation, and chemically-induced skin and forestomach papillomagenesis (Kapadia et al., 2013). Lawsone also could have strong cytotoxic properties due to its high antioxidant activity (Kavitha et al., 2013).

Furthermore, the potential of lawsone to undergo redox cycling and trace metal ion chelation are claimed to be partly responsible for some of its biological activities (Pradhan et al., 2012). The antioxidative activity of lawsone was found to be the highest when compared to vitamin E or tocopherol (Rani et al., 2013). It is also

established that lawsone produces greater antioxidant activity than the well-known standard ascorbic antioxidant acid or vitamin C. This would describe the mechanism of immunomodulatory action of henna, based on its capacity for antioxidants. Lawsone thus acts as free radical scavengers which prevent the peroxidation of the phospholipid membrane and protect immune-compromised cells from free radical damage (Mikhaeil et al., 2004).

Lawsone is responsible for the upregulation of antioxidant enzyme NQO1. The NQO1 gene is also regulated by Nrf2, which is known to protect against ROS (Lozza et al., 2019). Also, it was discovered that the naphthoquinone products, both lawsone and its derivatives, are the COX-2 antagonists that be able to reduce pain (Motz et al., 2012). Also, by reducing neutrophil infiltration and oxidative stress markers, treatment with lawsone significantly improved the severity of L-arginine induced pancreatic, which proves lawsone can act as a potential antioxidant (Biradar & Veeresh, 2013).

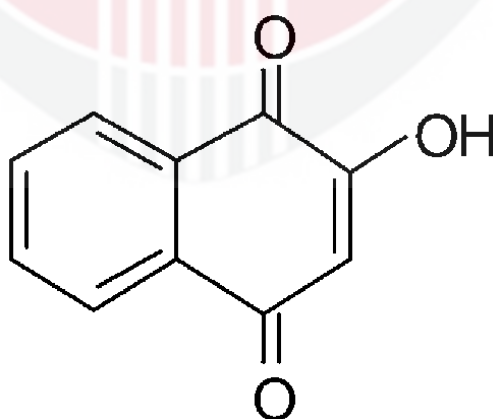


Figure 2.2 Chemical structure of lawsone (Adapted from ChemSpider)

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

Dulbecco's Modified Eagle Media (DMEM) culture media, penicillin-streptomycin, trypsin, ethylene diamine tetraacetic acid (EDTA), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were obtained from Nacalai Tesque Inc. (Kyoto, Japan). Phosphate buffer solution (PBS) was purchased from First Base (The Gemini, Singapore). Fetal bovine serum (FBS), trypan blue solution 0.4%, 2,4,6-tripyridyl-s-triazine (TPTZ), ferrous sulphate heptahydrate ($\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$) and sodium acetate anhydrous were acquired from Sigma-Aldrich (St. Louis, Missouri, USA). Dimethyl sulfoxide (DMSO) and glacial acetic acid were purchased from AMRESCO (Solon, Ohio, USA). Hydrochloric acid (HCl) and ferric chloride hexahydrate ($\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$) were purchased from Fisher Scientific (Loughborough, Leicestershire, UK). L-ascorbic acid was obtained from Santa Cruz Biotechnology (Dallas, Texas, USA) and sodium hydroxide (System, Classic Chemicals, Shah Alam, Selangor).

3.2 Compound

Powdered form of lawsone (2-hydroxyl-1,4-naphtoquinone) was purchased from Sigma-Aldrich (St. Louis, MO, USA).

3.3 Cell Lines

Human epidermoid carcinoma (A431) was a generous gift from Prof. Dr. Tan Wen Siang from Faculty of Biotechnology, Universiti Putra Malaysia and normal mouse fibroblast (3T3) cell lines were purchased from American Type Culture Collection (ATCC). All the cell lines were cultured in the culture flasks containing DMEM culture media, FBS and penicillin-streptomycin with the percentage of 89%, 10% and 1% respectively; by means of 44.5 mL of DMEM culture media, 5 mL of FBS and 500 μ L of penicillin-streptomycin.

3.4 Cell Culture

3.4.1 Thawing Frozen Cells

Firstly, the cryovials containing A431 and 3T3 cell lines were retrieved from the ultra-low temperature freezer (VIP Series, Sanyo Electrics Co., Ltd., Osaka, Japan). The cryovials were then swirled gently in the water bath for about two minutes until the content was thawed. The cryovials were then disinfected thoroughly with 70% alcohol before transferred to biosafety cabinet. The thawed content of the cryovials was immediately transferred into the T25 labelled culture flasks. The cells were incubated in 5% CO₂ incubator at 37°C for 24 hours before changing into a new media.

3.4.2 Changing Media

Changing of media was performed to maintain the cells to grow well by providing nutrients from the new media. The old media was removed from the culture flasks containing A431 and 3T3 cell lines. The cells were then washed thrice with 1mL of PBS. The flasks were swirled gently before PBS was removed after each wash. Then, 5mL of the complete media was added into culture flasks. The confluency of the cells was observed under the phase-contrast inverted microscope (Leica DM IL LED, Leica Microsystems, Wetzlar, Germany). The cells were incubated at in 5% CO₂ incubator (Galaxy 170R, Eppendorf AG, Hamburg, Germany) at 37°C.

3.4.3 Sub-culturing

Sub-culturing of cells was performed when the confluency of the cells reached 80%. The confluency of the cells was observed under the inverted microscope. The old media was removed from the culture flasks containing A431 and 3T3 cell lines. The cells were then washed thrice with 1mL of PBS. The flasks were swirled gently before PBS was removed after each wash. Then, 1mL of trypsin was added into the flask and incubated for 5 minutes. The cells were then observed under the fluorescent microscope to ensure the cells were detached from one another. The side of the culture flasks were tapped gently to increase the detachment of the cells. The media was added into the flasks with the ratio of media and trypsin of 2:1, by means adding 2mL of media. The content in the flasks were pipetted into the tubes. The tubes were then centrifuged (Rotofix 32, Jora-Aki Technology Sdn. Bhd, Selangor, Malaysia) at 12000rpm for 5 minutes. The supernatant was removed, and 1mL of media was added and resuspended with the pellet in the tubes. The volume of the cells needed for transferring into the new flasks was determined according to the number of the flasks to sub-culture. The confluency of the cell was observed under the phase-contrast inverted microscope before incubating the cells in the 5% CO₂ incubator at 37°C.

3.4.4 Preserving and Freezing Cells

In order to preserve stock, the cells were freeze, or retain in a long-term storage, with a minimum increase in generation number from initial freezing cells. Firstly, the old media was removed from the culture flasks containing A431 and 3T3 cell lines. The cells were then washed thrice with 1mL of PBS. The flasks were swirled gently before PBS was removed after each wash. Then, 1mL of trypsin is added into the flask and

incubated for 5 to 8 minutes. The cells were then observed under the fluorescent microscope to ensure the cells were detached from one another. The side of the culture flasks were tapped gently to increase the detachment the cells. The media was added into the flasks with the ratio of media and trypsin of 2:1. The content in the flasks were pipetted into the centrifuge tubes. The tubes were then centrifuged at 12000rpm for 5 minutes. The supernatant was removed and 100 μ L of media was added and resuspended with the pellet in the tubes. The content in the tubes were pipetted into the sterile labelled cryovials. Then, 850 μ L of FBS was added and resuspended before adding 50 μ L of DMSO into the cryovial. The cryovials were immediately sealed with parafilm and stored in the ultra-low temperature freezer.

3.5 Experimental Procedure

3.5.1 Cytotoxicity Assay

Cytotoxicity assay is done by using the method from Mosmann (1983) with slight modifications. Firstly, the cells from the flask were observed under microscope to check the confluency. The viability of the cells was determined by staining with trypan blue. The cells were harvested from the flask and counted by using haematocytometer. Next, 100µL of A431 and 3T3 cell lines were seeded into separate 96-well plates with the seeding density of 1×10^5 cells/mL. The plates were then incubated at 37°C in 5% CO₂ incubator for 24 hours. The working solution of lawsone and DMEM media were prepared in sterile petri dishes. After 24 hours, the cells in both plates were treated with 100µL of lawsone with 10 different concentrations (5000µM, 2500µM, 1250µM, 625µM, 312.5µM, 156.75µM, 78.13µM, 39.07µM, 19.53µM, 9.77µM). The well with complete media growth and without lawsone treatment was acted as the negative control. The plates were then wrapped with aluminium foil and incubated at 37°C in 5% CO₂ incubator for 48 hours. The MTT solution (5 mg/mL) was prepared by dissolving into PBS solution. After 48-hour incubation, 20µL of MTT solution was added into each well of 96-well plate. The plates were wrapped with aluminium foil and incubated at 37°C in 5% CO₂ incubator. After 4 hours of incubation, the medium of the wells was aspirated and 100µL of DMSO was replaced into each well. The plate was then read using microplate reader with wavelength of 570 nm and reference wavelength of 630nm after shake for 10 seconds. A graph of cell viability against lawsone concentration was plotted. IC₅₀ was determined from the concentration versus cytotoxicity graph. The cytotoxicity was calculated based on the formula:

$$\text{Cytotoxicity (\%)} = \frac{\text{optical density of sample (mean)}}{\text{optical density of control (mean)}} \times 100\%$$

3.5.2 Ferric Reducing Antioxidant Power (FRAP) Assay

Method from Benzie and Strain (1996) was used to perform biochemical FRAP assay, while a few modification was performed as reported by Hasiah et al. (2011) in order to perform FRAP assay in cell lines. Firstly, the FRAP reagent was prepared by mixing 300mM acetate buffer of pH 3.6, 10mM TPTZ, 20mM ferric chloride hexahydrate ($\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$) and distilled water with the ratio of 10:1:1:1.2 respectively. Also, the series of ferrous sulphate heptahydrate ($\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$) concentration from 100 μM to 1000 μM was prepared for obtaining the standard curve. Next, the FRAP assay was carried out in 2 conditions; as biochemical assay and in cell lines.

3.5.2.1 FRAP Assay in Cell Lines (Hasiah et al., 2011)

Both A431 and 3T3 cell lines were plated as in MTT assay in 96-well plate with the seeding density of 1×10^4 cells/mL. The plates then were incubated at 37°C in 5% CO_2 incubator for 24 hours. Then, the cells in both plates were treated with 50 μL of lawsone with eight different concentrations (200 μM , 100 μM , 50 μM , 25 μM , 12.5 μM , 6.25 μM , 3.13 μM , 1.56 μM). Positive control used in this assay was vitamin C (L-ascorbic acid). The plates were then wrapped with aluminium foil and incubated at 37°C in 5% CO_2 incubator for 24 hours. Then, the plates were sonicated by using the sonicator (Powersonic 410, Hwashin Technology Co., Seoul, South Korea) for 30 seconds. The plates were added with 150 μL freshly prepared FRAP reagent which was warmed at 37°C. The plates were then read on microplate reader with wavelength of 593nm. The absorbance reading was converted into FRAP value (μM).

3.5.2.2 Biochemical FRAP Assay (Benzie & Strain, 1996)

The biochemical FRAP assay on lawsone without the presence of the cell lines was also performed by adding FRAP reagent immediately to the lawsone solution in 96-well plate. The absorbance of various concentration of lawsone without the presence of the cell lines was also recorded. Three bar graphs of total antioxidant activities against lawsone concentration with and without the presence of A431 and 3T3 cell lines are plotted.



3.6 Statistical Analysis

In vitro results were analysed by using analysis of variance (ANOVA), followed by Dunnett's test as post-hoc test, which is significant at $p < 0.05$. All of this were analysed by using software package of IBM SPSS Statistical Package Version 26.



3.7 Molecular Docking

The molecular docking technique can be used to design the relationship at the atomic level between a small molecule and a protein that helps us to describe the actions of small molecules in the binding site of the target proteins but also to elucidate basic biochemical processes. The docking method includes two fundamental steps: the estimation of ligand conformation as well as its location and orientation at such sites (usually referred to as pose) and the determination of binding affinity (Meng et al., 2012). Thus, *in silico* study of molecular docking is performed to understand the chemical interaction between ligand (lawsone) and the protein (superoxide dismutase) in this study. The SOD protein was retrieved from the RSCB Protein Data Bank in the.pdb format file with PDB ID of 2C9V, atomic resolution of Cu-Zn Human Superoxide dismutase with a resolution of 1.07 Å.

3.7.1 Determination of Allosteric Site of SOD

According to Song et al. (2017), AlloSite Version 2.10 webserver with improved AllositePro (2016) method is a tool that predicts allosteric protein sites by integrating pocket features with perturbation analysis. The first step was to set the input format whether in PDB ID (2C9V) or upload the PDB file from the computer before submitting to the job by clicking “Run” button. All chains of 2C9V (chain “A” and “F”) were selected to determine the possible allosteric sites. The prediction of allosteric sites of 2C9V can be viewed from the result obtained from the webserver at the PDB Display Option. The report of results was downloaded and the PDB file of allosteric prediction site of 2C9V also can be observed by using PyMol software.



Figure 3.1 AlloSite 2.0 Webserver

3.7.2 Preparing Receptor and Ligand Input

AutoDock Tools (ADT) is used to create an independent structural framework which consists only SOD. PDB ID of 2C9V of SOD receptor was acquired from RSCB Protein Data Bank in '.pdb' file. Meanwhile, as for the ligand, the structure of lawsone was retrieved in '.sdf' format from PubChem which is then converted to '.pdb' format file by using Open Babel software. Both SOD and lawsone were required to be prepared into PDBQT format for the AutoDock Vina to evaluate and perform the molecular docking (Trott & Olson, 2010). SOD was prepared by deleting the crystal water as it can cause complication during the process of finding poses and binding affinity calculation in drug design (Wong & Lightstone, 2011). Next, the hydrogen atoms were added and merged with the polar and non-polar hydrogens. The charges

were computed using the Gasteiger method. The torsion was then assigned to the ligand.

3.7.3 Setting Up Docking Grid Box and Configuration Files

The docking grid box is essential in molecular docking to search for the ligand conformation as the grid box should cover the targeted area, by means, the two areas of allosteric sites which has been determined by using AlloSite webserver. The grid box size and coordinates were identified to operate as molecular docking parameters. Thus, the adjusted parameters used for SOD were as follows:

Protein	Area	Grid Size		Grid Coordinate	
SOD	A	x	14	x	12.407
		y	30	y	-4.285
		z	18	z	11.315
	B	x	8	x	32.034
		y	12	y	-0.611
		z	8	z	17.785

Table 3.1 The adjusted parameter of docking grid box

The parameters above were written in a text editor such as Notepad or Crimson Editor and saved as a configuration file in '.txt' format.

3.7.4 Molecular Docking via AutoDock Vina

The molecular docking between lawsone and SOD is carried out by utilizing AutoDock Vina software (Trott & Olson, 2010). The prepared PDB files of SOD and lawsone, configuration file in '.txt' format and AutoDock Vina software were included in one folder before proceeding to the command prompt for running the docking job. The output file would appear in the folder after the docking job completed.

3.7.5 Analysis on Docked Complexes

The docked ligand conformation produced nine best results of binding affinity with different pose predictions between the lawsone and SOD. Thus, the best docked ligand-protein complex (lawsone-SOD complex) with top binding affinity was then analysed by using PyMOL software (PyMOL Molecular Graphics System, Version 2.3 Schrödinger, LLC.), ProteinsPlus (Zentrum für Bioinformatik: Universität Hamburg - Proteins Plus Server) and PLIP webserver (Protein-Ligand Interaction Profiler, Biotechnology Center TU Dresden). The two-dimensional (2D) information of the interaction between the docked lawsone-SOD complex was obtained from ProteinsPlus.

CHAPTER 4

RESULTS

4.1 Effect of Lawsone on Cell Viability

4.1.1 IC₅₀ of Lawsone on Epidermoid Cell A431

MTT assay was used to determine toxicity profile of lawsone in the cells. Thus, IC₅₀ was obtained to determine the cytotoxicity of lawsone on the epidermoid A431 cell line. The cells were treated with a various dosage range of concentration from 5000 μ M, 2500 μ M, 1250 μ M, 625 μ M, 312.5 μ M, 156.75 μ M, 78.13 μ M, 39.07 μ M, 19.53 μ M, 9.77 μ M and 0 μ M. The incubation time of the treatment was 24 hours. Figure 4.1 showed the cytotoxicity result of lawsone on the percentage of cell viability of epidermoid A431 at a various group of treatments. By using the graph as a reference, the lawsone IC₅₀ was determined at 3650 μ M. The value of IC₅₀ was then used as the reference dose for the preceding FRAP assay.

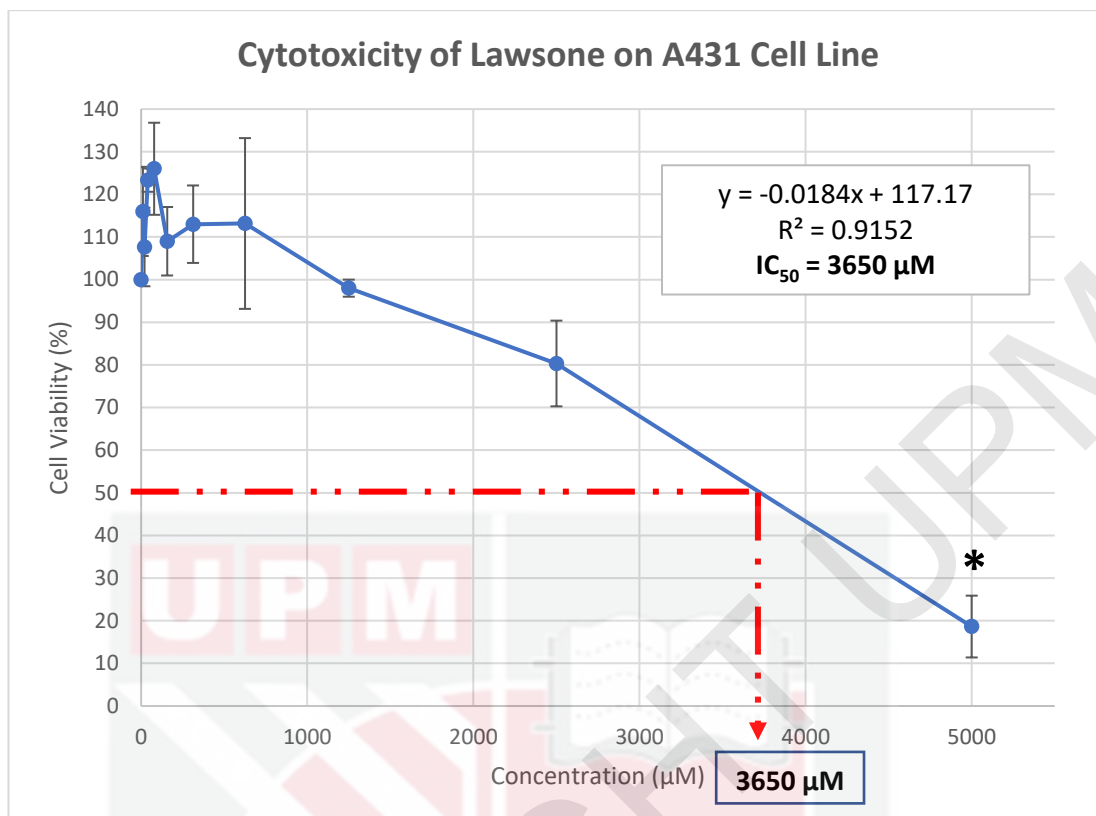


Figure 4.1 Graph of cell viability at various concentrations of lawsone treatment towards the A431 cell line. The graph above showed the cell viability of epidermoid A431 cell line after being treated with lawsone of various concentrations (5000µM, 2500µM, 1250µM, 625µM, 312.5µM, 156.75µM, 78.13µM, 39.07µM, 19.53µM, 9.77µM and 0µM) in 24 hours. IC_{50} of lawsone was determined at 3650µM. The values were expressed as mean $\pm$ SEM from three independent experiments, where * was indicated the significant difference ($p < 0.05$) relative to the untreated cells. One-way ANOVA was used to perform the statistical analysis followed by Dunnett's Test as the post-hoc test.

4.1.2 IC₅₀ of Lawsone on Fibroblast 3T3

IC₅₀ was obtained to determine the cytotoxicity of lawsone on fibroblast 3T3 cell line. The cells were treated with a various dosage range of concentration from 5000µM, 2500µM, 1250µM, 625µM, 312.5µM, 156.75µM, 78.13µM, 39.07µM, 19.53µM, 9.77µM and 0µM. The incubation time of the treatment was 24 hours. Figure 4.2 showed the cytotoxicity result of lawsone on the percentage of cell viability of fibroblast 3T3 at a various group of treatments. By using the graph as a reference, no IC₅₀ was detected for lawsone below the concentration of 5000µM.

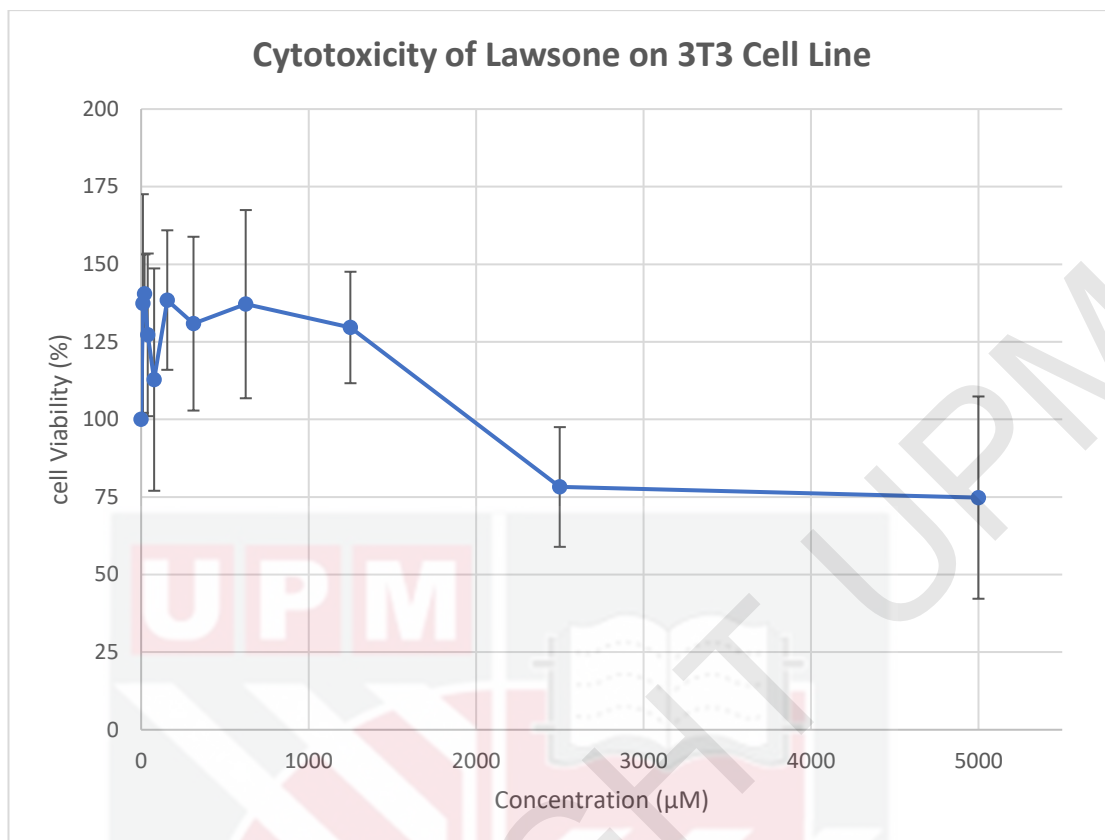


Figure 4.2 Graph of cell viability at various concentrations of lawsone treatment towards 3T3 cell line. The graph above showed the cell viability of fibroblast 3T3 cell line after being treated with lawsone of various concentrations (5000μM, 2500μM, 1250μM, 625μM, 312.5μM, 156.75μM, 78.13μM, 39.07μM, 19.53μM, 9.77μM and 0μM) in 24 hours. No IC₅₀ was detected for lawsone below the concentration of 5000μM in fibroblast 3T3 cell line.

4.2 FRAP Assay

4.2.1 Total Antioxidant Activity of Lawsone

Concentration (μM)	FRAP Value $\pm$ SEM (μM)	
	Vitamin C	Lawsone
0.00	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00
1.56	3.21 $\pm$ 0.30	0.46 $\pm$ 0.12
3.13	5.76 $\pm$ 0.43	0.52 $\pm$ 0.12
6.25	11.78 $\pm$ 0.26	0.71 $\pm$ 0.12
12.50	23.57 $\pm$ 0.41	0.77 $\pm$ 0.09
25.00	47.13 $\pm$ 1.57	1.27 $\pm$ 0.20
50.00	95.57 $\pm$ 2.87	1.82 $\pm$ 0.11
100.00	197.20 $\pm$ 5.47	2.36 $\pm$ 0.08
200.00	396.05 $\pm$ 5.00	4.04 $\pm$ 0.18

Table 4.1 FRAP values of vitamin C and lawsone at different concentrations. The total antioxidant activities of vitamin C (positive control) and lawsone in the absence of cells were reflected by FRAP values at various concentrations (200 μM , 100 μM , 50 μM , 25 μM , 12.5 μM , 6.25 μM , 3.13 μM , 1.56 μM and 0 μM) by FRAP assay. Data were presented as mean $\pm$ SEM from three independent experiments. The result from Figure 4.3 and Figure 4.4 depicted that vitamin C showed significant antioxidant properties at a concentration of 200, 100, 50, 25, 12.5 and 6.25 μM meanwhile lawsone showed significant antioxidant properties at concentration of 200, 100, 50 and 25 μM when compared to the negative control ($p < 0.05$). The total antioxidant of vitamin C and lawsone increased in a concentration-dependent manner.

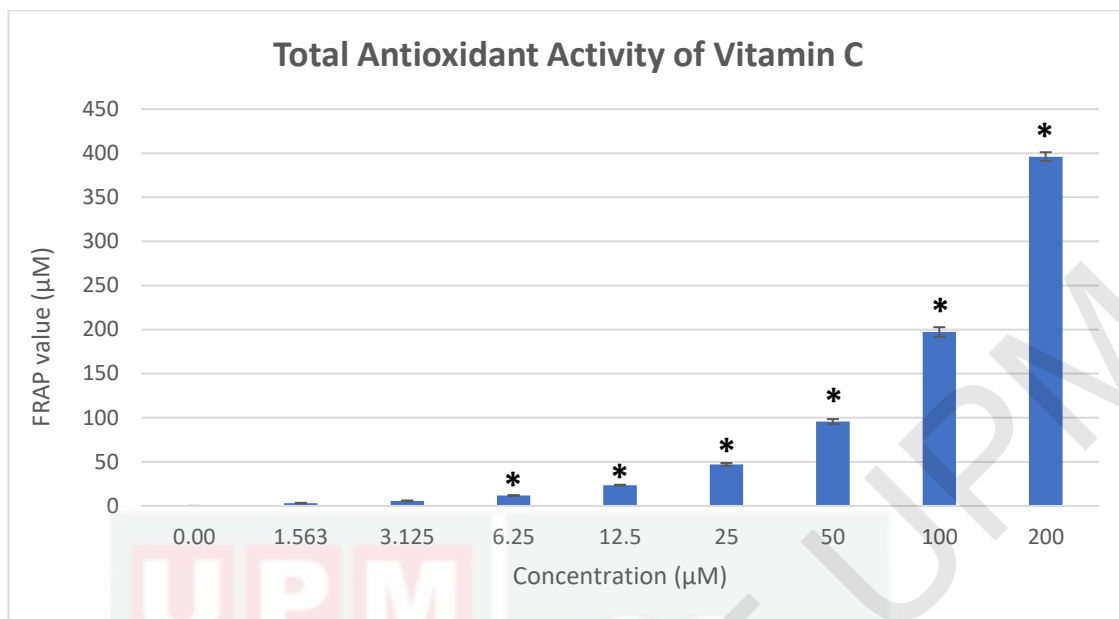


Figure 4.3 Graph of the total antioxidant activity of vitamin C in the absence of cells. The graph above showed the total antioxidant activity of lawsone with various concentrations (200μM, 100μM, 50μM, 25μM, 12.5μM, 6.25μM, 3.13μM, 1.56μM and 0μM). Data were represented as mean $\pm$ SEM from three independent experiments, where * was indicated the significant difference ($p < 0.05$) relative to the control group. One-way ANOVA was used to perform the statistical analysis followed by Dunnett's Test as the post-hoc test.

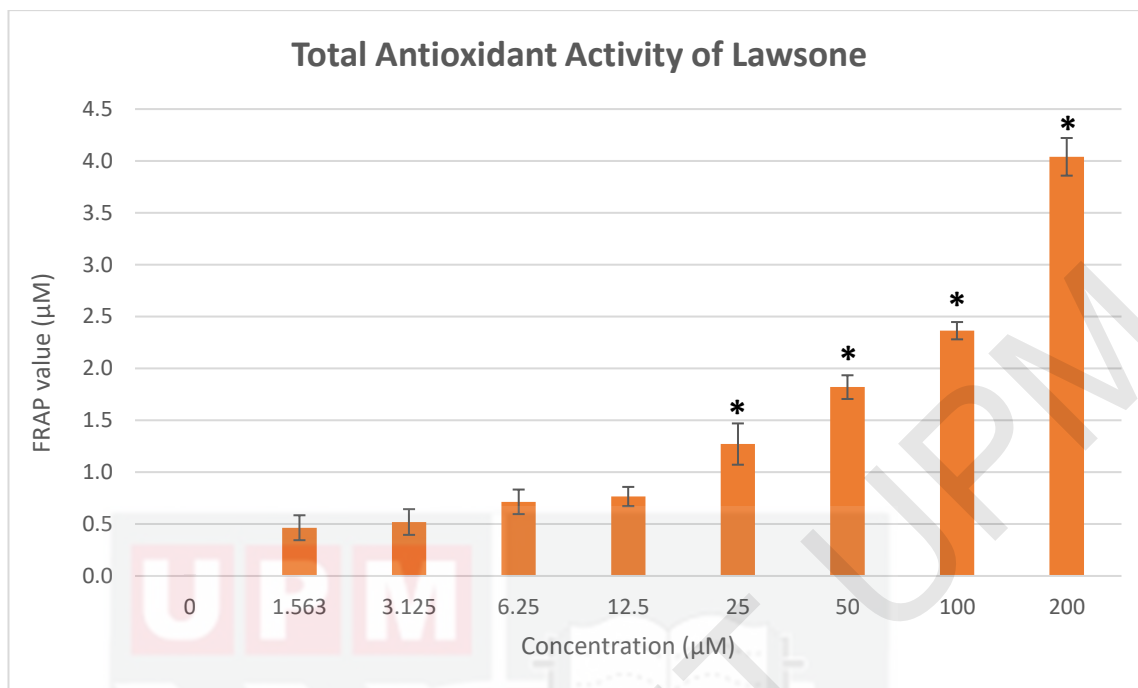


Figure 4.4 Graph of the total antioxidant activity of lawsone in the absence of cells. The graph above showed the total antioxidant activity of lawsone with various concentrations (200μM, 100μM, 50μM, 25μM, 12.5μM, 6.25μM, 3.13μM, 1.56μM and 0μM). Data were represented as mean $\pm$ SEM from three independent experiments, where * was indicated the significant difference ($p < 0.05$) relative to the control group. One-way ANOVA was used to perform the statistical analysis followed by Dunnett's Test as the post-hoc test.

4.2.2 Total Antioxidant Activity of Lawsone on Epidermoid Cell A431

Concentration (μM)	FRAP Value $\pm$ SEM (μM)	
	Vitamin C	Lawsone
0.00	80.15 $\pm$ 5.53	80.84 $\pm$ 5.01
1.56	86.00 $\pm$ 4.05	83.99 $\pm$ 8.67
3.13	89.00 $\pm$ 3.86	79.20 $\pm$ 4.93
6.25	92.48 $\pm$ 3.18	82.32 $\pm$ 1.07
12.50	95.01 $\pm$ 1.69	85.47 $\pm$ 1.09
25.00	96.29 $\pm$ 2.18	86.29 $\pm$ 2.21
50.00	100.91 $\pm$ 3.66	90.63 $\pm$ 0.88
100.00	110.56 $\pm$ 1.42	89.44 $\pm$ 1.23
200.00	123.74 $\pm$ 3.64	94.41 $\pm$ 1.21

Table 4.2 FRAP values of vitamin C and lawsone on epidermoid A431 cell line at different concentrations. The total antioxidant activities of vitamin C (positive control) and lawsone on epidermoid A431 cell line were reflected by FRAP values at various concentrations (200 μM , 100 μM , 50 μM , 25 μM , 12.5 μM , 6.25 μM , 3.13 μM , 1.56 μM and 0 μM) by FRAP assay. Data were presented as mean $\pm$ SEM from three independent experiments. The result from Figure 4.5 illustrated that lawsone showed no significant antioxidant properties on A431 cell line when compared to the negative control. In contrast, vitamin C showed significant antioxidant properties on A431 cell line at concentration of 200, 100, 50, 25, 12.5 μM when compared to the negative control ($p < 0.05$).

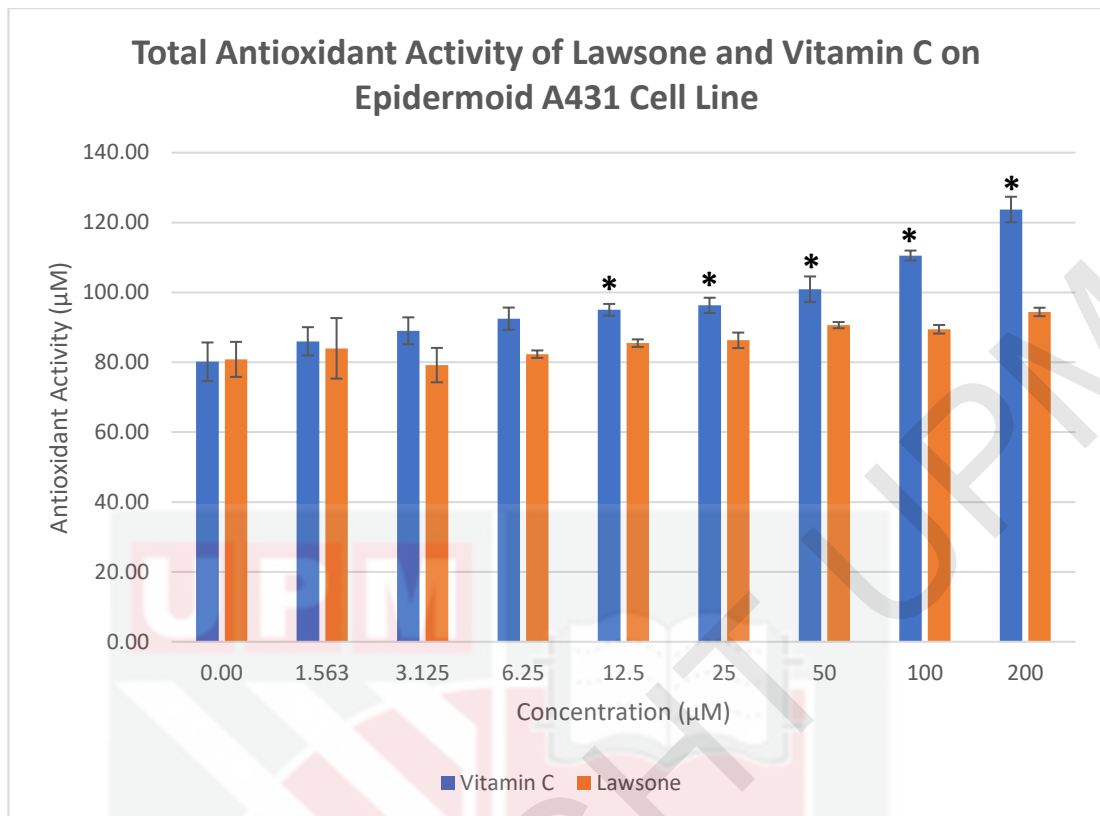


Figure 4.5 Graph of the total antioxidant activity of vitamin C and lawsone at various concentrations on the epidermoid A431 cell line. The graph above showed the total antioxidant activity of epidermoid A431 cell line after being treated with lawsone of various concentrations (5000μM, 2500μM, 1250μM, 625μM, 312.5μM, 156.75μM, 78.13μM, 39.07μM, 19.53μM, 9.77μM and 0μM). Data were represented as mean $\pm$ SEM from three independent experiments, where * was indicated the significant difference ($p < 0.05$) relative to the control group. One-way ANOVA was used to perform the statistical analysis followed by Dunnett's test as the post-hoc test.

4.2.3 Total Antioxidant Activity of Lawsone on Fibroblast 3T3

Concentration (μM)	FRAP Value $\pm$ SEM (μM)	
	Vitamin C	Lawsone
0.00	129.96 $\pm$ 8.76	80.22 $\pm$ 5.79
1.56	131.26 $\pm$ 9.49	82.33 $\pm$ 6.11
3.13	132.97 $\pm$ 9.80	81.91 $\pm$ 5.73
6.25	133.99 $\pm$ 8.20	82.26 $\pm$ 4.93
12.50	136.98 $\pm$ 9.11	82.91 $\pm$ 4.23
25.00	139.16 $\pm$ 8.42	84.16 $\pm$ 4.26
50.00	143.52 $\pm$ 4.81	84.89 $\pm$ 5.40
100.00	152.02 $\pm$ 0.85	87.28 $\pm$ 6.77
200.00	162.78 $\pm$ 5.19	93.50 $\pm$ 8.48

Table 4.3 FRAP values of vitamin C and lawsone on fibroblast 3T3 cell line at different concentrations. The total antioxidant activities of vitamin C (positive control) and lawsone on fibroblast 3T3 cell line were reflected by FRAP values at various concentrations (200 μM , 100 μM , 50 μM , 25 μM , 12.5 μM , 6.25 μM , 3.13 μM , 1.56 μM and 0 μM) by FRAP assay. Data were presented as mean $\pm$ SEM from three independent experiments. The result from Figure 4.6 described that lawsone showed no significant antioxidant properties on 3T3 cell line when compared to the negative control. However, vitamin C showed significant antioxidant properties on 3T3 cell line only at the concentration of 200 μM when compared to the negative control ($p < 0.05$).

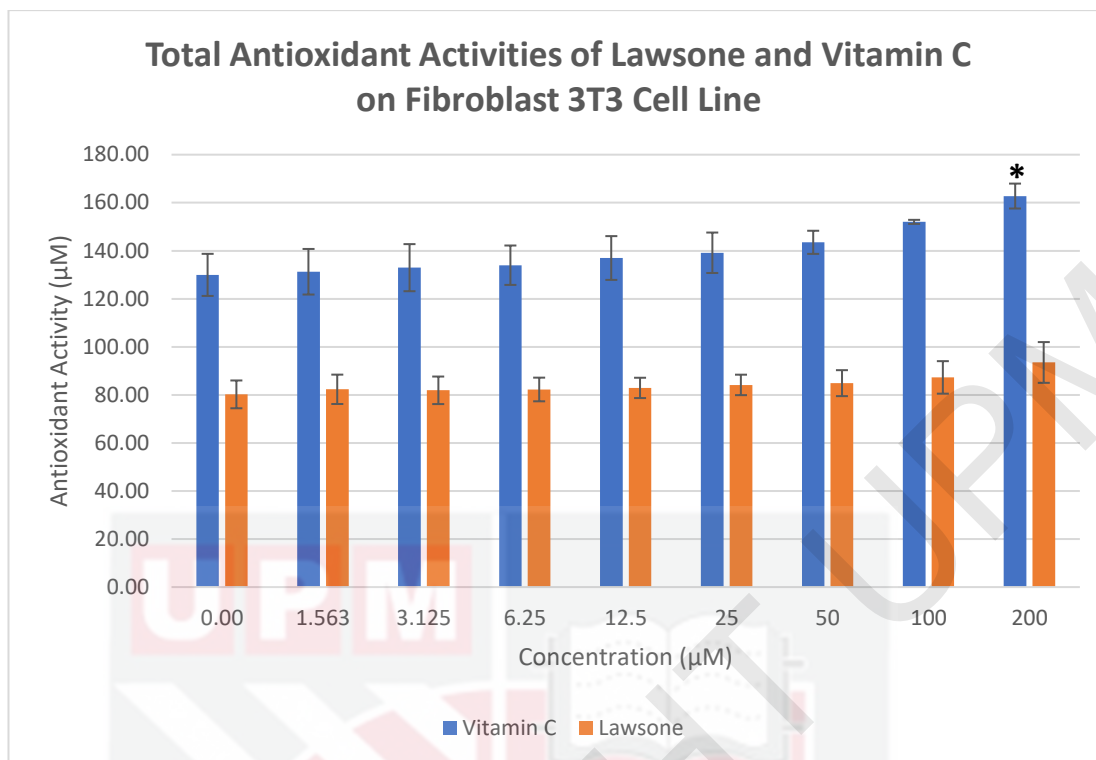


Figure 4.6 Graph of the total antioxidant activities of vitamin C and lawsone at various concentrations on fibroblast 3T3 cell line. The graph above showed the total antioxidant activity of 3T3 fibroblast cell line after being treated with lawsone of various concentrations (5000μM, 2500μM, 1250μM, 625μM, 312.5μM, 156.75μM, 78.13μM, 39.07μM, 19.53μM, 9.77μM and 0μM). Data were represented as mean $\pm$ SEM from three independent experiments, where * was indicated the significant difference ($p < 0.05$) relative to the control group. One-way ANOVA was used to perform the statistical analysis followed by Dunnett's test as the post-hoc test.

4.3 *In Silico* Analysis via Molecular Docking of Lawsone to SOD

4.3.1 Crystal Structure and Hydrophobic Pocket Residues of SOD

Figure 1 showed the crystal structure of SOD which was retrieved from PDB (PDB ID: 2C9V). This crystal structure consisted of 2 chains known as chain A and F with four ligand molecules, copper (Cu), sodium (Na), sulfate (SO_4) and zinc (Zn) which were attached to the hydrophobic pocket of SOD. SOD is a dimer that consists of only chain A, while, chain F is an exact copy of the latter. However, both chains were needed for this molecular docking study as the allosteric site was yet to determine in both chains.

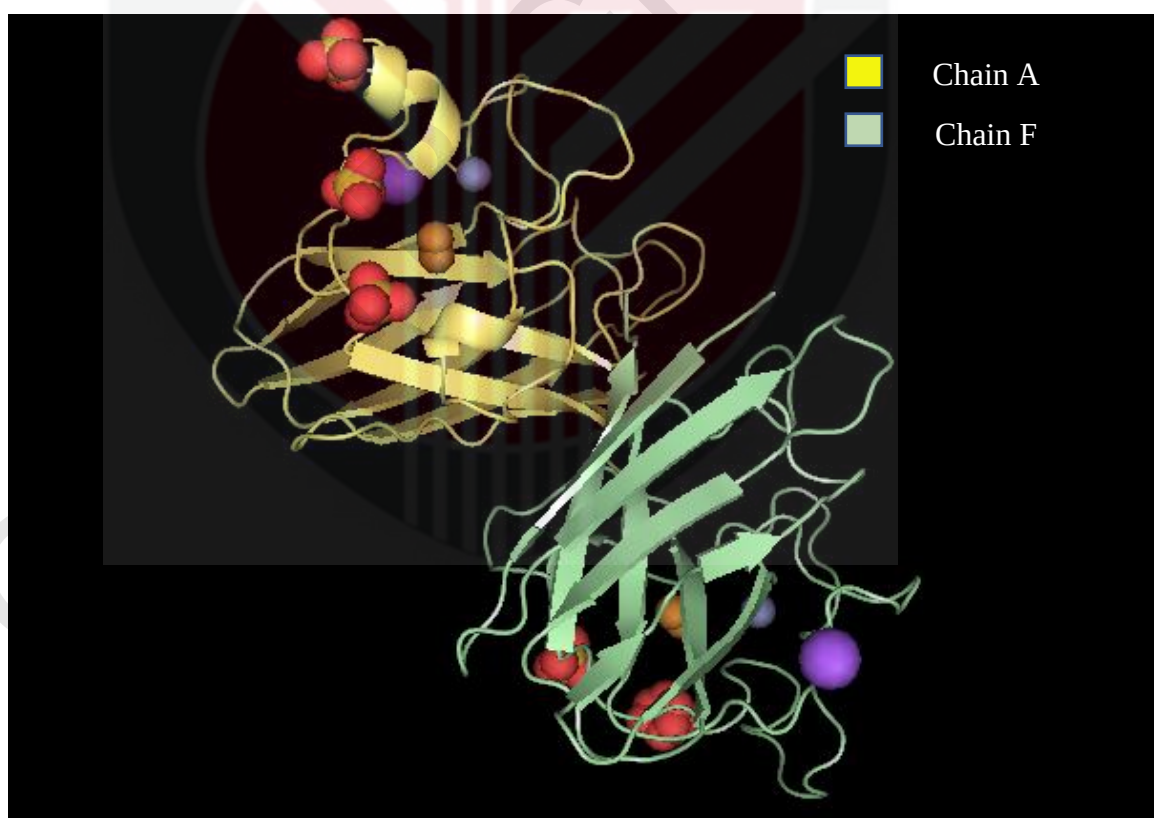


Figure 4.7 Crystal Structure of SOD retrieved from PDB (ID: 2C9V)

4.3.2 Determination of Allosteric Site of SOD

Allosteric site of SOD was determined by using AlloSitePro 2016. By using AlloSitePro 2016, a novel allosteric site in SOD also can be predicted. Figure 2 below showed the potential regions of the allosteric site in SOD, namely as Region A and Region B.

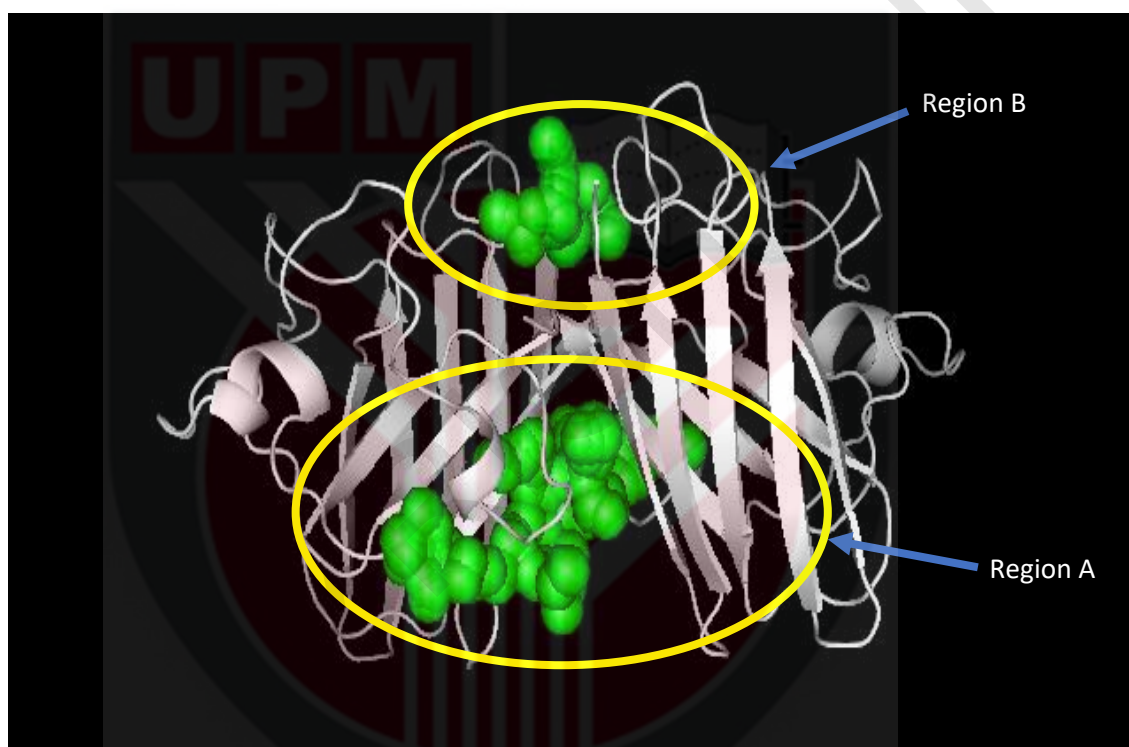


Figure 4.8 Allosteric sites of SOD. The allosteric sites (green spheres) was featured as the potential sites for the binding of small molecules.

4.3.3 AutoDock Vina Result

AutoDock Vina predicted nine best binding modes between lawsone and SOD as the poses were arranged into ranking from the highest binding affinity to lowest binding affinity. The most negative value was ruled as the best binding affinity. The best-docked configuration with the highest binding affinity was selected for advanced analysis. The tables below showed the results of molecular docking between lawsone and SOD for Region A and B.

Mode	Binding Affinity (kcal/mol)
1	-7.3
2	-7.2
3	-7.1
4	-6.9
5	-6.9
6	-6.8
7	-6.8
8	-6.8
9	-6.7

Table 4.4 Predicted binding modes of lawsone to SOD (Region A)

Mode	Binding Affinity (kcal/mol)
1	-6.6
2	-6.6
3	-6.5
4	-6.4
5	-6.3
6	-6.2
7	-6.2
8	-6.2
9	-6.2

Table 4.5 Predicted binding modes of lawsone to SOD (Region B)

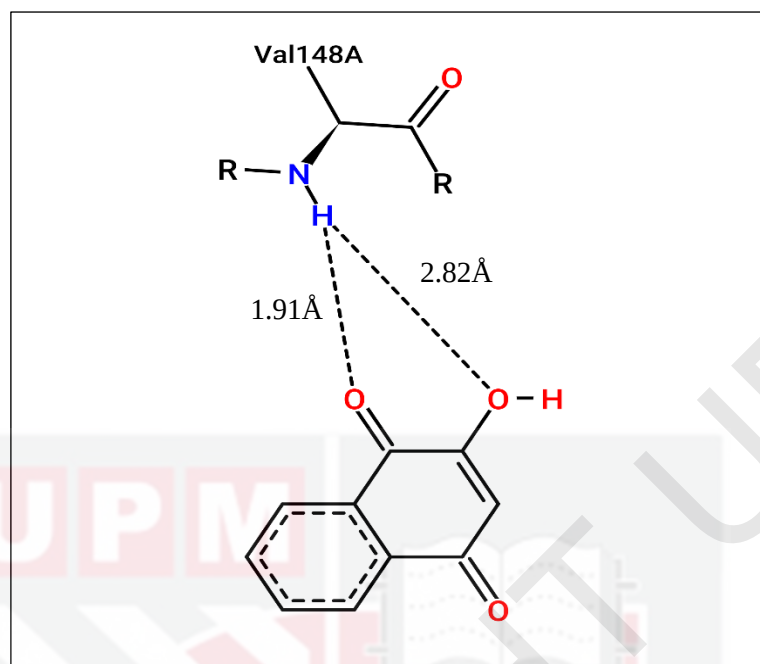
4.3.4 Analysis of Best Docked Complexes

4.3.4.1 Allosteric Site - Region A

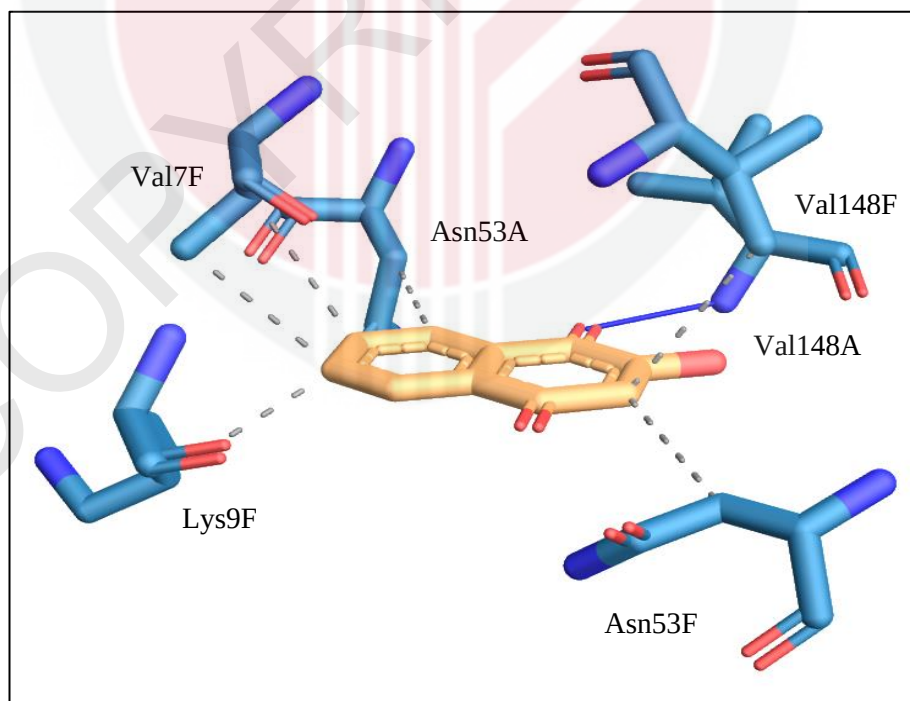
Figure 4.10 illustrated the best predicted binding results computed by AutoDock Vina. Lawsone bind to the Region A of the allosteric site of SOD with the highest binding affinity at -7.3 kcal/mol. The 2D result was analysed by using ProteinsPlus. From the Figure 4.10A, two hydrogen bonds were formed between hydroxyl group (distance 2.82Å) and a carbonyl group (distance 1.91Å) of lawsone and amide group of Valine 148 (Val148) residue at chain A. The distance of the hydrogen bonding was retrieved from PLIP webserver.

The 3D result was obtained from PLIP webserver. From the Figure 4.10B, there were six hydrophobic interactions between residues of SOD and lawsone. The hydrophobic interactions were formed between the aromatic ring of lawsone and the hydrophobic pockets of Valine 7 (Val7), Lysine 9 (Lys9) residues at chain F and Asparagine 53 (Asn53) residue at chain A. There was also hydrophobic interactions between the hydrophobic pockets of Asparagine 53 (Asn53) and Valine 148 (Val148) residues at chain F and the carbonyl group of lawsone. Simultaneously, a hydrogen bond was also established between the carbonyl group of lawsone and Valine 148 (Val148) residue at chain A.

[A]



[B]



[C]

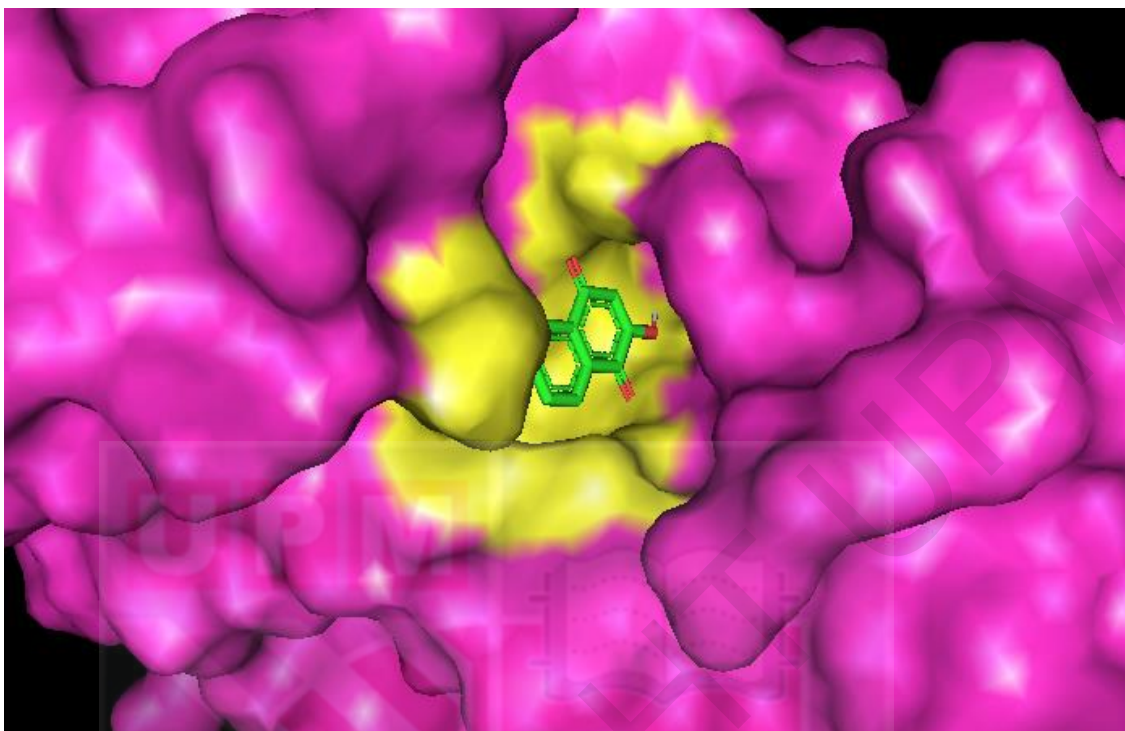


Figure 4.9 The best-docked complex between lawsone and SOD from region A.

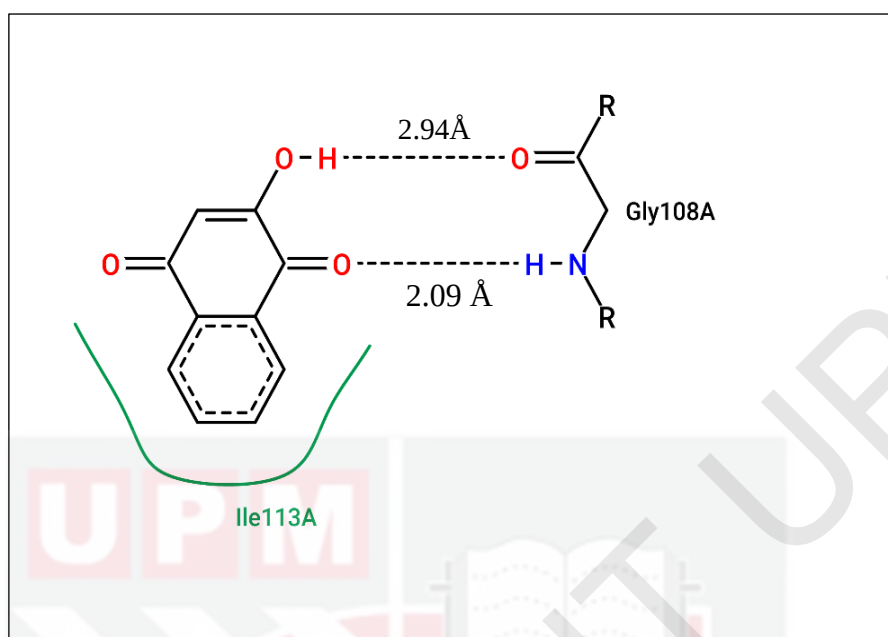
[A] Lawsone interact with Valine 148 residue at chain A. [B] The hydrophobic interactions (grey dashed line) and hydrogen bonding (blue line) between residues of SOD and lawsone analysed by using PLIP webserver. [C] The view from PyMol of lawsone best binding pose on SOD.

4.3.4.2 Allosteric Site - Region B

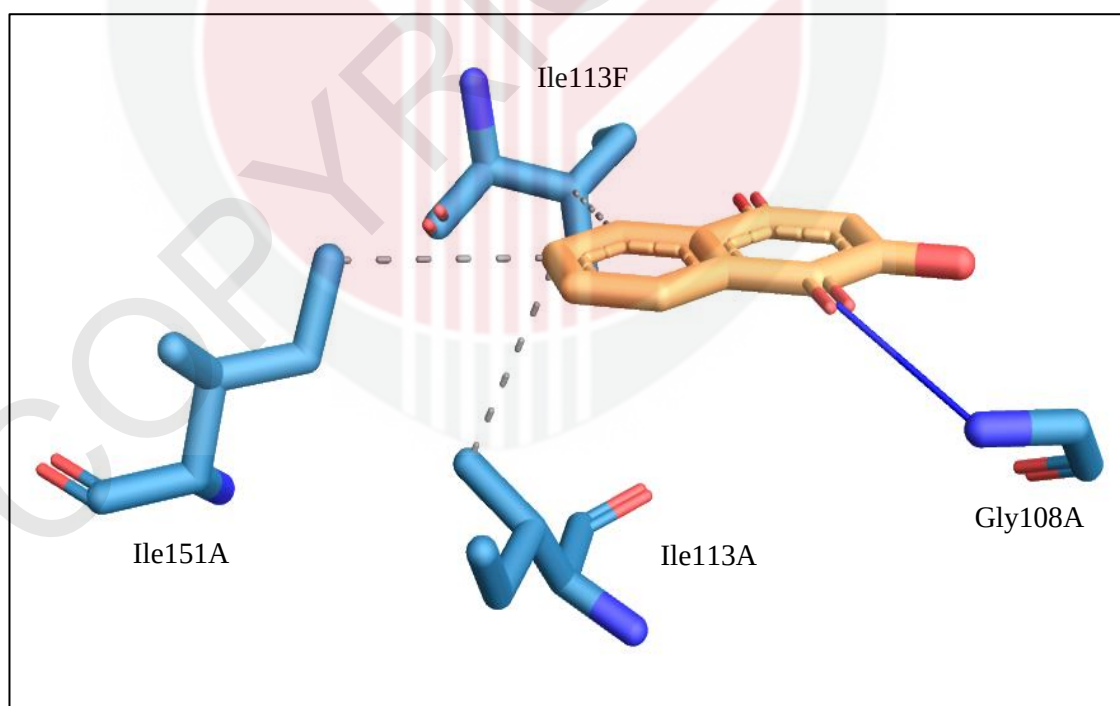
Figure 4.11 depicted the best predicted binding results computed by AutoDock Vina. Lawsone bind to the Region B of the allosteric site of SOD with the highest binding affinity at -6.6 kcal/mol. The 2D result was analysed by using ProteinsPlus. From Figure 4.11A, there was a presence of a hydrophobic pocket of Isoleucine 113 (Ile113) residue from chain A at the aromatic ring of lawsone. Concurrently, two hydrogen bonds were formed between carbonyl group (distance 2.09Å) and a hydroxyl group (distance 2.94Å) of lawsone and amide group and the carbonyl group of Glycine 108 (Gly108) residue at chain A respectively. The distance of the hydrogen bonding was retrieved from PLIP webserver.

The 3D result was obtained from PLIP webserver. From Figure 4.11B, there were three hydrophobic interactions between residues of SOD and lawsone. The hydrophobic interactions were formed between the aromatic ring of lawsone and the hydrophobic pockets of Isoleucine 113 (Ile113), Isoleucine 151 (Ile151) residues at chain A and Isoleucine 113 (Ile113) residue at chain F. Simultaneously, a hydrogen bond was also established between the carbonyl group of lawsone and Glycine 108 (Gly108) residue at chain A.

[A]



[B]



[C]

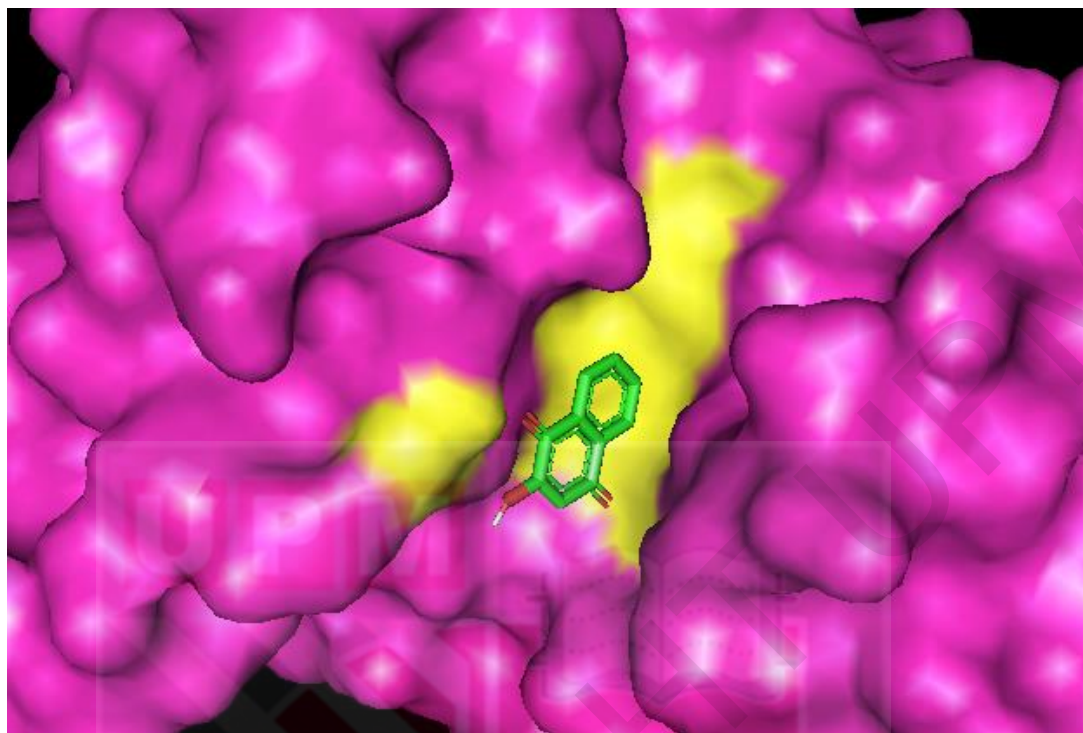


Figure 4.10 The best-docked complex between lawsone and SOD from region B.

[A] Lawsone interact with Isoleucine 113 and Glycine 108 residues at chain A. [B] The hydrophobic interactions (grey dashed line) and hydrogen bonding (blue line) between residues of SOD and lawsone analysed by using PLIP webserver. [C] The view from PyMol of lawsone best binding pose on SOD.

CHAPTER 5

DISCUSSION

5.1 Cytotoxicity and Total Antioxidant Activity of Lawsone

The cytotoxicity method is one of the biological assessments and screening experiments utilising tissue cells by using medical devices to investigate the developmental, replication, and morphological effects *in vitro* (Li, Zhou & Xu, 2015). The study of cytotoxicity is a meaningful “baby steps” study to determine the possible toxicity of a test substance, which includes biologically isolated active compound from the natural product or plant extracts. Cellular toxicity studies can be such an essential role for efficacious growth of pharmaceutical or cosmetic formulation with minimal to no toxicity (McGaw, Elgorashi & Eloff, 2014).

Thus, MTT assay was performed to determine the cytotoxicity of lawsone on A431 and 3T3 cell lines. This assay is a colourimetric reaction where the cell monolayers plated in the multiwell plates is measured. It is to assess the viability of cell; as MTT assay is dependent on mitochondrial respiration and serves to determine the cellular energy capacity of a cell indirectly. This assay is based on the enzymatic reduction into MTT-formazan from 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide (MTT) which is catalysed by mitochondrial succinate dehydrogenase (Chacon, Acosta & Lemasters, 1997). It is established upon the capability of cellular nicotinamide adenine dinucleotide phosphate (NADPH)-dependent cellular oxidoreductase enzymes to reduce tetrazolium dye MTT to its

insoluble purple-coloured formazan (Kuate, Karaosmanoğlu & Sivas, 2017). Overall, the cellular reduction of tetrazolium salts to formazan crystals is highlighted from the MTT assay. The optical density value obtained from the ELISA reader is directly reflected into the number of counted cells. Observed clinical correlation between *in vitro* and *in vivo* testing, reproducibility of the results and rapidity of the assay are one of the benefits of MTT assay (Winikoff et al., 2005).

In this study, the cytotoxicity of lawsone on A431 and 3T3 cell line was being assessed by determining IC_{50} from Figure 4.1 and 4.2 respectively. Based on the results, IC_{50} of lawsone on A431 cell line was determined at $3650\mu M$ while no IC_{50} was detected on 3T3 cell line when treated with various concentrations of lawsone. IC_{50} values of lawsone obtained from the graphs indicated that the cytotoxic effect of lawsone only occurs at high concentration. This demonstrated lawsone as a valuable candidate for potential antioxidant agents as it has low cytotoxic characteristics. This was supported by Lozza et al. (2019) which they reported that lawsone offered the clinical potential for managing skin conditions defined by hyperproliferation and inflammation, owing to its low cell toxicity. IC_{50} of lawsone from both cells was later used as a reference dose to determine the suitable range of concentration of lawsone for the preceding FRAP assay.

Antioxidant activity was characterised as “a restriction of the oxidation of proteins, lipids, DNA or other molecules that exists by preventing the propagation stage in oxidative chain reactions”. Antioxidant activity was also described as the capability of inhibiting the oxidation process. Primary antioxidants explicitly scavenge

free radicals, whereas secondary antioxidants indirectly inhibit free radicals from being generated by undergoing Fenton's reaction. Thus, many methods of determining total antioxidant activity define the capabilities of the tested compound or product to scavenge free radicals and complex metal ions that drive the oxidation reaction (Atasoy, Yesilnacar & Atasoy, 2019; Tirzitis & Bartosz, 2010).

Total antioxidant activity or total antioxidant capacity was a commonly used analyte to assess the antioxidant potential of biological samples. It may determine the antioxidant reaction against the free radicals generated in an illness. It was used to determine the antioxidant ability of the biological sample. It is also an assessment of the number of free radicals scavenged by a test solution (Rubio et al., 2016). A composite measure of the total activity of the biological fluid antioxidants is the combined effects of each scavenging antioxidant within the sample, providing a comprehensive overview of the overall prooxidant-antioxidant equilibrium (Choy, Benzie & Cho, 2000). According to Rubio et al. (2016), one of the assays that measure the total antioxidant activity based on the establishment of the potential of a sample to reduce a metal complex is ferric reducing antioxidant power (FRAP) assay.

FRAP assay is not based on hydrogen atom transfer but instead, it is focused on an electron transfer reaction. Electron transfer based assays evaluate the potential of an antioxidant to change colour when there is an occurrence of reduction (Huang, Ou & Prior, 2005). Thus, FRAP assay was a method that involves an electron transfer reaction in which portrayed the capability to reduce ferric ion (Fe^{3+}) to ferrous ion (Fe^{2+}). An acidic condition of FRAP reaction, which was at pH 3.6 is required to

maintain the solubility of iron. It caused the increment in redox potential and decrement in ionisation potential at the low pH. At the absorption at 593nm, the reaction formed Fe^{2+} coloured complex as the reduction of Fe^{3+} to ferrous ion Fe^{2+} happened in the presence of TPTZ (Cerretani & Bendini, 2010). This correlated to the FRAP mixture which is required to be prepared in an acidic condition by adding TPTZ and $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ in the acetic acid. The intensity of change in colour was associated with the antioxidant concentration level in the sample. It indicated that the test reaction mixture which comprises ferrous ion is directly correlated to the antioxidant concentration present in the sample (Nishaa et al., 2012). Thus, higher FRAP value denoted higher antioxidant capacity as the value depicted on the reducing ferric ions because the antioxidants were also known as the reducing agent.

In the present study, lawsone was used to determine the total antioxidant activity in two settings, firstly without cell line and secondly with the presence of cell lines which were A431 and 3T3 cell lines. Vitamin C or L-ascorbic acid was acted as the positive control because it can be easily oxidised, which can further accelerate its degradation due to the presence of light, heat and heavy metal cations. Thus, vitamin C can be a significant measure of food quality and contributed to the antioxidant attributes in food products (Pisoschi & Negulescu, 2011). Based on Figure 4.4, the total antioxidant activity of lawsone increased in a concentration-dependent manner from the concentration of $1.56\mu\text{M}$ up to $200\mu\text{M}$. This was supported by Majiene et al. (2019) in their study, where the concentration range of $50\mu\text{M}$ to $1000\mu\text{M}$ lawsone demonstrated a significant antioxidant activity by using dichloro-dihydro-fluorescein diacetate (DCFH-DA) assay on C6 glioblastoma cells statistically. This could be as a

strong supporting evidence in which lawsone possessed metal chelating and free radical scavenging features.

Furthermore, based on Figure 4.5 and Figure 4.6, the total antioxidant activity of lawsone towards A431 and 3T3 cell lines showed an increment in concentration up to 200 μ M, but in a very low range. Firstly, it might occur because the data from the result showed that there was a presence of antioxidant activities happened in the control group; utilising the untreated cell lines without lawsone treatment. It is supported by Hasiah et al. (2011) as they suggested that the cell lines might have their own endogenous antioxidant activity. In addition, this occurrence suggested that lawsone might be metabolised into an unstable and inactive form at the low concentration by both cell lines before it can exert its biological activity. According to Yang et al. (2018), lawsone can be reduced to 1,2,4-trihydroxynaphthalene. This metabolite was relatively in an unstable form as the presence of hydroxyl cloud in the metabolite can amplify the electron cloud density of the naphthalene ring. In contrast, Osman and van Noort (2003) suggested that lawsone is a non-redox-cycling quinone because lawsone is not a substrate for NADPH-cytochrome *c* reductase as such electron-transferring flavoenzymes cannot reduce lawsone and also it is considered a poor substrate for NAD(P)H:Quinone oxidoreductase₁ (NQO₁) *in vitro*. Also, Sauriasari et al. (2007) reported that lawsone has weak electrophilic features *in vitro*. Thus, it cannot react spontaneously due to the presence of anionic form of lawsone itself. This caused the lawsone does not undergo redox cycling *in vitro*. Another reason that could be the cause of insignificance was stated by Nakamura (2013) that 3T3 cell line displayed low saturation density, grow at high dilution and being highly sensitive to cell division inhibition after confluence although it was capable of dividing the cells

indefinitely. Thus, it is predicted that this study can be improved by increasing the concentration of lawsone when performing the cell-based FRAP assay up to 1000 μ M to observe the total antioxidant activity of lawsone in 3T3 and A431 cell lines at high concentration.

Nevertheless, many studies showed many pieces of evidence of the excellent antioxidant activity of lawsone despite the result obtained from the cell-based FRAP assay from this study. Kurtyka et al. (2016) reported that lawsone yielded higher production of H₂O₂ and activity of antioxidative enzymes such as SODs, catalases and peroxidases in coleoptile cells when compared to the juglone, an isolated metabolite from *Juglans nigra* or black walnut tree. Meanwhile, Zohourian et al. (2011) reported that, although lawsone did not mostly exert the antioxidant activity of henna but also due to the presence of other polyphenolic compounds from the extracts, pure lawsone already showed almost half of the antioxidant activity of the extract itself.

Moreover, although FRAP assay measured the potential of biological antioxidants, it also measured chemical reductant that reduced ferric complex to ferrous form; by means of the reduction of Fe³⁺-TPTZ to Fe²⁺-TPTZ. However, not all reductants were antioxidants such as glutathione as it cannot reduce Fe³⁺ to Fe²⁺. Therefore, FRAP assay does not justify for -SH antioxidant group. This determined that increment of ROS does not strongly interpret that there is an increment in antioxidant concentration or vice versa (Griffin & Bhagooli, 2004). Thus, antioxidant assay such as GSH reductase assay can be done for the future study to further investigate the antioxidant activity within the biological system such as in cell lines.

5.2 Molecular Docking of Lawsone on SOD

Oxidative stress is a biological process or disorder that arises in favour of oxidants because of the physiological imbalance between the amounts of antioxidants and oxidants such as free radicals or reactive species. The free radicals have apparently overcome the antioxidants. This occurred when the generation of free radicals surpasses the threshold with which the natural antioxidant defence mechanisms of a body can respond; thus, establishing a cellular oxidising atmosphere that makes important biomolecules such as DNA and proteins to oxidise. The molecules of antioxidants which comprise the antioxidant defence grid work at distinctive levels in a biological organism. One of them is first-line defence antioxidants which they have an ability to neutralise any potential molecules that can develop and induce the formation of free radicals. Superoxide dismutase (SOD) is one of the enzymes involved in this first line of defence (Ighodaro & Akinloye, 2018). These enzymes provide cellular defence against ROS as it can catalyse the dismutation of superoxide radicals ($O_2^{\cdot-}$) to hydrogen peroxide (H_2O_2) and oxygen molecules (Siddique, Deng & Ajroud-Driss, 2013). Moreover, SOD can be found in our body abundantly especially at the skin and its appendages which later provide excellent protection to the skin against ROS (Altobelli et al., 2020).

Thus, this study attempted to unbridle the potential activation mechanism of lawsone towards SOD to combat the oxidative stress condition caused by ROS. A study has been done by Kurtyka et al. (2016) described that lawsone played a significant role in the generation of hydrogen peroxide and ROS scavenger in maize coleoptile segments by increasing the antioxidant enzyme such as SOD.

Thus, the objective of this study is to investigate the activation mechanism of lawsone on superoxide dismutase (SOD) enzyme via molecular docking method. Molecular docking is among the highly admired structure-based strategies, commonly used for analysing molecular recognition, and intends to predict the binding mode and binding affinity of a complex composed of two or more respective molecules with established structures (Roy, Kar & Das, 2015).

Before proceeding with the molecular docking method, the allosteric site of SOD1 protein from the PDB (ID: 2C9V) was determined by using AlloSite 2.0. Allosite was available through the web server worldwide and it was a recently developed automatic contrivance to predict the allosteric regions in the protein of interest (Huang et al., 2013). Determination of the allosteric sites of SOD was done as SOD is known for its high stability in structure due to the presence of copper and zinc for redox and stabilising function respectively (Garrett & Kirkendall, 2000). Song et al. (2017) reported that targeting and identifying allosteric sites of a protein has turned into a quite attention as a novel strategy for allosteric-targeting drug development. Binding to the allosteric site may exploit specific features which characterise target protein from other homologous proteins. Also, the structural variety of allosteric site provides the allosteric modulators with better selectivity, fewer side effects and minor toxicity. Thus, lawsone was expected to exhibit antioxidant properties by binding to the allosteric site of SOD and enhance the SOD activity.

After determining the allosteric sites of SOD which namely as Region A and Region B, lawsone was then docked to the specific hydrophobic pockets of allosteric

sites of SOD. Based on the results provided by the AutoDock Vina, the docking lawsone-SOD complex showed a list of ranges of binding affinities from -6.7kcal/mol to -7.3kcal/mol for Region A and -6.2kcal/mol to -6.6kcal/mol. However, from the modes given, the study preferred to choose the first mode as the best predicted binding pose between lawsone and SOD in Region A as the search for ligand confirmation was always towards the negative values. It was supported by Das et al. (2016) emphasised that higher binding affinity signified by the negative score and weaker binding affinity signified by the positive score from the AutoDock Vina. As for Region B, the value of root-mean-square deviation (RMSD) for the second mode was higher compared to the first mode. Carugo (2003) stated that lower RMSD values were correlated with a higher resolution of protein structure. Thus, the first mode was chosen as the best predicted binding pose between lawsone and SOD although the second mode has the same binding affinity value with the first mode.

Based on the results in Figure 4.9 and 4.10, the best binding affinity of lawsone to SOD1 from Region A was recognised by the formation of hydrogen bonds between the hydroxyl group and the carbonyl group of lawsone and amide group of Valine 148 from chain A (Val148A) along with the hydrophobic interaction at the hydrophobic pockets of Valine 7 (Val7), Lysine 9 (Lys9), Asparagine 53 (Asn53) and Valine 148 (Val148) residues at chain F and Asparagine 53 (Asn53) residue at chain A. From Region B, it was recognised by the formation of hydrogen bonds between hydroxyl and carbonyl group of lawsone and amide group of Glycine 108 from chain A (Gly108) along with hydrophobic interaction with Isoleucine 113 (Ile113), Isoleucine 151 (Ile151) residues at chain A and Isoleucine 113 (Ile113) residue at chain F. The most substantial interactions between lawsone and SOD were hydrogen bonds and

hydrophobic interaction or known as Van der Waals force. The binding affinities for both regions were directly correlated to the analysis of docked lawsone-SOD complex. This was supported by Patrick (2017) stated that the potential for these two interactions in many other proteins are greater than ionic or covalent bond interactions, since there are numerous amino acid residues that are able to form hydrogen or hydrophobic interactions than other kinds of interactions. Furthermore, the presence of hydrophobic interactions between lawsone and SOD indicated the binding affinity between these target-protein interfaces. From the results, the study showed a quite number of hydrophobic interactions towards SOD. Patil et al. (2010) emphasised that the biological activity of the drug lead can be increased when the number of hydrophobic atoms in the active core of the drug-protein interface increases.

Besides, four hydrogen bonds were detected from the binding between SOD and lawsone. The distance of hydrogen bonds in Region A was 1.91Å and 2.82Å meanwhile in Region B, 2.09Å and 2.94Å. These mentioned distances between lawsone and the SOD consisted of moderate and strong binding interactions. It was supported by Jeffrey (1997) that the distance range of hydrogen bonding was reflected the strength of hydrogen bonds which has been categorised into strong interaction at 2.2Å to 2.5Å, moderate interaction at 2.5Å to 3.2Å and weak interaction at 3.2Å to 4.0Å.

In a nutshell, lawsone showed a good activation effect towards superoxide dismutase enzyme to reduce oxidative stress based on the result obtained from the molecular docking analysis. The activation of SOD enzyme could be an effective

indicator to be considered as a potential therapeutic target protein to treat inflammatory diseases caused by oxidative stress such as psoriasis and eczema. Hence, the potency of lawsone to trigger the activation mechanism of SOD can be developed and studied further for the development as a potential therapy for ROS related inflammatory diseases.



CHAPTER 6

CONCLUSION

6.1 Conclusion

The findings from this study illustrated that lawsone only causes toxic to both cell lines at the high concentration where the IC_{50} of lawsone was determined at 3650 μ M in A431 cell line and no IC_{50} was detected in 3T3 cell line. Next, the total antioxidant activity of lawsone increased in a concentration-dependent manner when performing FRAP assay without cells. As for cell-based FRAP assay, lawsone showed a slight insignificant increment of total antioxidant activity in both A431 and 3T3 cell lines. Furthermore, lawsone depicted an excellent binding affinity at Region A and Region B (the allosteric sites of SOD) through molecular docking method which was predicted to simultaneously trigger the activation of SOD enzyme. Lawsone has the potential to be developed into a promising pharmaceutical agent with low cytotoxicity and possesses antioxidant activity, with high binding affinity to SOD enzyme which increased its antioxidative activity. This highlighted the capability of lawsone as a promising therapeutic antioxidant for inflammatory reaction suppression.

6.2 Limitation

All the way through this project, some limitations have occurred. For instance, there was an emergence of COVID-19 cases in Malaysia in early March 2020. Thus, Malaysia government implemented an ad-hoc decision for preventive measure purposes, called Movement Control Order (MCO) by closing all the premises which include university, institute and laboratory starting on 18th March 2020 until 1st July 2020. Hence, this situation halted all the laboratory works especially on cell culture preparation for MTT and FRAP assay. Next, there was also a small likelihood of inaccuracy in the apparatus such as micropipette and weighing balance. Lastly, the determination of the mechanistic pathway of antioxidative property of lawsone on SOD enzyme via molecular docking was based on the prediction that was provided by the computational calculation; thus, the data need to be validated with a protein expression analysis of SOD enzyme on A431 and 3T3 cell lines (e.g. ELISA), or by a supporting powerful computational method (i.e. molecular dynamics method) for a robust justification on the analysis of the molecular docking results.

6.3 Subsequent Recommendations

Numerous recommendations can be done in the future for the advancement of this study which includes;

- a) Increase the concentration of lawsone up to 1000 μ M in cell-based FRAP assay for both cell lines.
- b) Perform glutathione reductase assay to consolidate the FRAP assay results.
- c) Perform FRAP assay on different types of skin cell lines specifically on non-carcinoma skin cell lines.
- d) Conduct *in vivo* studies to demonstrate the antioxidant properties of lawsone in an animal model.
- e) Carry out a study regarding the structure-activity relationship (SAR) of lawsone derivatives.
- f) Investigate on Molecular Dynamic (MD) study for further analysis of physical binding interaction between lawsone and SOD.

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APPENDIX

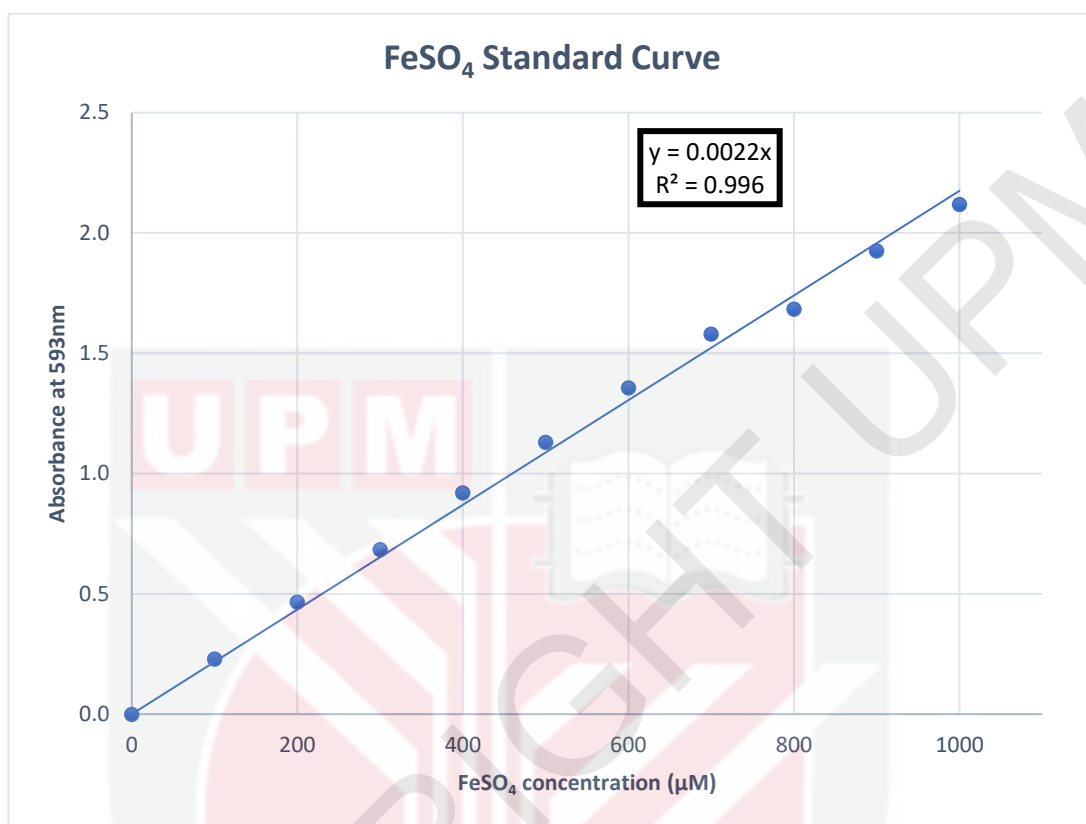


Figure 4.11 Calibration graph of FeSO₄. The graph above showed the standard curve of FeSO₄ from concentration of 0 μM to 1000μM with the equation of $y = 0.0022x$ and the coefficient of determination at $R^2 = 0.996$.