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COMPARISON OF ANTIOXIDANT ACTIVITY BETWEEN BENZIMIDAZOLE DERIVATIVES NN-1-10 AND NN-1-18

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FPSK2 2020 17

COMPARISON OF ANTIOXIDANT ACTIVITY BETWEEN BENZIMIDAZOLE DERIVATIVES NN-1-10 AND NN-1-18

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Introduction: Free radicals are molecules present in every human body that could cause damage to deoxyribonucleic acid (DNA) and proteins and are avoided by antioxidants. Benzimidazole compound and its derivatives have shown antioxidant activities in numerous studies, which further increases their potential as effective synthetic antioxidants. **General objective:** To evaluate the antioxidant activity of two of the benzimidazole derivatives; NN-1-10 and NN-1-18. **Specific objective:** To compare the antioxidant capacity of benzimidazole derivatives; NN-1-10 and NN-1-18 using FRAP, DPPH and PAP antioxidant assay. **Hypothesis:** There is a difference in the antioxidant capacity of benzimidazole derivative NN-1-10 and NN-1-18 in FRAP, DPPH and PAP antioxidant assay. **Methodology:** The antioxidant properties of NN-1-10 and NN-1-18 were determined through DPPH (2, 2-diphenyl-1-picrylhydrazyl) assay and Polybdenum Antioxidant Power (PAP) assay with concentration of 6.25 – 400 µM and Ferric ion Reducing Antioxidant Power (FRAP) assay with concentration of 3.125 – 200 µM along with controls of each respective assays. **Results:** For DPPH assay, the EC50 of NN-1-10 = 22.99 ug/ml, while the highest concentration of NN-1-18 (400uM) does not even yields 50% of the standard compound's DPPH radical scavenging activity. For FRAP assay, the antioxidant activity of NN-1-10 = 141 mmol Fe^{2+}/g while for NN-1-18 = 26.69 mmol Fe^{2+}/g. **Conclusion:** NN-1-10 shows a higher antioxidant activity than NN-1-18 through FRAP and DPPH assay, which also gives a comparable antioxidant value against the standard compounds.

Keywords: Antioxidant, Benzimidazole derivatives, DPPH assay, FRAP assay, NN-1-10, NN-1-18, PAP assay.

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PERBANDINGAN AKTIVITI ANTIOXIDAN ANTARA DERIVATIF BENZIMIDAZOLE NN-1-10 DAN NN-1-18

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Pendahuluan: Radikal bebas adalah molekul yang terdapat dalam setiap tubuh manusia yang dapat menyebabkan kerosakan pada asid deoksiribonukleik (DNA) dan protein, yang dihindari oleh antioksidan. Sebatian benzimidazole dan turunannya telah menunjukkan aktiviti antioksidan dalam banyak kajian, yang meningkatkan potensi mereka sebagai antioksidan sintetik yang berkesan. **Objektif umum:** Untuk menilai keupayaan antioksidan dua derivatif benzimidazol; NN-1-10 dan NN-1-18. **Objektif khusus:** Untuk membandingkan keupayaan antioksidan turunan benzimidazol; NN-1-10 dan NN-1-18 menggunakan ujian antioksidan FRAP, DPPH dan PAP. **Hipotesis:** Terdapat perbezaan keupayaan antara antioksidan derivatif benzimidazol NN-1-10 dan NN-1-18 dalam ujian antioksidan FRAP, DPPH dan PAP. **Metodologi:** Sifat antioksidan NN-1-10 dan NN-1-18 ditentukan melalui ujian DPPH (2, 2-diphenyl-1-picrylhydrazyl) dan Polybdenum Uji Kuasa Antioksidan (PAP) dengan kepekatan 6.25 - 400 µM dan Ferric Uji Kuasa Antioksidan Pengurangan ion (FRAP) dengan kepekatan 3.125 - 200 µM bersama dengan kawalan setiap ujian masing-masing. **Hasil:** Untuk ujian DPPH, EC50 NN-1-10 = 22,99 µg/ml, sementara kepekatan tertinggi NN-1-18 (400µM) bahkan tidak menghasilkan 50% dari aktiviti pengumpulan radikal DPPH sebatian standard. Untuk ujian FRAP, aktiviti antioksidan NN-1-10 = 141mmol Fe^{2+}/g sementara untuk NN-1-18 = 26.69 mmol Fe^{2+}/g. **Kesimpulan:** NN-1-10 menunjukkan aktiviti antioksidan yang lebih tinggi daripada NN-1-18 melalui ujian FRAP dan DPPH, yang juga memberikan nilai antioksidan yang setanding dengan sebatian piawai..

Kata kunci: Antioksidan, Derivatif Benzimidazole, ujian DPPH, ujian FRAP, NN-1-10, NN-1-18, ujian PAP.

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ACKNOWLEDGEMENT

In the name of Allah, the Most Gracious and the Most Merciful

All praises to the Almighty Allah *Subhanahu Wa Ta'ala*, for giving me strength, patience and good health condition throughout this project until the completion of this thesis. Nevertheless, this research project would not have been completed without the help from many people.

First and foremost, I would like to express my deepest gratitude to my supervisor, Assoc. Prof. Dr Abdah Md Akim, who played a big role for the success of this project. Thank you for your guidance, assistance, encouragement, and motivation, while all together being so considerate towards me throughout this study and project. Without your support, this research would have never been accomplished.

My deepest gratitude to the staff of the Laboratory of Biochemistry, Faculty of Medicine and Health Sciences and staff of the Laboratory of Pharmacotherapeutic which are En. Yuzairi and Miss Anoosha, for their help and contribution in this research by kindly guiding and allowing me to access all the facilities provided in the laboratory.

Besides, I would like to thank my supervisor's postgraduate student for his help and guidance throughout my project. Not to forget my FYP partner, Nur Syuhada Darol Napis for being there with me through thick and thin, in our FYP journey. I was able to stand strong because of you, Alhamdulillah we made it this far.

In addition, special thanks to my beloved parents and family for their unwavering love and trust, and for being my pillar of strength at each and every point of my life. My sincere thank you to all my friends, as their company had coloured and made my FYP and degree life memorable. I am truly blessed to have them all by my side. Finally, I would like to thank those who were directly or indirectly contributing to the completion of this project and thesis. Your kindness and involvement are highly appreciated.

Nur Hidayah Helmi Muhammad Fadil Helmi

Universiti Putra Malaysia

August 2020

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ABBREVIATIONS

ABTS	2, 2-azinobis (3-ethyl benzothiazoline-6-sulfonic acid)
AIDS	Acquired immune deficiency syndrome
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
COX-1	Cyclooxygenase-1 enzyme
COX-2	Cyclooxygenase-2 enzyme
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPPH	2, 2-diphenyl-1- picrylhydrazyl
EC50	Half maximal effective concentration
ET	Electron transfer
FRAP	Ferric ion Reducing Antioxidant Power
HAT	Hydrogen atom transfer
HBV	Human hepatitis B virus
HCL	Hydrochloric acid
HCT 116	Human colon carcinoma
HEPG2	Human hepatocellular carcinoma
HeLa	Henrietta lacks
HIV	Human immunodeficiency virus
IC50	Half maximal inhibitory concentration
IL	Interleukin
IUPAC	International Union of Pure and Applied Chemistry
LOX	Lipo-oxygenase
LPO	Lipid peroxidation
MAP	Mitogen-activated protein
MCF7	Human breast adenocarcinoma
MES	Maximum electroshock
MIC	Minimum inhibitory concentration
NH	Amines
ORAC	Oxygen radical absorption capacity
PAP	Phospholybdenum Antioxidant Power
PTZ	Pentylenetetrazole
REDOX	Oxidation-reduction
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SAR	Structure-activity relationship
TAR RNA	Trans-activation response of RNA
TPTZ	2,4,6-tripyridyl-s-triazine

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CHAPTER 1

INTRODUCTION

1.1 Background

Free radicals are any existing molecule with unpaired electrons at their outermost shell. These unpaired electrons give rise to the unstable and highly reactive properties of free radicals, as they can initiate both reduction and oxidation processes with other molecules (Lobo et al., 2010). When there is an excess in the amount of free radicals in the body, this leads to oxidative stress. Oxidative stress is extremely detrimental as it brings about countless diseases at several organ sites. This includes asthma in the lungs, hypertension in heart vessels, cataracts in eyes and even depression through the brain (Pham-Huy et al., 2008).

The human body uses antioxidants presence as a defensive mechanism in regard with free radicals. Antioxidants exert their action through two major mechanisms. First, antioxidants break the continuous free radical chain by donating their hydrogen atom or electrons. Next, antioxidants avert and stabilizes any initiator of free radical formation such as copper (Lobo et al., 2010; Pham-Huy et al., 2008). Benzimidazole compound is one of the endogenous compounds with potential as an antioxidant. Numerous studies throughout the years have proven that benzimidazole analogs exhibit various therapeutic activities, including antioxidants (Anastassova et al., 2018; Neochoritis et al., 2011; Padmavathi et al., 2012).

Even though free radicals are quite harmful, they are still present in the human body as a by-product of essential metabolic processes like respiration (Lobo et al., 2010). Just a slight increase in the number of free radicals could lead to oxidative stress. This ultimately escalates the possibility for individuals to be affected by numerous diseases. Hence, there is a need to find more compounds exhibiting antioxidant activity.

This study is interested in discovering the potential antioxidant activity of the 2 chosen novel benzimidazole derivatives. However, there is a limited current study regarding their antioxidant properties. As stated by Lobo et al. (2010), mutagenic activity of a compound could lead to oxidative damage. Azahar et al. (2019) conducted a study on the mutagenic activity of these two compounds, and the findings proved that they have no mutagenic activity. Therefore, these compounds are not free radical catalysts and could be tested to identify whether they have antioxidant properties.

1.2 Objective of Study

1.2.1 General Objective

To evaluate the antioxidant activity between benzimidazole derivatives NN-1-10 and NN-1-18.

1.2.2 Specific Objectives

- To compare the antioxidant capacity between benzimidazole derivatives NN-1-10 and NN-1-18 using Ferric ion Reducing Antioxidant Power (FRAP) assay.
- To compare the antioxidant capacity between benzimidazole derivatives NN-1-10 and NN-1-18 using DPPH (2, 2-diphenyl-1- picrylhydrazyl) assay.
- To compare the antioxidant capacity between benzimidazole derivatives NN-1-10 and NN-1-18 using Phospholybdenum Antioxidant Power (PAP) assay

1.3 Hypothesis

There is a difference between the antioxidant activity exhibited by benzimidazole derivatives NN-1-10 and NN-1-18 using FRAP, DPPH and PAP antioxidant assay.

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CHAPTER 2

LITERATURE REVIEW

2.1 Benzimidazole

The fusion of the Benzene compound to 4 and 5-position of the Imidazole ring compound produces a new bicyclic aromatic compound, Benzimidazole. The IUPAC name of benzimidazole is 1*H*-benzimidazole. Molecular weight of benzimidazole is 118.14 g/mol.

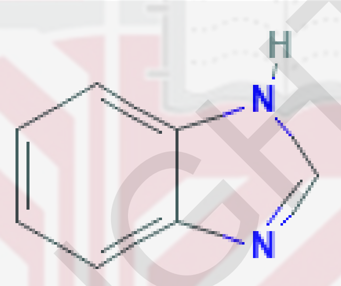

Figure 2.1: Structure of benzimidazole (*Benzimidazole | C7H6N2 - PubChem*, n.d.)

The boiling point of benzimidazole is >360 °C while its melting point is 170.5 °C (*Benzimidazole | C7H6N2 - PubChem*, n.d.). Benzimidazole is an amphoteric compound, however the presence of NH group in benzimidazole, which is a strong acid in nature causes the compound to have a slightly lower pH (strong acid, weak base) compared to its parent compound, Imidazole. N-ribosyl-dimethylbenzimidazole which functions as a cobalt ligand in vitamin B12 is the most prominent type of benzimidazole compound available in nature (Gaba et al., 2014).

There are numerous synthetic strategies for manufacturing benzimidazole. One of the strategies is by heating o-phenylenediamine with excess mono or di-basic acid could also synthesize benzimidazole, as fatty acid α-hydroxy acid and phenyl/diphenylacetic acid change to corresponding benzimidazole after the procedure. This step is further evolved by Philip modification, where o-phenylenediamine and monobasic acid are

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refluxed in 4 N hydrochloric acid. The resulting solution will then be neutralized with ammonium hydroxide in order to precipitate the benzimidazole product (Salahuddin et al., 2017).

2.2 Biological activity of benzimidazole derivatives

Various benzimidazole analogues or derivatives result in a wide range of pharmacological properties, exhibited by every different substitution of compound or group on the ring.

2.2.1 Antimicrobial activity

According to the study conducted by Fang et al. (2010), MIC value of one of the bis-azole compounds derived from benzimidazoles against *Pseudomonas aeruginosa* was 4 μg/mL, where its potency are 16-fold increased than chloramphenicol, the reference drug. Furthermore, Mungra et al. (2011) study also proved that benzimidazole-quinoline hybrids compounds produce significant antimicrobial activity against Gram-positive bacteria *Bacillus subtilis* compared to standard ampicillin. Novel 4-(1H-benz[d]imidazol-2yl)-1,3-thiazol-2-amines, which is synthesized from benzimidazole-thiazol-2-amine derivatives by (Reddy & Reddy, 2010) also showed similar potency of microbial inhibition against *Bacillus subtilis* compared to streptomycin and exhibit increased antimicrobial activity against *Fusarium oxysporum* compared to fluconazole.

2.2.2 Anti-inflammatory and analgesic

A review from Gaba et al. (2014) summarized the mechanism of actions shown by diverse benzimidazole derivatives in anti-inflammatory and analgesic activity. The pathways are illustrated as below.

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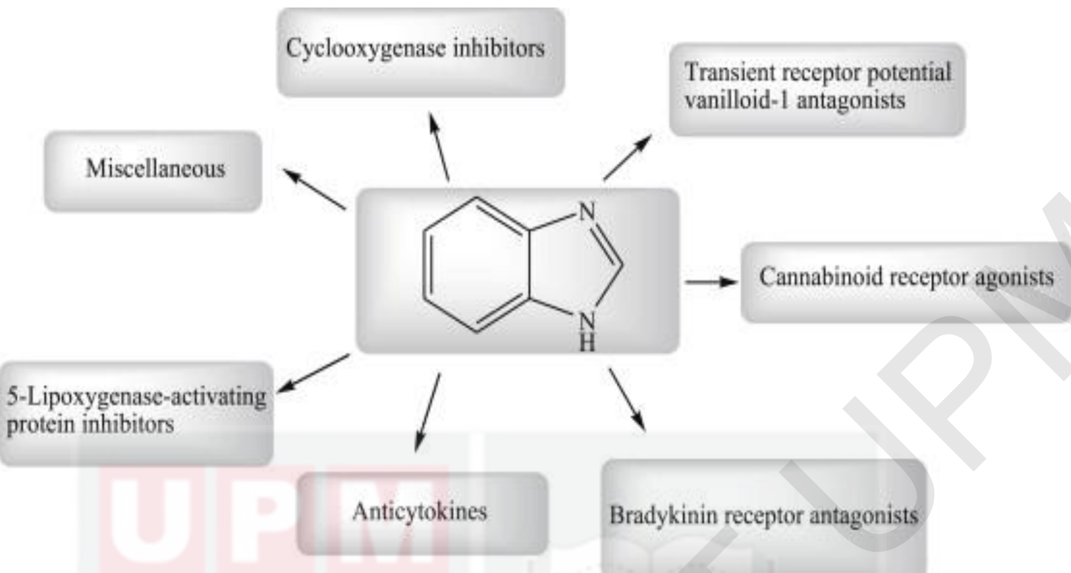

Figure 2.2: Various therapeutic targets acted by benzimidazole derivatives as anti-inflammatory and analgesic agents (Gaba et al., 2014).

Inhibition of cyclooxygenase-2 enzyme (COX-2) involved in inflammation is preferred rather than COX-1 which acts as a regulation of normal physiological inflammation. Synthesis of anacardic acid derivatives bearing benzimidazole scaffold yield compounds that was highly selective in its inhibition of COX-2 over COX-1 where there is 384-fold selectivity and comparable to established drug, celecoxib which shows 375-fold selectivity of COX-2 inhibitor (Paramashivappa et al., 2003) While, inhibition of cytokines on the other hand, productively decreases the inflammation process. de Dios et al., (2005) revealed 2-aminobenzimidazole and pyridinoyl-5-methoxybenzimidazole performs as a potent MAP kinase inhibitor and ultimately reduce the production of cytokines such as tumor necrosis factor-α, interleukin (IL)-1β and IL-6.

2.2.3 Anti-cancer

Refaat (2010) conducted the laboratory synthesis of several novel 2-substituted benzimidazole derivatives and screened them for anticancer activity. The results revealed good anticancer activity against human hepatocellular carcinoma (HEPG2), human breast adenocarcinoma (MCF7), and human colon carcinoma (HCT 116) cell lines, where the

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compounds IC50 were less than 10 μg/mL. The most potent compounds against these 3 cell lines were 2-thiazolylbenzimidazole derivative, benzylidene cyanomethylbenzimidazole and oxothiazolidin-2-ylide-necyanomethyl benzimidazole. Inhibition of human cancer cell lines proliferation was also tested by using schiff bases substituted into benzimidazole compounds in a study by Hranjec et al. (2011) One of the compounds showed the highest inhibition action of cancer growth and also affects HeLa and MCF-7 cell lines in a strong positive drug concentration and response manner.

2.2.4 Anticonvulsant

Anticonvulsant acts to arrest or decrease epilepsy, a neurological disorder caused by temporary abnormal brain activity which leads to seizure. Benzimidazole analogue and derivatives also exhibit this activity. This is proved by a study from Jain et al. (2010), where synthesized nitro-benzimidazole derivatives were administered in vivo. The mice were induced with maximum electroshock (MES) and pentylenetetrazole (PTZ) as the physical and chemical stimuli respectively. The stimuli should result in mice convulsions, however, most of the compounds managed to prevent epilepsy from occurring. Besides, in vivo testing of benzimidazole derivative for anticonvulsant activity was also done in the same MES mice model according to Shingalapur et al. (2010). A group of 4-thiazolidinones and 1,3,4-oxadiazoles containing benzimidazole moiety were incorporated into the study and two of the compounds produce potent anticonvulsant outcomes.

2.2.5 Antiviral

Benzimidazole derivatives exhibit antiviral activity against a wide range of viral strains. HIV, which is the most prominent viral strain causing AIDS and ultimately death, can also be treated by benzimidazole analogues. According to Ziółkowska et al. (2010), HIV-1 reverse transcriptase, an enzyme crucial for viral double strand DNA transcription, could be suppressed by synthetic nucleoside derivatives of *N*-benzyl-benzimidazole. Among the derivatives, methylene azide and methoxy groups substitution shows the

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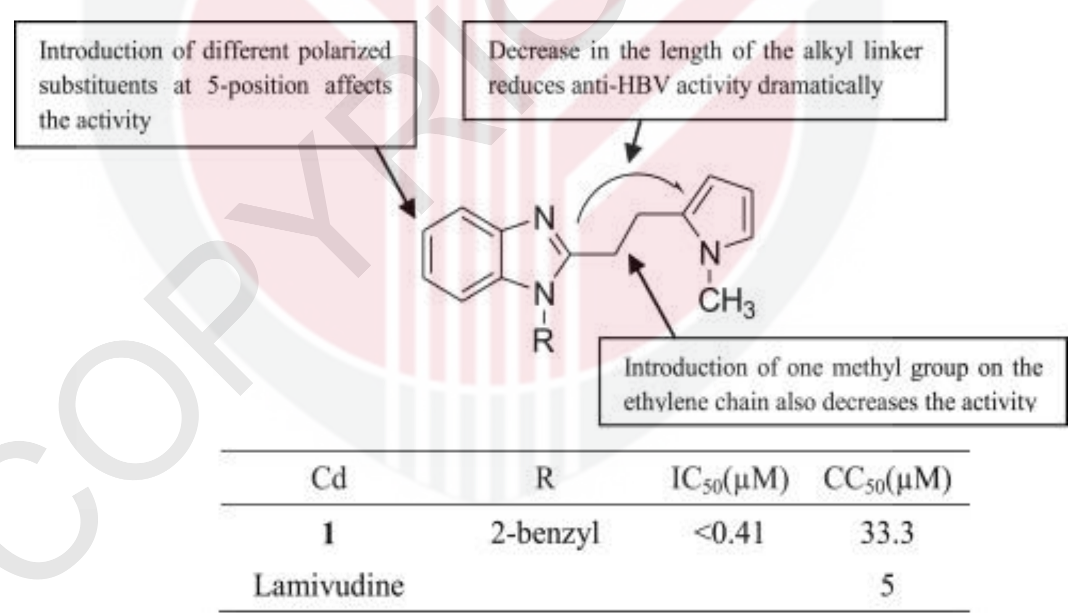
greatest activity. Moreover, TAR RNA of HIV stabilization that prevents the transactivation of viral promoter was increased in neomycin-benzimidazole conjugate compared to either neomycin or benzimidazole. The conjugate compound with the shortest linker length and lower triazole units also have higher affinity to TAR RNA (Ranjan et al., 2013).

Other than HIV, benzimidazole analogue for HBV antiviral activity was also recorded by numerous studies. Luo et al. (2010) developed a series of novel benzimidazole derivatives and tested the anti-HBV activity of the compounds in the HepG2.2.15 cell line. The most significant outcome is shown by the 2-pyrrolyl compound. This is due to its low cytotoxicity (CC50 = 33.3 μM), which reveals an elevated selectivity thrice the amount of common drug lamivudine. The structure of the compound and its relationship with anti-HBV activity is as illustrated below.



Cd	R	IC_{50}(μM)	CC_{50}(μM)
1	2-benzyl	<0.41	33.3
Lamivudine			5

Figure 2.3: SAR study of 2-pyrrolyl benzimidazole analogue (Yadav & Ganguly, 2015).

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2.3 Reactive oxygen species (ROS) and Antioxidant

Reactive oxygen species (ROS) are a byproduct of various cellular oxygen metabolism. Normal levels of ROS were maintained by diverse antioxidant enzymes. However, oxidative stress occurs when there is an imbalance between these two substances.

2.3.1 Reactive oxygen species (ROS)

In the aerobic respiration process, the mitochondrial electron transport chain shuttles the electrons for water molecule reduction and ATP energy production. This results in ROS byproduct synthesis. Two categories of ROS molecules are free radicals and non-radicals. The difference between these two groups is that the free radical contains unpaired electrons. The main physiological ROS are Superoxide anion, Hydroxyl radical and Hydrogen peroxide. ROS could also be present in other conditions or environments, such as in cigarette smoke, ionizing radiation and ozone exposure (Birben et al., 2012).

Table 2.1: Summary of several ROS (Birben et al., 2012)

Oxidant	Formula	Reaction Equation
Superoxide anion	O2-●	NADPH + 2O2 ↔ NADP+ + 2O2-● + H+ 2O2-● + H+ → O2 + H2O2
Hydrogen peroxide	H2O2	Hypoxanthine + H2O + O2 ⇋ xanthine + H2O2 Xanthine + H2O + O2 ⇋ uric acid + H2O2

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Hydroxyl radical	$\bullet OH$	$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + \bullet OH$
Hypochlorous acid	$HOCl$	$H_2O_2 + Cl^- \rightarrow HOCl + H_2O$
Peroxyl radicals	$ROO\bullet$	$R\bullet + O_2 \rightarrow ROO\bullet$
Hydroperoxyl radical	$HOO\bullet$	$O_2^- + H_2O \leftrightharpoons HOO\bullet + OH^-$

2.3.1 Antioxidants

Antioxidant functions as the scavenger of the ROS present in the body and also serves to suppress and inhibit the damage done by ROS in cells. Two types of the antioxidants are enzymatic antioxidants and non-enzymatic antioxidants.

Table 2.2: Summary of enzymatic antioxidants (Birben et al., 2012)

Name of Scavenger	Acronym	Catalyzed Reaction
Superoxide dismutase	SOD	$M^{(n+1)+}\text{-SOD} + O_2^- \rightarrow M^{n+}\text{-SOD} + O_2$ $M^{n+}\text{-SOD} + O_2^- + 2H^+ \rightarrow M^{(n+1)+}\text{-SOD} + H_2O_2$
Catalase	CAT	$2 H_2O_2 \rightarrow O_2 + 2 H_2O$ $H_2O_2 + Fe(III)\text{-}E \rightarrow H_2O + O = Fe(IV)\text{-}E(\bullet +)$ $H_2O_2 + O = Fe(IV)\text{-}E(\bullet +) \rightarrow H_2O + Fe(III)\text{-}E + O_2$
Glutathione peroxidase	GTPx	$2GSH + H_2O_2 \rightarrow GSSG + 2H_2O$ $2GSH + ROOH \rightarrow GSSG + ROH + H_2O$

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Thioredoxin	TRX	Adenosine monophosphate + sulfite + thioredoxin disulfide = 5'-adenylyl sulfate + thioredoxin Adenosine 3',5'-bisphosphate + sulfite + thioredoxin disulfide = 3'-phosphoadenyly + sulfate + thioredoxin
Peroxiredoxin	PRX	2 R'-SH + ROOH = R'-S-S-R' + H2O + ROH
Glutathione transferase	GST	RX + GSH = HX + R-S-GSH

Table 2.3: Summary of non-enzymatic antioxidants (Birben et al., 2012).

Chemical Name of Scavenger	Name of Scavenger	Structure
All trans retinol 2	Vitamin A	
Ascorbic acid	Vitamin C	

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α-Tocopherol	Vitamin E	
β-Carotene		
Glutathione		

2.3.2 Oxidative stress and its effect on the human body

Excessive buildup of ROS molecules or any significant reduction in antioxidant enzymes leads to a disruption in the ROS-antioxidant normal balance and resulting in oxidative stress. There are diverse impacts of oxidative stress towards humans, many of them causing lung diseases such as ischemia/perfusion, acute respiratory distress syndrome and asthma. The ROS mechanism of action towards pathological diseases involves numerous pathways, one of them is by inducing DNA strands breakage and base degradation. Furthermore, lipid peroxidation instigation, escalating proteolysis susceptibility and inducing inflammatory response through activation of several transcription factors are also oxidative stress mechanisms (Birben et al., 2012).

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2.4 Antioxidant assays

Evaluation of antioxidant activity exhibited by any compounds are done through various antioxidant assays available. These assays are mainly categorized into two groups: Hydrogen atom transfer (HAT) reaction-based assays and Electron transfer (ET) reaction-based assays.

HAT-based assays principle lies on the capacity of the antioxidant properties of a compound in quenching free radicals induced by the reagent of the specific assay. This assay measures the ability of the compound to donate hydrogen (H) atoms towards the free radicals, competitively with another H donor from the respective substrate assay. Meanwhile, ET-based assay principle lies on the capacity of the antioxidant properties of a compound in initiating simple redox reactions with reagent's free radicals. Antioxidant compounds reduce the free radicals while simultaneously being oxidized themselves. This reaction converts the colour of the reagent assay, thus absorbance is measured to establish the sample's antioxidant concentrations (Dontha, 2016; Huang et al., 2005).

2.4.1 FRAP assay

Ferric ion Reducing Antioxidant Power (FRAP) assay is grouped as one of the ET-based assays. This assay initially functions to assess human plasma capability as an antioxidant, then further applied for assessment of other molecules (Dontha, 2016). According to Gupta (2015), FRAP assay evaluates antioxidant activity in reducing ferric (III) tripyridyl triazine (Fe^{3+} TPTZ) complex to generate Fe^{2+} TPTZ. Binding of Fe^{2+} produces an intense navy blue colour, hence this could be monitored through absorbance change. The reference solution used for this assay is standard ferrous sulphate solution.

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2.4.2 DPPH assay

DPPH (2, 2-diphenyl-1- picrylhydrazyl) assay is classified as an ET-based assay. Dontha (2016) states that DPPH is widely used in measuring radical scavenger due to its very stable free radical, and presence of its unpaired electrons (DPPH●) produces purple colour at maximum absorption around 517nm. DPPH assay measures the reducing action of antioxidants towards DPPH● as represented by the equation below:

$$\text{(DPPH)} + \text{H-A (Antioxidant) [purple]} \rightarrow \text{DPPH-H} + \text{A [yellow]}$$

As DPPH● paired off with hydrogen due to reaction with antioxidant, the resulting DPPH form simultaneously decolours the purple colour to yellow. Hence, the absorbance value of DPPH declines linearly as the number of antioxidants increases. According to Pisoschi & Negulescu (2012), the standard antioxidant used for this assay is Trolox.

2.4.3 ORAC assay

Oxygen radical absorption capacity (ORAC) assay falls under the category of HAT-based antioxidant assays. Dontha (2016) articulated that this assay incorporates a fluorescent probe and a free radical reagent concurrently into the sample, which will be used to quantify antioxidant capacity in the samples. Decomposition of 2,2′-azobis (2-amidino-propane) dihydrochloride (AAPH) introduces free radicals to the sample, causing fluorescence signals from fluorescein probes to be damaged and consistently declined. However, the presence of antioxidant compounds are able to scavenge these free radicals thus loss of fluorescence signals could be decelerated.

ORAC assay measures the depletion of fluorescein's fluorescence signals over time in the sample. These values are compared against the standard curve generated by Trolox, the positive control of this assay with established ORAC values (Dontha, 2016; Gupta, 2015).

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2.4.4 PAP assay

Phosphomolybdenum antioxidant power (PAP) assay principle lies in the reduction of molybdenum 6+, Mo (VI) into molybdenum 5+, Mo(V) by antioxidant compounds found in the sample. The resulting Mo(V) compound produces green colour, hence changes in the colour could be evaluated through absorbance at a certain wavelength (Batool et al., 2019). According to Ishak et al. (2018), antioxidant concentration of the sample is quantified through comparison with antioxidant concentration of the control, ascorbic acid and its standard curve.

2.4.5 ABTS assay

ABTS: 2, 2-azinobis (3-ethyl benzothiazoline-6-sulfonic acid) assay is an example of the ET-based type of assay. This assay is also called the Trolox equivalence antioxidant capacity (TEAC II assay). ABTS will be first oxidized by treating with sodium/potassium persulphate to produce radical cation, (ABTS•+). ABTS•+ gives out a bluish-green colour at 743nm absorbance. Nonetheless, in presence of an antioxidant compound, ABTS•+ cation will be reduced and decolourized into a colourless form. Changes in the colour and absorbance of ABTS•+ will be recorded and serves as the method of quantifying antioxidant capacity in this assay. Standard antioxidant used for this assay is Trolox (Gupta, 2015; Pisoschi & Negulescu, 2012).

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2.5 Current studies of benzimidazole derivatives for antioxidant activity

Several studies of antioxidant action expressed by diverse benzimidazole analogues have been done throughout the years. Oxidative damage characterised by lipo-oxygenase (LOX) and lipid peroxidation (LPO) was successfully hindered by benzimidazole derivatives in Neochoritis et al. (2011) study. Benzimidazole was incorporated with schiff bases and 3-oxo-pyrimido to synthesize numerous novel analogs through a one-pot synthetic procedure, and all the analogues inhibited LOX and LPO while most of them have higher activity than trolox.

Moreover, Padmavathi et al. (2012) investigated the antioxidant activity of novel heterocycles containing benzimidazole like bis benzoxazolyl, benzothiazolyl and benzimidazolyl pyrazoles. This is done through various assays such as DPPH assay, ABTS assay and Nitric oxide. Among the heterocyclic compounds, bis-benzoxazolyl-isoxazole showed great antioxidant activity comparable to the standard compound, ascorbic acid and proved to have a significant potential as an antioxidant agent. The SAR study of bis-benzoxazolyl-isoxazole is as illustrated below

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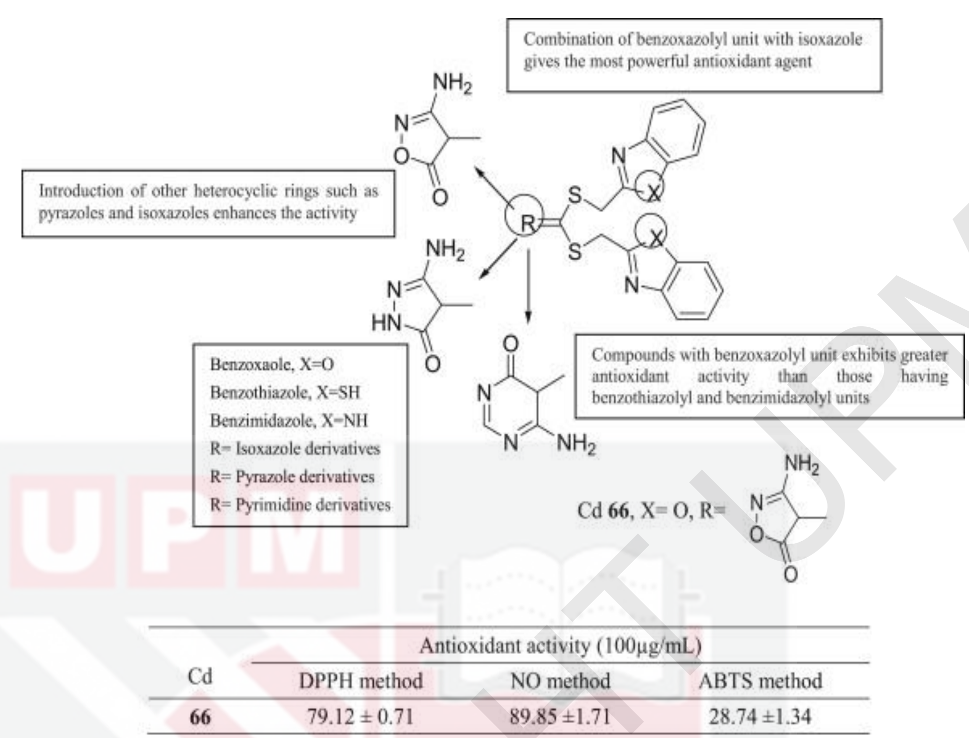

Cd	Antioxidant activity (100μg/mL)		
	DPPH method	NO method	ABTS method
66	79.12 ± 0.71	89.85 ±1.71	28.74 ±1.34

Figure 2.4: SAR study of bis-benzoxazolyl-isoxazole as an antioxidant (Yadav & Ganguly, 2015)

Besides that, antioxidant activity of benzimidazole derivatives could also lead to disease treatments. This is shown by a recent study of Anastassova et al. (2018), utilizing synthetic 1,3-disubstituted benzimidazole-2-thione compounds with hydrazone side chains for potential liver disorder therapy. In vivo testing of this compound involves rat hepatocytes that were induced with tert-butylhydroperoxide (tert-BOOH) as an oxidative stress agent. Result reveals that unsubstituted benzimidazole-2-thiones with methoxyphenyl moieties display cytoprotective effects like quercetin. The novel compounds were also tested for its radical scavenging mechanisms, and majority of them show a positive outcome in scavenging hydroxyl and alkoxyl radicals like melatonin, that ultimately prevents initiation of lipid peroxidation.

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2.6 The two chosen benzimidazole derivatives

Synthesis of these two novel benzimidazole derivatives was done by UiTM research team and was obtained as a commercialized powder form. The nomenclature of the first benzimidazole derivative is (E)-N'-(3,4-dihydroxybenzylidene)-4-(5,6-dimethyl-1h-benzo[d]imidazol-2-yl) benzohydrazine and termed as NN-1-10. The other benzimidazole derivative of interest is (E)-4-(5,6-dimethyl-1h-benzo[d]imidazol-2-yl-N'-(2-hydroxybenzylidine) benzohydrazine and termed as NN-1-18. The structure of both benzimidazole derivatives are displayed as below:

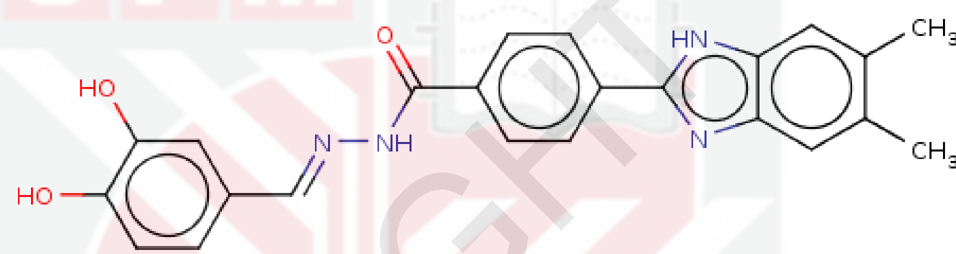

Figure 2.5 Structure of NN-1-10 benzimidazole derivative compound (*BDBM175312 - Binding DB*, n.d.)

Figure 2.6 Structure of NN-1-18 benzimidazole derivative compound (Azahar et al., 2019)

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CHAPTER 3

MATERIALS AND METHODS

3.1 Chemicals & Reagents

300mM Acetate buffer, 40mM Hydrochloric acid, 10mM 2,4,6-tripyridyl-s-triazine (TPTZ), Methanol (LiChrosolv®), 20 mM Iron(III) chloride hexahydrate (FeCl3.6H2O), 20mM Iron (II) sulphate heptahydrate (FeSO4.7H2O), 0.6M Sulphuric acid (J.T. Baker), 28mM Sodium phosphate, 4mM Ammonium molybdate (BAKER LTD), 0.1% Dimethyl sulfoxide (Riendemann Schmidt), Distilled water, Ascorbic acid, Trolox (ALDRICH), Gallic acid, Benzimidazole (ALDRICH), S4115323 504 Imidazole (MERCK), 1,1-diphenyl-2-picryl hydrazyl radical (DPPH) powder.

3.2 Instruments

Waver shaker MW-23 (Medigene), Heating block (DAIHAN Scientific), S00434702 / SAA55792 Analytical balance (Meitler Toledo), S00517203 Class II Biohazard Safety Cabinet (Malaysian Diagnostic Corp.), S00471303 / S1A174705 pH meter (Meitler Toledo), MS ISO/IEC 17025 Microplate reader (Tunable VERSA max).

3.3 Sample preparations

Compound samples NN-1-10 and NN-1-18 were obtained from collaborator, UiTM research team. These samples were in the form of commercialised powder and were stored in the chiller. Prior to antioxidant assays, 100mM stock solutions of both compounds were prepared by weighing the compound powders and diluting them with 0.1% DMSO. The stock solution was further diluted with DMSO to prepare the 400µM working solution of NN-1-10 and NN-1-18 for DPPH and PAP antioxidant assays.

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3.4 Methodology

3.4.1 Ferric reducing antioxidant power (FRAP) assay

The FRAP method was performed according to Ishak et al. (2018) with slight modification. 300mM of acetate buffer with pH3.6 and hydrochloric acid (HCL) was prepared as the first step of FRAP assay. 0.657ml of 40mM HCL stock was taken and adjusted until 200ml volume. Later, preparation of fresh FRAP reagent was done firstly by dissolving 10mM of 2,4,6-tripyridyl-s-triazine (TPTZ) solution in 40 mM HCl. Subsequently, acetate buffer and 20 mM FeCl3.6H2O was added into the dissolved solution at a ratio of 10:1:1(v:v:v), and heated in a 37°C water bath. The control used for this assay was Iron (II), Fe^{2+}. This assay was continued by mixing 20 µl of samples, standard and blank with 180 µl FRAP reagent in a 96-well microplate, then incubated at 37°C for 30 minutes. After 30 minutes, the absorbance of the microplate contents was read at 593nm and raw data were taken.

The graph for the standard calibration curve was plotted using values obtained from the absorbance of control, Iron (II) sulphate heptahydrate (FeSO4.7H2O) with a concentration range of 53.71µM - 1718.75µM. FRAP antioxidant activity of all samples and controls were shown based on the graph and expressed as mmol Fe2+/g.

3.4.2 1,1-diphenyl-2-picryl hydrazyl radical (DPPH) assay

The DPPH method was performed according to Ishak et al. (2018) with slight modification. DPPH powder was dissolved in methanol to prepare the DPPH reagent prior to this assay. Controls used for this assay were ascorbic acid, gallic acid, benzimidazole and imidazole while the blank used was dimethyl sulfoxide (DMSO). Afterwards, 100µl of all samples, controls and blank were pipetted into each 96 wells of the microplate. 2X serial dilution for samples and standards were done with initial concentration of 400µM, by adding DMSO until they reach 6.25µM at final volume of 50µl in each well. Then, 195µl of DPPH reagent was pipetted into each well, and finally the microplate was

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covered and left shaken for 30 minutes. 515nm of wavelength was used to read the absorbance of microplate contents, and raw data were taken.

DPPH antioxidant activity for each samples and controls were based on the percentage (%) of the DPPH free radical scavenging activity calculated using the formula:

DPPH scavenging activity (%):
(A blank - A sample or standard) / A control x 100

Where: A blank = absorbance of DPPH radicals without sample or control. A sample or standard = absorbance of DPPH radicals with sample or standard. Results of samples and controls were shown by calculating their respective EC50, which is the concentration value required for each test sample to inhibit 50% of the DPPH radical. This was expressed as µg/ml.

3.4.3 Phosphomolybdenum antioxidative power (PAP) assay

The PAP method was performed according to Ishak et al. (2018) with slight modification. PAP reagent was prepared as the first step of PAP assay. This was done by mixing 0.6M of sulphuric acid solution with 28mM sodium phosphate powder and 4mM ammonium molybdate powder, then added with distilled water until 5ml. Standards used for this assay were ascorbic acid, trolox, benzimidazole and imidazole while the blank used was dimethyl sulfoxide (DMSO) . After that, 50µl of all samples and standards was added with 500µl PAP reagent into respective microcentrifuge tubes. These microcentrifuge tubes were incubated in a heating block at 95°C for 90 minutes.

Microcentrifuge tubes were left cooled for a few minutes, then proceeded with pipetting 100µl of its content into a 96-well microplate. Blank was also pipetted into the wells. 2X serial dilution for samples and standards were done with initial concentration of 400µM, by adding DMSO until they reach 6.25µM at final volume of 50µl in each well.

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Immediately, the absorbance of the microplate contents was read at 695nm and raw data were taken.

The graph for the standard calibration curve was plotted using values obtained from the absorbance of control, ascorbic acid with a concentration range of 6.25µM - 400µM. PAP antioxidant activity of all samples and controls were calculated using the formula:

$$\text{Antioxidant capacity (AC)} = [(\text{AC per mL sample} \times \text{dilution factor} \times \text{total sample volume used}) / \text{sample weight}]$$

Where AC per mL sample = Absorbance value inserted into the linear equation derived from ascorbic acid standard calibration curve. Results of samples and controls were expressed as mg ascorbic acid equivalent (AAE) / mg.

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CHAPTER 4

RESULTS

4.1 Comparison of antioxidant activity of NN-1-10 and NN-1-18 using Ferric ion Reducing Power (FRAP) assay.

Antioxidant capacity using FRAP assay for NN-1-10, NN-1-18, Benzimidazole, Imidazole and Gallic acid and Trolox were determined through the standard calibration curve of control, Iron (II) sulphate heptahydrate. Results of these compounds were compared through a bar graph and expressed as mmol Fe2+/g. Figure 4.1 showed that NN-1-10 exhibited a higher potential to reduce Fe3+ in FRAP antioxidant assay than NN-1-18 and its parent compound: Benzimidazole & Imidazole. Though the FRAP antioxidant capacity of NN-1-10 is lower than Trolox and Ascorbic acid, the values are still comparable to these controls.

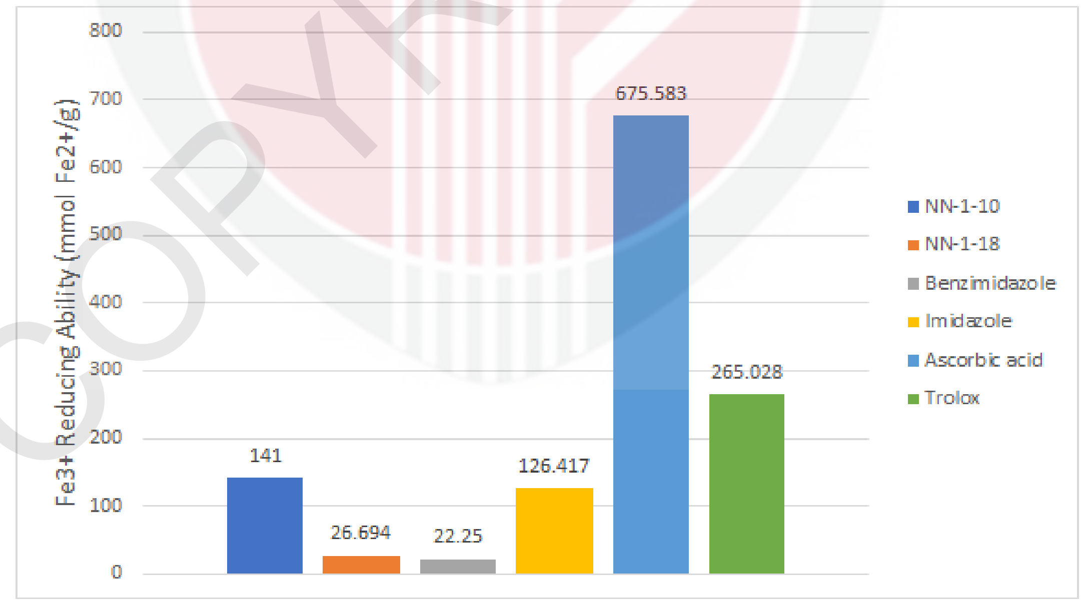

Figure 4.1: Fe3+ reducing capacity of samples and controls via FRAP assay. Samples and controls of 100µM concentration were added with FRAP reagent and incubated for 30 minutes at 37°C. Data was compared to Fe(II) and expressed as mmol Fe2+/g.

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4.2 Comparison of antioxidant activity of NN-1-10 and NN-1-18 using 1,1-diphenyl-2-picryl hydrazyl radical (DPPH) assay.

In order to determine the half maximal effective concentration (EC50) of NN-1-10, NN-1-18, Benzimidazole, Imidazole, Gallic acid and Ascorbic acid, the DPPH scavenging activity of each of the samples and controls were calculated based on the raw data and formula, and displayed in graph form. Figure 4.2 showed that NN-1-10 exhibited a higher DPPH inhibition capacity than NN-1-18, Benzimidazole and Imidazole. NN-1-10 also shows an approximate value of DPPH inhibition capacity against Gallic acid and Ascorbic acid controls, albeit lower.

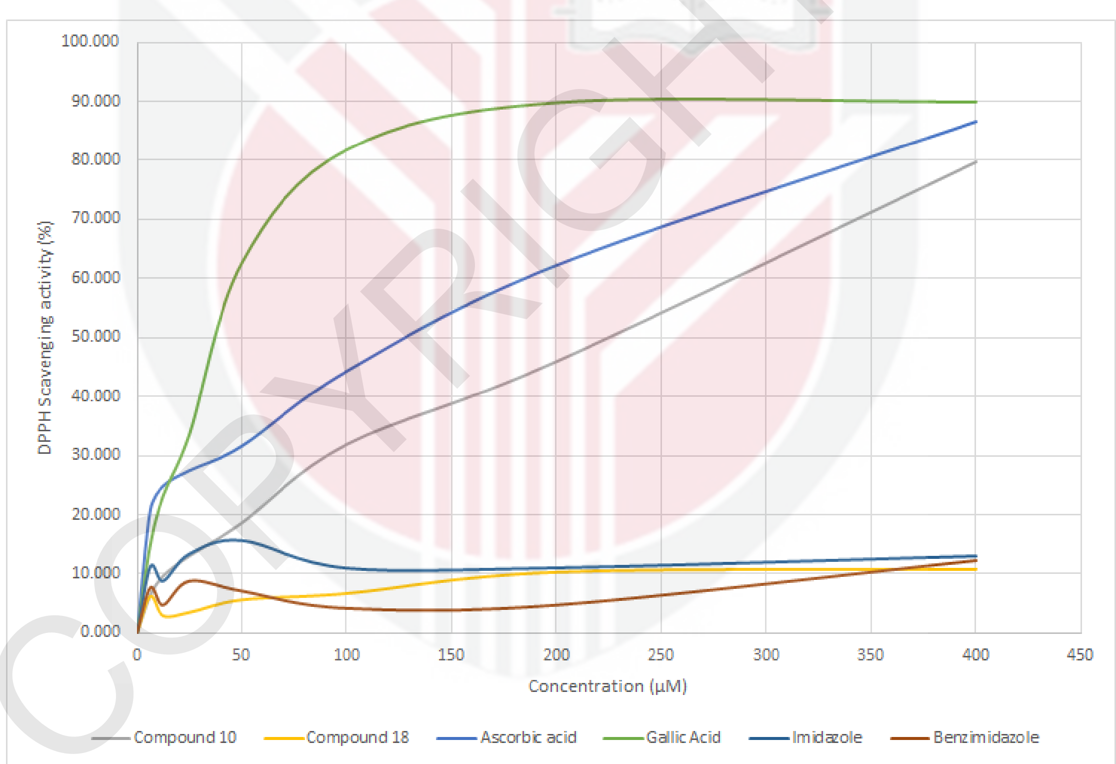

Figure 4.2: DPPH Inhibition capacity of samples and controls via DPPH assay. Samples and controls of different concentrations (6.25µM to 400µM) were added with DPPH reagent and incubated for 30 minutes at room temperature. Data was expressed as a mean of 3 DPPH inhibition capacity readings in percentage (%).

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After obtaining the percentage of DPPH inhibition capacity for each compound tested, the values of EC50 were calculated. Table 4.1 showed that the EC50 of NN-1-10 is lower than NN-1-18, Benzimidazole and Imidazole, however, is higher than Gallic acid and Trolox. The EC50 of NN-1-18, Benzimidazole & Imidazole are stated as not applicable as the highest concentration of these compounds did not exceed the value of 50% DPPH inhibition capacity obtained from EC50 of DPPH assay control, Gallic acid.

Table 4.1: EC50 of DPPH inhibition capacity of samples and controls.
EC50 value was derived from the linear equation of DPPH inhibition activity of every test compound and expressed as µg/ml.

	EC50 of DPPH inhibition capacity (µg/ml)
NN-1-10	217.76
NN-1-18	Not applicable
Benzimidazole	Not applicable
Imidazole	Not applicable
Gallic acid	38.28
Ascorbic acid	151.68

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4.3 Comparison of antioxidant activity of NN-1-10 and NN-1-18 using Phospholybdenum Antioxidant Power (PAP) assay.

Antioxidant capacity using PAP assay for NN-1-10, NN-1-18, Benzimidazole, Imidazole and Gallic acid compounds were determined through the standard calibration curve of control, Ascorbic acid. Results of these compounds were calculated through formula and expressed as Ascorbic acid equivalent (mg AAE/mg). Figure 4.3 showed that NN-1-10 exhibited the highest total antioxidant capacity, at a whopping 323 843 mg AAE/mg. It is also observed that all compounds exhibited an abnormal high value of total PAP antioxidant capacity (exceeding 100,000 mg AAE/mg) which indicates error and invalid readings.

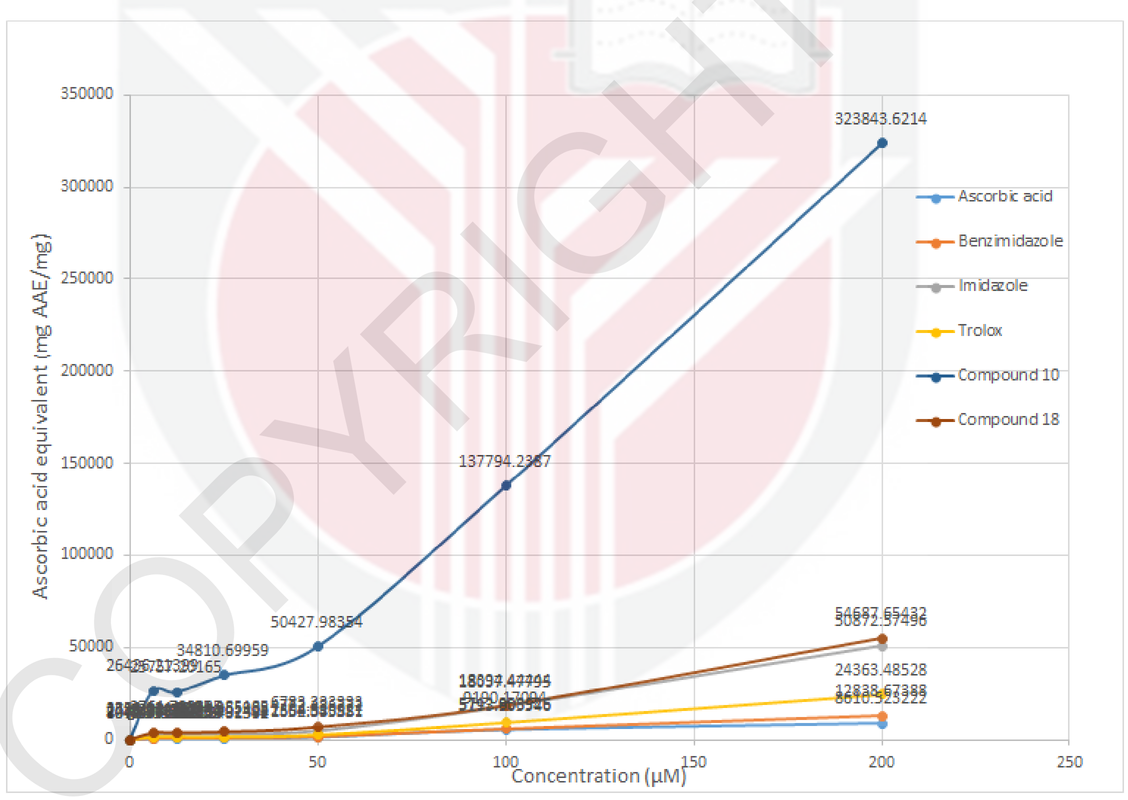

Figure 4.3: Total antioxidant capacity of samples and controls via PAP assay. Samples and controls of different concentrations (6.25µM to 400µM) were added with PAP reagent and heated for 90 minutes at 95°C. Data was expressed as a mean of 3 antioxidant values compared against ascorbic acid (mg AAE/mg).

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CHAPTER 5

DISCUSSION

Apak et al. (2018) explains one of the mechanisms exerted by antioxidants in order to prevent harmful effects of free radicals, which is by initiation of redox reactions. Redox reaction is defined as the paired action of reduction and oxidation in two components, as one of them becomes a reducing agent and is oxidized, while the other becomes the counter oxidizing agent and will be reduced. In the context of human antioxidants and free radicals, antioxidants serve as a reducing agent by donating their electrons or H atoms to reduce free radicals, hence breaking the chain of free radicals. All 3 assays in this experiment identify the reducing power of test compounds in order to quantify their antioxidant activity.

FRAP assays utilize ferric (III) ion, (Fe^{3+}) complex to mimic the iron (Fe) oxidants present in human physiological conditions. Iron is an active transition metal that could potentially be a catalyst for free radical formation, such as hydroxyl radical, and lead to lipid peroxidation (Apak et al., 2018). Hence, FRAP assay measures the reduced form of iron. PAP assays apply the same principle and indicative measures as FRAP assay. However, the difference is that PAP assay employs molybdenum, which was proven to stimulate oxidative stress and production of reactive oxygen species at high concentration (Terpilowska & Siwicki, 2019). DPPH assay also evaluates the reducing power of compounds, although this assay quantifies the declining oxidized form of DPPH, which is a stable free radical. Thus, reduction power based on these 3 assays by either NN-1-10 or NN-1-18 could lead to discovery of effective synthetic antioxidants.

Through results of both FRAP and DPPH assay shown in Figure 4.1 & Figure 4.2, NN-1-10 benzimidazole derivative exhibited higher antioxidant capacity and reducing power than NN-1-18 and parent compounds: Benzimidazole & Imidazole. This can be explained through the difference in compound structure as displayed below:

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Figure 2.5 Structure of NN-1-10 benzimidazole derivative compound (*BDBM175312 - Binding DB*, n.d.)

Figure 2.6 Structure of NN-1-18 benzimidazole derivative compound (Azahar et al., 2019)

The difference in both structures is the number of hydroxyl groups present in the respective novel benzimidazole compounds. According to Baldisserotto et al. (2018), the redox activity of a compound increases as the number of hydroxyl groups present in the compound increases. This statement is further supported by Neochoritis et al. (2011), by explaining the significance of hydroxyl groups in antioxidant activity mechanisms. Hydroxyl group is a functional group that is easily oxidized as they have the presence of an H atom that will be donated to stabilize the DPPH radicals. Therefore, the obtained findings are in accordance with both studies as NN-1-10 has a higher number of hydroxyl groups compared to other compounds.

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Furthermore, the difference in the position of the substituted hydroxyl groups could also be taken into consideration. Hydroxyl groups in NN-1-10 were substituted at the meta and para position of the ring while NN-1-18 has their hydroxyl group substituted at the ortho position of the ring. Bendary et al. (2013) described that activity is higher at the ortho position rather than meta and para due to its ability to form intramolecular hydrogen bonding (iHB). However, the findings were not consistent with this study as NN-1-18 exhibited a lower reducing power and antioxidant activity than NN-1-10, even though its hydroxyl group is positioned at the ortho position while the other compound has their hydroxyl group at meta and para. Hence, it can be concluded that the number of hydroxyl groups influence the activity more than its position of substitution.

EC50 of all compounds run through DPPH assay in Table 4.1 shows that NN-1-10 requires a lower concentration to inhibit 50% of DPPH radical among other test samples. The findings were consistent with a study done by Taha et al. (2019) where they stated that a compound with higher hydroxyl group will have higher potential to scavenge DPPH radical. However, NN-1-10 is less effective than standard compounds as they have a higher EC50. Regarding PAP assay, Figure 4.2 proves that the readings for test samples and controls were abnormally high. This differs drastically with a similar study by Ishak et al. (2018), where PAP results of the test extracts ranges only between 30 - 200mg AAE/g. Apart from that, an abnormality of the microplate containing test compounds was observed throughout this assay, as shown in the figure below

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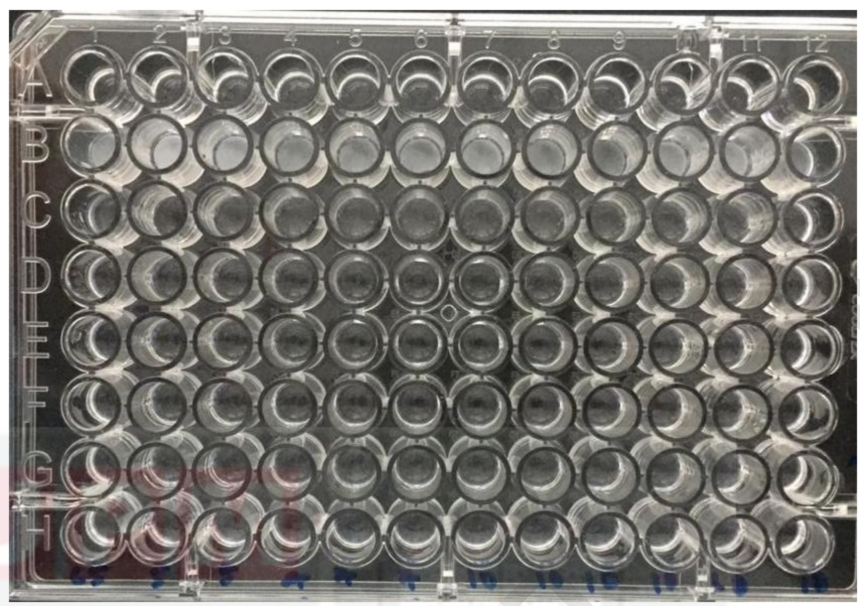

Figure 5.1 Condition of microplate containing sample and controls after PAP assay

Through Figure 5.1, observation of all test samples and controls shows that they remained colourless throughout this test, instead of turning green according to PAP assay principles. This condition is peculiar as all compounds were dissolved in an acid PAP reagent and had the right environment to change their colour as most compounds possess antioxidant properties. Thus, this further indicates that invalid readings were obtained for this assay. There might be some conditions causing these false readings in PAP assay procedures. This includes a much earlier preparation of PAP reagent and working solution of samples before proceeding which causes samples to be oxidized first. Moreover, samples might have been exposed and affected to exceeding UV rays during preparation as they are light-sensitive and very delicate. Miscalculations of sample and reagents concentrations or volumes needed could also account for the invalid results.

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CHAPTER 6

CONCLUSION & FUTURE RECOMMENDATION

6.1 Conclusion

Benzimidazole derivative of NN-1-10 displayed a higher antioxidant capacity and reducing power in both FRAP and DPPH assay compared to NN-1-18. This is due to the higher number of hydroxyl groups in NN-1-10 which increases the compound's potential in performing antioxidant mechanisms against induced radicals.

6.2 Future recommendation

The third objective of this study was not achieved due to invalid readings of test samples and controls using PAP assays. Hence, it is highly suggested to prepare the sample solutions and reagents needed for this assay briefly prior analysing the test compounds and only use them for about 2 days after preparation in order to preserve their chemical composition. Furthermore, steps of pipetting samples, controls and reagents into microwell plates should be done in a dark room with minimum light. Moreover, calculations for every preparation should be checked thoroughly and confirmed as accurate before starting any antioxidant assay.

Apart from that, this study only produces one independent value of results for every antioxidant assay, thus statistical analysis could not be done and the difference between antioxidant capacity could not be proven significant. Therefore, the experiment should be repeated at least thrice to obtain 3 independent values and for statistical procedures. One of the suggestions for statistical analysis is by using ANOVA to compare the mean between antioxidant activity of NN-1-10, NN-1-18 and controls. This is subsequently proceeded with a post-hoc test, to determine the specific significant readings.

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APPENDICES

A. Raw data of DPPH assay

	NN-1-10			MEAN	STDEV	CV%
400	0.146	0.141	0.138	0.141666667	0.004041452	2.852789565
200	0.373	0.442	0.32	0.378333333	0.061174613	16.16950136
100	0.481	0.584	0.366	0.477	0.109055032	22.86269014
50	0.537	0.63	0.543	0.57	0.052048055	9.13123767
25	0.669	0.823	0.609	0.700333333	0.110387197	15.76209386
12.5	0.640	0.626	0.633	0.633	0.007	1.105845182
6.25	0.660	0.672	0.635	0.655666667	0.018876794	2.879022905
BLANK	0.603	0.698	0.667	0.656	0.048445846	7.385037508

	NN-1-18			MEAN	STDEV	CV%
400	0.649	0.624	0.625	0.632666667	0.014153916	2.237183746
200	0.636	0.62	0.614	0.623333333	0.011372481	1.824462258
100	0.649	0.676	0.635	0.653333333	0.020840665	3.189897762
50	0.676	0.625	0.682	0.661	0.03132092	4.738414452
25	0.653	0.694	0.681	0.676	0.020952327	3.099456633
12.5	0.689	0.672	0.679	0.68	0.008544004	1.256471139
6.25	0.646	0.688	0.636	0.656666667	0.027592269	4.201868444
BLANK	0.630	0.701	0.650	0.660333333	0.036610563	5.544254892

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ASCORBIC ACID				MEAN	STDEV	CV%
400	0.118	0.077	0.086	0.093666667	0.021548395	23.00540407
200	0.246	0.236	0.311	0.264333333	0.040722639	15.40579032
100	0.504	0.418	0.362	0.428	0.071526219	16.7117334
50	0.557	0.355	0.525	0.479	0.108572556	22.66650446
25	0.522	0.457	0.545	0.508	0.045639895	8.984231266
12.5	0.544	0.591	0.509	0.548	0.041146081	7.508408982
6.25	0.539	0.523	0.571	0.544333333	0.024440404	4.489970062
BLANK	0.543	0.656	0.590	0.596333333	0.0567656	9.519105631

GALLIC ACID				MEAN	STDEV	CV%
400	0.073	0.071	0.067	0.070333333	0.00305505	4.343673645
200	0.068	0.074	0.072	0.071333333	0.00305505	4.282781023
100	0.208	0.085	0.089	0.127333333	0.069888006	54.88586831
50	0.263	0.266	0.256	0.261666667	0.005131601	1.961121569
25	0.453	0.476	0.466	0.465	0.011532563	2.480120988
12.5	0.501	0.551	0.563	0.538333333	0.032883633	6.108414838
6.25	0.593	0.564	0.631	0.596	0.033600595	5.637683764
BLANK	0.639	0.692	0.694	0.675	0.031192948	4.62117747

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	BENZIMIDAZOLE			MEAN	STDEV	CV%
400	0.571	0.633	0.638	0.614	0.037322915	6.078650686
200	0.633	0.693	0.675	0.667	0.030789609	4.616133229
100	0.655	0.674	0.684	0.671	0.01473092	2.195368087
50	0.663	0.611	0.677	0.650333333	0.03477547	5.34733013
25	0.591	0.666	0.658	0.638333333	0.041186567	6.452203769
12.5	0.667	0.659	0.675	0.667	0.008	1.1994003
6.25	0.676	0.697	0.566	0.646333333	0.070358605	10.88580793
BLANK	0.643	0.664	0.700	0.669	0.028827071	4.308979165

	IMIDAZOLE			MEAN	STDEV	CV%
400	0.63	0.586	0.61	0.608666667	0.022030282	3.619432999
200	0.642	0.599	0.627	0.622666667	0.021825062	3.505095618
100	0.621	0.632	0.616	0.623	0.008185353	1.313860798
50	0.581	0.599	0.724	0.634666667	0.07788667	12.27205932
25	0.706	0.783	0.606	0.698333333	0.088748709	12.70864567
12.5	0.685	0.625	0.605	0.638333333	0.04163332	6.522191121
6.25	0.657	0.599	0.608	0.621333333	0.031214313	5.023762815
BLANK	0.663	0.677	0.647	0.662333333	0.015011107	2.266397634

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B. Raw data of PAP assay

	NN-1-10			MEAN	STDEV	CV%
400	0.038	0.038	0.045	0.040333	0.004041452	10.02013
200	0.578	0.537	0.505	0.54	0.036592349	6.776361
100	0.328	0.321	0.337	0.328667	0.008020806	2.440408
50	0.096	0.096	0.083	0.091667	0.007505553	8.187877
25	0.059	0.06	0.053	0.057333	0.003785939	6.603382
12.5	0.056	0.054	0.051	0.053667	0.002516611	4.689338
6.25	0.052	0.053	0.048	0.051	0.002645751	5.187748
BLANK	0.043	0.04	0.042	0.041667	0.001527525	3.666061

	NN-1-18			MEAN	STDEV	CV%
400	0.04	0.039	0.038	0.039	0.001	2.564103
200	0.529	0.711	0.393	0.544333	0.159554	29.31173
100	0.17	0.209	0.185	0.188	0.019672	10.464
50	0.065	0.082	0.075	0.074	0.008544	11.54595
25	0.047	0.051	0.046	0.048	0.002646	5.511982
12.5	0.045	0.044	0.044	0.044333	0.000577	1.302294
6.25	0.043	0.043	0.041	0.042333	0.001155	2.727639
BLANK	0.042	0.041	0.041	0.041333	0.000577	1.396815

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	ASCORBIC ACID			MEAN	STDEV	CV%
400	0.038	0.038	0.045	0.040333	0.004041452	10.02013
200	0.578	0.537	0.505	0.54	0.036592349	6.776361
100	0.328	0.321	0.337	0.328667	0.008020806	2.440408
50	0.096	0.096	0.083	0.091667	0.007505553	8.187877
25	0.059	0.06	0.053	0.057333	0.003785939	6.603382
12.5	0.056	0.054	0.051	0.053667	0.002516611	4.689338
6.25	0.052	0.053	0.048	0.051	0.002645751	5.187748
BLANK	0.043	0.04	0.042	0.041667	0.001527525	3.666061

	TROLOX			MEAN	STDEV	CV%
400	0.043	0.046	0.039	0.042667	0.003512	8.230979
200	0.632	0.606	0.65	0.629333	0.022121	3.51497
100	0.23	0.245	0.251	0.242	0.010817	4.469692
50	0.072	0.072	0.07	0.071333	0.001155	1.618739
25	0.047	0.047	0.042	0.045333	0.002887	6.367834
12.5	0.044	0.044	0.044	0.044	0	0
6.25	0.042	0.048	0.043	0.044333	0.003215	7.250865
BLANK	0.042	0.041	0.042	0.041667	0.000577	1.385641

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	BENZIMIDAZOLE			MEAN	STDEV	CV%
400	0.062	0.044	0.041	0.049	0.011358	23.17922
200	0.658	0.692	0.77	0.706667	0.057422	8.125813
100	0.24	0.253	0.273	0.255333	0.016623	6.510422
50	0.068	0.076	0.075	0.073	0.004359	5.971094
25	0.052	0.049	0.048	0.049667	0.002082	4.191274
12.5	0.048	0.048	0.045	0.047	0.001732	3.685214
6.25	0.045	0.046	0.046	0.045667	0.000577	1.264271
BLANK	0.045	0.043	0.044	0.044	0.001	2.272727

	IMIDAZOLE			MEAN	STDEV	CV%
400	0.046	0.038	0.045	0.043	0.004358899	10.13697
200	0.594	0.584	0.527	0.568333	0.036143234	6.359513
100	0.244	0.275	0.252	0.257	0.016093477	6.262053
50	0.081	0.076	0.082	0.079667	0.00321455	4.035
25	0.049	0.049	0.049	0.049	0	0
12.5	0.048	0.042	0.045	0.045	0.003	6.666667
6.25	0.042	0.043	0.043	0.042667	0.00057735	1.353165
BLANK	0.043	0.044	0.041	0.042667	0.001527525	3.580137

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BIOGRAPHY

Nur Hidayah Helmi binti Muhammad Fadil Helmi was born on 20th November 1997 in a loving family. After attending lower secondary school, she was interested in science as her teacher effectively intrigued her in viewing the world through scientific perspective. Hence, this leads to her decision in taking pure science subjects for Sijil Pelajaran Malaysia (SPM). Alhamdulillah, learning science was both a joy and a blessing, that she went through her SPM smoothly and was accepted into Universiti Putra Malaysia for Foundation in Agricultural Science, a university which she had an eye for a long time. Through her foundation days, she discovered that Human biology was her muse, thus she decided to pursue her studies in Bachelor of Science (Biomedical Sciences) at the same university. Four years undergoing this degree had shaped her well, and finally she had come to the end of the path with the Final Year Project as her final task. With the help of her supervisor, Assoc. Prof. Dr. Abdah Md Akim, her FYP partner, Nur Syuhada Darol Napis, postgraduate students, laboratory staffs, lecturers, family and friends, this project was successfully finished. Thus, this thesis serves as a testament of her incredible degree journey and a constant inspiration for the beginning of her future life.

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