MICROFLUIDIC LAB-ON-DISC APPROACH OF 2,2-DIPHENYL-1-PICRYLHYDRAZYL ANTIOXIDANT ASSAY

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MICROFLUIDIC LAB-ON-DISC APPROACH OF 2,2-DIPHENYL-1-PICRYLHYDRAZYL ANTIOXIDANT ASSAY

ABSTRACT

This dissertation presents the integration of lab-on-disc (LoD) on centrifugal microfluidic platform for the 2,2-diphenyl-1-picrylhydrazyl (DPPH) antioxidant assay. In order to develop the centrifugal microfluidic device, a microfluidic compact disc (CD) was designed and fabricated where the different parallel concentrations, pipetting and loading in the conventional DPPH antioxidant assay procedures was integrated into a single microfluidic CD. The study used two antioxidant standards (ascorbic acid and quercetin) and three local plant extracts (*Areca catechu*, *Polygonum minus* and *Syzygium polyanthum*) to test the developed antioxidant platform. The results showed that, there were a significant difference of $p \leq 0.05$ between the conventional and LoD method at each concentration among all samples (standard antioxidant and the local plants). The higher the concentration of the extracts, the faster the reaction time for the LoD method to reach the same antioxidant activity level of conventional method. The developed microfluidic CD was able to decrease the conventional method incubation time (30 minutes) with four different parallel concentrations running simultaneously. For ascorbic acid, *P. Minus* and *S. polyanthum* it took only 5 minutes ($p \leq 0.01$) to reach the antioxidant activity level of conventional method, meanwhile for quercetin and *A. catechu* it takes10 minutes ($p \leq 0.01$). The developed centrifugal microfluidic CD have offered a rapid and automated platform for DPPH antioxidant assay, it simplified the meticulous and tedious procedures of the conventional DPPH antioxidant assay which can reduced the need of time-consuming and repetitive steps in the manual laboratory operations.

Keywords: lab-on-disc (LoD); centrifugal microfluidic CD; plant antioxidant activity; antioxidants; DPPH

PENDEKATAN MAKMAL-DIDALAM-CAKERA PADAT BAGI UJIAN ANTIOXIDA 2,2-DIPHENYL-1-PICRYLHYDRAZYL

ABSTRAK

Disertasi ini membentangkan sistem integrasi makmal-dalam cakera padat (ataupun dikenali sebagai lab-on-disc (LoD)) diatas platform mikrobendalir emparan bagi ujian antioksidan 2,2-diphenyl-1-picrylhydrazyl (DPPH). Demi membangunkan peranti mikrobendalir emparan ini, cakera padat mikrobendalir telah direka bentuk dan difabrikasi di mana ia mempunyai ciri-ciri yang mampu menjalankan ujian berkepekatan yang berbeza dan selari, menjalankan proses pempipetan dan memuat turun yang terdapat didalam prosedur ujian konvensional antioksidan DPPH boleh diintegrasikan ke dalam satu cakera padat mikrobendalir. Kajian ini telah dijalankan menggunakan dua standard antioksidan (asid askorbik dan quercetin) dan tiga ekstrak tumbuhan tempatan (*Areca catechu*, *Polygonum minus* dan *Syzygium polyanthum*) untuk menguji platform antioksidan yang dibina. Keputusan menunjukkan bahawa terdapat perbezaan yang signifikan antara $p \leq 0.05$ antara kaedah konvensional dan LoD pada setiap kepekatan di antara semua sampel (antioxidant standard dan tumbuhan tempatan). Semakin tinggi kepekatan ekstrak, lebih cepat masa tindak balas untuk kaedah LoD bagi mencapai tahap aktiviti antioksida yang sama dengan kaedah konvensional. CD mikrofluidik yang dibina dapat mengurangkan masa inkubasi kaedah konvensional (30 minit) dengan empat kepekatan selari dan berlainan yang dijalankan secara serentak. Untuk sampel asid askorbik, *P. Minus* dan *S. polyanthum* ianya hanya mengambil masa 5 minit ($p \leq 0.01$) untuk mencapai tahap aktiviti antioksidan bersamaan dengan kaedah konvensional, sedangkan untuk sampel quercetin dan *A. catechu* hanya10 minit ($p \leq 0.01$) diperlukan. Cakera padat mikrobendalir centrifugal yang dibangunkan dapat menawarkan platform yang pantas dan automatik untuk ujian antioksidan DPPH, ia juga mempermudahkan

prosedur ujian antiokidan DPPH konvensional yang memerlukan kelititian yang tinggi dan juga dapat mengurangkan keperluan masa dan langkah berulang-ulang di dalam operasi manual makmal.

Kata kunci: Makmal didalam cakera padat; cakera bendalir emparan; aktiviti antioksida tumbuhan; antioxida; DPPH

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LIST OF ABBREVIATIONS

µTAS	:	Micro Total Analysis System
A. catechu	:	*Areca catechu*
CD	:	Compact Disk
CNC	:	Computer Numerical Control
DNA	:	Deoxyribonucleic Acid
DPPH	:	2,2-diphenyl-1-picrylhydrazyl
LoC	:	Lab-on-Chip
LoD	:	Lab-on-Compact disk
P. minus	:	*Polygonum minus*
PCR	:	Polymerase Chain Reaction
PMMA	:	Poly Methyl Methacrylate
PSA	:	Pressure Sensitive Adhesive
S. polyanthum	:	*Syzygium polyanthum*
UV	:	Ultraviolet

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CHAPTER 1: INTRODUCTION

1.1 Overview

Free radicals are atoms with unpaired valence electrons that cause its chemical instability and reactivity. The valence electron of one atom accepts an electron from another molecule, which forms a new free radical. This process repeats itself, creating a free radical cascade. When free radical production becomes excessive in the body, it may cause oxidative stress, a condition whereby the body cannot counter-attack the production of free radicals, leading to cellular damage and cell death (Sinha, Das, Pal, & Sil, 2013). Free radicals have been implicated in many diseases, such as diabetes, hypertension, Parkinson's, Alzheimer's and heart diseases (Dias, Junn, & Mouradian, 2013; Dikalov & Ungvari, 2013; Matsuda & Shimomura, 2013; Vanessa Fiorentino, Prioletta, Zuo, & Folli, 2013; Yan, Wang, & Zhu, 2013). Additionally, free radical production increases as the individual gets older, thereby accelerating the aging process (Ashok & Ali, 1999).

Antioxidants are molecules that can donate electrons to stabilise free radical species. They play many important roles in daily life. For example, antioxidants are hypothesised to prevent the deleterious effects of free radicals on cells (Lee et al., 2014). In the medical field, antioxidants have been used as an alternative disease therapy for diabetes mellitus, reperfusion injury and inflammatory diseases. They also help prevent atherosclerosis and carcinogenesis in the human body (Maxwell, 1995). In the food industry, antioxidants can prevent the deterioration of food constituents and prevent it from spoiling. In addition, antioxidants absorb ultraviolet (UV) radiation, which minimises the risk of skin cancer (Pisoschi & Pop, 2015). There are many potential natural chemical constituents that can be exploited from plants, such as medical plants containing high antioxidant properties, which are scientifically significant in the human body (Li et al., 2013).

There are many methods to determine the antioxidant properties in a sample. Each of the methods work differently based on the chemical mechanism, among which, the DPPH

antioxidant activity test is the most common and easiest method used to determine the antioxidant activity in a sample (Gupta & Sharma, 2006). The DPPH antioxidant assay has been used widely to determine various antioxidant activities in food, beverages and plants. Furthermore, it is also used to evaluate the antioxidant activity of nutrients in the human body (Lee et al., 2014). DPPH is a stable, deep purple free radical powder, which will change to a pale yellow after reacting with the antioxidant. The free radical DPPH will react with the antioxidant, which acts as a hydrogen donor that stabilises DPPH.

Despite the simplicity of the DPPH antioxidant assay, DPPH itself is easily decomposed over time and is highly sensitive to changes in temperature, pH and light exposure (Pyrzynska & Pękal, 2013; J. Xie & K. M. Schaich, 2014). The DPPH solutions need to be diluted in organic solvent and prepared fresh before running the antioxidant activity test. According to Deng *et al* (Deng, Cheng, & Yang, 2011), DPPH solution needs to be used within two hours of preparation to prevent decomposition of the DPPH solution. It is also reported that the DPPH absorbance decreases within 90 minutes with changes in temperature. The absorbance of DPPH solution will also decrease when exposed to light within two hours, while in the dark, there are no significant changes in DPPH absorbance.

1.2 Problem statement

Many fundamental bio-assay methods are carried out manually. The experimental steps are often time-consuming and require expert skills. The amalgamation of human-prone error, especially in the pre-analytical phase, may also cause incorrect interpretations or false positive results in the analytical and post-analytical phases (Rana, 2012). One of the most common pre-analytical errors is caused by pipetting error. The pipetting steps are the most common repetitive tasks for laboratorians; however, this step is often taken for granted. The pipetting steps, especially the micro scale volumes, are very crucial and accuracy must be ensured to prevent erroneous results. Additionally,

organic solvents evaporate easily and may contribute to volume loss. Errors also happen due to repetitive steps and duplications when laboratorians are handling large amounts of samples. Hence, automation and miniaturisation technology is crucial to increase accuracy, decrease the test time and able to work in a minimal sample collection.

LoD which are also known as centrifugal microfluidic dics, are a part of the microfluidic device in the form of a disc with a spinning motor. LoD is a part of the Micro Total Analysis System (μTAS), which offers miniaturisation and automation of most of the chemical and biological analysis systems. LoD offers many advantages, such as simplicity of the assay procedure, fast results and cost efficiency. LoD has automated many bioassays, such as Enzyme-linked Immunosorbent Assay (ELISA) and the loop-mediated isothermal amplification (LAMP) assay (Hosseini et al., 2016; A. A. Sayad et al., 2016). Compared to lab-on-chips (LoCs) that require syringe pumps, LoD runs in a closed system and has an "all-in-one disc" assay procedure. LoD decreases the amount of reagent, sample usage and total processing time during the experiments. Subsequently, it also offers parallel or sequential loading of solutions. LoD skips multiple sample preparations and pipetting steps, which reduces human-prone handling error.

Jungwoo *et al*. (Lee et al., 2014) have demonstrated an LoC platform to evaluate the liver metabolism of antioxidants in food. He is focusing on mimicking the human liver metabolism and the detection of subsequent metabolised antioxidant food components. The design consists of a two microfluidic compartment; the first part contain human liver enzyme that mimic liver metabolism and the second part is DPPH detection of antioxidant activity of food components. In his design, the liver enzyme fractions were immobilised and the reaction with the DPPH solution were tested. Meanwhile, Xuhua *et al*. have used hydrogen peroxide techniques on a chip and to screen the antioxidant capacity by using herbal extracts (Wang et al., 2009). His focused is more on the fabrication of the chip by

using the thin-film organic photodiodes and the chemiluminescence detection of the antioxidant in the herbal extracts.

In this study, we present an approach of LoD method for antioxidant plant activities. As the conventional methods involved tedious procedures and dark environment to run the test. We offer automation and sample miniaturisation of the DPPH antioxidant activity test with parallel sequential sample loading and mixing. Our proposed LoD method has been tested on ascorbic acid, quercetin, *Areca catechu, Polygonus minus* and *Syzygium polyanthum* plant extract. Furthermore, with the use of black PMMA, our method protect the sample from the light and can be used in the normal laboratory conditioned. The incubation time has been compared between the proposed and conventional methods for validation.

1.3 Objective

The objective of this dissertation is to develop a microfluidic CD for the DPPH antioxidant assay. To achieve the objective the following approaches, need to be fulfil:

1. To identify the antioxidant activities of selected plant and standards antioxidant by using conventional DPPH antioxidant assay method.
2. To determine the optimised volume of reagent and sample for the development of the DPPH microfluidic CD.
3. To design, fabricate and test the DDPH microfluidic CD from the conventional methods.

1.4 Scope of Research

The research focuses on the development of the centrifugal microfluidic CD platform for antioxidant activity by integrating the conventional DPPH antioxidant assay into the microfluidic CD. The microfluidic CD is designed by using a computer aided software AutoCAD and being optimised by trial and error to obtain the best design for the

microfluidic CD. Henceforth, this dissertation will be fully covered on the sequential parallel mixing and loading of the sample and reagents on the design microfluidic CD. The results of the design of microfluidic CD is compared by 5 minutes time interval up to 30 minutes conventional DPPH antioxidant assay standard time.

1.5 Dissertation Outline

The dissertation is comprised of five chapters. **Chapter 1** presents the overview, objective and working scope of the dissertation.

Chapter 2 presents a concise literature review concerning the conventional methods for antioxidant assay, microfluidics methods for antioxidant assay and the valving technology in the LoD method.

Chapter 3 describes the whole methodology of the dissertation, including reagent and sample preparations, the design and fabrication details of the microfluidic CD and the microfluidic CD operations that includes parallel sequential loading and mixing.

Chapter 4 presents the test result of the microfluidic CD in comparisons with the conventional DPPH antioxidant assay method for each tested sample. The results will be compared by method used and will be further analysed by 5 minutes time interval. This chapter also presents the discussion and explanation on every result achieved.

Chapter 5 presents the overall conclusion of the research work and possibility of the research impact on future technology. Finally, this chapter mentions the current limitation of the developed microfluidic CD and proposes the future work for further improvement.

CHAPTER 2: LITERATURE REVIEW

2.1 Introduction

Free radicals have been identified to cause damage to the cells by chemically attacking the cell components such as lipid, protein and the DNA, thus causing instability to the cell to function normally (Dröge, 2002). Free radicals have also been discovered to cause many major chronic diseases such as cancer (Gupta et al., 2014), diabetes (Montane, Cadavez, & Novials, 2014), Alzheimer's (Wojtunik-Kulesza, Oniszczuk, Oniszczuk, & Waksmundzka-Hajnos, 2016) and hypertension (Baradaran, Nasri, & Rafieian-Kopaei, 2014). However, it is believed that dietary antioxidants may play a role in the prevention of these diseases (Halliwell & Gutteridge, 1985; Jiang, 2014).

Antioxidants are chemical compounds that can minimise the deleterious effect of free radicals. Antioxidants can be found naturally in daily food intake and supplements. There are many laboratory tests to access the content of antioxidant in food; each method has their own chemical principal and procedures and mechanism (Carocho & Ferreira, 2013). Moreover, antioxidant assessments are also important in the food industry and biomedical research field.

The past decade has seen the rapid development of Micro/ Nano-fluidics applications in many fields of research especially in biological, biomedical, chemical and molecular analysis. With the engineered manipulation of the fluids, the Micro/ Nano microfluidics plays a promising methods for variety of tasks for example manipulation of fluids sequences (Fu & Downs, 2017), preanalytical preparations (Cui, Rhee, Singh, & Tripathi, 2015), analyte screening (Windbergs & Weitz, 2011) and biosynthesis (Greener et al., 2012). Microfluidic and nanofluidic components is a part of micro total analysis systems (μTAS) that can practically simplify the conventional macroscale assay methods, cater cost effective device, serve miniaturised volume and serve faster separation of target

molecule with compact innovative device design and fabrication similar to the existing large-scale analysis equipment (Sackmann, Fulton, & Beebe, 2014).

The ability to perform antioxidant activity assays using minimal sample volumes confers a significant advantage, since some sample exist in scarce amounts. Moreover, microfluidics system is able to overcome the limitations of conventional antioxidant assay using complementary fluid mechanics principles.

This chapter provides the relevant literature review to gain more understanding of the study. It presents the relevant literature review on conventional antioxidant methods, application, DPPH antioxidant assay and its limitations. Furthermore, this chapter reviews the current microfluidic technology used to analyse the antioxidant in a sample.

2.2 Plants and antioxidant

Antioxidant has many benefits to human health, it can slow down the aging process and fight the free radicals in the cell. Currently, the public favour the natural based antioxidant rather than the artificial or synthetic produced. Natural product is one branch of the research that are increasing rapidly to discover the valuable hidden treasure of nature (Tan & Lim, 2015). For the past few decades, there been a great deal of attention towards the natural resources study, plant especially exhibit many phytochemical value such as antioxidant properties that are beneficial to human wellbeing (Bansal et al., 2013; Cartea, Francisco, Soengas, & Velasco, 2010). For centuries, India and China have been using plant especially medicinal plants for many diseases treatments and wellbeing purposes. Malaysia is a country which rich in the fauna diversity, thus there is a need for us to explore our natural resources. Extrapolation of the study will benefit the public and the scientific world.

2.2.1 Natural and synthetic antioxidant

As mention above, antioxidant has many advantages offered to human wellbeing. However, as the research intensified, there is an increased doubt on the synthetic used of antioxidant for example butylated hydroxyl anisole (BHA). Synthetic antioxidant are reported to be unstable and carcinogenic which are dangerous to human health. Moreover, the synthetics antioxidant cannot be recycled and reused in the body. When the antioxidant has been used in the body, they tend to turn to be hazardous to the body because they have change to be harmful metabolic products in the body (Caleja, Barros, Antonio, Oliveira, & Ferreira, 2017; Chandra et al., 2014; Lobo, Patil, Phatak, & Chandra, 2010; Tan & Lim, 2015). In addition, natural antioxidant have more nutritional value in compared to the synthetic antioxidants and it is safer.

2.3 Conventional antioxidant assay methods

To date, there are diverse antioxidant activity assays- each method has their own chemical target and mechanism of action. Table 2.1 summarises the current antioxidant activity assay and their chemical mechanism. All the antioxidant assay methods are *in vitro* methods which is to be tested in the laboratory, as the *in vivo* antioxidants methods are usually administered to animal subjects.

Table 2.1: Antioxidant assay and its chemical mechanism

Name of assay	Detection Mechanism	References
Hydrogen peroxide (H_2O_2) scavenging assay	Antioxidant scavenging ability of H_2O_2	(Saxena, Gautam, Arya, & Singh, 2016)
Ferric reducing-antioxidant power (FRAP) assay	Reducing ability of antioxidant from ferric ion (Fe^{3+}) to ferrous (Fe^{2+})	(Henderson, Nigam, & Owusu-Apenten, 2015)

Table 2.1 continued.

2,2 –azinobis (3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) assay	Antioxidant scavenging ability of ABTS•+	(de Souza et al., 2014)
Oxygen radical absorbance capacity (ORAC) Method	Antioxidant scavenging activity of peroxyl radical.	(Grajeda-Iglesias et al., 2016)
Cupric ion reducing antioxidant capacity (CUPRAC) method	Reducing ability of antioxidant from Cu (II) reduction to Cu(I)	(Koley, Kaur, Nagal, Walia, & Jaggi, 2016)
Xanthine oxidase method	Ability to inhibit xanthine oxidase	(Di Majo et al., 2014)
2,2-diphenyl-1-picrylhydrazyl (DPPH) assay	Antioxidant scavenging ability of DPPH•	(Achat, Rakotomanomana, Madani, & Dangles, 2016)

In the body, H_2O_2 decomposes into oxygen and water then produced hydroxyl radicals ($OH^{\cdot}$). It causes cell membrane damage leading to release of arachidonic acid which is long acting cell damaging molecule. Hydrogen peroxide antioxidant assay was used to test whether the sample have the ability to scavenge the H_2O_2 (Alam, Bristi, & Rafiquzzaman, 2013). Meanwhile, FRAP antioxidant assay is used to measure the reducing capability based upon the ferric ion concentration in the sample. This assay principle is to detect compounds with redox potentials. The principle mechanism is to reduce iron has the relationship to the radical quenching processes (hydrogen transfer) facilitated by the antioxidants (Prior, Wu, & Schaich, 2005). ABTS antioxidant assay measures the ability of antioxidants to scavenge the stable radical cation ABTS•+, a blue-green chromophore. The colour intensity of ABTS solution will decreases in the presence of antioxidants (Prior et al., 2005; Shahidi & Zhong, 2015). The ORAC antioxidant assay

measures the antioxidant's ability to break free radical chain reaction by monitoring the inhibition of peroxyl radical-induced oxidation. In this antioxidant method, fluorescein is used as the indicator of peroxyl radical decomposition by antioxidant (Pisoschi & Negulescu, 2011). Meanwhile, for CUPRAC antioxidant assay the reduction of Cu(II) to Cu(I) by the antioxidants in a sample is accompanied with changes of the chromogen colour. ORAP and CUPRAC antioxidant methods focuses on the reduction of copper rather than iron (Prior et al., 2005). Xanthine oxidase is an enzyme for the production of free radicals. In the xanthine oxidase antioxidant assay, the ability of the sample to inhibit the production of the enzyme is assessed. Among the available antioxidant assays, DPPH is the most widely-used antioxidant assay (Pisoschi & Negulescu, 2011). It is quite similar to ABTS, due to both are involved in scavenging the radical reagent ability by the sample. DPPH is a stable, purple-coloured free radical, in the antioxidant assay the ability of the sample to reduce the purple colour to pale yellow indicating the DPPH free radical has been scavenge (Molyneux, 2004).

2.4 Antioxidant applications

Antioxidants have numerous applications in industry and in medical-related fields. This subsection below will discuss the real-life applications of antioxidants application in detail.

Table 2.2: Antioxidant application

Application	Details	References
Industries		
Food Processing	Delay meat degradation	(B. M. Freedman, 2009)
Package food	Delay food spoilage	(Gómez-Estaca, López-de-Dicastillo, Hernández-Muñoz, Catalá, & Gavara, 2014)

Table 2.2 continued.

Medical		
Dentistry	Enhanced shear bond strength of cement to teeth enamel after bleaching	(Kılınç, Aslan, Kılıç, Er, & Kurt, 2016)
	Reduce bleaching deleterious effects on enamel	(Nair, Nesamani, Sanjeev, Sekar, & Renganathan, 2016)
Anti-cancerous agents	Antioxidant glutathione and thioredoxin kills cancer cells	(Benhar, Shytaj, Stamler, & Savarino, 2016)
	Selected plant derivatives have anti cancerous properties	(Roleira et al., 2015)
	Antioxidant lycopene inhibit gastric cancer	(Kim & Kim, 2015)
Red Blood cells	Antioxidant protect the red blood cells from oxidative stress generate by free radicals	(Hatia et al., 2014)
	Astragalin antioxidant in Mulberry plant extract protect the red blood cells from oxidative lysis	(Choi et al., 2013)
Hepatoprotective agents	Hepatoprotective against liver damage	(Mamat et al., 2013)
	Improve liver function test	(Murer et al., 2014)
Nervous system	Antioxidant stimulates brain nerve upon brain injury	(Steinbrenner & Sies, 2013)

Table 2.2 continued.

Therapeutic usage	Diabetic therapy	(Poole et al., 2015)
	Hypertension therapy	(Baradaran et al., 2014)
	Parkinson therapy	(Haney et al., 2015)
	Rheumatoid arthritis	(Marino et al., 2015)
	Psoriasis therapy	(Lin & Huang, 2016)
Dermatology	Enhanced the dermatologic changes	(Bruce M Freedman, 2009)
	Skin disorder prevention	(Działo et al., 2016)

2.4.1 Food Industries

Antioxidants are applied widely in the food industries. Nowadays, people are concerned about the nutrition in food and tend to eat healthily. Besides adding the nutrition value to the food, antioxidants have been a good natural preservative of the food. For example, in meat, antioxidants help to prevent the degradation of the lipid and proteins, which prevent the deterioration of the nutrition, preserve the flavour, texture and colour (Decker, Chan, Livisay, Butterfield, & Faustman, 1995). Another application of antioxidants in food industries are in packaged food. To preserve the food, antioxidants are added in the packaging to delay the food spoilage. The addition of antioxidants as additives in fats and oils and in food processing industries helps to prevent food spoilage.

2.4.2 Dentistry

Antioxidants have been applied in dentistry to strengthen the cement layer to enamel which help to strengthen the teeth treatment (Kılınç et al., 2016). As for aesthetics demands, people nowadays tend do go for bleaching treatments to whiten their teeth, however this treatment will cause teeth enamel erosion. However, currently researchers

have found that the application of antioxidants may help to preserve the tooth structure from the bleaching agents (Nair et al., 2016).

2.4.3 Anti-cancer properties

Free radicals have caused impairment in the cells and DNA functions, which lead to the damage and mutation in the cells. Antioxidants play an important role in repairing, protecting and preventing future damage to the cells. It is a nutritional recommendation to add more antioxidant intake in the daily diet to the prevent various oxidative stress such as cancer (Dai & Mumper, 2010). Recent study by Bhar *et, al* have found that combination of two powerful antioxidant of the glutathione and thioredoxin systems kills neoplastic cells (cancer cells) and decreases resistance to anticancer therapy (Benhar et al., 2016). Another group of researchers have also found that lycopene antioxidant is able to inhibit gastric cancer (Kim & Kim, 2015). Lycopene has been said to possess anticancer properties by increasing activities of antioxidant enzymes and reducing oxidative damage in gastric mucosa. Furthermore, antioxidants from medicinal plant derivatives have also anti-cancerous properties that can be further explored for cancer treatment.

2.4.4 Red Blood cells

Red blood cells transport the oxygen and carbon dioxide to the cells, the life span is about 120 days. Antioxidants have been found to protect the red blood cells from free radical lysis (Choi et al., 2013; Hatia et al., 2014).

2.4.5 Hepatoprotective agents

The liver has many functions, including detoxification of toxin, protein synthesis, and body metabolism, regulation of glycogen storage, decomposition of red blood cells and hormone production. Antioxidants help to protect and improve the liver function from free radical damage (Mamat et al., 2013; Murer et al., 2014).

2.4.6 Nervous system

The nervous system helps to carry signal and information from the brain and spinal cord to various parts of the body and vice versa. In their research, (Steinbrenner & Sies, 2013) have proved that selenium (antioxidant) helped to up-regulate the astrocytes (brain nerve) during brain injury.

2.4.7 Therapeutic usage

Consuming natural antioxidants in daily dietary diet and taking antioxidant supplements may help to prevent many chronic diseases. Antioxidant have been used in many disease therapies including diabetic therapy (Poole et al., 2015), hypertension therapy (Baradaran et al., 2014), Parkinson disease (Haney et al., 2015), rheumatoid arthritis (Marino et al., 2015), and psoriasis therapy (Lin & Huang, 2016).

2.4.8 Dermatology

The use of antioxidants among consumers are driven by the obsession towards maintaining a youthful appearance. Antioxidants protect the skin by reducing the production of free radicals, which can damage skin cells. Besides that, antioxidant can delay the skin aging process and protect it from the harm of ultraviolet light (UV). Furthermore, antioxidant will also help to protect the skin from disorder and improve the skin texture and appearance (Działo et al., 2016; Bruce M Freedman, 2009).

2.5 DPPH antioxidant assay

The 1, 1-diphenyl-2-picrylhydrazine (DPPH) radical scavenging assay was first described by Blois in 1958 and was later modified slightly by numerous researchers. It is one of the most extensively used antioxidant assays for plant samples. DPPH is a stable free radical that reacts with compounds that can donate a hydrogen atom. This method is based on the scavenging of DPPH through the addition of a radical species or an antioxidant that decolourises the DPPH solution. The antioxidant activity is then

measured by the decrease in absorption at 515 nm. In this method, a 0.1mM solution of DPPH in methanol is prepared, and 4ml of this solution are added to 1ml of the sample solution in methanol at varying concentrations. Thirty minutes later, the absorbance was measured at 517 nm. This reaction can be seen in the figure 2.1 below.

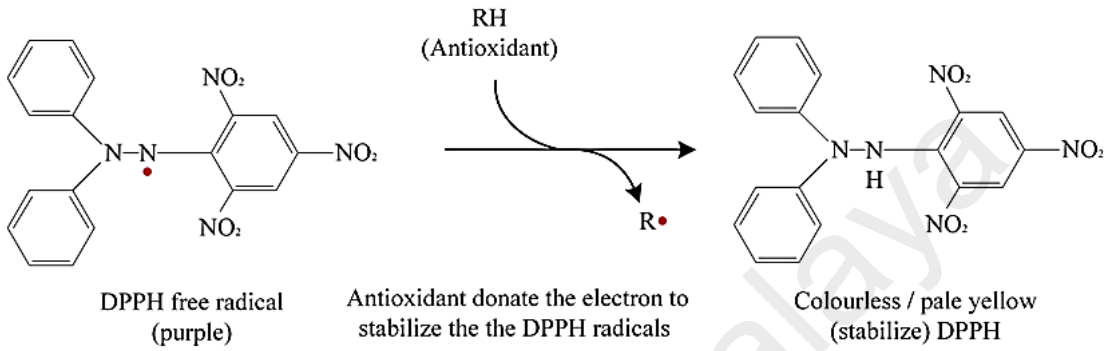

Figure 2.1: DPPH free radical reaction with antioxidant

2.5.1 DPPH antioxidant assay limitations

DPPH antioxidant method offers the first approach for evaluating the antioxidant potential of a compound, an extract or other biological sources. This is the simplest method, wherein the prospective compound or extract is mixed with DPPH solution and absorbance is recorded after a defined period. However, with the advancement and sophistication in instrumental techniques, the method has undergone various modifications to suit the requirements, even though the basic approach remains same in all of them (Kedare & Singh, 2011). Despite its simplicity, the DPPH antioxidant assay still has many limitations. DPPH is only soluble in organic solvents. The absorbance of DPPH in methanol and acetone decreased by 20% and 35%, respectively, for 120 min at 25^{o}C under light, while in the dark it did not change significantly for 150 min (Deng et al., 2011). According to Deng *et al.*, temperature plays an important role in DPPH antioxidant assay. Even though the DPPH assay is simple and does not require special sample treatment, its sensitivity may be affected by a number of factors, such as the type

and amount of solvent used, presence and concentration of hydrogen and metal ion and freshness of DPPH reagent (Shahidi & Zhong, 2015).

2.6 Microfluidic system

Microfluidic system or often referred as Micro total analysis system (μTAS) is a system that simplifies and automates the analytical steps and procedures in the assays or test. Microfluidic system plays a major role mainly in sample preparation, separation, dilution, detection and assay procedures automation. Many tedious assays have benefitted from the microfluidic system, for example, enzyme-linked immunosorbent assay (ELISA) (Giri, Liu, Nchocho, Corcoran, & Dutta, 2018; Verma et al., 2018; Yoshikawa, Yoshinaga, & Tamiya, 2018), Polymerase chain reaction (PCR) (Ahrberg, Manz, & Chung, 2016; Kim, Clark, Shahi, & Abate, 2018; Zhang & Jiang, 2016), Loop-mediated isothermal amplification (LAMP) (Chen, Liu, Wang, Fu, & Shih, 2018; Rane, Chen, Zec, & Wang, 2015; Abkar Ahmed Sayad et al., 2016), cancer biomarker detection (Mousavi et al., 2015; Reinholt & Craighead, 2018; Uliana, Peverari, Afonso, Cominetti, & Faria, 2018) and bacteria detection (Lee et al., 2015; Yasaki et al., 2018).

Microfluidic technologies employ small-scale volume of liquid, offer rapid reaction times and automates the fluidic process of materials and reagents in the analytical assays (Smith et al., 2016). These outlined advantages make microfluidic system more favourable to the scarce sample and resources. Most of the microfluidics platforms fall under four categories which are LoC (Guo, Feng, Fang, Xu, & Lu, 2015; Jamshaid et al., 2016; Sackmann et al., 2014), LOD (Duffy et al., 2018; Loo et al., 2017; Abkar Ahmed Sayad et al., 2016), Lab-on-Paper (LoP) (Gao, Liu, & Gu, 2016; San Park & Yoon, 2015) and droplet-based microfluidic (also known as droplet reactor) (Lignos et al., 2016; Shembekar, Chaipan, Utharala, & Merten, 2016). However, the widely discussed microfluidic technologies are LoC and LoD (LoD is also a part of LoC) (Guo et al., 2015;

Strohmeier et al., 2015; Tang, Wang, Kong, & Ho, 2016). Consequently, in the next following subsection, we will focus on the LoC and LoD.

2.6.1 Lab-on-Chip (LoC) Devices

LoC devices are a part of microfluidic division that offer many advantages to simplify many analytical methods. As the term "chip" implies, LoC exists in the form of integrated circuit chips where the chemical reactions to take place. LoC offer many advantages in the analytical analysis such as miniaturisation of the sample, materials, reagents or any liquid involved in the experimental procedures. LoC offer portability to the users as it can handle variety volume of liquid from nano to microliter. Moreover, due to its small size, diffusion and chemical reaction are easier to occur (small surface area) thus it is suitable to be used as a point–of-care-diagnostic tools.

The chips existed in the complex micro channel that is specially engineered for specific tests and analyses. Furthermore, the chips are connected with other additional appliances such as pumps, valves, sensors and electronics devices to make complement the chip itself. The pumps (usually syringe pump) is used to induce the movement of the liquids to the micro channel existed in the chips. While the sensors and the electronics devices are used for the detections of the target parameters in the test designed (Abgrall & Gue, 2007). Thus far, LoC device involves multidisciplinary fields such as mechanical systems, fluids mechanics, electronic system, biological and chemistry elements forms together to make a complete LoC device.

Due to the above discussed advantages of LoC, this microfluidic platform has been a great potential in many fields including medical diagnostic, environmental analysis and in the industry. With the cost effective and rapid time analysis LoC have a great potential to be the future devices for the analytical field.

2.6.2 Lab-on-Disc (LoD) Devices

Moving on now to the LOD devices, which is also one of the major platform of microfluidic devices. In comparison to LoC which is existed in the chips shape, LoD existed in the disc shape. Based on its name, LoD is also usually referred as centrifugal microfluidic or microfluidic compact disc (CD).

LoD shares many similarities of LoC devices. It minimises the sample and reagents volumes and automates the procedures or steps in the analytical procedures. Likewise LoC, LoD have been used in many multidisciplinary field such as medical diagnostic, environmental analysis, biotechnology and in the industry. However, although LoC and LoD incorporates the similar advantages, LoD is far more superior in terms of the inherent simplicity of the analytical system it provides.

LoD acts as all in one device which includes all the micro channels, valves and pumping system that have been used as an external appliance in the LoC devices, which make LoD far more cost effective and convenient to use. Additionally, LoD provide multiplexing of the sample which is one of the great advantage of the LOD that facilitates a few assay in one disc. From the reagent storage, sample preparations, sample processing, sample dilutions and detection strategies provided in the LOD devices, this steps have been provided in a single disc (Kong, Perebikovsky, Moebius, Kulinsky, & Madou, 2016).

In contrast to LoC devices, LoD devices use a motor to rotate the disc. Centrifugal force, Coriolis and Euler force have been combined in the rotating disc to propel the liquids outward from the CD centre. Fluids movements, pumping, mixing, and metering separations are effortlessly controlled by these forces together with the capillary action in the specific micro channel design in each CD (Hugo, Land, Madou, & Kido, 2014).

2.7 Comparisons of LoC and LoD microfluidic techniques

This subsection will critically discuss the features, similarities and the disadvantages between LoC and LoD devices. Table 2.3 summarises the features of LoC and LoD.

Table 2.3: Features of LoC and LoD

Microfluidic platform	Similarities	Disadvantages	References
LoC	• Miniaturise sample and reagents volume • Convenient Less human interface • Cost effective • Faster reaction time • Portable and programmable	• Need sealing Interconnected with microelectronic components • Requires syringe pump system for fluids movements • Liquid spreading out from target area	(Becker, 2009; Tantra & van Heeren, 2013; Temiz, Lovchik, Kaigala, & Delamarche, 2015)
LoD		Difficulty working with very small volumes (<1 nL)	(Kong et al., 2016)

In LoC devices, the problems such as restricting the solvents, reagents and the liquids from spreading out form the target wettable area on the chip is a major issue. Another issue faced by the researchers when making LoC devices is to minimise the evaporations of the samples and the reagents from the chips while protecting the target molecule. Evaporations may lead to the damage of the target cells/molecules and will cause inconsistency and interfere with the reading results. This will happen on the high volatile liquid such as organic solvents or alcohol. Sealing of the LoC devices are also important to protect the samples from dust and reduce the contaminations (Temiz et al., 2015). Moreover, LoC platform also need a syringe pump system to induce the liquid movements to the chips which is costly.

Meanwhile, the LOD devices have difficulties to work with volumes less than (<1 nL) which is hard to load and run in the microfluidic disc (Kong et al., 2016). However, the benefits have outshined this disadvantage. LOD devices offer "all load in run" processes in one close system which can eliminates the undesired factor such as the sample and reagents evaporations and contaminants. From sample preparations, processing, multiple steps parallelisation are processed in one disc (Gorkin et al., 2010). LOD devices can also run multiplexing assay in one disc. Multiple protein and biomarkers have been in tested in one single disc, which is conventionally existed in different separate assay test. By LOD devices, multiple immune assays have been combined together which can give rapid analysis and results in compared to the conventional methods. LOD devices can also run the thermal cycle and cold phases such as in PCR procedures. In conventional methods, the PCR procedures and machines are very costly and timely, with LOD devices, the cost effective and rapid analysis can be done (Amasia, Cozzens, & Madou, 2012; Park, Sunkara, Kim, Hwang, & Cho, 2012; Roy et al., 2015).

Conventionally in medical diagnostic, the bacteria detection and identification will take a long time for the analysis. Bacterial detection and analysis are very critical especially in the intensive care unit patients or any diseases cause by bacterial infections, such as food borne pathogen. With delay of the laboratory test, the conditions of a person may get worsen due to the mycotoxins released by the bacteria. With LOD devices, these problems can be solved. Fast sample processing such as DNA extractions, DNA amplification and real time detection offered by LOD devices have solved these problems, the process which usually takes 6-7 hours to be completed in conventional ways can be done within one hour by using LOD devices. (Kim, Park, Kim, & Cho, 2014; Park et al., 2012; Abkar Ahmed Sayad et al., 2016).

2.8 Microfluidics devices for antioxidant assay

There are a few microfluidic devices for the antioxidant assay. This subsection will cover the related work on antioxidant microfluidic system in terms of design, fabrication and type of test that have been used. Table 2.3 summarises the microfluidic technology available for the antioxidant assay.

Table 2.4: Microfluidics technology for antioxidant assay

Microfluidic platform	**Antioxidant activity assay principle**	**Details**	**References**
LoC	Peroxyoxalate chemiluminescence (PO-CL) assay detection with hydrogen peroxide	• Polydimethylsiloxane (PDMS) chip connected with syringe pump and chemiluminescence detector • Polydimethylsiloxane (PDMS) chip connected with syringe pump and thin-film organic photodiodes (OPDs) detector	(Amatatongchai, Hofmann, Nacapricha, Chailapakul, & deMello, 2007; Wang et al., 2009)
	Phenolic antioxidant measurement with potassium permanaganate as enhancer in detection system	Estimation of the antioxidant level by measuring phenolic content with potassium permanganate chemiluminescence (KmnO4-CL) detection system.	(Al Lawati, Al Haddabi, & Suliman, 2014)
	Liver metabolism enzyme reaction with DPPH	Hydrogel immobilised lived enzyme reaction with the antioxidant level in a serum	(Lee et al., 2014)

Table 2.4 continued.

LoP	Measurements of flavonoid, phenolic compounds and DPPH antioxidant activity	Based on the LoP platform integrated with antioxidant assay and technical drawing pen.	(Nuchtavorn & Macka, 2016)
LOD	Phenolic, Flavanoids and DPPH antioxidant assays	Integration of three antioxidant assay with complement optical fiber-coupled spectrometer	(Phonchai, Kim, Chantiwas, & Cho, 2016)

As been reviewed in table 2.4, The authors in (Amatatongchai et al., 2007) has used LoC microfluidic system incorporating chemiluminescence detector for measuring antioxidant capacity. In their paper, the detection is based on a peroxyoxalate chemiluminescence (PO-CL) assay with 9,10-bis- (phenylethynyl)anthracene (BPEA) as the fluorescent probe with hydrogen peroxide incorporated in the chip and tested on the plant based food and the pure pharmaceutical materials.

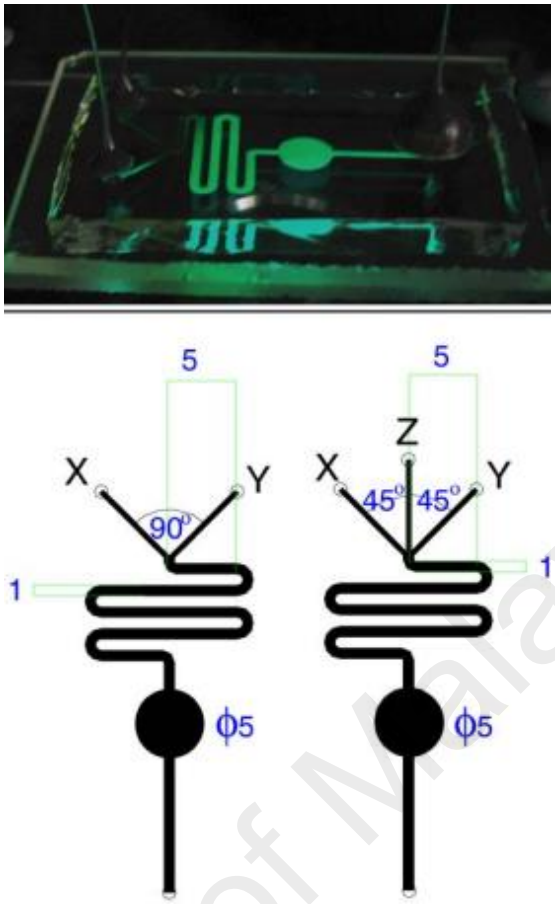

Figure 2.2: PDMS antioxidant lab on chip structure.

The diagram shows the two capillary channnel and three capillary channel on the right. The X, Y and Z inlet were connected to the mixture of regent, dye, catalyst of hydrogen peroxide and antioxidant. The outlet was connected to the waste (Amatatongchai et al., 2007).

Then later, this research design has been improved and integrated with photodiode on-chip chemiluminescence detection in (Wang et al., 2009). Basically in (Wang et al., 2009), the authors has replaced the detection system with the thin-film organic photodiodes (OPDs) as a detectors for antioxidant. In this research, the PDMS chips are still maintained, nonetheless only the detection system was replaced. In this paper, it can be seen that the authors are trying to find better and more cost-effective detection system

to complement the chips design for the antioxidant screening. Figure 2.2 illustrated the whole experiment set up for the chips and the OPD detection system.

In figure 2.2 in can be seen that the inlet 1 and inlet 2 is connected with the syringe pump. The syringe pump was loaded with the PO-CL that consist of the mixture of reagent, dye, catalyst and hydrogen peroxide respectively. After that, the antioxidants were shoot to the into the hydrogen peroxide stream by using syringe pump which result in the CL which later being detected by OPD.

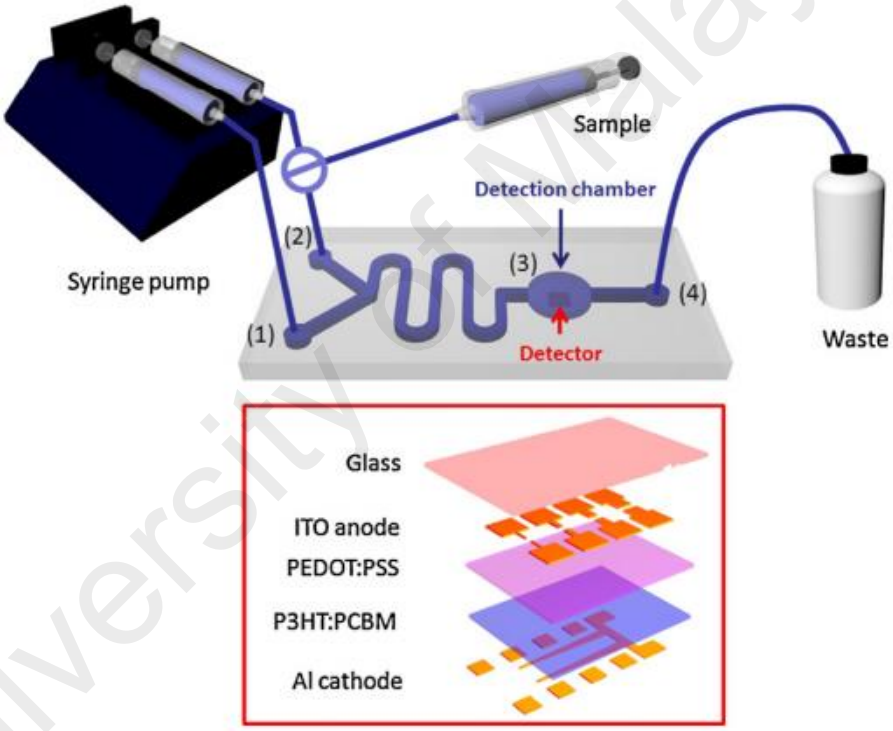

Figure 2.3: The whole experiment setup of antioxidant PDMS chip with OPD detection system

In Figure 2.3, (1 and 2) shows the two inlets of the microfluidics channel. (3), shows the outlet of the chips channel. (4) the outlet of the microfluidic chip and connected to the waste (Wang et al., 2009).

Another microfluidic work with antioxidant assay has been exemplified by the authors in their study in estimating the phenolic antioxidant level in honey by using a potassium permanganate chemiluminescence detection system (Al Lawati et al., 2014). The authors have been using LoC microfluidic platform with the chip design shown in figure 2.4

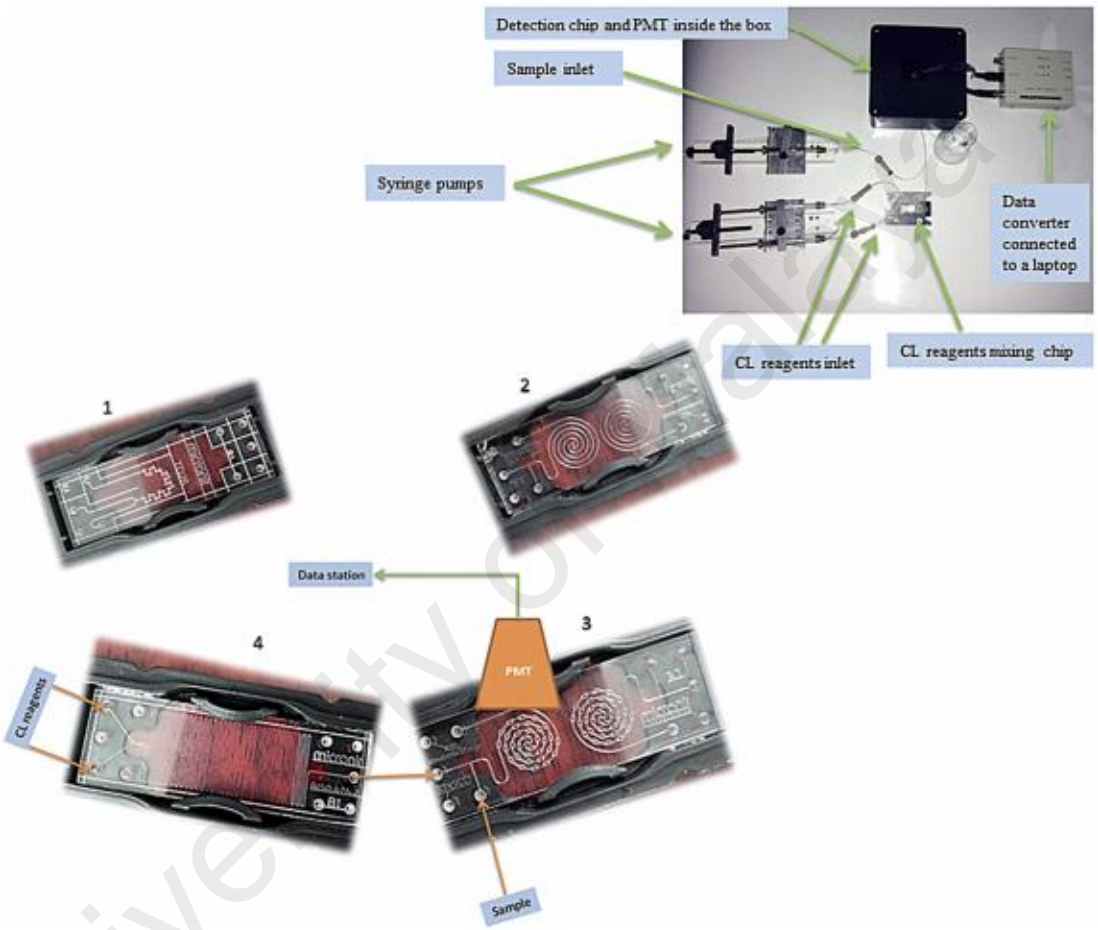

Figure 2.4: The whole experiment set up of phenolic antioxidant chip

In Figure 2.4, (1) tear drop chip, (2) 32 Splits chip, (3) spiral chip and (4) serpentine chip consecutively (Al Lawati et al., 2014).

In this study the phenolic antioxidant levels in honey were determined by using the $KMnO_4$-CL detection system and the final results are compared to the conventional method of Folin–Ciocalteu assay and DPPH assay. The authors have been using two syringe pumps to inject the reagents to the chips (one for permanganate and the other for

formaldehyde solution). The authors have been tested the design of four different chips in their study (refer figure 2.4) and found the spiral chip give the best results due to the centrifugal forces as the solution travels through the folding of spiral chips and the contact surface of the fluid are narrow that causes rapid diffusion.

Another microfluidic device for antioxidant assay has been used in (Lee et al., 2014). In their research, they also choose LoC microfluidic platform as a device design. The authors have tried to mimic the real body situations when reacting with the antioxidants. Their chip design has been divided into two part which are the first part consist of immobilised enzyme called poly (ethylene glycol) diacrylate (PEGDA) which mimic the liver metabolism. The second part is the (DPPH) absorbance measurement of the antioxidant. In their research, they have connected the chips with the custom-made LED spectrophotometer as a method of detections. This LED detectors detects the DPPH absorbance and act as a replacement of the conventional spectrophotometer. Figure 2.5 shows the experimental design of the chips that has integrated the DPPH detection principle in their chips.

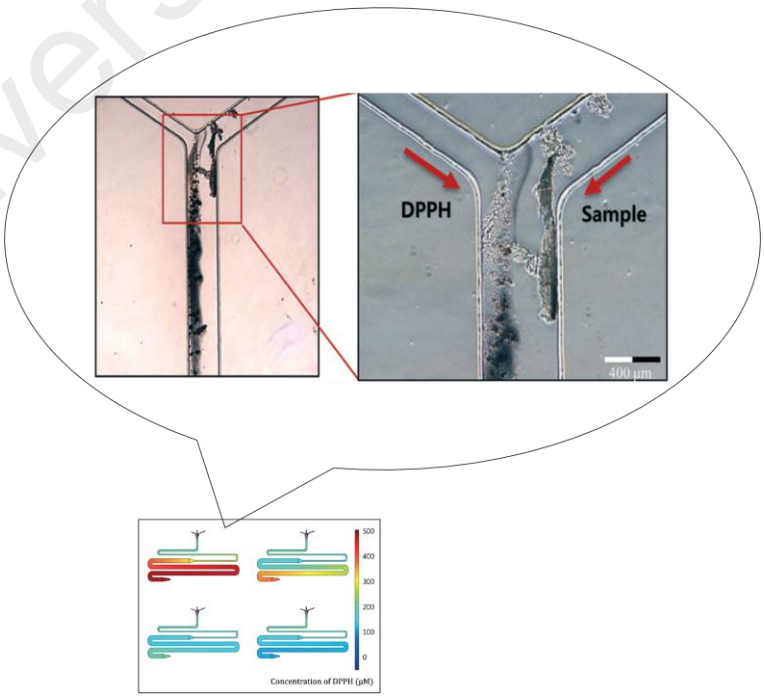

Figure 2.5: The chip design of the experiment (Lee et al., 2014)

Moving on now to another microfluidic devices for antioxidant detection as described in (Nuchtavorn & Macka, 2016). In (Nuchtavorn & Macka, 2016), the authors has used LoP microfluidic platform for the antioxidant detection. In their publication, the authors have combined three antioxidant assays which are; flavonoid, phenolic compounds and DPPH assay to detect the antioxidant level of the sample. The results can be detected qualitatively by the eyes with the change of ink colour. As for the quantitative measurement image analysis software has been used.

In LoP microfluidic devices, the capillary channel have been created by using a hydrophilic-hydrophobic reaction in an ink on a sheet of paper (Curto et al., 2013; Dungchai, Chailapakul, & Henry, 2011). The fibre and the paper cellulose has been manipulated to create a functional microfluidic channel that similar in the LoC devices technologies. In LoP, fabrication and the ink making techniques are crucial to determine the functionality of the LoP devices. In their paper, the authors has eliminated the LoP devices drawback such as expensive wax printer and heating step after wax polymerisation that cause protein denaturisation (Nuchtavorn & Macka, 2016). Figure 2.6 shows images of the LoP devices micro channel which consist of three antioxidant assays consecutively; total phenols (TP), total flavonoids (TF) and DPPH free radical scavenging activity. They have used their LoP devices in various beverages by dipping it and measuring it qualitatively and quantitatively with colour changes of the DPPH and in calorimetric detection of the level of the antioxidant in the beverages to scavenge the free radical DPPH.

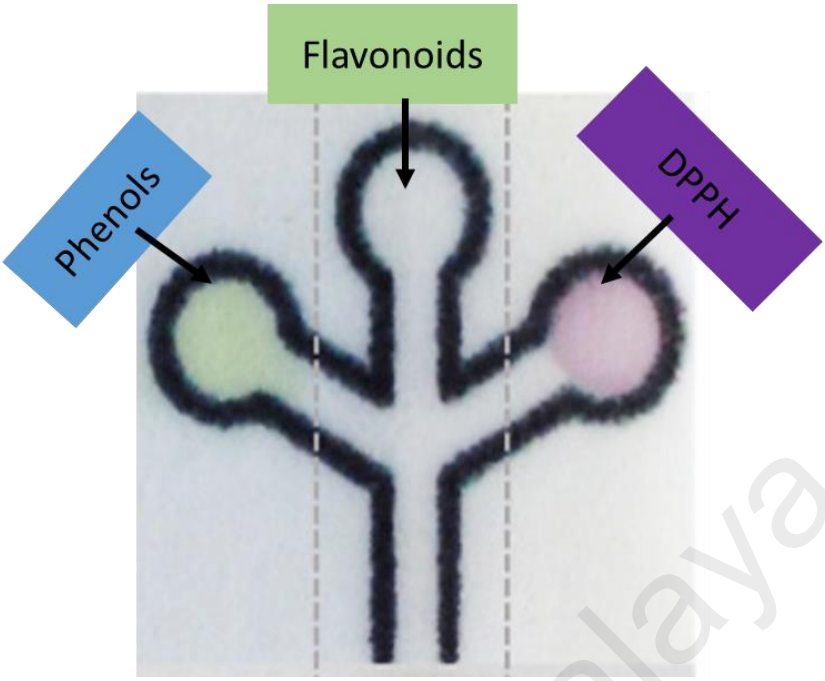

Figure 2.6: The microfluidics LoP integrated in the technical pen (Nuchtavorn & Macka, 2016)

The devices consist of three ink holes of the reagents of three antioxidant assay; TP, TF and DPPH antioxidant assay.

In (Phonchai et al., 2016), a LoD devices has been developed for the determination of the total phenolic content and antioxidant activity in beverages samples. The authors have integrated three antioxidant assays in their LoD devices which are; Folin–Ciocalteu assay, ferric reducing power assay and DPPH assay. Figure 2.7 shows the design of the LoD microfluidic CD. As can be seen in figure 2.7 (B) the engraved micro channel has different valves and capillary for different specified action; sample loading, metering, waste storage, sample dilution inlet, reagent storage, and optical detection. The number in 2.6 (B) represent the number of valves that will open during the spinning in order to allow the experimental steps procedures in the disc.

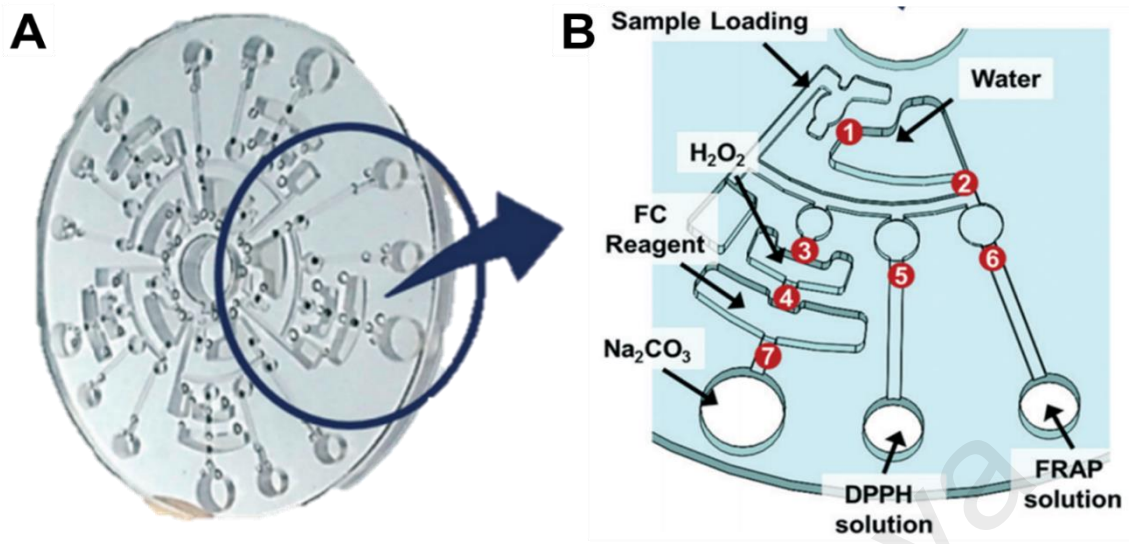

Figure 2.7: The LoD microfluidic CD design.

(A) The microfluidic CD (B) the engraved micro channel features on the CD.

2.9 Summary

In this chapter, an overview regarding the antioxidant assay, and its application has been illustrated. The current DPPH antioxidant assay have been outlined with critical analysis of its limitation. Moreover, the related work in antioxidant microfluidic technology have been outlined and summarised in the above-mentioned sub sectioned. After weighing the benefits of the LoD advantages and reviewed on lack of the availability of antioxidant microfluidic devices, it has been found there is a need for LoD antioxidant devices. Therefore, an alternative approach for overcoming such limitations is needed. An effective develop method for rapid response, cost-effective, simple operation, and with minimal solvent is required for the antioxidant detection in the sample.

CHAPTER 3: METHODOLOGY

3.1 Introduction

The chapter concentrates on the methodology of the design and development of the microfluidic CD for the DPPH antioxidant assay. In this chapter, all the methods and the procedure involved is discussed thoroughly from the initial of the study until the end of the experiment conforming to the subsection. The procedures of the plant materials preparation, collection and extraction are explained, then, the prepared plants materials along with the two standard antioxidant reagents (ascorbic acid and quercetin) used for the antioxidant activity assay of conventional method are described. Subsequently, the design and fabrication of the microfluidic CD are shown and elaborated. Finally, the integration of the antioxidant activity on microfluidic CD are described subtlety.

Complete project sequences and processes have been illustrated in figure 3.1, the project started with the plant extract preparation and screening of fifteen plants by using the conventional DPPH antioxidant assay test. By conducting the conventional method, the disadvantages of the conventional methods are determined, and are supported by further reading of the literature review. Thereafter, the optimisations and modifications of conventional DPPH antioxidant assay method for centrifugal microfluidic CD are done to overcome the drawback. Optimisation and modification of conventional DPPH antioxidant assay method for centrifugal microfluidic CD are done accordingly, followed by the design and fabrication of the centrifugal microfluidic CD. The developed centrifugal microfluidic CD is tested with the real reagent and at the end the statistical analysis and the validation of the results is done by comparing it to the DPPH antioxidant assay conventional method.

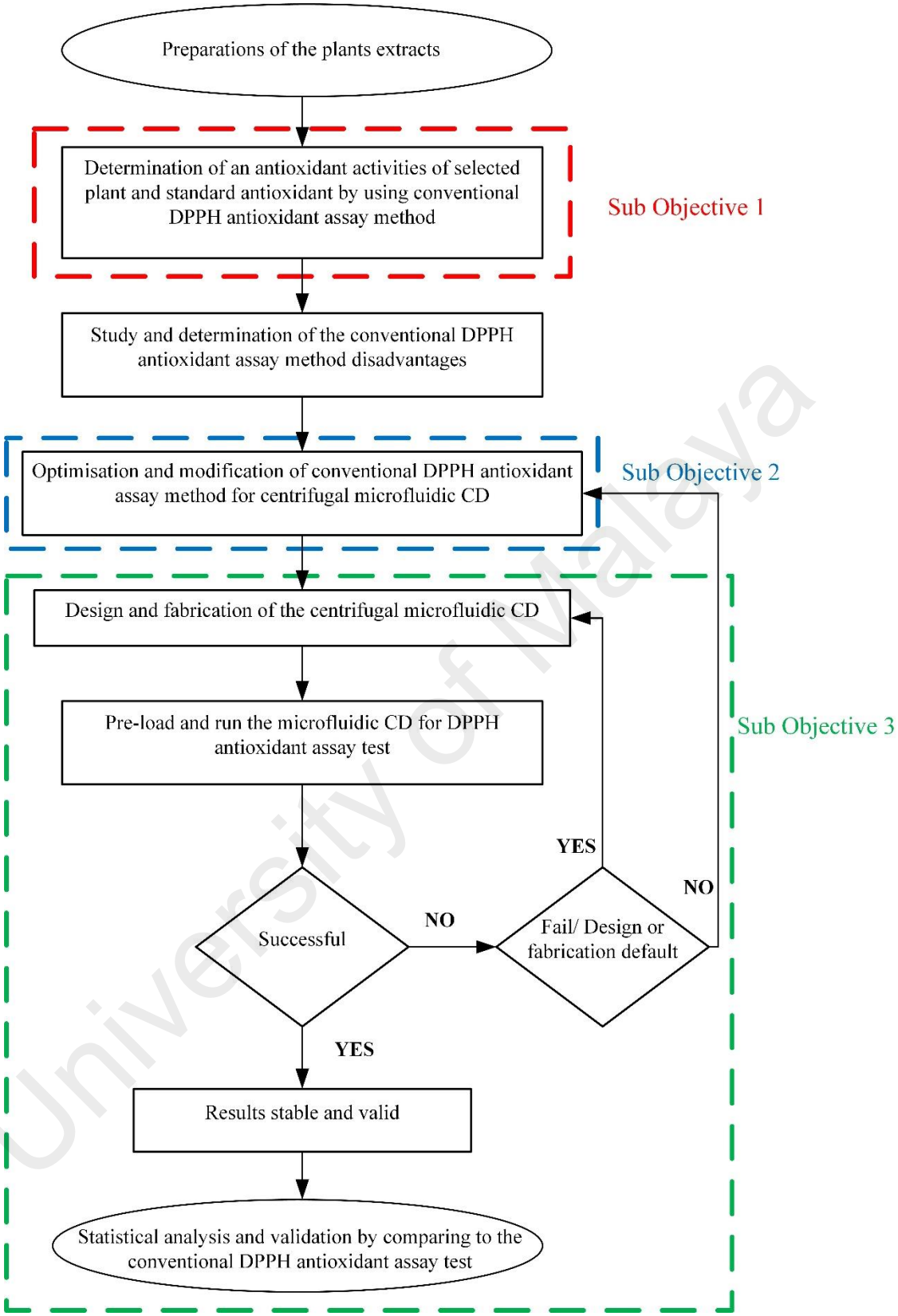

Figure 3.1: Overall flowchart of the project methodology

3.2 Plants extraction

The plants *A. catechu, P. minus* and *S. polyanthum* were collected from Selangor and Perak, Malaysia. The plants were chosen based on their reported high antioxidant activity among local plant species (George, Ng, O'Callaghan, Jensen, & Wong, 2014; Shilpa, Krishnakumar, & Sooryaprakash, 2014; Stohs & Bagchi, 2015). The plants were dried in a laboratory oven at 40 °C until crisp, then pulverised into a powder using a mechanical grinder. The ground powder was then soaked and shaken in 95 % ethanol for 48 hours. The solvent mixture was then filtered using Whatman No. 1 filter paper to eliminate the plant debris. After this, the excess solvents were eliminated using a rotary evaporator (Buchi Rotavapor R-114), then freeze-dried and stored for 20 °C until further use in the experiment.

3.3 DPPH conventional antioxidant activity assay

The radical scavenging activity of *A. catechu, P. minus* and *S. polyanthum* were determined using the method described by Saha *et al* (Saha et al., 2016), with some modification . The deep purple colorimetry reduction of DPPH was determined by using a spectrophotometer (Epoch, Biotek, US) at 517 nm with three repeated measurements. First, the plant extracts were diluted to four final concentrations of 25, 50, 75 and 100 mg/ml, to make 1 ml sample solutions in ethanol. Then, 1 mM of DPPH in methanol was prepared and added to the test solution and left to incubate for 30 minutes at room temperature. For the control experiment, ethanol was added with DPPH. Finally, the absorbance values were measured and the antioxidant activities were calculated using the following equation.

$$Antioxidant\ activity\ = \frac{Control - Test\ Sample}{Control} \tag{3.1}$$

3.4 DPPH microfluidic CD design and fabrication

The photoprotective microfluidic was designed using computer-aided design software (AutoCAD), as shown in figure 3.2. The photoprotective microfluidic CD consists of five layers: three black Polymethyl Methacrylate (PMMA) and two pressure sensitive adhesive (PSA) layers. The first layer is a transparent PMMA disc and contains injection/venting holes. A black adhesive film is used to cover the PMMA disc except the reaction chambers. The second layer is PSA and the third layer is a black PMMA disc with the microfluidic channel and chamber features engraved on it. The fourth layer is PSA and the fifth layer of the microfluidic CD is made of black PMMA. The CD features details can be seen in the figure 3.3.

All of the engraving of microfluidic channel features was done using a Computer Numerical Control (CNC) machine VISION 2525 by Vision Engraving and Routing Systems, USA, as shown in Figure 3.3. The engraved microfluidic channel features three chambers: a DPPH chamber, plant extract chamber, and reaction chamber. The DPPH chamber was connected to the valve to control the flow of DPPH solution to the reaction chamber. The inlet holes and microfluidic features in the PSA layers were cut using a cutter plotter machine (GCC P2-60/PUMA II, by GCC, Taiwan). Each layer of microfluidic CD is press-bonded together using a custom-made pressing tool.

Figure 3.4 shows the whole experimental set up, which consists of computer controlling systems and custom LoD spinning test systems. The centrifugal motor and the high-speed camera were connected to the computer to control the microfluidic CD rotating speed (rpm) and visualisation, respectively. The reflector attached to the microfluidic CD will provide the signal to the digital RPM meter to determine the CD's rotation speed.

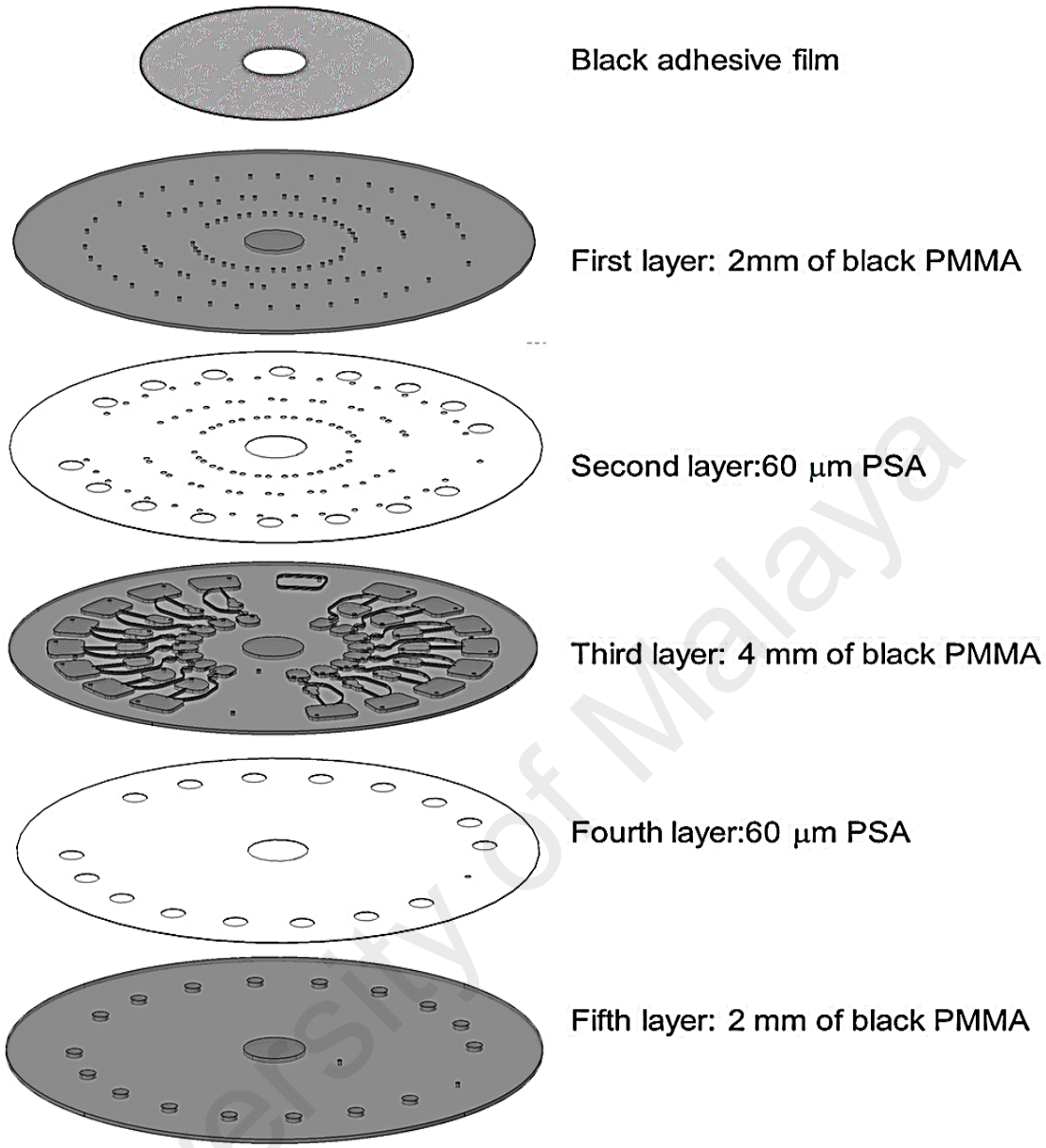

Figure 3.2: An exploded view of the photo-protective layers of photoprotective microfluidic CD. The CD consist of five layers. The first, third and the fifth layer was made from black PMMA layer. While the second and the third layer was made from PSA.

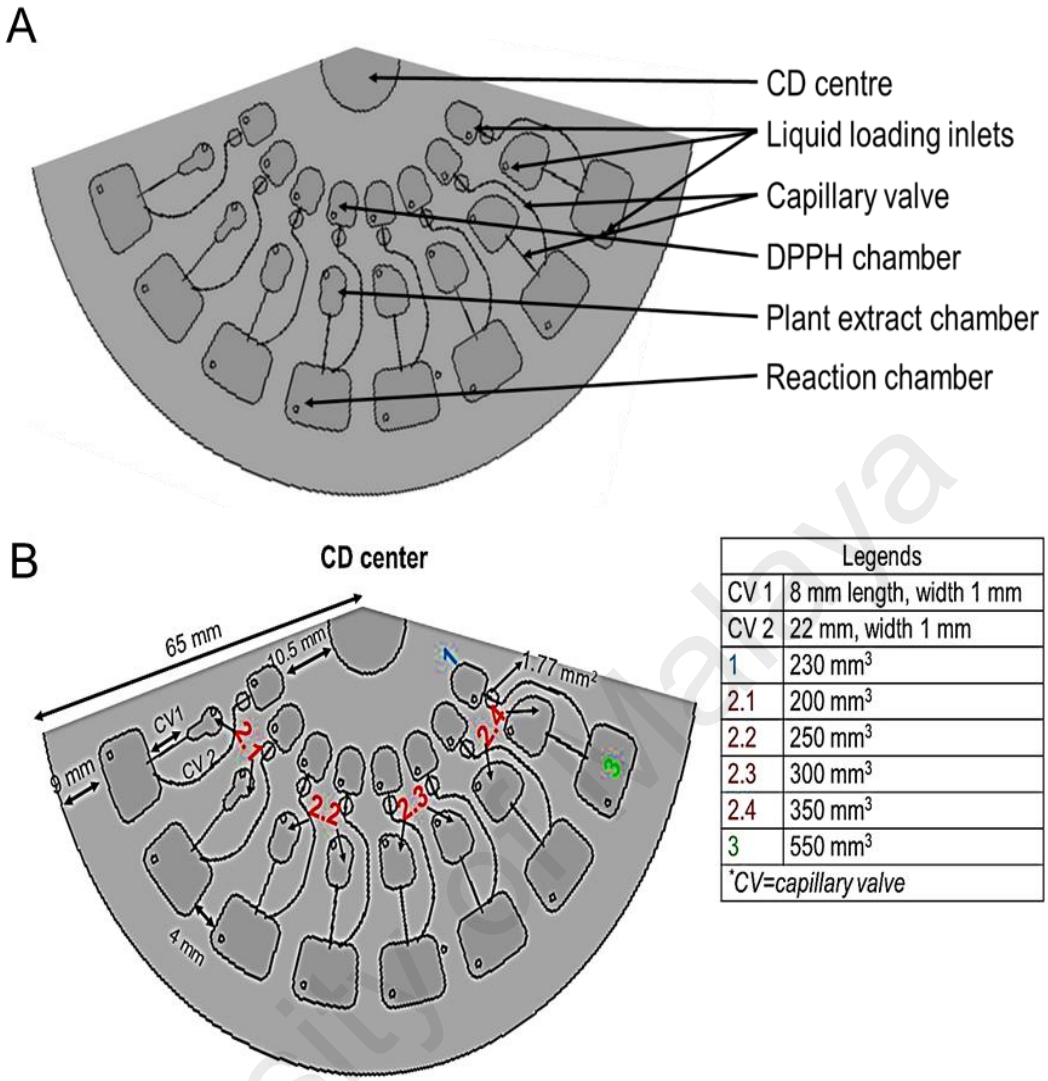

Figure 3.3: A top (A and B) view of the third layer of the photo-protective LoD with different specification of chamber.

The top figure shows the plant extract chamber was varied in four different sizes and duplicated for two consecutive concentrations. While, the figure below shows the details of each chamber in the microfluidic CD.

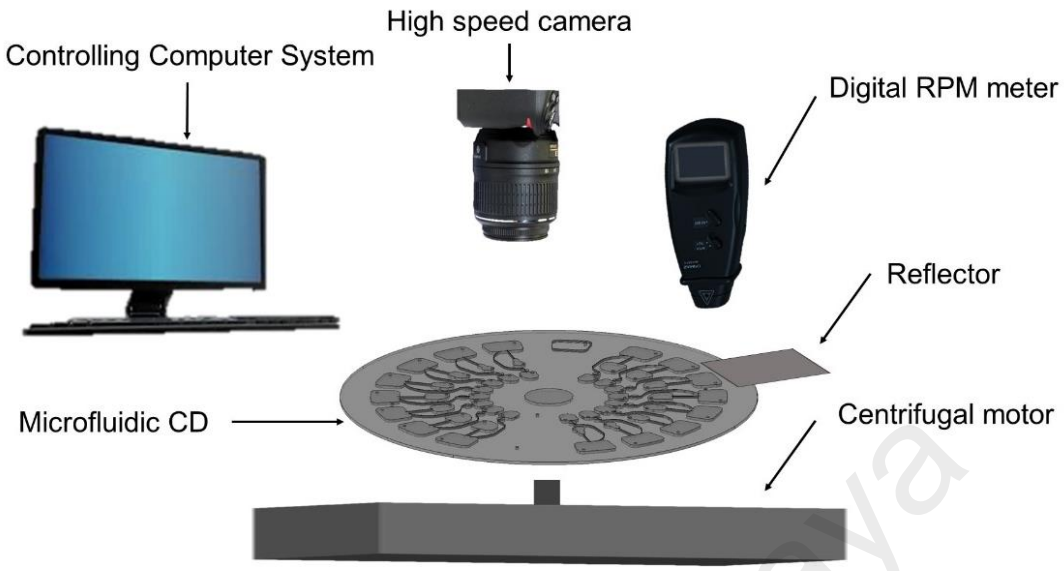

Figure 3.4: The whole experimental setup.

The whole experiment set up consists of the computer controlling systems and the custom LoD spinning test system. The centrifugal motor and the high-speed camera were connected to the computer to control the CD speed (rpm) and visualisation. The reflector will give the signal to the digital RPM meter to determine the speed of the CD spinning.

3.5 DPPH microfluidic CD optimisation

The optimisation processes were done by minimising and diversifying the liquid volume and the microfluidic CD design. Due to limited microfluidic CD area and to mitigate the fluid volume, the liquid volume has minimised to the microliter level.

3.6 Integrated DPPH microfluidic CD operations

3.6.1 Pre-loading reagents and sample preparations

Before running the microfluidic CD, the reagents and the sample for the pre-loading from the fridge and the stock solutions need to be diluted to four concentrations of 25, steps need to be prepared. The 1mM of DPPH need to be diluted in the methanol. The plants extracts need to be thawed 50, 75 and 100 mg/ml.

3.6.2 DPPH microfluidic CD operations

After preparing the plant extract (as discussed in the previous section), the plant extracts along with the two-standard reagent of antioxidant (ascorbic acid and quercetin) were then pre-loaded into the plant extract chambers. The plant extract chambers in the middle were designed in four different sizes in duplicate for 25, 50, 75 and 100 mg/ml plant extract. Meanwhile, as shown in figure 1b, the reaction chamber was located at the bottom of the microfluidic CD and filled with the required solvents. It is the final chamber for the mixture of the solvent, plant extract, and the DPPH. The DPPH chamber connected with a valve was designed to occupy 1 mM DPPH. The centrifugal microfluidic process starts after all the liquids have been pre-loaded in to the designated chamber. The spinning speed is increased slowly until all the fluids in the DPPH and plant extract chambers are emptied and mix in the reaction chamber. The spinning operation is summarised in table 3.1. The spinning is stopped after 30 minutes and the absorbance reading were measured.

Table 3.1: Spinning program of the microfluidic CD.

Step	**Spinning speed (rpm)**	**Time ***	**Spinning direction**	**Process**
1	0	1 minutes	nil	Sample preloading
2	300	30 seconds	Clockwise	Plant extracts flowing to the reaction chamber and emptied
3	800	15 seconds	Anticlockwise	DPPH solution flow out from the DPPH chamber to the capillary valve and reached reaction chamber

Table 3.1 continued.

4	1400	5 minutes interval to 30 minutes	Clockwise and anticlockwise	The disc is rotated for mixing purposes
* The time includes the acceleration time to reach the target speed and to change the rotation (2s)				

3.7 Absorbance reading

There are two devices used to read the antioxidant activity absorbance, which are a microplate reader and the CD reader. The microplate reader was used to compare the antioxidant absorbance activity for both the conventional and LoD method reading over 5 minute intervals up to 30 minutes. The final solutions obtained from the conventional and LoD reaction methods were then transferred to the 96 well plates for absorbance measurements.

Whereas, the CD reader (Figure 3.5) was used to measure the absorbance of the final biochemical solutions in the microfluidic CD (Thiha & Ibrahim, 2015; Uddin et al., 2015). The CD reader consisted of a 517 nm LED and photodiode to measure the light intensity passing through the solution. A microcontroller (ATmega328p) connected with the photodiode, calculates the absorbance of the light passing through the solutions. A stepper motor, a Hall Effect sensor and a magnet help to incorporate the alignment among the reaction chamber, LED and photodiode. The CD reader structure was built using black PMMA to reduce the optical noise.

3.8 Statistical analysis

The statistical analysis is done by using IBM SPSS 24 statistical software by using the repeated measure analysis of variance (ANOVA) statistical models to analyse the differences among group means among time interval and the concentrations.

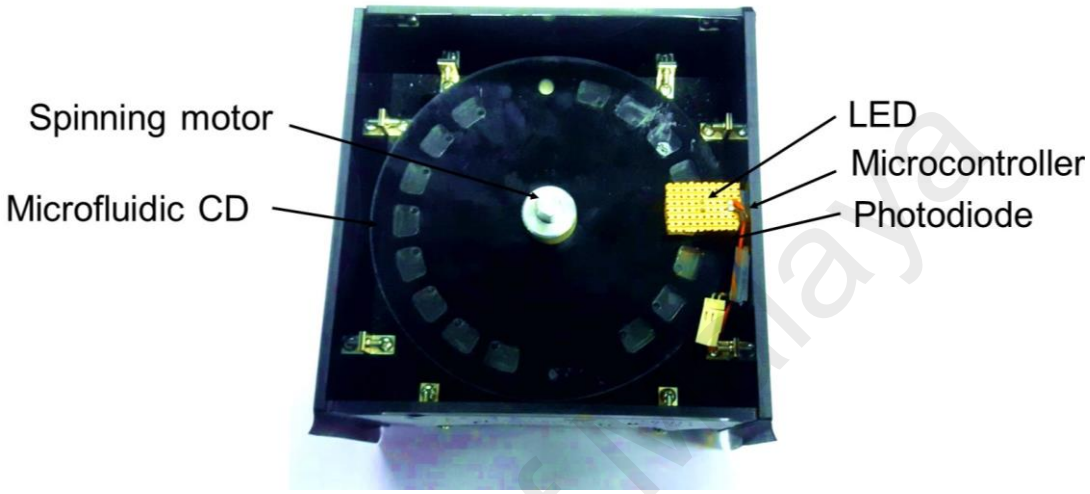

Figure 3.5: CD reader

The CD reader is used to read the final absorbance directly from the CD. The results were then compared to the microplate reader.

CHAPTER 4: RESULTS AND DISCUSSION

4.1 Introduction

This chapter presents the results and discussions of the project findings in continuation with the methodology described in chapter 3. Firstly, the microfluidic CD operations are elucidates in details with the sequential loading and mixing is shown and elaborated. Subsequently, the comparisons of conventional and LoD DPPH antioxidant activity method of in 5 samples which consists of ascorbic acid, quercetin, *A,catechu*, *P. minus* and *S. polyanthum* is presented and discuss accordingly. Finally, the comparisons of microfluidic CD reader and the conventional microplate reader have been illustrated in this chapter as a complementary endpoint detection system.

4.2 Optimisation of DPPH microfluidic CD

Most assays are volume dependant. Each liquid drop represents priceless reagents and solutions that contributes to the fundamental experiments results. Since DPPH antioxidant assays are very light sensitive. All experiments procedures need to be done in the absent of light. Another crucial factors in this experiment is the concentrations of the sample used in the assay.

At first, the experiments have been started with the concentrations of 5, 10, 15 and 20 mg/ml plants extracts. Nevertheless, we have found out that these concentrations are inadequate for the DPPH free radicals scavenging reactions with the plant extracts. We have found out that the spectrometers are unable to detect the any scavenging DPPH activity reaction. Furthermore, there are no significant changes of DPPH colour even though the standard antioxidant were used. Hence the concentrations were increased to 25, 50, 75 and 100 mg/ml respectively.

The optimised concentrations that have been used to carried out in the whole experiments for DPPH microfluidic CD has been shown in table 4.1. The final volume was set to 200µl as for optimum spectrometer reading by using the micro plate reader.

Table 4.1: Optimised volumes for the DPPPH microfluidics CD

Concentrations / Solutions (µl)	**25 mg/ml**	**50mg/ml**	**75mg/ml**	**100mg/ml**
Extract	3	4	5	6
Solvent	147	146	145	144
DPPH (1mM)	50	50	50	50
Total volume	200	200	200	200

4.3 Microfluidic CD operations

4.3.1 Sequential loading and mixing of the microfluidic CD

Figure 4.1 shows the actual images from the complete process of microfluidic CD operations, sequential loading, and mixing. For display purposes, the microfluidic CD was fabricated using clear PMMA. The schematic illustrations on the left side are drawn to show and explain the experiments, with corresponding images captures from the video of the high-speed camera. In the first step, Figure 3a has shown the liquids have been preloaded into the designated chambers i.e., DPPH solution in the DPPH chamber, plant extract solution in the plant extract chamber, and solvents in the reaction chamber. To start the experiment, the speed of the centrifugal motor was increased slowly. During this process, the plant extract solution started to flow into the capillary valve. This process is shown in Figure 3b. Subsequently, after the rotation speeds have reached 300 rpm, all the plant extract chambers have been emptied and the solution has been mixed in the reaction chamber with the solvent (as shown in Figure 3c). In Figure 3d, the speed was increased, which makes the DPPH solution flow out from its chamber to the

capillary valve. At 800 rpm, it was noticed that all the DPPH solution was emptied from the chamber as the solution moved to the reaction chamber, which is shown in Figure 3e. Finally, in Figure 3f the centrifugal speed was increased to 1400 rpm to ensure all the solutions were mixed properly.

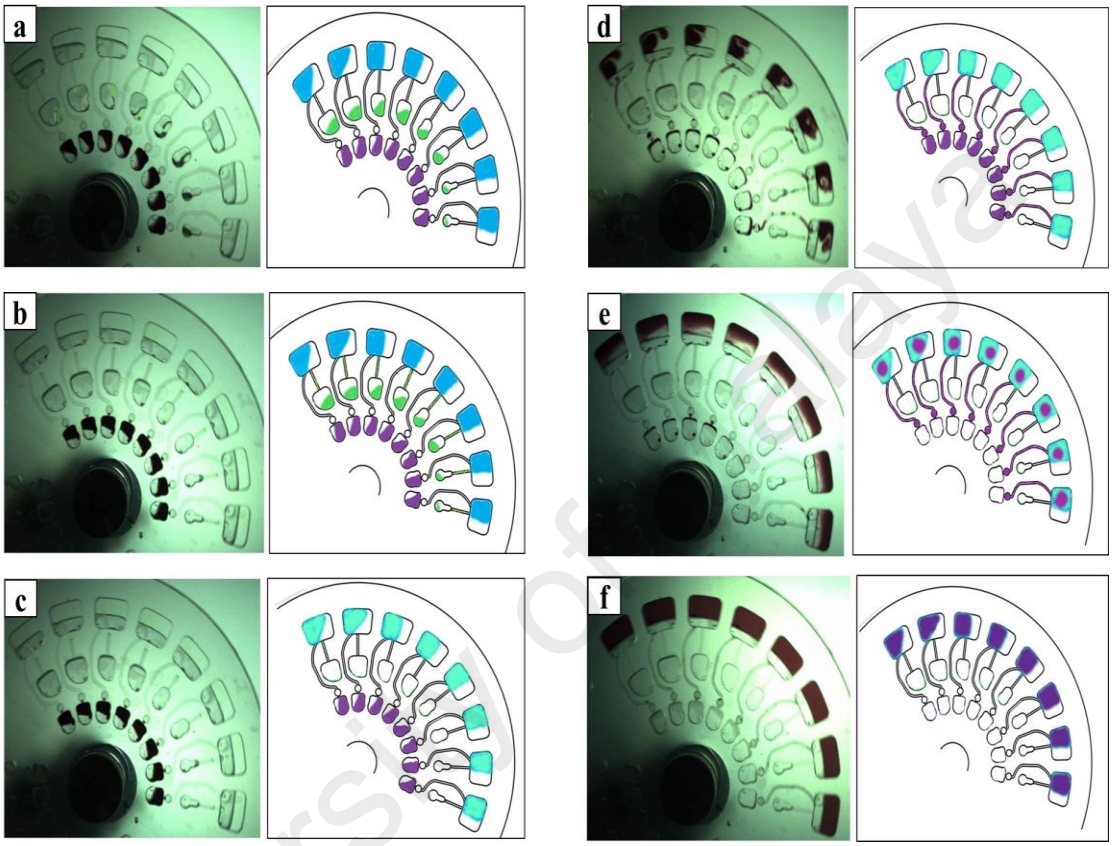

Figure 4.1: Entire sequences of the images and schematic illustrations in LoD antioxidant activity. (a) The initial of the experiment, speed 0. (b) The plant extract solution started to flow into the capillary valve; speed has been increased from 0 to 300 rpm. (c) The plant extract chambers have been emptied; speed 300 rpm. (d) DPPH solution flow out from its chamber to the capillary valve; speed has been increased slowly from 300 to 800 rpm. (e) The DPPH chamber was emptied; speed 800 rpm. (f) All of the solutions were mixed properly in the reaction chamber; speed 1400 rpm.

Our proposed method has minimised human operations in the DPPH antioxidant test, the repetitive pipetting, loading and mixing steps can be skipped by flow control

mechanism of the microfluidic CD. Basically, in our microfluidic CD design, the capillary passive valves were used to control the sequential fluidic flow and mixing of the liquids. The manipulation of the chamber position, centrifugal force and the capillary valve have determined the sequences of fluids flows to the reaction chamber, the details and analysis of the valves burst frequency is described by Thio *et al*. (Thio et al., 2013) and Kazemzadeh *et al.* (Kazemzadeh, Ganesan, Ibrahim, He, & Madou, 2013).

Thio *et al.* and Kazemzadeh *et al.* in their paper have been discussed extensively about the theory of the liquid flows inside the passive capillary valves toward the target chamber (reaction chamber). In order of the liquid to move from one chamber to another, the capillary pressure need to be overcome by increasing the speed of the motor (centrifugal pressure will have increased parallelly). In this study, the location and the geometry of the plant extract chamber and the DPPH chamber on the microfluidic CD have enable the liquid flows manipulation into the reaction chamber.

4.3.2 Assessments of quality system mixing

Figure 4.1(e) to 4.1(f) shows the homogenisation of a colored and non-colored liquid by shake-mode (or batch-mode) mixing progress technique on the microfluidic disc. Batch-mode mixing is based on inertia from abrupt changes in the disc spinning speed that creates chaotic advections inside the reaction chamber. To mix reagents in the disc spinning speed was alternated between 1400 rpm (clockwise) and -1400 rpm (anticlockwise) with acceleration for 2s. To visually assess the mixing efficiency of the system, we employed a stroboscope and a high-speed camera to observe the homogenisation degree of the coloured and non-coloured liquid during the shake-mode mixing (Figure 4.1(f)).

4.4 Comparisons of conventional and LoD DPPH antioxidant activity method

In this section, the comparison results of the conventional and LoD DPPH antioxidant methods are shown and discussed. Standard solutions of ascorbic acid and quercetin, were tested for antioxidant activity by using LoD method and compared with the conventional DPPH antioxidant activity test for 30 minutes (Brand-Williams, Cuvelier, & Berset, 1995). The LoD method was also compared to the conventional DPPH antioxidant activity test by using extracts from *A. catechu, P. minus* and *S. polyanthum.* Subsequently, the results were further analysed in 5 minute intervals up to 30 minutes. For the purposes of analysis, a repeated ANOVA measure by IBM SPSS statistical software version 24 was used to analyse the mean, standard error (SE), standard deviation (SD) and significant values between the time and concentrations of the conventional and LoD methods.

4.4.1 Ascorbic acid

In figure 4.2(a), the results showed that there was a significant difference of *$p \leq 0.05$ between the conventional and LoD method at each concentration. The result was then further analysed as presented in figure 4.2(b), which shows the comparisons of the conventional and LoD method for every 5 minute interval up to 30 minutes' time for each concentration. In figure 4.2, it can be seen that, the DPPH activity value with the LoD method at 10 minutes gave the same activity level as the conventional method for 25 mg/ml, 50 mg/ml and 75 mg/ml ascorbic acid at 30 minutes. On the other hand, 100 mg/ml with the LoD method of ascorbic acid took only 5 minutes to reach the antioxidant activity level of conventional method equal to 30 minutes. On average, the LoD method tested with ascorbic acid was able to produce similar measurement compared to the conventional method with an analysis reduction time of 21.25 minutes i.e. the LoD method duration was 5 (mins at 100mg/ml) whereas the conventional method took 30 (mins). The mean, SE and SD for the different concentrations and different time intervals were clearly seen in table 4.1. The small values of the SE and SD indicate that the results

is repeatable and precision of the mean value (Altman & Bland, 2005). The values highlighted in the table 4.1 show the significant mean of the LoD method compared to the conventional method at 30 minutes.

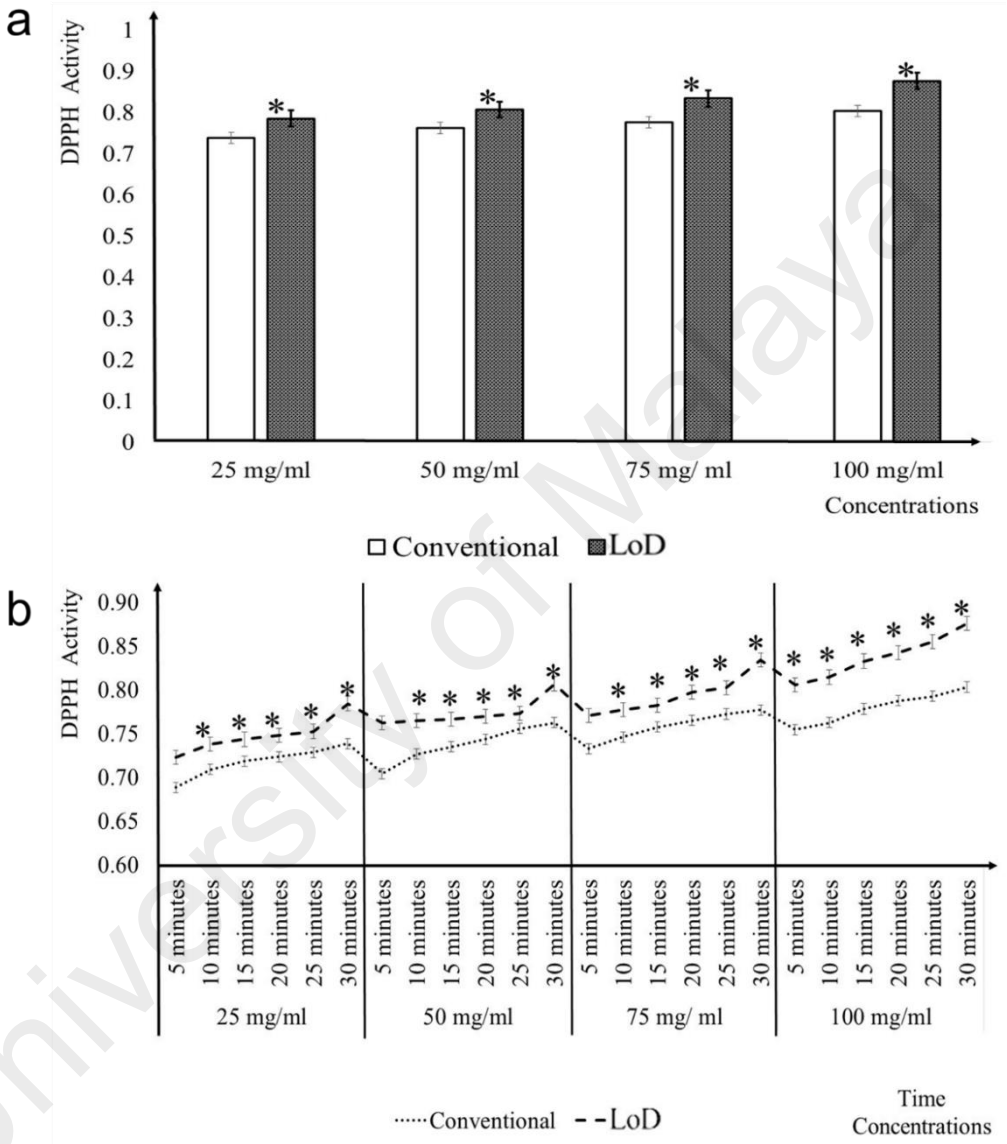

Figure 4.2: Comparison of ascorbic acid antioxidant activity in conventional and LoD method. (a) Comparisons of the ascorbic acid antioxidant activity between conventional and LoD method at each concentration. (b) Comparisons of ascorbic acid antioxidant activity in conventional and LoD method by 5 minute time interval.

Table 4.2: Mean, standard error and the standard deviation of ascorbic acid antioxidant activity

Concentrations		5 minutes		10 minutes		15 minutes		20 minutes		25 minutes		30 minutes		F value	P value
		Con	LoD	Con	LoD	Con	LoD	Con	LoD	Con	LoD	Con	LoD		
25 mg/ml	Mean	0.688	0.723	0.709	0.738	0.718	0.743	0.723	0.748	0.728	0.753	0.738	0.784		
	SE	0.001	0.001	0.000	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001		
	SD	0.001	0.002	0.001	0.002	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001		
50 mg/ml	Mean	0.704	0.760	0.726	0.765	0.735	0.767	0.744	0.769	0.756	0.773	0.762	0.807		
	SE	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001		
	SD	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.002	0.001	0.001	0.001	0.002	93.332	P≤ 0.05
75 mg/ml	Mean	0.733	0.771	0.746	0.777	0.758	0.782	0.765	0.797	0.772	0.802	0.777	0.834		
	SE	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001		
	SD	0.002	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.002	0.001		
100mg/ml	Mean	0.754	0.806	0.762	0.814	0.778	0.833	0.787	0.842	0.792	0.855	0.803	0.876		
	SE	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001		
	SD	0.002	0.002	0.001	0.001	0.002	0.002	0.001	0.001	0.001	0.001	0.001	0.001		

4.4.2 Quercetin

In figure 4.3(a), the results showed that there was a significant difference of *p≤ 0.05 between the conventional and LoD method at each concentration. The results were then further analysed in figure 4.3(b), which shows the comparisons of the conventional and LoD method for every 5 minute interval up to 30 minutes time for each concentration. The analysis shows that, its takes 20 minutes for 25 mg/ml and 15 minutes for 50 mg/ml and 75 mg/ml of quercetin to give the comparable antioxidant activity level of the DPPH conventional methods at 30 minutes. However, for 100 mg/ml quercetin it only took 10 minutes to give the same antioxidant activity level as the DPPH conventional methods at 30 minutes. On average, the LoD method accelerated the analysis time for quercetin by 15 minutes compared to the conventional methods at 30 minutes. The mean, SE and SD for the different concentrations and different time intervals can be clearly seen in table 4.2. The values highlighted in the table 2 show the significant mean of the LoD method compared to the conventional method at 30 minutes.

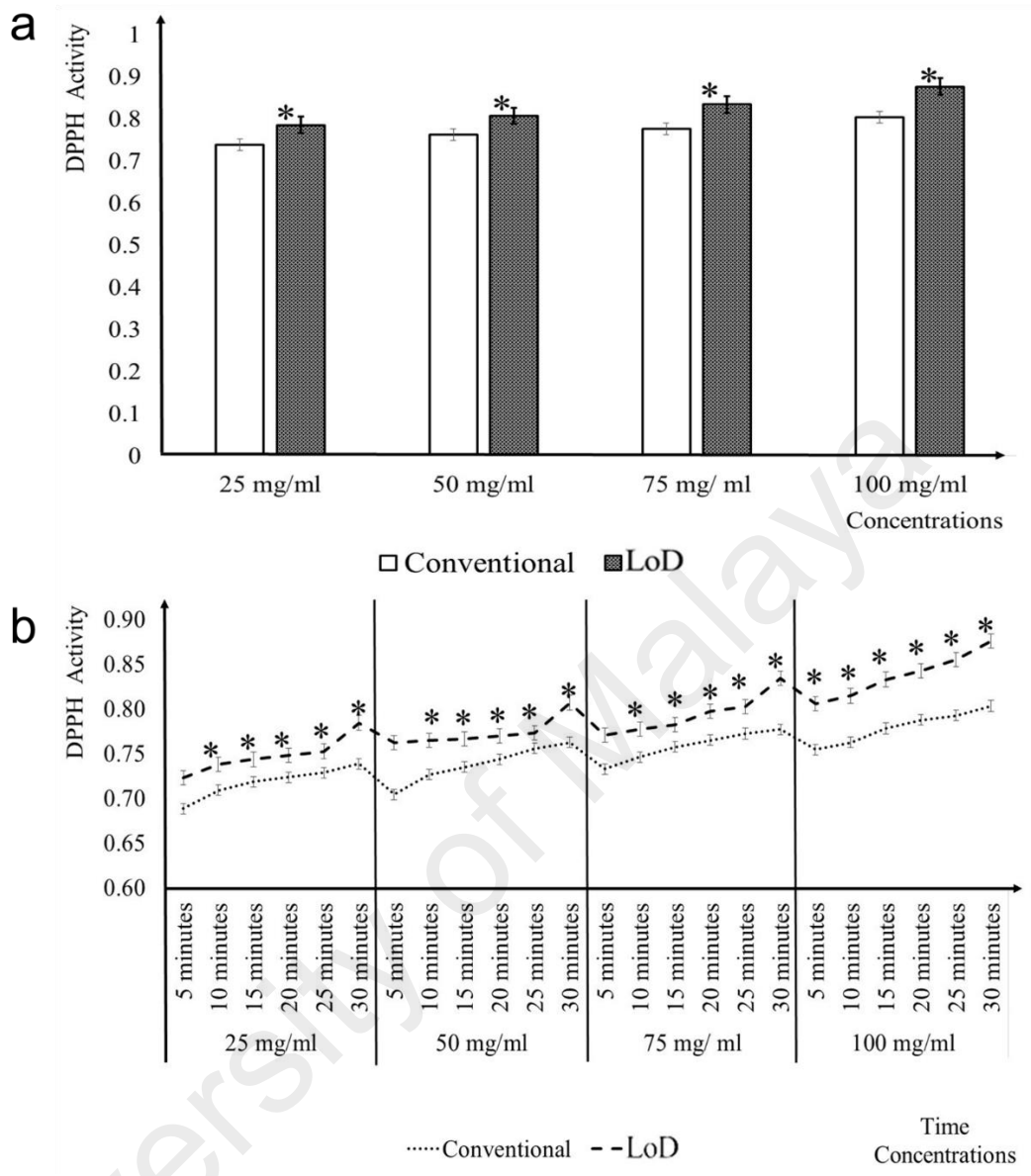

Figure 4.3: Comparison of quercetin antioxidant activity determined using conventional and LoD methods. (a) Comparisons of the quercetin antioxidant activity between conventional and LoD method at each concentration. (b) Quercetin antioxidant activity obtained using conventional and LoD methods at 5 minute time intervals

Table 4.3: Mean, standard error and the standard deviation of quercetin antioxidant activity

Concentrations		5 minutes		10 minutes		15 minutes		20 minutes		25 minutes		30 minutes		F value	P value
		Con	LoD	Con	LoD	Con	LoD	Con	LoD	Con	LoD	Con	LoD		
25 mg/ml	Mean	0.114	0.154	0.126	0.173	0.138	0.185	0.153	0.204	0.183	0.214	0.195	0.297	82.495	p≤ 0.05
	SE	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001		
	SD	0.001	0.002	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001		
50 mg/ml	Mean	0.346	0.356	0.343	0.376	0.351	0.384	0.358	0.392	0.371	0.403	0.377	0.424		
	SE	0.011	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001		
	SD	0.039	0.001	0.002	0.002	0.002	0.001	0.001	0.001	0.002	0.002	0.002	0.002		
75 mg/ml	Mean	0.352	0.362	0.357	0.381	0.362	0.392	0.374	0.398	0.380	0.416	0.391	0.431		
	SE	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001		
	SD	0.001	0.002	0.001	0.002	0.002	0.003	0.002	0.001	0.001	0.001	0.002	0.002		
100mg/ml	Mean	0.417	0.444	0.423	0.462	0.433	0.473	0.442	0.483	0.449	0.499	0.462	0.535		
	SE	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001		
	SD	0.001	0.002	0.002	0.002	0.002	0.001	0.001	0.002	0.001	0.003	0.002	0.002		

4.4.3 *A. catechu*

In figure 4.4(a), the results showed that there was a significant difference of *p≤ 0.05 between the conventional and LoD method at each concentration. The result was then further analysed in figure 4.4(b), which shows the comparisons of the conventional and LoD method every 5 minutes up to 30 minutes time for each concentration. The analysis showed that its took 20 minutes and 15 minutes for 25 mg/ml and 50 mg/ml, respectively, to give the same antioxidant activity level as the DPPH conventional methods at 30 minutes. Meanwhile, for 75 mg/ml and 100 mg/ml, at 10 minutes the LoD method was sufficient to give the same antioxidant activity as the DPPH conventional methods at 30 minutes. On average, the LoD method tested with *A. catechu* plant extract gave a result that was 16.25 minutes faster compared to the conventional methods at 30 minutes. The mean, standard error (SE) and standard deviation (SD) for the different concentrations and different time intervals can be clearly seen in table 4.3. The values highlighted in the table 4.3 show the significant mean of the LoD method compared to the conventional method at 30 minutes.

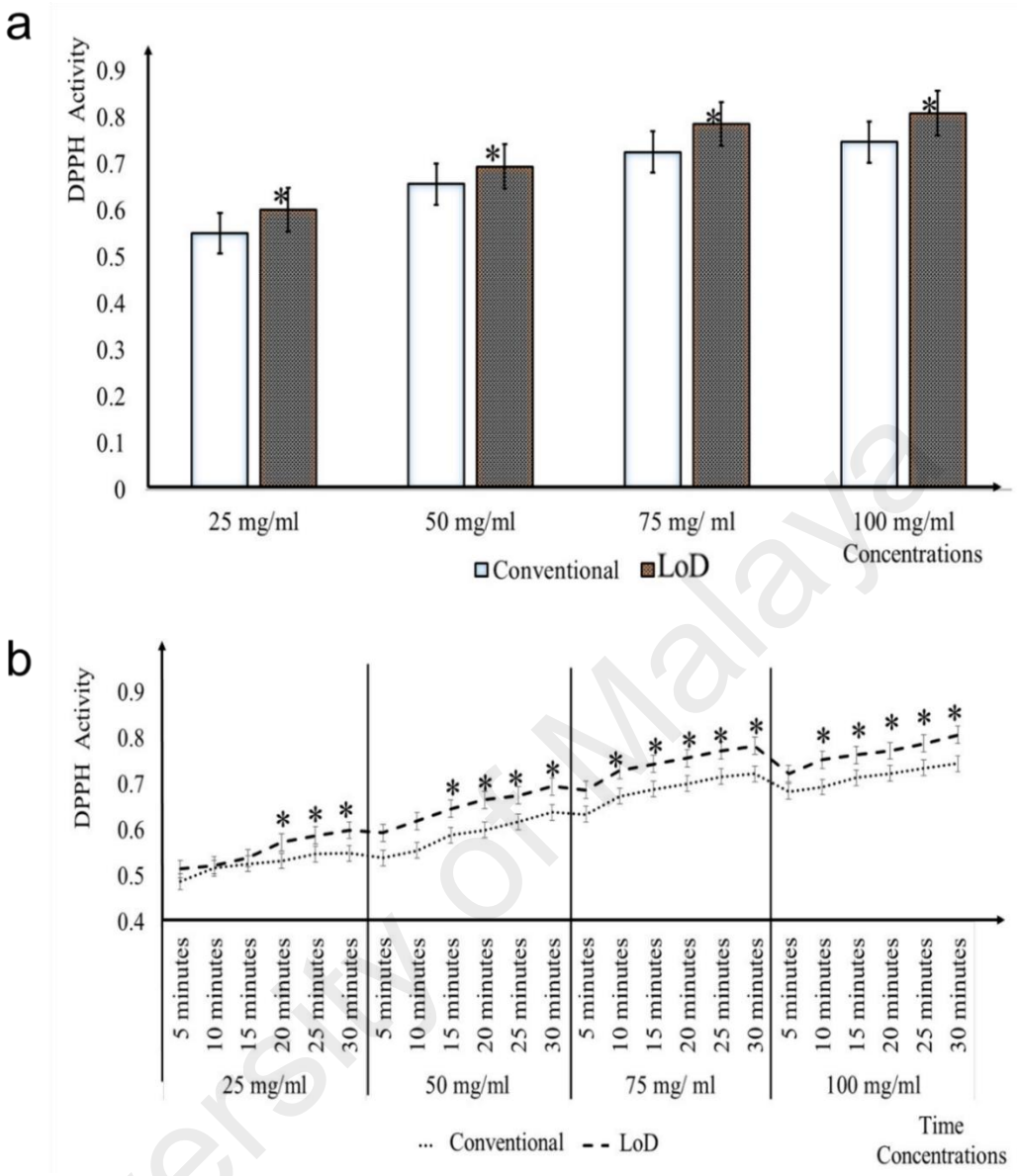

Figure 4.4: Comparison of *A. catechu* antioxidant activity determined using conventional and LoD methods. (a) Comparisons of the *A. catechu* antioxidant activity between conventional and LoD method at each concentration. (b) Comparison of *A. catechu* antioxidant activity in conventional and LoD method by 5 minutes time interval.

Table 4.4: Mean, standard error and the standard deviation of *A. catechu* antioxidant activity

Concentrations		5 minutes		10 minutes		15 minutes		20 minutes		25 minutes		30 minutes		F value	P value
		Con	LoD	Con	LoD	Con	LoD	Con	LoD	Con	LoD	Con	LoD		
25 mg/ml	Mean	0.487	0.515	0.516	0.523	0.526	0.539	0.533	0.573	0.547	0.588	0.549	0.599	45.528	p≤ 0.05
	SE	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001		
	SD	0.001	0.001	0.002	0.002	0.001	0.001	0.001	0.002	0.001	0.002	0.002	0.002		
50 mg/ml	Mean	0.539	0.593	0.555	0.619	0.588	0.646	0.600	0.666	0.617	0.675	0.638	0.694		
	SE	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001		
	SD	0.001	0.001	0.002	0.002	0.002	0.002	0.002	0.002	0.001	0.002	0.002	0.002		
75 mg/ml	Mean	0.634	0.687	0.673	0.730	0.689	0.743	0.700	0.756	0.716	0.772	0.722	0.783		
	SE	0.001	0.000	0.001	0.001	0.001	0.001	0.001	0.001	0.000	0.000	0.001	0.001		
	SD	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.001	0.001	0.002	0.004		
100mg/ml	Mean	0.684	0.722	0.693	0.752	0.713	0.763	0.723	0.772	0.735	0.788	0.744	0.807		
	SE	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001		
	SD	0.002	0.002	0.002	0.002	0.002	0.002	0.001	0.002	0.002	0.001	0.002	0.002		

4.4.4 *P. minus*

The results in figure 4.5(a) showed that there was a significant difference of *p≤ 0.05 between the conventional and LoD method at each concentration. The result was further analysed in figure 4.5(b), which shows the comparisons of the conventional and LoD method every 5 minutes for up to 30 minutes time for each concentration. The analysis shows that 5 minutes for 25 mg/ml, 50 mg/ml, 75 mg/ml and 100 mg/ml in the LOD method are sufficient to give the same antioxidant activity as the same concentrations at 30 minutes for the DPPH conventional methods. The mean, SE and SD for different concentrations and different time intervals can be clearly seen in table 4.4. The values highlighted in the table show the significance mean of the LoD method compared to the conventional method at 30 minutes.

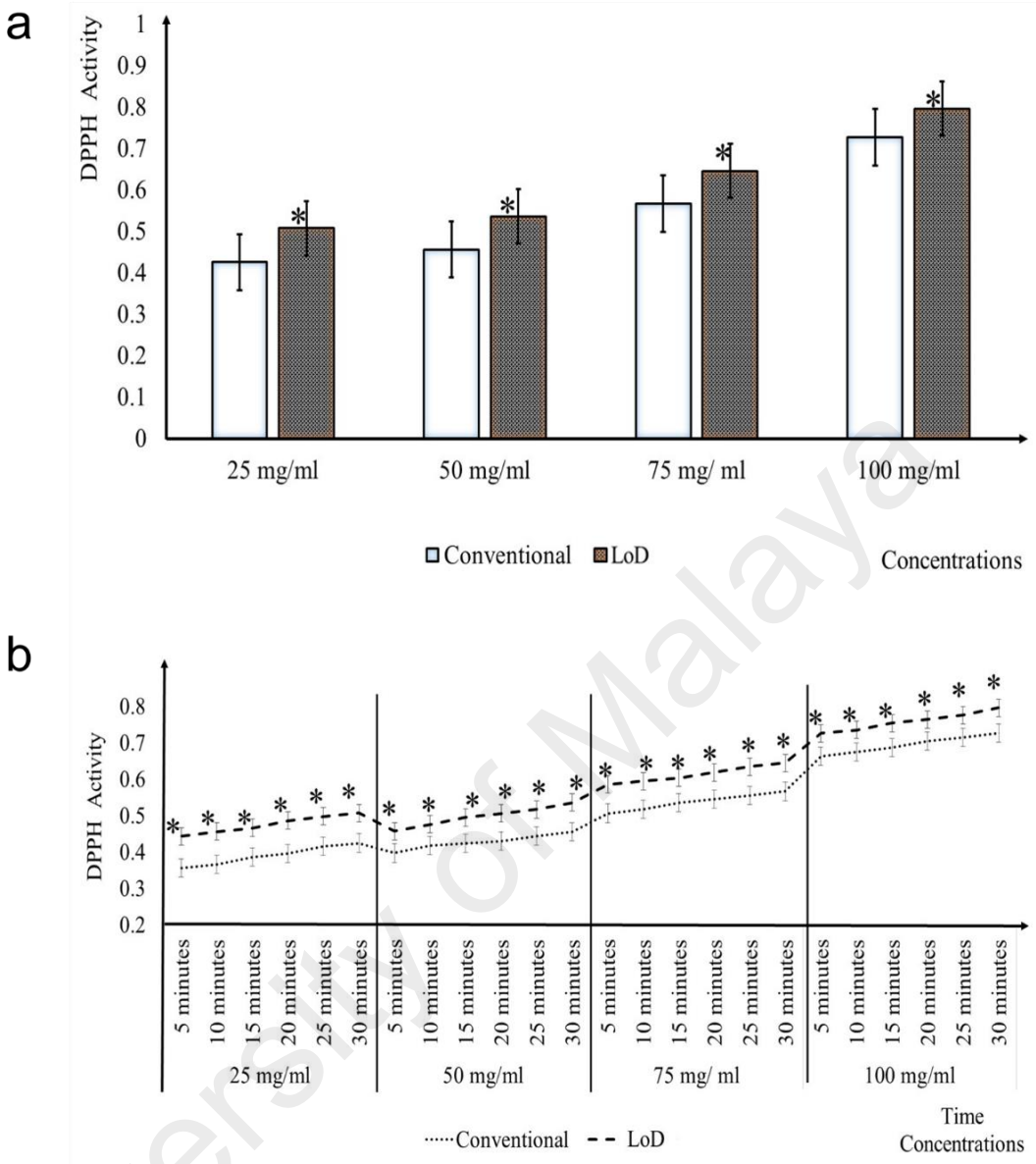

Figure 4.5: Comparison of P. minus antioxidant activity determined using conventional and LoD methods. (a) Comparisons of the P. minus antioxidant activity between conventional and LoD method by concentration. (b) Comparison of P. minus antioxidant activity in conventional and LoD method by 5 minutes time interval in each concentration

Table 4.5: Mean, standard error and the standard deviation of *P. minus* antioxidant activity

Concentrations		5 minutes		10 minutes		15 minutes		20 minutes		25 minutes		30 minutes		F value	P value
		Con	LoD	Con	LoD	Con	LoD	Con	LoD	Con	LoD	Con	LoD		
25 mg/ml	Mean	0.355	0.443	0.365	0.456	0.385	0.466	0.396	0.486	0.415	0.497	0.424	0.507	24.299	p≤ 0.05
	SE	0.000	0.001	0.001	0.000	0.001	0.001	0.000	0.001	0.000	0.023	0.000	0.000		
	SD	0.001	0.002	0.002	0.001	0.004	0.002	0.001	0.002	0.001	0.079	0.001	0.002		
50 mg/ml	Mean	0.397	0.457	0.417	0.476	0.424	0.495	0.435	0.505	0.443	0.516	0.455	0.535		
	SE	0.000	0.000	0.000	0.000	0.001	0.000	0.000	0.000	0.001	0.001	0.001	0.001		
	SD	0.001	0.001	0.001	0.001	0.003	0.002	0.002	0.002	0.002	0.002	0.002	0.002		
75 mg/ml	Mean	0.506	0.585	0.517	0.594	0.534	0.603	0.546	0.618	0.555	0.634	0.566	0.644		
	SE	0.001	0.000	0.001	0.000	0.000	0.001	0.001	0.001	0.001	0.001	0.001	0.001		
	SD	0.002	0.002	0.002	0.002	0.002	0.002	0.003	0.003	0.002	0.002	0.002	0.002		
100mg/ml	Mean	0.663	0.727	0.675	0.735	0.687	0.754	0.704	0.764	0.715	0.776	0.726	0.795		
	SE	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001		
	SD	0.002	0.004	0.003	0.004	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.003		

4.4.5 *S. polyanthum*

The results in figure 4.6(a) indicate that there was a significant difference of *$p \leq 0.05$ between the conventional and LoD method at each concentration. The result was then further analysed in figure 4.6(b), which shows the comparisons of the conventional and LoD method every 5 minutes for up to 30 minutes time for each concentration. Referring to table 4.5, the results show that the LoD method was able to give the same antioxidant level as early as 5 minutes at 50 mg/ml, 75 mg/ml and 100 mg/ml. By using the LoD method, it is believed with the increase of the concentrations, the time to reach the same activity level as the conventional method will be decreased. On average, the LoD method tested with *S. polyanthum* plant extract gave a result that was 21.25 minutes faster compared to the conventional methods.

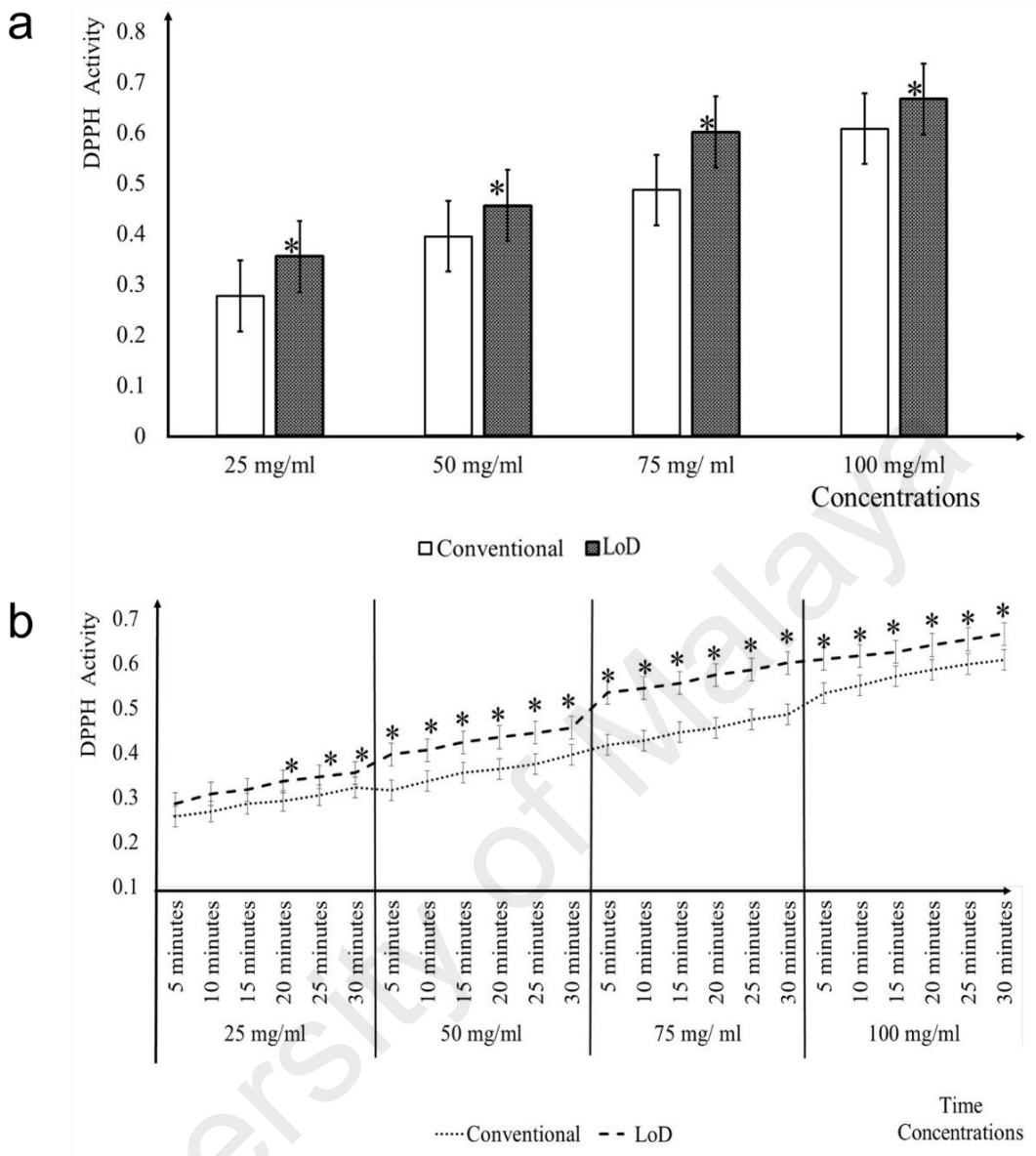

Figure 4.6: Comparison of S. polyanthum antioxidant activity in conventional and LoD method. (a) Comparisons of the S. polyanthum antioxidant activity between conventional and LoD method by concentration. (b) Comparison of S. polyanthum antioxidant activity in conventional and LoD method by 5 minutes time interval.

Table 4.6: Mean, standard error and the standard deviation of *S. polyanthum* antioxidant activity

Concentrations		5 minutes		10 minutes		15 minutes		20 minutes		25 minutes		30 minutes		F value	P value
		Con	LoD	Con	LoD	Con	LoD	Con	LoD	Con	LoD	Con	LoD		
25 mg/ml	Mean	0.256	0.284	0.267	0.307	0.284	0.316	0.291	0.335	0.304	0.345	0.321	0.354	20.366	P≤ 0.05
	SE	0.001	0.001	0.001	0.001	0.000	0.001	0.001	0.001	0.001	0.001	0.001	0.001		
	SD	0.002	0.002	0.002	0.002	0.001	0.002	0.002	0.002	0.002	0.002	0.003	0.003		
50 mg/ml	Mean	0.315	0.395	0.335	0.405	0.354	0.422	0.363	0.434	0.374	0.444	0.394	0.455		
	SE	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001		
	SD	0.002	0.002	0.002	0.003	0.002	0.003	0.003	0.002	0.002	0.002	0.003	0.002		
75 mg/ml	Mean	0.417	0.534	0.426	0.544	0.445	0.555	0.455	0.573	0.473	0.585	0.485	0.6		
	SE	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.000	0.001	0.001	0.001		
	SD	0.002	0.002	0.003	0.002	0.002	0.003	0.003	0.002	0.001	0.003	0.002	0.003		
100mg/ml	Mean	0.532	0.609	0.549	0.616	0.571	0.625	0.584	0.64	0.597	0.653	0.607	0.665		
	SE	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.000	0.001		
	SD	0.003	0.003	0.002	0.005	0.004	0.002	0.002	0.002	0.004	0.002	0.002	0.003		

In figure 4.2(a), 4.3(a), 4.4(a), 4.5(a) and 4.6(a), the comparisons of the ascorbic acid, quercetin, *A. catechu*, *P. minus* and *S. polyanthum* activity antioxidant has been presented between conventional and LoD method at each concentration. The results showed that there was a significant difference of $*p \leq 0.05$ between the conventional and LoD method at each concentration. What can be clearly observed in figure 4.2(a), 4.3(a), 4.4(a), 4.5(a) and 4.6(a), are the levels of the DPPH activity in LoD method are consistently higher than the conventional method. In conventional method, the reaction is let to stand for 30 minutes without any force applied on it. Whereas, in the LoD method, mixing scheme has been applied which involves the combination of centrifugal force, Euler force and Coriolis force. Centrifugal and Euler force is important for the automated liquid handling processing. While Coriolis force creates the stirring effect in the reaction chamber which lead to the better liquid homogenisation and diffusion of the particles.

Efficacious sample mixing accelerate the chemical reaction and decrease the time of assay (Tang et al., 2016). In chemical reaction, the ability to create a fast-homogenous reactant mixture is crucial especially in the small sample volume. For a rapid chemical reactions to occur, a fast kinetics reaction and high contact frequency between particles can be increased with a good mixing efficiency which this phenomena has been provided in the LoD platform (Demello, 2006; Mark, Haeberle, Roth, Von Stetten, & Zengerle, 2010). Our proposed method has introduced a passive mixer which maximise the area of the chemical reaction to occur in the reaction chamber. With the constant application of Coriolis stirring effect by the CD rotation, the chemical reaction between the plant extract and the DPPH has been enhanced which in contrast does not happen in the conventional method.

The results has been shown in figures 4.2(b), 4.3(b), 4.4(b), 4.5(b) and 4.6(b), where the reading does not reached plateau state in the microfluidics CD method, contrast the finding with Phonchai *et al*. which have shown a plateau DPPH activity reading with

respective mixing time (Phonchai et al., 2016). However, in contrast many researchers have also agreed that the kinetics reaction in DPPH assay may persist from minutes to hours. Although it is recommendable to allow the reaction and measurement until the plateau reading state, the diverse completion kinetic reaction time in DPPH have led us to use 30 minutes as a single standard measuring time (Brand-Williams et al., 1995; Molyneux, 2004; Papariello & Janish, 1966). Additionally, Xie *et al*. (J. Xie & K. Schaich, 2014) have illustrated that with regardless of the incubation time, the assay is definitely reflects the stoichiometry DPPH reaction with the antioxidant in the sample.

Referring to the figures 4.2(b), 4.3(b), 4.4(b), 4.5(b) and 4.6(b), it can be clearly seen that the time taken to measure the DPPH activity varies among different samples. For example, the analysis of 25mg/ml solutions of ascorbic acid and quercetin using LoD method was able to give the comparable DPPH antioxidant activity to the conventional method at 10 and 20 minutes respectively, meanwhile the LoD method reduced the time taken to analyse P. minus to 5 minutes, compared to the standard 30 minutes of the conventional method. This result may be explained by the fact that the purity, compound mixtures, different form and weight of molecules present in a sample did play a role for diverse antioxidant activity. In this study, the plant extract have been used to test the LoD method, the presence of compound such as phenol may cause interference and affect the reading compared to the actual activity which expound the different activity time between each plant (Lopez-Alarcon & Denicola, 2013; Schaich, Tian, & Xie, 2015).

The conventional DPPH method use a high concentration of the plant extract (i.e. 100, 200, 400, 800 mg/ml) which proportionally need large reagent volumes. The proposed method reduced the volume usage to the final of 200 µl to provide a good range of detection as the conventional method (Phonchai et al., 2016). In conventional method, the standard time to measure the DPPH activity is 30 minutes (Brand-Williams et al., 1995). As mentioned above, one of the major advantage of LoD method is to accelerate the

chemical reaction and decrease the time of assay. In our experiments, the time interval of 5 minutes up to 30 minutes has been chosen as the parameters to access at what time the LoD method will give the same results of DPPH conventional activity of 30 minutes.

However, in order to standardised our method (due to different analysis time), the method by Jung *et al* (Jung et al., 2006) where they had predicted the main antioxidant component present in the sample by comparing the reaction time to the standard pure antioxidant reaction time can be used. As mentioned in their paper, they are comparing the mugwort plant reaction time which is identical to the ascorbic acid reaction time. This method can be implemented in our proposed system by comparing the different time analysis in plants to the standard antioxidant time analysis. Nevertheless, more standard antioxidants should be tested by using our system to make a benchmark of the time analysis.

Figure 4.7 shows a fitted correlation of the original conventional method and the proposed method reduced volume. The decreased amount of reagent and sample used in the experiments offer an optimal advantage for the rare samples and limited reagent supply.

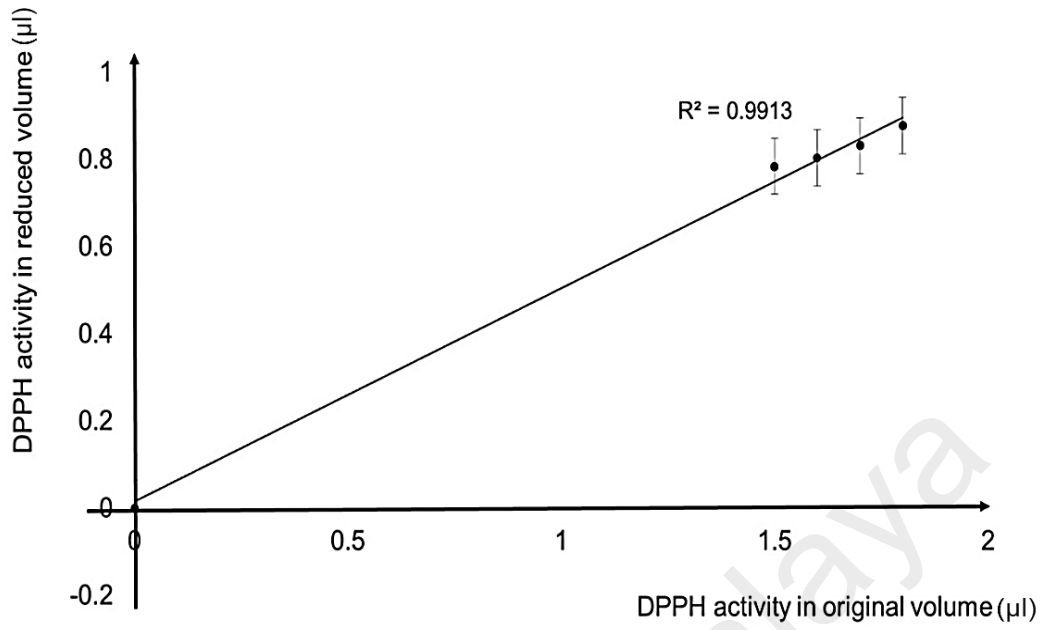

Figure 4.7: DPPH activity correlation between the original and reduced volume in proposed method.

4.5 Comparisons of CD reader and the conventional microplate reader

The experimental work was carried out with a modified version of custom-made CD spin test system (Thiha & Ibrahim, 2015; Uddin et al., 2015). The readings were taken after a 30 minutes incubation for both the proposed LoD method and the conventional DPPH method. The data in fig 9 shows a fitted coefficient and correlations (r2=0.96) between the reading from the CD reader and the microplate reader, indicating that a close relationship between the CD reader and the microplate reader. Unlike the conventional method, the CD reader can read the absorbance of the entire sample directly from the microfluidic CD. It is designed specially to work in complement with the microfluidic CD, and the pipetting process of transferring the solutions to the 96 well plate has be eliminated. The process of transferring the solutions to the 96 well plate risks losing volume, which can be rectified by a closed and automated system, such as that used in the LoD platform. This CD reader endpoint reading system complete and support to the development of an integrated platform for the photoprotective LoD system.

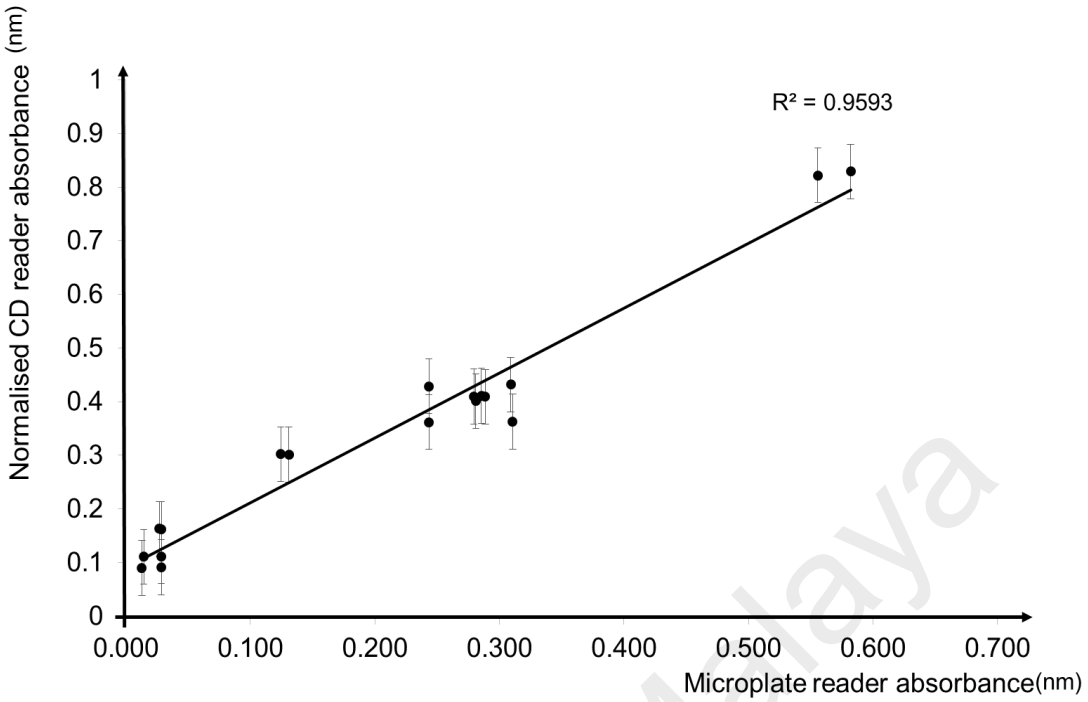

Figure 4.8: Graph of coefficient correlation between the CD plate reader and the microplate reader

4.6 Summary

In this chapter, the experimental results of the microfluidic CD for the DPPH assay were presented and analysed thoroughly. Five samples which consists of two standard antioxidant and three plant extract has been used in the integration of microfluidic CD-DPPH experiments. Generally, the results showed that the microfluidic CD technology can be used in the antioxidant assay. The system has simplified and automate the conventional DPPH assay with the "load and run" concept with shorter time advantage. Furthermore, the sequential liquid loading and mixing method proposed in the microfluidic CD-DPPH operation system has showed that the designed microfluidic CD is able work in a automate close system in a smaller volume in compare to the conventional method.

CHAPTER 5: CONCLUSION AND FUTURE WORK

5.1 Introduction

The aim of this study was to develop a microfluidic CD to expedite the DPPH antioxidant assay automation. Consequently, this dissertation served to develop "all in one close system antioxidant microfluidic CD" which helps to:

i. Reduced the incubation time of the test from routine duration of 30 minutes to minimum of 5 minutes
ii. Automate the conventional antioxidant assay into portable centrifugal microfluidic platform with smaller sample volume
iii. Minimised human handling in the test samples, especially reducing repetitive pipetting, mixing and loading with four parallel concentrations

5.2 Conclusion

The work describes the integration of DPPH antioxidant test procedure into the microfluidic compact disk (CD) with photoprotective features that protected the DPPH free radical solution and the antioxidant reagent. This combination has resulted in better reading and faster incubation time in compared to the conventional method. We had used ascorbic acid, quercetin, *A. catechu, P. minus,* and *S. polyanthum* plant extracts to compare the results of our proposed LoD method with the conventional method. Contrasted to the arduous laborious conventional method, our proposed method offers rapid analysis and simple determination of antioxidant.

The sequential sample loading and mixing in a closed LoD system minimises human error and volume loss due to manual pipetting procedures. The "load and run concept" in the proposed LoD method has omit the repetitive pipetting, mixing and loading steps in the DPPH conventional method. . With four parallel concentrations and duplications that able been run simultaneously on the microfluidic CD, this study has shown that the

proposed LoD method for antioxidant activities of the plant extracts procedures has been automated. Using this LoD technique not only it automates the processes, but also helps to reduce the incubation time to five minutes, to reach the same antioxidant activity level as the conventional method of 30 minutes. Moreover, we also found that higher antioxidant activity was obtained using the proposed method, compared to the conventional method. This indicated that the reduced experimental time and manual handling protected the quality of the DPPH solutions from degradation.

The issues of light sensitivity and stability faced by DPPH has been addressed by using black PMMA in the microfluidic CD fabrication. It can also be stated that by introducing the CD reader in place of the microplate reader helps the experiment to be more cost-efficient in analysing the outcome of the antioxidant activity test. This new approach can work as a fundamental study of antioxidant activity and will act as a platform for the determination of future antioxidant activity in a plants or clinical samples.

5.3 Recommendation for future work

In terms of directions for future research, some recommendations are listed below:

i. Improve the preloading steps in the experiments for easier implementations.

ii. Embedding powdered DPPH reagent in the microfluidic CD

iii. The research can be extended for another photoprotective assay to protect photosensitive reagent.

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LIST OF PUBLICATION AND PAPER PRESENTED

1. **Abd Rahman, N.**, Ibrahim, F., Aeinehvand, M. M., Yusof, R., & Madou, M. (2018). A Microfluidic Lab-on-a-Disc (LOD) for Antioxidant Activities of Plant Extracts. Micromachines, 9(4), 140. Q2, Impact Factor 2.22.

2. **Rahman, N. A.**, Ibrahim, F., Ainehvand, M. M., Yusof, R., & Madou, M. (2017, December). An Effect of Magnetic Beads to Boesenbergia rotunda Antioxidant Activity Using Photoprotective Microfluidic CD. In International Conference for Innovation in Biomedical Engineering and Life Sciences (pp. 139-144). Springer, Singapore. **Young Investigator Award by International Federation of Medical and Biological Engineering.**