MONASH University

DRYING EFFECTS ON THE BIOACTIVITY OF *PIPER NIGRUM* LINN. BERRIES

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DECLARATION

This thesis is an original work of my research and contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

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Every new project involves a journey that begins with the seed of an idea and travels its own unique path until its final creation in the world. I love the thrill of the journey, with its unexpected twists and turns, surprises, excitement and joy. But more than anything else, when I look back on the journey I have taken I am completely humbled by the number of incredible people who had played a tremendous role in bringing the project into the world. When I first sit down to write this wonderful thesis, the creation begins as a joyful yet solitary one; there is just UNIVERSE and ME. Gradually, the circle begins to widen, encompassing more and more people, all whom contribute their expertise from beginning to end, and yes, I would like to honour the extraordinary people who had made my dream possible. The following people were a part of the ever-widening circle of this amazing journey of a notable creation.

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ABSTRACT

Piper nigrum Linnaeus, also known as black pepper is an important commercial spice valued since early time for its pungency and flavour. It is now gaining popularity due to its health promoting benefits, especially based on its strong antioxidant, antimicrobial and anticancer properties. In this study, extraction of fresh berries of *P. nigrum* by 30, 50, 70, 100% ethanol and 50% methanol showed that 50% ethanol is the best extraction solvent. Seven drying methods were applied, namely oven 50°C and 100°C drying, ambient air drying, sun drying (with and without blanching in boiling water), microwave drying and freeze drying to assess their effects on the antioxidant, antibacterial properties and phytochemicals of *P. nigrum* berries extracts. Four *in vitro* antioxidant assays were used: Total Phenolic Content (TPC), Free Radical Scavenging (FRS), Ferric Reducing Power (FRP) and Ferrous Ion Chelating (FIC). Disc diffusion and micro-well dilution assays were employed in antimicrobial susceptible testing. Reversed-Phase High Performance Liquid Chromatography (RP-HPLC) was used in bioactive compound identification. This study revealed that microwave and oven 100°C treatments were the better drying method compared to other drying treatments as both increased significantly the antioxidant and antibacterial properties of *P. nigrum.* For the berries dried by the different methods, a significant positive correlation was observed between TPC vs Ascorbic Acid Equivalent Antioxidant Capacity (AEAC), 50% Inhibition concentration (IC_{50}) as well as FRP and 50% chelating (CC_{50}) activities.

The major compound focused in this project in all extracts, fresh and treated, was identified to be piperine by HPLC and Liquid Chromatography Mass Spectroscopy (LC-MS). A positive correlation between the TPC and piperine contents of the *P. nigrum* berries dried by various methods was observed.

The microwave treated berries showed more potent antibacterial activity when compared to the fresh berries with minimal inhibitory concentration (MIC) values ranging 31.25-250 µg/mL. Preliminary antibacterial screening test with disc diffusion assay and the MIC values determined using a 96-well microtitre plate serial dilution technique were tested against both gram-positive (*Staphylococcus epidermis* ATCC 25923) and gram-negative bacteria (*Escherichia coli* ATCC 25922*, Proteus mirabilis* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853. 50% ethanol extract of microwave treated berries showed strongest antibacterial activity against *S. epidermis* with a zone of inhibition 19.0 mm and 1.98 µg/mL

MIC value, followed by *E. coli* (21.7 mm; 3.91 µg/mL MIC value). *P. mirabilis* ATCC 25922 exhibited a moderate antibacterial activity (15.5 mm; 15.63 µg/mL MIC value) and the weakest activity was found against *P. aeruginosa* ATCC 27853 (11.5 mm; 62.5 µg/mL). The strong activity of microwave treated extract against *E. coli* ATCC 25922 was in marked constrast to the positive controls, vancomycin and penicilin which were recorded as not susceptible.

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ABBREVIATION

1D/2D NMR   One dimensional/two-dimensional nuclear magnetic resonance spectroscopy

AA   Ascorbic acid

AEAC   Ascorbic acid equivalent antioxidant capacity

ANOVA   One-way analysis of variance

ATCC   American Type Culture Collection

a_w Water activity

BSAC   British Society for Antimicrobial Chemotherapy

DMSO   Dimethyl sulfoxide

DNA   Deoxyribonucleic acid

DPPH   2,2-diphenyl-1-picrylhydrazyl

EEPN   Ethanolic Extract *Piper nigrum*

EUCAST   European Committee for Antimicrobial Susceptibility Testing

FC   Folin-Ciocalteau

FIC   Ferrous ion chelating

FRP   Ferric reducing power

FRS   Free radical scavenging

GAE   Gallic acid equivalent

LC-MS/MS   Liquid chromatography-tandem mass spectrometry

LDL   Low-density lipoprotein

MRSA   Methicilin-resistant *Staphylococcus aureus*

m/z   Mass to charge ratio

POD   Peroxidase

PPO   Polyphenol oxidase

RNS   Reactive nitrogen species

RP-HPLC   Reversed-phase high performance liquid chromatography

ROS   Reactive oxygen species

RTs   Retention times

TPC   Total phenolic content

Tukey's HSD   Tukey's Honestly Significant Difference

UPLC-Tof-MS   Ultra-performance liquid chromatography-time of flight mass spectrometry

CHAPTER 1

INTRODUCTION

1.1 Spices

A spice is a dried seed, fruit, root, bark or flower of a plant or herb used in small quantities for flavor, color or as a preservative. Spices are good sources of pharmaceutical compounds (Makkar et al., 2009). The use of spices has been challenged over the past century by the mainstream medicine practitioners due to lack of well-defined underlying mechanisms and scientific evidences. However, in recent years, herbs have been staging a comeback with herbal 'renaissance' occurring all over the world. Up till today, spices have not only been consumed as food source, but have been used for medicinal purposes and have been proven to be effective in treating certain diseases. The bioactivities of spices are generally ascribed to the presence of plant secondary metabolites (Makkar et al., 2009), which produce definite physiological actions on human body (Mohammedi and Atik, 2011).

The extracts from spices consist of numerous classes of compounds, among which are terpenoids, flavonoids and phenolics, etc., which are known to be active against bacteria, virus, protozoa and degenerative diseases. The quantities and phytochemical composition of the plant extracts are highly dependent and affected by the extraction method used, time and temperature; the nature, polarity and concentration of the solvent; along with the nature of the plant material, in particular with respect to fresh or dried condition. (Han et al., 2008; Saeed and Tariq, 2008; Jalal et al., 2009; Dai and Mumper, 2010; Tiwar et al., 2011).

1.2 Free Radicals and Antioxidants

Free radicals are usually generated in the body as by-products of turning food into energy or by sunlight's action on the skin and eyes (Serafini and Morabito, 2012). They all have a great appetite and ability to steal electrons from any nearby substances and alter their structures or functions. Although oxidation reactions are vital for life, they can also be damaging. Free radicals can damage and change the instructions of a deoxyribonucleic acid (DNA). They are capable to modify a cell's membrane and change the flow of what enters or leaves the cells. They may also change a circulating low-density lipoprotein (LDL) molecule so that it is

trapped easily in an artery wall (Knight, 2000). Cell damage caused by free radicals has been postulated to lead to such age dependent diseases as atherosclerosis, arthritis, cardiovascular disease, neurodegenerative disorders and cancer (Halliwell et al., 1992; Ames et al., 1993; Percival, 1998; Morsy et al., 2017)

One line of defence against free radical damage is the presence of antioxidants. Antioxidant means "substance against oxidation". An antioxidant is a molecule that preferentially oxidizes itself to neutralize ROS/RNS for protection against oxidative stress. Therefore, antioxidants contribute to the prevention of oxidation degradation and degenerative diseases (Gao et al., 2014). Antioxidants have come to attention since 1990s, when scientists discovered that free radicals were involved in atherosclerosis, vision loss, cancer and some other chronic diseases (Rafieian-Kopaei et al., 2013). They found that people with low intake of vegetables and fruits were at greater possibility for development of these diseases than others. Clinical trials began testing the effect of single antioxidative substances such as vitamin E, vitamin C and beta-carotene, etc, against cancer, heart disease and others.

Although free radicals contribute to chronic diseases such as cancer (Shirzad et al., 2009, Shirzad et al.,2011), diabetes (Behradmanesh et al., 2013), atherosclerosis (Shirzad et al., 2009, Behradmanesh et al., 2013), heart disease, nephrotoxicity, hepatotoxicity, cognitive (Behradmanesh et al., 2012) and vision loss (Bartlett and Eperjesi, 2007), a lot of researches, especially laboratory trials, showed benefits for antioxidants against these conditions; however, long clinical trials are inconclusive and do not show that antioxidant supplements have a substantial impact on these diseases. While it is believed that substances available naturally in vegetables, fruits, spices and grains act as natural antioxidants which help to prevent various critical disorders, it is unlikely that commercially available synthetic antioxidants in all of the conditions can do the same.

1.3 Microbes and Antibiotic Resistance

Another health associated disease of public concern is antibiotic resistance. Recently, many bacterial pathogens are becoming resistant to existing antibiotics due to their indiscriminate use in the treatment of infectious diseases (Morsy and El-Salam, 2017). Therefore, there is exigency to discover new and efficient antimicrobials from other source such as plants (Cordell, 2000; Karamaran et al., 2003). The emergence of multidrug resistance in microbes

and the nonavailability of antibiotics to combat and treat microbial infections have led to a constant search for new antimicrobial agents from natural sources. Previous research has shown that about **13 million deaths** are recorded throughout the world each year as a result of bacterial and fungal diseases that are often caused by multidrug-resistant pathogens (Kourtesi et al., 2013; Abreu et al., 2017). Natural products of plant origin include novel therapeutic and highly effective antimicrobial agents (Subramani, Narayanasamy and Feussner, 2017; Alviano and Alviano, 2009). Alternatively, bioactive compounds from natural sources could also be screened for leads to the discovery of new antibiotics to combat microbial resistance (Abreu et al., 2017; Yang et al., 2018; Mgbeahuruike et al., 2017). Bioactive compounds and extracts from plants can also potentiate the effect of antimicrobial drugs, thereby acting as antibiotic adjuvants (Abreu et al., 2017; Mgbeahuruike et al., 2017).

CHAPTER 2

LITERATURE REVIEW

2.1 Phytochemistry of *Piper* species

Piper species are rich in bioactive compounds and serve as a reservoir for drug discovery. Bioactive compounds such as amides, alkaloids, flavonoids, tannins, saponins, glycosides, terpenoids and phenolic compounds have been widely reported to be present in the seeds, leaves, and stem bark of *Piper* species (Scott et al., 2005, 2008; Tang et al., 2010; Vadlapudi and Kaladhar, 2012). The pharmacological activities of steroids, terpenes, chalcones, dihydrochalcones, and essential oils from *Piper* species indicate that they can serve as health promoting and pharmaceutical agents (Parmar et al., 1997). Amides and alkaloids are present in *Piper* species, these amides which are often referred to as piperamides are present in large quantity in their fruits (Bezerra et al., 2007; Bokesch et al., 2011). Most *Piper* species contain alkaloids, which have strong efficacy in cancer treatment, as documented in the literature (Bezerra al., 2005; Niu et al., 2015), serve as antioxidant and antimicrobial agents (Jeena et al., 2014; Mgbeahuruike et al., 2019). In addition, alkaloids and other bioactive compounds of *Piper* species could help to combat infections and solve the problem of increasing microbial resistance to antibiotics (Vadlapudi and Kaladhar, 2012). They are of great importance as therapeutic agents in drug discovery because of pharmacological discoveries concerning their bioactive compounds.

2.2 An Overview of *Piper nigrum* Linn

Piper nigrum L. (black pepper) belongs to the family Piperaceae. It is a monecious and decorous climbing vine native to southern India and Srilanka and is extensively cultivated there and elsewhere in tropical regions for its fruit (berry). The short climbing stems are very flexible with leathery blackish green leaves (Reshmi et al., 2010). The word "pepper" is derived from the Sanskrit pippali, the word for long pepper, via the Latin piper, which was used by the Romans to refer both to pepper and long pepper (as the Romans erroneously believed that both of these spices were derived from the same plant)(Kunnumakkara et al., 2009). Historically the sole use of black pepper is in the seasoning of food owing to its aroma

and pungency and this spice was exploited for its antimicrobial properties to preserve meat products. Only about 10% of all Piper species (112 of 1,000+ known species worldwide) have been phytochemically investigated. These 112 species have been found to produce 667 different compounds distributed as follows: 190 alkaloids/amides, 49 lignans, 70 neolignans, 97 terpenes, 39 propenylphenols, 15 steroids, 18 kavapyrones, 17 chalcones/ dihydrochalcones, 16 flavones, 6 flavanones, 4 piperolides (cinnamylidone butenolides) and 146 miscellaneous compounds (Dyer, Richards and Dodson, 2004). Secondary metabolites have been found in all parts of the plant, including leaves, stems, roots, and inflorescences (Dyer, Richards and Dodson, 2004). Al-Shahwany (2014) reported the presence of phenolic compounds such as gallic acid,   trans-p-feruloyl-beta-D-glucopyranoside, trans-p-sinapyl-beta-D-glucopyranoside,   quercetin   3-O-beta-D-glucopyranosyl-7-O-alpha-L-rhamnopyranoside,   quercetin   3-O-alpha-L-rhamnopyranoside,   luteolin   6-C-beta-D-glucopyranoside-8-C-alpha-L-arabinopyranoside,   luteolin   7-O-[2-(beta-D-apiofuranosyl)-4-(beta-D-glucopyranosyl),   and   luteolin   7-O-[2-(beta-D-apiofuranosyl)-beta-D-glucopyranoside] in *P. nigrum* extract.

Figure 1. *P. nigrum* studied in this project.

2.3 Pharmacological Properties of *P. nigrum*

P. nigrum Linn (PN), the king of spices, is one of the oldest and most consumed spices worldwide due to its pungent and tingling sensory impressions. The pungency of its dried berry (also known as black pepper or peppercorn) and alluring aroma is due to its nonvolatile alkaloid component, piperine (1-peperoyl piperidine) and a group of odor-active key volatiles (Dunkel et al., 2014).

Dried PN berry is loaded with numerous pharmacological activities as it is proved to exhibit antihypertensive, antiasthmatic, antimicrobial, antioxidant, anticancer, anti-inflammatory and immunomodulatory activities (Srinivasan, 2007; Jeena et al., 2014). These properties and the increase of demand for natural food additive make PN a suitable alternative for synthetic antioxidants and antibiotics (Morsy and El-Salam, 2017). Although promising results were also documented by *Piper longum* and *Piper bettle*, PN is the most commonly used spice in the world, especially India (Prashant et al., 2017).

Early research on the pungent principles of black peppercorns (Oersted, 1820; Landenburg and Scholtz, 1894) led to the isolation and identification of piperine (40-50 mg/100 g peppercorn), which is the predominate amide and major chemosensate of black peppercorns. Twenty five key amides responsible for pungent and tingling sensations of black pepper have been determined by liquid chromatography-tandem mass spectrometry (LC-MS/MS), ultra-performance liquid chromatography-time of flight-mass spectrometry (UPLC-Tof-MS) and one dimensional/two-dimensional nuclear magnetic resonance spectroscopy (1D/2D NMR) experiments as well as synthesis (Dawid et al., 2012).

According to recent literatures, black pepper oil has been reported to possess both *in vitro* and *in vivo* antioxidant activity. The study has further discovered/proved that oral administration of black pepper oil for a month, was found to significantly increase superoxide dismutase, glutathione and glutathione reductase enzyme levels in blood of mice and glutathione-S-transferase gluthathione peroxidase, catalase, superoxide dismutase and gluthathione in liver (Jeena et al., 2014). Singh et al (2008) reported that the petroleum extract of black pepper has high content of antioxidants which makes it a free radical scavenger.

Apart from antioxidant property, *P. nigrum* also has been vastly documented for its biovaluable antimicrobial properties. A study conducted by Morsy and El-Salam (2017) revealed ethanolic extract of black pepper displayed broad antimicrobial effect against gram-

positive microorganisms *Staphylococcus aureus* [RCMB 0100183], *Bacillus subtilis* [RCMB 010067] and gram-negative microorganisms *Pseudomonas aeruginosa* [RCMB 010043], *Escherichia coli* [RCMB 010052] and two fungi *Aspergillus flavus* [RCMB 02542], *Candida albicans* [RCMB 05036]. Several phytochemicals have been isolated from PN among which amide alkaloid (like piperine) and phytocannabinoid (like beta-caryophyllene) are considered to be most potent compounds exhibiting medicinal activities against several diseases including cancer (Selvendiran et al., 2003; Selvendiran, Banu and Sakthisekaran, 2004; Orav et al., 2004; Nashwa et al., 2017; Prashant A et al, 2017; Meza and Lehmann, 2017). The cytotoxicity studies of *P. nigrum* have been carried out using various cancer cell lines like human hepatocellular carcinoma (HepG2) and cervical carcinoma (HeLa), breast carcinoma (MCF-7), prostate carcinoma (PC-3), larynx carcinoma (Hep-2), colorectal carcinoma cell lines (HCT-116, HCT-15, and HT-29) (Morsy and El-Salam, 2017; Prashant et al.,2017)

Although much research has been done to determine the antioxidant, antibacterial and anticancer activities of commercial form of berries (sold in dried form) from PN, as far as I am aware, there is no reported work on the various biological activities of fresh berries and how the various drying methods used to dry the berries would affect their bioactivities.

2.3.1 Antioxidant Activity of *Piper nigrum* L.

Antioxidants are well-known for hydrogen donating property, thereby reducing active free radicals into stable nonradical forms and thus, block the chain of lipid peroxidation. It was proven that fifty percent ethanolic extract which contained amides and phenolics as the major phytochemicals was more reactive toward Fe (III)-TPTZ and reduce it into intense blue-colored Fe (II)-TPTZ, thus, indicating relatively higher reducing power in dose-dependent manner when compared to 70% and 100% ethanolic extracts. This study also showed that the radical scavenging activity (in terms of percentage inhibition) using 2,2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH) method was higher in 50% ethanolic extract of *P nigrum* (EEPN) when compared to 70% and 100% ethanolic extracts.

2.3.2 Antimicrobial Activity of *Piper nigrum* L.

Many plants and spices are used to eliminate microorganisms because of the increase of antibiotic resistance of microorganisms (Chattopadhyay et al., 2007). The essential oil of *P. nigrum* berries exhibited significantly (P<0.05) the largest zones of inhibition for *Candida albicans*, *Staphylococcus aureus*, *Bacillus subtilis* and *Escherichia coli*. It was reported that the minimum inhibitory concentration (MIC) value of the essential oil (1.95 µg/mL) from PN showed the strongest activity against *E. coli* compared with the MIC value (19.9 µg/mL) of the positive control, gentamicin (Morsy and El-Salam, 2017). The results of the study are in agreement with those reported by Dorman and Dean (2000). It was also concluded that *P. nigrum* essential oil antibacterial activity was due to its ability to affect cell membrane permeability resulting in the leakage of intracellular materials. This efficiency was attributed to the presence of sabinene which was found to be a potential modulator of bacterial resistance (Matias et al., 2016). The antimicrobial activity of piperine was vastly explored using different extraction solvents. Mohammed, Omran and Hussein (2016) reported methanolic extract of bioactive compounds of *P. nigrum* was assayed for *in vitro* antibacterial activity against *Escherichia coli*, *Pseudomonas aeruginosa*, *Proteus mirabilis*, *Staphylococcus aureus* and *Klebsiella pneumonia* by using the bored-well diffusion method in agar. The diameters of inhibition zones ranged from 5.00 ± 0.16 to 0.40 ± 0.12 mm for all treatments. Zarai et al (2013) reported that ethanolic extract was found to be the most effective solvent for extraction of *P. nigrum* compared to chloroform, ethyl acetate, methanol and water as it displayed a broad antimicrobial spectrum and exerted significant antibacterial effect against both gram-positive and gram-negative bacteria. The study also revealed that the most susceptible bacteria for ethanolic extracts were *S. aureus* and *B. subtilis* with a same MIC value of 156.3ug/mL, whereas the highest MIC value (1250 µg/mL) occurred with *E.coli* and *K. pneumonia*. Both aqueous and ethanolic extracts of black pepper have been screened for antibacterial activity against a penicillin G resistant strain of *Staphylococcus aureus* (Perez and Anesini, 1994), *Bacillus cereus* and *B. subtilis* (Singh et al., 2005). Blessy and Gopinath (2015) studied the antibacterial activity of ethanolic extract of black pepper against 10 clinical isolates of *S. aureus* and the results showed that the MIC value of ethanolic extract of black pepper was found to be 0.06 ->4% against all the clinical isolates. Ganesh, Kumar and Saranraj (2014) reported that the antimicrobial activity of *P. nigrum* ethanol extract is due to the presence of phytochemical compounds like alkaloids and tannins. The first report of piperine as an inhibitor of the NorA efflux pump in *S. aureus* came from

Mirza et al (2011). The study revealed piperine exhibited a two to fourfold reduction in the MIC of mupirocin for *S. aureus* American Type Culture Collection (ATCC) 2913 and Methicilin-resistant *S. aureus* (MRSA) isolates. The relative probability of the mupirocin and piperine combination to select resistant mutants was found to be significantly lower in comparison with mupirocin alone.

2.4 Biological Activities of Piperine – major compound in *P. nigrum*

The main compound responsible for the pungency of the green berries of pepper *P. nigrum L.* is piperine (Mohammed, Omran and Hussein, 2016). Piperine (1-piperoyliperidine; Fig 2), which belongs to the alkaloid family, is well-known as piperamide, and represents the major component in the dry fruit of *P. nigrum*. Generally the piperine content of black and white peppercorns lies within the range of 3-8g/100g, whereas the content of minor alkaloids piperyline and piperettine have been estimated to be 0.2-0.3 and 0.2-1.6g/100g respectively (Mohammed, Omran and Hussein, 2016). Piperine has been reported to have several pharmacological effects such as anti-diarrheal and hepatoprotective (Bajad et al., 2001; Koul and Kapil, 1993). Some studies have shown that piperine possesses an anti-inflammatory and an analgesic effect (Gupta et al., 2000). In addition, it has a high antioxidant activity and is used for treatment of Alzheimer diseases (Chonpathompikunlert, Wattanathorn and Muchimapura, 2010; Selvendiren et al., 2003). The chemical modification in the structure of piperine to piperic acid was confirmed by the appearance of the carboxyl group during the hydrolysis.
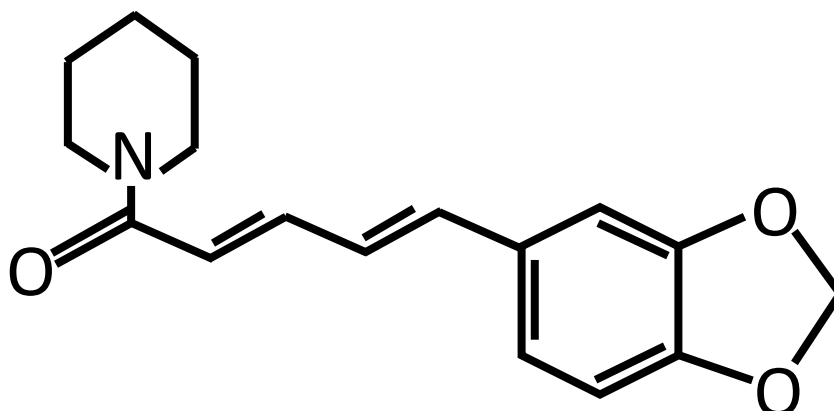
Figure 2: Chemical structure of Piperine

Piperic acid has a high anti-hyperlipidemic activity (Han et al., 2008). Recently, piperine and its derivatives have been evaluated for their inhibitory effects against epimastigote and amastigote (Ribeiro et al., 2004). A study proved that piperine and its analogues as potentiators of ciprofloxain activity against *S. aureus* and found that piperine reduced the MIC of ciprofloxacin by up to eightfold in *S. aureus* 1199B (a Nor A-hyperexpressing strain), indicating its role as a NorA inhibitor (Kumar et al., 2008). A concise mechanism of its bioavailability enhancing action is poorly understood. However, data suggests that piperine is absorbed very fast across the intestinal barrier; it may form non-polar complexes with drugs and solutes thus increasing permeability across the barriers (Khajuria et al., 2002). Chaudhry and Tariq (2006) showed that the spice mix of coriander, turmeric, red chili, black pepper and cumin favorably enhance the pancreatic lipase, chymotrypsin and amylase activity when consumed via diet.

2.5 Importance of Antioxidants

Antioxidants are defined as substances which delay or inhibit the oxidation of foods, and are therefore of great interest to food scientists. Antioxidants are naturally present in foods, but at a low level. Therefore, additional quantities are added to control oxidation, increase shelf life, and improve overall food quality. The mechanism by which this occurs is termed free radical termination, and is accomplished through the donation of an electron or hydrogen atom. Antioxidants can also protect food by the deactivation of metal ions and singlet oxygen. The most widely used synthetic antioxidants in foods are butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), propyl gallate (PG), and tert-butylhydroquinone (TBHQ). Due to the prooxidant effects of transition metal ions like magnesium, iron and copper, chelators are also used extensively in the food industry. Chelators such as citric acid (natural), ethylenediamintetraacetic acid (EDTA) and polyphosphates or their derivatives, are used to chelate metal ions. For many years there has been strong debate and concern regarding the safety of certain synthetic antioxidants as potential carcinogens. BHA, BHT, PG and TBHQ still remain on the GRAS (Generally Recognized As Safe) list, although limitations to their use have been implemented in the U.S., while BHT, PG and TBHQ still lack approval in many countries (Haworth, 2003). Therefore, there is growing interest by consumers and the food industry in replacing currently used synthetic compounds with natural alternatives which are perceived to be safer and have wider consumer acceptance. There are two main categories of antioxidants: primary and secondary. Primary antioxidants interrupt the free radical chain of oxidative reactions by contributing hydrogen from the phenolic hydroxyl groups, which form stable free radicals that do not initiate or propagate further oxidation of lipids. Secondary antioxidants trap radicals, chelate metals, regenerate primary antioxidants, or act as emulsifying agents. Synergism among different primary antioxidants and between primary and secondary antioxidants is often taken advantage of in food products and should be considered for all applications.

2.6 Antioxidant and Antimicrobial Properties of Phytochemicals

Plant secondary metabolites, or 'phytochemicals', are produced by plants for a myriad of functions, from UV protection, protection against pathogens and herbivores, pigmentation to improve chances of pollination, and other means of improving the plant's survivability and health, without being directly involved in crucial functions like the growth and reproduction (Bagniewska-Zadworna et al., 2008). In the past few decades, there has been a great increase in scientific interest around these compounds and their benefit to human health, as many exhibit considerable antioxidant and antibacterial activity (Bansal et al., 2013). Secondary metabolites are crucial for plant defences which have enabled plants to survive (Tan and Lim, 2015). Based on their biosynthetic origin, phytochemicals can be divided into several categories: phenolics, alkaloids, steroids, terpenes, saponins, etc. Phytochemicals could exhibit bioactivities such as antimutagenic, anticarcinogenic, antioxidant, antimicrobial, and anti-inflammatory properties (Abreu et al., 2017). These plant-derived phytochemicals with therapeutic properties could be used as single therapeutic agent or as combined formulations in drug development.

Plant phenolic compounds such as flavonoids and lignin precursors are important constituents of the human diet. These dietary phytophenolics have been recognized largely as beneficial antioxidants that can scavenge harmful active oxygen species including $O_2 \cdot^-$, H_2O_2, $\cdot OH$, and $^1O_2$. Phenolic compounds are well-known as secondary plant metabolites that possess in common one or more aromatic ring bearing one or more hydroxyl substituents. These compounds are water-soluble and may combine with a sugar molecule, as glycosides (Harbone, 1998). They are categorized into sub-groups and these include phenols, phenolic acids, phenylpropanoids, flavonoids, flavones, glycoflavonones and biflavonyls, aurones, flavonones, dihydrochalcones, isoflavones, xanthones and stilbenes, hydrolysable and condensed (proanthocyanidins) tannins and quinnes (Harborne, 1998).

The major chlorophylls in foods include chlorophyll a and b. Chlorophyll a degrades into chlorophyll derivatives (pheophytin a, pyrophyllium chlorophyll a and pheophorbide a) (Vezitskii 2000; Koca, Karadeniz and Burdulu, 2007), which is one of the important ways to participate in browning because chlorophyll a is sensitive to light, heat and pH. A current study conducted by Gu et al. (2018), discovered that phenolic compounds, chlorophyll and vitamin C are responsible to the blackening mechanisms of black pepper which was processed by different treatments: direct sun drying, blanching before sun drying, one-day

sun drying before mild fermentation, and two-day sun drying before mild fermentation. Natural antioxidants can also be obtained ubiquitously from certain crude, unrefined vegetable oils, including tocopherols and tocotrienols. These substances are present as constituents of unsaponifiable matter, and may occur together with phospholipids, carotenoids, chlorophylls and triterpenyl alcohols. Deodorization of these oils via molecular distillation yields a significant amount of purified tocopherols and tocotrienols. Most of the global supply of tocopherols originates from soybean oil processing, while tocotrienols are obtained from palm and rice bran oils.

Antioxidant efficacy is determined by the suitability of antioxidants in each food system. In general, more hydrophilic antioxidants are better at stabilizing bulk oil than oil-in-water emulsions. The activity of lipophilic antioxidants follows the opposite trend (Frankel, 1998). There are many other parameters which must be taken into account when selecting antioxidants for food applications.

The rise of antibiotic-resistant microorganisms has given extra impetus in the search for novel antibacterial compounds (Mgbeahuiruke et al., 2019). Numerous alkaloids, flavonoids, glycosides, terpenes, tannins and polyphenols from plant origins have been explored shown to exhibit antibacterial activity. Many have also been known to exhibit synergistic effects with existing antimicrobial drugs (Ncube et al., 2008). The novel alkaloids found in *Piper* species are piperine and piperlongumine. piperlongumine, also known as piplartin, is a piperamide compound found in Indian long pepper *Piper longum* L. (Piska et al., 2018). It is a bioactive compound of clinical importance that is active against multidrug-resistant pathogens such as *Pseudomonas aeruginosa* and *Staphylococcus aureus* (Naika, Prasanna and Ganapathy, 2010).

Phenolic compounds have diverse biological activities ranging from toxicity to hormonal mimicry and act as cell wall material, colorful attractants for birds and insects helping seed dispersal and pollination (Bhattacharya, Sood and Citovsky, 2010). Phenolic compounds also act as defence mechanisms of plants under different environmental stress conditions such as wounding, infection, excessive light or UV irradiation. (Harborne, 1998).

Among the wide variety of phytochemicals, phenolic compounds are by far the most commonly found potent antioxidants in nature. They are able to terminate free radicals and chelate metal ions that are capable of catalysing formation of oxygen reactive species that promote lipid peroxidation. Furthermore, they are capable to interfere with oxidation of lipids

and other free radicals by rapid donation of a hydrogen atom or electrons to the oxidized molecule radicals.

$$R. + POH \rightarrow RH + PO\cdot$$
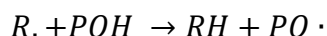
The resultant radical from the reaction of phenol with lipid radical is stabilized by the delocalisation of unpaired electrons around the aromatic ring (Ingold, 1960). Stability of the phenoxy radical intermediates ($PO\cdot$) reduces the rate of propagation of auto-oxidation chain reactions (Hudson, 2012).

It was understood that phenolic compounds induce the cellular antioxidant system; quercetin and flavonoids were found to increase the intracellular concentration of glutathione by approximately 50%. Flavonoids are important in the modulation of y-glutamylcysteine synthase in both cellular antioxidant defences and detoxification of xenobiotics. Glutathione is important in redox regulation of transcription factors and enzymes for signal transduction. It is therefore likely that polyphenols mediated regulation of glutathione alters cellular processes (Hudson, 2012; Yoshida et al., 1992).

2.7 Antioxidant Assays

There are various types of antioxidant assays that can be used to determine the respective antioxidant activity of the antioxidant compounds. The selection of antioxidant assay is based on the purpose of the study and also the nature of the analyte in the study. There are two general types of antioxidant assay which includes the assay that measures the total phenolic content and the assays that measure a specific group of antioxidant compounds. Naczk and Shahidi (2006) stated that the nature of the sample and the presence of some chemical substances will interfere with the quantification analysis of the antioxidant compounds. Generally, a few antioxidant assays will be chosen to quantify the antioxidant compounds in the sample due to the heterogeneity of the phenolic compounds in the sample and the possibility of interference by the presence of additional oxidizable compound in the sample. Various techniques such as cellular assay, chromatography assay and spectrophotometric assay can be used for phenolic content quantification. The spectrophotometric assay is a simple technique that can produce fast phenolic content quantification results on crude plant samples. Phenolic compounds quantification and profiling can also be done with the combination of modern high-performance liquid chromatography and instrument analysis. In this study, four antioxidant assays are used together with high-performance liquid chromatography in order to analyse and quantify the antioxidant compounds.

2.7.1 Folin-Ciocalteau (FC) Assay

Folin-Ciocalteau (FC) assay can be used to quantify the total phenolic content (TPC) amount of the plant samples. FC assay was based on the Folin-Denis (FD) assay developed by Folin and Denis with several improvements. FC assay and FD assay were initially used to determine the total protein concentration in the sample by measuring the amount of tyrosine and tryptophan in the sample. FC assay was then used to determine the total phenolic content in the wine sample by Singleton, Orthofer and Lamuela-Raventos (1999). The FC reagent consists of phosphotungstic acid ($H_3PW_{12}O_{40}$) and phosphomolybdic acid ($H_3PMo_{12}O_{40}$) where it relies on the transfer of electrons from phenolic compounds to form a blue chromophore comprised of the molybden-tungsten complex in alkaline solution (pH~10) using sodium carbonate. The blue chromophore has a maximum absorption at 765 nm

wavelength which depends on the concentration of the total phenolic content (Ainsworth and Gillespie 2007). Gallic acid is commonly used as a reference standard. The FC assay is simple, convenient, reproducible to quantify total phenolic content, and hence the assay has been commonly used (Huang, Ou and Prior 2005). However, this assay tends to overestimate the phenolic content because other reducing agents such as ascorbic acid, compounds containg thiols, amines groups and some others also respond to the FC reagent (Tan and Lim , 2015).

2.7.2 Ferrous Ion Chelating Assay

The role of primary antioxidant compounds is to reduce reactive oxygen or nitrogen species into harmless compounds and secondary antioxidant serves as a chelating agent that forms σ bonds with a metal ion. Chelated metal ions are more stable and have reduced redox potential. The secondary antioxidant compound can be quantified by using ferrous ion chelating (FIC) assay. Hydroxyl radical can be formed by the Fenton reaction which is catalysed by Fe^{2+} and results in many diseases. FIC assay is based on the reaction of divalent ferrous ions (Fe^{2+}) with ferrozine which then produces a purple ferrous-ferrozine complex that has a maximum absorption at the 562 nm wavelength (Stookey 1970). The secondary antioxidant will act as a competing chelating agent where it competes with ferrozine for ferrous ions which results in the decrease in the maximum absorption at 562 nm (Soler-Rivas, Espín and Wichers 2000). The strong chelating activity of the secondary antioxidants with ferrous ion hinders the transfer of electrons and leads to the arrest of the free radical chain reaction.

2.7.3 Ferric Reducing Power Assay

Ferric reducing power (FRP) assay is based on the reaction between antioxidants and the ferricyanide (Fe^{3+}) complexes. It is used to measure the ability of electron transfer from antioxidant compounds to Fe (III), resulting in a green Fe^{2+} complex. The increase of reduction ability of the antioxidant compounds will lead to more complexes to be reduced into the green Fe^{2+} complex which increases the intensity of the green colour of the reaction

solution with a maximum absorbance at 700 nm (Chu, Chang and Hsu 2000). Similarly to the FC assay, FRP assay also uses gallic acid as a reference standard.

2.7.4 Free Radical Scavenging Activity Assay

Free radical scavenging (FRS) activity assay is also known as the 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay where DPPH is a stable free radical that has an unpaired valence electron at one of the atoms at the nitrogen bridge (Blois, 1958). DPPH displays a maximum absorption at 517 nm wavelength which forms a dark purple violet solution. The FRS activity assay is based on the reaction between the antioxidant compounds with the DPPH free radical in the sample. When an antioxidant compound reacts with DPPH, it donates an electron or hydrogen to DPPH which then undergoes reduction and leads to the decolourisation of the violet colour solution. The changes in the colour reduction can then be monitored using a spectrophotometer (Kedare and Singh 2011). The higher the concentration of the antioxidant compound in the sample, the more DPPH will be reduced and leads to a greater decrease in the violet colour of the solution. This assay uses ascorbic acid as a reference standard.

2.8 Reverse-phase High Performance Liquid Chromatography

High performance liquid chromatography (HPLC) is one of the most reliable and popular methods for phenolic compounds fingerprinting. HPLC is usually coupled with various supporting instruments and mobile phases for antioxidant compounds analysis. Tan, Lim and Lee (2015) and Wu et al. (2013) have used HPLC to analyse phenolic compounds such as tannins, phenolic acids, anthocyanins and flavonoids. The use of reverse phase C18 column with HPLC can enhance the ability for different compounds separation. C18 column needs to be at a constant temperature to produce well separated and reproducible peaks. Compound separation can also be enhanced by using acidified mobile phase with formic acid to minimize gradient elution and peak tailing (Merken and Beecher 2000). Antioxidant compounds need to be injected into a detector for compound analysis. The commonly used detector is a UV/VIS detector or photodiode array detector as most phenolics/antioxidants show absorption in ultraviolet/visible region (Robbins, 2003). Photodiode array is most commonly used for complex sample mixture such as plant crude sample as it allows real time

scanning and ultraviolet visible (UV/VIS) spectral detection as the compounds pass through the detector. Besides photodiode array, mass spectrometric, electrochemical detection and nuclear magnetic resonance detection maybe used for antioxidant compounds analysis.

2.9 Principles and Applications of Liquid Chromatography Mass Spectrometry

Coupling of mass spectrometry (MS) to chromatographic techniques has always been desirable due to the sensitive and highly specific nature of MS compared to other chromatographic detectors. The coupling of MS with LC (LC-MS) was an obvious extension but progress in this area was limited for many years in the past due to the relative incompatibility of existing MS ion sources with a continuous liquid stream. Several interfaces were developed but they were cumbersome to use and unreliable, so uptake by clinical laboratories was very limited. This situation changed with the development of the electrospray ion source by Fenn in the 1980s (Fenn et al., 1989). Manufacturers rapidly developed instruments equipped with electrospray sources, which had a great impact on protein and peptide biochemistry. Fenn was awarded the Nobel Prize in 2002 with Koichi Tanaka who developed matrix assisted laser desorption ionisation, another extremely useful MS ionisation technique for the analysis of biological molecules. In the present work, we report for the first time the putative compounds obtained from *P. nigrum* berries which were subjected to microwave drying method.

2.9.1 Mass Spectrometry Instrumentation

Mass spectrometers operate by converting the analyte molecules to a charged (ionised) state, with subsequent analysis of the ions and any fragment ions that are produced during the ionisation process, on the basis of their mass to charge ratio (m/z). Several different technologies are available for both ionisation and ion analysis, resulting in many different types of mass spectrometers with different combinations of these two processes. In practice, some configurations are far more versatile than others and the following descriptions focus on the electrospray ion source which was used in this work. The reasons for choosing LC-MS over LC with conventional detectors are essentially the same as with GC-MS, namely high specificity and the ability to handle complex mixtures. Applications of electrospray MS were reviewed in The Clinical Biochemist Reviews in 2003 (Pitt, 2009). In the electrospray ionisation source of LC-MS, most molecules will be predominantly converted into singly charged ions, although multiple-charged ions are sometimes also formed. The charging process can involve the addition of a proton to the analyte to form $[M+H]^+$ when the ion

source is operated in positive ion mode or the loss of a proton to form [M-H]⁻ when operated in negative ion mode.

2.10 Antimicrobial Analysis Method

2.10.1 Disc Diffusion Method

One of the earliest and most widely-used antibacterial screening techniques is the disc diffusion technique; popular due to its ease of preparation, low cost and no need for specialized equipment (Osato, 2000). This method involves the loading of an antimicrobial testing disc with a compound or extract of choice which is then placed onto an agar medium inoculated with a lawn culture of bacteria. Following the diffusion of compounds from the disc into the surrounding agar, any antimicrobial property is visible by the presence of a 'clear zone', where the growth of bacteria has been inhibited (Lee et al., 2010). The diameter of this zone is directly related to the polarity of the compounds, its concentration and molecular weight. Thus, although disc diffusion is ideal for highly polar compounds, slightly soluble compounds can still be tested, albeit resulting in smaller clear zones. However, non-polar compounds may fail to diffuse, resulting in false negatives (Ahmed et al., 2013). Great care must be taken to not correlate the zone of diffusion with the strength of the antibacterial activity which remained as a misconception that still exists even in recent scientific literature. This would be true if the same compound(s) were tested, but even then it would only show comparative susceptibility of different bacterial species to that compound. This method would not be possible to compare the antibacterial activity of different samples, as the different compounds present will diffuse at different rates (Ncube, Afolayan, and Okoh, 2008).

Ultimately, the disc diffusion method is a qualitative test, and its results should not be used for quantitative purposes as it is impossible to determine the minimum inhibitory concentration (MIC), minimum bacteriocidal or minimum bacteriostatic concentrations using this method (MBC) (Ncube, Afolayan and Okoh, 2008). It does, however, remain a useful method for quick and simple qualitative screening (King, Dykes and Kristianti, 2008).

2.10.2 Broth Microdilution and Minimum Inhibitory Concentration

There is also a likelihood that potent antibacterial polyphenols may have a low rate of diffusion (Cushnie and Lamb, 2005). A suitable method for the quantification of antibacterial activity that has been gaining popularity is the broth microdilution method, which involves serial dilution of the tested sample in broth (typically Mueller-Hinton broth), which is then inoculated with bacteria in a 96-well microtitre plate (King, Dykes, and Kristianti, 2008). Unlike the disc diffusion assay, this assay can be used to determine the MIC of a sample and remains one of the few techniques able to determine the minimum bactericidal concentration (Osato, 2000). According to the British Society for Antimicrobial Chemotherapy (BSAC), the MIC can be defined as the lowest concentration capable of inhibiting visible growth of a microorganism after overnight incubation (Andrews, 2001); while Clinical and Laboratory Standards Institute (CLSI) (2006) and European Committee for Antimicrobial Suceptibility Testing (EUCAST) (2000) defined the MIC as the lowest concentration (mg/L) able to prevent growth under a defined period of time under defined conditions. The exact MIC breakpoints that define if a microorganism is susceptible, treatable or nigh-untreatable vary according to the particular species; taking into account clinical data and MIC distributions of relevant species, as well as the pharmacodynamics and pharmacokinetics of the antimicrobial agent used (Wiegand, Hilpert, and Hancock , 2008). Results between the disc diffusion and broth microdilution method are not always comparable (King, Dykes, and Kristianti, 2008), but the results obtained from the latter method are considered more accurate (Othman et al., 2011).

Dimethyl Sulfoxide (DMSO) is a popular alternative solvent given its comparatively lower toxicity. However, it is still important to determine the DMSO tolerance of the tested bacteria before proceeding with further MIC determination. Some bacteria may be inhibited by concentrations barely exceeding 2% DMSO (Miyasaki et al., 2013), although some may not be noticeably affected even at over 30% DMSO (Shah et al., 2014).

2.11 Drying of Plant Materials

Dehydration operations are important steps in the chemical and drying processing industries. The major challenge in drying process is the removal of water up to certain desired level without substantial loss of flavor, taste, colour and nutrients at which microbial spoilage and deterioration chemical reactions are greatly minimized. Today's consumer expectation for better quality, safety and nutritional value drives research and improvement of drying technologies. One of the most important ways to reduce the adverse influence of drying on food quality or to ensure basic quality properties of the final product is to carefully design the drying process and implement it consistently (Lewicki, 2006).

2.11.1 Thermal Drying

Thermal drying is a drying process which involves the application of artificial or natural heat. When a wet material is subjected to thermal drying two processes occur simultaneously; first, transfer of heat to raise the wet material temperature and to evaporate the moisture content and second is transfer of mass in the form of internal moisture to the surface of solid and its subsequent evaporation. The rate at which drying is accomplished is governed by the rate at which these two processes proceed. On the other hand, traditional thermal drying applies the direct drying method by placing the *P. nigrum* berries (the studied material in this project) under the sun until the berries are completely dry. Dried berries (spices) will then be packaged and exported. Drying is a time consuming, energy intensive and expensive process required in most industries, especially traditional sun-drying is very time consuming and the process is climate dependant where some of the areas do not have enough sun light during the day for the drying process. In order to overcome these drawbacks, manufacturers have applied modern equipment and techniques to dry spices and herbs faster and more efficient. However, till to date, the application of modern techniques of drying are not implemented to the spices due to lack of knowledge on their possible change in nutritional value when the fresh berries are subjected to these modern drying methods.

One such modern method is to use microwave to dry spices. Microwave drying (MD) has its own place in drying technology, due to the same transfer direction of temperature and moisture. This method can offer many advantages such as great energy efficiency and high

heat transfer rate. Vega-Mercado, Gongora-Nieto, and Barbosa-Canovas (2001) reported the use of MW as the fourth generation drying technology. Microwave oven uses electromagnetic waves which range from 3GHz-300GHz to dry the herbal plant materials (Dong et al. 2011). Microwaving the plant materials will cause water to be polarized where the polarization alters the dipole orientation of the water rapidly due to the bipolar properties of water, as the electromagnetic waves in the microwave region change with the frequency of billion times per second. The fast-moving molecules will cause friction and lead to the generation of heat which results in the increase of temperature in the plant cell within the spices.

Many conventional thermal methods, including airflow drying, vacuum drying, and freeze-drying, result in low drying rates in the falling rate period of drying (Clary, Wang, and Petrucci, 2005; Zhang, Li, and Ding, 2003, 2005).The long drying times at relatively high temperatures during the falling rate period of drying often lead to undesirable thermal degradation of the finished products (Mousa and Farid, 2002). MW drying offers opportunities to shorten the drying time and improves the final quality of the dried products (Schiffmann, 1992, Feng and Tang 1998). In some cases applying MW drying in the last stage of the dehydration process can also be very efficient in removing bound water from the product (Zhang et al., 2005) as well as prevent shrinkage of tissue structure (Feng et al., 2001).

Dielectric heating with MW energy has found industrial applications in drying food products such as fruits and vegetables. The rapid drying of the microwave enables this method to have high drying efficiency, high heat transmission, fast drying rate and produce high quality dried materials. Duan, Zhang and Mujumdar (2007) reported that the shorter drying duration of microwave drying method had resulted in less phenolic compounds loss in dried leaves and also able to prevent the browning process of the leaves as well as the occurrence of undesirable biotransformation in leaves. There are studies done on food material using microwave drying methods to study the sterilization of food, inactivation of enzymes and also the efficiency of the drying process (Bondaruk, Markowski and Błaszczak 2007). However, the microwave drying involves heat which poses a risk of phenolic compounds degradation when the drying process is set at high heat intensity and long duration (Jeni, Yapa and Rattanadecho 2010). Therefore, temperature and drying duration need to be optimized when the microwave drying process is applied.

Another method of thermal drying is oven-drying. Oven-drying is one of the most common drying methods that manufacturers used for herbal leaves drying as a large amount of leaves can be dried at the same time and this method is relatively inexpensive which will reduce the production time and cost. In the oven-drying process, hot air flows in the mechanical instrument while the fresh plant materials remain static or moving for the whole drying duration (Ramamoorthy and Bono, 2007). The hot air flows in various directions while the humidity and temperature remain constant throughout the drying process. There are some differences between oven-drying and traditional sun-drying where sun-drying requires the plant materials to be flipped around from time to time to achieve an even drying state whereas the materials in the oven can be static as the hot air flows are from multiple directions. In addition, the temperature of the oven during the drying process can be controlled and adjusted to ensure the materials do not receive extra heat which will cause the degradation of the nutrients.

2.11.2 Non–thermal drying

Non-thermal drying is defined as the drying method that allows water removal **without** the presence of applied heat. Ambient air-drying is similar to sun-drying method but it belongs to the non-thermal drying method as it does not involve applied heat. Light is an important factor that regulates the level of primary and secondary metabolites and also controls the plant growth (Agati and Tattini 2010). The biosynthesis of secondary metabolites, such as flavonoids, polyphenols etc, is light dependent. However, different plants will have different outcome on flavonoid and phenolic compounds biosynthesis under low light intensity. Matus et al. (2009) have reported that light intensity has an effect on morphology, physiology and the total phenolic compounds in the plant. Herbal spices will undergo photo inhibition under strong light intensity (Zhang and Scheller 2004). Ambient air-drying method is more suitable for shade-loving plant drying as ambient air-drying does not involve strong light intensity compared with sun-drying and this will prevent photo-decomposition of the herbal plant leaves thus improving the quality of the leaves. Traditional drying methods, such as drying in the shade or in the sun, have many drawbacks due to the inability to handle the large capacity of mechanical harvesters and to achieve the high quality standards required for medicinal plants. High ambient air temperature and relative air humidity during the harvesting season

promote insect and mold development in harvested crops (Rocha and Melo, 2011). Furthermore, intense solar radiation adversely affects quality, causing losses in essential oils or color changes in dried plants. Thus, traditional natural drying in the sun or in the shade does not meet the required standards or consumer demands.

Another alternative non-thermal drying treatment is freeze-drying method. Freeze drying method applies the lyophilisation process where the solvent medium, usually aqueous, will be frozen followed with the solvent sublimation under low temperature. This method has been widely used to dry herbal leaves in order to preserve the heat-sensitive biological compounds in the leaves. The typical freeze-drying process starts with a sample vial that is placed into the cold chamber of the freeze dryer machine. The chamber temperature will be decreased to the pre-set temperature to allow the sample to freeze. The air will be pumped out by a vaccum pump that will reduce the pressure in the chamber to start the primary drying process of the sample where the sublimation of the frozen water from the plant occurs. Then the chamber temperature will be increased to allow the removal of the physiosorbed water in the semi-dried sample. There are a few advantages of the freeze-drying method compared with some of the conventional methods where the freeze-drying method is able to preserve the physiological and biochemical properties, reduce the leaves weight, maintain the level of antioxidant compounds and also provide a longer shelf life of the leaves (Asami et al. 2003). However, the high energy consumption and maintenance cost of the freeze dryer makes this method relatively more expensive than the conventional methods.

CHAPTER 3
PROJECT DESCRIPTION AND OBJECTIVES

3.1 Research Question and Significance of Study

It is believed that the phytochemicals present in fresh sample of herbal plants will contribute tremendously to their therapeutic benefits. However, as plants are living organisms, they will undergo deterioration or loss in their active principle compounds when they are processed usually by drying methods such as oven-drying and sun-drying (as practised in black pepper processing industry). The reason for the expected different drying effects on fresh and dried berries of *P. nigrum* is not clear. Meghwal and Goswami (2011) reported heat treatment including domestic cooking processes caused piperine losses from *P. nigrum* ranging from 16 to 34%, with maximum loss observed in pressure cooking, indicating diminished availability of pepper bioactive constituents from cooked foods when the food ingredients have been subjected to either boiling or pressure cooking for only a few minutes. Drying of herbal plants by oven or ambient air-drying has been used widely as a common method in preserving bioactive compounds of herbal plants (Lim and Murtijaya, 2007). In addition, drying plant sample helps to remove moisture, as water will promote the growth of microorganisms (Anthony and Fontana, 2007). In the food/herbal industry, the fastest method in ensuring proper dryness without adversely affecting the herb's beneficial property would most likely to be used due to its time and cost saving properties. Although ambient air drying is the least expensive method, it is not the best choice due to its long drying time (Oh et al., 2019)

In general, antioxidant and antimicrobial compounds present within plants could be affected by the drying process they undergo, and many studies have found that dried and fresh samples could have different antioxidant and antibacterial activities. However, to date, it is not clear how the different drying methods will affect the properties of *P. nigrum* berries Subsequent questions raised relevant to this particular area are:

1. Is there a difference in antioxidant and antimicrobial activities of *P. nigrum* berries dried by different drying methods?
2. If the result obtained is positive, how does the bioactive compound content in differently dried *P. nigrum* berries differ from those fresh and commercial dried P. *nigrum* berries?

Literature search showed various pharmacological properties of *P. nigrum* including antioxidant and antibacterial properties have been studied on the commercial berries (in dry form) of *P. nigrum*. Due to lack of knowledge on how the drying processes could affect the bioactivity of *P. nigrum* berries, the primary focus of this current project is therefore to determine the effects of various drying techniques on the antioxidant and antimicrobial activities of fresh berries of *P. nigrum*. It is also aimed to find an appropriate drying method that would yield dried berries with better beneficial bioactivities than the commercial berries which are usually dried by sun-drying in open area (Ravindran and Kallupurackal, 2012).

The effects of different drying techniques of fresh *P. nigrum* berries have not been reported before in scientific literature. The results obtained will be helpful to processing of *P. nigrum* berries in industry by having an advanced understanding on a better drying process that will give maximum beneficial properties to black pepper.

3.2 Project Objectives

The core objectives of this project are:

1. To optimise the concentration of ethanol for antioxidant extraction and extraction efficiency of *P. nigrum* berries.
2. To assess the effects of drying methods (microwave drying , 100°C oven-drying, 50°C oven–drying, sun-drying (with different prior blanching treatments in boiling water), freeze drying, ambient air drying on the antioxidant properties of P. nigrum berries via total phenolic content (TPC), free radical scavenging (FRS), ferric reducing power (FRP) and ferrous ion chelating (FIC) assays.
3. To investigate the effects of drying methods on the antimicrobial activity of *P. nigrum* berries.
4. To identify and quantify the bioactive phytochemicals in the extracts of both fresh and dried *P. nigrum* berries using RP-HPLC.

CHAPTER 4

MATERIALS AND METHODS

4.1 Plant Sampling

The overall treatment steps of the fresh berries from BIOGREEN Good Acres Pepper Garden Sdn Bhd, Tambun, Perak are shown in Figure 3. Mature berries without insect bites and decolourisation were selected. Berries were then washed with distilled water to remove possible contaminants and dabbed gently with tissue paper. Triplicates of approximately 1g of berries were weighed and placed evenly on the petri dish for subsequent treatment or extraction. Extraction were done on the same day as harvesting/sampling to ensure freshness.
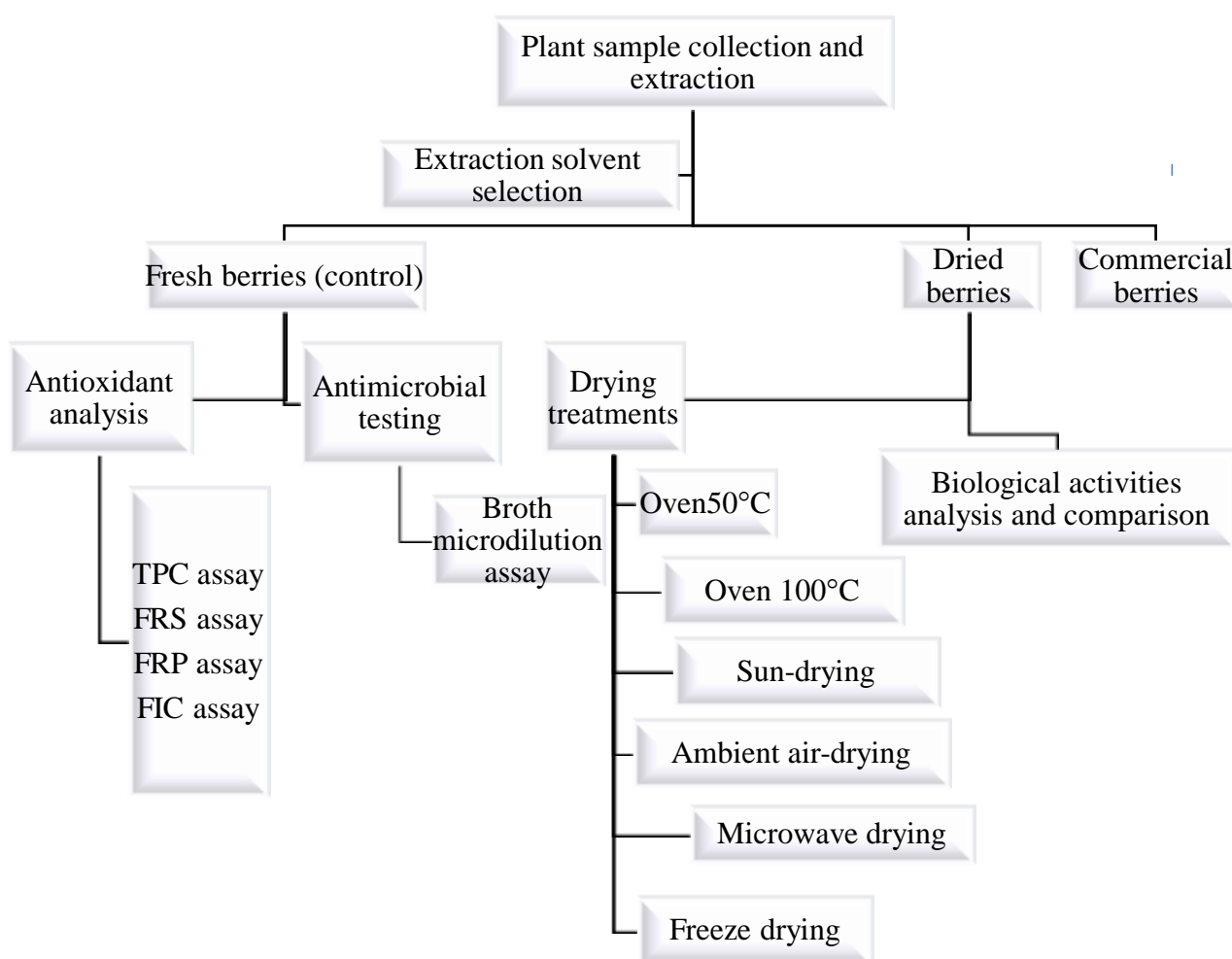
Figure 3. Project Description Overview

4.2 Drying Treatment Methods

4.2.1 Oven- Drying (50°C and 100°C)

Three sets of pre-weighed berries (~1.00 g) were incubated in an oven pre-set with the respective temperature of 50°C or 100°C. For each triplicate, the weight of berries was regularly monitored every hour until a constant weight was achieved. Every time after removing the berries from the oven, the berries were placed in a desiccator to cool down to room temperature before weighing to prevent water reabsorption in the berries. No further heating was required once a constant weight attained.

4.2.2 Ambient Air- Drying

The pre-weighed triplicates were dried under the laboratory environment condition with the average relative humidity of 65% and an average temperature of 23°C. The berries were evenly spread on the working bench which was minimally disturbed in the laboratory. The weight of the berries was measured every 24 hours until a constant mass was attained.

4.2.3 Microwave- Drying

Berries were placed into a glass petri dish and placed into the microwave oven (Sharp Model R-218L, ~50Hz). Highest setting (the instrument has low/medium/high settings) was used for the microwave drying treatment. Berries were dried and weighed every minute until the leaves reached a constant weight.

4.2.4 Freeze- Drying

P. nigrum berries were frozen using liquid nitrogen and then placed into the freeze dryer (Labconco FreeZone Plus 4.5 L Cascade Benchtop Freeze Dryer, USA) for 1-2 day before the extraction process. Berries were weighed after 24 h and placed back into the freeze dryer for another hour of drying to check the constancy of the weight of the dried berries.

4.2.5 Sun-drying

Pre-weighed berries samples (with or without boiled-water blanching) were left to sun dry for 1-2 days (equivalent to 6-12 h sunlight) until constant weight was obtained. Blanching was done by submerging berries in a beaker containing 80 ml of boiling distilled water for 1 minute and 15 minutes.

4.3 Water Activity Measurement

Fresh or dried berries were fitted into a sample cup. The cup was then inserted into the water activity meter (Aqualab model 3 TE; Pullman, WA) to measure the activity value. The result was expressed as water activity (a_w) at room temperature (°C).

4.4 Sample Extraction

4.4.1 Optimum Concentration of Ethanol for Extraction

The solvent selection for the phenolic content extraction of *P. nigrum* was first tested with ethanol in four different concentration (30%, 50%, 70% and 100%). To commence extraction, triplicates of pre-weighed fresh *P. nigrum* berries were powdered with a pestle and mortar in the presence of liquid nitrogen. The finely powdered sample was then transferred into a conical flask, added with 100% ethanol in a 1: 50 (w/v) ratio and the flask was capped with aluminium foil. The mixture in the flask was subjected to continuous swirling on an orbital shaker at a speed of 150 rpm at room temperature for an hour. After an hour shaking, the extracted solution was collected via vacuum filtration using filter paper. The extracted solution was then topped up to 50 mL using 100% ethanol before storing in a Schott bottle. These steps were repeated using 30, 50 and 70% ethanol. The extraction efficiency of these solvents was assessed based on the Total Phenolic Content (TPC) assay.

4.4.2 Best Extraction Solvent Selection between Methanol and Ethanol

A further extraction solvent selection was conducted between ethanol and methanol, using the solvent concentration reported for the highest TPC reading in section 4.4.1. All the

procedures were repeated as detailed in Section 4.4.1 for ethanol. The solvent with the high TPC content was used for all the subsequent studies.

4.4.3 Extraction Efficiency of first extraction

Once the extraction solvent was chosen based on procedures described in sections 4.4.1 and 4.4.2, the extraction efficiency of the first/single extraction (i.e. proportion of phenolic or phenolic-like content extracted in the first extraction) was determined by repeating three more times the extraction procedures as detailed in Section 4.4.1. For example, after the berries were extracted with the solvent, the residue powder on the filter paper was transferred back to the conical flask and re-extracted with another 50 mL of solvent using procedure as described in section 4.4.1. All the extracted solutions (4 x 50 mL) were stored at -20°C for further TPC analysis later. Extraction efficiency of the first extraction was determined based on the sum of TPC of first, second, third and fourth extractions according to the formula:

Extraction efficiency of first extraction (%) = (TPC of first extracted solution /Sum of TPC of 4 successive extracted solutions) x 100

4.4.4 Bulk Extraction for HPLC Analysis and Antibacterial Study

Berries samples (fresh and treated) were crushed into powder using liquid nitrogen with pestle and mortar and extracted using 50% ethanol with the ratio of 10 g sample: 100 ml 50% ethanol. The extraction procedure was repeated three times, similar to the procedure mentioned earlier. The solvent from the combined extracts was removed using a rotary evaporator followed by a freeze dryer. Samples were then kept at -20°C for further analysis.

4.5 Antioxidant Properties Analysis

All the following antioxidant assays performed in this study were taken from Chong and Lim (2012) with slight modifications.

4.5.1 Total Phenolic Content (TPC) Assay

Five-fold dilution of 0.3 mL plnt extract (triplicates) were added with 1.50 mL of FC reagent (10X dilution) followed by 1.2 mL of 7.5% w/v sodium carbonate.. The solution was then left in a dark for 30 minutes before the absorbance of the solution was taken at 765 nm at room temperature using a UV-Vis spectrophotometer (Unico 2100). TPC was expressed using the unit of gallic acid equivalent (GAE) in mg per 100 g of the fresh sample (mg GAE/ 100 g). The standard curve of the TPC has the equation of y= 0.0103x (r^2=0.9998) where y = absorbance at 765 nm and x = gallic acid concentration (mg/L).

4.5.2 DPPH Free Radical Scavenging (FRS) Assay

One mL of plant extract (triplicates) was added with 2.0 mL of DPPH (2.95 mg per 50 mL 100% methanol). The solution was then left in the dark to stand for 30 min before the absorbance of the reaction was taken at 517 nm at room temperature using a UV-vis spectrophotometer. The FRS activity was presented in ascorbic acid equivalent (AEAC) with the unit of mg AEAC /100 g of fresh sample. The standard curve of the FRS assay has the equation of y=12608x (R^2=0.9986) where y = scavenging activity percentage and x= ascorbic acid concentration (mg/mL). AEAC was calculated according to the following formula

$$\text{AEAC (mg AA/100g)} = \frac{IC_{50\,(ascorbic\,acid)}}{IC_{50\,(sample)}} \times 10^5$$

IC_{50} (ascorbic acid), concentration of ascorbic acid required to destroy 50% of the radical, was determined to be 0.00397 mg/mL.

4.5.3 Ferric Reducing Power (FRP) Assay

Various dilutions of the extract (1.0 mL in triplicate) were added to 2.5 mL of 0.2 M phosphate buffer pH 6 and 2.5 mL of potassium ferricyanide (1%w/v). The mixture was incubated for 20 min at 50ºC, after which 2.5 mL of 10% trichloroacetic acid was added. An aliquot of 2.5 mL of each mixture was diluted twice with deionised water, before adding 0.5 mL of 0.1% (w/v) iron (III) chloride ($FeCl_3$). Absorbance was measured at 700 nm after 30

min. A calibration curve was constructed using gallic acid. Results were expressed as mg gallic acid equivalent (GAE)/100g. The standard curve of the FRP assay has the equation of y=16.509x (R^2=0.9997) where y is absorbance and x is gallic acid concentration in mg/mL.

4.5.4 Ferrous Ion Chelating (FIC) Assay

Ferrous ion chelating measures the secondary antioxidative activity of the extracts. Different dilutions of samples amounting to 1.0 mL (triplicate) were added with 1.0 mL of 0.1 mM iron (II) sulfate (FeO_4S) followed by 1.0 mL of 0.25 mM ferrozine. The mixture was incubated 10 min at room temperature before absorbance was measured at 562 nm. The ability of extracts to chelate ferrous ion was calculated based on the equation shown below:

$$\text{Chelating ability } (\%) = \left(1 - \frac{A_{sample}}{A_{control}}\right) \times 100\%$$

Where $A_{control}$ is the absorbance of negative control and A_{sample} is the absorbance of sample. FIC is expressed as 50% chelating concentration in mg/mL (CC_{50}) which is the concentration of extract to chelate ferrous ion by 50%.

4.6 Identification and Quantification of Bioactive Compounds

4.6.1 RP-HPLC

The composition of bioactive compounds before and after drying was determined using RP-HPLC and LC-MS. For HPLC experiment, commercial standards were used for identification and quantification. The mobile phase condition was based on a literature procedure (Upadhyay et al., 2013) in which Acetonitrile: Water: Acetic acid (60: 39: 5: 0.5) mixed solvent was used as mobile phase in isocrastic mode. The HPLC instrument used was Agilent 1200 Infinity HPLC (Agilent Technologies, California, United States), with Ascentis® Express C18 (10 cm × 4.6 mm, 2.7 µm) (Supelco, USA) column; flow rate: 1.0 mL/min; concentration 1 mg/mL; detection wavelengths: 254, 340 and 360 nm.

4.6.2 LC-MS

Liquid chromatography‑mass spectrometry (LC‑MS) was performed using Agilent 1290 Infinity LC system with addition of 1% formic acid to the acetonitrile solvent. The compounds were putatively assigned based on their retention times (RTs) and m/z values

from the LC‑MS results. The mass spectrometer was run in positive mode with dual-ESI source and the compounds were putatively identified via Metlin database.

4.7 Antimicrobial Activity

4.7.1 Media Preparation

A 4.2 g of nutrient agar was weighed and added into Scott bottle. 150 mL of distilled water was added into Scott bottle and immediately autoclaved. After autoclaving, media was immediately poured into petri dishes. The media was allowed to completely solidify for 15 min. Once dried, petri dishes were stored appropriately in the refrigerator.

4.7.2 Antibacterial Assay

The extracts of *P. nigrum* were individually tested against a panel of microorganisms (Table 1). The antimicrobial activities of extracts were determined against four of human-pathogenic strains. The bacteria used were selected because they are connected with diseases related to skin, oral and intestinal tract of man. Three species of gram-negative bacteria and a species of gram-positive bacteria as shown in Table 1 were used in this study.

Table 1. Pathogenic bacteria used for the antimicrobial assay

Gram-negative bacteria	Strain
Escherichia coli	ATCC 25922
Proteus mirabilis	ATCC 25922
Pseudomonas aeruginosa	ATCC 27853
Gram-positive bacteria	
Staphylococcus epidermis	ATCC 25923

4.7.3 Bacteria Culture Preparation

A 0.56 g of nutrient broth was weighed and added into clean conical flask. Then 50 mL of distilled water was added in Scott bottle and immediately autoclaved. After autoclaving, the broth was allowed to cool for an hour. *Escherichia coli* was inoculated into the nutrient broth and left in shaker overnight and then adjusted to the turbidity of a 0.5 McFarland standard. The same step was repeated for *Staphylococcus epidermis, Pseudomonas aeruginosa and*

Proteus mirabilis. Controls without the test compounds were prepared (El-Baky and Hashem, 2016).

4.7.4 Disc Diffusion Assay

Antimicrobial activity was tested by using the disc diffusion method described by Mackeen et al. (2000). A swab of the test microorganism 1.5×10^8 CFU/mL based on McFarland turbidity standard 0.5 (Abachi et al., 2013; Barku et al., 2013) was spread onto petri dishes containing 4-mm-depth of Mueller-Hinton agar (once the bacterial suspension is adjusted, it was used within 30 min to avoid changes in the cell number). Each extract was dissolved in 20 % DMSO to a final concentration of 10 mg/mL. Sterile filter paper disc (6mm in diameter) impregnated with 1mg of the plant extract were placed on the cultured dishes. The dishes were inverted and incubated at 37ºC for 24 h. DMSO (20%) served as a negative control, while standard streptomycin (10 µg/mL) discs were used as the positive control. Antibacterial activity was evaluated by measuring the zone of inhibition in millimetres (mm). All experiments were done in triplicates. According to Clinical and Laboratory Standard Institutes (CLSI) (2012), the interpretative criteria for zone diameter (mm) in accordance to MIC (µg/mL) was shown in table below:

Table 2. Interpretive criteria for zone diameter (mm) in accordance to MIC (µg/mL)

	MIC (µg/mL)	Zone Diameter (mm)
Susceptible	≤4	≥20
Intermediate	8-16	15-19
Resistant	≥32	≤14

4.7.5 Micro-well Dilution Assay

Micro-well dilution assay was determined by using the method described by Baris et al. (2006).The minimal inhibition concentration (MIC) values were measured for the bacterial strains sensitive to the extracts in the disc diffusion assay. The inocula of the bacterial strains were prepared from 24 h broth cultures, and suspensions were adjusted to 0.5 McFarland standard turbidity. The extracts dissolved in 20% dimethylsulfoxide (DMSO) were first diluted to the highest concentration (500 µg/mL) to be tested, and then serial 2-fold dilutions were made in order to obtain a concentration range from 0.98 to 500 µg/mL in 10 mL sterile test tubes containing nutrient broth. MIC values of *P. nigrum* extracts against bacterial strains were determined based on a micro-well dilution method. The 96-well microtitre plates were prepared by dispensing into each well 95 µL of nutrient broth and 5 µL of inoculum. A 100

µL aliquot from the stock solutions of plants extracts initially prepared at the concentration of 500 µg/mL was added into the first wells. Then 100 µL from their serial dilutions were transferred into 6 consecutive wells. The last well, containing 195 µL of nutrient broth without compound and 5µL of the inoculum on each strip was used as a negative control. The final volume in each well was 200 µl. Vancomycin at the concentration range of 0.98–500 µg/mL was prepared in nutrient broth and used as standard drug for the positive control. The plate was covered with a sterile plate sealer and frozen at -20ºC. Contents of each well were mixed on plate shaker at 300 rpm for 20 s and then incubated at appropriate temperatures for 24 h. MIC was recorded as the lowest concentration of each plant extract that inhibited the bacterial growth as detected by the absence of visual turbidity. The turbidity was verified by measuring the absorbance of the suspension spectrophotometrically. The absorbance should be in the same range as that of the McFarland standard 0.5 (OD at 625 nm should be at 0.08–0.13).

4.8 Statistical Analysis

Analysis of comparison between the different drying treatment results was done using IBM SPSS statistics v23 software with one-way ANOVA and Tukey HSD post-hoc test analysis. The P value of <0.05 was considered statistically significant. All the data were presented as mean ± standard deviation where applicable.

CHAPTER 5

RESULTS AND DISCUSSION

5.1 Solvent Selection

Table 3 shows that the TPC value extracted using 50% ethanol has the highest extracted phenolic content (1174.5±9.2 mg GAE/100g), followed by 30 % ethanol (819.1±16.9 mg GAE/100g). and 100 % ethanol extracted the least amount of phenolic compounds.

Table 3. Comparison of TPC readings for 30%, 50%, 70% and 100% ethanol extractions

Solvent	TPC (mg GAE/ 100g leaves)
30% ethanol	819.1±16.9[b]
50% ethanol	1174.5±9.2[a]
70% ethanol	755.1±4.9[c]
100% ethanol	502.3±12.8[d]

based on first extraction.

*The values are presented as mean of three experiments ± standard deviation(SD). The values followed by the same superscripted letter are not statistically significant (P<0.05).

This shows that too much or too little of water content is not favourable to extract the antioxidant compounds present in berries.

Different solvent systems have been used to extract antioxidants and phytochemicals from plant materials such as fruits, vegetables, legumes, and other foodstuffs. Water, ethyl acetate, dimethylformamide (DMF), DMSO, ethanol, methanol, acetone, and their combinations are commonly used to extract antioxidants from plant foods. For extractions of antioxidants from different parts of plants the solvent systems used include absolute ethanol (Tao et al., 2014), DMSO/MeOH (Cano, Medina and Bermejo., 2008; Kawaii et al., 1999), ethyl acetate

(Goulas and Manganaris, 2012), 80 % methanol/water (Ramful et al.,2010; Abeysinghe et al., 2007), absolute methanol (Bocco et al., 1998), water (Guo et al,2003; Xu et al., 2008), 50% ethanol (Prashant et al., 2017), 70 % ethanol (Choi et al., 2003), 80 % acetone (Shin, 2012), and etc.

In addition, the property of different extracts from the same material depends on the extraction solvent. It is also apparent that different solvent extracts from the same plant material may vary widely with respect to their antioxidant concentrations and biological activities (Akuwuoh et al., 2005). In a comparative study of three different concentrations of ethanolic (50, 70 and 100%) extract of *Piper nigrum* (EEPN) berries, Prashant et al (2017) found that 50% ethanol was more effective than other concentrations in recovering total polyphenols from *Piper nigrum*. Also, in many studies, methanol and ethanol were chosen because they can deactivate enzymes such as polyphenol oxidase (PPO) and peroxidase (POD), which would destroy phenolics (Rocha and Melo, 2011).

Methanol and ethanol are well-known for their amphiphilic properties with middle polarity index, 5.1-5.2. Due to this notable charateristics, these solvents are commonly used in plant phytochemicals works as they can extract high amount of polar and non-polar compounds (IIoki-Assanga et al. 2015). For comparison purpose, 50% methanol was also used to extract and the result revealed that extraction using 50% ethanol had significantly higher TPC value (1174.5± 9.2 mg GAE/100g) compared to 50% methanol (853.9 ± 2.1 mg GAE/100g) as extraction solvent. Ethanol has also been reported in Han et al. (2008) as a suitable extraction solvent for extraction of phenolic compounds from *P. nigrum*. The result is in agreement with the work done by Prashant et al. (2017) who showed that 50% ethanolic extract of *P.nigrum* contained the highest amount of total phenolic value followed by 70% and 100% extract. This result is also in agreement with the findings obtained by Zarai et al. (2013) who reported that the highest levels of total phenolic content were found in the ethanol extract (45.08 µg of gallic acid equivalent/g of *P .nigrum*) followed by methanol, chlorform and ethyl acetate, while total phenolic content of the water extract was the lowest (1.09±0.10 µg of gallic acid equivalent/g of *P. nigrum*). Therefore based on our results, 50% ethanol was selected as extraction solvent for the subsequent studies.

5.2 Extraction Efficiency

Table 4 shows the extraction efficiency of sequential extraction using 50% ethanol as extraction solvent. The first extraction has the yield of 58.8% phenolic content followed by the second (25.0%), third (11.7%) and fourth (4.5%) extraction. The extraction efficiency based on the first extraction is lower than many of the plant leaves extraction (which are normally higher than 80%) that have been reported before (Tan, Lim and Lee, 2015; Lai and Lim, 2010; Lim and Quah, 2007). The reason could be due to the different nature of structural components of berries and leaves. The phenolics in berries could be more tightly bound to the plant matrix than that of leaves.

Table 4. Extraction efficiency of 50% ethanol of fresh *P.nigrum* with 4 succesive extractions.

Solvent	Extraction	TPC (mg GAE/100g)	Extraction Efficiency (%)
Ethanol (50%)	1st	1135.4 ± 14.7	58.8
	2nd	482.4 ± 4.0	25.0
	3rd	226.4 ± 3.1	11.7
	4th	87.9 ± 13.2	4.5

*The values are presented as mean of three experiments ± standard deviation(SD).

The extraction efficiency of the first extraction for the various treated samples were also determined. As shown in Table 5, the efficiency value varied according to the treated sample. It is found that microwave drying has the highest extraction efficiency of 86.4% while the two lowest extraction efficiencies are fresh and ambient air drying (58.8 and 52.7% respectively). Microwave treated sample has the highest extraction efficiency probably due to the more complete breakdown of the cellular structure as a result of intense heat generated within the cells.

Table 5. Extraction efficiency of 50% ethanol of P. nigrum for the various treated samples

Drying method	Extraction Efficiency (%)
Fresh control	58.8 ± 0.47^f
Microwave	86.4 ± 0.2^a
Oven 100°C	79.0 ± 0.4^b
Oven 50°C	62.1 ± 1.1^e
Freeze drying	70.4 ± 0.3^c
Sun drying (Blanching 1 min)	74.0 ± 0.9^c
Sun drying (Blanching 15 min)	60.3 ± 1.1^d
Sun drying (Without blanching)	68.9 ± 0.7^c
Sun-drying (Commercial berries with further sun dried)	71.5 ± 0.5^c
Ambient air drying	52.7 ± 1.0^{fg}

*The values are presented as mean of three experiment ± standard deviation(SD). The values followed by the same superscripted letter are not statistically significant (P<0.05).

5.3 Drying Duration of Berries under Various Drying Conditions

Fresh fruits have short shelf life and therefore need preservation. Fruits can extend their shelf life when used in dry form . The nutritional quality (determined by phytochemical content) and bioactivity are largely affected by the type of drying method employed (Pu and Sun, 2017). Several drying techniques have been used to dry plants by-products. In practice, sun-drying, hot air-drying, oven-drying, freeze-drying, and microwave-drying are the major drying methods with cost ranging from relatively inexpensive to moderately expensive.

This present report focussed on the fresh berries of *P. nigrum* which were subjected to eight drying treatments which include microwave drying , 100°C oven-drying, 50°C oven–drying, sun-drying (after 1-min blanched tretment), sun-drying (15 min blanched treatment), sun-drying (without blanching) freeze drying, and ambient air drying (23-25°C, average relative humidity 65%). The berries subsequent to drying treatments to a constant mass were subjected to antioxidant assay analysis. Two typical drying curves are shown in Figure 4 and 5. More drying curves are shown in Appendix.

As shown in Table 6, it is found that microwave drying has the shortest drying time of 4 min while the longest time required is the ambient air drying (5 days at ~23°C, at average relative humidity if 65%). The highest percentage of water loss was observed in microwave drying 90.0±0.8 (%) while the lowest percentage of water loss belonged to ambient air drying 39.1±1.8 (%). The percentage of water loss was mainly due to the moisture loss on drying.

Table 6. Percentage of water loss (%), water activity (a_w) and time required to completely dry the samples

Drying treatment	Time taken to obtain constant weight	Percentage of water loss (%)	Water activity (a_w)
Fresh			0.985 ± 0.001^a
Microwave	4 min	$90.0\pm0.8\,^a$	0.124 ± 0.003^h
Oven 100	4 h	$76.8\pm1.6\,^b$	0.238 ± 0.004^g
Oven 50	8 h	61.9 ± 1.0^{ef}	0.421 ± 0.005^c
Freeze	25 h	69.3 ± 0.9^{cd}	0.322 ± 0.005^e
Sun (Blanching 1min)	6 h	73.2 ± 2.8^{cd}	0.281 ± 0.004^e
Sun (Blanching 15min)	6 h	65.6 ± 0.9^{cd}	0.358 ± 0.003^d
Ambient air	5 days	39.1 ± 1.8^f	0.638 ± 0.261^b

*The values are presented as mean of three experiment $\pm$ standard deviation(SD). For each column, values followed by the same superscripted letter are not statistically significant ($P<0.05$).

Drying process is essential for herbal plants mainly because the cellular integrity is lost as cells die (the senescent process) causing the enzymes come in contact with substrates to which they are not normally exposed in living cells. Furthermore, the redox process, racemization, dimerization etc. also increase which in turn cause a degradative problem to bioactive compounds like phenolic compounds (Rickmann, Barrett, and Bruhn, 2007). The metabolic activities of the cells need to be curbed immediately after harvesting in order to control changes related to the enzyme activities. Various drying techniques at different temperatures can be performed (Baudry et al., 2004; Abascal, Ganora and Yarnell, 2005; Chan et al., 2009; Dorta, Lobo and Gonzalez, 2012).
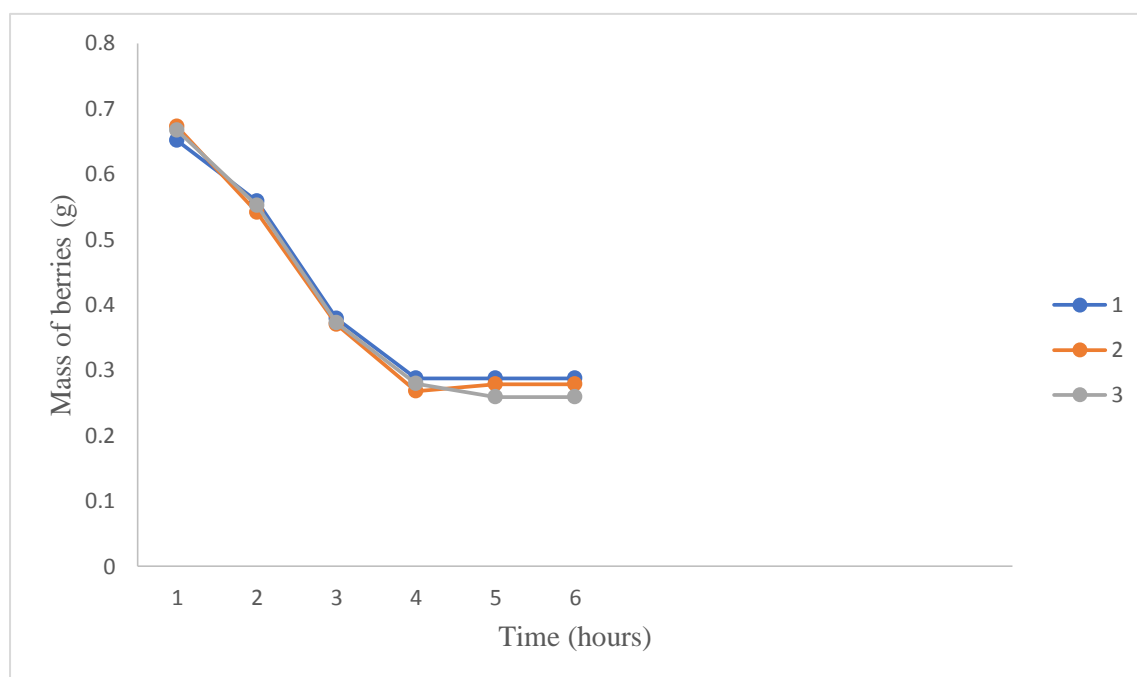
Figure 4. Drying curve of Oven 100ºC drying for the triplicate samples
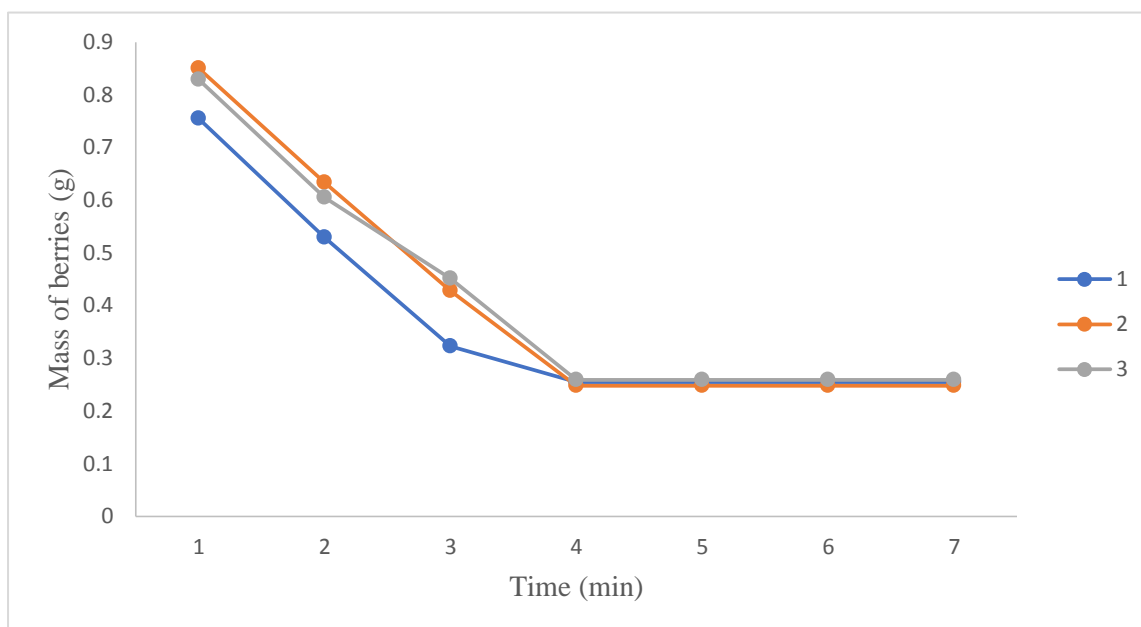
Figure 5. Drying curve of microwave drying for the triplicate samples

5.4 Drying Effect

5.4.1 Water activity of Treated Berries

Water activity is the water partial vapor pressure of a sample devided by the standard partial vapor pressure of pure water. In the food science field, the standard state is defined as the partial vapor pressure of pure water at the same temperature. Using this definition, the water activity of pure distilled water is one. Water activity describes the escaping tendency and energy status of water within a system. The unit of water activity is a_w where it represents how water is bounded chemically or structurally in a sample. a_w ranged from 1 (pure water) to 0 (bone dry). Beside moisture loss, water activity is also another important parameter to predict the total water content and microbial stability in the food product. Maltini et al. (2003) stated that water activity controls not only the microbial growth but also the enzymatic, physical and chemical nature of the food product. High level of water activity can initiate the degradation of the food product by dissolving and mobilizing chemical species thus increasing the chances of them reacting with each other and also hydrating ionic or polar group of some chemical compounds. In the dried samples with low water activity/moisture content, destructive enzymes were inactivated and high levels of phenolic compounds can remain in the extract (Hassain et al. 2010).Therefore, better food stability and preservation can be achieved by decreasing the water activity of the food product. Water behaves

differently at different water activity level. At high water activity, water serves as a pro-oxidant that promotes mobility by the effect of plasticization and exposing nutrient to the catalytic surfaces by matrix swelling (Labuza, Kaanane and Chen, 1985).

Prabhakar and Mallika (2014) reported that water activity lower than 0.6 a_w is suitable for preventing microorganisms growth. Based on the water activity-stability diagram propsed by Bone (1987), the threshold of a_w for food preservation is 0.4 as the food will be microbiogically stable (a_w<0.6) and most of the enzyme activities such as browning reaction and lipid peroxidation is inhibited when water activity is < 0.4 a_w. The food degradation rate will increase approximately by a factor of 50%-100% when the water activity increase by 0.1 a_w. In addition, Labuza, Kaanane and Chen (1985) also reported that the water activity of 0.2–0.4 a_w shows the lowest rate of food deterioration at the Brunauer, Emmett and Teller (BET) monolayer.

All drying treatments have shown a decrease in water activity ranging from 0.124 to 0.638. Largest decrease of water activity was observed in microwave, oven 100°C and sun-drying (1-min blanching) treatments (0.124 a_w, 0.238 a_w and 0.281 a_w respectively) followed by freeze-drying, sun-drying (15 min blanching), oven 50°C drying treatment (0.322 a_w , 0.358 a_w and 0.421 a_w respectively) and the least decrease of water activity was observed in ambient air-dry treatment (0.638 a_w). Generally, drying treatment with or without the presence of applied heat will cause a decrease in the water activity of the sample. In the food/herbal industry, the fastest method in ensuring proper dryness without adversely affecting the herb's beneficial property would most likely to be used due to its time- and cost- saving properties. Although ambient airdrying is the least expensive method, it is not the best choice due to its long drying time (Oh et al., 2019). Based on the result fromTable 6, most of the drying treaments have resulted in the water activity value of < 0.4 a_w except for oven 50°C and ambient air-dry treatment. The berries dried using oven 50°C has the water activity >0.4 a_w which indicates that oven drying at 50°C could have inhibited the growth of microorganism but could not suppress completely the enzyme activity of browning reaction and lipid peroxidation. In addtion, ambient air-dry treament has a water activity >0.6 a_w which suggests that ambient air-drying still allows the growth of microbes as well as the enzyme activities. It was further understood that the blackening of fresh green berries which were subjected to ambient air drying has provided major impact on the water activity. Berries obtained from ambient air drying have a duller black complexion compared to the other drying treatments.

An immediate drying treatment is required for *P. nigrum* berries to rupture cells, thus accelarating the escape of moisture from the inner core of the berries and enhancing its black color, due to the presence of resinoids inside the berry. For ambient air drying growth of fungal and mold can be observed on the outmost layer of berries. Hence, both oven 50°C and amibient air-dry treatments are not a good drying treatment to enhance the shelf life of *P. nigrum* berries.

5.5 Antioxidant Activities of Fresh and Dried Berries

As the extraction efficiency was different for different drying treatments, a fair and valid comparison of the antioxidant properties based on one single extraction (so called first extraction) cannot be achieved and therefore all the antioxidant parameters obtained are adjusted to 100% based on the extraction efficiency value (Table 7).

Table 7. TPC of first extraction and adjusted TPC values of various drying methods

Drying method	TPC of first extraction (mg GAE/ 100g)	TPC (adjusted to 100%) (mg GAE/ 100g)	Extraction Efficiency (%)
Fresh	1135.4±14.7[ef]	1932.1±25.0[e]	58.8±0.47[e]
Microwave	2351.9±14.2[a]	2722.8±15.7[a]	86.4±0.2[a]
Oven 100°C	2093.5±14.9[b]	2650.8±18.8[b]	79.0±0.4[b]
Oven 50°C	1232.2±16.9[e]	1984.9±26.4[e]	62.1±1.1[e]
Freeze drying	1588.5±19.1[c]	2257.4±23.0[c]	70.4±0.3[c]
Sun drying (Blanching 1 min)	1686.6±32.2[c]	2279.6 ±43.5[c]	74.0±0.9[c]
Sun drying (Blanching 15 min)	1352.3±29.7[d]	2241.7±49.3[c]	60.3±1.1[d]
Sun drying (Without blanching)	1567.9±22.0[c]	2274.3±31.9[c]	68.9±0.7[c]
Optimised Commercial	1621.4±15.1[c]	2267.8±24.2[c]	71.5±0.5[c]
Ambient air drying	1037.2±13.7[e]	1969.2±26.0[d]	52.7±1.0[fg]

*The values are presented as mean of three experiments ± standard deviation (SD). For each column data, the values followed by the same superscripted letter are not statistically significant ($P<0.05$) based on one way ANOVA with Tukey HSD analysis.

For most plant materials studied, the antioxidant (phenolic compounds, vitamins, etc.) contents of fresh plant materials were higher than those of dried plant materials and this is mainly due to the degradation process during drying. However, some recent studies have shown that dried plant materials could contain higher antioxidants such as polyphenolics, and antioxidant activity as compared to fresh plant materials (Pinela et al. 2012). It is argued that drying process can break down the cellular structures leading to releasing phenolic compounds from the food matrix (Chang et al. 2006; Arslan and Ozcan 2010).

In the present study, the highest total phenolic content (TPC) was obtained in microwave drying as extraction revealed 2722.8±15.7 mg GAE/ 100g, while the lowest one belonged to ambient air drying with TPC as 1969.2 ±26.0 mg GAE/ 100g which is similar to the fresh control 1932.1±25.0 mg GAE/ 100g. The TPC order was followed by oven drying (2650.8 ± 18.8mg GAE/ 100g) while sun drying and freeze drying had a significant increase in TPC. Apart from that, total phenolic content (TPC) was measured for commercial *P. nigrum* berries to obtain a comparison. However, this particular experiment was challenging due to large data variation obtained for the TPC value (probably caused by non-uniform drying of commercial samples). In oder to overcome this issue, we had sun-dried the commercial berries and found that the consistency of TPC reading was achieved.

The reason for the increased TPC in the present study could be due to an increase in piperine content (as shown by HPLC analysis to be discussed later in section 5.6) when the samples underwent drying treatments. To prove this point, the TPC of the pure piperine sample solution was measured and a suprisingly high TPC of 33.05 g GAE/100g was found. Thus, an increase in this compound will help to increase the TPC of the treated sample. Another interesting inference from the result is that even though piperine does not have phenolic group, it responds sensitively to F-C reagent test, probably because of the amide group in the compound.

Similar to the present study, Prathapan et al. (2009) reported that oven drying of fresh turmeric rhizome gave a higher amount of phenolic compounds among other drying methods. Their study showed that TPC values increased gradually when samples were heated from 60 to 100 °C. Moreover, polyphenol oxidase (PPO) was completely inactivated at 80 °C.

Generally, plants are often dried and stored for a long time before being used in manufacturing various types of products. Dehydration is an important preservation method for plant materials, as it inhibits enzymatic degradation and minimizes microbial growth of plants (Pinela et al. 2012). Natural drying has many disadvantages due to the inability to control large quantities and to achieve consistent quality standards (Fathi et al., 2016) In most previous studies, freeze-drying was considered as one of the more appropriate methods to preserve the quality of plants during processing (Arslan and Ozcan, 2010; Annegowda et al., 2014). Chan et al. (2009) had studied the effects of five different drying methods (microwave, oven, and sun drying) on antioxidant properties of *Alpinia zerumbet, Etlinger aelatior, Curcuma longa and Kaempferia galangal*. Air drying of ginger leaves resulted in significant losses in total phenolic content and antioxidant activity for all the four species (Chan et al., 2009). The various antioxidant activities of *P. nigrum* fresh and treated berries based on first extraction and the adjusted values are summarised in Table 8 and Table 9.

Table 8. Various antioxidant activity of *P. nigrum* fresh and treated berries based on first extraction

Drying method	IC_{50} (mg/mL)	AEAC (mg AA 100g)	CC_{50} (mg/mL)	FRP (mg GAE/g)
Fresh	0.87 ± 0.02^a	456.4 ± 51.4^a	2.87 ± 0.15^a	2.00 ± 0.03^e
Microwave	0.22 ± 0.01^e	1807.0 ± 82.2^e	1.63 ± 0.02^e	8.37 ± 0.21^a
Oven 100°C	0.27 ± 0.02^e	1492.1 ± 87.1^e	2.07 ± 0.15^e	5.77 ± 0.42^b
Oven 50°C	0.68 ± 0.01^b	487.8 ± 99.6^b	2.60 ± 0.38^b	2.27 ± 0.15^d
Freeze drying	0.42 ± 0.01^d	799.8 ± 24.9^c	2.24 ± 0.11^d	3.37 ± 0.25^c
Sun drying (Blanching 1 min)	0.35 ± 0.04^d	1153.20 ± 19.1^d	2.17 ± 0.27^d	3.67 ± 0.15^c
Sun drying (Blanching 15 min)	0.63 ± 0.01^b	583.9 ± 8.6^b	2.53 ± 0.10^b	2.63 ± 0.21^d
Sun drying (without blanching)	0.51 ± 0.04^c	969.6 ± 29.1^d	2.37 ± 0.15^c	3.28 ± 0.20^c
Ambient air drying	0.85 ± 0.02^a	467.1 ± 5.5^a	2.83 ± 0.26^a	2.07 ± 0.15^e

Table 9. Various adjusted antioxidant activities of fresh and dried *P. nigrum* berries

Drying method	IC_{50} (mg/mL)	AEAC (mg AA 100g)	CC_{50} (mg/mL)	FRP (mg GAE/g)
Fresh	0.52 ± 0.01^a	767.3 ± 8.7^e	1.71 ± 0.15^a	3.23 ± 0.02^f
Microwave	0.19 ± 0.01^d	2091.5 ± 95.2^a	1.30 ± 0.06^d	9.55 ± 0.06^a
Oven 100°C	0.21 ± 0.01^d	1888.7 ± 110.2^a	1.36 ± 0.01^d	6.79 ± 0.07^b
Oven 50°C	0.44 ± 0.01^a	910.9 ± 13.3^e	1.60 ± 0.01^{ab}	4.40 ± 0.05^e
Freeze drying	0.30 ± 0.05^{bc}	1160.9 ± 36.1^{cd}	1.45 ± 0.03^{bc}	4.92 ± 0.02^c
Sun drying (Blanching 1 min)	0.26 ± 0.03^{cd}	1558.4 ± 160.9^b	1.40 ± 0.06^{cd}	5.14 ± 0.02^c
Sun drying (Blanching 15 min)	0.38 ± 0.01^b	1045.2 ± 16.6^{cd}	1.58 ± 0.17^b	4.76 ± 0.06^e
Sun-drying (Without blanching)	0.35 ± 0.03^b	1387.4 ± 11.6^b	1.51 ± 0.02^b	4.98 ± 0.06^c
Ambient air drying	0.47 ± 0.01^a	886.3 ± 10.5^e	1.68 ± 0.02^a	4.09 ± 0.04^d

*The values are presented as mean of three experiments ± standard deviation (SD). For each column data, the values followed by the same superscripted letter are not statistically significant (P<0.05) based on one way ANOVA with Tukey HSD analysis.

The ability of 50 % ethanolic extracts of *P. nigrum* berries after drying treatments to quench DPPH free radical was measured. By plotting the graph of extract concentrations against the scavenging activity, a specific concentration of the sample that needed to provide 50 % inhibition (IC_{50}) was calculated (Table 8 based on the first extraction). Using the first extraction efficiency values in Table 7, the adjusted IC_{50}, AEAC, CC_{50} and FRP of different drying treatments of *P. nigrum* are summarised in Table 9. The IC_{50} of various drying treatments were in the range of 0.19±0.01 to 0.47±0.01 mg/ml. The order of IC_{50} among different drying treatments is as follows: Microwave ~ Oven 100 drying< Blanching 1 min< Freeze drying <Sun-drying (without blanching) <Blanching 15 min< Oven 50 drying~ Ambient drying< Fresh control. Based on the IC_{50}, the eight drying treatments studied can be classified into three groups, namely high level, average level and low level. Among

treatments, microwave and oven drying at 100 °C revealed the highest radical scavenging activity (the lowest $IC_{50} = 0.19 \pm 0.01$ mg/mL and $IC_{50} = 0.21 \pm 0.01$ mg/mL), while freeze drying and sun-drying (blanching 1 min) showed the medium scavenging activity of 0.30 ± 0.05 mg/mL and 0.26 ± 0.03 mg/mL respectively. The oven 50 °C , sun-drying (without blanching), sun-drying (15 min blanching), ambient air drying treatments and fresh control showed the lowest scavenging activity ($IC_{50} = 0.35 \pm 0.03$ mg/mL, 0.38 ± 0.01 mg/mL, 0.47 ± 0.01 mg/mL and 0.52 ± 0.01 mg/mL) (Table 9). The AEAC trend follows that of IC50 as is evident in the relationship between them which was discussed in section 4.5.2. As a comparison, previous research showed that the radical scavenging activity of commercial seaweeds of Sabah increased at higher drying temperatures using oven-drying treatments (Lee et al. 2013).

The ability of the antioxidant fraction to reduce Fe^{3+} to Fe^{2+} represents the reductive power of the antioxidant (Ardestani and Yazdanparast 2007). Color changes of the solution in the presence of the 50% ethanolic extract of *P. nigrum* were evaluated. Reducing capacities of ethanolic extracts of *P. nigrum* are shown in (Table 9). The reducing power of the extracts showed a dose dependence with increasing concentration of the samples. The strongest reducing power was obtained in microwave-drying method (FRP value 9.55 ± 0.06 mg GAE/g), while the weakest activity was observed in ambient air-drying and fresh control. Reducing capacity of an extract can serve as an indicator of its potential antioxidant activity. However, the antioxidant activity of antioxidants could be attributed to different mechanisms (Oktay, Gulcin and Kufrevioglu, 2003). For example, DPPH assay is mostly attributed to phenolic compounds that participate in scavenging free radicals, while in reducing power the reduction of the metal ions such as ferric ions is evaluated (Gharibi et al. 2013)

In general, higher TPC value corresponds to higher AEAC, FRP and lower IC_{50} and CC_{50} values. For an example, a good correlation ($r^2 = 0.9060$) between TPC and AEAC of different drying treatments was observed (Figure 6). A similar relationship was also observed in IC_{50} which correlated with TPC of different drying treatments ($r^2 = 0.8741$) (Figure 7) as well as FRP ($r^2 = 0.8095$) (Figure 8) and CC_{50} ($r^2 = 0.8274$) (Figure 9).
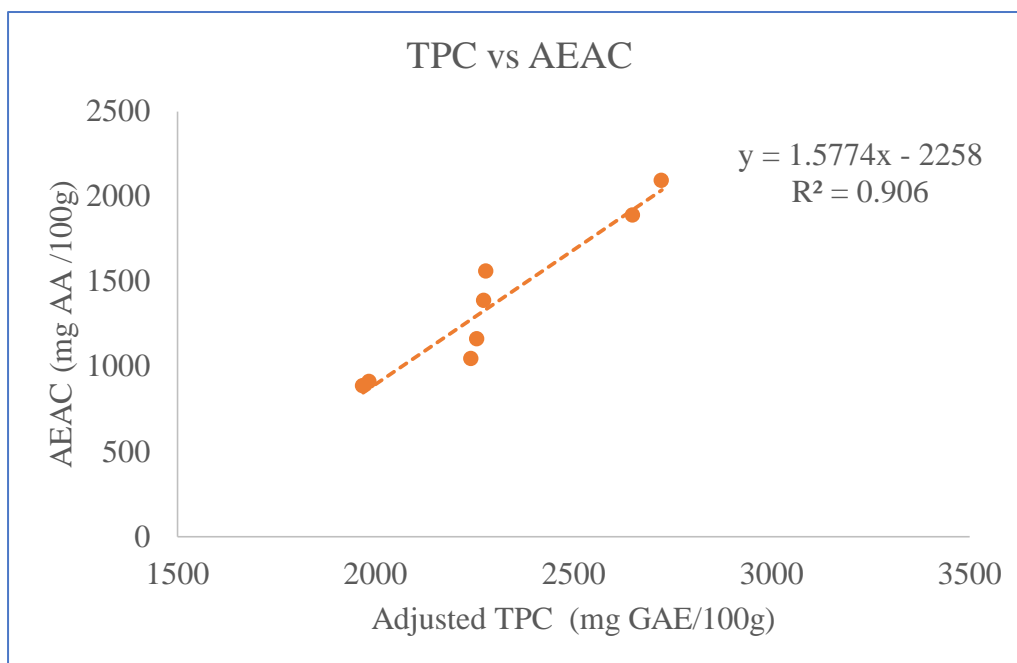
Figure 6. Correlation between TPC (mg GAE/100g) and AEAC (mg AA/100g)
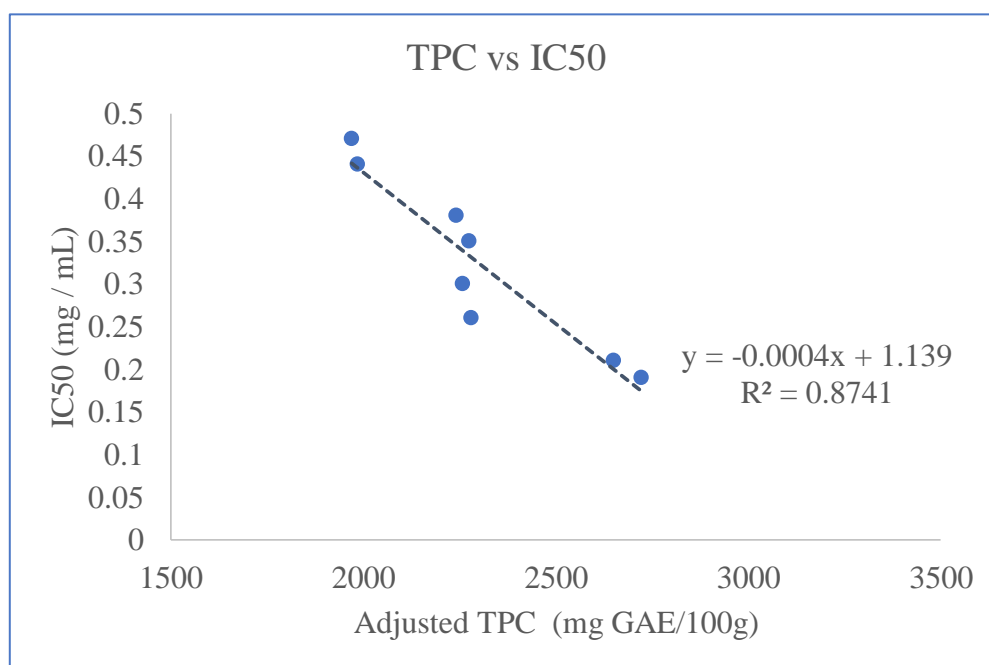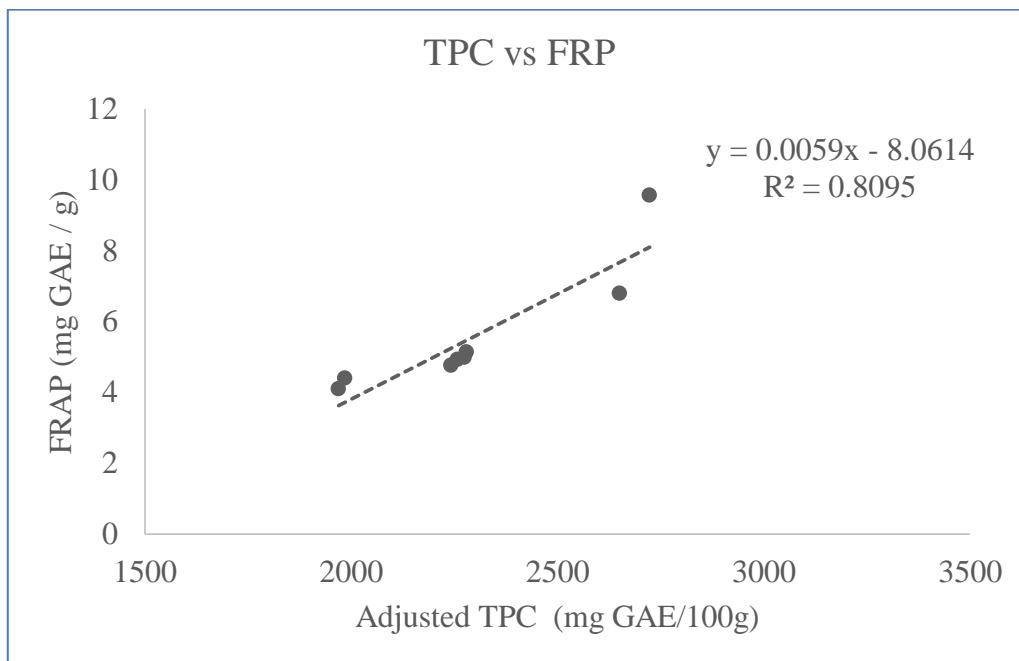

Figure 7. Correlation between TPC (mg GAE/100g) and IC_{50} (mg/mL)

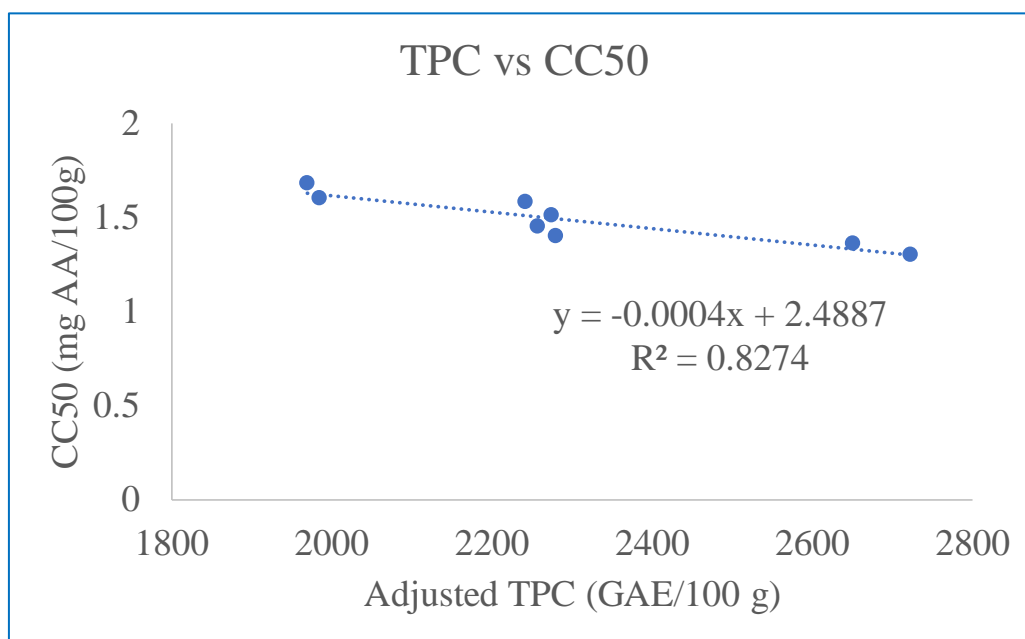Figure 8. Correlation between TPC (mg GAE/100g) and FRP (mg GAE/100g)

Figure 9. Correlation between TPC (mg GAE/100g) and CC_{50} (mg/mL)

5.6 HPLC and LC-MS Data Interpretation

5.6.1 Identification of Piperine

High performance liquid chromatography (HPLC) is a routine and popular method for phytochemical analysis because it possesses the outstanding advantages of good repeatability, wide applicability, and reasonable qualitative and quantitative capability. Among the common analytical practices, reversed-phase high performance liquid chromatography (RP-HPLC) has been commonly used for fingerprinting study of herbal plants (Martin and Guiochon, 2005; Fan et al., 2006), mainly due to its capability to handle compounds with diverse polarity and molecular mass (Prathap et al., 2016). Many previous studies have been reported on the characterization of secondary metabolites in plant extracts using RP-HPLC, chiefly phenol compounds, steroids, flavonoids and alkaloids (Boligon, Machado and Athayde, 2014). Phenolic compounds are frequently identified with the use of UV-VIS and photodiode array detectors at wavelengths 190-380 nm.

Piperine was confirmed to be present in the extracts by comparing the retention time, UV-Vis spectrum of the standard and spiking. Mohammed, Omran and Hussein, 2016 reported that piperine content of black or white peppercorns lies within the range of 3-8 g/100 g dried sample, whereas the content of minor alkaloids piperyline and piperettine have been estimated as 0.2-0.3 and 0.2-1.6 g/100g. Hamrapurkar, Jadhav and Zine, 2011 revealed that the % w/w yield of piperine in supercritical fluid extract was 8.76 for *P. nigrum* whereas in soxhlet extract % w/w yield for *P. nigrum* 8.13. This study showed that piperine content varied according to different drying treatments (Table 10). Microwave treatment gave 26.7 mg of piperine /g of extract or 1708.8 mg/100 g of berries compared to the fresh sample which gave 11.5 mg of piperine/ g of extract or 345.0 mg/100 g of fresh berries. Apart from that, some of the minor or unknown peak's areas were found to be increased and decreased according to different drying treatments. Other than the major piperine peak, a smaller peak at ~ 4.5 min was tentatively identified as piperyline (Figure 10 (c) microwave drying) based on the results reported by Wood et al., 1988. More chromatograms are shown in Appendix.
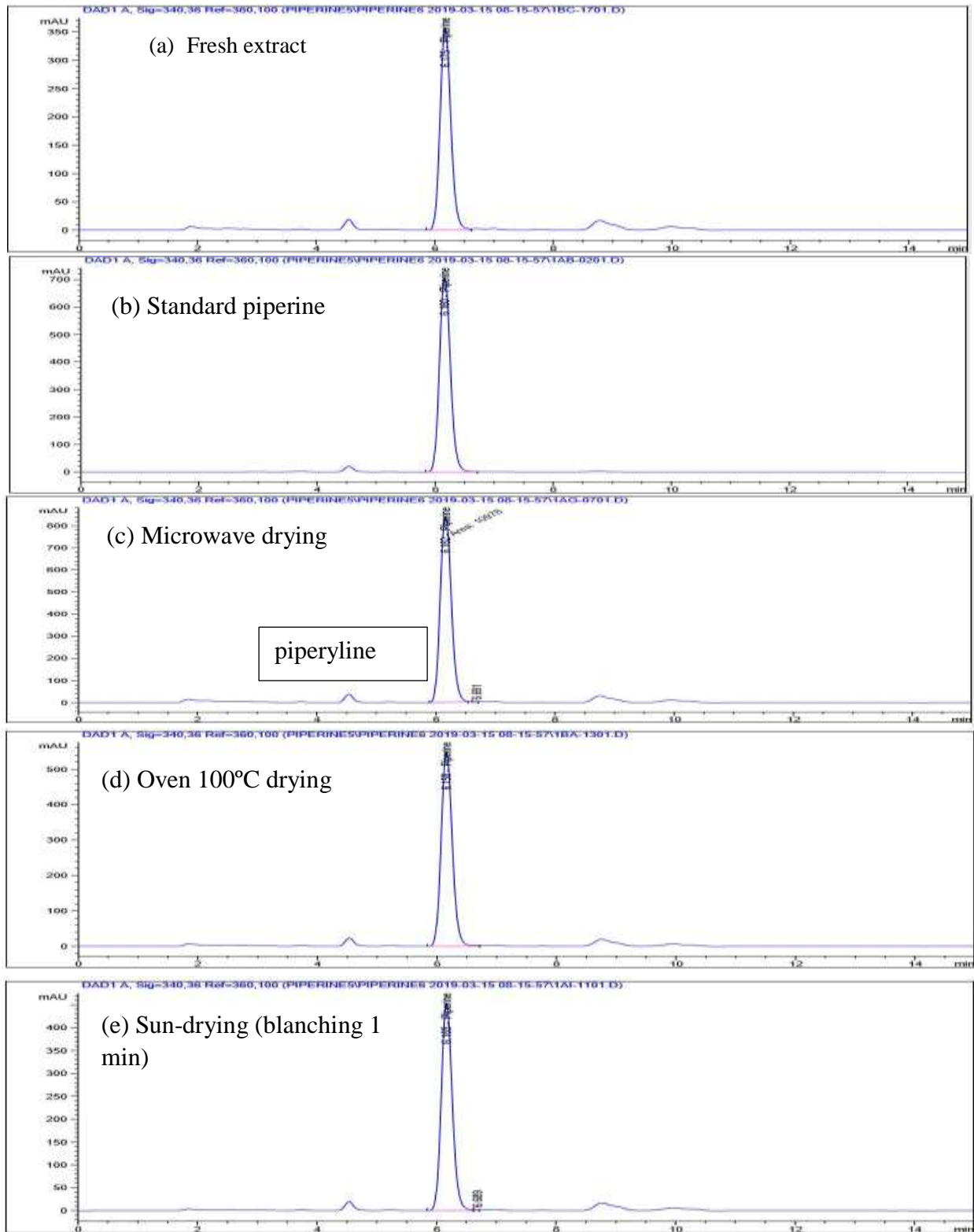
Figure 10. The chromatograms of (a) fresh extract, (b) standard piperine, (c) microwave, (d) oven 100ºC and (e) sun-drying (blanching 1 minute) drying treatments

Table 10. Adjusted TPC value and piperine content of *P. nigrum* berries subjected to various drying treatments

Drying Treatment	TPC (adjusted to 100%) (mg GAE/ 100g)	mg piperine/g of extract	mg/100g of *P. nigrum* berries
Fresh	1932.1±25.0[e]	11.50±0.02	345.0±0.6
Microwave	2722.8±15.7[a]	26.70±0.01	1708.8±0.9
Oven 100°C	2650.8±18.8[b]	17.95±0.01	1032.1±0.4
Oven 50°C	1984.9±26.4[e]	11.75±0.01	399.5±0.2
Freeze drying	2257.4±23.0[e]	13.20±0.01	627.0±0.4
Sun drying (Blanching 1 min)	2279.6±43.5[cd]	14.70±0.01	727.7±0.7
Sun drying (Blanching 15 min)	2241.7±49.3[cd]	12.71±0.02	599.2±0.8
Sun drying (Without Blanching)	2274.3±31.9[cd]	13.62±0.01	660.6±0.7
Ambient air drying	1969.2±26.0[e]	11.61±0.02	354.5±0.6

*The values are presented as mean of three experiments ± standard deviation (SD). For each column data, the values followed by the same superscripted letter are not statistically significant ($P<0.05$) based on one way ANOVA with Tukey HSD analysis.

This current finding also revealed that there is a good correlation ($r^2 = 0.9594$) between the TPC values of   berries which were subjected to different drying treatments and the piperine content observed (Figure 11). Thus, in general there is a large increase of piperine content when the TPC increases under various dryment treatments except for oven 50 C^o and ambient air drying which showed a slight or no increase in the amount of piperine.
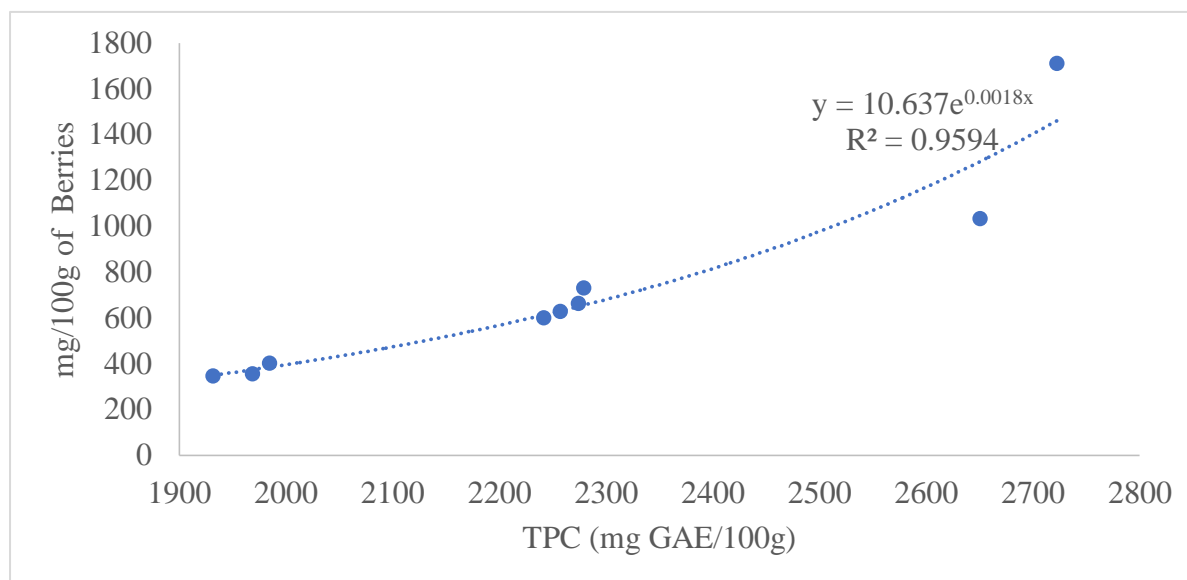
Figure 11: Correletion between TPC (mg GAE/100g) and piperine content (mg/100g) of berries

The reason for the increase in piperine content after drying is not clear at the moment. It may be due to some biochemical reaction(s) that could occur while drying or the drying process could have ruptured the cellular walls releasing piperine into the extracting solvent. A study reported that biosynthesis of piperine probably is initiated by sequence of reactions   i.e. condensation, decarboxylation, oxidative deamination and cyclization. The investigation suggests that biosynthetic pathway enroute to piperine would involve the amino acid precursor L-lysine (Chopra et al., 2016). The N-heterocycle piperidine is generated from the decarboxylation of amino acid L-lysine in the presence of pyridoxal phosphate (PLP) to cadaverine, which goes through oxidative deamination directly by the enzyme diamine oxidase to yield an amino aldehyde. Then the amino aldehyde further undergoes cyclization to give the imine, Δ1-piperideine, which further reduces to piperidine and then this generated piperidine reacts with piperoyl-CoA to produce piperine (Chopra et al., 2016).

Different drying treatments could have affected the enzymatic activity of biosynthetic pathway or degradation of piperine. Thus, more work needs to be done to investigate and explore the detailed pathways that *P. nigrum* berries undergo upon different drying conditions in future.

5.6.2 Identification of Piperine and other functional components of *P. nigrum*

The presence of phytochemical compounds from the fruits of *P. nigrum* was screened by LC-MS method. Eighteen bioactive phytochemical compounds were identified in the microwave treated 50 % ethanolic extract of *P. nigrum* whereas thirteen compounds were identified in fresh extract of *P. nigrum* extract (Table 11 and 12). The ion chromatograms are presented in Figure 12. Seven major alkaloid amides were identified in microwave treated extract; piperine, piperidine, piperlonguminine, piperyline, piperettine , piperolein A and piperanine whereas only four major alkaloid amides were detected in fresh extract: piperine, piperidine, piperolein A and piperanine. Few other compounds which were identified in microwave treated extract were not found in fresh extract: D-proline, cochlearine, senthoxydim and trilostane and this could partially contribute to the higher TPC value of microwave-dried extract compared to the fresh extract.

Table 11. Compounds detected from the fresh berries extract by LC-MS method

Phytochemical compounds	Chemical formula	m/z value/ [M+H]$^+$
(+)-2,3-Dihydro-3-methyl-1H-pyrrole	$C_5 H_9N$	84.08089
2-Amino-3-methyl-1-butanol	$C_5 H_{13}NO$	104.10687
Piperidine	$C_{14} H_{17}NO$	216.13834
N-3,7-Dimethyl-2,6-octadienylcyclopropylcarboxamide	$C_{14} H_{23}NO$	222.18518
(E,E)-2,4-Decadienoic isobutylamide	$C_{14} H_{25} NO$	224.20179
Piperine	$C_{17} H_{19}NO_3$	286.14486
Piperanine	$C_{17} H_{21}NO_3$	288.16141
Isoxsuprine	$C_{18} H_{23}NO_3$	302.17571
Nalorphine	$C_{19} H_{21}NO_3$	312.16014
Armepavine	$C_{19} H_{23}NO_3$	314.17539
Piperolein A	$C_{19} H_{25}NO_3$	316.1919
Simulenoline	$C_{20} H_{23}NO_3$	326.17634
Retrofractamide A	$C_{20} H_{25}NO_3$	328.19192

Table 12.Compounds detected from the microwave treated berries extract by LC-MS method

Phytochemical compound	Chemical Formula	m/z value/ [M+H]$^+$
2-Amino-3-methyl-1-butanol	C$_5$H$_{13}$NO	104.10719
Piperidine	C$_{14}$H$_{17}$NO	216.13834
N-3,7-Dimethyl-2,6-octadienylcyclopropylcarboxamide	C$_{14}$H$_{23}$NO	222.18551
D-proline	C$_5$H$_9$NO$_2$	138.05219
Cochlearine	C$_{15}$H$_{19}$NO$_3$	262.14481
Piperyline	C$_{16}$H$_{17}$NO$_3$	272.1293
Piperlonguminine	C$_{16}$H$_{19}$NO$_3$	274.14272
Piperine	C$_{17}$H$_{19}$NO$_3$	286.14486
Piperanine	C$_{17}$H$_{21}$NO$_3$	288.16141
Isoxsuprine	C$_{18}$H$_{23}$NO$_3$	302.17571
Nalorphine	C$_{19}$H$_{21}$NO$_3$	312.16014
Piperettine	C$_{19}$H$_{21}$NO$_3$	312.16149
Armepavine	C$_{19}$H$_{23}$NO$_3$	314.17539
Piperolein A	C$_{19}$H$_{25}$NO$_3$	316.1919
Simulenoline	C$_{20}$H$_{23}$NO$_3$	326.17634
Sethoxydim	C$_{17}$H$_{29}$NO$_3$S	328.19255
Trilostane	C$_{20}$H$_{27}$NO$_3$	330.2073
Nalmefene	C$_{21}$H$_{25}$NO$_3$	340.1923

Fragmentor Voltage 125 **Collision Energy** 0 **Ionization Mode** ESI

+ESI TCC Scan Frag=125.0V S1-MS-Pos-12_15-1uL.d

x10 7

Counts vs. Acquisition Time (min)

Fragmentor Voltage 125 **Collision Energy** 0 **Ionization Mode** ESI

+ESI TCC Scan Frag=125.0V S2-MS-Pos-12_15-1uL.d

x10 7

→
→ Piperine

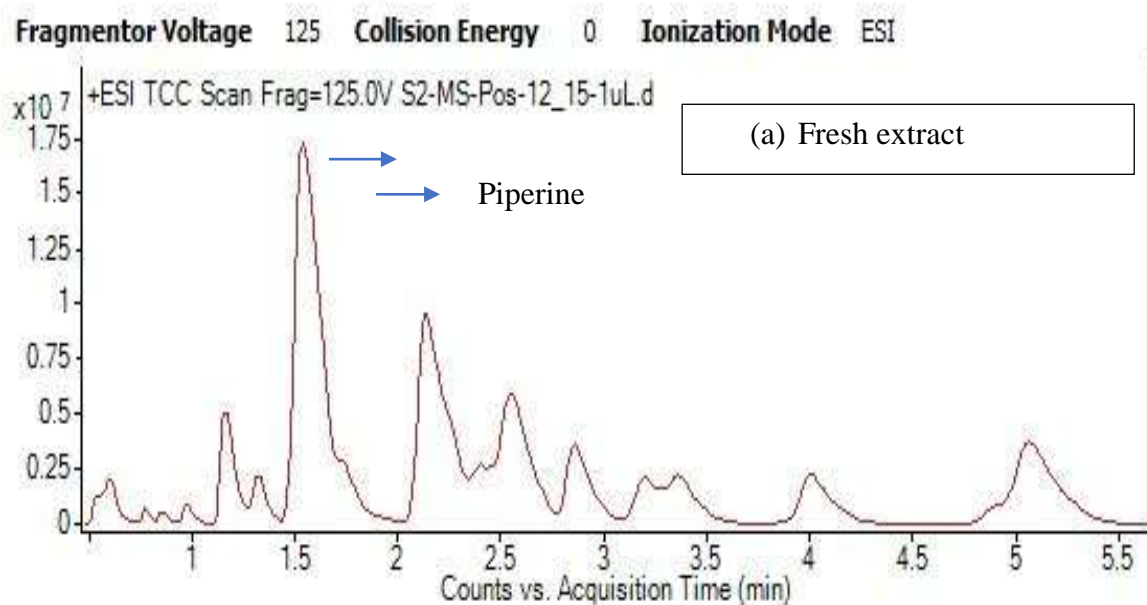Counts vs. Acquisition Time (min)
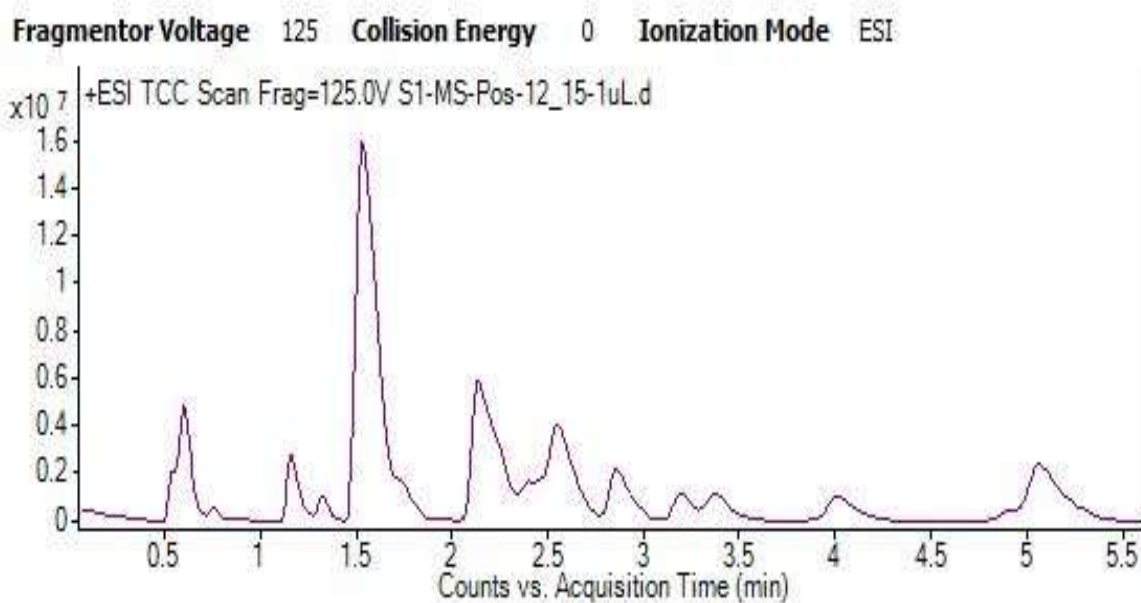
(a) Fresh extract

Figure 12. LC-MS chromatogram of (a) fresh and (b) microwave treated extracts of *P. nigrum* berries

(b) Microwave treated extract

Piperine

5.7 Antimicrobial Assay

Irrespective of the potentiality of *P. nigrum* not much work has been carried out to study the efficacy of 50% ethanol extract with various drying treatments as antimicrobial agent. We made an effort to evaluate the effect of the different drying treatments on the *P. nigrum* berries as an antimicrobial agent.

The antimicrobial activity of *P. nigrum* crude extract (fresh sample) and berries treated with microwave drying were examined against a gram-positive microorganism (*Staphyloccous epidermis* ATCC 25923) and three gram negative microorganisms (*Escherichia coli* ATCC 25922, *Proteus mirabilis* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 respectively). Their potency were qualitatively and quantitatively assessed with the presence and absence of inhibition zones and zone diameter (mm) and minimal inhibitory concentration (MIC) values. These extracts of fresh berries and microwave treated berries displayed varied antibacterial activities across the studied pathogens.

As can be seen from Tables 13 and 14, fresh extract exhibited weak activity against both Gram-positive and Gram-negative microorganisms. For the gram negative strains treated with fresh extract, the disc diameter zones of inhibition ranged from 7.5 mm-11.3 mm, along with MIC values ranging from 62.5-250 µg/mL whereas 13.0 mm zone of inhibition and 31.25 µg/mL MIC values were observed for Gram (+) microorganism.

Table 13. Inhibition zone diameter (mm) of the antibacterial effects of 50% ethanol fresh and microwave extract, piperine and streptomycin against *S. aureus*, *E.coli*, *P. mirabilis* and *P. aeruginosa*

Tested Strain	Zone of Inhibition (mm)			
	Fresh	Microwave	Piperine	Streptomycin
Staphyloccus epidermis	13.0±1.0 +	19.0±1.0 ++	22.0±1.0 +++	20.7±0.6 +++
Escherichia coli	11.3±0.6 +	21.7±0.6 +++	23.3±1.2 +++	13.7±0.3 +
Proteus mirabilis	8.2±0.3 +	15.5±0.5 ++	18.7±0.6 ++	16.5±0.5 ++
Pseudomonas aeruginosa	7.5±1.0 +	11.5±0.5 +	13.5±0.5 +	21.8±0.8 +++

Abbreviations: MIC, minimal inhibitory concentration (μg/mL). Values are means of triplicate measurements of inhibition zone diameter (mm) including diameter of disc (6 mm). Positive control: Piperine 1 mg, Streptomycin 10 μg. Negative control containing only solvent, did not show any activity. Antibacterial activity for zone of inhibition was categorized susceptible (+++), intermediate (++), resistant (+) according to Clinical and Laboratory Standard Institutes guidelines. Concentration used was 1 mg extract per disc.

Table 14. Minimum inhibitory concentrations (MIC) of fresh, microwave treated berries, piperine , vancomycin and penicillin against *S. aureus*, *E.coli*, *P. mirabilis* and *P. aeruginosa*

Tested Strain	Minimum Inhibitory Concentration (µg/mL)				
	Fresh	Microwave	Piperine	Vancomycin	Penicilin
Staphyloccus epidermis	31.25	1.98	0.98	1.98	1.98
Escherichia coli	62.5	3.91	0.98	NS	NS
Proteus mirabilis	125	15.63	7.815	3.91	NS
Pseudomonas aeruginosa	250	62.5	31.25	NS	NS

Abbreviations: NS, Not Susceptible; MIC, minimal inhibitory concentration (µg/mL). Results are mean from three sets of experiments, each set in triplicate. SD was + 0.00.

Among Gram (-) microorganisms, the strongest antibacterial activity of microwave treated *P. nigrum* berries was showed against *E. coli* ATCC 25922 (21.7 mm; 3.91 µg/mL MIC value) followed by *P. mirabilis* ATCC 25922 (15.5 mm; 15.63 µg/mL MIC value) and the weakest activity found against *P. aeruginosa* ATCC 27853 (11.5 mm; 62.5 µg/mL). It is interesting to find out that the MIC of microwave treated extract (3.91 µg/mL) showed strongest activity against *E.* ATCC 25922 which was not susceptible to the positive control, Vancomycin and Penicilin. (Table 14).

This present study also revealed that standard piperine has high potential to inhibit microbial growth. According to the results, piperine alone exhibited strongest activity against gram-negative microorganism: *E. coli*; 23.3 mm, 0.98 µg/mL MIC value and gram-positive microorganism: *S. epidermis*; 22.0mm, 0.98 µg/mL MIC value. Compared to fresh samples, the results obtained for microwave treated berries are closer to the MIC value of standard piperine. The susceptibility of *E. coli* to piperine may result from the *E. coli* having a highly permeable membrane in comparison to other gram negativity bacterial strains like *P. mirabilis* and *P. aeruginosa*. These results are in agreement with those reported by Aldaly

(2010). *P. mirabilis* and *P. aeruginosa* exhibited moderate to weak activity (15.5 mm; 15.63 µg/mL and 11.5 mm; 62.5 µg/mL). This generally higher resistance among Gram (-) bacteria could be ascribed to the nature of their outer membrane surrounding the cell wall, which restricts diffusion of hydrophobic compounds through its lipopolysaccharide covering (Morsy and El-Salam, 2017). The absence of this barrier in Gram (+) bacteria allows the direct contact of extract's hydrophobic constituents with the phospholipids bilayer of cell membrane, causing either an increase of ion permeability and leakage of vital intracellular constituents, or impairment of the bacterial enzyme systems. Differences in MIC values of bacteria could be related to the differential susceptibility of bacteria cell wall with minor differences present in the outer membrane in the cell wall composition.

The antimicrobial properties of microwave treated extract of *P. nigrum* are associated, in major part, with its high contents of alkaloid amides like piperine and its derivatives. Besides that, it could be also due to the presence of synergy, antagonism or additive effects of the major components of alkaloids other than piperine, which possess various potency of activity. It is most likely that numerous components of the extract play a crucial role in lipophilic or hydrophilic attraction and fixation on cell wall and membranes and cellular distribution. This feature is very important because, depending on their component, the distribution of the phytochemical compounds in the cell determines the different types of activities such as antibacterial, antifungal and cytoxicity.

CHAPTER 6

CONCLUSION AND PLAN FOR FUTURE WORK

6.1 Conclusion

Pepper (*Piper nigrum* Linnaeus) is an important commercial spice valued since early times for its pungency and flavour, and selecting the best drying treatment in respect to qualitative and nutritional characteristics of this product is very important.

In our study, four different concentrations of ethanol , i.e., 30%, 50%, 70%, and 100% and 50% methanol were used to extract the active compounds present in the plant. It was confirmed that 50% ethanol extract has the maximum TPC when compared to other solvents tested. Several different drying treatments were applied to *P. nigrum* berries. A significant positive correlation was observed between TPC vs AEAC and IC_{50} as well FRP and CC_{50}. This study revealed that microwave and oven $100^{\circ}C$ treatments as the better drying method compared to other drying treatments as they increased the antioxidant properties of *Piper nigrum.* The RP-HPLC chromatograms of all drying treatments showed a good correlation between the TPC and piperine content for the differently treated berries.

Our study can be considered as the first report of examining the effects of various drying methods of *P. nigrum* fresh green berries on the antimicrobial as well as antioxidant activities. The *in vitro* antimicrobial activity of the obtained results may be due to the thermal treatments which play the major role in influencing the phytochemical composition of treated *P. nigrum* berries.

Our results are a contribution to a better valorization of this medicinal plant as well as to pepper processing industries which are focussing on continuous new product innovations such as essential oil, fragnance, nutraceutical products and innovative packagaing of black pepper powder.

6.2 Plan for future work

<u>In-depth Analysis of Bioactive Compounds</u>

In this present experiment, fresh sample and extract processed from microwave drying treatment was analysed for the presence of bioactive constituents. Nonetheless, one should underscore that these preliminary LC-MS analysis only demonstrated the chemical profile of bioactive compounds from the extracts which had been freeze-dried prior to LC-MS analysis. In order not to overlook the other volatile compounds which may have contributed to the antioxidant and antibacterial activities, future work needs to be more focussed on the extracts obtained by supercritical carbon dioxide extraction or hexane extraction, which can extract the bioactive essential oil components. MS-MS analysis should be undertaken for identification of more compounds that may show novel antimicrobial activity.

<u>Synergistic Antibacterial Activity Analysis</u>

To combat microbial resistance, it is vital to explore the mode of activity of antimicrobial combination therapy as it differs from that of the same individual antibiotics when administered as single drugs. This synergistic study can be explored using the combination of the extracts of different spices with *P. nigrum* extract or in combination at some ratio with some conventional antimicrobials (tetracycline, rifampicin, itraconazole and etc) to provide new strategies for improving the use of antibiotics in the treatment of infectious diseases.

<u>Pharmacological and Biological Analysis of New Compounds</u>

The origin of the new compounds found in microwave treated sample is not known. This could be a subject of further research. Several other biological tests such as anticancer tests will be worthwhile to perform in future.

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Appendix A

Table A1: Chemical Reagents and Instruments

Assay	Chemical	Brand
Phenolic Extraction	Liquid nitrogen	Statebourne Cryostor 300
	Absolute Methanol (Analytical grade)	J. Kollin Chemicals
TPC assay	Folin-Ciocalteau reagent	Merck Millipore
	Anhydrous sodium carbonate	Fisher Scientific
	Gallic acid	RandM Marketing
FRS assay	2,2-diphenyl-1-picryl-hydrazyl-hydrate	Sigma-Aldrich
	Methanol (ACS grade)	Friendemann Schmidt
	L-Ascorbic acid	Fisher Scientific
FRP assay	Di-potassium hydrogen phosphate	Merck Milipore
	Potassium dihydrogen phosphate	Fisher Chemicals
	Potassium ferricyanide	Unilab Laboratory Reagent
	Trichloroacetic acid	Fisher Scientific
	Iron (III) chloride-6-hydrate	Fisher Scientific
FIC assay	Iron (II) sulphate	HmbG Chemicals
	Ferrozine	Acros Organics
HPLC	Acetonitrile (HPLC grade)	Friendemann Schmidt
	4% acetic acid	Friendemann Schmidt
	Piperine	Sigma-Aldrich

Instruments used	Instrument	Brand
Drying treatments	Oven drying at 50°C	Fine Tech SOV140B
	Oven drying at 100°C	Translab DHG-9030A
	Microwave	Sharp, Model R-218L
	Freeze-drying	Labconco Freezone 4.5 Plus
Sample preparation	Analytical balance	Kern ALS 120-4
Plant extraction	Orbital shaker	Protech Model 719
	Vacuum pump	Eyela aspirator A-3S
	Filter paper	Fisherbrand P5
Antioxidant assay	Spectrophotometer	Unico 2100
	UV-vis spectrophotometer	Unico 2100
HPLC	Rotary evaporator	Eyela N-1001S-W
	Freeze-dryer	Labconco Freezone 4.5 Plus
	Refrigerator (-80°C)	Sanyo MDF-U4086S
	Sonicator	Elma ultrasonic LC130H
	HPLC machine	Agilent Technologies 1260 Infinity
	C_{18} column	Agilent Poroshell 120 EC-C18 column (4.6×250 mm, 5 μm)
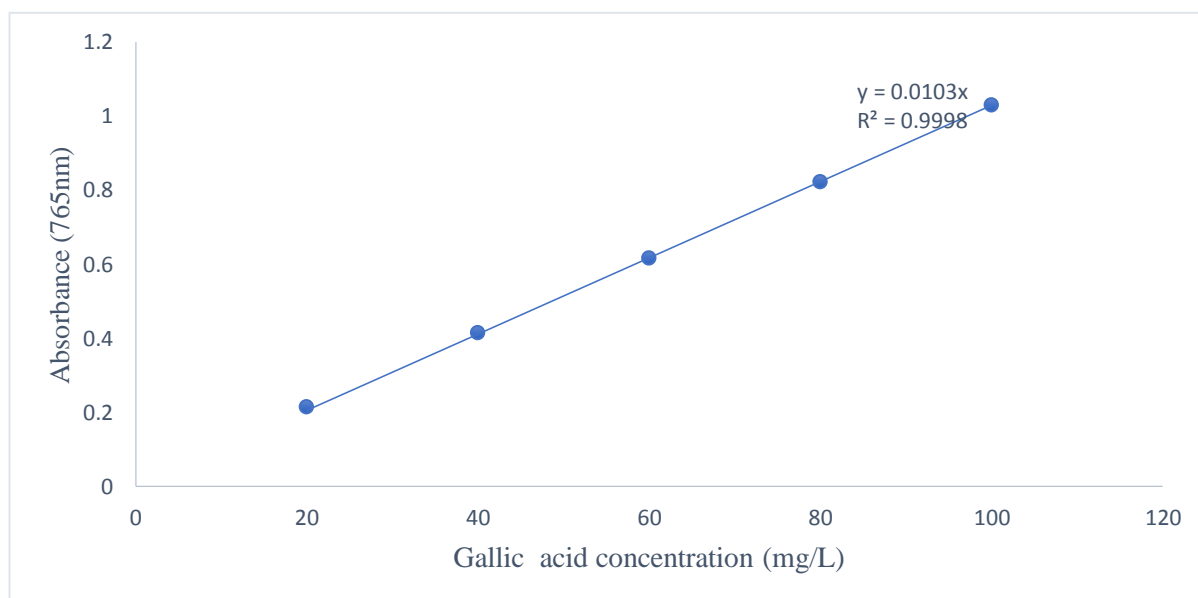
Standard Curves

Figure A1. Standard curve of absorbance at 765 nm for total phenolic content determination using gallic acid as standard
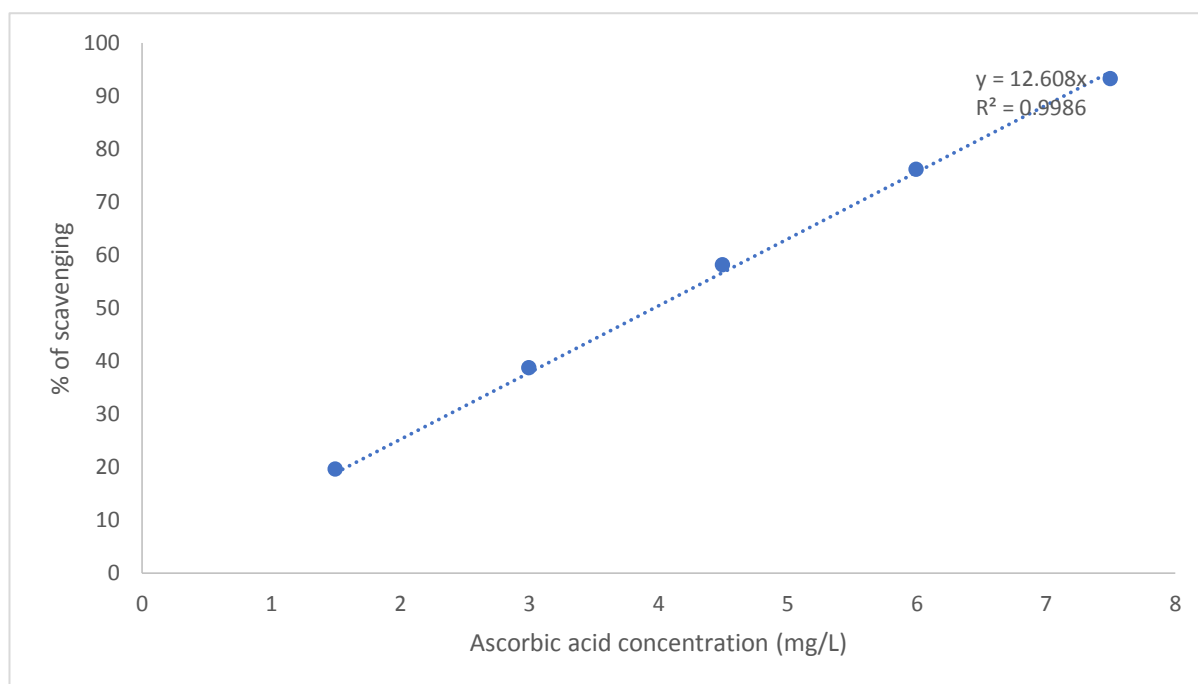
Figure A2. Standard curve of scavenging activity (%) against the ascorbic acid concentration (mg/L) for DPPH free radical scavenging activity.
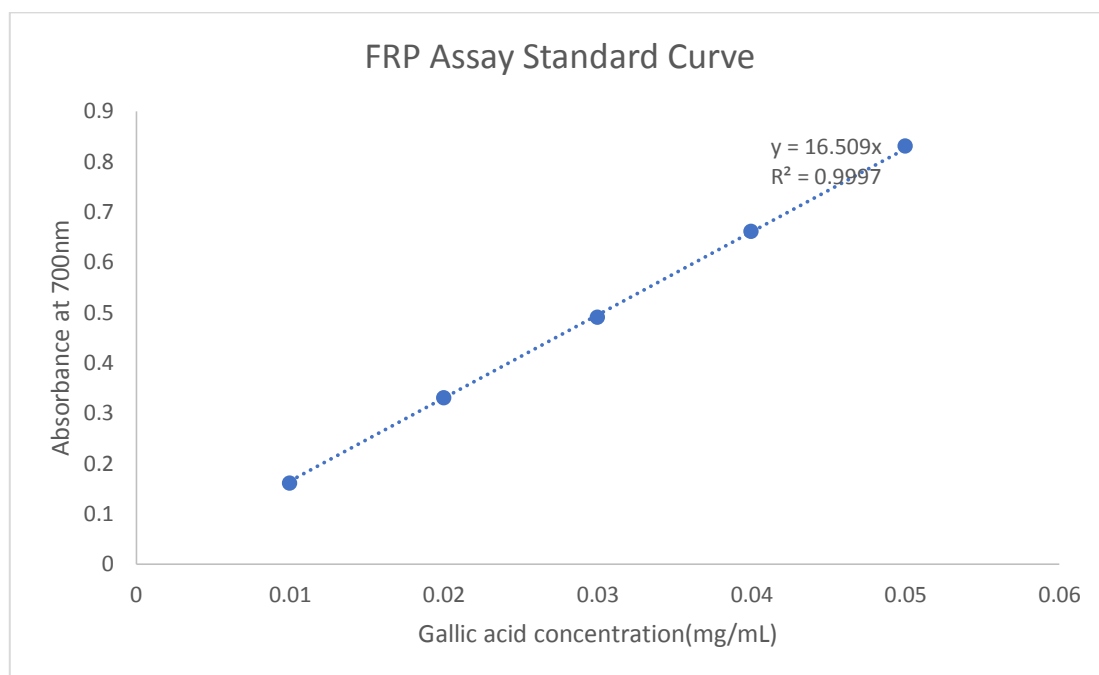
Figure A3.Standard curve of absorbance at 700 nm against gallic acid concentration (mg/mL) for ferric reducing power.

Appendix C

Raw Data

Table A2. TPC(mg GAE/100g) and peak area of piperine (mAU) of fresh and extract of various drying treatments

Drying method	TPC (mg GAE/ 100g)	Peak area of piperine (mAU)
Fresh control	1932.1 ± 25.0^d	3836.33 ± 6.4^h
Microwave	2722.8 ± 15.7^a	10971.0 ± 5.8^a
Oven 100°C	2650.8 ± 18.8^b	7132.5 ± 2.9^b
Oven 50°C	1984.9 ± 26.4^d	4372.8 ± 2.3^f
Freeze drying	2257.4 ± 23.0^c	5823.9 ± 4.0^d
Sun drying (Blanching 1 min)	2279.6 ± 43.5^c	5857.4 ± 5.8^c
Sun drying (Blanching 15 min)	2241.7 ± 49.3^c	4572.1 ± 5.8^e
Sun drying (Without Blanching)	2274.3 ± 31.9^c	5833.9 ± 5.8^d
Ambient air drying	1969.2 ± 26.0^c	3533.9 ± 5.8^g

Table A3. Yield of extract (%) of various drying treatments

Drying Treatment	Yield of Extract (%)
Fresh	6.0
Microwave	12.8
Oven 100°C	11.5
Oven 50°C	6.8
Freeze drying	9.5
Sun drying (Blanching 1 min)	9.9
Sun drying (Blanching 15 min)	8.8
Sun drying (Without Blanching)	9.7
Ambient air drying	6.2

```
RetTime Sig Type     Area      Amt/Area    Amount   Grp    Name
 [min]               [mAU*s]               [ppm]
-------|--|-------|-----------|-----------|----------|--|---------------
  6.162  1 MM      1.09780e4  5.02555e-3   55.17038      Piperine

Totals :                                   55.17038
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============================================================
                      Calibration Curves
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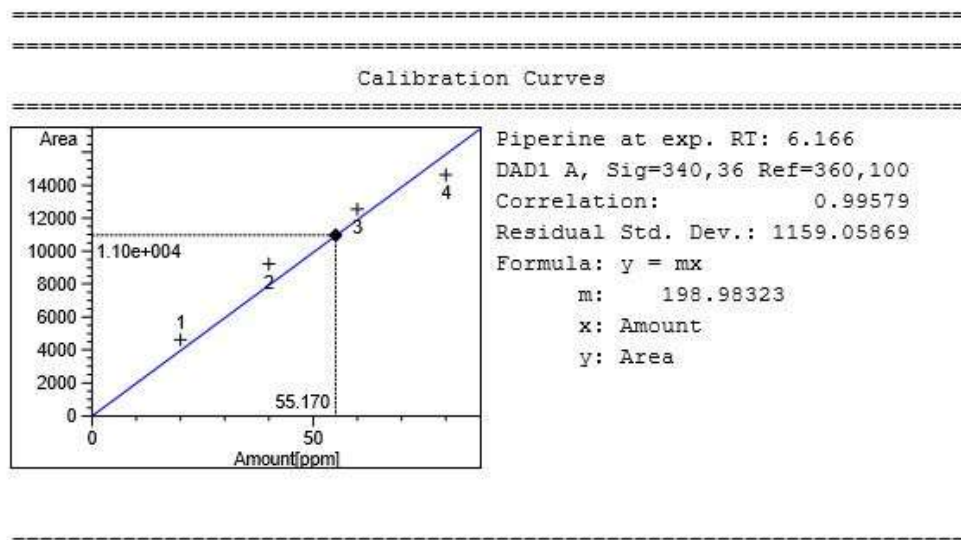

Figure A4. Calibration curve area vs piperine concentration (mg/L) of microwave treated extract

Calculation:

Area vs piperine concentration: 55.2 mg/L

1 mL= $\frac{55.2}{1000}$ mg piperine

1 g of extract= $\frac{1000}{2} \times \frac{55.2}{1000}$

$\qquad$ = 26.7 mg of piperine/ g of extract

1 g of berries gives 0.128g of extract

1 g of extract $= \frac{1}{0.128}$ g of berries

$\qquad$ = 7. 82 g of berries

100 g of berries give $= \frac{100}{7.82} \times 26.7$ mg

$\qquad$ = 341.76 mg

$\qquad$ = 341.76 × 5 (extract sample solution diluted 5 times)

$\qquad$ = 1708.8 mg/100g of berries
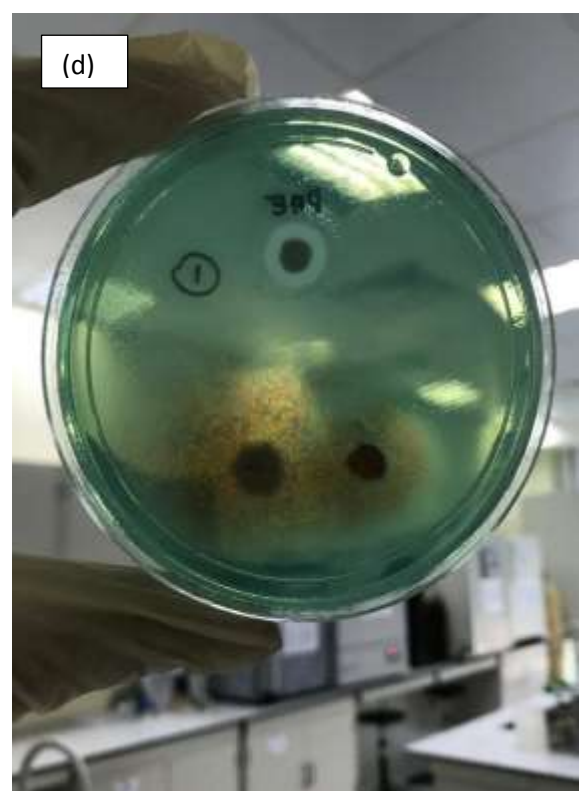
Figure A5. Zone of Inhibition (mm) of microwave treated extract, piperine and streptomycin of (a) *P. mirabilis*, (b) *E.coli* , (c) *S. epidermis* and (d) *P. aeruginosa*

APPENDIX D

SAMPLE CALCULATION

The following sample calculations are only shown for one replicate.

Calculation of total phenolic content (TPC)

Extraction	Triplicates absorbance			Average
1	0.479	0.466	0.491	0.479

Mass of berries sample = 1.0216g

Based on standard curve,

$$Absorbance = 0.0103 \, (Extract \ concentration)$$

$$Extract \ concentration = \frac{0.479}{0.0103} = 47.5 \, mg/L$$

$$Dilution \ factor = 47.9 mg/L \times 5 = 232.5 mg/L$$

$$In \ 50 mL \ of \ extract = 232.5 \times \frac{50}{1000} = 11.63 \, mg \ GAE$$

$$In \ 100g \ extract = \frac{11.63}{1.026} \times 100 = 1133.5 \, mg \ GAE/100 \, g$$

Calculation of DPPH free radical scavenging activity

Microwave (Highest setting)							
Mass	Sample Volume	mass/ml(mg)	Triplicates absorbance			Average	Scavenging
1021.5	50	0.3405	0.4411	0.4553	0.4501	0.4488	55.2
1021.5	100	0.681	0.3047	0.3051	0.3033	0.3044	69.6
1021.5	200	1.362	0.098	0.099	0.069	0.0887	93.8
	Negative control		0.9877	1.012	1.007	1.0022	

Calculation of extract concentration: $In \ 1mL \ of \ extract = \frac{1021.5}{50 \, mL} = 20.43 \, mg \ berry$

$$If \ 0.05 \, mL \ of \ extract \ volume \ was \ used, \ \frac{20.43 \times 0.05}{3} = 0.3405 mg/mL$$

Calculation of % scavenging:

$$\% \ scavenging = \frac{1.0022 - 0.4488}{1.0022} \times 100 = 55.2\%$$

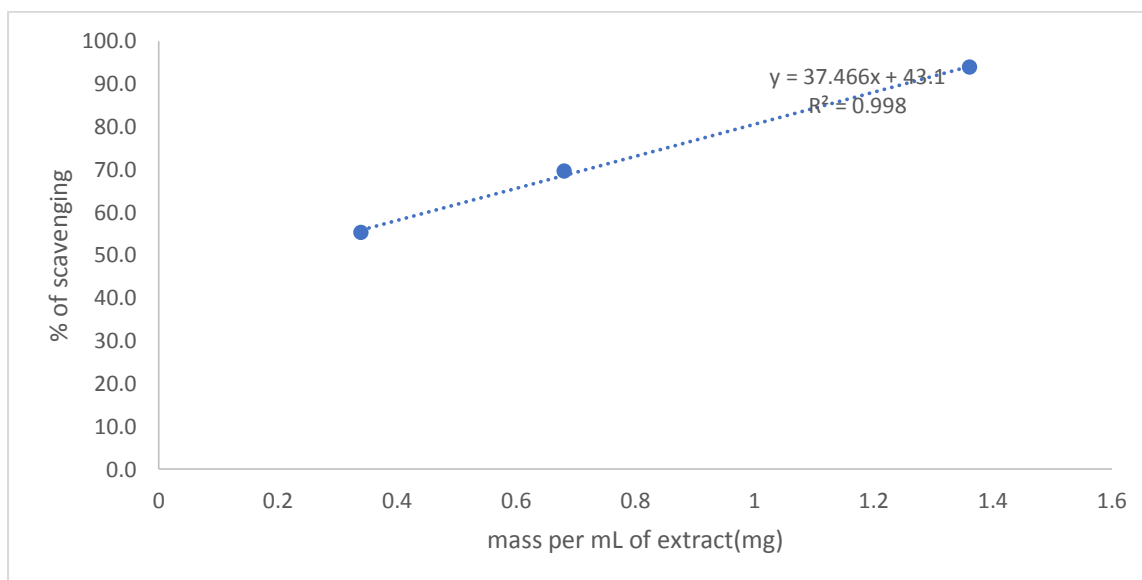Figure A6. Graph of % scavenging activity against extraction concentration of microwave treatment.

Calculation of IC$_{50}$:

$\text{IC}_{50} \text{ concentration} = \frac{50 - 43.1}{37.466} = 0.18$ mg/ml

Calculation of AEAC:

$\text{AEAC} = \frac{IC_{50(ascorbate)}}{IC_{50(sample)}} \times 10^5 = \frac{0.00397}{0.18} \times 100 = 2205.6$ **mg AA/ 100 g**

Calculation of FRP

Mass of berries (mg)	Solvent Volume (mL)	Sample Volume (uL)	Mass (mg)	Triplicates absorbance			Average	Gallic Acid (mg)
1021.6	50	400	8.1808	0.3683	0.3685	0.3672	0.368	0.0223
1021.6	50	200	4.0864	0.1756	0.1757	0.1721	0.2716	0.0106
1021.6	50	100	2.0432	0.1244	0.1311	0.1358	0.1304	0.0079

Fresh control:

50 ml extract has 1021.6 mg of sample

1 ml of extract $= \frac{1021.6}{50} = 20.452$ mg/ml

If extract volume is 0.4ml, the final leaves mass extracted $= 20.452 \times 0.4 = 8.1808$ mg

Based on the standard curve, Absorbance $= 16.509$ (GAE)

GAE$= \frac{0.3680}{16.509} = 0.0223$ mg
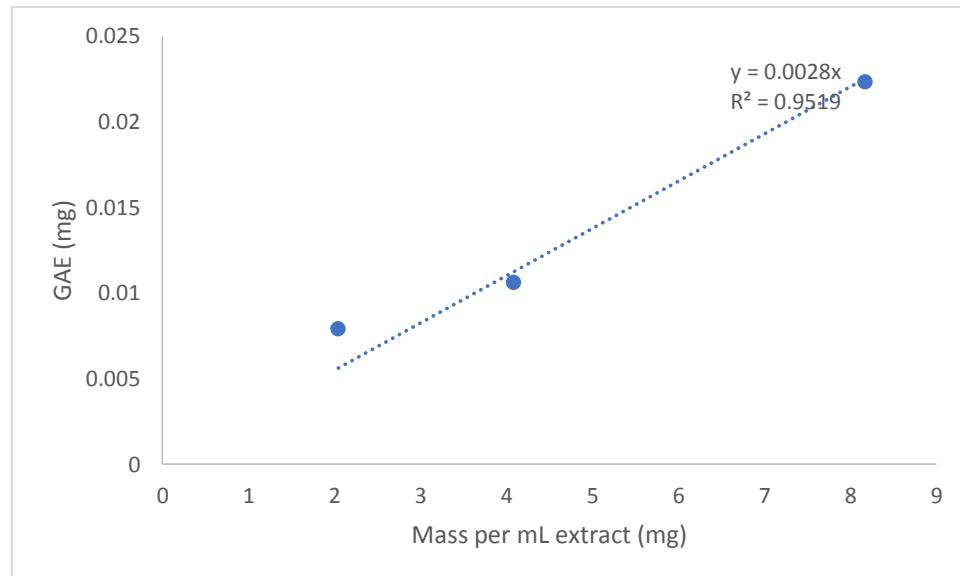
FRP value $= 0.0028 \times 1000 = $ **2.8 mg GAE/g**

Calculation of FIC

Fresh Extract (Tambun,Perak)								
Mass	Sample Volume (ul)	mass/ml(mg)	Triplicates absorbance			Average	Chelating (%)	mass/3 ml
1026.1	125	2.5653	0.7581	0.7588	0.7575	**0.7581**	25.0	0.8551
1026.1	250	5.1306	0.6212	0.6213	0.6208	**0.6211**	38.6	1.7102
1026.1	500	10.2612	0.4251	0.4225	0.4218	**0.4231**	58.2	3.42
	Negative control		1.008	1.011	1.015	**1.0113**		

Mass of leaves per ml $= \frac{1026.1}{50} \times 0.125 = 2.5653$ mg/ml

% chelating $= \frac{1.0113 - 0.4231}{1.0113} \times 100 = 58.2\%$
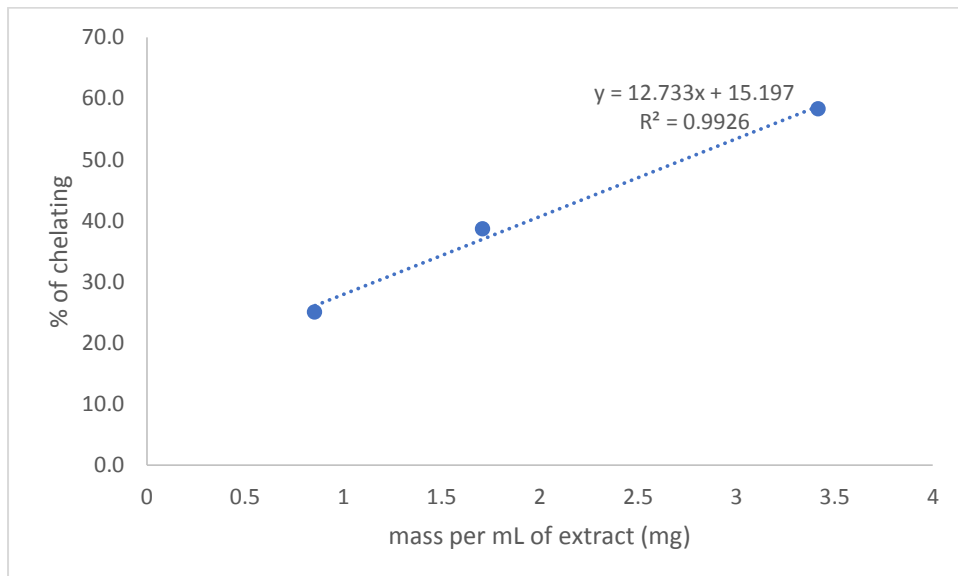
% chelating $= 12.733 \, (CC_{50}) + 15.197$

$CC_{50} = \frac{50 - 15.197}{12.733} = 2.7$ mg/ml
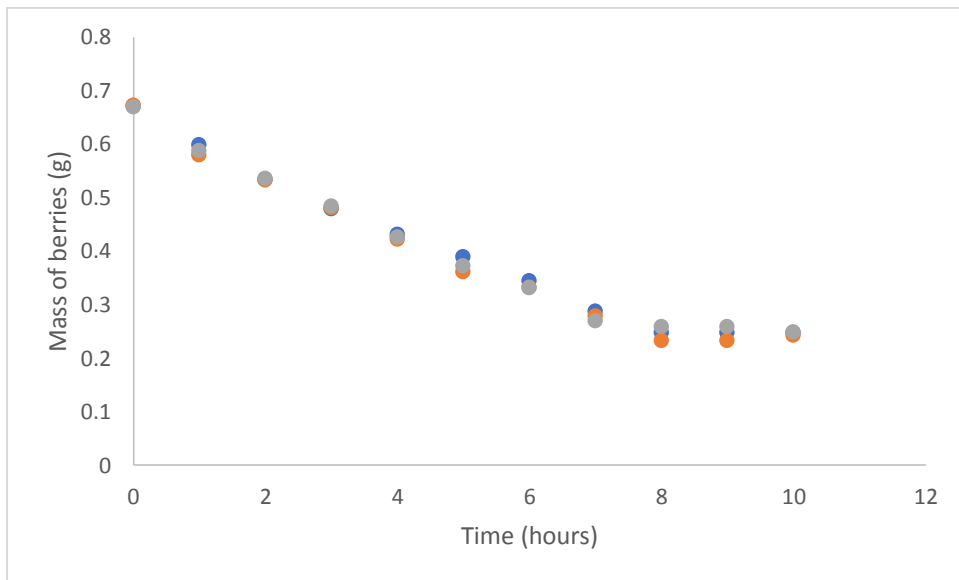
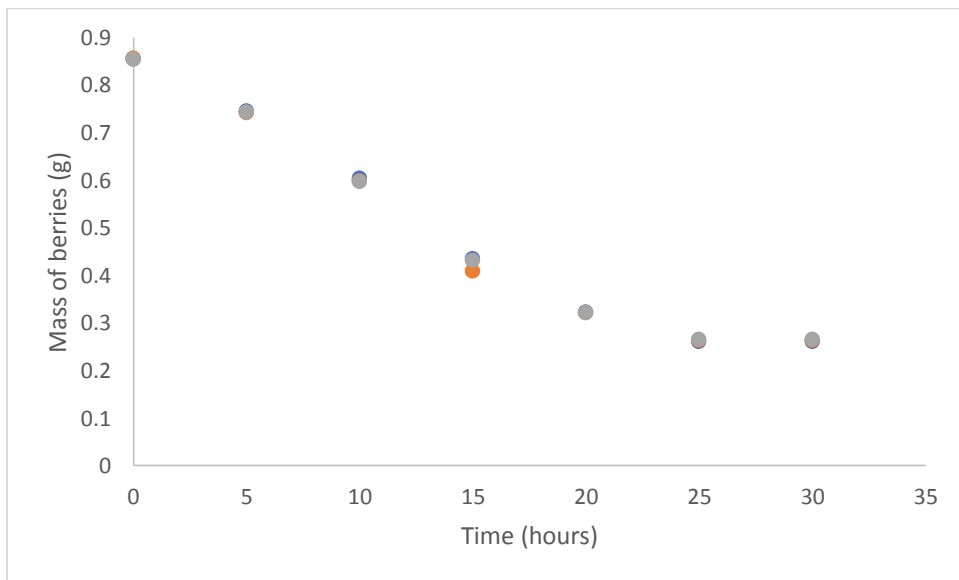Figure A7. Drying curve of oven 50°C for the triplicate samples

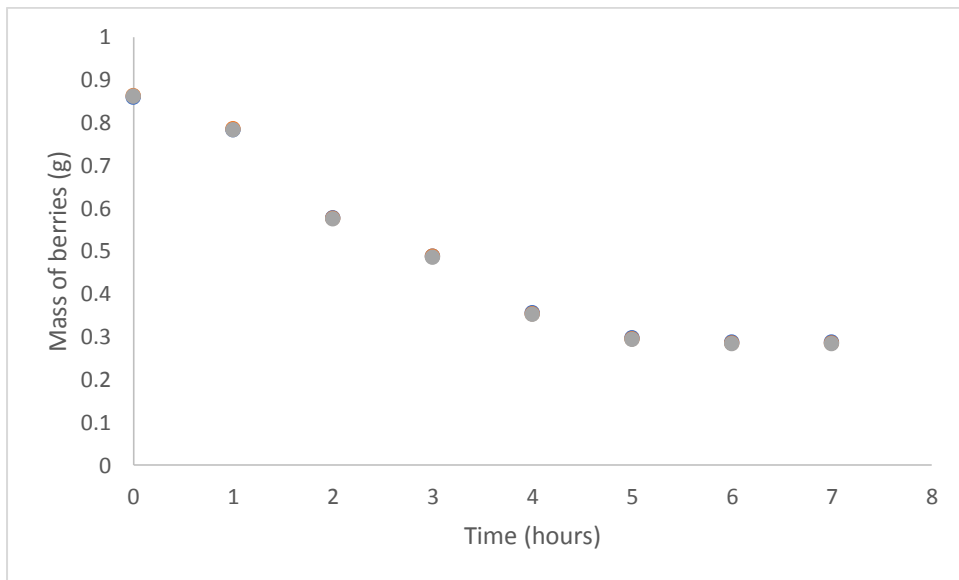Figure A8. Drying curve of freeze drying method for the triplicate samples

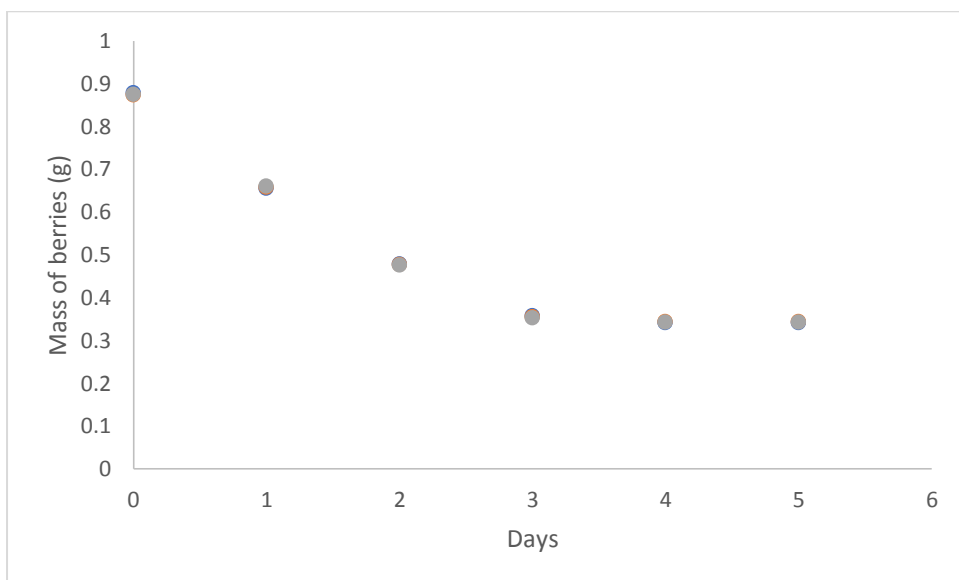Figure A9. Drying curve of sample (blanching 15 min) under sun drying for the triplicate samples

Figure A10. Drying curve of ambient air drying for the triplicate samples
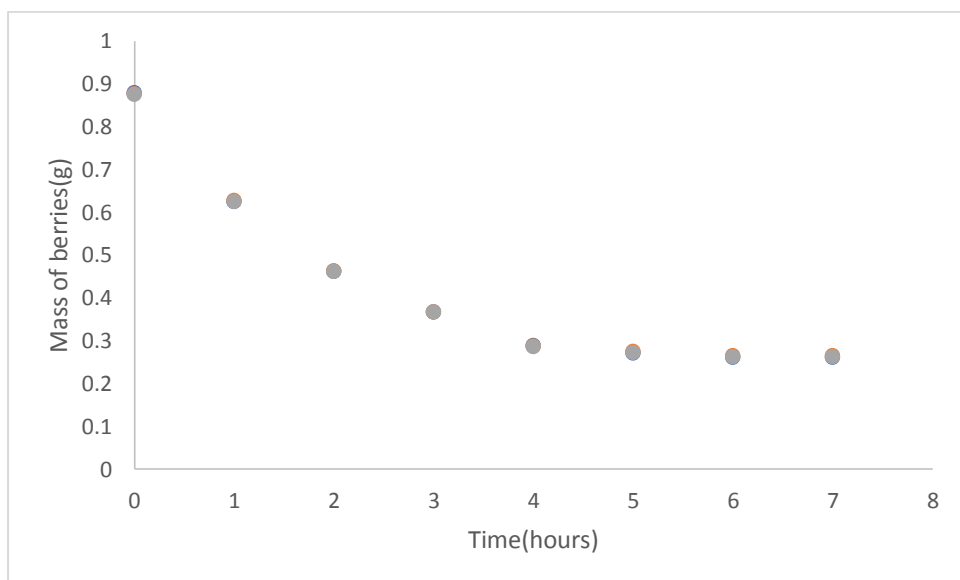
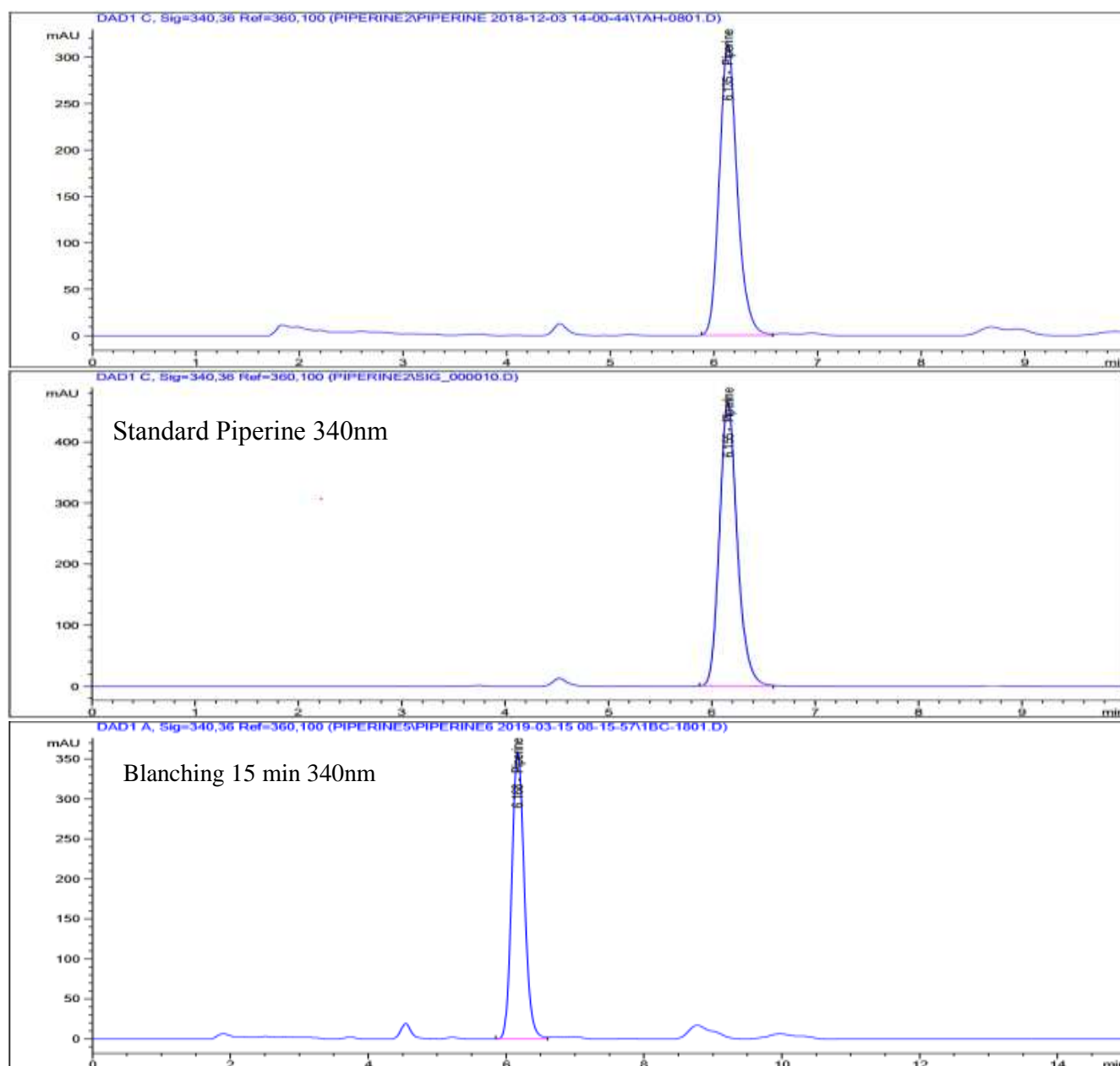Figure A11 Drying curve of (Blanching 1 min) under sun drying for the triplicate samples

Figure A12. The chromatogram of fresh extract, standard piperine and sun-drying (blanching 15 min) at 340 nm
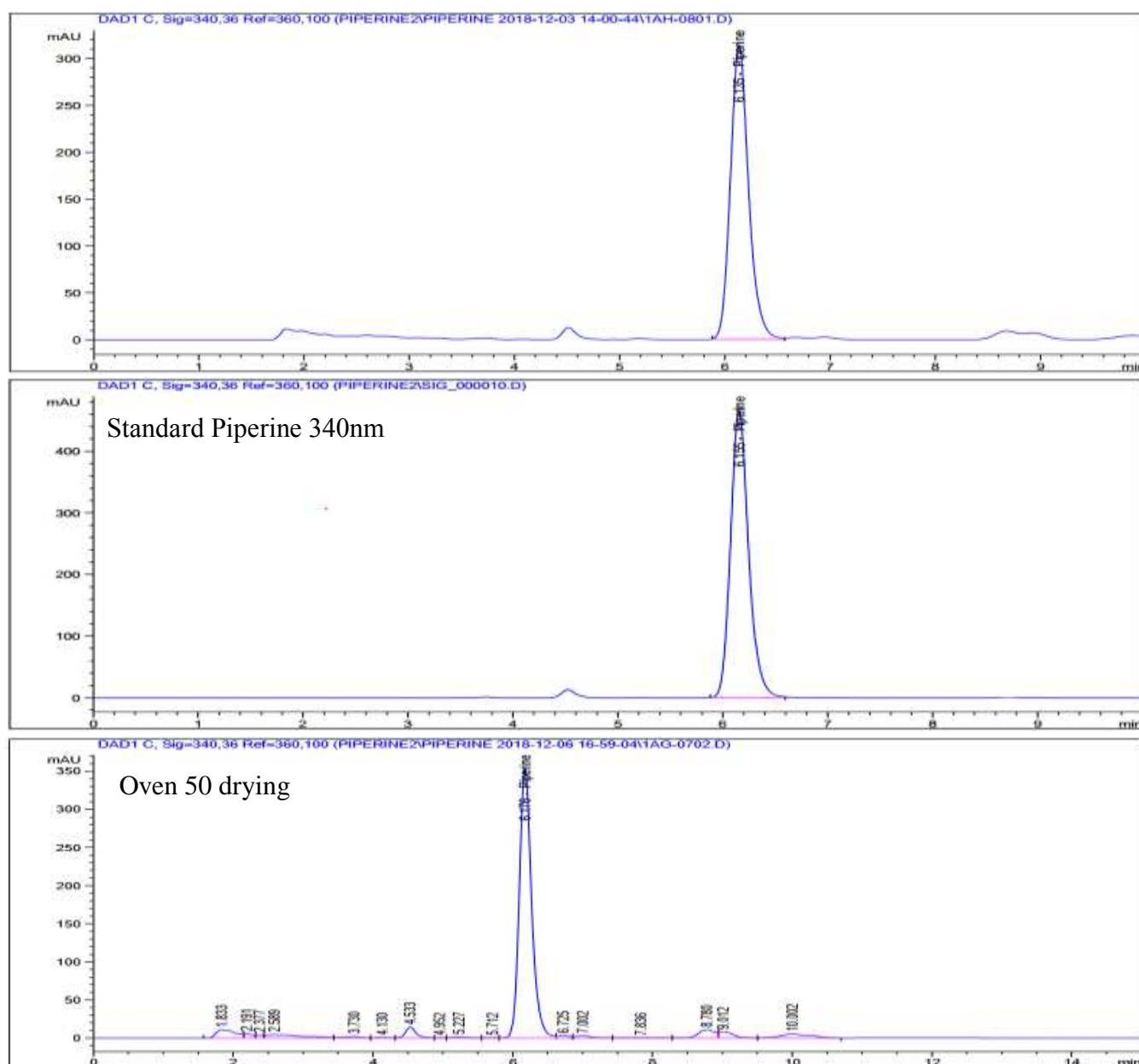
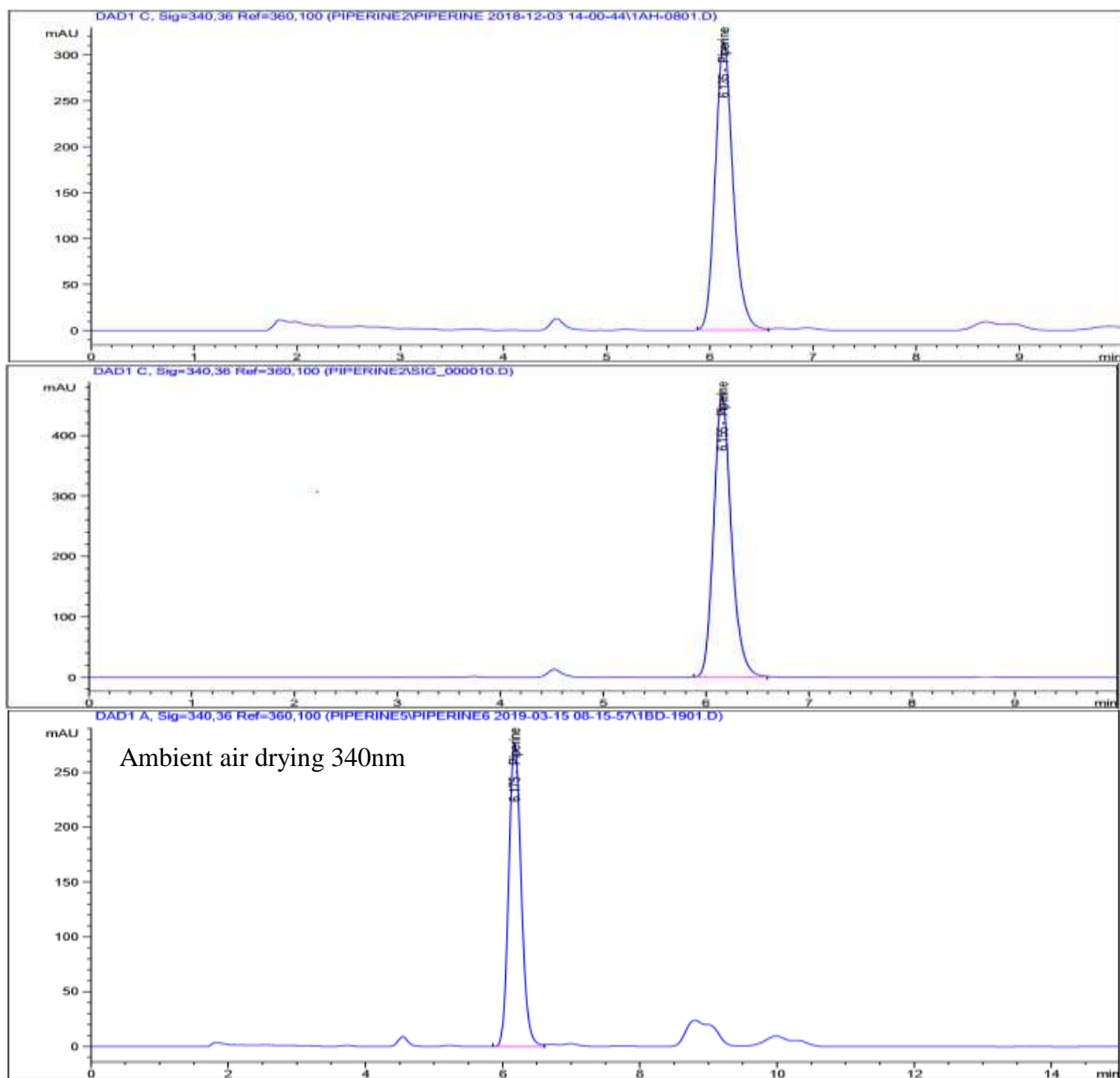Figure A13. The chromatogram of fresh extract, standard piperine and Oven 50ºC at 340 nm

Figure A14. The chromatogram of fresh extract, standard piperine and ambient air-drying at 340 nm