PRELIMINARY SCREENING OF *Piper nigrum* Linn FOR ITS ANTIOXIDANT ACTIVITY AND CYTOTOXICITY ON HUMAN COLON CANCER CELL LINE (LS 174T)

By

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ABSTRACT

PRELIMINARY SCREENING OF *Piper nigrum* Linn FOR ITS ANTIOXIDANT ACTIVITY AND CYTOTOXICITY ON HUMAN COLON CANCER CELL LINE (LS 174T)

Esther Thou Mun Huieh

To date, plants and its phytoconstituents are being widely screened for their medicinal properties for the development of new drugs. This is because since ancient times, plants and its biologically active compounds have played a significant role in the treatment of diseases and their therapeutic properties are undeniable. Hence, the present study was carried out to determine the antioxidant activity and to investigate the cytotoxicity of *Piper nigrum* Linn, which is commonly known as black pepper. Extraction of the plant was performed using solvents such as methanol, hydromethanol, ethyl acetate and hexane. The antioxidant activity of the extracts was evaluated based on their capability to scavenge free radicals via DPPH Free Radical Scavenging Assay. Hydromethanol extract was found to exhibit highest scavenging activity (EC_{50} of 746.8 µg/ml**)** as compared to other extracts. Besides, Folin-Ciocalteu Reagent Test and Aluminium Chloride Colourimetric Method were conducted to quantitatively measure the total phenolic and flavonoid content of the extracts. Methanol was found to be the most effective solvent in the extraction of phenolic compounds (237.79 µg GAE/mg), where hexane extract was shown to

contain highest flavonoid content (846.56 µg QE/mg). The cytotoxic properties of the three extracts (hydromethanol, ethyl acetate and hexane) were tested on human colon cancer cell line (LS 174T) via 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay using five different concentrations (20 to 320 µg/ml) and at three incubation periods (24, 48 and 72 hours). The results revealed that the crude extracts exhibited cytotoxic properties against LS 174T cells in a time-dependent and dose-dependent manner.

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DECLARATION

I hereby declare that the final year project entitled "**PRELIMINARY SCREENING OF *Piper nigrum* Linn FOR ITS ANTIOXIDANT ACTIVITY AND CYTOTOXICITY ON HUMAN COLON CANCER CELL LINE (LS 174T)**" is based on my original work. I have not copied from any student's work or from any sources, except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UTAR or other institutions.

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APPROVAL SHEET

This final year project entitled "**PRELIMINARY SCREENING OF *Piper nigrum* Linn FOR ITS ANTIOXIDANT ACTIVITY AND CYTOTOXICITY ON HUMAN COLON CANCER CELL LINE (LS 174T)**" was prepared by **ESTHER THOU MUN HUIEH** and submitted as partial fulfillment of the requirements for the degree of Bachelor of Science (Hons) Biomedical Science at Universiti Tunku Abdul Rahman.

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PERMISSION SHEET

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I hereby give permission to the University to upload the softcopy of my final year project in pdf format into the UTAR Institutional Repository, which may be made accessible to the UTAR community and public.

Yours truly,

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(ESTHER THOU MUN HUIEH)

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LIST OF ABBREVIATIONS

%	Percentage
ºC	Degree Celcius
$AlCl_3$	Aluminium (III) Chloride
ATCC	American Type Culture Collection
CAT	Catalase
CH_3COOK	Potassium Acetate
CO_2	Carbon dioxide
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic acid
DPPH	2,2-diphenyl-1-picrylhydrazyl
EC_{50}	Effective concentration at which 50% of activity is observed
EDTA	Ethylenediaminetetraacetic acid
FBS	Foetal Bovine Serum
FCR	Folin-Ciocalteu Reagent
G_1/S	Stage in the cell cycle at the boundary between the first gap phase and the synthesis phase
G_2/M	Stage in the cell cycle at the boundary between the second gap phase and mitotic phase
GAE	Gallic acid Equivalent
HCT-116	Human Colorectal Carcinoma Cell Line

HER2	Human Epidermal Growth Factor Receptor 2
HIV-1	Human Immunodeficiency Virus Type 1
HRT-18	Human Rectal Adenocarcinoma Cell Line
HT-1080	Human Fibrosarcoma Cell Line
IC_{50}	Inhibitory concentration to reduce 50% of cell viability
LS 174T	Human Colon Cancer Cell Line
MCF-7	Breast Cancer Cell Line
Mcl-1	Myeloid cell leukemia 1
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide
Na_2CO_3	Sodium Carbonate
NF-κB	Nuclear transcription factor kappa B
PBS	Phosphate Buffered Salts
QE	Quercetin Equivalent
Rpm	Revolutions per minute
SD	Standard Deviation
SOD	Superoxide Dismutase
UV	Ultraviolet
UV/VIS	Ultraviolet-visible
v/v	volume for volume
WHO	World Health Organization
w/w	weight for weight

CHAPTER 1

INTRODUCTION

1.1 Background Information

Natural products are defined as products from natural sources such as plants, animals and minerals. They have been discovered and studied since ancient times for their medicinal and therapeutic properties. Nowadays, the significance of natural products on human health can be seen clearly as they have been used to cure different types of diseases. In the olden days, some herbs were chewed to help to ease pain and some used leaves to wrap around the wound to enhance healing. With the help of modern chemistry, further investigation on natural products have been carried out and lots of important drugs or medicine were then synthesized. For an example, colchicine from *Colchicum autumale* (colchicum), caffeine from *Coffea Arabica* and nicotine from *Nicotiana tabacum* (Ji, Li and Zhang, 2009).

According to Lahlou (2013), natural products are suitable for new drug development because of the presence of their secondary metabolites or bioactive substances. Secondary metabolites are recognized as exhibiting more "drug-likeness" and biological friendliness as compared to fully synthetic compounds. Hence, more research is being carried out and high number of natural product-derived drugs have been rated worldwide selling ethical drugs.

Phytochemicals from plants were claimed to be safe, show less side effects and was reported to have many advantageous biological activities. The biologically active compounds or secondary metabolites from plants are usually obtained and identified through isolation, extraction and characterization (Sasidharan, et al., 2010). Secondary metabolite such as saponins from *Gymnema sylvestre* plant extract has been shown to exert strong anticancer and antioxidant activity (Arunachalam, et al., 2014). With the help of animal model, antioxidant components like total phenols and flavonoids in the aqueous extract of Aloe Vera leaves have been proven to increase antioxidant enzymes and significantly reduced the lipid peroxidation products (Raksha, Pooja and Babu, 2014). These clearly shows that their contribution and benefits to human health is indisputable.

Oxidation occurs naturally in the human body and free radicals will be produced whenever oxygen is metabolized. Free radicals, also known as reactive oxygen species can be oxygen derived or nitrogen derived and are known as pro-oxidants. Excessive amount of free radicals may cause cellular damage because they might attack the cells' deoxyribonucleic acid (DNA), lipid and protein. This will then give rise to oxidative stress if high amount of free radical is not eradicated from the body. In order to overcome the damaging effects on the cells, adequate amount of antioxidant is needed. Antioxidants can be synthesized in the body (superoxide dismutase, catalase, glutathione peroxidase and glutathione reductase) or acquired from external sources such as fruits and vegetables which are rich in minerals and vitamins (Amzad Hossain and Shah, 2015). Thus, it is clear that the reason behind the numerous exploitation of

bioactive compounds in different types of plants are due to their impressive antioxidant properties which can be taken into the body to quench free radicals.

High amount of free radicals in the body not only kill the cells but also possess the ability to cause cancer formation. Cells with damaged DNA will continue to proliferate and lead to continuous expansion and spreading of abnormal cells. So, antioxidant or free radical scavengers are crucial to neutralize the free radicals to lower the risk of cancer development. Hence, plant-derived compounds are being highly investigated for its cytotoxic properties to kill cancer cells (National Cancer Institute, 2015). Cytotoxicity refers to the ability of certain compounds or substances that are able to initiate cell death or cell damage (Golding, et al., 2013). To date, approximately 60% of chemotherapeutic drugs have been isolated from natural products and plant is one of the most important source (Solowey, et al., 2014).

1.2 Significance of Study

Since many plants possess potential biological activities, they are widely screened for their medicinal use and will continue to be immensely vital as sources of medicinal agents. Thus, this research was conducted to determine the antioxidant activity and cytotoxic effect of *Piper nigrum* Linn (*P. nigrum* L.).

The pungency of *P. nigrum* L. is due to the presence of alkaloidal constituent in its fruits, which is known as piperine (Damanhouri and Ahmad, 2014). *P.*

nigrum L. and its main active constituent (piperine) have been shown to have a few physiological effects such as in improving the cognitive effect and has an anti-depression like activity which may eventually improve brain function (Wattanathorn, et al., 2008).

Research on the cytotoxic effect of *P. nigrum* L. has been focused on few cancer cell lines such as human prostate cancer cells, breast cancer cells and human rectal adenocarcinoma cells. Piperine was reported to have an anti-proliferative effect on human prostate cancer cells by causing cell cycle arrest and by inducing apoptosis (Ouyang, et al., 2013). Besides, the anti-cancer effect of paclitaxel on breast cancer cell line (MCF-7) can be enhanced with the combination of piperine (Motiwala and Rangari, 2015). Therefore, in this research, the human colon adenocarcinoma cell line (LS 174T cells) was used to evaluate the cytotoxic potential of different crude extracts of the plant.

The preliminary screening was conducted by performing the extraction of *P. nigrum* L. using different types of solvents and various biochemical assays were carried out by using the crude extracts obtained.

1.3 Research Objectives

The objectives of this research were as follows:

i. To perform extraction of *Piper nigrum* L. using solvents of different polarity such as methanol, hydromethanol, ethyl acetate and hexane through maceration method.

ii. To investigate the antioxidant nature of crude extracts of *Piper nigrum* L. by performing DPPH Free Radical Scavenging Assay.

iii. To determine the total phenolic content and flavonoid content of crude extracts of *Piper nigrum* L. quantitatively by using Folin-Ciocalteu Reagent Test and Aluminium Chloride Colourimetric Method.

iv. To evaluate the cytotoxic activity of three crude extracts of *Piper nigrum* L. (hydromethanol, ethyl acetate and hexane) on human colon adenocarcinoma cell line (LS 174T) at different incubation periods (24, 48 and 72 hours) via MTT assay.

CHAPTER 2

LITERATURE REVIEW

2.1 Natural Products and Plants

2.1.1 Natural products

Natural products are substances produced by living organisms and originate in nature. They are shown to have numerous benefits to the society and have inspired researchers to undergo further investigation on their medicinal properties. Hence, natural products are used to serve as a source for the massive production of clinical drugs especially plants and marine organisms-based drugs. They are also well known for their anticancer and anti-infective properties. Most of the natural products have been marketed without chemical modification. However, in order improve drug properties, some natural products are required to be optimized via “semi-synthesis” (Kingston, 2011).

2.1.2 Plant-based Products

Plants are the great source for discovery and development of new drugs. They have been widely used properties since ancient times as healing agents due to their medicinal. The knowledge on Chinese traditional herbs have also led to further investigation and research on more medicinal plants for their therapeutic properties. Approximately 250,000 to 750,000 higher plants were found and 35,000 species of higher plants have been tested by National Cancer Institute in

the United States for their anticancer activity. A study has also been done using more than 60,000 plants extracts to test against lymphoblastic cells infected with HIV-1. In addition, one of the most famous breast cancer drug that has been widely used is paclitaxel, which is isolated from the bark of *Taxus brevifolia* (Pacific Yew) (Dias, Urban and Roessner, 2012). Different plant produces different types of bioactive compounds or secondary metabolites. These compounds serve as an important source of pharmaceuticals (Hussain, et al., 2012). Many researches have been done and have proved that secondary metabolites in plants possess anticancer, antioxidant, antimicrobial and antifungal properties.

2.2 Plant of Interest

2.2.1 General Description

Piper nigrum Linn or also known as black pepper is one of the most widely used spices in the world. The berry-like fruits, or peppercorns are 5 mm in diameter. It will turn yellowish-red when it ripens, bearing a single seed. Pepper has an aromatic odor and will give a hot, bitter and pungent taste. The plant is a flowering woody perennial climbing vine and may reach a height of 10 meters. The leaves are alternately arranged and the small flowers are in dense, slender spikes of about 50 blossoms each (Encyclopedia Britannica, 2015). *Piper nigrum* oil can be used to relief pain, increase circulation and reduce cold and flu. Besides, it also helps to enhance saliva and gastric secretion, increase appetite and improve digestion (Mother Herbs & Agro Products, 2016).

Figure 2.1: *Piper nigrum* Linn (Adapted from Encyclopedia Britannica, 2015).

2.2.2 Taxonomical Classification

Plantae is placed under the kingdom level, which is the first level in the taxonomical hierarchy for this plant. The subkingdom and superdivision for *Piper nigrum* Linn is *Tracheobionta* and *Spermatophyta* respectively. As we go down the hierarchy, it is then categorized under the division for flowering plants, *Magnoliophyta*. Different plants can be classified into various classes and this plant belongs to the class of *Magnoliopsida.* Moreover, it is placed under *Piperales* order and the family of *Piperaceae*. The genus of this plant is *Piper* L and lastly, the species level, *Piper nigrum* L (United States Department of Agriculture (USDA), 2016).

2.2.3 Distribution of Plant

Piper nigrum was cultivated in the tropics of Southeast Asia in the early historic times and became one of the most commonly used spices. The plant is now

widely grown throughout Indonesia, India, Brazil, Malaysia, Sri Lanka, Vietnam, China and has been brought into tropical areas of Africa and Western Hemisphere. Today, Western Europe, United States, Japan and Korea are said to be the biggest consumers of pepper (International Pepper Community, 2016).

2.2.4 Phytoconstituent and Chemistry of *Piper nigrum* L.

Different plants produce their own secondary metabolites including *Piper nigrum*. These metabolites have been shown to benefit human health. Therefore, researchers are screening on the biological activities of various secondary metabolites of different plants so that they can be used for biocontrol agents and drug development. Investigators from different field have discovered several compound from this plant such as phenolic, flavonoid, alkaloid, terpenes, chalcones and steroid. *Piper nigrum* contains 3% of essential oil and sesquiterpens make up about 20% of it. Piperamides is the active compound that is shown to possess insecticidal activity. Piperine is the main constituent (alkaloidal) which contributes to the pungency of *Piper nigrum.* Piperine not only contribute to the pungency of *Piper nigrum* but also proven to have some pharmacological activities such as antioxidant, anti-cancer, antimicrobial, anti-diarrheal and anti-inflammatory activity (Ahmad, et al., 2012).

On the other hand, *Piper nigrum* is an excellent source of manganese and iron. It also contains other nutrients such as copper, magnesium and calcium. Other nutritional composition like fiber, carbohydrates and protein can also be found

in *Piper nigrum.* In addition, it also contains vitamins like Vitamin K and A (Parthasarathy, Chempakam and Zachariah, 2008).

2.2.5 Previous Findings

2.2.5.1 Antioxidant Properties

Piper nigrum is well known for its antioxidant activity due to the presence of several bioactive compounds such as phenols, flavonoids and alkaloids. These phytoconstituents play a major role in scavenging free radicals and eliminating reactive oxygen species from the body. These extracellular antioxidants obtained from diet are crucial to the human body since the scavenging activity of endogenous antioxidants are not 100% efficient. The sufficient amount of antioxidants in the body is important to help lower the risk of certain diseases (Nahak and Sahu, 2011).

2.2.5.2 Cytotoxic properties

According to Wang, et al. (2014), 10 out of 2000 species of the genus *Piper* (Piperaceae) have been traditionally used as medicine to treat cancer. In addition, researches have been done to prove that 35 extracts from 24 *Piper* species and 32 compounds from *Piper* plants are shown to have cytotoxic effect. It has been reported that piperine, the main alkaloidal constituent in *P. nigrum* L., possessed an inhibitory effect on *HER2* gene expression at transcriptional level. This indicates a high possibility for it to act as a chemotherapeutic agent against human breast cancer

with *HER2* overexpression. Besides, piperine was shown to prevent human fibrosarcoma (HT-1080) cell expression of matrix metalloproteinase and also cause cytotoxic effect on human rectal tumor (HRT-18) cells.

2.2.6 Medicinal and Traditional Uses

The health-benefit properties and common usage of *P. nigrum* L. as flavoring in foods have led to further studies on its activity in the human body. It is also commonly used in the ayurvedic and traditional medicine system. In ayurvedic medicine, its powdered or decoction is used to improve digestion and also as a remedy for diarrhea, nausea, lack of appetite, and other dyspeptic complaints. Apart from that, a study has also proven the antidiarrheal, anti-motility, and anti-secretory effect of aqueous black pepper extract in Swiss albino mice (Shamkuwar, Shahi and Jadhav, 2012). The volatile compounds, tannins and phenols are said to be the compounds present in *P. nigrum* L. that are shown to have healing effect for cough, rheumatoid arthritis, peripheral neuropathy, melanoderma and leprosy (Shiva Rani, Saxena and Udaysree, 2013). It can be applied locally in certain types of skin disease, reduce pain and used externally for its rubefacient (Nahak and Sahu, 2011).

2.3 Extraction Process

2.3.1 Extraction of Biologically Active Constituents

Extraction is one of the most important step in the study of medicinal plant. It is usually done in order to obtain the chemically active compounds, also known

as secondary metabolites present in a particular plant that are soluble in solvents or liquid. Appropriate actions are vital in the process of extraction to prevent any distortion or loss of bioactive compounds (Sasidharan, et al., 2010). The bioactive compounds in plants showed favorable outcomes in treating human diseases. Therefore, pharmaceutical, food and chemical industries show great interest in searching and acquiring these constituents to use as medicine and supplements (Joana Gil-Chávez, et al., 2013).

2.3.2 Solvent System

Solvent system is a method used to extract certain compounds from materials such as plants. Breaking of tissue and cell integrity of plant by grinding is necessary before the exposure to solvents because the secondary metabolites are usually present in the plant cell. This may also help to increase the extraction yield. Solvent extraction has the advantage of moderate extraction condition because it can be done in room temperature. Selection of solvent largely depend on the nature of constituents being targeted. Solvents of different polarity such as methanol, ethanol, ethyl acetate, isopropanol, chloroform and hexane are commonly used in extraction (Meireles, 2009). In this research, polar solvents like methanol, hydromethanol and ethyl acetate were used in the extraction of hydrophilic compounds where hexane, a non-polar solvent was used to extract lipophilic compounds.

2.3.3 Maceration Method of Extraction

The aim of extraction is to separate the medicinally active portion of plants from the insoluble residues. Maceration is one of the commonly used method in medicinal plants analysis. The first step in maceration process is to soak the moderately coarse powdered form of plant material in a closed vessel with selected solvents. Powdered plant material should be used to enhance the surface area of plants to be exposed to the solvents. Closed vessel is required to prevent the evaporation of the solvents throughout the process (Azwanida, 2015). It is then properly mixed to make sure all of the plant materials are in contact with the solvent and is allowed to stand at room temperature for 72 hours (3 days) with regular agitation. The purpose of this step is to break the plant's cell wall to release its soluble phytoconstituents. After 3 days of soaking, filtration will be carried out to obtain the filtrate (Handa, et al., 2008). Fresh solvents will then be added to ensure exhaustive extraction. This process will be repeated for three times. In order to obtain concentrated crude extracts, the filtrates that have been collected will undergo evaporation with the help of rotary vacuum evaporator. Lastly, the crude extracts obtained can be dried in a drying incubator to remove excess solvents (Thangaraj, 2016).

2.4 Antioxidants

2.4.1 Plants as Source of Antioxidant

There are wide variety of naturally occurring antioxidants that can be found in nature which differ in their physical and chemical properties. Plants are said to be an excellent sources of exogenous antioxidant. Plant antioxidants like

phenolic have shown promising antioxidant activity *in vivo* as well as *in vitro* studies among most of the bioactive compounds (Kasote, et al.,2015). The wide range of non-enzymatic antioxidants are capable of reducing reactive oxygen species-induced oxidative damage that are seen to be the causative factor in the development of life threatening diseases. Besides having the ability to eliminate free radicals, plant antioxidants are also evaluated for properties such as metal chelating activity, acting as reducing agents or hydrogen donors (Gupta and Sharma, 2006).

2.4.2 Production of Free Radical and Its Effect on Human Body

Reactive oxygen species or free radicals are produced as necessary intermediates in normal biochemical reactions. However, excessive or uncontrollable formation of these products allows the destruction and damage of cells structure to occur including proteins, nucleic acids, lipids and membranes. There are two types of free radicals that can be generated. Endogenous free radicals formed from immune cell activation, inflammation and mental stress, where air pollution, cigarette smoking, alcohol, heavy metals are the sources of exogenous free radicals (Kabel, 2014). Radicals possess an unpaired electron, which makes them highly reactive. Damaged nucleic acids that are not properly restored may lead to cancer development and also providing chances for diseases to occur (Halliwell, 2012).

2.4.3 Antioxidants as Free Radical Scavenger

Nowadays, many form of diseases are caused by the overproduction of reactive oxygen species and reactive nitrogen species as they have been shown to damage the cellular components (Weber, et al., 2008). Hence, endogenous and exogenous antioxidants like superoxide dismutase (SOD), catalase (CAT), alpha-tocopherol, ascorbic acid, carotenoids, polyphenols and glutathione are vital to protect the human body against the damaging effects. Following that, researches on natural antioxidants are being extensively carried out to study on their capability in protecting organisms from any oxidative damage (Saleh, et al., 2010).

2.5 Cancer

2.5.1 Plants as Source of Anticancer Agent

Plant derivatives and secondary metabolites have been used to prevent and treat cancer over the last half century. There are some main classes of phytochemicals that have been discovered. These includes iridoids, phenols, phenolics, carotenoids, alkaloids, organosulfur compounds, and terpenoids. Scientists have discovered that these plant-derived phytoconstituents have played an important role in the development of clinically useful anticancer agent (Sahranavard, et al., 2009). Few chemotherapeutic drugs like paclitaxel, camptothecin, combrestatin, epipodophyllotoxin, vinblastine, vincristine were originated from herbs and are widely used these days. These have clearly shown that the cytotoxic properties of phytochemicals and bioactive compounds in plants are undeniable. Thus, this have led to further research to determine new plant

compounds with high efficiency in cancer prevention and treatment (Kaur, Kapoor and Kaur, 2011).

2.5.2 Overview of Cancer

Cancer is a broad term that is commonly used to describe a class of diseases. It is a condition where abnormally dividing cells grow continuously and capable of spreading to other parts of the body. Tumor is characterized as a group of cells that are proliferating uncontrollably and normally forms a solid mass in the body. Solid tumors can be found in most of the cancers, but not all such as cancers of the blood. Nevertheless, presence of tumor does not always indicate cancer. Only malignant tumors that possess the ability to invade surrounding tissues and disseminate are said to be cancerous, unlike benign tumors that only remain in a particular region in the body (MedlinePlus, 2016).

2.5.3 Worldwide Prevalence of Cancer

According to World Health Organization (WHO) (2016), cancer has been the major cause of death throughout the world. In year 2012, lung cancer has been reported to be the most common cancer, which is around 1.8 million followed by breast and colorectal cancer. However, the major causes of deaths are usually lung, liver and stomach cancer. WHO also stated that the most crucial risk factor for cancer is tobacco which had caused around 70% of worldwide lung cancer deaths. Additionally, Africa, Asia and Central and South America are reported to be the regions that account for 70% of the global cancer deaths.

2.5.4 Prevalence of Cancer in Malaysia

In Malaysia, an approximate number of 90,000 to 100,000 Malaysians living with cancer at any one time. The escalation in number of cancer cases is due to increasing population and longer life span. Childhood cancer are less common as compared to men and women who are 50 years and above. Besides, females are more prone to cancer than male with a ratio of male to female 1:1.2. Breast, colorectal and lung cancer are the top 3 cancers that are affecting both male and female in Malaysia and colorectal cancer is the second most common cancer. The three risk factors that are said to be the most common cause for colorectal cancer are ageing, eating habits and genetic inheritance (National Cancer Society Malaysia (NCSM), 2016).

2.6 Assays

2.6.1 Antioxidant Screening

2.6.1.1 DPPH Free Radical Scavenging Activity

Medicinal plants, herbs, vegetables, legumes and fruits are rich source of compounds like polyphenols. Polyphenols are naturally present phytochemicals that serves as natural antioxidants which aid in the eradication of harmful free radicals produced by the body during fat metabolism. The antioxidant activity of most plant-derived compounds is often associated with their phenolic contents. The elimination of free radicals and reactive oxygen species by

antioxidants can be extremely important in hindering oxidative mechanisms that lead to degenerative diseases (Proestos, et al., 2013).

2,2-diphenyl-1-picrylhydrazyl (DPPH) assay is commonly performed to analyze the free radical scavenging activity of plant extracts. It is an accurate and inexpensive method to examine the radical scavenging activity of antioxidants, as the radical compound is stable and need not be generated. It is also the simplest method where DPPH solution is added into the plant extracts and absorbance will be recorded after a specific duration (Nikolova, Evstatieva and Nguyen, 2011).

This method was developed by Blois, in which DPPH is used as the stable free radical. In the presence of antioxidants, the purple colour DPPH radical will be reduced to form a yellow product by accepting a hydrogen atom from the antioxidants (Kedare and Singh, 2011). The antioxidant properties of wheat grain and bran, vegetables, herbs, legumes, and flours in various solvent systems have also been successfully determined using this assay (Parry, et al., 2005). The structure of DPPH free radical and its stable form after receiving a hydrogen atom is shown in Figure 2.2.

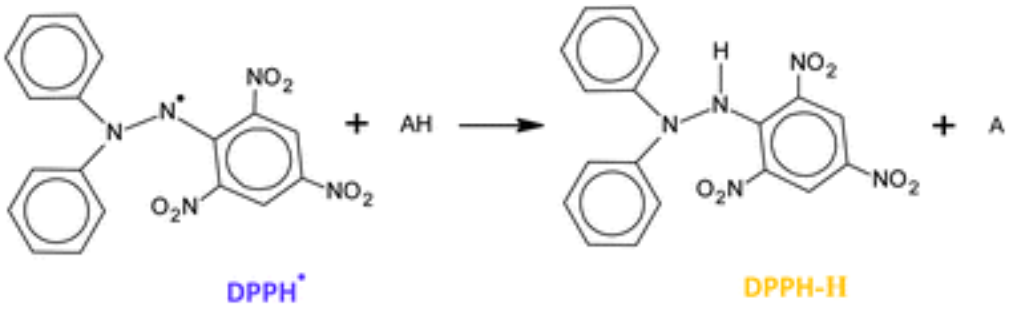

Figure 2.2: DPPH free radical conversion to DPPH by receiving a hydrogen atom (Adapted from Everette and Pekal, 2013).

2.6.1.2 Folin-Ciocalteu Reagent Test

In the plant kingdom, the most common type of secondary metabolite that can be found in plants are phenolics such as simple phenols, phenolic acids, coumarins, flavonoids, and condensed tannins, lignans, and lignins. These bioactive compounds play a role as antioxidant, protective agents against UV light and also contribute to plant pigmentation. Folin-Ciocalteu reagent can be applied to study the presence of these compounds (Blainski, Lopes and de Mello, 2013).

Colourimetric reactions are commonly used in the UV/VIS spectrophotometric method. It is a simple, fast and economic test to be carried out routinely in the laboratory and is suitable to be used in the measurement of total amount of phenolic hydroxyl groups that is present in plant extracts. The Folin-Ciocalteu Assay is one of the colourimetric method which was developed in year 1927 for the measurement of tyrosine. This method has been used to analysed more than 80 compounds (Blainski, Lopes and de Mello, 2013). The Folin-Ciocalteu reagent consists of a mixture of sodium molybdate, sodium tungstate and other

reagents. A blue chromophore which is constituted by a phosphotungstic-phosphomolybdenum complex will be formed when polyphenols present in plant extracts react with the reagent. The blue chromophore absorbs at 765 nm and the maximum absorption mainly depends on the alkaline solution and the concentration of phenolic compounds (Bueno, et al., 2012). This reaction is said to be precise and specific for measuring various groups of phenolic compounds because of the different colour change in different compounds due to differences in unit mass and reaction kinetics (Everette, et al., 2010).

2.6.1.3 Aluminium Chloride Colourimetric Method

The most common group of polyphenolic compound, flavonoids, are found in various types of plants. Flavonoids compounds from plants have been reported to show multiple biological activities including antimicrobial, mitochondrial adhesion inhibition, antiulcer, antiarthritic, antiangiogenic, anticancer and protein kinase inhibition. Their antioxidant and radical scavenging activity are depending on the position of hydroxyl groups and other features in its chemical structure. Besides, flavones and flavonols are the most commonly found among all the phenolics (Bag, Devi and Bhaigyabati, 2015).

In aluminium chloride ($AlCl_3$) colourimetric method, $AlCl_3$ forms acid stable complexes in the presence of C-4 keto groups and either the C-3 or C-5 hydroxyl group of flavones and flavonols. Other than that, $AlCl_3$ together with the ortho-dihydroxyl groups in the A- or B-ring of flavonoids will also lead to the formation of acid labile complexes (Bag, Devi and Bhaigyabati, 2015). In this

assay, quercetin is usually used as reference standard due to its high flavonol content. It possesses great antioxidant capacity due to the presence of all the right structural features required for free radical scavenging activity (Kalita, et al., 2013).

2.6.2 Cytotoxicity Screening

2.6.2.1 MTT Assay

Cell viability and cytotoxicity assays are generally used to determine if the test samples or chemicals have effects on cell proliferation or show cytotoxic effects that eventually lead to cell death. At the end of this assay, it is important to determine remaining number of viable cells in order to determine the cytotoxicity of certain compound or chemical. There are various types of assays that can be carried out to estimate the number of viable eukaryotic cells and one of the assay is known as the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide] assay method. This assay was first described by Mosmann and has been widely adopted in academic laboratories (Saravanan, et al., 2003).

In this assay, viable cells that are metabolically active will reduce the yellow tetrazolium MTT by the action of enzyme dehydrogenase present in the mitochondria, producing reducing equivalents such as NADH and NADPH. The reduced MTT will then form purple formazan crystals. After solubilization of the intracellular purple formazan, it will be quantified using a multiwell-spectrophotometer at 570 nm. The intensity of purple colour is directly proportional to the number of viable cells present (ATCC, 2011).

2.7 Samples

2.7.1 Cell Line

2.7.1.1 Human Colon Cancer Cell (LS 174T)

LS 174T is a human colon cancer cell line, which was derived from a 58 years old female patient (Caucasian) who was suffering from Dukes' type B colorectal adenocarcinoma. It is an adherent cell line, whereby the cells will attach onto the surface of the culture flask. As shown in Figure 2.3, the cells display an epithelial morphology after attaching to the surface of the flask (ATCC, 2016). Cells tend to grow in islands and usually pile on top of each other. Those that are not properly attached may eventually fall off into suspension and form a considerable amount of debris (Public Health England, 2016).

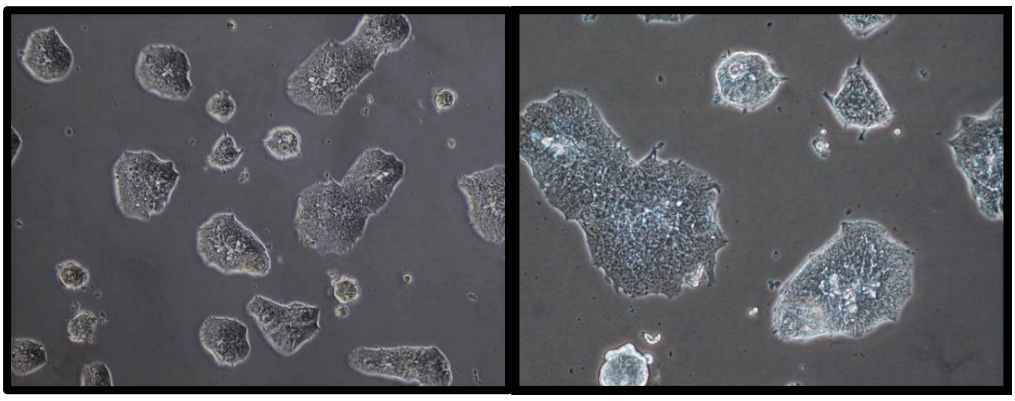

Figure 2.3: LS 174T cells under 10X (left) and 20X (right) magnification.

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

3.1.1 Chemicals and Solvents

Table 3.1: List of chemicals and solvents used throughout the research.

Materials	Company, Country
0.4% Trypan blue	Sigma Aldrich, China
0.25% Trypsin (1X)	Gibco, United States
2,2-diphenyl-1-picrylhydrazyl (DPPH) powder	Calbiochem, United States
3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) powder	Merck, Germany
Aluminium (III) Chloride, $AlCl_3$ powder	Sigma Aldrich, China
Ascorbic acid powder	Fisher Scientific, UK
Cisplatin	TCI, Tokyo
Dimethyl sulfoxide (DMSO)	Bio Basic, Canada
Dulbecco's Modified Eagle Medium (DMEM)	Millipore, U.S.
EDTA powder	Systerm, Malaysia
Ethyl acetate	IramaCanggih, Malaysia
Foetal Bovine Serum (FBS)	Biowest, USA
Folin-Ciocalteu reagent	Merck, Germany
Gallic acid powder	Bio Basic, Canada
Hexane	IramaCanggih, Malaysia
Methanol	IramaCanggih, Malaysia
PBS (Phosphate Buffered Salts) Tablets	Takara, Japan
Potassium Acetate powder	DAEJUNG, Korea
Quercetin powder	ACROS Organics, USA
Sodium carbonate	Systerm, Malaysia

3.1.2 Laboratory ware and Equipment

Table 3.2: List of laboratory ware and equipment used throughout the research.

Equipment / Laboratory ware	Company, Country
5% CO_2 humidified incubator (37ºC)	Binder, Germany
Autoclave machine	Hiramaya, Japan
Centrifuge machine	Heraeus, Germany
Drying incubator	Memmert, Germany
Electronic balance	Kern, Germany
Freezer (-20ºC)	Pensonic, Malaysia
Freezer (-80ºC)	Thermo Scientific
Haemacytometer	Hecht Assistant, Germany
Inverted phase contrast microscope	Olympus, United States
Laboratory blender	Waring Laboratory, USA
Laminar flow hood (cell culture)	Edamix Series, Germany
Microplate reader	Tecan, Switzerland
Refrigerator (4ºC)	Toshiba, Japan
Rotary vacuum evaporator	Buchi, Switzerland
Sonicator	Branson, USA
Vortex	Stuart, USA

3.2 Methods

3.2.1 Preparation of Crude Extract

3.2.1.1 Collection and Drying of Plant Material

One kilogram of fresh *Piper nigrum* L. were purchased from Sarikei, Sarawak in the month of October, 2015. They were blended into powder form using laboratory blender.

3.2.1.2 Plant Extraction

Eight hundred grams of the powdered form of *Piper nigrum* L. was divided equally and soaked in different solvents of varying polarity which were hexane, ethyl acetate, methanol and hydromethanol. The solvent-soaked plant materials were placed in an orbital shaker set at 90 rpm under room temperature for three consecutive days. The extracts were then filtered using filter paper and gauze with the aid of a filter funnel. Sufficient amount of filtrate was collected and a rotary vacuum evaporator was used to evaporate the filtrate. As a result of evaporation, concentrated crude extracts were obtained. They were stored in sample vials and further dried in a 37°C incubator to remove excess solvents. The crude extracts were measured from time to time using a weighing machine until a constant value was obtained.

3.2.2 Determination of Radical Scavenging Properties

3.2.2.1 DPPH free radical scavenging activity

3.2.2.2 Preparation of stock and test sample

Stock solution with a concentration of 1 mg/ml was prepared by dissolving 4 mg of crude extract in 4 ml of methanol. The solution was covered using aluminium foil and mixed properly using a vortex. Then, test samples of different concentrations (20, 40, 80, 160, 320 and 640 µg/ml) were prepared by performing serial dilutions.

3.2.2.3 Preparation of DPPH Solution

DPPH solution was freshly prepared on the day before the assay was performed. Eight milligrams of DPPH powder was dissolved in 8 ml of methanol to obtain a concentration of 1 mg/ml. The solution was covered with aluminium foil and mixed using a vortex.

3.2.2.4 Preparation of Ascorbic Acid (Positive Control)

Ascorbic acid was used as positive control. Four milligrams of ascorbic acid powder was dissolved in 4 ml of methanol to obtain a concentration of 1 mg/ml. The solution was mixed using a vortex, covered with aluminum foil and then stored at room temperature until future usage.

3.2.2.5 DPPH Assay

DPPH Assay was done using a 96-well plate and in the absence of light. Serial dilution was performed to prepare different concentrations of test sample. Dilution was done by adding 100 µl of methanol followed by another 90 µl of methanol. Finally, 10 µl of DPPH solution was added. The 96-well plate was wrapped with aluminium foil and then incubated for 30 minutes at room temperature. At the end of incubation period, the absorbance of the test samples was measured using a microplate reader at a wavelength of 517 nm (modified from Subedi, et al., 2014). Ascorbic acid was used as positive control and all of the steps mentioned above were repeated. A mixture of methanol and DPPH solution was used as negative control and as for blank, only methanol was used.

This assay was performed in triplicate. The DPPH free radical scavenging activity (%) of the crude extracts and ascorbic acid were calculated based on the following formula (Zarai, et al., 2013):

DPPH free radical scavenging activity (%) = $[1- (A_s/ A_c)] \times 100$

where A_s = Absorbance of sample

A_c = Absorbance of control

3.2.3 Determination of Total Phenolic Content

3.2.3.1 Folin-Ciocalteu Reagent Test

3.2.3.2 Preparation of Test Samples

Four milligrams of crude extract was dissolved in 4 ml of methanol. The solution was mixed using a vortex, covered with aluminium foil and stored in 4ºC fridge until future usage.

3.2.3.3 Preparation of Sodium Carbonate Solution, Na_2CO_3

Sodium carbonate solution (20%) was prepared by dissolving 4 g of sodium carbonate powder in 20 ml of deionized water. The solution was properly mixed using vortex until the powder dissolve completely (modified from Zarai, et al., 2013).

3.2.3.4 Preparation of Folin-Ciocalteu Reagent (FCR)

Folin-Ciocalteu reagent was diluted to 1:10 v/v with water. This can be obtained by diluting 2.5 ml of FCR in 22.5 ml of distilled water (modified from Stankovic, et al., 2011).

3.2.3.5 Preparation of Stock and Standard Solution of Gallic Acid

Stock solution with a concentration of 0.5 mg/ml was prepared by dissolving 5 mg of gallic acid powder in 1 ml of methanol and 9 ml of deionised water. The solution was covered with aluminium foil and properly mixed. The stock solution was used to prepare different concentrations (100, 200, 300, 400 and 500 µg/ml) of gallic acid standard solutions using methanol.

3.2.3.6 Folin-Ciocalteu Reagent Test

The stock solution of each crude extract prepared (1 mg/ml) was mixed with 100 µl of FCR in the absence of light and the microcentrifuge tube was thoroughly shaken. The mixture was allowed to react for about three minutes at room temperature and then 300 µl of Na_2CO_3 was added into the respective tubes. The reaction was allowed to stand at room temperature for two hours. Finally, the absorbance of each sample was measured at the end of incubation period. A volume of 100 µl of each sample was transferred into a 96-well plate for the measurement of absorbance using microplate reader at a wavelength of 765 nm (modified from Saeed, Khan and Shabbir, 2012). The same procedure was also applied to the standard solutions of gallic acid. A standard calibration

curve was generated using the absorbance values of gallic acid standard solutions. Total phenolic content of each extract was expressed as µg gallic acid equivalent per mg of the extract (µg GAE/mg of extract). This test was carried out in triplicate.

3.2.4 Determination of Total Flavonoid Content

3.2.4.1 Aluminium Chloride Colourimetric Method

3.2.4.2 Preparation of Quercetin

One hundred milligrams of quercetin powder was dissolved in 10 ml of methanol to obtain a concentration of 10 mg/ml. The solution was mixed properly, covered with aluminium foil and stored at room temperature until future usage.

3.2.4.3 Preparation of 1 M Potassium Acetate, CH_3COOK

Approximately 0.982 g of potassium acetate powder was dissolved in 10 ml of methanol. The solution was mixed properly and stored at room temperature until future usage.

3.2.4.4 Preparation of 1% Aluminium (III) Chloride, $AlCl_3$

Approximately 0.1 g of aluminium chloride powder was dissolved in 10 ml of methanol. The solution was mixed properly and stored at room temperature until future usage.

3.2.4.5 Preparation of Test Samples

Ten milligrams of crude extract was dissolved in 1 ml of methanol to obtain a concentration of 10 mg/ml. The solution was mixed properly, covered with aluminium foil and stored at room temperature until future usage.

3.2.4.6 Aluminium Chloride Colourimetric Method

Quercetin was diluted to a concentration of 1 mg/ml. Then, serial dilution was performed to obtain different concentrations of quercetin followed by the measurement of absorbance at a wavelength of 415 nm. This was done in order to generate a standard calibration curve of quercetin. A volume of 100 µl of stock solution of each crude extract prepared (10 mg/ml) was mixed with 300 µl of methanol. After that, 20 µl of 1% $AlCl_3$ solution was added followed by another 20 µl of 1 M Potassium Acetate solution and 560 µl of distilled water. The mixture was allowed to react for about fifteen minutes at room temperature. At the end of incubation period, a volume of 100 µl of each sample was transferred into a 96-well plate and a microplate reader was used to measure the absorbance at 415 nm. This test was carried out in triplicate and the results were expressed as µg quercetin equivalent per mg of the extract (µg QE/mg of extract).

3.2.5 Cell Culture

3.2.5.1 Complete Growth Medium Preparation

A volume of 36 ml of DMEM medium was supplemented with 4 ml of Foetal Bovine Serum (FBS) to make a total volume of 40 ml of complete growth medium. Complete growth medium preparation was done aseptically inside the laminar hood and then stored in the fridge at 4°C until future usage.

3.2.5.2 Preparation of EDTA Solution

Approximately 0.45 g of EDTA powder was fully dissolved in 500 ml of PBS solution in a 1 L Schott bottle. The EDTA-PBS solution was autoclaved and sealed with parafilm in the laminar hood. The solution was kept in fridge and used when needed.

3.2.5.3 Preparation of 0.1% Trypsin-EDTA Solution

A precise amount of 20 ml of 0.25% Trypsin was mixed with 30 ml of EDTA solution in a 50 ml falcon tube. The falcon tube was sealed with parafilm and kept in the fridge.

3.2.5.4 Frozen Cell Line Thawing

Cryovial containing the frozen cell line was taken out from the -80°C freezer. It was thawed by rolling the vial between palm of hands back and forth for about one minute. A volume of 9 ml of complete growth medium was added into a

75cm^3 culture flask by using a 10 ml disposable pipette and a pipette gun. Then, the defrost cell line in the cryovial was immediately poured into the culture flask. It was incubated for 6 hours in 5% CO_2 humidified incubator at a temperature of 37°C. After that, 5ml of 0.1% Trypsin-EDTA solution was added into the flask and kept in the CO_2 incubator for 5 minutes for the cells to detach. Following the incubation, the flask was viewed under an inverted phase contrast microscope to confirm the detachment of the cells. As soon as the cells were seen floating, 5 ml of complete growth medium was added to neutralise and stop the action of trypsin. The mixture in the flask was then entirely transferred into a 50 ml falcon tube and sealed with parafilm. The tube was then centrifuged at a speed of 1500 rpm for 10 minutes. The supernatant was discarded using a 10 ml disposable pipette and the pellet was resuspended gently with 1 ml of complete growth medium. A volume of 9 ml of complete growth medium was added into a new 75 cm^3 culture flask and finally, 1 ml of the cell suspension was transferred into the flask and it was incubated in 5% CO_2 humidified incubator at a temperature of 37°C (modified from ATCC, 2015a).

3.2.5.5 Subculturing Cell Line

The confluency of cells was checked from time to time through observation under the inverted phase contrast microscope. Subculture process was done once the cells have reached enough confluency, around 80% to 90%. Firstly, 5 ml of 0.1% Trypsin-EDTA solution was added into the flask and kept in the CO_2 incubator for 5 minutes for the cells to detach. Following the incubation, the flask was viewed under an inverted phase contrast microscope to confirm

the detachment of the cells. A little tap at the bottom of the flask helped the cells that have not detached to release themselves from the flask. As soon as the cells were seen floating, 5 ml of complete growth medium was added to neutralize and stop the action of trypsin. The mixture was then transferred into a 50 ml falcon tube by using a 10 ml disposable pipette and a pipette gun. The falcon tube was sealed with parafilm and centrifuged at a speed of 1500 rpm for 10 minutes. Then, the supernatant was discarded and the pellet was resuspended gently with 2 ml of complete growth medium. A volume of 9 ml of complete growth medium was added into two new 75 cm^3 culture flasks. The cell suspension was equally transferred into the two culture flasks and then incubated in 5% CO_2 humidified incubator at a temperature of 37°C to allow cell growth (modified from ATCC, 2015b).

3.2.5.6 Maintenance of Cell Line

Maintenance was done by checking the cells' condition regularly through observation under the inverted phase contrast microscope. Maintenance is a crucial step of cell culture as it allows the detection of any signs of contamination apart from checking and estimating the degree of confluency. Subculture was done whenever a confluency of 80% to 90% was reached. The old complete growth medium was changed each time during subculturing process.

3.2.5.7 Cryopreservation of Cell Line

The LS 174T cells were transferred into a 50 ml falcon tube and centrifuged at 1500 rpm for 10 minutes. After centrifugation, the supernatant was discarded. The pellet was then resuspended with 8 ml of DMEM medium, 1 ml of Foetal Bovine Serum (FBS) and finally 1 ml of Dimethyl Sulfoxide (DMSO) solution (modified from Thompson, Kunkel and Ehrhardt, 2014). The cell suspension was transferred into 10 cryovials with each cryovial containing 1 ml of the suspension. The cryovials were kept in a cryovial box and stored in a -80°C freezer for 24 hours. Finally, the box was transferred to a liquid nitrogen tank at -196°C for permanent storage.

3.2.5.8 Cell Count

Cell count is essential to estimate cell number, monitor growth rates and to determine the concentration of LS 174T cells required for MTT assay. A haemocytometer and cell counter are the devices used in the process of cell count. Firstly, a cover slip was placed onto a clean haemocytometer. Subculturing process was repeated and 10 µl of the cell suspension was transferred onto a piece of parafilm. Then, it was mixed gently with 10 µl of 0.4% trypan blue dye and left for a minute. After that, 10 µl of the suspension was loaded onto the haemocytometer. The magnification of the inverted phase contrast microscope was adjusted to 100X. The difference in staining helps in the detection of viable and dead cells, whereby dead cells are stained dark blue unlike viable ones due to their compromised cell membranes. As shown in Figure 3.1, the cells in all four counting grids were counted and recorded. The

formula used for the process of cell count is as shown below (Stephenson, et al., 2012):

1. Average number of viable cells = $\frac{\text{Number of viable cells}}{\text{Number of counting chambers}}$

2. Cell suspension concentration = $\frac{\text{Average number of viable cells} \times 2 \times 10^4}{10^3}$

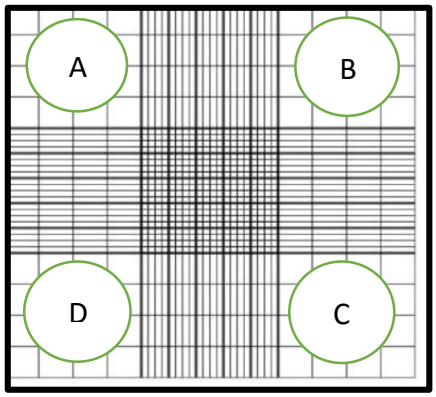

Figure 3.1: Squares labeled A, B, C and D which were used for cell count (Adapted from Grigoryev, 2013).

3.2.6 Determination of Cytotoxic Properties

3.2.6.1 MTT Assay

3.2.6.2 Preparation of Stock and Substock Solution and Test Samples

Approximately 100 mg of crude extract was weighed and added into a 1.5 ml microcentrifuge tube containing 1 ml of DMSO. The mixture was mixed using a vortex and a sonicator was used to fully dissolve the mixture. They were all filtered using a 1 ml syringe and 0.22 μm cellulose acetate syringe filter and left

overnight at 4°C. In order to prepare a substock solution with a concentration of 1000 µg/ml, 990 µl of complete growth medium and 10 µl of stock solution were mixed in a 1.5 ml microcentrifuge tube. Test samples of different concentrations (20, 40, 80, 160 and 320 µg/ml) were also prepared using substock solution and complete growth medium.

3.2.6.3 Preparation of MTT solution

MTT solution with a concentration of 5 mg/ml was prepared by adding 5 mg of MTT powder into a 1.5 ml microcentrifuge tube containing 1 ml of autoclaved phosphate buffered saline (PBS) solution. It was wrapped with aluminium foil and stored in the fridge at 4°C until future usage.

3.2.6.4 Preparation of 0.64% DMSO

A volume of 3.2 µl of DMSO was mixed with 497 µl of complete growth medium in a 1.5 ml microcentrifuge tube.

3.2.6.5 Cell Plating

Cell plating process was carried out by pipetting 100 µl of the cell suspension into each well. The plate was swirled gently side to side to allow equal distribution of cells. Then, it was incubated in the 5% CO_2 humidified incubator at 37°C for different incubation periods. In order to obtain 10,000 viable cells in 100 µl of cell suspension, the formula shown below was applied:

$$M_1V_1 = M_2V_2$$

where M_1 = Concentration of cell suspension (cells/µl) obtained from cell count

V_1 = Volume of cell suspension (µl) needed to obtain 1,000,000 cells

M_2 = Concentration of cells per well (cells/µl), 10,000 cells per well

V_2 = Total volume of cell suspension required per plate which was 10 ml

Based on the value obtained for V_1, complete growth medium was added to V_2 to make a total volume of 10,000 µl. The cell suspension now consisted of 1,000,000 cells which gave rise to 10,000 cells per well when 100 µl of the cell suspension was pipetted into each well. Three sets of 96-well plates were prepared. However, this calculation is only applicable for one plate.

3.2.6.6 Treatment of Cells with Crude Extracts and Positive Control

The 96-well plates that have been seeded with 100 µl of the cell suspension were treated with the crude extracts. The treatment was performed in triplicate at varying concentrations (20, 40, 80, 160 and 320 µg/ml) from well A to E respectively by adding 100 µl of filtered crude extracts. The plates were incubated at different incubation periods which were, 24, 48 and 72 hour intervals. Untreated cell suspension or standard, composed of 100 µl of cell suspension with a descending number of viable cells due to the serial dilution. The negative control used for the assay was the treated cell suspension, which composed of 100 µl of cell suspension and 100 µl of complete growth medium treated with 0.64% DMSO which reached a final concentration of 0.32% after

further dilution. As for the positive control, cisplatin was used. Five different concentrations (20, 40, 80, 160 and 320 μg/ml) of cisplatin were prepared and used to treat the cells. Besides, for sterility test, complete growth medium was used (modified from Chang, et al., 2013). The design of the plate was shown in Figure 3.2 with appropriate descriptions in Table 3.3.

3.2.6.7 MTT Assay

At the end of the incubation period (24, 48 and 72 hours respectively), 20 μl of MTT solution was added into each well in the absence of light. The plate was covered with aluminium foil and incubated for another four hours at 37°C in 5% CO_2 humidified incubator. After that, the solution in the wells were discarded and the insoluble purple formazan crystals were dissolved in 200 μl of DMSO. The plate was agitated using an orbital shaker for about ten minutes and finally, the absorbance was measured spectrophotometrically at 570 nm using a microplate reader (Jain and Jain, 2011). The percentage of cell viability was calculated using the following formula (Choudhari, et al., 2011):

$$\text{Percentage of cell viability (\%)} = A_s / A_c \times 100$$

where A_s = Absorbance of sample

A_c = Absorbance of standard

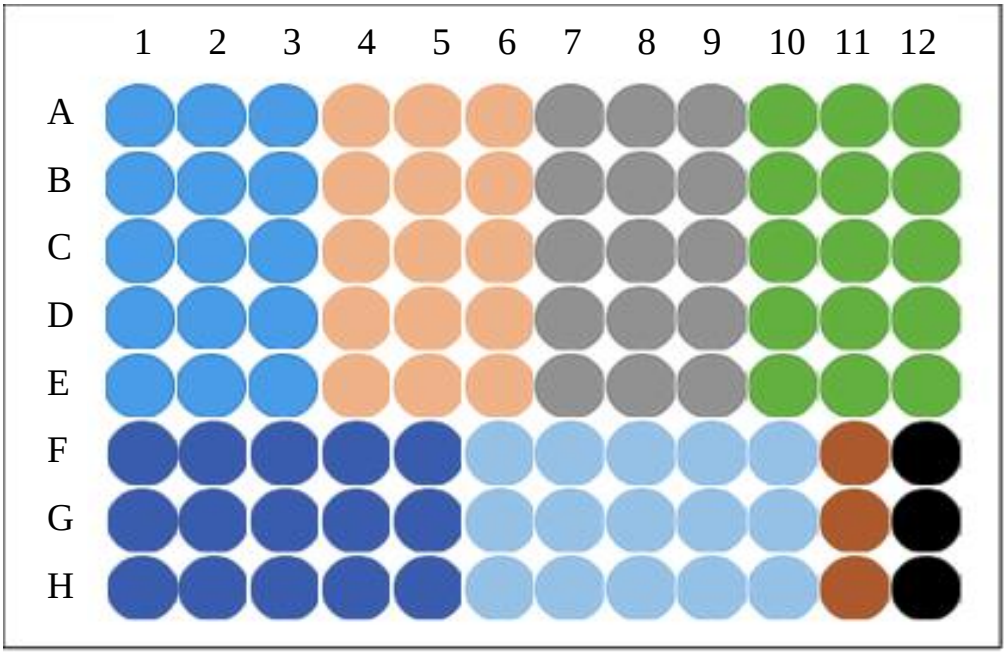

Figure 3.2: Layout of 96-well plate for MTT assay.

Table 3.3: Components and the colour it represents.

Component	Colour
1. Hydromethanol	
2. Ethyl acetate	
3. Hexane	
4. Positive control	
5. Negative control	
6. Standard (untreated cells)	
7. Complete Growth Medium (Sterility test)	
8. Empty wells	

CHAPTER 4

RESULTS

4.1 Extraction Yield of *Piper nigrum* L.

Four solvents of different polarity such as methanol, hydromethanol, ethyl acetate and hexane were utilized for extraction. The percentage of yield for each solvent have clearly shown their effectiveness in the extraction of compounds from *Piper nigrum* L. As shown in Table 4.1, hydromethanol extract of *Piper nigrum* L had the highest percentage of yield followed by methanol, ethyl acetate and hexane.

Table 4.1: Extract yields of *Piper nigrum* L. using different solvents.

Extract	Weight (g)	Percentage* (w/w) (%)
Methanol	20.07	10.04
Hydromethanol	22.25	11.13
Ethyl acetate	4.16	2.08
Hexane	13.05	6.53

*Percentage of yield was calculated based on the weight of the dried extract against 200 g dry weight of ground *Piper nigrum* L.

4.2 *In vitro* Antioxidant Assays

4.2.1 DPPH Radical Scavenging Activity of *Piper nigrum* L.

DPPH free radical scavenging assay was employed in the present study to evaluate the antioxidant nature of *Piper nigrum* L. crude extracts. The DPPH free radical scavenging activity of the crude extracts of *Piper nigrum* L. and ascorbic acid (positive control) was shown in Figure 4.1. Six different concentrations of ascorbic acid and crude extracts of *P. nigrum* L were prepared and used in this assay. From the graph plotted, it can be seen that the scavenging activity increased in response to increasing concentration which displayed a dose-dependent relationship.

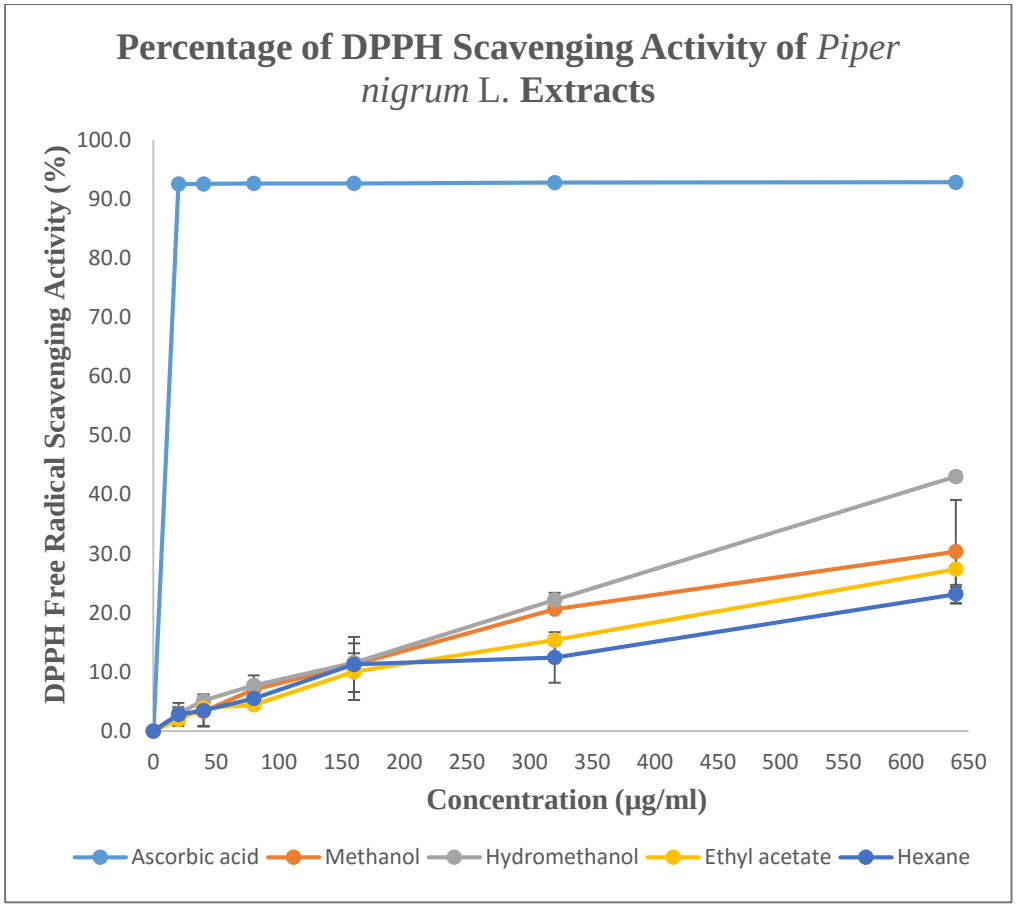

Figure 4.1: Graph showing the percentage of DPPH free radical scavenging activity of four different crude extracts of *Piper nigrum* L. (methanol, hydromethanol, ethyl acetate and hexane) and ascorbic acid at different concentrations.

The EC_{50} values of ascorbic acid can be determined directly from Figure 4.1 but not for crude extracts of *P. nigrum* L. due to their lower scavenging activity even at the highest concentration (640 µg/ml**)**. Hence, specific mathematical equation for each crude extracts were obtained from the graph and were used to calculate for their EC_{50} values respectively. As shown in Table 4.2, ascorbic acid has displayed highest radical scavenging activity followed by hydromethanol, methanol, ethyl acetate, and hexane extract.

Table 4.2: EC_{50} values of ascorbic acid and crude extracts of *Piper ngrum* L. based on DPPH free radical scavenging activity.

Test sample	EC_{50} (µg/ml)
Ascorbic acid	11.0
Methanol	1013.6
Hydromethanol	746.8
Ethyl acetate	1168.1
Hexane	1422.7

4.2.2 Total Phenolic Content of *Piper nigrum* L.

Folin-Ciocalteau Reagent Test was carried out using spectrophotometric measurement to quantify the total phenolic content of crude extracts of *Piper nigrum* L. Gallic acid of different concentrations were prepared and a standard curve, as shown in Figure 4.2, was generated using the absorbance values measured. The concentration of four crude extracts of *P. nigrum* L. were set at 1 mg/ml and the absorbance values were measured at 765 nm. As shown in Table 4.3, the absorbance readings were expressed as mean of absorbance values ± standard deviation (SD) of the triplicate measurement.

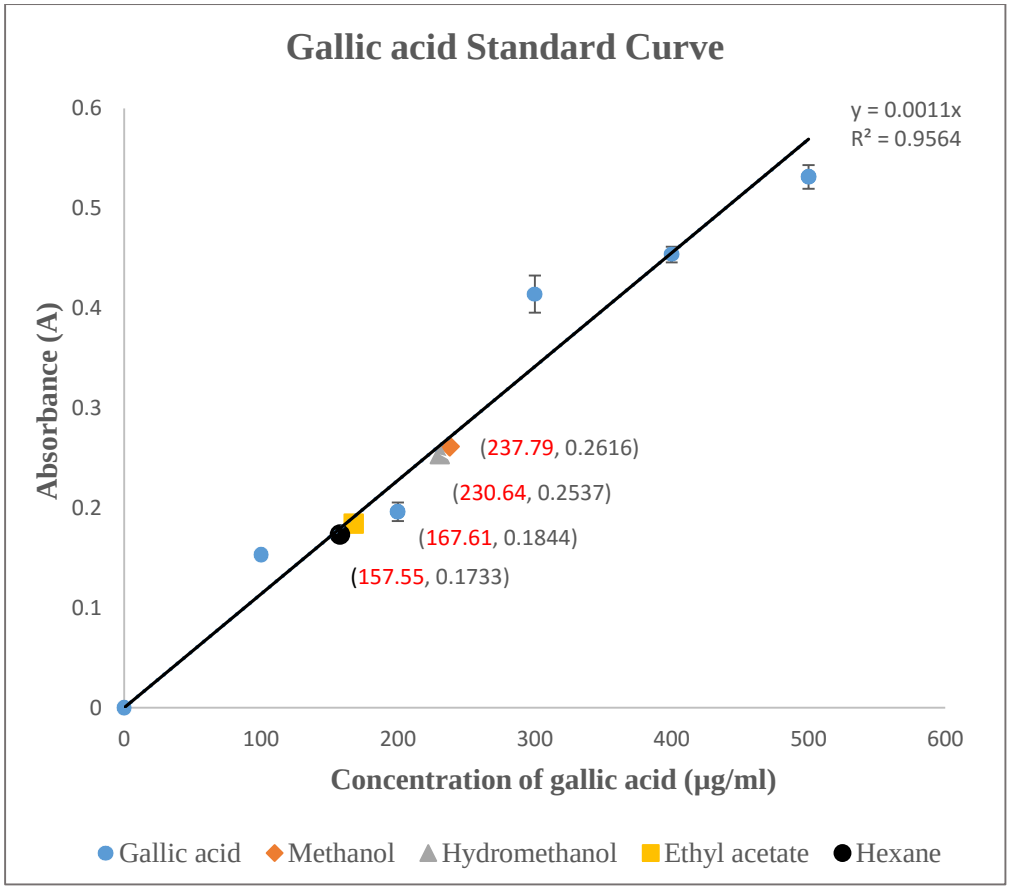

Figure 4.2: Graph showing the standard curve of gallic acid and the marked points representing different crude extracts of *Piper nigrum* L.

*The two values shown in each bracket are the concentration of gallic acid present in 1 mg/ml of the crude extracts of *P. nigrum* L. (shown in red) and the absorbance values of the crude extracts (shown in black) measured at 765 nm.

Table 4.3: Absorbance values of crude extracts of *Piper nigrum* L. (dosage of 1 mg/ml) measured at 765 nm.

Extract	Absorbance (A)
Methanol	0.2616 ± 0.025
Hydromethanol	0.2537 ± 0.040
Ethyl acetate	0.1844 ± 0.010
Hexane	0.1733 ± 0.011

The gallic acid standard curve generated was used to determine the total phenolic content of the crude extracts. The results were then calculated using the mathematical equation obtained from the standard calibration curve.

As shown in Table 4.4, methanol was found to be the most effective in the extraction of phenolic compounds from *Piper nigrum* L. followed by hydromethanol, ethyl acetate and lastly, hexane.

Table 4.4: Total Phenolic Content of the crude extracts of *Piper nigrum* L. expressed as (μg GAE/mg).

Extract	Total Phenolic Content (μg GAE/mg)
Methanol	237.79
Hydromethanol	230.64
Ethyl acetate	167.61
Hexane	157.55

4.2.3 Total Flavonoid Content of *Piper nigrum* L.

Aluminiun Chloride Colourimetric Method was carried out to quantify the total flavonoid content of crude extracts of *Piper nigrum* L. Six different concentrations of quercetin were prepared and the absorbance values measured were used to generate the standard curve of quercetin. The absorbance values of crude extracts of *P. nigrum* L. (dosage of 1 mg/ml) measured at 415 nm were recorded in Table 4.5. The absorbance readings were expressed as mean of absorbance values ± standard deviation (SD) of the triplicate measurement.

Table 4.5: Absorbance values of crude extracts of *Piper nigrum* L. (dosage of 1 mg/ml) measured at 415 nm.

Extract	Absorbance (A)
Methanol	0.4476 ± 0.008
Hydromethanol	0.4559 ± 0.003
Ethyl acetate	0.4940 ± 0.001
Hexane	1.3545 ± 0.036

The quercetin standard curve plotted was employed to determine the total flavonoid content of crude extracts of *P. nigrum* L. The results were then calculated using the mathematical equation obtained from the standard calibration curve.

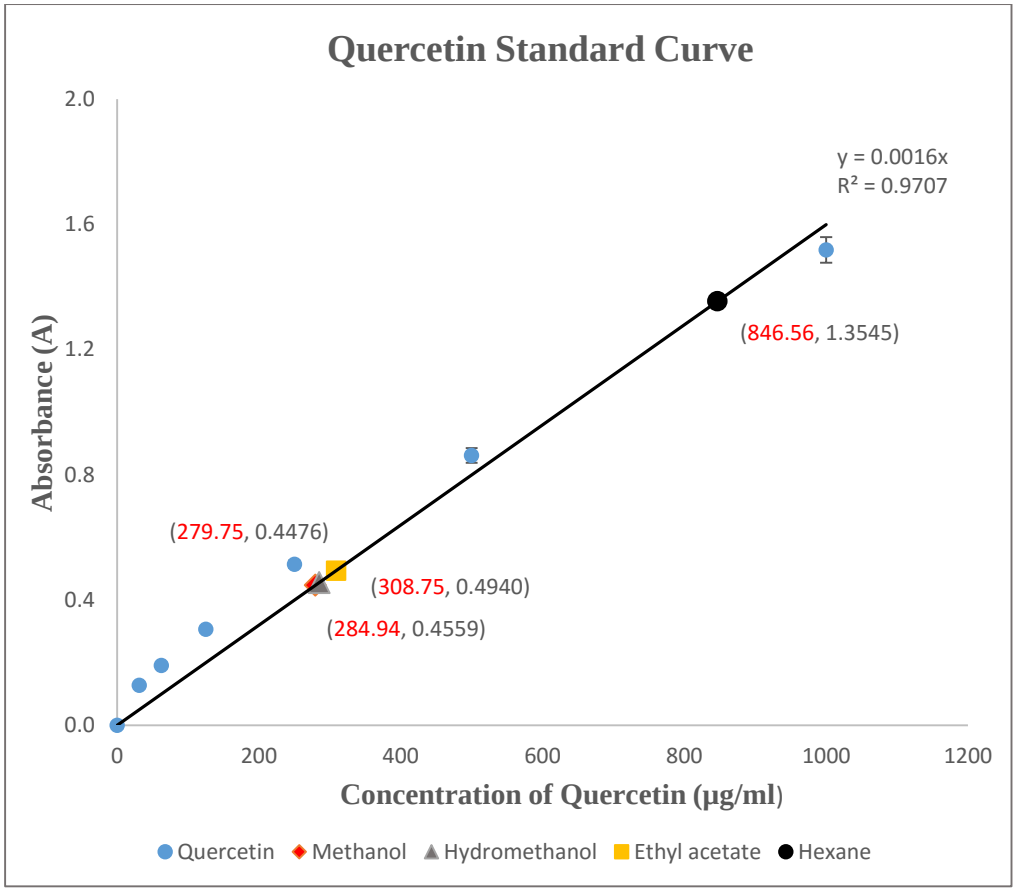

Figure 4.3: Graph showing the standard curve of quercetin and the marked points representing different crude extracts of *Piper nigrum* L.

*The two values shown in each bracket are the concentration of quercetin present in 1 mg/ml of the crude extracts of *P. nigrum* L. (shown in red) and the absorbance values of the crude extracts (shown in black) measured at 415 nm.

In Table 4.6, the concentration of total flavonoid content in each extracts of *P. nigrum* L. were recorded. This has clearly shown the effectiveness of different solvents in the extraction of flavonoid compounds from *P. nigrum* L. Among the four solvents used, hexane was the most potent in extracting flavonoid compounds from *P. nigrum* L. followed by ethyl acetate, hydromethanol and lastly, methanol.

Table 4.6: Total Flavonoid Content of the crude extracts of *Piper nigrum* L. expressed as (µg QE/mg).

Extract	Total Flavonoid Content (µg QE/mg)
Methanol	279.75
Hydromethanol	284.94
Ethyl acetate	308.75
Hexane	846.56

4.3 *In vitro* Cytotoxicity Screening

MTT assay was carried out to investigate the cytotoxic effects of crude extracts of *Piper nigrum* L. by determining the percentage of cell viability of LS 174T cells after treatment with the crude extracts. Percentage of cell viability varies due to varying effects of different types of crude extracts used, concentration of crude extracts and period of incubation.

4.3.1 MTT Assay (24 hours of treatment)

LS 174T cells were treated with different concentrations of crude extracts of *P. nigrum* L. and cisplatin for 24 hours. After incubation for 24 hours, absorbance values were measured and percentage of cell viability was calculated. The IC_{50} values of each crude extract was determined based on Figure 4.4 and results were recorded in Table 4.7. Among the four test sample, hexane extract showed greatest cytotoxic effect followed by hydromethanol extract, cisplatin and lastly, ethyl acetate extract.

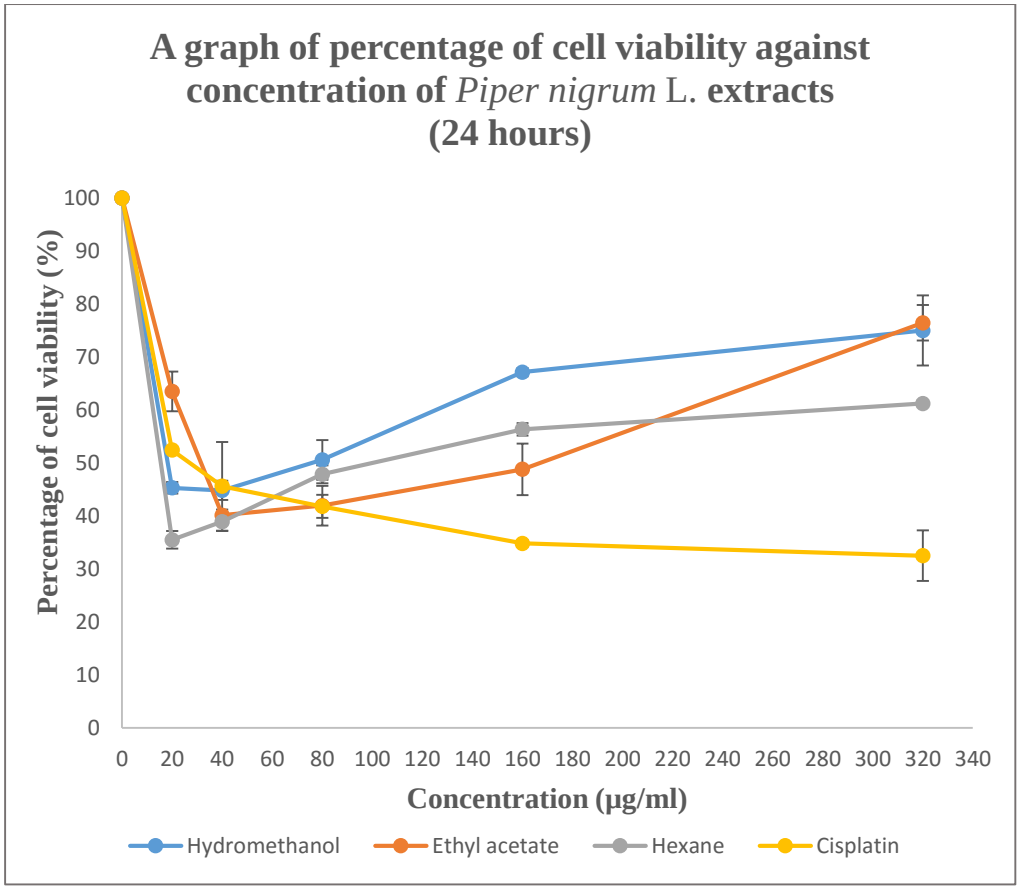

Figure 4.4: Cytotoxic effect of various concentrations (20-320 µg/ml) of *Piper nigrum* L. crude extracts and cisplatin on LS 174T cells after 24 hours of treatment.

Table 4.7: The IC_{50} values of cisplatin and *Piper nigrum* L. crude extracts after 24 hours of treatment.

Test sample	IC_{50} (µg/ml)
Cisplatin	26
Hydromethanol	18
Ethyl acetate	32
Hexane	16

4.3.2 MTT Assay (48 hours of treatment)

The duration of treatment was set for 48 hours and the whole procedure was repeated whereby LS 174T cells were treated with different concentrations of *Piper nigrum* L. crude extracts and cisplatin. After incubation for 48 hours, absorbance values were measured and percentage of cell viability was calculated. IC_{50} values were obtained from the graph shown in Figure 4.5 and results were recorded in Table 4.8. Based on the results obtained, hexane extract still displayed the highest cytotoxic activity followed by cisplatin, ethyl acetate extract and lastly, hydromethanol extract.

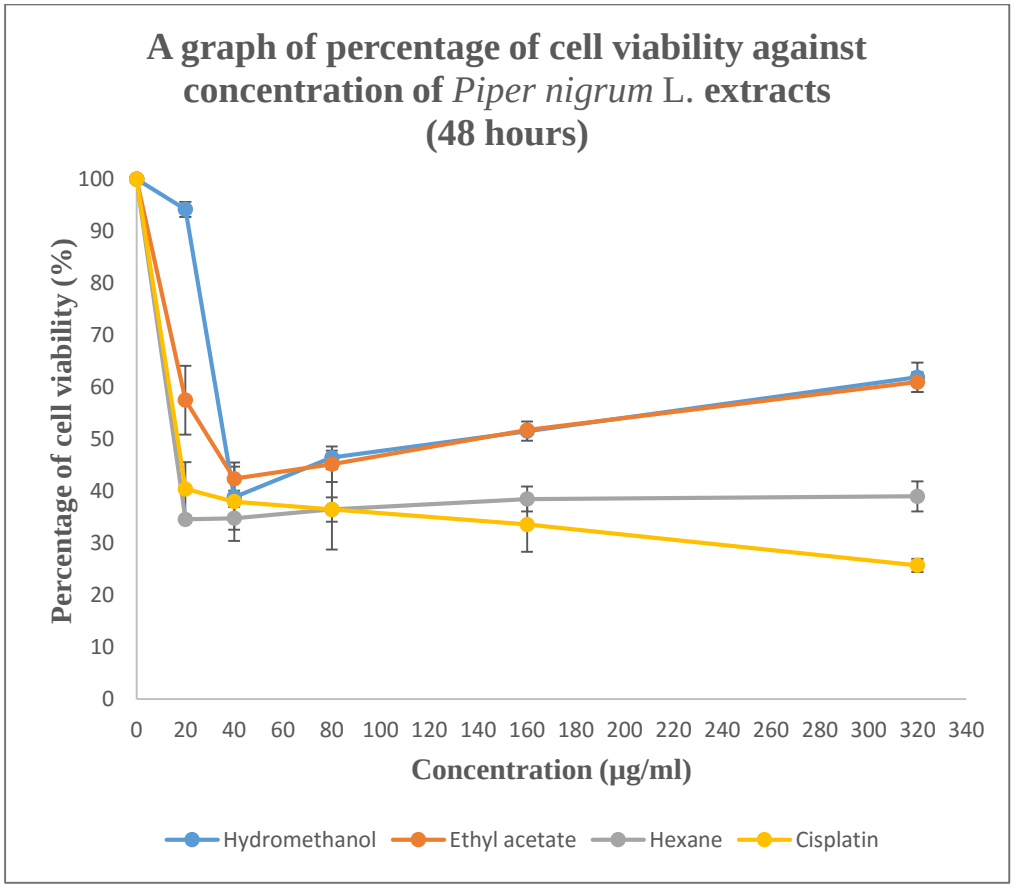

Figure 4.5: Cytotoxic effect of various concentrations (20-320 µg/ml) of *Piper nigrum* L. crude extracts and cisplatin on LS 174T cells after 48 hours of treatment.

Table 4.8: The IC_{50} values of cisplatin and *Piper nigrum* L. crude extracts after 48 hours of treatment.

Test sample	IC_{50} (µg/ml)
Cisplatin	17
Hydromethanol	36
Ethyl acetate	30
Hexane	16

4.3.3 MTT Assay (72 hours of treatment)

The duration of treatment was adjusted to 72 hours and the whole procedure was repeated again whereby LS 174T cells were treated with different concentrations of *Piper nigrum* L. crude extracts and cisplatin. After incubation, absorbance values were measured and percentage of cell viability was calculated. IC_{50} values were determined from Figure 4.6 and results were recorded in Table 4.9. Hexane extract again exhibited highest cytotoxic effect followed by hydromethanol and ethyl acetate extract which exhibited similar cytotoxic effect and cisplatin displayed lowest cytotoxicity.

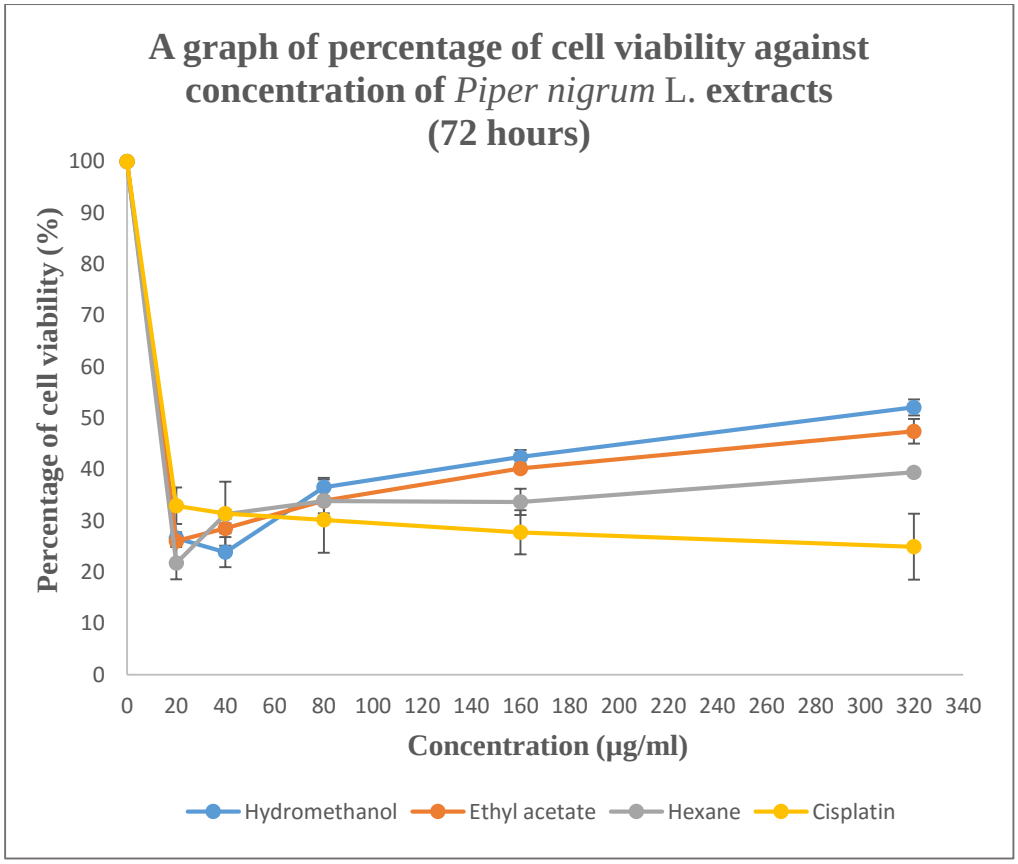

Figure 4.6: Cytotoxic effect of various concentrations (20-320 µg/ml) of *Piper nigrum* L. crude extracts and cisplatin on LS 174T cells after 72 hours of treatment.

Table 4.9: The IC_{50} values of cisplatin and *Piper nigrum* L. crude extracts after 72 hours of treatment.

Test sample	IC_{50} (µg/ml)
Cisplatin	15
Hydromethanol	14
Ethyl acetate	14
Hexane	13

CHAPTER 5

DISCUSSION

5.1 Plant Extraction Yield

The extraction of biologically active compounds from plants can be performed by using various extraction techniques. The most commonly employed method for isolation of plant phytochemicals is the solvent extraction technique. In spite of that, the nature of solvents used (polarity) and the chemical characteristics and polarities of plant active compounds are the common factors contributing to the amount of extract yields and the antioxidant activities (Sultana, Anwar and Ashraf, 2009). For instance, polar solvents like methanol and hydromethanol are normally employed to extract phenolic compounds in plants due to better solubility. Therefore, solvent polarity is said to be one of the most important parameter in extraction (Tomsone, Kruma and Galoburda, 2012).

In this study, hydromethanol extract of *Piper nigrum* L. had the highest percentage of extraction yield followed by methanol extract, hexane extract and lastly, ethyl acetate extract. These findings correlate with previous study done by Zarai, et al. (2013), whereby a higher extract yield of *P. nigrum* L was obtained using methanol, which is also a polar solvent, as compared to ethyl acetate and hexane (less polar solvents). The difference in yields between previous and present study could be due to the different polarities of the varying

compounds present in the spice. From the results obtained, it can be said that *P. nigrum* L. crude extracts contain mostly hydrophilic compound because polar solvents are more effective in extracting hydrophilic compounds than less polar solvents (Sasidharan, et al., 2010).

Other than the polarity of solvents, reducing the particle size of plant can also increase the extract yield. This is because smaller particle size will increase the surface area to volume ratio of plant that is exposed to the solvents and hence, allowing easier extraction of active compounds (Sarker, Latif and Gray, 2006). In this study, the dried fruit of *Piper nigrum* L. was pulverised into powdered form. The powdered form of *P. nigrum* L. eventually enhances the percentage of extraction yield due to higher surface area that is in contact with the solvents used. Besides, continuous addition of fresh solvents also plays an important role in affecting the yield. This procedure is usually done to ensure exhaustive extraction so that most chemically active constituents can be extracted out (Dai and Mumper, 2010).

5.2 Antioxidant Assays

5.2.1 Analysis of DPPH Free Radical Scavenging Activity

Free radicals are molecules or atoms that formed in the body and may eventually become the source of oxidative stress and damage to the cells when it is excessively accumulated in the body. Antioxidants which act as scavenger will help in the eradication of these radicals from the body to prevent diseases to occur. DPPH assay enables the determination of antioxidant activity of crude

extracts of *Piper nigrum* L. through the evaluation of DPPH free radical scavenging activity.

According to Singh, et al. (2014), different plants have various chemical profile and hence, an appropriate solvent is required for the extraction of specific type of antioxidant compound. This is because it is impossible to produce a universal solvent where all kinds of antioxidant compounds can be extracted. Based on the results obtained, hydromethanol extract has exhibited highest scavenging activity whereas hexane extract showed lowest scavenging activity. From here, a better antioxidant activity can be observed when a more polar solvent is used for extraction as compared to a less polar solvent. Polar solvents such as methanol, ethanol, and their aqueous mixtures are most preferable for extraction of antioxidant compounds from a plant matrix (Anwar and Przybylski, 2012).

However, the scavenging activity of methanol and hydromethanol extract of *P. nigrum* L. obtained in this study contradicted with the previous research done. Nahak and Sahu (2011) have reported a higher scavenging activity for methanolic extract (63.83%) than its aqueous extract (39.92%) of *P. nigrum* L. The present study showed a higher scavenging activity for hydromethanol extract then methanol extract of *P. nigrum* L. The difference might be due to geographical location in which the spice grows (Abrahim, Kanthimathi and Abdul-Aziz, 2012) and also the methods used in the preparation of crude extracts as some of the procedures may disrupt the active compounds present in the extracts.

The scavenging activity of each crude extracts might be due to the presence of phenolic compounds. Phenolic compounds from herbs and plants include phenolic acids, flavonoids, alkaloids, tannins, curcuminoids, lignans, quinones, and others. According to Symonowicz and Kolanek (2012), the number and location of free hydroxyl groups present on flavonoid skeleton poses a great influence on the free radical scavenging potential of natural polyphenolic compounds. Phenolic hydroxyl groups are excellent hydrogen donors. They usually react with free radicals in a termination reaction and thus hindering the generation of new reactive oxygen species (Brewer, 2011). Therefore, antioxidants with DPPH radical scavenging activity are able to donate hydrogen to DPPH free radicals which eventually leads to the change of purple to yellow colour observed in the assay (Sowndhararajan and Kang, 2013). In addition, the antioxidant properties of crude extracts of *P. nigrum* L. could also be due to the presence of alkaloid compound, piperine, which possess the ability to quench reactive oxygen species (Brewer, 2011).

In this assay, EC_{50} values for each crude extracts were determined. EC_{50} value represents the concentration of crude extracts of *P. nigrum* L. required to quench 50% of the DPPH free radicals. Acccording to Lu, et al. (2014), it is a typically employed parameter to express the antioxidant capacity and to compare the activity of different compounds. Compounds showing low EC_{50} values are said to possess good antioxidant properties. Based on the result, ascorbic acid showed highest antioxidant activity (lowest EC_{50} value) as compared to the

crude extracts. This is because crude extracts are mixture of compounds which are not as effective as pure compounds like ascorbic acid in exhibiting their scavenging activity.

5.2.2 Analysis of Total Phenolic Content

In this analysis, Folin-Ciocalteau reagent assay was utilized to quantitatively measure the concentration of phenolic compound present in the crude extracts of *Piper nigrum* L. This method measures the total concentration of phenolic hydroxyl groups in the plant extract. The formation of blue chromophore constituted by a phosphotungstic-phosphomolybdenum complex is due to the reaction of polyphenols in crude extracts with the redox reagent (Folin-Ciocalteu reagent). This blue colour complex can then be quantified spectrophotometrically (Agbor, Vinson and Donnelly, 2014). Moreover, the intensity of blue complex tends to increase as the concentration increases, resulting in higher absorbance values due to the greater availability of phenolic hydroxyl group to react with the redox reagent (Jadhav, et al., 2012).

Polyphenolics or phenolic compounds serve as a great source of antioxidant and are vital in the human diet (Balasundram, Sundram and Samman, 2006). Therefore, the purpose of this study is to assess the amount of phenolic content in each crude extracts of *P. nigrum* L. which may account for its total antioxidant activity, as it is a commonly used spice throughout the world.

Based on the results obtained, methanol extract of *P. nigrum* L. was found to contain the highest phenolic content followed by hydromethanol extract, ethyl acetate extract and lastly, hexane extract. The finding was supported by a research done by Zarai, et al. (2013), whereby total phenolic content of methanol and ethanol extracts of *P. nigrum* L. were higher than chloroform, ethyl acetate and water extracts of *P. nigrum* L. These could be explained through a research done by Galanakis, Goulas and Gekas (n.d.), which stated that phenols generally solubilized easier in polar solvents like ethanol and methanol due to the ability in formation of hydrogen bonds between phenol hydroxyl groups with the electronegative oxygen of polar solvents.

Gallic acid was used as standard in this assay because of its high phenolic content and possesses strong antioxidant activity (Natural Remedies, 2010). Hence, results were expressed as gallic acid equivalent (GAE). In this study, the overall total phenolic content values obtained were higher compared to previous researches done by Zarai, et al. (2013) and Uyoh, et al. (2013). Few possible factors that may cause the results to differ are the procedures employed in the processing of *P. nigrum* L. seed to form crude extracts, location or country of the plantation of spice and also the atmospheric conditions (Abrahim, Kanthimathi and Abdul-Aziz, 2012).

5.2.3 Analysis of Total Flavonoid Content

Flavonoids have been the focus of research in recent years due to its *in vitro* antioxidant properties. Flavonoids are polyphenolic compounds that can be

acquired from most fruits and vegetables and are able to hinder the occurrence of oxidative stress like most other antioxidants (Kumar and Pandey, 2013). Due to its numerous health benefits, present study was carried out to evaluate the total flavonoid content of *Piper nigrum* L. crude extracts. The quantification of total flavonoid content was done by using Aluminium Chloride Colourimetric Method.

Although hexane extract of *P. nigrum* L. displayed low extraction yield, DPPH scavenging activity and total phenolic content, but it was shown to have highest total flavonoid content. The least flavonoid content was found in methanol extract of *P. nigrum* L. The results obtained is in agreement with a study done by Zarai, et al. (2013), whereby lowest total flavonoid content was seen in methanol and ethanol extracts of *P. nigrum* L. and chloroform extracts of *P. nigrum* L. showed highest flavonoid content. Both chloroform and hexane are non-polar solvents and because of that, assumption can be made for hexane extract from chloroform extract of *P. nigrum* L. The results clearly showed the potential of solvents of decreasing polarity in extracting high amount of flavonoid compounds.

However, the overall flavonoid content of *P. nigrum* L. crude extracts were much higher than the values obtained in the previous study. According to Liu (2008), the chemical composition of extracts from the same plant can be quite different when different methods are employed in the preparation of extracts. Moreover, location or country of the plantation of spice and the atmospheric

conditions can also be the possible factors in causing dissimilarity of results obtained (Abrahim, Kanthimathi and Abdul-Aziz, 2012).

Quercetin is frequently used as standard in the measurement of total flavonoid content. This is attributable to its powerful antioxidant activity because it contains all the right structural features for the scavenging of free radicals (Prakash and Sharma, 2014). The strong antioxidant activity of quercetin can be one of the protective mechanism in preventing age dependent diseases such as cancer and neurodegenerative disorders (Rahman, 2007).

5.3 Cytotoxic Assay

Natural polyphenolic compounds which can be found largely in plants, spices and herbs are claimed to have important roles both *in vitro* and *in vivo*. The large group of phytoconstituents can be classified into several subgroups such as simple phenols, lignans, phenylpropanoids, flavonoids, coumarins and others (Jafari, Saeidnia and Abdollahi, 2014). Previous studies have shown the anticancer properties of phenolic compounds from several subgroups. They usually act by attenuating the initiation, progression and spread of cancers. In addition to that, angiogenesis that promotes tumor formation can also be inhibited by these compounds (Wahle, et al., 2010). Cancer can be caused by various external or internal factors. However, all these events are said to involve the excessive accumulation of free radicals in the body (Carocho and Ferreira, 2013). In this study, the evaluation on the cytotoxic properties of crude extracts of *Piper nigrum* L. towards LS 174T cancer cell line was done via MTT assay.

The LS 174T cells were treated with crude extracts and the cytotoxic activity was assessed.

5.3.1 Analysis of MTT Assay

Hexane extract of *P. nigrum* L. exhibited a much higher cytotoxic effect against LS 174T cells than hydromethanol and ethyl acetate extracts. In previous part of study, hexane extract was showed to have the highest concentration of total flavonoid content. This could possibly be one of the reason that it exerts greatest cytotoxic effect against LS 174T cells. According to Jaramillo-Carmona, et al., (2014), spices are one of the main sources of flavonoids. Besides, various studies have shown the anticancer properties of this compound by inducing cell cycle arrest and apoptosis in numerous types of cancer cell lines and colon cancer cell line was one of them. Mechanisms of apoptosis by flavonoids may also include the inhibition of DNA topoisomerase I/II activity, downregulation of nuclear transcription factor kappa B (NF-κB), activation of endonuclease, and suppression of Mcl-1 protein (Chahar, et al, 2011). Furthermore, flavonoids have the ability to induce cell cycle arrest by perturbing the checkpoints at G_1/S and G_2/M of the cell cycle in cancer cell lines (Batra and Sharma, 2013).

However, the cytotoxic activity for both hydromethanol and ethyl acetate extracts of *P. nigrum* L. were lower than hexane extract. This might be due to the polarity of solvents used in extraction of the biologically active compounds such as piperine and flavonoids from *P. nigrum* L. A study by Kolhe, Borole and Patel (2011) has reported that piperine tends to be more soluble in various

non-polar solvents but are insoluble in polar solvents. So, it can be said that the weaker cytotoxic activity of hydromethanol and ethyl acetate extracts (polar) of *P. nigrum* L. were due to its lower efficiency in extracting this compound. Meanwhile, extracts of non-polar solvents like hexane extract of *P. nigrum* L., exhibited a better cytotoxic effect against LS 174T and hence a lower IC_{50} value was obtained. The presence of piperine in hexane extract may also contribute to the low IC_{50} value. According to Duessel, Heuertz and Ezekiel (2008), piperine has shown a notable *in vitro* inhibition in proliferation of human colon cancer cells. Apart from the polarity of compounds, the amount of phytoconstituents extracted by different solvents might also play a role in their overall cytotoxicity. A study of Basri, et al., (2014) found that the lower cytotoxic activity of the methanol (polar solvent) extract as compared to acetone (less polar) extract could be due to more varieties of phytoconstituents found in methanol extract due to its ability to dissolve various polar compounds as well as some nonpolar compounds. The higher number of compounds extracted may act antagonistically to reduce its cytotoxic effect against HCT-116 cell line. Meanwhile, non-polar solvents allow only non-polar compounds to dissolve in it and hence, number of compounds extracted will be lesser, resulting in lower antagonistic effects. So, the lower cytotoxic activity exhibited by hydromethanol and ethyl acetate extracts of *P. nigrum* L. could be due to this as well. Polar solvents tend to be more efficient in extracting higher number of constituents which act antagonistically, suppressing the cytotoxicity of certain compounds.

Based on the result, the overall trend of the three graphs (3 different incubation period) were similar. When a lower concentration of crude extracts of *P. nigrum* L. (20-40 µg/ml) were used to treat LS 174T cells, the percentage of cell viability decreased, but it was found to rise again when higher concentrations (80-320 µg/ml) of crude extracts was used. This scenario might be due to the presence of multiple phytoconstituents in the crude extracts which cause them to act differently. In some cases, crude extracts did not show any activity but their pure compounds were proved to be active. This could be due to the presence of certain compounds which may act to inhibit the activity of others in a crude extract. Such antagonistic interactions can be possible in crude extracts and may eventually reduce their activity (Schinor, et al., 2007; Mazza, Shi and Le Maguer, 2002). This can be used to explain the results obtained in the present study. At lower concentration of crude extracts (less compounds present), there might be less constituents present to cause antagonistic effect towards those displaying cytotoxic activity. However, at higher concentration (more compounds present), the cytotoxic effects of certain phytoconstituents towards LS 174T cells might be repressed by higher number of compounds which act as antagonist in the crude extracts, reducing the overall cytotoxicity and hence leading to an increase in the percentage of cell viability (Odhiambo, et al., 2009). Therefore, a dose-dependent inhibitory effect of crude extracts of *P. nigrum* L. on LS 174T cells can be observed.

However, the cytotoxic effect of crude extracts of *P. nigrum* L. and cisplatin against LS 174T cells can be seen in a time-dependent manner, except for IC_{50} value of hydromethanol extract which showed fluctuation at 48 hours of

treatment. The overall IC_{50} values decreased as the incubation period increases. This has shown that the viability of LS 174T cells declined when they are exposed to the crude extracts of *P. nigrum* L. for a longer period of time. This phenomenon could be explained by the availability of more chance and time for phytochemicals in crude extracts to interact and exert its cytotoxic properties against the cancer cells and thus, lower IC_{50} values can be observed at longer period of treatment (Moyo and Mukanganyama, 2015)

In this study, hexane extract of *P. nigrum* L. consistently showed a lower IC_{50} values than of cisplatin (chemotherapeutic drug) as compared to hydromethanol and ethyl acetate extracts. At 24 hours of treatment, hexane and hydromethanol extracts of *P. nigurm* L. displayed lower IC_{50} values than cisplatin. At 48 hours of incubation, only hexane extract showed slightly lower IC_{50} value than cisplatin and in 72 hours of treatment, all the three crude extracts (hexane, hydromethanol and ethyl acetate extracts) shown to have slightly lower IC_{50} values than cisplatin. A study from Tantengco and Jacinto (2015) has reported that the cytotoxic activities of their plant extracts against HCT-116 cells (human colon cancer cell line) were almost similar to that of doxorubicin because there were no significant differences in their IC_{50} values. The low IC_{50} values have indicated the presence of potential anticancer compounds present in the plant extracts. Furthermore, Maqsood, et al. (2015) also reported that all of the six methanolic plant extracts tested (*Althaea rosea*, *Artemisia scoparia*, *Fagonia indica*, *Otostegia limbata*, *Schweinfurthia papillionacea* and *Withania coagulans*) possessed stronger and higher cytotoxic activity than the chemotherapeutic drugs used. Other than that, some Chinese herbal extracts also

shown to have similar response with conventional chemotherapeutic drugs against lung tumor cells (Monteiro, et al., 2014). Based on these findings, it shows that there is a possibility for crude extracts to possess higher or similar cytotoxic activity than chemotherapeutic drugs.

However, the cytotoxicity of the crude extracts of *P. nigrum* L. did not correspond to some of the previous works. In some studies, chemotherapeutic drugs have displayed a higher cytotoxicity than plant crude extracts. The type of solvents used in extraction, procedures in preparing crude extracts and the location of plantation of *P. nigrum* L. may contribute to the difference in results (Abrahim, Kanthimathi and Abdul-Aziz, 2012). Moreover, crude extracts contain multiple biologically active compounds which may react differently towards each other (synergistically or antagonistically) and also with the chemical components used in various assays, for an example MTT. All these factors might influence the consistency and accuracy of results. Therefore, in order to obtain a better result, few limitations in the current study should be taken into account.

Although MTT assay is widely employed to investigate the cell viability and cytotoxicity of certain compounds in drug discovery, yet there are some limitations which might affect the accuracy of results and may cause misinterpretation. A research has reported that MTT reduction is not only associated with the mitochondrial enzymatic activity of viable cells. Some other factors like non-mitochondrial enzymes, lysosomes and endosomes can also be

the sources contributing to the reduction activity (Jaszczyszyn and Gasiorowski, 2008). According to Han, et al. (2010), plant polyphenols like flavonoids and phenolic acids which consist of polyphenolic hydroxyl groups can also take part in the reduction of MTT. They can strongly reduce MTT in the absence of living cells. Therefore, the high cytotoxic effect exhibited by hexane extract of *P. nigrum* L. and the lower IC_{50} values of the three crude extracts (hexane, hydromethanol and ethyl acetate) of *P. nigrum* L. than cisplatin could be due to this as well.

5.4 Limitations of Study

5.4.1 Limitation of Method of Extraction

One of the limitations observed in this study was the large amount of organic waste produced when many different types of solvents were used. It requires appropriate management of the organic wastes. Besides, this method is also time-consuming and the exhaustive extraction process may lead to the loss of plant metabolites. Other than that, some biologically active compounds that have lower solubility in room temperature may not be extracted efficiently (Azwanida, 2015). According to Subramanian, et al. (2011), the double bypasses sidearm soxhlet apparatus (DBSA) is an excellent method to be used in the extraction of piperine from *Piper nigrum* L. It functions similarly as the conventional soxhlet but with increased extraction cycles and shorter extraction time. Thus, the more effective extraction method should have been employed in the current study.

5.5 Future Studies

Further studies should be carried out in order to investigate in more detail the types of bioactive compounds present in *Piper nigrum* L. crude extracts and also their biological activities. The compounds in each crude extracts that have contributed to the antioxidant and cytotoxic properties should be further explored. Other techniques like column chromatography, high performance liquid chromatography (HPLC) and thin layer chromatography (TLC) can be used to purify and isolate individual constituents from mixture of compounds present in *P. nigrum* L. crude extracts. This will allow the determination of the effectiveness of each constituent in their antioxidant and cytotoxic activity.

As for MTT assay, more cancer cell lines should be utilized for the assessment of cytotoxic properties of *P. nigrum* L. instead of using only one cancer cell line. The purpose of doing this is to determine whether the crude extracts of *P. nigrum* L. possess the ability to inhibit growth and progression of wide range of cancer cell lines. Apart from that, normal cell lines can also be tested. This is because some plant-derived compounds which are displaying cytotoxic effect towards cancer cell lines may also cause damage to normal tissues. By doing this, it would provide a useful information in telling if a plant is suitable to be developed and used as a chemotherapeutic drug (Sak, 2012).

Lastly, the mechanism of action, efficacy and toxicity of phytoconstituents extracted should be further analysed. By doing this, a more validate and

promising information can be obtained regarding the efficacy of the plant to serve as a powerful antioxidant and cytotoxic agent.

CHAPTER 6

CONCLUSION

In this study, solvents of varying polarity such as methanol, hydromethanol, ethyl acetate and hexane were used in the extraction of *Piper nigrum* L. The main purpose of doing this is to extract bioactive compounds from the plant which is said to have many physiological functions. Then, evaluation was done by conducting various biochemical assays using the crude extracts.

The choice of solvents has played a major role in the extraction process. Polar solvents such as hydromethanol obtained the highest percentage yield whereas ethyl acetate, a less polar solvent, obtained the lowest yield. The antioxidant nature of each crude extract were then evaluated based on DPPH free radical scavenging assay. Hydromethanol extract has displayed highest scavenging activity among the other extracts. Besides, phenolic and flavonoid content in each crude extract were also quantified since phenolic compounds are said to contribute to the antioxidant activity and flavonoids being one of the major phytoconstituents of the plant. Results have revealed that methanol extracts of *P. nigrum* L. contains highest total phenolic content whereas hexane extract was shown to have highest total flavonoid content. In addition, the cytotoxic properties of *P. nigrum* L. crude extracts on LS 174T cell line were assessed based on MTT assay. Results have shown that the growth of LS 174T cells was inhibited in a time-dependent and dose-dependent manner.

Even though the effectiveness of crude extracts of *P. nigrum* L. as potential source of antioxidant and cytotoxic agents could not be verified or supported by the data obtained in the present study, but the activities exhibited by this spice cannot be denied. Therefore, further studies should be conducted to purify and isolate individual active compounds from the crude extracts and assess their pharmacological properties.

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APPENDIX A

Table A: Percentage of DPPH scavenging activity of ascorbic acid and crude extracts of *Piper nigrum* L. (methanol, hydromethanol, ethyl acetate and hexane).

Test Sample Concentration (µg/ml)	DPPH Radical Scavenging Activity* (%)				
	Ascorbic acid	**Methanol**	**Hydromethanol**	**Ethyl Acetate**	**Hexane**
20	92.54 ± 0.022	2.67 ± 1.141	2.92 ± 1.819	1.91 ± 1.043	2.78 ± 1.263
40	92.57 ± 0.022	3.32 ± 2.489	5.13 ± 0.474	3.90 ± 0.701	3.46 ± 2.750
80	92.65 ± 0.006	6.99 ± 2.425	7.70 ± 0.278	4.41 ± 0.603	5.49 ± 1.426
160	92.65 ± 0.127	11.24 ± 0.122	11.54 ± 1.599	10.01 ± 4.810	11.25 ± 4.647
320	92.78 ± 0.075	20.64 ± 0.294	22.18 ± 1.190	15.37 ± 0.768	12.41 ± 4.279
640	92.82 ± 0.108	30.34 ± 8.721	43.06 ± 0.765	27.38 ± 3.009	23.15 ± 1.600

*The results were tabulated as mean of percentage of DPPH radical scavenging activity ± SD of triplicate.

APPENDIX B

Table B: Absorbance values of different concentration of gallic acid measured at 765 nm.

Concentration (µg/ml)	Absorbance* (765 nm)
100	0.1533 ± 0.003
200	0.1961 ± 0.009
300	0.4141 ± 0.019
400	0.4538 ± 0.008
500	0.5315 ± 0.012

*The results were tabulated as mean of absorbance ± SD of triplicate.

Table C: Absorbance values of different concentrations of quercetin measured at 415 nm.

Concentration (µg/ml)	Absorbance* (415 nm)
31.25	0.1278 ± 0.002
62.50	0.1912 ± 0.005
125.00	0.3066 ± 0.001
250.00	0.5146 ± 0.012
500.00	0.8618 ± 0.024
1000.00	1.5180 ± 0.041

*The results were tabulated as mean of absorbance ± SD of triplicate.

APPENDIX C

Table D: Percentage of cell viability of human colon cancer cell line (LS 174T) after treatment with cisplatin and crude extracts of *Piper nigrum* L. (hydromethanol, ethyl acetate and hexane) at 24, 48 and 72 hours.

Incubation Period (hours)	Test Sample Concentration (µg/ml)	Cell viability* (%)			
		Cisplatin	Hydromethanol	Ethyl acetate	Hexane
24	**20**	52.42 ± 0.216	45.30 ± 1.089	63.50 ± 3.756	35.50 ± 1.667
	40	45.59 ± 8.375	44.83 ± 1.792	40.13 ± 1.104	38.92 ± 1.765
	80	41.80 ± 2.164	50.59 ± 3.745	41.94 ± 3.732	47.86 ± 1.699
	160	34.82 ± 0.561	67.13 ± 0.785	48.80 ± 4.858	56.35 ± 1.176
	320	32.51 ± 4.795	75.00 ± 6.611	76.45 ± 3.372	61.22 ± 0.531
48	**20**	40.41 ± 5.151	94.19 ± 1.450	57.47 ± 6.615	34.55 ± 0.688
	40	37.97 ± 7.536	38.84 ± 3.395	42.38 ± 2.323	34.77 ± 2.183
	80	36.48 ± 7.727	46.44 ± 1.329	45.17 ± 3.430	36.46 ± 2.327
	160	33.57 ± 5.224	51.51 ± 1.846	51.73 ± 0.198	38.47 ± 2.389
	320	25.72 ± 1.268	61.89 ± 2.846	60.96 ± 1.930	38.99 ± 2.899
72	**20**	32.94 ± 3.542	26.64 ± 1.128	26.04 ± 0.853	21.77 ± 3.144
	40	31.40 ± 6.233	23.89 ± 2.949	28.53 ± 0.974	31.29 ± 0.849
	80	30.17 ± 6.391	36.55 ± 1.829	33.91 ± 4.164	33.88 ± 2.417
	160	27.77 ± 4.321	42.45 ± 1.342	40.19 ± 0.671	33.67 ± 2.552
	320	24.96 ± 6.416	52.11 ± 1.564	47.44 ± 2.427	39.44 ± 0.439

*The results were tabulated as mean of percentage of cell viability ± SD of triplicate.