

**PRELIMINARY SCREENING OF *Allium cepa* Linn FOR ITS
ANTIOXIDANT ACTIVITY, CYTOTOXICITY ON HUMAN
CHRONIC MYELOGENOUS LEUKAEMIA CELL LINE (K562)
AND ANTIBACTERIAL PROPERTIES**

By

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ABSTRACT

PRELIMINARY SCREENING OF *Allium cepa* Linn FOR ITS ANTIOXIDANT ACTIVITY, CYTOTOXICITY ON HUMAN CHRONIC MYELOGENOUS LEUKAEMIA CELL LINE (K562) AND ANTIBACTERIAL PROPERTIES

Parthiban a/l Murugaiyah

Due to their high medicinal qualities, plants are being massively explored in scientific researches, and in medical and pharmaceutical industries. Hence, the present study was conducted to determine the antioxidant activity, to investigate the cytotoxicity and to qualitatively screen for the antibacterial properties of *Allium cepa* Linn, which is commonly known as onion. Extraction of the plant was performed using solvents such as hexane, ethyl acetate, methanol, ethanol and hydromethanol. The antioxidant nature of the extracts was assessed based on their potential to scavenge free radicals and chelate metals like iron via DPPH Free Radical Scavenging Assay and Iron Chelating Assay. Ethyl acetate extract was found to exhibit good activity in both tests when compared to other extracts (IC₅₀ of 41.229 µg/ml and 55.419 µg/ml respectively). Folin-Ciocalteu Reagent Test and Aluminium Chloride Colourimetric Method were employed in order to quantify the total phenolic and flavonoid content of the extracts. Both the quantitative tests revealed the superiority of ethyl acetate in extracting phenolic compounds and flavonoids when compared to other extracts (70.10 µg GAE/mg and 101.28 µg GAE/mg

respectively). The cytotoxic properties of the extracts were tested on human chronic myelogenous leukaemia cell line (K562) via 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay at varying concentrations (20 to 320 µg/ml) and incubation periods (24, 48 and 72 hours). The results revealed that the crude extracts exhibited cytotoxic properties against K652 cells in both time-dependent and dose-dependent manner. Qualitative screening on the antibacterial properties of *A. cepa* L. extracts was carried out via Broth Microdilution Method by taking into account of their MIC and MBC values. Ethyl acetate extract was proved inhibitory and showed bactericidal activity towards *Staphylococcus aureus* and *Enterococcus faecalis* at 1.875 mg/ml and towards *Pseudomonas aeruginosa* and *Escherichia coli* at 7.5 mg/ml. Meanwhile, extracts like hexane and hydromethanol exerted bacteriostatic activity towards *E. coli*.

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I couldn't thank my family enough for their continuous support and care. They have always encouraged me to do my best. Last but not least, I thank the Lord for giving me the ultimate strength in completing the research and also for showering me with lots of blessings.

DECLARATION

I hereby declare that the final year project entitled “**PRELIMINARY SCREENING OF *Allium cepa* Linn FOR ITS ANTIOXIDANT ACTIVITY, CYTOTOXICITY ON HUMAN CHRONIC MYELOGENOUS LEUKAEMIA CELL LINE (K562) AND ANTIBACTERIAL PROPERTIES**” 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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This final year project entitled “**PRELIMINARY SCREENING OF *Allium cepa* Linn FOR ITS ANTIOXIDANT ACTIVITY, CYTOTOXICITY ON HUMAN CHRONIC MYELOGENOUS LEUKAEMIA CELL LINE (K562) AND ANTIBACTERIAL PROPERTIES**” was prepared by **PARTHIBAN A/L MURUGAIYAH** 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

It is hereby certified that, **PARTHIBAN A/L MURUGAIYAH** (ID No: **11ADB05976**) has completed the final year project entitled **“PRELIMINARY SCREENING OF *Allium cepa* Linn FOR ITS ANTIOXIDANT ACTIVITY, CYTOTOXICITY ON HUMAN CHRONIC MYELOGENOUS LEUKAEMIA CELL LINE (K562) AND ANTIBACTERIAL PROPERTIES”** under the supervision of Pn.Norliza binti Shah Jehan Muttiah from the Department of Biomedical Science, Faculty of Science.

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,

(PARTHIBAN A/L MURUGAIYAH)

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LIST OF ABBREVIATIONS

%	Percentage
°C	Degree Celcius
K562	Human Chronic Myelogenous Leukaemia Cell Line
AlCl ₃	Aluminium (III) Chloride
AP1	Activator protein-1
ATCC	American Type Culture Collection
ATM/ATR	Ataxia telangiectasia mutated/Ataxia telangiectasia and Rad3-related protein
B16F10	Melanoma Cell Line
Cdks	Cyclin-dependent kinases
CGM	Complete Growth Medium
CH ₃ COOK	Potassium Acetate
CO ₂	Carbon dioxide
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
ERK	Extracellular signal-regulated kinase
FBS	Foetal Bovine Serum
FCR	Folin-Ciocalteu reagent
FeCl ₂	Iron (II) Chloride
G1/S	Stage in the cell cycle at the boundary between the first gap phase and the synthesis phase

G2/M	Stage in the cell cycle at the boundary between the second gap phase and the mitotic phase
GAE	Gallic acid Equivalent
HepG2	Human Liver Carcinoma Cell Line
HPLC	High Performance Liquid Chromatography
IC ₅₀	Inhibitory concentration to reduce 50% of cell viability
INT	Iodonitrotetrazolium chloride
MAPK	Mitogen-activated protein kinase
MBC	Minimum Bactericidal Concentration
MH	Mueller-Hinton
MIC	Minimum Inhibitory Concentration
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
Na ₂ CO ₃	Sodium Carbonate
nm	Nanometer
OD	Optical Density
QE	Quercetin Equivalent
PBS	Phosphate Buffered Saline
p53	Tumour Suppressor Gene
Rb	Retinoblastoma
ROS	Reactive Oxygen Species
Rpm	Revolutions per minute
RPMI	Roswell Park Memorial Institute
SD	Standard Deviation
TB	Tuberculosis
TLC	Thin Layer Chromatography

UTI	Urinary Tract Infection
v/v	volume for volume
WHO	World Health Organisation

CHAPTER 1

INTRODUCTION

1.1 Background Information

Natural products are believed to possess biochemical and pharmacological properties that can promote many health-beneficial effects in treating diseases and hence, there is an increasing demand to seek for therapeutic drugs from these sources (Molinari, 2009). Due to the unmatched availability of chemical diversity and biodiversity, some of these natural products involve medicinal plants, either as pure compounds or standardised crude extracts. To date, studies involving natural products especially plants have increased tremendously throughout the world particularly in edible ones and a number of collected evidences prove the ability of plants to exert therapeutic effects (Sasidharan, et al., 2011). Their contribution towards different branches of study such as chemistry, medicine, pharmacology and drug discovery is undeniable.

Research and laboratory findings revealed that the pharmacological properties exhibited by plants are greatly due to the presence of active components present in the plants (Veilleux and King, 1996). These biologically active compounds tend to regulate many physiological functions in the human body and thus, they can be extracted or isolated out from plants and then be tested for the medicinal qualities that they possess. Extraction involves the isolation of medicinally and biologically active components of a plant from the inactive

portions through the usage of selective solvents in standard extraction procedures (Handa, et al., 2008).

The reason behind the exploitation of these active compounds found in plants is due to the outstanding level of antioxidants. Free radicals such as the reactive oxygen and nitrogen species are being produced in the human body as a result of cellular metabolism. These highly reactive molecules possess an unpaired electron and when they interact with macromolecules like lipid, protein and DNA, they cause adverse effects which may lead to the development of various diseases in the long run (Lobo, Patil and Chandra, 2010).

However, the harmful effects can be impeded by antioxidants. A balance between the production of free radicals and antioxidants inside the body is fundamental for proper physiological function and if the balance is disrupted, the production of free radicals overcome the body's ability to control them. This scenario is known as oxidative stress. Therefore, an antioxidant plays an important role as a defender by scavenging free radicals and confers protection to various systems in the human body (Mukesh, et al., 2008).

Besides, these free radicals tend to affect the cells as well, causing them to grow quickly and abnormally. This phenomenon may lead to cancer development. One of the factors that leads to carcinogenesis is the damage to the DNA of cells caused by excessive free radicals which in turn leads to mutation and transformation into cancerous cells (National Institutes of

Health, 2014). Nevertheless, the active compounds derived from plants are said to possess cytotoxic properties and they too help in the prevention of cancer. Cytotoxicity is the quality of being toxic to cells and the process results in either cell damage or cell death (Eldridge, 2013). A particular compound or substance is said to possess cytotoxic properties if it is capable of affecting the rate of replication of cells, exerting noticeable morphological changes, preventing cellular attachment or even reducing the overall cell viability (Niles, Moravec and Riss, 2009).

Furthermore, some of these antioxidants found in plants are able to exert cytotoxic properties as well. For instance, an antioxidant like Vitamin C is able to clearly differentiate cancerous and healthy cells, therefore killing them. Such substances possess the ability to manipulate themselves to either play their role as antioxidants or as pro-oxidants. This scenario greatly depends on the environment. In healthy cells, their function is to confer protection as antioxidants whereas in tumours, they produce hydrogen peroxide that attacks cancerous cells by acting as pro-oxidants (Hickey, 2013).

Apart from cancer, infectious diseases too, pose a great threat. These disorders arise due to the presence of pathogenic microorganisms. For instance, bacteria, fungi, parasites and even viruses (World Health Organisation, 2014). They are also considered as one of the world's leading cause of death. Approximately 50,000 people are killed annually due to various infections (Madduluri, Babu and Sitaram, 2013). Thus, the development of antibiotics and other therapies are of absolute necessity and it is not too far-fetched to say that there are

effective treatments or remedies that is readily available to treat different kinds of infections (Nhs, 2014). However, the phenomenon of drug resistance and the emergence of drug-resistant pathogens has triggered certain catastrophes that made pharmaceutical and scientific communities to look for other alternative treatments. This is where plant-based natural products comes into picture, whereby studies which focus on the potentials of active compounds found in plants to act as antimicrobial agents are being conducted (Savoia, 2012).

1.2 Significance of Study

Since plant-derived natural products are able to regulate many physiological and biochemical functions in the human body, they are being explored massively in many pharmaceutical and medical industry. Among the plants which are said to possess medicinal and therapeutic qualities, *Allium cepa* Linn stands out as one of the good sources of antioxidant, antibacterial and cytotoxic agent.

One of the major constituents found in *Allium cepa* L. is flavonoids. The outstanding flavonoid content has been discovered to possess great antioxidant activity such as free radical scavenging and metal chelating (Shenoy, et al., 2009). This active compound is also able to exert cytotoxic effect on cancer cells and inhibit microbial growth by acting as antimicrobial agent. Apart from that, the plant is also rich in phytochemicals such as disulfides, trisulfides, cepaene, vinyl dithiols and many other secondary metabolites which exhibit

biological activities within the human body (National Onion Association, 2011).

Cancer researches involving plants are mushrooming in an effort to come up with novel therapeutic agents that are able to minimise or reduce the toxic effects associated with current therapeutic agents (Mahavorasirikul, et al., 2010). Studies have focused on the cytotoxic properties of *A. cepa* L. on breast, colon and stomach cancer cell lines (National Onion Association, 2011). Hence, in this present study, the cytotoxic property of different crude extracts of the plant was tested on K562 human chronic myelogenous leukaemia cell line.

In addition, the emergence of pathogenic microorganisms which show multi-drug resistance has led to the search of plants with antimicrobial properties (Kirilov, Doycheva and Satchanska, 2014). *Allium cepa* L is proven to possess antibacterial and antifungal properties against some pathogenic microorganisms (Bakht, Khan and Shafi, 2013). The present analysis, however, focused on the evaluation of antibacterial properties of the plant crude extracts against *Staphylococcus aureus*, *Enterococcus faecalis*, *Pseudomonas aeruginosa* and *Escherichia coli*.

As a whole, keeping in view the role of *A. cepa* L. as a medicinal plant, the present study was conducted to investigate the antioxidant, antibacterial and cytotoxic activity of different solvent extracted samples of the plant.

Preliminary screening was carried out by performing the extraction of *A. cepa* L. and subjecting the crude extracts to various biochemical assays.

1.3 Research Objectives

The objectives of this research were as follows:

- i. To perform extraction of *Allium cepa* L. using solvents of varying polarity such as hexane, ethyl acetate, methanol, ethanol and hydromethanol via maceration method.
- ii. To determine the antioxidant activity of crude extracts of *Allium cepa* L. by performing DPPH Free Radical Scavenging Assay and Iron Chelating Assay.
- iii. To quantify the total phenolic content and total flavonoid content of crude extracts of *Allium cepa* L. via Folin-Ciocalteu Reagent Test and Aluminium Chloride Colourimetric Method.
- iv. To investigate the cytotoxic effect of crude extracts of *Allium cepa* L. on human chronic myelogenous leukaemia cell line (K562) by performing MTT assay at different incubation periods, 24, 48 and 72 hours respectively.
- v. To qualitatively screen for antibacterial properties of crude extracts of *Allium cepa* L. against two Gram-positive bacteria (*Staphylococcus aureus* and *Enterococcus faecalis*) and two Gram-negative bacteria (*Pseudomonas aeruginosa* and *Escherichia coli*) by performing Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC).

CHAPTER 2

LITERATURE REVIEW

2.1 Natural Products and Plants

2.1.1 Natural Products

Natural products have played a major role in the field of medicine, scientific research, pharmaceutical and applied science. For instance, higher plants, arthropods, marine invertebrates and the largest groups of taxonomically identified classes of organisms are being studied by researchers and explored in the medical field. Some appear to be established cancer chemotherapeutic agents, antimicrobial agents and antioxidants for over 40 years. They are found to be in either naturally occurring or synthetically modified forms (Kinghorn, Chin and Swanson, 2009).

2.1.2 Plant-based Products

World Health Organisation (WHO) reported that approximately 65% of the world's population are depending on plant-based therapeutic products and traditional medicines (Cragg, Grothaus and Newman, 2009). Furthermore, more than 250,000 species of plants have been discovered so far which are said to possess medicinal qualities due to the overwhelming presence of bioactive compounds. These plants have the potential to contribute to the field of science and medicine (Crozier, Clifford and Ashihara, 2008). Phytochemicals or secondary metabolites are natural active compounds that can be derived from a plant material. Studies have found out that these natural

compounds derived from plants are capable of participating in various bioactivities such as antioxidant, anti-inflammatory, and anticancer activities (Lee, Hwang and Lim, 2004).

2.2 Plant of Interest

2.2.1 General Description

Allium cepa Linn or commonly known as onion is considered to be one of the world's oldest cultivated vegetable. It is a herbaceous plant which has a pear-shaped underground bulb bearing fibrous roots at its base and is comprised of fleshy leaf sheaths which then forms a thin-skinned capsule (Partha and Mandal, 2001). The appearance of approximately one meter high scape occurs annually and is surmounted by large globular umbel of greenish-white flowers. As shown in Figure 2.1, the bulbs of this plant are used medicinally and they vary greatly in sizes (Drugs, 2000).



Figure 2.1: *Allium cepa* Linn (Adapted from Seror, 2000).

2.2.2 Taxonomical Classification

The taxonomic rank of the plant begins at the kingdom level, *Plantae*. As we go further, it is then placed under *Tracheophyta*, division for mostly vascular plants. Plants can then be classified into different classes and as for this

particular plant, it falls under the class of *Magnoliopsida* (ITIS, 2015). Furthermore, it belongs to the *Asparagales* order and is placed under the family of *Amaryllidaceae* (MDidea, 2014). The genus of this plant is *Allium* L and finally, the taxonomical hierarchy ends at the species level, *Allium cepa* L.

2.2.3 Distribution of Plant

In recent times, it has been reported that the *Allium* family has over 600 members, distributed all over North America, Europe, Asia and Northern Africa. The plant is cultivated throughout the country in Pakistan. According to Abbasi, et al. (2011), the plant is cosmopolitan in distribution, whereby it is found on the main islands of Japan, Taiwan, Indonesia, Korea, Malaysia, United Kingdom, Thailand, India and other regions of the world. Apart from the fact that each of the families differ in terms of taste, form and color, they are all also slightly dissimilar in terms of biochemical, phytochemical and nutraceutical content (Benkeblia and Lanzotti, 2007).

2.2.4 Phytoconstituent and Chemistry of *Allium cepa* L.

Allium cepa L. is a rich source in valuable phytonutrients such as flavonoids, thio-sulphinates, fructo-oligosaccharides and other sulfur compounds such as cepaenes; S-oxides; S,S-dioxides; mono-, di-, and tri-sulfides; and sulfoxides (Singh, 2013). Flavonoids are one of the active compounds found in excessive amount. They consist of quercetin, quercetrin, kaempferol and myricetin. This quercetin and its derivatives contribute to great antioxidant activities exhibited by this plant. In addition to that, disulfides, trisulfides, cepaene, and vinyl

dithiins are also some of the phytochemicals found in *A. cepa* L. (National Onion Association, 2011).

Apart from providing flavours which tend to be one of the main purposes of consumption, *A. cepa* L. is also well-known for its remarkable medicinal qualities and nutritive values which have been appreciated greatly (Szalay, 2014). It is rich in Vitamin C, dietary fiber and folic acid. It also has high protein quality, low in sodium and is fat-free. *Allium cepa* L. also contains considerable amount of calcium, riboflavin and iron. Furthermore, it also has carotene, niacin, phosphorus and thiamine in pocket-sized quantities apart from calcium as mineral and riboflavin as vitamin (National Onion Association, 2011).

2.2.5 Previous Investigation

2.2.5.1 Antioxidant Properties

Allium cepa L. is one of the plants that possess great amount of antioxidants. It contains phytoconstituents like anthocyanins, flavonoids (quercetin) and kaempferol. These active compounds found in the plant serve as antioxidants by scavenging free radicals, chelating transition metal ions, and terminating reactive oxygen species that brings harm to the body through the development of various diseases (National Onion Association, 2011). Shutenko, et al. (1999) reported that the antioxidant activity exhibited by onions as a result of daily consumption can lead to a reduced risk of neurodegenerative disorders and cancer.

2.2.5.2 Cytotoxic Properties

According to National Onion Association (2011), proliferation of cultured ovarian, breast, and colon cancer cells were inhibited when treated with *Allium cepa* L. extracts. Richter, Ebermann and Marian (1999) reported that quercetin, an active compound found in the plant was highly effective in inhibiting the proliferation of colorectal tumour cells by inducing apoptotic cell death while sparing normal cells. Furthermore, numerous *in vitro*, epidemiological and animal studies revealed that the plant and its extracts are able to prevent gastrointestinal cancer, skin cancer and ovarian cancer (Gupta, 2014).

2.2.5.3 Antibacterial Properties

The traditional uses of onion includes the prevention, control and treatment of infectious diseases. The plant confers protection against a few Gram-negative and Gram-positive microorganisms. Presence of active compounds such as flavonoids and organosulphur compounds contributes to its antibacterial activity. According to Packia, et al. (2015), the growth of *Staphylococcus aureus* species was inhibited by various solvent extracts of *Allium cepa* L. Moreover, organosulfur compounds found in onion extracts exert antibacterial effects against oral pathogenic bacteria (Kim,1997).

2.2.6 Medicinal and Traditional Uses

The health-beneficial qualities that *Allium cepa* L. possesses makes it one of the most used therapeutic medicines almost in all the medicinal systems. The different parts of the plant contribute to the prevention and treatment of

various diseases (Singh, 2015). Due to the therapeutic and pharmacological properties exhibited by the phytonutrients found in the plant, it is often included in the Mediterranean diet to treat various diseases such as cancer, obesity, coronary heart disease, diabetes, hypertension and hypercholesterolaemia. Besides its great antioxidant activity, it has also shown applications as antihelminthic, antiarthritic, antithrombotic, hypolipidaemic and hypoglycaemic agents (Lim, 2014). Each part of the plant has special properties that contributes to its high medicinal value. The raw bulb is helpful in improving eyesight besides its gastronomic purposes to treat cases like amenorrhoea, uterine and menstrual pains. Oral intake of the plant's root helps to facilitate the expulsion of placenta (Singh, 2015). Besides, the fresh bulb-essential oil can be used for the treatment of colds and gastrointestinal infections. In addition, the fresh bulb of the plant can also be made into juice and used as anti-inflammatory agent to treat insect bites, fungal infection and bronchitis (Singh, 2013).

2.3 Extraction Process

2.3.1 Extraction of Active Compounds

The isolation of medicinally active portions of plant from the inactive components with the aid of proper solvents in standard extraction procedures is defined as extraction (Mahdi and Altikriti, 2010). The medicinally active portions actually refers to all the constituents, secondary metabolites or active components which are able to solubilise in a liquid or solvent used to produce a particular extract. Extraction of these active components from a plant material is indeed crucial as their contribution to different commercial sectors

such as pharmaceutical, food and chemical industries is highly appreciated (Azmir, et al., 2013).

2.3.2 Solvent System

Solvent extraction is a process whereby desired substances are extracted from a plant material with the aid of selective solvents. The desired compounds or active components of a plant material are usually contained inside the cells and therefore, a particular solvent or liquid used for extraction must be able to penetrate into the cell to dissolve the desired compounds (Mahdi and Altikriti, 2010). In relation to that, the choice of solvent has to be considered to optimise the extraction of active components from plants. This is because the specific nature of the bioactive compound being targeted, characteristics of the constituents being extracted, cost and environmental issues impart great influence on the selection of solvent system (Raaman, 2006). Thus, different solvents can be utilised to extract the targeted compounds. In this study, polar solvents such as ethyl acetate, methanol, ethanol and hydromethanol was used for the extraction of hydrophilic compounds and extraction of hydrophobic compounds was done using non-polar solvent, hexane.

2.3.3 Maceration Method of Extraction

The desired substances found in plants are of different polarities and possess certain unique characteristics. They may be non-polar, semi-polar, polar, thermally labile or stable. Thus, the suitability of the method of extraction has to be taken into consideration (Sasidharan, et al., 2011). One of the commonly used extraction methods for the extraction of bioactive compounds found in

plants is the maceration method. As initial step, the plant of choice has to be washed, dried and ground into powder form to increase the surface area by rendering the sample more homogenous (Solanki and Nagori, 2012). Then, the pulverised plant material is left to soak in a closed vessel containing a selective solvent for effective penetration of the solvent into the plant material to extract the active compounds. To enhance better contact with the solvent, the extraction process can be done with occasional shaking for 72 hours (3 days) at room temperature (Trusheva, Trunkova and Bankova, 2007). Decanting process followed by filtration is done in order to separate the residual plant material from the solvent. Fresh solvent can then be added to the residual plant material to ensure exhaustive extraction. Usually, the extraction process will be repeated thrice. Finally, all the filtrates collected will be subjected to evaporation with the aid of rotary vacuum evaporator in order to obtain concentrated crude extracts. The final product can be further dried in an oven to get rid of excess solvent (Sarker and Nahar, 2012).

2.4 Antioxidants

2.4.1 Role of Plants as Antioxidants

Most natural antioxidants are being discovered from plants as they possess active compounds that exhibit strong antioxidant activity. These active compounds are polyphenolics or phenolic compounds which are found in various parts of the plant. They are responsible of conferring protection to the cells from oxidative stress caused by free radicals, hence protecting the body from chronic and degenerative diseases (Brewer, 2011). Apart from scavenging free radicals and terminating oxidative damage, these naturally

occurring antioxidants found in plants are also well-known for their other health-beneficial properties such as hydrogen donors, metal chelators, singlet oxygen quenchers and reducing agents (Narayanaswamy and Balakrishnan, 2011).

2.4.2 Free Radical Formation and Its Impact on Human Body

Free radicals, reactive oxygen and nitrogen species are constantly formed as a result of normal metabolic processes that occurs in the human body and also due to environmental factors such as air pollutants or cigarette smoke (Birben, et al., 2012). These harmful substances exert damaging effects by reacting with macromolecules such as proteins, nucleic acids, lipids or DNA found in living cells. In the long run, this scenario would lead to aging, cellular and metabolic damage and the development of diseases like cancer and neurodegenerative diseases (British Nutrition Foundation, 2008).

2.4.3 Antioxidants as Free Radical Scavenger

Harmful substances such as free radicals, reactive nitrogen species and reactive oxygen species can be scavenged and eliminated from the body with the aid of antioxidants. They have diverse physiological roles in the body even at a relatively low concentration by inhibiting the process of oxidation (Kumar, 2011). Production of free radicals and antioxidants should be balanced, if there is an imbalance and system goes haywire, this leads to a complicated condition known as oxidative stress (Angeline and Narendhirakannan, 2012).

2.5 Cancer

2.5.1 Role of Plants as Cytotoxic Agents

Natural compounds from plants have been considered for the treatment of cancer and a few examples were shown in Table 2.1. Screening of crude plant extracts for their inhibitory activities against different tumour cell lines has been made and according to National Cancer Institute, approximately 180,000 plant extracts from 2500 genera of plants have been screened for their cytotoxic properties (Cordell, 1977). Plant constituents or plant-derived substances which are able to kill cancer cells or exhibit the quality of being toxic to cells is termed as being cytotoxic. There are variety of classes of plant secondary metabolites such as flavonoids, lignans, quinones and alkaloids from which cytotoxic representatives have been discovered. These plant constituents or phytochemicals possess the potential to be cytotoxic against one or more tumour cell types in culture (Colegate and Molyneux, 1993).

2.5.2 Overview of Cancer

Cancer is characterised as a collection of related diseases whereby some of the body's cells undergo unregulated and abnormal proliferation. They begin to divide limitlessly, spread into surrounding tissues and form tumours. There are basically two types of tumour, benign which is localised and malignant which invades and spreads to neighbouring tissues. Unlike benign, malignant tumours can cause serious damage and is usually life-threatening (Worldwide Cancer Research, 2015). Self-sufficiency in growth signals, avoiding cell death, sensitivity towards growth suppressors, angiogenesis induction, limitless replicative potential, invasion and metastasis are a few biological

abilities of cancer cells. These capabilities are hallmarks of cancer (Hanahan and Weinberg, 2000).

2.5.3 Worldwide Prevalence of Cancer

The International Agency for Research on Cancer has shown that as a single entity, cancer is proven to be the biggest cause of mortality worldwide. This was issued with reference to the new global cancer report compiled by UN Agency on World Cancer Day 2014 (EACR, 2014). World Health Organisation has updated that around 60% of world's total new annual cancer cases are noticed in regions like Asia, Central and South America and Africa. Records also show that 70% of the world's cancer deaths are being reported frequently from those regions. Besides, these cases are expected to increase within the next two decades from 14 to 22 million in 2012 (WHO, 2015a).

2.5.4 Prevalence of Cancer in Malaysia

According to National Cancer Society Malaysia (2015), cancer is becoming a leading cause of death where approximately 90,000 to 100,000 people in Malaysia are suffering from cancer at any one time. One in four Malaysians are estimated to develop cancer by the age of 75. In children, the occurrence of cancer is less than 10%. However, 50% of men and 35% of women are estimated to develop cancer by the age of 50 and above, with a slightly higher prevalence in females (Urban Health, 2014). Breast, colorectal, cervical, lung and nasopharyngeal cancer are among the five most common cancers which affect both males and females in Malaysia. On the other hand, leukaemia or

blood cancer is the most common cancer which affects Malaysian children (National Cancer Society Malaysia, 2015).

Table 2.1: Examples of plants used in cancer research (Chavan, et al., 2013).

Plant	Active constituent	Class
<i>Catharanthus roseus</i>	vinblastine, vincristine, alstonine, ajmalicine and reserpine.	alkaloids
<i>Solanum nigrum</i>	solamargine, solasonine, solanin and quercetin	alkaloids, flavonoids
<i>Morinda citrifolia</i>	damnacanthal, alizarin, morindone and anthragallo-2,3-dimethyl ether, rubiadin-methyl ether	anthraquinones

2.6 Infectious Diseases

2.6.1 Role of Plants as Antimicrobial Agents

Due to the phenomenon of drug-resistance and the elevating number of drug-resistant pathogens, pharmaceutical and scientific communities are progressing towards studies which focus on the potential of plant-derived substances as antimicrobial agents (Savoia, 2012). A few examples were shown in Table 2.2. They are being utilised in traditional systems of medicine and considered as useful resources for the treatment of different infections in several communities of the developing world. Scientific studies and research centres throughout the world have discovered literally thousands of secondary metabolites or phytochemicals such as flavonoids, tannins, alkaloids and terpenoids (Cowan, 1999). These active compounds found in plants are proven to possess inhibitory effects on different types of microorganisms (Orth, et al., 2006).

2.6.2 Overview of Infectious Diseases

Pathogenic microorganisms such as bacteria, parasites, fungi and viruses give rise to many forms of infectious diseases that are detrimental to health (WHO, 2015b). According to Doughari and Manzara (2008), around 50,000 deaths are reported due to infectious diseases all over the world every year. There are various modes of transmission of these diseases. They can be transmitted from one person to another, acquired through ingestion of contaminated food or drinks and also through zoonotic transmission. Since our immune system acts as a protective barrier, most of the infectious agents do not harm our body. However, some are capable of breaching the immune system and evading all the defense mechanisms (Mayo Clinic, 2014).

2.6.3 Worldwide Prevalence of Infectious Diseases

The main cause of death worldwide in children and adolescents is due to infectious diseases (Smart Global Health, 2015). Besides, it is one of the leading causes of death in adults and falls in the category of top ten causes of death. Annually, 16% of all deaths are reported due to infectious diseases. For instance, tuberculosis (TB) is the greatest killer worldwide in 2013, in which about 9 million people suffered from TB and around 1.5 million died as a result of the disease (WHO, 2015c).

2.6.4 Prevalence of Infectious Diseases in Malaysia

Infectious diseases are emerging in Malaysia too, apart from the high prevalence in other regions of the world. According to the statistics of various types of infectious diseases reported in Malaysia in the year 2013,

tuberculosis, dengue and hand-foot-and-mouth diseases were reported to be high in incidence with a rate of 78.28, 143.27 and 78.52 per 1,000 live births respectively. However, highest rate of mortality was reported in the case of tuberculosis (Smart Global Health, 2015).

Table 2.2: Examples of plants with promising anti-infective activity (Iwu, Duncan and Okunji, 1999).

Plant	Active compound	Properties
<i>Origanum vulgare</i>	thymol, carvacrol, p-cymene and terpinene.	antibacterial, antifungal and antiparasitic
<i>Garcinia kola</i>	biflavonoids, xanthones and benzophenones	antiparasitic, antimicrobial and antiviral
<i>Cryptolepis sanguinolenta</i>	indo quinoline alkaloids	antibacterial and antiparasitic

2.7 Assays

2.7.1 Antioxidant Screening

2.7.1.1 DPPH Free Radical Scavenging Assay

Plants, green leafy vegetables and fruits possess a wide variety of bioactive, non-nutritive compounds known as phytochemicals. Beyond basic nutrition, these compounds impart a lot of health benefits. Some of these bioactive compounds found in natural products act as antioxidants or free radical scavengers that neutralise free radical species generated as a result of cellular metabolism in our body (Sivakanesan and Mathiventhan, 2013). Antioxidants are crucial substances, which has the ability to prevent deleterious effects and offer protection against free radical-induced oxidative stress (Doudach, et al., 2013).

A rapid, simple and inexpensive method used for the assessment of radical scavenging activity is the DPPH assay or 2,2-Diphenyl-1-picrylhydrazyl radical scavenging capacity assay. DPPH is one of the stable and commercially available organic nitrogen radicals (Huang, Ou and Prior, 2005). It is widely used to determine the capability of test compounds, for instance plant extracts to act as free radical scavenger or hydrogen donors, and thus, evaluating their antioxidant activity (Doudach, et al., 2013). This assay is judged on the basis of reducing ability of antioxidants toward DPPH, and the antioxidant activity can then be determined by measuring the decrease of its absorbance (Prior, Wu and Schaich, 2005).

Brand-Williams and co-workers first reported this widely used decolouration assay. DPPH radical bears a deep purple colour due to the virtue of the delocalisation of the spare electron over the molecule as a whole, thus causing the molecules not to dimerise. The loss of the deep purple colour of DPPH to pale yellow and eventually colourless upon reacting with test compounds is monitored and measured spectrophotometrically at 517 nm (Padmaja, Sravanthi and Hemalatha, 2011). Ascorbic acid is commonly used as the standard or reference material.

2.7.1.2 Iron Chelating Assay

There are other pharmacological properties that are underexplored in plants besides the common antioxidant properties. One of it is the iron chelating activity or the ability to chelate ferrous ions (Chai, et al., 2014). Transition elements like iron and copper are able to act as free radicals and strong

catalysts of oxidation reactions (Patel, 2013). This is due to the presence of one or more unpaired electrons. Besides, excessive iron is not good and may lead to iron-mediated oxidative stress, which in turn increases the risk of diabetes, stroke, cancer and neurodegenerative diseases. Since plant-derived phenolic compounds act as antioxidants and exhibit potential to chelate metal ions like iron, they are being studied and used as therapeutic agents (Mohan, et al., 2012).

Ferrozine or 1,10-phenanthroline are ferriin compounds and they can form stable magenta-coloured complexes with ferrous ion, Fe^{2+} . However, the complex formation is disrupted and there is a gradual decrease in the colour of complex in the presence of other chelating agents (Robu, et al., 2012). This ferrous ion chelating assay studies the ability of plants to act as metal chelators, especially iron and detects the presence of chelating agents in plants. EDTA is used as standard and crude extracts are assessed for their ability to act as metal chelators. Estimation of the metal chelating activity is done via measurement of colour reduction of the complex spectrophotometrically at 526 nm (Demirtas, et al., 2013).

2.7.1.3 Folin-Ciocalteu Reagent Test

Phenolic compounds such as flavonoids, saponins, alkaloids and polyphenols are commonly found in vegetables, fruits and other plant-based materials. These compounds are the most widely occurring secondary metabolites in the plant kingdom and one of the major groups contributing to the antioxidant activity (Othman, et al., 2011).

Phenolic compounds are present at varying amount in plants and thus, quantitative determination of these compounds can be done by performing the Folin-Ciocalteu Reagent Test. It is based on the reduction of phosphotungstic-phosphomolybolic reagent which happens in the presence of a slightly alkaline medium (Linskens and Jackson, 1999). This assay has been evaluated as the best among other approaches in determining the total phenolic content of plant extracts due to its rapid and routine laboratory use, low-cost and easy to perform nature. Gallic acid, prepared at different concentrations, is often used as standard and the total phenolic content of the extracts are expressed as gallic acid equivalents with reference to the standard calibration curve (Blainski, Lopes and Mello, 2013). The changes in colour of the Folin-Ciocalteu reagent from yellow to blue after the addition of plant extracts containing reducing compounds such as polyphenols is observable and this colourimetric reaction is measured spectrophotometrically at 765 nm (Spangenberg, Poole and Weins, 2011).

2.7.1.4 Aluminium Chloride Colourimetric Method

Bioactive compounds found in plants serve as antioxidants. These compounds can act as free radicals and reactive oxygen species scavengers and provide health beneficial effects (Khan, et al., 2012). Flavonoids, one of the secondary metabolites or phytochemical constituents found in plants, are said to be potent antioxidants. Due to their great influence on human health in combating diseases, flavonoids have aroused considerable attention and gained importance lately (Kiranmai, Kumar and Ibrahim, 2011).

Standard methods to test the presence of phytochemical constituents like flavonoids can be done by performing Shinoda's test and alkaline reagent test, therefore determining the qualitative measurement of total flavonoid content (Lallianrawna, et al., 2013). Besides, quantitative measurement are also being applied to quantify the total flavonoid content. One of the most commonly used method for the determination of total flavonoid content is the Aluminium Chloride Colourimetric Method (Pekal and Pyrzynska, 2014).

It is a spectrophotometric assay which involves the colourimetric measurement of aluminium complex formation. Basically, the addition of aluminium chloride results in the formation of acid-stable complexes and acid-labile complexes. The acid-stable complexes are formed with the C-4 keto group and either the C-3 or C-5 hydroxyl group of flavones and flavonols. On the other hand, the acid-labile complexes are formed with the ortho-dihydroxyl groups in the A- or B-ring of flavonoids (Kiranmai, Kumar and Ibrahim, 2011). Quercetin is often used as standard, whereby a standard calibration curve of quercetin is generated and the total flavonoid content of a plant extract is expressed as quercetin equivalent (Lallianrawna, et al., 2013).

2.7.2 Cytotoxicity Screening

2.7.2.1 MTT Assay

Studies related to proliferation and cell viability have been mushrooming over the past few years. One of the simplest, convenient and safest assays used to determine the metabolic activity of viable cells is the MTT assay and it was developed by Mossman (BioTek, 2009). MTT or chemically known as 3-(4,5-

dimethylthiazol-2-yl)-2,5-dipheyltetrazolium bromide is a standard colourimetric laboratory test which is used for the analysis of cell viability. Apart from that, this assay is also used to assess the cytotoxic properties of several potential toxic and medical agents against various cancer cell lines (Talupula, 2011).

This assay studies the ability of viable cells to reduce MTT. With the aid of active mitochondrial dehydrogenase enzymes present only in viable cells, the soluble, yellow tetrazolium salt or MTT is cleaved into insoluble, purple formazan crystals. The mitochondria-dependent reaction provides an indication of mitochondrial integrity and activity and hence, this in turn may be assessed as a measure of cell viability and proliferation (Beena, Purnima and Kokilavani, 2011). The insoluble purple formazan crystals are readily detected and quantified by a simple colourimetric method. It involves the measurement of absorbance which is done at a wavelength in between 500 nm to 600 nm using a spectrophotometer (Wallert and Provost Lab, 2007).

2.7.3 Antibacterial Screening

2.7.3.1 Broth Microdilution Method

Despite the evolving technology in the development of antimicrobial chemotherapy and supportive care, antibiotic resistance and insensitivity has turned out to be one of the crucial problems in the treatment of infectious diseases. Since natural products like plants possess active compounds that are capable of working in tandem with the available antibiotics and able to exert antibacterial effect against multiple drug-resistant bacteria, they are being

widely studied and utilised in the management of bacterial infections (Iqbal, et al., 2013). Hence, antibacterial assays serve as an ultimate tool to investigate and screen the myriad of compounds in plants. Natural products like plants and crude extracts can be screened for their antibacterial properties and inhibitory effects against various microorganisms (Bailey, 2013).

One of the simplest, inexpensive, and easy-to-perform antibacterial assays is the microtiter broth dilution method. It involves the usage of 96-well plate whereby each well has 100 µl broth followed by the addition of test material in serially descending concentrations as a result of two-fold serial dilutions (Gahlaut and Chhillar, 2013). Then, equal amounts of freshly prepared bacterial suspensions which meet the 0.5 McFarland standard (absorbance value of 0.08 to 0.1) will be added into all the wells. After a day of incubation in the 37°C incubator, 20 µl of INT (p-iodonitrotetrazolium violet) reagent is added into all the wells to indicate bacterial growth. Colour changes will be noted after incubation at 37°C for about 30 minutes. INT is a tetrazolium dye precursor that is often used as a growth indicator (Valgas, et al., 2007).

The quantitative estimation of susceptibility of microorganism to a plant crude extract is done by referring to the MIC (Minimal Inhibitory Concentration). It is defined as the lowest concentration required to inhibit bacterial growth (Kaur and Mondal, 2014). On the other hand, MBC (Minimum Bactericidal Concentration) is done as a confirmatory test to determine whether a plant extract is considered bacteriostatic or bactericidal. It is defined as the concentration at which no bacterial growth is observed. The test is performed

by loading 5 μ l of the well content from which MIC value has been determined onto a Mueller-Hinton agar (Ismail, et al., 2012).

2.8 Samples

2.8.1 Cell Line

2.8.1.1 Human Chronic Myelogenous Leukaemia (K562)

K562 is a human chronic myelogenous leukaemia cell line, which was derived from a 53 year old female patient who was suffering from terminal blast crisis of chronic myeloid leukaemia. It was established by Lozzio from pleural effusion of the particular patient (ATCC, 2014). Due to its unique properties, this cell line is being experimented in various fields or scopes such as haemoglobin synthesis, antitumour testing, differentiation, pharmacodynamics, cytotoxicity, cell biology, cellular effects of hyperthermia, cloning and natural killer assays (Abcam, 1998). The cell line exists in suspension form. As shown in Figure 2.2, the cells do not adhere to the surface of the flask, they are round and circular in shape and exhibit much less clumping (ATCC, 2014). The morphology of the cells bear some resemblance to lymphoblasts (Klein, et al., 1976).

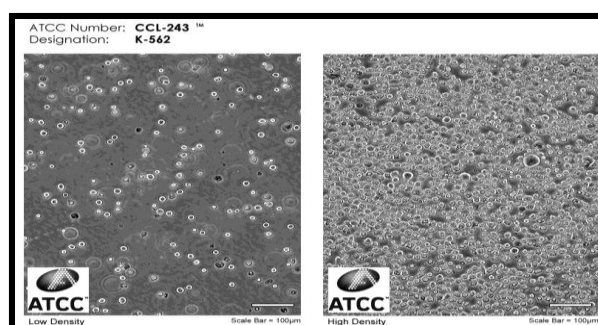


Figure 2.2: K562 cells at both low and high confluency (Adapted from ATCC, 2014).

2.8.2 Test Microorganisms

2.8.2.1 *Staphylococcus aureus*

Staphylococcus aureus is a Gram-positive bacteria. It bears coccus shape, exists in microscopic clusters resembling grapes which forms fairly large yellow colonies on a rich medium and is often haemolytic on blood agar. The microorganism is a vigorous catalase-producer, produces enzyme coagulase and is oxidase-negative (Todar, 2008b). Most of the time, *S. aureus* does not cause any harm. However, it can cause various infections and these infections can be fatal in healthcare settings. For example, *S. aureus* causes bacteraemia (sepsis), pneumonia, endocarditis which can lead to heart failure or stroke and osteomyelitis (Centers for Disease Control and Prevention, 2011).

2.8.2.2 *Enterococcus faecalis*

Enterococcus faecalis is a Gram-positive, spherical, non-motile bacterium. It can exist either singly, in pairs, or in short chains (Glick, 2005). This microorganism is a catalase-negative bacterium. The end product, which is the fermentation of glucose with L-lactic acid can be used to distinguish this species from other catalase-negative Gram-positive cocci. *Enterococcus faecalis* is a third-ranked nosocomial pathogen which causes nosocomial infections. It is able to cause clinical infections which include bacteraemia (sepsis), endocarditis, meningitis, infection in the urinary tract and peritonitis (Murray, Rosenthal and Pfaller, 2013).

2.8.2.3 *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is a Gram-negative, free-living aerobic rod-shaped bacteria that is commonly found in soil and water. It is often referred as 'blue pus' due to the production of pigment such as pyocyanin which gives out the blue colour and pyoverdine which fluoresces. It also causes haemolysis on blood agar and is an oxidase-positive microorganism which enables it to be differentiated from other *Enterobacteriaceae* (Todar, 2008a). Hospitalised patient and/or people with weakened immune systems are prone to *Pseudomonas* infections since it's an opportunistic pathogen. Pneumonia, infection in the blood (sepsis), infections following surgery (through the use of catheters) and severe burns can lead to severe illness and is highly fatal (Centers for Disease Control and Prevention, 2013).

2.8.2.4 *Escherichia coli*

Escherichia coli is a Gram-negative bacteria. It bears shape like rods or bacilli that exist either singly or in pairs. It is a facultative-anaerobic microorganism which possesses metabolism that is both fermentative and respiratory. It is non-motile or sometimes can be motile with the aid of peritrichous flagella. The microorganism produces dry and pinkish colonies on MacConkey agar. Besides, it is catalase-positive, indole-positive, oxidase-negative and citrate-negative (Huang, Chang and Chang, 2001). *Escherichia coli* is one of the important causes of common bacterial infections. For instance, urinary tract infection (UTI), bacteraemia, traveler's diarrhoea and other clinical infections such as pneumonia, neonatal meningitis, cholangitis and cholecystitis (Madappa, 1994).

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
1,10-phenanthroline powder	Merck, Germany
2,2-diphenyl-1-picrylhydrazyl (DPPH) powder	Calbiochem, United States
3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) powder	Bio Basic Inc., Canada
Aluminium (III) Chloride, AlCl ₃ powder	Sigma Aldrich, China
Ampicillin	Sigma Aldrich, China
Anhydrous sodium carbonate	Rdeh Laboratory, Malaysia
Ascorbic acid powder	R&M, United Kingdom
Cisplatin	Calbiochem, United States
Dimethyl sulfoxide (DMSO)	Fisher Scientific, UK
EDTA powder	R&M Marketing, UK
Ethanol	IramaCanggih, Malaysia
Ethyl acetate	Labmart, Malaysia
Foetal Bovine Serum (FBS)	JR Scientific Inc.
Folin-Ciocalteu reagent	Biobasic, Canada
Gallic acid powder	Biobasic, Canada
Hexane	Labmart, Malaysia
Iodonitrotetrazolium chloride, (INT) powder	Sigma, USA
Iron (II) chloride, FeCl ₂ powder	Bendosen Laboratory
Methanol	QreC®, Thailand
Mueller-Hinton agar	Media Laboratories, India
Mueller-Hinton broth	Scharlau, Spain
Penicillin G	Bio Basic Inc., Canada
Potassium Acetate powder	DAEJUNG, Korea
Quercetin powder	ACROS Organics, USA
Roswell Park Memorial Institute Medium (RPMI 1640)	Biowest, USA
Tetracycline	Bio Basic Inc., Canada

3.1.2 Labwares and Equipments

Table 3.2: List of labwares and equipments used throughout the research.

Labwares/Equipments	Company, Country
5% CO ₂ humidified incubator (37°C)	Binder, Germany
Autoclave machine	Hiramaya, Japan
Centrifuge machine	Sigma, United States
Drying incubator	Binder, Germany
Drying oven	Memmert, Germany
Electronic Balance	Kern ABJ, Australia
Freezer	Pensonic, Malaysia
Haemocytometer	Hecht-Assistant, Germany
Incubator (37°C)	Memmert, Germany
Inverted phase contrast microscope	Olympus, United States
Laboratory blender	Waring Laboratory, USA
Laminar flow hood (cell culture)	Edamix series, Germany
Laminar flow cabinet (microbiology)	Basic AS2243.8, Edamix series
Microplate reader	BMG Labtech, Germany
Orbital shaker	Yellowdine, OS 5 Basic
Refrigerator (4°C)	Toshiba, Japan
Rotary vacuum evaporator	Buchi, Switzerland
Sonicator	Branson
Vortex	VELP® Scientifica

3.2 Methods

3.2.1 Preparation of Crude Extract

3.2.1.1 Collection and Drying of Plant Material

Eight kilograms of fresh *Allium cepa* L. were purchased from Buntong market, Ipoh in the month of January, 2015. They were washed under running tap water, oven-dried at 40°C and then blended into powder form (Masibo and He, 2009).

3.2.1.2 Plant Extraction

One kilogram of the powder was divided equally and soaked in different solvents of varying polarity which were hexane, ethyl acetate, methanol, ethanol and hydromethanol (modified from Bharti, 2013). The solvent-soaked

plant materials were placed in an orbital shaker set at 150 rpm under room temperature for three consecutive days. The extracts were then filtered using gauze and filter paper with the aid of a filter funnel. Sufficient amount of filtrates were collected and a rotary vacuum evaporator was used to evaporate the filtrates (modified from Fidrianny, Rahmiyani and Wirasutisna, 2013). As a result of evaporation, concentrated crude extracts were obtained. They were stored in screw cap 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 Solution and Test Samples

Four milligrams of crude extract was dissolved in 4 ml of methanol to obtain a stock concentration of 1 mg/ml. The solution was mixed properly using a vortex. Then, test samples of different concentrations (7.5, 15, 30, 60, 120 and 240 µg/ml) were prepared by performing serial dilutions.

3.2.2.3 Preparation of DPPH Solution

Preparation was done one 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 mixed using a vortex and covered with aluminium foil.

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 carried out in 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 (Aksoy, et al., 2013). 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 (Rohman, et al., 2010):

$$\text{DPPH free radical scavenging activity (\%)} = 1 - (A_s / A_c) \times 100$$

where A_s = Absorbance of sample

A_c = Absorbance of control

3.2.3 Determination of Metal Chelating Properties

3.2.3.1 Iron Chelating Activity

3.2.3.2 Preparation of Iron (II) Chloride, FeCl₂

Approximately 0.6488 mg of iron (II) chloride powder was dissolved in 20 ml of methanol. The solution was mixed properly, covered with aluminium foil and stored at room temperature until future usage.

3.2.3.3 Preparation of 0.05% 1,10-phenanthroline

One milligram of 1,10-phenanthroline was dissolved in 20 ml of methanol. The solution was mixed properly, covered with aluminium foil and stored at room temperature until future usage.

3.2.3.4 Preparation of EDTA (Positive Control)

Five milligrams of EDTA powder was dissolved in 5 ml of methanol. The solution was mixed properly, covered with aluminium foil and stored at room temperature until future usage.

3.2.3.5 Preparation of Stock Solution and Substock Solution

Ten milligrams of crude extract was dissolved in 1 ml of methanol. The solution was mixed properly, covered with aluminium foil and stored at room temperature until future usage. Substock solution with a concentration of 1 mg/ml was freshly prepared from the stock solution prior usage.

3.2.3.6 Iron Chelating Assay

This assay was carried out in a 96-well plate. Firstly, 200 µl of substock solution was added into the first well and the subsequent wells were filled with 100 µl of methanol. Serial dilution was performed from the first well until the last well. Then, 100 µl of 1,10-phenanthroline was added into each well followed by 100 µl of FeCl₂. The plate was incubated at room temperature for 10 minutes. Finally, the spectrophotometric measurement was made using a microplate reader at a wavelength of 562 nm (modified from Shajiselvin and Muthu, 2011). Steps mentioned above were repeated for EDTA and as for negative control, the extracts were replaced with methanol. This assay was performed in triplicate. The iron chelating activity (%) of the extracts and EDTA was calculated based on the following formula (Rohman, et al., 2010):

$$\text{Iron chelating activity (\%)} = 1 - (A_s / A_c) \times 100$$

where A_s = Absorbance of sample

A_c = Absorbance of control

3.2.4 Determination of Total Phenolic Content

3.2.4.1 Folin-Ciocalteu Reagent Test

3.2.4.2 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 at room temperature until future usage.

3.2.4.3 Preparation of Sodium Carbonate Solution, Na₂CO₃

Four grams of sodium carbonate powder was dissolved in 20 ml of deionised water. The solution was properly mixed until the powder dissolve completely (modified from Raj, et al., 2005).

3.2.4.4 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 (Raj, et al., 2005).

3.2.4.5 Preparation of Stock and Standard Solution of Gallic Acid

Total 5 mg of gallic acid powder was dissolved in 1 ml of methanol and 9 ml of deionised water. The solution was mixed properly and covered with aluminium foil. The stock solution was used to prepare different concentrations (20, 40, 60, 80 and 100 µg/ml) of gallic acid standard solutions using deionised water.

3.2.4.6 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 Na₂CO₃ 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 (Padmaja, Sravanthi and Hemalatha, 2011). 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.

3.2.5 Determination of Total Flavonoid Content

3.2.5.1 Aluminium Chloride Colourimetric Method

3.2.5.2 Preparation of Quercetin

One hundred milligram 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 (modified from Pallab, et al., 2013).

3.2.5.3 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 (modified from Pallab, et al., 2013).

3.2.5.4 Preparation of 1% Aluminium (III) Chloride, AlCl_3

Approximately 10 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 (modified from Pallab, et al., 2013).

3.2.5.5 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.

3.2.5.6 Aluminium Chloride Colourimetric Method

Quercetin were 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% AlCl₃ 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 (Pallab, et al., 2013). 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).

3.2.6 Cell Culture

3.2.6.1 Complete Growth Medium Preparation

A volume of 45 ml of RPMI medium was supplemented with 5 ml of Foetal Bovine Serum (FBS) to make a total volume of 50 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.

3.2.6.2 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 14 ml of complete growth medium was added into a 75cm³ culture flask by using a 25 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% CO₂ humidified incubator at a temperature of 37°C. After that, the cell suspension was transferred into a 15 ml falcon tube. It was sealed properly with parafilm and centrifuged at a speed of 1000 rpm. The centrifugation process was carried out 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 14 ml of complete growth medium was added into a new 75 cm³ culture flask and finally, 1 ml of the cell suspension was transferred into the flask and it was incubated in 5% CO₂ humidified incubator at a temperature of 37°C (modified from Thompson, Kunkel and Ehrhardt, 2014).

3.2.6.3 Subculturing Cell Line

The confluency of cells was checked from time to time through observation under the inverted phase contrast microscope. Once the cells have reached enough confluency, around 80% to 90%, subculture process was done. Firstly, the cell suspension was transferred into a 15 ml falcon tube by using a 25 ml

disposable pipette and a pipette gun. The falcon tube was sealed with parafilm and centrifuged at a speed of 1000 rpm for 10 minutes. Then, the supernatant was discarded and the pellet was resuspended gently with 1 ml of complete growth medium. A volume of 14 ml of complete growth medium was added into two new 75 cm³ culture flasks. The cell suspension was equally transferred into the two culture flasks and then incubated in 5% CO₂ humidified incubator at a temperature of 37°C to allow cell growth (modified from ATCC, 2014).

3.2.6.4 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 (modified from ATCC, 2014).

3.2.6.5 Cryopreservation of Cell Line

The K562 cells were transferred into a 50 ml falcon tube and centrifuged at 1000 rpm for 10 minutes. After centrifugation, the supernatant was discarded. The pellet was then resuspended with 8 ml of RPMI 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.

3.2.6.6 Cell Count

Cell count is essential to estimate cell number, monitor growth rates and to determine the concentration of K562 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 haemacytometer. The magnification of the inverted phase contrast microscope was adjusted to 100×. 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 (Stephenson, et al., 2012). 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 = $\frac{\text{Average number of viable cells} \times 2 \times 10^4}{10^3}$

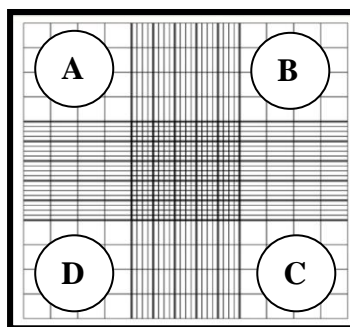


Figure 3.1: Squares labeled A, B, C and D which were used for cell count (Adapted from Grigoryev, 2013).

3.2.7 Determination of Cytotoxic Properties

3.2.7.1 MTT Assay

3.2.7.2 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 5 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 $\mu\text{g/ml}$, 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 $\mu\text{g/ml}$) were also prepared using substock solution and complete growth medium.

3.2.7.3 Preparation of MTT solution

A total of 5 mg of MTT powder was measured using a weighing machine and then added into 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.

3.2.7.4 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.

3.2.7.5 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.

3.2.7.6 Treatment of Cells with Crude Extracts and Positive Control

The 96-well plates that have been seeded with 100 μ l 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 μ g/ml) from well A to E respectively by adding 100 μ l of filtered crude extracts. The plates were incubated at different incubation periods which were, 24, 48 and 72 hours intervals. Untreated cell suspension or standard, composed of 100 μ l 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 μ l of cell suspension and 100 μ l 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 μ g/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 plates were shown in Figure 3.2 and Figure 3.3 with appropriate descriptions in Table 3.3.

3.2.7.7 MTT Assay

At the end of the incubation period (24, 48 and 72 hours respectively), 20 μ l 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% CO₂ humidified incubator. After centrifugation at 1,000 rpm for about ten minutes, the solution in the wells were discarded and the insoluble purple formazan crystals were dissolved in 200 μ l of DMSO. The plate was agitated using an orbital shaker for about fifteen minutes and finally, the absorbance

was measured spectrophotometrically at 550 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

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Figure 3.2: Layout of 96-well plate (1st set) for MTT assay.

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Figure 3.3: Layout of 96-well plate (2nd set) for MTT assay.

Table 3.3: Components and the symbol it represents.

Component	Symbol
1. Hexane	
2. Ethyl acetate	
3. Methanol	
4. Ethanol	
5. Hydromethanol	
6. Positive control (Cisplatin)	
7. Negative control (0.64% DMSO)	
8. Standard (untreated cells)	
9. Complete Growth Medium (Sterility test)	

3.2.8 Determination of Antibacterial Properties

3.2.8.1 Broth Microdilution Method

3.2.8.2 Preparation of Stock Solution and Test Samples

One hundred milligram of crude extract was dissolved in 20% DMSO. The solution was mixed using a vortex until it dissolved completely. Then, each of

the mixture was filtered using a 5 ml syringe and 0.22 µm cellulose acetate syringe filter. The filtered stock solutions were left overnight at 4°C. Test samples with a concentration of 30 mg/ml were prepared for all the extracts using stock solution and MH broth (modified from Jorgensen and Ferraro, 2009).

3.2.8.3 Preparation of Mueller-Hinton (MH) Broth

Nineteen grams of MH broth powder was dissolved in 500 ml of distilled water, then autoclaved. The autoclaved MH broth was sealed with parafilm and stored at room temperature.

3.2.8.4 Preparation of Mueller-Hinton (MH) Agar

Fourteen grams of MH agar powder was dissolved in 500 ml of distilled water, then autoclaved. The autoclaved MH agar was sealed with parafilm and stored in the 37°C incubator until future usage.

3.2.8.5 Preparation of Antibiotic (Positive Control)

The antibiotics used in this assay were tetracycline, ampicillin and penicillin G. Four milligrams of each antibiotic powder was dissolved in 1 ml of distilled water and mixed properly. Then, they were filtered using a 5 ml syringe and 0.22 µm cellulose acetate syringe filter. The filtered solutions (stock solutions) were left overnight at 4°C. Test samples with a concentration of 60 µg/ml were prepared for all antibiotics using stock solution and MH broth (modified from Jorgensen and Ferraro, 2009).

3.2.8.6 Preparation of INT Reagent

Four milligrams of INT powder was dissolved in 20 ml of distilled water to obtain a concentration of 0.2 mg/ml. The solution was mixed properly and filtered using a 5 ml syringe and 0.22 µm cellulose acetate syringe filter. The filtered solution was left overnight at 4°C (modified from Valgas, et al., 2007).

3.2.8.7 Preparation of Bacterial Suspension

Test bacterial strains, as shown in Table 3.4, were obtained from UTAR laboratory staff. Master plates were received and they were subcultured on MH agar. The agar plates were incubated in 37°C incubator for a day. Next, a number of distinct bacterial colonies were inoculated into sterile universal bottles containing 10 ml of MH broth. Then, the bottles were left in an orbital shaker for half an hour. Before using the suspensions for the assay, they were ensured to have achieved or met the 0.5 McFarland standard, which is tantamount to approximately 0.08 to 0.1 optical density (OD). The measurement of OD of the bacterial suspensions was done at 625 nm. However, the bacterial suspensions has to be freshly prepared prior usage each time if the assay is to be repeated (modified from Adeshina, et al., 2011).

Table 3.4: Tested bacterial strains.

Bacterial species	ATCC Number	Type
<i>Staphylococcus aureus</i>	25923	Gram-positive
<i>Enterococcus faecalis</i>	19433	Gram-positive
<i>Pseudomonas aeruginosa</i>	27853	Gram-negative
<i>Escherichia coli</i>	H70-20	Gram-negative

ATCC: American Type Culture Collection

3.2.8.8 Minimum Inhibitory Concentration (MIC) Assay

This assay was performed in the laminar hood to maintain sterility, using a 96-well plate. A volume of 100 μ l of MH broth was pipetted into all the wells except for row A. Then, 100 μ l of each test sample (extracts) was added into wells in row A and B. Two-fold serial dilution was performed from row B to G (treated with extracts). Finally, 100 μ l of freshly prepared bacterial suspension was added into all the wells. Row H served as negative control (untreated), whereby the wells contained MH broth diluted with 20% DMSO and bacterial suspension. As for the positive control (antibiotics), preparation of test samples and treatment was done in a similar way. The design of the plates were shown in Figure 3.4 and Figure 3.5 with appropriate descriptions in Table 3.5. The plate was then sealed properly with parafilm and incubated overnight in the 37°C incubator. The next day, 20 μ l of INT reagent was added into all the wells, followed by incubation for half an hour (modified from Adeshina, et al., 2011). At the end of the incubation period, observation on the colour changes was made and the results were recorded. All the steps mentioned above were repeated for the other bacterial strains used.

3.2.8.9 Minimum Bactericidal Concentration (MBC) Assay

Based on the MIC results, 5 μ l of the solution from the well with the lowest inhibitory concentration (which remained yellowish after the addition of INT reagent) was transferred to MH agar and then streaked using an inoculating loop. The labeled Petri dishes were sealed properly with parafilm and incubated overnight in a 37°C incubator (modified from Mahon, Lehman and Manuselis, 2014). The results were recorded the following day.




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Figure 3.4: Layout of 96-well plate (1st set) for antibacterial assay.

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B												
C												
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G												
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Figure 3.5: Layout of 96-well plate (2nd set) for antibacterial assay.

Table 3.5: Components and the symbol it represents.

Component	Symbol
1. Hexane	
2. Ethyl acetate	
3. Methanol	
4. Tetracycline	
5. Ampicillin/Penicillin G	
6. Ethanol	
7. Negative control	
8. Hydromethanol	

CHAPTER 4

RESULTS

4.1 Extraction Yield of *Allium cepa* L.

Five different solvents of varying polarity such as hexane, ethyl acetate, methanol, ethanol and hydromethanol were used for extraction and their effectiveness in extracting the active compounds responsible for the biological activity of *Allium cepa* L. were evaluated through the determination of extract yields. As shown in Table 4.1, ethanol extract of *A. cepa* L. had the highest percentage of yield followed by methanol, ethyl acetate, hydromethanol and hexane extract.

Table 4.1: Extract yields of *Allium cepa* L. using different solvents.

Extract	Weight (g)	Percentage* (w/w) (%)
Hexane	0.58	0.29
Ethyl acetate	7.96	3.98
Methanol	8.12	4.06
Ethanol	8.43	4.22
Hydromethanol	7.24	3.62

*Percentage of yield was determined based on the weight of the dried extract against 200 g dry weight of ground *Allium cepa* L.

4.2 *In vitro* Antioxidant Assays

4.2.1 DPPH Radical Scavenging Activity of *Allium cepa* L.

The antioxidant activity of the crude extracts of *Allium cepa* L. was determined via DPPH free radical scavenging assay whereby the antioxidant nature was assessed based on radical scavenging property. The results were

compared with ascorbic acid which was used as positive control. Figure 4.1 shows the DPPH free radical scavenging activity of the crude extracts of *Allium cepa* L. and ascorbic acid (standard) prepared at different concentrations. The antioxidant activity exhibited by the crude extracts varied considerably in relation to the different solvents used for extraction. A dose-dependent relationship was observed whereby the scavenging activity increased in response to increasing concentration.

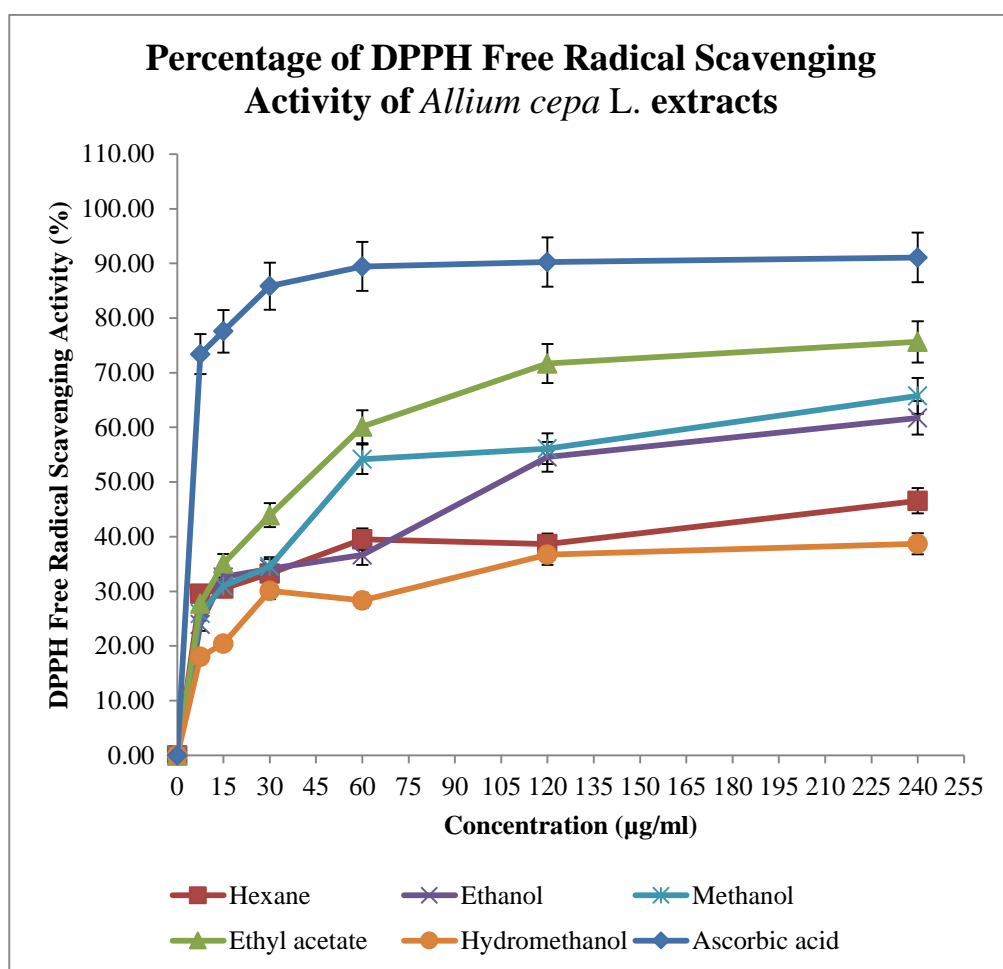


Figure 4.1: Percentage of DPPH free radical scavenging activity of ascorbic acid and crude extracts of *Allium cepa* L. (hexane, ethyl acetate, methanol, ethanol and hydromethanol) prepared at different concentrations.

The EC₅₀ values of ascorbic acid and crude extracts of *A. cepa* L. were determined based on Figure 4.1. As shown in Table 4.2, the radical scavenging activity of the crude extracts and ascorbic acid decreased in the following order: ascorbic acid > ethyl acetate > methanol > ethanol > hexane > hydromethanol.

Table 4.2: EC₅₀ values of ascorbic acid and crude extracts of *A. cepa* L. based on DPPH free radical scavenging activity.

Test Sample	EC₅₀ (µg/ml)
Ascorbic acid	5.108
Hexane extract	>240
Ethyl acetate extract	41.229
Methanol extract	53.639
Ethanol extract	104.640
Hydromethanol extract	>240

4.2.2 Iron Chelating Activity of *Allium cepa* L.

The antioxidant activity of *Allium cepa* L. extracts was also determined based on their abilities to chelate iron. The chelating activities of the crude extracts were compared with EDTA, positive control. Based on the results obtained, it was found that the chelating activity of the crude extracts varied considerably in relation to the different solvents used for extraction. Figure 4.2 shows the iron chelating activity of the crude extracts of *A. cepa* L. and EDTA at different concentrations. A dose-dependent relationship was observed whereby the chelating activities increased in response to increasing concentration of the crude extracts.

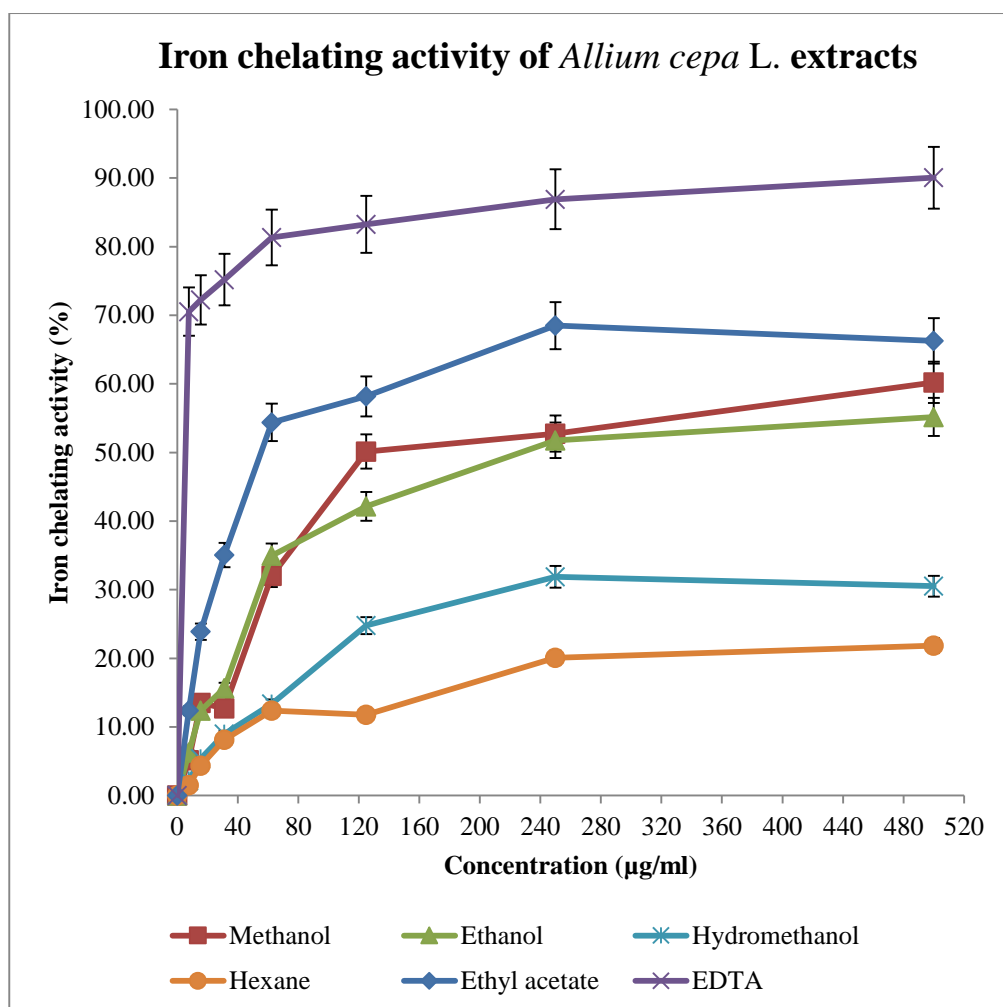


Figure 4.2: Iron chelating activity of EDTA and crude extracts of *Allium cepa* L. (hexane, ethyl acetate, methanol, ethanol and hydromethanol) prepared at different concentrations.

Based on Figure 4.2, the EC_{50} values of EDTA and crude extracts of *A. cepa* L. were determined. As shown in Table 4.3, the iron chelating activity of the crude extracts and EDTA decreased in the following order: EDTA > ethyl acetate > methanol > ethanol > hydromethanol > hexane.

Table 4.3: EC₅₀ values of EDTA and crude extracts of *A. cepa* L. based on iron chelating activity.

Test Sample	EC ₅₀ (µg/ml)
EDTA	5.539
Hexane extract	>500
Ethyl acetate extract	55.419
Methanol extract	124.552
Ethanol extract	227.025
Hydromethanol extract	>500

4.2.3 Total Phenolic Content of *Allium cepa* L.

The total phenolic content of crude extracts of *Allium cepa* L. was quantified by carrying out Folin-Ciocalteu Reagent Test which is based on spectrophotometric measurement. Gallic acid standard curve was generated through preparation of different concentrations of gallic acid and their respective absorbance values measured at 765 nm, as shown in Figure 4.3. A dosage of 1 mg/ml was set for the crude extracts. The absorbance values were measured at 765 nm. As shown in Table 4.4, the absorbance readings were expressed as mean of absorbance values measured at 765 nm ± standard deviation (SD) of the triplicate measurement.

Table 4.4: Absorbance values of crude extracts of *Allium cepa* L. (dosage of 1 mg/ml) at 765 nm.

Extract	Absorbance (765 nm)
Hexane	0.5880 ± 0.081
Ethyl acetate	1.6823 ± 0.025
Methanol	0.9953 ± 0.053
Ethanol	0.9187 ± 0.039
Hydromethanol	0.6840 ± 0.017

Total phenolic content of the crude extracts were determined based on the gallic acid standard curve. The results were expressed as μg GAE/mg of the extract using the mathematical equation obtained from the standard calibration curve.

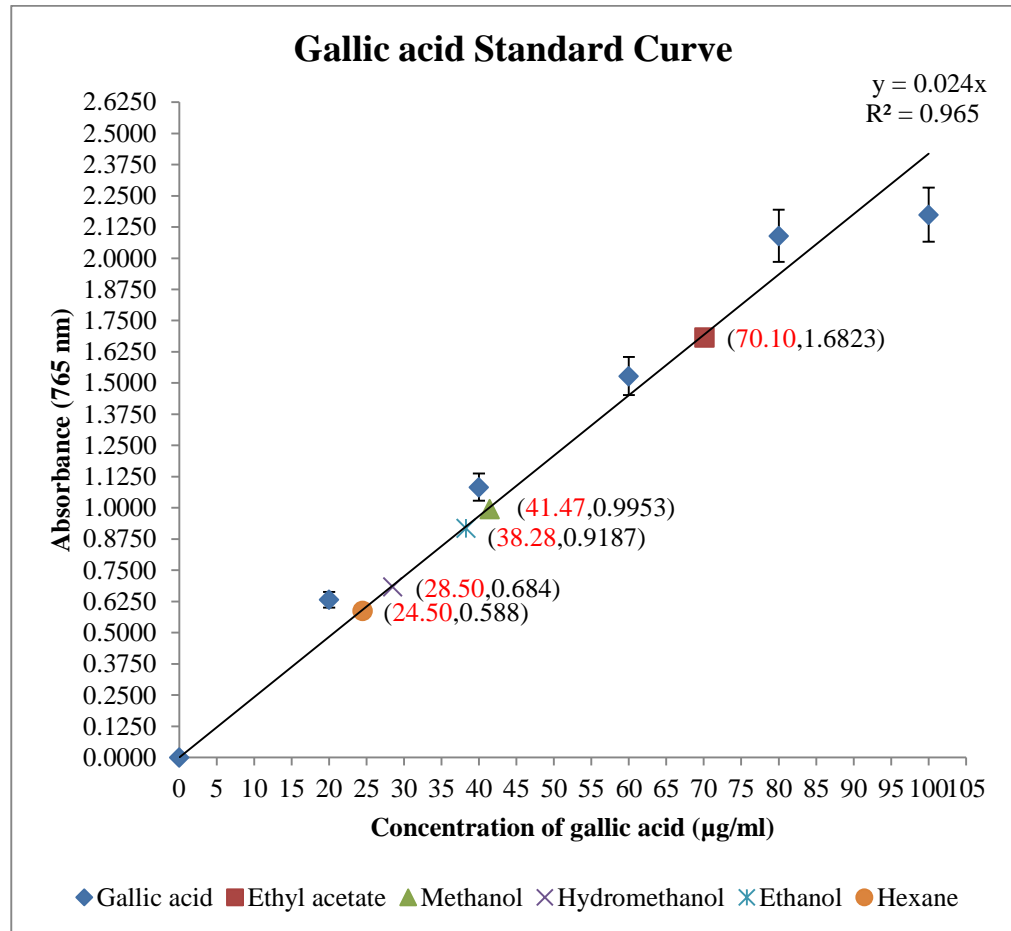


Figure 4.3: The standard calibration curve of gallic acid prepared at different concentrations and measured at 765 nm. The marked points on the standard curve represent the different extracts of *Allium cepa* L.

*The values in red represent the concentration of gallic acid present in 1 mg/ml of the crude extracts of *A. cepa* L. which were expressed as Gallic Acid Equivalent (GAE). The values in black represent the absorbance values of the crude extracts measured at 765 nm.

The quantitative analysis on the total phenolic content of the crude extracts of *Allium cepa* L was performed. As shown in Table 4.5, it was found that ethyl acetate extract was more potent in extracting phenolic compounds from *A. cepa* L. followed by methanol, ethanol, hydromethanol and lastly, hexane extract.

Table 4.5: Total Phenolic Content of the crude extracts of *Allium cepa* L. expressed as ($\mu\text{g GAE/mg}$).

Extract	Total Phenolic Content ($\mu\text{g GAE/mg}$)
Hexane	24.50
Ethyl acetate	70.10
Methanol	41.47
Ethanol	38.28
Hydromethanol	28.50

4.2.4 Total Flavonoid Content of *Allium cepa* L.

The total flavonoid content of crude extracts of *Allium cepa* L. was quantified by carrying out Aluminium Chloride Colourimetric Method. As shown in Figure 4.4, quercetin standard curve was plotted through the preparation of different concentrations of quercetin and their respective absorbance values measured at 415 nm. On the other hand, Table 4.6 demonstrates the absorbance value of crude extracts of *A. cepa* L. measured at 415 nm using a dosage of 1 mg/ml. The absorbance readings were expressed as mean of absorbance values measured at 415 nm \pm standard deviation (SD) of the triplicate measurement. This was done in order to express the results as quercetin equivalents ($\mu\text{g QE/mg sample}$).

Table 4.6: Absorbance values of crude extracts of *Allium cepa* L. (dosage of 1 mg/ml) at 415 nm.

Extract	Absorbance (415 nm)
Hexane	0.0947 ± 0.001
Ethyl acetate	0.4051 ± 0.006
Methanol	0.1140 ± 0.001
Ethanol	0.2096 ± 0.001
Hydromethanol	0.0662 ± 0.001

Total flavonoid content of the crude extracts were determined from the quercetin standard curve. The results were expressed as µg quercetin/mg of the extract using the mathematical equation obtained from the standard calibration curve.

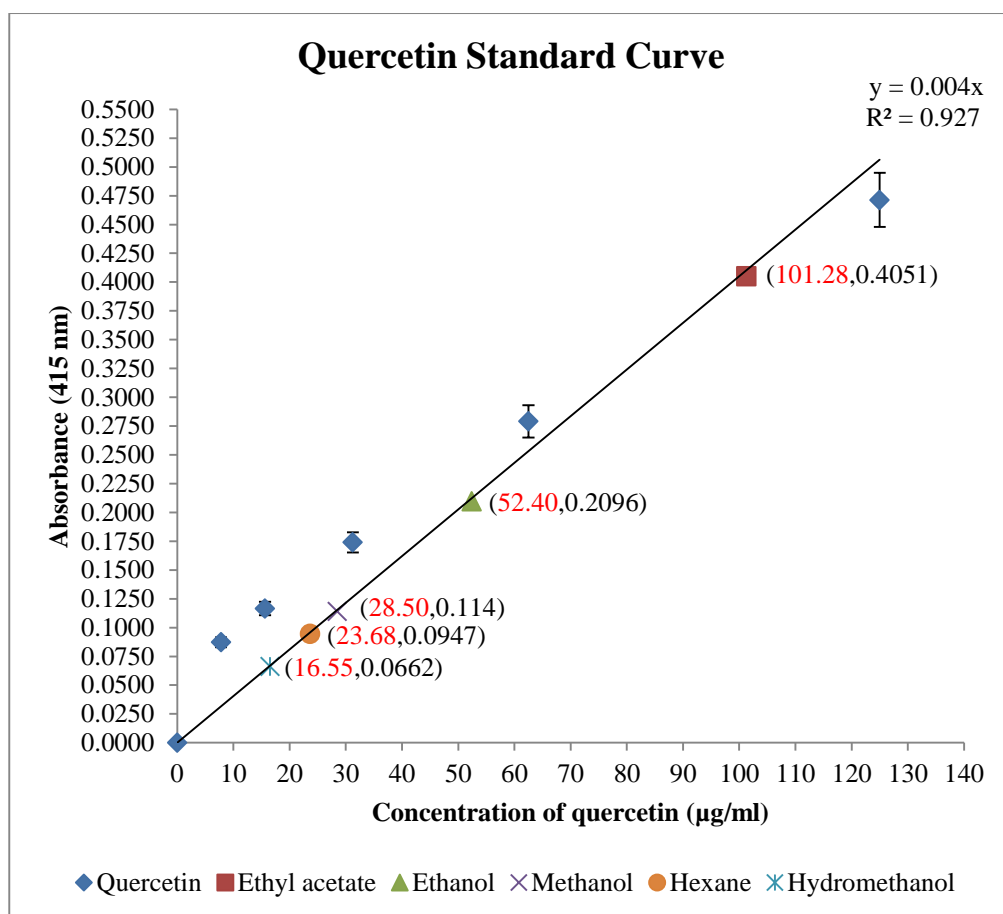


Figure 4.4: The standard calibration curve of quercetin prepared at different concentrations and measured at 415 nm. The marked points on the standard curve represent the different extracts of *Allium cepa* L.

*The values in red represent the concentration of quercetin present in 1 mg/ml of the crude extracts of *A. cepa* L. which were expressed as Quercetin Equivalent (QE). The values in black represent the absorbance values of the crude extracts measured at 415 nm.

The quantitative analysis on the total flavonoid content of different extracts of *A. cepa* L. was done and the results were shown in Table 4.7. Total flavonoid content of each extract varied and this scenario reflected the ability and effectiveness of different solvents used to extract flavonoids, one of the active compounds found excessively in *Allium cepa* L. Based on the results obtained, it was found that ethyl acetate extract was more potent in extracting flavonoids

from *A. cepa* L. followed by ethanol, methanol, hexane and hydromethanol extract.

Table 4.7: Total Flavonoid Content of the crude extracts of *Allium cepa* L. expressed as ($\mu\text{g QE/mg}$).

Extract	Total Flavonoid Content ($\mu\text{g QE/mg}$)
Hexane	23.68
Ethyl acetate	101.28
Methanol	28.50
Ethanol	52.40
Hydromethanol	16.55

4.3 *In vitro* Cytotoxicity Screening

MTT assay was performed in order to screen for the cytotoxic properties of the crude extracts of *Allium cepa* L. and also to determine the viability of K562 cells after treatment with the crude extracts. In this study, the percentage of cell viability varied considerably in relation to the different types of solvent used for extraction, concentration of crude extracts and period of incubation. The cytotoxicity screening of *A. cepa* L. crude extracts showed that the growth of K562 cells was inhibited both in a dose-dependent and time-dependent manner.

4.3.1 MTT Assay (24 hours of treatment)

Different concentrations of cisplatin (positive control) and crude extracts of *Allium cepa* L. were prepared and tested on K562 cells. The treatment was given for 24 hours and at the end of the incubation period, the percentage of cell viability was measured. Based on Figure 4.5, the IC_{50} value of each crude

extract was determined and the results were tabulated as shown in Table 4.8. The cytotoxic effect of the crude extracts and cisplatin decreased in the following order: cisplatin > ethyl acetate > methanol > ethanol > hexane > hydromethanol.

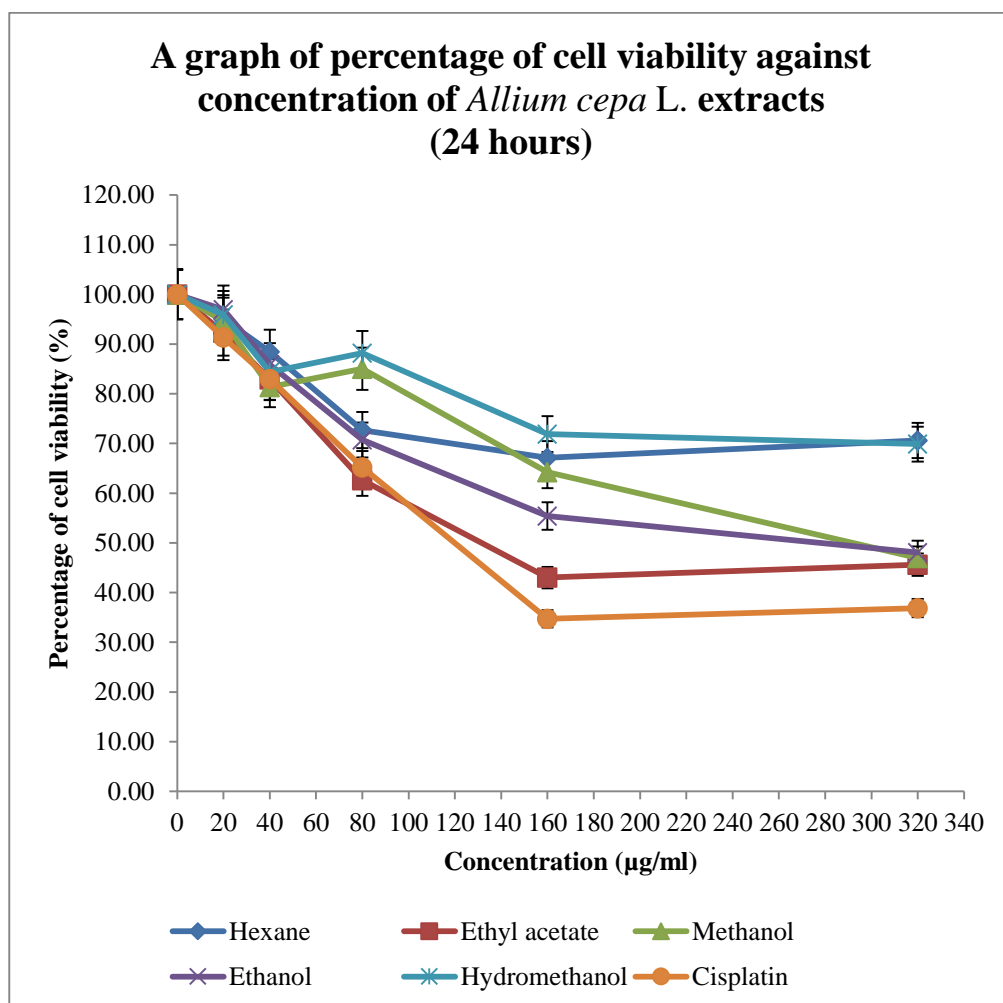


Figure 4.5: Cytotoxic effect of various concentrations (20-320 µg/ml) of *Allium cepa* L. crude extracts and cisplatin on K562 cells after 24 hours of treatment.

Table 4.8: The IC₅₀ values of cisplatin and *Allium cepa* L. crude extracts after 24 hours of treatment.

Test Sample	IC ₅₀ (µg/ml)
Cisplatin	119.93
Hexane extract	>320
Ethyl acetate extract	131.46
Methanol extract	291.67
Ethanol extract	277.83
Hydromethanol extract	>320

4.3.2 MTT Assay (48 hours of treatment)

The whole procedure was repeated whereby the K562 cells were treated with different concentrations of *Allium cepa* L. crude extracts and cisplatin. The duration of treatment was set for 48 hours. Then, percentage of cell viability was measured and the IC₅₀ value of each crude extract was determined based on Figure 4.6. The results were tabulated as shown in Table 4.9. The cytotoxic effect of the crude extracts and cisplatin (positive control) decreased in the following order: cisplatin > ethyl acetate > ethanol > methanol > hexane > hydromethanol.

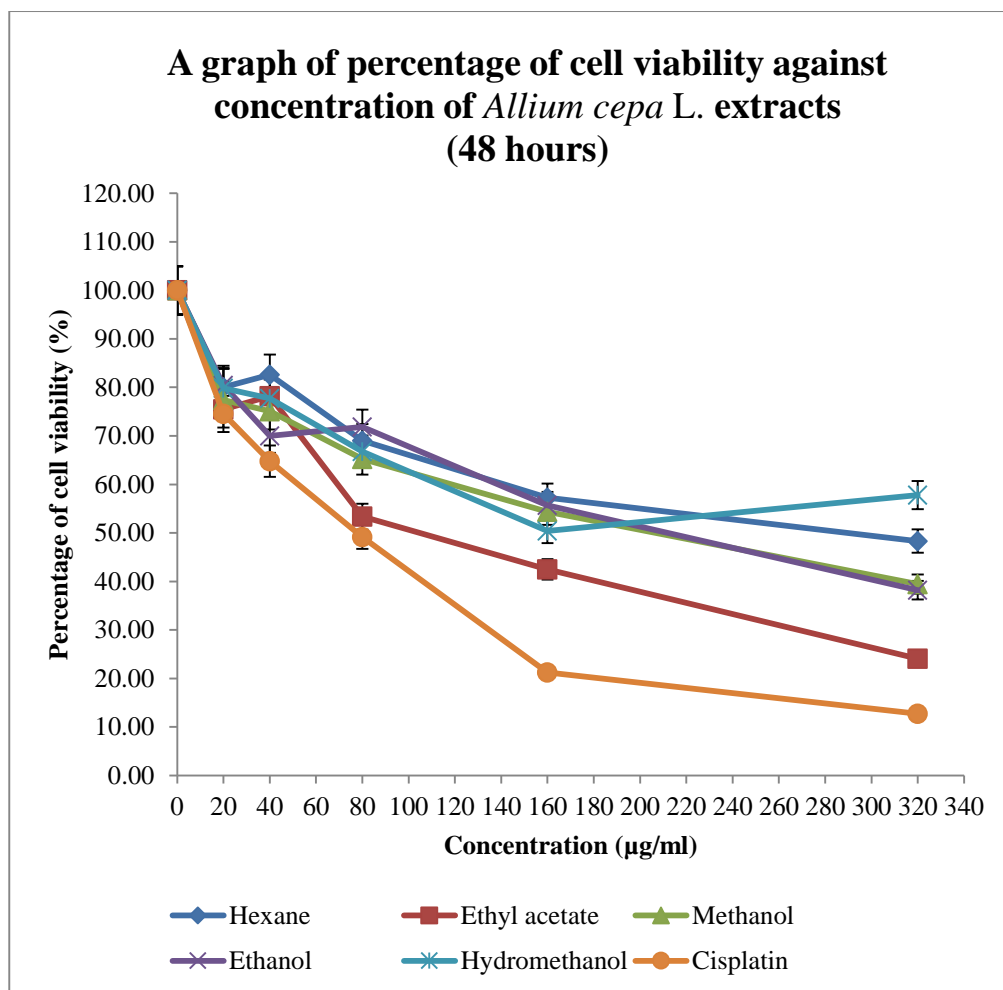


Figure 4.6: Cytotoxic effect of various concentrations (20-320 µg/ml) of *Allium cepa* L. crude extracts and cisplatin on K562 cells after 48 hours of treatment.

Table 4.9: The IC₅₀ values of cisplatin and *Allium cepa* L. crude extracts after 48 hours of treatment.

Test Sample	IC ₅₀ (µg/ml)
Cisplatin	77.90
Hexane extract	289.86
Ethyl acetate extract	104.75
Methanol extract	206.94
Ethanol extract	212.02
Hydromethanol extract	>320

4.3.3 MTT Assay (72 hours of treatment)

The whole procedure was again repeated whereby the K562 cells were treated with different concentrations of *Allium cepa* L. crude extracts and cisplatin. However, the duration of treatment was adjusted to 72 hours. Then, percentage of cell viability was measured and the IC₅₀ value of each crude extract was determined based on Figure 4.7. The results were tabulated as shown in Table 4.10. The cytotoxic effect of the crude extracts and cisplatin (positive control) decreased in the following order: cisplatin > ethyl acetate > methanol > ethanol > hexane > hexane > hydromethanol.

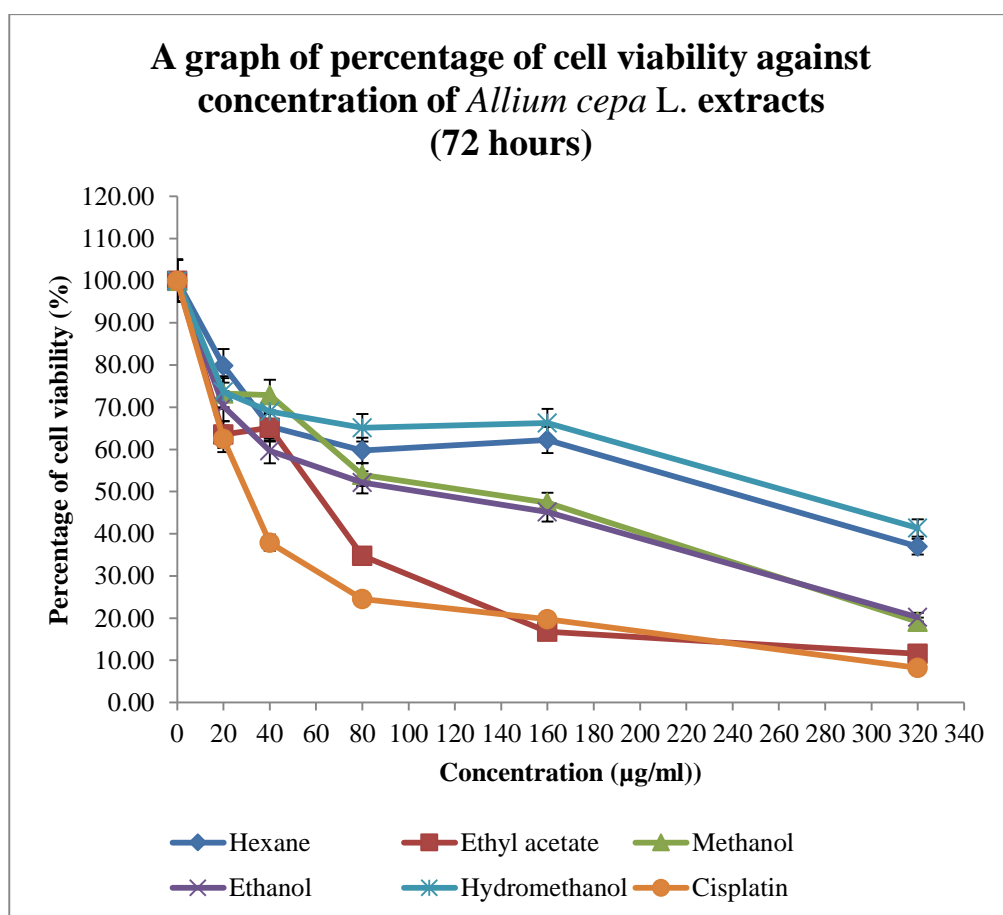


Figure 4.7: Cytotoxic effect of various concentrations (20-320 µg/ml) of *Allium cepa* L. crude extracts and cisplatin on K562 cells after 72 hours of treatment.

Table 4.10: The IC₅₀ values of cisplatin and *Allium cepa* L. crude extracts after 72 hours of treatment.

Test Sample	IC ₅₀ (µg/ml)
Cisplatin	30.16
Hexane extract	237.46
Ethyl acetate extract	59.91
Methanol extract	128.07
Ethanol extract	104.81
Hydromethanol extract	264.57

4.4 Antibacterial Assay (Broth Microdilution Method)

4.4.1 Minimum Inhibitory Concentration (MIC)

Allium cepa L. crude extracts (hexane, ethyl acetate, methanol, ethanol and hydromethanol) and antibiotics (ampicillin, penicillin G and tetracycline) were tested against two Gram-positive bacteria, *Staphylococcus aureus* and *Enterococcus faecalis*, as well as two Gram-negative bacteria, *Escherichia coli* and *Pseudomonas aeruginosa*. The MIC (mg/ml) values were shown in Table 4.11. All the four bacterial strains were susceptible to the positive control, tetracycline. Penicillin G exhibited greater inhibitory activity towards *S. aureus* and a lesser activity towards *E. faecalis*. On the other hand, ampicillin showed a lesser inhibitory activity towards both the microorganisms, *E. coli* and *P. aeruginosa*.

Ethyl acetate extract showed greater inhibitory activity against all the four bacteria that were tested when compared to other crude extracts of *Allium cepa* L. It exhibited a similar inhibitory activity towards *S. aureus* and *E. faecalis* with a MIC value of 1.875 mg/ml and also a similar inhibitory activity towards *E. coli* and *P. aeruginosa* with a MIC value of 7.5 mg/ml. Hexane extract showed similar inhibitory activity towards *S. aureus*, *P. aeruginosa* and *E. coli*

with a MIC value of 15 mg/ml and an even lower MIC value was achieved against *E. faecalis*. Apart from that, both methanol and ethanol extracts showed almost similar inhibitory activity against all the bacteria tested. As for the hydromethanol extract, higher inhibitory concentration (15 mg/ml) was needed to inhibit both *S. aureus* and *E. faecalis* and a concentration of 30 mg/ml was found inhibitory against *P. aeruginosa* and *E. coli*.

Table 4.11: Minimum inhibitory concentration (MIC) of crude extracts of *Allium cepa* L. and positive controls on four bacterial strains.

*Extracts	Bacterial strains			
	<i>Staphylococcus aureus</i>	<i>Enterococcus faecalis</i>	<i>Pseudomonas aeruginosa</i>	<i>Escherichia coli</i>
Hexane	15	7.5	15	15
Ethyl acetate	1.875	1.875	7.5	7.5
Methanol	7.5	15	7.5	15
Ethanol	7.5	7.5	7.5	15
Hydromethanol	15	15	30	30
¹ Ampicillin	-	-	0.06	0.06
¹ Tetracycline	0.00375	0.0075	0.03	0.00375
¹ Penicillin G	0.00375	0.03	-	-

Mean ± Standard deviation, n=3

*Concentration of extracts in mg/ml

¹Concentration of Ampicillin, Tetracycline and Penicillin G in mg/ml

(-): Not tested

4.4.2 Minimum Bactericidal Concentration (MBC)

Minimum bactericidal concentration (MBC) test was performed based on the results obtained for MIC test done previously. The results were reported as shown in Table 4.12. The wells which remained yellowish after the addition of INT reagent indicated that bacterial growth was inhibited. These wells were selectively chosen to carry out the MBC test. Hence, the lowest concentration

of the crude extract in which there was no any observable colony growth was taken as the MBC value. Moreover, there was no need to proceed with MBC test if any of the crude extracts did not possess minimum inhibitory concentration (MIC) value.

Based on Table 4.12, no activity was observed for ampicillin when it was tested on *Pseudomonas aeruginosa* and *Escherichia coli*. Penicillin G, on the other hand, exhibited bactericidal activity only towards *Staphylococcus aureus*. Tetracycline was the only antibiotic which was able to exhibit bactericidal activity against all the bacterial strains tested.

Ethyl acetate extract exhibited greater bactericidal activity against all the bacterial strains tested when compared to other crude extracts, just like tetracycline. Methanol and ethanol extracts exhibited almost similar activity against all the bacterial strains tested and similar MBC values were obtained for both the extracts. Hydromethanol extract exhibited bactericidal activity against *E. faecalis* and *P. aeruginosa* with MBC values of 15 mg/ml and 30 mg/ml respectively. However, no bactericidal activity was observed against *S. aureus* and *E. coli*. Apart from that, hexane extract also showed a slightly better bactericidal activity against *S. aureus*, *E. faecalis* and *P. aeruginosa* when compared to hydromethanol extract. Just like hydromethanol, hexane extract too, did not exhibit bactericidal activity against *E. coli*. This phenomenon indicates that hexane and hydromethanol extracts were being bacteriostatic towards *E. coli*.

Table 4.12: Minimum bactericidal concentration (MBC) of crude extracts of *Allium cepa* L. and positive controls on four bacterial strains.

*Extracts	Bacterial strains			
	<i>Staphylococcus aureus</i>	<i>Enterococcus faecalis</i>	<i>Pseudomonas aeruginosa</i>	<i>Escherichia coli</i>
Hexane	15	7.5	15	NA
Ethyl acetate	1.875	1.875	7.5	7.5
Methanol	7.5	15	7.5	15
Ethanol	7.5	7.5	7.5	15
Hydromethanol	NA	15	30	NA
¹ Ampicillin	-	-	NA	NA
¹ Tetracycline	0.00375	0.0075	0.03	0.00375
¹ Penicillin G	0.00375	NA	-	-

Mean± Standard deviation, n=3

*Concentration of extracts in mg/ml

¹Concentration of Ampicillin, Tetracycline and Penicillin G in mg/ml

NA: No activity

(-): Not tested

CHAPTER 5

DISCUSSION

5.1 Plant Extraction Yield

Depending on the nature of the active compounds, different solvents can be employed for the extraction of those targeted compounds. For instance, polar solvents such as methanol, ethanol or ethyl acetate can be used for the extraction of hydrophilic compounds. Extraction of hydrophobic compounds, can be done using non-polar solvents like hexane (Sasidharan, et al., 2011). Through the application of different solvent systems, the extraction yield and antioxidant activity of the extracts can be evaluated, which in turn reflects the quantitative and qualitative determination of the extracted bioactive compounds (Mohammedi and Atik, 2011).

In this study, ethanol extract of *Allium cepa* L. had the highest amount of extraction yield whereas hexane extract showed the least yield. These findings correlate with previous study done by Raman, et al. (2011), whereby a higher extract yield of *A. cepa* L. was obtained using ethanol when compared to hexane. Ethyl acetate, methanol and hydromethanol extracts obtained almost similar percentage of yields. The efficacy to extract phenolic compounds from *A. cepa* L. using ethyl acetate, methanol and ethanol have been demonstrated in other studies as well (Sultana, Anwar and Ashraf, 2009). Hence, we can say that *A. cepa* L. crude extracts contain mostly polar compounds.

Apart from choice of solvents, solvent ratio and sample extraction, the temperature at which extraction is performed, particle size of sample and the chemical composition of the sample can also influence the percentage of yield of plant extracts (Huda, et al., 2009). Moreover, the physical property of the test samples has a great influence on the yield of plant extracts. According to Choi, et al. (2006), dried leaves have greater percentage of yield than freshly used leaves. This is because drying process ruptures the cell wall and hence, increase the rate of diffusion of plant active components into the solvent. However, the temperature at which drying is done poses a great threat whereby exposure at high temperatures can result in loss of plant active components that are heat-labile (Yi and Wetzstein, 2011). In this study, the plant was dried and pulverised into powder. Powdered form gives a higher percentage and extraction yield due to the increase in surface area to volume ratio and the surface area that is subjected to the solvent (Ahmad, et al., 2009).

5.2 Antioxidant Assays

5.2.1 Analysis of DPPH Free Radical Scavenging Activity

Presence of free radicals may lead to biological damages if the balance between its production and antioxidants in the body is disrupted. Antioxidants are able to counteract the harmful effects caused by free radicals by scavenging them. The present analysis determines the antioxidant potential of crude extracts of *Allium cepa* L. through DPPH assay. The antioxidant nature was evaluated based on DPPH free radical scavenging activity, which in turn reflects the ability of the crude extracts to act as antioxidants.

According to Sultana, Anwar and Ashraf (2009), the amount of antioxidant components that can be effectively extracted from a plant material is dependent of the effectiveness of the extraction solvent used. In this study, ethyl acetate extract was found to exhibit highest scavenging activity whereas hexane extract displayed the lowest scavenging activity. Extraction using polar solvents exhibited better scavenging properties or antioxidant activity when compared to non-polar. According to Handa, et al. (2008), it is advisable to use polar solvents for the extraction of antioxidants as these compounds generally tend to be more polar and hence, they can be extracted effectively using solvents of similar polarity. These findings are in agreement with a previous research whereby, the results revealed that the antioxidant activity of ethyl acetate and ethanol (polar) extracts of *A. cepa* L. were greater than hexane (non-polar) extract (Fidrianny, Permatasari and Wirasutisna, 2013).

Active constituents or phenolic compounds found in plants such as phenolic acids, flavonoids and tannins are rich in hydroxyl groups, hence they are able to act as major free radical scavengers. Moreover, the notable antioxidant activity of the crude extracts might be due to the phenolic content, whereby the increase in the number of free hydroxyl groups helps to terminate reactive oxygen species and scavenge free radicals (Shoeb, Madkour and Refahy, 2014). Furthermore, Aksoy, et al. (2013) reported that these compounds which serve as antioxidants in the crude extracts could donate hydrogen to the DPPH free radicals and thus, the discolouration of the DPPH solution reflects the radical scavenging activity of the analysed extracts.

Moreover, the antioxidant activity of *A. cepa* L. extracts may also be attributed to the presence of mainly flavonoids, especially quercetin and its glycoside conjugates and sulphur compounds such as thiosulphinates and S-alk(en)yl-L-cysteine sulphoxides to a lesser extent (Lu, et al., 2011). Presence of compounds like flavonoids which are found excessively in *Allium cepa* L. possess the potential to stabilise free radicals and reactive oxygen species through electron transfer reactions (Nijveldt, et al., 2001).

One of the important parameters of this assay is the determination of EC₅₀ values, the concentrations of the crude extract to decrease the initial concentration of DPPH free radical by 50%. A lower EC₅₀ value indicates a higher antioxidant activity (Sowndhararajan and Kang, 2013). In this study, ascorbic acid exhibited the highest radical scavenging activity when compared to the crude extracts. Though much higher EC₅₀ values were obtained for all the crude extracts, their antioxidant activity cannot be neglected and can be considered relevant when compared to ascorbic acid. This is due to the fact that a crude extract may not be able to exert its antioxidant activity as effectively as pure antioxidant compounds such as ascorbic acid (Han, et al., 2004).

5.2.2 Analysis of Iron Chelating Activity

Transition metal species such as iron is associated with iron-mediated oxidative stress which is due to the production of reactive oxygen species (ROS) within living systems. Thus, the capacity of a substance to chelate iron can be evaluated as a valuable antioxidant capability (Chai, et al., 2014).

Different crude extracts of *Allium cepa* L. were tested for their abilities to chelate iron and hence, their antioxidant potential were studied.

In this study, the iron chelating activity was found to be higher in ethyl acetate extract, followed by ethanol, methanol, hydromethanol and lastly hexane extract. The chelating abilities of the extracts were modest when compared to EDTA, the positive control. According to the research done by Demirtas, et al. (2013), crude extracts of *Allium* species (ethyl acetate, methanol and hexane) were found to possess the ability to chelate iron and the results were comparable with the standard used, EDTA. Besides, Othman, et al. (2011) reported that *Allium cepa* L. can be considered as moderate metal chelator and its activities are approximately two times lesser than EDTA. Variation exist in the chelating activity of different crude extracts and this could be due to the different mechanism of action exerted by the active compounds involved and is also dependent on the solvents used for extraction (Yoo, et al., 2008).

The formation of 1,10-phenanthroline-Fe²⁺ complex is disrupted in the presence of chelating agent found in the extracts (Robu, et al., 2012). The chelating activity of crude extracts of *Allium cepa* L. may be attributed to the presence of phenolic compounds such as flavonoids. Apart from direct free radical scavenging activity, flavonoids also exert their antioxidant activity by interacting with transition metals like iron which are capable of generating free radicals and protect from iron-mediated oxidative stress (Malesev and Kuntic, 2007).

Moreover, EDTA is a broad-spectrum chelator. It interacts with a wide range of minerals and transition metal species including iron, thus binding and eliminating them from the body (Iron Disorders Institute, 2009). Hence, a lower EC₅₀ value was obtained for EDTA indicating its high chelating activity. As for the extracts, their EC₅₀ values were comparable with the standard. The chelating activity can be considered relevant because a crude extract, unlike pure compound, may not be able to exert its chelating effect as effectively as pure chelating agent or broad-spectrum chelator such as EDTA (Han, et al., 2004).

5.2.3 Analysis of Total Phenolic Content

The total phenolic content of *Allium cepa* L. crude extracts were investigated and quantified via Folin-Ciocalteu Reagent Test. Folin-Ciocalteu reagent is a bright yellow reagent which is sensitive to reducing compounds such as phenolic compounds and polyphenols. Upon reaction, it produces a blue colour which can be measured spectrophotometrically (Maurya and Singh, 2010). The colour intensity is proportional to the concentration of phenolic compounds found in the crude extracts (Blainski, Lopes and Mello, 2013). Furthermore, the increase in absorbance value in response to the increasing concentration explains the ability of the phenolic compounds found in the extract to reduce Folin-Ciocalteu reagent to blue oxides of tungsten and molybdenum.

Phenolic compounds are found abundantly in plants and they serve as antioxidants. Since antioxidant compounds generally exist in the phenolic

form, therefore the phenolic content can be quantified and this analysis may be helpful in the determination of antioxidant capacity of plant extracts (Aksoy, et al., 2013).

Total phenolic content of the tested crude extracts varied in accordance to the type of solvent used for extraction. According to Stankovic (2011), phenolic compounds are usually polar and hence, the solubility of these compounds are greater in polar solvents such as ethyl acetate, methanol and ethanol. In the present analysis, ethyl acetate extract of *A. cepa* L. had the highest content of phenolic compounds followed by methanol, ethanol, hydromethanol and lastly hexane extract. The total phenolic content of ethyl acetate extract was greater when compared to other crude extracts and this scenario could be attributed to the efficiency of the polar solvent used to effectively extract the phenolic compounds (Stankovic, 2011). This finding could be supported by other studies, for instance, research done by Horng, et al. (2005), whereby total phenolic content of ethyl acetate fraction of *A. cepa* L. was found to be greater than butanol and water fraction. Furthermore, studies done by Fidrianny, Permatasari and Wirasutisna (2013) also showed that ethyl acetate extract of *A. cepa* L. had greater phenolic content than hexane and ethanol extracts.

The phenolic content of *A. cepa* L. crude extracts were expressed as gallic acid equivalent (GAE). Gallic acid is an organic acid found in a variety of foods and plants. Due to its high phenolic content, it serves as powerful antioxidant and hence is used as standard in this test (Natural Remedies, 2010). However, the gallic acid equivalent (GAE) values of the extracts investigated in the

present analysis differ from previous reports. For instance, Choudhari, et al. (2011) reported a much higher value of the total phenolic content of *A. cepa* L. crude extracts whereas the present analysis showed a much lower value. This scenario can be attributed to geographical locations of the plant, seasonal cycles, climatic conditions where the plant is cultivated, drying method and also the mixing ratio of the solvent used (Abraham, Kanthimathi and Aziz, 2012).

5.2.4 Analysis of Total Flavonoid Content

Flavonoids are phenolic substances and their ability to act as antioxidant in plants has been the subject of studies in the past years (Pietta, 2000). Evaluation on the flavonoid content can be made based on complexation reaction of flavonoids found in a plant extract with aluminium chloride solution in the presence of acid or acetate solution (Pekal and Pyrzynska, 2014). In this study, the total flavonoid content of *Allium cepa* L. crude extracts were investigated and quantified via Aluminium Chloride Colourimetric Method.

Ethyl acetate extract of *A. cepa* L. obtained the highest flavonoid content and the least amount of flavonoids was found in the hydromethanol extract. The total flavonoid content of an extract may differ, depending on the type of solvent used for extraction. According to Harborne, Helga and Marby (2013), flavonoids can be extracted depending on their polarities. Semi-polar and polar flavonoids can be extracted using alcohol, water and extraction solvents such as ethyl acetate, benzene and chloroform.

Results obtained from the present analysis is in agreement with research done by Fidrianny, Permatasari and Wirasutisna (2013), whereby the total flavonoid content of ethyl acetate extract was found to be greater than ethanolic extract of *A. cepa* L. Besides, an antioxidant study by Singh, et al. (2009) also revealed that the total flavonoid content of *A. cepa* L. was higher in ethyl acetate fraction when compared to butanol and diethyl ether fractions.

Moreover, Gawad, et al. (2014) also reported that ethyl acetate fraction of *A. cepa* L. had the highest flavonoid content when compared to butanol and chloroform. Preliminary phytochemical screening of the plant then revealed the presence of steroids, terpenoids, flavonoids and saponins in high quantities. This could possibly be the reason for it to exhibit great antioxidant activity. However, the total flavonoid content of ethyl acetate extract in the present analysis is not in agreement with previous studies as a much lower value was obtained. This scenario could be due to factors such as the extract itself which existed in crude form and not as pure compound, geographical locations of the plant, climatic conditions where the plant is cultivated, drying method and mixing ratio of the solvent used (Abraham, Kanthimathi and Aziz, 2012).

The flavonoid content of the crude extracts were expressed as quercetin equivalent (QE). Quercetin was used as standard for this assay. It is one of the flavonoid compounds found in a plant which contribute to its colour. Flavonoids such as quercetin has strong antioxidant properties whereby it can scavenge free radicals and exert other antioxidant activities that prevent DNA

and cell damage (University of Maryland Medical Center, 2015). This could be another reason why ethyl acetate extract of *A. cepa* L. showed good radical scavenging and iron chelating activity previously.

5.3 Cytotoxicity Assay

Phenolic compounds found in plants are of great value as they help in the prevention of onset and progression of cancer. These compounds pose a great threat to cancer cells, but exhibit zero or a little less toxicity to normal cells. Hence, it plays a crucial role in cancer prevention (Florea and Büsselberg, 2011). A variety of diseases including cancer is said to arise from events such as lipid peroxidation, oxidative stress and damage to DNA. All these events are due to presence of free radicals and reactive oxygen species (ROS) and their activities can be halted by antioxidants by delaying or inhibiting the process of oxidation and lipid peroxidation (Kumar and Santhi, 2012). In this study, assessment on the cytotoxic activity of crude extracts of *Allium cepa* L. and the effect on cancer cell viability was done via MTT assay (Stockert, et al., 2012). The cytotoxic property of the crude extracts on K562 cells was evaluated for 24, 48 and 72 hours respectively at different doses.

5.3.1 Analysis of MTT Assay

Hexane extract of *Allium cepa* L. showed a much lower cytotoxic effect against the K562 cells. Hexane is a non-polar solvent just like petroleum ether and it can be used for the extraction of non-polar compounds. According to a research done by Wang, Tian and Ma (2012), petroleum ether exhibited the lowest cytotoxic effect on the proliferation of cancer cells and adipocytes for

24 hours when compared to polar solvents like ethanol, ethyl acetate and water extracts of *A. cepa* L. Besides, the IC₅₀ value obtained was higher, approximately 150 µg/ml. This finding is not in agreement with the present study whereby an even higher IC₅₀ value, more than 320 µg/ml was obtained. Handa, et al. (2008) reported that some antioxidants and phenolic compounds generally tend to be more polar and they can be extracted better in the presence of polar solvents. This could possibly be the reason for the inefficacy and inefficiency of the hexane extract to exhibit good cytotoxic activity.

Hydromethanol extract, too, exhibited a much lower cytotoxic activity. This finding is in agreement with previous studies by other researchers. For instance, Shrivastava and Ganesh (2010) reported that aqueous extract of different varieties of onion (*A. cepa*) have a certain cytotoxic effect when tested against B16F10 Melanoma cell line. Furthermore, the cytotoxic effect of the extract can only be observed at 72 hours of incubation and at higher concentrations. Votto, et al. (2010) reported that a significant cytotoxic effect exerted by the aqueous extract against the multi-drug resistant erythroleukaemic cell line could be observed only in the highest concentration after 72 hours of incubation. Hence, this could possibly be the reason why the observed cytotoxicity of hydromethanol extract were both time-dependent and dose-dependent. The IC₅₀ values obtained for the extract at 24 and 48 hours were much higher when compared to other crude extracts.

Cytotoxicity of the ethanol extract against the K562 cells could be attributed to the presence of active compounds like tannins. A polar solvent like ethanol

extracts polar compounds like tannins (Gregory, et al., 2013). Apart from the strong antioxidant and antibacterial activity of tannins, they participate in biological and physiological functions which are predominantly related to the modulation of carcinogenesis (Gulecha and Sivakuma, 2011). For instance, they are capable of inducing caspase-3-dependent apoptosis in cancer cell lines. This is done through various mechanisms like cell cycle arrest, extracellular signal-regulated kinase (ERK) and P38 mitogen-activated protein kinase (MAPK) pathway blockage, inhibiting transcription factors activation such as activator protein-1 (AP1), protein kinase C and growth factor-mediated pathways suppression (Dai and Mumper, 2010). However, the IC₅₀ value of the extract does not correlate with previous studies. Wang, Tian and Ma (2012) analysed the effect of ethanol extract of *A. cepa* L. on the proliferation of cancer cells and adipocytes for 24 hours. They found out that ethanol extract do possess cytotoxic and antiproliferative effects but they obtained a much lower IC₅₀ value, at 90 µg/ml.

On the other hand, methanol extract of *A. cepa* L. also exerted cytotoxic properties against the cancer cell line used. The extract has been shown to exhibit cytotoxic activity against human liver carcinoma cell line (HepG2) according to previous researches (Gawad, et al., 2014). Besides, the time course of treatment in which the extract could exhibit greater activity was observed at 72 hours of incubation. This finding can be supported by previous studies. Votto, et al. (2010) reported that methanolic extract showed a significant cytotoxic effect only in the highest concentration after 72 hours of incubation. However, a much higher IC₅₀ value was obtained for methanol

extract in this study. This finding is not in agreement with previous analysis done by Gawad, et al. (2014), whereby an IC₅₀ value of 10.9 µg/ml was obtained when the extract was tested against HepG2 cells. Nevertheless, the reason behind the capability of the extract to exert its cytotoxic effect could possibly be due to the presence of various concentrations of phenolic compounds. Recent studies have shown that antioxidant compounds such as flavonoids mainly quercetin and its derivatives were found in methanol extract of *A. cepa* L. Furthermore, flavonoids such as quercetin, apigenin, flavone, 7-hydroxyflavone, chrysin and luteolin have been shown to decrease the proliferation of K562 cells in a dose-dependent manner (Bhanot and Shri, 2010).

Among all the other crude extracts, ethyl acetate extract was found to exert greater cytotoxic activity against the cancer cell line used. The reason behind the cytotoxic effect observed in the extract could be possibly due to the presence of abundant flavonoids as well. Ethyl acetate is a semi-polar solvent which can extract out semi-polar compounds like certain flavonoids. This active compound is found excessively in ethyl acetate extract of *A. cepa* L. and it plays a significant role in carcinogenesis by interrupting the cell cycle in various cells. More than 90% of the total flavonoid content in *A. cepa* L. is comprised of quercetin and its derivatives (Slimestad, Fossen and Vagen, 2007). Quercetin was one of the major class of flavonoids which possesses antiproliferative activity *in vitro* against several cancer cells by arresting cell cycle progression either at the G1/S phase or at the G2/M transitional boundary (Elberry, et al., 2014).

Flavonoids are one the largest group of polyphenolic secondary metabolites found in plants that possess wide range of health-beneficial properties (Martinez, et al., 2014). Through its interaction with various kinds of genes and enzymes and involvement in many molecular mechanisms, flavonoids play a major role in the prevention of cancer. Its molecular mechanism of action includes the inactivation of carcinogen, cell cycle arrest, antiproliferative effect, apoptosis induction, antioxidation, angiogenesis inhibition and multidrug resistance reversal or a combination of these mechanisms (Chahar, et al., 2011). Besides, deregulated cell-cycle modulation of tumour cells received great attention in recent years. Among the natural agents, flavonoids also play very important role in the disruption of cell cycle by targeting regulator proteins such as cyclin-dependent kinases (Cdks) and their respective inhibitors, p53 and Rb protein, ATM/ATR and surviving transition-controlling points which are G1/S and G2/M (Hertzog and Tica, 2012).

However, the cytotoxic effect exhibited by ethyl acetate extract in the present analysis is not in agreement with previous studies. The IC₅₀ value obtained in this study is much higher. Gawad, et al. (2014) reported that ethyl acetate fraction of *A. cepa* L. obtained a much lower IC₅₀ value of 6.08 µg/ml when tested against human liver carcinoma cell line, HepG2. The variability in the cytotoxic effect could be attributed to cell-type specific cytotoxic property exhibited by a plant extract. Due to different specificities, plant extracts may respond towards different cancer cell lines in different ways (Sundaram, Verma and Dwivedi, 2011).

The efficacy of a crude extract to exhibit its activity on a cancer cell line vary considerably depending on certain conditions. Two factors which have great influence on the cytotoxic property of the extracts are the dose and time as these are the crucial variables of toxicity (Rozman and Doull, 2000). Dose-dependent activity can be assessed based on the preparation of different concentrations of the crude extracts whereas the time-dependent activity can be evaluated based on different incubation period or duration of treatment.

The cytotoxic property exhibited by *A. cepa* L. increased in a dose-dependent manner whereby the percentage of cell viability decreased in response to the increasing concentration of the crude extracts. This finding can be supported by other studies, for instance, research by Votto, et al. (2010), whereby lower concentrations of *A. cepa* L. extracts did not exhibit a significant difference. However, the cytotoxicity of the extracts increased in response to increasing dosage and the properties varied at different time course of treatment (Votto, et al., 2010). Based on the results obtained, all the crude extracts showed dose-dependent and time-dependent inhibitory effect against K562 cells. Hence, it can be roughly concluded that at lower concentration and shorter incubation period, there's an acute inhibitory effect on the viability of K562 cells and as the concentration of the extracts and exposure period increases, greater inhibitory activity is expressed.

A compound should possess an IC_{50} of less than 30 $\mu\text{g/ml}$ to be considered as a potent anticancer agent and this assessment is based on the criteria established by National Cancer Institute (Reddy, et al., 2012). It could be said

that the crude extracts possess somewhat cytotoxic properties against K562 cells, though their IC₅₀ values were higher than 30 µg/ml. Apart from that, cisplatin (positive control) also exhibited good cytotoxic properties on K562 cells. Cisplatin is one of the most potent chemotherapy drugs widely used for cancer treatment. The lesser IC₅₀ value of cisplatin in the present analysis revealed its potential as a cytotoxic agent. The cytotoxic effect of cisplatin works by interfering with DNA replication and transcription mechanisms, induction of apoptosis and damages tumours through activation of various signal transduction pathways and activation of mitochondrial pathways (Florea and Büsselberg, 2011).

The lower IC₅₀ values can also be attributed to the crude form of the extracts. Moreover, the cytotoxic properties of the extracts may or may not correlate with some of the previous works. This is due to the fact that a crude extract may not be able to exert strong cytotoxic effect unlike a pure compound which can exert better activity against the cancer cell line used. Besides, this scenario can also be due to the usage of late passage cells. It was reported that the proliferative ability of cancer cells increases in response to increasing passage number. Due to the increase in metabolic activity, the cells actively proliferate and this in turn leads to the increase in percentage of cell viability (Park, et al., 2008). Hence, better results can be obtained by taking into account of a few limitations in the current study.

Apart from that, this scenario may be attributed to a few limitations of the assay itself. The reduction of MTT is not solely dependent on the

mitochondrial activity as some other related components like the non-mitochondrial enzymes, endosomes and lysosomes are also involved. Alteration in the measurement of cell viability may occur due to the presence of some compounds that can directly react with MTT or interfere with the mitochondrial dehydrogenase activity of the living cells. According to Jaszczyszyn and Gasiorowski (2008), studies have proved that active compounds like flavonoids are able to react directly with MTT and induce strong reduction even in the absence of living cells. Hence, a better cytotoxic activity exhibited by the ethyl acetate extract perhaps may be due to this reason as well.

5.4 Antibacterial Assay (Qualitative Screening)

Due to the side effects and adverse reactions that occur from the usage of multispectrum antibiotics, phytomedicines or plant-based compounds are being evaluated for antimicrobial action and introduced through clinical and biological trials. Hence, in this study, extracts of *Allium cepa* L. were also tested for antibacterial properties apart from antioxidant and cytotoxic properties. However, only qualitative screening was done just to confirm whether the crude extracts possess antibacterial properties or not. In order to test the ability of the extracts to exert inhibitory properties on microorganisms and how it affects the growth, two types of tests were performed which were the Minimum Inhibitory Concentration (MIC) test and Minimum Bactericidal Concentration (MBC) test.

5.4.1 Analysis of MIC and MBC

Hexane extract showed moderate activity towards all the bacteria that were tested. However, MBC test revealed that no activity was observed against *Escherichia coli*. This finding could be supported by Penecilla and Magno (2011), whereby the hexane extract of *Allium cepa* L. showed inhibitory activities against *Staphylococcus aureus*, *Bacillus subtilis* and *Pseudomonas aeruginosa* except *Escherichia coli*. A non-polar solvent like hexane is only able to extract non-polar compounds (Nicholas, Morris and Judith, 2005). Preliminary chemical analysis of hexane extract of *A. cepa* L. revealed the presence of glycosides, steroids and proteins (Raman, et al., 2011). The little amount or weak non-polar antibacterial compounds present in the hexane extract could be the reason for its inefficiency to exert inhibitory activity against the tested microorganisms.

Hydromethanol extract, on the other hand, exhibited the least inhibitory activity when compared to the other extracts. This finding is in agreement with previous study by Penecilla and Magno (2011), whereby the aqueous extract of *A. cepa* L. showed moderate inhibitory activity when tested against *Staphylococcus aureus*, *Bacillus subtilis*, *Pseudomonas aeruginosa* and *Escherichia coli*. According to Packia, et al. (2015), alkaloids, saponins, terpenoids, tannins and reducing sugar were among the phytochemical constituents identified in the aqueous extract and these compounds were believed to be contributing to the moderate antibacterial activity exhibited by the hydromethanol extract.

Methanol and ethanol extracts of *A. cepa* L. showed almost similar inhibitory activity against all the bacteria that were tested. Nath, et al. (2010) reported that ethanol extract of *A. cepa* L. exhibited inhibitory activity against Gram-positive bacteria like *Staphylococcus aureus* and *Bacillus subtilis* and Gram-negative bacteria like *Escherichia coli* and *Klebsiella pneumoniae* strains even at a concentration of 1000 µg/ml. Furthermore, research done by Raman, et al. (2011) also revealed that the growth of *B. subtilis*, *B. pumilis*, *E. coli* and *P. aeruginosa* were inhibited significantly due to antibacterial activity exhibited by ethanol extracts of *A. cepa* L.

Phytochemical analysis of methanol extract showed the presence of active compounds such as flavonoids, anthroquinones, saponin, phenolics and reducing sugar (Packia, et al., 2015). Alkaloids, flavonoids, glycosides, tannins, terpenoid and saponin were the active compounds found in ethanolic extracts of *A. cepa* L. (Penecilla and Magno, 2011). These bioactive compounds could possibly be the reason behind the antibacterial activity exhibited by both the extracts. For instance, tannins found in the ethanol extract exhibit their antimicrobial activity through interaction with cell wall of bacteria which leads to morphological changes, tannin-induced membrane disruption, direct action on microbes metabolism and degradative extracellular enzymes that they secrete (Patra, 2012). Apart from that, saponins found in both methanol and ethanol extracts possess membranolytic properties. The detergent-like activity exhibited by saponins increase the permeability of bacterial cell membrane to allow the entrance of the active compounds (Borah, Das and Ahmed, 2013). Moreover, according to Grover, Bhandari and Rai

(2011), numerous organic sulfur compounds such as cycloalliin, S-methyl-cysteine sulfoxide, trans-S-(1-propenyl) cysteine sulfoxide and S-propyl-cysteine sulfoxide are also present in the methanol and ethanol extracts and these compounds too, contribute to their antibacterial activities.

In this study, ethyl acetate extract showed great activity towards all the four microorganisms. The MIC and MBC values were quite remarkable when compared to other crude extracts. This finding is in agreement with previous study done by other researchers. According to Bakht, Khan and Shafi (2013), ethyl acetate fraction of *A. cepa* L. showed inhibitory activity against all the eight microbes tested including bacteria and fungi. This scenario can be attributed to the overwhelming presence of flavonoids which was found to be the major phytochemical constituent in *Allium cepa* L. (Farhadi, et al., 2014).

In addition, Eltaweel (2013) also reported that the presence of active compounds such as flavonoids and polyphenols contribute to the antibacterial activity exhibited by *A. cepa* L. Furthermore, HPLC screening of flavonoids in ethyl acetate extract of *A. cepa* L. revealed the presence of flavonols such as quercetin, quercetin glycosides (isoquercetrin, quercetrin and rutin) and kaempferol as the active flavonoids (Olayeriju, et al., 2015). Flavonoid is one of the active compounds that possess many health-beneficial effects and has been identified to confer protection against various microbial invasions. It acts through various ways in fighting microorganisms which include nucleic acid synthesis inhibition, interference in energy metabolism and also interruption in

membrane-bound enzymes' function such as the ATPase (Li, Wang and Liu, 2003).

There are two categories of antibacterial agents. One is bacteriostatic whereby the agent exerts its inhibitory effect by halting the stationary phase and therefore, slowing down the growth of a microorganism. Bactericidal agent, on the other hand, is another category of antibacterial compound which kills the microorganism. So, there's one that exclusively kills the bacteria and another that slows the growth of bacteria (Shaik, Sujatha and Mehar, 2014). However, there are a wide array of mechanisms which influence the microbiological determination of an antibacterial agent *in vitro*. Growth conditions, test duration, bacterial density and decline in bacterial numbers are a few of them. Nevertheless, the clinical definition is even more arbitrary as certain compounds can be good at killing and also slowing the growth of microorganisms by exerting inhibitory properties. Hence, they can be better described as potentially being both bactericidal and bacteriostatic (Pankey and Sabath, 2004). The results of present analysis showed that the crude extracts of *Allium cepa* L. possess inhibitory properties against test microorganisms through either bactericidal or bacteriostatic action.

Ethyl acetate extract were proven to be bactericidal when tested with all the four bacterial strains whereas hexane and hydromethanol extracts were bacteriostatic towards the growth of *E. coli*. There are reasons on why a compound exerts different properties on the growth of a microorganism, be it bactericidal or bacteriostatic. For instance, intrinsic tolerance of a

microorganism and nature of the active compounds that present in an extract may contribute to the varying sensitivity of the tested bacterial strains towards the different types of crude extracts (Nanasombat and Lohasupthawee, 2005).

5.5 Limitations of Study

5.5.1 Limitation of Solvent System

There are various solvents that are capable of extracting different compounds which are made up of different compositions. Due to the absence of proper universal solvent for extraction, hence the total activities of the bioactive compounds found in the plant could not be measured.

5.5.2 Limitation of Method of Extraction

There are several disadvantages of maceration method of extraction. It is time-consuming. Through the usage of large volume of solvent, exhaustive maceration may lead to loss of plant materials. Furthermore, some compounds may not be properly extracted due to the fact that they dissolve poorly at room temperature (Sarker, Latif and Gray, 2005). Ahmad, et al. (2009) reported that Soxhlet extraction method is better than maceration method as it is simple, less time-consuming and gives a higher yield of extraction. Therefore, this method should have been used in the present study.

5.6 Future Studies

Further studies are needed in order to explore the bioactive compounds present in *Allium cepa* L. crude extracts. The effective constituents that contribute to the antioxidant, antibacterial and cytotoxic properties can be further

investigated. Analytical techniques such as high performance liquid chromatography (HPLC), thin layer chromatography (TLC) and column chromatography can be employed in future to isolate and purify the phytochemicals present in the plant. Besides, molecular biological methods and spectroscopy methods can also be performed in the future (Ghasemzadeh, Jaafar, and Rahmat, 2010).

Only one cancer cell line was used for the assessment of cytotoxic properties of the plant. Therefore, in future, the crude extracts of *A. cepa* L. can be tested on various cancer cell lines for a better investigation. Apart from testing the extracts on cancer cells, normal cell lines can also be utilised in future. This is due to the fact that the lacking in tumour specificity by a few plant-derived natural compounds may cause damage to normal cells as well. By doing this, it would indeed be helpful in future if a plant is going to be developed into an anticancer agent (Pramanik and Pandey, 2013). Human endothelial cells, for instance, can be used for the assessment of *in vitro* cytotoxic study of the crude extracts on normal cell line. Further studies on the antibacterial properties can also be performed with a wider range of bacterial strains and even fungi. Moreover, other parts of *Allium cepa* L. such as its outer skin layer or onion peel can be used for investigation on other properties as well.

Finally, analysis on the mechanism of action, efficacy and toxicity of the active compounds extracted should also be conducted. These studies can provide more convincing evidences on the efficacy of the plant to act as potent antioxidant, antibacterial and cytotoxic agent.

CHAPTER 6

CONCLUSION

Active compounds which are able to serve many physiological functions can be extracted through treatment of a particular plant with solvents of varying polarity. The present analysis emphasised on the extraction of *Allium cepa* L. using solvents of varying polarity such as hexane, ethyl acetate, methanol, ethanol and hydromethanol. Further studies on the crude extracts were performed by subjecting them to various biochemical assays.

The extraction process is influenced by the choice of solvents. Polar solvent such as ethanol obtained the highest percentage of yield whereas non-polar solvent like hexane obtained the lowest yield. The crude extracts were then tested for antioxidant properties to further clarify the existing information on the antioxidant level of the plant and the nature was assessed based on DPPH free radical scavenging and chelation of iron. Ethyl acetate extract showed good activity among all the other extracts signifying its great antioxidant potential. Since phenolic compounds contribute to the antioxidant activity of the crude extracts and flavonoids being one of the major active constituents of the plant, quantitation of both compounds were performed. The results showed that ethyl acetate extract of *A. cepa* L. was superior in extracting both phenolic compounds and flavonoids.

Besides, the cytotoxic activity of *A. cepa* L. crude extracts on K562 cell line were determined via MTT assay. The results proved that the growth of K562 cells was inhibited both in a time-dependent and dose-dependent manner. The crude extracts were believed to be able to exert cytotoxicity though much lower IC₅₀ values were obtained. Furthermore, the crude extracts were also assessed for antibacterial properties through qualitative screening and the results yielded MIC values which ranged from 1.875 to 60 mg/ml and MBC values which ranged from 1.875 to 30 mg/ml. With reference to the MIC and MBC values, ethyl acetate extract showed the best antibacterial activity towards all the bacterial strains tested when compared to other extracts whereby, it was found to exhibit bactericidal effect. Meanwhile, hexane and hydromethanol extracts showed no activity against *Escherichia coli* for MBC test and this scenario could be due to these crude extracts being bacteriostatic towards the microorganism but unable to kill it.

Although the present study involves usage of crude extracts rather than pure compounds of *A. cepa* L. and the data obtained may not be able to support the potency of the crude extracts as potential source of antioxidant, antibacterial and cytotoxic agents, nevertheless the activity exhibited by this plant cannot be opposed. Hence, further researches which involve the identification, isolation and testing the pharmacological properties of the isolated active compounds can be carried out in future with the availability of these primary information. These studies will certainly help to provide convincing information and further hold up the existing information obtained in the present analysis.

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APPENDIX A

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

Test Sample Concentration ($\mu\text{g/ml}$)	DPPH Radical Scavenging Activity* (%)					
	Ascorbic acid	Hexane	Ethyl acetate	Methanol	Ethanol	Hydro- Methanol
7.5	73.41 \pm 0.005	29.56 \pm 0.009	27.76 \pm 0.003	25.93 \pm 0.004	23.97 \pm 0.006	18.03 \pm 0.008
15	77.58 \pm 0.003	30.53 \pm 0.013	35.06 \pm 0.006	30.98 \pm 0.004	32.61 \pm 0.012	20.43 \pm 0.025
30	85.83 \pm 0.002	33.28 \pm 0.022	43.94 \pm 0.002	34.54 \pm 0.007	34.16 \pm 0.120	30.07 \pm 0.133
60	89.46 \pm 0.011	39.55 \pm 0.013	60.13 \pm 0.014	54.16 \pm 0.005	36.66 \pm 0.006	28.32 \pm 0.003
120	90.25 \pm 0.015	38.67 \pm 0.007	71.68 \pm 0.008	56.12 \pm 0.005	54.59 \pm 0.004	36.69 \pm 0.004
240	91.09 \pm 0.008	46.58 \pm 0.002	75.66 \pm 0.010	65.77 \pm 0.002	61.74 \pm 0.018	38.72 \pm 0.004

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

APPENDIX B

Table B: Percentage of iron chelating activity of EDTA and crude extracts of *Allium cepa* L. (hexane, ethyl acetate, methanol, ethanol and hydromethanol).

Test Sample Concentration ($\mu\text{g/ml}$)	Iron Chelating Activity* (%)					
	EDTA	Hexane	Ethyl acetate	Methanol	Ethanol	Hydro- Methanol
7.8125	70.53 \pm 0.006	1.48 \pm 0.012	12.36 \pm 0.004	5.14 \pm 0.012	6.25 \pm 0.007	2.15 \pm 0.002
15.625	72.24 \pm 0.011	4.37 \pm 0.023	23.89 \pm 0.001	13.51 \pm 0.011	12.37 \pm 0.012	5.36 \pm 0.001
31.25	75.19 \pm 0.002	8.12 \pm 0.004	35.05 \pm 0.003	12.69 \pm 0.003	15.63 \pm 0.009	8.97 \pm 0.011
62.5	81.34 \pm 0.005	12.36 \pm 0.008	54.38 \pm 0.010	32.00 \pm 0.001	34.96 \pm 0.003	13.34 \pm 0.032
125	83.24 \pm 0.002	11.75 \pm 0.016	58.17 \pm 0.019	50.13 \pm 0.005	42.14 \pm 0.023	24.78 \pm 0.008
250	86.91 \pm 0.014	20.09 \pm 0.007	68.48 \pm 0.005	52.75 \pm 0.016	51.77 \pm 0.006	31.89 \pm 0.005
500	90.05 \pm 0.009	21.85 \pm 0.002	66.24 \pm 0.003	60.20 \pm 0.005	55.18 \pm 0.004	30.51 \pm 0.014

*The results were tabulated as mean of percentage of iron chelating activity \pm SD of triplicate.

APPENDIX C

Table C: Absorbance values of different concentrations of gallic acid measured at 765 nm.

Concentration ($\mu\text{g/ml}$)	Absorbance* (765 nm)
20	0.6321 ± 0.004
40	1.0832 ± 0.002
60	1.5277 ± 0.001
80	2.0895 ± 0.006
100	2.1742 ± 0.002

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

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

Concentration ($\mu\text{g/ml}$)	Absorbance* (415 nm)
7.8125	0.0873 ± 0.002
15.625	0.1167 ± 0.013
31.25	0.1740 ± 0.006
62.5	0.2791 ± 0.004
125	0.4714 ± 0.011

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

APPENDIX D

Table E: Percentage of cell viability of human chronic myelogenous leukaemia cell line (K562) after treatment with cisplatin and crude extracts of *Allium cepa* L. (hexane, ethyl acetate, methanol, ethanol and hydromethanol) at 24, 48 and 72 hours.

Incubation Period (hours)	Test Sample Concentration (µg/ml)	Cell Viability* (%)					
		Cisplatin	Hexane	Ethyl acetate	Methanol	Ethanol	Hydro-methanol
24	20	91.38 ± 0.005	95.11 ± 0.007	92.32 ± 0.003	94.61 ± 0.022	96.93 ± 0.009	95.91 ± 0.003
	40	82.95 ± 0.012	88.45 ± 0.005	82.89 ± 0.006	81.39 ± 0.008	85.89 ± 0.004	84.40 ± 0.002
	80	65.23 ± 0.008	72.68 ± 0.023	62.60 ± 0.003	85.05 ± 0.005	70.71 ± 0.015	88.23 ± 0.026
	160	34.72 ± 0.110	67.15 ± 0.122	43.01 ± 0.045	64.22 ± 0.034	55.43 ± 0.006	71.90 ± 0.112
	320	36.86 ± 0.003	70.62 ± 0.007	45.61 ± 0.017	46.94 ± 0.007	48.06 ± 0.027	69.88 ± 0.041
48	20	74.55 ± 0.009	80.01 ± 0.014	75.46 ± 0.012	77.38 ± 0.010	80.42 ± 0.021	79.80 ± 0.011
	40	64.81 ± 0.004	82.63 ± 0.136	78.15 ± 0.006	75.09 ± 0.062	69.99 ± 0.007	77.73 ± 0.008
	80	49.18 ± 0.013	68.98 ± 0.004	53.36 ± 0.042	65.27 ± 0.140	71.82 ± 0.011	66.75 ± 0.006
	160	21.23 ± 0.033	57.28 ± 0.012	42.50 ± 0.008	54.38 ± 0.028	55.68 ± 0.003	50.39 ± 0.045
	320	12.76 ± 0.006	48.31 ± 0.002	24.11 ± 0.014	39.45 ± 0.007	38.21 ± 0.024	57.78 ± 0.123
72	20	62.51 ± 0.012	79.83 ± 0.014	63.51 ± 0.022	73.24 ± 0.037	70.24 ± 0.061	73.64 ± 0.027
	40	37.89 ± 0.008	65.44 ± 0.009	65.13 ± 0.118	72.85 ± 0.012	59.69 ± 0.034	68.97 ± 0.043
	80	24.52 ± 0.121	59.77 ± 0.021	34.74 ± 0.014	53.99 ± 0.017	52.18 ± 0.005	65.14 ± 0.120
	160	19.76 ± 0.045	62.23 ± 0.028	16.82 ± 0.016	47.35 ± 0.008	45.15 ± 0.011	66.26 ± 0.018
	320	8.23 ± 0.013	36.96 ± 0.009	11.56 ± 0.004	19.17 ± 0.003	20.23 ± 0.009	41.38 ± 0.005

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

APPENDIX E

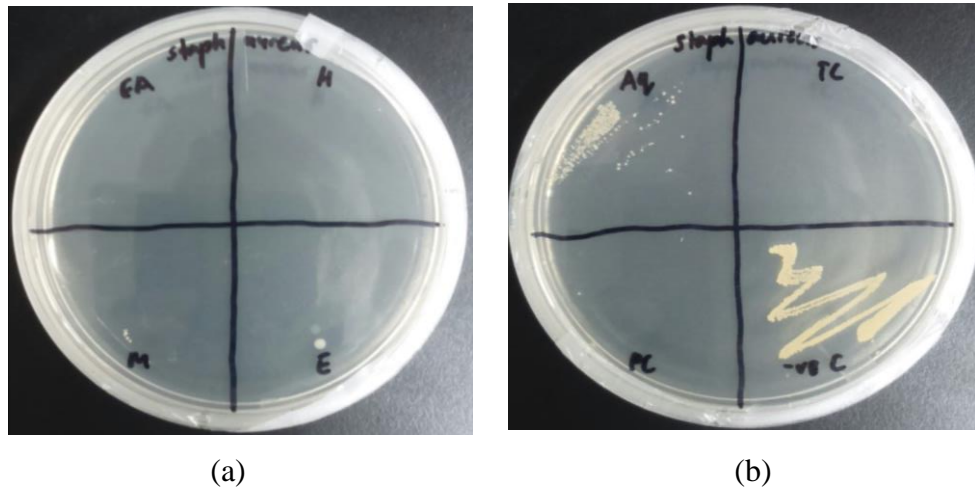


Figure A: The agar plate of minimum bactericidal concentration (MBC) test against *Staphylococcus aureus*. (a) Ethyl acetate, hexane, methanol and ethanol extract; (b) Hydromethanol extract, tetracycline, penicillin G and negative control.

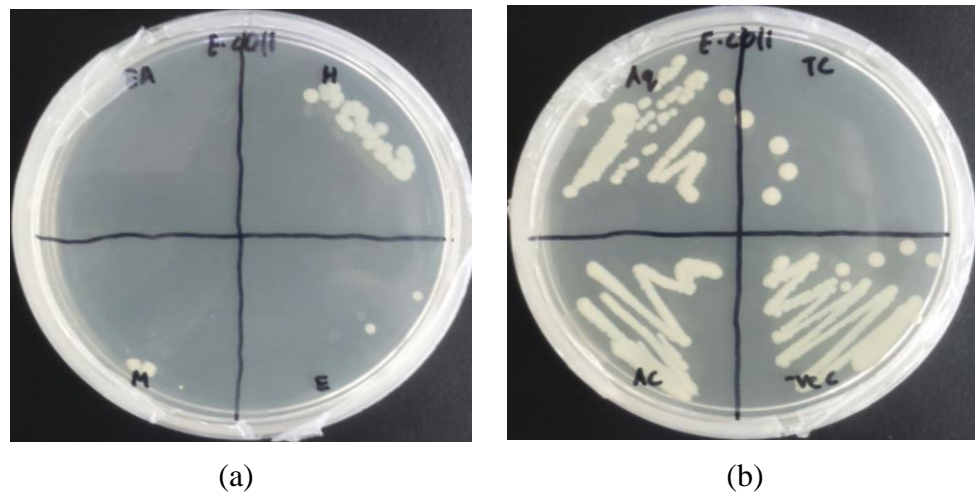


Figure B: The agar plate of minimum bactericidal concentration (MBC) test against *Escherichia coli*. (a) Ethyl acetate, hexane, methanol and ethanol extract; (b) Hydromethanol extract, tetracycline, ampicillin and negative control.