THE ANTIPROLIFERATIVE EFFECTS OF MORINGA OLEIFERA LEAF EXTRACTS AND SILVER NANOPARTICLES ON HUMAN LEUKEMIA KASUMI-1 CELLS

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by

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LIST OF SYMBOLS

°C	Celsius
IC_{50}	Half maximal inhibitory concentration
g	Gram
mg	Milligram
mL	Milliliter
nm	Nanometer
µg	Microgram
µL	Microliter
µm	Micrometer or micron
µM	Micromolar

LIST OF ABBREVIATIONS

DMSO	Dimethyl sulfoxide
FBS	Fetal bovine serum
RPMI	Roswell Park Memorial Institute
CGM	Complete growth media
H_2O	Water
ddH_2O	Deionized water
$AgNO_3$	Silver nitrate
hr	Hour
min	Minute
PBS	Phosphate buffered saline
S.D.	Standard deviation
SEM	Standard error of mean
MO-AgNPs	Moringa oleifera silver nanoparticles
MLEE	Moringa oleifera leaf ethanol extract
50% MLEE	Moringa oleifera leaf 50% ethanol extract
MLAE	Moringa leaf aqueous extract
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
TEM	TEM Transmission electron microscope
SEM	Scanning electron microscope
DLS	Dynamic Light Scaterring
USM	Universiti Sains Malaysia

LIST OF APPENDICES

KESAN ANTI-PROLIFERATIF BAGI EKSTRAK DAUN MORINGA OLEIFERA DAN NANOPARTIKEL PERAK KE ATAS SEL LEUKIMIA MANUSIA KASUMI-1

ABSTRAK

Moringa oleifera, yang juga dikenali sebagai 'moringa', ditanam secara meluas di kawasan tropika dan subtropika di seluruh dunia. Semua bahagian pokok moringa boleh digunakan dan banyak kajian telah menunjukkan bahawa pelbagai bahagian pokok moringa mempamerkan sifat-sifat anti kanser. Kajian ini adalah untuk menentukan kesan ekstrak daun moringa dan nanopartikel perak yang disintesis dari ekstrak daun moringa pada sel leukemia Kasumi-1. Serbuk daun moringa kering diekstrak dengan bantuan ultrasound bermula dengan etanol, diikuti oleh 50% etanol, dan akhirnya, air ternyah ion. Ekstrak air digunakan untuk mensintesis nanopartikel perak, di mana syarat-syarat optimum untuk menghasilkan nanopartikel perak moringa (MO-AgNPs) adalah lapan jam pengeraman pada 60 ° C dengan 1mM perak nitrat dan 1% moringa ekstrak air daripada pengekstrakan berturutan. Tiga jenis ekstrak dan MO-AgNPs digunakan untuk merawat sel-sel Kasumi-1 selama 24, 48, dan 72 jam dengan konsentrasi antara 400 dan 12.5 μg / mL, manakala daya maju sel ditentukan dengan 3 (4, 5-dimetythiazol-2 -il) -2, 5-diphenyltetrazolium bromide (MTT). Keputusan eksperimen menunjukkan bahawa selepas 72 jam rawatan, ekstrak etanol daun moringa menunjukkan kesan perencatan kuat pada sel-sel Kasumi-1 dengan IC_{50} 10 μg / mL, berbanding dengan daun moringa 50% ekstrak etanol (25 μg / mL) dan ekstrak air (> 400 μg / mL). MO-AgNPs menunjukkan kesan sitotoksik tertinggi pada sel-sel Kasumi-1 dengan IC_{50} 7.5 μg / mL. Kajian morfologi Kasumi-1 sel yang dirawat dengan IC_{50} ekstrak etanol daun moringa dan MO-AgNPs menunjukkan

peningkatan ketara dalam sel-sel yang mengecut dan mati, serta serpihan sel. Kajian kitaran sel menunjukkan peningkatan sel pada fasa G1 untuk ekstrak daun etanol, manakala MO-AgNPs menyebabkan penangkapan kitaran sel pada fasa S selepas rawatan dengan dos IC_{50} selama 24 jam. Ekstrak etanol bagi daun Moringa dan nanopartikel menginduksi apoptosis di dalam sel Kasumi-1 seperti yang ditunjukkan oleh Annexin V - FITC assays. Ekspresi gen bedasarkan qPCR mengesahkan hasil ini, kerana ekstrak etanol daun moringa membawa kepada peningkatan besar gen proapoptosis – *caspase 8*, manakala MO-AgNPs menyebabkan peningkatan ketara protein proapoptotic - *BID*. Kajian ini mendedahkan bahawa ekstrak daun etanol dan MO-AgNPs memacu kesan antiproliferatif yang kuat dalam sel-sel Kasumi-1 menerusi induksi apoptosis.

THE ANTIPROLIFERATIVE EFFECTS OF MORINGA OLEIFERA LEAF EXTRACTS AND SILVER NANOPARTICLES ON HUMAN LEUKEMIA KASUMI-1 CELLS

ABSTRACT

Moringa oleifera, also commonly known as 'moringa', is widely cultivated in tropical and subtropical regions across the globe. All parts of the moringa tree can be utilised and extensive studies have shown that various parts of the moringa tree exhibit anti-cancer properties. This study determined the effects of sequential moringa leaf extracts and silver nanoparticles synthesised from moringa leaf extract on Kasumi-1 leukemia cells. Dried moringa leaf powder was sequentially extracted with the assistance of ultrasound starting with absolute ethanol, followed by 50% ethanol, and finally, deionised water. The aqueous extract was incorporated to synthesise silver nanoparticles, whereby the optimum conditions to generate moringa silver nanoparticles (MO-AgNPs) were eight hours of incubation at 60 °C with 1 mM silver nitrate and 1% moringa aqueous extract from sequential extraction. The three various extracts and MO-AgNPs were used to treat Kasumi-1 cells for 24, 48, and 72 hours with concentrations ranging between 400 and 12.5 µg/mL, while cell viability was determined with 3(4, 5-dimethythiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. The experimental outcomes revealed that after 72 hours of treatment, the moringa leaf ethanolic extract displayed the strongest inhibitory effects on Kasumi-1 cells with IC_{50} of 10 µg/mL, in comparison to moringa leaf 50% ethanolic extract (25 µg/mL) and aqueous extract (>400 µg/mL). Interestingly MO-AgNPs exhibited the strongest cytotoxic effects on Kasumi-1 cells with an IC_{50} of 7.5 µg/mL. Morphological studies of Kasumi-1 cells treated with IC_{50} of moringa leaf ethanolic

extract and MO-AgNPs exemplified significant increment in shrinking and dying cells, as well as cell debris. Cell cycle studies displayed an increase in cells at the G1 phase for ethanol leaf extract, while MO-AgNPs caused cell cycle arrest at the S phase after treatment with IC_{50} dose for 24 hours. Moringa leaf ethanol extract and the nanoparticles induced apoptosis in Kasumi-1 cells as shown by Annexin V – FITC assays. Gene expression analysis by qPCR verified these outcomes, as the moringa leaf ethanol extract led to significant upregulation of proapoptotic gene *caspase 8*, whereas the MO-AgNPs caused a significant increase of proapoptotic protein *BID*. This study reveals that moringa ethanolic leaf extract and MO-AgNPs induced potent antiproliferative effects in Kasumi-1 cells by apoptosis.

CHAPTER 1

INTRODUCTION

1.1 Background of the Study

Leukemia is defined as cancer that starts in blood-forming tissue, such as bone marrow, that produces a substantial amount of abnormal blood cells and enters into the bloodstream (*Definition of COLPOSCOPY hemangioma - NCI Dictionary of Cancer Terms - National Cancer Institute NCI Dictionary of Cancer Terms*, 2019). Leukemia, or better known as blood cancer in layman terms, exerted a prevalence of 3.2% among all diagnosed cancer cases and caused 2.7% of cancer mortality worldwide (GLOBOCAN-WHO, 2018). A more recent figure on cases of leukemia recorded in America revealed that leukemia alone makes up 3.7% of all new cancer cases and contributes 4.1% to mortality due to cancer (National Cancer Institute, 2012). Although the prevalence and the mortality of leukemia appear to be rather low, in comparison to other more common cancers, such as breast and lung cancers, leukemia seems to be the most common cancer diagnosed amongst children. In pediatrics, leukemia accounts for approximately one third of all cancer cases, and it has been reckoned as the most common cancer among children and adolescents under the age of 20 (Leukemia and Lymphoma society, 2019). Leukemia patients are subjected to harsh chemotherapy regiments or bone marrow transplant as standard treatment. Despite availability of chemotherapy drugs with greater efficacy in improving survival rates amongst leukemia patients; long term side effects of these drugs on the patients remains a drawback. Many of these chemotherapy drugs are notorious for their cardio-toxic effects (Fulbright, 2011). Therefore, there is a pressing need to develop new drugs for treatment of

leukemia patients with less long-term side effects, as compared to conventional drugs. Compounds derived from plants known as phytochemicals have been a major source of anticancer drugs, hence making up around half of all conventional anticancer drugs (Amin *et al.*, 2009). These plant-derived compounds are safer, eco-friendly, cost-effective, and less toxic, in comparison to conventional treatment methods (Iqbal *et al.*, 2017).

Moringa oleifera, which is more commonly known as drumstick tree, horseradish tree or simply moringa, refers to a plant that has shown promising anticancer activities in numerous studies. All the different parts of the moringa tree, including roots, fruits, seeds, leaves, and roots, have been tested for anticancer activities *in vivo* with varying degrees of success. Most researches have focused on the leaves from the moringa tree since they possess the most phytochemicals and have displayed anticancer activities on differing cancer cell lines *in vitro* (Parvathy and Umamaheshwari, 2007; Tiloke, Phulukdaree and Chuturgoon, 2013; Jung, 2014; Suphachai, 2014; Madi *et al.*, 2016). Besides, several small-scale studies have proven that the moringa leaf extract exhibits anticancer activity *in vivo* (Rao, Uma Devi and Kamath, 2001; Jung, Lee and Kang, 2015). These studies highlight that moringa leaves have promising anticancer activities, thus emerging as an exceptional and promising candidate to test for anticancer activities.

Nanomedicine appears to be an emerging arena with a significant role in channeling the direction of alternative and more effective treatment strategies for cancer theranostics (Barabadi *et al.*, 2017). In recent times, green synthesis of biogenic nanoparticles that have been derived from plant extracts has gained popularity because it is safer, eco-friendlier, simpler, faster, more energy efficient, more cost-effective, and less toxic, when compared to chemical synthesis of nanoparticles. The relative ease of

green synthesis of nanoparticles that originate from plant extracts further makes it an attractive option to enhance the anticancer activities exhibited by these plant extracts.

1.2 Rationale of the Study

Given the current interest regarding research updates, mentioned in the previous section, this study investigated cytotoxic effects of *Moringa oleifera* leaf (MOL) extract and nanoparticles on Kasumi-1 cells, which was an Acute Myeloid Leukaemia (AML) cell line with t(8;21) chromosomal rearrangement. This cell line was chosen to be studied in view of the fact that numerous studies have tested MOL extracts on many different adherent cancer cell lines for its cytotoxic effects, while omitting suspension cells such as leukaemia cells. Furthermore, a comprehensive study on cytotoxic effects of MOL extract on Kasumi-1 cells is still lack in literature. With regard to MOL extract mediated nanoparticles, a scarcity is well noted, thus, suggesting a pressing need to test and determine whether or not MOL extract can be utilized to produce nanoparticles, as well as to verify nanoparticles exert cytotoxic effects upon Kasumi-1 cells.

1.3 Problem Statement

Conventional cancer drugs are costly and they have been proven to cause many side effects. Therefore, a dire need is present to develop new drugs derived from plants with fewer side effects for use against cancer. Based on several prior studies, *M. oleifera* leaf extract has displayed exceptional anticancer activities *in vitro* against various cancer cell lines. This study sought to investigate potential cytotoxic effects of moringa leaf extract and nanoparticles on leukemia cells – Kasumi-1 cells

1.4 Hypothesis

In this study, it was hypothesized that *Moringa oleifera* leaf extract and nanoparticles were cytotoxic and exert apoptosis in Kasumi-1 Cell lines

1.5 Main Objective

The main objective of this study was to evaluate antiproliferative effects of *Moringa oleifera* leaf extracts and nanoparticles on human leukemia Kasumi-1 cells.

1.6 Specific Objectives

The specific research objectives of this study were as listed below:

1. To prepare aqueous and ethanol extracts from *Moringa oleifera* leaves.
2. To prepare silver nanoparticles from *Moringa oleifera* leave aqueous extract.
3. To determine cytotoxic, cell death mode and cell cycle arrest of Kasumi-1 cells treated with *Moringa oleifera* leaf extracts and nanoparticles.
4. To study apoptosis induction by *Moringa oleifera* leaves extracts and nanoparticles on Kasumi-1 cells by means of annexin v assay, morphological parameters and gene expression.

CHAPTER 2

LITERATURE REVIEW

2.1 Cancer

Cancer is an escalating worldwide health threat. According to WHO statistics, cancer is rapidly increasing in both developed and developing countries (GLOBOCAN-WHO, 2018). Cancer is in fact a second leading cause of global mortality causing 8.8 million deaths in year 2015 alone (WHO, 2018). The cancer burden is disproportionately shouldered by developing countries which contribute 57% of overall cancer incidences and 67% of the entire mortality worldwide (GCO, 2019). The elevated mortality rate due to cancer in developing countries can be attributed to lack of proper medical intervention for the patients. In America, overall cancer death rate from year 1990 until 2014 has been significantly reduced by 25% (Simon, 2018) due to advancement in medical field whereby early detection and better treatment options have improved medical outcomes for patients suffering from cancers.

Although there is an improved chance of surviving cancer, it does not address the fact that there has also been a rapid increase in cancer incidences worldwide. Developing countries are expected to experience more rapid increases compared to developed countries with estimated up to 15 million new cases in year 2020 (Salminen, Izewska and Andreo, 2005). There are many reasons igniting cases of cancer in developing countries. The factors among others are prevalence of smoking and extensive environmental pollution in addition to lack of healthcare services to prevent and treat cancers, as well as infections such as hepatitis and human papilloma virus (HPV). All these factors contribute to the increment of cancer cases in developing

countries. All of the factors, coupled with lack of cancer healthcare will indeed lead to skyrocketing cancer mortality.

Even though there are many types of cancer, however, the most common ones are lung, breast, prostate and bowel cancers, composing more than 50% of all cancer cases (GCO, 2019). Among the less common cancer types, leukemia is a significant cancer due to its prevalence among children; it accounts for approximately one third of all reported pediatric cancer cases. In short, leukemia is a cancer of developing blood cells, caused by mutations, leading to either uncontrolled proliferation (class I) or lack of differentiation (class II) or both. Leukemia makes up roughly three percent of all diagnosed cancer cases (GCO, 2019). Leukemia is commonly diagnosed among adults more that 55 years of age and among youngsters below 21 years old of age. There are four main types of leukemia namely acute myeloid leukemia (AML), acute lymphocytic leukemia (ALL), chronic myeloid leukemia (CML) and chronic lymphocytic leukemia (CLL). Among them, AML affects both children and adults although it is more commonly detected among adults. AML also has the lowest survival rate within five years compared to other three types of leukemia (Leukemia and Lymphoma society, 2019).

2.2 Leukemia in Malaysia

Based on the most recent published study on cancer by the Ministry of Health – Malaysian Study on Cancer Survival (MySCan), published in October 2018, cancer has been acknowledged as the fourth leading cause of death in Malaysia (Ministry of Health Malaysia, 2018). In fact, cancer has been on the rise in Malaysia causing an increasing number of deaths year after year. For example, percentage of death from cancer contributed 11.3% to the entire fatality in year 2007 compared to 2016 which was higher at 12.6% (Ministry of Health Malaysia, 2017). According to the most recently published statistics on cancer in Malaysia, leukemia is the 7th most common cancer among males and 8th most common cancer among females witnessing 4.2 incidences over 100 000 males and 3.4 incidences over 100 000 females (Saleha and Hashimah, 2007). Leukemia cases might appear to be lower compared to other types of cancer such as colorectal and breast cancers, however, in pediatrics, leukemia is the most commonly diagnosed cancer. Among children between 0 and 14 years of age, leukemia accounts for almost half of overall reported cancer cases (Saleha and Hashimah, 2007).

2.3 Medicinal Plants as Anticancer Drugs

Many plants have been utilized since prehistoric times by humans as medicine to treat many types of ailment. Until today, the use of medicinal plants in traditional medicine is still widespread, taking ayurvedic and traditional Chinese medicines as examples. Medicinal plants have also become sources of medicinal compounds in development of drugs to treat many types of diseases. An excellent example is the discovery of antimalaria drug artemisinin by Tu Youyou in year 1972 who has been awarded the Nobel Prize for Medicine in year 2015 (Su and Miller, 2015). Artemisinin was discovered after Tu Youyou read through centuries old traditional Chinese medicine sources and discovered a text describing the use and preparation of qinghao to treat patients with symptoms of malaria. Artemisinin is isolated from qinghao and has been developed, making it one of the most effective antimalarial drugs available in market today.

According to Newman and Cragg (2012), up to 50% of approved drugs during last thirty years were either directly or indirectly derived from natural products (Newman and Cragg, 2012). In recent times, medicinal plants have also been studied for potential anticancer compounds that can be developed into anticancer drugs. In fact, there are many examples of anticancer drugs derived from plants in the market today. One of examples is camptothecin which is a product of isolation from bark and stem of *Camptotheca acuminate*, further modified and developed into a number of commercial anticancer drugs such as paclitaxel, vinorelbine and teniposide (Pezzuto, 1997). As another example, Vinflunine is a modification of vinblastine, initially isolated from *Catharanthus roseus* (Kruczynski *et al.*, 1998).

Although plant derived drugs are widely utilized today, only 5-15% of approximately 250,000 higher plants have ever been investigated for bioactive

compounds. More studies should be conducted on more diverse plant species as more and more people are aware of advantages of using such compounds for cancer treatment are their relatively non-toxic nature and availability in ingestible forms (Amin *et al.*, 2009). Besides, plants have potentially huge untapped reservoir of anticancer compounds which can provide limitless opportunities for the development of anticancer drugs.

2.4 Natural Products Currently Used for Treatment of Leukemia

There are various chemotherapeutic drugs successfully isolated from plants that have been used to treat leukemia. One of the most important naturally derived drugs is vinca alkaloid family which is earliest isolated from periwinkle – *Catharanthus roseus* which is native and endemic in Madagascar. Vinca alkaloids are the second most commonly utilized chemotherapeutic drugs; the most common derivatives are vinblastine, vinorelbine, vincristine and vindesine (Moudi *et al.*, 2013). One of the drugs from the vinca alkaloid family named vincristine was approved for use by the FDA in 1963 and has still been used until today. It is an important drug, thus, it is included in the World Health Organization's List of Essential Medicine ('WHO | WHO Model Lists of Essential Medicines', 2019). Vincristine is used to treat a number of different cancers but it is especially used for treatment of leukemia and improving leukemia cure rates (Heyn *et al.*, 1966).

Another chemotherapeutic drug used to treat leukemia is homoharringtonine (HHT). This plant alkaloid is initially isolated from *Cephalotaxus harringtonii* plant, commonly known as Japanese plum yew tree which is native in Japan. The compound HHT was first isolated and described in 1970 by Powell *et al.* (Powell *et al.*, 1970). The compound was under intensive research for the treatment of leukemia by the Chinese

in the 1970s which showed that HHT was an effective treatment for acute myeloid leukemia, acute promyelocytic leukemia and central nervous system (CNS) leukemia (Kantarjian, O'Brien and Cortes, 2013). However, it took another forty years of research and development before the semisynthetic purified HHT compound Omacetaxine mepesuccinate was finally approved for use by the FDA in 2012 for the treatment of chronic myeloid leukemia (CML) which were resistant to two or more tyrosine kinase inhibitors.

Currently, there is a new drug called flavopiridol which is still in the clinical trial phase which is being tested for the treatment of chronic lymphocytic leukemia. This drug is the semi-synthetic form of the plant alkaloid rohitukine, first isolated from *Amoora rohituka* and later from *Dysoxylum binectariferum*. Flavopiridol works by inhibiting cyclin dependant kinase (CDK) especially CDK9 which leads to cell cycle arrest at the G1 or G2 phase (De Azevedo, Canduri and Freitas da Silveira, 2002). In a phase II clinical trial, flavopiridol followed with 1-β-D-arabinofuranosylcytosine (ara-C) and mitoxantrone treatment produced 75% complete remissions in newly diagnosed secondary AML and first-relapse AML (Karp *et al.*, 2010). Although flavopiridol is still in the clinical trial phase, it was granted orphan drug status by the FDA for the treatment of AML in America ('FDA Grants Orphan Drug Status to Gevokizumab', 2013). This drug has demonstrated promising results in clinical trials and might be approved for commercial use in the near future.

2.5 *Moringa oleifera* : a potential souce for anticancer compounds

Moringa oleifera is native in the northwest of the Indian subcontinent, grows well in tropical and subtropical climates (Ramachandran, Peter and Gopalakrishnan, 1980). *Moringa oleifera* is the most cultivated species in the genus *Moringa* and a detail taxonomy hierarchy of moringa tree is shown in Table 2.1. It is known by many different names such as drumstick tree, horseradish tree, ben oil tree, wonder tree and moringa (*Taxonomy - GRIN-Global Web v 1.10.6.1*, 2020). Moringa plants are widely utilized in their native land of India as a source of food and medicine. All parts of the tree can be used, for example, leaves are consumed or used as a medicine, barks and roots are used in traditional medicines, fruits are consumed and enjoyed by people and seeds are pressed to produce edible and precious oil. The moringa leaves are rich in nutrients and antioxidants, motivated people to eat powder of dried moringa leaf as health supplement to improve overall wellbeing. Some related parties have also suggested adding moringa leaf powder in foodstuff as food aid to combat malnutrition (Oyeyinka and Oyeyinka, 2018).

Due to its worth, moringa has been widely planted in tropical and subtropical regions around the world, on all continents except Antarctica. It can be found in Mediterranean region, Africa, the Caribbean, South Pacific islands and tropical regions of Asia (Muluvi *et al.*, 1999). Many countries are promoting cultivation of moringa and incorporating it into local dietary to combat malnutrition. Moringa leaves and fruits are found to be rich in nutrients and scrumptious. Dried leaf powder goes well with bread, noodles and soups to get the most out of nutrients without compromising their authentic tastes.

Moringa seeds are pressed to produce ben oil which is high in antioxidants and nutrients similar to olive oil. Ben oil is not only edible, but also highly sought after in

manufacturing cosmetics and beauty products due to its resistance against oxidation which confers the products with a long stable shelf life. More interestingly, seed wastes from oil production are not as the name would suggest because they are still useable as a flocculating agent in water treatment (Ndabigengesere and Narasiah, 1998). The pressed seed wastes, called seed cake can efficiently bind to colloids in turbid water and clarify it for consumption. In short, moringa seeds not only able to provide valuable oil but the wastes from oil extraction process can be utilized for water purification.

The moringa plant is also widely utilized in traditional Indian medicine (ayurvedic) to treat a wide range of illnesses such as liver diseases, diabetes, heart diseases and many more. There are even current researches that have verified the use of moringa to treat certain diseases. For example, Pari and Kumar conducted an *in vivo* study on hepatoprotective effects of moringa leaf extract (Pari and Kumar, 2002). In the study, the researchers confirmed that moringa leaf extract was able to enhance recovery of hepatic damage, induced by antitubercular drugs. In another study, Nandave *et al*. evaluated cardioprotective effects of moringa on rats (Nandave *et al.*, 2009). Based on the results of the study, it was concluded that moringa leaf extract has significant cardioprotective effects and this may be due to antioxidant, antiperoxidative and myocardial preservative properties of moringa leaves. Another study was conducted to determine whether or not moringa was capable in treating diabetes on hyperglycemic rats (Jaiswal *et al.*, 2009). The study found that moringa leaf extract was capable of reducing blood sugar levels among hyperglycemic rats.

Moringa oleifera is a versatile plant that can be some sorts of food source, health supplement and medicine. Recent studies have verified medicinal uses of moringa leaf extract for treatments of liver and heart damages, among other diabetes. Moringa leaves are undoubtedly rich in antioxidants and have potential anticancer activties.

Table 2.1 Taxonomy Hierarchy of *Moringa oleifera* (Integrated Taxonomic Information System (ITIS), 2017)

Kingdom	*Plantae*	- Plant
Subkingdom	*Viridiplantae*	- Green plants
Infrakingdom	*Streptophyta*	
Superdivision	*Embryophyta*	- Terrestrial plants
Division	*Tracheophyta*	- Vascular plants
Subdivision	*Spermatophytina*	- Seed plants
Class	*Magnoliopsida*	- Dicotyledons
Superorder	*Rosanae*	
Order	*Brassicales*	
Family	*Moringaceae*	- Horse-radish tree family
Genus	*Moringa Adans.*	- Moringa
Species	*Moringa oleifera Lam.*	– Horseradish tree

Figure 2.1 The *Moringa oleifera* plant

2.6 *Moringa oleifera* Anticancer Activity

As discussed previously, moringa has a wide range of benefits in addition to a potential anticancer agent. In fact, there are a lot of *in vitro* and *in vivo* studies that confirmed anticancer activities shown by extracts of different parts of moringa plant such as leaves, bark, fruit and seeds. However, most researches have focused on moringa leaves because this part of tree has the richest nutrients and antioxidants. Moringa leaves have a very strong antioxidant activity as shown in a study conducted by Verma *et al*. (2009). In the study, five different fractions from moringa leaf extract were tested by means of eight different antioxidant tests such as β-carotene bleaching and linoleic acid assay (Verma *et al.*, 2009). Based on this study, it was determined that the polyphenolic fraction had the highest antioxidant activity, followed by crude extract, diethyl ether fraction, non-phenolic fraction, and aqueous fraction. Strong antioxidant activity is a good indication of anticancer activity as many of active compounds, for instance polyphenols and flavonoids have both antioxidant and anticancer properties.

There are numerous *in vitro* studies that proved anticancer activities of moringa leaf extract on a number of cancer cell lines. Charoensin (2014) conducted a study investigating anticancer activity of leaf extract among different cancer cell lines namely hepatocarcinoma (HepG2), colorectal adenocarcinoma (Caco-2), and breast adenocarcinoma (MCF-7) (Suphachai, 2014). In the study, methanol and dichloromethane extracts from moringa leaf were produced and tested on the three different cell lines. The results of the study indicated that dichloromethane leaf extract had better anticancer activity compared to methanol leaf extract. The researcher deduced that polyphenols, flavonoids and glucosinolate compounds were mainly responsible for anticancer activities. In another study by Berkovich *et al*.(2013), they tested effects of water-soluble leaf extract of M. oleifera on pancreatic cancer cells lines

Panc-1, COLO-357, and p34 (Berkovich *et al.*, 2013). The study showed that moringa leaf extract was the most cytotoxic to the Panc-1 cells. Berkovich *et al*. conducted more studies to determine effects of treating Panc-1 cells with a conventional anticancer drug cisplatin together with moringa leaf extract. They determined that the leaf extract had a synergistic effect when it has been used in conjunction with cisplatin against the Panc-1 cells. It appeared that moringa leaf extract sensitised the Panc-1 cancer cells to chemotherapeutic drug – cisplatin, therefore, increased the cytotoxicity of both substances when used together at a low inhibitory dose.

There are also several *in vitro* studies on anticancer activities of moringa leaf extract. A study by Jung, Lee and Kang (2015) examined cytotoxicity of aqueous moringa leaf extract on HepG2 and A549 (Jung, Lee and Kang, 2015). The experiment was conducted using the hollow fiber assay, in which cancer cells were cultured in hollow fibers and then they were implanted into immunodeficient mice. The mice were then fed moringa leaf extract. After the treatment was completed, the hollow fibers were recovered for analysis. Base on this *in vivo* study, it was discovered that at the maximum tested dose of 200 mg/kg, the survival of HepG2 and A549 cells decreased by 60% and 50%, respectively. In fact, the moringa leaf extract decreased the survival rate of HepG2 cells to certain levels lower than those achieved by conventional anticancer doxorubicin, used as a positive control.

Base on these studies, moringa leaf extract exhibited promising anticancer activities i*n vitro* and *in vivo*. However, there are no studies on effects of moringa leaf extract on leukemia cells (Kasumi-1). Therefore, this study would be able to provide new insights with regard to this knowledge gap and eventually bridge it.

2.7 Ultrasound Assisted Sequential Extraction

The active compounds from plant materials need to be extracted in order to test anticancer activities of the extract on cells. To do so, two main types of extraction methods are employed particularly conventional method such as Soxhlet or maceration and unconventional method such as ultrasound or microwave extraction (Devgun, Nanda and Ansari, 2012). Ultrasound assisted sequential extract (UASE) is an unconventional method for extracting plant extracts. As compared with conventional extraction methods such as Soxhlet extraction, UASE is a much more efficient process in addition to less solvent and time requirements to extract plant materials (Azwanida, 2015). UASE is based on a combination of two methods specifically ultrasound assisted extraction (UAE) and sequential extraction. Ultrasound extraction or sonication extraction involves the use of ultrasound ranging from 20kHz to 2000kHz for extraction (Handa *et al.*, 2008). Sequential extraction refers to extraction done on the same plant material successively in increasing order of polarity of the solvent (Altemimi *et al.*, 2017).

Ultrasound assisted extraction (UAE) uses ultrasound (sound waves) to break open cell walls and induce extraction of plant materials. It is able to effectively extract plant materials due to a cavitation phenomenon which is a physical process that creates and implodes microbubbles of gas presented in liquid medium. The ultrasound waves compress and refract the liquid medium to create a cavitation phenomenon. The cavitation process works on plant material surfaces by generating high pressure of approximately 1000 atm and temperature of about 5000 K, heating and cooling rates above 1010 K/s (Suslick *et al.*, 1999) that destroy cell walls and release cellular contents into medium. The extraction occurs via two main mechanisms which are diffusion across intact cell walls and releasing contents of cell after wall breakage (Mason,

Paniwnyk and Lorimer, 1996). This process is able to efficiently extract bioactive compounds presenting in plant materials. Besides, UAE is able to reduce extraction time, extraction solvent and energy needed to obtain plant extract from plant materials while increasing the extraction yield at the same time.

Sequential extraction involves the use of solvents with different polarities to extract plant materials. This process starts with extraction using the least polar solvent and it goes on with more polar solvent on the same plant materials (Liu, 2008). Bioactive compounds presented in plant materials have different polarities and they will be extracted by different solvents at different rates whereby each sequential extract will have a different composition of active compounds. Thus, sequential extraction leads to a selective and complete extraction of active compounds in plant materials. UAE allows for fast and efficient extraction of plant extracts from solid plant materials whereas sequential extraction enables the selective extraction of bioactive compound bases. Therefore, when UAE and sequential extraction are used in combination, bioactive compounds from *Moringa oleifer*a leaf will be extracted more efficiently and selectively.

2.8 Nanoparticles – as a powerful tool for scientific applications

A nanoparticle is defined as a microscopic particle with at least one dimension which is lesser than 100nm (Jarvie, King and Dobson, 2020). Currently nanoparticles are of great scientific interest as they have a variety of applications in the medical, optical and electronic fields. Nanoparticles are considered as the material that bridges bulk materials and atomic or molecular structures. They have unique physical properties when compared to bulk materials as percentages of atoms at a surface of the material become significant. For example, bulk copper wire is a malleable and ductile material, however, copper nanoparticles smaller than 50nm are super hard because bulk copper bends with movement of copper atom clusters of approximately 50nm (Visakh, 2016). Nanoparticles also have a high surface area to volume ratio when compared to bulk materials due to their small size. Nanosized metallic particles are special and can significantly change physical, chemical, and biological properties; therefore, they have been utilized for various purposes (Li *et al.*, 2001). Currently, nanoparticles are widely utilized in industries, household and healthcare.

The unique properties of nanoparticles due to their small size has allowed the nanoparticles to be utilized widely for scientific applications such as in the field of renewable energies in which carbon nanoparticles are being developed as electrodes for fuel cells (Lee *et al.*, 2013) and platinum -cobalt nanoparticles are developed as catalyst for fuel cells to increase energy outputs (Qiao and Li, 2011). However, some of the fields where nanoparticles have shown great potential and have been the main focus of researchers are biology and biomedicine. The nanoparticles have been utilized in wide ranging applications such as fluorescent biological labels, drug and gene delivery, probing DNA structure, tissue engineering, tumor destruction and anticancer applications (Salata, 2004). One of the reasons for the diverse utility of nanoparticles is

the diversity of materials that can be made into nanoparticles using many different methods which will be discussed further in the next subchapter.

2.9 Nanoparticle synthesis

There are a few methods can be utilized to produce nanoparticles namely physical, chemical and biological syntheses. The physical method of nanoparticle synthesis involves evaporation-condensation using a tube furnace at atmospheric pressure to produce nanoparticles, while chemical method utilizes chemicals as reducing agents to produce nanoparticles (Abou El-Nour *et al.*, 2010). The biological method uses biological compounds or systems in the synthesis of nanoparticles. Among the three different methods, the biological synthesis of nanoparticles is considered the most environmentally friendly because it does not require a large amount of energy or harsh chemicals to produce nanoparticles. Therefore, biological synthesis is also known as the green synthesis of nanoparticles (Makarov *et al.*, 2014).

Biological synthesis of nanoparticles occurs through reactions such as reduction, biosorption and enzyme mediation (Iravani *et al.*, 2014). The main reaction of nanoparticle synthesis is reduction and it can be done by different bacteria and fungi. Plant material and extracts can also be utilized to produce silver and gold nanoparticles. In this process, the metal salts of silver or gold are dissolved in water and active compounds presenting in plant extract will act as reducing agents to induce a formation of nanoparticles. These silver and gold nanoparticles are quite easily to be produced without the need of specialized equipment, thereby, making them some of the easiest and cheapest nanoparticles to produce at a small scale in a laboratory setting. Silver nanoparticles are attractive candidates for small scale production due to low cost as

compared to gold nanoparticles. The appealing properties of the silver nanoparticles will be described in the following subsection.

2.10 Silver Nanoparticles (AgNPs)

Silver nanoparticles are composed mainly from either elemental silver or silver oxides. They can be in many different shapes such as spherical, sheets, diamond or octagonal. As mentioned before, there are three main ways to produce nanoparticles specifically physical, chemical and biological methods. All three methods can also be utilized to produce AgNPs; however, biological method has emerged as the most eco-friendly and pollution free method when it comes to produce AgNPs (Gurunathan *et al.*, 2009). The biological method of producing silver nanoparticles is done via nucleation within a solution. The process involves reduction of silver ions from silver salt solutions such as AgNO3 (silver nitrate) into colloidal silver in a presence of a reducing agent. This method can also be utilized to produce silver nanoparticles from plant extracts in which active compounds in the plant extracts such as polyphenols and flavonoids act as reducing agents to form silver nanoparticles (Thakkar, Mhatre and Parikh, 2010). There are other advantages for the biological synthesis method such as incorporation of metabolites, proteins and amino acids into the AgNPs and the production of stable and soluble AgNPs (Kumar *et al.*, 2014).

Silver nanoparticles have been widely studied and they have shown to comprise antibacterial, antifungal, antiviral and anticancer activities. In the paper by Sondi and Salopek-Sondi, it was demonstrated that AgNPs were capable of killing *E.coli* by accumulating and forming pits on the bacterial cell walls (Sondi and Salopek-Sondi, 2004). There are also other studies that have demonstrated antibacterial activities of AgNPs on both gram positive and gram-negative bacteria. The main factor affecting

antibacterial activities of AgNPs is the size due to the fact that smaller AgNPs are more capable of penetrating the bacterial cell walls and this leads to cytotoxicity. AgNPs also have significant antifungal activities base on some researches. In one of the studies, AgNPs exhibited strong antifungal activity against *Trichophyton mentagrophytes* and *Candida species* (Kim *et al.*, 2008).

AgNPs exhibit a lot of useful characteristics with regards to the medical field as discussed. This study, however, will be focusing on the anticancer activity of the AgNPs. The anticancer activity of AgNPs will be further discussed in detail in the next subchapter.

2.11 Anticancer Activity of Silver Nanoparticles

In recent years, silver nanoparticles (AgNPs) have been extensively studied to determine its anticancer effects on a number of different cancer cell lines. These *in vivo* and *in vitro* studies have uncovered promising anticancer activity of AgNPs on many different cancer cell lines. There are also studies that demonstrate anti-inflammatory and antiangiogenic activities of AgNPs. Inflammation is known to contribute to a development of cancerous cells in human body while angiogenesis is a crucial factor for growth and expansion of solid tumors. It seems that silver nanoparticles are capable of attacking cancer cells through multiple pathways to achieve its anticancer effects (Kovács *et al.*, 2016).

Silver nanoparticles, formed from plant extract have other added advantages such as the incorporation of active compounds from the plant extract in synthesized silver nanoparticles. These silver nanoparticles could possibly have better anticancer activities compared to normal silver nanoparticles, merely composed out of silver. The presence of active compounds, together with silver could possibly have synergistic

effects contributing to better anticancer activities compared to individual plant extract or silver nanoparticles. Silver nanoparticles are superior candidate for this study due to ease of production, relatively low cost and strong anticancer activities according to previous studies (Lee and Jun, 2019).

There are a few *in vitro* studies that have demonstrated anticancer activities of biologically produced AgNPs using different plant extracts. In a study by Satyavani *et al*., the callus extract from *Citrullus colocynthis* was utilized to synthesize AgNPs. The AgNPs were then used to treat Human epidermoid larynx carcinoma (HEp -2) cells (Satyavani *et al.*, 2011). They performed a number of experiments which were MTT assay, caspase -3 assay, lactate dehydrogenase leakage assay and DNA fragmentation on treated cells. They determined that 500nM of the AgNPs was able to reduce viability of HepG2 cells to 50% with only five hours of treatment. Base on the caspase-3 assay, lactate dehydrogenase leakage assay, and DNA fragmentation results, they determined that AgNPs caused HepG2 cytotoxicity through induction of apoptosis. In another study, Sankar *et al*. produced AgNPs from herb called oregano - *Origanum vulgare*. The AgNps were evaluated *in vitro* against human lung cancer - A549 cells. It was determined based on this study that 100ug/ml of AgNPs was capable of inducing 50% cell death in the A549 cells. The cytotoxic effects of AgNPs were possibly due to presence of bioactive compounds such as carvacrol, quercetin, and apigenin as capping agents of the AgNPs (Sankar *et al.*, 2013).

There are even *in viv*o studies that show anticancer activity of AgNPs on mice models. For example, a study by Antony *et al.* used AgNPs synthesized from *Ficus religiosa* leaf extract to treat mice induced with Dalton's ascites lymphoma (DAL). In their study, swiss albino mice induced with DAL cells and treated with 50 ug/ml of *F. religiosa* derived AgNPs showed significant increment in lifespan compared to

untreated control (Antony *et al.*, 2013). Observation of blood vessel development of the mice treated with AgNPs showed that angiogenesis was inhibited compared to untreated mice which has high levels of angiogenesis. This suggested that *F. religiosa* derived AgNPs have antiangiogenesis effects on the mice. Base on AO/EB staining of DAL cells extracted from the mice, AgNPs treated mice had much more significant number of DAL cells which were undergoing apoptosis. The results of the study indicated that *F. religiosa* derived AgNPs have significant cytotoxic effects against DAL cells in the mice model as it could induce apoptosis of the DAL cells, also have antiangiogenesis effects and extended the lifespan of mice treated with AgNPs. Sukirtha *et al*. produced AgNPs using *Melia azedarach* extract and conducted *in vitro* and *in vivo* tests of its cytotoxicity (Sukirtha *et al.*, 2012). The *in vitro* test was conducted on human cervical cancer - HeLa cells and *in vivo* was conducted on dalton's ascites lymphoma (DAL) mice model. The AgNPs confirmed dose dependant cytotoxicity on HeLa cells and IC_{50} was determined to be 300ug/ml. The AgNPs also did not show significant cytotoxicity against normal HBL 100 cells while cytotoxicity only presented at high concentrations. The *in vivo* test showed that mice treated with AgNPs had a significantly higher lifespan and the DAL cells have increased apoptosis, compared to untreated mice (Sukirtha *et al.*, 2012).

Anticancer activities of AgNPs, synthesized from *Moringa oleifera* extract have also been demonstrated in a few studies. In a study by Vasanth *et al.*, synthesized AgNPs using *Moringa oleifera* stem bark extract was tested on human cervical carcinoma cells – HeLa. Based on their study, AgNPs caused cytotoxicity HeLa cells in dose dependant manner with 100 ug/ml of IC_{50} (Vasanth *et al.*, 2014). Further experiments showed that AgNPs induced cell death through apoptosis by increasing reactive oxygen species (ROS) in the HeLa cells. In a different study, Nayak *et al.*

synthesized three different AgNPs with three different plant extracts: *Cucurbita maxima* (petals), *Moringa oleifera* (leaves) and *Acorus calamus* (rhizome). Based on the *in vitro* study on A431 skin cancer cell line, IC_{50} values of AgNPs synthesised from *C. maxima, M. oleifera* and *A. calamus* were 82.39, 83.57 and 78.58 ug/ml (Nayak *et al.*, 2015). The moringa derived AgNPs showed significant anticancer activities on the A431 cells which were comparable to the AgNPs derived from *C.maxima* and *A.calamus*.

Based on these studies, it can be observed that AgNPs, derived from plant extracts exhibited significant anticancer activities, both *in vitro* and *in vivo* on many different cancer cell lines. There are also studies that demonstrated anticancer of AgNPs synthesized from *Moringa oleifera* plant extract. AgNPs derived from moringa plant extract showed promising potentials as an anticancer agent; therefore, it has been decided to further explore anticancer activities of moringa leaf extract synthesized AgNPs on Kasumi-1 cells in this study.

2.12 Cancer Biology

Uncontrolled cell growth leads to cancer. What distinguishes cancer cells from normal cells is the existence of multiple nuclei with coarse chromatin in them. The nature of cancer cells is just like any other normal cells. However, they are unique in the sense that they have the capacity for self-renewal and differentiation. Defective DNA structures in cancer cells may cause them to function and reproduce abnormally. This type of damage results in the mutations of certain DNA barriers. Consequently, the transfer and amplification of DNA may be affected and this can possibly trigger DNA transcription disorders. According to Croce (2008), cancer does not occur if there is only a single disorder in any event (Croce, 2008). Various steps are involved in the process which leads up to cancer occurrence (Ruddon, 2007). Proto-oncogene is a