EXTRACTING BIOACTIVE COMPOUNDS FROM *MORINGA OLEIFERA* LEAVES FOR ANTICANCER PRODUCT

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EXTRACTING BIOACTIVE COMPOUNDS FROM *MORINGA OLEIFERA* LEAVES FOR ANTICANCER PRODUCT

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Thesis submitted in partial fulfilment of the requirements
for the award of the degree of
Bachelor of Chemical Engineering

Faculty of Chemical & Natural Resources Engineering
UNIVERSITI MALAYSIA PAHANG

DECEMBER 2016

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Dedicated to my family

ACKNOWLEDGEMENT

I would like to express my deepest gratitude and appreciation to my beloved supervisor, Dr. Eman N. Ali for the endless motivation and guidance throughout my undergraduate research project. I am thankful for the suggestions, honest comments and advice on my research project as they served as a continuous encouragement in order for me to work harder and improve my research work. You have been a wonderful supervisor as well as a friend to me, always responsive to any question that I bring forward to you regardless on weekends or even public holidays. Without your support, my journey would have been a difficult one.

A special thanks to my family for showering me with love and support throughout my entire research project as well as keeping me in your prayers. Apart from that, I thank my friends and course mates who have helped me in one way or another in my research project in terms of knowledge sharing, inspiration and support towards achieving my goal.

I am also indebted to the Ministry of Higher Education and Universiti Malaysia Pahang for funding my study.

Besides that, I am sincerely grateful to the staffs of Chemical Engineering and Natural Resources Faculty who have been kind and resourceful in assisting me whenever I had any enquiries.

ABSTRACT

Cancer is one of the most dangerous illness in the world. It occurs when cells in the body divide at an uncontrolled rate which then produces lumps or masses of tissue known as tumour. In addition, it affects the digestive, nervous and circulatory systems, these tumours also produce hormones which disrupts body functions. A benign tumour which remains in the same spot is harmless. On the contrary, a malignant tumour is dangerous as it may destroy healthy cells. These malignant tumours spread to healthy cells through the blood or lymphatic system. This condition is known as metastasis. Nowadays, various modern cancer treatments are available including surgery, drugs, chemotherapy, radiation therapy, steroid medications, and hormonal therapy. However, there are numerous side effects that arise from these modern treatments such as heart, lung, endocrine system, bone, joint, soft tissue, brain, spinal cord, nerve, memory, dental, vision and digestion problems. Moreover, the patient may suffer from emotional difficulties, fatigue as well as develop secondary type of cancer. In order to prevent side effects which arise from modern cancer treatments, a more natural approach with no detrimental effects to human health is required to combat cancer. The objectives of this research is to extract the bioactive compounds from the leaves of *Moringa oleifera* and to test the effect of the extracted material on cancer cells. In this study, the bioactive compounds from *Moringa oleifera* leaves is extracted using two methods which are the Soxhlet extraction method using ethanol, 2-propanol, acetone, petroleum ether and water solvent and the soaking method using ethanol and boiling water as solvent. Sample *S2* which employed the Soxhlet extraction technique using water as the solvent exhibited significant cytotoxic activity against the human breast adenocarcinoma cancer cell line (MCF-7) in a concentration dependent manner with an IC_{50} value of 81.77±6.05μg/mL. The other samples showed no cytotoxic activity.

ABSTRAK

Kanser merupakan penyakit yang paling berbahaya di dunia ini. Kanser berpunca daripada penghasilan tumor melalui pembahagian sel-sel badan pada kadar yang tidak terkawal. Selain menjejaskan sistem pencernaan, sistem saraf dan sistem peredaran darah, tumor kanser turut menghasilkan hormon yang mengganggu fungsi badan. Tumor *benign* dianggap tidak membahayakan, tumor *malignant* pula sebaliknya kerana tumor ini mampu menghapuskan sel-sel yang sihat. Penyebaran tumor *malignan*t boleh berlaku melalui sistem peredaran darah dan limfa. Keadaan ini dikenali sebagai metastasis. Pada masa kini, terdapat pelbagai rawatan kanser yang moden seperti pembedahan, ubat-ubatan dadah, kemoterapi, terapi radiasi, ubat-ubatan steroid serta terapi hormon. Walau bagaimanapun, terdapat pelbagai kesan sampingan yang timbul melalui rawatan moden ini seperti masalah jantung, paru-paru, endokrin, tulang, sendi, tisu lembut, otak, saraf tunjang, saraf, ingatan, pergigian, penglihatan dan sistem pencernaan. Lebih-lebih lagi, pesakit mungkin menghadapi masalah emosi, keletihan dan masalah penghasilan kanser *secondary*. Bagi melawan penyakit kanser, kaedah semula jadi yang tidak memudaratkan kesihatan manusia patut digunakan bagi mengurangkan kesan negatif yang timbul akibat rawatan kanser moden. Objektif kajian ini adalah untuk mengekstrak kompoun bioaktif yang terkandung dalam daun *Moringa oleifera* serta untuk menguji aktiviti sitotoksik ekstrak terhadap kanser sel adenokarsinoma payudara manusia (MCF-7). Menerusi kajian ini, kompoun bioaktif yang terkandung dalam daun *Moringa oleifera* diekstrak melalui dua jenis kaedah iaitu kaedah pengekstrakan Soxhlet dengan menggunakan ethanol, 2-propanol, acetone, petroleum ether dan air sebagai pelarut serta kaedah rendaman menggunakan ethanol dan air sebagai pelarut. Sample *S2* yang mengaplikasikan kaedah pengekstrakan Soxhlet dengan menggunakan air sebagai pelarut menunjukkan aktiviti sitotoksik (nilai IC_{50} = 81.77±6.05µg/mL) yang positif terhadap kanser cell yang diuji. Sample yang lain tidak memaparkan activity sitotoksik.

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LIST OF ABBREVIATIONS

EBV	Epstein-Barr virus
HCT-8	ileocecal adenocarcinoma cancer cells of a 67 year old male
MDA-MB-231	breast mammary gland cancer cells
DPPH	2,2'diphenylpicrylhydrazyl assay
ALL	acute lymphoblastic leukemia
AML	acute myeloid leukemia
Panc-1	human pancreatic cancer cell lines
COLO-357	human pancreatic cancer cell lines
p34	human pancreatic cancer cell lines
A549	human lung cancer cell
MCF 7	epithelial breast cancer cell line
MDA MB 231	mesenchymal breast cancer cell line
CAE	chlorogenic acid equivalents
1QE	isoquercetin equivalents
SZ	Squeezing
DF	Decoction of fresh leaves
DD	Decoction of dried leaves
MF70	Maceration of fresh leaves with 70% ethanol
MD50	Maceration of dried leaves with 50 % ethanol
MD70	Maceration of dried leaves with 70% ethanol
PD50	Percolation of dried leaves with 50 ethanol
PD70	Percolation of dried leaves with 70% ethanol
SD50	Soxhlet extraction of dried leaves with 50 % ethanol
SD70	Soxhlet extraction of dried leaves with 70% ethanol
ND	Not detected
N/A	Not available
LE	Leucocyanidin equivalent
DE	Diosgenin equivalent.
IC50	50% inhibitory concentration
HepG2	human hepatocellular carcinoma
Caco-2	colorectal adenocarcinoma
MCF-7	breast adenocarcinoma
COS-7	African green monkey normal cells
TPC	Total phenolic content
TFC	Total flavonoid content
GAE	gallic acid equivalence
CE	catechin equivalence
QE	quercetin equivalence

CHAPTER 1

INTRODUCTION

1.1 Background of the Study

In adults, cells divide in order to replace dead cells and the worn out cells or to repair injured cells. Cancerous cells form when cells at a particular site of the body begin to divide at a high rate. The growth of cancer cells are unlike normal cell growth. These cancer cells continue to grow and form new, abnormal cells containing abnormal DNA. These abnormal cells which contain the same defective DNA as the original cancer cell may invade other tissues (Mandal, 2013). Cancer cells have the ability to invade and destroy surrounding normal tissue. In addition, they possess the ability to travel and grow in other parts of the body. This condition is known as metastasis and it is the most lethal attribute of cancer cells. More than 90% of the morbidity and mortality associated with cancer is due to metastasis (AACR Cancer Progress Report, 2012). Some of the common causes of cancer include smoking, obesity, alcohol, lack of physical activities, unhealthy eating habits, sun and UV, air pollution, infections, hormones and workplace conditions.

Many anticancer treatments are targeted to kill rapidly growing cells as cancer cells grow and divide at a faster pace compared to normal cells. One of these treatments is chemotherapy. In conjunction to this, chemotherapy may affect certain healthy cells that also multiplies rapidly including blood cells forming in the bone marrow, cells in the digestive tract (mouth, oesophagus, stomach, intestines),

reproductive system (sexual organs), and hair follicles. Anticancer drugs are used to treat cancer cells besides chemotherapy. However, vital organs such as the heart, lungs, kidneys, bladder and nervous system may be affected by anticancer drugs (National Cancer Institute, 2004). Anti-cancer antibiotics work by altering the DNA of cancer cells to prevent them from growing and multiplying. These drugs can permanently damage the heart if given in high doses (American Cancer Society, 2015). The development of more aggressive regimens as well as newer agents and combination chemotherapies have resulted in the significant increase of chemotherapy induced ocular side effects (Parul & Abhishek, 2012). In hospitals, conventional drugs are commonly prescribed to cancer patients. However, the research on medicinal plants and cancer has been intensified due to less toxic and adverse effects of phytochemicals (Johnson, 2007).

Herbal medicine uses plants or mixtures of plant extracts to treat illness. It aims to restore the body's ability to protect, regulate and heal from diseases. It is a whole body approach which focuses on the physical, mental and emotional well-being of an individual. Cancer patients turn to herbal medicine as one of the most commonly used alternative therapy. Certain studies have shown that in every 10 cancer patients, 6 of them use herbal remedies alongside conventional cancer treatments (Cancer Research U.K., 2015).

Many plants have been known to exhibit anticancer properties. Some of these anticancer herbs includes aloe vera. Aloe vera consists of a substance known as 1,8-dihydroxy-3-[hydroxymethyl]-anthraquinone which has been proven to induce cell death among T24 cells, human bladder cancer cell line (Lin et al., 2006). Besides that, *Artemisia annua* demonstrated anticancer activity. Artemisinin and its derivatives have been shown to induce apoptosis of prostate cancer cells and to possess activity against breast cancer, leukemia, colon cancer, and other cancer cells (Yoshiyuki et al., 2010). Apart from that, bitter melon (*Momordica charantia*) extract was effective against human breast cancer cells and primary human mammary epithelial cells. It was able to reduce the proliferation of cancer cells and induce cell death among breast cancer cells (Ray et al., 2010).

Moringa oleifera is a native plant in India. It is found growing in the Sub-Himalayan regions of Northern India. *Moringa oleifera* has become an important crop in India, Ethiopia, the Philippines and Sudan. It is being grown in Africa, Asia, Latin-America, the Caribbean, Florida and the Pacific Islands. Presently, it is a world-wide plant found in the tropics and non-tropics. *Moringa oleifera* is commonly known as the drumstick tree, miracle tree and horseradish tree (Centre for Jatropha Promotion & Biodiesel, 2007).

Moringa oleifera leaves contain nutrients such as essential amino acids, vitamins, minerals and β-carotene (Sabale et al., 2008; Sharma et al., 2012). The extract from its leaves have the potential for cancer chemoprevention and was claimed as a therapeutic target for cancer (Sreelatha et al., 2011). There are various benefits of *Moringa oleifera* for medical purposes. Some of these benefits includes the treatment of edema, stomach disorders, diabetes, herpes, asthma, urolithiasis, anemia, neurodegenerative diseases, nephrotoxicity, hypertension, sickle cell disease, cholesterol, and obesity besides being used for liver protection, cardiovascular protection, maintaining healthy bones, wound healing and as an antioxidant. *Moringa oleifera* possesses antibacterial and antimicrobial properties, immunosuppressive properties, anti-allergenic qualities, anti-fungal qualities, anti-fertility qualities, and is effective against the growth of disease-causing microbes (Organic Information Services Pvt Ltd, 2016).

Niazimicin is a thiocarbamate, an organic compound which consists of sulphur. It is one of the bioactive compounds found in the leaves of *Moringa oleifera* that have been recognized with significant anticancer activity (Guevaraa et al., 1999). Niazimicin inhibits the tumour promoter teleocidin B-4 which induces Epstein-Barr virus (EBV) activation (Murakami et al., 1998). EBV causes mononucleosis which leads to fatigue that can be prolonged for weeks or for months (Ratini, 2015). The virus is spread through direct contact with saliva from the mouth of an infected person and cannot be spread through blood contact. An individual can be exposed to the virus by a cough or sneeze, by kissing, or by sharing food or drinks with someone who has the virus. Apart from that, Niazimicin inhibited tumour promotion in a mouse. Niazimicin and compound 4-(4'-O-acetyl-a-L-rhamnopyranosyloxy)benzyl

isothiocyanate in *Moringa oleifera* showed potential as an inhibitor of phorbol ester in lymphoblastoid (Burkitt's lymphoma) cells (Fahey, 2005).

Epidemiological studies have shown that a consistent generous intake of flavonoids which are synthesised in response to microbial infections have positive effects against combating cancer (Kumar & Pandey, 2013). Flavonoid compounds are present in the leaves of *Moringa oleifera* plant. The main flavonoids present are myricetin, quercetin and kaempferol. Phenolic acid which exhibits anticancer properties are also present in *Moringa oleifera* leaves apart from other compounds with similar properties such as tannins and saponins. The concentration of these compounds vary in dried leaves and freeze dried leaves and it may be due to different environmental conditions, harvesting season, plant genetic, leaves maturity stage, drying method, extraction method and sensitivity of the analytical methods (Leone et al., 2015). Thus, further investigations are required in order to obtain substantial proof on the compounds in *Moringa oleifera* leaves which can be used to fight cancer.

Moringa oleifera has long been recognized by folk medicine practitioners as being valuable in tumour therapy. Modern medicine involve the use of crude extracts while neglecting the bioactive compounds. In order to achieve a level of proof required for full biomedical endorsement of *Moringa oleifera* as a cancer preventative plant, a more rigorous study needs to be conducted. Through extensive research, native plants like *Moringa oleifera* may be the key to cancer prevention and therapy (Fahey, 2005).

1.1.1 Botanical Description of *Moringa oleifera*

Moringa is a slender softwood tree that branches freely and can grow rapidly. Generally, it is considered a small to medium size tree although it can grow up to a height of 10m (Radovich, 2009). Its leaves are feathery with green to dark green elliptical leaflet 2cm long (Morton, 1991). Its flowers are pleasantly fragrant and 2.5cm wide are produced profusely in auxiliary, drooping panicles 10-25cm long. The flowers are generally white to cream colour, although some varieties can be

tinged with pink (Sachan et al., 2010). The fruit is referred to as a 'pod' and it is a tri-lobed capsule. The immature pods are green and some have a reddish colour (Burkill, 1966). Besides being drought tolerant, *Moringa oleifera* is able to tolerate poor soil, a wide rainfall range of 25-300cm per year and soil pH from 5.0-9.0. *Moringa oleifera* is recognized for being drought tolerant because it is deciduous during the dry season and it has an enlarged underground rootstock. The matured dried seeds are round or triangular in shape and the kernel is surrounded by a lightly wooded shell with three papery wings (Makkar and Becker, 1997). Figure 1, 2, 3, 4 displays the flowers, leaves, pods and seeds respectively of the *Moringa oleifera* plant.

Figure 1: *Moringa oleifera* flowers.

Figure 2: *Moringa oleifera* leaves.

Figure 3: *Moringa oleifera* pods.

Figure 4: *Moringa oleifera* seeds.

1.1.2 Nutritional value of *Moringa oleifera*

The leaves of *Moringa oleifera* can be freshly eaten, cooked or stored as dried powder for many months without refrigeration and loss of nutritional value. *Moringa oleifera* is an important source of food in the tropics as the leaves are full at the end of the dry season while other foods may be scarce. Vitamin A, B and C, calcium, iron and protein have been found in significant quantities through the analysis of the *Moringa oleifera* leaves composition.

According to optima of Africa Ltd, a group that has been working with the tree in Tanzania, 25 grams daily of *Moringa oleifera* leaf powder will give a child the following recommended daily allowances, protein 42%, calcium 125%,

magnesium 61%, potassium 41%, iron 71%, vitamin A 272%, and vitamin C 22%. The fresh leaves of *Moringa oleifera* contains seven times the vitamin C of oranges, four times the vitamin A of carrots, four times the calcium of milk, three times the potassium of bananas and two times the protein of yogurt. The micro nutrient content is even more in the dried leaves, it contains ten times the vitamin A of carrots, 17 times the calcium of milk, 15 times the potassium of bananas, 25 times the iron of spinach, and nine times the protein of yoghurt, however the vitamin C drops to half that of oranges (Academia, 2016).

1.1.3 Chemical composition of *Moringa oleifera* leaves

Tables 1, 2, 3 and 4 presents the chemical composition, amino acids composition, mineral contents and fatty acids composition of dried *Moringa oliefera* leaves respectively which were studied by Moyo and colleagues in 2011.

Table 1: Chemical composition of dried *Moringa oleifera* leaves.

Nutritive Value	Dry Leaf	Standard Error
Moisture (%)	9.533	0.194
Crude protein (%)	30.29	1.480
Fat (%)	6.50	1.042
Ash (%)	7.64	0.433
Neutral detergent fibre [*1] (%)	11.40	0.425
Acid detergent fibre [*2] (%)	8.49	0.348
Acid detergent lignin [*3] (%)	1.8	2.204
Acid detergent cellulose (%)	4.01	0.101
Condensed tannins (mg/g)	3.12	0.104
Total polyphenols (%)	2.02	0.390

[*1] **Neutral detergent fibre**: The portion of fibre that is composed of hemicellulose, cellulose and lignin.

[*2] **Acid detergent fibre**: The cell wall portions of the forage that are made up of cellulose and lignin. It often is used to calculate digestibility.

[*3] **Acid detergent lignin**: The percentage of plant material which is insoluble in 72 percent sulfuric acid. Lignin reduces digestibility and has been used to predict digestibility.

Table 2: Amino acids composition of dried *Moringa oleifera* leaves.

Amino Acid	Quantity (mean+/- %)	Standard Error
Arginine	1.78	0.010
Serine	1.087	0.035
Aspartic acid	1.43	0.045
Glutamic acid	2.53	0.062
Glycine	1.533	0.060
Threonine*	1.357	0.124
Alanine	3.033	0.006
Tyrosine*	2.650	0.015
Proline	1.203	0.006
HO-Proline	0.093	0.006
Methionine*	0.297	0.006
Valine*	1.413	0.021
Phenylalanine*	1.64	0.006
Isoleucine*	1.177	0.006
Leucine*	1.96	0.010
Histidine*	0.716	0.006
Lysine*	1.637	0.006
Cysteine	0.01	0.000
Tryptophan*	0.486	0.001

Table 3: Mineral contents of dried *Moringa oleifera* leaves.

Mineral	Dry Leaf	Standard Error
Macro-elements (%)		
Calcium %	3.65	0.036
Phosphorus %	0.30	0.004
Magnesium %	0.50	0.005
Potassium %	1.50	0.019
Sodium %	0.164	0.017
Sulphur %	0.63	0.146
Micro-elements (mg/kg)		
Zinc (mg/kg)	31.03	3.410
Copper (mg/kg)	8.25	0.143
Manganese (mg/kg)	86.8	3.940
Iron (mg/kg)	490	49.645
Selenium (mg/kg)	363.00	0.413
Boron (mg/kg)	49.93	2.302

Table 4: Fatty acids composition of dried *Moringa oleifera* leaves.

Fatty Acid	Quantity (mean+/- %)	Standard Error

Ether extract	6.50	0.041
Capric (C10:0)	0.07	0.064
Lauric (C12:0)	0.58	0.402
Myritic (C14:0)	3.66	1.633
Palmitic (C16:0)	11.79	0.625
Palmitoleic (C16:1c9)	0.17	0.056
Margaric (C17:0)	3.19	0.155
Stearic acid (C18:0)	2.13	0.406
Oleic (C18:1c9)	3.96	2.000
Vaccenic (C18:1c7)	0.36	0.038
Linoleic (C18;2c9,12(n-6)	7.44	0.014
α-Linolenic (C18:3c9,12,15(n-3)	44.57	2.803
g-Linolenic (C18:3c6,9,12 (n-6)	0.20	0.013
Arachidic (C20:0)	1.61	0.105
Heneicosanoic (C21:0)	14.41	0.194
Behenic (C22:0)	1.24	0.383
Tricosanoic (C23:0)	0.66	0.025
Lignoceric (24:0)	2.91	0.000
Total saturated fatty acids (SFA)	43.31	0.815
Total mono unsaturated fatty acids (MUFA)	4.48	1.984
Total poly unsaturated fatty acids (PUFA)	52.21	2.792
Total Omega-6 fatty acids (n-6)	7.64	0.012
Total Omega-3 fatty acids (n-3)	44.57	2.805
PUFA: SFA (PUFA:SFA)	1.21	0.096
n-6/n-3	0.17	0.016
PUFA: MUFA (PUFA:MUFA)	14.80	7.168

Table 5 exhibits the comparison of the nutritional value for the fresh raw leaves and leaf powder of *Moringa oleifera* plant per 100g of edible portion. The values were obtained from an article written by Lowell. The link to the article is provided in the reference.

*16g N which is equivalent to g/100g protein.

Table 5: The nutritional value of *Moringa oleifera*.

	Fresh raw leaves	**Leaf powder**
Moisture (%)	75.0	7.5
Calories	92.0	205
Protein (g)	6.7	27.1
Fat (g)	1.7	2.3
Carbohydrate (g)	13.4	38.2
Fiber (g)	0.9	19.2
Minerals (g)	2.3	-
Ca (mg)	440.0	2,003
Mg (mg)	24.0	368
P (mg)	70.0	204
K (mg)	259.0	1,324
Cu (mg)	1.1	0.57
Fe (mg)	7.0	28.2
S (mg)	137.0	870
Oxalic acid (mg)	101.0	1.6%
Vitamin A - B carotene (mg)	6.8	16.3
Vitamin B -choline (mg)	423.0	-
Vitamin B1 -thiamine (mg)	0.21	2.64
Vitamin B2 -riboflavin (mg)	0.05	20.5
Vitamin B3 -nicotinic acid (mg)	0.8	8.2
Vitamin C -ascorbic acid (mg)	220.0	17.3
Vitamin E -tocopherol acetate (mg)	-	113
Arginine (g/16g N) *	6.0	1.33%
Histidine (g/16g N) *	2.1	0.61%
Lysine (g/16g N) *	4.3	1.32%
Tryptophan (g/16g N) *	1.9	0.43%
Phenylanaline (g/16g N) *	6.4	1.39%
Methionine (g/16g N) *	2.0	0.35%
Threonine (g/16g N) *	4.9	1.19%
Leucine (g/16g N) *	9.3	1.95%
Isoleucine (g/16g N) *	6.3	0.83%
Valine (g/16g N) *	7.1	1.06%

1.2 Motivation

Many cancer patients around the world are in desperate need of new cancer treatment, especially those who are in the critical stage. For some of these patients, it may be their only hope for survival. Advanced cancer treatments such as chemotherapy have adverse effects on human health. Certain cancer cells find a way to grow back after treatment. These cancer cells have found a way to overcome modern treatment and they are no longer responding to the drugs that have previously worked. The human body is believed to have the ability to defend itself against various diseases including cancer. However, the body's immune system has to be triggered in order to strengthen its defenses against cancer cells. *Moringa oleifera* is a natural approach which may be the key to cure or at least to prevent cancer without side effects on the human body.

1.3 Problem Statement

Cancer is one of the most dangerous illness in the world. It has high mortality rate and it affects many people regardless of gender or age. Many modern cancer treatments which are currently available have side effects on the human body. In addition, some of these treatments require high cost over a long term period. This life threatening disease needs to be prevented and cured. Therefore, the search for new products to serve this purpose is of great importance, especially one of which is natural and non-detrimental to human health.

1.4 Objectives

The objectives of this research are:

1) To extract the bioactive compounds from the leaves of *Moringa oleifera* using different solvents.
2) To test the effects of the extracted material on cancer cells.

1.5 Scopes of Study

The scope of this research is to study the effects of *Moringa oleifera* on cancer cells by extracting the bioactive compounds from the leaves to be used for cancer cell treatment.

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

According to previous research, the *Moringa oleifera* leaves extract showed positive anticancer activity against various cancerous cell lines including breast, colorectal, leukemia, hepatocarcinoma, lung and pancreatic cancerous cells. The anticancer properties are due to the presence of bioactive compounds such as phenolics, flavonoids, tannins and saponins in the *Moringa oleifera* leaves.

2.2 Anticancer properties of *Moringa oleifera* leaves against various cancer cells

In the year 2015, Al-Asmari and his colleagues conducted a research to determine the effects of *Moringa oleifera* leaves on breast and colorectal cancer cell lines. Soxhlet apparatus was used to perform the extractions. Around 60g of dried leaves were grinded into coarse powder which was then added with 600ml of ethanol. The extraction process was continued for 6-8 hours. The soluble extracts were then filtered and the solvent was evaporated using a rotary evaporator. The resulting extracts were collected and stored at 4°C. Gas Chromatography-Mass Spectrometry was used to analyse the ethanol extracts of *Moringa oleifera* leaves. The analysis produced twelve peaks on the chromatogram, indicating the presence of twelve compounds. The extracts of the leaves were mainly comprised of thiocynates, hydrocarbons and fatty acids. The effect of *Moringa oleifera* leaves extract was tested on cancer cells HCT-8 which were derived from the ileocecal adenocarcinoma of a 67 year old male and MDA-MB-231 which were obtained from the breast mammary glands. When treated with the extract of the leaves, there was a significant decrease in the cell survival if MDA-MB-231 and HCT-8. However, this depended on the dosage of the extracts. Based on preliminary

studies on cell survival, it was discovered that a concentration of 250μg/ml and below did not manage to significantly reduce the cell survival. In addition, high concentrations above 500μg/ml lead to toxicity and high disruption on cell survival. The result of this study strongly implies that cancer cells survival mechanism are suppressed under the influence of *Moringa oleifera* leaves extracts.

Besides that, the active principle from *Moringa oleifera* lam leaves was found to be effective against two leukemias and a hepatocarcinoma by Mutasim and colleagues in the year 2010. For the extraction process, 1g of freeze-dried, powdered leaves were added in three different solutions which are 10ml hot water, cold water and 80% (v/v) ethanol. The extracts were mechanically stirred for 12 hours at room temperature except the hot water extract. Centrifugation was used to remove the solids and the supernatant was collected. The resulting extracts were completely dried in a rotary evaporator at 40°C, lyophilized and stored at 4°C for further process (El-Shemy et al., 2007; Khalafalla et al., 2009). 2,2'diphenylpicrylhydrazyl (DPPH) assay was used to evaluate the antioxidant activity of the plant extracts (Cuendet et al., 1997; Burits and Bucar, 2000). The extracts (5-20 μg in 50 μl) were added to 5 ml of a 0.004% (w/v) of DPPH in methanol (100% v/v). The cancer cells were harvested from adult leukemia patients admitted to the National Cancer Institute, Cairo University. A high percentage (70-86%) of abnormal cells (harvested from 10 patients suffering from acute lymphoblastic leukemia (ALL) and 15 with acute myeloid leukemia (AML)) were killed as well as a culture of hepatocarcinoma cells with 75% death. Hot water and ethanol extracts had the most significant results. After an incubation period of 24 hours, the mononuclear ALL cells with ethanolic extract had 82% of lymphoblast destruction and 86% of AML cells were destroyed. (Mutasim et al., 2010).

Another study conducted by Berkovich and colleagues in 2013 found that *Moringa oleifera* aqueous leaves extract down-regulates nuclear factor-kappa beta and increases cytotoxic effect of chemotherapy in pancreatic cancer cells (Panc-1, COLO-357 and p34). Human pancreatic cancer cell line, p34 which was developed from pleural effusion of a pancreatic cancer patient, was kindly provided by Dr. Alex Starr (Laboratory of Lung Biology, Lung and Allergy Institute, Tel Aviv Sourasky Medical Center, Israel). 1g of dried and powdered *Moringa oleifera* leaves were mixed with boiling water for 5 minutes. The mixture was then filtered with a 2μm pore sterile filter

paper. The aqueous extract was stored at 4°C. The cultured pancreatic cells were exposed to *Moringa oleifera* leaves extract for 72 hours at a concentration of 100-200 µg/mL. *Moringa oleifera* extract successfully inhibited the growth of all three tested cell lines. Panc-1 cells were more prone to the treatment at a concentration of 1.1 mg/ml compared to COLO-357 at 1.8 mg/ml and p34 cells at 1.5 mg/ml. Significant inhibition of Panc-1 cell survival occurred at a concentration of 0.75 mg/mL whereas a higher concentration of 1.5mg/mL was required in the other two cell lines. 98% reduction of Panc-1-cell survival was achieved at a concentration of 2mg/mL of *Moringa oleifera* extract.

In addition, a study from Jung in the year 2014 discovered a new anticancer activity in the soluble extract from *Moringa oleifera* leaves. Clonogenic survival assay was used to assess the surviving ability of human lung cancer cell A549 following the treatment with *Moringa oleifera* leaves extracts. 150g of dried *Moringa oleifera* leaves were suspended in 1mL of cold water at a temperature of 4°C. After applying 30 seconds of vigorous vortex, the suspension was refrigerated for 5 minutes to 24 hours. The suspension was then vigorously vortexed again for a minute at room temperature. Centrifugation at 12000rpm, 10min each were used to remove the insoluble parts of the suspension. A 0.2µm filter was used to obtain the supernatant by means of membrane filtration. After lyophilisation, the *Moringa oleifera* extracts produced were stored at a temperature of 20°C.Resuspension of the *Moringa oleifera* extracts in distilled water was required for the experimental purpose. The A549 cells were allowed to adhere for a time period of 24 hours before being treated with increasing concentrations of *Moringa oleifera* leaves extracts. The colonies were fixed and stained for a minute, washed with distilled water and then photographed. As the concentration of *Moringa oleifera* leaves increased, the fraction of surviving colonies significantly decreased. There was no colonies that survived above a concentration of 100µg/mL. Figure 5(A) and (B) displays the cytotoxic effect of the *Moringa oleifera* leaves extract against the A549 cancer cell line under microscopic view (×1000) and the number of viable colonies after treatment with the *Moringa oleifera* leaves extract respectively. Figure 6 shows of the percentage survival of the cancer cells and normal cells after they have been exposed to treatment with the *Moringa oleifera* leaves extract.

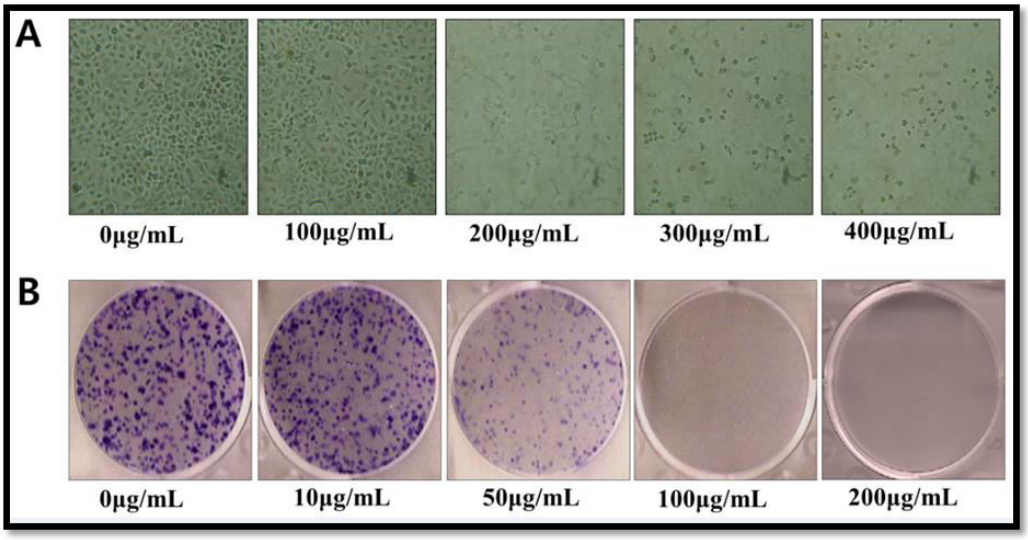

Figure 5(A): Microscopic view of the cytotoxic effect, (B): Number of viable colonies.

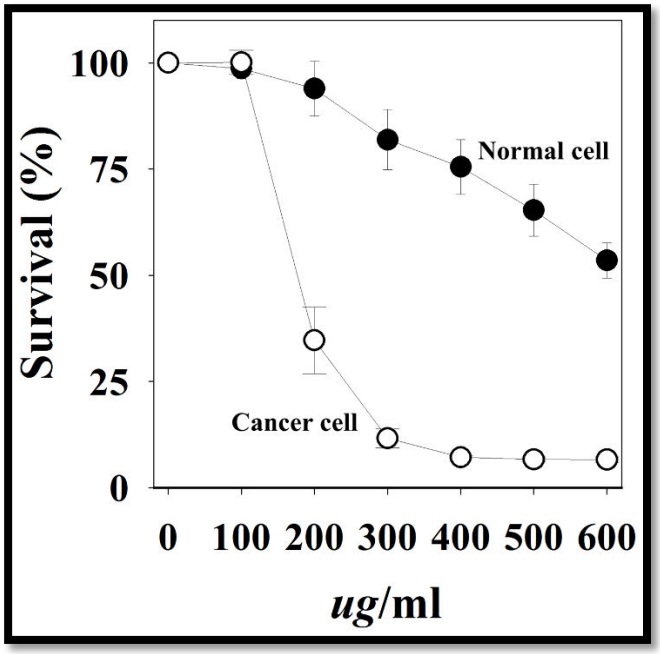

Figure 6: The percentage survival of cancer cells and normal cells after treatment.

According to Figure 6, the normal COS-7 cells are more resistant to the *Moringa oleifera* leaves extract as compared to the A549 cancer cells. At a concentration above 300µg/mL, the *Moringa oleifera* leaves extract managed to kill all the A549 cancer cells whereas the COS-7 normal cells exhibited a slow decrease in cell viability. At a high concentration of 600µg/mL, there is still more than 50% of COS-7 normal cell survival. It can be concluded that the *Moringa oleifera* leaves extract is highly specific of its cytotoxic ability against cancer cells.

In 2014, Nilanjana discovered the anticancer effect of *Moringa oleifera* leaves extract on human breast cancer cell. *Moringa oleifera* leaves collected were washed twice with distilled water and shade dried for a period of 72 hours. A domestic mixer grinder was used to powder the leaves into coarse powder. There were two methods of extraction in this experiment. The first method is the soxhlet extraction method. Using Soxhlet apparatus, 75g of the dried leaf powder was extracted in succession using petroleum ether, chloroform, ethyl acetate, methanol and water. Each extract that was obtained from the successive extraction was filtered with Whatman No.1 filter paper and dried to a semi-solid mass using water bath. The yield of each extract was then stored in a refrigerator at -20°C. The second method is the crude methanolic extraction. 100g of air dried *Moringa oleifera* leaf powder was mixed with 1000ml pure methanol in a round bottomed flask. The solution was kept at room temperature for 48 hours and was masticated with occasional stirring. Using suction apparatus, the supernatant was recovered after filtration through folded paper. The solvent was removed by rotary vacuum evaporator. The extract was freeze dried and stored in a -20°C freezer.
In this experiment, the cell lines tested on were epithelial breast cancer cell line, MCF 7 and mesenchymal breast cancer cell line, MDA MB 231. The effect on both cell lines indicated that it was time dependent and dose dependent. After 24 hours, the treated cells (30-40%) rounded up and was floating. After 48 hours, the percentage of rounded and floating cells increased (50-60%). Therefore, it can be predicted that *Moringa oleifera* leaves induce apoptosis in both MCF 7 and MDA MB 231.

Apart from that, in 2014, Suphachai discovered that *Moringa oleifera* leaves contained antioxidant and anticancer activities. The leaves of *M. oleifera* were collected from Lampang Herb Conservation, Lampang, Thailand. The air-dried leaves of *M. oleifera* were grounded into powder and stored at 4°C until extraction. 15g of leaf powder were extracted with 350 ml of methanol. Then, the liquid extract was filtered through Whatman no. 1 filter paper. The residue was subsequently extracted with 350 ml of dichloromethane. The solvent were evaporated and the extracts were lyophilized to obtain two crude extracts: methanol and dichloromethane extracts (ME and DE). Both extracts were kept in amber glass at -20°C until use. The antiproliferation was tested on HepG2, Caco-2, MCF-7. Each cancer cell type was incubated with various concentrations of extracts (0 – 250 μg/ml) for 48 h. It was found that both extracts (0 to 250 μg/ml) contributed to similar cancer cell viability patterns. DE was more cytotoxic

than ME. It showed a IC50 of 120.37±2.55, 112.46±3.74 and 133.58±2.47 µg/ml for HepG2, Caco-2 and MCF-7, respectively, while ME exhibited less cytotoxicity to all cancer cell lines (IC50 > 250 µg/ml).

In 2013, Charlette and colleagues conducted a study on *Moringa oleifera* crude aqueous leaves extract to determine its antiproliferative effect against cancerous human alveolar epithelial cells. The *Moringa oleifera* leaves were collected from Durban, South Africa. A pestle and mortar was used to crush 10g of the air dried leaves before subsequent addition of 10mL deionized water. The extract was produced by 20 minutes of boiling with continuous stirring. The, it was transferred into 50mL conical tubes where centrifugation took place for 10 minutes at room temperature. The upper layer removed was succumbed to lyophilisation and stored at a temperature of 4℃. The viability of A549 cell line after 24 hours of exposure to *Moringa oleifera* leaves extract at increasing concentrations is shown is Table 6. The lowest cell viability of 62.73% was detected at a concentration of 250µg/ml of extract.

Table 6: The A549 cell viability after exposure to *Moringa oleifera* leaves extract.

Concentration (µg/ml)	**Cell viability (%)**
0	100
1	80.123
10	76.242
50	68.108
100	81.756
150	79.646
200	65.725
250	62.730
500	66.950

2.3 Bioactive compounds extracted from *Moringa oleifera* leaves

Appropriate extraction methods were conducted by Boonyadist in 2012 to maximize the total phenolics, total flavonoids content and the antioxidant activity of *Moringa oleifera* leaves extract. The *Moringa oleifera* leaves were collected from Baan Klang Sub-District, Muang District, Pathum Thani Province, Thailand. Different extraction methods including squeezing, decoction of fresh leaves, decoction of dried leaves, maceration of fresh leaves, maceration of dried leaves, percolation of dried leaves and Soxhlet extraction of dried leaves was used to extract the bioactive compounds from the *Moringa oleifera* leaves. Folin-Ciocalteu procedure was used to determine the content of the total phenolic compound and the aluminium chloride colorimetric method was used to analyse the content of the total flavonoid compound (Pothitirat et al., 2009). The absorbance was measured using a UV–VIS spectrophotometer. The maximum contents of total phenolics and total flavonoids, are 13.23 g CAE/100 g extract and 6.20 g IQE/100 g extract, respectively as shown in Table 7. Maceration of the dried leaves with 70% ethanol produced the highest content of total phenolics and flavonoids.

Table 7: Yields of crude extracts, contents of total phenolics and flavonoids.

Method	Yield of crude extract (% dry weight)*	Total phenolics		Total flavonoids	
		(g CAE/100 g extract)*	(g CAE/100 g dry powder)*	(g IQE/100 g extract)*	(g IQE/100 g dry powder)*
SZ	21.96 ± 1.11^{a} (5.28 ± 0.27)	2.35 ± 0.18^{a}	0.25 ± 0.02^{a}	0.95 ± 0.07^{a}	0.10 ± 0.00^{a}
DF	60.95 ± 1.57^{b} (14.66 ± 0.38)	4.76 ± 0.04^{b}	1.39 ± 0.04^{b}	0.98 ± 0.04^{a}	0.29 ± 0.02^{b}
DD	59.24 ± 1.14^{b}	4.41 ± 0.05^{b}	2.61 ± 0.06^{c}	0.91 ± 0.10^{a}	0.54 ± 0.07^{c}
MF70	42.70 ± 2.16^{c} (10.27 ± 0.52)	9.23 ± 0.52^{c}	1.90 ± 0.19^{d}	4.89 ± 0.21^{b}	1.01 ± 0.09^{d}
MD70	**40.50 ± 1.24^{c}**	**13.23 ± 0.55^{d}**	**5.35 ± 0.09^{e}**	**6.20 ± 0.48^{c}**	**2.51 ± 0.11^{e}**
MD50	38.34 ± 1.17^{cdg}	7.22 ± 0.16^{e}	2.93 ± 0.15^{c}	3.03 ± 0.20^{d}	1.23 ± 0.11^{f}
PD70	32.75 ± 1.93^{efh}	10.91 ± 0.30^{f}	3.71 ± 0.36^{f}	5.29 ± 0.05^{e}	1.80 ± 0.23^{g}
PD50	34.47 ± 1.41^{efgh}	7.36 ± 0.22^{e}	3.28 ± 0.19^{c}	3.30 ± 0.15^{d}	1.46 ± 0.06^{h}
SD70	35.87 ± 1.12^{dfgh}	12.47 ± 0.24^{g}	4.55 ± 0.22^{g}	6.71 ± 0.22^{f}	2.45 ± 0.07^{e}
SD50	33.58 ± 1.58^{efgh}	13.25 ± 0.59^{d}	4.46 ± 0.39^{g}	3.77 ± 0.09^{g}	1.27 ± 0.06^{f}

In 2015, Leone and colleagues studied the cultivation, genetic, ethnopharmacology, phytochemistry and pharmacology of *Moringa oleifera* leaves. Tables 8 and 9 presents the contents of phenolic acids and flavonoids respectively in *Moringa oleifera* leaves.

Table 8: Phenolic acids content in *Moringa oleifera* leaves.

Bioactive Compound (Phenolic Acids)	**Leaves**	**Value Found in Literature**	**Extractive Method**
Caffeic acid	dried	ND	50% MeOH
	dried	0.409 mg/g	50% MeOH, 100% MeOH and water
	freeze-dried	0.536 mg/g	80% EtOH
Chlorogenic acid	dried	0.018 mg/g	50% MeOH
	dried	0.489 mg/g	Water at 80 °C for 2 h
o -Coumaric acid	freeze-dried	6.457 mg/g	80% EtOH
p -Coumaric acid	freeze-dried	ND	80% EtOH
Ellagic acid	dried	ND	50% MeOH, 100% MeOH and water
	dried	0.009 mg/g	50% MeOH
	dried	0.189 mg/g	Water at 80 °C for 2 h
Ferulic acid	dried	0.078 mg/g	50% MeOH
	dried	0.078 mg/g	50% MeOH, 100% MeOH and water
	dried	0.128 mg/g	Water at 80°C for 2 h
Gallic acid	dried	ND	50% MeOH, 100% MeOH and water
	dried	1.034 mg/g	50% MeOH
	dried	1.034 mg/g	Water at 80 °C for 2 h
Gentistic acid	freeze-dried	ND	80% EtOH
Sinapic acid	freeze-dried	ND	80% EtOH
Syringic acid	freeze-dried	ND	80% EtOH

Table 9: Flavonoids content in *Moringa oleifera* leaves.

Bioactive Compounds	Leaves	Value Found in Literature	Extractive Method
Myricetin	dried	5.804 mg/g	MeOH + 1% *v/v* HCl + TBHQ
Quercetin	dried	0.281 mg/g	MeOH + 1% *v/v* HCl + TBHQ
	dried	0.207 mg/g	50% MeOH
	dried	0.207 mg/g	50% MeOH, 100% MeOH and water
	dried	0.807 mg/g	Water at 80 °C for 2 h
	dried	0.90 mg/g	MeOH + HCl + 10 mg ascorbic acid
	dried	5.2 mg/g	70% MeOH + 0.1% acetic acid
		5.8 mg/g	
		7.57 mg/g	
	freeze-dried	3.21 mg/g [f]	70% MeOH
		4.16 mg/g [h]	
	freeze-dried	9.26 mg/g	80% MeOH
		6.34 mg/g	
		7.70 mg/g	
	freeze-dried	5.47 mg/g [b]	70% MeOH
		9.1 mg/g	
		15.2 mg/g	
		0.58 mg/g [c]	
		0.46 mg/g [d]	
Kaempferol	dried	0.04 mg/g	MeOH + 1% *v /v* HCl + TBHQ
	dried	ND	50% MeOH
	dried	2.360 mg/g	50% MeOH, 100% MeOH and water
	dried	0.198 mg/g	Water at 80°C for 2 h
	dried	0.36 mg/g	MeOH + HCl + 10 mg ascorbic acid
	dried	0.8 mg/g	70% MeOH + 0.1% acetic acid
		1.23 mg/g	
		4.59 mg/g	
	freeze-dried	0.98 mg/g [f]	70% MeOH
		0.54 mg/g [h]	
	freeze-dried	2.25 mg/g	80% MeOH
		1.75 mg/g	
		1.05 mg/g	
	freeze-dried	2.9 mg/g [d]	70% MeOH
		2.3 mg/g	
		3.5 mg/g	
		0.3 mg/g [c]	
		0.16 mg/g [d]	

In 2014, a research was conducted by Malliga and colleagues to analyse the phytochemicals present in *Moringa oleifera* leaves extract using petroleum ether, chloroform and aqueous extract as solvents. The collected leaves were washed thoroughly and shade dried at room temperature. A blender was used to blend the leaves into powdered form. 200g of dry powdered plant material was extracted with the solvent. Soxhlet extraction method was applied for this extraction process and the extraction was allowed to take place for 8 hours. A rotary vacuum evaporator was used to remove the solvent. The evaporation process was carried out at a temperature of 60˚C in a water bath. The frozen and freeze dried sample was then stored at -20˚C for further use. Colour reactions were used to detect the presence of the phytochemicals. Table 10 exhibits the various test conducted to determine the phytochemicals present in the *Moringa oleifera* leaves extracts. The petroleum ether and aqueous extract of *Moringa oleifera* leaves consists of phenols and flavonoids which are recognised to possess anti-cancer properties. The extracts also contained alkaloids and sugar. The chloroform extract only consists of sugars.

Table 10: Test conducted to identify the phytochemicals.

Type of Phytochemical	**Test Conducted**
Phenolic Compounds	**Ferric chloride test**: The extract is added to 5% $FeCl_3$ reagent and the formation of a deep blue colour was observed.
	Lead acetate test: The extract is mixed with 10% lead acetate solution and the formation of a white precipitate is observed.
Flavonoids	**Aqueous sodium hydroxide test**: The extract is treated with aqueous NaOH solution the formation of yellow-orange colour is observed.
	H_2SO_4 test: The extract is added to concentrated H_2SO_4 and the formation of orange colour is observed.

Ojiako conducted a study in 2014 in order to identify the phytochemicals present in *Moringa oleifera* leaves extract. The *Moringa oleifera* leaves collected in Nigeria were air dried at room temperature. A grinding machine was used to grind the leaves. Three different types of solvent which are ethanol, ethyl acetate and n-hexane were used in the extraction process and the extraction was carried out by Soxhlet extraction method. 50g of plant material was extracted with 300ml of solvent and the extraction was carried out for a period of 8 to 9 hours with the electronic hot plate set to the

boiling point of each solvent. When the refluxing solvent became clear, the extraction process was considered to be completed. Evaporation of the solvent was conducted by rotary evaporators and the sample was stored in air tight containers. The quantitative analysis revealed the presence of phytochemicals with known anti-cancer properties such as tannins (8.22%), saponins (1.75%) and phenols (0.19%). Other compounds present were alkaloids and phlobatannins. Table 11 displays the phytochemicals detected in the *Moringa oleifera* leaves extract from the extraction with three different types of solvent which are ethanol, ethyl acetate and n-hexane.

Table 11: The phytochemicals present from extraction using different solvents.

Phytochemicals	**Types of solvent**		
	Ethanol extract	**Ethyl acetate extract**	**N-hexane extract**
Tannins	Yes	Yes	Yes
Saponins	Yes	Yes	No
Phenols	Yes	Yes	No

A study was conducted in 2015 by Malliga and colleagues in order to determine the bioactive compounds present in the petroleum ether leaves extract of *Moringa oleifera* by using GC-MS analysis. The *Moringa oleifera* leaves were collected from Tamil Nadu, India. In order to preserve the essential oils, the leaves were shade dried at room temperature before being grounded with a grinding machine. Soxhlet extraction method was used to carry out the extraction process. A Soxhlet apparatus containing 250ml of the solvent was used to extract 200g of the powdered leaves. The extraction process was allowed to take place for a period of 8 hours. In order to produce a concentrated extract, a rotary vacuum evaporator was used to remove the solvent. The evaporation took place in a water bath at a temperature of 60˚C. The sample was then dried again aseptically with a drier before the spectroscopic analysis was carried out. Among the compounds detected in the GC-MS analysis of *Moringa oleifera* petroleum ether leaves extract are 6.268% of hentriacontane and 26.893% of D.L. α–Tocopherol (Malliga et al., 2015). Hentriacontane which is a C31-Saturated Fatty acid ester compound possesses anti-tumour and anti-cancer activities (Kim et al., 2011; Ramalakshmi and Muthuchelian, 2011). D. L. α-Tocopherol is an alcoholic compound which also exhibits anti-cancer and anti-tumour properties besides being anti-mutagenic.

2.4 Summary of literature review

The anticancer effect of bioactive compounds in *Moringa oleifera* leaves have been tested on various cancer cells such as ileocecal adenocarcinoma cells, breast mammary gland cells, acute lymphoblastic leukemia cells, acute myeloid leukemia cells, pancreatic cell lines, lung cell, epithelial breast cell line, mesenchymal cell line, hepatocellular carcinoma cells, colorectal adenocarcinoma cells and breast adenocarcinoma cells. *Moringa oleifera* leaves extracts have exhibited antiproliferative and antitumour properties on these cells. Based on the literature reviews, these experiments were conducted in various countries with different climates and environmental conditions. Besides that, many past research have been conducted to identify the bioactive compounds present in the leaves of *Moringa oleifera* which contain anticancer properties such as phenolic acid and flavonoids. The purpose of this research is to test the extracted bioactive compounds from the leaves of *Moringa oleifera* in Malaysia as different climate and environmental conditions may result in a variation of the bioactive compounds in terms of type and amount as well as a difference in its anticancer properties.

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CHAPTER 3

METHODOLOGY

3.1 Introduction

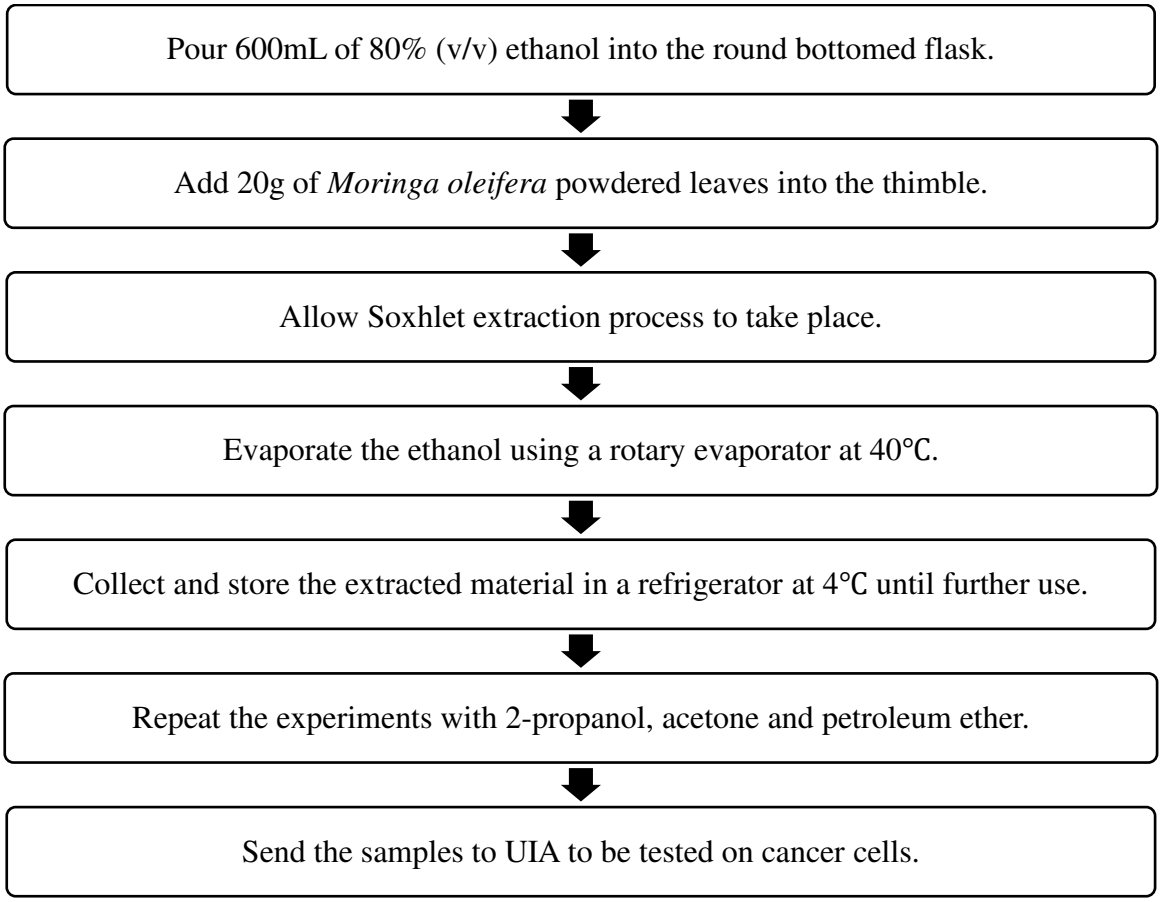
The powdered *Moringa oleifera* leaves were purchased from Mitomasa Sdn. Bhd., Kuala Lumpur and the solvents which were used for the extraction of the bioactive compounds from the leaves of *Moringa oleifera* plant were ordered from the Faculty of Chemical Engineering and Natural Resources laboratory of University Malaysia Pahang. The experiments were conducted using two different types of method which are the Soxhlet extraction method using ethanol, 2-propanol, acetone, petroleum ether and water as the solvent as well as the soaking method using ethanol and boiling water as the solvent. The extracted material were then delivered to Kulliyyah of Pharmacy International, Kuantan to be evaluated against cancer cells. Based on the information obtained from Table 6, 7, 8 and 9, (Leone et al., 2015), a decent amount of phenolic acids, flavonoids, tannins and saponins were extracted using 80% (v/v) of solvent. In 2015, Al-Asmari and his colleagues performed the extraction of *Moringa oleifera* leaves with 600mL of solvent which managed to significantly decrease the survival of ileocecal adenocarcinoma and breast mammary gland cancer cells. Therefore, in this research project using ethanol, 2-propanol, acetone and petroleum ether, 600mL of 80% (v/v) of solvent was used in the Soxhlet extraction process along with 20g of *Moringa oleifera* powdered leaves as it is the maximum amount which can be loaded into the thimble in the Soxhlet extraction chamber. As for the soaking method, 3500mL of solvent to fully submerge the *Moringa oleifera* powdered leaves was used to extract 350g (added to half of the soaking basin) of *Moringa oleifera* powdered leaves. However, for the water extracts obtained from the Soxhlet extraction method and boiling water method, lower amounts of water as solvent and *Moringa*

oleifera powdered leaves were used in order to test if it was possible to extract the bioactive compounds with water in a lower volume.

3.2 Extraction

Process Flow Chart

Soxhlet extraction using ethanol, 2-propanol, acetone and petroleum ether as solvents



Extraction by soaking method using ethanol as solvent

Pour 3500 mL of ethanol into a basin.

↓

Add 350g of *Moringa oleifera* powdered leaves into the basin and stir.

↓

Allow soaking to take place overnight.

↓

Store the extracted material in a refrigerator at 4℃ until further use.

↓

Send the sample to UIA to be tested on cancer cells.

Soxhlet extraction using water as solvent

Pour 200mL of water into the round bottomed flask.

↓

Add 15g of *Moringa oleifera* powdered leaves into the thimble.

↓

Allow Soxhlet extraction process to take place.

↓

Evaporate the water using a rotary evaporator.

↓

Collect and store the extracted material in a refrigerator at 4℃ until further use.

↓

Send the samples to UIA to be tested on cancer cells.

Extraction by soaking method using boiling water as solvent

Pour 1L of water into a basin.

↓

Add 150g of *Moringa oleifera* powdered leaves into the basin and stir.

↓

Allow soaking to take place overnight.

↓

Store the extracted material in a refrigerator at 4℃ until further use.

↓

Send the sample to UIA to be tested on cancer cells.

Soxhlet Extraction

Figure 7 shows the set-up of the Soxhlet extraction apparatus used in the actual experiment conducted under a fume hood in the Environmental Engineering Laboratory.

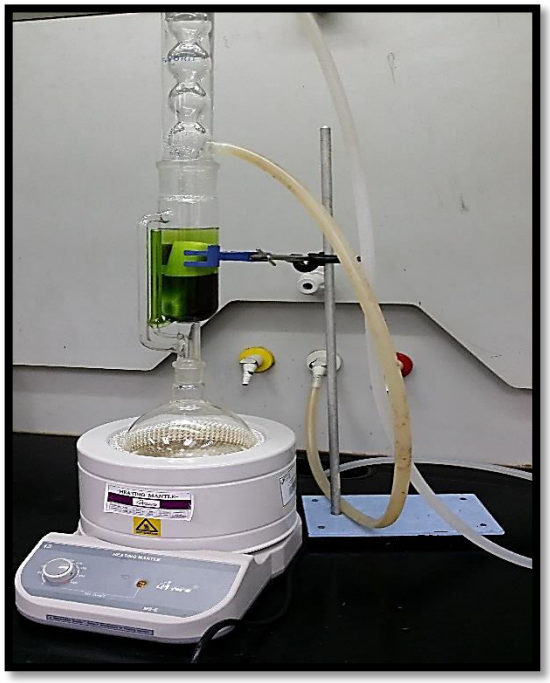


Figure 7: The set-up of Soxhlet extraction apparatus used in the actual experiment.

The basic components of a Soxhlet apparatus are a condenser, a thimble, a mantle and a round bottomed flask. The function of the condenser is to cool the solvent and condense its vapour into liquid form. The thimble contains the powdered *Moringa oleifera* leaves and it is where the condensed solvent passes through in order to facilitate the extraction of the bioactive compounds. The round bottomed flask contains the solvent and it serves as a reservoir to hold the concentrated solvent. The heating mantle heats the solvent. Sohxlet extraction is required when the desired compound has limited solubility in the solvent. The powdered *Moringa oleifera* leaves is placed inside the thimble which is loaded into the main chamber of the Soxhlet extractor. The Soxhlet extractor is placed onto the round bottomed flask containing the solvent. Then, the Soxhlet apparatus is equipped with the condenser. The solvent vapour is generated by heating the reservoir using the mantle. The solvent vapour travels up the distillation arm and condenses into the thimble containing the powdered *Moringa oleifera* leaves. The thimble is filled with warm solvent over time. The bioactive compounds then dissolves into the solvent. The thimble is automatically emptied by a siphon side arm and the solvent flows back into the round bottomed flask. The extracted material is then obtained by means of a rotary evaporator by evaporation of the solvent, leaving behind the residue of the *Moringa oleifera* leave extract. Soxhlet extraction method is employed because it allows the extraction of a solid sample into a liquid, leaving behind insoluble impurities. In addition, the solvent can be recycled (CHEMO Administrator, 2003).

Rotary Evaporator

Figure 8 displays a rotary evaporator which is used to evaporate the solvents under vacuum pressure.

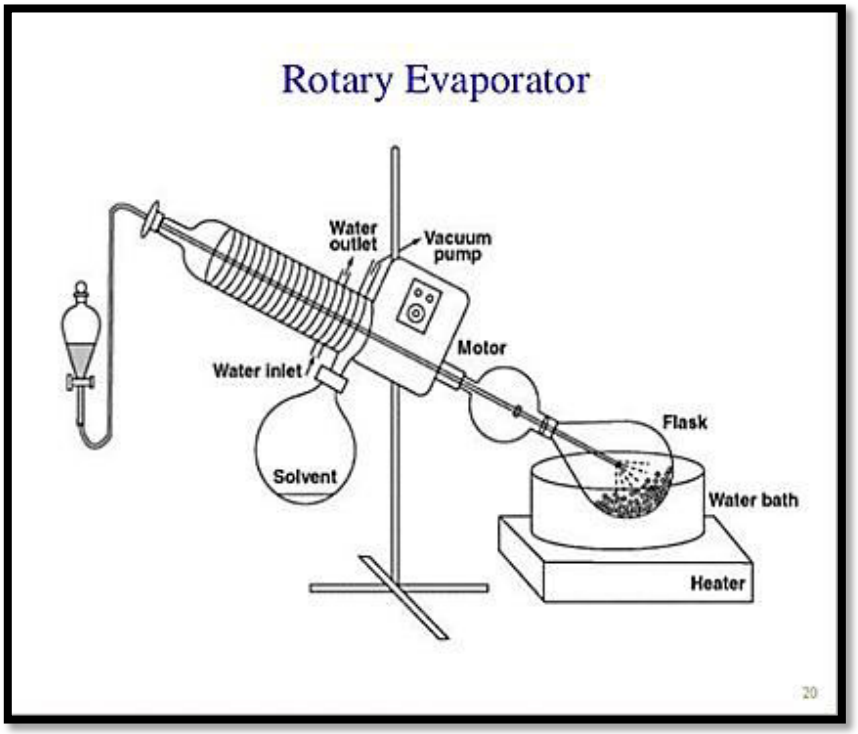


Figure 8: Rotary Evaporator.

Rotary evaporator is a technique used to remove large volumes of volatile solvents from solutions. The rotary evaporator rotates a flask containing the solution under reduced pressure. The rotation causes the solution to spread out forming a thin film. This thin film provides a large surface area which helps to speed up the rate of evaporation. The solute remains as the solvent is evaporated (The Interactive Lab Primer, 2016). A typical rotary evaporator has a water bath which is heated in order to prevent the solvent from cooling or even freezing during the evaporation process. The solvent removed under vacuum, is trapped by a condenser and is collected for easy reuse or disposal (Rob Toleki, 2015). The rotary evaporator increases the rate of evaporation by heating the solution, reducing the pressure to lower the solvent boiling point and rotating the solution to increase the surface area (Benoit, 2014).

Summary of Extraction Method

There are various methods that can be employed to extract the bioactive material from the *Moringa oleifera* leaves. However, this research focuses on Soxhlet extraction technique as it is the most suitable for the experiment. In addition, other methods have certain disadvantages that need to be taken into consideration as shown in Table 12. The disadvantages have been obtained from an article on natural products isolation written by Arthur. The link to the article is provided in the reference.

Table 12: Disadvantages of various extraction methods.

Method	Disadvantage
Percolation	1. Fine powders and materials such as resins and plants that swell excessively (e.g., those containing mucilages) can clog the percolator. 2. If the material is not distributed homogenously in the container, the solvent may not reach all areas and the extraction will be incomplete.
Maceration	The process can be quite time-consuming, taking from a few hours up to several weeks.
Reflux	Thermolabile components risk being degraded.
Microwave assisted extraction	The sample size should be limited to 0.5- 1.0 gram.
Ultrasound assisted extraction	It is mostly used for the initial extraction of a small amount of material.

3.3 Total phenolic content and total flavonoid content

Objective: To determine quantitatively the total phenolic content (TPC) and total flavonoid content (TFC) of *Moringa oleifera* leaves extracts.

Materials

Chemicals: Deionized water, sodium carbonate, aluminium trichloride, ascorbic acid, and Folin-Ciocalteau reagent. Quercetin was purchased from Thermo Fisher Scientific Inc. DPPH and gallic acid were purchased from Sigma-Aldrich Chemical Co. (St.

Louis, USA). Methanol was purchased from Merck, Germany.

Instruments: Microplate reader (Dynatech MR5000 TECAN, Tecan Group Ltd., Switzerland).

Disposable materials: Round-bottomed 96-well plate, aluminium foil, micropipette tips, and multichannel solution basin.

Methods

Total phenolic content of the S1, S2, S3, S4 and S5 extracts: The microplate TPC method was based on the 96-well microplate Folin-Ciocalteu method described by Bobo-Garcia et al., 2014. A total of 20µL of the diluted extract were mixed with 100µL of 1:4 diluted Folin–Ciocalteu reagent and shaken for 60s in a flat-bottom 96-well microplate. The mixture was left for 240 s and then 75µL of sodium carbonate solution (100g L^{-1}) were added and the mixture was shaken at medium-continuous speed for 1 min. After 2h at room temperature, the absorbance was measured at 750nm using the microplate reader. The absorbance of the same reaction with DMSO instead of the extract and the absorbance of 20µl of extract and 175µl water was subtracted from the absorbance of the reaction with the extract. Gallic acid dilutions (final concentration range of 3.205 – 102 mg L^{-1}) were used as standards for calibration.

Total flavonoid content of the S1, S2, S3, S4 and S5 extracts: Total flavonoid content was determined following the method described by Herald et al., 2014. Distilled water (100µL) was added to each of the 96 wells, followed by 10µL of 50g L^{-1} $NaNO_2$ and 25µL of sample solution. After 5min, 15µL of 100g L^{-1} $AlCl_3$ was added to the mixture; 6min later, 50µL of 1mol L^{-1} NaOH and 50µL of distilled water were added. The plate was shaken for 30s in the plate reader prior to absorbance measurement at 510nm. The sample was measured against a mixture of DMSO instead of the extract as blank and the absorbance was subtracted from the absorbance of 25µl of extract and 225µl water. Catechin at final concentration range of 9.375 – 300µg mL^{-1} were used to generate a calibration curve.

Total phenolic content of the water extracts: The method was modified from Ahmed et al. (2015) and Céspedes et al. (2008). 18µL of extract (1 mg/mL) were placed in each

well of a 96-well plate. Then, 90µL of diluted Folin–Ciocalteu reagent (20% v/v) was placed in each well and incubated at room temperature for 5 minutes. This was followed by the addition of 90µL of saturated sodium carbonate (75 mg/mL) and a further incubation for 2 hours at room temperature. The absorbance was then read at λ = 765nm using a microplate reader. The TPC was determined using gallic acid standard curve. All tests were conducted in triplicates. Results were expressed as mg gallic acid equivalence (GAE) per gram of extract. (Ahmed et al., 2015; Céspedes et al., 2008).

Total flavonoid content of the water extracts: The method was modified from Ahmed et al. (2015) and Meda et al. (2005). 100µL of 2 % aluminium trichloride in methanol was mixed with 100µL of extract (1 mg/mL). Absorption readings at λ = 415nm were taken after 10 minutes against blank samples consisting of 100µL of extract with 1 mL methanol only. All the blank values were subtracted from their respective extract. The TFC was determined using quercetin standard curve. All tests were conducted in triplicates. Results were expressed as mg quercetin equivalence (QE) per gram of extract (Ahmed et al., 2015; Meda et al., 2005).

3.4 DPPH radical scavenging activity

Objective: To determine quantitatively the DPPH radical scavenging activity of *Moringa oleifera* leaves extracts.

Materials

Chemicals: DPPH and ascorbic acid (from Sigma-Aldrich Chemical Co. St. Louis, USA), methanol and DMSO (from Merck, Germany) and deionized water.

Instruments: Microplate reader (Dynatech MR5000 TECAN, Tecan Group Ltd., Switzerland).

Disposable materials: Flat-bottomed 96-well plate, aluminium foil, micropipette tips, and multichannel solution basin.

Methods

DPPH radical scavenging activity of the S1, S2, S3, S4 and S5 extracts: The microplate antioxidant assay methodology was based on the 96-well plate assay described by Bobo-Garcia *et al.*, 2014. A total of 20µL of the diluted sample was added to 180µL of DPPH solution (150µmol L^{-1}) in methanol:water (80:20, v/v) and shaken for 60s in a 96-well microplate. After 40min in the dark at room temperature, the absorbance was measured at 515nm in the microplate reader. Ascorbic acid was used as a standard at 0.39 – 50µg/mL to generate a calibration curve. The % DPPH quenched was calculated using the following equation:

$$\% \text{ Inhibition of DPPH radicals} = (1 - [A/B]) \times 100\%$$

Where,

A = (Absorbance at 515nm of 20µL of sample with 180µL DPPH solution after 40min) - (Absorbance at 515nm of 20µL of sample with 180µL methanol:water (80:20, v/v) after 40min)

B = (Absorbance at 515nm of 20µL of DMSO with 180µL DPPH solution) - (Absorbance at 515nm of 20µL of DMSO with 180µL methanol:water (80:20, v/v)

The amount of extracts necessary to decrease the initial DPPH absorbance by 50 % (IC_{50}) was graphically determined by plotting the DPPH scavenging percentage as a function of extract concentration.

DPPH radical scavenging activity of the water extracts: The method was modified from Prieto (2012). The DPPH radical scavenging activity was measured by mixing 80µg/mL DPPH in methanol and a series of 2-fold dilutions of the extracts, which were then incubated in the dark at 37ºC for 30 minutes. After that, the absorption readings of the test mixtures were read at λ = 517nm. The percentage inhibition of DPPH radical will be calculated by comparison with the control using the formula:

$$\% \text{ DPPH scavenging} = \left(1 - \frac{A_0 - \text{A}}{A_0}\right) \times 100\ \%$$

Where,

A_0 = Absorbance DPPH (obtained from DPPH standard curve)

A = (Absorbance DPPH + sample) – (Absorbance sample without DPPH

The amount of extracts necessary to decrease the initial DPPH absorbance by 50 % (IC_{50}) was graphically determined by plotting the DPPH scavenging percentage as a function of extract concentration.

3.5 Cytotoxic activity

Objective: To determine the cytotoxic activity of *Moringa oleifera* leaves extracts on human breast adenocarcinoma (MCF-7) cancer cell line.

Methodology

Samples Preparation: The samples were dissolved in dimethyl sulfoxide (DMSO) which marks up to a concentration of 100mg/ml (stock solution).

Cell: The human breast adenocarcinoma (MCF-7) cancer cell line cells were cultured in complete DMEM culture medium supplemented with 10% (v/v) FBS and 5% of 50units/ml penicillin and 10µg/ml streptomycin. The cells were grown in a tissue culture flask and placed in a humidified incubator at 37°C and the presence of 5% CO_2 to allow cell growth. All the in vitro studies were performed in triplicates (n=3) in three different sets of experiment.

Cytotoxicity Assay: The cellular growth of MCF-7 was determined using a standard MTT ((3-(4,5)-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide)) colorimetric assay with some modifications (Mosmann, 1983; Sreejith et al., 2012). The reduction of yellow MTT substrate to purple formazan is directly related to activity of mitochondrial reductase enzymes and therefore the number of viable cells. The cells were exposed to a concentration of 100, 50, 25, 12.5, 6.25 and 3.125µg/ml of test samples for 72 hours. The percentage of cell viability was calculated using the following formula:

$$\text{Viability of cells (\%)} = \left(\frac{A,sample - A,blank}{A,untreated - A,blank}\right) \times 100\%$$

About 100mg/ml stock solution was prepared with dimethyl sulfoxide (DMSO). The control was the non-stimulated cells (pure cells). For the preparation of different concentrations, the test sample was diluted with complete growth medium. Serial

dilution was performed in order to obtain sample concentrations of 100, 50, 25, 12.5, 6.25 and 3.125µg/ml. The 5000 cells/well were seeded in 96 well of flat bottom plates and allowed to grow in a humidified incubator at 37°C and 5% CO_2 for 24 hours. Medium (edge effect), medium (blank) and various concentration of samples were added to the wells accordingly as shown in Figure 9. After 24 hours of incubation, the old medium in all of the wells were replaced by 150µl of diluted samples with respect to the concentration and medium only for blanks and untreated control. All plates were incubated for another 72 hours. Then, 15µl of 5 mg/ml MTT solution was loaded into all wells except wells containing medium (edge effect). After 4 hours of incubation, the medium was then removed from the wells. 100µl of 100% dimethyl sulfoxide (DMSO) was reloaded into the wells to solubilize the purple formazan crystal and were mixed thoroughly by pipetting technique. Then, their absorbance values were read at wavelength of 570nm and 630nm by using multi-detection plate reader (TECAN infinite M200) within 1 hour. The 50% reduction in cell viability relative to the control (IC_{50}) was established by extrapolation from graph of experimental data.

	1	2	3	4	5	6	7	8	9	10	11	12
A	M	M	M	M	M	M	M	M	M	M		
B	M	B	C	1	1	1	1	1	1	M		
C	M	B	C	2	2	2	2	2	2	M		
D	M	B	C	3	3	3	3	3	3	M		
E	M	B	C	4	4	4	4	4	4	M		
F	M	B	C	5	5	5	5	5	6	M		
G	M	B	C	6	6	6	6	6	6	M		
H	M	M	M	M	M	M	M	M	M	M		

Figure 9: Layout of 96-well plates for cytotoxicity study.

Definition:

- M is defined as edge effect which contains the media only.
- B is defined as blank which contains the medium only.
- C is defined as untreated control which contains the cells and medium only.
- B3-G9 is seeding cell.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Introduction

The results for the total phenolic content, total flavonoid content, DPPH radical scavenging activity as well as the cytotoxic activity of the *Moringa oleifera* leaves extracts against human breast adenocarcinoma cancer cell line were obtained from IIUM, Kuantan. Table 13 shows the different types of solvent and the extraction methods used in the extraction of the samples.

Table 13: The solvents and method used in the extraction of the samples.

Samples	Solvent used in extraction	Extraction method
S1	Ethanol	Soxhlet
S2	Ethanol	Soaking
S3	2-propanol	Soxhlet
S4	Acetone	Soxhlet
S5	Petroleum ether	Soxhlet
S1	Boiling water	Soaking
S2	Water	Soxhlet

4.2 Total phenolic content and total flavonoid content

Figure 10 shows the total phenolic content for S1, S2, S3, S4 and S5 whereas Figure 11 shows the total flavonoid content of the extracts. Figure 12 displays the total phenolic content of water extracts by Soxhlet extraction method and boiling method whereas Figure 13 displays the total flavonoid content of the extracts. The TPC and TFC of the extracts were conducted in triplicates (R1, R2, R3). The outcome of both of

the tests showed positive results which was indicated by the formation of a pale blue solution for TPC test and a yellow solution for TFC test.

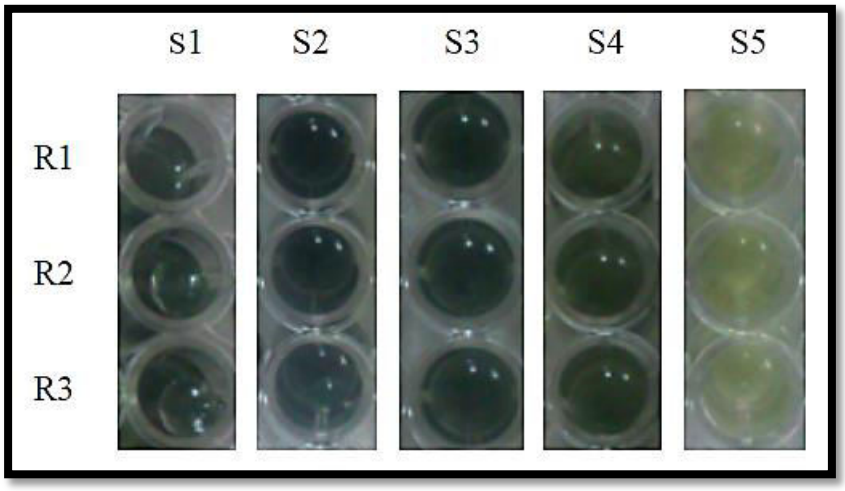


Figure 10: TPC test for S1, S2, S3, S4 and S5 extracts.

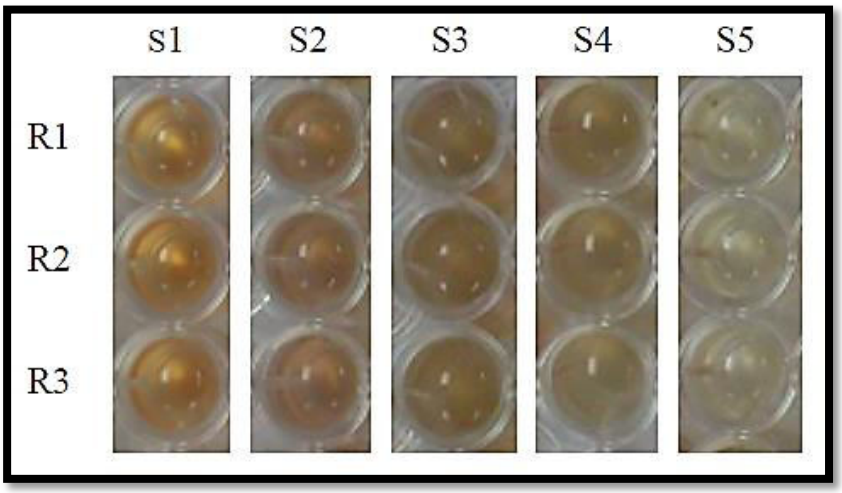


Figure 11: TFC test for S1, S2, S3, S4 and S5 extracts.

Figure 12: TPC test for water extracts by Soxhlet (left) and boiling (right).

Figure 13: TFC test for water extracts by Soxhlet (left) and boiling (right).

The total phenolic content and the total flavonoid content of the S1, S2, S3, S4 and S5 extracts are presented in Table 14. The results showed that the extracts contain appreciable amount of phenolics content but the flavonoids were only detected in S1, S2 and S3. The S1 extract possessed a higher TFC compared to the TPC. Flavonoids are a subclass of polyphenols. Theoretically during quantifying, the value of phenolics should be more than flavanoids. However, there are other factors which may affect the results including the reference standard used to determine the TPC and TFC as well as the presence of other compounds in the sample or extraction solvent. In addition, a single extraction or a single method to determine the compounds would be insufficient due to the complexity of compounds (Katsube et al., 2004; Wu et. al., 2004). Table 15 displays the total phenolic content and the total flavonoid content of the water extracts by extraction using Soxhlet method and boiling method. The total phenolic and flavonoid content in the water extract from the boiling method was found to be higher in comparison with the water extract from Soxhlet extraction method. Overall, the boiling water extract contained the highest TPC whereas S1 contained the highest TFC.

Table 14: TPC and TFC values of the S1, S2, S3, S4 and S5 extracts.

Sample	Total phenolic content (mg GAE/g dry extract)	Total flavonoid content (mg CE/g dry extract
S1	17.776 ± 0.530	38.717 ± 5.114
S2	26.799 ± 0.183	16.464 ± 0.889
S3	23.168 ± 0.982	20.494 ± 8.379
S4	16.294 ± 0.504	not detected
S5	10.326 ± 0.183	not detected

Table 15: TPC and TFC values of the water extracts.

Sample	Total phenolic content (mg GAE/g dry extract)	Total flavonoid content (mg QE/g dry extract
Water extract (Soxhlet)	17.042 ± 0.727	7.250 ± 0.284
Boiling water extract (soaking)	28.571 ± 2.174	9.900 ± 0.511

4.3 The DPPH radical scavenging activity

Results and Observation

The positive result for the DPPH assay was indicated by the purple colour reduction of the DPPH radicals to yellow colour as shown in Figure 14 and Figure 15. The *Moringa oleifera* leaves extracts were tested at a concentration range of 3.906µg/mL to 500µg/mL in triplicates. In terms of quality, the slight change of the colour intensity in each test indicated a weak antioxidant activity of the extract. The DPPH IC_{50} values of the extracts is presented in Table 16. According to the IC_{50} values, the tested *Moringa oleifera* leaves extracts possessed an appreciable antioxidant activity with *S1* as the most active extract and S5 as the least active extract. All of the extracts exhibited lower antioxidant activity in comparison to ascorbic acid. This result is in line with another study, which showed that the IC_{50} value of extracts demonstrates a negative relationship with the antioxidant activity (Hatamnia et al., 2014).

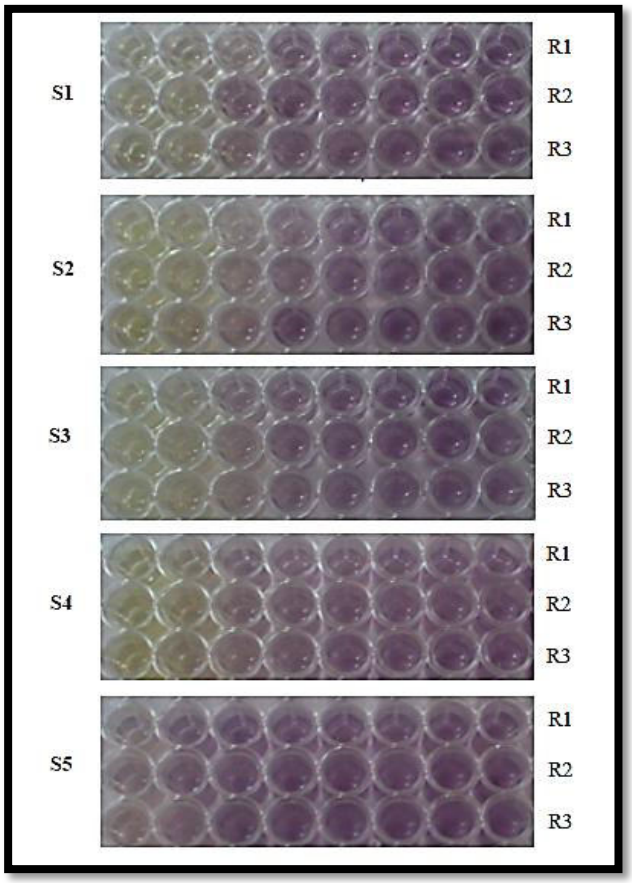


Figure 14: DPPH radical scavenging activity of S1-S5.

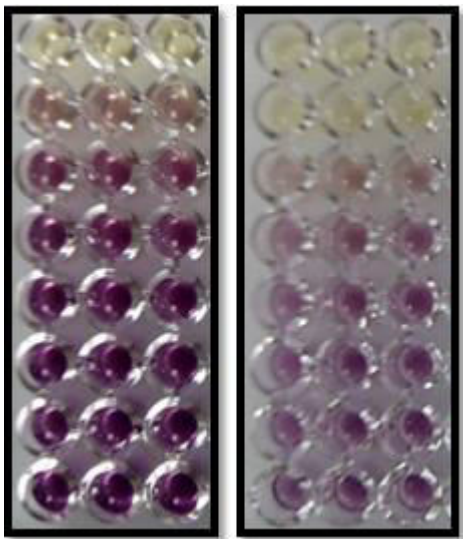


Figure 15: The DPPH test for water extracts by Soxhlet (left) and boiling (right).

Table 16: DPPH IC_{50} values of the samples.

Sample	**DPPH IC_{50} value (µg/mL)**
S1	53.889 ± 2.029
S2	47.752 ± 5.608
S3	119.809 ± 20.113
S4	93.314 ± 12.779
S5	404.768 ± 37.619
S1	14.902 ± 1.829
S2	83.366 ± 3.213
Ascorbic acid	2.834 ± 0.178

Figure 16 and Figure 17 displays the percentage inhibition of DPPH radicals by S1-S5 and the water extracts respectively. The IC_{50} of the extracts were calculated from the graphs shown in both of these figures.

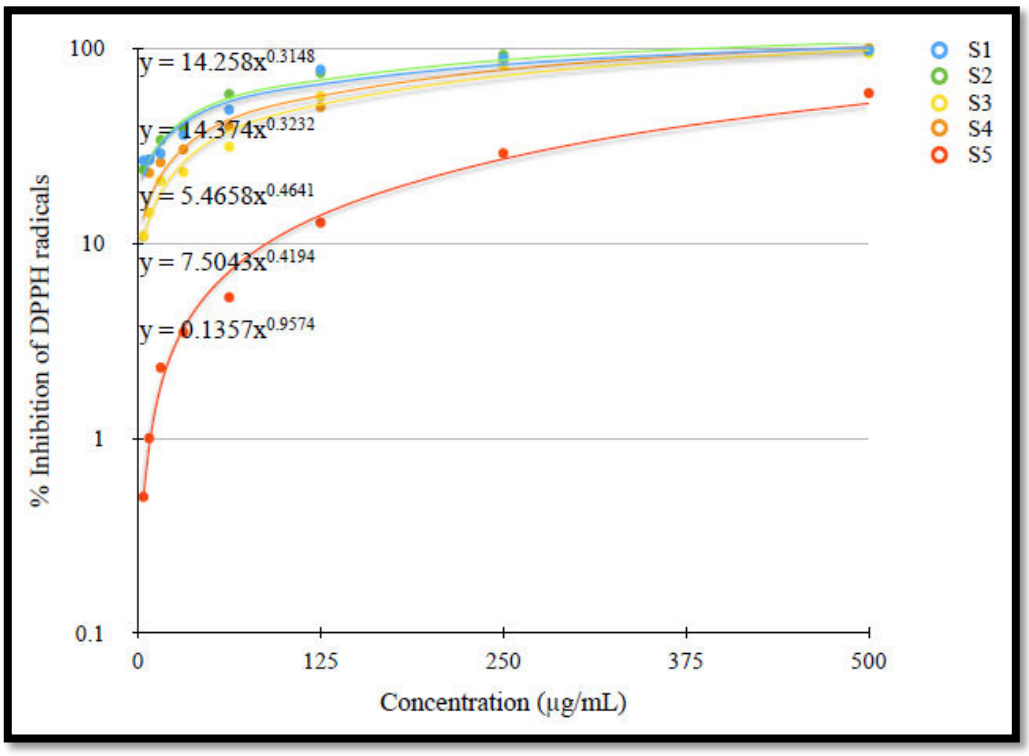


Figure 16: The % inhibition of DPPH radicals of S1-S5.

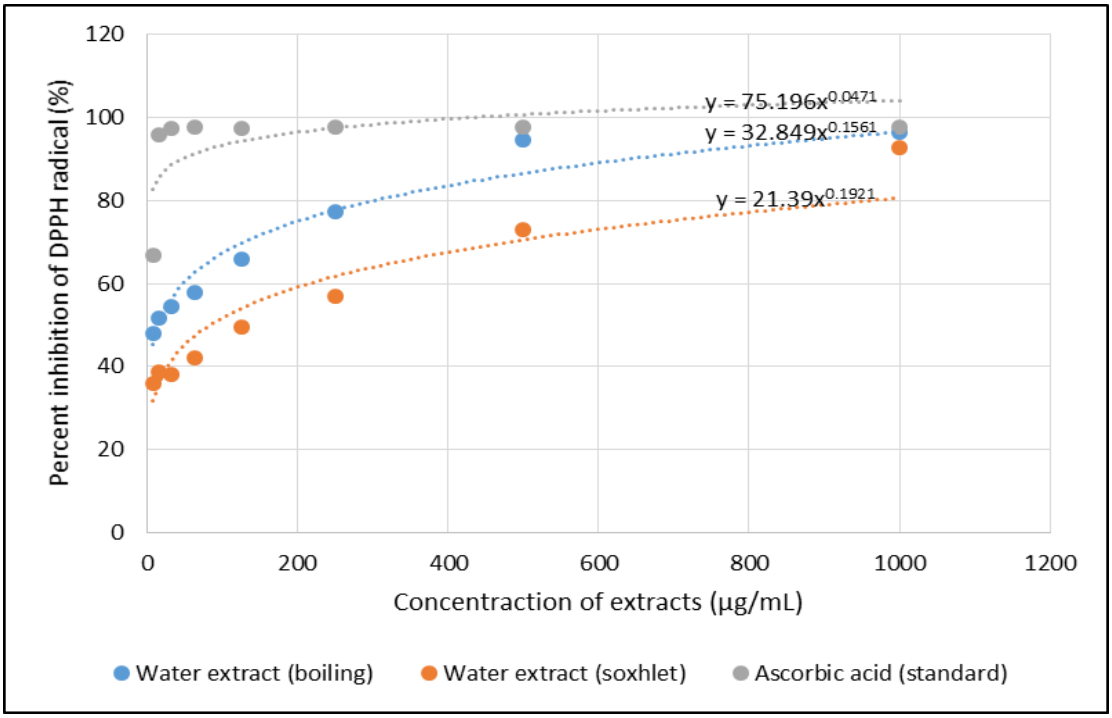


Figure 17: The % inhibition of DPPH radicals of the water extracts.

4.4 Cytotoxic activity of *Moringa oleifera* leaves extracts

Results

The growth of cells exposed to increasing concentrations of the extracts for 72 hours was examined using MTT assay. The cytotoxic effect of the extracts observed on the MCF-7 cell line is shown in Figure 18.

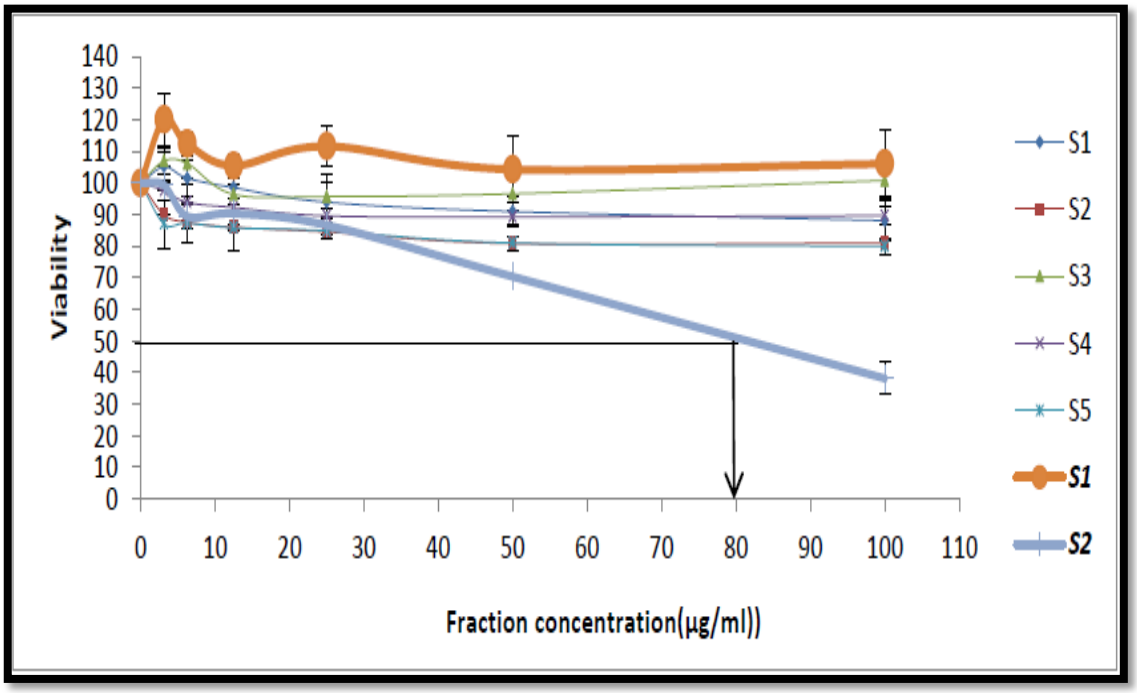


Figure 18: The effect of the extracts on MCF-7 cells after 72 hours of treatment.

According to Figure 19, the S1-S5 samples showed negative inhibition against the MCF-7 cell lines even at the highest concentration used. The samples were considered to be inactive towards the MCF-7 cell lines. In addition, the *S1* sample (did not manage to inhibit the MCF-7 cell lines. However, the *S2* sample (IC_{50} value of 81.77±6.05µg/mL) managed to inhibit the viability of MCF-7 cell lines in a concentration dependent manner.

CHAPTER 5

CONCLUSION AND RECOMMENDATION

5.1 Conclusion

In conclusion, the S1, S2, S3, S4, S5 and *S1* samples exhibited negative cytotoxic activity against the MCF-7 human breast adenocarcinoma cancer cell line. The cancer cell viability did not decrease significantly even as the highest concentration of the samples were used. However, the *S2* sample which employed the Soxhlet extraction technique using water as the solvent showed significant inhibition against the MCF-7 cancer cell line. The *S2* sample possessed an IC_{50} value of 81.77±6.05µg/mL and exhibited anticancer effect towards the cell lines tested upon in a concentration dependent manner. The cell viability decreased as the concentration of the sample fraction increased.

Although the results from previous research fail to support the results obtained in this research paper, it can be strongly suggested that Soxhlet extraction using water as solvent to extract the bioactive compounds of *Moringa oleifera* leaves should be further investigated.

5.2 Recommendation

In order to improve the results of this experiment, several recommendations may be considered. Since sample *S2* which used the Soxhlet extraction method with water as the solvent showed positive cytotoxic effect against the MCF-7 human adenocarcinoma cancer cell line, future research may focus on water as a better solvent to extract the bioactive compounds from the leaves of *Moringa oleifera* in comparison to ethanol, 2-propanol, acetone and petroleum ether. In addition, water is non-toxic to human health

in comparison to the other solvents used. Lesser purification efforts are required for the extraction of the bioactive compounds in *Moringa oleifera* leaves using water as the solvent. However, the variation of the results from previous research may be due to the difference in climate, weather and soil conditions in which the plants were grown in.

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