ANTI-CANCER EFFECTS OF *MORINGA OLEIFERA* ON HUMAN BREAST ADENOCARCINOMA CELL, MCF-7

BY

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LIST OF ABBREVIATION

ATCC	American Type Culture Collection
BLI	Beta Lactamase Inhibitor
DMSO	Dimethy Sulfoxide
GC-MS	Gas chromatography- Mass Spectrometre
GSH	Glutathione
HCT18	A designated nomenclature for a human Colon Adenocarcinoma Cell Line
HUVEC	Human Umbilical Vein Endothelial Cell
IC$_{50}$	Inhibitory Concentration at 50%
IL	Interleukin
LIF	Leukemia inhibitory factor
M.oleifera	*Moringa Oleifera*
MCF-7	A designated nomenclature for a human Breast Adenocarcinoma Cell Line
MDA	Malondialdehyde
MDA-MB-231	A designated nomenclature for an Invasive Ductal Carcinoma
MTT	Microtitrate Tetrazolium

NP	Natural product
OD	Optical Density
Panc-1	A designated nomenclature for Pancreas Ductal Adenocarcinoma cell lines
PGE2	Prostaglandin E2
PTH-RP	Parathyroid hormone-related protein
ROS	Reactive Oxygen Species
RPMI	Roswell Park Memorial Institute
SW480	A designated nomenclature of human Colon Carcinoma Cell Line
TNFα	Tumor Necrosis Factor Alpha
UAE	Ultrasound assisted Extraction
UASE	Ultrasound assisted Sound Extraction
WHO	World Health Organization

LIST OF SYMBOLS

µg	microgram
mL	microliter
mg	milligram
g	gram
±	plus minus

ABSTRAK

Pengenalan: Kanser payu darah adalah punca utama yang menyebabkan kematian di seluruh dunia, terutamanya dalam kalangan wanita. Berdasarkan World Health Organization (2015), kanser payu darah merupakan kanser paling kerap dan yang kedua tertinggi diikuti oleh kanser paru-paru di seluruh dunia dan juga paling kerap dalam kalangan wanita. *Moringa oleifera (M.oleifera)* merupakan sayuran yang tinggi khasiatnya dan terdapat banyak kepentingan terapeutik terutamanya fungsi anti-kanser. Oleh itu, para penyelidik menggunakan pelbagai kaedah pengekstrakan dan bahan larutan untuk mendapatkan banyak kompoun bioaktifnya.

Kaedah: Kajian semasa mengendalikan kaedah pengekstrakan berjujukan dengan gabungan ultrasound menggunakan tiga jenis bahan larutan (Etanol 99.7%, Etanol 50% and air ternyah ion) dengan tertiban polariti yang menaik. Ekstrak-ekstrak tersebut digunakan untuk mengkaji kesan anti-kanser terhadap sel kanser payu darah (MCF-7) dan sel normal, iaitu sel HUVEC melalui esei 3-(4, 5-dimethythiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT). Nilai IC_{50} bagi Etanol 99.7%, Etanol 50% dan Air deionised adalah 25, 200 dan 180 µg/ml masing-masing. Etanol 99.7% telah dikenal pasti sebagai bahan larutan yang terbaik disebabkan oleh nilai IC_{50} yang paling rendah.

Kesimpulan: *M.oleifera* dibuktikan sebagai agen pencegah yang amat berpotensi dan juga agen terapeutik bagi kanser payu darah sama ada yang berfungsi secara tunggal ataupun sebagai agen tambahan yang setanding dengan taraf modaliti kemoterapeutik.

ABSTRACT

Introduction: Breast cancer is the leading factor of mortality worldwide, especially among women. According to World Health Organization (2015), breast cancer is the second most common cancer worldwide next to lung cancer and is known as the most common cancer among females. *Moringa oleifera* (*M.oleifera*) is a highly nutritious vegetable that serves various kind of therapeutic benefits especially as anti-cancer agent, thus widely used by researcher through many types of extraction and solvents to reveal its bioactive ingredients.

Method: The current study carried out Ultrasound Assisted Sequential Extraction (UASE) using three solvents (Ethanol 99.7%. Ethanol 50% and Deionised Water) with ascending polarity. The resultant extracts were used to study their anti-cancer effects against human breast adenocarcinoma cell line, Mcf-7 and normal cell line, which is Human Umbilical Vein Endothelial Cell (HUVEC) with the aid of 3-(4, 5-dimethythiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. The IC_{50} value of the Ethanol 99.7%, Ethanol 50% and Deionised Water were 25, 200 and 180 μg/mL respectively. Ethanol 99.7% was found to be the best solvent for extracting as it gave the lowest IC50.

Conclusion: *M.oleifera* is useful as a potential preventative and/or therapeutic agent in breast cancers either alone or as an adjunct to standard chemotherapeutic modalities.

Chapter 1: Introduction

1.1 Background of the Study

According to World Health Organization (2016), cancer is defined as uncontrolled cell growth that able to invade and spread to nearby sites of the body (metastasis). The most prominent cancer that develops in women is the breast cancer followed by colorectal, lung, uterine cervix and stomach cancer. In fact, WHO stated that breast cancer is the second most common cancer worldwide next to lung cancer and known as the most usual tumor cases among females who establish nearly 1.7 million new cancer cases diagnosed in 2012 (Globocan, 2012). Every year 7.6 million human beings are dying due to cancer (Hossain et al., 2015). With long term therapies, cancer patients, unfortunately do develop drug resistance and side effects. Therefore, harmless ways of treatments are being introduced with the cooperation of integrative medicine involving herbal plants (Charoensin, 2014; Delgado *et al.*, 2011).

Based on the findings from numerous research studies on herbal plant possessing anti-cancer properties, *Moringa oleifera (M.oleifera)* is the frequent and dominating name found on those papers. Its' phytochemicals activity depends on the type of solvents and extraction method. Extracting non-conventionally with ascending polarity of solvents was reported to give great quantity of total phenolic contents (Azmir *et al.*, 2013; Lamalice *et al.*, 2007). *M.oleifera* is a perennial angiosperm plants which belongs to *Moringaceae* family reported to be successful anti-cancer candidate in the in vitro cancer studies as it

has the anti-proliferative and anti-oxidant traits (Al-Asmari *et al.*, 2015; Jung *et al.*, 2015;

Charoensin, 2014; Jung, 2014; Delgado *et al.*, 2011; Anwar *et al.*, 2007).

1.2 Rationale of the Study

Breast cancer is the death causing factor worldwide, especially among the women. The worldwide statistics estimates almost every woman has the risk of developing this cancer. Therapies or treatments for breast cancer, such as chemotherapy leaves side effects, which will worsen the condition (Adebayo *et al.*, 2017). Hence, researchers are competing to reveal the potency of natural products in anticancer drug discovery of various cancer (Diab *et al.*, 2015). In fact, it has been proven that natural products impressively have anticancer effects on many types of cancer. A lot of studies revealed that *M.oleifera* leaf extract provides radical scavenging activity that eventually helps in combating cancer occurrence and this is studied extensively in various cancer cell lines (Al-Asmari *et al*., 2015; Jung *et al*., 2015; Charoensin, 2014; Jung, 2014; Delgado *et al*., 2011; Anwar *et al*., 2007). Therefore, *M.oleifera* leaf *extract* was chosen in order to study its' anticancer effects on breast cancer cell line, MCF-7. Besides that, its' activity is made efficient by an Ultrasound Assisted Sequential Extraction (UASE) using solvents with the ascending polarity in the present study. Overall, this study provides evidence that *M.oleifera* leaf extract has anti-cancer properties over MCF-7.

1.3 Problem Statement

In present days, treatments for cancer are expensive with many side effects. Hence, there is a need for a plant's extract which is tolerable at higher dose and doesn't cause any adverse effects while being desirable for cancer therapeutic uses. Based on the previous studies, *M.oleifera* leaf extracts have anti-cancer properties. Therefore, in order to investigate whether the extract have the same effect on all cancer cells, human breast adenocarcinoma cell was used in the present study.

1.4 Objectives

General objective

To evaluate the anti-cancer effects of *M.oleifera* leaf extract on breast cancer cell line, MCF-7.

Specific objectives

1. To extract the *M.oleifera* leaf powder sequentially using solvents with ascending polarity with the aid of ultrasound (Ultrasound Assisted Sequential Extraction)
2. To investigate anti-proliferative activity by *M.oleifera* leaf extract using three type of solvents with ascending polarity on MCF-7 with the aid of microtitrate tetrazolium (MTT) assay

Chapter 2: Literature Review

2.1 Cancer

According to World Health Organization (2016), cancer is defined as an uncontrollable cell growth that is able to invade and spread to nearby sites of the body. Cancer has a number of health consequences and can be fatal. It is also stated that in men, the most common cancers are lung, prostate, colorectal and stomach cancers whereas in women are breast, colorectal, lung, uterine cervix and stomach cancer. In 2008, World Health Organization (WHO) declared that cancer occurrence is still escalating due to diet, environment and carcinogenic virus infection. Only malignant tumors are referred to as cancers and it is their tendency to invade and metastasize making cancer so threatening (Justus *et al.*, 2014; Lamalice *et al.*, 2007). Besides that, the unsuccessful treatment for metastasis also leads to a major number of death (Paul *et al.*, 2017). Basically, cancer cells establish abnormalities in the mechanism that operate normal cell growth, differentiate and affect one's lifespan. These properties determine the cellular level malignancy. American Cancer Society (2016) stated that environmental factors, such as lifestyle, imbalanced diet, infectious organisms and biological factors like genetics, hormones and immune status do lead to cancer. All these factors may act in sequence or simultaneously in order to cause cancer. There are plenty of treatments are being carried out, such as surgery, radiation, chemotherapy, targeted therapy (drug only acts on cancer cell), immune therapy and hormone therapy. However, interest are also being shown in alternative treatment using natural products (Veeresham, 2012).

2.2 Breast Cancer

Breast cancer is the leading factor of mortality worldwide, especially among women. According to World Health Organization (2015), breast cancer is the second most common cancer worldwide next to lung cancer and is known as the most common cancer among females where there were nearly 1.7 million new cancer cases diagnosed in 2012. 883,000 cases were found to be in less developed region whereas 794,000 of cases were in more developed regions. Despite the different regions breast cancer is still the most common cancer in women (Globocan, 2012). The highest mortality rate due to breast cancer was 1.38 million and at present 7.6 million human beings are dying every year due to cancer (Hossain *et al.*, 2015). The present statistics and estimation assume that both global prevalence of breast cancer and its death rate are on the rise. The pathological conditions of this cancer are diverse in kind in which some reveal as aggressive tumors whereas some establish slow growth with great prognosis. The American Cancer Society predicted that by 2050, the global incidence of female breast cancer will touch almost 3.2 million new cases each year (Tao *et al.*, 2015). The prevalence of this cancer is increasing drastically in developing countries. Meanwhile in developed countries, about 55% of occurrence is showed up. Another issue to be worried is the factors that contribute to breast cancer. It is very challenging to address these particular factors that have contributed in different populations globally (Ferlay *et al*., 2008).

2.3 Natural Products such as Medicinal Plants

Natural products (NPs) are vital sources of anti-cancer lead molecules and there are successfully becoming potential anti-cancer drugs (Du and Tang, 2014; Mondal *et al*., 2012). Historically, natural products have been the mainstay source of cancer chemotherapy for the past 30 years (Du and Tang, 201; Mondal *et al*., 2012; Mann, 2002). Unfortunately, in 1990's, they behave unexpectedly in pharmaceutical company because of the urge of targeted therapies that depends on antibodies or small synthetic molecules identified by high throughput screening (Basmadjian *et al*., 2014; Du and Tang, 2014). However, natural sources like medicinal plants are the potential and invulnerable alternative treatment for cancer patients.

Upon the toxicity or adverse effects of conventional drugs that are usually prescribed for cancer patients in hospitals, oncology research has shifted its focus to phytochemicals of medicinal plants (Charoensin, 2014). Some traditional medicines that have anti-proliferative effects such as *Sutherlandia frutescens*, known as cancer bush, is used to treat cancer by traditional healers in South Africa (Tiloke *et al.*, 2013). Several studies on effectiveness of *M.oleifera* extract proved that this plant is an anti- cancer agent (Al-Asmari *et al.*, 2015; Jung, 2014).

2.4 Taxonomy

Kingdom	*Plantae*
Subkingdom	*Viridiplantae*
Infrakingdom	*Streptophyta*
Superdivision	*Embryophyta*
Division	*Tracheophyta*
Subdivision	*Spermatophytina*
Class	*Magnoliopsida*
Superorder	*Rosanae*
Order	*Brassicales*
Family	*Moringaceae*
Genus	*Moringa Adans.*
Species	*Moringa oleifera Lam.*

Source: GBIF Backbone Taxonomy

Table 1: Taxonomy hierarchy of *M. oleifera* plant

2.5 *Moringa oleifera*

Retrieved from https://authoritynutrition.com/6-benefits-of-moringa-oleifera/

Moringa oleifera plant belongs to *Moringaceae* family and is a perennial angiosperm plants which is considered as an edible substance for centuries. The name *'Moringa'* originated from the Tamil name and that is the only genus belongs to *Moringaceae* family (Stimm, 2009). Being a native of the Himalayan region, it has the tendency to grow quickly and is widely cultivated throughout tropical and sub-tropical countries of the global including Saudi Arabia (Al-Asmari *et al*., 2015; Jung *et al*., 2015; Jung, 2014; Anwar *et al*., 2007).

Retrieved from https://www.pinterest.com/pin/376191375094406347/

It is grown extensively in Southeast Asian countries especially in India, Pakistan, Philippines and Thailand, well-known as food plant in Thai cuisine and have been used as an ingredient in Indian traditional medicine (Charoensin, 2014). Hossain (2015) mentioned that this plant has many names like 'drumstick tree', 'golden shower tree', 'horseradish tree', 'ben oil tree' and 'benzoil tree'. Being a diploid species with 28 chromosomes, *M.oleifera* and other species of *Moringa* serve as beneficial sources of medicinals, fiber, food and other products. Other species encompasses *M. concanensis*, *M. drouhardii*, *M. longituba*, *M. ovalifolia*, *M. peregrina* and *M. stenopetala* (Stimm, 2009).

Surprisingly, the taste of *M.oleifera* tender pots are almost the same as asparagus meanwhile the leaves do taste like watercress. *M.oleifera* leaves are vegetables with high nutrient content which can be cooked or pickled. The leaves are highly nutritious in the presence of essential amino acids, protein, minerals, iron, fats, beta-carotene, thiamin, riboflavin, and other vitamins, particularly vitamins A and C, benzyl isothiocyanate, niazimicin, pterygospermin and benzyl glucosinolate (Al-Asmari *et al*., 2015; Hossain *et al*., 2015; Charoensin, 2014). Consisting of vitamins like A and C, *M. oleifera* leaves can cure scurvy and respiratory ailments besides being use for emetic (Yamaguchi *et al.*, 2005). The leaf extract has medicinal properties, for instance antimalarial and antibacterial properties. Tropical application of the extract also aid in wound healing (Stimm, 2009).

2.6 Medicinal Values of *M. oleifera*

In ancient days, this plant had been used for its medicinal purpose widely in Asian and West African as it has numerous therapeutic effects such as anticancer, anti-ulcer, anti-viral, anti- atherosclerotic, immune-boosting, diuretic antimicrobial, anti-inflammatory, antispasmodic and for wound healing, anti-diabetic, antioxidant effects besides being used to treat cardiovascular diseases, skin diseases, hypertension, dental and ear infection, respiratory distress, diabetes, anemia, gastric discomfort, stomach ulcers, dysentery and cancer (Paul et al., 2017, Al-Asmari et al., 2015, Radwan et al., 2015; Handa, 2008; Yamaguchi et al., 2005). The diverse parts of *M.oleifera* tree are highly demanded for its therapeutic purposes such as for treatment of ascites, venomous bites, and rheumatism besides for cardiac and circulatory stimulants (Handa, 2008). As the entire parts of the *Moringa* tree (pods, seed and leaves) have served as medicinal source, hence, it was named "miracle vegetable" (Paul *et al*., 2017; Jung, 2014). *M.oleifera* leaves generally provide adequate nutrients for all and studies reported that it is helpful for expected mothers by accelerating uterine contraction during labor (Paul *et al.*, 2017). In ancient Sanskrit texts on medicine, it was stated that the roots, leaves and seeds serve some purposes in Ayurveda by treating an immense range of ailments (Handa, 2008).

Medicinal plants including those edible normally have phenolic compounds and they have been observed to possess diverse biological activities, including anti-oxidative activity. Antioxidants are crucial in scavenging free radical which causes oxidative stress to the bodily system in order to prevent any associated diseases or disorders which subsequently can increase out our life expectancy (Lamalice *et al.*, 2007). The major

phenolic that has this property are phenolic acid and flavonoids (Vaghora and Shukla, 2016; Lamalice *et al*., 2007; Yamaguchi *et al*., 2005). Naturally, flavonoids are those accumulated in the epidermal cells of plant organs such as fruits, flowers, seeds, root, leaves and stems which can be found in both glyosidic and non-glyosidic form. A numerous epidemiological studies have been carried out to verify the anti-cancer effects of flavonoids. These substances actions are related to deplete malignancy progression by affecting the cascade of immunological events involved in cancer (Vaghora and Shukla, 2016). This plant encompasses a numerous phytochemicals for instance, phenolic compounds like (Lamalice *et al.*, 2007; Radwan *et al.*, 2015).

Moreover, studies also reported saponins possess anti-cancer property on many cancer cells by ceasing neoplasm via cell arrest and apoptosis with IC50 values up to 0.2mM (Vaghora and Shukla, 2016). It was reported that a qualitative study has shown all the phytochemicals tested (flavonoids, anthraquinone, alkaloids, saponins, steroids, terpenoids, cardiac glycosides, anthocyanin, tannins and carotenoids) were present in *M.oleifera* leaves. It also has been used to treat conditions such as rheumatism, ascites, infection, hiccough influenza and internal abscess. In addition to that, the leaves contain nutrients specifically like essential amino acids, vitamins, minerals, β-carotene, benzyl isothiocyanate, niazimicin, pterygospermin and benzyl glucosinolate (Charoensin, 2014; Hossain *et al.,* 2015). Having those bioactive compounds made *M. oleifera* leaves do contribute to integrative medicines with numerous activities.

2.7 Anti-cancer Properties of *M. oleifera*

M. oleifera is reported to stave off diseases with its ability to reduce hyperglycemia and dyslipidemia (Charoensin, 2014). According to Charoensin (2014), finding showed the ethanol extract of the leaves avert cyclophosphamide-induced micronucleus generation and DNA destruction in mice. Besides that, this solvent extract improved hepatic glutathione restoration. *M.oleifera* leaf extract also was reported to possess anti-proliferative property and apoptosis promoting potential in tumor (KB) cell line besides elevating cytotoxicity upon chemotherapy on pancreatic cancer cells. However, there are only few proofs for *M.oleifera* leaf contributions in malignancy and treatments.

Accordingly, the benefits of administrating the *M.oleifera* leaf extract orally was assessed in a study to understand its potential as new anticancer medicine. It was proven in a study that *M.oleifera* leaves extract using a soluble cold Distilled Water extract (4°C; concentration, 300 µg/mL) highly leads to apoptosis, ceased cancer cell growth, and the amount of internal reactive oxygen species (ROS) was reduced in human lung cancer cells as well as other kind of tumor cells. This recommended the *M.oleifera* leaf extract treatment on cancer cells significantly lessened malignant cell uncontrolled growth and metastasis (Jung, 2014).

Furthermore, a novel study on the effectiveness of *M. oleifera* extracts proved this plant is an anti- cancer agent. This study successfully showed the leaves and bark have the anti-malignant properties and able to open door for new drugs development for breast and colorectal cancers. There was a significant reduction in cell survival in the cancer cell lines (MDA-MB-231 and HCT-8). A Gas Chromatograph-Mass Spectrometer (GC-MS) analysis

through this study has revealed many familiar anti-cancer compounds such as eugenol, D-allose, isopropyl isothiocynate, and hexadeconoic acid ethyl ester, all of which encompassed long chain hydrocarbons, sugar moiety and an aromatic ring. Hence, this analysis declared the anti-malignant properties of *M.oleifera* are contributed by the bioactive compounds in the plant extract (Al-Asmari et al., 2015).

2.8 Chemotherapeutic Agents for breast cancer

Death caused by cancer is epidemically increased now-a-days and 64% of death in developing world are attributed to cancer (Tacar *et al*., 2013).Thus, global scientists are continuously searching for new chemotherapeutic agents and trying to reduce the side effects of existing drugs. Synthetic drugs shows remarkable contribution to cure cancer for many years, but their use is being restraint due to serious side effects and some drugs even produce risks to death (Tacar *et al*., 2013; Mauri, 2008). Until now many chemotherapeutic agents are used to treat breast cancer where some are still under trial and some are popular in reducing cancer prognosis. Examples of chemotherapeutic drugs are cyclophosphamide, methotrexate, fluorouracil, doxorubicin, epirubicin, tamoxifen, taxanes, trastuzumab and raloxifene (Croxier *et al*., 2014).

Doxorubicin is the most effective and considered as frontline chemotherapeutic drugs to treat breast cancer. Doxorubicin, an anthracycline antibiotics, which constitute a group of vast spectrum therapeutic drugs is helpful in chemotherapy. However, the pitfall is it causes toxic effects (Tacar *et al*., 2013; Oz and IIhan, 2006). It binds with DNA associated enzyme and produces multiple molecular target as well as produce a wide range of cytotoxic effects. However, immune system is affected due to the damaged multiple cell lines which depressed the patient's immune system and developed viral or bacterial infection and also fatigue. Continuous administration can induce phlebosclerosis, cellulitis, and thrombophlebitis. Heart, brain, kidney and liver are also affected. Doxorubicin is responsible for cardiomyocytes and gives great impact on mitochondria in cardiac muscle. Long term doxorubicin therapy can cause a patient to undergo cognitive

impairment. Glomerulosclerosis occurs leading to hypertension, resistance to steroids and eventually resulting to renal failure. Besides these, several common side effects may appear such as nail bed, alopecia, itching, photosensitivity, rashes, vomiting, micosities etc (Tacar et al., 2013). Tamoxifen, a nonsteroidal, triphenylethylene antiestrogen type chemotherapeutic agent which increase the risk of developing endometrial cancer and risk is increased two- to three-fold when administered with tamoxifen. Most integral side effects are hot flushes, joint pains, headaches, and vaginal dryness (Mourits et al., 2001).

2.9 Extraction of Plant Material

Pharmaceutically, extraction means a separation of medicinally active constituents of plant from an inactive or inert substances using particular solvents in standard extraction procedures (Handa, 2008). Extraction is also known as a sample preparation technique (Azmir *et al.*, 2013). Usually the end products will be impure liquids, semisolids or powder intended for oral or external utilization (Handa, 2008). Pre-extraction preparation like grinding and drying have disparity in phytochemical preservation in the final (Brglez Mojzer *et al*., 2016; Azwanida, 2015; Azmir *et al*., 2013). The solvents used should make contact with the target analyte and the efficiency depends on how small the sample size is. That is the reason why powdered samples tend to have better surface contact with extraction solvents (Pandey and Tripathi, 2014). Moreover, powdered samples are more homogenized than grinded samples and therefore, good at making excellent contact with solvents (Azwanida, 2015). Medicinal plant studies involve both fresh and dried samples (Brglez Mojzer et al., 2016; Azwanida, 2015). Dried sample is widely used as it is time saving for an experiment. The fresh samples prone to get impaired quickly than dried samples. The fresh and dried samples of *M. oleifera* leaves have shown no noticeable effect in total phenolic. However, flavonoid content is found to be higher in dried samples (Brglez Mojzer *et al.*, 2016).

Extraction of plant material can be done in both non-conventional and conventional methods (Azwanida, 2015; Azmir et al., 2013; Handu, 2008; Vilkhu et al., 2008). Conventional methods are based on the extraction power of different solvents in the use and the application of heat and/ or mixing. The examples are Soxhlet extraction,

maceration and hydro distillation. Meanwhile, non-conventional techniques involve ultrasound, enzyme digestion, wave heating, ohmic heating, supercritical fluids and accelerated solvents (Azwanida, 2015; Azmir *et al*., 2013; Vilkhu *et al*., 2008).The most common conventional method used for extraction of *M. oleifera* has been Soxhlet extraction with heating time ranging up to 24 h (Laware, 2008). Generally, the major critical point of conventional extraction are longer extraction time, need expensive and highly pure solvents which might be hazardous and flammable, evaporation of the huge amount of solvent, low extraction selectivity, potential toxic emissions during extraction and thermal decomposition of thermos labile compounds (Azwanida, 2015; Azmir *et al*., 2013; Anwar *et al*., 2007). Normally, all the non-conventional methods are user friendly in every way unlike conventional ways as mentioned. Some of the non-conventional ways do comply with standards set by the Environmental Protection Agency, USA, therefore they are considered as "green techniques" The reason for choosing a non-conventional technique for this study is that these techniques use less hazardous chemical synthesis, less harmful chemicals and solvent auxiliaries, use renewable feedstock, lessen the derivatives, catalysis besides been designed for energy efficiency, reduce degradation and eventually pollution (Azmir *et al.*, 2013)

2.10 Ultrasound Assisted Extraction

In order to overcome the pitfalls by conventional methods, non-conventional methods are established in the upcoming studies. As a result, a non-conventional method called ultrasound assisted extraction (UAE) is done using *M. oleifera* leaves in this study. Ultrasound is an unusual type of sound wave with the range of 20 kHz to 100 kHz that is beyond human beings' hearing power. This wave penetrates medium by generating compression and expansion forming a phenomenon called cavitation (Azmir & Azwanida 2015). The entire procedure is untroublesome and consumes less technology regardless of how big the scale of phytochemical extraction is. UAE is user friendly as it reduces the working time and solvent usage.

Generally, UAE saves more time, energy and solvents. With the cavitation produced, UAE provides effective mixing, quick energy transfer, scaled down thermal gradients and extraction temperature besides giving quick response to extraction process. Furthermore, several findings reported UAE is basically the best choice for extracting bioactive materials from herbal plants (Azmir *et al.*, 2013). Authors stated that UAE that applied for thermo-labile compounds is able to reduce extraction time while preventing from being exposed to high temperature. Greater amount of phenolics was found in *Cratoxylum formosum* and high yield was gained from *Withania somnifera* through UAE. However, ultrasound energy which is more than 20 kHz might produce free radicals that can attack the active phytochemicals (Azwanida 2015).

Primary metabolites from leaf samples are routinely extracted and used in experiment to illuminate effects of diverse nutrients and stress conditions on plant growth