THE EFFECT OF *Moringa oleifera* LEAF EXTRACT ON CYTOTOXICITY AND APOPTOSIS PATHWAY IN BREAST CANCER CELL (MCF-7)

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by

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LIST OF SYMBOLS AND UNITS

cells/mL	Cell per millilitre
Cm	Centimetre
M	Metre
mg/g	Milligram per gram
Min	Minute
mL	Millilitre
Mm	Millimetre
mM	Milli molar
m/z	Mass per charge number of ions
Nm	Nanometre
Rpm	Rotation per minute
SD	Standard deviation
SI	Selectivity index
v/v	Volume per volume
w/v	Weight per volume
µg/mL	Microgram per millilitre
µL	Microlitre
µM	Micromolar
^{o}C	Degree Celsius
$°C.min^{-1}$	Degree Celsius per minute
$\Delta\Psi m$	Mitochondria potential

LIST OF ABBREVIATIONS

ABTS	2, 2'-azinobis (3-ethylbenzithiazoline-6-sulfonic acid) diammonium salt
ANOVA	One-way analysis of variance
APS	Ammonium persulphate
Aq	Aqueous
BSA	Bovine serum albumin
$CHCl_3$	Chloroform
CO_2	Carbon dioxide
DCM	Dichloromethane
ddH_2O	Deionized water
DPPH	1,1-diphenyl-2-picrylhydrazyl
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulphoxide
ECL	Enhanced chemiluminescence
EGF	Epidermal growth factor
FACS	Fluorescence-activated cell sorter
FBS	Fetal bovine serum
Hex	Hexane
GAE	Gallic acid equivalent
GC-MS	Gas chromatography mass spectrometry
IC_{50}	50 % cell inhibition
MCF-7	Breast cancer cell
MCF-10A	Normal breast cell
MTS	CellTiter 96® Aqueous One Solution Cell Proliferation Assay

n-BuOH	n-butanol
NIST	National Institute Standards and Technology
PBS	Phosphate Buffered Saline
PI	Propidium iodide
PS	Phosphatidylserine
PVDF	Polyvinylidene difluoride
QUE	Quercetin equivalent
RIPA	Radio-Immunoprecipitation Assay
RPMI-1640	Rosselle's Park Memorial Institute Media
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	Standard error of mean
TBST	Tris Buffered Saline with Tween-20
TEAC	Trolox-equivalent antioxidant capacity
TEMED	N, N, N', N'-tetramethylethylenediamine
TFC	Total flavanoid content
TG buffer	Tris-Glycine buffer
TPC	Total phenolic content
FITC	Fluorescein isothiocyanate
4-OH-TAM	4-hydroxytamoxifen
80 % MeOH	80 % methanol

KESAN EKSTRAK DAUN *Moringa oleifera* KE ATAS SITOTOSIK DAN LALUAN APOPTOSIS DALAM SEL KANSER PAYUDARA (MCF-7)

ABSTRAK

Moringa oleifera (MO) adalah daripada famili Moringaceae. Di Malaysia, MO dikenali sebagai pokok Munggai atau Kelor. MO terkenal dengan sifat-sifat terapeutik dan mempunyai pelbagai aktiviti biologi seperti antioksidan dan antikanser. Dalam kajian ini, daun MO telah diekstrak dengan pelarut yang mempunyai polariti berbeza. Kesan ekstrak 80 % metanol dan pecahan pelarut daun MO terhadap sitotosik sel MCF-7 telah diuji menggunakan ujian MTS. Sel normal payudara (MCF-10A) digunakan untuk perbandingan dengan sel MCF-7 dan memilih efektif ekstrak yang memberi kesan paling sitotosik pada sel MCF-7. Antara semua ekstrak, ekstrak diklorometan (DF-CME-MOL) telah dipilih berdasarkan IC_{50} terendah iaitu 5 µg/mL dan nilai selektif indeks (SI) tertinggi iaitu 9.5. Kemudian, ekstrak yang dipilih (DF-CME-MOL) digunakan untuk analisis seterusnya. Analisis fitokimia dilakukan dengan menggunakan ujian DPPH, ABTS, Folin-kiokalteu, aluminium klorida dan analisis GC-MS. Kandungan antioksidan dalam DF-CME-MOL untuk ujian DPPH dan ABTS adalah 10.52 ± 0.009 mg / g TEAC dan 4.32 ± 0.021 mg / g TEAC, masing-masing. Manakala, jumlah kandungan fenolik dan flavanoid adalah 34.96 ± 0.006 mg / g GAE dan 15.72 ± 0.050 mg / g QUE, masing-masing. Selepas itu, analisis kompoun oleh GC-MS mengesan beberapa potensi sebatian dalam DF-CME-MOL dinamakan; Fenol, 4-(metoksimetil)-, 2-Metoksi-4-vinilfenol, Fenol, 2-propil, Fenol, 2-metoksi-4-(1-propenil)-, (Z)-, 4-((1E)-3-Hidroksi-1-propenil)-2-metoksifenol, Asid Heksadekanoid, metil ester, Fenol, 2-[(4-hydroksifenil) metil], Fenol, 4, 4’-metilenebis-, Fenol, 2-etil-, Benzinetanol,

4-hidroksi-, Benzaldehad, 4-hidroksi-, Benzinasetonitril, 4-hidroksi- dan Benzaldehad, 3,4-dimetoksi-. Kemudian analisis apoptosis dijalankan dengan menggunakan ‘Annexin V-FITC’ melalui aliran analisis sitometri FACS dan seterusnya mengesahkan melalui ‘western blot’ untuk mengesan isyarat laluan apoptosis menggunakan protein terpilih seperti p53, bax, sitokrom c dan caspase 8. DF-CME-MOL sangat mendorong induksi apoptosis awal melalui laluan ekstrinsik sel MCF-7. Oleh itu, daun MO mempunyai potensi untuk dikembangkan sebagai agen antikanser pada kanser payudara.

THE EFFECT OF *Moringa oleifera* LEAF EXTRACT ON CYTOTOXICITY AND APOPTOSIS PATHWAY IN BREAST CANCER CELL (MCF-7)

ABSTRACT

Moringa oleifera (MO) is belonging to Moringaceae family. In Malaysia, MO was known as 'Munggai' or 'Kelor' tree. MO is well known for its therapeutic properties that possesses a wide range of biological activities such as antioxidant and anticancer properties. The impact of different solvents of various degree of polarities on the cytotoxicity of breast cancer cell line (MCF-7), were studied. The cytotoxicity effect of 80 % methanol and its solvent fractions of *Moringa oleifera* leave (MOL) extract against MCF-7 cell were determined by using MTS assay. The normal breast cell line (MCF-10A) was used to compare with MCF-7 cell. The effective extract that gave the most cytotoxic effect on MCF-7 cell had been chosen. Among of all extracts, dichloromethane extract (DF-CME-MOL) had been selected accordingly to its lowest IC_{50} (5 µg/mL) and highest selectivity index (SI) (9.5). Then, the chosen extract (DF-CME-MOL) was proceed for further analysis. The phytochemical analysis was performed using DPPH, ABTS, Folin-Ciocalteu, aluminium chloride and GC-MS analysis. An antioxidant contents in DF-CME-MOL for DPPH and ABTS assay were 10.52±0.009 mg/g TEAC and 4.32±0.021 mg/g TEAC, respectively. While, total phenolic and flavonoid content were 34.96±0.006 mg/g GAE and 15.72±0.050 mg/g QUE, respectively. After that, compound analysis by GC-MS detected some potential compounds in DF-CME-MOL named; Phenol, 4-(methoxymethyl)-, 2-Methoxy-4-vinylphenol, Phenol, 2-propyl, Phenol, 2-methoxy-4-(1-propenyl)-, (Z)-, 4-((1E)-3-Hydroxy-1-propenyl)-2-methoxyphenol, Hexadecanoid acid, methyl ester, Phenol, 2-[(4-hydroxyphenyl) methyl], Phenol, 4,

4’-methylenebis-, Phenol, 2-ethyl-, Benzeneethanol, 4-hydroxy-, Benzaldehyde, 4-hydroxy-, Benzeneacetonitrile, 4-hydroxy- and Benzaldehyde, 3,4-dimethoxy-. Then, apoptosis analysis was carried out by using Annexin V-FITC through flow cytometry FACS and further confirm by Western blot in detected the signalling of apoptosis pathway using selected proteins such as p53, Bax, Cytochrome c and Caspase 8. The DF-CME-MOL greatly induced early apoptosis through extrinsic pathway in MCF-7 cells. Thus, MOL has a potential to be developed into anticancer agent on breast cancer.

CHAPTER 1

INTRODUCTION

1.1 Background of study

The ductal carcinoma is a common type of breast cancer where it initiates either in the duct cell, lobules or tissues in the breast (Sharma et al., 2010). Once it starts to spread surround the tissue, it called invasive breast cancer. Commonly, breast cancer happens mostly among woman. However, it still happens rarely to man. According to American Cancer Society (2016) reported that the total of breast cancers occurrence in the USA are 246,660-woman and 2,600-man. Additionally, about 40,890 deaths caused by the disease. Nonetheless, it is worrying when breast cancer incidence is increasing in Malaysia and led as the main cancer disease where the statistics for breast cancer occurrence revealed an increasing about 30.4 % to 47.5 % (International Agency for Research on Cancer, 2018). Surprisingly, International Agency for Research on Cancer (2018) shown that the rate of breast cancer occurrence in both sexes about 17.3 % which is the highest cancer incidence followed by colorectal (14 %), lung (10.7 %), nasopharyngeal (4.8 %) and liver (4.4 %). The high number of breast cancer incidence has increased awareness and conducting a research needed for better understanding to promote the human health.

Treatment of breast cancer has several options to overcome that disease such as surgery, chemotherapy, radiation therapy, hormone therapy and targeted therapy (Komen, 2014). However, it depending on stage and type of cancer. Even so, each treatment option has possible risks and side effects. In addition, modern medicines and/or therapies offered nowadays are quite expensive and can be led to dangerous side effect toward patient's body system by causing of appetite loss, vomiting,

nausea, faintness, hair loss, bleeding, diarrhea, premature menopause, mouth soreness, and lowered resistance to infections (Siegel et al., 2012).

Therefore, studies must be carried out to overcome current side effect by identifying potential medicinal plants which can help in reducing the symptom of side effect. Besides that, the medicinal plant is commonly believed to promote human health that might prevent chronic diseases like cancers (Martin et al., 2011). Thus, most researchers have focused on potential medicinal plants and a more detailed study is required to seek the safety of compound that contented natural ingredients of bioactive foods, nutrients or phytochemicals, (Fahey et al., 2012). In this study, we also interested with beneficial of medicinal plant namely *Moringa oleifera* (MO).

In Malaysia, MO is known as Munggai or Kelor tree. However, it has a variety of names in different areas such as Horseradish tree, Drumstick tree, Miracle tree, Mother's best friend, and West Indian Ben (Fahey, 2005). The name given is based on the medicinal usage of *Moringa oleifera* (MO). MO is one of the medicinal plant that had been consumed by humans traditionally, especially in African and India as their folk remedies. This tree can be found around sub-Himalayan tracts of Africa, South, and Central American, Caribbean, Oceania, and Asia. Until now, it was widely grown at tropics and sub-tropics area either on loamy, sandy, or sandy-loam with drought-tolerance (Leone et al., 2015).

Virtually all part of this tree: leaf, flower, fruit (pods), seed, seed oil, bark, gum, and root have been used for numerous ailments as folk remedies when it has been proved to exhibit an amount of nutrition. This tree contains of minerals, amino acids, vitamins, β-carotene, alkaloids, flavonoids and numerous phenolic compound (Alhakmani et al., 2013). MO has been consumed because it poses some medicinal properties that act as antitumor, antipyretic, cardiac and circulatory stimulants, antiulcer, antiepileptic, anti-inflammatory, antispasmodic, diuretic, liver disease, antihypertensive, cholesterol lowering, antioxidant, antifungal, hepatoprotective, antibacterial, diabetes mellitus and antidiabetic activities (Kumar et al., 2010). MO also has been reported contains of compounds like glucosinolates, isothiocyanates, kaempferol, rhamnetin, quercetin, isorhamnetin, isoquercetin and more (Leone et al., 2015). However, the extensive research needs to be done to gain more information on the effect of MO that could be able to inhibit several major mechanisms in cancer development (Karim et al., 2016).

1.2 Problem statement

A few studies carried out abroad have proven the effectiveness of MOL as an anticancer agent on several type of cancer cells. However, there is still lack of information on apoptosis death mechanism pathway in MCF-7 cells. Therefore, this study would be beneficial to further explore the cytotoxicity effect of MOL extract on induction of death mechanism pathway in MCF-7 cells that could be act through induction of apoptosis pathways. Besides that, this study also focus on the effectiveness of fraction solvent extracts from various degree of polarities that had been not done yet on cytotoxicity effect of MCF-7 cells. The flow of research study can be referred to **Figure 1.1**.

1.3 Objectives of study

The objectives of this study are:

1. To investigate the cytotoxicity effect of MOL extracts on MCF-7 cells.
2. To analyze phytochemical contents of MOL extract by GC-MS.
3. To determine the death mechanism pathway through induction of apoptosis on MCF-7 cell treated with MOL extract by western blot analysis.

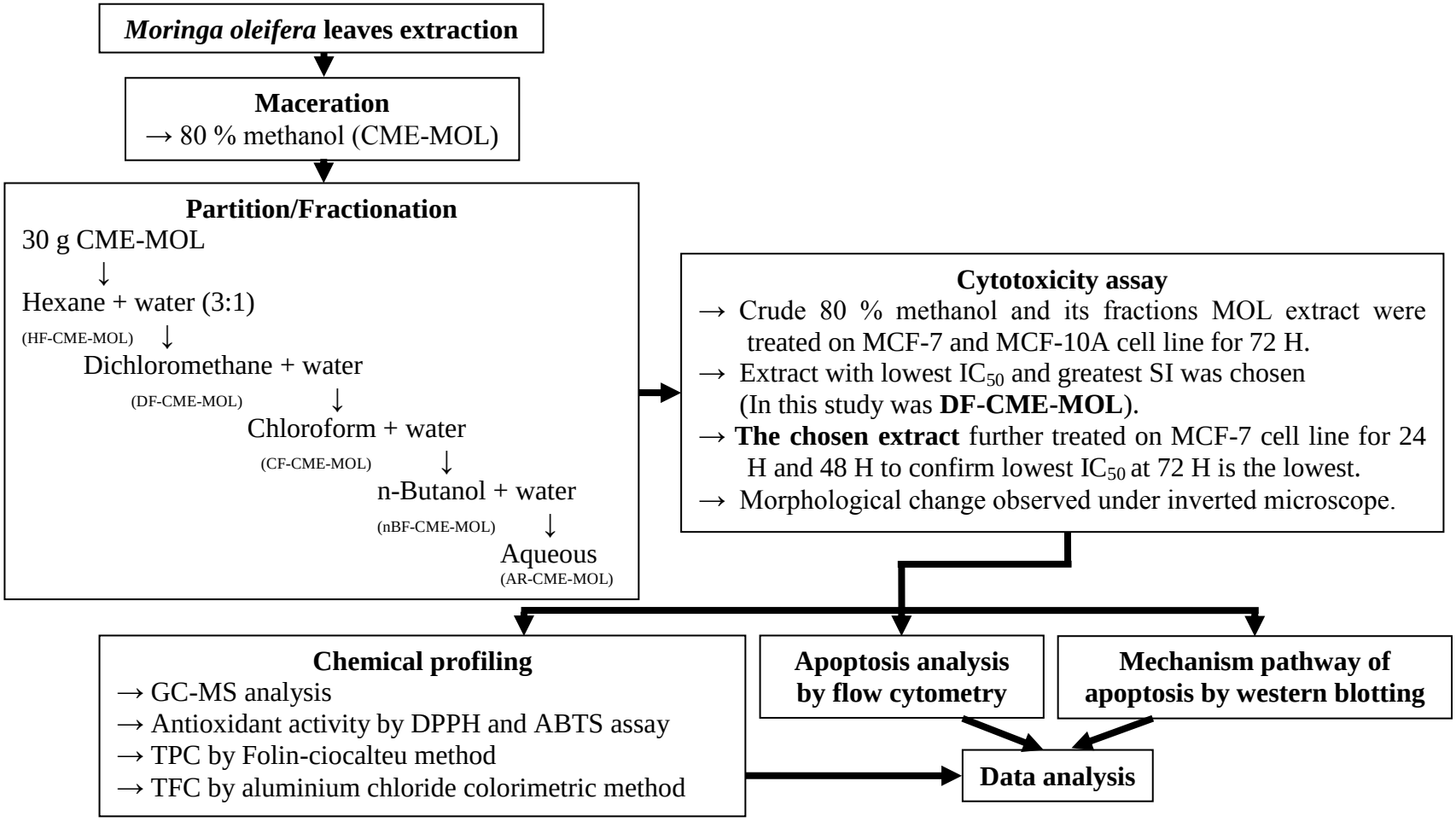

Figure 1.1 Flow chart of study

CHAPTER 2

LITERATURE REVIEW

2.1 *Moringa oleifera*

2.1.1 Introduction and its importance as a medicinal plant

Moringa oleifera Lam. (MO) is a well known species among thirteen species of the family Moringaceae (**Table 2.1**). It is usually recognized as Drumstick tree, Ben Oil tree, Miracle tree, Horseradish tree and West Indian Ben tree (Anwar et al., 2007), while in the Philippines, it is recognized as 'Mother's best friend' because it can be used as milk booster (Kumar et al., 2010). However, in Malaysia, it is known as Munggai or Kelor tree.

MO is a valuable plant that composed as various of medicinal properties. MO provide an important source of new drugs and potential pharmaceutical compounds because of containing several phytochemicals which beneficial in medicinal properties (Berkovich et al., 2013).

Table 2.1 Other well-known of varieties species of Moringa.

Family	Species	Reference
Moringacae	*M. arborea*	Daba, 2016
	M. borziana	
	M. concanensis	
	M. drouhardii	
	M. hildebrandtii	
	M. longitude	
	M. ovalifolia	
	M. peregrina	
	M. pygmaea	
	M. rivae	
	M. ruspoliana	
	M. stenopetala	

All parts of this plant: leaves, roots, seed, bark, fruit, flowers and immature pods contain a profile of important minerals and are a good source of protein, β-carotene, vitamins, amino acids, flavonoids and various phenolic (Moyo et al., 2011). As stated by Gopalakrishnan et al. (2016), the amounts of 11 elements in the MO leaves samples have been found specifically, which are the macronutrients: potassium, phosphorus, magnesium, calcium, and the micronutrients: copper, iron, manganese, zinc, boron, aluminium and sodium. This kind of mineral compounds can be extracted using solvent extraction (Zhao and Zhang, 2013).

There have many usage of every part of MO tree (**Table 2.2**) such as water purification from powdered seeds (Anwar et al., 2007, Sengupta et al., 2012 and Al-Anizi et al., 2014), medicine from all plant parts (Fahad et al., 2010) and malnutrient juice expressed from the leaves contain more iron than spinach, more vitamin C than oranges, more potassium than bananas and more calcium than milk which are usually used for infants and nursing mothers (Fahey, 2005). In Malaysia, the young tender pods of fruit are used for cooking which cut into small pieces and added to curries (Abdulkarim et al., 2005).

Table 2.2 The usage of each part of *Moringa oleifera* tree.

Parts of *Moringa oleifera*	Uses	References
Leaves	1. Natural medicines 2. Human food 3. Animal Fodder 4. Cleaning agent 5. Natural pesticide	Daba, 2016
Flowers	1. Source of nectar 2. Natural medicines 3. Human food	
Seeds	1. Oil production 2. Water purification 3. Natural medicines 4. Human food 5. Cosmetic 6. Lubricant 7. Fertiliser	
Pods	1. Natural medicines 2. Human food	
Bark	1. Natural medicines 2. Animal Fodder 3. Fuelwood 4. Rope/mats and paper	
Gum	1. Natural medicines 2. Human food	
Roots	1. Natural medicines 2. Human food 3. Alley cropping	
Resin	1. Textile industry	

Besides that, as stated by Kumar et al. (2010), MO also can act as antitumor, antibacterial, antifungal activities, antipyretic, antiepileptic, anti-inflammatory, antiulcer, antispasmodic, diuretic, antihypertensive, cholesterol lowering, antioxidant, antidiabetic, hepatoprotective, and can be effected for the treatment of various diseases in the indigenous system of medicine. MO also has potential to promote normal blood-glucose level, support immune system, enrich the anemic blood, improves eyesight, mental alertness, bone strength, burst lactating mothers, menopause, depression, osteoporosis (Mahmood et al., 2010). In addition, Rathi et al. (2006) concluded that the aqueous extract of MO had wound healing property.

MO also has been used in folk medicine revealed for centuries from generation to generation in many cultures around the world to treat anaemia, anxiety, skin infections, blackheads, asthma, bronchitis, blood impurities, chest congestion, infections, fever, glandular, cholera, swelling, scurvy, headaches, hysteria, pain in joints, psoriasis, pimples, respiratory disorders, abnormal blood pressure, semen deficiency, tuberculosis, sore throat, sprains, pregnancy and for intestinal worms (Mishra et al., 2012). Bakre et al. (2013) also reported that MO is traditionally used for the treatment of epilepsy and neurologic conditions. MO also has been suggested as a viable supplement of dietary minerals (Farooq et al., 2012).

2.1.2 Distribution of *Moringa oleifera*

MO is an important crop and grow wild especially in Sudan, India, Philippines, Ethiopia, and being grown in Tropical Asia, East, West and South Africa, Latin America, Florida, Caribbean and the Pacific Islands (**Figure 2.1** and **Figure 2.2**) (Fahey, 2005; Khalafalla et al., 2010; Paul and Didia et al., 2012). This plant can be propagated through sexual and/or asexual because it not demand for high soil nutrients and water hence, easy to handle and manage its planting (Adedapo et al., 2009). However, MO has been introduced as a field crop for production of biomass by propagating its seed (Nouman et al., 2012). It cultivates in many tropical and subtropical country with irregular environmental features with annual precipitation of 760 to 2500 mm, temperature between 18 to 28 °C and pH between 4.5 to 8, at an altitude up to 2000 m (Leone et al., 2015). It normally cultivated throughout the plains area especially in borders and in-house yards, grow well under the tropical climate and are abundant near the streams and sandy of rivers (Adedapo et al., 2009). In addition, it can cultivate well in hot dry lands or moist tropics and can survive on insolvent soils with little effect by drought tolerance (Zhao and Zhang, 2013).

Figure 2.1 Places of *M. oleifera* grows (Adapted from Trees for life, 2005).

Figure 2.2 Places of *M. oleifera* needed as food sources and medicinal drug treatment (Adapted from Trees for life, 2005).

2.1.3 Morphology of *Moringa oleifera* tree

This tree normally grows up between 10 to 12 m in height with whitish, soft, thick and corky bark (**Figure 2.3**). The tree spreading by an open crown of drooping with brittle branches, feathery foliage of tripinnate leaves and some parts such as flowers, fruits, roots and seed oil (Dao and Kabore, 2015). According to Zhigila et al. (2015), leaves shapes are tripinnate (with long up to 45 cm), alternate and spirally arranged on the twigs. Its structure is hairy, green on the upper surface, paler and hairless on the lower surface (**Figure 2.4 (A)**). The branches are finely hairy and green but turn brown when mature. According to Paliwal et al. (2011), the color of the flower is yellowish-white with a 10-25 cm long, five spathulate petals, five smaller stamens, five staminodes, and thinly veined (**Figure 2.4 (B)**). Paliwal et al. (2011) also described the fruits are linear, pendulous, three-sided pods with nine longitudinal ridges and usually long between 20 to 50 cm (**Figure 2.4 (C)**). However, its fruit can longer more than 1 m, and 2.0 to 2.5 cm broad. In pod's fruit, normally contains the seed about 26 seeds. The color of seed is dark green in early development and takes around three months to mature after flowering. They chance to brown during maturity and split open longitudinally along the three angles, spreading the dark brown of trigonous seeds (**Figure 2.4 (D)**). The measurement of seeds about 1 cm in diameter, with three whitish thin wings on the angles. Seed weights differ among variabilities, ranging between 3,000 to 9,000 seeds per kilogram (Paliwal et al., 2011).

Figure 2.3 *Moringa oleifera* tree (Adapted from Chaudhary and Chaurasia, 2017).

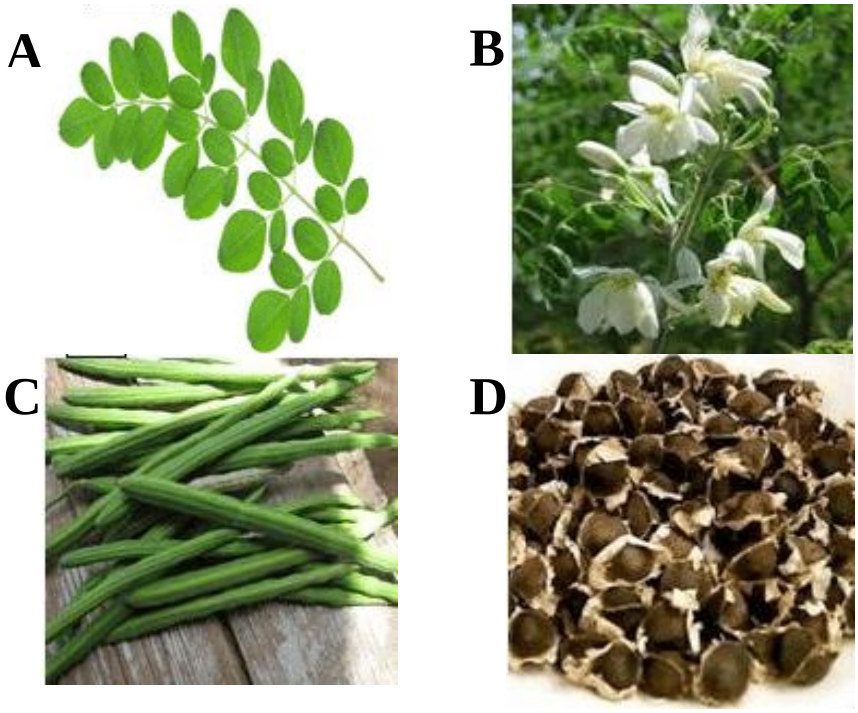

Figure 2.4 Part of *Moringa oleifera* tree: (A) Leaves (B) Flowers (C) Fruits and (D) Seeds (Adapted from Chaudhary and Chaurasia, 2017).

2.1.4 Bioactive compound of *M. oleifera* as medicinal plant

The bioactive compounds are usually referred as functional ingredients and responsible in the functional bioactivities (Ibañez et al., 2012). Bioactive compounds in the plant are known as secondary metabolites because it derived from the primary metabolites (Chikezie et al., 2015). The secondary metabolites gave a diverse range of toxicological or pharmacological effects in human and animal system (Bernhoft, 2010). Primary constituents include the common sugars, purines and pyrimidines of nucleic acids, amino acid of proteins, chlorophyll's etc., while secondary constituents include terpenes (a group of lipids), alkaloids (derived from amino acids), and phenolics (derived from carbohydrates) (Sen and Chakraborty, 2011). The production of secondary metabolites in specific pathway and site of synthesis depends on plant species (Yazdani et al., 2011). Secondary metabolites have played important role in various biological activities such as anticancer, antioxidant, antimicrobial, antihepatotoxic, antileprosy, antimalarial, anticholinergic, hypoglycemic, hepatoprotective, CNS depressant and mutagenicity (Negi et al., 2011).

Merina et al. (2012) and Sharma et al. (2011) reported that MO tree was used in folk remedies for tumors. The present study deals with the isolation of bioactive compounds by maceration and fractionation of extraction from MO leaves by using different polarity of solvents to make sure the possible of bioactive compounds which possess to anticancer and antioxidant were extract out. Phytochemical studies have shown that MO contains compounds with diverse chemical classes such as quercetin, kaempferol, niazimicin, beta-sitosterol, zeatin, vanillin, glucosinolates, isothiocyanate, saponins and tannins that are believed to have anticancer activities (Atawodi et al., 2010; Baraya et al., 2014). Isothiocyanate has antitumor activity in cancers of breast, lung, esophagus, skin, and pancreas (Merina et al., 2012). The

specific components in MO extract can be attributed to the anticancer property such as 4-(α-L-rhamnopyranosyloxy) benzyl isothiocyanate, 4-(α-L-rhamnopyranosyloxy) benzyl glucosinolates and benzyl isothiocyanate (Tiloke et al., 2013).

Ferreira et al. (2008) stated that all parts of MO are contain renewable sources like phenolic compounds, tocopherols (α and γ), vitamin C, ß-carotene, and total proteins, including the amino acids, sulfur, cysteine and methionine which acts as antioxidant compound. Al-Asmari et al. (2015) revealed numerous known anticancer compounds in MO leaves, bark and seed extract, namely isopropyl isothiocyanate, eugenol, hexadecanoic acid ethyl ester and D-allose. Karthika et al. (2013) reported that MO leaves extract can be acted against bacterial or fungal skin due to the presence of 1, 2-Benzene-dicarboxylic acid, diethyl ester, linalool oxide and palmitic acid. The present of bioactive compounds in plant extracts can be detected by using phytochemical screening assays, chromatographic techniques (TLC, HPLC, LC-MS, and GC-MS) and non-chromatographic techniques (immunoassay and FTIR) (Sasidharan et al., 2011).

2.1.5 Induction of apoptosis by *Moringa oleifera* in cancer cells

Solvent and aqueous extracts of MO have been reported to have significant antiproliferative effects on several type of cancer cells such as breast, colon, alveolar and pancreatic cell and induce apoptosis in KB, HeLa, HepG2, A549, MDA-MDB 231, HCT-8, and Panc-1 cells. For example, Charoensin (2014) carried out the study using two-stage of MO leaves extraction by using dichloromethane and methanol solvents. Both extracts were treated on breast cancer cell (MCF-7), colorectal cancer cell (Caco-2), and hepatocellular carcinoma (HepG2) to determine efficacy of growth inhibition. The extracts were significantly inhibit the proliferation of cancer cells in dose dependent manner but, unaffected on cell growth of normal human

fibroblast. However, their study lack of information about the knowledge of apoptosis inductions.

On the other hand, there have several pre-clinical research study had confirmed the ability of leave, seed, and bark of MO to induce apoptosis in various cancer cell leading their death mechanism pathways by activation or inhibition of apoptosis. According to Tiloke et al. (2013) study, hot water extract of MO leaves was induced apoptosis in A459 lung cancer cells by upregulating expression of pro-apoptotic proteins such as p53, Smac/DIABLO, downregulating expression of anti-apoptotic protein (Nrf2), and cleavage of PARP-1. Therefore, increased activities of caspase 3, 7 and 9 in treated A459 cells. In a related study, Jung (2014) proved that cold aqueous extract of MO leaves had been reported to trigger apoptosis in lung cancer cells (A549) through mitochondrial mediated pathway by activation of pro-caspase 3 to caspase 3. Additionally, the staining of propidium iodide (PI) on membrane integrity of A549 cells were also observed where PI was able to penetrate the membrane and attached to nucleic acid in the cytoplasm.

Additionally, according to the study by Sreelatha et al. (2011), hot water of MO leaves extract had triggered apoptosis in KB cells. PI and DAPI staining of treated KB cells revealed that the cells were permeable to PI which suggested that apoptosis had occurred. Moreover, DAPI staining was double confirmed the presence of nuclear changes peculiar to apoptotic cell death such as the appearance of condensed and fragmented chromatin. Also, study by Berkovich et al. (2013) reported that hot water of MO leaves extract induced apoptosis in Panc-1 cells by downregulating an expression of NF-kβ signalling pathway.

While, Al-Asmari et al. (2015) study showed that ethanolic extracts of MO leaves and barks induced apoptosis in MDA-MDB-231 cells and HCT8 cells. However, ethanolic extract of MO seeds mildly triggered apoptosis in HCT8 cells only. Their study were done by using double staining of annexin V and PI techniques where annexin V binds to translocated phosphatidylserine (PS) on the outer surface of cell membrane caused by apoptosis and PI passes through cell membrane when the membrane integrity has been compromised due to necrosis cell death and intercalate into nucleic acid of cell cytoplasm.

Hermawan et al. (2012) study was also proved that ethanolic of MO leaves extract treated on HeLa cells were detected apoptotic bodies. Then, the apoptosis cell death was further enhanced in the HeLa cells when treated with a combined drug of 100 nM doxorubicin and 250 µg/mL of MO leaves extract. Besides that, Jung et al. (2015) showed that a potential oral anticancer drug of cold water extract of MO leaves induced apoptosis on HepG2 cells by activating caspase 3 via cleavage and downregulating the expression of Bcl-xL anti-apoptotic protein.

However, further molecular study are required to understand the mechanism action of the plants parts by focusing on the cytotoxicity effect of different degree of solvent polarities extractions as continued in this present study.

2.2 Cancer

The field of this study known as oncology. However, there have certain fields can be related to cancer research such as cell and molecular biology, chemistry, physiology, epidemiology, anatomy, and other related fields (American Cancer Society, 2014).

Cancer proliferation involves multiple process (**Figure 2.5**) as following: 1) initiation, 2) promotion, 3) progression, 4) invasion, and 5) metastasis (Siddiqui et al., 2015). Cancer can occur when DNA damage is a failure to repair and lead to genetic mutation. This damaged cell caused by somatic mutation can be duplicated during division of mitosis, hence give chance to clone of mutated cells. Therefore, the promotion of tumor can further initiate cells to produce an active proliferation of multicellular premalignant tumor cell population that developed through a process of clonal expansion. Finally, cancer development, invasion, and metastasis happen in the late stages of cancer where tumor cells detach from primary tumor mass and migrate through neighboring tissues toward blood vessels or lymphatic vessels and create a second lesion. When this happens, it accumulates the cancer regulating genes become changing in oncogenes and/or tumor suppressor genes, resulting in decreased apoptosis, increase proliferation, cell maturation, and differentiation (Dai and Mumper, 2010).

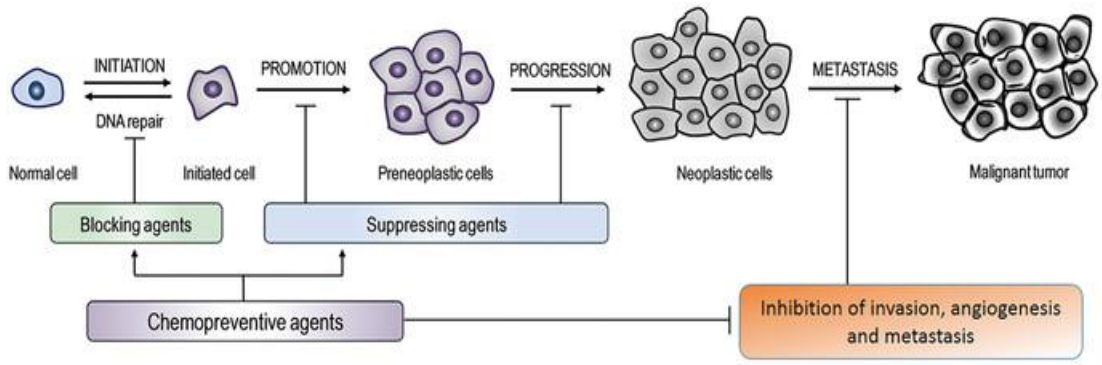

Figure 2.5 Carcinogenesis phases (Adapted from Siddiqui et al., 2015).

There are many types of cancer in this world. The most common cancer and widely occurs are lung cancer (1.82 million), breast cancer (nearly 1.67 million) and colorectal cancer (nearly 1.36 million) (Farley et al., 2014). However, breast cancer is the most common cancer among females followed by cervical cancer while colorectal cancer is increasing in dominance in both sexes (Pharmaceutical Association of Malaysia, 2009-2015).

2.2.1 Breast cancer

Breast cancer can be classified into different subgroups depending on the expression of progesterone receptor (PR), estrogen receptor (ER) and human epidermal growth factor receptor 2 (HER2) (Liu et al., 2014). Breast cancer is derived from carcinoma of breast and start growing in an abnormal way, thus it is causing to malignant tumor. The process happens is called as metastasis where cancer cell spreads out to other parts of the body and begin to destruct other tissues and organ usually includes the lungs, liver, bones or brain (Rahman and Mohammed, 2015).

There have several categories of a tumor; 1) non-invasive breast cancer benign stay in one spot without spread to other cell or organ and 2) invasive breast cancer spread other parts of the body. Somehow, breast cancer also can occur at the specific cell surface receptors which are progesterone receptor (PR), estrogen receptor (ER) and human epidermal growth factor receptor (HER)2/neu receptor (Zaha, 2014).

The types of breast cancer that caused by these receptors are known as HER2-positive breast cancer, HER2-negative breast cancer, hormone receptor-positive breast cancer and triple negative breast cancer (TNBC). HER2-positive breast cancer cell is express too much of a protein HER2/neu, HER2-negative do not overexpress a protein HER2/neu and hormone receptor-positive breast cancer in response to the hormones estrogen and progesterone (Iqbal, 2016). But TNBC does not respond or overexpress to receptors of estrogen, progesterone, and HER2/neu (Podo et al., 2010).

While, the symptoms of breast cancer are 1) new lump in or near breast or under arm, 2) swelling on a part of breast, 3) irritation, 4) redness on breast or nipple area, 5) nipple pulling inside or pain around nipple area, 6) liquid discharge other than breast milk that occurs without squeezing and 7) change in size or shape of breast (American Cancer Society, 2017).

2.2.2 Breast cancer cell line (MCF-7 cells)

In cancer research, cancer cell line is widely used and has been established as a model of human cancer for specific disease types since it is ubiquitous and used for our better understanding in cells and molecular activity, gene expression, its function or malfunction, interaction with other cells, physiology changes and therapeutic strategies (Keen, 2011). It was maintained by sub culturing under certain conditions in a laboratory. Cancer cell lines are used in research to study the biology of cancer and to test cancer treatments (*in vitro* study) (Gillet et al., 2013).

In the recent study, the type breast cancer cell line used is MCF-7 (adenocarcinoma type). MCF-7 has been established in 1973 at the Michigan Cancer Foundation-7 (MCF-7) (Soule et al., 1973). It is an invasive human breast cancer that expresses ER (Lee et al., 2015). It was predicted as MCF-7 ER-positive cell line where estrogen-stimulated tumor growth directly (Levenson and Jordan, 1997). According to Holliday and Speirs (2011), ER is a therapeutic target. Besides that, it is most commonly used in the world as an experimental tool in cancer research because they compromise an unlimited supply of a relatively standardized cell population where they capable of self-replication in standard cell culture medium. Zaha (2014) stated that PR, ER, HER2, Ki-67, and p53 are the most common used in immunohistochemical breast cancer prognostic and therapeutic markers.

2.3 Cancer metabolism pathway

The programmed cell death process happens naturally to control and balances cell proliferation by eliminating the damaged cells. In our body system, there are three type of program cell death and their mechanism are different. Apoptosis (known as Type I programmed cell death) is committing to suicide (**Figure 2.6**), autophagy (known as Type II programmed cell death) is maintaining cytoplasmic membranes and cell body and completely removed without any inflammation (**Figure 2.7**) while necrosis (known as Type III programmed cell death) is murdering the cells (**Figure 2.6**). These three distinct types of cell death are specifying for the fate of cell (Ouyang et al., 2012). In addition, the form of cell death depends on the genotype of cell, type of cell, type of DNA mutation and dose of sample used (Fulda and Debatin, 2006). All types of programmed cell death can be distinguishable based on morphological features as shown in **Table 2.3**.

Table 2.3 Morphology features of apoptosis, autophagy, and necrosis.

Type of cell death	Morphology features	References
Apoptosis	1. Nuclear changes (nuclear chromatin condensation and nuclear fragmentation) 2. Membrane blebbing (cause losing attachment with adjacent cells) 3. Cell contraction/shrinkage 4. Form apoptotic bodies.	Mollazadeh et al. (2015)
Autophagy	1. Formation of autophagosome, 2. Cytoplasmic macromolecules 3. Organelles surrounded by double membrane-bound structures.	Parzych and Kliansky (2014)
Necrosis	1. Swelling of cell 2. Dysfunction of organelle 3. Disrupted cell membrane 4. Cell lysis.	Nikoletopoulou et al. (2013)

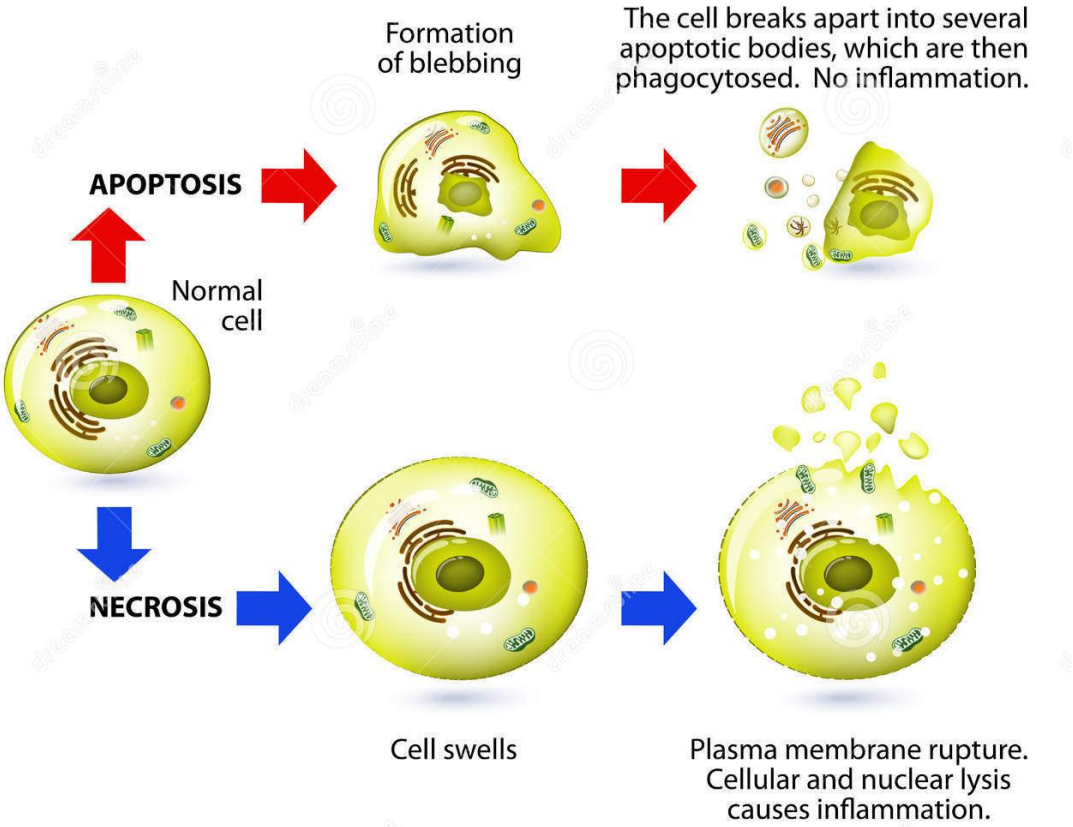

Figure 2.6 Diagram of apoptosis and necrosis (Adapted from Thinkstock, 2016).

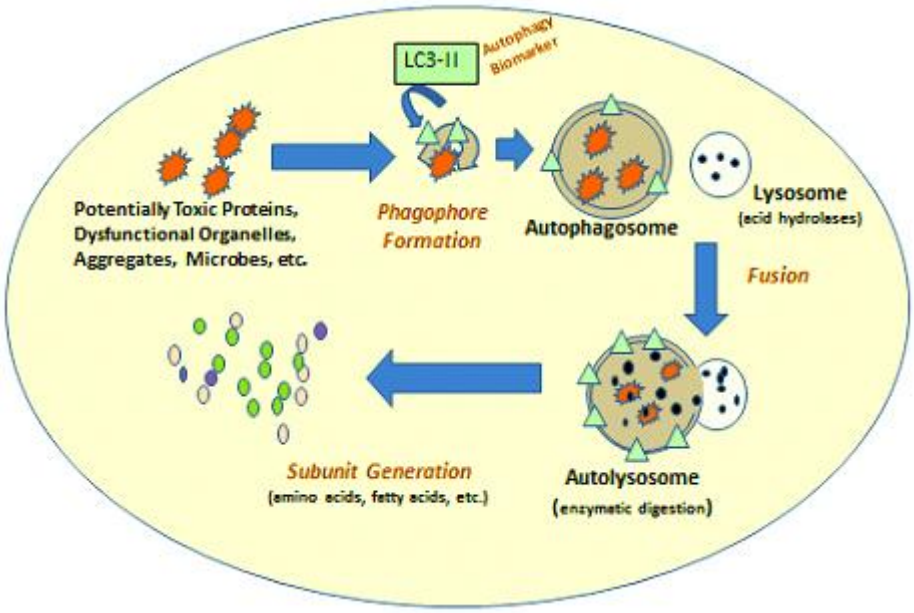

Figure 2.7 Diagram of autophagy (Adapted from Biophagy, 2013).