



*IN VITRO* CYTOTOXICITY STUDY OF *Melastoma malabathricum* Linn.: THE EFFECTS TOWARD MMP-13 EXPRESSION AND APOPTOSIS

BY

NURFARIZA AHMAD ROSLEN

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International Islamic University Malaysia

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## ABSTRACT

The search for natural anticancer remedies is one of the most prominent research in the cancer treatments. For that purpose, various plants from all over the world have been studied. The researchers are now more focusing to understand the mechanisms involved in the cancer treatment using the natural sources. *Melastoma malabathricum* Linn is known as a shrub that wildly grows in the tropical and subtropical regions. A number of studies have been conducted on this plant, but the reports on its anticancer properties are still limited. The main objective of this study is to study the effects of *M. malabathricum* extracts from different parts at various concentrations on three types of cancer cell lines *in vitro* (A375, HeLa and MCF-7) and its relation to the apoptosis mechanism and the expression of the target protein, MMP-13 in the treated cells. Liquid-liquid extraction protocol using methanol, petroleum ether and chloroform as the solvent systems were carried out on leaves, stems and flowers of *M. malabathricum*. Dimethyl sulfoxide, DMSO (1 %) was used in the extract dilution and serial dilutions were conducted to obtain eight different extract concentration, ranging from 0.078125 µg/mL to 10 µg/mL. The evaluation of cancer cells growth inhibition for 24, 48 and 72 hours of treatment was determined using MTT assay. The treated cancer cells were then tested for morphological apoptosis detection through TUNEL assay and detection of MMP-13 expression using Western Blot analysis. The result showed that petroleum ether extracts of stems (PeMMS) and leaves (PeMML) have the best growth inhibitory effects on A375 ( $EC_{50} = 0.185 \mu\text{g/mL}$  at 48 hours) and HeLa cell lines ( $EC_{50} = 0.368 \mu\text{g/mL}$  at 48 hours). The chosen extracts were also confirmed do not cause toxicity effect on normal human fibroblast cell lines (CCD-1090Sk). Further analysis revealed that PeMMS and PeMML caused a high percentage of apoptotic cells, around 26 % in A375 and HeLa cells respectively. The apoptosis results are comparable or in approximate with the percentage of apoptosis induced by commercialized anticancer drug, paclitaxel (26.4 %). Western blot analysis showed the reduction of MMP-13 expression in the A375 and HeLa cells treated with PeMMS and PeMML, respectively. Based from the outcomes, this study suggests that the reduced expression of MMP-13 correlates with the increasing level of apoptosis in the cells treated with PeMMS and PeMML. In conclusion, the petroleum ether extract of stems and leaves of *M. malabathricum* showed a promising anticancer properties toward skin melanoma and cervical cancer cell lines. Further investigation needs to be conducted in order to assess the probable bioactive compounds in the petroleum ether extract of *M. malabathricum* that may contribute to the significance cytotoxic effects to the respective cell lines.

## خلاصة البحث

حيث تمت دراسته علاقة هذا المستخلص باليه عمليه موت الخلايا الالي وبعمليه التمثيل البروتيني لبروتين الام ام بي ثلاثة عشر في الخلايا السرطانيه المعالجه. ولقد اتبع بروتوكول الاستخلاص السائل باستخدام الميثانول و البتروليوم ايثر مع الكلوروفورم كعوامل مذيبه كما واستخدمت مادة ثنائي ميثيل اوكسيد الكبريت في المستخلص كعامل مخفف وتم تخفيف المستخلص في سلسله من العمليات للحصول على ثمانية تراكيز مختلفه للمستخلص تتراوح من 0.078125 مايكروغرام\مل الى 10 مايكروغرام\مل. ولقد تم تقييم عمليه تثبيط نمو الخلايا السرطانيه بعد 24 و 48 و 72 ساعه من العلاج باستخدام تقنية الام تي تي ومن ثم تم اختبار الخلايا السرطانيه المعالجه للكشف عن التغيرات المجهرية في عمليه الموت الخلوي الالي باستخدام تقنية نتل وكما تم ايضا الكشف عن التمثيل البروتيني لبروتين الام ام بي ثلاثة عشر باستخدام طريقة الويستين بلوتينك المختبريه. لقد اظهرت النتائج ان مستخلصات العشبة باستخدام مادة البتروليوم ايثر ومستخلص السيقان واوراق العشبه كانت تمتلك افضل التأثيرات المثبطه لنمو الخلايا السرطانيه من بين الطرق الاخرى في خلايا سرطان البشره وسرطان عنق الرحم بعد 48 ساعه من العلاج. كما وتم التاكيد من عدم سميته هذا المستخلصات على الخلايا الطبيعيه للبشره في الانسان. واثبت ايضا عمليه البحث اللاحقه ان مستخلص السيقان والاراق للعشبه سبب نسبة كبيره من الموت الخلوي الالي بنسبه 26 بالمئه في خلايا سرطان البشره وخلايا سرطان عنق الرحم. ولقد تم مقارنة نتائج مستخلص العشبة في عمليه الموت الخلوي الالي مع نتائج عقار تجاري مضاد للسرطان يعرف بتاكسول وكانت النتائج متقاربه ومتشابهه. كما واطهرت نتائج تحليل الويستين بلوتينك انخفاض في عمليه التمثيل البروتيني لبروتين الام ام بي ثلاثة عشر في خلايا سرطان البشره وسرطان عنق الرحم المعالجه بمستخلص الساق واوراق العشبة وبناء على هذه النتائج فان هذه الدراسة ترجح ان النقصان التمثيل البروتيني لبروتين الام ام بي ثلاثة عشر له علاقته مع زياده مستوى الموت الخلوي الالي في الخلايا السرطانيه المعالجه بمستخلص السيقان والاوراق للعشبة.

## **APPROVAL PAGE**

I certify that I have supervised and read this study and that in my opinion, it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a thesis for the degree of Master of Health Sciences.

.....  
Mohammad Syaiful Bahari Abdull Rasad  
Supervisor

I certify that I have supervised and read this study and that in my opinion, it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a thesis for the degree of Master of Health Sciences.

.....  
Muhammad Ibrahim  
Examiner

I certify that I have read this study and that in my opinion, it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a thesis for the degree of Master of Health Sciences.

.....  
Venugopal Balakrishnan  
External Examiner

This thesis was submitted to the Department of Biomedical Science and is accepted as a fulfilment of the requirement for the degree of Master of Health Sciences.

.....  
Ibrahim Adham Taib  
Head, Department of Biomedical Science

This thesis was submitted to the Kulliyah of Allied Health Sciences and is accepted as a fulfilment of the requirement for the degree of Master of Health Sciences.

.....  
Wan Azdie Mohd Abu Bakar  
Dean, Kulliyah of Allied Health Sciences

## **DECLARATION**

I hereby declare that this thesis is the result of my own investigations, except where otherwise stated. I also declare that it has not been previously or concurrently submitted as a whole for any other degrees at IIUM or other institutions.

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Signature .....

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THE EFFECTS TOWARD MMP-13 EXPRESSION AND APOPTOSIS**

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*To my beloved husband and family,  
For your love, support and patience*

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## LIST OF ABBREVIATIONS

3LL	Lewis lung carcinoma cell lines
A375	Human skin melanoma cell lines
ANOVA	Analysis of variance
APS	Ammonium persulfate
ATCC	American Type Cell Culture
ATF-6	Activating transcription factor 6
Bak	Apoptosis regulator Bak
Bax	Apoptosis regulator Bax
Bcl-2	B-cell lymphoma 2
Bcl-xS	B-cell lymphoma-extra small
Bid	Apoptosis regulator Bid
Bim	Apoptosis regulator Bim
CAPS	<i>N</i> -cyclohexyl-3-aminopropanesulfonic acid
CCD-1090Sk	Human normal fibroblast cell lines
CHMMF	Chloroform <i>Melastoma malabathricum</i> flower extract
CHMML	Chloroform <i>Melastoma malabathricum</i> leaf extract
CHMMS	Chloroform <i>Melastoma malabathricum</i> stem extract
DAB	Diaminobenzidine
DISC	Death inducing signalling complex
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DU145	Prostatic adenocarcinoma cell lines
EC <sub>50</sub>	Half maximal effect concentration
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EMEM	Eagle's Minimum Essential Medium
ER	Endoplasmic reticulum
FADD	Fas-associated death domain
FasL	Fas ligand
FRIM	Forest Research Institute Malaysia
HCl	Hydrochloric acid
HeLa	Human cervical carcinoma cell lines
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPV	Human papilloma virus



Hrk	Apoptosis activator Hrk
IRE-1	Inositol requiring enzyme-1
K562	Chronic myeloid leukaemia cell lines
L1210	Leukaemic cell lines
MeOH	Methanol
MMMF	Methanol <i>Melastoma malabathricum</i> flower extract
MMML	Methanol <i>Melastoma malabathricum</i> leaf extract
MMMS	Methanol <i>Melastoma malabathricum</i> stem extract
MMP	Matrix metalloproteinases
MMP-13	Matrix metalloproteinases 13
MT1-MMP	Membrane type-1 matrix metalloproteinases
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NIH	National Institute of Health
NP-40	nonyl phenoxy polyethoxy ethanol
p53	Tumour protein 53
PBS	Phosphate-buffered saline
PeMMF	Petroleum ether <i>Melastoma malabathricum</i> flower extract
PeMML	Petroleum ether <i>Melastoma malabathricum</i> leaf extract
PeMMS	Petroleum ether <i>Melastoma malabathricum</i> stem extract
PERK	Protein kinase RNA-like endoplasmic reticulum kinase
PMSF	phenylmethanesulfonyl fluoride
RIPA	Radio-Immunoprecipitation Assay
SDS	Sodium dodecyl sulphate
TEMED	N,N,N',N'-tetramethylethane-1,2-diamine
TNF	Tumour necrosis factor
TNFR	Tumour necrosis factor receptor
TP53	Gene encodes for protein 53
TRADD	TMF receptor-associated death domain
U251	Glioblastoma cell lines
UPR	Unfold protein response
VEGF-2	Vascular endothelial growth factor 2
WHO	World Health Organization

# **CHAPTER ONE**

## **LITERATURE REVIEW**

### **1.1 CANCER**

As years passed by, cancer is still one of the leading death causes in the world. Its complexity makes the process of finding the most suitable cure becomes more difficult. This is due to the hundreds of mechanisms that can affect the cancer's behaviour. Researchers and scientist around the globe have discovered more than thousands of cancer-causing gene and the number can be increased in the future (Mandes, 2014). Currently, the cancer research has been focusing to understand its various mechanisms and to produce more natural and less destructive treatment.

Cancer has been categorized as genetic disease since it's involved the alterations of genetic arrangement in specific genes, but in most cases, it is not necessarily inherited (Karp, 2013). The genetic changes caused the human cells to behave differently. In this case, the cancer cells will grow continuously, denying any stop growth signals and producing a group of cancer cells known as a tumour. The tumour will invade the surrounding of normal tissue and may be able to spread to the other organs as well. This process is known as metastasis, where the malignant tumour will break away from its origin and enters lymphatic or vascular circulation and spread to other sites in the body.

According to the report by World Health Organization (WHO) (2014), cancer's death around the world have been estimated around 8.2 million, while more than 14.1 million new cases were recorded in the five years of their data collection. Until the year of 2012, it can be concluded that there are more than 32 million people that suffer

with cancers worldwide. The numbers are even much higher in the less developed countries. The most commonly diagnosed cancers worldwide were those of the lung (1.8 million, 13.0% of the total), breast (1.7 million, 11.9%), and colorectum (1.4 million, 9.7%). The most common causes of cancer death were cancers of the lung (1.6 million, 19.4% of the total), liver (0.8 million, 9.1%), and stomach (0.7 million, 8.8%) (WHO, 2012).

As in Malaysia, until the year of 2012, the most commonly diagnosed cancers in both sexes were breast (5,410, 14.5% of the total), colorectum (4,539, 12.1 %) and lung (4,403, 11.8%). However, the mortality rate was highest in lung (4,134, 19.1% of the total) followed by breast (2,572, 11.9%) and colorectum (2,300, 10.6%) (WHO, 2012). In men, of course, lung becomes the most common cancers, followed by colorectum and nasopharynx. While in women, the most common cancers were those of the breast, cervix uteri and colorectum. Overall, men possess slightly higher risk of getting cancer (15.4%) as compared to the women (14.6%). But it can be concluded that both sexes have risk around 15% of getting cancers before the age of 75 years old (WHO, 2012).

The statistics on cancer incidence in Malaysia in the year of 2002, which was ten years back showed different trends (National Cancer Registry, 2002). According to the report, Malaysia women tend to get cancers (55%) as compared to the men (45%). The most common cancers incidence among Malaysia men were lung, nasopharynx and nose. While common cancers in Malaysian women were breast and cervix uteri (National Cancer Registry, 2002). Colorectum cancer incidence was expected to be increasing in both sexes as time goes by. Today, colorectal cancer has become one of the most common cancers, especially in Malaysian men.

There are a lot of external and internal factors involved in the increasing incidence of cancers worldwide. External factors can always be referred as 'environmental factors' such as lifestyles, infection to viruses and radiations to the cancer-causing agents. Regular smoking of cigarette, high-fat diet intake and alcohol abuse are always becoming the common reasons for people to have a high risk of getting cancers such as lung, colon, bladder, mouth and much more (NIH, 2003).

Cancers can be divided into many types, basically based from where it originates. Carcinoma is the cancer that starts from the lining cells that cover external and internal surfaces in our body. It is the most frequent types of cancer incidence worldwide such as breast, colon and lung cancer. Secondly, the sarcomas that arise from the cells in the tissues such as muscle, bone and connective tissue. Lymphomas are the cancers that originate in the tissue of the immune system and lymph nodes as the name suggest. Another type of cancer is leukaemia that start in the immature blood cells produces in the bone marrow. It is likely to travel abundantly in the bloodstream to metastasize.

### **1.1.1 Skin Melanoma**

Skin melanoma is a type of skin cancer that originates from the melanin-producing pigment located in the basal layer of human skin epidermis known as melanocytes (Chodurek, Orchel, Gawlik, Kulczycka, Gruchlik, and Dzierzewicz, 2010) Although it can be considered as a less common type of cancer, (WHO, 2012), it is the most aggressive cancer so far. The low survival rates of five years due to the unresponsive treatment, mainly chemotherapy caused this type of cancer is hard to cure (Caputo, Maiorana, Vasta, Pezzino, Sunkara, Wynne and Kane, 2011).

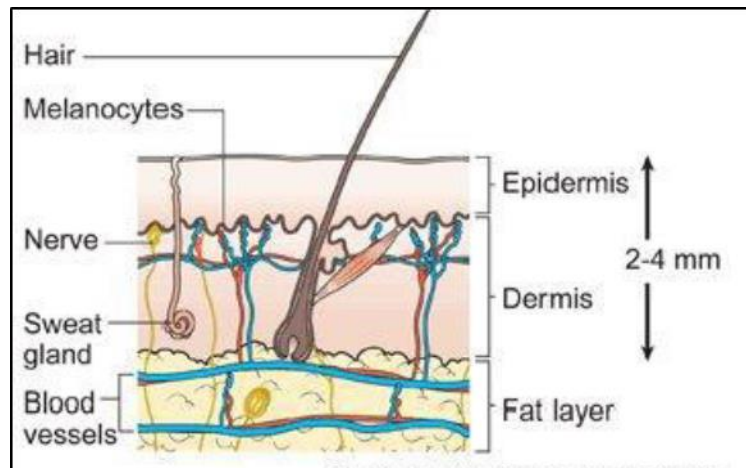


Figure 1.1 A schematic structure of a normal human skin (Adapted from Herbst and Ransburg, 2013).

It happens when the unrepaired DNA damage in the skin cells caused the mutation that facilitates the cells to grow more rapidly than normal cells. The overgrowth is often associated with the formation tumours. The tumours, if become malignant (metastasize) is where the skin melanoma becomes dangerous. The most important sign of this deadly cancer is the formation of spots (look like moles) that coloured black, brown, skin-coloured, pink, red, purple, blue or maybe white. Sometimes, the mole itself is also an indicator for skin melanoma (Herbst and Ransburg, 2013).

The causes of skin melanoma are mainly due to the excessive exposure to the ultraviolet radiation from sunshine, tanning equipment, and possibly, genetically inherited. Also, the person with the history of breast cancer may have a higher risk of developing skin melanoma (WHO, 2012).

At the early stage, surgical excision is the only treatment available to remove a thin layer of melanoma and may be some part of the skin with no further treatment. There were several treatments available for the patients with spreading cancers. Surgery can be done to remove the affected lymph nodes if it spreads to the nearby

lymph nodes. The other options are chemotherapy, radiation therapy and biological therapy. Biological therapy uses the substance that is naturally produced by the human body to boost the immune system to help fight the cancer.

### 1.1.2 Cervical Cancer

Cervical cancer is the second most common cancer among the women in the world. It starts from the cells lining the cervix, the lower part of the uterus and also known as the womb. Essentially, cervix acts as a connector between uterus and vagina. Cervix can be divided into two parts, exocervix and endocervix. Usually, the cancer will start at the point where these parts meet. A pre-cancerous form of cervical cancer does not means any harm and may go away without any treatment. However, once it becomes the invasive form, this is where the danger comes (American Cancer Society, 2013).

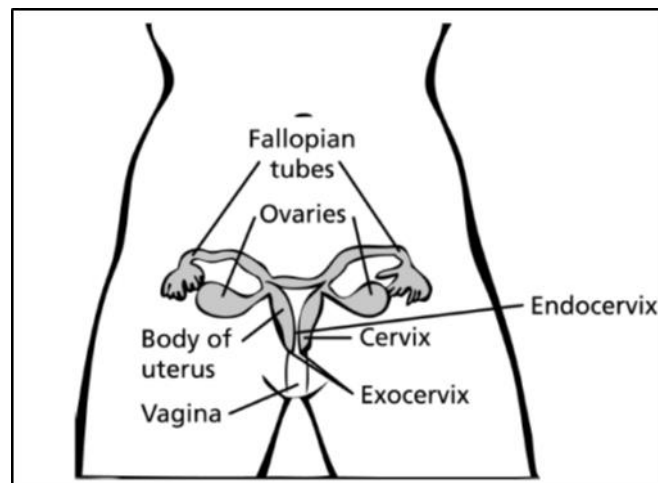


Figure 1.2 A schematic diagram of a woman's reproduction system (Adapted from American Cancer Society, 2013).

This cancer type is mainly caused by the prolonged infection with human papillomavirus (HPV). Women that involved in sex at an early age or having several sex partners are at higher risk of developing cervical cancer. This type of infection is quite typical in healthy women and it will resolved by our defence system. However, cervical cancer can develop when the immune system is blocked, increase number of childbirth and external factor such as smoking (tobacco or cigarette) (WHO, 2013).

The symptoms often develop when the cervical cancer is in invasive form, starting with abnormal vaginal bleeding and discharge. The bleeding may occur before, during or after the menstrual periods, sexual intercourse, douching or a pelvic exam. This may also occur to women that past menopause period. At the late stages, back pain, lethargy, nausea and renal failure are the symptoms that may appear. Like the other types of cancer, cervical cancer can be treated with the surgery, radiotherapy and chemotherapy.

### **1.1.3 Breast Cancer**

Women's breasts consist of glandular tissues, fats and connective tissues. Glandular tissues are made up of lobes, the place where the milk is produced and connected to the nipple through a network of milk ducts. Other than that, there are extensions of breast tissue under the armpit that contain lymph nodes. The lymph nodes also located beside the breastbone and behind the collarbone (American Cancer Society, 2013). It is the part of lymphatic systems that naturally function as body's defence systems against infections.

This type of cancer is the most common cancers affecting women around the world. It remains as the leading cancer-causing death in women (WHO, 2013). Usually, it starts from the lining cells in the glands (lobular carcinoma) and the ducts

(ductal carcinoma) of the breast (American Cancer Society, 2013). These cells tend to overgrowth and caused the formation of lumps around the breasts.

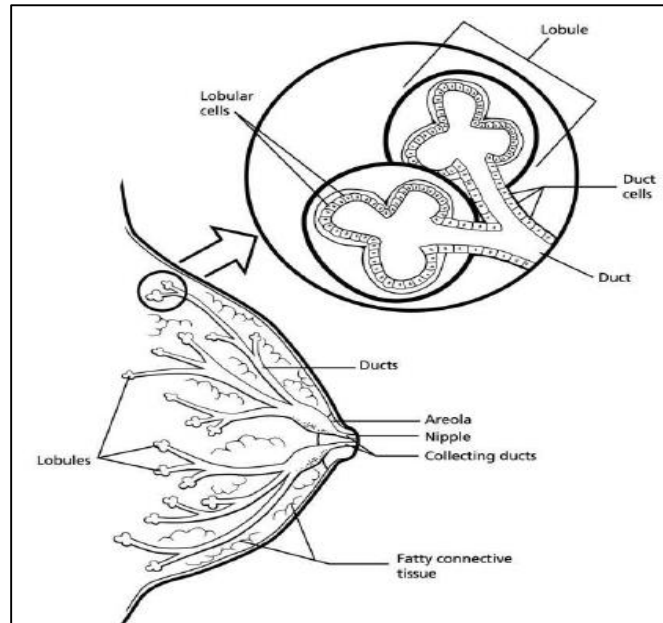


Figure 1.3 A schematic diagram of a normal woman breast (Adapted from American Cancer Society, 2012).

Normally, early detection will not be able to give any result. When the lump is formed, then only the other symptom may become apparent. This includes the change of size or shape of the breasts, dimpling of the skin or thickening in the breast tissue, inverted nipple (turned in), rash around the nipple, and liquid discharge from the nipple (American Cancer Society, 2013). The area under the armpit may also become swollen or lump formation that indicated the lymph nodes already infected.

The exact causes of this cancer are still unknown. Based on the studies and statistics conducted by WHO and NCI, gender, age and hormone may be among the factors that elevate the chance of getting this cancer. Other than that, it can be inherited. One may also have a higher risk of breast cancer when overexposed to the