UNIVERSITI PUTRA MALAYSIA

ANTI-BREAST CANCER EFFECT OF EUPATORIN In Vitro AND In Vivo

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IB 2018 30

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By

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Thesis submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirement for the Degree of Doctor of Philosophy

January 2018

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Doctor of Philosophy

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Chair: Associate Professor Noorjahan Banu Alitheen, PhD
Faculty: Biotechnology and Biomolecular Sciences

Triple-negative breast cancers (TNBC) represent the most aggressive form of breast cancers and their treatments are challenging due to the tumour heterogeneity and chemo-resistance which have been the major obstacle. Chemotherapy using synthesized drugs is commonly used to treat breast cancer. However, this kind of treatment may cause negative side effects to the patients. Recently, eupatorin had been reported to be a potent candidate to inhibit breast cancer cells proliferation. Eupatorin is a product that belongs to a flavonoid family. However, the detailed information on the efficacy and mechanisms of eupatorin as an anti-breast cancer agent *in vitro* and *in vivo* is very limited. In this study, the cytotoxic effect, anti-proliferative action, cell cycle arrest and apoptosis induction of eupatorin on the human breast carcinoma cell lines of MDA-MB-231 (estrogen-receptor negative), MCF-7 (estrogen-receptor positive) and on a cell line derived from normal mammary tissue, MCF-10a were studied. In addition, the *in vivo* antitumour effect of eupatorin was tested on the Balb/c mice challenged with 4T1 murine mammary cancer cells. MTT assay showed that eupatorin and tamoxifen (positive control) had cytotoxicity effects against the cancerous cell lines of MCF-7, MDA-MB-231 and 4T1 cells. At 48 hours, eupatorin at 14.52 µM inhibited cell proliferation of MCF-7 and MDA-MB-231 by 50%. However, the IC_{50} of tamoxifen at 48 hours to inhibit MCF-7 and MDA-MB-231 cells proliferation was 5.23 µM and 7.22 µM, respectively. In contrast, the IC_{50} value of eupatorin at 48 hours for MCF-10a was significantly ($p<0.05$) high with 58.09 µM. Through scratch assay, eupatorin (14.52 µM) prohibited the complete closure of scratched area in MDA-MB-231 cells after 24 hours incubation. In addition, Boyden chamber assay revealed that eupatorin at

14.52 µM inhibited the aggressiveness of MDA-MB-231 cells where less than 40% of the cells were migrating and invading the membrane in the Boyden chamber. Moreover, *ex vivo* model using aortic ring from Balb/c mouse suggested that eupatorin can act as anti-angiogeneic due to the inhibition of aortic sprouting activation in mouse aortic rings assay. In cell cycle analysis using MCF-7 and MDA-MB-231 cells, eupatorin enhanced the accumulation of cells in sub Gθ/G1. At 48 hours, the number of MCF-7 and MDA-MB-231 cells accumulated in sub Gθ/G1 was 27.52 ± 2.06% and 42.75 ± 4.67% respectively. Concurrently, the percentage of early apoptotic of respective MCF-7 and MDA-MB-231 cells were 28.38 ± 0.24% and 64.04 ± 0.66%. In addition, the population of late apoptotic cells of MCF-7 and MDA-MB-231 were 40.26 ± 0.33% and 18.27 ± 0.57%, respectively. In contrast, more than 95% of the untreated cells for both cell types were distributed in Gθ/G1 phase at 48 hours. In gene expression assay, eupatorin up-regulated the pro-apoptotic genes such as Bak1, Bax, cytochrome c, SMAC/Diablo and HIF1A in both cell lines and concurrently down-regulated the anti-apoptotic genes such as VEGFA and Bcl2L11. Furthermore, Western Blot analysis revealed that eupatorin could inhibit cells proliferation in MDA-MB-231 and MCF-7 cells through depletion of Cdc2 and Chk1 protein level respectively followed by the activation of Chk2 protein which lead to the Akt and MAPK signalling pathway blockage. Moreover, caspases activation confirmed that eupatorin initiated apoptosis via intrinsic pathway in MCF-7 and MDA-MB-231 cells due to high fold change number of activated caspase 9 compared to caspase 8. *In vivo* study showed that eupatorin at the dosage of 20 mg/kg was sufficient to delay the tumour development. Histologic assessment revealed that eupatorin (20 mg/kg) has significantly ($p<0.05$) enhanced the number of apoptotic cells in tumour. Additionally, clonogenic assay showed that eupatorin (20 mg/kg) has potently reduced the number of invaded 4T1 cells in lung where $30.70 \times 10^3 \pm 10.89$ blue colonies were detected whereas the untreated lung possessed $400.00 \times 10^3 \pm 28.28$ blue colonies. Besides, eupatorin (20 mg/kg) also has significantly ($p<0.05$) increased the NK cells and T-cell responses to 4T1 tumour cells in splenocytes assay. In immunophenotyping assessment, eupatorin (20 mg/kg) enhanced the stimulation of $NK1.1^+CD3$ and $CD8^+$ expression by 4.75 ±0.37% and 8.83 ±0.36% respectively when compared to the untreated ($NK1.1^+CD3$:3.57% ± 0.13; $CD8^+$:4.30%±0.07). Cytokine assay revealed that IL-1β was significantly ($p<0.05$) suppressed to 812.00 ± 57.50 pg/mL while the IFN-γ was enhanced to 1407.41 ± 25.66 pg/mL compared to the untreated (IL-1β :1045.33 ± 257.98 pg/mL; IFN-γ :1185.19 ± 187.86 pg/mL). Western blot analysis showed that eupatorin (20 mg/kg) could delay the tumour progression, invasion and migration through MAPK pathway blocking. Additionally, gene expression assay revealed that eupatorin (20 mg/kg) inhibited the regulation of gene MMP9, TNFα, and IL-1β expression sufficiently.

Taken together, eupatorin is a potent candidate as anti-breast cancer agent that has anti-proliferative and anti-metastasis activities on breast cancer cells which should be examined further in clinical study.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

KESAN EUPATORIN SEBAGAI ANTI-KANSER PAYUDARA *In Vitro* DAN *In Vivo*

Oleh

NURSYAMIRAH BINTI ABD RAZAK

Januari 2018

Pengerusi: Profesor Madya Noorjahan Banu Alitheen, PhD
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Kanser payudara ganda tiga-negatif (TNBC) telah dikenalpasti sebagai kanser payudara yang paling agresif serta kaedah rawatannya yang sangat mencabar berikutan kepelbagaian jenis tumor dan kimo-rintangan yang menjadi penghalang utama kepada keberkesanan rawatan. Kimoterapi menggunakan ubat sintesis lazim digunakan untuk merawat kanser payudara. Namun begitu, kaedah rawatan ini memberikan kesan negatif kepada pesakit. Baru-baru ini, eupatorin dilaporkan berpotensi menghalang pembiakan sel-sel kanser payudara. Eupatorin adalah produk dari kumpulan flavonoid. Walau bagaimanapun, maklumat lengkap keberkesanan dan kebolehan eupatorin menyirat sebagai agen anti-kanser payudara secara *in vitro* dan *in vivo* adalah sangat terhad. Dalam kajian ini, kesan sitotoksik, tindakan anti-proliferatif, rencatan kitaran sel dan rangsangan apoptosis oleh eupatorin terhadap sel-sel kanser karsinoma payudara seperti MDA-MB-231 (penerima estrogen negatif) dan MCF-7 (penerima estrogen positif) serta sel normal daripada tisu payudara iaitu MCF-10a telah dikaji. Tambahan lagi, kesan *in vivo* eupatorin sebagai anti-tumor juga diuji menggunakan tikus-tikus Balb/c yang disuntik dengan sel-sel kanser kelenjar mamma tikus 4T1. Asai MTT menunjukkan bahawa eupatorin dan tamoxifen (kawalan positif) mempunyai kesan sitotoksik terhadap sel-sel kanser MCF-7, MDA-MB-231 dan 4T1. Pada 48 jam, eupatorin pada kepekatan 14.52 µM telah merencat 50% pembiakan sel-sel MCF-7 dan MDA-MB-231. Walau bagaimanapun, nilai IC_{50} tamoxifen pada 48 jam untuk merencat pembiakan sel-sel MCF-7 dan MDA-MB-231 masing-masing adalah 5.23 µM dan 7.22 µM. Sebaliknya, nilai IC_{50} eupatorin pada 48 jam terhadap sel MCF-10a adalah signifikan ($p<0.05$) tinggi iaitu 58.09 µM. Melaui asai

gores, eupatorin (14.52 μM) telah menghalang penutupan lengkap kawasan gores pada sel-sel MDA-MB-231 selepas 24 jam inkubasi. Tambahan lagi, asai *Boyden chamber* menunjukkan eupatorin pada kepekatan 14.52 μM telah merencatkan kemasukan sel-sel kanser payudara (MDA-MB-231) secara agresif di mana kurang dari 40% sel-sel yang telah merentasi membran pada *Boyden chamber*. Seterusnya, model *ex vivo* menggunakan cincin aorta dari tikus Balb/c mencadangkan bahawa eupatorin mampu bertindak sebagai anti-angiogenik berikutan perencatan pertumbuhan aorta dalam asai cincin aorta tikus. Dalam analisis kitaran sel-sel MCF-7 dan MDA-MB-231, eupatorin telah meningkatkan taburan sel-sel di dalam sub Gθ/G1. Pada 48 jam, bilangan sel-sel MCF-7 dan MDA-MB-231 di dalam sub Gθ/G1 masing-masing adalah sebanyak 27.52 ± 2.06% dan 42.75 ± 4.67%. Sementara itu, peratusan sel-sel MCF-7 dan MDA-MB-231 pada awal apoptosis masing-masing adalah 28.38 ± 0.24% and 64.04 ± 0.66%. Tambahan lagi, populasi sel-sel MCF-7 dan MDA-MB-231 pada akhir apoptosis masing-masing adalah 40.26 ± 0.33% dan 18.27 ± 0.57%. Sebaliknya, lebih 95% sel bagi kedua-dua jenis sel yang tidak dirawat berada pada Gθ/G1 pada 48 jam. Melaui asai ekspresi gen, eupatorin meningkatkan regulasi gen pro-apoptotik seperti Bak1 ,Bax, cytochrom c, SMAC/Diablo dan HIF1A di kedua-dua jenis sel serta menghalang regulasi gen anti-apoptotik seperti VEGFA dan Bcl2L11. Selain itu, analisis sap western mendedahkan bahawa eupatorin mampu menghalang pembiakan sel-sel MCF-7 dan MDA-MB-231 dengan mengurangkan kandungan protein Cdc2 dan Chk1 seterusnya mengaktifkan protein Chk2 yang mengakibatkan terhalangnya isyarat tapak jalan Akt dan MAPK. Tambahan lagi, pengaktifan kaspase mengesahkan eupatorin memulakan apoptosis melaui tapak jalan intrinsik di dalam sel-sel MCF-7 dan MDA-MB-231 berikutan kadar pengaktifan kaspase 9 yang lebih tinggi dari kaspase 8. Kajian *in vivo* menunjukkan eupatorin pada dos 20 mg/kg mampu merencatkan pembiakan tumor. Penilaian histologi menunjukkan peningkatan signifikan ($p<0.05$) sel-sel apoptosis di dalam tumor oleh eupatorin (20 mg/kg). Tambahan lagi, asai klonogenik menunjukkan eupatorin (20 mg/kg) berpotensi mengurangkan bilangan sel-sel 4T1 yang merebak ke paru-paru di mana $30.70 \times 10^3 \pm 10.89$ koloni biru telah dikesan sementara paru-paru yang tidak dirawat mempunyai $400.00 \times 10^3 \pm 28.28$ koloni biru. Disamping itu, eupatorin (20 mg/kg) juga meningkatkan tindak balas sel-sel NK dan T terhadap sel-sel 4T1 secara signifikan ($p<0.05$) di dalam asai *splenocytes*. Melalui penilaian fenotipimun, eupatorin (20 mg/kg) meningkatkan stimulasi ekspresi $NK1.1^+CD3$ dan $CD8^+$ dengan peratusan masing-masing sebanyak 4.75 ± 0.37% dan 8.83 ± 0.36% berbanding yang tidak dirawat ($NK1.1^+CD3$:3.57% ± 0.13; $CD8^+$:4.30% ± 0.07). Asai sitokin juga menunjukkan pengurangan signifikan ($p<0.05$) IL-1β kepada 812.00 ± 57.50 pg/mL dan peningkatan IFN-γ kepada 1407.41 ± 25.66 pg/mL yang tidak dirawat (IL-1β :1045.33 ± 257.98 pg/mL; IFN-γ

:1185.19 ± 187.86 pg/mL). Analisis sap Western menunjukkan eupatorin (20 mg/kg) merencatkan pembiakan tumor, menghalang rebakan sel-sel tumor dengan menyekat tapak jalan MAPK. Seterusnya, ujian ekspresi gen mendedahkan eupatorin (20 mg/kg) menghalang regulasi gen MMP9, TNFα dan IL-1β dengan cekap. Berdasarkan hasil kajian, eupatorin adalah calon yang berpotensi tinggi sebagai agen anti-kanser payudara yang mempunyai sifat anti-pembiakan dan anti-metastasis terhadap sel-sel kanser payudara yang seharusnya dikaji dengan lebih lanjut dan terperinci secara klinikal.

ACKNOWLEDGEMENTS

In the name of Allah, the most gracious and the most merciful.

I wish to express my sincere gratitude to my supervisory committee Associate Professor Dr. Noorjahan Banu Mohamad Alitheen, Associate Professor Dr. Yeap Swee Keong, Dr. Tan Sheau Wei and Professor Dr. Tan Wen Siang for their technical guidance, encouragement and moral supports in carrying out this project work.

I sincerely thank Dr Kamariah Long for her motivation, inspiration and guidance and for encouraging me to embark this study. I may not be able to achieve this glory if it had not been to your kind heart to give the opportunity by introducing me to my supervisor and co-supervisors.

I also would like to acknowledge to the team members and staff in animal tissue culture laboratory (ATCL) who rendered their help during the period of my project. The completion of this project could not have been possible without the participation and assistance of so many people whose names may not all be enumerated. Their contribution are sincerely appreciated and gratefully acknowledge.

Thanks to my parents as well, Mr. Abd Razak Md Jais and Mrs. Latipah Ghani for their pray and countless times they helped me especially for babysitting my little princess Rania Alysha Ahmad Syakir throughout my journey in UPM. The appreciation also goes to my sibling Firdaus, Nur Syazni and Nurul Asyikin for their generous heart that always praying for my successful.

Not forgotten, my heartfelt thanks to my dear husband Ahmad Syakir Hariri for being my crying shoulder. His encouragement when the time get rough is much appreciated. It was a great relief to know that you are willing to provide everything you can give even though I know that you have struggled a lot. To my lovely daughter Rania Alysha Ahmad Syakir, I won't be this strong without you as my inspiration and motivation. You are the reason I keep pushing and struggling to face all the pains and hardship.

Finally, I would like to express my deepest gratitude to the Ministry of Higher Education for the honour of awarding the scholarship (MyPhd) during my study and also to Malaysian Agriculture Research and Development Institute (MARDI) for funding this project. Without the strong financial support, it would be impossible to complete this dissertation. Thank you for all your support.

This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Doctor of Philosophy. The members of the Supervisory Committee were as follows:

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Akt	Protein Kinase B
Apaf-1	Apoptosis Proteases Activating Factor 1
APC	Antigen Presenting Cells
Bad	Bcl2-antagonist of Cell Death
Bak1	Bcl2-antagonist/killer 1
Bax	Bcl2-associated X Protein
Bcl-2	B-cell Lymphoma 2
Bcl2L11	Bcl2-like 11 Protein
Bcl-X_L	B-cell Lymphoma Extra Large
BSA	Bovine Serum Albumine
BW	Body Weight Ratio
°C	Degree Celsius
CDC	Cell Division Cycle
Chk	Checkpoint Kinase
cm	Centimetre
CO_2	Carbon Dioxide
COX-2	Cyclooxygenase-2
CTLA-4	Cytotoxic T-lymphoscyte-associated Protein 4
CYP	Cytochrome P450
DC	Dendritic Cell
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
DTT	Diothreitol
ECM	Extracellular Matrix
EDTA	Ethylene Diamine Tetratacetate

EGFR	Epidermal Growth Factor Receptor
EGTA	Ethyleneglycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic Acid
ELISA	Enzyme-linked Immunosorbent Assay
ER	Estrogen Receptor
ERa	Estrogen Receptor Subtype a
FACS	Fluorescence-activated Cell Sorter
Fas	Tumour Necrosis Factor Receptor
FasL	Tumour Necrosis Factor Receptor Ligand
FBS	Foetal Bovine Serum
FGC	Fibroblast Growth Factor
FITC	Fluorescein Isothiocyanate
g	Gram
GAPDH	Glyceraldehyde-3-phosphate Dehydrogenase
G-CSF	Granulocyte-colony Stimulating Factor
H_2O_2	Peroxide
H_2SO_4	Sulphuric acid
HCl	Hydrochloric Acid
HER2	Human Epidermal Growth Receptor 2
HIF-1A	Hypoxia-inducible Factor-1 Alpha
IC_{50}	50% Inhibitory Concentration
IFN	Interferon
IgM	Immunoglobulin M
IL-6	Interleukin 6
IL-10	Interleukin 10
i.p	Intraperitoneal Injection

I.U	International Unit
kg	Kilogram
LD	Eupatorin Low Dosage (5 g/kg)
MAPKs	Mitogen-activated Protein Kinase Family Members
MCM	Maintenance Complex Component
MDSCs	Myeloid Derived Suppressor Cells
mA	Milliampere
µg	Microgram
mg	Milligram
mL	Millilitre
mm	Millimetre
µM	Micro molar
MMPs	Matrix Metalloproteinases
MMP2	Matrix Metallopeptidase 2
MMP9	Matrix Metallopeptidase 9
mRNA	Messenger Ribonucleic Acid
mTOR	Mammalian Target of Rapamycin
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide
N	Normality
NaCl	Sodium Chloride
NaF	Sodium Fluoride
NEM	N-ethylmaleimide
NF-κβ	Nuclear Factor Kappa B
ng	Nanogram
NK	Natural Killer Cell
NKT	Natural Killer T Cell

NPY	Neuropeptide Y
PAGE	Polyacrylamide Gel Electrophoresis
PARP	Poly(ADP-ribose) Polymerase
PBS	Phosphate Buffer Saline
PCA	Principal Component Analysis
PCNA	Proliferating Cell Nuclear Antigen
PCR	Polymerase Chain Reaction
PD-1	Programmed Cell Death Protein 1
PD-L1	Programmed Cell Death-ligand 1
PDGF	Platelet-derived Growth Factor
PMSF	Phenylmethylsulfonyl Fluoride
PR	Progesterone Receptor
PTEN	Phosphatase and Tensin Homolog
qPCR	Real-time Polymerase Chain Reaction
RBC	Red Blood Cell
RNA	Ribonucleic Acid
rpm	Revolutions per minute
s	Second
SD	Standard Deviation
SDS	Sodium Dodecyl Sulphate
SI	Selectivity Index
SMAC	The Second Mitochondria-derived Activator of Caspase
T_{reg}	Regulatory T Cell
TGF-β	Transforming Growth Factor β
TNBC	Triple Negative Breast Cancer
TNF	Tumour Necrosis Factor

p53	Tumour Protein (53 Kilo Dalton)
PI	Propidium Iodide
V	Voltage
VEGFA	Vascular Endothelial Growth Factor A
VEGFR	Vascular Endothelial Growth Factor Receptor

CHAPTER 1

INTRODUCTION

Cancer is a genetic disease, caused by changes of genes that control the cells growth and division. There are several types of cancer such as carcinoma, sarcoma, leukaemia, lymphoma, melanoma etc. (National Cancer Institute, 2015). Today, breast cancer is the second most common causes of cancer-related death in women worldwide, where delay diagnosis, rising incidence and high mortality rates make it essential to understand breast cancer literacy in women (Gupta et al., 2015).

Most patients are diagnosed with hormone-positive breast cancer (Steponaviciene et al., 2011) which can be classified into three subtypes based on the expression of the following hormone receptors: estrogen receptor (ER), progesterone receptor (PR), and cell surface receptor of human epidermal growth receptor 2 (HER2). However, breast cancer that do not express HER2, ER, and PR hormone is known as triple negative breast cancer (TNBC).

TNBC is more aggressive, difficult to treat and more likely to spread in diagnosed patients (National Breast Cancer Foundation, 2015). Women with TNBC have poor prognosis with few treatment options and therefore, new therapeutic agents for this aggressive tumour are critically needed (LaPorta & Welsh, 2014).

Unsuccessful TNBC treatment or late detection may lead to metastatic breast cancer, which is also known as advanced breast cancer or stage IV breast cancer. Metastatic TNBC may occur through lymphatic system or blood vessels which the aggressive breast cancer cells spread beyond breast to other organs such as brain, lung and liver to form secondary tumour through angiogenesis. Although metastatic breast cancer has invade to another part of body, it is still considered as breast cancer and treated with breast cancer drugs (Chang et al., 2014).

Previous studies have shown that matrix metalloproteinase-9 (MMP9) is involved in tumour metastasis and their elevated expression often lead to poor prognosis (Dodd et al., 2011; Kuei et al., 2012). In addition, high regulation of important angiogenic factor such asvascular endothelial growth factor A (VEGFA) also enhanced the tubular network formation (Karroum et al., 2012). Many studies repoted that the mitogen activated protein kinases (MAPKs) and protein kinase B (Akt) are commonly involved in promoting cancer cells invasion metastasis by activating the expression of MMP9 (Chun & Kim, 2013;

Chung et al., 2011; Dodd et al., 2011). MMP9 regulate the expression of VEGF to initiate angiogenesis (Tang et al., 2013). Hence, by inhibiting the signalling pathway of MAPK and Akt, expression of MMP9 and VEGF could be deactivated which lead to the inhibition of angiogenesis and metastasis.

Today, chemotherapy using synthesized drugs such as doxorubicin, cisplatin and epirubicin remains the only possible therapeutic option in the adjuvant setting in case of such cancers (Steponaviciene et al., 2011). In chemotherapy practice, the key success of the treatment is through induction of apoptosis and anti-metastatic event on cancer cells (Ferreira et al., 2014; Yixiang et al., 2012; Kuei et al., 2011). However, this kind of treatment may cause negative side effects to the patients such as low white blood cells count, hair loss, vomiting, and loss of weigh (McCarty, 2005; Rooijen et al., 2015; Vriens et al., 2013). Furthermore, although single pure chemical compounds can be very potent therapeutic agents, adverse side effects often lead to poor compliance, limited applications, and potential withdrawal from the market (Gosslau et al., 2011).

Besides the synthetic drugs, natural products such as flavonoids are also well known to have chemotherapeutics properties in treating cancer. Flavonoids may prevent cancer incidence and delay tumour development (Ledesma, 2011; Slattery et al., 2014). Additionally, it also help to reduce some negative side effects of synthesized compounds by reducing the cytotoxic effect and suppressing inflammation to patients (Gosslau et al., 2011).

Numerous researchers found that, flavonoids were capable to inhibit cancer cell proliferation and delay tumour progression (Androutsopoulos & Tsatsakis, 2013; Park et al., 2009) via supressing the metastasis, angiogenesis (Park et al., 2009) and by regulating many apoptosis related signalling pathways such as P13K/Akt/PTEN (Kim et al., 2014; Wróbel & Gregoraszczuk, 2015). Therefore, consuming natural products containing flavonoids may help patients with aggressive breast cancer to fight the disease.

Eupatorin (3',5-dihydroxy-4',6,7-trimethoxyflavone) is one of the potent candidate as anti-breast cancer agents (Androutsopoulos et al., 2008; Salmela et al., 2012). This bioactive compound belongs to flavonoid group and can be found in local herbs such as the leaves of Misai Kucing (*Orthosiphon stamineus*) (Yam et al., 2012). Previous research reported that eupatorin potently suppressed proliferation and induced apoptosis in multiple cancer cell lines such as HeLa cells and breast cancer cells (Salmela et al., 2012; Dolečková et al., 2012). However, the detail efficacy and mechanisms of eupatorin as anti-breast cancer agent *in vitro* and *in vivo* are very limited.

In vitro study, MCF-7 and MDA-MB-231 cells are widely used to study breast cancer disease. The MCF-7 cells is a human breast cancer cell line express estrogen-positive alpha receptor (ERα+). In breast cancer disease, approximately 70% patients highly express the ERα+ and therefore, the ERα+ has been proven to be one of the most important therapeutic targets in breast cancer over the last 30 years (Zheng et al., 2015). In contrast, MDA-MB-231 cell is triple-negative breast cancer cell line that represent estrogen receptor negative (ER-) (Hernández-Bedolla et al., 2014). This type of cell line is highly invasive and metastasize and therefore it is extensively used in breast cancer research (Hernández-Bedolla et al., 2014). Thus, this study was aimed to evaluate the cytotoxic effect and the mechanisms of eupatorin on MCF-7 and MDA-MB-231 breast cancer cell lines *in vitro*. In addition, the *in vivo* antitumor effect of eupatorin was tested on the murine mammary carcinoma 4T1 challenged Balb/c mice. The 4T1 cell is one of the most aggressive metastatic cancer cell lines that closely resemble the aggressive triple-negative breast cancer disease (Gao et al., 2011).

The hypotheses formulated in this study are as follows:

1. Eupatorin could show cytotoxic effects on MCF-7 and MDA-MB-231 cells by inhibiting the proliferation and induce apoptosis of cancer cell lines. Also, eupatorin could inhibit the angiogenesis and metastatic activity of breast cancer cells *in vitro*.
2. Eupatorin could enhance the pro-apoptotic genes regulation and concurrently reduced the activity of pro-survival and anti-apoptotic genes of cancer cells. Furthermore, eupatorin could also blocked the regulation of genes expression such as MMP9 and VEGFA which related to angiogenesis and thus prevented the cancer metastases.
3. Eupatorin could delay the tumour progression in 4T1-induced Balb/c mice by suppressing 4T1 cells proliferation and metastasis. Moreover, eupatorin at sufficient dosage could also enhanced the immune system of tumour mice by regulating T cells and natural killer (NK) cells activities that initiate antitumor cytokines production including interferon gamma (IFN-Y) and interleukin-1 beta (IL-1β).

The objectives of the study were:

1. To determine the *in vitro* cytotoxicity, anti-angiogenesis and anti-metastatic effects of eupatorin in MCF-7 and MDA-MB-231 cells.
2. To evaluate the differential regulation of target proteins and genes expression involve in metastases, angiogenesis and apoptosis of MCF-7 and MDA-MB-231 cells treated with eupatorin.
3. To determine the *in vivo* anti-tumour potential of eupatorin in Balb/c mice challenged with 4T1 cells.

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