ANTI-CANCER ACTIVITY AND IMMUNOADJUVANT PROPERTIES OF EDIBLE BIRD'S NEST EXTRACTS ON HUMAN BREAST CANCER CELL LINE

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DEDICATION

This thesis is dedicated to my beloved lord, family, supervisors and friends for their continued support and blessing throughout the journey of this research.

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ABSTRACT

Edible Bird's Nest (EBN) is well regarded as an immune enhancing nutraceutical food especially among the Chinese. Current cancer therapies bring adverse side effects, thus identification of a safe adjuvant medicine like EBN can provide a quality life for patients. Researches on the effect of EBN extract on the human breast cancer cell line (MCF-7) and human immune cells are still very limited. The aim of this study is to evaluate the anti-cancer activity and immunoadjuvant properties of EBN extract on MCF-7 cell line. Primarily, 3 types of EBN extracts coded as HMG, EHMG and pHMG were prepared using the water extraction method. These EBN extracts were then tested on their cytotoxicity level against MCF-7 and human immune cells (CD8+ and CD14+). Production of the key pro-apoptotic and anti-apoptotic molecules released in MCF-7, CD8+ and CD14+ cells before and after EBN treatment were measured through mRNA expression level, ELISA and Multiplex assay. Among the 3 EBN extracts, HMG showed the highest cytotoxic effect towards MCF-7 cells with IC_{50} of 15 μg/mL. However, HMG showed no harm towards CD8+ and CD14+ cells with cell viability of more than 90%. qRT-PCR results for non-activated and activated CD8+ and CD14+ cells showed increased of pro-apoptotic gene expression while lower anti-apoptotic gene expression after treated with HMG in single and co-culture. At the same time, supplementation of HMG increased the apoptosis through down regulation of anti-apoptotic genes and the up-regulation of pro-apoptotic genes in MCF-7 cells. Enhancement of pro-apoptotic and down regulation of anti-apoptotic soluble factors by non-activated and activated CD8+ and CD14+ cells in single and co-culture after treated with HMG also showed in ELISA and multiplex assay. In conclusion, the present study showed that HMG extract is a potential anti-cancer agent and causes no harm to human immune cells. qRT-PCR, ELISA and multiplex tests also verified that HMG acts as an immunoadjuvant by enhancing pro-apoptotic function in the human immune cells.

ABSTRAK

Sarang burung walit (EBN) dianggap sebagai satu makanan yang dapat meningkatkan imuniti khususnya di kalangan kaum Cina. Terapi kanser yang terdapat pada masa kini, boleh membawa kesan sampingan kepada pesakit. Oleh itu, pengenalpastian ubat adjuvan yang selamat seperti EBN dapat memberi kehidupan yang berkualiti untuk pesakit kanser. Penyelidikan mengenai kesan ekstrak EBN terhadap sel kanser payudara manusia (MCF-7) dan sel-sel pertahanan badan masih sangat terhad. Kajian ini bertujuan untuk menilai aktiviti anti-kanser dan sifat-sifat imunoadjuvan ekstrak EBN pada sel-sel MCF-7. Terutamanya, ekstrak EBN dikodkan sebagai HMG, EHMG dan pHMG telah disediakan melalui kaedah pengekstrakan air. Ekstrak-ekstrak ini kemudian diuji pada paras kesitotoksikkan mereka terhadap MCF-7 dan sel-sel pertahanan badan (CD8+ dan CD14+). Pengeluaran molekul pro-apoptotik dan anti-apoptotik dalam sel-sel MCF-7, CD8+ dan CD14+ sebelum dan selepas rawatan EBN diukur melalui tahap ungkapan mRNA, ujian ELISA dan multipleks. Antara 3 ekstrak EBN, HMG menunjukkan kesan kesitotoksikkan tertinggi terhadap sel MCF-7 dengan IC_{50} 15 µg/mL. Walau bagaimanapun, HMG menunjukkan tiada mudarat terhadap sel-sel CD8+ dan CD14+ dengan jumlah sel sihat lebih daripada 90%. Keputusan qRT-PCR untuk sel-sel tidak teraktif dan sel-sel yang diaktifkan CD8+ dan CD14+ menunjukkan tahap ungkapan gen pro-apoptotik yang meningkat dan anti-apoptotik yang menurun selepas dirawat dengan HMG bagi kultur tunggal dan bersama. Pada masa yang sama, penambahan HMG meningkatkan apoptosis melalui penurunan tahap ungkapan gen anti-apoptotik dan peningkatan gen pro-apoptotik dalam sel-sel MCF-7. Peningkatan faktor-faktor larut pro-apoptotik dan penurunan anti-apoptotik oleh sel-sel tidak teraktif dan sel-sel yang diaktifkan CD8+ dan CD14+ dalam kultur tunggal dan bersama, selepas dirawat dengan HMG telah dibuktikan melalui ujian ELISA dan multipleks. Kesimpulannya, kajian ini menunjukkan bahawa ekstrak HMG adalah agen anti-kanser yang berpotensi dan tidak memudaratkan sel-sel pertahanan badan. Ujian qRT-PCR, ELISA dan multiplex juga mengesahkan bahawa HMG bertindak sebagai imunoadjuvan dengan mempertingkatkan fungsi pro-apoptotik dalam sel-sel pertahanan badan.

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LIST OF ABBREVIATIONS

3T3	-	3-day transfer, inoculum 3×10^5 cells
10X TBE	-	1.0 Tris, 0.9M Boric acid, 0.01M EDTA
3D	-	Three Dimensional
7AAD	-	7-amino-actinomycin D
A431	-	Human squamous cell carcinoma cell line
AA	-	Antibiotic antimycotic
ABTS	-	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
ANOVA	-	One way analysis of variance
APAF-1	-	Apoptotic Protease Activating Factor 1
APC	-	Allophycocyanin
APCs	-	Antigen Presenting Cells
ATCC	-	American Type Culture Collection
BAX	-	Bcl-2-associated X protein
BCL2	-	B-Cell Lymphoma-2
BD	-	Becton, Dickinson and company
BJ1-hTERT	-	Human fibroblasts
BRCA	-	Breast Cancer
BSA	-	Bovine Serum Albumin
BXPC-3	-	Human Pancreatic Cancer Cell Line
CA	-	California

Caco-2	-	Human colonic adenocarcinoma cell line
CASP-7	-	Caspase 7
CCL2	-	Monocyte chemotactic protein-1
CD	-	Cluster of Differentiation
cDNA	-	Complementary DNA
CFSE	-	Carboxyfluorescein diacetate succinimidyl ester
CO_2	-	Carbon dioxide
CS&T	-	Cytometer Setup and Tracking
C_T	-	Threshold Cycle
CYCS	-	Cytochrome c
DC	-	Dendritic Cell
DD	-	Death Domains
DED	-	Death-Effector Domain
DEVD	-	Four amino acid peptide
DMEM	-	Dulbecco's modified eagle medium
DMSO	-	Dimethyl sulphoxide
DNA	-	Deoxyribonucleic acid
DR	-	Death receptors
DU145	-	Human prostate carcinoma cells
EBN	-	Edible Bird's Nest
EDTA	-	Ethylenediaminetetraacetic acid
EGF	-	Epidermal Growth Factor
ELISA	-	Enzyme-Linked Immune Sorbent Assay
ESR	-	Estrogen Receptor

FADD	-	Fas-Associated protein with Death Domain
Fas	-	Apoptosis Antigen 1
FasL	-	Fas ligand
FBS	-	Fetal bovine serum
FITC	-	Fluorescein Isothiocyanate
FRAP	-	Ferric Reducing Anti-oxidant Power assay
GAP	-	Guanosine TriPhosphatase Activating Protein
GAPDH	-	Glyceraldehyde-3-phosphate dehydrogenase
GAPH	-	Glyceraldehyde-3-phosphate
GM-CSF	-	Granulocyte-Macrophage Colony-Stimulating Factor
GPI	-	Glycosylphosphatidylinositol
GZMB	-	Granzyme B
HAC	-	Human Articular Chondrocytes
hADSC	-	Human Adipose-Derived Stem Cell
HCl	-	Hydrochloric acid
Hep2B	-	Liver cancer cells
HER2	-	Human Epidermal Growth Factor Receptor 2
HPAC	-	Homo sapiens Pancreas Adenocarcinoma
HPLC	-	High-Performance Liquid Chromatography
HRG	-	Histidine-Rich Glycoprotein
HTS	-	High Throughput Screening
IAP	-	Inhibitor of Apoptosis Proteins
IBM	-	International Business Machines Corporation
IC_{50}	-	Inhibitory Concentration

IEC	-	International Electrotechnical Commission
IFN-γ	-	Interferon-Gamma
IKB	-	Inhibitor of Kappa B
IL	-	Interleukin
LPS	-	Lipopolysaccharide
MACS	-	Magnetic Activated Cell Sorting
MACSiMAG	-	MACS magnetic
MANIS	-	Innovation Centre of Food Technology
MCF-10A	-	Normal mammary epithelial cells
MCF-7	-	Michigan Cancer Foundation-7 (Breast cancer cells)
MDCK	-	Madin-Darby Canine Kidney
MFI	-	Median Fluorescent Intensity
MHC	-	Major Histocompatibility Complex
MMP-9	-	Matrix metallopeptidase 9
mRNA	-	Messenger Ribonucleic Acid
MS	-	Magnetic Separation
MTT	-	3-(4,5-dimethylthiazol-2-Yl)-2,5-diphenyltetrazolium bromide
NADH	-	Reduced Nicotinamide Adenine Dinucleotide
NADPH	-	Reduced Nicotinamide Adenine Dinucleotide Phosphate
NAG	-	N-acetyl-D-glucosamine
NaOH	-	Sodium hydroxide
NF-KB	-	Nuclear Factor Kappa Beta
NHF	-	Normal Human Fibroblast
NHS	-	National Health Service

NJ	-	New Jersey
NK	-	Natural Killer
O.D.	-	Optical Densit
ORAC	-	Oxygen Radical Absorbance Capacity
p53	-	Tumour Phosphoprotein
PAMP	-	Pathogen-Associated Molecular Pattern
PARP	-	Poly (Adenosine diphosphate Ribose) Polymerase
PBMC	-	Peripheral blood mononuclear cells
PBS	-	Phosphate Buffer Saline
PE	-	Phycoerythrin
ppm	-	Parts per million
PRF-1	-	Perforin 1
PRR	-	Pattern-Recognition Receptor
qRT-PCR	-	Quantitative Real Time Reverse Transcriptase Polymerase Chain Reaction
QTOF LC-MS	-	Quadrupole Time of Flight Liquid Chromatography-Mass Spectrometry
RNA	-	Ribonucleic Acid
RPMI	-	Roswell Park Memorial Institute
RT-PCR	-	Reverse Transcriptase Polymerase Chain Reaction
SABC	-	HRP-Streptavidin Conjugate
SD	-	Standard Deviation
SDS PAGE	-	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SEM	-	Standard Error Mean
SH-SY5Y	-	Human derived cell line

SMAC	-	Second Mitochondria-derived Activator of Caspases
SPSS	-	Statistical Package for the Social Sciences
STAT3	-	Signal Transducer and Activator of Transcription-3
SYBR Green 1	-	Syber Green 1
TBE	-	Tris –Borate-EDTA
TCM	-	Traditional Chinese Medicine
TE	-	Trypsin-EDTA
TGF-β1	-	Transforming growth factor beta 1
Th	-	Helper T cells
Th1	-	T helper Cell Type 1
TLR	-	Toll-Like Receptor
TMB	-	3,3',5,5'-Tetramethylbenzidine
TNF	-	Tumor Necrosis Factor
TNF-α	-	Tumor Necrosis Factor Alpha
TPTZ	-	2,4,6-tri[2-pyridyl]-s-triazine
TRAIL	-	TNF-related Apoptosis-Inducing Ligand
UK	-	United Kingdom
UKM	-	Universiti Kebangsaan Malaysia
USA	-	United States of America
VEGF	-	Vascular Endothelial Growth Factor
WHO	-	World Health Organization
xMAP	-	Multi-Analyte Profiling

LIST OF SYMBOLS

–	-	Subtract or Negative
<	-	Less than
%	-	Percentage
+	-	Plus or Positive
=	-	Equal to
±	-	Plus minus
×	-	Multiple
≤	-	Less than or equals to
®	-	Registered sign
°C	-	Degree Celsius
µg	-	Microgram
µg/mL	-	Microgram per milliliter
µL	-	Microliter
µm	-	Micrometer
cells/mL	-	Cells per milliliter
g	-	Gram
hr	-	Hour
kDa	-	Kilo Daltons (molecular weight)
M	-	Molar
mg/mL	-	Milligram per milliliter
mg/kg	-	Milligram per kilogram

min	-	Minutes
mL	-	Milliliter
mM	-	Millimolar
mM/L	-	Millimolar per liter
mm	-	Millimeter
N	-	Normality
n	-	Population size
ng/mL	-	Nano gram per milliliter
nm	-	Nanometer
p	-	Significant level
pg/mL	-	Pico grams per milliliter
sec	-	seconds
™	-	Trade mark sign
V	-	Volts
w/v %	-	Weight/volume
w/w %	-	Weight/weight
x g	-	Relative centrifugal force
α	-	Alpha
β	-	Beta
γ	-	Gamma

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CHAPTER 1

INTRODUCTION

1.1 Background Information

National Cancer Institute (2015) of USA defines cancer as a disease in which abnormal replication of cells occurs without control and with the ability to invade other tissues. When this process occurs in breast cell linings, it is known as breast cancer. The formation of a cancerous cell is most likely to occur at milk producing glands and ducts that carry milk to the nipple. In the rarest occasions, stromal fatty tissues cells could turn cancerous too. Sometimes breast cancer is diagnosed around lymph nodes, especially the one at the armpit.

According to the Global Cancer Observatory (2018), there were around 43, 837 new cancer cases were diagnosed in Malaysia. Out of this figure, a total of 26, 395 cancer deaths (60%) were reported by the World Health Organization. In women, breast cancer is one of the leading killers among all ethnicities. Breast cancer recorded a 32.7% occurrence of all cancer incidents among females in Malaysia. Breast cancer cases were the highest among the Chinese followed by Indians and Malays. Women aged between 25 to 59 years are considered to be in the high risk category. In a rare occasion, men do develop breast cancer too (Azizah *et al.*, 2016). In the United States of America, it was projected that 34% cancer caused death will occur in the year 2019 out of the estimated number of new cancer cases which was around 1,762,450 cases according to the National Center for Health Statistics based on incidence data from 1991 to 2016 across 47 states and the District of Columbia (Siegel *et al.*, 2019).

National Cancer Institute (2015) of USA also reveals that surgery, chemotherapy, radiation, hormonal and targeted therapies are accredited treatments commonly known as allopathic medicine. However, these therapies involve synthetic drugs and medications which bring adverse side effects to cancer patients. Therefore, researchers are interested to develop safer options to avoid these side effects; as such many are exploring alternative natural remedies to treat cancer.

Researchers have discovered potential natural products that could boost the immune system. For example, polysaccharide compounds in Shitake mushrooms are able to increase the immune system and possible anticancer agent. Meanwhile, laboratory studies show lentinan, a beta-glucans, was able to initiate immune system by increasing macrophages, T cells and Natural Killer (NK) cells, and used in immunologic adjuvant therapies (Chen *et al.,* 2013).

Edible Bird's Nest (EBN) is a secretion from swiftlets (*Aerodramus fuciphagus*). It is a common animal by-product with medicinal values enjoyed mostly by the Chinese community. One of the main content of EBN is glycoprotein which plays a key role in nutritious and therapeutic functions (Kathan and Weeks, 1969). Sialic acid make up 9% of the carbohydrates in EBN and it may bring healthy attributes to infants intellectually and neurologically (Colombo *et al.,* 2003). Besides, EBN has other carbohydrates such as galactose (16.9%), galactosamine (7.2%), fucose (0.7%) and glucosamine (5.3%) (Kathan and Weeks, 1969).

Recent studies have shown that carbohydrates and lectins play an essential role in mediating the interaction between various types of cells and molecules that specifically interact with each other to initiate the host defense mechanism in the immune system. Lectins are structurally diverse carbohydrate-binding proteins of non-immune origin. The lectins and carbohydrate interaction are involved in activities like opsonization of microorganisms, phagocytosis, cell adhesion and migration, cell activation, cell differentiation and apoptosis (Ni and Tizard, 1996).

Moreover, some studies have validated several linkages to galactose or N-galactosamine in EBN and also indicated that birds' nests have abundant lectin-containing sugar chains (Wieruszeski *et al.*, 1987). Several such lectins from plants were shown to have anticancer properties *in vitro*, *in vivo* and human case studies. The lectins could bind with the cancer cell membrane and receptors. As a result, it inhibits tumor growth. Moreover, it could also cause agglutination or aggregation of the cancer cell. Therefore, it opens a new window for studying the effect of EBN extract to enhance human body's immune system to fight cancer cells (Elvira and Valentin, 2007).

One of the primary studies of the EBN aqueous extract showed mitogenic effects over human peripheral blood monocytes after being stimulated with proliferation agents namely, Concanavalin A and Phytohemagglutinin A. Thus, EBN extract could possess effects that help immune cells in division, therefore, exhibiting immune enhancing potential (Ng *et al.,* 1986).

A subsequent research provided further scientific evidence whereby the activity similar to Epidermal Growth Factor (EGF) was demonstrated in EBN aqueous extract in a dose dependent way *in vitro* that resulted on the synthesis of DNA in 3-day transfer, inoculum 3×10^5 cells (3T3) fibroblasts (Kong *et al.,* 1987). EGF appears to have an important role in cellular proliferation, differentiation and development process. EGF-like activity and mitogenic effect of EBN improves immunity, slows down ageing, and prolongs life expectancy (Yano *et al.,* 2003).

1.2 Problem Statement

Debates have been raised on the contribution of EBN towards cancer cells. Question on whether consuming EBN is beneficial for tumor patients still remains a myth, due to EBN being rich in amino acid and growth factors which would otherwise become the best nutrient to the tumor cells. This obstructs the exploration of EBN as an anti-cancer agent and immunoadjuvant against human cancer. This has also raised many doubts of EBN and its related products effect on cancer patients where it was meant to rejuvenate cells. Thus, the purpose of this study is to evaluate the potential of EBN extract as an anti-cancer agent and immunoadjuvant to treat breast cancer.

In this study, breast cancer was selected to be tested with EBN because breast cancer is the most common invasive cancer and leading cause of death among women of all ethnic backgrounds in Malaysia. Statistically, breast cancer recorded a 32.7% occurrence of all cancer incidents among females in Malaysia (Global Cancer Observatory, 2018). MCF-7 cell line used in this study as it is found to be useful for *in vitro* breast cancer studies because the cell line does retained several ideal characteristics similar to the mammary epithelium such being an estrogen receptor (ESR) positive cell line (Soule *et al.*, 1973). Current conventional treatment like chemotherapy is a systemic therapy to treat breast cancer causes serious side effects, as these therapies also damage healthy cells besides the cancer cells (Munden *et al.*, 2005). Therefore, a cancer patient might have a quality life with the identification of a safe adjuvant medicine like EBN which could reduce immune impairment and other side effects caused by chemotherapy.

Apart from that, a previous study has evaluated aqueous extract of the EBN prepared using enzyme treatment on cell viability on MCF-7 cells. No observable effect on neither cell proliferation nor cell cytotoxicity was found when comparing with the control group (Roh *et al.*, 2012). Therefore, this study determined to use EBN extract prepared using different specification in term of temperature and duration of extraction and without any additional enzyme treatment compare to the previous study to evaluate the cytotoxicity effect of the extract upon MCF-7 cells. Furthermore, the study of the specific effect of EBN extract on the human breast cancer cell line and human immune cells (CD8+ and CD14+) are still very limited (Zhao *et al.*, 2016).

1.3 Research Objective

To evaluate the anti-cancer activity and immunoadjuvant properties of EBN extract on the human breast cancer cell line.

1.4 Scope of Research

In order to achieve the above mentioned objective, the following scopes were covered:

I) To prepare 3 types of EBN extract using water extraction method:

There were 3 types of EBN extract used in this study namely, HMG, EHMG and pHMG. HMG and pHMG prepared using method adapted from Oda *et al.* (1998) while EHMG from Goh *et al.* (2000). These extracts were obtained from same batch of extracts to avoid variation in results.

II) To determine the cytotoxic effects of 3 types of EBN extracts on human breast adenocarcinoma cell line (MCF-7) and human immune cells (CD8+ T-lymphocytes and CD14+ monocytes):

There were 3 types of EBN extracts which are coded HMG, EHMG and pHMG with various concentrations used to treat MCF-7 cells for 72 hr and the cytotoxicity determined using MTT (3-(4,5-dimethylthiazol-2-Yl)-2,5-diphenyltetrazolium bromide) assay. IC_{50} is the inhibitory dose that causes a 50% reduction in cell growth compared to control. Extract that exhibited lowest IC_{50} was chosen to run in the subsequent test in this research which is the HMG extract. HMG extract was then used to treat CD8+ and CD14+ cells to identify the cytotoxicity of EBN upon them.

III) To investigate the chosen EBN extract in scope I (HMG) related to its effect on CD8+ and CD14+ isolated cells:

Level of messenger ribonucleic acid (mRNA) gene expression related to the pro-apoptotic and anti-apoptotic genes were measured in MCF-7, CD8+ and CD14+ cells before and after treatment with HMG extract. Respective controls were included for both MCF-7 and isolated CD8+ and CD14+ cells:

i) Non-activated CD8+ and CD14+ cells
ii) Activated CD8+ and CD14+ cells
iii) Non-activated CD8+ and CD14+ cells co-culture with MCF-7 cells
iv) Activated CD8+ and CD14+ cells co-culture with MCF-7 cells

This study tested both non-activated and activated CD8+ and CD14+ to determine the effect of HMG in both naive cells and activated cells after encounter with an antigen. The purpose of the co-culture experiment is mainly to measure the synergistic effect of HMG with CD8+ and CD14+ cells to kill MCF-7 cells.

IV) To evaluate the influence of EBN extract's (HMG) on pro-apoptotic and anti-apoptotic soluble factors in CD8+ and CD14+ cells:

The experiment involves the measurement of the level of pro-apoptotic and anti-apoptotic soluble factors released by the 4 tested groups of CD8+ and CD14+ cells (as stated in scope II) before and after treatment with HMG using Enzyme-linked immune sorbent assay (ELISA) and Multiplex assay.

1.5 Significance of Research

This study will contribute to the development of EBN extract as an anti-cancer agent and immunoadjuvant for human breast cancer. It will provide a fundamental research to explore the potential of EBN extract as an anti-cancer agent or functional food. Once the EBN is tested, it could be an alternative remedy for cancer without side effects which are commonly associated now with allopathic treatment. Overall, a novel product can arise from EBN for cancer patients. This will increase its demand which might result in higher sales revenue.

1.6 Thesis Overview Flowchart

This thesis is divided into 5 chapters and the sequences with brief contents. Figure 1.1 shows the flow chart of the overview for this study.

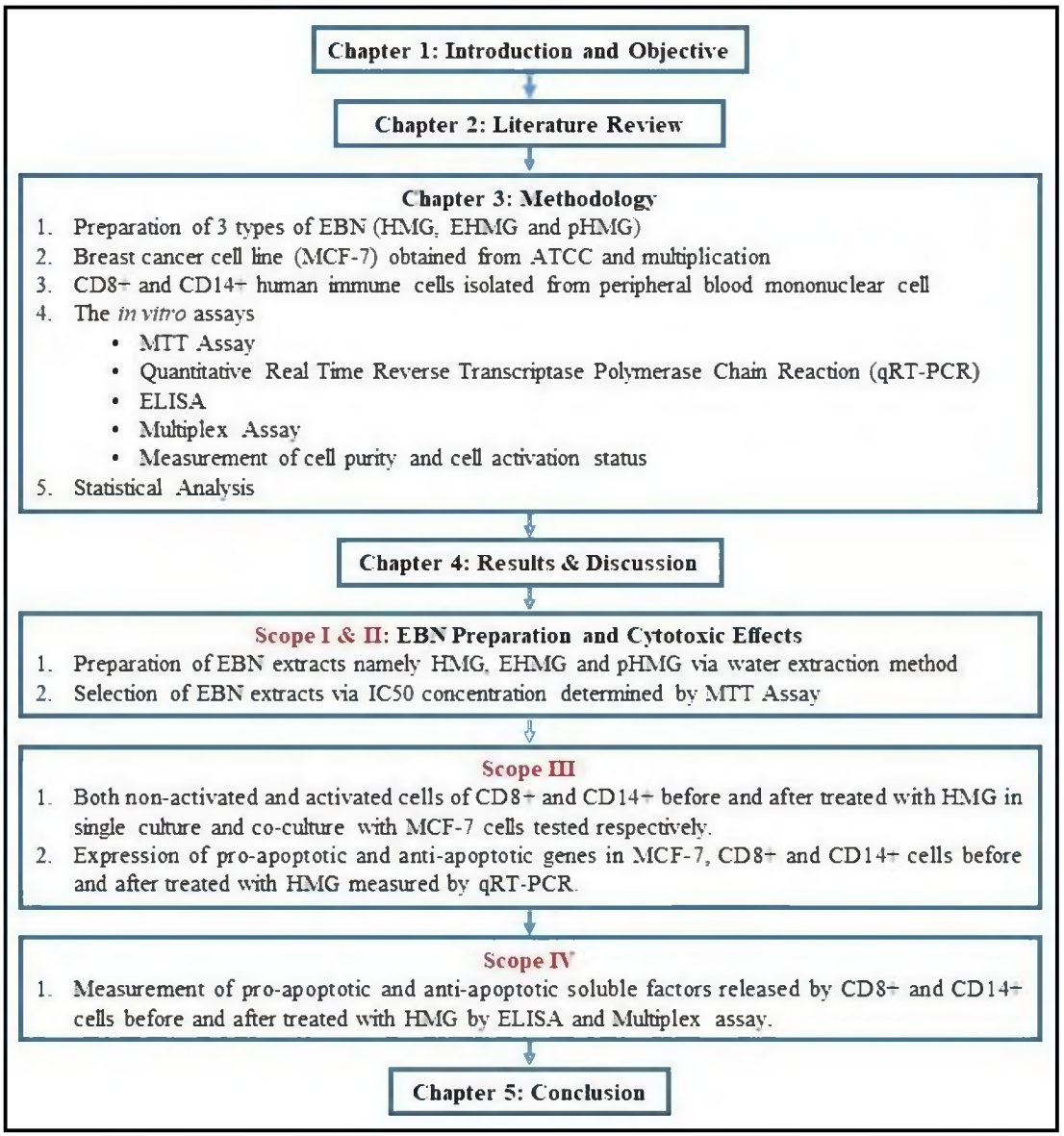

Figure 1.1 The overview flow chart for this study

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