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

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A thesis submitted in fulfilment of the requirements for the award of the degree of Master of Philosophy

School of Chemical and Energy Engineering
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SEPTEMBER 2019
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.
ACKNOWLEDGEMENT

Writing this thesis has been fascinating and extremely rewarding. I would like to thank a number of people who have contributed to the final result in many different ways. To commence with, I pay my obeisance to God, the almighty to have bestowed upon me good health, courage, inspiration, zeal and the light in order to accomplish my thesis project successfully within the time period. After God, I express my sincere and deepest gratitude to my supervisor, Dr. Lee Ting Hun who ploughed through several preliminary versions of my text, making critical suggestions and posing challenging questions. His expertise, invaluable guidance, constant encouragement, affectionate attitude, understanding, patience and healthy criticism added considerably to my experience. Without his continual inspiration, it would have not been possible to complete this study. I owe my special thanks to my co-supervisor, Asst. Prof. Dr. Chua Kien Hui and Dr. Nor Haslinda Abd Aziz for providing necessary laboratory facilities at Pusat Perubatan Universiti Kebangsaan Malaysia (PPUKM). Their assistance had brought me courage and interest all through the journey of accomplishment of my research and thesis. I feel a deep sense of gratitude to my father, Mr. Maruthai Subramaniam, my mother, Mrs. Vijaya Letchumy Pathmanathan, and also my siblings for providing me constant encouragement and financially supporting me for my success. My special thanks also extends to my friends who have been a boost when I was gloomy, a place where I throw all my frustrations and disappointments. I sincerely admire the contribution of all my seniors and laboratory assistant from Institute of Bioproduct Development (IBD) and PPUKM for extending their unstinted support, timely motivation, sympathetic attitude and unfailing help during the project. I also would like to take this moment to extend my gratitude to IBD and PPUKM staffs, for giving me the opportunity to conduct my work under their management. Last, but not the least thank you once again to everyone who had supported me directly or indirectly to successfully accomplish this study. Nothing can be said enough to express the gratitude.
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\textsubscript{50} of 15 \mu g/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.
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LIST OF ABBREVIATIONS

3T3 - 3-day transfer, inoculum $3 \times 10^5$ 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
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<tr>
<td>Caco-2</td>
<td>Human colonic adenocarcinoma cell line</td>
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<tr>
<td>CASP-7</td>
<td>Caspase 7</td>
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<td>CCL2</td>
<td>Monocyte chemotactic protein-1</td>
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<tr>
<td>CD</td>
<td>Cluster of Differentiation</td>
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<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>CFSE</td>
<td>Carboxyfluorescein diacetate succinimidyl ester</td>
</tr>
<tr>
<td>CO₂</td>
<td>Carbon dioxide</td>
</tr>
<tr>
<td>CS&amp;T</td>
<td>Cytometer Setup and Tracking</td>
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<tr>
<td>Cₜ</td>
<td>Threshold Cycle</td>
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<tr>
<td>CYCS</td>
<td>Cytochrome c</td>
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<tr>
<td>DC</td>
<td>Dendritic Cell</td>
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<tr>
<td>DD</td>
<td>Death Domains</td>
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<tr>
<td>DED</td>
<td>Death-Effector Domain</td>
</tr>
<tr>
<td>DEVD</td>
<td>Four amino acid peptide</td>
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<td>DMEM</td>
<td>Dulbecco’s modified eagle medium</td>
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<td>DMSO</td>
<td>Dimethyl sulphoxide</td>
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<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
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<td>DR</td>
<td>Death receptors</td>
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<td>DU145</td>
<td>Human prostate carcinoma cells</td>
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<td>EBN</td>
<td>Edible Bird’s Nest</td>
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<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
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<tr>
<td>EGF</td>
<td>Epidermal Growth Factor</td>
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<td>ELISA</td>
<td>Enzyme-Linked Immune Sorbent Assay</td>
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<td>ESR</td>
<td>Estrogen Receptor</td>
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<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>FADD</td>
<td>Fas-Associated protein with Death Domain</td>
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<td>Apoptosis Antigen 1</td>
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<tr>
<td>FasL</td>
<td>Fas ligand</td>
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<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
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<td>FITC</td>
<td>Fluorescein Isothiocyanate</td>
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<td>FRAP</td>
<td>Ferric Reducing Anti-oxidant Power assay</td>
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<tr>
<td>GAP</td>
<td>Guanosine TriPhosphatase Activating Protein</td>
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<td>GAPDH</td>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
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<td>GAPH</td>
<td>Glyceraldehyde-3-phosphate</td>
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<td>GM-CSF</td>
<td>Granulocyte-Macrophage Colony-Stimulating Factor</td>
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<td>Granzyme B</td>
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<td>HAC</td>
<td>Human Articular Chondrocytes</td>
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<td>hADSC</td>
<td>Human Adipose-Derived Stem Cell</td>
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<tr>
<td>HCl</td>
<td>Hydrochloric acid</td>
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<td>Hep2B</td>
<td>Liver cancer cells</td>
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<td>HER2</td>
<td>Human Epidermal Growth Factor Receptor 2</td>
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<td>HPAC</td>
<td>Homo sapiens Pancreas Adenocarcinoma</td>
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<td>HPLC</td>
<td>High-Performance Liquid Chromatography</td>
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<td>HRG</td>
<td>Histidine-Rich Glycoprotein</td>
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<td>HTS</td>
<td>High Throughput Screening</td>
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<td>IAP</td>
<td>Inhibitor of Apoptosis Proteins</td>
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<td>IBM</td>
<td>International Business Machines Corporation</td>
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<tr>
<td>IC₅₀</td>
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<tr>
<td>IEC</td>
<td>International Electrotechnical Commission</td>
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<tr>
<td>IFN-γ</td>
<td>Interferon-Gamma</td>
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<tr>
<td>IKB</td>
<td>Inhibitor of Kappa B</td>
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<tr>
<td>IL</td>
<td>Interleukin</td>
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<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
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<tr>
<td>MACS</td>
<td>Magnetic Activated Cell Sorting</td>
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<tr>
<td>MACSiMAG</td>
<td>MACS magnetic</td>
</tr>
<tr>
<td>MANIS</td>
<td>Innovation Centre of Food Technology</td>
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<tr>
<td>MCF-10A</td>
<td>Normal mammary epithelial cells</td>
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<tr>
<td>MCF-7</td>
<td>Michigan Cancer Foundation-7 (Breast cancer cells)</td>
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<td>MDCK</td>
<td>Madin-Darby Canine Kidney</td>
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<tr>
<td>MFI</td>
<td>Median Fluorescent Intensity</td>
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<tr>
<td>MHC</td>
<td>Major Histocompatibility Complex</td>
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<td>MMP-9</td>
<td>Matrix metallopeptidase 9</td>
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<tr>
<td>mRNA</td>
<td>Messenger Ribonucleic Acid</td>
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<tr>
<td>MS</td>
<td>Magnetic Separation</td>
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<tr>
<td>MTT</td>
<td>3-(4,5-dimethylthiazol-2-Yl)-2,5-diphenyltetrazolium bromide</td>
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<tr>
<td>NADH</td>
<td>Reduced Nicotinamide Adenine Dinucleotide</td>
</tr>
<tr>
<td>NADPH</td>
<td>Reduced Nicotinamide Adenine Dinucleotide Phosphate</td>
</tr>
<tr>
<td>NAG</td>
<td>N-acetyl-D-glucosamine</td>
</tr>
<tr>
<td>NaOH</td>
<td>Sodium hydroxide</td>
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<tr>
<td>NF-KB</td>
<td>Nuclear Factor Kappa Beta</td>
</tr>
<tr>
<td>NHF</td>
<td>Normal Human Fibroblast</td>
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<td>NHS</td>
<td>National Health Service</td>
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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

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<th>Description</th>
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<tr>
<td>SMAC</td>
<td>Second Mitochondria-derived Activator of Caspases</td>
</tr>
<tr>
<td>SPSS</td>
<td>Statistical Package for the Social Sciences</td>
</tr>
<tr>
<td>STAT3</td>
<td>Signal Transducer and Activator of Transcription-3</td>
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<tr>
<td>SYBR Green 1</td>
<td>Syber Green 1</td>
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<tr>
<td>TBE</td>
<td>Tris -Borate-EDTA</td>
</tr>
<tr>
<td>TCM</td>
<td>Traditional Chinese Medicine</td>
</tr>
<tr>
<td>TE</td>
<td>Trypsin-EDTA</td>
</tr>
<tr>
<td>TGF-β1</td>
<td>Transforming growth factor beta 1</td>
</tr>
<tr>
<td>Th</td>
<td>Helper T cells</td>
</tr>
<tr>
<td>Th1</td>
<td>T helper Cell Type 1</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-Like Receptor</td>
</tr>
<tr>
<td>TMB</td>
<td>3,3',5,5'-Tetramethylbenzidine</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor Necrosis Factor</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor Necrosis Factor Alpha</td>
</tr>
<tr>
<td>TPTZ</td>
<td>2,4,6-tri[2-pyridyl]-s-triazine</td>
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<tr>
<td>TRAIL</td>
<td>TNF-related Apoptosis-Inducing Ligand</td>
</tr>
<tr>
<td>UK</td>
<td>United Kingdom</td>
</tr>
<tr>
<td>UKM</td>
<td>Universiti Kebangsaan Malaysia</td>
</tr>
<tr>
<td>USA</td>
<td>United States of America</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular Endothelial Growth Factor</td>
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<tr>
<td>WHO</td>
<td>World Health Organization</td>
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<tr>
<td>xMAP</td>
<td>Multi-Analyte Profiling</td>
</tr>
<tr>
<td>Symbol</td>
<td>Description</td>
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<tr>
<td>-</td>
<td>Subtract or Negative</td>
</tr>
<tr>
<td>&lt;</td>
<td>Less than</td>
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<tr>
<td>%</td>
<td>Percentage</td>
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<tr>
<td>+</td>
<td>Plus or Positive</td>
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<tr>
<td>=</td>
<td>Equal to</td>
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<tr>
<td>±</td>
<td>Plus minus</td>
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<tr>
<td>×</td>
<td>Multiple</td>
</tr>
<tr>
<td>≤</td>
<td>Less than or equals to</td>
</tr>
<tr>
<td>®</td>
<td>Registered sign</td>
</tr>
<tr>
<td>°C</td>
<td>Degree Celsius</td>
</tr>
<tr>
<td>μg</td>
<td>Microgram</td>
</tr>
<tr>
<td>μg/mL</td>
<td>Microgram per milliliter</td>
</tr>
<tr>
<td>μL</td>
<td>Microliter</td>
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<tr>
<td>μm</td>
<td>Micrometer</td>
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<tr>
<td>cells/mL</td>
<td>Cells per milliliter</td>
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<tr>
<td>g</td>
<td>Gram</td>
</tr>
<tr>
<td>hr</td>
<td>Hour</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilo Daltons (molecular weight)</td>
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<tr>
<td>M</td>
<td>Molar</td>
</tr>
<tr>
<td>mg/mL</td>
<td>Milligram per milliliter</td>
</tr>
<tr>
<td>mg/kg</td>
<td>Milligram per kilogram</td>
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</table>
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 \textit{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 \textit{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 \textit{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.

![Flowchart of Thesis Overview](image)

Figure 1.1 The overview flow chart for this study
REFERENCES


