ANTIMETASTATIC AND IMMUNOMODULATORY POTENTIAL OF STROBILANTHES CRISPUS SUBFRACTION AND ITS SPECIFIC MAJOR COMPONENTS IN EXPERIMENTAL BREAST CANCER MODEL

YUSHA'U SHU'AIBU BARAYA

UNIVERSITI SAINS MALAYSIA

2018

ANTIMETASTATIC AND IMMUNOMODULATORY POTENTIAL OF STROBILANTHES CRISPUS SUBFRACTION AND ITS SPECIFIC MAJOR COMPONENTS IN EXPERIMENTAL BREAST CANCER MODEL

by

YUSHA'U SHU'AIBU BARAYA

Thesis submitted in fulfillment of the requirements

for the degree of

Doctor of Philosophy

February 2018

ACKNOWLEDGEMENT

In the name of Allah, the most gracious, the most merciful. I thank Allah for giving me the opportunity and strength to complete this study. I would like to express my sincere appreciation to those who have contributed in this study.

My profound appreciation goes to my supervisor, Professor Dr. Nik Soriani Yaacob, for her excellent support and encouragement, close supervision, and more eminently her patience, kindness and scholarly guidance demonstrated to me throughout the period of this research work. I will forever remain humble and grateful to her as my mentor that put in efforts for timely completion of this project despite her tight schedules. My special gratitude goes to my co-supervisor, Dr Wong Kah Keng for his invaluable contributions at every stage of this research project. Many thanks for all he has done to me.

I would like to acknowledge and appreciate Professor Norazmi Mohamad Nor, for being a source of inspiration to me during my research especially that his academic talks have helped me greatly to instill a lot of confidence in my abilities to carry out this study. My acknowledgment also goes to Drs Noor Fatmawati Mokhtar, Agustine Nengsih Fauzi, Zulkifli Mustapha, Muhammad Hassan Yankuzo, and the rest of NSY/NMN research group for assisting me in one way or another.

My sincere appreciation goes to all the staff of Chemical Pathology and Pathology Departments, ARASC, CRL, INFORMM and School of Dental Sciences, Universiti Sains Malaysia for helping me at some stage of this study. Notably among them, Mr.

Rosli Jusoh and Mrs. Jamaliah Lin, and Mr. Faizul Ismal Che Adam of Pathology Department and ARASC, respectively.

My special acknowledgement goes to my wife, Suwaiba Shehu and kids, Ahmad, Maimunatu, Ka'ab and Abdullah for their patience, support and prayers in the course of this study. My gratitude also goes to my friends and the entire family of my late father, Mal. Shu'aibu Baraya, particularly my mother, Hajiya Aishatu and elder brother Dr. Muhammad Shu'aibu Gobir for their invaluable support and encouragement.

Last but not least, I would like to acknowledge the TWAS-USM Fellowship Scheme and Usmanu Danfodiyo University Sokoto Nigeria for providing me with financial assistance and study leave, respectively to accomplish my PhD candidature.

TABLE OF CONTENTS

Acknowledgements ii

Table of Contents iv

List of Tables xiii

List of Figures xv

List of Abbreviations xix

Abstrak xxiv

Abstract xxvi

CHAPTER 1 – INTRODUCTION

1.1 Cancer 1

1.1.1 Cancer incidence 3

1.1.1(a) Cancer incidence in Malaysia 5

1.1.2 Cancer associated risk factors 7

1.1.3 Cancer nomenclature 8

1.1.4 Breast cancer 9

1.1.4(a) Classification of breast cancer 9

1.1.4(b) Pathogenesis of breast cancer 12

1.1.4(c) Diagnosis of breast cancer 15

1.1.4(d) Management of breast cancer 16

1.1.4(e) Staging of breast cancer 17

1.1.4(f) Surgery 17

1.1.4(g) Radiotherapy 19

1.1.4(h) Chemotherapy 20

1.1.4(i) Hormonal therapy 21

1.1.4(j) Immunotherapy 23

1.1.5 Breast cancer metastasis 25

1.1.5(a) Tumor cell invasion 30

1.1.5(b) Tumor cell angiogenesis 32

1.1.5(c) Tumor cell migration 33

1.1.6 *In vitro* models for breast cancer 35

1.1.7 Animal models for breast cancer 35

1.1.7(a) 4T1-induced breast cancer mouse model 37

1.1.8 Tumor microenvironment of breast cancer 39

1.2 The cadherin protein family 42

1.2.1 E-cadherin 42

1.2.2 N-cadherin 46

1.2.3 Vimentin 47

1.3 The EMT and cancer 49

1.4 Natural therapeutic agents and cancer 54

1.4.1 Medicinal plants as promising anticancer agents 56

1.4.1(a) Anticancer medicinal plants as immunotherapeutic agents 57

1.5 *Strobilanthes crispus* (*S. crispus*) 60

1.5.1 Background of *S. crispus* 60

1.5.1(a) Phytochemical components 63

1.5.1(b) Lutein 63

1.5.1(c) β-sitosterol 65

1.5.1(d) Traditional practices 66

1.5.1(e) Anticancer activities 66

1.5.1(f) Other pharmacological activities 67

1.6 Rationale of the study 69

1.7 Objectives of the study 70

CHAPTER 2 – MATERIALS AND METHODS

2.1 Materials 74

2.1.1 Chemicals and reagents 74

2.1.2 Laboratory equipments 74

2.1.3 Consumables 74

2.2 Study plan 80

2.3 *In vitro* study 80

2.3.1 Breast cancer cell (BCC) lines 80

2.3.2 Reagents and solutions for cell culture 81

2.3.2(a) Media 81

2.3.2(b) Fetal bovine serum (FBS) 81

2.3.2(c) Penicillin-streptomycin (Penstrep) solution 81

2.3.2(d) Preparation of growth media for cell lines 81

2.3.2(e) Preparation of assay culture media for cell lines 82

2.3.2(f) Preparation of phosphate buffered saline (PBS) 82

2.3.2(g) Trypsin-EDTA solution 82

2.3.2(h) Trypan blue solution 82

2.3.2(i) Preparation of cryopreservation medium 83

2.3.3 Retrieving frozen cell lines 83

2.3.4 Sub-culturing cell lines 84

2.3.5 Cell counting 84

2.3.6 Freezing down of cell lines 85

2.3.7 Preparation of stock solutions 87

2.3.7(a) 10 µg/ml SCS 87

2.3.7(b) 10 mM Lutein 87

2.3.7(c) 10 mM β-sitosterol 87

2.3.8 Cell proliferation assay 88

2.3.9 Migration assay 89

2.3.10 Invasion assay 90

2.3.11 Flow cytometric analysis 93

2.3.11(a) Preparation of buffers and stock solutions 93

2.3.11(b) FACS wash buffer 93

2.3.11(c) FACS fixation buffer 93

2.3.11(d) Dilution of antibody 94

2.3.11(e) Protocol of flow cytometry 94

2.4 *In vivo* study 96

2.4.1 Preparation of 4T1 cells for inoculation 96

2.4.2 Preparation of treatment stock solutions 96

2.4.2(a) 50 mg/ml SCS 96

2.4.2(b) 5 mg/ml Lutein 97

2.4.2(c) 5 mg/ml β-sitosterol 97

2.4.3 Experimental animals 98

2.4.4 Induction of breast cancer 98

2.4.5 Animal monitoring 100

2.4.6 Measurement of tumor volume 101

2.4.7 Experimental grouping and treatment plan 101

2.4.8 Necropsy and samples collection 102

2.4.9 Full blood count analysis 102

2.4.10 Histomorphological analysis 103

2.4.10(a) Reagents preparation for histomorphological analysis 103

2.4.10(b) 10 % neutral buffered formalin (NBF) 103

2.4.10(c) Harris Hematoxylin 103

2.4.10(d) Eosin Y, alcoholic 104

2.4.10(e) 1 % acid alcohol 104

2.4.10(f) 0.3 % ammonia water 104

2.4.10(g) 95 % ethanol 104

2.4.10(h) 80 % ethanol 104

2.4.10(i) 70 % ethanol 105

2.4.10(j) Xylene solution 105

2.4.10(k) Fixation and processing of tissue samples 105

2.4.10(l) Preparation of tissue sections for histology 107

2.4.10(m) Hematoxylin and Eosin (H&E) staining 107

2.4.10(n) Histomorphological examination of tissue sections 108

2.4.11 Immunohistochemical analysis 108

2.4.11(a) Reagents preparation for IHC analysis 110

2.4.11(b) 10 % NBF, ethanol and xylene solutions 110

2.4.11(c) Tris buffered saline and Tween 20 110

2.4.11(d) Tris-EDTA buffer 110

2.4.11(e) 3 % hydrogen peroxide 110

2.4.11(f) Antibody diluent 111

2.4.11(g) Ultravision Quanto detection system HRP 111

2.4.11(h) DAB Quanto Chromogen plus DAB Quanto Substrate 111

2.4.11(i) Preparation of IHC samples 112

2.4.11(j) Dilution of primary antibodies 112

2.4.11(k) Protocol of IHC staining 114

2.4.11(l) Optimization of antibody for IHC 115

2.4.11(m) Assessment of IHC scoring 116

2.5 Statistical analysis 117

CHAPTER 3 – EFFECTS OF SCS ON TUMOR GROWTH, HEMATOLOGICAL AND HISTOMORPHOLOGY OF ORGANS

3.1 Introduction 118

3.2 Effects of SCS, lutein and β-sitosterol on body weights of tumor-bearing mice 120

3.3 Effects of SCS, lutein and β-sitosterol on physical tumor growth parameters 122

3.4 Effects of SCS on hematological indices on normal mice 124

3.5 Effects of SCS on histomorphology of major organs in normal mice 126

3.6 Discussion 129

CHAPTER 4 – INHIBITION OF TUMOR METASTASIS BY SCS

4.1 Introduction 132

4.2 Effects of SCS on the proliferation of 4T1 cells 133

4.3 Effects of SCS on the proliferation of MDA-MB-231 cells 135

4.4 Effects of SCS on the migration of 4T1 cells 135

4.5 Effects of SCS on the migration of MDA-MB-231 cells 139

4.6 Effects of SCS on the invasion of 4T1 and MDA-MB-231 cells 139

4.7	Effects of SCS on the histomorphology of 4T1-induced mammary carcinoma	144
4.8	Effects of SCS on the tumor metastasis in major organs of 4T1-induced mammary carcinoma	144
4.9	Comparison of the expression of metastasis markers in normal and tumor bearing mice	148
4.10	Effects of SCS, lutein and β-sitosterol on the expression of tumor markers of metastasis	150
4.11	Discussion	156

CHAPTER 5 – INDUCTION OF ANTITUMOR IMMUNE RESPONSE BY SCS

5.1	Introduction	163
5.2	Effects of SCS on the expression of cell surface immune markers	165
5.3	Comparison of the expression of cellular immune markers in tumor-bearing and normal mice	168
5.4	Effects of SCS, lutein and β-sitosterol on cellular immune markers in tumor bearing mice	168
5.5	Discussion	177

CHAPTER 6 – GENERAL DISCUSSION AND CONCLUSION

6.1 General discussion 180

6.2 Conclusion 204

CHAPTER 7 – LIMITATIONS OF THE STUDY AND SUGGESTIONS FOR FUTURE RESEARCH 208

REFERENCES 211

APPENDICES

Appendix 1: Antimigration (A) and anti-invasion (B) effects of lutein and β-sitosterol on 4T1 BCCs

Appendix 2: Antimigration (A) and anti-invasion (B) effects of lutein and β-sitosterol on MDA-MB-231 BCCs

LIST OF PUBLICATIONS

LIST OF TABLES

		Page
Table 1.1	Characteristics of breast cancer molecular subtypes	13
Table 1.2	TNM breast cancer staging system	18
Table 1.3	Natural sources of anticancer agents	55
Table 1.4	Some selected medicinal plants with reported anticancer activity in Malaysia	61
Table 1.5	Anticancer studies on *S. crispus* (from 2010 upwards)	68
Table 2.1	List of cell lines, chemicals and reagents	75
Table 2.2	List of laboratory equipments	77
Table 2.3	List of consumables	79
Table 2.4	Tissue processing steps through automated Tissue Tek® VIP	106
Table 2.5	Hematoxylin and Eosin (H&E) staining steps	109
Table 2.6	List of antibodies and their description	113
Table 3.1	FBC indices in normal mice and normal mice treated with SCS	125
Table 4.1	Comparison of the expression of metastatic markers in TM and NM	149
Table 4.2	Effects of SCS on the expression of metastatic markers in 4T1-induced mice	153
Table 4.3	Effects of lutein on the expression of metastatic markers in 4T1-induced mice	154

Table 4.4	Effects of β-sitosterol on the expression of metastatic markers in 4T1-induced mice	155
Table 5.1	Effects of SCS on the expression of cell surface molecules on 4T1 cells	167
Table 5.2	Comparison of the expression of cellular immune marker in TM and NM	169
Table 5.3	Effects of SCS on the expression of cellular immune marker in TM and TM-S	173
Table 5.4	Effects of lutein on the expression of cellular immune marker in TM and TM-L	174
Table 5.5	Effects of β-sitosterol on the expression of cellular immune marker in TM and TM-β	175

LIST OF FIGURES

		Page
Figure 1.1	Overview of genotoxic and non-genotoxic causes of cancer	2
Figure 1.2	Estimated global numbers of new cancer cases and deaths in different continents of the world, all cancer cases included from both sexes, 2012	4
Figure 1.3	Estimated numbers of new cancer cases and deaths in Malaysia, 2012	6
Figure 1.4	Breast (A) ductal carcinoma *in situ* (DCIS) and (B) lobular carcinoma *in situ* (LCIS)	11
Figure 1.5	Hallmarks of cancer	26
Figure 1.6	Summary of the key molecular events in metastasis	28
Figure 1.7	Evolutionary roles of cadherins	43
Figure 1.8	E-cadherin in adherens junction	45
Figure 1.9	Representation picture of EMT/MET	50
Figure 1.10	Schematic representation of some plant-based immunomodulatory compounds	58
Figure 1.11	*S. crispus photo*	62
Figure 1.12	Overall experimental design (*in vitro*)	72

Figure 1.13	A summary of the *in vivo* study	73
Figure 2.1	Schematic representation of the cell counting chamber	86
Figure 2.2	Examination of invaded cells using 8 μm transwell cell cultures insert	92
Figure 2.3	Inoculation of 4T1 murine mammary carcinoma cells in female balb/c mice	99
Figure 3.1	Effects of SCS, lutein and β-sitosterol on body weight change during 30 days treatment periods	121
Figure 3.2	Effects of SCS, lutein and β-sitosterol on physical tumor growth parameters	123
Figure 3.3	Effects of SCS on the histomorphology of major organs in NM and NM-S after 30 days of SCS (100 mg/kg/day) administration	128
Figure 4.1	(A) Antiproliferative effects and (B) half maximal inhibitory concentration (IC_{50}) of SCS on 4T1 breast cancer cells	134
Figure 4.2	(A) Antiproliferative effects and (B) half maximal inhibitory concentration (IC_{50}) of SCS on MDA-MB-231 breast cancer cells	136
Figure 4.3	Antimigration effects of SCS on 4T1 breast cancer cells	137
Figure 4.4	Antimigration effects of SCS on 4T1 cells	138

Figure 4.5	Antimigration effects of SCS on MDA-MB-231 breast cancer cells	140
Figure 4.6	Antimigration effects of SCS on MDA-MB-231 cells	141
Figure 4.7	Anti-invasion effects of SCS on 4T1 (A) and MDA-MB-231 (B) cells	142
Figure 4.8	Anti-invasion effects of SCS on 4T1 and MDA MB231 cells	143
Figure 4.9	Histomorphology of mammary glands and 4T1-induced mammary tumor nodules in TM-S and TM, respectively after 30 days of SCS (100 mg/kg/day) treatment	145
Figure 4.10	Effects of SCS on tumor metastasis in major organs of TM and TM-S. TM-S received oral treatment of SCS (100 mg/kg/day) for 30 days	146
Figure 4.11	Representative photomicrographs showing increased expression of E-cadherin in tumor bearing mice treated with SCS (TM-S), tumor bearing mice treated with lutein (TM-L), tumor bearing mice treated with β-sitosterol (TM-β) and normal mice (NM) compared to the tumor bearing (TM) group	151
Figure 5.1	Flow cytometry analysis	166

Figure 5.2	Representative photomicrographs showing increased expression of CD4, CD8, CD45RO, CIITA, IL2, MHCI and MHCII in tumor bearing mice treated with SCS (TM-S), tumor bearing mice treated with lutein (TM-L) and tumor bearing mice treated with β-sitosterol (TM-β) compared to the tumor bearing (TM) group	170
Figure 6.1	An outline of the proposed mechanisms of action of SCS in 4T-induced mouse mammary tumor model	207

LIST OF ABBREVIATIONS

%	Percent
^{o}C	Degree Celsius
µg	Microgram
µl	Microlitre
27-HC	27-hydroxycholesterol
AIs	Aromatase inhibitors
APCs	Antigen-presenting cells
AR	Androgen receptor
ATCC	American Type Culture Collection
BCC	Breast cancer cell
BCS	Breast-conserving surgery
CBE	Clinical breast examination
CD	Cluster of differentiation
CIITA	Class II transactivator
CNS	Central nervous system
CTC	Circulating tumor cell
CTL	Cytotoxic T lymphocyte
DCIS	Ductal carcinoma in situ
DCs	Dendritic cells
DFS	Disease-free survival
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
E-cadherin	Epithelial cadherin

ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGCG	Epigallocatechin-3-gallate
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbent assay
EMH	Extramedullary hematopoiesis
EMT	Epithelial mesenchymal transition
ER	Estrogen receptor
FBC	Full blood count
FBS	Fetal bovine serum
FGF	Fibroblast growth factor
FOXP3	Forkhead box P3
g	Gram
GnRH	Gonadotropin releasing hormone
h	Hour
H_2O_2	Hydrogen peroxide
H&E	Hematoxylin and eosin
Hb	Hemoglobin
HER2	Human epidermal growth factor receptor 2
HPLC	High-performance liquid chromatography
IFN	Interferon
IHC	Immunohistochemistry
IL	Interleukin
IDC-NOS	Invasive ductal carcinoma not otherwise specified
IQR	Interquartile range

kg	Kilogram
L	Litre
LCIS	Lobular carcinoma in situ
LH-RH	Luteinizing hormone releasing hormone
Maspin	Mammary serine protease inhibitor
MCF-7	Michigan Cancer Foundation – 7
MCH	Mean corpuscular hemoglobin
MCHC	Mean corpuscular hemoglobin concentration
McTN	Microtentacles
MCV	Mean corpuscular volume
MFI	Mean fluorescence intensity
mg	Milligram
MHC	Major histocompatibility complex
Min	Minute
ml	Millilitre
mm	Millimetre
mmol/L	Millimole per litre
MMP	Matrix metalloproteinase
MTT	3-(4, 5-dimethythiazol-2-yl)-2, 5-diphenyl tetrazolium bromide
MUC1	Mucin 1
NBF	Neutral buffered formalin
N-cadherin	Nonepithelial cadherin
NCR	National Cancer Institute
NF-kB	Nuclear factor kappa B
NH4	Ammonia

NK	Natural killer cells
NP	Natural product
nm	Nanometer
NM	Normal mice
NM-S	Normal mice treated with SCS
NMU	N-methyl-N-nitrosourea
OD	Optical density
OS	Overall survival
PBS	Phosphate-buffered saline
PCV	Packed cell volume
PD1	Programmed cell death protein 1
PDGF	Platelet-derived growth factor
Penstrep	Penicillin-streptomycin
PI	Proliferation index
PLT	Platelet
PR	Progesterone receptor
RBC	Red blood cell
RDW	Red blood cells distribution width
RCF (g)	Relative centrifugal force
ROS	Reactive oxygen species
RPMI	Roselle's Park Memorial Institute Medium
RT	Room temperature
Sc	Subcutaneous
Sec	Second
SERM	Selective estrogen receptor modulator

SCS	*Strobilanthes crispus* subfraction
TAMs	Tumor-associated macrophages
TBS	Tris buffered saline
TM	Tumor bearing mice
TM-β	Tumor bearing mice treated with β-sitosterol
TM-L	Tumor bearing mice treated with lutein
TM-S	Tumor bearing mice treated with SCS
TNB	Triple negative breast
TNM	Tumor-node-metastasis
Tregs	Regulatory T cells
UK	United Kingdom
uPA	Urokinase plasminogen activator
US	United States
USM	Universiti Sains Malaysia
UT	Untreated tumor
V	Volume
VEGF	Vascular endothelial growth factor
WBC	White blood cell

POTENSI ANTIMETASTATIK DAN IMUNOMODULATORI SUB PECAHAN STROBILANTHES CRISPUS DAN KOMPONEN UTAMANYA YANG SPESIFIK DALAM MODEL KANSER PAYUDARA EKSPERIMENTAL

ABSTRAK

Strobilanthes crispus (*S. crispus*) adalah herba Malaysia yang dikenali sebagai Pecah kaca atau Jin batu yang telah mempunyai kesan antikanser yang poten dalam kedua-dua model *in vitro* dan *in vivo*. Namun begitu terdapat kekurangan maklumat mengenai perencatan metastasis dan potensi pengaktifan imun oleh *S. cripus* sebagai sebahagian mekanisma antikanser Dalam kajian ini, model kanser payudara *in vivo* dan *in vitro* digunakan untuk mengkaji kesan subpecahan *S. crispus* dan bahan utamanya iaitu lutein dan β-sitosterol ke atas aktiviti antimetastatik, imunomodulasi, dan mengurai mekanisma dasar yang terlibat. Dalam kajian *in vitro,* sel kanser payudara manusia dan murin, MDA-MB-231 dan 4T1 dirawat dengan SCS (25-200 µg/ml) dan penilaian aktiviti antimetastatik dibuat menggunakan asai proliferasi sel, penyembuhan luka dan asai invasi. Keputusan menunjukkan SCS merencatkan pertumbuhan sel selepas inkubasi selama 24 (100 µg/ml) dan 48 (75 µg/ml) jam dalam sel 4T1 dan MDA-MB-231 berbanding dengan sel kawalan yang tidak dirawat. SCS juga menyekat metastasis yang bebas daripada kesan sitotoksik secara rencatan migrasi dan invasi sel kanser di bawah separuh kepekatan rencatan berbanding dengan sel kawalan yang tidak dirawat. Selain daripada itu, analisis sitometri aliran juga dilakukan ke atas sel 4TI yang dirawat dengan SCS (50 µg/ml) selama 24 jam untuk menilai ekspresi penanda imun di permukaan sel (CIITA,

MHCI dan MHCII) dan hasil analisis menunjukkan berlaku peningkatan ekspresi semua protein yang diuji berbanding dengan isotip kawalan. Kajian *in vivo* telah menilai aktiviti antimetastatik dan imunomodulasi SCS, lutein dan β-sitosterol di dalam sel 4TI. Lima ekor tikus yang mempunyai tumor bersaiz 2 mm (TM) bagi setiap kumpulan dirawat dengan SCS (kumpulan TM-S), lutein (kumpulan TM-L) dan β-sitosterol (kumpulan TM-β) pada setiap hari secara oral selama 30 hari. Tindakbalas terhadap rawatan dinilai berdasarkan keputusan parameter pertumbuhan tumor, analisis hematologi dan histomorfologi. Keputusan menunjukkan regresi lengkap tumor (20%) dalam TM-S (100 mg/kg/sehari) dan regresi separa tumor dalam TM-S, TM-L dan TM-β (50 mg/kg/sehari). Tiada pembentukan tumor sekunder atau lesi berkaitan tumor dalam organ utama berbanding kumpulan TM lain. Regresi tumor yang berlaku adalah berkait dengan penyekatan invasi tumor dalam sistem imun dan pengaktifan sel T pemusnah tumor-perantara seperti yang ditunjukkan dengan peningkatan ekspresi CD4, CD8, CD45RO, CIITA, IL2, MHCI dan MHCII dan penurunan dalam ekspresi CD68 berbanding dengan kumpulan TM. SCS secara relatifnya memodulasi migrasi sel tumor, invasi dan angiogenesis melalui penurunan ekspresi MMP9, MUCI dan VEGF berbanding dengan kumpulan TM. TM-S juga menunjukkan peningkatan ekspresi E-cadherin yang ketara dan penurunan ekspresi N-cadherin, vimentin dan Twist berbanding dengan kumpulan TM. Secara amnya, keseluruhan hasil kajian jelas menunjukkan kesan antimetastatik SCS dan berpotensi menyokong sistem imun sebagai sebahagian daripada mekanisma antikanser dalam model tumor yang teraruh, 4TI. Hasil kajian ke atas bahan utama SCS iaitu lutein dan β-sitosterol juga mencadangkan peranan penting mereka dalam mekanisma antikanser SCS.

ANTIMETASTATIC AND IMMUNOMODULATORY POTENTIAL OF STROBILANTHES CRISPUS SUBFRACTION AND ITS SPECIFIC MAJOR COMPONENTS IN EXPERIMENTAL BREAST CANCER MODEL

ABSTRACT

Strobilanthes crispus (S. *crispus*), is a Malaysian herb locally known as '*Pecah kaca*' or '*Jin batu*' which have demonstrated potent anticancer effects in both *in vitro* and *in vivo* models. However, there is paucity of information on the inhibition of metastasis and immune activation potentials of *S. crispus* as part of its anticancer mechanism. Thus in this study, the antimetastatic and immunomodulatory activities of *S. crispus* subfraction (SCS) and its specific major constituents, lutein and β-sitosterol were investigated, particularly to unravel the underlying mechanisms involved, using *in vitro* and *in vivo* models of breast cancer. In the *in vitro* study, murine and human breast cancer cell (BCC) lines, 4T1 and MDA-MB-231, respectively, were treated with SCS and investigated for antimetastatic activity using cell proliferation, wound-healing and invasion assays. The results showed that SCS induced moderate cell growth arrest after 24h (100 µg/ml) and 48h (75 µg/ml) treatment of 4T1 and MDA-MB-231 BCC lines compared to the untreated control cells. SCS moderately inhibited metastasis independent of its cytotoxic effects due to inhibition of migration and invasion of cancer cells below the half maximal inhibitory concentration compared to the untreated control cells. In addition, flow cytometry was carried out to evaluate the expression of cell surface immune markers (CIITA, MHC I and MHC II) in 4T1 cells treated with SCS (50 µg/ml) for 24h, and the results showed considerable expression of all the tested proteins compared to the

isotype control. The *in vivo* study evaluated the antimetastatic and immunomodulatory activities of SCS, lutein and β-sitosterol in 4T1-induced mouse mammary carcinoma model. Beginning from 2 mm tumor size, five each (n = 5) from tumor bearing mice (TM) were administered SCS (TM-S group), lutein (TM-L group) and β-sitosterol (TM-β group) by oral gavage daily for 30 days, and the response to treatment was assessed based on the outcome of the tumor growth parameters, as well as hematological and histomorphological analyses. The results demonstrated a few complete regression of primary tumor (20%) in TM-S (100 mg/kg/day) and partial tumor regression in the rest of TM-S, TM-L and TM-β (50 mg/kg/day), and without secondary tumor formation or tumor-associated lesions in the major organs of treated groups compared to the TM group. The tumor regression was linked to blockage of tumor evasion of the immune system and activation of T cell-mediated tumor destruction, as indicated by significant increase in CD4, CD8, CD45RO, CIITA, IL2, MHCI and MHCII expression with concomitant decrease in CD68 expression compared to the TM group. In addition, SCS relatively modulated tumor cell migration, invasion and angiogenesis, through downregulation of MMP9, MUC1 and VEGF in comparison with the TM group. Additionally, TM-S showed significant increase in E-cadherin expression with resultant decrease in N-cadherin, vimentin and Twist expression SCS. In general, these results demonstrate for the first time that SCS induced antimetastasis and immune system activation effects as part of its anticancer mechanism in 4T1-induced mouse mammary tumor model, and SCS-related bioactive constituents, especially lutein could perhaps contribute significantly to the anticancer potential of SCS.

CHAPTER ONE

INTRODUCTION

1.1 Cancer

Cancer is a collection of diseases characterized by uncontrolled continuous growth and spread of abnormal cells throughout the body via metastasis. In consequence, it destroys normal cells and leads to the formation of malignant tumor cells which eventually results in death (Greenwell and Rahman, 2015; Shareef *et al*., 2016). Cancer of different parts of the body have been defined, namely lung, breast, prostate, colon, cervical, uterus, thyroid, liver, kidney, pancreas, testis, skin, oral and blood cancers (Siegel *et al*., 2016). Cancer evolves due to DNA-induced genomic instability which leads to abnormal mutation rate (Burrell *et al*., 2013; Tubbs and Nussenzweig, 2017). DNA anomalies are often detected for correction and elimination by several cellular proofreading mechanisms comprising of exonucleases, mismatch repair system, apoptosis of the mutated cells and activation of the immune system. However, if the abnormal DNA replication persists as in nucleotide changes and chromosome abnormalities, then this drives cancer development (Negrini *et al*., 2010; Leutholtz and Ripoll, 2011). Similarly, non-genetic (mutation-independent) cancer evolutionary dynamics exist, such as stress, protein imbalance and aging due to epigenetic processes, and these could confer cancer cells resistance to the anticancer agents and gives rise to tumor relapse (Huang, 2013; Horne *et al*., 2015; Pisco and Huang, 2015). An outline of genotoxic and non-genotoxic causes of cancer development is summarized in Figure 1.1.

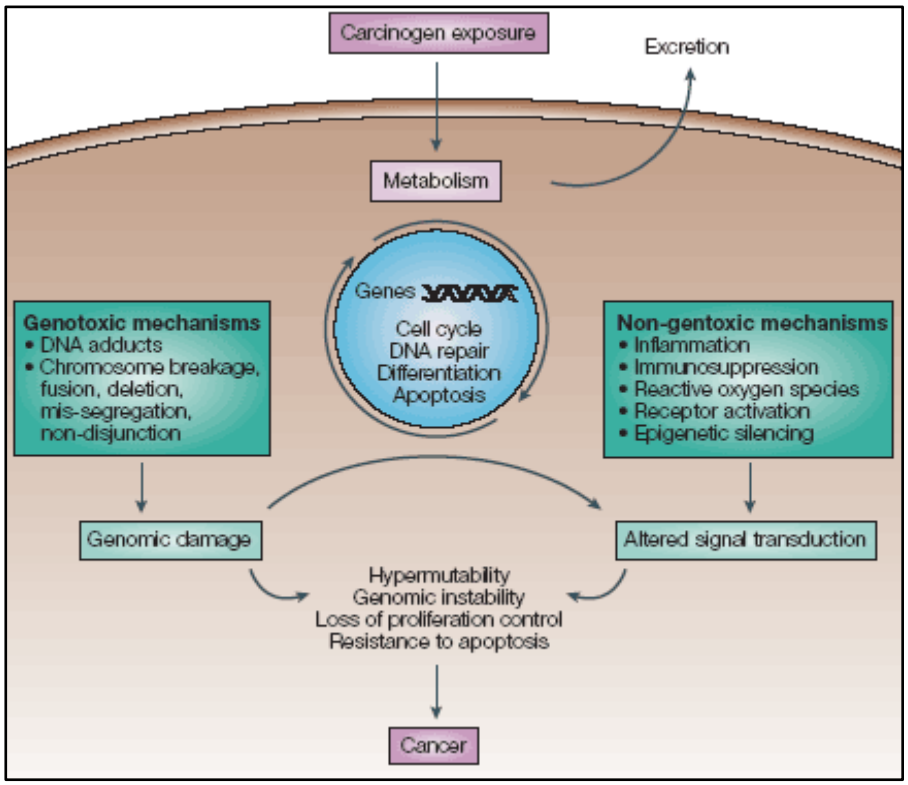

Figure 1.1 Overview of genotoxic and non-genotoxic causes of cancer (Source: Luch, 2005).

1.1.1 Cancer incidence

Cancer is the most frequently diagnosed cause of morbidity and mortality worldwide. There are about 14 million documented new cases of cancer in 2012, and these cases are predicted to increase by almost 70% in the next 2 decades (Bray *et al*., 2015). It is estimated that 65% of cancer-related deaths occurs in developing countries as compared to the death rates in developed countries (Ward *et al.*, 2015). Besides, 8.2 million cancer-related deaths have occurred all over the world in 2012. In that year alone, Asian countries constituted the regions with the highest number of new cancer cases and deaths estimated at 48% and 54.9%, respectively (Figure 1.2) (Ferlay *et al.*, 2014). The impact of cancer on world economy is enormous due to psychological distress, debility (Breitbart *et al*., 2015; Wilson *et al*., 2016) and high cost of cancer treatment. For example, the cost of cancer management in United States is estimated at $157 billion in 2010 (Mariotto *et al*., 2011).

More importantly, breast cancer is the second most commonly diagnosed cancer and by far the leading cause of cancer-related deaths in women globally with estimated 1.7 million and 522, 000 new cases and deaths respectively, in 2012 (Ferlay *et al*., 2014; Torre *et al*., 2015). Similarly, Siegel *et al*. (2016) showed that the three most common female cancers in the United States are breast, lung and bronchus, and colorectal which represent 50% of all cases; with breast cancer accounting for 29% of all cancers in women claiming approximately 246,660 and 40,450 new cases of breast cancer and deaths, respectively in 2016.

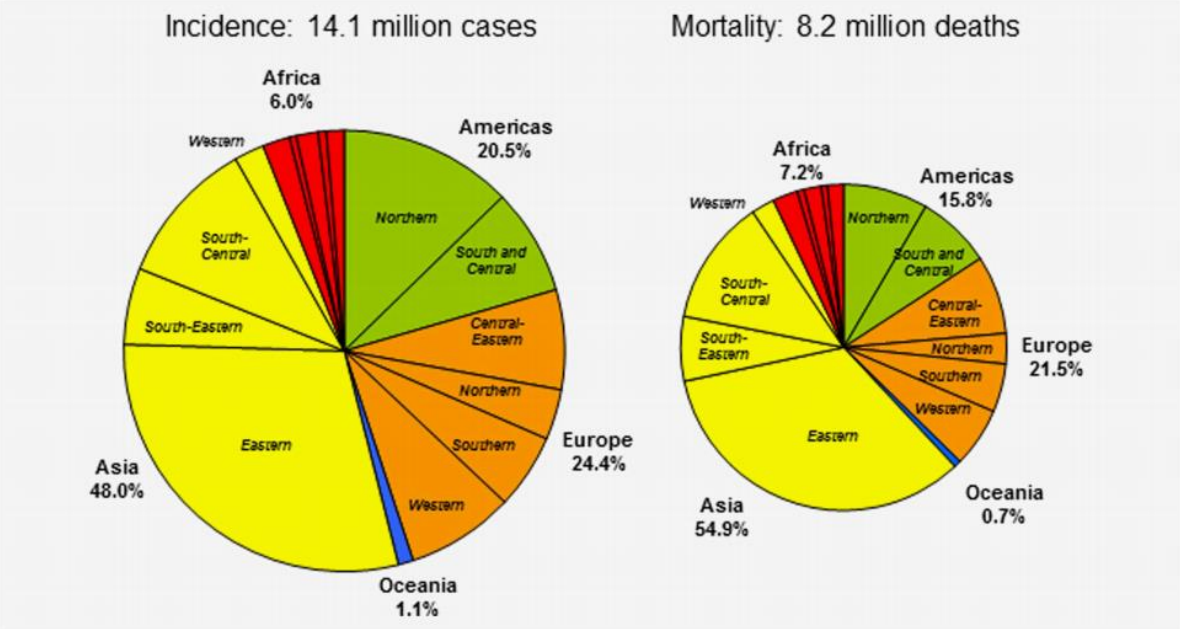

Figure 1.2 Estimated new cancer cases and deaths in different continents of the world, all cancer cases included from both sexes, 2012. (Source: Ferlay *et al*., 2014).

1.1.1(a) Cancer incidence in Malaysia

Cancer is the third prominent cause of death (15%) in both males and females in Malaysia after cardiovascular (36%) and infectious (16%) diseases (Omar and Tamin, 2011; WHO, 2014). Since 1970, Malaysia has witnessed massive industrial, economic and urban transformations which over the years resulted in the improvement of social wellbeing (Jaafar *et al*., 2012) and average life expectancy periods for Malaysian men and women from 61.6 years and 65.6 years, to 72 and 76 years, respectively (WHO, 2014). However, the impact of development such as occupational hazards and subsequent changes in people's lifestyle has led to increasing incidences of cancers (Jaafar *et al*., 2012). Based on the National Cancer Registry (NCR) of Malaysia, there were 18,219 new cancer cases diagnosed and registered at the NCR in 2007, with 3,292 (18.1%) cases of breast cancer, 2,246 (12.3%) cases of colorectal cancer (CRC), 1,865 (10.2%) cases of tracheal, bronchial and lung cancers, 940 (5.2%) and 847 (4.6%) cases of nasopharyngeal and cervical cancer, respectively (Omar *et al*., 2011).

Breast cancer has maintained a central position as an issue of national interest due to its high ranking position as the leading cause of cancer related morbidity and mortality. It was recognized that 1 in every 20 females in Malaysia may develop breast cancer in her life time (Dahlui *et al*., 2011). As shown in Figure 1.3, there were 5,410 verified breast cancer cases in 2012, which were higher than the number of new cases of male lung cancer in that year. Breast cancer resulted in 24% deaths (20 deaths per 100,000) out of the total deaths (10,300) associated with female cancers (WHO, 2014).

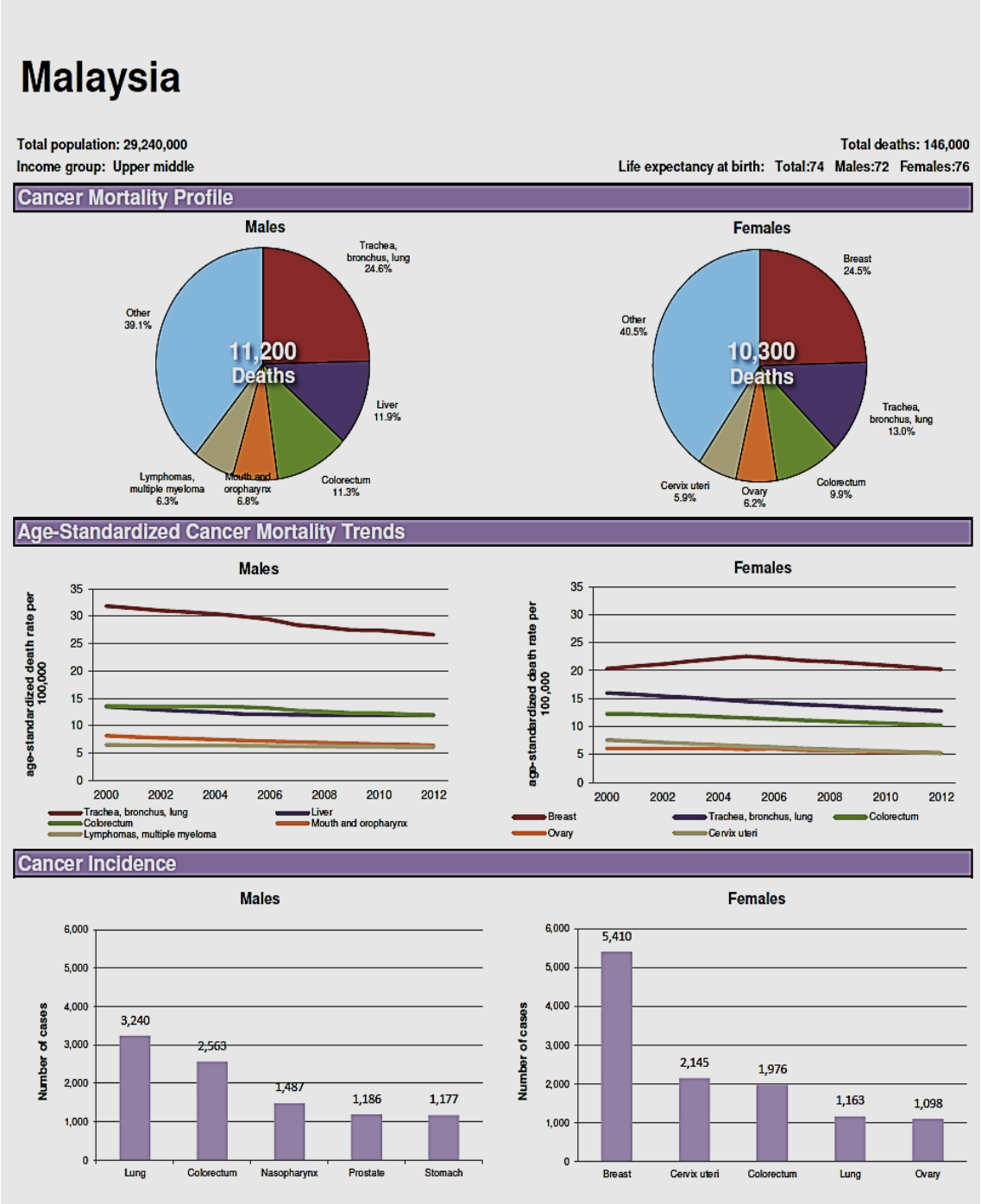

Figure 1.3 Estimated numbers of new cancer cases and deaths in Malaysia, 2012. (Source: WHO - cancer country profiles, 2014. [Accessed 28/06/17].

New cancer cases especially breast cancer are projected to increase in many folds by the year 2025 provided that adequate public awareness initiative has not been instituted as well as lack of early detection and provision of optimum care for breast cancer patient (Yip *et al*., 2014; Al-Naggar *et al*., 2015). In Malaysia, breast cancer is commonly found in the Chinese, followed by the Indians then the Malays (Dahlui *et al*., 2011), and peak age of breast cancer appearance is usually 40 - 49 years amongst Malaysians as compared to the 50 - 59 years of age in Europe and US (Ahmadian and Samah, 2012, Farid *et al*., 2014).

1.1.2 Cancer-associated risk factors

Cancer associated risk factors include ageing, tobacco smoking, alcohol use, poor diet, food additives, obesity, hazardous chemicals and radiation exposure (Bray *et al*., 2012). For example, tobacco smoking has been described as a major risk factor in lung cancer. Similarly, tobacco smoking is associated with other cancers, such as breast, esophagus, stomach, pancreas, liver, bladder, kidney, cervical and bones cancers. Tobacco contains potentially harmful chemicals such as polycyclic aromatic hydrocarbons and nitrosamines which over time accumulate and induce mutagenesis (Ezzati and Riboli, 2013; Klaunig, 2014; Grigoryeva *et al*., 2015).

Furthermore, lifestyle-associated eating behaviour accompanied by limited physical activity is considered as a risk factor in many cancers including breast cancer (Youlden *et al*., 2014; Donnenfeld *et al*., 2015). The consumption of food high in calories and animal (pig, cattle and chicken) fat with low fibre content is linked to colon cancer, breast cancer and prostate cancer. Lipids of animal origin have

significant amounts of polyunsaturated fatty acid which stimulates generation of free radical carcinogens via oxidative stress mechanism (Barendse, 2014; Troselj *et al*., 2016). On the other hand, consumption of fruits and vegetables as well as fish meat enhances anti-oxidant activity to prevent cancer (Moore, 2014).

In Malaysia, the government has put in place various strategies against the menace of male and female cancers through the provision of necessary early detection, diagnosis and treatment facilities in public health institution. Moreover, the government has instituted cancer prevention and control policy and strategy such as the establishment of the Malaysian National Cancer Registry and tobacco control measures with the aim of reducing tobacco burden and associated link with cancer particularly breast cancer (Shin *et al*., 2012; Moore, 2014). However, new cases of breast cancer are on the rise in Malaysia due to ageing society, people's lifestyle, diet, traditional beliefs, older age at first childbirth, absence or brief lifetime period of breastfeeding (Dahlui *et al*., 2011; Yip *et al*., 2014), and delayed hospital visit for clinical breast examination (CBE), timely detection and treatment of breast cancer (Farid *et al*., 2014).

1.1.3 Cancer nomenclature

Cancer of different parts of the body is mostly named according to the organ of origin. Even after metastasis, cancer maintained its original name. For examples, breast, brain, lung, and skin cancers. However, cancer can be classified according to the tissue it originated. Based on this, cancer is grouped into six major categories: Carcinoma accounts for 90% of all cancer cases, and this refers to malignant neoplasm of epithelial origin (e.g. breast, lung and colon cancers), in other words, it

is the cancer of internal or external body surfaces; Sarcoma, refers to cancer of supportive and connective tissues such as muscle, bone, cartilage, tendon, and fat; myeloma, refers to cancer originating from plasma cells of bone marrow; Leukemia, refers to cancer of bone marrow, in order word "blood cancer"; Lymphoma, refers to cancer which develops in glands or lymphatic system (spleen, tonsils, and thymus); mixed types, refers to cancer arising from multiple tissues such as adenosquamous carcinoma and carcinosarcoma (Kumar *et al*., 2014). Similarly, tumors are described as benign or malignant depending on several features, mainly the size, location, invasiveness and metastatic potential. A benign tumor of a tissue is usually designated with the suffix -oma, characterized by localized abnormal cell growth which does not invade adjacent normal tissue, unlike the malignant tumor (carcinoma) which is more clinically aggressive and can invade the normal surrounding tissue and metastasize to distant organs such as bones, lungs and liver via hematogenous and lymphatics routes (Bartuma *et al*., 2014).

1.1.4 Breast cancer

1.1.4(a) Classification of breast cancer

Breast cancers can be classified into subdivisions according to histological grade and histological type. Grade evaluates the degree of differentiation (e.g. tubule formation and nuclear pleomorphism), proliferative activity (mitosis) and its aggressiveness. Histological grade has been used to determine the prognosis and breast cancer therapy. However, histological type denotes the growth (morphological and cytological) patterns of the tumors. These patterns are termed histological types

including invasive ductal carcinoma not otherwise specified (IDC-NOS) or of no special type and invasive lobular carcinoma. IDC-NOS is the most common of all breast carcinomas and special types of breast cancer account for about 25% of all breast cancer cases such as tubular, cribriform and mucinous carcinomas. At least 17 distinct histological special types have been classified by World Health Organization (WHO). It is worth to mention that both tumor grade and type provides complementary information which forms the basis of prognosis (Lacroix-Triki *et al*., 2010; Weigelt *et al*., 2010; WHO, 2016). Similarly, breast cancer can be categorized histologically on the basis of tumor confinement within breast ducts and/or lobules. Ductal carcinoma *in situ* (DCIS) represents most frequently encountered by far (over 80%) compared to the lobular carcinoma *in situ* (LCIS) which accounts for 10 to 15% of all cases of breast cancer. Other histological breast cancer subtypes are infrequent and could represent less than 10% of cases reported annually (Hirata *et al*., 2014; Vuong *et al*., 2014). A photomicrograph of DCIS and LCIS is presented in Figure 1.4.

Furthermore, breast cancer is categorized into various molecular subtypes, namely luminal A and luminal B, basal-like and human epidermal growth factor receptor 2 (HER2)-positive breast cancers depending on the gene expression profiles of estrogen receptor (ER), progesterone receptor (PR) and HER2. These biomarkers have been used in determining normal biological processes, disease prognosis and chemotherapeutic regimen of breast cancer. Other non-routine biomarkers for breast cancer management include Ki-67, androgen receptor (AR) and p53. Luminal A tumor is ER positive and/or PR positive, HER2 negative and low proliferative marker Ki-67.

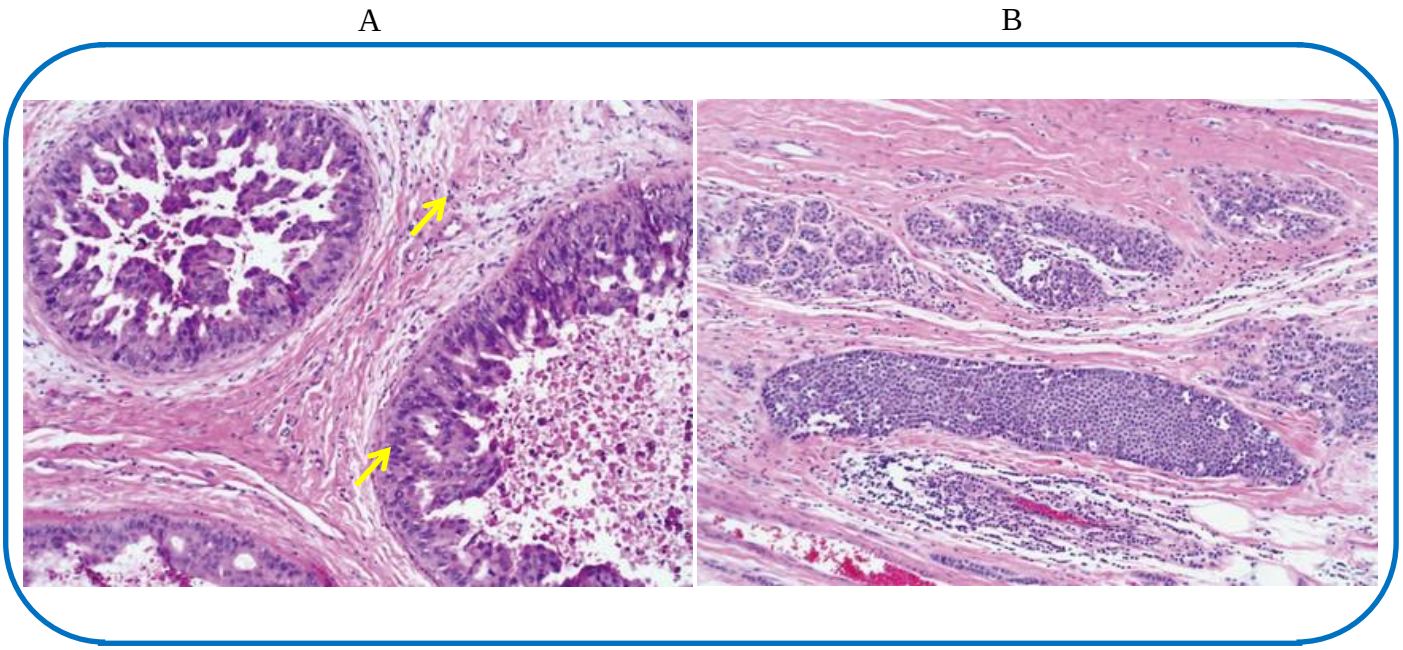

Figure 1.4 Breast (A) ductal carcinoma *in situ* (DCIS) and (B) lobular carcinoma *in situ* (LCIS), 100X magnification. (Source: Obeng-Gyasi *et al*., 2016). Arrows indicate invasive carcinoma.

Also, this subtype of breast cancer is the most frequently encountered, well differentiated with negative lymph node involvement, good prognosis and limited tumor relapse. Luminal B subtype is characterized by ER positive and/or PR positive, HER2 positive or negative, high Ki-67 and positive lymph node involvement. The HER2 breast cancer (15 - 20%) is a non-endocrine-responsive tumor displaying higher tumor grade, lymph node and brain metastases as well as higher proliferation index (PI) and tumor relapse. Basal-like subtype is defined by cancer cells which mimic those of outer (basal) layer of mammary epithelium, and represents a subset of triple-negative breast (TNB) cancer which accounts for 10 - 15% of breast cancers. TNB cancer lacks ER, PR and HER2 expression; it is grade 3 cancers with high mitotic count and lymphocyte infiltrate, therefore is more aggressive clinically than the luminal breast cancer subtype (Keam *et al*., 2011; Pracella *et al*., 2013; Reed *et al*., 2015). There is no standard therapy for basal-like breast cancer due to insufficiency of endocrine, HER2 and cytotoxic chemotherapeutic agents in this type of breast cancer and thus associated with poor prognosis (Hirata *et al*., 2014; Vuong *et al*., 2014). Table 1.1 shows the characteristics of breast cancer molecular subtypes.

1.1.4(b) Pathogenesis of breast cancer

Breast cancer is a mixed collection of neoplasms, forming within the breast ducts or lobules and has considerable variability at clinical, molecular and histomorphological stages due to complex interactions between the environment and host genetic makeup which can result in abnormal mutation in genes that regulate normal physiological functions of cell.

Table 1.1 Characteristics of breast cancer molecular subtypes

Subtypes	ER	PR	HER2	Ki-67	PI
Luminal A	ER (+) and/or PR (+)		(-)	(+)	Low
Luminal B	ER (+) and/or PR (+)		(+ or -)	(+)	High
HER2	(-)	(-)	(+)	(+)	High
Basal-like	(-)	(-)	(-)	(+)	High

Genomic instability constitutes the driven force in breast cancer evolution (Borresen-Dale *et al.*, 2010; Tubbs and Nussenzweig, 2017). The oncogenic miRNAs are small noncoding RNAs which have been implicated in the pathogenesis of several cancers including breast cancer via destabilizing role on tumor suppressor mRNAs to promote tumorigenesis and metastasis. Also, miRNAs confer breast cancer resistance to common chemotherapeutic agents through different molecular mechanism such as intracellular drug depletion, impaired cellular functions and induction of epigenetic changes (Shah and Chen, 2014).

Similarly, myoepithelial cells of the breast tissue have demonstrated susceptibility to chemical (e.g. polycyclic aromatic hydrocarbons and nitrosamines) carcinogen-induced DNA damage which can initiate carcinogenesis via genetic transformation as evidenced by variable hyperplasia (abnormal increase in the number of cells) and flat epithelial atypia (structural abnormality in epithelial cells). Consequently, the malformed cells proliferate in an uncontrolled manner in promotion phase of carcinogenesis, this stage is relatively longer and reversible by specific targeted therapy which could prevent tumor growth and development. Following the promotion phase of carcinogenesis is the progression phase provided that the cellular aberration is not hindered, this phase is characterized by conversion of pre-neoplastic cells into neoplastic ones which might later become malignant and metastatic (Burrell *et al*., 2013; Russo, 2016; Alvarado *et al*., 2017).

Furthermore, various risk factors and aetiological agents such as age, genetic predisposition, family history of breast cancer, early menarche, breast feeding, diet, alcohol consumption, obesity, lifestyle, physical inactivity, endocrine factors and age

at menopause have been implicated in the pathogenesis of breast cancer (Shah *et al*., 2014; Kaminska *et al*., 2015). For example, obesity is associated with both ER positive and ER negative breast cancers. Obesity causes derangement in biophysical properties of cell membrane as a result of defective cholesterol homeostasis which stimulates carcinogenesis via oxysterol 27-hydroxycholesterol (27-HC). The 27-HC is an ER agonist which promotes carcinogenesis and tumor progression in breast cancer. Also, obesity induces both intra-breast and systemic inflammation which could stimulate growth and metastasis of breast cancer. The impact of inflammation on development of several cancers including breast cancer is well documented. Understanding the molecular mechanism of carcinogenesis is vital to effective breast cancer prevention and treatment (Iyengar *et al*., 2013; McDonnell *et al*., 2014).

1.1.4(c) Diagnosis of breast cancer

The etiological risk factors which could influence breast cancer morbidity and mortality especially in developing countries include lack of early presentation for CBE, correct diagnosis and treatment modalities (Varughese and Richman, 2010; Jones *et al*., 2014). Effective method of early detection and diagnosis of carcinogenesis in breast tissue ensures that quick intervention is instituted in order to improve breast cancer outcome and survival. Screening mammography significantly decreases the rate of breast cancer-related deaths (by more than 30%) in females of 40 years and above. Diagnosis of breast cancer is based on clinical examination, imaging and pathological assessment. The clinical examination takes note of general health status of the patient such as history, menopausal status, physical examination, full blood count (FBC), liver and renal function tests. Imaging techniques comprises

bilateral mammography and ultrasound of the breast and regional lymph nodes. Magnetic resonance imaging is not routinely performed in breast cancer, however it is recommended in breast implants, large variations between conventional imaging and clinical examination and neoadjuvant chemotherapy to evaluate the response to treatment. The pre-treatment evaluation of breast cancer includes pathological analysis (e.g. fine needle aspiration or core needle biopsy) of primary tumor and affected lymph nodes. The final pathology report is detailing histological type and grade of breast cancer, and immunohistochemical (IHC) evaluation of ER, PR and HER2 status using standard assessment methods (e.g. Allred or H-score) (Senkus *et al*., 2015; Gohariyan *et al*., 2017).

1.1.4(d) Management of breast cancer

The current breast cancer management depends on many pathological prognostic factors such as histological type and grade, stage and biomarker (e.g. estrogen, progesterone and human epidermal growth factor) receptor status. Thus, correct diagnosis is paramount in categorizing breast cancer into clinically relevant subgroups with the ultimate goal of individualizing model of breast cancer management. Various therapeutic agents with better benefits and lesser side effects from different sources are currently being studied to improve potential outcome of breast cancer treatment (Alizart *et al*., 2012; Ali *et al*., 2016).

1.1.4(e) Staging of breast cancer

Breast cancer staging was established according to tumor size, involvement of regional lymph nodes and presence of distant metastasis, all together regarded as tumor-node-metastasis (TNM) staging system. On the basis of TNM features, breast cancer is categorized into five (0 to 4) main stages (Table 1.2). Most breast cancer cases in developing countries are diagnosed in advanced stages (3 to 4) due to medical access barriers compared to the developed countries where medical consultation and high quality management are adequate (Al-Muhrib *et al*., 2010; Unger-Saldana, 2014). After breast cancer has been diagnosed, the patient is clinically staged by TNM staging system for the purpose of establishing appropriate management modalities which include surgical treatment of breast cancer followed by adjuvant therapy such as radiotherapy, chemotherapy, hormonal therapy and immunotherapy (Senkus *et al.,* 2015).

1.1.4(f) Surgery

Surgical treatment of breast cancer includes breast-conserving surgery (BCS), such as partial mastectomy or lumpectomy in early breast cancer to more complex surgical procedure of mastectomy (removal of the whole breast) in advance breast cancer. BCS is as effective as radical mastectomy; it aims at preserving normal breast while removing cancerous part to reduce risk of local tumor relapse and commences additional treatment immediately. BCS followed by radiation treatment represent standard care for localized or regional cancers with increased survival rates as in mastectomy (Shah *et al*., 2014; Senkus *et al*., 2015).

Table 1.2 TNM breast cancer staging system (adapted from Al-Muhrib *et al*., 2010).

Stage	Definition
Stage 0 is carcinoma *in situ*	Tumors that have not grown beyond their site of origin and invaded the neighboring tissue. They include: - ductal carcinoma *in situ* - lobular carcinoma *in situ*
Stage 1	Tumor size < 2 cm, metastases to other organs and tissues not available.
Stage 2a	Tumor < 2 cm in cross-section with involvement of the lymph node or tumor from 2 to 5 cm without involvement of the axillary lymph nodes.
Stage 2b	Tumor > 5 cm in cross-section (the result of axillary lymph node research is negative for cancer cells) or tumor from 2 to 5 cm in diameter with the involvement of axillary lymph nodes.
Stage 3a	Also called spread of breast cancer: tumor > 5 cm with spread to axillary lymph nodes or any size with metastases in axillary lymph nodes, which are knitted to each other or with the surrounding tissues.
Stage 3b	Tumor of any size with metastases into the skin, chest wall or internal lymph nodes of the mammary gland (located below the breast inside of the chest).
Stage 3c	Tumor of any size with a more widespread metastases and involvement of more lymph nodes.
Stage 4	Defined as the presence of tumors (regardless of the sizes), spread to parts of the body that are located far removed from the chest (bones, lungs, liver, brain or distant lymph nodes).

Many patients require mastectomy (approximately 30 – 40% in the US) especially below 40 years of age due to tumor size (large or multiple aggressive tumors), tumor multicentricity, contraindication to radiation treatment and patient's choice. Mastectomy involves removal of the breast, overlying skin, nipple, areolar tissues as well as full axillary nodal clearance if axillary lymph node is positive. In skin-sparing mastectomy, skin covering is preserved for the purpose of breast reconstruction (Shah *et al*., 2014; Senkus *et al*., 2015). The success of multidisciplinary rehabilitation approach such as improvement in physical, psychological, occupational and general wellbeing of patient following surgery has been shown to increase survival rates in breast cancer in different part of the world including Malaysia (Loh and Musa, 2015).

1.1.4(g) Radiotherapy

Radiotherapy involves the use of high energy rays such as X-rays and gamma rays to destroy cancer cells using external beam or internal (brachytherapy) radiation. Recent advances in local or regional therapy for early stage breast cancer encourage the use of less extensive procedures such as radiotherapy. Therefore, axillary dissection could be safely avoided for patients with micrometastatic breast disease in sentinel nodes (first few lymph nodes into which tumor drains). Post-mastectomy radiotherapy is indicated in tumors greater than 5 cm irrespective of nodal status (Goldhirsch *et al*., 2013). Radiotherapy especially accompanying BCS has long demonstrated significant decrease by approximately 50% in local tumor recurrence (Shah *et al*., 2014). Similarly, radiotherapy of the whole breast could decrease 10 year threat of first tumor relapse by 15% and 15 year risk of breast cancer-related

deaths by 4%. Post-mastectomy radiotherapy is recommended in patients with four or more positive nodes, although this is contraindicated in those with one to three positive nodes, except in severe tumor-induced pathology. Furthermore, radiotherapy is not recommended in grade 3 luminal A-like disease, lymphovascular invasion and HER2 or TNB cancer. Chemotherapy could be combined with the radiotherapy where ever it is indicated in breast cancer management (Goldhirsch *et al*., 2013; Shah *et al*., 2014).

1.1.4(h) Chemotherapy

As far back as 1960, chemotherapy has been used as standard treatment against many cancers including ER negative breast cancer. It specifies the application of cytotoxic drugs in both local and advanced breast (stage 2 to 4) cancer to kill cancer cells via various anticancer mechanisms to improve overall survival. The aim of adjuvant chemotherapy is to increase the disease-free survival (DFS) and overall survival (OS) rates which are connected to breast cancer treatment by local surgery and/or radiotherapy alone. Accurate assessment of breast cancer stage including nodal status is critical to effective chemotherapeutic intervention. Doxorubicin (e.g. anthracycline) was first introduced into clinical trials in the late 1960s and was recognized as the most active chemotherapeutic drug against breast cancer. Currently, anthracyclines and taxanes are the most widely used treatments in ER negative, stage 2 HER2-positive and metastatic breast cancers. Various chemotherapeutic combinations are available in clinical practice and are administered for about 3 to 6 months to produce higher response rate and longer period of treatment failure. Combination-based chemotherapy includes

cyclophosphamide and doxorubicin, docetaxel and taxotere, as well as cyclophosphamide, methotrexate and fluorouracil combination (known as CMF). Similarly, cisplatin or methotrexate as one drug or lipoic acid and hydroxycitrate in combination or as single drug is not as effective as lipoic acid, hydroxycitrate and cisplatin or methotrexate combined together (Shah *et al*., 2014; Sledge *et al*., 2014).

Chemotherapy is associated with a wide range of short and long term consequences which could significantly affect the patient's quality of life. These side effects include impaired fertility, premature menopause, osteoporosis, neuropathy, cardiomyopathy and congestive heart failure (in anthracycline and HER2 therapies). Breast cancer survivors might also witness cognitive impairments and chronic fatigue. In order to lessen chemotherapy-related side effects, it is recommended that drug benefits should always outweigh its associated risks before commencing the chemotherapy (Miller *et al*., 2016).

1.1.4(i) Hormonal therapy

Hormonal therapy is used in treating hormone-receptor (ER and PR) positive breast cancer depending on the stage and menopausal status of the patient. Approximately 75% of females with breast cancer have ER positive breast cancer and 65% of these cancers are also PR positive (Burstein *et al*., 2014). The effects of estrogen and progesterone are mediated through ER and PR, and their overexpression is associated with growth of several breast cancers. Evaluation of ER, PR and HER2 status is needed for the assessment of tumor biology and determining the choice of hormonal therapy. TNB cancers lack expression of these prognostic markers, and

thus patients should not be treated with endocrine or HER2 targeted therapies (Ali *et al*., 2016).

There are different approaches to hormonal therapy such as inhibiting ovarian function (ovarian ablation), estrogen production and estrogen effects. Ovarian ablation restricts ovarian function of estrogen production in premenopausal women to inhibit estrogen effects on the breast. Inhibition of ovarian activity could be permanent removal (oophorectomy) or surgery and radiotherapy. Drugs which are gonadotropin (GnRH) or luteinizing hormone releasing hormone (LH-RH) agonists such as goserelin and leuprolide are used to achieve partial ovarian ablation. To achieve inhibition of estrogen production, drugs which are aromatase inhibitors (AIs) such as anastrozole, letrozole and exemestane are administered in postmenopausal women to prevent the effects of estrogen on cell growth. Aromatase enzyme plays a key role in the biosynthesis of estrogen in the ovaries and tissues (Ali *et al*., 2016).

There are various drugs which are used to counteract estrogen effect in stimulating the growth of breast cancer cells. Selective estrogen receptor modulator (SERM) has been used as an antagonist in breast tissue and agonist in other tissues, such as bone and uterus. For example, tamoxifen functions as an antagonist in the breast and agonist in the uterus. Drugs which are classified under SERM include tamoxifen (Nolvadex), raloxifene (Evista) and toremifene (Fareston). Antiestrogen drugs such as fulvestrant (Faslodex) can interact with ER to serve as an estrogen antagonist similar to SERMs, but the limiting factor in the use of fulvestrant was the potential destruction of receptor upon binding to ER. Nevertheless, fulvestrant is used in combination-based therapy with AIs such as anastrozole, letrozole and exemestane in

ER postmenopausal women with breast cancer metastasis (Ali *et al*., 2016). Tamoxifen is considered the gold standard therapy in ER positive breast cancer. However, chemoresistance to tamoxifen therapy develops in some patients, especially those with metastatic breast cancer due to the overexpression of HER2. Tamoxifen treatment is also associated with tumor relapse and breast cancer metastasis, endometrial cancer, thromboembolic disease as well as the development of undesirable side effects and toxicities such as drug resistance, liver and endometrial cancers. Hormonal therapy using AIs in postmenopausal women can cause osteoporosis, myalgia, arthralgia as well as menopausal symptoms, such as hot flushes, night sweats and atrophic vaginitis which can cause dyspareunia (Notas *et al*., 2015; Miller *et al*., 2016).

1.1.4(j) Immunotherapy

The aim of immunotherapy in cancer is to stimulate the patient's immune system in order to mount strong immune surveillance for effective destruction of invading cancer cells. Therefore, immune competence is an important factor to be considered in immunotherapeutic regimens (Hanahan and Weinberg, 2011). There are a number of studies which demonstrated that favorable outcome and chemotherapeutic response were suggestive of vital role played by immune infiltration particularly in TNB, HER2 and highly proliferative ER positive breast cancers. Immune infiltration is less prevalent in low-grade, luminal A type, ER positive breast cancers (Pusztai *et al*., 2016). HER2 is a tyrosine kinase-based cell receptor of epidermal growth factor (EGF), which stimulates cell growth and proliferation when it is overexpressed in

breast cancer. HER2 breast cancer has tendency of tumor relapse and fewer prognoses in about 25 to 30% cases where it is overexpressed (Ali *et al*., 2016).

Modern treatment strategies such as therapeutic monoclonal antibodies, and trastuzumab, take advantage of the human immune system for destruction of breast cancer cells. Herceptin® (trastuzumab) has been a standard anti-breast cancer drug which can interfere with HER2 involvement in tumor growth and metastasis. This drug is more relevant at stage 1 to 3 of breast cancer, it can binds to cancer cells overexpressing HER2 to effectively block the progression of breast cancer. Similarly, trastuzumab is used as a combination-based therapy with chemotherapy, including lapatinib, letrozole and capecitabine in breast cancer associated with HER2 alone or ER and HER2 mixed together. Pertuzumab and trastuzumab or pertuzumab, trastuzumab and docetaxel combination therapies are more effective than their single use due to more comprehensive signaling blockade. The beneficial effects of trastuzumab has been demonstrated in about 87% of patients over 5-year survival periods, however it is costly and associated with side effects especially cardiopathy (Nielsen *et al*., 2013; Swain *et al*., 2015; Ali *et al*., 2016). Furthermore, promising immunotherapeutic approaches include immune checkpoint inhibitors, such as cytotoxic T lymphocyte-associated antigen 4 (CTLA4) antibodies (the first to be approved in the US) and programmed cell death protein 1 (PD1). These have demonstrated antitumor effects and longer response in several clinical trials especially in metastatic and TNB cancers and to some extent lower response rates in ER positive breast cancers (Pusztai *et al*., 2016).