INVESTIGATION OF MOLECULAR MECHANISMS UNDERLYING THE ANTI-TUMOR AND ANTI-ANGIOGENIC ACTIVITIES OF ORTHOSIPHON STAMINEUS TOWARDS COLORECTAL CANCER

FOUAD SALEIHK RESQ AL-SUEDE

UNIVERSITI SAINS MALAYSIA

2016
INVESTIGATION OF MOLECULAR MECHANISMS UNDERLYING THE ANTI-TUMOR AND ANTI-ANGIOGENIC ACTIVITIES OF ORTHOSIPHON STAMINEUS TOWARDS COLORECTAL CANCER

by

FOUAD SALEIH RESQ ALSUEDE

Thesis submitted in fulfillment of the requirements for the degree of Doctor of Philosophy

September 2016
DEDICATION

This thesis is dedicated to

My beloved mother and my late father

To

Brothers, sisters

To

My beloved wife, sons and daughters
ACKNOWLEDGEMENT

{نَرْفَعُ دَرَجَاتٍ مّن نّشَآءٍ وَفَوْقَ كُلّ ذِي عِلْمٍ (76- سورة يوسف)}

All praises and thanks are due to ALLAH SUBHANH WA TAALA, the Lord of the world, for giving me the health, strength, knowledge and patience to complete this work. I would like to express my deep gratitude to my main supervisor Associate Professor Dr. Amin Malik Shah Abdul Majid for all his support, patience and guidance during this research. His contribution as a teacher has widened my horizon in conducting the research especially though his wisdom and relentless encouragement. Furthermore, my appreciation and sincere gratitude go to my co-supervisors Dr. Aman Shah Abdul Majid for his technical input and critical pointers to facilitate this work and Dr. Chern Ein Oon for providing valuable scientific input, constructive criticism, support and encouragement. I am privileged to be under the supervision of these supervisors during the PhD research years. I would like to thank Universiti Sains Malaysia and EMAN Biodiscoveries Sdn Bhd for giving me the opportunity and providing me with all the necessary facilities that made my study possible. I would like also to thank USM for the Graduate Assistant Award, which helped support my finances during my study. I would like to extend my gratitude to Natureceuticals Sdn. Bhd. for providing me financial assistance and scholarship throughout my stay here in Malaysia. I would like to express my gratitude and thanks to all School of Pharmaceutical Sciences faculty members, technicians, and administrative staff. My acknowledgement also goes to the Institute of Postgraduate Studies, and the university library for their help and support. I also would like to thank Professor Dr. Gurjeet Kaur for her help in the histopathology study interpretation and Mr. Shamasuddin for his help in the docking study.
As well as, I would like to express my gratitude to my friends and colleagues in the EMAN lab Dr. Mohamed Khadeer Ahamed, Dr. Sultan Ayesh Mohammed, Mr. Loiy Elsir Ahmed, Dr. Mahfuz, Mr. Hussin Baharetha, Mr. Mohammed Alsabri, Mr. Mohammed Asif, Mr. Mohammed Atta. Mr. Radwn, Mr. Saad, Ms. Norshirin Idris and Ms. Suzana Hashim and those whose names I may have missed to mention here for all their support and help during my PhD study. Last but not least, I would like to express my sincere gratitude to my family who are always in my heart; my beloved mother Amina, my beloved wife Samera, my wonderful children Abdul Rahman, Areg, Qusai, Rahf and Muhamnad, my uncles, my aunts, my dearest brothers and sisters for all their continuous prayers, support, love, inspiration and encouragement without which I would have not been able to complete my studies. This study was funded by Universiti Sains Malaysia (USM) under the Research University Team (RUT) Grant No.: 1001/PFARMASI/851001, Ministry of Agriculture, Malaysia, under NRGS (NKEA) grant No: 304/PFARMASI/650735/K123 and Nature Ceuticals Sdn Bhd.

_Fouad Saleih Resq Al-suede_

_Penang, Malaysia, September 2016_
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACKNOWLEDGEMENT ................................................................................. ii</td>
</tr>
<tr>
<td>TABLE OF CONTENTS ........................................................................ iv</td>
</tr>
<tr>
<td>LIST OF TABLES ........................................................................ xvii</td>
</tr>
<tr>
<td>LIST OF FIGURES ........................................................................ xx</td>
</tr>
<tr>
<td>LIST OF ABBREVIATIONS ................................................................ xxvii</td>
</tr>
<tr>
<td>LIST OF SYMBOLS ........................................................................ xxxi</td>
</tr>
<tr>
<td>ABSTRAK ......................................................................................... xxxii</td>
</tr>
<tr>
<td>ABSTRACT ....................................................................................... xxxiv</td>
</tr>
</tbody>
</table>

## CHAPTER ONE - INTRODUCTION AND LITERATURE REVIEW

1.1 Cancer ......................................................................................... 1

1.1.1 Cancer epidemiology ........................................................................ 2
1.1.2 Cancer in Malaysia ............................................................................. 4
1.1.3 Development and progression of cancer .............................................. 5
1.1.4 Cancer pathology and genetic events of tumorigenesis ..................... 9

1.2 Colorectal cancer ........................................................................... 11

1.2.1 Epidemiology of colorectal cancer .................................................. 12
1.2.2 Chemotherapeutics of colorectal cancer ......................................... 13

1.3 Tumor angiogenesis ....................................................................... 14

1.3.1 Physiologic and pathologic angiogenesis ...................................... 14
1.3.2 Angiogenesis cascade events ......................................................... 15
1.3.3 Regulation of angiogenesis ................................................. 17
1.3.4 Anti-angiogenic targets .................................................... 20
1.3.5 Anti-angiogenic therapies ................................................. 20

1.4 Correlation between cancer and angiogenesis .............................. 23
1.4.1 Pro and anti-angiogenic mediators ........................................ 23
1.4.1.(a) Vascular endothelial growth factor ................................... 24
1.4.1.(b) Hypoxia inducible factor-1 ............................................ 24
1.4.1.(c) Transforming growth factor .......................................... 25
1.4.1.(d) Basic fibroblast growth factor ....................................... 26
1.4.1.(e) Interferon ................................................................. 26
1.4.1.(f) Nerve growth factor .................................................... 27

1.5 Oxidative stress .......................................................................... 27

1.6 Inflammation and cancer development ........................................ 28
1.6.1 Prostaglandin synthesis, inflammation, and colorectal tumorigenesis .... 29
1.6.2 Cytokines in colorectal cancer ............................................. 30

1.7 Medicinal plants as a source for cancer therapy ............................... 30
1.8 *Orthosiphon stamineus* benth. ...................................................... 31
1.8.1 Traditional uses ...................................................................... 34
1.8.2 Phytochemical composition .................................................. 35
1.8.3 Biological and pharmacological effect ....................................... 36
1.8.3.(a) Anti-oxidant and anti-inflammatory ................................... 37
1.8.3.(b) Anti-cancer and anti-angiogenic study ............................... 37

1.9 Rosmarinic acid ....................................................................... 39

1.10 Justification of the research .......................................................... 41

1.11 Hypothesis ............................................................................... 42
1.12 Objectives of study............................................................................................................ 43
  1.12.1 General objective........................................................................................................ 43
  1.12.2 Specific objective........................................................................................................ 43

1.13 Flow chart of study ........................................................................................................ 44

CHAPTER TWO - MATERIALS AND METHODS

2.1 Chemicals and reagents.................................................................................................. 46

2.2 Equipments and apparatus............................................................................................... 49

2.3 Plant material and extraction........................................................................................... 51
  2.3.1 Plant collection and authentication............................................................................. 51
  2.3.2 Preparation of Orthosiphon stamineus extract......................................................... 51

2.4 Ex-vivo angiogenic screening study of various extract of Orthosiphon stamineus and standardization........................................................................................................ 51
  2.4.1 Ex-vivo angiogenic on rat aortic ring assay............................................................... 51
    2.4.1.(a) Experimental animals......................................................................................... 52
    2.4.1.(b) Preparation of aortic ring.................................................................................. 52
    2.4.1.(c) Preparation of the tissue culture plates............................................................. 52
    2.4.1.(d) Quantification of the blood vessels outgrowth............................................... 53
  2.4.2 Standardization and quantification of selected biomarkers in 50% ethanol extract of Orthosiphon stamineus.......................................................... 54
    2.4.2.(a) Preparation of standards compounds............................................................... 54
    2.4.2.(b) Preparation of 50% ethanolic extract of Orthosiphon stamineus for high performance liquid chromatography analysis ......................... 54
    2.4.2.(c) Instrumentation and chromatographic conditions ........................................... 54
    2.4.2.(d) Linearity .............................................................................................................. 56
    2.4.2.(e) Selectivity ............................................................................................................ 56
2.4.2.(f) Determination of eupatorin, sinensetin, rosmarinic acid and 3’-hydroxy-5, 6, 7, 4’-tetramethoxyflavone from 50% ethanol extract of Orthosiphon stamineus ........................................ 56

2.4.3 Total ash .................................................................................................................. 57
2.4.3.(a) Acid-insoluble ash .......................................................................................... 57
2.4.3.(b) Water-soluble ash ......................................................................................... 57

2.5 Anti-oxidant activity .................................................................................................. 58

2.5.1 Determination of total phenolic contents ................................................................. 58
2.5.2 Determination of total flavonoid contents ................................................................. 59
2.5.3 Ferric reducing anti-oxidant power assay ............................................................... 59
2.5.4 ABTS assay ............................................................................................................. 60
2.5.5 DPPH free radical scavenging assay ....................................................................... 61

2.6 Cell lines and cell culture maintenance .................................................................... 62

2.6.1 Cell lines .................................................................................................................. 62
2.6.2 Cells cryopreservation ........................................................................................... 63
2.6.3 Complete medium preparation .............................................................................. 63
2.6.4 Recovery of frozen cell line ................................................................................... 63
2.6.5 Subculture of adherent cell lines ............................................................................ 63
2.6.6 Cells counting ........................................................................................................... 64
2.6.7 Rosmarinic acid preparation .................................................................................. 65
2.6.8 Preparation of 50% ethanol extract of Orthosiphon stamineus ............................... 66
2.6.9 Reference standard preparation ............................................................................ 66

2.7 In vitro anti-inflammatory effect of 50% ethanol extract of Orthosiphon stamineus and rosmarinic acid ............................................................... 66

2.7.1 Cells proliferation assay ......................................................................................... 66
2.7.1.(a) Cell culture and treatment ................................................................................. 67
2.7.2 In vitro effect of 50% ethanol extract of Orthosiphon stamineus and rosmarinic acid on cytokine and nitric oxide concentration in human macrophage cells (U937) ......................................................... 67
2.7.3  *In vitro* effect of 50% ethanol extract of *Orthosiphon stamineus* on cyclooxygenase level................................................................. 68

2.8  Assessment of anti-angiogenic effect of 50% ethanol extract of *Orthosiphon stamineus* and rosmarinic acid .......................................................... 69

2.8.1  *Ex-vivo* anti-angiogenic assessment of the effect of 50% ethanol extract of *Orthosiphon stamineus* and rosmarinic acid on rat aortic ring assay ................................................................. 69

2.8.2  *In vitro* anti-angiogenic assessment of the effect of 50% ethanol extract of *Orthosiphon stamineus* and rosmarinic acid on human umbilical vein endothelial cells (HUVEC) .................................................. 69

2.8.2.(a)  *In vitro* anti-angiogenic assessment of the effect of 50% ethanol extract of *Orthosiphon stamineus* and rosmarinic acid on human umbilical vein endothelial cells proliferation........................................ 69

2.8.2.(b)  *In vitro* anti-angiogenic assessment of the effect of 50% ethanol extract of *Orthosiphon stamineus* on human umbilical vein endothelial cells migration ......................................................... 70

2.8.2.(c)  *In vitro* anti-angiogenic assessment of the effect of 50% ethanol extract of *Orthosiphon stamineus* and rosmarinic acid on human umbilical vein endothelial cells tube formation................................. 71

2.8.2.(d)  *In vitro* assessment of the effect of 50% ethanolic extract and rosmarinic acid on pro and anti-angiogenic growth factor using Luminex Multiplexing Platform ................................................................. 72

2.8.3  *In vivo* anti-angiogenic assessment of the effect of 50% ethanol extract of *Orthosiphon stamineus* and rosmarinic acid................................. 73

2.8.3.(a)  *In vivo* anti-angiogenic assessment of effect of 50% ethanol extract of *Orthosiphon stamineus* and rosmarinic acid on chick chorioallantoic membrane ................................................................. 73

2.8.3.(a) i - Preparation of chick membrane .................................................................. 73

2.8.3.(a) ii - Treatment of chick membrane .................................................................. 73

2.8.3.(b)  *In vivo* anti-angiogenic assessment of 50% ethanol extract of *Orthosiphon stamineus* on Matrigel plug.......................................................... 74

2.8.3.(b) i - Animals .................................................................................................. 74

2.8.3.(b) ii - Preparation of Matrigel plug .................................................................. 74

2.8.3.(b) iii - Establishment of the subcutaneous Matrigel plug assay .......... 75

2.8.3.(b) iv - Experimental design and treatment...................................................... 75
2.8.3. (b)  Hematoxylin and eosin staining of the blood vessels ............... 75

2.9  In vitro anti-cancer studies ................................................................. 77

2.9.1  Assessment of the effect of 50% ethanolic extract of Orthosiphon stamineus and rosmarinic acid on viability of various cell lines .......... 77

2.9.1. (a)  Preparation of cells ................................................................. 77

2.9.1. (b)  Treatment with different doses of 50% ethanolic extract of Orthosiphon stamineus and rosmarinic acid ......................... 77

2.9.1. (c)  MTT assay for assessment of cell viability ......................... 78

2.9.1. (d)  MTS assay for cell proliferation ........................................... 79

2.10  Anti-tumorigenicity ................................................................. 79

2.10.1  Cell invasion assay ................................................................. 79

2.10.2  Spheroids assay ................................................................. 80

2.11  In vivo anti-tumor studies of 50% ethanol extract of Orthosiphon stamineus and rosmarinic acid ......................................................... 81

2.11.1  Evaluation of the activity of 50% ethanol extract of Orthosiphon stamineus and rosmarinic acid on subcutaneous colorectal tumor growth in nude mice for 28 days (Ectopic model) ...................... 81

2.11.1. (a)  Animals ................................................................. 81

2.11.1. (b)  Preparation of HCT-116 cells ............................................ 81

2.11.1. (c)  Establishment of the subcutaneous tumors ......................... 82

2.11.1. (d)  Treatment and tumor size measurement ......................... 82

2.11.1. (e)  Euthanasia and tumor collection ....................................... 84

2.11.2  Evaluation of the activity of 50% ethanol extract of Orthosiphon stamineus and rosmarinic acid on colorectal cancer using nude mice for 35 days (Orthotopic xenograft tumor implantation model) ..... 84

2.11.2. (a)  Animals ................................................................. 84

2.11.2. (b)  Preparation of HCT-116 cells ............................................ 85

2.11.2. (c)  Establishment of the orthotopic tumors ......................... 85

2.11.2. (d)  Treatment ................................................................. 86

2.11.2. (e)  Histopathologic examination .......................................... 86

2.11.2. (f)  Biochemistry indexes .................................................... 87
2.11.2 (g) Tumor identification measurement using three dimensional fluorescence molecular tomography............................... 87

2.11.3 Evaluation of the effect of 50% ethanol extract of Orthosiphon stamineus and rosmarinic acid on subcutaneous colorectal tumor growth in nude mice for 26 weeks (Long-term survival xenograft ectopic model) ............................................................... 89

2.11.3.(a) Animals .................................................................................. 89
2.11.3.(b) Experimental design ................................................................. 89
2.11.3.(c) Euthanasia and tumor collection .............................................. 90

2.11.4 In vivo assessment of the preventive effect of 50% ethanol extract of Orthosiphon stamineus against subcutaneous colorectal tumor growth using nude mice (two weeks pre-treatment) ............................................ 90

2.11.4.(a) Animals .................................................................................. 90
2.11.4.(b) Experimental design ................................................................. 90
2.11.4.(c) Implantation of tumor ............................................................... 91

2.11.5 Evaluation of the anti-tumor activity of rosmarinic acid on subcutaneous colorectal tumor growth using nude mice for 28 days (Ectopic model) ......................................................................................... 91

2.11.5.(a) Animals .................................................................................. 91
2.11.5.(b) Experimental design ................................................................. 92

2.11.6 In vivo evaluation of the effect of 50% ethanolic extract and rosmarinic acid on pro and anti-angiogenic growth factor using Luminex Multiplexing Platform ............................................................... 92

2.11.6.(a) Sample preparation .................................................................. 92
2.11.6.(b) Investigation of protein level in tissue sample ....................... 93

2.11.7 In vivo evaluation of the effect of 50% ethanolic extract and rosmarinic acid on gene expression using quantitative Real Time Polymerase Chain Reaction ............................................................... 94

2.11.7.(a) RNA isolation ......................................................................... 94
2.11.7.(b) Quantitative Real Time Polymerase Chain Reaction ............... 95

2.12 In silico ligand binding and interaction studies of selected bioactive compounds in 50% ethanolic extract of Orthosiphon stamineus .................. 96

2.12.1 Ligand preparation for docking study ........................................... 96
2.12.2 Protein preparation for docking study ........................................... 98
2.12.3 Comparative molecular field analysis partial least-squares analysis...... 99
2.12.3.(a) FlexX docking .................................................................................................................. 99
2.13 Statistical analysis ................................................................................................................... 100

CHAPTER THREE - ANTI-ANGIOGENIC SCREENING AND PHYTOCHEMICAL STUDY OF ORTHOSIPHON STAMINEUS

3.1 Introduction .............................................................................................................................. 101
3.2 Materials and methods ............................................................................................................. 102
3.3 Results ...................................................................................................................................... 102
  3.3.1 Extraction method.................................................................................................................. 102
  3.3.2 Determination of total ash, water soluble and acid-insoluble ash ............. 103
  3.3.3 Ex- vivo angiogenic screening study of Orthosiphon stamineus ............. 103
  3.3.4 Quantification of rosmarinic acid, sinensetin, eupatorin and 3’-hydroxy-5, 6, 7, 4’-tetramethoxyflavone in Orthosiphon stamineus using high performance liquid chromatography .............................................. 106
  3.3.5 Anti-oxidant activity of 50% ethanol extract of Orthosiphon stamineus leaves .............................................................................................................................................................................. 109
            3.3.5.(a) Total flavonoid and phenolic contents of 50% ethanol extract of Orthosiphon stamineus leaves .............................................................................................................................................................................. 109
            3.3.5.(b) Ferric reducing anti-oxidant power ................................................................. 110
            3.3.5.(c) Free radicals scavenging assay......................................................................... 110
            3.3.5.(d) Effect of rosmarinic acid toward free radical scavenging .......................... 113
  3.3.6 Anti-inflammatory study ................................................................................................. 114
            3.3.6.(a) Effect of 50% ethanol extract of Orthosiphon stamineus and rosmarinic acid on viability of human macrophage cell line .......... 114
            3.3.6.(b) In vitro inhibitory effect of 50% ethanol extract of Orthosiphon stamineus on production of nitric oxide and cytokine in human macrophages cells ................................................................................................................. 115
            3.3.6.(c) In vitro inhibitory effect of rosmarinic acid on production of nitric oxide and cytokine in human macrophage cells .......... 116
3.3.6.(d) *In vitro* inhibitory effect of 50% ethanolic extract of *Orthosiphon stamineus* on cyclooxygenase activities.............. 118

3.4 Discussion .................................................................................................................. 119

3.5 Conclusion .................................................................................................................. 124

CHAPTER FOUR- *IN-VITRO AND IN-VIVO INVESTIGATION OF THE MOLECULAR MECHANISMS UNDERLYING THE ANTI-ANGIOGENIC ACTIVITY OF ORTHOSIPHON STAMINEUS AND ROSMARINIC ACID*

4.1 Introduction .................................................................................................................. 125

4.2 Materials and Methods .............................................................................................. 126

4.3 Result .......................................................................................................................... 126

4.3.1 *Ex-vivo* anti-angiogenic study using rat aortic ring assay.................. 126

4.3.1.(a) Dose-response curves of 50% ethanol extract of *Orthosiphon stamineus* and rosmarinic acid on rat aortic ring assay........... 126

4.3.2 *In vitro* anti-angiogenic study on Human Umbilical Vein Endothelial Cells........................................................................ 130

4.3.2.(a) Effect of 50% ethanol extract of *Orthosiphon stamineus* and rosmarinic acid on the proliferation of Human Umbilical Vein Endothelial Cells ................................................................. 130

4.3.2.(b) Effect of 50% ethanol extract of *Orthosiphon stamineus* on Human Umbilical Vein Endothelial Cells migration .............. 132

4.3.2.(c) Anti-angiogenic effect of 50% ethanol extract of *Orthosiphon stamineus* and rosmarinic acid on Human Umbilical Vein Endothelial Cells using tube formation assay .......................... 134

4.3.2.(d) *In vitro* effect of 50% ethanol extract of *Orthosiphon stamineus* and rosmarinic acid on protein expression.......................... 136

4.3.3 *In vivo* anti-angiogenic activity.............................................................................. 149

4.3.3.(a) Effect of 50% ethanol extract of *Orthosiphon stamineus* and rosmarinic acid on neovascularization in Chick Chorioallantoic Membrane assay .......................................................... 149
4.3.3.(b) *In vivo* anti-angiogenic effect of 50% ethanol extract of *Orthosiphon stamineus* on Matrigel plug assay in nude mice for 7 days ................................................................. 151

4.4 Discussion ........................................................................................................... 153

CHAPTER FIVE - *IN VITRO AND IN VIVO* INVESTIGATION OF THE MOLECULAR MECHANISMS UNDERLYING THE ANTI-TUMOR ACTIVITY OF *ORTHOSIPHON STAMINEUS* AND ROSMARINIC ACID IN A COLORECTAL CANCER MODEL

5.1 Introduction ........................................................................................................ 160

5.2 Materials and Methods ..................................................................................... 161

5.3 Result .................................................................................................................. 161

5.3.1 *In vitro* anti-cancer studies ........................................................................ 161

5.3.1.(a) Effect of 50% ethanol extract of *Orthosiphon stamineus* on the viability of various cancer cell lines ................................................................. 161

5.3.1.(b) Effect of rosmarinic acid on the viability of various cancer cell lines ................................................................. 162

5.3.1.(c) *In vitro* anti-tumorigenicity of 50% ethanol extract of *Orthosiphon stamineus* and rosmarinic acid on colorectal cancer cell line ................................................................. 163

5.3.2 *In vivo* tumor studies of 50% ethanol extract of *Orthosiphon stamineus* and rosmarinic acid on colorectal cancer cell line (HCT-116). ........................................................................................................ 167

5.3.2.(a) Effect of 50% ethanol extract of *Orthosiphon stamineus* on subcutaneous colorectal tumor growth in nude mice for 28 days (Ectopic Model) ................................................................. 167

5.3.2.(b) Effect of 50% ethanol extract of *Orthosiphon stamineus* on body weight ......................................................................................................................... 170

5.3.2.(c) Effect of 50% ethanol extract of *Orthosiphon stamineus* and rosmarinic acid on the growth of colorectal cancer in a metastatic model using nude mice orthotopic xenograft tumor implantation (short term study for 35 days) ........................................................................................................ 171
5.3.2 (c) iii - Effect of 50% ethanol extract of Orthosiphon stamineus and rosmarinic acid on Long-Term Survival (26 weeks) of nude mice using ectopic xenograft Model ........................................... 177

5.3.2 (d) Effect of 50% ethanol extract of Orthosiphon stamineus in nude mice using colorectal cancer ectopic model; a preventive study (2 weeks pre treatment) ......................................................... 179

5.3.3 \textit{In vivo} anti-tumor activity of rosmarinic acid on subcutaneous colorectal tumor growth using nude mice for 28 days (Ectopic model) ........................................................................................................ 185

5.3.4 \textit{In vivo} effect of 50% ethanol extract of Orthosiphon stamineus and rosmarinic acid on pro and anti-angiogenic growth factor protein expression using Luminex Multiplexing Platform ......................................................... 190

5.3.4 (a) \textit{In vivo} effect of 50% ethanol extract of Orthosiphon stamineus on vascular endothelial growth factor, fibroblast growth factor and granulocyte macrophage colony stimulating factor levels (short term study at 28 days in ectopic tumors) ........................................... 190

5.3.4 (b) \textit{In vivo} effect of 50% ethanol extract of Orthosiphon stamineus on interleukin-7, transforming growth factor-alpha and nerve growth factor- beta levels (short term study at 28 days in ectopic tumors) ........................................................................................................ 193

5.3.4 (c) \textit{In vivo} effect of 50% ethanol extract of Orthosiphon stamineus on interferon alpha, interferon beta and epidermal growth factor levels (short term study at 28 days in ectopic tumors) ....................... 195

5.3.4 (d) \textit{In vivo} effect of 50% ethanol extract of Orthosiphon stamineus on interferon gamma, tumour necrosis alpha, tumour necrosis beta and interleukin-2 growth factor levels (short term study for 28 days in ectopic tumors) ......................................................... 197

5.3.5 \textit{In vivo} effect of 50% ethanol extract of Orthosiphon stamineus on HIF-α, KDR, WNT and COX2 gene expression on human colorectal tumor tissue (Short term study for 28 days in ectopic tumors) ............... 199

5.3.6 \textit{In-silico} prediction of binding and interactions of selected bioactive compounds of 50% ethanol extract of Orthosiphon stamineus .......... 201

5.3.6 (a) Comparative molecular field analysis of selected bioactive compounds of 50% ethanol extract of Orthosiphon stamineus .... 201

5.3.6 (b) \textit{In silico} ligand binding and interaction studies of selected bioactive compounds in 50% ethanol extract of Orthosiphon stamineus to cyclooxygenase ................................................................. 203
5.3.6.(c) *In silico* ligand binding and interaction studies of selected bioactive compounds in 50% ethanol extract of *Orthosiphon stamineus* to epidermal growth factors ........................................ 205

5.3.6.(d) *In silico* ligand binding and interaction studies of selected bioactive compounds in 50% ethanolic extract of *Orthosiphon stamineus* to basic fibroblast growth factors ........................................ 207

5.3.6.(e) *In silico* ligand binding and interaction studies of selected bioactive compounds in 50% ethanolic extract of *Orthosiphon stamineus* to vascular endothelial growth factor A ........................................ 209

5.3.6.(f) *In silico* ligand binding and interaction studies of selected bioactive compounds in 50% ethanolic extract of *Orthosiphon stamineus* to granulocyte macrophage colony stimulating factor levels ........................................................................................................ 211

5.3.6.(g) *In silico* ligand binding and interaction studies of selected bioactive compounds in 50% ethanolic extract of *Orthosiphon stamineus* to interferon alpha 2 ........................................ 213

5.3.6.(h) *In silico* ligand binding and interaction studies of selected bioactive compounds in 50% ethanolic extract of *Orthosiphon stamineus* to interleukin-2 .................................................................................. 215

5.3.6.(i) *In silico* ligand binding and interaction studies of selected bioactive compounds in 50% ethanolic extract of *Orthosiphon stamineus* to tumor necroses factors alpha ........................................ 217

5.3.6.(j) *In silico* ligand binding and interaction studies of selected bioactive compounds in 50% ethanolic extract of *Orthosiphon stamineus* to tumor necrosis factor beta ........................................ 219

5.3.6.(k) *In silico* ligand binding and interaction studies of selected bioactive compounds in 50% ethanolic extract of *Orthosiphon stamineus* to vascular endothelial growth factor R1 ........................................ 221

5.4 Discussion ........................................................................................................................................ 223

CHAPTER SIX - GENERAL DISCUSSION .......................................................................................... 233
# CHAPTER SEVEN - CONCLUSION

7.1 Conclusion ........................................................................................................... 247

7.2 Limitations ........................................................................................................... 249

7.3 Future work ......................................................................................................... 249

REFERENCES ........................................................................................................... 250

APPENDICES ........................................................................................................... 275

List of PUBLICATIONS ........................................................................................... 307
LIST OF TABLES

Table 1.1  
List of oncogenes.

Table 1.2  
Some of tumor suppressor genes.

Table 1.3  
Example of pro and anti-angiogenic factors.

Table 1.4  
FDA-approved angiogenesis inhibitors

Table 2.1  
List of Chemicals and reagents

Table 2.2  
List of equipments and apparatus

Table 2.3  
HPLC mobile phase gradient elution program for separation of 50% ethanolic extract of Orthosiphon stamineus marker compounds

Table 2.4  
Types of cell lines used for in vitro cytotoxicity evaluation

Table 2.5  
Gene primers

Table 2.6  
Molecular structures and bioactivity (IC₅₀) of selected compound (PIC50 inhibitory potential)

Table 3.1  
Percentage yield of various extracts of Orthosiphon stamineus

Table 3.2  
Peak area, regression equation and percentage of marker compounds present in 50% ethanol extract of Orthosiphon stamineus leaves

Table 5.1  
Biochemical parameters of nude mice treated orally with various compounds for 35 days

Table 5.2  
In silico of ligand and cyclooxygenase interaction (interacted amino acids residues, number of hydrogen bonds, binding affinity and ligand efficiency
Table 5.3  *In silico* of ligand and epidermal growth factors interaction (interacted amino acids residues, number of hydrogen bonds, binding affinity and ligand efficiency)

Table 5.4  *In silico* of ligand (rosmarinic acid and Imatinib®) and fibroblast growth factors interaction (interacted amino acids residues, number of hydrogen bonds, binding affinity and ligand efficiency).

Table 5.5  *In silico* of ligand and vascular endothelial growth factor A interaction (interacted amino acids residues, number of hydrogen bonds, binding affinity and ligand efficiency)

Table 5.6  *In silico* of ligand and granulocyte macrophage colony stimulating factor interaction (interacted amino acids residues, number of hydrogen bonds, binding affinity and ligand efficiency).

Table 5.7  *In silico* of ligand and interferon alpha 2 interaction (interacted amino acids residues, number of hydrogen bonds, binding affinity and ligand efficiency)

Table 5.8  *In silico* of ligand and interleukin-2 (interacted amino acids residues, number of hydrogen bonds, binding affinity and ligand efficiency)

Table 5.9  *In silico* of ligand and tumor necrosis factors alpha interaction (interacted amino acids residues, number of hydrogen bonds, binding affinity and ligand efficiency)
| Table 5.10 | *In silico* of ligand and tumor necrosis factors beta interaction (interacted amino acids residues, number of hydrogen bonds, binding affinity and ligand efficiency) | 219 |
| Table 5.11 | *In silico* of ligand and vascular endothelial growth factor R1 interaction (interacted amino acids residues, number of hydrogen bonds, binding affinity and ligand efficiency) | 221 |
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 1.1</td>
<td>Ten hallmarks of cancer acquired during cancer progression.</td>
<td>10</td>
</tr>
<tr>
<td>Figure 1.2</td>
<td>Genetic alterations frequently associated with CRC progression</td>
<td>11</td>
</tr>
<tr>
<td>Figure 1.3</td>
<td>Angiogenesis Cascade.</td>
<td>16</td>
</tr>
<tr>
<td>Figure 1.4</td>
<td>Growth factors receptors.</td>
<td>23</td>
</tr>
<tr>
<td>Figure 1.5</td>
<td>Purple <em>Orthosiphon stamineus</em></td>
<td>33</td>
</tr>
<tr>
<td>Figure 1.6</td>
<td>White <em>Orthosiphon stamineus</em></td>
<td>33</td>
</tr>
<tr>
<td>Figure 1.7</td>
<td>Flow chart of study</td>
<td>45</td>
</tr>
<tr>
<td>Figure 2.1</td>
<td>Reaction mechanism of DPPH</td>
<td>62</td>
</tr>
<tr>
<td>Figure 0.1</td>
<td>Flow chart of subcutaneous tumors</td>
<td>82</td>
</tr>
<tr>
<td>Figure 3.1</td>
<td>Levels of total ash, water soluble and acid insoluble ash in <em>Orthosiphon stamineus</em> leaves.</td>
<td>103</td>
</tr>
<tr>
<td>Figure 3.2</td>
<td>Anti-angiogenic potency of different extract of <em>Orthosiphon stamineus</em> leaves.</td>
<td>105</td>
</tr>
<tr>
<td>Figure 3.3</td>
<td>Percentage inhibition of various extracts of <em>Orthosiphon stamineus</em> leaves on blood vessels growth of rat aortic ring at 100 µg/mL, (quantified after 5 days)</td>
<td>106</td>
</tr>
<tr>
<td>Figure 3.4</td>
<td>Chromatogram of rosmarinic acid, 3’-hydroxy-5,6,7,4’-tetramethoxyflavone, sinensetin, eupatorin and 50% ethanol extract of <em>Orthosiphon stamineus</em></td>
<td>108</td>
</tr>
<tr>
<td>Figure 3.5</td>
<td>Total flavonoid and total phenolic contents of 50% ethanol extract of <em>Orthosiphon stamineus</em></td>
<td>110</td>
</tr>
<tr>
<td>Figure 3.6</td>
<td>Scavenging activity of 50% ethanol extract of <em>Orthosiphon stamineus</em></td>
<td>112</td>
</tr>
<tr>
<td>Figure 3.7</td>
<td>Rosmarinic acid reducing radical scavenging activity of DPPH assay (A) ABTS assay (B)</td>
<td>113</td>
</tr>
<tr>
<td>Figure 3.8</td>
<td>Effect of 50% ethanol extract of <em>Orthosiphon stamineus</em> and rosmarinic acid on viability of U937 cell</td>
<td>114</td>
</tr>
<tr>
<td>Figure 3.9</td>
<td>Effect of 50% ethanolic extract of <em>Orthosiphon stamineus</em> on interleukin-1, tumor necrosis factor-alpha and nitric oxide</td>
<td></td>
</tr>
</tbody>
</table>

xx
synthesis by stimulation of human macrophage cells using Lipopolysaccharide

Figure 3.10  Effect of rosmarinic acid on interleukin-1, tumor necrosis factor-α and nitric oxide production by stimulation of human macrophage cells using Lipopolysaccharide

Figure 3.11  The percent inhibition of 50% ethanolic extract of Orthosiphon stamineus and aspirin for COX-1; celecoxib for COX-2 on the activities of cyclooxygenase enzymes

Figure 3.12  Summary of phytochemical study of Orthosiphon stamineus

Figure 4.1  Photomicrographs of anti-angiogenic activity of 50% ethanol extract of Orthosiphon stamineus and rosmarinic acid towards neovascularisation in rat aortic ring assay

Figure 4.2  The inhibition of 50% ethanolic extract of Orthosiphon stamineus (A) and rosmarinic acid (B) on spourting in the rat aortic tissue explants

Figure 4.3  Effect of 50% ethanol extract of Orthosiphon stamineus (A) and rosmarinic acid (B) on Human Umbilical Vein Endothelial Cells proliferation

Figure 4.4  Effect of 50% ethanol extract of Orthosiphon stamineus on HUVECs cell migration.

Figure 4.5  Anti angiogenic effect of 50% ethanol extract of Orthosiphon stamineus on HUVECs tube formation

Figure 4.6  Effect of 50% ethanol extract of Orthosiphon stamineus, rosmarinic acid and Imatinib® on pro and anti-angiogenic growth factor expression.

Figure 4.7  Effect of different doses of 50% ethanol extract of Orthosiphon stamineus, rosmarinic acid and Imatinib® on vesicular endothelial growth factor expression in Human Umbilical Vein Endothelial Cells after 24 h treatment

Figure 4.8  Effects of different doses of 50% ethanol extract of Orthosiphon stamineus, rosmarinic acid and Imatinib® on epidermal growth factor expression in endothelial cells (A) and basic fibroblast
growth factor (B) expression level in Human Umbilical Vein Endothelial Cells after 24 h treatment

**Figure 4.9** Effect of different doses of 50% ethanol extract of *Orthosiphon stamineus*, rosmarinic acid and *Imatinib*® on interferons expression levels in Human Umbilical Vein Endothelial Cells lysate after 24 h treatment

**Figure 4.10** Effect of different doses of 50% ethanol extract of *Orthosiphon stamineus*, rosmarinic acid and *Imatinib*® on interleukin-2 (A) and interleukin-7 (B) expression levels in Human Umbilical Vein Endothelial Cells lysate after 24 h treatment

**Figure 4.11** Effect of different doses of 50% ethanol extract of *Orthosiphon stamineus*, rosmarinic acid and *Imatinib*® on transfer growth factor (A) and nerve growth factor (B) expression levels in Human Umbilical Vein Endothelial Cells lysate after 24 h treatment

**Figure 4.12** Effect of different doses of 50% ethanol extract of *Orthosiphon stamineus*, rosmarinic acid and *Imatinib*® on granulocyte macrophage colony-stimulating factor expression level in Human Umbilical Vein Endothelial Cells lysate after 24 h treatment

**Figure 4.13** Effect of different doses of 50% ethanol extract of *Orthosiphon stamineus*, rosmarinic acid and *Imatinib*® on tumor necrosis factor alpha (A) and tumor necrosis factor beta (B) expression levels in Human Umbilical Vein Endothelial Cells lysate after 24 h treatment

**Figure 4.14** Inhibitory effect of different doses of 50% ethanol extract of *Orthosiphon stamineus* and rosmarinic acid on neovascularization in chorioallantoic membrane of chick embryo

**Figure 4.15** Anti-angiogenic effect of 50% ethanol extract of *Orthosiphon stamineus* on matrigel plug

**Figure 4.16** Summary of anti-angiogenic effects of 50% ethanol extract of *Orthosiphon stamineus*
Figure 5.1  Effect of the 50% ethanol extract of *Orthosiphon stamineus* on the viability of HCT-116, Skno-1, HL-60 and CCD-18Co cell lines  

Figure 5.2  Effect of rosmarinic acid on the viability of HCT-116, Skno-1, HL-60 and CCD-18Co cell lines  

Figure 5.3  Morphology of HCT-116 cell invasion after treatment with different doses of 50% ethanol extract of *Orthosiphon stamineus* and rosmarinic acid  

Figure 5.4  The principles of multicellular tumor spheroids preparation by the hanging-drop method  

Figure 5.5  Effects of 50% ethanolic extract of *Orthosiphon stamineus* and rosmarinic acid on *in-vitro* HCT-116 tumour in hanging drop assay  

Figure 5.6  Subcutaneous tumor in NCR nude mice  

Figure 5.7  Effect of 50% ethanol extract of *Orthosiphon stamineus* and the equivalent amount of rosmarinic acid on HCT-116 tumor size in nude mice  

Figure 5.8  Percentage inhibition of tumor growth in nude mice treated with different doses of 50% ethanolic extract of *Orthosiphon stamineus*, rosmarinic acid and Imatinib® compared with negative control  

Figure 5.9  Body weight of treated animals with 50% ethanol extract of *Orthosiphon stamineus*, rosmarinic acid and Imatinib® compared with negative control  

Figure 5.10  Imaging of tumor-bearing mice was implanted orthotopically into the cecal wall of nude mice, using fluorescence molecular tomography (FMT)  

Figure 5.11  Effect of 50% ethanol extract of *Orthosiphon stamineus* towards HCT-116 tumor implanted orthotopically in the cecal wall of nude mouse after treatment for 35 days  

Figure 5.12  Hematoxylin/eosin staining of crosses sections of tumor tissues  

Figure 5.13  Photographs of liver metastasis in untreated group
Figure 5.14 Effect of 50% ethanol extract of *Orthosiphon stamineus* and rosmarinic acid on animal survival rate, that was implanted ectopically (long term study for 26 weeks) 178

Figure 5.15 Animal survival rate during treatment with 50% ethanol extract of *Orthosiphon stamineus* and rosmarinic acid on nude mice (long term study for 26 weeks) 179

Figure 5.16 Chemoprevention effect of 50% ethanol extract of *Orthosiphon stamineus* towards HCT-116 tumor implanted ectopically in nude mice (2 weeks pre-treatment and 4 weeks after implanted the HCT-116 cells) 181

Figure 5.17 Tumor size of animals after treatment with different doses of 50% ethanol extract of *Orthosiphon stamineus* towards HCT-116 tumor implanted ectopically for 28 days 182

Figure 5.18 Body weight of animals treated with 50% ethanol extract of *Orthosiphon stamineus* compared with untreated. 183

Figure 5.19 Cross sections of tumor tissues stained with haematoxylin/eosin. The tumor cross sections were studied for the extent of apoptosis/necrosis 184

Figure 5.20 Effect of rosmarinic acid on colorectal tumors in nude mice 186

Figure 5.21 *In vivo* anti-tumor effect of rosmarinic acid on treated animals with different doses of rosmarinic acid for 28 days 187

Figure 5.22 Haematoxylin/eosin staining of the crosses sections of tumor tissues harvested from nude mice treated with rosmarinic acid for 28 days 189

Figure 5.23 Effect of 50% ethanol extract of *Orthosiphon stamineus*, rosmarinic acid and Imatinib® on vascular endothelial growth factor, basic-fibroblast growth factor and granulocyte macrophage colony stimulating factor concentration in human colorectal tumor tissue 192

Figure 5.24 Effect of 50% ethanol extract of *Orthosiphon stamineus* and rosmarinic acid on concentration of interleukin-7, transforming growth factor and nerve growth factor in human colorectal tumor tissue lysates 194
**Figure 5.25** Mean concentrations of IFN-α, IFN-β and EGF protein in the tumor tissue samples obtained from nude mice treated with varying doses of EOS, RA and Imatinib® for 28 day

**Figure 5.26** *In-vivo* effect of 50% ethanol extract of Orthosiphon stamineus at doses of 100, 200 and 400 mg/kg, rosmarinic acid and Imatinib® at doses of 30 mg/kg in level of interferon gamma, tumour necrosis alpha, tumour necrosis beta and interleukin-2 growth factor pathways in tissue lysates

**Figure 5.27** *In-vivo* effect of 50% ethanol extract of Orthosiphon stamineus at doses of 100, 200 and 400 mg/kg, rosmarinic acid and Imatinib® at 30 mg/kg in gene expression levels of HIF-α, KDR, WNT and COX2 in tissue lysates.

**Figure 5.28** Three dimension quantitative structure activity relationship and comparative molecular field analysis contour maps

**Figure 5.29** *In silico* ligand and cyclooxygenase interaction profile

**Figure 5.30** The favorable binding position of rosmarinic acid and Imatinib® with lowest binding free energy of epidermal growth factor as analyzed by molecular docking study

**Figure 5.31** *In silico* ligand and basic fibroblast growth factor interaction profile. (A) Surface visualization of proteins; (B) active site residues interaction of protein and hydrophobic interaction showed in green region

**Figure 5.32** *In silico* ligand and vascular endothelial growth factor interaction profile

**Figure 5.33** Predicted binding mode of rosmarinic acid and Imatinib® with granulocyte macrophage colony stimulating factor. (A) Surface visualization of proteins; (B) active site residues interaction of protein and hydrophobic interaction showed in green region

**Figure 5.34** *In silico* ligand and interferon alpha 2 interaction profile. (A); surface visualization of proteins, (B); active site residues interaction of protein and hydrophobic interaction showed in green region
Figure 5.35  In silico Ligand and interleukin-2 interaction profile. (A) Surface visualization of proteins; (B) active site residues interaction of protein and hydrophobic interaction showed in green region 216

Figure 5.36  In silico ligand and tumor necroses factors alpha interaction profile. (A) Surface visualization of proteins; (B) active site residues interaction of protein and hydrophobic interaction showed in green region 218

Figure 5.37  In silico ligand and tumor necrosis factors beta interaction profile 220

Figure 5.38  In silico ligand and vascular endothelial growth factor R1 interaction profile 222

Figure 5.39  Proposed signaling pathways underlying the effect of Orthosiphon stamineus in suppression of angiogenesis and human colorectal cancer 231

Figure 5.40  Summary of anti tumor activity of 50% ethanol extract of Orthosiphon stamineus and rosmarinic acid toward of colorectal cancer 232
LIST OF ABBREVIATIONS

5-FU 5-fluorouracil
ACS American Cancer Society
Ala Alanine
AlCl3 Aluminium chloride
Ang-2 Angiopoietin 2
APC Adenomatous Polyposis Coli
Are Arginine
Asp Asparagine
BA Beutilinic acid
BFGF Basic fibroblast growth factor
BM Basement membrane
Cap Capecitabine
CCD charge-coupled device
CIMP CpG island methylator phenotype
CIN Chromosomal instability
CoMFA Comparative molecular field analysis
COX Cyclooxygenases
CRCs Colorectal cancers
Cys Cysteine
DAPI 4',6-diamidino-2-phenylindole
Del-1 Developmental endothelial locus-1
DNA Deoxyribose nucleic acid
DEPC dissolved in diethyl pyrocarbonate
DQSAR Dimension quantitative structure activity relationship
DMSO Dimethyl sulfoxide
EC Endothelial cells
ECGS Endothelial cell growth supplements
ECM Endothelial cell medium
ELISA Enzyme-linked immunosorbutant assay
FDA Food and drug administration
FGF  Fibroblast growth factor
FTIR  Fourier transform infrared spectrometry
G-CSF  Granulocyte colony-stimulating factor
Glu  Glutamic acid
Gln  Glutamine
Gly  Glycine
H  Hour
HGF  Hepatocyte growth factor
HIF  Hypoxia-inducible factors
HIV  Human immunodeficiency virus
HIV-1  HIV-1 Human immunodeficiency virus type 1
His  Histidine
HMWK  High molecular weight kininigen
HPLC  High performance liquid chromatography
HUVEC  Human umbilical vein endothelial cells
IL1R1  Interleukin-1 receptor type 1
IL-1  Interleukin-1
IL-2  Interleukin-2
IL-7  Interleukin-7
Ile  Isoleucine
IP  Intraperitoneal injection
IP-10  Interferon-inducible protein-10
JEV  Japanese encephalitis virus
Leu  Leucine
LPS  Lipopolysaccharide
Lys  Lysine
MAPK  Mitogen-activated protein kinases
MCTS  Multicellular tumor spheroids
Met  Methionine
MMPs  Matrix metalloproteinase
MSI  Microsatellite instability
MTT  3-(4, 5-dimethylthiazol-2-yl)- 2,5 diphenyltetrazolium bromide
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>MTS</td>
<td>3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>NSAIDs</td>
<td>Nonsteroidal anti-inflammatory drugs</td>
</tr>
<tr>
<td>OX</td>
<td>Oxaliplatin</td>
</tr>
<tr>
<td>OA</td>
<td>Orthosiphon A</td>
</tr>
<tr>
<td>OLA</td>
<td>Oleanolic acid</td>
</tr>
<tr>
<td>PAs</td>
<td>Plasminogen activators</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>PBS-T</td>
<td>PBS with 0.1% tween 20</td>
</tr>
<tr>
<td>PC</td>
<td>Pericytes</td>
</tr>
<tr>
<td>PDB</td>
<td>Protein Data Bank</td>
</tr>
<tr>
<td>PD-ECGF</td>
<td>Platelet-derived endothelial cell growth factor</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet-derived growth factor</td>
</tr>
<tr>
<td>PDGFR</td>
<td>Platelet-derived growth factor receptors</td>
</tr>
<tr>
<td>PEDF</td>
<td>Pigment epithelium-derived factor</td>
</tr>
<tr>
<td>Pg</td>
<td>Picogram</td>
</tr>
<tr>
<td>PGE2</td>
<td>Prostaglandin E2</td>
</tr>
<tr>
<td>PLGF</td>
<td>Placental growth factor</td>
</tr>
<tr>
<td>Phe</td>
<td>Phenylalanine</td>
</tr>
<tr>
<td>PMA</td>
<td>Phorbol myristate acetate</td>
</tr>
<tr>
<td>Pro</td>
<td>Proline</td>
</tr>
<tr>
<td>P/S</td>
<td>Penicillin/streptomycin</td>
</tr>
<tr>
<td>RA</td>
<td>Rosmarinic acid</td>
</tr>
<tr>
<td>Ras-GAP</td>
<td>Guanosine triphosphatase-activating protein</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute medium</td>
</tr>
<tr>
<td>RT_PCR</td>
<td>Real-Time Polymerase Chain Reaction</td>
</tr>
<tr>
<td>Ser</td>
<td>Serine</td>
</tr>
<tr>
<td>SPARC</td>
<td>Secreted protein acidic and rich</td>
</tr>
<tr>
<td>sVEGFR1</td>
<td>Soluble VEGF receptor-1</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>TAMs</td>
<td>Tumor-associated macrophages</td>
</tr>
<tr>
<td>TGF</td>
<td>Transforming growth factor</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor beta</td>
</tr>
<tr>
<td>Thr</td>
<td>Threonine</td>
</tr>
<tr>
<td>TMF</td>
<td>3'-hydroxy-5,6,7,4'-tetramethoxyflavone</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor beta</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor alpha</td>
</tr>
<tr>
<td>Tris</td>
<td>Tris (hydroxymethyl) aminomethane</td>
</tr>
<tr>
<td>Trp</td>
<td>Tryptophan</td>
</tr>
<tr>
<td>TXA2</td>
<td>Thromboxane A2</td>
</tr>
<tr>
<td>Tyr</td>
<td>Tyrosine</td>
</tr>
<tr>
<td>UV-vis</td>
<td>Ultra-violet visible</td>
</tr>
<tr>
<td>Val</td>
<td>Valine</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>VEGFR-1,2</td>
<td>Vascular endothelial cell receptors -1,2</td>
</tr>
<tr>
<td>VEGI</td>
<td>Vascular endothelial growth inhibitor</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
</tr>
<tr>
<td>WNT</td>
<td>Wingless-type MMTV integration site family</td>
</tr>
</tbody>
</table>
## LIST OF SYMBOLS

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Meaning</th>
</tr>
</thead>
<tbody>
<tr>
<td>Å</td>
<td>Angstrom</td>
</tr>
<tr>
<td>γ</td>
<td>Gamma</td>
</tr>
<tr>
<td>β</td>
<td>Beta</td>
</tr>
<tr>
<td>α</td>
<td>Alpha</td>
</tr>
<tr>
<td>&lt;</td>
<td>Less than</td>
</tr>
<tr>
<td>&gt;</td>
<td>More than</td>
</tr>
<tr>
<td>μ</td>
<td>Micro</td>
</tr>
<tr>
<td>C</td>
<td>Celsius</td>
</tr>
<tr>
<td>%</td>
<td>Percent</td>
</tr>
</tbody>
</table>
PENYIASATAN MEKANISME MOLEKUL YANG MENDASARI AKTIVITI ANTI-TUMOR DAN ANTI-ANGIOGENIK ORTHOSIPHON STAMINEUS TERHADAP KANSER USUS

ABSTRAK

Teh Orthosiphon stamineus Benth. (Lamiaceae) digunakan secara meluas dalam perubatan tradisional. Kajian terbaru menunjukkan bahawa 50% ekstrak ethanolik daripada Orthosiphon stamineus (EOS) dan sebatian aktif, asid rosmarinik (RA), memaparkan kesan-kesan anti-angiogenik, anti-radang dan anti-tumor yang ketara dalam pelbagai model eksperimen. Walau bagaimanapun, mekanisme yang mendasari sifat-sifat ini tidak dinilai dengan sepenuhnya. Kajian yang dijalankan ini bertujuan untuk menilaikan lagi mekanisme molekul yang mendasari anti-tumor dan anti-angiogenik. Dalam model eksperimen penghijrahan, perkembangan dan pembentukan tiub, cerakin kedua-dua EOS dan RA aktif menyebabkan perencatan ketara terhadap fungsi sel endothelial manusia (HUVECs) yang penting bagi merangsang proses angiogenesis. Dalam kedua-dua kajian in vitro dan in vivo, penindasan besar neovaskularisasi dalam model aorta tikus, CAM dan plug matrigel juga diperhatikan. Kajian cerakin multipleks menunjukkan pengurangan faktor pertumbuhan utama bagi lata pro-angiogenik dan perkembangan tumor iaitu faktor pertumbuhan endothelial vaskular (VEGF), faktor pertumbuhan fibroblast asas (b-FGF), transformasi faktor pertumbuhan transformasi (TGF-α), faktor nekrosis tumor (TNF-β) dan interleukin-1, 2, 7. Induksi terhadap agen anti-tumor iaitu interferon (IFN-α, β) dan faktor perangsang koloni makrofaj granulosit (GM-CSF) secara in vitro dan in vivo juga diperhatikan. EOS dan RA juga menyebabkan penurunan yang ketara perantara-perantara radang pro-angiogenik, enzim cycloxygenase (COX), TNF-α, IL-1 dan tahap nitrik oksida (NO) yang penting untuk tumorigenesis. Lebih-
lebih lagi, EOS dan RA telah menghalang expresi gen secara signifikan dalam tisu tumor usus termasuk HIF-α, WNT, KDR dan COX2. Tambahan pula, EOS menghalang mercu tanda metastasis secara meluas iaitu pencerobohan dan pengagregatan tumor yang dibuktikan secara tomografi pendarfluor molekul (FMT) melalui pengimejan in vivo dan analisis histopatologi. Penemuan ini bertepatan dengan kesan rencatan pada tumor penggalak faktor angiogenesis dalam model mencit xenografit. Simulasi interaksi molekular dalam silico terhadap penanda biologi aktif EOS mengesahkan pertalian pengikat baik dan kesan modulatori kukuh terhadap faktor angiogenik dan tumorigenik. Ia mungkin disebabkan oleh kandungan fenolik dan flavonoid yang tinggi dalam EOS turut mengenakan kesan anti-tumor yang signifikan melalui modulasi pro-radang dan pengantara-pengantara angiogenesis melalui kesan hapse-sama radikal bebas yang ketara. Kesimpulannya, hasil keseluruhan menyokong dan mengesahkan bahawa sifat-sifat anti-angiogenik dan anti-tumor EOS dan RA dibuktikan melalui kesan pemodulasian signifikan terhadap faktor-faktor utama pertumbuhan dan perantara.
INVESTIGATION OF MOLECULAR MECHANISMS UNDERLYING THE ANTI-TUMOR AND ANTI-ANGIOGENIC ACTIVITIES OF ORTHOSIPHON STAMINEUS TOWARDS COLORECTAL CANCER

ABSTRACT

Orthosiphon stamineus Benth. (Lamiaceae) tea is widely consumed traditionally for its vast medicinal value. Recent studies revealed that 50% ethanolic extract of Orthosiphon stamineus (EOS) and its active compound, rosmarinic acid (RA), displayed significant anti-angiogenic, anti-inflammatory and anti-tumor effects in various experimental models. However, the mechanisms underlying these properties have not been fully evaluated. The present work aims to further evaluate the molecular mechanisms underlying its anti-tumour and anti-angiogenic properties.

In migration, proliferation and tube formation assay, both EOS and its active RA caused significant inhibition of human endothelial cell (HUVECs) functions crucial for promotion of angiogenesis. Both in vitro and in vivo studies revealed significant suppression of neovascularisation in rat aortic ring, CAM and matrigel plug. Multiplex array studies showed reduction of key growth factors for pro-angiogenic cascade and tumor development i.e. Vascular endothelial growth factor (VEGF), basic fibroblast growth factor (b-FGF), transforming growth factor alpha (TGF-α), tumor necrosis factor (TNF-β) and interleukin-1, 2, 7. Induction of anti-tumor agents i.e. interferon (IFN-α, β) and granulocyte macrophage colony stimulating factor (GM-CSF) both in vitro and in vivo was also noted. In addition, EOS and RA also caused a marked reduction of pro-angiogenic inflammatory mediators, cyclooxygenase (COX) enzyme, TNF-α, IL-1 and nitric oxide (NO) level vital for tumorigenesis. Moreover, EOS and RA significantly inhibited the genes expression
in colorectal tumor tissue including $HIF-\alpha$, $WNT$, $KDR$ and $COX2$. Furthermore, EOS extensively inhibited invasion and tumor aggregation evidenced by fluorescent molecular tomography (FMT) in vivo imaging and histopathological analysis. These findings coincide with its inhibitory effects on tumor promoting angiogenesis factors in nude mice xenograft. In silico molecular interaction simulations on EOS active biomarkers confirms good binding affinity and strong modulatory effect towards the angiogenic and tumorigenesis factors. It is likely the high phenolic and flavonoids content in EOS also exert a significant anti-tumor effect via modulating pro-inflammatory and angiogenesis mediators through their significant free radicals scavenging effect. In conclusion, overall results strongly substantiates EOS and RA anti-angiogenic and anti-tumor properties evidenced by their significant modulatory effect on key associated growth-factors and mediators.
CHAPTER ONE

INTRODUCTION AND LITERATURE REVIEW

1.1 Cancer

Cancer is a malignant disease, which affects different parts of the body resulting in pathologic changes, genetic and epigenetic disorders factors which may act together or in sequence to cause cancer. Cancer occurs when groups of normal cells grow abnormally fast, losing control of cell division or with slow cell death (apoptosis) consequently, transformed from normal cells into malignant cells (Vanhoecke et al., 2005; Giansanti et al., 2011). Cancer is not a single disease but syndrome which comprises of group of multiple diseases. There are more than 100 various types of cancer that are named according to the sites where a cancerous growth originates.

The cancer cells are characterized by their invasion of the nearby tissue and spreading through the blood stream and lymphatic system to other organs or tissue (metastasis) (Zhong and Bowen, 2006). Cancer that initiates in the organs such as breast is called breast cancer and cancer that starts in the lung is called lung cancer and so on.

Metastasis is the final step of cancer and the major cause of death resulting from cancer. Bone metastases are the most common cause of cancer pain. Usually, under normal conditions, cells grow and divided automatically in order to replace the damaged cells or produce new cells. At times this orderly process goes wrong probably due to problem with the genetic material (DNA). Mutations are generally
caused by internal or external cellular damage and thereby the normal cells are converted into malignant cells. To date, the resistance of cancer cells towards cancer therapy has recognized one of the major problems in treating the disease hence much research been made towards the understanding of cancer biology and treatment using advanced protocols like radiotherapy and chemotherapy. There are two types of tumors, classified based on their growth and spread. Tumors that do not spread to other parts of the body and are incapable of recurrence are referred to as benign tumors. However, tumors are called malignant when a tumor cell invades the surrounding tissues and spreads to other parts of the body (Hanahan and Weinberg, 2011).

1.1.1 Cancer epidemiology

Cancer is a major public health problem and the second killer disease after cardiovascular diseases which cause of illness and mortality worldwide. In 2002, an estimated 10.9 million new cases of cancer incidence and mortality were reported globally with 6.7 million deaths (Parkin et al., 2005). In 2013, Bray reported that about 29 million people were living with cancer (Bray et al., 2013) and there were an estimated 7.6 million deaths (13% of all deaths) in 2008 (Gutschner and Diederichs, 2012).

World Health Organization (WHO) reported that cancer incidence and cancer-related mortality has increased remarkably, with 14 million new cases and 8.2 million deaths in 2012. Among all the cancers, the five most commonly diagnosed cancers in men were lung cancer, followed by prostate, colorectal, stomach, and liver cancers. While for women, the five most commonly diagnosed cancers were breast, followed by colorectal, lung, cervix, and stomach cancers. In general, the most
frequent source of cancer death was lung cancer with an estimated mortality rate of 1.59 million cases followed by liver cancer with 745,000 cases, stomach cancer with 723,000 cases, colorectal cancer with 694,000 cases, breast cancer with 521,000 cases and oesophageal cancer with 400,000 cases. Incidence, morbidity and mortality of cancer is expected to rise by more than 70% in the next two decades, which means that the incidence of cancer cases will increased from 14.1 million in 2012 to 22 million within the next two decades (Organization, 2014).

Incident rate of cancer in more developed areas was highest compared with the least developed areas. On the other hand, the mortality cases were much higher in less developed region, because of the economic costs, lack of diagnosis, late detection and treatment (Torre et al., 2015). Incidences of cancer have been on rise in both developed and developing countries.

Cancer is the main cause of death among adults aged 40 to 79 years and is the first or second leading cause of death in every age group among women (Boffetta and Parkin, 1994; Siegel et al., 2015). In 2015, it is estimated that 1,658,370 new cancer cases will be diagnosed and 589,430 cancer deaths in the USA (Siegel et al., 2015). However, overall cancer death rates decreased in 2011 with 168.7 per 100,000 populations from 215.1 (per 100,000 populations) in 1991. The 22% decrease in cancer deaths from 1991 to 2011 was a result of early detection, decrease in smoking, new drugs and treatment. Advances in cancer prevention approaches have also been introduced (Siegel et al., 2015).

According to the third edition of International Classification of Diseases for Oncology (ICD-O), cancer can be divided into five categories based on the primary and initial tumor, as bellow;
a) Carcinoma starts in tissue that covers the internal organs or epithelial cell; it can be grouped into different subtypes such as, adenocarcinoma, squamous cell carcinoma, transitional carcinoma and basal cell carcinoma.

b) Lymphoma and myeloma that start in the cells of the immune system

c) Leukemia progresses in blood formation tissue like bone marrow.

d) Central nervous system cancers are cancers that originate in the tissues of the spinal cord and brain.

e) Sarcoma is initiated in cartilage, bone, blood vessels, fat, connective tissue and muscle (Fritz et al., 2000)

1.1.2 Cancer in Malaysia

In 2008, the WHO’s Globocan reported that cancer is one of the leading causes of death in Malaysia with an estimate of 30,000 annual cases. Based on the latest health facts 2013 reported by the Ministry of Health (MoH) of Malaysia, the incidence of cancer in Malaysia increased from 32,000 new cases in 2008 to 37,400 in 2012. This number may be expected to increase to 56,932 by 2025, if no proper prevention strategy or good lifestyle.

Breast cancer is the most common cancer among Malaysian followed by colorectal and lung cancer, with one in 19 Malaysians developing breast cancer, one in 33 developing colorectal cancer and one in 40 developing lung cancers. For men, lung cancer is the most frequent cancer followed by cancer of nasopharynx, colon, leukaemia, rectum and prostate. In women, the most frequent cancers are that of the breast followed by cervix, colon, ovary, leukaemia and lung (Lim et al., 2002).
1.1.3 Development and progression of cancer

To date, the causes of cancer are not completely understood. Cancer originates from single a mutated cell which starts to divides in uncontrolled manner exceeding normal cells, these aggressively proliferating cells can invade and destroy neighboring tissues and may spread to other parts of the body (metastasis), unlike normal cells which are self-regulated, restricted growth potential and on ability of metastasis.

The mutation may occur due to random genetic damage by endogenous factors, such as intrinsic chemicals of DNA bases, the abnormality or error in DNA replication which can be attributed to carcinogens such as infectious agent, chemicals, radiation, or free redials during metabolism (Ames, 1989; Hall and Angele, 1999; Bertram, 2000). The mutated cells grow fast until it form colony, these transformed cells divide more and more via altering the environment in a manner that favors the growth mutated cells over normal cells.

The first stage of transformed cells is a group of highly divided cell with normal appearance (hyperplasia). More transformation to hyperplastic leads to abnormal looking cells (dysplasia). The next stage of the transformation of mutated cells cancerous may take between 5-20 years for the transition of benign carcinogenic phase to the fully developed malignant stage where the neoplasia can be detected clinically.

The last stage is termed as ‘progression’, where further genetically changes take place resulting in the increase of proliferation and metastasis (Marshall, 1991; Weinberg, 1996; Compagni and Christofori, 2000; Kintzios and Barberaki, 2004). Genetic change (mutations) and external factors react together in sequence and target
two groups of normal regulatory genes (proto-oncogenes and tumor suppressor gene), which transfer to the cancer causing gene. Proto-oncogenes are genes encode proteins that are found in every cell, which stimulate cell proliferation, differentiation and development (Sherr, 2004). This normally helps in cells homeostasis. The genes that activated by mutation are called oncogenes, which can be produced by six major factors: growth factors, transcription factor, growth factors receptors, chromatin remodelers, apoptosis regulation, and signal transducers factors (Croce, 2008) (Table 1.1). In contrast, the gene of which the inhibition is by mutation is called the tumor-suppressor gene (Table 1.2). Oncogenes accelerate the tumor cells when activated by mutation. The normal cell process is a balance between tumor-suppressor genes and oncogenes. Tumour-suppressor genes are normal genes which inhibit tumor formation by controlling the cell division, apoptosis and repair DNA mistakes that occur during DNA replication. They act as the “brakes” for the cell cycle. Tumor-suppressor genes mutations lead to a growth of cancer by inactivating that inhibitory function of these genes. In addition, environment and lifestyle, including tobacco, obesity, infectious agents, alcohol, hyperglycemia, food carcinogens, sunlight, stress, and environmental pollutants are major causes of cancer which includes about 90-95% of cases and the remaining 5-10% are due to genetic defects (Anand et al., 2008).
### Table 1.1: List of oncogenes.

<table>
<thead>
<tr>
<th>Oncogenes</th>
<th>Activation/function</th>
<th>Cancer</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Abl</strong></td>
<td>Promote cell growth through tyrosine kinase activity</td>
<td>Chronic myelogenous leukemia (Croce, 2008)</td>
</tr>
<tr>
<td><strong>Myb</strong></td>
<td>Transcription factor</td>
<td>Colon carcinoma and leukemia</td>
</tr>
<tr>
<td><strong>Trk</strong></td>
<td>Receptor tyrosine kinase</td>
<td>Colon and thyroid carcinomas</td>
</tr>
<tr>
<td><strong>C-myc</strong></td>
<td>A transcription factor that promotes cell proliferation and DNA synthesis</td>
<td>Leukemia; breast, stomach, lung, cervical, and colon carcinomas; neuroblastomas and glioblastomas (Weber, 1987)</td>
</tr>
<tr>
<td><strong>HER2/neu</strong></td>
<td>Over-expression of signalling kinase due to gene amplification</td>
<td>Breast and cervical carcinomas (Weber, 1987)</td>
</tr>
<tr>
<td><strong>Af4 / hrx</strong></td>
<td>Fusion affects the hrx transcription factor / methyltransferase</td>
<td>Acute leukemias</td>
</tr>
<tr>
<td><strong>Akt-2</strong></td>
<td>Encodes a protein-serine / threonine kinase</td>
<td>Ovarian cancer</td>
</tr>
<tr>
<td><strong>KRAS</strong></td>
<td>Promoting cell survival and apoptosis suppression</td>
<td>Colorectal carcinomas and lung cancer (Croce, 2008)</td>
</tr>
<tr>
<td><strong>Alk/npm</strong></td>
<td>Translocation creates a fusion protein with nuclear phosphmin (npm)</td>
<td>Large cell lymphomas</td>
</tr>
<tr>
<td><strong>Aml1</strong></td>
<td>Encodes a transcription factor</td>
<td>Acute myeloid leukemia</td>
</tr>
<tr>
<td><strong>Aml1/mtg8</strong></td>
<td>A new fusion protein created by the translocation</td>
<td>Acute leukemias</td>
</tr>
<tr>
<td><strong>Axl</strong></td>
<td>Encodes a receptor tyrosine kinase</td>
<td>Hematopoietic cancers</td>
</tr>
<tr>
<td><strong>Bcl 2, 3, 6</strong></td>
<td>Block apoptosis (programmed cell death)</td>
<td>B-cell lymphomas and leukemias</td>
</tr>
<tr>
<td><strong>Dbl</strong></td>
<td>Guanine nucleotide exchange factor</td>
<td>Diffuse B-cell lymphoma</td>
</tr>
</tbody>
</table>
Table 1.2: Some of tumor suppressor genes.

<table>
<thead>
<tr>
<th>Tumor suppressor genes</th>
<th>Activation/function</th>
<th>Cancer</th>
</tr>
</thead>
<tbody>
<tr>
<td>APC (denomatous polyposis coli)</td>
<td>Signaling through adhesion molecules to the nucleus</td>
<td>Colorectal carcinomas (Santos, 2009)</td>
</tr>
<tr>
<td>BRCA1, BRCA2</td>
<td>DNA Damage Repair</td>
<td>breast cancers; ovarian cancers (Yoshida, 2004)</td>
</tr>
<tr>
<td>DCC</td>
<td>Netrin-1 receptor. Regulation of cell proliferation and apoptosis of intestinal epithelium.</td>
<td>Colorectal carcinomas</td>
</tr>
<tr>
<td>DPC4 (SMAD4)</td>
<td>Transcriptional factor involved in development; Implicated in metastasis and tumor invasiveness.</td>
<td>Colorectal tumors, pancreatic neoplasia</td>
</tr>
<tr>
<td>MADR2/JV18 (SMAD2)</td>
<td>Mediates signaling from growth factor receptors. Assists in transport of SMAD4 into nucleus.</td>
<td>Colorectal cancer</td>
</tr>
<tr>
<td>MLH1&amp;MSH2</td>
<td>DNA single-nucleotide mismatch-repair defect permitting the accumulation of oncogenic mutations and tumor-suppressor loss</td>
<td>Colorectal cancer (Sarrió, 2003)</td>
</tr>
<tr>
<td>NF1</td>
<td>RAS GTPase activating protein (RAS-GAP)</td>
<td>Neurofibromatosis type 1</td>
</tr>
<tr>
<td>p53</td>
<td>Cell cycle regulation, apoptosis</td>
<td>Bladder, breast, colorectal, esophageal, liver, lung, prostate, and ovarian carcinomas; brain tumors, sarcomas, lymphomas, and leukemias (Santos, 2009)</td>
</tr>
<tr>
<td>RB</td>
<td>Binds to, and inhibits, the E2F transcription factor. Halts cell cycle progression</td>
<td>Retinoblastoma, sarcomas; bladder, breast, esophageal, prostate, and lung carcinomas</td>
</tr>
<tr>
<td>TGFBR2</td>
<td>Receptor responsible for signaling pathways mediating growth arrest and apoptosis</td>
<td>Colorectal and ovarian cancer</td>
</tr>
</tbody>
</table>
1.1.4 **Cancer pathology and genetic events of tumorigenesis**

In 2000, Hanahan and Weinberg proposed 10 main cellular procedures based on transformation and development of normal cells to establish malignant neoplastic tissue (Hanahan and Weinberg; 2000; Negrini et al., 2010; Hanahan and Weinberg, 2011). These basic hallmarks capabilities acquired during tumor development are:

1- **Sustaining proliferatives signalling.** This is one of the characterise of cancer cells, which can be acquired by various pathways and is defined by the cells ability to grow constantly without external signals and produce their own growth factors, and the corresponding receptor molecules by the autocrine proliferative stimulation. In addition, they stimulate normal cells and tumor-associated cells by producing paracrine signals, in order to support the cancer cells by forming different growth factors (Gutschner and Diederichs, 2012).

2- **Inducing angiogenesis,** which activate quiescent endothelial cells in order to grow new blood cells. The growth and metastatic of tumors require the formation of new blood vessels. Therefore, during tumor development and progression the “angiogenic switch” is activated and maintained to support the neoplastic growth by suppling nutrient and oxygen throw the new blood vessels. Tumor cells activate the “angiogenic switch” by countervailing inhibitors, thus inducing and sustaining the angiogenesis substances will occur (Hanahan and Folkman, 1996).

3- **Evading growth suppressors (antigrowth).**

4- **Resisting of programmed cell death (apoptosis).**

5- **Limitless replication potential.** Cancer cells know how to renew themselves continuously.
6- Enhancement tissue metastasis and invasion. Benign tumors are not harmful if they do not spread to other parts of the body. While the malignant tumor which invade surrounding tissues and spread to the other parts of the body (Hanahan and Weinberg, 2011).

7- Genomic instability and mutation are characteristics of most of tumors which generate random mutations during DNA repair and drive tumor development (Negrini et al., 2010; Hanahan and Weinberg, 2011).

8- Evading immune surveillance, in particular by T and B lymphocytes, macrophages, and natural killer cells.

9- Tumor-promoting inflammation.

10- Deregulating cellular energetic (Figure 1.1).

**Figure 1.1:** Ten hallmarks of cancer acquired during cancer progression. Adapted from (Hanahan and Weinberg, 2011).
1.2 Colorectal cancer

Colorectal cancer (CRC) is a multistep process of epithelial cells transformation into malignant cells, which is caused by the sequential order of genetic, growth factors and epigenetic mutations (Pancione et al., 2012). Several researchers have been reported that development and rise of colorectal tumors associated with specific mutation including microsatellite instability (MSI), adenomatous polyposis coli (APC) gene, stabilization and translocation of β-catenin, chromosomal instability (CIN), Kirsten-rat sarcoma virus (KRAS), TP53, loss of the 18q21 gene cyclooxygenase-2 (COX-2), and mutations in transforming growth factor β II receptor (TGFβR2) (Markowitz and Bertagnolli, 2009; Kanthan et al., 2012) (Figure 1.2).

![Figure 1.2: Genetic alterations frequently associated with CRC progression Adapted from (Markowitz and Bertagnolli, 2009)](image)

Previous studies were illustrated the role of the APC suppressor gene in the early stages of colorectal carcinoma. Inactivation of APC gene is associated with accumulation of intracellular β-catenin which plays a central role in cell adhesion
and acts as a transcription factor of the Wnt signaling pathway (Yang et al., 2006). Stimulation of Wnt/β-catenin signaling pathway leads to activation of T-cell factor/lymphoid enhancing factor-1 (TCF/LEF1) transcription factors and subsequently to the expression of several target genes including COX-2, cyclin D1 and c-Myc that are concerned in tumorigenesis of colorectal carcinoma and several other cancers (MacDonald et al., 2009). In addition, oncogenic mutations in K-ras were found to play essential role in tumorigenesis of colorectal carcinoma and their presence indicates poor prognosis (Conlin et al., 2005). Besides the hereditary APC alteration and other acquired genetic changes there are other associated genetics, enzymes and antigenic that have been found to play a central role in the adenoma-carcinoma sequence. Various carcinogenic factors have been described which also contribute in the adenoma and carcinoma formation such as familial history of colonic neoplasia, acromegaly, ulcerative colitis, drinking, smoking and consumption of red meat.

1.2.1 Epidemiology of colorectal cancer

Colorectal cancer incidence in man is higher than women with an overall sex ratio of the age standard rate, of 1.4:1 (Ferlay et al., 2010). In man, it is the third most commonly diagnosed malignant neoplasm worldwide (663,000 cases, 10.0% of the total) (Siegel et al., 2015; Scholefield and Eng, 2014), and the second most common cancer in women (570,000 cases, 9.4% of the total), beside it is the third leading cause of cancer deaths, accounting for 600,000 deaths each year (Roper and Hung, 2013). In the United States, colorectal cancer is the third leading cause of cancer deaths (9% of estimated cancer deaths in both men and women in 2012) (Scholefield and Eng, 2014).
Globally, CRC is a burden; the incidence rate is ten times higher in regions with the highest rate, such as Australia and Canada than in regions with the lowest rates, such as India, while the mortality rate is five times higher in regions with the highest rates than it is in regions with the lowest rates (Scholefield and Eng, 2014). The American Cancer Society (ACS) estimates 132,700 new cases of colorectal cancer in women and men and that 49,700 will die as a result of it in the United States in 2015 (Smith et al., 2015), compared to the incidence 148,300 new cases and 56,600 deaths in 2002. CRC is the second most common malignancy in Malaysia. Life styles, heredity, diet and micronutrient malnutrition are few putative etiology in CRC. The incidence of CRC is higher in Malaysia compared to incidence in Indian subcontinent possibly due to low intake of dietary insoluble fibre, higher animal diet and red meat content of food. The epidemiological data on CRC in Malaysia are fragmentary and insufficient. Moreover there is no specific control or preventive measure taken by ministry of health to detect CRC early in Malaysia. Colonoscopy and Fecal occult blood test are rarely advocate for early detection. Therefore it is very rare to find CRC patients in early stages. The stage distribution of CRC patients in Malaysia is shifted to right with majority being presented in late stages (III & IV). In few earlier studies stage per stage survival of CRC are lower compared to western counterparts (Biswal et al., 2002).

1.2.2 Chemotherapeutics of colorectal cancer

Chemotherapy is a type of cancer treatment that uses one or more of medicinal drug to destroy the cancer cells. Up to know, no curative therapy is available for most types of cancer including colon cancer. The available treatments are used to prolong the life span of cancer patients. Previously, cytotoxic agents such as 5-
fluorouracil (5-FU), oxaliplatin (OX) and capecitabine (Cap) were used to treat colorectal cancer. The various combinations of these agents were extensively studied in phase II and phase III clinical trials such as IFL (irinotecan, 5-FU and LV), FOLFOX (5-FU, OX and LV), FOLFIRI (5-FU, LV and irinotecan) and CapOx (capecitabine/oxaliplatin). All of these combinations showed improvement in the therapeutic outcome than with mono-therapy (Cercek and Saltz, 2008). After the development of the monoclonal antibodies bevacizumab (anti-VEGF), panitumumab (human anti-EGFR) and Cetuximab (chimeric human-mouse anti-EGFR), several combinations of these agents, with the cytotoxic drugs have been studied in phase II and phase III clinical trials. In general, the results show that the combination of anti-angiogenic factors either anti-VEGF or anti-EGFR antibodies with cytotoxic agents resulted in increased therapeutic outcomes than each individual therapy (Cercek and Saltz, 2008).

1.3 Tumor angiogenesis

1.3.1 Physiologic and pathologic angiogenesis

Angiogenesis or neovascularization is the multistep physiological process of generating new blood vessels from pre-existing vasculature. It is an essential requirement for normal physiological conditions such as the process during the development of the organs in new-borns, during wound healing, vascular system in embryonic development of the placenta during pregnancy and for the reproductive function of adults (Tonnesen et al., 2000; Auerbach et al., 2003; Sagar et al., 2006). Nevertheless, angiogenesis play fundamental role in numerous pathologic disorders of many diseases, such as rheumatoid arthritis, psoriasis, cardiovascular, blindness, obesity, ischemia, cornel neovascularisation, diabetic retinopathy, tumor growth,
tumor propagation, metastasis formation and inflammatory diseases (Folkman, 1971; Folkman, 1995; Auerbach et al., 2003; Hanyu et al., 2009; Prager et al., 2011). In 2004, Hoeben and others reported angiogenesis in adults as being tightly controlled by a physiological balance between the stimulatory (pro-angiogenic) and inhibitory (anti-angiogenic) signals.

Several studies reported that angiogenesis may be an excellent therapeutic target for the treatment of tumor and other angiogenesis dependent disease (Ferrara, 2002; Carmeliet, 2005). Therefore, insufficiency of angiogenesis may occur in reduction the tumor growth, invasion and metastasis (Folkman, 1974; Chia et al., 2010).

Therapeutically, targeting angiogenesis has been widely regarded as an attractive approach for cancer therapy (Hoeben et al., 2004). For that reason, determining the pro-angiogenic or anti-angiogenic effects of the molecules currently used in cancer treatment is crucial (Folkman, 1971; Carmeliet, 2005).

1.3.2 Angiogenesis cascade events

Angiogenesis depends on the interactions of the endothelial cell with the extracellular matrix compounds. There are multistep processes to develop effective angiogenesis. Regulated steps, which involve the formation of blood vessels, are initiation by biological signals, which lead to the activation of the receptors on the endothelial cell by the angiogenic growth factors (Gupta and Qin, 2003). The activated cells, which cover the blood vessel walls, start to release proteases enzymes (Matrix Metalloproteinase such as MMP9) that cause pericytes to detach and degrade the extracellular matrix and basement membrane, which allows the underlying
endothelial cells to escape from the blood vessel walls. Following this, the front lines of endothelial cells migrate toward the angiogenic stimulus (Fischer et al., 2006). The migrating cells start proliferation to form solid sprouts that link to adjacent vessels using adhesion molecules called integrins.

The sprouts fuse with other sprouts to form loops. The new blood vessels formed are lined by vascular basal lamina. Finally, blood starts to flow through the new vessels (Eliceiri and Cheresh, 2001; Hoeben et al., 2004). Targeting any of these steps can inhibit the formation of new blood vessels, thus it could be striking approach for treating angiogenesis-related diseases, most notably cancer (Cardenas et al., 2011) (Figure 1.3).

**Figure 1.3:** Angiogenesis Cascade. (a): Blood vessels (b): Pericytes (PC) detach, blood vessels dilate before basement membrane (BM) and extracellular matrix (ESM) gets degraded (c) The underlying endothelial cells escape from the blood vessels wall to allows endothelial cells (EC) to migrate into perivascular space towards angiogenic stimuli, (d) after that, the endothelial cells proliferate, following each other, guided by pericytes, (e) endothelial cells adhere to each other and formed a lumen which is accompanied by basement membrane formation and attachment by pericytes. Finally, the blood vessels sprouting fuse with other sprouts to form loops which formation of new circulatory systems. Adapted from (Bergers & Benjamin 2003).
1.3.3 Regulation of angiogenesis

Angiogenesis is a tightly regulated process that is controlled by balance between pro-angiogenic (stimulators) and anti-angiogenic (inhibitors) molecules. In their review article, Liekens and his co-workers narrate the angiogenesis process in three steps: the first step is the degradation of the extracellular matrix, the second is the regulation of angiogenic modulators, including the growth factors and the cytokines and enzymes, and the third level is the cell-cell and the cell-matrix interactions (Liekens et al., 2001).

The first step in the formation of new vessels is the proteolytic breakdown of the basement membrane underlying endothelial cells, in order for them to migrate and invade the stroma of surrounding tissues. This process requires the activity of the plasminogen activators (PAs) and the matrix metalloproteinase MMPs (Mignatti and Rifkin, 1996). The activity of both PAs and MMPs is controlled either at their expression level, at the activation level by the proteolytic enzymes, or at the level of their inhibitors, such as the tissue inhibitor of metalloproteinase and the plasminogen activator inhibitors (Liekens et al., 2001).

Subsequent to the proteolytic degradation of the extracellular matrix and under the influence of a variety of growth factors, the frontline endothelial cells start to proliferate and migrate through the degraded matrix towards angiogenesis stimuli. Several modulators of angiogenesis, including inducers and inhibitors, have been described so far: the vascular endothelial growth factor (VEGF), fibroblast growth factor-2 (FGF-2), the angiopoietins 1 and 2 (Ang-1 and 2), angiostatin, endostatin, interferons α and γ (IFN-α and γ) and several other growth factors (Liekens et al., 2001). The regulation of angiogenesis depends on the balance between the
stimulators and inhibitors of the process; when the pro-angiogenic growth factors predominate, then proliferation and migration of endothelial cells is increased and this consequently leads to the formation of new blood vessels and angiogenesis can be halted when anti-angiogenic modulators dominate pro-angiogenic mediators (Table 1.3). The cell adhesion molecules, besides the proteases enzymes and growth factors, play a critical role in the regulation of the angiogenesis cascade of events.

Cell adhesion molecules are classified into four families such as immunoglobulin supergene family, cadherins, and the integrins. The integrins, for example, mediate the interaction of endothelial cells with the extracellular matrix during invasion and migration. Also, the cell adhesion molecules are required for cell–cell and cell– extracellular matrix interactions, which are required for lumen formation and the construction of functional capillary loops (Bischoff, 1997).
Table 1.3: Example of pro and anti-angiogenic factors.

<table>
<thead>
<tr>
<th>Pro-angiogenic (stimulators)</th>
<th>Anti-angiogenic (Inhibitors)</th>
</tr>
</thead>
<tbody>
<tr>
<td>VEGF</td>
<td>Interferons a/b</td>
</tr>
<tr>
<td>bFGF/aFGF</td>
<td>Canstatin</td>
</tr>
<tr>
<td>PDGF</td>
<td>VEGI</td>
</tr>
<tr>
<td>PIGF</td>
<td>Tumstatin</td>
</tr>
<tr>
<td>TGFα/β</td>
<td>Angiostatin</td>
</tr>
<tr>
<td>Del-1</td>
<td>IL-12</td>
</tr>
<tr>
<td>TNF-a</td>
<td>Vasostatin</td>
</tr>
<tr>
<td>IL-8</td>
<td>Platelet factor-4</td>
</tr>
<tr>
<td>HGF</td>
<td>Thrombospondin</td>
</tr>
<tr>
<td>PD-ECGF</td>
<td>Endostatin</td>
</tr>
<tr>
<td>Angiogenin</td>
<td>16-kd prolactin fragment</td>
</tr>
<tr>
<td>IL-3</td>
<td>PEDF</td>
</tr>
<tr>
<td>Midkine</td>
<td>2 methoxyestradiol</td>
</tr>
<tr>
<td>Leptin</td>
<td>53-kd antithrombin III</td>
</tr>
<tr>
<td>Follistatin</td>
<td>Prothrombin fragments 1 and 2</td>
</tr>
<tr>
<td>G-CSF</td>
<td>Domain 5 of HMWK</td>
</tr>
<tr>
<td>Proliferin</td>
<td>Restin</td>
</tr>
<tr>
<td>Pleiotrophin</td>
<td>Maspin</td>
</tr>
<tr>
<td>HIV Tat</td>
<td>SPARC</td>
</tr>
<tr>
<td>Plasminogen activators, MMPs</td>
<td>IP-10</td>
</tr>
<tr>
<td>----</td>
<td>IL-18</td>
</tr>
</tbody>
</table>

**Abbreviations:** FGF, fibroblast growth factor; PlGF, placental growth factor; PDGF, platelet-derived growth factor; TGF, transforming growth factor; Del-1, developmental endothelial locus-1; TNF-α, tumor necrosis factor alfa; VEGI, vascular endothelial growth inhibitor; IL, interleukin; HGF, hepatocyte growth factor; PD-ECGF, platelet-derived endothelial cell growth factor; PEDF, pigment epithelium-derived factor; HMWK, high molecular weight kininogen; G-CSF, granulocyte colony-stimulating factor; HIV Tat, human immunodeficiency virus TAT; IP-10, interferon-inducible protein-10; SPARC, secreted protein acidic and rich in cysteine.
1.3.4 Anti-angiogenic targets

The formation of new blood vessels is a complicated multistep process. Agents that suppress or stop neovascularization often do so by interfering with an essential step in this process, such as: (a) Reduction of endothelial cell activation, which may be achieved via the inhibition of growth factor signal production, inhibition of receptors production or inhibition of the binding between signals and receptors, (b) Targeting of endothelial cell proliferation, (c) inhibition of endothelial cell migration, (d) inhibition of endothelial cell differentiation to form a three dimensional tube-like structure and (e) stimulation of apoptosis in endothelial cells (Zhang and Bicknell, 2001).

1.3.5 Anti-angiogenic therapies

Angiogenesis is the hallmark of cancer, which plays an important role in tumor growth, invasion, and metastasis, so blockade of angiogenesis has been viewed as an effective strategy for the therapy of tumor growth and progression. Therefore, targeting angiogenesis became of great therapeutic value to cancer and other angiogenesis related diseases, it work by starving the tumor and suppress its growth rather than targeting neoplastic cells (Folkman, 1971; Quesada et al., 2006).

There are several current strategies for the inhibition of angiogenesis, which include antisense mRNA, monoclonal antibodies, receptor antagonists, and soluble receptors. More than forty anti-angiogenic drugs are being tested in human cancer patients in clinical trials all over the world. These can be divided into three groups according to the target point. The first group includes drugs that inhibit the growth of endothelial cells, such as endostatin and combretastatin A4, which induce the
apoptosis of endothelial cells (Kerbel and Folkman, 2002), whereas curcumin is an inhibitor of proliferation and cell cycle progression of endothelial cell (Singh et al., 1996). The second group includes drugs that block angiogenesis signaling, such as Avastin® and Interferon-alpha, which inhibits the production of basic fibroblast growth factor (b-FGF) and VEGF (Zhang and Bicknell, 2003). The third group consists of drugs that block extracellular matrix breakdown, such as inhibitors of matrix metalloproteinase (MMPs) and Pericytes (PC), which work by inhibiting the breakdown of extracellular matrix and thus interfere with the invasion and migration of endothelial cells.

Other new drugs, such as the tyrosine kinase inhibitors (Erlotinib, Sorafenib and Sunitinib) block the activity of multiple growth factor receptors, such as VEGF and platelet-derived growth factor receptors (PDGFRs) (Tabernero, 2007; Gotink and Verheul, 2010). Currently, seventeen anti-angiogenic agents have been approved as anti-cancer therapies by the American Food and Drug Administration (FDA) (Bodnar, 2014) (Table 1.4). These include small molecule tyrosine kinase inhibitors directed against pro-angiogenic growth factor receptors and monoclonal antibodies directed against specific pro-angiogenic growth factors or their receptors (Samant and Shevde, 2011; Bodnar, 2014).
<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Trade name</th>
<th>Type of drug</th>
<th>Target</th>
<th>Clinical usage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bevacizumab</td>
<td>Avastin</td>
<td>Monoclonal antibody</td>
<td>VEGFR1–2 tyrosine kinase</td>
<td>Metastatic CRC, NSCLC, glioblastoma, metastatic RCC</td>
</tr>
<tr>
<td></td>
<td>(Genentech)</td>
<td></td>
<td>EGFR tyrosine kinase</td>
<td></td>
</tr>
<tr>
<td>Cetuximab</td>
<td>Erbitux</td>
<td>Monoclonal antibody</td>
<td>EGFR tyrosine kinase</td>
<td>Metastatic CRC, RCC</td>
</tr>
<tr>
<td></td>
<td>(Bristol-Meyers Squibb)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Panitumumab</td>
<td>Vectibix</td>
<td>Monoclonal antibody</td>
<td>EGFR tyrosine kinase</td>
<td>Metastatic CRC</td>
</tr>
<tr>
<td></td>
<td>(Amgen)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RanibizumabLucentis</td>
<td>Lucentis</td>
<td>Monoclonal antibody</td>
<td>VEGF-A</td>
<td>Wet age-related macular degeneration</td>
</tr>
<tr>
<td>(Genentech)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trastuzumab</td>
<td>Herceptin</td>
<td>Monoclonal antibody</td>
<td>HER2</td>
<td>Advanced RCC</td>
</tr>
<tr>
<td>(Axitinib)</td>
<td>Inlytan</td>
<td>Small-molecule inhibitor</td>
<td>VEGFR1–3 tyrosine kinase</td>
<td>Advanced RCC</td>
</tr>
<tr>
<td></td>
<td>(Pfizer)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cabozantinib</td>
<td>Cometriq</td>
<td>Small-molecule inhibitor</td>
<td>VEGFR2</td>
<td>c-Met Metastatic medullary thyroid cancer</td>
</tr>
<tr>
<td></td>
<td>(Exelixis)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Erlotinib</td>
<td>Tarceva</td>
<td>Small-molecule inhibitor</td>
<td>EGFR tyrosine kinase</td>
<td>Advanced or metastatic NSCLC</td>
</tr>
<tr>
<td></td>
<td>(Genentech)</td>
<td></td>
<td>mTOR, PI3/AKT pathway</td>
<td></td>
</tr>
<tr>
<td>Everolimus</td>
<td>Afinitor</td>
<td>Small-molecule inhibitor</td>
<td>VEGFR1–3 tyrosine kinase</td>
<td>Advanced RCC, pancreatic neuroendocrine tumor, SEGA</td>
</tr>
<tr>
<td></td>
<td>(Novartis)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Imiquimod</td>
<td>Aldara</td>
<td>Small-molecule inhibitor</td>
<td>TLR-7</td>
<td>Actinic keratosis, basal cell carcinoma</td>
</tr>
<tr>
<td></td>
<td>(Medicis)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pazopanib</td>
<td>Votrient</td>
<td>Small-molecule inhibitor</td>
<td>VEGFR, PDGFR, c-Kit</td>
<td>Advanced RCC</td>
</tr>
<tr>
<td></td>
<td>(GlaxoSmithKline)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Regorafenib</td>
<td>Stivarga</td>
<td>Small-molecule inhibitor</td>
<td>VEGFR1–3 tyrosine kinase</td>
<td>Metastatic CRC</td>
</tr>
<tr>
<td></td>
<td>(Bayer)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sunitinib</td>
<td>Sutent</td>
<td>Small-molecule inhibitor</td>
<td>VEGFR1–3 tyrosine kinase</td>
<td>Advanced RCC, GIST, pancreatic neuroendocrine tumor</td>
</tr>
<tr>
<td></td>
<td>(Pfizer)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sorafenib</td>
<td>Nexavar</td>
<td>Small-molecule inhibitor</td>
<td>VEGFR1–3 tyrosine kinase</td>
<td>Advanced RCC, advanced HCC</td>
</tr>
<tr>
<td></td>
<td>(Bayer/Onyx)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Temsirolimus</td>
<td>Torisel</td>
<td>Small-molecule inhibitor</td>
<td>VEGFR1–3 tyrosine kinase</td>
<td>Advanced RCC</td>
</tr>
<tr>
<td></td>
<td>(Wyeth)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vandetanib</td>
<td>Caprelsa</td>
<td>Small-molecule inhibitor</td>
<td>VEGFR, FGFR</td>
<td>Medullary thyroid cancer</td>
</tr>
<tr>
<td></td>
<td>(AstraZeneca)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pegabtanib</td>
<td>Macugen</td>
<td>Pegylatedaptamer</td>
<td>VEGF</td>
<td>Wet age-related macular degeneration</td>
</tr>
<tr>
<td></td>
<td>(OSI Pharmaceuticals)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
1.4 Correlation between cancer and angiogenesis

1.4.1 Pro and anti-angiogenic mediators

Pro-angiogenic factors are one of the most critical tumor markers that play an important role in neoplastic transformation and the progression of microvessel growth in cancer. It is initiated by the secretion of growth factors with angiogenic properties, such as vascular endothelial growth factor (VEGF), transforming growth factor-α (TGF-α), basic fibroblast growth factor (b-FGF) and epidermal growth factor (EGF), nerve growth factor (NGF) platelet-derived growth factor (PDGF), Interleukin 1, 2 &7, Interferon (IFN) and Granulocyte macrophage colony stimulating factor (GM CSF), (Prager et al., 2011). These growth factors stimulate angiogenesis via the binding to their relevant receptors which are mainly expressed in endothelial cells (Figure 1.4).

![Growth factors receptors](image)

**Figure 1.4:** Growth factors receptors.
1.4.1.(a) Vascular endothelial growth factor

VEGFA is the prototype member of a gene family that also includes VEGFB, VEGFC, VEGFD and placenta growth factor (PLGF) (Figg and Folkman, 2008). It is a dimeric glycoprotein that binds strongly with vascular endothelial cell receptors called VEGF receptor-2 (VEGFR-2), which is a member of a receptor tyrosine kinase family (Shibuya, 2011). This is an important protein involved in developing a new blood supply, like the formation of new blood vessels from pre-existing ones (angiogenesis) and the formation of new blood vessels from non-pre-existing ones (vasculogenesis). In addition to a secreted endothelial-specific growth factor that is strongly VEGFR-2-implicated in all aspects of pathological vascular-endothelial-cell biology, dimerization of the receptor is followed by autophosphorylation, which leads to the activation of the angiogenic cascade (Olsson et al., 2006; Koch and Claesson-Welsh, 2012). Since, a close relationship between several pathologies and angiogenesis has been clarified; various angiogenic inhibitors have been studied. Many of these inhibitors are directed against VEGF or its receptors, which are considered to play a key role in angiogenesis (Niu and Chen, 2010). Thus targeting angiogenesis could be a strategy to combat angiogenesis-dependent diseases. In the case of cancer, most tumors require a more extensive blood supply to provide nutrition in order to support rapid growth (Veeravagu et al., 2007; Wang et al., 2015).

1.4.1.(b) Hypoxia inducible factor-1

Hypoxia inducible factor-1 (HIF-1) is a transcription factor which plays critical role in the regulation of multiple aspects of tumorigenesis such as nutritional stress,