

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

FOUAD SALEIH 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

{تَرْفَعُ دَرَجَاتٍ مِّنْ نَّشَأٍ وَفَوْقَ كُلِّ ذِي عِلْمٍ عَلِيمٌ} (سورة يوسف -76)

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 Muhannad, 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

	Page
ACKNOWLEDGEMENT	ii
TABLE OF CONTENTS	iv
LIST OF TABLES	xvii
LIST OF FIGURES	xx
LIST OF ABBREVIATIONS	xxvii
LIST OF SYMBOLS	xxxi
ABSTRAK	xxxii
ABSTRACT	xxxiv
 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	<i>Orthosiphon stamineus</i> 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 <i>Orthosiphon stamineus</i> extract.....	51
2.4	<i>Ex- vivo</i> angiogenic screening study of various extract of <i>Orthosiphon stamineus</i> and standardization.....	51
2.4.1	<i>Ex-vivo</i> 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 <i>Orthosiphon stamineus</i>	54
2.4.2.(a)	Preparation of standards compounds.....	54
2.4.2.(b)	Preparation of 50% ethanolic extract of <i>Orthosiphon stamineus</i> 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 <i>Orthosiphon stamineus</i>	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.....	64
2.6.6	Cells counting	65
2.6.7	Rosmarinic acid preparation	65
2.6.8	Preparation of 50% ethanol extract of <i>Orthosiphon stamineus</i>	66
2.6.9	Reference standard preparation	66
2.7	<i>In vitro</i> anti-inflammatory effect of 50% ethanol extract of <i>Orthosiphon stamineus</i> and rosmarinic acid	66
2.7.1	Cells proliferation assay.....	66
2.7.1.(a)	Cell culture and treatment	67
2.7.2	<i>In vitro</i> effect of 50% ethanol extract of <i>Orthosiphon stamineus</i> and rosmarinic acid on cytokine and nitric oxide concentration in human macrophage cells (U937)	67

2.7.3	<i>In vitro</i> effect of 50% ethanol extract of <i>Orthosiphon stamineus</i> on cyclooxygenase level.....	68
2.8	Assessment of anti-angiogenic effect of 50% ethanol extract of <i>Orthosiphon stamineus</i> and rosmarinic acid	69
2.8.1	<i>Ex-vivo</i> anti-angiogenic assessment of the effect of 50% ethanol extract of <i>Orthosiphon stamineus</i> and rosmarinic acid on rat aortic ring assay.....	69
2.8.2	<i>In vitro</i> anti-angiogenic assessment of the effect of 50% ethanol extract of <i>Orthosiphon stamineus</i> and rosmarinic acid on human umbilical vein endothelial cells (HUVEC)	69
2.8.2.(a)	<i>In vitro</i> anti-angiogenic assessment of the effect of 50% ethanol extract of <i>Orthosiphon stamineus</i> and rosmarinic acid on human umbilical vein endothelial cells proliferation.....	69
2.8.2.(b)	<i>In vitro</i> anti-angiogenic assessment of the effect of 50% ethanol extract of <i>Orthosiphon stamineus</i> on human umbilical vein endothelial cells migration	70
2.8.2.(c)	<i>In vitro</i> anti-angiogenic assessment of the effect of 50% ethanol extract of <i>Orthosiphon stamineus</i> and rosmarinic acid on human umbilical vein endothelial cells tube formation.....	71
2.8.2.(d)	<i>In vitro</i> 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	<i>In vivo</i> anti-angiogenic assessment of the effect of 50% ethanol extract of <i>Orthosiphon stamineus</i> and rosmarinic acid.....	73
2.8.3.(a)	<i>In vivo</i> anti-angiogenic assessment of effect of 50% ethanol extract of <i>Orthosiphon stamineus</i> 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)	<i>In vivo</i> anti-angiogenic assessment of 50% ethanol extract of <i>Orthosiphon stamineus</i> 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)	v - Hematoxylin and eosin staining of the blood vessels	75
2.9	<i>In vitro</i> anti-cancer studies	77
2.9.1	Assessment of the effect of 50% ethanolic extract of <i>Orthosiphon stamineus</i> 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 <i>Orthosiphon stamineus</i> 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	<i>In vivo</i> anti-tumor studies of 50% ethanol extract of <i>Orthosiphon stamineus</i> and rosmarinic acid	81
2.11.1	Evaluation of the activity of 50% ethanol extract of <i>Orthosiphon stamineus</i> 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 <i>Orthosiphon stamineus</i> 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 <i>Orthosiphon stamineus</i> 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 <i>In vivo</i> assessment of the preventive effect of 50% ethanol extract of <i>Orthosiphon stamineus</i> 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 <i>In vivo</i> 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 <i>In vivo</i> 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 <i>In silico</i> ligand binding and interaction studies of selected bioactive compounds in 50% ethanolic extract of <i>Orthosiphon stamineus</i>	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	<i>Ex- vivo</i> angiogenic screening study of <i>Orthosiphon stamineus</i>	103
3.3.4	Quantification of rosmarinic acid, sinensetin, eupatorin and 3'-hydroxy-5, 6, 7, 4'-tetramethoxyflavone in <i>Orthosiphon stamineus</i> using high performance liquid chromatography	106
3.3.5	Anti-oxidant activity of 50% ethanol extract of <i>Orthosiphon stamineus</i> leaves	109
3.3.5.(a)	Total flavonoid and phenolic contents of 50% ethanol extract of <i>Orthosiphon stamineus</i> 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 <i>Orthosiphon stamineus</i> and rosmarinic acid on viability of human macrophage cell line	114
3.3.6.(b)	<i>In vitro</i> inhibitory effect of 50% ethanol extract of <i>Orthosiphon stamineus</i> on production of nitric oxide and cytokine in human macrophages cells	115
3.3.6.(c)	<i>In vitro</i> inhibitory effect of rosmarinic acid on production of nitric oxide and cytokine in human macrophage cells	116

3.3.6.(d)	<i>In vitro</i> inhibitory effect of 50% ethanolic extract of <i>Orthosiphon stamineus</i> 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	<i>Ex-vivo</i> anti-angiogenic study using rat aortic ring assay.....	126
4.3.1.(a)	Dose-response curves of 50% ethanol extract of <i>Orthosiphon stamineus</i> and rosmarinic acid on rat aortic ring assay.....	126
4.3.2	<i>In vitro</i> anti-angiogenic study on Human Umbilical Vein Endothelial Cells.....	130
4.3.2.(a)	Effect of 50% ethanol extract of <i>Orthosiphon stamineus</i> and rosmarinic acid on the proliferation of Human Umbilical Vein Endothelial Cells	130
4.3.2.(b)	Effect of 50% ethanol extract of <i>Orthosiphon stamineus</i> on Human Umbilical Vein Endothelial Cells migration.....	132
4.3.2.(c)	Anti-angiogenic effect of 50% ethanol extract of <i>Orthosiphon stamineus</i> and rosmarinic acid on Human Umbilical Vein Endothelial Cells using tube formation assay	134
4.3.2.(d)	<i>In vitro</i> effect of 50% ethanol extract of <i>Orthosiphon stamineus</i> and rosmarinic acid on protein expression.....	136
4.3.3	<i>In vivo</i> anti-angiogenic activity.....	149
4.3.3.(a)	Effect of 50% ethanol extract of <i>Orthosiphon stamineus</i> and rosmarinic acid on neovascularization in Chick Chorioallantoic Membrane assay.....	149

4.3.3.(b)	<i>In vivo</i> anti-angiogenic effect of 50% ethanol extract of <i>Orthosiphon stamineus</i> 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	<i>In vitro</i> anti-cancer studies.....	161
5.3.1.(a)	Effect of 50% ethanol extract of <i>Orthosiphon stamineus</i> 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)	<i>In vitro</i> anti-tumorigenicity of 50% ethanol extract of <i>Orthosiphon stamineus</i> and rosmarinic acid on colorectal cancer cell line.....	163
5.3.2	<i>In vivo</i> tumor studies of 50% ethanol extract of <i>Orthosiphon stamineus</i> and rosmarinic acid on colorectal cancer cell line (HCT-116).....	167
5.3.2.(a)	Effect of 50% ethanol extract of <i>Orthosiphon stamineus</i> on subcutaneous colorectal tumor growth in nude mice for 28 days (Ectopic Model)	167
5.3.2.(b)	Effect of 50% ethanol extract of <i>Orthosiphon stamineus</i> on body weight.....	170
5.3.2.(c)	Effect of 50% ethanol extract of <i>Orthosiphon stamineus</i> 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 <i>Orthosiphon stamineus</i> 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 <i>Orthosiphon stamineus</i> in nude mice using colorectal cancer ectopic model; a preventive study (2 weeks pre treatment)	179
5.3.3 <i>In vivo</i> anti-tumor activity of rosmarinic acid on subcutaneous colorectal tumor growth using nude mice for 28 days (Ectopic model).....	185
5.3.4 <i>In vivo</i> effect of 50% ethanol extract of <i>Orthosiphon stamineus</i> and rosmarinic acid on pro and anti-angiogenic growth factor protein expression using Luminex Multiplexing Platform.....	190
5.3.4.(a) <i>In vivo</i> effect of 50% ethanol extract of <i>Orthosiphon stamineus</i> 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) <i>In vivo</i> effect of 50% ethanol extract of <i>Orthosiphon stamineus</i> 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) <i>In vivo</i> effect of 50% ethanol extract of <i>Orthosiphon stamineus</i> on interferon alpha, interferon beta and epidermal growth factor levels (short term study at 28 days in ectopic tumors).....	195
5.3.4.(d) <i>In vivo</i> effect of 50% ethanol extract of <i>Orthosiphon stamineus</i> 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 <i>In vivo</i> effect of 50% ethanol extract of <i>Orthosiphon stamineus</i> on <i>HIF-α</i> , <i>KDR</i> , <i>WNT</i> and <i>COX2</i> gene expression on human colorectal tumor tissue (Short term study for 28 days in ectopic tumors).....	199
5.3.6 <i>In-silico</i> prediction of binding and interactions of selected bioactive compounds of 50% ethanol extract of <i>Orthosiphon stamineus</i>	201
5.3.6.(a) Comparative molecular field analysis of selected bioactive compounds of 50% ethanol extract of <i>Orthosiphon stamineus</i>	201
5.3.6.(b) <i>In silico</i> ligand binding and interaction studies of selected bioactive compounds in 50% ethanol extract of <i>Orthosiphon stamineus</i> to cyclooxygenase	203

5.3.6.(c)	<i>In silico</i> ligand binding and interaction studies of selected bioactive compounds in 50% ethanol extract of <i>Orthosiphon stamineus</i> to epidermal growth factors	205
5.3.6.(d)	<i>In silico</i> ligand binding and interaction studies of selected bioactive compounds in 50% ethanolic extract of <i>Orthosiphon stamineus</i> to basic fibroblast growth factors.....	207
5.3.6.(e)	<i>In silico</i> ligand binding and interaction studies of selected bioactive compounds in 50% ethanolic extract of <i>Orthosiphon stamineus</i> to vascular endothelial growth factor A.....	209
5.3.6.(f)	<i>In silico</i> ligand binding and interaction studies of selected bioactive compounds in 50% ethanolic extract of <i>Orthosiphon stamineus</i> to granulocyte macrophage colony stimulating factor levels	211
5.3.6.(g)	<i>In silico</i> ligand binding and interaction studies of selected bioactive compounds in 50% ethanolic extract of <i>Orthosiphon stamineus</i> to interferon alpha 2	213
5.3.6.(h)	<i>In silico</i> ligand binding and interaction studies of selected bioactive compounds in 50% ethanolic extract of <i>Orthosiphon stamineus</i> to interleukin-2.....	215
5.3.6.(i)	<i>In silico</i> ligand binding and interaction studies of selected bioactive compounds in 50% ethanolic extract of <i>Orthosiphon stamineus</i> to tumor necroses factors alpha.....	217
5.3.6.(j)	<i>In silico</i> ligand binding and interaction studies of selected bioactive compounds in 50% ethanolic extract of <i>Orthosiphon stamineus</i> to tumor necrosis factor beta.....	219
5.3.6.(k)	<i>In silico</i> ligand binding and interaction studies of selected bioactive compounds in 50% ethanolic extract of <i>Orthosiphon stamineus</i> 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

	Page
Table 1.1	List of oncogenes. 7
Table 1.2	Some of tumor suppressor genes. 8
Table 1.3	Example of pro and anti-angiogenic factors. 19
Table 1.4	FDA-approved angiogenesis inhibitors 22
Table 2.1	List of Chemicals and reagents 46
Table 2.2	List of equipments and apparatus 49
Table 2.3	HPLC mobile phase gradient elution program for separation of 50% ethanolic extract of <i>Orthosiphon stamineus</i> marker compounds 55
Table 2.4	Types of cell lines used for <i>in vitro</i> cytotoxicity evaluation 62
Table 2.5	Gene primers 95
Table 2.6	Molecular structures and bioactivity (IC ₅₀) of selected compound (PIC50 inhibitory potential) 97
Table 3.1	Percentage yield of various extracts of <i>Orthosiphon stamineus</i> 102
Table 3.2	Peak area, regression equation and percentage of marker compounds present in 50% ethanol extract of <i>Orthosiphon stamineus</i> leaves 109
Table 5.1	Biochemical parameters of nude mice treated orally with various compounds for 35 days 177
Table 5.2	<i>In silico</i> of ligand and cyclooxygenase interaction (interacted amino acids residues, number of hydrogen bonds, binding affinity and ligand efficiency) 203

Table 5.3	<i>In silico</i> of ligand and epidermal growth factors interaction (interacted amino acids residues, number of hydrogen bonds, binding affinity and ligand efficiency)	205
Table 5.4	<i>In silico</i> of ligand (rosmarinic acid and Imatinib [®]) and fibroblast growth factors interaction (interacted amino acids residues, number of hydrogen bonds, binding affinity and ligand efficiency).	207
Table 5.5	<i>In silico</i> of ligand and vascular endothelial growth factor A interaction (interacted amino acids residues, number of hydrogen bonds, binding affinity and ligand efficiency)	209
Table 5.6	<i>In silico</i> of ligand and granulocyte macrophage colony stimulating factor interaction (interacted amino acids residues, number of hydrogen bonds, binding affinity and ligand efficiency).	211
Table 5.7	<i>In silico</i> of ligand and interferon alpha 2 interaction (interacted amino acids residues, number of hydrogen bonds, binding affinity and ligand efficiency)	213
Table 5.8	<i>In silico</i> of ligand and interleukin-2 (interacted amino acids residues, number of hydrogen bonds, binding affinity and ligand efficiency)	215
Table 5.9	<i>In silico</i> of ligand and tumor necrosis factors alpha interaction (interacted amino acids residues, number of hydrogen bonds, binding affinity and ligand efficiency)	217

Table 5.10	<i>In silico</i> 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	<i>In silico</i> 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

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

	synthesis by stimulation of human macrophage cells using Lipopolysaccharide	116
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	117
Figure 3.11	The percent inhibition of 50% ethanolic extract of <i>Orthosiphon stamineus</i> and aspirin for COX-1; celecoxib for COX-2 on the activities of cyclooxygenase enzymes	118
Figure 3.12	Summary of phytochemical study of <i>Orthosiphon stamineus</i>	124
Figure 4.1	Photomicrographs of anti-angiogenic activity of 50% ethanol extract of <i>Orthosiphon stamineus</i> and rosmarinic acid towards neovascularisation in rat aortic ring assay	128
Figure 4.2	The inhibition of 50% ethanolic extract of <i>Orthosiphon stamineus</i> (A) and rosmarinic acid (B) on sprouting in the rat aortic tissue explants	129
Figure 4.3	Effect of 50% ethanol extract of <i>Orthosiphon stamineus</i> (A) and rosmarinic acid (B) on Human Umbilical Vein Endothelial Cells proliferation	131
Figure 4.4	Effect of 50% ethanol extract of <i>Orthosiphon stamineus</i> on HUVECs cell migration.	133
Figure 4.5	Anti angiogenic effect of 50% ethanol extract of <i>Orthosiphon stamineus</i> on HUVECs tube formation	135
Figure 4.6	Effect of 50% ethanol extract of <i>Orthosiphon stamineus</i> , rosmarinic acid and Imatinib [®] on pro and anti-angiogenic growth factor expression.	136
Figure 4.7	Effect of different doses of 50% ethanol extract of <i>Orthosiphon stamineus</i> , rosmarinic acid and Imatinib [®] on vesicular endothelial growth factor expression in Human Umbilical Vein Endothelial Cells after 24 h treatment	137
Figure 4.8	Effects of different doses of 50% ethanol extract of <i>Orthosiphon stamineus</i> , 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	139
Figure 4.9	Effect of different doses of 50% ethanol extract of <i>Orthosiphon stamineus</i> , rosmarinic acid and Imatinib [®] on interferons expression levels in Human Umbilical Vein Endothelial Cells lysate after 24 h treatment	141
Figure 4.10	Effect of different doses of 50% ethanol extract of <i>Orthosiphon stamineus</i> , 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	143
Figure 4.11	Effect of different doses of 50% ethanol extract of <i>Orthosiphon stamineus</i> , 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	145
Figure 4.12	Effect of different doses of 50% ethanol extract of <i>Orthosiphon stamineus</i> , rosmarinic acid and Imatinib [®] on granulocyte macrophage colony-stimulating factor expression level in Human Umbilical Vein Endothelial Cells lysate after 24 h treatment	146
Figure 4.13	Effect of different doses of 50% ethanol extract of <i>Orthosiphon stamineus</i> , 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	148
Figure 4.14	Inhibitory effect of different doses of 50% ethanol extract of <i>Orthosiphon stamineus</i> and rosmarinic acid on neovascularization in chorioallantoic membrane of chick embryo	150
Figure 4.15	Anti-angiogenic effect of 50% ethanol extract of <i>Orthosiphon stamineus</i> on matrigel plug	152
Figure 4.16	Summary of anti-angiogenic effects of 50% ethanol extract of <i>Orthosiphon stamineus</i>	159

Figure 5.1	Effect of the 50% ethanol extract of <i>Orthosiphon stamineus</i> on the viability of HCT-116, Skno-1, HL-60 and CCD-18Co cell lines	162
Figure 5.2	Effect of rosmarinic acid on the viability of HCT-116, Skno-1, HL-60 and CCD-18Co cell lines	163
Figure 5.3	Morphology of HCT-116 cell invasion after treatment with different doses of 50% ethanol extract of <i>Orthosiphon stamineus</i> and rosmarinic acid	164
Figure 5.4	The principles of multicellular tumor spheroids preparation by the hanging-drop method	165
Figure 5.5	Effects of 50% ethanolic extract of <i>Orthosiphon stamineus</i> and rosmarinic acid on <i>in-vitro</i> HCT-116 tumour in hanging drop assay	166
Figure 5.6	Subcutaneous tumor in NCR nude mice	168
Figure 5.7	Effect of 50% ethanol extract of <i>Orthosiphon stamineus</i> and the equivalent amount of rosmarinic acid on HCT-116 tumor size in nude mice	169
Figure 5.8	Percentage inhibition of tumor growth in nude mice treated with different doses of 50% ethanolic extract of <i>Orthosiphon stamineus</i> , rosmarinic acid and Imatinib [®] compared with negative control	169
Figure 5.9	Body weight of treated animals with 50% ethanol extract of <i>Orthosiphon stamineus</i> , rosmarinic acid and Imatinib [®] compared with negative control	170
Figure 5.10	Imaging of tumor- bearing mice was implanted orthotopically into the cecal wall of nude mice, using fluorescence molecular tomography (FMT)	172
Figure 5.11	Effect of 50% ethanol extract of <i>Orthosiphon stamineus</i> towards HCT-116 tumor implanted orthotopically in the cecal wall of nude mouse after treatment for 35 days	173
Figure 5.12	Hematoxylin/eosin staining of crosses sections of tumor tissues	175
Figure 5.13	Photographs of liver metastasis in untreated group	176

Figure 5.14	Effect of 50% ethanol extract of <i>Orthosiphon stamineus</i> 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 <i>Orthosiphon stamineus</i> and rosmarinic acid on nude mice (long term study for 26 weeks)	179
Figure 5.16	Chemoprevention effect of 50% ethanol extract of <i>Orthosiphon stamineus</i> 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 <i>Orthosiphon stamineus</i> towards HCT-116 tumor implanted ectopically for 28 days	182
Figure 5.18	Body weight of animals treated with 50% ethanol extract of <i>Orthosiphon stamineus</i> 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	<i>In vivo</i> 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 <i>Orthosiphon stamineus</i> , 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 <i>Orthosiphon stamineus</i> 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	196
Figure 5.26	<i>In-vivo</i> effect of 50% ethanol extract of <i>Orthosiphon stamineus</i> 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	198
Figure 5.27	<i>In-vivo</i> effect of 50% ethanol extract of <i>Orthosiphon stamineus</i> at doses of 100, 200 and 400 mg/kg, rosmarinic acid and Imatinib [®] at 30 mg/kg in gene expression levels of <i>HIF-α</i> , <i>KDR</i> , <i>WNT</i> and <i>COX2</i> in tissue lysates.	200
Figure 5.28	Three dimension quantitative structure activity relationship and comparative molecular field analysis contour maps	202
Figure 5.29	<i>In silico</i> ligand and cyclooxygenase interaction profile	204
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	206
Figure 5.31	<i>In silico</i> 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	208
Figure 5.32	<i>In silico</i> ligand and vascular endothelial growth factor interaction profile	210
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	212
Figure 5.34	<i>In silico</i> 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	214

Figure 5.35	<i>In silico</i> 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	<i>In silico</i> 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	<i>In silico</i> ligand and tumor necrosis factors beta interaction profile	220
Figure 5.38	<i>In silico</i> ligand and vascular endothelial growth factor R1 interaction profile	222
Figure 5.39	Proposed signaling pathways underlying the effect of <i>Orthosiphon stamineus</i> in suppression of angiogenesis and human colorectal cancer	231
Figure 5.40	Summary of anti tumor activity of 50% ethanol extract of <i>Orthosiphon stamineus</i> and rosmarinic acid toward of colorectal cancer	232

LIST OF ABBREVIATIONS

5-FU	5-fluorouracil
ACS	American Cancer Society
Ala	Alanine
AlCl ₃	Aluminium chloride
Ang-2	Angiopoietin 2
APC	Adenomatous Polyps Coli
Arg	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 immunosorbant 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 kininogen
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 diphenyltetrazolim bromide

MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
NO	Nitric oxide
NSAIDs	Nonsteroidal anti-inflammatory drugs
OX	Oxaliplatin
OA	Orthosiphon A
OLA	Oleanolic acid
PAs	Plasminogen activators
PBS	Phosphate-buffered saline
PBS-T	PBS with 0.1% tween 20
PC	Pericytes
PDB	Protein Data Bank
PD-ECGF	Platelet-derived endothelial cell growth factor
PDGF	Platelet-derived growth factor
PDGFR	Platelet-derived growth factor receptors
PEDF	Pigment epithelium-derived factor
Pg	Picogram
PGE2	Prostaglandin E2
PLGF	Placental growth factor
Phe	Phenylalanine
PMA	Phorbol myristate acetate.
Pro	Proline
P/S	Penicillin/streptomycin
RA	Rosmarinic acid
Ras-GAP	Guanosine triphosphatase-activating protein
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute medium
RT_PCR	Real -Time Polymerase Chain Reaction
Ser	Serine
SPARC	Secreted protein acidic and rich
sVEGFR1	soluble VEGF receptor-1

TAMs	Tumor-associated macrophages
TGF	Transforming growth factor
TGF- β	Transforming growth factor beta
Thr	Threonine
TMF	3'-hydroxy-5,6,7,4'-tetramethoxyflavone
TNF- α	Tumor necrosis factor alpha
Tris	Tris (hydroxymethyl) aminomethane
Trp	Tryptophan
TXA2	Thromboxane A2
Tyr	Tyrosine
UV-vis	Ultra-violet visible
Val	Valine
VEGF	Vascular endothelial growth factor
VEGFR-1,2	Vascular endothelial cell receptors -1,2
VEGI	Vascular endothelial growth inhibitor
WHO	World Health Organization
WNT	Wingless-type MMTV integration site family

LIST OF SYMBOLS

Symbol	Meaning
Å	Angstrom
γ	Gamma
β	Beta
α	Alpha
<	Less than
>	More than
μ	Micro
C	Celsius
%	Percent

**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 cyclooxygenase (COX), TNF- α , IL-1 dan tahap nitrik oksida (NO) yang penting untuk tumorigenesis. Lebih-

lebih lagi, EOS dan RA telah menghalang ekspresi gen secara signifikan dalam tisu tumor usus termasuk *HIF- α* , *WNT*, *KDR* dan *COX2*. Tambahan pula, EOS menghalang merca 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 xenograft. 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 hapus-sisa 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- α* , *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.

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

Table 1.2: Some of tumor suppressor genes.

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

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 proliferative signalling. This is one of the characteristics of cancer cells, which can be acquired by various pathways and is defined by the cell's 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 activates quiescent endothelial cells in order to grow new blood vessels. The growth and metastasis 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 supplying nutrient and oxygen through 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).

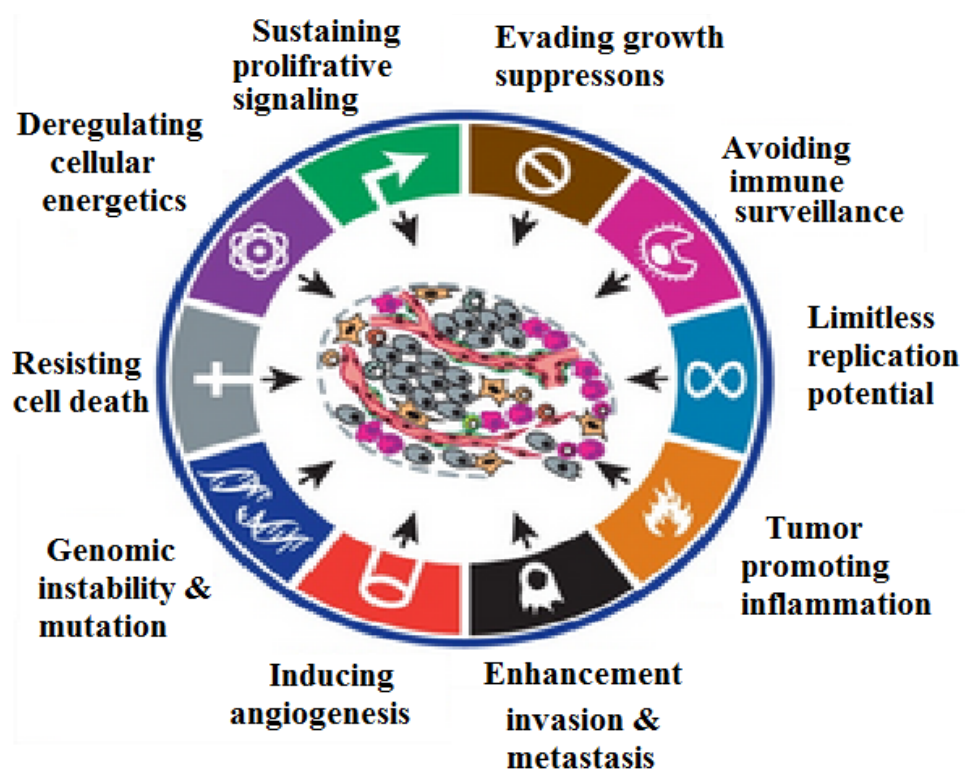


Figure 1.1: Ten hallmarks of cancer acquired during cancer progression. Adapted from (Hanahan and Weinberg, 2011).

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 (TGF β R2) (Markowitz and Bertagnolli, 2009; Kanthan et al., 2012) (Figure 1.2).

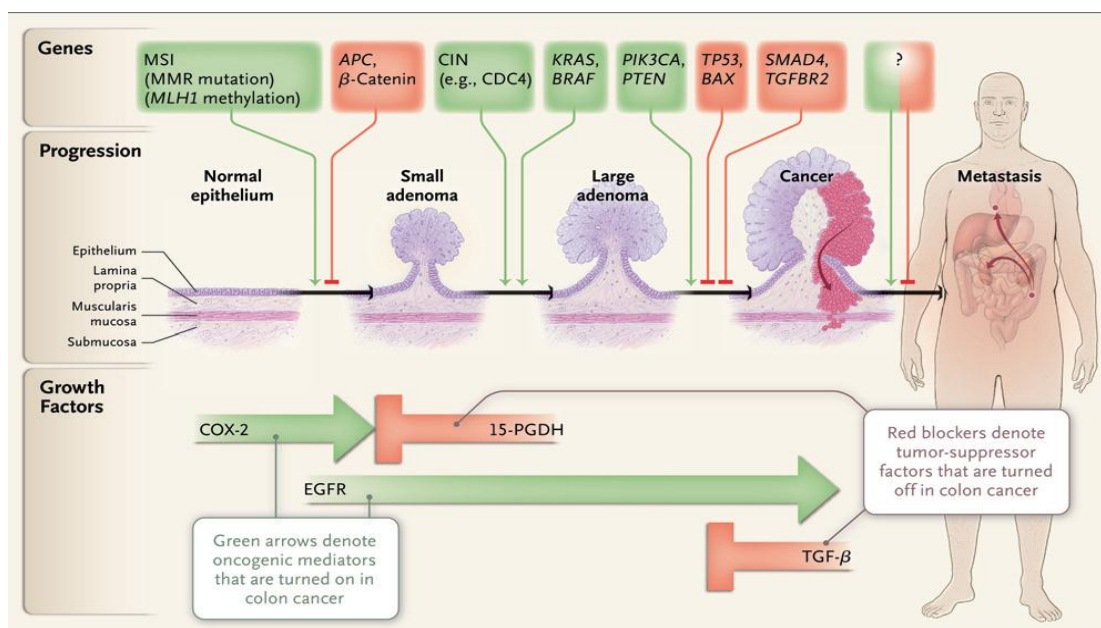


Figure 1.2: Genetic alterations frequently associated with CRC progression
Adapted from (Markowitz and Bertagnolli, 2009)

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) (Figure1.3).

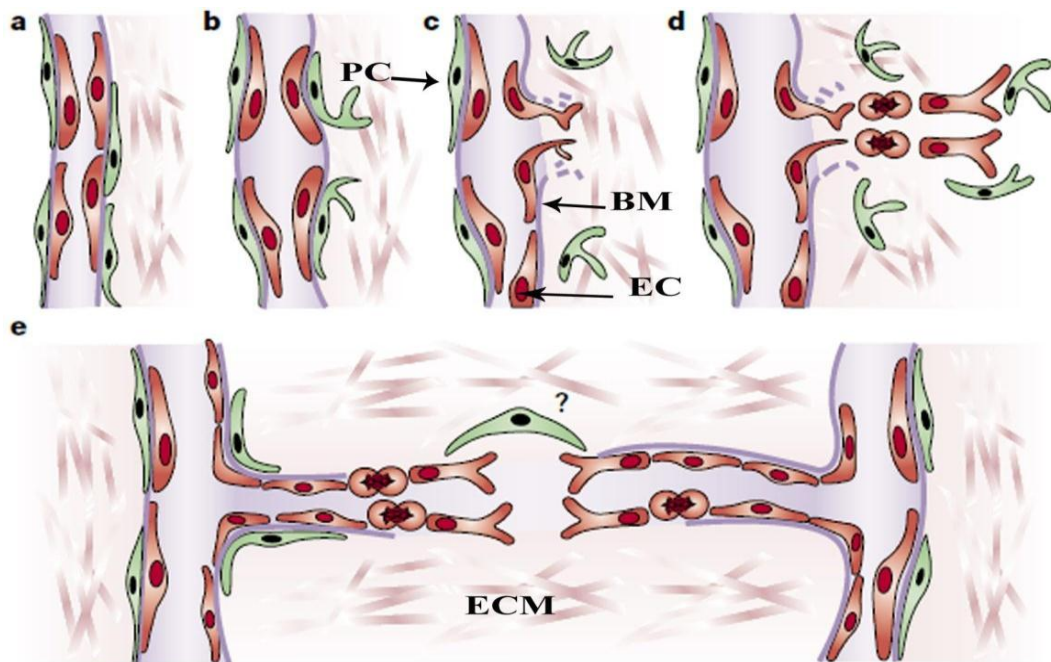


Figure 1.3: Angiogenesis Cascade. (a): Blood vessels (b): Pericytes (PC) detach, blood vessels dilate before basement membrane (BM) and extracellular matrix (ECM) gets degraded (c) The underlying endothelial cells escape from the blood vessels wall to allows endothelial cells (EC) to migrate into perivascular 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 tightly regulated process that 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.

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

Abbreviations: FGF, fibroblast growth factor; PIGF, 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 1.4: FDA-approved angiogenesis inhibitors (Adapted from Bodnar, 2014)

Inhibitor	Trade name (manufacturer)	Type of drug	Target	Clinical usage
Bevacizumab	Avastin (Genentech)	Monoclonal antibody	VEGFR1–2 tyrosine kinase	Metastatic CRC, NSCLC, glioblastoma, metastatic RCC
Cetuximab	Erbitux (Bristol-Myers Squibb)	Monoclonal antibody	EGFR tyrosine kinase	Metastatic CRC, RCC
Panitumumab	Vecitbix (Amgen)	Monoclonal antibody	EGFR	Metastatic CRC
Ranibizumab	Lucentis (Genentech)	Monoclonal antibody	VEGF-A	Wet age-related macular degeneration
Trastuzumab	Herceptin (Genentech)	Monoclonal antibody	HER2	Advanced RCC
(Axitinib)	Inlytan (Pfizer)	Small-molecule inhibitor	VEGFR1–3	Advanced RCC
Cabozantinib	Cometriq (Exelixis)	Small-molecule inhibitor	VEGFR2	c-Met Metastatic medullary thyroid cancer
Erlotinib	Tarceva (Genentech)	Small-molecule inhibitor	EGFR tyrosine kinase	Advanced or metastatic NSCLC
Everolimus	Afinitor (Novartis)	Small-molecule inhibitor	mTOR, PI3/AKT pathway	Advanced RCC, pancreatic neuroendocrintumor, SEGA
Imiquimod	Aldara (Medicis)	Small-molecule inhibitor	TLR-7	Actinic keratosis, basal cell carcinoma
Pazopanib	Votrient (GlaxoSmithKline)	Small-molecule inhibitor	VEGFR, PDGFR, c-Kit	Advanced RCC
Regorafenib	Stivarga (Bayer)	Small-molecule inhibitor	VEGFR1–3, PDGFR, FGFR, Kit, Raf, RET	Metastatic CRC
Sunitinib	Sutent (Pfizer)	Small-molecule inhibitor	VEGFR1–3, PDGFRb, RET	Advanced RCC, GIST, pancreatic neuroendocrine tumor
Sorafenib	Nexavar (Bayer/Onyx)	Small-molecule inhibitor	VEGFR1–3, PDGFRb, Raf-1	Advanced RCC, advanced HCC
Temsirolimus	Torisel (Wyeth)	Small-molecule inhibitor	mTOR	Advanced RCC
Vandetanib	Caprelsa (AstraZeneca)	Small-molecule inhibitor	VEGFR, FGFR	Medullary thyroid cancer
Pegabtanib	Macugen (OSI Pharmaceuticals)	Pegylated aptamer	VEGF	Wet age-related macular degeneration

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 (IFNs) 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).

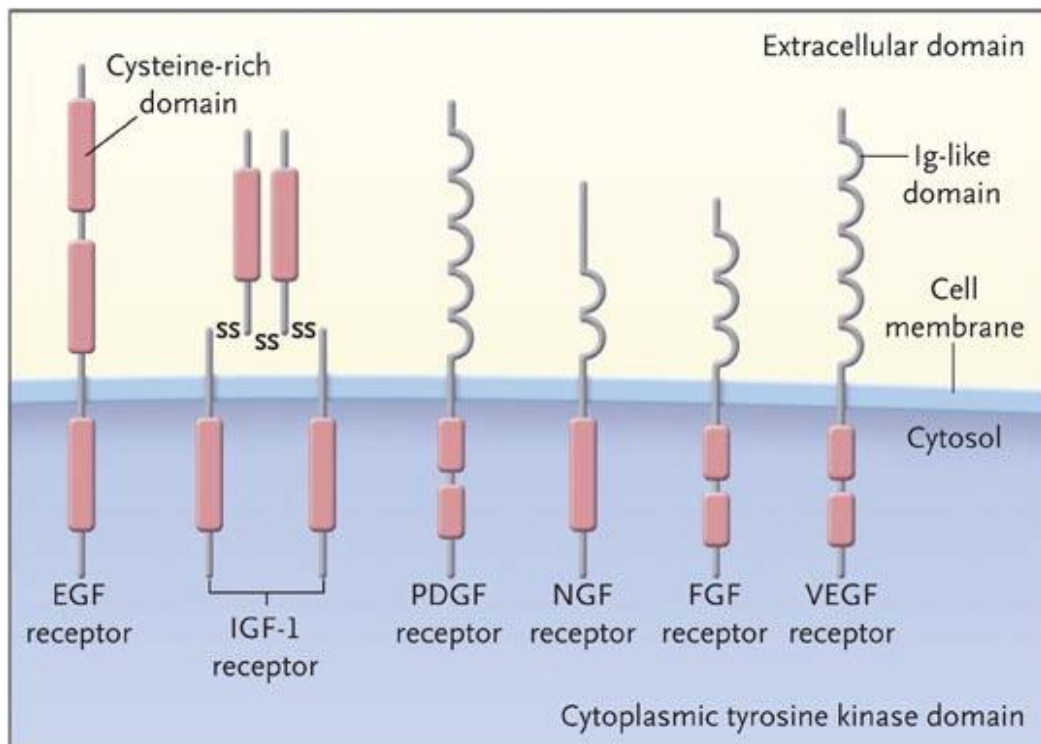


Figure 1.4: Growth factors receptors.

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,