Anti-angiogenic Effects and Mechanisms of the Chinese Herbs Rhizoma Rhei, Fructus Alpiniae and Rhizoma Kaempferiae

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Abstract of thesis entitled:

Anti-angiogenic Effects and Mechanisms of the Chinese Herbs Rhizoma Rhei, Fructus Alpiniae and Rhizoma Kaempferiae

Submitted by Zhiheng HE for the degree of Doctor of Philosophy at The Chinese University of Hong Kong

Cancer is a generic term for a large group of diseases that can affect any part of the body, which causes a vast medical problem and is a leading cause of death worldwide nowadays. However, for many years the main methods of treating cancer have been surgery, radiotherapy and chemotherapy. Among these treatments, chemotherapy has played a major role in cancer therapy for half a century. Despite improving managements and efforts, it is not surprising that the prognosis has not greatly improved because of the limitations of current therapies, such as toxicity, inherent and acquired resistance, and metastatic spread. This calls for novel cancer therapies and new group of anticancer agents for selectively targeting cancers without or with lower toxicity to normal tissues.

Angiogenesis refers to the formation of new blood capillaries from pre-existing ones, and is essential in a series of normal physiological processes such as embryonic development and pathological responses. However, persistent unregulated angiogenesis causes "angiogenic diseases" such as diabetic retinopathy, tumor growth and metastasis, rheumatoid arthritis, and inflammatory diseases. The linkage between angiogenesis, tumor growth and metastasis was first hypothesized by Dr. Judah Folkman in the 1970s, and now this controversial idea is widely accepted and the inhibition of angiogenesis, or anti-angiogenesis, is considered as a promising anticancer therapeutic strategy. Bevacizumab (Avastin® by Genentech Inc.), the first

approved anti-angiogenic drug by U.S. FDA in 2004, is a humanized monoclonal antibody to inhibit endothelial cell proliferation and angiogenesis for the treatment of metastatic colorectal cancer, non-small cell lung cancer, advanced breast cancer, glioblastoma, metastatic renal cell cancer.

Anti-angiogenic therapy in cancer treatment has led to the development of compounds designed to control a tumor's growth by blocking its ability to develop a blood supply. The development of agents with different mechanisms of action requires powerful preclinical models for the analysis and optimization of the therapy. Some *in vitro* and *in vivo* anti-angiogenic assays are already developed, for example, Human Umbilical Vein Endothelial Cell (HUVEC) assay, Chorioallantoic Membrane assay, Matrigel plug assay et al. Zebrafish, as a relatively new model organism, is firmly established as a powerful research platform for many areas of biology and drug discovery, allowing the testing of bioactive compounds in a whole organism and in cells undergoing normal cell-cell and cell-matrix interactions. Many anti- and pro-angiogenic molecules tested in zebrafish demonstrated similar effects to those observed in humans or other mammalian models. Besides providing a powerful platform for drug screening, zebrafish model can also be used for probing biological processes, and generate insights into mechanisms.

Traditional Chinese medicines (TCMs) have long been recognized as a rich source for discovering drugs, and various TCMs and their components have shown anti-angiogenic properties. In this thesis study, as a continuing pursuit for elucidating the anti-angiogenic properties of TCMs, our attention is focused on those with effects of anti-inflammation, anti-rheumatoid arthritis and anti-cancer. On zebrafish screening model, three of the selected TCMs, *Rheum palmatum* (大黄), *Alpinia oxyphylla* (seeds) (益智仁), and *Kaempferia galanga* (沙姜) showed potential

anti-angiogenic activity, indicating the existence of potent anti-angiogenic components in these herbs. The ethyl acetate fraction of *R. palmatum* showed strong inhibition of vessel formation in zebrafish embryos. Further testing of the anthraquinones of this herb showed three of them displayed potent anti-angiogenic activities. The most potent compound—rhein could inhibit HUVEC migration and affect the mRNA expression of *vegfa*, *kdr*, *angiopoietin1/2* and *tie1/2*; The *n*-hexane and ethyl acetate fractions of *A. oxyphylla* and *K. galangal* showed anti-angiogenic potentials both in zebrafish and HUVEC assays. The *n*-hexane and ethyl acetate fractions of *A. oxyphylla* could both inhibit the proliferation, migration and tube formation processes of HUVEC. And the most potential component, *trans*-ethyl-*p*-methoxycinnamate from *K. galanga*, could inhibit HUVEC migration and tube formation, and reduce all gene expressions involved in angiogenesis process except for *vegfa*.

All the results showed that TCMs can provide a source for discovering anti-angiogenic agents for the treatment of cancer, and all these experiments in the zebrafish and mammalian models further confirmed the value of zebrafish model in anti-angiogenic drug discovery.

摘要

癌症作為一類疾病的總稱可以影響全身任何組織、器官的健康。目前，癌症是造成全球人類死亡的最主要原因之一，同時也帶來了巨大的醫療問題。但是，多年來用於治療癌症的方法僅限於外科手術、放射療法和化學療法。其中，近半個世紀以來化學療法一直是最主要的癌症治療方法之一。儘管不斷的努力，但由於目前治療方法的局限性，如毒副作用、先天或後天的抗藥性以及術後擴散，使得其預後的效果都沒有實質性的進展。這就迫切需要開發出一種新的抗癌方法或一類新的抗癌藥物來選擇性的作用於癌症病灶，與此同時對正常組織沒有或僅有微量的毒副作用。

血管新生是指從現有的血管生長出新的血管分支，其對於一系列正常的生理活動例如：胚胎髮育、病理反應是至關重要的。 但是，持續的毫無控制的血管新生則會導致諸如糖尿病視網膜病變、腫瘤生長和轉移、風濕性關節炎以及炎症類疾病等"血管新生類疾病"的發生。Judah Folkman 博士早在20世紀70年代假設了血管新生與腫瘤生長和轉移之間存在的聯繫，現在這個飽受爭議的觀點已經得到廣泛的認可。而且，抑制血管新生療法被認為是非常有前景的癌症治療方法。Bevacizumab (Avastin®) 是由美國基因泰克公司開發的第一個被美國食品藥品監督管理局批准的血管抑製劑，它是一種單克隆抗體，能抑制內皮細胞的增殖以及血管新生，已被用於治療轉移性結直腸癌、非小細胞肺癌、晚期乳腺癌、惡性膠質瘤和轉移性腎細胞癌。

能適用於通過抑制血管新生來治療癌症的藥物要求具備擁有阻斷癌組織誘發血管新生從而達到控制腫瘤生長的能力。對於開發具有不同作用機理的該類藥物需要有效的臨床模型來分析與優化。很多體內、外的抗血管新生的方法模型，例如人類臍靜脈內皮細胞模型、雞胚絨毛尿囊膜模型和Matrigel塞模型等都已被

用於篩選研究工作。斑馬魚作為相對較新的動物模型，現在已經被視為一個強大的研究模型而用於諸多生物學領域研究和藥物篩選研究。該模型既能夠進行活性候選物質在生物整體水平上的研究，又能在具有細胞間及其基質相互影響的正常細胞水平上的研究。許多具有抑制或是促進血管新生的活性物質能夠在斑馬魚上產生與在人類或是其他哺乳動物模型上相似的效果。斑馬魚除了能用於藥物的篩選工作外，同時也能用於各種相關生物過程的研究和機理的探討。

中草藥長久以來就是藥物篩選的一個重要來源，很多中草藥及其成分都具有抗血管新生的功效。在本論文研究中，作為對源於中草藥抗血管新生藥物的繼續研究，我們的篩選工作主要集中在具有抗炎、抗風濕性關節炎和抗癌功效的中草藥。通過斑馬魚模型的篩選，三味中草藥：大黃、益智仁和沙姜顯示出一定的抗血管新生的活性，表明這三味中草藥含有相關活性成分。大黃的乙酸乙酯提取部位具有較強的抑制血管生長的功效。隨後對其主要活性組分——蒽醌類化合物進行篩選，發現其中三個化合物具有不同程度的血管生長抑制活性。化合物rhein是其中活性在高濃度時最強的一個，它能抑制人類臍靜脈內皮細的遷移以及影響相關基因的表達 (*vegfa*, *kdr*, *angiopoietin1/2* 和*tie1/2*)；益智仁和沙薑的正己烷和乙酸乙酯的提取部位同時在斑馬魚和人類臍靜脈內皮細胞模型上具有抑制血管新生的功效。其中，益智仁的兩個提取部位對於人類臍靜脈內皮細胞的增殖、遷移和血管的形成都具有不同程度的抑制作用。反式對甲氧基肉桂酸乙酯 (*trans*-ethyl-*p*-methoxycinnamate) 是沙薑中主要抗血管新生的活性物質，它能抑制人類臍靜脈內皮細胞的遷移和血管的形成，同時能降低除*vegfa*之外與血管新生相關基因的表達。

現有結果不僅表明中草藥的確能夠作為尋找用於癌症治療的抗血管新生藥物的一個重要來源，而且進一步確立了斑馬魚這個模型用於抗血管新生藥物篩選的強大潛力。

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Table of Contents

Chapter 2 Materials and Methods

Chapter 3 Results, Discussion and Conclusion

Chapter 4 General Discussion

List of figures and tables

Abbreviations

ANGPT, *angpt*: *angiopoietin*

Aq: aqueous

BuOH: *n*-butanol

CAM: chick chorioallantoic membrane

CT: computed tomography

D-MEM/F-12: Dulbecco's modified eagle medium: nutrient mixture F-12

DMSO: dimethylsulfoxide

EA: ethyl acetate

EAP: endogenous alkaline phosphatase

ECs: endothelial cells

EE: ethanol extract

EGFP: enhanced green fluorescent proteins

EGM-2: endothelial growth medium-2

FBS: fetal bovine serum

FDA: Food and Drug Administration

GFP: green fluorescent protein

Hex: *n*-hexane

hpf: hours post-fertilization

HUVECs: human umbilical vein endothelial cells

ISVs: intersegmental blood vessels

MMLV: moloney murine leukemia virus

MMPs: matrix metalloproteinases

MRI: magnetic resonance imaging

mTOR: mammalian target of rapamycin

MTT: 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

NBT/BCIP: nitro blue tetrazolium chloride/5-bromo-4-chloro-3- indolyl phosphate, toluidine salt

NP: vinorelbine (Navelbine®)-cis-platinum/carboplatin

NRP: neuropilins

NTMT: 100mM Tris pH 9.5, 100mM NaCl, 50mM MgCl2, 0.1% Tween-20

PBS: phosphate buffered saline

PBST: phosphate-buffered saline + 0.1% tween-20

PCR: polymerase chain reaction

PET: positron emission tomography

PlGF: placenta growth factor

P/S: penicillin-streptomycin

PTU: 1-phenyl-2-thiourea

RT: reverse transcription

SAR: structure–activity relationship

S.E.M.: standard error of the mean

SD: standard deviation

SIVs: subintestinal vessels

TCM: traditional Chinese medicine

TKIs: tyrosine kinase inhibitors

VEGF, *vegf*: vascular endothelial growth factor

WHO: World Health Organization

Chapter 1

General Introduction

1.1. Cancer and management

Cancer is a vast medical problem and a leading cause of death worldwide. According to World Health Organization (WHO) report in February, 2009 (Fact sheet N° 297), cancer accounted for 7.4 million deaths (around 13% of all deaths) in 2004, with an estimated 12 million deaths in 2030. In the Western world the commonest cancers are of the lung, breast, skin, gut and prostate gland.

Cancer is a generic term for a large group of diseases that can affect any part of the body. The cancer cells that can overcome the boundaries impeding unrestrained division will multiply, and in turn sustain the opportunity to acquire further aberrations that fuel growth, survival, invasion, and migration to, and establishment in, distant organs (DeVita et al., 2008). Many steps (Fig. 1-1) should occur before the early events that set a cell off on the path to become a cancer are finally expressed in the form of an invasive metastasizing malignancy. The biological characteristics of a cancer includes: self-sufficient proliferation of growth, refractory to inhibitory signals, survival without survival signals, unlimited replicative potential, recruitment of blood supply, invasion and metastasis, and loss of genomic stability (Hanahan and Weinberg 2000).

For many years the main methods of treating cancer have been surgery, radiotherapy and chemotherapy. Surgery is quick and effective, but there is no guarantee of complete removal and it is ineffective for metastasis. Radiotherapy is the use of high-energy radiation to kill cancer cells and shrink tumors, which is useful in the treatment of localized cancers, but may cause a significant amount of irradiation to the healthy normal tissues. Chemotherapy refers to the use of medications to treat

cancer. Among these treatments, chemotherapy has played a major role in cancer treatment for half a century. Chemotherapy is sometimes the first choice for treating advanced cancers for its being a systemic treatment. However, the majorities of current cancer chemotherapeutic agents in clinic use are not very specific and can affect DNA replication or cell division in any cell. Doses, and therefore cure rates, are limited by toxicity. Cure rates are also limited by inherent and acquired resistance to many forms of chemotherapy (King, 2000). The control of the primary tumor is indeed a concern, since this is usually responsible for the patient's symptoms. There may be unpleasant symptoms due to local spread, and failure to control the disease locally means certain death. Despite improving managements and efforts, the prognosis has not greatly improved because the most important cause of mortality is metastatic spread (Souhami and Tobias, 2005). This calls for novel cancer therapies and new group of anticancer agents for selectively targeting cancers without or with lower toxicity to normal tissues.

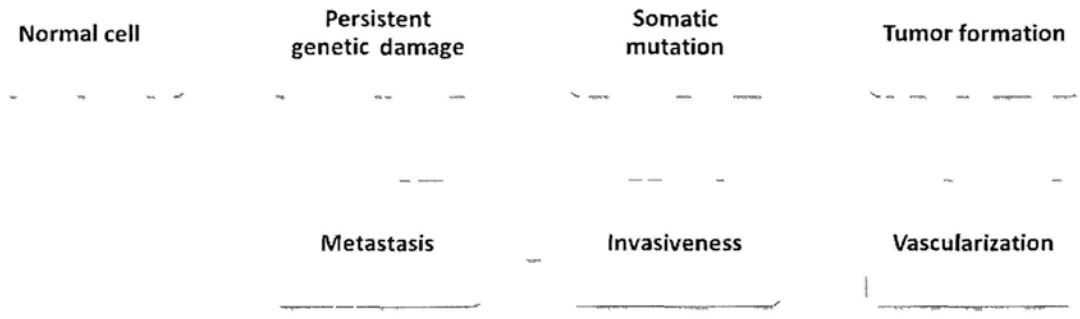


Figure 1-1* Steps in the development of a metastasing cancer
(*modified from Souhami and Tobias, 2005, Fig 3.1)

1.2. Anti-angiogenic cancer therapy

1.2.1. Angiogenesis

Angiogenesis refers to the growth of new blood vessels from pre-existing ones

(Fig. 1-2), and it is essential during fetal development, female reproductive cycle, and tissue repair. In contrast, uncontrolled angiogenesis promotes the 'angiogenic diseases' such as diabetic retinopathy, tumor growth and metastasis, rheumatoid arthritis, inflammatory diseases (Folkman, 1995) and retinopathies, while inadequate angiogenesis can lead to coronary artery disease. A balance between pro-angiogenic and anti-angiogenic growth factors and cytokines tightly controls angiogenesis (Gupta and Zhang, 2005).

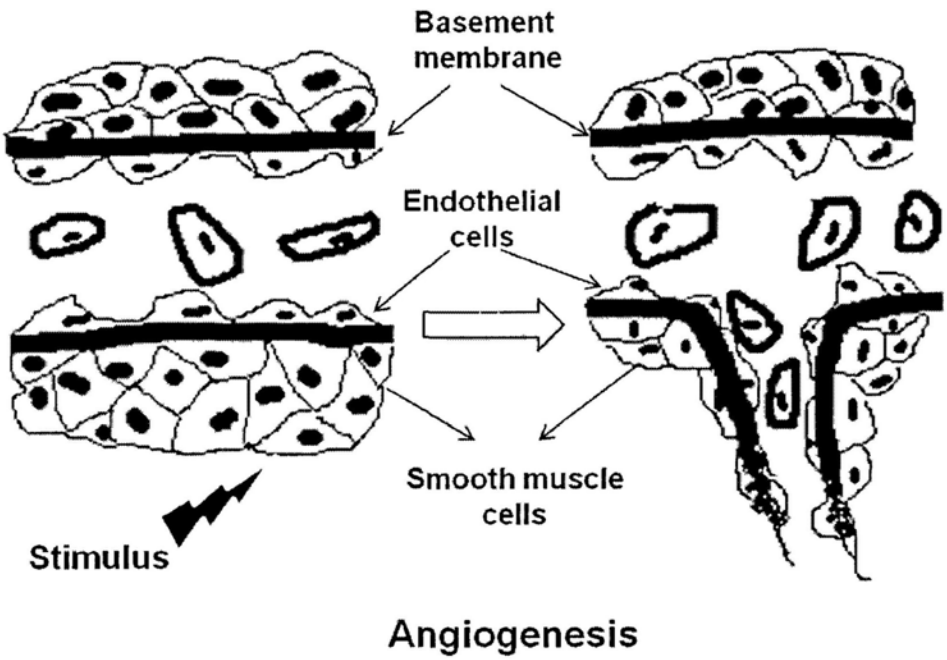


Figure 1-2. Angiogenesis demonstration

Ten sequential steps are involved in angiogenesis: (1) in response to hypoxia, injured or diseased tissues synthesize and release angiogenic factors; (2) angiogenic factors bind to their receptors on endothelial cells (ECs); (3) receptor binding leads to EC activation; (4) proteases are released to dissolve the basement membrane; (5) ECs migrate and proliferate; (6) adhesion molecules (e.g. integrin $\alpha v\beta 3$ and $\alpha v\beta 5$) help to pull the sprouting blood vessel forward; (7) matrix metalloproteinases (MMPs) are produced to dissolve the extracellular matrix and to initiate remodeling; (8) sprouting

endothelial cells roll up to form a blood vessel tube; (9) the EphB–ephrinB system regulates loop formation and (10) finally, pericytes are incorporated to stabilize the newly formed blood vessel (Fan et al., 2006). Blood flow then begins. Angiogenesis stimulators and inhibitors target one or more of these steps (Fig. 1-3).

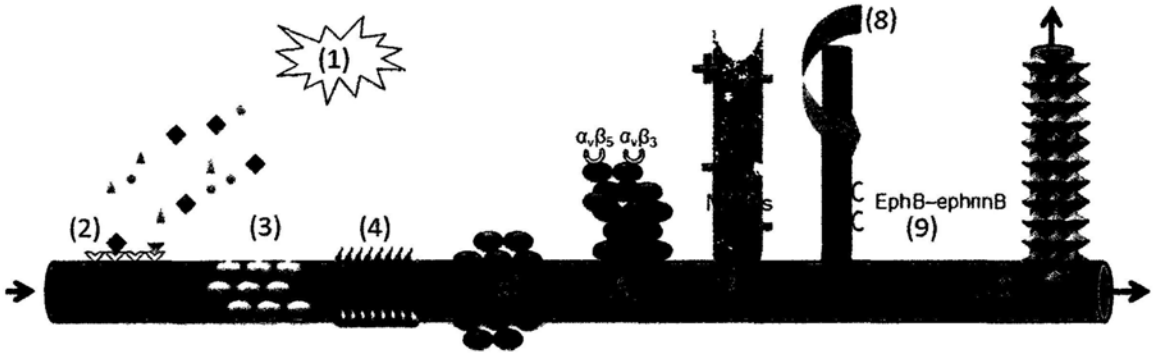


Figure 1-3*. Ten sequential steps of angiogenesis
(*modified from Fan et al., 2006).

1.2.2. Anti-angiogenic therapy

Angiogenesis has always been linked to the name of Dr. Judah Folkman who founded the entire field nearly 40 years ago. In a single hypothesis paper, Folkman (1971) was the first to hypothesize a linkage between angiogenesis, tumor growth and metastasis. Since the inflammation and cancer are closely linked with angiogenesis (Kobayashi and Lin, 2009), and the role of inflammation in the promotion of carcinogenesis was originally proposed by Virchow in 1863. Chronic and persistent inflammation contributes to cancer development and may be responsible for a substantial portion of tumor vascularization in "inflammatory angiogenesis", indicating that combating inflammation with appropriate drugs or substances could prevent inflammatory angiogenesis in carcinogenesis (Albini et al., 2005; Allavena et al., 2008; Bisacchi et al., 2003). The inhibition of angiogenesis, or anti-angiogenesis, is considered as a promising anticancer therapeutic strategy (Folkman, 1971; Ruegg

and Mutter, 2007; Bar and Onn, 2008; Griffioen, 2008; Cao, 2009). Folkman's original hypothesis has opened a new era in today's biomedical research and changed the face of cancer medicine, and his hypothetical publication remains one of the most cited publications in the research field of cancer and vascular biology, which has been cited over 3000 times according to the citation report by the Web of Sciences (Cao, 2010).

Angiogenesis plays a crucial role in the growth of both primary and metastatic tumors. Without its own blood supply, a tumor is restricted in its growth by the capacity of oxygen and nutrients to diffuse, which is consequently limited in size. In contract, a tumor that is able to generate its own blood supply can theoretically grow to any size. The tumor vasculature offers a potential target for treatment.

The process of angiogenesis is complex and involves soluble pro-angiogenic and anti-angiogenic factors that can be produced by the tumor clone itself or by non-neoplastic cells that are present in the tumor microenvironment. Angiogenic factors act on vascular endothelial cells, resulting in their migration or proliferation, or both. Endothelial cells, in turn, stimulate tumor cell growth. A gram of tumor may contain 10-20 million endothelial cells that not themselves neoplastic. Vascular endothelial growth factor (VEGF) appears to be an important factor in angiogenesis of malignancies because it is highly expressed in a variety of solid and hematologic tumors. Application of neutralizing antibodies to these pro-angiogenic factors and their corresponding receptors resulted in suppression of human tumors in murine models. It should also be noted that many of the soluble factors that are involved in angiogenesis have nonangiogenic effects, including but not limited to induction of proliferation of the tumor clone itself (Haskel, 2001).

Currently seven anti-cancer drugs with recognized anti-angiogenic properties in oncology have been approved by U.S. Food and Drug Administration (FDA) (Table

1-1). These agents comprise three categories: 1) monoclonal antibodies directed against specific proangiogenic growth factors and/or their receptors; and 2) small molecule tyrosine kinase inhibitors (TKIs) of multiple proangiogenic growth factor receptors; 3) inhibitors of mTOR (mammalian target of rapamycin). Moreover, two other FDA-approved angiogenic agents may indirectly inhibit angiogenesis through unknown mechanisms. In China, a recombinant human endostatin (Endostar, Simcere) has been approved by Chinese FDA for the treatment of patients with stage III/IV Non-Small Cell Lung Cancer in combination with NP (vinorelbine (Navelbine®)-cis-platinum/carboplatin) chemotherapy regimen. The role of such treatment in the overall schema of cancer treatment still need to be further elucidated, but anti-angiogenesis therapy will function in an ancillary role to conventional and potentially novel anticancer therapies.

1.2.3. Rationale and limitations of angiogenesis based therapy

Despite the tremendous heterogeneity of different cancers, increased angiogenesis is the only one common feature of different cancers, which provides an excellent target to fight with cancers.

Some of the major advantages of angiogenesis based treatment over others, such as surgery, radiotherapy and chemotherapy, are: (1) a single vessel provides the nutrition for thousands of tumor cells and only a damage at one point can block blood flow upstream and downstream. (2) The endothelial cell is a normal diploid cell, which is unlikely to acquire genetic mutations that render it drug resistant (Gupta and Zhang, 2005). (3) Endothelial cells are more easily accessed by anti-angiogenic targeting agents compared with drugs that act on tumor cells directly and have to penetrate large bulky masses. (4) Angiogenesis occurs in very limited circumstances in adults (wound healing and ovulation), thus anti-angiogenic therapies targeting

specific receptors on proliferating tumor endothelium potentially are safe and should avoid normal tissue toxicities (Wachsberger et al., 2003).

However, most of the angiogenesis based treatments that have worked well in experimental rodent models have not been successful in clinical trials. First of all, the target of anti-angiogenesis treatments is actively proliferating ECs. The number of proliferation ECs in human tumors is relative smaller than in rodent tumor models. Thus, additional markers associated specifically with specific pathological angiogenesis need to be identified. Secondly, angiogenesis process involves numerous molecules and biochemical pathways. Ordinarily, a drug only blocks a few specific molecules, while other molecules and pathways in the angiogenesis process continue to function. Therefore, for an individual drug, inhibition of angiogenesis did not reach 100 %. Thirdly, the drug delivery to the ischaemic site can be another problem. In addition, angiogenesis based therapy is more effective in small primary tumors than advanced cancers. Moreover, the normal physiological forms of angiogenesis, such as wound healing, reproductive angiogenesis, etc. would be adversely affected in a cancer patient receiving anti-angiogenic drugs. Despite these limitations, angiogenesis based treatments still hold promise in clinical trials combined with other therapies (Gupta and Zhang, 2005).

1.2.4. Preclinical models for discovering anti-angiogenic agents

The development of angiogenic inhibitors with different mechanisms of action relies on a range of preclinical models that mimic various steps of the angiogenic cascade. Knowledge of the mechanisms of action of the tested compound will dictate the choice of assay. Alternatively, the behavior of the compound in different assays may indicate its mechanism of action (Taraboletti and Giavazzi, 2004). Through decades of investigation, *in vitro* and *in vivo* anti-angiogenic models and assays are

well developed. However, numerous challenges still exist in properly modeling each of the steps involved in angiogenesis both *in vitro* and *in vivo*. Rakesh Jain and colleagues (1997) delineated aspects of an ideal angiogenesis model, which includes (1) a known release rate and spatial and temporal concentration distribution of angiogenic factors and inhibitors being studied for forming dose-response curves; (2) the assay should be able to quantify the structure of the new vasculature; (3) the assay should be able to quantify the function of the new vasculature (this includes ECs migration rate, proliferation rate, canalization rate, blood flow rate, and vascular permeability); and (4) *in vitro* responses should be confirmed *in vivo*. This final point is especially challenging as many models are carried out in two dimensions and may not take into account the more complex three-dimensional arrangements involved in cell and extracellular environment interactions (Ucuzian et al., 2007).

Table 1-1. Anti-angiogenic drugs

Monoclonal Antibody Therapies	Small Molecule Tyrosine Kinase Inhibitors (TKIs)	Inhibitors of mammalian target of rapamycin (mTOR)	Other Angiogenic Agents
Bevacizumab (Avastin®) Genentech	Erlotinib (Tarveca®) Genentech OSI Roche	Temsirolimus (Torisel®) Wyeth	Lenalidomide (Revlimid®) Celgene
Cetuximab (Erbitux®) Bristol-Myers Squibb ImClone	Sorafenib (Nexavar®) Bayer Onyx	Everolimus (Afinitor') Novartis	Thalidomide (Thalomid®) Celgene
	Sunitinib (Sutent®) Pfizer		rhEndostatin (Endostar/Endu- available only in China) Simcere

*modified from http://www.angio.org/understanding/inhib.php (The Angiogenesis Foundation)

1.2.4.1. In vitro models

In 1980, bovine capillary ECs were found to spontaneously form tubes when cultured in gelatin *in vitro*. Since then, many *in vitro* models have been designed mimicking many of the basic steps of the *in vivo* process (Ucuzian et al., 2007). *In*

vitro assays are designed to recapitulate each of the multiple events that constitute the angiogenic process. Some of them are very specific in analyzing a single event (proliferation, apoptosis, migration, production of proteases), whereas others provide a more complex picture of the process, involving multiple aspects of cell functions and interactions with the environment (Auerbach et al., 2003; Jain et al., 1997; Vailhe et al., 2001).

In vitro assays for the activity of anti-angiogenic compounds are usually based on the use of ECs, and focused predominantly on proliferation, migration and tubule formation. The choice of suitable ECs is a critical issue in setting up an *in vitro* assay. Immortalized endothelial cells are sometimes used because they provide an 'unlimited' source of cells. ECs from the human umbilical vein are most commonly used, as the source (the umbilical cord) is easily available and cell isolation is relatively simple. When developing inhibitors of tumor angiogenesis, it would be ideal to use tumor-derived ECs (Alessandri et al., 1999). However, practical difficulties in their isolation from tumor tissues and maintenance in culture have limited their use in preclinical studies (Taraboletti and Giavazzi, 2004).

1.2.4.1.1. EC proliferation assay

Cell proliferation assays are easy to perform and highly reproducible. ECs established in culture are capable of cell division, thus, the effects of compounds on cell proliferation can be measured by mitogenic assays (incorporation of thymidine, 5-bromodeoxyuridine). Although EC proliferation is often considered the leading assay for anti-angiogenic activity, the ability of a compound to affect proliferation is not sufficient to prove such activity. Indeed, any cytotoxic compound can inhibit the proliferation of endothelial cells, but only EC-specific antiproliferative compounds can be considered selectively anti-angiogenic. This issue has been particularly

relevant in the definition of the anti-angiogenic activity of cytotoxic chemotherapeutics. Also, it should be kept in mind that the inhibition of endothelial cell proliferation is not a requisite for anti-angiogenic activity. On the contrary, a lack of antiproliferative activity may indicate low toxicity for anti-angiogenic compounds acting via non-anti-proliferative mechanisms (Taraboletti and Giavazzi, 2004).

1.2.4.1.2. EC migration assay

The Boyden chamber assay is the most commonly used test for ECs migration (Alessandri et al., 1983). It can be performed in modified or unmodified chambers, or using disposable membrane inserts. The lower compartment of the chamber (containing the attractant) is separated from the upper (containing the endothelial cells) by a matrix-coated polycarbonate filter with pores small enough to allow only the active passage of the cells (5-12 mm pore size). Migration occurs rapidly, and in 4-6 h the cells have migrated through the filter and can be stained and counted. The effects of the test agents can be evaluated by staining and counting the cell number that migrated through the filter (Falk et al., 1980).

1.2.4.1.3. EC tube formation assay

Following their proliferation and migration, ECs align and establish contact with each other, resulting in capillary-like structures. The ECs, seeded on a permissive matrix substrate (such as Matrigel), can rapidly form capillary-like structures to mimic this event (Vailhe et al., 2001). The presence of a lumen and the formation of tight junctions between the ECs confirmed by electron microscopy are considered proofs that these assays truly recapitulate the morphogenetic events leading to capillary formation (Auerbach et al., 2003). Depending on whether the formed capillaries lie in one plane or are spatially organized within the whole matrix, the

assays can be two- or three-dimensional. Image-analysis techniques are used to quantify the response by measuring the number and length of the tubes, the area covered by the capillary-like network, and/or the number and complexity of the connections (Taraboletti and Giavazzi, 2004).

1.2.4.2. In vivo models

Due to the complex cellular and molecular activities of angiogenic reactions, *in vivo* studies are more informative than *in vitro* studies. The ideal *in vivo* assay for evaluating the efficacy of anti-angiogenic compounds should permit the scrutiny of vessel formation and maturation in a natural environment, and, at the same time, be suitable for rapid, easy and possibly quantitative analysis. Although several ingenious *in vivo* assays have been developed for measuring angiogenic processes, no single assay that is optimal for all situations has been described due to the heterogeneity of tissues and the molecular and cellular complexities of angiogenic reactions. Most of them have the disadvantage of requiring surgical skills, of being difficult to quantitate, of inducing angiogenesis in irrelevant sites, and of lacking reproducibility. Finally, because tissue is often injured in the process, inflammatory reactions, which in turn cause vasoproliferation, can disturb the results.

Indeed, many workers with expertise in this area have expressed certain disillusionment with the available assays. Some assays allow for the detection and evaluation of vessels throughout the duration of the experiment, as angiogenesis is induced in sites that are naturally (cornea) or artificially (chambers) accessible. In other tests (Matrigel plug), the evaluation is made at one time-point only. In some cases, new vessels can be induced in a previously avascular site (such as the cornea, which is naturally avascular, or in exogenous supports such as Matrigel or sponges) (Norrby, 2006; Taraboletti and Giavazzi, 2004).

1.2.4.2.1. The corneal micropocket assay

This is perhaps the most traditional assay of angiogenesis. Folkman and associates introduced the corneal micropocket assay in 1974 (Gimbrone et al., 1974). Angiogenesis is induced by the implantation of slow-release pellets containing the test substances (including the angiogenic growth factors) into micropockets produced surgically in the avascular cornea of rabbits, rats or mice. It is possible to evaluate the interaction of multiple factors by either incorporating different substances into the same pellet or by implanting separate pellets into two adjacent pockets in the same cornea. Angiogenesis is quantified by subjective or computer-assisted analysis of the number and growth rate of newly formed vessels, using a slit-lamp stereomicroscope. An advantage of this assay is that the cornea is naturally avascular and hence the interpretation of result is clearer than in other bioassays such as the chorioallantoic membrane. Moreover, given the accessibility of the site, the angiogenic response can be monitored daily, and permits non-invasive observation and long-term monitoring. The assay is regarded as technically difficult and surgery becomes more difficult as eye size decreases in animals smaller than the rabbit. The difficulty in quantifying the response, the high costs and the limited number of performable tests, especially when using rabbits, are also its limitations. Importantly, ethical problems can be encountered when using an assay that involves a major sensory organ (Norrby, 2006; Taraboletti and Giavazzi, 2004). Additional disadvantages of the assay include (1) the short duration of the assay (less than 7 days) (Knighton et al., 1991); (2) the time required; (3) the cost associated with the use of rabbits; and (4) the low number of substances that can be tested (Ribatti and Vacca, 1999). Since the surgical technique is demanding and time-consuming, relatively few animals (~20 mice) can be grafted in a single setting (Auerbach et al., 2003; Knighton et al., 1991). One additional disadvantage is that tumors other than those that originate from an animal of the same

genetic background as the test animal may induce an immune response once they are vascularized (Ziche, 2001).

1.2.4.2.2. The chick chorioallantoic membrane (CAM) assay

Folkman and associates (Gimbrone et al., 1974) also introduced the CAM assay in 1974. Originally used by embryologists to study the developmental potential of embryonic tissue grafts, the assay has been adopted for testing angiogenic and/or anti-angiogenic compounds in the naturally occurring vasculature of the CAM (Ribatti et al., 2001; Ribatti and Vacca, 1999). Test compounds are added to a support (such as collagen or gelatin sponges or synthetic polymers such as Elvax 40 or Hydron) and placed on the extra-embryonic membrane. The angiogenic response is measured by subjective or computer-assisted counting of the new vessels, or by histological analysis of the membrane.

Largely due to its simplicity and low cost, it is currently the most widely used *in vivo* model for the study of angiogenesis (Staton et al., 2004). Some skill in evaluating the response is required, as the presence of natural vessels can interfere with the results. Any irritant, such as the shell dust generated during the excision of a rectangular opening in the shell, and any sliver of shell membrane that protrudes and touches the CAM will cause an inflammation-mediated angiogenic reaction (Jakob et al., 1978; Knighton et al., 1999). The membrane is also extremely sensitive to changes in oxygen tension, so careful control of the pO2 level is essential. Drugs that require metabolic activation cannot be assessed using this method (Auerbach et al., 2000). Attempts have been made to make this assay more quantitative (Nguyen et al., 1994; Seandel et al., 2001).

1.2.4.2.3. The sponge implant assay

Andrade and associates (1987) introduced an assay in which compounds of interest were injected directly into a sterile polyester sponge that was implanted subcutaneously in the rat followed by measurement of blood flow in the implants as they become vascularized, which enables objective, continuous and reproducible studies on angiogenesis (Andrade et al., 1987). Different kinds of modified sponge implant assay have been described. Due to its replicating the hypoxic tumor microenvironment, this assay is suitable for the study of tumor angiogenesis (Hasan et al., 2004).

A common problem is that the matrix implants can cause non-specific inflammatory host responses (Jain et al., 1997), and sponge composition can vary, making inter-experimental comparisons difficult (Staton et al., 2004). In addition, the use of radioactive gas is a complication and the animals have to be housed singly (Andrade, 2001).

1.2.4.2.4. The Matrigel plug assay

Passaniti and co-workers (1992) introduced this assay, which takes advantage of the peculiar characteristics of Matrigel, an extract of the Engleberth-Holm-Swarm tumor consisting of extracellular matrix components and growth factors. Although liquid at 4 ℃, Matrigel reconstitutes into a gel or plug at body temperature when injected subcutaneously into mice, where it is progressively surrounded by granulation tissue, allowing the slow release of the substance. The plug supports an intense vascular response when supplemented with angiogenic factors. Some 5-10 days after implantation, the angiogenic response is quantified either by measuring the haemoglobin content of the Matrigel (as an index of functional, blood-containing vessels) or by immunohistochemical analysis of the pellet (i.e. by counting structures

positive for CD31, a specific marker of ECs). This is an assay that does not require any surgical procedures and is not difficult to administer, although it is considered by some workers to be time-consuming (Staton et al., 2004). In addition, this assay is suitable for large-scale screening and can carry out rapid quantitative analysis in chambers (Norrby, 2006).

The limitations of the Matrigel assay concern partly that the composition of Matrigel is not chemically defined and it is difficult to make plugs uniform in 3-dimensional shape (except in chambers). In addition, the subcutaneous tissue is not highly relevant for tumor growth, which poses questions about the significance of the results for oncological applications. However, the technical simplicity, versatility and use of the mouse as the recipient host make this assay a convenient *in vivo* model (Norrby, 2006).

1.2.4.2.5. Whole small animal angiogenesis models: zebrafish

Zebrafish are small tropical freshwater fish (approximately 3–4 cm long as adults) with a short generation time (about 3 months) that can be housed in large numbers and in a relatively small space (Norrby, 2006).

Traditionally, zebrafish has been mainly used as a model organism in the fields of molecular genetics and developmental biology of vertebrates. Many zebrafish homologues of mammalian genes have been cloned and found to have similar functions. More recently, its value as a model organism for drug target discovery, target validation, drug discovery strategies and toxicological studies has begun to be recognized for its fecundity, morphological and physiological similarity to mammals and the ease with which large phenotype-based screens can be performed. Therefore, zebrafish could be a useful and cost-effective alternative to some mammalian models, such as rodents, dogs and pigs (Crawford et al., 2008;

Langheinrich, 2003; Zon and Peterson, 2005). Many drugs tested in zebrafish have demonstrated similar effects to those observed in humans or other mammalian models. Indeed, more and more evidence shows that anti- and pro-angiogenic molecules that are effective in mammals are likely to exert corresponding effects in the zebrafish (Langheinrich, 2003; Parng et al., 2002). Moreover, as a proximate for the human system, zebrafish is the only vertebrate system currently used for rapid *in vivo* compound screening, and addition to the *in vivo* models for studying anti-angiogenic agents (Taraboletti and Giavazzi, 2004). Besides providing a powerful platform for drug screening, zebrafish model can also be used for probing biological processes to identify the molecular pathways, so as to generate insights into mechanisms and testable hypotheses about how a compound works (He et al., 2009a).

Unlike the traditional drug screens using cell lines or *in vitro* protein binding assays—neither of which represents the normal physiology of multi-cellular organisms, the use of zebrafish would allow the selection of bioactive compounds in a whole organism and in cells undergoing normal cell–cell and cell–matrix interactions (Stern and Zon, 2003). Compared with the existing assays, the major advantages of a zebrafish-based assay are obvious: (1) hundreds of compounds can be tested simultaneously using a microplate format, (2) the assay is relatively cost-effective, fast, truly quantitative and suitable for large-scale screening, and (3) embryo maintenance, compound addition and embryo assessment are technically simple (Serbedzija et al., 1999; Zon and Peterson, 2005; Norrby, 2006). In addition, the optical transparency and ability to survive for 3–4 d without functioning circulation make the zebrafish embryo amenable for direct and continuous microscopic inspections of vascularization. Its transparency becomes even more useful when fluorescent markers are used to label specific populations of cells, including ECs (Lawson and Weinstein, 2002; Tran et al., 2007). These features make zebrafish

embryos an attractive model for identification of anti- or pro-angiogenic agents.

However, given the heterogeneity of tissues and the molecular and cellular complexities of angiogenic reactions, it is not surprising that no single assay is optimal for all situations. There are clearly also limitations in working with the zebrafish model. First, zebrafish is an embryonic, non-mammalian model and the vasculature in zebrafish follows a similar but not entirely identical plan to that of higher vertebrates (Norrby, 2006; Serbedzija et al., 1999). Second, due to differences in molecular weight and hydrophobicity, not all small molecules are readily absorbed by zebrafish embryos, possibly leading to false negative results. Third, as zebrafish are vertebrates, country-specific animal rights legislations and institutional bioethics regulations should be included in planning experiments (Crawford et al., 2008).

1.2.4.3. Assays for studying anti-angiogenesis in tumor models

Preclinical investigations of the antineoplastic activity of angiogenic inhibitors and vascular targeting compounds require final validation in tumor models. Murine tumors in syngeneic mice or rats and human tumors xenografted into immunodeficient mice can be used.

The classical model of the Lewis lung carcinoma has been extensively used to test anti-angiogenic compounds because it is rapid, reproducible and metastatic. In addition, the host response, including the vasculature, in a syngeneic mouse is intact. However, the subcutaneous transplantation does not reflect its original site.

Xenograft models have the advantage of reflecting the biology of human tumors, allowing continuous delivery of angiogenic factors produced by tumor cells, thus mimicking the initial stages of tumor angiogenesis and metastasis. Tumors are often transplanted orthotopically in the organ site of origin. Unfortunately, xenografts do grow in a xerogeneic and immunodeficient recipient, with consequent impairment of

the host response (Taraboletti and Giavazzi, 2004).

Recent studies have shown the feasibility of injecting human melanoma cells in zebrafish embryos to follow their fate and study their impact on zebrafish development (Topczewska et al., 2006), indicating the feasibility of using zebrafish as a biosensor for tumor-derived signals. Nicoli and Presta (2007) described a novel experimental procedure to study tumor angiogenesis based on the grafting of mammalian tumor cells in the proximity of the developing subintestinal vessels (SIVs) plexus at 48 hours post-fertilization (hpf) zebrafish embryos. Pro-angiogenic factors released locally by the tumor graft will induce a neovascular response originating from the developing SIVs by stimulating the migration and growth of sprouting vessels toward the implant. This can be observed at macroscopic and microscopic levels after whole-mount alkaline phosphatase staining of wild-type zebrafish embryos, or by fluorescence microscopy in transgenic embryos in which endothelial cells express the green fluorescent protein (GFP). Angiogenesis inhibitors added to the injected cell suspension or to the fish water prevent tumor-induced neovascularization. The assay is rapid and inexpensive, and allows a simple and unambiguous identification of newly formed ectopic vessels driven by the tumor graft in live embryos, which represents a novel tool for investigating tumor angiogenesis and for anti-angiogenic drug discovery. Also, it is suitable for identifying genes involved in tumor angiogenesis by antisense morpholino oligonucleotide strategy.

1.2.4.4. Imaging of angiogenesis

Advances in imaging are transforming our understanding of angiogenesis and providing a way to accurately and reliably assess changes in angiogenic vasculature in preclinical models and human disease. The number and spacing of blood vessels, blood flow and vascular permeability can be quantified. And it is possible to analyze

cellular and molecular abnormalities in blood vessel walls (McDonald and Choyke, 2003).

Microscopic imaging methods ranging from fluorescence, confocal and multiphoton microscopy to electron microscopy are particularly useful for elucidating structural and functional abnormalities of angiogenic blood vessels, as well as cellular and molecular features of the microvasculature. When applied intravitally, these techniques are particularly powerful for the high-resolution analysis of tissue specimens. Intravital microscopy has provided unprecedented insights into various molecular and cellular processes *in vivo* with high spatial and temporal resolution down to the subcellular level and has revealed new approaches to improved detection and treatment (Jain et al., 2002). Fluorescent (the GFP reporter system) and bioluminescent (the luciferase reporter system) imaging techniques are of particular value for mapping specific molecular events and tracking cells. The zebrafish GFP reporter system has shown the feasibility of *in vivo* endothelial imaging in living animals. GFP transgenic zebrafish driven by a murine *tie2* promoter and zebrafish *fli1* promoter in ECs and stable germline transgenic lines have been prepared (Lawson and Weinstein, 2002; Motoike et al., 2000). The establishment of vascular-specific transgenic zebrafish with fluorescently "tagged" blood vessels enables long-term, time-lapse analysis of the ECs and facilitates high-resolution imaging studies of developing blood vessels *in vivo* (Cha and Weinstein, 2007).

Clinical methods, such as magnetic resonance imaging (MRI), computed tomography (CT), positron emission tomography (PET), ultrasonography and optical imaging provide noninvasive, functionally relevant images of angiogenesis in animals and humans. Compared with microscopy, however, they give images of living tissues deep within the body with much lower resolution and specificity, and generally cannot resolve vessels of the microcirculation. Future challenges include developing

new imaging methods with higher resolution and specificity, and choosing the suitable benchmarks of microscopic techniques to interpret clinical images (McDonald and Choyke, 2003).

1.3. Medicinal herbs for discovering of anti-angiogenic agents

Traditional Chinese medicine (TCM) has long been recognized as a rich source for discovering drugs (Tang et al., 2003), and so far, only a few Chinese herbs have been reported with anti-angiogenesis properties, including *Berberis paraspecta* Andrent, *Catharanthus roseus* (L.) G. Don., *Coptis chinensis* Franch., *Polygonum cuspidatum* Siebold & Zucc., *Scrophularia ningpoensis* Hemsl., *Scutellaria baicalensis* Georgi, *Scutellaria barbata* Don., *Taxus chinensis* (Pilger) Rehd. (Wang et al., 2004), *Rabdosia rubescens* (Hemsl.) Hara (Sartippour et al., 2005), *Ganoderma lucidum* (Leyss.ex Fr.) Karst. (Yuen and Gohel, 2005) and *Ulmus davidiana* var. *japonica* Rehder (Jung et al., 2007).

Several anti-angiogenic components have also been reported from TCM (Fig.1-4), including curcumin from the rhizome of *Curcuma longa* Linn (Sreejayan and Rao, 1997; Arbiser et al., 1998; Garcia-Cardena and Folkman, 1998; Gururaj et al., 2002; Shim et al., 2003), camptothecin from the Chinese tree *Camptotheca acuminate* Decaisne (Clements et al., 1999; Tsuchida et al., 2003; Kamiyama et al., 2005), quercetin from *Sophora flavescens* Alt. and *Hovenia dulcis* Thunb. as well as many fruits such as apples, onions, raspberries, red grapes, citrus fruit, cherries, broccoli and leafy greens (Igura et al., 2001; Banerjee et al., 2002; Huynh et al., 2003; Tan et al., 2003; Ma et al., 2004; O'Leary et al., 2004), resveratrol from *Polygonum cuspidatum* Sieb. & Zucc. as well as in red wine, peanuts and mulberries (Kimura and Okuda, 2001; Cao et al., 2002), ginkgolide B from *Ginkgo biloba* L. (Zhang et al., 2002; DeFeudis et al., 2003), baicalein, baicalin from *Scutellaria baicalensis* Georgi

(Liu et al., 2003), erianin from *Dendrobium chrysotoxum* Lindl. (Gong et al., 2004), geniposide from the fruits of *Gardenia jasminoides* Ellis (Koo et al., 2004a, b), ginsenosides Rb1 and Rg3 from ginseng (Sengupta et al., 2004; Zhang et al., 2006), pseudolarix acid B from *Pseudolarix kaempferi* (Tan et al., 2004), 2',4'-dihydroxy-6'-methoxy-3',5'-dimethylchalcone from the dried flower *Cleistocalyx operculatus* (Roxb.) Merr.. et Perry (Zhu et al., 2005), tanshinone IIA and cryptotanshinone from *Salvia miltiorrhiza* Bunge (Hur et al., 2005), heyneanol A from the roots of *Vitis amurensis* Rupr. (Lee et al., 2006) and triptolide from *Tripterygium wilfordii* Hook. f. (He et al., 2010).

1.4. The project aims

As mentioned above, quite a few TCMs and components possess anti-angiogenic activity. These active herbs and their components may provide a good source for discovering new potential anti-angiogenic agents and developing new anti-cancer drugs. Our objectives were to (1) identify potential anti-angiogenic compounds from selected TCMs for the treatment of cancer, (2) elucidate their preliminary anti-angiogenesis mechanism, and the efficacy of the selected TCMs can be further confirmed by the anti-angiogenic compounds.

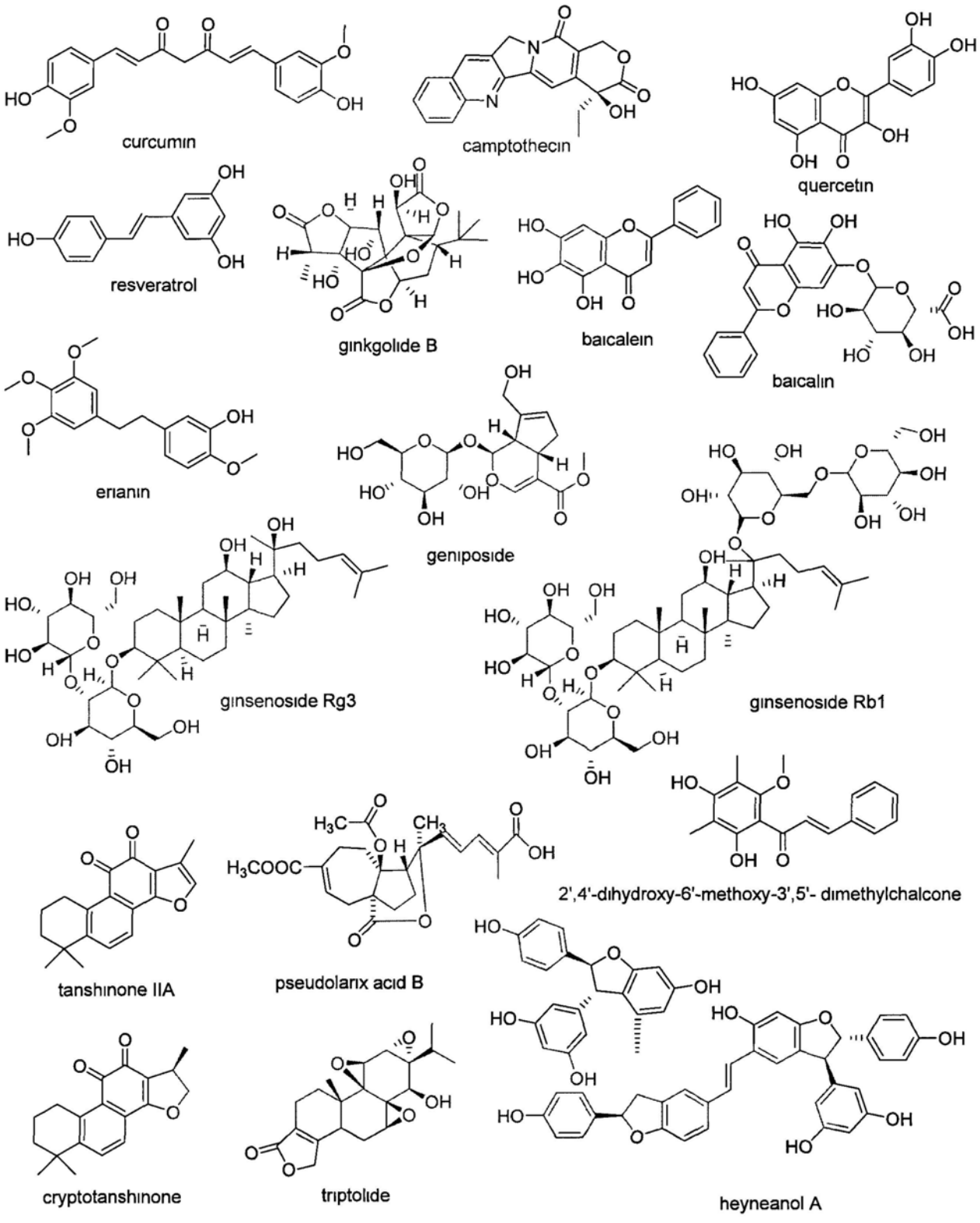


Figure 1-4 Anti-angiogenic components from TCMs

Chapter 2
Materials and Methods

2.1. Animals, medicinal herbs, cell culture and chemicals

Zebrafish embryos used for screening were obtained from natural spawning of wild type fish bought from local pet stores in Hong Kong, and the *Tg(fli1a:EGFP)y1* line was obtained from the Zebrafish International Resource Center (5274, University of Oregon, Eugene). The zebrafish were both maintained in flow-through aquaria (36 L) at 28.5 ℃ on a 14/10-h (light/dark) photoperiod. The handling of fish was licensed by the Government of the Hong Kong Special Administrative Region and endorsed by the Animal Experimentation Ethics Committee of The Chinese University of Hong Kong.

Alpinia officinarum and *Alpinia katsumadai* were purchased from a herb shop in Hong Kong, and *Rheum palmatum* was purchased from a herb shop in Changde, Hunan Province, PR China. All other fifteen herbs were purchased from an herb shop in Shenzhen, Guangdong Province, P. R. China, in February 2008. All voucher specimens (Table 2-1) were deposited in the Museum of Chinese Medicine, Institute of Chinese Medicine, The Chinese University of Hong Kong.

Primary HUVECs were purchased from American Type Culture Collection (ATCC®, Manassas, VA). HUVECs at early passages (passages 10-15) were used in all the experiments, and were cultured in gelatin-coated plates with Complete Growth Medium [Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (D-MEM/F-12), 10% fetal bovine serum (FBS)] (GIBCO, Gaithersburg, MD) containing 0.1 mg/ml heparin, 0.03-0.05% mg/ml endothelial cell growth supplement, and 1% penicillin-streptomycin (P/S) (SIGMA-ALDRCH, St. Louis, MI). Cells were incubated at 37 ℃ in 5% CO_2 in air.

For *Alpinia oxyphylla,* primary HUVEC and endothelial growth medium-2 (EGM-2) were purchased from Lonza Group Ltd (Walkersville, MD). HUVEC at early passages (passages 2-7) were used in all the experiments. HepG2 and MCF7 cells were obtained from American Type Culture Collection (ATCC®, Manassas, VA). HepG2 cells were propagated in Eagle's minimum essential medium (MEM, ATCC®, Rockville, MD) with 10% heat-inactivated FBS and 1% P/S (Life Technologies, Grand island, NY). MCF7 cells were propagated in RPMI-1640 medium (Gibco, Gaithersburg, MD) with 10% FBS, 1% P/S and 0.01mg/ml bovine insulin (SIGMA-ALDRCH, St. Louis, MI). All cells were maintained in a 5% CO_2 humidified atmosphere at 37 ℃.

Endogenous alkaline phosphatase (EAP) staining was assayed with phosphatase substrate kit (Pierce, Rockford, IL) and nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate, toluidine salt (NBT/BCIP) ready-to-use tablets (Roche Diagnostics GmbH, Germany). Anthraquinone derivatives, *trans*-ethyl *p*-metnoxycinnamate (KG-1) and kaemperol (KG-2) were obtained from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China).

2.2. Preparation of ethanol extracts, fractions and thin layer chromatography (TLC) identification

The pulverized herbs were extracted with 95% ethanol three times (reflux, 2 h each time), and then the ethanol extract (EE) was obtained after evaporation under reduced pressure. For solvent fractionation, EE was suspended in distilled water and extracted successively with equal volumes of *n*-hexane (Hex), ethyl acetate (EA) and *n*-butanol (BuOH), leaving a residual aqueous fraction (Aq). Each fraction was evaporated under reduced pressure to yield the extracts of Hex, EA, BuOH and Aq fractions, respectively.

Thin layer chromatography (TLC Silica gel 60, Merck KGaA, Darmstadt, Germany) was used to identify the presence of anthroquinone derivatives. Solution spot containing the sample was applied to a plate, about one centimeter from the base. Then the plate was dipped in to a suitable solvent with an appropriate ratio and placed in a sealed container. Due to capillary action, the solvent moved up the plate and met the sample mixture, which was dissolved and carried up the plate by the solvent. Different compounds in the sample mixture traveled at different rates due to the differences in their attraction to the stationary phase, and their solubility in the solvent. After the solvent reaching the top of the plate, the plated was taken out and dried. Spots on TLC plates were visualized by spraying Vanillin-H_2SO_4 solvent (15 g vanillin + 250 mL ethanol + 2.5 mL sulfuric acid), or under a blacklight (UV_{254}). The adsorbent layer will thus fluorescence light green by itself, but spots of analyte quench this fluorescence.

2.3. Embryo handling

Zebrafish embryos were generated by natural pairwise mating as described by Westerfield (1993). The embryos were maintained in embryo water (0.2 g/L Instant Ocean® Salt, Spectrum Brands, Madison, WI) at 28.5 ℃ incubator. Any dead or unfertilized embryos should be cleaned out in the first few hours of development and periodically thereafter to prevent developmental delay. They were manually dechorionated with forceps at 24 hpf immediately prior to drug treatment.

2.4. Drug administration

24 hpf zebrafish embryos were arrayed in 96-well plate (Fig. 2-1), one embryo per well, and incubated with 100 μl of embryo water per well containing various

concentrations of an extract at 28.5 ℃ for 48 h. Dimethylsulfoxide (DMSO, Sigma) was used as carrier control (0.2 %). For chrysophanol and physcion, the mixture of 0.5% DMSO and 0.2 mM NaOH was used as carrier control.

Table 2-1. Selected TCMs

Family	Species	Vouch No.
Annonaceae	*Fissistigma glaucescens* (烏骨藤)	2010-3261
Aquifoliaceae	*Ilex cornuta* (苦丁茶)	1065
Asteraceae	*Xanthium sibiricum* (蒼耳子)	2010-3254
Cucurbitaceae	*Gynostemma pentaphyllum* (絞股藍)	2010-3259
Gramineae	*Lophatherum gracile* (淡竹葉)	2010-3253
Meliaceae	*Melia toosendan* (川楝子)	2010-3252
Orchidaceae	*Gastrodia elata* (天麻)	2010-3257
Paeoniaceae	*Paeonia lactiflora* (赤芍)	2010-3258
Paeoniaceae	*Paeonia suffruticosa* (牡丹皮)	2010-3251
Poaceae	*Imperata cylindricl* (白茅根)	2010-3260
Polygonaceae	*Rheum palmatum* (大黃)	2008-3023
Ruscaceae	*Polygonatum odoratum* (玉竹)	2010-3255
Sapindaceae	*Litchi chinenesis* (荔枝核)	2010-3250
Solanaceae	*Lycium chinense* (地骨皮)	2010-3256
Zingiberaceae	*Alpinia katsumadai* (草豆蔻)	2010-3264
Zingiberaceae	*Alpinia officinarum* (高良姜)	2010-3262
Zingiberaceae	*Alpinia oxyphylla* (益智仁)	2009-3230
Zingiberaceae	*Kaempferia galanga* (沙姜)	2010-3263

2.5. Quantitative EAP assay on zebrafish embryo

During zebrafish development, the stage between 24 and 72 hpf has the highest angiogenic activity and quantitative EAP assay was performed as described (Parng et al., 2002). Drug-treated embryos at 72 hpf were dehydrated by increasing concentration of ethanol. Then the embryos were washed three times with diethanolamine buffer (Pierce, Rockford, IL). Next, the embryos were stained according to the protocol described in phosphatase substrate kit. After staining, 50 μl 2 M NaOH was added to stop the reaction. The optical density of soluble end product was measured at 405 nm using a microplate reader. Vessel growth was presented as percentage in optical density compared with control [% vessel formation = (OD treated day 3 − OD control day 1) / (OD control day 3 − OD control day 1) ×100%]. Each assay was repeated at least three times. No additional maintenance was required as the embryo yolk sac supplies nutrients during the process of the experiment.

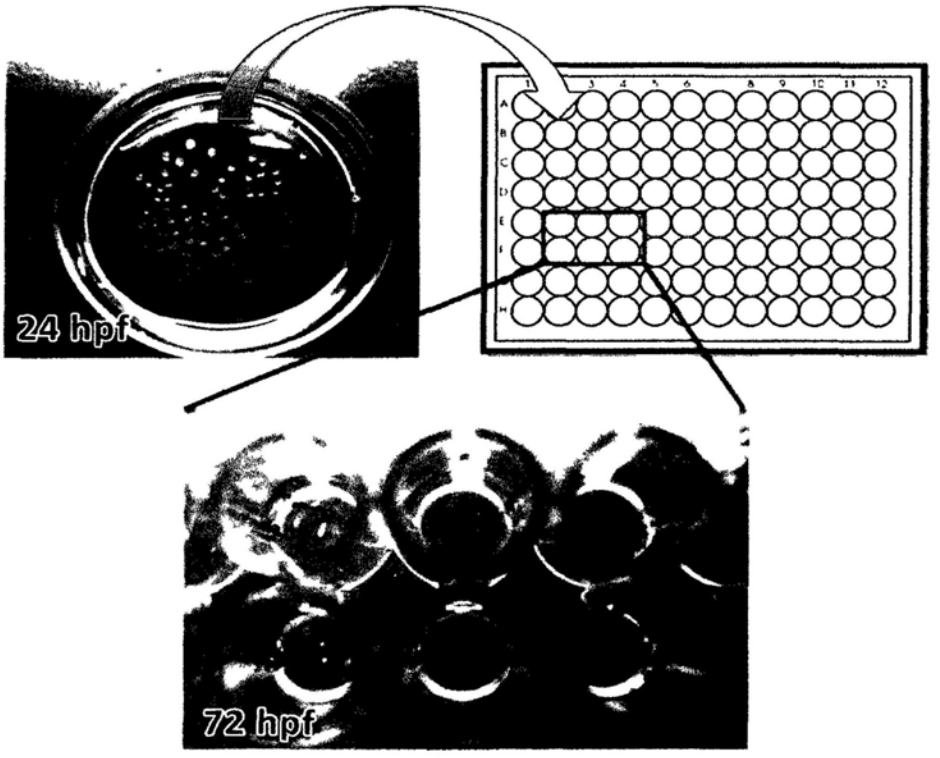


Figure 2-1 Zebrafish embryos arrangement in 96-well plate

2.6. Microscopic imaging

For better documentation of the blood vessels in embryos, both wild-type and *Tg(fli1a:EGFP)y1* zebrafish embryos were used. On wild-type zebrafish embryos, we used EAP staining for visual inspection of intersegmental blood vessels (ISVs) with NBT/BCIP substrate to stain the blood vessels. Embryos at 24 hpf were incubated with embryo water containing 1-phenyl-2-thiourea (PTU, final concentration 0.2 mM) before drug administration. At 72 hpf, embryos were fixed with 4% paraformaldehyde in PBST (phosphate-buffered saline + 0.1% tween-20) for 30 min. Then the embryos were dehydrated with ethanol, rinsed with PBST, and equilibrated with NTMT (100 mM Tris pH 9.5, 100 mM NaCl, 50 mM MgCl2, 0.1% Tween-20). The staining reaction was started by incubating embryos with NBT/BCIP solution for about 15–30 min according to the protocol described for the NBT/BCIP ready-to-use tablets. After staining was completed, the embryos were washed with PBST.

On *Tg(fli1a:EGFP)y1* zebrafish embryos, observation was made on Enhanced Green Fluorescent Proteins (EGFP) expressed in all endothelial cells of the vasculature in ISVs and SIVs plexus at 48 and 72 hpf, respectively, after drug treatment at 24 hpf (Lawson et al., 2002; Raghunath et al., 2009; Tran et al., 2007). The embryos at 24 hpf were incubated with embryo water containing 0.2 mM PTU before drug administration in order to prevent pigment development. The embryos were examined for blood vessel development using an Olympus IX71S8F-2 inverted microscope (Olympus, Tokyo, Japan). Dead or unhealthy embryos were removed.

2.7. Total RNA isolation, reverse transcription and semi-quantitative polymerase chain reaction (PCR)

Total RNA samples were isolated from three embryos using Tri-Reagent (Molecular Research Center, Cincinnati, OH) according to the manufacturer's

protocol. Reverse transcription (RT) was performed at 37 ℃ for 2 h in a total volume of 10 μl reaction solution containing 1×moloney murine leukemia virus (MMLV) reverse transcriptase buffer, 0.5 mM of each dNTP, 0.5 μg oligo(dT), and 100 U MMLV reverse transcriptase (Invitrogen, Carlsbad, CA). The primers were synthesized by Invitrogen. The cycle numbers were optimized according the methods described by Wang and Ge (2003) (Fig. 2-2), and the cycle numbers of half maximal amplification were used for subsequent semi-quantitative RT-PCR (Table 2-2). The PCR was carried out in a volume of 15 μl containing 1:15 diluted RT reaction product, 1× PCR buffer, 0.1 μM of each primer, and 0.375 U *Taq* polymerase, using the iCycler Thermal Cycler (Bio-Rad) with an annealing temperature of 61 ℃. All the PCR products were visualized in 1.6% agarose gel stained with ethidium bromide.

2.8. MTT assay

MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] assay was performed as described (Kim et al., 2009). HUVECs (4000 cells/well) were seeded in 96-well plate in the Complete Growth Medium for 12 h for attachment. Then cells were treated for 48 h with growth medium containing vehicle 0.1% DMSO and indicated compounds. 30 μl per well of 5 mg/ml MTT in phosphate buffered saline (PBS, Invitrogen) was added and incubation continued for another 2 h at 37 ℃. The culture medium was removed and 100 μl DMSO per well was added. Then after complete dissolution, absorbance was detected at 540 nm by a Benchmark microtiter plate reader (Bio-Rad Laboratories, Hercules, CA). The mean absorbance percentage ratio was calculated from 3 independent experiments (each time with 5 wells) and control wells (regarded as 100%), and expressed as % proliferation ± standard deviation (SD).

2.9. In vitro proliferation assay

Assay was performed as described (Kim et al., 2009). HUVECs (4000 cells/well) were seeded in 96-well plate in the Complete Growth Medium for 12 h for attachment. Then cells were treated for 48 h with growth medium containing vehicle 0.1% DMSO and indicated compounds. At the end of 48 h incubation, 10 μCi of tritiated thymidine (GE Healthcare, Piscataway, NJ) in 20 μl of the growth medium was added to each well for incubation at −20 ℃ overnight and then thawed at room temperature before the cells were harvested onto unifilter-96 GF/C plates (PerkinElmer, Waltham, MA) with a unifilter-96 harvester (PerkinElmer). 30 μl per well of MicroScint TM 40 scintillation cocktail (PerkinElmer) was added and the plates were subjected to measurement using the TopCount 2 detector 96-format liquid scintillation analyzer (PerkinElmer). The mean absorbance percentage ratio was calculated from 3 independent experiments (each time with 5 wells) and control wells (regarded as 100%), and expressed as % proliferation ± SD.

For *Alpinia oxyphylla,* proliferation assay was performed as described (He et al., 2010). HUVEC (8000 per well), HepG2 (5000 per well) and MCF7 (8000 per well) were seeded in 96-well plate in the appropriate growth medium for 12 h for attachment. Then cells were treated for 24 h with growth medium containing vehicle 0.1% DMSO and indicated extracts. Cell proliferation was measured by CellTiter 96® Aqueous One Solution (Promega, Madison, WI). Each treatment was performed in triplicate.

Table 2-2 Primers used in RT-PCR

Gene	Primer sequence	Cycle no. used	Expected size (bp)	Accession no.
ef1a	5'-GGCTGACTGTGCTGTGCTGATTG-3'	28	382	NM_131263.1
	5'-CTTGTCGGTGGGACGGCTAGG-3'			
vegfa	5'-GAGAGCCAGCGACTCACCGCAACAC-3'	28	623 for $vegfa_{165}$	AF016244 1
	5'-GTTCGCTCGATCATCATCTTGGC-3'		491 for $vegfa_{121}$	AF059661 1
kdr	5'-GCCTGATCCACAACTGCTTCC-3'	31	150	BC129158.1
	5'-CTCTCCTCACACGACTCAATGC-3'			
angpt1	5'-ACAGCAGTGGAACCGAACAG-3'	30	169	NM_131813 1
	5'-AGCCTCCGCCAGCAGAC-3'			
angpt2	5'-AGGTGGAGGCTGGACTGTC-3'	28	141	NM_131814 1
	5'-GTGGTGAGCAGGTGGATGAC-3'			
tie1	5'-GCGATGGATGGCTATTGAGTCTCTA-3'	31	348	AF053633 1
	5'-GCATCTATTCCAGCATA-3'			
tie2	5'-CTACCCAGTGACCAACGC-3'	31	117	AF053632 1
	5'-GCTCTACAGCTCCTGACGAT-3'			

2.10. In vitro migration assay

HUVEC migration assay was performed in a modified Boyden chamber (Transwell®, CORNING, NY) (He et al., 2010). 100 µl Complete Growth Medium containing 1% FBS and indicated compounds together with 0.1% DMSO (vehicle) was added to the upper compartment of each chamber. 500 µl Complete Growth Medium containing 10% FBS was added to the lower compartments. 100 µl HUVEC suspension (5×10^4 cells/well) in Complete Growth Medium containing 1% FBS was added to the upper compartments. SU5416, a selective VEGF receptor-2 inhibitor, was used as positive control (He et al., 2010). After 3-5 h incubation in an incubator, the non-migrated cells on the upper of the membrane were removed by cotton swab. The migrated cells on the bottom of the membrane were fixed with 1% parafomaldehyde for 3 minutes and then stained with hematoxylin (Merck, Germany)

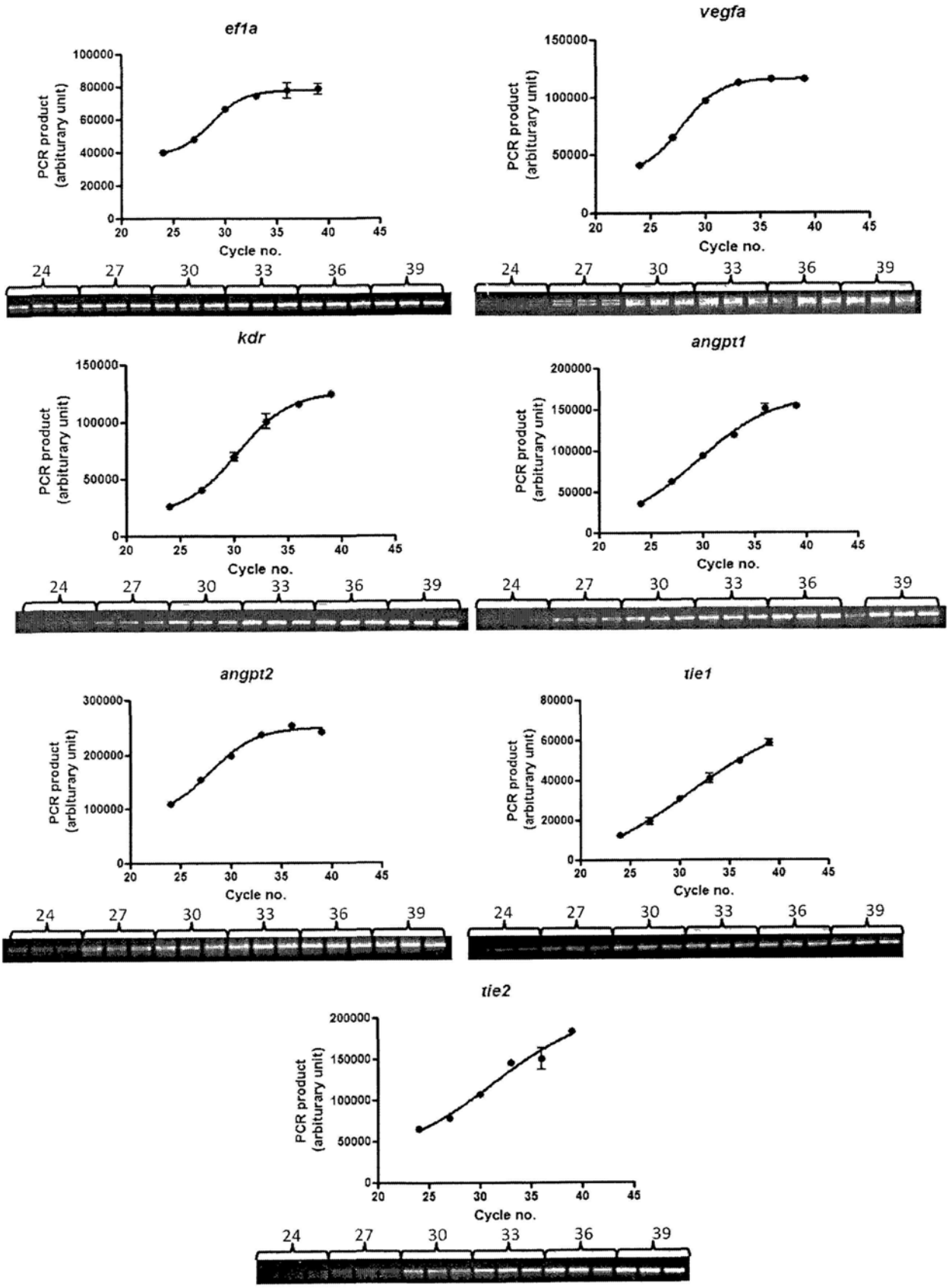


Figure 2-2. Optimization of cycle number for semi-quantitative RT-PCR. The values are the mean ± S.E.M. (n=3).

for 5 minutes. Each treatment was done in triplicate and four randomly selected fields under 100 × magnification of each well were scored for migrated cells.

For *Alpinia oxyphylla,* HUVEC migration assay was performed in a modified Boyden chamber (Neuroprobe, Cabin John, MD) (He et al., 2010). 27 μl of M199 (Gibco, Gaithersburg, MD) supplemented with 0.1% bovine serum albumin (BSA, SIGMA-ALDRCH) plus 1% P/S (Life Technologies, Grand island, NY) containing VEGF-A (10 ng/ml) (PeproTech, London, UK) together with 0.1% DMSO (vehicle) or indicated extracts was added to the lower compartment of each chamber. Gelatin-precoated membrane was used to separate each chamber into upper and lower compartments. HUVEC suspension (4×10^4 cells/well) in M199 medium with 0.1% BSA and 1% P/S was added to the upper compartments. After 8-h incubation in an incubator, the non-migrated cells from the upper face of the membrane were removed by PBS-soaked cotton swab. The migrated cells on the bottom face of the membrane were fixed with methanol and stained with Giemsa solution (Merck, Germany). Each treatment was done in triplicate and five randomly selected fields under 100 × magnification of each well were scored for migrated cells.

2.11. In vitro network formation assay

HUVECs (1.5×10^4 cells/well) in Complete Growth Medium were seeded in 96-well plate precoated with growth factor-reduced Matrigel (60 μl/well, BD Biosciences, Bedford, MA) and exposed to 0.1% DMSO and indicated compounds for 18-24 h (He et al., 2010). SU5416 was used as positive control. The network formation was visualized and imaged under inverted microscope (Nikon, Tokyo, Japan) at 100 × magnification, and ach treatment was done in four times. The network length was quantified by Image-Pro Plus software (Media Cybermetics, Silver Spring, MD).

For *Alpinia oxyphylla,* HUVEC (2×10^4 cells/well) in EGM-2 medium were seeded in 48-well plate precoated with Matrigel (100 μl/well, 8 mg/ml, BD Biosciences, Bedford, MA) and exposed to 0.1% DMSO and indicated extracts for 18 h (He et al., 2010). The network formation was visualized and imaged under inverted microscope (Nikon, Tokyo, Japan) at 100 × magnification. The network length was quantified by Image-Pro Plus software (Media Cybermetics, Silver Spring, MD).

2.12. Statistics

All experiments were repeated at least three times. Values are given as means ± the standard error of the mean (S.E.M.). Data were analyzed using GraphPad Prism 5.0 software (GraphPad Software Inc., La Jolla, CA). Statistical significance was assessed by one-way ANOVA or Student's t-test. *P* values less than 0.05 were considered significant.

Chapter 3

Results, Discussion and Conclusion

3.1. Anti-angiogenic effects of selected TCMs

3.1.1. Results

Under the zebrafish-based anti-angiogenic assay, 95% ethanol crude extracts of the rhizomes of *Rheum palmatum* (Rhizoma Rhei), the fruits of *Alpinia oxyphylla* (Fructus Alpiniae), and the rhizomes of *Kaempferia galanga* (Rhizoma Kaempferiae) showed potential bioactivity at 10μg/ml (Fig. 3-1), which indicates the existence of potent anti-angiogenic components in these herbs.

3.1.2. Discussion

Rheum palmatum, *Alpinia oxyphylla*, and *Kaempferia galanga* are used both as food and/or medicine. *Rheum palmatum* (Polygonaceae), one of best-known TCMs, has been widely used for thousands of years in China for treatment of many diseases, including constipation, jaundice, gastrointestinal hemorrhage, and ulcers (Chinese Pharmacopoeia Commission, 2005). On the other hand, it is often included as an ingredient in traditional Chinese compound prescriptions for the treatment of inflammation, such as acute appendicitis, acute cholecystitis and rheumatoid arthritis (Qiu, 1994; Anon., 1976). In recent years, rhubarb has been shown to have good antitumor (Dorsey and Kao, 2007; Huang et al., 2007; Lee et al., 2001; Shi et al., 2001) and anti-inflammation effects (Cuellar et al., 2001). *Alpinia oxyphylla* (Zingiberaceae) is an important herb commonly used in East Asian traditional medicine. Its fruits, called Yizhi, Yakuchi and Ikji in Chinese, Japanese and Korean, respectively, are widely used to treat dyspepsia, diarrhea, abdominal pain, spermatorrhea, 'kidney' asthenia and poor memory (But, 1997; Pharmacopoeia

Committee, 2005). The main chemical components include fruit oil, flavonoids, diarylheptanoids and sesquiterpenes. Its antineoplastic properties have long attracted scientific attention (Itokawa, 1979; Lee et al., 1998; Surh et al., 1998). A methanol extract of the herb suppressed skin carcinogenesis in mice and induced apoptotic death in human promyelocytic leukemia (HL)-60 cells (Lee et al., 1998); while an 80% aqueous acetone extract and a series of sesquiterpenes isolated from the ethyl acetate-soluble fraction of the herb inhibited nitric oxide production in lipopolysaccharide-activated mouse peritoneal macrophages (Morikawa et al., 2002; Muraoka et al., 2001). Yakuchinones A and B, two major pungent diarylheptanoid ingredients isolated from the ethyl acetate-soluble fraction of the fruit of *A. oxyphylla* (Xu et al., 2009), have inhibitory effects on phorbol ester-induced inflammation (Chun et al., 2002a) and anti-tumor promotional properties (Chun et al., 1999; 2002b). Since inflammation and cancer are closely linked with angiogenesis (Kobayashi and Lin, 2009), and combating inflammation with appropriate drugs or substances could prevent inflammatory angiogenesis in carcinogenesis (Albini et al., 2005; Allavena et al., 2008; Bisacchi et al., 2003), anti-angiogenesis is considered as a promising anticancer therapeutic strategy (Folkman, 1971; Ruegg and Mutter, 2007; Bar and Onn, 2008; Griffioen, 2008; Cao, 2009). All these backgrounds suggest anti-angiogenic functions in this herb. *Kaempferia galanga* (Zingiberaceae) is a popular spice cultivated in South and South-East Asia. It is a perennial aromatic herb with very fragrant underground rhizome, which has been a popular condiment in Asian cuisine. It is also good for dyspepsia, leprosy, skin diseases, rheumatism, asthma, cough, bronchitis, wounds, ulcers, helminthiasis, fever, malarial fever, splenopathy, cephalalgia, inflammatory tumor, nasal obstruction, halitosis, strangury, urolithiasis and haemorrhoids (Warrier et al., 2003). Its rhizome contains about 2.5-4% of essential oil. The main components are ethyl cinnamate, *trans*-ethyl

p-methoxycinnamate, *cis*-ethyl *p*-methoxycinnamate and *p*-methoxycinnamic acid and a monoterpene ketone compound, 3-carene-5-one. The other constituents are borneol, 1, 8-cineole, 3-carene, and (*E*)-cinnamaldehyde, eucalyptol, kaempferol, methyl *p*-coumaric acid ethyl ester, and pentadecane (Kiuchi et al., 1987; Namba, 1993; Peter, 2004; Wong et al., 1992). The rhizome has also been reported to display antitumor, cytotoxic properties, and induce human cell apoptosis and DNA damage (Muangnoi et al., 2007; Peter, 2004). The ethanolic rhizome extract and hexane fraction of *K. galanga* have been studied by the Hippocratic screening test, acute and subacute toxicities in rats, and dermal irritation in rabbits. Pathologically, neither gross abnormalities nor histopathological changes were observed. Also, no sign of irritation was observed during the dermal irritation test of the hexane fraction of *K. galanga* (Kanjanapothi et al., 2004). Because of its anti-rheumatism, anti-inflammatory and anti-tumor effects, *K. galanga* may have the value to further investigate its anti-angiogenic potentials.

The potential anti-angiogenic activity of these three herbs on zebrafish angiogenic assay prompted us to identify their anti-angiogenic agents and the underling mechanism.

3.2. Anti-angiogenic effects and mechanisms of *Rheum palmatum* (Rhizoma Rhei)

3.2.1. Results

3.2.1.1. Quantitative EAP assay on zebrafish embryo

The 95% ethanol crude extract and Hex, EA, BuOH, and Aq fractions obtained from the 95% ethanol extract of rhubarb (Rhizoma Rhei) were examined with zebrafish angiogenic assay. As shown in Fig. 3-2, the ethanol extract of rhubarb inhibited vessel formation by 46% at 20 μg/ml. Successive fractionation showed that the Hex and EA fractions at 20 μg/ml potently inhibited vessel formation by 46 and

52%, respectively, in the embryos, indicating the presence of anti-angiogenic components in the two fractions. The main bioactive constituents of EA fraction are anthraquinone derivatives including aloe-emodin, chrysophanol, emodin, physicon and rhein (Fig. 3-3). Their existence in EA fraction was further confirmed by comparing with authentic chemical markers on TLC (Fig.3-4)

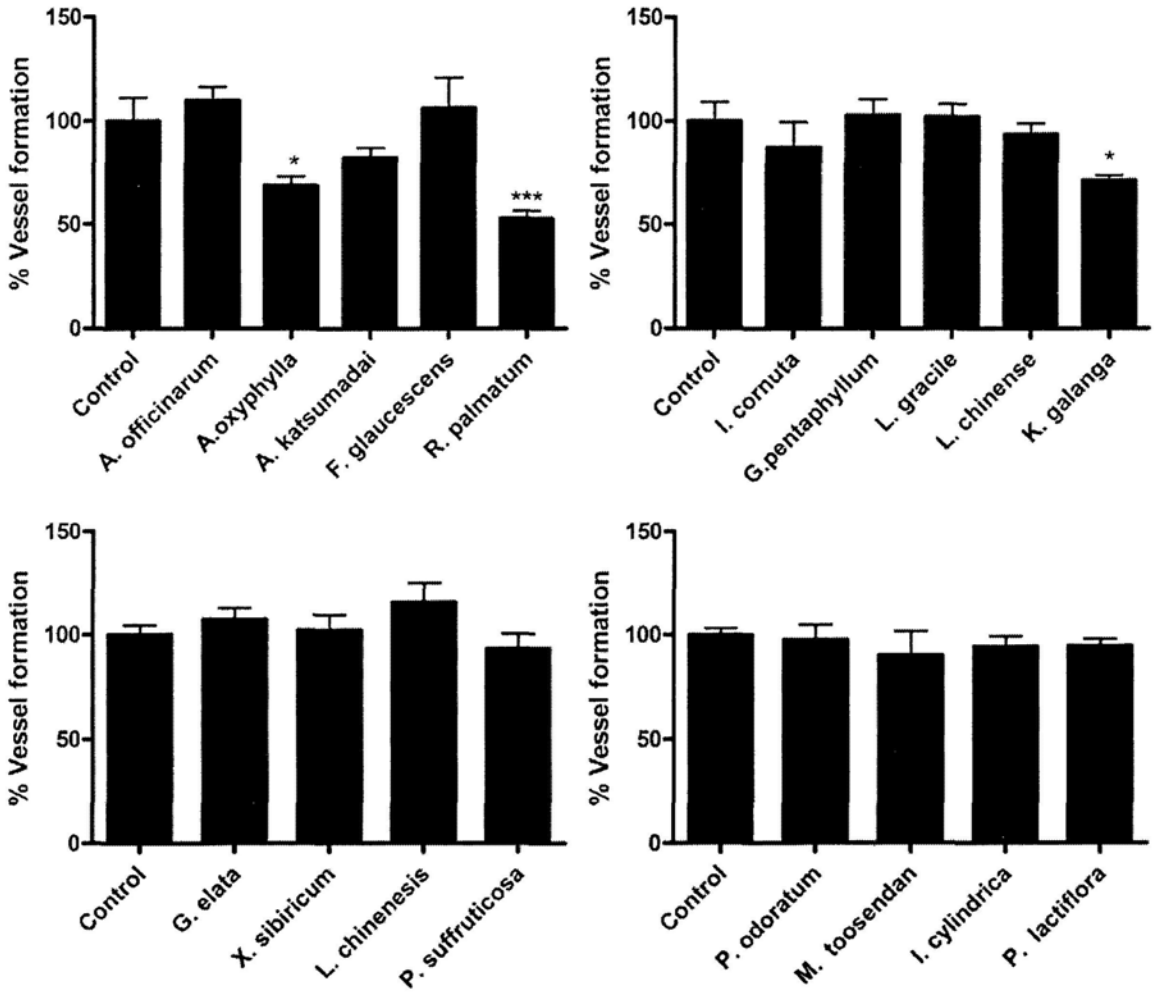


Figure 3-1. Anti-angiogenic activity of the ethanol extracts of selected TCMs. Fractions were dissolved in DMSO and then added directly to the embryo water (final concentration 10 μg/ml). 0.2% DMSO was used as carrier control. After drug treatment, embryos were processed for quantitative EAP assay. Each value represents the mean ± S.E.M. (n = 10) from a representative experiment. * represents $P < 0.05$, *** represents $P < 0.001$ in one-way ANOVA followed by the Dunnett's multiple comparison tests.

The anti-angiogenic activities of these anthraquinone derivatives were evaluated with zebrafish angiogenic assay. As shown in Fig. 3-5, three of the anthraquinone derivatives, aloe-emodin, emodin and rhein displayed anti-angiogenic activities in the zebrafish model. Among them, only emodin inhibited 29% of vessel formation at 10 μM, and the inhibition reached 38% at 25 μM. However both at 25 and 50 μM, rhein displayed the highest anti-angiogenic activity and inhibited 49 and 68% of vessel formation, respectively in the zebrafish model. Aloe-emodin inhibited 41 and 47% of vessel formation at 25 and 50 μM, respectively. The remaining two compounds, however, did not show any significant effect in zebrafish angiogenic model. According to the results, aloe-emodin and emodin elicited comparable responses on anti-angiogenic activities in zebrafish model to those *in vitro* and *in vivo* models (Cardenas et al., 2006; Kwak et al., 2006).

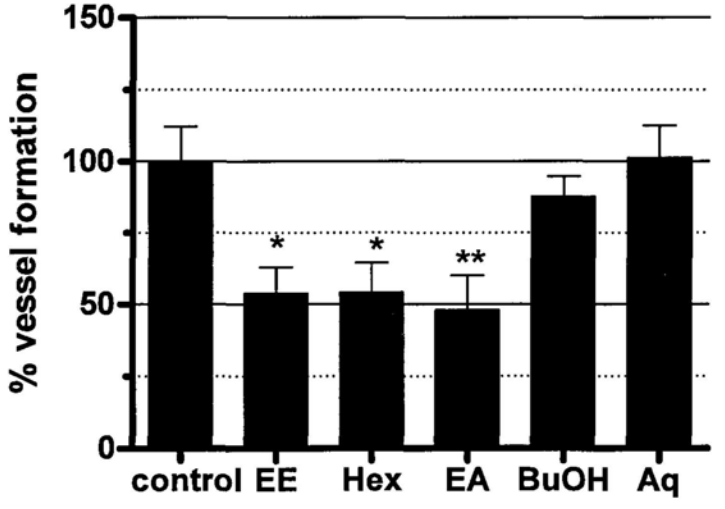


Figure 3-2. Anti-angiogenic activity of the fractions of rhubarb. Fractions were dissolved in DMSO and then added directly to the embryo water (final concentration 20 μg/ml). 0.2% DMSO was used as carrier control. After drug treatment, embryos were processed for quantitative EAP assay. Each value represents the mean ± S.E.M. (n = 10) from a representative experiment. * represents $P < 0.05$, ** represents $P < 0.01$ in one-way ANOVA followed by the Dunnett's Multiple Comparison Test. Ethanol extract (EE) *n*-hexane fraction (Hex), ethyl acetate fraction (EA) and *n*-butanol fraction (BuOH), aqueous fraction (Aq).

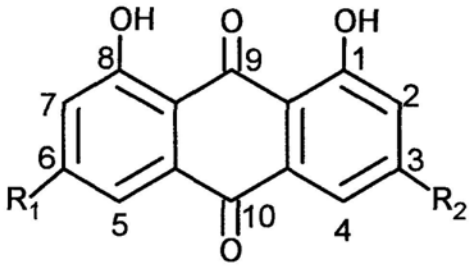


Figure 3-3. Structures of the five anthraquinone derivatives tested. Aloe-emodin R_1 = H, R_2 =CH_2OH; chrysophanol R_1 = H, R_2 = CH_3; emodin R_1 = OH, R_2 = CH_3; physcion R_1 = CH_3O, R_2 = CH_3; rhein R_1 = H, R_2 = COOH.

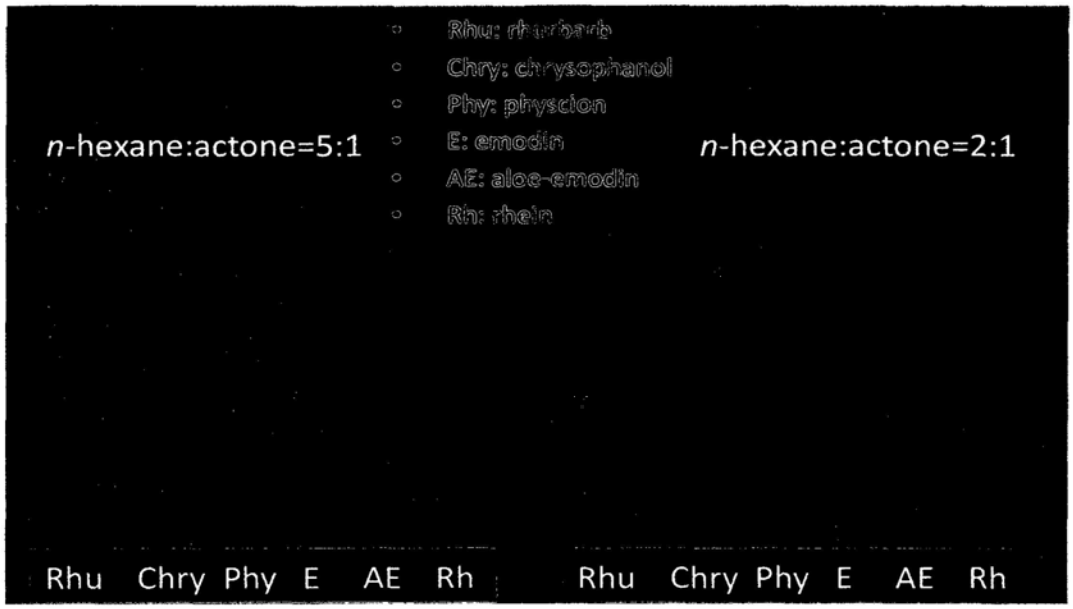


Figure 3-4. TLC results of the five anthraquinone derivatives tested.

3.2.1.2. Microscopic imaging

The loss of vessel formation in the embryos treated with 2.5–50 μM of rhein was further confirmed using microscopic imaging on both wild-type and *Tg(fli1a:EGFP)y1* zebrafish embryos. As shown in Fig. 3-5, the activities of rhein showed a dose-dependent manner. The ISVs were the most easily observed angiogenic vessels in the embryos (Fig. 3-6 and 3-8A). Treatment with 0.2% DMSO had no effect on the vessel formation and served as a vehicle control (Fig. 3-6A and 3-8A). On wild-type, rhein at 25 μM partially inhibited ISVs formation (Fig. 3-6E)

and at 50 μM completely blocked ISV formation (Fig. 3-6F). However, on *Tg(fli1a:EGFP)y1* type, 20 μM rhein could almost block ISV formation at 48 hpf. The SIV plexus, appearing as a smooth basket-like structure with 5-6 arcades, is also easily observed on *Tg(fli1a:EGFP)y1* zebrafish at 72 hpf (Fig. 3-8A'). Rhein at 20 μM could completely inhibit SIV formation compared to control group after 48-h treatment. Complete blockage of ISV formation is often associated with the appearance of pericardial edema (Figs. 3-6F, 3-7, 3-8B and B'). Similar pericardial edema was found in the embryos treated by PTK787/ZK222584, which is a novel anilinophthalazine compound with a high affinity for VEGF receptors to block their signaling, and currently under phase II clinical trial for the treatment of metastatic gastrointestinal stromal tumors (Chan et al., 2002; Joensuu et al., 2008). Meanwhile, the overall morphology and structure of treated embryos were generally normal by 72 hpf. These may indicate the potential selectivity of rhein for molecule(s) involved in angiogenic signaling pathways.

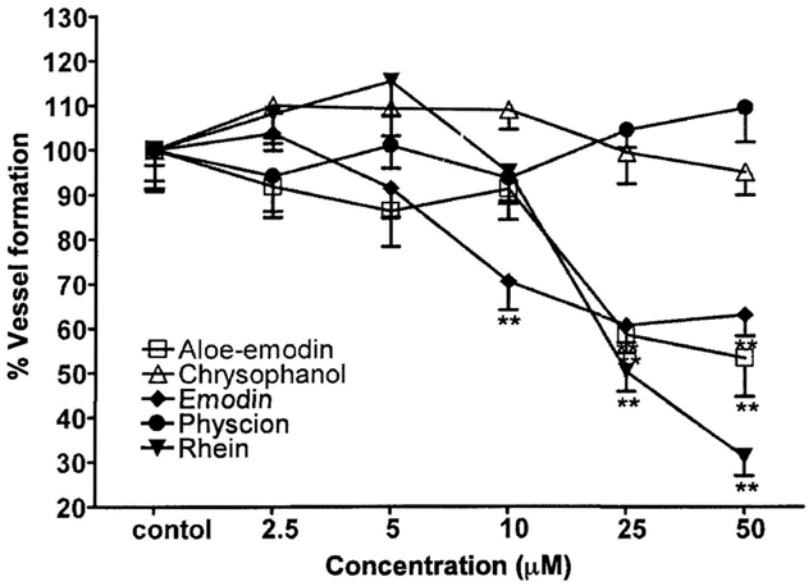


Figure 3-5. Anti-angiogenic activity of anthraquinone derivatives in zebrafish model. Each value represents the mean ± S.E.M. (n = 10) from a representative experiment. * represents $P < 0.05$, ** represents $P < 0.01$ in one-way ANOVA followed by the Dunnett's Multiple Comparison Test.

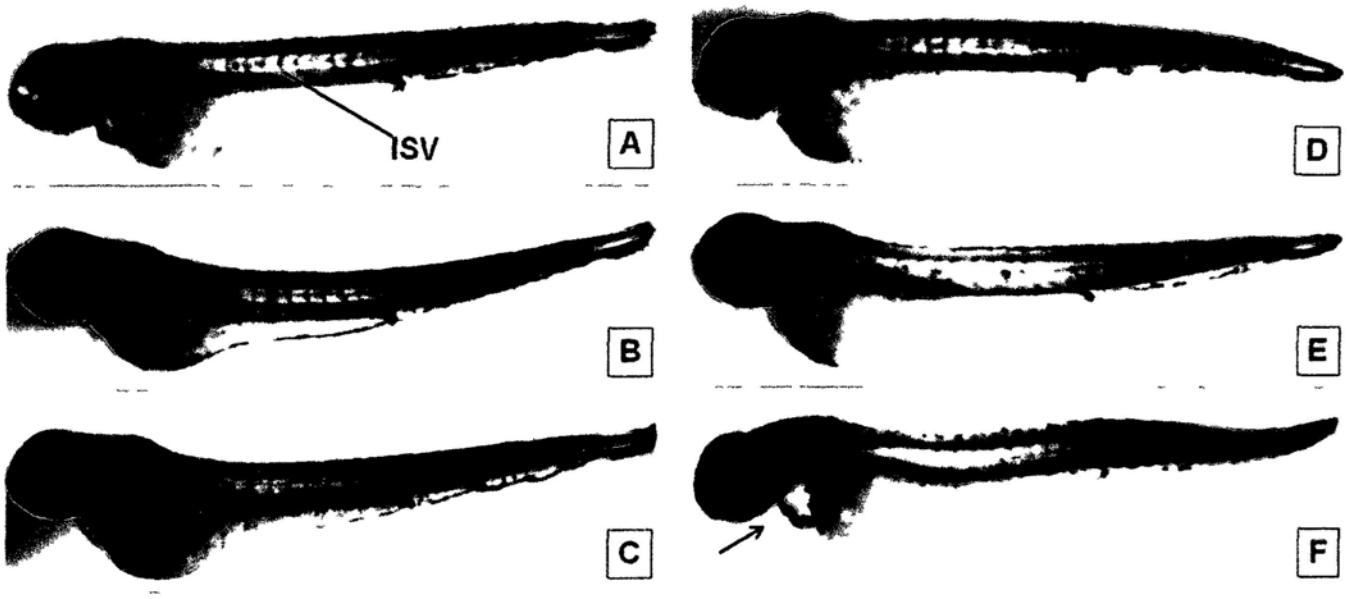


Figure 3-6. Lateral view of EAP stained zebrafish embryos at 72 hpf showing embryo vessel formation after rhein treatment. A=control group given 0.2% DMSO; B–F=rhein concentrations added 2.5, 5, 10, 25 and 50μM, respectively. ISVs= intersegmental vessels; the arrow on F points at pericardinal edema.

Figure 3-7. Lateral view of zebrafish embryos at 72 hpf showing pericardinal edema (indicated by an arrow) after high-dose rhein group (50 μM) treatment.

3.2.1.3. Molecular mechanism of aloe-emodin, emodin and rhein on zebrafish angiogenesis

Among these anthraquinone derivatives, aloe-emodin, emodin and rhein showed potent anti-angiogenic activities on zebrafish model, however their action mechanisms are still not fully understood, and deserved further investigations.

VEGFA-VEGFR and ANGIOPOIETIN (ANGPT)-TIE are two major studied signaling pathways involved in angiogenesis (Ellis et al., 2008; Thomas et al., 2009; Thurston, 2003). VEGFA (commonly referred to as VEGF) is a potent angiogenic factor that mediates most biological functions via KDR (VEGFR2), and expressed as various isoforms due to alternative splicing which leads to mature 121-, 165-, 189- and 206-amino-acid proteins. $VEGFA_{165}$ is the predominant isoform and is commonly overexpressed in many human solid tumors (Ellis et al., 2008; Donnini et al., 2004). The ANGPT-TIE system acts as vascular specific ligand/receptor system essential for blood vessel formation, and it consists of four ligands (ANGPT1-4) and two corresponding tyrosine kinase receptors (TIE1 and TIE2). The best characterized ligands are ANGPT1 and ANGPT2 (Gale and Yancopoulos, 1999; Thomas et al., 2009). So the major molecules participating in these two signaling pathways, including $VEGFA_{165}$, KDR, ANGPT1, ANGPT2, TIE1 and TIE2 were thus investigated. Because zebrafish embryos were treated with drugs at 24 hpf and evaluated at 72 hpf, the time frame between 24 and 72 hpf was selected for the mechanism study.

In the time course study of compound aloe-emodin (Fig. 3-9A, A'), the mRNA expression encoded by $vegfa_{165}$ were consistently up-regulated before 48 hpf, and then restored to the control level at 60 hpf. However, the isoform $vegfa_{121}$ expression stayed normal until 60 hpf, and then was up-regulated after 48-h treatment at 72 hpf. The expression encoded by *angpt1* and *tie1*were up-regulated at the early course (36 hpf) after 12-h treatment, and then down-regulated after 48- and 36-h treatment at 72 and 60 hpf, respectively. The expression of the rest three genes (*kdr*, *angpt2* and *tie2*) was down-regulated after 36-h treatment at 60 hpf.

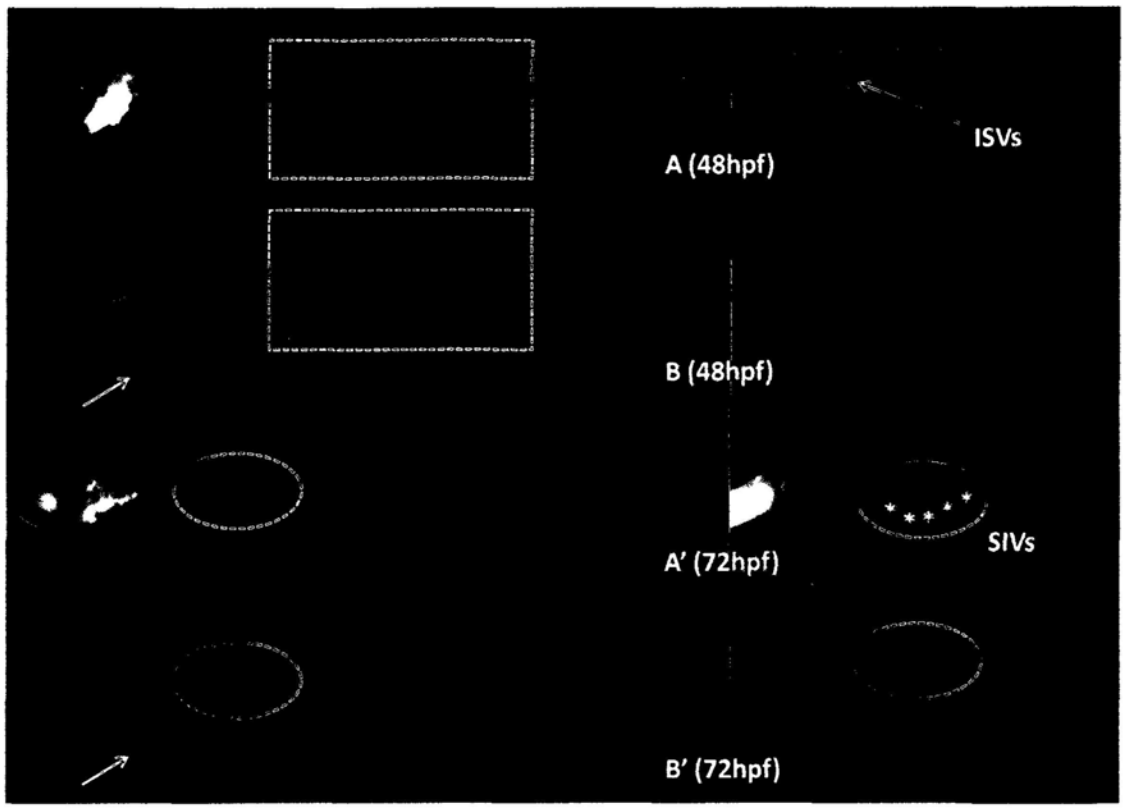


Figure 3-8. Lateral view of *Tg(fli1a:EGFP)y1* zebrafish embryos at 48 and 72 hpf. (A, A') Control: live fluorescence microscopy hightlights EGFP expressing ISVs (arowhead) and the subintestinal vessel plexus (SIVs), which appears as a smooth basket-like structure with 5-6 arcades (asterisks); (B,B') the ISVs and SIVs failed to form at the treatment of 20 μM rhein.

In the time course study of compound emodin (Fig. 3-9B, B'), the gene expression of $vegfa_{165}$, $vegfa_{121}$ and *angpt2* was slightly down-regulated after 48-h treatment at 72 hpf. The expression encoded by *angpt1* was consistently up-regulated until 48 hpf, then down-regulated after 48-h treatment at 72 hpf. The gene expression of the rest three (*kdr*, *tie1* and *tie2*) were up-regulated at 36 hpf, and then down-regulated after 48-h treatment at 72 hpf.

In the time course study of compound rhein (Fig. 3-9C, C'), the expression encoded by $vegfa_{165}$, *kdr*, *angpt1* an *tie1*were first up-regulated after 12-h treatment at 36 hpf, then down-regulated after 48- or 36-h treatment. However, the isoform $vegfa_{121}$ expression was kept up-regulating until 60 hpf. The gene expression of

angpt2 and *tie2* were dramatically down-regulated after 24- and 36-h treatment at 48 and 60 hpf, respectively. According to the further dose-response investigation after 48-h treatment with rhein (5-20 μM) (Fig. 3-10), *angpt2* and *tie 2* seemed to be the major molecular targets.

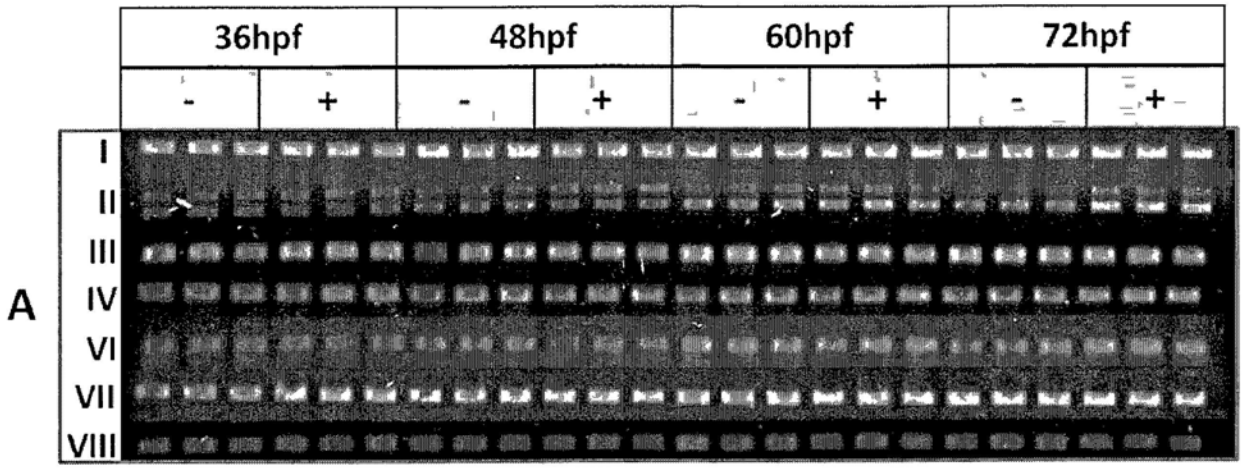
3.2.1.4. Effects of rhein on HUVEC proliferation, migration and tube formation

In order to further confirm the anti-angiogenic effect of rhein, HUVECs was used in the MTT-based cytotoxicity test and the tritiated thymidine incorporation assay (Kim et al., 2009). In MTT assay (Fig. 3-11A), rhein showed no obvious cytotoxicity on HUVECs at the range from 1.6 to 25 μM. Using tritiated thymidine incorporation assay, rhein also had no inhibitory effect on the proliferation of HUVECs at the corresponding concentrations. Migration is a key step for angiogenesis, and compound rhein could dose-dependently inhibit HUVEC migration induced by FBS concentration gradient, achieving 70% inhibition at 25 μM (Fig. 3-12). Tube formation is another key step associated with cell migration and differentiation, however, rhein showed no obvious inhibitory effect on HUVECs (Fig. 3-13). All the results revealed that rhein could specifically inhibit the migration step of HUVECs to prevent the angiogenesis progress.

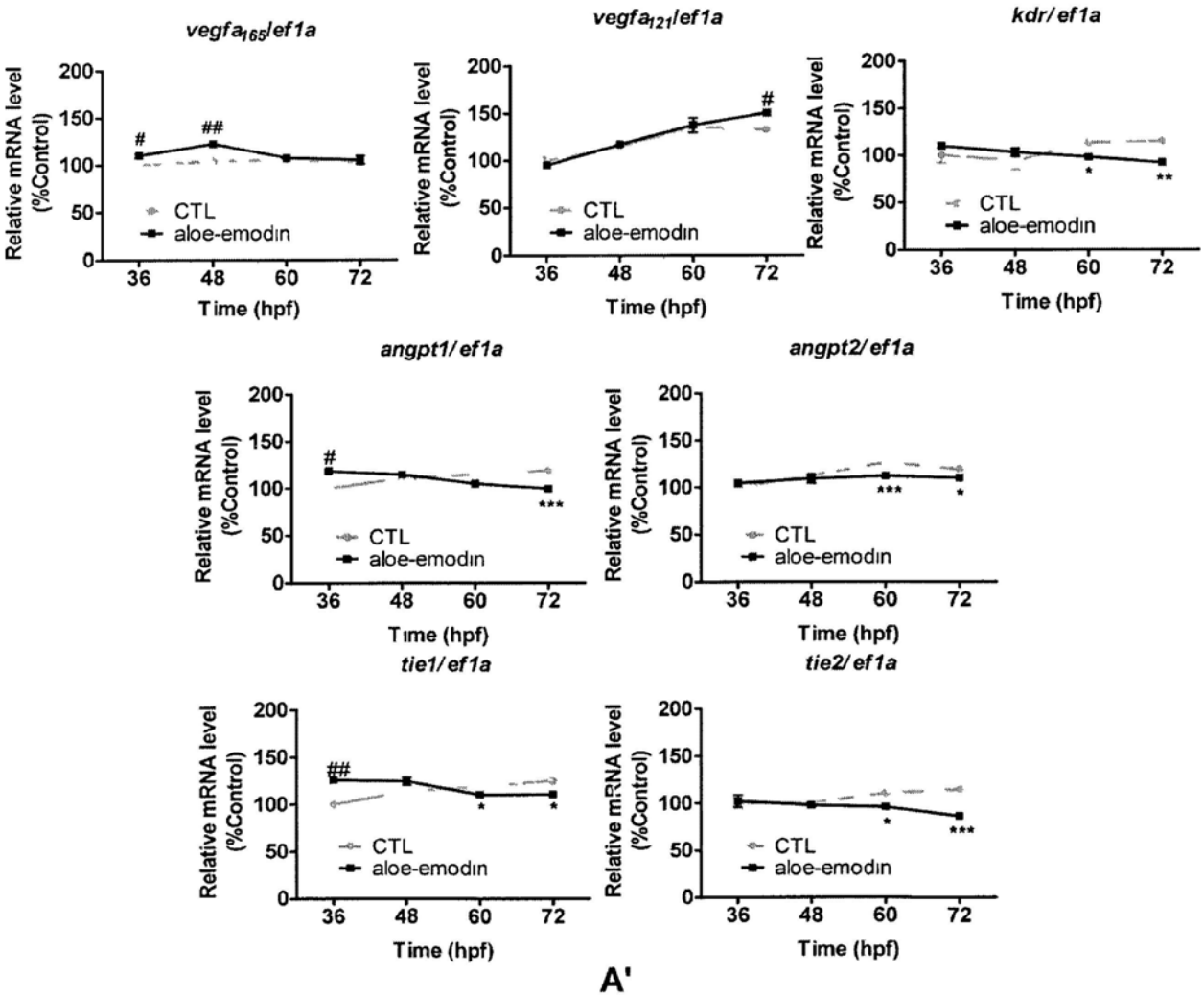
3.2.2. Discussion and conclusion

Anthraquinone derivatives are the main bioactive constituents in rhubarb. Based on the position of the hydroxyl group substitution, anthraquinones are divided into two types, the emodin-type (1, 8-dihydroxy-anthraquinones) and alizarin-type (1, 2-dihydroxyanthraquinones). The five compounds (Fig. 3-3) tested here all belong to the emodin-type. According to the results, different substitution of functional groups

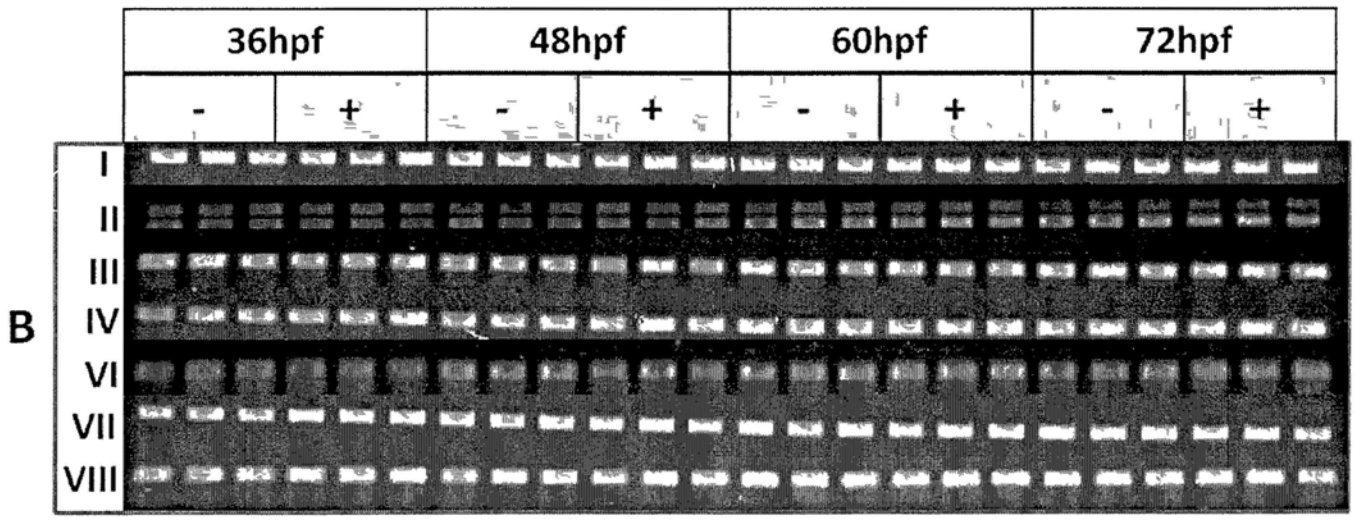
can affect the anti-angiogenic activity, and some structure–activity relationship (SARs) can be deduced.



A



A'


36hpf
48hpf
60hpf
72hpf
-
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+
-
+
I
II
III
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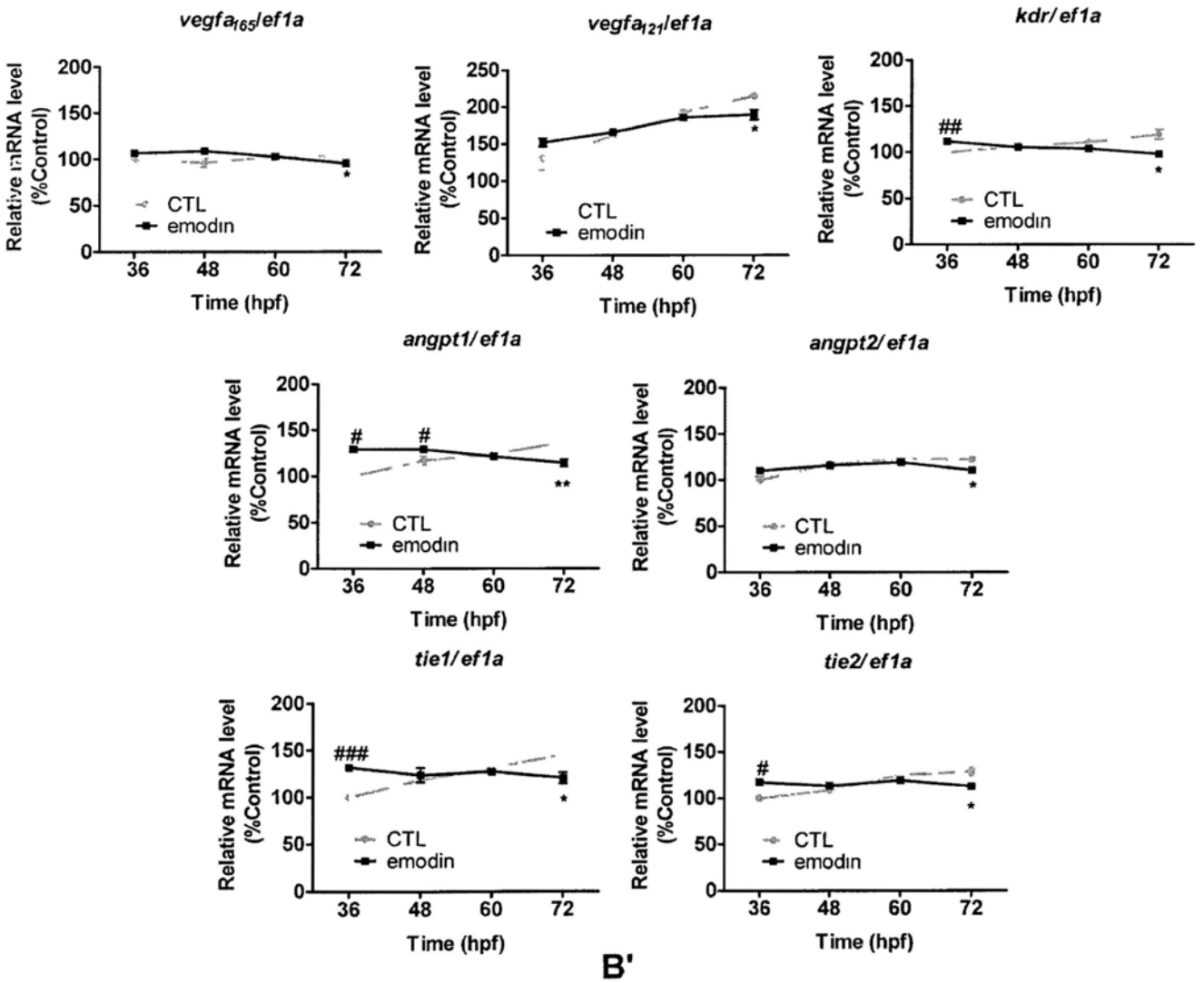
VII
VIII

B


vegfa165/ef1a
vegfa121/ef1a
kdr/ef1a
angpt1/ef1a
angpt2/ef1a
tie1/ef1a
tie2/ef1a
Relative mRNA level (%Control)
Time (hpf)
CTL
emodin
36
48
60
72
#
##
###
*
**

B'

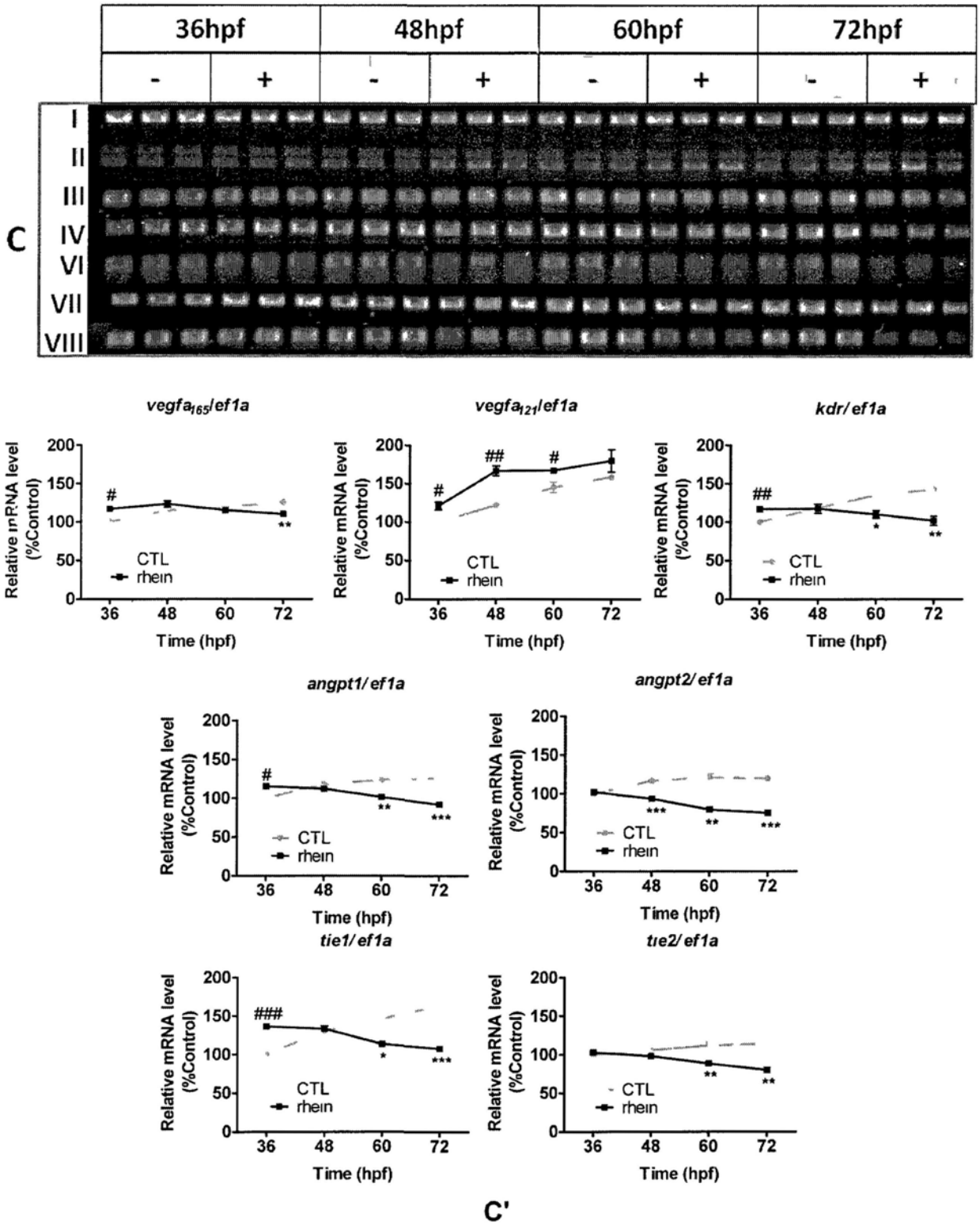


Figure 3-9. Time course of aloe-emodin (A, A', 20 μM), emodin (B, B', 20 μM) and rhein (C,C', 20 μM) effects on the expression of *vegfa* (II), *kdr* (III), *angpt1* (IV), *angpt2* (V), *tie1* (VI) *and tie2* (VII) in zebrafish embryos. *ef1a* (I) was used as internal control to normalize the expression levels. Each value represents the mean ± S.E.M. (n = 3) from a representative experiment. *, # Represents $P < 0.05$, **, ## represents $P < 0.01$, ***, ### represents $P < 0.001$ in Student's t-test.

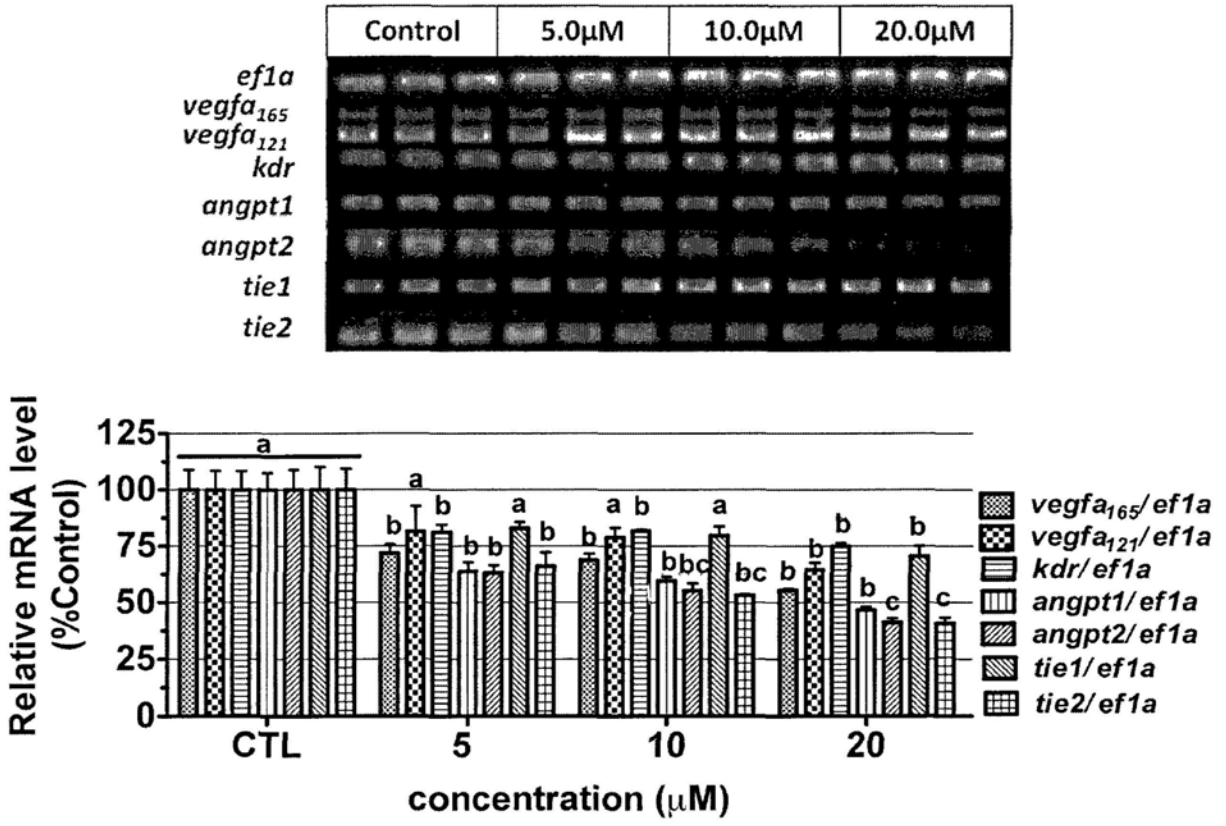


Figure 3-10. Dose–response of rhein effects on the expression of *vegfa, kdr, angpt1, angpt2, tie1 and tie2* in zebrafish embryos at 72 hpf. *ef1a* was used as internal control to normalize the expression levels. Each value represents the mean ± S.E.M. (n = 3) from a representative experiment. The data were analyzed by one-way ANOVA followed by the Newman–Keuls test for comparisons of all pairs of groups. Different letters indicate statistical significance among groups ($P < 0.05$).

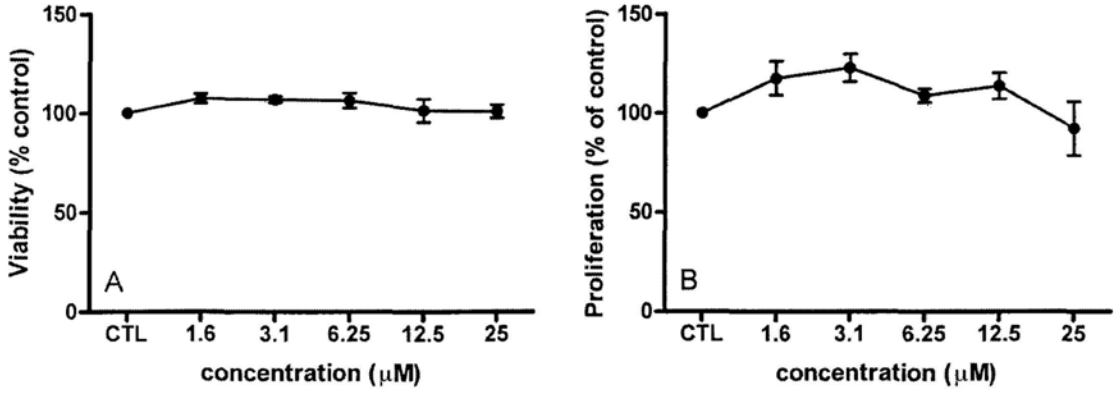


Figure 3-11. MTT (A) and tritiated thymidine (B) assay of rhein on HUVECs. Each point represents mean ± S.E.M. (n = 5) from 3 independent experiments. * represents $P < 0.05$ in one-way ANOVA followed by the Dunnett's test for comparisons of all treated groups with respective control group.

Chrysophanol, emodin and physcion possess a methyl group at the C-3 position, and only differ from one another at the C-6 position. Chrysophanol with no substitution and physcion with a methoxy group substitution did not show any anti-angiogenic activity in the zebrafish model, while emodin with a hydroxyl group at C-6 position showed high activity. On the other hand, aloe emodin, chrysophanol and rhein all have no substitution at the C-6 position, but the differences in oxidation state of the methyl group or absence of substitution at C-3 position led to dramatic differences. Among them, rhein with a carboxylic group displayed the strongest anti-angiogenic activity. Due to their planar chemical structure, the C-3 and C-6 positions of anthraquinones can be convertible in the emodin-type. Comparing the structural characteristics of these three anthraquinones, acidic substitution with a phenolic or carboxylic group at C-3 or C-6 positions, or polar, hydrophilic substitution such as hydroxymethyl group at C-3 position may contribute to the anti-angiogenesis potency.

A few studies have also explored the anti-angiogenic potentials of aloe-emodin and emodin. Aloe-emodin could inhibit tumor cell proliferation and showed anti-angiogenic activity through inhibiting the proliferation, urokinase secretion and tubule formation of endothelial cells (Cardenas et al., 2006). At a high concentration of 100 μM, it inhibited the VEGFR-2 expression or VEGF-induced VEGFR-2 (KDR) phosphorylation (Kimura et al., 2008), which is corresponding to our result of down-regulation of *kdr* mRNA expression after 36-h treatment at 60 hpf (Fig.3-9A, A'). Emodin, on the other hand, preferentially inhibits VEGFA-induced angiogenesis *in vivo* and *in vitro*, possibly through blocking the phosphorylation of KDR/Flk-1, which is similar to our finding of *kdr* mRNA expression at the 72 hpf (Fig.3-9B, B'), and downstream effector molecules, including FAK, ERK1/2, p38, Akt, and endothelial nitric oxide synthase (Kwak et al., 2006; Ljubimov et al., 2004; Wang et

al., 2004). Rhein has received much less attention. Huang et al. (2007) reported that it could effectively inhibit the uptake of glucose in tumor cells and induce cell necrosis. Rhein also showed anti-inflammation effects through inhibiting the inducible nitric oxide synthase (Wang et al., 2002). Recently, Lin's group (2009) reported that rhein might inhibit the invasion and migration of human nasopharyngeal carcinoma cells in part through the suppression of matrix metalloproteinases-9 and VEGF expression via the modulation of NF-κB signaling pathway. This also agrees with the down-regulation of *vegfa*$_{165}$ mRNA expression at 72 hpf in our study (Fig. 3-9C, C'). Our findings suggested that these anthraquinone derivatives might have multiple molecular targets of angiogenic factors. Some of these factors can be up-regulated at early developmental stage and then restored to the normal level or even down-regulated later to cause an opposite effect. However, the final effects of these anthraquinone derivatives are due to the overall functions of these angiogenic factors. Especially, the compound rhein could completely inhibit the ISV formation at 48 hpf, when only *angpt2* expression was down-regulated despite that *vegfa*$_{121}$ was up-regulated at the same time. The *angpt2* and *tie 2* seemed to be the major molecular targets of rhein according to our results. ANGPT*2* is produced by endothelial cells and acts as an autocrine antagonist of ANGPT1-mediated TIE2 activation, which is required to maintain the quiescent resting state of the endothelium. ANGPT2 induces vascular destabilization at higher concentrations through antagonizing ANGPT1-TIE2 signaling. Also, ANGPT2 is strongly expressed in the vasculature of many tumors and may act synergistically with other cytokines such as VEGF to promote the tumor-associated angiogenesis and tumor progression. So both reduced expression of *angpt2* and *vegf*$_{165}$ caused by rhein could synergistically avoid vascular destabilization to block angiogenesis. Since TIE2 activation is related to endothelia cell migration and promotes vessel assembly, rhein's inhibition effect on HUVEC migration (Fig.

3-12) might be due to its down-regulation effect on *tie2* expression (Fig. 3-10). Our results supports that ANGPT2 and TIE2 is a potential target for manipulative therapies (Fiedler and Augustin, 2006; Thomas and Augustin, 2009).

In conclusion, rhein blocked angiogenesis mainly through *angpt2* and *tie2* signaling pathway, and specifically inhibited HUVEC migration, which may play a role in antitumor and anti-inflammatory actions. At the same time, our findings also suggested that inflammation inhibitors might have the potential to be angiogenesis inhibitors. Further search for anti-angiogenic agents should therefore include Chinese herbs used for inflammatory diseases.

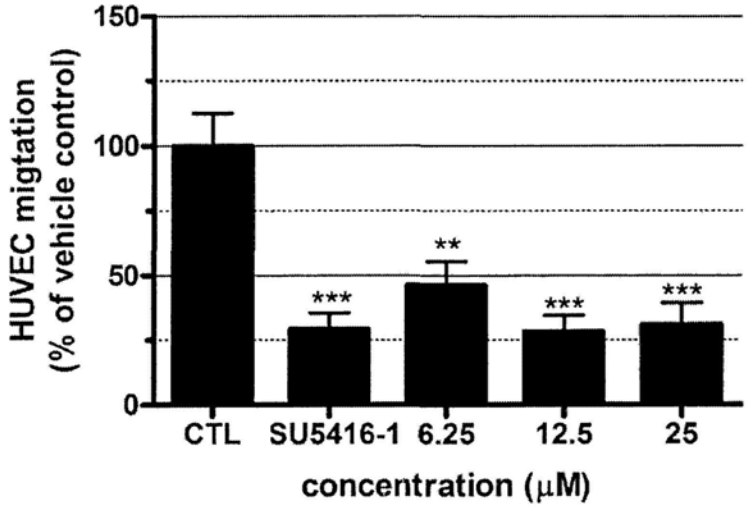


Figure 3-12. Migration assay of rhein. Representative images depicting effect of rhein treatment (3 h) on HUVEC migration; Diagrams represent percentage of HUVEC migration following a 3-h exposure to the indicated concentration of rhein relative to control. SU5416 (1μM) was used as positive control. Each point represents mean ± S.E.M. (n = 3) from a representative experiment. **represents $P < 0.01$, *** represents $P < 0.001$ in one-way ANOVA followed by the Dunnett's test for comparisons of all treated groups with control group.

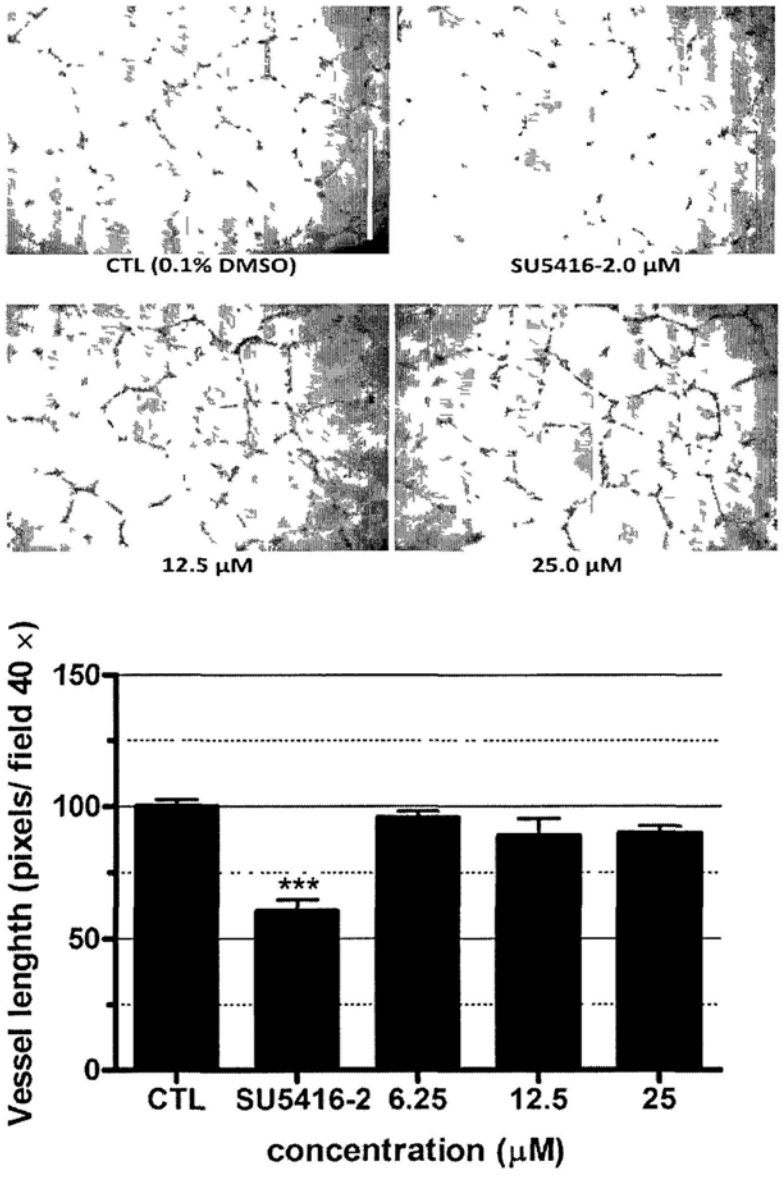


Figure 3-13. Tube formation assay of rhein. Representative images and diagram depicting formation of capillary-like tube structures by HUVECs following 24 h of treatment with indicated concentration of rhein. SU5416 (2 μM) was used as positive control. Each point represents mean ± SEM (n = 4) from a representative experiment. *** represents $P < 0.001$ in one-way ANOVA followed by the Dunnett's test for comparisons of all treated groups with control group. Scale bar=1000 μm.

3.3. Anti-angiogenic effects and mechanisms of *Alpinia oxyphylla* (Fructus Alpiniae)

3.3.1. Results

3.3.1.1. Anti-angiogenic effect of A. oxyphylla on zebrafish model

Quantitative EAP assay showed the 95% ethanol extract inhibited vessel formation by 31% at 10 μg/ml. Two subsequent fractions, the Hex and EA fractions, at 10 μg/ml potently inhibited vessel formation by 45% and 23%, respectively, in the embryos (Fig. 3-14). The Hex fraction also dose-dependently inhibited vessel formation at 5 and 10 μg/ml, but the EA fraction did not show significant activity at 5 μg/ml (Fig. 3-15).

The suppression effect of vessel formation in the embryos treated with different concentrations of Hex fraction was further confirmed using *Tg(fli1a:EGFP)y1* zebrafish. As shown in Fig. 4-3, the ISVs were the most easily observed angiogenic vessels in the embryos at 48 hpf. Treatment with 0.2% DMSO had no effect on the vessel formation and served as a vehicle control (Fig. 3-16A, A'). The Hex fraction showed potent inhibition on ISV formation at 10 μg/ml (Fig. 3-16C, C'), but no obvious effect at 1 μg/ml (Fig. 3-16B, B'). At 72 hpf, the SIVs developed as a smooth basket-like structure with approximately 5–6 arcades in the vehicle control group (Fig. 3-17A). In the experimental groups, SIVs only partially developed at 5 μg/ml treatment (Fig. 3-17B), but almost failed to form after 10 μg/ml treatment (Fig. 3-17C). The overall morphology and structure of the treated embryos were generally normal by 72 hpf.

3.3.1.2. Inhibition of HUVEC, HepG2 and MCF7 proliferation

The Hex fraction of the ethanol extract of *A. oxyphylla* at 30–50 μg/ml significantly inhibited HUVEC and HepG2 proliferation in a dose-dependent manner. The IC_{50} of Hex fraction for HUVEC proliferation inhibition was 45 μg/ml. Hex fraction at 50 μg/ml had much higher inhibitory effect on HUVEC proliferation than on HepG2 cells (Fig. 3-18A). However, the EA fraction of *A. oxyphylla* at 30–50 μg/ml showed weaker inhibitory effects on both HUVEC and HepG2 proliferation as

compared to the Hex fraction. MCF7 cells were resistant to the treatment of both Hex and EA fraction treatments at all concentrations.

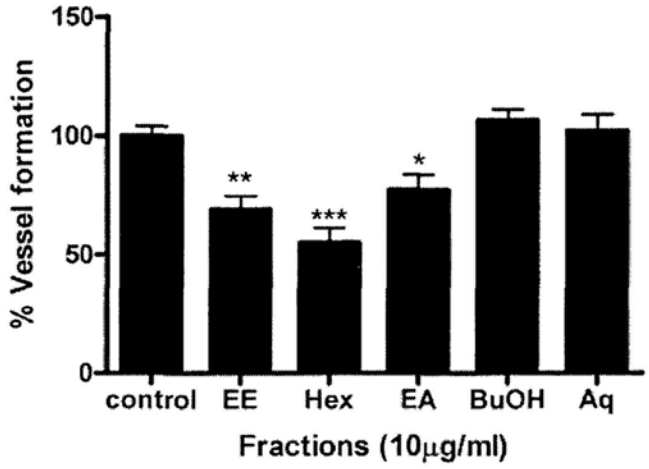


Figure 3-14. Anti-angiogenic activity of the fractions of *A. oxyphylla*. Fractions were dissolved in DMSO and then added directly to the embryo water (final concentration 10 μg/ml). 0.2% DMSO was used as carrier control. After drug treatment, embryos were processed for quantitative EAP assay. Each value represents the mean ± S.E.M. (n = 10) from a representative experiment. * represents $P < 0.05$, ** represents $P < 0.01$, *** represents $P < 0.001$ in one-way ANOVA followed by the Dunnett's multiple comparison test. Ethanol extract (EE) *n*-hexane fraction (Hex), ethyl acetate fraction (EA) and *n*-butanol fraction (BuOH), aqueous fraction (Aq).

3.3.1.3. Inhibition of HUVEC migration

Migration is a key step required for angiogenesis. VEGF-A is a potent stimulator for HUVEC migration and thus was used as a chemoattractant in the control group (0.1% DMSO) and drug treatment groups. Hex and EA fractions of *A. oxyphylla* dose-dependently inhibited HUVEC migration induced by VEGF-A, achieving 80% and 90% inhibition at 50 μg/ml, respectively (Fig. 3-19). SU5416, a selective VEGF receptor-2 inhibitor, possessed inhibitory effect with 88% at 1 μM. EA fraction at 50 μg/ml showed a comparable effect with SU5416. These results suggest that both fractions inhibited HUVEC migration possibly through VEGF-A-mediated signaling

pathways.

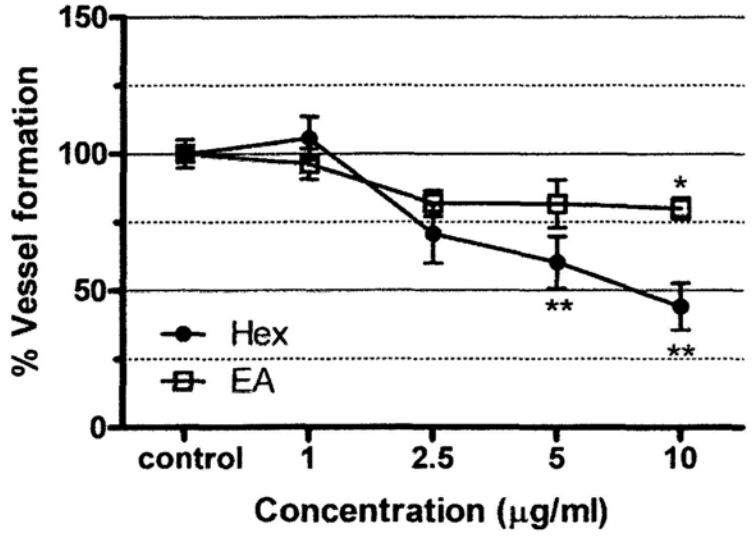


Figure 3-15. Dose response of the anti-angiogenic activity of Hex and EA fractions of *A. oxyphylla*. Fractions were dissolved in DMSO and then added directly to the embryo water. 0.2% DMSO was used as carrier control. After drug treatment, embryos were processed for quantitative EAP assay. Each value represents the mean ± S.E.M. (n = 10) from a representative experiment. * represents $P < 0.05$, ** represents $P < 0.01$ in one-way ANOVA followed by the Dunnett's multiple comparison test.

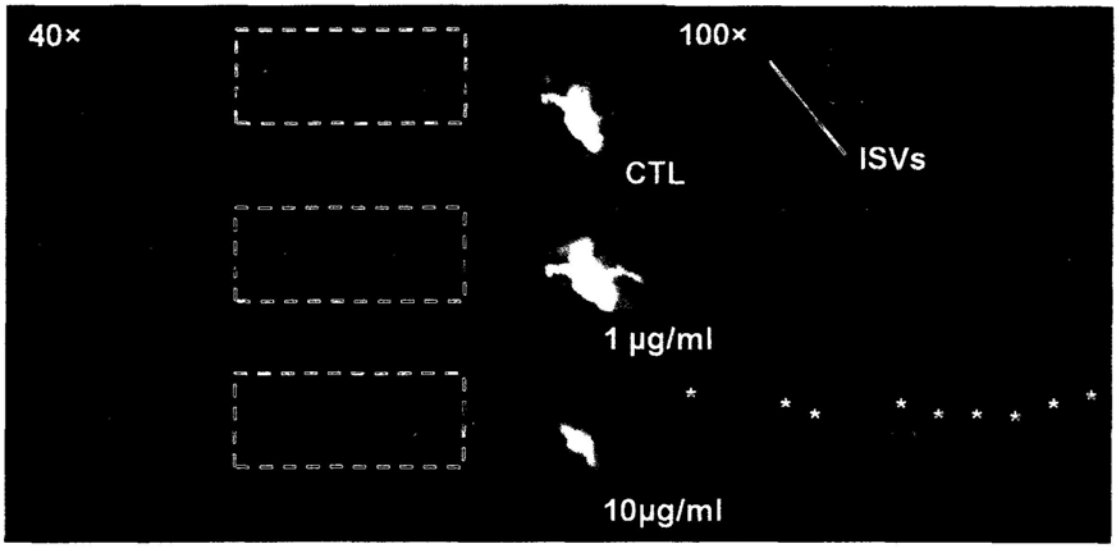


Figure 3-16. Lateral view of *Tg(fli1a:EGFP)y1* zebrafish embryos at 48 hpf immersed in *n*-hexane fraction of *A. oxyphylla* at different concentrations. CTL: live fluorescence microscopy highlights EGFP expressing intersegmental blood vessels (ISVs); 1 μg/ml: the ISVs appeared normal as those of the control group; 10 μg/ml: most of the ISVs failed to form at the highest dosage treatment (asterisks).

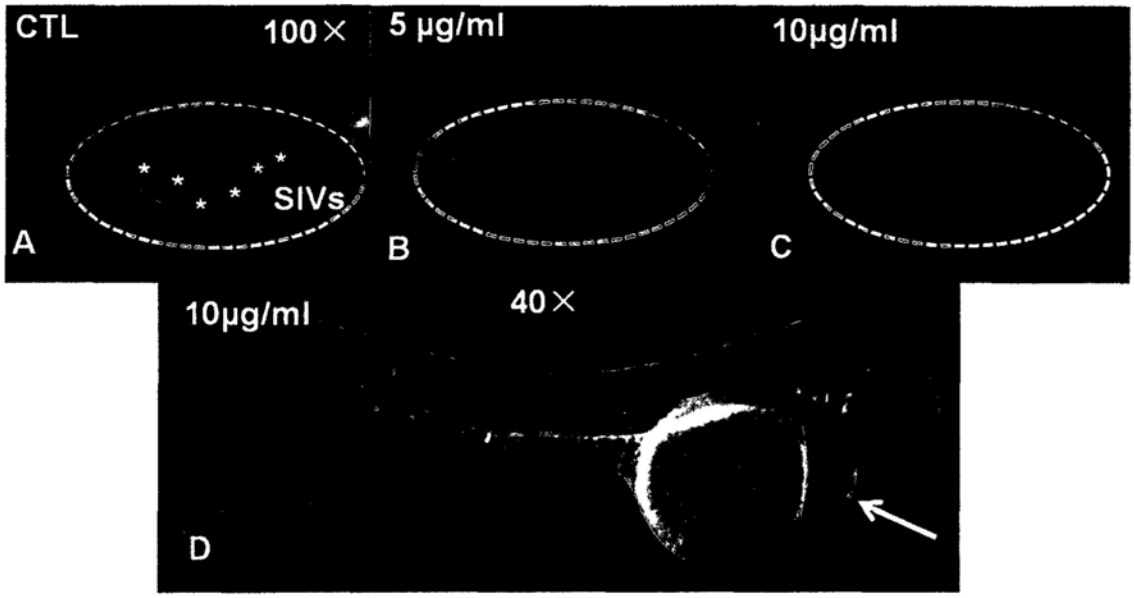


Figure 3-17. (A~C) Lateral view of *Tg(fli1a:EGFP)y1* zebrafish embryos at 72 hpf immersed in *n*-hexane fraction of *A. oxyphylla* at different concentrations. CTL: live fluorescence microscopy highlights EGFP expressing the subintestinal vessel plexus (SIVs), which appears as a smooth basket-like structure with 5–6 arcades (asterisks); 5μg/ml: the SIVs were highly inhibited; 10 μg/ml: the SIVs almost failed to form at this high dosage. (D) Lateral view of *Tg(fli1a:EGFP)y1*zebrafish embryos at 72 hpf showing pericardinal edema (arrow) after 10μg/ml treatment.

3.3.1.4. Inhibition of HUVEC network formation

When seeded on Matrigel, HUVEC can elongate and form capillary like-structure mimicking the *in vivo* neoangiogenesis process (Taraboletti and Giavazzi, 2004). HUVEC at 18-h post seeding had peak network formation in the control group. SU5416 at 1 μM marginally inhibited network formation while both Hex and EA fractions of *A. oxyphylla* at 30–50 μg/ml significantly inhibited network formation in dose-dependent manners. Hex fraction at 50 μg/ml almost completely blocked network formation by 97% (Fig. 3-20), and showed much higher potency than EA fraction.

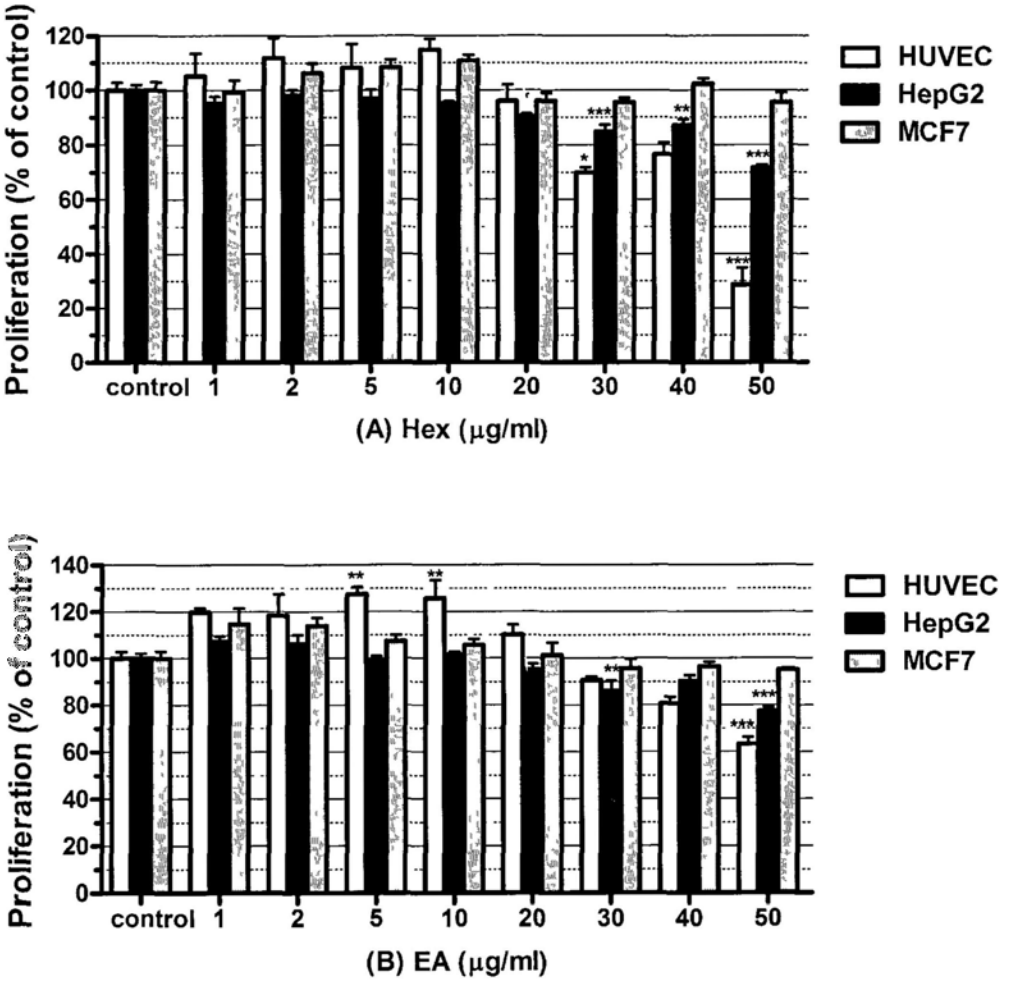


Figure 3-18. Anti-proliferation effect of Hex (A) and EA (B) fractions of *A. oxyphylla* on HUVECs, HepG2 and MCF7 at 24h. Each point represents mean + S.E.M. (n=3) from a representative experiment. * represents $P < 0.05$, ** represents $P < 0.01$, *** represents $P < 0.001$ in one-way ANOVA followed by the Dunnett's multiple comparison test.

3.3.2. Discussion and conclusion

Alpinia species are often used for the treatment of inflammatory conditions and as an anti-cancer agent in East Asian medicine. Four *Alpinia* species (*A. galanga* (L.) Willd., *A. katsumadai* Hayata, *A. officinarum* Hance and *A. oxyphylla* Miq.) are listed in the Chinese Pharmacopoeia for various therapeutic applications (Pharmacopoeia Committee, 2005; Tewari et al., 1999). A methanol extract of *A. officinarum* exhibited

remarkable antitumor-promoting activity *in vivo*, and its diarylheptanoids, for example, 7-(4'-hydroxy-3'-methoxyphenyl)-1-phenylhept-4-en-3-one, showed marked anti-inflammatory effects (Yadav et al., 2003), while 1-(4-hydroxy-3-methoxyphenyl) 7-(3,4-dihydroxyphenyl)-4E-en-3-heptanone induced cytotoxic and apoptotic effects against tumor cells (Tian et al., 2009). The ethanol extract of *A. katsumadai* also showed anti-inflammatory potential (Choi et al., 2009; Yang et al., 2009), and two flavonoids derived from the herb exhibited potent cytotoxic effect against tumor cells (Hahm et al., 2003; Hua et al., 2009). An 80% aqueous acetone extract from the rhizomes of *A. galanga* showed NO production inhibitory activity in mouse peritoneal macrophages (Morikawa et al., 2005), while ethyl 4-methoxy-*trans*-cinnamate and 1'S-1'-acetoxychavicol acetate from the herb, respectively, exhibited anti-carcinogenic effects (Zheng et al., 1993) and anti-inflammatory effects (Ando et al., 2005). Anti-cancer or anti-inflammatory effects are reported from *A. blepharocalyx* (K.) (Ali et al., 2001a; Ali et al., 2001b; Kadota et al., 2003; Tezuka et al., 2001), *A. Calcarata* Rosc. (Kong et al., 2002), *A. conchigera* Griff (Lee et al., 2006b; Sulaiman et al., 2009), *A. densespicata* Hayata (Kuo et al., 2009), and *A. pricei* Hayat (Yang et al., 2008; Yu et al., 2009).

Our results revealed that the Hex and EA fractions of *A. oxyphylla* could both reduce the vessel formation of zebrafish and also block network formation in HUVEC. Furthermore, both fractions also inhibited the proliferation of HepG2 tumor cells, suggesting the anti-cancer activity may be partly mediated through anti-angiogenesis. A series of sesquiterpenes and diarylheptanoids, showing anti-inflammatory or anti-tumor potentials, have been isolated from the fruit of *A. oxyphylla*; they very likely exist in Hex and EA fractions according to their molecular polarities. Further studies on diarylheptanoids, such as yakuchinones A and B, could possibly provide more details on the anti-angiogenic effects of this herb.

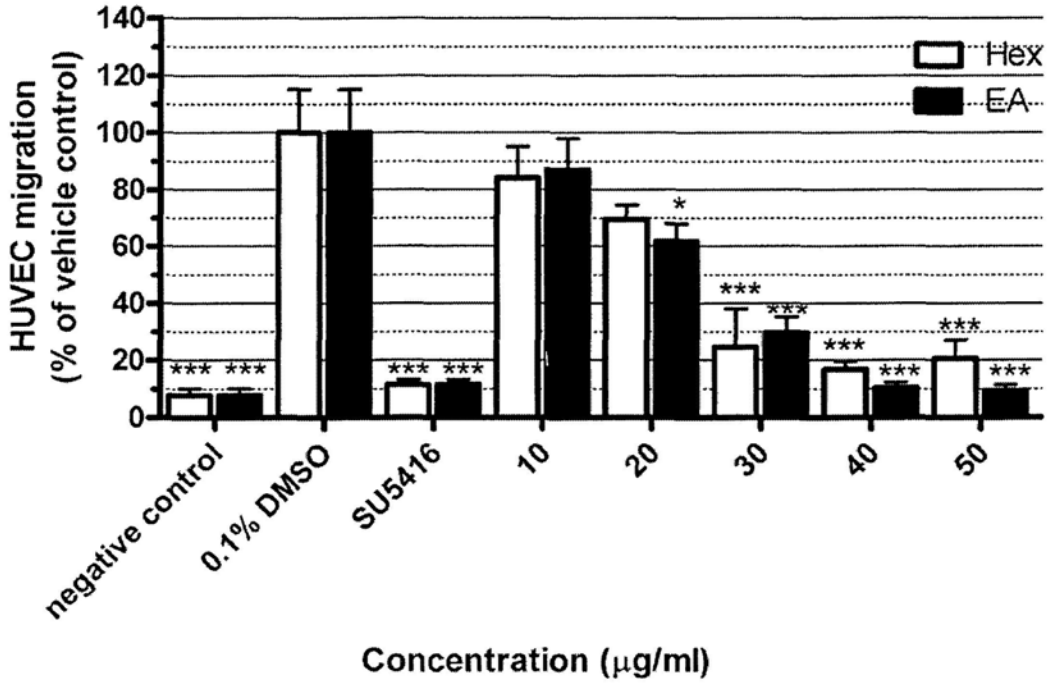


Figure 3-19. Inhibitory effects of Hex and EA fractions of *A. oxyphylla* on HUVEC migration mediated by VEGF-A (10 ng/ml). Each point represents mean +S.E.M. (n=3) from a representative experiment. * represents $P < 0.05$, ** represents $P < 0.01$, *** represents $P < 0.001$ in one-way ANOVA followed by the Dunnett's multiple comparison test.

3.4. Anti-angiogenic effects and mechanisms of *Kaempferia galanga* (Rhizoma Kaempferiae)

3.4.1. Results

3.4.1.1. Quantitative EAP assay on zebrafish embryo

The 95% ethanol crude extract and Hex, EA, BuOH, and Aq fractions obtained from the 95% ethanol extract of *K. galanga* were examined with zebrafish angiogenic assay. As shown in Fig. 3-21, the ethanol extract inhibited vessel formation by 42% at 10 μg/ml. Successive fractionation showed that the Hex and EA fractions at 10 μg/ml potently inhibited vessel formation by 47% and 35%, respectively, in the embryos, and both fractions showed does-dependent manner (Fig. 3-22), indicating the presence of anti-angiogenic components in the two fractions. The existence of compounds

KG-1 and KG-2 was confirmed by comparing with authentic chemical markers on thin layer chromatography (Fig 3-23)

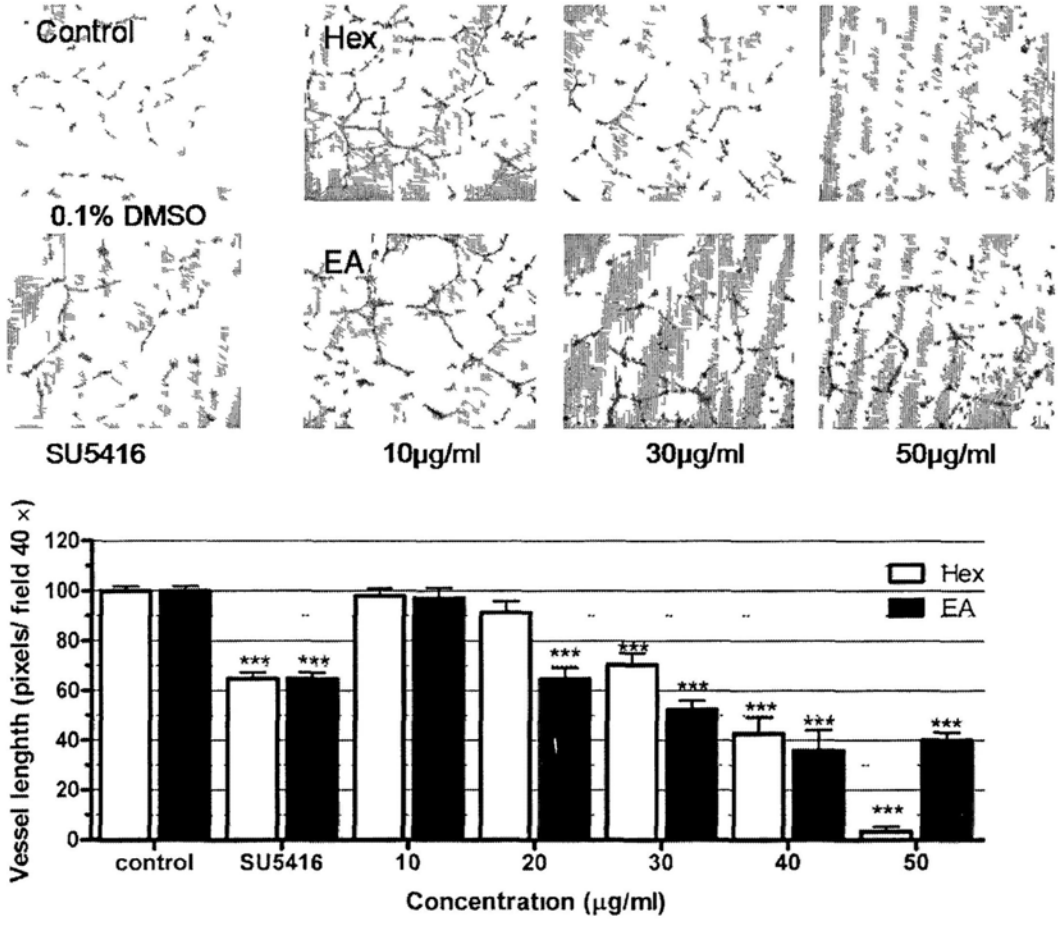


Figure 3-20 Inhibitory effects of Hex and EA fractions of *A oxyphylla* on HUVEC network formation Images were taken under inverted microscope after 18 h incubation The network length in each treatment conditions were quantified in the lower panel Each point represents mean +S E M (n=4) from a representative experiment * represents $P < 0.05$, ** represents $P < 0.01$, *** represents $P < 0.001$ in one-way ANOVA followed by the Dunnett's multiple comparison test

The anti-angiogenic activity of compounds KG-1 and KG-2 were evaluated with zebrafish angiogenic assay. As shown in Fig. 3-24, both compounds displayed anti-angiogenic activity in the zebrafish model, starting to inhibit vessel formation by about 20% at 2.5 μM. At the highest concentration (20 μM), the inhibition of KG-1

could reach about 60%. However, the effect of KG-2 was less than 25% at all doses tested. The inhibitory effect showed a dose-dependent manner and KG-1 was a more potent candidate than KG-2 for further anti-angiogenic investigation.

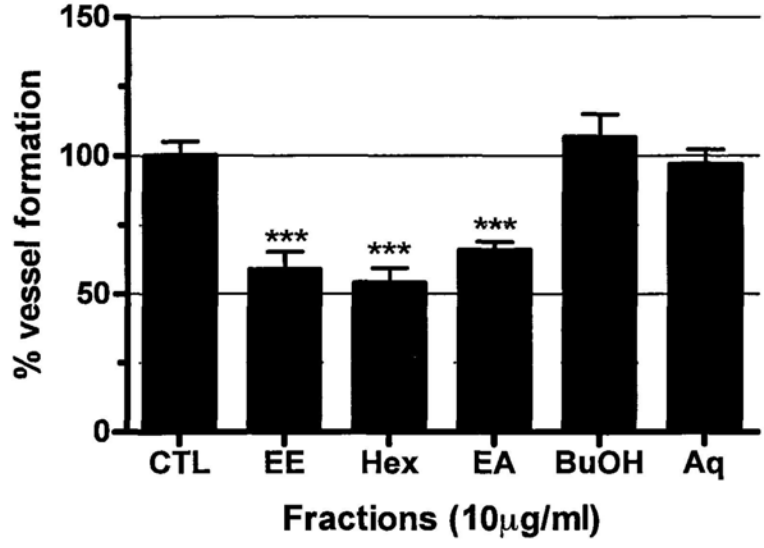


Figure 3-21 Anti-angiogenic activity of the fractions of *K galanga* Fractions were dissolved in DMSO and then added directly to the embryo water (final concentration 10 μg/ml) 0 2% DMSO was used as carrier control After drug treatment, embryos were processed for quantitative EAP assay Each value represents the mean ± S E M (n = 10) from a representative experiment *** represents $P < 0\ 001$ in one-way ANOVA followed by the Dunnett's multiple comparison test Ethanol extract (EE) *n*-hexane fraction (Hex), ethyl acetate fraction (EA) and *n*-butanol fraction (BuOH), aqueous fraction (Aq).

3.4.1.2. Microscopic imaging

The loss of vessel formation in the embryos treated with 20 μM of compounds KG-1 and KG-2 were further confirmed using microscopic imaging on *Tg(fli1a EGFP)y1* zebrafish embryos. The ISVs were the most easily observed angiogenic vessels in the embryos at 48 hfp, and treatment with 0.2% DMSO had no effect on the vessel formation and served as a vehicle control (Fig. 3-25, CTL). After treatment, both KG-1 and KG-2 could partially block ISVs formation at 48 hpf (Fig. 3-25, asterisks). The SIVs, appearing as a smooth basket-like structure with 5-6

arcades, is also easily observed on *Tg(fli1a:EGFP)y1* zebrafish at 72 hpf (Fig. 3-27, CTL). At 20 μM, both KG-1and KG-2 could completely inhibit SIVs formation compared to control group after 48-h treatment (Fig. 3-27). However, only KG-1 caused pericardinal edema both at 48 and 72 hpf (Fig. 3-25, 3-26, arrowheads), and the similar phenomenon was also found in the embryos treated by PTK787/ZK222584, which is a novel anilinophthalazine compound, a high affinity inhibitor of VEGF receptors, and currently under phase II clinical trial for the treatment of metastatic gastrointestinal stromal tumors (Chan et al., 2002; Joensuu et al., 2008). Meanwhile, the overall morphology and structure of treated embryos were generally normal by 72 hpf. All these results suggested the necessity of further investigation on KG-1 of its action mechanism.

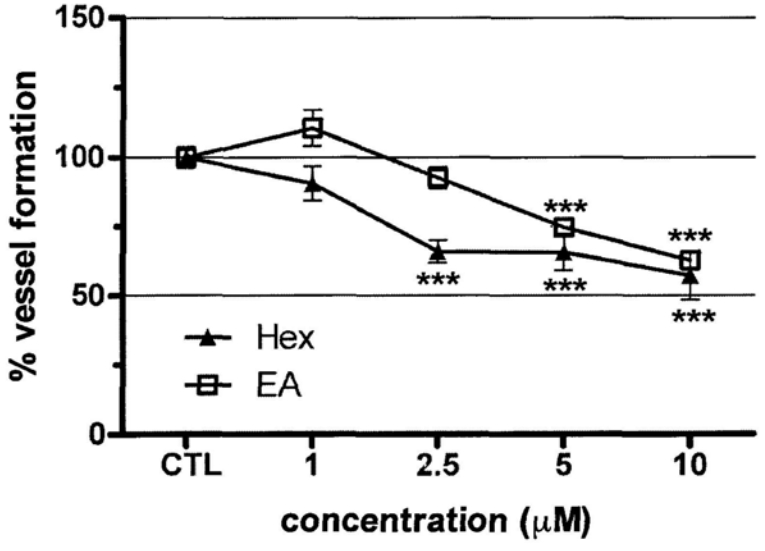


Figure 3-22. Dose response of the anti-angiogenic activity of Hex and EA fractions of *K. galanga*. Fractions were dissolved in DMSO and then added directly to the embryo water. 0.2% DMSO was used as carrier control. After drug treatment, embryos were processed for quantitative EAP assay. Each value represents the mean ± S.E.M. (n = 10) from a representative experiment. *** represents $P < 0.001$ in one-way ANOVA followed by the Dunnett's multiple comparison test.

3.4.1.3. Molecular mechanism of KG-1 on zebrafish angiogenesis

KG-1 showed potent anti-angiogenic activities on zebrafish model, however its action mechanism is unknown. VEGFA-VEGFR and ANGIOPOIETIN (ANGPT)-TIE are two major studied signaling pathways involved in angiogenesis (Ellis et al., 2008; Thomas et al., 2009; Thurston, 2003). VEGFA (commonly referred to as VEGF) is a potent angiogenic factor that mediates most biological functions via KDR (VEGFR2), and expressed as various isoforms due to alternative splicing which leads to mature 121-, 165-, 189- and 206-amino-acid proteins. $VEGFA_{165}$ is the predominant isoform and is commonly overexpressed in many human solid tumors (Ellis et al., 2008; Donnini et al., 2004). The ANGPT-TIE system acts as vascular specific ligand/receptor system essential for blood vessel formation, and it consists of four ligands (ANGPT1-4) and two corresponding tyrosine kinase receptors (TIE1 and TIE2). The best characterized ligands are ANGPT1 and ANGPT2 (Gale and Yancopoulos, 1999; Thomas et al., 2009). So the major molecules participating in these two signaling pathways, including $VEGFA_{165}$, KDR, ANGPT1, ANGPT2, TIE1

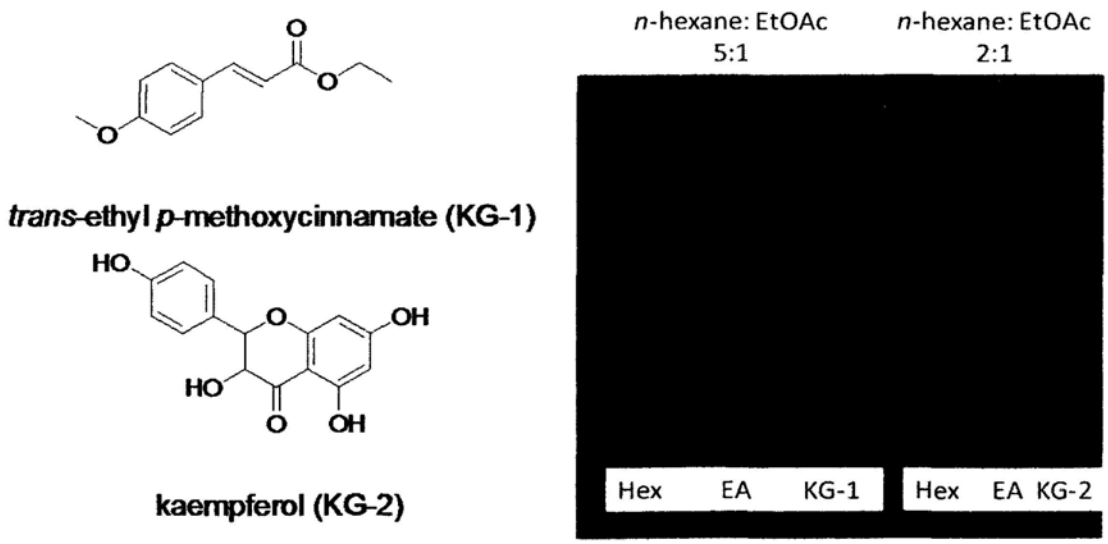


Figure 3-23. Structure and TLC identification of compounds KG-1 and KG-2

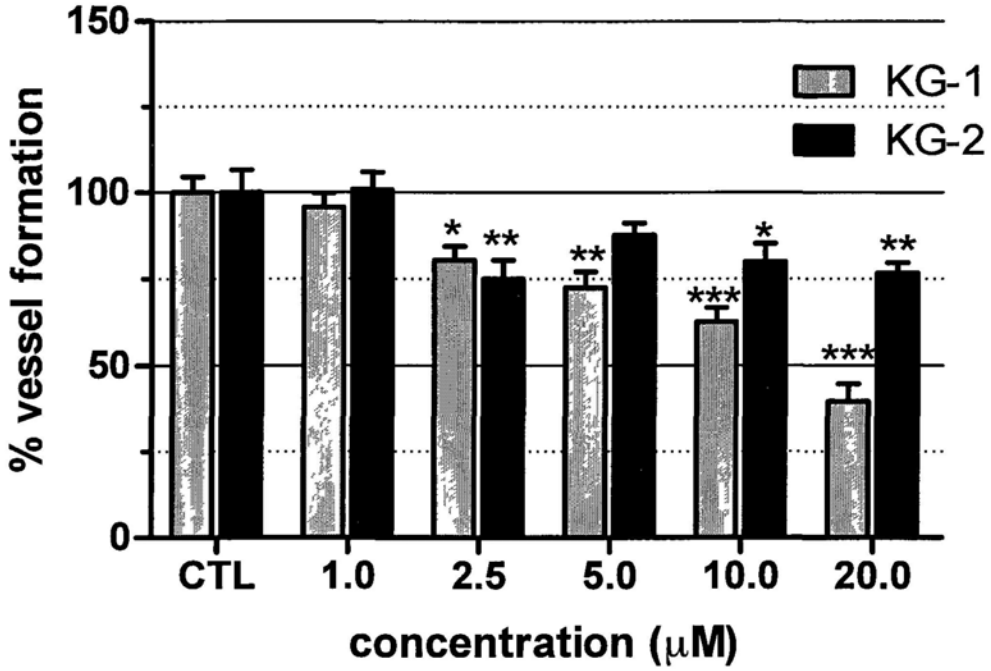


Figure 3-24. Dose response of the anti-angiogenic activity of compounds KG-1 and KG-2. 0.2% DMSO was used as carrier control. After drug treatment, embryos were processed for quantitative EAP assay. Each value represents the mean ± S.E.M. (n = 10) from a representative experiment. * represents $P < 0.05$, ** represents $P < 0.01$, *** represents $P < 0.001$ in one-way ANOVA followed by the Dunnett's Multiple Comparison Test.

and TIE2 were thus investigated. Because zebrafish embryos were treated with drugs at 24 hpf and evaluated at 72 hpf, the time frame between 24 and 72 hpf was selected for the mechanism study.

In the time course study after treatment with 10 μM KG-1 at 24 hpf (Fig. 3-29A), the mRNA expression of five genes (*kdr*, *angpt1*, *angpt2*, *tie1* and *tie2*) was down-regulated at the early developmental stage at 36 or 48 hpf after 12- or 24-h treatment. By comparison, the expression of $vegfa_{165}$ and $vegfa_{121}$ remained unchanged even at 20 μM (Fig. 3-29A'). The reduced effect of KG-1 after longer treatment on relative gene expression may be due to its short half-life or other unknown reasons. So we carried out another experiment by adding drugs at 24 and 48

hpf to see the responses of the target genes. As shown in Fig. 3-30, the result was similar to the single treatment at 24 hpf (Fig. 3-29A), suggesting that some other reasons may cause the transient response but not short half-life. In the dose-response study after 18-h treatment at 42 hpf (Fig. 3-31), the mRNA expression encoded by *kdr*, *angpt1*, *angpt2*, *tie1* and *tie2* were reduced in a dose-dependent manner. At the highest concentration of 20 μM, the mRNA expression of all the five genes could be reduced significantly by about 30%.

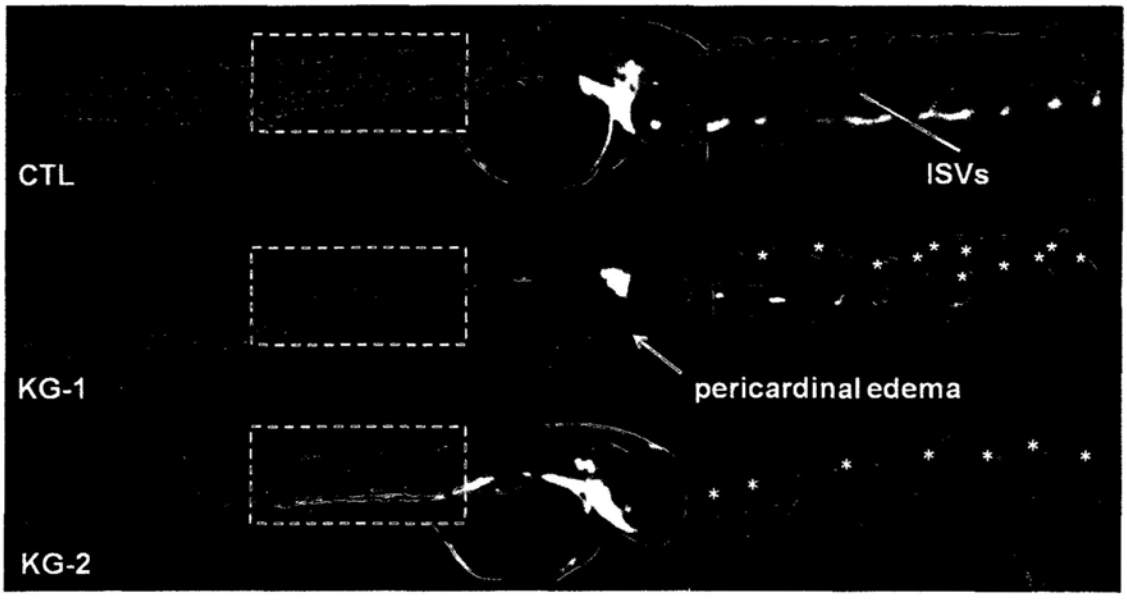


Figure 3-25. Lateral view of the intersegmental blood vessels (ISVs) on *Tg(fli1a:EGFP)y1* zebrafish embryos at 48 hpf immersed in compounds KG-1 and KG-2 at 20 μM concentration.

Figure 3-26. Lateral view of zebrafish embryo at 72 hpf showing pericardinal edema (arrow) after KG-1 treatment at 20 μM concentration.

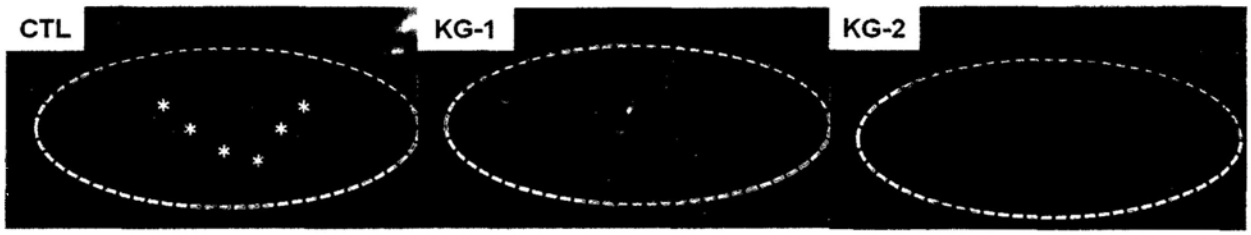


Figure 3-27. Lateral view of the subintestinal vessel plexus (SIVs) on *Tg(fli1a:EGFP)y1* zebrafish embryos at 72 hpf immersed in compounds KG-1 and KG-2 at 20 μM concentration.

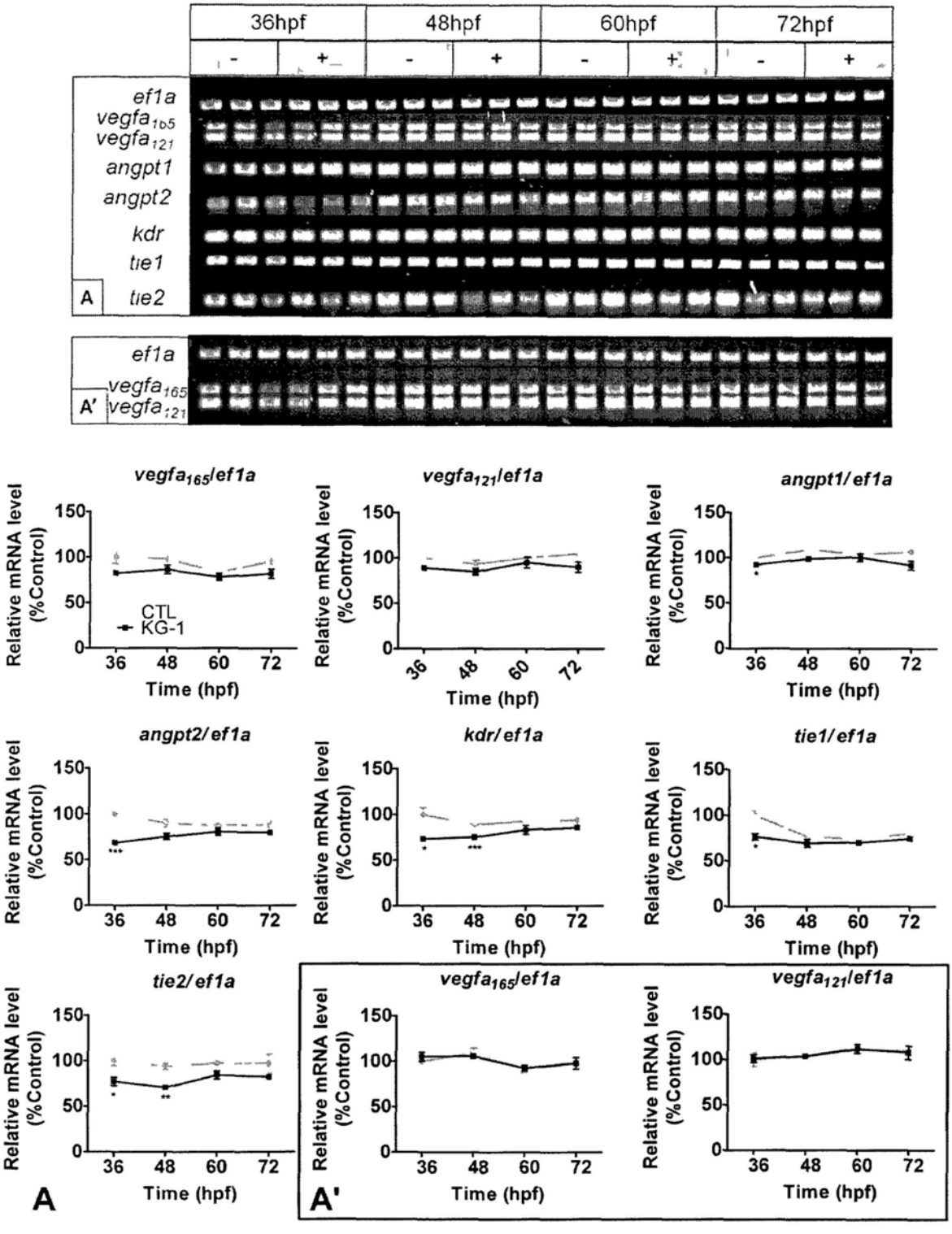


Figure 3-28. The time course effects of KG-1 on the expressions of *vegfa, kdr, angpt1, angpt2, tie1 and tie2* genes in zebrafish embryos after drug treatment at 24 hpf (A. 10 μM, A': 20 μM). *ef1a* was used as internal control to normalize the expression levels. Each value represents the mean ± S.E.M. (n = 3) from a representative experiment. * represents $P < 0.05$, ** represents $P < 0.01$, *** represents $P < 0.001$ in Student's t-test.

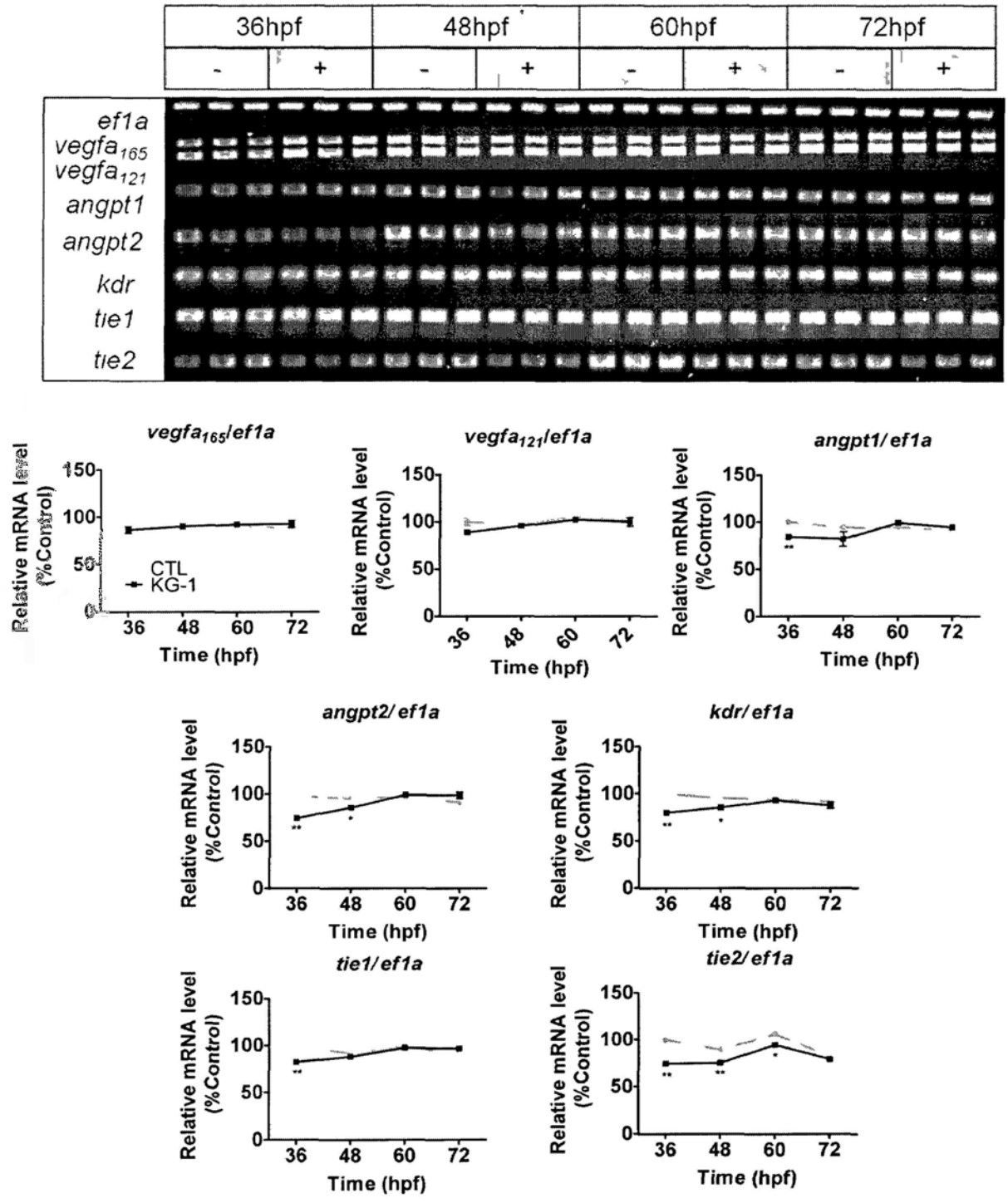


Figure 3-29 The time course effects of KG-1 on the expressions of *vegfa, kdr, angpt1, angpt2, tie1 and tie2* genes in zebrafish embryos after repeated drug treatments at 24 and 48 hpf (10 μM). *ef1a* was used as internal control to normalize the expression levels. Each value represents the mean ± S E.M (n = 3) from a representative experiment. * represents $P < 0.05$, ** represents $P < 0.01$ in Student's t-test

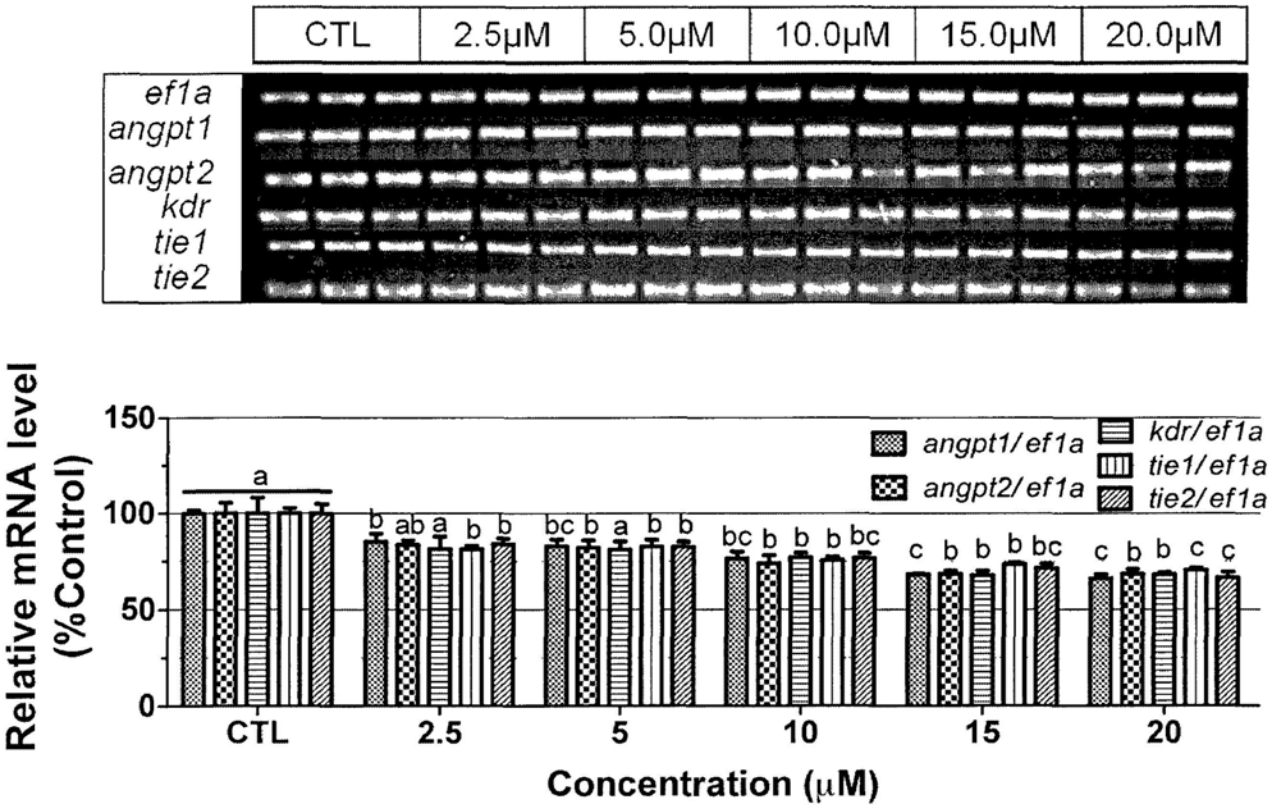


Figure 3-30. The dose–response effects of KG-1 on the expressions of *vegfa, kdr, angpt1, angpt2, tie1 and tie2* genes in zebrafish embryos at 42 hpf. *ef1a* was used as internal control to normalize the expression levels. Each value represents the mean ± S.E.M. (n = 3) from a representative experiment. The data were analyzed by one-way ANOVA followed by the Newman–Keuls test for comparisons of all pairs of groups. Different letters indicate statistical significance among groups ($P < 0.05$).

3.4.1.4. Effects of KG-1 on HUVEC proliferation, migration and tube formation

In order to further confirm the anti-angiogenic effect of KG-1, HUVECs were used in the MTT-based cytotoxicity test and the tritiated thymidine incorporation assay (Kim et al., 2009). In MTT assay (Fig. 3-11, A), KG-1 showed no obvious cytotoxicity on HUVECs at the range from 2.5 to 40 μM. Using tritiated thymidine incorporation assay, it also had no inhibitory effect on the proliferation of HUVECs at the same concentration range. Migration is a key step for angiogenesis, and KG-1

could dose-dependently inhibit HUVEC migration induced by FBS concentration gradient, achieving about 30-40 % inhibition at 20 and 40 μM (Fig. 3-33). Tube formation is another key step usually associated with cell migration and differentiation, and it showed inhibitory effect by about 30% at 20 and 40 μM on HUVECs (Fig. 3-34). All the results revealed that KG-1 could specifically inhibit the migration and tube formation steps of HUVECs to prevent the angiogenesis progress.

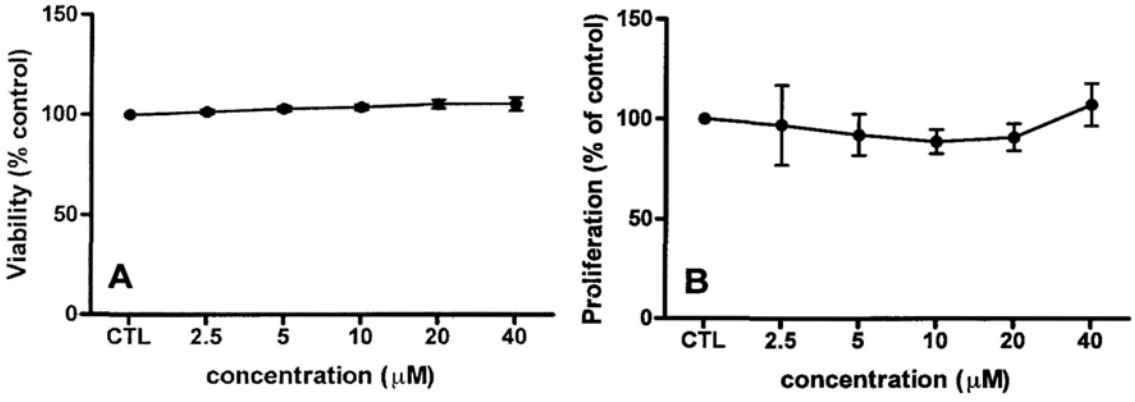


Figure 3-31. MTT (A) and tritiated thymidine (B) assay of KG-1 on HUVECs. Each point represents mean ± S.E.M. (n = 5) from 3 independent experiments. * represents $P < 0.05$ in one-way ANOVA followed by the Dunnett's test for comparisons of all treated groups with respective control group.

3.4.2. Discussion and conclusion

Kaempferia galanga can be used as food seasoning and medicinal herb with various effects, especially anti-rheumatism, anti-inflammatory and anti-tumor (Warrier et al., 2003). Rheumatoid arthritis is closely related to angiogenesis, which is characterized by destruction of peripheral joints, and the cartilage and bone are destroyed by proliferative synovitis, involving inflammatory cells infiltration and new vessel formation. Since the early stage of the disease, angiogenesis occurs and supports the arthritis progression (Roccaro et al., 2005). The results demonstrated that medicinal herb used to treat anti-inflammatory diseases may have the potential to be

the angiogenesis inhibitors because the anti-inflammatory herb and its components, KG-1 and KG-2, also showed potent anti-angiogenic activity. Thus, medicinal Chinese herb for the treatment of inflammatory diseases may be a good source for discovering anti-angiogenic agents.

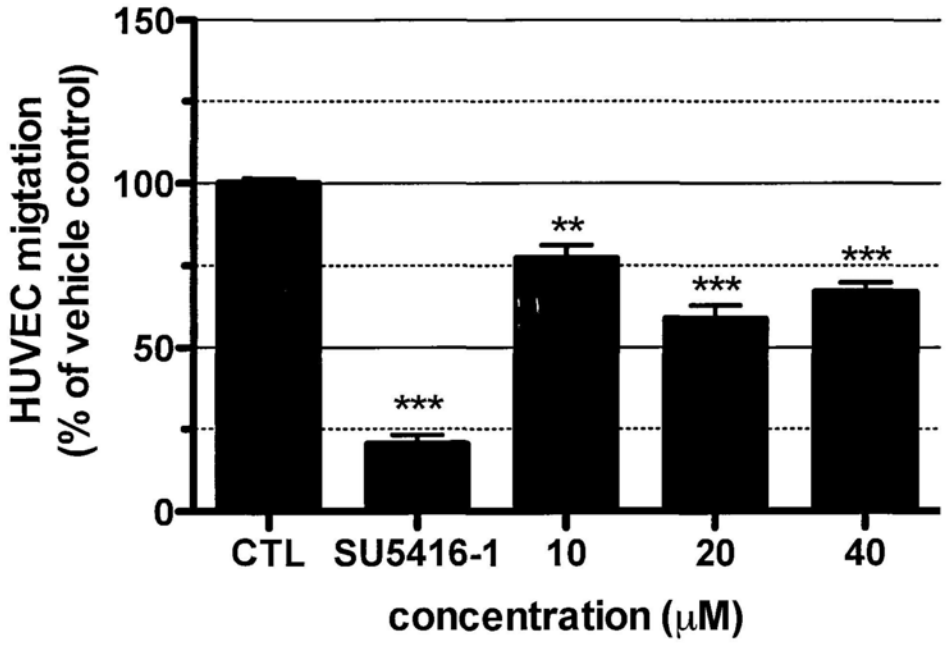


Figure 3-32. Migration assay of KG-1 on HUVECs. Representative images depicting the treatment effect (3 h) on HUVEC migration; Diagrams represent percentage of HUVEC migration following a 3-h exposure to the indicated concentration of KG-1 relative to control. SU5416 (1 μM) was used as positive control. Each point represents mean ± S.E.M. (n = 3) from a representative experiment. ** represents $P < 0.01$, *** represents $P < 0.001$ in one-way ANOVA followed by the Dunnett's test for comparisons of all treated groups with control group.

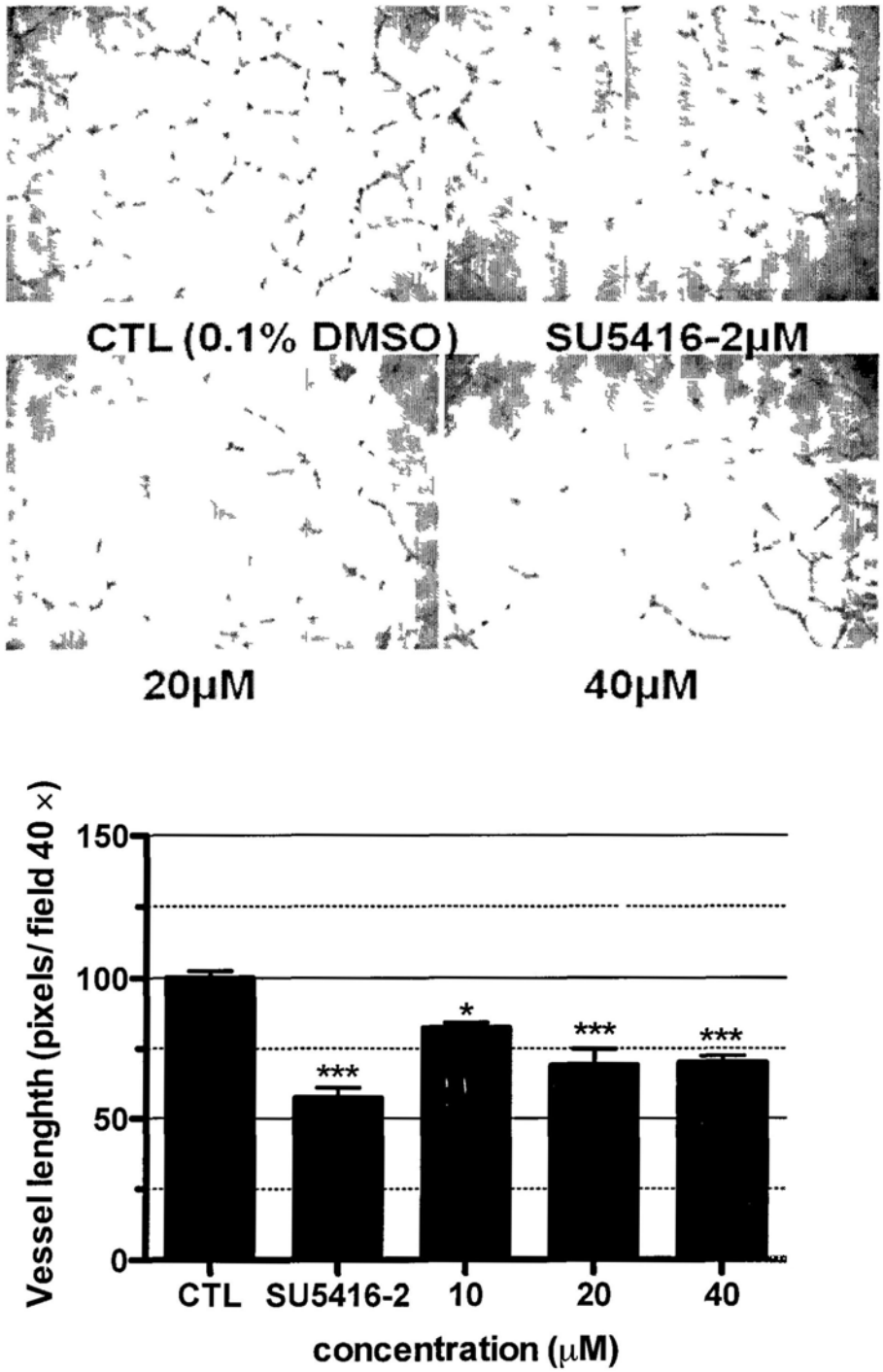


Figure 3-33 Tube formation assay of KG-1 on HUVECs Representative images and diagram depicting formation of capillary-like tube structures by HUVECs following 17-h treatment with indicated concentration SU5416 (2 μM) was used as a positive control Each point represents mean ± SEM (n = 4) from a representative experiment * Represents $P < 0.05$, *** represents $P < 0.001$ in one-way ANOVA followed by the Dunnett's test for comparisons of all treated groups with control group Scale bar=1000 μm

Our work has successfully used an *in vivo* zebrafish model for both the screening of anti-angiogenic agents from medicinal herb and subsequent discovery for the drug targets. As shown in Fig. 3-21, both Hex and EA fractions had anti-angiogenic activity, and the Hex fraction had much stronger effect than EA fraction according to the dose-dependent result (Fig. 3-22). In rhizomes, KG-1(*trans*-ethyl *p*-methoxycinnamate) is the major bioactive component with anti-cancer effect. Results showed that both *cis*- and *trans*-ethyl *p*-methoxycinnamate in *K. galang* had a relatively strong anti-carcinogenic potential with IC_{50} of 5.5 and 9.5 mol/L, respectively (Xue et al., 2002a; b). KG-1 had inhibitory activity against HeLa cells (Kosuge et al., 1985) and showed promise as potential chemopreventive agent due to its induction of increased glutathione *S*-transferase (GST) activity, which is believed to be a major mechanism for chemical carcinogen detoxification and has been recognized as one of the characteristics of the action of anticarcinogens (Zheng et al., 1993). KG-1 (51.6-59.24%) in the rhizome of *K. galanga* is the major component (Fan et al., 2005; Jirovetz et al., 2001; Wong et al., 1992). Due to its low polarity, the essential oil should be extracted mostly in *n*-hexane (Hex) solvent, and therefore KG-1 may be the most potential component to inhibit vessel formation. The TLC identification result proved our hypothesis that KG-1 mainly existed in Hex fraction and also in EA fraction (Fig. 3-23), suggesting that this compound should be the major bioactive component for Hex fraction. The assay on zebrafish model revealed that its dose-dependent anti-angiogenic effect was mainly mediated through blocking ISVs and SIVs formation (Fig. 3-25 and 3-27).

KG-2 (kaempferol) is another major bioactive component existing in EA fraction only (Fig. 3-23) with various biological activities including inhibition of lipoxygenase and cyclooxygenase, antiaggregatory, antibacterial and anticancer (Kim et al., 2006). KG-2 could inhibit tumor necrosis factor-α-induced endothelial cell selectin

(E-selectin) expression on HUVECs (Yuko et al., 2003). KG-2 also had anti-inflammatory property through modulating pro-inflammatory genes and blocking signaling molecules NF-κ and activator protein-1 (AP-1) induced by a cytokine mixture in HUVECs (Crespo et al., 2008). With low cytotoxicity, KG-2 could suppress the VEGF-stimulated HUVEC tubular structure formation by 15% (Kim et al., 2006), and reduced VEGF secretion from breast cancer cells at 0.1 μM (Schindler and Mentlein, 2006). Its inhibition of angiogenesis and VEGF expression in human ovarian cancer cells was mediated through both HIF-dependent (Akt/HIF) and HIF-independent (ESRRA) pathways (Luo et al., 2009; Matsuo et al., 2005). Our results further confirmed its potential application as an anti-angioenic agent on zebrafish model.

Because no anti-angiogenic activity has been reported on KG-1 and it showed much stronger effect than KG-2, we decided to use zebrafish as the model to elucidate its molecular targets. KG-1 could reduce multi-target expression, including *kdr*, *angpt1*, *angpt2*, *tie1* and *tie2*, in time- and dose-dependent manners during zebrafish angiogenesis (Fig. 3-28, 5-29 and 5-30). Although the expression reduction of these five genes occurred only at the early stage (36 or 48 hpf), the inhibition of vessel formation could last up to 72 hpf (Fig. 3-27). This drug target pathway has not been reported before. Because cancer is a multi-genetic disease and the process of carcinogenesis also requires a permissive microenvironment to facilitate the tumor development and maintenance. In this perspective, the use of multiple-targeting compounds able to target both the tumor and the microenvironment cells might represent a more efficient strategy in fighting cancer. Moreover, multi-targeted anti-angiogenic drug like Sorafenib and Sunitinib has been recently approved by FDA to treat advanced cancer (Petrelli and Giordano, 2008). In VEGFA-VEGFR pathway, the ligand of predominant isoform-$VEGFA_{165}$ binds to and activates tyrosine kinase-

VEGFR2 (KDR) to regulate the angiogenesis. KDR expression is restricted primarily to the vasculature and is the key mediator of VEGF-induced angiogenesis (Elli and Hicklin, 2008). In ANGPT1-TIE2 pathway, ANGPT1 and ANGPT2 are specific ligands of TIE2 with similar affinity. TIE2 activation promotes vessel assembly and maturation. ANGPT2 is produced by endothelial cells and acts as an autocrine antagonist of ANGPT1-mediated TIE2 activation, which is required to maintain the quiescent resting state of the endothelium. ANGPT2 induces vascular destabilization at higher concentrations through antagonizing ANGPT1-TIE2 signaling. Also, ANGPT2 is strongly expressed in the vasculature of many tumors and may act synergistically with other cytokines such as VEGF to promote the tumor-associated angiogenesis and tumor progression. However, the function of ANGPT1 in tumor-associated angiogenesis remains controversial. The promoting or inhibiting functions are dependent on the tumor cell type, the dosage and possibly on the amount of ANGPT2 in the tumors. TIE1 is almost exclusively expressed by endothelial cells and critical for vascular development, however, it is still largely considered as an orphan receptor and the function remains enigmatic (Fiedler and Augustin, 2006; Thomas and Augustin, 2009; Thurston, 2003).

Zebrafish homologues of mammalian genes have been cloned and found to have similar functions. Its value as a model organism for drug screening and target discovery has begun to be recognized (Langheinrich, 2003; Crawford et al., 2008). More and more evidence shows that anti-angiogenic compounds effective in mammals elicit similar effects in zebrafish (Langheinrich, 2003; Parng et al., 2002). Further tests on human model confirmed that *trans*-ethyl *p*-methoxycinnamate could inhibit the progress of HUVEC migration and tube formation (Fig. 3-32 and 3-33).

In conclusion, *K. galanga* and its *trans*-ethyl *p*-methoxycinnamate and kaemperol are potent angiogensis inhibitors with potential application against

angiogenesis in cancer treatment, and the anti-angiogenic effect of this herb may partially contribute to its anti-cancer effect. Both *vegfa-vegfr* and *angpt-tie* signaling pathways are potential targets for *trans*-ethyl *p*-methoxycinnamate-induced anti-angiogenesis. Our results also demonstrated that zebrafish platform is excellent not only for screening potential anti-angiogenic agents but also for molecular target identification.

Chapter 4

General Discussion

As essential in a series of normal physiological processes such as embryonic development and pathological responses, angiogenesis also causes "angiogenic diseases" such as diabetic retinopathy, tumor growth and metastasis, rheumatoid arthritis, and inflammatory diseases under persistent unregulated situation (Folkman, 1995). Among these angiogenesis dependent diseases, cancer is a large group of diseases that cause a vast medical problem and death worldwide nowadays. However, the prognosis has not greatly improved due to the limitations of current therapies, such as toxicity, inherent and acquired resistance caused by chemotherapy, and metastatic spread caused by surgery. This calls for novel cancer therapies and new group of anticancer agents for selectively targeting cancers without or with lower toxicity to normal tissues. The inhibition of angiogenesis or anti-angiogenic therapy may represent a new promising anticancer therapeutic strategy, and the U.S. National Cancer Institute and other researchers have integrated the anti-angiogenic therapeutic approaches to their preclinical anticancer drug research (Folkman, 1971; Ruegg and Mutter, 2007; Bar and Onn, 2008; Griffioen, 2008; Cao, 2009). Bevacizumab (Avastin®) developed by Genentech Inc. is the first humanized monoclonal antibody to inhibit endothelial cell proliferation and angiogenesis for the treatment of metastatic colorectal cancer, non-small cell lung cancer, advanced breast cancer, glioblastoma, metastatic renal cell cancer. Up to now, there have been seven anti-cancer drugs with recognized anti-angiogenic properties in oncology approved by U.S. FDA. Although anti-angiogenesis therapy still function in an ancillary role in clinical trials combined with conventional and potentially novel anticancer therapies due to its limiations (Gupta and Zhang, 2005), it provides another novel choice and

method to treat cancer.

4.1. Zebrafish as an in vivo preclinical model for anti-angiogenic agents screening

The development of anti-angiogenic agents requires powerful preclinical models for the analysis and optimization of the therapy. Various *in vitro* and *in vivo* preclinical models have been used for the discovery of angiogenic inhibitors. *In vitro* assays for the activity of anti-angiogenic compounds are usually based on the use of ECs, such as HUVECs, and focused predominantly on proliferation, migration and tubule formation. Among *in vivo* assays, the CAM assay is currently the most widely used model for the study of angiogenesis largely due to its simplicity and low cost (Staton et al., 2004).

Zebrafish is a small tropical freshwater fish with a short generation time. As a relatively new model organism, it is being applied in more and more areas of biological studies, such as drug target discovery, target validation, drug discovery strategies and toxicological studies, due to its fecundity, morphological and physiological similarity to mammals, and the ease with which large phenotype-based screens can be performed (Stern and Zon, 2003; Zon and Peterson, 2005). Unlike the traditional drug screens using cell lines or *in vitro* protein binding assays, the use of zebrafish would allow the selection of bioactive compounds in a whole organism and in cells undergoing normal cell–cell and cell–matrix interactions (Stern and Zon, 2003). Many anti- and pro-angiogenic molecules tested in zebrafish demonstrated similar effects to those observed in humans or other mammalian models. Besides providing a powerful platform for drug screening, zebrafish model can also be used for probing biological processes to generate insights into mechanisms. In addition, the optical transparency and ability to survive for 3–4 d without functioning circulation

make the zebrafish embryo amenable for direct and continuous microscopic inspections of vascularization. Its transparency becomes even more useful when fluorescent markers are used to label specific populations of cells, including ECs (Lawson and Weinstein, 2002; Tran et al., 2007). Zebrafish is the only vertebrate system currently used for rapid *in vivo* compound screening, and addition to the *in vivo* models for studying anti-angiogenic agents (Taraboletti and Giavazzi, 2004). These features make zebrafish embryos an attractive model for identification of anti- or pro-angiogenic agents.

Under the zebrafish model screening, our laboratory has successfully found out the anti-angiogenic components from a traditional Chinese medicine, *Tripterygium wilfordii*, which is a popular herb in China for the treatment of chronic inflammations (Brinker et al., 2007). The most potent component from *T. wilfordii*, triptolide, could dose- and time-dependently reduce the mRNA expression of *angpt2* and *tie2* in zebrafish. Subsequent investigation of triptolide on *in vitro* HUVEC assay and *in vivo* Matrigel plug and murine tumorigenesis assays showed comparable effects to those observed in zebrafish model (He et al., 2009a and 2010). In this thesis study, three TCMs, *Rheum palmatum*, *Alpinia oxyphylla*, and *Kaempferia galanga* showed potential anti-angiogenic activity on zebrafish model, and aloe-emodin, emodin and rhein from *R. palmatum*; *trans*-ethyl-*p*-methoxycinnamate and kaemperol from *K. galanga*, were found to be their bioactive components. Three compounds, aloe-emodin, emodin and kaemperol could cause comparable effects both on zebrafish model and other *in vitro* and *in vivo* models as reported (Cardenas et al., 2006; Kim et al., 2006; Kimura et al., 2008; Kwak et al., 2006; Ljubimov et al., 2004; Wang et al., 2004). A GFP transgenic zebrafish—*Tg(fli1a:EGFP)y1* driven by a zebrafish *fli1* promoter in ECs has also been used to facilitate the observation of vessel formation affected by these potential agents. The underlying action

mechanisms of the most potential compounds, rhein and *trans*-ethyl-*p*-methoxycinnamate, were also successfully elucidated on zebrafish model.

All these further demonstrated that zebrafish angiogenic model could be an excellent platform not only for screening anti-angiogenic drugs, but also for probing biological processes and generate insights into mechanisms (He et al., 2009a). In addition, vascular-specific transgenic zebrafish with fluorescently "tagged" blood vessels enables long-term, time-lapse analysis of the ECs and facilitates high-resolution imaging studies of developing blood vessels *in vivo*.

4.2. Traditional Chinese medicines as a source for discovering anti-angiogenic agents

Traditional Chinese medicines have long been recognized as a rich source for drug discovering and developing. Various TCMs and their components have shown anti-angiogenic properties. Increasing investigation into medicinal herbs for anti-angiogenic agents leads to the identification of many phytochemicals with anti-angiogenic activity. Due to the close linkage between angiogenesis, tumor growth and metastasis, rheumatoid arthritis, and inflammatory diseases (Folkman, 1995), TCMs possessing effects of anti-"angiogenic diseases", such as anti-inflammation and anti-rheumatoid arthritis may have the potential to treat cancer. Some TCMs that have traditionally been used to treat tumor and inflammation diseases may also act through anti-angiogenesis. As a continuing pursuit in our laboratory for elucidating the anti-angiogenic properties of TCMs, our attention is focused on those with anti-inflammation, anti-rheumatoid arthritis and anti-cancer effects.

Three herbs, *Rheum palmatum*, *Alpinia oxyphylla*, and *Kaempferia galanga*, out of eighteen selected TCMs that are traditionally used to treat inflammation, rheumatoid arthritis and tumor, showed potential anti-angiogenic activity on zebrafish

model (Fig. 3-1.). Several bioactive components have been found from *R. palmatum* and *K. galanga*. Among them, compounds rhein from *R. palmatum* and *trans*-ethyl-*p*-methoxycinnamate from *K. galanga* were the most potential ones. Their action mechanisms were further investigated through semi-quantitative RT-PCR and HUVEC assays. The work on a Chinese herb, *Tripterygium wilfordii* (雷公藤), was another good example in our lab. *T. wilfordii* (Celastraceae family), a woody vine native to Eastern and Southern China, has long been used in China to treat swelling, fever, chills, sores, joint pain and immuno-inflammatory diseases. Assayed on the zebrafish and other *in vitro* and *in vivo* models, compound triptolide was found to be the most potent anti-angiogenic component (He et al., 2009a and 2010).

So far, seven anti-cancer drugs with anti-angiogenic effects have been approved by U.S. FDA (Table 1-1.). However, they all belong to biological products, such as monoclonal antibodies, small molecule tyrosine kinase inhibitors; none of them were derived from herbs. Our findings suggested that searching for anti-angiogenic agents should definitely include Chinese herbs used for inflammation, rheumatoid arthritis and cancer diseases.

4.3. Signaling pathways related to angiogenesis

VEGF-VEGFR and ANGPT-TIE are two major studied signaling pathways (Fig.3-14) involved in angiogenesis (Ellis et al., 2008; Thomas et al., 2009; Thurston, 2003). The best characterized molecules $VEGF_{165}$-KDR ANGPT1/ANGPT2-TIE1/TIE2 were thus investigated.

VEGFA (commonly referred to as VEGF) is a potent angiogenic factor that mediates most biological functions via KDR (VEGFR2), and expressed as various isoforms. $VEGF_{165}$ is the predominant isoform and is commonly overexpressed in many human solid tumors (Ellis et al., 2008; Donnini et al., 2004). KDR expression is

restricted primarily to the vasculature and is the key mediator of VEGF-induced angiogenesis (Elli and Hicklin, 2008).

The ANGPT-TIE system acts as vascular specific ligand/receptor system essential for blood vessel formation, which is consisted of four ligands (ANGPT1-4) and two corresponding tyrosine kinase receptors (TIE1 and TIE2). The best characterized ligands are ANGPT1 and ANGPT2 (Gale and Yancopoulos, 1999; Thomas et al., 2009). In ANGPT1-TIE2 pathway, ANGPT1 and ANGPT2 are specific ligands of TIE2 with similar binding affinity. TIE2 activation promotes vessel assembly and maturation. ANGPT2 is produced by endothelial cells and acts as an autocrine antagonist of ANGPT1-mediated TIE2 activation, which is required to maintain the quiescent resting state of the endothelium. ANGPT2 induces vascular destabilization at higher concentrations through antagonizing ANGPT1-TIE2 signaling. Also, ANGPT2 is strongly expressed in the vasculature of many tumors and may act synergistically with other cytokines such as VEGF to promote the tumor-associated angiogenesis and tumor progression. However, the function of ANGPT1 in tumor-associated angiogenesis remains controversial. The promoting or inhibiting functions are dependent on the tumor cell type, the dosage and possibly on the amount of ANGPT2 in the tumors. TIE1 is almost exclusively expressed by endothelial cells and critical for vascular development, but later in embryonic development than TIE2. However, no specific ligand has been identified for TIE1 and it is still largely considered as an orphan receptor and the function remains enigmatic. Intriguingly, at high concentrations, ANGPT1 binds to TIE1 through integrins (Fiedler and Augustin, 2006; Thomas and Augustin, 2009; Thurston, 2003).

In this thesis study, rhein from *R. palmatum* affected multiple molecular targets and had a special action manner on the angiogenic factors. Part of these factors can be up-regulated at the early developmental stage and then restored to the normal level or

even down-regulated later to cause an opposite effect (Fig. 3-9 C, C'). The overall effects are likely due to the combined functions of these angiogenic factors. Among these factors, *angpt2* and *tie2* seemed to be the major molecular targets (Fig. 3-10.).

At the same time, *trans*-ethyl-*p*-methoxycinnamate from *K. galanga* reduced multi-targets expression, including *kdr, angpt1*, *angpt2*, *tie1* and *tie2*, in time- and dose-dependent manners during zebrafish angiogenesis. Although, the down-regulation level of each affected factor seemed similar and not so strong, and the lasting time was also short only at the early developmental stage at 36 or 48 hpf after 12- or 24-h treatment (Fig.3-28 and 3-30), the inhibition of vessel formation still lasted to 72 hpf on quantitative EAP assay (Fig. 3-24.) and *Tg(fli1a:EGFP)y1* zebrafish embryos (Fig. 3-27).

Our results suggested that when investigating the molecular targets in angiogenesis, other potential factors might also be included. Currently, the VEGF family consists of VEGFA, PlGF (placenta growth factor), VEGFB, VEGFC, VEGFD, VEGFE and snake venom VEGF. Beside VEGFA, other ligands also deserve to be examined. VEGFC shares approximate 30% amino acid identity with $VEGF_{165}$; however, it is more closely related to VEGFD. Both VEGFC and VEGFD are mitogenic for cultured endothelial cells, and can bind and activate VEGFR3 (a member of the VEGFR family that does not bind VEGF-A) as well as VEGFR2. VEGFC also binds to neuropilins (NRP)-2. Overexpression of VEGFC in the epidermis of transgenic mice caused a hyperplastic lymphatic vessel network. VEGFD led to the formation of lymphatics within tumors and promoted the metastasis of tumor cells. *In vitro*, both VEGFC and VEGFD stimulated the migration and mitogenesis of cultured endothelial cells. VEGFE, a collective term for a group of proteins, have been shown to have VEGFA-like activities and seems to be as potent as $VEGF_{165}$ in stimulating endothelial cell proliferation. K14-driven $VEGFE_{NZ-7}$

transgenic mice showed a significant increase in angiogenesis at subcutaneous tissue without clear side effects (Kiba et al., 2003; Takahashi et al., 2005). Neuropilins, as receptors for VEGF, have implicated by many studies in playing key roles in tumor biology. Recent evidence has shown that manipulating neuropilin function can regulate tumor growth and metastasis through effects on vascular biology (Bagri et al., 2009). VEGF receptor-1 (VEGFR1), another VEGF receptor, is expressed not only on the vasculature but also on several other types of cells. VEGFR1 has much higher binding affinity to VEGF, but less activation of intracellular signaling intermediates than KDR. It can function as a negative regulator of angiogenesis through binding VEGF and preventing its binding to KDR. Nevertheless, the exact role of VEGFR1 on tumor endothelium remains to be elucidated (Ellis et al., 2008).

4.4 Conclusion

In summary, the present study provides evidence that zebrafish angiogenic model can assist the anti-angiogenic agents' discovery, and action mechanism investigation. Among eighteen TCMs traditionally used to treat inflammation, rheumatoid arthritis and cancer, three TCMs, *Rheum palmatum*, *Alpinia oxyphylla*, and *Kaempferia galanga* were found to possess anti-angiogenic effects on zebrafish model. Rhein from *R. palmatum* and *trans*-ethyl-*p*-methoxycinnamate from *K. galanga* were found to show anti-angiogenic effect for the first time, whose preliminary action mechanisms were also elucidated through semi-quantitative RT-PCR and *in vitro* HUVEC assays. So when searching for anti-angiogenic agents, Chinese herbs used for inflammation, rheumatoid arthritis and cancer diseases should definitely be included.

However, there are still some limitations of our current results. The underlying action mechanisms of these compounds were only investigated at the cell and RNA

level. More assays, such as Western Blotting, *in vivo* tumor models, should be carried out to confirm their activities. So we will further elucidate the anti-angioenic effect of the most potential compounds, such as *trans*-ethyl-*p*-methoxycinnamate from *K. galanga*, using Matrigel gel plug assay. In addition, when investigating the molecular targets of these potential agents, besides the most studies factors, other factors involved in angiogenesis should be taken into accounted as well.

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