ANTI-ANGIOGENESIS OF HOAN-NGOC

(*PSEUDERANTHEMUM PALATIFERUM* (NEES)

RADLK.) EXTRACT ON B16F10-INOCULATED CAM

Benjamas Sala-Ngam

A Thesis Submitted in Partial Fulfillment of the Requirements for the

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การยับยั้งการสร้างหลอดเลือดใหม่ของสารสกัดฮว่านง็อกต่อเซลล์มะเร็ง
B16F10 บน CAM

นางสาวเบญจมาศ ศาลางาม

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Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for a Master's Degree.

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เบญจมาศ ศาลางาม : การยับยั้งการสร้างหลอดเลือดใหม่ของสารสกัดฮ่วนง็อกต่อ
เซลล์มะเร็ง B16F10 บน CAM (ANTI-ANGIOGENESIS OF HOAN-NGOC
(*PSEUDERANTHEMUM PALATIFERUM* (NEES) RADLK.) EXTRACT ON B16F10-
INOCULATED CAM. อาจารย์ที่ปรึกษา : ผู้ช่วยศาสตราจารย์ ดร.เบ็ญจมาศ จิตรสมบูรณ์,
92 หน้า.

Pseuderanthemum palatiferum (Nees) Radlk. หรือ ฮ่วนง็อก (พญาวานร) เป็นหนึ่งในพืช
สมุนไพรที่ชาวไทยนิยมใช้ในการรักษาโรคต่าง ๆ ที่เกี่ยวข้องกับการอักเสบรวมทั้งโรคมะเร็ง การ
สร้างหลอดเลือดใหม่เป็นปัจจัยสำคัญของการลุกลามและแพร่กระจายของโรคมะเร็งในคน
การศึกษาครั้งนี้มีวัตถุประสงค์เพื่อตรวจสอบฤทธิ์ของ WEP (สารสกัดน้ำจากใบสดฮ่วนง็อกที่แยก
ส่วนจากสารสกัดเอทานอล 95%) ต่อการสร้างหลอดเลือดใหม่ที่เกิดตามธรรมชาติและที่เกิดจาก
การชักนำโดยเซลล์มะเร็งเมลาโนมา B16F10 โดยใช้โมเดลแคม (CAM; chorioallantoic
membrane) ผลการทดสอบพบว่า WEP ประกอบด้วยสารประกอบฟีนอลลิกรวม และฟลาโวนอยด์
รวมในปริมาณสูงร่วมกับความสามารถในการต้านอนุมูลอิสระ DPPH การศึกษาในแคมพบว่า
ปริมาณ 1×10^6-6×10^6 เซลล์ของ B16F10 สามารถชักนำการเกิดหลอดเลือดใหม่ได้สูงกว่า   100
มิลลิกรัมต่อมิลลิลิตรของโกรทแฟกเตอร์ bFGF อย่างมีนัยสำคัญ ($p < 0.05$) การได้รับสารสกัด
WEP ที่ความเข้มข้น 100-1,500 ไมโครกรัมต่อมิลลิลิตร เป็นระยะเวลา 24 และ 48 ชม. ไม่ก่อให้เกิด
การตายของตัวอ่อนในไข่แต่อย่างใด ฤทธิ์ต้านการสร้างหลอดเลือดใหม่ของ WEP ขึ้นกับระดับ
ความเข้มข้นและระยะเวลา สารสกัด WEP ที่ความเข้มข้น 100 และ 300 ไมโครกรัมต่อมิลลิลิตรใน
ระยะเวลา 48 ชม. มีฤทธิ์ยับยั้งการสร้างหลอดเลือดใหม่เฉพาะชนิดที่เกิดจากการชักนำโดย
เซลล์มะเร็ง B16F10 ประมาณ 50-90% อย่างมีนัยสำคัญ ($p < 0.05$) โดยไม่ส่งผลกระทบต่อการ
สร้างหลอดเลือดใหม่ที่เกิดตามธรรมชาติในแคม โดยรวมการศึกษาครั้งนี้ชี้แนะว่าสารสกัด WEP
อาจสามารถยับยั้งการแพร่กระจายของเซลล์มะเร็ง B16F10 โดยกลไกการออกฤทธิ์บางส่วนอาจเกิด
ผ่านการต้านการสร้างหลอดเลือดใหม่ของเซลล์มะเร็ง

สาขาวิชาเภสัชวิทยา   ลายมือชื่อนักศึกษา_____
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BENJAMAS SALA-NGAM : ANTI-ANGIOGENESIS OF HOAN-NGOC
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HOAN-NGOC/ *PSEUDERANTHEMUM PALATIFERUM* (NEES) RADLK./ ANTI-
ANGIOGENESIS/ B16F10

Pseuderanthemum palatiferum (Nees) Radlk. (*P. palatiferum*) known as
Hoan-Ngoc or Payawanon is one of the most frequently used medical plants in
Thailand for treating a variety of inflammatory diseases including cancer.
Angiogenesis is a key process in human cancer progression and metastasis. This study
aimed to investigate the effect of WEP (the water fresh leaf extract of *P. palatiferum*
fractionated from 95% ethanol extract) on angiogenesis occurring naturally or induced
by tumor melanoma B16F10 using the *in ovo* CAM (chorioallantoic membrane)
model. The results showed that WEP contained high total phenolic and total
flavonoid contents in conjunction with high DPPH radical scavenging activity.
B16F10 at 1×10^6-6×10^6 cell pellets induced a stronger angiogenic response than that
of 100 ng/ml of angiogenic cytokine bFGF (basic fibroblast growth factor) ($p < 0.05$).
No lethality of chick embryos was observed post exposure to WEP at 100-1,500
µg/ml for 24 and 48 hours. The anti-angiogenesis activity of WEP was dose and time
dependent. WEP at 100-300 µg/ml selectively and significantly suppressed the
B16F10-induced angiogenesis by about 50 to 90% at 48 hr. ($p < 0.05$) and did not
inhibit the normal neovascularization on the CAM. Overall, the data suggest that

WEP might inhibit B16F10 melanoma metastasis, at least in part, through its anti-angiogenesis activity.
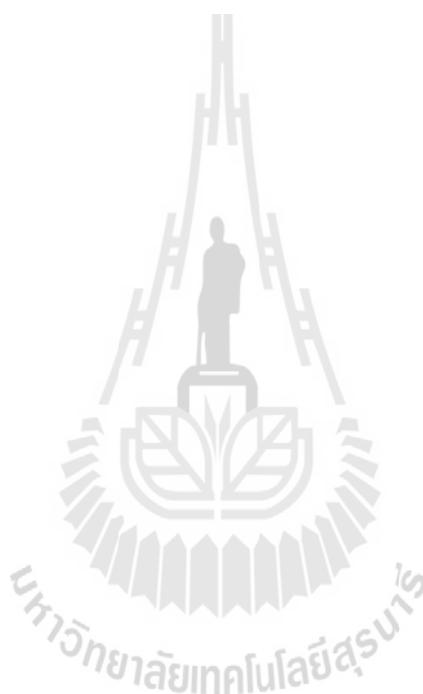
School of Pharmacology   Student's Signature_____

Academic Year 2014   Advisor's Signature_____

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LIST OF ABBREVIATIONS

µg/ml	=	Microgram per milliliter
µl	=	Microliter
µM	=	Micromolar
g	=	Gravitational acceleration
ANOVA	=	Analysis of variance
BSA	=	Bovine serum albumin
bFGF	=	basic fibroblast growth factor
CAE	=	Catechin equivalent
CAM	=	Chorioallantoic membrane
DI water	=	Distilled water
DMEM	=	Dulbecco's modified eagle medium
DMSO	=	Dimethylsulfoxide
DPPH	=	2,2-diphenyl-1-picrylhydrazyl
EDTA	=	Ethylenediaminetetraacetic acid
FBS	=	Fetal bovine serum
FRAP	=	Ferric reducing antioxidant power
g	=	Gram
GAE	=	Gallic acid equivalent
HEPES	=	4-(2-hydroxylethyl)-1-piperazineethanesulfonic acid
IC_{50}	=	Median inhibition concentration

LIST OF ABBREVIATIONS (Continued)

kg	=	Kilogram
L	=	Liter
LD_{50}	=	Median lethal dose
M	=	Molar
min	=	Minute
mg	=	Milligram
mg/ml	=	Milligram per milliliter
ml	=	Milliliter
mM	=	Millimolar
MTT	=	3-(4,5-dimethythiazol-2-yl)-2,5-diphenyltetrazolium bromide
nm	=	Nanometer
OD	=	Optical density
PBS	=	Phosphate buffered saline
PP	=	*Pseuderanthemum palatiferum*
ROSs	=	Reactive oxygen species
rpm	=	Revolution per minute
RT	=	Room temperature
SD	=	Standard deviation
TFC	=	Total flavonoid contents
TPC	=	Total phenolic contents
VH	=	Vehicle

LIST OF ABBREVIATIONS (Continued)

v/v   =   volume by volume

WEP   =   Water extract of fresh leaves of *P. palatiferum*

w/v   =   weight by volume

CHAPTER I

INTRODUCTION

1.1 Introduction

Cancer is a major cause of morbidity and mortality in the coming decades in every region of the world. According to GLOBOCAN 2012 estimates, there were 14.1 million new cases and 8.2 million deaths in 2012. The most commonly diagnosed cancers were lung (1.82 million), breast (1.67 million), and colorectal (1.36 million). The most common causes of cancer death were lung cancer (1.6 million deaths), liver cancer (745,000 deaths), and stomach cancer (723,000 deaths) (Ferlay et al., 2015).

Angiogenesis is critical for various growth and development-relevant events including embryogenesis, tissue remodeling, and wound healing. The sophisticated process is usually regulated in a spatial and temporal manner via active interactions between angiogenic factors, extracellular matrix components, and various types of cells. Imbalanced angiogenesis will lead to angiogenic disorders and destructive process of diseases, such as cardiovascular diseases, inflammation, and tumor growth/metastasis. (Hong et al., 2014).

Recently, many researchers have taken a great interest in medicinal plants for their phenolic concentrations and related biological activities. It is reported that some medicinal plants contain a wide variety of natural antioxidants, such as phenolic acids, flavonoids, and tannins, which possess more potent antioxidant activity than dietary

plants. The health-promoting effects of antioxidants from plants are thought to arise from their protective effects by counteracting with reactive oxygen species (Bouayed et al., 2007; Choi et al., 2002; Wootton, 2011). Acacetin is a flavonoid compound commonly present in several plants, seeds, and flowers. It has been reported to inhibit proliferation, cell cycle progression, invasion, and migration of cancer cells (Liu et al., 2011). Scopoletin, one of the main coumarin constituents in the stems of *Erycibeobtusifolia* Benth, is usually used for the treatment of various rheumatoid diseases in traditional Chinese medicines. Scopoletin possesses a wide range of biological activities, such as anti-inflammatory, hypouricemic, and antioxidant activities. Recently, several coumarin-type compounds were reported to block angiogenesis by inhibiting endothelial cell growth (Pan et al., 2010).

Angiogenesis, the development of new microvascular networks, is required for invasive tumor growth and metastasis and constitutes a key point in controlling the development and progression of a variety of cancer. Accumulating evidences have demonstrated that vascular endothelial growth factor (VEGF) and hypoxia-inducible factor-1α (HIF-1α) play a critical role in tumor angiogenesis. VEGF is a hypoxia-inducible secreted glycoprotein that is a predominant regulator of angiogenesis. It belongs to a family that includes platelet derived growth factor, and a group of proteins closely related to the primary structure of VEGF: placental growth factor and VEGF homologous VEGF B, C, D, and E. VEGF acts via high affinity binding to three tyrosine kinase receptors, VEGFR-1, VEGFR-2, and VEGFR-3. Studies involving gene activation or inhibition have demonstrated that VEGFR-2 transmits essential angiogenic signals in response to VEGF. Hypoxia inducible factor 1 (HIF-1) is a heterodimeric transcription factor composed of HIF-1α and HIF-1β subunits,

and is a major regulator of VEGF expression in response to hypoxia. HIF-1 is a key factor in carcinogenesis, tumor angiogenesis, tumor growth, invasion, and metastasis and can be induced by hypoxia and growth factors. HIF-1α is often up regulated in human cancers to regulate VEGF expression by binding to the hypoxia response element of VEGF promoter. It has been well demonstrated that AKT is one of the important upstream regulators of HIF-1α, and this signaling pathway is one of the major pathways in regulating tumor angiogenesis and tumor growth in ovarian, prostate, and lung cancer cell (Liu et al., 2011).

Growth of new blood vessels is intrinsic to inflammation and is associated with structural changes, including activation and proliferation of endothelial cells and capillary and venues remodeling, all of which result in an expansion of the tissue microvascular bed. A potential functional consequence of this expansion is the promotion of inflammation through various mechanisms. First, influx of inflammatory cells may increase; second, there is an increased nutrient supply that keeps feeding a metabolically active immune process; and third, the activated endothelium contributes to the local production of cytokines, chemokines, and matrix metalloproteinase. Therefore, the anatomic expansion of the microvascular bed combined with its increased functional activation can foster further recruitment of inflammatory cells, and angiogenesis and inflammation may become chronically codependent processes (Leng et al., 2006). Inflammation may promote angiogenesis in a number of ways. Inflammatory tissue is often hypoxia, and hypoxia can induce angiogenesis through up regulation of factors such as VEGF. Extravagated plasma proteins such as fibrinogen products may stimulate neovascularization. Inflammatory cells such as macrophages, lymphocytes, mast cells, and fibroblasts, and the

angiogenic factors they produce, can stimulate vessel growth. Many pro-inflammatory cytokines, such as tumor necrosis factor (TNF)-α, may have antigenic activity in addition to pro-inflammatory activity. Increased blood flow itself may stimulate angiogenesis through shear stresses on the endothelium. Inflammation may also up regulate the expression of angiogenic growth factors such as VEGF and fibroblast growth factor-1 (FGF-1) by resident fibroblasts (Wang et al., 2001). IL-8 expression has profound biological consequences. It is a potent angiogenesis, mitogenic and chemotactic factor in several malignancies including breast and prostate cancer. Still others have suggested that FGF-2, acting in an autocrine and paracrine, can induce IL-6 expression through p38. It has also been reported that IL-1α promotes angiogenesis by activating the VEGF-2 signaling pathway between inflammatory cells and blood vessel endothelial cells (Kenneth et al., 2002).

Pseuderanthemum palatiferum (Nees) Radlk is a new medicinal plant belonging to the Acanthaceafamily. Its vernacular names are "Hoan-Ngoc" or "Xuan-Hoa". Hoan-Ngoc was found in the latter half of the 1990's in Cuc Phuong forest in northern Vietnam (Padee et al., 2010). This plant has been used in Vietnamese both as a medicinal and an ornamental plant. It has been used to treat or prevent many diseases and symptoms including diarrhea, wound, trauma, stomach-ache, colitis, hypertension, nephritis, and tumors (Khonsung et al., 2011). This plant has already been proved to have some pharmacological properties such as antioxidant, antibacterial, and antifungal activities (Dieu et al., 2005).

The leaf extract of Hoan-Ngoc showed high antioxidant activity against hydrogen peroxide radical in the human blood. The ethyl acetate extract of the leaves showed strong antibacterial activity towards *Salmonella* typhi158, *Shigellaflesineri*

,and *Escherichia coli*. The leaf extract also exhibited an active antifungal activity against *Candida albicansand* and *Candida stellatoides*. *Pseuderanthemum palatiferum* (Nees) Radlk is used widely as a medicinal plant in Thailand to treat various diseases such as hypertension, diabetes, and tumor (Padee et al., 2009).

Hoan-Ngoc leaves have been reported to contain many important compounds such as flavonoids, β-sitosterol, and apigenin-7-0-β-glucoside which have various pharmacological properties, including high efficiency against cancer through apoptosis induction (Chayarop et al., 2011; Buncharoen et al., 2010). Previous work from this laboratory has revealed anti-inflammatory activity of Hoan-Ngoc leaves the water extract fractionated from 95% ethanol extract of fresh (WEP) as evidenced by decreased nitric oxide production and suppression of inducible nitric oxide synthase and cyclooxygenase-2 in lipopolysaccharide and interferon gamma-activated RAW 264.7 cells (Dieu et al., 2008).

As angiogenesis is essential in almost all tumor growth, progression and metastasis, and it is related and promoted by inflammation, WEP which possesses anti-inflammatory property may exert its activity against various cancers through targeting the angiogenesis pathway.

1.2   Research objectives

1. To investigate the effect of WEP on natural neovacularization in CAM assay.

2. To determine cytotoxicity of WEP against B16F10 cells.

3. To investigate the effect of WEP on B16F10-induced angiogenesis in CAM assay.

1.3   Scope and limitations of the study

Hoan-ngoc leaves were purchased from local area in Yasothon province, during October to February of 2012. This thesis was only focused the study of cytotoxicity and anti-angiogenesis activity of crude extracts from Hoan-ngoc leaves. Cytotoxicity was investigated on mouse melanoma cell line (B16F10) using MTT assay. The anti-angiogenic activity was investigated using the *in vivo* chick CAM model.

1.4   Expected results

The WEP may have possess anticancer activity mediated through anti-angiogenesis mechanism. This information can be used as a basic pharmacological data for a consideration of its therapeutic potential in the future.
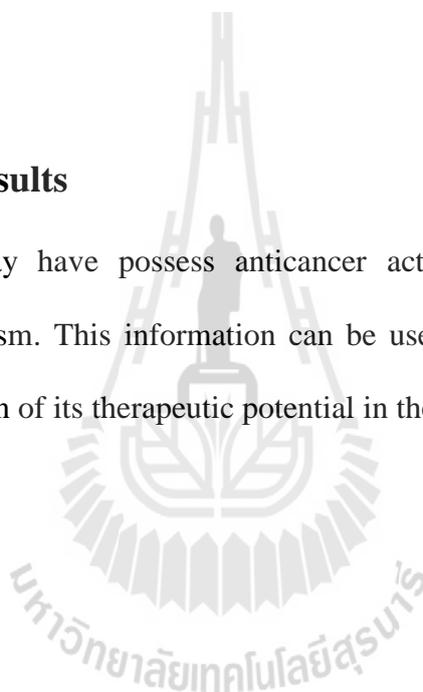
CHAPTER II

LITTERATURE REVIEWS

2.1 Cancer

Cancer is a leading cause of death worldwide and accounted for 7.4 million deaths (around 13% of all deaths) in 2004. The main types of cancer are lung (1.3 million deaths), stomach (803,000 deaths), colorectal (639,000 deaths), liver (610,000 deaths), and breast (519,000 deaths). More than 70% of all cancer deaths occurred in low- and middle-income countries. Deaths from cancer worldwide are projected to continue rising, with an estimated 11.5 million deaths in 2030 (GLOBOCAN, 2012). Cancer results from a series of molecular events that fundamentally alter the normal properties of cells. In cancer cells, the normal control systems that prevent cell overgrowth and the invasion of other tissues are disabled. These altered cells divide and grow in the presence of signals that normally no activation or inhibit cell growth; therefore, they no longer require special signals to induce cell growth and division (Hamilton, 2000). As these cells grow they develop new characteristics, including changes in cell structure, decreased cell adhesion, and production of new enzymes. These heritable changes allow the cell and its progeny to divide and grow, even when in contact with the neighboring normal cells that typically leads to cell contact inhibition. Such changes allow the cancer cells to spread and invade other tissues (Christine et al., 1989). The abnormalities in cancer cells usually result from mutations in protein-encoding genes that regulate cell division. Over time more genes

become mutated. This is often because the genes that make the proteins that normally repair DNA damage are themselves mutated. Consequently, mutations begin to increase in the cell, causing further abnormalities in that cell and its daughter cells (Thompson, 2009). Some of these mutated cells die, but other alterations may give the abnormal cell a selective advantage that allows it to multiply much more rapidly than the normal cells. This enhanced growth describes most cancer cells, which have gained functions that are normally repressed in the normal, healthy cells. As long as these abnormal cells remain in their original location, they are considered benign; if they become invasive, they are considered malignant. Cancer cells in malignant tumors can often metastasize, sending cancer cells to distant sites in the body where new tumors may form. Only a small number of the approximately 35,000 genes in the human genome have been associated with cancer (Alison et al., 2010). Alterations in the same gene often are associated with different forms of cancer. These malfunctioning genes can be broadly classified into three groups. The first group, called proto-oncogenes, produces protein products that normally enhance cell division or inhibit normal cell death. The mutated forms of these genes are called oncogenes. The second group, called tumor suppressors, makes proteins that normally prevent cell division or cause cell death. The third group contains DNA repair genes, which help prevent mutations that lead to cancer. Proto-oncogenes and tumor suppressor genes work much like the accelerator and brakes of a car, respectively. The normal speed of a car can be maintained by controlled use of both the accelerator and the brake. Similarly, controlled cell growth is maintained by regulation of proto-oncogenes, which accelerate growth, and tumor suppressor genes, which slow cell growth (Kolodecik et al., 2014). Mutations that produce oncogenes accelerate growth

while those that affect tumor suppressors prevent the normal inhibition of growth. In either case, uncontrolled cell growth occurs. Although tumor cells are no longer dependent on the control mechanisms that govern normal cells, they still require nutrients and oxygen in order to grow. All living tissues are amply supplied with capillary vessels (Bar-Sela et al., 2010), which bring nutrients and oxygen to every cell. As tumors enlarge, the cells in the center no longer receive nutrients from the normal blood vessels. To provide a blood supply for all the cells in the tumor, it must form new blood vessels to supply the cells in the center with nutrients and oxygen. In a process called angiogenesis, tumor cells make growth factors which induce formation of new capillary blood vessels (Fabienne et al., 2010). The cells of the blood vessels that divide to make new capillary vessels are inactive in normal tissue; however, tumors make angiogenic factors, which activate these blood vessel cells to divide. Without the additional blood supplied by angiogenesis, tumors can grow no larger than about half a millimeter. Without a blood supply, tumor cells also cannot spread, or metastasize, to new tissues. Tumor cells can cross through the walls of the capillary blood vessel at a rate of about one million cells per day. However, not all cells in a tumor are angiogenic. Both angiogenic and non-angiogenic cells in a tumor cross into blood vessels and spread; however, non-angiogenic cells give rise to dormant tumors when they grow in other locations. In contrast, the angiogenic cells quickly establish themselves in new locations by growing and producing new blood vessels, resulting in rapid growth of the tumor (Gacche et al., 2015).

2.2   Angiogenesis

The word angiogenesis is first named by Hertig in 1935 and the mechanism was revealed by Folkman in studying tumor angiogenesis. Angiogenesis refers to the growth of new capillaries from pre-existing capillaries and post-capillary venules. It is a tightly controlled process that rarely occurs under normal conditions, except for instances of wound healing, embryonic development and development of the corpus luteum. Many diseases, however, are driven by persistent unregulated angiogenesis (Wang et al., 2004).

2.2.1   Normal angiogenesis in children

In addition to its role in tumors, angiogenesis occurs normally in the human body at specific times in development and growth. For example, a developing child in a mother's womb must create the vast network of arteries, veins, and capillaries that are found in the human body as shown in Figure 2.1. A process called vasculogenesis creates the primary network of vascular endothelial cells that will become major blood vessels. Later on, angiogenesis remodels this network into the small new blood vessels or capillaries that complete the child's circulatory system (Naoyo, 2006).

Figure 2.1   Normal angiogenesis in children (Naoyo, 2006)**.**

2.2.2   Normal angiogenesis in adults

Proliferation of new blood vessels also takes place in adults, although it is a relatively infrequent event. In women shown in Figure 2.2, angiogenesis is active a few days each month as new blood vessels form in the lining of the uterus during the menstrual cycle. Also, angiogenesis is necessary for the repair or regeneration of tissue during wound healing (Naoyo, 2006).
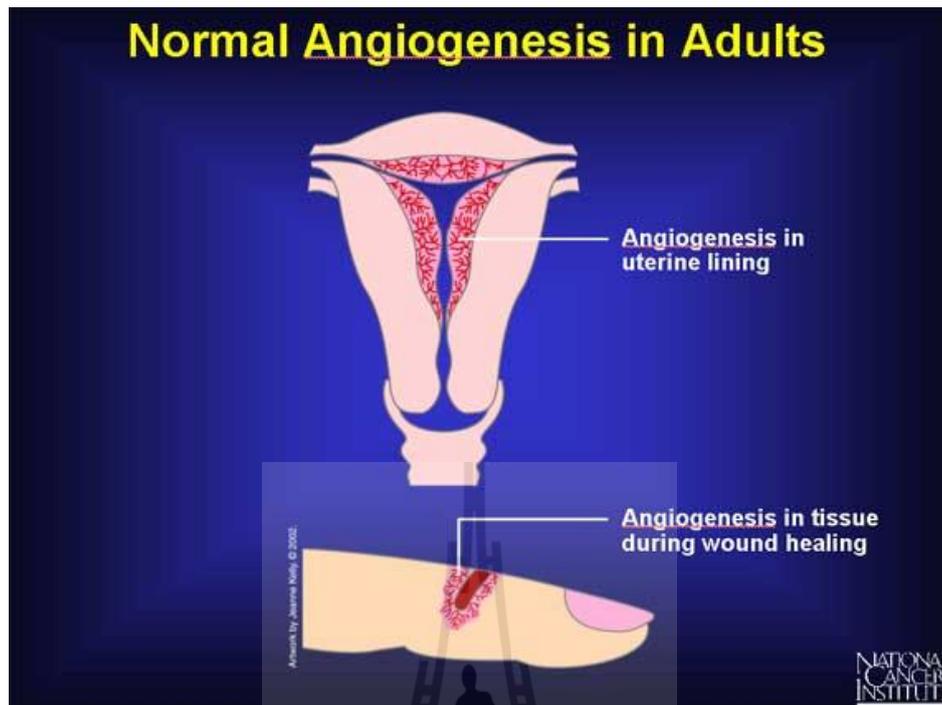
Figure 2.2   Normal angiogenesis in adults (Naoyo, 2006)**.**

2.2.3 Angiogenesis in cancer

Tumor growth and metastasis depend on angiogenesis and lymphangiogenesis triggered by chemical signals from tumor cells in a phase of rapid growth. In a previous study, Naoyo (2006) compared the behavior of cancer cells infused into different regions of the same organ. One region was the iris with blood circulation; another was the anterior chamber without circulation. The cancer cells without blood circulation grew to 1-2 mm^3 in diameter and then stopped, but grew beyond 2 mm^3 when placed in an area where angiogenesis was possible. In the absence of vascular support, tumors may become necrotic or even apoptotic. Therefore, angiogenesis is an important factor in the progression of cancer. Neovascularization, including tumor angiogenesis, is basically a four-step process. First, the basement membrane in tissues is injured locally. There is immediate

destruction and hypoxia. Second, endothelial cells activated by angiogenic factors migrate. Third, endothelial cells proliferate and stabilize. Fourth, angiogenic factors continue to influence the angiogenic process. Vascular endothelial cells divide only about every 1000 days on average. Angiogenesis is stimulated when tumor tissues require nutrients and oxygen. Angiogenesis is regulated by both activator and inhibitor molecules. However, up-regulation of the activity of angiogenic factors is itself not sufficient for angiogenesis of the neoplasm. Negative regulators or inhibitors of vessel growth need to also be down-regulated (Figure 2.3) (Naoyo, 2006).

a) "SWITCH OFF"   b) "SWITCH ON"
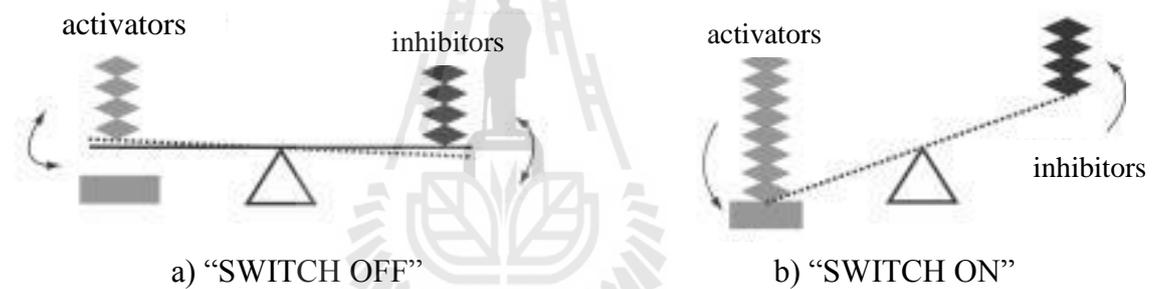
Figure 2.3   Angiogenesis is regulated by a balance between activators and inhibitors (a) When tumor tissues require fuel (nutrients and oxygen), angiogenesis is stimulated. However, up-regulation of by the activity of angiogenic activators alone is not sufficient for angiogenesis of the neoplasm. Negative regulators or inhibitors of vessel growth need also to be down-regulated (b) (Naoyo, 2006)**.**

2.2.4 Angiogenesis and growth factor

Angiogenesis is defined as the physiological process by which new blood vessels are formed from pre-existing ones. Formation of new blood vessels is necessary to supply nutrients and oxygen to support tumor growth. Angiogenesis is accomplished by the organized release of angiogenic factors from the tumor cells, such as vascular endothelial growth factor (VEGF), epidermal growth factor (EGF) and fibroblast growth factor (FGF) as shown in Figure 2.4. These angiogenic factors bind their corresponding receptors on the endothelial cell surface leading to augmented vascular permeability, which results in the extravasation of plasma proteins and the dissociation of pericyte coverage. Endothelial cell proliferation and migration to initiate the formation of new blood vessels follows. Localized degradation of the extracellular matrix is a necessary step in the formation of the new blood vessels (Weon-Kyoo et al., 2015).

This degradation is executed by the matrix metalloproteinases, cathepsin B and other degradation enzymes, in addition to increased matrix protein expression including fibronectin and laminin. Tumor cells or cancer-associated fibroblast are the main source of these essential extracellular matrix proteins. Tumor-associated angiogenesis in intrahepatic cholangiocarcinoma has been observed by the immunohistochemical study of microvessel density and lymphatic microvessel density. Lower 5-year survival rates, higher recurrence rates, and increased nodal spread were seen in patients that had tumors with increased microvessel density. The angiogenic factors, nerve growth factor-β (NGF-β), and vascular endothelial growth factor-C (VEGF-C) are overexpressed by 57.1% and 46.4%, respectively in cholangiocarcinoma samples. VEGF-A and VEGF receptors (VEGFRs), angiogenic

factors angiopoietin-1, -2, and thrombospondin-1, EGF, EGF receptors (EGFR), and basic fibroblast growth factor are also overexpressed in human cholangiocarcinoma cell lines and samples. Secretion of these factors may individually or cooperatively increase angiogenesis, evidenced by increased microvessel density. An example of this is VEGF-A, which contributes to the neovascularization of extrahepatic cholangiocarcinoma (Leyva-Illades et al., 2012).
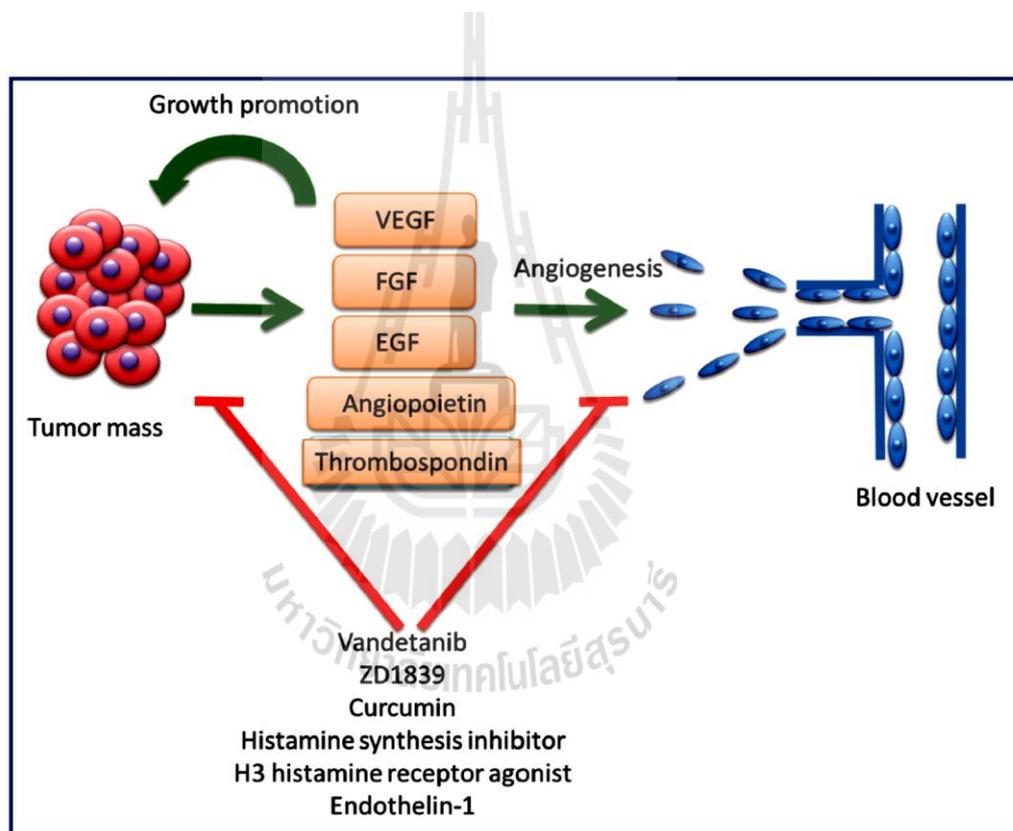
Figure 2.4   Interactions between cholangiocarcinoma and angiogenic factors that regulate proliferation and angiogenesis. VEGF (vascular endothelial growth factor), FGF (fibroblast growth factor), EGF (epidermal growth factor) (Leyva-Illades et al., 2012).

2.3   Chick chorioallantoic membrane structure

The chicken is a well-known experimental model to study embryonic development. Chick embryos are readily accessible for visualization *in ovo* and experimental manipulation. Chick embryo development lasts 21 days before hatching. Three extraembryonic membranes are formed during development: the yolk sac membrane, the amnion, and the chorioallantoic membrane (CAM) as shown in Figure 2.5. The CAM is formed on day 3-4 of incubation by the fusion of the chorion and the allantois and it consists of three layers, ectoderm (from the chorion), mesoderm, and endoderm (from the allantois). The CAM has a rich vascular system that develops within the mesodermal layer and is served by paired allantoic arteries and paired allantoic veins. By 16 days of incubation, the CAM has become so large that it covers most yolk sac, and become closely pressed against the shell membrane, which enables it to act as a gas-exchange organ receiving oxygen and eliminating carbon dioxide through the pores in the shell. The surface area of the CAM increases from approximately 6 cm^2 at day 6 to 65 cm^2 by day 14 (Ribatti, 2014).
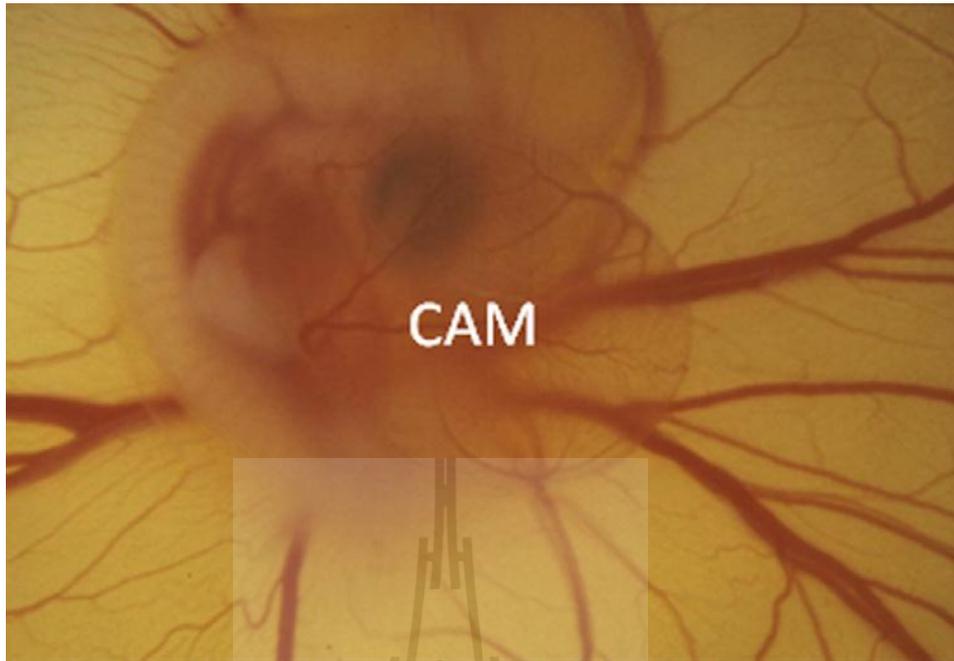
Figure 2.5   Macroscopic *in ovo* features of the chick chorioallantoic membrane
(CAM) at day 5 of incubation (Ribatti, 2014).

Chick chorioallantoic membrane (CAM) is a specialized and highly
vascularized extraembryonic membranes mediating gas and nutrients exchange
between the embryo and the atmosphere surrounding the egg and hence performs the
lung function during an embryonic life. During chick embryo incubation, CAM is
formed between day 4 and 5 by partial fusion of the adjacent mesodermal layer of the
chorion and the allantois, and the CAM central part is fully developed by day 8 to 10.
By that time, CAM is capable of sustaining tissue grafts, while its outskirts are still
developing and expanding until the membrane is fully wrapped around the embryo by
day 12 of the development. CAM is composed of three germ layers; ectoderm,
mesoderm, and endoderm. The ectoderm attaches to the shell membrane and is
underlined by the dense, honeycomb like network of respiratory capillary plexus

formed by both angiogenesis and vasculogenesis between day 5 and 6 of the development (Figure 2.5). The mesoderm is a collagen rich connective tissue enriched in blood vessels and stromal components from arteriolar and venous systems. The thin endoderm saparates the CAM from the allantoic cavity (Brakenhielm et al., 2004; Miller et al., 2004; Peifer and Dannhard, 2004; Deryugina and Quigley, 2008).

2.3.1 CAM as *an in vivo* model for cancer studies

Among available *in vivo* models for tumor development, invasion, metastasis and angiogenesis, CAM is one of the most popular, well established and useful tools especially for screening pro- and anti-angiogenesis substances. Up till day 11 or 12, the blood vessel system of the CAM is highly angiogenic, therefore, between day 8 and 10, the developing CAM vasculature is highly responsive to either pro-or anti-angiogenic stimuli. The response of the CAM to pro-or anti-angiogenic stimuli is relatively rapid and most assays takes only 3-5 days. The highly vascularized nature of the CAM and the naturally immunoincompetent until embryonic day 17 also make the CAM an ideal model for grafting tumor cells of different species origin. Test substances or tumor cells can be inoculated *in ovo* through a small window cut in the shell above the lowered CAM. Test substances can be sustained release using various biologically inert polymers or collagen gels. Inoculated xenogenic tumor cells can proliferate and grow up to a certain size of 500-600 mg within 6-7 days, depending on the origin of tumors and inoculated cell number. Not only the aggressive tumor cells can reach sizable tumors, but they can also escape from the inoculated site, invade surrounding stroma, intravasate into blood vessels and disseminate to distal portions of the CAM and internal organs forming micro metastasis foci. This enable the similarity of the CAM assay to the

multistep of murine tumor carcinogenesis model, but only occurs within much shorter time in the CAM assay (Deryugina and Quigley, 2008; Richardson and Singh, 2003). At present, there are several modification of the original CAM assay allowing for various ways of quantitation of the pro-or anti-angiogenic potential of test substances. Various growth factors such as TGF-β, VEGF, and bFGF are shown to induce angiogenesis, whereas certain steroids, polysaccharides, vasostatin and anti-integrin antibodies are anti-angiogenesis in the CAM assay (Hasan et al., 2004). In addition, several variations of the CAM assays have been developed to assess spontaneous tumor cell intravasation and metastasis (Chen et al., 2010; Deryugina and Quigley, 2008; Peifer and Dannhardt, 2004). Therefore, the CAM model has several advantages, including inexpensive, easy preparation, absence of a mature immune system, and can be exploited in many aspects of research in cancer such as assessment of tumor growth, inflammation or cytokine-induced angiogenesis, tumor cells intravasation and metastasis.

As disrupting angiogenesis in neoplatic diseases may suppress tumor growth and metastasis with less systemic toxicity than conventional cytotoxic therapeutics, and anti-angiogenic therapeutic approaches have recently been proven to be benefit in various cancer treatments, the present study aims to investigate the anti-tumor growth and anti-angiogenic activity of HLE using the embryonic chick CAM as the *in vivo* assay model.

2.4   *Pseuderanthemum palatiferum* (Nees) **Radlk.**

Pseuderanthemum palatiferum (Nees) Radlk known as Hoan-Ngoc is a native plant of Vietnam found in Cuc Phuong forest in the northern Vietnam during the lalter half of the 1990´s. After the discovery, the plant has been cultivated throughout the country as both a medicinal and ornamental plant (Dieu et al., 2006). Hoan-Ngon was taken through northeast of Thailand about 20 years ago (Surin, Burirum, and Sisaket provinces) by a Vietnam Era veteran.   Its Thai names are "Wan ling" or "Payawanorn". Hoan-Ngoc is a shrub tree of 1-3 m high. Stem is quadrangular, glabrous, and green in color. Leaf arrangement is opposite, simple and green foliage color. Shape of leaf is lanceolate to elliptic, 3-5 x 5-15 cm, acuminate terminal, attenuate at base and entire margin. Flower is inflorescence and irregular. Corolla is white-violet in color. In Vietnam, Hoan-Ngoc has been used for both treatment and prevention of many diseases such as hypertension, diarrhea, arthritis, pharyngitis, hemorrhoids, tumors, colitis, bleeding, wounds, cancer, and so on. In addition, Hoan-Ngoc has also been used for treatment and prevention of various diseases in animals (Dieu et al., 2005; Dieu et al., 2006). Hoan-Ngoc has become popular among Thai people. According to folkloric medicine, its fresh leaves are claimed to cure various diseases, including diarrhea, peptic ulcer, hepatitis, nephritis, hypertension and diabetes. In addition, Hoan-Ngoc has gained its reputation for alleviating or curing various cancers, the number one cause of death among Thai people (Health Information System Development Office, 2005). At present, Hoan-Ngoc products, including dried powder for decoctions, herbal tea bags, and capsules have been developed and commercialized without quality control. A report on constituents of Hoan-Ngoc leaves have been recently established (Chayarop et al., 2011). The

phytochemical screenings revealed that this plant contains flavonoids, phenolic compounds, unsaturated lactone rings, and steroid nuclei. Dieu et al. (2008) reported that the chemical composition of Hoan-Ngoc was a mixture of β-sitosterol, β-sitosterol-3-0-β-glucoside, apigenin-7-0-β-glucoside, 1-triacontanol, and salicylic acid. In addition, the leaves of Hoan-Ngoc contained 30.8% of the dry matter as crude protein, minerals such as Ca, Mg, Fe and Cu, and amino acids such as lysine, methionine and threonine. The 80% ethanol extract of Hoan-Ngoc did not induce acute or sub-acute toxicity in male rats at doses of 2,000 and 1,000 mg/kg by oral administration. This extract was not cytotoxic to Vero cells at a concentration of 50 μg/ml (Padee et al., 2009). Khonsung et al. (2011) reported the water extract of fresh Hoan-Ngoc leaves exhibited hypotensive and bradycardic effects in both normotensive and N-nitro-L-arginine methyl ester-induced hypertensive anesthetized rats. Besides, Hoan-ngoc leave extract at the dose of 250 mg/kg showed a beneficial hypoglycemic effect in streptozotocin-induced diabetic rats (Padee et al., 2010).

2.5   Antiangiogenesis and antioxidant

Free radicals are implicated in the initiation of variety of human ailments. Generation of free radicals or reactive oxygen species (ROS) during metabolism and other activities beyond the antioxidant capacity of a biological system gives rise to oxidative stress. Body cells and tissues are continuously threatened by the damages caused by free radicals and ROS, which are either produced during normal oxygen metabolism or are induced by exogenous damages. Oxidative stress is now perceived as a prominent feature of many acute and chronic diseases including cancer and leukemias (Gacche et al., 2015). Recent studies validated that oxidative stress, along

with influencing development and progression of breast cancer eventually lead to induce angiogenesis (Majidian Eydgahi et al., 2015). Therefore, exploring antioxidant properties of therapeutic agents can pave new avenues in understanding the role of oxidative stress in concern to cancer therapy.

Edible berry anthocyanins possess a broad spectrum of therapeutic and anti-carcinogenic properties. Berries are rich in anthocyanins, compounds that provide pigmentation to fruits and serve as natural antioxidants. Flavonoid constituents of the berry extracts may have been responsible for the observed effect on inducible VEGF expression and release (Bagchi et al., 2004).

Flavonoids, sulphated carbohydrates, or triterpenoids have been suspected to be the active anti-angiogenic components of plant products (Paper,1998). Catechins and polyphenols from plant extracts such as green tea show potent anticancer activity. Although there is a general agreement that certain plant products may possess anti-angiogenic properties, the underlying mechanisms are not well characterized. A recent report has shown that resveratrol, a phytoalexin found in grapes, berries, and peanuts, is one of the most promising agents for cancer prevention. Previous study was observed that the antitumor activity of resveratrol occurs through p53-mediated apoptosis. Both ERKs and p38 kinase mediated resveratrol-induced activation of p53 and apoptosis through phosphorylation of p53 at serine 15 (She et al., 2001). The current work presents first evidence showed that berry extracts potently inhibit inducible VEGF expression. Some antioxidants have been observed to have anti-angiogenic effects (Sashwati et al., 2002).

Flavonoids are phenolic compounds, widely distributed as secondary metabolites in plant kingdom. The biological, pharmacological, and medicinal

properties of flavonoids have been extensively reviewed (Czaplinska, 2012). The best-described property of almost every group of flavonoids is their capacity to act as antioxidants. They are powerful chain-breaking antioxidants. Their antioxidant effectiveness depends on the stability in different systems, as well as number and location of hydroxyl substitutions (Gacche, 2015).

In many *in vitro* studies, phenolic compounds demonstrated higher antioxidant activity as compared to vitamin C and carotenoids. The flavonoids display a remarkable array of biochemical and pharmacological actions, some of which suggest that certain members of this group of compounds may significantly affect the function of various mammalian cellular systems. The flavones and catechins are described as the most powerful flavonoids for protecting the body against ROS and thus potentially possess anti-neoplastic activities. The anti-inflammatory, antioxidant, antiallergic, hepatoprotective, antithrombotic, antiviral and anticarcinogenic attributes of flavonoids are well established (Muslim et al., 2012).

CHAPTER III

MATERIALS AND METHODS

3.1   Materials

3.1.1   Plant

Pseuderanthemum palatiferum (Nees) Radlk or Hoan-Ngoc was purchased from Yasothon province, Thailand. It was harvested in July 2011. Specimen was identified by Dr. Kongkanda Chayamarit of forest Herbarium, Royal Forest Department, Bangkok, Thailand. A voucher specimen (BKF 174009) was deposited at the Forest Herbarium, Royal Forest Department, Bangkok, Thailand (Sittisart and Chitsomboon, 2014).

Figure 3.1   The leaves of *Pseuderanthemum palatiferum* (Nees) Radlk (kusherb, www, 2012).

3.1.2 Cell lines

B16-F10 (mouse melanoma cell line) was purchased from American Type Culture Collection (ATCC, USA). B16-F10 cells were cultured in Dulbecco's Modified Eagle's medium (DMEM) with high glucose supplemented with 10% FBS and 100 U/ml penicillin and 100 µg/ml streptomycin without HEPES. The cells was maintained at 37 ℃ in 5% CO_2 and 95% humidity.

3.1.3 Chemicals and instruments

The chemicals, materials and instruments employed in the present studies were summarized in Tables 3.1 and 3.2.

Tables 3.1 List of chemicals.

Name	Source
Absolute ethanol	Carlo erba
Aluminum chloride hexahydrate	Sigma
Ascorbic acid (Vitamin C)	Fluka
basic fibroblast growth factor (bFGF)	Sigma
Catechin	Fluka
Dimethyl sulfoxide (DMSO)	Amresco
Dulbecco's Modified Eagle's Medium - high glucose	Invitrogen
EDTA (Ethylenediaminetetraacetic acid)	Sigma
Folin & Ciocalteu's Phenol Reagent	Sigma
Gallic acid	Riedel-de Haen®
Hexane	Carlo erba
Methyl alcohol	Fisher Scientific

Table 3.1   List of chemicals (Continued).

Name	Source
MTT [3(4,5-dimethylthiazol-2-yl) ,5-diphenyltetrazolium bromide]	Invitrogen
Penicillin G	Sigma-Aldrich
Sodium hydroxide	Carlo erba
Sodium nitrite	Sigma
Streptomycin solution sulfate	Sigma-Aldrich
Trolox (6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid)	Sigma
Trypsin	Gibco

Tables 3.2   List of instruments.

Name	Source
Centrifuge (modelCT15RT)	Techcomp
Inverted microscope (model CKX41)	Olympus
Class II Biohazard safety cabinet	ESCO
Light microscope (model CX21)	Olympus
Lyophilizer(model Freeze-zone 12 plus)	Labconco Corporation
Microcentrifuge	Sorvall
Microplate spectrophotometer	Bio-Rad
pH meter	Selecta
Rotary evaporator with vacuum (model R205)	Buchi

3.2   Methods

3.2.1   Preparation of water crude Hoan-Ngoc leave extracts

The preparation of crude Hoan-Ngoc leave extracts was described by Sittisart and Chitsomboon, 2014. Briefly, fresh leaves (4 kg) were blended in 95% ethanol and filtered through gauze. The extract was centrifuged at $3,500 \times g$ for 10 min, and then the supernatant was filtered through a Whatman No.1 filter paper. After that, the ethanolic filtrate was concentrated using a vacuum rotary evaporator (Buchi Labortechnik AG, Flawil, Switzerland) and dried by lyophilization (Freeze-Zone 12 plus, Labconco Corporation, Missouri, USA) into powder of 95% ethanol crude extract. Then, the 95% ethanol crude extract (40 g) was partitioned between hexane and water (1:1, v/v). The water fraction was collected, centrifuged at $14,000 \times g$ for 10 min at 4 ^{o}C, and then the supernatant was filtered through a Whatman No.1 filter paper. After that, the water fraction was evaporated and lyophilized into powder of water fraction of 95% ethanol crude extract (WEP). The WEP was stored in a refrigerator at -20 ^{o}C till use in subsequent experiments. The WEP was dissolved in water when used in experiments. For cell culture experiments, the WEP was dissolved in cell culture medium directly.

3.2.2   Determination of phytochemicals and antioxidant activity

3.2.2.1   Total Phenolic Content (TPC)

The total phenolic content of individual extract was determined colorimetrically using Folin-Ciocalteau method (Singleton, Orthofer, and Lamuela-Ravent´os, 1998). This method is based on a chemical reduction of the reagent, a mixture of tungsten and molybdenum oxides. Briefly, 0.1 ml of the extracts was added to 2 ml of 2% Na_2CO_3 solution and mixed thoroughly. After 2 min, 0.1 ml of 50%

Folin-Ciocalteau reagent was added, mixed and incubated for 30 min at room temperature. Then, the absorbance of the extracts was measured at 750 nm using a Cecil 1000 series spectrophotometer (Cecil Instruments, Cambridge, UK). Gallic acid solutions ranging from 0.05 to 0.3 mg/ml were used to prepare a standard curve. The phenolic contents of the extracts were expressed as milligrams of gallic acid equivalent (GAE) per gram of dried extract.

3.2.2.2 Total Flavonoid Content (TFC)

Total flavonoid content was determined using the aluminium trichloride colorimetric assay (Liu et al., 2002). The reaction is based on formation of acid stable complexes between aluminium chloride with the C-4 keto group and either the C-3 or C-5 hydroxyl group of flavones and flavonols. Aluminium chloride also forms acid labile complexes with the ortho-dihydroxyl groups of flavonoids. The intensity of yellow color of the kelate formed by the flavonoids, when treated with $AlCl_3$ in acetate buffer, was spectrophotometrically determined (Constanta and Rodica, 2010). Briefly, 0.25 ml of the extracts was diluted with 1.25 ml of distilled water. Then, 0.075 ml of 5% $NaNO_2$ solution was added to the mixture. After 6 min, 0.15 ml of 10% $AlCl_3 \cdot 6H_2O$ solution was added, mixed and incubated for 5 min. Then, 0.5 ml of 1 M NaOH was added and the total volume was made up to 2.5 ml with distilled water. The absorbance of the extracts was measured immediately at 510 nm using a spectrophotometer. The catechin solution (0.05-0.4 mg/ml) was used as a standard for the calibration curve. Total flavonoid contents of the extracts were expressed as milligrams of catechin equivalent (CAE) per gram of dried extract.

3.2.2.3 The 2, 2-diphenyl-1-picrylhydrazyl (DPPH) assay

This assay is based on the measurement of the scavenging ability of antioxidants towards the stable radical DPPH, and hence the decreasing absorbance at 515-528 nm. The free radical DPPH (purple) is reduced to the corresponding hydrazine (no color) when it reacts with hydrogen donors from antioxidant compounds as shown in Figure 3.2 (Sánchez-Moreno, Larrauri, and Saura-Calixto, 1999).
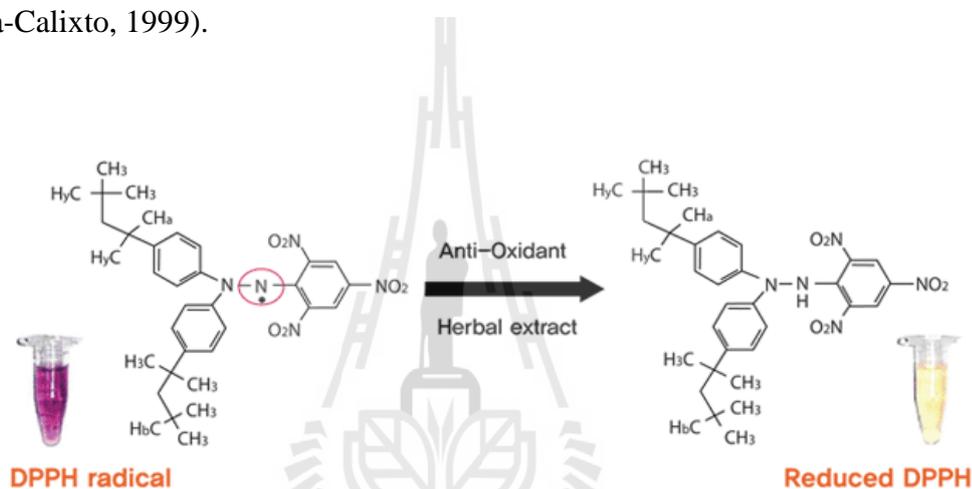
Figure 3.2 Structure of DPPH before and after reaction with antioxidant (Damo, 2010).

The DPPH scavenging activity was determined by following the method of Sánchez-Moreno et al., (1999). Briefly, one milliliter of the extracts at different concentrations was added to 3.9 ml of DPPH solution (63 mM). The mixture was shaken vigorously at room temperature for 45 min in the dark and measured the absorbance at 515 nm using a spectrophotometer. The free radical scavenging activity was calculated as shown below. The IC_{50} of DPPH was determined from a dose

response curve using linear regression analysis. Decreasing DPPH solution absorption indicates an increase of DPPH radical scavenging activity.

$$\text{DPPH inhibition (\%)} = \frac{[(A_{control}-A_{sample})] \times 100}{A_{control}}$$

Where $A_{control}$ = The absorbance of control

A_{sample} = The absorbance of different concentrations of sample extracts

3.2.3 *In vitro* cytotoxic test (MTT assay)

3.2.3.1   Cytotoxic effect of WEP against B16F10

The cytotoxic effect of WEP on cell proliferation was determined by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay (Chun et al., 2007). Briefly, B16-F10 was seeded in a 96-well plate at a density of 1×10^4 cells/well. The cells were allowed to adhere overnight, and then treated with various concentrations of WEP for 24 h. After incubation, the cultured medium was removed and 0.5 mg/ml (final concentration) MTT was added. Then, cells were further incubated for 4 h at 37 °C. Formazan crystal formed by viable cells was dissolved in DMSO, and absorbance was measured at 540 nm with a microplate spectrophotometer (Benchmark Plus, Bio-Rad, Japan).

3.2.4   CAM model

Five-day-old chick embryos were purchased from Suranaree University of Technology Farm. Chick CAM assays were performed as previously described (Vargas et al., 2007). Briefly, a hypodermic needle was used to make a small hole in the blunt end of the egg, and a second hole was made on the broad side of the egg, directly over an avascular portion of the embryonic membrane. An approximately 2 cm^2 window was cut in the shell over the false air sac, allowing access to the CAM. Sterile disk (5 mm diameter of No.1 Whatman filter paper) were then suspended in DMEM and placed on CAM (1 disk/CAM). Then, 30 µl 100 U/ml penicillin and 100 µg/ml streptomycin were added on the disk. CAM was incubated at 37 °C and 70% relative humidity for 24 and 48 hours. After incubation, the number of neovascular around the disk was quantified under a stereomicroscope as   shown in Figure 3.3.
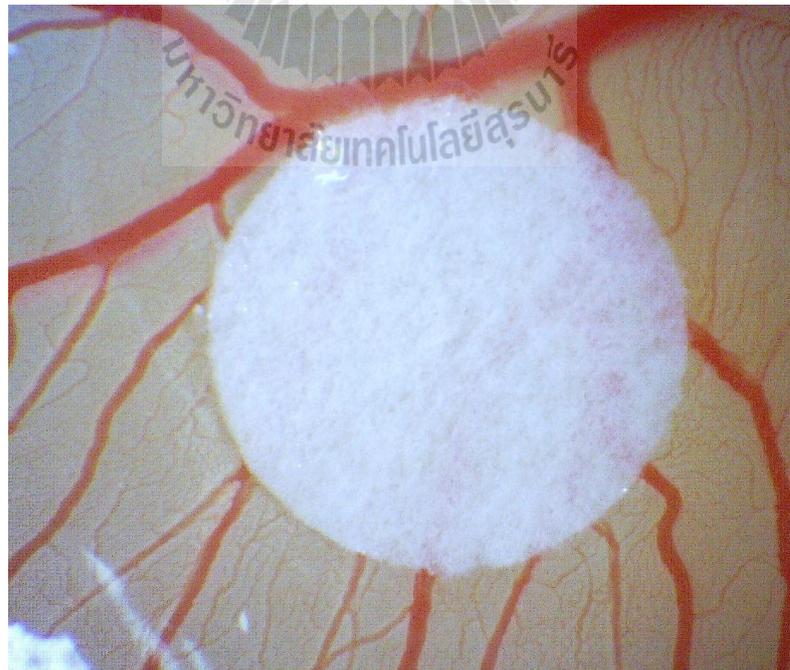
Figure 3.3   Chorioallantoic membrane (CAM) in 5 days (magnified×6.7).

3.2.4.1   B16F10 and bFGF induce angiogenesis in CAM model

A group of fertilized chicken eggs were maintained at 37 °C and 70% humidity for 5 days in an egg incubator. On day 5, eggs were created a window of 2 cm^2. Angiogenesis occurred naturally on induced by adding basic-fibroblast growth factor (bFGF)-soaked on filter disks which were placed onto the CAM. For VH control, 10 µl DMEM was added onto a disk. Pellets of 1×10^6, 3×10^6 or 6×10^6 B16F10 cells were placed directly onto the CAM, then, 30 µl 100 U/ml penicillin was added prior covering cells with a paper disk. The exposed hole in the shell of each egg was closed with tape and further incubated for 24 hours and 48 hours. Angiogenesis were quantified by using the number of neovascularization contact to the circular disk viewed under a stereomicroscope (10X magnification, and photograph (Figure 3.4). From the taken photograph, each circular disk was roughly subdivided into 4 quarters (use main blood vessel as a landmark) and all numbers of major and minor blood vessels in direct contact to the marker disk, placed on the CAM, were counted for each quarter, one at a time. (Figure 3.5).
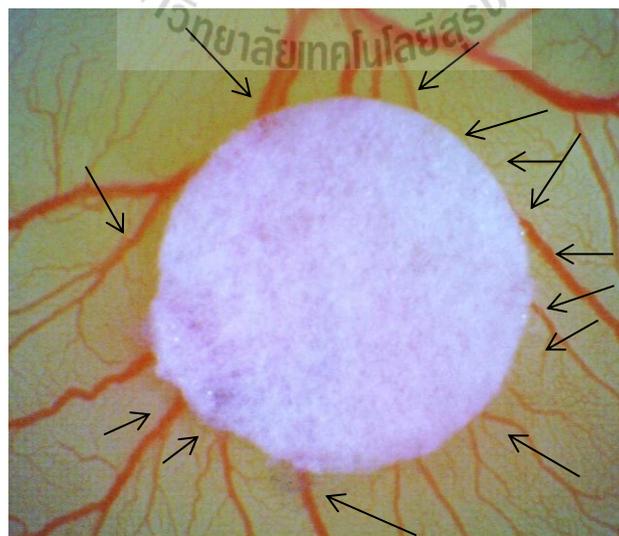
Figure 3.4   Blood vessels on CAM. Arrows indicate total blood vessels around the CAM. (magnified×6.7).
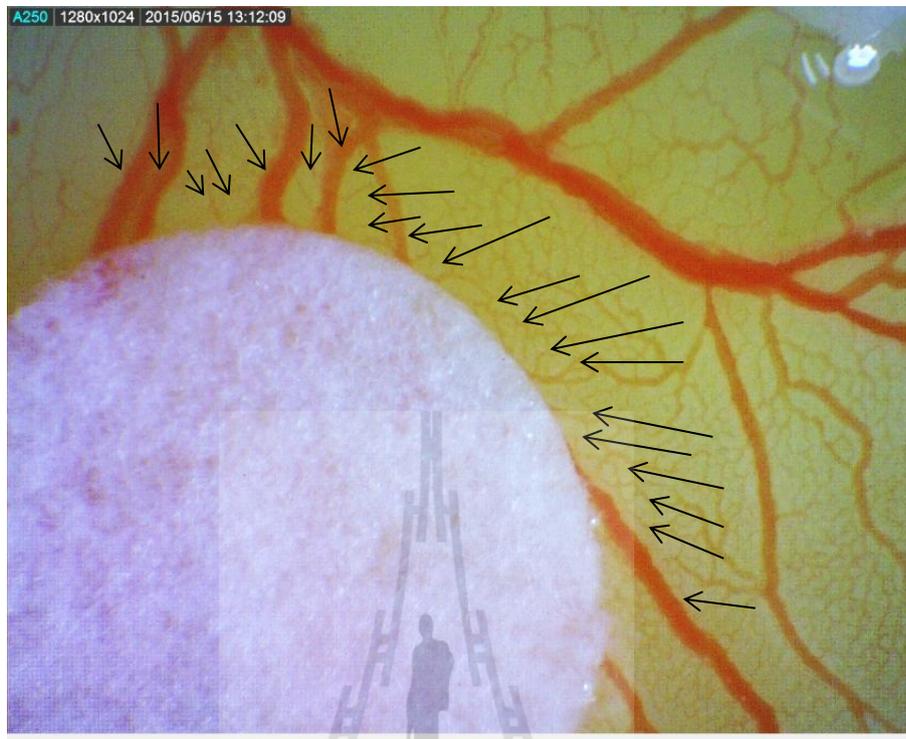
Figure 3.5 Blood vessels on CAM. Arrows indicate blood vessels around CAM. (magnified×10).

The alteration of neovascularization around the disk was calculated as following:

$$\%\ \text{Neovascularization for 24 hr.} = \frac{[(V_{24}-V_0)]\times100}{A_{24}}$$

Where V_{24} = Vessel numbers around the disk at 24 hr. post B16F10 inoculation

V_0 = Vessel Numbers around the disk at 0 hr. post B16F10 inoculation

$$\%\ \text{Neovascularization for 48 hours} = \frac{[(V_{48}-V_0)]\times100}{A_{48}}$$

Where V_{48}= Vessel Numbers around the disk at 48 hours post B16F10 inoculation

V_0 = Vessel Numbers around the disk at 0 hours post B16F10 inoculation

3.2.4.2 The toxicity of WEP on chick chorioallantoic membrane (CAM)

Fertilized chicken eggs were used after incubating them for 5 days. A 2 cm^2 window was made in the shell to create a pocket to expose the CAM. Filter discs in the presence or absence of WEP was then placed upon the CAM and adds immediately added 30 µl 100 U/ml penicillin. The exposed hole in each egg was closed with tape and further incubated for 24 hours and 48 hours. Angiogenesis was quantified at each time point by using the number of neovascularization under a stereomicroscope. Angiogenesis was quantitated by counting the number of neovessels in direct contact with the filter disk.

3.2.4.3 The anti-angiogenesis of WEP on chick chorioallantoic membrane (CAM)

Fertilized chicken eggs were used after incubating them for 5 days. A 2 cm^2 window was made in the shell to create a pocket to expose the CAM. A Pellet of 3×10^6 B16F10 cells were placed directly onto the CAM, then a filter disc in the presence or absence of WEP was then placed upon the CAM using 6 eggs per group and add immediately added 30 µl 100 U/ml penicillin. The exposed hole in each egg was closed with tape and further incubated for 24 hours and 48 hours. Angiogenesis was quantified at each time point by using the number of neovascularization under a stereomicroscope. Angiogenesis was quantitated by counting the number of neovessels in direct contact with the filter disk.

3.2.5 Statistical analysis

All statistical analyses were conducted using SPSS software (Statistics Package for the Social Sciences, version 11). Homogenous data from DPPH was analyzed by one-way analysis of variance (ANOVA) with a post hoc Tukey's analysis

to determine differences between treatment and control groups (Nascimento et al., 2013). Values were considered statistically significant when $p < 0.05$. Non-homogenous data were evaluated by using a non-parametric analysis of variance. Significance differences were compared between the experimental groups and the vehicle a control groups by using Dunett T3.
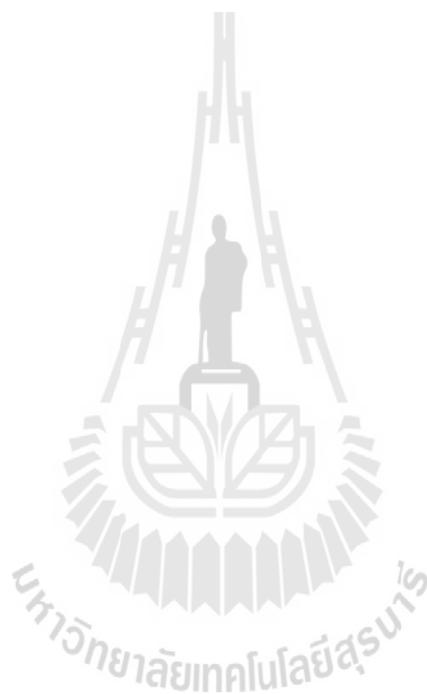
CHAPTER IV

RESULTS

4.1 Phytochemicals and antioxidant activity

4.1.1 Extraction yield

Pseuderanthemum palatiferum or Hoan-ngoc fresh leave was extracted by 95% ethanol and then partitioned between hexane and water (1:1, v/v), giving the water extract of 95% ethanol extract (WEP). WEP exhibited a percentage of recovery of 1.15% from fresh leaves of *P. palatiferum* (Table 4.1). WEP that was prepared from the water fraction of 95% ethanol extracts partitioned with hexane and water (1:1, v/v) showed a percentage of 52.5% recovery.

Table 4.1 The percentage yields of crude extracts of *P. palatiferum* leave extracts.

Extract	Amount and source of preparation	Yield (g)	Percentage of recovery (%)
WEP	40 g of 95% ethanol extract	21.00	52.5 (from 95% ethanol extract)
	(1,826 g from fresh leaves)		1.15 (from fresh leaves)

4.1.2 Total phenolic and flavonoid content

Initial studies conducted to investigate and determine the total phenolic and total flavonoid contents from WEP were shown in Figure 4.1. The total phenolic content (TPC) was analyzed by fitting the calibration curve of gallic acid (R^2 = 0.9928). The total flavonoid content (TFC) was calculated using the calibration curve of catechin (R^2 = 0.9973). The results exhibited that the TPC and TFC of WEP were 338.12±6.54 mg GAE/g and 310.33±1.56 mg CAE/g, respectively.
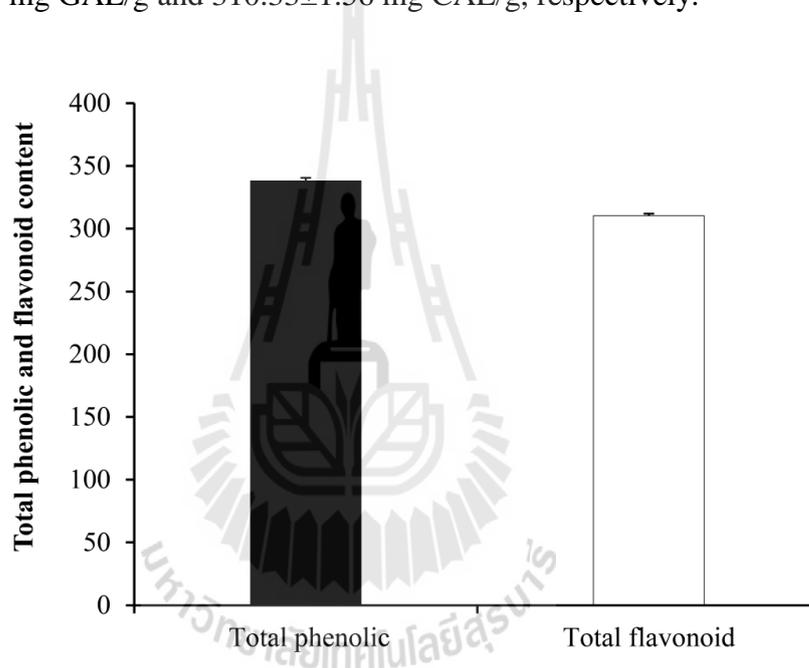
Figure 4.1 Total phenolic and flavonoid contents of WEP. Values are mean ± SD (n = 3) and are representative of three independent experiments with similar results.

4.1.3 The 2, 2-diphenyl-1-picrylhydrazyl (DPPH) assay

The free radical-scavenging activity of WEP and reference standards (vitamin C and trolox) was evaluated by the DPPH assay, and the results were expressed as IC_{50} values. A lower value of IC_{50} indicates a higher antioxidant activity.

As shown in Figure 4.2, the IC_{50} values of DPPH radical scavenging activity of WEP was 8.27±0.07 µg/ml. The positive antioxidant controls, ascorbic acid and trolox showed the scavenging activity with IC_{50} values of 3.41±0.03 and 3.44±0.03 µg/ml, respectively. Therefore, the results suggested that WEP demonstrated the ability to scavenge DPPH free radicals. However, the radical scavenging activity of WEP was not as effective as the other positive antioxidant controls.
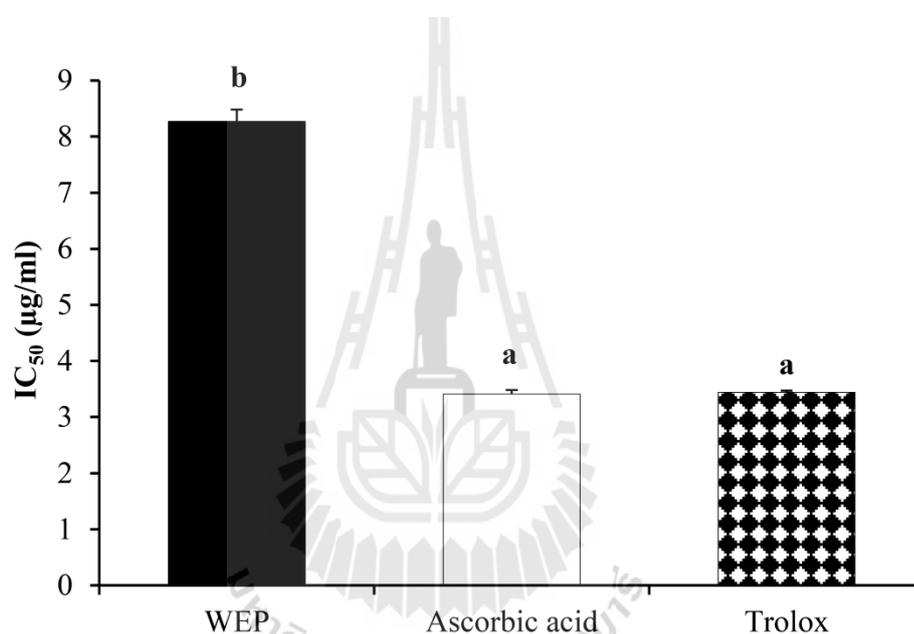
Figure 4.2   DPPH radical scavenging activity of WEP and positive controls (ascorbic acid and trolox). Values are expressed as means ± SD (n = 3) and are representative of three independent experiments with similar results. Bars marked with different letters are significantly different at $p < 0.05$ as determined by one-way ANOVA.

4.2   Cytotoxic effect of WEP against B16F10

To rule out the possibility of direct cytotoxicity of WEP towards B16F10, the range finding test was performed to evaluate the cytotoxicity of WEP towards B16F10 using the MTT assay. The MTT assay has been widely used for measuring cell survival and proliferation. The reduction of tetrazolium dye (MTT) to purple coloured formazan products depends on mitochondrial dehydrogenase activity in the living cells (Mosmann, 1983). The cells were incubated for 24 hours with various concentrations of WEP (50, 100, 300, 600, 900, 1,200, or 1,500 µg/ml). Figure 4.3 showed that B16F10 cells exhibited different susceptibility to WEP in a dose dependent manner. WEP at the concentrations of 50 and 100 µg/ml had no toxicity towards B16F10 cells ($p < 0.05$). However, 300 µg/ml of WEP displayed slightly decreased the viability of B16F10 by 8.97% ($p < 0.05$). At high concentration (600-1,500 µg/ml), WEP showed the cytotoxicity of B16F10 by 30-70%. The *in vitro* cytotoxic effect of WEP against B16F10 cell lines after 24 hours of exposure were expressed as the concentration of the extract causing 50% of cancer cell death (LC_{50}). The LC_{50} was calculated from a dose response curve using linear regression analysis. The LC_{50} of cytotoxic activity of WEP against B16F10 cell lines was 995.59±35.95 µg/ml. Based on this range finding result, the concentration ranged from nontoxic till the maximum tolerated concentration of WEP (50-300 µg/ml) was selected for further investigated on the effect of WEP on the induction of angiogenesis by B16F10 in the CAM model.
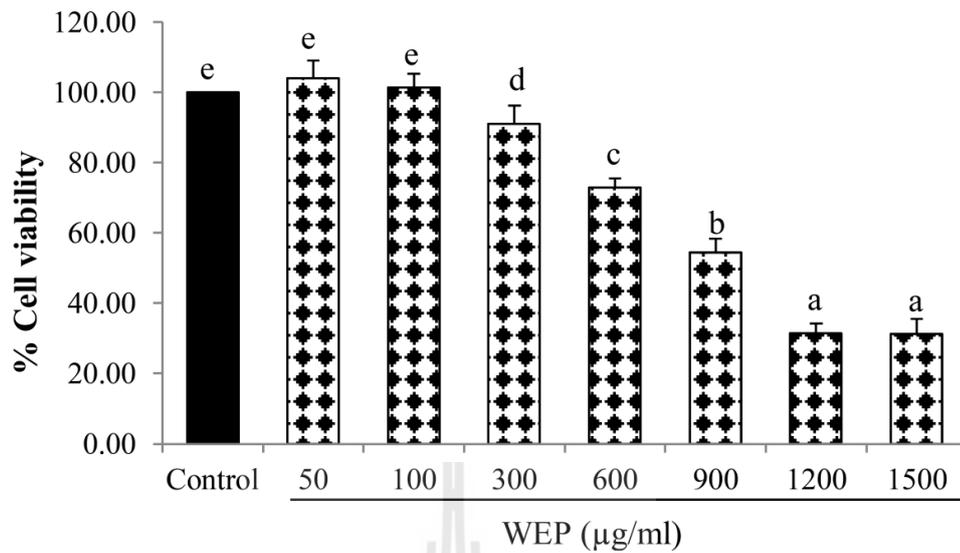
Figure 4.3   Cytotoxic effect of WEP against B16F10 cell lines. The cells were exposed to various concentrations of WEP for 24 hour. The cells were assessed for cell viability by MTT assay. Reported means ± SD values (n=4) are from a representative of three independent experiments. Bars marked with different letters are significantly different at $p < 0.05$ as determined by one-way ANOVA.

4.3   CAM assay

4.3.1 Angiogenesis induction by B16F10 and bFGF

The results of angiogenesis induction in CAM after inclubation with B16F10 melanoma and bFGF for 24 and 48 hours were shown Figure 4.4 and 4.5, respectively. As expected, both B16F10 and bFGF were strong inducers of angiogenesis in the CAM model as suggested by significantly increased neovascular vessels ($p < 0.05$) when compared to the uninduced control group both at 24 and 48 hours. After incubation of B16F10 on CAM, the number of neovascular vessels around the disk placed on CAM was significantly increased in both dose- and time-

dependent manners ($p < 0.05$). When compared to the uninduced control, the numbers of neovasculars were increased by 16, 26, and 30 folds after 24 hour inoculation with 1×10^6, 3×10^6 and 6×10^6 cells of B16F10, respectively. After 48 hour of B16F10 inoculation, the number of neovascular vessels was increased by 5, 6, and 8 fold ($p < 0.05$) at 1×10^6, 3×10^6 and 6×10^6 cells of B16F10, respectively. The increased neovascular vessels were 12 and 4 folds upon exposure to the positive control 100 ng/ml of bFGF for 24 and 48 hours, respectively. Therefore, the increased number of neovascular vessels induced by B16F10 was significantly greater ($p < 0.05$) than that of 100 ng/ml of bFGF in both time points. Furthermore, the results also exhibited that the angiogenesis induction by both B16F10 and bFGF were significantly higher at 48 hours compared to 24 hours (Figure 4.6). As doubling the cell number of B16F10 from 3×10^6 to 6×10^6 cells increased less neovascularization (neovascularization were 15.77% and 16.23% for 24 and 48 hours, respectively) than that of increasing cells from 1×10^6 to 3×10^6 (neovascularization were 63.96% and 31.66 for 24 and 48 hour, respectively). The cell number of 3×10^6 cells of B16F10 was selected for further evaluation or anti-angiogenesis activity of bFGF on the CAM model in subsequent studies.
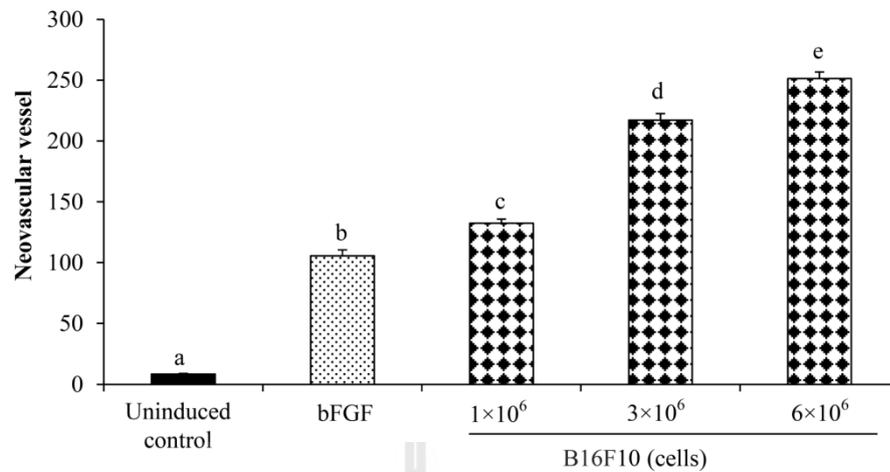
Figure 4.4 Induction of angiogenesis by B16F10 and bFGF in CAM model for 24 hours. Values are expressed as means $\pm$ SD (n = 4) and are representative of two independent experiments with similar results. Bars marked with different letters are significantly different at $p < 0.05$ as determined by one-way ANOVA.
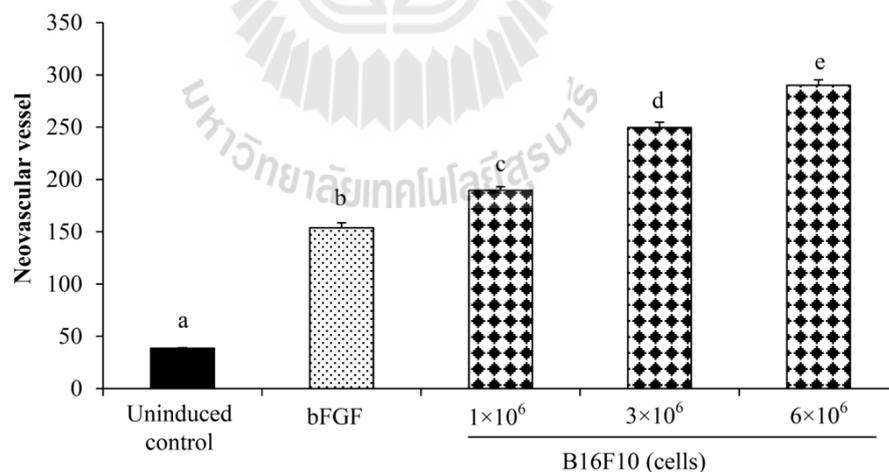
Figure 4.5 Induction of angiogenesis by B16F10 and bFGF in CAM model for 48 hours. Values are expressed as means $\pm$ SD (n = 4) and are representative of two independent experiments with similar results. Bars marked with different letters are significantly different at $p < 0.05$ as determined by one-way ANOVA.
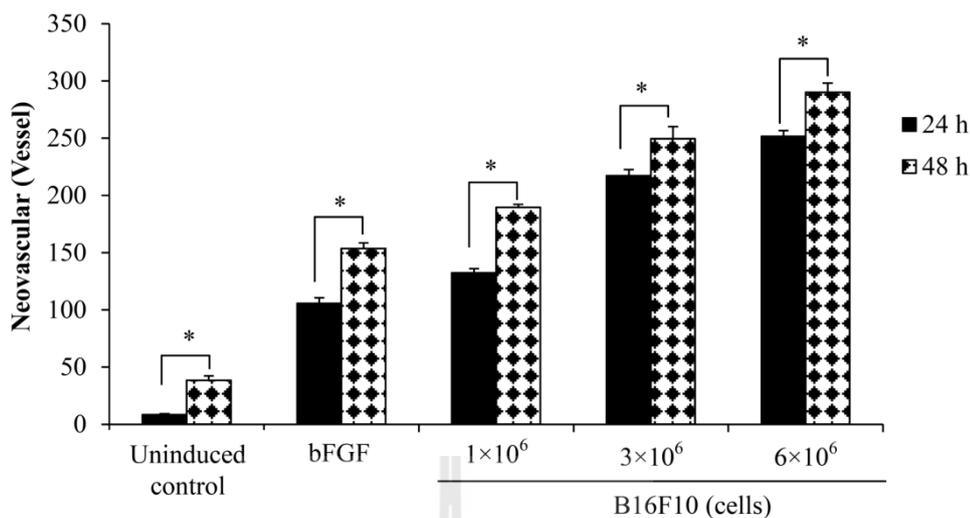
Figure 4.6   Comparison of angiogenesis induction by bFGF and B16F10 in the CAM model at 24 and 48 hours. Values are mean ± SD (n = 4) and are representative of two independent experiments with similar results. Statistical analysis was performed by Student's *t*-test. The asterisk indicates $p < 0.05$.

4.3.2   Toxicity of WEP in the CAM model

To find out the optimum concentration of WEP used in the CAM model, the effects of WEP on natural angiogenesis in the CAM was evaluated at 24 and 48 hours post exposure. The results in Figure 4.7 and 4.8 showed that exposure to WEP at 900 µg/ml for 24 hours had no significant alteration in the number of neovascular formation compared to the unexposed control. However, the number of neovascular was significantly increased after exposed to 100 and 300 µg/ml of WEP and was significantly decreased after exposure to 1,500 µg/ml of WEP for 24 hours, when compared to the control group ($p < 0.05$). At 48 hours post exposure, the number of neovascular was significantly decreased from 39±4 vessels (control) to

8 ± 4, -4 ± 12, and -19 ± 7 vessels after exposure to WEP at 600, 900, and 1,500 µg/ml of WEP, respectively. In contrast, WEP at the concentration range of 100-300 µg/ml had no effect on angiogenesis at the same time point. Notably, the number of neovascular vessels after exposed to WEP at 100 and 300 µg/ml for 48 hours was significantly higher ($p < 0.05$) than at 24 hours (Figure 4.9), and the increased angiogenesis was about 2.5-3 folds which was comparable to a 4 fold increase observed in the unexposed control. Therefore, the results suggested that, exposure to WEP up to 600 µg/ml for 24 hours and up to 300 µg/ml after 48 hours did not decrease the angiogenesis formation in the CAM. In contrast, exposure to higher normal concentration of WEP (600-1,500 µg/ml) for 48 hour could suppress the normal angiogenesis formation. In spite of some toxicity on neovascular formation at 48 hour, there was still no lethality of all chick embryos was observed even at the highest treatment group of 1,500 µg/ml WEP. Consequently, the concentration up to 300 µg/ml of WEP which had no suppression on natural angiogenesis generation was chosen for subsequent anti-angiogenesis study.
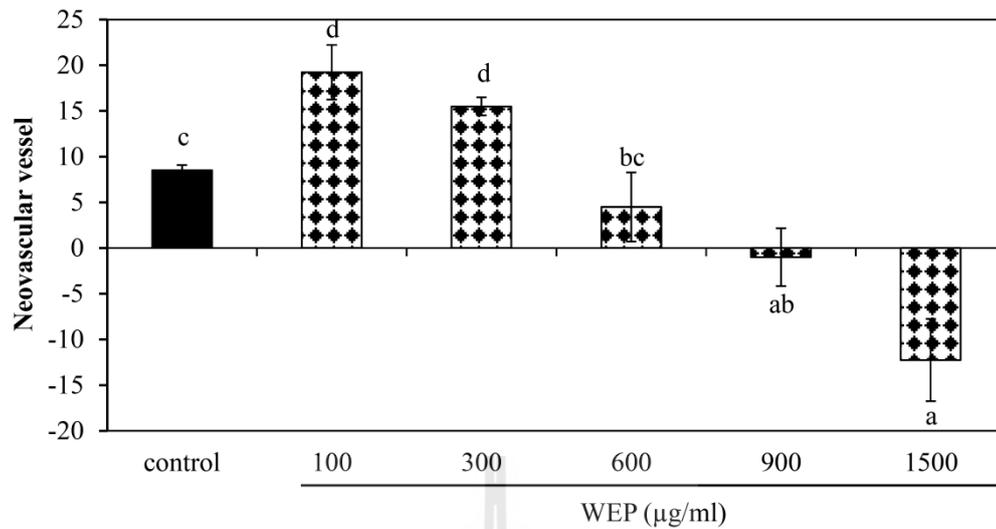
Figure 4.7   The effect of WEP on normal angiogenesis in the CAM model at 24 hours. Values are expressed as means ± SD (n = 4) and are representative of two independent experiments with similar results. Bars marked with different letters are significantly different at $p < 0.05$ as determined by a non-parametric analysis of variance.
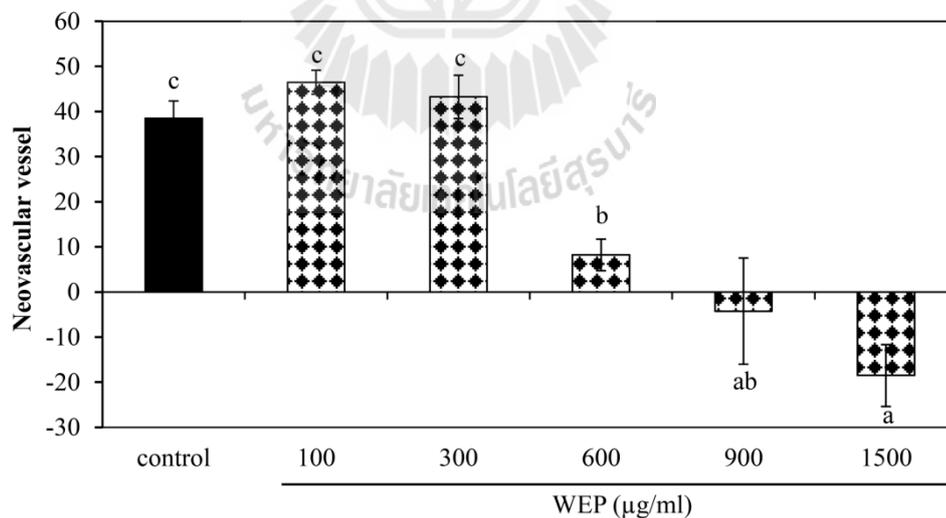
Figure 4.8   The effect of WEP on normal angiogenesis in the CAM model at 48 hours. Values are expressed as means ± SD (n = 4) and are representative of two independent experiments with similar results. Bars marked with different letters are significantly different at $p < 0.05$ as determined by one-way ANOVA.
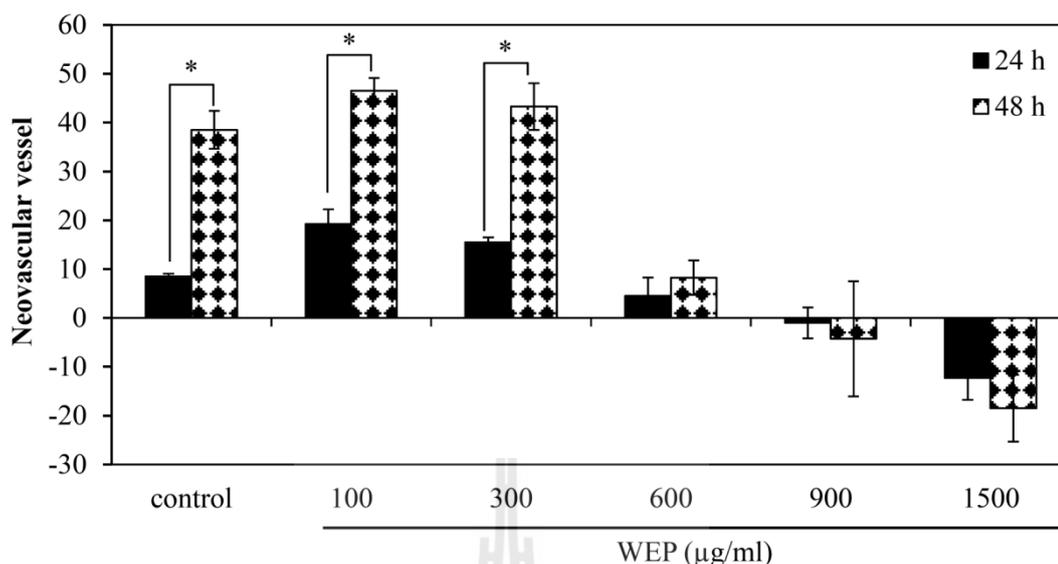
Figure 4.9 Comparison of the effect of WEP on normal angiogenesis between 24 and 48 hours. Values are means ± SD (n = 4) and are representative of two independent experiments with similar results. Statistical analysis was performed by Student's *t*-test. Different letters indicate significant differences ($p < 0.05$).

4.4.3 Anti-angiogenic activity of WEP on B16F10-induced angiogenesis in the CAM

Anti-angiogenesis activity of WEP on B16F10-induced angiogenesis in the CAM at 24 and 48 hour time points are shown in Figure 4.10 and 4.11, respectively. The results indicated that WEP clearly produced a dose dependent suppression of B16F10-induced angiogenesis in the CAM model ($p < 0.05$). Concomitantly exposure of B16F10 with WEP on the soaked paper disk for 24 hours caused the reduction of tumor-induced neovascularization by 20.48%, 50.90% and 87.65% at 30, 100 and 300 µg/ml of WEP, respectively. Likewise, After 48 hours the number of neovascularization induced by of B16F10 was also reduced by 14.7%,

49.8% and 90.8% upon exposure to WEP at 30, 100 and 300 µg/ml, respectively (Figure 4.11). Furthermore, the results in Figure 4.12 suggested that as well as the unexposed control, the number of neovascular vessels in B16F10-induced angiogenesis on CAM after exposed to WEP at 30 and 100 µg/ml for 48 hours was significantly greater ($p < 0.05$) than that of 24 hours. Interestingly, there was significant inverse relationship ($p < 0.05$) between increases of neovascular vessels of greater exposure time increasing concentration of WEP (Figure 4.13).
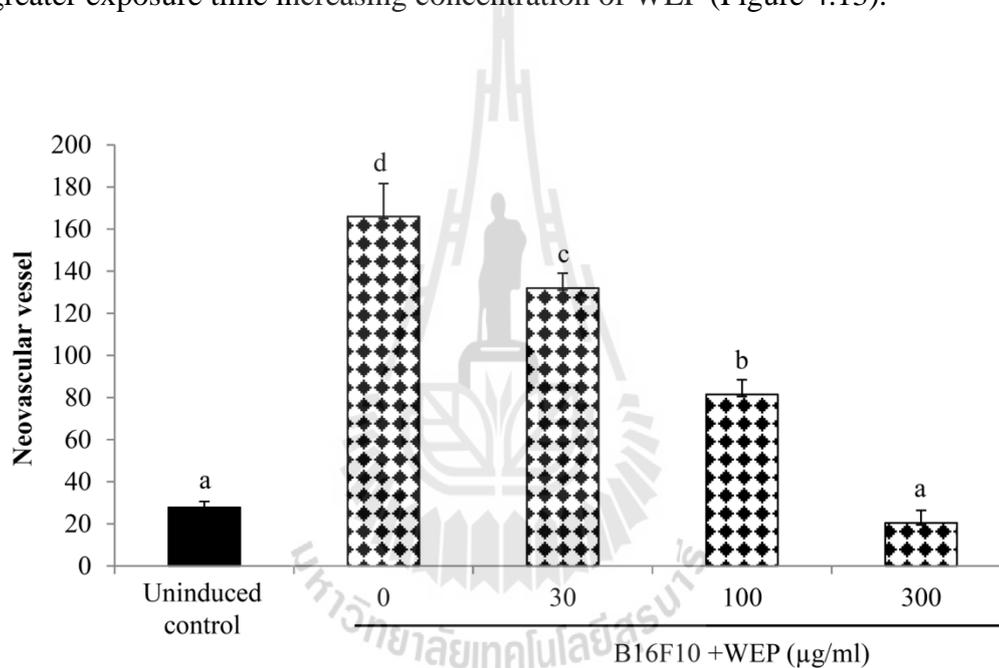
Figure 4.10   Anti-angiogenic activity of WEP on B16F10-induced angiogenesis in the CAM model at 24 hours. Values are expressed as means ± SD (n = 6) and are representative of two independent experiments with similar results. Bars marked with different letters are significantly different at $p < 0.05$ as determined by one-way ANOVA.
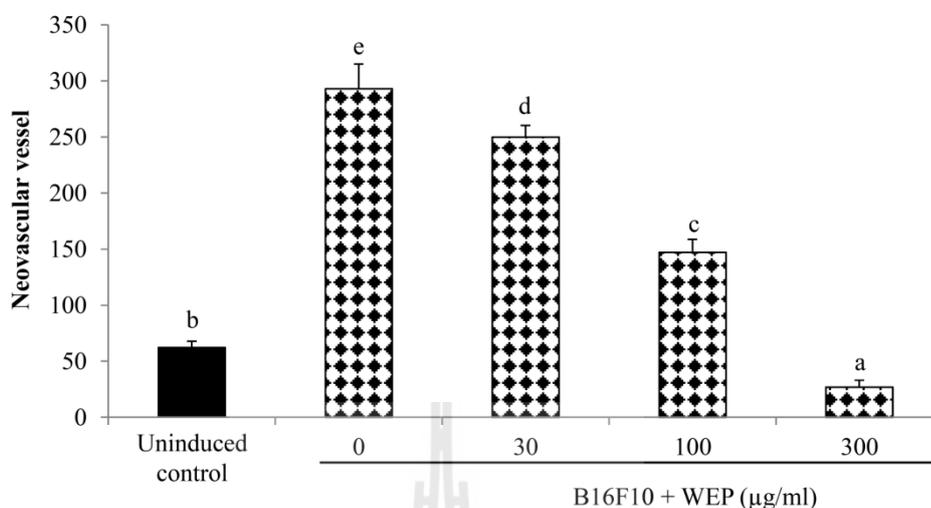
Figure 4.11   Anti-angiogenesis activity of WEP on B16F10-induced angiogenesis in the CAM model at 48 hours. Values are expressed as means ± SD (n = 6) and are representative of two independent experiments with similar results. Bars marked with different letters are significantly different at $p < 0.05$ as determined by a non-parametric analysis of variance.
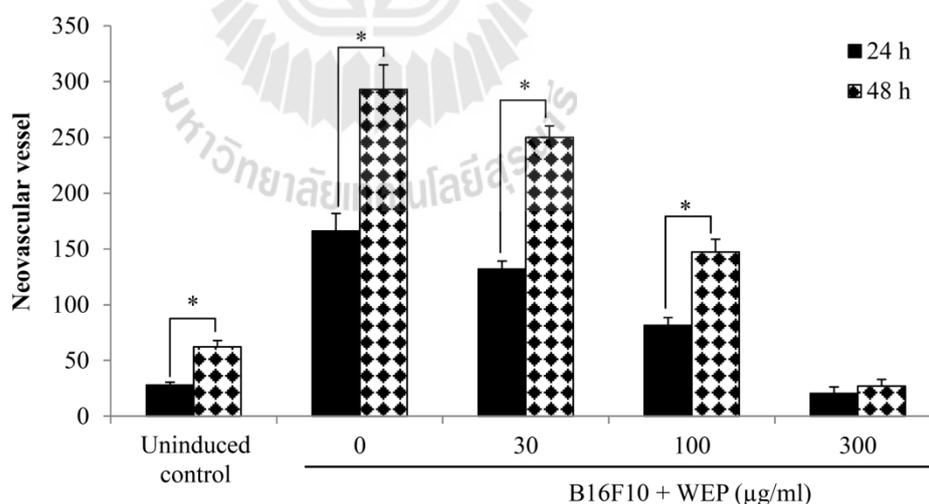
Figure 4.12   Comparison of anti-angiogenesis activity between 24 and 48 hours of WEP on B16F10 induced angiogenesis in the CAM model. Values are expressed as means ± SD (n = 6) and are representative of two independent experiments with similar results. Bars marked with different letters are significantly different at $p < 0.05$ as determined by one-way ANOVA.
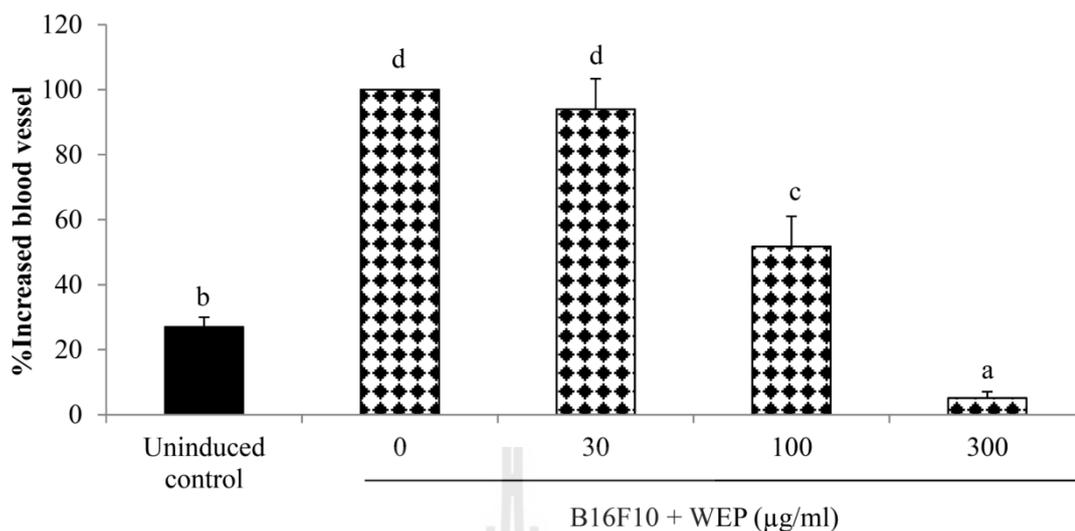
Figure 4.13   Percentages increase of anti-angiogenesis activity of WEP on B16F10-induced angiogenesis in the CAM model for 24 hours compared to 48 hours. Values are expressed as means ± SD (n = 6) and are representative of two independent experiments with similar results. Bars marked with different letters are significantly different at $p < 0.05$ as determined by a non-parametric analysis of variance.

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CHAPTER V

DISCUSSION

Pseuderanthemum palatiferum (Nees) Radlk. or Hoan-Ngoc is a new medicinal plant that widely used in both Vietnamese and Thais as a medicinal and ornamental plant. Several studies have described the beneficial effects of *P. palatiferum* such as antioxidant, antidiabetic, anti-inflammatory, hypotensive and antiproliferative activities (Chayarop et al., 2011; Khonsung et al., 2011; Pamok et al., 2012; Sittisart and Chitsomboon, 2014). The present study aimed to investigate the potential of water leave extract of *P. palatiferum* as an important source of food supplement for anticancer therapeutics in the future.

This study found phenolics and flavonoids in the water extract fractioned from 95% ethanol extract of *P. palatiferum* (WEP). As reported by Nguyen and Eun (2011), phenolics and flavonoids were found in *P. palatiferum* leaves extracted with methanol, ethanol, acetone, and water when assessed with Folin-Ciocalteu and aluminum trichloride methods. Dieu et al. (2008) and Chayarop et al. (2011) reported that the major chemical compositions of *P. palatiferum* leaves are flavanoids, apigenin, stigmasterol, β-sitosterol, β-sitosterol-3-0-β-glucoside, and apigenin-7-0-β-glucoside. In addition, Sittisart and Chitsomboon (2014) found high levels of phenolics and flavonoids in both 95% ethanol extract and the water extract of *P. palatiferum*, which were correlated to DPPH radical scavenging activity.

The DPPH model was widely used in the model system to investigate the scavenging activities of various natural compounds such as phenolic compounds, anthocyanins, or crude mixtures such as ethanolic extract of plants (Panchawat and Sisodia, 2010). The results of the current study demonstrated that WEP possessed high levels of phenolic and flavonoid contents which were correlated to high radical scavenging activities when assessed by DPPH assay. This study agreed with Sittisart and Chitsomboon (2014), who reported that the IC_{50} values of 95% ethanol extract and water extract fractionated from 95% ethanol extract of *P. palatiferum* were 23.45±0.12 and 21.55±0.06 μg/ml, respectively. Moreover, Nguyen and Eun (2011) reported DPPH scavenging activity of ethanol extract and water extract of *P. palatiferum* with IC_{50} values of 23.30±0.46% and 7.44±0.56%, respectively. In addition, researchers have found a correlation between total phenolic and flavonoid contents and antioxidant activity. Saeed et al. (2012) also found that the total phenolic and flavonoid contents of *Torilis leptophylla* were related to its free radical scavenging activity. Fernandes de Oliveira et al. (2012) observed a strong correlation between total polyphenol contents and antioxidant activity of the crude extracts of *Sidastrum micranthum* and *Wissadula periplocifolia*. Bunea et al. (2011) demonstrated a high correlation between total polyphenol content and antioxidant activity in cultivated blueberry varieties from Romania. Therefore, high total phenolic and flavonoid contents in WEP could be associated with the scavenging activity against DPPH radicals.

The current study showed a dose-related cytotoxic effect of WEP against murine melanoma B16F10 cells. Several medicinal plants containing polyphenol compounds have been reported to have cytotoxicity against various types of cancer

cell lines such as T cell leukemia Jurkat cells (Musika and Indrapichate, 2014), gastric (AGS, SNU-668 and SNU-638) and breast (MDA-MB-231, MCF-7 and SK-BR-3) cancer cell lines. Therefore, the antiproliferative activity of WEP towards cancer cell lines might be due to phytochemical constituents that dissolved in water fraction such as flavanoids, apigenin, catechin, gallic acid, and tannic acid (Dieu et al., 2008; Das et al., 2009; Chayarop et al., 2011). Importantly, a highly desirable property of chemotherapeutic compounds is selectively toxic to just cancer cells and not normal cells to avoid damages to normal cells or healthy tissues (American Cancer Society, 2013). At low concentration (50-100 µg/ml) WEP had no directed cytotoxicity against B16F10, whereas the high dose (600-1500 µg/ml) induced about 26.97-69.63% cell death. The concentration of 300 µg/ml WEP could be considered as a maximum tolerated concentration as this level caused less than 10% cell death in B16F10. To compromise for an exclusion of the concentration that was cytotoxic to B16F10 and maximize the concentration of WEP in the study, the dose range of 50-300 µg/ml of WEP was selected for further investigation of the anti-angiogenic activity of WEP in the CAM model.

Basic fibroblast growth factor (bFGF) plays a pivotal role in the proliferative response of tumor angiogenesis (Sun, 2013). Many types of tumors expressed potent angiogenic cytokines which have been regarded as positive factors for enhancing tumor growth, metastasis, and correlated with poor prognosis (Claffcy et al., 2001). bFGF is one of tumor-derived angiogenic cytokines that is strongly correlated with vascular density, metastatic potential and poor survival in human colon, breast, prostate and melanoma (Ribatti et al., 2012) This study used bFGF to mimic the angiogenic activity of tumor-derived cytokines. As expected, bFGF at 100 ng/ml

strongly induced neovascularization by 4-12 folds, when compared to the uninduced control.

B16F10 melanoma cell metastasize through the induction of neovascularization (Gacche, 2015). In this study, the pellets of 1×10^6- 6×10^6 cells of B16F10 melanoma strongly induced neovascularization within 24 and 48 hours after inoculation on the CAM. Notably, all concentrations of B16F10 used in the studies provided greater angiogenic response than that of bFGF. Ribatti et al. (2013) also reported that B16F10 melanoma can induce a strong angiogenic response comparable to the basic fibroblast growth factor-2 (bFGF). The easily observed black nodules of B16F10 in conjunction with its strong angiogenic property that mediating metastasis makes it an ideal candidate for the study of tumor-induced angiogenesis in the CAM assay. In this study, 3×10^6 cells of B16F10 were chosen to be implanted on the CAM in the tumor-induced angiogenesis model. In fact, B16F10 melanoma has been used in several spontaneous metastasis models including the CAM model (Ribalti et al., 2012; Chen et al., 2009). More than 80% of B16F10 can survive, extravasate and metastasize efficiently from the CAM. Moreover, B16F10 are capable of forming visible tumor mass within 2-5 days after implantation on CAM (Ribatti et al., 2013).

Angiogenesis is a strictly controlled process in normal human body and is regulated by a variety of endogenous angiogenic and angiostatic factors (Folkman and Klagsbrun, 1987). Pathological angiogenesis occurs, for example, in cancer, chronic inflammation, or atherosclerosis (Gacche et al., 2015). Angiogenesis inhibitors are able to interfere with various steps of angiogenesis, on the other hand, angiogenesis promoters can stimulate angiogenesis occurring by local destruction of preexisting blood vessel, activating endothelial cell proliferation and migration

(Koutrafouri et al., 2001). As angiogenesis is essential for most, if not all, tumor growth and metastasis, compounds with anti-angiogenic activity can inhibit metastasis or the progression of cancer. Thus, anti-angiogenesis is one of the most potential and promising strategy in battling cancer. This study suggested that WEP suppressed neovascularization in a dose dependent manner. WEP at the concentration range of 30-300 μg/ml could suppress the tumor-induced angiogenesis by about 20-87% after 24 hours and suppressed by about 15-91% after 48 hours of exposure. The anti-angiogenic activity of WEP was not due to direct cytotoxicity to the tumor B16F10 cells as the concentrations used (30-100 μg/ml) were not cytotoxic to the cells. Interestingly, almost 90% suppression of angiogenesis by WEP was observed at 300 μg/ml, the level that induced only about 9% cell death in B16F10. Obviously, this great magnitude of angiogenic suppression by 1,500 μg/ml WEP was not solely explained by the direct cytotoxic effect of WEP towards B16F10. The mechanism mediating anti-angiogenic effect of WEP is needed to be further elucidated. The data also suggested that WEP was more efficient as anti-angiogenic inhibitor at the concentration that was slightly toxic to the tumor cells. The anti-angiogenesis activity of WEP was further confirmed by a significantly dose-related suppression of increased neovascularization from 24 to 48 hours after WEP exposure (Figure 4.13) ($p > 0.05$). When compared to the unexposed control, 100 and 300 μg/ml of WEP significantly decreased the tumor-induced angiogenesis formation from 24 to 48 hours by about 51% and 88%, respectively. Importantly, 100-300 μg/ml of WEP only selectively inhibited the tumor-induced angiogenesis without targeting the normal neovascularization on the CAM (Figure 4.7 and Figure 4.8). In addition, the wide ranges of concentration of WEP (50-1,500 μg/ml) also exert no toxicity towards the

chick embryos suggesting that WEP could be used as tumor angiogenesis inhibitor at high concentration. The anti-angiogenic activity of WEP might be related to the free radical scavenging property and other anti-oxidant activities of the extract. To date, many natural products or plant extracts have been demonstrated to possess antioxidant properties in conjunction with potent anti-angiogenic activity in many *in vivo* models including in the CAM assay. Examples of plant extracts or their chemical constituents that possess both antioxidant and anti-angiogenic activities are ethanolic extracts of *Pithecellobium* Jiringae, leaf extracts of *Nuelumbo nucifera*, methanolic extracts of *Sphenocentrum jollyanum* Pierre, green tea, the dietary anthocyanidin delphinidin, flavones and catechins (Muslim et al., 2012; Lee et al., 2015; Nia et al., 2005; Lamy et al., 2006; Lamy et al., 2002; Cao and Cao, 1999; Gacche et al., 2015). In fact, it is well recognized that ROS can play important roles in angiogenesis activation. The major source of ROS in endothelial cells that involved in angiogenesis induction are the enzyme NADPH oxidase and endothelial nitric oxide synthase. The higher level of H_2O_2 also produced by tumor cells and can induce angiogenesis signaling through VEGF receptors, mainly VEGFR2 and angiopoietin-I/Tie-2 receptors, up-regulating NADPH oxidase 1 via hypoxia inducible factor 1, or through up-regulation of VEGF expression. In addition, oxidized phospholipids and metabolized products from arachidonic acid have also been reported to participate in activation of angiogenesis. (Prauchner, 2014; Bochkov et al., 2006). Therefore, it is very plausible that antioxidants or free radical scavengers could inhibit angiogenesis.

Angiogenesis is normally regulated by a balance between activators and inhibitors. The major stimuli that can activate endothelial cells to undergoing the

process of "angiogenic switch" are VEGF, angiopoietin-1, angiopoietin-2, IL-8, bFGF, platelet-derived growth factor, angiotensin II, matrix metalloproteinases-2 (MMP-2) and metalloproteinase-9 (MMP-9) (Prauchner 2014; Pepper, 2001). Several antioxidants from natural products can mediate their inhibitory mechanism of angiogenesis induction *in vitro* and *in vivo* through regulating these pro-angiogenic pathways. For example, flavonoid precursor 4-hydroxychalcone (Q797) was able to modulate both VEGF- and bFGF-induced phosphorylation of extracellular signal regulated kinase (ERK)-1/-2 and Akt kinase. The potent inhibitory effect of Q797 on bFGF-driven neovascularization was also demonstrated *in vivo* using the CAM assay (Varinska et al., 2012). EGCG was shown to inhibit angiogenesis induction in several *in vitro* and *in vivo* models including the CAM assay. The reported various inhibitory mechanisms of angiogenesis induction mediated by EGCG in different assays were following: suppression of IL-8 production, inhibition of urokinase or MMPs activity, removing ROS, inhibition of VEGF induction, and blocking neutrophil mediated angiogenesis (Tang and Meydani 2001; Zhang et al., 2000; Prauchner, 2014). In addition, EGCG and theaflavins from teas also inhibited the binding of AP-1 to DNA, and hence the suppression of angiogenesis as AP-1 is the transcription factor that up-regulates MMP-1 and urokinases (Prauchner, 2014). Resveratrol, the polyphenolic compound in grapes, was also shown to inhibit angiogenesis in the CAM model in a dose-dependent manner. Its mechanism of anti-angiogenic activity involves disruption of VEGF-dependent ROS production in the step of Src kinase activation and the subsequent vascular endothelial-cadherin tyrosine phosphorylation (Lin et al., 2003). Liu et al. (2005) also found that apigenin significantly inhibited tumor growth in nude mice through inhibition of HIF-1 and

VEGF expression in the tumor tissues, suggesting an inhibitory effect of apigenin on angiogenesis. In addition, curcumin derivatives inhibited angiogenesis in CAM assay by inhibition of VEGF and angiopoietin 1 and 2 signaling in EAT cells, to VEGF and angiopoietin 1 in NIH3T3 cells, and to tyrosine kinase Flk-1/KDR in HUVECs. Furthermore, the reduction of expressions of epidermal growth factor receptor, VEGF and MMP-9, and the reduction level of MMP-2 by curcumin were also been reported (Prauchner, 2014). Lycopene is also another natural compound that could down regulate angiogenesis in both rat aortic ring and in the CAM models. The anti-angiogenic activity of lycopene was accompanied by reduced activites of MMP-2, urokinase-type plasminogen activator, attenuating VEFG-2 signaling, and enhancing the expression of tissue inhibitors of metalloproteinase-2 and plasminogen activator inhibitor-1 (Chen et al., 2012; Prauchner, 2014). Therefore, several antioxidants from natural products could exert their anti-angiogenic activity via distinct signaling pathways, either redox sensitive or insensitive mechanisms, depending on the investigated cell types and/or the test systems. Likewise, it is plausible that WEP could exert anti-angiogenic activity via its strong free radical scavenging and other antioxidant activities. In addition to its antioxidant property, both water and ethanol extracts of *Pseuderanthemum palatiferum* also possess an anti-inflammatory property. Sittisart and Chitsomboon (2014) demonstrated the suppression of both extracts on NO• production by down regulation of iNOS expression in LPS and IFN-γ–stimulated RAW264.7 cells. The suppression of COX-2 was also concomitantly observed in the same stimulated RAW264.7 cells by the extracts. Since VEGF can induce the expression of eNOS mRNA and protein, and increase NO• production in endothelial cells (Hood et al., 1998), and NO• is known to participate in angiogenesis

induction (Murohara and Asahara, 2002), WEP which is iNOS inhibitor and suppression of NO• production would be likely to repress angiogenesis. Furthermore, the inducible COX-2 is considered as an important mediator in angiogenesis and tumor development. COX-2 is up-regulated in many types of cancers and are related to increased VEGF production and angiogenesis (Gately and Li, 2004; Toomey et al., 2009). Gately and Li (2004) reported that the proangiogenic activity of COX-2 is mediated mainly by thromboxane A (2), prostaglandin E (2), and prostaglandin I (2). In addition to increase VEGF, the proangiogenic effects of these three eicosanoid products also include the induction of matrix metalloproteinases, activation of EPGF receptor-mediated angiogenesis, promoting endothelial cell survival by increased BCl-2 expression and Akt signaling, and enhancing of vascular sprouting, migration and tube formation (Gately and Li, 2004). Therefore, the down regulation of COX-2 as a redox insensitive mechanism of anti-angiogenic effect of WEP should not be excluded. However, what specific pro-angiogenic stimuli or inhibitors in the angiogenesis pathway are targeted by WEP in the CAM assay still needs further investigation. As angiogenesis stimulation in cancer promotes metastasis, the anti-angiogenic therapy is an important adjunct treatment in battling cancer. Whether the inhibition of angiogenesis of WEP could actually inhibit B16F10 metastasis should be further investigated. Nevertheless, this inhibition of tumor induced angiogenic activity in WEP could explain, at least in part, the claimed property of anti-cancer activity of Hoan-Ngoc leaves in the folk medicine.

CHAPTER VI

CONCLUSION

The water extract fractionated from 95% extract of fresh leaves from *Pseuderanthemum palatiferum* (Nees) Radlk (WEP) contained the comparable levels of TPC and TFC as determined by using Folin-Ciocalteu method and aluminium trichloride colorimetric assay, respectively. The results revealed that WEP possessed high antioxidant activity as estimated by the DPPH assay.

WEP exerted a direct *in vitro* cytotoxicity against B16F10 cells in a dose-dependent manner. The extract at the concentration of 50 and 100 µg/ml had no toxicity towards B16F10 cells. However, 300 µg/ml of WEP displayed slightly decreased the viability of B16F10 cells (less than 10%). To rule out the direct cytotoxicity towards B16F10 cells, the concentration up to 300 µg/ml of WEP was used as the maximum concentration in further *in vivo* anti-angiogenesis study in the CAM model.

From cell titer study, the pellet of 3×10^6 cells of B16F10 was optimum to be used in the model of tumor-induced angiogenesis in the CAM assay. WEP at the concentration ranging from 100-1,500 µg/ml did not exert lethality in chick embryos. Nevertheless, the normal angiogenesis on CAM was reduced at 1,500 µg/ml of WEP at 24 hours and at 600-1,500 µg/ml of WEP at 48 hours post exposure. Therefore, to avoid damaging healthy normal cells, 30-300 µg/ml WEP was used to investigate the

anti-angiogenic property in the CAM assay. The anti-angiogenesis study revealed that WEP at 100-300 µg/ml possessed strong anti-angiogenic activity in dose and time dependent manners. WEP was a highly selective and efficient inhibitor of angiogenesis induced by B16F10 melanoma cells but spared normal neovascularization. The inhibition of neovascularization induced by tumor cells was more pronounced at the concentration that was slightly toxic towards tumor cells. Overall, the data suggested that WEP might inhibit B16F10 melanoma, at least in part, through its anti-angiogenesis activity.
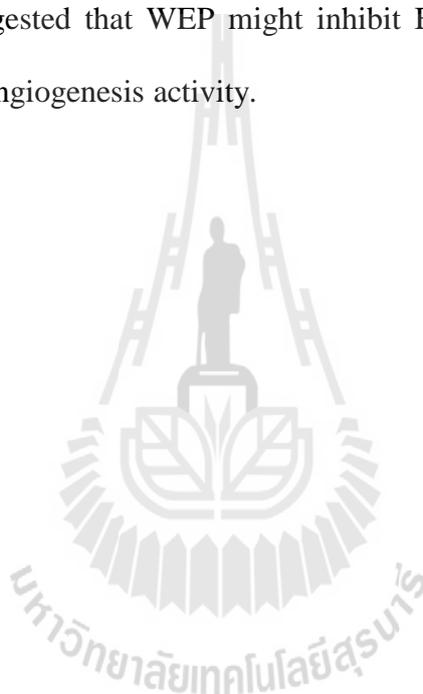
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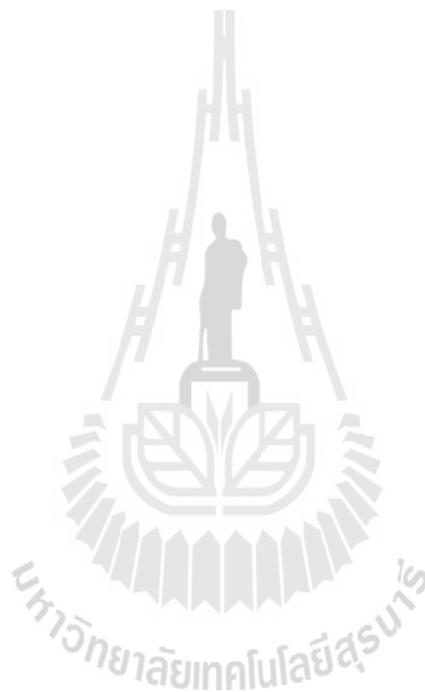
APPENDICES

APPENDIX A

PREPARATION OF REAGENTS FOR CHEMICAL

ANALYSIS

A.1 Folin-Ciocalteu method

- Gallic acid stoCk solution (1mg/ml)

  - Gallic acid   0.011 g

  - 10% Ethanol   10 ml

- 2% Na_2CO_3

  - Na_2CO_3 2 g

  - DI water   100 ml

- 50% Folin-Ciocalteu reagent

  - Folin-Ciocalteu reagent   1 ml

  - Methanol   1 ml

A.2 Aluminium trichloride method

- 10% $AlCl_3.6H_2O$

  - $AlCl_3.6H_2O$ 1 g

  - DI water   10 ml

- 5% $NaNO_2$

  - $NaNO_2$ 0.5 g

  - DI water   10 ml

1 N NaOH

- NaOH   4 g

- DI water   100 ml

A.3 DPPH assay

- DPPH solution (63 mM)

  - DPPH   0.004 g

  - DI water   10 ml

APPENDIX B

PREPARATION OF REAGENTS FOR CELL CULTURE

B.1 Phosphate buffer saline (PBS), 1X, pH 7.4

- KH_2PO_4 0.144 g
- $Na_2HPO_4.7H_2O$ 0.795 g
- NaCl   9.0 g
- DI water   1 L

Adjust pH to 7.2 ± 0.1 and filter sterile (store at 4 °C).

B.2 Trypsin/EDTA preparation

- Trypsin   0.25 g
- EDTA   0.04 g
- PBS, 1X   100 ml

Filter sterile and aliquot (store at 4 °C).

B.3 Culture media preparation

- FBS (heat inactivated)

  - Slowly thaw the frozen FBS in a beaker filled with water.

  - Put in water bath at 37 °C till completely thaw.

  - Heat inactivate (56 °C, 20 min), gentle mix every 10 min.

  - Aliquot 45 ml into conical tubes.

    (store at -20 °C).

- HEPES buffer, 1M

- HEPES	23.83 g
- DI water	100 ml

Filter sterile and aliquot (store at -20 °C).

- Penicillin/Streptomycin, 100X

- Penicillin	0.6 g
- Streptomycin	1.34 g
- PBS, 1X	100 ml

Filter sterile and aliquot (store at -20 °C).

- DMEM, high glucose, 1X (incomplete medium)

- DMEM, high glucose, 1X with L-glutamine and phenol red	1 pack
- NaHCO$_3$	3.7 g
- DI water	1 L

Adjust pH to 7.2-7.4 and filter sterile (store at 4 °C).

- DMEM, high glucose, 1X (complete medium)

- Inactivated FBS	20 ml
- Penicillin/Streptomycin	2 ml
- HEPES buffer, 1M	3 ml

Adjust volume to 200 ml with DMEM, high glucose, 1X (incomplete medium). (store at 4 °C).

APPENDIX C

DATA ANALYSIS

D.1   Standard curves

- **Total phenolic content**

Standard curve of gallic acid

$y = 2.9173x - 0.0562$
$R^2 = 0.9928$

- **Total flavonoid content**

Standard curve of catechin

$$y = 3.153x - 0.0068$$
$$R^2 = 0.9954$$
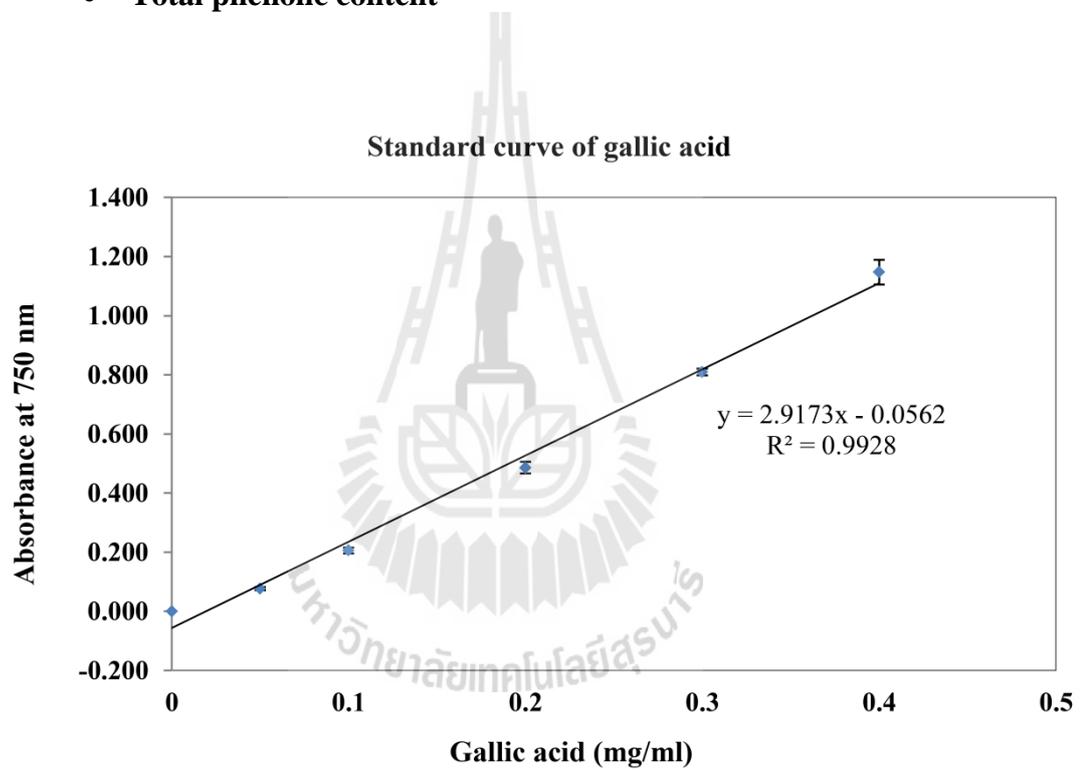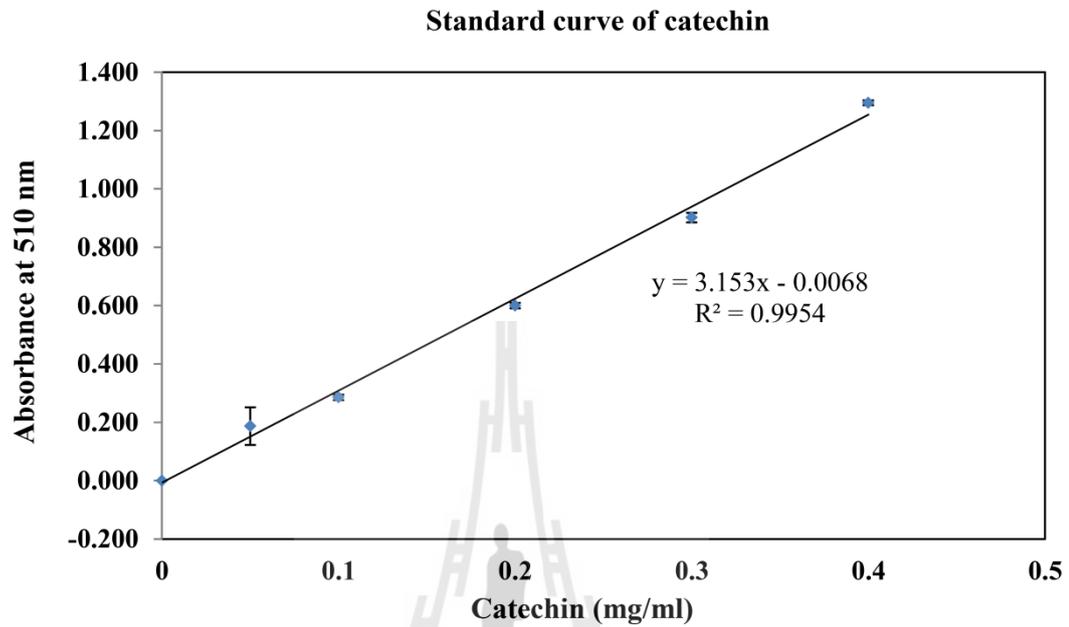
- **DPPH radical scavenging activity of *P. palatiferum* leaf extracts (EEP and WEP) and positive controls (ascorbic acid and trolox)**

% DPPH radical scavenging activity
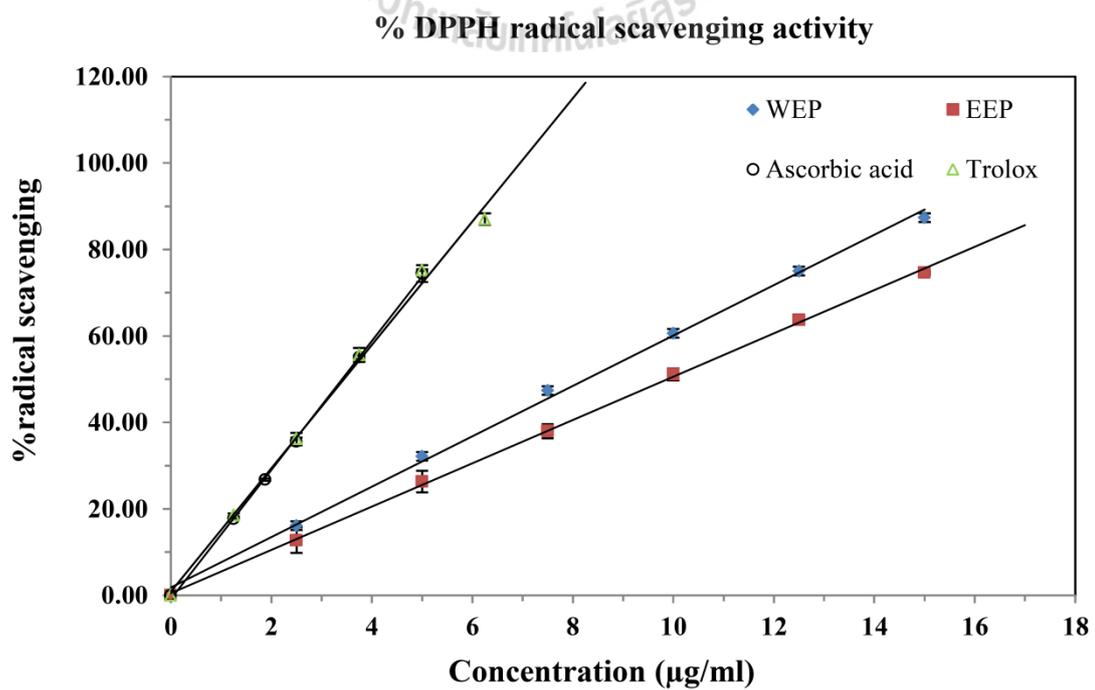
- **In vivo chick chorioallantoic membrane (CAM) angiogenesis assays**

  - **B16F10 and bFGF induce angiogenesis in CAM model**
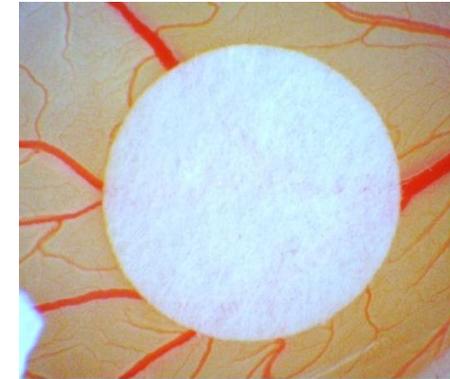
	0 hours	24 hours	48 hours
Uninduced control			
bFGF 100 ng /ml			

	0 hours	24 hours	48 hours

B16F10
1×10^6 cells
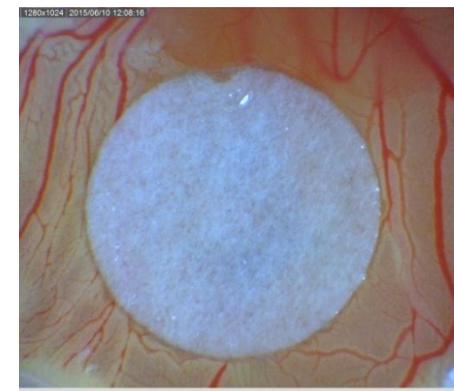
B16F10
3×10^6 cells
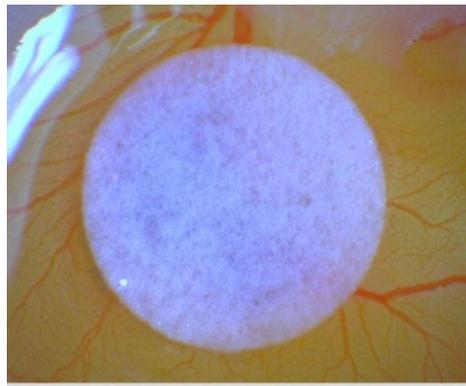
	0 hours	24 hours	48 hours

B16F10
6×10^6 cells
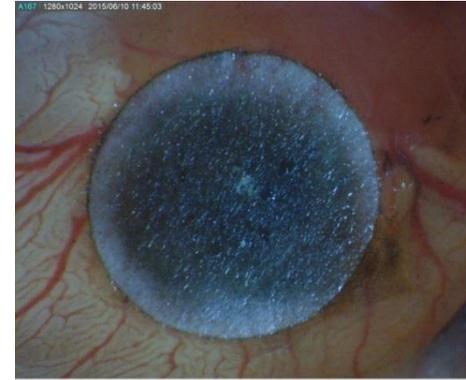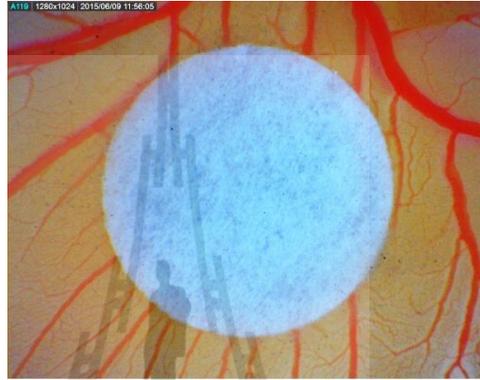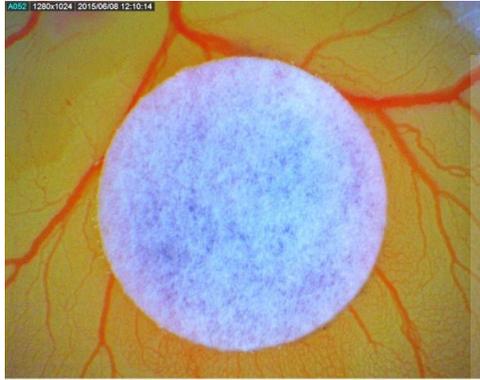
- **The toxicity of WEP on chick chorioallantoic membrane (CAM)**

	0 hours	24 hours	48 hours
Uninduced control			
WEP 100µg/ml			
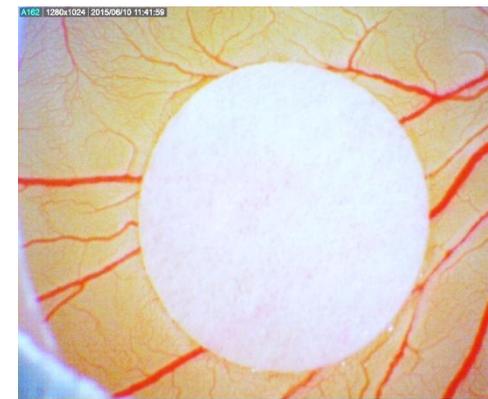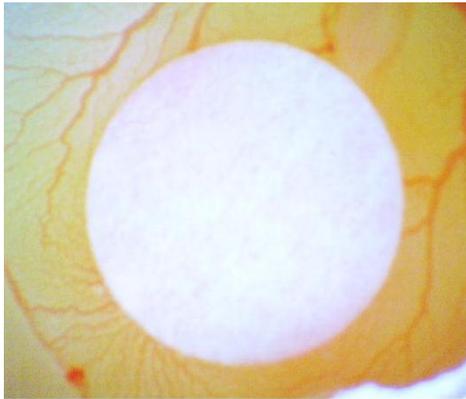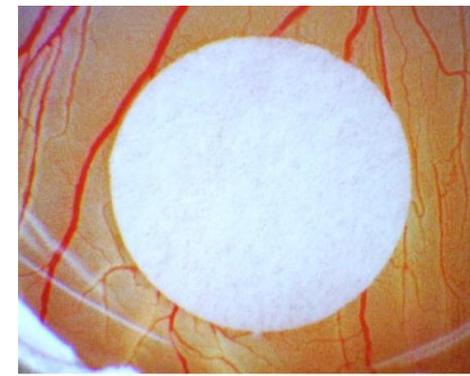
	0 hours	24 hours	48 hours
WEP 300µg/ml			
WEP 600µg/ml			
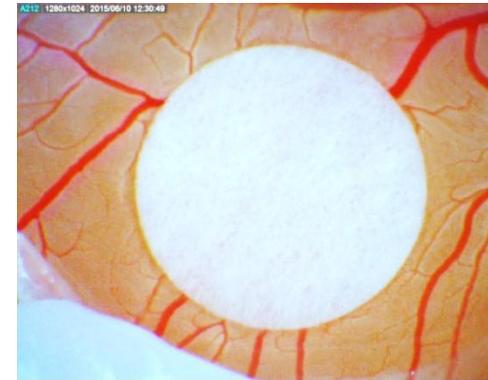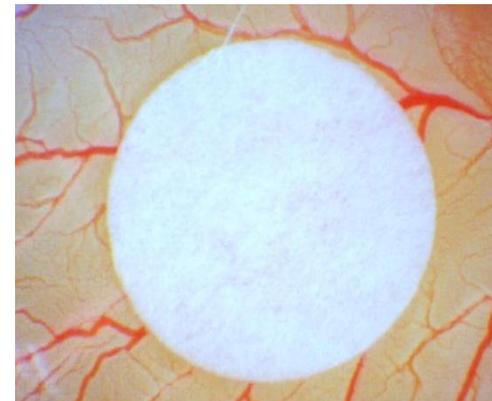
	0 hours	24 hours	48 hours
WEP 900µg/ml			
WEP 1500µg/ml			
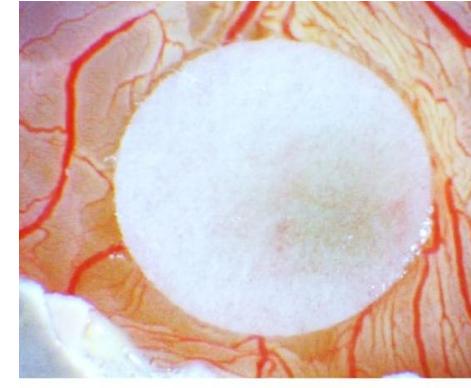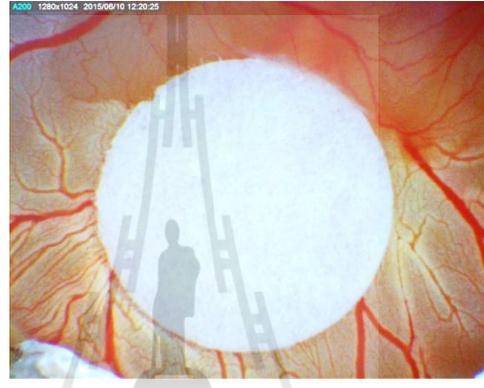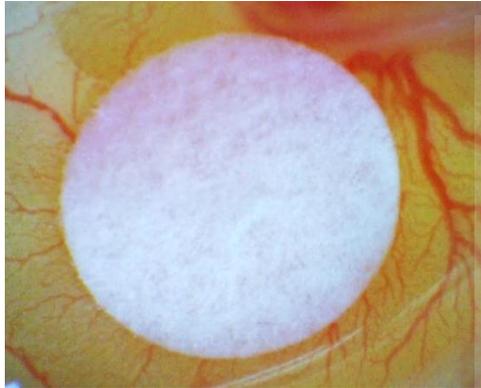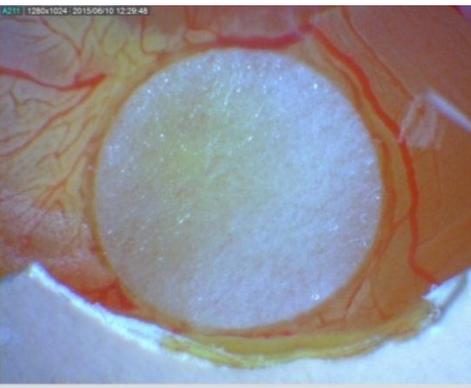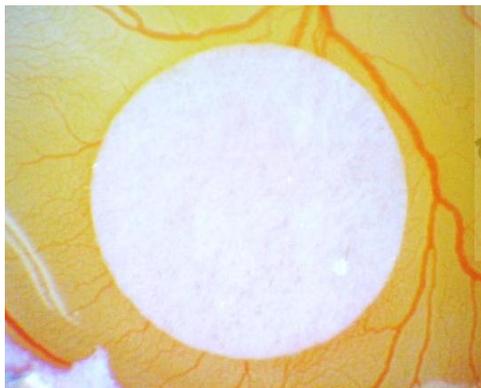
- **The anti-angiogenesis of WEP on chick chorioallantoic membrane (CAM)**

	0 hours	24 hours	48 hours

Uninduced control
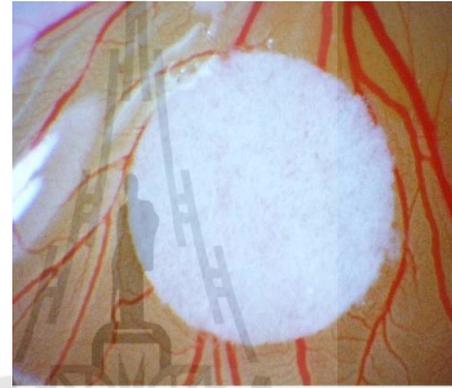
B16F10 (3×10^6 cells)
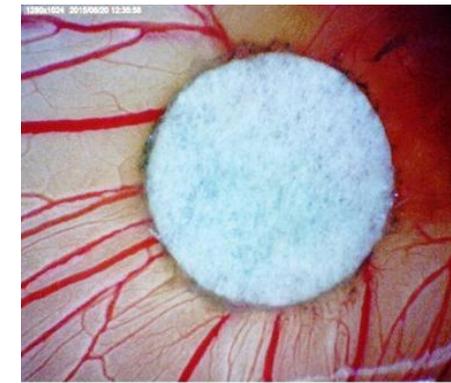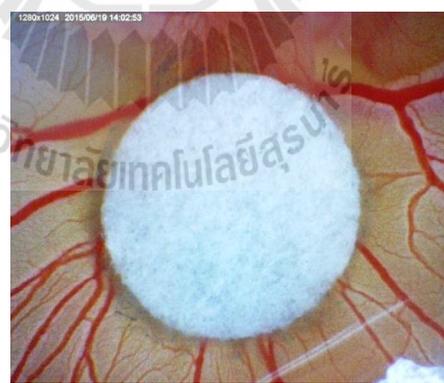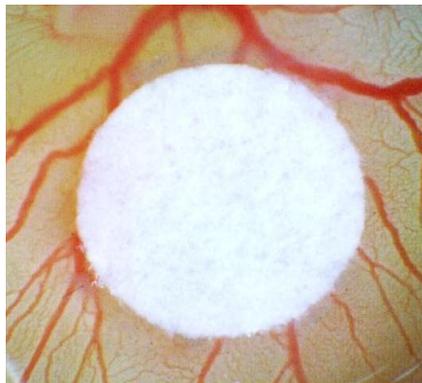
	0 hours	24 hours	48 hours

WEP 30μg/ml + B16F10

WEP 100μg/ml + B16F10
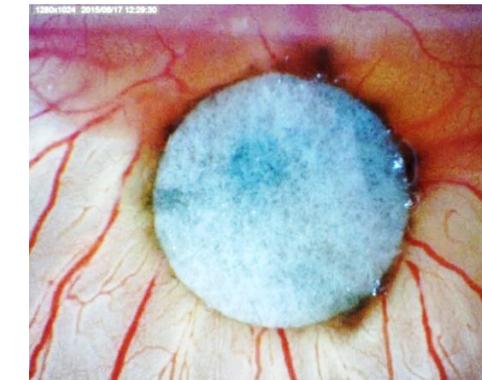
	0 hours	24 hours	48 hours

WEP 300μg/ml
+ B16F10
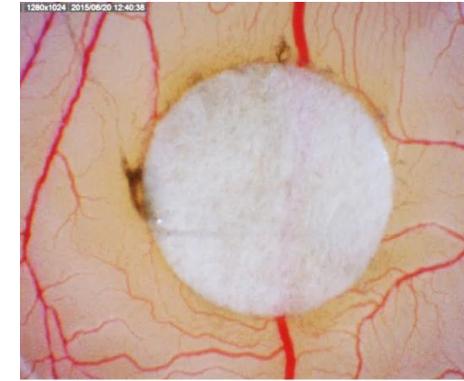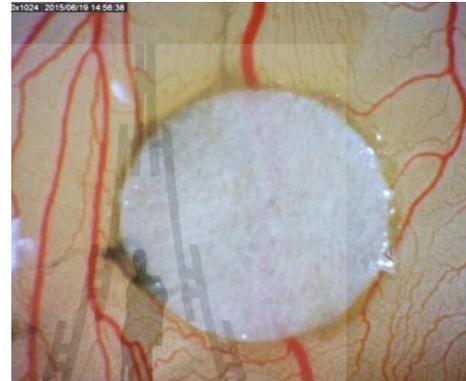
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