In vitro Antioxidant and Anti-angiogenic Effects of Mushroom Water Extracts

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Abstract

Tumor angiogenesis refers to the characteristic feature of persistent endothelial cells growth and blood vessels formation. It is critical for tumor cells to obtain nutrients, oxygen and growth factors. It has been reported that tumor cells generate more reactive oxygen species (ROS) than their normal counterparts. Treatment of tumor cells and endothelial cells with ROS resulted in an increase in the production of vascular endothelial growth factor (VEGF) which is a potent angiogenic promoter and the enhancement of the functions of endothelial cells, respectively. Both of these situations result in tumor angiogenesis. It has been reported recently that antioxidants from plant foods could suppress VEGF-induced tumor angiogenesis. Antioxidants in mushrooms have also been extensively studied for their biological functions. Based on the above findings, antioxidants in mushrooms could be a potential inhibitor of tumor angiogenesis and hence the anti-angiogenic effect of mushroom water extracts was investigated in this study.

This study was divided into two parts. The first one was a screening of the antioxidant activity and the phenolic content of four locally edible mushrooms. They were: *Agrocybe aegerita* (*Aa*), *Pleurotus ostreatus* (*Po*), *Pleurotus eryngii* (*Pe*) and *Pholiota nameko* (*Pn*). The scavenging activities against DPPH, superoxide anion radical, hydroxyl radical and hydrogen peroxide of the water extracts from these mushrooms were evaluated. While a relatively higher overall antioxidant activity was found in the *Aa* water extract, the highest total phenolic content ($p < 0.05$) measured by the Folin-Ciocalteu method was also found in the *Aa* water extract. A total of five phenolic acids were identified in the *Aa* water extract by the Fourier transform-ion cyclotron resonance mass spectrometry.

Since a strong *in vitro* antioxidant activity was found in the *Aa* water extract, its anti-angiogenic properties was studied in part two. Significant ($p < 0.05$) *in vitro* anti-angiogneic effects of the *Aa* water extract at a concentration of 12.5 μg/ml were

observed in terms of reduction in intracellular ROS level and VEGF production in Caco-2 cells as well as suppression in VEGF-induced proliferation, migration and tubule formation in HUVECs. A treatment of the *Aa* water extract at a concentration of 3.125 μg/ml significantly inhibited ($p < 0.05$) the outgrowth of microvessels in VEGF-induced rat aortic ring. The anti-anigiogenic properties of phenolic acids were also studied. Similar to that of the *Aa* water extract, a treatment of 5 mM of gallic acid, caffeic acid or protocatechuic acid also significantly reduced ($p < 0.05$) the VEGF-induced proliferation, migration and tubule formation in HUVECs, as well as completely inhibited the outgrowth of microvessels in VEGF-induced rat aortic ring. These results suggested that the suppression of the functions in the VEGF-induced HUVECs and the microvessels outgrowth in rat aortic ring by the *Aa* water extract might be related to their antioxidant activities and be partly explained by the presence of phenolic acids.

摘要

持久性的內皮細胞生長和血管形成是腫瘤血管生成的表徵。腫瘤血管生成對腫瘤細胞養分，氧氣和生長因子獲得過程中起了很重要的作用。在眾多的血管生成因子中，血管內皮生長因子 (VEGF) 是其中很重要的一類。有研究顯示腫瘤細胞所產生的活性氧 (ROS) 比正常細胞為多，而 ROS 能增加腫瘤細胞 VEGF 的產生及內皮細胞的功能。這兩種情況均能導致腫瘤血管生成。據報導，抗氧化劑可以抑制因血管內皮生長因子誘導而引起的腫瘤血管生成。食用菌的抗氧化能力近來亦被廣泛研究。基於以上的認識，食用菌內的抗氧化劑很可能抑制腫瘤血管生成。因此，本研究旨在了解食用菌水相提取液在對抗血管生成方面的作用。

本研究分為兩部份。首先是篩選四種常見食用菌 (包括茶薪菇、平菇、杏鮑菇及滑子菇)，通過採用清除 DPPH 自由基、超氧陰離子自由基、羥自由基和過氧化氫的測定法，對其水相提取液的抗氧化活性進行評估。研究顯示，茶薪菇的水相提取液擁有相對較高的整體抗氧化活性。斐林酚類測定方法顯示茶薪菇的水相提取液含有最高總酚含量 ($p < 0.05$)，而傅葉變換離子回旋共振質譜儀亦肯定了茶薪菇的水相提取液內有五個酚酸存在。

基於第一部份結果顯示茶薪菇水相提取液有較好的抗氧化能力，它因而被挑選用在第二部份抗體外腫瘤血管生成的研究。在 12.5 微克/毫升的濃度下，茶薪菇的水相提取液能明顯地 ($p < 0.05$) 降低大腸腺癌細胞 (Caco-2) 內的 ROS 水平和 VEGF 的生產以及抑制 VEGF 誘導的人臍靜脈內皮細胞的增生、遷移和管狀結構的形成，而在 3.125 微克/毫升的濃度下，茶薪菇的水相提取液亦能顯

著地 ($p < 0.05$) 降低 VEGF 誘導的大鼠動脈環外微血管生成。此外，本研究亦對酚酸類物質之抗血管生成進行了實驗，發現在 5 毫摩爾/升濃度下，沒食子酸、咖啡酸及原兒茶酸也能明顯地 ($p < 0.05$) 降低 VEGF 誘導的人臍靜脈內皮細胞的增生、遷移和管狀結構的形成，以及完全抑制 VEGF 誘導的大鼠動脈環外微血管生長。這些結果表明，茶薪菇水相提取液的抗氧代活性可能能抑制體外腫瘤血管的生成而茶薪菇水相提取液中存在的酚酸可部份解釋其所具有的抗血管生成能力。

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List of abbreviations

Aa	*Agrocybe aegerita*
ANOVA	One-way analysis of variance
AOS	Active oxygen species
AP-1	Activator protein 1
ARP	Antiradical power
ATCC	American Type Culture Collection
bFGF	Basic fibroblast growth factor
CAM	Chorioallantoic membrane
DCF	2',7'-dichlorofluorescein
DCFH	2'7'-dichlorofluorescin
DCFH-DA	Dichlorofluorescin diacetate
DMEM/F12	Dulbecco's modified Eagle's medium/Ham's Nutrient Mixture F12
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPPH	2,2-diphenyl-1-picrylhydrazyl
EC	Epicatechin
EC_{50}	50% effective concentration
ECG	Epicatechin gallate
ECGC	Epigallocatechin gallate
ECGS	Endothelial cell growth supplement
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGC	Epigallocatechin
ELISA	Enzyme-linked immunosorbent assay
eNOS	Endothelial nitric oxide synthase
ERK	Extracellular signal-regulated kinase
ERW	Electrolyzed reduced water
FBS	Fetal bovine serum
FC	Folin-Ciocalteu
FGF	Fibroblast growth factor
FGFRs	Fibroblast growth factor receptors
FOX	Ferrous ion oxidation-xylenol orange
FT-ICR MS	Fourier transform ion cyclotron resonance mass spectrometry
GAE	Gallic acid equivalent
HCEC	Human cerebral endothelial cells

HESFM	Human endothelial serum free medium
HIMEC	Microvascular endothelial cells
HOCl	Hypochlorous acid
HOO·	Hydroperoxyl radical
HPLC	High performance liquid chromatography
HR	Hydroxyl radical
HUVEC	Human umbilical vein endothelial cells
IL-8	Interleukin-8
IP-10	Interferon inducible protein
LAL	*Limulus* amebocyte lysate
LC-MS	Liquid chromatography-mass spectrometry
L-NAME	N-nitro-L-arginine methyl ester
LOS	Lipooligosaccharide
LPS	Lipopolysaccharide
MDA	Malondialdehyde
MEM	Minimum Essential Medium
MTT	Colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
NADH	-nicotinamide adenine dinucleotide
NBT	Nitroblue tetrazolium
NF- κB	Nuclear factor kappa B
PBS	Phosphate buffer saline
PCTE	Polycarbonate
PD-ECGF	Platelet-derived endothelial cell growth factor
PDGF-BB	Platelet-derived growth factor-BB
Pe	*Pleurotus eryngii*
PIGF	Placenta growth factor
PMS	Phenazine methosulfate
Pn	*Pholiota nameko*
Po	*Pleurotus ostratus*
PTN	Pleiotrophin
ROI	Reactive oxygen intermediates
ROS	Reactive oxygen species
SAR	Superoxide anion radical
SOD	Superoxide dismutase
SPSS	Statistical Package for Social Sciences
TBA	Thiobarbituric acid
TGF-α/ β	Transforming growth factor-alpha/ beta
TIMPs	Metalloproteinase

TIMP-1	Tissue inhibitor of metalloproteinase-1
TIMP-2	Tissue inhibitor of metalloproteinase-2
TIMP-3	Tissue inhibitor of metalloproteinase-3
TMB	3,3',5,5' tetramethyl benzidine
TNF-α	Tumor necrosis factor-alpha
Tris-HCl	Tris(hydroxymethyl)aminomethane hydrochloride
u-PA	urokinase plasminogen activaton
RA	Rosmarinic acid
RPMI 1640	Roswell park memorial institute-1640
VEGF	Vascular endothelial growth factor
VEGFRs	Tyrosine kinase receptors

Chapter 1: Introduction

1.1 Food market trends in Hong Kong and world's mushroom production

In Hong Kong, there is a trend for healthy eating in recent years. This leads to an increasing demand on healthier, fresher as well as more nutritious foods. Customers are now seeking naturally occurring foods with low calories, low fat, low sugar but with high fiber (USDA, 2006a; USDA, 2006b; USDA, 2006c). On the other hand, the world production on cultivated edible mushrooms had increased sharply between 1975 and 1997 (Chang and Miles, 2004). Starting from 1975, the number of ten most popular cultivated mushroom species gradually increased from 0.90 million tons to 1.26 million tons in 1981. This number was kept on increasing to 2.18 million tons and 3.76 million tons in 1986 and 1990, respectively. After 1990, this number started to increase rapidly to 4.91 million tons in 1994 and finally to 6.16 million tons in 1997 (Chang and Miles, 2004).

Due to this dramatic increase in the production as well as consumption of mushrooms, active research on the nutritional values of various edible mushrooms becomes very popular. Generally, mushrooms provide a considerable amount of fiber, protein, vitamins and minerals. Dried mushrooms usually contain ~ 63% carbohydrate including dietary fiber, ~22% protein including most of the essential amino acids, ~5% fat with linoelic acid being the most abundant one and ~10% minerals in the form of ash. They are considered as good sources for several vitamins like thiamin, riboflavin, niacin and biotin (Mattila and others, 2000).

1.1.1 *Agrocybe aegerita*

The classification of *Agrocybe aegerita* is shown below. Kingdom: Fungi; Class: Agaricomycetes; Order: Agaricales; Family: Strophariaceae and Genus: *Agrocybe*. The common name of *Agrocybe aegerita* is black poplar mushroom. It belongs to white rot fungi and is medium-sized agaric having an open and covex cap. *A.*

aegerita is usually cultivated in regions of warm or mild climates and therefore it can be found in North America, Europe and Asia (Ullrich and others, 2004). It grows on tufts on logs and holes in the poplars and other trees of large leaves. Because of its rich and woody flavor, it goes well in soups or stir-fried.

It has been reported that *A. aegerita* possesses several important bioactivities like antioxidant (Lo and Cheung, 2005), anticancer (Xu and others, 2005; Yang and others, 2009) and antifungal activities (Zhong and Xiao, 2009). It was found that different extracts (such as methanol extract) of *A. aegerita* demonstrated a strong *in vitro* antioxidant activity (Lo and Cheung, 2005). Besides, water extract from *A. aegerita* gave a remarkable protective effect against hydrogen peroxide-induced oxidative damage (Ho, 2007). Treating several human cell lines (such as SW480 and HL60) with lectin isolated from *A. aegerita* resulted in a potent tumor suppression (Yang and others, 2009) while feeding tumor-bearing mouse with *A. aegerita* inhibited the viability of tumor cells *in vivo* (Xu and others, 2005). It was also reported that a secondary metabolite of *A. aegerita*, agrocybenine, possessed antifungal activity (Zhong and Xiao, 2009).

1.1.2 *Pleurotus spp*

Pleurotus ostreatus, and *Pleurotus eryngii* both have the following classification. Kingdom: Fungi; Class: Basidiomycetes; Order: Agaricales; Family: Tricholomataceae and Genus: *Pleurotus*. The common name of *Pleurotus* mushroom is oyster mushroom due to their shell-like, spatulate cap and lateral, eccentric stipe. *Pleurotus* mushrooms naturally grow in subtropical areas on rotting trees like oak and have a fragrant odor and delicious flavor (Chang and Miles, 2004). The fruiting body of *P. eryngii* is similar to that of *P. ostreatus* but more pleasant aroma and better culinary qualities are found in *P. eryngii* (Zadrazil, 1978). The pleasant aroma in *P. eryngii* is mainly contributed by volatiles and taste components (Mau and others, 1998).

Pleurotus ostreatus was first cultivated in 1900 (Chang and Miles, 2004). In 1997, *Pleurotus* species ranked the third in the most popular cultivated edible mushrooms (Huang, 1997). From 1986 to 1991, the production of *Pleurotus spp.* increased by 442% from 169 x 10^3 tons to 917 x 10^3 tons (Chang, 1993).

Similar to *Agrocybe aegerita*, *Pleurotus spp.* demonstrated antioxidant (Jayakumar and others, 2009) as well as antitumor effects (Lavi and others, 2006). Antiviral (Noda-shokukin, 1998), antibacterial (Karacsonyi and Kuniak, 1994), and immunomodulation activities (Paulik and others, 1996) have also been reported in *Pleurotus*. The ethanolic extract of *P. ostreatus* demonstrated scavenging effects towards hydroxyl and superoxide radicals (Jayakumar and others, 2009) as well as anti-proliferative and pro-apoptotic activities on colon cancer cells *in vitro* (Lavi and others, 2006). The presence of ubiquitin-like protein in *P. ostreatus* contributed to its antiviral effect (Wang and Ng, 2000) while the antibacterial and immunomodulatory effects in *P. ostreatus* were found to be related to the presence of β-D-glucan (Karacsonyi and Kuniak, 1994; Paulik and others, 1996).

1.1.3 *Pholiota nameko*

The classification of *Pholiota nameko* is shown below. Kingdom: Fungi; Class: Basidiomycetes; Order: Agaricales; Family: Strophariaceae and Genus: *Pholiota*. Its common name in English is nameko mushroom. It is a viscid mushroom and has sticky cap with glutinous substance. Besides sticky, its cap is smooth and shiny. The center of cap is yellowish brown and becomes lighter towards the margin. *Pholiota* mushroom is usually cultivated in regions of temperate climate (Lou and others, 1983) because low temperature is required for fruiting to occur. It grows on trucks, logs and sawdust of coniferous trees. It can also be cultivated on broad leaf trees sawdust medium from beech and oak. Because of its good flavor and viscous nature, it is widely consumed in Japan and used to make miso soup (Chang and Miles, 2004).

Pholiota nameko was first cultivated in 1958 (Chang and Miles, 2004). In 1997, it ranked the ninth in the total worldwide production of edible mushrooms (Chang, 1999). From 1986 to 1991, the production of *P. nameko* was increased by 60% from 25 x 10^3 tons to 40 x 10^3 tons (Chang, 1993).

Studies on *Pholiota nameko* include its antioxidant activity (Li and others, 2008) and hypolipidemic effect (Li and others, 2010). It was reported that *P. nameko* polysaccharides could effectively scavenge various reactive oxygen species (ROS) including superoxide anion, hydroxyl radical, lipid-derived radicals and singlet oxygen (Li and others, 2008). Treatment of hyperlipidemic Wistar rats with *P. nameko* polysaccharides decreased serum low-density lipoproteins, triacylglycerol, and phospholipids but increased serum high-density lipoprotein cholesterol as well as lowered hepatic total lipids, total cholesterol, triacylglycerol and phospholipids (Li and others, 2010). Although the number of studies on *P. nameko* were smaller than those of *A. aegerita* and *Pleurotus spp.*, the findings on its antioxidative and hypolipidemic effects had strongly suggested the beneficial health effects by consuming *Pholiota nameko*.

1.2 Objectives

Due to the unique flavor and texture of mushrooms, they have been long used as common ingredients in many dishes. In the past ten years, there was a rapid increase in mushroom production, both in quantity and quality. Numerous studies have already demonstrated the potent bioactivities of edible mushrooms, especially their antioxidant and antitumor effects. In the present study, four edible mushrooms were chosen, namely *Agrocybe aegerita*, *Pleurotus ostreatus*, *Pleurotus eryngii* and *Pholiota nameko*, for investigating their antioxidant (Chapter 2) and anti-angiogenic (Chapter 3) effects due to their popularity in the local market.

The objectives of this project are:

1. To compare the antioxidant activities of these four mushroom water extracts towards DPPH radical, superoxide anion radical, hydroxyl radical and hydrogen peroxide scavenging activities and to screen out the most potent mushroom water extract in radical scavenging activity.
2. To measure the total phenolic content of these mushroom water extracts using the Folin-Ciocalteu method and to characterize the phenolic acids present in them using chromatographic technique.
3. To evaluate the effect of a selected mushroom water extract on tumor angiogenesis by studying its *in vitro* effects towards intracellular reactive oxygen species (ROS) levels and vascular endothelial growth factor (VEGF) production in a colon cancer cell line (Caco-2).
4. To evaluate the effect of the selected mushroom water extract and some phenolic acids on tumor angiogenesis by studying its *in vitro* effects against the functions of endothelial cells (HUVECs) including proliferation, migration and tubule formation.
5. To determine whether the phenolic acids present in the selected mushroom water extract are related with the anti-angiogenic effects observed.

Chapter 2: Chemical assays for *in vitro* antioxidative properties of mushroom extracts

2.1 Introduction

2.1.1 Reactive oxygen species (ROS)

2.1.1.1 Definition of ROS

Reactive oxygen species (ROS) are also termed as active oxygen species (AOS) or reactive oxygen intermediates (ROI). They refer to a group of reactive, short-lived, oxygen containing species that are energetically more reactive than molecular oxygen (Hancock and others, 2001). ROS include both oxygen radicals like superoxide anion radical ($O_2^{\cdot -}$), hydroxyl radical ($OH^{\cdot}$), peroxyl radical ($ROO^{\cdot}$) and hydroperoxyl radical ($HOO^{\cdot}$) as well as easily radical-converting non-radical oxygen agents like hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl) and ozone (O_3) (Bayr, 2005).

2.1.1.2 Sources of ROS

2.1.1.2.1 Endogenous source of ROS

ROS can be produced in our bodies by both endogenous and exogenous sources (Loschen and Flohe, 1971). Among all biochemical reactions that might generate ROS, mitochondrial oxidative phosphorylation, which is a vital and normal mechanism in generating ATP, the energy source, in our bodies, is the most well-known reaction involved in the production of ROS (Chance and others, 1979).

There are two major pathways to reduce oxygen to water biologically, the first and the most common one is via the electron transport chain and the second one is via the one-electron oxidation-reduction pathway. In the electron transport chain of oxidative phosphorylation, four electrons are required to reduce oxygen to water. This pathway accounts more than 95% in converting oxygen to water (Thannickal and Fanburg, 2000). However, sometimes oxygen may escape the normal electron transfer process and enter the one-electron oxidation-reduction pathway before being

converted to water accidentally with the formation of various ROS with different toxicities (Scandalios, 2005). This conversion of oxygen into water is in a stepwise manner as shown in Table 2.1. Oxygen is first incompletely reduced to superoxide anion radical ($O_2\cdot^-$) by adding a single electron to ground state oxygen (O_2). Under physiological pH, this superoxide anion radical will exist in the form of hydrogen peroxide (H_2O_2) by taking up an additional electron while around 1% of $O_2\cdot^-$ is protonated to the more reactive peroxyl radical (HO_2^-) (Borg, 1993). Finally, the H_2O_2 produced is converted to hydroxyl radicals ($OH\cdot$) through the Haber-Weiss/ Fenton reaction in the presence of a transition metal catalyst especially iron in particular. Water (H_2O) is finally produced (Cadenas and Davies, 2000). This latter pathway contributes 1-2% in the biological reduction of oxygen to water (Thannickal and Fanburg, 2000).

Table 2.1 Generation of ROS in the one-electron oxidation-reduction pathway

1	Production of superoxide anion $O_2 + e^- \rightarrow O_2\cdot^-$
2	Production of peroxyl radical $3O_2\cdot^- + 2H_2O + e^- \rightarrow 4HO_2^-$
3	Production of hydrogen peroxide $HO_2^- + e^- + H \rightarrow H_2O_2$
4	Production of hydroxyl radical $H_2O_2 + e^- \rightarrow OH^- + OH\cdot$ $Mn^+ + H_2O_2 \rightarrow Mn^{+1} + OH\cdot + OH^-$

Besides, endogenous ROS can also be produced in the cytochrome P450 metabolism, peroxisomes, inflammatory cell activation like neutrophils, eosinophils and macrophages activations (Table 2.2). ROS production in cytochrome P450 "futile cycling" is produced following the breakdown or uncoupling of the P450 catalytic cycle (Parke and Sapota, 1996). For example, ROS are released during the oxygenation of ethanol by the enzyme P4502E1 (Eksrom and Ingelman-Sundberg, 1989) while superoxide anion is produced during the metabolism of phenobarbital by the enzyme P4502B (Rice and others, 1994). Peroxisomes is an enzyme-containing organelle. The presence of enzymes help removing hydrogen atoms to produce H_2O_2

in an oxidative manner while the H_2O_2 produced is further reduced to water by catalase or to other substrates by peroxidation reaction (Tsaftaris and others, 1983). The proliferation of peroxiosome increase peroxisomal enzyme activities, resulting in the escape of H_2O_2 and hence the oxidative stress within a cell (Wade and others, 1992). Generation of endogenous ROS is one of the defense mechanism adopted by our immune system. For instance, activated macrophage increase O_2 uptake via respiratory burst in order to produce more $O_2\cdot^-$ and H_2O_2, which in turns, kill foreign organisms (Babior and Woodman, 1990).

2.1.1.2.2 Exogenous source of ROS

Besides endogenous production, ROS can also be produced exogenously (Table 2.2). Exposure to various xenobiotics, nongenotoxic carcinogens, chlorinated compounds directly or indirectly generate ROS in cells and hence, induce oxidative stress and damage (Rice-Evans and Burden, 1993). Besides, cigarette smoking and radiation are also shown to increase oxidative states (Klaunig and others, 1997).

Table 2.2 Sources of endogenous and exogenous ROS

	Sources	Oxidative species
Endogenous ROS	Mitochondria	$O_2\cdot^-$, H_2O_2 , $OH\cdot$
	Cytochrome P450	$O_2\cdot^-$, H_2O_2
	Peroxisomes	H_2O_2
	Inflammatory cells	$O_2\cdot^-$, H_2O_2
Exogenous ROS	Xenobiotics	$O_2\cdot^-$
	Radiation	$OH\cdot$
	Smoking	$OH\cdot$, H_2O_2

Adapted from Klaunig and Kamendulis, 2004

2.1.1.3 Damaging effects of ROS

Being a highly reactive species, ROS can attack macromolecules like proteins, lipids as well as DNA, leading to protein modification, lipid peroxidation and DNA damage, respectively as shown in Table 2.3 (Freeman and Crapo, 1982).

Oxidative attack on proteins can result in an irreversible modification of polypeptide chain, including site-specific amino acid modifications, fragmentation of peptide chain, aggregation of cross-linked reaction products, altered electrical charge and increased susceptibility to proteolysis. Protein functions would be compromised with any changes in the corresponding protein structure (Cecarini and others, 2007). Since many receptors, enzymes and transport proteins in our body are protein-based, cell structure, cellular signaling as well as various metabolisms would be affected by protein oxidation (Cecarini and others, 2007).

Oxidative degradation of lipids is sometimes noted as lipid peroxidation. Due to the presence of unsaturated fatty acids in the membrane phospholipid bilayer, cellular membrane is a common target for lipid peroxidation. Lipid peroxidation of cellular membrane would lead to changes in the membrane permeability as well as activities of membrane receptors. It would also increase the membrane rigidity and decrease the activity of membrane-bound enzymes such as sodium pumps. These changes finally affect cellular functions, leading to membrane leakage or even cell death (Halliwell and Chirico, 1993).

Attack of ROS to nucleic acids induces numerous lesions, including DNA strand breaks, abasic sites, modified DNA bases and DNA-protein cross-links (Dizdaroglu, 1991). As genetic materials are encoded in DNA, any damage in nucleic acid has to be identified and repaired by DNA repair proteins. Failure in DNA repairing results in genetic mutation and diseases like cancer.

Table 2.3 Examples of ROS damages to proteins, lipids and nucleic acids

Macromolecules	Damaging effects	References
Proteins	Site-specific amino acid modifications Fragmentation of peptide chain Aggregation of cross-linked reaction products Altered electrical charge Increase susceptibility to proteolysis	Cecarini and others, 2007
Lipids	Changes in membrane permeability Changes in activities of membrane receptors Increase membrane rigidity Decrease activities of membrane-bound enzymes	Halliwell and Chirico, 1993
Nucleic acids	DNA strand break Loss of DNA base site Modification of DNA bases DNA-protein cross-links	Dizidaroglu, 1991

2.1.2 Antioxidants

As its name implies, ROS are highly reactive and therefore, they are usually toxic as well. Antioxidants refer to any substances, which present in a low concentration in comparison with oxidized substances, delay or inhibit the oxidation of these substances significantly (Halliwell and Gutteridge, 1995). Oxidation may result in the production of free radicals, which are generally unstable and highly reactive. As mentioned in section 2.1.1.3, these free radicals are capable to start chain reaction and attack lipids, proteins, sugars and DNAs, resulting in diseases, cancers and aging through membrane damages, protein modifications, enzyme deactivations and DNA damages.

Antioxidants should effectively inhibit the generation of free radicals or quench free radicals once they are generated. It should also be capable to chelate redox metal ions and inhibit other oxidation reactions by acting as a reducing agent.

2.1.2.1 Mechanism of action

Antioxidants can be classified into two groups, primary antioxidants and secondary antioxidants (Ingold, 1968).

Primary antioxidants, also known as chain-breaking antioxidants, can react with lipid radicals and convert them into more stable products. Any molecule that is able to donate a hydrogen atom to a lipid radical readily and cause the formation of stable products or an antioxidant radical that is more stable than that lipid radical can be regarded as primary antioxidants. Phenols, for example, belong to this group of antioxidants. After the reaction of the lipid radical and phenol, a stable phenolic radical is formed because of the delocalization of the unpaired electron around the aromatic benzene ring as shown in Figure 2.1 (Gordon, 1990).

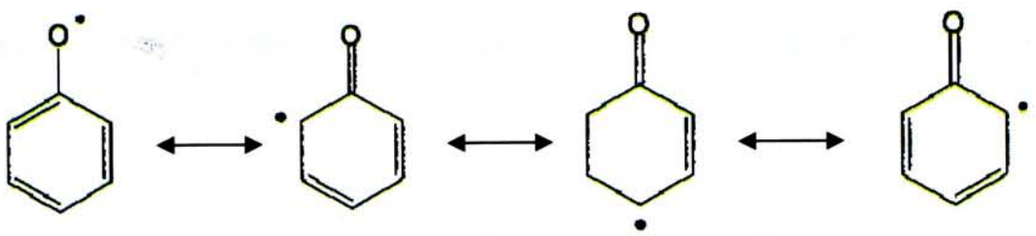


Figure 2.1 Delocalization of phenolic radical

Secondary antioxidants, also known as preventive antioxidants, can retard the rate of chain initiation through different mechanisms like binding with metal ions, scavenging oxygen, degrading hydroperoxides to non-radical species, absorbing UV radiation or deactivating singlet oxygen. Ethylenediaminetetraacetic acid (EDTA), for example, is a good metal-chelating type of antioxidant which forms thermodynamically stable complexes with all transition metal ions (Gordon, 1990).

2.1.2.2 Sources of antioxidants

2.1.2.2.1 Dietary antioxidants

A wide variety of antioxidants can be found in foods and foodstuffs. Vitamins and vitamin precursors like β-carotene, vitamin C, vitamin E; plant polyphenolic compounds like tannic acid are common dietary antioxidants.

β-carotene is the precursor of vitamin A and it belongs to the group of carotenoid, which contain more than 500 species including hydrocarbon carotenoids, like β-carotene, and oxygenated carotenoids, like lutein. After β-carotene reacts with the

free radical, a stable structure is formed by the delocalization of unpaired electron within its conjugated double-bond (El-Agamey and others, 2004).

Vitamin C is a water soluble radical-scavenging antioxidant and helps to remove free radicals in aqueous medium (Niki and others, 1995). Ascorbic acid is one form of vitamin C. Due to its electron-rich property, it can donate its electrons in the form of hydrogen to free radicals and form a resonance stabilized tricarbonyl ascorbate free radical (Schuler, 1990). In our bodies, vitamin C can also work with glutathione peroxidase to revitalize vitamin E. Therefore, besides acting as radical-scavenging antioxidant in aqueous solutions, it also helps antioxidants in lipids.

Vitamin E, on the other hand, is a fat soluble, lipophilic radical scavenging antioxidant. There are eight forms of vitamin E and α-tocopherol is one of them. α-tocopherol donates its phenolic hydrogen to free radicals, especially peroxyl radicals, to prevent chain propagation from occurring with the formation of a α-tocopheroxyl radical, which can react with vitamin C to regenerate α-tocopherol (Niki, 1991).

Polyphenolic compounds refer to those chemical substances having more than one phenol unit or building block in each molecule. They can be classified into different subclasses like hydrolysable tannins, condensed tannins, flavonoids, coumarins, lignans, lignins, quinoids and phenolic compounds of small molecule according to their structures (Okuda, 1997). These polyphenolic compounds may act through terminating chain reactions and chelating redox-active metal ions.

2.1.2.2.2 Antioxidants in edible mushrooms

Antioxidants present in mushrooms may include phenolic compounds, sterols and polysaccharides. Edible mushrooms are generally high in their total phenolic content (Vinson and others, 1998). Phenolic compounds like *trans*-cinnamic acid, *p*-hydroxy-benzoic acid, protocatechuic acid, caffeic acid were found in *Agaricus*

bisporus and *Lentinus edodes* (Mattila and others, 2001); asiaticusin A and B were found in *Boletinus asiaticus* (Wada and others, 1996); and flavoglaucin was found in *Eurotium chevalieri* (Ishikawa and others, 1984). Sterols like ergesterol were found in *Agrocybe bisporus, Pleurotus ostreatus and Lentinus edodes* (Mattila and others, 2002). Polysaccharides in some mushrooms also show superoxide radical scavenging activities (Liu and others, 1997).

2.1.2.2.3 Phenolic compounds in mushrooms

Polyphenol antioxidants contain a common polyphenolic structure that can be classified into several types including phenolic acids, flavonoids, tannins, coumarins, lignans, quinones, stilbenes and curcuminoids. Although they are present in traditional Chinese medicine herbs, not all of them are commonly found in common edible mushrooms (Cai and others, 2004). For example, no flavonoids could be detected in 16 different Portuguese wild mushroom species (Barros and others, 2009). It has been suggested that only higher plants possess the biosynthetic pathways to produce flavonoids but not animals and fungi (Iwashina, 2000) and mushrooms contain phenolic acids only (Barros and others, 2009) while some researchers could still find the presence of flavonoids in some common mushrooms like *Agaricus bisporus*. Table 2.4 summarizes the different polyphenols found in mushrooms.

Naturally-occurring phenolic acids can be subdivided into two groups, the hydroxycinnamic acids like *p*-coumaric, caffeic, ferulic, and sinapic acids and hydroxybenzoic acids, like *p*-hydroxybenzoic, protocatechuic, vanillic, syringic, and gallic acids (Table 2.5) (Lodovici and others, 2001). Both hydroxycinnamic acids and hydroxybenzoic acids have the same basic skeleton but are different in the numbers and positions of hydroxyl groups on the aromatic ring (Figure 2.2) (Robbins, 2003). Hydroxycinnamic acids are commonly esterified with small molecules such as tartaric acids and linked to cell wall structural components such as cellulose (Liu, 2004) while hydroxybenzoic acids are commonly found in the bound form and linked to sugar derivatives or organic acids in plant foods (Barros and others, 2009).

Table 2.4 Summary of polyphenols identified in mushroom fruiting bodies

	Agaricus bisporus	*Boletinus asiaticus*	*Eurotium chevalieri*	*Flammulina velutipes*	*Lentinus edodes*	*Lentinus sajor caju*	*Pleurotus ostreatus*
Flavonoid							
Flavoglaucin			√				
Myricetin	√						√
Naringin	√						√
Quercetin				√			
Phenolic acids							
Caffeic acid	√			√	√	√	
Chlorogenic acid				√			√
Trans-cinnamic acid	√				√		
Coumaric acid						√	
Ferulic acid				√			
Gallic acid	√			√	√	√	
Homogentisic acid				√			√
p-hydroxybenzoic acid	√				√		
Protocatechuic acid	√			√	√	√	√
5-sulfosalicyclic acid				√			
Syringic acid						√	
Tannic acid						√	
Vanillic acid						√	
Phenolic Compounds							
Asiaticusin A		√					
Asiaticusin B		√					
Catechin					√		
Pyrogallol	√			√			
References	Mattila and others., 2001; Kim and others, 2008	Wada and others, 1996	Ishikawa and others, 1984	Kim and others, 2008	Mattila and others, 2001; Kim and others, 2008	Puttaraju and others, 2006	Kim and others, 2008

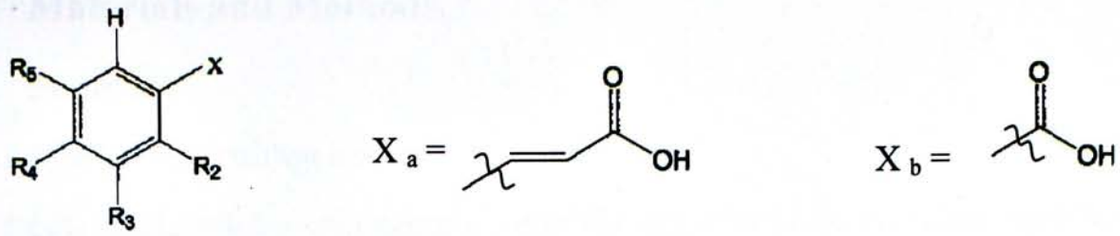


Figure 2.2 Structures of common naturally-occuring phenolic acids (Modified from Robbins, 2003)

Table 2.5 Structures of common naturally-occurring phenolic acids

R_2	R_3	R_4	R_5	Common name	
				hydroxycinnamic acid (a)	hydroxybenzoic acid (b)
H	H	H	H	cinnamic acid	benzoic acid
-OH	H	H	H	*o*-coumaric acid	salicyclic acid
H	-OH	H	H	*m*-coumaric acid	---
H	H	-OH	H	*p*-coumaric acid	*p*-hydrozybenzoic acid
H	-OH	-OH	H	caffeic acid	protocatechuic acid
H	$-OCH_3$	-OH	H	ferulic acid	vanillic acid
H	$-OCH_3$	OH	$-OCH_3$	sinapic acid	syringic acid
-OH	H	H	-OH	---	gentisic acid
-OH	-OH	-OH	-OH	---	gallic acid

2.2 Materials and Methods

2.2.1 Materials

2.2.1.1 Mushroom fruiting bodies

Four fresh, edible mushrooms namely *Agrocybe aegerita* (*Aa*), *Pleurotus ostreatus* (*Po*), *Pleurotus eryngii* (*Pe*) and *Pholiota nameko* (*Pn*) (Figure 2.3) were purchased from local market in Hong Kong.

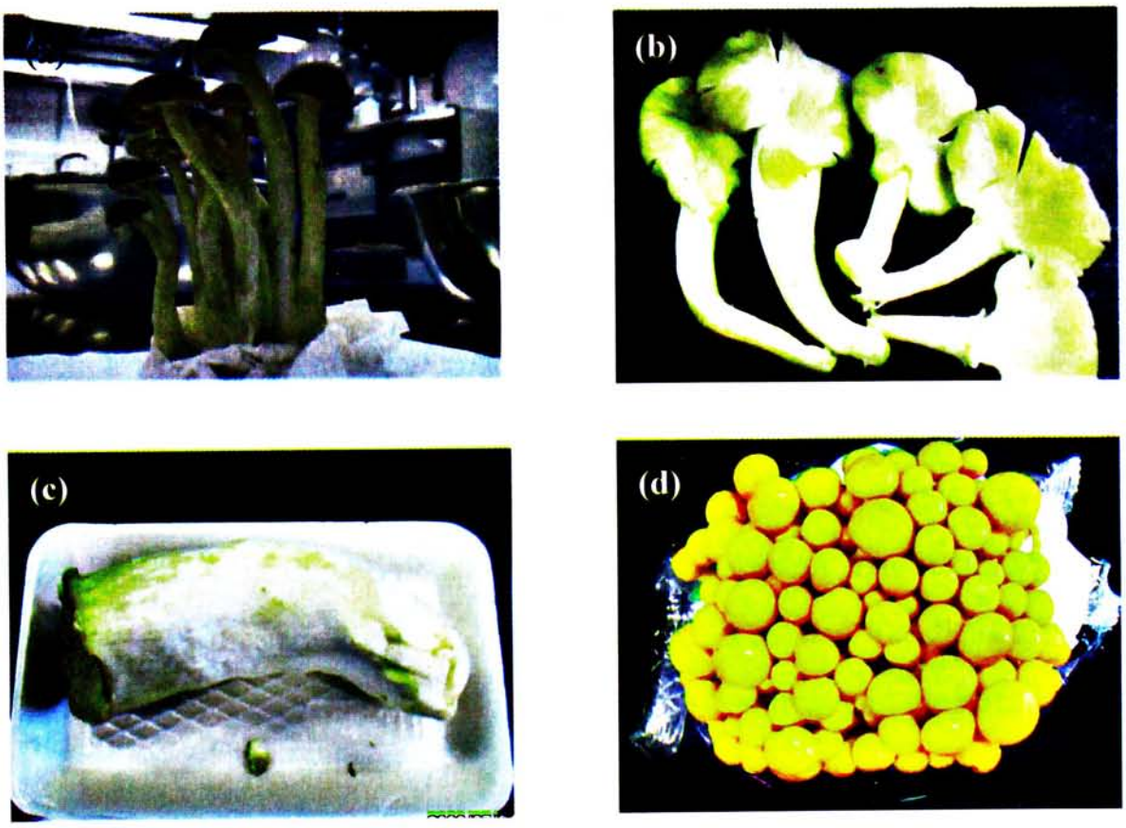


Figure 2.3 Fresh fruiting mushroom bodies (a) *Agrocybe aegerita*; (b) *Pleurotus ostreatus*; (c) *Pleurotus eryngii*; (d) *Pholiota nameko*

2.2.2 Principles of Methods and Experimental Protocols

2.2.2.1 Sample preparation

After purchasing, these mushrooms were first cleaned with brushes to remove any adherent foreign materials and then cut into small pieces. These cut mushroom samples were weighed and put into an ultra-low freezer (MDF-U50V, Sanyo, Japan) at -80°C overnight and lyophilized by a freeze-dryer (Labconco, England). The dried mushrooms were weighed and then milled into powder with a hammer mill (0.5 mm sieve size) (MF 10, IKA-WERKE, Germany).

Dried mushroom powders were then extracted with distilled water at room temperature (~25°C) for 3 h with continuous stirring as shown in Figure 2.4. The extraction ratio was 1 g sample: 20 ml water. After extraction, the water-soluble fraction was separated from the insoluble residue by centrifugation using a high speed centrifuge (J2-M1 centrifuge, Beckman, USA). The centrifuge speed used was 3838 *g* and the centrifuge time was 5 min. The mushroom water extracts were then further filtered through 2 pieces of filter papers (Whatman 41, diameter 110 mm, Whatman, USA) by vacuum filtration. The filtrate was frozen in the ultra-low freezer at -80°C and then lyophilized. These water crude extracts were weighed to obtain the extraction yield, which was expressed as grams of extract obtained per gram of dried matter of mushroom sample. All extracts were stored inside dark airtight plastic bottles and put inside a desiccator at room temperature prior to analysis.

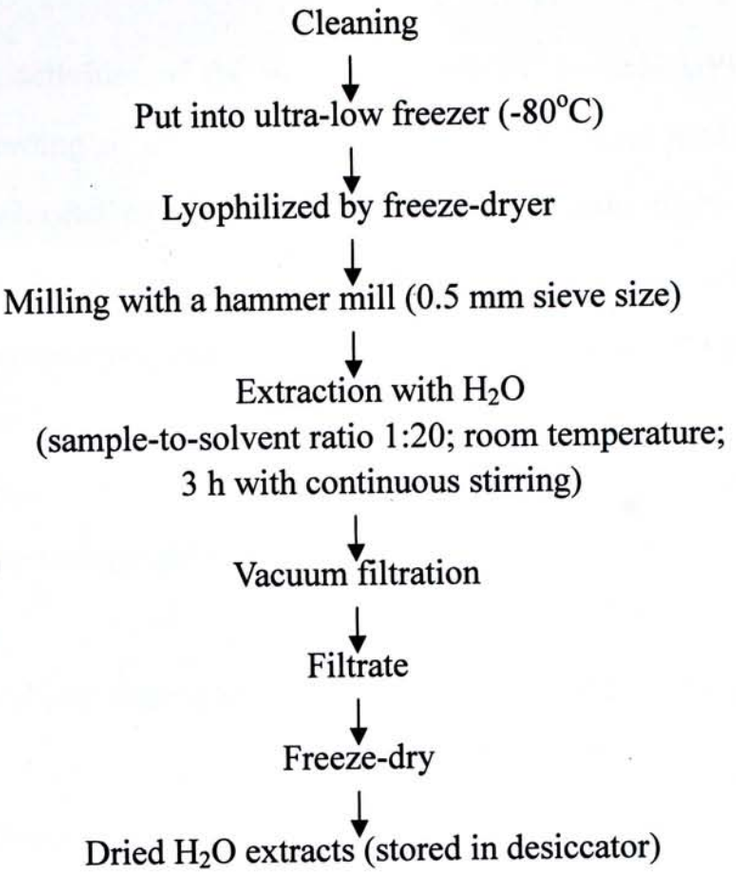


Figure 2.4 Extraction method of mushroom water extracts

2.2.2.2 Evaluation of antioxidant capacity

2.2.2.2.1 DPPH radical scavenging activity

Principle

The measurement of 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging activity is a simple, rapid and sensitive method for the evaluation of antioxidant activity of a compound (Ozcelik and others, 2003). When the odd electron of nitrogen atom in the violet DPPH, which has a maximum absorbance at 517 nm, received a hydrogen atom from the antioxidants, this atom is reduced and hence the violet color fades. Good antioxidants referring to those compounds that are capable to reduce the violet DPPH quickly by donating their hydrogen atoms (Cotelle and others, 1996; Ancerewicz and others, 1998; Koleva and others, 2002). By measuring the decrease of the absorbance at 517 nm in sample and comparing with that in the control, the inhibition percentage, which is proportional to the antioxidant concentrations, could be obtained. The result could be expressed as EC_{50} referring to the concentration of sample that causes a 50% decrease of the activity concerned.

Procedures

The scavenging activities of the mushroom extracts towards DPPH radicals were determined according to Chu and others (2000) with some modifications. Half a milliliter of mushroom extract with various concentrations (0.25 – 4 mg/ml) was added into an eppendorf with 1 ml 0.1 mM DPPH (Sigma, Cat # D9132). The mixture was vortex-mixed and incubated in dark for 30 min. It was then transferred into curvette and the absorbance at 517 nm was measured using a UV-VIS spectrophotometer (Genesys5, Spectronic Instruments, USA). Negative control was prepared by using water instead of mushroom extracts.

The DPPH radical scavenging activity (%) was calculated by the following equation:

$$\text{Scavenging activity (\%)} = [1-(Abs_{517sample}/ Abs_{517control}]x100\%$$

The EC_{50} value (mg/ml) refers to the effective concentration to scavenge the DPPH radicals by 50%.

2.2.2.2.2 Superoxide anion radical scavenging activity

Principle

The superoxide anion radical scavenging activity is based on the PMS-NADH-NBT system (Nishikimi and others, 1972). In this method, superoxide anions are generated by mixing β-nicotinamide adenine dinucleotide (reduced NADH) and phenazine methosulfate (PMS) under aerobic condition. The superoxide anion produced will then reduce nitroblue tetrazolium (NBT) to form a purple colored formazan which has the highest absorbance at 560 nm (Sarkar and others, 2009). Therefore, presence of superoxide anion radical scavenger will lower the superoxide anion availability to react with NBT and hence the chromogen formed. As a result, by measuring the decrease of the absorbance at 560 nm in sample and comparing it with that of the control, the inhibition percentage, which is proportional to the antioxidant concentrations, can be obtained and the EC_{50} can then be found.

Procedures

The scavenging activity of superoxide radical was determined according to the method of Liu and others (1997) with some modifications. Briefly, 1 ml 312 μM NADH (Sigma, Cat # 8129) was added into a test tube followed by the addition of 1 ml 200 μM NBT (Sigma, Cat # N-6876) solution, which is prepared by dissolving NBT in 16 mM Tris-HCl buffer (Sigma, Cat # T3253) (pH 8.0). One milliliter of mushroom extract with various concentrations (0.3125-10 mg/ml) dissolved in the Tris-HCl buffer was then added. Finally, 1 ml 50 μM PMS (Sigma, Cat # P-9625) in buffer was added and the absorbance at 560 nm was immediately measured using a spectrophotometer. Negative control was prepared by using Tris-HCl buffer instead of mushroom extracts.

The superoxide anion radical scavenging activity (%) was calculated by the following equation:

Scavenging activity (%) = $[1-(Abs_{560sample}/ Abs_{560control})]x100\%$

The EC_{50} value (mg/ml) refers to the effective concentration to scavenge the superoxide anion radicals by 50%.

2.2.2.2.3 Hydroxyl radical scavenging activity

Principle

This assay is known as deoxyribose method and was first described in 1987 by Halliwell and others. In this system, hydroxyl radicals are produced by mixing iron (III)-EDTA complex with hydrogen peroxide and ascorbic acid according to the following equations.

Fe^{3+}-EDTA + ascorbic acid → Fe^{2+}-EDTA + oxidized ascorbic acid

Fe^{2+}-EDTA + O_2 → Fe^{3+}-EDTA + O_2^-

$2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$

Fe^{2+}-EDTA + H_2O_2 → OH^- + · OH + Fe^{3+}-EDTA

The hydroxyl radical produced then quickly attacks the deoxyribose (2-deoxy-D-ribose) to form malondialdehyde (MDA) under acidic condition. Upon the addition of thiobarbituric acid (TBA), the MDA formed will form a pink MDA-TBA- chromogen having a highest absorbance at 532 nm.

$$\cdot OH + \text{deoxyribose} \rightarrow \text{fragment} \xrightarrow[\Delta]{\text{TBA with acid}} \text{MDA}$$

TBA + MDA → MDA-TBA-chromogen

Therefore, presence of hydroxyl radical scavenger will lower the hydroxyl radical availability to attack the deoxyribose and hence the extent of the later series of reactions, resulting in a lower formation of MDA as well as the MDA-TBA-chromogen. Therefore, by measuring the decrease of the absorbance at 532 nm in sample and comparing with that in the control, the inhibition percentage, which is proportional to the antioxidant concentrations, can be obtained and the EC_{50} can then be found.

Procedures

The scavenging activity of hydroxyl radical was determined according to the method of Liu and others (1997) with some modifications. One hundred microliter of mushroom extract with various concentrations (0.625 – 20 mg/ml) was mixed with 690 µl 2.5 mM 2-deoxy-D-ribose (Aldrich, Cat # 121649) in 0.2 M phosphate buffer saline (pH 7.4). One hundred microliter of iron (III) chloride-EDTA mixture, which was prepared by mixing 2.0 mM iron (III) chloride (Unilab, Cat # 011533) in degassed water and 2.08 mM EDTA (Fluka, Cat # 03685) in 0.2 M phosphate buffer

saline (1:1, v/v), was added to the sample mixture. After that, 100 µl 1.0 mM ascorbic acid (Sigma, Cat # A5960) and 10 µl 0.1 M H_2O_2 in degassed water were added and the mixture was incubated at 37°C water bath for 10min. After the incubation, 1.0 ml cold (4°C) 2.8% (w/v) trichloroacetic acid (Riedel-delHaën, Cat # 27242) was added followed by 0.5 ml 1% thiobarbituric acid (Sigma, Cat # T-5500). The reaction mixture was incubated in a boiling (100°C) water bath for 8 min. After rapid cooling to room temperature with running tap water, the absorbance at 532 nm was measured using a spectrophotometer. Negative control was prepared by using distilled water instead of mushroom extracts.

The hydroxyl radical scavenging activity (%) was calculated by the following equation:

Scavenging activity (%) = $[1-(Abs_{532sample}/ Abs_{532control})]x100\%$

The EC_{50} value (mg/ml) refers to the effective concentration to scavenge the hydroxyl radicals by 50%.

2.2.2.2.4 Hydrogen peroxide scavenging activity

Principle

This assay is known as ferrous ion oxidation-xylenol orange (FOX) method and was first described in 1973 by Gupa and was modified by Jiang and others in 1990. The system is based on the peroxyl-mediated oxidation of ferrous to ferric ion under acidic condition in the presence of xylenol orange and sorbitol, which acts as a catalyst. Peroxyl radical is first produced by hydrogen peroxide and sorbitol. This peroxyl radical formed then oxidizes the Fe^{2+} to Fe^{3+}. The Fe^{3+} produced quickly complexes with xylenol orange under acidic environment due to the presence of sulphuric acid to form a purple chromogen having the highest absorbance at 595 nm. Therefore, presence of hydrogen peroxide scavenger will result in a lower peroxyl radical formation and hence a decrease in the absorbance measured due to the

lowered hydrogen peroxide availability to react with sorbitol. By measuring the decrease of the absorbance at 595 nm in sample and comparing it with that in the control, the inhibition percentage, which is proportional to the antioxidant concentrations, can be obtained and the EC_{50} can then be found.

Procedures

The scavenging activity of hydrogen peroxide was determined using a commercial kit (PeroXOquant™ Quantitative Peroxide Assay Kits, Pierce, Cat # 23280) according to manufacture's instruction. Working reagent was prepared by mixing Reagent A (25 mM ammonium ferrous (II) sulphate, 2.5 M H_2SO_4) with Reagent B (100 mM sorbitol, 125 μM xylenol orange in water) (1:100, v/v). Sample were prepared by mixing mushroom extracts of various concentrations (0.02 – 2 mg/ml for *Aa* and *Po* and 0.5 – 2 mg/ml for *Pe* and *Pn*) with 0.1 mM H_2O_2 (Wako, Cat # 080-01206) for 15 min before assay while standard was prepared by diluting 30% H_2O_2 to various concentrations (0 – 1000 μM) with water. Twenty microliter of sample or standard was added to a 96 well plate and 200 μl working reagent was added using a multiple channel to each well. The plate was then incubated at room temperature for 20 min for the reaction to reach an endpoint. The absorbance at 595 nm was measured using a spectrophotometer.

The concentration of H_2O_2 present in the sample was determined by interpolating from the sample absorbance value using the standard curve.

The hydrogen peroxide scavenging activity (%) was calculated by the following equation:

$$\text{Scavenging activity (\%)} = \{1-([H_2O_2]_{\text{in sample}}/\ [H_2O_2]_{\text{in control}})\}\text{x}100\%$$

2.2.2.3 Determination of phenolic compounds

2.2.2.3.1 Total phenolic content

Principle

This assay is known as Folin-Ciocalteu (FC) method and was first described in 1951 by Lowry and others for protein analysis. It was then modified by Singleton and Rossi in 1965 to extent its use to measure the concentrations of phenolic compounds in foods.

The Folin-Ciocalteu's phenol reagent used does not contain phenol. Instead, phenols and non-phenolic reducing substances in samples transfer electrons at basic pH and reduce the phosphotunsgtates-phosphomolybdates complexes present in the FC reagent to form an intense blue molybdenum and tungsten oxides, with maximum absorbance at 750 nm (Bray and Thorpe, 1954). Therefore, the higher the absorbance measured at 750 nm, the more the abundance of the reducing compounds present in the sample. By comparing the result with that of gallic acid, an antioxidant standard, the result can be expressed as gallic acid equivalent (GAE (μM)/mg extract), representing the amount of gallic acid (μM) having the activity as 1 mg of sample extract.

In this assay, saturated sodium carbonate is added since the reaction only take place under alkaline conditions. The phenolic compound will dissociate to form phenolate anion and reduce the FC reagent under basic condition by an electron-transfer mechanism.

Procedures

The total phenolic content in the mushroom extracts were determined according to Singleton and Rossi (1965) with some modifications. Half of a milliliter of Folin-Ciocalten phenol reagent (BDH, Cat # 19058) was first added to a test-tube, followed by an addition of equal volume of mushroom extract. After incubating at room temperature in dark for 3 min, 0.5 ml of 35% saturated Na_2CO_3 (AnalaR, Cat #

102404H) was added to the test tube together with 3.5 ml distilled water. The reaction mixture was then incubated in dark for 90 min. The absorbance at 725 nm was read using a spectrophotometer afterwards by transferring 1 ml of sample mixture to curvette. The reading was compared with the absorbance recorded in the calibration curve, which is obtained by various known gallic acid (Sigma, Cat # G8647) concentration (0.005 mM - 2 mM) and expressed as GAE value.

2.2.2.3.2 Identification of phenolic acids

Principle

The characterization of phenolic compounds in mushroom samples can be performed using fourier transform-ion cyclotron resonance mass spectrometry (FT-ICR MS system). This FT-ICR MS system is actually similar to liquid chromatography-mass spectrometry (LC-MS). Sample was injected by the sample injector and then is carried through a column by a single degassed solvent or degassed solvent mixture. In the column, sample is separated into different components based on its polarity and molecular weight and eluted out in different retention time. The compounds eluted were then detected in the detector and spectral data is produced. The major difference in this FT-ICR MS system is that after individual components are eluted, they will be quickly ionized to their ionization products and gave a corresponding m/z ratio. Therefore, by matching the retention time and comparing the observed mass with the theoretical mass of the detected ionization product, the presence of a particular compound could be confirmed.

Procedures

Sample preparation

The sample preparation method used in this study was according to Kim and others (2008) with some modifications. Prior to analysis the phenolic acids present in the mushroom water extracts were extracted by mixing with acetonitrile and 0.1 N hydrochloric acid at 5:1 ratio by volume while the extraction ratio was 1 g sample: 6ml extraction solution. The mixture was stirred for 2 h at room temperature. After

that, the solution was centrifuged for 5 min at 180 *g*. The suspension was then filtered through 0.2 μm syringe filter (Minisart, Cat # 17821K). The extract was freeze-dried below -50°C and the residues were redissolved in 10 ml 20% aqueous methanol. The extracts were stored at -80°C before analysis.

FT-ICR MS analysis

The Apex Ultra 7.0 Hybride Qh-FTMS (Bruker Daltonics Inc., USA) equipped with an Apollo II ion source and Dionex Ultimate 3000 2D Nanoflow LC system (Bruker Daltonics Inc., USA) was employed for the analysis. The autosampler, pump and flow manager system (Ultimate 3000) were monitored and controlled by the computer.

The system was connected to an analytical (2.1 x 150 mm) C18 column (5 μm) (XBridge™, Ireland). Two solvents were used as mobile phases: Solvent A was 0.1% formic acid in milliQ water and solvent B was 100% acetonitrile (Scharlau, Cat # AC03402500). Table 2.6 shows the elution profile. The flow rate was 0.1 ml/min and the temperature of column was kept at 30°C. Twenty microliters of sample solution was injected into the system using an auto-sampler.

Table 2.6 Elution profile used in the FT-ICR MS analysis

Time (min)	0.1% formic acid (%)	100% acetonitrile (%)
0	95	5
30	70	30
40	70	30
50	50	50
60	50	50
60.01	95	5
65.00	95	5

Eleven phenolic acids standards: caffeic acid (Sigma, Cat # C0625), chlorogenic acid (Sigma, Cat # C3878), cinnamic acid (Sigma, Cat # C8,085-7), ferulic acid (Sigma, cat # F3500), folic acid (Sigma, Cat # F8758), gallic acid (Sigma, Cat # G8647), protocatechuic acid (Sigma, Cat # P5630), salicyclic acid (Fluka, Cat # 84210), sinapic acid (Chemika, Cat # 85430), syringic acid (Sigma, Cat # S6881) and *tert*-butylhydroxyquinone (Aldrich, Cat # 112941) were used as references.

2.2.3 Statistical analysis

All experiments were done in triplicate and data were expressed as mean ± standard derivation (SD). Multiple comparisons between the antioxidant activities and phenolic contents in these four mushroom extracts were investigated by one-way ANOVA with Tukey's method while the dose-dependent responses within the same mushroom extract at various concentrations was investigated by Pearson correlation coefficient in bivariate correlations. All these statistical analysis were performed by the Statistical Package for Social Sciences (SPSS 16.0, 2007). Differences with $p < 0.05$ were considered statistically significant.

2.3 Results and Discussions

2.3.1 Extraction yield

The water extraction yield of *Aa*, *Po*, *Pe* and *Pn* at room temperature was determined. The highest extraction yield on dried weight basis was found in *Po* (48.22%) with a decreasing order of *Pn* (41.89%) > *Pe* (38.93%) > *Aa* (32.28%). Generally, all these four mushrooms gave a high water extraction yield (> 30%), indicating that most of the compounds in them are water soluble.

The moisture content in these mushrooms determined by freeze-drying method in section 2.2.2.1 was found to be higher than 70% (Table 2.7), indicating that dried matter in the mushroom was about 30%. In fact, high moisture content in fresh mushrooms has been reported previously. Lin and others (2006) reported the moisture content in fresh *A. aegerita* to be 89.4% while Ho (2007) reported a similar value of 90.8%. These two values were comparable to the moisture content found in the present study.

Table 2.7 Water content (% by weight), water soluble fraction (% by weight) and water insoluble residues (% by weight) of *A. aegerita*, *P. ostreatus*, *P. eryngii*, and *P. nameko*

Sample	Water content	Water soluble fraction	Water insoluble residues
Agrocybe aegerita (*Aa*)	88.00	8.13	3.87
Pleurotus ostreatus (*Po*)	74.29	13.31	12.40
Pleurotus eryngii (*Pe*)	80.93	11.65	7.42
Pholiota nameko (*Pn*)	74.16	15.02	10.82

In the present study, water extraction was performed at room temperature. Actually, it has been reported that extracting mushroom under higher temperature would result in a higher extraction yield (Ho, 2007). However, some studies suggested that thermal treatment would decrease the anti-radical activities as well as lower the content of phenolic compounds in vegetables (Roy and others, 2007). Moreover, it had also been reported that organic solvent mushroom extracts such as methanol,

ethyl acetate and butanol possessed a higher antioxidant activity than its aqueous extract (Lo, 2003). However, it is more conveniently to use aqueous extracts in cell culture study due to its water solubility in the culture medium. Therefore, in the present study, mushroom water extracts obtained at room temperature were used for all the cell culture experiments.

2.3.2 Evaluation of antioxidant capacity

2.3.2.1 DPPH radical scavenging activity

Figure 2.5 shows the DPPH scavenging activities of four edible mushroom water extracts at a concentration between 0.25 – 4 mg/ml. Generally speaking, the scavenging activities increased while the rate of the increase in their scavenging activities actually decreased with increasing concentration of mushroom extracts with the exception for *Pe*. Actually, a linear correlation between the mushroom extract concentrations and DPPH scavenging activities were found in all mushroom extracts when the extracts concentrations were low. At a concentration below 1 mg/ml, the correlation coefficient between the scavenging activity and extract concentration found in *Aa* was 0.991 while a higher concentration of *Aa* water extract could no longer cause an effective increase in its DPPH scavenging activity. It is possibly because of the saturation of scavenging power at high extract concentration as mentioned by previous study (Ma, 2006). Same phenomenon was also observed in *Po* and *Pn* when the extracts concentration reached 2 mg/ml. When the concentration of *Po* and *Pn* were below 2 mg/ml, the correlation coefficients found were 0.9945 and 0.9666, respectively. The only exception was found in *Pe* water extract, which had a linear correlation (0.9987) up to 4 mg/ml.

Table 2.8 shows the DPPH scavenging activities in mushroom water extracts at different concentrations. The greatest increase in DPPH scavenging activities were found when the concentrations of mushroom extracts used were between 0.25 mg/ml and 1 mg/ml. All mushroom extracts gave a 3-fold increase (28.12 ± 0.68% to 90.36 ± 0.72% in *Aa*; 12.40 ± 0.73% to 45.49 ± 2.43% in *Po*; 6.23 ± 0.16% to 21.53 ±

1.25% in *Pe* and 15.53 ± 0.64% to 60.36 ± 2.65% in *Pn*) in scavenging activities when the concentration used increased from 0.25 mg/ml to 1 mg/ml.

The EC_{50} value used in here refers to the effective concentration that gives 50% DPPH scavenging activity. As its name implies, a lower EC_{50} indicates a higher scavenging activity. The EC_{50} values of DPPH scavenging activity in these mushroom extracts were shown in Table 2.13. The lowest EC_{50} value was found in *Aa* (0.46 mg/ml) followed by *Pn* (0.82 mg/ml) while the third and fourth were found to be in *Po* (1.21 mg/ml) and *Pe* (2.67 mg/ml), respectively. Since *Aa* and *Pn* both possessed a very low EC_{50} value (< 1 mg/ml), it indicated that they were excellent DPPH scavengers.

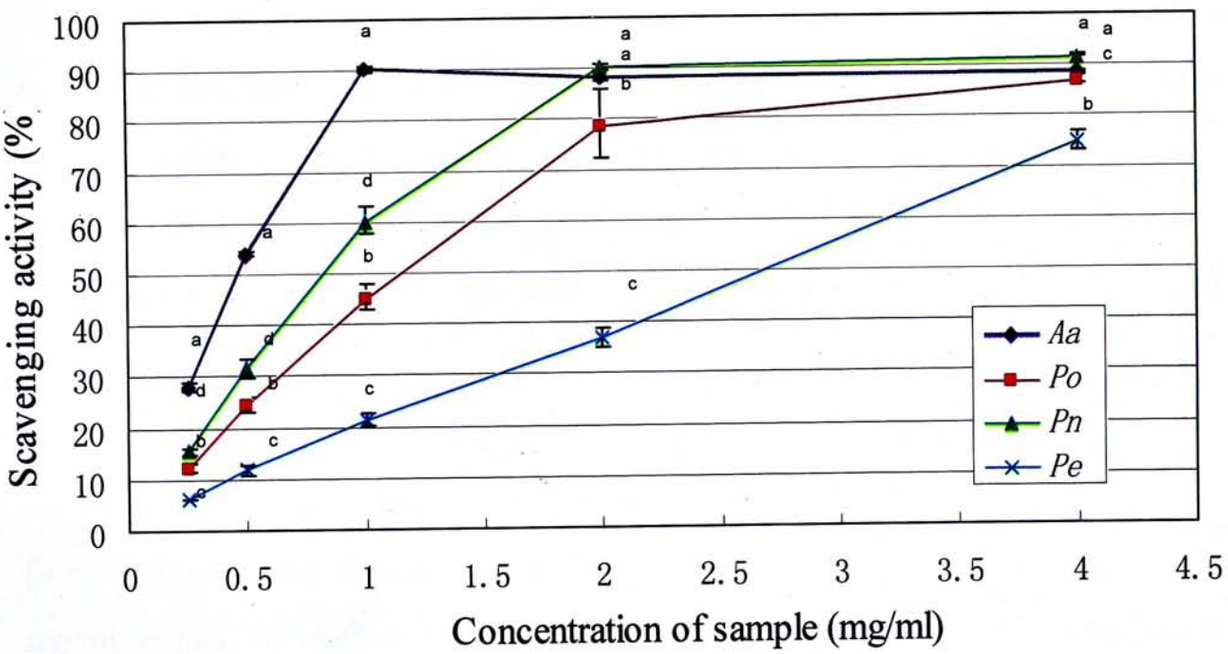


Figure 2.5 DPPH scavenging activities (%) of the mushroom water extracts at different concentrations

Each value is expressed as mean ± SD ($n = 3$)

Different superscript letters indicate significant difference between different mushroom water extracts at specific concentration ($p < 0.05$, ANOVA, Tukey's multiple comparison)

Table 2.8 DPPH scavenging activities (%) of the mushroom water extracts at different concentrations

Concentration (mg/ml)	DPPH scavenging activities (%) in different mushroom extracts			
	Aa	*Po*	*Pe*	*Pn*
0.25	28.12 ± 0.68^{a}	12.40 ± 0.73^{b}	6.23 ± 0.16^{c}	15.53 ± 0.64^{d}
0.5	54.11 ± 0.43^{a}	24.66 ± 1.36^{b}	11.86 ± 0.99^{c}	31.64 ± 1.72^{d}
1	90.36 ± 0.72^{a}	45.49 ± 2.43^{b}	21.53 ± 1.25^{c}	60.36 ± 2.65^{d}
2	88.54 ± 0.99^{a}	79.11 ± 6.66^{b}	37.06 ± 1.79^{c}	90.30 ± 0.81^{a}
4	88.69 ± 0.30^{a}	87.17 ± 0.86^{a}	75.00 ± 1.88^{b}	91.91 ± 0.25^{c}

Each value is expressed as mean ± SD ($n = 3$)

Different superscript letters in the same row indicate significant difference among the means ($p < 0.05$, ANOVA, Tukey's multiple comparison)

2.3.2.2 Superoxide anion radical scavenging activity

Figure 2.6 shows the superoxide anion radical (SAR) scavenging activity of the four mushroom water extracts at a concentration between 0.3125 – 10 mg/ml. When comparing the SAR scavenging activity to the DPPH scavenging activity (section 2.3.2.1), it was obvious that all four mushroom extracts possessed a much lower SAR scavenging activity than DPPH scavenging activity at the same concentration. Therefore, a boarder range of mushroom extract concentration up to 10 mg/ml was used in this assay.

Aa water extract was found to have a significantly higher SAR scavenging activity ($p < 0.05$) than other three mushroom extracts in a concentration range of 1.25 to 10 mg/ml. In fact, *Aa* was the only mushroom water extract having an EC_{50} value lower than 10 mg/ml while the EC_{50} value for other 3 mushrooms were all > 10 mg/ml. Although *Pn* ranked the second in DPPH scavenging activity assay (section 2.3.2.1), it was found to have the weakest SAR scavenging activity. Even when the concentration applied reached 10 mg/ml, the SAR scavenging activity of *Pn* was just around 10%, which was significantly lower ($p < 0.05$) than all other three mushroom extracts. In fact, there was not much difference in the SAR scavenging activity of *Pn* within a concentration range of 0.3125 to 10 mg/ml (Table 2.9).

The SAR scavenging activity in these four mushroom extracts, especially in *Po*, *Pe* and *Pn*, were not as high as expected. Scavenging activity was expected to increase linearly with an increase in sample concentration as more antioxidants (ie. mushroom extracts) available for radical removal should result in a higher scavenging activity. However, when calculating the correlation coefficient to check if the SAR scavenging activities by *Po*, *Pe* and *Pn* occurred in a dose-dependent manner, it was found that all the correlation coefficients were below 0.8 (0.7925, 0.7735 and 0.7918 for *Po*, *Pe* and *Pn*, respectively). This indicated that the concentration of these three mushroom extracts and the corresponding inhibition percentage were not strongly correlated with each other in this assay.

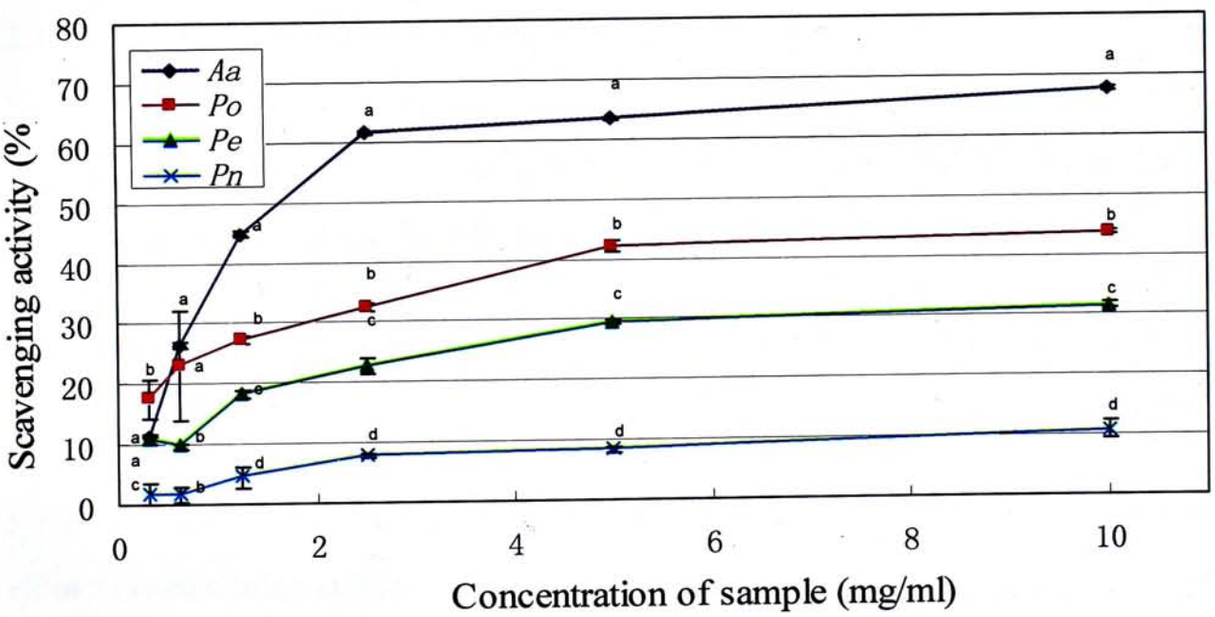


Figure 2.6 Superoxide anion radical scavenging activities (%) of the mushroom water extracts at different concentrations

Each value is expressed as mean ± SD ($n = 3$)

Different superscript letters indicate significant difference between different mushroom water extracts at specific concentration ($p < 0.05$, ANOVA, Tukey's multiple comparison)

Table 2.9 Superoxide anion radical scavenging activities (%) of the mushroom water extracts at different concentrations

Concentration (mg/ml)	Superoxide anion radical scavenging activities (%) in different mushroom water extracts			
	Aa	*Po*	*Pe*	*Pn*
0.3125	10.87 ± 0.87^{a}	17.53 ± 3.20^{b}	10.58 ± 0.66^{a}	1.73 ± 1.83^{c}
0.625	26.38 ± 0.55^{a}	23.15 ± 9.07^{a}	9.57 ± 0.38^{b}	1.67 ± 1.20^{b}
1.25	44.86 ± 0.50^{a}	27.17 ± 0.75^{b}	18.11 ± 0.70^{c}	4.49 ± 1.76^{d}
2.5	61.59 ± 0.33^{a}	32.39 ± 0.78^{b}	22.68 ± 1.20^{c}	7.75 ± 0.33^{d}
5	63.33 ± 0.13^{a}	42.10 ± 0.82^{b}	29.42 ± 0.25^{c}	8.33 ± 0.55^{d}
10	67.75 ± 0.25^{a}	43.77 ± 0.25^{b}	31.52 ± 0.43^{c}	10.72 ± 1.45^{d}

Each value is expressed as mean ± SD ($n = 3$)

Different superscript letters in the same row indicate significant difference among the means ($p < 0.05$, ANOVA, Tukey's multiple comparison)

2.3.2.3 Hydroxyl radical scavenging activity

Hydroxyl radical (HR) is one of the most active radicals attacking macromolecules like DNA and proteins leading to mutagenesis and carcinogenesis (Lee and Jang, 2004). Figure 2.7 shows the HR scavenging activities of four edible mushroom water extracts at a concentration between 0.625 – 20 mg/ml. Generally speaking, HR scavenging activity of all four mushroom extracts was found to increase with concentration, with the correlation coefficient 0.9253 for *Aa*, 0.8691 for *Po*, 0.8224 for *Pe* and 0.8287 for *Pn*, suggesting a linear relationship between the mushroom extract concentration and the HR scavenging activities.

Although a high correlation coefficient was found in all mushroom water extracts, the HR scavenging activity of these four mushroom water extracts indeed was relatively similar to each others in the concentrations under tested (19.14 ± 1.03% to 80.11 ± 1.43% for *Aa*, 15.84 ± 1.24% to 75.22 ± 0.90% for *Po*, 14.19 ± 0.60% to 75.53 ± 0.47% for *Pe* and 11.04 ± 1.48% to 65.62 ± 0.34% for *Pn*) (Table 2.10). Besides, no single mushroom water extract always possessed the highest scavenging activity among the concentration tested. For example, *Aa* showed a significantly higher ($p < 0.05$) scavenging activity at a concentration of 0.625 mg/ml than others while at 1.25 mg/ml, no significant difference ($p < 0.05$) could be found between *Aa*

and *Pe* although it still possessed the highest scavenging activity. *Pe* water extract had the highest HR scavenging activity ($p < 0.05$) at a concentration between 2.5 – 10 mg/ml while *Aa* water extract gave the highest ($p < 0.05$) HR scavenging activity at a concentration of 20 mg/ml.

The lowest EC_{50} value for HR scavenging activity was found in *Pe* (5.10 mg/ml), followed by *Po* (5.53 mg/ml), *Aa* (5.94 mg/ml) and *Pn* (7.55 mg/ml). It was found that the EC_{50} value in these mushroom water extracts was actually very close to each other except *Pn*.

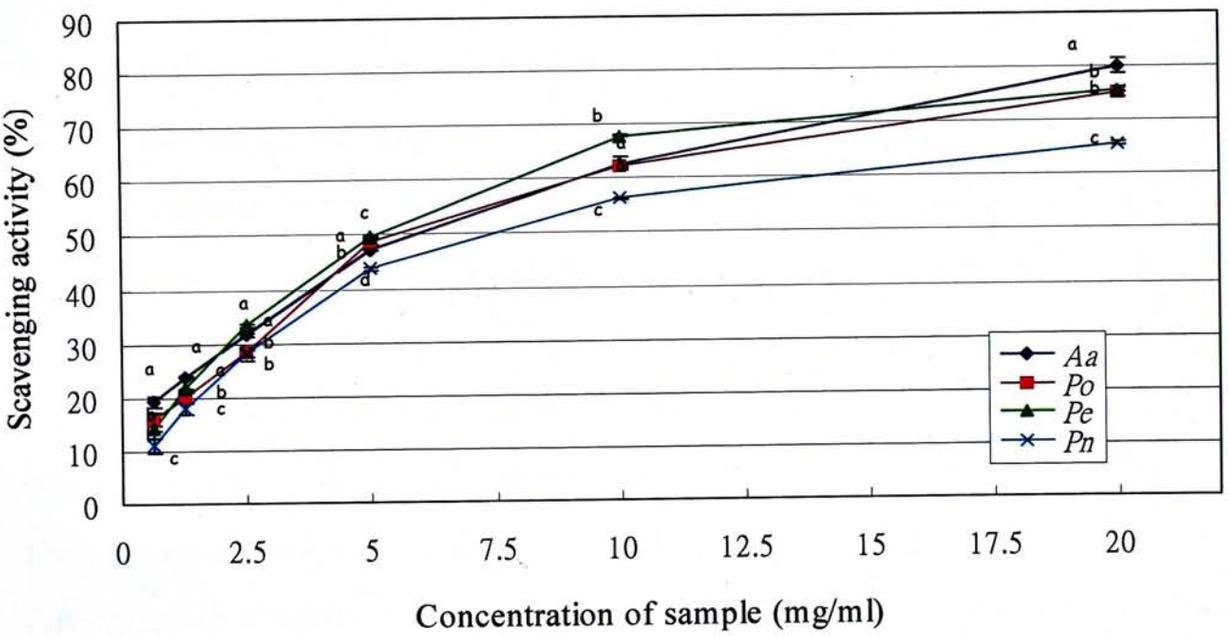


Figure 2.7 Hydroxyl radical scavenging activities (%) of the mushroom water extracts at different concentrations

Each value is expressed as mean ± SD ($n = 3$)

Different superscript letters indicate significant difference between different mushroom water extracts at specific concentration ($p < 0.05$, ANOVA, Tukey's multiple comparison)

Table 2.10 Hydroxyl radical scavenging activities (%) of the mushroom water extracts at different concentrations

Concentration (mg/ml)	Hydroxyl radical scavenging activities (%) of different mushroom water extracts			
	Aa	*Po*	*Pe*	*Pn*
0.625	19.14 ± 1.03^{a}	15.84 ± 1.24^{b}	14.19 ± 0.60^{b}	11.04 ± 1.48^{c}
1.25	23.80 ± 0.26^{a}	19.97 ± 1.06^{b}	21.55 ± 0.26^{a}	17.72 ± 1.02^{c}
2.5	31.61 ± 0.47^{a}	28.00 ± 1.32^{b}	33.18 ± 0.34^{a}	28.23 ± 0.79^{b}
5	47.07 ± 0.00^{a}	48.57 ± 0.57^{b}	49.62 ± 0.13^{c}	43.54 ± 0.26^{d}
10	62.61 ± 1.19^{a}	62.09 ± 0.13^{a}	67.72 ± 0.13^{b}	56.23 ± 0.26^{c}
20	80.11 ± 1.43^{a}	75.22 ± 0.90^{b}	75.53 ± 0.47^{b}	65.62 ± 0.34^{c}

Each value is expressed as mean ± SD ($n = 3$)

Different superscript letters in the same row indicate significant difference among the means ($p < 0.05$, ANOVA, Tukey's multiple comparison)

2.3.2.4 Hydrogen peroxide scavenging activity

Figure 2.8 shows the hydrogen peroxide scavenging activities of four edible mushroom water extracts at a concentration between 0.5 – 2 mg/ml towards 0.1 mM hydrogen peroxide. Indeed, all mushroom extracts except *Pn* showed a strong scavenging activity (> 50%) towards 0.1 mM H_2O_2 at a range of 1 – 2 mg/ml. Although *Pe* gave a relatively lower scavenging activity at 0.5 mg/ml, its activity was comparable to *Aa* and *Po* when its concentration increased to 1 mg/ml while further increase to 1.5 mg/ml and 2 mg/ml did not result in any significant differences ($p > 0.05$) with *Aa* (Table 2.11). Incubation of 0.1 mM H_2O_2 with *Aa* water extract at the designated concentration for 15 min resulted in almost 100% scavenging activity. It suggested that *Aa* water extract possessed a higher hydrogen peroxide scavenging capacity when compared with others, giving an EC_{50} value lower than 0.5 mg/ml. However, we could not simply deduce that *Aa* water extract had the highest hydrogen peroxide scavenging activity since the EC_{50} value of *Po* water extract was also lower than 0.5 mg/ml (Figure 2.8). Based on this finding, lower concentrations of *Aa* and *Po* extracts were used to confirm which one of them would give a lower EC_{50} value and the result was shown in Table 2.12.

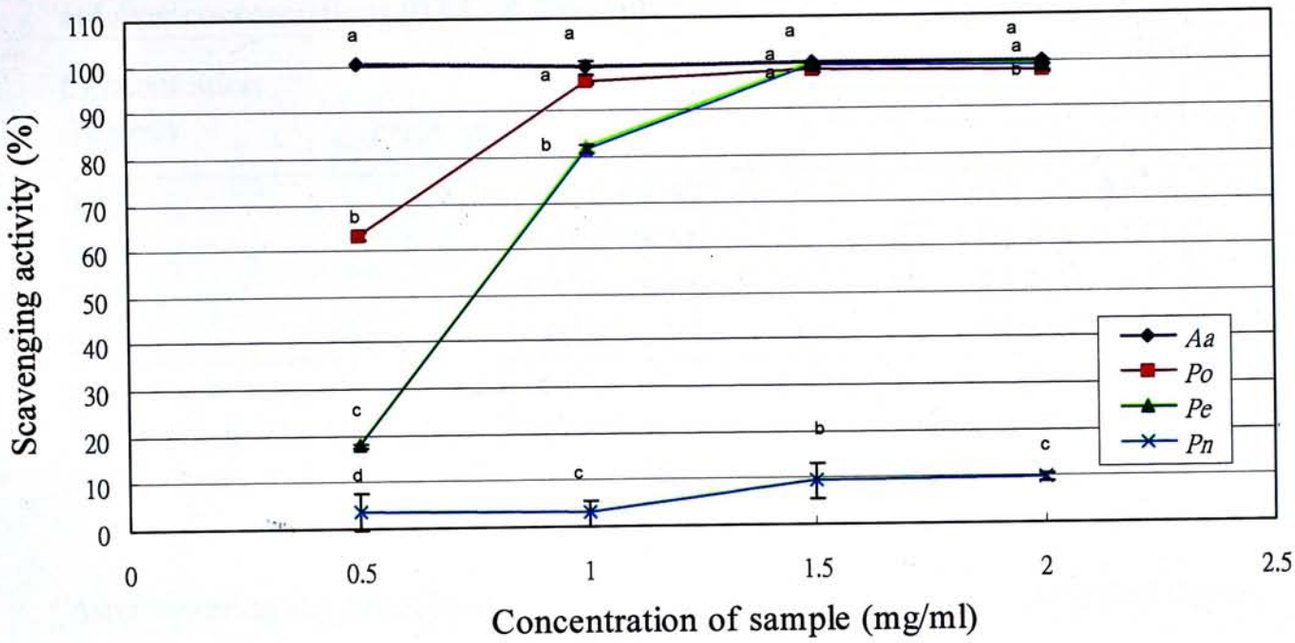


Figure 2.8 Hydrogen peroxide scavenging activities (%) of four edible mushroom water extracts at different concentrations (0.5-2mg/ml)

Each value is expressed as mean ± SD ($n = 3$)

Different superscript letters indicate significant difference between different mushroom water extracts at specific concentration ($p < 0.05$, ANOVA, Tukey's multiple comparison)

Table 2.11 Hydrogen peroxide scavenging activities (%) of the mushroom water extracts at different concentrations

Concentration (mg/ml)	Hydrogen peroxide scavenging activities (%) of different mushroom water extracts			
	Aa	*Po*	*Pe*	*Pn*
0.5	100.24 ± 0.05^{a}	63.12 ± 0.76^{b}	17.72 ± 0.52^{c}	3.56 ± 4.03^{d}
1	99.24 ± 1.35^{a}	95.86 ± 0.14^{a}	81.65 ± 0.66^{b}	2.77 ± 2.91^{c}
1.5	100.05 ± 0.00^{a}	98.13 ± 0.28^{a}	99.29 ± 0.16^{a}	9.23 ± 3.87^{b}
2	99.79 ± 0.09^{a}	97.52 ± 0.08^{b}	98.95 ± 0.08^{a}	9.73 ± 0.76^{c}

Each value is expressed as mean ± SD ($n = 3$)

Different superscript letters in the same row indicate significant difference among the means ($p < 0.05$, ANOVA, Tukey's multiple comparison)

Table 2.12 Hydrogen peroxide scavenging activities (%) of *Aa* and *Po* water extracts at lower concentrations (0.05 - 0.2 mg/ml)

Concentration (mg/ml)	Hydrogen peroxide scavenging activities (%) of different mushroom water extracts	
	Aa	*Pe*
0.05	98.28 ± 1.53	45.05± 0.3*
0.1	94.84 ±5.11	50.76 ± 1.14*
0.15	97.79 ± 0.00	52.71 ± 1.19*
0.2	100.25 ± 1.7	55.34 ± 2.41*

Each value is expressed as mean ± SD ($n = 3$)

* indicates significant difference between the two samples at the same concentration ($p < 0.05$, student's t test)

After lowering the extracts concentration to 0.05 - 0.2 mg/ml, we could find that the hydrogen peroxide scavenging activity of *Aa* was still nearly 100% even when the concentration lowered to 0.05 mg/ml while that of *Po* decreased to below 50% at such concentration. Therefore, we suggested that *Aa* water extract possessed a more powerful scavenging activity towards hydrogen peroxide than that of *Po* water extract.

2.3.2.5 Comparison of the EC_{50} of mushroom water extracts in different antioxidant assays

Effective concentration (EC_{50}) was used to screen out the most potent mushroom water extract in scavenging the ROS in each assay and their values were summarized in Table 2.13.

Table 2.13 The EC_{50} value of four edible mushroom water extracts in different antioxidant assays

Sample	EC_{50} value (mg/ml) in different assays			
	DPPH	O_2^-	$OH^·$	H_2O_2
Aa	0.46	1.63	5.94	<0.05
Po	1.21	>10	5.53	0.093
Pe	2.67	>10	5.10	0.75
Pn	0.82	>10	7.55	>2

: The mushroom extract possessed the highest antioxidant activity (ie the lowest EC_{50} value) in each assay

From the above table, we could see that *Aa* mushroom water extract generally showed the highest scavenging activity in the antioxidant assays used in this study. It ranked first in three out of four tests (scavenging activity towards DPPH radical, superoxide anion radical and hydrogen peroxide). Although it only ranked third in the hydroxyl radical assay, its EC_{50} value (5.94 mg/ml) was very close to *Pe* (5.10 mg/ml), which ranked first in the corresponding assay. Based on these results, *Aa* water extract was suggested to possess a relatively higher antioxidant activity than that of the others.

2.3.3 Determination of phenolic compounds

2.3.3.1 Total phenolic content

The total phenolic content of mushroom water extracts was shown in Table 2.14. The amount of total phenolic content was expressed as gallic acid equivalent (GAE) representing the amount of gallic acid (μM) having the activity as 1 mg of sample extract. Therefore, a higher GAE value indicates a larger amount of phenolic compounds present in the sample extract.

In the mushroom extracts, the phenolic content of *Aa* was the highest (13.67 ± 0.21 GAE (μM)/ml) among the others (Table 2.14). This suggested that the strong scavenging activity towards DPPH radical, superoxide anion radical, hydroxyl radical and hydrogen peroxide in *Aa* water extract might be related to its high phenolic content. In fact, the antioxidant activities of mushrooms have long been suggested to be contributed by their phenolic compounds (Ma, 2006) and some research findings have shown a strong correlation ($r > 0.99$) between the total phenolic content in mushroom extracts and their antioxidant activity (Huang and others, 2005).

Table 2.14 The gallic acid equivalent (GAE(μM)/mg extract) in four edible mushroom water extracts

Sample	GAE(μM)/mg extract
Aa	13.67 ± 0.21^{a}
Po	11.25 ± 0.29^{b}
Pe	6.53 ± 0.15^{c}
Pn	7.37 ± 0.11^{d}

Each value is expressed as mean ± SD ($n = 3$)

Different superscript letters in the same row indicate significant difference among the means ($p < 0.05$, ANOVA, Tukey's multiple comparison)

The highest GAE value was found in *Aa* suggesting that it might contain the highest amount of phenolic materials. However, this Folin-Ciocalteu method was not specifically measuring the absolute amounts of phenolic materials present in the sample but was actually measuring the chemical reducing capacity relative to an equivalent reducing capacity of gallic acid (McDonald and others, 2001). Therefore, results in this assay also reflected the reducing capacity of different mushroom extracts while the accuracy in measuring the total phenolic content was compromised. The presence of ascorbic acid, citrate and sulfite can easily interfere the result obtained (Ma and Cheung, 2007). To solve this problem, further analysis using FT-ICR MS on the identification of the phenolic compounds present in mushroom extracts was performed (section 2.3.3.2).

2.3.3.2 Identification of phenolic acids

The antioxidant activities in the extracts from the four mushrooms were determined in section 2.3.2. Because phenolics are known to contribute largely to antioxidant activities (Puttaraju and others, 2006), the total phenolic content in these mushroom extracts were determined in section 2.3.3.1. The total phenolic content determined by the Folin-Ciocalteu method was not accurate enough since the presence of interfering compounds like ascorbic acid could greatly affect its accuracy and different phenolic compounds have different responses in the Folin-Ciocalteu method (Gazzani and others, 1998; Heinonen and others, 1998; Kahkonen and

others, 1999). In this section, FT-ICR MS was used to characterize the different compounds, especially the phenolic acids, in the mushroom water extracts in reference to the retention time of 11 phenolic acid standards.

The retention time of these phenolic acid standards were between 5.9 - 34.4 min (Figure 2.9). The characterization of the phenolic acids present in mushroom extracts was done by comparing the retention time and the mass accuracy (deviation between the theoretical mass and observed mass to be < 5 ppm). After matching the retention time and mass accuracy of the peaks in the mushroom water extracts with those in the phenolic acid standards, 5 phenolic acids (gallic acid, protocatechuic acid, chlorogenic acid, ferulic acid and sinapic acid) were identified in the *Aa* water extract and 2 phenolic acids (protocatechuic acid and folic acid) were found in the *Pe* water extract. There was only 1 phenolic acid (cinnamic acid) identified in the *Po* water extract while no major peaks in the *Pn* water extract could match with the phenolic acid standards (Figure 2.9). The amount of each phenolic acid identified in the mushroom water extracts were expressed as the relative percentage of total peak area in the FT-ICR MS chromatogram (Table 2.15).

Table 2.15 Phenolic acids and their % of total peak area identified in mushroom extracts

Mushroom water extract	Phenolic acids identified	Retention time (min)	Detected ionization form	Theoretical mass (Da)	Observed mass (Da)	Relative % of total peak area
Agrocybe aegerita (*Aa*)	Gallic acid	3.3	$[M+Na_2-H]^+$	214.99269	214.99482	3.1
	Protocatechuic acid	5.9	$[M+Na_2-H]^+$	198.99777	199.00007	3.9
	Chlorogenic acid	9.4	$[M+H]^+$	355.10236	355.10236	4.7
	Ferulic acid	14.8	$[M+Na_2-H]^+$	239.02908	239.03010	4.4
	Sinapic acid	16.7	$[M+H]^+$	225.07575	225.07580	6.7
Pleurotus ostreatus (*Po*)	Cinnamic acid	18.6	$[M+H]^+$	149.05971	149.06354	0.9
Pleurotus eryngii (*Pe*)	Protocatechuic acid	5.9	$[M+CH_3CN+H]^+$	196.06040	196.06460	7.3
	Folic acid	8.8	$[M+H]^+$	442.14696	442.14378	4.6

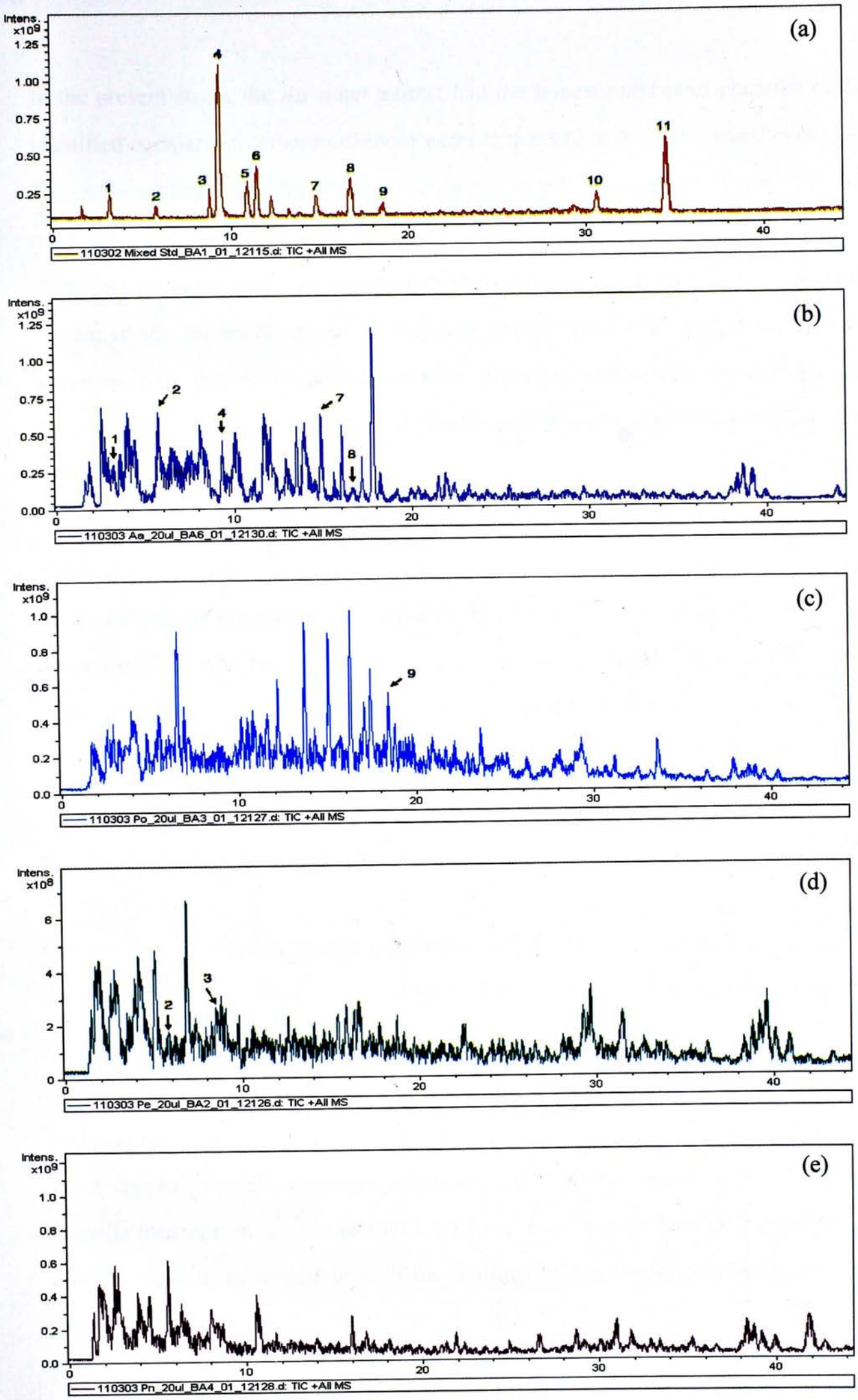


Figure 2.9 FT-ICR MS chromatogram of phenolic acid profiles in (a) standard; (b) *Aa* water extract; (c) *Po* water extract; (d) *Pe* water extract and (e) *Pn* water extract
1, gallic acid; 2, Protocatechuic acid; 3, folic acid; 4, chlorogenic acid; 5, caffeic acid; 6, syringic acid; 7, ferulic acid; 8, sinapic acid; 9, cinnamic acid; 10, *tert*-butylhydroxy quinine; 11, salicyclic acid

In the present study, the *Aa* water extract had the largest number of phenolic acids identified compared to other mushroom water extracts (2 in *Pe*, 1 in *Po* and even 0 in *Pn*). After adding up all the relative percentage of total peak area in these phenolic acids, *Aa* water extract had the largest value of 22.78%, indicating that these five identified phenolic acids accounted for more than one-fifth of those compounds present in the *Aa* water extract. It had been found that DPPH radical scavenging activities was positively correlated with phenolic acids such as gallic acid, protocatechuic acid, caffeic acid and ferulic acid found in mushrooms (Kim and others, 2008) while individual phenolic acid such as gallic acid, caffeic acid, p-coumaric acid and benzoic acid in mushrooms also demonstrated a positive correlation with the total phenolic content measured ($r > 0.99$) (Kim and others, 2008). Hence, the present results suggested that the antioxidant activities found in *Aa* water extract could be at least partly contributed by these five phenolic acids. Antioxidant activity of phenolic compounds includes their ability to chelate metals, inhibit lipoxygenase, and scavenge free radicals (Decker, 1997), all of which are believed to be related to their biological actions in human bodies like anti-inflammatory actions (Gryglewski and others, 1987).

Our result in this section is quite consistent with the antioxidant activities as well as the total phenolic content measured in section 2.3.2 and 2.3.3.1, respectively. *Aa* water extract was suggested to possess the strongest antioxidant properties and it ranked first in DPPH, superoxide anion and hydrogen peroxide scavenging assays while for the *Pn* water extract, it was found to be the weakest in the hydroxyl radical and hydrogen peroxide scavenging assays (Table 2.16) (section 2.4). For their total phenolic content measured in section 2.3.3.1, *Aa* was found to have the highest GAE, which was again in consistent with the findings in this section. However, *Po* was found to possess the second highest GAE value, followed by *Pn* and *Pe* in section 2.3.3.1 while in this section, only 2 phenolic acids were identified in *Pe* and none was identified in *Pn*. These results seemed to be not consistent when only the number peaks or the % of the total peak area of identified phenolic acids in these water

extracts was considered. In fact, although 1 phenolic acid was being identified in *Po*, there were many major unidentified peaks present which might represent phenolic acids but could not match with the 11 phenolic acid standards used in the present study. For *Pe* and *Pn*, their total phenolic content measured by FC method was indeed very close with each other (only 0.84 GAE difference), and by referring to their FT-ICR MS chromatograms, the total peak area in *Pe* and *Pn* were similar as well.

Besides identifying the presence of phenolic acids, it was also observed even though *Po* and *Pe* are from the same genus, their phenolic acids profiles were totally different. This suggested that analysis of phenolic acids profile might be useful in mushroom taxonomic studies up to the species level. However, difference in cultivation techniques, growing conditions or even the storage conditions may result in different levels of phenolic compounds produced even in the same species (Naczk and Shahidi, 2006). For example, Kim and others (2008) reported the presence of gallic acid, protocatechuic acid and chlorogenic acid in *Po* while only cinnamic acid was found in *Po* in the present study which was probably due to different cultivation conditions. Therefore, strict protocols in mushroom cultivation have to be followed if phenolic acid profile of the same mushroom species is to be used for matching.

2.4 Summary

Table 2.16 summarizes the relative rankings of the total phenolic content determined by the Folin-Ciocalteu method which is expressed as gallic acid equivalent (GAE(μM)/mg extract) for comparison, as well as antioxidant capacities towards the DPPH, superoxide anion radical, hydroxyl radical and hydrogen peroxide scavenging activities, based on the effective concentration (EC_{50}) value of these mushrooms water extracts.

Table 2.16 Rankings of relative antioxidant activities determined by four antioxidant assays and relative abundance of total phenolic content in mushroom water extracts

Sample	Assays					No. of phenolic acids identified by FT-ICR MS
	DPPH	O_2^-	$OH^·$	H_2O_2	TPC	
Aa	1	1	3	1	1	5
Po	3	---	2	2	2	1
Pe	4	---	1	3	4	2
Pn	2	---	4	4	3	0

Ranking of *Po*, *Pe* and *Pn* was denoted as "--" as their EC_{50} value were all higher than 10 mg/ml which was the highest concentration used in the experiment

Based on the above results, we suggested that *Aa* water extract generally demonstrated the strongest antioxdative properties due to its high ranking in DPPH, superoxide anion and hydrogen peroxide scavenging assays. It also contained the largest amount of total phenolic compounds with five phenolic acids being identified. Due to its relative higher antioxidant activity, *Aa* water extract was selected for further study on its anti-angiogenic effect to be discussed later in chapter 3.

Chapter 3: Anti-angiogenic properties of the *Aa* water extract

3.1 Introduction

3.1.1 Angiogenesis

The word angiogenesis was first introduced by Hertig in 1935. It refers to the proliferation of endothelial cells and the formation of new blood capillaries (Folkman, 1972). Angiogenesis is a vital and normal process during growth and development such as in wound healing, fetal development and corpus luteum development (Folkman and Shing, 1992). Angiogenesis is tightly regulated by a wide variety of angiogenic promoting and angiogenic inhibiting regulators. Once there is an imbalance of these regulators, blood vessel formation will be either in excess or deficit. Pathologically, angiogenesis regulation imbalance has been associated with many diseases. For example, in diabetes mellitus, angiogenesis imbalance can lead to diabetic retinopathy and nephropathy because of the excessive angiogenesis and impaired wound healing due to the insufficient angiogenesis (Martin and others, 2003). Abnormal angiogenesis is also found in the development of tumor and is named as tumor angiogenesis (Folkman, 1971).

3.1.1.1 Process of angiogenesis

Angiogenesis is a complex process. For angiogenesis to take place, endothelial cells of blood vessels must first be activated by those angiogenic factors secreted by nearby tissues or cells. Activated endothelial cells then produce proteases to degrade the surrounding basement membrane forming dissolved holes in the blood vessels. After that, endothelial cells start to proliferate and migrate towards the angiogenic factors through those dissolved holes to create a capillary sprout. Sprout endothelial cells roll up to form a new blood vessel tube. With the help of pericytes, new basement membrane is produced. The process of angiogenesis is finally completed when individual blood vessel tubes fuse with each other to form blood vessel loops and blood flow starts (Folkman and Haudenschild, 1980; Folkman, 1984).

3.1.1.2 Regulations of angiogenesis

As mentioned above, angiogenesis is tightly regulated by a large variety of factors and some common angiogenic promoters and inhibitors are listed in Table 3.1.

Table 3.1 Common regulators of angiogenesis

Angiogenic promoters	Angiogenic inhibitors
Angiopoietin-1	Angiostatin
Angiogenin	Endostatin
Basic fibroblast growth factor (bFGF)	Interferon alpha/beta/gamma
Endothelin	Interferon inducible protein (IP-10)
Interleukin-8 (IL-8)	Interleukin-12
Placental growth factor	Metalloproteinase inhibitors (TIMPs)
Platelet-derived endothelial cell growth factor (PD-ECGF)	Thrombospondin
Platelet-derived growth factor-BB (PDGF-BB)	Tissue inhibitor of metalloproteinase-1 (TIMP-1)
Pleiotrophin (PTN)	Tissue inhibitor of metalloproteinase-2 (TIMP-2)
Proliferin	Tissue inhibitor of metalloproteinase-3 (TIMP-3)
Transforming growth factor-alpha/ beta (TGF-α/ β)	Vasculostatin
Tumor necrosis factor-alpha (TNF- α)	Vasostatin
Vascular endothelial growth factor (VEGF)	

Among the dozen regulators, it is found that both bFGF and VEGF are the two most important angiogenic promoters in sustaining tumor growth (Goto and others, 1993).

3.1.1.2.1 Fibroblast growth factor (bFGF)

bFGF is also known as FGF-2 that belongs to the family of FGF (fibroblast growth factor). There are 22 members found in FGF family till now and they bind to different receptors for signaling (Itoh, 2007). Among these 22 members, FGF-1 to FGF-10 are more well-characterized than the others and they all bind to fibroblast growth factor receptors (FGFRs), which contains four members, FGFR-1, FGFR-2, FGFR-3 and FGFR-4.

bFGF can be found in the basement membrane as well as sub-endothelial extracellular matrix of blood vessels in normal tissues. It can be activated by heparin sulfate-degrading enzymes and leads to angiogenesis (Dowd and others, 1999).

3.1.1.2.2 Vascular endothelial growth factor (VEGF)

VEGF is a signaling molecule released by cells to induce angiogenesis. It is a family with several members including VEGF-A, VEGF-B, VEGF-C, VEGF-D and placenta growth factor (PIGF). VEGF-A is the most important member and sometimes it is just called as VEGF since it was the first member being discovered in this family (Senger and others, 1983). Different members of VEGF play different roles in angiogenesis. For example, VEGF-A is responsible for angiogenesis (Li, 2006); VEGF-B is for embryonic angiogenesis (Claesson-Welsh, 2008); VEGF-C is for lymphangiogenesis (Hirakawa and others, 2007); VEGF-D is for lymphatic vasculature (Starcker and others, 2001) while PIGF is for vasculogenesis (Li and others, 2006).

Members in the VEGF family binds to tyrosine kinase receptors (VEGFRs) on cell surface in order to generate cellular responses. Upon binding, VEGFRs will dimerize and transphosphorylate, resulting in activation (Gabhann and Popel, 2007). There are three related tyrosine kinase receptors for VEGFs, namely VEGFR-1 (fins-like tyrosine kinase receptor, Flt or Flt1), VEGFR-2 (kinase insert domain-containing receptor, KDR or Flk1) and VEGFR-3 (fms-like tyrosine kinase receptor, Flt4). Different members in the VEGF family bind to different VEGFRs with different affinities (Ferrara, 2002). For example, VEGF-A has a higher affinity towards VEGFR-1 and VEGFR-2; VEGF-B has a higher affinity towards VEGFR-1 while VEGF-C and VEGF-D have a higher affinity towards VEGFR-2 and VEGFR-3. Figure 3.1 shows the relationship between VEGFs and their corresponding VEGFRs.

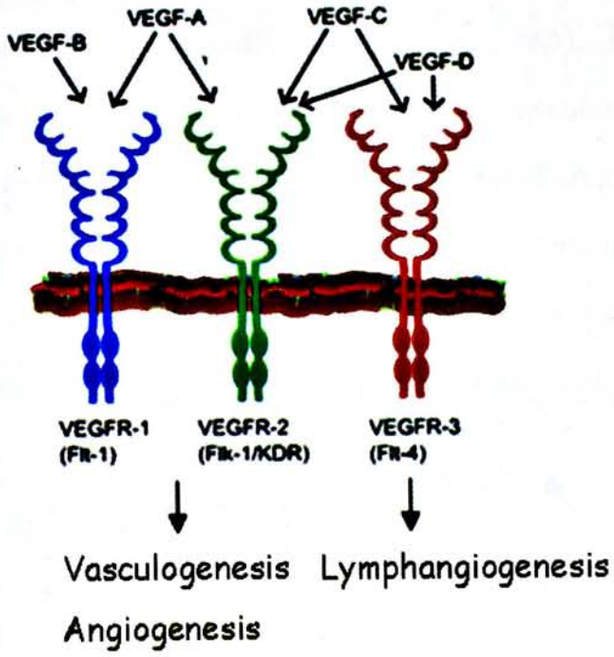


Figure 3.1 VEGFs and their corresponding VEGFRs
(Modified from Hicklin and Ellis, 2005)

3.1.2 Tumor angiogenesis

An adequate supply of blood, which provides nutrients, oxygen and growth factors is essential for tumor growth and development due to their rapid and uncontrolled growth.

In the past, the blood supply to tumor cells was thought to be simply by the dilation of pre-existing blood vessels. In 1971, Folkman first proposed that angiogenesis is necessary for the growth of both primary and metastastic tumors (Folkman, 1971). Figure 3.2 shows the processes involved in tumor angiogenesis. When tumor is first formed, its size is relatively small (less than 1 – 2 mm in diameter) and the exchange of nutrients, oxygen and waste materials can be achieved by pre-existing blood vessels and no angiogenesis is required. Such tumor is named as avascular tumor (Gimbrone and others, 1972). However, once tumor grows beyond the size of 1 – 2 mm in diameter, those pre-existing blood vessels can no longer provide adequate oxygen and nutrients. As a result, tumor cells secrete angiogenic factors in order to form new blood capillaries from existing ones and this process is denoted as tumor angiogenesis (Folkman, 1986). With tumor angiogenesis, tumor cells can be

transported by the circulation system or the lymphatic system to distant sites where they reinvade and grow at new locations (Folkman, 1986). Tumor angiogenesis, therefore, marks the pivotal transition from avascular to vascular tumor growth and a point, beyond which, makes treatment difficult and survival chance decreases. Hence, controlling tumor angiogenesis is one of the key issues in treating cancer as it helps reducing the chance of metastasis, which accounts for more than 90% of cancerous death and the development of secondary cancer (American Cancer Society).

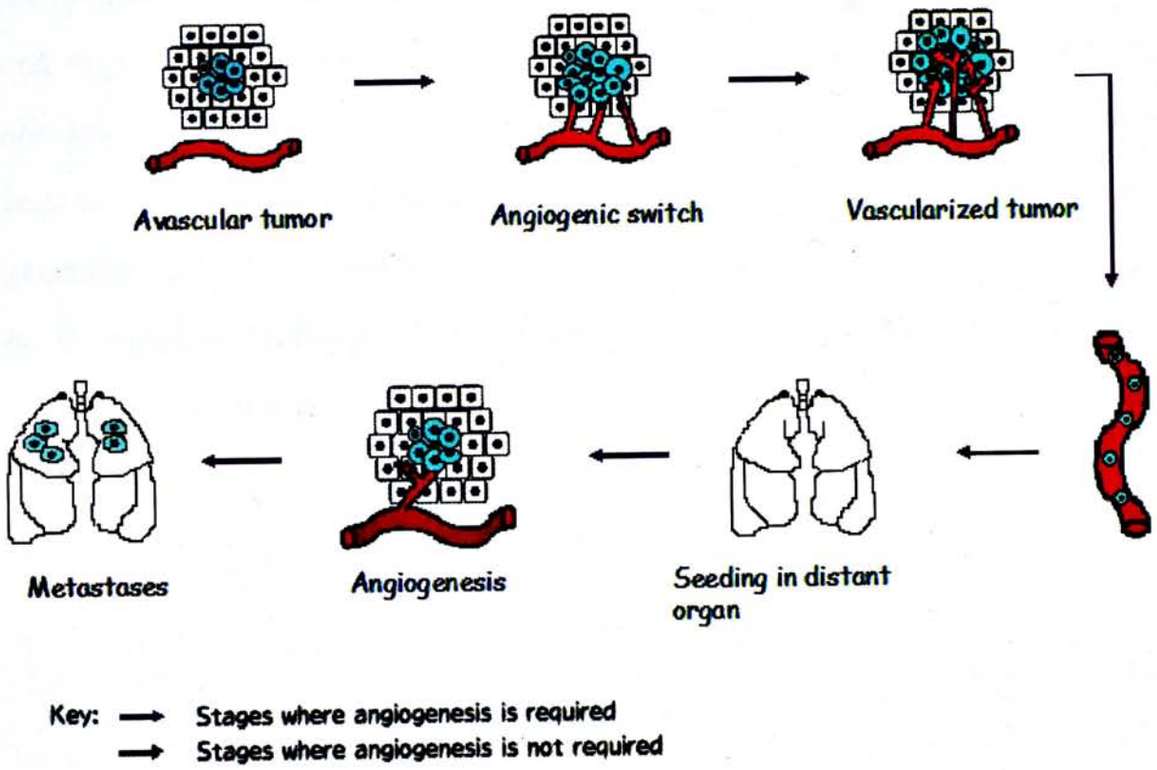


Figure 3.2 Different stages involved in metastasis
(Modified from Ellis and Fidler, 1996)

3.1.2.1 ROS generation in tumor cells

It has been reported that tumor cells are under higher oxidative stress than normal cells. *In vitro* studies have demonstrated that the production of ROS, especially hydrogen peroxide, is much higher in several tumor cells, including breast carcinoma, colon carcinoma, and ovarian carcinoma, than their normal counterparts (Szatrowski and Nathan, 1991). Human tumor cells can generate ROS up to a rate of 0.5 nmol/ 10^4 cells/ h, equivalent to the amount generated by phorbol ester-triggered neutrophils (Szatrowski and Nathan, 1991). This constitutive excess production of

hydrogen peroxide was believed to be associated with tumor heterogeneity, invasion and metastasis (Szatrowski and Nathan, 1991). Recently, the excessive production of hydrogen peroxide in tumor cells is found to be linked with tumor angiogenesis (Zhu and others, 2002).

3.1.2.2 Hydrogen peroxide and VEGF

Vascular endothelial growth factor (VEGF) is a stimulator of angiogenesis as mentioned in section 3.1.1.2.2. In the presence of VEGF, endothelial cells proliferate and migrate. Upon binding to its receptor (VEGFR-2), a signaling cascade begins and leads to the production of factors to stimulate vessel permeability, endothelial cells proliferation and migration (Prior, 2004). VEGF expressions in tumor cells are generally elevated (Ferrara and Davis-Smyth, 1997). Besides, its expression can also be regulated by pathological processes like hypoxia (Detmar and others, 1997), cytokines like interleukin 6 (Huang and others, 2004), growth factors like epidermal growth factor (Goldman and others, 1993) as well as hydrogen peroxide (Kubo and others, 2007).

Under high dose of hydrogen peroxide treatment (> 0.1 mM), cell growth is hampered due to the excessive oxidative stress. However, moderate administration (< 0.1 mM) of exogenous hydrogen peroxide can increase the migration of endothelial cells induced by cancer cells (Zhu and others, 2002). Previous study showed that bone marrow cells pretreated with various hydrogen peroxide concentrations (< 5 μM) had an increase in VEGF mRNA expression, VEGF production, and microvessel density (Kubo and others, 1997) while treatment of human microvascular endothelial cells (HMVEC) with H_2O_2 (100 – 500 μM) led to microvascular tubule formation in culture (Shono and others, 1996).

In fact, the relationship between H_2O_2 and VEGF is like a positive feedback mechanism. In vascular cells, ROS, which plays a role in VEGF-induced angiogenesis, can be derived from growth factor-stimulated receptors. It was found

that VEGF-treated endothelial cells generated elevated level of H_2O_2, which in turns, could increase the production of VEGF (Lin and others, 2003).

3.1.2.3 Previous studies on tumor angiogenesis

3.1.2.3.1 ROS and endothelial cells proliferation

There are many lines of evidence showing that ROS can trigger the activation of endothelial cells *in vitro* (Stone and Collins, 2002) and that intracellular signaling, for example the up-regulation of IL-8 production, can result in enhanced angiogenesis *in vivo* (Koch and others, 1992; Maulik and Das, 2002). ROS can increase endothelial cells proliferation directly by acting on endothelial cells leading to up-regulation of transcriptional factors responsible for cells proliferation, like nuclear factor kappa B (NF- κB) and activator protein 1 (AP-1) (Barchowsky and others, 1995) or indirectly on cancer cells resulting in over-expression of vascular endothelial growth factor (VEGF) which is the most important angiogenic promoter in endothelial cells sprouting (Carmeliet, 2004).

NF- κB is a protein complex that controls the transcription of DNA. This protein complex is made up of 3 sub-units, a DNA binding 48 – 65 kD protein (p50) (Kawakami and others, 1988), a DNA binding 65 – 68 kD protein (p65) and an inhibitory subunit Iκ (Baeuerle and Baltimore, 1989), which inhibit the DNA binding of NF- κB.

Activator protein 1 (AP-1) is a transcription factor containing a homo-dimer, Jun-Jun or a hetero-dimer, Jun-Fos (Young and others, 1999). As its name implies, it is a positive control in regulating the gene expression, which in turns, increases the rate of transcription.

It was found that when treating low passage porcine aortic endothelial cells with H_2O_2, both NF- κB-DNA complex and AP-1-DNA binding complex increased over 1 – 2 h and resolved by 24 h when comparing with the control (Barchowsky and

others, 1995). This increase occurred in a dose-dependent manner when H_2O_2 added was ranged from 25 μM to 200 μM (Barchowsky and others, 1995), indicating that treatment of low concentration of ROS can increase endothelial cell proliferation directly by acting on endothelial cells leading to the up-regulation of NF- κB and AP-1.

3.1.2.3.2 VEGF and endothelial cells functions

The positive correlation between VEGF and endothelial cells functions has been demonstrated previously. For example, Rafiee and others (2004) had shown that incubating human intestinal microvascular endothelial cells (HIMEC) with 50 ng/ml VEGF for 24 h resulted in a significant increase in cell number compared with the control. Besides, enhanced human umbilical vein endothelial cells (HUVEC) invasion stimulated by 20 ng/ml VEGF was observed by Kamath and others (2001).

When human cerebral endothelial cells (HCEC) was treated with 20 ng/ml VEGF, formation of microvessel-like tubes, which was defined as endothelial cord formations was induced (Fan and others, 2008). In another study using HIMEC as endothelial cell model, similar findings were observed (Rafiee and others, 2004). These results together suggested that VEGF does not specifically act on a particular endothelial cell type. Instead, a wide range of endothelial cells can give similar responses towards the induction of VEGF.

3.1.3 Use of antioxidants in cancer treatment

3.1.3.1 Antioxidant use in cancer therapy

Whether the use of antioxidant supplement during cancer therapy has improved efficacy is still controversial with divided opinions. While it has been suggested that antioxidant supplements may reduce the effectiveness of chemotherapeutical agents by eradicating the free radicals produced (Labriola and Livingston, 1999), there were findings that indicated the synergistic effect between antioxidant supplements and

anticancer drugs as well as the reduction of side effects given by chemotherapy and radiotherapy (Prasad and others, 1999).

3.1.3.2 Antioxidant and endothelial cells functions

Although the role of antioxidants in cancer therapy is still unknown, the use of antioxidants is believed to inhibit the development of tumor angiogenesis mediated by ROS as ROS (especially H_2O_2) plays an important role in regulating the production of VEGF, which is linked to *in vitro* endothelial cells growth, migration, invasion and tubule formation, in tumor cells. Many studies have found out that the use of antioxidants can greatly inhibit human endothelial cells functions by inhibiting VEGF expression and production mainly due to their hydrogen peroxide scavenging activities. One of the mechanisms is believed to act through down-regulating endothelial nitric oxide synthase (eNOS) activity (Polytarchou and Papadimitriou, 2005). Nitric oxide is an important modulator for the expression of VEGF and bFGF (Dulak and Jozkowicz, 2000). Since VEGF is positively correlated with angiogenesis, control of VEGF production can hence control the extent of angiogenesis and metastasis. Since VEGF can be up-regulated by H_2O_2, any chemical species that can strongly inhibit the formation of H_2O_2 or removing the existed H_2O_2 can be potential agents in decreasing endothelial cells functions. Epigallocatechin gallate, an antioxidant in green tea, was found to inhibit VEGF expression in human colon cancer cell line (HT29) (Jung and others, 2001) while electrolyzed reduced water, an antioxidant produced in the cathode during water electrolysis, was found to reduce VEGF expression in human lung cancer cell line (A549) (Ye and others, 2008). Table 3.2 shows the effects of some common antioxidants, including both dietary antioxidants and preventive antioxidants found in biological systems, on angiogenesis.

Table 3.2 Literature review on antioxidants and their anti-angiogenesis effect

Antioxidants	Effect on angiogenesis	References
4-(2-aminoethyl-benzenesulfonyl fluoride)	↓ HUVEC proliferation and migration	Polytarchou and Papadimitriou, 2005
	↓ endothelial NOS (eNOS) activity	
Apocynin	↓ HUVEC proliferation and migration	Polytarchou and Papadimitriou, 2005
	↓ endothelial NOS (eNOS) activity	
Catalase	↓ HUVEC proliferation and migration	Polytarchou and Papadimitriou, 2005
	↓ endothelial NOS (eNOS) activity	
Electrolyzed reduced water (ERW)	↓ HUVEC tube formation induced by A549 human lung adenocarcinoma cells	Ye and others, 2008
	↓ VEGF gene expression in A549 human lung adenocarcinoma cells	
	↓ VEGF extracellular secretion in A549 human lung adenocarcinoma cells	
L-NAME (N-nitro-L-arginine methyl ester)	↓ endothelial cell functions	Polytarchou and Papadimitriou, 2005
NADPH oxidase inhibitors	↓ HUVEC proliferation and migration	Polytarchou and Papadimitriou, 2005
	↓ endothelial NOS (eNOS) activity	
Rosmarinic acid (RA)	↓ HUVEC proliferation, migration, adhesion and tube formation	Huang and Zheng, 2005
	↓ intracellular ROS level	
	↓ H_2O_2-dependent VEGF expression of endothelial cells	
	↓ IL-8 release of endothelial cells	
Sodium pyruvate	↓ HUVEC proliferation and migration	Polytarchou and Papadimitriou, 2005
	↓ endothelial NOS (eNOS) activity	
Superoxide dismutase (SOD)	↓ HUVEC proliferation and migration	Polytarchou and Papadimitriou, 2005
Tempol (membrane permeable SOD mimetic)	↓ endothelial NOS (eNOS) activity	Polytarchou and Papadimitriou, 2005
	↓ HUVEC proliferation and migration	
	↓ endothelial NOS (eNOS) activity	
Vitamin E	↓ HMVEC tube formation	Tang and Meydani, 2001
	↓ H_2O_2-induced IL-8 production	

3.1.3.3 Anti-angiogenic effects of polyphenols

As mentioned in chapter 2, polyphenols possess excellent antioxidative power, it is not surprising that polyphenols can effectively inhibit tumor angiogenesis induced by ROS. Besides its anti-angiogenic effect mediated by its antioxidant activity, polyphenols can also act as potent angiogenesis inhibitors against angiogenesis although the exact mechanism is not known and believed to be complex (Cao and others, 2002). Table 3.3 summarized the anti-angiogenic effects of some phenolic compounds on endothelial cells functions.

3.1.3.3.1 Phenolic acids

Caffeic acid, one of the common phenolic acids, has reported to have antioxidant activities (Chen and Ho, 1997) and anti-inflammatory properties (Fernandez and others, 1998). Caffeic acid has been found to reduce the VEGF level secreted by human renal cell carcinoma by inhibiting VEGF promoter recruitment as well as HUVECs capillary-like networking formations (Jung and others, 2007) while its derivatives, caffeic acid phenethyl ester, also inhibited HUVECs tubule formation and cell invasion induced by colon carcinoma through down-regulating the MMP-2 and MMP-9 expression as well as VEGF production (Liao and others, 2003).

Besides caffeic acid, ellagic acid is another naturally occurring phenolic acids in fruits (Daniel and others, 1990). Ellagic acid is found to possess inhibitory activity towards VEGF-induced endothelial cells migration and endothelial cells morphogenic differentiation into capillary-like structures by specifically inhibiting the VEGF-induced tyrosine phosphorylation of VEGFR-2 (Labrecque and others, 2005). Its anti-angiogenic activity is also proved by the inhibition of HUVECs tubule formation and proliferation on a reconstituted extracellular matrix, which is linked to the decreased levels of MMP-2, MMP-9 and $VEGF_{165}$ production (Losso and others, 2004).

3.1.3.3.2 Tea catechin

Polyphenols in tea have been long believed to be related to cancer prevention and tumor inhibition. Among the tea polyphenols, epigallocatechin gallate (ECGC) is the most abundant catechin found. Studies have found that ECGC is powerful in inhibiting endothelial cells growth, including *in vitro* capillary endothelial cells proliferation, *in vivo* blood vessel formation and sprouting in developing chick embryos and corneal neovascularization in animal model (Cao and Cao, 1999). The anti-angiogenic mechanism behind is thought to be related to the inhibition of MMP-2 and MMP-9 (Garbisa and others, 2001) as well as urokinase plasminogen activator (u-PA) (Jankun and others, 1997), the down regulation of VEGF production in tumor cells (Jung and others, 2001) and the repression of AP-1, NFκB and STAT-1 transcription factor pathways (Lin and others, 1999).

3.1.3.3.3 Resveratrol

Resveratrol which can be found in high levels in seeds and skin of grapes is the major bioactive component in red wine. Recent studies have found that red wine is related to the reduction in endothelin-1 production in endothelial cells (Corder and others, 2001) while resveratrol reduces *in vitro* VEGF-induced endothelial cells tubule formation and migration (Brakenhielm and others, 2001), and induces apoptosis in endothelial cells (Szende and others, 2000). *In vivo* studies have found that resveratrol is also effective in suppressing FGF-2 and VEGF-induced neovascularization in chorioallantoic membrane (CAM) and reducing vascular density in mouse tumor model (Brakenhielm and others, 2001). Molecular studies have shown that the resveratrol-induced endothelial cells apoptosis may be related to p-53 and caspase-mediated pathways while the reduced VEGF-induced angiogenic effects in HUVECs is through the blockage of Src kinase-dependent tyrosine phosphorylation of VE-cadherin (Lin and others, 2003).

3.1.3.3.4 Genistein

Genistein, a soybean isoflavone, is linked to the inhibition of human breast cancer cells growth (Peterson and Barnes, 1991) as well as to the prevention of mammary cancer (Lamartiniere and others, 1995). It is found that genistein inhibits *in vitro* vascular endothelial cells proliferation derived from bovine adrenal cortex or aorta, endothelial cells migration and tubule formation (Fotsis and others, 1995). Besides *in vitro* studies, genistein is found to inhibit corneal neovascularization induced by FGF-2 in rabbits in *in vivo* studies (Kruse and others, 1997). The anti-angiogenic effect is thought to involve the down-regulation of MMP-9 and VEGF as well as the up-regulation of tissue inhibitor of metalloproteinases (TIMP)-1 resulted in the decrease in tumor invasion and blood vessel growth (Shao and others, 1998).

Table 3.3 Phenolic compounds with anti-angiogenic effects towards endothelial cell functions

Compound	Sources	Cell proliferation	Cell migration	Tube formation	References
Catechins					
Epicatechin (EC)	Tea	√			Yang and others, 1997
Epicatechin gallate(ECG)	Tea	√			Yang and others, 1997
Epigallocatechin (EGC)	Tea	√			Yang and others, 1997
Epigallocatechin gallate (EGCG)	Tea	√		√	Cao and Cao, 1999
Flavnoids					
Quercetin	Grapes	√	√	√	Morrow and others, 2001
Myricetin	Cranberry	√			Fotsis and others, 1997
Silibinin	Milk thistle	√			Zhao and Agarwal, 1999
Flavones					
Apigenin	Celery roots	√	√	√	Wei and others, 1990
Luteolin	Fruit	√	√		Wei and others, 1990
Flavanones					
Eriodictyol	Beans	√			Wei and others, 1990
hesperetin	Citric fruits	√			Wei and others, 1990
Isoflavones					
Genistein	Soybeans	√	√		Fotsis and others, 1995
Resveratrol	Grapes	√	√	√	Brakenhielm and others, 2001
Phenolic acids and derivatives					
Caffeic acid	Coffee			√	Jung and others, 2007
Ellagic acid	Fruits		√	√	Labrecque and others, 2005
Gallic acid	Tea			√	Lu and others, 2010
Sodium caffeate	Synthetic	√			Xu and others, 2004

3.2 Principles of Methods and Experimental Protocols

3.2.1 Sample preparation

All the *Aa* water extract used in the cell line models were dissolved in their respective media (RPMI 1640 for Vero; MEM for Caco-2; and DMEM/F12 for HUVEC) and sterilized by filtering through a 0.2 μm syringe filter (Minisart, Cat # 17821K) before applied to cells.

3.2.2 Toxicity of the *Aa* water extract

3.2.2.1 *Limulus* amebocyte lysate (LAL) test

Principle

The presence of endotoxin in the *Aa* water extract was determined by E-TOXATE™ [*Limulus* amebocyte lysate (LAL)] test (Sigma, Cat # ET0200). The E-TOXATE Reagent is prepared from a lysate of the circulating amebocytes of the horseshoe crab, *Limulus polyphemus*. Upon the exposure to trace amount of endotoxin, the opacity and viscosity increase or may even form gel if the endotoxin concentrations present in samples are high enough. Although the underlying mechanism for this reaction is not fully understood, it is believed to be analogous to the mammalian blood clotting involving the activation of trypsin-like preclotting enzymes in the presence of calcium ions and the modification of a "coagulogen" by the activated enzymes to form a clottable protein (Solum, 1973; Sullivan and others, 1975; Tai and Liu, 1977). Therefore, the formation of a hard gel (complete inversion of the tube or vial without disruption of the gel) indicates the presence of endotoxin while all other results like formation of soft gels, increase in turbidity or viscosity are considered as a negative result.

Procedures

The endotoxin level in the *Aa* water extract was detected by E-TOXATE™ according to manufacture's instruction. All equipments and ultra-pure water were autoclaved before use. In brief, designated volume of the *Aa* water extract (25 μg/ml and 50

μg/ml), endotoxin-free LAL reagent water and Endotoxin Standard Dilutions (0 – 400 EU/ml) were added to non-siliconized glass culture tubes. The E-TOXATE Working Solution was then added and the tube was covered with parafilm. After that, the tube contents were mixed gently for 10 s and were incubated in a 37°C water bath for 1 h without disturbance. The test tubes were then removed and were slowly inverted by 180° to observe the presence of gelation.

3.2.2.2 Toxicity towards normal cells

3.2.2.2.1 Cell line and its subculture

The normal kidney epithelial cell line, Vero (CCL-81, American Type Culture Collection (ATCC)) as shown in Figure 3.3 was used for evaluating the cytotoxic effects of the *Aa* water extract on normal cells.

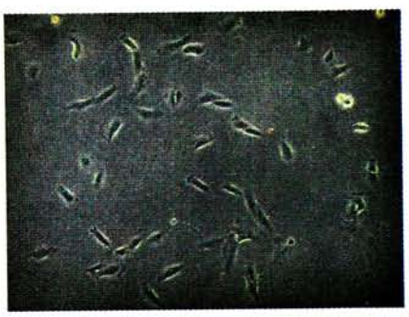


Figure 3.3
Photomicrograph of Vero

Vero cells were cultured in Roswell Park Memorial Institute-1640 (RPMI 1640) medium (Gibco, Cat # 31800-022) supplemented with 1.5 g/L sodium bicarbonate and the pH was adjusted to 7.2. The complete growth medium was prepared by adding heat-inactivated fetal bovine serum (FBS) (HyClone, Cat # 2SV30160.03) to a final concentration of 10% (v/v) and 1% penicillin-streptomycin (HyClone, Cat # SV30010) to the base medium. Cells were cultured in adhesive culture flask and were grown in a humidified atmosphere with 5% CO_2 at 37°C. The cells were passed to a new flask with fresh complete medium three times a week.

To subculture, the medium in the culture flask was first removed and discarded. The tissue culture flask was then rinsed with phosphate buffer saline (PBS, pH 6.8) to remove traces of serum that contains trypsin inhibitor. Cells were then treated with Trypsin-EDTA solution (HyClone, Cat # SH30042.01) for 1-3 min until cells were dispersed and the flask was then tapped gently to detach the cells. Several milliliters of medium were added to facilitate centrifugation (180 *g*, 5 min). After that, medium were discarded and cell pellets was resuspended in fresh complete growth medium.

Appropriate aliquot of cell suspensions was added to a new culture flask and was incubated at 37°C water jacketed CO_2 incubator (Forma Series II 3121).

3.2.2.2.2 Colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay

Principle

MTT, which measures the formation of a purple formazan by viable cells, was first described by Mosmann (1983). It is based on the color changes of the MTT, a tetrazolium salt, from pale yellow to a purple formazan product in the mitochondria of living cells. Mitochondrial dehydrogenases of viable cells cleave the tetrazolium ring in MTT, tetrazole, and form the purple MTT formazan crystals, which can then be solubilized by dimethyl sulfoxide (DMSO) or isopropanol and can be measured by spectrophotometer at a wavelength of 570 nm. Therefore, increase in viable cells will be resulted in the increase of purple formazan production, which in turns, increase in the absorbance measured. The conversion of MTT to formazan is shown in Figure 3.4

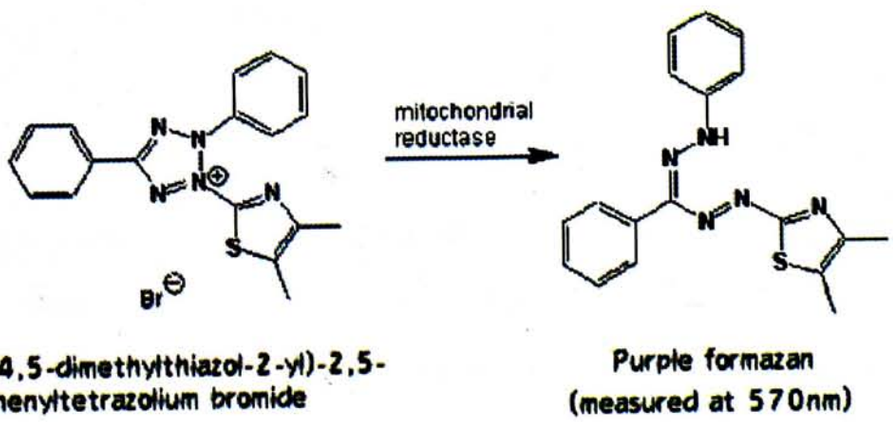


Figure 3.4 Conversion of MTT to formazan

Procedures

MTT assay was determined according to Mosmann (1983) with some modifications. Fifty microliters of cells were seeded in a 96-well plate at a density of $1x10^4$ cells/ well for 24 h in complete growth medium. After 24 h, 50 µl various concentrations of the *Aa* water extract (final concentration of 1.6125 – 800 µg/ml) in fresh medium or

fresh medium only (served as negative control) were added and cells were incubated for an additional 24, 48 and 96 hours. After treatments, 10 µl 5 mg/ml MTT (Sigma, Cat # M5655) solution in filtered PBS was added to each well. The plate was then incubated in 37°C for 4 h. Medium was then removed by gentle pipetting. One hundred microliters DMSO (Fluka, Cat # 41650) was added and the plate was gently shaken by rotator (Scientific Industries SI-1101) for 5 min to dissolve the purple formazan crystals formed. The absorbance at 570 nm was then measured using the UV-VIS spectrophotometer (Genesys5, Spectronic Instruments, USA).

Cell viability was expressed as % of control = ($Abs_{570sample}$/ $Abs_{570control}$) x 100%

3.2.3 Effect of the *Aa* water extracts on cancer cells

3.2.3.1 Cell line and its subculture

Adult human colorectal cancer cell line, Caco-2 (HTB-37, ATCC) as shown in Figure 3.5, was used for evaluating the effects of the *Aa* water extract on cancer cells.

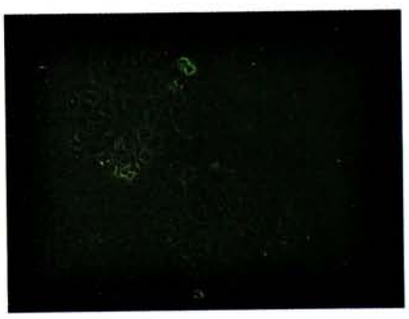


Figure 3.5
Photomicrograph of Caco-2

Caco-2 was cultured in Minimum Essential Medium (MEM) (Gibco, Cat # 41500-034) supplemented with 1.5 g/L sodium bicarbonate and the pH was adjusted to 7.2. The complete growth medium was prepared by adding heat-inactivated FBS (HyClone, Cat # 2SV30160.03) to a final concentration of 20% (v/v) and 1% penicillin-streptomycin (HyClone, Cat # SV30010) to the base medium. Cells were cultured in adhesive culture flask and were grown in a humidified atmosphere with 5% CO_2 at 37°C. The cells were passed to a new flask with fresh complete medium twice a week. The subculturing protocol was same as the one used in Vero.

3.2.3.2 Redox status

Principle

The measurement of intracellular reactive oxygen scavenging activity of Caco-2 was

performed using the dichlorofluorescein assay for flow cytometry (Bass and others, 1983). Dichlorofluorescin diacetate (DCFH-DA) was first reported to be used for measuring hydrogen peroxide in aqueous solution (Brandt and Keston, 1965 and Keston and Brandt, 1965). It was then modified to test the intracellular redox status as this stable, non fluorescent DCFH-DA is able to penetrate through the cell membrane and rapidly be deacetylated by cytosolic enzymes, esterases, to 2'7'-dichlorofluorescin (DCFH) and trapped within the cell (Figure 3.6). This non-fluorescent DCFH then undergoes rapid oxidation in the presence of hydrogen peroxide to a highly fluorescent 2',7'-dichlorofluorescein (DCF) and this fluorescent signal can then be measured by fluorescence microscopy (Yang and others, 2005), video microscopy (Leach and others, 2001), flow cytometry (Takada and others, 2002) or microtiter plate analysis (Wan and others, 2003). In this study, flow cytometry is used because it can integrate signals from individual cells (Davey and Kell, 1996).

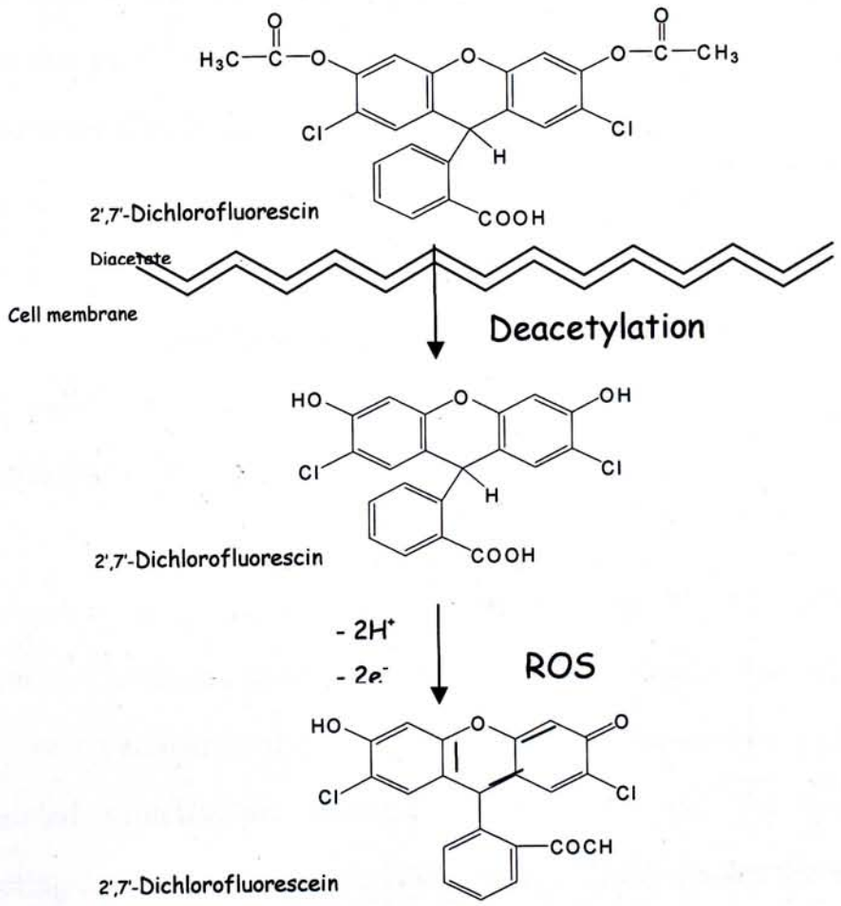


Figure 3.6 Mechanism of the entry of DCFH-DA into cells (Modified from Bass and others, 1983)

Procedures

Cells ($2.5x10^5$/ flask) were seeded in a T-25 culture flask two days before treating with various concentrations of the *Aa* water extract (final concentration of 6.25 – 50 μg/ml) for 24 h. After the treatment, culture supernatants were filtered and stored in aliquots of 1 ml in a -80°C deep freezer (U85-18m, So-low, USA) for later use on the measurement of VEGF secretion (Section 3.2.3.3). Flask was then rinsed twice with ice-cold PBS. After rinsing, cells were harvested with Trypsin/EDTA solution and were washed with 2 ml ice-cold PBS. After washing, cell numbers were counted and approximately $3x10^5$ cells from each treatment were transferred to a 15 ml centrifuge tube and made up to 1ml with PBS. After collection, cells were centrifuged for 5 min, 180 *g* and were then washed with 2 ml ice-cold PBS twice again with centrifugation (180 *g*, 5 min, 4°C). After that, 1 ml DCHF-DA (0.5 μg/ml) (Sigma, Cat # D6883) was added to resuspend the cells and was incubated at 37°C water bath for 10 min. After incubation, cells were centrifuged (180 *g*, 5 min, 4°C) and washed with 5 ml ice-cold PBS three times. After the final centrifugation step, cells were resuspended in flow tubes with 0.6 ml PBS and the fluorescent signal was then measured by a CXP 500 flow cytometer (Beckman Coulter, Miami, FL).

Intracellular ROS scavenging activity was expressed as % of control

$= [1-(\text{fluorescent signal}_{\text{sample}}/ \text{fluorescent signal}_{\text{control}})] \times 100\%$

3.2.3.3 VEGF secretion

Principle

The VEGF level released in the culture supernatants can be measured by an enzyme-linked immunosorbent assay (ELISA) method. With the anti-human $VEGF_{165}$ antibody being coated on the microplate, VEGF in the samples can then be captured. Unbounded proteins are washed and removed and the binding of biotinylated detecting antibody on the second site on the VEGF causes the formation of a yellow colored product after the addition of streptavidin-horseradish peroxidase and 3,3',5,5' tetramethyl benzidine (TMB).

Procedures

The VEGF secretions in the Caco-2 medium were determined using a Human VEGF ELISA kit (Thermo Scientific, Cat # EHVEGF) according to manufacture's instructions. In each well of the anti-human VEGF coated 96-well plate, 50 µl culture supernatants collected from the flow cytometric assay (Section 3.2.3.2) or VEGF standard (0 – 2000 pg/ml) was added and the plate was incubated for 2 h at room temperature (RT). After incubation, the plate was washed vigorously for three times using the wash buffer. One hundred microliters biotinylated antibody reagent (contains 0.1% sodium azide) was then added to each well and the plate was incubated for an additional 1 at RT. It was then again washed vigorously for three times with the wash buffer. One hundred microliters streptavidin-HRP reagent (with 0.1% sodium azide) was added. After incubating for 30 min and washed the plate with wash buffer for three times, 100 µl TMB substrate was added to each well. The plate was then incubated in dark for 30 min at RT and 100 µl stop solution (contains 0.16 M sulfuric acid) was added to stop the reaction. The absorbance at 450 nm and 550 nm was measured using an automated multifunctional monochromator reader (Safire Tecan F129013).

The amount of human VEGF in the culture supernatant was determined by interpolating from the sample absorbance value using the VEGF standard curve.

3.2.4 *In vitro* cell culture anti-angioenesis analysis

3.2.4.1 Cell line and its subculture

The human umbilical vein endothelial cell, HUVEC-C (CRL-1730, ATCC) as shown in Figure 3.7, was used for evaluating the effects of the *Aa* water extract on endothelial cells.

Figure 3.7
Photomicrograph of HUVEC

HUVEC-C was cultured in Dulbecco's modified Eagle's medium/Ham's Nutrient Mixture F12 (1:1) (DMEM/F12) (Gibco, Cat # 12400-024) supplemented with 1.5

g/L sodium bicarbonate and the pH was adjusted to 7.2. The complete growth medium was prepared by adding 0.1 mg/ml heparin (Sigma, Cat # H3393), 0.03 mg/ml endothelial cell growth supplement (ECGS) (Sigma, Cat # E2759) and heat-inactivated FBS to a final concentration of 10% (v/v) and 1% penicillin-streptomycin to the base medium. Cells were cultured in 0.1% gelatin (BBL, Cat # 02-158) coated adhesive culture flask and were grown in a humidified atmosphere with 5% CO_2 at 37°C. Medium were renewed every two days and the cells were passed to a new flask with fresh complete medium once a week. The subculturing protocol was same as the one used in Vero, and cells with a numbered passage from 6 to 12 were used.

3.2.4.2 Endothelial cells proliferation

Principle

As mentioned above, the action of MTT is determined by mitochondrial dehydrogenases, MTT, therefore, can not only used for measuring cell viability, it can also help in determining the extent of proliferation after treatment with samples.

Procedures

The MTT assay used here is similar to the one used in measuring the cell viability in Vero except some modifications (section 3.2.2.2.2). In brief, 50 µl cells were seeded in a 0.1% gelatin-coated 96-well plate at a density of $1x10^4$ cells/ well for 24 h in complete growth medium. After 24 h, cells were washed with PBS twice and the medium was changed to containing only 0.5% FBS (starve medium) to starve the cells for 24 h. Cells were then treated with samples (co-treated with 50 µl various concentrations of the *Aa* water extract (final concentration of 1.6125 – 25 µg/ml) or 5 mM gallic acid (Sigma, Cat # G8647), caffeic acid (Sigma, Cat # C-0625) and protocatechuic acid (Sigma, Cat # P-5630) and 20 ng/ml VEGF (Sigma, Cat # V7259) in fresh medium) or treated with fresh medium only (served as control vehicle) or 20 ng/ml VEGF only (served as control) for 24 h. Steps after sample treatment were same as those used in determining the viability of Vero.

Cell proliferation was expressed as % of control vehicle

$= (Abs_{570sample} / Abs_{570control\ vehicle}) \times 100\%$

3.2.4.3 Endothelial cells migration

3.2.4.3.1 Wound healing assay

Principle

In the presence of angiogenic factor or antiangiogenic factor, endothelial cells move, in a manner of chemotaxis, towards or away accordingly. Evaluation of the endothelial cells chemotaxis can be done in several methods and the simplest and inexpensive method is called the wound healing assay (Rodriguez and others, 2005).

Migration of endothelial cells determined by wound healing method is achieved by producing a "wound" scraped by a scraping tool in a confluence monolayer. This method is based on the idea that in *in vivo* wound healing, endothelial cells migration into a denuded area is a pivotal process. As endothelial cells have a tendency to migrate, the scraped area will then be refilled by migrated endothelial cells and the monolayer will be re-formed. Therefore, by measuring the distance between the wound edges, the rate and extent of cells migration can be calculated (Pepper and others, 1990).

Procedures

The wound healing method was determined according to Sato and Rifkin (1988) with some modifications. Three hundred microliters cells were seeded in a 0.1% gelatin-coated 24 well plate at a density of $3x10^5$ cells/ well for 24 h in complete growth medium. It was then washed with PBS twice and the cells were starved for another 24 h by changing the medium to starve medium. The HUVECs were then scraped away horizontally in each well using a sterilized P100 pipette tip. After washing with PBS twice, the cells were treated with 300 µl samples (co-treated with various concentrations of the *Aa* water extract (final concentration of 1.6125 – 25 µg/ml) or 5 mM gallic acid, caffeic acid and protocatechuic acid and 20 ng/ml VEGF

in fresh medium) or treated with fresh medium only (served as control vehicle) or 20 ng/ml VEGF only (served as control) for 24 h. Three randomly views along the line were selected and photographed using an inverted microscope (Nikon ECLIPSE TS-100F) at 0 h. After 24 h, another set of images were taken by the same method.

Image analysis for determining the extent of migration was performed by Gwyddion data analysis software (freeware, GNU General Public License). The average distance between the two wound edges under each condition was measured and the percentage of wound size after 24 h was calculated using the following equation:

Wound size reduction percentage was expressed as % of control vehicle

$= (\text{Wound size reduction}_{\text{sample}} / \text{Wound size reduction}_{\text{control vehicle}}) \times 100\%$

3.2.4.3.2 Transwell culture insert assay

Principle

Besides the wound healing assay, migration of endothelial cells can be achieved using transwell culture insert method, which is highly sensitive to small differences in concentration gradients (Falk and others, 1980). As shown in Figure 3.8, there are two compartments present in the transwell which are separated by a porous filter membrane (0.4, 3 or 8 μm) coated in the cell culture insert. It can closely mimic an *in vivo* environment for monitoring cell migrations. Cells are first placed on the upper chamber while the chemical signal or angiogenic promoter or inhibitor is placed in the lower chamber. Only those actively migrating cells can penetrate through the porous filter and move from the upper to the lower compartment (Bird and others, 2009). The number of cells migrated to the lower compartment can then be directly counted under a microscope or indirectly measured by trypan blue exclusion method or MTT assay.

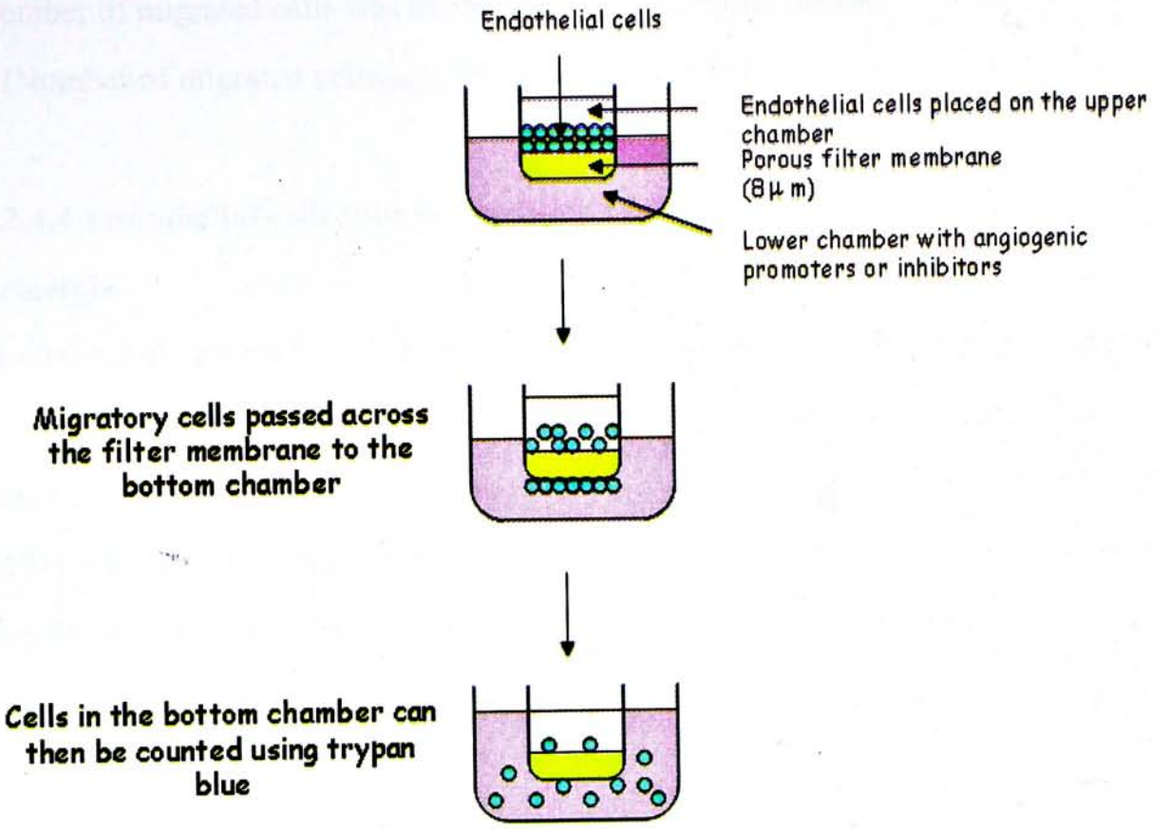


Figure 3.8 Principle of tranwell culture insert method (Modified from http://www.biocat.com/cgi-bin/page/sub1.pl?sub1=cell_migration&main_group=cell_biology)

Procedures

Endothelial cells chemotaxis assay measured by transwell culture insert assay was carried out according to Kim and others (2003) with some modifications. In this method, the 24 well plate insert (Polycarbonate (PCTE) membrane with 6.5 mm diameter and 8.0 μm pore size) (SPL Life Science) was used. The lower chamber of the transwell was filled with 600 μl starved medium with samples (co-treated with various concentrations of the *Aa* water extract (final concentration of 1.6125 – 25 μg/ml) or 5 mM gallic acid, caffeic acid and protocatechuic acid and 20 ng/ml VEGF in starved medium) or with starved medium only (served as control vehicle) or 20 ng/ml VEGF only (served as control). Cells on the upper chamber (culture inserts) was prepared using starved medium. $1x10^4$ cells (in 100 μl) were seeded on the upper chamber and the assembled transwell was then incubated in incubator for 6 h. The number of migrated cells was counted under an inverted microscope.

Number of migrated cells was expressed as % of control vehicle

= (Number of migrated $cells_{sample}$/ Number of migrated $cells_{control\ vehicle}$) x 100%

3.2.4.4 Endothelial cells tubule formation

Principle

In vitro tubule assays are regarded as a critical and representative feature in the later stages of angiogenesis. There is an increasing popularity in determining the stimulated formation of capillary-like tubules by endothelial cells (Staton and others, 2004). The cells differentiation and tubule formation can be measured in an *in vitro* way by culturing endothelial cells on matrices with fibrin, collagen or Matrigel as capillary-like tubes are formed on these matrices when endothelial cells are cultured on them (Kanzawa and others, 1993).

Matrigel is made from extracellular and basement membrane proteins from mouse Engelreth-Holm-Swarm sarcoma (Kubota and others, 1988). It is liquid at 4°C but solidifies at 37°C. Seeding endothelial cells on this solidified matrix results in the rapid formation of tubes. Tubule formation assay is a simple and rapid method in the determination of angiogenesis. After allowing Matrigel to gel at 37°C and seeding endothelial cells together with angiogenic promoters or inhibitors, the tube network will be formed within 20 h depending on the number of cells seeded and the health status of cells.

Procedures

The tubule formation assays was determined according to Lee and others (1999) with some modifications. All plates and pipette tips in this experiment were chilled to -20°C before use. In each of the 96 well, 50 µl of 4°C Matrigel (BD Biosciences, Cat # 354234) was coated and the plate was incubated in 37°C incubator for 1 h to solidify the Matrigel. About $1x10^4$ cells (in 50 µl) prepared by starved medium was seeded onto the layer of Matrigel. After that, 50 µl samples (co-treated with various concentrations of the *Aa* water extract (final concentration of 1.6125 – 25 µg/ml) or

5mM gallic acid, caffeic acid and protocatechuic acid and 20ng/ml VEGF in starved medium) or with starved medium only (served as control vehicle) or 20 ng/ml VEGF only (served as control) prepared in starved medium were immediately added to each well containing cells. The plate was then incubated for 5 h in 37°C. After that, images of three randomly selected fields were captured using the inverted microscope and the total tubule length, total tubule area and the total number of junctions formed was analyzed and quantified using an automated image analysis tool, MATLAB®-based program (AngioQuant) (The MathWorks, Natick, MA).

Total tubule length was expressed as % of control vehicle

$= (\text{Total tubule length}_{\text{sample}} / \text{Total tubule length}_{\text{control vehicle}}) \times 100\%$

Total tubule area was expressed as % of control vehicle

$= (\text{Total tubule area}_{\text{sample}} / \text{Total tubule area}_{\text{control vehicle}}) \times 100\%$

Total number of junctions was expressed as % of control vehicle

$= (\text{Total number of junctions}_{\text{sample}} / \text{Total number of junctions}_{\text{control vehicle}}) \times 100\%$

3.2.5 *In vitro* organ culture anti-angiogenesis analysis

3.2.5.1 Aortic ring assay

Principle

Organ culture represents various aspects of angiogenesis from endothelial cells proliferation, migration and microvessel formation to invasion through extracellular matrices (Chau and Figg, 2006). Tissues from various origins and types like rat aorta (Auerbach and others, 2003), chick embryonic aorta arch (Muthukkaruppan and others, 2000), and porcine (Stiffey-Wilusz and others, 2001), could be used for organ culture outgrowth assay. Among them, the most widely used assay is called the rat aortic ring assay (Auerbach and others, 2003).

In these outgrowth assays, segments of specific tissue type are placed on matrix-coated wells and are covered with an additional layer of the matrix, resulting in the embedment of the tissue in a three-dimensional matrix. They are then cultured in a basal nutrient growth medium. After a designated time period, the outgrowths of microvessels occurred spontaneously at a basal rate from the cut surfaces are monitored and the number of microvessels outgrowth could be determined.

Procedures

The aortic ring assay was carried out according to Matsubara and others (2005) with some modifications. Briefly, the thoracic rat aorta was removed from a male Sprague Dawley rat (body weight 200 – 250 g). After the removal of fibroblast and fatty tissues, the aorta was cut into short segments of about 1 – 1.5 mm in length. It was then washed six times with human endothelial serum free medium (HESFM) (Invitrogen, Cat # 11111044) and placed in the center of a 100 µl Matrigel coated well on a 48 culture well and covered with an additional 100 µl Matrigel. The gel was incubated at 37°C for 45 min to solidify. After 45 min, the gel was overlaid with 750 µl of HESFM and incubated for 24 h to avoid dehydration of the arotic ring. After 24 h, the medium was changed to a conditioned medium. The conditioned medium included the followings: (i) HESFM co-treated with various concentrations of the *Aa* water extract (final concentration of 1.6125 – 25 µg/ml); (ii) 5 mM gallic acid, caffeic acid and protocatechuic acid and 20 ng/ml VEGF; (iii) with HESFM only (served as control vehicle); and (iv) with 20 ng/ml VEGF only (served as control). The conditioned medium was renewed every two days and the images of microvessels outgrowths were captured using an inverted microscope and the number of microvessels formed was counted at day 7 after implantation of the aortic ring.

Vessel outgrowth percentage was expressed as % of control vehicle

= (Number of vessel outgrowth$_{sample}$/ Number of vessel outgrowth$_{control\ vehicle}$) x 100%

3.2.6 Statistical analysis

All experiments were done in triplicate and data were expressed as mean ± standard derivation (SD).

For analyzing the cytotoxicity effect, student's *t*-test was used to investigate if there is any significant decrease in cell viability at different concentrations of the *Aa* water extract while one-way ANOVA with Tukey's method was used for multiple comparisons on the effect of intracellular ROS level and VEGF production in Caco-2 among various treatment concentrations.

In evaluating the anti-angiogenic effects towards HUVECs proliferation, migration and tubule formation, the term 'control vehicle' refers to the group without any treatment of VEGF, *Aa* water extract and phenolic acids while the term 'control' refers to the group with VEGF addition but no *Aa* water extract and phenolic acids. Moreover, the proliferation, migration and tubule formation, including total tubule length, total tubule area and the total number of junctions formed, of HUVECs in the control vehicle were all normalized to 100% for comparison. Three statistical analyses were performed in each experiment. First of all, student's *t*-test was used to investigate the variability between control vehicle and the control to determine if significant increase was observed. Secondly, the variability between the control and the *Aa* water extract treatment groups (or phenolic acid treatment groups) was compared using student's *t*-test as well. Finally the multiple comparisons among various treatment concentrations were investigated by one-way ANOVA with Tukey's method.

All these statistical analysis was performed by the Statistical Package for Social Sciences (SPSS 16.0, 2007). Differences with $p < 0.05$ were considered statistically significant.

3.3 Results and Discussions

3.3.1 Toxicity of the *Aa* water extract

3.3.1.1 *Limulus* amebocyte lysate (LAL) test

Endotoxin is a complex lipopolysaccharide (LPS) or lipooligosaccharide (LOS) found in the outer membrane of most gram-negative bacteria like *E. coli*. The presence of endotoxin is very important for these bacteria to cause diseases (Tzeng and others, 2002). Studies of endotoxin have shown that the presence of endotoxin may affect the performance of cell culture and therefore contributing experimental variation. Hence, it is necessary to confirm the absence of endotoxin contamination in any sample before carrying out cell culture models. The Limulus Amebocyte Lysate assay used in this study is a very sensitive assay for detecting endotoxin due to the powerful amplification through an enzymatic cascade. Very low levels of LPS could cause coagulation of the limulus lysate.

The presence of any endotoxin in the *Aa* water extract was checked by E-TOXATE™. LPS was used as the reference standard in this endotoxin test. At a concentration above 0.25 EU/ml, LPS gave a positive result by causing the clotting of lysate leading to the formation of hard gels. The *Aa* water extract could only form soft gel at a concentration of 50 μg/ml while a clear liquid was found at a concentration of 25 μg/ml. This indicated that the level of endotoxin in the *Aa* water extract was extremely low (< 0.25 EU/ml) when compared to the sensitivity limit of the kit and therefore it was not a concern.

3.3.1.2 Toxicity towards normal cells

When a study involved a cell culture model, it is quite important to test if the sample applied only affects the targeted population but not others. For example, in carcinogenesis studies, the potential drug applied should only selectively affect the proliferation and growth of cancer cells and not to normal cells. Similarly, in the present tumor angiogenesis study, the sample applied (ie the *Aa* water extract)

should only affecting cancer cells and endothelial cells but not the normal cells. Therefore, testing Vero viability after the *Aa* water extract application is needed to confirm only the targeted cell populations is being affected.

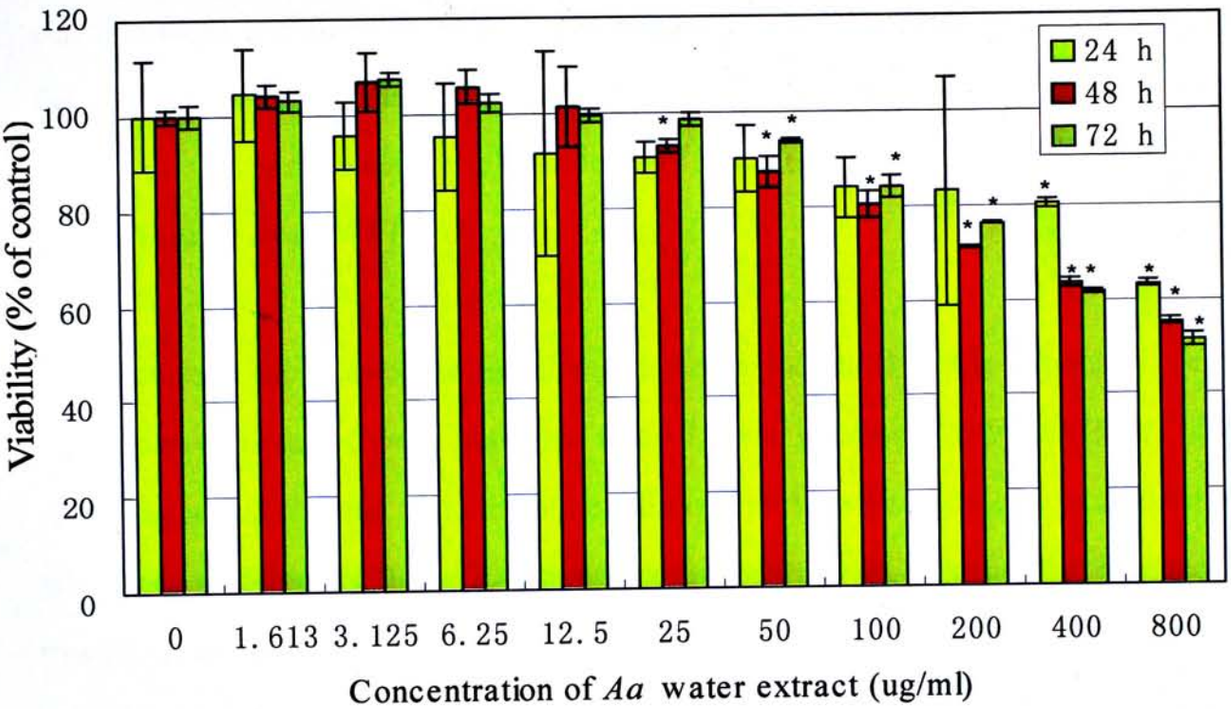


Figure 3.9 Toxic effect of the *Aa* water extract on Vero measured MTT assay

Each value is expressed as mean ± SD ($n = 3$)

* Indicate significantly lower than non-treatment group in the same time point ($p < 0.05$, student's t test)

As shown in Figure 3.9, the *Aa* water extract only inhibits the growth of Vero at very high concentrations and long incubation time. When Vero was incubated with the *Aa* water extract at a concentration of 800 μg/ml for 72 h, the survival rate dropped to around 50% (51.28 ± 1.40%). This finding suggested that even when treating cancer cells or endothelial cells at such high concentration of mushroom extract and extended period of time could inhibit their proliferation, other normal cells were very likely to be affected similarly. Therefore, only low concentrations (1.6125 – 50 μg/ml) of the *Aa* water extract were used for the present cell culture experiments as they did not substantially affect the growth of Vero.

3.3.2 Effect the of *Aa* water extracts on cancer cells

3.3.2.1 Redox status

Although there are many chemical assays to test the antioxidative effect of a sample, whether the antioxidants present in the sample could enter or take effect within the cell is always questionable (Zhang and others, 2010). Therefore, besides checking the antioxidant capacities based on chemical methods, a more biologically relevant assay was also performed in the present study based on the use of a non-fluorescent, cell permeable dye, DCFH-DA.

A reduction in the fluorescent signal was detected, indicated by the left-shifted peak signal curve compared with untreated Caco-2 cells, as shown in Figure 3.10. This result suggested that the antioxidant activity of the *Aa* water extract could possibly take place within cells. Table 3.4 shows the intracellular ROS levels (%) in Caco-2 treated with the *Aa* water extract measured by flow cytometry based on the determination of the intensity of the fluorescence relative to that of control cells (ie cells without the *Aa* water extract treatment). Generally speaking, the intracellular ROS scavenging activity increased with increasing concentrations of the *Aa* water extract (Table 3.4), with a 0.9107 correlation coefficient, indicating a linear relationship between *Aa* water extract concentration and the intracellular ROS scavenging activities. Besides, significant difference ($p < 0.05$) was found in the intracellular ROS scavenging activity between different *Aa* water extract concentrations with 44.59 ± 3.85% found when the concentration of *Aa* water extract reached 50 μg/ml (Table 3.4).

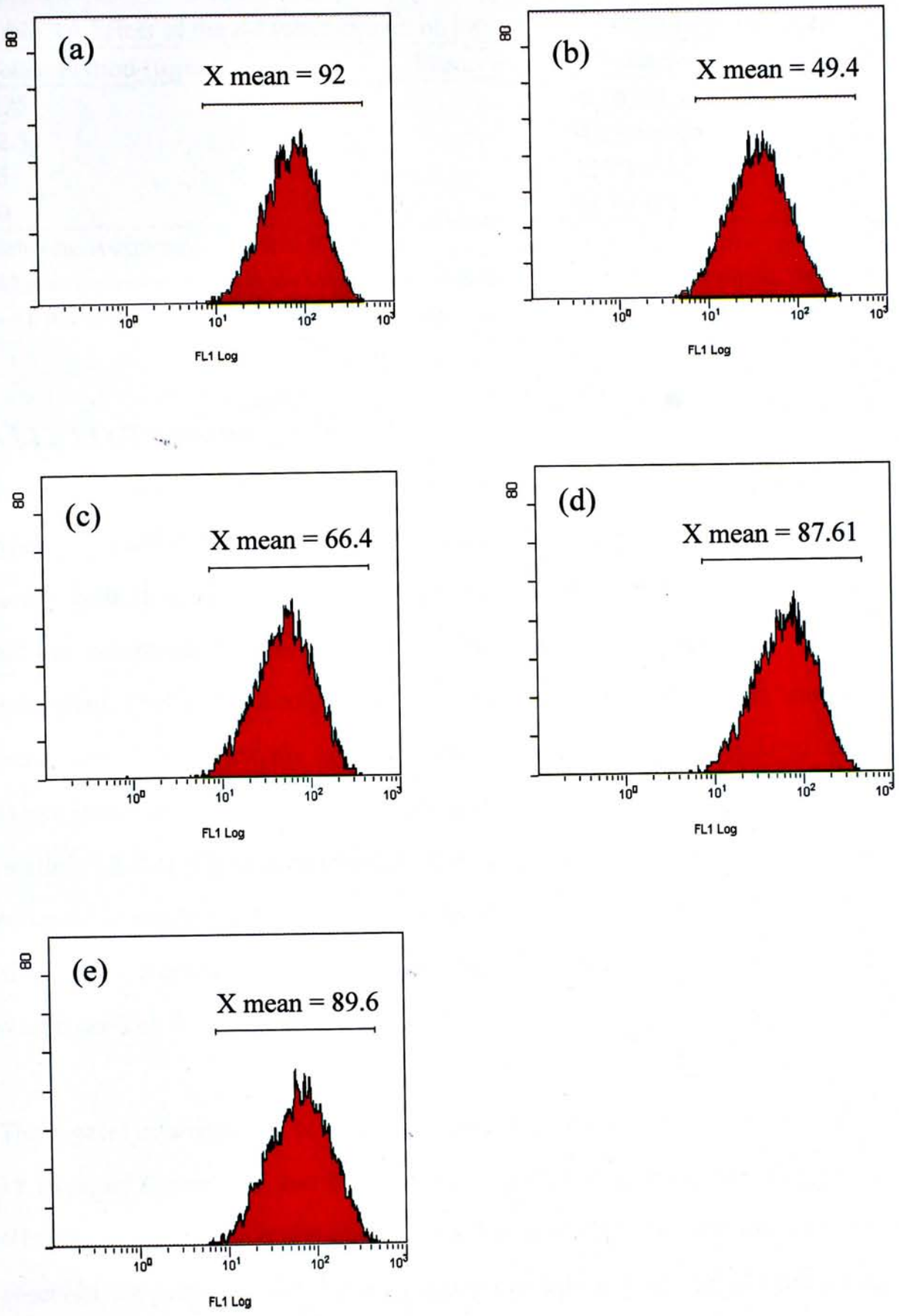


Figure 3.10 Fluorescent signals detected in Caco-2 measured by flow cytometry after treating cells with the *Aa* water extract at a concentration of 0 µg/ml (control) (a); 50 µg/ml (b); 25 µg/ml (c); 12.5 µg/ml (d) and 6.25µg/ml (e) after 24 h

Table 3.4 Effect of the *Aa* water extract on intracellular ROS release in Caco-2

Concentration (μg/ml)	Intracellular ROS scavenging activity (%)
6.25	6.66 ± 5.97^{a}
12.5	16.3 ± 0.24^{b}
25	33.73 ± 1.56^{c}
50	44.59 ± 3.85^{d}

Each value is expressed as mean ± SD ($n = 3$)

Different superscript letters in the same column indicate significant difference among the means ($p < 0.05$, ANOVA, Tukey's multiple comparison)

3.3.2.2 VEGF secretion

Since the production of ROS could stimulate the induction of VEGF in various cell types (Ruef and others, 1997; Chua and others, 1998) and the production of ROS in cancer cells is usually high (Szatrowski and Nathan, 1991), the ability of cancer cells to metastasis has been shown to link with its ROS production and VEGF generation (Ye and others, 2008). In this study, the *Aa* water extract was demonstrated to effectively scavenge various types of free radicals in chemical assays performed (section 2.3.2) and intracellular ROS in colon cancer cell, Caco-2 (section 3.3.2.1). These data together suggested that the *Aa* water extract might be powerful in regulating VEGF secretion in Caco-2 mediated by ROS. To verify this idea, culture supernatants collected from the flow cytometric assay (section 3.2.3.2) were examined for their VEGF content.

The highest concentration of VEGF was found in control cells which was 435.81 ± 15.54 pg/ml (Table 3.5), and treating Caco-2 with the *Aa* water extract lowered the VEGF secretion while a significant reduction ($p < 0.05$) in intracellular ROS was observed when the *Aa* water extract applied as low as 6.25 μg/ml (Table 3.4), no significant difference ($p > 0.05$) could be found in the secreted VEGF accumulations at such low concentration (Table 3.5). Only when the concentration of the *Aa* water extract applied was increased to 25 μg/ml, significant reduction ($p < 0.05$) in the VEGF secretion was then be observed (Table 3.5).

Table 3.5 Effect of the *Aa* water extract on VEGF secretion in Caco-2

Concentration (μg/ml)	VEGF secretion (pg/ml)
0 (Control)	435.81 ± 15.54^{a}
6.25	363.38 ± 13.35^{ab}
12.5	372.48 ± 48.45^{ab}
25	359.71 ± 33.96^{b}
50	248.48 ± 24.80^{c}

Each value is expressed as mean ± SD ($n = 3$)

Different superscript letters in the same column indicate significant difference among the means ($p < 0.05$, ANOVA, Tukey's multiple comparison)

3.3.2.3 Relationship between intracellular ROS and VEGF secretion detected

The above results suggested that the *Aa* water extract could reduce the intracellular ROS level as well as the VEGF production in a human colon cancer cell line, Caco-2. An increase in the ROS scavenging activity was correlated with a decrease in VEGF secretion only at a high concentration of the *Aa* water extract as shown in Figure 3.11.

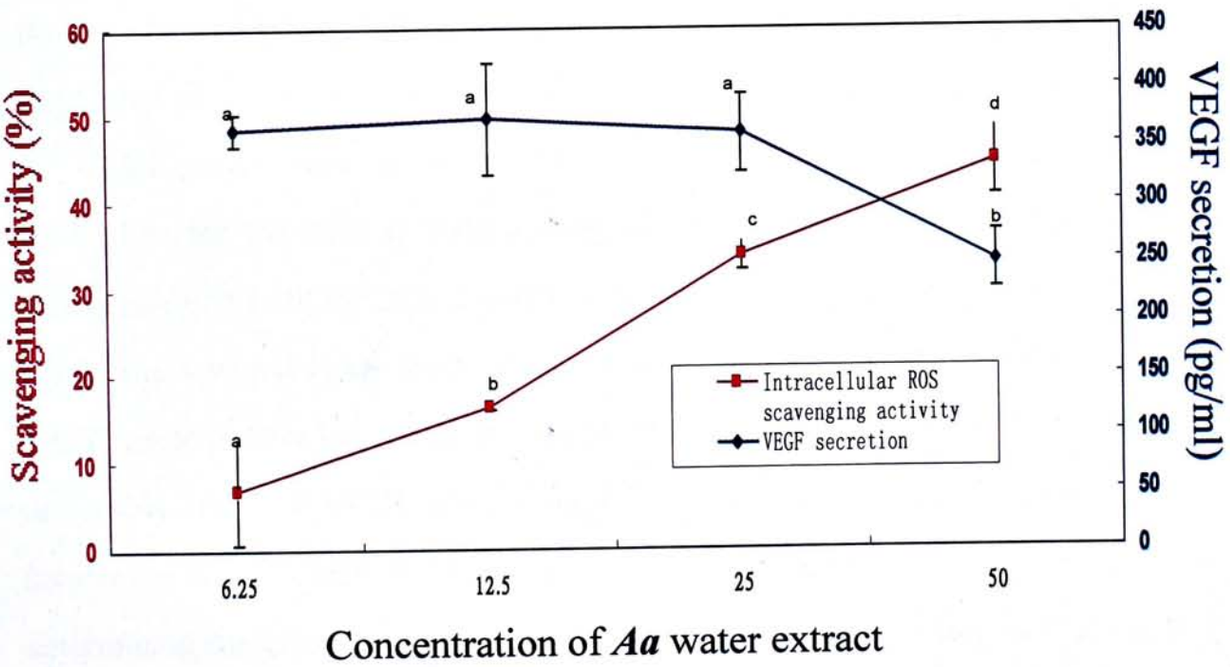


Figure 3.11 Effect of the *Aa* water extract on intracellular ROS release and VEGF secretion in Caco-2

Each value is expressed as mean ± SD ($n = 3$)

Different letters indicate significant difference among the *Aa* water extract treatment groups within the same assay ($p < 0.05$, ANOVA, Tukey's multiple comparison)

Previous *in vitro* studies have demonstrated that the production of ROS, especially hydrogen peroxide, is significantly higher in several tumor cells, including breast carcinoma, colon carcinoma, and ovarian carcinoma, than their normal counterparts (Szatrowski and Nathan, 1991) and small amount of hydrogen peroxide (< 5 μM) could actually increase VEGF mRNA expression and hence VEGF produced in cells (Kubo and others, 2007). Therefore, the production of ROS in cancer cells was thought to be linked to the triggering of angiogneic process in those cancer cells (Monte and others, 1997; Stone and Collins, 2002; Qian and others, 2003). Therefore, the blockage of H_2O_2 release and decrease in VEGF secretion from cancer cells should result in an anti-angiogenic effect (Ye and others, 2008).

The present study demonstrated that the *Aa* water extract scavenged endogenous H_2O_2 at low concentration as well as decreased VEGF production in Caco-2, showing its potential for therapeutic purposes. In addition, at low concentrations (6.25 - 12.5 μg/ml) of the *Aa* water extract, it was found that the decrease in VEGF protein secreted in the culture medium was not significant when compared to the significant decrease in intracellular ROS level. One possible interpretation was that the VEGF protein level assayed were cumulative instead of time-point levels and time taken for the ROS to induce a signal transduction pathway to stimulate the production of VEGF in Caco-2 might be longer than the incubation time used. As a result, the lowered H_2O_2 level could not instantly cause a significant decrease in VEGF protein secretion tested in the culture medium. It was predicted that a more noticeable result in VEGF protein secretion could be obtained by increasing the incubation time. VEGF mRNA expression might serve as a better indicator in determining the effect of the *Aa* water extract in VEGF production in Caco-2. It is because transcription is the first step in gene expression and the transcribed mRNA further undergoes translation to create a protein. Any changes, therefore, will be firstly reflected in the VEGF mRNA level and longer time is required to be reflected in the accumulated protein levels in VEGF protein secretion.

3.3.3 Effect of the *Aa* water extract on angiogenesis

3.3.3.1 Endothelial cells proliferation

As mentioned in the introduction, it has been well recognized that all successful tumors must undergo neovascularization (angiogenesis) in order to acquire adequate nutrients and oxygen for continuous growth (Folkman, 1971) and vascular endothelial growth factor (VEGF) is the best characterized angiogenic cytokine and the most important angiogenic factor in sustaining tumor growth (Goto and others, 1993) by inducing proliferation, migration and tubule formation of endothelial cells (Waltenberger and others, 1994; Cai and others, 2006; Belloni and others, 2007).

Many angiogenic inhibitors, such as tea catechin, epigallocatechin gallate (EGCG) and resveratrol, suppress endothelial cells proliferation *in vitro* (Matsubara and others, 2005). To determine the anti-angiogenic activity of the *Aa* water extract *in vitro*, its inhibitory effect on VEGF-induced HUVECs proliferation was first examined. Treatment of HUVECs with 20 ng/ml VEGF resulted in a significant increase ($144.06 \pm 4.20\%$, $p < 0.05$) in cells proliferation while this increase was significantly decreased by co-incubation together with the *Aa* water extract at various concentration (1.6125 – 25 μg/ml) for 24 h in a dose-dependent manner (Figure 3.12).

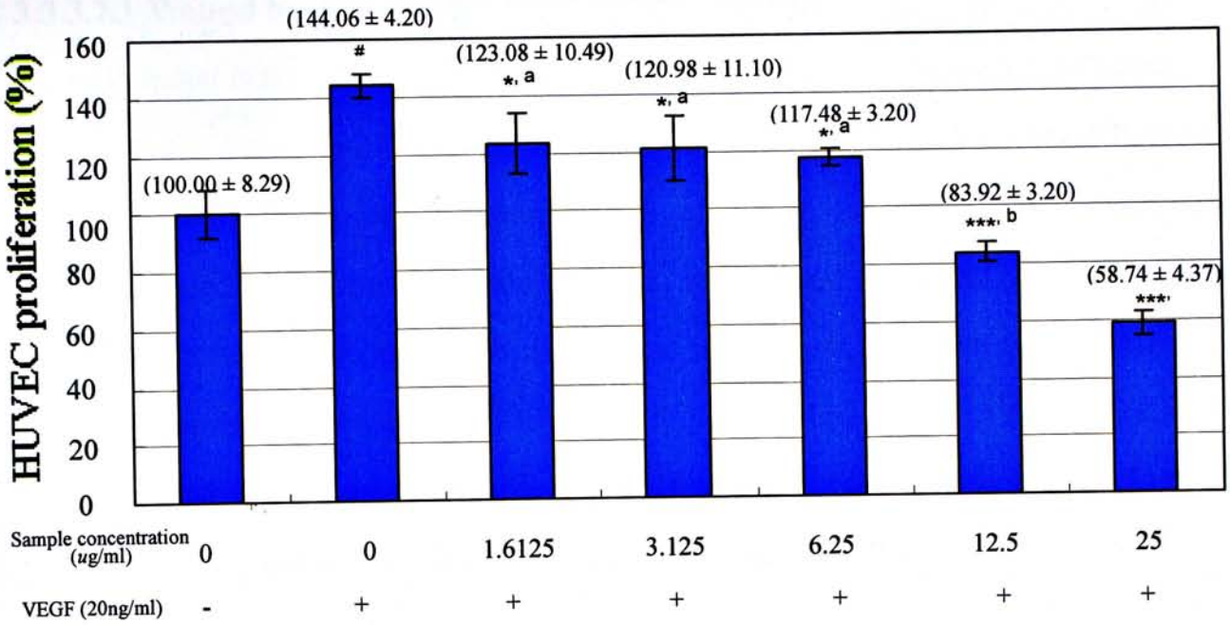


Figure 3.12 Effect of the *Aa* water extract on VEGF-induced HUVECs proliferation

Each value is expressed as mean ± SD ($n = 3$)

Figures in parentheses indicates the numeric value

Indicate significant difference between control vehicle and control ($p < 0.05$, student's t test)

* Indicate significant difference between control and the *Aa* water extract treatment groups ($p < 0.05$, student's t test)

*** Indicate significant difference between control and the *Aa* water extract treatment groups ($p < 0.001$, student's t test)

Different letters indicate significant difference among the *Aa* water extract treatment groups ($p < 0.05$, ANOVA, Tukey's multiple comparison)

Control vehicle was normalized to 100% for comparison

Although significant inhibition ($p < 0.05$) in VEGF-induced HUVECs proliferation was observed when the *Aa* water extract concentration applied as low as 1.6125 μg/ml, insignificant difference ($p > 0.05$) in the inhibiting ability was observed when the extract concentration ranged from 1.6125 to 6.25 μg/ml. This suggested that applying the *Aa* water extract at a concentration of 1.6125 to 6.25 μg/ml did not affect HUVECs proliferation differently and treating HUVECs with 1.6125 μg/ml of the *Aa* water extract was enough to induce the same effect on its proliferation as that with 6.25 μg/ml of the *Aa* water extract.

3.3.3.2 Endothelial cells migration

3.3.3.2.1 Wound healing assay

As endothelial cells migration is critical for the process of angiogenesis, the effect of the *Aa* water extract on HUVECs migration *in vitro* was found by wound healing and transwell culture insert method. The effect of the *Aa* water extract on VEGF-induced HUVECs migration was first determined using the wound healing method. Figure 3.13 illustrates the wound size in different treatment groups after 24 h. Since this assay measures the ability of HUVECs migration after a denuded area was produced using a scraping tool, the smaller the wound size indicates the higher extent of wound closure and therefore, the higher rate of cell migration.

It was found that with the addition of 20 ng/ml VEGF, the denuded region was almost refilled by HUVECs after 24 h while a small gap was still remained in the control vehicle group. Treatment of VEGF-induced HUVECs with different concentrations of the *Aa* water extract (1.6125 – 25 μg/ml) prohibited significantly the increment in migration induced by VEGF to different extents (Figure 3.14). Significant inhibition ($p < 0.005$) in HUVECs migration started when the *Aa* water extract applied was as low as 1.6125 μg/ml (Figure 3.14). When cells were treated with 25 μg/ml of the *Aa* water extract, the wound size reduction was 46.14 ± 8.53%, which was significantly smaller than that of the control (149.86 ± 4.32%, $p < 0.001$) (Figure 3.14).

In this wound healing method, the quantification was only arbitrary and there were difficulties to ensure the consistency of identical growth conditions of confluence and the scarped area between control and different treatment groups (Auerbach and others, 1991). Therefore, an alternative type of migration assay, transwell culture insert method, was also performed in this study (section 3.3.3.2.2) to further confirm the inhibitory effect of VEGF-induced HUVECs migration by the *Aa* water extract.

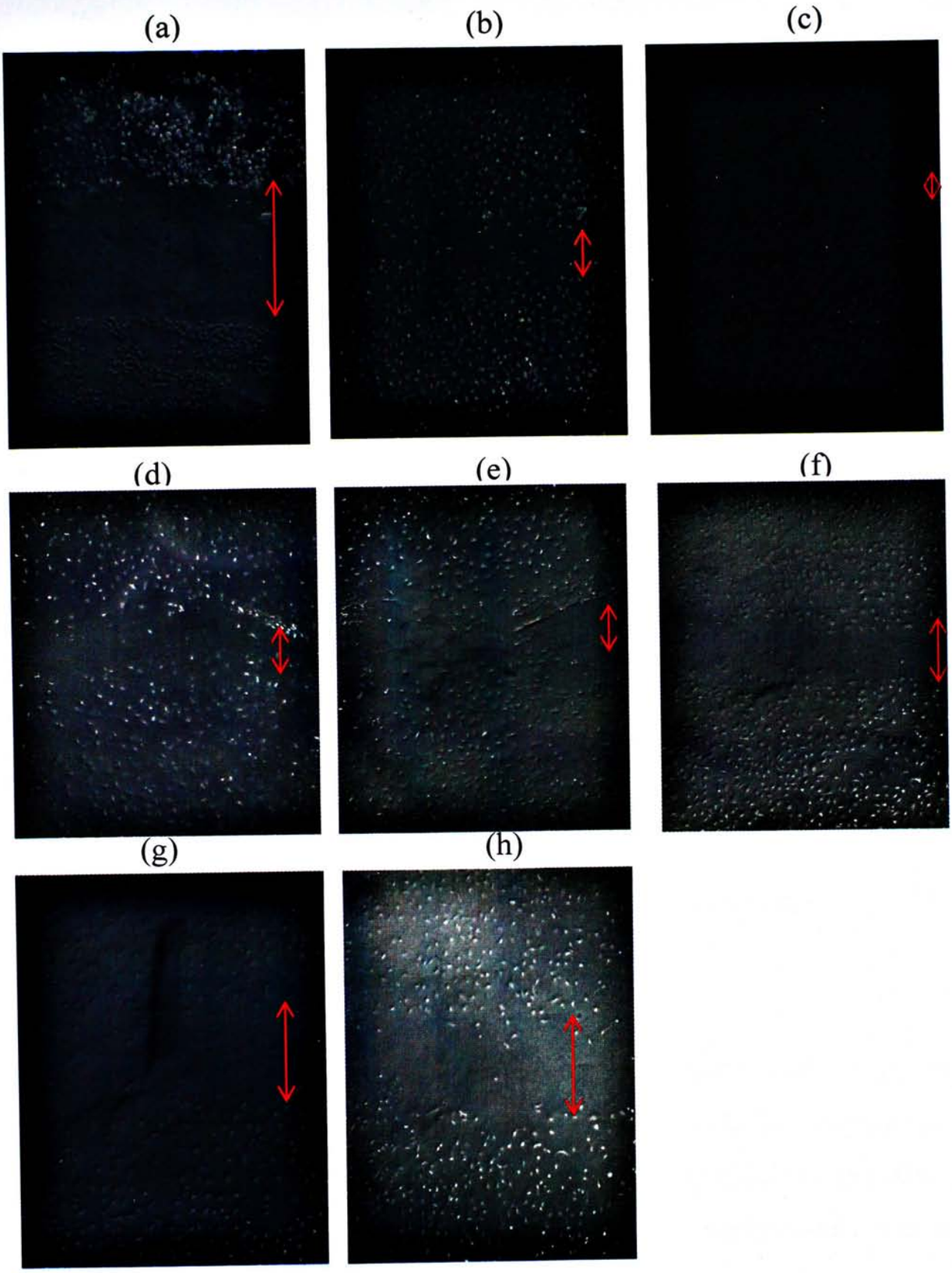


Figure 3.13 Inhibition of VEGF-induced HUVECs migration by the *Aa* water extract in wound healing method. (a) wound size at 0h; (b) wound size at 24 h without VEGF and *Aa* water extract; (c) with VEGF and without *Aa* water extract at 24 h; (d) with VEGF and 1.6125μg/ml at 24 h, (e) 3.125μg/ml at 24 h, (f) 6.25μg/ml at 24 h, (g) 12.5μg/ml at 24 h and (h) 25μg/ml at 24 h of the *Aa* water extract. Length in between two arrows indicated the wound length

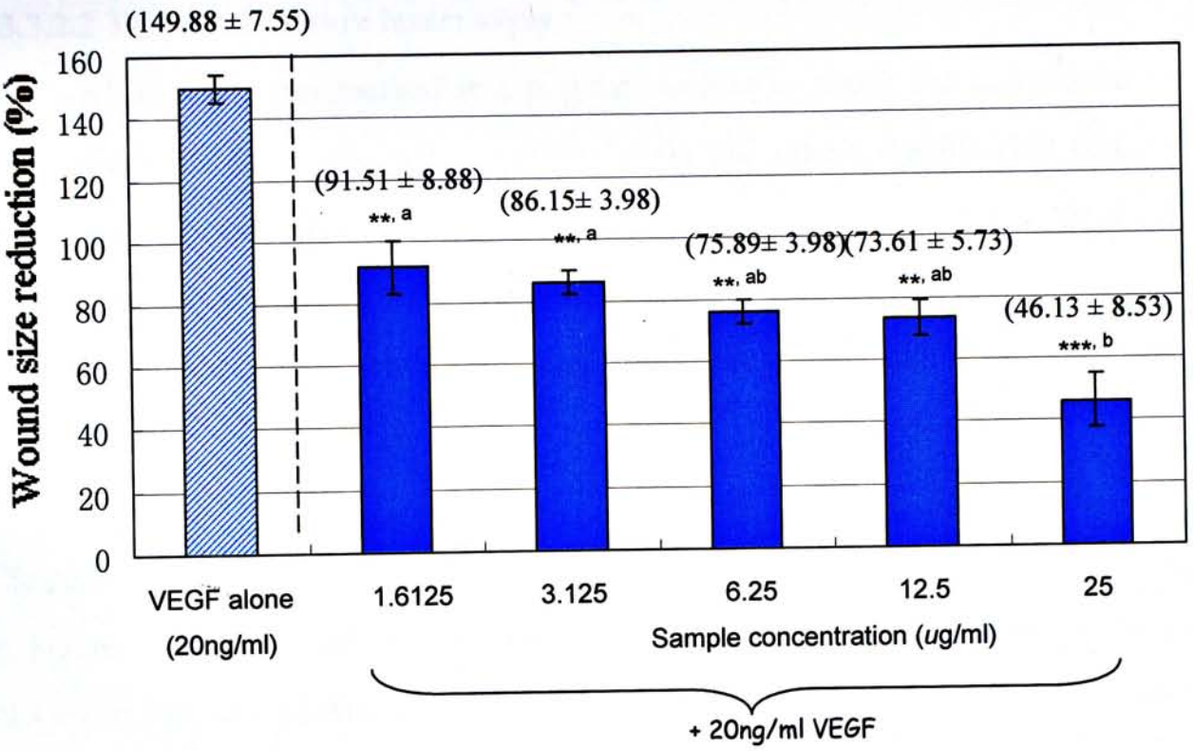


Figure 3.14 Effect of the *Aa* water extract on VEGF-induced HUVECs migration measured by wound healing method

Each value is expressed as mean ± SD ($n = 3$)

Figures in parentheses indicates the numeric value

** Indicate significant difference between control and the *Aa* water extract treatment groups ($p < 0.005$, student's *t* test)

*** Indicate significant difference between control and the *Aa* water extract treatment groups ($p < 0.001$, student's *t* test)

Different letters indicate significant difference among the *Aa* water extract treatment groups ($p < 0.05$, ANOVA, Tukey's multiple comparison)

Control vehicle was normalized to 100% for comparison

Similar to VEGF-induced HUVECs proliferation, the dose-response effect in VEGF-induced HUVECs migration measured by this wound healing method was not clearly demonstrated. It was observed that no significant difference ($p < 0.05$) could be observed in the wound size reduction when the *Aa* water extract concentration applied at a concentration of 1.6125 to 12.5 μg/ml. In other words, HUVECs migration measured by wound healing method was not affected in a different manner when treating cells with 1.6125 to 12.5 μg/ml of the *Aa* water extract. Significant difference ($p > 0.05$) could only be seen when treating HUVECs with a higher *Aa* water extract concentration (25 μg/ml).

3.3.3.2.2 Transwell culture insert assay

Transwell culture insert method is a popular method to check the chemotaxis of endothelial cells due to its high sensitivity (Falk and others, 1980). This method mainly based on measuring the migration of cells in response to a chemical gradient and is extremely sensitive to small changes in concentration making it very useful in studying angiogenesis when determining endothelial cells migration (Falk and others, 1980).

The presence of VEGF (20 ng/ml) strongly stimulated HUVECs migration as shown in Figure 3.15. Migrated cells increased significantly ($p < 0.05$) from 100.00 ± 19.49% to 186.70 ± 15.84% after cells were treated with VEGF (20 ng/ml). With the addition of various concentrations of the *Aa* water extract (1.6125 – 25 μg/ml), this VEGF-induced cells migration was suppressed significantly ($p < 0.05$) in a dose-dependent way (Figure 3.15). When the concentration of the *Aa* water extract reached to 12.5 and 25 μg/ml, cells that could migrate from the upper to the lower chamber resumed to almost the same level as in the control vehicle, 95.10 ± 9.46% and 96.50 ± 25.54%, respectively. These values were significantly lower ($p < 0.001$) than that of the control.

In this transwell culture insert assay, although the number of migrated cells showed significant difference ($p > 0.05$) in the treatment of HUVECs with 6.25 μg/ml of the *Aa* water extract from that with 1.6125 μg/ml of the *Aa* water extract, this significant difference could not be find anymore when the concentration of the *Aa* water extract increased to 25 μg/ml (Figure 3.15).

Based on the result obtained in these two migration methods, wound healing method (section 3.3.3.2.1) and transwell culture insert method (section 3.3.3.2.2), it is suggested that the dose-response effect of the *Aa* water extract concentration and HUVECs migration could not be clearly demonstrated at the concentrations used in the present study.

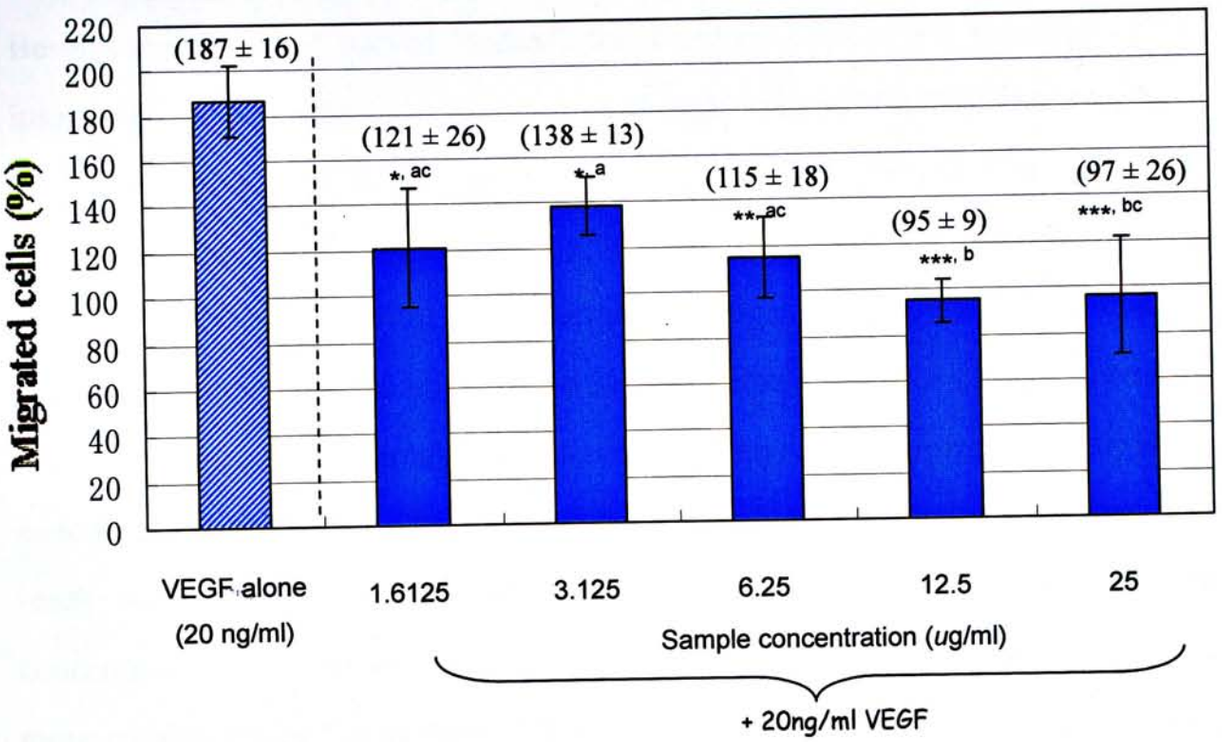


Figure 3.15 Effect of the *Aa* water extract on VEGF-induced HUVECs migration measured by transwell culture insert method

Each value is expressed as mean ± SD ($n = 3$)

Figures in parentheses indicates the numeric value

* Indicate significant difference between control and the *Aa* water extract treatment groups ($p < 0.005$, student's *t* test)

** Indicate significant difference between control and the *Aa* water extract treatment groups ($p < 0.005$, student's *t* test)

*** Indicate significant difference between control and the *Aa* water extract treatment groups ($p < 0.001$, student's *t* test)

Different letters indicate significant difference among the *Aa* water extract treatment groups ($p < 0.05$, ANOVA, Tukey's multiple comparison)

Control vehicle was normalized to 100% for comparison

The medium used in this assay was the starved medium with 0.5% instead of 10% FBS, because some studies had found that although no detectable VEGF existed in FBS, 20 ng/ml VEGF was less effective in inducing the migration of some endothelial cells than FBS did (Zhang and others, 2006). This suggested that results in the transwell culture insert assay using complete growth medium might be more related to inhibition in FBS-induced cells migration rather than VEGF-induced cells migration. Therefore, in order to eliminate cells migration contributed by FBS, starved medium was used in this assay to obtain a more reliable result.

Besides the choice of starved medium, the treatment time in this transwell culture insert method was selected to be 6 h. Although transwell culture insert assay is highly sensitive and is useful in applying to low concentration gradients, maintaining the transfilter gradient for prolonged periods is a challenge (Keenan and Folch, 2008). It is mainly due to the fact that angiogenic promoters or inhibitors are placed only at the lower chamber while the upper chamber is free of these chemicals to create a concentration gradient in attracting endothelial cells migration. Due to the concentration difference between chambers, chemicals are tended to diffuse and reach equilibrium between chambers over time, resulting in a deviation of concentration gradients at different time points. Therefore, in order to achieve a more reliable result, the treatment time in the present transwell culture insert assay should be as short as possible (probably less than 6 h) to avoid concentration gradient changes.

Despite the difficulty in maintaining chemical concentration gradient, transwell culture insert method is thought to illustrate the endothelial cells migration in a better way than wound healing method does. It is because cells are not moving freely inside our body but they have to continuously cross matrix and/ or cellular barriers (Albini and Benelli, 2007). One of the crucial steps in metastasis and angiogenesis is the invasion of basement membranes in order to disseminate or to form new blood vessels. Therefore, with the presence of the barrier in this assay, it is more suitable than wound healing assay for illustrating cells migration in studying tumor angiogenesis.

3.3.3.3 Endothelial cells tubule formation

Capillary formation starts with endothelial cells differentiation and tube formation is a consequence of endothelial cells differentiation (Zhang and others, 2006). There were several *in vivo* differentiation assays developed for the purpose of evaluating angiongenesis like chick chorioallantoic membrane (CAM) assay, Matrigel plug assay and corneal angiogenesis assay, allowing a more realistic assessment of angiogenic response (Zhang and others, 2006). These *in vivo* systems, however, are all time-consuming (Taylor and Weiss, 1984), and technically difficult (Norrby, 2006), some may be even ethically questionable (Staton and others, 2004).

One rapid assessment of endothelial cells differentiation is based on their ability to form tubules on Matrigel. With the use of this extracellular matrix (ECM), HUVECs tubule formation is allowed and vascular network visualization is also permitted at the same time. The endothelial cells tubule formation on ECM gel can also reasonably reflect the *in vivo* situation (Crabtree and Subramanian, 2007).

In this assay, the volume of Matrigel used per well was 50 μl. Actually, the volume of Matrigel used between different research groups varied considerably. The smallest volume of Matrigel used was found to be 0.17 $\mu l/mm^2$ (Staton and others, 2004) while the largest volume was found 3.53 $\mu l/mm^2$ (Zhong and others, 2005). It was also found that using too small volume of Matrigel resulted in pooling of cells to the center of the well due to the surface tension of the Matrigel (Smith and Staton, 2006). Therefore, to avoid such centering effect, 50 μl Matrigel application to each well was selected in this study.

Besides the Matrigel volume consideration, seeding density was another concern. It was reported that too low plating density of endothelial cells prevented tubule formation as cells do not make contact while too high plating density of cells resulted in large areas of clustered and treating cells with angiogenic factors under this condition resulted in a decrease in "cell and tubule area" (Liu and others, 2002).

Therefore, after assessing the optimal plating cell density (data not shown), $1x10^4$ cells were used in each well.

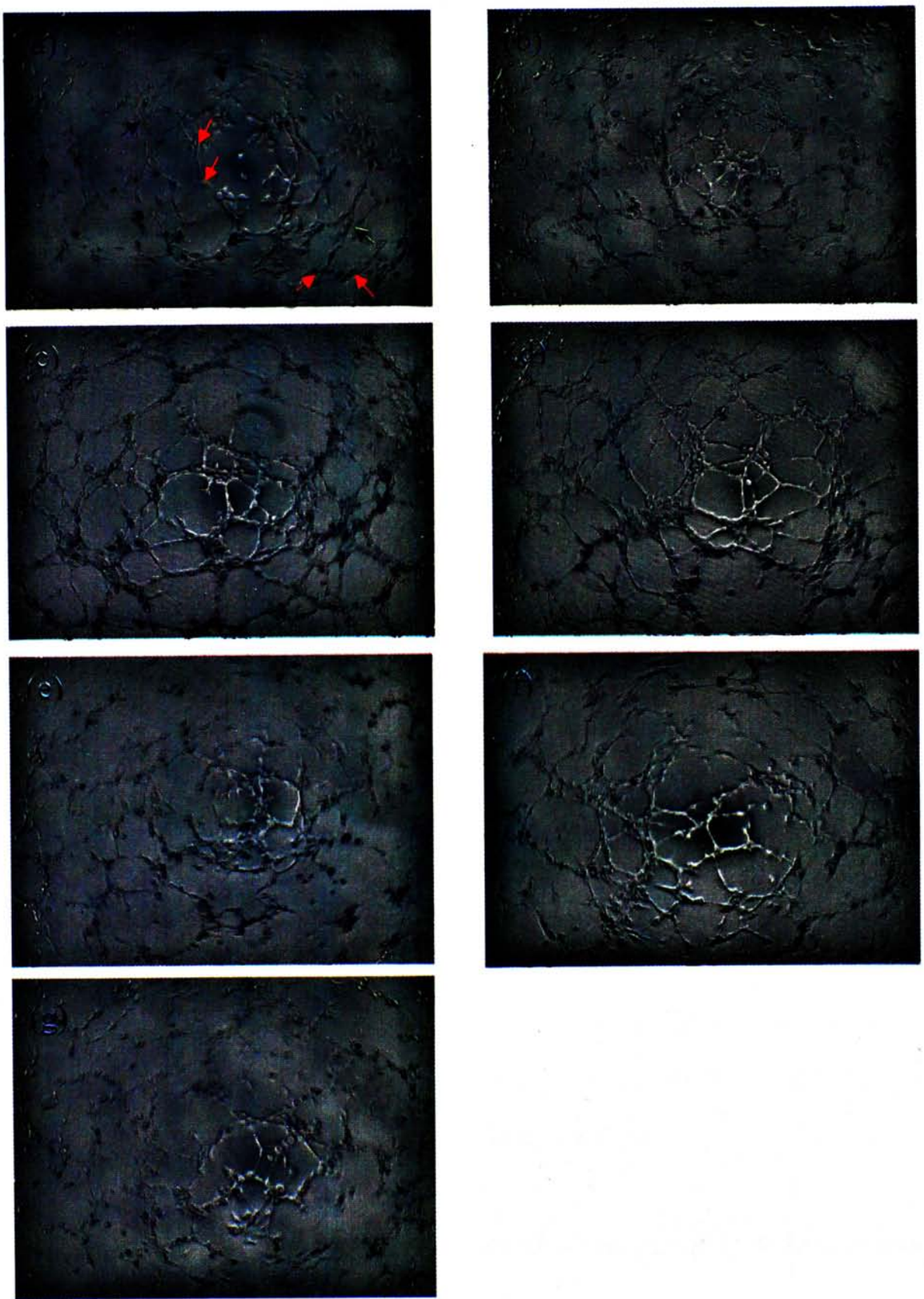


Figure 3.16 Inhibition of VEGF-induced HUVECs tubule formation by the *Aa* water extract after 5 h incubation. (a) without VEGF and the *Aa* water extract; (b) with VEGF and without the *Aa* water extract; (c) with VEGF and 1.6125μg/ml, (d) 3.125μg/ml, (e) 6.25μg/ml, (f) 12.5μg/ml and (g) 25μg/ml of the *Aa* water extract Dark arrow indicates the branching points (junctions between tubules) while distance between two red arrows indicate the tubule length

Figure 3.16 (a) shows that HUVECs formed a mesh of tubes within 5 h on Matrigel in the control while the tube formation was more extent if cells were incubated with 20 ng/ml VEGF (Figure 3.16 (b)). To test whether the *Aa* water extract could decrease the VEGF-induced formation of tubules by HUVECs in Matrigel *in vitro*, cells were co-treated with 20 ng/ml VEGF and various concentrations of the *Aa* water extract (final concentration of 1.6125 – 25 μg/ml). The tubule formation pattern of HUVECs treated with low concentrations (1.6125 and 3.125 μg/ml) of the *Aa* water extracts were similar to those in the control group (Figure 3.16 (b) to (d)) whereas those treated with higher concentrations (6.25 – 25 μg/ml) mostly remained dotted on the Matrigel without extensive network of tubule formation could be seen (Figure 3.16 (e) to (g)).

It was also observed that after co-treating HUVECs with VEGF and the *Aa* water extract, the average tubule length or area was increased due to the decrease in the branching points as shown in Figure 3.16 (d). As the average tubule length and tubule area is heavily relied on the number of tubules formed, it cannot strictly be seen as a separate independent variable (Smith and Staton, 2006). Moreover, the three primary variables commonly used for tubule formation determination, namely the total tubule length, total tubule area and total number of junctions, have their own advantages and limitations. Determining the extent of tubule development based on only one parameter might result in bias (Smith and Staton, 2006). Therefore, in the present study, the total tubule length, total tubule area and number of tubules were used as variables to determine the extent of tubule formation.

Total tubule length has been extensively used to assess tubule formation in many studies probably because it directly measures the formation of tubules and therefore provides a very good representation towards the extent of tubule formation (Isaji and others, 1997; Hernandez and others, 2004). Table 3.6 shows that the *Aa* water extract significantly reduced ($p < 0.005$) the VEGF-induced tubule length of tubules formed on Matrigel at a concentration of 3.125 μg/ml from 129.57 ± 12.90% to 87.55 ±

12.56%. Further increase in the concentrations of the *Aa* water extract from 6.25 to 25 μg/ml further decreased the tubule length significantly ($p < 0.001$) from 77.33 ± 4.99% to 71.77 ± 4.61%, respectively.

Table 3.6 Effect of the *Aa* water extract on VEGF-induced HUVECs total tubule length

Concentration (μg/ml)	With (+) or without (-) VEGF (20ng/ml)	Total tubule length (%)
0	-	100.00 ± 8.62
0	+	129.57 ± 12.90#
1.6125	+	120.23 ± 16.50 a
3.125	+	87.55 ± 12.56**,b
6.25	+	77.33 ± 4.99***,b
12.5	+	70.76 ± 6.39***,b
25	+	71.77 ± 4.61***,b

Each value is expressed as mean ± SD ($n = 3$)

Indicate significant difference between control vehicle and control ($p < 0.05$, student's *t* test)

** Indicate significant difference between control and the *Aa* water extract treatment groups ($p < 0.005$, student's *t* test)

*** Indicate significant difference between control and the *Aa* water extract treatment groups ($p < 0.001$, student's *t* test)

Different letters indicate significant difference among the *Aa* water extract treatment groups ($p < 0.05$, ANOVA, Tukey's multiple comparison)

Control vehicle was normalized to 100% for comparison

Besides measuring the total tubule length, the tubule area is another commonly used variable to assess the tubule development (Leopold and others, 2003).

Based on Table 3.7, treating HUVECs with 20 ng/ml VEGF induced a significant increase ($p < 0.05$) in the total tubule area by nearly 140% while co-incubating 20 ng/ml VEGF with different concentrations of the *Aa* water extract reduced this induced tubule area increase in different extents. No significant decrease ($p > 0.05$) in the total tubule area when compared with the control was found when HUVECs were incubated with 1.6125 μg/ml of the *Aa* water extract, which was consistent with the results of total tubule length. Significant decrease ($p < 0.05$) in total tubule area was found only when the concentration of the *Aa* water extract increased to 3.125

μg/ml. Further increase in the *Aa* water extract concentrations to 6.25, 12.5 and 25 μg/ml resulted in a highly significant reduction ($p < 0.001$) on the total tubule area to 73.48 ± 6.97%, 70.54 ± 1.59% and 81.64 ± 11.73%, respectively (Table 3.7).

Table 3.7 Effect of the *Aa* water extract on VEGF-induced HUVECs total tubule area

Concentration (μg/ml)	With (+) or without (-) VEGF (20ng/ml)	Total tube area (%)
0	-	100.00 ± 14.45
0	+	135.97 ± 19.80#
1.6125	+	119.61 ± 2.90a
3.125	+	88.40 ± 5.06**,b
6.25	+	73.48 ± 6.97***,b
12.5	+	70.54 ± 1.59***,b
25	+	81.64 ± 11.73***,b

Each value is expressed as mean ± SD ($n = 3$)

Indicate significant difference between control vehicle and control ($p < 0.05$, student's *t* test)

** Indicate significant difference between control and the *Aa* water extract treatment groups ($p < 0.005$, student's *t* test)

*** Indicate significant difference between control and the *Aa* water extract treatment groups ($p < 0.001$, student's *t* test)

Different letters indicate significant difference among the *Aa* water extract treatment groups ($p < 0.05$, ANOVA, Tukey's multiple comparison)

Control vehicle was normalized to 100% for comparison

The final parameter used in this tubule formation assay was to evaluate the tubule formation by counting the total number of junctions formed. This parameter is widely used in assessing the tubule formation and its popularity was probably because it is relatively easy to assess with or without image analysis software (Wu and others, 1997; Smith and Hoffman, 2005).

Supplementing HUVECs with 1.6125 – 25 μg/ml suppressed the VEGF-induced tubule formation measured by the total number of junctions (Figure 3.16 (f) and (g)). The reduction in the number of junctions observed was not as significant as those measured by total tubule length and total tubule area as incubating HUVECs with 1.6125 – 6.25 μg/ml of the *Aa* water extract, there was no significant difference ($p > 0.05$) in the reduction of tubule junctions (Figure 3.17) when compared with the

control. Significant reduction ($p < 0.05$) in total tubule junctions only found when the *Aa* water extract in the medium reached to a concentration of 12.5 and 25 μg/ml (Table 3.8).

Table 3.8 Effect of the *Aa* water extract on VEGF-induced HUVECs total number of junctions

Concentration (μg/ml)	With (+) or without (-) VEGF (20ng/ml)	Total number of junctions (%)
0	-	100.00 ± 26.78
0	+	176.63 ± 31.45$^{\#}$
1.6125	+	156.06 ± 24.92^{a}
3.125	+	113.81 ± 14.02^{b}
6.25	+	104.70 ± 13.82^{b}
12.5	+	88.16 ± 15.67^{*,b}
25	+	70.56 ± 10.76^{**,b}

Each value is expressed as mean ± SD ($n = 3$)

Indicate significant difference between control vehicle and control ($p < 0.05$, student's *t* test)

* Indicate significant difference between control and the *Aa* water extract treatment groups ($p < 0.005$, student's *t* test)

** Indicate significant difference between control and the *Aa* water extract treatment groups ($p<0.005$, student's *t* test)

Different letters indicate significant difference among the *Aa* water extract treatment groups ($p < 0.05$, ANOVA, Tukey's multiple comparison)

Control vehicle was normalized to 100% for comparison

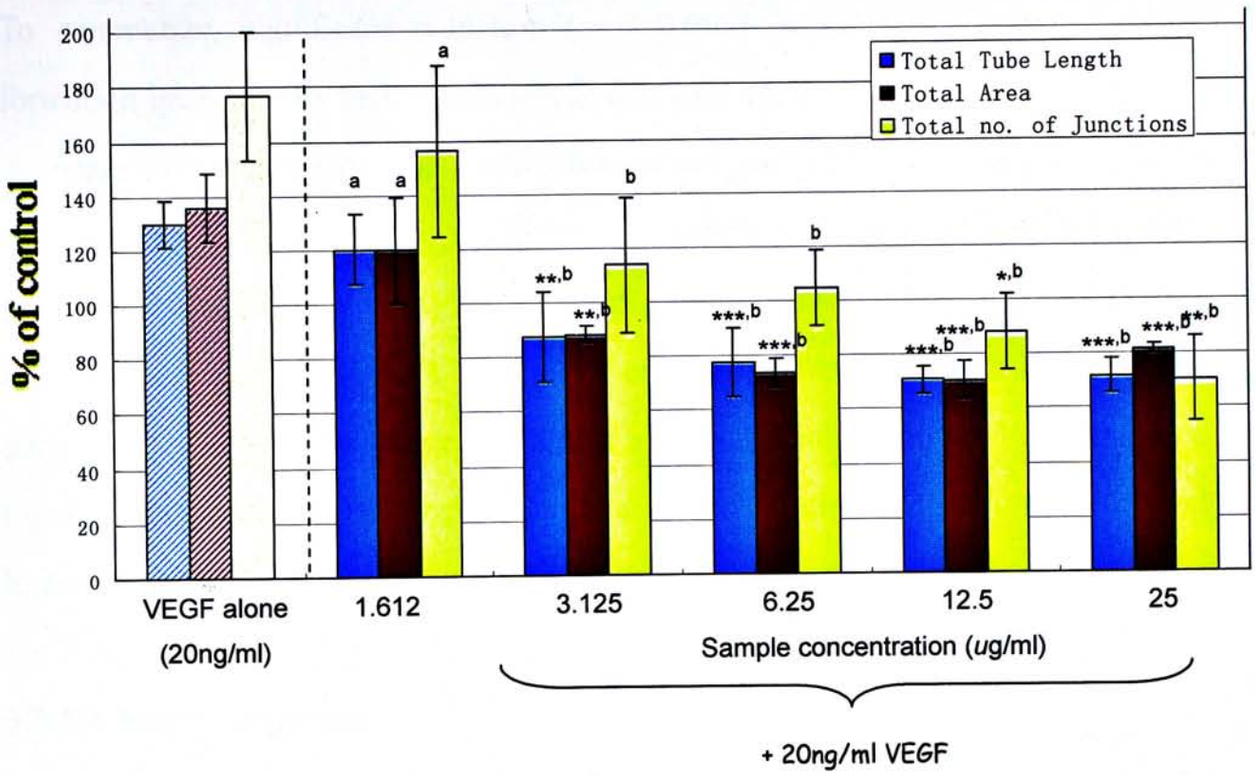


Figure 3.17 Effect of the *Aa* water extract on VEGF-induced HUVECs tubule formation

Each value is expressed as mean ± SD ($n = 3$)

* Indicate significant difference between control and the *Aa* water extract treatment groups ($p < 0.05$, student's *t* test)

** Indicate significant difference between control and the *Aa* water extract treatment groups ($p < 0.005$, student's *t* test)

*** Indicate significant difference between control and the *Aa* water extract treatment groups ($p < 0.001$, student's *t* test)

Different letters indicate significant difference among the *Aa* water extract treatment groups ($p < 0.05$, ANOVA, Tukey's multiple comparison)

Control vehicle was normalized to 100% for comparison

No matter in measuring the effect of the *Aa* water extract in the total tubule length, total tubule area or the total number of junctions formed by HUVECs induced by VEGF on the Matrigel, significant difference ($p < 0.05$) was found when the *Aa* water extract concentration increased from 1.6125 μg/ml to 3.125 μg/ml (Figure 3.17). However, further increase in extract concentration did not result in significant reduction in these three parameters suggesting that the inhibitory effect of the *Aa* water extract on VEGF-induced HUVECs tubule formation might not be in a dose-dependent manner.

To summarize, significant reduction ($p < 0.005$) in the VEGF-induced tubule formation measured by total tubule length and total tubule area were found when the *Aa* water extract concentration applied was as low as 3.125 μg/ml while significant reduction ($p < 0.05$) in the VEGF-induced tubule formation measured by total number of junction could only be found at a higher concentration of the *Aa* water extract (12.5 μg/ml) (Figure 3.17). Taken the results measured by all these parameters together, we suggested that a significant inhibition in VEGF-induced tubule formation could only be observed at a concentration of the *Aa* water extract higher than 12.5 μg/ml.

3.3.3.4 Aortic ring assay

Organ culture outgrowth assay represents the *in vivo* situation better than *in vitro* endothelial cell culture due to the existence of surrounding non-endothelial cells like smooth muscle cells and pericytes and a supporting matrix (Staton and others, 2004). Besides, the endothelial cells within the aorta are not proliferating at the time of aortic ring implantation. This gives a more representative picture of the *in vivo* tumor angiogenesis as quiescent endothelial cells is triggered by angiogenic factors, resulting in proliferation and migration from existing vessels and differentiation into tubules (Staton and others, 2004).

Figure 3.18 shows the microvessels outgrowth from an aortic ring at day 7 after implantation. Without the addition of 20 ng/ml VEGF, nearly no tubules could be formed as shown in Figure 3.18 (a), suggesting that in the absence of exogenous sera or growth factors, the microvessels proliferation and outgrowth was minimal. Addition of 20 ng/ml VEGF stimulated the microvessels outgrowth as shown in Figure 3.18 (b) while this induced microvessels outgrowth was suppressed by co-incubating the rat aortic ring with various concentrations of the *Aa* water extract (Figure 3.18 (c) – (g)).

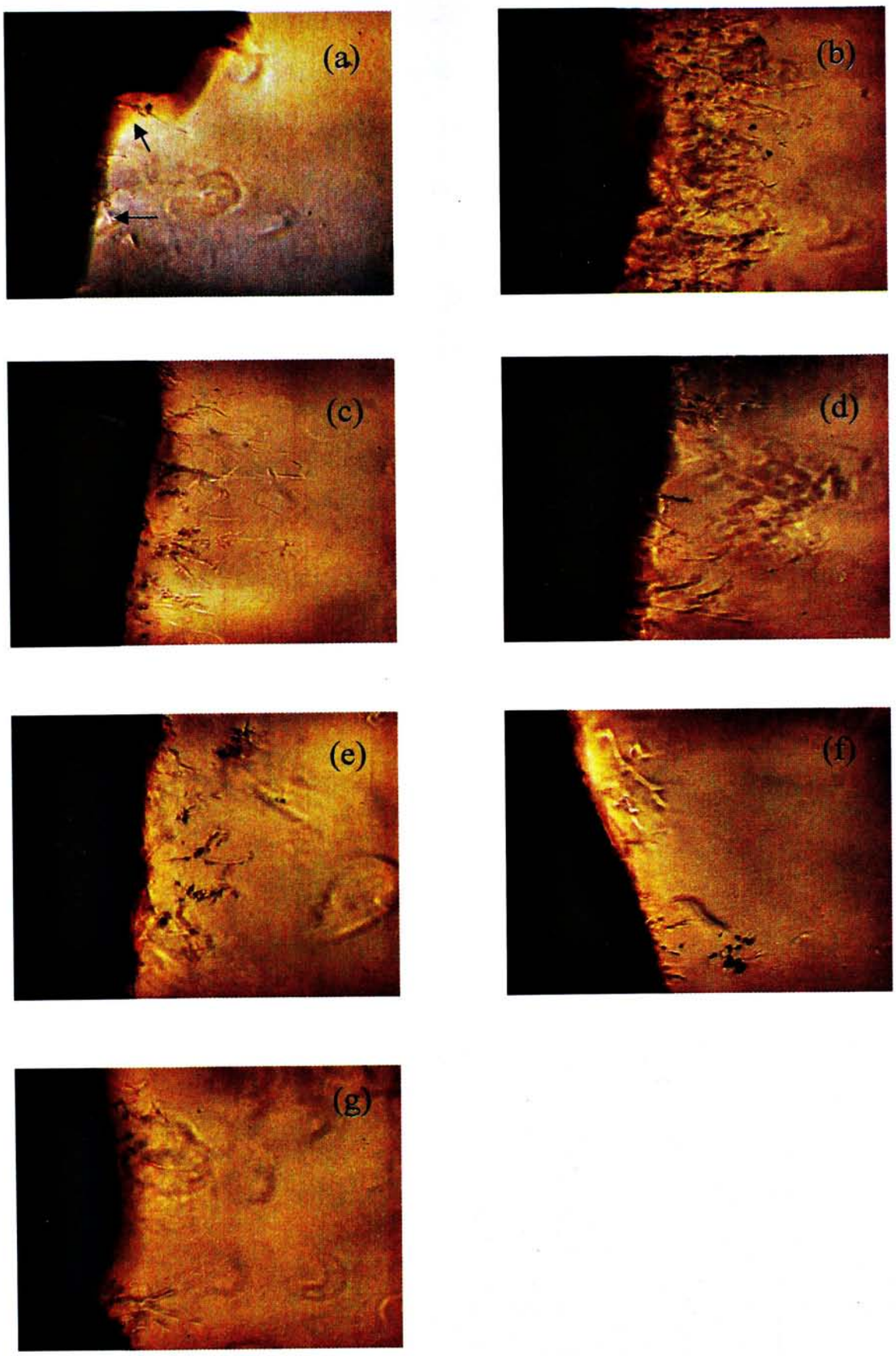


Figure 3.18 Inhibition of VEGF-induced aortic ring microvessels outgrowth by the *Aa* water extract at day 7 after implantation. (a) without VEGF and the *Aa* water extract; (b) with VEGF and without the *Aa* water extract; (c) with VEGF and 1.6125μg/ml, (d) 3.125μg/ml, (e) 6.25μg/ml, (f) 12.5μg/ml and (g) 25μg/ml of the *Aa* water extract. Dark arrow shows the position of microvessels outgrowth

Treatment of VEGF-induced rat aortic ring with different concentrations of the *Aa* water extract (1.6125 – 25 μg/ml) suppressed the microvessels outgrowth to different extents. When the treatment concentration was as low as 1.6125 μg/ml, no significant reduction ($p > 0.05$) could be observed. However, significant decrease (842.42 ± 161.69% to 242.42 ±59.15%, $p < 0.005$) in VEGF-induced aortic ring microvessels outgrowth was observed when the *Aa* water extract concentration increased to 3.125 μg/ml (Figure 3.19). Further increase in the *Aa* water extract concentration could further decrease the number of microvessels outgrowth from the aortic ring.

Similar to those results found in *in vitro* cell culture anti-angiogenesis analysis (section 3.3.3.1 – 3.3.3.3), the dose-response effect in this aortic ring assay was not obvious. Although significant difference in microvessels outgrowth was found when the concentration of the *Aa* water extract increased from 1.6125 μg/ml to 3.125 μg/ml, further increase in the *Aa* water extract concentration did not result in any significant difference ($p > 0.05$) in the number of microvessels outgrowth formed (Figure 3.19).

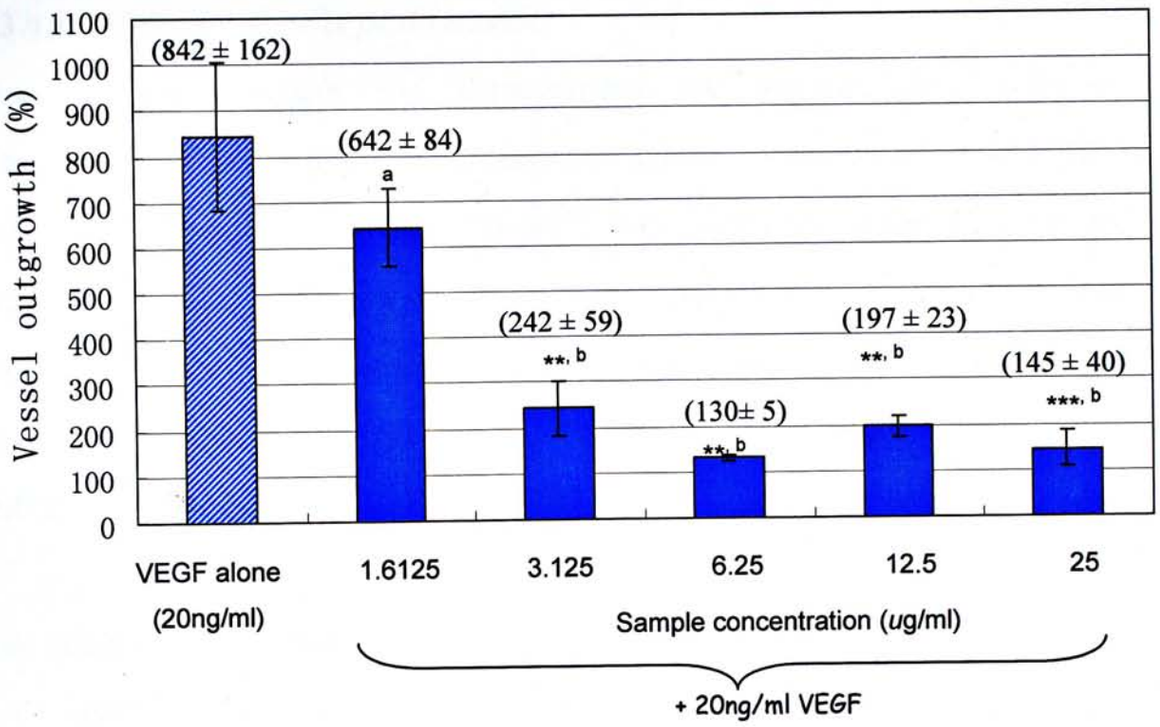


Figure 3.19 Effect of the *Aa* water extract on VEGF-induced aortic ring microvessels outgrowth

Each value is expressed as mean ± SD ($n = 3$)

Figures in parentheses indicates the numeric value

** Indicate significant difference between control and the *Aa* water extract treatment groups ($p < 0.005$, student's *t* test)

*** Indicate significant difference between control and the *Aa* water extract treatment groups ($p < 0.001$, student's *t* test)

Different letters indicate significant difference among the *Aa* water extract treatment groups ($p < 0.05$, ANOVA, Tukey's multiple comparison)

Control vehicle was normalized to 100% for comparison

3.3.4 Effect of phenolic acids on endothelial cells

3.3.4.1 Endothelial cells proliferation

The *Aa* water extract had demonstrated an anti-angiogenic response to VEGF-induced proliferation, migration and tubule formation of endothelial cells (section 3.3.3) and there were 5 phenolic acids identified in the *Aa* water extract (section 2.3.3.2). With the knowledge that phenolic acids may also possess anti-angiogenic effect (section 3.1.3.3), the present study also investigated the effects of three phenolic acids, gallic acid, caffeic acid and protocatechuic acid, on endothelial cells functions.

The effects of these phenolic acids on the proliferation of VEGF-induced endothelial cells proliferation were evaluated. Similar to the findings in section 3.3.3.1, incubating HUVECs with 20 ng/ml VEGF caused a significant increase (139.16 ± 7.37%, $p < 0.05$) in the endothelial cells proliferation (Figure 3.20). Such increase in cells proliferation was significantly decreased ($p < 0.05$) by co-incubation together with 5 mM of either gallic acid, caffeic acid or protocatechuic acid, indicating that these three phenolic acids were potent in inhibiting VEGF-induced endothelial cells proliferation (Figure 3.20).

Comparison between these three phenolic acids towards VEGF-induced HUVECs proliferation was performed using ANOVA. No significant difference ($p > 0.05$) in the HUVECs proliferation (%) was found between the treatment groups of gallic acid, caffeic acid and protocatechuic acid, indicating that the inhibitory power of these three phenolic acids towards VEGF-induced HUVECs proliferation were similar to each other.

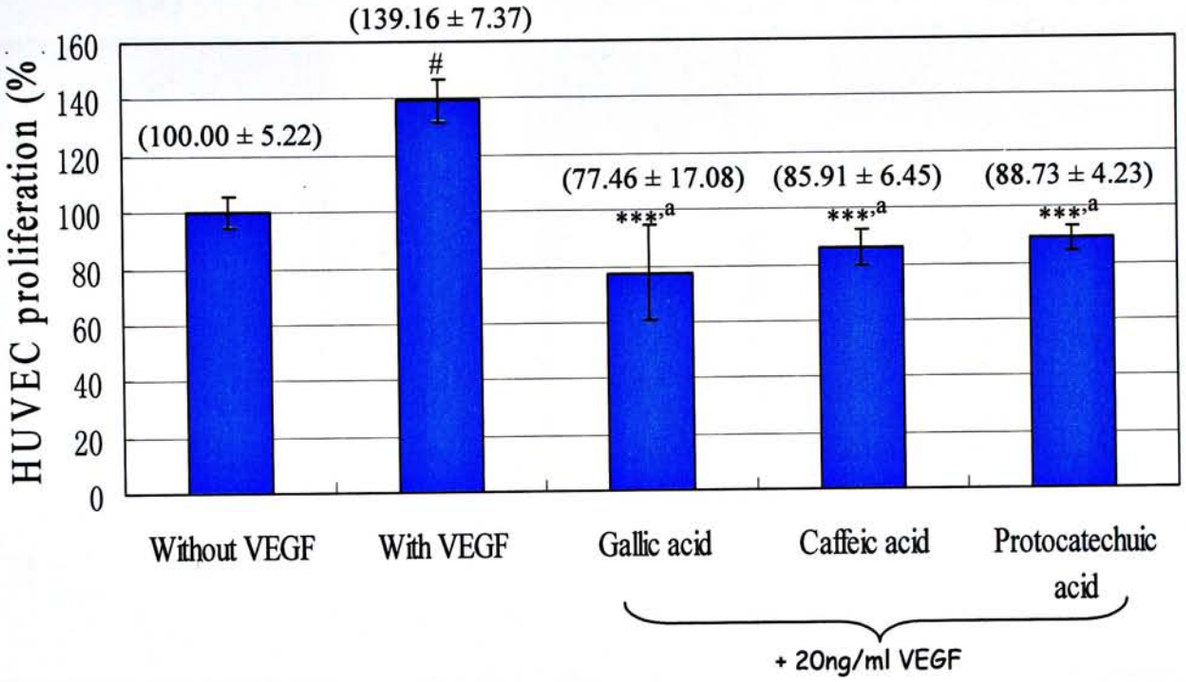


Figure 3.20 Effect of phenolic acids on VEGF-induced HUVECs proliferation

Each value is expressed as mean ± SD ($n = 3$)

Figures in parentheses indicate the numeric value

Indicate significant difference between control vehicle and control ($p < 0.05$, student's t test)

*** Indicate significant difference between control and phenolic acid treatment groups ($p < 0.001$, student's t test)

Different letters indicate significant difference among phenolic acid treatment groups ($p < 0.05$, ANOVA, Tukey's multiple comparison)

Control vehicle was normalized to 100% for comparison

3.3.4.2 Endothelial cells migration

3.3.4.2.1 Wound healing assay

Figure 3.21 shows the wound size after HUVECs were co-incubated with 20 ng/ml VEGF and 5 mM of either gallic acids, caffeic acid or protocatechuic acid, respectively.

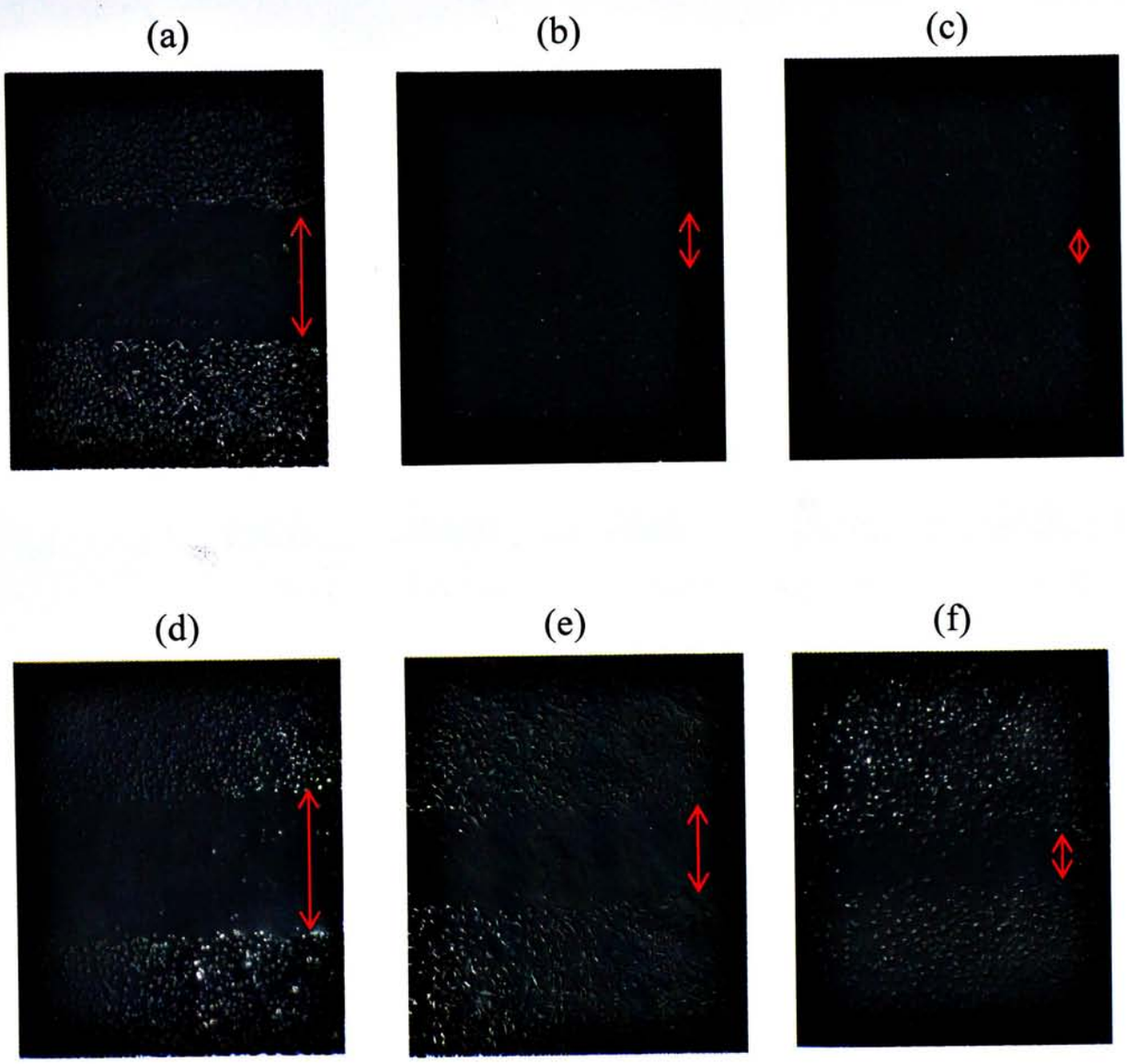


Figure 3.21 Inhibition of VEGF-induced HUVECs migration by phenolic acids in wound healing method. (a) Wound size at 0h; (b) wound size at 24 h without VEGF and phenolic acids; (c) with VEGF and without phenolic acids at 24 h; (d) with VEGF and 5 mM gallic acid at 24 h; (e) caffeic acid at 24 h and (f) protocatechuic acid at 24 h. Length in between two arrows indicated the wound length

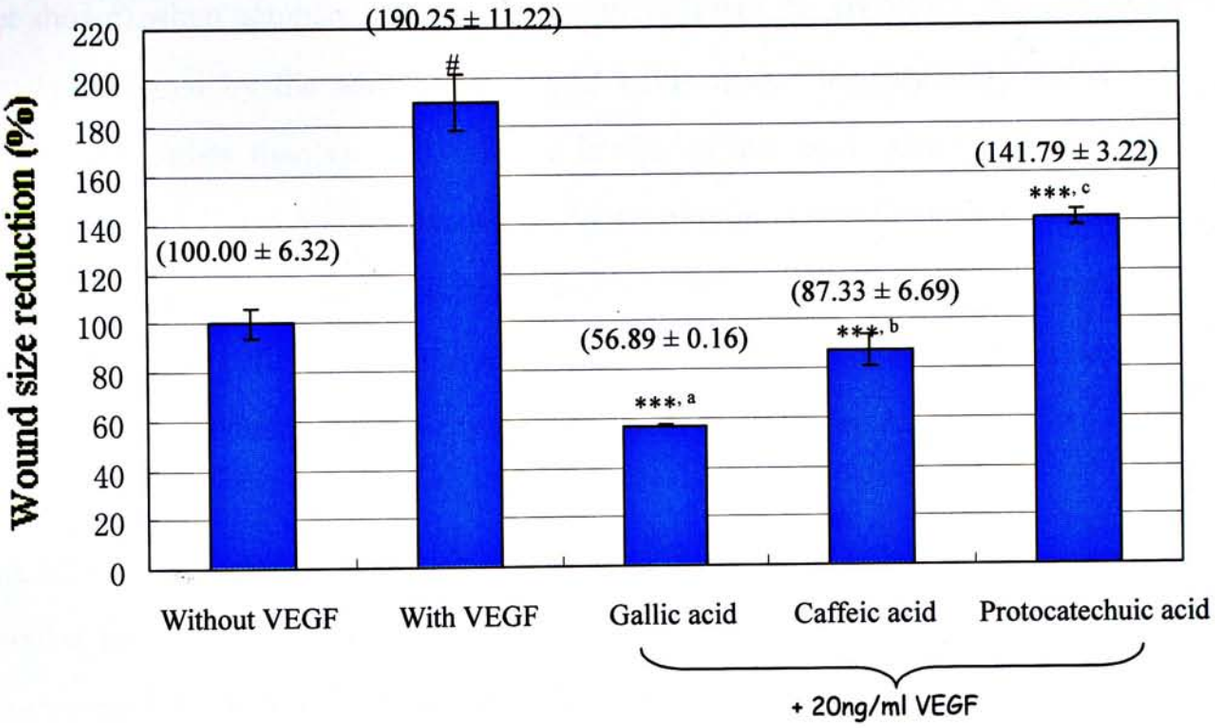


Figure 3.22 Effect of phenolic acids on VEGF-induced HUVECs migration measured by wound healing method

Each value is expressed as mean ± SD (n = 3)

Figures in parentheses indicate the numeric value

Indicate significant difference between control vehicle and control ($p < 0.05$, student's t test)

*** Indicate significant difference between control and phenolic acid treatment groups ($p < 0.001$, student's t test)

Different letters indicate significant difference among phenolic acid treatment groups ($p < 0.05$, ANOVA, Tukey's multiple comparison)

Control vehicle was normalized to 100% for comparison

Co-treatment of HUVECs with 20 ng/ml VEGF and 5 mM phenolic acids resulted in a significant decrease ($p < 0.001$) in the VEGF-induced HUVECs migration, from 190.25 ± 11.22% to 56.89 ± 0.16%, 87.33 ± 6.69% and 141.79 ± 3.22% in the treatment group of gallic acid, caffeic acid and protocatechuic acid, respectively (Figure 3.22). This result suggested that these three phenolic acids were potent in inhibiting VEGF-induced HUVECs migration.

Unlike in the VEGF-induced HUVECs proliferation, significant difference ($p < 0.05$) was found in the reduction of wound size after cells were treated with gallic acid, caffeic acid and protocatechuic acid. In the gallic acid treatment group, there was nearly no migration in the HUVECs, the wound size at 24 h was 97.47 ± 0.28% (data

not shown) when compared to 0 h. This suggested that the HUVECs migration was totally inhibited by the addition of 5 mM gallic acid. This inhibitory effect was significant higher than ($p < 0.05$) that in the caffeic acid. Although significant decrease ($p < 0.05$) in VEGF-induced HUVECs migration was found in the treatment group with 5 mM protocatechuic acid, its inhibitory effect might not be comparable to that of 5 mM caffeic acid as significant difference ($p < 0.05$) was found between the wound size reduction in these two treatment groups (Figure 3.22).

3.3.4.2.2 Transwell culture insert assay

Similar to the results obtained in the wound healing method in section 3.3.4.2.1, co-treating HUVECs with 20 ng/ml VEGF and 5 mM phenolic acids resulted in a significant decrease ($p < 0.001$) in the VEGF-induced migration measured by the transwell culture insert assay. Besides significant difference ($p < 0.05$) in the inhibitory effect on the VEGF-induced HUVECs migration, significant difference ($p < 0.05$) was found between treatment groups treated with gallic acid, caffeic acid and protocatechuic acid, which was similar to that measured by the wound healing assay (Figure 3.23).

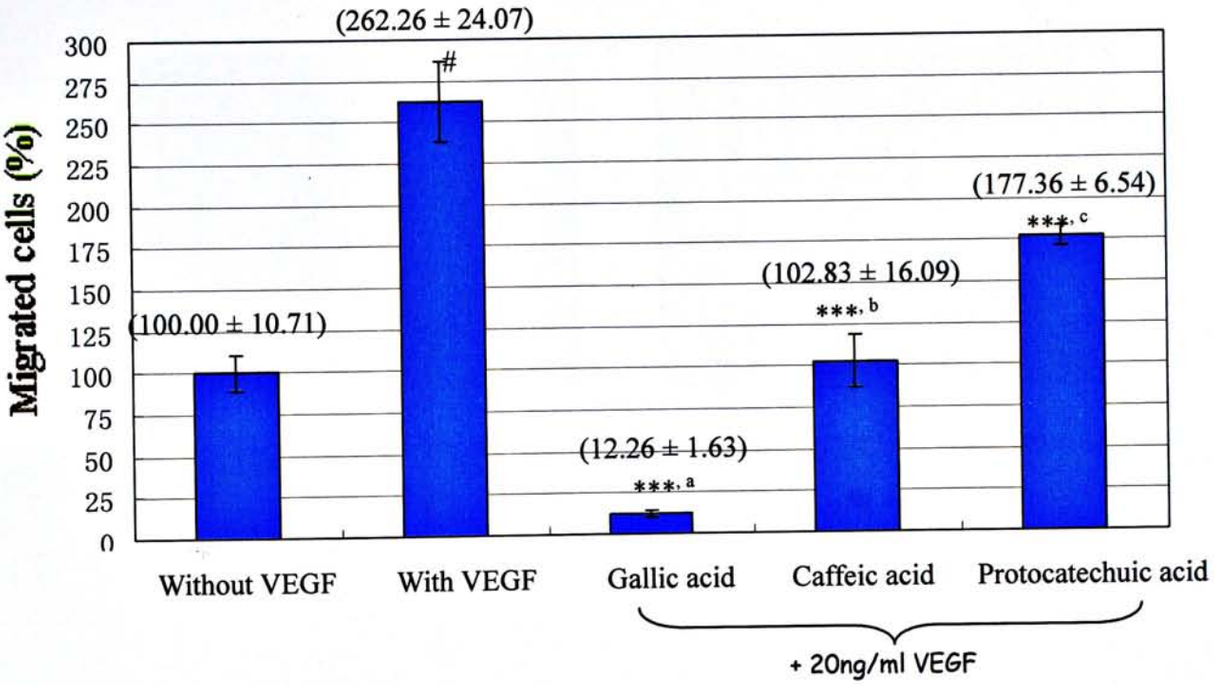


Figure 3.23 Effect of phenolic acids on VEGF-induced HUVECs migration measured by transwell culture insert method

Each value is expressed as mean ± SD ($n = 3$)

Figures in parentheses indicate the numeric value

Indicate significant difference between control vehicle and control ($p < 0.05$, student's t test)

*** Indicate significant difference between control and phenolic acid treatment groups ($p < 0.001$, student's t test)

Different letters indicate significant difference among phenolic acid treatment groups ($p < 0.05$, ANOVA, Tukey's multiple comparison)

Control vehicle was normalized to 100% for comparison

3.3.4.3 Endothelial cells tubule formation

The anti-angioenic effect of phenolic acids on endothelial cells function was also tested with its inhibitory power towards VEGF-induced HUVECs tubule formation. As shown in Figure 3.24 (c), gallic acid-treated HUVECs remained dotted and almost no tubule could be formed on the Matrigel. Similar tubule formation patterns were found when HUVECs were treated with either caffeic acid or protocatechuic acid. Although some tubules could still be found in these two treatment groups, no extensive network of tubule formation could be seen as shown in Figure 3.24 (d) and (e).

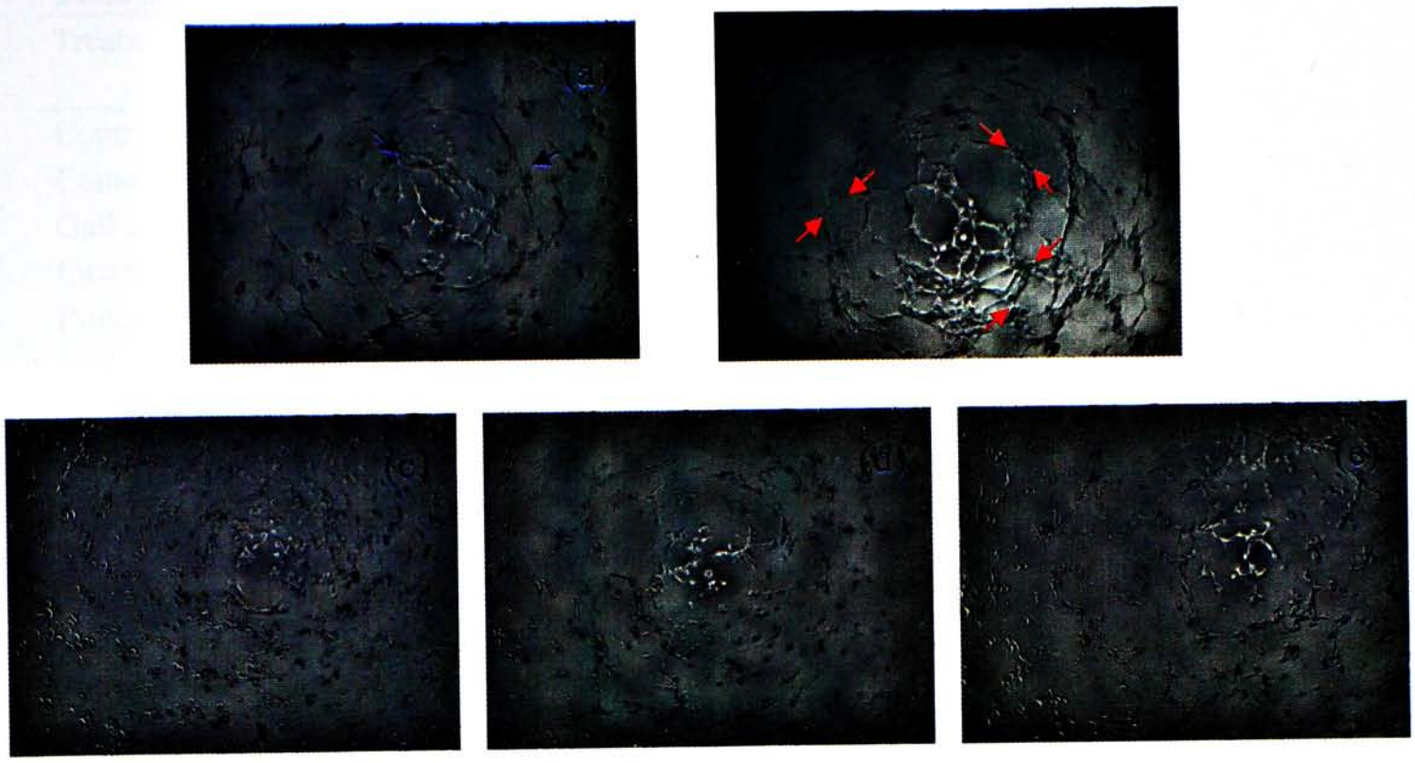


Figure 3.24 Inhibition of VEGF-induced HUVECs tubule formation by phenolic acids after 5 h incubation. (a) without VEGF and phenolic acids; (b) with VEGF and without phneolic acids; (c) with VEGF and 5 mM gallic acid, (d)caffeic acid, (e) protocatechuic acid
Dark arrow indicates the branching points (junctions between tubules) while distance between two red arrows indicate the tubule length

Concerning the length of the tubules developed on the Matrigel, co-treatment of gallic acid, caffeic acid or protocatechuic acid with 20 ng/ml VEGF all demonstrated a significant reduction ($p < 0.001$) in the VEGF-induced tubules formation at a concentration of 5 mM (Table 3.9). The total tubule length formed on the Matrigel decreased from 129.57 ± 12.90% to 74.79 ± 4.55% in protocatechuic acid (Table 3.9). A greater extent, which is significantly different ($p < 0.05$) from the protocatechic acid and caffeic acid, in the total tubule length measured was found in the treatment group of gallic acid (Table 3.9).

Table 3.9 Effect of phenolic acids on VEGF-induced HUVECs total tubule length

Treatment	With (+) or without (-) VEGF (20ng/ml)	Total tubule length (%)
Control vehicle	-	100.00 ± 8.62
Control	+	129.57 ± 12.90#
Gallic acid	+	23.98 ± 5.35***,a
Caffeic acid	+	65.99 ± 4.63***,b
Protocatechuic acid	+	74.79 ± 4.55*,b

Each value is expressed as mean ± SD ($n = 3$)

Indicate significant difference between control vehicle and control ($p < 0.05$, student's t test)

* Indicate significant difference between control and phenolic acid treatment groups ($p < 0.05$, student's t test)

*** Indicate significant difference between control and phenolic acid treatment groups ($p < 0.001$, student's t test)

Different letters indicate significant difference among phenolic acid treatment groups ($p < 0.05$, ANOVA, Tukey's multiple comparison)

Control vehicle was normalized to 100% for comparison

Similar results were found in measuring the total tubule area and the number of junctions of these tubules formed on the Matrigel induced by HUVECs (Table 3.10 and Table 3.11). Regarding to the total tubule area, after analyzing by the software, co-incubating 20 ng/ml VEGF with different phenolic acids reduced the VEGF-induced tubule area increase in different extents. Significant decrease ($p < 0.001$) in total tubule area was found in all the treatment groups (Table 3.10).

Table 3.10 Effect of phenolic acids on VEGF-induced HUVECs total tubule area

Treatment	With (+) or without (-) VEGF (20ng/ml)	Total tubule area (%)
Control vehicle	-	100.00 ± 14.45
Control	+	135.97 ± 19.80#
Gallic acid	+	19.53 ± 4.42***,a
Caffeic acid	+	72.58 ± 1.30***,b
Protocatechuic acid	+	80.79 ± 1.13***,b

Each value is expressed as mean ± SD (n = 3)

Indicate significant difference between control vehicle and control ($p < 0.05$, student's t test)

*** Indicate significant difference between control and phenolic acid treatment groups ($p < 0.001$, student's t test)

Different letters indicate significant difference among phenolic acid treatment groups ($p < 0.05$, ANOVA, Tukey's multiple comparison)

Control vehicle was normalized to 100% for comparison

The most potent inhibition in the VEGF-induced total tubule area was found in the gallic acid-treated group (Table 3.10). Although significant reduction ($p < 0.05$) in the VEGF-induced total tubule area was observed in both the caffeic acid and protocatechuic acid treatment groups comparing to the control, no significant difference ($p > 0.05$) was found between them, indicating that the inhibitory effects on VEGF-induced total tubule area given by caffeic acid and protocatechuic acid might be similar to each other (Table 3.10).

For the total number of junctions formed, reduction in the number of junctions was observed in all treatment groups. Supplementing HUVECs with 5 mM gallic acid, caffeic acid and protocatechuic acid significantly suppressed ($p < 0.05$) the VEGF-induced tubule formation measured by the number of total tubule junctions formed from 176.63 ± 31.45% to 2.50 ± 0.54%, 75.74 ± 6.13% and 85.60 ± 8.47%, respectively (Table 3.11).

Table 3.11 Effect of phenolic acids on VEGF-induced HUVECs total number of junctions

Treatment	With (+) or without (-) VEGF (20ng/ml)	Total number of junctions (%)
Control vehicle	-	100.00 ± 26.78
Control	+	176.63 ± 31.45#
Gallic acid	+	2.50 ± 0.54***,a
Caffeic acid	+	75.74 ± 6.13***,b
Protocatechuic acid	+	85.60 ± 8.47***,b

Each value is expressed as mean ± SD (n = 3)

Indicate significant difference between control vehicle and control ($p < 0.05$, student's t test)

*** Indicate significant difference between control and phenolic acid treatemtn groups ($p < 0.001$, student's t test)

Different letters indicate significant difference among phenolic acid treatment groups ($p < 0.05$, ANOVA, Tukey's multiple comparison)

Control vehicle was normalized to 100% for comparison

To summarize the inhibitory effect of phenolic acids (gallic acid, caffeic acid and protocatechuic acid) on VEGF-induced HUVECs tubule formation, significant difference ($p < 0.001$) were found between the control and phenolic acid treatment groups in terms of the total tubule length, total tubule area and the total number of junctions formed by HUVECs induced by VEGF on the Matrigel (Figure 3.25), indicating that 5 mM of gallic acid, caffeic acid and protocatechuic acid are all effective in suppressing the tubule formation of HUVECs induced by 20 ng/ml VEGF.

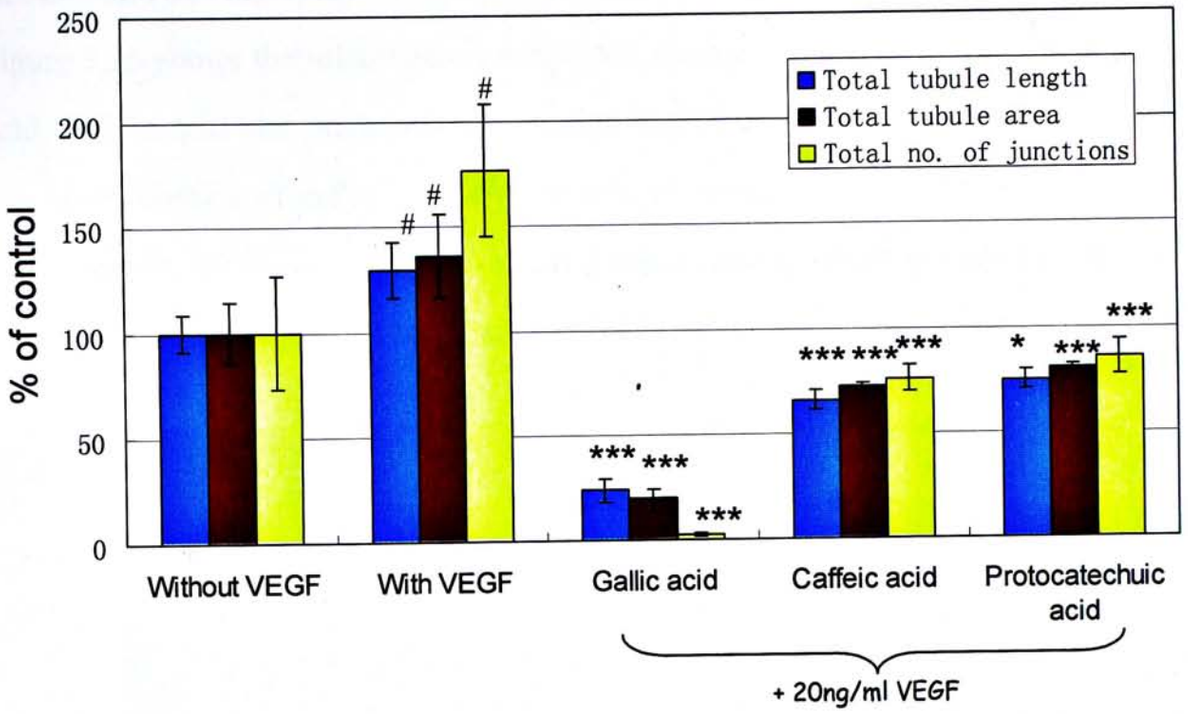


Figure 3.25 Effect of phenolic acids on VEGF-induced HUVECs tubule formation

Each value is expressed as mean ± SD ($n = 3$)

indicate significant difference between control vehicle and control ($p < 0.05$, student's t test)

* Indicate significant difference between control and phenolic acid treatment groups ($p < 0.005$, student's t test)

*** Indicate significant difference between control and phenolic acid treatment groups ($p < 0.001$, student's t test)

Different letters indicate significant difference among phenolic acid treatment groups ($p < 0.05$, ANOVA, Tukey's multiple comparison)

Control vehicle was normalized to 100% for comparison

3.3.4.4 Aortic ring assay

Figure 3.26 shows the microvessels outgrowth from an aortic ring treated with gallic acid, caffeic acid and protocatechuic acid at day 7 after implantation. It was found that co-incubation of gallic acid, caffeic acid or protocatechuic acid with 20 ng/ml VEGF totally inhibited the VEGF-induced microvessels outgrowth from aortic ring as no microvessel could be seen in all these treatment groups (Figure 3.26).

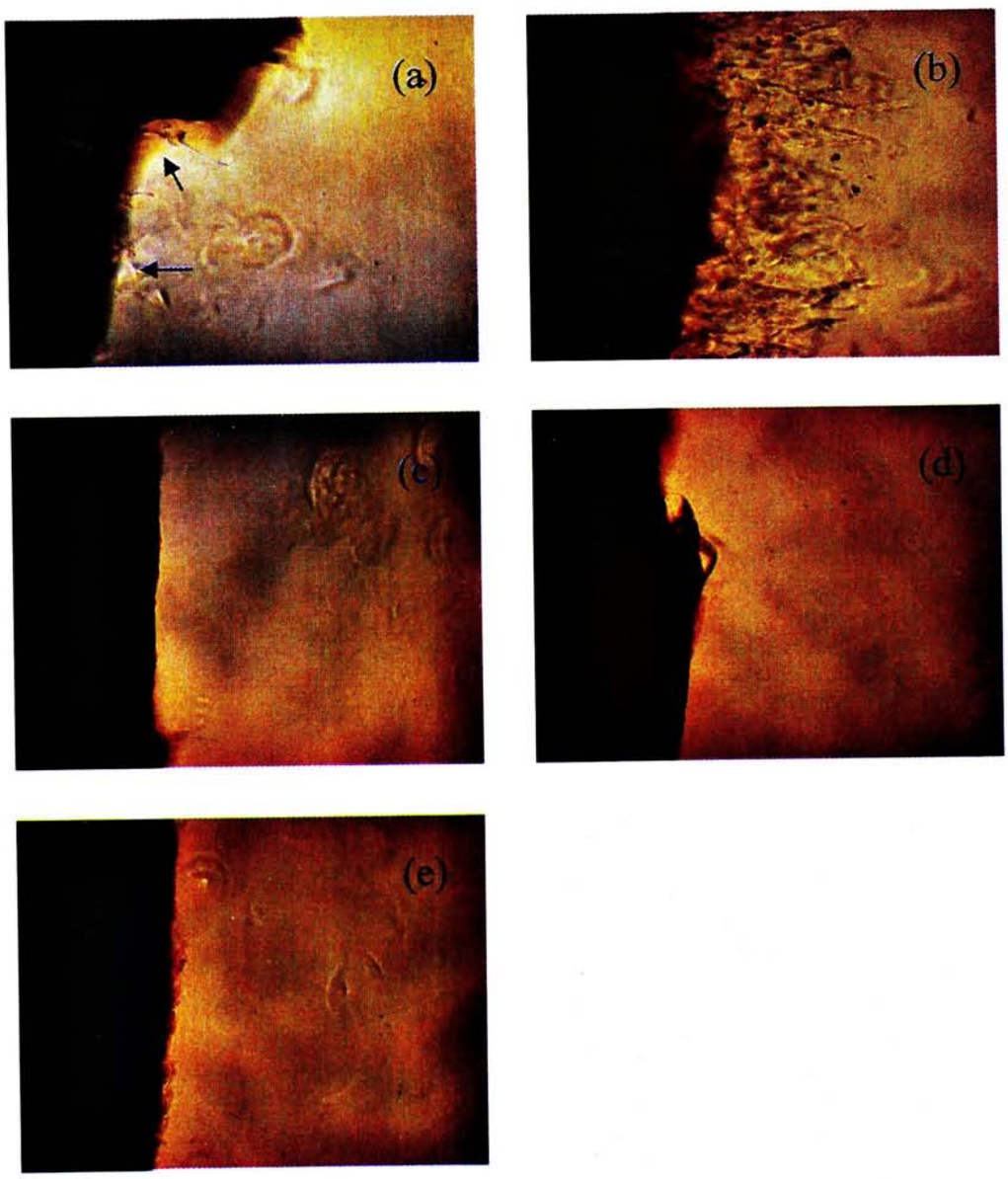


Figure 3.26 Inhibition of VEGF-induced aortic ring microvessels outgrowth by phenolic acids at day 7 after implantation. (a) without VEGF and phenolic acids; (b) with VEGF and without phenolic acids; (c) with VEGF and 5 mM gallic acid; (d) caffeic acid; (e) protocatechuic acid
Dark arrow shows the position of microvessels outgrowth

In this section, we found out that gallic acid, caffeic acid and protocatechuic acid were effective in inhibiting the VEGF-induced endothelial cells proliferation, migration as well as tubule formation. These three compounds are all belong to phenolic acid, a group that possesses strong antioxidant activities. In section 3.1.2.3.1, we have already discussed the importance of ROS in endothelial cells functions, and the use of antioxidants as therapeutical agents to inhibit tumor angiogenesis (section 3.1.3.2) while in section 2.1.2.2.3, the antioxidant activities of phenolic acids were mentioned as well. Combining all these results together, it was obvious that the anti-angiogenic effects of these three phenolic acids might be related to their antioxidative activities.

Although gallic acid, caffeic acid and protocatechuic acid all belong to the group of phenolic acid, their anti-angiogenic effects towards endothelial cells functions varied greatly. The highest anti-angiogenic activity was found in gallic acid, followed by caffeic acid and protocatechuic acid.

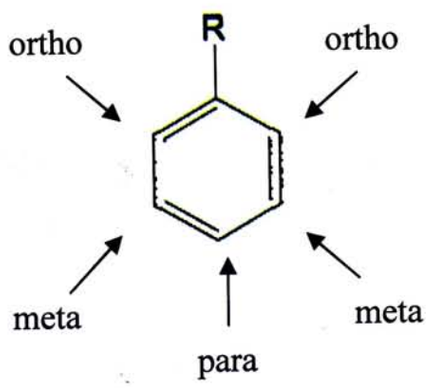
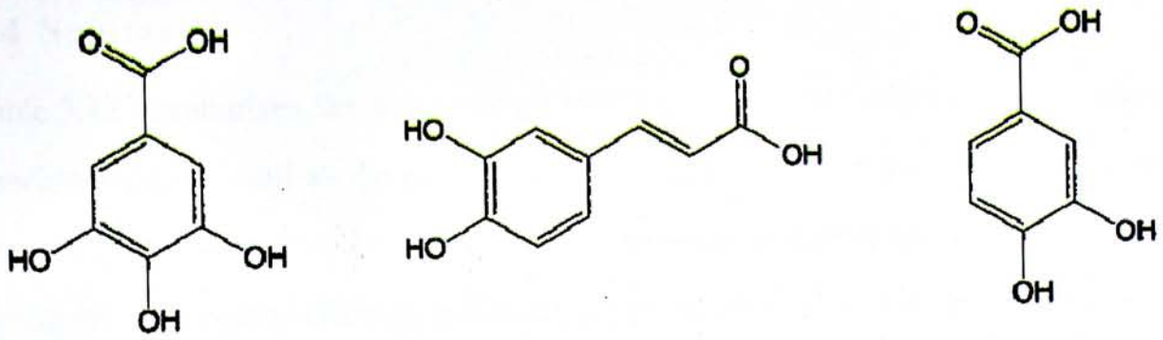


Figure 3.27 Substitution patterns in the benzene ring

The antioxidant activity of phenolic acids depends on the number of hydroxyl groups in the molecule, the position of the hydroxyl groups present, the chain length as well as the functional group present in the R position (Figure 3.27) (Von Gadow and others, 1997). The more the hydroxyl group, the stronger the antioxidant activity is. For example, polyphenols are found to have a stronger antioxidant activity than

monophenols (Rice-Evans and others, 1996). Besides, the position of the hydroxyl group also plays a role in the antioxidant activity. The carboxylate group present in the hydroxybenzoic acids possesses an electron-withdrawing property that decreases their hydrogen donating abilities. Such influence of the electron-withdrawing potential by this carboxylate group was much less when its position is not adjacent to the hydroxyl groups. In hydroxybenzoic acids with dihydroxylation, it was found that a higher antioxidant activity was found in the *meta*-position than that in the *ortho-meta* positions, which was in turn higher than that in the *meta-para* positions or in the *ortho*-positions (Rice-Evans and others, 1996). Finally, hydroxycinnamic acid was found to have a higher antioxidant activity than their hydroxybenzoic acid counterparts as the presence of –CH=CH-COOH groups in the R position of hydroxycinnamic acid strengthen the hydrogen-donating power and resulted in greater radical stabilization ability by resonance (Cuvelier and others, 1992). However, the presence of –COOH group in the R position of hydroxybenzoic acid possesses electron-withdrawing properties, which resulted in decreasing the hydrogen donating abilities as mentioned before (Rice-Evans and others, 1996).

In this study, the structures of the three phenolic acids used including gallic acid, caffeic acid and protocatechuic acid, are shown in Figure 3.28. Gallic acid and protocatechuic acid belong to hydroxybenozic acid while caffeic acid belongs to hydroxycinnamic acid. Gallic acid contains three –OH groups while caffeic acid and protocatechuic acid contain only two –OH groups. Since gallic acid is a trihydroxybenzoic acid while protocatechuic acid is only a dihydroxybenzoic acid, the antioxidant activity of gallic acid is expected to be higher than that of protocatechuic acid. Moreover, as hydroxycinnamic acid should have a higher antioxidant activity than their hydroxybenzoic acid counterparts, the antioxidant activity of caffeic acid should be higher than that of protocatechuic acid. Therefore, the antioxidant power of these three phenolic acids should be in an order of gallic acid > caffeic acid > protocatechuic acid.



(a) (b) (c)

Figure 3.28 Structures of (a) gallic acid, (b) caffeic acid and (c) protocatechuic acid

In fact, the antioxidant activity ranking in these three phenolic acids has been confirmed previously. Brand-Williams and others (1995) have tested the antioxidant activities of these phenolic acids using the DPPH radical as a model. They found out that the antiradical power (ARP), which is expressed in terms of $1/EC_{50}$, was 12.5, 9.1 and 7.14 in gallic acid, caffeic acid and protocatechuic acid, respectively, indicating that gallic acid was the most efficient antioxidant followed by caffeic acid and protocatechuic acid. Besides, the total antioxidant activities relative to Trolox of gallic acids, caffeic acid and protocatechuic acid were found to be 3.01 ± 0.05 mM, 1.26 ± 0.01 mM and 1.19 ± 0.03 mM, respectively (Rice-Evans and others, 1996). This result also indicated that the antioxidant activity found in gallic acid was much stronger than that in caffeic acid, which in turns, was stronger than that in protocatechuic acid.

With the knowledge on the mechanism on the enhancement of endothelial cells functions induced by VEGF and the antioxidative activity in these three phenolic acids, the anti-angiogenic effects by phenolic acids observed in the present study might be contributed by its antioxidative action. Hence, the highest anti-angiogenic effect was observed when HUVECs were co-treated with VEGF and gallic acid, followed by VEGF and caffeic acid co-treatment and VEGF and protocatechuic acid co-treatment due to the difference of the antioxidant activities in the phenolic acids.

3.4 Summary

Table 3.12 summarizes the anti-angiogenic effects of the *Aa* water extract at various concentrations as well as the anti-angiogenic effects of gallic acid, caffeic acid and protocatechuic acid. Based on the table, the *Aa* water extract generally could have a strong anti-angiogenic effect in inhibiting the production of VEGF in cancer cells as well as inhibiting the functions in endothelial cells and tubule formations in rat aortic ring. Treated with the *Aa* water extract at a concentration of 12.5 μg/ml or above, the intracellular ROS and VEGF production in Caco-2 were significantly reduced and the proliferation, migration, tubule formations in VEGF-induced HUVECs as well as the microvessels outgrowth in rat aortic ring were suppressed as well. All these findings suggested that the *Aa* water extract possessed a strong anti-angiogenic activity.

Although the effects of phenolic acids on the intracellular ROS level and VEGF secretion in Caco-2 had not been tested in this study, applying either 5 mM gallic acid, caffeic acid or protocatechuic acid resulted in a significant inhibition ($p < 0.05$) on VEGF-induced HUVECs proliferation, migration and tubule formation as well as the microvessels outgrowth in the rat aortic ring assay. These results suggested that all phenolic acids tested in this study are strong anti-angiogenic inhibitors. Further investigation is needed to understand the underlying mechanism of the anti-angiogenic effects observed in this study.

Table 3.12 Summary of the anti-angiogenic effects of the *Aa* water extract and phenolic acids

	Concentration of the *Aa* water extract (μg/ml)					Phenolic acids		
	1.6125	3.125	6.25	12.5	25	Gallic acid	Caffeic acid	Protocatechuic acid
Effect on cancer cells								
Intracellular ROS scavenging activity	√	√	√	√	√	---	---	---
VEGF secretion	X	X	X	√	√	---	---	---
Effects on endothelial cell functions								
Proliferation	√	√	√	√	√	√	√	√
Migration								
Wound healing method	√	√	√	√	√	√	√	√
Transwell culture insert	√	√	√	√	√	√	√	√
Tubule formation								
Total tubule length	X	√	√	√	√	√	√	√
Total tubule area	X	√	√	√	√	√	√	√
Total number of junctions	X	X	X	√	√	√	√	√
Effect on organ culture								
Rat aortic ring assay	X	√	√	√	√	√	√	√

√ Indicates significant difference between control and the *Aa* water extract treatment groups or phenolic acid treatment groups

X Indicates no significant difference between control and the *Aa* water extract treatment groups or phenolic acid treatment groups

--- Indicates no experiments had been performed

Chapter 4: Conclusion and future works

The antioxidant activity of four mushroom water extracts, *Aa*, *Po*, *Pe* and *Pn* were investigated by four chemical antioxidant activity assays. The highest scavenging activities towards DPPH radical, superoxide anion radical as well as hydrogen peroxide were found in the *Aa* water extract. The highest total phenolic content measured by the FC method was also found in the *Aa* water extract. Using FT-ICR MS, five phenolic acids including chlorogenic acid, ferulic acid, gallic acid, protocatechuic acid and sinapic acid were identified in the *Aa* water extract. Since the total phenolic content measured and the phenolic acids being identified using FT-ICR MS was the highest in the *Aa* water extract, the strong scavenging activity measured in the *Aa* water extract is suggested to be partly contributed by its phenolic compounds, the phenolic acids present.

As ROS plays an important role in tumor angiogenesis, which marks the pivotal transition from avascular to vascular tumor growth, removing excess ROS should result in an anti-angiogenic effect and increase the life expectancy in cancer patients. In the present study, the anti-angiogenic activity of the *Aa* water extract was demonstrated. The *Aa* water extract reduced the intracellular ROS level measured in Caco-2 in a concentration dependent manner. It also significantly suppressed the VEGF production in Caco-2 at a concentration of 25 μg/ml. Applying different concentrations of the *Aa* water extract inhibited the functions including HUVECs proliferation, migration and tubule formation of VEGF-induced HUVECs on Matrigel to different extents as well as the microvessels outgrowth in VEGF-induced rat aortic ring. Treating HUVECs with three phenolic acids, namely gallic acid, caffeic acid and protocatechuic acid, at a concentration of 5 mM also resulted in a significant suppression in VEGF-induced proliferation, migration and tubule formation in HUVECs and rat aortic ring microvessels outgrowth.

The positive results found in the anti-angiogenic activity of the *Aa* water extract was in agreement with its strong antioxidant activity and phenolic acids profile. Our findings suggested that the anti-angiogenic effects by the *Aa* water extract is partly related to the presence of phenolic acids and the anti-angiogenic activity of phenolic acids is related to their strong antioxidant activity. However, it is worth to note that the amount of phenolic acids present in the *Aa* water extract was not very high when compared with the concentration of phenolic acids used in this study. Therefore, the anti-angiogenic effects observed in the *Aa* water extract could also be possibly due to the actions of other biological compounds. Other anti-angiogenic mechanisms include down-regulating some signaling pathways such as the suppression of ERK phosphorylation in HUVECs (Labrecue and others, 2005; Chikaraishi and others, 2010). Cell cycle and matrix metalloproteinase that are coupled with angiogenesis are also possible targets for inhibiting angiogenesis (Fotsis and others, 1997). Further investigation is required to delineate the underlying mechanism of this anti-angiogenic effect.

Besides, the antioxidant and anti-angiogenic effect was only evaluated in the *Aa* cold water crude extract. Since cooking for mushroom involves heat treatment, hot water extracts instead of cold water extracts might be considered for future studies. It is anticipated that hot water extraction could also extract more water soluble compounents from the mushroom but the degree of damage to the potent components should not be overlooked. Furthermore, the reduction in VEGF level was only evaluated in Caco-2 which is a colon cancer cell line. A wider range of cancer cell lines should be tested in order to ascertain whether the lowering of VEGF secretion in tumor cells is cell-specific or not. Last but not the least, the endothelial cell line used in this study was HUVEC, which is derived from large blood vessel, while tumor angiogenesis takes place in microvessels. It is suggested that the evaluation on endothelial cells functions should be done with different types of endothelial cells as some previous findings have indicated that *in vitro* assays should be carried out using endothelial cells from more than one source whenever possible (Staton and others,

2004).

Bioactivities of mushrooms include antitumor, hypolipidemic and immunomodulatory activities. It is widely accepted that the antioxidant activity of phenolic compounds can remove oxidative stress and prevent diseases like cancer in humans. This study has shown that the *Aa* water extract might be pharmacologically potent and *A. aegerita* might serve as a source of potential anti-angiogenic inhibitors in human diets against tumor angiogenesis.

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