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CYTOPROTECTIVE EFFECT OF *CISSUS QUADRANGULARIS* EXTRACT ON HUMAN UMBILICAL VEIN ENDOTHELIAL CELLS

Mr. Tarat Sapsrithong

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Biomedicinal Chemistry
Department of Biochemistry and Microbiology
Faculty of Pharmaceutical Sciences
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Thesis Title	CYTOPROTECTIVE EFFECT OF *CISSUS QUADRANGULARIS* EXTRACT ON HUMAN UMBILICAL VEIN ENDOTHELIAL CELLS
By	Mr. Tarat Sapsrithong
Field of Study	Biomedicinal Chemistry
Thesis Advisor	Associate Professor Duangdeun Meksuriyen, Ph.D.
Thesis Co-Advisor	Assistant Professor Punnee Nusuetrong, Ph. D.

Accepted by the Faculty of Pharmaceutical Sciences, Chulalongkorn University in Partial Fulfillment of the Requirements for the Master's Degree

…….. Dean of the Faculty of Pharmaceutical Sciences
(Associate Professor Pintip Pongpech, Ph.D.)

THESIS COMMITTEE

…….. Chairman
(Associate Professor Nongluksna Sriubolmas, Ph.D.)

……..……. Thesis Advisor
(Associate Professor Duangdeun Meksuriyen, Ph.D.)

…….. Thesis Co-advisor
(Assistant Professor Punnee Nusuetrong, Ph. D.)

…….. Examiner
(Associate Professor Thitima Pengsuparp, Ph.D.)

…….. External Examiner
(Assistant Professor Wisit Tangkeangsirisin, Ph.D.)

ธรัชต์ ทรัพย์ศรีทอง: ฤทธิ์ปกป้องเซลล์บุผิวหลอดเลือดจากรกของสารสกัดเพชรสังฆาต. (CYTOPROTECTIVE EFFECT OF *CISSUS QUADRANGULARIS* EXTRACT ON HUMAN UMBILICAL VEIN ENDOTHELIAL CELLS) อ. ที่ปรึกษาวิทยานิพนธ์หลัก : รศ.ภญ.ดร.ดวงเดือน เมฆสุริเยนทร์, อ. ที่ปรึกษาวิทยานิพนธ์ร่วม : ผศ.ดร.พรรณี หนูซื่อตรง, 121 หน้า.

งานวิจัยนี้มีวัตถุประสงค์เพื่อศึกษาฤทธิ์ของสารสกัดเพชรสังฆาตในการต้านอนุมูลอิสระ และในการปกป้องเซลล์บุผิวหลอดเลือดจากรกของมนุษย์อีซีวี 304 ที่ถูกกระตุ้นด้วยไฮโดรเจนเปอร์ออกไซด์ (H_2O_2) ผลการทดลองพบว่าน้ำคั้นจากเพชรสังฆาตสดและนำมาพ่นแห้ง (CQWS1) สามารถต้านอนุมูลอิสระ superoxide anion, H_2O_2 และ hydroxyl radical ได้ดีกว่าเพชรสังฆาตที่สกัดด้วยเอทานอลโดยวิธี soxhlet เป็นเวลา 48 ชั่วโมง (CQES48) ซึ่งสอดคล้องกับฤทธิ์ต้านอนุมูลอิสระของสารสำคัญ quercetin และ resveratrol ที่พบในเพชรสังฆาต จึงมีความเป็นไปได้ว่าสารสกัดเพชรสังฆาตจะสามารถปกป้องเซลล์อีซีวี 304 จากอนุมูลอิสระ จึงศึกษาหาความเข้มข้นของสารสกัดเพชรสังฆาตที่ไม่เป็นพิษต่อเซลล์ด้วยวิธี MTT โดยบ่มเซลล์อีซีวี 304 ด้วยสารสกัดเพชรสังฆาตเป็นเวลา 24 ชั่วโมง พบว่า CQWS1 และ CQES48 ที่ความเข้มข้นไม่เกิน 1 มิลลิกรัมต่อมิลลิลิตรไม่เป็นพิษต่อเซลล์ จึงใช้ความเข้มข้นช่วงดังกล่าวในการศึกษาฤทธิ์ปกป้องเซลล์ การทดสอบฤทธิ์ลดอนุมูลอิสระภายในเซลล์โดยวิธี DCFH-DA พบว่าการบ่มเซลล์ด้วย CQES48 เป็นเวลา 24 ชั่วโมง สามารถลดปริมาณอนุมูลอิสระภายในเซลล์เมื่อได้รับ H_2O_2 (100 ไมโครโมลาร์) ในขณะที่ CQWS1 (1 มิลลิกรัมต่อมิลลิลิตร) ต้องใช้ความเข้มข้นที่สูงกว่า CQES48 (0.1 มิลลิกรัมต่อมิลลิลิตร) ถึง 10 เท่าในการลดปริมาณอนุมูลอิสระภายในเซลล์ได้อย่างมีนัยสำคัญ ผลการทดลองสอดคล้องกับกลุ่มเซลล์ที่ได้รับ quercetin และ resveratrol (5 ไมโครโมลาร์) แสดงให้เห็นว่าฤทธิ์ลดอนุมูลอิสระภายในเซลล์ของสารสกัดเพชรสังฆาตน่าจะเกิดจาก quercetin และ resveratrol ซึ่งอาจเกี่ยวข้องกับการแสดงออกของเอนไซม์ต้านอนุมูลอิสระและ endothelial nitric oxide synthase (eNOS) การศึกษากลไกการลดอนุมูลอิสระภายในเซลล์ด้วยวิธี Western blot พบว่า CQES48 ที่ความเข้มข้น 0.5 มิลลิกรัมต่อมิลลิลิตร รวมทั้ง quercetin และ resveratrol สามารถเพิ่มการแสดงออกของเอนไซม์ที่เร่งปฏิกิริยากำจัดอนุมูลอิสระ copper/zinc superoxide dismutase (Cu/Zn-SOD), manganese superoxide dismutase (Mn-SOD) และ glutathione peroxidase (GPx) และเมื่อบ่มเซลล์ด้วย CQES48 เป็นเวลา 24 ชั่วโมงก่อนกระตุ้นเซลล์ด้วย H_2O_2 ที่ความเข้มข้น 100 ไมโครโมลาร์สามารถเพิ่มการแสดงออกของโปรตีน Cu/Zn-SOD, Mn-SOD, GPx และ eNOS ได้ ผลจากงานวิจัยสรุปได้ว่าเพชรสังฆาตที่สกัดด้วยเอทานอลโดยวิธี soxhlet เป็นเวลา 48 ชั่วโมง สามารถลดการเกิดอนุมูลอิสระภายในเซลล์อีซีวี 304 โดยเพิ่มการแสดงออกของเอนไซม์ต้านอนุมูลอิสระและ eNOS และมีความสัมพันธ์กับฤทธิ์ต้าน H_2O_2 ที่ทดสอบในหลอดทดลอง อันเป็นผลจากสารสำคัญในเพชรสังฆาต quercetin และ resveratrol

ภาควิชา ชีวเคมีและจุลชีววิทยา ลายมือชื่อนิสิต.....

สาขาวิชา ชีวเวชเคมี ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก.....

ปีการศึกษา 2553 ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม.....

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KEYWORDS : *CISSUS QUADRANGULARIS* / CYTOPROTECTION / REACTIVE OXYGEN SPECIES / ANTIOXIDANT ENZYMES / HUMAN UMBILICAL VEIN ENDOTHELIAL ECV304 CELLS /

TARAT SAPSRITHONG: CYTOPROTECTIVE EFFECT OF *CISSUS QUADRANGULARIS* EXTRACT ON HUMAN UMBILICAL VEIN ENDOTHELIAL CELLS. ADVISOR: ASSOC. PROF. DUANGDEUN MEKSURIYEN, Ph.D., CO-ADVISOR: ASST. PROF. PUNNEE NUSUETRONG, Ph. D., 121 pp.

The aims of the present study are to determine whether *Cissus quadrangularis* (CQ) could protect against hydrogen peroxide (H_2O_2)-induced oxidative injury in human umbilical vein endothelial ECV304 cells and also whether the protective potential may have any relation to the free radical scavenging activities in cell-free system. In cell-free system, superoxide anion, H_2O_2, and hydroxyl radical scavenging activities of the spray drying of compressed fresh CQ (CQWS1) were stronger than ethanolic CQ extract using soxhlet apparatus for 48 h (CQES48). These results were correlated with quercetin and resveratrol, active constituents in CQ, which exhibited the free radical scavenging activities in cell-free system. Based on the MTT assay, no cytotoxicity on ECV304 cells was observed after 24-h exposure to CQWS1 and CQES48 at the concentration not more than 1 mg/mL. Hence, the concentrations up to 1 mg/mL of CQWS1 and CQES48 were chosen for the cytoprotective study from oxidative stress using DCFH-DA assay. Pretreatment of the cells with CQES48 reduced H_2O_2-induced reactive oxygen species (ROS) generation in a concentration-dependent manner, as examined by a decrease of DCF fluorescence intensity induced by H_2O_2 (100 µM). Meanwhile, CQWS1 (1 mg/mL) significantly restored the generation of the intracellular ROS at 10-fold higher concentration than CQES48 (0.1 mg/mL). Additionally, quercetin and resveratrol (5 µM) significantly reduced H_2O_2-induced ROS generation in the cells. The results indicated that the attenuation of intracellular ROS of CQES48 might be due to the presence of quercetin and resveratrol, and might involve in the increase of antioxidant enzymes and endothelial nitric oxide synthase (eNOS) expression. Western blot analysis revealed that treatment of the cells with CQES48 (0.5 mg/mL) as well as quercetin and resveratrol (50 µM) significantly up-regulated the protein expression of antioxidant enzymes, copper/ zinc superoxide dismutase (Cu/Zn-SOD), manganese superoxide dismutase (Mn-SOD) and glutathione peroxidase (GPx), as compared with control. However, treatment with CQES48 for 24 h before exposure to 100 µM H_2O_2 significantly increased the protein expression of Cu/Zn-SOD, Mn-SOD, GPx and eNOS. The results showed that CQES48 attenuated the intracellular ROS level in H_2O_2-induced ECV304 cells injury via the up-regulation of the antioxidant enzymes and eNOS, which was also correlated with H_2O_2 scavenging activity in cell-free system, resulting from its constituents, quercetin and resveratrol.

Department : Biochemistry and Microbiology Student's Signature .....

Field of Study : Biomedicinal Chemistry Advisor's Signature .....

Academic Year : 2010 Co-advisor's Signature .....

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LIST OF ABBREVIATIONS

ANOVA	analysis of variance
BHA	butylated hydroxyanisole
CAT	catalase
cGMP	cyclic guanosine monophosphate
CO_2	carbon dioxide
CQ	*Cissus quadrangularis* Linn.
CQER7	ethanolic extract of CQ using reflux for 7 h
CQES7	ethanolic extract of CQ using soxhlet apparatus for 7 h
CQES48	ethanolic extract of CQ using soxhlet apparatus for 48 h
CQWF1	aqueous extract of CQ and further freeze-dried
CQWS1	spray drying of freshly compressed CQ
Cu/Zn-SOD	copper/zinc superoxide dismutase
^oC	degree celsius
DCF	dichlorofluorescein
DCFH	dichlorodihydrofluorescein
DCFH-DA	2′, 7′-dichlorodihydrofluorescein diacetate
DMPO	5,5-dimethyl-1-pyproline *N*-oxide
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DPPH	2, 2-diphenyl-1-picrylhydrazyl
EC_{50}	half maximal effective concentration
ECV304	transformed human umbilical vein endothelial cells
EDTA	ethylenediaminetetraacetic acid
eNOS	endothelial nitric oxide synthase
ER	endoplasmic reticulum
ESR	electron spin resonance
et al.	*et alii*, and others
ETC	electron transport chains
Fe^{2+}	ferrous ion
GPx	glutathione peroxidase
GR	glutathione reductase

GSH	glutathione
GSSG	glutathione disulfide
GTP	guanosine triphosphate
h	hour
H_2O_2	hydrogen peroxide
HCl	hydrochloric acid
HO-1	heme oxygenase 1
HRP	horseradish peroxidase
HUVECs	human umbilical vein endothelial cells
IC_{50}	half maximal inhibitory concentration
iNOS	inducible nitric oxide synthase
M	molar
MDA	malondialdehyde
mg	milligram (s)
min	minute (s)
mL	millilitre (s)
mM	millimolar
Mn-SOD	manganese superoxide dismutase
mRNA	messenger ribonucleic acid
MTT	3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide
NaCl	sodium chloride
NADH	nicotinamide adenine dinucleotide
NBT	nitrotetrazolium blue chloride
ng	nanogram (s)
nm	nanometer (s)
nNOS	neuronal nitric oxide synthase
NO	nitric oxide
$NO^{\bullet}$	nitric oxide radical
NOS	nitric oxide synthase
$O_2^{-\bullet}$	superoxide radical
OD	optical density
$OH^{\bullet}$	hydroxyl radical
$OONO^-$	peroxynitrite

%	percentage
PBS	phosphate-buffered saline
pH	the negative logarithm of hydrogen ion concentration
PMS	phenazine methosulfate
PVDF	polyvinylidene fluoride
R_f	retention factor
RNS	reactive nitrogen species
ROOH	hydroperoxide
ROS	reactive oxygen species
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SEM	standard error of mean
SNP	sodium nitroprusside
SOD	superoxide dismutase
TBA	2-thiobarbituric acid
TBARS	thiobarbituric acid reactive substances
TBST	tris-buffered saline, 0.05% tween 20
TCA	trichloroacetic acid
TLC	thin layer chromatography
v/v	volume by volume
µL	microliter (s)
µM	micromolar
UV	ultraviolet
w/w	weight by weight

CHAPTER I
INTRODUCTION

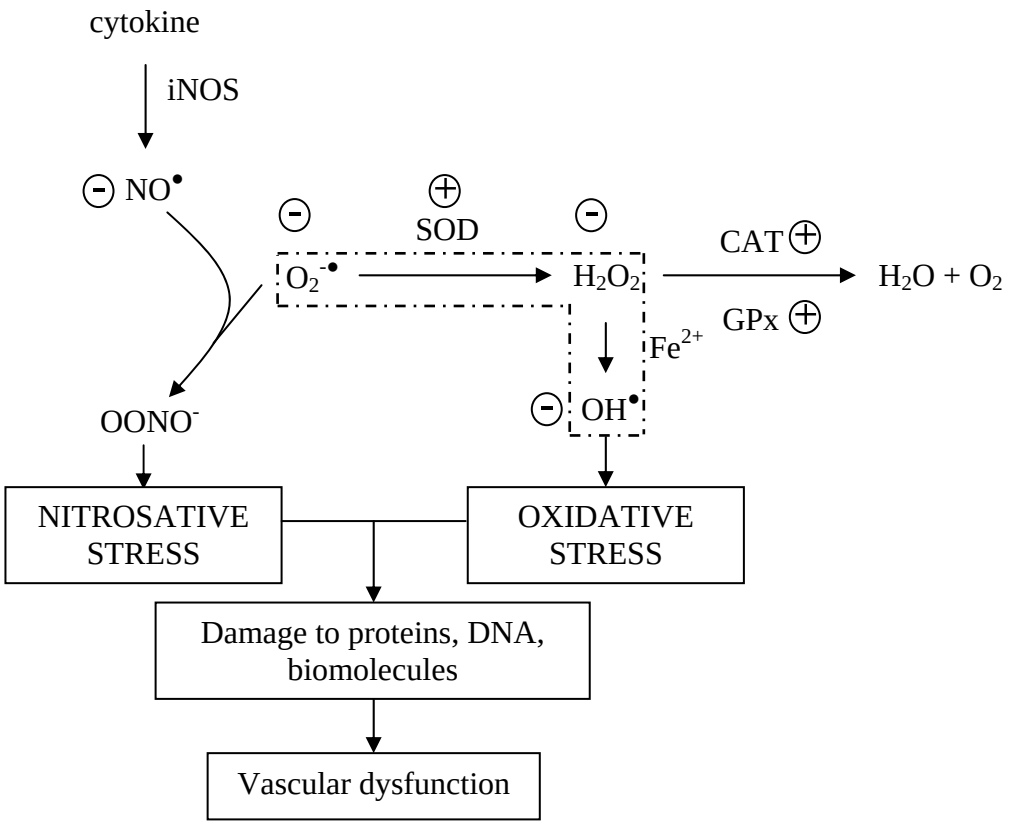
Nowadays, most people take an interest in their health. Thus, natural products prefer to be used as supplementary food, cosmetic, drink and herbal medicine. Medicinal plants have been considered to be less side effects, less toxic and lower cost than synthetic chemicals. In order to apply the medicinal plants to be used worldwide, scientific evidences in various fields are needed. There are also other vascular diseases, although not fatal, but it demonstrates the weakness of the blood vessel such as hemorrhoid. Hemorrhoid, a pathologic dilation of the venous plexus, is a common problem worldwide. It occurs at any age and can affect both men and women especially pregnancy. Routine activities may lead to this symptom including increasing intra-abdominal pressure, sitting for long periods of time, pregnancy, low-fiber diet and aging. This symptom is not crucial but it is bothering and has a serious impact on patients' quality of life. The treatment of hemorrhoid is always prevention by consuming a diet high in fiber. The hemorrhoid surgery was the last option for extreme cases or cases that could not heal by simultaneously. According to Ministry of Public Health, Thai herbs used for the treatment of hemorrhoid are *Anacyclus pyrethrum*, *Clerodendrum serratum* Moon., *Iresine herbstii* Hook f., *Catharanthus roseus*, *Streblus asper* Lour., *Pluchea indica* Less. and *Cissus quadrangularis* Linn (CQ) (ประกาศกระทรวงสาธารณสุข, 2542: 116). CQ, a climbing herb in Vitaceae family, is an easily approached herb in India, Africa and Arab countries. CQ, known in Thai as Phet Sang Khat, San Chakhat, Samroikho, Tamleung Tong, is widely used in Thai traditional over-the-counter drug for the treatment of hemorrhoid, bone healing and carminative (Pongboonrod, 1995) including scurvy and nose bleed. Based on scientific evidence, CQ possesses many biological activities, for example analgesic, anti-inflammatory, venotonic effects (Panthong *et al*., 2006; Srisook *et al*., 2011) and antioxidant activities (Badami and Channabasavaraj, 2007; Jainu and Devi, 2006). CQ contains various antioxidants such as quercetin, resveratrol (Adesanya *et al*., 1999; Singh *et al*., 2007), quercitrin and iso-quercitrin (Jakikasem *et al*., 2000; Singh *et al*., 2007). Hence, CQ may protect blood vessels from oxidative stress or to strengthen them.

The pathogenesis of hemorrhoid is swollen or inflamed of the veins around the anus or lower rectum by free radicals and inflammatory substances (Buckshee *et al*., 1997; Weissmann *et al*., 1980) which released from stasis and stagnation of the blood in the vein. Free radicals has been proven to be involved in the pathogenesis of hemorrhoid (Glowinski and Glowinski, 2002; Wali *et al*., 2002). Thus, the protection of the vein endothelial cells from free radicals may lead to the prevention from hemorrhoid. Human umbilical vein endothelial ECV304 cells has been used to determine the protection from oxidative stress in many experiments (Chiou *et al*., 2008; Hou *et al*., 2004; Kosem *et al*., 2007; Lin *et al*., 2007; Wang *et al*., 2007). Therefore, the aim of this study is to determine the protective role of CQ on human umbilical vein endothelial ECV304 cells and its underlying mechanism.

Conceptual framework

Active constituents in ethanolic CQ extract, quercetin and resveratrol, possessed the scavenging activities of superoxide radical ($O_2^{-\bullet}$) (Hazra *et al*., 2008; Leonard *et al*, 2003), hydrogen peroxide (H_2O_2) (Gülçin, 2010), hydroxyl radical ($OH^{\bullet}$) (Gao *et al*., 1999; Leonard *et al*, 2003) and nitric oxide radical ($NO^{\bullet}$) (Choi *et al*., 2007) in cell-free system. These activities may further encourage the protective activities in cell-based system through the regulation of antioxidant enzyme expression which was superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT). In Thai traditional medicine, CQ was boiled with water and further used to cure many diseases. The aqueous CQ extract may contain flavonoid glycosides which also possessed the free radical scavenging activities (Lee *et al*., 2001) and cytoprotective effect (Choi, 2010; Yan *et al*., 2002). Thus, the aqueous CQ extract as well as the ethanolic CQ extract might reveal the free radical scavenging activities against reactive oxygen species (ROS) and reactive nitrogen species (RNS) and cytoprotective effect. After the determination of the free radical scavenging activities in cell-free system, the non-toxic concentrations of CQ extracts were used to investigate the protective effect from the excessive ROS generation. These activities were used as a guideline to study the effects of the CQ extracts on the protein expression of antioxidant enzymes and nitric oxide synthase (NOS) in endothelial cells.

Conceptual framework



⊖ CQ extracts scavenge ROS and RNS.

⊕ CQ extracts up-regulate the protein expression of antioxidant enzymes and NOS.

Objectives

1. To determine scavenging activities against ROS and RNS of CQ extracts in cell-free system.
2. To investigate the effect of CQ on viability of human umbilical vein endothelial ECV304 cells in the absence or presence of H_2O_2 as compared with quercetin and resveratrol.
3. To investigate the effect of CQ on ROS accumulation in ECV304 cells in the absence or presence of H_2O_2 as compared with quercetin and resveratrol.
4. To evaluate the effect of CQ on protein expression of antioxidant enzymes and NOS in ECV304 cells in the absence or presence of H_2O_2 as compared with quercetin and resveratrol.

Scope of study

In this study, ROS and RNS scavenging activities of CQ extracts in cell-free system were investigated by using DPPH, $O_2^{-\bullet}$, H_2O_2, $OH^{\bullet}$ and $NO^{\bullet}$ scavenging assay. The non-toxic concentrations of CQ extracts were determined using MTT assay. The attenuation of intracellular ROS in the H_2O_2-treated ECV304 cells was determined using 2′, 7′-dichlorodihydrofluorescein diacetate (DCFH-DA) assay. The underlying mechanism of the intracellular ROS attenuation of the CQ extracts was determined using Western blot analysis.

Experimental design

In this study, the qualitative and semi-quantitative analysis of antioxidative constituent in CQ extracts was investigated by TLC densitometric method. The free radical scavenging activities of CQ extracts in cell-free system were investigated by using DPPH, $O_2^{-\bullet}$, H_2O_2, $OH^{\bullet}$ and $NO^{\bullet}$ scavenging assay. To determine the effect of CQ extracts in cell-based system, ECV304 cells were used. The non-toxic concentrations of CQ extracts were used to determine the ROS attenuation and cytoprotection on H_2O_2-injured ECV304 cells using DCFH-DA and MTT assays, respectively. The alteration of the protein expression of antioxidant enzymes and NOS was analyzed by Western blot.

Contributions of the study

1. Information for the antioxidant activities of CQ, which might involve in the protective effect on endothelial cells
2. Understanding the mechanism underlying the endothelial cytoprotection of CQ, involving in the protein expression of antioxidant enzymes and NOS.

CHAPTER II
LITERATURE REVIEW

2.1 Reactive oxygen species and reactive nitrogen species

Free radicals are ions, atoms or molecules with unpaired electrons, which are unstable and highly reactive. The free radicals are divided into two groups: the first are ROS such as $O_2^{-\bullet}$, H_2O_2 and $OH^{\bullet}$, and the second, are RNS such as $NO^{\bullet}$ and peroxynitrite ($OONO^-$). Free radicals are generated by various conditions e.g. cellular metabolism, inflammation, ultraviolet (UV) light and pollution. In biological processes, free radicals reveal some necessary activities, such as intracellular killing of bacteria and cell signaling processes (Dröge, 2002; Nordberg and Arner, 2001), whereas ROS at high concentrations can readily react with most biomolecules, nucleic acids, lipids, proteins leading to damage cell structure followed by cell death (Martindale and Holbrook, 2002; Willcox *et al*., 2004). Moreover, massive ROS production has been involved in the endothelial dysfunction, which has been used to refer to vascular diseases such as atherosclerosis, stroke and hemorrhoid (Cai and Harrison, 2000; Dröge, 2002). In order to stop the chain reaction, the unpaired electrons must be eliminated or react with free radical scavengers.

Nitric oxide (NO) is enzymatically formed from L-arginine in the presence of NOS, which is divided into three isoforms: constitutively expressed endothelial nitric oxide synthase (eNOS), inducible nitric oxide synthase (iNOS), and neuronal nitric oxide synthase (nNOS) (Cepinskas *et al*., 2002). NO generated by eNOS is implicated with a vasodilation and antiplatelet aggregation in blood vessel (Loscalzo, 2001). The activation of iNOS, usually induced by inflammation-mediating cytokines, leads to the production of $NO^{\bullet}$ in higher level (micromolar level) and longer duration than constitutive eNOS or nNOS (nanomolar level). Thus, the high level of inducible $NO^{\bullet}$ is a major factor in cytotoxicity and endothelial dysfunction (Achike and Kwan, 2003). Morevoer, the excessive $NO^{\bullet}$ can interact with $O_2^{-\bullet}$ via a reaction that proceeds at more rapidly rate than dismutation by SOD and forms powerful oxidants, such as $OONO^-$, which are capable of toxic nitrosylation or nitration of some amino acid residues such as tyrosine and cysteine (Achike and Kwan, 2003; Cai and Harrison, 2000). The toxicity of sodium nitroprusside (SNP) and/or paraquat in cerebral endothelial cells of Sprague-Dawley rats exhibited by the increase of lactate dehydrogenase level. The toxicity of the combination of $NO^{\bullet}$ and $O_2^{-\bullet}$ suggested a

synergic toxicity (Gobbel *et al*., 1997), which was a consequence of the increase of nitrotyrosine formation by $OONO^-$ (Beckman *et al*., 1992). Hence, $NO^\bullet$ has been found to be involved in pathophysiological conditions, as well as inflammatory conditions that lead to tissue injury (Wink and Mitchell, 1998) (Figure 1).

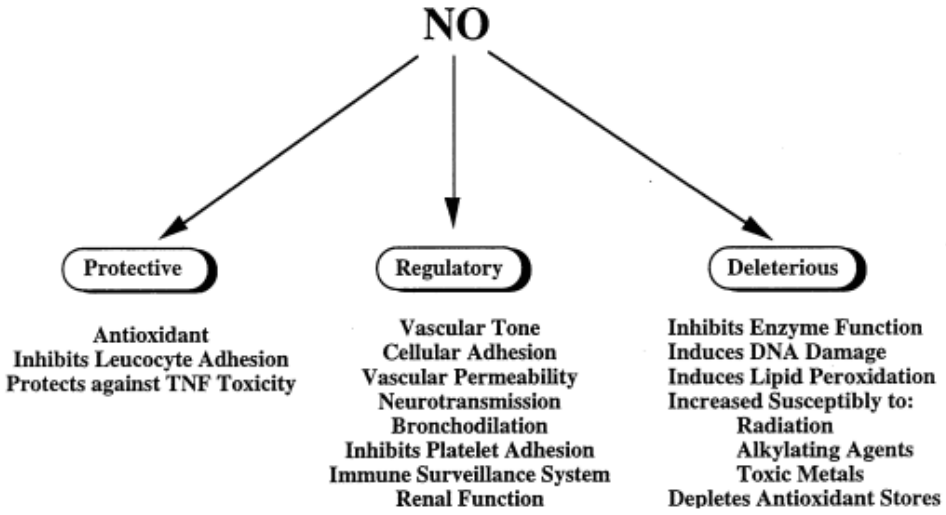


Figure 1. The biological effect of NO (Wink and Mitchell, 1998).

2.2 Role of free radicals in hemorrhoid

Hemorrhoid is one of vascular diseases that showing an infirmity of blood vessel. These symptoms are not crucial but bothering and decreasing hygiene in daily life. Moreover, the symptoms reveal the blood vessel weakness which can involve in other vascular diseases. The veins around the anus or lower rectum are swollen or inflamed by increasing intra-abdominal pressure, sitting for long periods of time, mostly found in pregnancy. There are two types of typical symptoms, internal hemorrhoid and external hemorrhoid. Internal hemorrhoid occurs inside the rectum, which is painless than external hemorrhoid and most patients are ignoring. External hemorrhoid appears outside of the anal verge (Kann and Whitlow, 2004; Moore, 2000). The pathogenesis of hemorrhoid involves in stasis and stagnation of the blood in the vascular plexuses of anal cushion, which the venous stasis lead to inflammation of the vessel by increase the permeability, fragility, necrosis and resulting in bleeding. During the inflammatory response, free radicals are released and lead to acute development of hemorrhoid. Lipid peroxidation, which can be induced by free radicals such as $O_2^{-\bullet}$, correlated with the progression of the varicose vein disease. This was determined by the determination of malondialdehyde (MDA) production in

the wall of patient veins using thiobarbituric acid reactive substances (TBARS) assay. Due to the generation of $O_2^{-\bullet}$ in varicose vein of patients, SOD level was increased to scavenge $O_2^{-\bullet}$ as a general protection (Wali *et al*., 2002). Nevertheless, varicose vein with superficial thrombophlebitis revealed to increase the free radical generation through an increased content of xanthine oxidase, a free radical generator (Glowinski and Glowinski, 2002). These results suggested that $O_2^{-\bullet}$ played an important role in the pathogenesis of varicose veins such as hemorrhoid.

2.3 Cellular model for the study of endothelial dysfunction under oxidative stress

The pathogenesis of hemorrhoid involves a degeneration of the anchoring supporting tissue of the anal cushions, their descent, and venous distention and stasis due to lack of support. The venous stasis releases the free radicals and inflammatory substances and further leads to inflammation on the luminal surface of the vein (Buckshee *et al*., 1997; Weissmann *et al*., 1980).

Human umbilical vein endothelial cells (HUVECs) were used as a model for the investigation of endothelial dysfunction under oxidative stress. H_2O_2 has been reported to induce oxidative stress and cell dysfunction in HUVECs (Chen *et al*., 2010; Gong *et al*., 2010). Thus, antioxidant, which can slow or prevent the oxidation, may protect endothelial cells from injury. Ophiopogonin D isolated from *Ophiopogon japonicas* reduced H_2O_2-induced lipid peroxidation, protein carbonylation and also attenuated mitochondrial ROS generation and cell apoptosis in HUVECs. In addition, ophiopogonin D restored cellular total antioxidative capacity and inhibited the release of inflammatory cytokines (Qian *et al*., 2010). Moreover, HUVECs have been reported in the expression of antioxidant enzymes that involved in the cellular protection (Kuo *et al*., 2009). Oxidized low-density lipoprotein induced oxidative stress in HUVECs through the imbalance between intracellular ROS and antioxidant enzymes. ROS was inactivated by decrease of copper/zinc SOD (Cu/Zn-SOD) and eNOS expression. These activities were reversed after pretreatment with *Solanum lyratum* extract, which contained a lot of antioxidant compounds (Kuo *et al*., 2009).

ECV304 cells, a spontaneously transformed human umbilical vein endothelial cells, was used to determine the protection from oxidative stress as well as inflammation, which involved in many vascular diseases including hemorrhoid (Cai and Harrison, 2000; Dröge, 2002). ECV304 cells expressed the antioxidant enzymes

such as SOD (Liu *et al*., 2009), GPx, CAT (Hou *et al*., 2004; Wang *et al*., 2007) including eNOS and iNOS (Chiou *et al*., 2008; Gil'yano *et al*., 2009; Xia *et al*., 2006). These enzymes played an important role to protect cells from the excessive ROS formation and vasodilation (Loscalzo, 2001; Nordberg and Arner, 2001). The protection of ECV304 cells from H_2O_2-induced cell injury was attenuated by chitosan oligosaccharides (Liu *et al*., 2009) through the attenuation of intracellular ROS leading to increase in the viability in H_2O_2-injured ECV304 cells. The protection against oxidative stress included the restoring activities of antioxidant enzymes, SOD and GPx, along with the capacity of increasing levels of NOS. Moreover, many researches were supported to use ECV304 cells in terms of cellular protection from oxidative stress (Chiou *et al*., 2008; Hou *et al*., 2004; Kosem *et al*., 2007; Lin *et al*., 2007; Wang *et al*., 2007) including inflammation (Luo *et al*., 2008). Thus, ECV304 cells are a tidy model to determine the protective effect of CQ from oxidative stress.

2.4 Antioxidant systems and protein expression in endothelial cells under oxidative stress

Defense mechanisms against free radical-induced oxidative stress contribute to three step: first, the preventive antioxidant enzymes to inhibit the generation of excessive free radicals; second, the free radical scavenging effect of non-enzymatic antioxidants such as vitamin C and vitamin E; and third, de novo enzymes act as the repairing damage and reconstituting of lipases, proteases, DNA repair enzymes, and transferases (Figure 2). The antioxidant enzymes, SOD, GPx and CAT, worked within the cells in the preventive mechanisms to remove free radicals such as $O_2^{-\bullet}$ and H_2O_2 before they reacted with metal ions to form more potent reactive free radical, $OH^{\bullet}$ (Valko *et al*., 2007; Willcox *et al*., 2004) (Figure 3). SOD is a major cellular defense system against $O_2^{-\bullet}$ in all cells. This enzyme contains redox metals in the catalytic center and catalyzes the reduction of $O_2^{-\bullet}$ to H_2O_2 and oxygen (O_2) (Figure 3). Three different isoforms of SOD have been identified: the mitochondrial manganese SOD (Mn-SOD), the cytosolic Cu/Zn-SOD and the extracellular SOD. The extracellular SOD is also a Cu/Zn-containing enzyme which is mainly secreted by vascular smooth muscle cells and binds to glycosaminoglycans in the vascular extracellular matrix on the endothelial cell surface (Wassmann *et al*., 2004). The cytosolic and mitochondrial

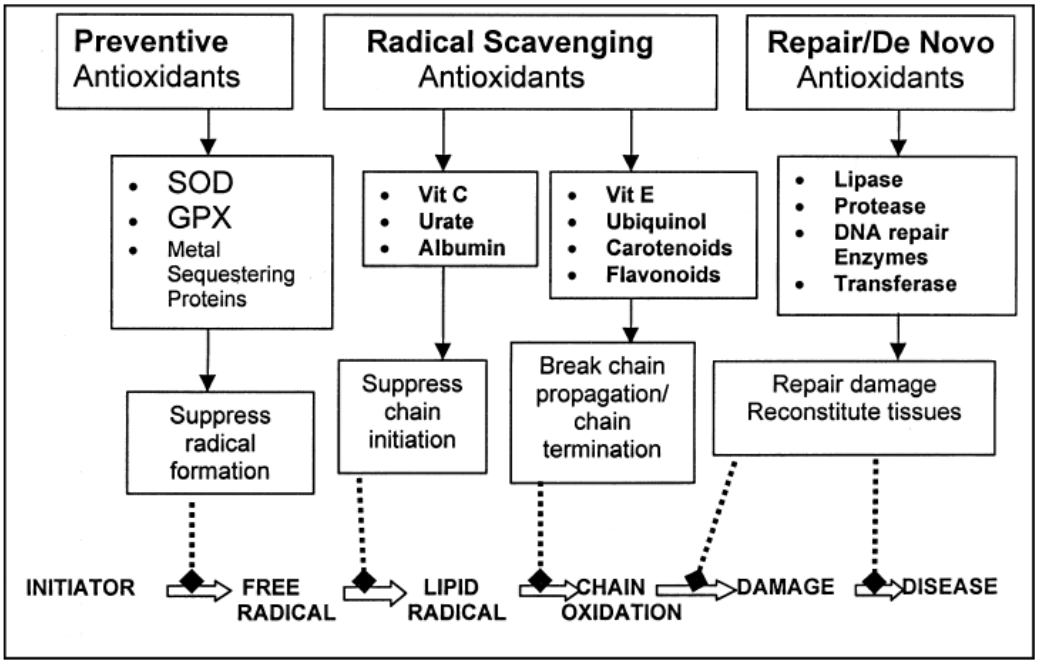


Figure 2. Defense mechanisms against free radical-induced oxidative stress (Willcox *et al*., 2004)

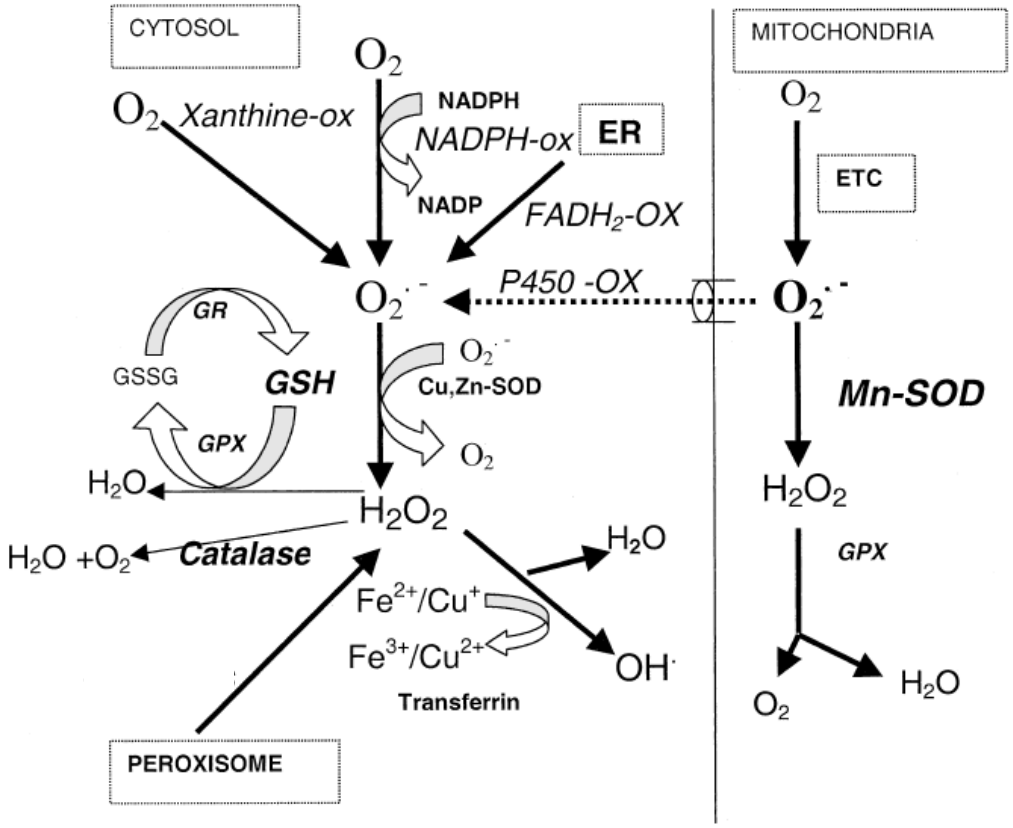


Figure 3. Generation of ROS and endogenous antioxidant mechanisms (modified from Willcox *et al*., 2004).

GPx is a selenium-containing antioxidant enzyme that catalyzes the reduction of H_2O_2 and lipid peroxides to water and lipid alcohols, respectively (Figure 3), and also oxidizes glutathione (GSH) to glutathione disulfide (GSSG). In the absence of GPx or GSH levels, H_2O_2 and lipid peroxides may react with metal ions (Fe^{2+}) to form $OH^{\bullet}$ and lipid peroxyl radicals, respectively, that involve in the vascular disease risk (Wassmann *et al*., 2004). CAT, mainly located in cellular peroxisomes and cytosol, catalyzes the reduction of H_2O_2 to water and O_2 (Figure 3). CAT is especially important in the case of limited GSH content or reduced GPx activity and plays a significant role in the development of tolerance to oxidative stress in the cellular adaptive-response (Wassmann *et al*., 2004).

Under normal physiological condition, there is a balance between the level or activities of antioxidant enzymes and free radicals, which is essential for the survival of organisms and their health (Valko *et al*., 2007). However, at higher levels of free radicals, cell injury may occur when the balance of antioxidant enzymes and free radicals is annihilated. This lead to oxidative damage to all types of biomolecules including DNA, proteins and lipids that have been associated the endothelial dysfunction and causes of many vascular diseases (Cai and Harrison, 2000; Diaz *et al*., 1997; Dröge, 2002; Østerud and Bjørklid, 2003; Vanhoutte *et al*., 2009; Willcox *et al*., 2004).

2.5 Natural antioxidants

A non-enzymatic antioxidant, such as tocopherols and ascorbic acid (Elango *et al*., 2006), is a molecule that can slow or prevent the oxidation such as biological oxidation processes, ionizing, UV irradiation and a variety of oxidizing pollutants. Antioxidant compounds, especially from fruits and herbal extracts, are not only doing as free radical scavengers but also modulating the antioxidant enzymes. For example, α-tocopherol as a well-known exogenous antioxidant involves in modulating entire antioxidant defense systems in HUVECs by scavenging free radicals and also increases mRNA and protein level of Cu/Zn-SOD and CAT (Nakamura and Omaye, 2008). While the activities of SOD, CAT and glutathione *S*-transferase in rabbit blood plasma were increased after the administration of ascorbic acid (Yousef *et al*., 2007). Thus, herbal extract containing antioxidant compounds might increase the entire antioxidant defense systems. For example, *Lycium chinense* fruit extract containing a lot of flavonoids possessed abilities to scavenge free radicals and induced the protein

expression of antioxidant enzymes. The extract scavenged $OH^{\bullet}$ and $O_2^{-\bullet}$ in cell-free system and also deceased intracellular ROS in H_2O_2-injured human Chang liver cells. Hence, the extract revealed the protective effect against H_2O_2-induced cell death that may come from the consequence of the recovery of Cu/Zn-SOD, Mn-SOD, GPx and CAT (Zhang *et al*., 2010). This result revealed that the antioxidant enzyme expression in endothelial cells was correlated with the cellular protection under oxidative stress condition. Therefore, the antioxidant compounds are used to maintain the balance of the antioxidant defenses and free radicals in biological system, which can be obtained from drug, herb or antioxidant compound.

Quercetin and resveratrol are bioactive compounds from fruits, vegetables and herbs (Figure 4). Quercetin is a strong antioxidant and has been demonstrated free radical scavenging activities such as $O_2^{-\bullet}$, which was created by phenazine methosulfate-nicotinamide adenine dinucleotide (PMS-NADH) and measured by the reduction of NBT (Hazra *et al*., 2008). The $OH^{\bullet}$, which was generated in Fenton reaction trapped by 5, 5-dimethyl-1-pyproline *N*-oxide (DMPO) forming spin adduct, was scavenged by quercetin after the detection by electron spin resonance (ESR) spectrometer (Gao *et al*., 1999; Lee *et al*., 2001). Thus, the free radical scavenging activity of quercetin in cell-free system may further encourage the protective effect in cell-based system. Quercetin can inhibit the hexanal formation during copper-catalysed human low-density lipoprotein oxidation *in vitro* (Meyer *et al*., 1997) and can also protect blood healthy volunteer from damage by decrease of copper binding to low-density lipoprotein (Roland *et al*., 2001). Administration of quercetin inhibited the oxidative stress by the decrease of MDA level on homocysteine-injured ECV304 cells (Lin *et al*., 2007). Quercetin not only directly scavenged free radicals but also indirectly increased the expression and activities of antioxidant enzymes, SOD, CAT, GPx and glutathione *S*-transferase, in cirrhotic rat induced by carbon tetrachloride (Amália *et al*., 2007). Moreover, quercetin attenuated phenylephrine-induced contraction in rat aortic vessels through the stimulation of eNOS phosphorylation and the increase of nitrite level, indicative of eNOS derived NO production in bovine aortic endothelial cells (Khoo *et al*., 2010). NO, produced by eNOS, has been discovered as a vasodilator product to relax the vascular smooth muscle and resulting arterial vasodilation, which leads to improved perfusion and O_2 delivery (Loscalzo, 2001).

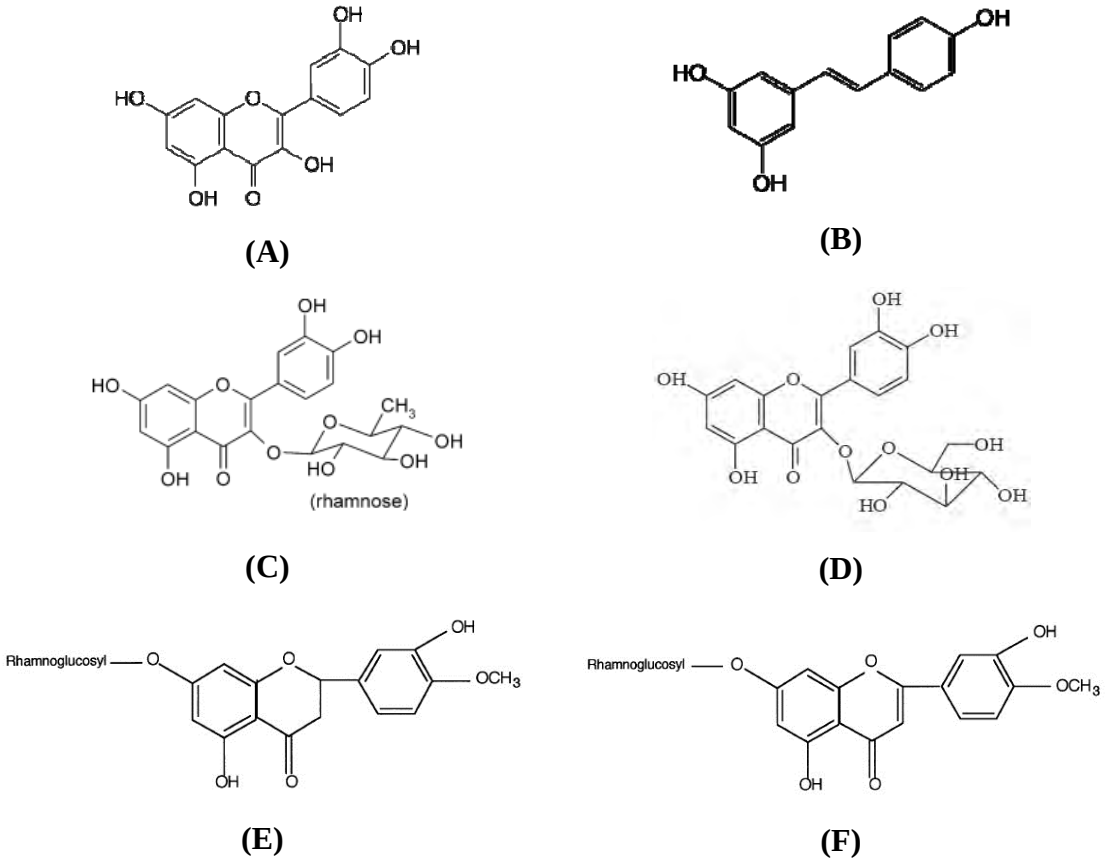


Figure 4. Chemical structures of (A) quercetin (B) resveratrol (C) quercitrin (D) isoquercitrin (E) hesperidin and (F) diosmin.

Flavonoids are mostly present as their glycosides (Murota and Terao, 2003). Glycoside form of quercetin (Figure 4), such as quercetin-3-rhamnoside (quercitrin) and quercetin-3-glucoside (iso-quercitrin), is also presented in plants including CQ (Jakikasem *et al*., 2000; Singh *et al*., 2007). Water soluble quercetin glycosides (Formica and Regelson 1995) were poorly absorbed in the small bowel, meanwhile the most likely route of intestinal transport was by passive uptake of the relatively lipophilic aglycone in the colon after hydrolysis of the glycosides by the large intestinal microflora (Manach *et al*. 1995, 1997). Quercitrin and iso-quercitrin possessed the free radical scavenging activities, which was similar to quercetin. Administration of quercitrin exhibited to scavenge $OH^{\bullet}$, which was generated by the Fenton reaction and detected by ESR spectrometer (Lee *et al*., 2001). Moreover, quercitrin treatment protected murine osteoblastic MC3T3-E1 cells from the cytotoxic H_2O_2 and decreased the production of MDA, protein carbonyl and nitrotyrosine on H_2O_2-injured osteoblasts (Choi, 2010; Yan *et al*., 2002). The ROS scavenging activities in cell-free system were also involved in the biological activities. However, the $O_2^{-\bullet}$ scavenging activity of quercitrin and iso-quercitrin, determined by using the cellular xanthine/xanthine oxidase system as a superoxide source, was lower than quercetin (Lu and Yeap Foo, 2000). Hence, the activities of quercetin and its glycosides may encourage to the prevention of vascular diseases and strengthen the blood vessel.

Resveratrol, a stilbene derivative (Figure 4B), was presumed to be beneficial for human health. Similarly to quercetin, resveratrol exhibited antioxidant property to scavenge $O_2^{-\bullet}$ and $OH^{\bullet}$ that was generated by xanthine/xanthine oxidase system and the Fenton reaction, respectively (Leonard *et al*., 2003). The scavenging activity of resveratrol was revealed through a decrease in the absorbance upon oxidation of H_2O_2 (Gülçin, 2010). Thus, the free radical scavenging activities in cell-free system may be encouraged to the biological activities of resveratrol. Administration of resveratrol decreased the H_2O_2 production on linoleic acid hydroperoxide-induced damage in human colon carcinoma HT29 cells (Kaindl *et al*., 2008) and decreased MDA level in the brain of healthy rats (Mokni *et al*., 2007). Resveratrol possessed a vasorelaxant through a stimulation of Ca^{2+}-activated K^{+} channels and enhanced $NO^{\bullet}$ signaling in the endothelium (Baur and Sinclair, 2006). Moreover, the incubation with 100 µM resveratrol for 72 h increased the expression and activity of eNOS in HUVECs

derived EA.hy 926 cells (Wallerath *et al*., 2002) and increased the activities of SOD, CAT and peroxidase in the brain of healthy rats (Mokni *et al*., 2007).

The free radicals are the major cause of blood vessel weakness resulting in many vascular diseases including hemorrhoid (Cai and Harrison, 2000; Diaz *et al*., 1997; Østerud and Bjørklid, 2003). Fortunately, quercetin, resveratrol and quercetin glycosides possessed abilities to scavenge free radicals in both cell-free and cell-based systems, which may perform as antihemorrhoidal agent like hesperidin and diosmin (Figure 4). Thus, herbal extracts containing the antioxidants may prevent blood vessels from vascular diseases and/or strengthen them.

2.6 Agents used in the treatment of hemorrhoid

Medical treatment of hemorrhoid is aimed to relieve the symptoms through analgesics relieve pain, anti-itch and anti-burn. Corticosteroids have been used to cure hemorrhoid by reducing inflammation and can relieve itching. Unfortunately, long time used of corticosteroids can damage to the skin permanently (Odukoya *et al*., 2009).

The combination of hesperidin (10%) and diosmin (90%) (Figure 4), a well-known antihemorrhoidal drug (Bouskela *et al*., 1997; Sarabia *et al*., 2001), relieved acute symptom in 66% patients (Buckshee *et al*., 1997). Hesperidin and diosmin exhibited anti-inflammatory in clinical trial of pregnancy (Buckshee *et al*., 1997; Sarabia *et al*., 2001) and venotonic effect in human umbilical vein (Panthong *et al*., 2007). According to previous study, anti-inflammation drugs are located at sites of inflammation at concentrations capable of interfering with oxidant production, which contribute to their anti-inflammatory activity (Halliwell *et al*., 1988). Diosmin (90%) and hesperidin (10%) (Daflon® 500) also revealed the inhibition of oxidant-induced plasma leakage from postcapillary venules in the hamster cheek pouch microcirculation (Bouskela *et al*., 1997). Nevertheless, hesperidin exhibits $O_2^{-\bullet}$, $OH^{\bullet}$ and $NO^{\bullet}$ scavenging activities in cell-free system (Kalpana *et al*., 2009). In cell-based system, hesperidin administration decreased extensive damage from bacterial lipopolysaccharide-induced lipid peroxidation in liver of male Wistar rats due to increased production of ROS. Hesperidin-administered rats restored the levels of glutathione reductase (GR) and SOD resulting in the decrease in ROS production and effectively protected liver of rats (Kaur *et al*., 2006). Hesperidin also decreased MDA level on H_2O_2-induced lipid peroxidation in red blood cells (Kalpana *et al*., 2009).

According to DCFH-DA assay, hesperidin possessed ability to eliminate the intracellular ROS accumulation in cyclic strain-induced oxidative stress in HUVECs. Nevertheless, hesperidin enhanced NO production and NOS activity that can be considered as the protective effects in vascular vessels (Chiou *et al*., 2008). Diosmin treatment protected liver microsomes from carrageenan or carbon tetrachloride-induced lipid peroxidation (Melin *et al*., 1996) and reduced the inflammation in clinical trial (Sarabia *et al*., 2001). Thus, other antioxidants, which revealed similar activities as hesperidin and diosmin, may protect the cells from injury.

2.7 *Cissus quadrangularis* and its biological activities

CQ, a climbing herb in Vitaceae family, has quadrangular fleshy stem with internodes 8 to 10 cm long and 1.2 to 1.5 cm wide (Figure 5). The plant is an easily found in India, Africa, Arab countries and Thailand. In India, the powdered dry shoot has been used for indigestion treatment (Chopra *et al*., 1965). The leaves have been used against internal ulceration (Reddy *et al*., 1989). In Arab countries, the aqueous extract of dried entire plant has been used as “cure-all” medicine. In Thai traditional medicine, CQ is widely used as bone healing, carminative and anti-hemorrhoid (Pongboonrod, 1995). Based on scientific evidence, CQ possessed gastroprotective effect involving in the role of proinflammatory cytokines and oxidative damage (Table 1). The methanolic CQ extract decreased interleukin-1β, tumor necrosis factor-α and iNOS in gastric mucosal in rat induced injury by aspirin. Besides, the CQ extract inhibited gastric damage by enhancement in SOD, CAT and GSH, and also decreased the lipid peroxidation level of gastric mucosal tissues (Jainu and Devi, 2006). The gastroprotective effect may be accorded to the free radical scavenging activities of methanolic CQ extract in cell-free system, revealing an approximately EC_{50} values of 200-400 µg/mL for $OH^{\bullet}$ and $O_2^{-\bullet}$ (Badami and Channabasavaraj, 2007) and 373.33 ± 1.66 µg/mL for H_2O_2 (Jainu and Devi, 2005). The edema induction in rat by ethyl phenylpropiolate, carrageenan and acetic acid decreased after administration of methanolic CQ extract, revealing by the contraction of vascular smooth muscle of human umbilical vein when compared with well-known anti-hemorrhoid reagents diosmin and hesperidin (Panthong *et al*., 2007). Moreoever, ethyl acetate CQ extract possessed an anti-inflammatory effect in murine macrophage cell line RAW264.7 through the induction of heme oxygenase 1 (HO-1) expression.

(A)

(B)

Figure 5. Aerial part of *Cissus quadrangularis*. (A) Stems and (B) flowers of *Cissus quadrangularis*.

Table 1. *Cissus quadrangularis* and its biological activities.

Extract	Fraction	Compound (% Content)	Pharmacological/ Biological activity	Reference
80 % Ethanol	Dichloromethane	Quercetin (0.00210%)	-	Adesanya *et al*., 1999.
	Ethyl acetate	Resveratrol (0.00050%)	-	
Ethanol	Ethyl acetate	Quercitrin	-	Jakikasem *et al*., 2000.
		Iso-quercitrin		
	-	Quercetin Quercitrin	-	Singh *et al*., 2007.
	-	-	Antiosteoporotic	Shirwaikar *et al*., 2003.
Methanol	-	-	DPPH, H_2O_2 scavenging / lipid peroxidation	Badami and Channabasavaraj, 2007.
			$O_2^{-\bullet}$, $OH^{\bullet}$ scavenging, antioxidant enzyme activities	Jainu and Devi, 2005.
			Analgesic, anti-inflammatory and venotonic	Panthong *et al*., 2007.
Ethyl acetate	-	-	Inhibition of $NO^{\bullet}$ production by suppression of iNOS expression	Srisook *et al*., 2011

The CQ extract also suppressed the mRNA and protein expression of iNOS induced by lipopolysaccharide (Srisook *et al*., 2011). In clinical trial, two capsules of 500 mg dry powder of CQ taken twice daily were effective to reduce pain, inflammation and the size of hemorrhoid (Segsunviriya and Choomprabutra, 1989).

CQ contains various antioxidant agents such as quercetin found in dichloromethane (0.0021%) (Adesanya *et al*., 1999) or ethyl acetate fraction of ethanolic extract (Patarapanich *et al*., 2004) (Table 1). Resveratrol can also be found in ethyl acetate fraction of ethanolic extract (0.0005%) (Adesanya *et al*., 1999). Moreover, quercetin glycosides, such as quercitrin and iso-quercitrin, have been found in ethyl acetate fraction of ethanolic extract (Jakikasem *et al*., 2000). Nevertheless, quercetin glycosides were previous reported to be extracted by water (Formica and Regelson 1995) such as iso-quercitrin from broccoli (Price *et al*., 1998), and quercitrin from *Albizia julibrissin* (Ekenseair *et al*., 2006) and *Eucalyptus globulus* Labill. (Almeida *et al*., 2009). Thus, the ethanolic and aqueous extracts of CQ, which contain of quercetin, resveratrol, quercitrin and iso-quercitrin, may strengthen and prevent blood vessel damage from the free radicals.

2.8 Quality control of herbal extracts

Medicinal plants become to take more part in protecting and curing roles. At the beginning, medicinal plants are using in small area for traditional uses that have no scientific evidence to prove its activities. The herbal medicine must be examined and further developed to reduce the variation before going to be used as worldwide drugs. Due to the variability of herbal cultivation, such as landscape, climate and harvest administration, this may involve in the inaccuracy of herbal extracts activity through the uncertainty of the amount of active constituents. According to the WHO guideline about the assessment of crude plant materials, the active constituents in the extracts should be determined. The qualitative and quantitative of active constituents are detected by following the validation of analytical methods of International Conference on Harmonisation (ICH) guidelines (International Conference on Harmonisation, 1994).

The determination of the phenolic compounds in the ethanolic extract of *Hypericum venustum* contained active constituents, such as hypericin, pseudohypericin, and quercetin, more than in the aqueous extract. Moreover, the content of phenolic compounds in the extracts was correlated with antioxidant

activities (Spiteller *et al.*, 2008). The variable of herbal cultivation also revealed the different amount of active constituents. For example, thirteen samples of *Garcinia mangostana* fruit rind were collected in different harvesting period and locations, East and South of Thailand. The qualitative and quantitative analysis of thirteen ethanolic extract were all exhibited the mangostins, active constituent, with a range from 30.19 ± 0.16 to 45.61 ± 0.09 % w/w, while in the dried powder were 8.51 ± 0.05 to 11.50 ± 0.02 % w/w (Pothitirat and Gritsanapan, 2008). The samples from the South, where it rains all year, contained a higher yield of total mangostins (36.92 ± 5.55 % w/w in the extract and 10.39 ± 1.04 % w/w in the dried powder) than the samples from the East (35.68 ± 3.79 % w/w in the extract and 9.55 ± 0.45 % w/w in the dried powder). These revealed that the amount of active constituents in the herbal extract were variable. Thus, the quantitative and qualitative analysis of quercetin in the CQ extract is necessary.

CHAPTER III
MATERIALS AND METHODS

Materials

Equipments

- CAMAG Linomat 5 applicator (022.7808, CAMAG, Muttenz, Switzerland)
- CAMAG TLC Scanner 3 (027.6481, CAMAG, Muttenz, Switzerland)
- 5% CO_2 incubator (3121, Forma Scientific Inc, Massachusetts, USA)
- Gel documentation (GeneGnome5, Syngene, Cambridge, UK)
- Hemocytometer (Bright-line, Hausser Scientific, Pennsylvania, USA)
- Microplate reader (Perkin Elmer, Victor 3, Massachusetts, USA)
- Phase-contrast inverted microscope (CK30, Olympus, Tokyo, Japan)
- Rotary evaporator (RE120, Buchi, Flawil, Switzerland)
- Sieve number 40 (RETSCH 5657 HAAN 0.425 nm Mesh No. 40, Germany)
- Silica gel 60 F_{254} (1.05554.0001, Merck, Darmstadt, Germany)
- Spray dried apparatus (B-290, Buchi, Flawil, Switzerland)

Reagents

- Ascorbic acid (95210, Fluka Chemicals, Steinheim, Germany)
- Butylated hydroxyanisole (BHA) (B1253, Sigma, St. Louis, USA)
- 2-Deoxy-D-ribose (31170, Fluka Chemicals, Steinheim, Germany)
- 2′, 7′-Dichlorodihydrofluorescein diacetate (DCFH-DA) (D6883, Sigma, St. Louis, USA)
- Dimethyl sulfoxide (DMSO) (60153, Merck, Darmstadt, Germany)
- 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) (M2128, Sigma, St. Louis, USA)
- Diosmin (D3525, Sigma, St. Louis, USA)
- 2, 2-Diphenyl-1-picrylhydrazyl (DPPH) (43180, Fluka Chemicals, Steinheim, Germany)
- Enhanced chemiluminescence (ECL) prime Western blotting detection reagent (RPN2232, Amersham, Buckinghamshire, UK)
- Ethyl acetate (E/0900/17, Fisher Scientific, California, USA)
- Ethylenediaminetetraacetic acid (EDTA) (180, Univar, Sydney, Australia)

- Fetal bovine serum (FBS) (CH30160.02, Hyclone, Utah, USA)
- Formic acid (1779, Univar, Sydney, Australia)
- Goat polyclonal secondary antibody to rabbit IgG - H&L (HRP) (ab6721, Abcam, Cambridge, England)
- Griess reagent (sulfanilamid/N-(1-naphthyl)-ethylene-diamine dihydrochloride) (G4410, Sigma, St. Louis, USA)
- Guaiacol (G5502, Sigma, St. Louis, USA)
- Hesperidin (H5254, Sigma, St. Louis, USA)
- Hydrogen peroxide (H_2O_2) (H/1750/17, Fisher Scientific, California, USA)
- Iron (III) chloride ($FeCl_3$) (UN1773, Merck, Darmstadt, Germany)
- D(-)-mannitol (5980, Merck, Darmstadt, Germany)
- Medium 199 (31100-027, Gibco BRL Life Technologies, New York, USA)
- Mouse monoclonal to β-actin, horseradish peroxidase conjugated (ab20272, Abcam, Cambridge, England)
- β-Nicotinamide adenine dinucleotide reduced disodium salt hydrate (NADH) (N6005, Sigma, St. Louis, USA)
- Nitrotetrazolium blue chloride (NBT) (N6876, Sigma, St. Louis, USA)
- Penicillin streptomycin (15140, Gibco BRL Life Technologies, New York, USA)
- Peroxidase type I from horseradish (HRP) (P8125, Sigma, St. Louis, USA)
- Phenazine methosulfate (PMS) (P9625, Sigma, St. Louis, USA)
- Protease inhibitor (80-6501-23, Amersham Biosciences, Buckinghamshire, England)
- Quercetin (Q0125, Sigma, St. Louis, USA)
- Rabbit polyclonal CAT (ab16731, Abcam, Cambridge, England)
- Rabbit polyclonal to eNOS (ab5589, Abcam, Cambridge, England)
- Rabbit polyclonal to iNOS (ab3523, Abcam, Cambridge, England)
- Rabbit polyclonal to glutathione peroxidase 1 (GPx) (ab22604, Abcam, Cambridge, England)
- Rabbit polyclonal to Cu/Zn-SOD (ab16831, Abcam, Cambridge, England)
- Rabbit polyclonal to Mn-SOD (ab13533, Abcam, Cambridge, England)
- Resveratrol (R5010, Sigma, St. Louis, USA)
- Sodium nitroprusside (SNP) (71778, Fluka Chemical, Steinheim, Germany)

- 2-Thiobarbituric acid (TBA) (T5500, Sigma, St. Louis, USA)
- Toluene (8325, Merck, Darmstadt, Germany)
- Trichloroacetic acid (TCA) (641730, Merck, Darmstadt, Germany)

Methods

Plant material

Aerial parts of CQ were collected in Ayuthaya, Thailand in December 2008 and identified by Associate Professor Dr. Rutt Suttisri, Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Thailand. A voucher specimen (No. RS-160651) was deposited in Department of Biochemistry and Microbiology, Faculty of Pharmaceutical Sciences, Chulalongkorn University.

Preparation of CQ extracts

The aerial parts of CQ (11.79 kg) were dried at 60°C and grinded into powder (1.24 kg). The sieve number 40 was used to sift CQ powder. The powder was extracted with 95% ethanol (25 g of dried powder per 400 mL of ethanol) using soxhlet apparatus for 7 h (CQES7) and 48 h (CQES48) or reflux for 7 h (CQER7). Each extract was evaporated to dryness under reduced pressure at 40°C. The extracts were aliquot and stored in well-closed containers protected from light and kept at -20°C.

For the preparation of aqueous extracts, the CQ powder was boiled for 1 h and further concentrated (25 g of dried powder per 400 mL of water) under reduced pressure at 60°C and freeze dried (CQWF1). The extract was stored in capped vials, at room temperature.

Another aqueous extract of CQ was acquired from fluid of the compressed fresh CQ (1 kg). The fluid was boiled for 1 h and spray dried (CQWS1). The extract was aliquot and stored in well-closed containers at room temperature.

The yields of the CQ extracts were calculated by the following equation: $(Y_a/Y_b) \times 100$, where Y_a is the weight of CQ extract, Y_b is the weight of CQ powder or fluid of the compressed fresh CQ.

TLC densitometric analysis of quercetin in CQ extracts

A solvent system of toluene-ethyl acetate-formic acid (6:4:1, v/v/v) was used and detected at the absorbance of 254 and 365 nm. The mobile phase was allowed to run a distance of 100 mm in a saturated tank. Standard quercetin (100, 200, 300, 400 and 500 ng/spot) was applied on a TLC plate (silica gel 60 F_{254}) and developed under the above conditions to assess the standard calibration curve. CQES7 and CQER7 (199 µg/spot) were spotted on each TLC plate to investigate the amount of quercetin in the extracts. After the development, the peak height of the spot on the plate was integrated by CAMAG TLC Scanner 3. For every sample, the procedure was repeated three times.

Free radical scavenging activities in a cell-free system

DPPH free radical scavenging assay

DPPH, which can generate a stable radical, was determined to reveal the total scavenging activity. The extracts or standards were allowed to react with DPPH in order to evaluate the free radical scavenging activity, which changed the color of DPPH from purple to yellow.

The method was slightly modified from Brand-Williams *et al.* (1994). Briefly, various concentrations of the aqueous extracts (0.025, 0.05, 0.1, 0.2, 0.3, 0.4 and 0.5 mg/mL) or the ethanol extracts (0.2, 0.3, 0.4, 0.5, 0.75 and 1 mg/mL) were thoroughly mixed with freshly prepared DPPH (78 µM) ethanol solution to give a final volume of 200 µL. The mixture was then kept in the dark at room temperature for 90 min. The absorbance was measured at 510 nm using a microplate reader. Ascorbic acid was used as a positive control. The percentage of scavenging activity was calculated by the following equation: $[(A_a-A_b)/A_a] \times 100$, where A_a is the absorbance of the DPPH solution without addition of samples, A_b is the absorbance of the DPPH solution after reacting with the samples. The half maximal effective concentration (EC_{50}) values were assessed from the curve-fitting to the above mentioned equation.

Scavenging of $O_2^{-\bullet}$

$O_2^{-\bullet}$ was generated in a non-enzymatic PMS-NADH system. The reduction of NBT, which was interacted with $O_2^{-\bullet}$ and revealed blue color, was determined using spectrophotometric measurement as described by Valentão *et al.* (2001). Various

concentrations of the extracts (0.0125, 0.025, 0.05, 0.1, 0.2, 0.4, 0.6, 0.8 and 1 mg/mL) were incubated at room temperature for 5 min with a reaction mixture containing a final volume of 200 µL of 166 µM NADH, 43 µM NBT and 2.7 µM PMS in 0.1 M potassium phosphate buffer. The absorbance was measured at 560 nm. Quercetin was used as a positive control. The percentage of $O_2^{-\bullet}$ scavenging activity was calculated by the following equation: $[(A_a-A_b)/A_a] \times 100$, where A_a is the absorbance of the reaction mixture without addition of samples, and A_b is the absorbance of the reaction mixture after reacting with the samples.

Scavenging of H_2O_2

H_2O_2 scavenging activity was measured using the guaiacol method (Michot *et al*., 1985). The peroxidase catalyzed the oxidation of guaiacol (colorless) in the presence of H_2O_2 to tetraguaiacol (brown color) by the following equation.

$$4\text{guaiacol} + 4H_2O_2 \xrightarrow{\text{peroxidase}} \text{tetraguaiacol} + 8H_2O$$

The method was slightly modified from Choi *et al*. (2007). Briefly, the reaction mixture contained a final volume of 200 µL of 0.01% guaiacol solution, 1 U/mL of HRP, 0.5 mM H_2O_2 and the extracts at various concentrations (0.1, 0.2, 0.4, 0.6, 0.8 and 1 mg/mL) in 0.1 M potassium phosphate buffer pH 7.4. The reaction mixture was further incubated at room temperature for 60 min. The absorbance was measured at 450 nm. Ascorbic acid was used as a positive control. The percentage of H_2O_2 scavenging activity was calculated by the following equation: $[(A_a-A_b)/A_a] \times 100$, where A_a is the absorbance of the reaction mixture without addition of samples, and A_b is the absorbance of the reaction mixture after reacting with the samples.

Scavenging of $OH^\bullet$

$OH^\bullet$ scavenging ability was determined by measuring the formation of TBARS using 2-deoxy-D-ribose as substrate. Fe^{3+}-ascorbate-EDTA-H_2O_2 system (Fenton reaction) generated the $OH^\bullet$, which degraded the 2-deoxy-D-ribose. The activity was monitored by a decrease in an absorbance at 532 nm.

The method was slightly modified from Hsu *et al*. (2006). Briefly, the reaction mixture contained a final volume of 1 mL of 2.8 mM 2-deoxy-D-ribose, 0.1 mM H_2O_2, 100 µM $FeCl_3$, 104 µM EDTA, 0.1 mM ascorbic acid and the extracts at

various concentrations (0.1, 0.2, 0.4, 0.6, 0.8 and 1 mg/mL) in 0.1 M potassium phosphate buffer pH 7.4. After incubation at 37°C for 1 h, 1 mL of 1% TBA and 1 mL of 2.8% TCA were added and incubated at 100°C for 15 min. The absorbance of the clear supernatant was measured at 532 nm. Mannitol was used as a positive control. The percentage of $OH^{\bullet}$ scavenging activity was calculated by the following equation: $[(A_a-A_b)/A_a] \times 100$, where A_a is the absorbance of the reaction mixture without addition of samples, and A_b is the absorbance of the reaction mixture after reacting with the samples.

Scavenging of $NO^{\bullet}$

$NO^{\bullet}$ was generated by SNP and further interacted with oxygen to create nitrite ions that can be detected with Griess reagent (Jagetia *et al*., 2004). Briefly, various concentrations of the extracts (0.1, 0.2, 0.4, 0.6, 0.8 and 1 mg/mL) were incubated with 4 mM SNP at room temperature under light. After 2.5-h incubation, Griess reagent was added, and the mixture was color-developed for 10 min. The absorbance of pink solution was measured at 560 nm. Quercetin was used as a positive control. The percentage of $NO^{\bullet}$ scavenging activity was calculated by the following equation: $[(A_a-A_b)/A_a] \times 100$, where A_a is the absorbance of the reaction mixture without addition of samples, and A_b is the absorbance of the reaction mixture after reacting with the samples.

Cell culture

Human umbilical vein endothelial ECV304 cells were purchased from CLS Germany (Lot. No. 600560-2). The cells with a seeding density of 1×10^5 cells/mL were cultured in M199 medium containing 10% FBS and 1% penicillin-streptomycin at 37°C in 5% CO_2 incubator. Medium was changed every 2-3 days and subcultured every 4-5 days using 1 mM EDTA and 0.25% trypsin in PBS. DMSO (0.5% final concentration) was used as a vehicle control in all experiments of cell system.

Determination of viability of ECV304 cells

Determination of the effect of CQ or H_2O_2 on cell viability

The cytotoxicity evaluation was determined using MTT assay. The mitochondrial dehydrogenase in viable cells reduced MTT to purple color of formazan crystal, which can be dissolved with DMSO. The intensity of purple solution can be measured at 570 nm.

Method was slightly modified from Carmichael *et al*. (1987). Sub-confluent cells were trypsinized and seeded into 96-well plates at a density of 1×10^5 cells/mL and incubated for 24 h before treatment. Thereafter, the cells were exposed to various concentrations of the extracts (0.5, 1, 2, 5, 8 and 10 mg/mL) or H_2O_2 (12.5, 25, 50, 100 and 200 µM) for 0.5, 6, 12 and 24 h. The medium was replaced by the MTT solution (0.4 mg/mL). After 4-h incubation, the MTT solution was replaced by 100% DMSO. The optical density (OD) was measured using a microplate reader at 570 nm. The percentage of cell survival was calculated by the following equation: $(OD_b/OD_a) \times 100$, where OD_a is the absorbance of control and OD_b is the absorbance of the treated cells. The half maximum inhibitory concentration (IC_{50}) was determined from % cell viability versus concentration curve.

Determination of the effect of CQ on viability of H_2O_2–treated cells

MTT assay was slightly modified from Kosem *et al*. (2007). Cells were seeded to 96-well plates and incubated for 24 h. The cells were treated with CQES48 and CQWS1 at various concentrations (0.05, 0.1, 0.2, 0.5 and 1 mg/mL) for 24 h followed by the addition of 100 µM H_2O_2 for 0.5, 2 and 6 h. The medium was removed and MTT (0.4 mg/mL) in medium was added. After 4-h incubation, the MTT solution was replaced by 100% DMSO. The OD was measured with a microplate reader using wavelength of 570 nm. The percentage of cell survival was calculated by the following equation: $(OD_b/OD_a) \times 100$, where OD_a is the absorbance of control and OD_b is the absorbance of the treated cells.

Measurement of intracellular ROS

Effect of CQ or H_2O_2 on intracellular ROS

The generation of intracellular ROS was determined by DCFH-DA, a non-polar dye which can be permeated into the cell and interacted with cytoplasmic esterase to generate dichlorodihydrofluorescein (DCFH). In the presence of cytosolic

esterase, DCFH was rapidly oxidized to a highly fluorescent dichlorofluorescein (DCF) (Eruslanov and Kusmartsev, 2009; Girard-Lalancette *et al*., 2009; Wang and Joseph, 1999). The intensity of DCF implied the amount of intracellular ROS such as H_2O_2, $OH^{\bullet}$ and hydroperoxides (ROOH).

The accumulation of ROS in the cells determined by DCFH-DA method was slightly modified from Shirai *et al*., 2002. The cells were trypsinized and seeded into 96-well plates at 1×10^5 cells/mL and incubated for 24 h before treatment. The cells were then exposed to CQ extracts (0.05, 0.1, 0.2, 0.5, 1 mg/mL) or H_2O_2 (12.5, 25, 50, 100 and 200 µM) for 0.5, 2, 6, 12 or 24 h. Thereafter, the cells were washed twice with cold-PBS and incubated with 5 µM DCFH-DA for 30 min. The absorbance was measured by a fluorescent microplate reader with excitation at 485 nm and emission at 535 nm. The DCF fluorescence (% control) was calculated by the following equation: $(OD_b/OD_a) \times 100$, where OD_a is the absorbance of control, the untreated cells, and OD_b is the absorbance of the cells that pre-incubated with samples for 24 h.

Effect of CQ on intracellular ROS in H_2O_2-treated cells

The effect of CQ extracts on the accumulation of ROS in H_2O_2-induced cell injury was determined by DCFH-DA assay. The cells were trypsinized and seeded into 96-well plates at 1×10^5 cells/mL and incubated for 24 h. The cells were then pretreated with CQ extracts at the concentrations of 0.05, 0.1, 0.2, 0.5, 1 mg/mL for 24 h. Thereafter, the cells were washed twice with cold-PBS and incubated with 5 µM DCFH-DA for 30 min. After DCFH-DA was removed, the cells were washed twice with cold-PBS and incubated with 100 µM H_2O_2 for 30 min. The absorbance was measured by a fluorescent microplate reader with excitation at 485 nm and emission at 535 nm. The DCF fluorescence (% control) was calculated by the following equation: $(OD_c/OD_a) \times 100$, where OD_a is the absorbance of control, the untreated cells, and OD_c is the absorbance of the cells that pre-incubated with samples for 24 h and followed by the incubation of 100 µM H_2O_2 for 30 min.

Western blotting analysis

Western blotting analysis was used to determine the specific proteins using gel electrophoresis to separate proteins by the size. The proteins on the gel were then transferred to PVDF membrane and the specific antibodies were probed.

The cells were pretreated with CQES48 at various concentrations (0.05, 0.1, 0.5 and 1 mg/mL) in 6-well plates for 24 h in the absence or presence of 100 µM H_2O_2 for 6 h. Treated cells were washed with ice-cold PBS then collected and centrifuged at 1500 rpm for 8 min. The protein was extracted by lysis buffer (300 mM NaCl, 40 mM Tris HCl (pH 7.4), 2 mM Pefabloc and protease inhibitor) at 4°C for 30 min. After centrifugation at 12000 rpm for 10 min, the supernatant was collected and protein concentrations were determined by Bradford method. The protein extract was mixed with loading buffer (60 mM Tris-base, pH 6.8, 2% SDS, 25% glycerol, 14.4 mM β-mercaptoethanol and 0.1% bromophenol blue) and further incubated at 95°C for 5 min. Proteins (30 µg) of each sample were loaded onto 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) for Cu/Zn-SOD, Mn-SOD, CAT, GPx, and 8% SDS-PAGE for eNOS and iNOS. The gel was further transferred onto PVDF membrane. The membrane was blocked with 5% skimmed milk in TBST buffer (10 mM Tris-base, pH 7.5, 0.1 M NaCl and 0.05% Tween 20) at room temperature for 1 h. Blots were probed with primary antibodies (Cu/Zn-SOD, Mn-SOD, GPx, CAT, eNOS, iNOS and β-actin) at 4°C overnight. After washing with TBST buffer, the membranes were blotted with goat polyclonal secondary antibody with horseradish peroxidase at room temperature for 1 h. The proteins bands (Cu/Zn-SOD, Mn-SOD, GPx and CAT) were detected by enhanced chemiluminescence detection reagent and exposed to X-ray films while eNOS and iNOS were determined using gel documentation. The intensities of the bands were then visualized and computed by ImageJ 1.41o software (NIH, Bethesda, MD, USA).

Statistical analysis

All values were presented as mean ± SEM calculated from at least three independent experiments performed in triplicate. The data were analyzed by one-way analysis of variance (ANOVA) and followed by LSD post hoc test. Values of $p < 0.05$ were considered statistically significant.

CHAPTER IV
RESULTS AND DISCUSSION

4.1 Preparation of CQ extracts

The aerial parts of CQ (11.79 kg) were dried at 60°C and grinded into powder. The powder was extracted with 95% ethanol using soxhlet apparatus for 7 h (CQES7) and 48 h (CQES48) or reflux for 7 h (CQER7). Each ethanolic extract was evaporated to dryness under reduced pressure at 40°C to give average yield of CQES7 (2.93%), CQES48 (3.48%) and CQER7 (3.07%) (Table 2). The aqueous extract was prepared by boiling CQ powder with water which was further freeze-dried to give average yield of CQWF1 (2.52%) (w/w) (Table 2). On the other hand, the compressed fresh CQ was boiled and further spray-dried to give an average yield of CQWS1 (0.45%) (w/w).

Yield of CQER7 was more than of CQES7 probably due to the increase in solubility of substance. The reflux extraction was more direct heat than soxhlet extraction. However, the main disadvantage of conventional reflux is possible degradation of target bioactive compounds due to local overheating effect (Arias *et al.*, 2009). Thus, CQES48 gave the highest yield due to longer extraction time. The freeze drying is a preservation technique by dehydration and freezing process. After the freezing step, the ice, which was inside the frozen extract, was sublimated by reducing of the pressure and using of suitable heat. This step is called as primary drying. Then, the secondary drying was performed to remove the unfrozen water by using of higher temperature and lower pressure, when compared with primary drying (Franks, 1998). On the other hand, spray drying is a method of producing a dry powder from a liquid or slurry by rapidly drying with a hot gas, which the thermal degradation of active constituents may occur. Moreover, CQWF1 was prepared by boiling CQ powder with water for 1 h while CQWS1 was acquired by compressing fluid from fresh CQ, which was further boiled for another 1 h. Thus, average yield of CQWF1 was more than CQWS1. In Thai traditional medicine, the fresh whole CQ was eaten with another fruit such as banana, to avoid the irritation effect of calcium oxalate. This was the rational for the preparation of compressed fresh CQ.

Table 2. Percent yield of CQ extracts from various extraction procedures.

Extract	**% Yield (w/w) (extract/fresh plant)**
CQES7	2.93
CQES48	3.48
CQER7	3.07
CQWS1	0.45
CQWF1	2.52

4.2 TLC densitometric analysis of quercetin in CQ extracts

According to the amount of active constituent(s) depending on the difference in the extraction procedures (Liu *et al*., 2010), determination of the existence of quercetin content in CQES7 and CQER7 was performed using TLC densitometer. Quercetin is a well-known free radical scavenger (Gao *et al*., 1999; Hazra *et al*., 2008) and has been reported the protective activities in cell-based system (Lin *et al*., 2007; Meyer *et al*., 1997; Roland *et al*., 2001). Due to the variable of herbal cultivation, the uncertainty of the amount of quercetin may exhibit which this may also affect the antioxidant and protective activities of CQ extracts. To find the appropriate extraction of CQ, the determination of quercetin in CQ extracts is necessary.

Our preliminary study found that the ethanolic extract contained quercetin which 40% ethanolic and aqueous extract cannot be observed. Quercetin content in the ethanolic CQ extract using heat (0.37 ± 0.0035 µg/mg extract) gave more yield than soaking without heat (0.23 ± 0.0040 µg/mg extract). The result was correlated with previous extraction reported by Liu *et al*., 2010. Quercetin content was therefore analyzed only in CQES7 and CQER7. For quantitative analysis using TLC-densitometric method, quercetin exhibited R_f value equal to standard quercetin (R_f 0.52) in a solvent system of toluene-ethyl acetate-formic acid (6:4:1, v/v/v) and detected at the absorbance of 254 and 365 nm. The TLC-densitometric calibration curve (Figure 6) used to calculate the quercetin content in the extracts was expressed by the following linear equation: $y = 0.611x - 75.714$; $R^2 = 0.9887$. Linearity was observed between quercetin content and peak height. The result revealed that quercetin content in CQES7 (1.82 ± 0.09 µg/mg of extract) more than CQER7 (1.46 ± 0.06 µg/mg of extract) (Table 3), demonstrating a good correlation with a previous study that soxhlet extraction obtained chlorogenic acid, an active constituent in *Folium eucommiae*, more than reflux extraction (Liu *et al*., 2010). Thus, the different extraction procedure is most likely to be involved in the amount of active constituents in the extract and its biological activities.

4.3 Free radical scavenging activities in a cell-free system

4.3.1 DPPH radical scavenging activity

The model of scavenging the stable DPPH radical is widely used to evaluate the free radical scavenging ability of various extracts (Chen *et al*., 2007; Maksimović, 2008; Sandoval *et al*., 2002; Shi *et al*., 2009). It was found that the radical scavenging

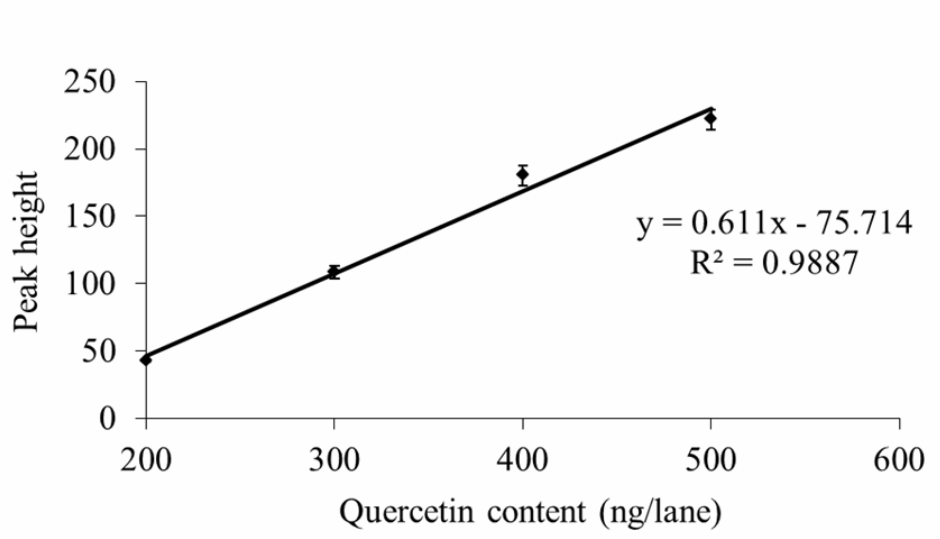


Figure 6. Calibration curve for the determination of quercetin by TLC densitometry (n=3).

Table 3. TLC densitometric analysis of quercetin content in CQ extracts using soxhlet as compared with reflux extraction**.**

Extract	Quercetin content	
	ng/lane	µg/mg of extract
CQES7	362.38	1.82 ± 0.09
CQER7	291.09	1.46 ± 0.06

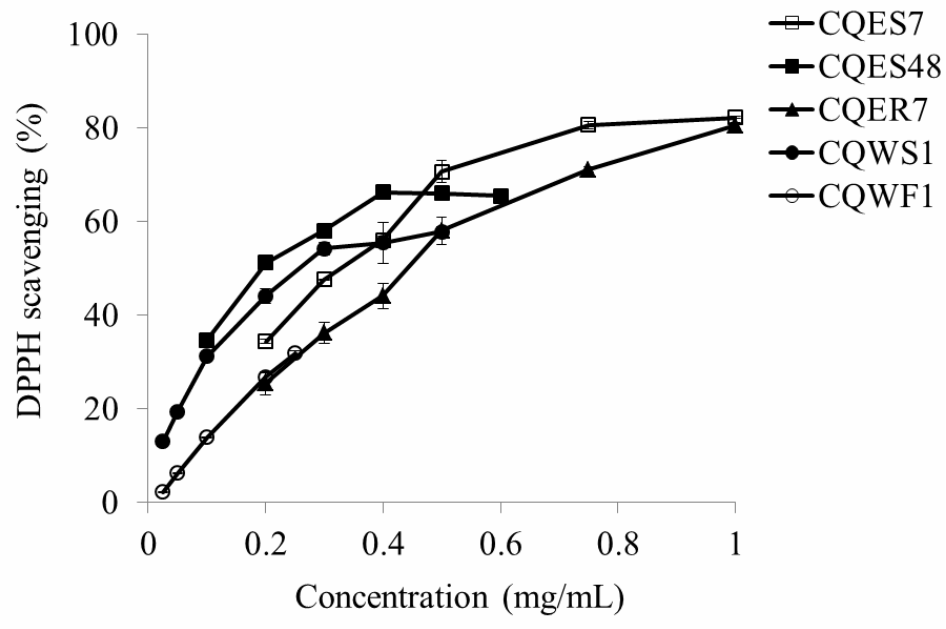
activity of all CQ extracts increased with increasing concentrations (Figure 7A). The DPPH radical scavenging activity (EC_{50}, mg/mL) was in the order: CQES48 (0.223 ± 0.010) > CQWS1 (0.256 ± 0.003) > CQES7 (0.333 ± 0.005) > CQER7 (0.443 ± 0.030) (Figure 7B). Since the maximum solubility of CQWF1 in water was found at a final concentration of 0.250 mg/mL, its EC_{50} value was therefore not able to determine.

Quercetin and resveratrol, well-known antioxidants (Boots *et al*., 2008, Gülçin, 2010), were isolated from the aerial parts of CQ (Adesanya *et al*., 1999; Singh *et al*., 2007). Our results demonstrated that quercetin and resveratrol exhibited the DPPH radical scavenging activity in a concentration-dependent manner (Figure 8B, 8C) and expressed their EC_{50} values of 6.23 ± 0.10 µM (2.11 ± 0.03 µg/mL) and 109.70 ± 2.27 µM (25.03 ± 0.52 µg/mL), respectively (Figure 8D), while ascorbic acid used as a positive control (Maksimović, 2008) expressed an EC_{50} value of 19.66 ± 0.13 µM (3.46 ± 0.02 µg/mL) (Figure 8A).

The DPPH radical scavenging activity of CQES7 was higher than CQER7 which may be due to the presence of more quercetin in CQES7 (Table 3). Based on the assumption that the longer time of soxhlet extraction may give the higher content of active constituents, resulting that the free radical scavenging activity of CQES48 was more than CQES7. The dried stem of CQ extracted with methanol for 48 h by maceration exhibited DPPH radical scavenging activity with an EC_{50} approximately 0.2 - 0.4 mg/mL (Jainu and Devi, 2005), similarly to CQES48. Our results indicated that quercetin was 3-fold more potent than ascorbic acid. According to a previous report, quercetin possessed an ability to scavenge DPPH free radical better than ascorbic acid and resveratrol (Villaño *et al*., 2007). These implied that quercetin can be used as a potential biomarker of CQ for free radical scavenging activity.

Comparing of the DPPH free radical scavenging activity and quercetin content, the EC_{50} value of quercetin was 2.11 ± 0.03 µg/mL (6.23 ± 0.10 µM) (Figures 8D) while quercetin content was 1.82 ± 0.09 µg/mg of CQES7 (Table 3). If quercetin was the single antioxidative constituent in CQES7, the CQ used in the testing would be equal to 1.16 mg/mL (2.11 µg/mL ÷ 1.82 µg/mg). However, the calculated EC_{50} value is higher than the experimental data of CQES7, which was 0.333 ± 0.005 mg/mL (Figure 7B). This implied that there is not only quercetin but also other antioxidative constituents such as resveratrol and kaempferol in CQ (Adesanya *et al*., 1999; Thakur *et al*., 2009). The result was accorded to previous EC_{50} value of resveratrol (123.3 µM) (Lee *et al*., 2004).

(A)



(B)

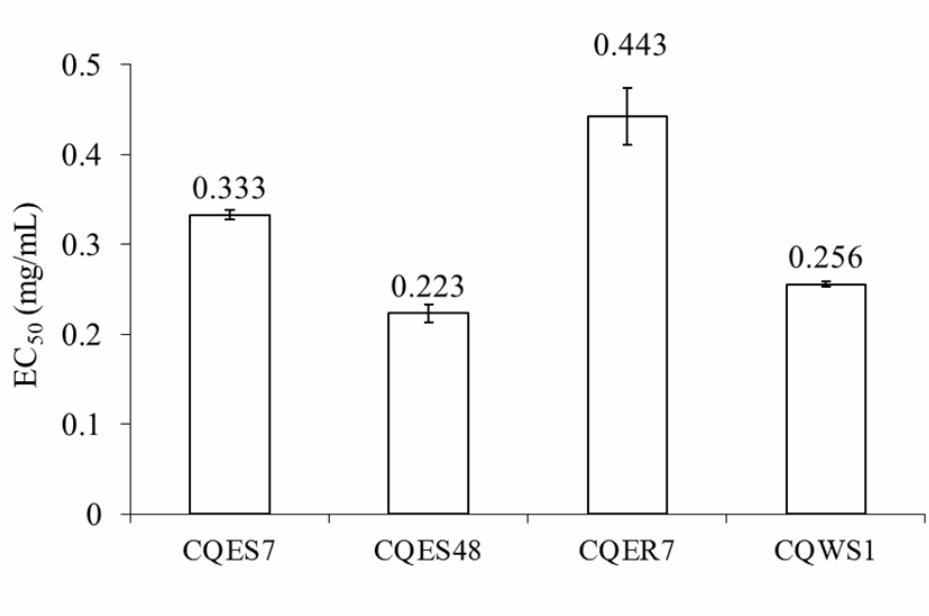


Figure 7. DPPH radical scavenging activity of CQ. (A) Concentration dependent curve of CQES7, CQES48, CQER7, CQWS1 and CQWF1 and (B) EC_{50} values of CQES7, CQES48, CQER7 and CQWS1. Data were expressed as mean ± SEM of three independent experiments (n=3). Each performed in triplicate.

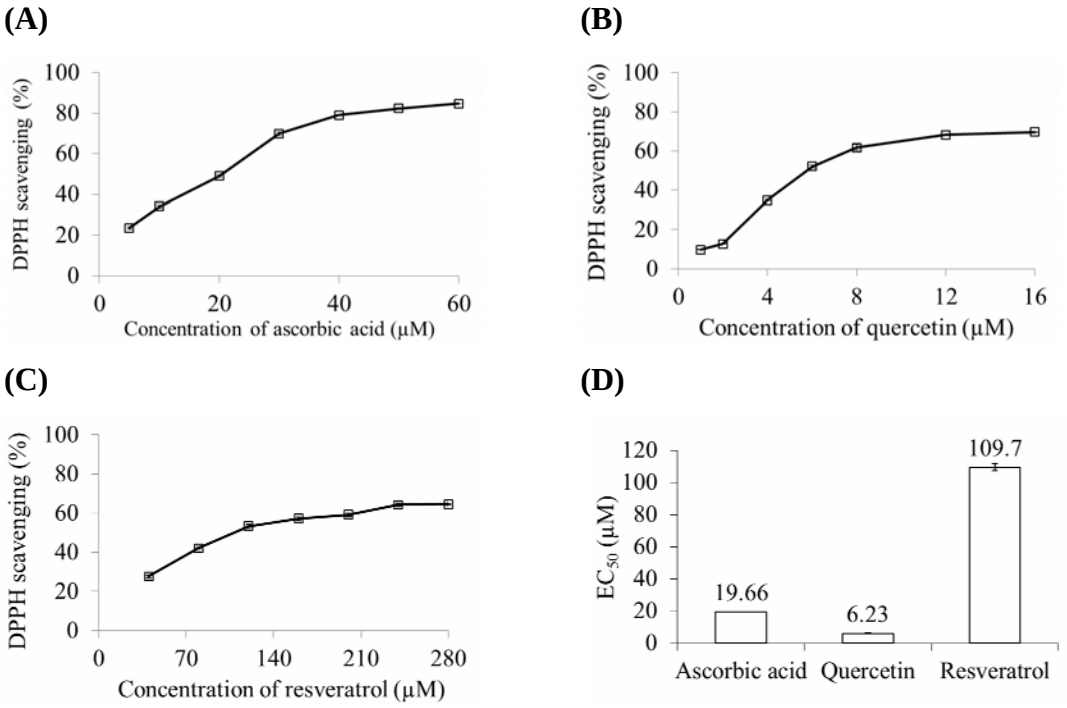


Figure 8. Concentration-dependent curve for the DPPH radical scavenging activity of (A) ascorbic acid used as a positive control, (B) quercetin, (C) resveratrol and (D) EC_{50} values. Data were expressed as mean ± SEM of three independent experiments (n=3). Each performed in triplicate.

Our results demonstrated the ability of CQ from different extraction procedures to scavenge stable DPPH radical. To characterize ROS and RNS scavenging activities of CQ, the scavenging assays of $O_2^{-\bullet}$, H_2O_2, $OH^{\bullet}$ and $NO^{\bullet}$ in cell-free systems were further evaluated.

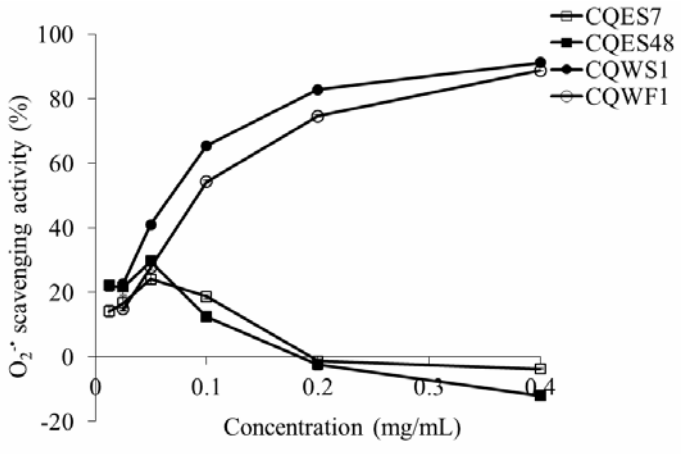
4.3.2 Scavenging of ROS

4.3.2.1 Scavenging of $O_2^{-\bullet}$

Among CQ from various extraction procedures, scavenging activity of $O_2^{-\bullet}$ by CQWS1 with the increase in concentration was higher than CQWF1 (Figure 9A). The EC_{50} values of CQWS1 and CQWF1 were 0.068 ± 0.004 and 0.086 ± 0.007 mg/mL, respectively (Figure 9B). Due to color interference, $O_2^{-\bullet}$ scavenging activity of the CQES7 and CQES48 are only observed at the concentration of 0.05 mg/mL, which displayed 24.11 ± 1.54% and 29.57 ± 0.90%, respectively (Figure 9C). Quercetin, one of flavonoids in CQ, is a strong $O_2^{-\bullet}$ scavenger (Hazra *et al*., 2008). Our result demonstrated that quercetin (EC_{50} 23.41 ± 0.62 µM; 7.92 ± 0.21 µg/mL) possessed the ability to scavenge $O_2^{-\bullet}$ more than resveratrol (EC_{50} 159.42 ± 5.97 µM; 36.38 ± 1.36 µg/mL) in a concentration-dependent manner (Figure 10).

The methanolic extract of CQ possessed ability to scavenge $O_2^{-\bullet}$ with an approximately EC_{50} value of 0.2 – 0.4 mg/mL using the photoreduction of riboflavin (Jainu and Devi, 2005). The contradiction of the $O_2^{-\bullet}$ scavenging activity among these extracts may result from the different testing system. Our method was based on the generator of $O_2^{-\bullet}$ using PMS/NADH system and measured by the reduction of NBT (Hazra *et al*., 2008). The difference in the $O_2^{-\bullet}$ scavenging activity of CQWS1 and CQWF1 may result from the amount of active constituents such as quercitrin and iso-quercitrin. Quercitrin and iso-quercitrin were isolated from ethyl acetate fraction of the ethanolic CQ extract (Jakikasem *et al*., 2000). Quercitrin and iso-quercitrin were also reported as $O_2^{-\bullet}$ scavengers using xanthine/xanthine oxidase system (Lu and Yeap Foo, 2000). Quercitrin possessed the ability to scavenge $O_2^{-\bullet}$ using PMS/NADH system (Robak and Gryglewski, 1988). Moreover, the amount of active constituents in the extracts may be due to different extraction technique. The ethanolic extract of CQ contained aglycone such as quercetin and resveratrol (Adesanya *et al*., 1999, Singh *et al*., 2007, Thiangtham, 2003) while the aqueous extract contained glycosides corresponding to their aglycones such as quercitrin and iso-quercitrin (Jakikasem *et al*., 2000). The flavonol quercetin occurs in plants predominantly in the

(A)



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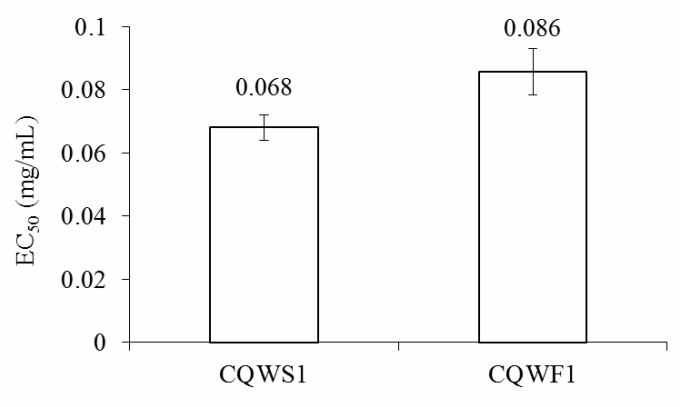


(C)

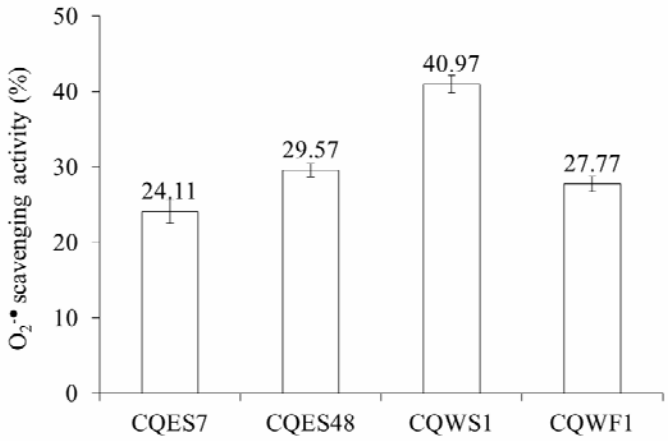
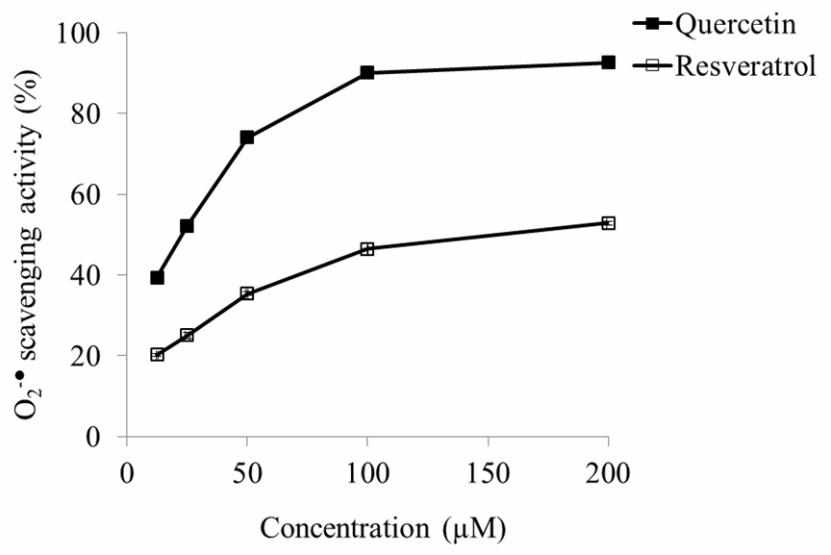


Figure 9. The $O_2^{-\bullet}$ scavenging activity of CQ extracts. (A) Concentration dependent curve of CQ at various concentrations (0.0125, 0.025, 0.05, 0.1, 0.2, 0.4, 0.6, 0.8 and 1 mg/mL), (B) EC_{50} values of CQWS1 and CQWF1 and (C) scavenging activity of CQ extracts at the concentration of 0.05 mg/mL. Results were expressed as mean ± SEM (n=3). Each performed in triplicate.

(A)



(B)

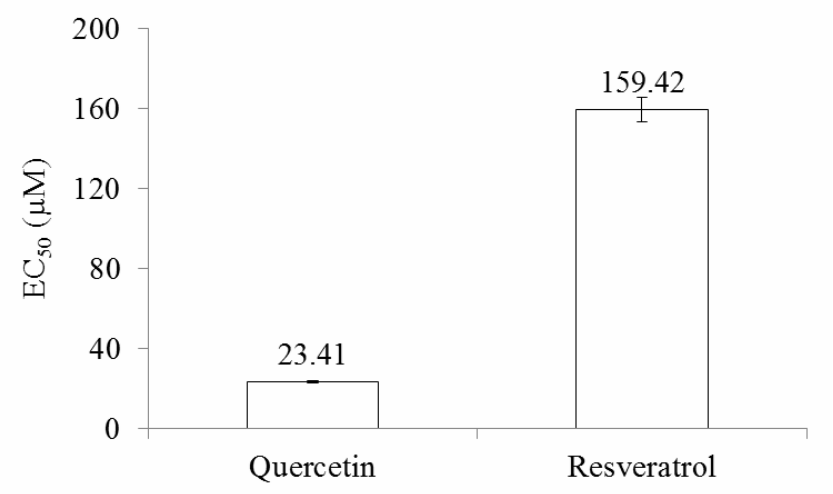


Figure 10. The $O_2^{\bullet-}$ scavenging activity of quercetin and resveratrol. (A) Concentration dependent curve of quercetin and resveratrol at various concentrations (12.5, 50, 100, 200 and 400 µM). (B) EC_{50} values. Results were expressed as mean ± SEM (n=3). Each performed in triplicate.

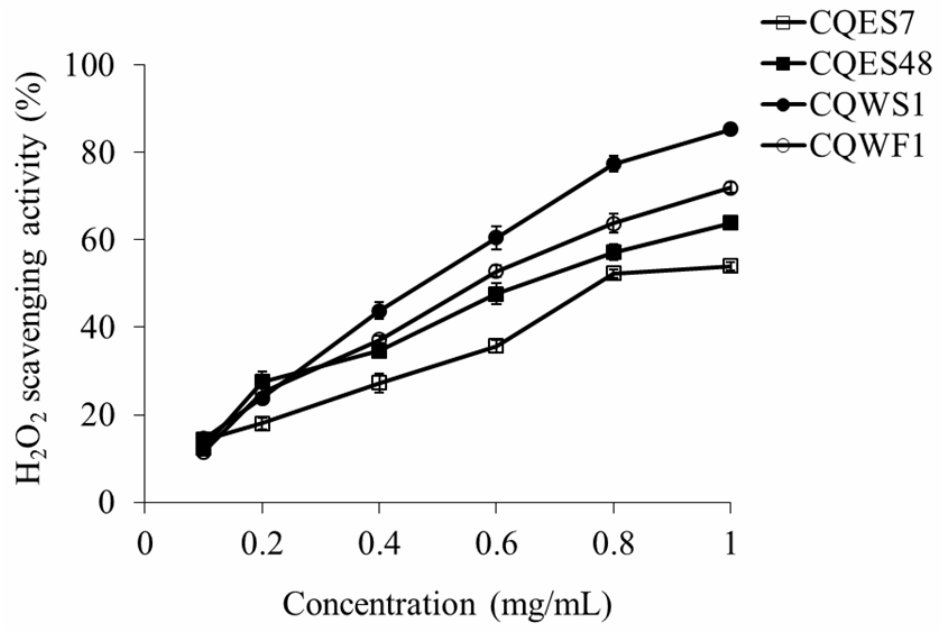
form of glycosides, which are water soluble (Formica and Regelson, 1995). Aqueous extract of broccoli contained quercetin glycoside such as iso-quercitrin (Price *et al*., 1998). Nevertheless, quercitrin was extracted by water from many herbs such as *Albizia julibrissin* (Ekenseair *et al*., 2006) and *Eucalyptus globulus* Labill. (Almeida *et al*., 2009).

4.3.2.2 Scavenging of H_2O_2

The CQ extracts revealed the scavenging activity against H_2O_2 in a concentration-dependent manner (Figure 11A) by exhibiting the EC_{50} values in order of 0.488 ± 0.017 (CQWS1), 0.587 ± 0.026 (CQWF1), 0.661 ± 0.034 (CQES48) and 0.805 ± 0.030 mg/mL (CQES7) (Figure 11B). Quercetin possessed the ability to scavenge H_2O_2 in a concentration-dependent manner (Figure 12A) by exhibiting an EC_{50} value of 46.34 ± 2.28 µM (15.67 ± 0.77 µg/mL) (Figure 12B). Meanwhile, the scavenging ability of resveratrol at the concentration of 300 µM displayed 45.97 ± 1.13% as compared with corresponding control (Figure 12A). Ascorbic acid used as a positive control exhibited an EC_{50} value of 87.04 ± 4.91 µM (Figure 12B) which accorded with an EC_{50} value of 46.3 µM (Choi *et al*., 2007).

The EC_{50} values of CQES7 and CQES48 were higher than a previous report, a methanolic CQ extract, obtained by maceration with occasional shaking for 7 days at room temperature, which exhibited an EC_{50} value of 0.373 ± 0.002 mg/mL (Badami and Channabasavaraj, 2007). The different EC_{50} value might be due to different testing method and the extraction solvent, which affected on the type and amount of active constituents (Formica and Regelson, 1995; Liu *et al*., 2010). The solvent effect on chlorogenic acid extraction revealed that various solvents obtained the different amount of chlorogenic acid, an active constituent in *Folium eucommiae* (Liu *et al*., 2010). CQWS1 and CQWF1 exhibited better activity to scavenge H_2O_2 than CQES48 and CQES7 probably due to the presence of quercitrin and iso-quercitrin, which were previously isolated from the ethyl acetate fraction of CQ (Table 1) (Jakikasem *et al*., 2000; Singh *et al*., 2007). Quercitrin was reported to decrease the damage from H_2O_2 by reducing the deoxyribose degradation which revealed the H_2O_2 scavenging activity (Wagner *et al*., 2006). Additionally, CQES48 displayed the H_2O_2 scavenging activity higher than CQES7 probably due to the presence of higher quercetin content.

(A)



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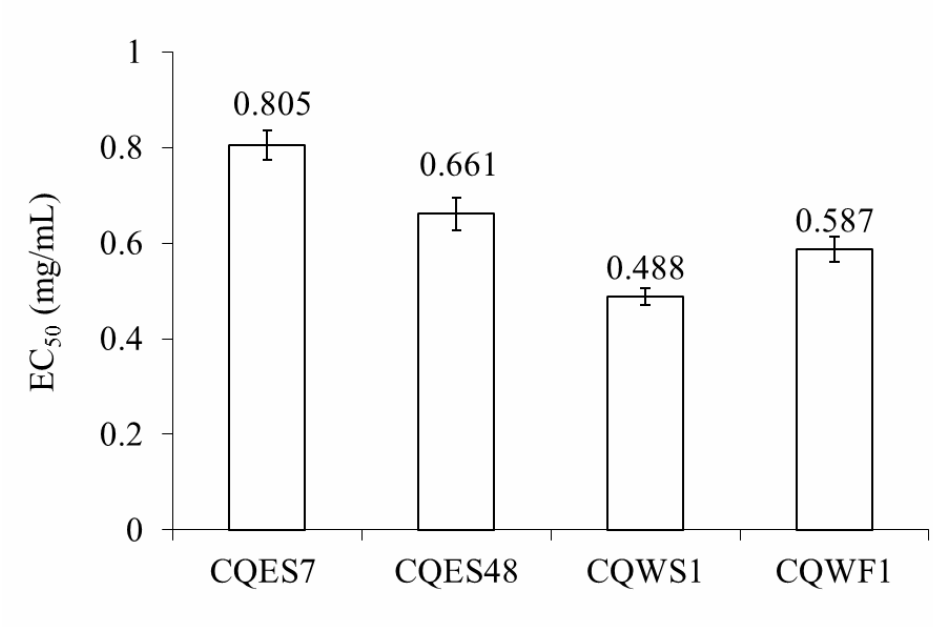
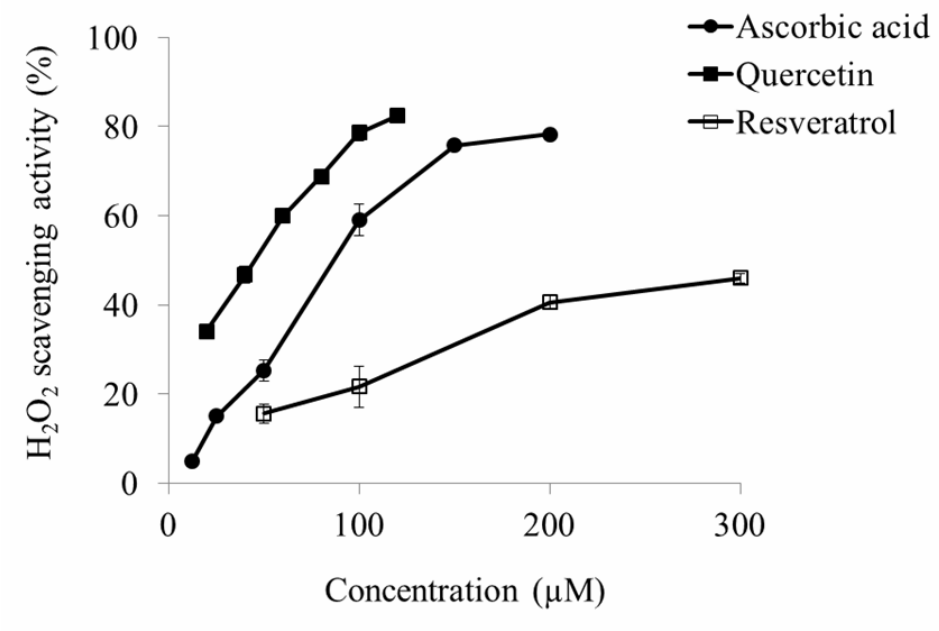


Figure 11. The H_2O_2 scavenging activity of CQ extracts. (A) Concentration dependent curve of CQ at various concentrations (0.1, 0.2, 0.4, 0.6, 0.8 and 1 mg/mL). (B) EC_{50} values. Results were expressed as mean ± SEM (n=3). Each performed in triplicate.

(A)



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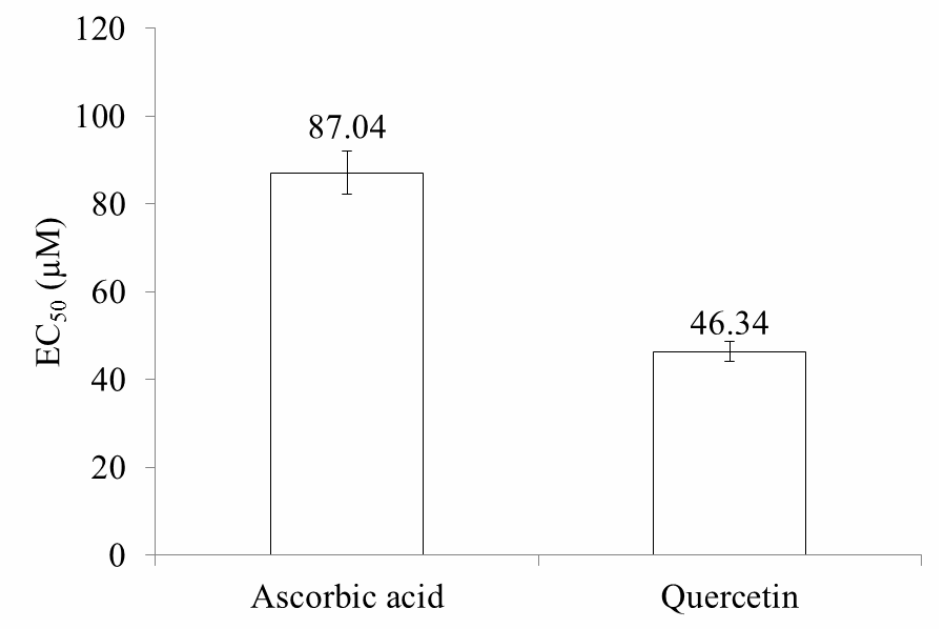


Figure 12. The H_2O_2 scavenging activity of quercetin and resveratrol. (A) Concentration dependent curve of quercetin and resveratrol at various concentrations. (B) EC_{50} values of quercetin and ascorbic acid (positive control). Results were expressed as mean ± SEM (n=3). Each performed in triplicate.

4.3.2.3 Scavenging of $OH^{\bullet}$

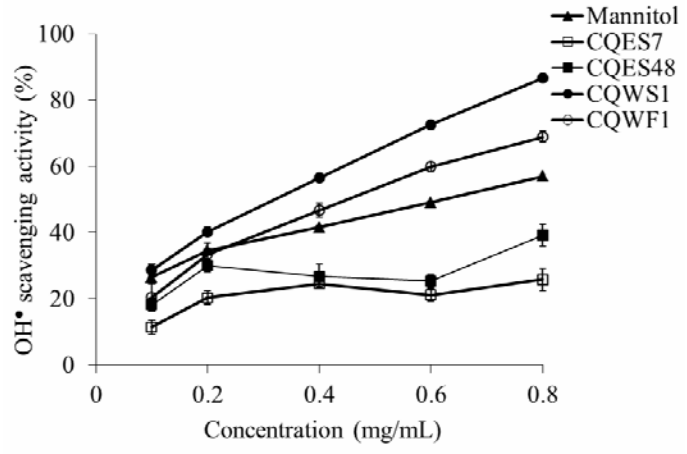
CQWS1 and CQWF1 revealed the $OH^{\bullet}$ scavenging activity in a concentration-dependent manner (Figure 13A) with their EC_{50} values of 0.329 ± 0.018 and 0.460 ± 0.036 mg/mL, respectively (Figure 13B). The results indicated the $OH^{\bullet}$ scavenging ability of CQWS1 was slightly stronger than of CQWF1. Unfortunately, the $OH^{\bullet}$ scavenging testing of CQES7 and CQES48, were only observed at the concentrations of 0.2 mg/mL and displayed 20.28 ± 2.06 and 29.98 ± 1.86% (Figure 13C), respectively. The EC_{50} values cannot be determined due to the color interference. Mannitol, as a positive control, was also performed and exhibited an EC_{50} value of 0.611 ± 0.011 mg/mL (3.354 ± 0.060 mM) (Figure 13B), which was correlated to the EC_{50} value of a previous report 0.571 ± 0.020 mg/mL (Hazra *et al*., 2008).

Previous study revealed that the $OH^{\bullet}$ scavenging activity of methanolic CQ extract could be measured and revealed an approximately EC_{50} 0.2 – 0.4 mg/mL (Jainu and Devi, 2005). This might be due to the different testing procedure which was based on formaldehyde formation during the oxidation of the DMSO by the Fe^{3+}–ascorbic acid system (Jainu and Devi, 2005). Our $OH^{\bullet}$ scavenging assay was based on the formation of TBARS by the degradation of 2-deoxy-D-ribose, resulting in the problem of color interference of CQES7 and CQES48. Noticeably, DMSO, used to dissolve CQES7, CQES48, quercetin and resveratrol in our assay system also scavenged $OH^{\bullet}$ by itself (Bruck *et al*., 1999; Repine *et al*., 1979). To avoid the solvent interference, only 0.5% DMSO was therefore used but the problem still occurred from the partial solubility of CQES7 and CQES48.

4.3.3 Scavenging of RNS: $NO^{\bullet}$

Apart from the role of NO in physiological processes, $NO^{\bullet}$ is also a pro-oxidant and implicated in inflammation and other pathological conditions (Wink and Mitchell, 1998). We therefore determined whether CQ possessed an ability to scavenge $NO^{\bullet}$. The result demonstrated that only CQWF1 scavenged $NO^{\bullet}$ in a concentration-dependent manner (Figure 14A) with the EC_{50} value of 0.776 ± 0.027 mg/mL (Table 4). Meanwhile, CQES7 and CQES48 at the concentration of 0.4 mg/mL revealed 32.90 ± 1.33% and 34.25 ± 2.14% scavenging of $NO^{\bullet}$ (Figure 14B), respectively. The EC_{50} values of CQES7 and CQES48 were not obtained due to color interference. Additionally, CQWS1 was not a good scavenger of $NO^{\bullet}$. Quercetin, the

(A)



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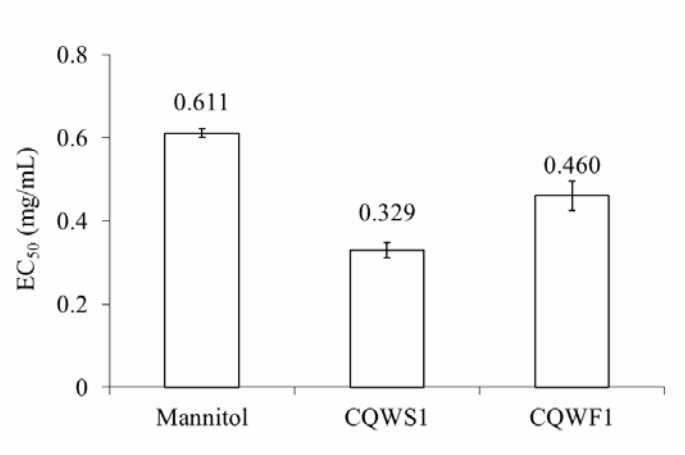


(C)

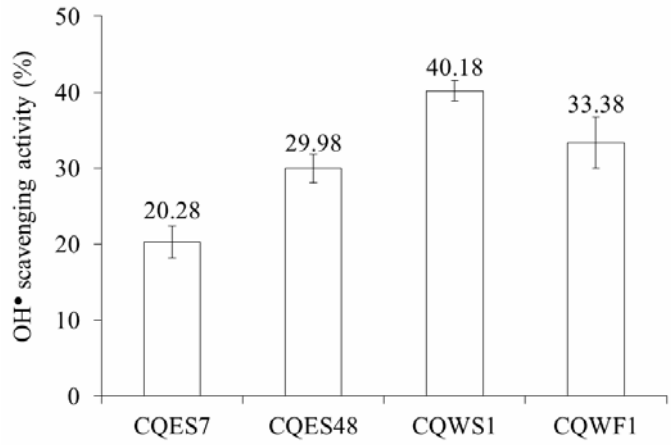
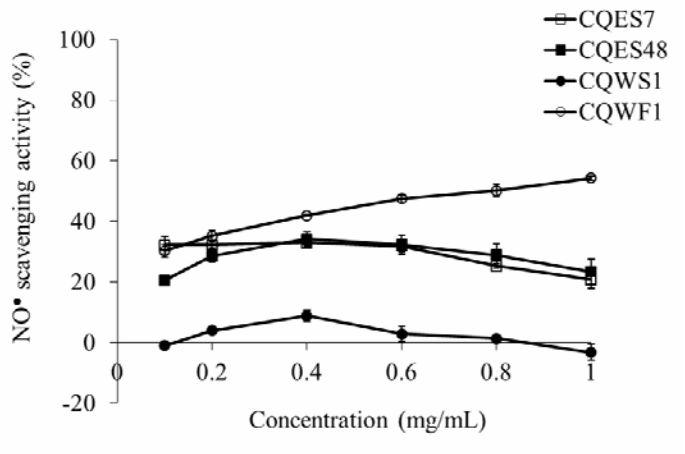
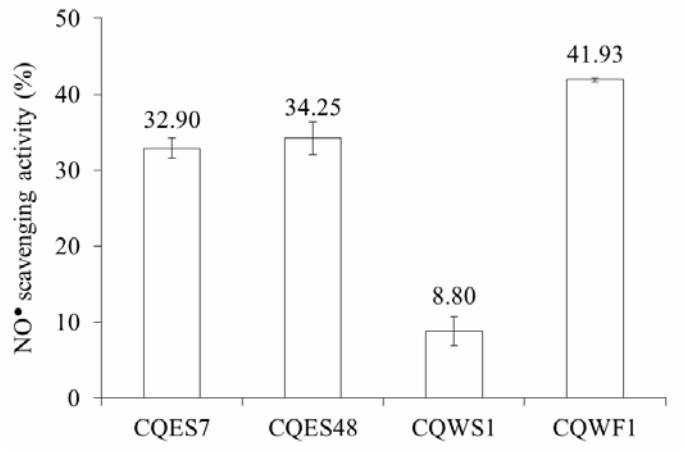


Figure 13. The $OH^{\bullet}$ scavenging activity of CQ extracts. (A) Concentration dependent curve of CQ at various concentrations (0.1, 0.2, 0.4, 0.6, 0.8 and 1 mg/mL). (B) EC_{50} values of CQWS1 and CQWF1. (C) Scavenging activity of CQ extracts at the concentration of 0.2 mg/mL. Mannitol was used as a positive control. Results were expressed as mean ± SEM (n=3). Each performed in triplicate.

(A)



(B)



(C)

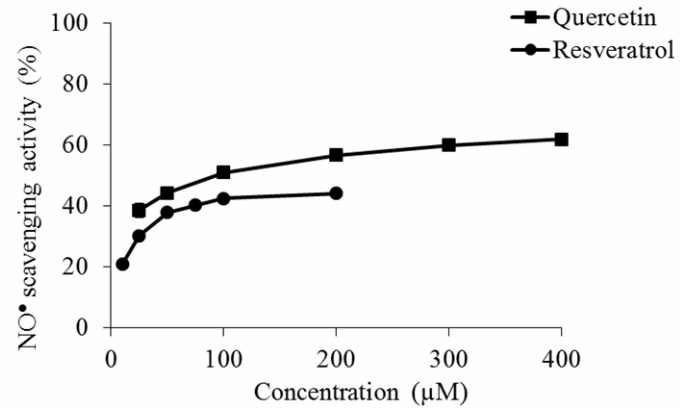


Figure 14. The NO• scavenging activity of CQ extracts, quercetin and resveratrol. Concentration dependent curve of (A) CQ at various concentrations (0.1, 0.2, 0.4, 0.6, 0.8 and 1 mg/mL). (B) Scavenging activity of CQ extracts at the concentration of 0.4 mg/mL and (C) quercetin and resveratrol. Results were expressed as mean ± SEM (n=3). Each performed in triplicate.

active constituent in CQ and also used as a positive control, had an ability to scavenge $NO^{\bullet}$ in a concentration-dependent manner (Figure 14C) and exhibited the EC_{50} value of 97.69 ± 14.46 µM (33.045 ± 4.891 µg/mL) (Table 4), which was resembled to a previous report (EC_{50} 91.4 µM) (Choi *et al*., 2007). Unfortunately, resveratrol was partially dissolved in DMSO at the maximum solubility of 200 µM and exhibited the $NO^{\bullet}$ scavenging activity of 44.09 ± 1.30% as compared with vehicle control (Figure 14C, Table 4).

The DPPH radical scavenging activity of the ethanolic CQ extracts in a cell-free system could be partly due to the presence of ascorbic acid (Jainu and Mohan, 2008; Mishra *et al*., 2010), quercetin and resveratrol, which were isolated from CQ (Adesanya *et al*., 1999; Singh *et al*., 2007; Thiangtham, 2003). Moreover, the CQ extract contained glycosides corresponding to their aglycones, quercitrin and iso-quercitrin (Jakikasem *et al*., 2000; Singh *et al*., 2007), which may be obtained from aqueous extraction (Formica and Regelson, 1995). Quercitrin and iso-quercitrin were reported to scavenge $O_2^{-\bullet}$ using xanthine/xanthine oxidase system (Lu and Yeap Foo, 2000). The $OH^{\bullet}$, which was generated by the Fenton reaction to form DMPO-OH adducts and detected by ESR spectrometer, was scavenged after administration of quercitrin (Lee *et al*., 2001). The different scavenging activity of CQ from various extraction procedures might be related to solvent, extraction method (Liu *et al*., 2010) and time of the extraction (Boonsiripiphat and Theerakulkait, 2009), resulting in the difference of the amount and type of active constituents (Liu *et al*., 2010; Nikolić *et al*., 2008). CQES7 comprised of quercetin (1.82 ± 0.09 µg/mg of extract) more than CQER7 (1.46 ± 0.06 µg/mg of extract) (Table 3). The reflux extraction was more direct heat than soxhlet extraction, resulting in the thermal degradation of active constituents. A previous study demonstrated that the soxhlet extraction of *Folium eucommiae* gained more chlorogenic acid than reflux extraction (Liu *et al*., 2010). The amount of quercetin was well correlated to the DPPH radical scavenging activity, showing that CQES7 was more effective than CQER7 (Tables 3, 4). Especially, CQWS1, obtained from the fluid of compressed CQ, also exhibited strong DPPH and ROS scavenging activities in cell-free system (Table 4). The time of extraction also involved in the amount of active constituents (Boonsiripiphat and Theerakulkait, 2009) as revealed in the discrepancy of DPPH radical and H_2O_2 scavenging activities between CQES7 and CQES48 (Table 4). Solvent was also affected the amount and type of active constituents. Methanol, ethanol and water were used to extract

Table 4. The EC_{50} values of free radical scavenging activities in a cell-free system of CQ from various extraction procedures in comparison with its active constituents. Each value represents the mean ± SEM (n=3).

Test compound/extract	**EC_{50} value**				
		Oxidative stress			**Nitrosative stress**
	DPPH	$O_2^{-\bullet}$	H_2O_2	$OH^{\bullet}$	$NO^{\bullet}$
Ascorbic acid (µM)	19.660 ± 0.131	ND	87.040 ± 4.910	ND	ND
Quercetin (µM)	6.230 ± 0.098	23.413 ± 0.618	46.340 ± 2.280	ND	97.690 ± 14.460
Resveratrol (µM)	109.700 ± 2.274	159.420 ± 5.973	> 300	ND	> 200
Mannitol (mM)	ND	ND	ND	3.354 ±0.060	ND
CQES7 (mg/mL)	0.333 ± 0.005	CD	0.805 ± 0.030	CD	CD
CQES48 (mg/mL)	0.223 ± 0.010	CD	0.661 ± 0.034	CD	CD
CQER7 (mg/mL)	0.443± 0.031	ND	ND	ND	ND
CQWS1 (mg/mL)	0.256 ± 0.003	0.068 ± 0.004	0.488 ± 0.017	0.329 ± 0.018	NA
CQWF1 (mg/mL)	> 0.250	0.086 ± 0.007	0.587 ± 0.026	0.460 ± 0.036	0.776 ± 0.027

NA = no activity ND = not determine CD = color disturbance

chlorogenic acid from *Folium eucommiae*, showing that the amount of chlorogenic acid decreased in the order: methanol > ethanol > water (Liu *et al*., 2010). Moreover, the flavonol quercetin in plants is usually found in the form of glycosides which can be extracted by water (Formica and Regelson, 1995). For example, iso-quercitrin was isolated from aqueous extract of broccoli (Price *et al*., 1997), quercitrin isolated from aqueous extract of *Albizia julibrissin* (Ekenseair *et al*., 2006) and *Eucalyptus globulus* Labill. (Almeida *et al*., 2009).

Methanolic CQ extract possessed $O_2^{-\bullet}$, H_2O_2, $OH^{\bullet}$ and $NO^{\bullet}$ scavenging activities in cell-free system (Badami and Channabasavaraj, 2007; Jainu and Devi, 2005). This was the first report of free radical scavenging activities of the aqueous CQ extract. According to Thai traditional medicine, the ethanolic and aqueous CQ extracts have been used. Therefore, CQES48 and CQWS1, which exhibited strong DPPH and H_2O_2 scavenging activities in cell-free system (Table 4), were chosen for further evaluation of cell viability and molecular mechanism underlying their cytoprotective effect.

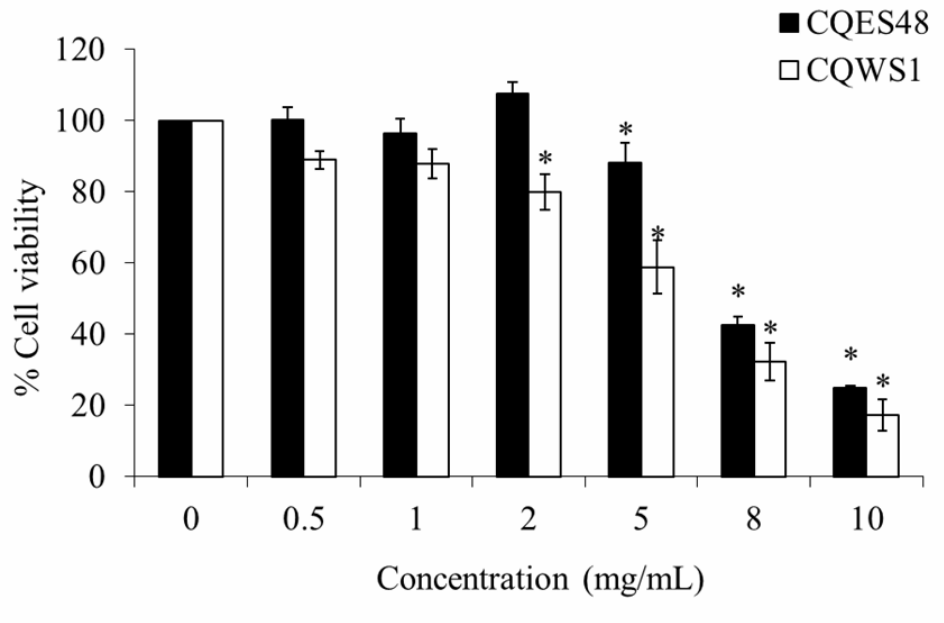
4.4 Viability of ECV304 cells

4.4.1 Effect of CQ on cell viability

Hemorrhoid occurs in the veins around the anus or lower rectum which the venous stasis leads to inflammation of the vessel. Then, free radicals are released by the inflammatory response and lead to acute development of hemorrhoid (Glowinski and Glowinski, 2002; Wali *et al*., 2002). In order to determine whether there was any correlation between H_2O_2 scavenging activity in cell-free system and protective effects of CQ against H_2O_2-induced oxidative damage on endothelial cells, human umbilical vein endothelial ECV304 cells were used for further investigation.

To determine whether CQ alone was cytotoxic, ECV304 cells were treated with CQES48 or CQWS1 for 24 h and viable cells were measured by MTT assay. The number of viable cells was not significantly altered by CQES48 and CQWS1 at the concentration below 1 mg/mL (Figure 15A). Furthermore, CQES48 and CQWS1 decreased the cell viability in a concentration-dependent manner with the IC_{50} values of 7.49 ± 0.20 and 5.85 ± 0.75 mg/mL, respectively (Figure 15B). The result indicated that CQWS1 was slightly more toxic than CQES48. Quercetin, resveratrol, hesperidin (Figure 16A) and diosmin (Figure 16B) affected cell viability in a concentration-

(A)



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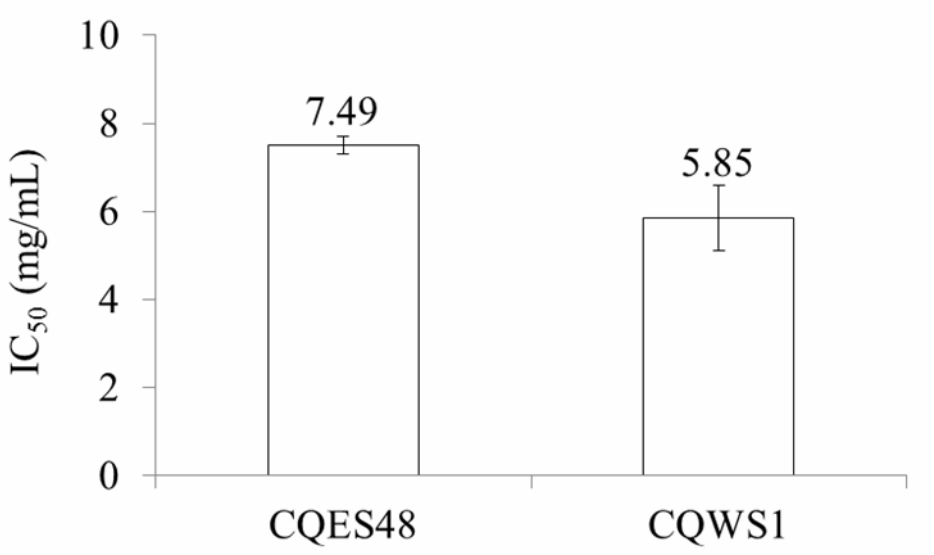
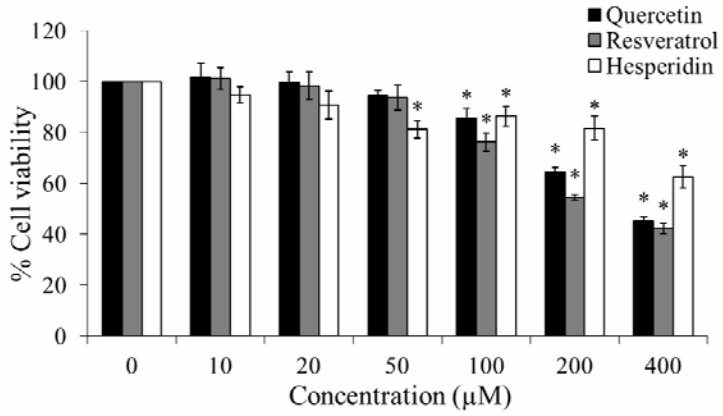


Figure 15. Effect of CQES48 and CQWS1 on cell viability. ECV304 cells were treated with (A) CQES48 and CQWS1 at various concentrations (0.5, 1, 2, 5, 8 and 10 mg/mL) for 24 h. (B) IC_{50} values. The cell viability was determined by MTT assay. *$p < 0.05$ compared to the untreated control. Results were expressed as mean ± SEM (n=3). Each performed in triplicate.

(A)



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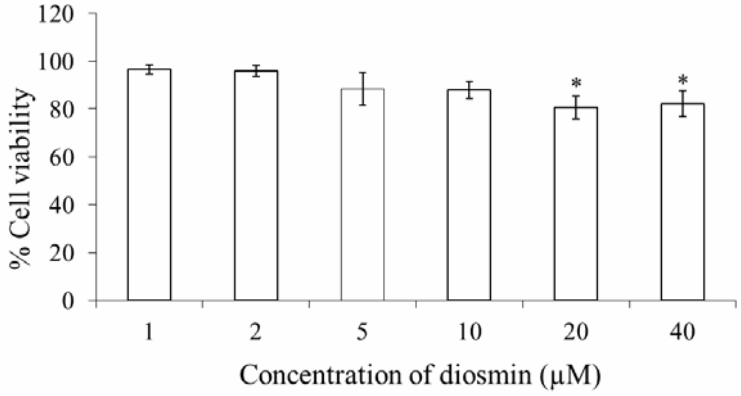

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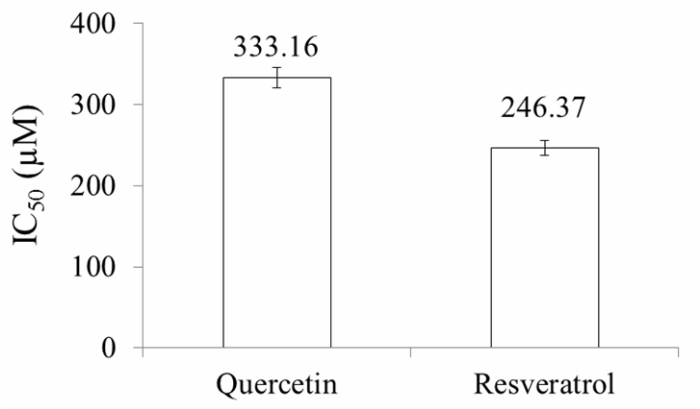


Figure 16. Effect of quercetin, resveratrol, hesperidin and diosmin on cell viability. Concentration-dependent effect of (A) quercetin, resveratrol, hesperidin and (B) diosmin. (C) IC_{50} values of quercetin and resveratrol. The cell viability was determined by MTT assay. *$p < 0.05$ compared to the untreated control. Results were expressed as mean ± SEM (n=3). Each performed in triplicate.

dependent manner. According to the above data and their IC_{50} values (Figure 16C), the cytotoxicity was in the following way: diosmin > resveratrol > quercetin > hesperidin. The IC_{50} values of resveratrol and quercetin were 246.37 ± 8.80 and 333.16 ± 12.90 µM, respectively. Diosmin and hesperidin did not reach the IC_{50} value at our tested concentrations.

From previous reports, 48-h incubation of 30 µM quercetin significantly decreased viability of bovine aortic endothelial cells (Jackson and Venema, 2006) as well as 48-h incubation of 50 µM resveratrol in human microvascular endothelial cells (Trapp *et al*., 2010). In our study, treatment of ECV304 cells with quercetin and resveratrol at the concentration of 100 µM for 24 h significantly decrease cell viability (Figure 16A). These contradictions may result from the incubation time and the difference of cell type. Combination of hesperidin and diosmin (Daflon®) is a well-known antihemorrhoidal agent (Buckshee *et al*., 1997; Sarabia *et al*., 2001). Hesperidin exhibited the free radical scavenging activities in cell-free system against $O_2^{-\bullet}$, $OH^{\bullet}$ and $NO^{\bullet}$ scavenging (Kalpana *et al*., 2009). In the cell-based system, hesperidin at a concentration of 10 µM significantly inhibited cyclic strain-induced ROS formation in HUVEC measured by DCFH-DA (Chiou *et al*., 2008) and also inhibited H_2O_2-induced oxidative stress in rat pheochromocytoma PC12 cells (Hwang *et al*., 2008). Moreover, hesperidin and diosmin inhibited the increase of luminol chemiluminescence induced by H_2O_2, and also reduced H_2O_2 formation in polymorphonuclear neutrophils (Cypriani *et al*., 1993).

CQ, composed of quercetin and resveratrol, may act like antihemorrhoidal agent in terms of cell protection. Hence, the non-toxic concentrations of CQES48 and CQWS1 (below 1 mg/mL), quercetin, resveratrol, hesperidin (below 50 µM) and diosmin (below 20 µM) were chosen for the cytoprotective study.

4.4.2 Effect of H_2O_2 on cell viability

H_2O_2 is one of the ROS that can induce cell death in many different cell types including ECV304 cells (Kosem *et al*., 2007; Liu *et al*., 2009; Wang and Huang, 2005). Treatment of mouse Leydig TM3 cells with H_2O_2 for 24 h revealed an IC_{50} value of 100 µM (Chang *et al*., 2008) as well as in mouse macrophage RAW264.7 cells exhibited an IC_{50} value of 400 µM (Chow *et al*., 2005). The 16-h incubation of 400 µM H_2O_2 in rat pheochromocytoma PC12 cells displayed 68% cell viability

(Hwang *et al*., 2008). The difference of cell type and incubation time affected the IC_{50} value.

In order to select an appropriate concentration of H_2O_2 to induce cell injury, the effect of H_2O_2 on the viability of ECV304 cells was therefore determined by MTT assay. The result demonstrated that H_2O_2 decreased cell viability in concentration- and time-dependent manner (Figure 17A). The IC_{50} values after 6-, 12- and 24-h incubation of H_2O_2 were 71.27 ± 1.81, 50.40 ± 0.53 and 41.73 ± 14.30 μM, respectively (Figure 17B). Meanwhile, the IC_{50} value after 0.5-h incubation of H_2O_2 was not observed while H_2O_2 at the concentration of 100 μM revealed 61.15 ± 1.43% viable cells.

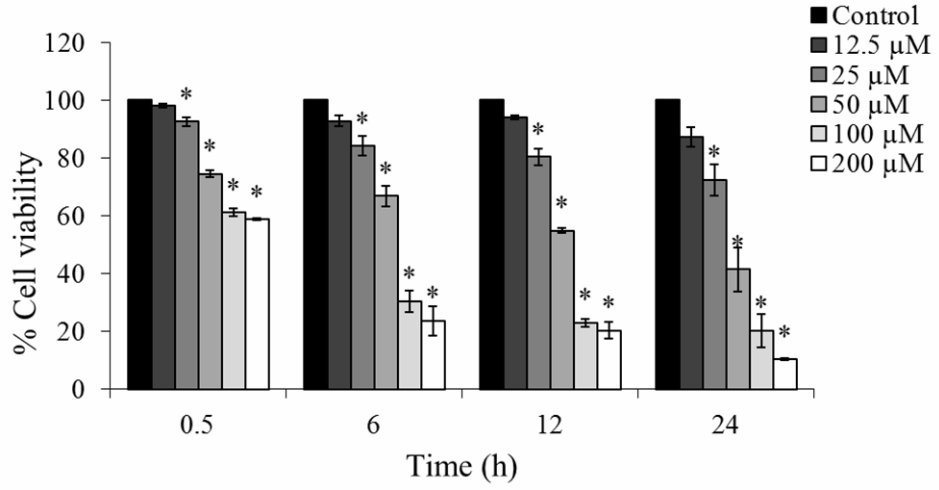
Previous studies of the cytotoxicity of H_2O_2-treated ECV340 cells showed various degree of cell death. H_2O_2 at a concentration of 1 mM after 6-h incubation exhibited 49.7% cell viability (Kosem *et al*., 2007), and 750 μM H_2O_2 after 18-h incubation exhibited 29.3% cell viability (Wang and Huang, 2005). The cytotoxicity of 12-h incubation of 300 μM H_2O_2 revealed 71.9% cell viability (Liu *et al*., 2009). The difference of IC_{50} values of H_2O_2-treated ECV304 cells may came from the seeding density. The previous reports seeded the cells into 96-well with a density of 5×10^5 cells/mL. While our study was performed at a density of 1×10^5 cells/mL reaching 80-90% cell confluence after 24-h incubation.

Therefore, H_2O_2 at the concentration of 100 μM, which significantly displayed viable cells 61.15 ± 1.43% (0.5-h incubation) and 30.44 ± 6.42% (6-h incubation), was used to induce cell injury.

4.4.3 Effect of CQ on the viability of H_2O_2-injured cells

Our study demonstrated that CQES48 and CQWS1 had a potential ability to scavenge H_2O_2 in cell-free system (Table 4). We therefore determined whether CQ could protect the cells from oxidative stress induced by H_2O_2. After pretreatment the cells with CQES48 and CQWS1 at the non-toxic concentrations for 24 h followed by exposure to H_2O_2 at a concentration of 100 μM for 0.5, 2 and 6 h, the cell viability was determined by MTT assay. The result revealed that CQES48, CQWS1, (Figure18) quercetin and resveratrol (Figure 19) did not significantly increase the viable cells as compared with H_2O_2 alone. This may result from 100 μM H_2O_2 generating the extreme oxidative stress, which CQES48, CQWS1, quercetin and resveratrol cannot

(A)



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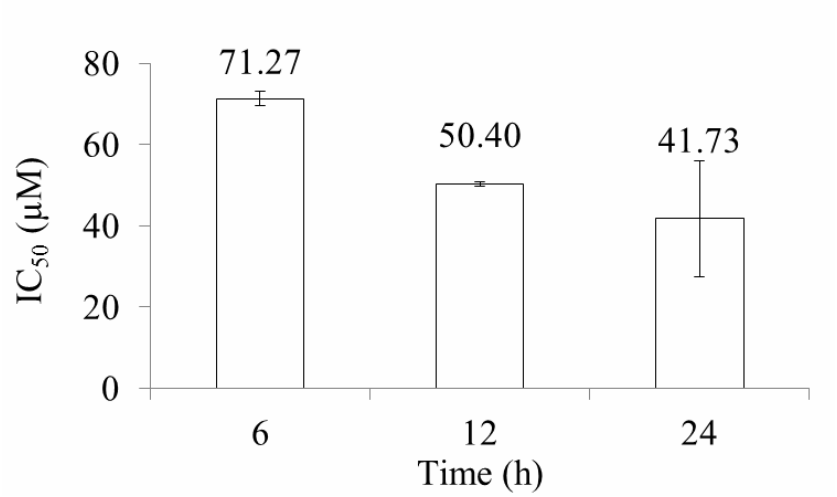
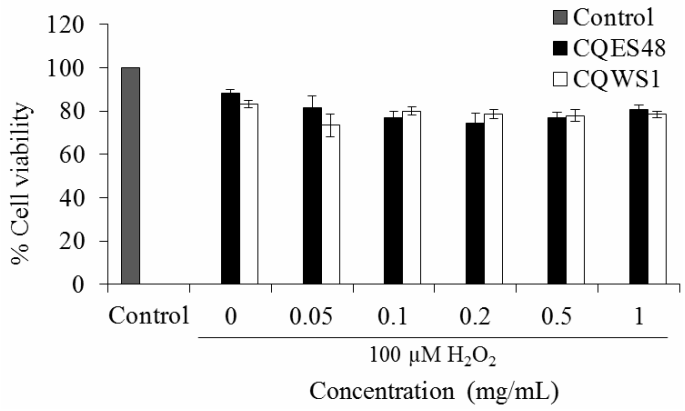


Figure 17. Effect of H_2O_2 on cell viability. (A) Concentration- and time-dependent effect of H_2O_2 on the cell viability. (B) IC_{50} values at the indicated time. The cell viability was measured by MTT assay. *$p < 0.05$ compared to the untreated control. Results were expressed as mean ± SEM (n=3). Each performed in triplicate.

(A)



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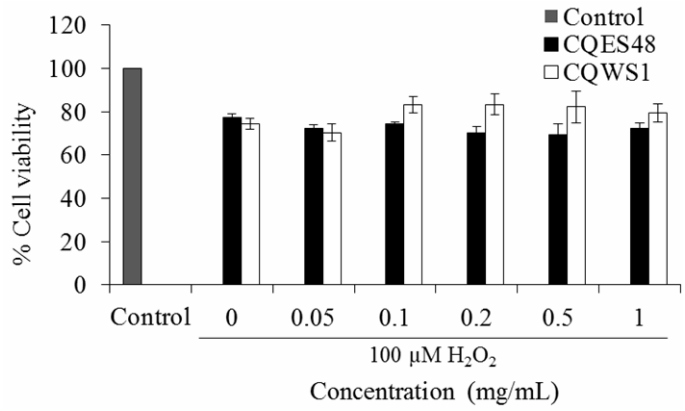


(C)

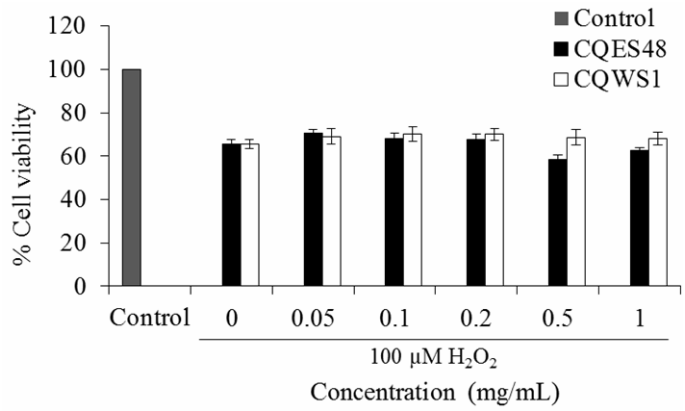
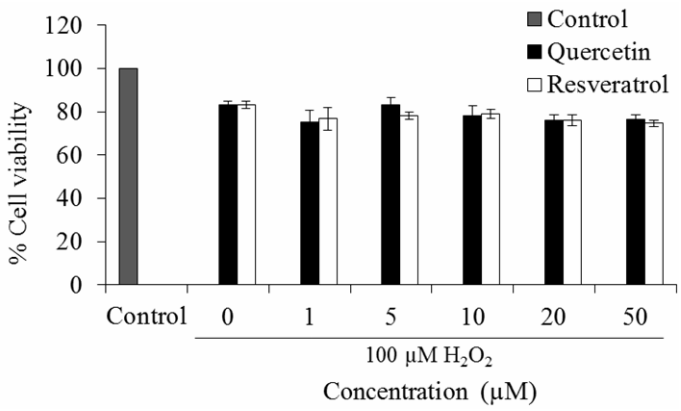


Figure 18. Effect of CQES48 and CQWS1 on the viability of H_2O_2-injured cells. The pretreatment of CQES48 and CQWS1 for 24 h prior to H_2O_2 (100 µM) exposure for (A) 0.5 h, (B) 2 h and (C) 6 h. The cell viability was measured by MTT assay. *p < 0.05 compared to the untreated control. Results were expressed as mean ± SEM (n=3). Each performed in triplicate.

(A)



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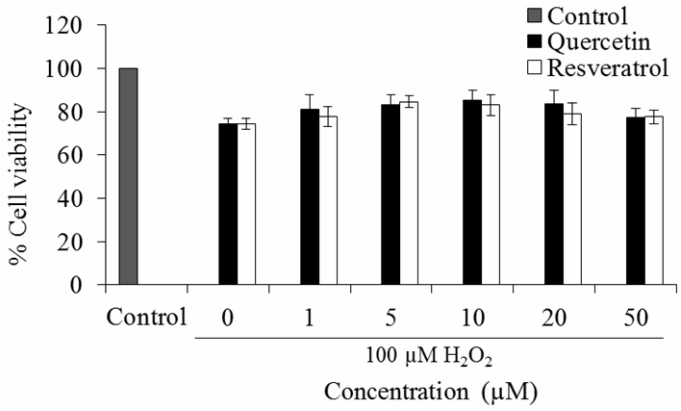

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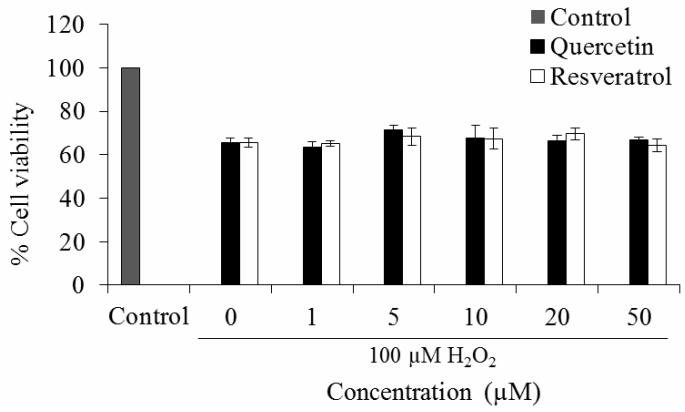


Figure 19. Effect of quercetin and resveratrol on the viability of H_2O_2-injured cells. The pretreatment of quercetin and resveratrol for 24 h prior to H_2O_2 (100 µM) exposure for (A) 0.5 h, (B) 2 h and (C) 6 h. The cell viability was measured by MTT assay. *$p < 0.05$ compared to the untreated control. Results were expressed as mean ± SEM (n=3). Each performed in triplicate.

maintain the balance between antioxidant system and excessive free radicals, resulting in cell death.

4.5 Intracellular ROS

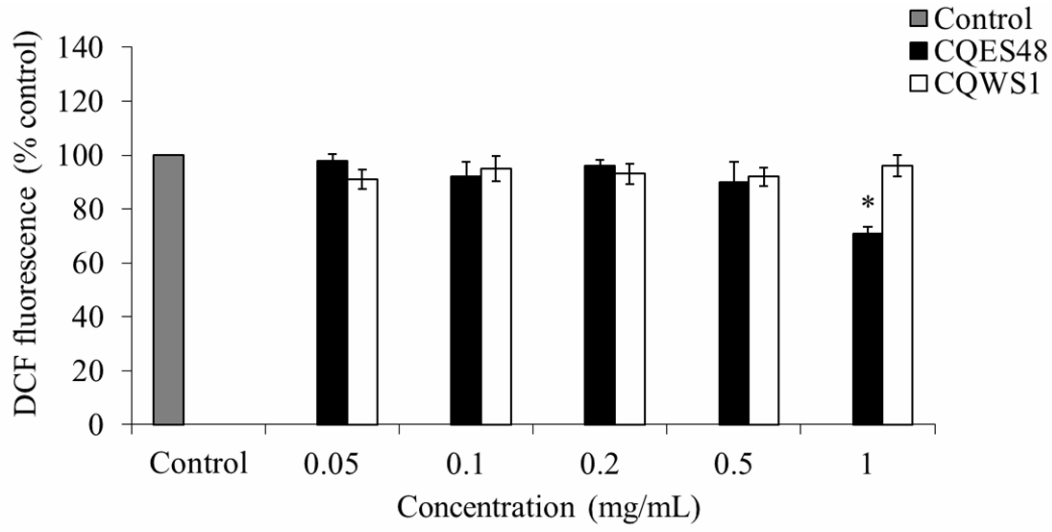
4.5.1 Effect of CQ on the intracellular ROS generation

To guarantee whether CQ itself affected ROS level in ECV304 cells, the intracellular ROS level was then analyzed using DCFH-DA fluorescence dye. Quantification of the corresponding fluorescence intensity indicated that treatment of cells with CQES48, CQWS1, quercetin, resveratrol, hesperidin or diosmin at the non-toxic concentrations did not significantly increase the intracellular ROS when compared to control (Figure 20). Moreover, CQES48 (1 mg/mL) and quercetin (50 µM) significantly decreased the intracellular ROS when compared to control.

4.5.2 Effect of H_2O_2 on the intracellular ROS generation

H_2O_2 has been extensively used as an inducer of oxidative stress by increasing the accumulation of ROS as determined by DCFH-DA assay in cell-based system (Hong and Liu, 2004; Kosem *et al*., 2007; Liu *et al*., 2009; Wang and Joseph, 1999). The results revealed that the cells treated with H_2O_2 at various concentrations for 0.5 h and 2 h rapidly increased intracellular ROS in a concentration-dependent manner (Figure 21). While the intracellular ROS level after 2-h exposure to H_2O_2 was lower than 0.5-h. This resulted from the elimination constant rate of DCF after 2-h incubation which decreased approximately 10% (Taguchi *et al*., 1996). However, 6-, 12- and 24-h exposure to H_2O_2 was not significantly different when compared with unstressed control. According to previous report, the DCF fluorescence intensity gradually decreased with a half-life of approximately 5.5 h (Taguchi *et al*., 1996). Based on our experimental procedure, DCFH-DA was prestrained for 0.5 h prior to H_2O_2 exposure for 6, 12 and 24 h, resulting that the DCF fluorescence intensity was not different when compared with control (Figure 21). Additionally, H_2O_2 at a concentration of 100 µM for 0.5-h incubation clearly increased intracellular ROS approximately 2-fold (Figure 21) and the viable cells was 61.15 ± 1.43 % (Figure 17A) when compared with control. Therefore, H_2O_2 at the concentration of 100 µM was selected for the study of H_2O_2-induced oxidative stress in ECV304 cells.

(A)



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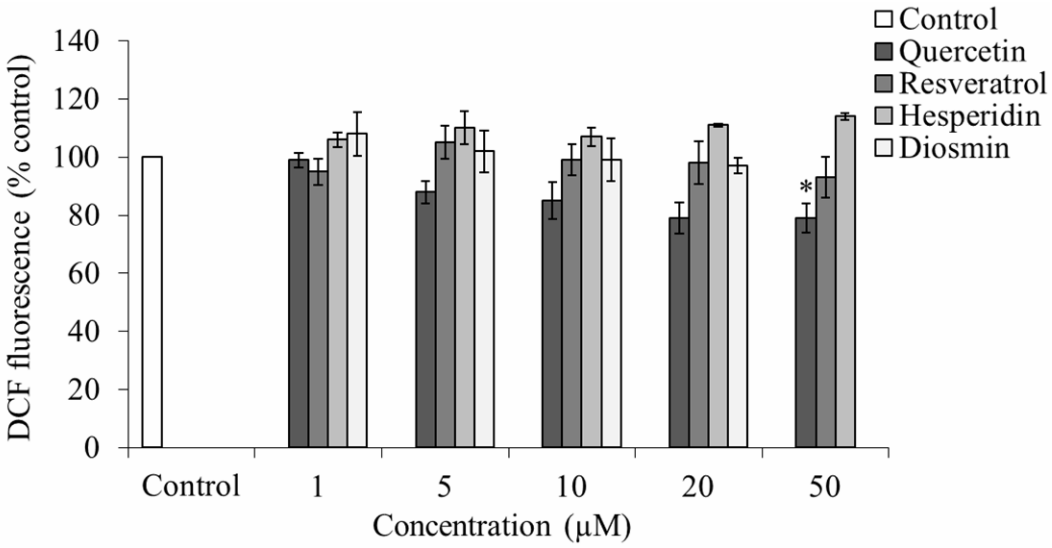


Figure 20. Effect of CQ on intracellular ROS generation in ECV304 cells measured by DCFH-DA assay. ECV304 cells treated with (A) CQES48 and CQWS1 (B) quercetin, resveratrol, hesperidin and diosmin at the indicated concentrations for 24 h. The cells were further incubated with 5 μM DCFH-DA in PBS for 30 min. The absorbance was measured at excitation at 485 nm and emission 535 nm. *p < 0.05 compared to the vehicle control. Results were expressed as mean ± SEM (n=3). Each performed in triplicate.

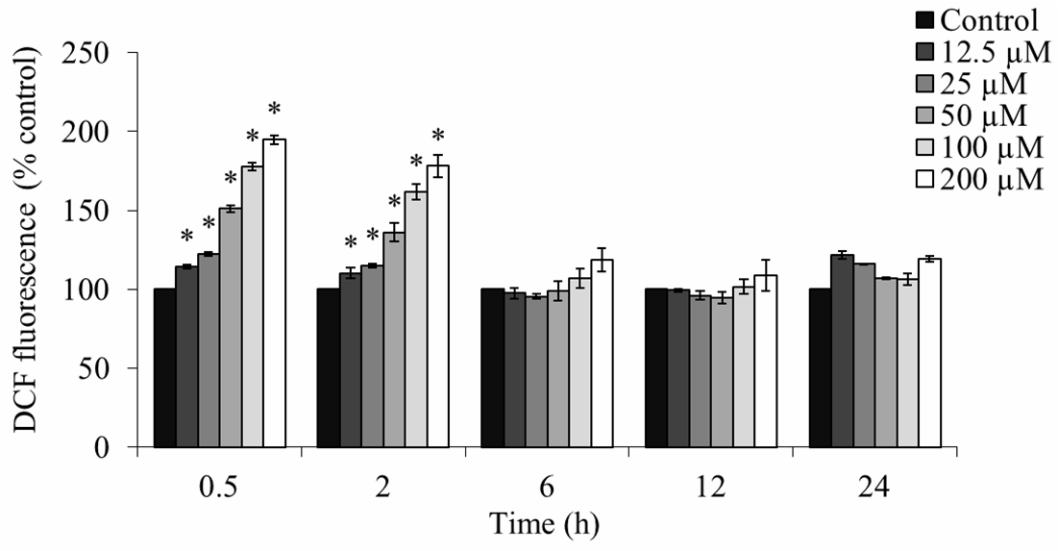


Figure 21. The effect of H_2O_2 on intracellular ROS generation in ECV304 cells measured by DCFH-DA assay. The cells were incubated with 5 µM DCFH-DA in PBS for 30 min followed by various concentrations of H_2O_2 (12.5, 25, 50, 100 and 200 µM) for indicated times. The absorbance was measured by fluorescence plate reader at excitation 485 nm and emission 535 nm. *$p < 0.05$ compared to the vehicle control. Results were expressed as mean ± SEM (n=3). Each performed in triplicate.

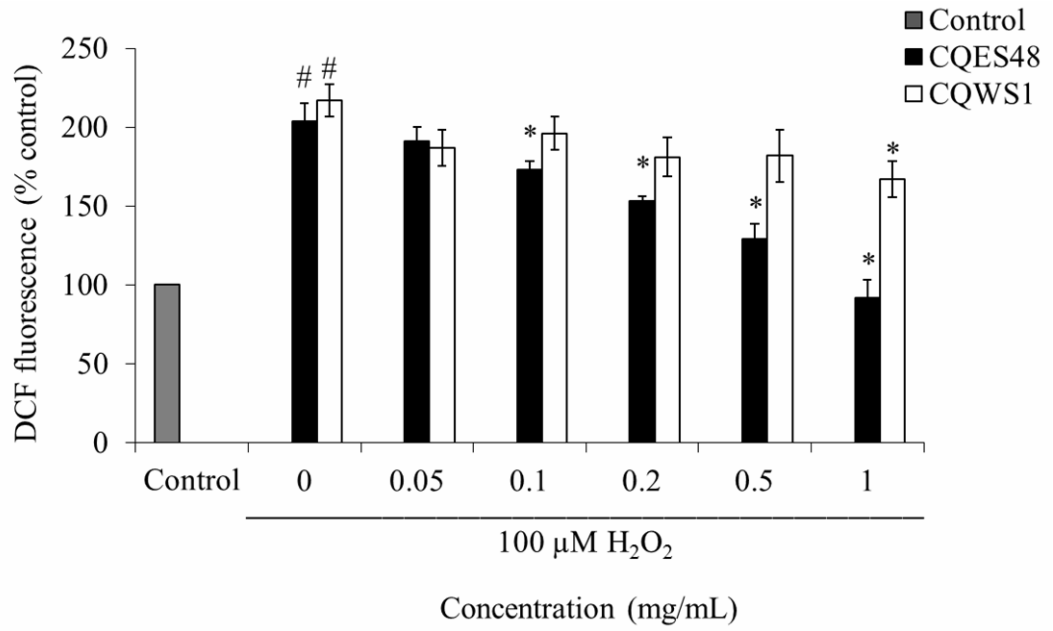
4.5.3 Effect of CQ on the intracellular ROS in H_2O_2-treated cells

In order to further explore the biochemical basis correlating to CQ scavenged H_2O_2 in cell-free system (Table 4), intracellular ROS scavenging effect of CQES48 and CQWS1 against H_2O_2-induced oxidative stress in ECV304 cells were then examined. The result demonstrated that an increase in the production of H_2O_2 was significantly attenuated in a concentration-dependent manner when the cells were pretreated with CQES48 (0.1 mg/mL) and CQWS1 (1 mg/mL) for 24 h (Figure 22A). Moreover, the excessive intracellular ROS was significantly decreased by quercetin and resveratrol at a concentration of 5 μM (Figure 22B). Diosmin, but not hesperidin, at a concentration of 10 μM significantly decreased intracellular ROS.

Pretreatment with CQES48 reduced the DCF fluorescence signal more than CQWS1 (Figure 23). The result may come from the difficulty of the cellular uptake of aglycone in CQES48 less than of glycoside in CQWS1 (Murota and Terao, 2003). Previous study demonstrated that quercetin at the concentration of 1 μM inhibited the increase of intracellular ROS in H_2O_2-induced injury in mouse fibroblast cells (Shirai *et al*., 2002). The intracellular ROS was also attenuated after 1-h incubation of 100 μM resveratrol in H_2O_2-induced damage in HUVEC (Liu *et al*., 2003). In contrast, 6-h incubation of hesperidin decreased the intracellular ROS in H_2O_2-induced rat pheochromocytoma PC12 cells injury (Hwang *et al*., 2008). The contradiction may result from the pre-incubation time of hesperidin for 24 h in the present study. Quercetin and resveratrol, active constituents in CQES48, effectively attenuated intracellular ROS more than diosmin including hesperidin, an antihemorrhoidal drug (Bouskela *et al*., 1997; Sarabia *et al*., 2001). The result suggested that quercetin, resveratrol and CQES48, but not CQWS1, might be a potential candidate to treat endothelial dysfunction injured by H_2O_2.

Administration of H_2O_2, one of the models producing an increase of intracellular ROS generation, results in oxidative stress and induces cell death. Antioxidants from medicinal plants, such as flavonoid, were used to protect the cells from ROS and cell injury (Diaz *et al*., 1997, Maron, 2004, Siekmeier *et al*., 2007, Vanhoutte *et al*., 2009). Thus, CQES48 and CQWS1, which contained various potent antioxidants such as quercetin, resveratrol, quercitrin and iso-quercitrin, were used to determine the effect on viability of H_2O_2-treated cells. Unfortunately, CQES48 and CQWS1 did not reveal the cytoprotective effect against H_2O_2-induced cell injury

(A)



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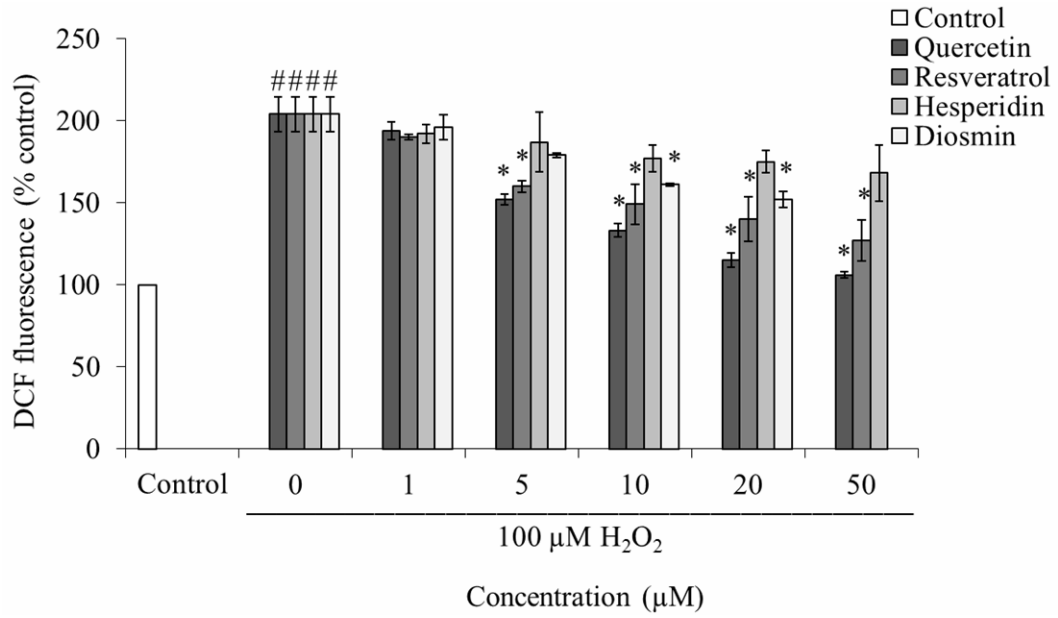


Figure 22. DCF fluorescence intensity of ECV304 cells treated with (A) CQES48 and CQWS1 as well as (B) quercetin, resveratrol, hesperidin and diosmin. After 24-h incubation with samples, the cells were incubated with 5 μM DCFH-DA in PBS for 30 min and then incubated with 100 μM H_2O_2 for 30 min. The absorbance was measured at excitation 485 nm and emission 535 nm. $^{\#}p < 0.05$ compared to the untreated control. $^{*}p < 0.05$ compared to the H_2O_2-treated control. Results were expressed as mean ± SEM (n=3). Each performed in triplicate.

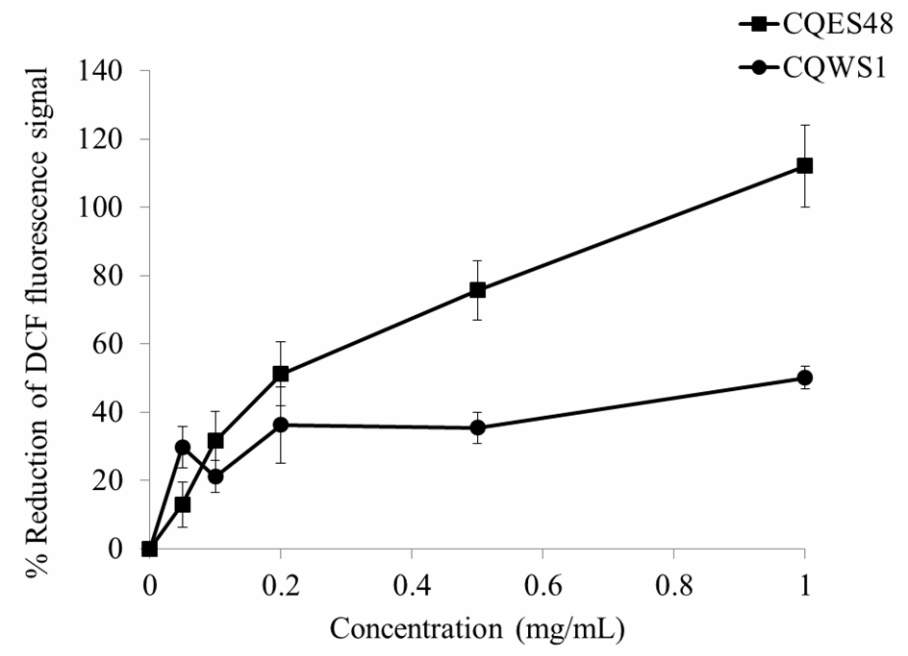


Figure 23. Percent reduction in DCF fluorescence by CQES48 and CQWS1 compared with H_2O_2-treated cells.

(Figure 18). H_2O_2 at the concentration of 100 µM generated oxidative stress so extreme that CQES48 and CQWS1 cannot maintain homeostasis leading to cell death. However, CQES48 and CQWS1 decreased the accumulation of intracellular ROS in H_2O_2-treated cells which was correlated to the antioxidant activity in cell-free system. Comparison between toxicity and the ability to attenuate intracellular ROS revealed that CQES48 (IC_{50}:EC_{50} = 32.57) was more effective and safety than CQWS1 (IC_{50}:EC_{50} > 5.85) (Table 5).

The results of DCFH-DA assay (Figure 21) and the MTT assay (Figure 17) revealed that H_2O_2 induced the excessive ROS generation and further damaged the cells. Additionally, pretreatment with CQES48 attenuated the intracellular ROS in the cells injured by H_2O_2 (Figure 22A). Thus, treatment the cells with CQES48 may increase the protein expression of antioxidant enzymes and NOS. Intracellular antioxidant enzymes such as cytosolic Cu/Zn-SOD, mitochondrial Mn-SOD, GPx and CAT maintain the balance between antioxidant enzymes and free radicals and also play important roles in cellular protection by oxidative stress-induced cell damage (Diaz *et al*., 1997; Nordberg and Arner, 2001; Østerud and Bjørklid, 2003).

Taken all results together, CQES48 was then chosen for further experiments in order to assess the possible mechanism involving in the protein expression of antioxidant enzymes and NOS using Western blotting analysis.

4.6 Effect of CQ on protein expression in ECV304 cells

4.6.1 Effect of CQ on protein expression of antioxidant enzymes

To verify whether CQES48 could regulate the protein expression of antioxidant enzymes in ECV304 cells, Western blot analysis was performed. The results revealed that treatment of the cells with CQES48 alone significantly increased the protein expression of Cu/Zn-SOD, Mn-SOD and GPx, but not CAT, at a concentration of 0.05, 0.1 and 0.5 mg/mL, when compared to control (Figure 24). Quercetin at the concentration of 50 µM, which was nearly equal to the EC_{50} value of H_2O_2 scavenger (Table 4), significantly up-regulated the expression of Cu/Zn-SOD, Mn-SOD, GPx, CAT, while resveratrol (5 µM) also increased the Mn-SOD, GPx and CAT expression (Figure 25). Thus, pretreatment of the cells with CQES48 increased the expression of antioxidant enzymes before encountering with H_2O_2. Treatment with H_2O_2 alone significantly decreased the protein expression of the Cu/Zn-SOD, Mn-SOD and GPx but not CAT (Figure 26B). Pretreatment the cells with CQES48 for

Table 5. Comparison between the toxicity, measured by MTT assay, and the ability to reduce intracellular ROS, measured by DCFH-DA assay, of CQES48, CQWS1, quercetin, resveratrol, hesperidin and diosmin.

Samples	IC_{50}	EC_{50}	IC_{50}: EC_{50}
Quercetin (µM)	246.37 ± 8.80	4.87 ± 0.17	50.59
Resveratrol (µM)	333.16 ± 12.90	8.04 ± 0.24	41.44
Hesperidin (µM)	> 400	> 50	> 8
Diosmin (µM)	> 40	> 20	> 2
CQES48 (mg/mL)	7.49 ± 0.20	0.23 ± 0.08	32.57
CQWS1 (mg/mL)	5.85 ± 0.75	> 1	> 5.85

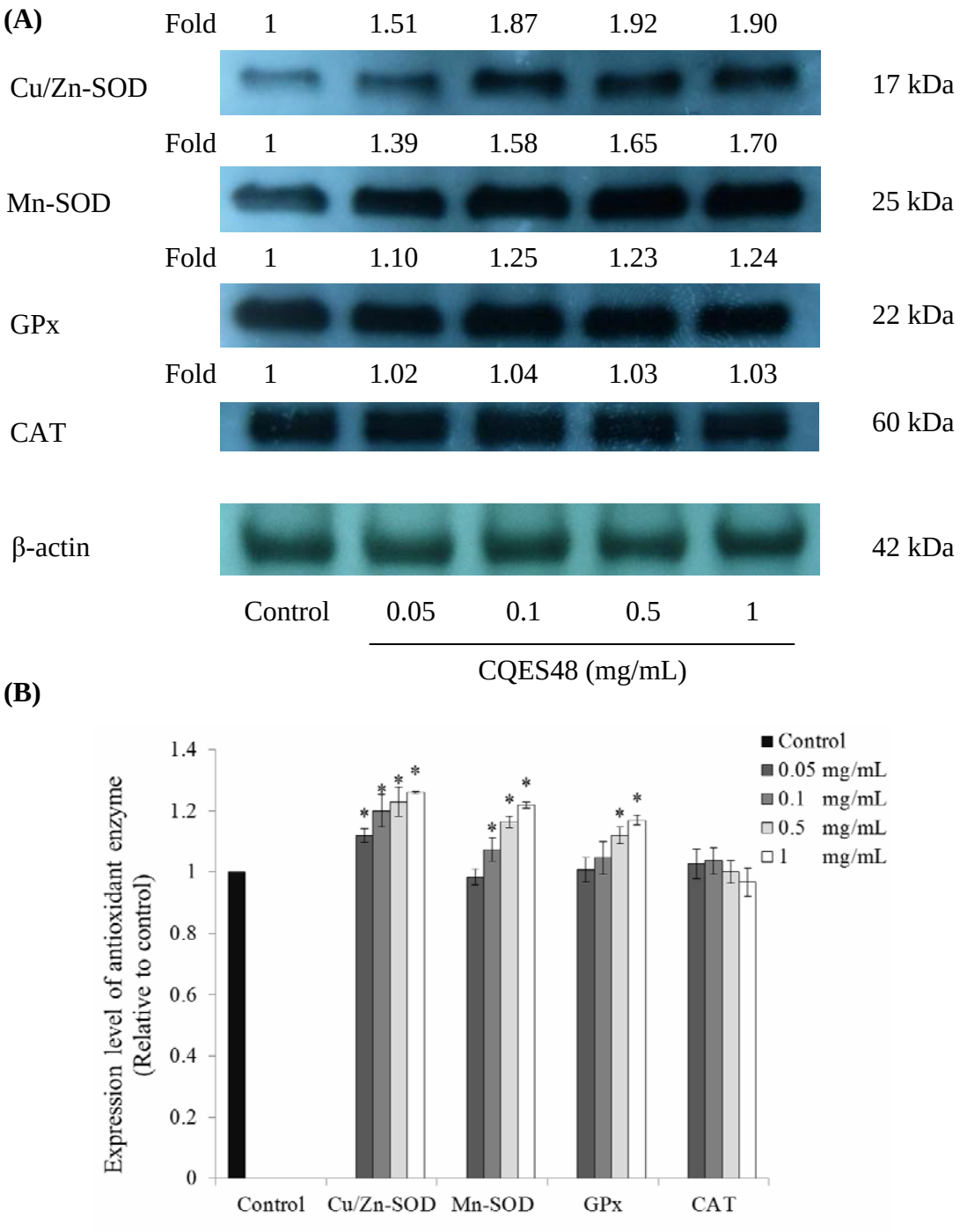


Figure 24. Effect of CQES48 on protein expression of antioxidant enzymes in ECV304 cells. Cells were incubated with CQES48 at various concentrations for 24 h and were determined by Western blot analysis. (A) Cu/Zn-SOD, Mn-SOD, GPx and CAT protein levels. (B) The intensity of each band was quantified and data were normalized using β-actin signal. The protein expression from control group was designated as 1. *$p < 0.05$ compared to the untreated control. Results were expressed as mean ± SEM (n=3).

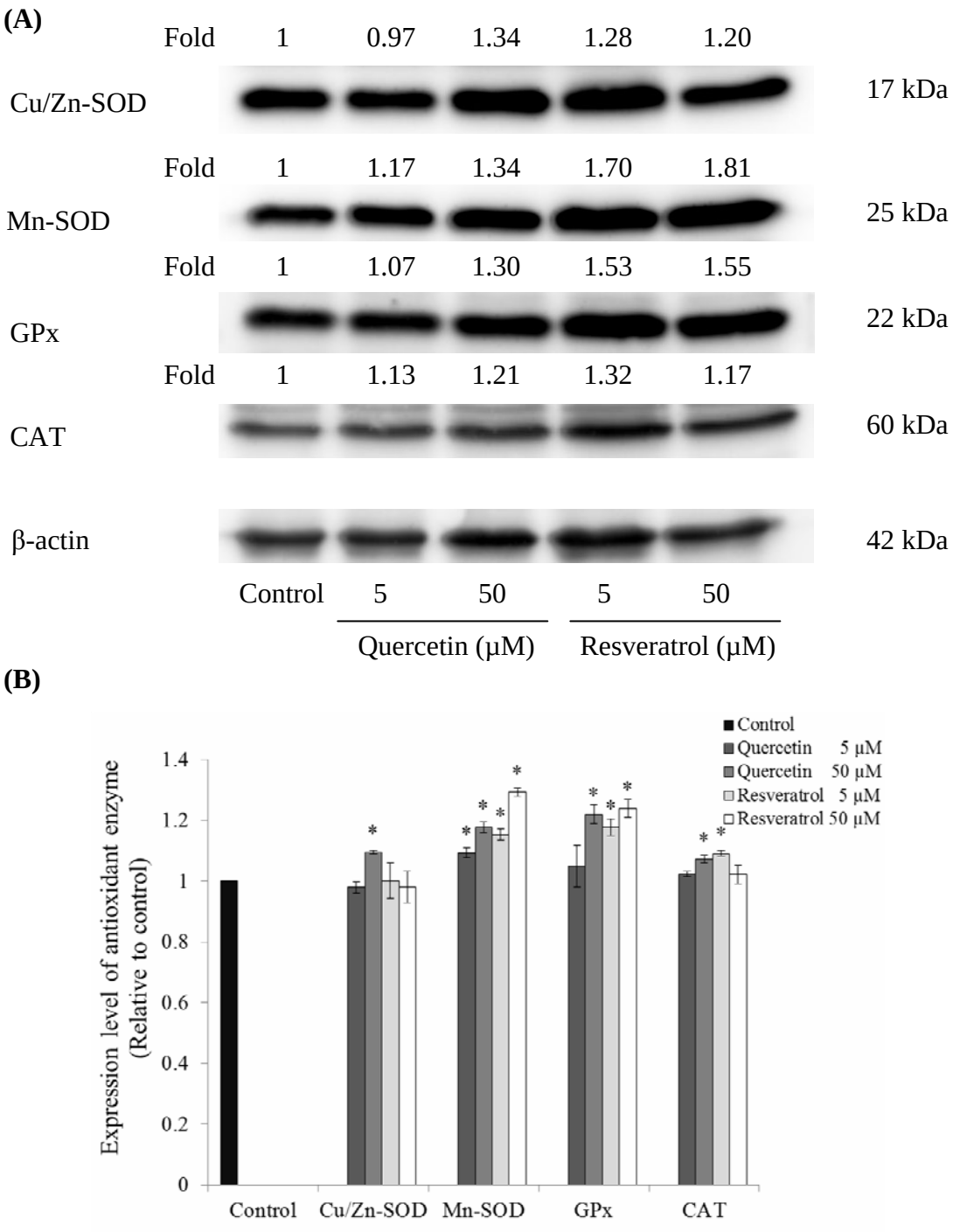


Figure 25. Effect of quercetin and resveratrol on protein expression of antioxidant enzymes in ECV304 cells. Cells were incubated with quercetin or resveratrol at 5 and 50 μM for 24 h and were determined by Western blot analysis. (A) Cu/Zn-SOD, Mn-SOD, GPx and CAT protein levels. (B) The intensity of each band was quantified and data were normalized using β-actin signal. The protein expression from control group was designated as 1. *$p < 0.05$ compared to the untreated control. Results were expressed as mean ± SEM (n=3).

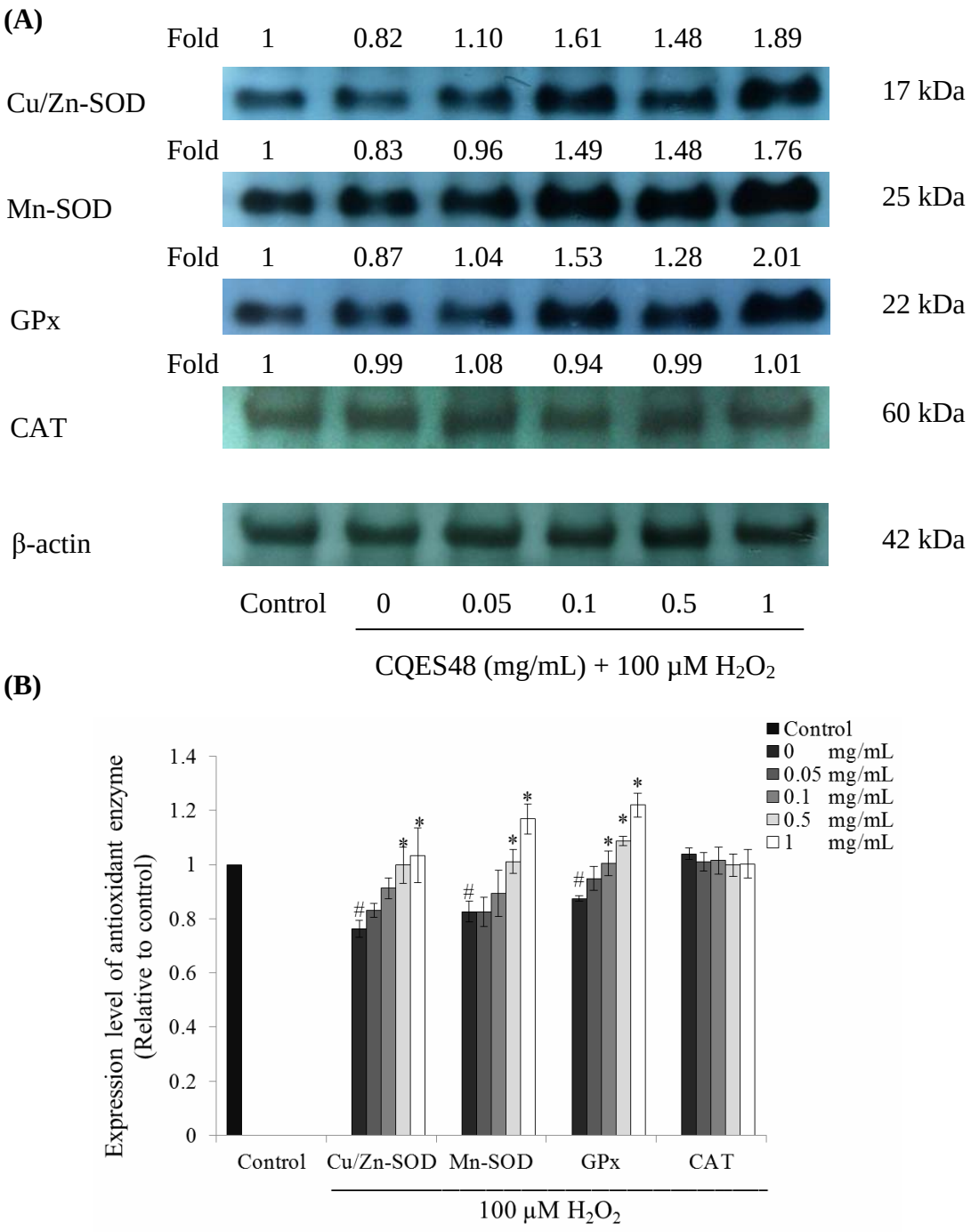


Figure 26. Effect of CQES48 on protein expression of antioxidant enzymes in H_2O_2-treated ECV304 cells. ECV304 cells were incubated with CQES48 at various concentrations for 24 h followed by 100 μM H_2O_2 for 6 h and were determined by Western blot analysis. (A) Cu/Zn-SOD, Mn-SOD, GPx, and CAT protein levels. (B) The intensity of each band was quantified and data were normalized using β-actin signal. The protein expression from control group was designated as 1. $^{\#}p < 0.05$ compared to the untreated control. $^{*}p < 0.05$ compared to the H_2O_2-treated control. Results were expressed as mean ± SEM (n=3).

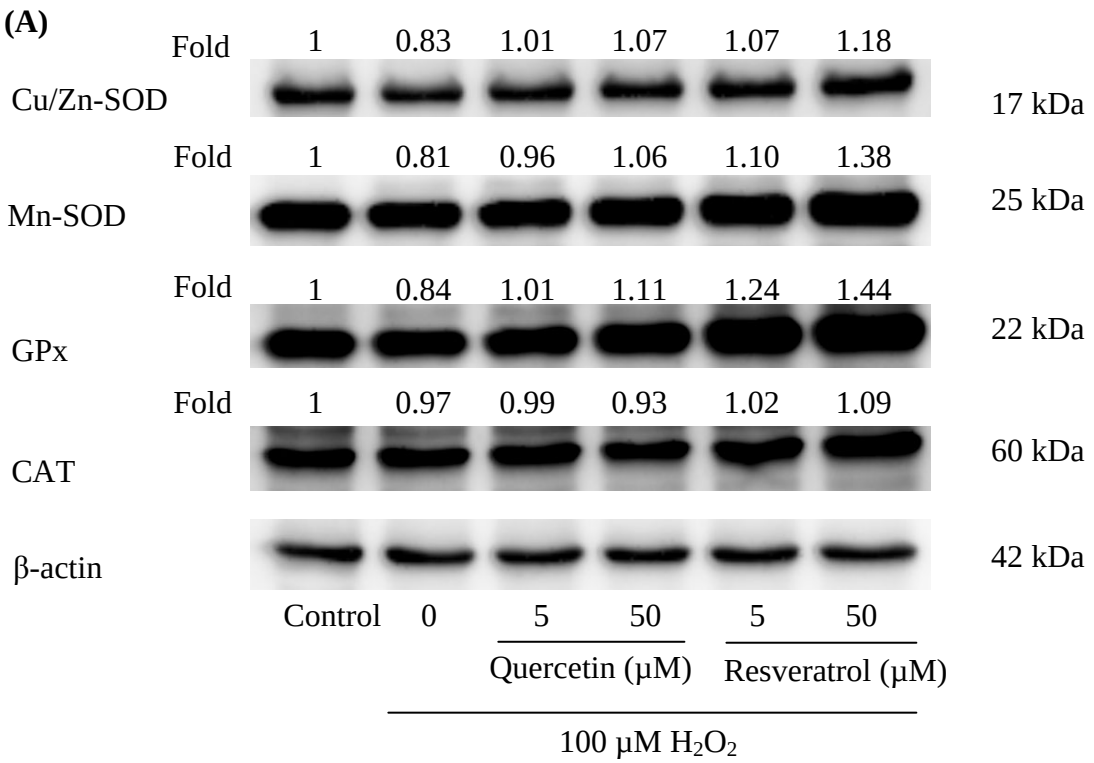
24 h prior to H_2O_2 exposure for 6 h could reverse this effect (Figure 26A). The protein levels of Cu/Zn-SOD, Mn-SOD and GPx, but not CAT, in cells treated with CQES48 at the concentration of 0.5 mg/mL were significantly up-regulated when compared to H_2O_2-treated control (Figure 26B). Additionally, pretreatment with quercetin and resveratrol at the concentration of 5 μM restored the expression of Cu/Zn-SOD, Mn-SOD and GPx in H_2O_2-treated cells (Figure 27).

The result suggested that the up-regulation of antioxidant enzymes by CQES48 might come from quercetin and resveratrol. The phenolic compounds, quercetin and resveratrol play an important role in the free radical scavenging activity in cell-free system (Hazra *et al*., 2008; Leonard *et al*, 2003) and in the attenuation of intracellular ROS level (Cheng *et al*., 2004; Yokomizo and Moriwaki, 2006) including the up-regulation of antioxidant enzyme expression (Galisteo *et al*., 2004; Jackson *et al*., 2010; Spanier *et al*., 2009) and activity (Amália *et al*., 2007; Lee *et al*., 2003; Lin *et al*., 2007). Thus, the antioxidant activities of CQES48 were well-correlated with quercetin and resveratrol. Pretreatment of CQES48 did not observe any change in the protein expression of CAT in H_2O_2-treated cells. CAT is especially important in the case of limited glutathione content or reduced GPx activity (Wassmann *et al*., 2004). Our study exhibited that GPx was up-regulated after pretreatment with CQES48 in H_2O_2-treated cells. The results advocated that the scavenging of intracellular ROS in ECV304 cells injured by H_2O_2 involved in the up-regulation of antioxidant enzymes.

Cells can normally deal with oxidative stress by up-regulating the synthesis of these antioxidant enzymes. The antioxidants were used to maintain the balance of the antioxidant defenses and high level of free radicals. Pretreatment of *Lycium chinense* (0.5 mg/mL), which contained quercetin, can protected Chang liver cells against oxidative stress-induced cell damage by enhancing the expression of Cu/Zn-SOD, Mn-SOD, CAT and GPx (Zhang *et al*., 2010). Thus, CQES48, which also contained quercetin and resveratrol, may protect endothelial cells from oxidative stress by an increase the expression of antioxidant enzymes.

4.6.2 Effect of CQ on protein expression of NOS

NOS plays a major role in maintaining homeostasis in endothelial cells. eNOS is constitutively expressed and generates NO, which is involved in the regulation of



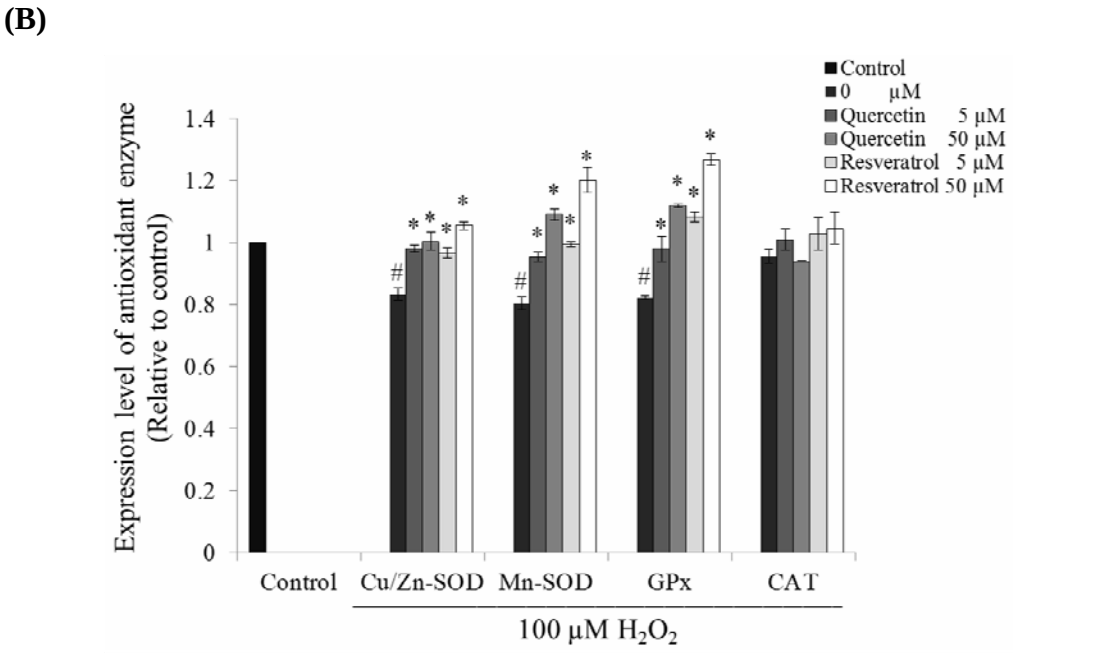


Figure 27. Effect of quercetin and resveratrol on protein expression of antioxidant enzymes in H_2O_2-treated ECV304 cells. ECV304 cells were incubated with quercetin or resveratrol at 5 and 50 μM for 24 h followed by 100 μM H_2O_2 for 6 h and were determined by Western blot analysis. (A) Cu/Zn-SOD, Mn-SOD, GPx, and CAT protein levels. (B) The intensity of each band was quantified and data were normalized using β-actin signal. The protein expression from control group was designated as 1. $^{\#}p < 0.05$ compared to the untreated control. $^{*}p < 0.05$ compared to the H_2O_2-treated control. Results were expressed as mean ± SEM (n=3).

vasodilation and platelet aggregation (Loscalzo, 2001). Unfortunately, eNOS can be reduced by oxidative stress leading to vascular diseases (Bao and Lou, 2006). iNOS is induced only in response to various stimuli including oxidative stress (Chen *et al*., 2002; Gosgnach *et al*., 2000), leading to the production of $NO^{\bullet}$ in high level which further involves in the cytotoxicity and endothelial dysfunction (Achike and Kwan, 2003). Thus, the protein expression of eNOS and iNOS were investigated.

We therefore determined the role of NOS expression in H_2O_2 mediating oxidative stress in ECV304 cells pretreated with CQES48. The result revealed that CQES48 alone for 24-h incubation did not significantly up-regulate the protein expression of eNOS in ECV304 cells (Figure 28) while H_2O_2 alone for 6-h incubation significantly decreased the protein expression of eNOS (Figure 29). In contrast, pretreatment with CQES48 at a concentration 0.05 mg/mL significantly increased the protein expression of eNOS, but not iNOS, in a concentration-dependent manner when compared to H_2O_2-treated cells (Figure 29).

The activation of iNOS usually induced by inflammation-mediating, cytokines such as interleukin-1β (Mendes *et al*., 2003), TNF-α (Xia *et al*., 2006). Macrophage, a potent producer of cytokines, was used to determine the protein expression of iNOS which was decreased after the treatment of antioxidant such as quercetin (Čiž *et al*., 2008). In addition, the protein expression of iNOS in lipopolysaccharide-stimulated RAW 264.7 macrophage cells were suppressed by the ethyl acetate extract of CQ (Srisook *et al.,* 2010). The methanolic extract of CQ was demonstrated to inhibit iNOS activity of gastric mucosa in aspirin-administered rats (Jainu and Devi, 2006). The result suggested that the oxidative stress inducer used in our condition may not be suitable to induce iNOS expression in ECV304 cells.

The increase of oxidative stress mostly attributes to the reduction of antioxidant enzymes (Kuo *et al*., 2009; Zhang *et al*., 2010) and eNOS (Calò *et al*., 2006). Thus, the balance between antioxidant enzymes and ROS is necessary for NO function. According to the protective effect of *Solanum lyratum* extract that reversed the decrease of Cu/Zn-SOD and eNOS expression in oxidized low-density lipoprotein-induced oxidative stress in HUVECs (Kuo *et al*., 2009). CQ was not only maintenance the balance of antioxidant enzymes and ROS but also up-regulation the eNOS expression in ECV304 cells, leading to the production of NO and vasodilation. Therefore, CQES48 may be useful for treatment of the progression of the hemorrhoid.

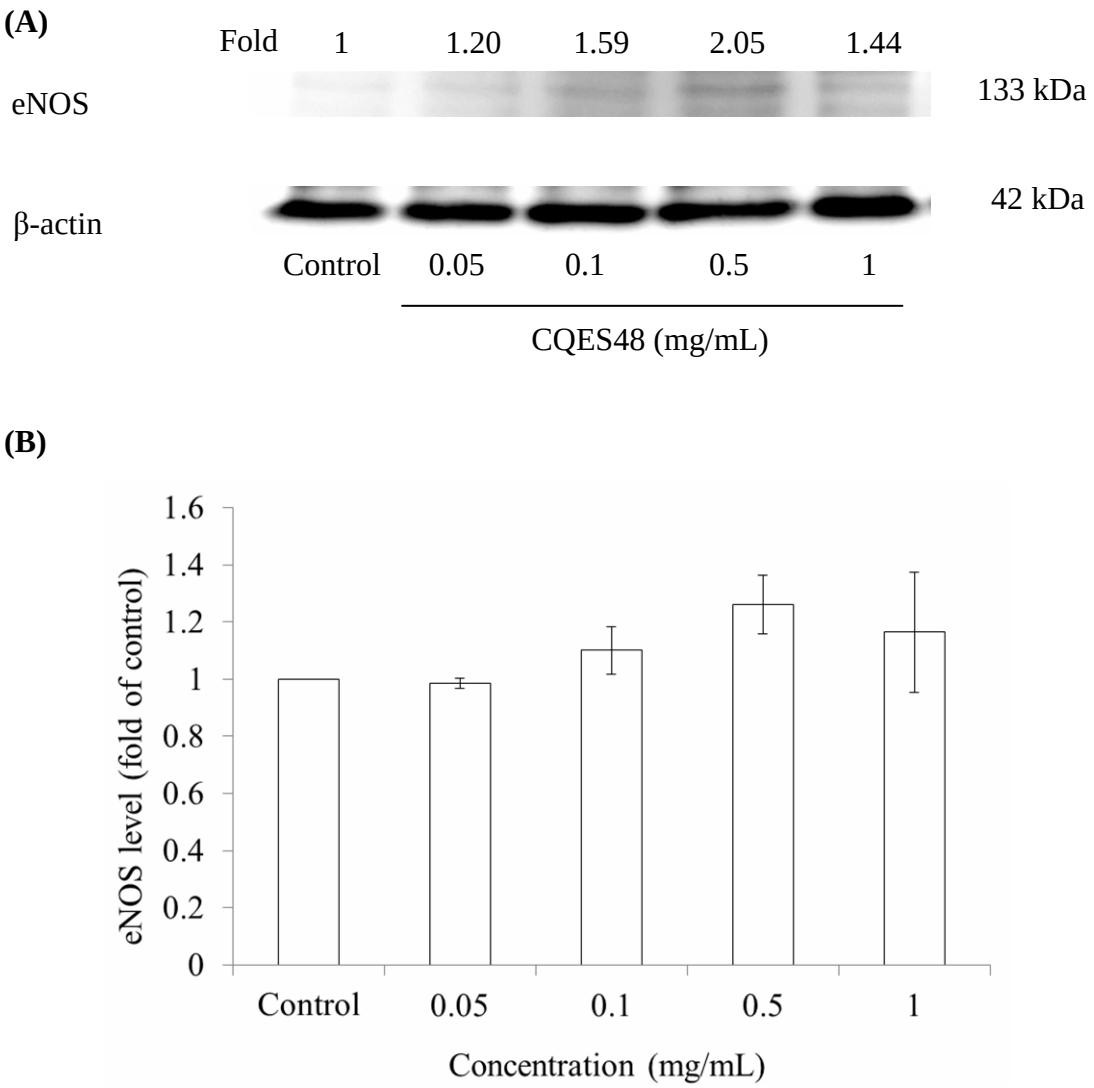
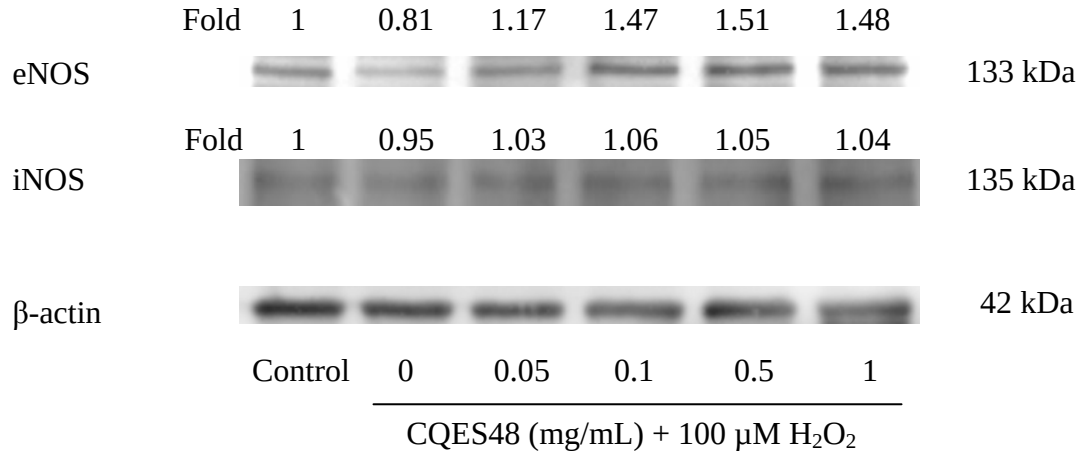


Figure 28. Effect of CQES48 on protein expression of eNOS in ECV304 cells. Cells were incubated with CQES48 at various concentrations for 24 h and were determined by Western blot analysis. (A) eNOS protein levels. (B) The intensity of each band was quantified and data were normalized using β-actin signal. The protein expression from control group was designated as 1. Results were expressed as mean ± SEM (n=3).

(A)



(B)

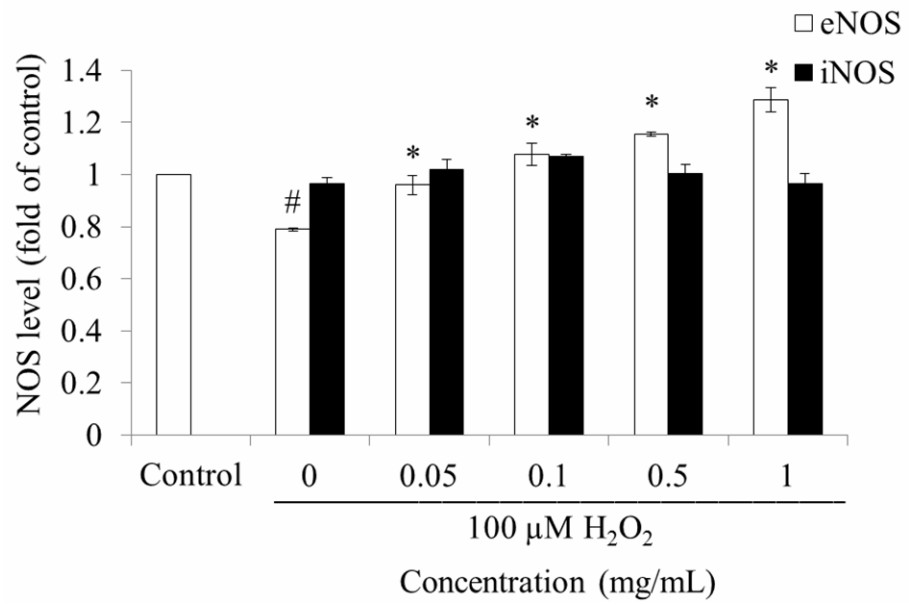


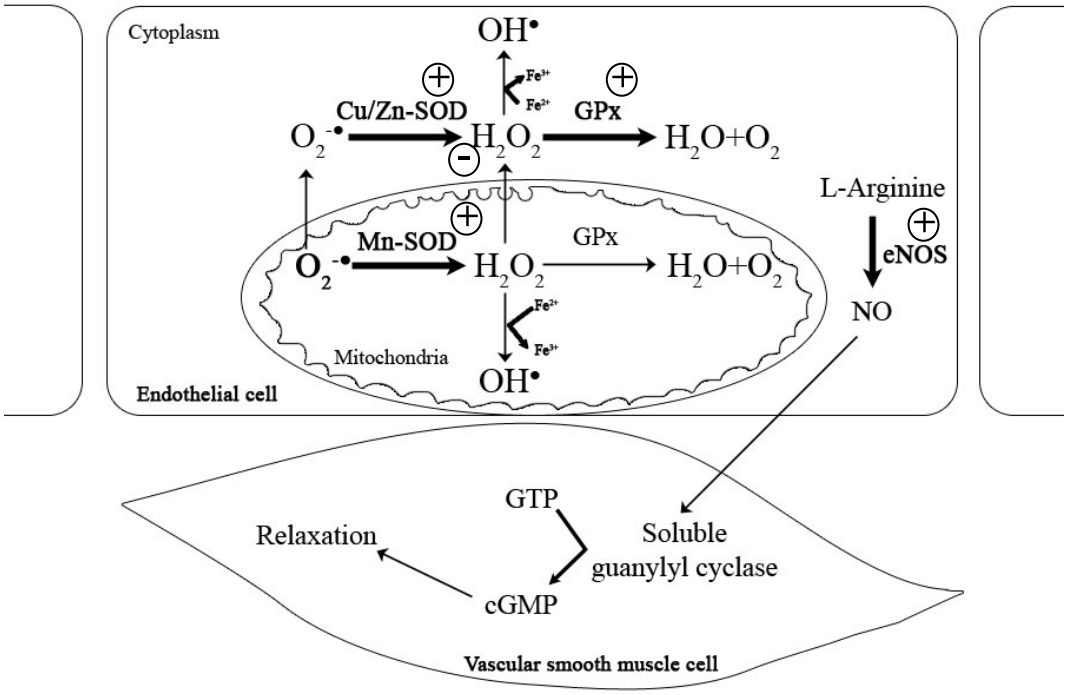
Figure 29. Effect of CQES48 on protein expression of eNOS and iNOS in H_2O_2-treated ECV304 cells. Cells were incubated with CQES48 at various concentrations for 24 h followed by 100 μM H_2O_2 for 6 h and were determined by Western blot analysis. (A) eNOS protein levels. (B) The intensity of each band was quantified and data were normalized using β-actin signal. The protein expression from control group was designated as 1. $^{\#}p < 0.05$ compared to the untreated control. $^{*}p < 0.05$ compared to the H_2O_2-treated control. Results were expressed as mean ± SEM (n=3).

CHAPTER V
CONCLUSION

In the present study, the free radical scavenging and cytoprotective activities of CQ extracts as well as its bioactive constituents, quercetin and resveratrol, were determined. Qualitative and semi-quantitative analysis of quercetin in the CQ extracts using TLC densitometric method found that ethanolic extraction using heat gained more quercetin than maceration at room temperature but not 40% ethanolic and aqueous extract. Thus, the soxhlet and reflux extraction were performed. Aqueous extract (CQWS1), obtained from the fluid of compressed fresh CQ, exhibited stronger scavenging activities against DPPH, $O_2^{-\bullet}$, H_2O_2 and $OH^{\bullet}$ in cell-free system than ethanolic CQ extract using soxhlet apparatus for 48 h (CQES48). These free radical scavenging activities might depend on either quercetin and resveratrol in ethanolic CQ extract or quercitrin and iso-quercitrin in aqueous CQ extract. H_2O_2 has been shown to induce oxidative stress in endothelial cells and antioxidant treatments were used to protect the cells from ROS-induced cell injury. Using DCFH-DA assay, both CQES48 and CQWS1 at the non-toxic concentrations (below 1 mg/mL) significantly attenuated intracellular ROS in H_2O_2-induced ECV304 cell damage, which were well correlated with the antioxidant activities in cell-free system. CQWS1 (1 mg/mL) significantly reduced the intracellular ROS at 10-fold less than CQES48 (0.1 mg/mL) eventhough CQWS1 (EC_{50} 0.488 ± 0.017 mg/mL) exhibited stronger H_2O_2 scavenging ability than CQES48 (EC_{50} 0.661 ± 0.034 mg/mL) in the cell-free system. According to quercetin and resveratrol found in CQ, both also significantly reduced intracellular ROS in H_2O_2-induced cell injury. Thus, CQES48 was more effective than CQWS1 in cell-based system. This might result from the cellular uptake of quercetin aglycone, more than its glycosides such as quercitrin and iso-quercitrin. Intracellular ROS level is caused by an imbalance between free radicals and the level and activities of antioxidant enzymes including Cu/Zn-SOD, Mn-SOD, GPx and CAT. Treatment with CQES48 (0.5 mg/mL) for 24 h significantly up-regulated the protein expression of Cu/Zn-SOD, Mn-SOD and GPx, as compared with control. Moreover, preincubation with CQES48 (0.5 mg/mL) restored the protein expression of Cu/Zn-SOD, Mn-SOD and GPx in H_2O_2-induced cell injury. The up-regulation of

Cu/Zn-SOD, Mn-SOD and GPx catalyzed the reduction of $O_2^{-\bullet}$ to H_2O_2 and O_2, and further catalyze H_2O_2 to H_2O and O_2 to prevent the formation of $OH^{\bullet}$, leading to the normal level of intracellular ROS. The result was correlated with H_2O_2 scavenging activity of CQES48 in cell-free system (EC_{50} 0.661 ± 0.034 mg/mL). Additionally, CQES48 (0.05 mg/mL) significantly up-regulated eNOS but not iNOS expression in H_2O_2-treated cells. The result suggested that CQES48 might increase NO production to stimulate vasodilation, resulting in lowering the risk of vascular dysfunction including hemorrhoid.

In conclusion, CQES48 acts as not only a H_2O_2 scavenger but also a cytoprotective agent. The underlying mechanisms of the protective effect are partly contributed to the attenuation of intracellular ROS and restoring the protein expression of antioxidant enzymes, Cu/Zn-SOD, Mn-SOD and GPx, including eNOS in H_2O_2-induced ECV304 cell injury (Figure 30). These activities are at least partly due to the presence of quercetin in CQ analyzed by TLC densitometry. This is the first report to demonstrate the role of the protein expression of antioxidant enzymes and eNOS in the cytoprotection of the ethanolic CQ extracts as compared with its constituents, quercetin and resveratrol. Since intracellular ROS are involved in vascular diseases partially through the oxidative damage, our finding suggests that the antioxidant activity of CQ partly hold a benefit for the prevention of oxidative-induced diseases such as hemorrhoid and other vascular diseases.



⊖ Decrease in the intracellular ROS in H_2O_2-induced cell injury.

⊕ Up-regulation of the antioxidant enzymes and eNOS.

Figure 30. Proposed signaling pathways of CQES48-attenuated intracellular ROS in human umbilical vein endothelial ECV304 cells.

REFERENCES

ภาษาไทย

ประกาศกระทรวงสาธารณสุข พ.ศ. 2542. เรื่อง ยาสามัญประจำบ้านแผนโบราณ. *ราชกิจจานุเบกษา เล่ม 116 ตอนที่ 67 ง* (24 สิงหาคม 2542): 29-56.

ภาษาอังกฤษ

Achike, F.I., and Kwan, C.Y. 2003. Nitric oxide, human diseases and the herbal products that affect the nitric oxide signalling pathway. *Clinical and Experimental Pharmacology and Physiology* 30 (9): 605-615.

Adesanya, S.A., *et al*. 1999. Stilbene derivatives from *Cissus quadrangularis*. *Journal of Natural Products* 62 (12): 1694-1695.

Almeida, I.F., *et al*. 2009. Oxygen and nitrogen reactive species are effectively scavenged by *Eucalyptus globulus* leaf water extract. *Journal of Medicinal Food* 12 (1): 175-183.

Amália, P.M., Possa, M.N., Augusto, M.C., and Francisca, L.S. 2007. Quercetin prevents oxidative stress in cirrhotic rats. *Digestive Diseases and Sciences* 52 (10): 2616-2621.

Arias, M., *et al*. 2009. Fast supercritical fluid extraction of low- and high-density polyethylene additives: comparison with conventional reflux and automatic soxhlet extraction. *Journal of Supercritical Fluids* 50 (1): 22-28.

Badami, S., and Channabasavaraj, K.P. 2007. In vitro antioxidant activity of thirteen medicinal plants of India's Western Ghats. *Pharmaceutical Biology* 45 (5): 392-396.

Bao, M., and Lou, Y. 2006. Flavonoid from Seabuckthorn protect endothelial cells (EA.hy926) from oxidized low-density lipoprotein induced injuries via regulation of LOX-1 and eNOS expression. *Journal of Cardiovascular Pharmacology* 48: 834-841.

Baur, J.A., and Sinclair, D.A. 2006. Therapeutic potential of resveratrol: the in vivo evidence. *Nature Reviews Drug Discovery* 5 (6): 493-506.

Beckman, J.S., Beckman, T.W., Chen, J., Marshall, P.A., and Freeman, B.A. 1990. Apparent hydroxyl radical production by peroxynitrite: Implications for endothelial injury from nitric oxide and superoxide. *Proceedings of the National Academy of Sciences of the United States of America* 87 (4): 1620-1624.

Boonsiripiphat, K., and Theerakulkait, C. 2009. Extraction of rice bran extract and some factors affecting its inhibition of polyphenol oxidase activity and browning in potato *Preparative Biochemistry and Biotechnology*. 39 (2): 147-158.

Boots, A.W., Haenen, G.R.M.M., and Bast, A. 2008. Health effects of quercetin: from antioxidant to nutraceutical. *European Journal of Pharmacology* 585 (2-3): 325-337.

Bouskela, E., Cyrino, F.Z.G.A., and Lerond, L. 1997. Microvascular reactivity after ischemia/reperfusion in the hamster cheek pouch: Beneficial effects of different oral doses of S-5682 (Daflon 500 mg). *Angiology* 48 (1): 33-37.

Brand-Williams, W., Cuvelier, M.E., and Berset, C. 1995. Use of a free radical method to evaluate antioxidant activity. *LWT - Food Science and Technology* 28 (1): 25-30.

Bruck, R., *et al*. 1999. The hydroxyl radical scavengers dimethylsulfoxide and dimethylthiourea protect rats against thioacetamide-induced fulminant hepatic failure. *Journal of Hepatology* 31 (1): 27-38.

Buckshee, K., Takkar, D., and Aggarwal, N. 1997. Micronized flavonoid therapy in internal hemorrhoids of pregnancy. *International Journal of Gynecology and Obstetrics* 57 (2): 145-151.

Cai, H., and Harrison, D.G. 2000. Endothelial dysfunction in cardiovascular diseases: the role of oxidant stress. *Circulation Research* 87 (10): 840-844.

Calò, L.A., *et al*. 2006. Antioxidant effect of L-carnitine and its short chain esters: Relevance for the protection from oxidative stress related cardiovascular damage. *International Journal of Cardiology* 107 (1): 54-60.

Carmichael, J., Degraff, W.G., Gazdar, A.F., Minna, J.D., and Mitchell, J.B. 1987. Evaluation of a tetrazolium-based semiautomated colorimetric assay: assessment of chemosensitivity testing. *Cancer Research* 47 (4): 936-942.

Cepinskas, G., Rui, T., and Kvietys, P.R. 2002. Interaction between reactive oxygen metabolites and nitric oxide in oxidant tolerance. *Free Radical Biology and Medicine* 33 (4): 433-440.

Chang, M.S., *et al*. 2008. Cytoprotective effects of *Morinda officinalis* against hydrogen peroxide-induced oxidative stress in Leydig TM3 cells. *Asian Journal of Andrology* 10 (4): 667-674.

Chen, H.W., Chien, C.T., Yu, S.L., Lee, Y.T., and Chen, W.J. 2002. Cyclosporine A regulate oxidative stress-induced apoptosis in cardiomyocytes: mechanisms via ROS generation, iNOS and Hsp70. *British Journal of Pharmacology* 137 (6): 771-781.

Chen, J., Gu, Y., Shao, Z., Luo, J., and Tan, Z. 2010. Propofol protects against hydrogen peroxide-induced oxidative stress and cell dysfunction in human umbilical vein endothelial cells. *Molecular and Cellular Biochemistry* 339 (1-2): 43-54.

Chen, Y.H., *et al*. 2007. Identification of phenolic antioxidants from Sword Brake fern (*Pteris ensiformis* Burm.). *Food Chemistry* 105 (1): 48-56.

Cheng, T.H., *et al*. 2004. Inhibitory effect of resveratrol on angiotensin II-induced cardiomyocyte hypertrophy. *Naunyn-Schmiedeberg's Archives of Pharmacology* 369 (2): 239-244.

Chiou, C.S., *et al*. 2008. Effects of hesperidin on cyclic strain-induced endothelin-1 release in human umbilical vein endothelial cells. *Clinical and Experimental Pharmacology and Physiology* 35 (8): 938-943.

Choi, E.H., Chang, H.J., Cho, J.Y., and Chun, H.S. 2007. Cytoprotective effect of anthocyanins against doxorubicin-induced toxicity in H9c2 cardiomyocytes in relation to their antioxidant activities. *Food and Chemical Toxicology* 45 (10): 1873-1881.

Choi, E.M. 2010. Protective effect of quercitrin against hydrogen peroxide-induced dysfunction in osteoblastic MC3T3-E1 cells. *Experimental and Toxicologic Pathology*. Article in Press.

Chopra, R.N., Nayar, S.L., and Chopra, I.C. 1965. I.C. *Glossary of Indian Medicinal foant* 66-67. India: Catholic Press.

Chow, J.M., Shen, S.C., Huan, S.K., Lin, H.Y., and Chen, Y.C. 2005. Quercetin, but not rutin and quercitrin, prevention of H_2O_2-induced apoptosis via anti-oxidant activity and heme oxygenase 1 gene expression in macrophages. *Biochemical Pharmacology* 69 (12): 1839-1851.

ČÍŽ, M., *et al*. 2008. The influence of wine polyphenols on reactive oxygen and nitrogen species production by murine macrophages RAW 264.7. *Physiological Research* 57: 393-402.

Cypriani, B., *et al*. 1993. Antioxidant activity of micronized diosmin on oxygen species from stimulated human neutrophils. *Biochemical Pharmacology* 45 (7): 1531-1535.

Diaz, M.N., Frei, B., Vita, J.A., and Keaney Jr., J.F. 1997. Antioxidants and atherosclerotic heart disease. *New England Journal of Medicine* 337 (6): 408-416.

Dröge, W. 2002. Free radicals in the physiological control of cell function. *Physiological Reviews* 82 (1): 47-95.

Ekenseair, A.K., Duan, L., Carrier, D.J., Bransby, D.I., and Clausen, E.C. 2006. Extraction of hyperoside and quercitrin from mimosa (*Albizia julibrissin*) foliage. *Applied Biochemistry and Biotechnology* 130 (1-3): 382-391.

Elango, N., Samuel, S., and Chinnakkannu, P. 2006. Enzymatic and non-enzymatic antioxidant status in stage (III) human oral squamous cell carcinoma and treated with radical radio therapy: influence of selenium supplementation. *Clinica Chimica Acta* 373 (1-2): 92-98.

Eruslanov, E., and Kusmartsev, S. 2009. *Identification of ROS using oxidized DCFDA and flow-cytometry*. pp 57 – 72. United States: Humana Press.

Formica, J.V., and Regelson, W. 1995. Review of the biology of quercetin and related bioflavonoids. *Food and Chemical Toxicology* 33 (12): 1061-1080.

Franks, F. 1998. Freeze-drying of bioproducts: putting principles into practice. *European Journal of Pharmaceutics and Biopharmaceutics* 45 (3): 221-229.

Galisteo, M., *et al*. 2004. Effect of quercetin treatment on vascular function in deoxycorticosterone acetate-salt hypertensive rats. Comparative study with verapamil. *Planta Medica* 70: 334-341.

Gao, Z., Huang, K., Yang, X., and Xu, H. 1999. Free radical scavenging and antioxidant activities of flavonoids extracted from the radix of *Scutellaria baicalensis* Georgi. *Biochimica et Biophysica Acta* 1472 (3): 643-650.

Gil'yano, N.Ya., Semenova, E.G., Fedortseva, R.F., and Konevega, L.V. 2009. Characteristics of spontaneously transformed human endothelial ECV304 cell line: II. Functional responses of ECV304 cells. *Cell and Tissue Biology* 3 (3): 274-282.

Girard-Lalancette, K., Pichette, A., and Legault, J. 2009. Sensitive cell-based assay using DCFH oxidation for the determination of pro- and antioxidant properties of compounds and mixtures: Analysis of fruit and vegetable juices. *Food Chemistry* 115 (2): 720–726.

Glowinski, J., and Glowinski, S. 2002. Generation of reactive oxygen metabolites by the varicose vein wall. *European Journal of Vascular and Endovascular Surgery* 23 (6): 550-555.

Gobbel, G.T., Chan, T.Y.Y., and Chan, P.H. 1997. Nitric oxide- and superoxide-mediated toxicity in cerebral endothelial cells. *Journal of Pharmacology and Experimental Therapeutics* 282 (3): 1600-1607.

Gong, G., *et al*. 2010. Rutin inhibits hydrogen peroxide-induced apoptosis through regulating reactive oxygen species mediated mitochondrial dysfunction pathway in human umbilical vein endothelial cells. *European Journal of Pharmacology* 628 (1-3): 27-35.

Gosgnach, W., Messika-Zeitoun, D., Gonzalez, W., Philipe, M., and Michel, J.B. 2000. Shear stress induces iNOS expression in cultured smooth muscle cells: Role of oxidative stress. *American Journal of Physiology - Cell Physiology* 279: 1880-1888.

Gülçin, I. 2010. Antioxidant properties of resveratrol: A structure-activity insight. *Innovative Food Science and Emerging Technologies* 11 (1): 210-218.

Halliwell, B., Hoult, J.R., and Blake, D.R. 1988. Oxidants, inflammation, and anti-inflammatory drugs. *FASEB Journal* 2 (13): 2867-2873.

Hazra, B., Biswas, S., and Mandal, N. 2008. Antioxidant and free radical scavenging activity of *Spondias pinnata. BMC Complementary and Alternative Medicine* 8, art. no. 63.

Hong, H., and Liu, G.Q. 2004. Protection against hydrogen peroxide-induced cytotoxicity in PC12 cells by scutellarin. *Life Sciences* 74 (24): 2959-2973.

Hou, Y.Z., *et al*. 2004. Protective effect of *Ligusticum chuanxiong* and *Angelica sinensis* on endothelial cell damage induced by hydrogen peroxide. *Life Sciences* 75 (14): 1775-1786.

Hsu, B., Coupar, I.M., and Ng, K. 2006. Antioxidant activity of hot water extract from the fruit of the Doum palm, *Hyphaene thebaica*. *Food Chemistry* 98 (2): 317-328.

Hwang, S.L., and Yen, G.C. 2008. Neuroprotective effects of the citrus flavanones against H_2O_2-induced cytotoxicity in PC12 cells. *Journal of Agricultural and Food Chemistry* 56 (3); 859-864.

International Conference on Harmonisation. 1994. Validation of analytical procedures: text and methodology Q2(R1). International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use. Washington D.C.

Jackson, J.R., Ryan, M.J., Hao, Y., and Always, S.E. 2010. Mediation of endogenous antioxidant enzymes and apoptotic signaling by resveratrol following muscle disuse in the gastrocnemius muscles of young and old rats. *American Journal of Physiology* 299: 1572-1581.

Jackson, S.J.T., and Venema, R.C. 2006. Quercetin inhibits eNOS, microtubule polymerization, and mitotic progression in bovine aortic endothelial cells. *Journal of Nutrition* 135 (5): 1178-1184.

Jagetia, G.C., Rao, S.K., Baliga, S.M., and Babu, K.S. 2004. The evaluation of nitric oxide scavenging activity of certain herbal formulations in vitro: a preliminary study. *Phytotherapy Research* 18 (7): 561-565.

Jainu, M., and Devi, C.S.S. 2005. In vitro and in vivo evaluation of free radical scavenging potential of *Cissus quadrangularis*. *African Journal of Biomedical Research* 8: 95–99.

Jainu, M., and Devi, C.S.S. 2006. Gastroprotective action of *Cissus quadrangularis* extract against NSAID induced gastric ulcer: Role of proinflammatory cytokines and oxidative damage. *Chemico-Biological Interactions* 161 (3): 262-270.

Jainu, M., and Mohan, K.V. 2008. Protective role of ascorbic acid isolated from *Cissus quadrangularis* on NSAID induced toxicity through immunomodulating response and growth factors expression. *International Immunopharmacology* 8 (13-14): 1721-1727.

Jakikasem, S., Limsiriwong, P., Kajsongkarm, T., and Soontorntanasart, T. 2000. Phytochemical study of *Cissus quadrangularis* Linn. *Thai Journal of Pharmaceutical Sciences* 24: 25.

Kaindl, U., Eyberg, I., Rohr-Udilova, N., Heinzle, C., and Marian, B. 2008. The dietary antioxidants resveratrol and quercetin protect cells from exogenous pro-oxidative damage. *Food and Chemical Toxicology* 46 (4): 1320-1326.

Kalpana, K.B., Srinivasan, M., and Menon, V.P. 2009. Evaluation of antioxidant activity of hesperidin and its protective effect on H_2O_2 induced oxidative damage on pBR322 DNA and RBC cellular membrane. *Molecular and Cellular Biochemistry* 323 (1-2): 21-29.

Kann, B.R., and Whitlow, C.B. 2004. Hemorrhoids: diagnosis and management. *Techniques in Gastrointestinal Endoscopy* 6 (1): 6-11.

Kaur, G., Tirkey, N., and Chopra, K. 2006. Beneficial effect of hesperidin on lipopolysaccharide-induced hepatotoxicity. *Toxicology* 226 (2-3): 152-160.

Khoo, N.K.H., *et al*. 2010. Dietary flavonoid quercetin stimulates vasorelaxation in aortic vessels. *Free Radical Biology and Medicine* 49 (3): 339-347.

Kosem, N., Han, Y.H., and Moongkarndi, P. 2007. Antioxidant and cytoprotective activities of methanolic extract from *Garcinia mangostana* hulls. *Science Asia* 33 (3): 283-292.

Kuo, W.W., *et al*. 2009. Crude extracts of *Solanum lyratum* protect endothelial cells against oxidized low-density lipoprotein-induced injury by direct antioxidant action. *Journal of Vascular Surgery* 50 (4): 849-860.

Lee, H.J., Seo, J.W., Lee, B.H., Chung, K.H., and Chi, D.Y. 2004. Syntheses and radical scavenging activities of resveratrol derivatives. *Bioorganic and Medicinal Chemistry Letters* 14 (2): 463-466.

Lee, M.H., *et al*. 2001. Monoamine oxidase B and free radical scavenging activities of natural flavonoids in *Melastoma candidum* D. Don. *Journal of Agricultural and Food Chemistry* 49 (11): 5551-5555.

Lee, S.E., Hwang, H.J., Ha, J.-S., Jeong, H.-S., and Kim, J.H. 2003. Screening of medicinal plant extracts for antioxidant activity. *Life Sciences* 73 (2): 167-179.

Leonard, S.S., *et al*. 2003. Resveratrol scavenges reactive oxygen species and effects radical-induced cellular responses. *Biochemical and Biophysical Research Communications* 309 (4): 1017-1026.

Lin, R., Liu, J., Gan, W., and Ding, C. 2007. Protective effect of quercetin on the homocysteine-injured human umbilical vein vascular endothelial cell line (ECV304). *Basic and Clinical Pharmacology and Toxicology* 101 (3): 197-202.

Liu, H.T., *et al*. 2009. Chitosan oligosaccharides attenuate hydrogen peroxide-induced stress injury in human umbilical vein endothelial cells. *Pharmacological Research* 59 (3): 167-175.

Liu, J.C., Chen, J.J., Chan, P., Cheng, C.F., and Cheng, T.H. 2003. Inhibition of cylic strain-induced endothelin-1 gene expression by resveratrol. *Hypertension* 42: 1198-1205.

Liu, Q.M., Yang, X.M., Zhang, L., and Majetich, G. 2010. Optimization of ultrasonic-assisted extraction of chlorogenic acid from *Folium eucommiae* and evaluation of its antioxidant activity. *Journal of Medicinal Plant Research* 4 (23): 2503-2511.

Liu, S.C., Lin, J.T., Wang, C.K., Chen, H.Y., and Yang, D.J. 2009. Antioxidant properties of various solvent extracts from lychee (*Litchi chinenesis* Sonn.) flowers. *Food Chemistry*. 114 (2): 577-581.

Loscalzo, J. 2001. Nitric oxide insufficiency, platelet activation, and arterial thrombosis. *Circulation Research* 88 (8): 756-762.

Lu, Y., and Yeap Foo, L. 2000. Antioxidant and radical scavenging activities of polyphenols from apple pomace. *Food Chemistry* 68 (1): 81-85.

Luo, P., *et al*. 2008. Scutellarin isolated from *Erigeron multiradiatus* inhibits high glucose-mediated vascular inflammation. *Yakugaku Zasshi* 128 (9): 1293-1299.

Maksimović, Z. 2008. In vitro antioxidant activity of ragweed (*Ambrosia artemisiifolia* L., Asteraceae) herb. *Industrial Crops and Products* 28 (3): 356-360.

Manach, C., *et al*. 1995. Quercetin metabolites in plasma of rats fed diets containing rutin or quercetin. *Journal of Nutrition* 125 (7): 1911-1922.

Manach, C., *et al*. 1997. Bioavailability of rutin and quercetin in rats. *FEBS Letters* 409 (1): 12-16.

Maron, D.J. 2004. Flavonoids for reduction of atherosclerotic risk. *Current Atherosclerosis Reports* 6 (1): 73-78.

Martindale, J.L., and Holbrook, N.J. 2002. Cellular response to oxidative stress: Signaling for suicide and survival. *Journal of Cellular Physiology* 192 (1): 1-15.

Melin, A.M., Perromat, A., and Clerc, M. 1996. In vivo effect of diosmin on carrageenan and CCl_4-induced lipid peroxidation in rat liver microsomes. *Journal of Biochemical Toxicology* 11 (1): 27-32.

Mendes, A.F., Caramona, M.M., Carvalho, A.P., and Lopes, M.C. 2003. Hydrogen peroxide mediates interleukin-1β-induced AP-1 activation in articular chondrocytes: inplications for the regulation of iNOS expression. *Cell Biology and Technology* 19: 203-214.

Meyer, A.S., Heinonen, M., and Frankel, E.N. 1998. Antioxidant interactions of catechin, cyanidin, caffeic acid, quercetin, and ellagic acid on human LDL oxidation. *Food Chemistry* 61 (1-2): 71-75.

Michot, J.L., Virion, A., and Deme, D. 1985. NADPH oxidation catalyzed by the peroxidase/H_2O_2 system. guaiacol-mediated and scopoletin-mediated oxidation of NADPH to $NADP^+$. *European Journal of Biochemistry* 148 (3): 441-445.

Mishra, G., Srivastava, S., and Nagori, B.P. 2010. Pharmacological and therapeutic activity of *Cissus quadrangularis*: an overview. *International Journal of PharmTech Research* 2 (2): 1298-1310.

Mokni, M., Elkahoui, S., Limam, F., Amri, M., and Aouani, E. 2007. Effect of resveratrol on antioxidant enzyme activities in the brain of healthy rat. *Neurochemical Research* 32 (6): 981-987.

Moore, R.A. 2000. Hemorrhoid review. *Current Surgery*. 57 (2): 103-106.

Murota, K., and Terao, J. 2003. Antioxidative flavonoid quercetin: implication of its intestinal absorption and metabolism. *Archives of Biochemistry and Biophysics* 417 (1): 12-17.

Nakamura, Y.K., and Omaye, S.T. 2008. α-Tocopherol modulates human umbilical vein endothelial cell expression of Cu/Zn superoxide dismutase and catalase and lipid peroxidation. *Nutrition Research* 28 (10): 671-680.

Nikolić, N.Č., Cakić, S.M., Novaković, S.M., Cvetković, M.D., and Stanković, M.Z. 2008. Effect of extraction techniques on yield and composition of soybean oil. *Macedonian Journal of Chemistry and Chemical Engineering* 28 (2): 173-179.

Nordberg, J., and Arnér, E.S.J. 2001. Reactive oxygen species, antioxidants, and the mammalian thioredoxin system. *Free Radical Biology and Medicine* 31 (11): 1287-1312.

Odukoya, O.A. *et al*. 2009. Hemorrhoid therapy with medicinal plants: astringency and inhibition of lipid peroxidation as key factors. *International Journal of Biological Chemistry* 3: 111-118.

Østerud, B., and Bjørklid, E. 2003. Role of monocytes in atherogenesis. *Physiological Reviews* 83 (4): 1069-1112.

Panthong, A., Supraditaporn, W., Kanjanapothi, D., Taesotikul, T., and Reutrakul, V. 2007. Analgesic, anti-inflammatory and venotonic effects of *Cissus quadrangularis* Linn. *Journal of Ethnopharmacology* 110 (2): 264-270.

Patarapanich, C., Thiangtham, J., Saifah, E., Laungchonlatan, S., and Janthasoot, W. 2004. Determination of antioxidant constituents in the herb *Cissus quadrangularis* Linn. *Iranian Journal of Pharmaceutical Research* 2: 77-77.

Pongboonrod, S. 1995. *Mai Thed Mung Muang Thai*. Bangkok. pp 428-429. Thailand. Kasembanakij Printing

Pothitirat, W., and Gritsanapan, W. 2008. Quantitative analysis of total mangostins in *Garcinia mangostana* fruit rind. *Journal of Health Reseach* 22 (4): 161-166.

Price, K.R., Casuscelli, F., Colquhoun, I.J., and Rhodes, M.J.C. 1998. Composition and content of flavonol glycosides in broccoli florets (*Brassica olearacea*) and their fate during cooking. *Journal of the Science of Food and Agriculture* 77 (4): 468-472.

Qian, J., *et al*. 2010. Ophiopogonin D prevents H_2O_2-induced injury in primary human umbilical vein endothelial cells. *Journal of Ethnopharmacology* 128 (2): 438-445.

Reddy, M.B., Reddy, K.B., and Reddy, M.N. 1989. A survey of plant crude drugs of Anantapur district, Andhra Pradesh, India. *International Journal of Crude Drug Research* 27 (3): 145-155.

Repine, J.E., Eaton, J.W., and Anders, M.W. 1979. Generation of hydroxyl radical by enzymes, chemicals, and human phagocytes in vitro. Detection with the anti-inflammatory agent, dimethyl sulfoxide. *Journal of Clinical Investigation* 64 (6): 1642-1651.

Robak, J., and Gryglewski, R.J. 1988. Flavonoids are scavengers of superoxide anions. *Biochemical Pharmacology* 37 (5): 837-841.

Roland, A., Patterson, R.A., and Leake, D.S. 2001. Measurement of copper-binding sites on low density lipoprotein. *Arteriosclerosis, Thrombosis, and Vascular Biology* 21 (4): 594-602.

Sandoval, M., *et al*. 2002. Anti-inflammatory and antioxidant activities of cat's claw (*Uncaria tomentosa* and *Uncaria guianensis)* are independent of their alkaloid content. *Phytomedicine* 9 (4): 325-337.

Sarabia, M., *et al*. 2001. Calcium dobesilate versus purified flavonoid fraction of diosmin in the treatment of hemorrhoidal crises: A randomized, controlled study with an initial double-blind, double-dummy period. *Current Therapeutic Research - Clinical and Experimental* 62 (7): 524-529.

Segsunviriya, C., and Choomprabutra, S. 1989. A clinical study of *Cissus quadrangularis* Linn. in hemorrhoid patients. In: Seminar of Research and Development of Medicinal Plant. Division of Medical Research, Department of Medical Sciences, Ministry of Public Health, Thailand, pp. 54–55.

Shi, J., Gong, J., Liu, J., Wu, X., and Zhang, Y. 2009. Antioxidant capacity of extract from edible flowers of *Prunus mume* in China and its active components. *LWT - Food Science and Technology* 42 (2): 477-482.

Shirai, M., Yamanishi, R., Moon, J.-H., Murota, K., and Terao, J. 2002. Effect of quercetin and its conjugated metabolite on the hydrogen peroxide-induced intracellular production of reactive oxygen species in mouse fibroblasts. *Bioscience, Biotechnology and Biochemistry* 66 (5): 1015-1021.

Shirwaikar, A., Khan, S., and Malini, S. 2003. Antiosteoporotic effect of ethanol extract of *Cissus quadrangularis* Linn. on ovariectomized rat. *Journal of Ethnopharmacology* 89 (2-3): 245-250.

Siekmeier, R., Steffen, C., and März, W. 2007. Role of oxidants and antioxidants in atherosclerosis: results of in vitro and in vivo investigations. *Journal of Cardiovascular Pharmacology and Therapeutics* 12 (4): 265-282.

Singh, G., Rawat, P., and Maurya, R. 2007. Constituents of *Cissus quadrangularis*. *Natural Product Research* 21 (6): 522-528.

Spanier, G. et al. 2009. Resveratrol reduces endothelial oxidative stress by modulating the gene expression of superoxide dismutase 1 (SOD1), glutathione peroxidase 1 (GPX1) and NADPH oxidase subunit (NOX4). *Journal of Physiology and Pharmacology* 60 (4): 111-116.

Spiteller, M., Özen, T., Smelcerovic, A., Zuehlke, S., and Mimica-Dukić, N. 2008. Phenolic constituents and the in vitro antioxidant activity of the flowers of *Hypericum venustum*. *Fitoterapia* 79 (3): 191-193.

Srisook, K., Palachot, M., Mongkol, N., Srisook, E., and Sarapusit, S. 2011. Anti-inflammatory effect of ethyl acetate extract from *Cissus quadrangularis* Linn may be involved with induction of heme oxygenase-1 and suppression of NF-κB activation. *Journal of Ethnopharmacology* 133 (3): 1008-1014.

Taguchi, H., Ogura, Y., Takanashi, T., Hashizoe, M., and Honda, Y. 1996. In vivo quantitation of peroxides in the vitreous humor by flurophotometry. *Investigative Ophthalmology and Visual Science* 37 (7): 1444-1450.

Thakur, A.K., Jain, V., Hingorani, L., and Laddha, K.S. 2009. Improved high-performance liquid chromatography-DAD method for the simultaneous analysis of quercetin and kaempferol in the stems of *Cissus quadrangularis* Linn. *Acta Chromatographica* 21 (1): 95-103.

Thiangtham, J. 2003. Determination of antioxidant constituents in medicinal plant *Cissus quadrangularis* Linn. M. Sc. Thesis. Department of Pharmaceutical Chemistry. Faculty of Pharmaceutical Sciences. Chulalongkorn University.

Trapp, V., Parmakhtiar, B., Papazian, V., Willmott, L. and Fruehauf, J.P. 2010. Anti-angiogenic effects of resveratrol mediated by decreased VEGF and increased TSP1 expression in melanoma-endothelial cell co-culture. *Angiogenesis*. Article in Press.

Valentão, P., *et al*. 2001. Antioxidant activity of *Centaurium erythraea* infusion evidenced by its superoxide radical scavenging and xanthine oxidase inhibitory activity. *Journal of Agricultural and Food Chemistry* 49 (7): 3476-3479.

Valko, M., *et al*. 2007. Free radicals and antioxidants in normal physiological functions and human disease. *International Journal of Biochemistry and Cell Biology* 39 (1): 44-84.

Vanhoutte, P.M., Shimokawa, H., Tang, E.H.C., and Feletou, M. 2009. Endothelial dysfunction and vascular disease. *Acta Physiologica* 196 (2): 193-222.

Villaño, D., Fernández-Pachón, M.S., Moyá, M.L., Troncoso, A.M., and García-Parrilla, M.C. 2007. Radical scavenging ability of polyphenolic compounds towards DPPH free radical. *Talanta* 71 (1): 230-235.

Wagner, C., *et al*. 2006. Quercitrin, a glycoside form of quercetin, prevents lipid peroxidation in vitro. *Brain Research* 1107 (1): 192-198.

Wali, M.A., Suleiman, S.A., Kadoumi, O.F., and Nasr, M.A. 2002. Superoxide radical concentration and superoxide dismutase (SOD) enzyme activity in varicose veins. *Annals of Thoracic and Cardiovascular Surgery* 8 (5): 286-290.

Wallerath, T., *et al*. 2002. Resveratrol, a polyphenolic phytoalexin present in red wine, enhances expression and activity of endothelial nitric oxide synthase. *Circulation* 106 (13): 1652-1658.

Wang, B., Peng, L., Zhu, L., and Ren, P. 2007. Protective effect of total flavonoids from *Spirodela polyrrhiza* (L.) Schleid on human umbilical vein endothelial cell damage induced by hydrogen peroxide. *Colloids and Surfaces B: Biointerfaces* 60 (1): 36-40.

Wang, H., and Joseph, J.A. 1999. Quantifying cellular oxidative stress by dichlorofluorescein assay using microplate reader. *Free Radical Biology and Medicine* 27 (5-6): 612-616.

Wang, Y.K., and Huang, Z.Q. 2005. Protective effects of icariin on human umbilical vein endothelial cell injury induced by H_2O_2 in vitro. *Pharmacological Research* 52 (2): 174-182.

Wassmann, S., Wassmann, K., and Nickenig, G. 2004. Modulation of oxidant and antioxidant enzyme expression and function in vascular cells. *Hypertension* 44 (4): 381-386.

Weissmann, G., Smolen, J.E., and Korchak, H.M. 1980. Release of inflammatory mediators from stimulated neutrophils. *New England Journal of Medicine* 303 (1): 27-34.

Willcox, J.K., Ash, S.L., and Catignani, G.L. 2004. Antioxidants and prevention of chronic disease. *Critical Reviews in Food Science and Nutrition* 44 (4): 275-295.

Wink, D.A., and Mitchell, J.B. 1998. Chemical biology of nitric oxide: insights into regulatory, cytotoxic, and cytoprotective mechanisms of nitric oxide. *Free Radical Biology and Medicine* 25 (4-5): 434-456.

Xia, Z., *et al*. 2006. *N*-acetylcysteine attenuates TNF-α-induced human vascular endothelial cell apoptosis and restores eNOS expression. *European Journal of Pharmacology* 550 (1-3): 134-142.

Yan, X., Murphy, B.T., Hammond, G.B., Vinson, J.A., and Neto, C.C. 2002. Antioxidant activities and antitumor screening of extracts from Cranberry fruit (*Vaccinium macrocarpon*). *Journal of Agricultural and Food Chemistry* 50 (21): 5844-5849.

Yokomizo, A., and Moriwaki, M. 2006. Effects of uptake of flavonoids on oxidative stress induced by hydrogen peroxide in human intestinal Caco-2 cells. *Bioscience, Biotechnology and Biochemistry* 70 (6): 1317-1324.

Yousef, M.I., Awad, T.I., Elhag, F.A., and Khaled, F.A. 2007. Study of the protective effect of ascorbic acid against the toxicity of stannous chloride on oxidative damage, antioxidant enzymes and biochemical parameters in rabbits. *Toxicology* 235 (3): 194-202.

Zhang, R., *et al*. 2010. Cytoprotective effect of the fruits of *Lycium chinense* Miller against oxidative stress-induced hepatotoxicity. *Journal of Ethnopharmacology* 130 (2): 299-306.

APPENDICES

APPENDIX A
PREPARATION OF REAGENTS

Acrylamide Gel

To make 100 mL of 50% acrylamide, 49.2 g of acrylamide and 0.8 g of N, N'-methylenebisacrylamide were dissolved in ultrapure water. The solution was stirred until completely solubilized, then adjusted volume to 100 mL and stored in dark bottles at room temperature.

4X separating buffer (100 mL)

1.5 M Tris-HCl (pH 8.8)

0.4% SDS

Adjust volume with ultrapure water to 100 mL

4X stacking buffer (100 mL)

0.5 M Tris-HCl (pH 6.8)

0.4% SDS

Adjust volume with ultrapure water to 100 mL

Ammonium persulfate (APS) 10% APS in ultrapure

N, N, N', N'-tetremethylenediamine (TEMED)

1. **Preparation of separating gel (main gel)**

To make two plates of acrylamide gel, the ingredients of separating gel were

	Acrylamide gel		
	8%	12%	
Ultrapure water	5.9	5.1	mL
4X separating buffer	2.5	2.5	mL
50% acrylamide	1.6	2.4	mL
10% APS	50	50	µL
TEMED	10	10	µL

The ingredients were thoroughly mixed and immediately pour to the glass plates. Then, DDW was layered on the top of the separating gel (4-5 mm. thick). The gels were leaved for approximately 20-30 min to polymerize.

2. Preparation of stacking gel (top gel)

Since the separating gels has completely polymerized, DDW was removed from the top of the gels. To make stacking gel, the ingredients were

Ultrapure water	2.6	mL
4X stacking buffer	1.0	mL
50% acrylamide	0.4	mL
10% APS	30	µL
TEMED	5	µL

The ingredients were thoroughly mixed and immediately pour to the glass plates. Then, the combs were inserted on the top of the gels. The gels were leaved for approximately 20-30 min to polymerize.

3. Application of samples

Since the stacking gel has completely polymerized, the combs were gently removed. The wells were flushed out with electrophoresis buffer. The chamber was filled out with electrophoresis chamber and loaded samples to the wells.

Bradford reagent

To make 1 liter of Bradford reagent, the ingredients including 50 mg Coomasie Brilliant Blue G-250, 25 mL of methanol and 50 mL of 85% phosphoric acid were mixed and adjusted volume to 500 mL with ultrapure water. The solution was filtrated through Whatman No 93 and kept in tight cap can avoid from light at 4ºC.

Growth medium of ECV304 cells

M199 powder (1 package) was dissolved with ultrapure water and the 2.2 g sodium hydrogen carbonate was added. The medium was mixed and adjusted pH to 7.2 with HCl. The medium was then adjusted volume to 1000 mL and further

sterilized by filtration with 0.22 μm millipore filter membrane. The medium was added with 10% FBS and 0.1% penicillin-streptomycin.

Lysis buffer for Western blot analysis

To make 30 mL of 2X lysis buffer (40 mM Tris HCl (pH 7.4), 300 mM NaCl, 2% Triton X-100, 2% sodium deoxycholate, 20 mM NaF, 2 mM Pefabloc, 2 mM sodium orthovanadate) for stock solution, the ingredients were dissolved in ultrapure water. The solution was adjusted volume to 30 mL. Before use, 990 μL of the solution was added with 10 μL of protease inhibitor.

Phosphate buffered saline (PBS)

The ingredients for 1 liter of PBS, 8 g NaCl, 0.2 g KCl, 0.2 g KH_2PO_4 and 1.15 g Na_2HPO_4, were dissolved in ultrapure water and adjusted the pH to 7.4 with NaOH. The solution was adjusted volume to 1 liter.

Running and transfer solution

To make 1 liter of 10X running and transfer solution (250 mM Tris (pH 8.3), 1.92 M glycine) for stock solution, the ingredients were dissolved in ultrapure water and adjusted volume to 1 liter.

1. **Running buffer for Western blot analysis**

To make 1 liter of 1X running buffer, 100 mL of 10X running and transfer solution was mixed with 10 mL of 10% SDS. The solution was adjusted volume to 1 liter with ultrapure water.

2. **Transfer buffer for Western blot analysis**

To make 1 liter of 1X transfer buffer, 80 mL of 10X running and transfer solution was mixed with 220 mL of methanol. The solution was adjusted volume to 1 liter with ultrapure water.

Sample buffer for Western blot analysis

To make 50 mL of 5X sample buffer (60 mM Tris-HCl (pH 6.8), 2% SDS, 25% glycerol, 14.4 mM β-mercaptoethanol and 0.1% bromophemol blue) for stock

solution, the ingredients were dissolved in ultrapure water. The solution was adjusted volume to 50 mL. 5X sample buffer was aliquot into 1mL/tube and storeat -20 ºC.

Tris-buffered saline (TBS) for Western blot analysis

To make 1 liter of 10X TBS (100 mM Tris (pH 7.5), 1 M NaCl) for stock solution, 50 mL of 2 M Tris and 87.6 g of NaCl were dissolved in ultrapure water. The solution was adjusted volume to 1 liter.

Tris-buffered saline, 0.05% Tween 20 (TBST)

To make 1 liter of 1X TBST, 100 mL of 10X TBS was mixed with 0.5 mL of Tween 20. The solution was adjusted volume to 1 liter with ultrapure water.

APPENDIX B
TABLES OF EXPERIMENTAL RESULTS

Table 6. Quantitative analysis of quercetin. Each value represented the mean ± SEM of three experiments. Each performed in triplicate.

Quercetin content (ng/lane)	Peak height
200	42.17 ± 0.53
300	108.35 ± 4.72
400	180.17 ± 7.18
500	221.91 ± 7.46

Table 7. The percentage of DPPH scavenging activity of CQES7. Each value represented the mean ± SEM of three experiments. Each performed in triplicate.

CQES7 (mg/mL)	% Scavenging
0.2	34.31 ± 0.51
0.3	47.56 ± 0.11
0.4	55.97 ± 0.78
0.5	70.54 ± 2.34
0.75	80.52 ± 0.70
1	82.13 ± 0.23

Table 8. The percentage of DPPH scavenging activity of CQES48. Each value represented the mean ± SEM of three experiments. Each performed in triplicate.

CQES48 (mg/mL)	% Scavenging
0.1	34.64 ± 0.76
0.2	51.13 ± 0.76
0.3	57.94 ± 1.17
0.4	66.21 ± 0.43
0.5	65.97 ± 0.25
0.6	65.40 ± 0.08

Table 9. The percentage of DPPH scavenging activity of CQER7. Each value represented the mean ± SEM of three experiments. Each performed in triplicate.

CQER7 (mg/mL)	% Scavenging
0.2	25.37 ± 2.38
0.3	36.11 ± 2.27
0.4	44.06 ± 2.71
0.5	57.96 ± 3.00
0.75	71.06 ± 0.56
1	80.41 ± 0.74

Table 10. The percentage of DPPH scavenging activity of CQWS1. Each value represented the mean ± SEM of three experiments. Each performed in triplicate.

CQWS1 (mg/mL)	% Scavenging
0.025	12.89 ± 0.31
0.05	19.38 ± 0.40
0.1	31.18 ± 0.93
0.2	44.02 ± 1.54
0.3	54.13 ± 1.37
0.4	55.36 ± 4.33
0.5	57.75 ± 1.17

Table 11. The percentage of DPPH scavenging activity of CQWF1. Each value represented the mean ± SEM of three experiments. Each performed in triplicate.

CQWF1 (mg/mL)	% Scavenging
0.025	2.14 ± 0.16
0.05	6.2 ± 0.07
0.1	13.79 ± 0.20
0.2	26.72 ± 0.89
0.25	31.84 ± 0.591

Table 12. The EC_{50} values of DPPH scavenging activity of CQ extracts. Each value represented the mean ± SEM of three experiments. Each performed in triplicate.

CQ extracts	EC_{50} (mg/mL)
CQES7	0.333 ± 0.005
CQES48	0.223 ± 0.010
CQER7	0.443 ± 0.030
CQWS1	0.256 ± 0.003

Table 13. The percentage of DPPH scavenging activity of ascorbic acid. Each value represented the mean ± SEM of three experiments. Each performed in triplicate.

Ascorbic acid (μM)	% Scavenging
5	23.55 ± 0.51
10	34.17 ± 0.84
20	49.07 ± 0.74
30	69.89 ± 0.90
40	79.02 ± 0.27
50	82.32 ± 0.46
60	84.76 ± 0.02

Table 14. The percentage of DPPH scavenging activity of quercetin. Each value represented the mean ± SEM of three experiments. Each performed in triplicate.

Quercetin (μM)	% Scavenging
1	9.76 ± 0.14
2	12.78 ± 0.30
4	34.86 ± 0.50
6	52.05 ± 0.90
8	61.74 ± 1.37
12	68.13 ± 0.16
16	69.74 ± 0.16

Table 15. The percentage of DPPH scavenging activity of resveratrol. Each value represented the mean ± SEM of three experiments. Each performed in triplicate.

Resveratrol (μM)	% Scavenging
40	27.68 ± 0.69
80	42.03 ± 0.39
120	53.33 ± 1.25
160	57.33 ± 0.83
200	59.18 ± 0.71
240	64.23 ± 0.71
280	64.64 ± 0.36

Table 16. The EC_{50} values of DPPH scavenging activity of ascorbic acid, quercetin and resveratrol. Each value represented the mean ± SEM of three experiments. Each performed in triplicate.

Samples	EC_{50} (μM)
Ascorbic acid	19.66 ± 0.13
Quercetin	6.23 ± 0.10
Resveratrol	109.7 ± 2.27

Table 17. The percentage of $O_2^{-\bullet}$ scavenging activity of CQ extracts. Each value represented the mean ± SEM of three experiments. Each performed in triplicate.

CQ extracts (mg/mL)	% Scavenging			
	CQES7	CQES48	CQWS1	CQWF1
0.0125	14.14 ± 1.82	22.10 ± 1.75	-	-
0.025	16.68 ± 2.24	21.62 ± 2.39	22.74 ± 1.13	14.81 ± 0.56
0.05	24.11 ± 1.54	29.57 ± 0.90	40.97 ± 1.15	27.77 ± 0.98
0.1	18.65 ± 0.73	12.49 ± 0.70	65.47 ± 0.44	54.30 ± 0.44
0.2	-1.27 ± 0.29	-2.36 ± 1.71	82.83 ± 0.05	74.64 ± 0.25
0.4	-3.75 ± 0.29	-12.02 ± 1.27	91.16 ± 0.07	88.64 ± 0.05
0.6	-7.06 ± 0.29	5.83 ± 7.37	93.45 ± 0.06	92.03 ± 0.05
0.8	-11-70 ± 0.33	-2.48 ± 0.33	95.68 ± 0.05	92.96 ± 0.13
1	-13.30 ± 0.51	2.42 ± 0.48	95.84 ± 0.08	92.87 ± 0.07

Table 18. The EC_{50} values of $O_2^{-\bullet}$ scavenging activity of CQ extracts. Each value represented the mean ± SEM of three experiments. Each performed in triplicate.

CQ extracts	EC_{50} (mg/mL)
CQWS1	0.068 ± 0.004
CQWF1	0.086 ± 0.007

Table 19. The percentage of $O_2^{-\bullet}$ scavenging activity of quercetin and resveratrol. Each value represented the mean ± SEM of three experiments. Each performed in triplicate.

Samples (µM)	% Scavenging	
	Quercetin	Resveratrol
12.5	39.39 ± 0.98	20.26 ± 0.30
25	52.24 ± 0.46	25.00 ± 0.85
50	74.07 ± 0.57	35.40 ± 0.41
100	90.03 ± 0.33	46.44 ± 0.23
200	92.58 ± 0.31	52.83 ± 0.43
400	94.45 ± 0.04	62.25 ± 0.25

Table 20. The EC_{50} values of $O_2^{-\bullet}$ scavenging activity of quercetin and resveratrol. Each value represented the mean ± SEM of three experiments. Each performed in triplicate.

Samples	EC_{50} (µM)
Quercetin	23.41 ± 0.62
Resveratrol	159.42 ± 5.97

Table 21. The percentage of H_2O_2 scavenging activity of CQ extracts. Each value represented the mean ± SEM of three experiments. Each performed in triplicate.

CQ extracts (mg/mL)	% Scavenging			
	CQES7	CQES48	CQWS1	CQWF1
0.1	14.29 ± 0.58	12.55 ± 1.73	14.51 ± 0.96	11.55 ± 0.81
0.2	18.04 ± 1.29	27.49 ± 2.36	23.83 ± 0.92	24.95 ± 1.34
0.4	27.30 ± 2.21	34.70 ± 1.66	43.83 ± 1.98	37.16 ± 0.88
0.6	35.69 ± 1.40	47.66 ± 2.33	60.53 ± 2.66	52.87 ± 1.39
0.8	52.28 ± 1.04	57.15 ± 1.78	77.42 ± 1.88	63.78 ± 2.14
1	53.97 ± 0.89	63.93 ± 1.66	85.26 ± 0.21	72.00 ± 1.16

Table 22. The EC_{50} values of H_2O_2 scavenging activity of CQ extracts. Each value represented the mean ± SEM of three experiments. Each performed in triplicate.

CQ extracts	EC_{50} (mg/mL)
CQES7	0.805 ± 0.030
CQES48	0.661 ± 0.034
CQWS1	0.488 ± 0.017
CQWF1	0.587 ± 0.026

Table 23. The percentage of H_2O_2 scavenging activity of ascorbic acid. Each value represented the mean ± SEM of three experiments. Each performed in triplicate.

Ascorbic acid (µM)	% Scavenging
12.5	4.93 ± 0.37
25	15.06 ± 0.74
50	25.31 ± 2.35
100	59.03 ± 3.55
150	75.85 ± 0.48
200	78.28 ± 0.29

Table 24. The percentage of H_2O_2 scavenging activity of quercetin. Each value represented the mean ± SEM of three experiments. Each performed in triplicate.

Quercetin (µM)	% Scavenging
20	34.02 ± 1.15
40	46.73 ± 1.83
60	59.92 ± 1.40
80	68.79 ± 1.43
100	78.61 ± 1.86
120	82.47 ± 1.64

Table 25. The percentage of H_2O_2 scavenging activity of resveratrol. Each value represented the mean ± SEM of three experiments. Each performed in triplicate.

Resveratrol (µM)	% Scavenging
50	15.61 ± 2.15
100	21.64 ± 4.58
200	40.60 ± 1.68
300	45.97 ± 1.13
400	48.04 ± 0.83
500	40.62 ± 2.29

Table 26. The EC_{50} values of H_2O_2 scavenging activity of ascorbic acid, quercetin and resveratrol. Each value represented the mean ± SEM of three experiments. Each performed in triplicate.

Samples	EC_{50} (µM)
Ascorbic acid	87.04 ± 4.91
Quercetin	46.34 ± 2.28

Table 27. The percentage of $OH^{\bullet}$ scavenging activity of CQ extracts. Each value represented the mean ± SEM of three experiments. Each performed in triplicate.

Samples (mg/mL)	% Scavenging			
	CQES7	CQES48	CQWS1	CQWF1
0.1	11.44 ± 2.19	18.19 ± 2.05	28.58 ± 1.84	20.46 ± 3.97
0.2	20.28 ± 2.06	29.98 ± 1.86	40.18 ± 1.34	33.38 ± 3.39
0.4	24.52 ± 0.85	26.75 ± 3.75	56.50 ± 1.09	46.64 ± 2.14
0.6	21.10 ± 1.83	25.34 ± 1.90	72.54 ± 0.49	59.91 ± 1.42
0.8	25.75 ± 3.27	39.17 ± 3.39	86.61 ± 0.06	68.85 ± 1.81
1	-	-	89.90 ± 1.34	76.41 ± 2.00

Table 28. The percentage of $OH^{\bullet}$ scavenging activity of mannitol. Each value represented the mean ± SEM of three experiments. Each performed in triplicate.

Mannitol (mg/mL)	% Scavenging
0.1	26.45 ± 1.26
0.2	34.58 ± 0.77
0.4	41.56 ± 0.62
0.6	49.11 ± 0.36
0.8	56.90 ± 0.57
1	65.96 ± 0.33

Table 29. The EC_{50} values of $OH^{\bullet}$ scavenging activity of mannitol and CQ extracts. Each value represented the mean ± SEM of three experiments. Each performed in triplicate.

Samples	EC_{50} (μM)
Mannitol	0.611 ± 0.011
CQWS1	0.329 ± 0.018
CQWF1	0.460 ± 0.036

Table 30. The percentage of $NO^{\bullet}$ scavenging activity of CQ extracts. Each value represented the mean ± SEM of three experiments. Each performed in triplicate.

Samples (mg/mL)	% Scavenging			
	CQES7	CQES48	CQWS1	CQWF1
0.1	32.21 ± 2.87	20.51 ± 1.29	-1.07 ± 0.90	30.16 ± 2.03
0.2	32.26 ± 1.22	28.41 ± 1.94	3.98 ± 0.54	35.23 ± 1.85
0.4	32.90 ± 1.33	34.25 ± 2.14	8.80 ± 1.90	41.93 ± 0.25
0.6	31.89 ± 2.04	32.31 ± 3.21	2.83 ± 2.54	47.46 ± 1.30
0.8	25.22 ± 0.77	28.90 ± 3.49	1.28 ± 1.37	50.16 ± 2.02
1	20.69 ± 2.76	23.39 ± 4.04	-3.23 ± 2.62	54.26 ± 0.72

Table 31. The percentage of $NO^{\bullet}$ scavenging activity of quercetin. Each value represented the mean ± SEM of three experiments. Each performed in triplicate.

Quercetin (μM)	% Scavenging
25	38.51 ± 2.48
50	44.18 ± 1.75
100	50.96 ± 1.24
200	56.57 ± 1.40
300	59.92 ± 1.00
400	61.85 ± 1.07

Table 32. The percentage of $NO^{\bullet}$ scavenging activity of resveratrol. Each value represented the mean ± SEM of three experiments. Each performed in triplicate.

Resveratrol (μM)	% Scavenging
10	20.78 ± 1.33
25	30.16 ± 1.31
50	37.80 ± 0.84
75	40.25 ± 1.16
100	42.50 ± 0.55
200	44.09 ± 1.30

Table 33. The percentage of cell viability of CQES48 and CQWS1 at various concentrations for 24 h measured by MTT assay. Each value represented the mean ± SEM of three experiments. Each performed in triplicate. *$p < 0.05$ compared to the untreated control.

Samples (mg/mL)	% Cell viability	
	CQES48	CQWS1
0	100.00 ± 0.00	100.00 ± 0.00
0.5	100.18 ± 3.57	88.94 ± 2.48
1	96.27 ± 4.36	87.80 ± 4.09
2	107.66 ± 3.09	79.97 ± 5.02*
5	88.06 ± 5.75*	58.85 ± 7.46*
8	42.52 ± 2.46*	32.25 ± 5.29*
10	24.84 ± 0.58*	17.25 ± 4.54*

Table 34. The IC_{50} values of CQES48 and CQWS1 treatment on ECV304 cells. Each value represented the mean ± SEM of three experiments. Each performed in triplicate.

CQ extracts	IC_{50} (mg/mL)
CQES48	7.489 ± 0.200
CQWS1	5.852 ± 0.747

Table 35. The percentage of cell viability of quercetin, resveratrol and hesperidin at various concentrations for 24 h measured by MTT assay. Each value represented the mean ± SEM of three experiments. Each performed in triplicate. *$p < 0.05$ compared to the untreated control.

Samples (µM)	% Cell viability		
	Quercetin	Resveratrol	Hesperidin
0	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00
10	101.63 ± 5.69	101.21 ± 4.30	94.71 ± 3.11
20	99.54 ± 4.27	98.33 ± 5.28	90.71 ± 5.41
50	94.39 ± 1.99	93.63 ± 4.94	81.08 ± 3.49*
100	85.54 ± 3.82*	76.16 ± 3.66*	86.30 ± 3.83*
200	64.42 ± 1.82*	54.39 ± 1.25*	81.52 ± 4.77*
400	45.11 ± 1.59*	42.16 ± 2.01*	62.43 ± 4.33*

Table 36. The percentage of cell viability of diosmin at various concentrations for 24 h measured by MTT assay. Each performed in triplicate. *$p < 0.05$ compared to the untreated control.

Samples (µM)	% Cell viability
	Hesperidin
0	100.00 ± 0.00
1	96.50 ± 1.95
2	95.82 ± 2.25
5	88.58 ± 6.86
10	87.94 ± 3.46
20	80.77 ± 4.78*
40	82.10 ± 5.38*

Table 37. The IC_{50} values of quercetin, resveratrol and hesperidin treatment on ECV304 cells. Each value represented the mean ± SEM of three experiments. Each performed in triplicate.

Sample	IC_{50} (µM)
Quercetin	333.16 ± 12.90
Resveratrol	246.37 ± 8.80

Table 38. The percentage of cell viability of H_2O_2 at various concentrations for 24 h measured by MTT assay. Each value represented the mean ± SEM of three experiments. Each performed in triplicate. *p < 0.05 compared to the untreated control.

H_2O_2 (µM)	% Cell viability			
	0.5 h	6 h	12 h	24 h
0	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00
12.5	98.10 ± 1.12	92.79 ± 3.08	93.97 ± 1.44	87.31 ± 5.91
25	92.49 ± 2.57*	84.08 ± 5.74*	80.37 ± 4.94*	72.34 ± 9.12*
50	74.48 ± 2.13*	66.82 ± 6.20*	54.91 ± 1.70*	41.49 ± 13.24*
100	61.15 ± 1.43*	30.44 ± 6.42*	23.01 ± 2.16*	20.30 ± 9.79*
200	58.70 ± 0.50*	23.71 ± 8.88*	20.40 ± 5.10*	10.44 ± 0.59*

Table 39. The IC_{50} values of H_2O_2 treatment on ECV304 cells for various time points. Each value represented the mean ± SEM of three experiments. Each performed in triplicate.

Time (h)	IC_{50} (μM)
6	71.27 ± 1.81
12	50.40 ± 0.53
24	41.73 ± 14.30

Table 40. The percentage of cell viability of pretreatment with CQES48 and CQWS1 for 24 h prior to 100 μM H_2O_2 for 0.5 h measured by MTT assay. Each value represented the mean ± SEM of three experiments. Each performed in triplicate. *p < 0.05 compared to the untreated control.

Samples (mg/mL)	% Cell viability	
	CQES48	CQWS1
Control	100.00 ± 0.00	100.00 ± 0.00
0	88.28 ± 1.44	83.23 ± 1.76
0.05	81.60 ± 5.46	73.40 ± 5.22
0.1	76.65 ± 3.27	79.99 ± 1.69
0.2	74.29 ± 4.46	78.52 ± 2.05
0.5	76.85 ± 2.45	77.80 ± 2.68
1	80.49 ± 2.42	78.36 ± 1.46

Table 41. The percentage of cell viability of pretreatment with CQES48 and CQWS1 for 24 h prior to 100 µM H_2O_2 for 2 h measured by MTT assay. Each value represented the mean ± SEM of three experiments. Each performed in triplicate.

Samples (mg/mL)	% Cell viability	
	CQES48	CQWS1
Control	100.00 ± 0.00	100.00 ± 0.00
0	74.90 ± 1.32	74.30 ± 2.57
0.05	69.72 ± 1.57	70.31 ± 4.02
0.1	73.66 ± 0.47	83.11 ± 3.60
0.2	69.86 ± 2.81	83.34 ± 4.94
0.5	73.94 ± 4.84	82.19 ± 7.45
1	77.02 ± 2.29	79.30 ± 4.22

Table 42. The percentage of cell viability of pretreatment with CQES48 and CQWS1 for 24 h prior to 100 µM H_2O_2 for 6 h measured by MTT assay. Each value represented the mean ± SEM of three experiments. Each performed in triplicate.

Samples (mg/mL)	% Cell viability	
	CQES48	CQWS1
Control	100.00 ± 0.00	100.00 ± 0.00
0	65.42 ± 2.06	65.42 ± 2.06
0.05	70.61 ± 1.82	69.08 ± 3.40
0.1	68.16 ± 2.34	70.00 ± 3.34
0.2	67.77 ± 2.32	70.12 ± 2.71
0.5	58.33 ± 2.12	68.60 ± 3.53
1	62.69 ± 1.07	68.06 ± 2.74

Table 43. The percentage of cell viability of pretreatment with quercetin and resveratrol for 24 h prior to 100 µM H_2O_2 for 0.5 h measured by MTT assay. Each value represented the mean ± SEM of three experiments. Each performed in triplicate.

Samples (µM)	% Cell viability	
	Quercetin	Resveratrol
Control	100.00 ± 0.00	100.00 ± 0.00
0	83.23 ± 1.76	83.23 ± 1.76
0.05	75.21 ± 1.84	76.79 ± 2.82
0.1	83.19 ± 1.43	78.20 ± 0.55
0.2	78.07 ± 0.50	78.95 ± 1.36
0.5	75.98 ± 1.25	76.05 ± 2.23
1	76.26 ± 0.91	74.69 ± 3.43

Table 44. The percentage of cell viability of pretreatment with quercetin and resveratrol for 24 h prior to 100 µM H_2O_2 for 2 h measured by MTT assay. Each value represented the mean ± SEM of three experiments. Each performed in triplicate.

Samples (µM)	% Cell viability	
	Quercetin	Resveratrol
Control	100.00 ± 0.00	100.00 ± 0.00
0	74.30 ± 2.57	74.30 ± 2.57
0.05	80.97 ± 6.63	77.84 ± 4.62
0.1	83.26 ± 4.67	84.44 ± 2.69
0.2	85.30 ± 4.59	82.94 ± 4.72
0.5	83.45 ± 6.27	78.88 ± 5.04
1	77.09 ± 4.57	77.51 ± 3.11

Table 45. The percentage of cell viability of pretreatment with quercetin and resveratrol for 24 h prior to 100 μM H_2O_2 for 6 h measured by MTT assay. Each value represented the mean ± SEM of three experiments. Each performed in triplicate.

Samples (μM)	% Cell viability	
	Quercetin	Resveratrol
Control	100.00 ± 0.00	100.00 ± 0.00
0	65.42 ± 2.06	65.42 ± 2.06
0.05	63.57 ± 2.41	65.00 ± 1.33
0.1	71.55 ± 2.13	68.43 ± 3.99
0.2	67.79 ± 5.63	67.27 ± 4.78
0.5	66.40 ± 2.54	69.55 ± 2.76
1	66.75 ± 1.51	64.40 ± 3.02

Table 46. The percentage of DCF fluorescence of CQES48 and CQWS1 treatment for 24 h measured by DCFH-DA assay. Each value represented the mean ± SEM of three experiments. Each performed in triplicate. $*p < 0.05$ compared to the untreated control.

Samples (mg/mL)	% DCF fluorescence	
	CQES48	CQWS1
0	100.00 ± 0.00	100.00 ± 0.00
0.05	97.86 ± 2.26	91.49 ± 3.49
0.1	92.07 ± 5.41	94.79 ± 4.56
0.2	96.22 ± 2.35	92.84 ± 3.77
0.5	90.32 ± 7.63	91.53 ± 3.45
1	71.18 ± 2.25*	95.52 ± 3.96

Table 47. The percentage of DCF fluorescence of quercetin, resveratrol, hesperidin and diosmin treatment for 24 h measured by DCFH-DA assay. Each value represented the mean ± SEM of three experiments. Each performed in triplicate. *p < 0.05 compared to the untreated control.

Samples (µM)	% DCF fluorescence			
	Quercetin	Resveratrol	Hesperidin	Diosmin
0	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00
1	99.23 ± 2.58	94.67 ± 4.50	106.45 ± 2.48	108.41 ± 7.62
5	88.48 ± 3.86	104.73 ± 5.73	109.71 ± 5.73	101.66 ± 7.25
10	85.38 ± 6.40	98.79 ± 5.27	107.00 ± 3.26	99.02 ± 7.35
20	79.27 ± 5.41	97.92 ± 7.35	110.72 ± 0.48	96.87 ± 2.60
50	79.50 ± 4.88*	92.97 ± 6.99	114.03 ± 1.23	-

Table 48. The percentage of DCF fluorescence of H_2O_2 treatment for 24 h measured by DCFH-DA assay in concentration- and time-dependent manner. Each value represented the mean ± SEM of three experiments. Each performed in triplicate. *p < 0.05 compared to the untreated control.

H_2O_2 (µM)	% DCF fluorescence				
	0.5 h	2 h	6 h	12 h	24 h
0	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00
12.5	114.33 ± 1.31*	110.29 ± 3.29	97.55 ± 3.32	99.28 ± 1.20	121.58 ± 2.51
25	122.50 ± 1.08*	115.07 ± 1.13	95.47 ± 1.62	96.06 ± 2.89	116.04 ± 0.44
50	151.05 ± 2.18*	136.07 ± 5.80	99.10 ± 6.07	94.78 ± 3.73	107.16 ± 0.62
100	177.55 ± 2.39*	161.72 ± 4.86	107.19 ± 6.08	101.71 ± 4.33	106.55 ± 3.78
200	194.90 ± 2.73*	178.06 ± 7.32	118.70 ± 7.42	108.99 ± 9.95	119.22 ± 1.85

Table 49. The percentage of DCF fluorescence of pretreatment with CQES48 and CQWS1 for 24 h prior to 100 µM H_2O_2 for 0.5 h measured by DCFH-DA assay. Each value represented the mean ± SEM of three experiments. Each performed in triplicate. *$p < 0.05$ compared to the untreated control.

Samples (mg/mL)	% DCF fluorescence	
	CQES48	CQWS1
Control	100.00 ± 0.00	100.00 ± 0.00
0	204.21 ± 10.72#	204.21 ± 10.72#
0.05	191.28 ± 11.08	187.49 ± 10.36
0.1	172.64 ± 8.82*	196.08 ± 11.49
0.2	153.03 ± 5.57*	180.98 ± 10.55
0.5	128.57 ± 3.05*	181.84 ± 12.32
1	92.18 ± 9.61*	167.13 ± 16.59*

Table 50. The percentage of DCF fluorescence of quercetin, resveratrol, hesperidin and diosmin treatment for 24 h prior to 100 µM H_2O_2 for 0.5 h measured by DCFH-DA assay. Each value represented the mean ± SEM of three experiments. Each performed in triplicate. *$p < 0.05$ compared to the untreated control.

Samples (µM)	% DCF fluorescence			
	Quercetin	Resveratrol	Hesperidin	Diosmin
Control	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00
0	204.21 ± 10.72#	204.21 ± 10.72#	204.21 ± 10.72#	204.21 ± 10.72#
1	194.37 ± 5.43	189.54 ± 1.38	192.26 ± 5.72	195.98 ± 7.77
5	151.89 ± 3.37*	159.65 ± 3.49*	187.27 ± 18.26	178.67 ± 1.24
10	132.64 ± 4.12*	149.18 ± 12.04*	177.39 ± 7.94	161.41 ± 0.79*
20	114.88 ± 4.40*	139.86 ± 13.75*	175.12 ± 6.79	151.78 ± 4.83*
50	105.87 ± 1.87*	126.69 ± 12.68*	167.92 ± 17.31	-

Table 51. The percentage of reduction of fluorescence signal by CQES48 and CQWS1 treatment for 24 h prior to 100 μM H_2O_2 for 0.5 h.

Samples (mg/mL)	% Reduction of fluorescence signal	
	CQES48	CQWS1
0	0.00 ± 0.00	0.00 ± 0.00
0.05	12.92 ± 6.63	29.72 ± 6.04
0.1	31.56 ± 8.62	21.13 ± 4.58
0.2	51.18 ± 9.41	36.23 ± 11.11
0.5	75.63 ± 3.05	35.37 ± 4.49
1	112.02 ± 11.93	50.09 ± 3.42

Table 52. The relative ratio of CQES48 on protein expressions of antioxidant enzymes in ECV304 cells normalized by β-actin and quantitated by Western blot analysis. Each value represented the mean ± SEM of three experiments. Each performed in triplicate. *$p < 0.05$ compared to the untreated control.

CQES48 (mg/mL)	Relative ratio			
	Cu/Zn-SOD	Mn-SOD	GPx	CAT
Control	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00
0.05	1.12 ± 0.02*	0.98 ± 0.03	1.01 ± 0.04	1.03 ± 0.05
0.1	1.20 ± 0.05*	1.07 ± 0.04*	1.05 ± 0.05	1.04 ± 0.04
0.5	1.23 ± 0.05*	1.16 ± 0.02*	1.12 ± 0.03*	1.00 ± 0.04
1	1.26 ± 0.00*	1.22 ± 0.01*	1.17 ± 0.02*	0.97 ± 0.05

Table 53. The relative ratio of quercetin and resveratrol on protein expressions of antioxidant enzymes in ECV304 cells normalized by β-actin and quantitated by Western blot analysis. Each value represented the mean ± SEM of three experiments. Each performed in triplicate. $^{\#}p < 0.05$ compared to the untreated control. $^{*}p < 0.05$ compared to the untreated control.

Samples (μM)	Relative ratio			
	Cu/Zn-SOD	Mn-SOD	GPx	CAT
Control	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00
Quercetin (5 μM)	0.98 ± 0.02	1.09 ± 0.02*	1.05 ± 0.07	1.02 ± 0.01
Quercetin (50 μM)	1.10 ± 0.00*	1.18 ± 0.02*	1.22 ± 0.03*	1.07 ± 0.01*
Resveratrol (5 μM)	1.00 ± 0.06	1.15 ± 0.02*	1.18 ± 0.03*	1.09 ± 0.01*
Resveratrol (50 μM)	0.98 ± 0.05	1.29 ± 0.01*	1.24 ± 0.03*	1.02 ± 0.03

Table 54. The relative ratio of CQES48 on protein expressions of antioxidant enzymes in H_2O_2-treated ECV304 cells normalized by β-actin and quantitated by Western blot analysis. Each value represented the mean ± SEM of three experiments. Each performed in triplicate. $^{\#}p < 0.05$ compared to the untreated control. $^{*}p < 0.05$ compared to the untreated control.

CQES48 (mg/mL)	Relative ratio			
	Cu/Zn-SOD	Mn-SOD	GPx	CAT
Control	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00
0	0.76 ± 0.03$^{\#}$	0.83 ± 0.04$^{\#}$	0.87 ±0.01$^{\#}$	1.04 ±0.02
0.05	0.83 ± 0.03	0.83 ± 0.05	0.95 ±0.05	1.01 ±0.03
0.1	0.91 ± 0.04	0.89 ± 0.09	1.00 ±0.05	1.01 ±0.05
0.5	1.00 ± 0.07*	1.01 ± 0.04*	1.09 ±0.02*	1.00 ±0.04
1	1.03 ± 0.10*	1.17 ± 0.06*	1.22 ±0.04*	1.00 ± 0.05

Table 55. The relative ratio of quercetin and resveratrol on protein expressions of antioxidant enzymes in H_2O_2-treated ECV304 cells normalized by β-actin and quantitated by Western blot analysis. Each value represented the mean ± SEM of three experiments. Each performed in triplicate. $^{\#}p < 0.05$ compared to the untreated control. $^{*}p < 0.05$ compared to the untreated control.

Samples (μM)	Relative ratio			
	Cu/Zn-SOD	Mn-SOD	GPx	CAT
Control	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00
0	0.83 ± 0.02$^{\#}$	0.80 ± 0.02$^{\#}$	0.82 ± 0.01$^{\#}$	0.95 ± 0.02
Quercetin 5 μM	0.98 ± 0.01*	0.95 ± 0.02*	0.98 ± 0.04*	1.01 ± 0.03
Quercetin 50 μM	1.00 ± 0.03*	1.09 ± 0.02*	1.12 ±0.00*	0.94 ± 0.00
Resveratrol 5 μM	0.97 ± 0.02*	0.99 ± 0.01*	1.08 ± 0.01*	1.03 ± 0.05
Resveratrol 50 μM	1.05 ±0.01*	1.20 ± 0.04*	1.27 ± 0.02*	1.05 ± 0.05

Table 56. The relative ratio of CQES48 on protein expressions of eNOS in ECV304 cells normalized by β-actin and quantitated by Western blot analysis. Each value represented the mean ± SEM of three experiments. Each performed in triplicate.

Samples (μM)	Relative ratio
	eNOS
Control	1.00 ± 0.00
0.05	0.98 ± 0.02
0.1	1.10 ± 0.08
0.5	1.26 ± 0.10
1	1.16 ± 0.21

Table 57. The relative ratio of CQES48 on protein expressions of eNOS and iNOS in H_2O_2-treated ECV304 cells normalized by β-actin and quantitated by Western blot analysis. Each value represented the mean ± SEM of three experiments. Each performed in triplicate. $^{\#}p < 0.05$ compared to the untreated control. $^{*}p < 0.05$ compared to the untreated control.

Samples (μM)	Relative ratio	
	eNOS	iNOS
Control	1.00 ± 0.00	1.00 ± 0.00
0	0.79 ± 0.01$^{\#}$	0.96 ± 0.02
0.05	0.96 ± 0.04	1.02 ± 0.04
0.1	1.08 ± 0.04	1.07 ± 0.01
0.5	1.16 ± 0.01*	1.00 ± 0.04
1	1.29 ± 0.05*	0.97 ± 0.04

VITA

Mr. Tarat Sapsrithong was born on December 15, 1985 in Bangkok, Thailand. He received his Bachelor of Science from the Faculty of Science, Chulalongkorn University in 2008. Since graduation, he entered the Master's degree program in Biomedicinal Chemistry at the Faculty of Pharmaceutical Sciences, Chulalongkorn University.