ฤทธิ์ต้านออกซิเดชันและฤทธิ์ปกป้องเซลล์ของสารสกัดเปลือกเมล็ดมะขาม

นางสาวอรนุช นาคชาติ

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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ANTIOXIDANT AND CYTOPROTECTIVE EFFECTS OF *TAMARINDUS INDICA* SEED COAT EXTRACTS

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A Dissertation Submitted in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy Program in Biomedicinal Chemistry
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อรนุช นาคชาติ : ฤทธิ์ต้านออกซิเดชันและฤทธิ์ปกป้องเซลล์ของสารสกัดเปลือกเมล็ดมะขาม (ANTIOXIDANT AND CYTOPROTECTIVE EFFECT OF *TAMARINDUS INDICA* SEED COAT EXTRACTS) อ. ที่ปรึกษาวิทยานิพนธ์หลัก : รศ.ภญ.ดร.สุนันท์ พงษ์สามารถ, อ. ที่ปรึกษาวิทยานิพนธ์ร่วม : รศ.ภญ.ดร. ดวงเดือน เมฆสุริเยนทร์, 181 หน้า.

งานวิจัยนี้มีวัตถุประสงค์เพื่อศึกษาฤทธิ์ของสารสกัดเปลือกเมล็ดมะขามในการต้านออกซิเดชันและฤทธิ์ในการปกป้องเซลล์ไฟโปรบลาส์ต ซีซีดี1064เอสเค ที่เหนี่ยวนำให้เกิดออกซิเดชันด้วยไฮโดรเจนเปอร์ออกไซด์ (H_2O_2) ทดสอบฤทธิ์ต้านอนุมูลอิสระในหลอดทดลองของสารสกัดเปลือกเมล็ดมะขามครั้งที่สกัดด้วยตัวทำละลายที่แตกต่างกัน ได้แก่ สกัดด้วย 70% เอทานอล (TSCE-E) สกัดด้วยเอทิลอะซิเตท (TSCE-EA) สกัดด้วย 70% เอทานอลต่อด้วยคลอโรฟอร์มและเอทิลอะซิเตท (TSCE-EEA) สกัดด้วยน้ำร้อนต่อด้วยเอทิลอะซิเตท (TSCE-W) พบว่า TSCE-W มีสารประกอบฟีนอลิกมากที่สุดและสามารถต้านอนุมูลอิสระ $DPPH^{\bullet}$ และ $ABTS^{\bullet+}$ ได้ดีที่สุด นอกจากนี้ TSCE-W ยังมีฤทธิ์ในการต้านอนุมูลอิสระซุปเปอร์ออกไซด์ แอนอิออน ($O_2^{\bullet-}$) อนุมูลอิสระไฮดรอกซิล ($HO^{\bullet}$) ไฮโดรเจนเปอร์ออกไซด์ และ ไนตริก ออกไซด์ ($NO^{\bullet}$) รวมถึงมีความสามารถในการรีดิวซ์และต้านการเกิดลิพิดเปอร์ออกซิเดชันได้ดีใกล้เคียงกับสารสกัดเปลือกเมล็ดมะขามที่สกัดด้วยตัวทำละลายอื่น ๆ ศึกษาฤทธิ์ต้านอนุมูลอิสระในเซลล์ซีซีดี1064เอสเค พบว่า TSCE-W ที่ความเข้มข้นไม่เกิน 1 มิลลิกรัมต่อมิลลิลิตร ไม่เป็นพิษต่อเซลล์เมื่อทดสอบด้วยวิธี MTT, neutral red, ย้อมด้วย Hoechst 33342 และ trypan blue นอกจากนี้ TSCE-W ที่ความเข้มข้นดังกล่าวไม่ก่อให้เกิดอนุมูลอิสระชนิดออกซิเจน (ROS) และลิพิดเปอร์ออกซิเดชันภายในเซลล์ อีกทั้ง TSCE-W ยังสามารถเพิ่มปริมาณกลูตาไธโอนและเพิ่มการทำงานของเอนไซม์ซุปเปอร์ออกไซม์ดิสมิวเทส (SOD) และคะตะเลส (CAT) ด้วย ศึกษาฤทธิ์ในการปกป้องเซลล์ที่เหนี่ยวนำให้เกิดภาวะเครียดด้วย H_2O_2 พบว่า TSCE-W สามารถลดปริมาณ ROS ลดการเกิดลิพิดเปอร์ออกซิเดชันและเพิ่มปริมาณกลูตาไธโอนภายในเซลล์ที่มีชีวิต ศึกษากลไกการกำจัดอนุมูลอิสระภายในเซลล์ของ TSCE-W ผ่านการแสดงออกของโปรตีนด้วยวิธี Western blot พบว่าเมื่อบ่มเซลล์ด้วย TSCE-W เป็นเวลา 24 ชั่วโมงก่อนเหนี่ยวนำด้วย H_2O_2 เป็นเวลานาน 3 ชั่วโมง TSCE-W สามารถเพิ่มการแสดงออกโปรตีนของเอนไซม์ต้านอนุมูลอิสระ copper, zinc superoxide dismutase (Cu,ZnSOD), glutathione peroxidase (GPx) และ glutathione-*S*-transferase (GST) อย่างมีนัยสำคัญทางสถิติเมื่อเทียบกับกลุ่มควบคุมที่ไม่ได้รับสารสกัดและH_2O_2 และเมื่อเหนี่ยวนำด้วย H_2O_2 เป็นเวลานาน 6 ชั่วโมง พบว่า TSCE-W สามารถควบคุมการแสดงออกของโปรตีนของเอนไซม์ Cu,ZnSOD, GPx และ CAT ให้กลับสู่สมดลเทียบกับกลุ่มควบคุมที่ไม่ได้รับสารสกัดและH_2O_2 ผลการวิจัยสรุปได้ว่า การสกัดด้วยน้ำเดือดเป็นวิธีที่มีราคาถูกและง่าย ได้สารสกัดธรรมชาติของสารต้านอนุมูลอิสระที่มีฤทธิ์ต้านอนุมูลอิสระได้ดีทั้งในหลอดทดลองและภายในเซลล์ สารสกัด TSCE-W สามารถปกป้องเซลล์จากภาวะออกซิเดชันผ่านการควบคุมการแสดงออกของเอนไซม์ต้านอนุมูลอิสระต่าง ๆ สารสกัดมีศักยภาพสามารถนำไปประยุกต์ใช้ในผลิตภัณฑ์อาหารเสริมสุขภาพและผลิตภัณฑ์เวชสำอางได้

ภาควิชา ชีวเคมีและจุลชีววิทยา ลายมือชื่อนิสิต.....

สาขาวิชา ชีวเวชเคมี ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก.....

ปีการศึกษา 2554 ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม.....

5076958233 : MAJOR BIOMEDICINAL CHEMISTRY
KEYWORDS : *TAMARINDUS INDICA* / TAMARIND SEED COAT / ANTIOXIDANT / CYTOPROTECTIVE / SKIN FIBROBLASTS

ORANUCH NAKCHAT : ANTIOXIDANT AND CYTOPROTECTIVE EFFECT OF *TAMARINDUS INDICA* SEED-COAT EXTRACTS. ADVISOR : ASSOC. PROF. SUNANTA PONGSAMART, Ph.D., CO-ADVISOR : ASSOC. PROF. DUANGDEUN MEKSURIYEN, Ph.D., 181 pp.

This study aimed to determine antioxidant activities of *Tamarindus indica* seed coat extracts and evaluate its protective activities against hydrogen peroxide (H_2O_2)-induced oxidative stress in human foreskin fibroblast CCD-1064Sk cells. The roasted seed coat of tamarind was extracted with various solvent systems: 70% ethanol (TSCE-E), ethyl acetate (TSCE-EA), 70% ethanol followed by partition with chloroform and then ethyl acetate (TSCE-EEA), and boiling-water followed by partition with ethyl acetate (TSCE-W). The highest phenolic content was observed in TSCE-W which revealed the free radical scavenging activities in cell-free system. TSCE-W possessed the highest $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ scavenging activities. TSCE-W was also exhibited superoxide anion radical, hydroxyl radical, H_2O_2, and nitric oxide scavenging activities, including reducing power and anti-lipid peroxidation activities comparable with other TSCEs. In cell-based assay, TSCE-W up to 1 mg/mL did not show cytoxicity against CCD-1064Sk cells as determined by various standard assays: MTT, neutral red, Hoechst 33342 staining and trypan blue dye exclusion assay. Cells treated with TSCE-W alone at the non-toxic concentration did not generate reactive oxygen species (ROS) and lipid peroxidation. Additionally, TSCE-W significantly increased intracellular glutathione (GSH) level and the activity of superoxide dismutase (SOD) and catalase (CAT). TSCE-W reduced the generation of ROS and lipid peroxidation as well as increased intracellular GSH level in H_2O_2-treated cells. The antioxidant mechanism of TSCE-W via protein expression was performed using Western blot analysis. The result showed that treatment of TSCE-W before H_2O_2 exposure for 3 h significantly up-regulated the protein expression of antioxidant enzymes including copper, zinc superoxide dismutase (Cu,ZnSOD), glutathione peroxidase (GPx), and glutathione-*S*-transferase (GST) as compared with vehicle control. Treatment of TSCE-W prior to H_2O_2 exposure for 6 h maintained the protein expression of Cu,ZnSOD, GPx, and CAT comparable with vehicle control. The results suggest that an inexpensive and simple boiling-water extraction of TSCE-W may provide a good source of natural antioxidant. TSCE-W protected oxidative stress in cells by the regulation of antioxidant enzymes expression, TSCE-W potentially useful for health food additives and cosmeceuticals.

Department : Biochemistry and Microbiology Student's Signature .....
Field of Study : Biomedicinal Chemistry Advisor's Signature .....
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LIST OF ABBREVIATIONS

$ABTS^{\bullet+}$	2,2/-azino-bis(3-ethylbenzthiazolin)-6-sulfonic acid action radical
AH	A primary antioxidant
ANOVA	Analysis of variance
ARE	Antioxidant-response element
BHA	Butylated hydroxyanisole
BSA	Bovine serum albumin
BTB	Broad complex, tramtrack, and bric-a-brac
CCD-1064Sk	Human foreskin fibroblast cells
Cu, Zn-SOD	Zinc superoxide dismutase
DCF	Dichlorofluorescin
DCFH	Dichlorofluorescin diacetate
DGR	Double glycine repeat
$DPPH^{\bullet}$	2,2-Diphenyl-1-picrylhydrazyl radical
DTNB	Dithiobis-2-nitrobenzoic acid
EC_{50}	Half maximal effective concentration
EC-SOD	Extracellular-SOD
EpRE	Electrophile-responsive element
GAE	Gallic acid equivalents
GCL	Glutamate cysteine ligase
GPx	Glutathione peroxidase
GSH	Glutathione (γ-glutamylcysteinylglycine)
GST	Glutathione-*S*-transferase
H_2O_2	Hydrogen peroxide
HO-1	Heme oxygenase 1
Keap 1	Kelch-like erythorid CNC homologue (ECH)-associated protein 1
$L^{\bullet}$	Allyl radical
$LO^{\bullet}$	Alkoxyl radical
$LOO^{\bullet}$	Lipid peroxyl radical
LOOH	Lipid hydroperoxides
Maf	Musculo-aponeurotic fibrosarcoma

MDA	Malondialdehyde
Mn-SOD	Manganese superoxide dismutase
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
Na_2EDTA	Ethylenediaminetetraacetic acid di-sodium salt
NBT	Nitroblue tetrazolium
NED	Napthylethylenediamine dihydrochloride
$NH_4Fe(SO_4)_2.12H_2O$	Ferrous ammonium sulfate dodecahydrate
$NO^{\bullet}$	Nitric oxide radical
NQO1	Quinine oxidoreductase
NR	Neutral red
Nrf2	Nuclear factor-erythroid 2 p45-related factor
$O_2^{\bullet -}$	Superoxide anion radical
$OH^{\bullet}$	Hydroxyl radical
$ONOO^-$	Peroxynitrite anion
OPC	Oligomeric proanthocyanidin
PBS	Phosphate-buffered saline
RNS	Reactive nitrogen species
$ROO^{\bullet}$	Peroxyl radicals
ROOH	Organic peroxides
ROS	Reactive oxygen species
s	second
SOD	Superoxide dismutase
TAC	Total antioxidant capacity
TBARS	Thiobarbituric acid reactive substance
TEAC	Trolox equivalent antioxidant capacity
TEP	Tetramethoxypropane
TKP	Tamarind seed kernel powder
TNB	Nitro-5-thiobenzoic acid
TSC	Tamarind seed coat
TSCE-E	TSC extracted with 70% ethanol
TSCE-EA	TSC extracted with ethyl acetate
TSCE-EEA	TSC extracted with 70% ethanol/ chloroform/ ethyl acetate

TSCEs	Tamarind seed coat extracts
TSCE-W	TSC extracted with boiling water
TSCP	Tamarind seed coat powder
TSP	Tamarind seed polysaccharide

CHAPTER I
INTRODUCTION

Pollutants, microorganisms, xenobiotic, and stress from work are well known agents that lead to the free radicals in body. The harmful effect of the free radicals is related with biological damage including lipid, protein, and DNA damage, which is involving many diseases such as cancer, cardiovascular disease, atherosclerosis, hypertension, ischemia, diabetes mellitus, neurodegenerative disease, rheumatoid arthritis as well as ageing (Droge, 2002; Sorg, 2004; Valko *et al.*, 2007)**.** Recently, there are reported that the phytochemicals such as phenolic compounds (flavonoid, phenolic acid, tannin, anthocyanins) from various parts of plant having antioxidant, antibacterial, anti-carcinogenesis and anti-cancer properties (Choudary and Swarnkar, 2011) can be used as an ingredient in medicinal, pharmaceutical and health product.

Tamarindus indica L (Leguminosae), commonly known as tamarind, is a tree originally native to Africa. Tamarind is now cultivated in many tropical countries worldwide. In Thailand, there are sweet and sour types of tamarind, it has been widely used in traditional medicine to treate cold, fever, stomach disorder, diarrhea, jaundice, skin cleanser (Farnsworth and Bunyapraphatsara, 1992; Doughari, 2006) and also used as seasoning food. Tamarind seeds have long been used to adulterate coffee (Sarma, Mallick, and Ghosh, 2010). A seed comprises a kernel and seed coat. Tamarind seed kernel powder (TKP) is used as a sizing material in the textile, paper and jute industries (Kumar & Bhattacharya, 2008) while the seed coat, a by-product of the TKP industry, is a rich source of polyphenols and tannins (Tsuda *et al.*, 1994; Pumthong, 1999; Caluwé, Halamová, and Damme, 2010). The phenolic compounds and antioxidant activities of tamarind seed coat (TSC), which can be extracted with various solvent systems, have been reported. The ethyl acetate extract composes of phenolic compounds, such as 2-hydroxy-3$^{/}$, 4$^{/}$-dihydroxyacetophenone, methyl 3,4-dihydroxybenzoate, 3,4-dihydroxyphenyl acetate and (-)-epicatechin and those compounds have strong antioxidant activity (Tsuda *et al*., 1994). The 70% ethanol extract contains polyphenols including tannins, anthrocyanidin and oligomeric anthrocyanidins. The extracts exhibit scavenging activity against peroxyl radicals ($ROO^{\bullet)}$, hydroxyl radical ($OH^{\bullet}$), and superoxide anion radical ($O_2^{\bullet -}$) (Pumthong,

1999) and 95% ethanol extract have also possessed DPPH$^{\bullet}$ scavenging activity.(Maisuthisakul, Suttajit, and Pongsawatmanit, 2007), the methanolic and aqueous acetone extracts of dry-heated of TSC exhibited antioxidant activities on the $O_2^{\bullet-}$, $OH^{\bullet}$, 2,2-diphenyl-1-picrylhydrazyl radical (DPPH$^{\bullet}$) and 2,2$^{/}$-azino-bis(3-ethylbenzthiazolin)-6-sulfonic acid action radical (ABTS$^{\bullet+}$) scavenging assays (Siddhuraju, 2007) and the methanolic extracts from the seed coat of five major tamarind types cultivated in Thailand have also composed of phenolic compounds and exhibited antioxidant properties (Suksomtip, Ukrisdawithid, Bhusawang, and Pongsamart, 2010). Addition, hexane has also used to extract the phenolic compounds and tested antioxidant activities of seed and skin of tamarind (Razali, Mat-Junit, Abdul-Muthalib, Subramaniam, and Abdul-Aziz, 2012).

A number of studies on antioxidant activities of TSC extracted with various solvent extraction methods were widely investigated in cell-free system. However, the antioxidant activities of TSC extracted with water, a cheap and simple solvent, including its toxicity and cytoprotective activity in cell-based system remains unexplored. Therefore, this work is aimed to study antioxidant activity of TSC extracted with boiling water (TSCE-W) to compare with the other organic solvent extracts. The assay using various standard assays in cell-free system and study the antioxidant activities including the protective role of TSCE-W on human foreskin fibroblast CCD-1064Sk cells and its underlying mechanism.

Hypothesis

Tamarind seed coat extracted with boiling water having antioxidant properties and, can be used to protect against oxidative stress in CCD-1064Sk cells induced by hydrogen peroxide (H_2O_2).

Objectives

1. To prepare TSC extracted with various solvent systems.
2. To determine phenolic contents in TSCEs.
3. To compare antioxidant activities of various TSCEs in cell-free system.

4. To study the effect of TSCE, having the highest antioxidant activities on viability and intracellular ROS level in human foreskin fibroblast CCD-1064Sk cells.
5. To study the effect of TSCE on antioxidant enzyme activities and intracellular GSH level in CCD-1064Sk cells and H_2O_2-treated cells.
6. To study an effect of TSCE on protein expression of antioxidant enzymes using Western blot analysis.

Conceptual framework

TSCE from various solvents such as 70% ethanol (Pumthong, 1999); ethyl acetate (Tsuda *et al*., 1994); 70% ethanol partition with chloroform and ethyl acetate, respectively (Suksomtip *et al.*, 2010); and hot water may be have scavenge free radicals and has antioxidant activities by assay in cell-free system. The TSCE which has the highest antioxidant activity was further investigated the antioxidant and protective activities in cell system. The TSCE at non-toxicity dose may be an antioxidant and may be has protective activities, including the regulation of the expression of antioxidant enzymes and nuclear transcription factor Nrf2 in CCD-1064Sk cells.

Scope of the study and experiment design

1. Study the phenolic compounds contents including free radicals scavenging and antioxidant activities such as $DPPH^{\bullet}$, $ABTS^{\bullet+}$, $O_2^{-\bullet}$, $OH^{\bullet}$, H_2O_2, $NO^{\bullet}$ scavenging, reducing power, metal chelating and anti-lipid peroxidation activity of TSCEs, which assay in cell-free system.

2. The TSCE which has the highest antioxidant activity was further used to determine the antioxidant activities including cell viability, intracellular ROS, intracellular glutathione level, antioxidant enzymes activity such as superoxide dismutase, glutathione peroxidase, catalase, including the protein expression of Nrf2, SOD, GPx, CAT, GST and HO-1 in the absence and presence of H_2O_2.

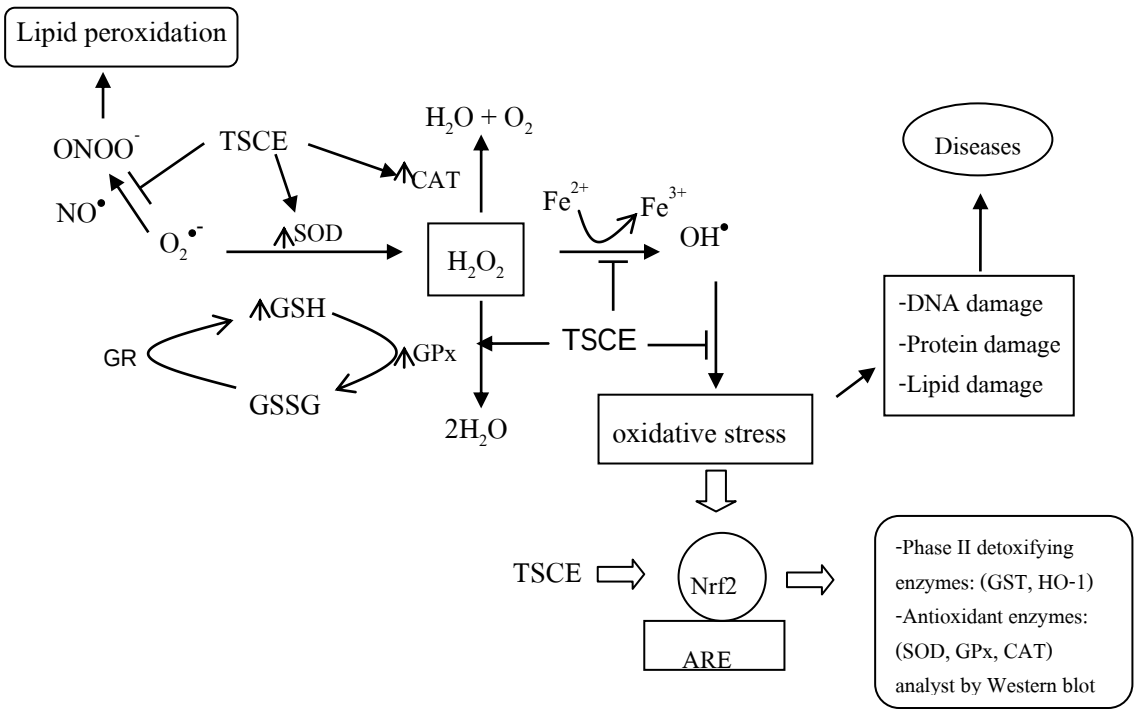


Figure 1. Conceptual framework

Remark:

TSCE = tamarind seed coat extract

TSCE having antioxidant properties will be tested by $O_2^{-\bullet}$, $NO^{\bullet}$ and $OH^{\bullet}$ scavenging assay, chelate Fe^{2+}, reducing power, inhibit lipid peroxidation and increase antioxidant enzymes activity.

TSCE activated expression of phase II detoxifying and antioxidant enzymes.

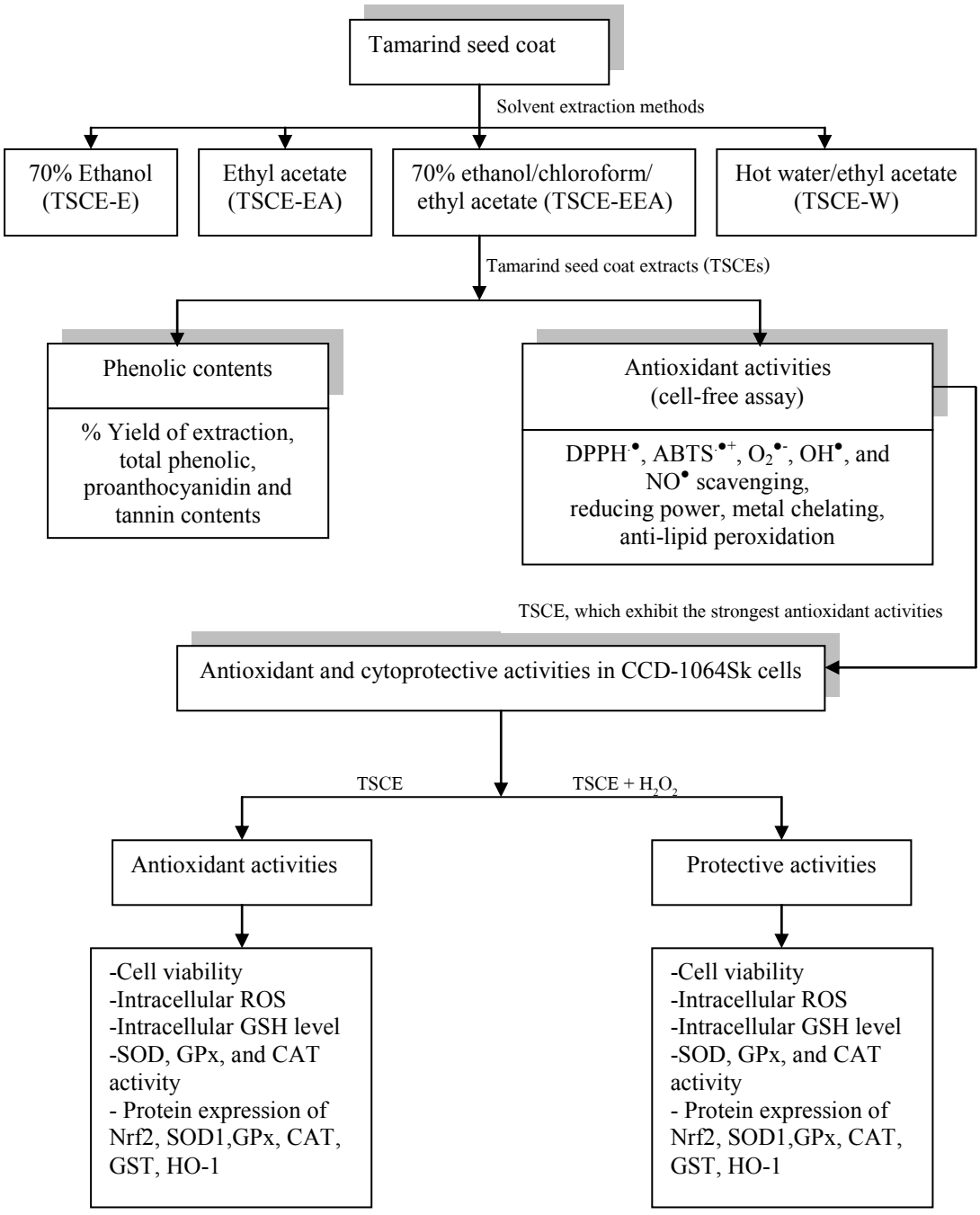


Figure 2. Experimental design

Contributions of the study

1. Know free radicals scavenging and antioxidant activities of TSC by assay in cell-free system.
2. Know the ability of TSC on an antioxidant and the protective activities in CCD-1064 Sk cells.
3. Understanding the mechanism of TSC on the regulation of antioxidant enzymes and cellular defenses expression in CCD-1064 Sk cells.
4. Know the more information about the suitable solvent for using to extract the antioxidative compounds in TSC.
5. TSC can be derived to a new good natural source of antioxidants for neutraceutical and cosmeceutical used.

CHAPTER II
LITERATURE REVIEW

1. Tamarind

Tamarind (*Tamarindus indica* Linn.) is a member of the dicotyledonous family Fabaceae (Leguminosae). It grows in more than 50 countries worldwide. The major areas of production are in Asia countries like India, Bangladesh, Sri Lanka, Thailand and Indonesia, and in the Africa and the American continents (Kumar and Bhattacharya, 2008).

1.1 Scientific classification of tamarind (Soemardji, 2007)

Kingdom	: Plantae
Sub Kingdom	: Tracheobionta
Division	: Spermatophyta
Sub Division	: Magniliophyta
Class	: Magnoliopsida
Family	: Fabaceae
Subfamily	: Caesalpiniaceae
Genus	: *Tamarindus* L.
Species	: *Tamarindus indica* Linn.

1.2 Description and usage of tamarind

1.2.1 Tree

The tamarind tree is a long-lived, large evergreen or semi-green tree, grows wild, through cultivated to a limited extent. A mature tree may attain a maximum height of 30 m. The tamarind tree has the ability to grow in poor soils because of their nitrogen fixing capacity (Kumar and Bhattacharya, 2008).

1.2.2 Leaves and flowers

Tamarind leaves and flowers can be eaten as vegetables and are prepared in a variety of dishes. They are used to make curries, salads, and soup in many countries. Before consumption, leaves are sometimes boiled in water and prepared as tamarind fruit (Caluwé *et al*., 2010).

1.2.3 Fruit pulp

The fruit contains about 55% pulp, 34% seed, and 11% shell and the fiber in a pod. The fruit is pendulous, the pods are oblong or sausage shaped, curved or straight, with rounded ends. The pods contain 1-10 seeds. The shell is light greenish or scruffy brown and minutely scaly, often irregularly constricted between seeds, brittle, and easily broken, if pressed. It is filled with firm soft pulp surrounding the seed cavities. Pulp is thick and blackish-brown in color. The fruit pulp is the chief souring agent for curries, sauces, and certain beverages (Kumar and Bhattacharya, 2008). Tamarind fruit pulp is eaten fresh and often made into a juice, infusion or brine, and also be processed into jam and sweet. Sometime pulp is fermented into an alcoholic beverage (Caluwé *et al.*, 2010).

1.2.4 Seed

The seed are very hard, shiny, reddish, or purplish brown. They are embedded in the pulp and constitutes about 30-34% of whole fruit. Tamarind seed is by-product of the tamarind pulp industry. The seed contain kernel or endosperm (70-80%) and seed coat or testa (20-30%). The major industrial product of tamarind seed is the tamarind kernel powder (TKP) which is an important sizing material used in the textile, paper, and jute industries (Kumar and Bhattacharya, 2008).

1.2.5 Seed kernel

Tamarind decorticated seed kernels contains 46-48% of gel forming substance. Tamarind seed polysaccharide (TSP) (so-called “jellose” or “polyose”) is found to be superior to other methods of fruit preservation. The substance gelatinizes with sugar concentrates even in cold water or milk (Kumar and Bhattacharya, 2008), and has been recommended for used as a stabilizer in ice cream, mayonnaise and cheese, and as an ingredient or agent in a number of pharmaceutical products (Caluwé *et al*., 2010).

1.2.6 Seed coat or testa

The seed coat is removed from kernel either by roasting or by soaking the seed in water followed by drying. The seed coat contains 38-40% of water soluble of which 80% is a mixture of tannins and coloring agents. It can be used in dyeing and tanning of several materials such as leather and textiles (Kumar and Bhattacharya, 2008). The pods, seeds and seed coat of tamarind are shown in Figure 3.

(a)

(b)

(c)

Figure 3. The pods (a), seeds (b) and seed coat (c) of *Tamarindus indica* L. "Priao-Yak".

1.3 Ethnomedicinal usage (Farnsworth and Bunyapraphatsara, 1992)

1.3.1 Roots

Roots are used for wound healing, treatment of herpes simplex infection, as an astringent.

1.3.2 Barks

Barks are used for wound healing, treatment of herpes simplex infection, external ulcers, abscesses and diseases of the oral cavity, as an astringent.

1.3.3 Leaves

Leaves are used for improvement of menstrual blood quality, treatment of coughs, the common cold, conjunctivitis and dimness of vision, as a diaphoretic, anti-dysenteric, laxative, carminative, and expectorant.

1.3.4 Fruit pulps

Fruit pulps are used for improvement of menstrual blood quality, treatment of constipation, coughs, severe disorders of three origins occurring during exposure to open fire after giving birth, abscesses, insect bites, arising in the ear, fever, bleeding from the vagina, as a laxative, expectorant, enema, carminative and blood tonic.

1.3.5 Unripe pods

Unripe pods are used for improvement of menstrual blood quality, as an antiobesity, laxative and antipyretic.

1.3.6 Seeds and seed coat

Seeds are used as an anthelmintic, antidiarrheal, emetic and tonic. Seed coats are used for wound healing, treatment of wounds, burns, and wounds in diabetic patients, as a detoxicant and antidysenteric.

1.4 Chemical and Biological study in *Tamarindus indica*

The chemical and biological studies in various parts of *T. indica* are investigated (Table 1).

1.4.1 The barks and the leaves

The barks and the leaves of *T. indica* were extracted with various solvent extractions possesses antioxidant activities (Romos *et al*., 2003; Choudhary and Swarnkar, 2011; Razali *et al*., 2012) and antimicrobial activities (Muthu, Nandakumar,

and Rao, 2005; Doughari, 2006; Meléndez and Capriles, 2006). The leave extracted with water showed protective activity against paracetamol induced hapatotoxicity in rat (Pimple, Kadam, Badgujar, Bafna, and Patil, 2007). The ethanolic extract of the bark of *T. indica* at low dose was no toxicity and in the other hand, it showed toxicity at a high dose in Brine shrimp and chicken embryos (Nwodo *et al.*, 2011).

1.4.2 Flowers

The flower of tamarind showed strong antioxidant, antimicrobial and cytotoxic activities (Al-Fatimi, Wurster, Schröder, and Lindequist, 2007).

1.4.3 Pulps

The pulp of fruit has strongly acid test, composes of tartaric acid, potassium tartrate, invert sugar, amino acids, fatty acids, and minerals (Glew *et al*., 2005). The extract of pulp exhibited co-stimulatory effects on *N*-nitroso *N*′-methyl urea-induced colonic cell proliferation (Shivshankar and Shyamala Devi, 2004), possesses antioxidant activities, reduced cholesterol in hamster (Martinello *et al*., 2006; Librandi *et al.*, 2007), protective activity against paracetamol induced hapatotoxicity in rat (Pimple *et al.*, 2007), devoid of clastogenic and genotoxic activities (Silva *et al*., 2009), possessed potential antinociceptive activity (Khalid *et al.*, 2010) and prevent free radical against fluoride-induced oxidative stress (Ekambaram *et al.*, 2010).

1.4.4 Seeds

The tamarind seed composes of seed meal (kernels) and seed coat (testa). The seed meal consists of polysaccharide and protein (Savur and Sreenivasan, 1947). The tamarind seed polysaccharide (TSP) had non-toxicity and lack carcinogenicity in $B6C3F_1$ mice (Sano *et al*., 1996), including it showed 77% decreasing a number of *Raillietina'*s egg of *Taenia* helminthes in native chicken (Sukprasert *et al*., 2006) and 1% formulation of TSP exhibited beneficial used to treated dry eye syndrome (Rolando and Valente, 2007). The extract of seed composed of polyphenolic compounds and exhibited antioxidant activities (Soong and Barlow, 2004; Vadivel *et al*., 2011; Razali *et al.*, 2012), anti-snake venom properties (Ushanandini *et al*., 2006). The aqueous extract of the seed of tamarind is decreased hyperglycemia and hyperlipidemia (Maiti, Das, and Ghosh, 2005) and restores pancreatic beta cells in Streptozotocin-induced diabetic rats (Mahmoudzadeh-Sagheb *et al*., 2010).

Moreover, the un-roasted seed extract with water showed protective activity against paracetamol induced hepatotoxicity in rat (Pimple *et al.*, 2007).

1.4.5 Tamarind seed coat

The extract of seed coat of tamarind is studied by many researcher, The first report of Tsuda *et al*. (1994), they reported that the TSCEs consists of four antioxidative compounds such as, 2-hydroxy-3′,4′-dihydroxyacetophenone; methyl 3,4-dihydroxybenzoate; 3,4-dihydroxyphenyl acetate; and (-) epicatechin. The seed coat of tamarind showed antioxidant activities with various assay methods such as, $DPPH^{\bullet}$ scavenging, superoxide anion scavenging, $OH^{\bullet}$ scavenging, and anti-lipid peroxidation (Tsuda *et al*., 1994; Pumthong, 1999; Luengthanaphol *et al*., 2004; Sudjaroen *et al.*, 2005; Siddhuraju, 2007; Razali *et al*., 2012). Beside, the seed coat of tamarind had effects to rumen fermentation activity and exhibited digestibility of nutrients of crossbred diary cows (Bhatta, Krishnamoorthy, and Mohammed, 2001). Kalra *et al.* (2011) reported the methanolic extract of the seed coat of tamarind exhibited antiulcer potential by reduce the total volume of gastric juice, free and total acidity of gastric secretion. Razali *et al*. (2012) studied antioxidant activity of the methanol, ethyl acetate, and hexane extraction of leaves, seeds, veins and skins of *T. indica* from Malasia. They reported that the methanol was the most effective solvent for extraction of antioxidant phenolic compounds.

Table 1. Solvent extracts and bioactivity of various part of *T. indica*

Part (tested)	Solvent extracts	Bioactivity assay	Site of collection	References
Barks	Hydroalcoholic	The extracts showed antimutagenicity and antioxidant activity in $DPPH^{\bullet}$, $HO^{\bullet}$and inhibition of lipid peroxidation	Cuba	Romos *et al*., 2003
Barks	Ethanol	The extract at high dose showed toxicity in *Artemia salina* and chicken embryos and hepatotoxicity in wistar rats	Nigeria	Nwodo *et al.*, 2011
Stem bark, Leaves	80% methanol	The extract showed $DPPH^{\bullet}$and $O_2^{\bullet-}$ scavenging activity	India	Choudhary and Awarnkar, 2011
Leaves	Methanol	The extracts showed antibacterial activity	Puerto Rico	Meléndez and Capriles, 2006
Flowers	Successively extracted with dichloromethane, methanol, and water	The extracts showed $DPPH^{\bullet}$ scavenging, antibacterial and toxicity activities in human amniotic epithelial (FL-cells) cells using neutral red assay	Yemen	Al-Fatimi *et al*., 2007
Pulp	70% ethanol	Tamarind pulp showed hypolipemic, decrease lipid peroxidation, $DPPH^{\bullet}$, and $O_2^{\bullet-}$scavenging	Brazil	Martinello *et al*., 2006
Pulp	70% ethanol	Tamarind pulp can blocked the increase of complementary activity in hamsters submitted to a cholesterol-enrich diet	Brazil	Librandi *et al*., 2007
Pulp	70% ethanol	The extract devoid clastogenic and genotoxic activities in the cells of rodents	Brazil	Silva *et al*., 2009
Pulp	Boiling water	The extract showed antinociceptive activities in rodent	Malasia	Khalid *et al*., 2010
Seed polysaccharide	-	TSP lake carcinogenicity in $B6C3F_1$ mice	Japan	Sano *et al*., 1996

Table 1. Solvent extracts and bioactivity of various part of *T. indica* (cont.)

Part (tested)	Solvent extracts	Bioactivity assay	Site of collection	References
Seed polysaccharide	-	Study tamarind seed polysaccharide in treating dry eye syndrome	Italy	Rolando and Valente, 2007
Seeds	50% ethanol	The extract showed antioxidant activity in ABTS, FRAP and FCR	Singapore	Soong and Barlow, 2004
Seeds	Aqueous (hot water)	The extract attenuated hyperglycemia and hyperlipidemia in STZ-induced diabetic rats	India	Maiti *et al*., 2005
Seeds	95% ethanol	The extract showed anti-snake venom properties	India	Ushanandini *et al*., 2006
Fruits, Leaves, Unrosted seeds	-Marceration and decoction in water	The extracts showed protective effect against paracetamol-induced hepatotoxicity in rat	India	Pimple *et al*., 2007
Seeds	Aqueous (hot water)	The extracts can protect against STZ-induced damages in pancreatic islets in rats	Iran	Mahmoudzadeh-Sagheb *et al*., 2010
Seed, Seed coat	-Ethyl acetate -Ethyl acetate:ethanol (1:1) -Ethanol -Methanol	The ethyl acetated extracts showed antioxidative components and exhibited antioxidant activity in linoleic acid system	Japan	Tsuda *et al*., 1994
Seed coat	Successively extracted with 70% ethanol, chloroform, and the remaining of aqueous fraction was used	TSC consisted of oligomeric proanthocyanidin (OPC) and antioxidant activities against $ROO^{\bullet}$, $O_2^{\bullet-}$, $HO^{\bullet}$ and lipid peroxidation	Thailand	Pumthong, 1999

Table 1. Solvent extracts and bioactivity of various part of *T. indica* (cont.)

Part (tested)	Solvent extracts	Bioactivity assay	Site of collection	References
Seed coat	Successively extracted with 70% ethanol, chloroform, ethyl acetate, and methanol	TSC exhibited NO inhibition in vitro and in vivo testing, and no effect on cell viability using MTT assay	Thailand	Kumutarind *et al*., 2004
Seed coat	-Supercritical CO_2, -Supercritical CO_2 with 10% ethanol -Ethanol -Ethyl acetate	The ethanol extract has the highest (-)-epicatechin content and the most antioxidant activity in term peroxide value	Thailand (Sweet tamarind)	Luengthanaphol *et al*., 2004
Seeds, Pericarp	Methanol	Tamarind seed composed of 8 major flavonoids and pericarp composed of 12 major flavonoids. They showed antioxidant activity in hypoxanthine/xanthine oxidase assay and 2-deoxyguanosin assay	Thailand	Sudjaroen *et al*., 2005
Seed, Seed skin	95% ethanol	The extracts showed $DPPH^{\bullet}$scavenging activity	Thailand	Maisuthisakul *et al*.,2007
Seed coat (roasted and unrosted)	-Methanol -70% acetone	The methanolic extracts showed antioxidant activities on $DPPH^{\bullet}$, $O_2^{\bullet-}$, $HO^{\bullet}$, FRAP and $ABTS^{\bullet+}$	India	Siddhuraju, 2007
Seed coat	-Successively extracted with 70% ethanol, chloroform, and ethyl acetate -Ethyl acetate -95% ethanol	The ethyl acetate fraction was the most potent on $DPPH^{\bullet}$ scavenging activity	Thailand (Sweet tamarind)	Lourith, Kanlayavattanakul, and Chanpirom, 2009

Table 1. Solvent extracts and bioactivity of various part of *T. indica* (cont.)

Part (tested)	Solvent extracts	Bioactivity assay	Site of collection	References
Seed coat	95% ethanol and precipitated with non-fat milk	The extract showed antibiotic activity on productive performance of broilers	Thailand	Aengwanich *et al.*, 2009
Seed coat	95% ethanol5% ethanol and precipitated with non-fat milk	The extracts reduce heat stress and oxidative stress, improve the growth rate of heat-stressed boilers	Thailand	Aengwanich *et al.*, 2010
Seed coat (sweet and sour type)	Successively extracted with 70% ethanol, chloroform, and ethyl acetate	The extracts composed of phenolic compound, tannin and proanthocyanidin and has antioxidant activities on $DPPH^{\bullet}$, $HO^{\bullet}$, reducing power and anti-lipid peroxidation	Thailand	Suksomtip *et al*., 2010
Seed coat	Methanolic	The extracts exhibited antiulcer, reduce total volume of gastric juice, free and total acidity of gastric secretion in rat	India	Kalra *et al.,* 2010
Seed coat	95% ethanol5% ethanol and precipitated with non-fat milk	The extracts reduce GPx activity and bilirubin in feces, and increase red blood cell parameters in heat-stressed boilers	Thailand	Aengwanich *et al.*, 2012
Leaves, Veins, Seeds, Skins	-Methanol -Ethyl acetate -Hexane	The methanolic extracts possessed the most antioxidant activity on $DPPH^{\bullet}$, $O_2^{\bullet-}$, FRAP and $ABTS^{\bullet+}$	Malasia	Razali *et al.*, 2012

1.5 Antioxidant activity in Thai tamarind seed coat

The first study of antioxidant activities of seed coat of Thai tamarind by Pumthong in 1999 reported that the seed coat extracted with 70% ethanol followed by chloroform and the remaining extract of aqueous fraction of Thai tamarind contained polyphenolic compounds, so-called oligomeric proanthocyanidin (OPC) and exhibited antioxidant activities against peroxyl radicals, hydroxyl radicals, superoxide anions, and showed effectively prevent lipid oxidation.

Luengthanapol *et al*. (2004) studied the antioxidants in various solvent extractions in sweet Thai tamarind seed coat. The seed coats are extracted with supercritical CO_2, supercritical CO_2 with 10% ethanol co-solvent, ethanol, and ethyl acetate. They reported that ethanol has the highest (-) epicatechin content and show the most active of antioxidants in term of peroxide value. In addition, Kumutarind *et al*. (2004) reported Thai tamarind seed coat extracted with 70% ethanol, partition with chloroform, and ethyl acetate, the ethyl acetate fraction exhibited nitric oxide ($NO^{\bullet}$) inhibition in macrophage by *in vitro* and *in vivo* tests.

Sudjaroen *et al*. (2005) have studied the isolation and structure elucidation of phenolic antioxidants in Thai tamarind seeds and pericarp extracted with methanol. They reported that the Thai tamarind seeds containing eight major components, such as procyanidin B_2, (-)-epicatechin, procyanidin trimer, procyanidin tetramer, procyanidin pentamer, procyanidin hexamer, and polymeric tannins, and tamarind pericarp showed twelve major components, such as (+)-epicatechin, procyanidin B_2, (-)-epicatechin, procyanidin trimer, procyanidin tetramer, procyanidin pentamer, procyanidin hexamer, taxifolin, apigenin, eriodictyol, luteolin, naringenin (Fig. 4). Moreover, the extract showed antioxidant activity in hypoxanthine/xanthine oxidase assay.

Maisuthisakul *et al*. (2007) studied phenolic contents and antioxidant activity of Thai tamarind seed and seed coat extracted with 95% ethanol. They found that the seed coat of Thai tamarind has phenolics content and $DPPH^{\bullet}$ scavenging activity than the seed of tamarind.

Suksomtip and Pongsamart (2008) reported that the methanolic extract of sour type of Thai tamarind seed coat showed protective effect against oxidation of human low-density lipoprotein and plasmid DNA strand scission. Moreover, in year 2010,

they studied phenolic compound content, antioxidant and radical-scavenging properties in methanolic extracts of seed coat of five cultivars of Thai tamarind both sweet and sour types including Srichomphu, Sithong-nak, Sithong-bao, Priao-yak and Khanti. They reported that the extracts of Priao-yak, sour type of tamarind, showed the highest tannin content and showed the strongest reducing power, while the extracts of Khanti showed the highest proanthocyanidin content revealed high scavenging ability on both $DPPH^{\bullet}$ and $OH^{\bullet}$. The extracts of Sithong-bao having high content of total phenols, anthocyanidin and tannin, shows strong antioxidant activity with most of the assays.

Aengwanich *et al.* (2009) reported that the TSC extracted with 95% ethanol and precipitated with non-fat milk and adjusted pH to 6 to reduce the effect of high molecular weigh tannin having antibiotic effect in broiler, additionally, it could reduce heat stress, oxidative stress and improve the growth rate of heat-stressed broilers (Aengwanich and Suttajit, 2010).

Lourith, Kanlayavattanakul, and Chanpirom (2009) studied free radical scavenging activity and total phenolics content of Thai tamarind seed coat extracted with 70% ethanol and partition with dichloromethane and ethyl acetate, successively, and maceration of ethyl acetate and 95% ethanol. They found that TSC extracted with the ethyl acetate partition and maceration showed high $DPPH^{\bullet}$ scavenging and high total phenolic content.

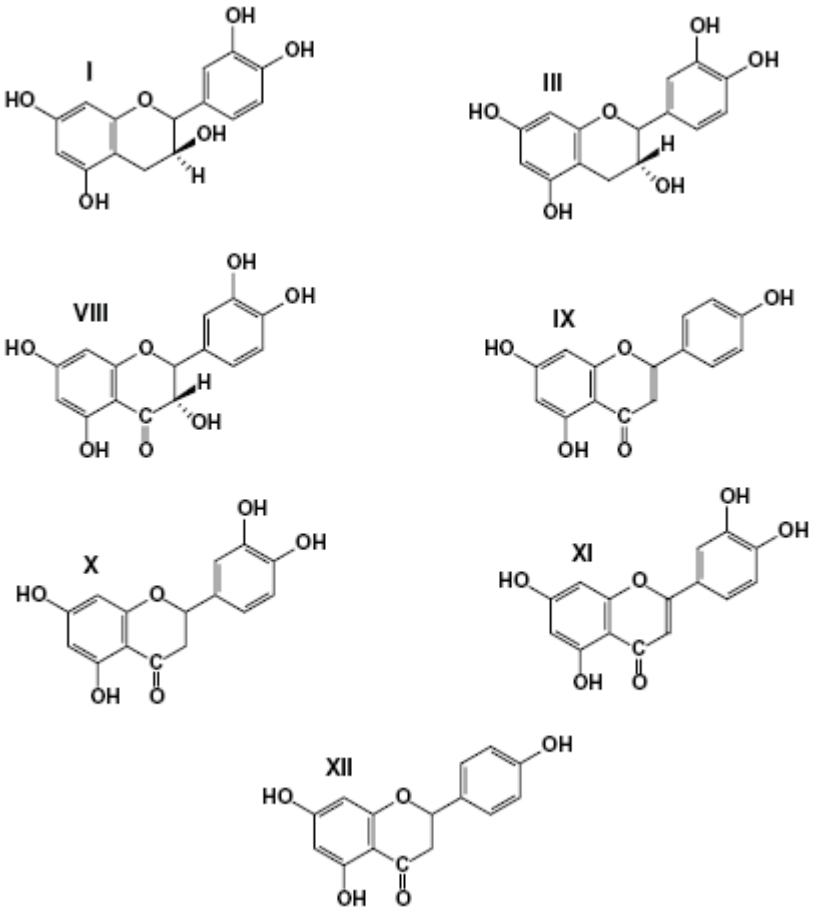


Figure 4. The structures of the monomeric flavonoids in seed coat of *T. indica*; (+)-catechin (I), (-)-epicatechin (III), taxifolin (VIII), apigenin (IX), eriodictyol (X), luteolin (XI), and naringenin (XII) (Sudjaroen *et al*., 2005)

2. Free radicals

Free radicals are any chemical species capable of independent existence, processing one or more unpaired electrons. Biological free radicals are thus highly unstable molecules that have electrons available to react with various substrates (Somogyi *et al*., 2007). Free radicals are divided into 2 groups, such as ROS and reactive nitrogen species.

2.1 Reactive oxygen species

Reactive oxygen species (ROS) can be produced from both endogenous, such as mitochondria, cytochrome P450 metabolism, peroxisomes and inflammatory cell activation; and exogenous substances including environmental agents, microorganisms, xenobiotic substances. ROS are composed of $O_2^{\bullet-}$, H_2O_2, $HO^{\bullet}$, singlet oxygen, alkoxyl radicals, $ROO^{\bullet}$ (Valko *et al*., 2007)**.**

Superoxide anion ($O_2^{\bullet-}$) is created from molecular oxygen by the addition of an electron is, in spite of being a free radical, not highly reactive. The formation of superoxide takes place spontaneously, especially in the electron-rich aerobic environment in vicinity of the inner mitochondria membrane with the respiratory chain and two molecules of superoxide rapidly dismutate to H_2O_2 and oxygen by superoxide dismutase (SOD) (Fig. 5). Superoxide arising either through metabolic processes or following oxygen activation by physical irradiation, is considered the *primary* ROS and $O_2^{\bullet-}$ can further interact with other molecules to generate secondary ROS (Nordberg and Arner, 2001; Valko *et al*., 2007).

Hydroxyl radical ($OH^{\bullet}$) is the neutral form of the hydroxide ion, it has a high reactivity, which making a very dangerous radical in living cells. Hydroxyl radicals are mostly produced through Fenton reaction (1) and in the Haber-Weiss reaction (2) (Valko *et al.*, 2007; Singh *et al.,* 2008).

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{\bullet} + OH^{-} \quad (1)$$

$$O_2^{\bullet-} + H_2O_2 \rightarrow O_2 + OH^{\bullet} + OH^{-} \quad (2)$$

Hydrogen peroxide (H_2O_2) is not a free radical but is nonetheless highly much important because of its ability to penetrate biological membrane. It plays a radical forming role as an intermediate in the production of more reactive ROS molecule such as $OH^{\bullet}$ (Fig. 3) and hypochlorus acid (HOCl) (Nordberg and Arner,

2001). In biological cell, H_2O_2 is produced by peroxisome, which is a major sites of oxygen consumption in the cell and participate in several metabolic functions that use oxygen. Oxygen consumption in the peroxisome leads to H_2O_2 and then decomposed by catalase, which contains in peroxisome to prevent accumulation of this toxic compound. When peroxisomes are damaged and their H_2O_2 consuming enzymes down regulated, H_2O_2 releases into cytosol and then it used to oxidize a variety of molecules (Valko *et al.*, 2007).

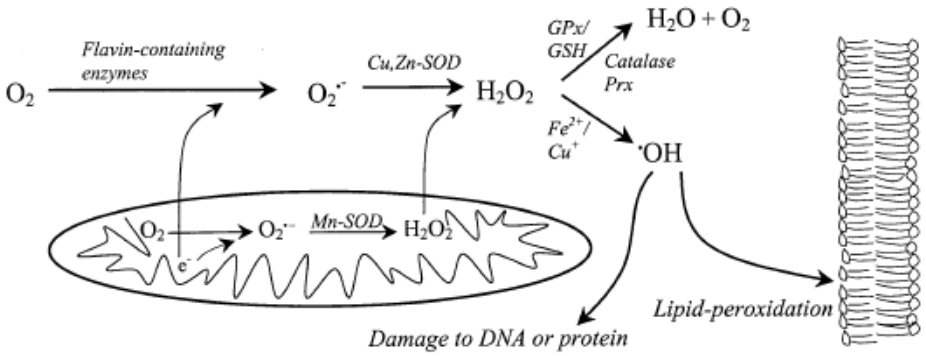


Figure 5. Free radical generation and radical scavenging in living cell. Super oxide radical generated from respiratory reaction in mitocondria can dismutate to H_2O_2 by MnSOD in mitochondria and Cu,Zn SOD in cytosol. Hydrogen peroxide can neutralized to O_2 and H_2O by CAT and GPx or convert to $OH^{\bullet}$, the high reactive radical, which leading to lipid peroxidation and DNA or protein damage.

Singlet oxygen ($^1O_2$) is the first excited electronic state of O_2, and is a non-radical ROS with one of the strongest activities. It directly damage onto biological lipids, proteins, and DNA (Nishida, Yamashita, and Miki, 2007). It can be produced by energy transfer from triplet state of sensitizers according the following scheme 3 (Li, Ahmed, and Bernstein, 2010) and formed chemically, enzymatically, and photochemically which show in Figure 6 (Min and Boff, 2002).

$$^3\text{Sensitizer} + {}^3O_2 \text{ ---------} {}^1\text{Sensitizer} + {}^1O_2 \qquad (3)$$

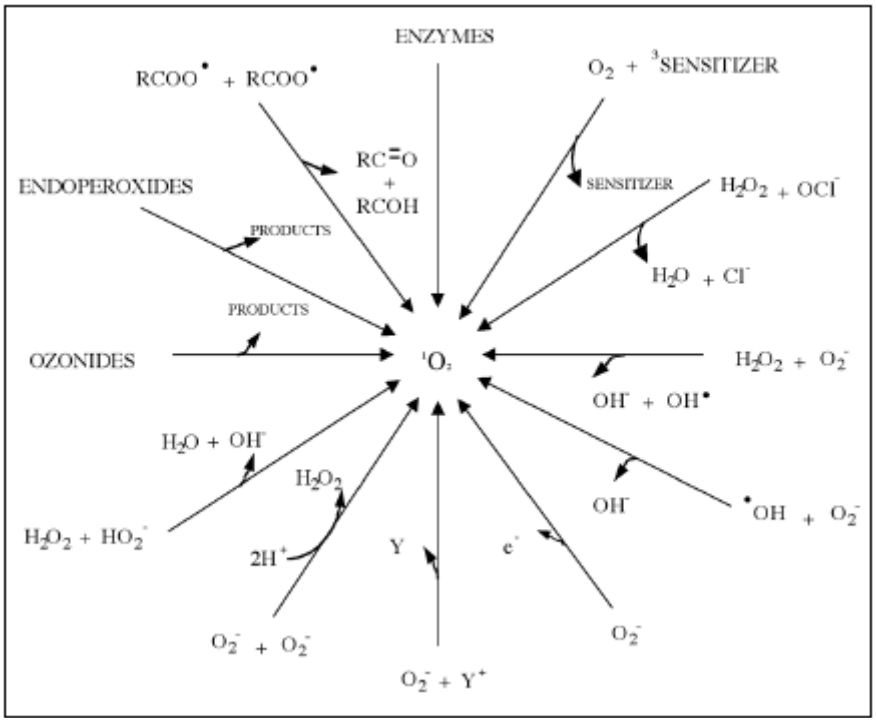


Figure 6. Singlet oxygen formation (Min & Boff, 2002)

Peroxyl radicals ($ROO^{\bullet}$) are reactive radicals derived from oxygen, particularly through the reaction of $OH^{\bullet}$ with unsaturated lipid (Valko *et at*., 2007; Singh *et al*., 2008). The overproduction or excess ROS in cellular is called "oxidative stress", which can damage cellular lipid, protein, and DNA.

2.2 Reactive nitrogen species

Reactive nitrogen species (RNS) are composed of $NO^{\bullet}$ and peroxynitrite.

Nitric oxide ($NO^{\bullet}$) is a small molecule that contains a single unpaired electron. $NO^{\bullet}$ is generated in biological tissues by specific nitric oxide synthases, which metabolize arginine to citruline with the formation of $NO^{\bullet}$ (4). Nitric oxide is abundant reactive radical that acts as important oxidative biological signaling molecule in a large variety of diverse physiological processes, including neurotransmission, blood pressure regulation, defense mechanisms, smooth muscle relaxation and immune regulation.

$$O_2 + \text{arginine} + NADPH \xrightarrow{\text{Nitric oxide syntase}} NO^{\bullet} + \text{citruline} + H_2O + NADP^{+} \quad (4)$$

Peroxynitrite anion ($ONOO^-$), a highly active molecule, is produced from the reaction of $NO^{\bullet}$ and $O_2^{\bullet-}$ in the immune system (5). It is an oxidizing free radical that can cause DNA fragmentation and lipid peroxidation.

$$O_2^{\bullet-} + NO^{\bullet} \rightarrow ONOO^- \quad (5)$$

The overproduction of reactive nitrogen species in cellular are called "nitrosative stress", which may lead to unwanted nitrosylation reactions that alter the structure and functions of certain proteins (Valko *et at*., 2007; Singh *et al*., 2008).

3. The classical free radical route (Antolovich *et al.,* 2002)

The classical free radical route leads to initiation of rapidly progressing, destructive chain reactions. The essential features of oxidation *via* a free radical-mediated chain reaction are initiation, propagation, branching and termination steps.

The process may be initiated by the action of external agents, such as heat, light or ionizing radiation or by chemical initiation involving metal ions or metalloproteins (6).

Initiation $LH + R^{\bullet} \rightarrow L^{\bullet} + RH \quad (6)$

Where, LH represents the substrate molecule, for example, a lipid, with $R^{\bullet}$ as the initiating oxidizing radical. The oxidation of the lipid generates a highly reactive allyl radical ($L^{\bullet}$) that can rapidly react with oxygen to form a lipid peroxyl radical ($LOO^{\bullet}$) (7).

Propagation $L^{\bullet} + O_2 \rightarrow LOO^{\bullet} \quad (7)$

$LOO^{\bullet} + LH \rightarrow L^{\bullet} + LOOH \quad (8)$

The peroxyl radicals are the chain carriers of the reaction that can further oxidize the lipid, producing lipid hydroperoxides (LOOH) (8), which in turn break down to a wide range of compounds including alcohols, aldehydes, alkyl formats, ketones and hydrocarbons and radicals including the alkoxyl radical ($LO^{\bullet}$)(9-10).

Branching $LOOH \rightarrow LO^{\bullet} + HO^{\bullet}$ (9)

$2LOOH \rightarrow LOO^{\bullet} + LO^{\bullet} + H_2O$ (10)

The breakdown of LOOH often involves transition metal ion catalaysis, in reactions analogous to that with H_2O_2, yielding lipid peroxyl and lipid alkoxyl radicals (11-12).

$LOOH + M^{n+} + H^{+} \rightarrow LO^{\bullet} + M^{(n+1)} + H_2O$ (11)

$LOOH + M^{(n+1)} + OH^{-} \rightarrow LOO + M^{n+} + H_2O$ (12)

Termination reactions involve the combination of radicals to form non-radical products (13-15).

Termination $LO^{\bullet} + LO^{\bullet} \rightarrow$ (13)

$LOO^{\bullet} + LOO^{\bullet} \rightarrow$ (14) non-radical products

$LO^{\bullet} + LOO^{\bullet} \rightarrow$ (15)

4. Beneficial and harmful effect of free radicals

The beneficial effect of ROS is occur at low or moderate concentrations and involve physiological roles in cellular responses to noxia, as for example in defense against infectious agents, in the induction of a mitogenic response and in the function of a number of cellular signaling systems (Valko *et at*., 2007).

The harmful effect of free radicals is causing potential biological damage and produces oxidative stress and nitrosative stress. They cause DNA, lipid, and protein, damage and loss of function. The effect of free radical in cells involving many diseases such as, cancer, cardiovascular disease, atherosclerosis, hypertension, ischemia, diabetes mellitus, neurodegenerative diseases (Alzheimer's disease and Parkinson's disease), rheumatoid arthritis, and ageing (Droge, 2002; Sorg, 2004; Valko *et al*., 2007).

5. Oxidative stress

Somogyi *et al.,* 2007 defied that "oxidative stress" is the imbalance between the pro-oxidant and antioxidant. Oxidative stress is basically caused by two mechanisms, (i) the concentration of antioxidants is reduced and (ii) the number of oxygen /nitrogen reactive species is increased. Following the oxidative stress, the

activity of a given signal-transducing molecules is either reduced or increased, additionally the function of the molecules (lipid, protein, and DNA) may also change, which lead to a variety of diseases as shown in Figure 7.

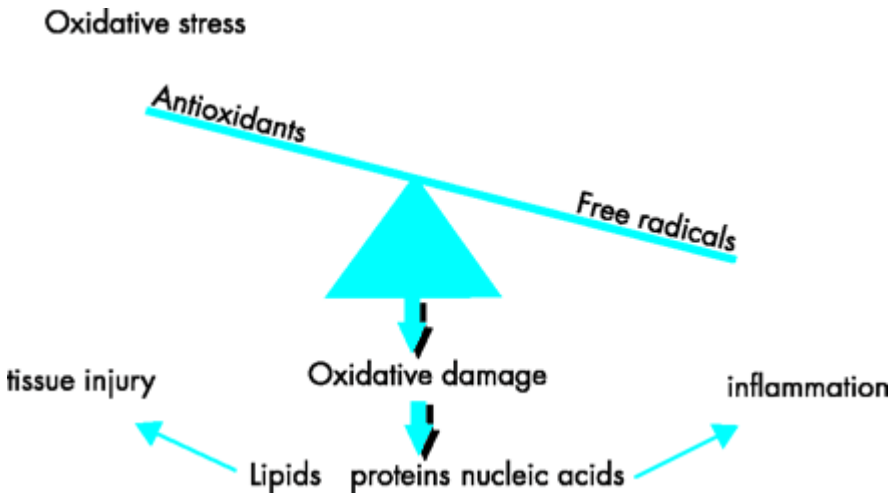


Figure 7. Oxidative stress exists when the living cells have free radicals more than antioxidants which leading to lipids, proteins and nucleic acids damage (Kelly, 2003).

6. Antioxidants

They have various defied the meaning of antioxidant, such as:

Halliwell (1996) defied that *"antioxidant"* is a molecules which, when present in small concentrations compared to the biomolecules they are supposed to protect, can prevent or reduce the extent of oxidative destruction of biomolecules.

Huang, Ou, and Prior (2005) collected the definition of antioxidant from the various sources, such as from the Webster's third new international dictionary (2004) defied that *"antioxidant"* is a substance that opposes oxidation or inhibits reactions promoted by oxygen or peroxides. In biological field defied that *"antioxidant"* is synthetic or natural substances added to products to prevent or delay their deterioration by action of oxygen in air. In biochemistry and medicine defied that *"antioxidant"* is enzymes or other organic substances, such as vitamin E or A, that are capable of counteracting the damaging effects of oxidation in animal tissues. In the chemical industry, *"antioxidant"* refers to compounds that retard autoxidation of a

chemical product. In food science, *"antioxidant"* is a substance in foods that significantly decreases the adverse effects of reactive species, such as reactive oxygen and nitrogen species, on normal physiological functions in humans.

Brainina *et al*. (2007) defied that *"antioxidants"* are molecules that can neutralize free radicals by donating an electron to free radicals for eliminate the unpaired condition of free radicals. After electron donating, the antioxidant molecule becomes a free radical in the process of neutralizing a free radical molecule to a non-free-radical molecule, but the antioxidant molecule will usually be a much less reactive free radical than the free radical neutralized. The antioxidant molecule may be very large, it may be readily neutralized by another antioxidant and/or it may have another mechanism for terminating its free radical condition.

Antolovich *et al*. (2002) divided antioxidant into 2 classes, primary or chain breaking antioxidants and secondary or preventive antioxidants.

Primary or chain breaking antioxidants. A primary antioxidant (AH), when present in trace amounts, may either delay or inhibit the initiation step by reacting with a lipid radical or inhibit the propagation step by reacting with peroxyl or alkoxyl radicals (16-18).

$$L^{\bullet} + AH \rightarrow LH + A^{\bullet} \quad (16)$$

$$LOO^{\bullet} + AH \rightarrow LOOH + A^{\bullet} \quad (17)$$

$$LO^{\bullet} + AH \rightarrow LOH + A^{\bullet} \quad (18)$$

The antioxidant free radical may further interfere with chain propagation reactions by forming peroxy antioxidant compounds (19-20).

$$A^{\bullet} + LOO^{\bullet} \rightarrow LOOA \quad (19)$$

$$A^{\bullet} + LO^{\bullet} \rightarrow LOA \quad (20)$$

The activation energy of the above reaction increasing AH and LH bond dissociated energy. Therefore, the efficiency of the antioxidant increases with decreasing A-H bond strength. These consist of the reported of Maisuthisakul *et al.* (2007) which reported that the primary antioxidant can inhibit or retard oxidation by scavenging free radical by donating hydrogen atom or electron to radicals, which converts them to more stable product.

Secondary or preventive antioxidants are compounds that retard the rate of oxidation. This may be achieved in a number of ways including removal of substrate or singlet oxygen quenching. Moreover, Maisuthisakul *et al.* (2007) reported that a secondary antioxidnts has many machanisms, including binding metal ions, scavenging oxygen, and converting H_2O_2 to non-radical species.

7. Antioxidant defense system

Free radicals are neutralized by antioxidant defense. The antioxidant defense system composes of 2 groups such as, enzymatic antioxidants or primary antioxidant defense and non-enzymatic antioxidants or secondary antioxidant defense (Matés and Sánchez-Jiménez, 1999; Nordberg and Arnér, 2001). The antioxidant defenses are show in Table 2.

7.1 Enzymatic antioxidants

7.1.1 Superoxide dismutase (SOD, EC 1.15.1.1) is the primary enzymatic antioxidant defense. The enzyme catalyzed two molecules of superoxide radicals to H_2O_2 and oxygen (21) (Valko *et al*., 2006). In human, there are three forms of SOD, manganese superoxide (Mn-SOD), copper,zinc superoxide dismutase (Cu, Zn-SOD), and extracellular-SOD (EC-SOD) (Matés and Sánchez-Jiménez, 1999).

$$2O_2^{\cdot -} + 2\,H \xrightarrow{SOD} H_2O_2 + O_2 \qquad (21)$$

Mn-SOD or SOD-2 is a homotetramer containing one manganese atom per subunit (96 kDa). Mn-SOD is primary antioxidant enzyme that function to remove the superoxide radical, which found in mitochondria.

Cu, Zn-SOD or SOD-1, containing a metal cluster, the active site, constituted by a copper and a zinc atom bridged by a common ligan (32 kDa). This enzyme is found in cytosol.

EC-SOD or SOD-3 is a secretory, tetrameric, copper and zinc containing glycoprotein. It found in the intersticial spaces of tissue and also in extracellular fluids. EC-SOD, the enzyme is not induced by its substrate or others oxidants and its regulation in mammalian tissues primarily in a manner coordinated by cytokines, rather than as a response of individual cells to oxidants.

7.1.2 Catalase (CAT, EC 1.11.1.6) is a tetrameric heme-containing enzyme (60 kDa). Catalase is located in a cell organelle called peroxisome. The enzyme can catalyse the dismutation of H_2O_2 to water and oxygen (22) (Nordberg and Arnér, 2001).

$$2H_2O_2 \xrightarrow{CAT} O_2 + 2\,H_2O \quad (22)$$

7.1.3 Glutathione peroxidases (GPx, EC 1.11.1.19) are selenocysteine containg enzyme (80 kDa). They are at least four different GPx in mammals (GPx1-GPx4). GPx1 and GPx4 are both cytosolic enzymes abundant in most tissue while GPx2 (gastrointestinal GPx) and GPx3 (plasma GPx) are mainly expressed in gastrointestinal tract and kidney, respectively. GPx acts in conjunction with the tripeptide glutathione (GSH), which is the substrate for the catalytic reaction of GPx is H_2O_2 or an organic peroxides (ROOH) to water or alcohol (23-24) (Nordberg and Arnér, 2001).

$$2GSH + H_2O_2 \xrightarrow{GPx} GSSG + 2\,H_2O \quad (23)$$

$$2GSH + ROOH \xrightarrow{GPx} GSSG + ROH + H_2O \quad (24)$$

Although, GPx shares the substrate (H_2O_2) with CAT, this enzyme alone can react effectively with lipid and other organic hydroperoxides. The glutathione redox cycle is a major source of protection against low levels of oxidant stress, whereas CAT becomes more significant in protecting against severe oxidant stress (Matés and Sánchez-Jiménez, 1999).

Table 2. Enzymatic and non-enzymatic antioxidants (Sorg, 2004).

Antioxidant	Phase	Action
Superoxide dismutases (SOD)	Hydrophilic	Dismutation of O_2^- into H_2O_2 and O_2
Catalase	Hydrophilic	Dismutation of H_2O_2 into H_2O and O_2
Glutathione peroxidases (GPX)	Hydrophilic or lipophilic	Reduction of R–OOH into R–OH
Glutathione reductase (GSR)	Hydrophilic	Reduction of oxidised glutathione
Glutathione-S-transferases (GST)	Hydrophilic	Conjugation of R–OOH to GSH (→ GS–OR)
Metallothioneins	Hydrophilic	Binding to transition metals (= neutralisation)
Thioredoxins	Hydrophilic	Reduction of R–S–S–R into R–SH
Glutathione	Hydrophilic	Reduction of R–S–S–R into R–SH Free radical scavenger Cofactor of GPX and GST
Ubiquinol	Lipophilic	Free radical scavenger (prevents LPO)
(Dihydro)lipoic acid	Amphiphilic	ROS scavenger Increases antioxidant and phase II enzymes
Ascorbic acid (vitamin C)	Hydrophilic	Free radical scavenger Recycles tocopherols (vitamin E) Maintains enzymes in their reduced state
Retinoids (vit. A) and carotenoids	Lipophilic	Free radical scavengers Singlet oxygen ($^1O_2$) quencher
Tocopherols (vitamin E)	Lipophilic	Free radical scavenger (prevents LPO) Increases selenium absorption
Selenium	Amphiphilic	Constituent of GPX and thioredoxins

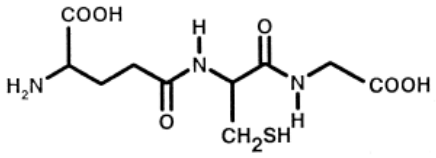
7.1.4 Phase II detoxifying enzymes are contribute to biosynthesis/recycling of thiols or facilitate excretion of oxidized through reduction/conjugation reactions of Nrf2 (nuclear factor-erythroid 2 p45-related factor)-antioxidant response elements pathway during xenobiotic detoxification. They are represented by glutathione-*S*-transferase (GST) isozyme and NADP(H): quinine oxidoreductase (NQO1) (Cho and Kleeberger, 2009).

7.2 Non-enzymatic antioxidants

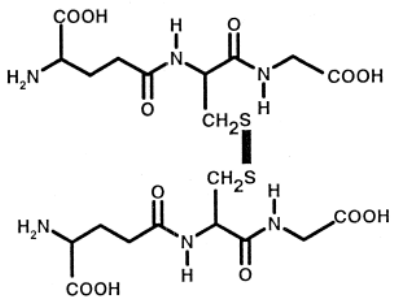
Non-enzymatic antioxidant defense, such as GSH, vitamin C, vitamin E, carotenoids and phenolic compounds, which include tannins and flavonoids, are found in fruits, vegetables, and food. Their antioxidant properties can protect cell damage and against cell death.

Glutathione (γ-glutamylcysteinylglycine, GSH) is a thiol-containing tripeptide, glutamic acid-cysteine-glycine. It is play a major role thiol antioxidant and redox buffer in cell. They have 2 forms, such as glutathione reduced form (GSH) and the oxidized form (GSSG; glutathione disulfiide), in which 2 GSH molecules join via the oxidation of the –SH groups of the cysteine residue to form a disulphide bridge (Fig. 8) (Kohen and Nyska, 2002). GSH is present in humans, animals, plants, and aerobic bacteria at a high concentration such as, 3-5 mM in nuclei, 1-11 mM in

cytosol, and 5-11 mM in mitochondria. Reduced glutathione acts as a cofactor for the enzyme peroxidase, thus serving as an indirect antioxidant donating the electrons necessary for its decomposition of H_2O_2 and converted it to the oxidized form. Once oxidized, GSH can be reduced back by glutathione reductase, using NADPH as an electron donor (Fig. 9). Oxidized glutathione is accumulated inside the cells and the ratio of GSH/GSSG is a good measure of oxidative stress of an organism. Too high a concentration of GSSH may be a sensitive indicator of oxidative stress and may damage many antioxidant enzymes. In the healthy cell GSSG, is rear exceeds 10 percent of total glutathione (Kidd, 1997). The protective roles of glutathione against oxidative stress are (i) glutathione is a cofactor of several detoxifying enzymes against oxidative stress; (ii) GSH participates in amino acid transport through the plasma membrane; (iii) GSH scavenges $OH^{\bullet}$ and singlet oxygen directly, detoxifying H_2O_2 and lipid peroxides by the catalytic action of glutathione peroxidase; and (iv) it is able to regenerate vitamin C and E back to their active form (Valko *et al.,* 2007).



GSH



GSSG

Figure 8. Structure of reduced glutathione (GSH) and oxidized glutathione (GSSG) (Havel, Pritts, and Wielgos, 1999).

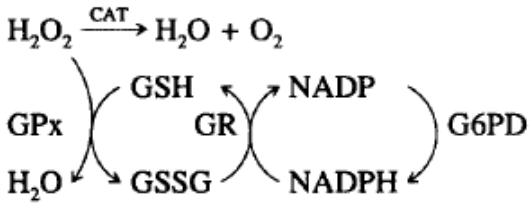


Figure 9. Glutathione redox cycle. The GSH converted to GSSG by GPx and can reduced back by GR and NADPH (Sun, 1990).

L-Ascorbic acid (ascorbate, vitamin C) is a water-soluble low molecule weight antioxidant can be synthesized by plants and some animals. It is required for collagen synthesis, iron absorption and maintenance of the redox status of cells (Sorg, 2004). Ascorbic acid is an antioxidant, it is an efficient scavenger or reducing antioxidant, capacity of donation its electron to ROS and eliminating them (Kohen and Nyska, 2002). The structure of ascorbic acid is shown in Figure 10.

Tocopherols (Vitamin E) are the main antioxidant in the lipophilic phase. It can scavenge ROO to inhibit the lipid peroxidation process in biological membrane. When the vitamin E is oxidized, they become radicals, and then converted to their functional reduced state by ascorbic acid (Sorg, 2004). The structure of tocopherol is shown in Figure 10.

Carotenoids (terpenoid molecules synthesized by plants, among which vitamin A precursors such as β-carotene (Fig. 10) are free radical scavengers, and most importantly, singlet oxygen quenchers (Sorg, 2004).

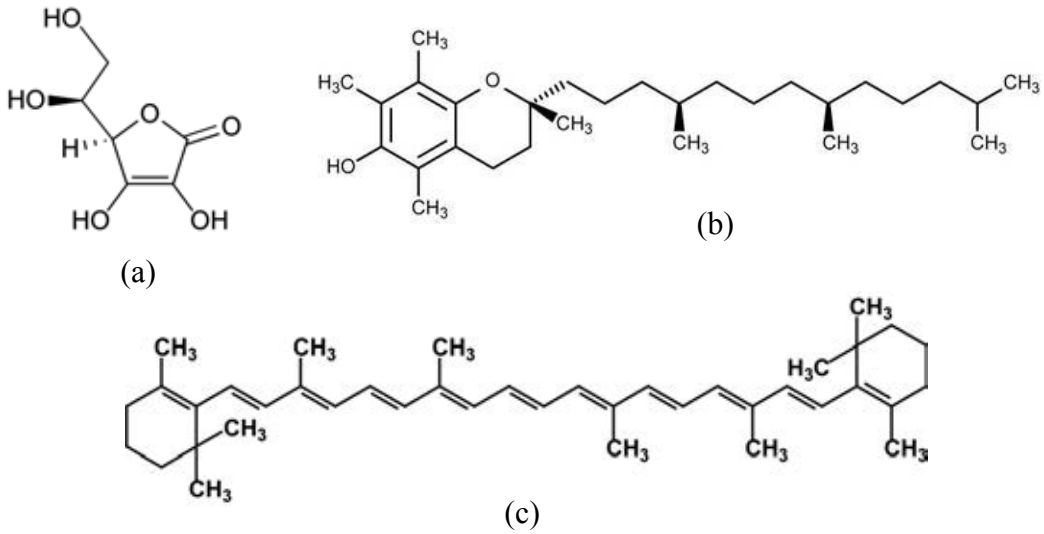


Figure 10. Structure of non-enzymatic antioxidants; a) L-Ascorbic acid, b) α–Tocopherol, and c) β-Carotene.

Polyphenols, one of the major difficulties of elucidating the beneficial effects of polyphenols is the large number of polyphenolic compounds found in fruits, vegetables, and beverages and the even larger numbers of their metabolites. The polyphenols have antioxidants and free radical scavenging activities mainly contribute to their beneficial effects. Polyphenols can be broadly divided into two categories, flavonoids and nonflavonoid polyphenols (Singh *et al.*, 2008) as show in Figure 11.

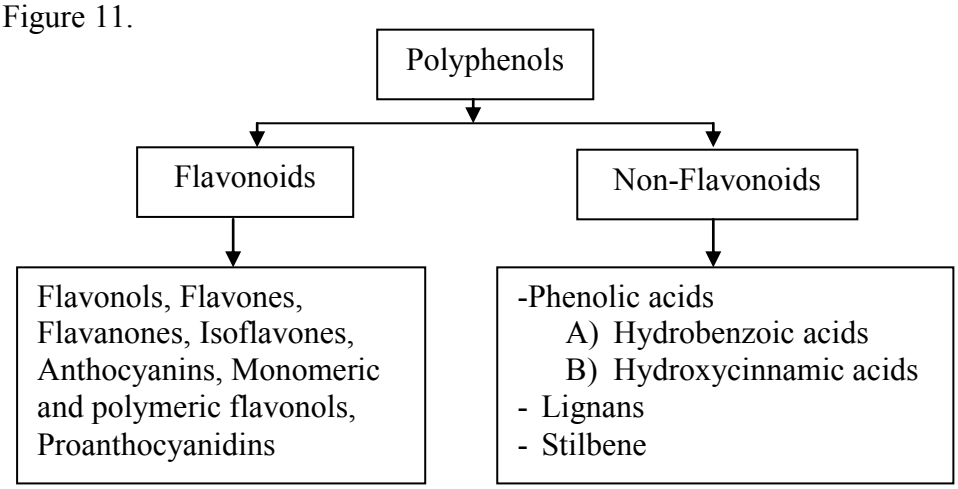


Figure 11. Classification of polyphenols, modifired from Singh *et al.* (2008).

Flavonoids, are benzo-γ-pyrone derivatives consisting of phenolic and pyrane ring (Figure 12) and are classified according to substitutions (Fig. 13), which found in various part of plants. Dietary flavonoids differ in the the arrangements of hydroxyl, methoxy, and glycosidic side groups, and in the conjugation between the A-and B- rings.

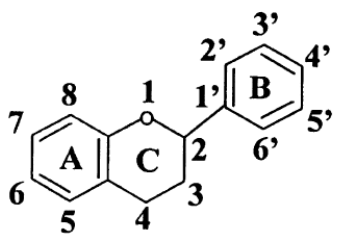


Figure 12. Nuclear structure of flavonoids (Heim, Tagliaferro, and Bobilya, 2002).

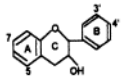
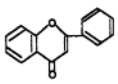
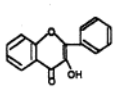
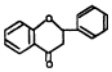
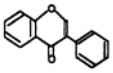
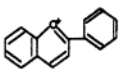
Class	General structure	Flavonoid	Substitution Pattern	Dietary Sources	TEAC (mM)
Flavanol		(+)-catechin	3,5,7,3′,4′-OH	Tea (camellia sinensis)[6]	2.4
		(-)-epicatechin	3,5,7,3′,4′-OH	Tea[6]	2.5
		Epigallocatechin gallate	3,5,7,3′,4′,5′-OH,3-gallate	Tea[6]	4.75
Flavone		chrysin	5,7-OH	Fruit skins	1.43
		apigenin	5,7,4′-OH	Parsley, celery	1.45
		rutin	5,7,3′,4′-OH, 3-rutinose	Red wine[5] , buckwheat[7] citrus, tomato skin[8]	2.4
		luteolin	5,7,3′,4′-OH	Red pepper[11]	2.1
		luteolin glucosides	5,7,3′-OH, 4′-glucose		1.74
			5,4′-OH, 4′,7-glucose		0.79
Flavonol		kaempferol	3,5,7,4′-OH	Leek, broccoli, endives grapefruit, black tea	1.34
		quercetin	3,5,7,3′,4′-OH	Onion, lettuce, broccoli tomato, tea, red wine berries, olive oil, appleskin	4.7
		myricetin	3,5,7,3′,4′,5′-OH	Cranberry grapes, red wine	3.1
		tamarixetin	3,5,7,3′-OH,4′-OMe		
Flavanone (dihydroflavon		naringin	5,4′-OH,7-rhamnoglucose	Citrus, grapefruit	0.24
		naringenin	5,7,4′-OH	Citrus fruits	1.53
		taxifolin	3,5,7,3′,4′-OH	Citrus fruits	1.9
		eriodictyol	5,7,3′,4′-OH	Lemons[6-4]	1.8
		hesperidin	3,5,3′-OH,4′-OMe, 7-rutinose	Oranges[9]	1.08
Isoflavone		genistin	5,4′-OH, 7-glucose	Soybean[10]	1.24
		genistein	5,7,4′-OH	Soybean[10]	2.9
		daidzin	4′-OH, 7-glucose	Soybean[10]	1.15
		daidzein	7,4′-OH	Soybean[10]	1.25
Anthocyanidin		apigenidin	5,7,4′-OH	Colored fruits	2.35
		cyanidin	3,5,7,4′-OH,3,5-OMe	Cherry, raspberry, strawberry	4.42

Figure 13. Classification, structure and food sources of dietary flavonoids (Heim, Tagliaferro, and Bobilya, 2002).

The antioxidant activity of flavoniods and their metabolites in vitro depends upon the arrangement of functional groups about the nuclear structure. Free radical scavenging capacity of flavonoids is primarily attributed to the reactivities of hydroxyl substituents that participate in the following reaction:

$$F\text{-}OH + R^{\bullet} \rightarrow F\text{-}O^{\bullet} + RH$$

Hydroxyl grops on the B-ring can donate hydrogen and electron to hydroxyl, peroxyl, and peroxynitrite radicals, stabilizing them and giving rise to relatively stable flavonoids radical (Heim, Tagliaferro, and Bobilya, 2002).

Tannins are the most abundant secondary metabolites made by plants. They are defined as water-soluble polymeric phenolics. Tannins are found in other plant tissues, including twigs, wood, bark, seeds (Barbehenn and Constabel, 2011) and a wide variety in foods such as apples, berries, chocolate, red wines and nuts (Beecher, 2003). Tannins are divided into two groups: hydrolysable tannins and condensed tannins

Hydrolysable tannins are galloyl glucose and ellagitannins (Fig. 14). Their structure contain a polyol core (commonly glucose), which is esterified with galloyl groups. They are more susceptible to enzymatic and non-enzymatic hydrolysis than proanthocyanidins.

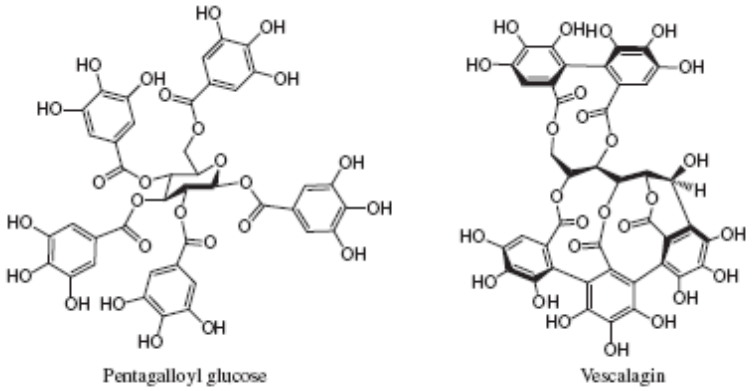


Figure 14. Structures of some hydrolysable tannins (Barbehenn and Constabel, 2011).

Condensed tannins also known as proanthocyanidins, are oligomers or polymers of two or more flavan-3-ols, usually catechin and epicatechin, and frequently the corresponding trihydroxylated gallocatechins. Two of the most common condensed tannins are the procyanidins and the prodelphinidins (Barbehenn and Constabel, 2011). The nomenclature for proanthocyanidins is derived from the acid–catalyzed oxidation reaction that produces the red anthocyanidins upon heating proanthocyanidins in acidic alcohol solutions (Reed, 1995). Condensed tannins are many types which classified based on the propotion of *cis* and *trans* monomers, reflecting the ratio of epicatechin to catechin, such as proanthocyanidin B1 (a epicatechin (4β→8) catechin dimmer) and proanthocyanidin B2 (a epicatechin

(4β→8) epicatechin dimmer). The structure of condensed tannins is show in Figure 15.

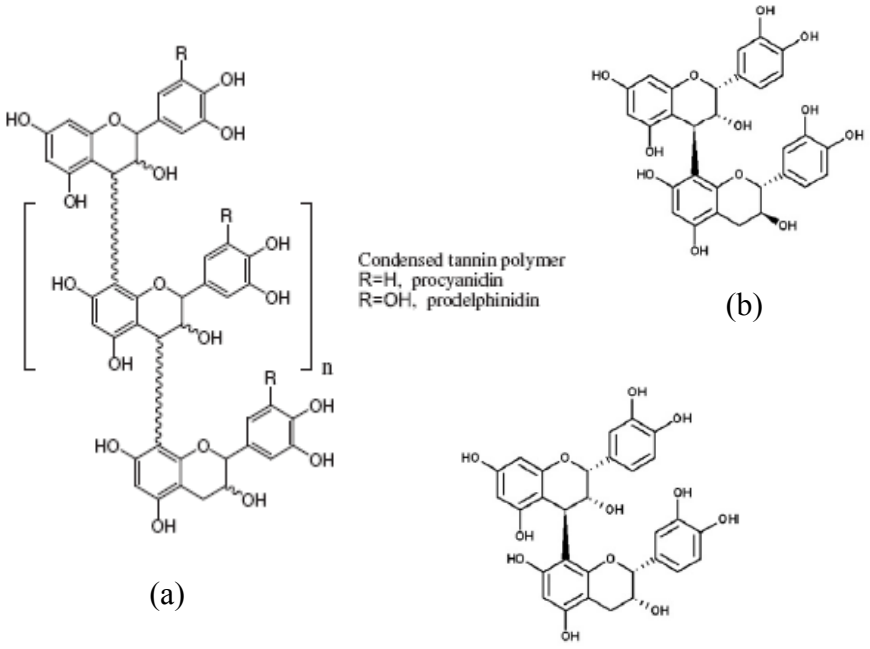


Figure 15. Structure of condensed tannins (a), proanthocyanidin B1 (b), proanthocyanidin B2, and (c) (Barbehenn and Constabel, 2011).

Tannins act as antioxidants, prooxidant, or toxin. If tannins scavenge free radicals, or reduce other oxidized compounds, and form relatively stable semiquinone radicals they can act as antioxidant (Fig. 16). Tannins are often to act as antioxidants in vertebrate gut and show antioxidant property *in vitro* study (Bagchi *et al*., 2000; Labieniec and Gabryelak, 2005; Kim *et al*., 2006). However, tannins act as prooxidant activity and toxicity in herbivores is likely when tannin oxidized to form high level of semiquinone radicals and quinine.

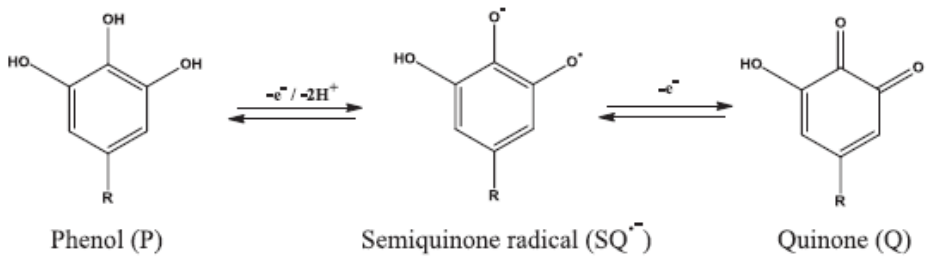


Figure 16. Autoxidation of a trihydroxy phenolic side group via a semiquinone radical which also catalyzed by peroxidase in the presence of H_2O_2, whereas quinones can be produced directly by the two-electron oxidation of phenolic (Barbehenn and Constabel, 2011).

8. Mechanism in the cellular defense

Excessive oxidative stress and toxicants are major cause cell damage; they may contribute to atherosclerosis, coronary artery disease, stroke, Alzheimer's disease and cancer (Lee *et al.*, 2011).

A major mechanism in the cellular defense against oxidative or electrophilic stress is an activation of Nrf2 antioxidant response element signaling pathway, which controls the expression of genes that producing protein or enzymes involved in the detoxification and elimination of reactive oxidants and electrophillic agents and by enhancing cellular oxidant capacity of the induction of phase II detoxifying enzymes such as, GPx, glutamate cysteine ligase (GCL), GST and NQO1, and cellular defensive enzymes such as, heme oxygenase 1 (HO-1) and GST (Owuor and Kong, 2002; Beyer *et al*., 2007; Surh, 2008; Harvey *et al*., 2009).

Under basal or quiescent condition, the transcription factor Nrf2 interacts with 2 molecules of the Kelch-like erythorid CNC homologue (ECH)-associated protein 1 (Keap1), which suppresses Nrf2 transcription activity by coupling and retaining Nrf2 in the cytoplasma (Owuor and Kong, 2002; Surh, 2008). Keap1 molecule contains two major domains, a BTB domain (broad complex, tramtrack, and bric-a-brac) through which it is dimerized to another Keap1 molecule and a Kelch (DGR; double glycine repeat) domain which is anchored to the F-actin cytoskeleton to form Keap1 complex. Keap1 complex also recruits the ubiquitin machinery to Nrf2, leading to proteasomal degradation of Nrf2. However, encounter with reactive oxygen species

brings about a change in the conformation of Keap1 which results in the dissociation of Nrf2 from this inhibitory subunit and then the Nrf2 can translocates to the nucleus (Fig. 17) (Negi *et al.*, 2011).

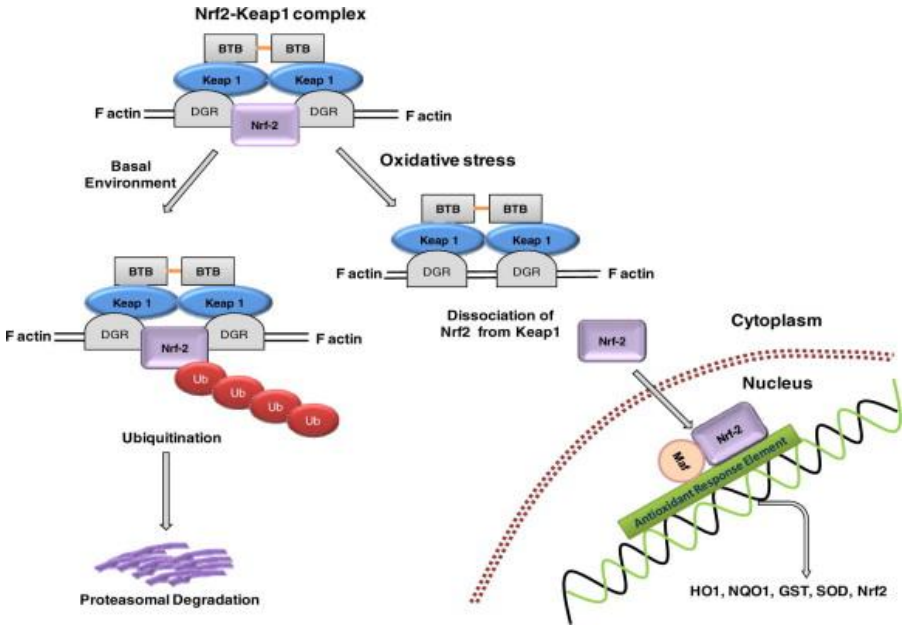


Figure 17. Diagrammatic representation of Nrf2-Keap1 interaction and Nrf2 activation by oxidative stress (Negi *et al.*, 2011).

Diminution the affinity of Keap1 for Nrf2 can activate by oxidative stress (Owuor and Kong, 2002) or electrophillic substances (Durchdewald *et al*., 2007) or antioxidant inducer (Surh, 2008) or chemoprotective agents (Thangapazham *et al*., 2006) via two pathway such as oxidation of Keap1 (direct pathway) and/or stimulation by phosphorylation by protein kinase C, mitogen-activated kinase, or phosphoinositide-3-kinase (indirect pathway) (Wolf, 2001; Durchdewald *et al*., 2007).

In the nucleus, activated Nrf2 form a heterodimer with members of small musculo-aponeurotic fibrosarcoma (Maf) transcription factor family. These Nfr2/Maf binds to the specific consensus *cis*-element called the antioxidant-response element (ARE) or the electrophile-responsive element (EpRE) that are present in the promoter region of genes encoding many antioxidant enzymes which results in the increased expression of antioxidant enzymes and phase II detoxyfing enzymes such as SOD (Negi *et al.*, 2011), NQO1, GST, GPx, CAT, HO-1 (Fig. 18) (Lee *et al.*, 2011).

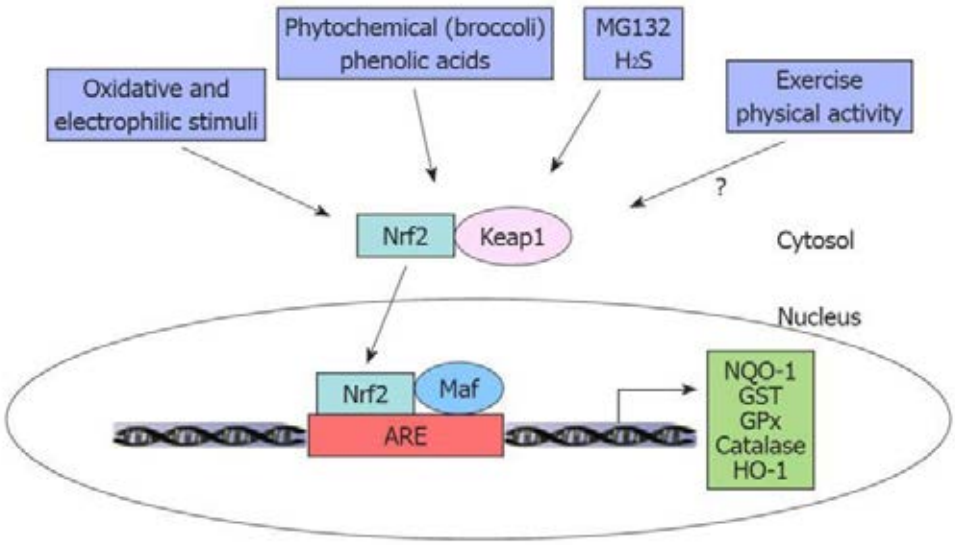


Figure 18. Transcription factor Nrf2 and the antioxidant response element in the modulation of antioxidant enzymes and phase II detoxifying enzymes expression (Lee *et al.*, 2011)

Phytochemicals are bioactive non nutrient components of various plant parts such as seeds, leaves, and rhizomes (Thangapazham *et al*., 2006). They are containing phenolic compounds (including flavonoid, tannin), which exhibit antioxidant property. The antioxidants exert their protective effects not only by scavenging ROS, but also by induction de novo expression of genes that encode detoxifying or defensive proteins, including phase II enzyme. A number of phytochemicals have been shown to induce expression of antioxidant and phase II enzymes via Nrf2 in different organs or cultured cells such as epigallocatechin-3-gallate from green tea up-regulates HO-1 expression in endothelial cells (Wu *et. al*., 2006). Bilberry extract up-regulates the oxidative stress defense enzymes HO-1 and GST-pi in cultured human retinal pigment epithelial cells (Milbury *et al.*, 2007). Blackberry extract elevates the protein expression levels of Nrf2, CuZnSOD, MnSOD, GPx-1/2, and HO-1 in rat liver (Cho *et al.*, 2011). The 6-Shogalo-rich extract from ginger up-regulates the antioxidant defenses such as Nrf2, HO-1, SOD2, GPx, and CAT in HepG2 cells and mice (Bak *et al.*, 2012). Moreover, curcumin, sulforaphane, and gingerol, resveratrol have been

also shown to induce expression of antioxidant and phase II enzymes (Surh, 2003; Na and Surh, 2008).

9. Fibroblast cells

Fibroblastic cell systems are useful model for general toxicological evaluation of chemicals relevant to environmental contamination (Stammati *et al*., 1981; Cereser *et al.*, 2001). Human skin fibroblasts are recently used to investigate the effect of drugs, toxic substances and ultraviolet radiation on common basic biochemical process in living cells (Keyse and Tyrrell, 1989; Moysan *et al*., 1993; Lee and Ho, 1995; Basu-Modak *et al*., 2003; Campo *et al*., 2004; Jeoung *et al*., 2004, Adetutu *et al.*, 2011). In addition, they are also used to investigate the bioactivity or protective effect of plant, herb, and fruit in the cells such as human skin fibroblasts (Foreskin ATCC CRL-1635) were used to study the ability of germinated seeds extract on respiratory activity, cell viability and proliferation of human skin fibroblasts, and their uselfulness as active cosmetic ingredient (Benaiges *et al.*, 2001). Normal human skin fibroblasts (AG13145) were used an in vitro model for study the effects of green tea extract on H_2O_2 induced necrosis (Silverberg *et al.*, 2011) and a primary human dermal fibroblast cells were used to study the antioxidant and anti-inflammatory activity of extracts and formulation of white tea, rose, and witch hazel (Thring, Hili and Naughton, 2011).

Human foreskin fibroblast cells (CCD-1064Sk, Cat No. CRL-2076) are widely use as a normal model in cells-based assay for study the effect of substances and drug on cells (Funk and Krise, 2006; Fuchs, 2009) and wound healing assay (Chen *et al*., 2001), then this cell was chosen in this study.

CHAPTER III
MATERIALS AND METHODS

Materials

Chemicals	Cat No.	Manufacturer or Distributer
Albumin, from bovine serum	A7906	Sigma-Aldrich**,** St. Louis, MO, USA
Ascorbic acid	A7506	Sigma-Aldrich**,** St. Louis, MO, USA
2,2′-azino-bis (3-ethylbenzo-thiazoline-6-sulfonic acid) diammonium salt (ABTS)	A1888	Sigma-Aldrich**,** St. Louis, MO, USA
n-Butanol	UN1120	UNIVAR, APS, NSW, Australia
Butylate hydroxyanisole (BHA)	B1253	Sigma-Aldrich**,** St. Louis, MO, USA
Butylated hydroxytoluene (BHT)	B1378	Sigma-Aldrich**,** St. Louis, MO, USA
Copper (II) chloride ($CuCl_2$)	CAS7447-39-4	Sigma-Aldrich, Steinheim, Germany)
Cu,ZnSOD from porcrine erythrocytes	S7571	Sigma-Aldrich**,** St. Louis, MO, USA
2-Deoxy-D-ribose	31170	Fluka, Steinheim, Germany
2′, 7′-Dichlorodihydrofluorescein diacetate (DCFH-DA)	D6883	Sigma-Aldrich**,** St. Louis, MO, USA
Dimethyl sulfoxide (DMSO)	60153	Merck, Darmstadt, Germany
3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT)	M2128	Sigma-Aldrich**,** St. Louis, MO, USA

Chemicals	Cat No.	Manufacturer or Distributer
2,2-Diphenyl-1-picrylhydrazyl (DPPH)	43180	Fluka, Steinheim, Germany
5-5′-Dithiobis-(2-nitrobenzoic acid) (DTNB)	D8130	Sigma Chemical Company, USA
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
Ferrous ammonium sulfate dodecahydrate ($FeNH_4(SO_4)_2.12H_2O$)	-	Ajax Finechem, NSW, Australia
Ferrous (III) chloride ($FeCl_3$)	UN1773	Merck, Darmstadt, Germany
Fetal bovine serum (FBS)	CH30160.02	Hyclone, Utah, USA
Folin-Ciocalteu's phenol reagent	F9252	Sigma-Aldrich, St. Louis, MO, USA
Gallic acid	G7384	Sigma-Aldrich, St. Louis, MO, USA
Glutathione (GSH), reduced form	G4251	Sigma-Aldrich, St. Louis, MO, USA
Glutathione reductase, from bakers yeast	G3664	Sigma-Aldrich, St. Louis, MO, USA
Goat polyclonal secondary antibody to rabbit IgG-H&L (HRP)	ab6721	Abcam, Cambridge, England
Hide powder, non-chromated	H0162	Sigma-Aldrich, St. Louis, MO, USA

Chemicals	Cat No.	Manufacturer or Distributer
Hydrogen peroxide (H_2O_2)	H/1750/17	Fisher Scientific, California, USA
6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox)	23,881-3	Sigma-Aldrich, Steinheim, Germany
Iscove's modified Dulbecco's medium	12200-036	Gibco, Invitrogen Corporation, NY, USA
Mouse monoclonal [mAbcam 8226] to beta actin, horseradish peroxidase conjugated	ab20272	Abcam, Cambridge, England
Napthylethylenediamine dihydrochloride (NED)	33461	Riedel-de Haen : trademark under licence form Riedel-de Haen GmbH
β-Nicotinamide adenine dinucleotide 2′-phosphate reduced tetrasodium salt hydrate (NADPH)	N1630	Sigma-Aldrich, St. Louis, MO, USA
β-Nicotinamide adenine dinucleotide reduced disodium salt hydrate (NADH)	N6005	Sigma-Aldrich, St. Louis, MO, USA
Nitrotetrazolium blue chloride (NBT)	N6876	Sigma-aldrich, St. Louis, MO, USA
Penicillin streptomycin	15140	Gibco, Invitrogen Corporation, NY, USA
Peroxidase	P-8000	Sigma chemical company, MO, USA
Phenazine methosulfate (PMS)	P9625	Sigma-Aldrich, St. Louis, MO, USA
Phosphoric acid	-	UNIVAR, Ajax Finechem, NSW, Australia

Chemicals	Cat No.	Manufacturer or Distributer
Phosphotungstric acid	GMB262910	Lobo Chemie, Mumbai, India
Potassium ferricyanide	-	Merk, Darmstadf, Germany
Potassium persulfate ($K_2S_2O_8$)	21, 622-4	Sigma, St. Louis, MO, USA
3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-$4^{/},4^{//}$-disulfonic acid monosodium salt (ferrozine)	82950	Fluka, USA
Pyrogallol	P0381	Sigma-Aldrich, Steinheim, Germany
Rabbit polyclonal catalase (CAT)	ab16731	Abcam, Cambridge, England
Rabbit polyclonal to glutathione peroxidase 1 (GPx)	ab22604	Abcam, Cambridge, England
Rabbit polyclonal to glutathione-S-transferase (GST)	ab6613	Abcam, Cambridge, England
Rabbit polyclonal heme oxygenase 1 (HO-1)	ab13243	Abcam, Cambridge, England
Rabbit polyclonal Nfr2	ab31163	Abcam, Cambridge, England
Rabbit polyclonal to superoxide dismutase 1 (Cu/Zn-SOD)	ab16831	Abcam, Cambridge, England
Sulphanilamide	404248	Fluka, Switzerland
Sodium carbonate (Na_2CO_3)	A463	Ajax Finechem, NSW, Australia
Sodium dodecyl sulfate (SDS)	1241	Ajax chemicals, Auburn, Australia

Chemicals	Cat No.	Manufacturer or Distributer
1,1,3,3-tetramethoxypropane (TEP)	T9889	Sigma-Aldrich, St. Louis, MO, USA
2-Thiobarbituric acid (TBA)	T5500	Sigma-Aldrich, St. Louis, MO, USA
Trichloroacetic acid	33731	Sigma-Aldrich, St. Louis, MO, USA
Triton X-100	93420	Fluka Chemie AG, Switzerland
Trypsin, from porcine pancreas	T4799	Sigma-Aldrich, St. Louis, MO, USA
Xanthine	X7375	Sigma-Aldrich, St. Louis, MO, USA
Xanthine oxidase, from bovine milk	X4500	Sigma-Aldrich, St. Louis, MO, USA

Equipments	Manufacturer or Distributer
Centrifuge (Allegra X-12R centrifuge)	BECKMAN COULTER, USA
Centrifuge (EBA 12R)	Hettich Zentrifugen, Tuttlingen
5% CO_2 incubator (3121)	Forma Scientific Inc, Massachusetts, USA
Hemocytometer (Bright-line)	Hausser Scientific, Pennsylvania, USA
Lyophilizer	FTS system, Dura Dry MD, Newyork
Microplate reader (Wallac 1420)	Perkin Elmer, Massachusetts, USA
Obital shaker	IKA-Schutter MTS4, Germany
Phase-contrast inverted microscope (CK30)	Olympus, Tokyo, Japan
Rotary evaporator (RE120)	Buchi, Flawil, Switzerland
Sonicate bath (S 30H)	Elmasinic, Germany
Spectrofluorometer (Jusco model FP-777)	Jusco corporation, Japan
Spectrophotometer (Spectronic Genesys 5)	Rochester, NY
Ultrasonic disintegrator (SONIPREP 150)	SANYO, UK

Plant sample

Ripened tamarind pods of the sour type, *Tamarindus indica* L. "Priao-Yak", were collected from Chanika Farm, Phetchabun province, Thailand. Herbarium specimens were preserved at the Museum of Natural Medicine, Faculty of Pharmaceutical Sciences, Chulalongkorn University.

Cell culture

Human foreskin fibroblasts CCD-1064Sk cells derived from newborn skin were purchased from American Type Culture Collection (CRL-2076, VA, USA).

Methods

1. Preparation of tamarind seed coat

The tamarind seeds were separated from the pulp and roasted in a pre-acid washed sand-bath at 150 °C for 1 h. The seeds were then quickly washed and dried in a hot air oven at 50°C for 24 h. The tamarind seed-coats were removed from the kernels, pulverized into a tamarind seed coat powder (TSCP) and stored in a desiccator until used.

2. Tamarind seed coat extraction

Tamarind seed coat powder (3 g) was extracted using four different solvent extraction systems:

TSCE-E was extracted with 70% ethanol by stirring the TSCP in 30 mL of 70% ethanol with magnetic stirrer for 4 h, centrifuged at 1,500 rpm for 10 min. The clear extract was collected and the residues were re-extracted with 30 mL aliquot of 70% ethanol until colorless extract was obtained. The pooled extract was filtered through Whatman No.1 filter paper and concentrated by using rotary evaporator at 40°C. The extracts were dried by using lyophilizer.

TSCE-EA was extracted with ethyl acetate by stirring the TSCP in 30 mL ethyl acetate with a magnetic stirrer for 4 h. The mixture was centrifuged at 1,500 rpm for 10 min and the clarified extract was collected. The residue was then re-extracted with 30 mL aliquot of ethyl acetate until a colorless extract was obtained. The pooled

extract was filtered and concentrated by rotary evaporator at 40°C, the extract was dried under a flow of nitrogen gas.

TSCE-EEA was extracted with 70% ethanol by stirring the TSCP in 30 mL of 70% ethanol with a magnetic stirrer for 4 h. The mixture was then centrifuged at 1,500 rpm for 10 min. The clarified extract was collected and the residue was re-extracted with 30 mL aliquot of 70% ethanol until a colorless extract was obtained. The pooled extract was filtered and concentrated by rotary evaporator at 40°C until half of the original volume was obtained. The resulting aqueous ethanol extract was partitioned with chloroform (1:1 by volume) to discard lipid and the upper layer of aqueous ethanol was collected and partitioned with ethyl acetate (1:1 by volume). The ethyl acetate layer was collected and concentrated by rotary evaporator at 40°C, and the extract was dried under a flow of nitrogen gas.

TSCE-W was extracted with boiling-water by adding TSPC into a cloth sack and dip in 200 mL boiling-water and boiled for 2 min. The sack was pressed and the red water extract was collected. The residue was then re-extracted with 200 mL aliquot of boiling-water until a colorless extract was obtained. The pooled extract was filtered, the clarified extract was partitioned with ethyl acetate (1:1 by volume). The ethyl acetate layer was collected and concentrated by rotary evaporator at 40°C and the extract was dried under a flow of nitrogen gas.

The tamarind seed coat extracts (TSCEs) were stored at 4°C until uses. TSCEs were dissolved in methanol to prepare a stock solution at 100 mg/mL and serial dilutions were prepared using distilled water before analysis.

3. Determination of the polyphenolic compounds

3.1 Total phenol content

The total phenol content was determined using a modified Folin-Ciocalteu method (Yu *et al*., 2008). Briefly, 0.5 mL of 50 μg/mL TSCEs solution was mixed with 0.5 mL of 1 N Folin-Ciocalteu reagent for 3 min, added 0.5 mL of 1% Na_2CO_3 and incubated for 2 h in a dark room at ambient temperature. The absorbance of the reaction mixture was measured at 760 nm using spectrophotometer (Spectronic Genesys 5, Rochester, NY). The total phenol content was calculated from a

calibration curve prepared using gallic acid as a standard, at concentrations ranging from 6.25 to 200 μg/mL. The total phenol content was expressed as grams of gallic acid equivalents (GAE) per 100 grams of the dry extract.

3.2 Tannin content

The tannin content was examined using a method described in the European Pharmacopoeia (2002). In brief, 100 μL of the test stock solution (100 mg/mL) was diluted to 25 mL with distilled water and then filtered. The first 5 mL of the filtrate was discarded and the rest of the filtrate was collected. Five mL of this filtrate was diluted to 25 mL with distilled water and 2 mL of this diluted solution was mixed with 1 mL Folin-Ciocalteu reagent and 10 mL of distilled water was added. This solution was then mixed and 12 mL of 29% Na_2CO_3 was added, mixed, and incubated until the reaction was completed (30 min). The absorbance (A_1) of the total polyphenols was measured at 760 nm.

The amount of polyphenol not absorbed by hide powder was examined by adding 10 mL of the test filtrate to 0.1 g hide powder, stirring for 60 min and filtering. Two mL of this filtrate was mixed with Folin-Ciocalteu reagent, distilled water and Na_2CO_3 as described above. The mixture was incubated for 30 min and the absorbance (A_2) was recorded. A solution of 2 mL pyrogallol (0.025 mg/mL) was used as a standard and the absorbance (A_3) was recorded. The percentage tannin content, expressed as pyrogallol, was calculated using the following equation:

$$\% \text{ Tannin} = (62.5 \times [A_1 - A_2] \times m_2)/A_3 \times m_1$$

Where m_1 and m_2 were the weight (g) of the sample and pyrogallol, respectively.

3.3 Proanthocyanidin content

Quantification of the total proanthocyanidin content was carried out using the alchohol-HCl-Fe^{3+} method (Hsieh, Shen, Kuo and Hwang, 2008). In brief, 0.25 mL of 500 μg/mL of TSCEs was added into 3 mL of *n*-butanol-HCl (95:5 v/v), 1 mL of 10% ferrous ammonium sulfate dodecahydrate ($NH_4Fe(SO_4)_2.12H_2O$) in HCl solution was added. The reaction mixture was shaken and incubated at 95°C for 40 min, a red coloration was developed and the reaction mixture was cooled to room temperature. The absorbance at 500 nm was measured against a reagent blank

(reagent without sample)**.** The proanthocyanidin content was expressed as the observed absorbance value.

4. HPLC analysis of phenolic compounds

Analysis of phenolic compounds in TSCE-W was performed according to the method described by Sudjaroen *et al.,* (2005). Analysis was carried out on a C-18 hypersil gold column (Thermo Electron Corporation, UK, internal diameter 4.6 x 250 mm, particle size 5 μm) using HPLC instrument with a UV spectrophotometric detector (Shimadzu, Japan) at 278 mm. A mobile phase consisted of 2% acetic acid in double distilled water (A) and methanol (B). The 0.5 mL of TSCE-W (2.5 mg/mL) was applied on the column and eluted by gradient elution with 1 mL/min flow rate, 50 min total run time and a complete run was monitored as the following: 95% A for 10 min, 90% A for 10 min, 85% A for 10 min, 80% A for 10 min, 60% A for 5 min and 0% A for 5 min. Compounds were identified by co-injection with standard reference mixture of phenolic compounds.

5. Determination of free radicals scavenging in a cell-free system

5.1 DPPH radical scavenging activity

The antioxidant activity was assessed in terms of their ability in hydrogen donating or radical scavenging activity, the method using the stable 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical (Singh *et al*., 2007). Briefly, the reaction mixture contained 300 μL of various concentrations (0-100 μg/mL) of TSCEs and 2 mL of 0.1 mM DPPH in methanol. The mixture was incubated in a dark room at ambient temperature for 30 min. The decolorized purple to yellow mixture was observed, and the absorbance was measured at 517 nm against a methanol blank. Butylated hydroxyanisole (BHA) and Trolox (a water soluble vitamin E analogue) were used as reference antioxidant. The DPPH radical scavenging activity (%) was calculated using the following equation:

$$\text{DPPH radical scavenging (\%)} = [A_D\text{-}A_S/A_D] \times 100$$

Where A_D was an absorbance of DPPH solution and A_S was an absorbance of TSCEs/reference antioxidant. The antioxidant activity was expressed as EC_{50} (half maximal effective concentration) by using a linear regression analysis.

5.2 ABTS$^{\bullet+}$ scavenging activity

ABTS$^{\bullet+}$ scavenging activity was estimated the total antioxidant capacity (TAC) for TSCEs using the Trolox equivalent antioxidant capacity (TEAC) assay, as described by Gođevac, Vujisić, Mojović, Ignjatović, Spasojević and Vajs (2008). This assay is based on the scavenging of the stable blue-green ABTS (2,2'-azino-bis(3-ethylbenzo-thiazoline-6-sulfonic acid) diammonium salt radical (ABTS$^{\bullet+}$) into a colorless product, with the activity measured in terms of electron or hydrogen donation. Briefly, a stock solution of ABTS$^{\bullet+}$ was prepared from the reaction of 7 mM ABTS and 2.45 mM potassium persulfate in 0.01 M phosphate-buffered saline (PBS) at pH 7.4. This mixture was allowed to stand in a dark room for 12-16 h before use. The ABTS$^{\bullet+}$ stock solution was diluted with PBS to obtain an absorbance of 0.70±0.02 at 734 nm and then used for further analysis. One hundred ml of the TSCEs (12 μg/mL) were added to 900 μL of diluted ABTS$^{\bullet+}$ solution, mixed and allowed to stand for 5 min. The absorbance at 734 nm was measured. Trolox was used for calibration. BHA was used as a reference antioxidant. The results were recorded as μmol of TEAC per milligram of sample. Higher values indicate stronger antioxidant activity.

5.3 Superoxide anion scavenging activity

The superoxide anion scavenging activity was evaluated by measuring the reduction of nitroblue tetrazolium (NBT) in a PMS-NADH system, using a method modified by Choi, Chang, Cho and Chun (2007). Generation of the superoxide anion was measured in the reaction mixture containing 20 μL of various concentrations (0-1,000 μg/mL) of TSCEs, 60 μL of 553 μM NADH, 60 μL of 143 μM NBT, and 60 μL of 9 μM PMS in 0.1 M phosphate buffer (pH 7.4), incubated at room temperature for 8 min. The superoxide anion was measured as the reduction of yellow NBT to the purple formazan, using a microplate reader at 540 nm (Wallac 1420, PerkinElmer Ltd.). The scavenging ability of TSCEs on the superoxide anion was calculated as the inhibition of NBT reduction using the following equation:

$$\text{Superoxide anion scavenging (\%)} = [A_B\text{-}A_S/A_B] \times 100$$

Where A_B was an absorbance of the reagent blank and A_S was an absorbance of the TSCEs/reference antioxidant. The EC_{50} values were determined.

5.4 Hydrogen peroxide scavenging activity

The Hydrogen peroxide scavenging activity was evaluated based on the scavenging of H_2O_2 in a ABTS-peroxidase system, as described by Athukorala, Kim and Jeon (2006). The reaction mixture consisted of 20 μL of various concentrations (0-200 μg/mL) of TSCEs, 20 μL of 10 mM H_2O_2 and 100 μL of 0.1 M phosphate buffer (pH 5) in a 96 microwell plates and incubated at 37^{o}C for 5 min. The 30 μL of 1.25 mM ABTS (freshly prepared) and 30 μL of peroxidase (1 U/mL) were added and incubated at 37°C for a further 10 min. The reduction of the blue-green color was measured as an absorbance at 405 nm using a microplate reader. Hydrogen peroxide scavenging activity (%) was calculated using the following equation:

$$\text{Hydrogen peroxide scavenging (\%)} = [A_B\text{-}A_S/A_B] \times 100$$

Where A_B was an absorbance of the reagent blank and A_S was an absorbance of the TSCEs/reference antioxidant. The EC_{50} values were determined.

5.5 Hydroxyl radical scavenging activity

The hydroxyl radical scavenging activity was evaluated by measuring the formation of thiobarbituric acid reactive substance (TBARS), using 2-deoxyribose as a substrate, as described by Suksomtip *et al*. (2010). The reaction mixture contained 250 μL of various concentrations (0-128 μg/mL) of TSCEs, 250 μL of 100 mM potassium phosphate buffer (pH 7.4), 100 μL of 28 mM 2-deoxyribose, 200 μL of Fe^{3+}-EDTA (100 μM $FeCl_3$ + 104 μM Na_2EDTA in 1:1 ratio by volume), 100 μL of 1 mM H_2O_2 and 100 μL of 1 mM ascorbic acid. The reaction was mixed and incubated at 37°C for 1 h, then 1 mL of 2.8% TCA and 1 mL of 1% TBA were added to stop the reaction. The reaction mixture was heated at 100°C for 15 min, cooled and centrifuged at 1,500 rpm for 10 min. The absorbance of the colored product was measured at 532 nm against a reagent blank. Hydroxyl radical scavenging activity (%) was calculated using the following equation:

$$\text{Hydroxyl radical scavenging (\%)} = [A_B\text{-}A_S/A_B] \times 100$$

Where A_B was an absorbance of the reagent blank and A_S was an absorbance of the TSCEs/reference antioxidant. The EC_{50} values were determined.

5.6 Nitric oxide scavenging activity

The ability of TSCEs to scavenge NO was examined using the method described by Gurav *et al.* (2007) with slight modification. Nitric oxide was generated from sodium nitroprusside in an aqueous solution (spontaneous at physiological pH) via interaction with oxygen to produce nitrite ions that can be examined using Griess reagent. Briefly, a solution of 1.5 mL of 5 mM sodium nitroprusside in PBS (pH 7.4) was mixed with 0.5 mL of TSCEs at various concentrations (0-100 μg/mL), and the mixture was incubated at 25°C for 150 min, mixed 1 mL of clear upper layer of the reaction mixture with 1 mL of Griess reagent (1% sulphanilamide, 2% phosphoric acid and 0.1% napthylethylenediamine dihydrochloride (NED). The chromophore generated by diazotization of nitrite ions with sulfanilamide and subsequent coupling with NED was measured an absorbance at 546 nm against a reagent blank. Nitric oxide scavenging activity (%) was calculated using the following equation:

$$\text{Nitric oxide scavenging (\%)} = [A_B\text{-}A_S/A_B] \times 100$$

Where A_B was an absorbance of the reagent blank and A_S was an absorbance of the TSCEs/reference antioxidant. The EC_{50} values were determined.

6. Antioxidant activities in a cell-free system

6.1 Reducing power activity

The reducing power was determined based on the measurement of a Fe^{3+}-Fe^{2+} transformation. This transformation occurs in the presence of reductones, which exert antioxidant action by breaking the free radical chain through the donation of a hydrogen atom. The reducing power was evaluated by slightly modified the method described by Jiang *et al.* (2005). Briefly, 0.5 mL of various concentrations (0-200 μg/mL) of the TSCEs, 1 mL of 0.2 M phosphate buffer (pH 6.6), and 1 mL of 1% potassium ferricyanide was mixed and incubated at 50°C for 20 min, then the reaction was quenched by adding 1 mL of 10% TCA and then centrifuged at 3,600 rpm for 10 min. The clear upper layer of 2 mL was pipetted into 2 mL of distilled water and mixed with 0.5 mL of 0.1% $FeCl_3$ (freshly prepared), leading to the generation of a green product. An absorbance was measured at 700 nm against a reagent blank. Higher absorbance indicates a stronger reducing power, and the reducing power was

expressed as an effective concentration at the absorbance 0.5 by using linear regression analysis.

6.2 Metal chelating activity

The metal chelating activity was evaluated by measuring the ability of the TSCEs to chelate ferrous ion (Fe^{2+}), following the method described by Geckil *et al.* (2005). The reaction mixture composed of 1.7 mL distilled water, 50 μL of 2 mM $FeCl_2.4H_2O$ and 50 μL of various concentrations (0-1,000 μg/mL) of TSCEs and the mixture was vigorously stirred for 1 min. The reaction was initiated by adding 0.2 mL of 5 mM ferrozine and then vigorously stirring the reaction mixture for 10 min. The disruption of the Fe^{2+}-ferrozine complex was measured the absorbance at 562 nm. The chelating agent ethylenediaminetetraacetic acid di-sodium salt (Na_2EDTA) was used as a positive control. The percent inhibition of the Fe^{2+}-ferrozine complex against a reagent blank was calculated using the following equation:

$$\text{Metal chelation (\%)} = [(A_B-A_S)/A_B] \times 100$$

Where A_B was an absorbance of the reagent control and A_S was an absorbance of the TSCEs/reference antioxidant or Na_2EDTA.

6.3 Anti-lipid peroxidation activity

The inhibition of lipid peroxidation was evaluated by measuring the formation of malondialdehyde (MDA), a secondary end product from the oxidation of polyunsaturated fatty acid, as described by Jiang *et al.* (2005). A total volume of 500 μL of yolk suspension (previously prepared by adding an equal volume of PBS (pH 7.4) and egg–yolk, stirring vigorously and the mixture was diluted to 40 times the volume with PBS) was mixed with 250 μL of various concentrations (0-1,000 μg/mL) of TSCEs and 500 μL of 24 mM $FeSO_4$ in PBS and incubated at 37°C for 15 min, added 500 μL of 20% TCA to stop the reaction and 1 mL of 0.8% TBA was mixed and heated at 100°C for 15 min, cooled and centrifuged at 3,600 rpm for 10 min to precipitate protein. An absorbance of the colored upper layer of the reaction mixture was measured at 532 nm. The inhibition of lipid peroxidation (%) was calculated using the following equation:

Inhibition of lipid peroxidation (%) = $[(A_B-A_S)/A_B] \times 100$

Where A_B was an absorbance of the reagent blank and A_S was an absorbance of TSCEs/reference antioxidant. The EC_{50} values were determined.

7. Cell culture

CCD-1064Sk cells were grown and maintained in Iscove's modified Dulbecco's medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin and 100 μg/mL streptomycin and maintained at 37°C in a humidified atmosphere of 5% CO_2 incubator. The culture medium was changed every 2-3 days and the cells were sub-cultured every 3-4 days with 0.25% trypsin and 1 mM EDTA. The cells between 10 to 20 passages grown to 80% confluence were used in all experiment tested. The vehicle control was the cells treated with 0.25% DMSO in medium.

8. Determination of cell viability

8.1 Effect of TSCE-W on cell viability using MTT assay

The MTT reduction assay is used to evaluate cell viability based on the reduction of a yellow tetrazolium salt to the blue or purple colored formazan salt by mitochondrial succinate dehydrogenase in living cells (Basu-Modak *et al.*, 2003). The cells were seeded into 96-well plates at a density of 1 x 10^5 cells/mL and incubated for 24 h. After removal of medium, the cells were treated with various concentrations of TSCE-W (0.05, 0.1, 0.2, 0.4, 0.8 and 1 mg/mL in final concentration of 0.25% DMSO) for 24 h. After incubation time, the medium was removed and the cells were washed twice with 0.01 M PBS (pH 7.4) to prevent color interference of the extracts. The cells were incubated with 100 μL of MTT solution (0.4 mg/mL in incomplete medium) for 4 h. After removal of medium, the purple formazan salt was dissolved in 100 μL of 100% DMSO and then shaken for 15 min. The absorbance of purple color was measured at 570 nm by using a microplate reader. The percentage of cell viability was calculated by the equation:

Cell viability (%) = [(As-Asb)/Ac] x 100

Where As was an absorbance of cells treated with TSCE-W, Asb was an absorbance of sample blank containing TSCE-W without cells (to prevent interference of the extracts), Ac was an absorbance of vehicle control.

8.2 Effect of TSCE-W on cell viability using neutral red assay

The neutral red assay is used to evaluate cell viability based on the uptake and accumulation of neutral red in the lysosome of living cell. Neutral red is a water soluble dye, weakly basic that passively passes through the plasma membrane and accumulates in the lysosome in living cell. In damage cells, neutral red is no longer retained by the lysosome and lost from the cells, as the plasma membrane does not act as a barrier (Basu-Modak *et. al.,* 2003). The assay was modified from the method of Chiba *et al.* (1998) and Babich *et al*. (2005). The CCD-1064Sk cells were seeded in 96- well plates at a density of 1 x 10^5 cells/mL and incubated for 24 h. After removal of medium, the cells were treated with 100 μL of 0.05-1 mg/mL concentration of TSCE-W for 24 h and then washed twice with 100 μL of PBS (pH 7.4). The 100 μL of neutral red medium (40 μg/mL neutral red in medium) and incubated at 37°C for 3 h. The neutral red medium was prepared (4 mg/mL aqueous stock suspension of neutral red) following by a foil wrapped stored at room temperature, and neutral red stock suspension was diluted to a working concentration at 40 μg/mL neutral red in medium and incubated overnight at 37°C. The neutral red medium was centrifuged at 3,000 rpm for 10 min to remove the fine dye crystals before used. After incubated cell with neutral red medium at 37°C for 3 h, the neutral red medium was removed and rapidly washed and fixed with 100 μL of fixing solution (0.5% formalin-1% $CaCl_2$ (v/v)) The dye was extracted from the cells by adding 100 μL of extraction solution (1% acetic acid-50% ethanol), incubated at 37°C for 20 min, and then agitated on a rocking platform for 5 min. An absorbance was measured at 570 nm using micropalte reader. The cell viability was calculated by the equation:

$$\text{Cell viability (\%)} = [(As-Asb)/(Ac-Acb)] \times 100$$

Where As was the absorbance of cells treated with various concentration of TSCE-W, Asb was the absorbance of sample blank containing only TSCE-W without cell, Ac was the absorbance of cell vehicle control and Acb was the absorbance of vehicle control without cell.

8.3 Effect of TSCE-W on cell viability using Hoechst 33342 staining

The nuclear morphology of the cells was evaluated using cell-permeable DNA dye (Hoechst 33342). Cells with homogeneously stained were considered viable, whereas the presence of chromatin condensation and/or fragmentation was indicative of apoptosis according to the slightly modified method of Heo *et al.* (2008). The CCD-1064Sk cells were seeded in 96-well plates at a density of 1 x 10^5 cells/mL and incubated for 24 h. After removal of medium, the cells were treated with 100 µL of 0.05-1 mg/mL TSCE-W for 24 h. The 100 µL of Hoechst 33342 (stock 1 mg/mL), a DNA-specific fluorescent dye, were added, followed by 10 min of incubation at 37°C. The stained cells were then observed under a fluorescence microscope in order to examine the degree of nuclear condensation.

8.4 Effect of TSCE-W on cell viability using trypan blue exclusion assay

Trypan blue dye, a large molecule, can penetrate cells that have damaged membranes, described by Arrigo *et al*. (2005). The CCD-1064Sk cells were seeded in 96-well plates at a density of 1 x 10^5 cells/mL and incubated for 24 h, the medium was removed and then the cells were treated with 100 µL of 0.05-1 mg/mL TSCE-W for 24 h. The cells were trypsinized and stained with trypan blue, and the viable cells were counted on a hemocytometer in triplicate wells. Three replicates of counting were performed for each well.

8.5 Effect of H_2O_2 on cell viability using MTT assay

The cells were seeded into 96-well plates at a density of 1 x 10^5 cells/mL and incubated for 24 h. After removal of medium, the cells were treated with H_2O_2 at concentrations ranging from 0.1 to 10 mM for 0.5, 1, 3, 6 and 12 h. The MTT solution (0.4 mg/mL in incomplete medium) was added and incubated for 4 h, 100% DMSO was added and then shaken. The absorbance of purple color was measured at 570 nm. The percentage of cell viability was calculated by the equation:

$$\text{Cell viability (\%)} = [As/Ac] \times 100$$

Where As was an absorbance of cell treated with H_2O_2 and Ac was an absorbance of vehicle control.

8.6 Cytoprotective effect of TSCE-W on viability of H_2O_2-treated cells

The protective of TSCE-W on cell viability in H_2O_2-induced oxidative stress was determined using MTT and neutral red assay. The cells were seeded into 96-well plates at a density of 1 x 10^5 cells/mL for 24 h. After removal of medium, the cells were pre-treated with TSCE-W (0.05-1 mg/mL) for 24 h. The cells were washed twice with PBS and incubated with 2 mM H_2O_2 for 30 min. The medium was removed and MTT solution or neutral red solution was added. The reaction was incubated and carried out as previously described. The percentage of cell viability was calculated by the equation:

$$\text{Cell viability (\%)} = [(As-Asb)/Ac] \times 100$$

Where As was an absorbance of cells pre-treated with TSCE-W for 24 h and exposure to 2 mM H_2O_2 for 30 min, Asb was an absorbance of sample blank containing TSCE-W without cells, Ac was an absorbance of cells vehicle control.

9. Determination of intracellular ROS using DFH-DA assay

9.1 Effect of TSCE-W or H_2O_2 on intracellular ROS

The intracellular ROS level was investigated using DCFH-DA assay as described by Girard-Lalancette *et al*. (2009). DCFH-DA, a non-polar and non-ionic compound, that readily diffuses through the cell membrane and is hydrolyzed to 2′,7′ dichlorofluorescin diacetate (DCFH) by intracellular esterase. The non-fluorescence DCFH interacts with intracellular ROS or oxidants and converts to the fluorescence 2′,7′ dichlorofluorescin (DCF) (Heo *et al*., 2008; Zhang *et al*., 2008; Girard-Lalancette *et al.,* 2009). Hence, the intracellular production of ROS is determined by measuring the intensity of DCF fluorescence.

The cells were seeded into 96-well plates at a density of 1 x 10^5 cells/mL and allowed to grow for 24 h. The cells were treated with TSCE-W (0.05-1 mg/mL) for 24 h or H_2O_2 (0.1-2 mM) for 0.25, 0.5, 1 and 3 h. The cells were then washed twice with 100 μL of cold PBS and 100 μL of 0.25% DMSO in medium containing 5 μM of DCFH-DA was added, allowed to stand for 30 min. After removal of DCFH-DA medium, the cells were washed once with 100 μL of cold PBS and 100 μL of cold PBS was added. The fluorescence intensity of DCF was measured by using a

microplate reader at an excitation wavelength of 485 nm and an emission wavelength of 535 nm. The percentage of intracellular ROS was calculated following an equation:

$$\text{Intracellular ROS (\%)} = [1-(F_s/F_c)] \times 100$$

Where F_s was the fluorescence intensity of cells treated with TSCE-W or H_2O_2, and F_c was the fluorescence intensity of vehicle control.

9.2 Effect of TSCE-W on intracellular ROS in H_2O_2-treated cells

The cells were seeded into 96-well plates at a density of 1 x 10^5 cells/mL and incubated for 24 h. After removal of medium, the cells were pre-treated with TSCE-W (0.05-1 mg/mL) for 24 h. The cells were washed twice with cold PBS and 5 μM of DCFH-DA was added and incubated for 30 min. After removal of DCFH-DA, the cells were washed with cold PBS and 1 mM H_2O_2 was added and further incubated at 37 °C for 15 min for generating intracellular ROS. The fluorescence intensity of DCF was measured by using a microplate reader at an excitation wavelength of 485 nm and an emission wavelength of 535 nm. The percentage of intracellular ROS was calculated following an equation:

$$\text{Intracellular ROS (\%)} = [1-(F_{-s}/F_c)] \times 100$$

Where F_s was the fluorescence intensity of the cells pre-treated with TSCE-W for 24 h and followed by 1 mM H_2O_2 for 15 min, F_c was the fluorescence intensity of vehicle control.

10. Determination of lipid peroxidation in the cells using TBARS assay

10.1 Effect of TSCE-W on lipid peroxidation in the cells

Lipid peroxidation was determined by measuring a level of MDA, an end product of lipid peroxidation, using a TBARS assay by following the slightly modified method of Chirdchupunseree and Pramyothin (2010). The cells were seeded into 6-well plates at a density of 1 x 10^5 cells/mL and incubated for 24 h. The cells were treated with TSCE-W (0.05-1 mg/mL) and then incubated for 24 h. The cells were washed twice with 1 mL of cold PBS and lysed on ice with 300 μL of 2% SDS for 30 min. The cell lysates were scraped and transferred into a glass test tube and adjusted to 1 mL with 2% SDS. Three milliliter of the reaction mixture contained 950 μL of cell lysates, 50 μL of 4% BHT, 1 mL of 10% phosphotungstric acid, and 1 mL

of 0.7% TBA. The reaction mixture was mixed and heated at 100°C for 1 h, the reaction was stopped in cooling water and 4 mL of *n*-butanol was added, mixed and centrifuged at 3,600 rpm for 10 min. The fluorescence of the supernatant was measured by using a spectrofluorometer with an excitation wavelength at 515 nm and an emission wavelength at 553 nm. MDA level was calculated from the standard curve of 1,1,3,3-tetramethoxypropane (TEP). The values were expressed as nmol MDA/mg protein. Protein content was determined by Bradford method (Bradford, 1976).

10.2 Effect of H_2O_2 on cells lipid peroxidation

The cells were seeded into 6 well plates at a density of 1 x 10^5 cells/mL and incubated for 24 h. The medium was removed and 2 mL of 0.25% DMSO in medium was added in each well and incubated for 24 h. The cells were added with H_2O_2 (1 and 2 mM) for 0.25, 0.5, 1, 3 and 6 h. The cells were washed twice with 1 mL of cold PBS, lysed and MDA level was determined as previously described**.** Briefly, the 3 mL of reaction mixture contained 950 µL of cell lysates, 50 µL of 4% BHT, 1 mL of 10% phosphotungstic acid, and 1 mL of 0.7% TBA. The reaction mixture was mixed and heated at 100°C for 1 h. After incubation, the reaction was stopped in cooling water and 4 mL of *n*-butanol was added, mixed and centrifuged at 3,600 rpm for 10 min. The fluorescence of the supernatant was measured by using a spectrofluorometer with an excitation wavelength at 515 nm and an emission wavelength at 553 nm. MDA level was calculated from standard curve of 1,1,3,3-tetramethoxypropane (TEP).

10.3 Effect of TSCE-W on lipid peroxidation in H_2O_2-treated cells

The cells were seeded into 6 well plates at a density of 1 x 10^5 cells/mL and incubated for 24 h. The cells were pre-treated with TSCE-W (0.05-1 mg/mL) for 24 h and treated with 2 mM H_2O_2 for 1 h. The cells were washed, lysed and MDA level in cells was determined according to the previously described. Briefly, 3 mL of the reaction mixture contained 950 µL of cell lysates, 50 µL of 4% BHT, 1 mL of 10% phosphotungstric acid, and 1 mL of 0.7% TBA. The reaction mixture was mixed and heated at 100°C for 1 h. The reaction was stopped in cooling water and 4 mL of

n-butanol was added, mixed and centrifuged at 3,600 rpm for 10 min. The fluorescence of the supernatant was measured by using a spectrofluorometer with an excitation wavelength at 515 nm and an emission wavelength at 553 nm. MDA level was calculated from standard curve of 1,1,3,3-tetramethoxypropane (TEP).

11. Determination of total intracellular GSH level

11.1 Effect of TSCE-W on total intracellular GSH level

The level of total intracellular GSH was measured based on enzymatic recycling assay (Tietze, 1969). In circumstances of oxidative stress, GSH (reduced form) is converted to GSSG (oxidized form). The GSSG is catalysed by glutathione reductase in the presence of NADPH to generate a high intracellular GSH level. The GSH is reacted with Ellman's reagent (5,5'-dithiobis-2-nitrobenzoic acid; DTNB) to produce a yellow colored product of the 2-nitro-5-thiobenzoic acid (TNB) (Eady *et al*., 1995). The rate of TNB can be measured by using spectrophotometer. This recycling reaction improves the sensitivity of total GSH (the sum of reduced GSH and oxidized GSH) detection.

The cells were seeded into 6 well plates at a density of 1 x 10^5 cells/mL and incubated for 24 h. Thereafter, the cells were treated with TSCE-W (0.05-1 mg/mL) for 24 h. After removal of medium, the cells were washed twice with 1 mL of cold PBS, the cells were trypsinized (Susanto *et al.*, 1998) and followed by centrifugation at 225 x *g* at 4°C for 5 min (Gille *et al.*, 1987). The cell pellets were washed twice with 1 mL of cold PBS to remove exogenous GSH (Jeoung *et al*., 2004). The cell pellets were lysed in 100 µL of 0.05% Triton X-100 with 0.5 mM EDTA in PBS by vigorously mixing for 10 s (Gille *et al,* 1987) and sonicated on ice using ultrasonic bath for 2 s. The protein content in cell lysates (10 µL) was determined by Bradford method. The rest of the cell lysates were deproteinized by adding 90 µL of 5% TCA, vigorously mixing for 10 s and centrifuged at 10,000 x *g* at 4°C for 5 min. The supernatant was stored at -80°C until assay.

Total intracellular glutathione GSH was determined by using the modified method of Chirdchupunseree and Pramyothin (2010). The cell lysates (10 µL) were neutralized with 90 µL of 0.1 M sodium phosphate buffer containing 1 mM EDTA

(pH 7.5). Thereafter, the 10 μL of 10 U GR, 80 μL of 0.25 mM NADPH and 10 μL of 1.5 mM DTNB were added, respectively. The rate of TNB production was measured at 405 nm by using a microplate reader at 30 s intervals for 10 min. The total intracellular GSH level was analyzed by the kinetic method from a linear standard curve of GSH. The value was expressed in μM per mg protein.

11.2 Effect of H_2O_2 on total intracellular GSH level

The cells were seeded into 6 well plates at a density of 1 x 10^5 cells/mL and incubated for 24 h. The medium was removed and 2 mL of 0.25% DMSO in medium was added and incubated for 24 h. The cells were added with H_2O_2 (1 and 2 mM) for 0.25, 0.5 and 1 h. The cells were collected and the total intracellular GSH level was determined according to the previously described. Briefly, the reaction mixture contained 10 μL of cell lysates, 90 μL of 0.1 M sodium phosphate buffer containing 1 mM EDTA (pH 7.5), 10 μL of 10 U GR, 80 μL of 0.25 mM NADPH and 10 μL of 1.5 mM DTNB. The rate of TNB production was measured at 405 nm by using a microplate reader at 30 s intervals for 10 min. The total intracellular GSH level was analyzed by the kinetic method from a linear standard curve of GSH. The value was expressed in μM per mg protein.

11.3 Effect of TSCE-W on total intracellular GSH level in H_2O_2-treated cells

The activity of TSCE-W on the level of total intracellular glutathione in H_2O_2-treated cells was measured as described previously. After removal of the medium, the cells were seeded into 6 well plates at a density of 1 x 10^5 cells/mL and incubated for 24 h. The cells were pre-treated with various concentrations of TSCE-W (0.05-1 mg/mL) for 24 h. Thereafter, the cells were exposed with 1 mM H_2O_2 for 30 min. The cells were collected and total intracellular GSH level was determined. Briefly, the reaction mixture contained 10 μL of cell lysates, 90 μL of 0.1 M sodium phosphate buffer containing 1 mM EDTA, pH 7.5, 10 μL of 10 U GR, 80 μL of 0.25 mM NADPH and 10 μL of 1.5 mM DTNB. The rate of TNB production was measured at 405 nm using a microplate reader at 30 s intervals for 10 min. The total

intracellular GSH level was analyzed by the kinetic method from a linear standard curve of GSH. The value was expressed in μM per mg protein.

12. Determination of SOD activity by using NBT reduction assay

12.1 Effect of TSCE-W on SOD activity

SOD activity was evaluated by the method based on the inhibition of NBT reduction by the xanthine-hypoxanthine oxidase system (Sun, Oberley and Li, 1988). The cells were seeded into 6 well plates at a density of 1 x 10^5 cells/mL and incubated for 24 h. After removal of medium, the cells were added with TSCE-W (0.05-1 mg/mL) for 24 h and then washed twice with 1 mL of cold PBS. The cells were harvested by trypsinization according to the method modified by Wijeratne *et al*.(2005). Cells were centrifuged at 130 x *g* at 4°C for 5 min. Cell pellets were washed twice with 1 mL of cold PBS and resuspened in 600 μL of 50 mM $NaHCO_3$ buffer, pH 10.2. Thereafter, the cells were lysed on ice by using ultrasonic disintegrator at 5 amplitude microns every 2 s for 10 s. Cell lysates were centrifuged at 20,000 x *g* at 4°C for 10 min. The supernatant was stored at -80°C until assay.

Total SOD activity was evaluated according to the method of Sun *et al.* (1988). The reaction mixture contained 2.45 mL of SOD assay reagent (0.12 mM xanthine, 0.12 mM EDTA, 30 μM NBT, 48 mM Na_2CO_3 (pH 10.2) and 0.06 g/L bovine serum albumin) and 0.5 mL of cell lysate. The reaction was initiated by adding 50 μL of xanthine oxidase (167 U/L, a final concentration) and incubated at 25°C for 20 min and the reaction was stopped by adding 1 mL of 0.8 mM $CuCl_2$ and then mixed. The reduction of NBT was measured at 560 nm. The inhibition (%) of NBT reduction was calculated from the equation:

$$\text{Inhibition (\%)} = [A_b\text{-}A_s/A_b] \times 100$$

Where A_b was an absorbance of reagent blank and A_s was an absorbance of TSCE-W. The percent inhibition was calculated from the standard curve of Cu,ZnSOD (0-75 ng/mL). A specific SOD activity was expressed as units per milligram protein, where 1 unit of enzyme activity was defined as the amount of enzyme that inhibits the rate of NBT reduction by 50 %.

12.2 Effect of H_2O_2 on SOD activity

The cells were seeded into 6 well plates at a density of 1 x 10^5 cells/mL and incubated for 24 h. The medium was removed and 2 mL of 0.25% DMSO in medium was added and incubated for 24 h, the medium was removed, the cells were added with H_2O_2 (1 and 2 mM) for 0.25, 0.5, 1 and 3 h. The cells were collected and SOD activity was determined as previously described. Shortly, the 0.5 mL of cell lysate was added into 2.45 mL of SOD assay reagent. The 50 μL of xanthine oxidase (167 U/L, a final concentration) was added and then incubated at 25°C for 20 min. One milliliter of 0.8 mM $CuCl_2$ was added, mixed, and the reduction of NBT was measured at 560 nm. The inhibition (%) of NBT reduction was calculated from the equation:

$$\text{Inhibition (\%)} = [A_b\text{-}A_s/A_b] \times 100$$

Where A_b was an absorbance of reagent blank and A_s was an absorbance of H_2O_2-treated cells.

12.3 Effect of TSCE-W on SOD activity in H_2O_2-treated cells

The cells were seeded into 6 well plates at a density of 1 x 10^5 cells/mL and incubated for 24 h. After removal of medium, the cells were pre-treated with TSCE-W (0.05-1 mg/mL) for 24 h. Thereafter, the cells were exposed with 2 mM H_2O_2 for 15 min. The cells were collected and SOD activity was determined as previously described. Shortly, the 0.5 mL of cell lysate was added into 2.45 mL of SOD assay reagent. The 50 μL of xanthine oxidase (167 U/L, a final concentration) was added and then incubated at 25°C for 20 min. The 1 mL of 0.8 mM $CuCl_2$ was added, mixed, and the reduction of NBT was measured at 560 nm. The inhibition (%) of NBT reduction was calculated from the equation:

$$\text{Inhibition (\%)} = [A_b\text{-}A_s/A_b] \times 100$$

Where A_b was an absorbance of reagent blank and A_s was an absorbance of the cells pre-treated with TSCE-W for 24 h and followed by 2 mM H_2O_2 for 15 min

13. Determination of GPx activity

13.1 Effect of TSCE-W on GPx activity

GPx activity was measured according to the method of Vives-Bauza, Starkov, and Garcia-Arumi (2007). The cells were seeded into 6 well plates at a density of 1 x 10^5 cells/mL and incubated for 24 h. After removal of medium, the cells were added with TSCE-W (0.05-1 mg/mL) for 24 h and then washed twice with 1 mL of cold PBS. The cells were harvested by trypsinization and centrifuged at 130 x *g* at 4°C for 5 min. Cell pellets were washed twice with 1 mL of cold PBS and then resuspened in 200 μL of PBS (1 mL PBS per 40 million cells). Cell pellets were stored at -80°C until assay.

Immediately before the assay, cell pellets were thawed and resuspened in ice-cold 0.013% sodium cholate (20 million cells/mL). The cells were sonicated on ice every 2 s for 10 s and then centrifuged at 12,000 x *g* at 4°C for 10 min. The 0.1 mL of cell supernatant (phosphate buffer was used in blank) was added into the reaction mixture contained 0.1 mL of 50 mM phosphate buffer (pH 7.0), 0.1 mL of 1 mM EDTA, 0.2 mL of 5 mM NaN_3 (for blocking activity of CAT), 0.2 mL of 1 mM NADPH, 6.4 μL of glutathione reductase (from a stock of 0.312 U/ml), 0.2 mL of 5 mM GSH. The reaction mixture was mixed by gentle agitation and incubated for 5 min in order to eliminate the nonspecific degradation of NADPH. Then, 0.2 mL of 0.25 mM H_2O_2 (freshly prepared) was added to initiate the reaction. The decay of NADPH was monitored for 5 min at 340 nm. The rate of the decreasing of the $\Delta A_{340\ nm}$ is directly proportional to the GPx activity in the sample. The linear rate was used to calculate the activity, which was reported as units per mg of protein according to the equation:

$$\text{Units/mg protein} = \Delta A/\text{min}_s \times (V_t/V_s) \times 1/\varepsilon \times (1/\text{prot})$$

Where $\Delta A/\text{min}_s$ was changed in absorbance per minute for the sample, V_t was the total volume (mL) of the assay, V_s was the sample volume (mL), ε was the extinction coefficient of NADPH (6.22 $nM^{-1}cm^{-1}$). One enzyme unit of GPx activity was defined as 1 μmol of NADPH oxidized per minute at 37°C.

13.2 Effect of H_2O_2 on GPx activity

The cells were seeded into 6 well plates at a density of 1 x 10^5 cells/mL and incubated for 24 h. The medium was removed and 2 mL of 0.25% DMSO in medium was added and incubated for 24 h. After removal of medium, the cells were exposed with 2 mM H_2O_2 for 0.25, 0.5, 1 and 3 h. and then washed twice with 1 mL of cold PBS. The cells were harvested by trypsinization and collected as previously described. Briefly, cell pellets were thawed and resuspened in ice-cold 0.013% sodium cholate and then sonicated on ice every 2 s for 10 s and centrifuged at 12,000 x *g* at 4°C for 10 min. Total volume of 0.1 mL of cell supernatant was added into the reaction mixture containing 0.1 mL of 50 mM phosphate buffer (pH 7.0), 0.1 mL of 1 mM EDTA, 0.2 mL of 5 mM NaN_3, 0.2 mL of 1 mM NADPH, 6.4 μL of GR, 0.2 mL of 5 mM GSH. The reaction assay was mixed by gently agitation and incubated for 5 min. Then, 0.2 mL of 0.25 mM H_2O_2 (freshly prepared) was added to initiate the reaction. The decay of NADPH was monitored at 340 nm for 5 min. The linear rate was used to calculate the activity according to the equation:

$$\text{Units/mg protein} = \Delta A/\text{min}_s \times (V_t/V_s) \times 1/\varepsilon \times (1/\text{prot})$$

Where $\Delta A/\text{min}_s$ was changed in absorbance per minute for the sample, V_t was the total volume (mL) of the assay, V_s was the sample volume (mL), ε was the extinction coefficient of NADPH (6.22 $nM^{-1}cm^{-1}$). One enzyme unit of GPx activity was defined as 1 μmol of NADPH oxidized per minute at 37°C.

13.3 Effect of TSCE-W on GPx activity in H_2O_2-treated cells

The cells were seeded at a density of 1 x 10^5 cells/mL into 6 well plates and incubated for 24 h. The medium was removed and added with TSCE-W (0.05-1 mg/mL) for 24 h. After removal of medium, the cells were exposed with 2 mM H_2O_2 for 0.5 h. and then washed twice with 1 mL of cold PBS. The cells were harvested by trypsinization and collected as previously described. Briefly, cell pellets were thawed and resuspened in ice-cold 0.013% sodium cholate and then sonicated on ice every 2 s for 10 s and centrifuged at 12,000 x *g* at 4°C for 10 min. The 0.1 mL of cell supernatant was added into the reaction mixture contained 0.1 mL of 50 mM phosphate buffer (pH 7.0), 0.1 mL of 1 mM EDTA, 0.2 mL of 5 mM NaN_3, 0.2 mL of

1 mM NADPH, 6.4 μL of GR, 0.2 mL of 5 mM GSH. The reaction assay was mixed by gently agitation and incubated for 5 min. Then, 0.2 mL of 0.25 mM H_2O_2 (freshly prepared) was added to initiat the reaction. The decay of NADPH was monitored for 5 min at 340 nm. The linear rate was used to calculate the activity according to the equation:

$$\text{Units/mg protein} = \Delta A/\text{min}_s \times (V_t/V_s) \times 1/\varepsilon \times (1/\text{prot})$$

Where $\Delta A/\text{min}_s$ was changed in absorbance per minute for the sample, V_t was the total volume (mL) of the assay, V_s was the sample volume (mL), ε was the extinction coefficient of NADPH (6.22 $nM^{-1}cm^{-1}$). One enzyme unit of GPx activity was defined as 1 μmol of NADPH oxidized per minute at 37°C.

14. Determination of CAT activity

14.1 Effect of TSCE-W on CAT activity

CAT activity was measured according to the method of Vives-Bauza *et al.* (2007). The cells were seeded into 6 well plates at a density of 1 x 10^5 cells/mL and incubated for 24 h. After removal of medium, the cells were added with TSCE-W (0.05-1 mg/mL) for 24 h and then washed twice with 1 mL of cold PBS. The cells were harvested by trypsinization and centrifuged at 130 x *g* at 4°C for 5 min. Cell pellets were washed twice with 1 mL of cold PBS and then resuspened in 200 μL of PBS (1 mL PBS per 40 million cells). Cell pellets were stored at -80°C until assay.

Immediately before the assay, cell pellets were thawed and resuspened in ice-cold 0.013% sodium cholate (20 million cells/mL). The cells were sonicated on ice every 2 s for 10 s and then centrifuged at 12,000 x *g* at 4°C for 10 min. The 2.9 mL of 10.3 mM H_2O_2 in 50 mM phosphate buffer, pH 7.0 (freshly prepared) was added into 3 mL quartz cuvette, the reaction was started by adding 100 μL of cell supernatant. The absorbance reading was taken against blank (100 μL of cell supernatant and 2.9 mL of 50 mM phosphate buffer without H_2O_2). The decay in absorbance at 240 nm was monitored for 2 min. The linear rate was used to calculate the activity, which was presented in *k* (the rate of a first-order reaction) per mg of protein according to the equation:

$$k/\text{ mg protein} = 69/\Delta t \times \log (A_1/A_2)/\text{ prot}$$

Where $\Delta t = t_2 - t_1$ was measured in time interval (in second), A_1 was an absorbance at t_1, A_2 was an absorbance at t_2.

14.2 Effect of H_2O_2 on CAT activity

The cells were seeded into 6 well plates at a density of 1 x 10^5 cells/mL and incubated for 24 h. The medium was removed and 2 mL of 0.25% DMSO in medium was added and incubated for 24 h. After removal of medium, the cells were exposed with 2 mM H_2O_2 for 0.25, 0.5, 1 and 3 h. and then washed twice with 1 mL of cold PBS. The cells were harvested by trypsinization and collected as previously described. Briefly, cell pellets were thawed and resuspened in ice-cold 0.013% sodium cholate and then sonicated on ice every 2 s for 10 s and centrifuged at 12,000 x *g* at 4°C for 10 min. The 100 μL of cell supernatant was added into 2.9 mL of 10.3 mM H_2O_2 in 50 mM phosphate buffer, pH 7.0 (freshly prepared) and gently mixed. The decay in absorbance at 240 nm was monitored for 2 min. The linear rate against blank was used to calculate the activity according to the equation:

$$k/\text{ mg protein} = 69/\Delta t \text{ x } \log (A_1/A_2)/\text{ prot}$$

Where $\Delta t = t_2 - t_1$ was measured in time interval (in second), A_1 was an absorbance at t_1, A_2 was an absorbance at t_2.

14.3 Effect of TSCE-W on CAT activity in H_2O_2-treated cells

The cells were seeded at a density of 1 x 10^5 cells/mL into 6 well plates and incubated for 24 h. The medium was removed and added TSCE-W (0.05-1 mg/mL) for 24 h. After medium was removed, the cells were exposed with 2 mM H_2O_2 for 0.5 h. and then washed twice with 1 mL of cold PBS. The cells were harvested by trypsinization and collected as previously described. Briefly, cell pellets were thawed and resuspened in ice-cold 0.013% sodium cholate and then sonicated on ice every 2 s for 10 s and centrifuged at 12,000 x *g* at 4°C for 10 min. The 100 μL of cell supernatant was added into 2.9 mL of 10.3 mM H_2O_2 in 50 mM phosphate buffer, pH 7.0 (freshly prepared) and gently mixed. The decay in absorbance at 240 nm was monitored for 2 min. The linear rate against blank was used to calculate the activity according to the equation:

$$k/\text{ mg protein} = 69/\Delta t \text{ x } \log (A_1/A_2)/\text{ prot}$$

Where $\Delta t = t_2 - t_1$ was measured in time interval (in second), A_1 was an absorbance at t_1, A_2 was an absorbance at t_2.

15. West blotting analysis

Western blotting analysis is an analytical technique used to determine the specific proteins. This technique uses gel electrophoresis to separate proteins by molecular size. The protein on the gel was then transferred to a membrane and probed with the antibodies specific to the target peotein.

15.1 Effect of TSCE-W on protein expression of Cu,Zn SOD, GPx, CAT, HO-1, GST, and Nrf2

The cells were seeded at a density of 1 x 10^5 cells/mL into 6 well plates and incubated for 24 h. The medium was removed and then added with TSCE-W at various concentrations (0.2, 0.4, and 0.8 mg/mL) for 24 h. The vehicle control was treated with 0.25% DMSO in medium for 24 h, while the negative control was treated with 1 mM H_2O_2 for 3 and 6 h. The cells were washed twice with 1 mL of ice-cold PBS, and then scrapped, collected, and centrifuged at 1500 rpm for 8 min. The protein was extracted by RIPA lysis buffer (50 mM Tris HCl, pH 8, 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, and 0.1% SDS) on ice for 30 min. After centrifuged at 12,000 rpm for 10 min, the supernatant was collected and protein concentration was measured by the 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% brophenol blue), boiled at 95°C for 5 min. The amount of 30 mg proteins of each sample were loaded onto 12% SDS-polyacrylamide gel and electrophoresed at 90 volts for 1.5 h. The gel further transferred onto PVDF membrane by electroblotting at 45 volts for 2 h. The membrane was incubated with with 5% bovine serum albumin (BSA) in TBST (10 mM Tris-base, pH 7.5, 0.1 M NaCl and 0.05% Tween 20) for 2 h at room temperature for blocking non-specific binding protein. Blots were probed with primary rabbit antibodies (Cu/Zn SOD or GPx or CAT or HO-1 or GST or Nrf2 from Abcam, Cambridge, England) at 4°C overnight. The membrane was washed 6 times with TBST and then blotted with goat anti-rabbit polyclonal secondary antibody with horseradish peroxidase conjugated for

1 h at room temperature. The protein bands were detected by enhanced chemiluminascence detection reagent and exposed to Kodak X-ray films. The intensities of the bands were visualized and computed by Image J 1.43u software (NIH, Bethesda, MD, USA).

15.2 Effect of H_2O_2 on protein expression of Cu,Zn SOD, GPx, CAT, HO-1, GST, and Nrf2

The cells were seeded at a density of 1 x 10^5 cells/mL into 6 well plates and incubated for 24 h. The medium was removed and 0.25% DMSO in medium was added and incubated for 21 and 18 h. The medium was removed and added with 1 mM H_2O_2 for 3 and 6 h, respectively. The cells were collected and blotted according the previously described protocol.

15.3 Effect of TSCE-W on protein expression of Cu,Zn SOD, GPx, CAT, HO-1, GST, and Nrf2 in H_2O_2-treated cells

The cells were seeded at a density of 1 x 10^5 cells/mL into 6 well plates and incubated for 24 h. The cells were pretreated with TSCE-W at 0.4 and 0.8 mg/mL for 24 h. After removal of medium, the cells were exposed with 1 mM H_2O_2 for 3 and 6 h and then collected and blotted according the previously described protocol.

16. Statistical analysis

SPSS program (version 15) was used for statistical analysis. Data were expressed as means ± S.E.M. Statistical analysis differences between two groups were determined by Student's *t*-test. For comparison of multiple groups were performed using one-way analysis of variance (ANOVA) followed by Tukey post hoc mean comparison. A level of $p< 0.05$ was considered statistically significant.

CHAPTER IV
RESULTS AND DISCUSSION

1. Extraction yield and phenolic content in TSCEs

The extraction yields of the total phenolic, tannin and proanthocyanidin contents of the TSCEs are shown in Table 3. The TSCE-E possessed the highest yield (45.09 ± 2.35%), while the yield of TSCE-W (1.54 ± 0.32%), TSCE-EEA (1.54 ± 0.46%) and TSCE-EA (0.54 ± 0.10%) were not significantly different. The highest phenolic content was found in TSCE-W followed by TSCE-EA, TSCE-E, and TSCE-EEA with the values of 89.21 ± 0.25, 74.21 ± 0.45, 69.24 ± 0.58, 52.17 ± 0.84 GAE (g)/100 g of extracts, respectively. The tannin content of TSCE-W (20.91 ± 1.43% pyrogallol equivalent) was comparable with that of TSCE-E (21.93 ± 0.54% pyrogallol equivalent) and TSCE-EA (18.81 ± 1.06% pyrogallol equivalent), while TSCE-EEA contained the lowest tannin content (15.68 ± 1.74% pyrogallol equivalent). However, TSCE-EEA contained the highest proanthocyanidin content (1.298 ± 0.03) followed by TSCE-E (0.888 ± 0.01), TSCE-W (0.741 ± 0.01), and TSCE-EA (0.662 ± 0.02), respectively. The extraction yield of TSCE-EEA and its chemical content in this study were found to be higher than those of the previous studies that using the same process of extraction (Suksomtip *et al*., 2010). Our results showed that TSCEs from boiling-water extraction or infusion contained phenolic content at a higher level than the organic solvent extractions. Hot-water may be a suitable solvent for the extraction of polar polyphenols from tamarind seed coat and the water extracts potentially having a high antioxidant activity.

2. HPLC analysis

The HPLC profile of TSCE-W was demonstrated the peaks identical with the standard phenolic compounds including (+)-catechin, (-)-epicatechin and procyanidin B_2 (Fig. 19), which was in agreement with the previous study (Sudjaroen *et al.,* 2005). The result suggested that hot-water, an inexpensive polar and environmentally friendly solvent, was suitable for the extraction of polar polyphenols from the tamarind seed coat which possessed the high antioxidant activities.

Table 3. Extraction yield and phenolic contents in the four TSCEs extracted by different solvent systems from *Tamarindus indica* "Priao-Yak". Data are mean ± SEM of three independent experiments.

Extract	% Yield of extract	Phenolic content (GAE(g)/100g of extract)	Tannin content (% pyrogallol equivalent)	Proanthocyanidin content (A_{550})
TSCE-E	45.09 ± 2.35^{a}	69.24 ± 0.58^{c}	21.93 ± 0.54^{a}	0.89 ± 0.01^{b}
TSCE-EA	0.54 ± 0.10^{b}	74.21 ± 0.45^{b}	18.81 ± 1.06^{a}	0.66 ± 0.02^{c}
TSCE-EEA	1.54 ± 0.46^{b}	52.17 ± 0.84^{d}	15.68 ± 1.74^{b}	1.30 ± 0.03^{a}
TSCE-W	1.54 ± 0.32^{b}	89.21 ± 0.14^{a}	20.91 ± 1.43^{a}	0.74 ± 0.01^{c}

a,b,c,d are significant difference between extraction groups in each experiment.

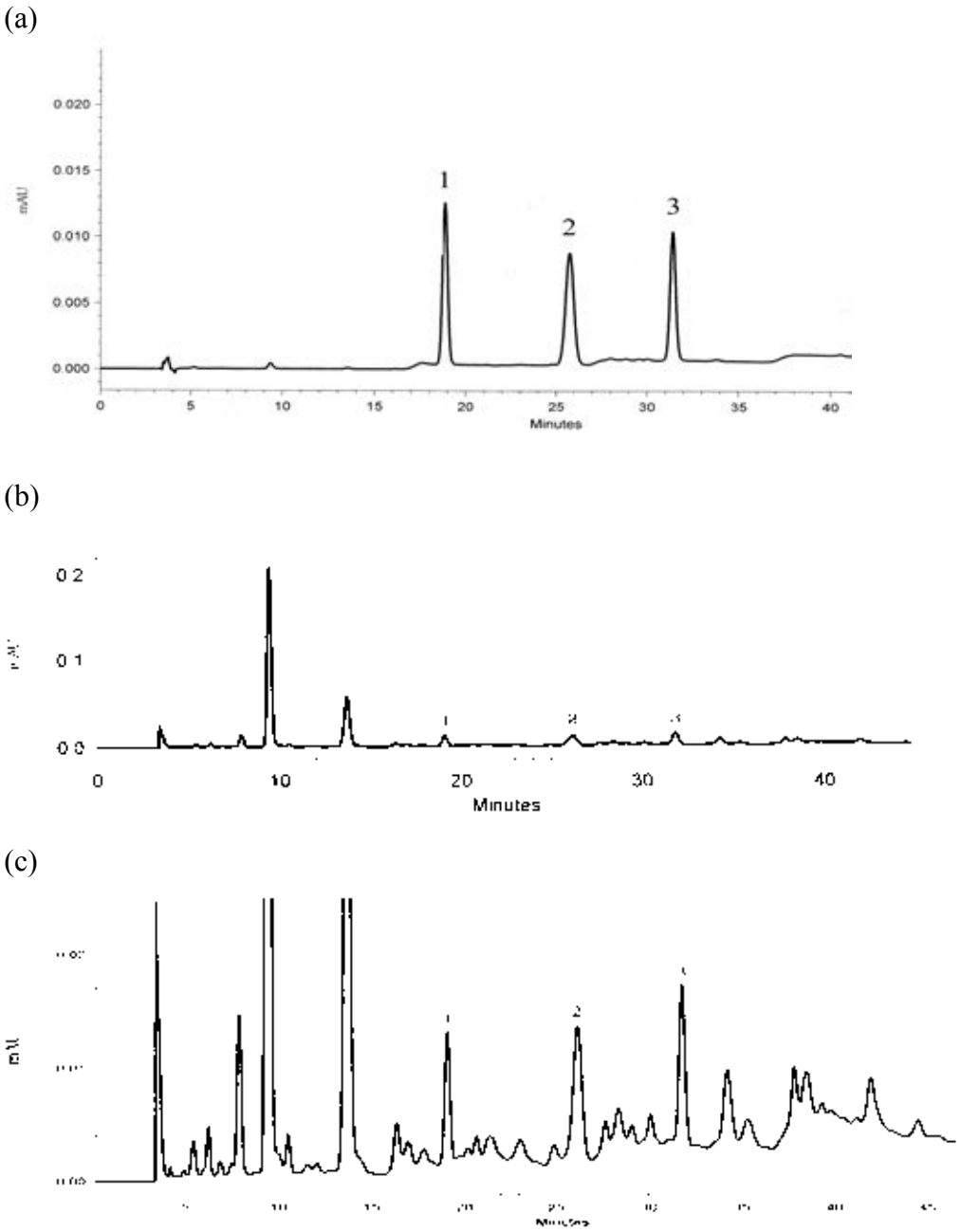


Figure 19. HPLC profile of standard phenolic compounds (a), TSCE-W (b), and the expanded of b showing peaks identical with standard references (+)-catechin (1), (-)-epicathechin (2), and procyanidin B2 (3).

3. Free radicals scavenging and antioxidant activity

3.1 DPPH radical scavenging activity

A stable non-biological radical, DPPH, is widely used for screening the antioxidant activity of hydrogen-donating of antioxidant substances. DPPH free radical scavenging activity of the four TSCEs in Figure 20a showed that TSCE-W exhibited the highest scavenging ability for the DPPH radical (90.75%) followed by TSCE-E (83.38%), TSCE-EEA (81.16%), BHA (75.66%), Trolox (67.64%) and TSCE-EA (62.17%) at a tested concentration of 5.22 μg/mL. The EC_{50} values in Figure 20b showed that TSCE-W possessed the highest DPPH radical scavenging capability, as the lowest EC_{50} value (2.44 μg/mL) was obtained. The increasing EC_{50} values were found in TSCE-E (3.01 μg/mL), TSCE-EEA (3.02 μg/mL), BHA (3.23 μg/mL), Trolox (3.80 μg/mL) and TSCE-EA (4.38 μg/mL), respectively. The DPPH radical scavenging activity of TSCEs was corresponding with their phenolic contents in the extract. All of the TSCEs in this study showed high DPPH radical scavenging activity, these results have found agreeable with the previous reports (Maisuthisakul *et al*., 2007; Siddhuraju, 2007; Suksomtip *et al*., 2010).

3.2 $ABTS^{\bullet+}$ scavenging activity

The total antioxidant activity of TSCEs was also evaluated by measuring the free radical scavenging ability using an assay involved the decolorization of a solution of the stable free radical $ABTS^{\bullet+}$. The $ABTS^{\bullet+}$ scavenging ability of TSCEs was expressed as Trolox equivalent by using Trolox standard curve (Fig. 21a). The results in Figure 21b showed that TSCE-W exhibited higher $ABTS^{\bullet+}$ scavenging activity (9.64 ± 0.45 μmol TEAC/mg of extract) than the standard BHA (8.23 ± 0.11 μmol TEAC/mg of extract), TSCE-EA (7.28 ± 0.11 μmol TEAC/mg of extract), and TSCE-EEA (4.97 ± 0.13 μmol TEAC/mg of extract), respectively, where its activity was comparable with TSCE-E (8.83 ± 0.15 μmol TEAC/mg of extract). The $ABTS^{\bullet+}$ scavenging activity of TSCE-W, TSCE-E and TSCE-EA exhibited the higher activity than the values previously reported (Siddhuraju, 2007) where TSCE-EEA was comparable consistent, the auther demonstrated that the $ABTS^{\bullet+}$ scavenging activity of the dry-heated seed coat of tamarind extracted with methanol and 70% acetone are

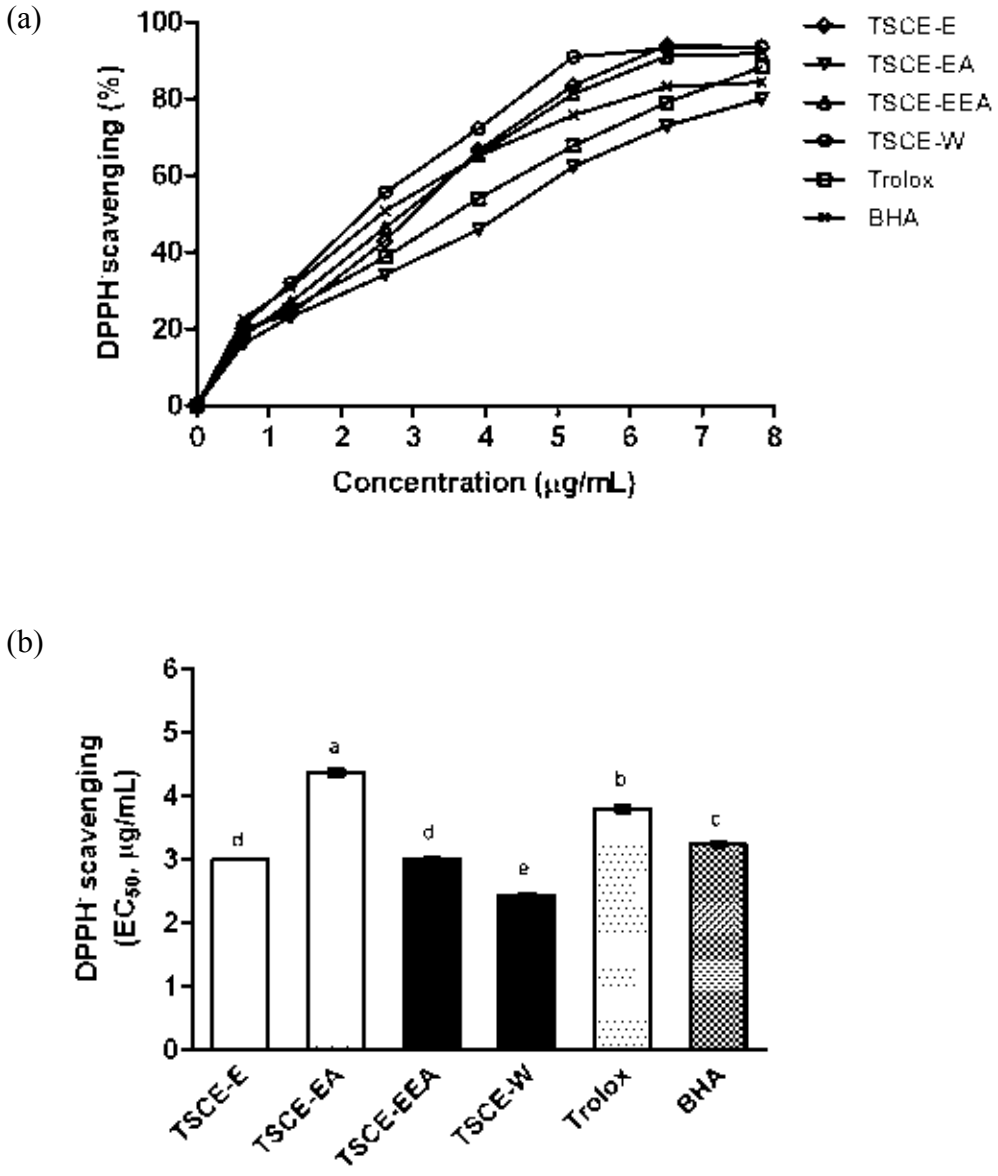


Figure 20. DPPH radical scavenging activity of the four TSCEs extracted by different solvent systems from *Tamarindus indica* "Priao-Yak". (a) % scavenging and (b) EC_{50} values (μg/mL). Trolox and BHA were used as reference antioxidants. Data are mean ± SEM of three independent experiments. a, b, c, d, e are significant difference between groups.

4389.7 and 3496.0 mmol TEAC/kg of extract (4.3897 and 3.4960 μmol TEAC/mg of extract), respectively. The $ABTS^{\bullet+}$ of TSCEs was correlated with their proanthocyanidin contents in the extract (r^2=0.905). These results suggested that TSCEs possessed antioxidant activity on scavenging $ABTS^{\bullet+}$ depends on its ability to donate hydrogen to the free radical, which converts them into stable compounds and terminates the radical chain reaction.

3.3 Superoxide anion scavenging activity

The superoxide anion is a free radical that plays an important role in the formation of other reactive oxygen species such as hydrogen peroxide and the hydroxyl radical (Jiao, Liu, and Wang, 2005). The superoxide anion radical scavenging activity of the four TSCEs in Figure 22a showed no significantly different of their activity. The TSCEs tested at a concentration 100 μg/mL exhibited higher scavenging activity against superoxide anion radicals than Trolox, while BHA at the same concentration did not show this activity. The EC_{50} values were 23.53 ± 1.19, 25.39 ± 2.48, 19.07 ± 0.18, 21.44 ± 0.23, and 352.70 ± 15.44 μg/mL for TSCE-E, TSCE-EA, TSCE-EEA, TSCE-W, and Trolox, respectively (Fig. 22b). The four TSCEs showed higher superoxide scavenging activity than methanol and 70% acetone extracts of dry heated tamarind seed coat as previously reported (Siddhuraju, 2007).

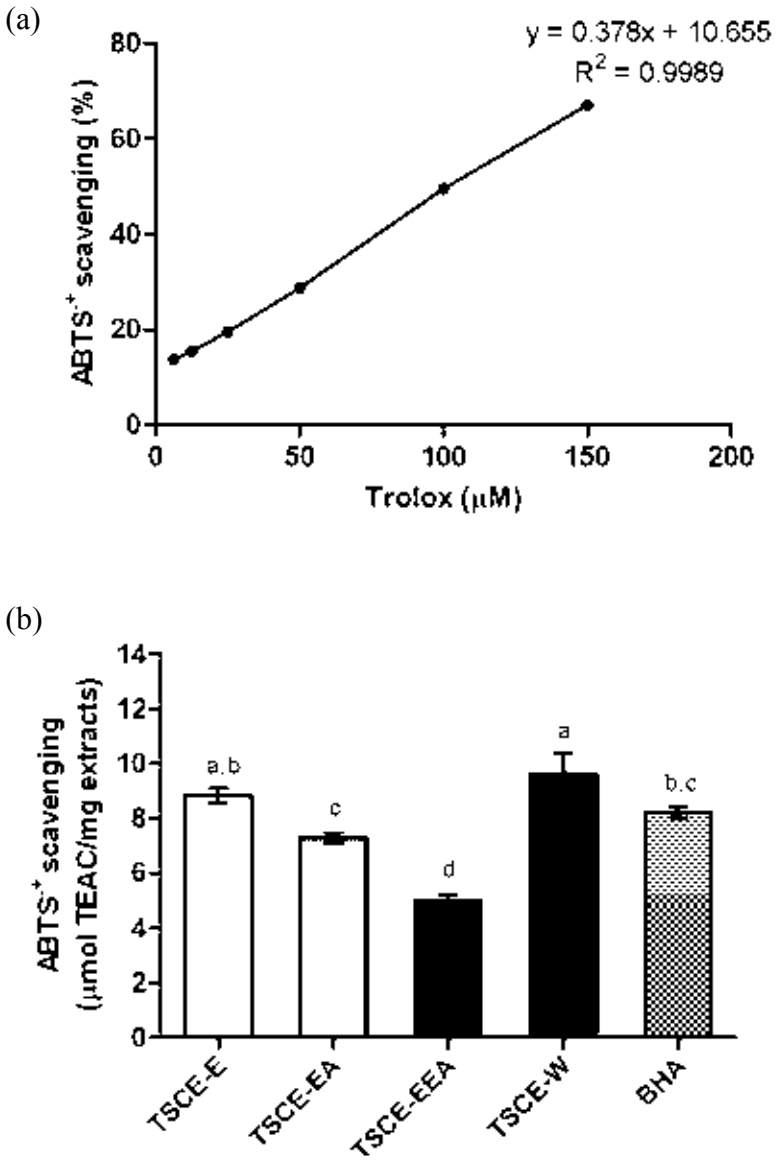


Figure 21. ABTS$^{\bullet+}$scavenging activity of Trolox (a), and the four TSCEs extracted by different solvent systems from *Tamarindus indica* "Priao-Yak" and BHA (a reference antioxidants) in all tested at 12 μg/mL (b). Data are mean ± SEM of three independent experiments. a, b, c, d are significant difference between groups.

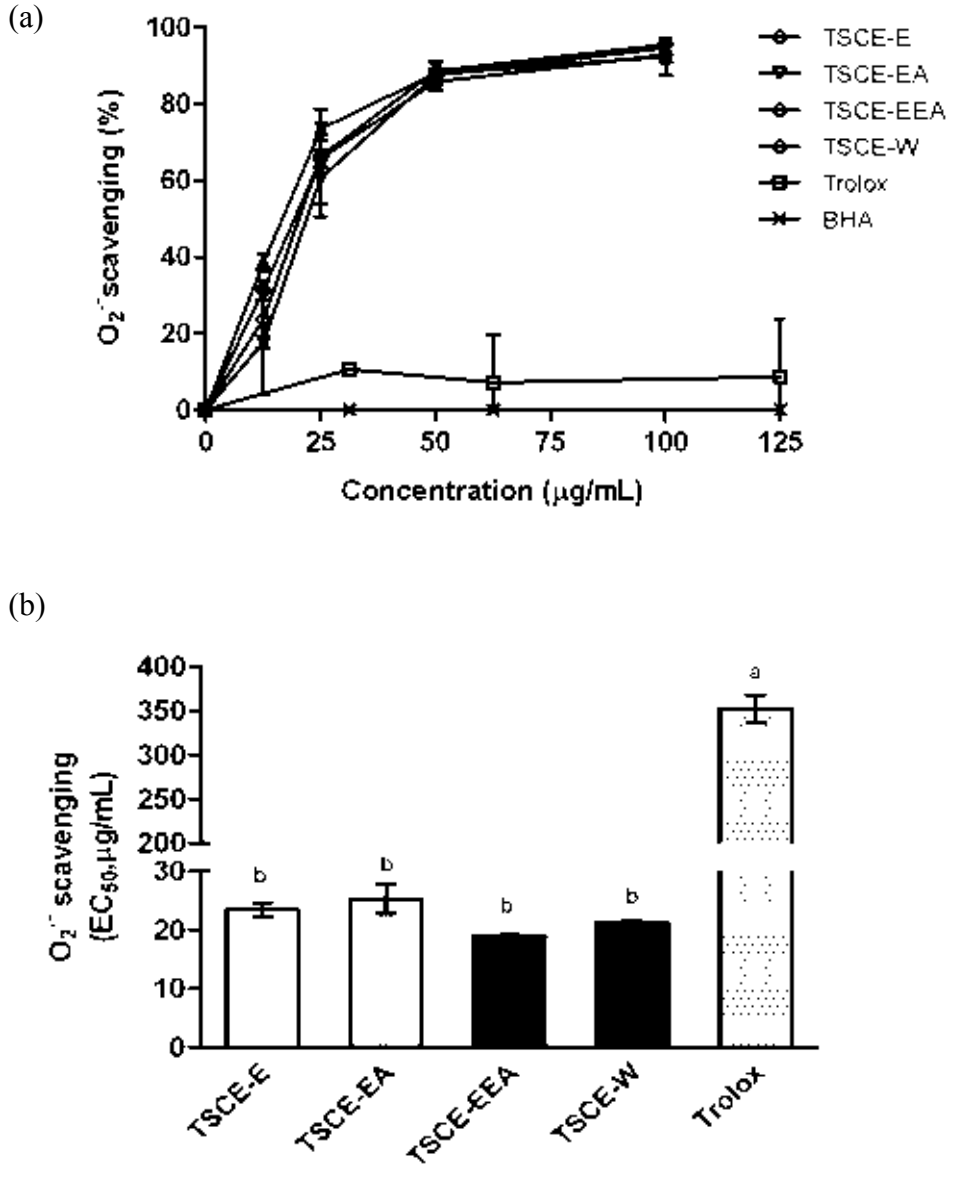


Figure 22. Superoxide anion radical scavenging activity of the four TSCEs extracted by different solvent systems from *Tamarindus indica* "Priao-Yak". (a) % scavenging and (b) EC_{50} values (μg/mL). Trolox was used as reference antioxidants. Data are mean ± SEM of three independent experiments. a, b are significant difference between groups.

3.4 Hydrogen peroxide scavenging activity

A non-radical H_2O_2 is naturally produced as a by-product of oxygen metabolism. It can readily crosses the cellular membranes giving rise to the highly reactive radicals and has ability to react with macromolecules (DNA, protein and lipids) leading to cell damage (Heo *et al.*, 2008). Capability of the TSCEs to scavenge hydrogen peroxide is shown in Figure 23a. TSCE-E and TSCE-EA showed a higher activity of scavenge hydrogen peroxide (97.20% and 94.81%) than TSCE-EEA (43.57%), TSCE-W (31.82%), BHA (13.28%) and Trolox (0.25%), at a tested concentration (20 μg/mL). The EC_{50} values are shown in Figure 23b. The four TSCEs tested showed higher hydrogen peroxide scavenging activity than the reference standards, Trolox and BHA. The EC_{50} values for TSCE-E, TSCE-EA, TSCE-EEA, TSCE-W, Trolox and BHA were 11.33 ± 0.75, 12.88 ± 0.11, 20.79 ± 1.62, 26.03 ± 1.99, 52.53 ± 1.51 and 53.31 ± 2.42 μg/mL, respectively. So far, the H_2O_2 scavenging activity of tamarind seed coat has not been reported. This study was first reported and suggested that TSCEs possessed H_2O_2 scavenging activity similar with the phenolic compound having H_2O_2 scavenging activity in buckwheat hulls and flour (Quettier-Deleu *et al.*, 2000) and in *Foeniculum vulgare* seed (Oktay, Gülçin, and Küfrevioğlu, 2003). Hydrogen peroxide itself not reactive, but it can give rise to hydroxyl radical, a highly reactive radical, which is toxic to cells. Hence, the scavenging of H_2O_2 is very important for antioxidantive defense in cell (Oktay, *et al.*, 2003).

3.5 Hydroxyl radical scavenging activity

Hydroxyl radicals are the most reactive free radical of the oxygen radical species, therefore, it is considered to be particularly dangerous to living cells (Nordberg & Arner, 2001). The percentage of $OH^{\bullet}$ scavenging of TSCE-EEA (79.70%), TSCE-W (79.04%), TSCE-EA (78.47%), and TSCE-E (73.55%) were comparable with BHA (78.57%) and Trolox (74.54%) at the tested concentration (4 μg/mL) as showed in Figure 24a. The TSCE-EEA exhibited the highest, where TSCE-E exhibited the lowest $OH^{\bullet}$ scavenging activity. The activity of BHA, TSCE-EA, Trolox, and TSCE-W was comparable. The EC_{50} values were 0.21 ± 0.02, 0.24 ±

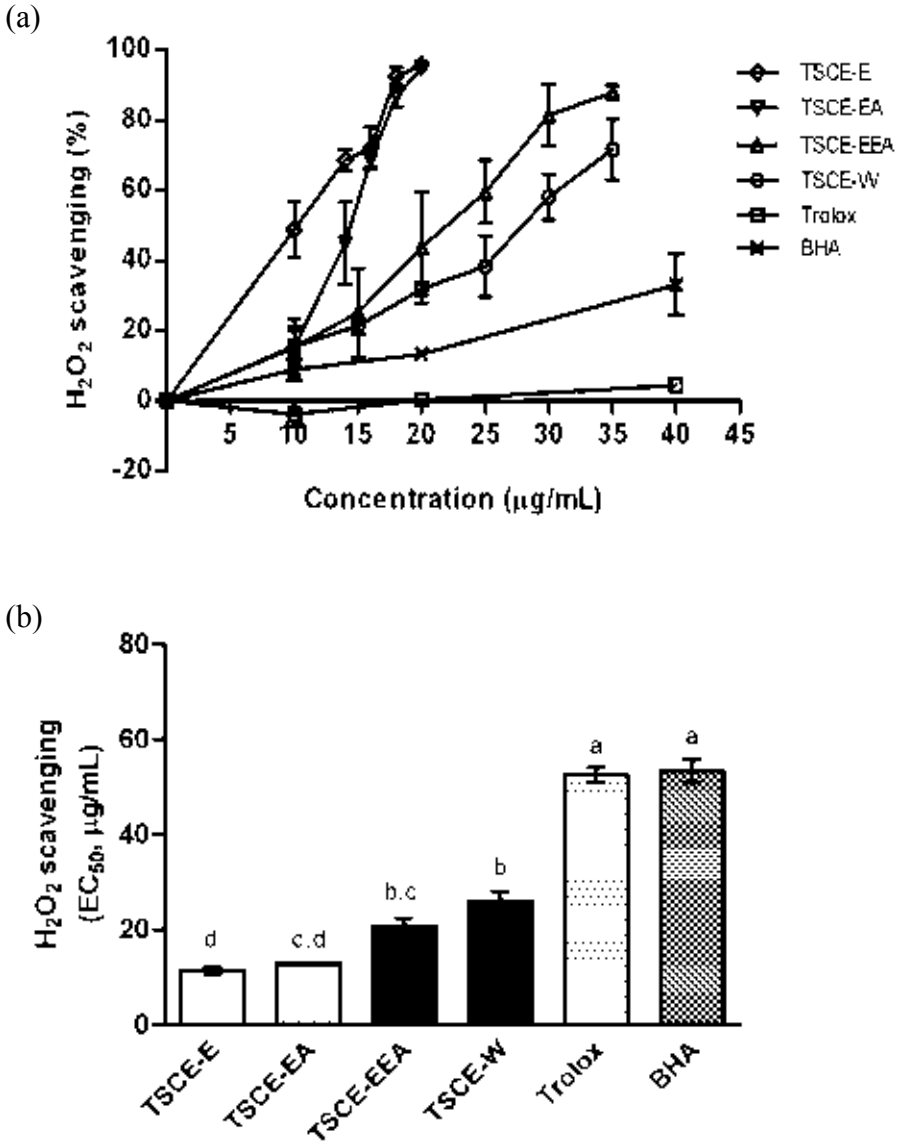


Figure 23. Hydrogen peroxide scavenging activity of the four TSCEs extracted by different solvent systems from *Tamarindus indica* "Priao-Yak". (a) % scavenging and (b) EC_{50} values (µg/mL). Trolox and BHA were used as reference antioxidants. Data are mean ± SEM of three independent experiments. a, b, c, d are significant difference between groups.

0.01, 0.34 ± 0.02, 0.38 ± 0.01, 0.42 ± 0.03 and 0.58 ± 0.04 μg/mL for TSCE-EEA, BHA, TSCE-EA, Trolox, TSCE-W, and TSCE-E, respectively (Fig. 24b). The high hydroxyl radical scavenging activity of organic solvent extracts of TSCEs, such as methanolic and ethanolic extracts, has been reported previously (Siddhuraju, 2007; Suksomtip *et al*., 2010). These results indicate that almost of TSCEs possessed scavenging activity for hydroxyl radicals as good as the reference antioxidant, Trolox and BHA.

3.6 Nitric oxide scavenging activity

Nitric oxide is an abundant reactive radical that plays an important role in inflammatory processes in living systems. NO scavenging activity can help to arrest the reaction chain initiated by excess generation of NO causing damage to human health (Yu *et al.,* 2008). The TSCE-W showed higher percentage NO scavenging (72.55%) than TSCE-E (69.36%), TSCE-EA (64.70%), TSCE-EEA (63.32%), Trolox (59.18), and BHA (56.29%) at a tested concentration 25 μg/mL (Fig. 25a.). The EC_{50} values of TSCE-W, TSCE-EA and TSCE-E were much higher nitric oxide scavenging activity than TSCE-EEA and BHA, while Trolox showed the lowest NO scavenging activity. The EC_{50} values were 1.48 ± 0.15, 1.74 ± 0.21, 1.76 ± 0.34, 4.05 ± 0.23, 5.17 ± 0.78, and 10.26 ± 0.59 μg/mL for TSCE-W, TSCE-EA, TSCE-E, TSCE-EEA, BHA, and Trolox, respectively (Fig. 25b). The NO scavenging activity of the four TSCEs were correlated with their phenol and tannin contents (r^2 =0.78). The activity of TSCEs on inhibiting NO production in a cell-free system has found agreeable with the study previously reported (Kumutarin *et al.*, 2004).

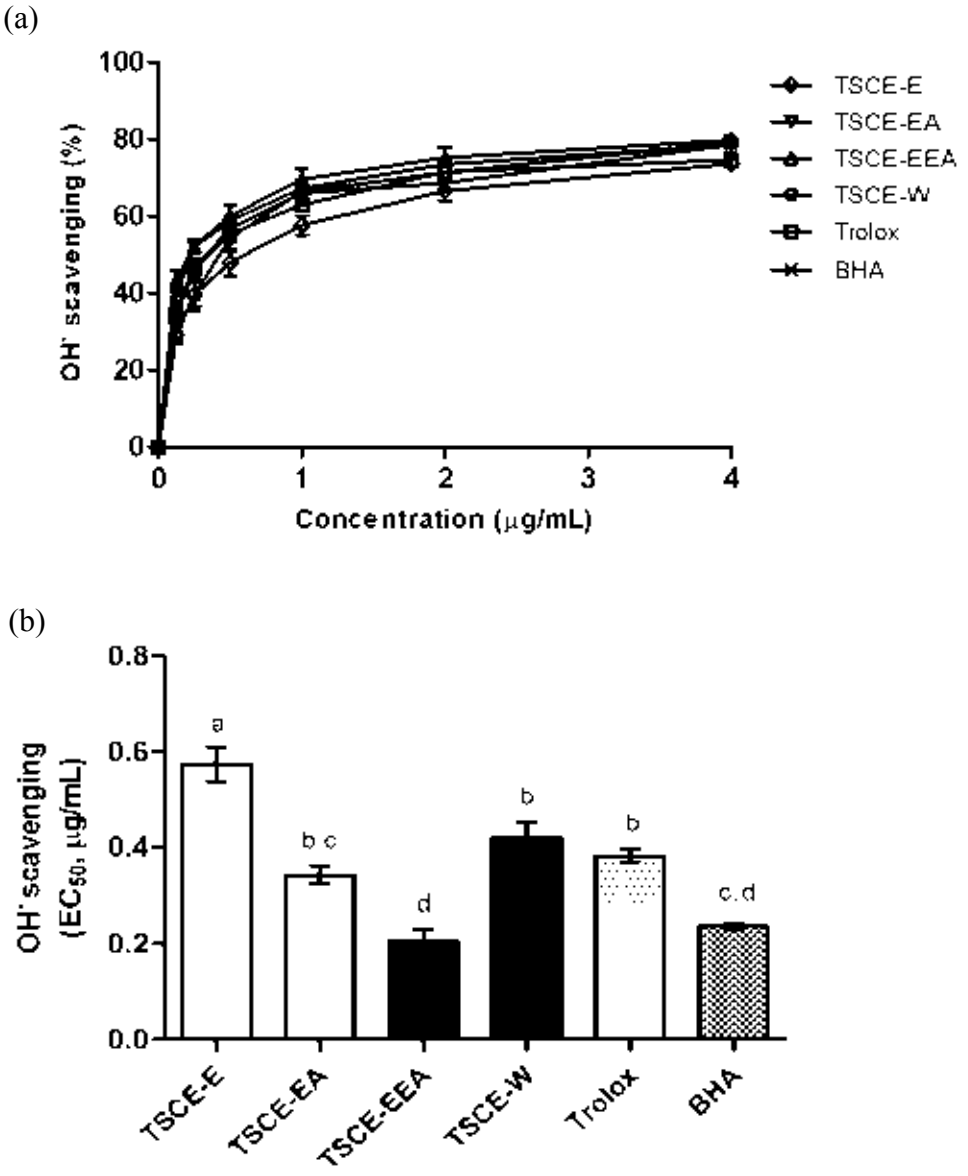


Figure 24. Hydroxyl radical scavenging activity of the four TSCEs extracted by different solvent systems from *Tamarindus indica* "Priao-Yak". (a) % scavenging and (b) EC_{50} values (μg/mL). Trolox and BHA were used as reference antioxidants. Data are mean ± SEM of three independent experiments. a, b, c, d are significant difference between groups.

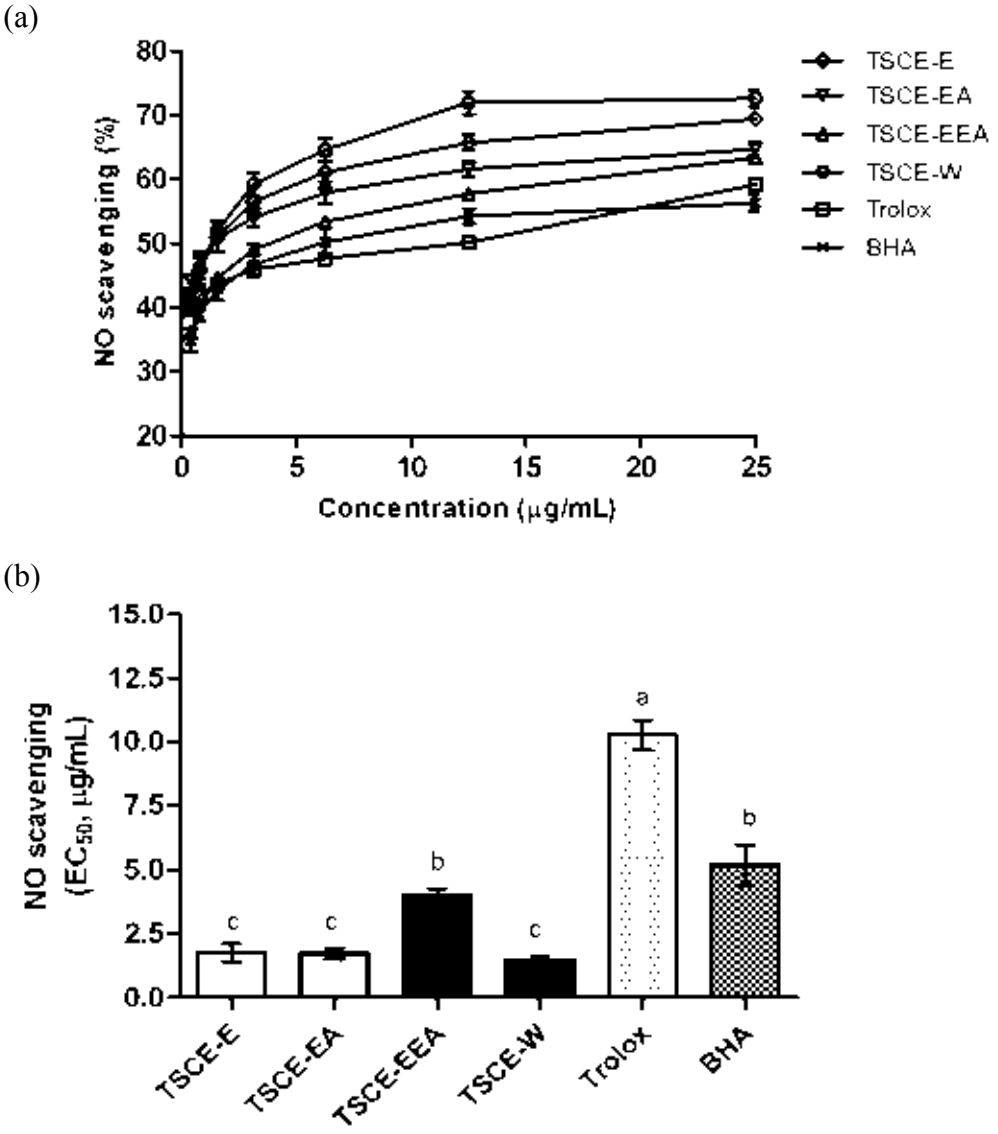


Figure 25. Nitric oxide scavenging activity of the four TSCEs extracted by different solvent systems from *Tamarindus indica* "Priao-Yak". (a) % scavenging and (b) EC_{50} values (μg/mL). Trolox and BHA were used as reference antioxidants. Data are mean ± SEM of three independent experiments. a, b, c are significant difference between groups.

3.7 Reducing power activity

The reducing power capacity of the TSCEs was evaluated by measuring the reduction of the Fe^{3+} ferricyanide complex into its reduced form (Fe^{2+}). In the presence of antioxidant substances, the yellow color of the reaction mixture becomes green and the absorbance is measured at 700 nm. The higher absorbance values indicate the higher reducing power. The reducing power is generally associated in the presence of the reductone, which exert antioxidant action by breaking the free radical chain on donating a hydrogen atom (Kumaran and Joel Karunakaran, 2007). The reducing power of the TSCEs is shown in Figure 26a. TSCE-E exhibited the highest reducing power (1.09), followed by BHA (0.93), TSCE-EEA (0.77), TSCE-W (0.69), Trolox (0.67) and TSCE-EA (0.65) at a concentration 40 μg/mL of TSCEs tested. The EC_{50} values were 18.14 ± 0.19, 21.61 ± 0.77, 25.67 ± 0.99, 28.60 ± 0.40, 30.22 ± 0.74, and 30.50 ± 0.50 μg/mL for TSCE-E, BHA, TSCE-EEA, TSCE-W, Trolox, and TSCE-EA, respectively (Fig. 26b). These results show that TSCE-E exhibited a reducing power activity higher than the reference antioxidants Trolox and BHA, while TSCE-EEA and TSCE-W showed a comparable activity with Trolox. All of the TSCEs in this study exhibited higher reducing power activity than of the TSCE previously studied (Suksomtip *et al*., 2010). These results indicated that reducing power capacity of TSCEs works as electron donors.

3.8 Metal chelating activity

The metal chelating capacity of the TSCEs was evaluated by measuring their chelating ability with Fe^{2+}. The results showed that the four TSCEs exhibited very low ability to chelate Fe^{2+} compared with the reference chelating agent Na_2EDTA (100% chelating activity at 10 μg/mL). TSCEs were tested at a concentration of 25 μg/mL, TSCE-W and TSCE-E possessed significantly higher Fe^{2+} chelating activity (26.33% and 24.63%) than TSCE-EA (15.73%), but their activity was comparable with TSCE-EEA (22.05%). The reference antioxidants Trolox and BHA at the same concentration did not exhibit Fe^{2+}chelating activity in this assay (Fig. 27). The four TSCEs exhibited low metal chelating ability which was the same as the levels of tannin from faba beans previously reported (Carbonaro *et al.,* 1996).

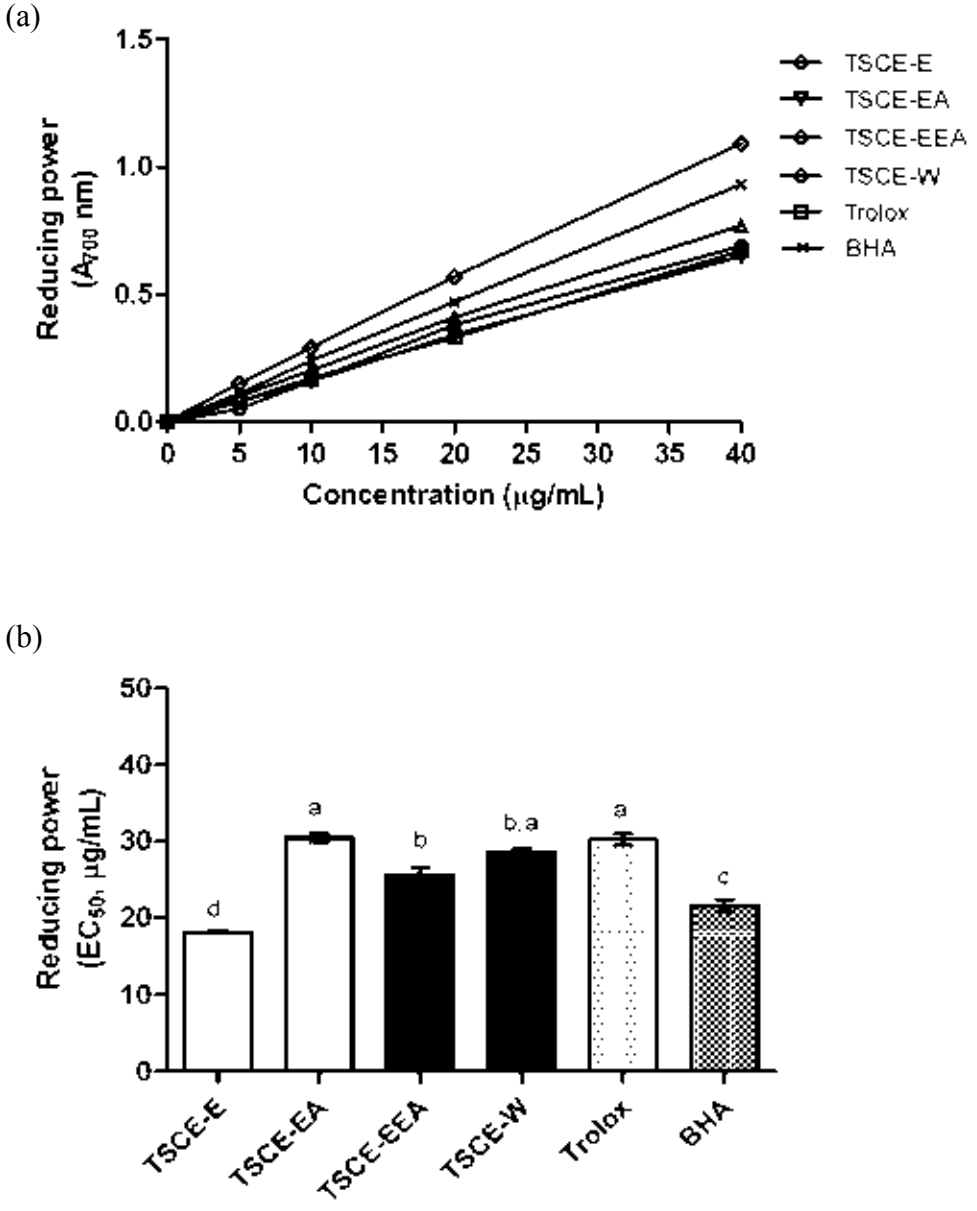


Figure 26. Reducing power activity of the four TSCEs extracted by different solvent systems from *Tamarindus indica* "Priao-Yak". (a) % scavenging and (b) EC_{50} values (μg/mL). Trolox and BHA were used as reference antioxidants. Data are mean ± SEM of three independent experiments. a, b, c, d are significant difference between groups.

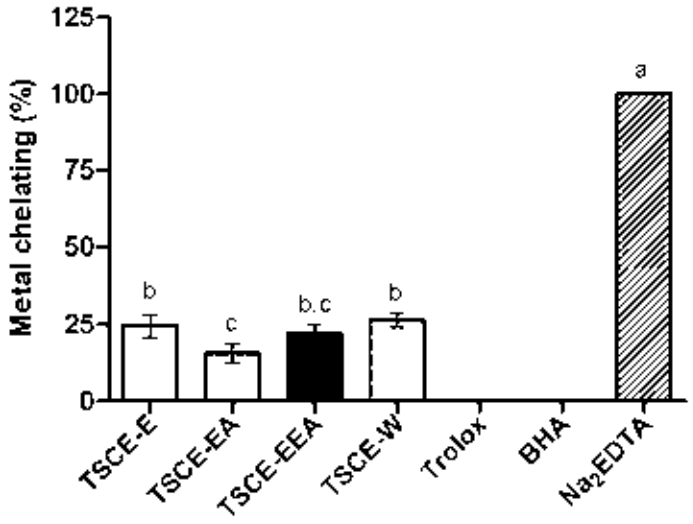


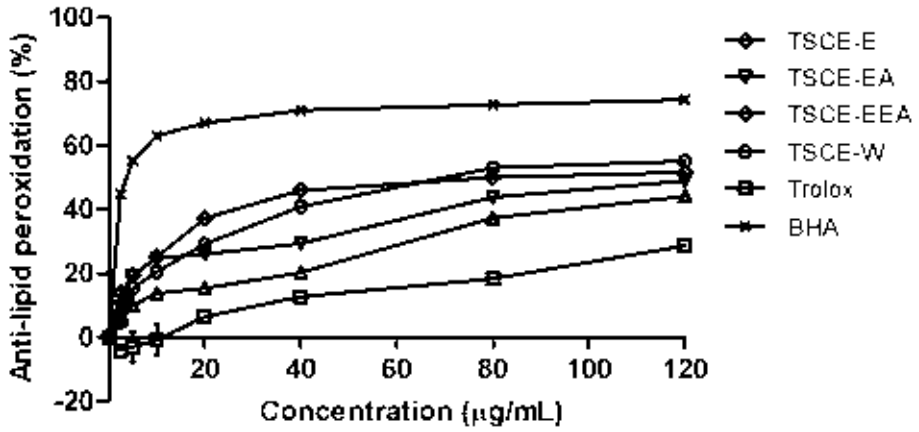
Figure 27. Metal chelating activity at 25 μg/mL of the four TSCEs extracted by different solvent systems from *Tamarindus indica* "Priao-Yak", and Trolox and BHA (as reference antioxidants). Na_2EDTA as a chelating agent was used at 10 μg/mL. Data are mean ± SEM of three independent experiments. a, b, c are significant difference between groups.

3.9 Anti-lipid peroxidation activity

The activity of the four TSCEs on lipid peroxidation inhibition was estimated by measuring the inhibitory activity of egg-yolk lipid peroxidation. The results in Figure 28a showed that TSCE-W exhibited a higher lipid peroxidation inhibition (57.29%) than TSCE-E (52.50%), TSCE-EEA (46.34%), TSCE-EA (45.13%) and the reference antioxidant Trolox (44.90%), but lower than BHA (69.46%) at a test concentration of 160 μg/mL. The EC_{50} values were 2.70 ± 0.24, 78.17 ± 1.21, 80.99 ± 2.00, 177.32 ± 1.69, 192.03 ± 16.40 and 420.48 ± 0.24 μg/mL for BHA, TSCE-W, TSCE-E, Trolox, TSCE-EA and TSCE-EEA, respectively (Fig. 28b). These results showed that the TSCE obtained from boiling-water extraction or infusion possessed two-times higher anti-lipid peroxidation than ethyl acetate extraction and comparable with 70% ethanol extraction. The lipid peroxidation inhibition observed may be related to the high phenol content in the four extracts (r^2=0.70). The TSCE from 70% ethanol extraction obtained here exhibited lower inhibitory activity compared with the study previously reported (Suksomtip *et al*., 2010).

EC_{50} values of TSCEs and antioxidant standards on free radicals scavenging and antioxidant activities were showed in Table 4. The result found that TSCE-W possess antioxidant activities and free radicals scavenging as well as TSCE-E, however TSCE-W showed higher scavenge $DPPH^{\bullet}$ (Table 4) and total phenolic content (Table 3) than TSCE-E. A high $DPPH^{\bullet}$ scavenging activity in extracts is mean that the extract can high hydrogen atom or electron donating to radical and a high amount of phenolic content in extracts may explain their high antioxidant activity (Ebrahimzadeh *et al.*, 2010). Moreover, TSCE-W has not been study in cell-based system. Therefore, the toxicity, antioxidant and cytoprotective activities of TSCE-W were further study in cells.

(a)



(b)

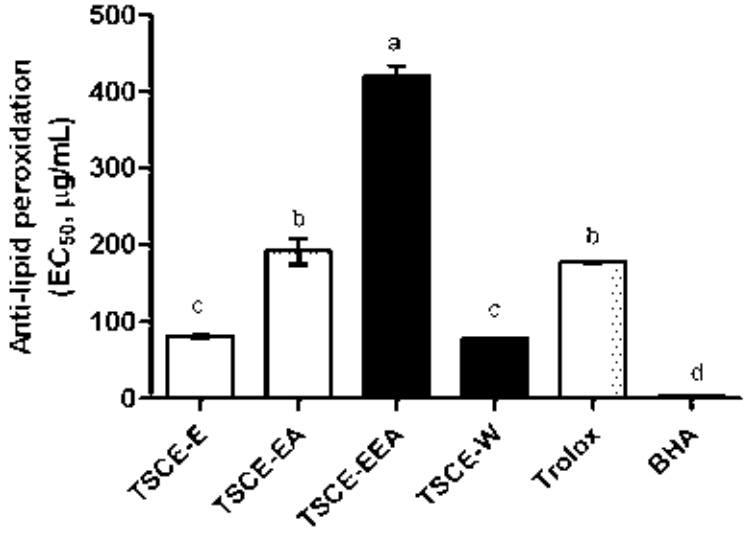


Figure 28. Anti-lipid peroxidation activity of the four TSCEs extracted by different solvent systems from *Tamarindus indica* "Priao-Yak". (a) % scavenging and (b) EC_{50} values (μg/mL). Trolox and BHA were used as reference antioxidants. Data are mean ± SEM of three independent experiments. a, b, c, d are significant difference between groups.

Table 4. EC_{50} (μg/mL) values of TSCEs and antioxidant standards on free radical and antioxidant activities. Data are mean ± SEM of three independent experiments. a, b, c, d, e are significant difference between groups.

Sample	Free radical scavenging activities						Antioxidant activities		
	$ABTS^{\bullet+}$*	$DPPH^{\bullet}$	$O_2^{\bullet-}$	H_2O_2	$OH^{\bullet}$	$NO^{\bullet}$	Reducing power	Metal chelating	Anti-lipid peroxidation
TSCE-E	$8.83^{a,b}$	3.01^{d}	23.53^{b}	11.33^{d}	0.58^{a}	1.76^{c}	18.14^{d}	24.63^{b}	80.99^{c}
TSCE-EA	7.28^{c}	4.38^{a}	25.39^{b}	$12.88^{c,d}$	$0.34^{b,c}$	1.74^{c}	30.50^{a}	15.73^{c}	192.03^{b}
TSCE-EEA	4.97^{d}	3.02^{d}	19.07^{b}	$20.79^{b,c}$	0.21^{d}	4.05^{b}	25.67^{b}	$22.05^{b,c}$	420.48^{a}
TSCE-W	9.64^{a}	2.44^{e}	21.44^{b}	26.03^{b}	0.42^{b}	1.48^{c}	$28.60^{b,a}$	26.33^{b}	78.17^{c}
Trolox	-	3.80^{b}	352.70^{a}	52.53^{a}	0.38^{b}	10.26^{a}	30.22^{a}	NA	177.32^{b}
BHA	$8.23^{b,c}$	3.23^{c}	NA	53.31^{a}	$0.24^{c,b}$	5.17^{b}	21.61^{c}	NA	2.70^{d}

*μmole TEAC/mg of extract, NA= No activity

4. Viability of CCD-1064Sk cells

4.1 Effect of TSCE-W on cell viability

Tamarind seed coat, composed of phenolic compounds including flavonoid, tannin, proanthocyanidin, having antioxidant activity in cell-free system have been widely studied (Tsuda *et al.,* 1994; Pumthong,1999; Sudjaroen *et al*., 2005; Siddhuraju, 2007; Suksomtip *et al.,* 2010). However, the cytotoxicity, antioxidant and reported. The antioxidant activity and cytoprotectice effect of TSCE-W in CCD-1064Sk cells were evaluated in the present studies.

The effect of TSCE-W on the cell viability was examined by using MTT, neutral red, Hoechst 33342 staining, and trypan blue exclusion assays. Although the MTT assay is widely used to determine cell viability, cell proliferation and cytotoxicity of drug and herbal extracts (Han *et al.,* 2010), however, phenolic compounds especially tannin can reduce MTT directly in the absence of living cells, leading to a false-positive or overestimated result (Peng, Wang, and Ren, 2005; Han, *et al.*, 2010). Therefore, the extract was completely washed before adding MTT reagent to avoid the false positive result. While, neutral red assays was used to confirm the effect of TSCE-W on cell viability which have not been reported about the interference of the assay. Hoechst 33342 (a cell-permeable DNA dye) is using to determine apoptotic cells which cause cell death, then it can also used to determine the viability of cells. Trypan blue exclusion assay is a traditional assay for determination of the cells viability, however, quantitation has historically required tedious, time-consuming cell counting prone to investigator bias and sampling error (Uliasz and Hewett, 2000).

Cells were treated with TSCE-W at various concentrations of 0.05, 0.1, 0.2, 0.4, 0.8 and 1 mg/mL for 24 h. The result demonstrated that the cytotoxicity was not observed after treated cells with TSCE-W at the concentrations ranging from 0.05 to 1 mg/mL by using the four assays (Fig. 29). MTT assay showed the highest percentage of viable cells compared with vehicle-treated control (0.25% DMSO in medium). The viable cells increased in a concentration-dependent manner, the values were 103.57, 105.98, 112.97, 118.32% as treated with TSCE-W at 0.05, 0.1, 0.2, 0.4 mg/mL, respectively. Cell viability was significantly increased after treated with TSCE-W at the concentrations of 0.8 and 1.0 mg/mL (132.42% and 139.47%). Neutral red assay

exhibited the cells viability comparable to Hoechst 33342 staining. Meanwhile, Trypan blue assay possessed the lowest percentage of viable cells in concentration-dependent manner.

The cytotoxicity of water extract of seed coat of tamarind has not been reported. However, there has been reported that the ethanolic extracts of the heated tamarind seed coat (20-200 μg/mL) did not affect to the viability of a murine macrophage RAW 264.7 cells (Kumutarin *et al.*, 2004), which was similar to our study. In addition, the methanolic extracts of the flower part of *T. incida* exhibited toxicity against a human amniotic epithelium FL-cells (IC_{50} = 9 μg/mL) (Al-Fatimi *et al.*, 2007). These studies confirmed that TSCE-W was not cytotoxic to CCD-1064Sk cells.

4.2 Effect of H_2O_2 on cell viability

H_2O_2, one of the biologically ROS, is naturally produced as a by-product of oxygen metabolism. It is widely used as an inducer of oxidative stress in various cell-based models (Sen, 2003; Jamshidzadeh and Mehrabadi, 2010; Adetutu *et al.*, 2011). It readily crosses the cellular membranes giving rise to the highly reactive hydroxyl radical, which has ability to react with macromolecules such as DNA, protein and lipids, leading to cell damage (Heo *et al*., 2008). Then, H_2O_2 was used to induce oxidative stress in this study.

When cells treated with H_2O_2 at various concentrations (0.1-10 mM) for 0.5, 1, 3, 6, and 12 h decreased cell viability in a concentration- and time-dependent manner (Fig. 30a), exhibiting the IC_{50} values of 2.20 ± 0.06, 2.18 ± 0.13, 1.73 ± 0.13, 1.40 ± 0.27, and 1.44 ± 0.15 mM H_2O_2, respectively (Fig. 30b). Our study showed that the cells treated with 2 mM of H_2O_2 for 0.5 h significantly decreased cell viability (55.56%) as compared with vehicle control. Variation of H_2O_2 concentrations and time of incubation were tested concentration and time which caused 48-57% cell viability, were used to induce oxidative stress in cells (Iwata *et al*., 1998; Benedi *et al*., 2004; Crispo *et al*., 2010). Therefore, 2 mM H_2O_2 and incubated for 0.5 h, which caused nearly 50% cell viability, were used for further study the induction of oxidative stress in CCD-1064Sk cells**.**

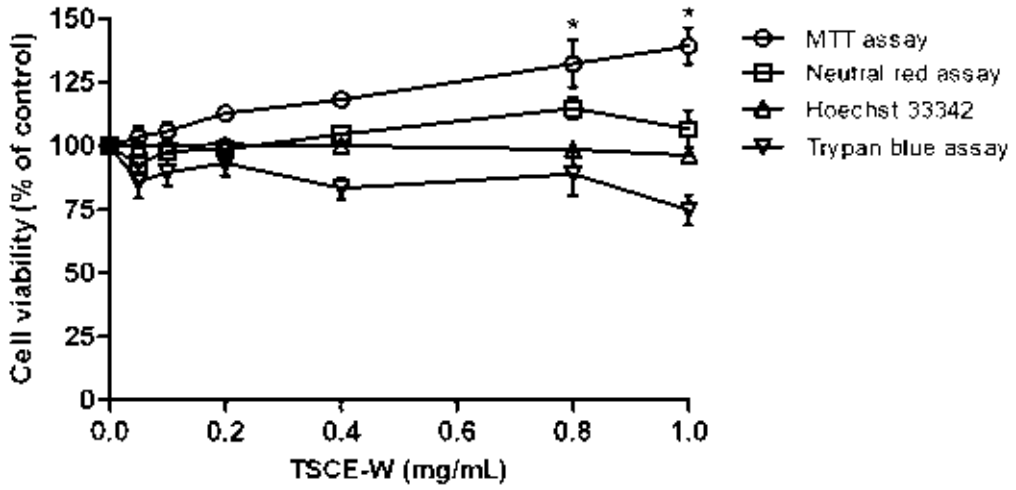
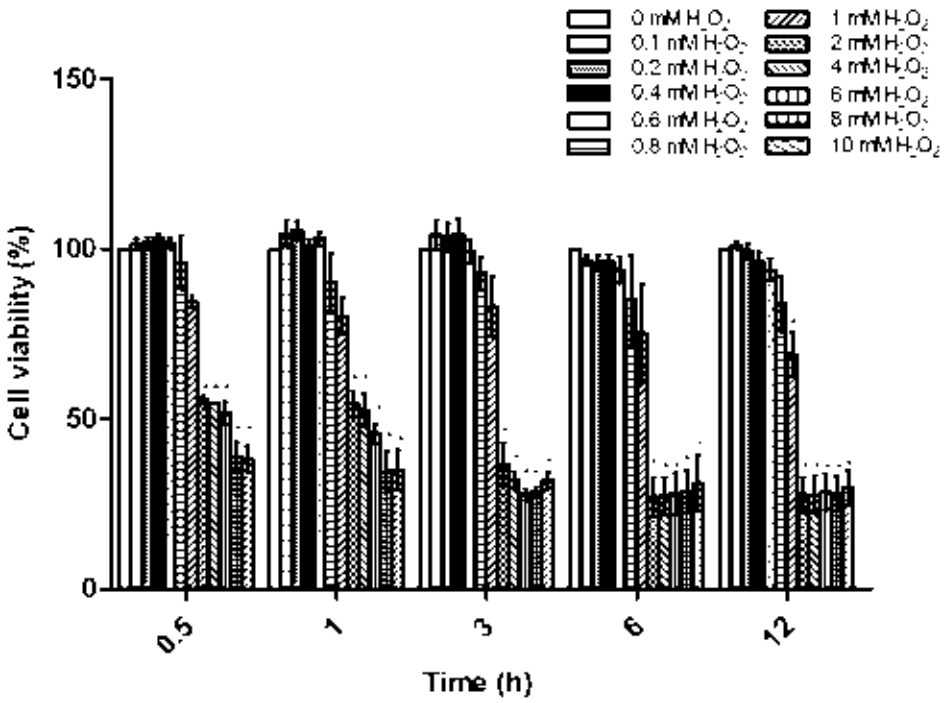


Figure 29. Effect of TSCE-W on the viability of CCD-1064Sk cells. The cells were treated with TSCE-W at different concentrations (0.05-1 mg/mL) for 24 h. The cell viability was assessed by MTT assay, Neutral red assay, Hoechst 33342, and Trypan blue assay. Data are expressed as the mean ± SEM of three replicate values in three independent experiments. *p< 0.05 compared to the vehicle control.

(a)



(b)

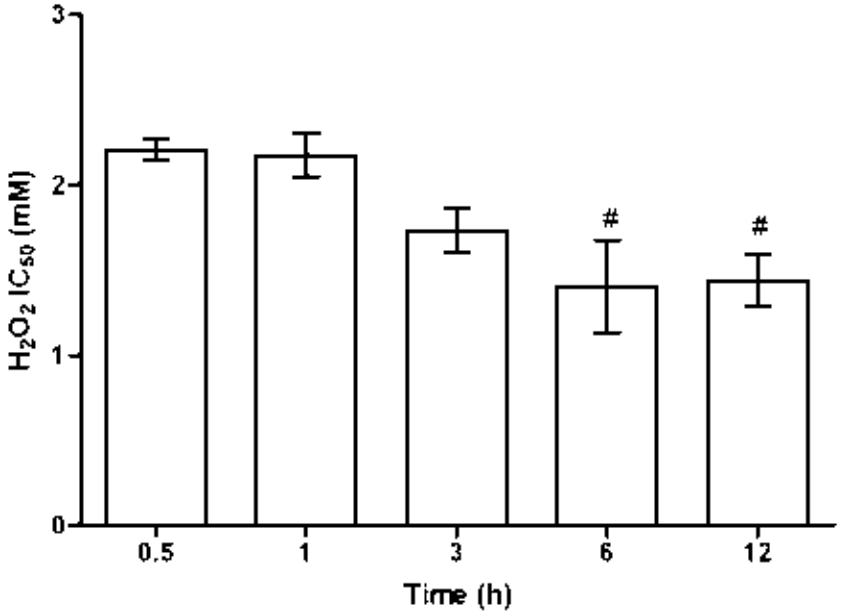


Figure 30. Effect of H_2O_2 on the viability of CCD-1064Sk cells. The cell viability of H_2O_2 at 0.1-10 mM for 0.5,1, 3, 6, and 12 h (a) and IC_{50} values at the indicated times (b). Cell viability was assessed by MTT assay. Data are expressed as mean ± SEM. of three replicate values in three independent experiments. *p< 0.05 compared to the vehicle control, and $^{\#}p$< 0.05 compared to 0.5 h.

4.3 Effect of TSCE-W on viability of H_2O_2-treated cells

TSCE-W had ability to scavenge H_2O_2 in cell-free system (Fig. 31). Therefore, TSCE-W was further studied on its property to protect the cells from oxidative stress induced by H_2O_2. The protective activity was determined by MTT and neutral red assay. The cells were pre-treated with TSCE-W at 0.05-1 mg/mL for 24 h prior to exposure with 2 mM H_2O_2 for 0.5 h to induce oxidative stress. The result showed that TSCE-W at the concentrations of 0.4, 0.8, and 1 mg/mL was significantly increased cell viability compared to the cells treated with 2 mM H_2O_2 without TSCE-W, as determined by MTT assay. The cell viability was 77.64, 98.58, and 100.07%, respectively. In contrast, TSCE-W at a concentration of 0.05 to 1 mg/mL did not increase the viability of cells, as determined by using neutral red assay. The viability of the cells treated with 2 mM H_2O_2 without TSCE-W was similar (47.12 and 44.33%, respectively). TSCE-W at high concentration increased cell viability in H_2O_2-treated cells as determined by MTT assay. The phenolic compound containing in TSCE-W may reduce MTT directly, causing overestimated result. The assay using neutral red showed no increment of H_2O_2 induced oxidative stress cells by TSCE-W. This result suggested that no protective activity of TSCE-W against H_2O_2 treated cells. The high concentration of 2 mM H_2O_2 may not suitable to be used to induce cells oxidative stress by this study.

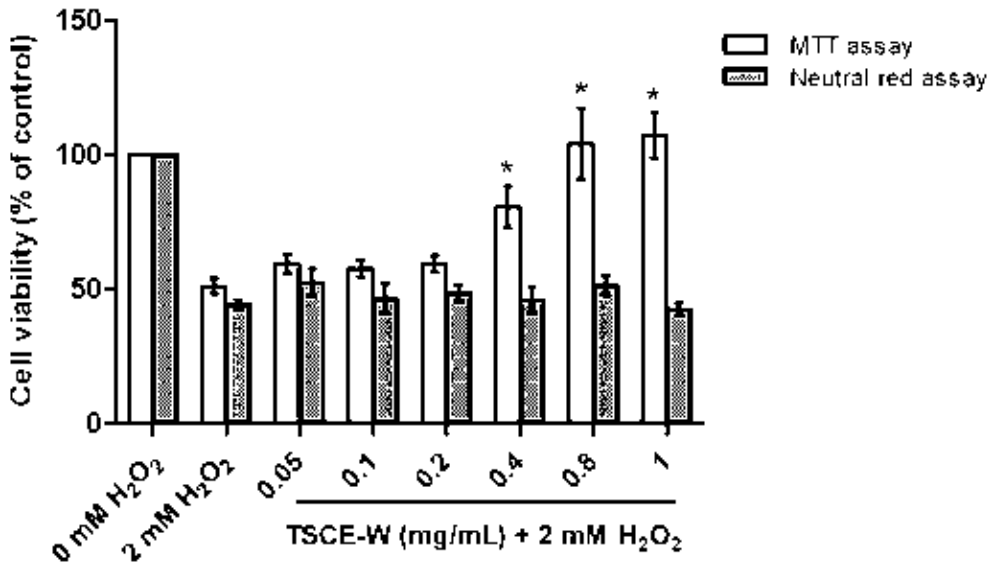


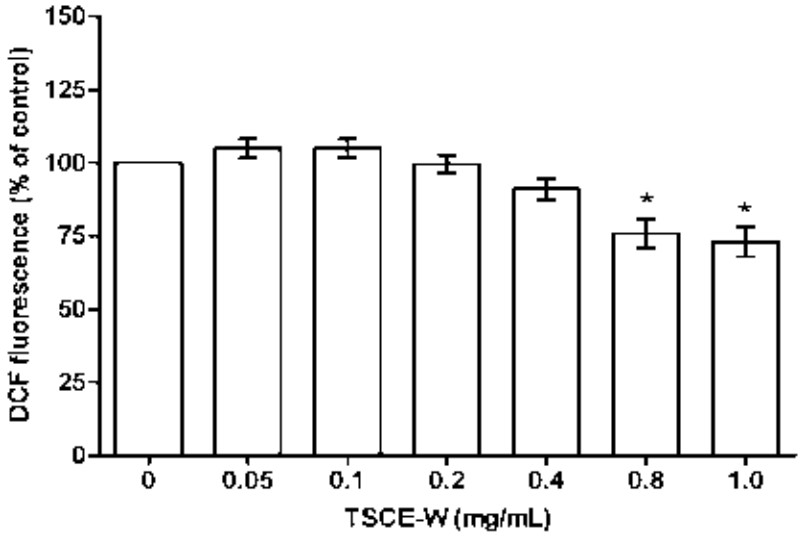
Figure 31. Effect of TSCE-W on cell viability in H_2O_2-treated cells. The CCD-1064Sk cells were pre-treated with TSCE-W (0.05-1 mg/mL) for 24 h. and then exposed with 2 mM H_2O_2 for 0.5 h: Cell viability was assessed by MTT and Neutral red assay. Data are expressed as mean ± SEM of three independent experiments. Each performed in triplicate, *p< 0.05 compared to H_2O_2-treated cells without TSCE-W.

5. Intracellular ROS

5.1 Effect of TSCE-W on intracellular ROS

ROS are highly reactive molecules produced from aerobic metabolism (Chu *et al*., 2010) and can damage the cellular membrane and intracellular molecules (DNA, protein), leading to a number of pathology, such as cancer, diabetes, cardiovascular diseases, neurodegenerative diseases as well as aging. (Campo *et al*., 2004; Girard-Lalancette *et al.*, 2009; Chu *et al.*, 2010). The DFCH-DA assay is recently used to indirectly determine the intracellular ROS level in cells. (Benedí *et al*., 2004; Heo *et al*., 2008; Girard-Lalancette *et al*., 2009). The effect of TSCE-W on intracellular ROS was evaluated by using DCFH-DA assay. The values expressed as DCF fluorescence (% of control), a high DCF fluorescence represented a high intracellular ROS. The CCD-1064Sk cells were added with TSCE-W (0.05-1 mg/mL) for 24 h. The results showed that the intracellular ROS in cells-treated with TSCE-W at 0.05 and 0.1 mg/mL exhibited slightly not significantly increased intracellular ROS (104.89 and 107.63 %) while TSCE-W at 0.2-0.4 mg/mL of TSCE-W not significantly decreased intracellular ROS (99.42 and 90.93 %) (Figure 32a). However, TSCE-W at 0.8 and 1 mg/mL significantly decreased intracellular ROS (75.73 and 72.95 %) compared to the vehicle control (100 %). Although, TSCE-W at 0.8 and 1 mg/mL decreased intracellular ROS but these concentrations did not affective to cell viability which determined using neutral red assay (Figure 32b). This result confirmed that TSCE-W was not toxicity and can be attenuated intracellular ROS in CCD-1064Sk cells.

(a)



(b)

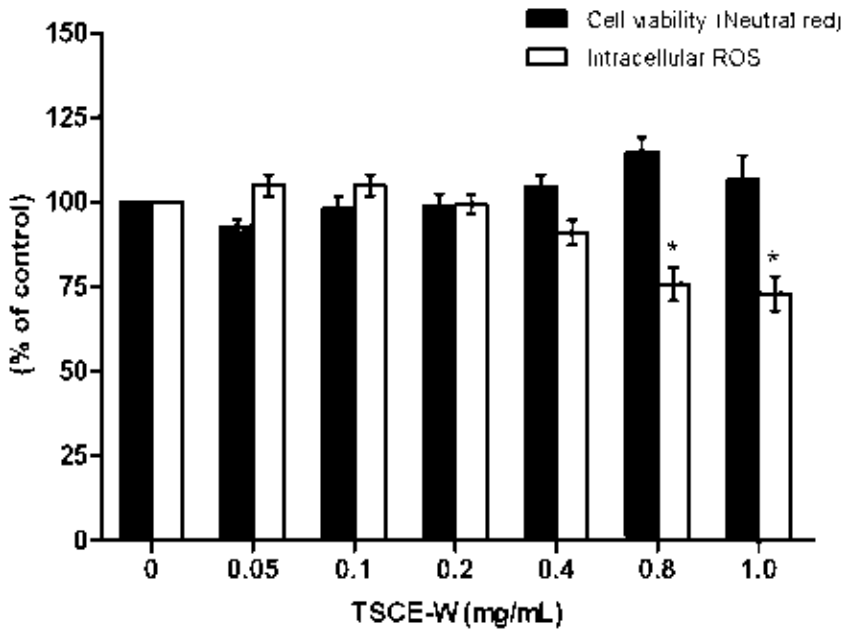


Figure 32. Effect of TSCE-W on intracellular ROS in CCD-1064Sk cells. a) The cells were treated with TSCE-W (0.05-1 mg/mL) for 24 h. and then incubated with 5 μM DCFH-DA in PBS for 30 min. Intracellular ROS in cells was determined by DFCH-DA assay. Data are expressed as mean ± SEM of four independent experiments. Each performed in triplicate. b) A comparison of cell viability (Neutral red assay) and intracellular ROS in cells treated with TSCE-W. *p< 0.05 compared to the vehicle control.

5.2 Effect of H_2O_2 on intracellular ROS

Various concentrations of H_2O_2 and incubation times have been used to induce oxidative stress in difference cell lines (Wang and Joseph, 1999; Sen, 2003; Yao *et al.*, 2008; Zhang *et al.*, 2008). The suitable concentrations and incubation times of H_2O_2 on the induction of intracellular ROS in CCD-1064Sk cells have never been reported. Therefore, suitable concentrations of H_2O_2 and incubation times to induce intracellular ROS in CCD-1064Sk cells were studied. The results showed that the cells exposure with 0.1 and 0.2 mM H_2O_2 for 0.25-3 h were not increased intracellular ROS. However, cells treated with 0.4, 0.6 and 0.8 mM H_2O_2 for 0.25 and 0.5 h showed potentially increased intracellular ROS and then continuously decreased intracellular ROS after incubated for 1 and 3 h. in a concentration and time-dependent manner. The cells treated with 1 mM and 2 mM H_2O_2 for 0.25 h rapidly increased intracellular ROS and then intracellular ROS decreased after incubated for 0.5-3 h in a concentration and time-dependent manner. In addition, the cells treated with 0.6-2 mM for 3 h showed significantly decreased intracellular ROS compared to the vehicle control (Fig. 33). Although, intracellular ROS was not significantly increased in H_2O_2 treated cells more than control in this study. However, treated cells with 1 mM H_2O_2 for 0.25 h showed the highest intracellular ROS accumulation (126.97 % of control), but not toxic to the cells, the cell viability by the MTT assay was 86.10 %. While, cells treated with 2 mM H_2O_2 for 0.5 h showed the decrease of intracellular ROS in cells, which was the caused of cell death in MTT assay at 55.56 % cell viability. Therefore, at 1 mM H_2O_2 and 0.25 h incubation time was used to induce oxidative stress in CCD-1064Sk cells.

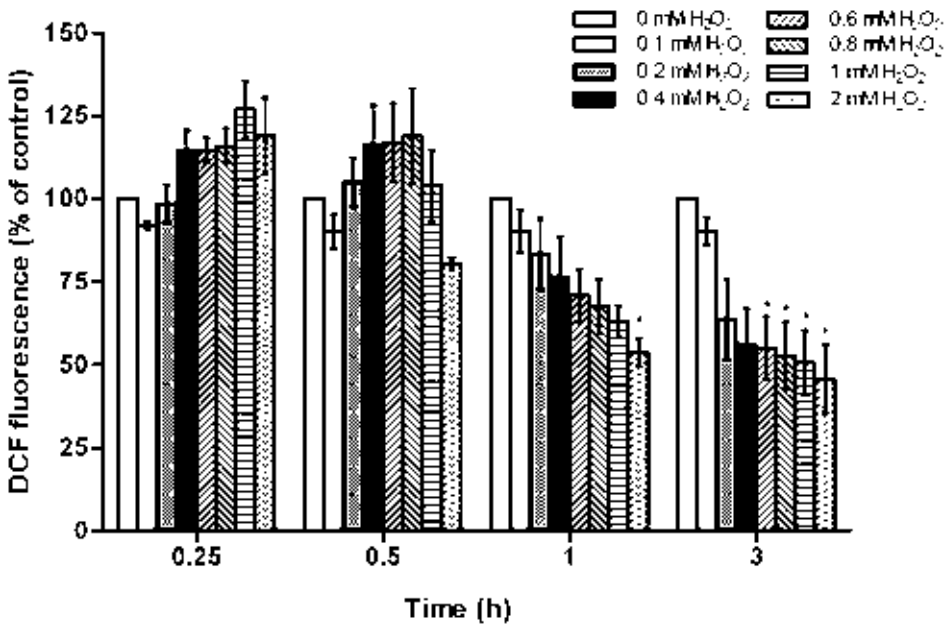


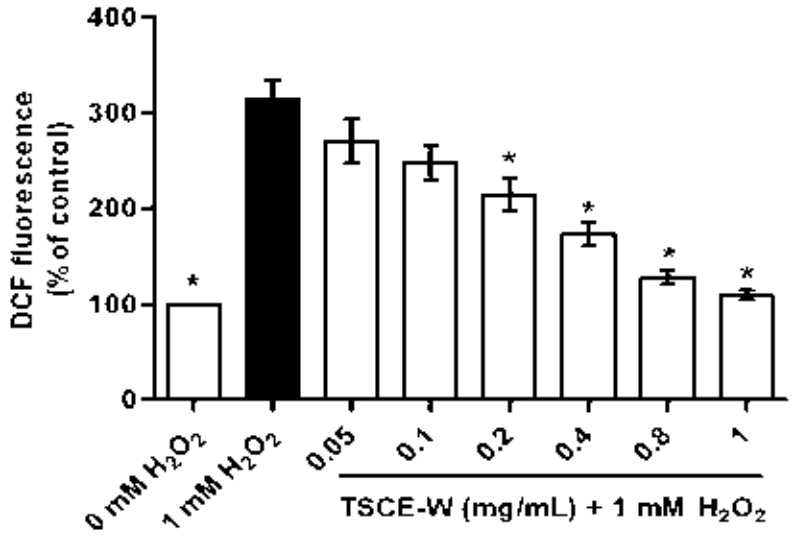
Figure 33. Effect of H_2O_2 on intracellular ROS in CCD-1064Sk cells. The cells were treated with various concentrations of H_2O_2 (0.1-2 mM) for 0.25, 0.5, 1, and 3 h and then incubated with 5 μM DCFH-DA in PBS for 30 min. Intracellular ROS in cells was determined by DFCH-DA assay. Data are expressed as mean ± SEM of three independent experiments. Each performed in triplicate. *p< 0.05 compared to the vehicle control.

5.3 Effect of TSCE-W on intracellular ROS in H_2O_2-treated cells

Effect of TSCE-W on reducing intracellular ROS in H_2O_2-induced oxidative stress in CCD-1064Sk cells was evaluated. The cells were pre-treated with various concentrations of TSCE-W (0.05-1 mg/mL) for 24 h and then incubated with 5 μM DCFH-DA for 30 min. Thereafter, the cells were exposed with 1 mM H_2O_2 for 0.25 h, which intracellular ROS was significantly increased (314.42 %) compared with the vehicle control (100 %). The cells treated with TSEC-W at 0.05-1 mg/mL decreased intracellular ROS accumulation in a concentration-dependent manner. TSCE-W at 0.2-1 mg/mL significantly decreased intracellular ROS in a concentration-dependent manner compared with H_2O_2-treated without TSCE-W. Although, the highest concentration (1 mg/mL) of TSCE-W exhibited the highest reduction of intracellular ROS, but the value was not significantly different compared with the vehicle control (Fig. 34a). The DCF fluorescence in the cells treated with TSCE 4 at 0.05, 0.1, 0.2, 0.4, 0.8, and 1 mg/mL were 270.55, 247.39, 213.95, 172.59, 127.92, and 109.59 %, respectively. The percentage intracellular ROS scavenging activity of TSCE-W compared with 1 mM H_2O_2-treated cells without TSCE-W was 13.90, 21.09, 31.91, 45.07, 59.29, and 65.12 %, respectively (Fig. 34b). The EC_{50} was 0.48 mg/mL. This result suggested that TSCE-W did not increased intracellular ROS. But, it potentially reduced intracellular ROS against-H_2O_2 induced accumulative intracellular ROS in CCD-1064SK cells.

The ability of TSCE-W on the reduction intracellular ROS may related with the antioxidant phenolic compounds in the seed coat of tamarind, similar to the other previous studied of Kang *et al*. (2006), they reported that the caffeic acid at 0.5-50 μM showed protective effect on H_2O_2 treated in WI-38 cells. In addition, cells treated with kahweol and cafestol, an antioxidative agents in coffee beans, at 5-10 μM showed protective potential against-H_2O_2 induced production of intracellular ROS in NIH3T3 cells (Lee and Jeong, 2007) and a marine natural carotenoid fucoxanthin from *Sargassum siliquastrum* at 5-100 μM showed intracellular ROS scavenging activity against-H_2O_2 induced oxidative stress in Vero cells (Heo *et al.*, 2008).

(a)



(b)

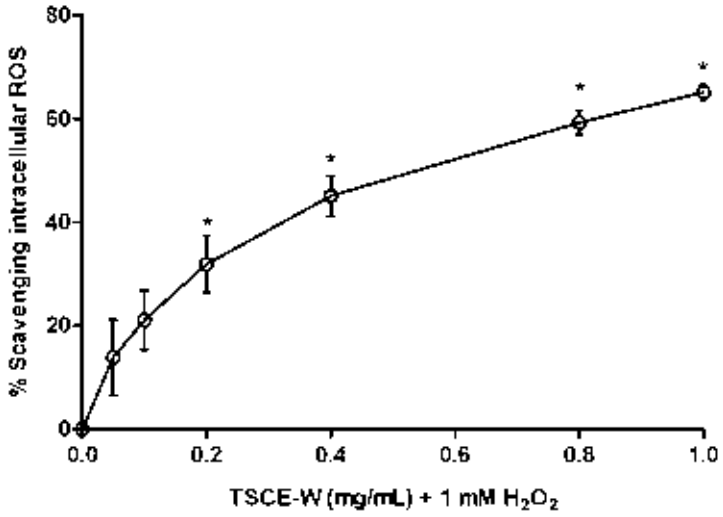


Figure 34. Intracellular ROS scavenging activity of TSCE-W in H_2O_2-treated cells. The CCD-1064Sk cells were pre-treated with various concentrations of TSCE-W for 24 h and then incubated with 5 μM DCFH-DA for 30 min. Thereafter, the cells were exposed with 1 mM H_2O_2 for 0.25 h to induce accumulation of intracellular ROS. a) intensity of DCF fluorescence (%) and b) intracellular ROS scavenging (%). Data are expressed as mean ± SEM of five independent experiments. Each performed in triplicate. *p< 0.05 compared to 1 mM H_2O_2-treated cells without TSCE-W.

6. Lipid peroxidation

6.1 Effect of TSCE-W on lipid peroxidation

Lipid peroxidation is the major lesion in the membrane that can cause from the free radicals and oxidants and lead to the loss of cell viability (Cereser *et al.*, 2001; Zhang, 2008). The effect of free radicals on lipid peroxidation was evaluated by measuring TBARS production (a marker for membrane lipid peroxidation) which is the most commonly used in laboratory assay (Cereser *et al.*, 2001) and the MDA is the end product of lipid peroxidation detected.

The effect of drugs, chemicals or the extracts from plants, herbs and fruits on lipid peroxidation have been studied (Krishnamoorthy *et al*., 2007; Lee and Jeong, 2007; Zhang *et al*., 2008). However, the effect of TSCE-W on lipid peroxidation in cells has not been reported. Then, the effect of TSCE-W on cells lipid peroxidation was investigated in the present study by measuring a level of MDA. The high MDA level represented the high lipid peroxidation. The CCD-1064Sk cells were treated with TSCE-W (0.05-1 mg/mL) for 24 h. The result indicated that treated cells with TSCE-W up to 1 mg/mL did not significantly increased MDA level compared with vehicle control (Fig. 35). However, TSCE-W at 0.8 mg/mL significantly increased MDA level more than at 0.05-0.2 mg/mL TSCE-W treated cells.

The MDA level of TSCE-W at 0.05, 0.1, 0.2, 0.4, 0.8, and 1 mg/mL were 0.08 ± 0.01, 0.07 ± 0.01, 0.09 ± 0.02, 0.12 ± 0.02, 0.19 ± 0.03, 0.15 ± 0.02 nmol MDA/mg protein, respectively. While, MDA level of the vehicle control was 0.13 ± 0.02 nmol MDA/mg protein. This result indicated that TSCE-W did not effect on lipid peroxidation to cell membrane of CCD-1064Sk cells. The similar results have been observed as previously reported of Lee and Jeong (2007), the authors found that the kahweol and cafestol at 10 μM did not change the degree of TBARS formation in NIH3T3 cells and Zhang *et al.* (2008) have also reported that 10 μg/mL of butin did not increased lipid peroxidation in V79-4 cells.

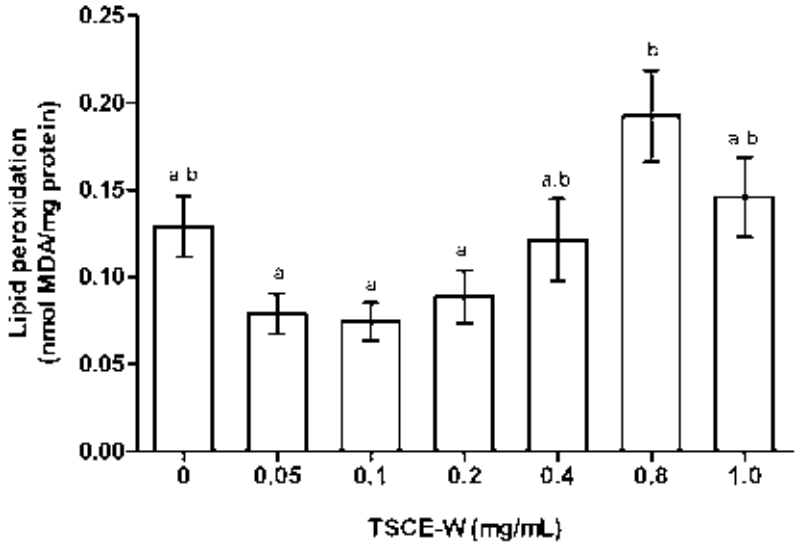


Figure 35. Effect of TSCE-W on lipid peroxidation in CCD-1064Sk cells. The cells were treated with various concentrations of TSCE-W (0.05-1 mg/mL) for 24 h. Lipid peroxidation was determined by TBARS assay. Data are expressed as mean ± SEM of four independent experiments. a, b are significant difference between groups.

6.2 Effect of H_2O_2 on lipid peroxidation

H_2O_2 was widely used to induce membrane damage to cell (Horáková *et al*., 2003; Lee *et al.,* 2003; Campo *et al.*, 2004; Kang *et al.*, 2006). When the cells exposed with H_2O_2 or ROS, it caused to cell membrane damage and devastated to the membrane function, which lead to cell death (Chen *et al.*, 2009).

The effect of H_2O_2 on lipid peroxidation in CCD-1064Sk cells has not been reported. Thus, the effect of H_2O_2 on lipid peroxidation in CCD-1064Sk cells was evaluated following the previously described assay. The CCD-1064Sk cells were exposed with 1 mM and 2 mM H_2O_2 for 0.25-6 h. The results showed that treated cells with 1 mM H_2O_2 for 0.25-6 h did not significantly increased lipid peroxidation more than the vehicle control. While 2 mM H_2O_2 treated for 1 h significantly increased MDA level higher than the vehicle control (Fig. 36). The further study, H_2O_2 at 2 mM concentration and time at 1 h incubation were used to induce lipid peroxidation in CCD-1064Sk cells.

6.3 Effect of TSCE-W on lipid peroxidation in H_2O_2-treated cells

From our previous results, TSCE-W showed inhibitory effect on lipid peroxidation activity in cell-free system (Fig. 28). Then, the ability of TSCE-W on inhibition of lipid peroxidation in H_2O_2-treated cells was investigated in cell-based system. The CCD-1064Sk cells were pre-treated with 0.05-1 mg/mL of TSCE-W for 24 h and then exposed with 2 mM H_2O_2 for 1 h. The results showed that TSCE-W at 0.05-1 mg/mL significantly highly decreased MDA level compared with H_2O_2-treated cells without TSCE-W, however, it did not significantly decreased lower than the vehicle control (Fig. 37). The MDA level of the cells pre-treated with TSCE-W at 0.05, 0.1, 0.2, 0.4, 0.8 and 1 mg/mL were 0.23 ± 0.03, 0.20 ± 0.01, 0.20 ± 0.05, 0.16 ± 0.03, 0.21 ± 0.07, 0.14 ± 0.03 nmol MDA/mg protein, respectively. While, The MDA level in the vehicle control and the cells treated with 2 mM H_2O_2 alone were 0.29 ± 0.05 and 0.73 ± 0.12 nmol MDA/mg protein, respectively. This result clearly indicated that TSCE-W possesses anti-lipid peroxidation property by decreasing the generation of TBARS products in cells with H_2O_2-induced lipid peroxidation compared with the control H_2O_2-treated cells without TSCE-W.

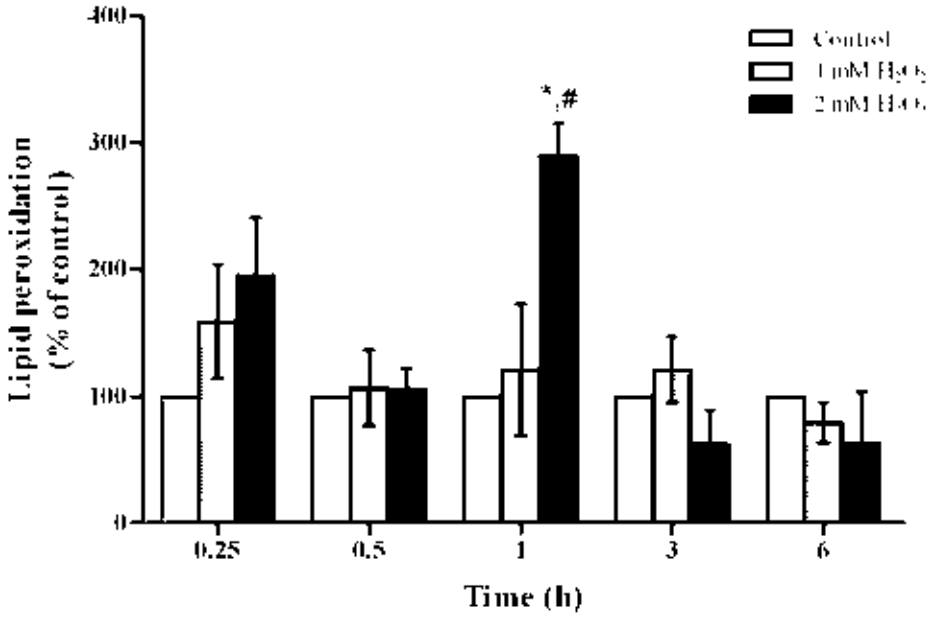


Figure 36. Effect of H_2O_2 on lipid peroxidation in CCD-1064Sk cells. The cells were treated with 1mM and 2 mM H_2O_2 for 0.25-6 h. Lipid peroxidation was determined by TBARS assay. Data are expressed as mean ± SEM of three independent experiments. *p< 0.05 compared to the vehicle control, and $^{\#}p$< 0.05 compared to 1 mM H_2O_2-treated cells alone.

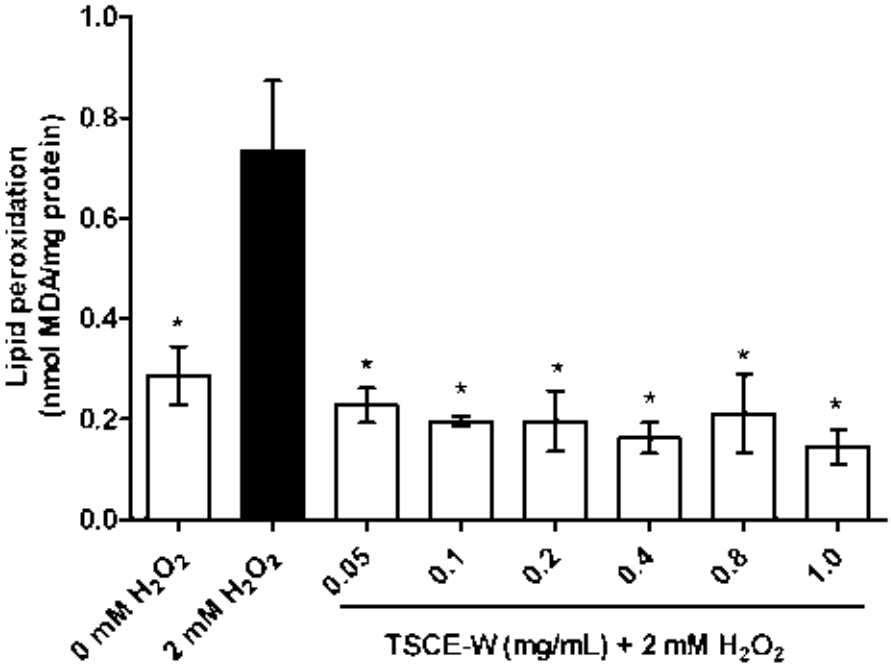


Figure 37. Effect of TSCE-W on inhibition of lipid peroxidation in H_2O_2-treated cells. The CCD-1064Sk cells were pre-treated with various concentrations of TSCE-W (0.05-1 mg/mL) for 24 h and then exposed with 2 mM H_2O_2 for 1 h. Lipid peroxidation was determined by TBARS assay. Data are expressed as mean ± SEM of three independent experiments. *p< 0.05 compared to H_2O_2-treated cells without TSCE-W.

Similar to the previous studied of Lee, Ju, and Kim (2002) who reported that the ethyl acetate fraction of *Euryale ferox* at 100 μg/mL reduced lipid peroxidation in H_2O_2-treated V79-4 cells, including the standardized flavoniod extracts of *Ginkgo biloba* at 160 μg/mL showed protective effect on lipid peroxidtion against-H_2O_2 induced oxidative stress in PC12 cells (Horáková *et al.*, 2003), the methanolic extracts of Chinese medicinal plants at 100 μg/mL shows inhibitory effect on lipid peroxidation in H_2O_2 induced oxidative stress in V79-4 cells (Lee *et al*., 2003), the caffeic acid at 0.5-50 μM decreases lipid peroxidation on H_2O_2-treated in WI-38 cells (Kang *et al*., 2006), the kahweol and cafestol at 5-10 μM prevent TBARS production induced by H_2O_2 in NIH3T3 cells (Lee and Jeong, 2007; Zhang *et al.,* 2008), they reported that 10 μg/mL of butin reduce lipid peroxidation in V79-4 cells.

7. Total glutathione level

7.1 Effect of TSCE-W on total GSH level

Glutathione, a non-protein thiol, and a part of non-enzymatic antioxidant, which is an important component of anti-oxidant defenses in tissues (Gille, Wortelboer, and Joenje, 1987; Susanto *et al*., 1998). GSH is oxidant-scavenger and having redox-regulating capacities including detoxification of the toxic metabolite property (Chen *et al.*, 2009). GSH is the major intracellular reductant and analysis ratio of GSSG: GSH can provide a valid index of cellular oxidative stress. Too high concentration of GSSG indicated the high oxidative stress, which may be damage many antioxidant enzymes. Furthermore, since there is evidence that GSSG rapidly exportes from some cells, a detection of a fall in total GSH is also supportive of an increased oxidative stress in cells (Jackson, 1999).

The effect of plant, herb, and fruit extracts on intracellular GSH level have been studied (Maity, Vedasiromoni, and Ganguly, 1998; Du, Guo and Lou, 2007). However, the effect of TSCE-W on intracellular GSH have not been reported. Then, the effect of TSCE-W on intracellular GSH was investigated in this study by measuring the level of total intracellular glutathione by using the Tietze enzymatic recycling assay. The TSCE-W at 0.05-1 mg/mL was added to the cells for 24 h. The total intracellular GSH level was increased in a concentration-dependent manner.

Meanwhile, TSCE-W at 0.2-1 mg/mL treated cells showed significantly increased total intracellular GSH compared to vehicle control (Fig. 38). The total intracellular GSH level of TSCE-W at 0.05, 0.1, 0.2, 0.3, 0.4, 0.8, and 1 mg/mL and vehicle control were 23.57 ± 1.01, 25.44 ± 1.71, 29.39 ± 2.74, 38.86 ± 0.81, 53.17 ± 4.57, 59.76 ± 2.88 and 22.03 ± 1.26 μM/mg protein, respectively. This result suggested that TSCE-W, which composed polyphenolic compounds, elevated total intracellular GSH level. The similar result to the previously reported (Du *et al.,* 2007), they found that catechin and proanthocyanidin B4, the polyphenols isolated from grape seed extract at 100 μM elevated the intracellular GSH (approximately 1.6 fold from control) in rat heart (H9C2) cells.

7.2 Effect of H_2O_2 on total intracellular GSH level

H_2O_2 was widely used to induce oxidative stress in cells by the depletion of intracellular GSH level. Campo *et al.* (2004) used 0.2 mM H_2O_2 for 1.5 h induced the depletion of GSH level in CRL 2056 cells (decreased 3 fold of control), Qu *et al.* (2009) used 0.75 mM H_2O_2 for 18 h induced the depletion of GSH level in human hepatocyte (HL-7702) cells (decreased 1.44 fold from control) and Jamshidzadeh and Mehrabadi (2010) used 20 mM H_2O_2 for 1 h in erythrocyte. However, the effect of H_2O_2 on intracellular GSH in CCD-1064Sk cells was never reported. Therefore, the effect of H_2O_2 on intracellular GSH in CCD-1064Sk cells was determined by following previous assay.

The cells were treated with 1 mM and 2 mM H_2O_2 for 0.25-1 h. The results showed that the cells treated with 1 mM and 2 mM H_2O_2 for 0.25 h showed not significant difference in total intracellular GSH level from vehicle control. Whereas, cells treated with 1 mM and 2 mM H_2O_2 for 0.5-1 h significantly decreased in total intracellular GSH level compared with vehicle control, the cells treated with 2 mM H_2O_2 for 1 h exhibited the lowest intracellular GSH level (Fig. 39). Although, cells treated with 2 mM H_2O_2 for 1 h possessed the highest deplete intracellular GSH level (decreased 3 fold from control), but it also showed cytotoxicity to cells, the cell viability with the MTT assay was 54.49 %. Cells treated with 1 mM H_2O_2 showed the lowest intracellular GSH compared at 0.5 h incubation time (decreased 2 fold from control) and at this concentration did not showed toxic effect in cells, the cell viability

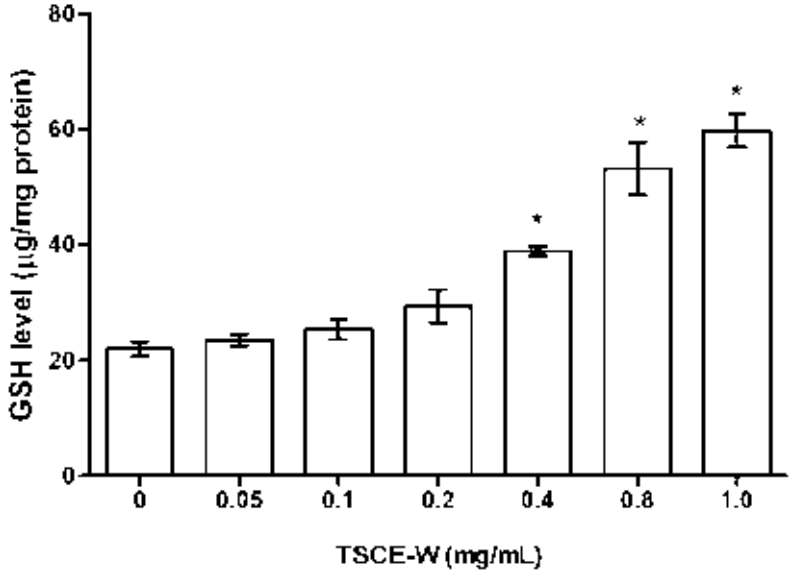


Figure 38. Effect of TSCE-W on intracellular GSH level in CCD-1064Sk cells. The cells were treated with TSCE-W at different concentrations (0.05-1 mg/mL) for 24 h. Data are expressed as mean ± SEM of four independent experiments. *p< 0.05 compared to the vehicle control.

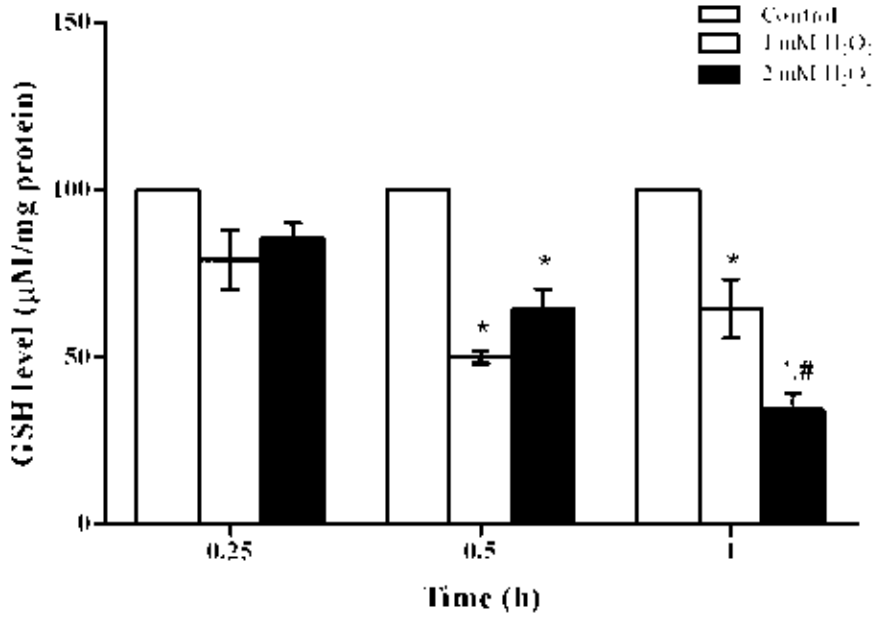


Figure 39. Effect of H_2O_2 on intracellular GSH level in CCD-1064Sk cells. The cells were treated with 1mM and 2 mM H_2O_2 for 0.25-1 h. Data are expressed as mean ± SEM of three independent experiments. *p< 0.05 compared to the vehicle control, and $^{\#}p$< 0.05 compared to 1 mM H_2O_2 at 1 h.

with the MTT assay was 84.56 %. Therefore, 1 mM H_2O_2 and 0.5 h incubation time were used to induce the depletion of total intracellular GSH level for further study.

7.3 Effect of TSCE-W on total intracellular GSH level in H_2O_2-treated cells

The ability of TSCE-W on total intracellular GSH level in H_2O_2-treated cells was evaluated. The CCD-1064Sk cells were pre-treated with 0.05-1 mg/mL of TSCE-W for 24 h and then exposed with 1 mM H_2O_2 for 0.5 h. The results showed that TSCE-W at 0.2-1 mg/mL significantly increased intracellular GSH level in H_2O_2-treated cells. In addition, TSCE-W at 0.8 and 1 mg/mL significantly increased intracellular GSH level compared with vehicle control (Fig. 40). The total intracellular GSH level of TSCE-W at 0.05, 0.1, 0.2, 0.4, 0.8, and 1 mg/mL were 9.47 ± 1.29, 12.57 ± 1.10, 16.95 ± 3.95, 22.34 ± 2.52, 34.53 ± 5.66, and 35.60 ± 0.57 μM/mg protein, respectively, whereas, the GSH level of the vehicle control and H_2O_2 treated without TSCE-W were 14.37 ± 2.22 and 4.24 ± 0.41 μM/mg protein. The protective activity of TSCE-W on intracellular GSH in H_2O_2-treated cells was similar to the previous studied of Campo *et al.* (2004), they reported that hyaluronic acid treated cells at 1-2 mg/mL can increase GSH level against H_2O_2-induced oxidative stress in CRL 2056 cells, Qu *et al.* (2009) found that the homoplantaginin isolated from *Salvia plebeia* at 1-100 μg/mL increases GSH level against H_2O_2-induced oxidative stress in HL-7702 cells, Hong and Lee (2009) reported that the ethyl acetate fraction of *Artemisia capillaries* at 100 μg/mL increases GSH level against H_2O_2-induced oxidative stress in V79-4 cells and Jamshidzadeh and Mehrabadi (2010) reported that quercetin at 15 mM increases GSH level against H_2O_2-induced oxidative stress in erythrocyte. This result indicates that TSCE-W showed cytoprotective activity against H_2O_2-induced oxidative stress by enhanced on elevation intracellular GSH level in CCD-1064Sk-cells.

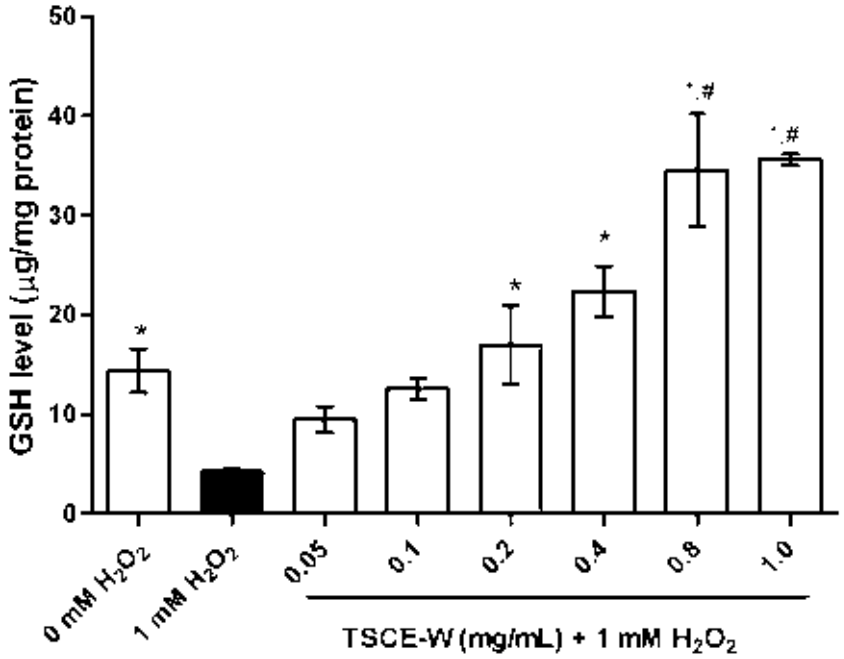


Figure 40. Effect of TSCE-W on intracellular GSH level in H_2O_2-treated cells. The CCD-1064Sk cells were pre-treated with various concentrations of TSCE-W (0.05-1 mg/mL) for 24 h and then exposed with 1 mM H_2O_2 for 0.5 h. Data are expressed as mean ± SEM of three independent experiments. *p< 0.05 compared to 1 mM H_2O_2-treated cells alone, and $^{\#}p$< 0.05 compared to the vehicle control.

8. SOD activity

8.1 Effect of TSCE-W on SOD activity

Superoxide dismutase, a member of enzymatic anti-oxidative defenses that play an important role in protecting cells from ROS by catalysis the dismutation of $O_2^{\bullet-}$ into H_2O_2 and O_2 (Sun and Oberley, 1988). There are three forms of SOD in living cells. One form, found primarily in the cytoplasm, is a homodimer containing Cu and Zn (Cu,ZnSOD). The two other forms, the one that found predominantly in mitochondria, is a tetramer containing Mn (MnSOD) and the second, extracellular form (EC-SOD), is a tetramer containing Cu,Zn as well as sugar residues (Kankofer, 2002).

This study we determined an effect of TSCE-W on total SOD (Cu,ZnSOD and MnSOD) activity. The result showed that TSCE-W at 0.05-1 mg/mL did not reduced SOD activity, while TSCE-W at 0.8 mg/mL significantly elevated the SOD activity compared to vehicle control (Fig. 41). The SOD activity of TSCE-W at 0.05, 0.1, 0.2, 0.4, 0.8, 1 mg/mL and vehicle control were 1.71 ± 0.26, 2.35 ± 0.06, 2.65 ± 0.31, 2.83 ± 0.24, 3.34 ± 0.28, 2.78 ± 0.42, and 1.76 ± 0.03 units/mg protein, respectively. The 50 % inhibition of NBT from pure bovine liver Cu,ZnSOD standard curve in this study was about 10 ng/mL, which was similar to the previous report of Sun and Oberley (1988).

The elevated SOD activity of TSCE-W in cells may be correlated with the antioxidant phenolic compounds in the seed coat of tamarind as described by the other previous studied, they found that the ethyl acetate fraction of *Euryale ferox* seed (Lee *et al.*, 2002), the methanol extracts of medicinal plants from Chinese (Lee *et al*., 2003), the methanol extracts of *Betula platyphylla* var. *japonica* (Ju *et al*., 2004), the green tea and other herb extracts at 100 μg/mL (Yoo *et al.*, 2008), and the butin at 10 μg/mL (Zhang *et al*., 2008), those compound can enhanced SOD activity in V79-4 cells. Moreover, the catechin and proanthocyanidin B4 from grape seed extract at 100 μM increased SOD activity (approximately 1.2 fold from control) in H9C2 cells (Du *et al.,* 2007).

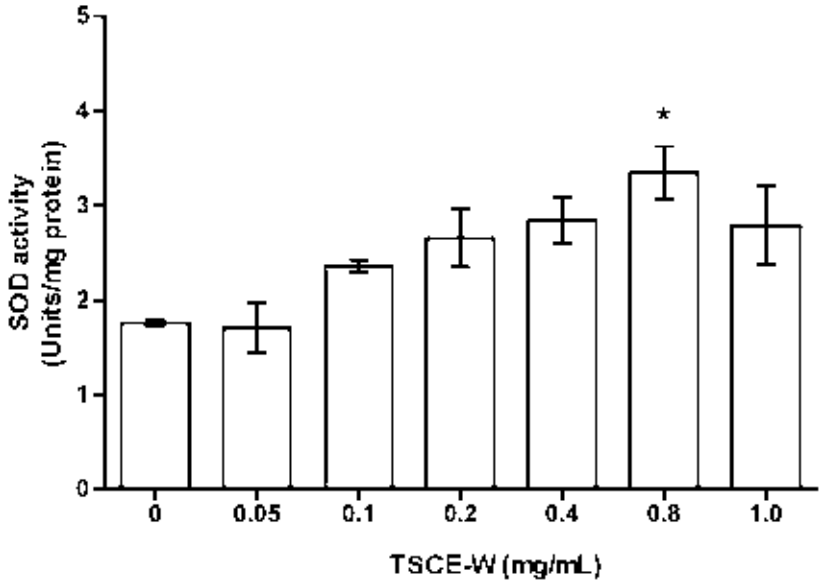


Figure 41. Effect of TSCE-W on SOD activity in CCD-1064Sk cells. The cells were treated with TSCE-W at different concentrations (0.05-1 mg/mL) for 24 h. Data are expressed as mean ± SEM of three independent experiments. *p< 0.05 compared to the vehicle control.

8.2 Effect of H_2O_2 on SOD activity

According to the previously studied, various concentrations of H_2O_2 and times incubation on SOD activity were used to induced oxidative stress in various cell lines by observing the decrease SOD activity, such as an exposure at 0.2 mM H_2O_2 for 1.5 h in CRL 2056 cells (Campo *et al.*, 2004); 150-250 μM H_2O_2 for 30 min in human colon carcinoma (Caco-2) cells (Wijaratne, Cuppett, and Schlegel, 2005); 1 mM H_2O_2 for 1 h in V79-4 (Zhang *et al*., 2008)**;** 0.75 mM H_2O_2 for 18 h in HL-7702 cells (Qu *et al.*, 2009). However, the effect of H_2O_2 on SOD activity in CCD-1064Sk cells was never reported. Hence, the effect of H_2O_2 on SOD activity in CCD-1064Sk cells was investigated. The CCD-1064Sk cells were treated with H_2O_2 at the concentrations of 1 and 2 mM for 0.25-3 h. The results in Figure 42 indicated that H_2O_2 did not significantly increased SOD activity in H_2O_2-treated CCD-1064Sk cells. This result was similar to the previous report of Roig *et al.* (2002), they found that 0.6 mM H_2O_2-treated cells for 3 and 12 h was not affected to hepatocyte (Fao) cells. In fact, the primary role of SOD is to maintain a steady state of $O_2^{\bullet-}$ by catalyzes the dismutation of $O_2^{\bullet-}$ into H_2O_2 and O_2, and then H_2O_2 was converted to H_2O and O_2 by catalase. This reason supported that SOD seem not to response against the oxidative stress in some cell types (Lee and Ho 1995).

Although, H_2O_2 did not give significantly different of SOD activity from vehicle control in this study, but treated cells with 2 mM H_2O_2 for 0.25 h exhibited the lowest SOD activity. Thus, this concentration and time incubation were used for further study.

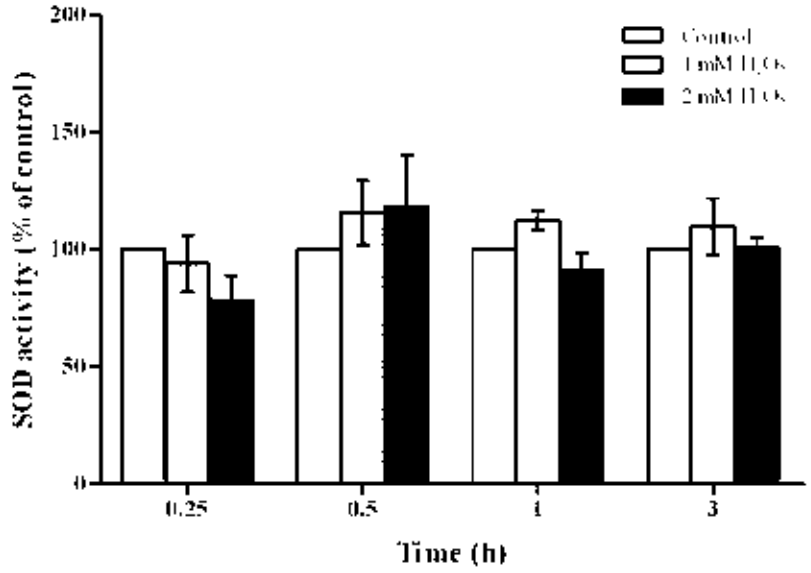


Figure 42. Effect of H_2O_2 on SOD activity in CCD-1064Sk cells. The cells were treated with 1 mM and 2 mM H_2O_2 for 0.25-3 h. Data are expressed as mean ± SEM of three independent experiments.

8.3 Effect of TSCE-W on SOD activity in H_2O_2-treated cells

The protective activity of TSCE-W on the decreased of SOD activity in H_2O_2-induced oxidative stress in CCD-1064Sk cells was evaluated. The cells were pre-treated with various concentrations of TSCE-W (0.05-1 mg/mL) for 24 h and then exposed with 2 mM H_2O_2 for 0.25 h. The results showed that TSCE-W at 0.05-1 mg/mL did not significantly increased SOD activity compared with vehicle control and 2 mM H_2O_2-treated cells without TSCE-W. However, TSCE-W treated cells showed potentially higher SOD activity. The SOD activity of TSCE-W at 0.05, 0.1, 0.2, 0.4, 0.8, 1 mg/mL were 0.80 ± 0.08, 0.89 ± 0.15, 0.91 ± 0.19, 0.81 ± 0.29, 0.88 ± 0.23, and 0.89 ± 0.34 units/mg protein, respectively, whereas the vehicle control and 2 mM H_2O_2-treated cells without TSCE-W were 0.74 ± 0.07 and 0.63 ± 0.05 units/mg protein (Fig. 43). Although, TSCE-W at 0.8 mg/mL alone possessed increase SOD activity (Fig. 41), but it did not increased SOD activity when H_2O_2-treated cells. This result may be at this too high concentration of H_2O_2 did not activate SOD activity. There have been reported that the concentrations of H_2O_2 higher than 10 μM having less effective to activate intermembrane space of Cu,ZnSOD (Inarrea *et al*., 2005) and the major role of SOD is to maintain a steady state of $O_2^{\bullet-}$ to H_2O_2 (Roig *et al.*, 2002) but not scavenge H_2O_2. This result is similarly to the studied of Roig *et al.* (2002), they reported that the flavonoids from red wine, such as catechine, epicatechin, quercetin and proanthocyanidins do not increased SOD activity in H_2O_2-treated Fao cells. The result suggested that TSCE-W contained a high proanthocyanidins was not affected to increase the SOD activity, but they possessed the $O_2^{\bullet-}$ scavenging activity.

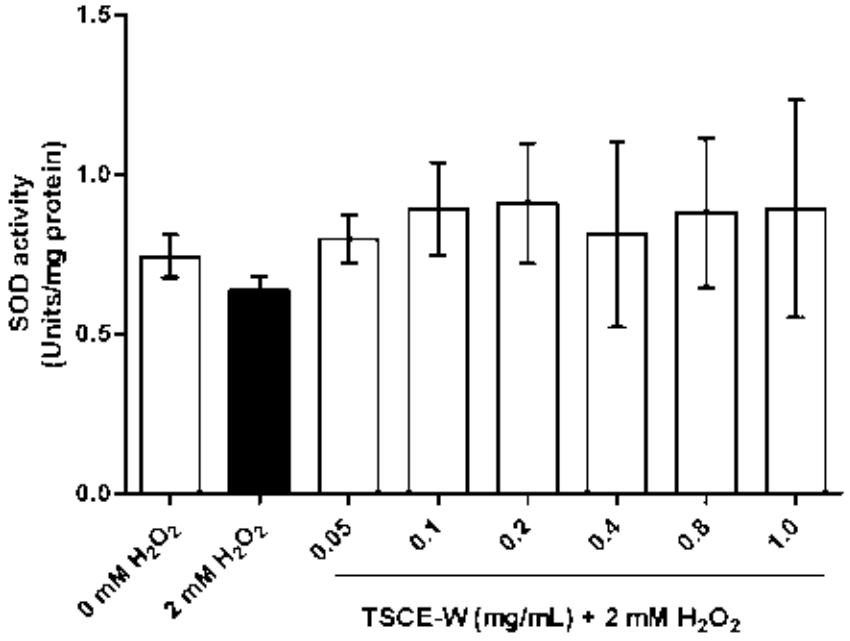


Figure 43. Effect of TSCE-W on SOD activity in H_2O_2-treated cells. The CCD-1064Sk cells were pre-treated with various concentrations of TSCE-W (0.05-1 mg/mL) for 24 h and then exposed with 2 mM H_2O_2 for 0.25 h. Data are expressed as mean ± SEM of three independent experiments.

9. GPx activity

9.1 Effect of TSCE-W on GPx activity

Glutathione peroxidase is antioxidant enzyme which catalyst H_2O_2 to O_2 and H_2O using GSH as a substrate. The cells were treated with TSCE-W at a concentration of 0.05-1 mg/mL for 24 h. The result showed that TSCE-W up to 1 mg/mL did not significantly increase GPx activity in CCD-1064Sk cells compared to vehicle control (Fig. 44). This result was similarly to the reported of Du *et al.* (2007), they found that catechin and proanthocyanidin B4, the polyphenols isolated from grape seed extract at 25-100 μM do not increased GPx activity in H9C2 cells. This result suggested that TSCE-W did not reduce the activity of GPx in normal condition. However, it may have protective effect against H_2O_2-treated cells.

9.2 Effect of H_2O_2 on GPx activity

The previous studied on evaluation of GPx activity, various concentrations of H_2O_2 and times incubation were used to induced oxidative stress in various cell lines, the values show the decrease of GPx activity, such as 0.6 mM H_2O_2 for 3 and 21 h in Fao cells (Roig *et al.*, 2002); 7.5 mM H_2O_2 for 15 min (Sen *et al.*, 2003) and 1 mM H_2O_2 for 24 h in V79 fibroblast cells (Hong and Lee, 2009). However, the effect of H_2O_2 on GPx activity in CCD-1064Sk cells was never reported. The effect of H_2O_2 on GPx activity in CCD-1064Sk cells was investigated. The CCD-1064Sk cells were treated with H_2O_2 at 2 mM for 0.25-3 h. The results showed that H_2O_2-treated cells gradually increased GPx activity with a time-dependent manner (Fig. 45). Although, 2 mM H_2O_2-treated cells for 0.25 h and 0.5 h showed not significantly decrease GPx activity compared to control and GPx activity was lower than at 1 and 3 h incubation time, but they possessed lesser cytotoxic than at 1 and 3 h incubation time. Hence, at these incubation times were used for further study.

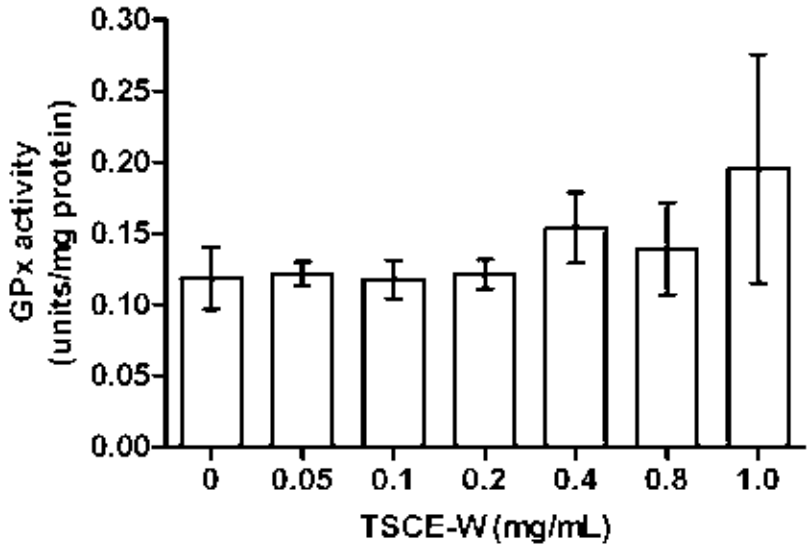


Figure 44. Effect of TSCE-W on GPx activity in CCD-1064Sk cells. The cells were treated with TSCE-W at different concentrations (0.05-1 mg/mL) for 24 h. Data are expressed as mean ± SEM of three independent experiments.

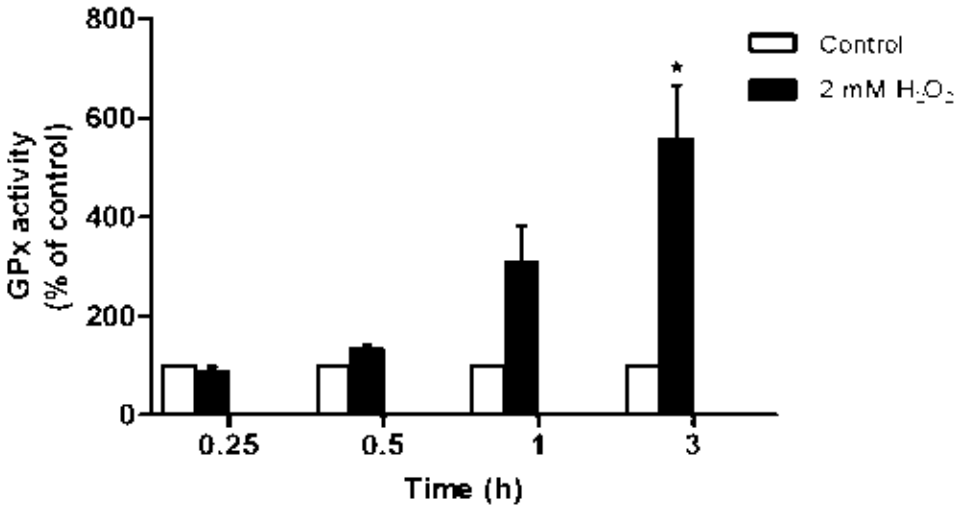


Figure 45. Effect of H_2O_2 on GPx activity in CCD-1064Sk cells. The cells were treated with 2 mM H_2O_2 for 0.25-3 h. Data are expressed as mean ± SEM of three independent experiments. * $p< 0.05$ compared to the vehicle control.

9.3 Effect of TSCE-W on GPx activity in H_2O_2-treated cells

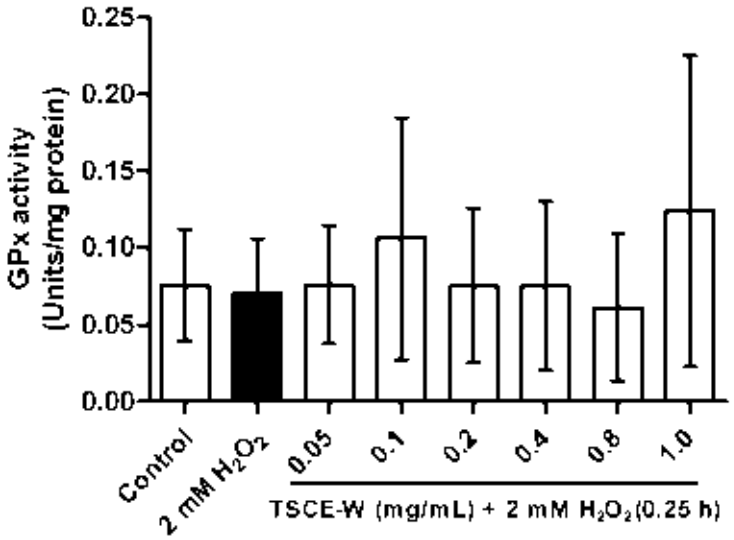
The cells were pre-treated with various concentrations of TSCE-W (0.05-1 mg/mL) for 24 h and then exposed with 2 mM H_2O_2 for 0.25 h and 0.5 h. The results showed that TSCE-W at 0.05-1 mg/mL did not significantly increase GPx activity in H_2O_2-treated cells in both 0.25 h (Fig. 46a) and 0.5 h (Fig. 46b) incubation times. TSCE-W did not showed protective effect on increasing the GPx activity against H_2O_2-induced damage cells. This may be result from 2 mM H_2O_2 generating severe oxidative stress, causing cell death. The viability of CCD-1064Sk cells after exposed with 2 mM H_2O_2 for 0.25 h and 0.5 h was 56.10% and 55.56%, respectively. In addition, GPx is not a major source for protection against severe oxidative stress, it only protects against the low level of oxidant stress including GPx alone can react effectively with lipid and other organic hydroperoxides (Yan and Harding, 1997). This result is similarly to the studied of Roig *et al.* (2002), they reported that the flavonoids from red wine, such as catechin, epicatechin, and procyanidins did not increase GPx activity in 0.6 mM H_2O_2-treated Fao cells for 3 h.

10. CAT activity

10.1 Effect of TSCE-W on CAT activity

Catalase is antioxidant enzyme which catalyst H_2O_2 to O_2 and H_2O. After the cells were treated with TSCE-W at a concentrations of 0.05-1 mg/mL for 24 h, the result showed that TSCE-W at 0.5-0.8 mg/mL did not significantly increase CAT activity, while TSCE-W at 1 mg/mL possessed significantly increase in CAT activity in CCD-1064Sk cells compared to vehicle control (Fig. 47). This result is agrees with previously reported, they found that medicinal plants and phenolic compound in plants have potential to increase the activity of CAT. (Lee *et al.*, 2002; Chae *et al.*, 2004; Ju *et al.,* 2004; Du *et al*., 2007).

(a)



(b)

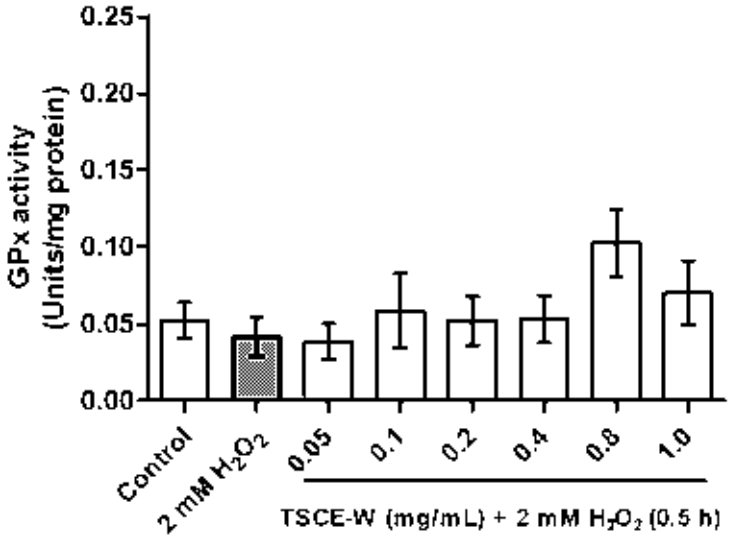


Figure 46. Effect of TSCE-W on GPx activity in H_2O_2-treated cells. The CCD-1064Sk cells were pre-treated with various concentrations of TSCE-W (0.05-1 mg/mL) for 24 h and then exposed with 2 mM H_2O_2 for 0.25 h (a) and 0.5 h (b). Data are expressed as mean ± SEM of three independent experiments.

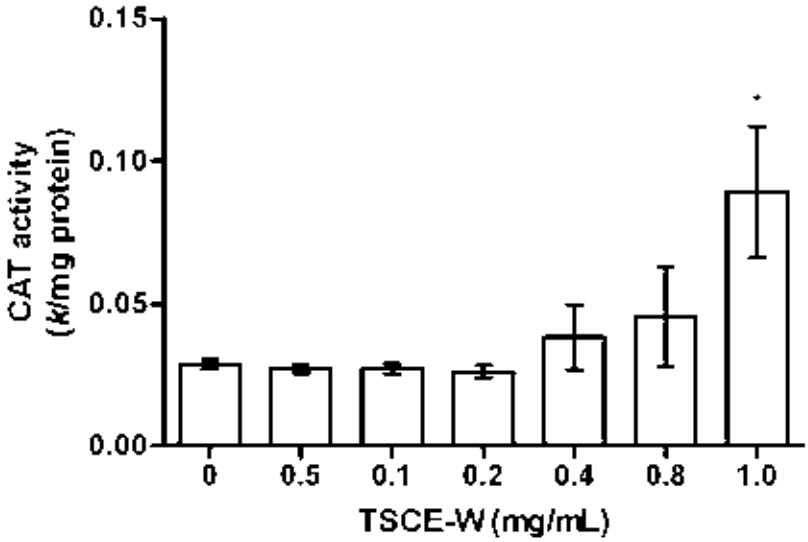


Figure 47. Effect of TSCE-W on CAT activity in CCD-1064Sk cells. The cells were treated with TSCE-W at different concentrations (0.05-1 mg/mL) for 24 h. Data are expressed as mean ± SEM of three independent experiments. *p< 0.05 compared to the vehicle control.

10.2 Effect of H_2O_2 on CAT activity

There are reported of various concentrations of H_2O_2 and times incubation were used to induced oxidative stress in various cell lines to evaluate the decrease of CAT activity, such as 1 mM H_2O_2, for 1 h in V79-4 cells (Zhang *et al.,* 2008), 1 mM H_2O_2 for 24 h in V79 fibroblast cells (Hong and Lee, 2009), while 0.6 mM H_2O_2 and incubated for 3 and 21 h was used to induced oxidative stress in Fao cells (Roig *et al.*, 2002), the result showed the increasment of CAT activity. However, the effect of H_2O_2 on CAT activity in CCD-1064Sk cells was never reported. Hence, the effect of H_2O_2 on CAT activity in CCD-1064Sk cells was evaluated.

The CCD-1064Sk cells were treated with H_2O_2 at 2 mM for 0.25-3 h. The 2 mM H_2O_2-treated cells for 0.25 h and 0.5 h did not significantly decrease CAT activity compared to control, whereas 1 h- incubated cells exhibited the highest CAT activity and 3 h-incubated cells possessed the lowest CAT activity (Fig. 48). However, treated cells with 2 mM H_2O_2 for 1 and 3 h were more toxic to cells than the 0.25 and 0.5 h-incubated cells, in addition 0.5 h-incubated time possessed more decrease of CAT activity than 0.25 h-incubated time. Then, this incubation time was used for further study.

10.3 Effect of TSCE-W on CAT activity in H_2O_2-treated cells

The cells were pre-treated with various concentrations of TSCE-W (0.05-1 mg/mL) for 24 h and then exposed with 2 mM H_2O_2 for 0.5 h. The results showed that TSCE-W at 0.05-1 mg/mL did not significantly increase CAT activity compared with H_2O_2-treated cells without TSCE-W (Fig. 49). This may be result of the treatment cells with 2 mM H_2O_2 for 0.5 h generated severe oxidative stress and causing cell death (55.56% cell viability), so TSCE-W can not increase CAT activity in this study.

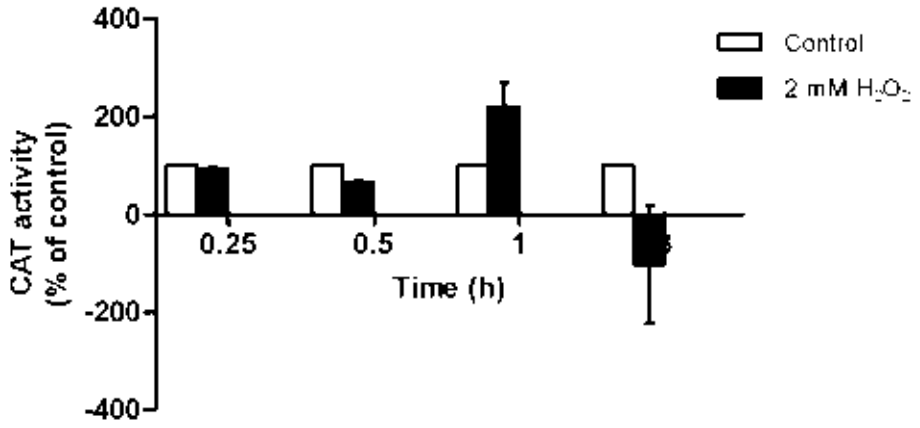


Figure 48. Effect of H_2O_2 on CAT activity in CCD-1064Sk cells. The cells were treated with 2 mM H_2O_2 for 0.25-3 h. Data are expressed as mean ± SEM of three independent experiments.

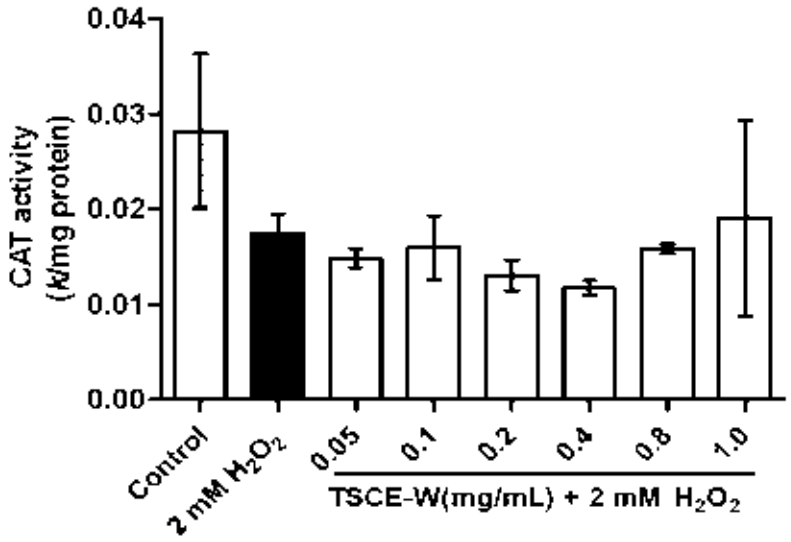


Figure 49. Effect of TSCE-W on CAT activity in H_2O_2-treated cells. The CCD-1064Sk cells were pretreated with various concentrations of TSCE-W (0.05-1 mg/mL) for 24 h and then exposed with 2 mM H_2O_2 for 0.5 h. Data are expressed as mean ± SEM of three independent experiments.

11. Protein expression of antioxidant enzymes in CCD-1064Sk cells

11.1 Effect of TSCE-W and H_2O_2 on protein expression of Cu,ZnSOD, GPx, and CAT

The effect of TSCE-W and H_2O_2 on protein expression of antioxidant enzymes: Cu,ZnSOD, GPx, and CAT in CCD-1064Sk cells was investigated by using Western blot analysis. The cells were treated with TSCE-W at 0.2-0.8 mg/mL for 24 h. These concentrations did not cause cytotoxic effect, attenuated intracellular ROS, increased intracellular GSH level, and increased the activity SOD and catalase activity. The 1 mM H_2O_2-treated cells for 3 and 6 h were compared. The result showed that all tested concentrations of TSCE-W did not significantly up-regulate protein expression of Cu,ZnSOD, GPx, and CAT enzymes compared with vehicle control. Meanwhile, 1 mM H_2O_2-treated cells for 3 and 6 h, which is a non toxic dose (83.06 and 75.17% cell viability, respectively), did not significantly up-regulate protein expression of these enzymes compared with the vehicle control (Fig. 50).

11.2 Effect of TSCE-W on protein expression of Cu,ZnSOD, GPx, and CAT in H_2O_2-treated cells

Pretreatment the cells with TSCE-W at 0.4 mg/mL and 0.4-0.8 mg/mL before treated cells with 1 mM H_2O_2 for 3 h, significantly up-regulated the protein expression of Cu,ZnSOD and GPx compared with the vehicle control, respectively. However, all tested concentrations of TSCE-W did not effect to increase protein expression of CAT (Fig. 51). In contrast, treated cells with 1 mM H_2O_2 for 6 h significantly up-regulated the protein expression of Cu,ZnSOD, GPx, and CAT, meanwhile, pretreatment the cells with TSCE-W at 0.8 mg/mL before exposured cells with 1 mM H_2O_2 for 6 h restored the balance of protein expression of Cu,ZnSOD, GPx, and CAT to the vehicle control.

This is the first reported on the effect of TSCE-W on the protein expression of antioxidant enzymes. The result suggested that TSCE-W, containing phenolic compounds possessed protective effect against H_2O_2 induced oxidative stress via the regulation of the protein expression of antioxidant enzymes: Cu,ZnSOD, GPx, and CAT similarly to the other previously studied (Zang *et al.*, 2008).

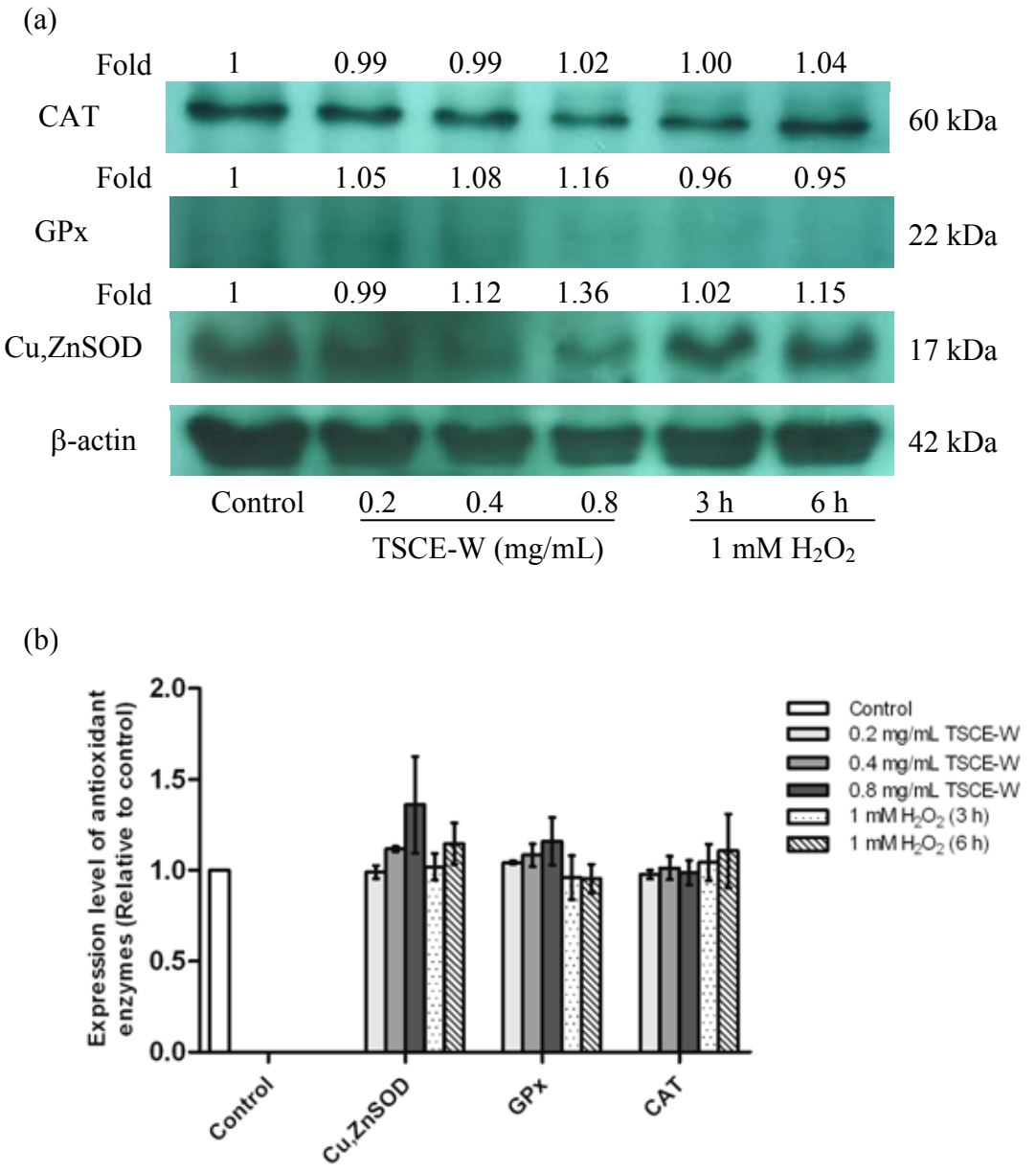


Figure 50. Effect of TSCE-W and H_2O_2 on protein expression of Cu,ZnSOD, GPx, and CAT. The CCD-1064Sk cells were treated with various concentrations of TSCE-W (0.2-0.8 mg/mL) for 24 h and treated with 1 mM H_2O_2 for 3 and 6 h. a) Cu,ZnSOD, GPx, and CAT protein levels were determined by Western blot analysis. b) The intensity of each band was normalized using β-actin. Data are expressed as mean ± SEM of three independent experiments.

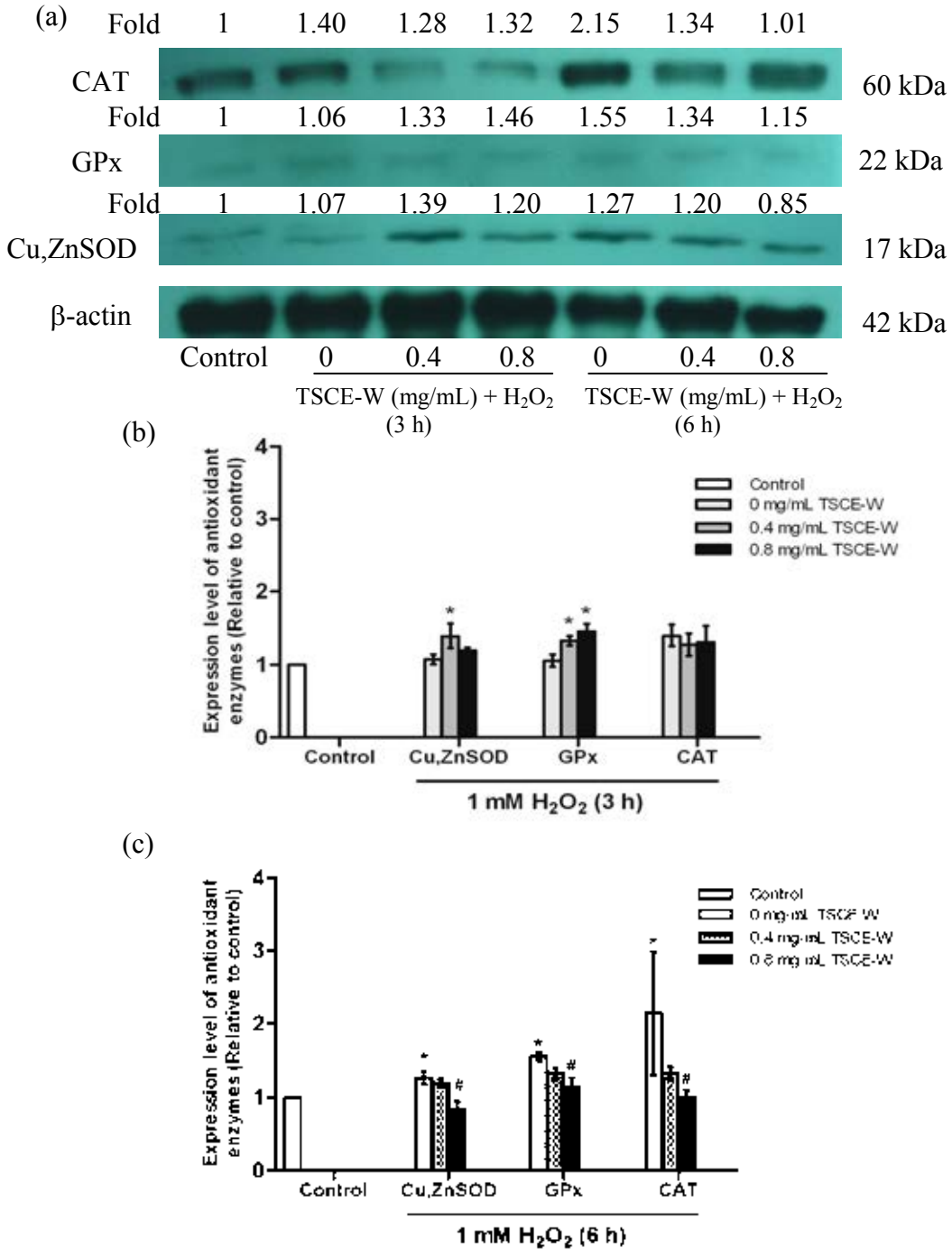


Figure 51. Effect of TSCE-W on protein expression of Cu,ZnSOD, GPx, and CAT in H_2O_2-treated cells. The cells were treated with TSCE-W at 0.4-0.8 mg/mL for 24 h followed by 1 mM H_2O_2 for 3 and 6 h. a) Cu,ZnSOD, GPx, and CAT protein levels. The intensity of each band after incubated with 1 mM H_2O_2 for 3 h (b) and 6 h (c). Data are expressed as mean ± SEM (n=3). *, $p< 0.05$ compared to the vehicle control and #, $p< 0.05$ compared to H_2O_2-treated alone.

11.3 Effect of TSCE-W and H_2O_2 on protein expression of Nrf2, HO-1, and GST

Nrf2 is a transcription factor that regulates the expression of HO-1 (a cytoprotective enzyme) and GST (a phase II detoxifying enzyme) via antioxidant response element (Thangapazham, Sharma, and Maheshwari, 2006). Therefore, the effect of TSCE-W and H_2O_2 on protein expression of Nrf2, HO-1, and GST in CCD-1064Sk cells was carried out. The cells were treated with TSCE-W at 0.2-0.8 mg/mL for 24 h. The result showed that all tested concentrations of TSCE-W did not significantly up-regulate the protein expression of Nrf2 and GST, but TSCE-W at 0.8 mg/mL significantly up-regulated the protein expression of HO-1 compared with the vehicle control. Whereas, 1 mM H_2O_2-treated cells for 3 and 6 h did not significantly up-regulate the protein expression of these enzymes compared with the vehicle control (Fig. 52). The result suggested that TSCE-W up-regulated HO-1 protein expression similarly to the bilberry extract (Milbury *et al.*, 2007) and blackberry extract (Cho *et al.*, 2011).

11.4 Effect of TSCE-W on protein expression of Nrf2, HO-1, and GST in H_2O_2-treated cells

The protective effect of TSCE-W on protein expression of these antioxidant enzymes was evaluated. The cells were pre-treated with TSCE-W at 0.4-0.8 mg/mL for 24 h followed by exposured with 1 mM H_2O_2 for 3 h. The result showed that TSCE-W at 0.8 mg/mL significantly up-regulated the protein expression of GST and HO-1 compared to the vehicle control and H_2O_2-treated cells, respectively. However, all tested concentrations of TSCE-W did not affect on protein expression of Nrf2 (Fig 53a). Meanwhile, 1 mM H_2O_2-treated cells without TSCE-W for 3 h did not significantly up-regulate the protein expression of Nrf2, HO-1 and GST as compared with the vehicle control (Fig. 53b). However, pretreatment the cells with TSCE-W at 0.4-0.8 mg/mL for 24 h before treated cells with 1 mM H_2O_2 for 6 h and H_2O_2-treated cells alone for 6 h, did not significantly affect on protein expression of Nrf2, HO-1 and GST as compared with the vehicle control.

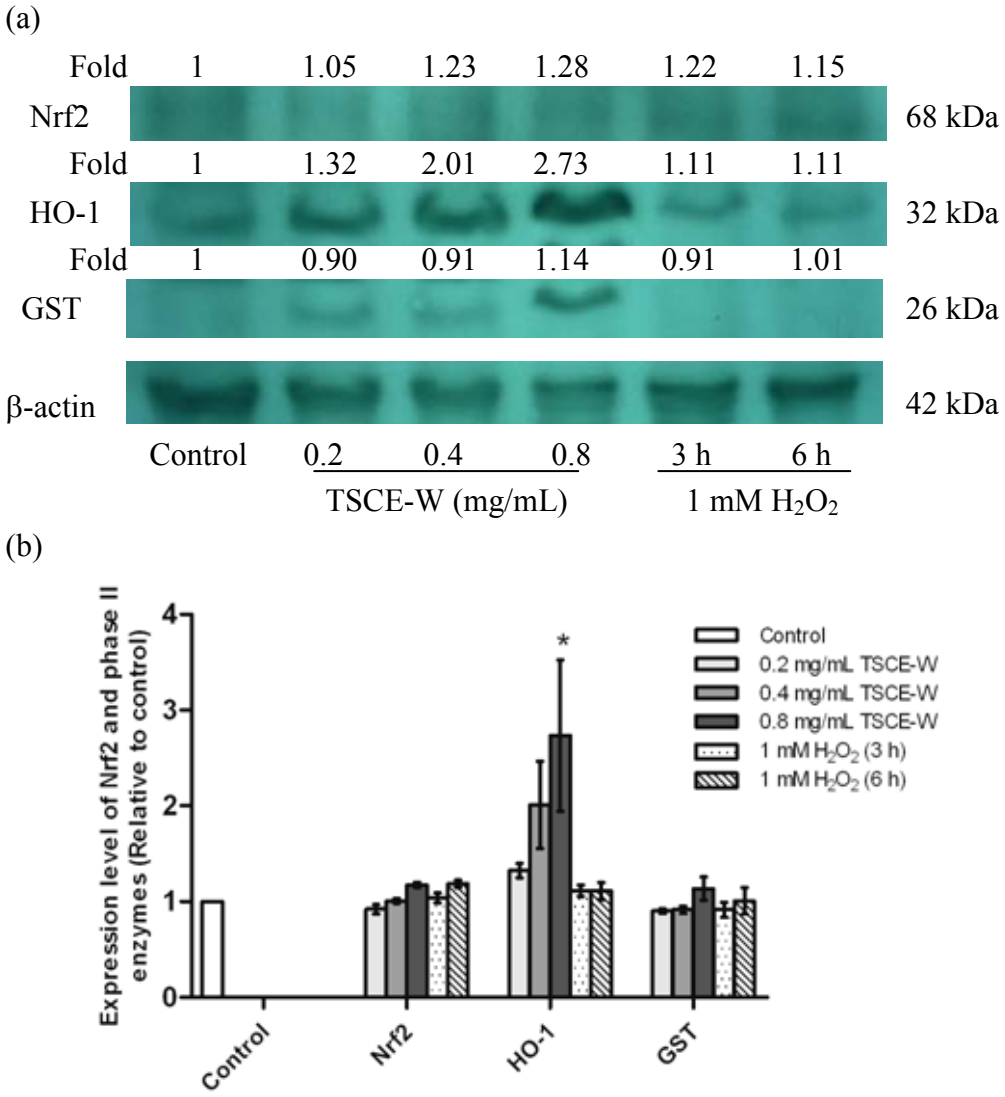


Figure 52. Effect of TSCE-W and H_2O_2 on protein expression of Nrf2, HO-1 and GST. The CCD-1064Sk cells were treated with various concentrations of TSCE-W (0.2-0.8 mg/mL) for 24 h and treated with 1 mM H_2O_2 for 3 and 6 h. a) Nrf2, HO-1 and GST protein levels were determined by Western blot analysis. b) The intensity of each band was normalized using β-actin. Data are expressed as mean ± SEM of three independent experiments. *, $p< 0.05$ compared to the vehicle control.

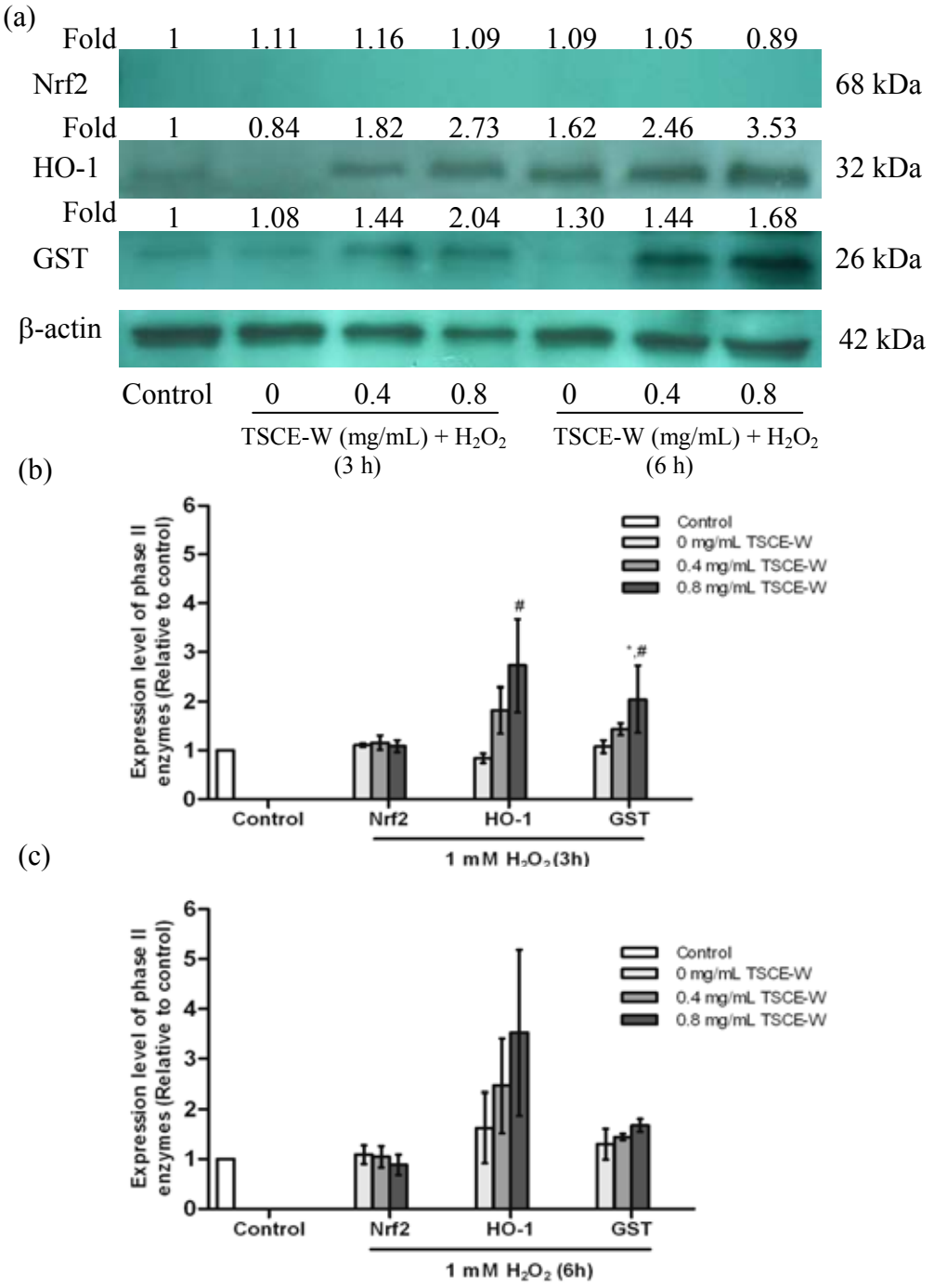


Figure 53. Effect of TSCE-W on protein expression of Nrf2, HO-1 and GST in H_2O_2-treated cells. The cells were treated with TSCE-W at concentration of 0.4-0.8 mg/mL for 24 h followed by 1 mM H_2O_2 for 3 and 6 h. a) GST and HO-1 protein levels. The intensity of each band after incubated with 1 mM H_2O_2 for 3 h (b) and 6 h (c). Data are expressed as mean ± SEM (n=3). *, $p< 0.05$ compared to the vehicle control and #, $p< 0.05$ compared to H_2O_2-treated alone.

This is a first reported on the effect of TSCE-W on the protein expression of HO-1 and GST antioxidant enzymes. The result suggested that TSCE-W, containing phenolic compounds possessed protective against H_2O_2 induced oxidative stress via the regulation of the protein expression of HO-1 and GST, which was similarly to the phenolic compound in other medicinal plants (Roland Wolf, 2001; Lima, Pereira-Wilson, and Rattan, 2011; Qi *et al*., 2011), bilberry extract (Milbury *et al.*, 2007) and ginger extract (Bak *et al*., 2012).

The protective effect of TSCE-W may be function via the regulation of Nrf2-Keap1 pathway. Nrf2 regulates the expression of HO-1 and GST via antioxidant response element. Nrf2 activated by Keap1, which releases of Nrf2 occurred in the presence of antioxidants and chemoprotective agents, thereby leading to activation of ARE and the expression of phase II enzyme and other protective enzymes for protecting cells from oxidative stress induced by the free radicals (Thangapazham *et al.,* 2006). Although, this study, TSCE-W did not increase the protein expression of Nrf2 but it increased the protein expression of antioxidant proteins. These may be the reason of this experiment we measured protein expression of Nrf2 in whole cells extract (nucleus and cytosol) not only in nucleus extract and the Nrf2 protein was diluted, then the Nrf2 protein expression could not be detected. The protein expression of Nrf2 in nucleus was further determined.

CHAPTER V
CONCLUSION

The four extracts of tamarind seed coat: TSCE-E, TSCE-EA, TSCE-EEA, and TSCE-W were evaluated the free radical scavenging, antioxidant activities, including phenolic compound, tannin, and proanthocyanidin contents in cell-free system. The result showed that TSCE-E possessed the highest yield of the extract as well as the highest H_2O_2 scavenging and reducing power activity. TSCE-EEA exhibited the highest proanthocyanidin content with the highest $OH^{\bullet}$ scavenging activity. TSCE-W possessed the highest phenolic content with the highest $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ scavenging activities. Moreover, TSCE-E exhibited the highest tannin content with the high anti-lipid peroxidation activity comparable with TSCE-W. Additionally, TSCE-E exhibited the highest $NO^{\bullet}$ scavenging activity comparable to TSCE-EA and TSCE-W. All TSCEs tested showed not significantly different in $O_2^{\bullet-}$ scavenging activity. All TSCEs tested possessed antioxidant activities comparable with antioxidant standards. This result demonstrated that TSCE-W possessed the most effective antioxidant. Chemical fingerprint of TSCE-W revealed the phenolic content, containing (+)-catechin, (-)-epicathechin, and procyanidin B2 in the extract as analyzed by using HPLC analysis. Then, TSCE-W was further used to study the antioxidant activity in CCD-1064Sk cells.

The effect of TSCE-W in CCD-1064Sk cells showed that TSCE-W up to 1 mg/mL did not have cytotoxic effect against CCD-1064Sk cells as examined by using various standard assays: MTT, neutral red, Hoechst 33342 staining and trypan blue assay. TSCE-W at the non-toxic dose did not generate ROS and lipid peroxidation in cells. However, TSCE-W at 0.8-1 mg/mL attenuated ROS in cells. Moreover, TSCE-W at 0.4-1 mg/mL increased intracellular GSH level, including TSCE-W at 0.8 and 1 mg/mL increased the enzyme activity of SOD and CAT, respectively. Because of these antioxidant properties, TSCE-W exhibited protective activities against H_2O_2-induced oxidative stress in CCD-1064Sk cells.

The oxidative stress in CCD-1064Sk cells was induced by H_2O_2. TSCE-W did not show protective activity in H_2O_2-damaged cells, as the cells did not increase the cell viability after exposed with 2 mM H_2O_2 for 30 min. However, the cells reduced

the generation of ROS and lipid peroxidation, as well as increased intracellular GSH level in H_2O_2-treated cells.

The antioxidant mechanism of TSCE-W via protein expression was performed by using Western blot analysis. The result showed that incubation of TSCE-W alone at 0.2-0.8 mg/mL for 24 h did not significantly up-regulate protein expression of Cu,ZnSOD, GPx, CAT, Nrf2, and GST, but TSCE-W at 0.8 mg/mL significantly up-regulated the protein expression of HO-1 compared with the vehicle control. Whereas at 1 mM H_2O_2-treated cells for 3 and 6 h did not significantly up-regulate protein expression of these enzymes compared with the vehicle control.

The protective effect of TSCE-W on the regulation of antioxidant protein expression in H_2O_2-treated cells exhibited that pretreatment the cells with TSCE-W at 0.4 and 0.8 mg/mL before treated cells with 1 mM H_2O_2 for 3 h, significantly up-regulated the protein expression of Cu,ZnSOD, GPx, and GST was obtained more than the vehicle control. TSCE-W at 0.8 mg/mL significantly up-regulated the protein expression of HO-1 more than the H_2O_2-treated cells, but it was not significantly different from the vehicle control. However, all concentrations of TSCE-W did not effect on protein expression of CAT. In contrast, H_2O_2-treated cells alone for 6 h showed significantly up-regulate the protein expression of Cu,ZnSOD, GPx, and CAT compared with the vehicle control. Meanwhile, pretreatment the cells with TSCE-W at 0.8 mg/mL before treated cells with 1 mM H_2O_2 for 6 h maintained the protein expression of Cu,ZnSOD, GPx, and CAT comparable to control.

TSCE-W possessed not only antioxidant activity and also showed protective activities against H_2O_2-damaged cells by maintain the balance between free radicals and antioxidant defense in cells. TSCE-W clearly scavenged intracellular ROS, inhibited lipid peroxidation, elevating intracellular GSH level and may regulated the protein expression of antioxidant enzymes (SOD, GPx, and CAT) and phase II detoxifying enzymes (HO-1 and GST) (Fig. 54). The antioxidant activities of TSCE-W in cells were correlated with the free radicals scavenging activity in cell-free system. These antioxidant capacities of TSCE-W may be due to the present of the phenolic compound content in the extract of tamarind seed coat.

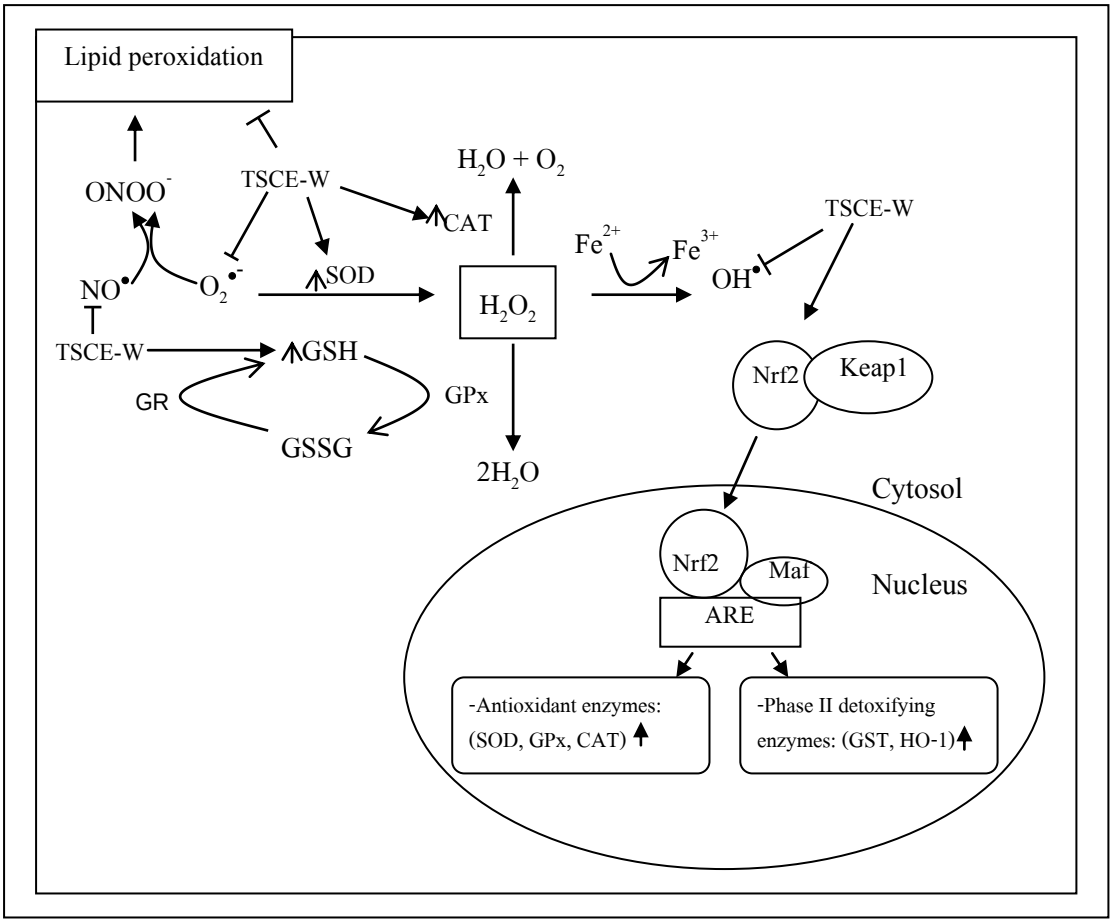


Figure 54. Free radicals scavenging, antioxidant activity and proposed signaling pathways of TSCE-W in CCD-1064Sk cells.

In conclusion, the results in the present study suggest that an inexpensive and simple boiling-water extraction of TSCE-W may provide a good source of natural antioxidant having high antioxidant activity in both cell-free and in CCD-1064Sk cells. TSCE-W protected oxidative stress in cells via the regulation of antioxidant enzymes expression, TSCE-W can be derived into products that useful for health food additives and cosmeceuticals.

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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 12% Acrylamide gel

Ultrapure water	5.1	mL
4X separating buffer	2.5	mL
50% acrylamide	2.4	mL
10% APS	50	µL
TEMED	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 CCD-1064Sk cells

Iscove's modified Dulbecco's medium (1 package) was dissolved with ultrapure water and the 1.432 g sodium hydrogen carbonate was added. The medium was mixed and 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 stored at -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 RESULES

Table 5. DPPH radical scavenging activity and EC_{50} values of the four TSCEs of the seed coat of *Tamarindus indica* "Priao-Yak" compared with the reference antioxidants (Trolox and BHA). Data are mean ± SEM of three independent experiments. a, b, c, d, e are significant difference between groups.

Concentration (μg/mL)	DPPH• scavenging (%)					
	TSCE-E	TSCE-EA	TSCE-EEA	TSCE-W	Trolox	BHA
0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
0.65	20.06 ± 0.27	16.24 ± 0.36	18.24 ± 0.46	21.01 ± 2.55	18.77 ± 0.40	22.59 ± 1.40
1.30	23.68 ± 0.41	23.06 ± 0.71	26.78 ± 1.05	31.76 ± 1.27	25.06 ± 0.91	30.82 ± 1.15
2.61	42.90 ± 0.25	34.04 ± 0.36	46.30 ± 0.10	55.47 ± 1.63	38.6 ± 1.62	50.71 ± 0.89
3.91	66.49 ± 0.11	45.84 ± 1.28	65.25 ± 0.49	72.10 ± 1.13	53.95 ± 1.59	65.07 ± 1.75
5.22	83.38 ± 1.09	62.17 ± 0.53	81.16 ± 0.25	90.75 ± 0.76	67.64 ± 0.34	75.66 ± 0.69
6.52	93.78 ± 1.06	72.87 ± 0.72	91.07 ± 0.54	93.05 ± 0.41	78.95 ± 0.13	83.12 ± 0.85
7.83	93.37 ± 0.21	79.92 ± 0.32	91.73 ± 0.32	93.34 ± 0.18	88.12 ± 0.30	84.22 ± 0.89
EC_{50} (μg/mL)	3.01 ± 0.00^{d}	4.38 ± 0.06^{a}	3.02 ± 0.02^{d}	2.44 ± 0.03^{e}	3.80 ± 0.06^{b}	3.23 ± 0.04^{c}

Table 6. $ABTS^{\bullet+}$ scavenging activity of the four TSCEs of the seed coat of *Tamarindus indica* "Priao-Yak" compared with the reference antioxidant (BHA). Data are mean ± SEM of three independent experiments. a, b, c, d are significant difference between groups.

Extract	$ABTS^{\bullet+}$ scavenging (μmole TEAC/mg of extract)
TSCE-E	$8.83 \pm 0.15^{a,b}$
TSCE-EA	7.28 ± 0.11^{c}
TSCE-EEA	4.97 ± 0.13^{d}
TSCE-W	9.64 ± 0.45^{a}
BHA	$8.23 \pm 0.11^{b,c}$

Table 7. Superoxide radical scavenging activity of the four TSCEs of the seed coat of *Tamarindus indica* "Priao-Yak" compared with the reference antioxidant (Trolox). Data are mean ± SEM of three independent experiments. a, b are significant difference between groups.

Extract	$O_2^{\bullet-}$ scavenging (EC_{50}, μg/mL)
TSCE-E	23.53 ± 1.19^{b}
TSCE-EA	25.39 ± 2.48^{b}
TSCE-EEA	19.07 ± 0.18^{b}
TSCE-W	21.44 ± 0.23^{b}
Trolox	352.70 ± 15.44^{a}

Table 8. Hydrogen peroxide scavenging activity of the four TSCEs of the seed coat of *Tamarindus indica* "Priao-Yak" compared with the reference antioxidants (Trolox and BHA). Data are mean ± SEM of three independent experiments. a, b, c, d are significant difference between groups.

Extract	H_2O_2 scavenging (EC_{50}, μg/mL)
TSCE-E	11.33 ± 0.75^{d}
TSCE-EA	$12.88 \pm 0.11^{c,d}$
TSCE-EEA	$20.79 \pm 1.62^{b,c}$
TSCE-W	26.03 ± 1.99^{b}
Trolox	52.53 ± 1.51^{a}
BHA	53.31 ± 2.42^{a}

Table 9. Hydroxyl radical scavenging activity and EC_{50} values of the four TSCEs of the seed coat of *Tamarindus indica* "Priao-Yak" compared with the reference antioxidants (Trolox and BHA). Data are mean ± SEM of three independent experiments. a, b, c, d are significant difference between groups.

Concentration (μg/mL)	$OH^{\bullet}$ scavenging (%)					
	TSCE-E	TSCE-EA	TSCE-EEA	TSCE-W	Trolox	BHA
0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
0.125	29.11 ± 0.85	35.57 ± 1.08	43.11 ± 1.58	29.01 ± 1.20	34.18 ± 1.31	39.98 ± 0.45
0.25	39.57 ± 1.78	46.35 ± 0.36	52.21 ± 0.94	39.27 ± 2.27	46.24 ± 1.12	51.84 ± 0.24
0.5	47.89 ± 1.96	56.75 ± 1.28	59.89 ± 1.62	54.28 ± 2.06	55.30 ± 1.17	58.69 ± 1.10
1	57.54 ± 1.44	65.87 ± 1.15	69.46 ± 1.55	66.23 ± 1.76	62.76 ± 0.46	67.10 ± 0.81
2	66.46 ± 1.54	68.55 ± 0.73	75.15 ± 1.48	71.31 ± 1.57	71.41 ± 1.04	73.37 ± 1.06
4	73.55 ± 0.80	78.47 ± 1.33	79.70 ± 0.45	79.04 ± 0.57	74.54 ± 0.53	78.57 ± 0.16
EC_{50} (μg/mL)	0.58 ± 0.04^{a}	$0.34 \pm 0.02^{b,c}$	0.21 ± 0.02^{d}	0.42 ± 0.03^{b}	0.38 ± 0.01^{b}	$0.24 \pm 0.01^{c,b}$

Table 10. Nitric oxide scavenging activity and EC_{50} values of the four TSCEs of the seed coat of *Tamarindus indica* "Priao-Yak" compared with the reference antioxidants (Trolox and BHA). Data are mean ± SEM of three independent experiments. a, b, c are significant difference between groups.

Concentration (μg/mL)	NO scavenging (%)					
	TSCE-E	TSCE-EA	TSCE-EEA	TSCE-W	Trolox	BHA
0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
0.39	40.81 ± 1.20	44.19 ± 0.41	36.04 ± 0.23	41.05 ± 0.74	39.93 ± 0.20	34.86 ± 1.01
0.78	45.80 ± 1.36	47.69 ± 0.55	40.42 ± 0.34	46.13 ± 0.99	41.88 ± 0.33	39.02 ± 0.65
1.56	50.99 ± 1.34	50.74 ± 0.50	44.64 ± 0.46	51.97 ± 0.84	43.69 ± 0.26	42.76 ± 0.97
3.13	56.47 ± 1.41	54.02 ± 0.81	48.97 ± 0.52	59.22 ± 0.99	45.94 ± 0.38	46.67 ± 1.07
6.25	61.09 ± 1.58	57.86 ± 0.99	53.35 ± 0.48	64.59 ± 1.03	47.62 ± 0.38	50.16 ± 1.30
12.50	65.70 ± 0.68	61.58 ± 0.71	57.73 ± 0.19	71.94 ± 1.04	50.12 ± 0.36	54.21 ± 0.70
25.00	69.36 ± 0.49	64.70 ± 0.66	63.32 ± 0.43	72.55 ± 0.75	59.18 ± 0.16	56.29 ± 0.77
EC_{50} (μg/mL)	1.76 ± 0.34^{c}	1.74 ± 0.21^{c}	4.05 ± 0.23^{b}	1.48 ± 0.15^{c}	10.26 ± 0.59^{a}	5.17 ± 0.78^{b}

Table 11. Reducing power activity and EC_{50} values of the four TSCEs of the seed coat of *Tamarindus indica* "Priao-Yak" compared with the reference antioxidants (Trolox and BHA). Data are mean ± SEM of three independent experiments. a, b, c, d are significant difference between groups.

Concentration (μg/mL)	Reducing power (Absorbance at 700 nm)					
	TSCE-E	TSCE-EA	TSCE-EEA	TSCE-W	Trolox	BHA
0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
2.50	0.07 ± 0.00	0.03 ± 0.01	0.05 ± 0.01	0.00 ± 0.00	0.05 ± 0.00	0.06 ± 0.02
5.00	0.15 ± 0.01	0.08 ± 0.01	0.10 ± 0.01	0.05 ± 0.00	0.08 ± 0.00	0.11 ± 0.01
10.00	0.29 ± 0.01	0.16 ± 0.01	0.20 ± 0.00	0.16 ± 0.00	0.17 ± 0.00	0.24 ± 0.01
20.00	0.57 ± 0.00	0.34 ± 0.00	0.41 ± 0.02	0.38 ± 0.04	0.33 ± 0.02	0.47 ± 0.00
40.00	1.09 ± 0.01	0.65 ± 0.01	0.77 ± 0.03	0.69 ± 0.01	0.67 ± 0.07	0.93 ± 0.04
EC_{50} (μg/mL)	18.14 ± 0.19^{d}	30.50 ± 0.50^{a}	25.67 ± 0.99^{b}	$28.60 \pm 0.40^{b,a}$	30.22 ± 0.74^{a}	21.61 ± 0.77^{c}

Table 12. Metal chelating activity of the four TSCEs of the seed coat of *Tamarindus indica* "Priao-Yak" compared with the reference antioxidants (Trolox and BHA) and chelating agent (Na_2EDTA). Data are mean ± SEM of three independent experiments. a, b, c significant difference between groups.

Extract	Metal chelating (% chelating at 25 μg/mL)
TSCE-E	24.63 ± 2.15^{b}
TSCE-EA	15.73 ± 1.72^{c}
TSCE-EEA	$22.05 \pm 1.61^{b,c}$
TSCE-W	26.33 ± 1.24^{b}
Trolox	NA
BHA	NA
Na_2EDTA^{*}	100.00 ± 0.00^{a}

*concentration at 10 μg/mL

Table 13. Anti-lipid peroxidation activity and EC_{50} values of the four TSCEs of the seed coat of *Tamarindus indica* "Priao-Yak" compared with the reference antioxidants (Trolox and BHA). Data are mean ± SEM of three independent experiments. a, b, c, d are significant difference between groups.

Concentration (μg/mL)	Anti-ipid peroxidation (%)					
	TSCE-E	TSCE-EA	TSCE-EEA	TSCE-W	Trolox	BHA
0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
2.50	13.86 ± 1.79	9.15 ± 0.67	5.37 ± 0.24	4.71 ± 0.24	-4.04 ± 1.10	44.83 ± 0.71
5.00	19.34 ± 1.10	19.20 ± 0.50	9.91 ± 0.51	15.31 ± 2.14	-3.07 ± 4.68	55.26 ± 0.49
10.00	25.18 ± 0.62	24.84 ± 0.90	13.75 ± 0.81	20.60 ± 1.85	-0.81 ± 4.68	63.07 ± 0.88
20.00	37.22 ± 0.31	26.03 ± 0.76	15.36 ± 0.20	29.16 ± 1.18	6.47 ± 0.75	67.07 ± 0.78
40.00	45.97 ± 0.56	29.31 ± 0.46	20.22 ± 1.60	40.84 ± 0.44	12.77 ± 1.30	71.02 ± 0.14
80.00	50.08 ± 0.49	43.84 ± 1.26	37.37 ± 0.31	53.07 ± 0.86	18.35 ± 1.47	72.72 ± 0.31
120.00	51.62 ± 0.12	48.98 ± 0.26	44.13 ± 0.37	55.18 ± 0.37	28.69 ± 0.90	74.38 ± 0.26
160.00	52.50 ± 0.23	45.13 ± 1.32	46.34 ± 0.41	57.29 ± 0.32	44.90 ± 1.28	69.46 ± 2.25
EC_{50} (μg/mL)	80.99 ± 2.00^{c}	192.03 ± 16.40^{b}	420.48 ± 13.74^{a}	78.17 ± 1.21^{c}	177.32 ± 1.69^{b}	2.70 ± 0.24^{d}

Table 14. The percentage of cell viability of TSCE-W treatment in CCD-1064Sk cells in a concentration-dependent manner for 24 h. The cell viability was determined by MTT assay, Neutral red assay, Hoechst 33342 straining, and Trypan blue exclusion assay. Each value demonstrated in the mean ± SEM of three independent experiments. Each performed in triplicate. * $p< 0.05$ compared to the vehicle control.

TSCE-W (mg/mL)	Cell viability (% of control)			
	MTT	Neutral red	Hoechst 33342	Trypan blue
0	100.00 ± 0.00	100.00 ±0.00	100.00 ± 0.00	100.00 ± 0.00
0.05	103.57 ± 4.07	92.69 ± 2.03	85.83 ± 6.41	99.82 ± 0.56
0.1	105.98 ± 3.63	98.07 ± 3.49	89.67 ± 5.18	100.46 ± 0.09
0.2	112.97 ± 1.14	98.79 ± 3.75	93.30 ± 4.96	100.66 ± 0.04
0.4	118.32 ± 1.76	104.75 ± 3.23	83.42 ± 4.23	100.18 ± 0.34
0.8	132.42 ± 9.26*	114.90 ± 4.24	89.05 ± 8.61	98.67 ± 1.14
1.0	139.47 ± 7.10*	106.65 ± 7.22	74.76 ± 5.86	96.38 ± 2.33

Table 15. The percentage of cell viability of H_2O_2-treated in CCD-1064Sk cells in various concentration and time. The cell viability was determined by MTT assay. Each values expressed in the mean ± SEM of three independent experiments. Each performed in triplicate. *p< 0.05 compared to the vehicle control.

H_2O_2 (mM)	Cell viability (% of control)				
	0.5 h	1 h	3 h	6 h	12 h
0	100.00 ± 0.00	100.00 ±0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00
0.1	101.32 ± 1.63	104.50 ± 3.90	104.12 ± 4.37	96.65 ± 1.42	101.12 ± 1.13
0.2	101.87 ± 1.56	105.45 ± 2.75	103.51 ± 4.13	96.01 ± 2.24	99.45 ± 1.94
0.4	103.21 ± 1.26	100.57 ± 2.25	104.23 ± 4.54	96.49 ± 1.80	96.32 ± 2.68
0.6	101.66 ± 1.59	102.96 ± 2.08	99.24 ± 3.57	93.84 ± 3.90	93.89 ± 3.25
0.8	96.04 ± 7.81	90.28 ± 8.34	92.84 ± 4.97	85.02 ± 13.23	83.78 ± 8.16
1	84.56 ± 1.58	80.35 ± 5.30	83.06 ± 8.75	75.17 ± 14.26	68.91 ± 6.68*
2	55.56 ± 1.16*	54.49±3.66*	36.76 ± 6.15*	27.14 ± 5.64*	27.67 ± 5.20*
4	54.69 ± 0.15*	52.51 ± 4.83*	32.14 ± 2.31*	27.26 ± 5.58*	27.75 ± 5.50*
6	51.65 ± 3.24*	45.68 ± 2.86*	27.70 ± 1.79*	27.94 ± 6.13*	28.56 ± 5.26*
8	38.88 ± 4.27*	34.50 ± 5.86*	28.41 ± 1.64*	28.65 ± 6.27*	27.87 ± 5.17*
10	38.26 ± 3.80*	35.04 ± 5.79*	31.86 ± 2.50*	31.12 ± 8.31*	29.75 ± 5.30*

Table 16. The IC_{50} values of H_2O_2 treatment in a concentration and time-dependent manner on CCD-1064Sk cells. Each values expressed in the mean ± SEM of three independent experiments. Each performed in triplicate. *p< 0.05 compared to IC_{50} at 0.5 h.

Time (h)	IC_{50} (mM)
0.5	2.20 ± 0.06
1	2.18 ± 0.13
3	1.73 ± 0.13
6	1.40 ± 0.27*
12	1.44 ± 0.15*

Table 17. The percentage of cell viability of CCD-1064Sk cells pre-treated with various concentrations of TSCE-W for 24 h prior to 2 mM H_2O_2-induced cells injury for 0.5 h. The cell viability was determined by MTT and Neutral red assay. Data are expressed as mean ± SEM of three independent experiments. Each performed in triplicate. *p< 0.05 compared to H_2O_2-treated cells alone.

TSCE-W (mg/mL)	Cell viability (% of control)	
	MTT	Neutral red
Control	100.00 ± 0.00*	100.00 ± 0.00*
0	47.12 ± 3.99	44.33 ± 1.66
0.05	57.18 ± 4.96	52.56 ± 4.98
0.1	54.27 ± 3.95	46.51 ± 5.57
0.2	53.94 ± 4.07	48.53 ± 2.94
0.4	77.64 ± 4.43	45.96 ± 4.85
0.8	98.58 ± 10.23*	51.47 ± 3.49
1	100.07 ± 6.63*	42.53 ± 2.25

Table 18. The percentage of DCF fluorescence of TSCE-W in CCD-1064Sk cells. The cells were treated with TSCE-W (0.05-1 mg/mL) for 24 h. and then incubated with 5 μM DCFH-DA in cold PBS for 0.5 h. Intracellular ROS in cells was determined by DFCH-DA assay. Data are expressed as mean ± SEM of four independent experiments. Each performed in triplicate. *p< 0.05 compared to the vehicle control.

TSCE-W (mg/mL)	DCF fluorescence (% of control)
0	100.00 ± 0.00
0.05	104.89 ± 3.20
0.1	107.63 ± 5.89
0.2	99.42 ± 3.00
0.4	90.93 ± 3.63
0.8	75.73 ± 4.94*
1	72.95 ± 5.40*

Table 19. The percentage of DCF fluorescence of H_2O_2 in CCD-1064Sk cells. The cells were treated with various concentrations of H_2O_2 (0.1-2 mM) for 0.25, 0.5, 1, and 3 h. Data are expressed as mean ± SEM of three independent experiments. Each performed in triplicate. *p< 0.05 compared to vehicle control.

H_2O_2 (mM)	DCF fluorescence (% of control)			
	0.25 h	0.5 h	1 h	3 h
0	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00
0.1	92.07 ± 1.09	89.96 ± 5.24	90.22 ± 6.43	90.17 ± 4.14
0.2	98.47 ± 5.68	104.90 ± 7.34	83.36 ± 10.75	63.58 ± 12.04
0.4	114.79 ± 5.96	116.33 ± 11.71	76.47 ± 11.97	56.08 ± 10.58
0.6	114.65 ± 3.76	116.98 ± 11.73	70.66 ± 8.02	55.05 ± 9.25*
0.8	115.94 ± 5.15	118.86 ± 14.43	67.50 ± 8.15	52.64 ± 9.99*
1	126.97 ± 8.62	103.92 ± 10.83	62.85 ± 4.66	50.68 ± 9.78*
2	118.92 ± 11.51	80.42 ± 1.75	53.81 ± 4.32*	45.86 ± 10.29*

Table 20. The percentage of DCF fluorescence in CCD-1064Sk cells pre-treated with various concentrations of TSCE-W for 24 h prior to 1 mM H_2O_2-induced accumulation of intracellular ROS for 0.25 h. Data are expressed as mean ± SEM of five independent experiments. Each performed in triplicate. *p< 0.05 compared to vehicle control, and $^{\#}p$< 0.05 compared to 1 mM H_2O_2-treated cells.

TSCE-W (mg/mL)	DCF fluorescence (% of control)
Control	100.00±0.00
0	314.23±19.70*
0.05	270.55±23.07*
0.1	247.39±18.07*
0.2	213.95±17.19$^{*,\#}$
0.4	172.59±12.38$^{*,\#}$
0.8	127.92±7.21$^{\#}$
1	109.59±4.94$^{\#}$

Table 21. The % scavenging intracellular ROS of TSCE-W in 1 mM H_2O_2-treated cells. The CCD-1064Sk cells were pre-treated with various concentrations of TSCE-W for 24 h prior to 1 mM H_2O_2 for 0.25 h. The % scavenging intracellular ROS was compared to the cells treated with H_2O_2 alone. Data are expressed as mean ± SEM of five independent experiments. Each performed in triplicate.*p< 0.05 compared to 1 mM H_2O_2-treated cells alone.

TECE W (mg/mL)	% Scavenging intracellular ROS
0	0.00±0.00
0.05	13.90±7.34
0.1	21.09±5.75
0.2	31.91±5.47*
0.4	45.07±3.94*
0.8	59.29±2.30*
1	65.12±1.57*

Table 22. Effect of TSCE-W on lipid peroxidation in CCD-1064Sk cells. The cells. were treated with various concentrations of TSCE-W (0.05-1 mg/mL) for 24 h. Data are expressed as mean ± SEM of four independent experiments. a, b are significant difference between groups.

TSCE-W (mg/mL)	Lipid peroxidation (nmol MDA/mg protein)
0	$0.13\pm0.02^{a,b}$
0.05	0.08 ± 0.01^{a}
0.1	0.07 ± 0.01^{a}
0.2	0.09 ± 0.02^{a}
0.4	$0.12\pm0.02^{a,b}$
0.8	0.19 ± 0.03^{b}
1	$0.15\pm0.02^{a,b}$

Table 23. Effect of H_2O_2 on lipid peroxidation in CCD-1064Sk cells. The cells were treated with 1 mM and 2 mM H_2O_2 in various times (0.25-6 h). Data are expressed as mean ± SEM of three independent experiments. $^{*}p< 0.05$ compared to the vehicle control, and $^{\#}p< 0.05$ compared to 1 mM H_2O_2-treated cells alone.

H_2O_2 (mM)	Lipid peroxidation (% MDA of control)				
	0.25 h	0.5 h	1 h	3 h	6 h
0	100.00±0.00	100.00±0.00	100.00±0.00	100.00±0.00	100.000±.00
1	159.15±44.57	107.13±29.94	120.67±52.10	121.28±25.85	79.65±16.26
2	194.97±45.84	106.21±16.02	$289.20\pm25.33^{*,\#}$	62.33±27.34	63.40±40.54

Table 24. Anti-lipid peroxidation of TSCE-W in H_2O_2-treated cells. The cells were pre-treated with various concentrations of TSCE-W for 24 h prior to 2 mM H_2O_2 for 1 h for induced lipid peroxidation in cells. Data are expressed as mean ± SEM of three independent experiments. *p< 0.05 compared to 2 mM H_2O_2-treated cells alone.

TSCE-W (mg/mL)	Lipid peroxidation (nmol MDA/mg protein)
Control	0.29±0.05*
0	0.73±0.12
0.05	0.23±0.03*
0.1	0.20±0.01*
0.2	0.20±0.05*
0.4	0.16±0.03*
0.8	0.21±0.07*
1	0.14±0.03*

Table 25. Effect of TSCE-W on total intracellular GSH level in CCD-1064Sk cells. The cells were treated with various concentrations of TSCE-W (0.05-1 mg/mL) for 24 h. Data are expressed as mean ± SEM of four independent experiments. *p< 0.05 compared to the vehicle control.

TSCE-W (mg/mL)	GSH level (μM/mg protein)
0	22.03±1.26
0.05	23.57±1.01
0.1	25.44±1.71
0.2	29.39±2.74
0.4	38.86±0.81*
0.8	53.17±4.57*
1	59.76±2.88*

Table 26. Effect of H_2O_2 on total intracellular GSH level in CCD-1064Sk cells. The cells were treated with 1mM and 2 mM H_2O_2 in various times (0.25-1 h). Data are expressed as mean ± SEM of three independent experiments. *p< 0.05 compared to the vehicle control, and $^{\#}p$< 0.05 compared to 1 mM H_2O_2 at 1 h.

H_2O_2 (mM)	GSH level (% of control)		
	0.25 h	0.5 h	1 h
0	100.00±0.00	100.00±0.00	100.00±0.00
1	79.12±8.90	50.07±1.96*	64.50±1.96*
2	85.65±4.40	64.33±6.04*	34.25±4.76*,#

Table 27. Effect of TSCE-W on total intracellular GSH level in H_2O_2-treated cells. The cells were pre-treated with various concentrations of TSCE-W for 24 h prior to 1 mM H_2O_2 for 0.5 h for induced oxidative stress in cells. Data are expressed as mean ± SEM of three independent experiments. *p< 0.05 compared to 1 mM H_2O_2-treated cells alone, and $^{\#}p$< 0.05 compared to the vehicle control.

TSCE-W (mg/mL)	GSH level (μM/mg protein)
Control	14.37±2.22*
0	4.24±0.41
0.05	9.47±1.29
0.1	12.57±1.10
0.2	16.95±3.95*
0.4	22.34±2.52*
0.8	34.53±5.66*,#
1	35.60±0.57*,#

Table 28. Effect of TSCE-W on SOD activity in CCD-1064Sk cells. The cells were treated with various concentrations of TSCE-W (0.05-1 mg/mL) for 24 h. Data are expressed as mean ± SEM of three independent experiments. *p< 0.05 compared to the vehicle control.

TSCE-W (mg/mL)	SOD activity (Units/mg protein)
0	1.76±0.03
0.05	1.71±0.26
0.1	2.35±0.06
0.2	2.65±0.31
0.4	2.83±0.24
0.8	3.34±0.28*
1	2.78±0.42

Table 29. Effect of H_2O_2 on SOD activity in CCD-1064Sk cells. The cells were treated with 1mM and 2 mM H_2O_2 in various times (0.25-3 h) Data are expressed as mean ± SEM of three independent experiments.

H_2O_2 (mM)	SOD activity (% of control)			
	0.25 h	0.5 h	1 h	3 h
0	100.00±0.00	100.00±0.00	100.00±0.00	100.000±.00
1	94.03±11.80	115.84±13.69	112.21±3.97	109.55±12.03
2	78.42±10.25	118.09±21.89	91.05±8.56	100.67±5.23

Table 30. Effect of TSCE-W on SOD activity in H_2O_2-treated cells. The cells were pre-treated with various concentrations of TSCE-W for 24 h prior to 2 mM H_2O_2 for 0.25 h for induced oxidative stress in cells. Data are expressed as mean ± SEM of three independent experiments.

TSCE-W (mg/mL)	SOD activity (Units/mg protein)
Control	0.74±0.07
0	0.63±0.05
0.05	0.80±0.08
0.1	0.89±0.15
0.2	0.91±0.19
0.4	0.81±0.29
0.8	0.88±0.23
1	0.89±0.34

Table 31. Effect of TSCE-W on GPx activity in CCD-1064Sk cells. The cells were treated with various concentrations of TSCE-W (0.05-1 mg/mL) for 24 h. Data are expressed as mean ± SEM of three independent experiments.

TSCE-W (mg/mL)	GPx activity (Units/mg protein)
0	0.12±0.02
0.05	0.12±0.01
0.1	0.12±0.01
0.2	0.12±0.01
0.4	0.15±0.02
0.8	0.14±0.03
1	0.20±0.08

Table 32. Effect of H_2O_2 on GPx activity in CCD-1064Sk cells. The cells were treated with 2 mM H_2O_2 in various times (0.25-3 h). Data are expressed as mean ± SEM of three independent experiments. *p< 0.05 compared to the vehicle control.

H_2O_2 (mM)	GPx activity (% of control)			
	0.25 h	0.5 h	1 h	3 h
0	100.00±0.00	100.00±0.00	100.00±0.00	100.000±.00
2	89.85±9.17	132.81±9.40	310.64±72.84	557.95±109.37*

Table 33. Effect of TSCE-W on GPx activity in H_2O_2-treated cells. The cells were pre-treated with various concentrations of TSCE-W for 24 h prior to 2 mM H_2O_2 for 0.25 h and 0.5 h for induced oxidative stress in cells. Data are expressed as mean ± SEM of three independent experiments.

TSCE-W (mg/mL)	GPx activity (Units/mg protein)	
	0.25 h	0.5 h
Control	0.08±0.04	0.05±0.01
0	0.07±0.03	0.04±0.01
0.05	0.08±0.04	0.04±0.01
0.1	0.11±0.08	0.06±0.02
0.2	0.08±0.05	0.05±0.02
0.4	0.08±0.05	0.05±0.02
0.8	0.06±0.05	0.10±0.02
1	0.12±0.10	0.07±0.02

Table 34. Effect of TSCE-W on CAT activity in CCD-1064Sk cells. The cells were treated with various concentrations of TSCE-W (0.05-1 mg/mL) for 24 h. Data are expressed as mean ± SEM of three independent experiments. *p< 0.05 compared to the vehicle control.

TSCE-W (mg/mL)	CAT activity (k/mg protein)
0	0.03±0.00
0.05	0.03±0.00
0.1	0.03±0.00
0.2	0.03±0.00
0.4	0.04±0.01
0.8	0.05±0.02
1	0.09±0.02*

Table 35. Effect of H_2O_2 on CAT activity in CCD-1064Sk cells. The cells were treated with 2 mM H_2O_2 in various times (0.25-3 h). Data are expressed as mean ± SEM of three independent experiments.

H_2O_2 (mM)	CAT activity (% of control)			
	0.25 h	0.5 h	1 h	3 h
0	100.00±0.00	100.00±0.00	100.00±0.00	100.000±.00
2	94.09±2.40	65.863±3.79	220.05±49.17	-102.56±120.38

Table 36. Effect of TSCE-W on CAT activity in H_2O_2-treated cells. The cells were pre-treated with various concentrations of TSCE-W for 24 h prior to 2 mM H_2O_2 for 0.5 h for induced oxidative stress in cells. Data are expressed as mean ± SEM of three independent experiments.

TSCE-W (mg/mL)	CAT activity (*k*/mg protein)
Control	0.03±0.01
0	0.02±0.00
0.05	0.01±0.00
0.1	0.02±0.00
0.2	0.01±0.00
0.4	0.01±0.00
0.8	0.02±0.00
1	0.02±0.01

Table 37. The relative ratio of TSCE-W and 1 mM H_2O_2 on protein expressions of antioxidant enzymes in CCD-1064Sk cells normalized by β-actin, which determined using Western blot analysis. Data are expressed as mean ± SEM of three independent experiments.

TSCE-W (mg/mL)	Relative ratio		
	Cu,ZnSOD	GPx	CAT
0	1.00±0.00	1.00±0.00	1.00±0.00
0.2	0.99±0.04	1.05±0.01	0.98±0.02
0.4	1.12±0.01	1.08±0.06	1.01±0.06
0.8	1.36±0.26	1.16±0.13	0.98±0.07
H_2O_2 (3h)	1.02±0.07	0.96±0.12	1.04±0.10
H_2O_2 (6h)	1.15±0.12	0.95±0.08	1.11±0.20

Table 38. The relative ratio of TSCE-W on protein expressions of Cu,ZnSOD, GPx, and CAT in 1 mM H_2O_2-treated cells for 3 h normalized by β-actin, which determined using Western blot analysis. Data are expressed as mean ± SEM of three independent experiments. *p< 0.05 compared to the vehicle control.

TSCE-W (mg/mL)	Relative ratio		
	Cu,ZnSOD	GPx	CAT
Control	1.00±0.00	1.00±0.00	1.00±0.00
0	1.07±0.07	1.00±0.08	1.40±0.14
0.4	1.39±0.16*	1.33±0.06*	1.28±0.15*
0.8	1.20±0.04	1.46±0.10*	1.32±0.21*

Table 39. The relative ratio of TSCE-W on protein expressions of Cu,ZnSOD, GPx, and CAT in 1 mM H_2O_2-treated cells for 6 h normalized by β-actin, which determined using Western blot analysis. Data are expressed as mean ± SEM of three independent experiments. *p< 0.05 compared to the vehicle control, $^{\#}p$< 0.05 compared to 1 mM H_2O_2 for 6 h.

TSCE-W (mg/mL)	Relative ratio		
	Cu,ZnSOD	GPx	CAT
Control	1.00±0.00	1.00±0.00	1.00±0.00
0	1.27±0.08*	1.55±0.05*	2.15±0.84*
0.4	1.20±0.06	1.34±0.08	1.34±0.08
0.8	0.85±0.10$^{\#}$	1.15±0.12$^{\#}$	1.01±0.09$^{\#}$

Table 40. The relative ratio of TSCE-W and 1 mM H_2O_2 on protein expressions of Nrf2, GST and HO-1 in CCD-1064Sk cells normalized by β-actin, which determined using Western blot analysis. Data are expressed as mean ± SEM of three independent experiments. *p< 0.05 compared to the vehicle control.

TSCE-W (mg/mL)	Relative ratio		
	Nrf2	GST	HO-1
0	1.00±0.00	1.00±0.00	1.00±0.00
0.2	1.05±0.09	0.90±0.02	1.32±0.08
0.4	1.23±0.20	0.91±0.04	2.01±0.45
0.8	1.28±0.08	1.14±0.12	2.73±0.79*
H_2O_2 (3h)	1.22±0.17	0.91±0.08	1.11±0.10
H_2O_2 (6h)	1.15±0.012	1.01±0.14	1.11±0.15

Table 41. The relative ratio of TSCE-W on protein expressions of Nrf2, GST and HO-1 in 1 mM H_2O_2-treated cells for 3 h normalized by β-actin, which determined using Western blot analysis. Data are expressed as mean ± SEM of three independent experiments. *p< 0.05 compared to the vehicle control.

TSCE-W (mg/mL)	Relative ratio		
	Nrf2	GST	HO-1
Control	1.00±0.00	1.00±0.00	1.00±0.00
0	1.11±0.04	1.08±0.13	0.84±0.09
0.4	1.16±0.14	1.44±0.12	1.82±0.47
0.8	1.09±0.12	2.04±0.68*	2.73±0.95

Table 42. The relative ratio of TSCE-W on protein expressions of Nrf2, GST and HO-1 in 1 mM H_2O_2-treated cells for 6 h normalized by β-actin, which determined using Western blot analysis. Data are expressed as mean ± SEM of three independent experiments.

TSCE-W (mg/mL)	Relative ratio		
	Nrf2	GST	HO-1
Control	1.00±0.00	1.00±0.00	1.00±0.00
0	1.09±0.19	1.30±0.31	1.62±0.71
0.4	1.05±0.21	1.44±0.07	2.46±0.95
0.8	0.89±0.21	1.68±0.13	3.53±1.66

VITA

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