ฤทธิ์ปกป้องเซลล์และรักษาแผลของสารสกัดเปลือกเมล็ดมะขามต่อเซลล์เพาะเลี้ยงของมนุษย์

นางสาวศิริพร คนซื่อ

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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ปีการศึกษา 2554




# CYTOPROTECTIVE AND WOUND HEALING EFFECTS OF *TAMARINDUS INDICA* SEED COAT EXTRACTS ON HUMAN CELL LINES

Miss Siriporn Konsue

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Biomedicinal Chemistry
Department of Biochemistry and Microbiology
Faculty of Pharmaceutical sciences
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| Thesis Title | CYTOPROTECTIVE AND WOUND HEALING EFFECTS OF *TAMARINDUS INDICA* SEED COAT EXTRACTS ON HUMAN CELL LINES |
| By | Miss Siriporn Konsue |
| Field of Study | Biomedicinal Chemistry |
| Thesis Advisor | Associate Professor Sunanta Pongsamart, Ph.D. |

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

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ศิริพร คนซื่อ : ฤทธิ์ปกป้องเซลล์และรักษาแผลของสารสกัดเปลือกเมล็ดมะขามต่อเซลล์เพาะเลี้ยงของมนุษย์ (CYTOPROTECTIVE AND WOUND HEALING EFFECTS OF *TAMARINDUS INDICA* SEED COAT EXTRACTS ON HUMAN CELL LINES) อ. ที่ปรึกษาวิทยานิพนธ์หลัก: รศ. ภญ. ดร. สุนันท์ พงษ์สามารถ, 139 หน้า.

เปลือกเมล็ดมะขามใช้เป็นยาแผนโบราณเพื่อรักษาแผลไฟไหม้และแผลเรื้อรังในผู้ป่วยเบาหวานมาเป็นเวลานาน มีการศึกษาสารประกอบฟีโนลิคในเปลือกเมล็ดมะขามอย่างกว้างขวางเกี่ยวกับฤทธิ์ในการต้านอนุมูลอิสระ งานวิจัยนี้ออกแบบเพื่อประเมินฤทธิ์ของสารสกัดเปลือกเมล็ดมะขาม (TSCEs) สามสายพันธุ์ ทั้งมะขามหวานและเปรี้ยวในการปกป้องและการรักษาแผลต่อเซลล์เพาะเลี้ยงของมนุษย์ที่ถูกกระตุ้นให้เกิดการถูกทำลายด้วยไฮโดรเจนเปอร์ออกไซด์ ($H_2O_2$) โดยเปลือกเมล็ดมะขามถูกสกัดด้วยน้ำร้อน (สารสกัด 1) จากนั้นนำสารสกัดน้ำมาสกัดแยกต่อด้วยเอธิลอะซิเตดอัตราส่วน 1:1 (สารสกัด 2) ส่วนกากเปลือกเมล็ดมะขามนำมาสกัดต่อด้วย 70% อะซีโตน (สารสกัด 3) นำสารสกัดทั้งสามส่วนไประเหยแห้ง วิเคราะห์หาปริมาณสารประกอบฟีโนลิกและประเมินความเป็นพิษต่อเซลล์ไฟโบรบลาสต์ของผิวหนังซีซีดี 1064 เอสเคและเซลล์มะเร็งเยื่อบุกระเพาะอาหารเอจีเอสด้วยวิธี MTT และการย้อมสีดีเอ็นเอ พบว่าสารสกัดเปลือกเมล็ดมะขามทั้งสามสารสกัดมีปริมาณสารประกอบฟีโนลิกแตกต่างกันและสารสกัดเอธิลอะซิเตด (สารสกัด 2) ให้ผลความเป็นพิษต่อเซลล์ทดสอบทั้งสองต่ำสุด จึงทำการประเมินผลสารสกัดน้ำที่สกัดแยกต่อด้วยเอธิลอะซิเตด (สารสกัด 2) จากเปลือกเมล็ดมะขามสามสายพันธุ์ต่อการปกป้องและการรักษาแผลในเซลล์ของมนุษย์ทั้งสองชนิดที่ใช้ทดสอบ โดยวิเคราะห์ปริมาณสารประกอบฟีโนลิกพบว่ามะขามเปรี้ยวมีปริมาณแทนนินและโปรแอนโทไซยานินสูงกว่ามะขามหวาน นอกจากนี้โครมาโทแกรม HPLC profile ของสารสกัดเปลือกเมล็ดมะขามทั้งสามสายพันธุ์ชี้บ่งตรงกับสารฟลาวานอยด์มาตราฐานทั้งสาม ได้แก่ คาเทชิน, เอพิคาเทชินและโปรไซยานิดินบีทู ส่วนผลของการปกป้องเซลล์ของสารสกัดเปลือกเมล็ดมะขามทั้งสามสายพันธุ์ที่ทดสอบวิธี NRU และ DCFH-DA ชี้ให้เห็นว่าสารสกัดเปลือกเมล็ดมะขามมีผลลดจำนวนเซลล์ที่ถูกทำลายและลดการสร้างสารอนุมูลอิสระในเซลล์ทดสอบทั้งสองชนิดที่กระตุ้นให้เกิดการทำลายเซลล์ด้วย $H_2O_2$ นอกจากนี้พบว่าสารสกัดเปลือกเมล็ดมะขามทั้งสามสายพันธุ์ที่ความเข้มข้นต่ำมีผลต่อการเพิ่มจำนวนเซลล์ทดสอบทั้งสองชนิดด้วยวิธี NRU โดยความเข้มข้นที่มีผลต่อการเพิ่มจำนวนเซลล์เมื่อทดสอบด้วยวิธี scratch พบว่าไม่มีผลต่อการเร่งการปิดของแผลในเซลล์ทดสอบชนิดทั้งสอง แต่พบว่าสารสกัดเปลือกเมล็ดมะขามมีผลต่อการปกป้องเซลล์ซีซีดี 1064 เอสเคและเซลล์เอจีเอส ที่ชักนำให้เกิดแผลและมีการเหนี่ยวนำให้เกิดการทำลายด้วย $H_2O_2$ โดยช่วยเพิ่มอัตราเร็วการปิดของแผลในเซลล์ทดสอบทั้งสองชนิดที่กระตุ้นให้อัตราการปิดของแผลช้าลงด้วย $H_2O_2$ โดยสารสกัดเปลือกเมล็ดมะขามสายพันธุ์หวานที่ความเข้มข้นต่ำมีผลต่อการเพิ่มอัตราการปิดของแผลในเซลล์ซีซีดี 1064 เอสเคได้ดีกว่ากลุ่มเซลล์ที่ไม่ได้รับสารสกัดและถูกเหนี่ยวนำด้วย $H_2O_2$ ซึ่งเป็นผลจากการต้านสารอนุมูลอิสระของสารสกัดเปลือกเมล็ดมะขามไปลดปริมาณสารอนุมูลอิสระที่เป็นสาเหตุของการทำให้แผลปิดช้าลง ส่วนสารสกัดเปลือกเมล็ดมะขามสายพันธุ์เปรี้ยวมีผลต่อการต้านสารอนุมูลอิสระได้ดีในเซลล์ทดสอบเอจีเอส โดยช่วยเร่งอัตราการปิดของแผลได้ดีกว่ากลุ่มเซลล์ที่ไม่ได้รับสารสกัดและถูกเหนี่ยวนำด้วย $H_2O_2$

ภาควิชา ชีวเคมีและจุลชีววิทยา ลายมือชื่อนิสิต ศิริพร คนซื่อ

สาขาวิชา ชีวเวชเคมี ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก

ปีการศึกษา 2554

## 5276598133 : MAJOR BIOMEDICINAL CHEMISTRY
KEYWORDS: TAMARIND SEED-COAT / PHENOLIC COMPOUNDS / CYTOPROTECTIVE / WOUND HEALING

SIRIPORN KONSUE : CYTOPROTECTIVE AND WOUND HEALING EFFECTS OF *TAMARINDUS INDICA* SEED COAT EXTRACTS ON HUMAN CELL LINES. ADVISOR : ASSOC. PROF. SUNANTA PONGSAMART, Ph.D, 139 pp.

Tamarind seed-coats have long been used in traditional medicine for treatment of burn and chronic wound in diabetic patients. Phenolic compounds in tamarind seed-coat extract have also widely studied for antioxidant activity. This study was designed to evaluate the cytoprotective and wound healing effect of phenolic compounds in the tamarind seed-coat extracts (TSCEs) from the three tamarind cultivars including sweet and sour tamarinds against hydrogen peroxide ($H_2O_2$)-induced oxidative injury in two human cell lines. Tamarind seed-coat was extracted with boiling water (fraction1) and then the water extract was partitioned with an equal volume of ethyl acetate (fraction 2) and the seed-coat residues were re-extracted with 70% acetone (fraction 3). Three fractions of tamarind seed-coat extracts were dried, the total phenolic contents were analyzed and cytotoxic effect was evaluated on human foreskin fibroblasts (CCD-1064Sk) and human gastric adenocarcinoma epithelial cells (AGS) by MTT and DNA stain assays. The three fractions of tamarind seed-coat extracts composed of various amount of phenolic contents and the ethyl acetate fraction (fraction 2) exhibited the lowest cytotoxic effect in both of the human cells tested. The ethyl acetate fraction (fraction 2) of the tamarind seed-coat extracts (TSCEs) from the three tamarind cultivars were used to evaluated for their cytoprotective and wound healing effects. The phenolic compounds in the sour tamarinds composed of tannin and procyanidin which were higher than the sweet tamarind. The chromatogram of HPLC profile of TSCEs from the three tamarind cultivars showed the peaks that identical with the three standard flavonoids including (+)-catechin, procyanidin B2 and (-)-epicatechin. The cytoprotective effect of TSCEs from the three tamarind cultivars was evaluated by NRU and DCFH-DA assays. The TSCEs exhibited cytoprotective effect by decreasing the number of damaged cells and reducing the intracellular ROS generations against $H_2O_2$-induced cells damage in both human cells tested. At the lower concentrations of the three TSCEs were used to evaluate the proliferative effect on the tested human cells by using NRU assay. Wound healing effect of TSCEs at the proliferative concentrations was evaluated by using the scratch assay, TSCEs did not show wound healing effect on both of the treated human cells. However, TSCEs exhibited cytoprotective effect on $H_2O_2$-induced oxidative stress in the scratch wound of CCD-1064Sk and AGS cells. TSCEs increased the rate of wound closure against $H_2O_2$–induced the delayed rate of wound closure. The lower concentrations of TSCEs from the sweet tamarind accelerated the rate of wound repair in CCD-1064Sk cells better than $H_2O_2$–treated cells (untreated with TSCEs), which was due to the scavenging effect of the TSCE against ROS-caused the delay of wound closure. The TSCEs of the sour tamarinds possessed the ROS scavenging effect in AGS cells, a better acceleration the percent wound closure than their $H_2O_2$-treated cells (untreated with TSCEs) was observed.

Department : Biochemistry and Microbiology Student's Signature Siriporn Konsue

Field of Study : Biomedicinal Chemistry Advisor's Signature Sunanta Pongsamart

Academic Year : 2011

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# LIST OF ABBREVIATIONS

| | |
|---|---|
| % | Percentage |
| Δh | Change of times |
| °C | Degree Celsius (centigrade) |
| µg | Microgram (s) |
| µl | Microliter |
| µm | Micrometer |
| $ABTS^{\bullet+}$ | 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) radical |
| AGS | Human gastric adenocarcinoma epithelial cell line |
| AIA | Adjuvant-induced arthritis |
| AIDS | Acquired immune deficiency syndrome |
| ANOVA | Analysis of variance |
| ATTC | American type culture collection, Maryland, USA. |
| CCD-1064Sk | Human foreskin fibroblast cell line |
| CCD-986Sk | Normal human skin fibroblast cell line |
| cm | Centimeter |
| $CO_2$ | Carbon dioxide |
| $Cu^{2+}$ | Copper(II) complex ions |
| DCFH-DA | 2',7'-dichlorofluorescin diacetate |
| DMSO | Dimethylsulfoxide |
| DNA | Deoxyribonucleic acid |
| $DPPH^{\bullet}$ | 2,2-diphenyl-1-picrylhydrazyl free radical |
| EGCG | (-)-Epigallocatechin-3-gallate |
| ERK | Extracellular signal-regulated kinases |
| et al. | et alii, and other |
| EtOH | Ethanol |
| $Fe^{2}$ | Iron (II) complex ions |
| $Fe^{3+}$ | Iron (III) complex ions |
| $FeNH_4(SO_4)\cdot 12H_2O$ | Ferric ammonium sulfate dodecahydrate |
| FGF | Fibroblast growth factor |
| FRAP | Ferric reducing antioxidant power |

| | |
|---|---|
| g | Gram (s) |
| GAE | Gallic acid equivalents |
| GSH | Glutathione |
| GSPE | Grape seed proanthocyanidin extract |
| GSSG | Glutathione disulfide |
| $H_2O$ | Dihydrogen Monoxide (water) |
| $H_2O_2$ | Hydrogen peroxide |
| $H_2S$ | Hydrogen Sulfide |
| HaCaT | Human keratinocyte cell line |
| HCl | Hydrochloric acid |
| HPF-1 | Human lung fibroblasts |
| HPLC | The high performance liquid chromatography |
| $IC_{50}$ | 50% inhibitory concentration |
| IL6 | Interleukin-6 |
| kg | Kilogram (s) |
| LDL | Low-density lipoprotein |
| m | Meter (s) |
| min | Minute (s) |
| MKN 28 | Human well differentiated gastric tubular adenocarcinoma cell line |
| ml | Mililiter (s) |
| mM | Milimolar |
| mm | Millimeter (s) |
| MTCC | Microbial Type Culture Collection |
| MTT | 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide |
| N | Normality |
| NaHS | Sodium hydrogen sulfide |
| NCIM | National Collection of Industrial Microorganisms |
| NIH3T3 | mouse fibroblast |
| nm | Nanometer |
| NRU | Neutral red uptake |
| $O_2^{\bullet-}$ | Superoxide radical |

| | |
|---|---|
| $OD_{570}$ | The optical density at a wavelength of 570 nm |
| $OH^{\bullet}$ | Hydroxyl radical |
| PBS | Phosphate buffer saline |
| PC12 | Rat pheochromocytoma cells |
| PDGF | Platelet derived growth factor |
| PGE2 | Prostaglandin E2 |
| PGs | Prostaglandins |
| pH | Potential of Hydrogen ion |
| ROOH | Hydroperoxide |
| ROS | Reactive oxygen species |
| rpm. | Revolutions per minute |
| SPSS | Statistical package for social sciences |
| TGF-β | transforming growth factor-β |
| TI-P | *Tamarindus indica* "Priao-Native" |
| TI-PK | *Tamarindus indica* "Priao-Kradan" |
| TI-SP | *Tamarindus indica* "Srichomphu" |
| TSCEs | Tamarind seed-coat extracts |
| UV | Ultraviolet |
| v/v | Volume per volume |
| VEGF | Vascular endothelial growth factor |
| w/v | Weight per volume |

# CHAPTER I

# INTRODUCTION

## Background and rationale

Reactive oxygen species (ROS) are generated in cells metabolism or received from exogenous such as irradiation by UV light, smoking and food. At high concentrations of ROS can damage to cells structure including lipids, proteins and nucleic acids and induce cells oxidative stress. Oxidative stress has been reported as a key role in the development of diseases such as aging, cancers, arteriosclerosis, neurodegenerative disorders and other diseases. (Thannickal and Fanburg, 2000; Valko *et al*., 2006). In the role of wound repair, ROS may be involved in stage of wound healing process at the low level such as angiogenesis, proliferation, and apoptosis. However, excessive ROS concentrations are harmful due to their high reactivity, resulting in the presence of oxidative stress in skin wound, in particular the non healing stage or chronic wounds (Mohammad *et al*., 2008; Schäfer and Werner, 2008). Moreover, the gastric mucosa damage by various factors is mediated through the ROS generation (Das *et al*., 1997; Demir *et al*., 2003). Thus, the antioxidants have an important role in health promotion, disease prevention due to the antioxidant activity on scavenging ROS or free radicals.

The phenolic compounds, a large group of aromatic phenols have been found more than 4,000 different types in plants such as flavonoids, phenolic acids, tannins and phenolic diterpenes (Hollman *et al*, 1996; Rao *et al*, 2007). Nowadays, the phenolic compounds are popular compounds having a positive effect on a human health (Veberic *et al*, 2008). These compounds are often found in vegetables, fruits, and certain beverages. The biological activities of these compounds have been studied for antioxidant, anticancer, anti-inflammatory and cardioprotective activity (Wojdyl *et al*, 2007; Dufour *et al*, 2007; Cook and Sammam, 1996). The phenolic compounds in the natural source have been reported the protective effect on oxidative stress in various cells.

This study interested in wound healing effect of tamarind seed-coat on skin fibroblasts and gastric cells, due to both cells can contact to factor-induced ROS

generations that risk to wound more than other cells. The dietary antioxidants compounds showed to protect gut epithelial cells by decreased cells death of gut epithelium cells on $H_2O_2$ and hypoxanthine-xanthine oxidase induced apoptosis (Miller *et al*., 2001). Moreover, the procyanidin from grape seeds have been reported gastroprotective effect on HCL/EtOH solution-induced gastric mucosal in rat (Saito *et al*., 1998) and the plant flavonoid compounds exhibited gastroprotective and ulcer healing activities (Lira Mota *et al*., 2005). On the other hand, the polyphenol from leaves of *Chromolaena odorate* have been reported the proliferative effect on fibroblasts, endothelials and keratinocytes cells, increased migration of keratinocytes cells and exhibited protective effect of these cells against $H_2O_2$ and hypoxanthine-xanthine oxidase induced cells damage (Thang *et al*., 2000).

Tamarind (*Tamarindus indica* L.) is a tropical fruit found and cultivated in Thailand. Virtually, every part of tamarind has nutritional and medicinal value. Tamarind seed-coats were evaluated in this present study, it is a byproduct of tamarind gum or jellose and candy manufacturing. Tamarind seed-coat extract has been reported that its compose of antioxidative compounds such as monomeric, oligomeric procyanidin, (+)-catechin, (-)-epicatechin, taxifolin, apigenin, eriodictyol, luteolin and naringenin (Tsuda *et al*., 1994; Sudjaroen *et al.,* 2005). These compounds contain the phenolic groups, its bioactivity are related to antioxidative properties (Tanrioven and Eksi, 2005). In traditional medicine has reported that tamarind seed-coats are used to treat wound burns and chronic wounds in diabetic patient (Farnsworth and Bunyapraphatsara, 1992) and the extracts from tamarind seed-coat have been reported the anti-inflammatory activity by inhibiting nitric oxide production in murine macrophage (Komutarin *et al*.,2004). However, the compounds found in tamarind seed-coat extract have not been reported the protective and wound healing effects in skin fibroblasts and gastric epithelial cells. This study, we expect that tamarind seed-coat may involve the cytoprotective effect and accelerate the rate of wound of human skin fibroblast and gastric epithelial cells.

Two types of tamarind including the sweet and sour types are cultivated in Thailand. The phenolic contents and antioxidant activities of tamarind seed-coats from tamarind different cultivars were investigated *in vitro*. The seed-coat extracts from different tamarind cultivar exhibited different antioxidant properties, which may

be partly because of the difference in the phenolic compounds (Suksomtip *et al*., 2010). Thus, evaluation of the effects of seed-coat extract from different tamarind cultivars on human skin fibroblasts and gastric epithelial cells are investigating to evaluate their function in cells. So far, the efficacy of tamarind seed-coat extracts on cytoprotective and accelerating the rate of wound healing has not been studied. These properties of tamarind seed-coat extracts from different tamarind cultivars were evaluated in this study.

**Objective**s

1. To investigate a cytoprotective effect of tamarind seed-coat extracts from three tamarind cultivars on human skin fibroblasts and human gastric epithelial cells.

2. To study an effect of tamarind seed-coat extracts from three tamarind cultivars on acceleration the rate of wound closure on human skin fibroblasts and human gastric epithelial cells.

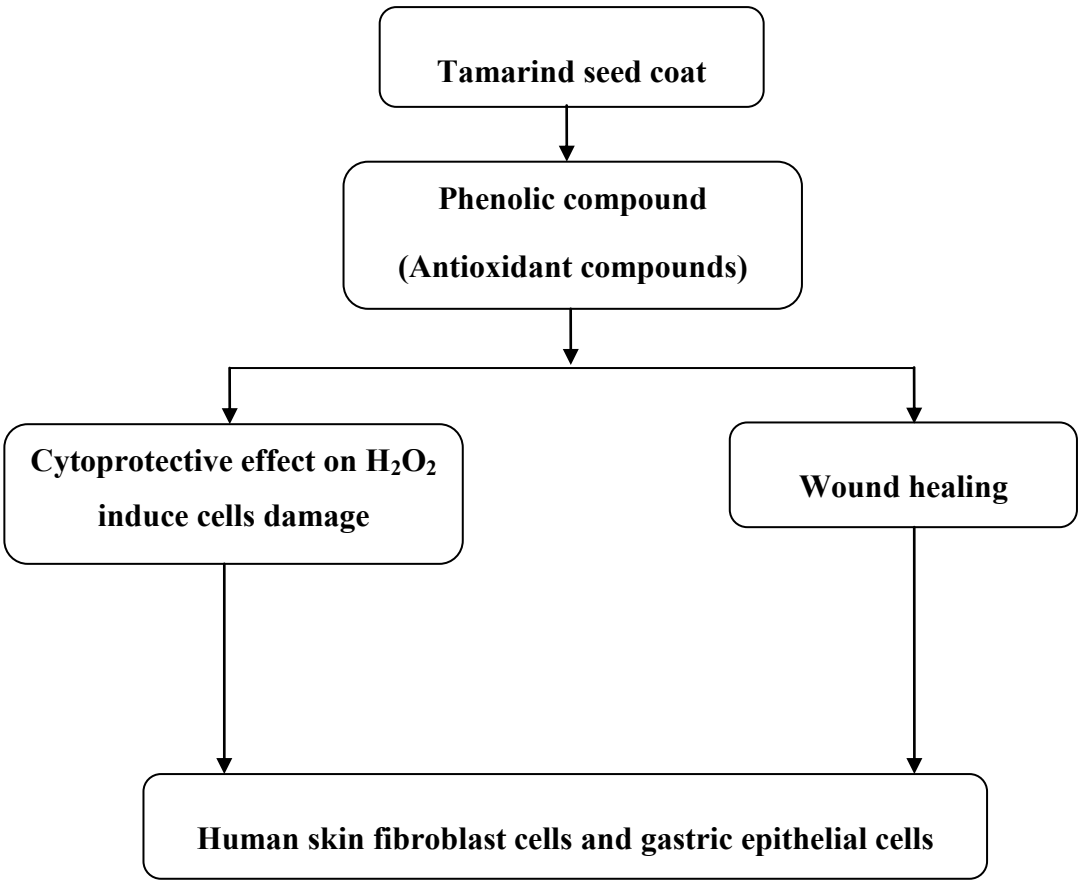


**Figure 1** The conceptual framework of this study

**Scope of study**

In this study present the *in vitro* assay of tamarind seed-coat extract from the three tamarind cultivars on the cytoprotective and wound healing effects in human skin fibroblasts and gastric epithelial cells. Firstly, tamarind seed-coat was extracted by different solvent extractions and examined the phenolic contents by using spectrophotometric method and estimated the cytotoxic effect on human skin fibroblasts and gastric epithelial cells. The least toxic fraction was used for further study the protective and wound healing effects on cells.

The tamarind seed-coat extracts from different cultivars of the fraction exhibited the least toxic effect was carried out for the chemical analysis and bioactivities. The chemicals analysis including the phenolic contents and HPLC fingerprint were analyzed from tamarind seed-coat of the three different tamarind cultivars. Bioactivities including the cytoprotective and wound healing effects were studied. The cytotoxic effect on human skin fibroblasts and gastric epithelium cells was estimated by using standard method (MTT and NRU assay). The non-toxic concentrations were used to evaluate the cytoprotective effect against $H_2O_2$ induced cells damage and oxidative stress by measuring the cells viability and intracellular ROS production in human cells. Moreover, the rate of wound healing effect of tamarind seed-coat extracts from the three tamarind cultivars on human skin fibroblasts and gastric epithelial cells was investigated by using the scratch assay, in conditions untreated and treated with $H_2O_2$.

**Contribution of the study**

1. The information about the protective effect of tamarind seed-coat extracts from different cultivars on human fibroblasts and human gastric epithelial cells against hydrogen peroxide-induced damage. These data may be supporting for developing an inexpensive nature antioxidative compounds.

2. The information about the wound healing effect of tamarind seed-coat extracts from different cultivars on human fibroblasts and human gastric epithelial cells may contribute a natural agent to treat acute and chronic skin and gastric wound repairs.

# CHAPTER II

# LITERATURE REVIEWS

## 1. Tamarind

Tamarind (*Tamarindus indica* Linn.) is a semi-evergreen tree member of the dicotyledonous family *Leguminosa* (*Fabaceae*) in subfamily *Caesalpinioideae*, which is the third largest family of flowering plants with 727 genera and 19,327 species (Chant, 1993; Lewis *et al*., 2005). Tamarind grows naturally in tropical and subtropical area in more than 50 countries of worldwide, the major areas are in Asia such as India, Bangladesh, Sri Lunka, Indonesia and Thailand (Kumar and Bhattacharya, 2008). In Thailand, the tamarind is divided into two types, the so-called sweet and sour tamarinds. Virtually, every part of tamarind is one of the most important plant resources as food material and medicine, which essential for industrial and commercial applications.

Taxonomical classification (Bhadoriya *et al*., 2010)

| | |
|---|---|
| Kingdom | : Plantae |
| Phylum | : Spermatophyte |
| Class | : Angiosperm |
| Sub class | : Dicotyledone |
| Family | : Leguminosae |
| Subfamily | : Caesalpiniaceae |
| Genus | : *Tamarindus* |
| Species | : *indica* |

**1.1 Plant description and morphology** (El-Sidding *et al*., 2006; Bhadoriya *et al*., 2010)

### 1.1.1 Tree

Tamarind is a long-lived, large in size evergreen or semi-evergreen tree, up to 24 m in height and 7 m in circumference. The bark is brownish-grey, rough and scaly.

### 1.1.2 Root

The root of tamarind is deep tap root and extensive lateral root system. The tap root is flexuous and lateral roots. The tap root may be stunted in badly drained or compacted soils.

### 1.1.3 Leaves

Leaves are alternate, compound in length 5-15 cm with 10-18 pairs of opposite leaflets. The leaflet is narrowly oblong, asymmetric with the tuft of yellow hairs, shortly petiolated (up to 1.5 cm long) and rachis finely haired, midrib and net veining more or less conspicuous on both surfaces.

### 1.1.4 Flowers

Flowers are bisexual and the bone in lax racemes which a few to several flowered (up to 18), borne at the ends of branches and are shorter than the leaves, the lateral flowers are drooping. Flowers are attractive pale yellow or pinkish, in small, lax spikes about 2.5 cm in width.

### 1.1.5 Fruits

Fruit is a pod, subcylindrical, oblong, curved or straight. The pod is 5-16 cm long and 2 cm broad. The outer pericarp of pod has light grey or brown color. In the pod has the pulp, the outer of the pulp has three tough branched fibers.

### 1.1.6 Seeds

The seeds contained in the pod, about 3-12 seeds, approximately 1.3 cm long, which are irregularly shaped, flattened covered with a hard, shiny and smooth testa. Seeds are hard, the color is red to purple brown.

**1.2 Phytochemistry and medicinal usage** (El-Sidding *et al*., 2006; Soemardji, 2007; Bhadoriya *et al*., 2010; Caluwé *et al*., 2010)

### 1.2.1 Wood and Bark

The bark contains phlobatannine for 35%, phenols, flavonoids and up to 70% tannin. The wood or bark is used for furniture such as house frame, kitchen tools and toys. The bark has been used in the tannin industry and the

preparation of ink and for fixing dyes. The bark of tamarind has been reported antioxidant activity on DPPH radical (Ramos *et al*., 2003) and used for the production of anti-inflammatory agents, against leucorrhoea, and for skin disorders (Algabal *et al*., 2011). In traditional medicine, the bark is used for asthmatic, pyretic, amenorrhea, colic, scorbutic and treated stomachache and wounds (Libman *et al*., 2006).

### 1.2.2 Pulp

The fruit pulp contains organic acids such as Tartaric- (3-10 %), acetic-, citric-, formic-, malic-, succinic acid; amino acids (alanine, leucin, phenylalanine, proline, serine), invert or reducing sugar (25-30 %), pectin, protein (87.9 g/kg), fat (19.1 g/kg), some pyrazines, trans-2-hexenal, and some thiazoles (2-ethylthiazole, 2-methylthiazole) as fragrant and high polyphenolic compounds. Moreover, the pulp is found volatile oil such as furan derivatives (44.4%) and carboxylic acid (33.3%), rich in minerals (high in potassium, phosphorus and calcium, and fair in iron) and high content of vitamin B (thiamine, riboflavin and niacin), but low amounts of carotene and vitamin C. The pulp is used for a wide variety of domestic and industrial because its compose energy or nutrition sources with benefits effective for healthy. The pulps are used for seasoning, juice, jam, syrup and candy. The acidic pulp is used for ingredient in culinary preparations such as curries, sauces and ice cream. Moreover, the fruit pulps have been reported to exhibit antioxidant, anti-hepatoxic, anti-mutagenic, anti-hypercholesterolemic, anti-inflammatory, anti-fungal and anti-bacterial activities. In folk medicinal, the fruit pulps are used for cure digestive or constipate, carminative, laxative, expectorant, blood tonic, pyretic, dysentery, loss of appetite, alcohol toxicity, vomit, worm infection, jaundice, nausea and vomit in pregnant, asthmatic, breast inflammation urticaria allergic, morbilli, thirsty and applied on inflammations.

### 1.2.3 Leaves and flowers

The leaves contain proteins (4.0-5.8%) while the flowers contain only 2-3% of proteins. The leaves are a fair source of vitamin C and ß-carotene, high source of the minerals (potassium, phosphorous, calcium and magnesium). Leaves are found tartaric acid, maleic acid and oxalic acid, two triterpenes, lupanone and lupeol, leaves oil (limonene and benzyl benzoate were most

predominant) and rich in tannin. The leaves are also used for domestic animals and wild animals. The leaves and flowers are mordant in drying, prepare an essential oil and used to make curries, salads, stews and soups. The leaves and flowers have been reported of antioxidant, anti-bacterial activity. In traditional medicines, the leaves are used to cure cough, pyretic, rheumatism, jaundice, worm infection, sores, ulcer and insomnia while the flowers are used to treat pulmonary tuberculosis, cough with blood, chronic pharinkhitis, rheumatism, locally edema and wound.

#### 1.2.4 Seeds

Tamarind seeds are by-product of the commercial utilization of the fruit pulp. The seeds are rich source of protein, crude fiber, carbohydrate and high minerals (potassium and magnesium). The seed kernels are high protein (13-20%), while the seed-coats are high fiber (20%) and tannins (20%). The seed is consisting of the seed-coat or testa approximately 20-30% and kernel or endosperm approximately 70-75%. The seed kernel contains polysaccharides are found with main chain consisting of ß-1,4- connected glucose molecules together with xylose (α-1,6) and galactose (ß-1,2), like fruit pectin. The seed is good source of fatty acids, unsaturated (55.6%) and saturated (44.4%) fatty acid. The tamarind seeds are high containing of tamarind oil especially palmitic, stearic and linoleic acid and rich source of minerals such as calcium, phosphorus, magnesium and potassium. The kernel of tamarind seed is used as source of carbohydrate as the adhesive or binding agent in paper and textile sizing and used to produce the jellose, used for stabilizer in ice cream, mayonnaise, cheese and as an ingredient or agent in pharmaceutical products. The seeds have been reported the various bioactivities such as antioxidant, anti-inflammatory, anti-diabetic anti-venom and wound healing activities. In addition, the tamarind seed is used in folk medicinal for cure chronic diarrhea, dysentery, snake bite, wound or ulcer, and drop off hair.

### 2. Tamarind seed-coat or testa

**2.1 Phytochemistry and medicinal usage** (Sudjaroen *et al*., 2005; El-Sidding *et al*., 2006; Soemardji, 2007; Bhadoriya *et al*., 2010; Caluwé *et al*., 2010)

The tamarind-seed coats are by-product of manufacturing tamarind gum. Tamarind seed-coats are rich in fiber (20%) and tannin (20%) and its compose with polyphenolic compound, are dominated by proanthocyanidins (73.4 %) in form of catechin (2.0 %), epicatechin (9.4 %), procyanindin B2 (8.2 %), procyanidin trimer (11.3%), procyanidin tetramers (22.2%), procyanidin pentamer (11.6%), procyanidin hexamers (12.8) and the flavonoids taxifolin (7.4%), apigenin (2%), eriodictyol (6.9%), luteolin (5%), and naringenin (1.4%). Moreover, phytochemical such as furfural, tetrazene, levuglucosan, cyclohaxasiloxane, dioxolanebutanediol etc., saponins, steroids, flavonoid, isoflavonoid are found in tamarind seed-coat (Waghmare *et al*., 2010; Bhadoriya *et al*., 2011). In traditional medicine, the seed-coats are used to treat chronic diarrhea, dysentery and as astringent for treat burn and aid in wound of diabetic patients.

## 2.2 Bioactivity research of tamarind seed-coat

### 2.2.1 Antioxidant activity

The tamarind seed-coat contain phenolic compounds have been reported strongly antioxidant activity. The 2-hydroxy-3',4'-dihydroxyacetophenone, methyl 3,4-dihydroxybenzoate, 3,4-dihydroxyphenyl acetate, and (-)-epicatechin are isolated as antioxidant compounds from tamarind seed-coat. These compounds exhibit strong antioxidative activity in the linoleic acid auto-oxidation system (Tsuda *et al*., 1994; Tsuda *et al*.,1995). The sweet Thai tamarind seed-coat extracts consisted with high levels of (-)-epicatechin show very effective as an antioxidant (Luengthanaphol *et al*., 2004). Moreover, Sudjaroen *et al*. (2005) investigated that the antioxidative compounds of methanolic tamarind seed pericarp show antioxidant activity on hypoxanthine/xanthine oxidase and 2-deoxyguanosine assay. The main antioxidative compounds including proanthocyanidin in various form (+)-catechin, procyanidin B2, (-)-epicatechin, procyanidin trimer, procyanidin tetramer, procyanidin pentamer, procyanidin hexamer, taxifolin, apigenin, eriodictyol, luteolin and naringenin. The raw and dry heated seed-coat of tamarind exhibit good antioxidant activity against the linoleic acid emulsion system on $O_2^{\bullet -}$, $OH^{\bullet}$, $\alpha,\alpha$-diphenyl-$\beta$-picrylhydrazyl ($DPPH^{\bullet}$), $ABTS^{\bullet +}$ and FRAP (Siddhuraju, 2007). Different cultivar of methanolic tamarind seed-coat extracts show different phenolic compounds resulting in different

antioxidative activity on the 2,2-diphenyl-1-picrylhydrazyl ($DPPH^{\bullet}$) and hydroxyl radical, anti-lipid peroxidation and reducing power assay (Suksomthip *et al*.2010). The free radical scavenging activity of phenolic compounds from Thai tamarind seed-coat extracts on the 2,2-diphenyl-1-picrylhydrazyl ($DPPH^{\bullet}$) radical assay are applied on anti-wrinkle cosmetic such as lotion (Lourith *et al*., 2009).

### 2.2.2 Protective effect on low-density lipoprotein and DNA damage

Suksomthip and Pongsamart (2008) found methanolic extract of Thai tamarind seed-coat compose with polyphenol, procyanidin and (-)-epicatechin exhibit the protective effect against $Cu^{2+}$-induced LDL oxidation by the chelation of transition metal ion. Moreover, methanolic extract from Thai tamarind seed-coat show protective effect on supercoiled DNA against hydroxyl radical-induced DNA scission.

### 2.2.3 Anti-inflammatory and immunomodulatory activity

The polyphenols extract from tamarind seed-coat reduce heat stress, oxidative stress and improve the growth rate of heat-stressed broilers, increase lymphocyte and basophil levels of broilers, decrease heterophil, monocyte and both increase and decrease eosinophil, show antibiotic feed additives and improve average daily weight gain of broilers, relative bursa of Fabricius weight, reduce lesion scores of bursa of Fabricius, reduce the impact of heat stress to broilers immune system and reduce GPx activity and bilirubin in feed and protect red blood cells from free radicals in heat-stressed broilers (Aengwanich *et al*., 2009 and 2010; Srikhun *et al*., 2010; Aengwanish and Suttajit, 2012). The study of Komutarin (2004) found the effect of polyphenolic flavonoid from tamarind seed-coat extract on the suppression of nitric oxide production, attenuated nitric oxide production by lipopolysaccharide and interferon gamma in murine macrophage-like cell line, RAW 264.7 and freshly isolated B6C3F1 mouse peritoneal macrophages. Morever, Babaria *et al*. (2011) investigated that the phenolic compound from tamarind seed-coat show antiarthritic activity in Wister rats, induced arthritis by Freund's Complete Adjuvant by inhibit the disease progression of AIA and protect the affected joints against cartilage destruction and bone erosion in rats and inhibit the production of two important pro-inflammatory mediators, IL6 and $PGE_2$ (inflammatory activity).

### 2.2.4 Antimicrobial activity

The phytochemicals present in tamarind seed-coat extract exhibit the antimicrobial activity. The active phytochemical in tamarind seed-coat such as furfural, tetrazene, levuglucosan, cyclohaxasiloxane, dioxolanebutanediol etc show the antimicrobial activity against *Staphylococcus aureus* NCIM -5021, *Pseudomonas aeruginosa* NCIM-2036 and *Salmonella typhimurium* NCIM-2501. (Waghmare *et al*., 2010). Morover, Bhadoriya *et al*. (2011) found tannins, saponins, steroids, carbohydrates and rich with flavonoid, isoflavonoid and polyphenolic compounds in tamarind seed-coat exhibit antimicrobial activity against Gram-positive bacterial strains: *Staphylococcus aureus* (MTCC-3160) and *Bacillu ssubtilis* (MTCC-1790), Gram-negative bacterial strains: *Escherichia coli* (MTCC-2960), *Pseudomonas auruginosssa* (MTCC-4676), *Klebsiella pneumonia* (MTCC-3030) and yeast *Candida albicans* (MTCC-183) by agar well diffusion method.

### 2.2.5 Anthelmintic activity

The rich flavonoid extract from tamarind seed-coat show strong anthelmintic activity against tapeworms (*Taeniasolium*) and earthworms (*Eisoniafatida*), but do not show prominent lethal action against round worms (*Ascaridiagalli*) and earthworm (*Pheretimaposthuma*) (Bhadoriya *et al*., 2011).

## 3. Phenolic compounds

### 3.1 Definitions of phenolic compounds (Vermerris and Nicholson, 2006)

Phenolic compounds are compounds that comprise of one or more hydroxyl groups directly attached to aromatic ring, in case is benzene. Polyphenols are compounds that have one or more phenolic hydroxyl groups attached to one or more benzene aromatic ring. Phenolic compounds are second metabolite found in plants tissues such as fruit and vegetables and usually found as ester or glycoside more than as free compounds.

### 3.2 Classification of phenolic compounds (Vermerris and Nicholson, 2006)

Phenolic compounds are very large and various group of chemical compounds. The classification of these compounds can investigate in various ways such as based on the number of carbons in molecule or based on group of phenols.

The type of phenolic compound are constitute simple phenolics, phenolic acids and aldehydes, acetophenones and phenylacetic acids, cinnamic acids, coumarins, flavonoids, biflavonyls, benzophenones, xanthones and stilbenes, benzoquinones, anthraquinones and naphthaquinones, betacyanins, lignans, lignin, tannins and phlobaphenes.

**3.3 Effects on human health** (Vermerris and Nicholson, 2006**)**

The phenolic compounds have been reported that using in folk medicinal application such as antiseptic, oral anesthetic, antioxidant etc. These activities of phenolic compounds are used for beneficial effects on health. Phenolic compounds can scavenge radicals act as antioxidant activity. These activities are resulting in protective effect on various diseases such as polyphenol from grape prevent cardio-vascular diseases, flavonoid show benefit health in protection against cardiovascular diseases, antimutagenic effects, anti-carcinogenic effects, anti-inflammatory, anti-allergic and antiviral.

**4. Flavonoids**

**4.1 Definitions and classification of flavonoids** (Vermerris and Nicholson, 2006; Yao *et al*., 2004**)**

Flavonoids comprise the one class of phenolic compounds. Flavonoids have six-member heterocyte and have A-, B- and C-ring (on the left-hand side of structure is A-ring). Flavonoids are $C_{15}$ compound in structure of $C_6$-$C_3$-$C_6$, compose of two benzene ring are linked together between a group of three carbons **(Figure 2)**.

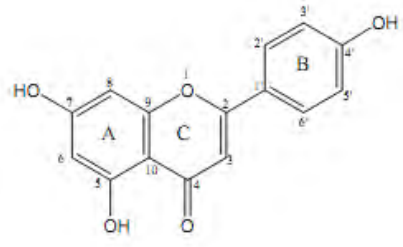


**Figure 2** The chemical structure of flavonoid

Flavonoids are major coloring component of flowing plant. These compounds are rich in seed citrus fruit, olive oil, tea and red wine. Flavonoids can be subdivided into six classes according to the present of an oxy group at position 4, a

double bond between carbon atoms 2 and 3, or hydroxyl group in position 3 of the C middle ring. The chemical structure of examples flavonoids is shown in **Figure 3**. The subclasses of flavonoids as

- Flavanones are the heterocycle of flavanones contains a ketone group, but there is no unsaturated carbon-carbon bond, as in naringenin.

- Flavanonols are also known dihydroflaonols such as taxifolin-leucoanthocyanidins are referred to as flavan-3, 4-*cis*-diols. These compounds are also present in wood. The examples are leucocyanidin, catechins.

- Flavones are heterocycle of flavones cantains a ketone group and have an unsaturated carbon-carbon bond. These compounds are common found in angiosperms such as quercetin, kaemferol.

- Anthocyanidins and deoxyanthocyandins are pyriliumkation. These compounds are present in vacuoles of color plant tissue such as leaves or flower petals. The examples are cyanidin, luteolinidin.

- Anthocyanins are water-soluble glycosides of anthocyanidins such as petanin.

**4.2 Biological activity of flavonoids** (Middleton *et al*, 2000; Yao *et al*., 2004)

Flavonoids are important for human health, exhibit high pharmacological activity. The biochemical activities of flavonoid depend on the chemical structure of these compounds. Flavonoids are easily oxidized at B ring, resulting in the opening of this ring at the oxygen atom. These compounds have binding affinity to biological polymers and heavy metal ions ($Fe^{2+}$,$Fe^{3+}$, $Cu^{2+}$) and the ability to catalyze electron transport and to scavenge free radicals. Flavonoids are the group of compounds containing the most powerful antioxidant activities, these activities as radical scavenger of flavonoids are correlate to health-promoting properties. The flavonoids have been investigated to possess anti-inflammatory, anti-allergic, cytoprotective, antithrombotic, antivirus, anti-carcinogenic, enzyme modulator activities. The flavonoids suggest protective effects against cardio-vascular diseases, cancers, and other age-related diseases in the epidemiological studies.

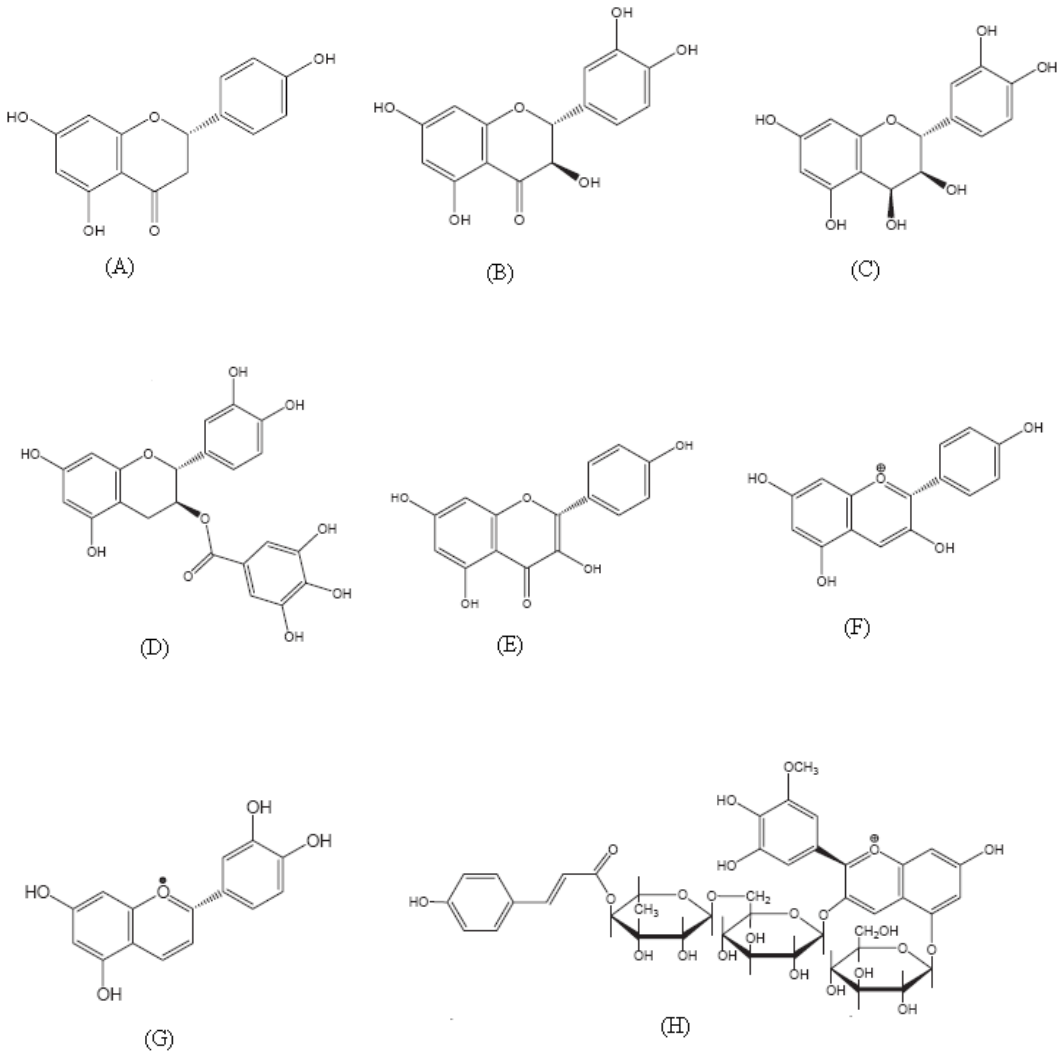


**Figure 3** The chemical structure of various flavonoid compounds. (A) naringenin, (B) taxifolin, (C) leucocyanidin, (D) catechins, (E) kaemferol, (F) cyanidin, (G) luteolinidin and (H) petanin (Vermerris and Nicholson, 2006).

## 5. Tannins

### 5.1 Definitions and classification of tannin (Vermerris and Nicholson, 2006)

Tannins comprise a group of phenolic compounds, much diversity in structure. Tannins show their ability to bind and precipitate proteins molecules. Tannins protect against infection and herbivory in plant tissue such as leaves, bark and fruits. The classifications of tannin are divided into three groups of tannins as condensed tannins, hydrolysable tannins and complex tannins. The chemical structure of sample tannins is shown in **Figure 4**. The subclasses of tannins as:

- Condensed tannins are also referred to as proanthocyanidins. These compounds are oligomeric or polymeric flavonoids containing of flavan-3-ol (catechin) units. Procyanidin B2 (epicatechin-(4β→8')-epicatechin) is an example of condensed tannins.

- Hydrolysable tannins are divided into 2 groups as:

- Gallotannins are hydrolysable tannins with a polyol core (referring to a compound with multiple hydroxyl groups) substituted with 10-12 gallic acid residues. The D-glucose is the commonly of polyol. An example of gallotannins is the hexagalloylated compound 2-O-digalloyl-1,3,4,6-tetra-O-galloyl-β-D-glucopyranose

- Ellagitannins are hydrolysable tannins derived from pentagalloylglucose, but different from gallotannins. These compounds contain with C-C bonds between adjacent galloyl moieties in the pentagalloylglucose molecule. An example is valoneoyl.

- Complex tannins are containing with catechin unit, bound glycosidically to either a gallotannin or an ellagitannin unit. The structure of these compounds are very complex, acutissimin is an example.

### 5.2 Biological activity of tannins (Chung *et al*., 1998; Fine, 2000; Vermerris and Nicholson, 2006)

Tannins have been used as anti-inflammatory and antiseptic compounds in Japan and Chinese. The effect of tannin compounds has been investigated antioxidant, anti- carcinogen, antimutagenic, immunomodulating and

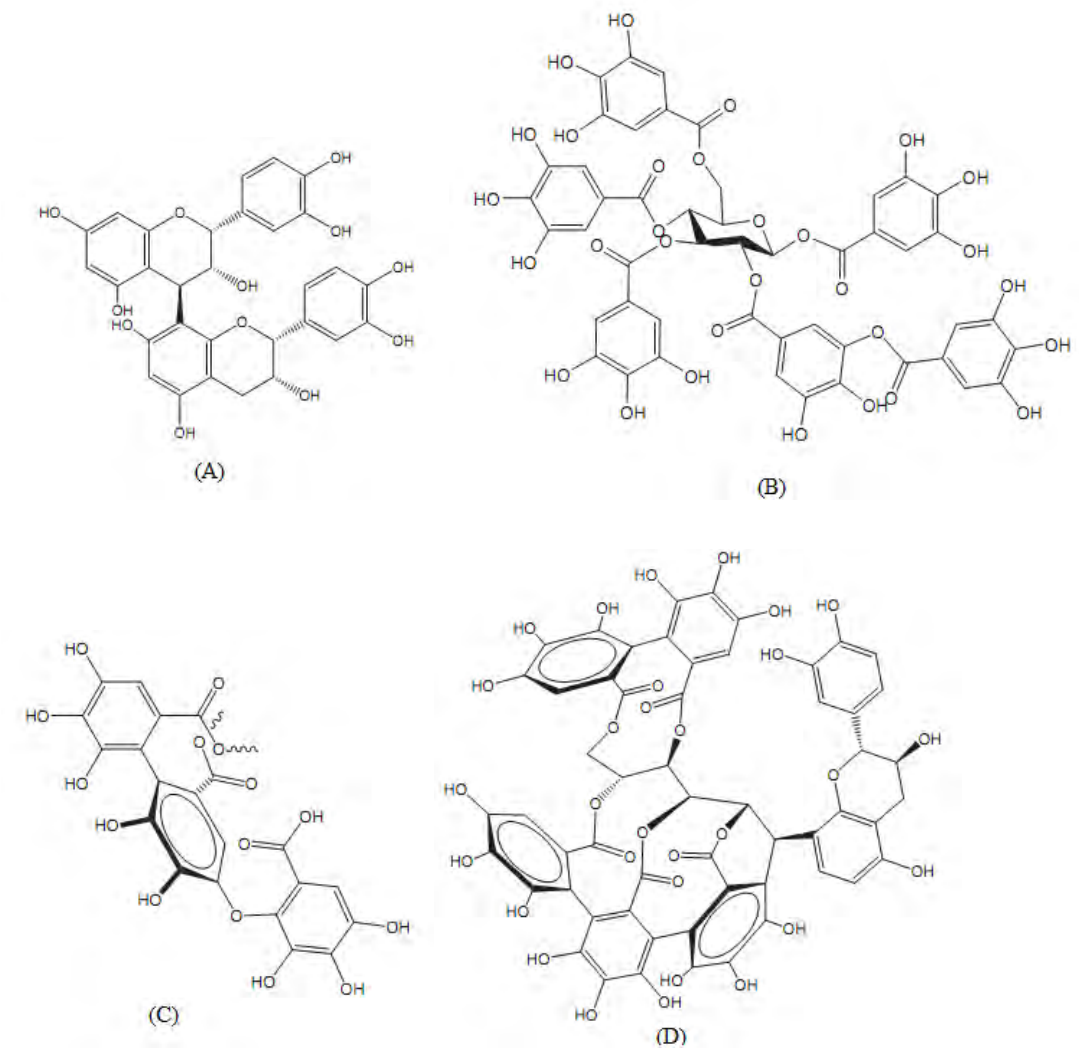


**Figure 4** The chemical structure of examples tannin compounds. (A) procyanidin B2, (B) 2-O-digalloyl-1,3,4,6-tetra-O-galloyl-β-D-glucopyranose, (C) valoneoyl and (D) acutissimin (Vermerris and Nicholson, 2006).

antimicrobial activities. Moreover, these compounds have been used to treat various illnesses such as diarrhea and tumors in stomach or duodenum. Proanthocyanidins demonstrate antibacterial, antiviral, anti-carcinogenic, anti-inflammatory, anti-allergic and antioxidant activities (good reducing agent), may participate in prevention of cancers of digestive tract and inner organs and preventive of cardiovascular diseases by protect low density lipids (LDLs) against oxidation and inhibit platelet aggregation. The proanthocyanidins use in the pharmaceutical application such as prevention of cardiovasceular disease, treatment of hypercholesterolemia, reduced adverse allergic and inflammatory responses, enhance immune function and wound healing. Moreover, ellagitannins is one group of hydrolysable tannins act as cancer chemo-preventive agent.

## 6. Reactive oxygen species (ROS)

**6.1 Definition and chemistry of ROS** (Thannickal and Fanburg, 2000; Valko *et al*., 2006.)

Molecular oxygen ($O_2$) is the final electron acceptor in oxidation energy of mitochondrial electron transport, an essential for the survival of aerobic organisms. These $O_2$ metabolites include superoxide anion ($O_2^{\bullet -}$), and hydrogen peroxide ($H_2O_2$), formed by one- and two-electron reductions of $O_2$, respectively. In the presence of transition metal ions, the even more reactive hydroxyl radical ($OH^{\bullet}$) can be formed. These partially reduced metabolites of $O_2$ are often referred to as reactive oxygen species (ROS) due to their higher reactivities relative to molecular $O_2$. Reactive oxygen species (ROS) are known as oxygen-free radicals, as molecules or molecular fragments containing one or more unpaired electrons. These radicals can be produced from both endogenous and exogenous substances. The endogenous sources are metabolic pathways in cellular activities such as byproducts of mitochondria-catalyzed electron transport reactions, inflammation process by neutrophils and macrophages. ROS are generated during irradiation by UV light, X-rays gamma-rays, metal ions, induced by pollutants in atmosphere, foods, tobacco smoke, organic solvent, infection of microorganism and strain conditions as exogenous process. These radicals exhibit high toxic agents on tissue damage by

initiating free radicals chain reactions. The mechanism of damage involves DNA damage, lipid peroxidation, protein damage and oxidation of important enzymes.

**6.2 Antioxidant defences and ROS detoxification** (Turrens, 2003; Powers and Jackson, 2008)

Under the normal condition of cells, the ROS are eliminated by antioxidant defense systems of cells. Antioxidant systems are divided into three groups of enzyme and non-enzyme-mediated system.

- Enzymatic antioxidants such as

- Superoxide dismutase is metalloenzymes can convert $O_2^{\bullet-}$ to $H_2O_2$ (Weisiger and Fridovich, 1973).

$$O_2^{\bullet-} + O_2^{\bullet-} + 2H^+ \rightarrow H_2O_2 + O_2$$

-Glutathione peroxidase can catalyze hydroperoxide to hydrogen peroxide by oxidation of glutathione or other free radicals such as lipid hydroperoxide (Takahashi and Cohen, 1986).

$$ROOH + 2GSH \rightarrow GSSG + H_2O + ROH$$

- Catalase is major $H_2O_2$ detoxifying enzyme (Kirkman, 1987)

$$\text{catalase–Fe(III)} + H_2O_2 \rightarrow \text{compound I}$$

$$\text{compound I} + H_2O_2 \rightarrow \text{catalase–Fe(III)} + 2H_2O + O_2$$

- Non enzymatic antioxidants are small molecules. These donors or receptors of electron molecules convert into stable substances such as carotenoids, flavonoids, Ubiquinol10, vitamin E, vitamin C.

- Transition metal binding proteins are importance in antioxidant system, containing ferritin, transferrin, lactoferrin and caeruloplasmin. An example is caeruloplasmin can convert $Fe^{2+}$ into $Fe^{3+}$ by oxidation of Fenton reaction.

$$4Fe^{2+} + O_2 + 4H^+ \rightarrow 4Fe^{3+} + 2H_2O$$

**6.3 Oxidative stress** (Turrens, 2003; McCord, 2000)

The ROS are continuously produced as byproducts of metabolic pathways in cells. These radicals are eliminated by antioxidant defences system. The imbalance between the excessive formation ROS from endogenous and exogenous

substances and limited antioxidant defences of cells is induced to “Oxidative stress”. The low level of ROS may actually play a role in intracellular signaling, uncontrolled (excessive ROS) increases in concentrations of these radicals lead to free radicals mediated chain reactions with indiscriminately target proteins, lipids and DNA. These reactions of ROS lead to the various human diseases such as inflammatory diseases (arthritis, vasculitis, glomerulonephritis, lupus erythematosus, adult respiratory distress syndrome), ischemic diseases (heart disease, stroke, intestinal ischemia), hemochromatosis, acquired immune deficiency syndrome (AIDS), emphysema, organ transplantation, gastric ulcers, hypertension and preeclampsia, neurologic diseases multiple sclerosis (Alzheimer’s disease, Parkinson disease, amyotrophic lateral sclerosis, muscular dystrophy), alcoholism, smoking-related diseases and many others.

## 7. Wound

The wound is a break in the continuity of the tissue in the body. The wound is found both internal and external of the body, involves the damage of skin or mucous membrane.

### 7.1 Classification of wounds (Velnar *et al*., 2009)

Wound is divided according to various criteria. Times are important factor for wound repair process. In clinical, the wound can be categorized as acute and chronic wound according to times criteria.

- Acute wounds are wounds, which repair themselves in times and orderly normal healing process. Acute wounds are performed in the results of traumatic loss of tissue or surgical procedure.

- Chronic wounds are abnormal wounds or non-healing wounds, which fail in process of wound healing. These wounds cannot be repaired in time and order of wound healing process. The various factor are induced in chronic wounds such as the prolong times on or more process of wound healing, infection, tissue hypoxia, necrosis, exudates and excess levels of inflammatory cytokines. Chronic wounds may result from naturopathic, pressure, arterial and venous insufficiency, burns and vasculitis.

### 7.2 Gastric ulcer

Stomach is an important organ of digestive tract, its located between esophagus and small intestine. The stomach is composed of 4 layers, from inside to outside as mucosa consists of the epithelium and lamina propria, submucosa consists of the fibrous connect tissue, muscularis externa consists of the smooth muscle and serosa consists of the connective tissue continuous with the peritoneum (Tangaumnoy, 1997).

Gastric ulcer is disorder result from an imbalance between endogenous aggressive factors such as hydrochloric acid, pepsin, refluxed bile, leukotrienes, reactive oxygen species (ROS) and cytoprotective factors such as mucus-bicarbonate barrier, surface active phospholipids, prostaglandins (PGs), mucosal blood flow, cell renewal and migration, nonenzymatic and enzymatic antioxidants and some growth factors (Lira Mota *et al*., 2009). Gastric mucosa injury arises in daily life during digestion. The diverse factors are damaged gastric mucosa cells including stress lifestyle, smoking, alcohol, aspirin and/or non-steroidal anti-inflammatory compounds, the infection of *Helicobacter pylori* (Cho and Wang, 2002; Lira Mota *et al*., 2009). The expose of factors are induced the acute ulcer become chronic ulcer. The process of ulcer healing including inflammation and proliferation, re-epithelialize, angiogenesis and tissue remodeling. The associate tissues of gastric healing are ulcer margin and granulation tissue including fibroblasts, macrophages and proliferating endothelial cells (Tarnawski *et al*., 1990).

### 7.3 Skin wound

The skin is outer covering organ, can covers and protects the body from abrasion, bacterial attack, ultraviolet radiation and dehydration and controls internal temperature of body. The skin structure can be divided into two classes as epidermis, which thin layer consists of epithelial and keratinocyte cells and dermis is a thicker layer of the skin underlying the epidermis consists of connective tissue (collagen, elastic fibers), fibroblasts, macrophages, occasional fat cells, nerve ending and blood vessels (Wynsberghe *et al*., 1995). Loss of the integrity is induced to injury or illness of skin, lead to major incapacitate of skin. The causes of skin wounds usually result from external physical forces such as fall, cut in body tissues, jagged,

pointed objects and burn. After the tissue injury, the wound healing is occurred. The skin wound healing process including inflammation phase, which arise after hemostasis and consists of the inflammatory cells, new tissue formation phase, which occurs 2-10 days after injury and consists of different cells type (fibroblasts, keratinocytes, endothelial cells) and remodeling phase begins 2-3 week after injury and long times (Singer and Clark, 1999; Gurtner *et al*., 2008).

## 8. Wound healing

### 8.1 Wound healing process (Velnar *et al*., 2009)

Wound healing is a complex and dynamic process respond to tissue damage or injury. The wound healing process is involved with soluble mediators, blood cells, extracellular matrix and parenchymal cells. The main phase of wound healing process including coagulation and hemostasis phase, inflammation phase, proliferation phase and remodeling phase.

1. Coagulation and hemostasis phase begin immediately after tissue damage or injury. The blood spills into site of injury and then the blood component and platelets come in and contract with collagen and extracellular matrix components. Moreover, the platelets are released the platelet cytokines (platelet derived growth factor (PDGF), transforming growth factor-β (TGF-β), epidermal growth factor and insulin-like growth factors) to promote inflammatory cells.

2. Inflammation phase occurs during the late of coagulation phase. The neutrophils are promoted into injury within 24-36 hours to destroy and remove bacteria foreign particles and damages tissue by phagocytosis. After injury for 48-72 hours, the macrophages appear in wound area and continue of the phagocytosis. The monocytes and macrophages are release chemoattractive agents such as TGF-β TGF-α, heparin binding epidermal growth factor, fibroblast growth factor (FGF), collagenase to activate keratinocytes, fibroblasts and endothelial cells. The late of inflammatory phase, lymphocytes are attracted after injury for 72 hours.

3. Proliferation phase arise on the third day after injury and lasts for about 2 weeks. This process associated with the migration and proliferation

of fibroblast cells, the collagen synthesis, angiogenesis and granulation tissue formation and epithelialization.

4. Remodeling phase is a final phase of wound healing. This process is responds for the development of new epithelium, synthesis and breakdown of collagen and scar tissue formation. This phase may last up to 1 or 2 years.

The factors are affected for wound healing including local factors (remodeling process growth factors, edema and ischemia, low oxygen tension, and infection), regional factors (arterial insufficiency, venous insufficiency, and neuropathy), systemic factors (inadequate perfusion and metabolic disease) and other factors such as nutritional state, preexisting illnesses, exposure to radiation therapy, and smoking. The acute wound should heal within three weeks and remodeling phase arising for long times. If the wound does not follow the normal process it may imbalance of repair wound process resulting in chronic wound or non-healing wound.

**8.2. ROS on wound healing** (Soneja *et al*., 2005)

ROS are generated in the wound healing process. Innate immune cells (neutrophils, monocytes and macrophages) are secreted large amount of ROS or superoxide radical anions. Moreover, the fibroblast cells can be promoted by pro-inflammatory cytokines to generate ROS.

**8.2.1 Positive effect of ROS in wound healing**

The low levels of ROS are essential on microorganism protection and investigate the mediators of intracellular signaling such as stimulate angiogenesis of wound (Keller *et al*., 2006; Schäfer and Werner, 2008), increase DNA synthesis, increase expression of proliferative gene, stimulate migration and proliferation of keratinocyte cells (Loo *et al*., 2011), activate the clot formation of platelets, activate reepithelization by activating collagenase expression (Soneja *et al*., 2005).

**8.2.2 Negative effect of ROS in wound healing**

The effect of ROS on gastric ulceration is suggests that gastric mucosal damage by ethanol, non steroidal anti-inflammatory drugs and *Helicobacter pylori* infection is mediated through the ROS, induce to oxidative stress (Das *et al*.,

1997). These radicals can be promoting mucosal damage by the degradation of epithelial basement component, change the cell metabolism, damage of DNA (Demir *et al*., 2003). In addition, $H_2O_2$ has been reported that induce the delayed epithelial migration (Choi *et al*., 2008).Thus, ROS are caused a pathological role in peptic ulcer and gastritis. The $H_2O_2$ reduce the migration and proliferation of fibroblast cells (Thomas *et al*., 2009). In chronic wound, an excess of oxidants can be damage fibroblast cells into abnormal morphology and inhibit the migration and proliferation of keratinicytes (Soneja *et al*., 2005). The high levels of ROS are harmful on wound and wound healing process.

**9. Research of natural antioxidant compounds on oxidative stress conditions and wound healing effect**

The excess oxidant radicals result from the imbalance of oxidant and antioxidant system. These compounds can be destroying proteins, lipids and DNA, which induce to various diseases. The nature antioxidant compounds are important for the scavenging of excess oxidants in cells, prevent the cells damage from excess oxidants and induce beneficial effects on human health and disease prevention. The nature antioxidant compounds have been reported the cytoprotective and wound healing effect on various cells. The flavonoid compounds exhibit protective effect the rat hepatocytes against hypoxia-reoxygenation injury (Moridani *et al*., 2003) and induce gastric mucosal production, decrease the secretion of hydrochloric acid by mucosal cells, inhibit pepsinogen production and antiulcer in gastric of rat (La Casa *et al*., 2000). Grape seed proanthocyanidin extract are greater protective effect against free radicals-induced lipid peroxidation and DNA damage. Moreover, grape seed proanthocyanidin extract demonstrate protective effect on tobacco-induced oxidative stress in human oral keratinocytes (Bagchi *et al*., 2000), accelerate the rate of wound healing and stimulate the expression of VEGF (Vascular endothelial growth factor) in keratinocytes (Khanna *et al*., 2001). Anthocyanidin from black soybean seed coats stimulates migration and proliferation, inhibit ROS accumulation on human keratinocytes and fibroblasts (Nizamutdinova *et al*., 2009). The phenolic compounds of *Chromolaena odorata* and antioxidant compounds from curcumin show protective

effect against $H_2O_2$-induced oxidative damage on human keratinocytes and fibroblasts (Phan *et al*., 2001).

# CHAPTER III

# MATERIALS AND METHODS

## Materials

### 1. Chemicals

| Chemical | Grade | Supplier/manfacturer |
|---|---|---|
| (-)-Epicatechin | HPLC grade | Sigma, USA. |
| (+)-Catechin hydrate | HPLC grade | Sigma, USA. |
| 2',7'-dichlorofluorescin diacetate (DCFH-DA) | Cell culture grade | Sigma, USA. |
| 2N Folin-Ciocalteu's phenol reagent | Analytical reagent grade | Sigma, USA. |
| Acetone | Analytical reagent grade | Fisher Scientific, Leicestershire, UK. |
| Bis-benzimide H 33324 | Cell culture grade | Sigma, USA. |
| Butanol | Analytical reagent grade | MERCK, Germany |
| Calcium chloride dehydrate | Analytical reagent grade | MERCK, Germany |
| Dimethyl sulfoxide (DMSO) | Analytical reagent grade | Sigma-Aldrich, Germany |
| Ethanol | Analytical reagent grade | J.T.Baker, USA |
| Ethyl acetate | Analytical reagent grade | J.T. Baker, USA. |
| F-12 nutrient mixture (Ham) | Cell culture grade | GIBCO, USA. |
| Ferric ammonium sulfate dodecahydrate | Analytical reagent grade | MERCK, Germany |
| Fetal bovine serum | Cell culture grade | Hyclone, UK. |
| Formaldehyde | Analytical reagent grade | Thailand |
| Gallic acid | Analytical reagent grade | Sigma, China |
| Glacial acetic acid | Analytical reagent grade | Lab-scan, Thailand |
| Hide powder | Analytical reagent grade | Sigma, UK. |
| Hydrochloric acid | Analytical reagent grade | J.T. Baker, USA. |
| Hydrogen peroxide ($H_2O_2$) | Analytical reagent grade | Fisher Scientific, Leicestershire, UK. |

| Chemical | Grade | Supplier/manfacturer |
|---|---|---|
| Iscove's Modified Dulbecco's Medium | Cell culture grade | GIBCO, USA. |
| Methanol | Analytical reagent grade | Sigma, USA. |
| Neutral red | Analytical reagent grade | BDH chemical, England |
| Nitrogen gas | Industrial grade | TIG, Thailand |
| Penicillin – streptomycin | Cell culture grade | GIBCO, USA. |
| Procyanidin B2 | HPLC grade | Sigma, USA. |
| Pyrogallol | Analytical reagent grade | Sigma, Germany |
| Sodium bicarbonate | Analytical reagent grade | Fisher Scientific, UK**.** |
| Sodium sulphate anhydrous | Analytical reagent grade | RFCL, India |
| Sodium carbonate | Analytical reagent grade | UNIVAR, Australia |
| Titriplex III, EDTA | Analytical reagent grade | Ajax Finechem, |
| Trypan blue | Analytical reagent grade | Australia |
| Trypsin | Cell culture grade | Sigma-Aldrich, Germany |

**2. Equipments**

| Equipments | Model | Supplier/manfacturer |
|---|---|---|
| 15, 50 ml conical tube | | Axygen, California, USA. |
| 200 µl, 1,000 µl Tips | | Corning Inc., USA. |
| 24, 96-well plate | | Corning Inc., USA. |
| 25, 75 $cm^3$ T-flask | | Corning Inc., USA. |
| Autoclave | HA-300 MD | Hirayama, Japan |
| Balance | | Mettler Toledo, Switzerland |
| | | International Scientific, Thailand |
| Biosafety laminar flow hood class II | BV-2225 | Waringcomercial, USA. |
| Blender | LB 20 EG | Corning Inc., USA. |
| Centrifuge | Allegra X-12 R | Beckman, Germany |
| Centrifuge | EBA 12 | Hettichzentrifugen, Germany |
| $CO_2$ incubator | 3121 | Thermo Fisher Scientific,USA. |

| **Equipments** | **Model** | **Supplier/manfacturer** |
|---|---|---|
| Desiccator | | |
| Disposable syringe filter 13 mm NYL (0.45 µm) | | Xiboshi, China |
| Filter Paper | | Whatman, UK. |
| Filter Sartolon polyamid (0.45 µm) | | Sartorius, Germany |
| Glassware apparatus | | Pyrex, USA. |
| Hemocytometer | | Hausser Scientific, Bright Line, Pennsylvania, USA. |
| Hot air oven | Memmert | Becthai, Thailand |
| Hot plate | | Becthai, Thailand |
| HPLC system | Class VP software 6.1 | Shimazu, Japan |
| Hypersil GOLD C18 250 x 4.6mm ID 5µm | | Thermo Fisher Scientific, UK. |
| Inverted microscope | IX-50 | Olympus, Japan |
| Inverted phase-contrast microscope | CK 30 | Olympus, Japan |
| Lyophilize | FTS system | Dura Dry MD, Newyork |
| Magnetic stirrer | Hermolyne | Branstead, USA. |
| Microcentrifuge tube | | Corning Inc., USA. |
| Microplate reader | Victor 3 | Perkin Elmer, Thailand |
| Rotary evaporator | R-200 | Buchi, Switzerland |
| Shakers | | Schuttler, Germany |
| Sonicator | | Elma, Transsonic, Germany |
| Spectrophotometer | Spectronic | Becthai, Thailand |
| Suction apparatus | Buchner Funnel, Aspirator, SIBATA circulating aspirator WJ-20 | Sibata, Japan |
| Vacuum suction | | Sibata, Japan |

| Equipment | Model | Supplier/manfacturer |
|---|---|---|
| Vortex | | Scientific, USA. |
| Water bath | Memmert | Becthai, Thailand |

**3. Plant Materials**

The ripen tamarind pods of *Tamarindus indica* L. "Priao-native" (TI-P) and "Priao-Kradan" (TI-PK) of the sour tamarind and the sweet tamarind -"Srichomphu" (TI-SP) were collected from Nakorn Ratchasima province, Thailand. The seeds of each cultivar were separated from the pulps, and the seed-coat was used for the preparation of tamarind seed-coat extracts.

**4. Cell lines and culture**

The human foreskin fibroblast cell line (CCD-1064Sk, CRL-2076) obtained from the American Type Culture Collection (ATTC). The cells were maintained in Iscove's Modified Dulbecco's Medium supplemented 10%(v/v) fetal bovine serum and 1%(v/v) penicillin-streptomycin solution under standard culture conditions (95% humidified air and 5% $CO_2$ at 37˚C), the culture medium was changed every 2-3 day.

The human gastric adenocarcinoma epithelial cell line (AGS, CRL-1739) was purchased from the American Type Culture Collection (ATCC). The cell was grown in Ham F-12 nutrient medium supplemented with 10%(v/v) fetal bovine serum, and 1%(v/v) penicillin-streptomycin solution, incubated in a humidified atmosphere of 5% $CO_2$, 95% air at 37˚C. The passage number range for cell line was maintained between 45-60 passages (Zheng *et al*., 1996), the culture medium was changed every 2 days.

**Method**

**1. Preparation of tamarind seed-coats**

The seeds of each tamarind cultivar were separated from the pulps and roasted in acid washed sand, quickly washed and dried in hot air oven at 50˚C for 6 hours. The seed-coats were separated from the kernel and the dried seed-coats were grounded into a fine powder by using the blender. The tamarind seed-coat powders were stored in the desiccator at room temperature until used.

**2. Extraction and determination of tamarind seed-coat extracts**

**2.1 Extraction of tamarind seed-coats using various solvents**

The total of 3 g tamarind seed-coat powder of sweet tamarind - "Srichomphu" (TI-SP) was extracted by boiling with 200 ml of distilled water for 1 min, allowed to stand at room temperature for 45-60 min and repeated extraction was continued until colorless filtrate was obtained. All of the aqueous extracts were pooled and filtered through the filter paper (Whatman No.4) and then partitioned with ethyl acetate (ratio 1:1). The mixture was vigorously shaken in the separating funnel for 10-15 min, the upper layer containing ethyl acetate fraction was separated and concentrated by using rotary evaporator at 40˚C and then dried under blowing nitrogen gas. The lower layer containing aqueous fraction was dried by using lyophilizer. The tamarind seed-coat residues from boiling water extraction were re-extracted with 200 ml of 70% acetone by vigorously shaken for 30-45 min, filtered and repeat extracted until the filtrate was colorless, and then all filtrates were collected and the acetone fraction was concentrated by using rotary evaporator at 50˚C and dried under blowing nitrogen gas. Percent yield of tamarind seed-coat extracts (TSCEs) of each fraction was calculated and the extracts were kept in the desiccator at room temperature until used.

**2.2 Extraction of tamarind seed-coat extracts (TSCEs) from the three tamarind cultivars**

The powder of tamarind seed-coats (3g) of the three tamarind cultivars were extracted by boiling in 200 ml of water for 1 min and allowed to stand at room temperature for 45-60 min, filtered through Whatman No.4 filter paper and then repeated extraction until the water extract became colorless. The filtrates were collected and partitioned with ethyl acetate (ratio 1:1) in the separating funnel. The mixture was shaken well and allowed to stand until the 2 layers were separated. The ethyl acetate upper layers were combined, the solvent was removed under reduced pressure by using a rotary evaporator at 40˚C, and the extracts were dried by blowing with nitrogen gas. The three TSCEs dry extracts were kept in the desiccator until used.

## 2.3 Determination of phenolic compounds

### 2.3.1 Total phenolic contents

Total phenolic contents of TSCEs from each fraction were examined using Folin-Ciocalteu colorimetric method as previously described by Spanos and Wrolstad (1990) and Suksomtip *et al*. (2010) with some modification. Briefly, the TSCEs were dissolved in distilled water to make 1 mg/ml concentration, 100 µl of 1 mg/ml TSCEs was mixed with 8.4 ml of distilled water and 500 µl of 2N Folin-Ciocalteu's phenol reagent, mixed by using vortex mixer and allowed to stand at room temperature for 3 minute. One milliliter of 20% sodium carbonate solution was added to the mixture, mixed and incubated at room temperature for 1 hour. After incubation, the absorbance of the resulting blue color was recorded at 765 nm against reagent blank by using spectrophotometer. The amount of total phenolic content was expressed as gallic acid equivalents (GAE) from the calibration curve as milligrams per gram of TSCEs dry weight.

### 2.3.2 Tannin contents

Tannin contents of TSCEs were estimated using the reaction of phosphomolybdotungstic reagent method described in the European Pharmacopoeia $4^{th}$ edition (2002).

#### 2.3.2.1 TSCEs sample

The TSCEs powder were dissolved in distilled water to make 100 mg/ml concentration of TSCEs, the TSCEs solutions were mixed with 250 ml of distilled water and filtrated through filter paper (Whatman No 4.), discarded the first 50 ml of the filtrate and then all filtrate was collected for further analysis. Total volume of 5 ml of TSCEs filtrate was diluted with distilled water to make volume to 25 ml, and then 2 ml of this solution was mixed with 1 ml of 2N Folin-Ciocalteu's phenol reagent and 10 ml of distilled water. The mixture was added with sodium carbonate solution (29% w/v) to make volume to 25 ml, votexed and allowed to stand at room temperature for 30 min, and then the absorbance of total phenols was measured at 760 nm against reagent blank. The absorbance of total phenols was expressed as $A_1$.

The amount of phenol not absorbed by the hide powder was examined by adding 0.1 g of hide powder into 10 ml of TSCEs filtrate, the mixture was shaken for 60 min and filtrated through filter paper (Whatman No.4). Five milliliter of the filtrate was diluted to 25 ml with distilled water and 2 ml of this solution was added with 1 ml of 2N Folin-Ciocalteu's phenol reagent and 10 ml of distilled water, mixed and diluted to 25 ml with sodium carbonate solution (29% w/v). The mixture was allowed to stand at room temperature for 30 min and the absorbance of phenol not absorbed by the hide powder was measured at 760 nm against reagent blank. The absorbance of total phenols was expressed as $A_2$.

**2.3.2.2 Standard**

Pyrogallol standard was used, 2 ml of 0.025 mg/ml of pyrogallol was mixed with 1 ml of 2N Folin-Ciocalteu's phenol reagent and 10 ml of distilled water and then 29% w/v sodium carbonate solution was added to make volume to 25 ml and allowed to stand at room temperature for 30 min. The absorbance of pyrogallol was measured at 760 nm against reagent blank. The absorbance of pyrogallol was expressed as $A_3$.

The percentage of tannin content expressed as pyrogallol was calculated by the following equation:

$$\% \text{ Tannin} = \frac{62.5(A_1 - A_2)\, m_2}{A_3 \times m_1}$$

$m_1$ and $m_2$ are weight of TSCEs powder and pyrogallol, in grams, respectively.

**2.3.3 Proanthocyanidin contents**

The proanthocyanidin contents of TSCEs were determined by the butylalcohol-HCl-$Fe^{3+}$ method described by Rathee, Hassarajani and Chattopahyay (2006).

The acid butanol reagent was prepared by mixing n-butanol with concentrated HCl in ratio 95:5 (v/v). The iron reagent was prepared by dissolving 0.5 g of ferric ammonium sulfate dodecahydrate ($FeNH_4(SO_4)\cdot 12H_2O$) in 25 ml of 2N HCl, this solution was kept in a bottle and protected from light. One

milliliter of 1 mg/ml TSCEs was added into a screw cap tube, mixed with 6 ml of acid butanol reagent and 0.2 ml of iron reagent, boiled in water bath for 50 min (the screw cap tubes of these mixtures were loosely closed). After boiling, the mixture was cooled down at room temperature and the absorbance was measured at 550 nm against reagent blank.

**3. Separation and identification of flavonoid compounds in TSCEs (ethyl acetate fraction) by High Performance Liquid Chromatography (HPLC)**

**3.1 Preparation of standard flavonoid compounds and TSCEs (ethyl acetate fraction) for HPLC analysis**

The high performance liquid chromatography of TSCEs was carried out following the method reported by Sudjaroen *et al*. (2005) with some modification.

(+)-Catechin, (-)-epicatechin and procyanidin B2 were used as the standard flavonoid compounds. Standard flavonoid compounds and TSCEs were dissolved in methanol as stock solutions at concentrations 100 μg/ml and 5,000 μg/ml, respectively. The standard flavonoids and TSCEs stock solutions were diluted with methanol to make a working solution at concentrations 25 μg/ml and 2,500 μg/ml, respectively. After dilution, the standard flavonoids and TSCEs working solutions were filtered through the 13 mm NYL (pore size 0.45 μm) into the vial before HPLC analysis.

The mobile phase consisted of 2% acetic acid (A) (diluted glacial acetic acid in ultrapure water) and methanol (B). Before analysis, the mobile phase was filtrated through 0.45 μm filter membrane.

**3.2 HPLC conditions**

Reversed-phase analytical HPLC was performed on a reversed phase C-18 column (Hypersil GOLD C18, 250 x 4.6 mm, 5 μm) using HPLC instrument with the system of LC-10AD VP pump, SPD-10AD VP UV-Vis detector, SIL-10AD VP injector and CTO-10AD VP column oven. The mobile phase consisted of 2% acetic acid (A) and methanol (B) by using the gradient system, the injection volume of sample was 10 μl, the flow rate of mobile phase was 1 min/ml, the run time was 50

min at 30˚C and the flavonoid compounds were detected with SPD-10AD VP UV-Vis detector set at 278 nm. The run time and the gradient system were monitor as the following:

| Run time (min) | 2% acetic acid (Solvent A) | methanol(Solvent B) |
|---|---|---|
| 0-10 | 95% | 5% |
| 10-20 | 90% | 10% |
| 20-30 | 85% | 15% |
| 30-40 | 80% | 20% |
| 40-45 | 60% | 40% |
| 45-50 | 0% | 100% |

**4. Preliminary studies the effect of TSCEs on human foreskin fibroblast cell and human gastric adenocarcinoma cell line using different standard assay.**

**4.1 TSCEs sample preparation**

**4.1.1 The tamarind seed-coat extracts (TSCEs) of sweet tamarind "Srichomphu" (TI-SP) from different solvent fractions**

The tamarind seed-coat extracts (TSCEs) of sweet tamarind "Srichomphu" (TI-SP) including boiling water extract (fraction 1), ethyl acetate extract (fraction 2) and 70% acetone extract (fraction 3) were dissolved in DMSO to make 100 mg/ml stock solution and diluted with DMSO to make 0.5% concentration in complete medium to make a final concentration at 500 μg/ml TSCEs working solution.

**4.1.2 The tamarind seed-coat extracts (TSCEs) of ethyl acetate fraction from different tamarind cultivars**

The tamarind seed-coat extracts (TSCEs) of ethyl acetate fraction from different tamarind cultivars were dissolved in DMSO to make 400

mg/ml stock solution and diluted with DMSO to make 0.5% concentration in complete medium to make a final concentration at 2 mg/ml (2000 μg/ml) TSCEs working solution.

**4.2 Cell culture preparation**

The cells were trypsinized in a solution of 0.25% trypsin at humidified atmosphere of 5% $CO_2$, 95% air at 37˚C for 5 min and centrifuged at 130 rpm. at 4˚C for 6 min and the cells were resuspended and made to final concentration of $1x10^5$ cells/ml. The cells were cultured into 96-well plate (100 μl/well) in Iscove's Modified Dulbecco's Medium for CCD-1064Sk cells or Ham F-12 nutrient medium for AGS cells supplemented 10%(v/v) fetal bovine serum and 1%(v/v) penicillin-streptomycin solution and incubated under standard culture conditions (95% humidified air and 5% $CO_2$ at 37˚C) for 24 hours. After 24 hours, the cells were treated with various concentrations of TSCEs and incubated under standard culture conditions for 24 hours.

**4.3 Cell viability assay**

**4.3.1 Inhibition of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide reduction (MTT assay)**

MTT assay was applied to determine the effect of TSCEs on cell viability of CCD-1064Sk and AGS cells. The principle of MTT assay has been described by Mossmann (1983). The colorimetric method of analysis was used for detecting the cellular oxidative metabolism by measuring the reduction of yellow 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) by mitochondrial enzyme succinate dehydrogenase in the living cells. The MTT enters the cells and then into the mitochondria, where it is reduced by the mitochondrial enzyme to form a colored (dark purple) insoluble formazan crystal. The organic solvent is used to dissolve the crystal, then the released and solubilised formazan is measured spectrophotometrically. Since the reduction of MTT can only occur in metabolically active cells, then the level of the activity measured is corresponding to the viability of the cells. The assay which was slightly modified from the method described by Fotakis and Timbell (2006) was used in this study.

After treated with various concentrations of TSCEs, the cells were washed with 100 µl phosphate buffer saline (PBS) and incubated with 0.4 mg/ml of MTT solution which was previously dissolved in serum free medium, for 4 hours at 37˚C, and then the MTT solution was removed and added 100 µl of DMSO, the plates were shaken gently for 15 min to dissolve the formazan crystals completely. An absorbance at 570 nm was recorded by using the microplate reader spectrophotometer. Each experiment was performed in triplicate. The effect of TSCEs on this cell was expressed as a relatively cell viability which was calculated by using the following formula: Percent viability = ($OD_{570}$ (TSCEs treated cells) / $OD_{570}$ (Non-treated cells)) x 100 (Kim *et al*., 2006). The $IC_{50}$ values (concentration that produces a 50% inhibitory effect on the evaluated parameter) were graphically obtained from the concentration-response curves with CurveExpert software (version 1.40). The percent viability was presented as the percentage of control values.

### 4.3.2 Neutral red uptake assay (NRU assay)

The NRU assay was carried out as described by Borenfreund and Puerner (1984). The assay was slightly modified. Briefly, the cells were treated overnight with various concentrations of TSCEs in a humidified atmosphere of 5% $CO_2$ and 95% air at 37˚C for 24 hours and washed with 150 µl of PBS buffer, incubated with 100 µl of serum free medium containing 40 µg Neutral red/ml (which was pre-incubated overnight at 37˚C and centrifuged for 10 min at 600xg (1800 rpm.) to remove the fine crystal precipitates before used). The cell cultures were incubated in a humidified atmosphere of 5% $CO_2$, 95% air at 37˚C for 3 hours. After incubation, the neutral red solution was aspirated and the attached cells were rinsed with PBS buffer, the cell was fixed with 150 µl of fixative solution (1% calcium chloride, 1% formaldehyde in ultrapure water) for 1-2 min. The fixative solution was removed and then the cells were solubilised in 150 µl of neutral red destain solution (containing 50% of 96% ethanol, 49% deionized water and 1% glacial acetic acid) to detach the neutral red within lysosomes of the viable cells by rapidly shaking for at least 10 min, or until the neutral red was completely extracted from the cells and formed a homogeneous solution. Absorbance at 570 nm was measured by using microplate reader spectrophotometer. All experiments were performed in triplicate. The effect of

TSCEs on cell viability was expressed as a relatively cell viability which was calculated by using the following formula: Percent viability = ($OD_{570}$ (TSCEs treated cells) / $OD_{570}$ (Non-treated cells)) x 100, the $IC_{50}$ values (concentration for TSCEs reflecting a 50% inhibition) were calculated (Repetto *et al*., 2008). The $IC_{50}$ values were graphically obtained from the concentration-response curves with CurveExpert software (version 1.40). The results were presented as the percentage of control values.

**4.4 Cell death assay by DNA staining with Hoechst 33342 dye**

The DNA staining with Hoechst 33342 dye is generally used to detect the nuclear morphological changes in apoptotic cells**.** This assay was used to determine an effect of TSCEs on CCD-1064Sk and AGS cells. Hoechst is a bis-benzimidazole derivative compound which binds to the minor groove of DNA which is often used in fluorescence microscopy for DNA staining. Hoechst stains appear yellow when dissolved in aqueous solutions and emit blue light under UV excitation (excited by ultraviolet light at around 350 nm, and both emit blue/cyan fluorescence light around an emission maximum at 461 nm). Hoechst 33342 contains an ethyl substitution on the terminal hydroxyl group making it more hydrophobic for easier plasma membrane passage. The Hoechst stains may be used on live or fixed cells, and are often used as a substitute for another nucleic acid stain (Chen *et al*., 2011).

After the cells were treated with various concentrations of TSCEs under standard culture conditions for 24 hours, the cells were washed with 100 µl of phosphate buffer saline (PBS), 100 µl of media containing 10 µg/ml of Hoechst 33342 was added and the cells were immediately analyzed under an inverted fluorescence microscope by using blue filter and the photographs were taken. Five microscopic fields were counted (in each cell culture) for the proportion of viable and death cells and the percentage of cell death compared with control was calculated, using the following formula: Percentage of cell death = (Amount of death cell/ Total cell) x 100.

**5. The cytoprotective effect of TSCEs (ethyl acetate fraction) from different tamarind cultivars on hydrogen peroxide induced cell injury**

**5.1 Evaluation of the effect of hydrogen peroxide ($H_2O_2$) on cells cytotoxicity using NRU assay**

Hydrogen peroxide ($H_2O_2$) is a reactive oxygen species compound generated in living cells by superoxide dismutase (SOD), the enzyme catalyze superoxide into hydrogen peroxide and oxygen. Hydrogen peroxide ($H_2O_2$), an inducer of oxidative stress in cell, has been reported (Erol-Dayi, Ardaand Erdem 2011). It can generate serious free radical as hydroxyl radical through Fenton reaction ($H_2O_2 + Fe^{2+} \rightarrow Fe^{3+} + {}^{\bullet}OH + {}^{-}OH$). Thus, the cytotoxicity of hydrogen peroxide occurred by a result from this reaction, it can be permeable into cell membrane and has long half-life within cells (Powers and Jackson, 2008).

CCD-1064Sk and AGS cells were applied to determine the cytotoxic effect of hydrogen peroxide ($H_2O_2$) in cells. The cells were seeded in 96-well plate at density $1x10^5$ cells/ml (100 µl/well). After 24 hours of incubation, various concentrations of $H_2O_2$ were added to the cells and incubated at 37˚C for different times as indicated, and the cell viability was determined by NRU assay as previously described. The percentage of the cell survival from the control value was calculated. The $IC_{50}$ values were graphically obtained from the concentration-response curves with CurveExpert software (version 1.40). The value of $IC_{50}$ was selected to use for the study in cytoprotective effect.

**5.2 Protective effect of TSCEs on cells injury**

The protective effect of TSCEs on cells injury was evaluated by the methods using $H_2O_2$ to induce cell damage as previously described (Zheng *et al*., 1996; Miller, 2001). Human foreskin fibroblasts (CCD-1064Sk) or human gastric adenocarcinoma epithelial (AGS) cells were seeded into 96-well plates at density $1x10^5$ cells/ml (100 µl) and incubated at 37˚C in a 95% air atmosphere, 5% $CO_2$ for 24 hours. After incubation, the cells were treated with TSCEs for 24 hours and then exposed to $H_2O_2$ by adding 1 mM $H_2O_2$ solution for 60 min and $H_2O_2$ solutions were removed. The cell survival was evaluated by NRU assay.

### 5.3 Effect of TSCEs on intracellular reactive oxygen species

The generation process of reactive oxygen species can be monitored by using the fluorescence method. The intracellular ROS generation of cells can be examined using the 2',7'-dichlorofluorescein-diacetate (DCFH-DA) which is a well-established compound used to detect and quantify the produce intracellular $H_2O_2$. DCFH-DA is widely used for the analysis of reactive oxygen species (ROS) in cells. Cells can be loaded with $DCFH_2$ (non-fluorescent) by incubation with a diacetylated derivative of the compound. DCFH-DA is converted to $DCFH_2$ by intracellular esterases enzyme and non-fluorescent DCFH is oxidized to DCF (fluorescent dichlorofluorescein) in present of ROS. The increasing fluorescence may be a useful indicator of the increased ROS generation. Thus, DCF fluorescence is now more appropriately considered a measurement of ROS formation rather than being specific for $H_2O_2$.

The effect of TSCEs on intracellular ROS generation in CCD-1064Sk and AGS cells was evaluated by the methods as previously described (Martin *et al*., 2008). The cells were trypsinized and seeded in 96-well plate at density $1x10^5$ cells/ml (100 µl/well) and incubated under standard condition for 24 hours, cells were treated with TSCEs at various concentrations and incubated for 24 hours. The cells were washed with 100 µl of PBS buffer solution, discarded the buffer solution and added 100 µl of 5 µM DCFH-DA solution and incubated for 30 min. Subsequently, the fluorescence intensity was monitored by excitation at 485 nm and emission 535 nm. The relative of the percentage of ROS production was calculated according to the following equation: ROS (%) = $(F_1/F_0)$ x 100% (where $F_0$ is the florescence intensity of untreated control group, $F_1$ is the florescence intensity of experimental group) (Erol-Dayi *et al*., 2011).

### 5.4 Evaluation the effect of $H_2O_2$ concentration on increasing intracellular reactive oxygen species (ROS) in cells

The experiment was carried out to select the concentration of $H_2O_2$ that generate of the highest intracellular ROS in CCD-1064Sk and AGS cells. The cells were trypsinized and seeded in 96-well plate at density $1x10^5$ cells/ml (100 µl/well) and incubated for 24 hours, cells were treated with various concentrations of $H_2O_2$ in

ranges 0.1-2 mM and incubated for 15, 30 and 60 min. The cells were washed with 100 µl of PBS buffer solution and then added 100 µl of 5 µM DCFH-DA solution and incubated for 30 min. The fluorescence intensity was monitored by excitation at 485 nm and emission 535 nm by using microplate reader. The relative of percentage of ROS production was calculated according to the following equation: ROS (%) = ($F_1/F_0$) x 100% (where $F_0$ is the florescence intensity of untreated control group, $F_1$ is the florescence intensity of experimental group) (Erol-Dayi *et al*., 2011).

### 5.5 Protective effect of TSCEs on $H_2O_2$-induced intracellular ROS in cells

Scavenging ability of TSCEs on $H_2O_2$-induced intracellular ROS in cells (CCD-1064Sk and AGS) was examined based on the DCFH-DA method. In 96-well plate, $1x10^5$ cells/ml were cultured, and incubated at 37˚C for 24 hours. The cells were treated with various concentrations of TSCEs and incubated for 24 hours. After incubation, the cells were washed with 100 µl of PBS buffer solution, added 100 µl of 5 µM DCFH-DA solution and incubated for 30 min. Subsequently, the cells were washed with 100 µl of PBS buffer and exposed to $H_2O_2$ by adding 1 mM $H_2O_2$ and 2 mM $H_2O_2$ for CCD-1064Sk and AGS cells, respectively, for 15 min to stimulate oxidative stress in cells. The cells were washed with 100 µl of PBS buffer and measured the fluorescence intensity by excitation at 485 nm and emission 535 nm by using microplate reader. The relative of percentage of ROS production was calculated according to the following equation: ROS (%) = ($F_1/F_0$) x 100% (where $F_0$ is the florescence intensity of untreated control group, $F_1$ is the florescence intensity of experimental group) (Erol-Dayi *et al*., 2011).

## 6. Effect of TSCEs from different tamarind cultivars on wound healing rate by scratch assay using CCD-1064Sk and AGS cells

### 6.1 Effect of TSCEs on the rate of wound closure

The rate of wound healing of TSCEs was determined by scratch assay (Chi-Liang *et al*., 2007; Yue *et al*., 2010). The human foreskin fibroblasts (CCD-1064Sk) or human gastric adenocarcinoma (AGS) cells were seeded in to 24-well plate in volume 1 ml/well at concentration of $1x10^5$or $2x10^5$ cells/ml, respectively, and cells were cultured in complete medium to nearly confluent cell monolayer. Then,

one to two liner wound were created in the cell monolayer by using a sterile 200 µl pipette tip, after which detached and the cells debris were removed by washing with 500 µl of phosphate buffer saline (PBS) and 1 ml of complete medium with dimethyl sulfoxide (0.5%) was added in vehicle control group. TSCEs (1 ml/well) were added in the scratched cells monolayer. Four representative images from of the scratched wound from each well were captured under phase-contrast microscopy at time zero (t = 0 h) to record the initial area of wounds immediately and normally after 6, 12, 18 and 24 hours (t = Δh) for AGS cells and 6, 12, 18, 24, 36 and 48 hours (t = Δh) for CCD-1064Sk cells of incubation at 37˚C to evaluate the area of wound closure. The area of wound was determined by Java's Image J software (1.43u) and calculated the migration of cells toward the wounds was expressed as percentage of wound closure by using the following formula (Yue *et al*., 2010):

$$\% \text{ of wound closure} = [(A_{t=0\,h} - A_{t=\Delta\,h})/A_{t=0\,h}] \times 100\%$$

where, $A_{t=0h}$ is the area of wound measured immediately after scratching, and $A_{t=\Delta h}$ is the area of wound measured at various times after scratching.

### 6.2 Effect of $H_2O_2$-induced oxidative stress on the delay of wound repair in cells

#### 6.2.1 The effect of $H_2O_2$ on the rate of wound closer of wounded monolayer

Hydrogen peroxide can reduce both migration and proliferation of cell in wound area has been reported (Gregory *et al*., 2009). $H_2O_2$ can increase oxidative stress at wound area and the migration of cells was delayed (Choi *et al*., 2008). Thus, in this study, $H_2O_2$ was used to induce oxidative stress in wound to evaluate the scavenging effect of TSCEs on ROS in wound area.

Effect of $H_2O_2$ concentrations on the delay of wound repair was evaluated. The cells were trypsinized and seeded in 24-well plate at concentration of 1x $10^5$ cells/ml for CCD-1064Sk or 2x$10^5$ cells/ml for AGS and the cells were cultured in complete medium to nearly confluent cell monolayer. The cell monolayer was scratched by using a sterile 200 µl pipette tip, washed out the detached and debris cells with 500 µl of phosphate buffer saline (PBS) and then the scratch cells were

treated with 1 ml of various concentrations of $H_2O_2$ for 15, 30 and 60 min and incubated at standard condition. After incubation, the cells were washed with 500 µl of phosphate buffer saline (PBS) and added 1 ml of complete medium. The image at time zero were captured to record the initial area of the wounds at zero time, and the recovery of the wounded monolayer due to cell migration toward the denuded area was evaluated at 24 hours by using an inverted phase-contrast microscope. The image was analyzed and calculated the percent of wound closure compared with control (Choi *et al*., 2008, Gregory *et al*., 2009).

**6.2.2 Effect of TSCEs on increasing the rate of wound repair on $H_2O_2$–induced oxidative stress cells**

Hydrogen peroxide can delay cell migration in wounded healing. The effect of TSCEs on wound healing was determined in $H_2O_2$induced the reduction of cell migration. The cells were seeded into 24-wellplate at concentration of 1x $10^5$ cells/ml for CCD-1064Sk or 2x$10^5$ cells/ml for AGS and the cells were cultured to nearly confluent cell monolayer. One to two liners were generated in the monolayer by using a sterile 200 µl pipette tip. Cellular debris was removed by washing with 500 µl of phosphate buffer saline (PBS) and then the scratch cells were treated with 1 ml of 2 mM $H_2O_2$ for 15 min in CCD-1064Sk cells and 30 min in AGS cells and incubated at standard condition. After incubation, the cells were washed with 500 µl of phosphate buffer saline (PBS). One milliliter of complete medium with dimethyl sulfoxide (0.5%) was added in vehicle control plates and 1 ml of various concentrations of TSCEs was added in experimental plates. The images of scratched areas at time zero were photographed to record the initial area of the wounds and the recovery of the wounded monolayer due to cell migration toward the denuded area was evaluated at 24 hours by using an inverted phase-contrast microscope. The areas were analyzed by Java's Image J software (1.43u) and calculated the percent of wound closure as previously described (Choi *et al*., 2008, Gregory *et al*., 2009).

**7. Statistical analysis**

Each experiment was performed at least three times. Results were expressed as mean ± SEM. Data were analyzed by one-way analysis of variance (ANOVA) with post hoc test was conducted. The $P$ value < 0.05 was regarded as significant.

# CHAPTER IV

# RESULTS AND DISCUSSIONS

## 1. Preparation and determination of tamarind seed-coats extracts from different solvent extraction

Tamarind seed-coat from sweet tamarind "Srichomphu" (TI-SP) was evaluated its phenolic contents in various solvent fractions. The percent yield of the dry extracts from boiling water, ethyl acetate and 70% acetone fraction was 25.67 ± 2.58, 1.18 ± 0.22 and 15.76 ± 1.58%, respectively. The highest yield was obtained in boiling water fraction. The percent yield of the extract in 70% acetone and ethyl acetate fraction was less than the water fraction. Phenolic contents in each fraction from the extracts of TI-SP are shown in **Table 1**. The ethyl acetate fraction exhibited the highest amount of total phenolic contents (502.533 mg GAE/g dry extract) with the lowest amount of proanthocyanidin, while tannin and proanthocyanidin were the highest in 70% acetone fraction at 32.78 ± 0.24 and 2.78 ± 0.01, respectivly. The lowest tannin and total phenolic contents were found in the boiling water fraction.

Total yields, phenolic and tannin contents of various tamarind seed-coat extracts have been reported (Siddhuraju, 2007). The different solvent extractions show the different the phenolic content. Moreover, the percent yield and tannin contents in 70% acetone fraction are 13.10% and 23.85%, respectively, these values are comparable with this study.

Lourith *et al*. (2009) studies the total phenolic contents of Thai tamarind seed-coat extract using 70% ethanol and then partitioned with chloroform and ethyl acetate. They found percent yield of the extract from ethyl acetate fraction approximately 1.03%, which was comparable with the present study. However, the phenolic content in the ethyl acetate fraction (636.91 mg GAE/g dry extract) was found higher than the present study.

**Table 1.** The phenolic compounds of TSCEs from different fractions of *Tamarindus indica* "Srichomphu" (TI-SP). Data are mean ± standard error (n=3), GAE = gallic acid equivalent.

| TSCEs Fraction | Phenolic content | | |
|---|---|---|---|
| | Total phenol (mg GAE/g dry extract) | Tannin (%) (as pyrogallol equivalents) | Proanthocyanidin ($A_{550}$) |
| Boiling water | $267.82 \pm 12.79^{a}$ | $14.57 \pm 0.42^{a}$ | $1.76 \pm 0.04^{b}$ |
| Ethyl acetate | $502.53 \pm 8.99^{c}$ | $28.25 \pm 0.87^{b}$ | $0.93 \pm 0.05^{a}$ |
| 70% Acetone | $437.44 \pm 4.65^{b}$ | $32.78 \pm 0.24^{c}$ | $2.78 \pm 0.01^{c}$ |

a,b,c = significantly different between group ($P < 0.05$)

**2. The chemical analysis and fingerprint of TSCEs from ethyl acetate fraction of different tamarind cultivars**

The TSCEs from the sweet tamarind "Srichomphu" (TI-SP) and the sour tamarind "Priao-native" (TI-P) and "Priao-Kradan" (TI-PK) were extracted by boiling in hot water and partitioned with ethyl acetate as described. TSCEs from ethyl acetate fraction of the three tamarind cultivars were used in this analysis.

**2.1 Chemical analysis of phenolic compounds of the TSCEs**

The percent yield and total phenolic compound of the three TSCEs from the sweet tamarind "Srichomphu" (TI-SP), "Priao-native" (TI-P) and "Priao-Kradan" (TI-PK) of the sour tamarind are shown in **Table 2**. The percent yields of TSCEs from the three cultivars were not significant difference. The total phenolic contents were expressed in mg GAE/g dry extract, the results in **Table 2** showed that the total phenolic contents of TSCEs of the three tamarind cultivars were not significantly different which was found in ranges 374.37 - 400 mg GAE/g dry extract. The percent tannin was expressed as pyrogallol, the TSCEs from sour tamarind (TI-P and TI-PK) was significantly higher the percent tannin than the sweet tamarind (TI-SP) at $P < 0.05$. The highest tannin content was found at 35.96 ± 1.43% in TSCE from TI-PK which was not significantly higher than TI-P. The proanthocyanidin contents in the TSCEs from the three different tamarind cultivars were significantly different at $P < 0.05$. Proanthocyanidin in the TSCE from TI-PK was found higher than that of TI-P and TI-SP, the proanthocyanidin contents were 1.99 ± 0.02, 1.76 ± 0.01 and 1.02 ± 0.04 for TSCEs from TI-PK, TI-P and TI-SP, respectively.

Tsuda *et al*. (1994) studied the antioxidant activity of ethanol and ethyl acetate extracts from tamarind seed-coats and germs by using the thiocyanate and thiobarbituric acid method. The result suggested that the antioxidative compounds found only in tamarind seed-coats. The tamarind seed-coat extract from ethyl acetate extraction exhibited the strongest antioxidative activity. Their study indicated that

**Table 2** The percent yield and phenolic compounds of TSCEs from ethyl acetate fraction of different tamarind cultivars. Data are mean ± standard error (n=3), GAE =gallic acid equivalent.

| Cultivar | % Yield of extract | Phenolic content | | |
|---|---|---|---|---|
| | | Total phenol (mg GAE/g dry extract) | Tannin (%) (as pyrogallol equivalents) | Proanthocyanidin ($A_{550}$) |
| Sweet-tamarind | | | | |
| TI-SP | 1.31±0.12 | 374.37±6.97 | 29.36±0.32[a] | 1.02±0.04[a] |
| Sour-tamarind | | | | |
| TI-P | 0.93±0.09 | 394.28±6.81 | 33.04±0.93[a,b] | 1.76±0.01[b] |
| TI-PK | 1.21±0.17 | 400.00±8.18 | 35.96±1.43[b] | 1.99±0.02[c] |

a,b,c = significantly different between group ($P < 0.05$)

tamarind seed-coat extract from ethyl acetate fraction showed high antioxidant activity. Moreover, the tamarind seed-coat extract from ethyl acetate extraction has been reported the total amount was 740 mg/100 g of seed-coat (0.74 % w/w) (Tsuda *et al*., 1995). The extract from ethyl acetate fraction from this present study showed higher the percent yield of extract than Tsuda *et al*. (1995).

Furthermore, Suksomtip *et al*. (2010) studied the content of phenolic compounds and antioxidative activity of methanolic seed-coat extracts from five tamarind cultivars in Thailand, including "Srichomphu", "Sithong-nak", "Sithong-bao", "Priao-yak" and "Khanti". The contents of phenolic compounds were found in "Khanti" > "Sithong-bao" > "Priao-yak" > "Sithong-nak" > "Srichomphu" (36.30 ± 0.64, 35.94 ± 0.98, 29.63 ± 0.98, 24.07 ± 0.37 and 20.74 ± 0.74%, respectively). The total tannin content in "Priao-yak" > "Sithong-bao" > "Khanti" > "Sithong-nak" > "Srichomphu" (27.16 ± 4.94, 21.83 ± 1.05, 18.87 ± 1.05, 16.87 ± 0.17 and 10.08 ± 0.51, respectively). The proanthocyanidin content was found the highest in "Sithong-bao" and "Khanti" (0.18 ± 0.01), followed by Sithong-nak and Priao-yak (0.13 ± 0.01) and the lowest was found in Srichomphu (0.08 ± 0.01). The similar result to this study was found, the result indicated that the tannin and proanthocyanidin contents of sour tamarind seed-coat extracts were higher than the extracts from sweet tamarind seed-coat.

**2.2 HPLC analysis of Phenolic compounds in the TSCEs**

In this study, high performance liquid chromatography was applied by using Hypersil GOLD C18 column (250 x 4.6 mm, 5µm) and 2% acetic acid and methanol were used as the mobile phase to separate flavonoid compounds following the method described by Sudjaroen *et al*. (2005). The standard flavoniods, (+)-catechin, (-)-epicatechin and procyanidin B2 were used. The standard flavonoid mixture and TSCEs from different cultivars were applied in total volume of 10 µl at final concentration at 25 µg/ml and 2500 µg/ml, respectively, and UV detector at 278 nm was monitored. The chromatograms of TSCEs from different cultivars presented the peaks of the three flavonoid compounds identical with the standard flavonoids compounds including (+)-catechin, procyanidin B2 and (-)-epicatechin with the retention time of 18.59, 25.20 and 31.14, respectively. The HPLC chromatograms of

standard flavonoids and TSCEs from the three different tamarind cultivars are shown in **Figure 5**. The HPLC analysis has performed for separation and identification of phenolic compounds. The similar chemical fingerprint of the three TSCEs was observed. However, the (+)-catechin peak of the sweet tamarind (TI-SP) was much higher than the sour tamarind (TI-P and TI-PK).

The different classes of phenolic compounds are separated by using the reversed-phase column. The conditions of HPLC analysis for phenolic compound are most recommended at ambient column temperature, but moderately higher temperature between 30-40˚C is also used. The mobile phases are used both isocratic and gradient elution. The organic solvents used in HPLC system are acetonitrile and methanol. The pH range 2-4 is recommended for HPLC system, controlled by using small amount of acid (acetic, formic, phosphoric and perchloric acid). The high absorption of phenolic compounds is measure by the UV detector at maximum absorption ranges 240–285 nm, due to the A-ring structure in phenolic compounds (Stalikas, 2007).

According to the studies of Razali *et al*. (2012), the reversed-phase HPLC is used to identify the flavonoid compounds of the extracts from tamarind. The tamarind leaves extracted with methanol, ethyl acetate and hexane are analyzed the flavoniod compounds by using gradient reverse phase HPLC analysis performed on the acid-hydrolysed samples. The compounds are separated on NovaPak C18 reversed-phase column, % trifluoroacetic acid and acetonitrile are used in the mobile phase, eluted at flow rate of 0.5 ml/min under controlled temperature (40˚C) at wavelength 260 nm. Epicatechin is found in all three extracts while catechin is presented in methanolic leaves extract. Quercetin and isorhamnetin are detected in ethyl acetate and hexane leaves extract.

The HPLC chromatogram of tamarind seed-coat extract from ethyl acetate was demonstrated by Tsuda *et al*. (1994), the sample was separated on Develosil ODS-10 column with UV detector at 280 nm. The mobile phase was 0.1% trifluoroacetic acid and 17% acetonitrile at flow rate of 5 ml/min. The 2-hydroxy-3',4'-dihydroxyacetophenone, methyl 3,4-dihydroxybenzoate, 3,4-dihydroxyphenyl acetate and (-)-epicatechin were separated on this system. Moreover, only epicatechin

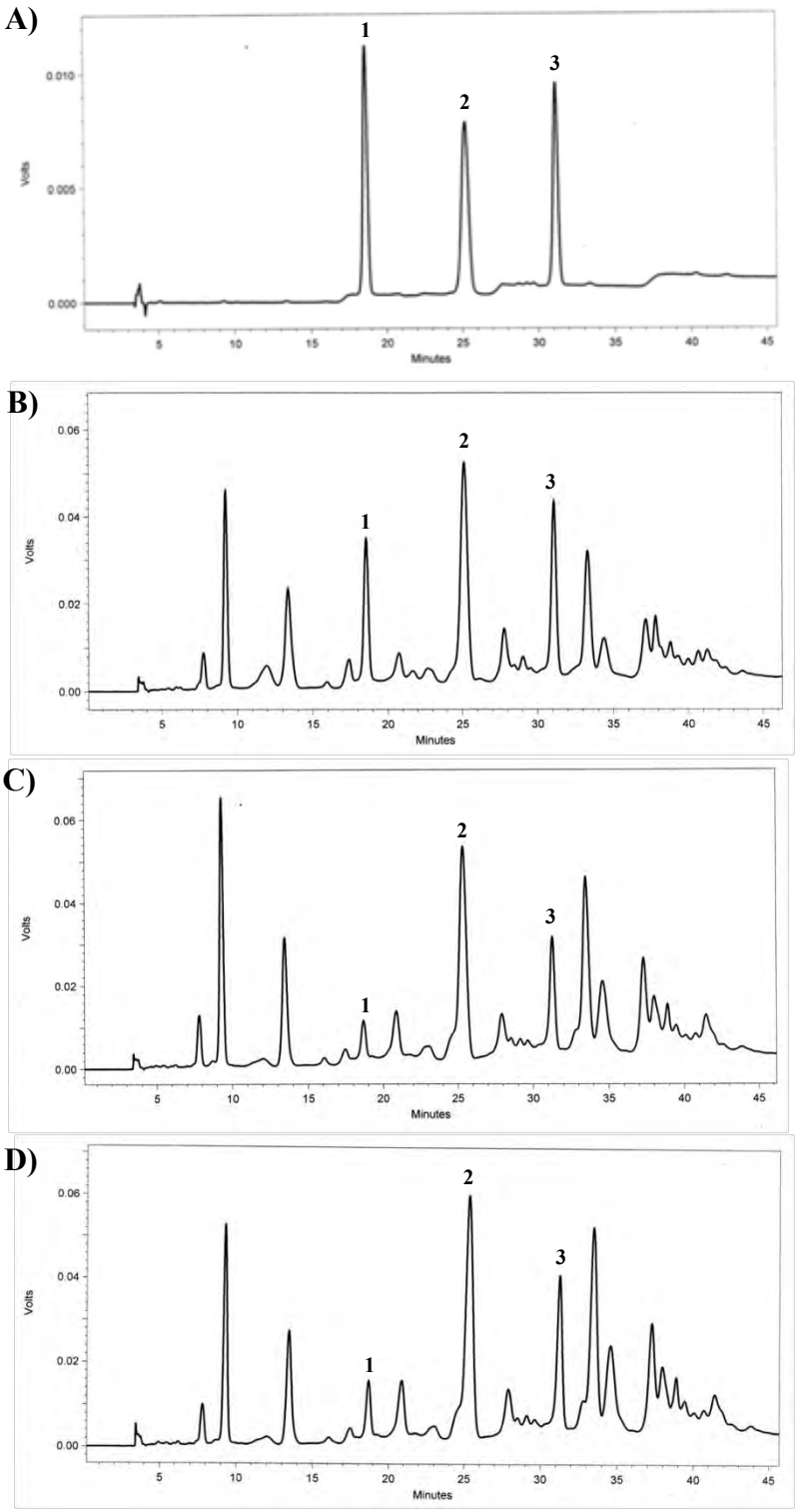


**Figure 5** HPLC chromatogram of standard flavonoids (A) and TSCEs, (B) = sweet tamarind (TI-SP), (C) = TSCE from sour tamarind (TI-P) and (D) = TSCE from sour tamarind (TI-PK), the three peaks are the standard flavonoids including (+)-catechin (1), procyanidin B2 (2) and (-)-epicatechin (3), respectively.

approximately 25 mg/100 g of seed-coat was found in tamarind seed coat from ethyl acetate fraction. Sample is also separated by using HPLC analysis in the same mobile phase at a flow rate of 1 ml/min, column HiQsil ODS-5 micron, UV-visible detector at wavelength 280 nm (Luengthanaphol *et al*., 2004).

Furthermore, Sudjaroen *et al*. (2005) found that methanolic extract of tamarind pericarp composed of twelve major compounds of phenolic compounds by using reversed-phase HPLC. The gradient system of HPLC analysis consisted of 2% acetic acid and methanol as the mobile phase, running time for 50 min. The (+)-catechin, procyanidin B2, (-)-epicatechin, procyanidin trimer, procyanidin tetramer, procyanidin pentamer, procyanidin hexamer, taxifolin are detected at 278 nm of UV-diode array and apigenin, eriodictyol, luteolin and naringenin are detected at 340 nm of UV-diode array. This report shows the similar results as the present study, the three peaks of TSCEs from three tamarind cultivars were found identical with the standards (+)-catechin, procyanidin and (-)-epicatechin.

**3. Effect of TSCEs from different fractions of TI-SP on human cell lines**

**3.1 Effect of TSCEs on human foreskin fibroblast cells**

The MTT assay and DNA staining with Hoechst 33342 dye were applied to examine the effect of TSCEs from the three fractions of boiling water, ethyl acetate and 70% acetone fraction on viability of human foreskin fibroblast cells (CCD-1064Sk). The MTT assay in **Figure 6** showed that the three TSCEs fractions increased the percent cell survival with the increasing TSCEs concentrations. It was observed that the fractions of the boiling water and 70% acetone were precipitated in the tested medium, whereas this precipitation was not observed with the fraction from ethyl acetate. In **Figure 7**, the cell morphology of CCD-1064Sk cells was examined under the inverted microscope after treated cells with TSCEs for 24 hours. The results showed that the morphology of the cells was changed after treated with the fractions of TSCEs from boiling water and 70% acetone. It might be noted that the increasing percent cell survival of TSCEs from the boiling water and 70% acetone fractions by MTT assay was the false positive results occurred by the interfering precipitation of TSCEs in the test medium.

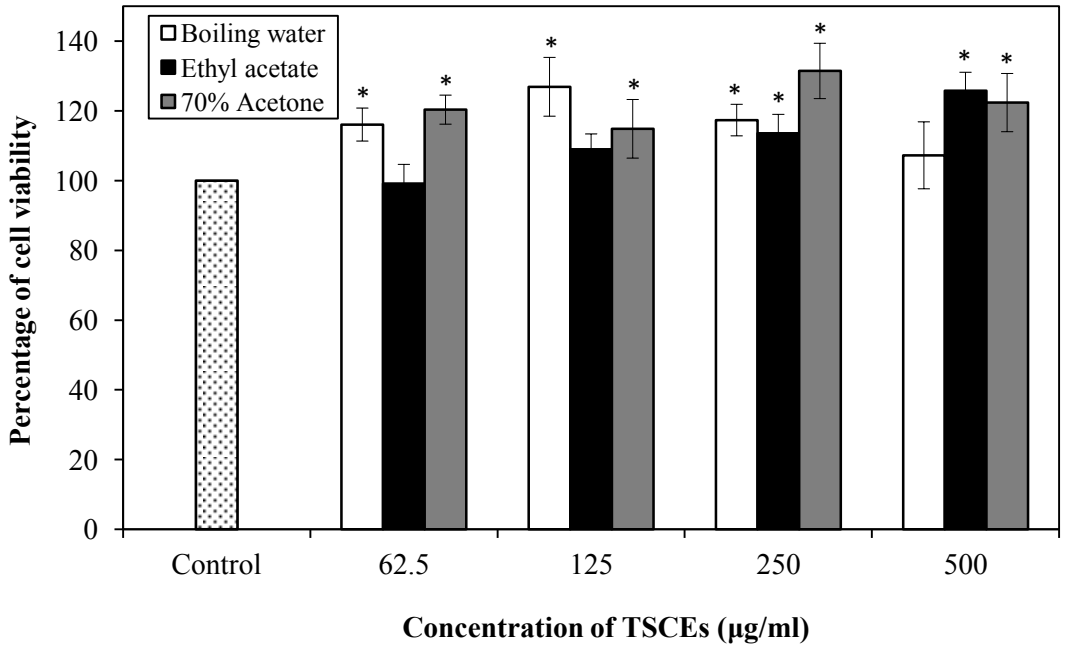


**Figure 6** Effects of TSCEs from the three different solvent fractions on cell survival in human foreskin fibroblast cells (CCD-1064Sk). The cells were treated with various concentrations of TSCEs for 24 hours as evaluated by MTT assay. Data are mean ± standard error,*$P< 0.05$ compared to control.

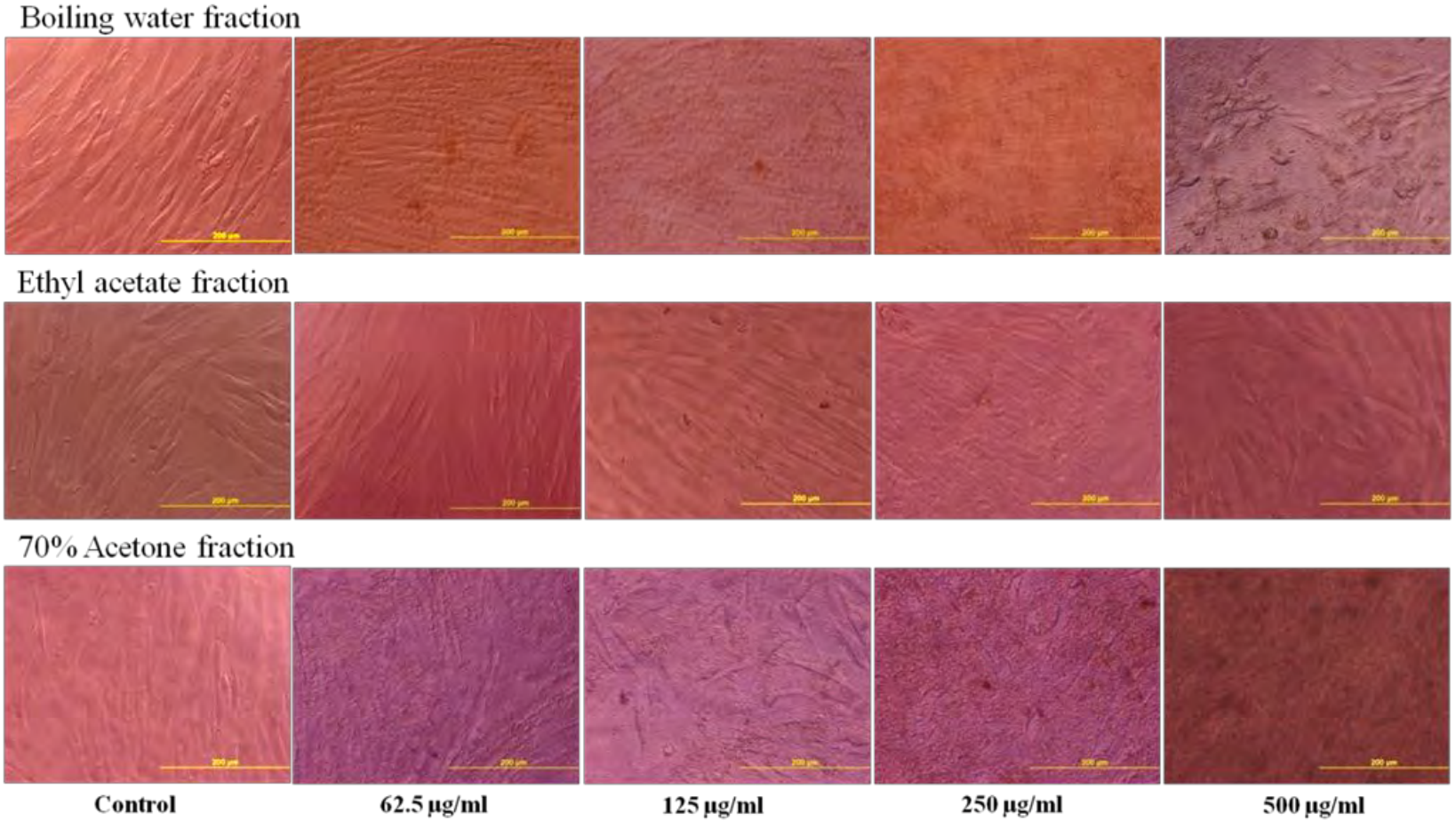


**Figure 7** Morphology of human foreskin fibroblasts (CCD-1064Sk) under inverted microscope after treated with TSCEs from the three different solvent fractions at concentrations ranges 62.5-500 µg/ml for 24 hours (original magnification × 20).

DNA staining assay with Hoechst 33342 dye indicated the nuclear morphological changes of the CCD-1064Sk cells. After the cells were treated with TSCEs for 24 hours, TSCE from 70% acetone fraction exhibited the DNA fragmentation of cells apoptosis (arrow) higher than TSCEs from the boiling water fraction, whereas TSCE from ethyl acetate fraction did not cause DNA fragmentation of cells apoptosis compared with the vehicle control cells (0.5% DMSO, untreated with TSCEs) as shown in **Figure 8**. The results in **Figure 9** showed that at concentrations 125-500 μg/ml of TSCE from 70% acetone fraction gave the highest percent cell death compared with the TSCEs from boiling water and ethyl acetate fractions. TSCEs from 70% acetone fraction at concentrations 125-500 μg/ml and boiling water fraction at concentration 500 μg/ml probably induced DNA damage, the percent cell death was significantly increased, compared with their vehicle control. The TSCE at concentrations 62.5-500 μg/ml from the ethyl acetate fraction showed no significant difference in percent cell death compared with the vehicle control cells.

### 3.2 Effect of TSCEs on human gastric adenocarcinoma epithelial cells

The results of MTT assay and DNA staining with Hoechst 33342 dye of TSCEs on human gastric adenocarcinoma epithelial cells (AGS) indicated that TSCE from 70% acetone fraction possessed the highest cytotoxic effect on the AGS cells. The MTT assay in **Figure 10** indicated that the cytotoxic effect of TSCEs from the three solvent fractions increased (the percentage of survival cell decreased) with the increasing TSCEs concentrations. The concentration that produces a 50% inhibitory effect ($IC_{50}$) of TSCEs from boiling water and 70% acetone were 203.5 ± 2.89 and 106.50 ± 14.38 μg/ml, respectively, while the ethyl acetate fraction showed the $IC_{50} > 500$ μg/ml. The $IC_{50}$ values of TSCEs from boiling water and 70% acetone fractions were significant difference from ethyl acetate fraction. These result showed that TSCE from 70% acetone fraction possessed higher cytotoxic effect than boiling water and ethyl acetate fraction.

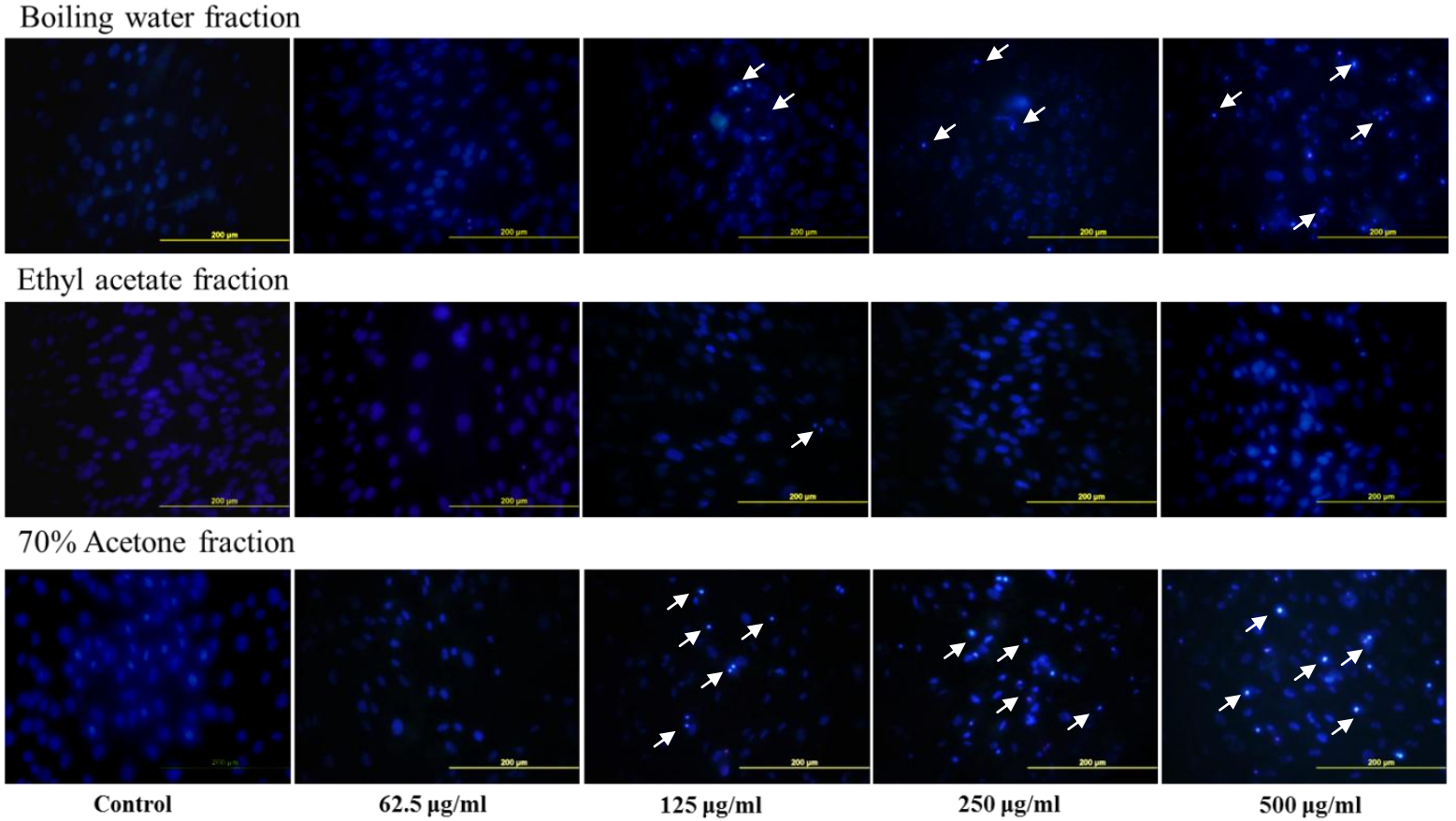


**Figure 8** The DNA damage using DNA staining with Hoechst 33342 dye of the cells human foreskin fibroblasts (CCD-1064Sk), under inverted microscope. The cells were treated for 24 hours with TSCEs from the three different solvent fractions at concentrations ranges 62.5-500 µg/ml (original magnification × 20).

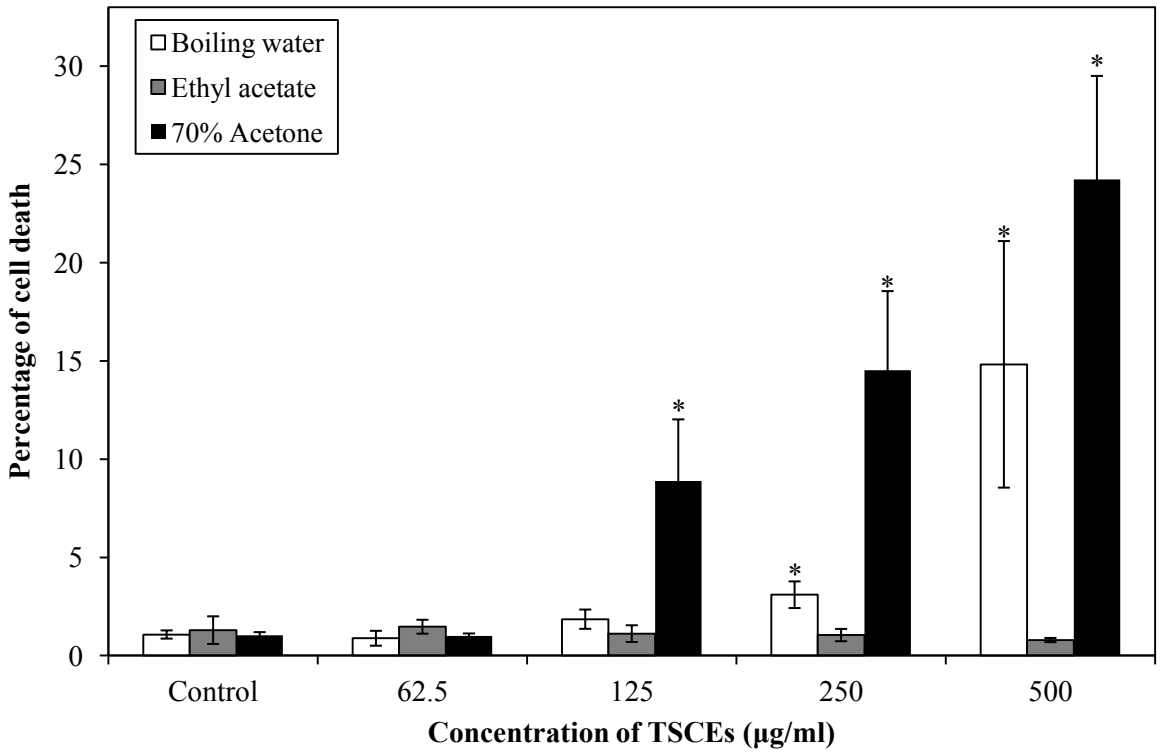


**Figure 9** Effect of TSCEs on percent cell death in the human foreskin fibroblasts (CCD-1064Sk) as evaluated by DNA staining with Hoechst 33342 dye. The cells were treated for 24 hours with 62.5-500 µg/ml TSCEs from the three solvent fractions and the control was without TSCEs. Data are mean ± standard error,*$P< 0.05$ compared to their control.

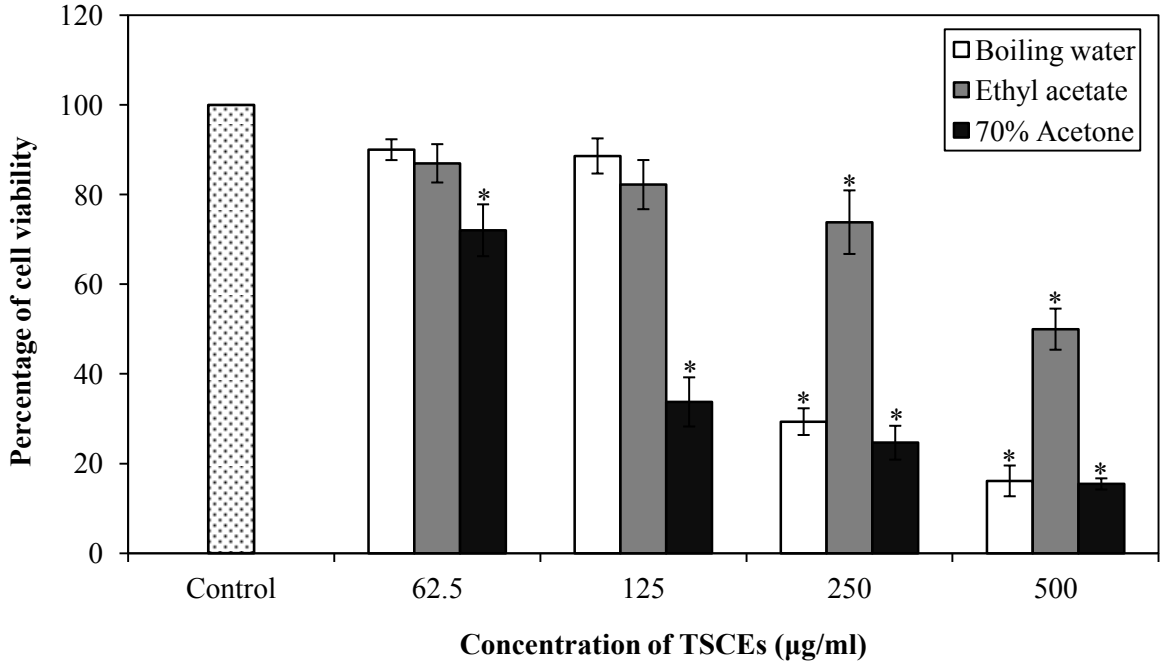


**Figure 10** Effects of TSCEs from the three different solvent fractions on cell survival in human gastric adenocarcinoma epithelial cells (AGS). The cells were treated with various concentrations of TSCEs for 24 hours as evaluated by MTT assay. Data are mean ± standard error, $*P< 0.05$ compared to control.

The DNA staining with Hoechst 33342 dye indicated the cytotoxic effect of TSCEs from the three solvent fractions on DNA damage. The morphology of the cells was changed as shown in **Figure 11**, after treated with TSCEs from the three solvent fractions. The results of DNA damage (arrow) in **Figure 12** by the DNA staining assay with Hoechst 33342 dye was related with the results of cytotoxic effect by MTT assay. The results in **Figure 13** indicated the TSCE from 70% acetone fraction showed higher toxic effect than the boiling water and ethyl acetate fractions, since the TSCE from 70% acetone fraction showed the highest percent death cells (96.17 ± 0.74) at 500 µg/ml concentration. The percent death cells treated with TSCE from 70% acetone fraction and boiling water fraction in ranges 62.5-500 µg/ml and 250-500 µg/ml concentrations showed cytotoxic effect at 27.57-96.19% and 39.41-93.30%, respectively while TSCE from ethyl acetate fraction did not show cytotoxic effect on AGS cells compared with vehicle control cells.

It may be noted that the percent cell death may be related with the proanthocyanidin content in TSCEs. Extracts from 70% acetone and boiling water fractions exhibited higher cytotoxic effect than the extract from ethyl acetate fraction in both human cell lines.

The high contents of tannin and proanthocyanidin in the higher polarity of solvents (70% acetone and boiling water) may lead to the precipitation reaction with the tested medium. Mechanism of protein precipitation of tannin probably occurred by forming cross-link between protein molecules (Hangerman *et al*., 1998).

Proanthocyanidin, a form of condensed tannin, has been reported that it precipitates and has strong affinity with proline-rich proteins and polymers (Hagerman and Butler, 1981). The proanthocyanidin oligomers demonstrated interaction with protein by both hydrophobic and hydrophilic interactions (Artz *et al*., 1987). The polyphenol tannins are an effective precipitating agent than procyanidin dimers and momomer (Baxter *et al*., 1997; Sarni-Manchado *et al*., 1999; Freitas and Mateus, 2001). Tannins precipitate protein and interact with dietary proteins and digestive enzymes in the gut, resulting in the antinutritive and toxic effect of the compounds (Baxter *et al*., 1997).

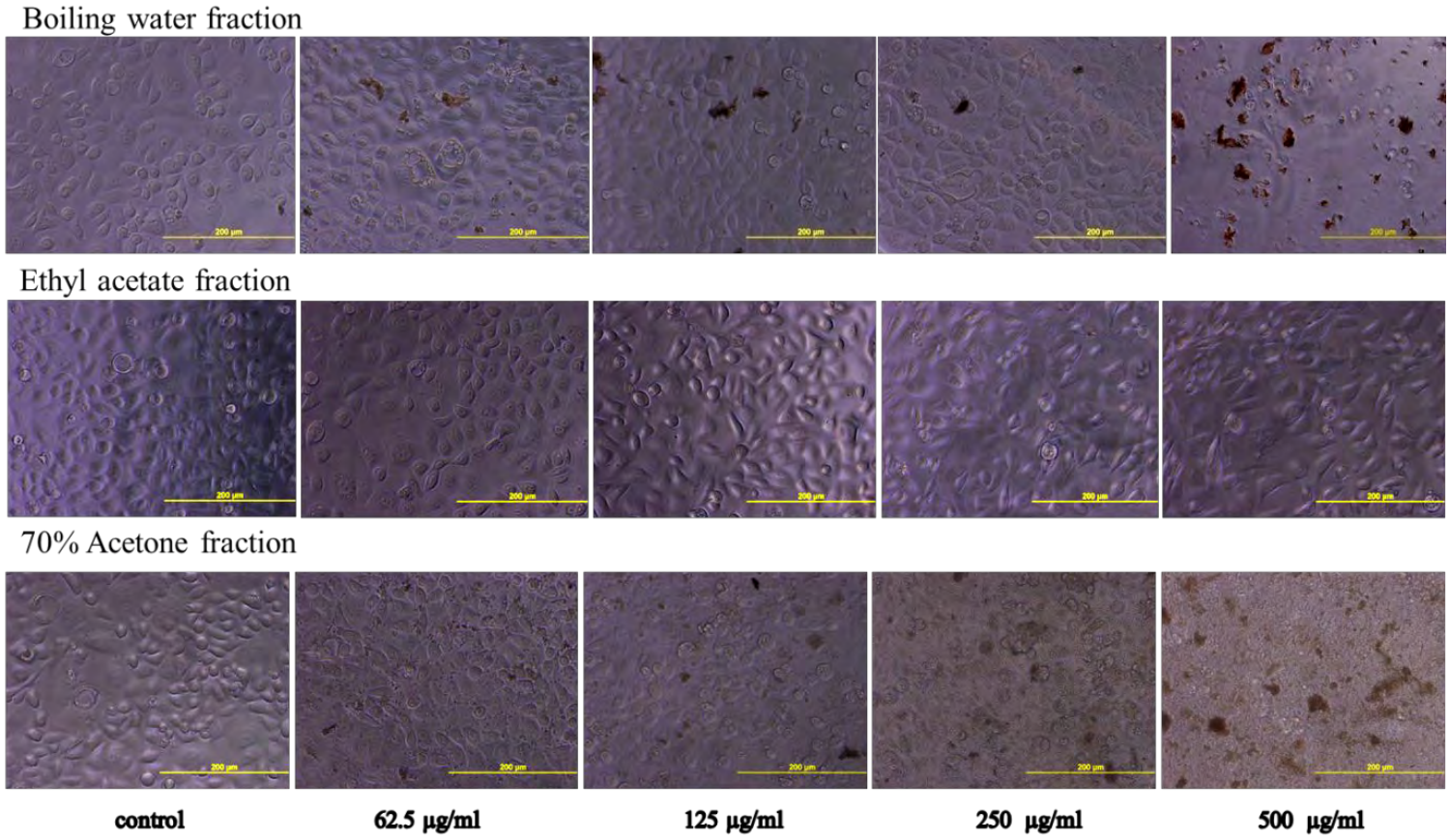


**Figure 11** Morphology of human gastric adenocarcinoma epithelial cells (AGS) under inverted microscope after treated cells for 24 hours with TSCEs from the three different solvent fractions at concentration in ranges 62.5-500 µg/ml (original magnification × 20).

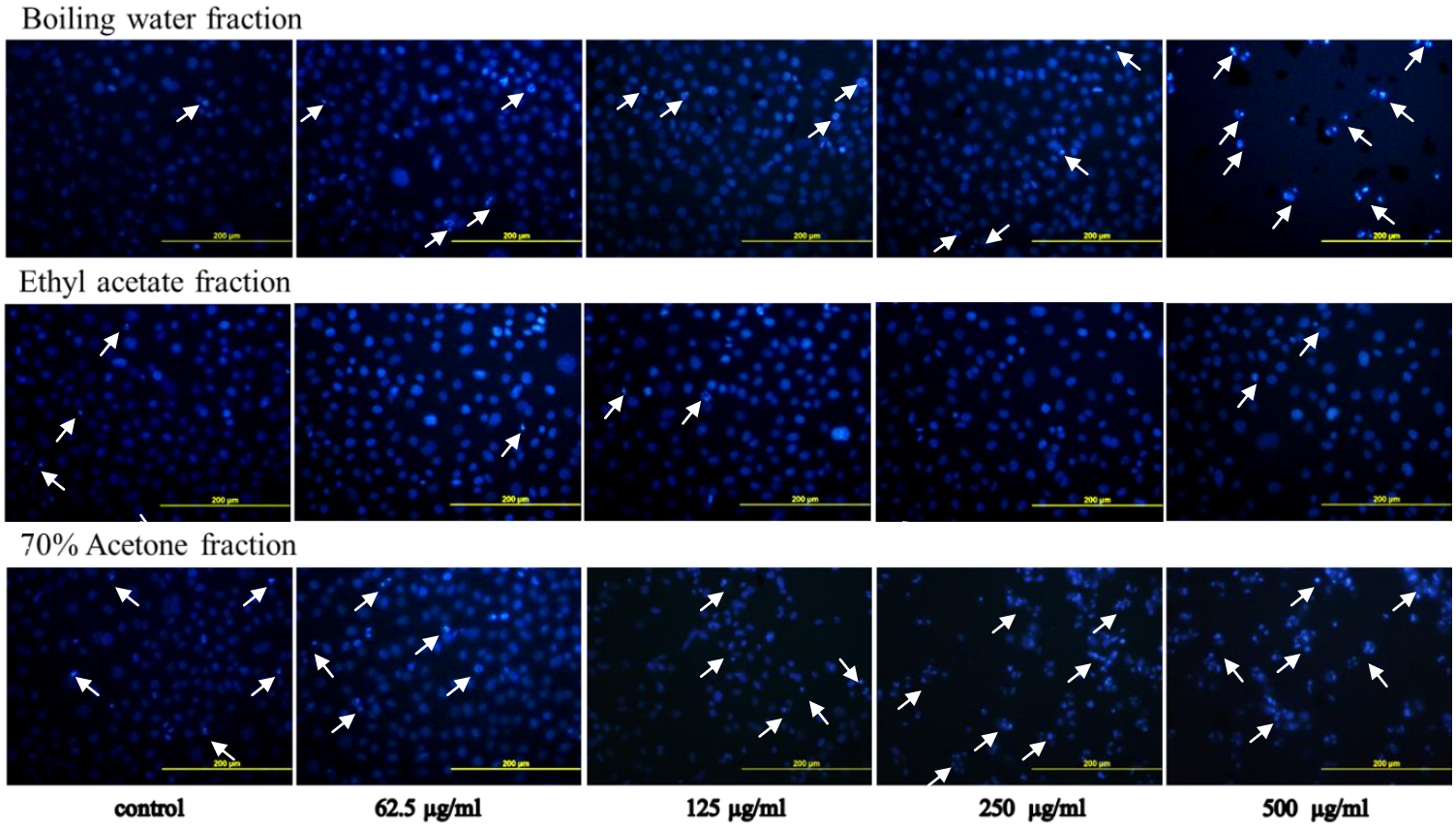


**Figure 12** The DNA damage of human gastric adenocarcinoma epithelial cells (AGS) under inverted microscope using DNA staining with Hoechst 33342 dye. The cells were treated for 24 hours with TSCEs from the three different solvent fractions at concentrations ranges 62.5-500 µg/ml (original magnification × 20).

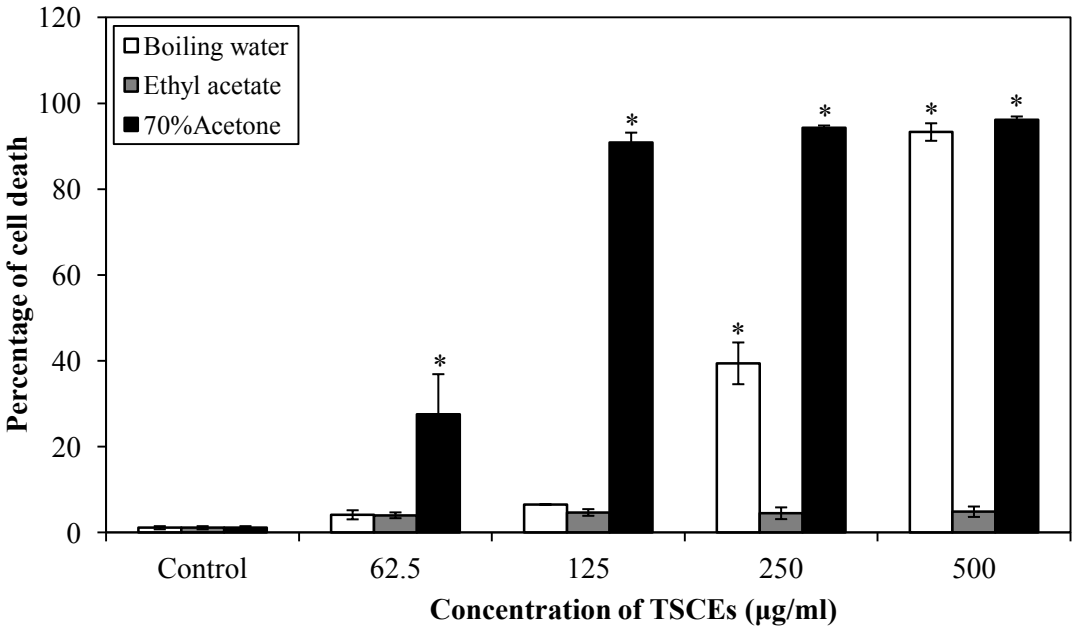


**Figure 13** Effect of TSCEs on the percent cell death in human gastric adenocarcinoma epithelial cells (AGS) as evaluated by DNA staining with Hoechst 33342 dye. The cells were treated for 24 hours with 62.5-500 µg/ml TSCEs from the three solvent fractions and control was without TSCEs. Data are mean ± standard error, *$P< 0.05$ compared to their control.

Labieniec and Gabryelak (2003) studied the cytotoxic and genotoxic effects of tannins such as tannic, ellagic and gallic acids in the concentration ranges 15-240 μM for 1 hour on Chinese hamster fibroblast cells (cell line B14). Tannin decreases the viability of cells and induces DNA stand breaks at concentration 60 μM by using MTT and comet assay. Moreover, the tannins induced the fluidity changes in plasma membrane by decreasing in lipid packing density in lipid core, but no changes in the surface polar head group region of plasma membrane.

Condensed and hydrolysable oligomeric tannins from Witch hazel (*Hammamelis* V*irginiana*) bark, soluble in both ethyl acetate and water were estimated the cytotoxicity on human keratinocyte cell line (HaCaT) and mouse fibroblast cell line 3T3. These compounds were exhibited mildly cytotoxic effect on 3T3 fibroblasts and HaCaT keratinocytes. The $IC_{50}$ of these compounds to 3T3 fibroblasts and HaCaT keratinocytes were 51 ± 3 and 41 ± 2 μg/ml, respectively. In addition, the antioxidant activity of these extract was observed at lower than cytotoxic concentrations (Touriño *et al*., 2008).

The results from these experiment indicated that the TSCE from ethyl acetate fraction, which was partitioned from boiling water exhibited the lowest cytotoxic effect on CCD-1064Sk and AGS cells in comparison with the fraction of 70% acetone and boiling water. The TSCEs from ethyl acetate fraction of different tamarind cultivars were used for further study in CCD-1064Sk and AGS cells according to the lowest cytotoxic effect of ethyl acetate fraction.

**4. The effects of TSCEs from ethyl acetate fraction of different tamarind cultivars on human foreskin fibroblast cell and human gastric adenocarcinoma epithelial cell lines**

The TSCEs from the sweet tamarind “Srichomphu” (TI-SP) and sour tamarinds “Priao-native” (TI-P) and “Priao-Kradan” (TI-PK) were extracted by boiling in hot water and partitioned with ethyl acetate were used in this study.

**4.1 Effect of tamarind seed-coat extracts (TSCEs) on human foreskin fibroblast cells**

The toxic effect of TSCEs was evaluated on human foreskin fibroblast cells (CCD-1064Sk) by using MTT and NRU assays. Both of these assays presented

the percent cell viability of the TSCEs treated cells for 24 hours and the concentration of the fifty percent inhibition ($IC_{50}$) was calculated. The toxic effect of TSCEs from the three different tamarind cultivars on CCD-1064Sk cells by using MTT assay are shown in **Figure 14**. The results of 10 fold dilution series at concentrations in ranges 0.02-2000 µg/ml are shown in **Figure 14A**. The results indicated that TSCEs showed no cytotoxic effects on CCD-1064Sk cells at concentrations in ranges 0.02-200 µg/ml. In addition, TSCEs at concentration ranges 20-200 µg/ml increased the cells viability and at concentration 2000 µg/ml decreased the cells viability compared with vehicle control cells. According to these results the concentrations ranges 62.5-2000 µg/ml of TSCEs were used for the study to screen cytotoxicity of TSCEs on cells. Cytotoxic effect using two fold dilution series at concentration ranges 62.5-2000 µg/ml TSCEs is shown in **Figure 14B**. The result showed that TSCEs increased the percent cell viability with the increasing concentrations of TSCEs at ranges 62.5-1000 µg/ml, except for the TSCE from TI-PK at 1000 µg/ml concentration, and the cell viability decreased at TSCEs concentration 2000 µg/ml, the $IC_{50}$ of TSCEs on CCD-1064Sk cells by this assay was not calculated. This result indicated that TSCEs of the sour tamarinds (TI-P and TI-PK) possessed higher toxic effect on CCD-1064Sk cells than the sweet tamarind (TI-SP).

The result of NRU assay indicated that TSCEs from the three cultivars showed no cytotoxic effect on CCD-1064Sk cells treated with TSCEs at concentration ranges 0.02-200 µg/ml (10 fold dilutions series), but at concentration 2000 µg/ml of TSCEs exhibited cytotoxic effect on CCD-1064Sk cells (**Figure 15A**). From these results, concentration ranges 62.5-2000 µg/ml of TSCEs were used for the screening cytotoxic effect on cells as shown in **Figure 15B**. The result showed the percent cell viability was decreased with increasing concentrations of TSCEs at ranges 1000-2000 µg/ml except for the TSCE from TI-PK showed cytotoxic effect of concentration 500 µg/ml. From this result, the $IC_{50}$ value of TSCEs from the three cultivars was calculated. The TSCE from TI-PK exhibited higher cytotoxic effect than the TSCEs

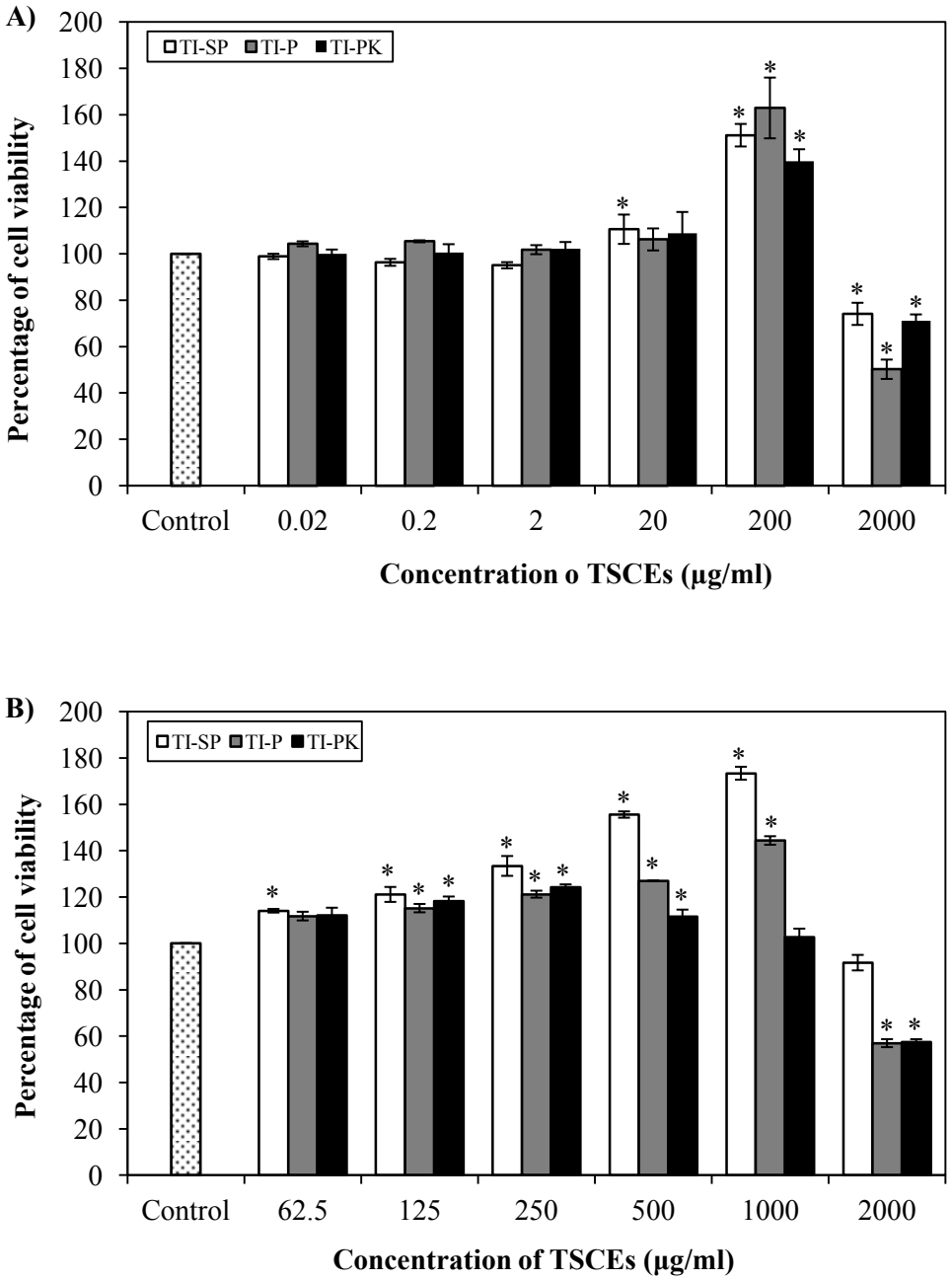


**Figure 14** Cytotoxic effect of TSCEs from different tamarind cultivars on human foreskin fibroblast cells (CCD-1064Sk) treated with TSCEs for 24 hours as evaluated by MTT assay. (A) Treated cells with TSCEs at concentration 10 fold dilutions series. (B) Treated cells with TSCEs at concentration 2 fold dilutions series. Data are mean ± standard error, **P< 0.05* compared to control.

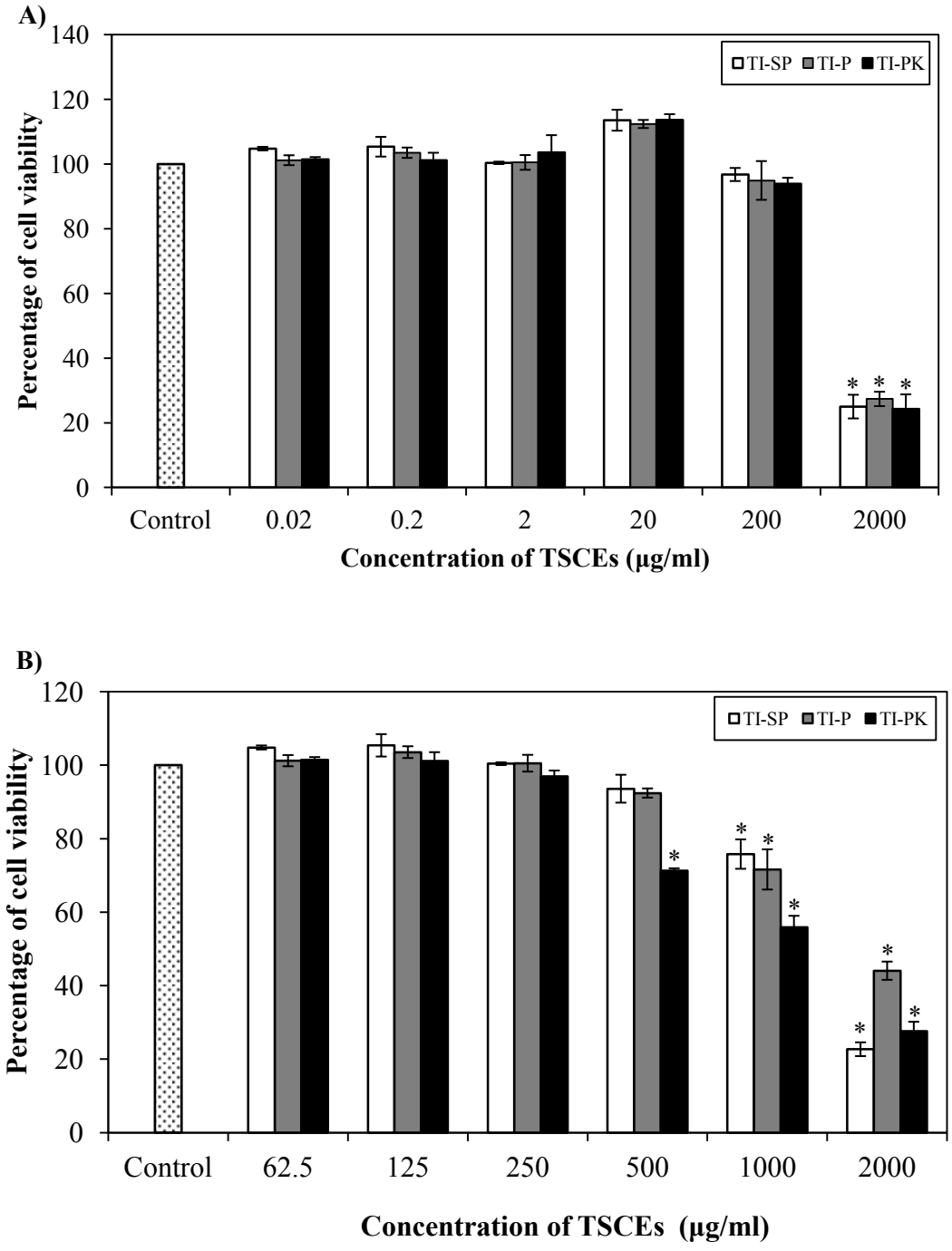


**Figure 15** Effect of TSCEs from different cultivars on human foreskin fibroblast cells (CCD-1064Sk) after treated cells with TSCEs for 24 hours as evaluated by NRU assay. (A) Effect of TSCEs on CCD-1064Sk after treated cells with TSCEs at concentration 10 fold dilution series. (B) Effect of TSCEs on CCD-1064Sk after treated cells with TSCEs at concentration 2 fold dilution series. Data are mean ± standard error, *$P< 0.05$ compared to control.

from TI-P and TI-SP, the $IC_{50}$ values were 1042 ± 13, 1707 ± 136 and 1448 ± 48 μg/ml for TSCEs from TI-PK, TI-P and TI-SP, respectively. However, the percent cell viability of TSCEs treated cells was increased at the lower concentrations when cells were treated with TSCEs at concentration ranges 6.25-200 μg/ml. The result in **Figure 16** showed that the percent cell viability increased at the lower TSCEs concentrations, but the increasing the percent cell viability was not observed at higher TSCEs concentrations. The TSCE from sweet tamarind (TI-SP) exhibited significantly increased cell viability 12-14% of control at TSCE concentration ranges 6.25-12.5 μg/ml while the TSCEs from sour tamarind including TI-P and TI-PK were presented significantly increased cell viability 15 and 13% of control, respectively, of TSCEs concentration 6.25 μg/ml compared with vehicle control cells ($P < 0.05$). The result of the increasing the cells may due the cells proliferative effect of TSCEs.

The cytotoxic effect of phenolic compounds have been studied, Ugartondo *et al*. (2007) reported that the cytotoxic effect of procyanidin fractions from grape and pine by NRU assay on human keratinocyte cell line (HaCaT) and mouse fibroblast cell line 3T3 was correlated with the degree of polymerization and the percentage of galloylation. Antioxidant activities are exhibited at nontoxic concentration 500 μg/ml as reported by Kim *et al*. (2010). Cytotoxic effect of phenolic compounds from the aqueous and ethanolic extracts from Malaysian plants at 500 μg/ml has not observed on normal lung fibroblast cells (Qader *et al*., 2011). The similar results were found in this studied, the TSCEs showed no toxic effect in the same concentration ranges (lower than 500 μg/ml).

**4.2 Effect of tamarind seed-coat extracts from different cultivars on human gastric adenocarcinoma epithelial cells**

The cytotoxic effect of TSCEs from different tamarind cultivars on human gastric adenocarcinoma epithelial cells (AGS) was evaluated by using MTT and NRU assays. After treated cells with TSCEs for 24 hours, the percent cell viability was evaluated by these assays. The fifty percent inhibitory concentration ($IC_{50}$) was calculated. The results from MTT and NRU assays were the same. The percent cell viability was decreased in concentration-dependent manner. The toxic effect of TSCEs from the three different tamarind cultivars on AGS cells by using

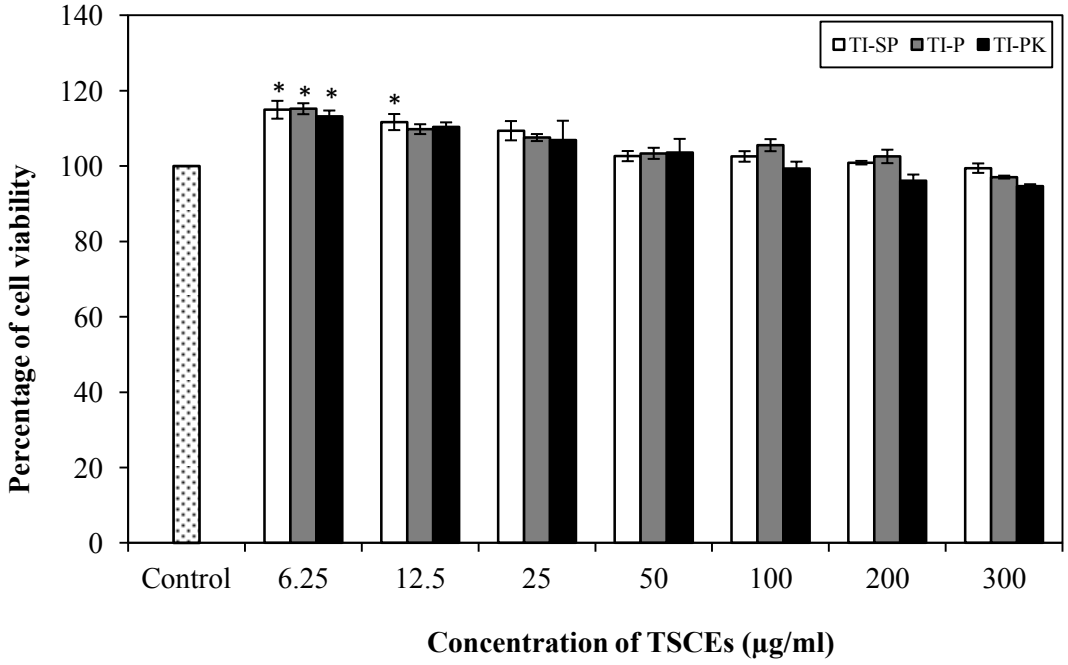


**Figure 16** The proliferative effect of TSCEs from different tamarind cultivars on human foreskin fibroblast cells (CCD-1064Sk) after treated cells with TSCEs for 24 hours as evaluated by NRU assay. Data are mean ± standard error, $*P< 0.05$ compared to control.

MTT assay are shown in **Figure 17**. The results of TSCEs at concentrations 0.02-2000 µg/ml (10 fold dilution series) are shown in **Figure 17A**. TSCEs showed high toxic effect on cells at 200-2000 µg/ml, but the lower concentrations (up to 20 µg/ml) showed no cytotoxic effect on AGS cells. The toxic concentrations of TSCEs were used for the study cytotoxic effect on AGS cells. The results of 2 fold dilutions series concentrations ranges 62.5-2000 µg/ml of TSCEs are shown in **Figure 17B**. The results indicated that the percent cell survival rapidly decreased in the concentration response manner. The extracts from the three different tamarind cultivars exhibited strongly cytotoxic effect on AGS cells. The percent cells survival was significantly decreased after treated with TSCEs of TI-SP, TI-P at 125-2000 µg/ml, but TI-PK at 62.5-2000 µg/ml. The $IC_{50}$ values of TSCEs from the three cultivars were 147.10 ± 1.35, 110.30 ± 10.49 and 90.97 ± 6.87 µg/ml for TI-SP, TI-P and TI-PK, respectively. The result of $IC_{50}$ values indicated that TSCEs from the sour tamarinds showed significantly higher cytotoxic effect than the sweet tamarind ($P < 0.05$). The TSCE from TI-PK exhibited the highest cytotoxic effect on AGS cells. In addition, the result of 10 fold dilutions series (**Figure 17A)** indicated that TSCEs may effect on increasing cell proliferation at the low concentrations ranges 0.2-2 µg/ml.

The result of cytotoxic effect of TSCEs by NRU assay showed in **Figure 18** that TSCEs reduced the percentage of AGS cells viability in concentration-dependent manner. The results of TSCEs at concentration ranges 0.02-2000 µg/ml (10 fold dilution series) are shown in **Figure 18A**. TSCEs showed no cytotoxic effect on AGS cells at concentrations ranges 0.02-20 µg/ml, but at the higher concentrations ranges 200-2000 µg/ml of TSCEs showed high cytotoxic effect on AGS cells. From these results, concentrations range 62.5-2000 µg/ml of TSCEs were used for the screening the cytotoxic effect on AGS cells as showed in **Figure 18B**. The decreasing percent cell survival in the concentrations response curve was observed. Percent cell survival was significantly decreased after treated cells with 62.5-2000 µg/ml of TSCEs from the three cultivars in comparison with the vehicle control cells ($P < 0.05$). The $IC_{50}$ values of TSCEs from the three cultivars were calculated, the $IC_{50}$ values of TSCEs from TI-SP, TI-P and TI-PK were 97.44 ± 4.78, 88.00 ± 2.46 and 86.47 ± 2.41 µg/ml, respectively. The $IC_{50}$ value indicated that TSCEs from sour tamarinds possessed higher toxic effect than the TSCE from sweet tamarind.

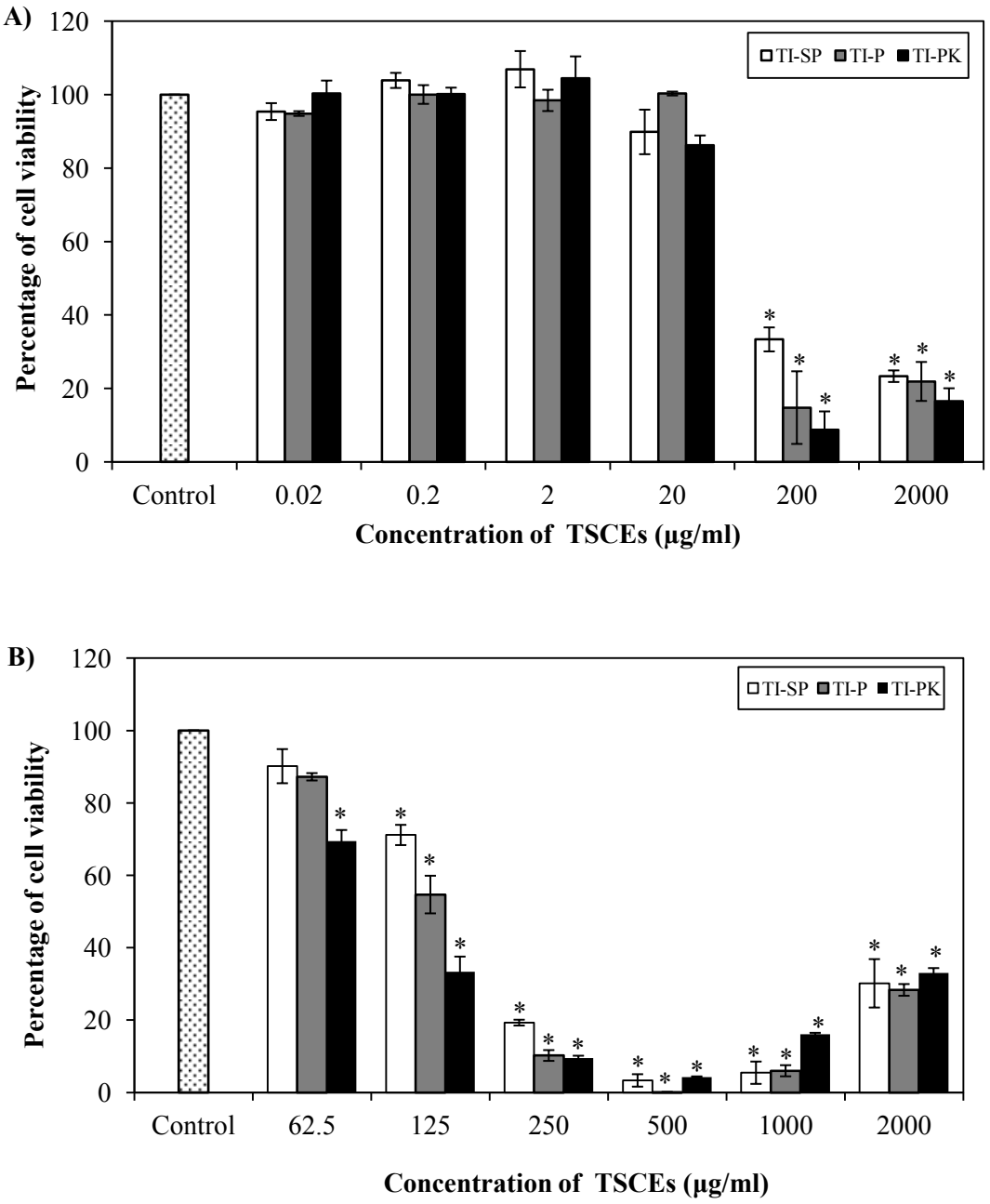


**Figure 17** Effect of TSCEs from different tamarind cultivars on human gastric adenocarcinoma epithelial cells (AGS) after treated cells with TSCEs for 24 hours as evaluated by MTT assay. (A) Effect of TSCEs on AGS after treated cells with TSCEs at concentration 10 fold dilution series. (B) Effect of TSCEs on AGS after treated cells with TSCEs at concentration 2 fold dilution series. Data are mean ± standard error, *$P< 0.05$ compared to control.

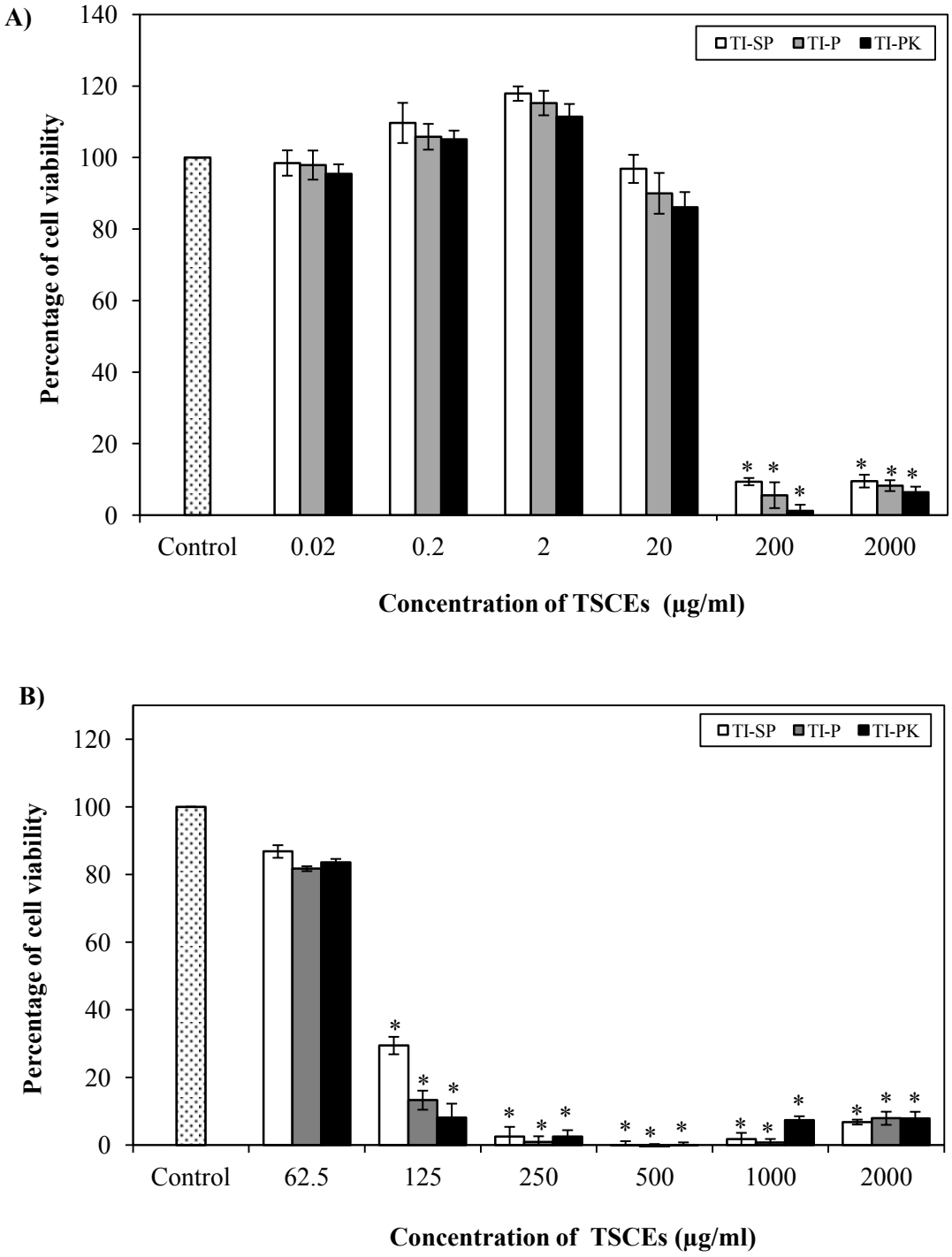


**Figure 18** Effect of TSCEs on human gastric adenocarcinoma epithelial cells (AGS) after treated cells with TSCEs for 24 hours as evaluated by NRU assay. (A) AGS cells after treated with TSCEs at concentration 10 fold dilution series. (B) AGS cells after treated with TSCEs at concentration 2 fold dilution series. Data are mean ± standard error, *$P< 0.05$ compared to control.

The cytotoxicity of TSCEs from different cultivars was not significantly different by using NRU assay ($P < 0.05$). The percent cell viability of TSCEs treated cells at higher concentrations increase was due to the effect of interfering color from TSCEs (**Figure 18)**. However, the percent cell viability was then confirmed by the assay with dye exclusion (trypan blue) and the cells count was recorded. The results showed that the TSCEs exhibited strongly cytotoxic effect on AGS cells. The percent cell viability of TSCEs treated cells was not observed at higher concentrations (data shown in Table 17 at appendix). In addition, the percent cell viability of TSCEs treated cell was increased at concentration ranges 0.2-2 µg/ml (using 10 fold dilution series). Therefore, the concentration effects on increasing cell viability may due to the effect of cell proliferation, this concentrations were used for studying the effect TSCEs on AGS cell proliferation by using 2 fold dilution series of TSCEs. Cells were treated with TSCEs at concentration ranges 0.625-20 µg/ml. The result in **Figure 19** represented the percent cell viability increased at the lower concentrations of TSCEs. The extract from sweet tamarind "TI-SP" at concentration ranges 0.625-2.5 µg/ml, sour tamarind "TI-P" at concentration ranges 1.25-2.5 µg/ml and "TI-PK" at concentration 2.5 µg/ml significantly increased cell proliferation compared with vehicle control cells ($P < 0.05$). The percent of cell proliferation in AGS cells with TSCEs from TI-SP, TI-P and TI-PK were 15-17%, 15% and 13% of control, respectively. These result suggested that the extract from sweet tamarind exhibited the cell growth or proliferation in AGS cells better than the sour tamarind.

The cytotoxic effect of phenolic compounds on AGS cells has been studied, (Chung *et al*., 2011) the phenolic compounds from Adlay (*Coixlachryma-jobi* L. var. *ma-yuen* Stapf) seeds extracts at concentrations 50-200 µg/ml exhibited inhibitory effects on growth of AGS cells by MTT assay with the $IC_{50}$ less than 100 µg/ml. Ye *et al*. (1999) suggested that grape seed proanthocyanidin extract (GSPE) at concentration ranges 25-50 µg/ml inhibit cell growth of AGS cells in concentration and time-dependent manner. In addition, GSPE exhibits the increasing cell growth of normal human gastric mucosal cells at 25-50 µg/ml of GSPE. The results are similarly with this present study. TSCEs at high concentrations (125-2000 µg/ml) exhibited cytotoxic effect on AGS cells. The lower concentrations of TSCEs (0.625-2.5 µg/ml)

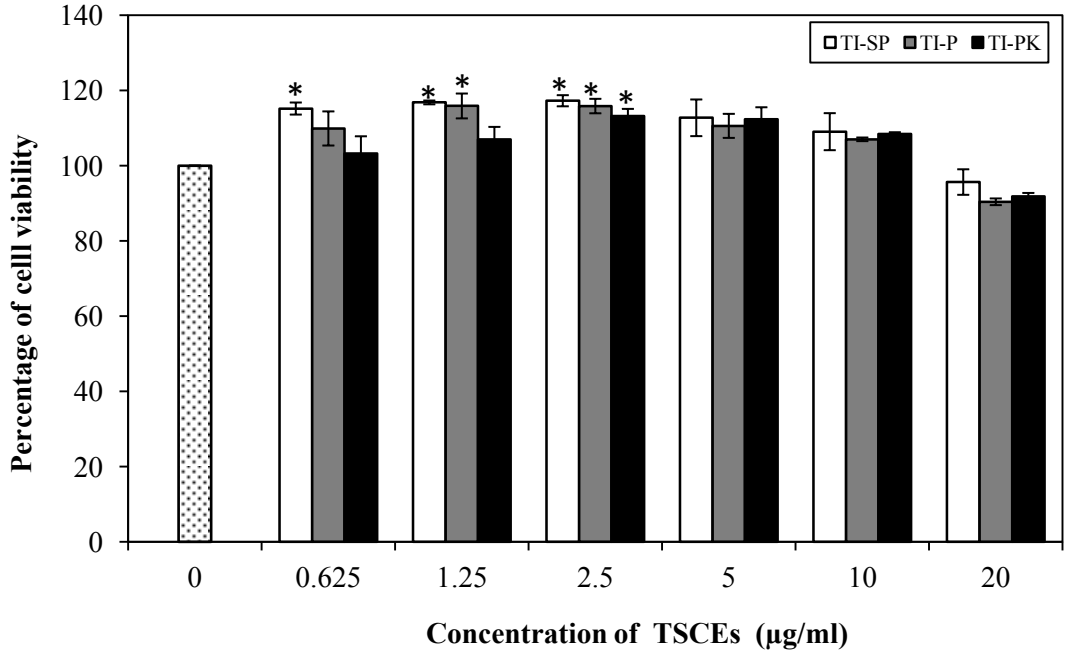


**Figure 19** The proliferative effect of TSCEs from different tamarind cultivars on human gastric adenocarcinoma epithelial cells (AGS) after treated cells with TSCEs for 24 hours as evaluated by NRU assay. Data are mean ± standard error, $*P< 0.05$ compared to control (untreated TSCEs).

stimulated cells growth of AGS cells, but this cell growth stimulating effect was not observed in the study of Ye *et al*. (1999). Effect on cell proliferation in AGS cells may result in disadvantage, this cell line is adenocarcinoma cells. However, the concentrations greater than 20 µg/ml of TSCEs showed antiproliferative effect on AGS cells, but these concentrations did not show antiproliferative effect on CCD-1064Sk (human foreskin fibroblasts).

The chemical analysis indicated that TSCEs from sour tamarinds consisted of higher proanthocyanidin contents than the sweet tamarind. The cytotoxic effect of TSCEs from sour tamarinds was higher than sweet tamarind. These results indicated that the cytotoxic responses of CCD-1064Sk cells and AGS cells against the given TSCEs were different. TSCEs at higher concentrations exhibited cytotoxic effect on both human cell lines. In addition, TSCEs exhibited higher cytotoxic effect on AGS cells than CCD-1064Sk cells. The $IC_{50}$ values of TSCEs on AGS cells presented lower than CCD-1064Sk cells. The TSCEs exhibited strongly cytotoxic effect on AGS cells. Moreover, the ethyl acetate fraction partitioned from methanolic fraction exhibited less toxic ($IC_{50} > 100$ µg/ml) on normal human foreskin fibroblast cell line (MRC-5) by using NRU assay, but high toxic on tumor cells (Malek *et al*, 2008). The phenolic compounds from natural source exhibit antiproliferative or cytotoxic effect on tumor cells (Kim *et al*., 2010; Rao *et al*., 2007; Sun *et al*., 2002). Thus, TSCEs exhibited the inhibition growth of human gastric adenocacinoma epithelial cells.

The increased percent cell viability in TSCEs treated cells (**Figure 14, 17**) was due to the false positive reaction of MTT assay. Shoemakere *et al*. (2004) has reported that the extracts from herbs are rapidly formed direct reaction with MTT test and developed formazan crystal formation. Antioxidant compounds and the botanical extracts that contained free thiol (SH) groups have also been reported that it can reduce MTT in the cell free assay, thus the MTT assay may lead to false positive results when treated with natural compounds.(Natarajan *et al*.,2000; Bruggisser *et al*., 2002).

A comparison of $IC_{50}$ value of phenolic compounds as determined between MTT and NRU assay has been reported in mouse 3T3 fibroblast cells. The result shows the value of $IC_{50}$ from MTT assay is higher than that of NRU assay

(Zapõr, 2004; Skowroń and Zapõr, 2004) which was corresponding with the present study. The NRU assay was applied in this study to measure the cell viability study.

TSCEs at lower concentrations exhibited proliferative effect on both of human cell lines. The result of HPLC analysis showed that TSCEs composed of the flavoniod compounds including (+)-catechin, procyanidin B2 and (-)-epicatechin. These compounds have been reported having an effect on cell proliferation. (+)-Catechin and (-)-epicatechin from methanol extract of stems of *Actinidia arguta* promoted cell proliferation of bone marrow cells and stimulated the formation of myeloid colonies (Takano *et al*., 2003). In mice which was orally administered (-)-epigallocatechin-3-gallate (EGCG), a major catechin of green tea, for 4 weeks before stained the brain section was found to increase the cell proliferation and the number of neuroblasts in mice hippocampal dentate gyrus (Yoo *et al*., 2010). EGCG can enhance the epidermal keratinocytes cell proliferation (Chung *et al*., 2003; Hsu *et al*., 2003). Moreover, Takahashi *el al*. (1999) found that procyanidin oligomers and monomer (+)-catechin and (-)-epicatechin exhibited to promote the proliferative effect of hair follicular epithelium cell and mouse epidermal keratinocytes. Procyanidin dimer and timer showed higher activity than the monomer in both cells.

Moreover, Jie *et al*. (2006) found that the major compounds such as catechin and gallic acid of the Pu-erh tea extracts from ethyl acetate fraction increased cell viability in human fibroblast cell (HPF-1) was about 18.9% by using MTT assay.

The standard references (+)-catechin, (-)-epicatechin were used to examine the proliferative effect on CCD-1064Sk and AGS cells in this study. The results exhibited that (+)-catechin at concentration ranges 0.01-100 µg/ml significantly increased the number of CCD-1064Sk cells for 13-22%. On the other hand, (-)-epicatechin at concentration ranges 0.001-0.1µg/ml significantly increased the cell proliferation of AGS cells at 11-15% in comparison with vehicle control cells ($P < 0.05$) by using NRU assay (data shown in Table 22 at appendix B). The TSCEs compound of both (+)-catechin and (-)-epicatechin that stimulated the cell proliferation on both CCD-1064Sk and AGS cells, high content of (+)-catechin in the sweet tamarind (TI-SP) exhibited higher cell proliferative effect on CCD-1064Sk than sour tamarind.

In this present study, the AGS cell was used to study in cytoprotective and wound healing effects. The AGS cell line was derived from human stomach adenocarcinoma consisting of mucus-secreting epithelial cells. This cell line displays some characterizations of normal gastric epithelial cells such as epithelial-like morphology, microvilli and production of mucus. AGS cells can be continuously maintained by subculturing for longer time when compared with the primary culture (Zeng *et al*., 1996). This cell was used to study an *in vitro* model for determination of gastroprotective effect of nature compound or drugs (Hall *et al*., 2006; Jang *et al*., 2008), to study the protective mechanism of compounds on *in vitro* gastric epithelial cells invasion by *Helicobacter pylori* (Park *et al*., 2005; Shih *et al*., 2007) and wound healing effect (Choi *et al*., 2008; Kim *et al*., 2012). Thus, this present study was used the AGS cells for study the cytoprotective and wound healing effects of TSCEs on gastric epithelial cells.

**5. Protective effect of tamarind seed-coat extracts from ethyl acetate fraction of different tamarind cultivars on hydrogen peroxide induced human cell lines injury**

**5.1 Effect of hydrogen peroxide on cell viability of human cell lines CCD-1064Sk and AGS cells by NRU assay**

Hydrogen peroxide is one of the main ROS that can generate in the cells. $H_2O_2$ can induce to oxidative stress and enhance apoptotic in many cells through lipid peroxidation and DNA damage (Li *et al*., 2003). In this study, $H_2O_2$was applied to induce oxidative stress in both human cell lines to assess the protective potential of TSCEs against $H_2O_2$-induced cell injury.

The results in **Figure 20** showed that hydrogen peroxide ($H_2O_2$) which was used to induce oxidative stress in CCD-1064Sk and AGS cells decreased the cell viability in concentration and time-dependent manners. The inhibitory concentration of the fifty percentage ($IC_{50}$) value of $H_2O_2$ was 1.08 ± 0.13 and 1.16 ± 0.45 mM for CCD-1064Sk and AGS cells, respectively. The $IC_{50}$ value was used to evaluate the protective effect of TSCEs on the tested cells. Therefore, $H_2O_2$ at the concentration of 1 mM for 60 min, which significantly displayed viable cells 55.41 ± 4.78% and 52.82

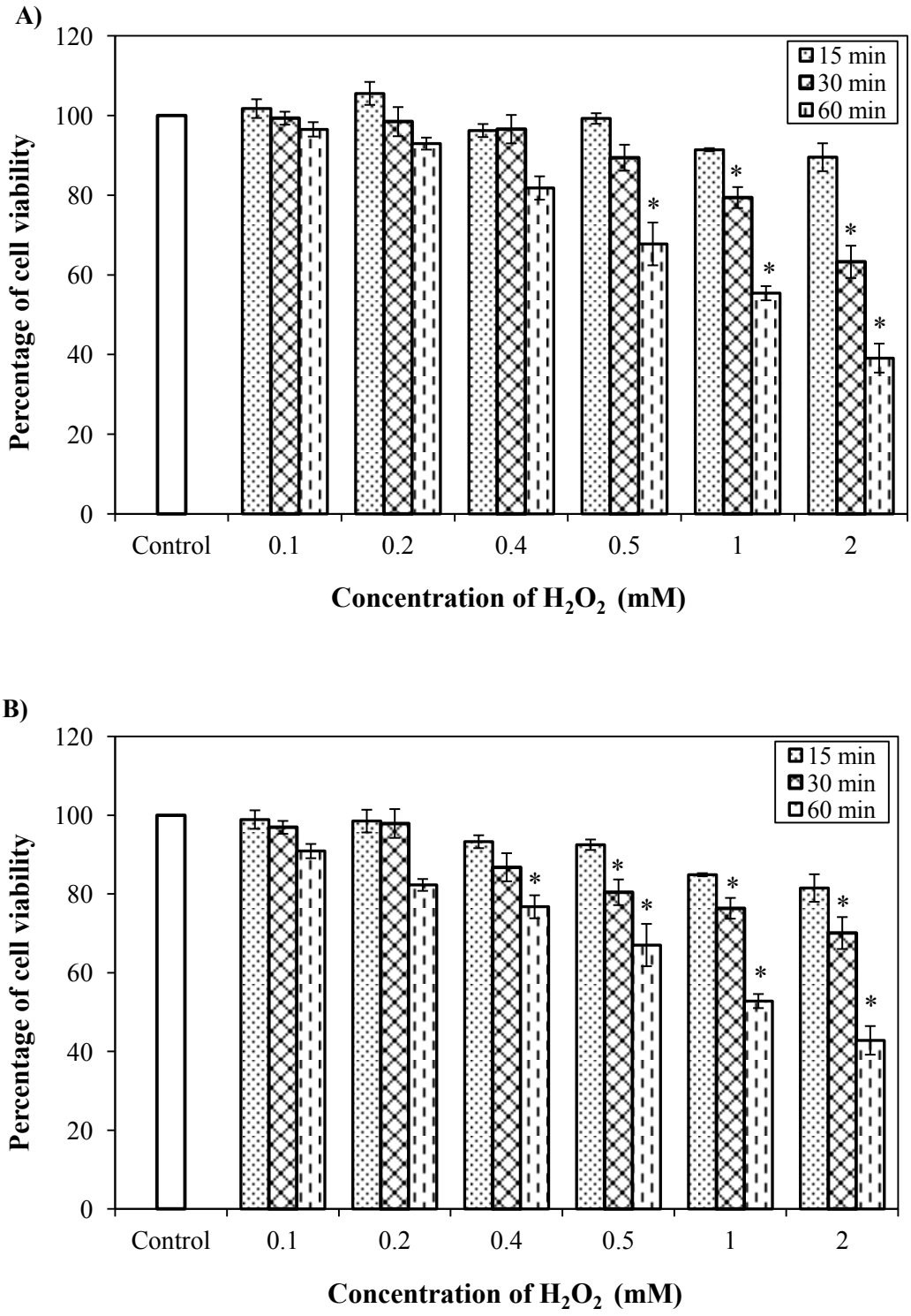


**Figure 20** Effect of hydrogen peroxide ($H_2O_2$) on (A) human foreskin fibroblast cells (CCD-1064Sk), (A) and (B) human gastric adenocarcinoma epithelial cells (AGS) after treated cells with various concentrations $H_2O_2$ for 15-60 min. Data are mean ± standard error, *$P< 0.05$ compared to control.

± 1.79% for CCD-1064Sk and AGS cells, respectively, were used to induce cell injury.

### 5.1.1 Protective effect of tamarind seed-coat extracts on cells viability of human foreskin fibroblast cells

The protective effect of TSCEs against $H_2O_2$-induced cell injury on the viability of CCD-1064Sk cells was evaluated by using the NRU assay. The cells were pretreated with the non-toxic dose of TSCEs at concentrations ranges 6.25-300 µg/ml for 24 hours and then treated with 1 mM $H_2O_2$ for 60 min. The result in **Figure 21A** showed that the number of viable cells was significantly decreased approximately 50% (55.41 ± 4.78%) in $H_2O_2$ treated cells compared to the vehicle control cells ($P < 0.05$). The number of viable cells in TSCEs pretreated cells increased in concentration-dependent manner compared with $H_2O_2$ treated cells. The TSCEs significantly increased the number of viable cells at concentrations ranges 200-300 µg/ml (76.99-82.68%) compared with $H_2O_2$ treated cells without TSCEs ($P < 0.05$). TSCEs exhibited protective effect against $H_2O_2$-induced oxidative damaged cells. However, the protective effect of the TSCEs of the three different cultivars on cell viability was not significantly different between cultivars.

The protective effect of antioxidant compounds on human fibroblast cells was previously observed. Jie *et al*. (2006) studied the cytroprotective effect of different Pu-erh tea extrects on $H_2O_2$-incued oxidative stress in human fibroblast cell (HPF-1). The ethyl acetate and *n*-butanol extract fractions comprised the major catechin and gallic acid showed protective effect on HPF-1 cells pretreated with various concentrations of extracts for 1 hour and exposed to 600 µM $H_2O_2$ for 24 hours. Hu *et al*. (2011) reported that 2 mM $H_2O_2$ exposured for 3 hours decreased percent cell viability of CCD-986Sk cells to 55.39%. Pretreatment cells with methanolic extract from *Duchesnea indica* at concentrations ranges 25-200 µg/ml increased percent cell viability in concentration-dependent manner. The phenolic compounds exhibited cytoprotective effect on human fibroblast cells which was corresponding with the present study.

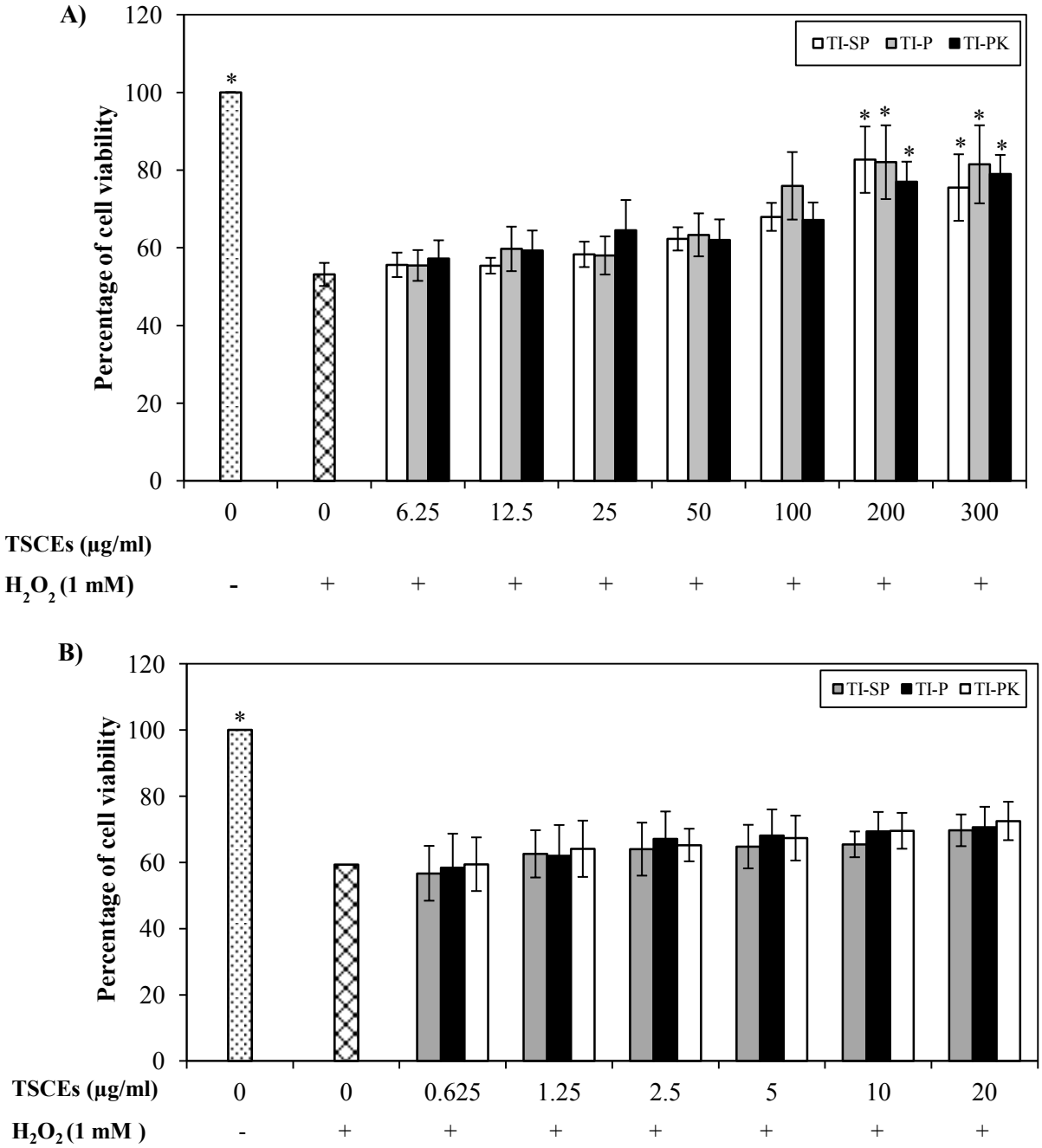


**Figure 21** Protective effect of TSCEs on $H_2O_2$-induced injury human foreskin fibroblast cells (CCD-1064Sk) (A) and human gastric adenocarcinoma epithelial cells (AGS) (B). Cells were pretreated with TSCEs for 24 hours and exposed 1 mM $H_2O_2$ for 60 min. The number of viable cell was measured by NRU assay. Data are mean ± standard error, *$P< 0.05$ compared to $H_2O_2$-treated cells (untreated with TSCEs).

### 5.1.2 Protective effect of tamarind seed-coat extracts on cells viability of human gastric adenocarcinoma epithelial cells

The protective effect of TSCEs against $H_2O_2$-induced cell injury on the viability of AGS cells was determined by using NRU assay. The $IC_{50}$ value of $H_2O_2$ in AGS cell was 1 mM $H_2O_2$, exposed for 60 min (52.82 ± 1.79%). The cells were pretreated with the non-toxic concentrations of TSCEs at concentration ranges 0.625-20 µg/ml for 24 hours and exposured to 1 mM $H_2O_2$ for 60 min. The result in **Figure 21B** showed that the number of viable cells in TSCEs pretreated cells did not significantly increase in concentration-dependent manner differences compared with $H_2O_2$ treated cells ($P < 0.05$). The cells were treated with 2 mM $H_2O_2$ for 30 min, this treatment induced approximately 30% cell death (70.09 ± 4.06%). The result in **Figure 22** showed the number of viable cells in the three TSCEs pretreated cells increased in concentration-dependent manner compared with $H_2O_2$ treated cells without TSCEs. The TSCEs significantly increased cell viability of $H_2O_2$-induced AGS cells injury at concentrations 10-20 µg/ml (79.20-86.57%) and 20 µg/ml (82.38%) for TSCE from the sour and the sweet tamarind cultivar, respectively, compared with $H_2O_2$ treated cell without TSCEs ($P < 0.05$). TSCEs exhibited protective effect by decreased the damaged cells exposure to $H_2O_2$. The extract from sour tamarind showed the protective effect against $H_2O_2$ induced damage AGS cells better than sweet tamarind.

The protective effect of antioxidant compounds on gastric epithelial cells has been studied by Miller *et al*. (2001), the protective effect of dietary antioxidants compound from cat's claw and green tea were observed and found that 50 µM $H_2O_2$, exposed for 24 hours increased necrosis in AGS cells at 34%. The NaHS, a $H_2S$ donor exhibited strongly suppressed 1mM $H_2O_2$-caused cell death for 4 hours in rat normal gastric epithelial RGMI cells by using LDH release (Yonezawa *et al*., 2007). $H_2O_2$ was used to induce oxidative stress and cell death in gastric epithelial cells in protective effect study.

The apple extracts decreased the effect of xanthine-hypoxanthine oxidase or indomethacin-induced injury to gastric epithelial cells (MKN 28) and prevented indomethacin injury to the rat gastric mucosa. Moreover, the major

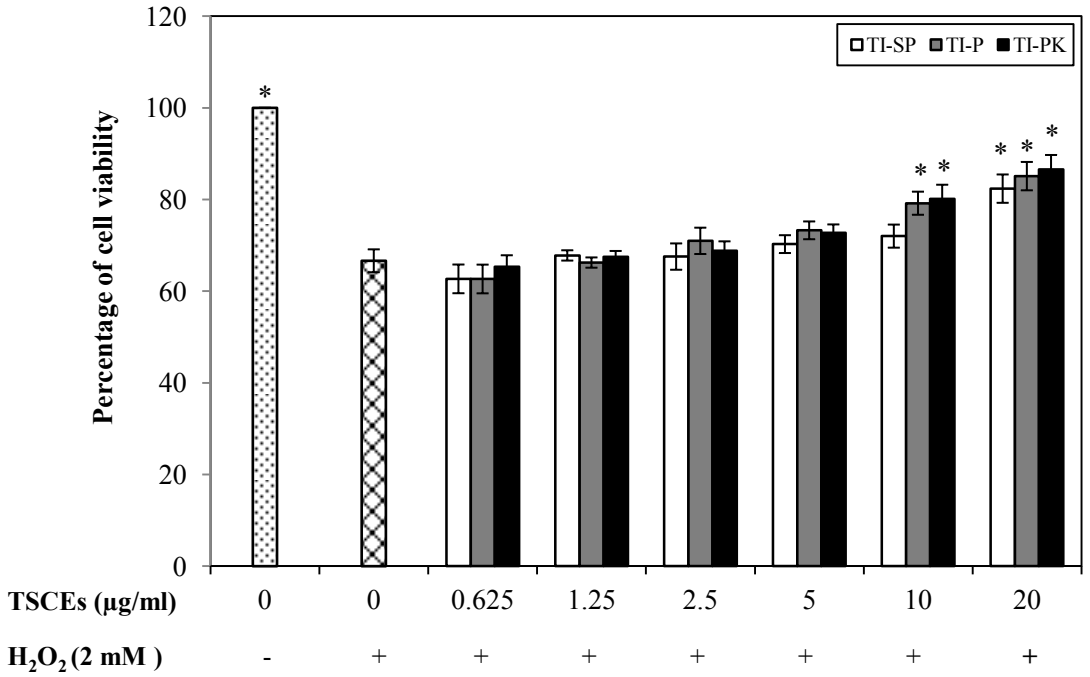


**Figure 22** Protective effect of TSCEs on $H_2O_2$-induced injury human gastric adenocarcinoma epithelium cells (AGS) approximately 30% cell death ($IC_{30}$) was observed. The pretreated TSCEs cells exposed 2 mM $H_2O_2$ for 30 min. The number of cell viability of treated cells was measured by NRU assay. Data are mean ± standard error, *$P< 0.05$ compared to $H_2O_2$-treated cells (untreated with TSCEs).

compounds, catechin and chlorogenic acid showed effective preventing oxidative injury to gastric cells (Graziani *et al*., 2012).

The tannins and flavonoids fraction in *Mouriri pusa* exhibit the gastroprotective effect in rat induced ulcer by oral ethanol and acetic acid. The flavonoids fraction to be involved in anti-inflammatory, induce angiogenesis and cell proliferation while tannins fraction may act to promote a mechanic barrier to protect the stomach from ulcer formation (Vasconcelos *et al*., 2010). The protective effect of phenolic compounds is found in human gastric epithelial cells, this proliferative effect was corresponding with the present study.

### 5.2 Effect of tamarind seed-coat extracts on intracellular ROS in human cell lines-CCD-1064Sk and AGS cells

#### 5.2.1 Effect of tamarind seed-coat extracts on intracellular ROS in human foreskin fibroblast cells

The effect of TSCEs on intracellular ROS in CCD-1064Sk was evaluated. Cells were treated with non-toxic concentrations of TSCEs at concentration ranges 6.25-300 µg/ml for 24 hours and then the intracellular ROS levels of cells were measured by using DCFH-DA assay. Effect on the intracellular ROS in CCD-1064Sk at low concentrations of TSCEs was not observed **(Figure 23A)**. The TSCEs from sour tamarinds (TI-P, TI-PK) were significantly reduced the intracellular ROS in CCD-1064Sk cells at concentration ranges 200-300 µg/ml (66.48-75.84%) while the TSCE from sweet tamarind showed significant reduction the intracellular ROS in CCD-1064Sk cells at concentration 300 µg/ml (71.31%) ($P < 0.05$). The results suggest that the TSCEs from sour tamarinds reduced intracellular ROS levels in CCD-1064Sk cells more than the TSCE from sweet tamarind.

The results were consistent with the report of Jie *et al*. (2006), the monomeric polyphenols of catechins and gallic acid extract fractions from Pu-erh tea and catechin, (-)-epigallocatechin-3-gallate (EGCG) decreased intracellular ROS in embryonic human lung fibroblasts (HPF-1) cell. The concentrations of TSCEs exhibited decreasing the intracellular ROS levels higher than the concentrations of extracts reported by Jie *et al*. (2006).

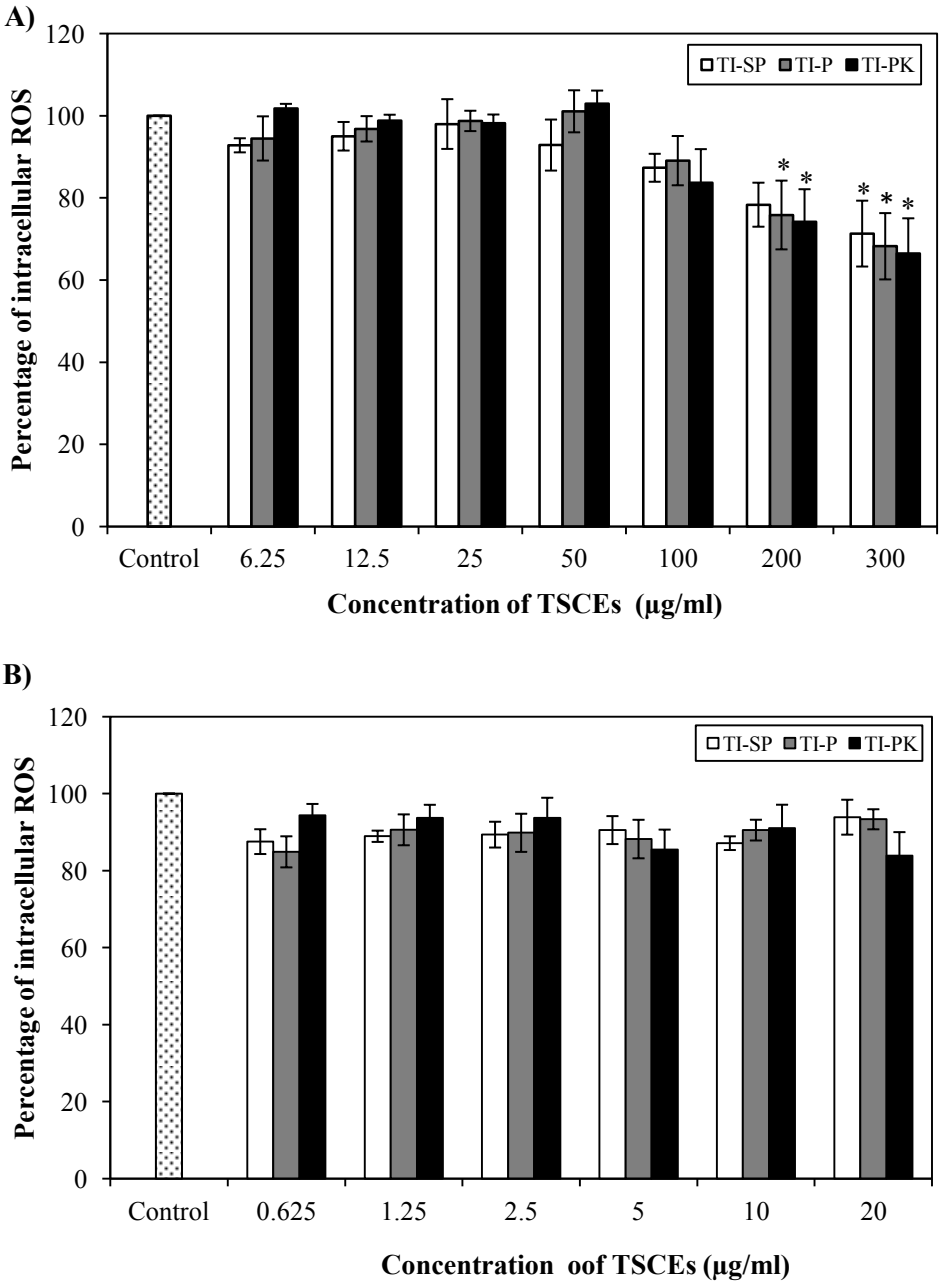


**Figure 23** Effect of TSCEs on intracellular ROS levels in human foreskin fibroblast cells (CCD-1064Sk) (A) and human gastric adenocarcinoma epithelial cells (AGS) (B). Cells were treated with TSCEs for 24 hours at concentrations ranges 6.25-300 and 0.625-20 µg/ml for CCD-1064Sk and AGS cells, respectively. The intracellular ROS levels were evaluated by DCFH-DA assay. Data are mean ± standard error, **P*< *0.05* compared to control.

### 5.2.2 Effect of tamarind seed-coat extracts on intracellular ROS in human gastric adenocarcinoma epithelial cells

An effect of TSCEs on intracellular ROS in AGS cells was examined by using DCFH-DA method. Cells were treated with TSCEs at concentration ranges 0.625-20 μg/ml for 24 hours. The result in **Figure 23B** showed that the TSCEs not significantly decreased the intracellular ROS in AGS cells compared to vehicle control cells. This result indicated the effect of TSCEs on intracellular ROS levels was not observed at concentration ranges 0.625-20 μg/ml. in AGS cells

## 5.3 Protective effect of tamarind seed-coat extracts on $H_2O_2$-induced intracellular ROS in human cell lines- CCD-1064Sk and AGS cells

### 5.3.1 Effect of $H_2O_2$ on intracellular levels of ROS in CCD-1064Sk and AGS cells

Hydrogen peroxide ($H_2O_2$) can increase intracellular ROS in human cell lines. In this study, $H_2O_2$ was used to induce oxidative stress in human cell lines. Cells were treated with $H_2O_2$ at concentration ranges 0.1-2 mM for 15, 30 and 60 min. The intracellular ROS was determined by using DCFH-DA assay. The intracellular ROS levels are shown in **Figure 24**. The intracellular ROS levels of treated cells increased in comparison with vehicle control cells. The result demonstrated that intracellular ROS levels increased in concentration and time-independent manners at 15 min as shown in **Figure 24**. At longer time incubation with $H_2O_2$, the result of ROS levels did not increase in concentration-dependent manner due to the decreasing of cell viability (**Figure 20A,B**) resulting in decreasing intracellular ROS levels in tested cells. The intracellular ROS level was the highest at 1 mM $H_2O_2$ exposured for 15 min (225.55 ± 5.63%) and 2 mM $H_2O_2$ exposured for 15 min (267.09 ± 4.73%) in CCD-1064Sk and AGS cells, respectively. This treatment condition was used to study the protective effect of TSCEs on the tested cells in this study.

The DCFH-DA assay was used to detect intracellular ROS generation in tested cells. The DCFH-DA assay using microplate reader has been reported that

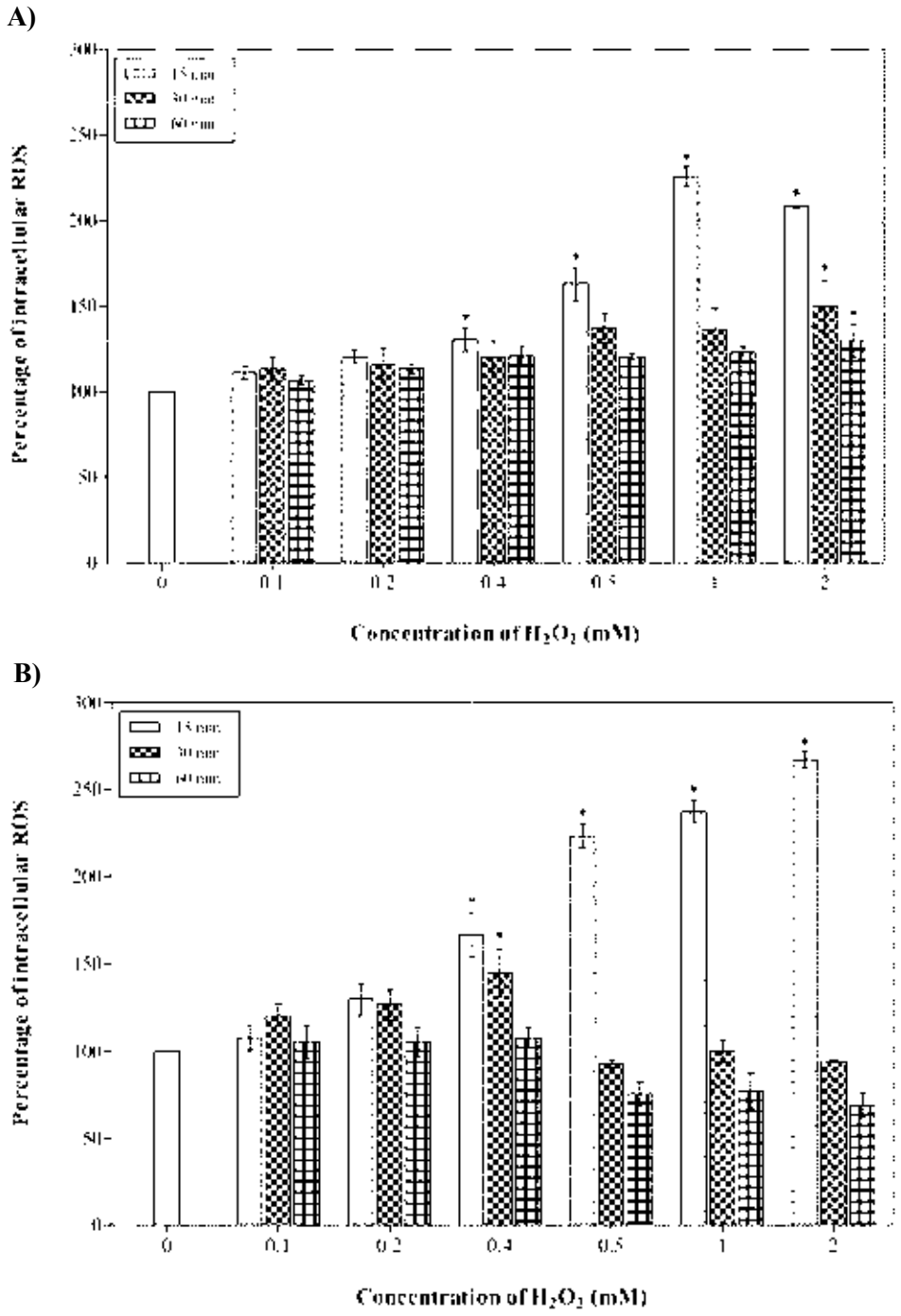


**Figure 24** Effect of $H_2O_2$ on the intracellular ROS generation in human foreskin fibroblast cells (CCD-1064Sk) (A) and human gastric adenocarcinoma epithelial cells (AGS) (B). Cells were treated with $H_2O_2$ for 15-60 min at 0.1-2 mM concentrations. The intracellular ROS was evaluated by DCFH-DA assay. Data are mean ± standard error, *$P< 0.05$ compared to control.

the method is an easy and efficient method with low variability. This method is used to detect the potency of pro-oxidants and evaluate the efficiency of antioxidants against ROS in various cell lines. The DCFH-DA assay is examined the oxidative stress or the generation of intracellular ROS in cells induced with various free radical generators. The $H_2O_2$ is the standard generator used to induce oxidative stress**.** Cells were exposed to 0.1-1 mM $H_2O_2$ for 30 min to induce the increasing of intracellular ROS in concentration dependent manner (Wang and Joseph, 1999). The DCFH-DA assay is used to study antioxidant and protective properties of compounds against $H_2O_2$ or another generator-induced oxidative stress in cells including fibroblasts and gastric mucosal cells (Cozzi *et al*., 1997; Fujii *et al*., 2000; Jie *et al*., 2006; Esmaeili *et al*., 2010). Thus, the DCFH-DA assay was used to evaluate the efficiency of TSCEs in protective effect against $H_2O_2$-induced intracellular ROS generation.

### 5.3.2 Protective effect of tamarind seed-coat extracts on $H_2O_2$-induced intracellular ROS in human foreskin fibroblast cells

The protective effect of TSCEs on $H_2O_2$-induced intracellular ROS generation in CCD-1064Sk cells was evaluated. Cells were pretreated with non-toxic concentrations of TSCEs in concentration ranges 6.25-300 µg/ml for 24 hours before exposured to 1 mM $H_2O_2$ for 15 min. Intracellular ROS levels were measured by DCFH-DA assay. The result was expressed as percent intracellular ROS as shown in **Figure 25A**. The $H_2O_2$ treated cells without TSCEs exhibited the highest intracellular ROS levels (225.55 ± 5.63%) compared vehicle control cells ($P < 0.05$). Pretreated cells with TSCEs reduced the intracellular ROS levels in concentration-dependent manner (compared to $H_2O_2$-treated cells without TSCEs). The cells pretreated with 300 µg/ml TSCE from the sweet tamarind (149.72%) and 50-300 µg/ml TSCEs from sour tamarinds (129.53-189.56%) showed significant reduction of the intracellular ROS levels compared to $H_2O_2$-treated cells without TSCEs ($P < 0.05$). The results indicated that TSCEs from the sour tamarinds reduced the intracellular ROS levels in $H_2O_2$-induced intracellular ROS in CCD-1064Sk cells better than the TSCE from sweet tamarind.

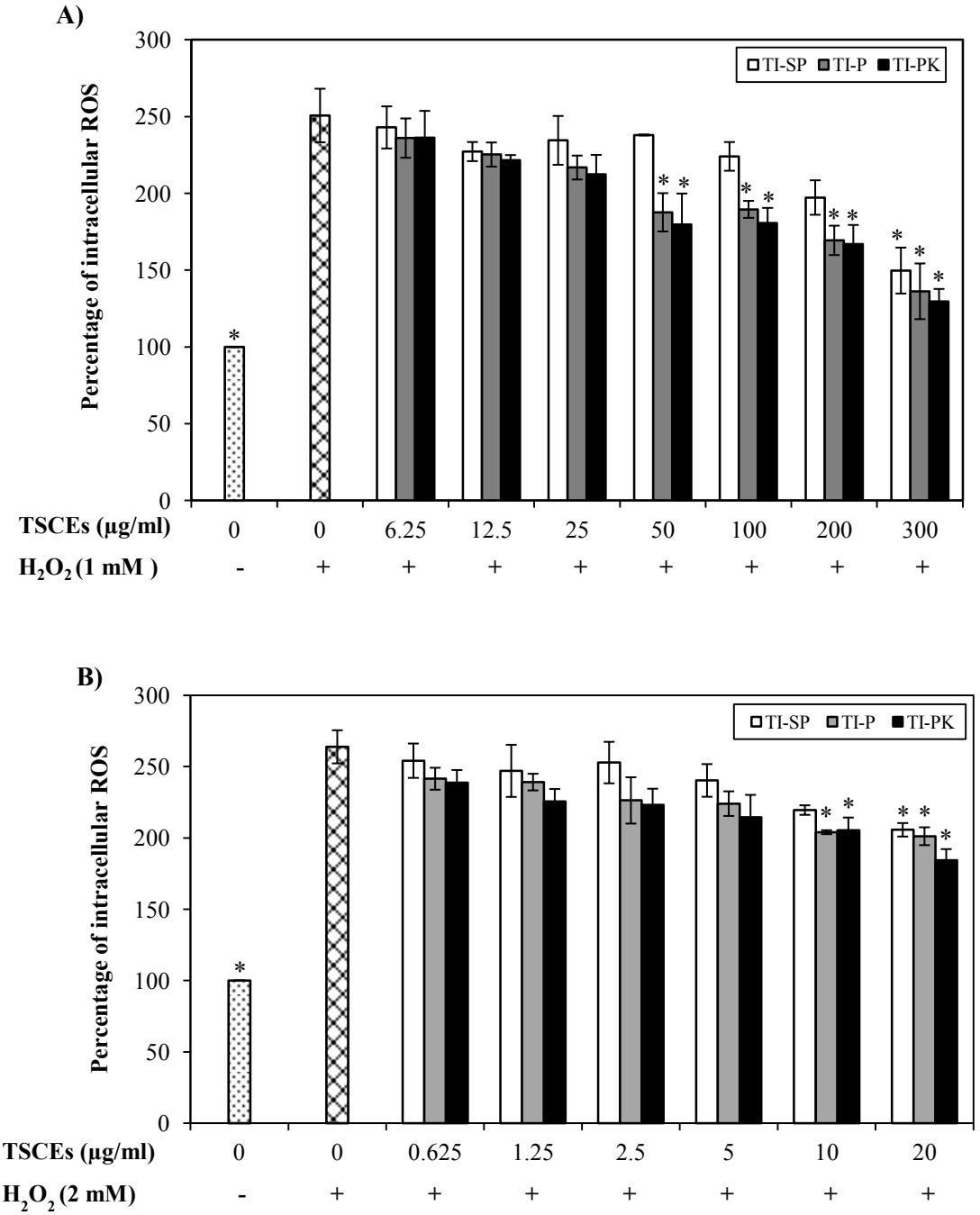


**Figure 25** Protective effect of TSCEs on $H_2O_2$-induced intracellular ROS levels in human foreskin fibroblast cells (CCD-1064Sk) (A) and human gastric adenocarcinoma epithelium cells (AGS) (B). Cells were pretreated with TSCEs for 24 hours and exposed to $H_2O_2$. The intracellular ROS was evaluated by DCFH-DA assay. Data are mean ± standard error, **P*< *0.05* compared to $H_2O_2$-treated cells (untreated with TSCEs).

### 5.3.3 Protective effect of tamarind seed-coat extracts on $H_2O_2$-induced intracellular ROS in human gastric adenocarcinoma epithelial cells

The protective effect of TSCEs on $H_2O_2$-induced intracellular ROS generation in AGS cells was evaluated by using DCFH-DA assay. Cells were pretreated with TSCEs in concentration ranges 0.625-20 μg/ml for 24 hours, and then treated with 2 mM $H_2O_2$ for 15 min. The result showed in **Figure 25B** that the intracellular ROS generation in $H_2O_2$-treated cells without TSCEs (267.09 ± 4.73%) showed significantly higher intracellular ROS levels than the vehicle control cells ($P < 0.05$). Pretreated cells with TSCEs decreased the intracellular ROS levels in concentration-dependent manner. The cells pretreated with 20 μg/ml TSCE from sweet tamarind (TI-SP) (205.71%) and 10-20 μg/ml TSCEs from sour tamarinds (184.43-205.30%) showed significant reduction the intracellular ROS levels compared to $H_2O_2$-treated cells without TSCEs ($P < 0.05$). These results suggested that TSCEs from sour tamarinds reduced the intracellular ROS levels against $H_2O_2$-induced intracellular ROS in AGS cells better than the TSCE from sweet tamarind.

The phenolic compounds have been reported the high efficiency of antioxidant activity for protective effect on oxidative stress in various cells. Procyanidins and proanthocyanidins from grape seeds exhibited protective effect against generator-induced oxidative stress and genotoxic damage by decreasing the intracellular ROS of mouse fibroblast (NIH3T3) and chick embryonic ventricular myocytes cells (Shao *et al*., 2003 and Lu *et al*., 2004). Anthocyanidins from purple sweet potato showed reducing the enhancement of intracellular ROS levels by amyloid-beta peptide-induced oxidative stress in rat pheochromocytoma PC12 cells (Ye *et al*., 2010). Moreover, the polyphenol extracts of Pu-erh tea are composed (+)-catechin and (-)-epigallocatechin-3-gallate are decreased the accumulation of intracellular reactive oxygen species in $H_2O_2$-induced embryonic human lung fibroblasts (HPF-1) (Jie *et al*., 2006).

The results from this study indicated that TSCEs exhibited protective effect against $H_2O_2$-induced damage cells by the decreasing of cell death or damaged cells and the reducing intracellular ROS levels in both of treated cells. TSCEs from the sour tamarinds showed better the protective effect than TSCE from sweet tamarind on both of CCD-1064Sk and AGS cells.

**6. Effect of tamarind seed-coat extracts on wound healing rate in human cell lines-CCD-1064Sk and AGS cells**

**6.1 Effect of tamarind seed-coat extracts on wound healing rate in human foreskin fibroblast cells**

The cell proliferation is an importance process for wound healing steps. At the wound edge, fibroblast cells are attracted into the wound area site and initiate the proliferation and migration for wound healing. The lower concentrations of TSCEs on stimulating cell proliferation of CCD-1064Sk cells were observed. The extract at concentrations 6.25-12.5 μg/ml TSCE from TI-SP stimulated proliferative effect on CCD-1064Sk cells at 12-14% while the extracts at concentration 6.25 μg/ml of TSCEs from TI-P and TI-PK stimulated proliferative effect on CCD-1064Sk cells with the values of 15 and 13%, respectively. Therefore, these concentrations were used for studying an effect of TSCEs on wound healing rate. The scratch assay was used to evaluate an effect of proliferative concentration of TSCEs on wound healing rate in CCD-1064Sk cells. The monolayer of CCD-1064Sk cells was scratched and then treated with the TSCEs at concentrations in ranges 1.5625-50 μg/ml and the pictures were captured at 6, 12, 18, 24, 36 and 48 hour, respectively. The result represented the percent wound closure is shown in **Figure 26**. The percent wound closure increased in time dependent manner. TSCEs-treated cells exhibited not significantly different the rate of wound closure compared to vehicle control cells ($P < 0.05$). Thus, TSCEs possessed low effect on the wound healing comparable with control in CCD-1064Sk cells.

**6.2 Effect of tamarind seed-coat extracts on wound healing rate in human gastric adenocarcinoma epithelial cells**

The lower concentrations of TSCEs stimulated the proliferation on AGS cells. These concentrations of TSCEs may accelerate the rate of wound closure. TSCEs from TI-SP, TI-P and TI-PK exhibited proliferative effect at concentrations 0.625-2.5 μg/ml, 1.25-2.5 μg/ml and 2.5 μg/ml, respectively, with the percent of cell proliferation in AGS cells at 15-17%, 15% and 13% for TSCEs from TI-SP, TI-P and TI-PK, respectively. These concentrations were used for studying an effect of TSCEs

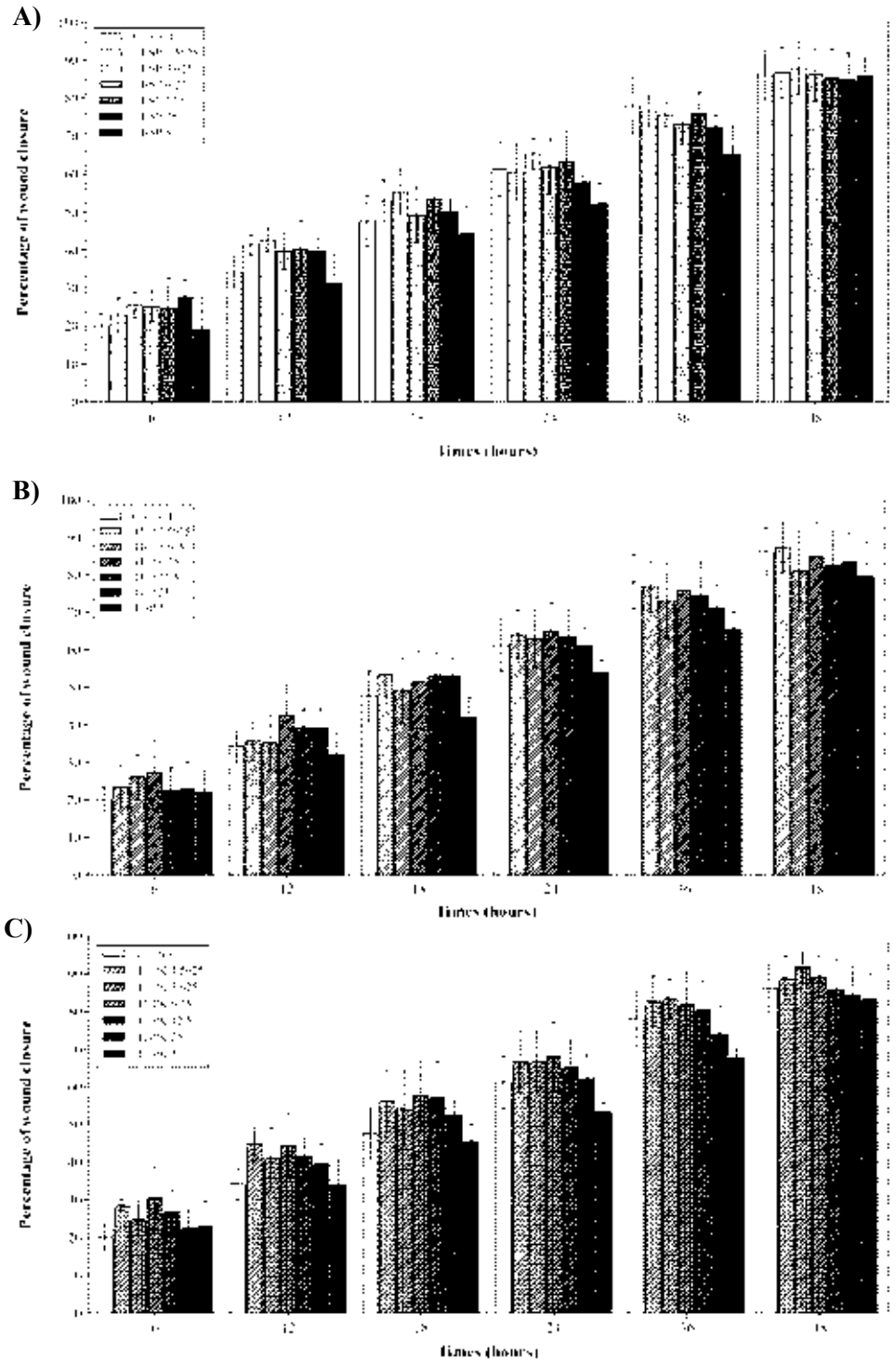


**Figure 26** Effect of TSCEs on the percentage of wound closure in human foreskin fibroblast cells (CCD-1064Sk) after scratched cells were treated at proliferative concentrations of TSCEs for 6-48 hours. (A) TI-SP-treated cells, (B) TI-P-treated cells and (C) TI-PK-treated cells. Data are mean ± standard error.

on the rate of AGS wound healing by using the scratch assay. The AGS cells monolayer was scratched and treated with the concentrations stimulating cell proliferation (0.625-20 μg/ml). The wound area of scratched cells was recorded by using the inverted microscope at 6, 12, 18 and 24 hours. The complete wound closure was observed at 24 hours. The wound area decreased with time dependent manner. The percent wound closure is shown in **Figure 27**. In proliferative concentrations of TSCEs, the percent wound closure of TSCEs-treated cells were not significantly different the rate of wound closure compared to vehicle control cells ($P < 0.05$). Thus, the effect on the stimulating the rate of wound closure of TSCEs on AGS cells was not observed. The results indicated that the stimulating on the rate of wound healing of proliferative concentrations of TSCEs was not observed in CCD-1064Sk and AGS cells.

Burlando *et al*. (2009) reported that the wound healing is correlated with the stimulation of motility (cell migration) rather than of mitosis (cell proliferation). The compound stimulated slightly cell proliferation cannot clearly show the rapid role on wound closure. The high cytotoxic and not stimulate of cell proliferation compound can exhibit strong promotion of wound closure by inducing the migration of keratinocytes (HaCaT) cells. The present studied was also showed the similar result, the proliferative concentrations of TSCEs were not stimulated the increasing the rate of wound healing on CCD-1064Sk and AGS cells.

The result of their study is correlated with the present study. Phan *et al*. (2001) found that the phenolic compounds from *Chromolaena odorata* may be one of the potential to enhance cutaneous wound healing. The phenolic compounds from *Chromolaena odorata* exhibited powerful antioxidant compounds against $H_2O_2$-induced damage cultured skin cells. Moreover, antioxidant compound from curcumin showed good protective effect against $H_2O_2$-induced fibroblast injury. This study suggests that curcumin compounds can be used to develop a preparation for the treatment of burns and chronic wounds (Phan *et al*., 2001). These results suggested that the phenolic and antioxidant compounds have benefit for treatment of wounds. The effect of TSCEs on the rate of wound repair against $H_2O_2$-induced the delay rate of wound closure will be evaluated in the further study.

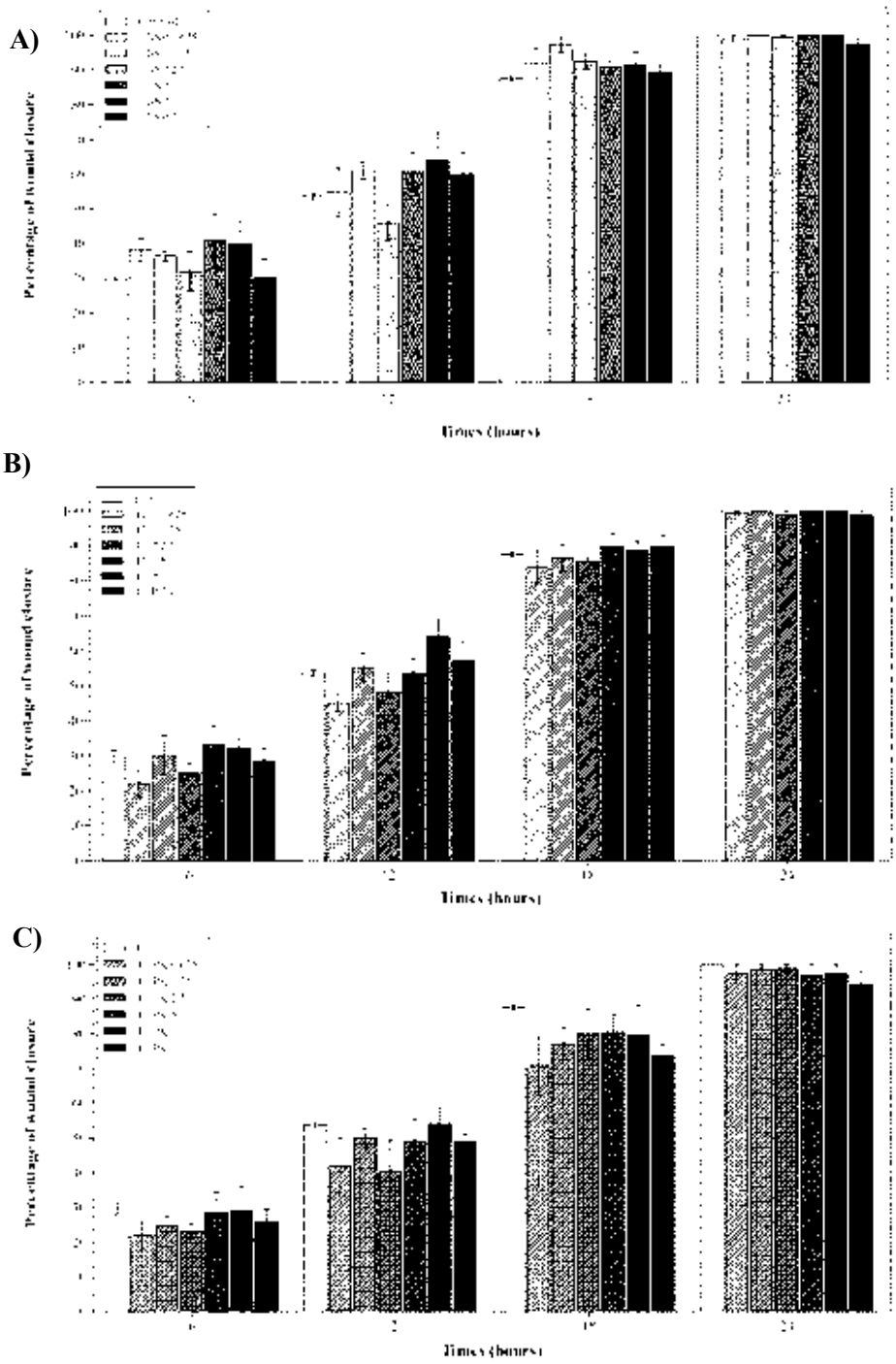


**Figure 27** Effect of TSCEs on the percentage of wound closure in human adenocarcinoma gastric adenocarcinoma epithelial cells (AGS) after scratched cells were treated at proliferative concentrations of TSCEs for 6-24 hours. (A) TI-SP-treated cells, (B) TI-P-treated cells and (C) TI-PK-treated cells. Data are mean ± standard error.

## 7. Protective effect of TSCEs on the rate of wound repair in $H_2O_2$–induced oxidative stress cells in human cell lines-CCD-1064Sk and AGS cells

### 7.1 Effect of hydrogen peroxide ($H_2O_2$) on the delayed rate of wound repair in human cell lines-CCD-1064Sk and AGS cells

Hydrogen peroxide ($H_2O_2$) reduces both migration and proliferation of cells at wound edge. Moreover, $H_2O_2$ can increase oxidative stress in wound area that affects the delay rate of wound repair. In Prolonged inflammation, inflammatory cells (neutrophils and macrophages) can migrate into the damaged tissues and generate superoxide anion radicals resulting in oxidative stress in the wound and produce the amounts of excessive ROS. The excessive amounts of ROS exhibit a major key on cells damage and oxidative stress in wounds. The enhance ROS can induce the acute wound to the chronic wound or non-healing wound (Mohammad *et al*., 2008; Schäfer and Werner, 2008). In this experiment, $H_2O_2$ was used to induce oxidative stress in wound and delayed the rate of wound repair (chronic wound). The cell monolayer was scratched and then exposured to 0.1-2 mM $H_2O_2$ for 15, 30 and 60 min, respectively, and the scratched cell was incubated for 24 hours in complete medium. The wound area of the scratched cell was recorded and analyzed as the percent wound closure compared to vehicle control cells. In **Figure 28** showed that $H_2O_2$ reduced the percent wound closure at high concentration and for long time incubations in both cells (CCD-1064Sk and AGS cells). At the higher concentrations and longer time incubations of $H_2O_2$ showed significantly lower the rate of wound closure than vehicle control cells ($P<0.05$). $H_2O_2$ exposured at concentration 2 mM for 15 min in CCD-1064Sk cells (52.75 ± 6.47%) and 30 min in AGS cells (45.19 ± 7.63%) were used in the present study to induce oxidative stress in scratched wound and the delayed rate of wound repair. The bright images of CCD-1064Sk and AGS cells were shown in **Figure 29 and 30**, respectively.

Loo *et al*., (2011) demonstrated that at low concentrations of $H_2O_2$ can stimulate proliferation and migration of keratinocytes in the scratch assay through the ERK pathway. At high concentrations and long time incubations with $H_2O_2$ can inhibit the migration of keratinocyte cells at wound edge by disruption ERK1/2 signaling.

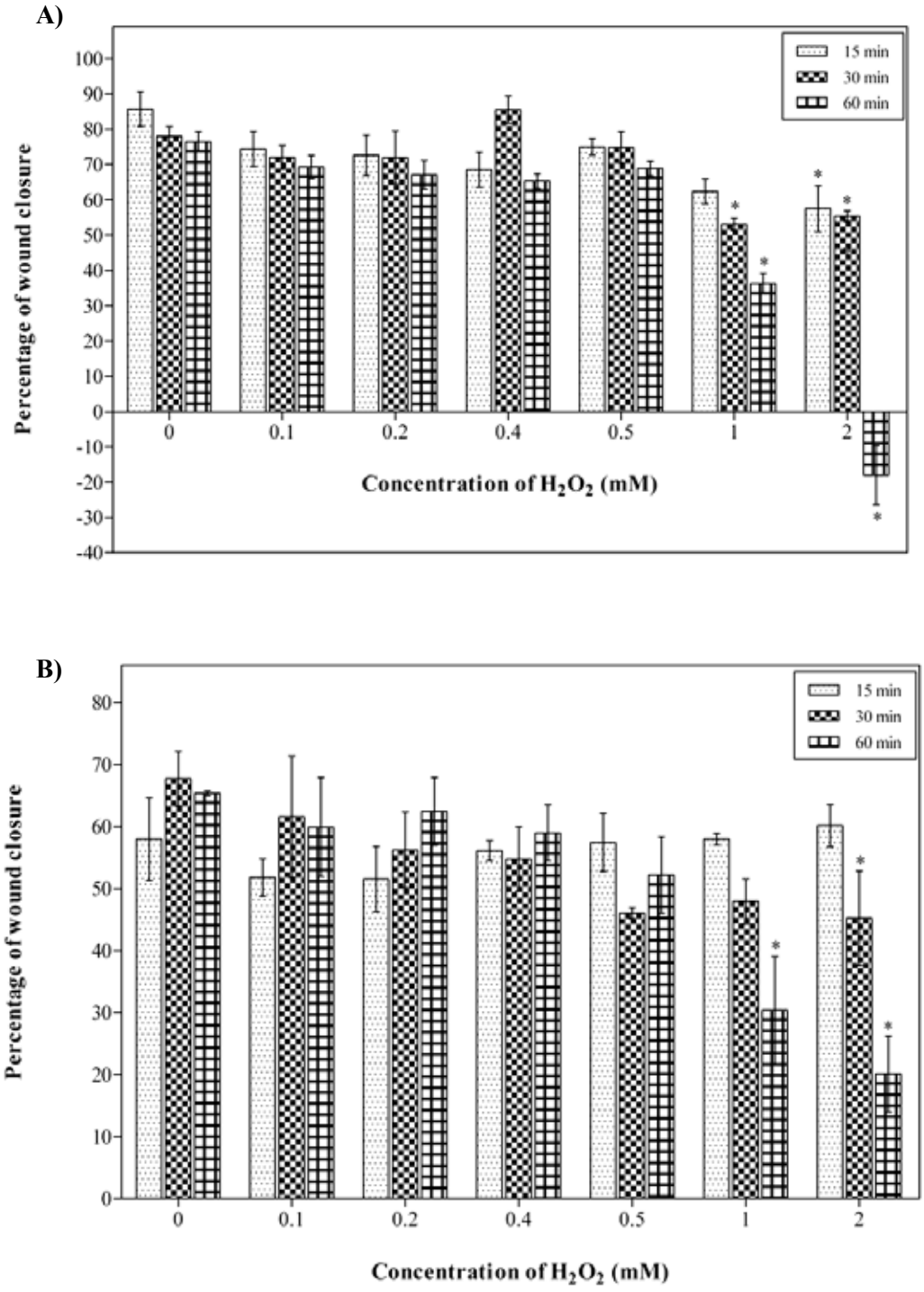


**Figure 28** Effect of $H_2O_2$ on the delay of wound closure on the scratched cells. (A) the scratched human foreskin fibroblast cells (CCD-1064Sk), (B) the scratched human gastric adenocarcinoma epithelial cells (AGS). The scratched cells were exposed to $H_2O_2$ at wound area concentration ranges 0.1-2 mM for 15-60 min. Data are mean ± standard error, *$P< 0.05$ compared to control.

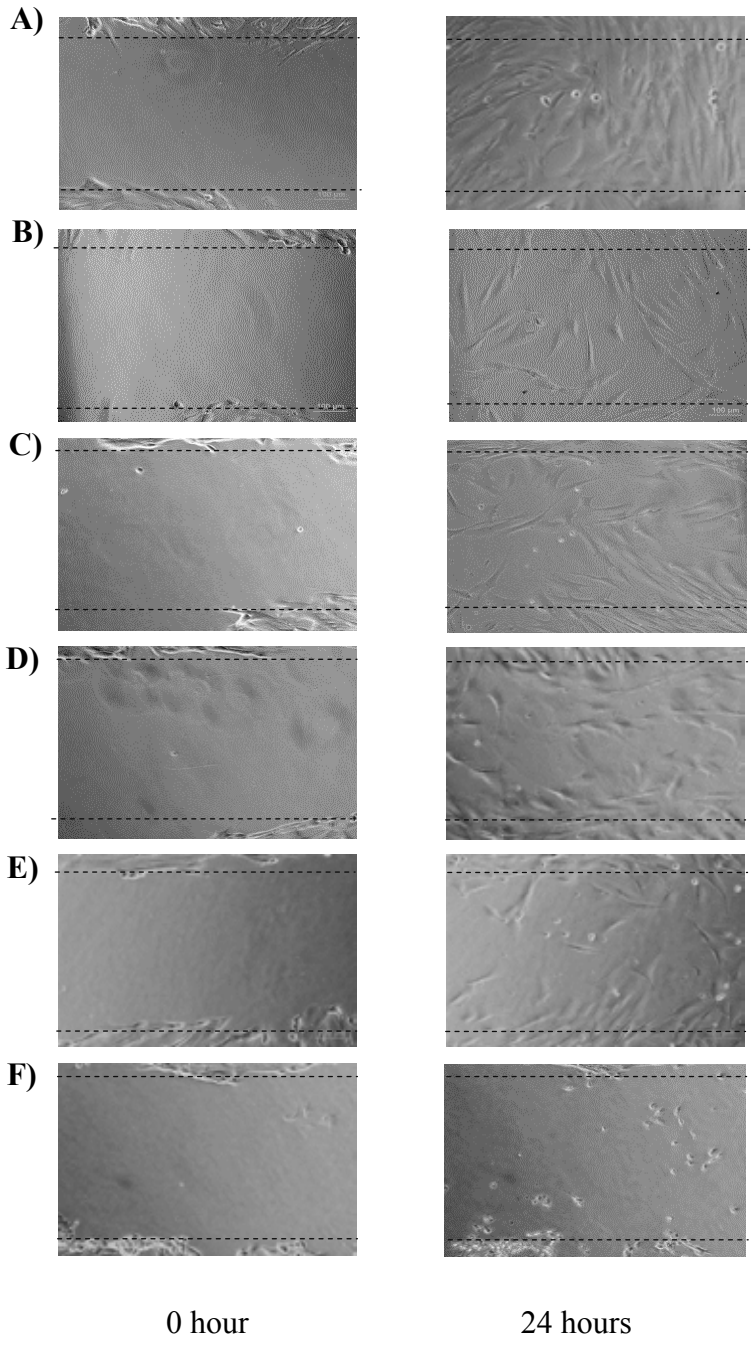


**Figure 29** Images of wound areas of CCD-1064Sk cells taken at 0 and 24 hours, after incubation with various concentrations of $H_2O_2$ for different times. (A) Control (untreated with $H_2O_2$), (B) treated cells with 2 mM $H_2O_2$ for 15 min, (C) treated cells with 1 mM $H_2O_2$ for 30 min, (D) treated cells with 2 mM $H_2O_2$ for 30 min, (E) treated cells with 1 mM $H_2O_2$ for 60 min and (F) treated cells with 2 mM $H_2O_2$ for 60 min.

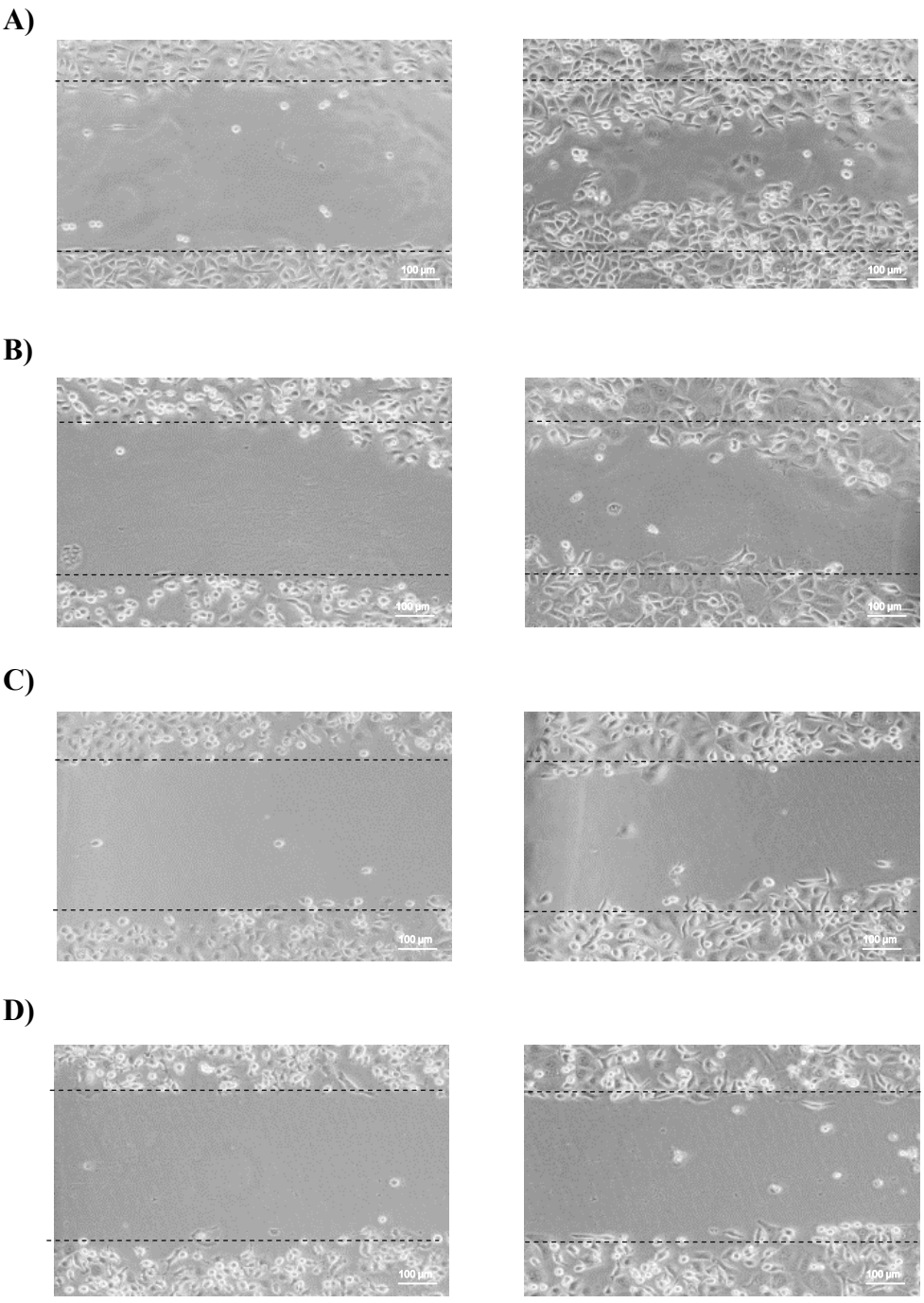


**Figure 30** Images of wound areas of AGS cells taken at 0 and 24 hours, after incubation with various concentrations of $H_2O_2$ for different times. (A) Control (untreated with $H_2O_2$), (B) treated cells with 2 mM $H_2O_2$ for 30 min, (C) treated cells with 1 mM $H_2O_2$ for 60 min and (D) treated cells with 2 mM $H_2O_2$ for 60 min.

The similar result was found in the present study. Incubation at low concentrations of $H_2O_2$ for short times showed the rate of wound closure not significant faster than control group.

Thomas *et al*. (2009) found that at very low concentrations of $H_2O_2$ can contribute the wound healing process of fibroblast cells, while high concentrations of $H_2O_2$ is reduced both migration and proliferations of fibroblast cells in concentration-dependent fashion by using scratch assay. Moreover, $H_2O_2$ significantly delayed epithelial migration in wound edge by using scratch assay (Choi *et al*., 2008). Thus, $H_2O_2$ is suitable for inducing the delayed rate of wound closure by using scratch assay.

**7.2 Protective effect of TSCEs on the rate of wound repair in human foreskin fibroblast cells**

The scavenging effect of TSCEs on ROS in wound was evaluated, the cell monolayer of CCD-1064Sk was scratched and exposed with 2 mM for 15 min and then treated with 3.625-300 μg/ml of TSCEs for 24 hour. The wound area of scratched cell was captured after treated cells with TSCEs for 24 hour. The result in **Figure 31A** showed that $H_2O_2$-treated cells exhibited significantly lower the rate of wound repair than the vehicle control cells approximately 30% ($P < 0.05$). TSCE of TI-SP treated cell exhibited the significantly increasing rate of wound closer at concentration ranges 6.25-12.5 μg/ml (**Figure 31A**). At the higher concentrations of TSCEs (25-200 μg/ml), the rate of wound closure showed not significantly higher than the $H_2O_2$-treated cells without TSCEs, where at 300 μg/ml TSCEs inhibited the wound closure. In addition, 6.25-12.5 μg/ml of TSCE from TI-SP showed the significantly higher in the percent wound closure (63.40-64.91%) than the $H_2O_2$-treated cells without TSCE ($P<0.05$), the bright fields of wound areas are shown in **Figure 32**.

The low ROS levels has been reported to be the key factor in wound healing process (Keller *et al*., 2006; Schäfer and Werner, 2008). The result of TSCEs on intracellular ROS supported that high concentrations of TSCEs showed decreasing intracellular ROS effect in CCD-1064Sk cells. Thus, the increasing the rate of wound

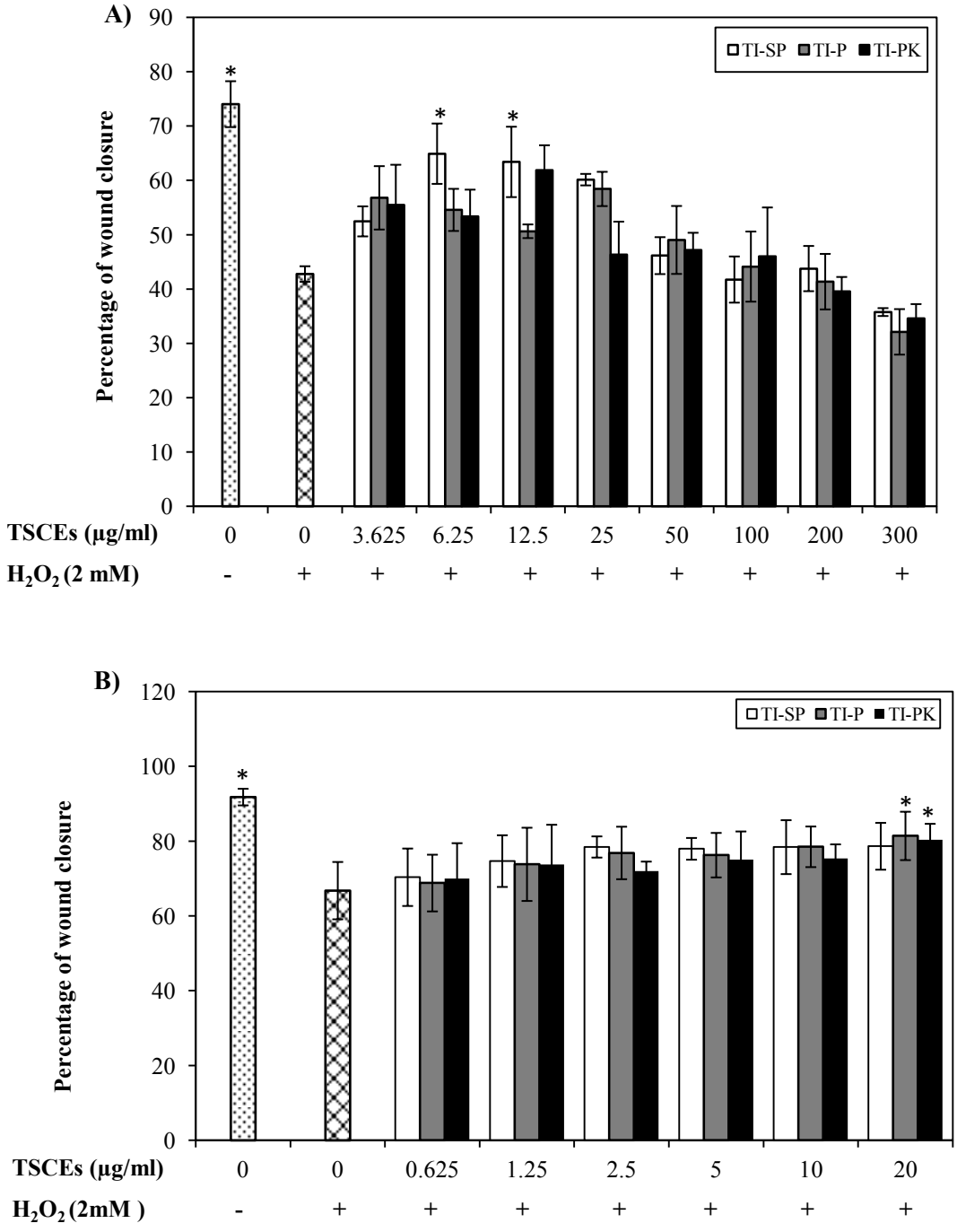


**Figure 31** Effect of TSCEs on the percentage of wound closure in $H_2O_2$-induced wound injury. (A) the human foreskin fibroblast cells (CCD-1064Sk), (B) the human gastric adenocarcinoma epithelium cells (AGS). Cells were exposed with $H_2O_2$ and treated with TSCEs for 24 hours. Data are mean ± standard error, *$P< 0.05$ compared to control (untreated with TSCEs).

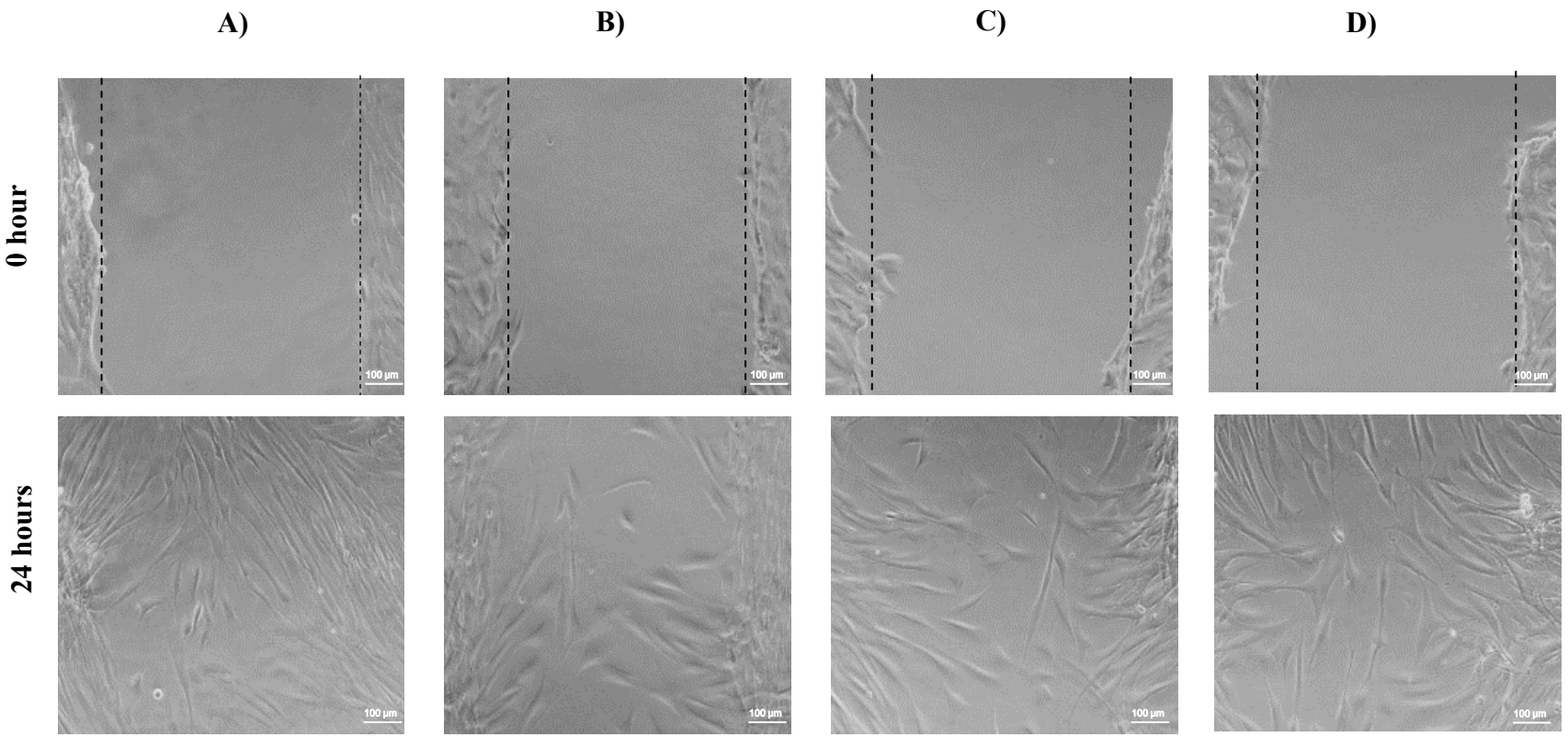


**Figure 32** Images of wound areas of CCD-1064Sk cells taken at 0 and 24 hours. Cells were exposed with 2 mM $H_2O_2$ for 15 min and treated with TSCEs for 24 hours.(A) control (non-treated cells), (B) treated cells with 2 mM $H_2O_2$ for 15 min, (C) treated cells with 2 mM $H_2O_2$ for 15 min and 6.25 µg/ml of TI-SP and (D) treated cells with 2 mM $H_2O_2$ for 15 min and 12.5 µg/ml of TI-SP(original magnification × 10)

closure at the low TSCEs concentrations may be due to the strongly free radical scavenging effect of TSCEs, resulting in decreasing the ROS in cells.

The study of Shetty *et al*. (2007) supported this study. The antioxidant compounds are importance for wound healing effect in oxidative stress conditions. The antioxidant compounds from alcoholic and aqueous *Ocimum sanctum* extracts exhibited faster skin wound healing, increasing antioxidant enzyme activity and decreasing lipid peroxidation of wound in rats.

**7.3 Protective effect of TSCEs on the rate of wound repair in human gastric adenocarcinoma epithelium cell**

The scavenging effect of TSCEs on ROS in wound was evaluated by using scratch assay. The monolayer cell was scratched and exposed to 2 mM $H_2O_2$ for 30 min and then treated with 0.625-20μg/ml of TSCEs and incubated for 24 hours, the wound area of scratched cells was recorded and calculated in the percent wound closure. The delay rate of wound closure in **Figure 31B** was observed in $H_2O_2$ treated cells compared to the vehicle control cells ($P<0.05$) approximately 20%. TSCEs-treated cells increased the rate of wound closure in the concentration-dependent manner. The 20μg/ml of TSCEs from the sour tamarind (TI-P, TI-PK) exhibited the significantly increase the rate of wound closure (81.42 ± 6.47, 80.37 ± 4.29%), whereas the TSCE from sweet tamarind (TI-SP) showed not significantly increased the wound closure rate compared to $H_2O_2$-treated cells without TSCEs ($P<0.05$), the bright fields of wound areas are shown in **Figure 33**

The results suggest that, TSCEs from sour tamarind potentiate the ROS scavenging effect in AGS cells to accelerate the percent wound closer.

Choi *et al*. (2008) studied the effect of eupatilin against oxidative damage on gastric adenocarcinoma epithelium cells (AGS) by using scratch assay. The stimulation of cell proliferation and migration of eupatilin did not observe, but this compound prevented the reduction of epithelium migration induced by $H_2O_2$. This compound suggested the acts as antioxidant and may be developed for repair the gastric ulcer. The result supports the present study, TSCEs showed antioxidant activity against $H_2O_2$-induced the delayed rate of wound closure on AGS scratched cells.

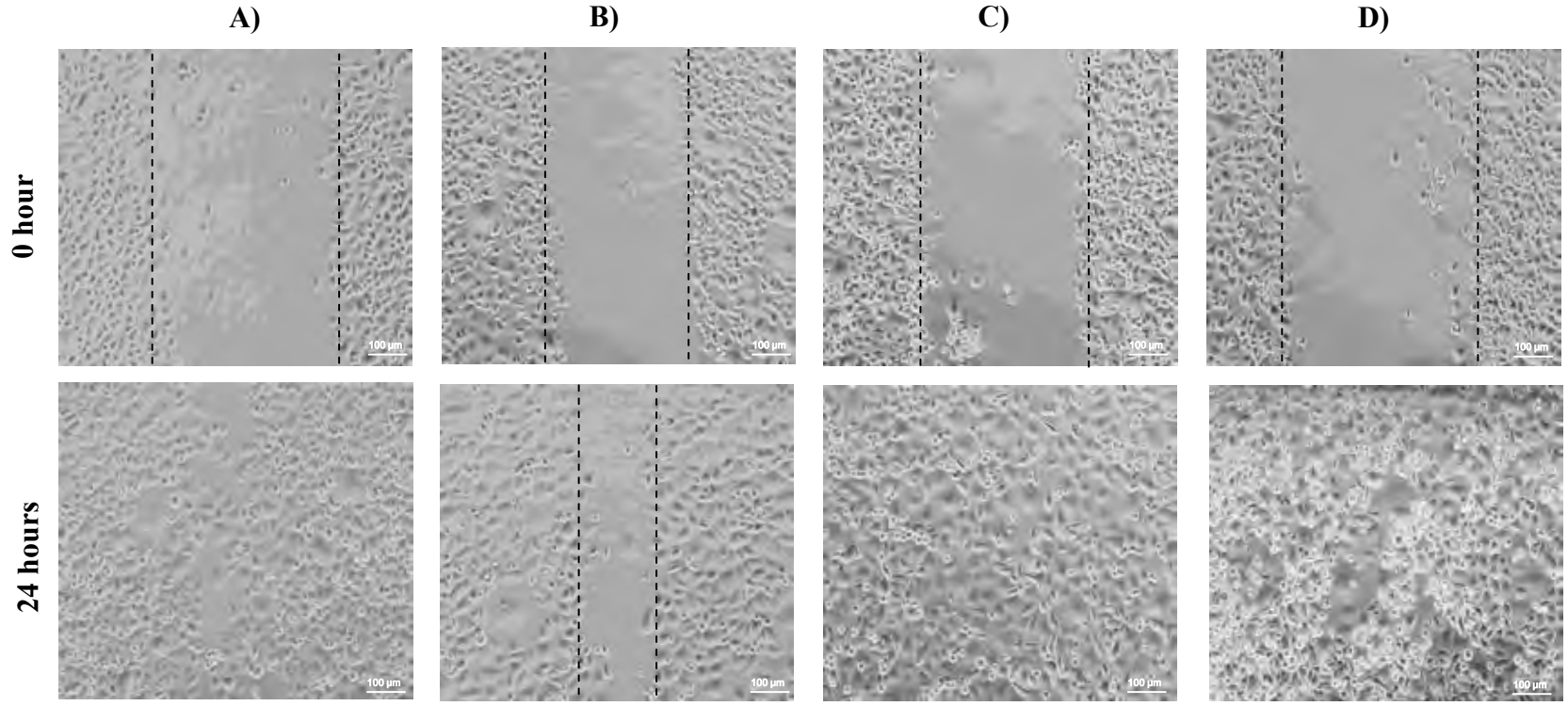


**Figure 33** Images of wound areas of AGS cells taken at 0 and 24 hours. Cells were exposed with 2 mM $H_2O_2$ for 30 min and treated with TSCEs for 24 hours. (A) control (non-treated cells), (B) treated cells with 2 mM $H_2O_2$ for 30 min, (C) treated cells with 2 mM $H_2O_2$ for 30 min and 20 µg/ml of TI-P and (D) treated cells with 2 mM $H_2O_2$ for 30 min and 20 µg/ml of TI-PK (original magnification × 10).

Moreover, the antiulcer effect of methanolic extract from tamarind seed-coat in rats was investigated. The methanolic tamarind seed-coat extract shows cytoprotective effect in ibuprofen, alcohol, and pylorus-ligation-induced ulcer models. The cytoprotective effect may be due to the free radical scavenging activity of tamarind seed-coat. This reported supported the potential of TSCEs on antiulcer activity (Kalra *et al*., 2011).

The antioxidant activity has been reported the involvement on scavenging of oxygen anion-radicals and correlated with cytoprotective activity (Potapovich and Kostyuk, 2003). The different cultivars are influenced the concentration of active compounds, resulting in the different antioxidant activity (Danesi *et al*., 2008 and Saravanan and Aradhya, 2011). The results of this study suggested that the phenolic compounds of TSCEs exhibited anitioxidant activity. Antioxidant activity showed protective effect against oxidative stress by $H_2O_2$ in CCD-1064Sk and AGS cells and the antioxidant activity of TSCEs from different cultivars was differed. The capacity of antioxidant compound of TSCEs from different cultivars in cultured cells was correlated with the study of Suksomtip *et al*. (2010). TSCEs from sour tamarinds exhibited better antioxidant activity than the sweet tamarind due to the sour tamarinds composed higher polymeric compound than sweet tamarind. Moreover, this study supported that the effect of TSCEs on traditional medicine, the tamarind seed-coats have been reported in using as an astringent for treat burn and chronic wound in diabetic patients.

# CHAPTER V

# CONCLUSIONS

The solvents extraction of tamarind seed-coat extracts (TSCEs) including boiling water, ethyl acetate and 70% acetone showed different amount of the phenoilc content. TSCE from 70% acetone fraction having the highest tannin and proanthocyanidin content and exhibited more cytotoxic effect than boiling water and ethyl acetate fractions on human foreskin fibroblast cell (CCD-1064Sk) and human gastric adenocarcinoma epithelial cell line (AGS). The ethyl acetate fraction showed the highest the total phenolic content and showed the least cytotoxic effect on both human cell lines.

Tamarind seed-coat from different cultivars including TSCEs from the sweet tamarind “Srichomphu” (TI-SP) and the sour tamarinds “Priao-native” (TI-P) and “Priao-Kradan” (TI-PK) were extracted by using boiling water and partitioned with ethyl acetate. The chemical analysis of TSCEs was determined. The TSCEs from sour tamarind showed higher the percent tannin than the sweet tamarind and the TSCE from TI-PK showed highest proanthocyanidin content. The HPLC fingerprint of TSCEs demonstrated the profile of three peaks identical with the standard (+)-catechin, (-)-epicatechin and procyanidin B2. The chromatograms of TSCEs showed peaks at the same retention time of standard flavonoid compounds.

The cytotoxic effect of TSCEs from ethyl acetate fraction of different tamarind cultivars on CCD-1064Sk and AGS cells was studied. These results indicated that the responses of CCD-1064Sk cells and AGS cells to the given TSCEs were different. At higher concentrations of TSCEs showed cytotoxic effect on both human cell lines, TSCEs exhibited higher cytotoxic effect on AGS cells than CCD-1064Sk cells.

The cytoprotective effect of TSCEs on CCD-1064Sk and AGS cells was examined. TSCEs potentiated protective effect of both human cells against oxidative stress by using $H_2O_2$-induced cell injury. TSCEs showed protective effect on CCD-1064Sk and AGS cells by decreasing the number of damaged cell and reducing the intracellular ROS levels in both tested human cells. TSCEs from the sour tamarind

exhibited protective effect better than the sweet tamarind. In addition, at higher concentrations of TSCEs directly decreased the intracellular ROS in CCD-1064Sk cells.

At lower concentrations of TSCEs exhibited proliferative effect on CCD-1064Sk and AGS cells. The sweet tamarind showed proliferative effect in concentration ranges more than the sour tamarinds. These concentrations were used for study the effect on the rate of wound closure by using scratch assay. TSCEs at proliferative concentrations were not effective on accelerated on the rate of wound closure on both human cell lines.

TSCEs exhibited protective effect on $H_2O_2$-induced oxidative stress in wound area of CCD-1064Sk and AGS cells. TSCEs increased the rate of wound closure of $H_2O_2$-induced the delay rate of wound repair. At the lower concentration of TI-SP potentiated the scavenging effect of TSCEs on ROS in wound to increase the wound repair in CCD-1064Sk. TSCEs from sour tamarinds potentiate the ROS scavenging effect in AGS cells to accelerate the percent wound closure.

Antioxidant compound in TSCEs effectively pretreated cells from oxidative stress on human cells and can be applied as a therapeutic agent for chronic wound management.

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# APPENDICES

# APPENDIX A

# PREPARATION OF REAGENTS

**Growth medium of human fibroblast cells**

Iscove's Modified Dulbecco's medium powder was dissolves with ultrapure water and the 3.024 g of sodium bicarbonate was added. The medium was mixed and adjusted volume to 1,000 ml with ultrapure water. The medium was sterilized by filtration by using 0.2 μm Bottle-Top Vacuum Filters. Before using, the medium was supplement with 10% FBS and 1% penicillin and streptomycin.

**Growth medium of human gastric epithelium cells**

Ham's F-12 nutrient medium powder was dissolves with ultrapure water and the 1.176 g of sodium bicarbonate was added. The medium was mixed and adjusted the pH to 7.2-7.4 with HCl. After that, the medium was adjusted volume to 1,000 ml with ultrapure water. The medium was sterilized by filtration by using 0.2 μm Bottle-Top Vacuum Filters. Before using, the medium was supplement with 10% FBS and 1% penicillin and streptomycin.

**Phosphate Buffer Saline (PBS)**

To make 1 L of PBS, ingredients of PBS solution including 8.00 g of NaCl, 0.20 g of KCl, 1.15 g of $Na_2HPO_4$ and 0.20 g of $KH_2PO_4$ were dissolved in 800 ml of ultrapure water and then adjusted the pH to 7.2-7.4 with HCl. After that, the solution was adjusted the volume to 1000 ml and sterilized by autoclave before stored at room temperature.

# APPENDIX B

# TABLES OF EXPERIMENTAL RESULTS

**Table 3** The percentage of CCD-1064Sk cell viability in response to various concentrations of TSCEs from the three different solvent fractions by MTT assay.

| Concentration (µg/ml) | Fraction | | |
|---|---|---|---|
| | Boiling water | Ethyl acetate | 70%Acetone |
| 0 | 100 ± 0.00 | 100 ± 0.00 | 100 ± 0.00 |
| 62.5 | 116.10 ± 4.73 | 99.13 ± 5.54 | 120.37 ± 4.17 |
| 125 | 126.92 ± 8.43 | 108.99 ± 4.38 | 114.88 ± 8.40 |
| 250 | 117.37 ± 4.53 | 113.57 ± 5.46 | 131.47 ± 7.94 |
| 500 | 107.25 ± 9.60 | 125.81 ± 5.28 | 122.39 ± 8.34 |

Each value represents as mean ± SEM of three independent experiments.

**Table 4** The percentage of AGS cell viability in response to various concentrations of TSCEs from the three different solvent fractions by MTT assay.

| Concentration (µg/ml) | Fraction | | |
|---|---|---|---|
| | Boiling water | Ethyl acetate | 70%Acetone |
| 0 | 100 ± 0.00 | 100 ± 0.00 | 100 ± 0.00 |
| 62.5 | 90.02±2.32 | 86.97 ± 4.27 | 72.05 ± 5.78* |
| 125 | 88.62 ± 3.91 | 82.23 ± 5.48 | 33.75 ± 5.48* |
| 250 | 29.35 ± 2.98* | 73.85 ± 7.09* | 24.65 ± 3.77* |
| 500 | 16.13 ± 3.43* | 49.98 ± 4.58* | 15.46 ± 1.24* |

Each value represents as mean ± SEM of three independent experiments. *$P > 0.05$ versus non treated control by one way ANOVA.

**Table 5** The inhibitory concentration of fifty percentage ($IC_{50}$) values of TSCEs from the three different solvent fractions on CCD-1064Sk and AGS cells.

| Fraction | $IC_{50}$ (μg/ml) | |
|---|---|---|
| | CCD-1064Sk | AGS |
| Boiling water | > 500 | 203.5±2.89[a] |
| Ethyl acetate | > 500 | > 500[b] |
| 70%Acetone | > 500 | 106.50 ± 14.38[a] |

Each value represents as mean ± SEM of three independent experiments.
a,b,c = significantly different between group ($P < 0.05$)

**Table 6** The percentage of CCD-1064Sk cell death in response to various concentrations of TSCEs from the three different solvent fractions by DNA staining with Hoechst 33342 dye.

| Concentration (μg/ml) | Fraction | | |
|---|---|---|---|
| | Boiling water | Ethyl acetate | 70%Acetone |
| 0 | 1.07 ± 0.21 | 1.30 ± 0.70 | 1.03 ± 0.16 |
| 62.5 | 0.88 ± 0.38 | 1.47 ± 0.35 | 0.99 ± 0.13 |
| 125 | 1.85 ± 0.49 | 1.12 ± 0.42 | 8.89 ± 3.14* |
| 250 | 3.10 ± 0.68* | 1.04 ±0.31 | 14.52 ± 4.03* |
| 500 | 14.83 ± 6.28* | 0.79 ± 0.11 | 24.23 ± 5.28* |

Each value represents as mean ± SEM of three independent experiments. *$P > 0.05$ versus non treated control by one way ANOVA.

**Table 7** The percentage of AGS cell death in response to various concentrations of TSCEs from the three different solvent fractions by DNA staining with Hoechst 33342 dye.

| Concentration (μg/ml) | Fraction | | |
|---|---|---|---|
| | Boiling water | Ethyl acetate | 70%Acetone |
| 0 | 1.11 ± 0.37 | 1.11 ± 0.37 | 1.11 ± 0.37 |
| 62.5 | 4.12±1.06 | 4.01 ± 068 | 27.57 ± 9.32* |
| 125 | 6.52 ± 0.11 | 4.66 ± 0.78 | 90.85 ± 2.31* |
| 250 | 39.41 ± 4.86* | 4.49 ± 1.37 | 94.27 ± 0.55* |
| 500 | 93.30 ± 2.04* | 4.84 ± 1.21 | 96.19 ± 0.74* |

Each value represents as mean ± SEM of three independent experiments. *$P > 0.05$ versus non treated control by one way ANOVA.

**Table 8** The percentage of CCD-1064Sk cell viability in response to 10 fold concentration series of TSCEs from the three different cultivars by MTT assay.

| Concentration (μg/ml) | Cultivar | | |
|---|---|---|---|
| | TI-SP | TI-P | TI-PK |
| 0 | 100 ± 0.00 | 100 ± 0.00 | 100 ± 0.00 |
| 0.02 | 98.88 ± 1.15 | 104.32 ± 1.07 | 99.95 ± 1.91 |
| 0.2 | 96.34 ± 1.50 | 105.41 ± 0.44 | 100.52 ± 3.64 |
| 2 | 95.08 ± 1.33 | 101.79 ± 1.96 | 102.19 ± 2.92 |
| 20 | 110.61 ± 6.32* | 106.22 ± 4.75 | 108.91 ±9.15 |
| 200 | 151.17 ± 4.87* | 162.93 ± 13.10* | 140.00 ± 5.12* |
| 2000 | 74.11 ± 4.78* | 50.21 ± 4.17* | 71.13 ± 2.75* |

Each value represents as mean ± SEM of three independent experiments. *$P > 0.05$ versus non treated control by one way ANOVA.

**Table 9** The percentage of CCD-1064Sk cell viability in response to 2 fold concentration series of TSCEs from the three different cultivars by MTT assay.

| Concentration (μg/ml) | Cultivars | | |
|---|---|---|---|
| | TI-SP | TI-P | TI-PK |
| 0 | 100 ± 0.00 | 100 ± 0.00 | 100 ± 0.00 |
| 62.5 | 114.05 ± 0.79* | 111.73± 1.87 | 112.11 ± 3.24 |
| 125 | 121.08 ± 3.23* | 115.17 ± 1.80* | 118.3 ± 1.88* |
| 250 | 133.38 ± 4.28* | 121.18 ± 1.51* | 124.31 ± 1.11* |
| 500 | 155.55 ± 1.36* | 126.94 ± 1.84* | 111.57 ± 2.96 |
| 1000 | 173.38 ±2.97* | 144.36 ± 1.84* | 102.74 ± 3.57 |
| 2000 | 91.65 ± 3.35 | 56.91 ± 1.72* | 57.47 ± 1.14* |

Each value represents as mean ± SEM of three independent experiments. *$P > 0.05$ versus non treated control by one way ANOVA.

**Table 10** The percentage of AGS cell viability in response to 10 fold concentration series of TSCEs from the three different cultivars by MTT assay.

| Concentration (μg/ml) | Cultivar | | |
|---|---|---|---|
| | TI-SP | TI-P | TI-PK |
| 0 | 100 ± 0.00 | 100 ± 0.00 | 100 ± 0.00 |
| 0.02 | 95.40 ± 1.62 | 94.86 ± 0.46 | 100.37 ± 2.46 |
| 0.2 | 103.89 ± 1.55 | 100.05 ±1.80 | 100.18 ± 1.22 |
| 2 | 106.93 ± 3.50 | 98.45 ± 2.05 | 104.46 ± 4.21 |
| 20 | 89.86 ± 4.29 | 100.32 ± 0.38 | 86.21± 1.89 |
| 200 | 33.35 ± 2.31* | 14.77 ± 6.99* | 8.69 ± 3.56* |
| 2000 | 23.33 ± 1.12* | 21.89 ± 3.753* | 16.51 ± 2.48* |

Each value represents as mean ± SEM of three independent experiments. *$P > 0.05$ versus non treated control by one way ANOVA.

**Table 11** The percentage of AGS cell viability in response to 2 fold concentration series of TSCEs from the three different cultivars by MTT assay.

| Concentration (µg/ml) | Cultivar | | |
|---|---|---|---|
| | TI-SP | TI-P | TI-PK |
| 0 | 100 ± 0.00 | 100 ± 0.00 | 100 ± 0.00 |
| 62.5 | 90.16 ± 4.72 | 87.22 ± 1.02 | 69.43 ± 3.09* |
| 125 | 71.15 ± 2.80* | 54.69 ± 5.20* | 33.32 ± 4.22* |
| 250 | 19.31 ±0.81* | 10.22 ± 1.49* | 9.53 ± 0.64* |
| 500 | 3.34 ± 1.72* | 0.09 ± 0.08* | 4.22 ± 0.36* |
| 1000 | 5.47 ± 3.08* | 6.01 ± 1.54* | 16.13 ± 0.36* |
| 2000 | 30.17 ± 6.69* | 28.34 ± 1.61* | 32.99 ± 1.38* |

Each value represents as mean ± SEM of three independent experiments. *$P > 0.05$ versus non treated control by one way ANOVA.

**Table 12** The percentage of CCD-1064Sk cell viability in response to 10 fold concentration series of TSCEs from the three different cultivars by NRU assay.

| Concentration (µg/ml) | Cultivar | | |
|---|---|---|---|
| | TI-SP | TI-P | TI-PK |
| 0 | 100 ± 0.00 | 100 ± 0.00 | 100 ± 0.00 |
| 0.02 | 104.79 ± 0.54 | 101.21 ± 1.53 | 101.46 ± 0.72 |
| 0.2 | 105.38 ± 3.05 | 103.53 ± 1.60 | 101.17 ± 2.35 |
| 2 | 100.39 ± 0.37 | 100.52 ± 2.28 | 103.63 ± 5.32 |
| 20 | 113.59 ± 3.24 | 112.407 ± 4.75 | 113.66 ± 1.79 |
| 200 | 96.79 ± 2.03 | 94.95 ± 5.99 | 93.88 ± 1.89 |
| 2000 | 25.02 ± 3.66* | 27.39 ± 2.22* | 24.31 ± 4.48* |

Each value represents as mean ± SEM of three independent experiments. *$P > 0.05$ versus non treated control by one way ANOVA.

**Table 13** The percentage of CCD-1064Sk cell viability in response to 2 fold concentration series of TSCEs from the three different cultivars by NRU assay.

| Concentration (µg/ml) | Cultivar | | |
|---|---|---|---|
| | TI-SP | TI-P | TI-PK |
| 0 | 100 ± 0.00 | 100 ± 0.00 | 100 ± 0.00 |
| 62.5 | 104.80 ± 0.54 | 101.21 ± 1.53 | 101.46 ± 0.72 |
| 125 | 105.38 ± 3.05 | 103.53 ± 1.60 | 101.17 ± 2.35 |
| 250 | 100.39 ± 0.39 | 100.52 ± 2.28 | 96.97 ± 1.57 |
| 500 | 93.59 ± 3.80 | 92.41 ± 1.25 | 71.32 ± 0.60* |
| 1000 | 75.79 ± 4.00* | 71.61 ± 5.47* | 55.89 ± 3.11* |
| 2000 | 22.69 ± 1.87* | 44.05 ±2.49* | 27.65 ± 2.51* |

Each value represents as mean ± SEM of three independent experiments. *$P > 0.05$ versus non treated control by one way ANOVA.

**Table 14** The percentage of AGS cell viability in response to 10 fold concentration series of TSCEs from the three different cultivars by NRU assay.

| Concentration (µg/ml) | Cultivar | | |
|---|---|---|---|
| | TI-SP | TI-P | TI-PK |
| 0 | 100 ± 0.00 | 100 ± 0.00 | 100 ± 0.00 |
| 0.02 | 98.46 ± 3.54 | 97.89 ± 4.07 | 95.45 ± 2.66 |
| 0.2 | 109.67 ± 5.62 | 105.81 ± 3.61 | 105.12 ± 2.41 |
| 2 | 117.87 ± 2.02 | 115.23 ± 3.43* | 111.43 ± 3.54 |
| 20 | 96.82 ± 3.93 | 89.98 ± 5.71 | 86.08 ± 4.25 |
| 200 | 9.37 ± 1.02* | 5.58 ± 3.61* | 1.19 ± 1.72* |
| 2000 | 9.51 ± 1.78* | 8.25 ± 1.52* | 6.38 ± 1.57* |

Each value represents as mean ± SEM of three independent experiments. *$P > 0.05$ versus non treated control by one way ANOVA.

**Table 15** The percentage of AGS cell viability in response to 2 fold concentration series of TSCEs from the three different cultivars by NRU assay.

| Concentration (µg/ml) | Cultivar | | |
|---|---|---|---|
| | TI-SP | TI-P | TI-PK |
| 0 | 100 ± 0.00 | 100 ± 0.00 | 100 ± 0.00 |
| 62.5 | 86.81 ± 1.87 | 81.73 ± 0.72 | 83.56 ± 1.06 |
| 125 | 29.43 ± 2.58* | 13.27 ± 2.82* | 8.13 ± 4.11* |
| 250 | 2.46 ± 2.93* | 0.89 ± 1.72* | 2.47 ± 1.93* |
| 500 | 0.00 ± 1.15* | 0.00 ± 1.07* | 0.00 ± 0.82* |
| 1000 | 1.73 ± 1.89* | 0.73 ± 1.06* | 7.36 ± 1.17* |
| 2000 | 6.80 ± 0.73* | 7.93 ±1.94* | 7.89 ± 1.96* |

Each value represents as mean ± SEM of three independent experiments. *$P > 0.05$ versus non treated control by one way ANOVA.

**Table 16** The percentage of CCD-1064Sk cell viability in response to 2 fold concentration series of TSCEs from the three different cultivars by dry exclusion (trypan blue).

| Concentration (µg/ml) | Cultivar | | |
|---|---|---|---|
| | TI-SP | TI-P | TI-PK |
| 0 | 100 ± 0.00 | 100 ± 0.00 | 100 ± 0.00 |
| 62.5 | 110.35 ±18.17 | 111.59 ±8.44 | 108.62 ± 6.07 |
| 125 | 108.66 ± 7.44 | 85.04 ± 4.70 | 89.10 ± 7.20 |
| 250 | 87.66 ±15.99 | 76.91 ±9.89* | 78.67 ± 11.04* |
| 500 | 67.50 ±16.14* | 51.70 ± 10.14* | 54.29 ± 2.55* |
| 1000 | 36.47 ±10.39* | 36.14 ±12.22* | 31.29 ± 2.98* |
| 2000 | 2.39 ± 1.21* | 3.81 ± 1.01* | 5.37 ± 0.82* |

Each value represents as mean ± SEM of three independent experiments. *$P > 0.05$ versus non treated control by one way ANOVA.

**Table 17** The percentage of AGS cell viability in response to 2 fold concentration series of TSCEs from the three different cultivars by dry exclusion (trypan blue).

| Concentration (μg/ml) | Cultivar | | |
|---|---|---|---|
| | TI-SP | TI-P | TI-PK |
| 0 | 100 ± 0.00 | 100 ± 0.00 | 100 ± 0.00 |
| 62.5 | 85.4 ± 16.56 | 65.77 ± 12.34* | 77.17 ±11.50* |
| 125 | 20.71 ±14.05* | 23.26 ± 7.83* | 18.18 ± 14.06* |
| 250 | 2.01 ± 1.45* | 1.78 ± 0.89* | 4.13 ± 2.09* |
| 500 | 1.21 ± 0.69* | 0.00 ±0.00* | 0.32 ± 0.32* |
| 1000 | 1.21 ± 1.21* | 0.00 ± 0.00* | 0.31 ± 0.31* |
| 2000 | 0.80 ± 0.40* | 0.00 ±0.00* | 0.00 ± 0.00* |

Each value represents as mean ± SEM of three independent experiments. *$P > 0.05$ versus non treated control by one way ANOVA.

**Table 18** The inhibitory concentration of fifty percentage ($IC_{50}$) values of TSCEs from the three different cultivars on CCD-1064Sk by different cytotoxic assays.

| Cultivar | $IC_{50}$(μg/ml) | | |
|---|---|---|---|
| | MTT assay | NRU assay | Trypan Blue |
| TI-SP | > 2000 | 1447.53 ± 48.24 | 739.94 ± 186.58[a] |
| TI-P | > 2000 | 1707.22 ± 135.85 | 592.02 ± 125.93[b] |
| TI-PK | > 2000 | 1042.22 ± 12.78 | 569.42 ± 59.36[b] |

Each value represents as mean ± SEM of three independent experiments.

a,b,c = significantly different between group ($P < 0.05$)

**Table 19** The inhibitory concentration of fifty percentage ($IC_{50}$) values of TSCEs from the three different cultivars on AGS by different cytotoxic assays.

| Cultivar | $IC_{50}$(μg/ml) | | |
|---|---|---|---|
| | MTT assay | NRU assay | Trypan Blue |
| TI-SP | 147.10 ± 1.35[a] | 97.44 ± 4.78[a] | 88.06 ± 18.89 |
| TI-P | 110.30 ± 10.49[b] | 88.00 ± 2.64[b] | 80.37 ± 18.51 |
| TI-PK | 90.97 ± 6.87[c] | 86.47 ± 2.41[b] | 90.24 ± 23.84 |

Each value represents as mean ± SEM of three independent experiments.
a,b,c = significantly different between group ($P < 0.05$)

**Table 20** The proliferative effect of TSCEs from the three different cultivars on CCD-1064Sk cells by NRU assay.

| Concentration (μg/ml) | Cultivar | | |
|---|---|---|---|
| | TI-SP | TI-P | TI-PK |
| 0 | 100 ± 0.00 | 100 ± 0.00 | 100 ± 0.00 |
| 6.25 | 114.94 ± 2.37* | 115.22 ± 1.45* | 113.21 ±1.53* |
| 12.5 | 111.57 ± 2.13* | 109.79 ± 1.34 | 110.39 ± 1.20 |
| 25 | 109.38 ± 2.56 | 107.56 ± 0.93 | 106.93 ± 5.09 |
| 50 | 102.64 ± 1.35 | 103.37 ± 1.48 | 103.59 ± 3.62 |
| 100 | 102.55 ±1.39 | 105.54 ± 1.59 | 99.37 ± 1.79 |
| 200 | 100.89 ± 0.48 | 102.55 ±1.78 | 96.09 ± 1.65 |
| 300 | 99.44 ± 1.26 | 97.07 ± 0.39 | 94.65 ± 0.53 |

Each value represents as mean ± SEM of three independent experiments. *$P > 0.05$ versus non treated control by one way ANOVA.

**Table 21** The proliferative effect of TSCEs from the three different cultivars on AGS cells by NRU assay.

| Concentration (μg/ml) | Cultivar | | |
|---|---|---|---|
| | TI-SP | TI-P | TI-PK |
| 0 | 100 ± 0.00 | 100 ± 0.00 | 100 ± 0.00 |
| 0.625 | 115.21 ±1.60* | 109.91 ± 1.19 | 103.29 ± 4.54 |
| 1.25 | 116.84 ±0.51* | 115.90 ± 1.27* | 107.02 ± 3.31 |
| 2.5 | 117.29 ± 1.48* | 115.87 ± 3.15* | 113.19 ± 1.92* |
| 5 | 112.75 ± 4.87 | 110.60 ± 1.29 | 112.36 ± 3.19 |
| 10 | 109.06 ± 4.92 | 107.03 ± 4.69 | 108.43 ± 0.48 |
| 20 | 95.67 ± 3.93 | 90.42 ± 2.91 | 91.88 ± 0.89 |

Each value represents as mean ± SEM of three independent experiments. *$P > 0.05$ versus non treated control by one way ANOVA.

**Table 22** The proliferative effect of (+)-catechin and (-)-epicatechin on CCD-1064Sk and AGS cells by NRU assay.

| Concentration (μg/ml) | (+)-Catechin | | (-)-Epicatechin | |
|---|---|---|---|---|
| | CCD 1064Sk | AGS | CCD 1064Sk | AGS |
| 0 | 100 ± 0.00 | 100 ± 0.00 | 100 ± 0.00 | 100 ± 0.00 |
| 0.0001 | 103.09 ± 0.54 | 100.00 ± 2.43 | 96.62 ± 1.76 | 108.29 ± 1.39 |
| 0.001 | 107.93 ± 2.03 | 107.35 ± 8.57 | 104.23 ± 3.81 | 115.44 ± 1.49* |
| 0.01 | 114.59 ± 1.26* | 102.43 ± 3.78 | 98.83 ± 2.89 | 112.97 ± 2.67* |
| 0.1 | 114.98 ± 5.06* | 103.15 ± 8.05 | 98.79 ± 2.44 | 111.21 ± 2.67* |
| 1 | 120.39 ± 2.39* | 101.89 ± 4.76 | 105.86 ± 0.79 | 108.98 ± 1.43 |
| 10 | 122.43 ± 0.45* | 98.25 ± 2.45 | 108.13 ± 1.20 | 107.54 ± 3.22 |
| 100 | 113.42 ± 0.81* | 97.04 ± 1.26 | 86.79 ± 3.54 | 98. 67 ± 1.46 |

Each value represents as mean ± SEM of three independent experiments. *$P > 0.05$ versus non treated control by one way ANOVA.

**Table 23** The percentage of CCD-1064Sk cell viability in response to various concentrations of $H_2O_2$ at various time points by NRU assay.

| Concentration (mM) | Time | | |
|---|---|---|---|
| | 15 min | 30 min | 60 min |
| 0 | 100 ± 0.00 | 100 ± 0.00 | 100 ± 0.00 |
| 0.1 | 101.76 ± 3.76 | 99.35 ± 2.16 | 96.55 ± 2.47 |
| 0.2 | 105.58 ± 3.47 | 98.48 ± 5.33 | 92.97 ± 2.60 |
| 0.4 | 96.25 ± 2.50 | 96.59 ± 2.96 | 81.83 ± 3.13 |
| 0.5 | 99.27 ± 5.78 | 89.43 ± 0.79 | 67.78 ± 5.79* |
| 1 | 91.44 ± 1.03 | 79.42 ± 1.41* | 55.41 ± 4.78* |
| 2 | 89.55 ± 4.48 | 63.28 ± 4.14* | 38.12 ± 0.56* |

Each value represents as mean ± SEM of three independent experiments. *$P > 0.05$ versus non treated control by one way ANOVA.

**Table 24** The percentage of AGS cell viability in response to various concentrations of $H_2O_2$ at various time points by NRU assay.

| Concentration (mM) | Time | | |
|---|---|---|---|
| | 15 min | 30 min | 60 min |
| 0 | 100 ± 0.00 | 100 ± 0.00 | 100 ± 0.00 |
| 0.1 | 98.92 ± 2.35 | 96.94 ± 1.63 | 90.91 ± 1.81 |
| 0.2 | 98.53 ± 2.88 | 97.92 ± 3.66 | 82.31 ± 1.51 |
| 0.4 | 93.29 ± 1.64 | 86.81 ± 3.57 | 76.76 ± 2.93* |
| 0.5 | 92.52 ± 1.33 | 80.44 ± 3.26 | 67.04 ± 5.38* |
| 1 | 84.90 ± 0.40 | 76.37 ± 2.64* | 52.82 ± 1.79* |
| 2 | 81.53 ± 3.49 | 70.09 ± 4.06* | 42.83 ± 3.64* |

Each value represents as mean ± SEM of three independent experiments. *$P > 0.05$ versus non treated control by one way ANOVA.

**Table 25** The percentage of CCD-1064Sk cell viability in response to various concentrations of TSCEs from the three different cultivars pretreatment prior to exposure with 1mM $H_2O_2$ for 60 min, determined by NRU assay.

| Treatment | Cultivar | | |
|---|---|---|---|
| | TI-SP | TI-P | TI-PK |
| 0 | 100 ± 0.00 | 100 ± 0.00 | 100 ± 0.00 |
| 1mM $H_2O_2$ | 53.15 ±2.97 | 53.15 ±2.98 | 53.15 ±2.99 |
| 6.25 µg/ml TSCE + 1mM $H_2O_2$ | 55.62 ±1.15 | 55.46 ± 3.97 | 57.24 ± 4.70 |
| 12.5 µg/ml TSCE + 1mM $H_2O_2$ | 55.40 ± 2.04 | 59.72 ±5.72 | 59.31 ± 5.14 |
| 25 µg/ml TSCE + 1mM $H_2O_2$ | 58.31 ± 3.27 | 58.03 ± 4.91 | 64.54 ± 7.76 |
| 50 µg/ml TSCE + 1mM $H_2O_2$ | 62.30 ± 2.99 | 63.34 ± 5.53 | 62.02 ± 5.30 |
| 100 µg/ml TSCE + 1mM $H_2O_2$ | 67.97 ±3.60 | 75.96 ± 8.69 | 67.12 ±4.55 |
| 200 µg/ml TSCE + 1mM $H_2O_2$ | 82.68 ± 8.54* | 82.04 ± 9.50* | 76.99 ± 5.18* |
| 300 µg/ml TSCE + 1mM $H_2O_2$ | 75.51 ± 8.56* | 81.50 ± 10.04* | 79.00 ± 4.92* |

Each value represents as mean ± SEM of three independent experiments. *$P > 0.05$ versus $H_2O_2$-treated cells (untreated TSCEs) by one way ANOVA.

**Table 26** The percentage of AGS cell viability in response to various concentrations of TSCEs from the three different cultivars pretreatment prior to exposure with 1 mM $H_2O_2$ for 60 min, determined by NRU assay.

| Treatment | Cultivar | | |
|---|---|---|---|
| | TI-SP | TI-P | TI-PK |
| 0 | 100 ± 0.00 | 100 ± 0.00 | 100 ± 0.00 |
| 1mM $H_2O_2$ | 59.30 ± 7.81 | 53.15 ±2.98 | 53.15 ±2.99 |
| 0.625 µg/ml TSCE + 1mM $H_2O_2$ | 56.68 ± 8.11 | 58.42 ± 8.28 | 59.42 ± 10.24 |
| 1.25 µg/ml TSCE + 1mM $H_2O_2$ | 62.55 ± 8.50 | 61.96 ± 7.14 | 64.08 ± 9.31 |
| 2.5 µg/ml TSCE + 1mM $H_2O_2$ | 63.98 ± 4.93 | 67.06 ± 8.00 | 65.22 ± 8.29 |
| 5 µg/ml TSCE + 1mM $H_2O_2$ | 64.74 ± 6.77 | 68.12 ± 6.58 | 67.32 ± 7.85 |
| 10 µg/ml TSCE + 1mM $H_2O_2$ | 65.43 ± 5.42 | 69.33 ± 3.90 | 69.51 ± 5.88 |
| 20 µg/ml TSCE + 1mM $H_2O_2$ | 69.66 ± 5.81 | 70.61 ± 5.87 | 72.49 ± 6.16 |

**Table 27** The percentage of AGS cell viability in response to various concentrations of TSCEs from the three different cultivars pretreatment prior to exposure with 2 mM $H_2O_2$ for 30 min, determined by NRU assay.

| Treatment | Cultivar | | |
|---|---|---|---|
| | TI-SP | TI-P | TI-PK |
| 0 | 100 ± 0.00 | 100 ± 0.00 | 100 ± 0.00 |
| 1mM $H_2O_2$ | 66.64 ± 2.50 | 66.64 ± 2.51 | 66.64 ± 2.52 |
| 0.625 μg/ml TSCE + 1mM $H_2O_2$ | 62.69 ± 2.51 | 62.67 ± 3.13 | 65.33 ± 2.59 |
| 1.25 μg/ml TSCE + 1mM $H_2O_2$ | 67.81 ± 1.30 | 66.25 ± 1.12 | 67.48 ± 4.51 |
| 2.5 μg/ml TSCE + 1mM $H_2O_2$ | 67.55 ± 2.05 | 70.99 ± 2.87 | 68.83 ± 4.73 |
| 5 μg/ml TSCE + 1mM $H_2O_2$ | 70.27 ± 1.81 | 73.28 ± 1.94 | 72.76 ± 4.87 |
| 10 μg/ml TSCE + 1mM $H_2O_2$ | 72.02 ± 3.09 | 79.20 ± 2.52* | 80.15 ± 1.66* |
| 20 μg/ml TSCE + 1mM $H_2O_2$ | 82.38 ± 3.16* | 85.11 ± 3.09* | 86.57 ± 1.74* |

Each value represents as mean ± SEM of three independent experiments. *$P > 0.05$ versus $H_2O_2$-treated cells (untreated TSCEs) by one way ANOVA.

**Table 28** The percentage of intracellular ROS levels of CCD-1064Sk cells response to various concentrations of TSCEs from the three different cultivars, determined by DCFH-DA assay.

| Concentration (μg/ml) | Cultivar | | |
|---|---|---|---|
| | TI-SP | TI-P | TI-PK |
| 0 | 100 ± 0.00 | 100 ± 0.00 | 100 ± 0.00 |
| 6.25 | 92.80 ±1.72 | 94.48 ± 5.38 | 101.78 ± 1.12 |
| 12.5 | 95.01 ± 3.48 | 96.82 ± 3.09 | 98.78 ± 1.47 |
| 25 | 97.98 ± 6.06 | 98.74 ± 2.48 | 98.18 ± 2.13 |
| 50 | 92.87 ± 6.21 | 101.09 ± 5.12 | 102.96 ± 3.17 |
| 100 | 87.33 ± 3.40 | 89.07 ± 6.00 | 83.70 ± 8.17 |
| 200 | 78.34 ± 5.36 | 75.84 ± 8.37* | 74.22 ± 7.90* |
| 300 | 71.31 ± 8.02* | 68.22 ± 8.07* | 66.48 ±8.56* |

Each value represents as mean ± SEM of three independent experiments. *$P > 0.05$ versus non treated control by one way ANOVA.

**Table 29** The percentage of intracellular ROS levels of AGS cells response to various concentrations of TSCEs from the three different cultivars, determined by DCFH-DA assay.

| Concentration (μg/ml) | Cultivar | | |
|---|---|---|---|
| | TI-SP | TI-P | TI-PK |
| 0 | 100 ± 0.00 | 100 ± 0.00 | 100 ± 0.00 |
| 0.625 | 87.54 ± 3.22 | 84.89 ± 4.04 | 94.38 ± 2.94 |
| 1.25 | 88.93 ± 1.47 | 90.61 ± 4.01 | 93.72 ± 3.39 |
| 2.5 | 89.36 ± 3.35 | 89.83 ± 4.95 | 93.66 ± 5.28 |
| 5 | 90.54 ± 3.63 | 88.21 ± 5.00 | 85.45 ± 5.21 |
| 10 | 87.14 ± 1.78 | 90.53 ± 2.70 | 91.00 ± 6.13 |
| 20 | 93.89 ± 4.53 | 93.35 ± 2.61 | 83.90 ± 6.11 |

**Table 30** The percentage of intracellular ROS levels of CCD-1064Sk cells in response to various concentrations of $H_2O_2$ at various time points, determined by DCFH-DA assay.

| Concentration (mM) | Time (min) | | |
|---|---|---|---|
| | 15 | 30 | 60 |
| 0 | 100 ± 0.00 | 100 ± 0.00 | 100 ± 0.00 |
| 0.1 | 110.90 ± 3.54 | 113.10 ± 6.60 | 106.61 ± 2.55 |
| 0.2 | 120.05 ± 3.63 | 115.54 ± 9.30 | 113.28 ± 2.83 |
| 0.4 | 130.35 ± 7.03* | 119.94 ± 9.26 | 120.35 ± 6.21 |
| 0.5 | 162.91 ± 9.82* | 137.07 ± 8.57 | 120.08 ± 1.72 |
| 1 | 225.55 ± 5.63* | 136.27 ± 12.20 | 122.96 ± 2.68* |
| 2 | 208.04 ± 0.78* | 149.40 ± 15.61* | 129.78 ± 9.64* |

Each value represents as mean ± SEM of three independent experiments. *$P > 0.05$ versus non treated control by one way ANOVA.

**Table 31** The percentage of intracellular ROS levels of AGS cells in response to various concentrations of $H_2O_2$ at various time points, determined by DCFH-DA assay.

| Concentration (mM) | Time (min) | | |
|---|---|---|---|
| | 15 | 30 | 60 |
| 0 | 100 ± 0.00 | 100 ± 0.00 | 100 ± 0.00 |
| 0.1 | 107.81 ± 7.64 | 120.51 ± 6.22 | 105.47 ± 9.72 |
| 0.2 | 129.35 ± 8.81 | 126.35 ± 8.63 | 115.53 ± 8.30 |
| 0.4 | 166.56 ± 12.88* | 144.37 ± 14.03* | 107.69 ± 6.00 |
| 0.5 | 223.19 ± 7.01* | 92.92 ± 2.23 | 75.57 ± 6.80 |
| 1 | 237.05 ± 6.35* | 99.84 ± 6.22 | 77.32 ± 10.16 |
| 2 | 267.09 ± 4.73* | 94.45 ± 0.58 | 68.98 ± 6.87 |

Each value represents as mean ± SEM of three independent experiments. *$P > 0.05$ versus non treated control by one way ANOVA.

**Table 32** The percentage of intracellular ROS levels of CCD-1064Sk cells in response to various concentrations of TSCEs from the three different cultivars pretreatment prior to exposure with 1 mM $H_2O_2$ for 15 min, determined by DCFH-DA assay.

| Treatment | Cultivar | | |
|---|---|---|---|
| | TI-SP | TI-P | TI-PK |
| 0 | 100 ± 0.00 | 100 ± 0.00 | 100 ± 0.00 |
| 1mM $H_2O_2$ | 250.67 ± 17.44 | 250.67 ± 17.45 | 250.67 ± 17.46 |
| 6.25 µg/ml TSCE + 1mM $H_2O_2$ | 242.92 ± 13.70 | 235.97 ± 12.80 | 236.20 ± 17.48 |
| 12.5 µg/ml TSCE + 1mM $H_2O_2$ | 227.19 ± 6.21 | 225.27 ±7.85 | 221.61 ± 3.31 |
| 25 µg/ml TSCE + 1mM $H_2O_2$ | 234.48 ± 15.90 | 216.80 ± 7.79 | 212.33 ± 12.70 |
| 50 µg/ml TSCE + 1mM $H_2O_2$ | 237.99 ± 0.32 | 187.64 ± 12.47* | 179.67 ± 20.20* |
| 100 µg/ml TSCE + 1mM $H_2O_2$ | 224.05 ± 9.35 | 189.56 ± 5.53* | 180.69 ± 9.85* |
| 200 µg/ml TSCE + 1mM $H_2O_2$ | 197.27 ±11.22 | 169.37 ± 9.58* | 166.96 ± 12.45* |
| 300 µg/ml TSCE + 1mM $H_2O_2$ | 149.72 ±14.94* | 136.22 ± 18.14* | 129.53 ± 8.23* |

Each value represents as mean ± SEM of three independent experiments. *$P > 0.05$ versus $H_2O_2$-treated cells (untreated TSCEs) by one way ANOVA.

**Table 33** The percentage of intracellular ROS levels of CCD-1064Sk cells in response to various concentrations of TSCEs from the three different cultivars pretreatment prior to exposure with 2 mM $H_2O_2$ for 15 min, determined by DCFH-DA assay.

| Treatment | Cultivar | | |
|---|---|---|---|
| | TI-SP | TI-P | TI-PK |
| 0 | 100 ± 0.00 | 100 ± 0.00 | 100 ± 0.00 |
| 2mM $H_2O_2$ | 263.88 ± 11.61 | 263.88 ± 11.62 | 263.88 ± 11.63 |
| 0.625 μg/ml TSCE + 2mM $H_2O_2$ | 254.15 ± 12.04 | 241.51 ± 7.70 | 238.71 ± 8.91 |
| 1.25 μg/ml TSCE + 2mM $H_2O_2$ | 247.04 ± 18.24 | 239.16 ±5.84 | 225.60 ± 8.71 |
| 2.5 μg/ml TSCE + 2mM $H_2O_2$ | 252.83 ± 14.54 | 226.31 ± 16.24 | 223.20 ± 11.31 |
| 5 μg/ml TSCE + 2mM $H_2O_2$ | 240.32 ± 11.44 | 224.03 ± 8.63 | 214.50 ± 15.68 |
| 10 μg/ml TSCE + 2mM $H_2O_2$ | 219.53 ± 3.35 | 204.02 ± 1.38* | 205.30 ± 9.00* |
| 20 μg/ml TSCE + 2mM $H_2O_2$ | 205.71 ± 4.73* | 201.20 ± 6.26* | 184.43 ± 7.73* |

Each value represents as mean ± SEM of three independent experiments. *$P > 0.05$ versus $H_2O_2$-treated cells (untreated TSCEs) by one way ANOVA.

**Table 34** The percentage of wound closure of CCD-1064Sk cells in response to various concentrations of TSCE from TI-SP at various time points.

| Time | Concentration (μg/ml) | | | | | | |
|---|---|---|---|---|---|---|---|
| (hour) | Control | 1.5625 | 3.625 | 6.25 | 12.5 | 25 | 50 |
| 6 | 20.12 ± 3.24 | 22.98 ± 4.38 | 25.48 ± 3.42 | 25.19 ± 3.98 | 24.91 ± 7.85 | 27.63 ± 4.56 | 18.93 ± 8.72 |
| 12 | 34.35 ± 4.15 | 41.31 ± 2.69 | 42.62 ± 3.04 | 39.74 ± 4.75 | 40.39 ± 7.19 | 39.69 ± 3.48 | 31.09 ± 7.75 |
| 18 | 47.66 ± 6.59 | 53.07 ± 5.28 | 55.32 ± 5.92 | 49.34 ± 7.16 | 53.39 ± 9.01 | 49.93 ± 3.21 | 44.40 ± 7.07 |
| 24 | 61.26 ± 6.98 | 60.58 ± 7.29 | 65.39 ± 3.99 | 61.99 ± 7.17 | 63.30 ± 8.15 | 57.91 ± 1.82 | 52.20 ± 5.27 |
| 36 | 78.05 ± 7.23 | 76.56 ± 3.92 | 75.59 ± 3.06 | 73.22 ± 5.39 | 75.82 ± 5.64 | 72.39 ± 3.05 | 65.31 ± 7.25 |
| 48 | 86.19 ± 6.38 | 86.71 ± 6.69 | 87.93 ± 6.96 | 86.26 ± 6.92 | 85.27 ± 7.61 | 84.86 ± 7.04 | 85.78 ± 4.55 |

**Table 35** The percentage of wound closure of CCD-1064Sk cells in response to various concentrations of TSCE from TI-P at various time points.

| Time | Concentration (μg/ml) | | | | | | |
|---|---|---|---|---|---|---|---|
| (hour) | Control | 1.5625 | 3.625 | 6.25 | 12.5 | 25 | 50 |
| 6 | 20.12 ± 3.24 | 23.49 ± 5.98 | 26.19 ± 6.04 | 27.44 ± 8.61 | 22.75 ± 5.75 | 22.99 ± 7.15 | 22.12 ± 5.82 |
| 12 | 34.35 ± 4.15 | 35.68 ± 5.10 | 35.41 ± 7.40 | 42.69 ± 8.14 | 39.52 ± 4.58 | 39.24 ± 5.07 | 32.27 ± 5.68 |
| 18 | 47.66 ± 6.59 | 53.39 ± 6.01 | 49.24 ± 8.78 | 51.39 ± 8.28 | 53.10 ± 6.22 | 53.13 ± 4.73 | 42.05 ± 4.99 |
| 24 | 61.26 ± 6.98 | 64.19 ± 6.53 | 63.08 ± 7.75 | 64.99 ± 7.67 | 63.39 ± 7.48 | 61.05 ± 4.72 | 53.78 ± 3.49 |
| 36 | 78.05 ± 7.23 | 76.89 ± 6.84 | 73.18 ± 9.98 | 75.95 ± 7.94 | 74.65 ± 8.73 | 71.23 ± 6.13 | 65.51 ± 4.87 |
| 48 | 86.19 ± 6.38 | 86.46 ± 6.70 | 81.14 ± 10.77 | 84.99 ± 9.19 | 82.53 ± 9.28 | 83.57 ± 7.75 | 79.65 ± 9.40 |

**Table 36** The percentage of wound closure of CCD-1064Sk cells in response to various concentrations of TSCE from TI-PK at various time points.

| Time | Concentration (μg/ml) | | | | | | |
|---|---|---|---|---|---|---|---|
| (hour) | Control | 1.5625 | 3.625 | 6.25 | 12.5 | 25 | 50 |
| 6 | 20.12 ± 3.24 | 28.63 ± 1.38 | 24.83 ± 4.97 | 30.51 ± 8.41 | 26.90 ± 5.85 | 22.44 ± 4.70 | 23.13 ± 6.64 |
| 12 | 34.35 ± 4.15 | 44.83 ± 4.50 | 41.39 ± 7.75 | 44.41 ± 8.53 | 41.69 ± 4.79 | 39.51 ± 5.38 | 34.02 ± 6.65 |
| 18 | 47.66 ± 6.59 | 56.30 ± 8.34 | 54.20 ± 9.97 | 57.14 ± 9.29 | 57.14 ± 9.48 | 52.47 ± 4.00 | 45.67 ± 4.49 |
| 24 | 61.26 ± 6.98 | 64.19 ± 6.53 | 66.84 ± 7.79 | 67.97 ± 9.20 | 65.14 ± 7.35 | 62.30 ± 6.27 | 53.40 ± 2.41 |
| 36 | 78.05 ± 7.23 | 82.69 ± 6.75 | 83.56 ± 5.07 | 81.79 ± 8.88 | 80.55 ± 7.77 | 74.11 ± 7.40 | 67.78 ± 2.49 |
| 48 | 86.19 ± 6.38 | 88.83 ± 5.74 | 91.77 ± 4.12 | 89.27 ± 5.61 | 85.75 ± 8.01 | 84.41 ± 7.60 | 83.18 ± 6.72 |

**Table 37** The percentage of wound closure of AGS cells in response to various concentrations of TSCE from TI-SP at various time points.

| Time | Concentration (μg/ml) | | | | | | |
|---|---|---|---|---|---|---|---|
| (hour) | Control | 0.625 | 1.25 | 2.5 | 5 | 10 | 20 |
| 6 | 29.77 ± 1.71 | 38.18 ± 3.31 | 36.41 ± 1.49 | 32.00 ± 5.81 | 40.90 ± 7.67 | 39.92 ± 6.51 | 30.11 ± 5.33 |
| 12 | 53.68 ± 0.82 | 54.76 ± 6.93 | 61.02 ± 2.61 | 46.15 ± 5.52 | 60.68 ± 5.62 | 63.94 ± 7.91 | 60.06 ± 6.15 |
| 18 | 87.71 ± 0.71 | 91.83 ± 4.55 | 97.56 ± 2.14 | 92.56 ± 2.14 | 90.95 ± 1.88 | 91.57 ± 3.65 | 89.33 ± 2.04 |
| 24 | 100.00 ± 0.00 | 99.07 ± 0.93 | 99.57 ± 0.43 | 99.57 ± 0.43 | 100.00 ± 0.00 | 100.00 ± 0.00 | 97.32 ± 1.74 |

**Table 38** The percentage of wound closure of AGS cells in response to various concentrations of TSCE from TI-P at various time points.

| Time (hour) | Concentration (µg/ml) | | | | | | |
|---|---|---|---|---|---|---|---|
| | Control | 0.625 | 1.25 | 2.5 | 5 | 10 | 20 |
| 6 | 29.77 ± 1.71 | 22.17 ± 3.90 | 25.18 ± 2.68 | 30.29 ± 5.46 | 33.22 ± 5.13 | 32.39 ± 2.35 | 28.63 ± 3.54 |
| 12 | 53.68 ± 0.82 | 45.12 ± 2.54 | 48.21 ± 5.50 | 55.32 ± 3.94 | 53.80 ± 3.92 | 64.23 ± 4.65 | 57.17 ± 5.67 |
| 18 | 87.71 ± 0.71 | 84.00 ± 4.84 | 85.64 ± 1.16 | 86.44 ± 3.85 | 89.85 ± 3.78 | 88.54 ± 2.71 | 89.87 ± 3.11 |
| 24 | 100.00 ± 0.00 | 99.54 ± 0.46 | 98.78 ± 1.22 | 99.78 ± 0.22 | 100.00 ± 0.00 | 100.00 ± 0.00 | 98.78 ± 1.22 |

**Table 39** The percentage of wound closure of AGS cells in response to various concentrations of TSCE from TI-PK at various time points.

| Time (hour) | Concentration (µg/ml) | | | | | | |
|---|---|---|---|---|---|---|---|
| | Control | 0.625 | 1.25 | 2.5 | 5 | 10 | 20 |
| 6 | 29.77 ± 1.71 | 21.99 ± 4.04 | 24.80 ± 2.59 | 23.14 ± 2.21 | 28.63 ± 5.56 | 29.29 ± 6.84 | 26.05 ± 3.40 |
| 12 | 53.68 ± 0.82 | 42.02 ± 7.88 | 50.51 ± 2.64 | 40.63 ± 8.81 | 49.26 ± 6.16 | 54.10 ± 4.82 | 48.91 ± 2.24 |
| 18 | 87.71 ± 0.71 | 70.85 ± 8.35 | 77.29 ± 4.72 | 80.00 ± 7.22 | 80.48 ± 5.13 | 79.57 ± 8.80 | 73.72 ± 3.43 |
| 24 | 100.00 ± 0.00 | 97.77 ± 2.23 | 98.95 ± 1.18 | 98.95 ± 1.18 | 96.92 ± 3.08 | 97.39 ± 2.61 | 94.39 ± 3.82 |

**Table 40** The percentage of wound closure of CCD-1064Sk cells at 24 hours in response various concentrations of $H_2O_2$ at various time points.

| Concentration (mM) | Time (min) | | |
|---|---|---|---|
| | 15 | 30 | 60 |
| 0 | 85.72 ± 4.89 | 77.94 ± 2.78 | 76.44 ± 2.79 |
| 0.1 | 74.41 ± 4.91 | 72.00 ± 3.39 | 69.27 ± 3.27 |
| 0.2 | 72.60 ± 5.70 | 71.96 ± 7.49 | 67.05 ± 4.03 |
| 0.4 | 68.51 ± 4.90 | 85.53 ± 3.75 | 65.28 ± 2.07 |
| 0.5 | 75.01 ± 2.39 | 74.84 ± 4.40 | 68.88 ± 2.04 |
| 1 | 62.35 ± 3.50 | 53.07 ± 1.63* | 36.19 ± 2.90* |
| 2 | 52.75 ± 6.47* | 55.37 ± 1.50* | (-)18.04 ± 8.33* |

Each value represents as mean ± SEM of three independent experiments. *$P > 0.05$ versus non treated control by one way ANOVA.

**Table 41** The percentage of wound closure of AGS cells at 24 hours in response various concentrations of $H_2O_2$ at various time points.

| Concentration (mM) | Time (min) | | |
|---|---|---|---|
| | 15 | 30 | 60 |
| 0 | 57.96 ± 5.75 | 67.70 ± 3.81 | 65.37 ± 0.29 |
| 0.1 | 51.79 ± 3.01 | 61.58 ± 9.76 | 59.90 ± 8.00 |
| 0.2 | 51.53 ± 5.27 | 56.22 ± 6.11 | 62.44 ± 5.48 |
| 0.4 | 56.08 ± 1.61 | 54.77 ± 5.21 | 58.98 ± 4.51 |
| 0.5 | 57.42 ± 4.90 | 48.35 ± 0.61 | 52.17 ± 6.17 |
| 1 | 57.95 ± 0.95 | 47.90 ± 3.63 | 30.39 ± 8.64* |
| 2 | 60.16 ± 3.38 | 45.19 ± 7.63* | 20.10 ± 6.10* |

Each value represents as mean ± SEM of three independent experiments. *$P > 0.05$ versus non treated control by one way ANOVA.

**Table 42** The percentage of wound closure of CCD-1064Sk cells after exposed with 2 mM $H_2O_2$ and various concentrations of TSCEs from the three different cultivars at 24 hours.

| Treatment | Cultivar | | |
|---|---|---|---|
| | TI-P | TI-SP | TI-PK |
| 0 | 74.03 ± 4.23 | 74.03 ± 4.24 | 74.03 ± 4.25 |
| 2mM $H_2O_2$ | 42.76 ± 1.43 | 42.76 ± 1.44 | 42.76 ± 1.45 |
| 2mM $H_2O_2$ + 3.625 µg/ml TSCE | 52.45 ± 2.76 | 56.79 ± 5.84 | 55.45 ± 7.43 |
| 2mM $H_2O_2$ + 6.25 µg/ml TSCE | 64.91 ± 5.56* | 54.56 ± 3.88 | 53.35 ± 4.49 |
| 2mM $H_2O_2$ + 12.5 µg/ml TSCE | 63.40 ± 6.48* | 50.63 ± 3.88 | 61.86 ± 4.61 |
| 2mM $H_2O_2$ + 25 µg/ml TSCE | 60.13 ± 1.06 | 58.42 ± 3.16 | 46.30 ± 6.09 |
| 2mM $H_2O_2$+ 50 µg/ml TSCE | 46.14 ± 3.40 | 49.03 ± 6.26 | 47.18 ± 3.17 |
| 2mM $H_2O_2$ + 100 µg/ml TSCE | 41.74 ± 4.25 | 44.13 ± 6.45 | 46.02 ± 8.99 |
| 2mM $H_2O_2$ + 200 µg/ml TSCE | 43.76 ± 4.16 | 41.35 ± 5.11 | 39.54 ± 2.67 |
| 2mM $H_2O_2$+ 300 µg/ml TSCE | 35.75 ± 0.73 | 32.11 ± 4.19 | 34.57 ± 2.65 |

Each value represents as mean ± SEM of three independent experiments. *$P > 0.05$ versus $H_2O_2$-treated cells (untreated TSCEs) by one way ANOVA.

**Table 43** The percentage of wound closure of AGS cells after exposed with 2 mM $H_2O_2$ and various concentrations of TSCEs from the three different cultivars at 24 hours.

| Treatment | Cultivar | | |
|---|---|---|---|
| | TI-P | TI-SP | TI-PK |
| 0 | 91.81 ± 2.23 | 91.81 ± 2.23 | 91.81 ± 2.23 |
| 2mM $H_2O_2$ | 66.79 ± 7.67 | 66.79 ± 7.67 | 66.79 ± 7.67 |
| 2mM $H_2O_2$ + 0.625 µg/ml TSCE | 70.36 ± 7.68 | 68.82 ± 5.84 | 70.03 ± 9.45 |
| 2mM $H_2O_2$ + 1.25 µg/ml TSCE | 74.69 ± 6.91 | 73.82 ± 9.79 | 73.74 ± 10.66 |
| 2mM $H_2O_2$ + 2.5 µg/ml TSCE | 78.46 ± 2.83 | 76.86 ± 7.02 | 71.99 ± 2.57 |
| 2mM $H_2O_2$ + 5 µg/ml TSCE | 77.97 ± 2.90 | 76.28 ± 5.95 | 75.10 ± 7.52 |
| 2mM $H_2O_2$ + 10 µg/ml TSCE | 78.42 ± 7.21 | 78.51 ± 5.43 | 75.40 ± 3.76 |
| 2mM $H_2O_2$ + 20 µg/ml TSCE | 78.66 ± 6.27 | 81.42 ± 6.47* | 80.37 ± 4.29* |

Each value represents as mean ± SEM of three independent experiments. *$P > 0.05$ versus $H_2O_2$-treated cells (untreated TSCEs) by one way ANOVA.

# VITA

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