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PROTECTIVE EFFECTS OF *GLYCINE MAX* SEED EXTRACT AND *CHRYSANTHEMUM INDICUM* FLOWER EXTRACT AGAINST CISPLATIN-INDUCED RENAL CELL DEATH

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ยาต้านมะเร็งซิสพลาทินได้รับการยอมรับว่าเป็นยาเคมีบำบัดที่มีประสิทธิภาพในการใช้รักษามะเร็งชนิดต่างๆ แต่เนื่องจากผลข้างเคียงที่ทำให้เกิดพิษต่อไต ทำให้การใช้ยาซิสพลาทินถูกจำกัดลงในผู้ป่วยหลายราย การศึกษานี้มีวัตถุประสงค์เพื่อศึกษาผลของสารสกัดเมล็ดถั่วเหลืองและสารสกัดดอกเก็กฮวยในการปกป้องการตายของเซลล์ไตแบบอะพอพโทซิสจากการได้รับซิสพลาทิน การวิจัยนี้พบว่าซิสพลาทินจะเหนี่ยวนำให้ระดับของสารอนุพันธ์ออกซิเจนที่ว่องไวภายในเซลล์ไตสูงขึ้น ซึ่งถือว่าเป็นกลไกสำคัญที่ทำให้เกิดการตายของเซลล์ไตแบบอะพอพโทซิส การให้สารสกัดเมล็ดถั่วเหลืองและสารสกัดดอกเก็กฮวยก่อนการให้ยาซิสพลาทินนั้นมีผลยับยั้งการเพิ่มขึ้นของระดับสารอนุพันธ์ออกซิเจนที่ว่องไวภายในเซลล์ไตและทำให้การตายของเซลล์แบบอะพอพโทซิสลดลง ในการศึกษานี้ได้ชี้ให้เห็นว่าการทำให้เกิดพิษต่อไตของซิสพลาทินนั้น เกิดจากการที่ซิสพลาทินทำให้ระดับของไฮโดรเจนเพอร์-รอกไซด์และไฮดรอกซิลแรดดิคอลภายในเซลล์เพิ่มสูงขึ้น จากการทดลองเพื่อหากลไกของสารสกัดเมล็ดถั่วเหลืองและสารสกัดดอกเก็กฮวยในการปกป้องการตายของเซลล์ไตจากซิสพลาทินนั้น พบว่าเกี่ยวข้องกับคุณสมบัติในการต้านอนุมูลอิสระของสารสกัดทั้งสอง โดยสารสกัดเมล็ดถั่วเหลืองสามารถต้านการเพิ่มขึ้นของระดับไฮโดรเจนเพอร์รอกไซด์ในเซลล์ไตได้ดี ในขณะที่สารสกัดดอกเก็กฮวยมีผลต้านการเพิ่มขึ้นของทั้งไฮโดรเจนเพอร์รอก-ไซด์และไฮดรอกซิลแรดดิคอล นอกจากนี้จากผลการศึกษายังแสดงให้เห็นว่าสารสกัดเมล็ดถั่วเหลืองและสารสกัดดอกเก็กฮวยสามารถปกป้องการตายของเซลล์ไตจากซิสพลาทิน โดยไม่มีผลรบกวนฤทธิ์ของซิสพลาทินในการฆ่าเซลล์มะเร็งปอด (เอช. 460) และเซลล์มะเร็งผิวหนัง (จี.361) จากผลการวิจัยที่ได้ค้นพบขึ้นใหม่นี้ชี้ให้เห็นถึงหน้าที่ของสารสกัดเมล็ดถั่วเหลืองและสารสกัดดอกเก็กฮวยในการปกป้องการตายของเซลล์ไตจากซิสพลาทิน ซึ่งจะเป็นประโยชน์ต่อการพัฒนาเพื่อใช้เป็นสารสำหรับปกป้องเซลล์ไตต่อไป

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KANITTHA PONGJIT : PROTECTIVE EFFECTS OF *GLYCINE MAX* SEED EXTRACT AND *CHRYSANTHEMUM INDICUM* FLOWER EXTRACT AGAINST CISPLATIN-INDUCED RENAL CELL DEATH. THESIS ADVISOR : ASST. PROF. PITHI CHANVORACHOTE, Ph.D., 84 pp.

Although cisplatin has been accepted as one of the most efficient chemotherapeutic agents for the treatment of solid tumors, frequently observed nephrotoxicity has limited its use in several patients. The purpose of this study was to investigate the protective effect of *Glycine max* seed extract (GM) and *Chrysanthemum indicum* flower extract (CM) on cisplatin-induced apoptosis in human proximal tubular epithelial HK-2 cells. Cisplatin was shown to induce cellular reactive oxygen species (ROS) up-regulation playing a key role in HK-2 cell apoptosis. Pretreatment of the cells with GM or CM and cisplatin resulted in the inhibition of ROS induction and a decrease of cell apoptosis. Cisplatin induced renal cell toxicity via its ability to increase cellular hydrogen peroxide (H_2O_2) and hydroxyl radical ($^{\bullet}OH$) levels. GM protects cisplatin-induced apoptosis by the mechanism which involves its anti-oxidant activity against only intracellular hydrogen peroxide, while CM could scavenge both of hydrogen peroxide and hydroxyl radical. Furthermore, our results demonstrated that GM and CM selectively protect renal cells without significant interfering effect on cisplatin toxicity in non-small cell lung cancer H460 and melanoma G361 cells. These findings indicate a novel activity for GM and CM extracts protection of cisplatin-induced nephrotoxicity which could benefit the development for nephroprotective approaches.

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CONTENTS

	Page
ABSTRACT (Thai)……..	iv
ABSTRACT (English).....	v
ACKNOWLEDGEMENTS.....	vi
CONTENTS.....	vii
LIST OF TABLES……	viii
LIST OF FIGURES……..	x
LIST OF ABBREVIATIONS……..	xii
CHAPTER	
I INTRODUCTION……..	1
II LITERATURE REVIEW……...	4
1. Cisplatin……..	4
2. Ciplatin-induced nephrotoxicity……..	11
3. Natural products against cisplatin nephrotoxicity…….....	16
III MATERIALS AND METHODS……..	21
IV RESULTS…….	30
V DISCUSSION AND CONCLUSION…….	61
REFERENCES…….	65
APPENDIX……..	74
VITA…….	84

LIST OF TABLES

Page

Table 1 Summary of endogenous antioxidants 7
Table 2 The percentage of HK-2 cell viability in response to various concentrations of GM, determined by MTT assay (dose dependency)……75
Table 3 The percentage of HK-2 cell viability in response to various concentrations of CM, determined by MTT assay (dose dependency)……76
Table 4 The percentage of HK-2 cell viability in response to various concentrations of cisplatin, determined by MTT assay (dose dependency)…… 77
Table 5 The percentage of HK-2 cell viability in response to 50 μM cisplatin treatment at various time points, determined by MTT assay (time dependency) 77
Table 6 Antioxidant effects on HK-2 cell viability in response to 50 μM of cisplatin treatment, determined by MTT assay 78
Table 7 The percentage of HK-2 cell viability in response to various concentrations of GM pretreatment prior to exposure with 50 μM of cisplatin, determined by MTT assay……78
Table 8 The percentage of HK-2 cell viability in response to various concentrations of CM pretreatment prior to exposure with 50 μM of cisplatin, determined by MTT assay 79
Table 9 The effects of various ROS scavengers on cisplatin-induced HK-2 cell death, determined by MTT assay……79
Table 10 The percentage of H460 cell viability in response to various concentrations of cisplatin, determined by MTT assay (dose dependency)..... 80
Table 11 The percentage of H460 cell viability in response to 100 μM of cisplatin treatment at various time points, determined by MTT assay (time dependency) 80
Table 12 The percentage of G361 cell viability in response to various concentrations of cisplatin, determined by MTT assay (dose dependency)..... 81
Table 13 The percentage of G361 cell viability in response to 100 μM of cisplatin treatment at various time points, determined by MTT assay (time dependency) 81

Table 14 The percentage of H460 cell viability in response to GM and CM pretreatment prior to exposure with 100 μM of cisplatin, determined by MTT assay 82
Table 15 The percentage of G361 cell viability in response to GM and CM pretreatment prior to exposure with 100 μM of cisplatin, determined by MTT assay 83

LIST OF FIGURES

Page

Figure 1 Chemical structure of cisplatin……4
Figure 2 The binding of cisplatin to DNA……..5
Figure 3 Simplified of oxidative and cellular antioxidant systems 8
Figure 4 Role of mitochondria in apoptosis 10
Figure 5 Overall of apoptic pathways activated by cisplatin in renal tubular epithelial cells……...13
Figure 6 The morphological changes of apoptotic and necrotic cell death..... 14
Figure 7 Chemical structure of isoflavone 17
Figure 8 Soybeans : legumes, commercial products, and extracts……...18
Figure 9 Chrysanthemum indicum products and extracts. 19
Figure 10 Chemical structures of flavone (A), and flavonol (B)…… 20
Figure 11 Effects of GM on HK-2 cell viability as a function of dose. 30
Figure 12 Effects of CM on HK-2 cell viability as a function of dose. 31
Figure 13 Cytotoxic effect of cisplatin on HK-2 in dose-dependent study…… 33
Figure 14 Time-dependent effect of cisplatin on HK-2 cells……...34
Figure 15 Effect of cisplatin on intracellular ROS level in HK-2 cells in time-dependent study……... 35
Figure 16 Effects of anti-oxidants on intracellular ROS induced by cisplatin…….37
Figure 17 Effects of anti-oxidants on cisplatin-induced renal cell death. 39
Figure 18 Protective effect of GM on cisplatin-induced renal cell death..... 41
Figure 19 Protective effect of CM on cisplatin-induced renal cell death..... 43
Figure 20 Effects of GM and CM on intracellular ROS induced by cisplatin 45
Figure 21 Cisplatin-induced renal cell death mediated by specific ROS..... 47
Figure 22 Effect of hydrogen peroxide (A) and ferrous sulphate (hydroxyl radical generator) (B) on ROS level in HK-2 cells in time-dependent study 48
Figure 23 Anti-oxidant activities of GM and CM against hydrogen peroxide and hydroxyl radical in HK-2 cells 50
Figure 24 Cytotoxic effect of cisplatin on lung (H460) cancer cells in dose-dependent study 52

Figure 25 Time-dependent effect of cisplatin on lung (H460) cancer cell viability 53
Figure 26 Cytotoxic effect of cisplatin on melanoma (G361) cancer cells in dose-dependent study 55
Figure 27 Time-dependent effect of cisplatin on melanoma (G361) cancer cell viability 56
Figure 28 Effects of GM and CM on cisplatin-treated lung (H460) cancer cells 58
Figure 29 Effects of GM and CM on cisplatin-treated melanoma (G361) cancer cells 60

LIST OF ABBREVIATIONS

%	= percentage
°C	= Degree Celsius
µM	= Micromolar
%v/v	= percentage of volume by volume
ATP	= adenosine triphosphate
CO_2	= carbon dioxide
CM	= *Chrysanthemum indicum* extract
$DCFH_2$-DA	= 2,7-dichlorofluorescein diacetate
DFO	= deferoxamine
DMEM	= Dulbecco's modified Eagle's medium
DMSO	= Dimethyl sulfoxide
DNA	= deoxyribonucleic acid
ER	= endoplasmic reticulum
et al.	= et alibi, and others
$FeSO_4$	= ferrous sulphate
g	= gram
GM	= *Glycine max* extract
GPx	= glutathione peroxidase
GSH	= glutathione
h	= hour, hours
H_2O_2	= hydrogen peroxide
IC_{50}	= 50% inhibitory concentration
LPO	= lipid peroxidation
MDA	= malondialdehyde
min	= minute (s)
ml	= milliliter
mM	= millimolar
MnTBAP	= Mn(III)tetrakis (4-benzoic acid) porphyrin chloride
MTT	= 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NAC	= *N*-acetlycysteine
$O_2^{\bullet-}$	= superoxide anion radical

$^{\bullet}OH$	= hydroxyl radical
PBS	= Phosphate buffer saline
ROS	= reactive oxygen species
RPMI	= Roswell Park Memorial Institute's medium
S.D.	= standard deviation
U	= unit

CHAPTER I

INTRODUCTION

The undesirable toxic effects from anti-cancer drugs are the major limitation in cancer therapy. Therefore, novel less toxic anti-cancer agents as well as additive or supplementary treatments which effectively attenuate such toxicity are considerate very of interest nowadays.

Among various anti-cancer agents, *cis*–diamminedichloroplatinum (II) (cisplatin) is one of the most frequently prescribed drugs and has been reported for its high efficiency against various solid tumors including ovarian, head and neck, testicular and lung cancers (Sleijfer, Meijer, and Mulder, 1985). However, nephrotoxicity found during cisplatin-based chemotherapy due to cisplatin-induced epithelial cell apoptosis in an area of S3 segment of proximal tubule has limits in the dose and usage of this agent (Kuhlmann, Burkhardt, and Kohler, 1997; Okuda *et al*., 2000; Tsuruya *et al*., 2003; Pabla and Dong, 2008). Cisplatin is able to generate the reactive oxygen species (ROS) namely superoxide anion radical, hydrogen peroxide, and hydroxyl radical in many cells, and such increase cellular ROS has been shown to be a vital event participating in cisplatin-induced various cell damages (Kawai *et al*., 2006; Santos *et al*., 2007; Kim *et al*., 2010). Recent studies have shown that cisplatin-induced nephrotoxicity is closely associated with an increase in lipid peroxidation (Hannemann and Baumann, 1988; Yang *et al*., 2002) and also reduce in anti-oxidant enzymes in renal tissue including superoxide dismutase (SOD), catalase, and glutathione peroxidase (GPx) (Schrier, 2002). This evidence led to the introducing of cellular anti-oxidant likes reduced glutathione (GSH) together with cisplatin treatment in order to attenuate cisplatin-induced renal toxicity (Zunino, 1989; Luo, 2008). However, the consequent results that GSH protected both normal and cancerous cells from cisplatin-induced cell death had attenuated the further development of this combination therapy (Kartalou and Essigmann, 2001; Chen and Kuo, 2010). So far, no substance has been found to possess a renoprotective effect, well-tolerated by the patients and did not interfering with anti-cancer action of cisplatin.

A large number of natural products possessing anti-oxidant property are promising sources of additive treatment that may improve cisplatin intolerance (Ali and Moundhri, 2006; Ateşşahín *et al*., 2007; Hsu *et al*., 2007). Among them, extract of *Glycine max* (GM) contains

high protein and isoflavone content which have been shown to benefit patients with cardiovascular diseases, hyperlipidemia, osteoporosis, and cancers (Watanabe, Uesugi, and Kikuchi, 2002; Carson, 2003; Davis *et al*., 2008). Likewise, Chrysanthemum extract (CM), an extract from *Chrysanthemum indicum*, has long been used as a traditional medicine. Its pharmacological properties have been continuously reported such as anti-oxidant, anti-tumour, anti-inflammation, and neuroprotective effects (Cai *et al*., 2004; Sucher, 2006; Lee *et al*., 2009; Li *et al*., 2009).

Evidence from several studies suggested that cancer cells, compared to normal cells, produce a higher rate of ROS. Therefore, they had to develop themselves to survive even under highly oxidative stress (Sun, 1990; Jaruga *et al*., 1994; Kondo *et al*., 1999). From this reason, the possibility of GM and CM in the prevention of cisplatin-induced renal cell death is based on the truth that renal cells are shown to be highly susceptible to oxidative stress-induced cell damage (Paller, Hoidal, and Ferris, 1984), whereas frequently proliferated cancer cells are more sensitive to DNA-adduct induced cell apoptosis (Reedijk and Lohman, 1985). Since both plant extracts not only have anti-oxidant effects but also benefit cancer patients in many ways, and the evidence regarding renoprotective property of these extracts on cisplatin-induced renal cell damage is still unclear. The present study thus evaluated GM and CM for their potential to be developed for further use as a nephroprotective agent.

Since, cisplatin nephrotoxicity is the predominant side effect limits its clinical usage and the dose that can be applied to patients. Overcoming this limitation is a major goal that could subsequently improve quality of lives and clinical outcomes of cancer patients. This aim will most likely achieved by rational targeted investigations based on the anti-oxidant properties of GM and CM as well as a detailed knowledge of the difference between cancer and normal cells. This study provides the evidences supporting the significant protective effect of GM and CM and also reveals their underlying mechanism in the protection of renal cell death induced by cisplatin. Furthermore, the interfering effects of GM and CM on cisplatin-induced cancer cell death are examined in this study. Using human renal proximal tubular HK-2, human lung carcinoma H460 and human melanoma G361 cells as

models, these findings obtained from the present study may provide the initial evidence necessary for the further development of these extracts to be used as renoprotective agents.

Hypothesis

Due to the anti-oxidant property of GM and CM, their specific ROS scavenging activities may protect renal tubular cells from cisplatin-induced oxidative stress and consequently cisplatin-induced cell death.

Objectives

1. To study the protective effect of GM and CM on cisplatin-induced renal cell death
2. To identify the mechanism based on the anti-oxidant activity of GM and CM in the protection of cisplatin-induced renal cell death

CHAPTER II

LITERATURE REVIEW

1. Cisplatin

cis–diamminedichloroplatinum (II) (Cisplatin), a simple inorganic molecule as shown in figure 1, is one of the most effective chemotherapeutic agents. It has been widely used alone or in combination with other therapeutic agents for the treatment of various solid tumors such as ovarian, head and neck, testicular, skin, bladder, cervical and lung cancer (Sleijfer, Meijer, and Mulder, 1985; Cohen and Lippard, 2001). Despite the effectiveness of cisplatin in cancer therapy, its use is mainly limited by the severe toxic effects in normal tissues, including nephrotoxicity, neurotoxicity and ototoxicity. Among them, nephrotoxicity which usually occurs either acutely or after repeated treatments is a major problem limiting its dose and usage. Despite maintaining hydration and giving appropriate diuretics, this toxicity could not be completely prevented (Daugaard and Abildgaard, 1989; Pabla and Dong, 2008).

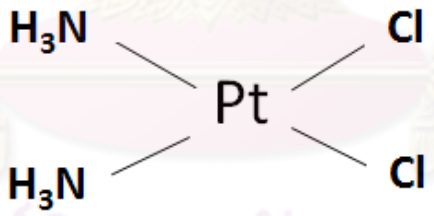


Figure 1 Chemical structure of cisplatin

The cytotoxic mechanisms of cisplatin can be specified into two major pathways.

- DNA-adduct

This is the major event responsible for cisplatin antitumor properties. Upon entering the cell, the binding of cisplatin to DNA has occurred after its two labile chloride groups have been displaced by water molecules through hydrolysis or aquation of cisplatin. The aquated form of cisplatin is a potent electrophile that can react with a variety of nucleophile centers of

biomolecules, especially at the N7 sites of DNA purine bases. The binding of cisplatin to genomic DNA in the nucleus, leading to the formation of inter- and intrastrand cross-links. Cross-linking results in defective DNA templates and inhibition of DNA synthesis and replication (Eastman, 1987; Cepeda *et al*., 2007; Rebillard *et al*., 2008) as shown in figure 2. These events have a greatly impact on the rapidly dividing cells, such as cancer cells results in DNA damage, irreversible injury and cell death (Reedijk and Lohman, 1985; Pabla and Dong, 2008).

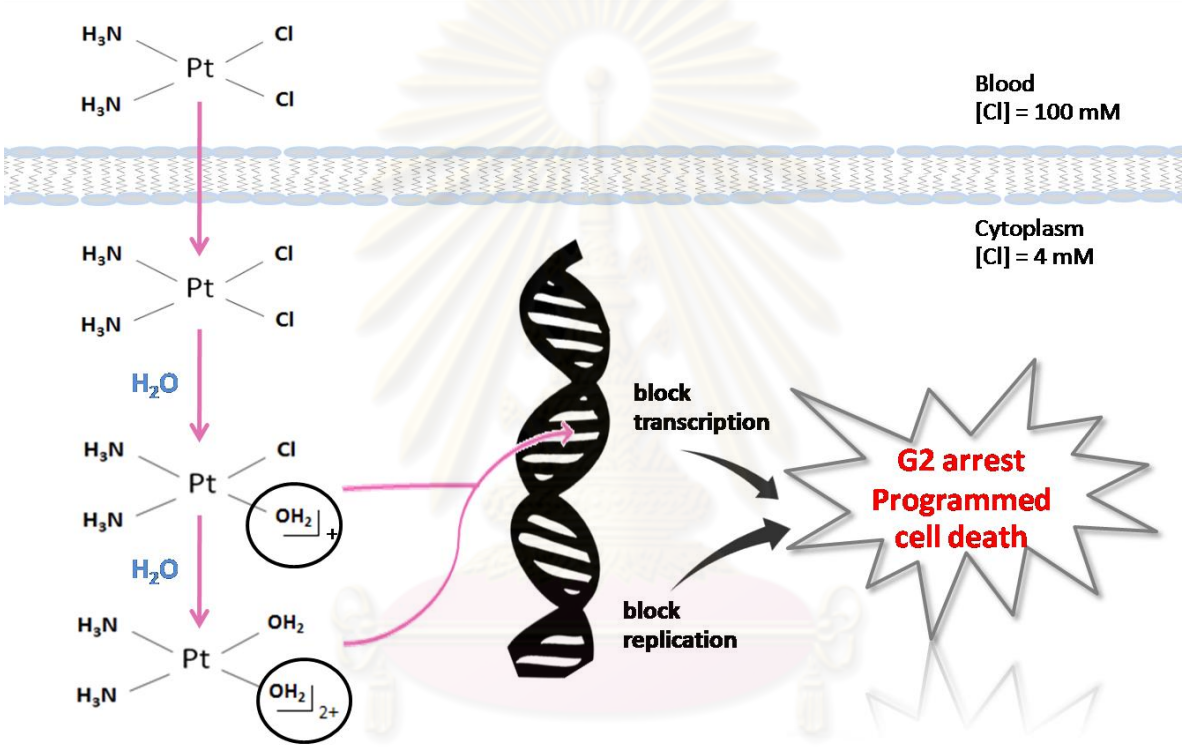


Figure 2 The binding of cispltin to DNA, one of the major mechanisms of the anti-cancer activity of cisplatin

- **Cisplatin induced oxidative stress**

Increasing evidences have shown that cisplatin-induced oxidative stress is one of the primarily mechanism of cisplatin cytotoxicity. There are various approaches have attempted to elucidate the biochemical mechanisms of this event in order to understand the other pathway of cisplatin insulting the cells.

Oxidative stress

Oxidative stress corresponds to an imbalance between the rate of oxidant production and their removal or degradation (Sies, 1997). This event may be a result of an overproduction of these oxidants or by the reduction of anti-oxidant defense mechanisms (Halliwell, 2007). The cellular oxidants mediate most of the reactions leading to oxidative stress called Reactive Oxygen Species (ROS). ROS, chemically reactive molecules containing oxygen and also constantly produced in our cells, which can be described as O_2-derived free radicals such as superoxide anion radical ($O_2^{\bullet -}$), hydroxyl radical ($^{\bullet}OH$), peroxyl ($RO_2^{\bullet}$), and alkoxyl ($RO^{\bullet}$) radicals, as well as O_2-derived non-radical species such as hydrogen peroxide (H_2O_2). Moreover, the reactive molecules are represented in form of reactive nitrogen species (RNS), including nitric oxide ($NO^{\bullet}$), nitrogen dioxide ($NO^{\bullet}_2$) and peroxynitrite ($ONOO^{\bullet}$) as well (Salganik, 2001; Circu and Aw, 2010).

Normally, ROS are produced during cellular metabolic processes especially through electron transport chain in mitochondria, phagocytosis in immune system and from metabolic pathway of exogenous drugs and toxins (Salganik, 2001). ROS are known as mediators of intracellular signaling cascades as well as triggers or executioners of essential defense mechanisms. Excessive production of ROS can lead to oxidant injury results from the shift in oxidant/anti-oxidant balance. Oxidant-induced irreversible oxidative modification of DNA, lipids, and proteins contributes to cell and organ dysfunction (Nordberg and Arner, 2001; Valko *et al*., 2007). In human, oxidative stress is involved in many diseases for example, atherosclerosis, Parkinson's disease, heart failure, myocardial infraction, Alzheimer's disease, and diabetes mellitus (Sorg, 2004; Valko *et al*., 2007). According to the harmfulness of unbalanced redox status, anti-oxidant defense mechanisms have played a key role in cellular protection. Endogenous anti-oxidant systems can be divided into two major groups: enzymatic and non-enzymatic anti-oxidant as listed in Table 1 (Salganik, 2001; Chirino and Pedraza-Chaverri, 2009).

Table 1 Summary of endogenous anti-oxidants

Antioxidant
Enzymatic antioxidants
Superoxide dismutase (SOD)
Catalase (CAT)
Glutathione peroxidase (GPx)
Glutathione reductase (GR)
Glutathione-S-transferase (GST)
Non-enzymatic antioxidants
Glutathione (GSH)
Thioredoxins (Trx)
Ubiquinol
Ascorbic acid (vitamin C)
Retinol (vitamin A)
Tocopherols (vitamin E)
Selenium compounds
lipoic acid

ROS is generated from a few main sources in our bodies. Among them, the leakage of electron from mitochondrial electron transport chain is considered as a major source of ROS production as demonstrated in Figure 3. Superoxide anion radical ($O_2^{\bullet-}$) is the first species generated from this pathway which is dismutateded by superoxide dismutase (SOD), then transformed into the much less reactive hydrogen peroxide (H_2O_2). H_2O_2 can be catalyted by catalase and glutathione peroxidase (GPx), the latter uses reduced glutathione (GSH) as a substrate, and then turn into non toxic substances (H_2O and O_2). However, when hydrogen peroxide interacts with ferrous ion (Fe^{2+}) or copper ion (Cu^{+}), the most reactive ROS, hydroxyl radicals ($^{\bullet}OH$) are formed, this reaction is called Fenton reaction (Salganik, 2001). Furthermore, superoxide anion radical also participates in generation of $^{\bullet}OH$ through Haber-Weiss reaction. Actually, Haber-Weiss reaction is the combination between Fenton reaction

and the reduction of Fe^{3+} by $O_2^{\bullet-}$ as shown in the reaction below (Nordberg and Arner, 2001; Valko *et al*., 2007).

Fenton reaction : $H_2O_2 + Fe^{2+} / Cu^{+} \longrightarrow {}^{\bullet}OH + OH^{-} + Fe^{3+} / Cu^{2+}$

Reduction of metal ions : $O_2^{\bullet-} + Fe^{3+} / Cu^{2+} \longrightarrow O_2 + Fe^{2+} / Cu^{+}$

Haber-Weiss reaction : $H_2O_2 + O_2^{\bullet-} \longrightarrow {}^{\bullet}OH + OH^{-} + O_2$

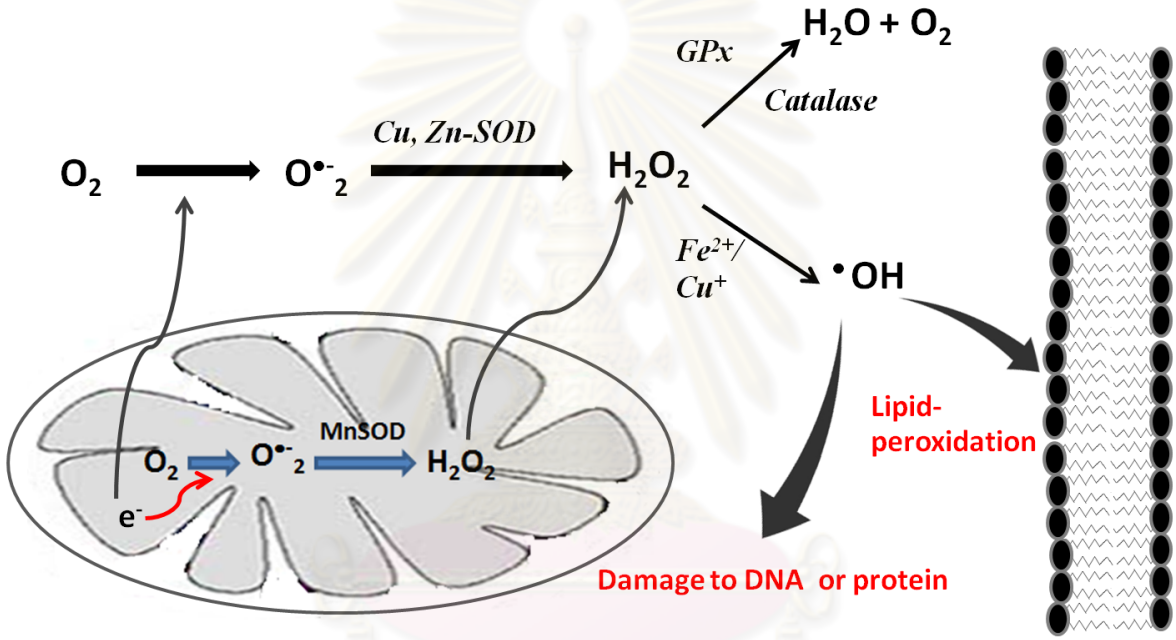


Figure 3 Simplified of oxidative and cellular anti-oxidant systems

Oxidative damage to biomolecules and cellular components are summarized as followed

- *DNA*

ROS, especially ${}^{\bullet}OH$ can cause DNA damage by cleavage, DNA-protein cross linking, oxidation of amino acid base and deoxyribose backbone, and also inhibit repairing process of damaged DNA. These events lead to the disruption of protein synthesis and gene expression,

mutagenesis, carcinogenesis, apoptosis, and cell death (Valko *et al*., 2007; Reuter *et al*., 2010).

➢ *Lipid*

The multiple double bonds in polyunsaturated fatty acid (PUFA) are excellent targets for free radical attack. They are oxidized by ROS, and then generate lipid radicals which can, in turn, initiate and self-sustained lipid peroxidation (LPO) resulting in a reduction in membrane fluidity, increase in membrane permeability, and damage to membrane proteins as well (Sorg, 2004). In addition, many studies showed that this process could be important for glomerular proteinuria (Neal *et al*., 1994; Holthofer, *et al*., 1999).

➢ *Protein*

ROS can damage protein directly or indirectly through lipid peroxidation. The side chain of amino acid residues of protein, especially at cysteine and methionine are highly susceptible to attack by $^{\bullet}OH$ and $O_2^{\bullet-}$ (Valko *et al*., 2007). Additionally, oxidative modification of protein residues can accelerate the loss of the scaffolding function of structural proteins, inactivate enzymes, and finally alter the degradation and clearance of these molecules (Fligiel *et al*., 1984).

➢ *ROS-induced apoptotic cell death*

ROS- induced apoptotic cell death can occur through several ways; for instance, death receptor – mediated apoptosis, mitochondrial pathway, transcriptional activation via p53 activation, and JNK signaling (Simon, Haj-Yehia, and Levi-Schaffer, 2000; Yang *et al*., 2004; Circu and Aw, 2010). Among them, mitochondrial pathway is generally accepted as a prominent way in ROS - regulated apoptosis. Mitochondria is considered as both source and target of ROS. As shown in figure 4, ROS cause the defect of mitochondria membrane potential resulting from the oxidative modification of permeability transition pore (PTP) contributes to the release of cytochrome *c* and others apoptogenic molecules to cytosol. They further trigger caspase-dependent or caspase-independent apoptotic pathway (Karl and Suzanne, 2000; Simon, Haj-Yehia, and Levi-Schaffer, 2000; Tsujimoto and Shimizu,

2007; Circu and Aw, 2010). Moreover, ROS also induced mitochondrial DNA damage leading to compromise of electron transport chain and enhancing the burst of ROS (Circu and Aw, 2010). In the other way, necrotic cell death can be stimulated by high concentration of ROS generation (Teramoto *et al*., 1999).

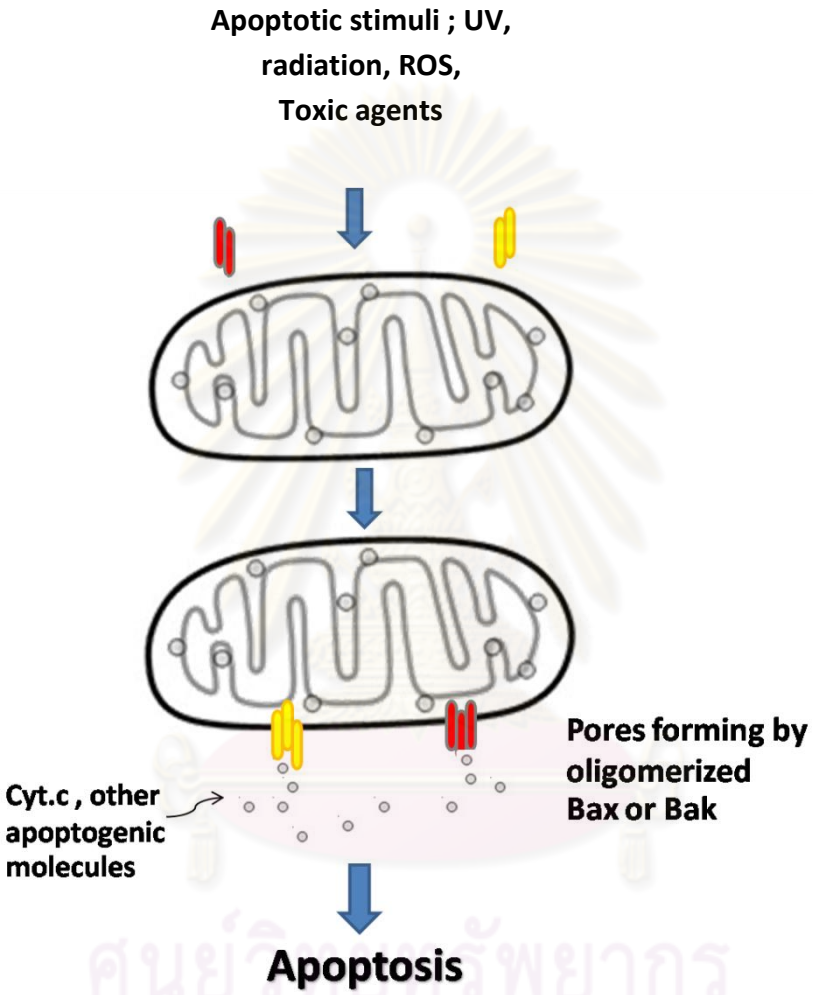


Figure 4 Role of mitochondria in apoptosis

In term of cisplatin cytotoxicity, there is growing evidence that cisplatin treatment induces not only DNA damaging stress, but also oxidative and endoplasmic reticulum stress. As mentioned above, aquated cisplatin is a highly reactive form, which can rapidly react with many nucleophilic targets, especially the negatively charged of thiol- containing species including glutathione (GSH), a well known anti-oxidant. As a result of the disruption or inactivation of GSH function and the other endogenous anti-oxidants by cisplatin, the cellular

redox status is shifted. This contributes to the accumulation of intracellular ROS and oxidative stress inside the cells (Istvan and Robert, 2003). In addition, the reactive form of cisplatin also induces mitochondrial dysfunction resulting in the increase of ROS production via the disrupted respiratory chain (Kruidering *et al*., 1996).

Due to the fact that cancer cells can produce large amounts of ROS than non-transformed cells resulting from a higher rate of their proliferation. This phenomenon means that cancer cells are persistently exposed to oxidative stress leading to remarkably adaptation and resistance to ROS-induced apoptosis. As demonstrated in several studies that tumor cells are more durable to ROS-induced cell death (Sun, 1990; Jaruga *et al*., 1994; Toyokuni *et al*., 1995; Kondo *et al*., 1999; Brown and Bicknell, 2001). Consequently, cisplatin-induced oxidative stress seem to have a specifically influence on normal cells. Increasing evidences have showed that cisplatin-generated ROS is one of the primarily mechanisms and also plays a key role in several normal cell damages (Weijl, Cleton, and Osanto, 1997; Jiang *et al*., 2008; Kim *et al*., 2010).

2. Cisplatin induced nephrotoxicity

Despite intensive prophylactic measures, acute renal failure and irreversible renal damage, occurs about 25-30% of cisplatin treated patients, even since the first dose of cisplatin injection (Daugaar and Abildgaar, 1989). Cisplatin-mediated renal cell death is a major limitation of cisplatin-based chemotherapy and frequently impaired renal function (Pabla and Dong, 2008). The clinical manifestations including, reduction in glomerular filtration rate (GFR), increase of serum creatinine (Scr) and blood urea nitrogen (BUN), hypomagnesemia and hypokalemia (Gonzalez-Vitale, 1977; Launay-Vacher, 2008). Pharmacokinetics data show that kidney is the main route of cisplatin excretion. More than 50 percent of the drug is excreted in the urine in the first 24 hours following cisplatin administration (Chirino and Pedraza-Chaverri, 2009). Additionally, the concentration of platinum achieved in kidney is several folds greater than that in plasma and other organs, probably through mediated transporters (Gately and Howell, 1993; Ishida *et al*., 2002). The

S3 segment of proximal tubule is well recognized as a major site of cisplatin nephrotoxicity (Kuhlmann, Burkhardt, and Kohler, 1997).

Mode of cisplatin-induced tubular cell death has been identified in many studies. The amount of evidence suggested that the concentration of cisplatin may determine whether the cells die by apoptosis or necrosis. Necrotic cell death was occurred when a high concentration of cisplatin was used (more than 200 μM to millimolar), whereas lower concentration of cisplatin (less than 200 μM) contributes to apoptosis (Lieberthal *et al*., 1996; Okuda *et al*., 2000). However, gathering some more evidence showed that the role played by apoptosis in acute renal injury has become an important issue (Norishi, Gur, and Sudhir, 2000), for example, in ischemic renal injury (Schumer *et al*., 1992) or partial ureteral obstruction (William *et al*., 1997). Also, a various kinds of cellular stresses applied at intensity lower than the threshold for necrosis, therefore apoptosis seems to be an important mechanism of cisplatin nephrotoxicity.

During the last few years, several pathways of cisplaitn-induced tubular cell apoptosis have been investigated as shown in figure 5, including extrinsic pathway mediated by death receptors (Tsuruya *et al*., 2003), mitochondria apoptotic pathway: caspase 3-dependent (Kaushal *et al*., 2001; Park, Leon, and Devarajan, 2002) and caspase 3-independent (Liu *et al*., 2010), and the endoplasmic reticulum (ER)-stress pathway (Liu and Baliga, 2005).

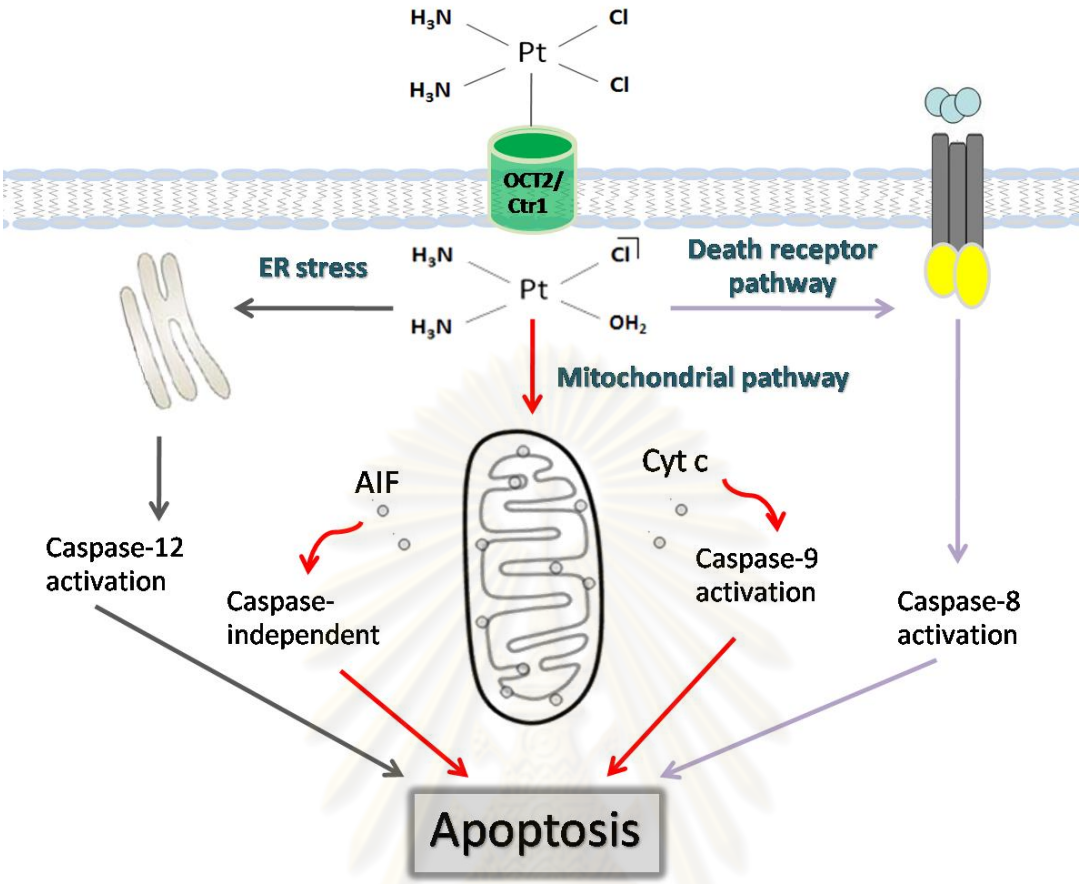


Figure 5 Overall of the apoptotic pathways activated by cisplatin in renal tubular epithelial cells

Apoptotic cell death is characterized by distinct morphological changes consisting of cell shrinkage, plasma and nuclear membrane blebbing, chromatin condensation, DNA fragmentation, membrane-bound condensing and form as apoptotic bodies. While necrotic cell death is performed in differential morphological changes including loss of membrane integrity, cell swelling, no vesicle formation, and cell lysis (Norishi, Gur, and Sudhir, 2000). The illustration of the morphological features of apoptosis and necrosis is shown in figure 6.

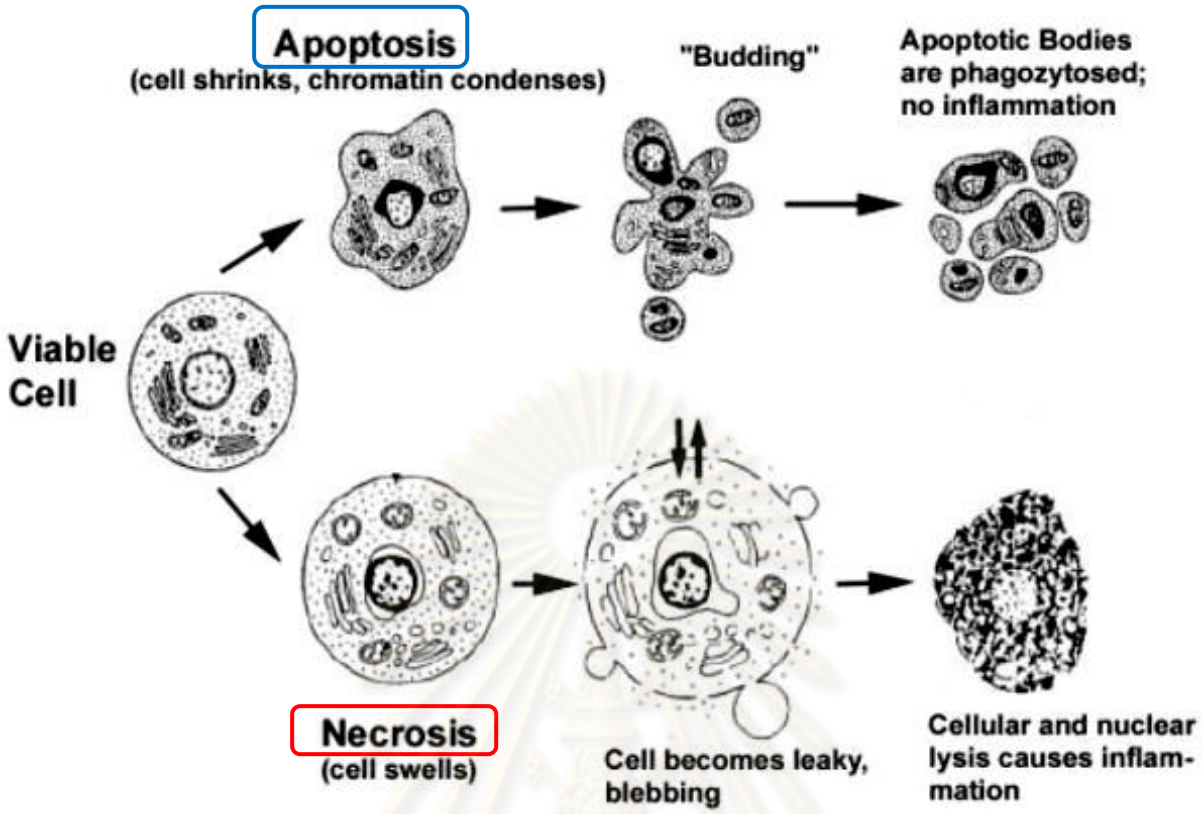


Figure 6 The morphological changes of apoptotic and necrotic cell death

Comparing to other organs, kidney has long been studied as an organ that can generate ROS and vulnerable to ROS-induced organ damage (Paller, Hoidal, and Ferris, 1984). Several renal diseases, including glomerulonephritis, vasculitis, toxic nephropathies, acute renal failure, and others are likely to be mediated, at least in part, by oxidative injury (Andreoli, 1991; Thadhani, Pascual, and Bonventre, 1996; Shah, 2008). One of the major factors resulting in the susceptibility of kidney to severe oxidant stresses is kidney transport process. Due to oxygen consumption during kidney transport, $O_2^{\bullet-}$ and H_2O_2 are produced by mitochondria. Therefore, the more kidney transport work, the more decrease in cellular GSH storages (Paller, 1988). Among the parts of kidney, the S3 segment of proximal tubule has received much attention because it is selectively susceptible to various types of oxidative injury such as ischemia-reperfusion and toxicant-induced nephropathies (Venkatachalam *et al.*, 1978; Tsuraya *et al.*, 2003). The recent study showed that ROS reduced the regeneration of proximal tubular cells, while promote the proliferation of interstitial cells (Kim, Jung, and

Park, 2010). The conceivable mechanisms may involve in an early fall in ATP levels when renal tubular epithelial cells are insulted from oxidant injury, while cell detachment and lytic injury occur later (Andreoli and McAteer, 1990). In addition, ROS also have a great impact on membrane potential and cellular morphology of renal tubular by disruption the actin cytoskeleton function (Scott *et al*., 1987).

Owing to the fact that cisplatin is able to elevate the intracellular ROS level in many cells, such many studies have suggested that cisplatin-induced nephrotoxicity is ascribed by oxidative stress resulting from free radical generation (Kawai *et al*., 2006; Santos *et al*., 2007). Recently report has exhibited that cisplatin treatment caused xanthine oxidase activation and cellular anti-oxidant systems impairment resulting in significantly increase oxidant damage to kidney. Therefore, the oxidative reactions were accelerated in kidney tissues such as increase in renal lipid peroxidation, and oxidative modification at sulfhydryl moieties of proteins (Cetin *et al*., 2006).

There is much evidence trying to perform that free radicals are responsible for the development of cisplatin-induced acute renal failure. The protective effects of specific ROS scavengers as well as various natural or synthetic anti-oxidants on cisplatin nephrotoxicity are investigated by given them prior or together with cisplatin treatment. For instance, dimethylthiourea (DMTU; $^{\bullet}$OH scavenger) pretreatment was able to reduce tubular cell apoptosis and nephrotoxicity (Jiang *et al*., 2007), selenium combined with a high dose of vitamin E preserved GSH concentration, up-regulated GPx, and reduced MDA level in Wistar rat kidneys (Naziroglu, Karaoglu, and Aksoy, 2004), garlic powder which is well-known its anti-oxidant property prevented cisplatin-induced nephrotoxicity and lipid peroxidation in Wistar rats (Razo-Rodriguez *et al*., 2008), renal impairment after cisplatin administration was prevented by GSH pretreatment (Zunino *et al*., 1989), pretreatment with *N*-acetylcysteine (NAC) attenuated acute renal failure in cisplatin-treated rats (Luo *et al*., 2008). Although ROS have been considered to play a central role in cisplatin nephrotoxicity, the exact roles of free radicals and the underlying mechanisms through the beneficial effects of specific free radical scavengers have not been completely evaluated. There is concerned that anti-oxidants might interfere with the anti-cancer activity of cisplatin. For example, several studies showed that GSH has compromised cisplatin cytotoxicity and also a major cause of cisplatin resistant in

cancer cells (Kartalou and Essigmann, 2001; Chen and Kuo, 2010). So far, no one has been elucidated to be a renoprotective agent and not attenuation anti-tumor activity of cisplatin.

3. Natural products against cisplatin nephrotoxicity

Many natural products contain powerful components that fight against various kinds of diseases. Among them, natural products possessing anti-oxidant property have been tried against nephrotoxicity of cisplatin. It has been shown that these dietary anti-oxidants may detoxify ROS, and reduce cisplatin toxic effects (Ali and Moundhri, 2006). Polyphenolic constituents, the prominent compounds which are gained more interesting in their anti-oxidant effect against various diseases and health problems such as cancer, cardiovascular disease, hyperlipidemia and anti-inflamatory as well (Soobrattee *et al*., 2005; Perron and Brumaghim, 2009). The beneficial point that highly relevant to this study is the renoprotective effect of these polyphenolic compounds (Rodrigo and Bosco, 2006). For instance, they played an important role against the glomerular inflammatory processes (Ishikawa and Kitamura, 2000), suppressed proteinuria (Nihei, Miura, and Yagasaki, 2001) and also restricted on glomerular mesangial cell apoptosis (Kitamura and Ishikawa, 1999). Polyphenol contains many groups of chemical substance for example, tannin, flavonoid, and lignins. Among them, flavonoids are the largest and best studied polyphenols which are classified into many compounds, including flavonols, flavones, flavanones, isoflavone, flavanols, catechins, and anthocyanidins (Rice-Evans, Miller, and Paganga, 1996). Therefore, in this study *Glycine max* and *Chrysanthemum indicum* which have high isoflavones and flavonoids content respectively are selected to investigate their anti-oxidant activity and also their renoprotective effect against cisplatin-induced tubular injury.

- **Soybean extract (*Glycine max* extract; GM)**

Soybean is a crucial crop in Asia. Chinese people has long been used this plant as food and traditional medicine as well. It belongs to Fabaceae family and the scientific name is *Glycine max* (L.) *Merrill*. *Glycine max* has been well-recognized as an excellent source of high quality proteins and lipids. It has various biologically active phytochemicals such as

isoflavones, coumestrols, phytosterols, tocopherols, and saponins. Among these substances, isoflavones have provided several health benefits through the protection against oxidative stress (Kay *et al*., 2003). Isoflavones (structure is shown in figure 7) represent the most studied class of phytoestrogen. Their two major bioactive aglycone components are genistein and daidzein (Cederroth and Nef, 2009). The amount of isoflavones in soybeans are varied according to the type of soybean, area of cultivation, culture conditions, and

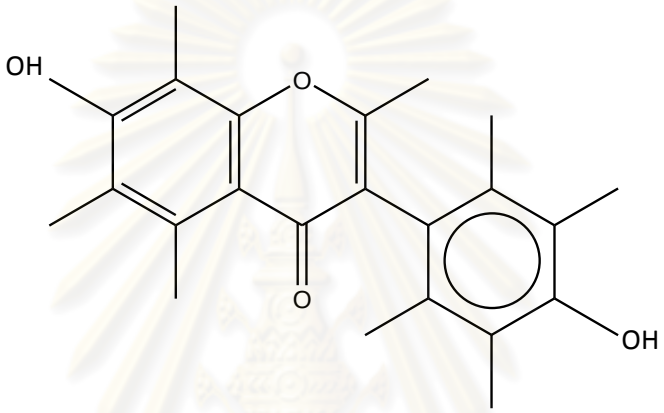


Fig. 7 Chemical structure of isoflavone

processing technique or method. Indeed, alcohol extraction appears to be the method which greatly dissociates isoflavones from soy-proteins (Bhathena and Velasquez, 2002). The amount of isoflavone containing in soybeans and commercially available soy products are around 1 to 5 mg isoflavone/g of soy protein (Wang and Murphy, 1994; Bhathena and Velasquez, 2002). The soybean seeds and their products are shown in figure 8.

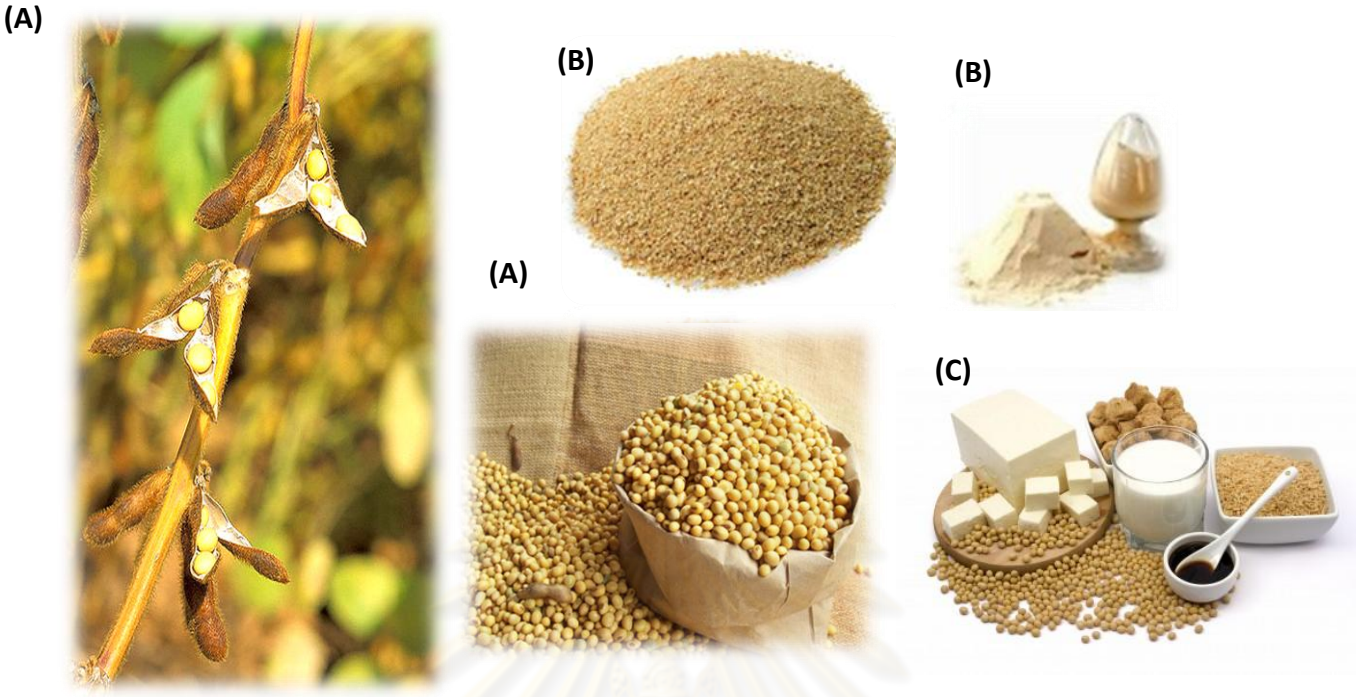


Fig. 8 Soybeans : legumes (A), extracts (B), and commercial products (C)

According to their anti-oxidant property, soybeans have been benefited especially in oxidative stress-induced pathological injuries and diseases for example, cardiovascular disease, osteoporosis, dyslipidemia and cancer (Watanabe, Uesugi, and Kikuchi, 2002; Carson, 2003; Davis *et al*., 2008). Soybean products also exhibited the protective effect in chronic renal disease both in animal models and humans including slowly progressive polycystic kidney disease, reduced proteinuria, glomerular sclerosis, and tubular atrophy (Ranich, Bhathena, and Velasquez, 2001). In addition, the recent study reported that GM can attenuate gentamicin-induced renal tubular injury (Ekor, Farombi, and Emerole, 2006). Although it seems to have a highly potential in oxidant-induced renal cell damage, the relevant mechanism through scavenging properties related with cisplatin nephrotoxicity have not been established.

- **Chrysanthemum flower extract (*Chrysanthemum indicum* extract; CM)**

Chrysanthemum indicum L. which belongs to Asteraceae (Compositae) family is a traditional herb in East Asia. Prepared as tea using dried chrysanthemum flowers is the most popular form of consuming this plant. *Chrysanthemum indicum* flowers as well as extracts and their use are shown in Figure 9. The health benefits of CM in China and Korea herbal medicine are well-known for thousands of year to treat various immune – related

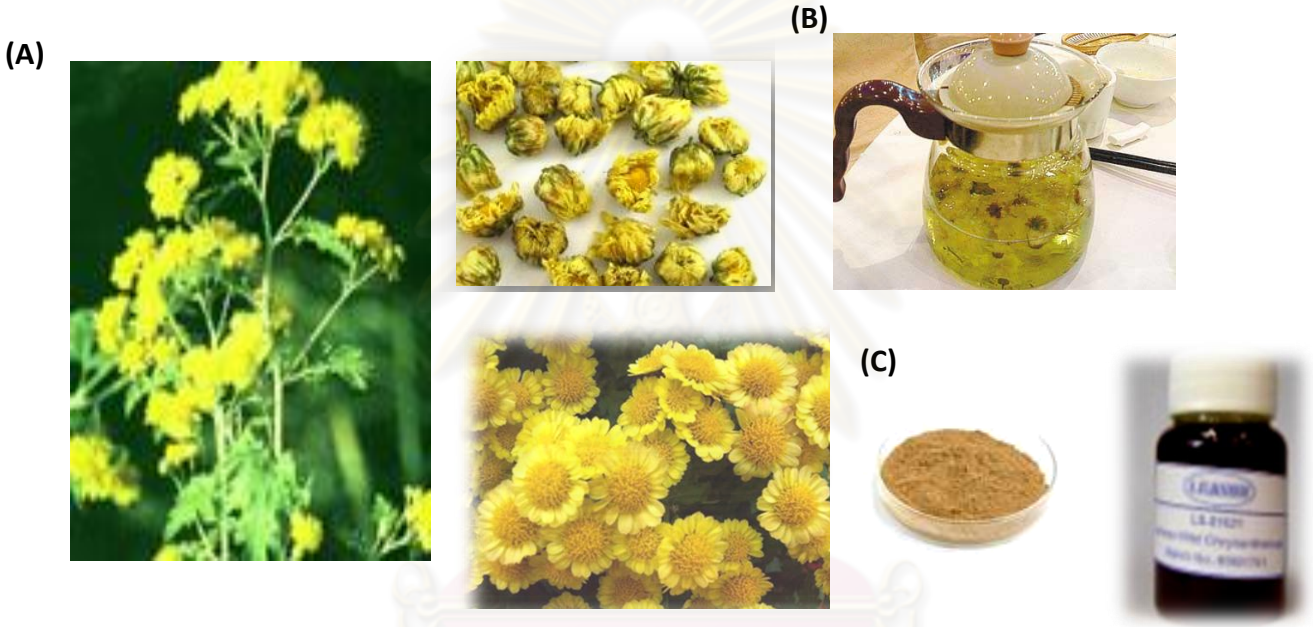


Fig. 9 *Chrysanthemum Indicum* products: flowers (A), chrysanthemum tea (B), and extracts (C)

disorders, hypertensive symptoms and several infection diseases (Shunying *et al*., 2005). Nutritional analysis shows that CM contains choline, vitamin A, vitamin B1, glycosides, adenine, flavonoid, volatile oil, and other amino acid. Several studies reported that CM has powerful anti-oxidants resulting from the highly amount of flavonoid contents. The analysis of chemical compositions also found that the flavonoid groups containing in CM are flavones; (luteolin, apigenin, vitexin) and flavonols (quercetin, myricetin, kaempferol**)** (the structures are shown in figure 10) (Cai *et al*., 2004; Wu *et al*., 2010). The extraction processes that generally use to dissociate flavonoid from chrysanthemum flowers are solvent extraction. Recently, the much evidence reported its pharmacological properties which have been

associtated with the antioxidative effect, including anti-cancer (Li *et al*., 2009), anti-inflamation (Lee *et al*., 2009), and neuroprotective therapy for seizures (Sucher, 2005). Furthermore, the essential oil from CM exhibit strongly anti-microbial activity (Shunying *et al*., 2005; Jung, 2009) and also increase the effect of ampicillin or gentamicin against oral bacteria, when exerted in combination therapy (Jung, 2009). Despite many beneficial effects, the role of CM on cisplatin-induced renal cell death is currently unknown.

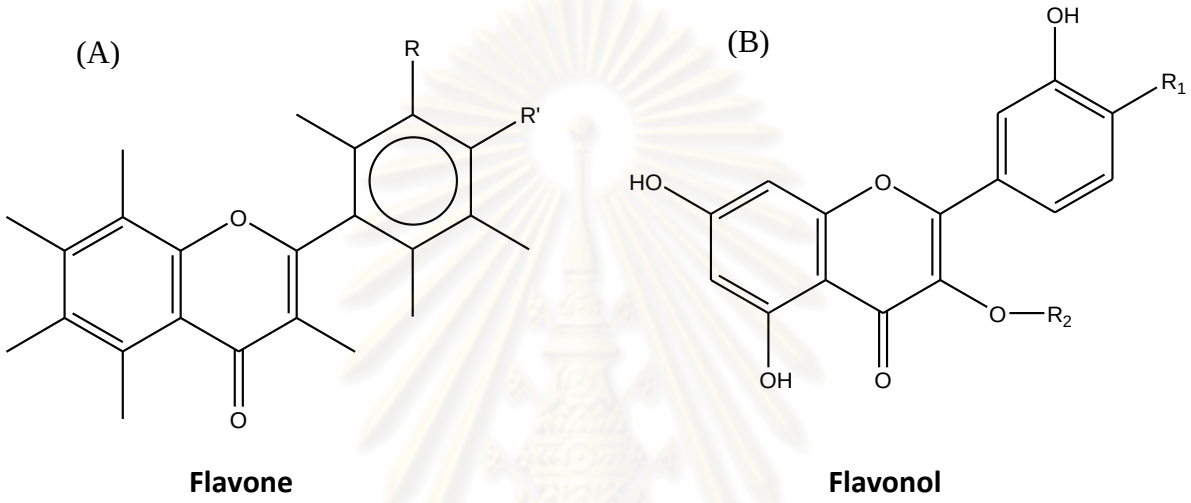


Fig. 10 Chemical structures of flavone and flavonol; the major flavonoid contents in CM

CHAPTER III

MATERIALS AND METHODS

Materials

Glycine max extract (GM) was purchased from Xi'an Huarui Bio-Engineering Co.,Ltd. (Shaanxi, China) . *Chrysanthemum indicum* extract (CM) was purchased from Shanghai Leasun Chemical Co., Ltd. (Shanghai, China). *N*-acetylcysteine (NAC), reduced glutathione (GSH), cisplatin, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 2,7-dichlorofluorescein diacetate ($DCFH_2$-DA), Hoechst 33342, propidium iodide (PI), deferoxamine (DFO), catalase (CAT), and ferrous sulphate were obtained from Sigma Chemical, Inc. (St. Louis, MO, USA). Mn(III)tetrakis (4-benzoic acid) porphyrin chloride (MnTBAP) was obtained from Calbiochem (San Diego, CA, USA). The human proximal tubular epithelial HK-2, human lung cancer epithelial H460, and human melanoma G361 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA).

Methods

1. Cells culture

HK-2 cells were cultured in DMEM medium, while H460 and G361 cells were cultured in RPMI 1640 medium in a 5% CO_2 environment at 37°C. All media are supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine and 100 units/ml of penicillin, and 100 μg/ml streptomycin.

2. Sample preparation

GM and CM were prepared by diluting with deionized water to obtain the desired concentrations.

3. Cytotoxicity assay

Cell viability was determined by MTT assay which measures cellular capacity to reduce 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (yellow) to purple formazan crystal by mitochondria dehydrogenase enzyme. Briefly, cells in 96-well plates were incubated with 500 μg/ml of MTT for 4 h at 37° C. The supernatant was removed and replaced with 100 μl of DMSO to dissolve the formazan crystal. The intensity of formazan product was measured at 570 nm using an ELISA microplate reader. All analyses were performed in at least three independent replicate cultures. Optical density (OD) ratio of treated to non-treated control cells was calculated and presented as relative cell viability.

cell viability was calculated as followed

$$\text{Relative cell viability} = \frac{OD_{570} \text{ of treatment}}{OD_{570} \text{ of control}}$$

4. Apoptosis and necrosis assay

Mode of cell death is determined by Hoechst 33342 and propidium iodide (PI) co-staining assay. Hoechst 33342 is a non-cytotoxic DNA dye that preferentially binds to triplet adenine and thymine base pairs in the minor groove outside of the double helix. Nuclear morphological changes of apoptotic cells using the fluorescence dye Hoechst 33342 were determined. PI is an intercalating agent which binds to DNA. It is membrane impermeant and generally excluded by viable cells. PI is commonly use for identifying necrotic cell death in a population and as a counterstain in multicolor fluorescence techniques. Cells were seeded onto 96-well plates. After 1 h pretreatment and incubation with cisplatin for optimal time, cells were incubated with 10 μM of the Hoechst 33342 and 5 μg/mL PI dye for 2 min at 37°C. The apoptotic cells having condensed chromatin and/or fragmented nuclei and PI-positive necrotic cells were visualized and photographed under a fluorescence microscope. At least three replicate wells were analyzed for each treatment of five random fields in each well.

5. ROS detection

Intracellular ROS level was measured by two methods

- **Measurement of intracellular ROS by fluorescence microscope**

Cells were seeded in a 96-well plate and pretreated with known anti-oxidants (NAC, GSH), GM, and CM or incubated with IC_{50} of cisplatin or specific ROS (H_2O_2)/ROS generators ($FeSO_4$). After incubation for 1 h, cells were washed and incubated with 15 μM fluorescence probe; $DCFH_2$ - DA for 30 min, then detected for intracellular ROS under fluorescence microscope. The results were compared to non-treated cells.

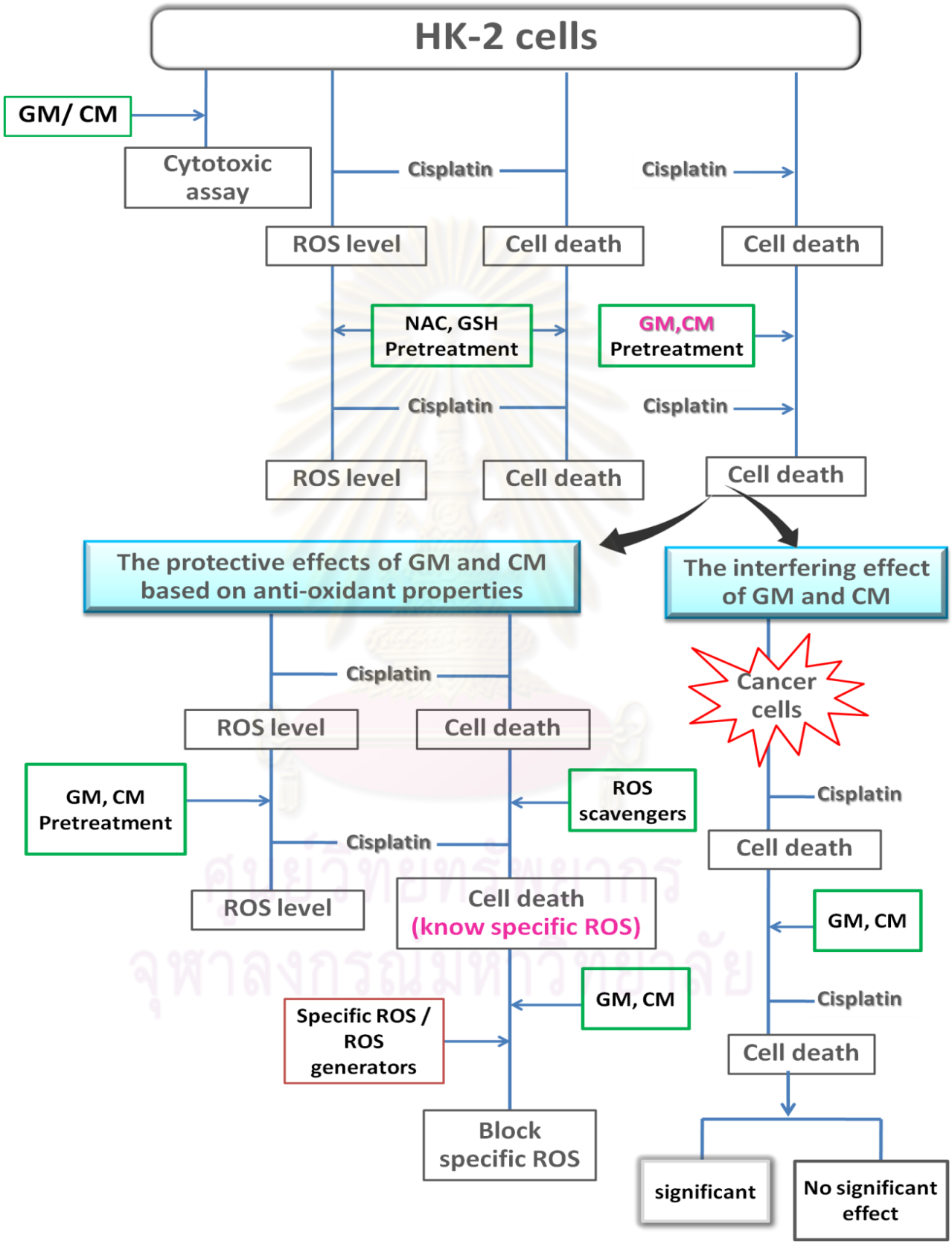
- **Measurement of intracellular ROS by flow cytometry**

Cells were seeded in a 6-well plate and pretreated with known anti-oxidants (NAC, GSH), GM, and CM. After that, cells were treated with IC_{50} of cisplatin, specific ROS (H_2O_2) or ROS generator ($FeSO_4$) for 1 h. Cells were washed three times with ice-cold phosphate buffer saline (PBS) (pH 7.4). Then cells were incubated with 15 μM $DCFH_2$-DA probe for 30 min at 37°C, after which they were washed three times with PBS, trypsinized, and re-suspended in 1 ml of DMEM supplemented with 10% FBS. Fluorescence intensity were immediately examined by flow cytometry using a 485 nm excitation beam and a 538 nm band-pass filter (FACSort, Becton Dickinson, Rutherford, NJ, USA). The mean fluorescence intensity is quantified by CellQuest software (Becton Dickinson) analysis of the recorded histograms.

6. Statistics

Values were expressed as the means ± SEM. The reproducibility of the results was confirmed in at least three independent sets of experiments. Data shown in figures were from a representative set of experiments. Statistical differences between two groups were determined by Student's *t* test. For comparison of multiple groups, analysis of variance (ANOVA) with Scheffe as a post hoc test was conducted. $p < 0.05$ was selected to reflect significance.

Conceptual framework



7. Experimental design

This study aimed to investigate possible protective effect of GM and CM against cisplatin-induced renal cell death by the evaluation of cell viability, cell death, and cellular ROS level. Human proximal tubular epithelial HK-2 cells were used as a cell model for cisplatin-induced renal toxicity. Human lung cancer epithelial H460 cells and human melanoma G361 cells for studying the interfering effect of these extracts on anti-cancer activity of cisplatin.

7.1 Effects of GM and CM on HK-2 cell viability

The proliferative or cytotoxic effects of GM and CM on HK-2 cells were first determined by cell viability MTT assay. HK-2 cells were seeded at the density of 1 x 10^5 cells per well in a 96-well plate and left untreated or incubated with various concentrations of GM and CM (0, 0.025, 0.05, 0.1, 0.2, 0.5, and 1.0 %v/v) for 24 h. Then cell viability was determined.

7.2 Cisplatin induced HK-2 cell death

The effect of cisplatin on HK-2 cell viability was determined in dose and time-dependent responses.

For dose-dependent study, cells were treated with various concentrations of cisplatin (0, 5, 25, 50, and 100 μM) and incubated for 24 h. After treatment, cell viability was determined by MTT assay and IC_{50} was then calculated as the concentration of cisplatin that caused 50% reduction in cell viability.

For time-dependent study, cisplatin-induced renal cell death was investigated by leaving the cells untreated or treated with 50 μM of cisplatin. Cell viability was then determined at various time points (0, 6, 15, and 24 h) by MTT assay. In addition, mode of cisplatin-induced renal cell death in time-dependent manner was further identified by Hoechst 33342/PI nuclear staining assay. Cells were similarly treated with 50 μM of cisplatin for various time points (0, 6, 15, and 24 h). Until the period of time, cells were stained with Hoechst 33342/PI fluorescence dye. The apoptotic cells stained by Hoechst 33342 and PI-positive necrotic cells were detected under fluorescence microscope.

7.3 Cisplatin induced renal cell death through ROS-dependent mechanism

The exact role of ROS generated by cisplatin in renal cell death was determined in this study. Cisplatin-induced ROS generation in HK-2 cells was first detected. Subsequently, the effects of known anti-oxidants (*N*-acetylcysteine; NAC and glutathione; GSH) on cellular ROS and HK-2 cell death in response to cisplatin treatment were evaluated.

7.3.1 Cisplatin induced oxidative stress in HK-2 cells

Time-dependent study was performed to estimate the minimal time that intracellular ROS was distinctively increased in response to cisplatin treatment. Cells were treated with 50 μM of cisplatin and incubated for various time points (0, 15, 30 min, 1, and 2 h). Until the period of time, cells were washed and incubated with $DCFH_2$-DA. Intracellular ROS was detected under fluorescence microscope.

7.3.2 Effect of anti-oxidants on intracellular ROS level generated by cisplatin

The effects of anti-oxidants (NAC and GSH) on cellular ROS induced by cisplatin were determined by two methods.

- *Fluorescence microscope* : Cells were pretreated with 5 mM NAC and 5 mM GSH for 1 h, then treated with 50 μM of cisplatin. After incubation for 1 h, cells were washed and incubated with $DCFH_2$-DA. Intracellular ROS was detected under fluorescence microscope.

- *Flow cytometry* : Cells were similarly pretreated with NAC and GSH for 1 h, then incubated with 50 μM of cisplatin for 1 h. After the incubation period, cells were washed with PBS, and then incubated with $DCFH_2$-DA. The fluorescence intensity was examined by flow cytometry.

7.3.3 Effects of anti-oxidants on HK-2 cell viability in response to cisplatin treatment

Cells were pretreated with 5 mM NAC and 5 mM GSH for 1 h, and then incubated with 50 μM of cisplatin. After incubation for 24 h, cell viability was determined by MTT assay. Hoechst 33342 and PI staining assay were also used to investigate the protective effect of these anti-oxidants on cell apoptosis and necrosis.

7.4 GM and CM protected renal cells from cisplatin-induced cytotoxicity

Protective effects of GM and CM on cisplatin-induced renal cell death were evaluated in both terms of cell death and intracellular ROS detection.

7.4.1 Effects of GM and CM on HK-2 cell viability in response to cisplatin treatment

HK-2 cells were pre-incubated with various concentrations of GM and CM (0.025, 0.05, 0.1, and 0.2 %v/v). After an hour, cells were treated with 50 μM of cisplatin for 24 h. To determine the protective effect of these extracts, cell viability and cell death detection were performed by MTT and Hoechst 33342/ PI staining assay, respectively.

7.4.2 Effects of GM and CM on cellular ROS level generated by cisplatin

Cells were pretreated with 0.1, and 0.2%v/v of GM and CM for 1 h, and then treated with 50 μM of cisplatin. After incubation for 1 h, cellular ROS levels were detected by fluorescence microscope and flow cytometry with $DCFH_2$-DA, the specific fluorescence dye.

7.5 The specific ROS which are responsible for cisplatin-mediated cytotoxicity in renal cells

In order to determine the specific ROS which are responsible for cisplatin-induced renal toxicity, cells were pre-incubated with various ROS scavengers; 50 μM MnTBAP, 1 mM deferoxamine (DFO) and 5000 U/ml catalase. After 1 h incubation, cells were treated with 50 μM of cisplatin for 24 h. Cell viability was determined by MTT assay. Moreover, apoptotic and necrotic cell death were detected under fluorescence microscope by Hoechst 33342 and PI staining assay.

7.6 Specific ROS scavenging activities of GM and CM

In this study, hydrogen peroxide and ferrous sulphate were used as specific ROS and ROS generator, respectively. Cellular ROS induced by these two compounds in time-dependent manner were first detected in HK-2 cells. Subsequently, the specific scavenging

activity of GM and CM against hydrogen peroxide and ferrous sulphate were evaluated in this section.

7.6.1 Intracellular ROS generation induced by specific ROS and ROS generator treatment

Time-dependent study was performed to estimate the minimal time that intracellular ROS was distinctively elevated in response to hydrogen peroxide and ferrous sulphate treatment. Cells were exposed to hydrogen peroxide (specific ROS) or ferrous sulphate (hydroxyl radicals generator) and incubated for various time points (0, 15, 30 min, 1 h, and 2 h). The intracellular ROS was detected under fluorescence microscope with a specific fluorescence probe; $DCFH_2$-DA.

7.6.2 Scavenging activities of GM and CM against specific ROS or ROS generator treatments

The anti-oxidant effect of GM and CM against hydrogen peroxide and hydroxyl radical induced oxidative stress was evaluated. Cells were pretreated with 0.1 and 0.2 %v/v of GM and CM prior to the exposure with hydrogen peroxide or ferrous sulphate. After incubation for 1 h, intracellular ROS levels were determined by flow cytometry with $DCFH_2$-DA.

7.7 The interfering effect of GM and CM on cisplatin-induced cancer cell death

The major concern of this study was the interfering effect of GM and CM on cisplatin anti-cancer activity. Therefore, human lung (H460) and human melanoma (G361) cancer cells which are susceptible to cisplatin treatment were used as models. After pretreatment with GM and CM, the changes in cancer cell viability as well as apoptosis and necrosis induced by cisplatin were evaluated, comparing to group that treated with cisplatin.

7.7.1 Cisplatin induced lung (H460) and human melanoma (G361) cancer cell death

Cisplatin induced H460 and G361 cell death were first determined in terms of dose and time-dependent effect.

For dose-dependent study, cells were treated with various concentrations of cisplatin (0, 5, 25, 50, 100, 200, and 300 μM) for 24 h, then cell viability was determined by MTT assay.

For time-dependent study, H460 and G361 cells were incubated with 100 μM of cisplatin for various time points (0, 6, 15, and 24 h). After that, cell viability and morphology of apoptosis and necrosis were analyzed by MTT and Hoechst 33342/PI nuclear staining assay, respectively.

7.7.2 Effects of GM and CM on cisplatin induced lung (H460) and melanoma (G361) cell death

The purpose of this study was to investigate whether GM and CM are able to reduce anti-cancer activity of cisplatin, H460 and G361 cells were left untreated or pretreated with 5 mM NAC, 5 mM GSH, 0.1 and 0.2%v/v of GM and CM for 1 h and then exposed with 100 μM of cisplatin. After 24 h incubation, cell viability was determined by MTT assay as well as apoptotic and necrotic cell death was detected under fluorescence microscope by Hoechst 33342 and PI staining assay.

CHAPTER IV

RESULTS

1. Effects of GM and CM on HK-2 cell viability

1.1 Effect of GM on HK-2 cell viability

This study first determined the effect of GM on HK-2 cell viability by MTT assay. Cells were incubated with various concentrations of GM (0.025, 0.05, 0.1, 0.2, 0.5, and 1.0%v/v) and cell viability was determined after 24 h incubation. The results clearly showed that 0.025, 0.05, 0.1, and 0.2%v/v of GM provided non-significant alteration in HK-2 cell viability (figure 11) (96.32 ± 2.86%, 97.29 ± 1.33%, 101.71 ± 3.25%, and 102.73 ± 1.92%, respectively), whereas the percentage of cell viability was significantly increased at 0.5, and 1.0%v/v of GM (107.43 ± 3.19%, and 107.97 ± 3.14%, respectively). This result implied that GM had no significant toxic effect on HK-2 cells.

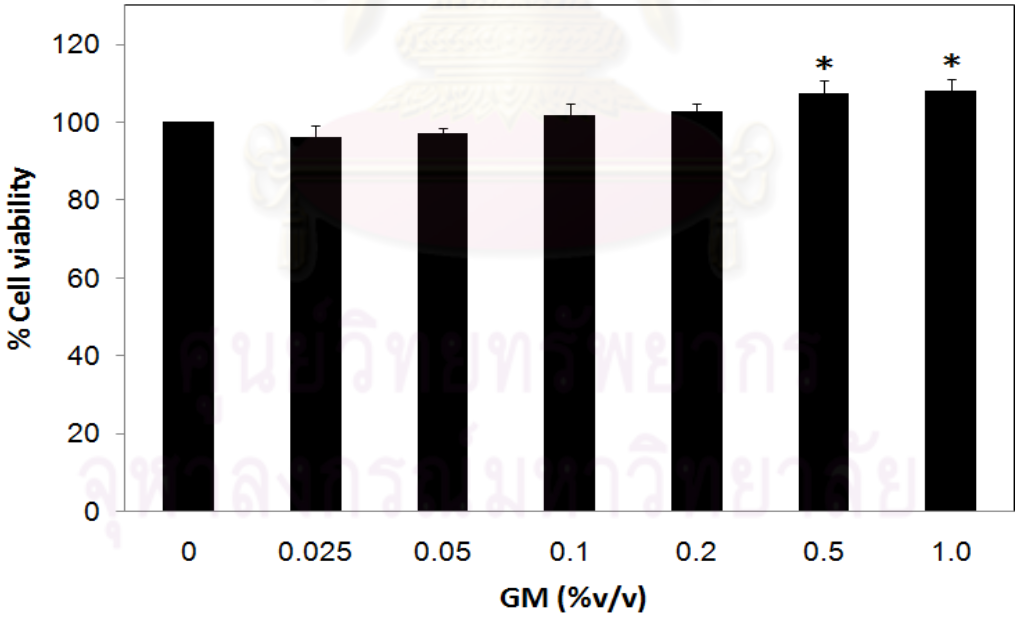


Figure 11 Effects of GM on HK-2 cell viability as a function of dose. Cells were treated with various concentrations of GM (0-1.0 %v/v) for 24 h, and then cell viability was determined by MTT assay. Values were represented as relative cell viability compared to control cells and plots were mean ± SEM. (n=3). * $P < 0.05$ versus non-treated control.

1.2 Effect of CM on HK-2 cell viability

HK-2 cell viability in response to CM treatment was determined by leaving the cells untreated or treated with various concentrations of CM (0.025, 0.05, 0.1, 0.2, 0.5, and 1.0%v/v) and cell viability was evaluated after 24 h incubation. Similarly, these concentrations of CM did not have toxic effect on HK-2 cells (figure 12). The percentage of cell viability in response to 0.025, 0.05, 0.1, and 0.2%v/v of CM treatment were no significant alteration compared to non-treated controls (94.45 ± 2.80%, 97.25 ± 1.63%, 101.37 ± 1.50%, and 102.81 ± 1.91%, respectively), whereas 0.5 and 1.0%v/v of CM caused a significant increase in HK-2 cell viability (108.31 ± 1.52%, and 109.22 ± 3.45%, respectively).

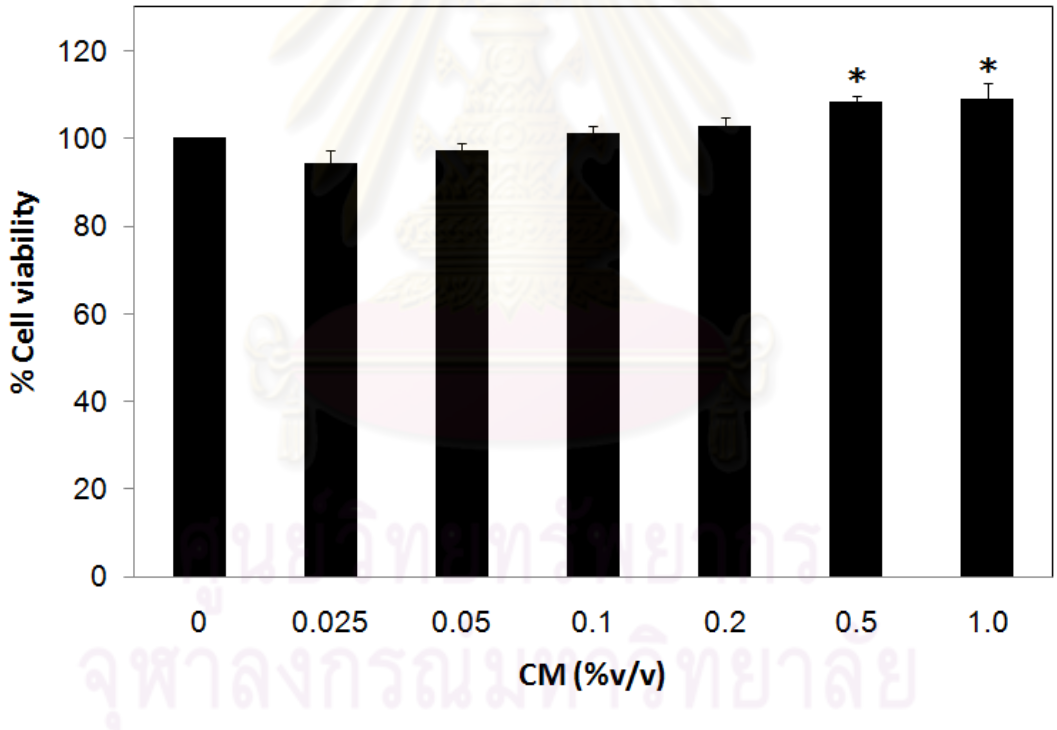


Figure 12 Effects of CM on HK-2 cell viability as a function of dose. Cells were left untreated or treated with various concentrations of CM (0-1.0 %v/v) for 24 h, and then cell viability was determined by MTT assay. Values are represented as relative cell viability compared to control cells and plots are mean ± SEM. (n=3). * $P < 0.05$ versus non-treated control.

2. Cisplatin induced HK-2 cell death

In order to investigate the cytotoxic effect of cisplatin on HK-2 cells, dose and time-dependent responses of HK-2 cells to cisplatin treatment were determined.

For dose dependent study, cells were left untreated or treated with various concentrations of cisplatin (0, 5, 25, 50, and 100 μM) for 24 h. The results showed that cisplatin treatment caused a dose-dependent decrease in cell viability. The reduction was first significantly observed at 25 μM of cisplatin (71.66 ± 2.93% cell viability) (figure 13A) and continuously decreased to approximately 50% at the concentration of 50 μM (55.77 ± 2.24% cell viability). Since apoptosis and necrosis have been shown to be two major mechanisms of cell death, mode of cell death in response to various concentrations of cisplatin treatment was identified by Hoechst 33342 and PI staining assay. The results showed that the decrease in cell survival as presented in figure 13A was mainly due to apoptosis. Treatment of the cells with increasing concentration of cisplatin caused a dose-dependent increase in cell apoptosis over control level, as indicated by the increase in nuclear fluorescence, DNA condensation, chromatin fragmentation, and apoptotic bodies. Necrotic cell death, the cells stained with red fluorescence, in response to low concentration of cisplatin (5-50 μM) was presented in a very less quantity and increased in 100 μM of cisplatin treatment (figure 13B).

For time-dependent study, HK-2 cells were incubated with 50 μM of cisplatin for various time points (0, 6, 15, and 24 h) and cell viability was determined by MTT assay. The results exhibited that HK-2 cell viability was significantly reduced as early as 15 h (74.97 ± 2.39 %), then further decreased to 53.23 ± 2.95% at 24 h (figure 14A). The morphology of apoptosis and necrosis at various time points in response to cisplatin treatment was also identified by Hoechst 33342 and PI staining assay. Treatment of the cells with increasing cisplatin incubation time caused a time-dependent increase in cell apoptosis over control level, whereas necrotic cell death was presented in a very less quantity as indicated by the cells exhibiting red fluorescence staining (figure 14B). The nuclear morphology of apoptosis and necrosis cells in various time points strongly correlated with the percentage of cell viability from MTT assay.

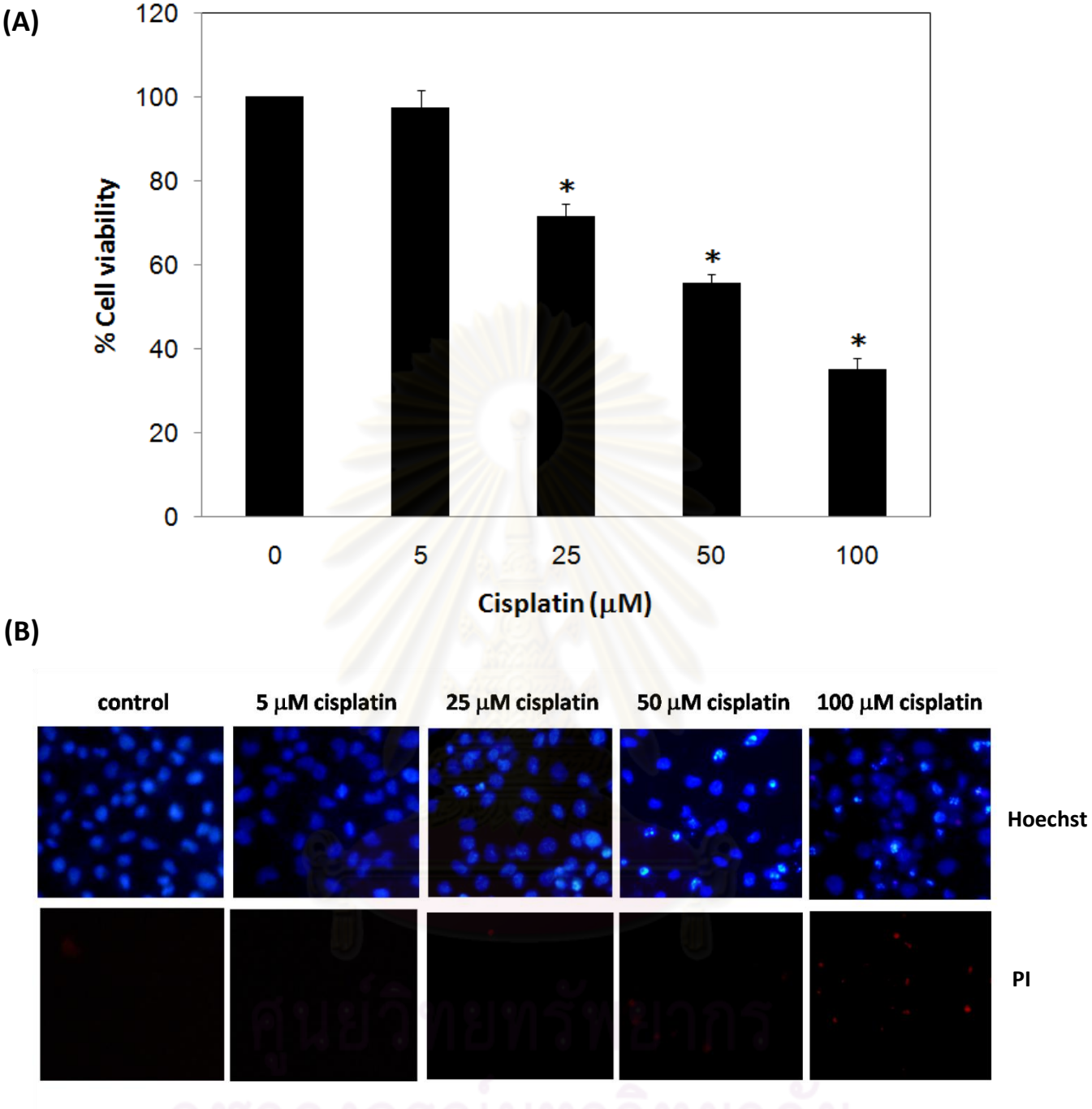


Figure 13 Cytotoxic effect of cisplatin on HK-2 cells in dose-dependent study. (A) Cells were left untreated or treated with various concentrations of cisplatin (0, 5, 25, 50, and 100 μM) for 24 h, cell viability was determined by MTT assay. Values are represented as relative cell viability compared to control cells and plots are mean ± SEM. (n=3). * $P < 0.05$ versus non-treated control. (B) Morphological analysis of apoptotic and necrotic cell death were detected by Hoechst 33342 and PI staining assay. Representative photographs are shown from three independent experiments.

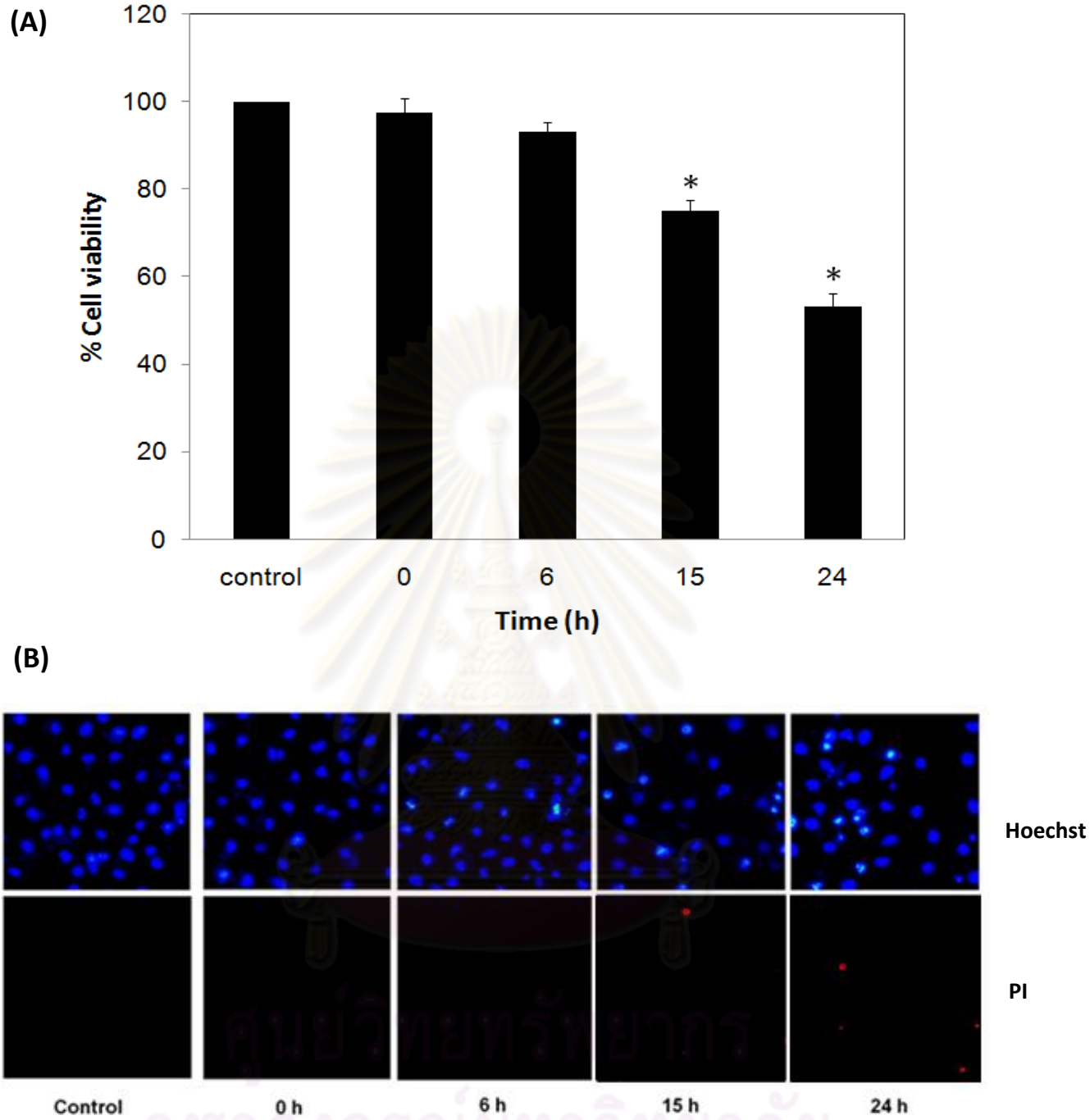


Figure 14 Time dependent effect of cisplatin on HK-2 cells. (A) Cells were left untreated or treated with 50 μM of cisplatin for various incubation times (0, 6, 15, and 24 h). Cell viability was determined by MTT assay. Values are represented as relative cell viability and plots are mean ± SEM. (n=3). * $P < 0.05$ versus non-treated control. (B) Morphological analysis of apoptotic and necrotic cell death were detected by Hoechst 33342 and PI staining assay. Representative photographs are shown from three independent experiments.

3. Cisplatin induced renal cell death through ROS-dependent mechanism

3.1 Cisplatin induced oxidative stress in HK-2 cells

To provide supporting evidence for the correlation of ROS and cisplatin-induced cytotoxicity, intracellular ROS levels in response to 50 μM cisplatin treatment at various time points were detected by ROS-specific fluorescence dye, $DCFH_2$-DA. The results of fluorescence microscope clearly showed that cisplatin caused a significant induction of intracellular ROS in HK-2 cells as early as 30 min and peaked at 1 h and also sustained to 2 h (figure 15).

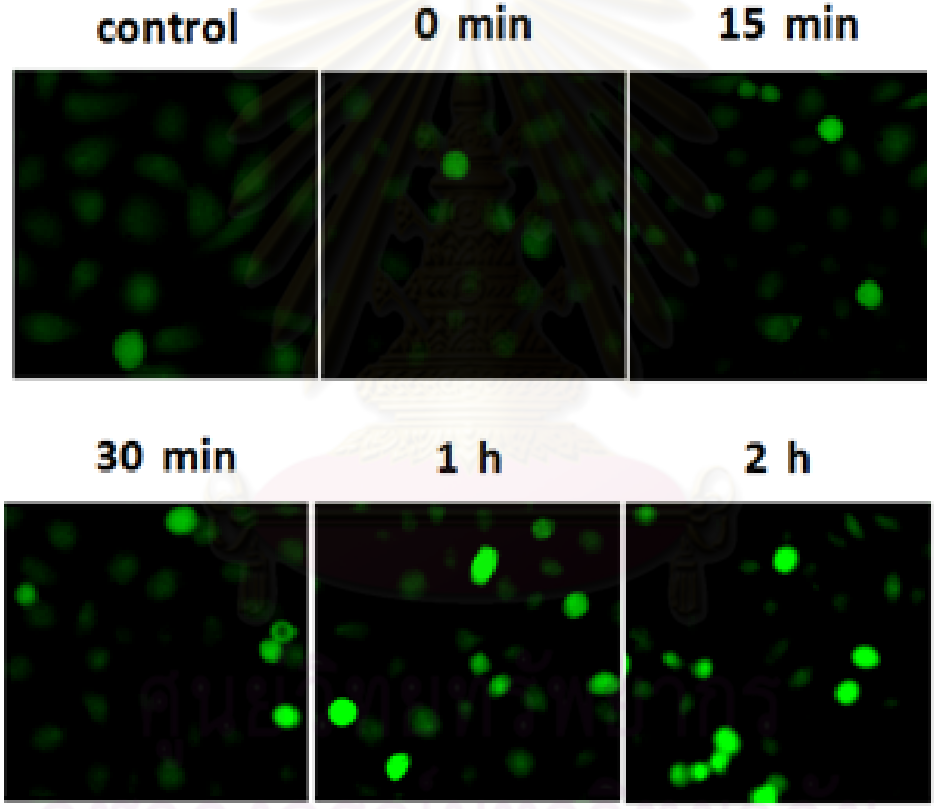


Figure 15 Effect of cisplatin on intracellular ROS levels in HK-2 cells in time-dependent study. Cells were treated with 50 μM of cisplatin and incubated for various time ponits (0, 15, 30 min, 1, and 2 h). The increase of fluorescence intensity was detected by fluorescence microscope with a specific probe,$DCFH_2$-DA.

3.2 Effect of anti-oxidants on intracellular ROS levels generated by cisplatin

The role of anti-oxidant in the blockage of oxidative stress in HK-2 cells induced by cisplatin was evaluated by leaving the cells untreated or pre-incubated with NAC and GSH prior to 50 μM cisplatin exposure. After 1 h, cellular ROS levels were detected by fluorescence microscope and flow cytometry, using $DCFH_2$-DA as a specific fluorescence dye.

Intracellular ROS detected under fluorescence microscope clearly demonstrated that cellular ROS levels were dramatically increased in response to cisplatin treatment and markedly reduced by NAC and GSH pretreatment (figure 16A).

In addition, intracellular ROS levels were evaluated using flow cytometry in order to confirm the results of fluorescence microscope. As demonstrated in figure 16B, treatment of the cells with 50 μM of cisplatin caused an elevation of cellular ROS levels over non-treated control (1.47 folds). Pretreatment with NAC and GSH completely blocked cisplatin-induced ROS generation in HK-2 cells to 0.57, and 0.46 folds, respectively (figure 16B).

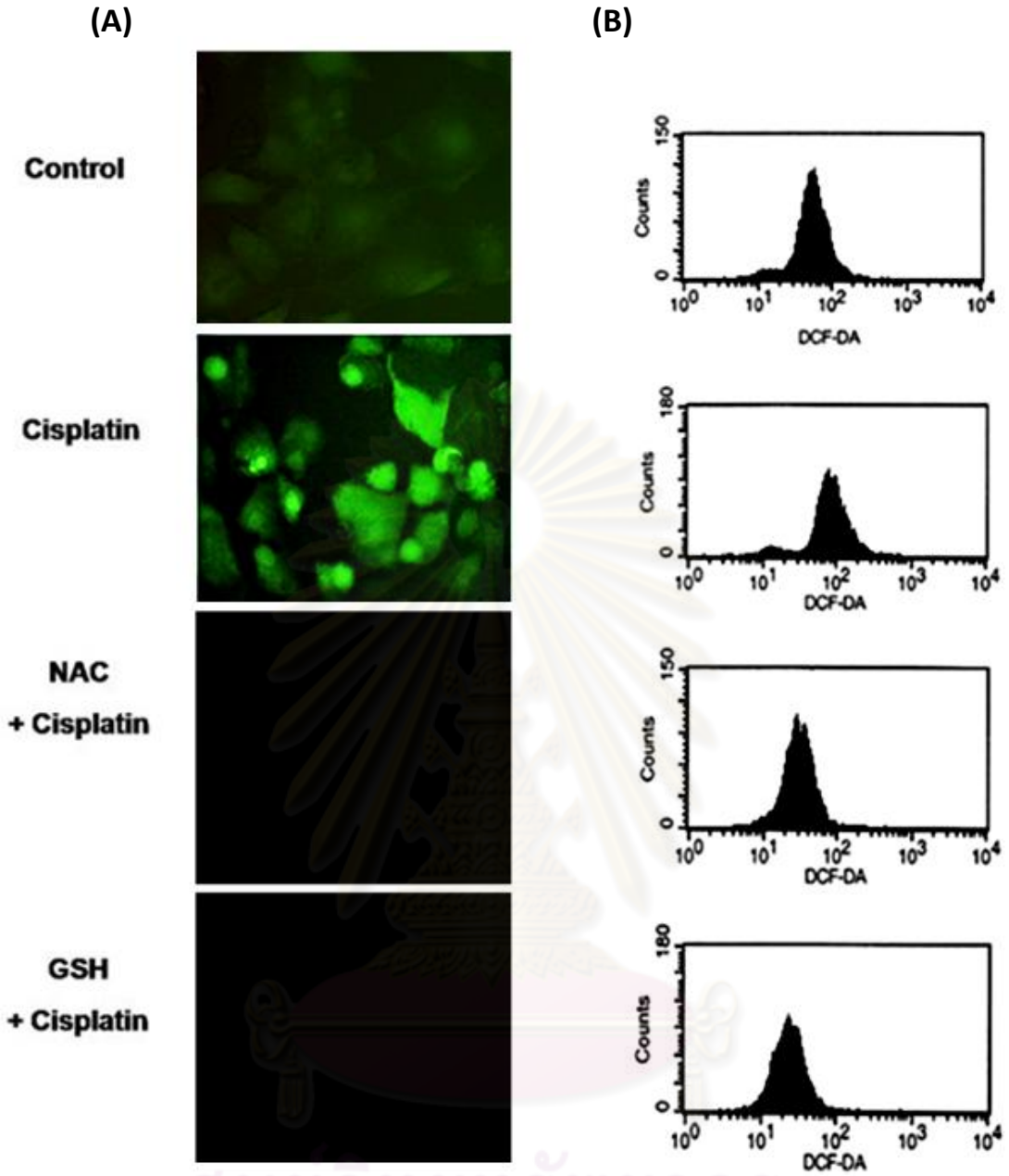


Figure 16 Effect of anti-oxidants on intracellular ROS induced by cisplatin. (A) Cells were left untreated or incubated with 5 mM NAC and 5 mM GSH for 1 h prior to 50 μM cisplatin treatment. After 1 h cisplatin incubation, intracellular ROS was detected by fluorescence microscope with $DCFH_2$-DA. (B) Cells were similarly treated and then intracellular ROS levels were detected using flow cytometry with $DCFH_2$-DA as a fluorescence probe.

3.3 Effects of anti-oxidants on HK-2 cell death induced by cisplatin

After treatment with 50 μM of cisplatin for 24 h in the presence or absence of NAC and GSH, cell viability was determined by MTT assay. The result exhibited that 50 μM of cisplatin caused a reduction in HK-2 cell survival approximately 50% (54.81 ± 2.07%). Pretreatment of the cells with either NAC or GSH dramatically abolished the toxic effect of cisplatin on HK-2 cell viability (81.45 ± 1.56% and 93.31 ± 1.83%, respectively).

The protective effects of NAC and GSH were further investigated by Hoechst 33342 and PI nuclear staining assay. Very few dead cells were detected in the untreated control (figure 17B). In contrast, 50 μM of cisplatin-treated cells exhibited the typical morphologies of apoptosis, including nuclear condensation, chromatin fragmentation, and apoptotic bodies, whereas necrotic cell death was observed in a very less amount (figure 17B). Cisplatin-mediated cell apoptosis and necrosis were significantly decreased in NAC and GSH pretreatment groups as compared to untreated control (figure 17B).

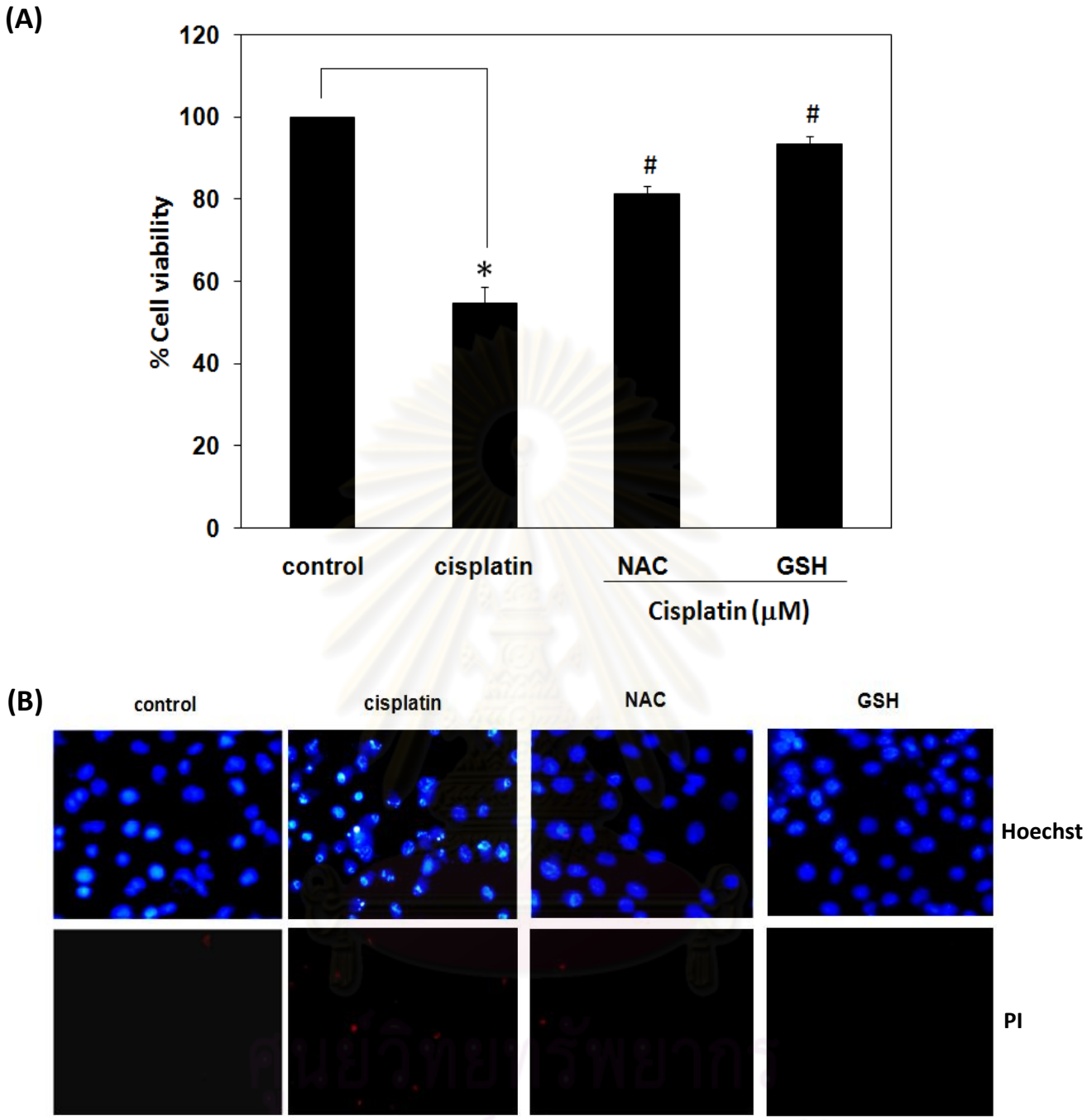


Figure 17 Effects of anti-oxidants on cisplatin-induced renal cell death. (A) HK-2 cells were left untreated or incubated with known anti-oxidant (5 mM NAC, and 5 mM GSH) for 1 h prior to 50 μM cisplatin treatment. After 24 h, cell viability was determined by MTT assay. Values are represented as relative cell viability and plots are mean ± SEM. (n=3). * $P < 0.05$ versus non-treated control and # $P < 0.05$ versus cisplatin-treated control. (B) Morphological analysis of apoptotic and necrotic cell death were detected by Hoechst 33342 and PI staining assay. Representative photographs are shown from three independent experiments.

4. GM and CM protected renal cells from cisplatin-induced cytotoxicity

4.1 Cell death detection of GM and CM pretreatment

4.1.1 Effects of GM on HK-2 cell viability in response to cisplatin exposure

To investigate the protective effect of GM on cisplatin-induced renal cell death as a function of dose, HK-2 cells were left untreated or pre-incubated with various concentrations of GM (0.025-0.2%v/v) for 1 h prior to 50 μM of cisplatin exposure. After 24 h incubation, cell viability and cell death were determined.

The result of MTT assay indicated that cisplatin caused a significant reduction in cell survival approximately 50% (55.09 ± 2.07%). GM pretreatment, at the concentrations of 0.1 and 0.2%v/v, could prevent the loss of cell viability in response to cisplatin treatment (67.82 ± 2.04 % and 78.86 ± 2.63 %, respectively) (figure 18A)

This study also used Hoechst 33342 and PI staining assay to further investigate the protective effect of GM on cisplatin-induced renal cell death. The nuclear staining results exhibited the normal morphology of living cells in control (figure 18B). Treatment of the cells with 50 μM cisplatin caused an increase in cell apoptosis, as indicated by increase in nuclear fluorescence, chromatin condensation, and apoptotic bodies, whereas positive necrotic cell death was exhibited very less amount (figure 18B). Cisplatin-mediated renal cell apoptosis could be reduced in GM pretreatment groups in dose-dependent manner (figure 18B). These results provided the evidence that 0.1 and 0.2%v/v of GM could significantly prevent cisplatin-induced HK-2 cell damage.

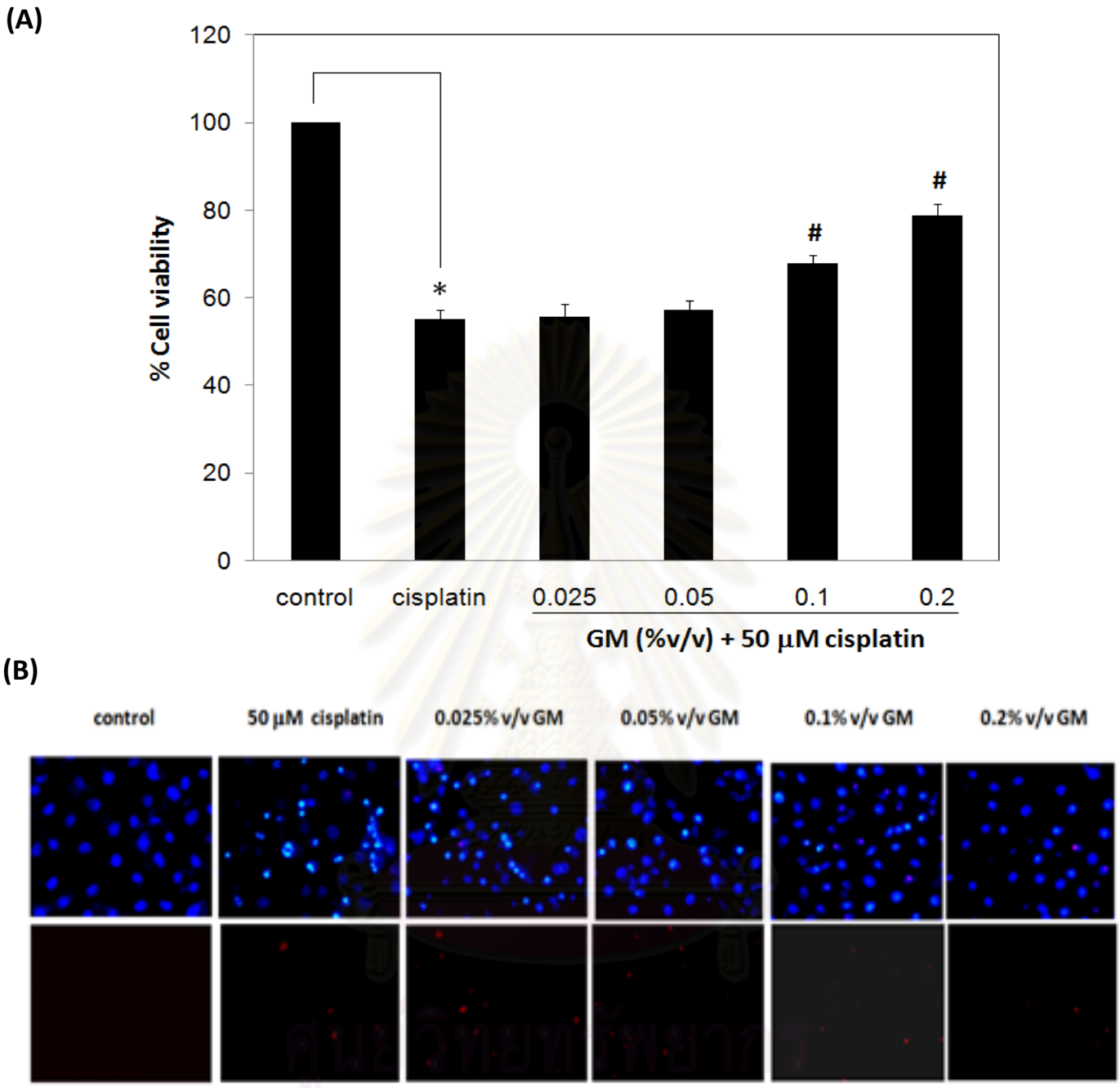


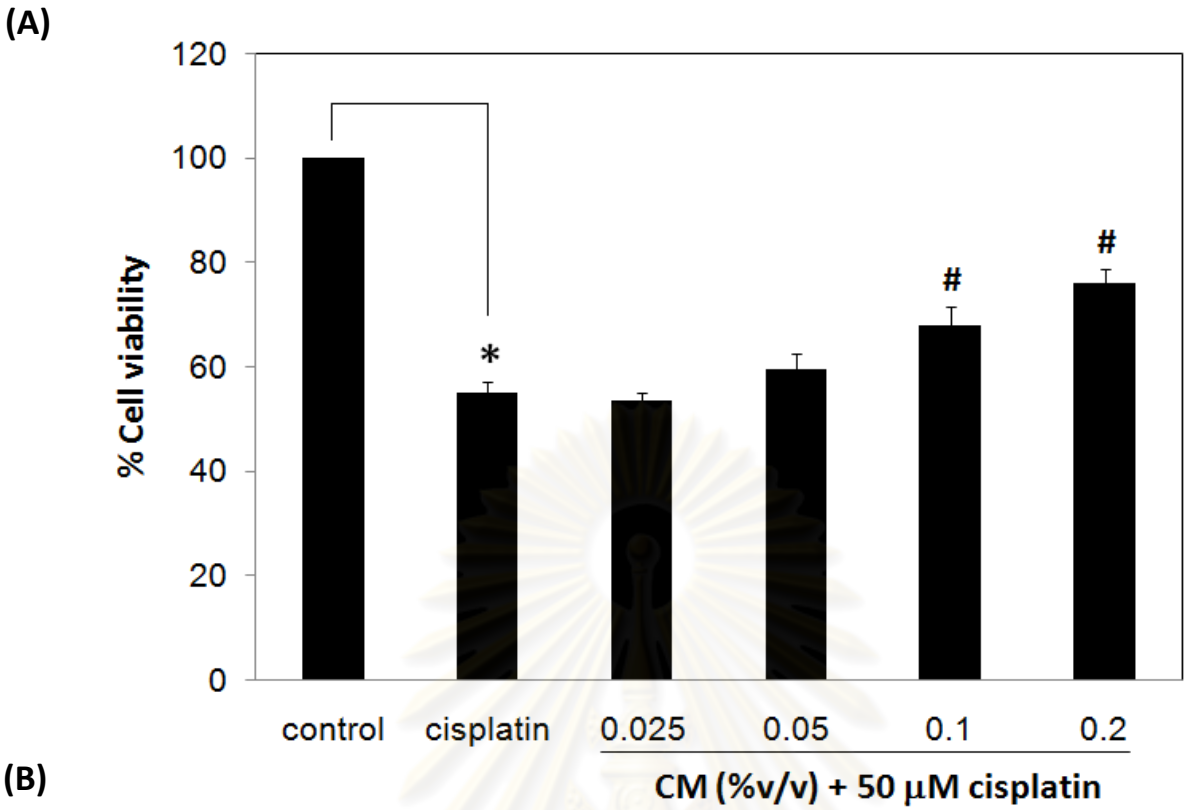
Figure 18 Protective effect of GM on cisplatin-induced renal cell death. (A) HK-2 cells were left untreated or incubated with various concentrations of GM (0.025, 0.05, 0.1, and 0.2%v/v) for 1 h prior to 50 µM cisplatin treatment. After 24 h, cell viability was determined by MTT assay. Values are represented as relative cell viability and plots are mean ± SEM. (n=4). * $P < 0.05$ versus non-treated control and # $P < 0.05$ versus cisplatin-treated control. (B) Morphological analysis of apoptotic and necrotic cell death were detected by Hoechst 33342 (top) and PI (below) staining assay. Representative photographs are shown from three independent experiments.

4.1.2 Effects of CM on HK-2 cell viability in response to cisplatin exposure

HK-2 cells were left untreated or pretreated with various concentrations of CM (0.025-0.2%v/v) for 1 h and treated with 50 μM of cisplatin. After 24 h incubation, cell viability and cell death were analyzed.

The result indicated that HK-2 cell viability was significantly decreased approximately 50% (55.09 ± 2.07%) in response to cisplatin exposure. The dose-dependent effect of CM pretreatment on HK-2 cell viability indicated that 0.1 and 0.2%v/v of CM significantly prevented HK-2 cell death induced by cisplatin (67.84 ± 3.70% and 76.15 ± 2.58%, respectively).

The results of cell death detected by Hoechst 33342/ PI staining assay showed that apoptotic cell death were increased in response to 50 μM cisplatin treatment. Whereas necrotic cell death stained with red fluorescence was exhibited in a very less amount (figure 19B). Cisplatin-mediated renal cell apoptosis could be markedly reduced in CM pretreatment groups in dose-dependent manner (figure 19B). These results indicated that cisplatin-induced HK-2 cell death could be prevented by 0.1 and 0.2%v/v of CM pre-incubation.



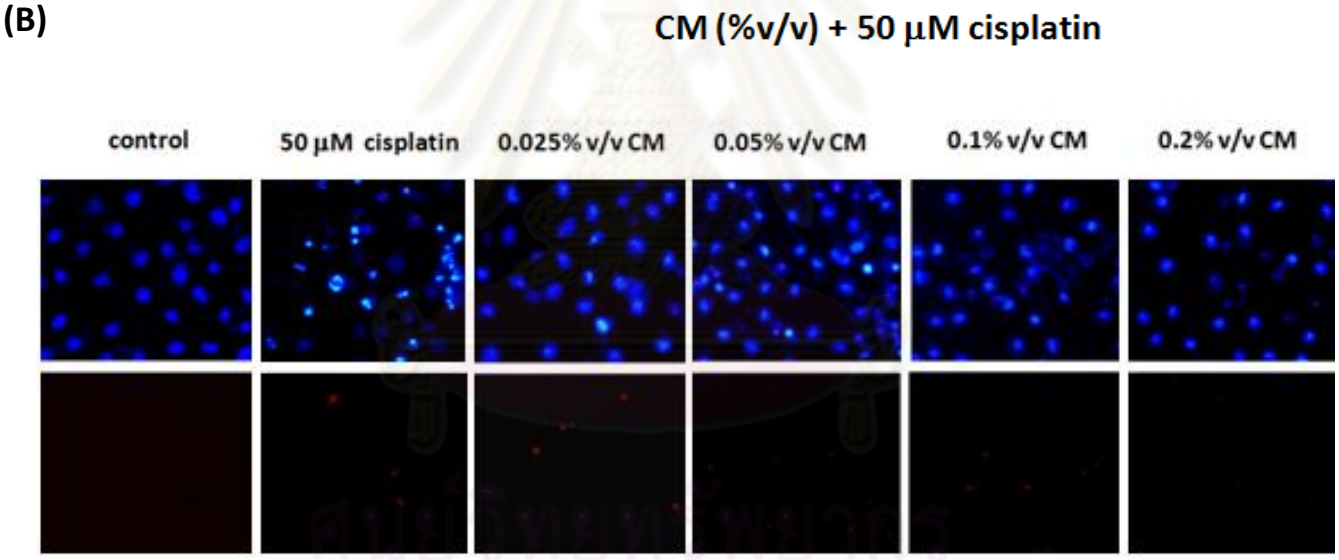


Figure 19 Protective effect of CM on cisplatin-induced renal cell death. (A) HK-2 cells were left untreated incubated or with various concentrations of CM (0.025, 0.05, 0.1, and 0.2%v/v) for 1 h prior to 50 µM cisplatin treatment. After 24 h, cell viability was determined by MTT assay. Values are represented as relative cell viability and plots are mean ± SEM. (n=4). * $P < 0.05$ versus non-treated control and # $P < 0.05$ versus cisplatin-treated control. (B) Morphological analysis of apoptotic and necrotic cell death were detected by Hoechst 33342 (top) and PI (below) staining assay. Representative photographs are shown from three independent experiments.

4.2 ROS detection for anti-oxidant property of GM and CM on cisplatin-treated HK-2 cells

To clarify whether the protective effect of GM and CM was due to their anti-oxidant properties, cells were left untreated or pre-incubated with 0.1 and 0.2%v/v of GM and CM for 1 h prior to 50 μM cisplatin exposure. After 1 h, cellular ROS levels were evaluated by fluorescence microscope and flow cytometry, using $DCFH_2$-DA as a specific fluorescence dye.

The results of fluorescence microscope demonstrated that cellular ROS was extremely increased in response to 50 μM cisplatin treatment, comparing to basal level in control. The addition of 0.1 and 0.2%v/v of GM and CM markedly decreased the fluorescence intensity induced by cisplatin (figure 20A)

From flow cytometric analysis, cisplatin caused an induction of cellular ROS levels over non-treated control (1.47 folds) (figure 20B). The elevation of cellular ROS could be reduced to 0.73, and 0.59 folds in 0.1 and 0.2 %v/v GM pretreatment, respectively (figure 20B). Likewise, the addition of 0.1 and 0.2 %v/v of CM was able to inhibit cellular ROS originated by cisplatin to 0.53, and 0.42 folds, respectively (figure 20B).

These results suggested that GM and CM exhibited anti-oxidant properties against cisplatin-induced oxidative stress in HK-2 cells.

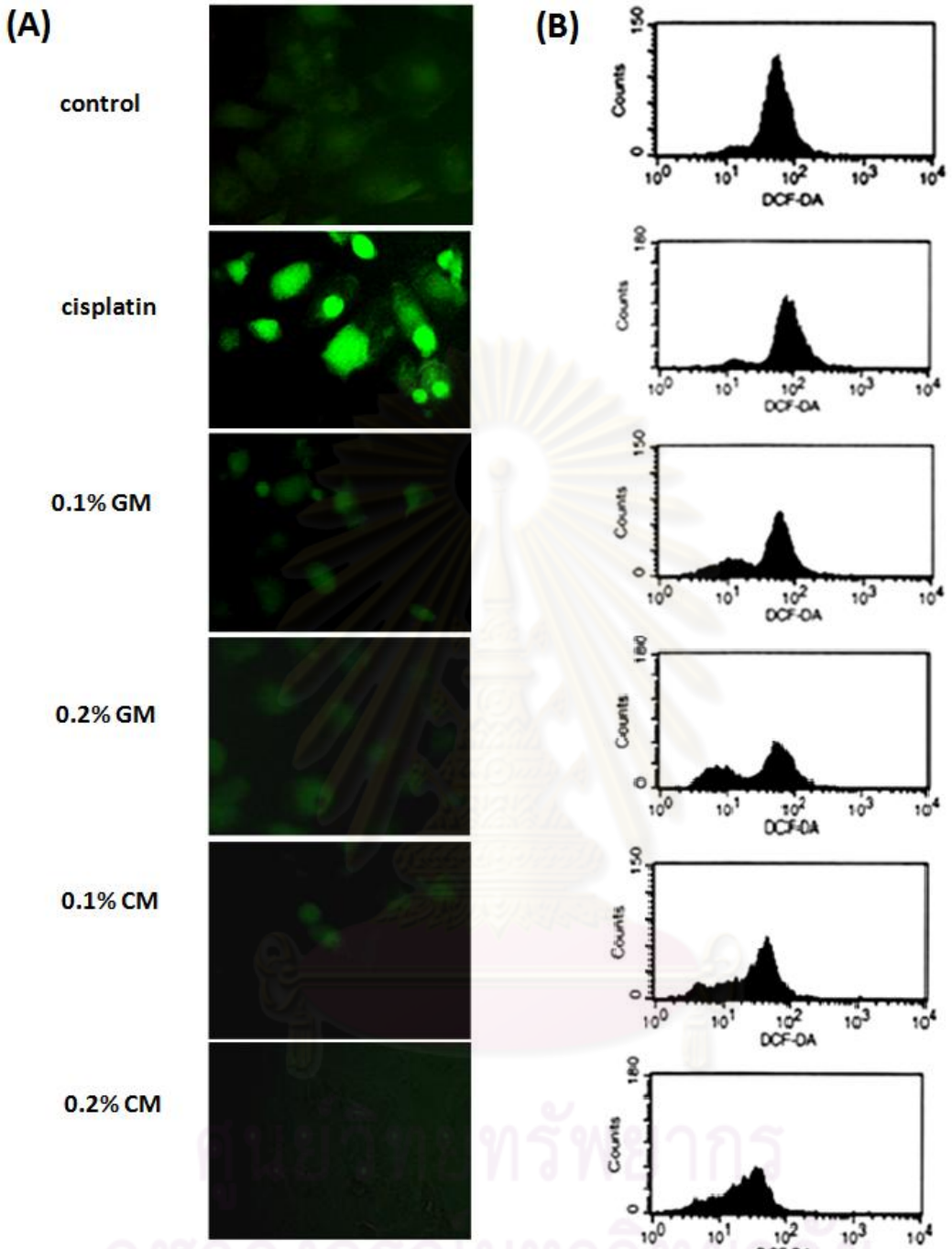


Figure 20 Effects of GM and CM on intracellular ROS induced by cisplatin. (A) HK-2 cells were left untreated or incubated with 0.1 and 0.2%v/v of GM and CM for 1 h prior to 50 μM cisplatin treatment. After 1 h cisplatin incubation, intracellular ROS levels were detected by fluorescence microscope with $DCFH_2$-DA. (B) Cells were similarly treated and then intracellular ROS levels were evaluated using flow cytometry with $DCFH_2$-DA as a fluorescence probe.

5. The specific ROS which are responsible for cisplatin-mediated cytotoxicity in renal tubular cells

Having shown that ROS mediated by cisplatin plays an important role in cisplatin-induced renal cell death, the present study further identified the specific ROS which are responsible for such cytotoxicity. Accordingly, specific ROS scavengers for superoxide anion radical, hydrogen peroxide, and hydroxyl radical (50 μM MnTBAP, 5000 U/ml catalase, and 1 mM deferoxamine, respectively) were used to treat prior to 50 μM cisplatin exposure. After 24 h, cell viability and morphology of apoptosis and necrosis were evaluated by MTT and Hoechst 33342/ PI nuclear staining assay, respectively.

The percentage of cell viability as shown in figure 21A exhibited that 50 μM of cisplatin reduced approximately 50% of cell survival (51.92 ± 1.66%). Only catalase and deferoxamine pretreatment were able to protect HK-2 cells from cisplatin-induced cell death (58.23 ± 2.41%, and 65.87 ± 1.61% cell viability, respectively). However, MnTBAP pretreatment showed non-significant protective effect when compared to group that treated with cisplatin (52.52 ± 0.13% cell viability) (figure 21A).

The morphological studies of apoptotic and necrotic cells clearly demonstrated the protective effect of catalase and deferoxamine pretreatment against cisplatin induce apoptosis in HK-2 cells. The nuclear staining results exhibited the normal morphology of living cells in control (figure 21B). Treatment of the cells with 50 μM of cisplatin caused an increase in morphological changes of apoptosis (figure 21B). Pretreatment with catalase and deferoxamine significantly reduced apoptotic cell death induced by cisplatin, while MnTBAP did not show this protective effect over group that treated with only cisplatin (figure 21B).

The evidence indicated that catalase and deferoxamine pretreatment were able to protect HK-2 cells from cisplatin cytotoxicity, suggesting the significant role of hydrogen peroxide and hydroxyl radical in cisplatin-induced renal cell death.

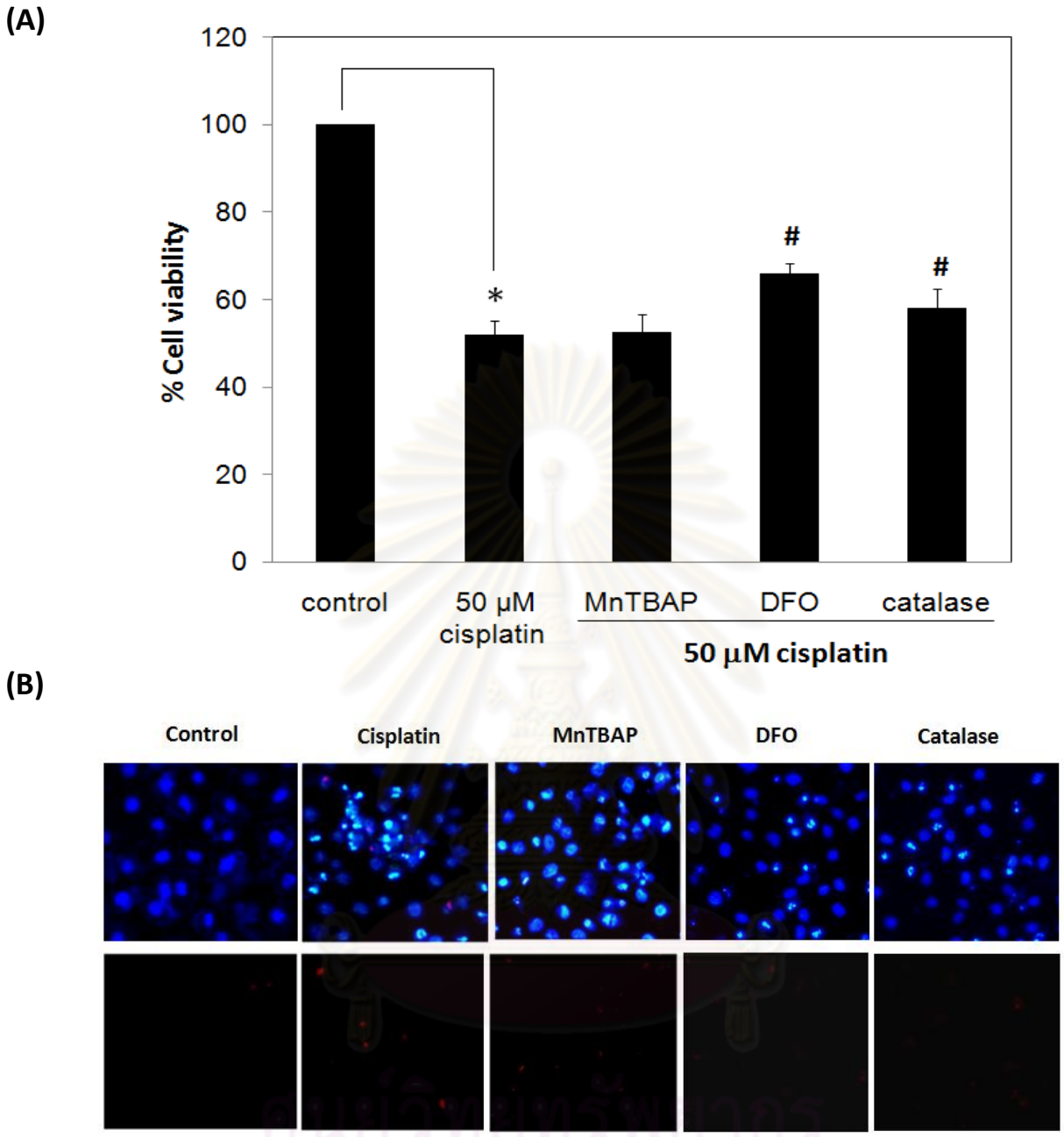


Figure 21 Cisplatin-induced renal cell death mediated by specific ROS. (A) HK-2 cells were left untreated or incubated with various specific ROS scavengers; 50 µM MnTBAP, 1 mM DFO, and 5000 U/ml catalase) for 1 h prior to 50 µM cisplatin treatment. After 24 h, cell viability was determined by MTT assay. Values are represented as relative cell viability and plots are mean ± SEM. (n=4). * $P < 0.05$ versus non-treated control and # $P < 0.05$ versus cisplatin-treated control. (B) Morphological analysis of apoptotic and necrotic cell death were detected by Hoechst 33342 (top) and PI (below) staining assay. Representative photographs are shown from three independent experiments.

6. Specific ROS scavenging activities of GM and CM

6.1 Intracellular ROS induced by specific ROS and ROS generator

In order to determine the time-dependent effect of hydrogen peroxide (specific ROS) and ferrous sulphate (ROS generator) on intracellular ROS levels in HK-2 cells, cells were incubated with 200 μM hydrogen peroxide or 100 μM ferrous sulphate for various time points (0, 15, 30 min, 1, and 2 h). Intracellular ROS was detected under fluorescence microscope with $DCFH_2$-DA. The results of fluorescence microscope exhibited that both hydrogen peroxide and ferrous sulphate caused an elevation of cellular ROS in HK-2 cells which peaked at 1 h (figure 22A, 22B).

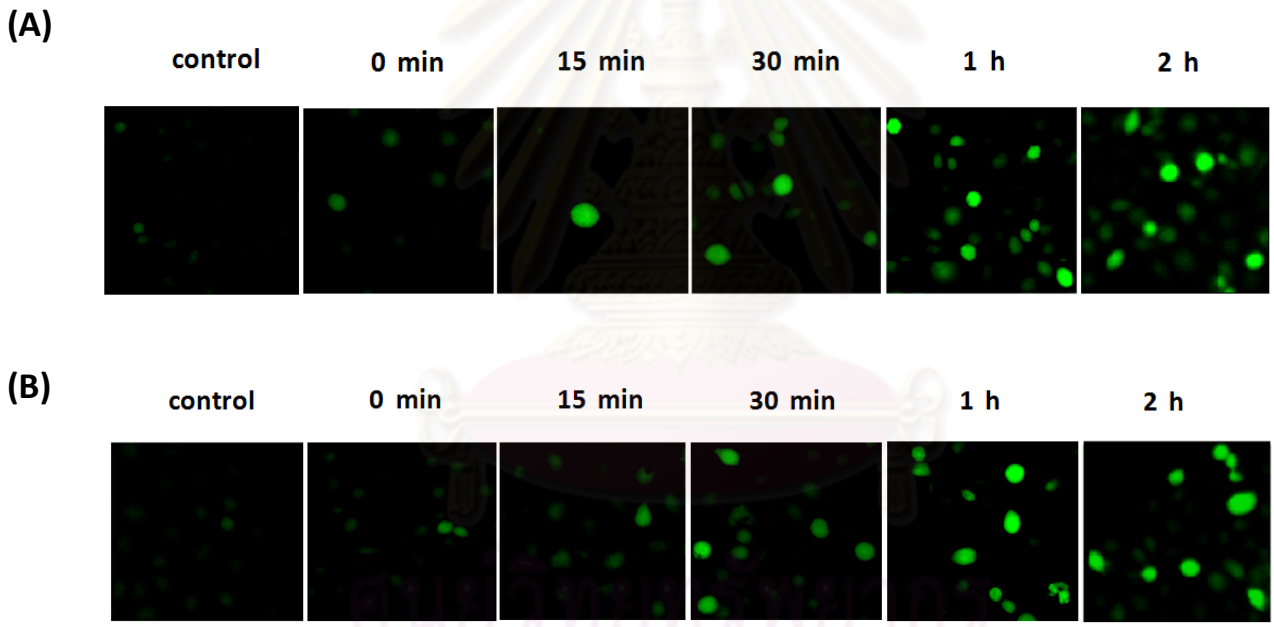


Figure 22 Effect of hydrogen peroxide (H_2O_2) and ferrous sulphate ($FeSO_4$; hydroxyl radicals generator) on ROS level in HK-2 cells in time-dependent study. (A) Cells were treated with 200 μM of H_2O_2, (B) cells were treated with 100 μM of $FeSO_4$ and incubated for various time points (0, 15, 30 min, 1, and 2 h). The increase of fluorescence intensity was detected by fluorescence microscope with $DCFH_2$-DA as an oxidative probe.

6.2 Anti-oxidant activities of GM and CM against hydrogen peroxide and hydroxyl radical

In an attempt to investigate whether GM and CM could protect renal cell death via the anti-oxidant activities against hydrogen peroxide and hydroxyl radical, cells were left untreated or pretreated with 0.1 and 0.2%v/v of GM and CM for 1 h followed by hydrogen peroxide or ferrous sulphate treatments. After 1 h incubation, cellular ROS levels were detected by flow cytometry with specific fluorescence dye, $DCFH_2$-DA.

The results showed that hydrogen peroxide dramatically increased cellular ROS in HK-2 cells over the basal level in non-treated control (2.83 folds) (figure 23A). Pretreatment with 0.1 and 0.2%v/v of GM caused a dose-dependent reduction in cellular ROS levels in response to hydrogen peroxide treatment to 2.43, and 1.96 folds, respectively. Cellular ROS induced by hydrogen peroxide in HK-2 cells was also blocked by 0.1 and 0.2%v/v of CM pretreatment in dose-dependent manner to 1.39, and 0.96 folds, respectively (figure 23A).

Likewise, treatment of the cells with ferrous sulphate caused an elevation in cellular ROS levels over non-treated control (1.63 folds). The results demonstrated that only CM pretreatment could exhibit a strong anti-oxidant effect against hydroxyl radical generated by ferrous sulphate. Cellular ROS levels in 0.1 and 0.2%v/v of CM pretreatment groups were reduced to 1.01, and 0.98 folds, respectively (figure 23B). In contrast, similar concentrations of GM did not block cellular ROS elevation induced by ferrous sulphate (1.70, and 1.78, respectively) (figure 23B). These results suggested that GM and CM could, at least in part, protect cisplatin-induced renal cell death by their specific scavenging activities against hydrogen peroxide and hydroxyl radical.

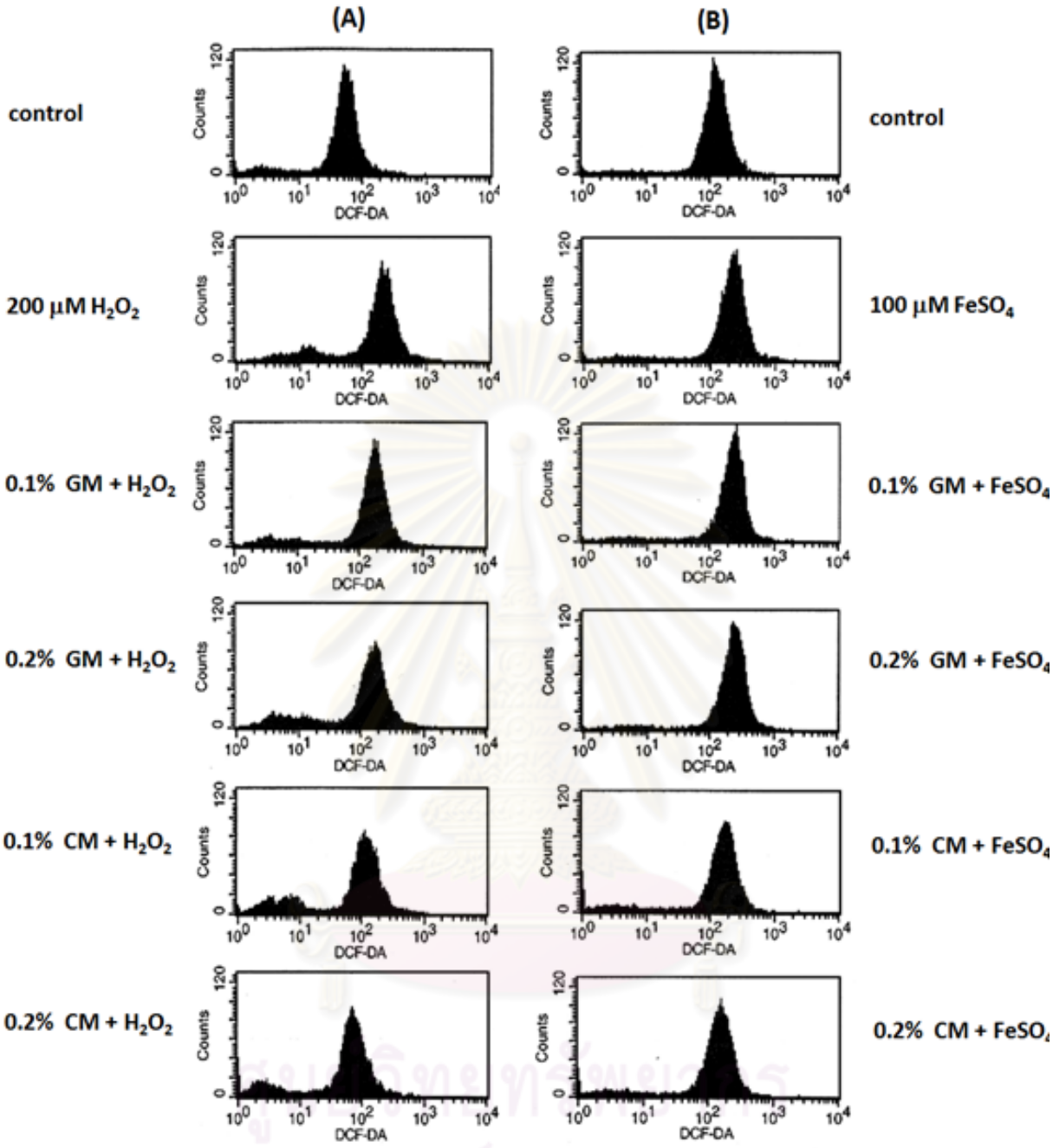


Figure 23 Anti-oxidant activities of GM and CM against hydrogen peroxide and hydroxyl radical in HK-2 cells. (A) Cells were left untreated or pretreated with 0.1 and 0.2 %v/v of GM and CM for 1 h, and then exposed to 200 μM of H_2O_2. After incubation for 1 h, cellular ROS levels were evaluated by flow cytometry with $DCFH_2$-DA. (B) Cells were similarly pretreated with GM and CM prior to 100 μM of $FeSO_4$ exposure for 1 h. Cellular ROS levels were evaluated by flow cytometry.

7. The interfering effect of GM and CM on cisplatin-induced cancer cell death

7.1 Cytotoxic effect of cisplatin on cancer cells

7.1.1 Cisplatin induced human lung cancer cell death (H460)

For dose-dependent study, H460 cells were treated with various concentrations of cisplatin (5-300 μM) for 24 h. The results of MTT assay showed that cisplatin treatment caused a dose-dependent decrease in cell viability of H460 cells (figure 24). The reduction of cell survival was first significantly observed at 25 μM of cisplatin treatment and further decreased to approximately 50% (52.04 ± 2.32%) at the concentration of 100 μM (figure 24A). For apoptosis and necrosis assay, the results showed that the numbers of apoptotic and necrotic cells resulted from cisplatin treatment were increased in dose-dependent manner. As shown in figure 24B, H460 cell apoptosis was first observed at 50 μM of cisplatin treatment and further increased at the concentration of 100-300 μM. Likewise, PI positive H460 cells could be detected as the dose increased up to 100 μM of cisplatin (figure 24B).

This study also performed the time-dependent response (0, 6, 15, and 24 h) of H460 cells to 100 μM cisplatin treatment. The results demonstrated that the reduction of cell viability in response to cisplatin treatment was observed as early as 15 h (75.62 ± 1.76 %) and markedly decreased to approximately 50% (51.71 ± 3.37 %) at 24 h (figure 25A). In order to confirm the time-dependent result of MTT assay, the morphology of H460 cells in terms of apoptosis and necrosis after cisplatin treatment at various time points was determined by Hoechst 33342 and PI staining assay. Figure 25B demonstrated that 100 μM of cisplatin caused a time-dependent increase in cell death. The number of cell apoptosis were clearly observed as early as 15 h and dramatically presented at 24 h (figure 25B). Likewise, late apoptosis that exhibited as necrotic cell death was also detected at 24 h (figure 25B). From these results, the apoptosis and necrosis cells in response to time-dependent cisplatin treatment was strongly correlated with the percentage of H460 cell viability from MTT assay.

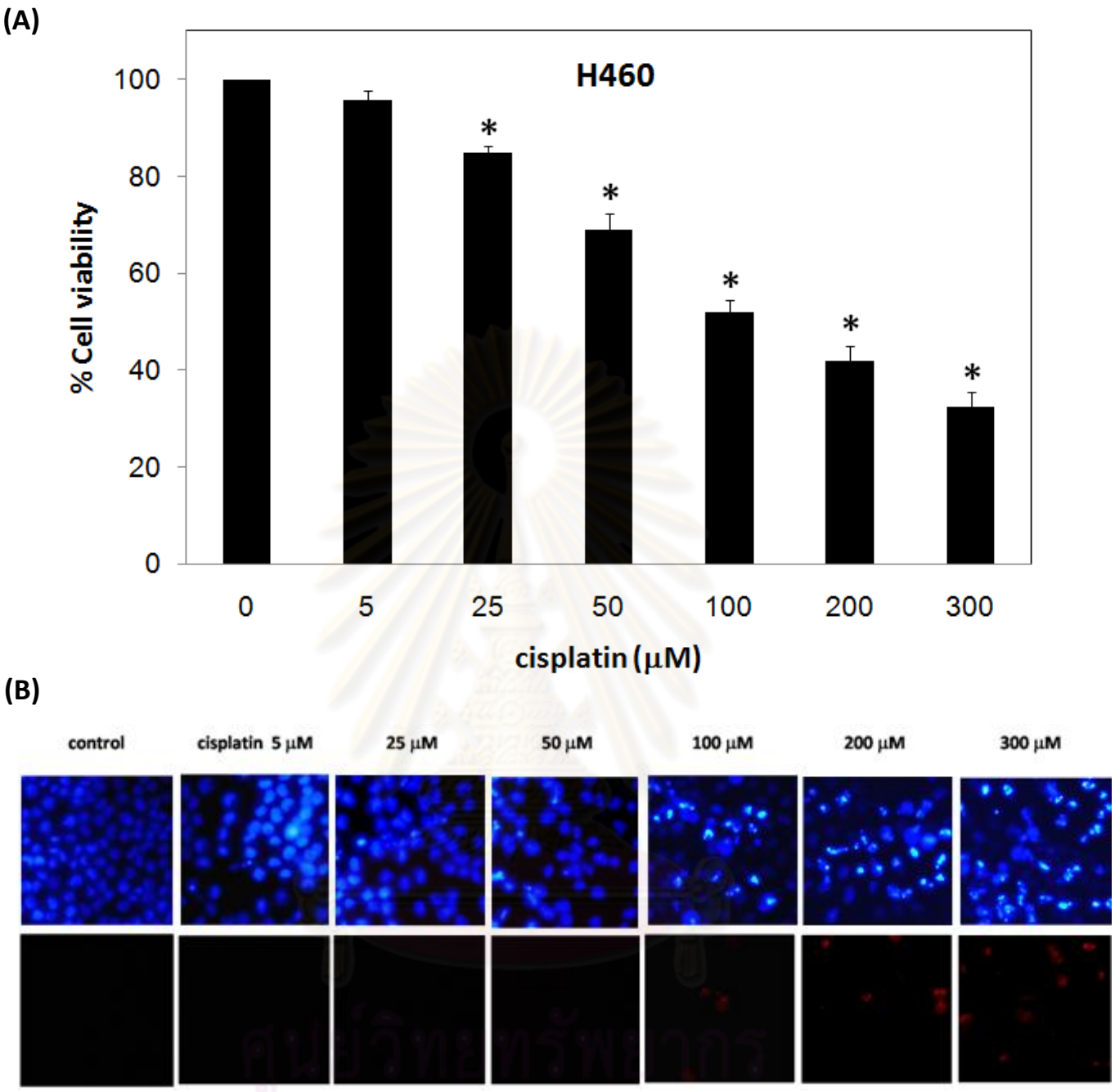


Figure 24 Cytotoxic effect of cisplatin on human lung cancer cells (H460) in dose-dependent study. (A) Cells were left untreated or treated with various concentrations of cisplatin (0, 5, 25, 50, 100, 200, and 300 μM) for 24 h, cell viability was determined by MTT assay. Values are represented as relative cell viability compared to control cells and plots are means ± SEM. (n=4). * $P < 0.05$ versus non-treated control. (B) Morphological analysis of apoptotic and necrotic cell death were detected by Hoechst 33342 (top) and PI (below) staining assay. Representative photographs are shown from three independent experiments.

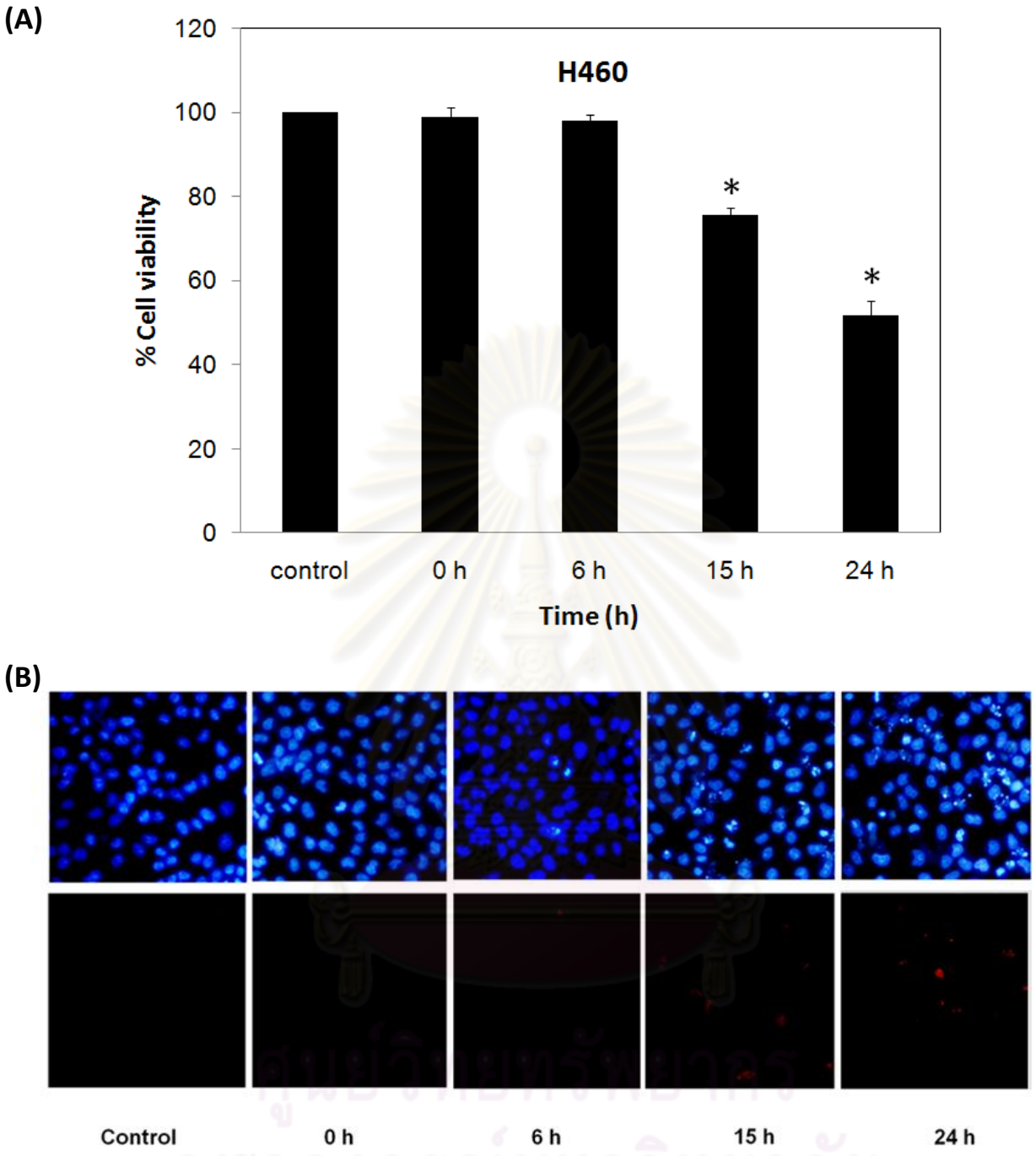


Figure 25 Time-dependent effect of cisplatin on H460 cell viability. (A) Cells were left untreated or treated with 100 μM of cisplatin for various incubation times (0, 6, 15, and 24 h). Cell viability was determined by MTT assay. Values are represented as relative cell viability and plots are mean ± SEM. (n=3). * $P < 0.05$ versus non-treated control. (B) Morphological analysis of apoptotic and necrotic cell death were detected by Hoechst 33342 (top) and PI (below) staining assay. Representative photographs are shown from three independent experiments.

7.1.2 Cisplatin induced human melanoma cancer cell death (G361)

The effects of cisplatin on G361 cells were determined as a function of dose and time. For dose-dependent study, G361 cells were treated with various concentrations of cisplatin (5-300 μM) for 24 h and then cell viability was analyzed by MTT assay. The results showed that cisplatin caused a dose-dependent decrease in G361 cell viability. The reduction of cell survival was first significantly observed at 5 μM of cisplatin treatment (82.41 ± 3.44%) and further decreased to approximately 50% (53.65 ± 1.78%) at 100 μM of cisplatin (figure 26A). The dose-dependent effect of cisplatin on G361 cell death was detected by Hoechst 33342 and PI nuclear staining assay. The results exhibited that G361 cell apoptosis was elevated in response to higher concentrations of cisplatin treatment. The number of apoptotic cell death was detected in the concentration starting from 25 μM of cisplatin treatment and continuously increased at the concentration of 50-300 μM (figure 26B), whereas necrotic cell death was presented in a very a less amount (figure 26B).

In order to determined the time-dependent response of G361 cells to cisplatin exposure, cells were treated with 100 μM of cisplatin for various time points (0, 6, 15, and 24 h). The result of MTT assay as shown in figure 27A exhibited that the percentage of cell viability was significantly reduced as early as 6 h (71.57 ± 3.03%) and continuously decreased to approximately 50% at 24 h after cisplatin treatment (52.78 ± 1.86%). In addition, the morphological studies of apoptosis and necrosis were detected by Hoechst 33342/ PI staining. Data from fluorescence microscope exhibited that cisplatin caused a time-dependent increase in G361 cell apoptosis. The apoptosis characteristics were observed as early as 6 h and further increased at 24 h. While, PI positive that indicated as necrotic cell death did not present in G361 treated with cisplatin (figure 27B).

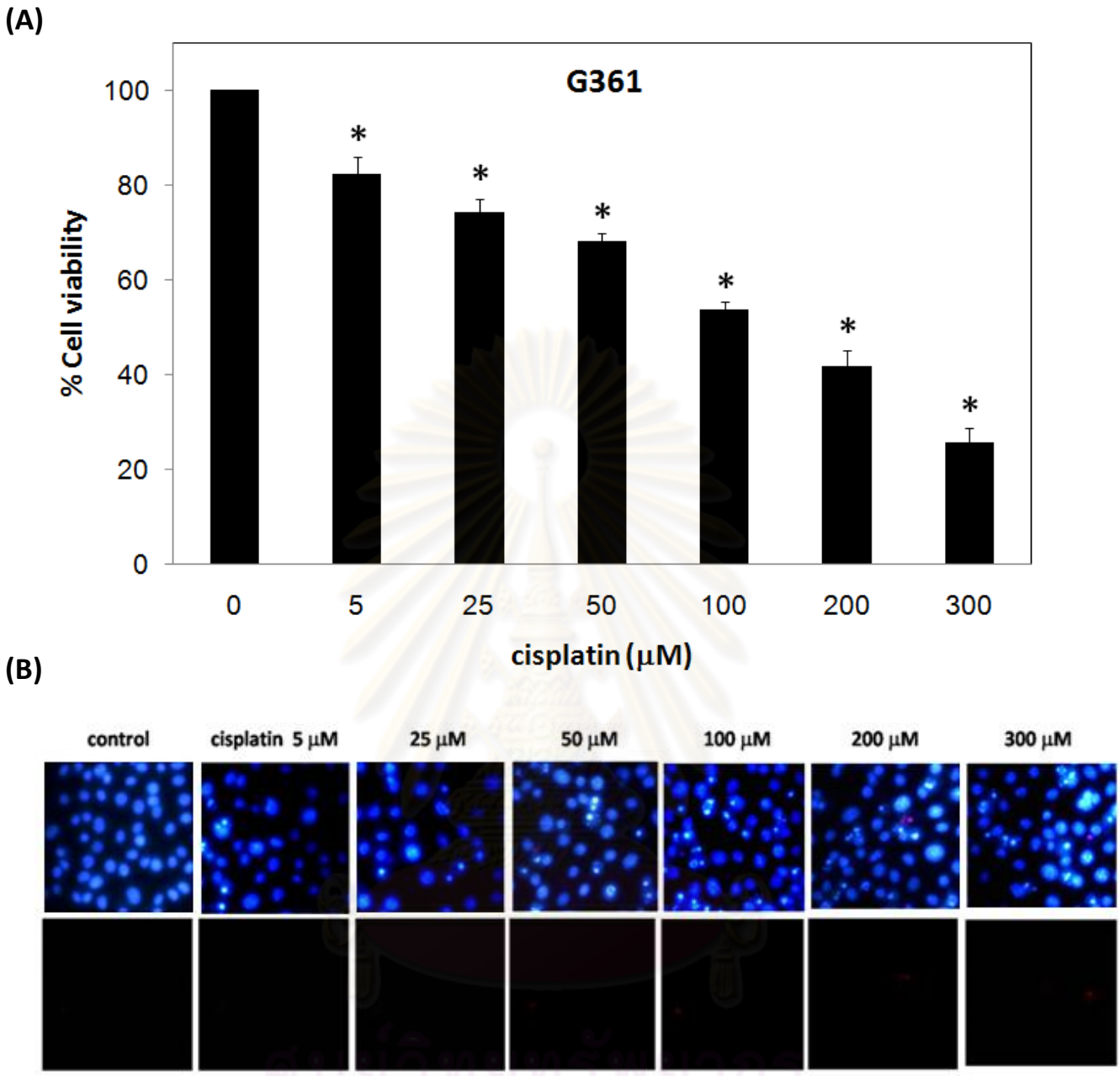


Figure 26 Cytotoxic effect of cisplatin on human melanoma cancer cells (G361) in dose-dependent study. (A) Cells were left untreated or treated with various concentrations of cisplatin (0, 5, 25, 50, 100, 200, and 300 μM) for 24 h, cell viability was determined by MTT assay. Values are represented as relative cell viability compared to control cells and plots are means ± SEM. (n=4). * $P < 0.05$ versus non-treated control. (B) Morphological analysis of apoptotic and necrotic cell death was detected by Hoechst 33342 (top) and PI (below) staining assay. Representative photographs are shown from three independent experiments.

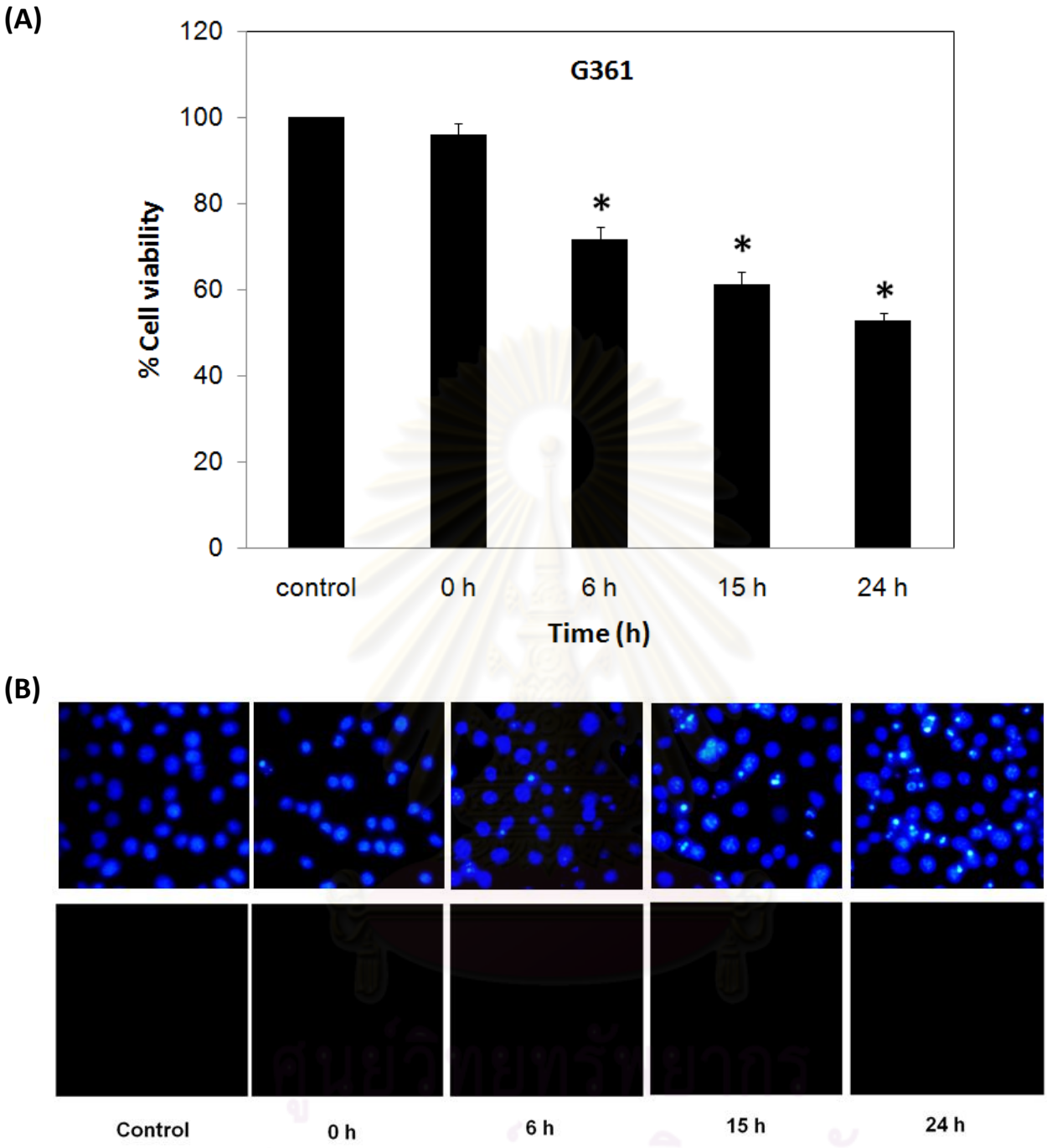


Figure 27 Time-dependent effect of cisplatin on G361 cell viability. (A) Cells were left untreated or treated with 100 μM of cisplatin for various incubation times (0, 6, 15, and 24 h). Cell viability was determined by MTT assay. Values are represented as relative cell viability and plots are mean ± SEM. (n=3). * $P < 0.05$ versus non-treated control. (B) Morphological analysis of apoptotic and necrotic cell death was detected by Hoechst 33342 (top) and PI (below) staining assay. Representative photographs are shown from three independent experiments.

7.2 Effects of GM and CM on cisplatin anti-cancer activity

7.2.1 Effect of GM and CM on cisplatin-induced human lung cancer (H460) cell death

The interfering effects of anti-oxidants (NAC and GSH) as well as GM and CM on anti-cancer activity of cisplatin were investigated. H460 cells were left untreated or pretreated with 5 mM NAC, 5 mM GSH, 0.1 and 0.2%v/v of GM and CM for 1 h prior to 100 μM cisplatin exposure. After 24 h, cell viability and the morphology of apoptosis and necrosis were determined.

The result of MTT assay in figure 28A exhibited that treatment with 100 μM of cisplatin caused a reduction of H460 cell viability approximately 50% (52.08 ± 2.83%) which was significantly increased by NAC and GSH pretreatment (86.19 ± 3.90%, and 90.04 ± 1.67%, respectively). Importantly, co-treating the cells with 0.1 and 0.2%v/v of GM had no significant effect on the anti-cancer activity of cisplatin, comparing to group that treated with only cisplatin (52.64 ± 2.23%, and 50.96 ± 3.08%, respectively) (figure 28A). Similarly, cisplatin-induced H460 cell death could not interfere by pre-incubation with 0.1 and 0.2%v/v of CM (53.06 ± 2.13%, and 53.29 ± 3.01%, respectively) (figure 28A).

The morphological studies of apoptosis and necrosis by Hoechst 33342 and PI staining assay also showed that 0.1 and 0.2%v/v of GM and CM pre-incubation had no significant changes in mode and quantity of cell death in response to cisplatin treatment (figure 28B).

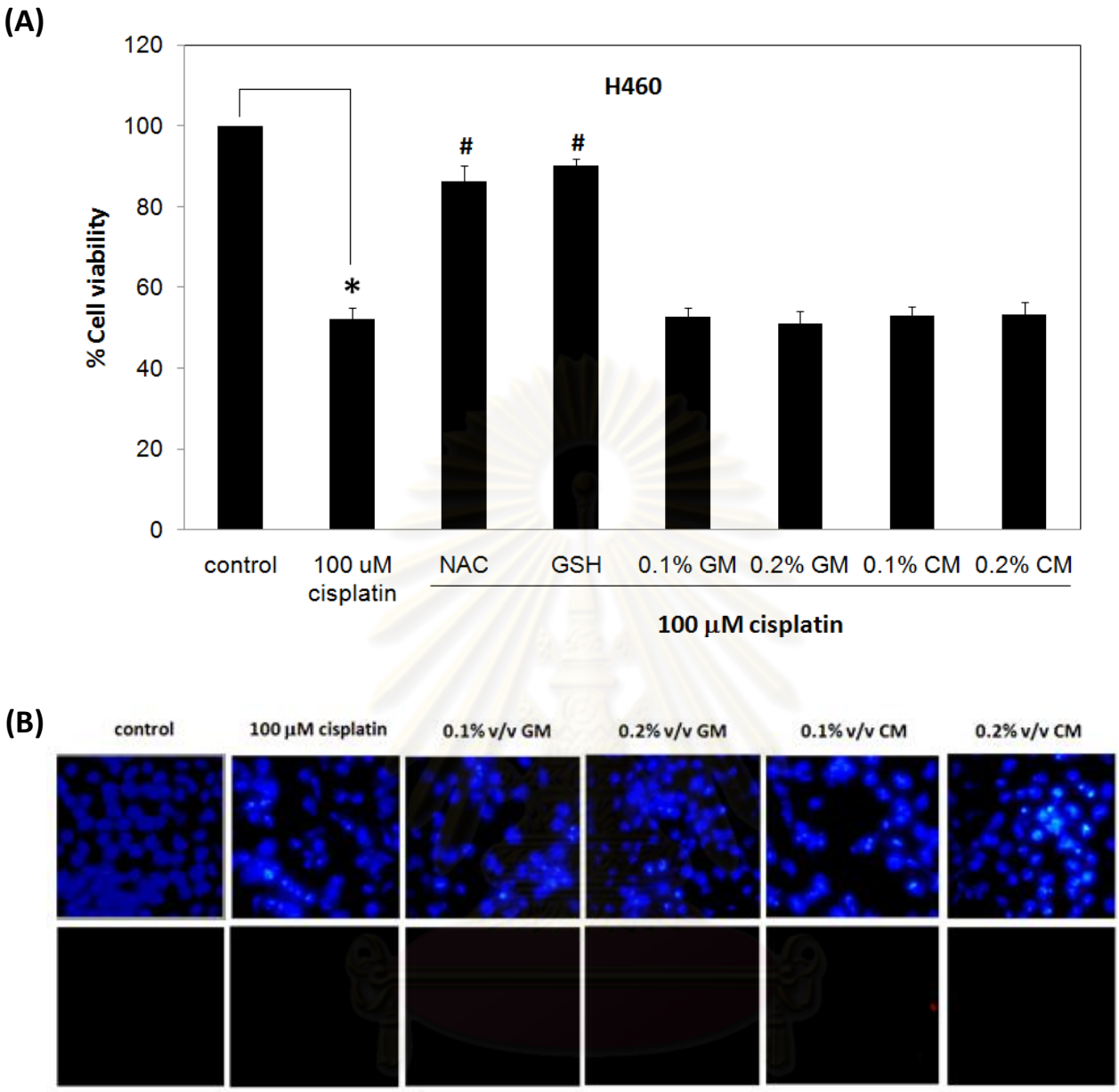


Figure 28 Effects of GM and CM on cisplatin-treated lung (H460) cancer cells. (A) Cells were left untreated or pretreated with 5 mM NAC and GSH, 0.1 and 0.2 %v/v of GM and CM for 1 h prior to treatment with 100 µM of cisplatin. After 24 h incubation, cell viability was determined by MTT assay. Values are represented as relative cell viability and plots are mean ± SEM. (n=5). * $P < 0.05$ versus non-treated control and # $P < 0.05$ versus cisplatin-treated control. (B) Morphological analysis of apoptotic and necrotic cell death was detected by Hoechst 33342 (top) and PI (below) staining assay. Representative photographs are shown from three independent experiments.

7.2.2 Effect of GM and CM on cisplatin-induced human melanoma cancer (G361) cell death

To evaluate the interfering effect of know anti-oxidants (NAC and GSH) as well as GM and CM on cisplatin-induced G361 cell death, G361 cells were left untreated or pretreated with 5 mM NAC, 5 mM GSH, 0.1 and 0.2 %v/v of GM and CM for 1 h prior to 100 μM cisplatin exposure. After 24 h, cell viability and the morphology of apoptosis and necrosis were determined.

The percentage of cell viability as shown in figure 29A demonstrated that 100 μM of cisplatin caused a significant decrease in G361 cell survival approximately 50% (52.68 ± 2.65%) which could be inhibited by NAC and GSH pretreatment (69.20 ± 3.06%, and 76.77 ± 2.53%, respectively). Pretreatment with 0.1 and 0.2 %v/v of GM did not alter cell viability (52.74 ± 1.75%, and 51.66 ± 1.57%, respectively). Likewise, cisplatin-induced G361 cell death could not compromise by pre-incubation with 0.1 and 0.2%v/v of CM (51.36 ± 1.87%, and 51.07 ± 2.23% cell viability, respectively) (figure 29A).

The morphology of apoptosis and necrosis exhibited that the apoptotic characteristics, as indicated by nuclear condensation, chromatin fragmentation, and apoptotic bodies, induced by 100 μM cisplatin still remained in GM and CM pretreatment (figure 29B).

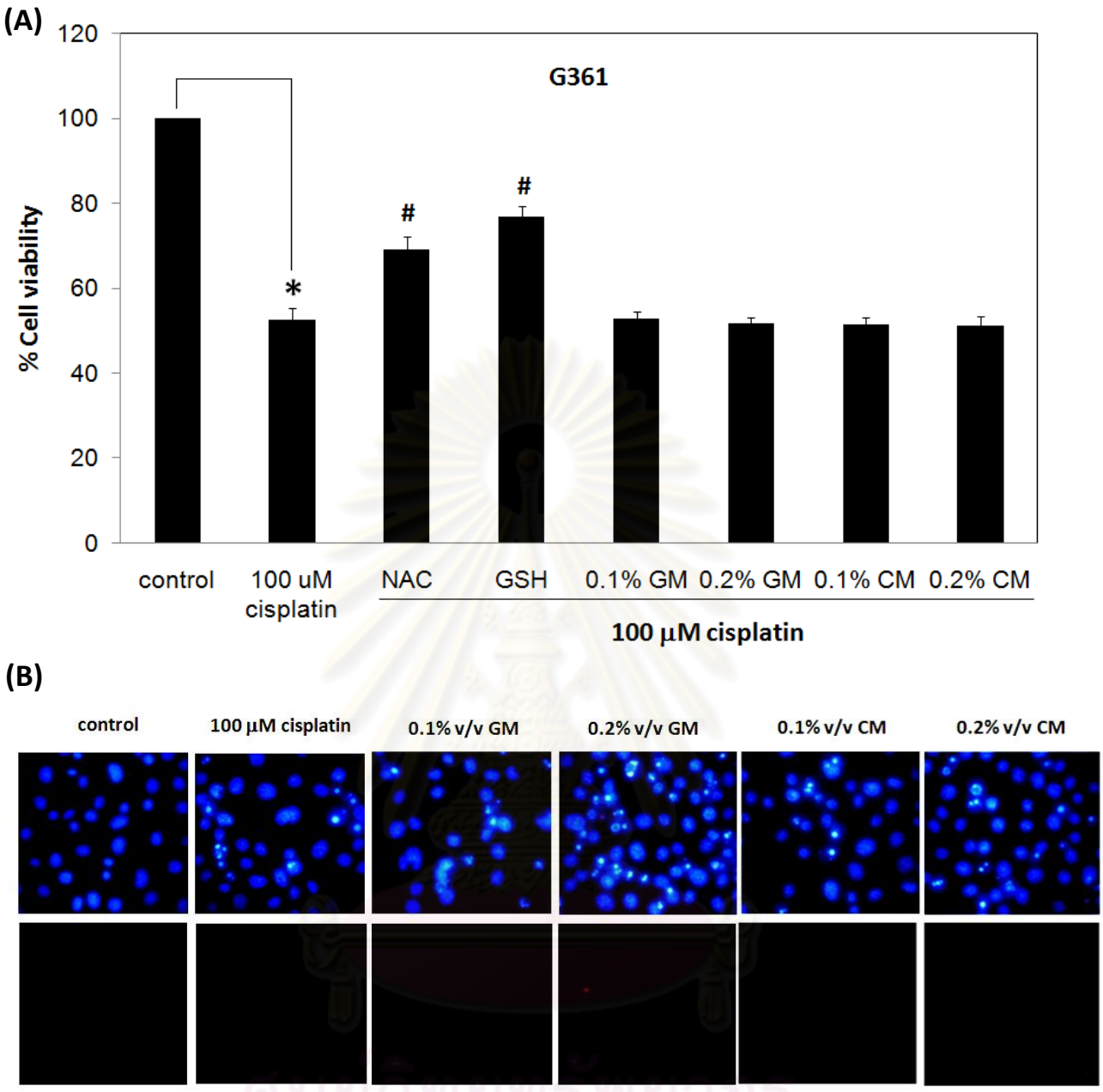


Figure 29 Effects of GM and CM on cisplatin-treated melanoma (G361) cancer cells. (A) Cells were left untreated or pretreated with 5 mM NAC and GSH, 0.1 and 0.2 %v/v of GM and CM for 1 h prior to treatment with 100 µM of cisplatin. After 24 h incubation, cell viability was determined by MTT assay. Values are represented as relative cell viability and plots are mean ± SEM. (n=5). * $P < 0.05$ versus non-treated control and # $P < 0.05$ versus cisplatin-treated control. (B) Morphological analysis of apoptotic and necrotic cell death was detected by Hoechst 33342 (top) and PI (below) staining assay. Representative photographs are shown from three independent experiments.

CHAPTER V

DISCUSSION AND CONCLUSION

Cisplatin-induced nephrotoxicity is a major side effect which limits the use of cisplatin in many patients. Since the amount of evidence shows that oxidative stress is the primary factor that contributes to cisplatin nephrotoxicity, much effort has been directed toward the search for natural products that are capable of attenuating renal damage. Cisplatin-induced renal cell apoptosis, especially at the site of proximal tubule, was a major event resulting in renal impairment and in some cases may lead to renal failure (Lau, 1999; Pabla and Dong, 2008). Consistent with the results of current study that cisplatin caused a dramatic decrease of human proximal cell (HK-2) viability. We further indentified mode of cell death and found that apoptosis was the main mode of cisplatin-mediated cell death. Regarding mechanism of cisplatin in mediating cell toxicity, sufficient evidence indicated that ROS or oxidative stress generated by cisplatin played a prominent role in cisplatin-induced renal cell death and such oxidative stress induction was proved to be associated with acute renal failure in vivo (Matsushima *et al*., 1998; Kawai *et al*., 2006; Santos *et al*., 2007; Chirino and Pedraza-Chaverri, 2009). Consistently, the results of this study strengthen the above evidence that cisplatin caused a significant induction of cellular ROS in renal cells (figure 15, and 16) and consequently increased the number of cell apoptosis (figure 13B, and 14B). In order to confirm the role of ROS on cisplatin-induced cell death, we pretreated the cells with known anti-oxidants (NAC and GSH) prior to cisplatin treatment and found that NAC and GSH could be able to reduce both ROS induction as well as renal cell apoptosis mediated by cisplatin (figure 16, and 17).

Previous studies have reported that soy bean extract was able to relieve signs and symptoms in chronic renal disease (Ranich, Bhathena, and Velasquez, 2001) and its phenolic compositions were able to attenuate gentamicin-induced renal injury (Ekor, Farombi, and Emerole, 2006). Likewise, *Chrysanthemum indicum*, the Chinese herbal medicine, is composed of flavonoids which exhibit anti-oxidant activity (Cai *et al*., 2004; Wu *et al*., 2010). However, the roles on *Glycine max* seed extract (GM) and *Chrysanthemum indicum*

flower extract (CM) on cisplatin-induced renal toxicity are largely unknown. This study demonstrated herein for the first time that GM and CM significantly prevented renal cell apoptosis in response to cisplatin treatment (figure 18, and 19) by the mechanism involved anti-oxidant activities of these extract. The present study found that the induction of intracellular ROS induced by cisplatin in HK-2 cells could be significantly attenuated by GM and CM pretreatment (figure 20).

In normal physiology and pathology, three principal ROS namely superoxide anion radical ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($^{\bullet}OH$) are formed continuously by the energy producing process in mitochondria (Sorg, 2004; Circu and Aw, 2010). Indeed, the first oxidative species resulted from mitochondrial chain reaction is $O_2^{\bullet-}$, and $O_2^{\bullet-}$ can be converted to H_2O_2 by the catalytic activity of superoxide dismutase (SOD). Further, H_2O_2 is detoxified to water and oxygen by activity of catalase or glutathione peroxidase. However, in the presence of reduced transition metals, H_2O_2 prefers to be transformed to highly toxic $^{\bullet}OH$ (Halliwell and Gutteridge, 1984; Salganik, 2001). Although these ROS have a proximal correlation and has been observed intracellular at almost the same time, role of each specific ROS in controlling cellular events and behaviors is various (Fligiel *et al*., 1984; Halliwell and Gutteridge, 1984; Salganik, 2001; Valko *et al*., 2007). Regarding cisplatin-mediated cytotoxicity, especially in human renal cells, the specific ROS playing a key role on cytotoxic mode of cisplatin was unknown. In an attempt to elucidate key ROS which are responsible for cisplatin-induced nephrotoxicity, cells were pretreated with various specific ROS scavengers, including MnTBAP, catalases, and deferoxamine, prior to cisplatin exposure. MnTBAP is cell-permeable SOD mimetic functioning in transform $O_2^{\bullet-}$ to H_2O_2. Catalase is specific for H_2O_2 detoxification, the formation of $^{\bullet}OH$ can be inhibited by deferoxamine through the chelation with cellular iron ion (Fe^{2+}) (Halliwell and Gutteridge, 1984; Salganik, 2001; Zahmatkesh et al., 2005). The results of the present study indicated that pretreatment with either catalase or deferoxamine significantly reduced cell death induced by cisplatin whereas MnTBAP showed non-significant alteration (figure 21). Thus the blockage of either hydrogen peroxide or hydroxyl radical generated by cisplatin could be able to protect renal HK-2 cells from cisplatin-induced injury. This result implied that some specific ROS like superoxide anion radical may not play a role in killing renal cells.

Having shown that hydrogen peroxide and hydroxyl radical play a key role in cisplatin-mediated HK-2 cell death. The present study further investigated for anti-oxidant activity of GM and CM whether these extracts could be able to detoxify named specific ROS generated by cisplatin. The results showed that GM and CM exhibited different anti-oxidant characteristics. From flow cytometric analysis, GM inhibited only cellular hydrogen peroxide up-regulation, whereas CM possessed both hydrogen peroxide and hydroxyl radical scavenging activities (figure 23). Since the ability of both extracts at same concentrations in inhibition of cisplatin-induced cell damage was quite equal quantitatively. There was a possibility that anti-oxidant activity against hydrogen peroxide of GM and CM may be sufficient to protect renal cells from cisplatin-induced death.

Major concern that anti-oxidants possess ability to attenuate cisplatin-mediated both renal and cancerous cells death was clarified in the present study using cisplatin-susceptible cancer cell lines (H460 and G361 cells). The present study provided evidence in human lung (H460) and melanoma (G361) cancer cells that pretreatment of known anti-oxidants (NAC and GSH) prior to cisplatin exposure could compromise anti-cancer activity of cisplatin, whereas GM and CM had no significant impact on cisplatin-induced H460 and G361 cell death (figure 28A, 29A). In addition, the remaining of H460 and G361 cell apoptosis confirmed that GM and CM had no effect on these cancer cell death induced by cisplatin (figure 28B, 29B). The explanations for such selectivity of GM and CM in attenuating toxic action of cisplatin only in renal cells may be (i) cisplatin mediates cell death by 2 major pathways which are ROS and DNA-adduct formation and renal cells are more susceptible to oxidative stress-induced damages (Venkatachalam *et al*., 1978; Paller, Hoidal, and Ferris, 1984; Baud and Ardaillou, 1986; Tsuruya *et al*., 2003) and (ii) the frequently proliferated cells including cancer cells are reported to be more sensitive to cisplatin-mediated DNA-adduct induced cell apoptosis (Reedijk and Lohman, 1985; Wang and Lippard, 2005). Therefore, an introduction of these extracts which focus on specific ROS inhibition may allow the selectivity in protecting only renal cells but not interfering with the cisplatin sensitivity of cancer cells.

In summary, the present study demonstrated that cisplatin-induced human proximal tubular cell apoptosis via a ROS-dependent mechanism. GM and CM showed strong anti-oxidant activities against cisplatin-induced ROS production and consequently protected renal

cell damage. Importantly, this study reported for the first time that in renal epithelial cells hydrogen peroxide and hydroxyl radical are the principal ROS playing a key role in cisplatin-induced cytotoxicity. Unlike other anti-oxidants, GM and CM selectively protected renal cells with no significant interfering effect on cisplatin-induced cancer cell death demonstrated in lung carcinoma H460 and melanoma G361 cells. These findings may at least provide the initial evidence necessary for the further development of these extracts to be used as renoprotective agents.

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APPENDIX

APPENDIX

TABLE OF EXPERIMENTAL RESULTS

Table 2. The percentage of HK-2 cell viability in response to various concentrations of GM (dose dependency), and determined by MTT assay.

GM (% v/v)	% Cell viability
0	100.00 ± 0.00%
0.025	96.32 ± 2.86%
0.05	97.29 ± 1.33%
0.1	101.71 ± 3.25%
0.2	102.73 ± 1.92%
0.5	107.43 ± 3.19% *
1.0	107.97 ± 3.14% *

Each value represents as the mean ± SEM. of three independent experiments. Asterisks refer significant difference from the control group (non-treated control): * $P < 0.05$ determined by One-way ANOVA.

Table 3. The percentage of HK-2 cell viability in response to various concentrations of CM (dose dependency), and determined by MTT assay.

CM (% v/v)	% Cell viability
0	100.00 ± 0.00%
0.025	94.45 ± 2.80%
0.05	97.25 ± 1.63%
0.1	101.37 ± 1.50%
0.2	102.81 ± 1.91%
0.5	108.31 ± 1.52% *
1.0	109.22 ± 3.45% *

Each value represents as the mean ± SEM. of three independent experiments. Asterisks refer significant difference from the control group (non-treated control): * $P < 0.05$ determined by One-way ANOVA.

Table 4. The percentage of HK-2 cell viability in response to various concentrations of cisplatin (dose dependency), and determined by MTT assay.

cisplatin (μM)	% Cell viability
0	100.00 ± 0.00%
5	97.54 ± 4.26%
25	71.66 ± 2.93% *
50	55.77 ± 2.24% *
100	35.14 ± 2.88% *

Each value represents as the mean ± SEM. of three independent experiments. Asterisks refer significant difference from the control group (non-treated control): * $P < 0.05$ determined by One-way ANOVA.

Table 5. The percentage of HK-2 cell viability in response to 50 μM cisplatin treatment at various time points (time dependency), and determined by MTT assay.

Time (h)	% Cell viability
control	100.00 ± 0.00%
0	97.48 ± 3.12%
6	93.12 ± 2.02%
15	74.97 ± 2.39% *
24	53.23 ± 2.95% *

Each value represents as the mean ± SEM. of three independent experiments. Asterisks refer significant difference from the control group (non-treated control): * $P < 0.05$ determined by One-way ANOVA.

Table 6. Anti-oxidant effects on HK-2 cell viability in response to 50 μM cisplatin treatment, determined by MTT assay.

Treatment	% Cell viability
control	100.00 ± 0.00%
50 μM cisplatin	54.81 ± 3.79% *
5 mM NAC + 50 μM cisplatin	81.45 ± 1.92% *#
5 mM GSH + 50 μM cisplatin	93.31 ± 1.97% *#

Each value represents as the mean ± SEM. of three independent experiments. Asterisks and sharp refer significant difference from each control group: * $P < 0.05$ versus non-treated control and # $P < 0.05$ versus cisplatin-treated control determined by Student' s *t*-test.

Table 7. The percentage of HK-2 cell viability in response to various concentrations of GM pretreatment prior to exposure with 50 μM cisplatin, determined by MTT assay.

Treatment	% Cell viability
control	100.00 ± 0.00%
50 μM cisplatin	55.09 ± 2.07% *
0.025 %v/v GM + 50 μM cisplatin	55.57 ± 3.01% *
0.05 %v/v GM + 50 μM cisplatin	57.19 ± 2.14% *
0.1 %v/v GM + 50 μM cisplatin	67.82 ± 2.04% *#
0.2 %v/v GM + 50 μM cisplatin	78.86 ± 2.63% *#

Each value represents as the mean ± SEM. of four independent experiments. Asterisks and sharp refer significant difference from each control group: * $P < 0.05$ versus non-treated control and # $P < 0.05$ versus cisplatin-treated control determined by One-way ANOVA.

Table 8. The percentage of HK-2 cell viability in response to various concentrations of CM pretreatment prior to exposure with 50 μM cisplatin, determined by MTT assay.

Treatment	% Cell viability
control	100.00 ± 0.00%
50 μM cisplatin	55.09 ± 2.07% *
0.025 %v/v CM + 50 μM cisplatin	53.69 ± 1.48% *
0.05 %v/v CM + 50 μM cisplatin	59.70 ± 2.95% *
0.1 %v/v CM + 50 μM cisplatin	67.84 ± 3.70% *#
0.2 %v/v CM + 50 μM cisplatin	76.15 ± 2.58% *#

Each value represents as the mean ± SEM. of four independent experiments. Asterisks and sharp refer significant difference from each control group: * $P < 0.05$ versus non-treated control and # $P < 0.05$ versus cisplatin-treated control determined by One-way ANOVA.

Table 9. The effects of various ROS scavengers on cisplatin-induced HK-2 cell death, determined by MTT assay.

Treatment	% Cell viability
control	100.00 ± 0.00%
50 μM cisplatin	51.92 ± 3.20% *
50 μM MnTBAP + 50 μM cisplatin	52.52 ± 4.07% *
1 mM DFO + 50 μM cisplatin	65.87 ± 2.44% *#
5000 U/ml catalase + 50 μM cisplatin	58.23 ± 4.14% *#

Each value represents as the mean ± SEM. of four independent experiments. Asterisks and sharp refer significant difference from each control group: * $P < 0.05$ versus non-treated control and # $P < 0.05$ versus cisplatin-treated control determined by Student' s *t*-test.

Table 10. The percentage of H460 cell viability in response to various concentrations of cisplatin (dose dependency), determined by MTT assay.

cisplatin (μM)	% Cell viability
0	100.00 ± 0.00%
5	95.73 ± 1.99%
25	84.76 ± 1.65% *
50	68.85 ± 3.39% *
100	52.04 ± 2.32% *
200	42.03 ± 2.99% *
300	32.39 ± 3.11% *

Each value represents as the mean ± SEM. of four independent experiments. Asterisks refer significant difference from the control group (non-treated control): * $P < 0.05$ determined by One-way ANOVA.

Table 11. The percentage of H460 cell viability in response to 100 μM cisplatin treatment at various time points (time dependency), determined by MTT assay.

Time (h)	% Cell viability
Control	100.00 ± 0.00%
0	98.86 ± 2.27%
6	98.04 ± 1.32%
15	75.62 ± 1.76% *
24	51.71 ± 3.37% *

Each value represents as the mean ± SEM. of three independent experiments. Asterisks refer significant difference from the control group (non-treated control): * $P < 0.05$ determined by One-way ANOVA.

Table 12. The percentage of G361 cell viability in response to various concentrations of cisplatin (dose dependency), determined by MTT assay.

cisplatin (μM)	**% Cell viability**
0	100.00 ± 0.00%
5	82.41 ± 3.44% *
25	74.19 ± 2.97% *
50	68.13 ± 1.71% *
100	53.65 ± 1.78% *
200	41.98 ± 3.23% *
300	25.76 ± 3.12% *

Each value represents as the mean ± SEM. of four independent experiments. Asterisks refer significant difference from the control group (non-treated control): * $P < 0.05$ determined by One-way ANOVA.

Table 13. The percentage of G361 cell viability in response to 100 μM cisplatin treatment at various time points (time dependency), determined by MTT assay.

Time (h)	**% Cell viability**
Control	100.00 ± 0.00%
0	95.92 ± 2.80%
6	71.57 ± 3.03% *
15	61.14 ± 2.94% *
24	52.78 ± 1.86% *

Each value represents as the mean ± SEM. of three independent experiments. Asterisks refer significant difference from the control group (non-treated control): * $P < 0.05$ determined by One-way ANOVA.

Table 14. The percentage of H460 cell viability in response to GM and CM pretreatment prior to exposure with 100 μM cisplatin, determined by MTT assay.

Treatment	% Cell viability
control	100.00 ± 0.00%
100 μM cisplatin	52.08 ± 2.83% *
5 mM NAC + 100 μM cisplatin	86.19 ± 3.90% *#
5 mM GSH + 100 μM cisplatin	90.04 ± 1.67% *#
0.1 %v/v GM + 100 μM cisplatin	52.64 ± 2.23% *
0.2 %v/v GM + 100 μM cisplatin	50.96 ± 3.08% *
0.1 %v/v CM + 100 μM cisplatin	53.06 ± 2.13% *
0.2 %v/v CM + 100 μM cisplatin	53.29 ± 3.01% *

Each value represents as the mean ± SEM. of five independent experiments. Asterisks and sharp refer significant difference from each control group: * $P < 0.05$ versus non-treated control and # $P < 0.05$ versus cisplatin-treated control determined by One-way ANOVA.

Table 15. The percentage of G361 cell viability in response to GM and CM pretreatment prior to exposure with 100 μM cisplatin, determined by MTT assay.

Treatment	% Cell viability
control	100.00 ± 0.00%
100 μM cisplatin	52.68 ± 2.65% *
5 mM NAC + 100 μM cisplatin	69.20 ± 3.06% *#
5 mM GSH + 100 μM cisplatin	76.77 ± 2.53% *#
0.1 %v/v GM + 100 μM cisplatin	52.74 ± 1.75% *
0.2 %v/v GM + 100 μM cisplatin	51.66 ± 1.57% *
0.1 %v/v CM + 100 μM cisplatin	51.36 ± 1.87% *
0.2 %v/v CM + 100 μM cisplatin	51.07 ± 2.23% *

Each value represents as the mean ± SEM. of five independent experiments. Asterisks and sharp refer significant difference from each control group : * $P < 0.05$ versus non-treated control and # $P < 0.05$ versus cisplatin-treated control determined by One-way ANOVA.

Vita

Ms. Kanittha Pongjit was born of July 29, 1983 in Bangkok. She received her B.Pharm (2^{nd} class honor) from the Faculty of Pharmacy, Chiang Mai University in 2006.