ผลของไซลิมารินในการปกป้องการตายของเซลล์ไตจากซิสพลาทิน และผลต่อการฆ่าเซลล์มะเร็งของซิสพลาทิน

นางสาวชวนพิศ นิลสันเทียะ

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาเภสัชศาสตรมหาบัณฑิต
สาขาวิชาเภสัชวิทยา ภาควิชาเภสัชวิทยาและสรีรวิทยา
คณะเภสัชศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2553

EFFECTS OF SILYMARIN IN PROTECTION AGAINST CISPLATIN-INDUCED RENAL CELL DEATH AND IN CANCER-KILLING EFFECT OF CISPLATIN

Miss Chuanpit Ninsontia

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science in Pharmacy Program in Pharmacology
Department of Pharmacology and Physiology
Faculty of Pharmaceutical Sciences
Chulalongkorn University
Academic Year 2010

Thesis Title	EFFECTS OF SILYMARIN IN PROTECTION AGAINST CISPLATIN-INDUCED RENAL CELL DEATH AND IN CANCER-KILLING EFFECT OF CISPLATIN
By	Miss Chuanpit Ninsontia
Field of Study	Pharmacology
Thesis Advisor	Assistant Professor Pithi Chanvorachote, Ph.D.

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

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

THESIS COMMITTEE

……..…..... Chairman
(Assistant Professor Suree Jianmongkol, Ph.D.)

…….. Thesis Advisor
(Assistant Professor Pithi Chanvorachote, Ph.D.)

…….. Examiner
(Assistant Professor Rataya Luechapudiporn, Ph.D.)

…….. External Examiner
(Sureerut Porntadavity, Ph.D.)

ชวนพิศ นิลสันเทียะ: ผลของไซลิมารินในการปกป้องการตายของเซลล์ไตจากซิสพลาทินและผลต่อการฆ่าเซลล์มะเร็งของซิสพลาทิน. (EFFECTS OF SILYMARIN IN PROTECTION AGAINST CISPLATIN-INDUCED RENAL CELL DEATH AND IN CANCER-KILLING EFFECT OF CISPLATIN) อ. ที่ปรึกษาวิทยานิพนธ์หลัก: ผศ.ภก.ดร.ปิติ จันทร์วรโชติ, 88 หน้า.

แม้ว่าซิสพลาทินเป็นยาเคมีบำบัดที่มีประสิทธิภาพดีในการรักษามะเร็งหลายชนิดแต่มักทำให้เกิดพิษต่อไตที่รุนแรงระหว่างทำการบำบัดรักษาด้วยยาดังกล่าว เป็นสาเหตุให้ผู้ป่วยจำนวนมากต้องหยุดการรักษา ไซลิมารินเป็นสารที่สกัดจากเมล็ดของต้นมิลค์ทิสเทิล มีองค์ประกอบของฟลาโวโนลิกแนนหลายชนิดซึ่งมีฤทธิ์ทางเภสัชวิทยาหลายประการ อย่างไรก็ตามยังไม่มีข้อมูลที่แน่ชัดว่าไซลิมาริ น สามารถปกป้องการตายของเซลล์ไตจากยาซิสพลาทินโดยไม่ลดประสิทธิภาพในการฆ่าเซลล์มะเร็งของยาซิสพลาทิน การศึกษานี้มีวัตถุประสงค์เพื่อศึกษาศักยภาพของไซลิมารินในการปกป้องการตายของเซลล์ไตจากยาซิสพลาทินโดยไม่ลดประสิทธิภาพในการฆ่าเซลล์มะเร็งของยาซิสพลาทิน ผลจากการทดลองชี้ให้เห็นว่ายาซิสพลาทินเหนี่ยวนำให้เซลล์ไตตายทั้งแบบอะพอพโทซิสและเนคโครซิสโดยการตายจะเพิ่มขึ้นตามความเข้มข้นของยาซิสพลาทิน แต่เมื่อเซลล์ไตได้รับไซลิมาริน ในความเข้มข้น 50-200 ไมโครโมลต่อลิตร ก่อนที่จะสัมผัสกับยาซิสพลาทินจะส่งผลให้การตายของเซลล์ไตลดน้อยลงอย่างมีนัยสำคัญทางสถิติ และความสามารถในการปกป้องนั้นจะเพิ่มขึ้นตามระดับความเข้มข้นของไซลิมาริน ตรงกันข้ามเมื่อเซลล์มะเร็งได้รับไซลิมารินก่อนที่จะสัมผัสกับยาซิสพลาทินพบว่าไม่ส่งผลกระทบต่อประสิทธิภาพในการฆ่าเซลล์มะเร็งปอด แต่ช่วยเพิ่มประสิทธิภาพในการฆ่าเซลล์มะเร็งผิวหนังอย่างมีนัยสำคัญทางสถิติ โดยกลไกความเป็นพิษของยาซิสพลาทินต่อเซลล์ไต , เซลล์มะเร็งปอด และเซลล์มะเร็งผิวหนัง นั้น เกี่ยวข้องกับการเหนี่ยวนำให้มีการเพิ่มขึ้นของไฮโดรเจนเปอร์ออกไซด์และไฮดรอกซิล แรดดิคอลภายในเซลล์ งานวิจัยนี้พบว่าไซลิมารินมีความจำเพาะเจาะจงในการปกป้องการตายของเซลล์ไตจากซิสพลาทินด้วยคุณสมบัติที่ไซลิมารินเป็นแอนตี้ออกซิแดนท์ต่อไฮโดรเจนเปอร์ออกไซด์ และไฮดรอกซิล แรดดิคอลที่เกิดจากยาซิสพลาทิน โดยที่ไซลิมารินไม่ลดประสิทธิภาพในการฆ่าเซลล์มะเร็งปอดของยาซิสพลาทิน อีกทั้งยังช่วยเพิ่มประสิทธิภาพในการฆ่าเซลล์มะเร็งผิวหนังของ ยาซิสพลาทินด้วย ซึ่งเนื่องมาจากการที่ไซลิมารินมีฤทธิ์โดยตรงในการฆ่าเซลล์มะเร็ง การศึกษานี้แสดงให้เห็นว่าไซลิมารินสามารถปกป้องการตายของเซลล์ไตจากยาซิสพลาทินได้อย่างจำเพาะเจาะจง และน่าจะเป็นประโยชน์สำหรับการพัฒนาไซลิมารินเพื่อการนำไปใช้เป็นสารสำหรับปกป้องไตต่อไป เนื่องจากเป็นสารที่ได้รับการทดสอบแล้วว่ามีความปลอดภัย

ภาควิชา เภสัชวิทยาและสรีรวิทยา ลายมือชื่อนิสิต.....

สาขาวิชา เภสัชวิทยา ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก.....

ปีการศึกษา 2553

5276562533: MAJOR Pharmacology
KEYWORDS : cisplatin / nephrotoxicity / renoprotection / selectivity / silymarin

CHUANPIT NINSONTIA: EFFECTS OF SILYMARIN IN PROTECTION AGAINST CISPLATIN-INDUCED RENAL CELL DEATH AND IN CANCER-KILLING EFFECT OF CISPLATIN. ADVISOR: ASST PROF. Pithi Chanvorachote, Ph.D., 88 pp.

Cisplatin-induced nephrotoxicity has been accepted as an important obstacle for efficient cisplatin-based chemotherapy. Silymarin, a mixture of flovonolignans extracted from seeds of milk thistle, has been shown to possess various potential pharmacological properties; however, whether or not this agent selectively protects renal cells form cisplatin-induced cell death with no interfering effect on cancer cells is not clear. Therefore, potential of silymarin in protection of cisplatin-induced renal cell death without compromising effect on anti-cancer activity of cisplatin was demonstrated in this study. The results indicated that cisplatin induced both apoptosis and necrosis in HK-2 cells and caused a decrease in cell viability ~ 40% and 60% at the doses of 25 and 100 μM. Pretreatment with 50–200 μM of silymarin significantly protected against cisplatin-induced cell death in a dose-dependent manner. In contrast, pretreatment of silymarin caused no significant change on cisplatin-induced cell death in H460 cells but significantly potentiated cisplatin-induced apoptosis in G361 cells. Although, cisplatin induced renal, lung cancer and melanoma cell death through the induction of hydrogen peroxide (H_2O_2) and hydroxyl radical ($^{\cdot}OH$), silymarin still selective protected only renal cell by its anti-oxidant activites against H_2O_2 and $^{\cdot}OH$ without an interfering effect on cisplatin-induced cytotoxicity in human lung cancer H460 cells. In addition, silymarin showed enhancement of cisplatin-induced melanoma G361 cell death due to the direct anti-cancer activity of silymarin. These findings revealed the selectivity of silymarin in protection of renal cells from cisplatin-induced cell death and could be beneficial for the development of this considerately safe compound as a renoprotective agent.

Department : Pharmacology and Physiology Student's Signature .....
Field of Study : Pharmacology Advisor's Signature .....
Academic Year : 2010 .....

ACKNOWLEDGEMENTS

My sincere and heartfelt thanks to my advisor, Assistant Professor Pithi Chanvorachote for his great understandings, excellent advices, encouragements, guidance and supporting, which have given me throughout this study. Without his kindness and understanding, this work could not be accomplished.

I would like to express my deep appreciate to my partner, Miss Kanittha Pongjit for valuable advices, as well as her kindness and helpfulness. She always solves many problems together with me.

My special thanks go to all members in my laboratory; Mr. Hasseri Halim, Mr. Chatchai choatham, Mr. Preedakorn Chunhacha, Mr. Wongsakorn Suchaoin, and Miss Thitiporn Songserm for valuable comments and their great friendships I had received.

My thankfulness is also expressed to my best friend, Miss Ratjika Wongwanakul and Mr Sitthipong Kanokewanjumrus for their helpful and encouragement. They always motivate me to work to the best of my ability.

I would like to thank to all persons of the Department of Pharmacology and Physiology, Chulalongkorn University. Their advices and helpfulness are also appreciated.

Eventually, the extremely gratitude is expressed to my parents and to my brothers for their love, understanding, helping, supporting and encouraging with care, which enable me to conduct this research successfully.

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LIST OF ABBREVIATIONS

%	= percentage
°C	= degree Celsius
μM	= micromolar
ATP	= adenosine triphosphate
CAT	= catalase
CO_2	= carbon dioxide
$DCFH_2$-DA	= 2,7-dichlorofluorescein diacetate
DFO	= deferoxamine
DHE	= dihydroethidium
DMEM	= Dulbecco's Modified Eagle's Medium
DMNQ	= 2,3-dimethoxy-1,4-naphthoquinone
DMSO	= dimethyl sulfoxide
DNA	= deoxyribonucleic acid
ER	= endoplasmic reticulum
et al.	= et alibi, and others
Fe_2SO_4	= ferrous sulphate
g	= gram
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-buffered saline
ROS	= reactive oxygen species
RPMI	= Roswell Park Memorial Institute's medium
S.E.M.	= standard error of means
SOD	= superoxide dismutase
U	= unit

CHAPTER I

INTRODUCTION

Nephrotoxicity induced by cisplatin [*cis*-diaminedichloplatinum (II)] has been shown to be an important obstacle for efficient cisplatin-based chemotherapy in many patients (Patrick and Lawrence, 1984; Launay-Vacher *et al*., 2008; Pabla and Dong, 2008). Because cisplatin is accepted as one of the most prescribed drugs for the treatment of many solid tumors such as testicular, head and neck, lung, melanoma, and ovarian cancers (Patrick and Lawrence, 1984; Atallah and Flaherty, 2005; Kelland, 2007). Co-administration of cisplatin and effective nephroprotective agents protecting renal cells from cisplatin-induced damages while having minimal effect on cisplatin actions against cancer cells, which should be a recommended part of the treatment regimen of named cancers (Pabla and Dong, 2008).

Silymarin, a flovonolignan extracted from milk thistle (*Silybum marianum*), has been long used for the treatment of various liver diseases and the hepatoprotection against several toxic substances (Muriel *et al*., 1992; Flora *et al*.,1998; Aller, Meier, and Brignoli, 2001 *et al*, 2001; Saller, Meier, and Brignoli, 2001; Mansour, Hafez, and Fahmy, 2006). Silymarin has been shown to possess various potential pharmacological properties including: scavenging reactive oxygen species (Mira, Silva, and Manso, 1994; Dehmlow, Murawski, and Groot, 1996; Asghar and Masood, 2008; Jadhav, Upasani, and Pingale, 2009), inhibiting lipid peroxidation (Velenzuela *et al*., 1985; Letteron *et al*., 1990; Bosisio, Benelli, and Pirola, 1992; Muriel *et al*., 1992; Svobodová, Walterová, and Psotová, 2006), increasing in glutathione and superoxide dismutase levels (Feher *et al*., 1987; Velenzuela *et al*., 1989; Lang *et al*., 1993), inducing tissue regeneration, and promoting DNA, RNA as well as protein synthesis (Sonnenbichler *et al*., 1976, 1986, 1999). In addition, silymarin exerts cancer-suppressing activity in many kinds of tumor cells, such as lung, prostate, cervical, leukemia, and breast cancers (Zi, Feyes, and Agarwal, 1998; Sharma *et al*., 2003; Huang *et al*., 2005; Deep *et al*., 2006; Zhong *et al*., 2006).

Although silymarin has potential activities namely antioxidant and tissue stimulating effects, whether this agent selectively protects renal cells with no interfering effect of cisplatin on cancer cells is not known. However, the possible reasons for selectivity of silymarin in the protection of renal cells without interfering the mode of cisplatin action in cancer cells may be : (i) silymarin possesses direct anticancer activity; (ii) renal cells have been shown to be highly susceptible to oxidative stress-induced cell damage, whereas many cancer cells have shown to resist ROS-induced cell death (Andreoli, 1991; Nath and Norby, 2000; Galle, 2001; Djamali, 2007; Valko *et al.*, 2007; Pabla and Dong, 2008; Gibellini *et al.*, 2010), and the main mechanism of cisplatin-induced cancer cell death is through DNA-adduct formation (Reedijk and Lohman, 1985; Wang and Lippard, 2005). For these reasons lead to the purposes of this study which aim to investigate (i) the effect of silymarin on the protection of cisplatin-induced cell damage in human proximal tubular HK-2 cells, (ii) the effect of silymarin on anticancer activity of cisplatin in cancer cells and (iii) the possible mechanism of silymarin in preventing cisplatin cytotoxicity. This study may advocate the necessity of developing silymarin, a relatively safe compound, for its potential use in clinical cancer therapy in combination with anticancer agents.

Research questions

1. Whether or not silymarin could selectively protect renal cells from cisplatin cytotoxicity without interfering anticancer effect of cisplatin on cancer cells.
2. What were the possible mechanisms of silymarin in the selective protection against cisplatin-induced renal cell death?

Hypothesis

Silymarin could selectively protect human renal cell from cisplatin-induced cell death without compromising effect on anticancer activity of cisplatin in cancer cells.

Objectives

1. To investigate the effect of silymarin on the protection of cisplatin-induced cell death in human proximal tubular HK-2 cells.
2. To investigate the possible mechanisms of silymarin in the selective protection against cisplatin-induced renal cell death.
3. To evaluate the effect of silymarin on anticancer activity of cisplatin in cancer cells.

CHAPTER II

LITERATURE REVIEWS

Cisplatin

Cisplatin [*cis*-diammine-dichloroplatinum (II)] is a potent cytotoxic drug commomly used in cancer chemotherapy for over 30 years. It is highly effective in the treatment of many solid tumors including testiscular, head and neck, lung, melanoma, and ovarian cancers (Patrick and Lawrence, 1984; Atallah and Flaherty, 2005; Kelland, 2007). Although, its structure is a small and simple inorganic molecule formed by an atom of platinum surrounded by ammonia and chlorine atoms in the *cis* position of horizontal plane as shown in the figure 1.1, it can strongly react with DNA (Reedijk and Lohman, 1985). Because of low concentrations of chloride ion inside the cells, when cisplatin enters into the cells mainly by passive diffusion through the plasma membranes, the chloride groups of cisplatin are substituded by hydroxyl groups or water, creating the positive charge molecule that has a capability to react with DNA (Reedijk and Lohman, 1985; Wang and Lippard, 2005; Launay-Vacher *et al*., 2008).

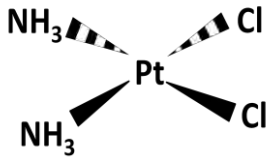


Figure 1.1 structure of cisplatin

However, it has been widely accepted that cisplatin is able to induce cytotoxicity by two major mechanisms involved in DNA-adduct formation and intracellular reactive oxygen species (ROS) induction.

- DNA-adduct formation

After entry into the cells, cisplatin undergoes aquation to form $[Pt(NH_3)_2Cl(OH_2)]^+$ or $[Pt(NH_3)_2(OH_2)]^{2+}$ called aquated molecule which is more reactive to DNA. The platinum of cisplatin forms covalent bonds to the N7 position of purine bases to afford primaryly intrastand crosslinks and a small number of interstand crosslinks. Then, a plenty of cellular responses, such as replication arrest, translation inhibition, cell-cycle arrest, DNA repair process, and apoptosis are activated by cisplatin-DNA adduct as demonstrated in figure 1.2. It is known that DNA-adduct formation always occurs in the proliferated cells, therefore, cancer cells are more sensitive to cisplatin induced cell damage via this mechanism than normal cells (Patrick and Lawrence, 1984; Reedijk and Lohman, 1985; Wang and Lippard, 2005).

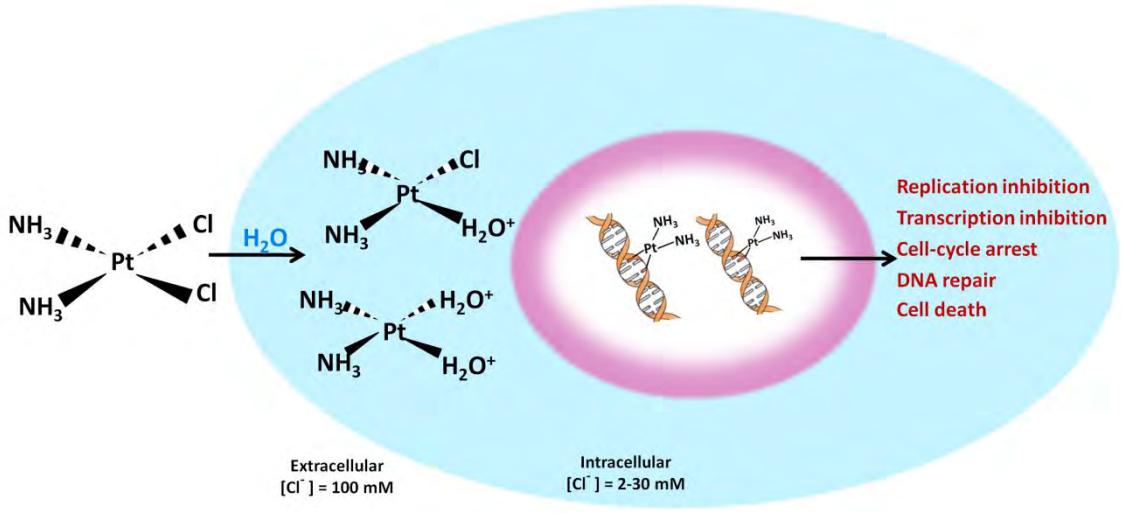


Figure 1.2 Mechanism of cisplatin involved DNA-adduct formation

- ROS induction

An amount of evidence suggest that cisplatin increases many kinds of ROS production such as hydrogen peroxide, superoxide anion radical and hydroxyl radical in cultured cells and animal tissues, which has been shown the relationship between such alteration of intracellular ROS level and activation of cell death signaling mechanisms including apoptosis and necrosis (Kruidering, 1996; Matsushima *et al*., 1998; Baek *et*

al., 2002; Yang *et al*., 2002; Kawai *et al*., 2005; Lu and Cederbaum, 2006; Kim *et al*., 2010). On the other hand, a variety of antioxidants such as vitamin C, vitamin D, *N*-acetylcysteine and genistein have been shown to inhibit cell damage induced by cisplatin (Zunino *et al*., 1983; sheikh-hamad, Timmins, and Jalali, 1997; Ajith, Usha, and Nivitha, 2007; Sung *et al*., 2007). It is implicated that cisplatin-induced ROS has been considered to play a critical role in cisplatin-induced cytotoxicity, especially in normal cells. Because cancer cells have abnormally active metabolism, they always generate high level of intracellular ROS which contributes to persistent oxidative stress. However, cancer cells have evolved mechanism to protect themselves from intrinsic oxidative stress and have developed a sophisticated adaptation system involving antioxidant defenses such as increase of intracellular superoxide dismutase or glutathione levels. Therefore, cancer cell are likely to resist oxidative stress while normal cells are very sensitive to oxidative stress induced cell damage (Valko *et al*., 2007; Gibellini *et al*., 2010).

Reactive Oxygen Species

Reactive Oxygen Species (ROS) are known as mediators of intracellular signaling pathway when present at the low or moderate concentrations in normal condition. Formation of ROS generally takes place in the cells by enzymatic and non-enzymatic reaction. Superoxide anion radical ($O_2^{\cdot-}$) is mainly produced from non-enzymatic process during oxidative phosphorylation of electron transport chain in mitrochondria and also from oxidation reaction of xanthine oxidase enzyme. Although, superoxide anion radical lacks an ability to penetrate lipid membranes, with accelerated by superoxide dismutase (SOD) two molecules of superoxide anion radical are rapidly dismutated to hydrogen peroxide (H_2O_2), which can penetrate cell membranes. Because hydrogen peroxide is able to cross the cell membranes, an important function of hydrogen peroxide is to act as inter- and intracellular signaling molecules. Besides, hydrogen peroxide can be inactivated by antioxidant enzymes, namely catalase and glutathione peroxidase, or catalyzed by metal ions (Fe^{2+} or Cu^{+}) to form hydroxyl radical ($^{\cdot}OH$) called Fenton reaction. In addition to hydrogen peroxide, superoxide anion radical is another intermediate in the production of hydroxyl radical via reduction

of metal ions (Fe^{3+} or Cu^{2+}). The summation of these two reactions as mentioned above is the Haber-weiss reaction which demonstrates below.

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

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

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

These reactions indicate that the transition metals play a principal role in the formation of hydroxyl radical which is more reactive and has a capability to cause more damage than other ROS.

According to the concentration of ROS, the low or moderate level of ROS can be beneficial for biomolecular functions while the high concentrations of ROS seem to be harmful to the cells. Several studies have been stated that overproduction of ROS may lead to oxidative stress which is imbalance between the formation of ROS and antioxidant defenses. The excess ROS interact with biomolecules in the body such as lipid, protein, and DNA (Hancock, Desikan, and Neill, 2001; Nordberg and Arner, 2001; Young and Woodside, 2001; Valko *et al*., 2007; Pham-Huy LA, He, and Pham-Huy C, 2008) causing the loss of cell function and ultimately apoptosis or necrosis as indicated in figure 1.3.

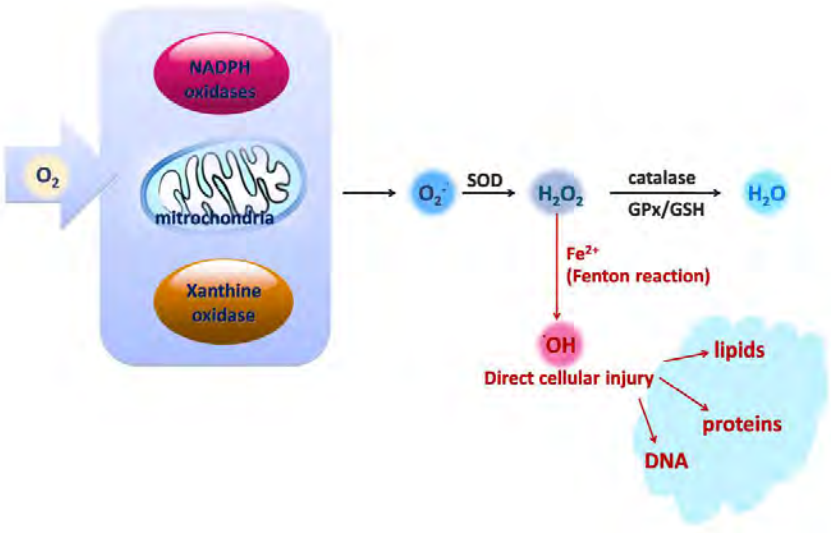


Figure 1.3 Reactive Oxygen Species (ROS) system

- Lipid

Polyunsaturated fatty acid is a biomolecular molecule commonly presenting at the cell membranes and very sensitive to oxidation reaction. Many previous studies have been provided the evidence that the interaction of ROS, especially hydroxyl radical, with lipid results in a chain reaction of lipid peroxidation, thus damaging plasma membrane, causing further oxidation of membrane lipid, and finally leading to cell injury (Gutteridge, 1982; Nordberg and Arner, 2001; Teiero *et al*., 2007).

- Protein

ROS has been shown to react with several amino acids, peptides and proteins resulting in structural changes and enzyme inactivation such as DNA repairing enzyme, DNA polymerase, and membrane transporter proteins, which contribute to cellular dysfunction or damage and finally develops pathophysiology of various diseases (Nordberg and Arner, 2001; Butterfield *et al*., 1998).

- DNA

It is well established that the interaction between ROS and DNA not only causes DNA damage but also inhibits DNA-repairing process, which enhances the mutagenesis and carcinogenesis. Moreover, the damaged DNA induced by ROS is able to activate cell death signaling mechanism (Marnett, 2000; Nordberg and Arner, 2001; Valko *et al*., 2004).

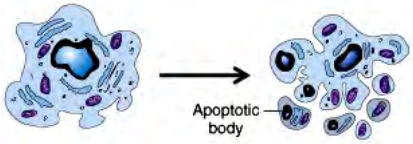
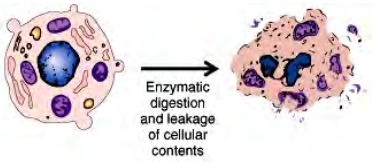
Interestingly, it has been reported that some toxic substances, including cisplatin is able to induce many kinds of ROS that play a crucial role in its cytotoxicity (Kruidering, 1996; Matsushima *et al* 1998; Baek *et al*., 2002; Yang *et al*., 2002; Kawai *et al*., 2005; Lu and Cederbaum, 2006; Kim *et al*., 2010)

Cisplatin-induced apoptosis and necrosis

As apoptosis and necrosis have been shown to be two distinct forms of cell death, which induced by cisplatin (Matsushima *et al* 1998; Baek etal., 2002; Yang *et al*., 2002), the differences of features between them have to be defined. Apoptotic cell death is characterized on the basis of the following criteria: cellular shrinkage, condensation

of nuclear chromatin, DNA fragmentation and formation of apoptotic bodies, whereas necrotic cell death is likely to demonstrate the morphology of cellular swelling, loss of cell membrane integrity, nucleus and cell lysis (Raffray and Cohen, 1997; McConkey, 1998; Haddad, 2004; Prayong, Weerapreeyakul, and Barusrux, 2007) as shown in table 1. Even though the mechanism of cisplatin-induced both apoptosis and necrosis are remain poorly understood, there are sufficient evidence indicating that cisplatin induces apoptosis or necrosis in renal tubular cells in vitro depending on the concentration and the time of cisplatin exposure. Necrotic cell death is induced by the high concentrations of cisplatin exposure (300-1000 μM) for a short period of time (less than 6 hr), whereas apoptosis is elicited by long time exposure to low concentrations of cisplatin (3-50 μM) (Shino *et al.*, 2003; Yono *et al.*, 2007). In addition, different kinds of ROS generated by cisplatin seem to cause the death of cell in different ways. Bake and colleagues reported that necrosis induced by cisplatin resulted from superoxide anion radical and hydrogen peroxide, while apoptosis caused by superoxide anion radical and hydroxyl radical (Bake *et al.*, 2003), which contradicts the studies of Matsushita and co-workers as well as Yang and colleagues that cisplatin generated hydroxyl radical resulting in lipid peroxidation and cell necrosis (Matsushita *et al.*, 1998; Yang *et al.,* 2002).

Table 1.1 Morphological and functional distinctions between apoptosis and necrosis

	Apoptosis	Necrosis
Cytoplasm	Condensation/blebbing/fragmentation Cytoskeleton protein breakdown	Cytoplasm and mitochondria Swelling
Nucleus	pyknotic	No
Nuclear protein breakdown	Yes	No
DNA fragmentation	Yes	Random digestion
Morphological change	Cell shrinkage and cell fragmentation into smaller bodies	Plasma membrane lysis
		
ATP requirement	Yes	No
Inflammatory response	No	Yes

Cisplatin-induced nephrotoxicity

Cisplatin is one of the most effective chemotherapeutic agents against various solid tumors (Patrick and Lawrence, 1984; Atallah and Flaherty, 2005; Kelland, 2007), the development of nephrotoxicity during treatment has been shown to be the major limitation of efficient therapy. A number of experimental and clinical studies have revealed that cisplatin can induce both acute and chronic renal failure (Patrick and Lawrence, 1984; Launay-Vacher *et al*., 2008; Pabla and Dong, 2008); however, the

stratergies of nephrotoxicity prevention and the mechanisms of cisplatin-induced renal toxicity are not fully clear.

Nowadays, the prevention of cisplatin nephrotoxicity still relies on drug dosage decrease, specific measures of hydration and active screening for renal abnormalities as part of pre-therapeutic usual biological work up in patients treated with cisplatin. Nevertheless, these approaches have been shown to be partially successful as acute renal failure occuring at low dose and uncertain clinical application (Launay-Vacher *et al*., 2008). Because of the low molecular weight and uncharge of cisplatin, it is freely filtered at the glomerulus and may be accumulated in the tubular epithelial cells, mainly at the S3 segment of the renal proximal tubule (Patrick and Lawrence, 1984; Launay-Vacher *et al*., 2008; Pabla and Dong, 2008). In the present of cisplatin in renal cells, cisplatin may induce the generation of various ROS via induction of mitochondrial dysfunction (Kruidering *et al*., 1997), interaction with microsomal cytochrome P450 (Liu and Baliga, 2003) or activation of NADPH oxidase (Kawai *et al*., 2006). An increase of intracellular ROS induced by cisplatin may affect cell function by reacting with cell components including lipid, protein as well as DNA resulting in cellular damage. Besides, such ROS appear to be involved in the activation of several signaling mechanisms during cisplatin nephrotoxicity including p38, p53 and NF-κB activation, leading to renal cell injury (Jiang and Dong, 2008). Therefore, an elevation of intracellular ROS induced by cisplatin resulting in oxidative stress may play a key role in pathophysiology of cisplatin related- nephrotoxicity.

For these reasons, the introduction of antioxidant could be beneficial in preventing renal cell damage during cisplatin therapy. Moreover, it has been reported that various ROS scavengers or antioxidants show protective effect on cisplatin nephrotoxicity both in vivo and in vitro such as vitamin D, vitamin C, genistein, *N*-acetylcysteine, and Glutathione (Zunino *et al*., 1983; Sheikh-hamad, Timmins, and Jalali, 1997; Ajith, Usha, and Nivitha, 2007; Sung *et al*., 2007). Nevertheless, a number of antioxidants fail to be further developed for the use in clinical treatment because their antioxidant activities frequently attenuate the cytotoxicity mechanism of cisplatin in cancerous cells (Eastman, 1987; Meijer *et al*., 1990; Roller and Weller, 1998; Miyajima *et al*., 1999; Wu, Muldoon, and Neuwelt, 2005; Chanvorachote, 2006).

Silymarin

Silymarin, a flavonolignan mixture compound originated from seed of Milk thistle (*Silybum marianum)*, consists of four flavonolignan isomers ($C_{25}H_{22}O_{11}$), namely silibinin (silybin), isosilibinin (isosilybin), silydianin and silychristin as shown in figure 1.4. Among them silibinin is the major and most active component and represents approximately 50-60%, followed by silychristin (20%), silydrianin (10%) and isosilibinin (5%) (Ghosh A, Ghosh T,and Jain, 2010).

Figure 1.4 Silymarin (*Silybum marianum*)

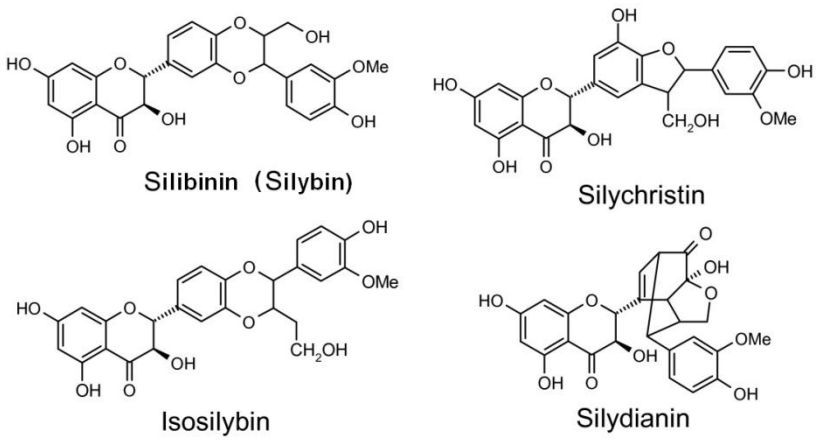


Figure 1.5 Structures of four isomers of silymarin (Sonnenbichler *et al*., 1999)

Silymarin is not able to dissolve in water and usually administrated in capsule or tablet forms with the standard extract containing 70-80% silibinin. After single oral administration of a standardized-dose of silibinin 100-360 mg in healthy volunteers, about 23-47% of them are absorbed and a peak plasma concentration is reached within 2 h. For total silibinin, an elimination half-life of 6 h is estimated. Approximately 20-40% of an oral dose is recovered from enterohepatic circulation as glucoronide and sulfate conjugates, while 3-8% is excreated in urine (Saller, Meier, and Brignoli, 2001; Dixit *et al*., 2007).

Silymarin has been used for over 2000 years not only to treat various liver diseases including cirrhosis, hepatitis as well as alcoholic liver disease (Flora *et al*., 1998; Saller, Meier, and Brignoli, 2001) but also to detoxify a wide range of toxic substances such as amanita phalloides toxin, carbontetrachloride, phenylhydrazine, thioacetamide, galactosamine, halothane, paracetamol and erythromycin estolate as hepatoprotective agent (Vogel *et al*., 1984; Muriel *et al*., 1992; Saller, Meier, and Brignoli, 2001; Dixit *et al*., 2007). These abilities of silymarin are mainly due to its diversity of pharmacological properties including antioxidant activity, stimulation of tissue regeneration, anti-inflammatory, and anticancer activity.

The antioxidant effect of silymarin results from its ROS-scavenging activity and its ability to inhibit lipid peroxidation. Many studies suggest that silymarin is not a good scavenger of superoxide anion radical. However, a reaction with hydrogen peroxide is detected and silymarin rapidly reacts with hydroxyl radical and effectively inhibits lipid peroxidation in cell-free system (Velenzuela *et al*., 1985; Letteron *et al*., 1990; Muriel *et al*., 1992; Mira, Silva, and Manso, 1994; Dehmlow, Murawski, and Groot, 1996; Asghar and Masood, 2008; Jadhav, Upasani, and Pingale, 2009). On the other hand, lipid peroxidation induced by some toxic stimuli such as ethanol, paracetamol and carbontetrachloride in rat (Velenzuela *et al*., 1985; Letteron *et al*., 1990; Muriel *et al*., 1992) as well as UVA in human keratinocytes (Svobodova *et al*., 2006) is blocked by silymarin. Likewise, silymarin inhibits lipid peroxidation in rat liver microsome and isolated hepatocytes (Bosisio, Benelli, and Pirola, 1992). Moreover, it has been reported that silymarin markedly increases an expression and activity of superoxide dismutase of

lymphocytes (Feher *et al*., 1987; Lang *et al*., 1993) as well as glutathione level in liver, stomach and intestine of rat (Valenzuela *et al*., 1989).

Numerous investigations performed by Sonnenbichler and colleagues state that the capability of silymarin to induce tissue regeneration may be result from stimulating effects of silymarin on protein, RNA and DNA synthesis in injured liver, hepatocytes and kidney cells (Sonnenbichler *et al*., 1976, 1986, 1999). The molecular basis of anti-inflammatory of silymarin is not fully known; it might be related to the inhibition of leukotriene formation (Dehmlow, Murawski, and Groot, 1996). A number of evidence indicated that silymarin exerts the anticancer activity against various cancerour cell types such as lung, prostate, cervical, leukemia and breast cancers by induction of cell growth inhibition and promotion of cancer cell apoptosis as well as necrosis (Zi, Feyes, and Agarwal, 1998; Sharma *et al*., 2003; Huang *et al*., 2005; Deep *et al*., 2006; Zhong *et al*., 2006). This activity of silymarin has been documented in different animal models and human cancer cells. Silymarin induces cell cycle arrest and suppresses proliferation of cancer cells by increase in an expression of cyclin-dependent kinase inhibitors (CDKIs), such as Cip/p21 and Kip1/p27 and decrease in kinase activity of cyclin-dependent kinase (CDK) as well as associated cyclins (Agarwal *et al.*,2006; Comelli *et al.*, 2007).

In addition to the several pharmacological properties, silymarin is considered to be safe and well-tolerated with gastrointestinal upset, a mild laxative effect and rare allergic reaction, which are the only adverse events reported when taken within the recommended dose (Saller, Meier, and Brignoli, 2001). Acute toxicity tests of silymarin in animal support that silymarin has a very low toxicity with LD_{50} values of silymarin are 400 mg/kg for mice, 385 mg/kg for rats and 140 mg/kg for rabbits and dogs after intravenous infusion (Radko and Cybulski, 2007). Importantly, silymarin was approved by US FDA since 2001 as a herbal supplement in the treatment of alcoholic liver diseases (420 mg/day), *Amanita phalloides* poisoning (20-50 mg/kg/day), viral hepatitis (480 mg/day), and liver cirrhosis (420-600 mg/day) (Saller, Meier, and Brignoli, 2001; Tamayo and Diamond, 2007).

CHAPTER III

MATERIALS AND METHODS

Materials

1. Chemicals and Reagents

Cisplatin, silymarin, trypsin, Hoechst 33342, propidium iodide (PI), 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 2,7-dichlorofluorescein diacetate (H_2DCF-DA), dimethysulfoxide (DMSO), deferoxamine (DFO), catalase (CAT), dihydroethidium (DHE), DMNQ (2,3-dimethoxy-1,4-naphthoquinone), hydrogen peroxide, and ferrous sulphate ($FeSO_4$) 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).

2. Equipments

Laminar flow cabinet, carbon dioxide incubator, autopipette: 2-10 µl, 10-100 µl, 20-200 µl and 200-1,000 µl, pipette tips for 2-10 µl, 10-100 µl, 20-200 µl, and 200-1,000 µl, cell culture plate: 96-well and 6-well (Nunc), conical tube: 15 ml and 50 ml (Neptune) , bottle: 100 ml, 250ml, 500 ml, and 1,000 ml (Duran) disposable pipette: 1ml and 5ml, hemocytometer, pH meter, vertex mixer, balance, ELISA reader (Anthros, Durham, NC, USA), Flow cytometer (FACSort, Becton Dickinson, Rutherford, NJ, USA), and fluorescence microscope (Olympus IX51 with DP70).

Methods

1. Sample preparation

Various samples of silymarin were prepared by dissolving in ethanol and diluted by phosphate buffer saline (PBS) to the indicated concentrations with less than 0.1% ethanol.

2. Cell culture

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

3. Cytotoxicity Assay

Cell viability was determined by MTT colorimetric assay. Briefly, cells were seeded in 96-well plate for 24 h prior to the addition of test compounds. After specific treatment, cells in 96-well plate were incubated with 500 μg/ml of MTT for 4 h at 37°C. The supernatant was then removed and DMSO was added to dissolve the formazan product. The optical density (OD) was spectrophotometrically measured at 570 nm using an ELISA reader (Anthros, Durham, NC, USA). As viable cells could convert yellow MTT to purple formazan by mitochondria reductase, the absorbance of crystal formazan was referred to amount of living cells.

Cell viability was calculated as follow:

$$\text{Percentage of cell viability} = \frac{OD_{570} \text{ of treatment}}{OD_{570} \text{ of control}} \times 100$$

4. Apoptosis and necrosis assay

Apoptotic and necrosic cell death were detected by Hoechst 33342 and propidium iodide (PI) co-staining. After specific treatments, cells were stained with 10 μM of the Hoechst and 5 μg/ml PI dyes for 30 min at 37°C and analyzed under a fluorescence microscope using blue filter for Hoechst 33342 and red filter for propidium iodide. The apoptotic cells having condensed chromatin and/or fragmented nuclei stained by Hoechst 33342 and PI-positive necrotic cells were visualized and scored under a fluorescence microscope (Olympus IX51 with DP70).

5. ROS detection

Intracellular ROS was analyzed by 2 methods**;**

5.1. Fluorescence microscope

Intracellular ROS were detected by fluorescence microscope using dihydrodichloro-fluorescein diacetate (H_2DCF-DA) as a fluorescent probe for hydrogen peroxide and hydroxyl radical as well as dihydroethidium (DHE) as a fluorescent probe for superoxide anion radical. After specific treatments, cells were incubated with 10 μM of H_2DCF-DA or DHE for 30 min at 37°C, after which they were analyzed under a fluorescence microscope (Olympus IX51 with DP70). The fluorescence intensity represents the intracellular ROS.

5.2. Flow cytometry

Intracellular ROS were determined by flow cytometry using H_2DCF-DA as a fluorescent probe. Briefly, after specific treatment cells were incubated with 10 μM of H_2DCF-DA for 30 min at 37°C, after which cells were washed, resuspended in phosphate-buffered saline (PBS), and immediately analyzed for fluorescence intensity by FACScan flow cytometer (Beckton Dickinson, Rutheford, NJ) using a 488-nm excitation beam and a 538-nm band-pass filter. Intracellular superoxide anion radical was similarly determined but using 10 μM of DHE as a probe. The fluorescence intensity was analyzed on the FACScan flow cytometer using a 488-nm excitation beam and a 610-nm band-pass filter. Mean fluorescence intensity was quantified by CellQuest software (Becton–Dickinson) analysis of the recorded histograms. Relative fluorescence was calculated as a ratio of the treated to the non-treated control fluorescence intensity.

6. Statistical Analysis

All data were expressed as the means ± S.E.M. from three or more independent experiments. Statistical differences between two groups were performed by Student's *t* test. Multiple comparisons were examined for significant differences of multiple groups, using analysis of variance (ANOVA), followed by individual comparisons with the Scheffe's post-hoc test. Statistical significance was set at $P<0.05$.

7. Experimental designs

7.1. Conceptual framework

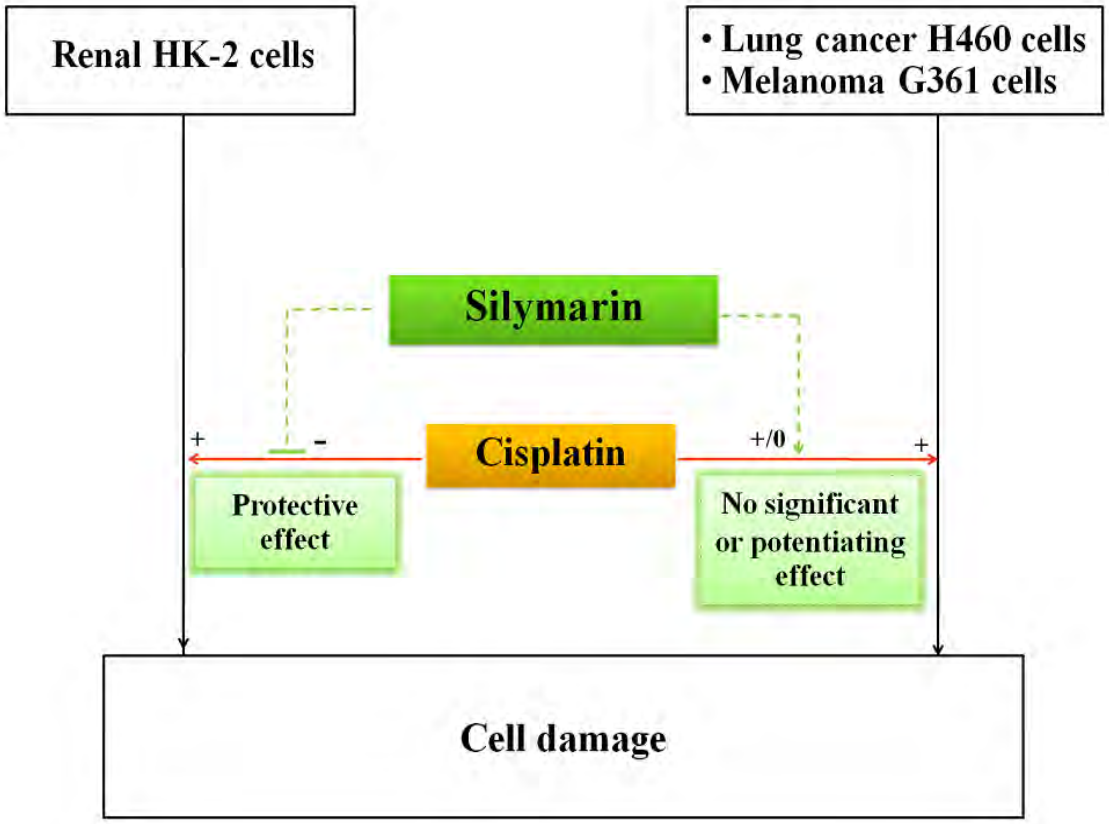


Figure 3.1 Conceptual frame work of this study

7.2. Selective effect of silymarin in the protection of cisplatin-induced renal cell death

7.2.1. Effect of silymarin on HK-2 cell viability

To determine whether or not silymarin has direct effect on HK-2 cells and to comfirm that silymarin has no toxicity to HK-2 cells. Sub-toxic concentrations of silymarin were used in further experiments. Cells were incubated with various concentrations of silymarin (25, 50, 100, and 200 μM) or left untreated for 24 h and cell viability and cell death detection were evaluated. Sub-toxic concentrations of silymarin causing the reduction of HK-2 cell viability less than 95% were analyzed.

7.2.2. Cisplatin induced cell death

To determine the cytotoxic effect of cisplatin on HK-2, H460 and G361 cells and to define the patterns of cisplatin-induced cell death, for dose-dependent experiment, cisplatin-induced cell death was determined by leaving the cells untreated or treated with various concentrations of cisplatin (5, 25, 50, 100, 200, and 300 μM). After 24 h, cell viability was then measured by MTT assay. Then, in each cell IC_{50} of cisplatin-induced cell death was estimated. For time-dependent study, cisplatin-induced cell death was analyzed by leaving the cells untreated or treated with IC_{50} of cisplatin. Cell viability was then measured after 0, 6, 15, and 24 h by MTT assay.

As apoptosis and necrosis have been shown to be two major modes of cisplatin-induced cell death (Matsushima *et al* 1998; Baek etal., 2002; Yang *et al*., 2002), type of cell death in response to cisplatin treatment in these cells were further identified by the fluorescence dye co-staining. Hoechst 33342 assay was performed for apoptosis detection, and propidium iodide staining assay was used for necrosis detection. Cells were treated or left untreated with various concentrations of cisplatin (25, 50, and 100 μM). After 24 h, cells were stained with Hoechst 33342 and propidium iodide for 30 min at 37°C. For time-dependent assay, cells were treated or left untreated with cisplatin (IC_{50}). After incubation 0, 6, 15, and 24 h, cells were stained with Hoechst 33342 and propidium iodide as described. The apoptotic cells with condensed

chromatin and/or fragmented nuclei stained by Hoechst 33342 and PI-positive necrotic cells were visualized and scored under a fluorescence microscope.

7.2.3. Protective effect of silymarin against cisplatin-induced renal cell death

To investigate the potential of silymarin in the protection of cisplatin-induced renal cell death, HK-2 cells were pre-incubated or left untreated with various concentrations of silymarin (25, 50, 100, and 200 μM) for 1 h and treated with cisplatin (25, 50, and 100 μM). After 24 h, cell viability and cell death detection were performed.

7.2.4. Effect of silymarin on anticancer activity of cisplatin in cancer cells

A limitation of using antioxidants for the protection of normal cell toxicities in chemotherapy was that antioxidants frequently attenuated anticancer activities of drugs on tumor cells (Eastman, 1987; Meijer *et al*., 1990; Roller and Weller, 1998; Miyajima *et al*., 1999; Wu, Muldoon, and Neuwelt, 2005).

To investigate whether silymarin can reduce efficacy of cisplatin in the induction of cancer cell death, H460 and G361 cells were left untreated or pretreated with various concentrations of silymarin (25, 50, 100, and 200 μM) for 1 h and treated with cisplatin (25, 50, and 100 μM). After 24 h, cell viability, apoptosis, and necrosis detection were determined.

7.3. Effect of silymarin on ROS generated by cisplatin and cisplatin-induced cytotoxicity

7.3.1. Cisplatin induced renal cell death via an ROS-dependent pathway

To clarify whether nephrotoxicity induced by cisplatin was involved in oxidative damage. Effects of known antioxidants on cisplatin-induced renal cell death were evaluated by leaving the cells untreated or pretreated with known antioxidants (5 mM glutathione and 5 mM *N*-acetylcysteine) for 1 h and treated with 50 μM cisplatin. After 24 h, cell viability and modes of cell death were determined.

7.3.2. Cisplatin induced specific ROS generation in HK-2, H460 and G361 cells

To identify the specific ROS generation induced by cisplatin in different cells, time-dependent study was performed in order to estimate the optimal time that intracellular ROS was distinctively increase in response to cisplatin treatment for using in the further experiment. Cells were treated with IC_{50} of cisplatin and intracellular ROS detection was then performed at various time points (0, 15, 30, 60, and 120 min) by fluorescence microscope using H_2DCF-DA as a fluorescent probe for superoxide anion radical, hydrogen peroxide and hydroxyl radical as well as DHE as a probe for superoxide anion radical.

For cisplatin-induced specific ROS generation analysis, cells were pretreated with or without various specific ROS scavengers, such as MnTBAP (superoxide anion radical scavenger), catalase (hydrogen peroxide scavenger), and deferoxamine (hydroxyl radical inhibitor) for 1 h prior to cisplatin (IC_{50}) treatment. After 1 h, ROS detection was performed by fluorescence microscope using the fluorescent probes as described.

7.3.3. Hydrogen peroxide and hydroxyl radicals play a role on cytotoxic mode of cisplatin.

To further clarify which specific ROS induced by cisplatin were responsible for cisplatin cytotoxicity, cells were pretreated with or without various specific ROS scavengers, such as MnTBAP (superoxide anion radical scavenger), catalase (hydrogen peroxide scavenger), and deferoxamine (hydroxyl radical inhibitor) for 1 h prior to cisplatin (IC_{50}) treatment. After cisplatin treatment for 24 h, cell viability was determined by MTT assay.

7.3.4. Specific scavenging activity of silymarin on ROS in HK-2 cells

To clarify mechanism of silymarin in reduction of cellular oxidative stress and further identified specific ROS scavenging activity of silymarin, intracellular ROS level was determined in HK-2 cell exposed standard ROS inducers which were DMNQ (superoxide anion radical generator), hydrogen peroxide and combination of

hydrogen peroxide and ferrous sulphate (hydroxyl radical generator) in the presence or absence of silymarin **(**50 μM**).** Cells were seeded in a 6-well plate and pre-incubated with silymarin for 1 h and then treated with standard ROS inducers. After treatment for 1 h, intracellular ROS level was determined by flow cytometry using H_2DCF-DA as a fluorescent probe for hydrogen peroxide and hydroxyl radical as well as DHE as a probe for superoxide anion radical.

7.4. Direct anticancer activity of silymarin on H460 and G361 cells

To support the ideal of selective protection of silymarin, direct anticancer activity of silymarin on H460 and G361 cells were evaluate. The effect of silymarin-induced cell death was determined by leaving the cells untreated or treated with various concentrations of silymarin (25, 50, 100, and 200 μM). After 24 h, cell viability and modes of cell death assay were performed.

For time-dependent study, cells were left untreated or treated with silymarin (200 μM) and cell viability and modes of cell death assay were then evaluated after 0, 6, 15, and 24 h.

CHAPTER IV

RESULTS

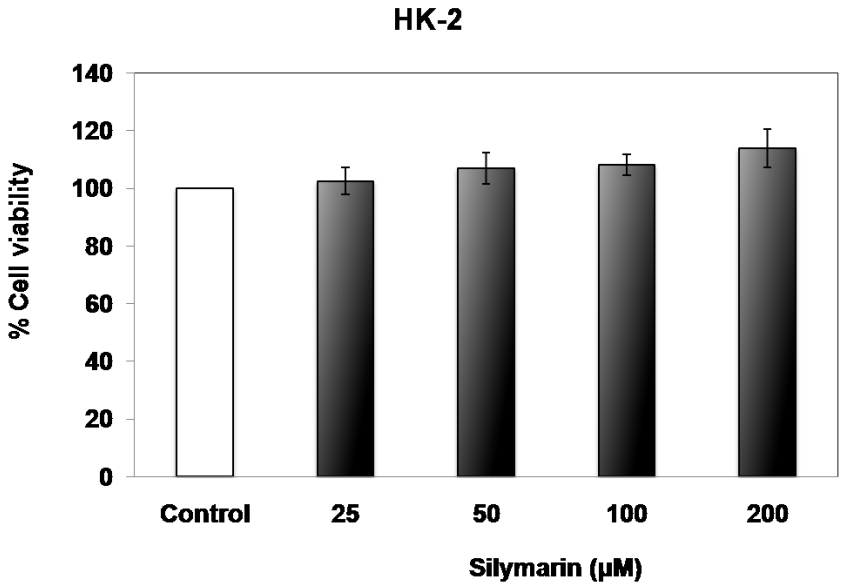
1. Silymarin protected cisplatin-induced renal cell death

1.1. Effect of silymarin on renal HK-2 cell viability

To investigate the cytoprotective effect of silymarin, this study first examined the effect of silymarin on HK-2 cell by cytotoxicity, apoptosis, and necrosis assays. Cells were left untreated or treated with silymarin at the concentrations of 25, 50,100, and 200 μM. After 24 h, cell viability and cell death detection were determined.

The result showed that the treatment of silymarin at the concentration range from 25 to 200 μM had no significant effect on HK-2 cell viability. (As shown in figure 4.1A) In addition, the nuclear morphology study supported that no apoptotic and necrotic cell death were detected in response to 25-200 μM of silymarin treatment. (As shown in figure 4.1B) These results suggested that those concentrations of silymarin had neither cytotoxic nor proliferative effects on HK-2 cells.

A



B

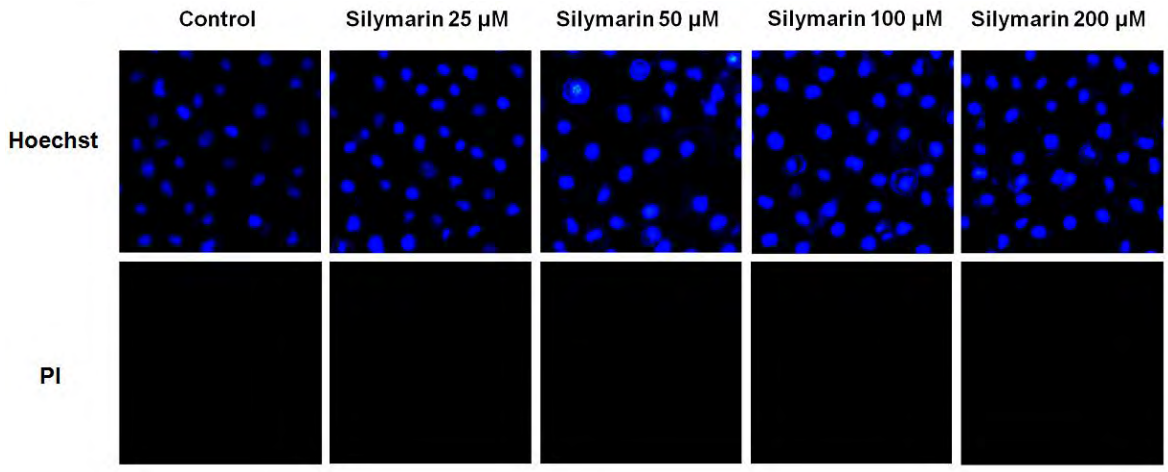


Figure 4.1 Effect of silymarin on HK-2 cells. A) Cells were treated with various concentrations of silymarin (0, 25, 50, 100, and 200 μM) for 24 h. Cell viability was measured by MTT assay. B) Nuclear morphology of apoptosis and necrosis were detected by Hoechst 33342 and PI assays. Values were means ± S.E.M. of three-independent experiments, $*P < 0.05$ versus non-treated control.

1.2. Cytotoxic effect of cisplatin on renal, lung cancer and melanoma cells.

1.2.1. Cisplatin induced renal cell death.

This study characterized cisplatin-induced cell death in HK-2 cells in dose- and time-dependent manners. As apoptosis and necrosis have been shown to be two major modes of cisplatin-induced cell death, this study further identified modes of cell death in response to cisplatin treatment in HK-2 cells. Hoechst 33342 assay was performed for apoptosis detection, and propidium iodide staining assay was used for necrosis detection.

For dose-dependent study, cells were left untreated or treated with cisplatin at the concentrations of 5, 25, 50, 100, 200, and 300 μM. After 24 h, cell viability, apoptosis, and necrosis were determined.

The results suggested that in HK-2 cell cisplatin treatment caused a decrease in cell viability with approximately 50% of the cells remaining viable at cisplatin concentrations of 50 μM for 24 h (50.17 ± 1.88%) and the significant loss of cell viability caused by cisplatin was first observed at 25 μM (66.56 ± 3.32%). However, the dose-dependent decrease in HK-2 cell viability was observed only at the low concentration of cisplatin treatment (5-50 μM). (As indicated in figure 4.2A)

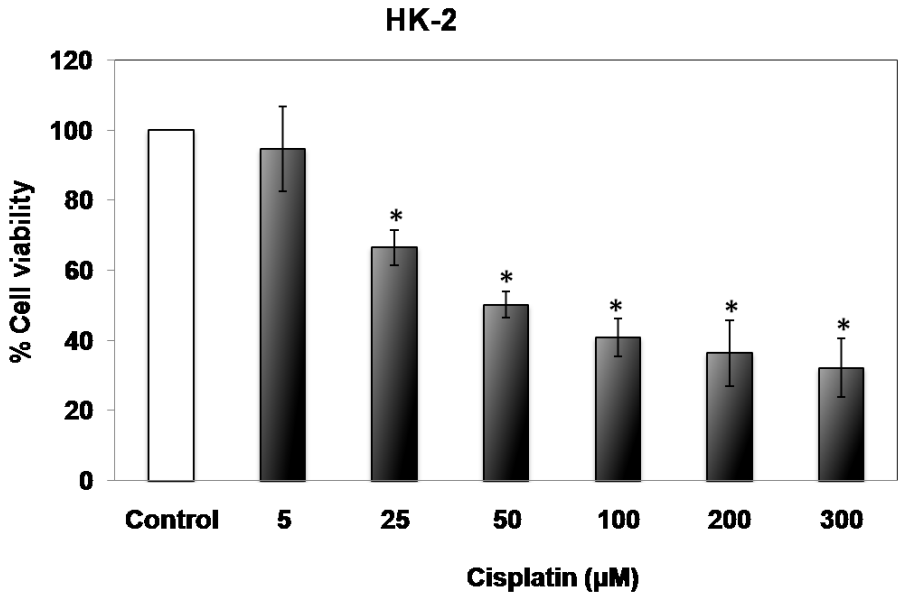
The nuclear staining results clearly showed that the number of apoptotic cells exhibiting intense nuclear fluorescence and DNA condensation and necrotic cells with positive PI staining were increased dose- and time-dependently in response to cisplatin treatment in HK-2 cell. At low concentration of cisplatin treatment (25 μM) the number of apoptosis was mainly detected and continuously increased as the dose was increased up to 100 μM. For PI staining assay, the number of necrosis was detected in response to cisplatin treatment in the dose starting from 50 μM and apparently presented at the concentration of 100 μM (figure 4.2B).

For time-dependent experiment, cells were left untreated or treated with with 50 μM cisplatin. After treatment, cell viability, apoptosis, and necrosis were evaluated at 0, 6, 15, and 24 h.

The result of cell viability in figure 4.3A indicated that cisplatin induced the reduction of cell viability in time-dependent manner with significant reduction as early as 15 h after 50 μM of cisplatin treatment (73.55 ± 2.82%) and the viability of treated cell continuously decreased until reached 50% at 24 h.

The nuclear staining results clearly showed that, apoptosis and necrosis were detected as early as 15 h after cisplatin treatment and the number of apoptosis and necrosis gradually increased until saturated at 24 h (figure 4.3B).

A



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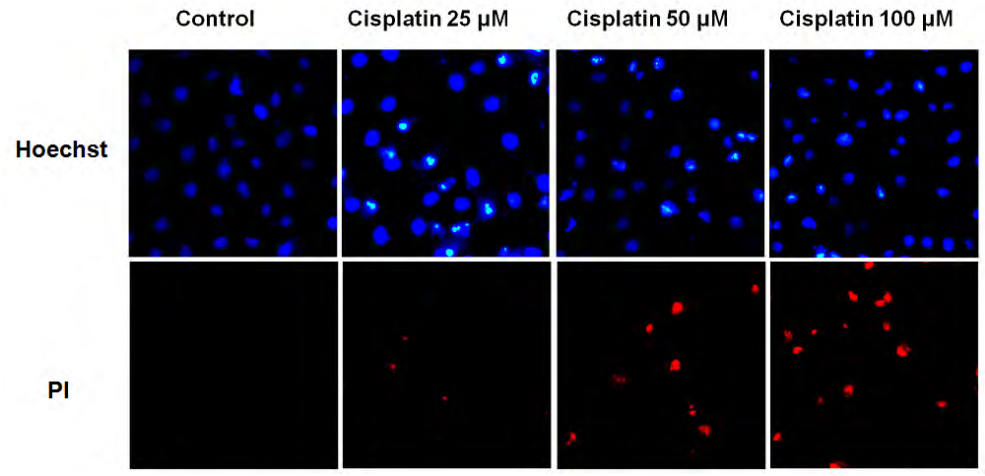
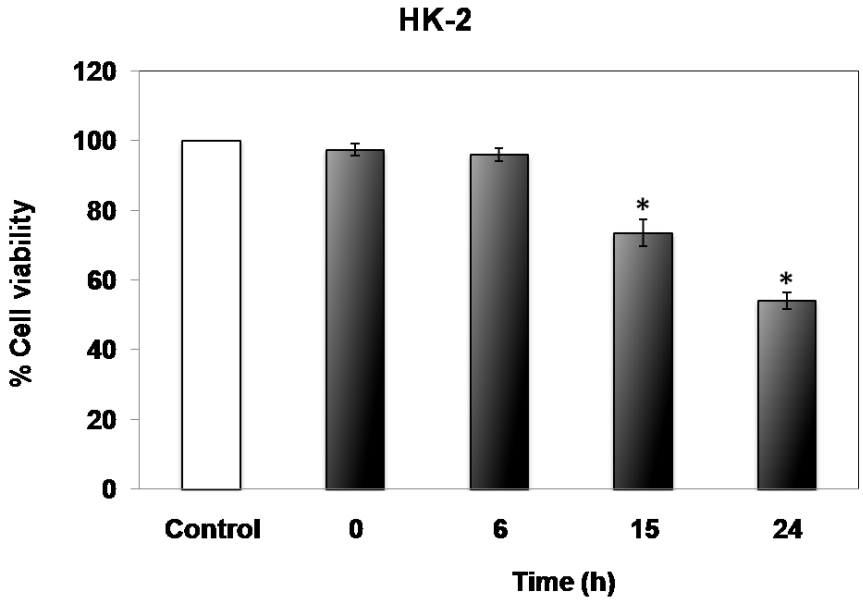


Figure 4.2 Cytotoxic effect of cisplatin on HK-2 cell in dose-dependent study. A) Cells were treated with various concentrations of cisplatin (0, 5, 25, 50, 100, 200, and 300 μM) for 24 h and cell viability was measured by MTT assay. Values were means ± S.E.M. of three-independent experiments, $*P < 0.05$ versus non-treated control. B) Apoptotic and necrotic cells were detected by Hoechst 33342 and propidium iodide co-staining assay and examined under a fluorescence microscope.

A



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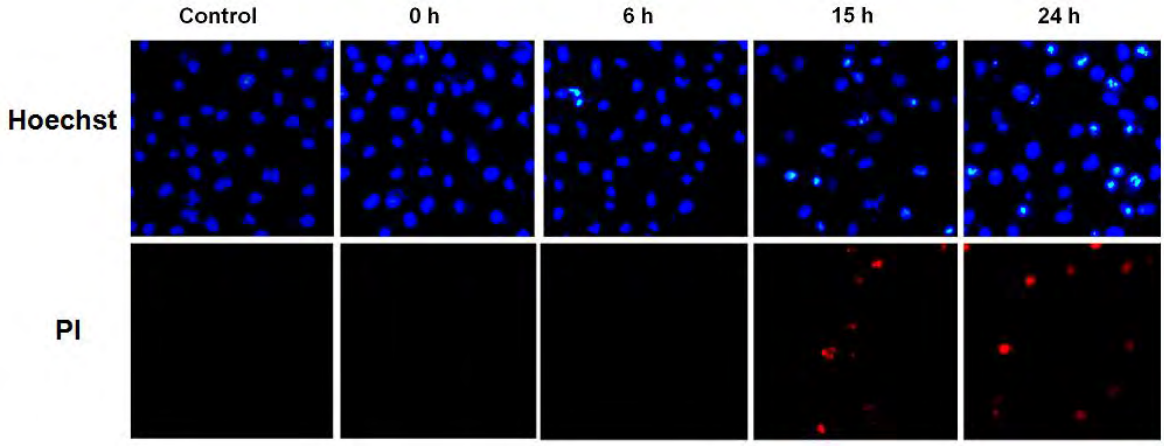


Figure 4.3 Cytotoxic effect of cisplatin on HK-2 cell in time-dependent study. A) Cells were treated with 50 μM of cisplatin and cell viability was measured by MTT assay at 0, 6, 15, and 24 h. Values were means ± S.E.M. of three-independent experiments, $*P < 0.05$ versus non-treated control. B) Apoptotic and necrotic cells were detected by Hoechst 33342 and propidium iodide co-staining assay and examined under a fluorescence microscope at the indicated time points.

1.2.2. Cisplatin induced human lung cancer cell death.

This study characterized cisplatin-induced cell death in H460 cells in dose- and time-dependent manners. For dose-dependent study, cells were left untreated or treated with cisplatin at the concentrations of 5, 25, 50, 100, 200, and 300 μM. Cell viability and mode of cell death were determined after 24 h.

The results showed that in H460 cell cisplatin treatment caused a dose-dependent decrease in cell viability, which significantly observed at 25 μM. (86.71 ± 2.69%) and the loss of cell viability continuously reduced as the dose was increased up with approximately 50% of the cells remaining viable at cisplatin concentrations of 100 μM for 24 h (51.84 ± 1.64%) (figure 4.4B).

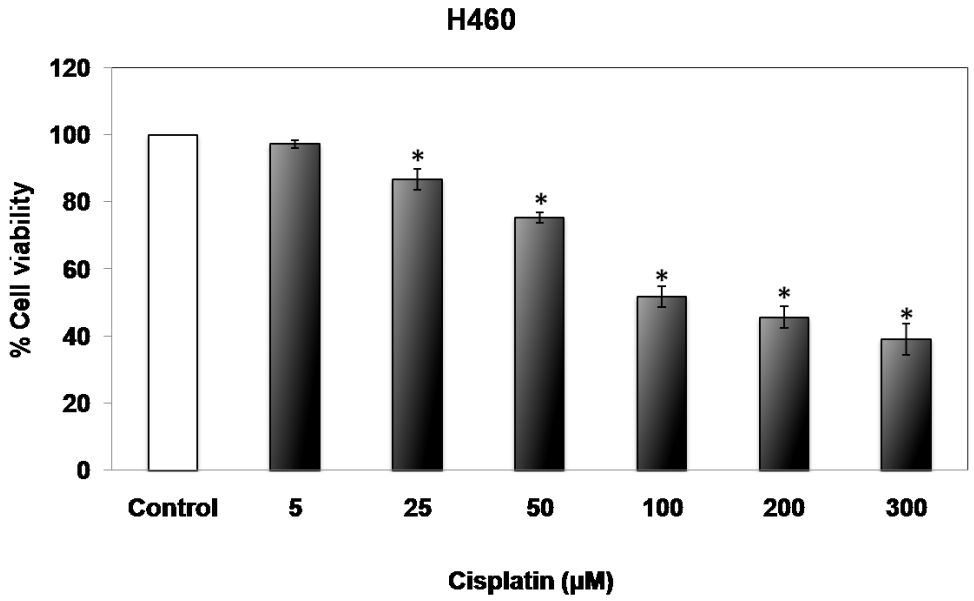
For apoptosis and necrosis assay in H460 cell, the results clearly showed that the number of apoptotic cells and necrotic cells were increased in response to cisplatin treatment in the dose-dependent manners. At low concentration of cisplatin treatment (25 μM) a few number of apoptosis and necrosis were detected; however, as the dose was increased up to 50 and 100 μM a large number of both apoptosis and necrosis were detected in response to cisplatin treatment (figure 4.4B).

For time-dependent experiment, after treatment the cells with 100 μM cisplatin cell viability, apoptosis, and necrosis detection were evaluated at 0, 6, 15, and 24 h.

The results demonstrated that cisplatin caused a decreased in H460 cell viability in time-dependent manner. The reduction of cell viability was significant as early as 15 h after treatment with 100 μM cisplatin (74.15 ± 3.16%) and continuously decreased until reached 50% of the viable cells at 24 h (figure 4.5A).

The nuclear staining results clearly showed that the number of apoptosis was detected as early as 15 h and gradually increased in response to time of cisplatin exposure, whereas necrosis clearly presented as early as 24 h after cisplatin treatment as shown in figure 4.5B.

A



B

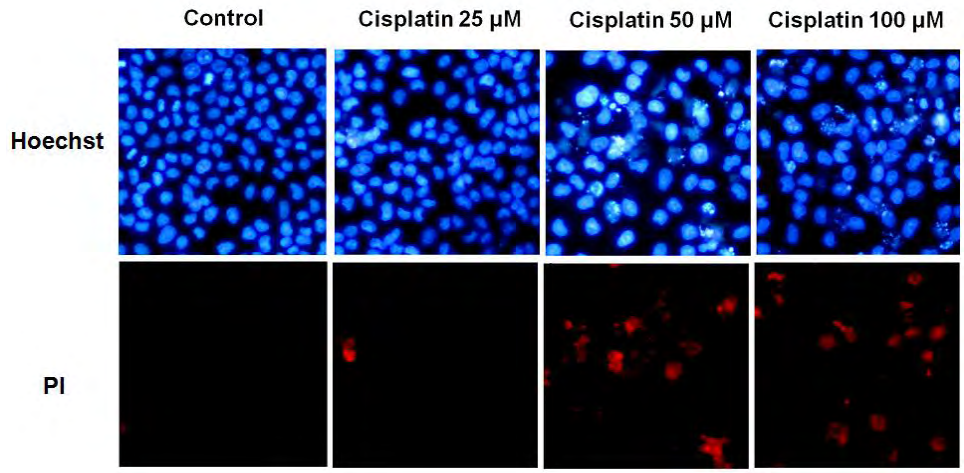
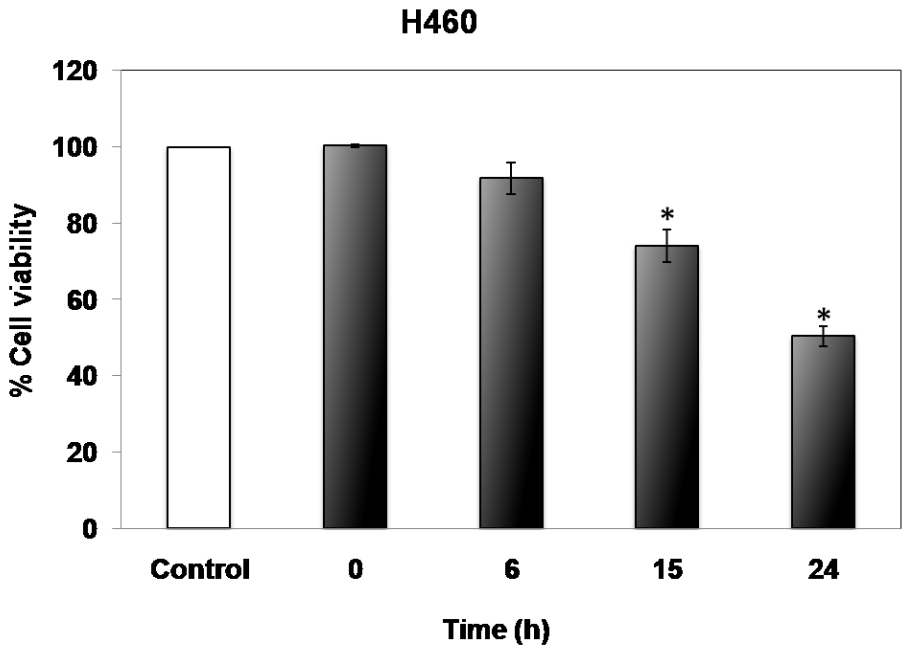


Figure 4.4 Cytotoxic effect of cisplatin on H460 cell in dose-dependent study. A) Cells were treated with various concentrations of cisplatin (0, 5, 25, 50, 100, 200, and 300 μM) for 24 h and cell viability was measured by MTT assay. Values were means ± S.E.M. of three-independent experiments, $*P < 0.05$ versus non-treated control. B) Apoptotic and necrotic cells were detected by Hoechst 33342 and propidium iodide co-staining assay and examined under a fluorescence microscope.

A



B

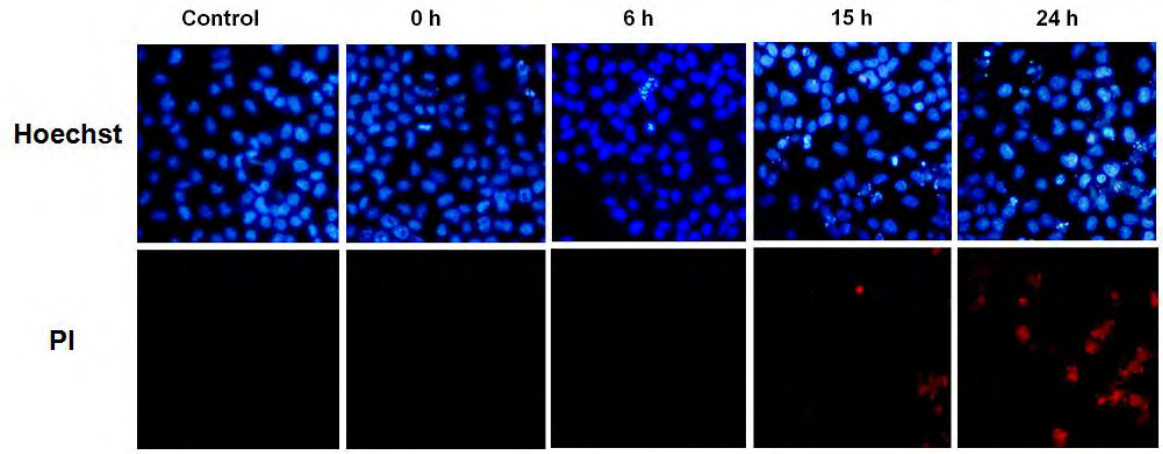


Figure 4.5 Cytotoxic effect of cisplatin on H460 cell in time-dependent study. A) Cells were treated with 100 μM of cisplatin and cell viability was measured by MTT assay at 0, 6, 15, and 24 h. Values were means ± S.E.M. of three-independent experiments, $*P < 0.05$ versus non-treated control. B) Apoptotic and necrotic cells were detected by Hoechst 33342 and propidium iodide co-staining assay and examined under a fluorescence microscope at the indicated time points.

1.2.3. Cisplatin induced human melanoma cell death.

This study characterized cisplatin-induced cell death in G361 cells in dose- and time-dependent manners. For dose-dependent study, cells were left untreated or treated with cisplatin at the concentrations of 5, 25, 50, 100, 200, and 300 μM. Cell viability and mode of cell death were determined after 24 h.

The results suggested that in G361 cell cisplatin treatment caused a dose-dependent decreased in cell viability, which was significantly observed at 5 μM (77.09 ± 2.68%) and percentage of viable cells was continuously reduced with approximately 50% of the cells remaining viable at cisplatin concentrations of 100 μM (50.72 ± 2.48%) as shown in figure 4.6A.

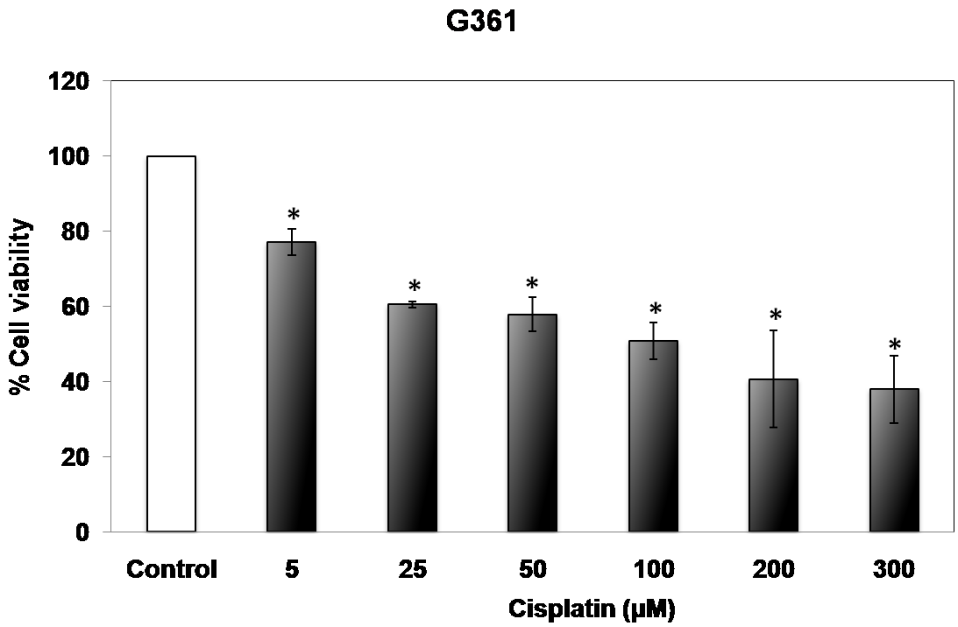
The results of apoptosis and necrosis assays clearly showed that the number of apoptotic cells was increased in response to cisplatin treatment in the dose-dependent manners. Notably, only Hoechst positive cells were observed in G361 cells. The number of apoptotic cells was detected in the concentration starting from 25 μM of cisplatin and continuously increased until the total number of cell death reached approximately 50% of viable cell at the concentration of 100 μM after 24 h of cisplatin treatment as indicated in figure 4.6B.

For time-dependent experiment, after treatment with 100 μM cisplatin cell viability, apoptosis, and necrosis were evaluated at 0, 6, 15, and 24 h.

The results indicated that the viability of melanoma cell was significantly reduced as early as 6 h after treatment with 100 μM cisplatin (72.46 ± 2.26%) and continuously decreased until reached approximately 50% at 24 h as shown in figure 4.7A)

The nuclear staining results clearly showed that apoptosis was detected as early as 15 h after 100 μM cisplatin treatment and gradually increased until the total number of cell death reached approximately 50% of viable cell at 24 h as shown in figure 4.7B.

A



B

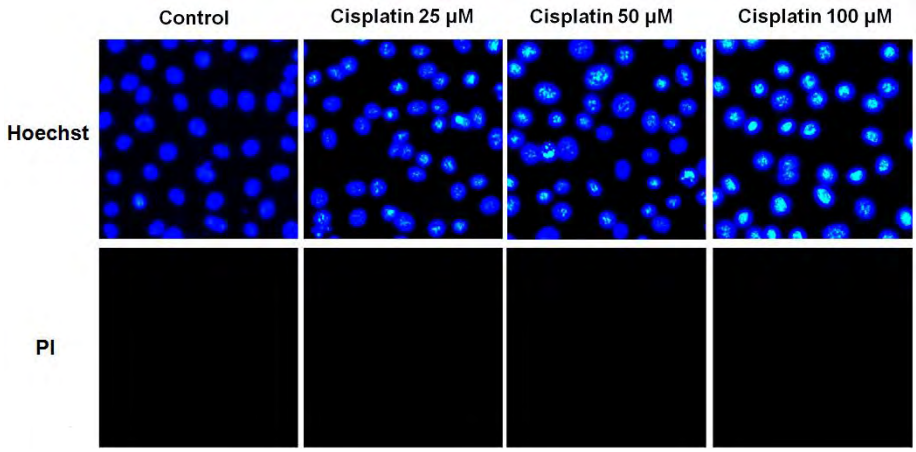
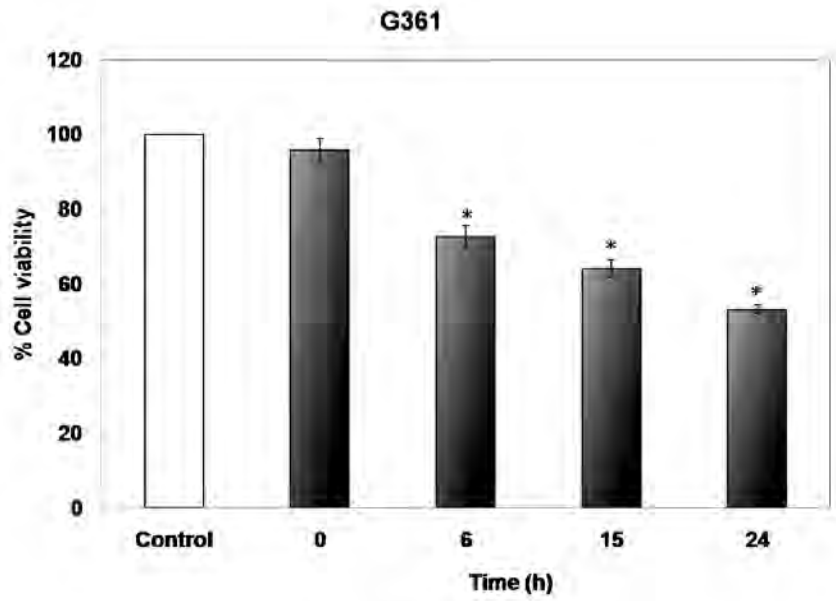


Figure 4.6 Cytotoxic effect of cisplatin on G361 cell in dose-dependent study. A) Cells were treated with various concentrations of cisplatin (0, 5, 25, 50, 100, 200, and 300 μM) for 24 h and cell viability was measured by MTT assay. Values were means ± S.E.M. of three-independent experiments, $*P < 0.05$ versus non-treated control. B) Apoptotic and necrotic cells were detected by Hoechst 33342 and propidium iodide co-staining assay and examined under a fluorescence microscope.

A



B

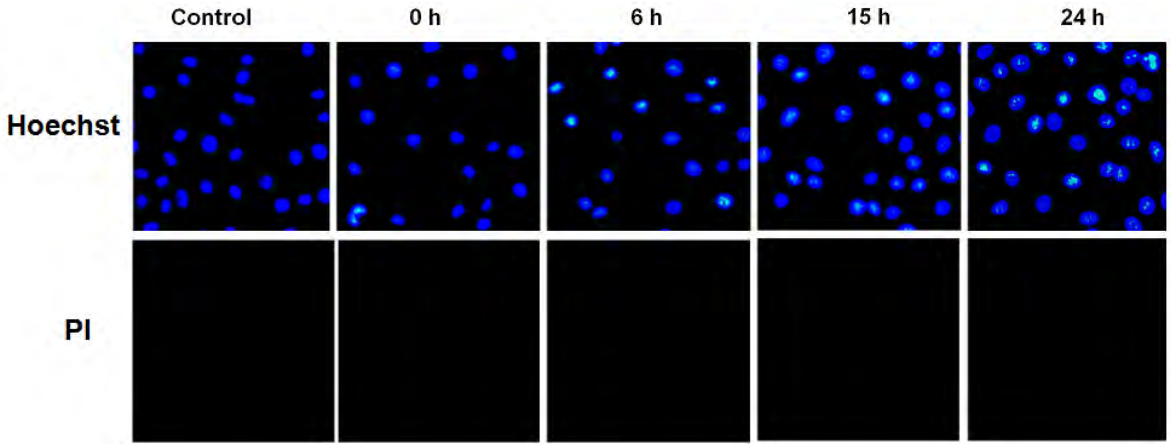


Figure 4.7 Cytotoxic effect of cisplatin on G361 cell in time-dependent study. A) Cells were treated with 100 μM of cisplatin and cell viability was measured by MTT assay at 0, 6, 15, and 24 h. Values were means ± S.E.M. of three-independent experiments, $*P < 0.05$ versus non-treated control. B) Apoptotic and necrotic cells were detected by Hoechst 33342 and propidium iodide co-staining assay and examined under a fluorescence microscope at the indicated time points.

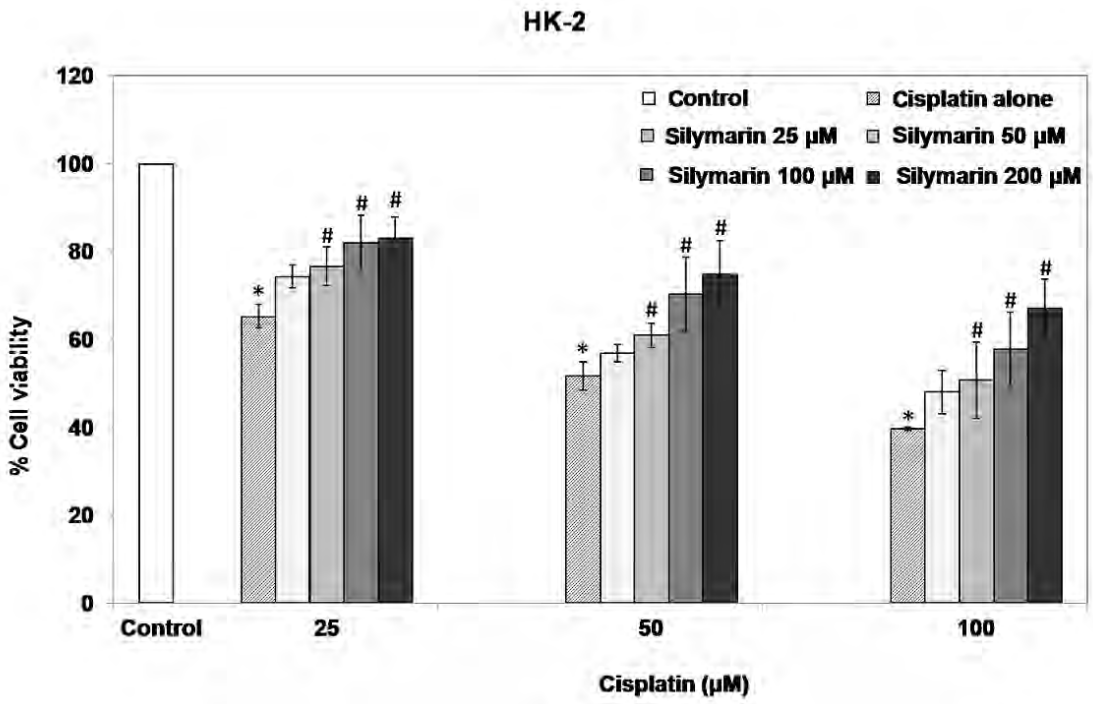
1.3. Silymarin protected renal cells from cisplatin-induced cytotoxicity.

To investigate the potential of silymarin in protection of cisplatin-induced renal cell death, HK-2 cells were pre-incubated with various concentrations of silymarin (25, 50,100, and 200 μM.) for 1 h and treated with various concentrations of cisplatin (25, 50, and 100 μM). After 24 h incubation, cell viability, apoptosis, and necrosis were determined.

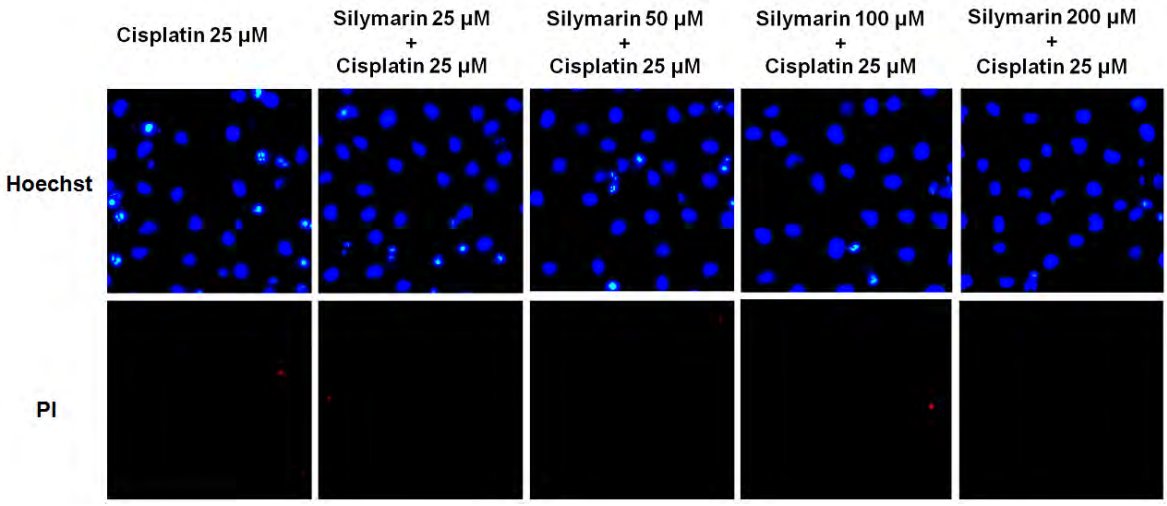
The results indicated that treatment of the cells with cisplatin alone at the concentrations of 25, 50, and 100 μM caused a reduction in cell survival approximately 40, 50, and 60%, respectively. However, pretreatment of HK-2 cells with silymarin (50-200 μM) significantly prevented the loss of cell viability induced by cisplatin while pretreatment with 25 μM silymarin had minimal effect on the cisplatin-induced reduction of HK-2 cell viability as shown in figure 4.8A**.**

Furthermore, the results of Hoechst 33342 and PI assays also supported that silymarin succeeded in decreasing both apoptosis and necrosis induced by cisplatin in HK-2 cells. Treatment of the cells with cisplatin alone at the concentrations of 25 μM resulted in only apoptotic cell death while at the concentrations of 50 and 100 μM caused the increased in both apoptotic and necrotic cell death. Addition of silymarin (25, 50,100, and 200 μM) dramatically reduced cisplatin-induced apoptosis and completely eradicated necrosis induced by cisplatin as indicated in figure 4.8B-D. These results provided the evidence that silymarin was able to ameliorate cisplatin-induced HK-2 cell damage.

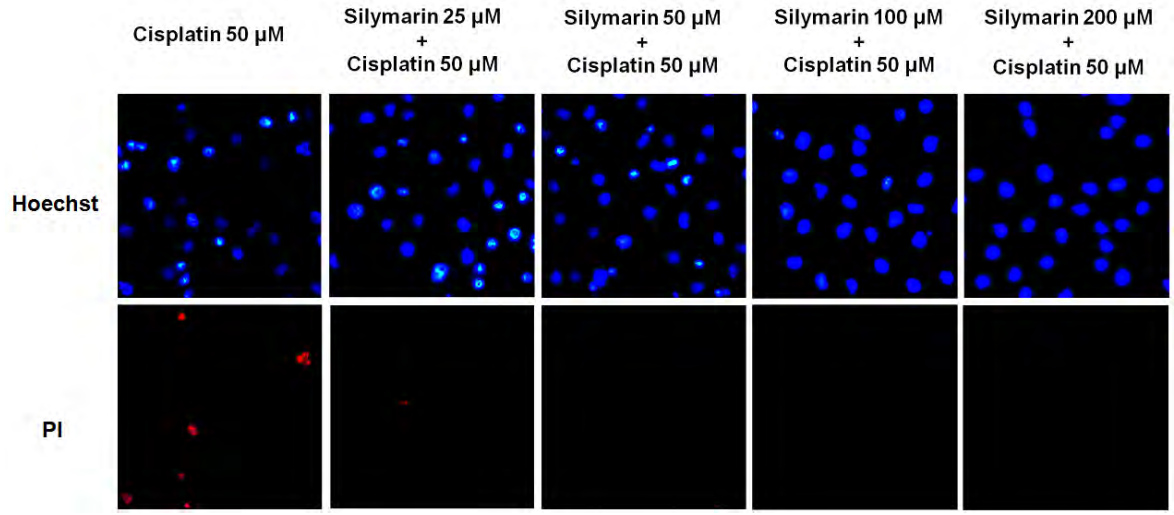
A


HK-2
Control
Cisplatin alone
Silymarin 25 μM
Silymarin 50 μM
Silymarin 100 μM
Silymarin 200 μM
% Cell viability
120
100
80
60
40
20
0
Control
25
50
100
Cisplatin (μM)

B


Cisplatin 25 μM
Silymarin 25 μM + Cisplatin 25 μM
Silymarin 50 μM + Cisplatin 25 μM
Silymarin 100 μM + Cisplatin 25 μM
Silymarin 200 μM + Cisplatin 25 μM
Hoechst
PI

C



D

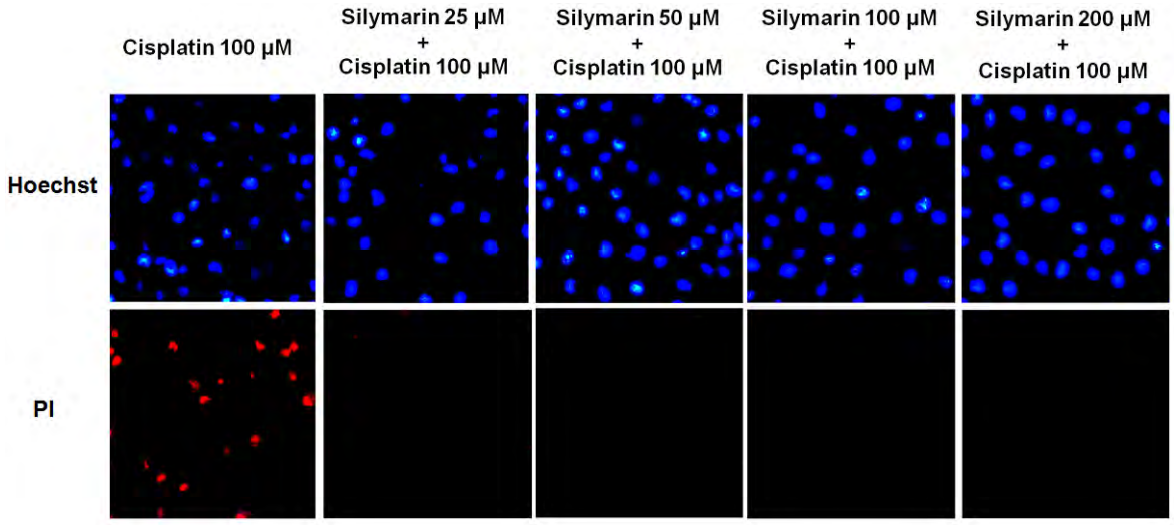


Figure 4.8 Protective effect of silymarin on cisplatin-induced HK-2 cell death. A) Cells were pretreated with various concentrations of silymarin (25, 50,100, and 200 µM) for 1 h prior to 24-h cisplatin exposure (25, 50, and 100 µM). Cell viability was measured by MTT assay. Values were means ± S.E.M. of three-independent experiments, $*P < 0.05$ versus non-treated control, and $\#P < 0.05$ versus cisplatin-treated control. B-D) Nuclear morphology of apoptosis and necrosis detected by Hoechst 33342 and PI assay.

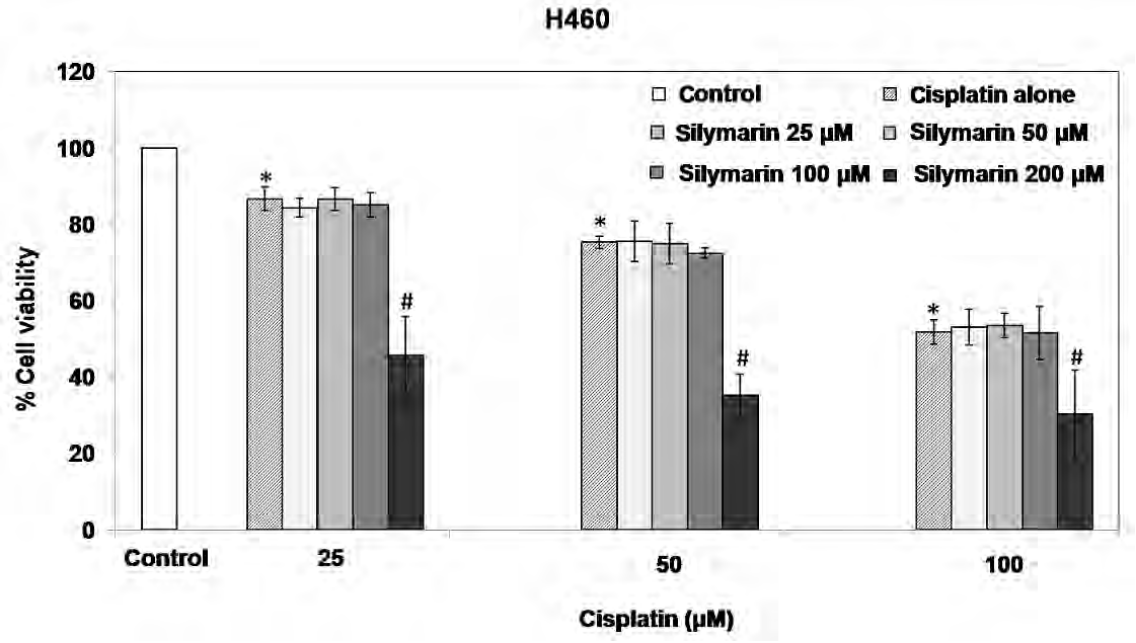
1.4. Silymarin caused minimal effect on cisplatin-induced lung cancer cell death.

This study further investigated whether silymarin could reduce efficacy of cisplatin in the induction of human lung cancer cell death. Cells were left untreated or pretreated with various concentrations of silymarin (25, 50,100, and 200 μM.) for 1 h and treated with various concentrations of cisplatin (25, 50, and 100 μM). After 24 h of incubation, cell viability, apoptosis, and necrosis were determined.

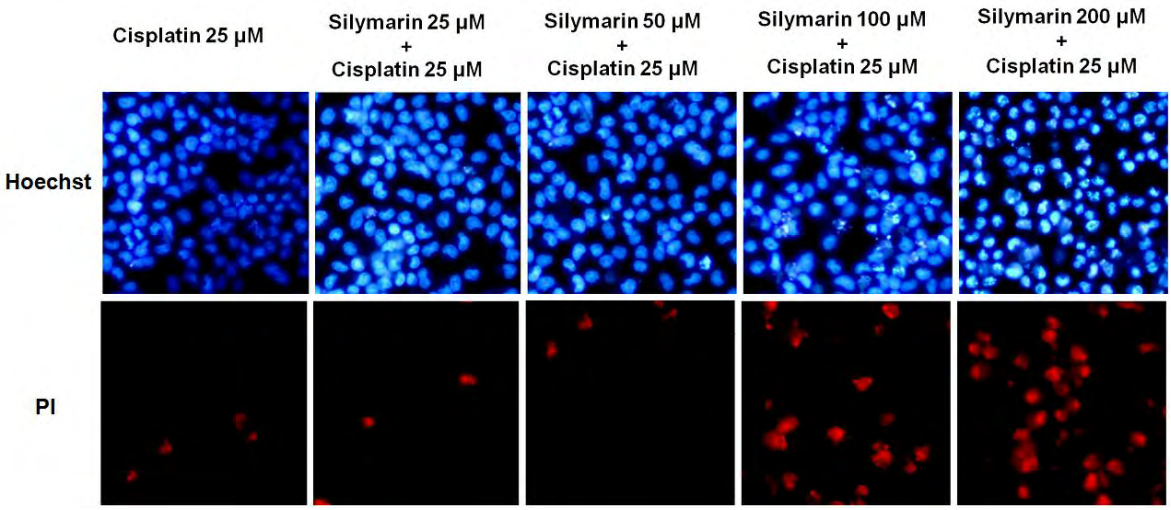
The results clearly showed that silymarin at the concentrations of 100 μM and lower caused no significant change on lung cancer cell viability after cisplatin treatment. As the dose of silymarin was increased up to 200 μM, silymarin dramatically enhanced the cytotoxic effect of cisplatin with approximately a 2-fold reduction as compared to the cisplatin-treated control (figure 4.9A).

The result of Hoechst 33342 and PI staining assays confirmed that in H460 cells, pretreatment of low concentrations silymarin caused no significant change in mode and quantity of cell death in response to cisplatin treatment. Consistently, at 200 μM silymarin, the number of both apoptotic and necrotic cells significantly increased (figure 4.9B-D).

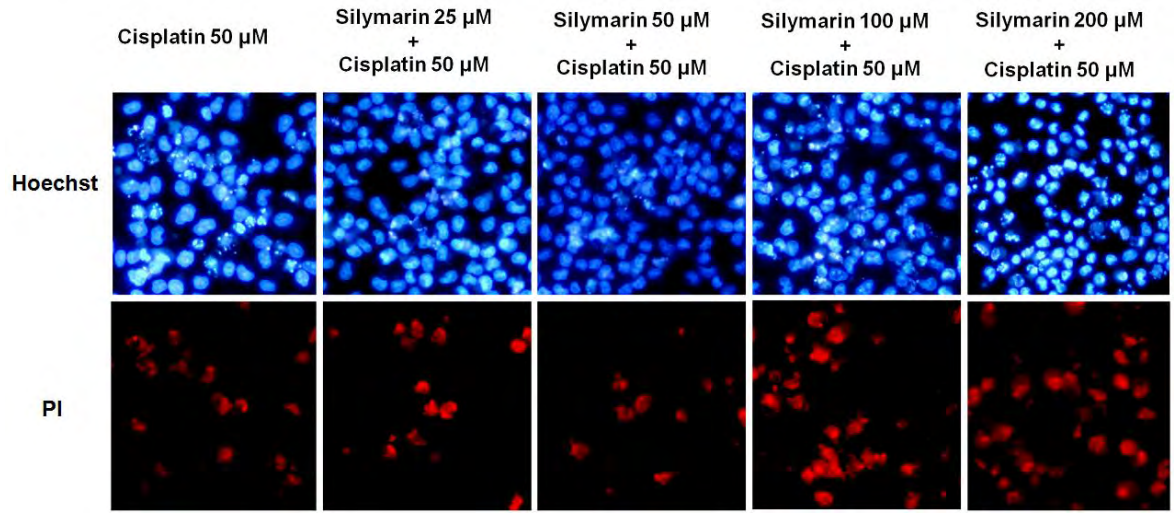
A


H460
120
100
80
60
40
20
0
% Cell viability
Control
Cisplatin alone
Silymarin 25 µM
Silymarin 50 µM
Silymarin 100 µM
Silymarin 200 µM
*
#
Control
25
50
100
Cisplatin (µM)

B


Cisplatin 25 µM
Silymarin 25 µM + Cisplatin 25 µM
Silymarin 50 µM + Cisplatin 25 µM
Silymarin 100 µM + Cisplatin 25 µM
Silymarin 200 µM + Cisplatin 25 µM
Hoechst
PI

C



D

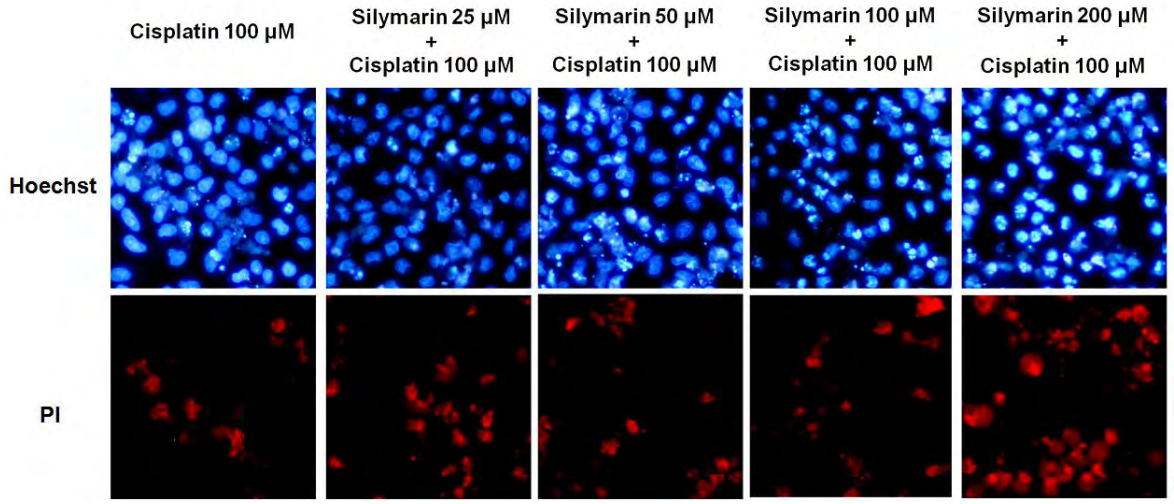


Figure 4.9 Effect of silymarin on cisplatin-induced H460 cell death. A) Cells were pretreated with various concentrations of silymarin (25, 50,100, and 200 μM.) for 1 h prior to 24-h cisplatin treatment (25, 50, and 100 μM). Cell viability was measured by MTT assay. Values were means ± S.E.M. of three-independent experiments, $*P < 0.05$ versus non-treated control, and $\#P < 0.05$ versus cisplatin-treated control. B-D) Nuclear morphology of apoptosis and necrosis detected by Hoechst 33342 and PI assay.

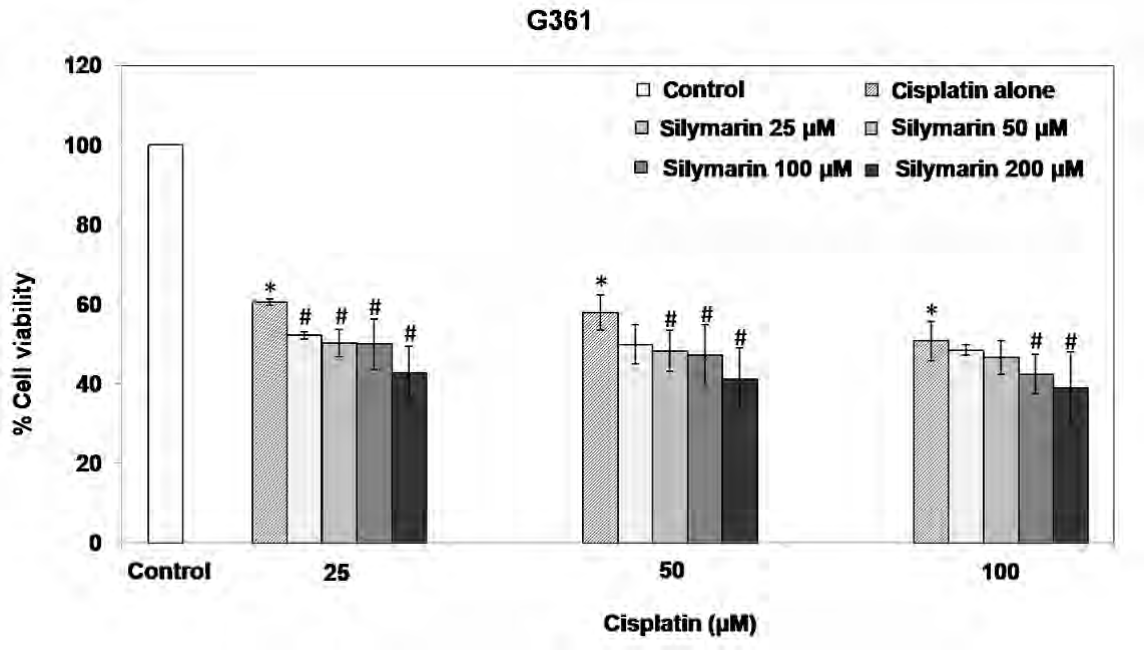
1.5. Silymarin potentiated cisplatin-induced melanoma cell death

This study further investigated whether silymarin could reduce efficacy of cisplatin in the induction of human melanoma cell death. Cells were left untreated or pretreated with various concentrations of silymarin (25, 50,100, and 200 μM.) for 1 h and treated with various concentrations of cisplatin (25, 50, and 100 μM). After 24 h of incubation, cell viability, apoptosis, and necrosis were determined.

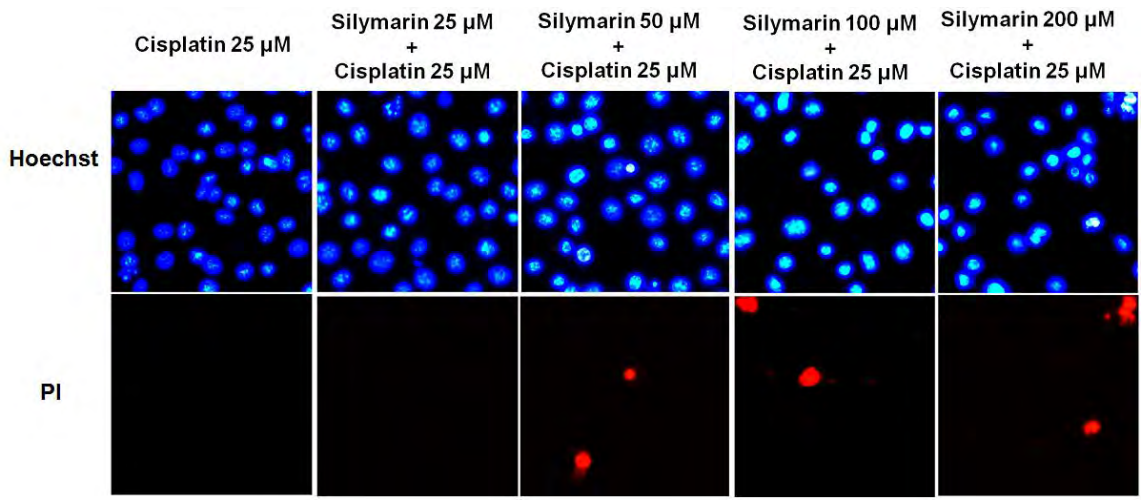
In melanoma cells, treatment with cisplatin alone caused cell survival reduction by approximately 40-50% at the concentration range of 25-100 μM. Addition of silymarin in cisplatin-treated melanoma cells significantly potentiated cisplatin-induced toxicity in a concentration-dependent manner (figure 4.10A).

The result of Hoechst 33342 and PI staining assays confirmed that unlike H460 cells, co-treating the G361 cells with silymarin and cisplatin mainly caused an increase in apoptotic cell death as indicated in figure 4.10B-D. These findings suggested that the addition of silymarin in cisplatin treatment not only protected against human renal cell damage, but also, at least, either did not interfere with the cytotoxic mechanisms of cisplatin or enhanced the response of cancer cells to cisplatin-induced cell death.

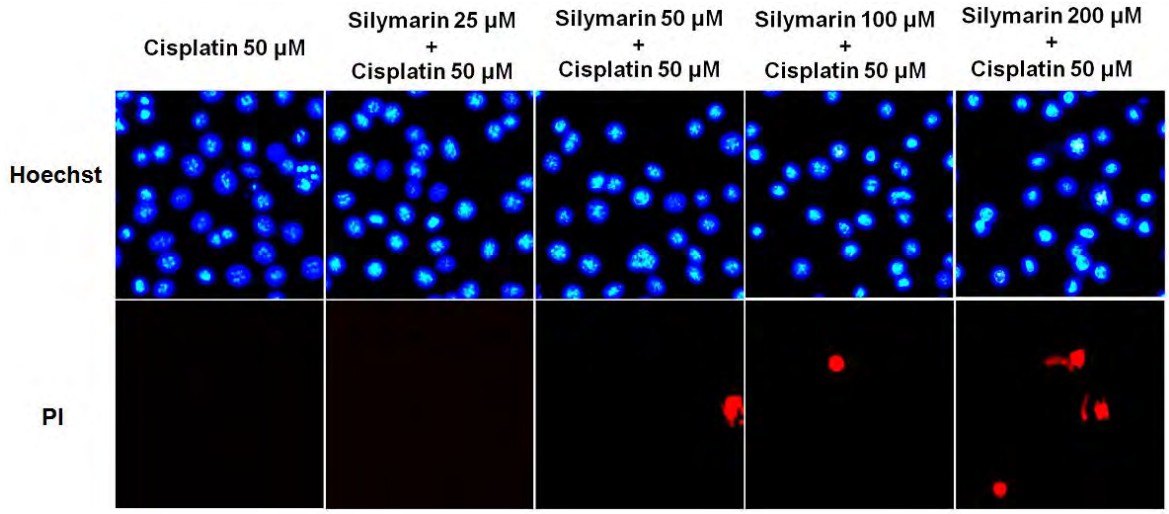
A


G361
Control
Cisplatin alone
Silymarin 25 μM
Silymarin 50 μM
Silymarin 100 μM
Silymarin 200 μM
% Cell viability
120
100
80
60
40
20
0
Control
25
50
100
Cisplatin (μM)

B


Cisplatin 25 μM
Silymarin 25 μM + Cisplatin 25 μM
Silymarin 50 μM + Cisplatin 25 μM
Silymarin 100 μM + Cisplatin 25 μM
Silymarin 200 μM + Cisplatin 25 μM
Hoechst
PI

C



D

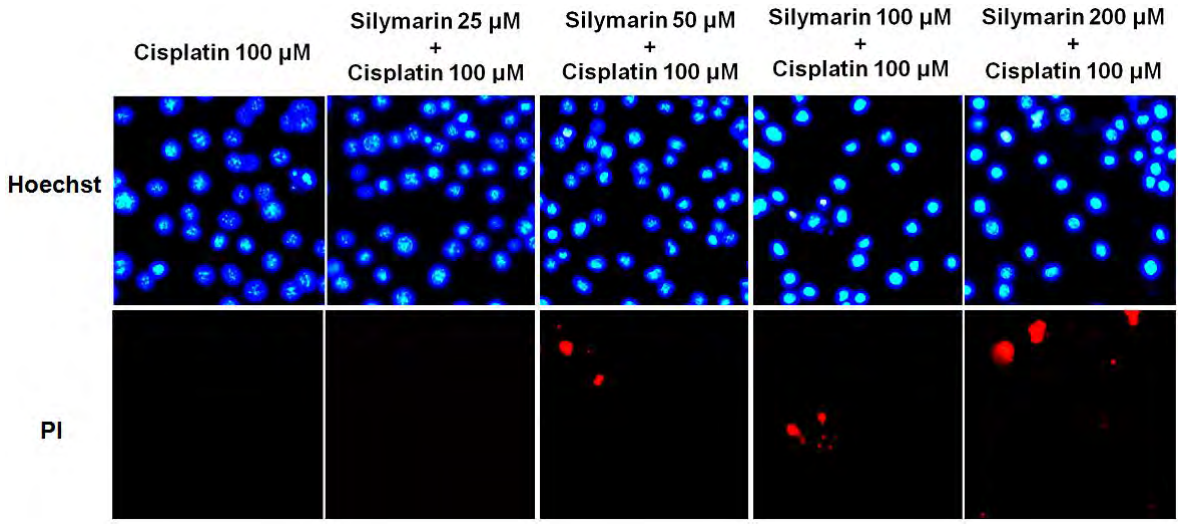


Figure 4.10 Effect of silymarin on cisplatin-induced G361 cell death. A) Cells were pretreated with various concentrations of silymarin (25, 50,100, and 200 μM.) for 1 h prior to 24-h cisplatin treatment (25, 50, and 100 μM). Cell viability was measured by MTT assay. Values were means ± S.E.M. of three-independent experiments, $*P < 0.05$ versus non-treated control, and $\#P < 0.05$ versus cisplatin-treated control. B-D) Nuclear morphology of apoptosis and necrosis detected by Hoechst 33342 and PI assay.

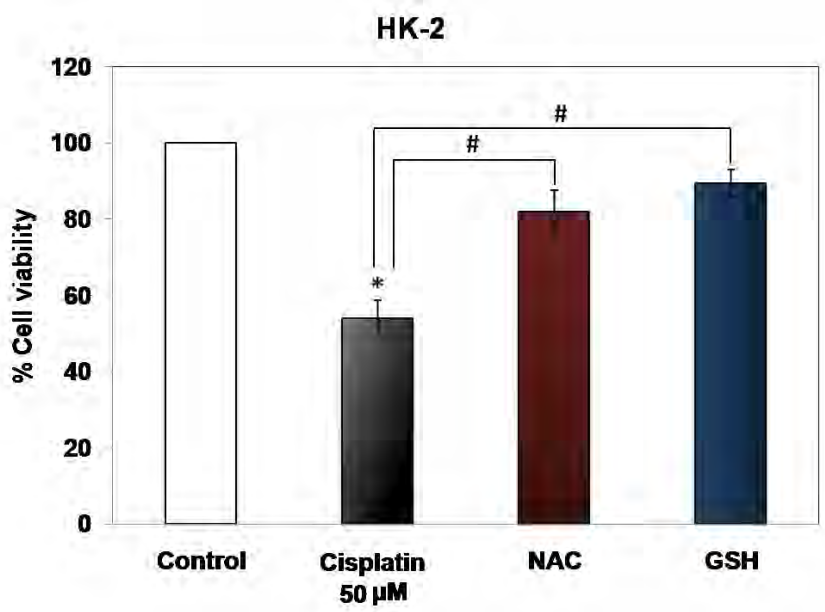
2. Effect of silymarin on ROS generated by cisplatin and cisplatin-induced cytotoxicity

2.1. Cisplatin induced renal cell death via an ROS-dependent mechanism.

Since previous studies have shown that cisplatin induced cell death via an ROS-dependent mechanism (Miyajima *et al*., 1997; Wu, Muldoon, and Neuwelt, 2005; Chanvorachote *et al*., 2006; Bragado *et al*., 2007), this study also clarifed cisplatin-induced ROS could play a key role in renal cell damage by evaluating the ROS inhibition effect of known antioxidants *N*-acetylcysteine (NAC) and reduced glutathione (GSH) on cisplatin-induced renal cell death. Cells were pre-incubated with NAC (5 μM), GSH (5 μM), or left untreated for 1 h and treated with 50 μM cisplatin. Then, cell viability, apoptosis, and necrosis were analyzed after 24 h incubation.

The results showed that 50 μM cisplatin treatment alone caused approximately 50% reduction of cell survival; however, the addition of 5 mM NAC and GSH dramatically attenuated the cisplatin cytotoxicity (figure 4.11A). Consistently, Hoechst 33342 staining results indicated that the addition of antioxidants significantly reduced both apoptosis and necrosis induced by cisplatin (figure 4.11B).

A



B

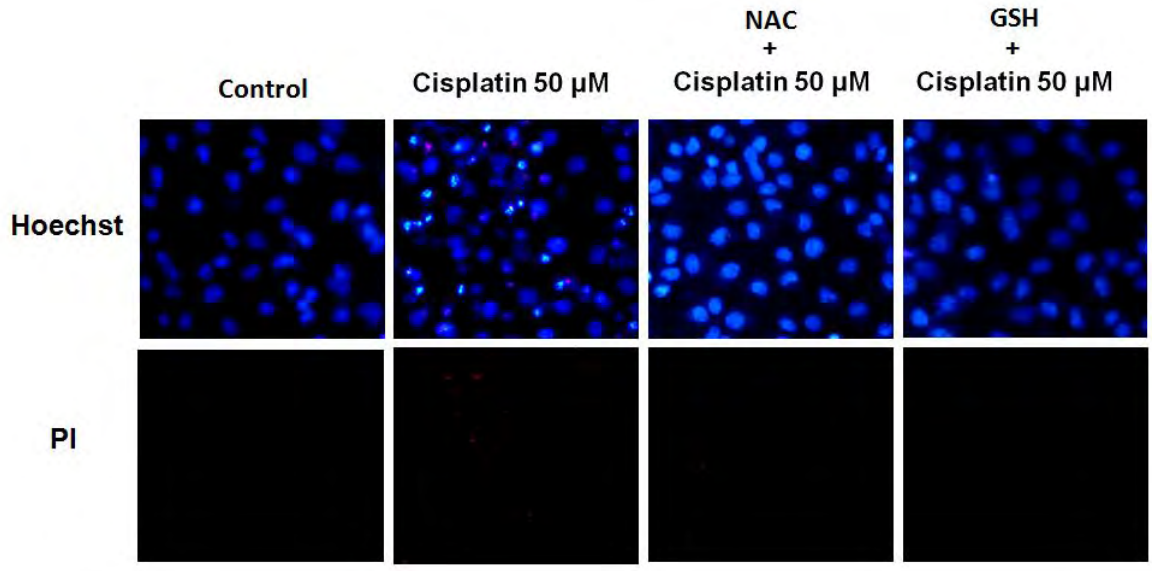


Figure 4.11 Effect of antioxidants on cisplatin-induced cell death in HK-2. A) Cells were pretreated with various antioxidants; NAC (5 µM) and GSH (5 µM) for 1 h prior to 50 µM cisplatin treatment. A) After cisplatin treatment for 24 h, Cell viability was determined by MTT assay. Values were means on triplicate samples ± S.E.M. $*P < 0.05$ versus non-treated control and $\#P < 0.05$ versus cisplatin-treated control. B) Nuclear morphology of apoptosis and necrosis detected by Hoechst 33342 and PI assay.

2.2. Cisplatin induced specific ROS generation in HK-2, H460, and G361 cells

To identify specific ROS generation induced by cisplatin in different cells, this study first evaluated intracellular ROS induction in response to cisplatin treatment in renal and cancer cells using dihydrodichloro-fluorescein diacetate (H_2DCF-DA) as a fluorescent probe for total ROS and using dihydroethidium (DHE) as a selective probe for intracellular superoxide anion radical.

The results of time-dependent study clearly showed that in all cells intracellular ROS levels were differently increased as early as 1 h in response to cisplatin treatment (IC_{50}) as shown in figure 4.12A-C.

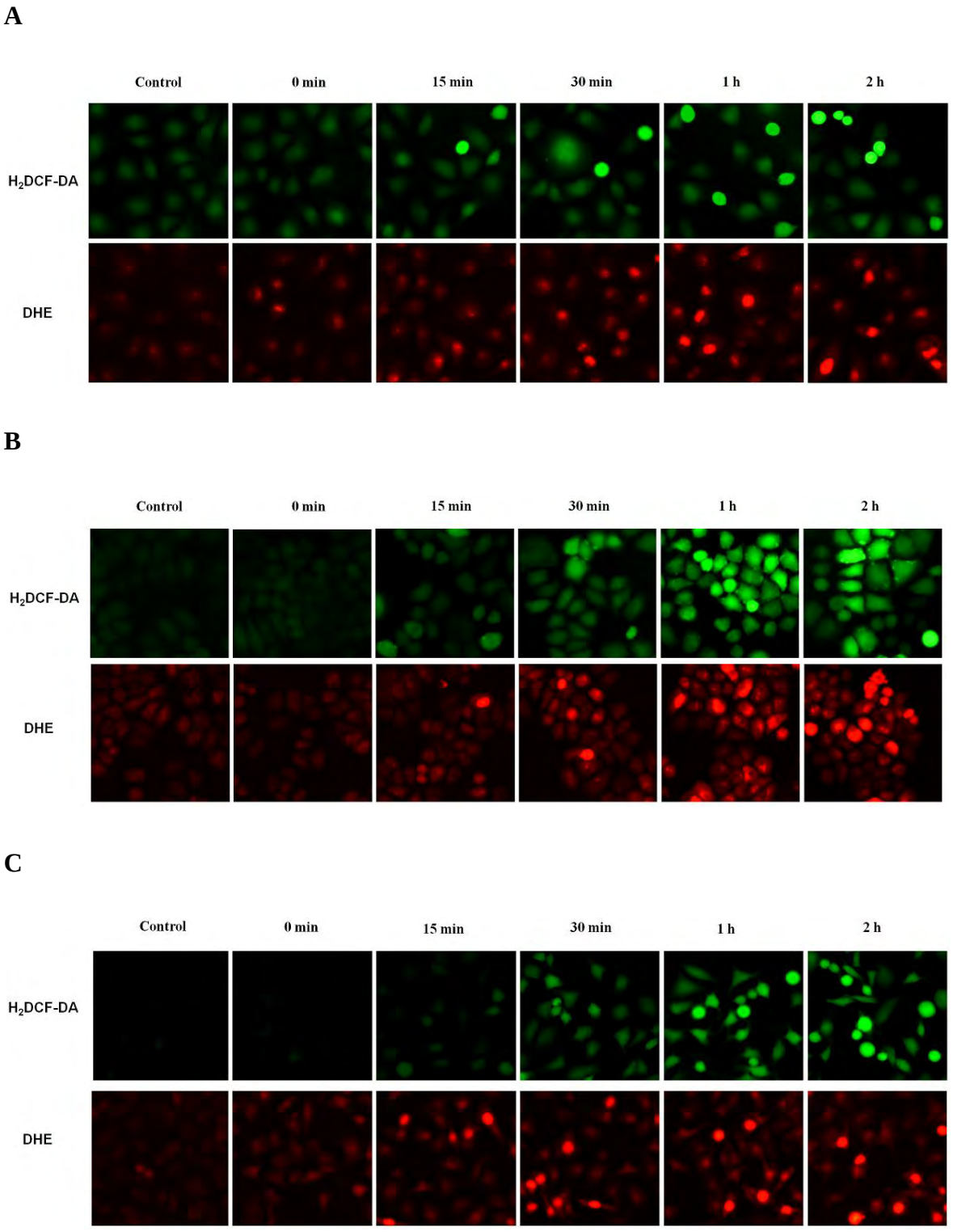


Figure 4.12 Cisplatin induced ROS generation in A) HK-2, B) H460, and C) G361 cells. Cells were treated with cisplatin (IC_{50}) and intracellular ROS was detected under fluorescence microscope at 0, 15, 30, 60, and 120 min.

2.2.1. Specific ROS generation in cisplatin-induced renal HK-2 cell damage.

To identify the specific ROS generation induced by cisplatin in HK-2 cells, cells were pre-incubated with various specific ROS scavengers, such as MnTBAP (superoxide anion radical scavenger), catalase (hydrogen peroxide scavenger), and deferoxamine (hydroxyl radical inhibitor) for 1 h prior to 50 μM of cisplatin treatment. After incubation with cisplatin for 1 h, intracellular ROS were detected by H_2DCF-DA and intracellular superoxide anion radical was detected by DHE.

The fluorescence result indicated that the fluorescence intensity of ROS was markedly increased after 50 μM cisplatin treatment. However, the addition of MnTBAP, catalase, and deferoxamine dramatically decreased the fluorescence intensity of ROS originated from cisplatin treatment, suggesting that superoxide anion radical, hydrogen peroxide, and hydroxyl radical were produced in response to cisplatin treatment as shown in figure 4.13.

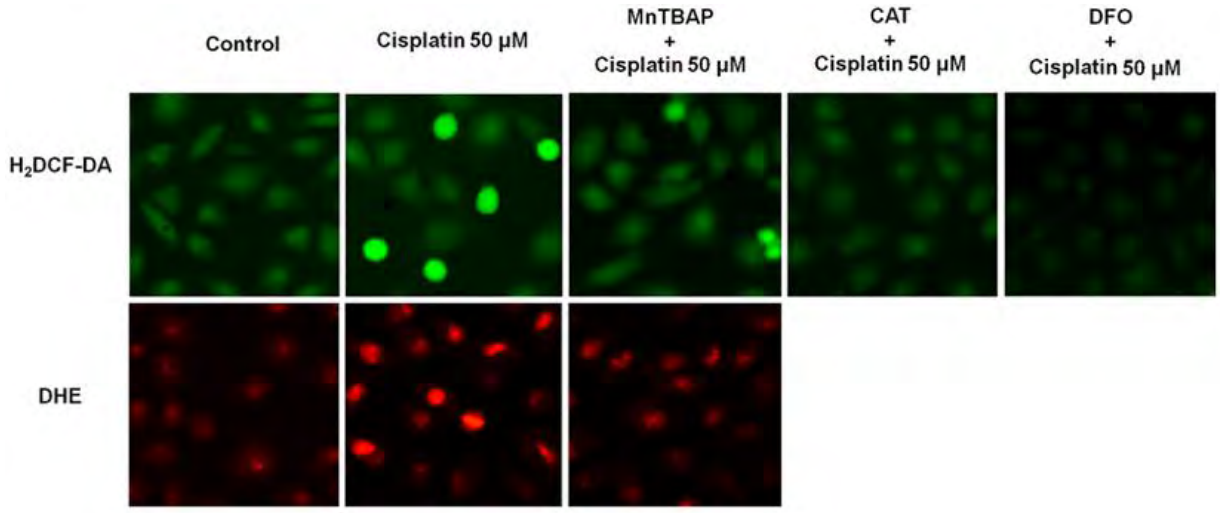


Figure 4.13 Effect of ROS scavengers on cisplatin-induced intracellular ROS level in HK-2 cell. Cells were pretreated with various specific ROS scavengers; MnTBAP (50 μM), catalase (5000U/ml), and DFO (1 mM) for 1 h prior to cisplatin treatment (50 μM). After cisplatin treatment for 1 h, intracellular ROS levels were evaluated under a fluorescence microscope with H_2DCF-DA or DHE.

2.2.2. Specific ROS generation in cisplatin-induced human lung cancer cell damage.

To identify the specific ROS generation induced by cisplatin in H460 cells, cells were pre-incubated with various specific ROS scavengers, such as MnTBAP (superoxide anion radical scavenger), catalase (hydrogen peroxide scavenger), and deferoxamine (hydroxyl radical inhibitor) for 1 h prior to 100 μM of cisplatin treatment. After incubation with cisplatin for 1 h, intracellular ROS were detected by H_2DCF-DA and Intracellular superoxide anion radical was similarly detected by DHE.

In H460 cells, the fluorescence result indicated that the fluorescence intensity of ROS was markedly increased after expose to 100 μM cisplatin for 1 h. However, the addition of MnTBAP, catalase, and deferoxamine dramatically reduced the fluorescence intensity of ROS caused by cisplatin, suggesting that superoxide anion radical, hydrogen peroxide, and hydroxyl radical were produced in response to cisplatin treatment (figure 4.14).

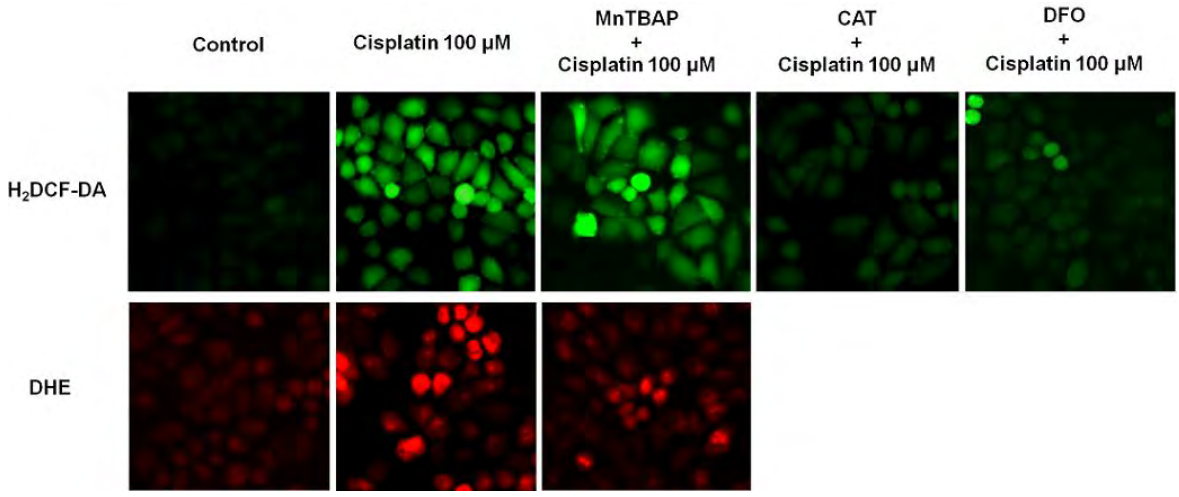


Figure 4.14 Effect of ROS scavengers on cisplatin-induced intracellular ROS level in H460 cell. Cells were pretreated with various specific ROS scavengers; MnTBAP (50 μM), catalase (5000U/ml), and DFO (1 mM) for 1 h prior to cisplatin treatment (100 μM). After cisplatin treatment for 1 h, intracellular ROS levels were evaluated under a fluorescence microscope with H_2DCF-DA or DHE.

2.2.3. Specific ROS generation in cisplatin-induced human melanoma cell damage.

To identify the specific ROS generation induced by cisplatin in G361 cells, cells were pre-incubated with various specific ROS scavengers, such as MnTBAP (superoxide anion radical scavenger), catalase (hydrogen peroxide scavenger), and deferoxamine (hydroxyl radical inhibitor) for 1 h prior to 100 μM of cisplatin treatment. After incubation with cisplatin for 1 h, intracellular ROS were detected by H_2DCF-DA and intracellular superoxide anion radical was similarly detected by DHE.

Similar to HK-2 and H460 results, in G361 cell the fluorescence intensity of ROS was markedly increased after treatment with 100 μM cisplatin. However, the addition of MnTBAP, catalase, and deferoxamine dramatically decreased the fluorescence intensity of ROS caused by cisplatin, suggesting that superoxide anion radical, hydrogen peroxide and hydroxyl radical were produced in response to cisplatin treatment (figure 4.15).

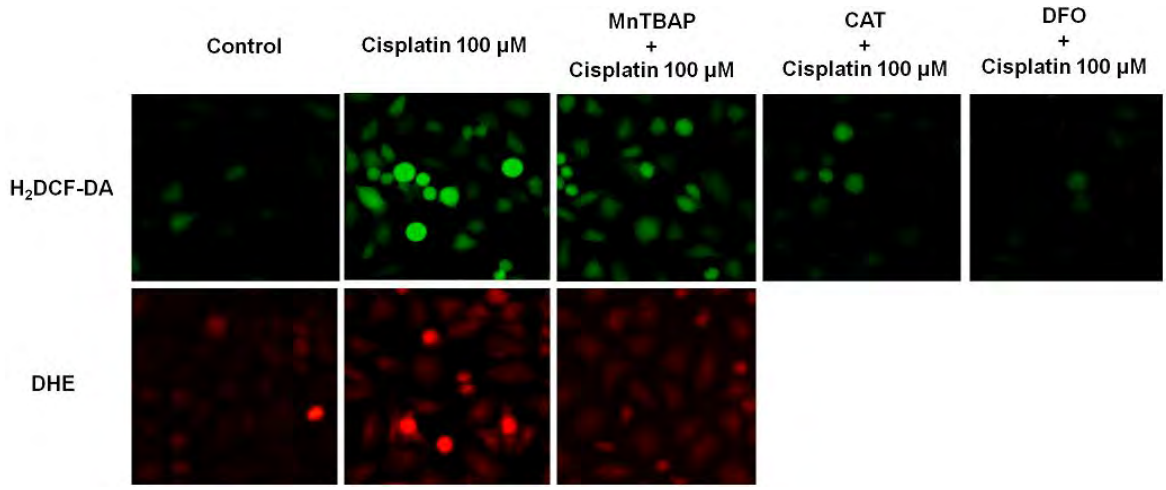


Figure 4.15 Effect of ROS scavengers on cisplatin-induced intracellular ROS level in G361 cell. Cells were pretreated with various specific ROS scavengers; MnTBAP (50 μM), catalase (5000U/ml), and DFO (1 mM) for 1 h prior to cisplatin treatment (100 μM). After cisplatin treatment for 1 h, intracellular ROS levels were evaluated under a fluorescence microscope with H_2DCF-DA or DHE.

2.3. Hydrogen peroxide and hydroxyl radicals play a role on cytotoxic mode of cisplatin.

2.3.1. Hydrogen peroxide and hydroxyl radicals were the key ROS in cisplatin-induced renal HK-2 cell damage.

To evaluate the key ROS which were responsible for cisplatin cytotoxicity in HK-2 cells, cells were pre-incubated with various specific ROS scavengers, such as MnTBAP (superoxide anion radical scavenger), catalase (hydrogen peroxide scavenger), and deferoxamine (hydroxyl radical inhibitor) for 1 h prior to 50 μM of cisplatin treatment. After incubation with cisplatin for 24 h, cell viability was determined by MTT assay.

The cell viability result indicated that 50 μM cisplatin caused the loss of HK-2 cell viability approximately 50% of cell remaining viable; however, the addition of catalase and deferoxamine pretreatment were significantly reversed cisplatin-induced renal cell death (56.29 ± 2.14% and 64.59 ± 2.55%, respectively) while no significant change was observed in MnTBAP pre-treated group (46.69 ± 1.56%), suggesting that hydrogen peroxide and hydroxyl radical were key mediators in cisplatin-induced renal cell damage (figure 4.16).

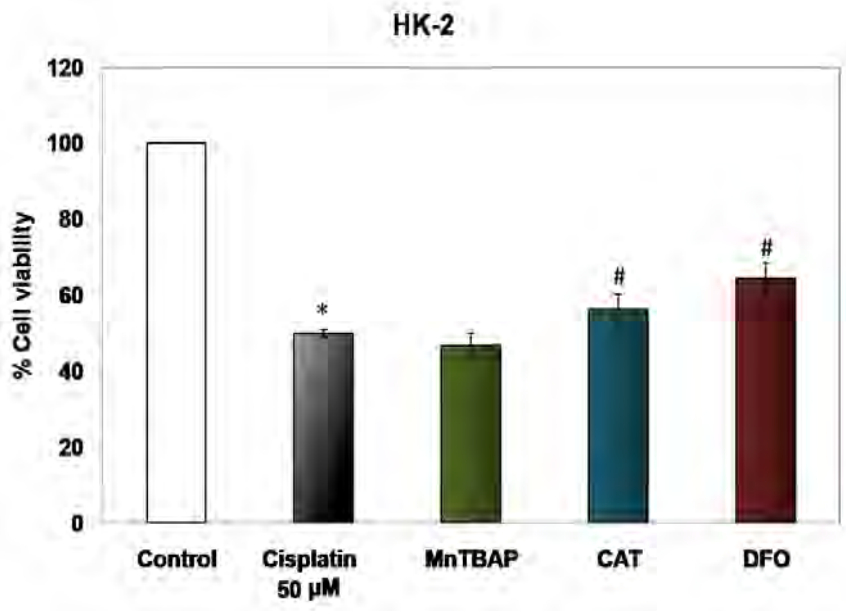


Figure 4.16 Effect of ROS scavengers on cisplatin-induced cell death in HK-2 cell. Cells were pretreated with various specific ROS scavengers; MnTBAP (50 µM), catalase (5000U/ml), and DFO (1 mM) for 1 h prior to cisplatin treatment (50 µM). After cisplatin treatment for 24 h, Cell viability was determined by MTT assay. Values were means on triplicate samples ± S.E.M. ∗P<0.05 versus non-treated control and #P<0.05 versus cisplatin-treated control.

2.3.2. Hydrogen peroxide and hydroxyl radicals were the key ROS in cisplatin-induced human lung cancer cell damage.

To evaluate the key ROS which were responsible for cisplatin cytotoxicity in H460 cells, cells were pre-incubated with various specific ROS scavengers, such as MnTBAP (superoxide anion radical scavenger), catalase (hydrogen peroxide scavenger), and deferoxamine (hydroxyl radical inhibitor) for 1 h prior to 100 μM of cisplatin treatment. After incubation with cisplatin for 24 h, cell viability was determined by MTT assay.

The MTT result showed that 100 μM cisplatin caused the significant reduction of cell viability approximately 50% of cell remaining viable, which could be blocked by pretreatment with either catalase or deferoxamine (68.56 ± 0.62% and 67.16 ± 1.08%, respectively) whereas MnTBAP had no significant effect (56.42 ± 0.79%), suggesting that hydrogen peroxide and hydroxyl radical were key mediators in cisplatin-induced human lung cancer cell damage (figure 4.17).

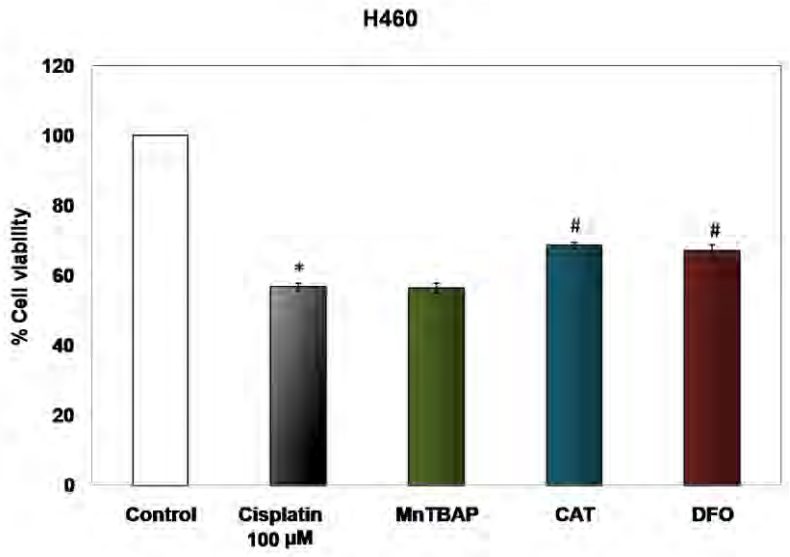


Figure 4.17 Effect of ROS scavengers on cisplatin-induced cell death in H460 cell. Cells were pretreated with various specific ROS scavengers; MnTBAP (50 μM), catalase (5000U/ml), and DFO (1 mM) for 1 h prior to cisplatin treatment (100 μM). After cisplatin treatment for 24 h, Cell viability was determined by MTT assay. Values were means on triplicate samples ± S.E.M. $*P < 0.05$ versus non-treated control and $\#P < 0.05$ versus cisplatin-treated control.

2.3.3. Hydrogen peroxide and hydroxyl radicals were the key ROS in cisplatin-induced human melanoma cell damage.

To evaluate the key ROS which were responsible for cisplatin cytotoxicity in G361 cells, cells were pre-incubated with various specific ROS scavengers, such as MnTBAP (superoxide anion radical scavenger), catalase (hydrogen peroxide scavenger), and deferoxamine (hydroxyl radical inhibitor) for 1 h prior to 100 μM of cisplatin treatment. After incubation with cisplatin for 24 h, cell viability was determined by MTT assay.

The cell viability result demonstrated that the treatment of 100 μM cisplatin resulted in an increase in cell viability approximately 50% of cell remaining viable, which could be prevented by addition of catalase and deferoxamine (74.38 ± 6.37% and 61.34 ± 0.98%, respectively) while MnTBAP could not prevent (56.35 ± 1.68%), suggesting that hydrogen peroxide and hydroxyl radical were key mediators in cisplatin-induced human melanoma cell damage (figure 4.18).

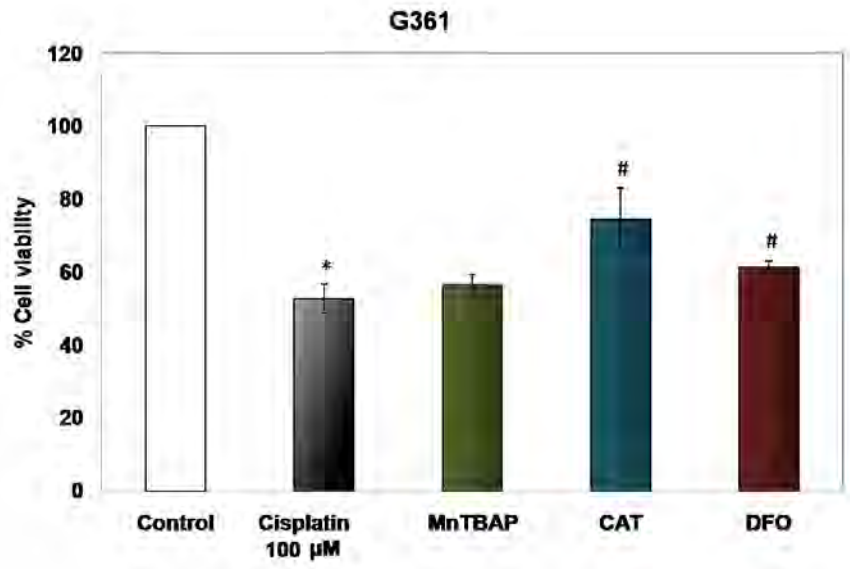


Figure 4.18 Effect of ROS scavengers on cisplatin-induced cell death in G361 cell. Cells were pretreated with various specific ROS scavengers; MnTBAP (50 μM), catalase (5000U/ml), and DFO (1 mM) for 1 h prior to cisplatin treatment (100 μM). After cisplatin treatment for 24 h, Cell viability was determined by MTT assay. Values were means on triplicate samples ± S.E.M. $*P < 0.05$ versus non-treated control and $\#P < 0.05$ versus cisplatin-treated control.

2.4. Silymarin prevented cisplatin-induced renal cell death by its hydrogen peroxide and hydroxyl radical scavenging activities.

To clarify mechanism of silymarin in modulation of cellular oxidative stress and further identified specific ROS scavenging activities of silymarin, this study determined intracellular ROS level in HK-2 cells exposed with ROS generators: H_2O_2, DMNQ (2, 3-dimethoxy-1, 4-naphthoquinone) for superoxide anion radical generation, and combination of hydrogen peroxide and ferrous sulfate for hydroxyl radical generation, in the presence or absence of silymarin (50 μM). After treatment, the intracellular ROS level was determined by flow cytometry using H_2DCF-DA as a fluorescent probe for total ROS and using DHE as a selective probe for intracellular superoxide anion radical.

The results showed that all ROS generators could increase intracellular ROS level in HK-2 cells. However, pretreatment with silymarin for 1 h caused a significant reduction of cellular ROS in H_2O_2 and combination of H_2O_2 and $FeSO_4$ ($^{\cdot}OH$) treated cells whereas had a minimal effect on $^{\cdot}O_2$- level in DMNQ treated cells. These results suggested that silymarin could, at least in part, exert antioxidant activities against hydrogen peroxide and hydroxyl radicals as shown in figure 4.19.

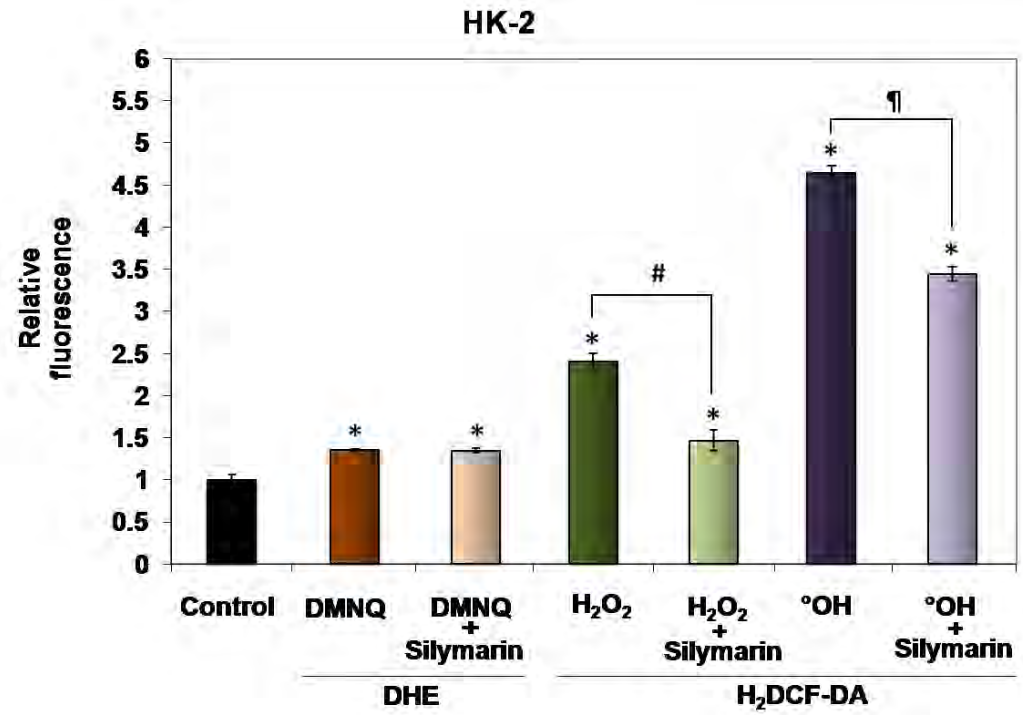


Figure 4.19 Antioxidant activity of silymarin against hydrogen peroxide and hydroxyl radical in HK-2 cells. Cells were pretreated with 50 μM of silymarin for 1 h, then cell were exposed to 5 μM DMNQ, 200 μM hydrogen peroxide, as well as combination with 50 μM ferrous sulphate and 100 μM hydrogen peroxide, for 1 h. Cellular ROS levels were evaluated by flow cytometry with H_2DCF-DA or DHE. Values were means on triplicate samples ± S.E.M. ∗$P < 0.05$ versus non-treated control and #$P < 0.05$ versus H_2O_2-treated control and ¶$P < 0.05$ versus ˙OH -treated control.

3. Silymarin exhibited anticancer activity against lung cancer H460 and melanoma G361 cells.

3.1. High-dose silymarin exhibited anticancer activity against lung cancer H460

To determine whether silymarin had direct anticancer activity on H460, cells were incubated with silymarin at the concentrations of 25, 50, 100, and 200 μM for 24 h, and cell viability as well as mode of cell death were then analyzed by MTT, Hoechst 33342, and PI staining assays.

The results demonstrated that treatment of the H460 cells with low concentrations of silymarin (25-50 μM) showed only a minimal effect on cell viability. As the dose of silymarin was increased up to 100 and 200 μM, silymarin caused a significant toxic effect on H460 cells with approximately 10 and 30% reduction of cell viability (figure 4.20A).

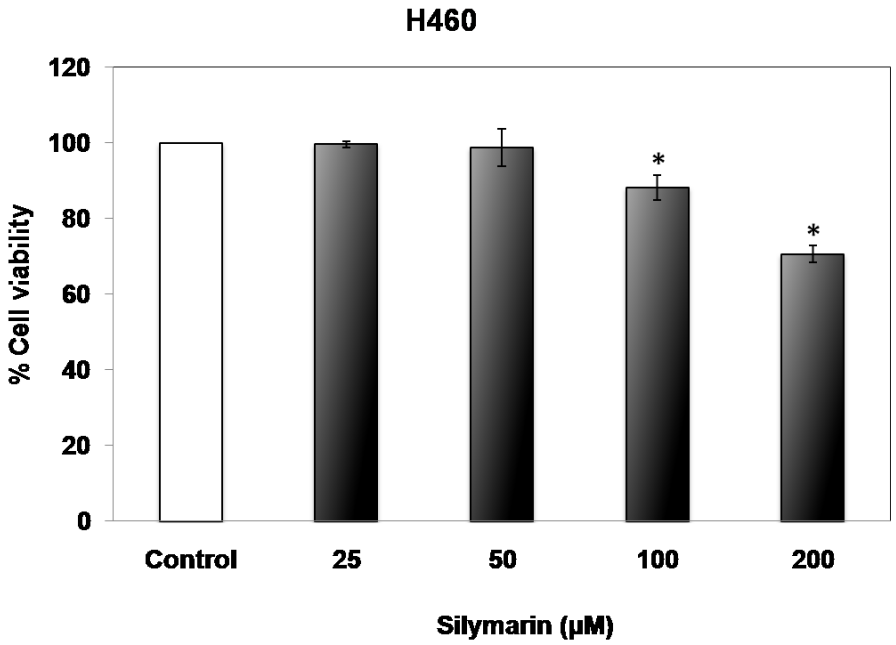
The results of Hoechst 33342 and PI co-staining assay showed that at concentration of 25, 50, and 100 μM, treatment of silymarin alone caused no significant apoptosis and necrosis in H460 cells. Notably, at a high concentration (200 μM), significant necrosis was detected as indicated in figure 4.20B.

For time-dependent experiment, after treatment the cells with 200 μM silymarin cell viability and mode of cell death were determined at 0, 6, 15, and 24 h.

The results showed that the viability of H460 cells was significantly decreased as early as 6 h after 200 μM silymarin treatment (79.73 ± 7.55%). and the reduction of viable cells was continuously decreased until reached approximately 30% at 24 h as indicated in figure 4.21A.

Accordingly, the nuclear staining results clearly showed that the number both apoptosis and necrosis seemed to increase in time-dependent manner in response to 200 μM of silymarin treatment. Apoptosis was clearly observed as early as 15 h after treatment with silymarin whereas necrosis was detected as early as 6 h as indicated in figure 4.21B.

A



B

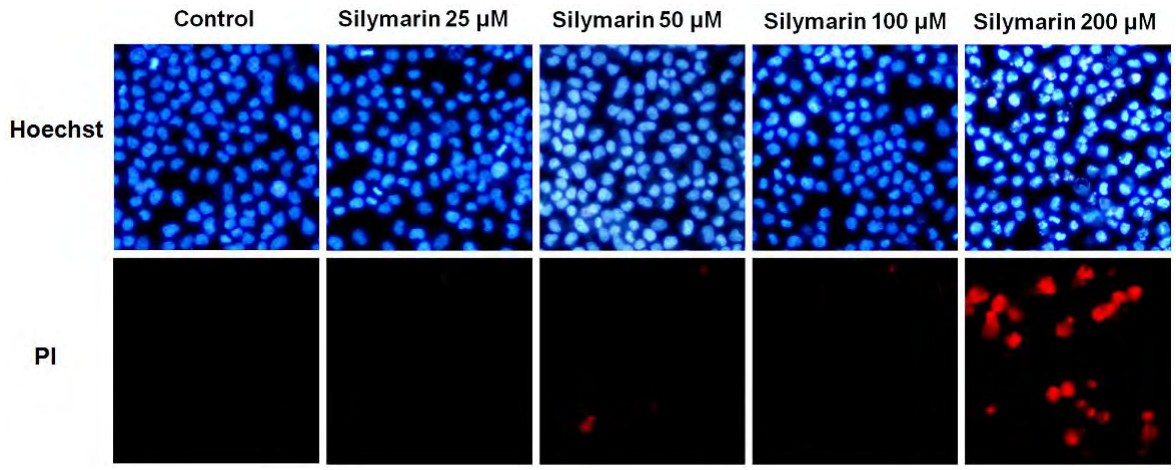
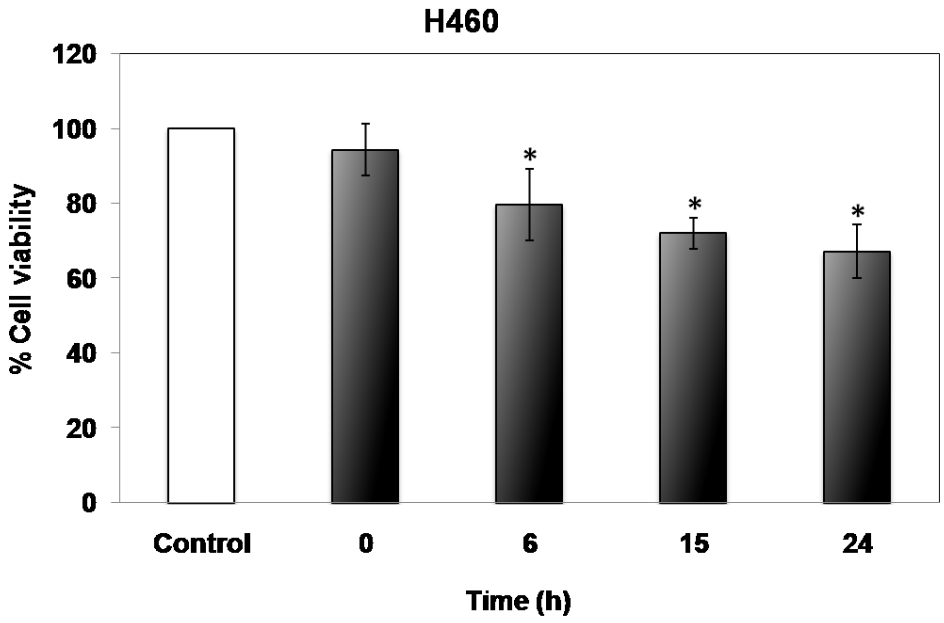


Figure 4.20 Effect of silymarin on H460 cell in dose-dependent manner. A) Cells were treated with various concentrations of silymarin (25, 50,100, and 200 μM.) for 24 h and cell viability was measured by MTT assay. Values were means ± S.E.M. of three-independent experiments, $*P < 0.05$ versus non-treated control. B) Nuclear morphology of apoptosis and necrosis detected by Hoechst 33342 and PI assay.

A



B

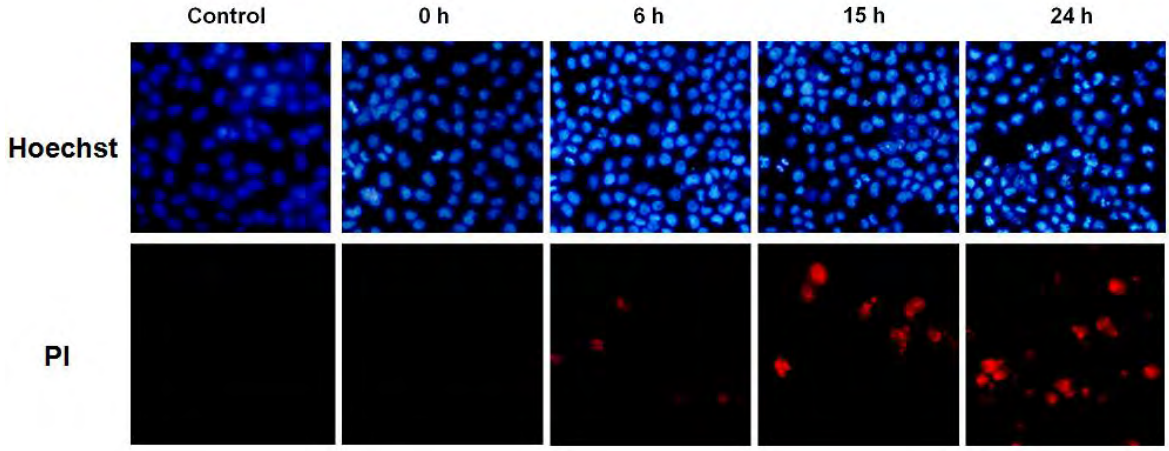


Figure 4.21 Effect of silymarin on H460 cell in time-dependent manner. A) Cells were treated with 200 μM of silymarin and cell viability was measured by MTT assay at 0, 6, 15, and 24 h. Values were means ± S.E.M. of three-independent experiments, $*P < 0.05$ versus non-treated control. B) Nuclear morphology of apoptosis and necrosis detected by Hoechst 33342 and PI assay at the indicated time points.

3.2. Direct anticancer activity of silymarin on melanoma G361 cells

To determine whether silymarin had direct anticancer activity on G361 cells, cells were incubated with silymarin at the concentrations of 25, 50, 100, and 200 μM for 24 h. Cell viability and mode of cell death were then evaluated by MTT, Hoechst 33342, and PI staining assays.

The results indicated that silymarin exhibited dose-and time-dependent cytotoxic effects with approximately 50% reduction in cell viability in response to 200 μM of silymarin treatment (49.22 ± 5.69%). Silymarin caused a significant reduction of G361 cell viability in the dose starting from 25 μM (87.40 ± 4.99%) as indicated in figure 4.22A.

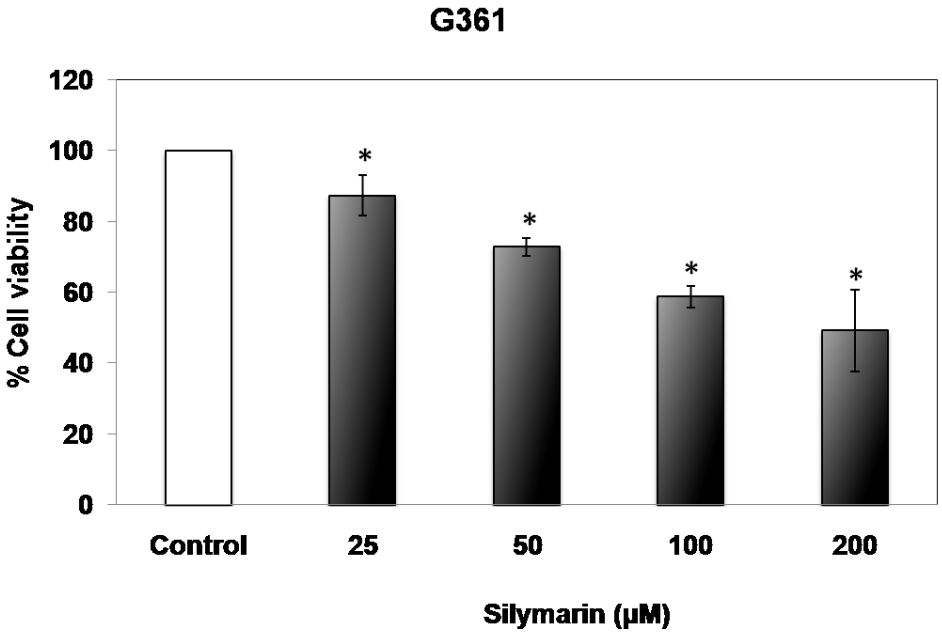
Consistantly, the results of Hoechst 33342 and PI co-staining assay showed that treatment with silymarin alone at the concentration of 25, 50, 100, and 200 μM caused the dose-dependent increase in the number of apoptotic cells while only minimal necrosis was detected in G361 cells (figure 4.22B).

For time-dependent experiment, after treatment the cells with 200 μM silymarin cell viability and mode of cell death were determined at 0, 6, 15, and 24 h.

The results indicated that silymarin caused a time-dependent decrease in G361 cell viability, which significantly reduced as early as 6 h in response to 200 μM of silymarin treatment (87.53 ± 3.97%) and the reduction of viable cells was continuously decreased until reached approximately 50% at 24 h (figure 4.23A).

Moreover, the nuclear staining results showed that apoptotic cells were clearly observed as early as 15 h after treatment with 200 μM silymarin while very less number of necrotic cells was detected as early as 24 h as indicated in figure 4.23B.

A



B

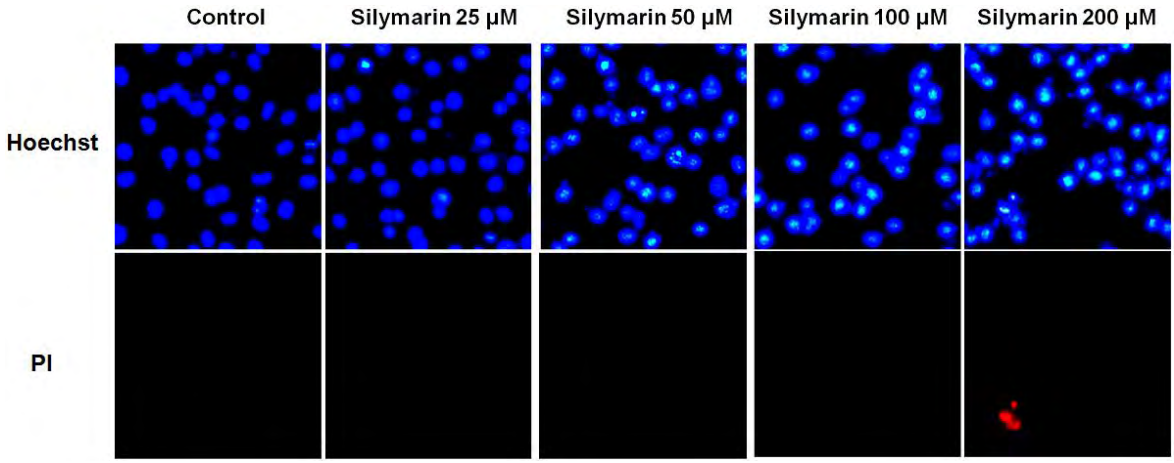
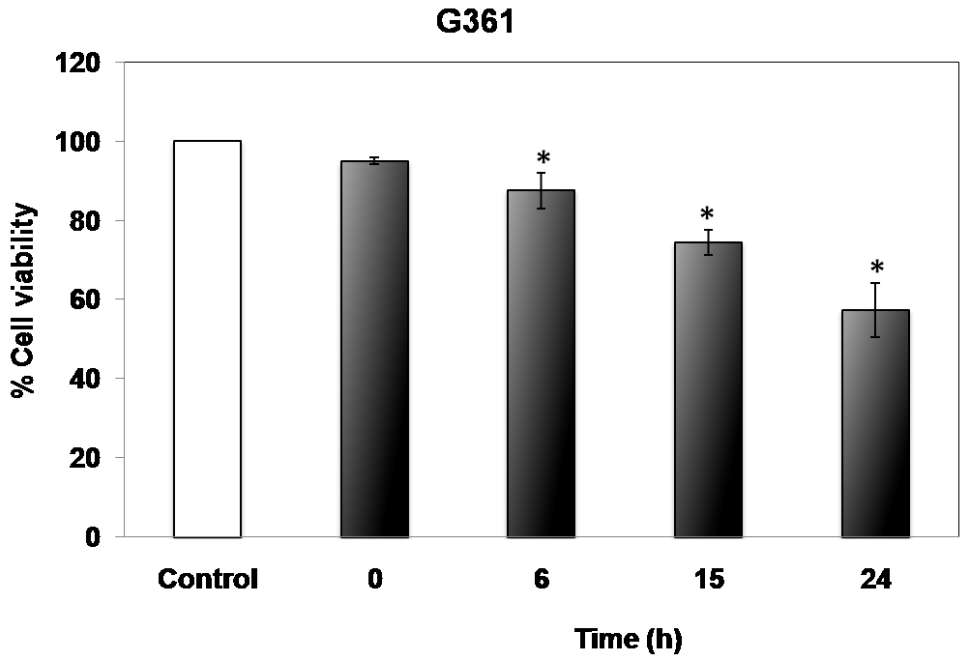


Figure 4.22 Effect of silymarin on G361 cell in dose-dependent manner. A) Cells were treated with various concentrations of silymarin (25, 50,100, and 200 μM.) for 24 h and cell viability was measured by MTT assay. Values were means ± S.E.M. of three-independent experiments, $*P < 0.05$ versus non-treated control. B) Nuclear morphology of apoptosis and necrosis detected by Hoechst 33342 and PI assay.

A



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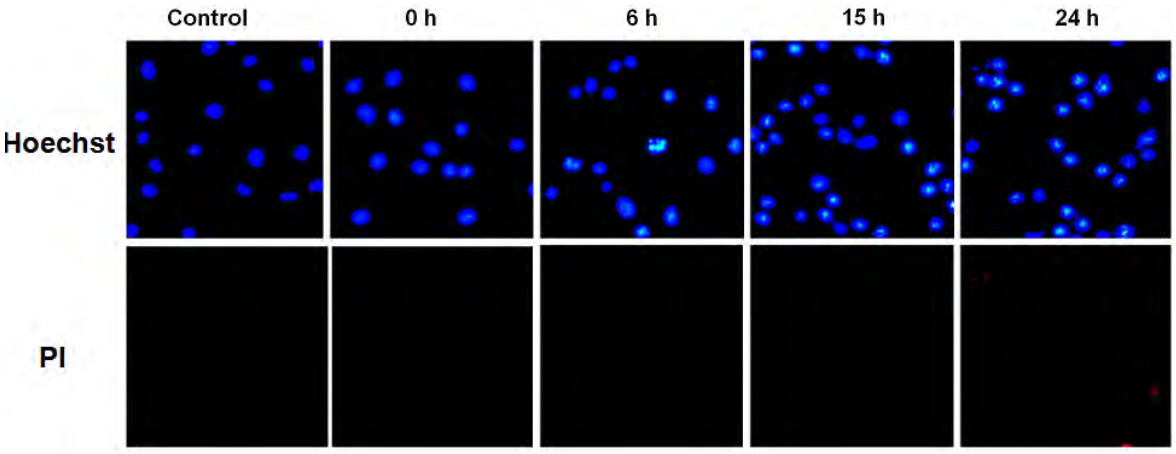

Figure 4.23 Effect of silymarin on G361 cell in time-dependent manner. A) Cells were treated with 200 μM of silymarin and cell viability was measured by MTT assay at 0, 6, 15, and 24 h. Values were means ± S.E.M. of three-independent experiments, $*P < 0.05$ versus non-treated control. B) Nuclear morphology of apoptosis and necrosis detected by Hoechst 33342 and PI assay at the indicated time points.

CHAPTER V

DISCUSSION AND CONCLUSION

The development of nephrotoxicity during cisplatin treatment has been shown to be a major limitation of efficient therapy (Patrick and Lawrence, 1984; Launay-Vacher *et al*., 2008; Pabla and Dong, 2008). Cisplatin induces cytotoxicity by the mechanisms involved in DNA-adduct formation and/or intracellular reactive oxygen species (ROS) induction (Reedijk and Lohman, 1985; Miyajima *et al*. 1997; Wang and Lippard, 2005; Bragado *et al*., 2007). Because a sufficient number of studies have indicated that cisplatin-induced renal cell damage is mainly due to its ability to generate oxidative stress (Matsushima *et al*., 1998; Yang *et al*., 2002; Baek *et al*., 2003). An introduction of antioxidant could be beneficial in preventing renal cell damage during cisplatin therapy (Ajith, Usha, and Nivitha, 2007; Sung *et al*., 2008). Various antioxidants have shown a protective effect on cisplatin nephrotoxicity both in *vivo* and in *vitro* experiments (Zunino *et al*., 1983; Sheikh-hamad, Timmins, and Jalali, 1997; Ajith, Usha, and Nivitha, 2007; Sung *et al*., 2007). Unfortunately, many antioxidants fail to be further developed for the use in clinical treatment since their antioxidant activities frequently attenuate the cytotoxic mechanisms of cisplatin in cancerous cells (Eastman, 1987; Roller and Weller, 1998; Miyajima *et al*., 1999). However, many researchers still make an effort to search for some natural products or antioxidants that could be able to prevent renal damage induced by cisplatin without compromising its anticancer activity.

A number of evidence have stated that silymarin has been shown to have antioxidant activity and capability to protect normal cells including liver and renal cells from several death stimuli (Muriel *et al*., 1992; Sonnenbichler *et al*., 1999; Mansour, Hafez, and Fahmy, 2006). In addition, silymarin is able to increase glutathione level in liver, stomach and intestine of rat (Valenzuela *et al*., 1989) and superoxide dismutase level in lymphocytes (Fehér *et al*., 1987). However, whether or not this compound could be able to inhibit cisplatin-induced renal cell death without interfering with the effects of cisplatin on the cancer cells is still largely unknown.

The results of this study indicated that silymarin selectively protected only human renal tubular HK-2 cells from cisplatin-induced cytotoxicity without compromising the anticancer activity of cisplatin as demonstrated in lung carcinoma and melanoma cells (figure 4.8-10). The mechanism by which silymarin protects HK-2 cells from cisplatin-induced cell damage is not fully understood. The results of this work provided the evidence that the mechanism of cisplatin-induced renal cell damage via ROS-dependent pathway because known anti-oxidants (NAC and GSH) had a capability to prevent renal toxicity of cisplatin (figure4.11). Besides, The results revealed that treatment with cisplatin resulted in renal cell death due to apoptosis and necrosis mechanism, but necrosis seemed to be the main mode of cell death in this condition (figure 4.2).

Previous studies reported that hydroxyl radicals could induce lipid peroxidation of the cell membrane, finally resulting in necrosis (Gutteridge, 1984; Salahudeen, 1995; Matsushima *et al*., 1998; Yang *et al*., 2002). Numerous studies asserted that silymarin succeeded in preventing lipid peroxidation induced by several drugs and toxic compounds, such as paracetamol, adriamycin, carbon tetrachloride, Ferric nitrilotriacetate, and ethanol (Letteron *et al.,* 1990; Valenzuela *et al.,* 1985; El-Shitany, El-Haggar, and El-desoky, 2008; Kaur, Athar, and Alam, 2009; Muriel *et al*., 1992). However, the scavenging activities of silymarin in the reduction of cellular oxidative stress in renal cell were still unclear. The results of the present study indicated that silymarin exerted hydrogen peroxide and hydroxyl radical scavenging activities whereas caused a minimal effect on cellular superoxide anion radical up-regulation (figure 4.19). As the result, the reduction of hydroxyl radicals caused by silymarin may inhibit lipid peroxidation of the cell membrane, which consequently protectd cisplatin-induced necrosis in HK-2 cells.

As cisplatin-induced ROS has been considered to play a critical role in cisplatin-induced renal toxicity and many studies have reported that cancer cell are likely to resist oxidative stress because cancer cells have evolved some mechanisms to protect themselves from intrinsic oxidative stress and also have improved a sophisticated adaptation system involving antioxidant defenses, such as increase in cellular superoxide dismutase or glutathione levels in response to persistent oxidative stress

originated from abnormal metabolism (Valko *et al*., 2007; Gibellini *et al*., 2010). For this reason, this concept seems to advocate the approach of antioxidant usage in preventing nephrotoxicity caused by cisplatin. However, the consequent results that a number of antioxidants could protect both normal and cancerous cells from cisplatin-induced cell death had attenuated the further development of these agents. Furthermore, a sufficient number of evidence indicated that oxidative stress also played a significant role in cisplatin-induced cancer cell death (Miyajima *et al*., 1997; Bragado *et al*., 2007; Wu, Muldoon, and Neuwelt, 2005; Chanvorachote, 2006), which was consistent with the results of this study that treatment with cisplatin caused an apparent induction of intracellular ROS at least three species: superoxide anion radical, hydrogen peroxide, and hydroxyl radical, in both renal and cancer cells.

Interestingly, pretreatment with catalase and deferoxamine could be able to both reduce ROS generation and cell death induced by cisplatin, whereas pretreatment with MnTBAP had only non-significant alteration of cell death. These results implied that some specific ROS like superoxide anion radical could not play a role in killing renal, lung cancer and melanoma cells, while hydrogen peroxide and hydroxyl radical were the key ROS that mediated cisplatin-induced cell death in these cells. Even though in the present study the key ROS mediated cisplatin-induced cell death in renal and cancer cells were similar, silymarin still selective protected only renal tubular HK-2 cells from cisplatin-induced cell damage without compromising the anticancer activity of cisplatin in lung carcinoma and melanoma cells. It was implicated that silymarin may have other direct effects on cancer cells**.**

Previous reports have suggested that silymarin exerts antitumor activities in some cancer cells (Zi, Feyes, and Agarwal, 1998; Sharma *et al*., 2003; Huang *et al*., 2005; Deep *et al*., 2006; Zhong *et al*., 2006). This activity of silymarin has been documented in different animal models and human cancer cells. Silymarin induces cell cycle arrest and suppresses proliferation of cancer cells by increasing in an expression of cyclin-dependent kinase inhibitors (CDKIs), such as Cip/p21 and Kip1/p27 and decreasing in kinase activity of cyclin-dependent kinase (CDK) as well as associated cyclins (Agarwal *et al.*, 2006; Comelli *et al.*, 2007). The results of this study confirmed that silymarin had a direct anticancer activity against melanoma G361 cells, whereas it

seemed to have only minimal anticancer activity on non-small-lung cancer H460 cells. Moreover, the addition of silymarin in cisplatin treatment not only protected against human renal cell damage, but also, at least, either did not interfere with the cytotoxic mechanisms of cisplatin or enhanced the response of cancer cells to cisplatin-induced cell death.

Unlike normal cells, cancer cells have an ability to enter into cell cycle phase independently, resulting in rapid and uncontrollable proliferation. Over-activity of CDKs/cyclins or inactivation of essential CDKIs leads to the independent cell cycle progression of cancer cells (Senderowicz, 2000, 2002; Schwartz and Shah, 2005; Ramasamy and Agarwal, 2008). Therefore, treatment with silymarin alone could have a deleterious impact on lung cancer H460 and melanoma G361 cells, while have no significant effect on renal HK-2 cell.

The possible reasons for silymarin selectivity in the protection of renal cells while not interfering with the mode of cisplatin action in cancer cells include the following: (i) renal cells were shown to be highly susceptible to oxidative stress-induced cell damage (Andreoli, 1991; Nath and Norby, 2000; Galle, 2001; Djamali, 2007), and (ii) silymarin possessed direct anticancer activity (Zi, Feyes, and Agarwal, 1998; Sharma *et al*., 2003; Huang *et al*., 2005; Deep *et al*., 2006; Zhong *et al*., 2006).

In summary, this study reports herein for the first time that silymarin exhibited a protective effect against cisplatin-induced human renal cells damage by its scavenging activities against hydrogen peroxide and hydroxyl radical, whereas had no significant effect on cisplatin-induced lung carcinoma H460 cells death, and enhanced cisplatin-induced melanoma G361 cell death. These findings may advocate the necessity of developing silymarin, a considerately safe compound, for its potential use in clinical cancer therapy in combination with anticancer agents.

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APPENDIX

APPENDIX

TABLES OF EXPERIMENTAL RESULTS

Table 2. The percentage of HK-2 cell viability was determined by MTT assay after treatment with various concentration of silymarin (dose dependency).

Silymarin (μM)	Cell viability (%)
Control	100.00 ± 0.00
25	102.50 ± 4.83
50	106.92 ± 5.89
100	108.19 ± 3.89
200	113.93 ± 7.63

Value represents means ± S.E.M. of three-independent experiments, $*P < 0.05$ versus non-treated control.

Table 3. The percentage of HK-2 cell viability was determined by MTT assay after treatment with various concentration of cisplatin (dose dependency).

Cisplatin (μM)	Cell viability (%)
Control	100.00 ± 0.00
5	94.70 ± 11.40
25	66.56 ± 3.32∗
50	50.17 ± 1.88∗
100	40.83 ± 2.17∗
200	36.43 ± 3.43∗
300	32.22 ± 2.72∗

Value represents means ± S.E.M. of three-independent experiments, $*P < 0.05$ versus non-treated control.

Table 4. The percentage of HK-2 cell viability was determined by MTT assay after treatment with 50 μM cisplatin at various time points (time dependency).

Time (h)	Cell viability (%)
Control	100.00 ± 2.50
0	97.40 ± 1.66
6	96.01 ± 1.77
15	73.55 ± 2.82∗
24	54.02 ± 1.26∗

Value represents means ± S.E.M. of three-independent experiments, $*P < 0.05$ versus non-treated control.

Table 5. The percentage of H460 cell viability was determined by MTT assay after treatment with various concentration of cisplatin (dose dependency).

Cisplatin (μM)	Cell viability (%)
Control	100.00 ± 0.00
5	97.26 ± 1.10
25	86.71 ± 2.69∗
50	75.33 ± 1.21∗
100	51.84 ± 1.64∗
200	45.66 ± 1.50∗
300	39.07 ± 1.78∗

Value represents means ± S.E.M. of three-independent experiments, $*P < 0.05$ versus non-treated control.

Table 6. The percentage of H460 cell viability was determined by MTT assay after treatment with 100 μM cisplatin at various time points (time dependency).

Time (h)	Cell viability (%)
Control	100.00 ± 0.00
0	100.29 ± 0.37
6	91.76 ± 3.83
15	74.15 ± 3.16∗
24	50.47 ± 1.33∗

Value represents means ± S.E.M. of three-independent experiments, $*P < 0.05$ versus non-treated control.

Table 7. The percentage of G361 cell viability was determined by MTT assay after treatment with various concentration of cisplatin (dose dependency).

Cisplatin (μM)	Cell viability (%)
Control	100.00 ± 0.00
5	77.09 ± 2.68∗
25	60.46 ± 0.47∗
50	57.87 ± 2.59∗
100	50.72 ± 2.48∗
200	40.56 ± 5.24∗
300	37.90 ± 3.40∗

Value represents means ± S.E.M. of three-independent experiments, $*P < 0.05$ versus non-treated control.

Table 8. The percentage of G361 cell viability was determined by MTT assay after treatment with 100 μM cisplatin at various time points (time dependency).

Time (h)	Cell viability (%)
Control	100.00 ± 0.00
0	89.14 ± 0.42
6	72.46 ± 2.26∗
15	64.02 ± 1.52∗
24	53.10 ± 0.62∗

Value represents means ± S.E.M. of three-independent experiments, $*P < 0.05$ versus non-treated control.

Table 9. The percentage of HK-2 cell viability was determined by MTT assay after pretreatment with various concentration of silymarin prior to cisplatin treatment.

Treatment	Cell viability (%)
Control	100.00 ± 0.00
Cisplatin 25 μM	65.23 ± 1.76∗
Silymarin 25 μM + Cisplatin 25 μM	74.34 ± 1.88
Silymarin 50 μM + Cisplatin 25 μM	76.68 ± 3.32#
Silymarin 100 μM + Cisplatin 25 μM	81.96 ± 5.10#
Silymarin 200 μM + Cisplatin 25 μM	83.05 ± 4.08#
Cisplatin 50 μM	51.67 ± 1.71∗
Silymarin 25 μM + Cisplatin 50 μM	56.92 ± 1.12
Silymarin 50 μM + Cisplatin 50 μM	60.91 ± 1.67#
Silymarin 100 μM + Cisplatin 50 μM	70.24 ± 5.99#
Silymarin 200 μM + Cisplatin 50 μM	74.76 ± 5.83#
Cisplatin 100 μM	39.67 ± 0.16∗
Silymarin 25 μM + Cisplatin 100 μM	48.05 ± 2.37
Silymarin 50 μM + Cisplatin 100 μM	50.76 ± 4.42#
Silymarin 100 μM + Cisplatin 100 μM	57.77 ± 4.92#
Silymarin 200 μM + Cisplatin 100 μM	67.07 ± 4.43#

Value represents means ± S.E.M. of three-independent experiments, $*P < 0.05$ versus non-treated control, and $\#P < 0.05$ versus cisplatin-treated control.

Table 10. The percentage of H460 cell viability was determined by MTT assay after pretreatment with various concentration of silymarin prior to cisplatin treatment.

Treatment	Cell viability (%)
Control	100.00 ± 0.00
Cisplatin 25 μM	86.71 ± 2.96∗
Silymarin 25 μM + Cisplatin 25 μM	84.38 ± 2.06
Silymarin 50 μM + Cisplatin 25 μM	86.72 ± 2.61
Silymarin 100 μM + Cisplatin 25 μM	85.23 ± 2.73
Silymarin 200 μM + Cisplatin 25 μM	45.64 ± 4.68#
Cisplatin 50 μM	75.33 ± 1.21∗
Silymarin 25 μM + Cisplatin 50 μM	75.64 ± 4.05
Silymarin 50 μM + Cisplatin 50 μM	74.96 ± 4.00
Silymarin 100 μM + Cisplatin 50 μM	72.48 ± 1.00
Silymarin 200 μM + Cisplatin 50 μM	35.25 ± 1.92#
Cisplatin 100 μM	51.84 ± 1.64∗
Silymarin 25 μM + Cisplatin 100 μM	53.18 ± 2.50
Silymarin 50 μM + Cisplatin 100 μM	53.61 ± 1.73
Silymarin 100 μM + Cisplatin 100 μM	51.52 ± 3.58
Silymarin 200 μM + Cisplatin 100 μM	30.30 ± 3.49#

Value represents means ± S.E.M. of three-independent experiments, $*P < 0.05$ versus non-treated control, and $\#P<0.05$ versus cisplatin-treated control.

Table 11. The percentage of G361 cell viability was determined by MTT assay after pretreatment with various concentration of silymarin prior to cisplatin treatment.

Treatment	Cell viability (%)
Control	100.00 ± 0.02
Cisplatin 25 μM	60.46 ± 0.47∗
Silymarin 25 μM + Cisplatin 25 μM	52.16 ± 0.46#
Silymarin 50 μM + Cisplatin 25 μM	50.18 ± 1.71#
Silymarin 100 μM + Cisplatin 25 μM	49.93 ± 3.19#
Silymarin 200 μM + Cisplatin 25 μM	42.68 ± 2.82#
Cisplatin 50 μM	57.87 ± 2.59∗
Silymarin 25 μM + Cisplatin 50 μM	49.84 ± 2.47
Silymarin 50 μM + Cisplatin 50 μM	48.19 ± 2.47#
Silymarin 100 μM + Cisplatin 50 μM	47.27 ± 3.61#
Silymarin 200 μM + Cisplatin 50 μM	41.10 ± 3.26#
Cisplatin 100 μM	50.72 ± 2.48∗
Silymarin 25 μM + Cisplatin 100 μM	48.42 ± 0.64
Silymarin 50 μM + Cisplatin 100 μM	46.61 ± 1.96
Silymarin 100 μM + Cisplatin 100 μM	42.42 ± 2.14#
Silymarin 200 μM + Cisplatin 100 μM	38.97 ± 3.51#

Value represents means ± S.E.M. of three-independent experiments, $*P < 0.05$ versus non-treated control, and $\#P<0.05$ versus cisplatin-treated control.

Table 12. The percentage of HK-2 cell viability was determined by MTT assay after pretreatment with antioxidants NAC and GSH prior to 50 μM of cisplatin treatment.

Treatment	Cell viability (%)
Control	100.00 ± 0.00
Cisplatin 50 μM	53.96 ± 0.97∗
NAC	81.97 ± 5.25#
GSH	89.49 ± 7.41#

Value represents means ± S.E.M. of three-independent experiments, ∗$P < 0.05$ versus non-treated control, and #$P<0.05$ versus cisplatin-treated control.

Table 13. The percentage of HK-2 cell viability was determined by MTT assay after pretreatment with various specific ROS scavengers prior to 50 μM of cisplatin treatment.

Treatment	Cell viability (%)
Control	100.00 ± 0.00
Cisplatin 50 μM	49.94 ± 0.45∗
MnTBAP	46.69 ± 1.56
CAT	56.29 ± 2.14#
DFO	64.59 ± 2.55#

Value represents means ± S.E.M. of three-independent experiments, ∗$P < 0.05$ versus non-treated control, and #$P<0.05$ versus cisplatin-treated control.

Table 14. The percentage of H460 cell viability was determined by MTT assay after pretreatment with various specific ROS scavengers prior to 100 μM of cisplatin treatment.

Treatment	Cell viability (%)
Control	100.00 ± 0.00
Cisplatin 100 μM	56.72 ± 0.06∗
MnTBAP	56.42 ± 0.79
CAT	68.56 ± 0.62#
DFO	67.16 ± 1.08#

Value represents means ± S.E.M. of three-independent experiments, ∗$P < 0.05$ versus non-treated control, and #$P<0.05$ versus cisplatin-treated control.

Table 15. The percentage of G361 cell viability was determined by MTT assay after pretreatment with various specific ROS scavengers prior to 100 μM of cisplatin treatment.

Treatment	Cell viability (%)
Control	100.00 ± 0.00
Cisplatin 100 μM	52.72 ± 2.04∗
MnTBAP	56.35 ± 1.68
CAT	74.38 ± 6.37#
DFO	61.34 ± 0.98#

Value represents means ± S.E.M. of three-independent experiments, ∗$P < 0.05$ versus non-treated control, and #$P<0.05$ versus cisplatin-treated control.

Table 16. The Relative fluorescence intensity in HK-2 was quantified by flow cytometry in response to specific ROS inducers in the present and absent of silymarin.

Treatment	Relative fluorescence
Control	1.00 ± 4.12
DMNQ 5 μM	1.35 ± 0.90∗
Silymarim 50 μM + DMNQ 5 μM	1.35 ± 2.63∗
H_2O_2 200 μM	2.41 ± 13.83∗
Silymarim 50 μM + H_2O_2 200 μM	1.47 ± 12.00∗ #
$FeSO_4$ 100 μM + H_2O_2 100 μM	4.64 ± 41.16∗
Silymarim 50 μM + $FeSO_4$ 100 μM + H_2O_2	3.45 ± 27.83∗ ¶

Value represents means ± S.E.M. of three-independent experiments, ∗ $P < 0.05$ versus non-treated control and # $P < 0.05$ versus H_2O_2-treated control and ¶ $P < 0.05$ versus ˙OH-treated control.

Table 17. The percentage of H460 cell viability was determined by MTT assay after treatment with various concentration of silymarin (dose dependency).

Silymarin (μM)	Cell viability (%)
Control	100.00 ± 0.00
25	99.63 ± 0.82
50	98.68 ± 4.86
100	88.17 ± 3.01*
200	70.63 ± 1.64∗

Value represents means ± S.E.M. of three-independent experiments, ∗$P < 0.05$ versus non-treated control.

Table 18. The percentage of H460 cell viability was determined by MTT assay after treatment with 200 μM silymarin at various time points (time dependency).

Time (h)	Cell viability (%)
Control	100.00 ± 0.00
0	94.35 ± 6.56
6	79.73 ± 7.55∗
15	72.05 ± 2.92∗
24	67.18 ± 4.86∗

Value represents means ± S.E.M. of three-independent experiments, $*P < 0.05$ versus non-treated control.

Table 19. The percentage of G361 cell viability was determined by MTT assay after treatment with various concentration of silymarin (dose dependency).

Silymarin (μM)	Cell viability (%)
Control	100.00 ± 0.00
25	87.40 ± 4.99∗
50	72.90 ± 1.82∗
100	58.75 ± 1.84∗
200	49.22 ± 5.69∗

Value represents means ± S.E.M. of three-independent experiments, $*P < 0.05$ versus non-treated control.

Table 20. The percentage of G361 cell viability was determined by MTT assay after treatment with 200 μM silymarin at various time points (time dependency).

Time (h)	Cell viability (%)
Control	100.00 ± 0.00
0	94.93 ± 0.82
6	87.53 ± 3.97∗
15	74.36 ± 2.36∗
24	57.39 ± 3.92∗

Value represents means ± S.E.M. of three-independent experiments, $*P < 0.05$ versus non-treated control.

VITA

Miss. Chuanpit Ninsontia was born on August 29, 1985 in Nakhonratchasima. She received her B.Pharm (2^{nd} honor) from the faculty of Pharmacy, Chiang Mai university in 2006.