การเพิ่มการละลายของซิไลมารินโดยเทคนิคโซลิดดิสเพอร์ชันและผลยับยั้งความเป็นพิษต่อไตที่
เกิดจากซิสพลาทินของซิไลมาริน

นางสาวพัชราภรณ์ เจียมไชยศรี

ENHANCED DISSOLUTION OF SILYMARIN BY SOLID DISPERSION
TECHNIQUE AND INHIBITORY EFFECT OF SILYMARIN AGAINST
CISPLATIN-INDUCED NEPHROTOXICITY

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พัชราภรณ์ เจียมไชยศรี : การเพิ่มการละลายของซิไลมารินโดยเทคนิคโซลิดดิสเพอร์ซัน และผลยับยั้งความเป็นพิษต่อไตที่เกิดจากซิสพลาทินของซิไลมาริน.　(ENHANCED DISSOLUTION OF SILYMARIN BY SOLID DISPERSION TECHNIQUE AND INHIBITORY EFFECT OF SILYMARIN AGAINST CISPLATIN-INDUCED NEPHROTOXICITY) อ.ที่ปรึกษาวิทยานิพนธ์หลัก : อ.ดร.อังคณา ตันติธุวานนท์, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: อ.ดร.ปิติ จันทร์วรโชติ, ผศ.ดร.กุลวรา เมฆสวรรค์, 114 หน้า.

ซิไลมารินมีคุณสมบัติที่ดีในการนำมาใช้ป้องกันและรักษาโรคตับ อย่างไรก็ตามการที่ซิไลมารินมีค่าการละลายน้ำที่น้อย จึงส่งผลจำกัดต่อชีวประสิทธิผลและการออกฤทธิ์ของซิไลมาริน การเตรียมซิไลมารินด้วยเทคนิคโซลิดดิสเพอร์ซันสามารถเพิ่มการละลายของยาได้ ผลดังกล่าวเกิดจากอันตรปฏิกิริยาระหว่างยาและพอลิเมอร์ที่ละลายน้ำได้ดี โดยที่ชนิดของพอลิเมอร์และอัตราส่วนของยาต่อพอลิเมอร์ส่งผลต่อรูปแบบการละลายของยา ดังนั้นหนึ่งในวัตถุประสงค์ของการศึกษานี้คือการศึกษาผลของพอลีเอธิลีนไกลคอล 4000, 6000 และ พอลีไวนิลไพโรลิโดล เค 30 ที่อัตราส่วนของซิไลมารินต่อพอลิเมอร์ คือ 1 : 2, 1 : 4 และ 1 : 8 ต่อการละลายของซิไลมารินที่เตรียมโดยเทคนิคโซลิดดิสเพอร์ซันจากการพ่นเคลือบ ผลการศึกษาพบว่าซิไลมารินที่เตรียมโดยเทคนิคโซลิดดิสเพอร์ซันสามารถเพิ่มการละลายของซิไลมารินได้ดีกว่าการละลายของผงซิไลมาริน และการใช้พอลีเอธิลีนไกลคอล 6000 ในทุกอัตราส่วน สามารถเพิ่มการละลายของซิไลมารินได้ดีกว่าพอลีเอธิลีนไกลคอล 4000 และพอลีไวนิลไพโรลลิโดล เค 30 ที่อัตราส่วนเดียวกัน นอกจากการนำมาใช้รักษาโรคตับแล้วพบว่า ซิไลมารินสามารถเสริมฤทธิ์ในการรักษามะเร็งเมื่อให้ร่วมกับยาซิสพลาทิน ซิสพลาทินทำให้เกิดความเป็นพิษในเซลล์ปกติ โดยเฉพาะอย่างยิ่งต่อเซลล์ไต และพบว่าซิไลมารินช่วยลดความเป็นพิษของซิสพลาทินในเซลล์ปกติ แต่ผลของซิไลมารินในการยับยั้งความเป็นพิษต่อเซลล์ไตที่เกิดจากซิสพลาทินยังไม่ทราบเป็นที่แน่ชัด ดังนั้นในการศึกษานี้จึงมีวัตถุประสงค์อีกข้อหนึ่งคือศึกษาผลของซิไลมารินในการยับยั้งความเป็นพิษของซิสพลาทินต่อเซลล์ไตของมนุษย์ ผลการศึกษาพบว่าซิไลมารินสามารถยับยั้งความเป็นพิษของซิสพลาทินต่อเซลล์ไตได้ โดยลดจำนวนการตายของเซลล์ซึ่งเกิดจากซิสพลาทิน ซึ่งผลดังกล่าวเกิดจากคุณสมบัติของซิไลมารินในการกำจัดไฮโดรเจนเพอร์ออกไซด์ และไฮดรอกซิล แรดิคอล ซึ่งเป็นอนุมูลอิสระที่เกิดจากซิสพลาทินและมีผลทำให้เกิดความเป็นพิษต่อเซลล์

ภาควิชา วิทยาการเภสัชกรรมและเภสัชอุตสาหกรรม	ลายมือชื่อนิสิต	
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PACHARAPORN JIAMCHAISRI: ENHANCED DISSOLUTION OF SILYMARIN BY SOLID DISPERSION TECHNIQUE AND INHIBITORY EFFECT OF SILYMARIN AGAINST CISPLATIN-INDUCED NEPHROTOXICITY. ADVISOR: ANGKANA TANTITUVANONT, Ph.D., CO-ADVISORS: PITHI CHANVORACHOTE, Ph.D., ASST. PROF. KULWARA MEKSAWAN, Ph.D., 114 pp.

Silymarin has beneficial effects in the prevention and the treatment of liver disease, however, the bioavailability of silymarin is limited by its poor solubility in water. The preparation of silymarin by solid dispersion technique may improve the dissolution of silymarin. The enhancement of dissolution is caused by an interaction between drugs and hydrophilic carriers. The dissolution characteristics of the drug depend on the type and ratio of carrier used. For this reason, one of the objectives of this study was to investigate the effect of polyethylene glycol 4000, 6000 and polyvinylpyrrolidone K 30 at the ratio silymarin to carrier for 1 : 2, 1 : 4 and 1 : 8 on the dissolution of silymarin prepared by solid dispersion technique using fluidized-bed coating technique. The results suggested that percent dissolved of silymarin solid dispersion pellets were higher than silymarin powder. Silymarin solid dispersion pellets using polyethylene glycol 6000 a t all ratios provided the higher percent dissolved of silymarin than polyethylene glycol 4000 and polyvinylpyrrolidone K 30 at the same ratio. In addition for the treatment of liver disease, silymarin was found to be synergist with cisplatin in the treatment of cancers. Cisplatin caused cytotoxicity in normal cells especially kidney cells. Silymarin reduced the cisplatin-induced cytotoxicity in normal cells but this protective effect is unclear. For this reason, another objective in this study was to investigate the inhibitory effect of silymarin against cisplatin-induced cytotoxicity in human kidney cells (HK-2 cells). The results suggested that silymarin showed protective effect against cisplatin-induced cytotoxicity in HK-2 cells by decreasing the number of cell death. The protective effect of silymarin may be due to its ability to scavenge hydrogen peroxide and hydroxyl radical. Cisplatin-induced cytotoxicity is caused by the production of these radicals.

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Field of Study : Pharmaceutics	Advisor's Signature ....
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	Co-Advisor's Signature ....

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LIST OF ABBREVIATIONS

ANOVA	=	analysis of variance
°C	=	degree Celsius
CI	=	confidence interval
conc	=	concentration
cm	=	centimeter
CV	=	coefficient of variation
df	=	degree of freedom
DPPH	=	1,1-diphenyl-2-picryl-hydrazyl radical
et al.	=	*et alii*, 'and others'
g	=	gram
hr	=	hour
HPLC	=	high performance liquid chromatography
mg	=	milligram
min	=	minute
ml	=	milliliter
mmol/l	=	millimolar
n	=	sample size
nm	=	nanometer
P188	=	poloxamer 188
pH	=	the negative logarithm of the hydrogen ion concentration
R^2	=	coefficient of determination
ROS	=	reactive oxygen species
rpm	=	round per minute
s	=	second
SD	=	standard deviation
SEM	=	Scanning Electron Microscope
SLS	=	sodium lauryl sulfate
μg	=	microgram
USP/NF	=	The United States Pharmacopoeia/National Formulary

UV	=	ultraviolet
w/v	=	weight by volume
w/w	=	weight by weight

CHAPTER I

INTRODUCTION

The experiments conducted in this study were divided into 2 parts; the first one concerning the enhancement of dissolution of silymarin by solid dispersion technique and the second one pertaining to the inhibitory effect of silymarin against cisplatin-induced nephrotoxicity.

Silymarin is a standardized extract obtained from the seeds of *silybum marianum* L. It contains 70-80% of flavonolignans and 20-30% of polyphenolic compounds (Kren and Walterova, 2005). Silymarin is widely used as a supportive therapy for liver disorders such as cirrhosis and hepatitis (Ulbricht and Basch, 2005). It has been shown to protect liver and other organs from toxins or drugs such as carbon tetrachloride (Letteron et al., 1990), ethanol (Singha et al., 2007), acetaminophen (Nencini et al., 2007), adriamycin (El-Shitany et al., 2008), rifampicin and pyrogallol (Upadhyay et al., 2007). The protective effect of silymarin may be due to its antioxidant activity (Kiruthiga et al., 2007), its ability to prevent the depletion of glutathione (Alidoost et al., 2001) and its potential to stimulate protein synthesis (Sonnenbichler et al., 1999). Although silymarin has several advantages as previous described, the bioavailability of silymarin is limited by its poor solubility in water (Gazak et al., 2004). The solubility of poorly water soluble drug is often a rate limiting step for the permeation and the oral bioavailability. Improvement of the solubility can increase the rate and the extent of drug dissolution. Enhanced the dissolution extent resulted in the increment of bioavailability (Fernandez et al., 1993; Horter and Dressman, 1997; Leuner and Dressman, 2000). Many researchers have been tried to improve the dissolution of silymarin by using solid dispersion technique (Wu et al., 2006; Sun et al., 2007). The solid dispersion technique is used to improve the dissolution rate of poorly water-soluble drugs by changing the physical properties of the drugs such as transformation of the drug crystallinity to an amorphous form (Leuner and Dressman, 2000). The enhancement of the dissolution is due to an interaction between drugs and carriers such as polyethylene glycol (PEG) and

polyvinylpyrrolidone (PVP). These polymers are freely water soluble, non-toxic and compatible with the drug (Horter and Dressman, 1997; Leuner and Dressman, 2000). The dissolution characteristics of the dispersed drugs depends on type and ratio of the carrier used (Fernandez et al., 1993; Leuner and Dressman, 2000; Nair et al., 2002; Konno et al., 2008). The solid dispersion technique can be prepared by melting method and solvent method. However, the degradation of thermolabile drugs and the insufficiency of solvent removing are the disadvantages of these two methods, respectively. (Leuner and Dressman, 2000; Sun et al., 2007). Fluid-bed coating technique has been applied for solid dispersion preparation in order to solve the mentioned problems (Sun et al., 2007; Ho, et al., 1996). To date, only a few studies have used this technique for solid dispersion preparation. For this reason, the enhancement of silymarin dissolution using fluid-bed coating technique for solid dispersion preparation was investigated in this study.

From these beneficial effects as previously described, several studies have been attempted to use silymarin for the prevention of cancers (Bongiovanni et al., 2007; Ramakrishnan, 2006). In addition, silymarin has been used in combination with cisplatin in the treatment of cancer patients (Scambia et al., 1996).

Cisplatin is an effective chemotherapeutic agent for the treatment of cancers including ovary, bladder, head and neck cancers (Chabner, 2006). However, the clinical application of cisplatin is limited because of its dose-dependent nephrotoxicity, a severe adverse effect. Long term used of cisplatin would lead to an acute renal injury and renal failure (Ali and Al Moundhri, 2006). Previous studies found that the cytotoxicity of cisplatin in normal cells is due to the induction of oxidative stress and the depletion of antioxidant such as glutathione leading to apoptosis and necrosis cell death (Sadzuka et al., 1994; Ali and Al Moundhri, 2006; Okuda et al., 1999; Atessahin et al., 2005). Silymarin is found to be synergist with the cisplatin in the treatment of cancer (Scambia et al., 1996). In addition, the cytotoxicity of cisplatin in normal cells is reduced after the treatment with silymarin (Neuman et al., 1999; Mansour et al., 2006). However, the protective effect of silymarin against cisplatin-induced nephrotoxicity is unclear but its mechanism may be due to an antioxidant activity of silymarin. For this reason, the protective effect of silymarin against cisplatin was investigated in this study.

As described above, the poor water solubility of silymarin affected its bioavailability and the protective effect of silymarin against cisplatin-induced nephrotoxicity remains unclear.

The purposes of this study were as follows:

1. To investigate the effect of PEG 4000, PEG 6000 and PVP K 30 at various ratios on the dissolution of silymarin from solid dispersion pellets prepared by fluid-bed coating technique.

2. To investigate the inhibition effect of silymarin against cisplatin-induced nephrotoxicity in HK-2 cells.

CHAPTER II

LITERATURE REVIEW

A. Silymarin

Silymarin is a flavonoid complex extracted from the seeds of milk thistle (*silybum marianum* L.). Silymarin consists of approximately 70-80% flavonolignans and 20-30% polyphenolic compounds (Figure 1). Silibin (silibinin) is the main flavonolignans component of silymarin. The other components of silymarin are isosilybin, silydianin, silychristin, querctin and taxifolin (Kren and Walterova, 2005; Svobodova et al., 2006). Silymarin has been used for more than 2000 years in the treatment of liver diseases including hepatitis and cirrhosis (Mayer et al., 2005; Saller et al., 2001). It has been shown to protect the liver against toxins or drugs such as carbon tetrachloride (Letteron et al., 1990), ethanol (Singha et al., 2007), rifampicin and pyrogallol (Upadhyay et al., 2007). The protective effects of silymarin may be due to several mechanisms including, scavenging free radical activity (Kiruthiga et al., 2007), prevention of glutathione depletion (Alidoost et al., 2001), membrane stabilizing effect (Breschi et al., 2002) and stimulation of protein synthesis (Sonnenbichler et al., 1999). Recently, silymarin is received an attention due to these protective effects not only in the liver but also in heart (Rao and Viswanath, 2007), brain (Nencini et al., 2007), kidney (El-Shitany et al., 2008) and skin (Svobodova et al., 2003; Svobodova et al., 2007). In addition, silymarin has been used in the prevention and treatment of cancers (Bongiovanni et al., 2007; Kren and Walterova, 2005; Ramakrishnan et al., 2006). Silymarin has been shown to synergist with chemopretherapeutic agents such as doxorubicin and cisplatin in the treatment of cancers (Scambia et al., 1996). Moreover, the cytotoxicity of chemotherapeutic agents such as methotrexate (Neuman et al., 1999) and cisplatin (Mansour et al., 2006) is reduced after the treatment of silymarin.

2 R, 3 R, 10 R, 11 R
Silybin A

2 R, 3 R, 10 S, 11 S
Silybin B

Isosilybin A

Silydianin

Silychristin

(+)-Taxifolin

Quercetin
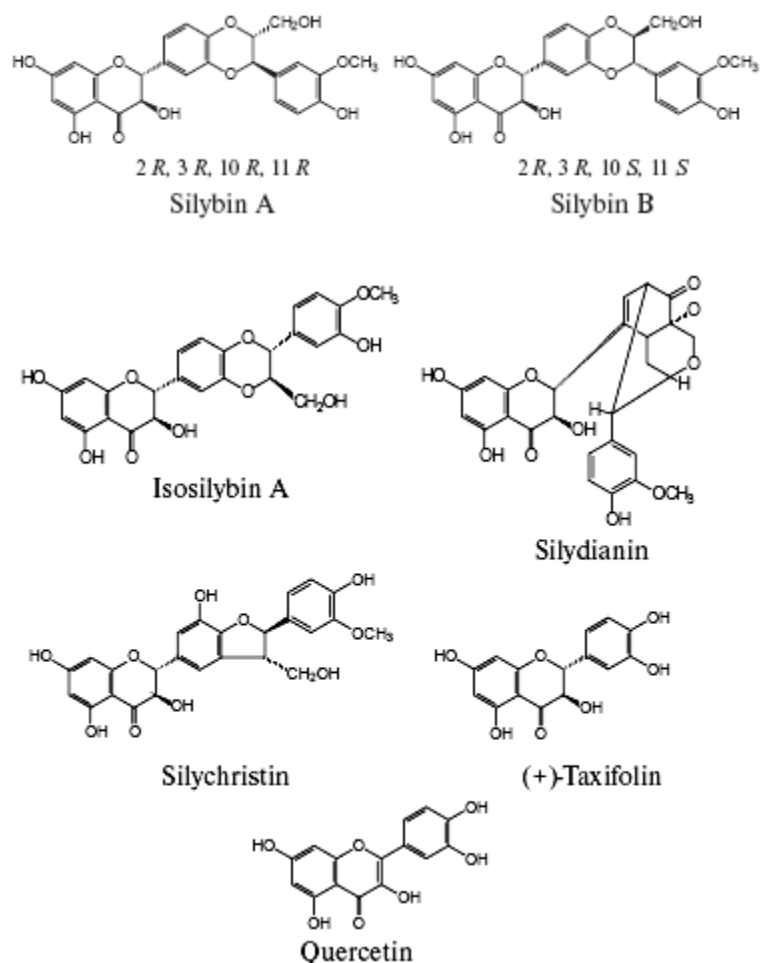
Figure 1. Structure of silymarin mixture (Kren and Walterova, 2005)

B. Cisplatin

cisplatin (*cis*-diamminedichloroplatinum (II)) is a water-soluble planar member of the platinum coordination complex class of anticancer drugs. It is an organic complex formed by an atom of platinum surrounded by chloride and ammonium atoms in the cis position of a horizontal plane (Figure 2). Cisplatin enter cells by diffusion and by active Cu^{2+} transporter. Inside the cell, the chloride atoms are replaced by water, yielding a p ositively charged molecule that interacts with nucleophilic sites on DNA and proteins (Chabner, 2006). Cisplatin causes intrastrand and interstrand crosslinking probably between N7 and O6 of the adjacent guanine molecules, which results in local denaturation of the DNA chain (Jamieson and Lippard, 1999). Cisplatin is widely used for the treatment of many types of cancers

including ovary, bladder, head and neck cancer (Chabner, 2006). However, the clinical application of cisplatin is limited because of its dose-dependent nephrotoxicity, a severe adverse effect. Long term used of cisplatin would lead to an acute renal injury and renal failure (Ali and Moundhri, 2006; Atessahin et al., 2005; Sadzuka et al., 1994).
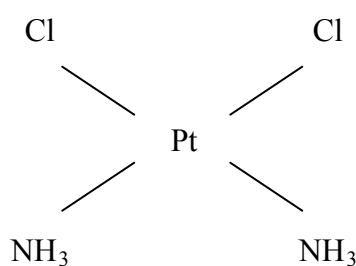
Figure 2. Structure of cisplatin

The exact mechanism of cisplatin-induced nephrotoxicity is not completely understood but current studies have been suggested a role for p53 in tubular cell apoptosis (Jiang et al., 2007; Pabla and Dong, 2008; Seth et al., 2005; Yang et al., 2007). On the other hand, there are well-known evidences that oxidative and nitrosative stress is involved in kidney damage after cisplatin administration. (Chirino et al., 2008; Daugaard and Abildgaard, 1989; Fukutomi et al., 2006; Kuhad et al., 2006). Many researchers have been tried to reduce the cisplatin cytotoxicity by using less intensive treatment, the replacement of cisplatin analogue and the combination of cisplatin and antioxidant agents such as lycopene (Atessahin et al., 2005), melatonin (Hara et al., 2001) and vitamin E (Naziroglu et al., 2004).

C. Reactive Oxygen Species (ROS)

Free radicals can be defined as molecules or molecular fragments containing one or more unpaired electrons in atomic or molecular orbitals. This unpaired electron usually gives a considerable degree of reactivity to the free radical. Radicals derived from oxygen, for examples, superoxide anion, hydroxyl radical and peroxyl radical. represent the most important class of radical species generated in living systems. Other reactive molecules such as molecular oxygen, singlet oxygen and hydrogen

peroxide are not free radicals but are capable of initiating oxidative reactions and generating free radical species. Together, these free radicals and reactive oxygen species are called ROS (Pinnell, 2003; Valko et al., 2007). The generation of ROS in biological systems may be endogenous, through metabolic processes or by exogenous sources like ultraviolet light (Wlaschek et al., 2001), ionising radiation (Leach et al., 2001), drugs (Atessahin et al., 2005) or exposure to environmental contaminants of pollution (Becker et al., 2002). Pathways of ROS formation are shown in Figure 3 (Valko et al., 2007).

ROS are well recognised for playing a dual role as both deleterious and beneficial species, since they can be either harmful or beneficial to living systems. Beneficial effects of ROS occur at low or moderate concentrations and involve physiological roles in cellular responses as for example in defense against infectious agents (Kazura et al., 1981) and in the function of a number of cellular signaling systems (Lander, 1997). The harmful effect of free radicals causing potential biological damage is termed oxidative stress. Under normal conditions, there is a balance between both the ROS and the intracellular levels of the antioxidants. The balance is essential for the survival of organisms and their health. The unbalance of oxidative stress and the antioxidant systems can cause cell damage and finally cell death (Valko et al., 2007). The excess ROS can damage cellular lipids, proteins, or DNA inhibiting their normal functions (Bandyopadhyay et al., 1999; Valko et al., 2004). Mitochondria represent the first target. Alterations of mitochondrial membrane are the consequence of lipid peroxidation, causes harm to cell membranes and thiol residue damage that lead to aging, atherosclerosis and other diseases (Batandier et al., 2002; Suematsu et al., 2003; Wei et al., 2006).
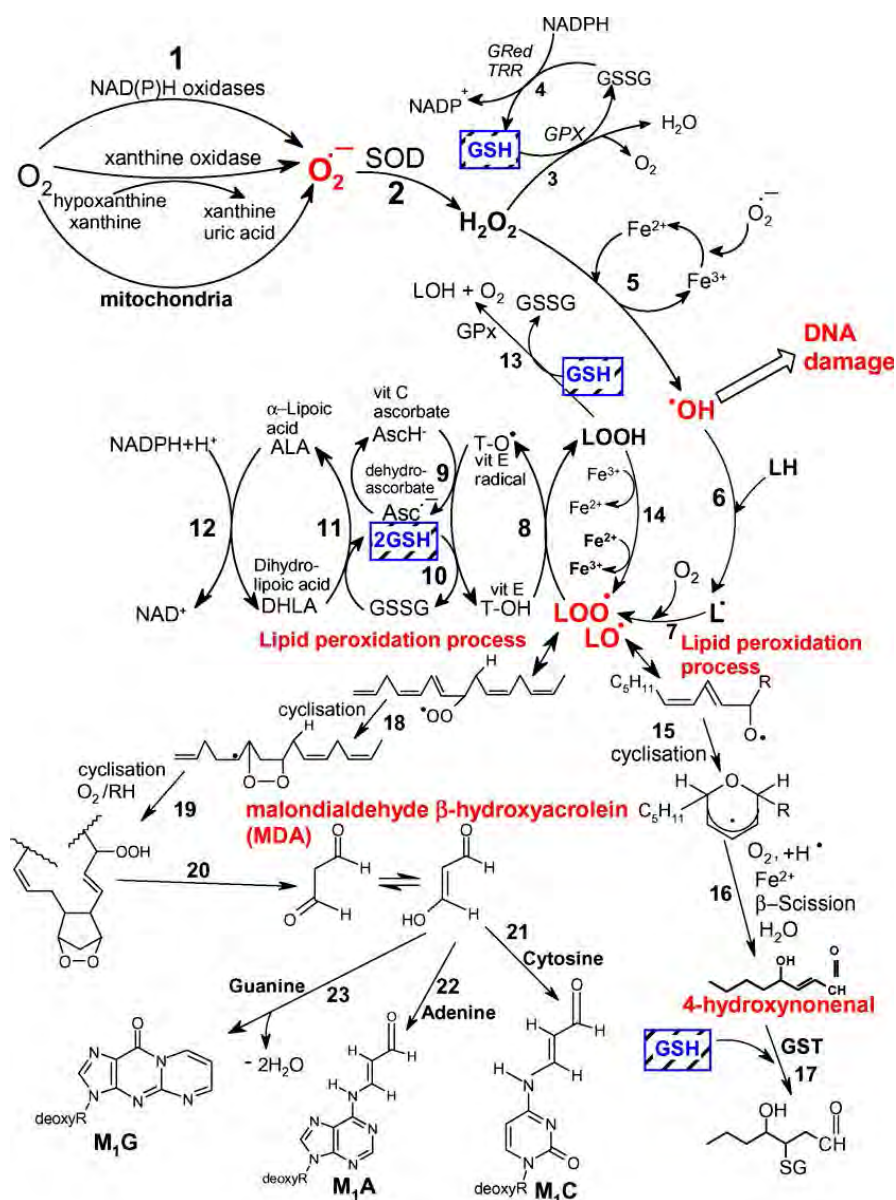
Figure 3 Pathways of ROS formation (Valko et al., 2007)

1. Superoxide anion radical

The addition of one electron to dioxygen forms the superoxide anion radical ($O_2^{\bullet-}$). Superoxide anion is produced within the mitochondria and is considered as a primary ROS, and can further interact with other molecules to generate secondary ROS, either directly or prevalently through enzyme- or metal-catalysed processes This superoxide anion is scavenged by intramitochondrial Super Oxide Dismutase (Mn SOD) leading to hydrogen peroxyde (H_2O_2) (Fridovich 1983; Valko et al., 2007)..

2. Hydrogen peroxide

Hydrogen peroxide is not a radical, for it has no unpaired electrons, and it displays a moderate chemical reactivity. However, this chemical reactivity is substantially enhanced by two features of hydrogen peroxide: First, hydrogen peroxide can cross freely biological membrane. Second, hydrogen peroxide is required for the formation of more potent oxidants, such as the hydroxyl radical ($^{\bullet}OH$) The released Fe^{2+} and hydrogen peroxide can participate in the Fenton reaction, generating highly reactive hydroxyl radical ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^{\bullet}OH + OH^{-}$). The superoxide radical also participates in the Haber–Weiss reaction ($O_2^{\bullet-} + H_2O_2 \rightarrow O_2 + {}^{\bullet}OH + OH^{-}$), generating reactive hydroxyl radical (Batandier et al., 2002; Cadenas and Davies, 2000; Valko et al., 2007).

3. Hydroxyl radical

The hydroxyl radical is the neutral form of the hydroxide ion. The hydroxyl radical has a high reactivity, making it a very dangerous radical with a very short in vivo half-life of approximately 10^{-9} s. The hydroxyl radical is known to react with all components of the DNA molecule, damaging both the purine and pyrimidine bases and also the deoxyribose backbone (Cader et al., 1999; Valko et al., 2007).

D. Detection of ROS

The three reactive oxygen species superoxide anion radical, hydrogen peroxide and hydroxyl radical are scavenged spontaneously by enzymatic dismutation. Hydroxyl radical is the most unstable with a half-life of 10^{-9} sec. Hydrogen peroxide is more stable, but it is highly metabolized either enzymatically by catalase and glutathione peroxidase, or low concentrations of transitional metals such as iron. Thus, the lifetime of hydrogen peroxide in the cell is depending on the concentration of protective enzymes in the closed environment and its high diffusibility of hydrogen peroxide crossing the membranes. The lifetime of superoxide anion radical cannot be calculated without a precision of local concentration of SOD and other substrates. However, the lifetime of superoxide anion radical in a cellular environment is expected to be very short. Therefore the highly sensitive analytical techniques for ROS detection is required (Batandier et al., 2002).

Hydroethidine (dihydroethidium, HE) is a cell-permeant compound that can undergo a two-electron oxidation to form the DNA-binding fluorophore ethidium bromide or a structurally similar product. The reaction is relatively specific for superoxide, with minimal oxidation induced by hydrogen peroxide (Budd et al., 1997; Tarpey et al., 2004). The oxidation of 2-7-dichlorofluorescin diacetate (DCFH) to the fluorescent compound 2-7-dichlorofluorescein (DCF) was initially thought to be a relatively specific indicator of hydrogen peroxide formation. The diacetate form of DCFH-DA is taken up by cells, where DCFH-DA is metabolized by intracellular esterases to DCFH, which has been suggested to trap intracellularly. In the presence of hydrogen peroxide, DCFH is oxidized to DCF; fluorescence is measured with excitation at 498 nm and emission at 522 nm. (Shen et al., 1996; Tarpey et al., 2004).

E. Defence mechanism against ROS

Exposure to free radicals from a variety of sources has led organisms to develop a series of defence mechanisms. Defence mechanisms against free radical-induced oxidative stress involve in antioxidant defences (Pinnell, 2003; Valko et al., 2007). Various kinds of antioxidants with different functions play an important role in these defense systems. The preventive antioxidants such as catalase and glutathione peroxidase acting in the first defense line suppress the formation of ROS (Yabe et al., 2001). The radical scavenging antioxidants such as flavonoids are responsible in the second defense line and inhibit chain initiation and break the chain propagation (Salah et al., 1995). The antioxidant enzymes such as phospholipases and DNA repair enzymes act as the third line defense in repairing the damage (Barnett and King, 1995). Antioxidants can be classified into two major groups: enzymatic and non enzymatic antioxidants. Enzymatic antioxidant defences are mainly endogenously produced including superoxide dismutase, glutathione peroxidase and catalase. Non-enzymatic antioxidants are mainly obtained from dietary sources. Dietary antioxidants can be classified into various classes, polyphenols is the largest class. Polyphenols consist of phenolic acids and flavonoids. (Ratnam et al., 2006).

F. Flavonoids

Flavonoids are benzo-γ-pyrone derivatives consisting of phenolic and pyrane rings (Figure 4) and are classified according to substitutions. Dietary flavonoids differ in the arrangement of hydroxyl, methoxy, glycosidic side groups and in the conjugation between the A- and B-rings (Heim et al., 2002). There is great interest in flavonoids because of their potential role as cancer chemopreventive agents such as quercetin (Yang et al., 2006) and epigallocathecin-3 gallate (Pianetti et al., 2002). This beneficial effect is considered to be mainly due to their antioxidant and chelating activities. The antioxidant activity of flavonoids and their metabolites in vitro depends on the arrangement of functional groups of the nuclear structure (Heim et al., 2002; Rice-Evans et al., 1996). The B-ring hydroxyl configuration is the most significant in scavenging of ROS. Flavonoids with 2,3 double bond in conjugation with a 4-carbonyl group exhibit stronger antioxidant activity compared to those saturated with a 4-carbonyl group (Heim et al., 2002). However, some flavonoids have been reported to be mutagenic or co-carcigenic. These effects may due to their pro-oxidant activities of flavonoids generating ROS under certain conditions such as in the presence of metal ions (Cao et al. 1997; Rietjens, et al., 2002)
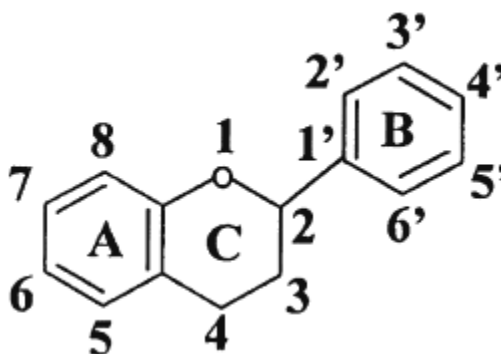
Figure 4. The nuclear structure of flavonoids (Heim et al., 2002)

G. Apoptosis and Necrosis Cell Death

Cell death is part of the normal development, maturation cycle, maintenance of tissue homeostasis, and the response patterns of living tissues to xenobiotic agents. Abnormalities of the cell death are important in cancer development, cancer prevention and cancer therapy (Kanduc et al., 2002). Cell death can be divided into

two classes, apoptosis and necrosis. Apoptosis is usually called programmed cell death as it is a cell-intrinsic mechanism for suicide that is regulated by a variety of cellular signaling pathways such as caspase. Apoptosis involves cellular shrinkage, condensation of nuclear chromatin, DNA fragmentation and the formation of apoptotic bodies and engulfment of apoptotic fragments by phagocytic cell. Theses alterations occur before membrane integrity is lost. Necrosis is usually called accidental type of death that cells receive a structural or chemical stimulation which cell are damaged and cannot recover. In contrast to apoptosis, early event of necrosis is the loss of plasma membrane integrity and allows water into the dying cell causing it to swell so that plasma and membrane burst. As a result, cells are released from the cytoplasmic contents into the surroundings and produces local inflammation. (Edinger and Thompson, 2004; Krysko et al., 2008; Lee et al., 2009; Schulze-Bergkamen et al., 2006).

Oxidative stress is one of the major factors associated with cell death. The intensity of the oxidative stress may determine the selection between apoptosis and necrosis (Baigi et al., 2008). It has been shown that cells divert the mode of cell death from apoptosis to necrosis after exposure with hydrogen peroxide (Gardner et al., 2007) or some alkylating compounds (Zong et al., 2004) .

The detection of apoptosis and necrosis generally used the fluorescent dyes, Hoechst 33342 and Propidium iodide (PI). During apoptosis, the plasma membrane undergoes multiple changes. Hoechst 33342, a blue fluorescence dye can diffuse through intact cell membrane stains the condensed chromatin in apoptotic cells more brightly than chromatin in normal cells. Propidium iodide, a red fluorescence dye cannot diffuse through intact cell membrane but only permeant to dead cell that membrane integrity was lost. The staining pattern from these dyes make the distinguish of normal, apoptosis and necrosis cell death (Sgonc and Gruber, 1998).

H. Bioavailability of Silymarin

The absorption and the solubility of the drug are the keys determinants of its oral bioavailability. Since the absorption of orally solid dosage forms occur when drugs were dissolved in the gastrointestinal tract. The solubility of poorly water soluble drug is a rate limiting step for permeation and oral bioavailability.

Improvement of the solubility increases the rate and extent of drug dissolution. An enhancement in the dissolution rate and extent of poorly water soluble drugs should increase the absorption and the bioavailability (Horter and Dressman, 1997; Leuner and Dressman, 2000).

The effectiveness of silymarin is limited because of its poor water solubility (430 mg/l) leads to low bioavailability after oral administration (Gazak et al., 2004). The oral absorption of silymarin is only about 23-47% and peak plasma concentration is about 6-8 hr. Eighty percent of absorbed silymarin is excreted via bile as glucuronide and sulfate conjugates and only 3-8% is excreted in the urine (Dixit et al., 2007). Many researchers have been investigated the preparations to improve the dissolution of silymarin including self-microemulsifying (Wu et al., 2006) and solid dispersion technique (Sun et al., 2007).

I. Solid Dispersion

Solid dispersion is the dispersion of the drug compound in a hydrophilic carrier and when the solid dispersion was exposed to water or gastrointestinal fluids, the soluble carrier would dissolve rapidly and the finely dispersed drug particles would then be released in very fine almost in micron or submicron range. There are many mechanisms for increasing dissolution of drug: the reduction of particle size of the drug within the dispersion, increasing wettability and dispersibility of a drug, formation of soluble compound or complex between drug and carrier, and transformed the crystalline from of the drug to an amorphous form (Craig, 2002; Leuner and Dressman, 2000; Sethia and Squillante, 2004; Verheyen et al., 2002).

There are two conventional methods of preparation solid dispersion, melting (fusion) and solvent method. Melting method is prepared by melt the drug and carrier and cool to form a homogeneous solid dispersion. This method is simplicity and economy but the disadvantages of melting method are immiscibility between drug and carrier may occur during fusion, only low melting point drugs or carriers can be used due to the problems of thermal degradation, and the solid dispersion product may be tacky and hard to fill into capsule. Solvent method is prepared by dissolve drug and carrier in suitable organic solvent and evaporate the solvent, finally, pulverize the product. The advantages of solvent method is the prevention of thermal degradation

of drugs or carriers but the disadvantages of this method is the difficulty in completely removing liquid solvent which may lead to toxicity problem (Leuner and Dressman, 2000; Sun et al., 2007). Sun et al., 2007 prepared the solid dispersion using fluidized-bed coating. The fluidized-bed coating method is more advantages than both conventional methods that the solid dispersions from fluidized-bed coating are easy to fill into capsule and the fluidized-bed coating is highly efficient for remove the solvent.

J. Carriers

The selection of the carrier has influence on the dissolution characteristics of the dispersed drug. Using a water soluble carrier combined with poorly water soluble drug resulted in a fast release of drug from the matrix (Khan and Zhu, 1999). Whereas using a poorly water soluble carrier combined with a good water soluble drug leads to a retardation of drug release from the matrix (Tiwari, et al., 2003). To increase the dissolution of drug, hydrophilic carrier chosen for the preparation of solid dispersion should meet the following criteria: freely water soluble, non-toxic, compatible with the drug, and pharmacologically inert (Leuner and Dressman, 2000). The interesting hydrophilic carriers which are widely used are polymers, especially, polyethylene glycols (PEGs) and polyvinylpyrrolidone (PVP). Other hydrophilic carriers used are urea, bile acids and surfactants.

1. Polyethylene glycols (Macrogols, Polyoxyethylene glycols, PEGs)

Polyethylene glycols are mixture of condensation polymers of ethylene oxide and water. The molecular weight, indicated the number in the name, vary from 200 to 300000 and the molecular weight used for solid dispersions vary from 1500 to 20000. Their solubility in water is good but decrease with molecular weight. PEGs of molecular weight 4000-6000 are the most frequently used for solid dispersion preparation because in these molecular weights the water solubility is still very high (Craig, 1995; Leuner and Dressman, 2000). Many researchers have been improve the dissolution of poorly water soluble drug by using PEGs 4000-6000 as carrier in solid dispersion preparation including alkyl p-aminobenzoates (Saers and Craig, 1992), diazepam (Rabasco et al., 1991), fenofibrate (Sheu et al., 1994), ketoprofen (Margarit

et al., 1994), nifedipine (Law et al., 1992), phenytoin (Franco et al., 2001), and piroxicam (Fernandez et al., 1993). An advangtage of PEGs for the formation of solid dispersions is that they have good solubility in many organic solvents. In addition, the benefits of the PEGs including their ability to solubilize some compounds and also improve compound wettability.

2. Polyvinyl pyrrolidone (Povidone, Polyvidone, Kollidon, PVP)

Polyvinyl pyrrolidone is the polymerized of vinylpyrrolidone. Molecular weights of PVP ranging from 2500-3000000. PVP can be classified to the K value (Table 1). Likewise PEGs, PVP is good water solubility and can improve the wettability of the dispersed drug. Molecular weights of PVP have an influence on the dissolution of the drug. The solubility in water of PVP decreases with increasing molecular weight due to their much higher viscosity (Leuner and Dressman, 2000). Most studied of PVP solid dispersion have used PVP of molecular weight ranging from 2500-50000 (K12-K30) and especially, PVP K30. The enhancement of poorly water soluble drugs using PVP as carrier in solid dispersion including indomethacin (Hilton and Summer, 1986), frusemide (Doherty and York, 1987), albendazole (Torrado et al., 1996) and silymarin (Sun et al., 2007)

Table 1 K values and the molecular weights of PVP (Leuner and Dressman, 2000)

K value	Approximate molecular weight
12	2500
15	8000
17	10000
25	30000
30	50000
60	400000
90	1000000
120	3000000

CHAPTER III

MATERIALS AND METHODS

Cell cultures

Normal human kidney cell, HK-2 cells (ATCC$^®$, USA, Lot no. 4738634)

Cell cultures medium

1. Antibiotic Antimycotic Solution 100X (10,000 units penicillin, 10 m g streptomycin and 25 μg amphotericin B per ml) (Sigma-Aldrich Inc., USA, Lot no.028K2402)

2. Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen Corporation, USA, Lot no.1384657)

3. Fetal Bovine Serum (FBS) (Research grade) EU approved triple 0.1 μm sterile filtered (Thermo Fisher Scientific Inc., USA, Lot no.CSE0441)

4. Phosphate Buffered Saline (PBS)10X pH 7.4 (Invitrogen Corporation, USA, Lot no.1395685)

5. Trypsin (1:250) porcine parvovirus tested (Invitrogen Corporation, Canada, Lot no.1256246)

Accessories for Cell cultures

1. Disposable Sterile Pipette 5 m l (Corning Incorporated, USA, Lot no.09008003)

2. Disposable Sterile Pipette 10 m l (Corning Incorporated, USA, Lot no.08108002)

3. 96-well Cell Cultured plate (Corning Incorporated, USA, Lot no.1208022)

4. 25 cm^2 Cell Culture Flask, Canted Neck with 0.2 μm Vent Cap (Corning Incorporated, USA)

5. Cryogenic Vial (Corning Incorporated, USA, Lot no.15508044)

6. Microcentrifuge Tube 1.5 ml (Corning Incorporated, USA)

7. Centrifuge tube 15 ml, Plug Seal Cap (Corning Incorporated, USA)

8. Centrifuge tube 50 ml, Plug Seal Cap (Corning Incorporated, USA)

9. Pipette Tips, 1-200 l Universal Fit Pipette Tips (Corning Incorporated, USA)

10. Pipette Tips, 100-1000 l Universal Fit Pipette Tips (Corning Incorporated, USA)

Chemicals for cell cultures tests

1. 2,3-Dimethoxy-1,4-naphthoquinone (DMNQ) (Sigma-Aldrich Inc., USA, Lot no.086K1983)

2. 2$^{'}$, 7$^{'}$-dichlorofluorescein diacetate (DCF-DA) (Sigma-Aldrich Inc., USA, Lot no. 115K4039)

3. biz Benzimide H33342 trihydrochloride (Sigma-Aldrich Inc., USA, Lot no.G46K4017)

4. Catalase from bovine liver (Sigma-Aldrich Inc., USA, Lot no.G34K7064)

5. Cisplatin (Sigma-Aldrich Inc., USA)

6. Dihydroethidium (DHE) (Fluka, USA)

7. Dimethylsulfoxide (DMSO) Analytical Grade (Labscan Asia., Ltd., Thailand, Lot no.07030033)

8. Ferrous sulphate (FeSO$_4$.7H$_2$O) (Ajax Finechem, Australia, Lot no.AF511300)

9. Hydrogen peroxide solution (3%w/v) (Siribancha, Thailand)

10. Mn (III) tetrakis (4-benzoic acid) porphyrin (MnTBAP) (EMD Chemicals Inc., Germany, Lot no.D0030652)

11. Propidium Iodide (Sigma-Aldrich Inc., USA, Lot no.037K3676)

12. Sodium formate (Sigma-Aldrich Inc., USA, Lot no.O5227CE)

13. Thiazolyl Blue Tetrazolium Bromide, approx. 98% TLC (Sigma-Aldrich Inc., USA, Lot no.085K5304)

14. Trypan blue stain 0.4% (Invitrogen Corporation, USA, Lot no.1368311)

Chemicals for preparation of solid dispersions and dissolution testing

1. 14/18 CH Sugar Spheres, NF White (CHR HANSEN, USA, Lot no.2534480)
2. Absolute Ethanol, AR grade (Labscan Asia., Ltd., Thailand, Lot no.07080175)
3. Avicel PH101 (Ajax Finechem, Australia)
4. Methanol, HPLC grade (Lab Scan Co., Ltd., Thailand, Lot no.06080013)
5. Polyethylene glycol 4000 (Srichand United Dispensary Co., Ltd., Thailand, Lot no.PHF05700)
6. Polyethylene glycol 6000 (Ajax Finechem, Australia, Lot no.AF401212)
7. Polyvinylpyrrolidone K30 (BASF, Germany)
8. Silymarin Soluble (Powdered Milk Thistle Extract) (Berlin Pharmaceutical Industry Co., Ltd., Thailand, Lot no. 0710220)
9. Sodium dihydrogen phosphate (Ajax Finechem, Australia, Lot no.0710220)
10. Sodium hydroxide (Merck, Germany, Lot no.B020089880B)
11. Sodium lauryl sulphate (Ajax Finechem, New Zealand, Lot no.0802175)

Apparatus

1. Analytical balance (Model AX105, Mettler Toledo, Switzerland)
2. CO_2 Incubator (Model 311, Thermo Electron Corp., USA)
3. Dissolution testing station (Model VK7000, Vankel Industries, Inc., USA)
4. Flow cytometry (Becton Dickinson, USA)
5. Fluid bed coating (Aeromatic Fielder AG, Germany)
6. Laminar hood biosafety cabinet class II (Model ABS1200CL32MK2, ASTEC Microflow, UK)
7. Magnetic stirrer (Model RCT basic, KIKA® Works Guangzhou, China)
8. Micropipette (Biohit, Finland)
9. Microplate reader (Anthos Labtec, UK)
10. Microscope (Model CKX41, OLYMPUS®, Germany)
11. pH meter (Orion model 420A, Orion Research Inc., USA)
12. Reversed microscope (Model IX, OLYMPUS®, Germany)
13. Scanning Electron Spectroscopy (SEM) (Shimadzu, Japan)
14. Sonicator (Model TP680DH, Elma, Germany)
15. UV-Vis spectophotometer (UV-1601, Shimadzu, Japan)
16. Vortex mixer (Vortex Ginies-2, Scientific Industries, USA)

17. Water bath (Model WB22, Memmert, Germany)

Methods

A. Preparation of Silymarin Solid Dispersion by Fluidized-Bed Coating Technique

1. Calculation of silymarin amount for fluid-bed coating

Non-pareil sugar beads in the amount of 60 g were used as cores of solid dispersion. Silymarin in the amount of 8.4 m g was accurately weighed to give an equivalent amount of silymarin 70 mg in 500 mg of non-pariel sugar beads.

2. Preparation of silymarin and carrier solution

The carriers used for this study were PEG 4000, PEG 6000 and PVP K30. The ratios between silymarin and carriers were varied as shown in Table 2. An accurately weighed amount of carriers were melted at 50°C in a water bath. Silymarin and melted carrier were dissolved in 1200 ml of 40% absolute ethanol and stirred continuously to give a homogeneous solution. Avicel PH101 was added at 6%w/v as a glidant in the preparation of silymarin solid dispersion by fluidized-bed coating.

Table 2. Ratios varied and carrier used in preparation of silymarin solid dispersions

Formulation	Carrier	Silymarin:Carrier (%w/w)
F1	PEG 4000	1:2
F2		1:4
F3		1:8
F4	PEG 6000	1:2
F5		1:4
F6		1:8
F7	PVP K30	1:2
F8		1:4
F9		1:8

3. Fluidized-bed coating condition

Preparation of silymarin and carriers solid dispersion on non -pariel sugar beads were performed in a fluid-bed coater. The solution of silymarin and carriers were sprayed through a nozzle onto the fluidized non-pariel sugar beads. The operating conditions were as follow:

Inlet air temperature: 47°C

Fan capacity: 90-130 m^3/h

Atomizing air pressure: 2 bar

Spray rate: 2 ml/min

After finished spraying, the silymarin solid dispersion pellets were dried for 15-20 min at 30°C in a coating chamber. Pellets were stored in sealed containers until analysis.

4. Characteristics of solid dispersion

The surface and cross-section morphology of silymarin solid dispersion pellets were investigated under Scanning Electron Microscopy.

B. Determination of Silymarin Content in Solid Dispersions Pellets

UV-VIS spectrophotometric method was used for analyzing of silymarin content in solid dispersion pellets.

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1. Preparation of standard solutions

Silymarin in the amount of 2.41 m g was accurately weighed and then transferred to a 25 ml volumetric flask. Methanol was used to dissolve and adjust the volume to make the final concentration of silymarin stock solution at 200 μmol/l. Standard solutions of silymarin were prepared by pipetting 250, 375, 50 0, 625, 750, 875, 1000 a nd 1125 μl of silymarin stock solutions into 5 m l volumetric flasks, diluted and adjusted to volume with methanol to give the final concentrations of the solution to 10, 15, 20, 25, 30, 35, 40 a nd 45 μmol/l respectively. Then, standard

solutions prepared were analyzed spectrophotometrically at the wavelength 288 nm. Methanol was used as a blank. The standard curve was plotted between concentration and absorbance.

2. Validation of UV-vis spectrophotometric method

The analytical parameters used for the assay validation were specificity, linearity, accuracy and precision.

2.1 Specificity

Specificity is the ability to assess non-interference of an analyte in the presence of components which may be expected to be present. Silymarin and other components of solid dispersion pellets: non-pariel sugar beads, PEG and PVP K 30 in methanol were analyzed by UV-VIS spectrophotometric method. Under the condition selected for study, the absorbance of silymarin must not be interfered by the absorbance of other components in the sample.

2.2 Linearity

The linearity of an analytical procedure is its ability to obtain test results, which are directly proportional to the concentration of analyte in the sample. Three sets of nine standard solutions of silymarin ranging from 10 to 45 μmol/l were prepared and analyzed. Linear regression analysis of the absorbances versus their concentrations was performed. The linearity was determined from the coefficient of determination (R^2).

2.3 Accuracy

The accuracy of an analytical procedure expresses the closeness of agreement between the value, which is accepted as a conventional true value and the value found. Five sets of three concentrations of silymarin in methanol at 17.5, 27.5 and 37.5 μmol/l were prepared and analyzed. These solutions were prepared by pipetting stock solution of silymarin for 875, 1375 and 1875 μl respectively into 10 ml volumetric flasks, diluted and adjusted to volume with methanol. The accuracy of this method was determined from the percentage of recovery. The percentage of recovery

of each concentration was calculated from the estimated concentration to known concentration multiplied by 100. Acceptance criteria for the accuracy, the percentage of recovery should be within 98%-102% (USP 29, 2006).

2.4 Precision

The precision of an analytical procedure expresses the closeness of agreement between a series of measurements obtained from multiple sampling of the same homogeneous sample under the same conditions. Precision may be considered at two levels: within run precision and between run precision. Acceptance criteria for the accuracy, the percent coefficient of variation should be less than 2% (USP 29. 2006).

1). Within run precision

Within run precision expresses the precision under the same operating conditions over a short interval of time. The within run precision was determined by analyzed five sets of three concentrations (low, medium and high) of silymarin at 17.5, 27.5 and 37.5 μmol/l in the same day. Concentration of silymarin was calculated and the percent of coefficient of variation (%CV) of each concentration was determined.

2). Between run precision

Between run precision expresses within-laboratories variations: different days, different analysts, different equipment, etc. The precision during the operation run was determined by analyzing three concentrations (low, medium and high) of silymarin at 17.5, 27.5 and 37.5 μmol/l on five different days. Concentration of silymarin was calculated and the percent of coefficient of variation of each concentration was determined.

3. Assay of silymarin content in solid dispersions pellets

Silymarin solid dispersions pellets in the amount of 500 mg were accurately weighed and transferred to 25 ml volumetric flask. Methanol was used to dissolve and adjust to volume. The solution was sonicated for 30 min. Then, solution was diluted and adjusted to volume with methanol. The solution was filtered through 0.45 μm of

filtered membrane and analyzed by UV-VIS spectrophotometric method. The content of silymarin was calculated from standard curve. The percent recovery was calculated from the estimated concentration to known concentration multiplied by 100.

C. Dissolution Testing of Silymarin Solid Dispersions

1. Preparation of dissolution medium (USP 29, 2006)

1.1 Preparation of dissolution medium pH 7.5

Monobasic sodium phosphate in the amount of 27.6 g, sodium hydroxide in the amount of 6.08 g, and sodium lauryl sulfate in the amount of 80 g were dissolved in water and diluted with water to 4000 ml.

1.2 Preparation of dissolution medium pH 6.8

Fifty ml of 0.2 m ol/l monobasic potassium phosphate solution was placed in a 200 ml volumetric flask, 22.4 ml of 0.2 mol/l sodium hydroxide solution was added. The mixture was diluted with water to volume. Monobasic potassium phosphate at the concentration of 0.2 m ol/l was prepared by dissolve 27.22 g of monobasic potassium phosphate in water and diluted with water to 1000 ml. Sodium hydroxide solution at the concentration of 0.2 mol/l was prepared by dissolve 32.4 g of sodilum hydroxide in 150 ml of water and dilute to 1000 ml with water.

1.3 Preparation of dissolution medium pH 1.2

Fifty ml of 0.2 mol/l potassium chloride solution was placed in a 200 ml volumetric flask, 85 ml of 0.2 mol/l hydrochloric acid was added. The mixture was diluted with water to volume. Potassium chloride at the concentration of 0.2 mol/l was prepared by dissolved 14.91 g potassium chloride in water and diluted with water to 1000 ml. Hydrochloric acid at the concentration of 0.2 M was prepared by aliquot hydrochloric acid for 85 ml and dilute with water to 1000 ml.

2 Validation of UV-VIS spectrophotometric method

The analytical parameters used for the assay validation method in dissolution media pH 7.5, 6.8 a nd 1.2 w ere specificity, linearity, accuracy and precision as previously described.

3. Dissolution testing of silymarin solid dispersions

Silymarin pellets (500 mg or equivalent to 70 m g of silymarin) were transferred to capsule no.2. Dissolution test of silymarin solid dispersions were carried out in 900 ml of dissolution medium equilibrated at 37 ± 0.5°C. Dissolution tests were performed with USP apparatus type II (paddle) at the rate of 100 r pm. Silymarin capsule (n=6) were random by introduced in each vessel. Three milliliter of samples were collected at 10, 25, 45, 60, 120, 24 0 and 480 m in and filtered through 0.45 μm of membrane filter. The volume withdrawn each time was then replaced by equivalent amount of dissolution medium to maintain a constant volume of dissolution medium during the test. The concentration of drug dissolved was analyzed using UV-VIS spectrophotometric method and calculated from standard curve. No less than 75% of silymarin is dissolved in 45 minutes (USP 29, 2006).

D. Cytoprotectivity of Silymarin against ROS in HK-2 Cells

1. Cell cultures

Human Kidney cells (HK-2) were grown in DMEM supplemented with heat-inactivated FBS (10% v/v), penicillin (10,000 units/ml), streptomycin (10 mg/ml) and amphotericin B (25 μg/ml). Cells were cultured in a humidified atmosphere with 5% CO_2 at 37 ± 0.5 °C. For all experiments in this section, cells were seeded in 96-well cell cultured plates at density of 1×10^5 cells/well and grew near 70-80% confluence in 24 hr.

2. Sample preparation

2.1 Preparation of silymarin solution

Silymarin in the amount of 12.05 m g was dissolved in 1 m l of methanol to give a stock solution at concentration of 40 mmol/l and diluted with PBS to give concentrations of 250, 500, 1000, 2000 and 4000 μmol/l.

2.2 Preparation of ROS generators solutions

1). Preparation of superoxide anion radical solution

DMNQ (2, 3-Dimethoxy-1,4-naphthoquinone) was used to generate superoxide anion radical in cells. One milligram of DMNQ was dissolved in 1 ml DMSO to make 4 mmol/l of stock solution. The solution was then diluted with PBS to give concentrations of 100, 200, 300 and 400 μmol/l.

2). Preparation of hydrogen peroxide solution

Hydrogen peroxide 3% v/v was used to generate hydrogen peroxide in cells. Forty-five μl of hydrogen peroxide 3% v/v was diluted with PBS to give a stock solution at concentration of 40 mmol/l and diluted with PBS to give concentrations of 1000, 2500, 5000 and 10000 μmol/l.

3). Preparation of hydrogen peroxide/ferrous sulfate solution

Ferrous sulfate and 3% v/v hydrogen peroxide were used to generate hydroxyl radical in cells. Ferrous sulfate in the amount of 11.2 mg and 45 μl of 3% v/v hydrogen peroxide were dissolved with 1 ml of PBS to give a stock solution at concentration of 40 mmol/l. and diluted with PBS to give concentrations of 1000, 2500, 5000 and 10000 μmol/l.

3. Cytotoxicity of ROS generators in HK-2 cells

Cells were treated with either silymarin or ROS generators (10 μl/well) at various concentrations and were placed in 5% CO_2 at 37 ± 0.5 °C for 24 hr. Control cells contained only medium with the same aliquot of DMSO/PBS or methanol/PBS instead of test compounds. The final concentration of DMSO and methanol in medium were 0.5%. The cytotoxicity after treatment was examined using MTT assay. Briefly, after treatment incubation, medium containing test compounds were removed and washed with 100 μl PBS for 2 times. Then the medium was replaced by MTT solution for 50 μl/well. This MTT solution was freshly prepared in serum free medium to give a final concentration of 0.45 mg/ml. The plates with added MTT solution were then wrapped in aluminium foil and were placed in 5% CO_2 at 37 ± 0.5 °C incubator for 4 hr. The MTT solution was removed and 100 μl of DMSO was added to each well to dissolve the formazan crystals. Read the absorbance at wavelength 570 nm and 620 nm as a background wavelength using microplate reader. All experiments were performed with three replicates for each sample. The cytotoxicity of silymarin and ROS generators were expressed as the percentage of cell viability. The percentange cell viability was calculated using equation 1. The non-toxic concentration of silymarin and the half-maximal concentration of inhibitory concentration (IC_{50}) of ROS generators were chosen for evaluating the cytoprotective effect of silymarin against ROS.

% cell viability   = $\dfrac{\text{absorbance of treatment} \times 100}{\text{absorbance of control}}$ (equation1)

4. Cytoprotective effect of silymarin against ROS generators in HK-2 cells

Cells were pretreated with silymarin solution at non-toxic concentration for 30 min followed by ROS generator solutions at IC_{50} and were placed in 5% CO_2 at 37 ± 0.5 °C incubator for 24 hr. After treatment, the protective effect of silymarin was estimated using MTT assay as previously described. The cytoprotectivity of silymarin against ROS was expressed as the percent cell viability.

E. ROS Scavenging Activity of Silymarin against ROS Generators-Induced ROS Production in HK-2 Cells

1. Cell cultures

Cells were cultured in a humidified atmosphere with 5% CO_2 at 37 ± 0.5 °C. For all experiments in this section, cells were seeded in 96-well cultured plate at density of 1×10^5 cells/well or were cultured in 25-cm^2 cultured flask and grew near 70-80% confluence in 24 hr.

2. Preparation of fluorescent compounds for ROS detection

2.1 Detection of superoxide anion radical generation

Dihydroethidium (DHE) was used as a fluorescent probe for detecting superoxide anion radical. One mg of DHE was freshly prepared in 1 ml of DMSO to give a stock at concentration of 500 μmol/l and diluted with serum-free medium to final concentration of 50 μmol/l.

2.2 Detection of hydrogen peroxide and hydroxyl radical generation

2', 7' dichlorodihydrofluorescein diacetate (DCFH-DA) was used as a fluorescent probe for detecting hydrogen peroxide and hydroxyl radical. DCFH-DA in the amount of 0.5 mg was freshly prepared in 1 ml of DMSO to give a stock solution

at concentration of 1 mmol/l and diluted with PBS to give a final concentration of 100 μmol/l.

3. Investigation intracellular ROS response from ROS generators in HK-2 cells

Medium were removed, cell were washed with 1 ml PBS for 2 times and incubated with 5 μmol/l of DHE or 10 μmol/l of DCFH-DA at 4 °C for 30 min. After incubation, cells were placed on ice, the DHE or DCFH-DA solution were removed, washed with 1 ml PBS for 3 times and added with 12 ml of serum-free medium. Cells were scrapped from 25 cm^2-cell cultured flask using cell scrapper and suspended in microcentrifuge tube for 1 ml of serum free medium/tube. ROS generators (10 μl) were added and were placed in 5% CO_2 at 37 $\pm$ 0.5 °C incubator for 30 min for hydrogen peroxide and hydrogen peroxide/ferrous sulphate and 3 hr of DMNQ. After incubation, ROS production from ROS generators was investigated as fluorescent intensity of DHE or DCFH-DA using reversed microscope and flow cytometer.

4. Investigation of ROS scavenging activity of silymarin in HK-2 cells

Medium were removed, cell were washed with 1 ml PBS for 2 times and incubated with either 5 μmol/l of DHE or 10 μmol/l of DCFH-DA at 4 °C for 30 min. After incubation, Cells were placed on ice, the DHE or DCFH-DA solution were removed, washed with 1 ml PBS for 3 times and added 12 ml serum-free medium. Cells were scrapped from 25 cm^2-cell cultured flask and suspended in microcentrifuge tube for 1 ml/tube. Cells were pre-treated with non-toxic concentration of silymarin solution for 30 min followed by ROS generators at mentioned time in 5% CO_2 at 37 $\pm$ 0.5 °C incubator. After incubation, the ROS scavenging activity of silymarin was evaluated as the fluorescent intensity of DHE or DCFH-DA using reversed microscope and flow cytometer.

F. Cytoprotectivity of Silymarin against Cisplatin-Induced Cytotoxicity in HK-2 Cells

1. Cell cultures

Cells were cultured in a humidified atmosphere with 5% CO_2 at 37 ± 0.5 °C. For all experiments in this section, cells were seeded in 96-well cell cultured plates at density of 1×10^5 cells/well and grew near 70-80% confluence in 24 hr.

2. Sample preparation

2.1 Preparation of cisplatin solution

Cisplatin in the amount of 3 mg was dissolved in 1 ml of DMSO to give a stock solution at concentration of 10 mmol/l and diluted with PBS to give concentrations of 200, 400, 600, 800 and 1000 μmol/l.

3. Investigation the cytotoxicity of cisplatin in HK-2 cells

Cells were treated with cisplatin solution at various concentrations for 10 μl/well and were placed in 5% CO_2 at 37 ± 0.5 °C incubator for 24. Control cells contain only medium with the same aliquot of DMSO/PBS or methanol/PBS instead of the test compounds. The final concentration of DMSO and methanol in medium were 0.5%. The cytotoxicity of cisplatin was examined using MTT assay as previously described. The cytotoxicity of cisplatin was expressed as the percent cell viability. The IC_{50} of cisplatin was selected for determining the cytoprotectivitiy of silymarin against cisplatin.

4. Investigation the cisplatin-induced apoptosis and necrosis in HK-2 cells

Cells were treated with cisplatin at IC_{50} and were incubated for 24 hr in 5% CO_2 at 37 ± 0.5 °C. After incubation, medium were removed and cells were washed with PBS (100 μl/well). Apoptosis and necrosis cell death were investigated using hoescht 33342 and propidium iodide (PI) staining respectively. Hoescht 33342 and PI

were added (100 μl/well) and kept in the dark for 15 min. Then, the apoptosis and necrosis were characterized by the fluorescent intensity of hoescht 33342 and PI using reversed microscope.

5. Investigation of the cytoprotectivity of silymarin against cisplatin in HK-2 cells

Cells were pre-treated with silymarin solution at non-toxic concentration for 30 min followed by cisplatin at IC_{50} and were placed in 5% CO_2 at 37 ± 0.5 °C incubator for 24 hr. After treatment, the protective of silymarin was estimated using MTT assay as previously described. The cytoprotectivity of silymarin against cisplatin was expressed as the percent cell viability.

6. Effect of silymarin against cisplatin-induced apoptosis and necrosis in HK-2 cells

Cells were pre-treated with silymarin solution at non-toxic concentration for 30 min followed by cisplatin at IC_{50} and were were placed in 5% CO_2 at 37 ± 0.5 °C incubator for 24 hr. After incubation, cells were removed medium and washed with PBS 100 μl/well. Apoptosis and necrosis cell death were investigated using hoescht 33342 and PI staining as previously described.

G. Antioxidant Activity of Silymarin against Cisplatin-Induced ROS Production in HK-2 Cells

1. Cell cultures

Cells were cultured in a humidified atmosphere with 5% CO_2 at 37 ± 0.5 °C. For all experiments in this section, cells were seeded in 96-well cell cultured plates at density of 1×10^5 cells/well or were cultured in 25-cm^2 cultured flask and grew near 70-80% confluence in 24 hr.

2. Sample preparation

2.1 Preparation of ROS scavengers solution

1). Preparation of MnTBAP solution

MnTBAP was used as a superoxide anion scavenger. MnTBAP in the amount of 4.23 m g was dissolved in 1 m l of DMSO to make a stock solution at concentration of 5 mmol/l and diluted with PBS to give a concentration of 50 μmol/l.

2). Preparation of catalase solution

Catalase was used as a hydrogen peroxide scavenger. Catalase in the amount of 3 m g was dissolved in 1 m l of PBS to make a stock solution at concentration of 150,000 units/ml and diluted with PBS to give a concentration of 15,000 units/ml.

3). Preparation of sodium formate solution

Sodium formate was used as a hydroxyl radical scavenger. Sodium formate in the amount of 3.4 m g was dissolved in 1 m l of PBS to make a stock solution at concentration of 500 mmol/l and diluted with PBS to give a concentration for 50 mmol/l

4). Preparation of deferoxamine solution

Deferoxamine was used as an iron chelator, inhibit conversion of hydrogen peroxide to hydroxyl radical. Deferoxamine in the amount of 3.3 mg was dissolved in 1 ml of PBS to make a stock solution at concentration of 1000 μmol/l and diluted with PBS to give a concentration of 100 μmol/l

3. Investigation of cisplatin-induced ROS production in HK-2 cells

Medium were removed, cell were washed with 1 ml PBS for 2 times and incubated with 10 μmol/l of DCFH-DA at 4 °C for 30 m in. After incubation, cells were placed on ice, the DCFH-DA solution were removed, washed with 1 ml PBS for 3 times and added 12 ml serum-free medium. Cells were scrapped from 25 cm^2-cell

cultured flask using cell scrapper and suspended in microcentrifuge tube for 1 ml of serum free medium/tube. Cisplatin at IC_{50} were added for 10 μl and were placed in 5% CO_2 at 37 ± 0.5 °C incubator for 2 hr. After incubation, ROS production from cisplatin was investigated as fluorescent intensity of DCFH-DA using reversed microscope and flow cytometer.

4. Investigation the cytotoxicity of ROS scavengers

Cells were pre-treated with MnTBAP, catalase, deferoxamine and sodium formate solution at concentrations above for 10 μl/well and were were placed in 5% CO_2 at 37 ± 0.5 °C incubator for 24 and 48 hr. After incubation, the cytotoxicity of ROS scavengers were evaluated by the MTT assay as previously described.

5. Determination of primary mechanism of cisplatin-induced cell death

Cells were pre-treated with MnTBAP, catalase, deferoxamine and sodium formate solution at concentrations above for 10 μl/well and placed in 5% CO_2 incubator for 30 min followed by cisplatin at IC_{50} were added for 10 μl/well and were placed in 5% CO_2 at 37 ± 0.5 °C incubator for 24 and 48 hr. After incubation, the MTT assay was used for determination the percent cell viability as previously described. The types of ROS production from cisplatin were determined.

6. Antioxidant activity of silymarin against cisplatin in HK-2 cells

Medium were removed, cell were washed with 1 ml PBS for 2 times and incubated with 10 μmol/l of DCFH-DA at 4 °C for 30 min. After incubation, cells were placed on ice, the DCFH-DA solution were removed, washed with 1 ml PBS for 3 times and added 12 ml serum-free medium. Cells were scrapped from 25 cm^2-cell cultured flask using cell scrapper and suspended in microcentrifuge tube for 1 ml of serum free medium/tube. Cells were pre-treated with non-toxic concentration of silymarin for 30 min followed by cisplatin at IC_{50} for 10 μl and were placed in 5% CO_2 at 37 ± 0.5 °C incubator for 2 hr. After incubation, ROS production from cisplatin was investigated as fluorescent intensity of DCFH-DA using reversed microscope and flow cytometer.

H. Statistical Analysis

Data are represented as mean ± SD (standard deviation). Statistical analysis was performed using one-way analysis of variance (ANOVA). p-value of less than 0.05 was considered significantly.

CHAPTER IV

RESULTS AND DISCUSSION

A. Analytical Method Validation of Silymarin by UV-VIS Spectrophotometry

The silymarin contents in methanol, dissolution media pH 1.2, 6.8 a nd the medium pH 7.5 w ith 2% SLS were determined using a validated UV-VIS spectrophotometric method. The analytical method of silymarin was validated in various media to ensure suitability and reliability of the test method. The analytical parameters were specificity, linearity, accuracy and precision.

1. Specificity

The UV absorption spectra of silymarin in methanol and dissolution media are shown in Figures 5-8. The maximum absorption was found at the wavelength of 288 nm. The blank solution (non-pariel sugar beads and PEG or PVP K 30) did not show any peak at 288 nm suggesting that the components in the formulation was not interfered with the peak of silymarin in all media. These results demonstrated that UV-VIS spectrophotometric method used in this study was able to detect and separate silymarin from other components in the formulation.

(A)

(B)

(C)
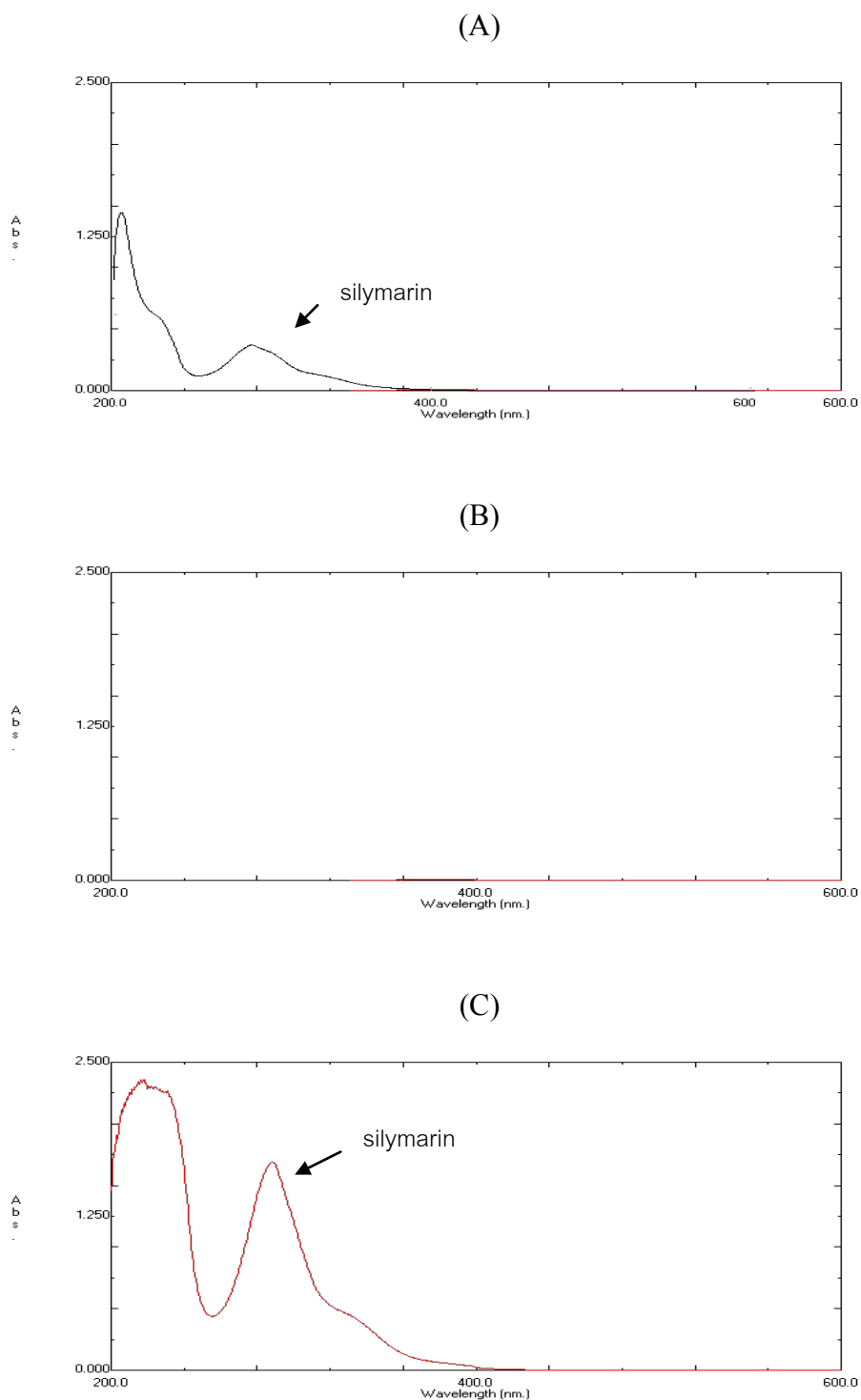
Figure 5 Absorption spectra of (A) 0.7 mg/ml of silymarin in methanol, (B) blank solution: non-pareil sugar beads and PEG or PVP K30 in methanol (C) silymarin solid dispersion pellets in methanol.

(A)

(B)

(C)

Figure 6 Absorption spectra of (A) 0.7 mg/ml of silymarin in dissolution medium pH 1.2, (B) blank solution: non-pareil sugar beads and PEG or PVP K30 in dissolution medium pH 1.2 (C) silymarin solid dispersion pellets in dissolution medium pH 1.2.

(A)

(B)

(C)

Figure 7 Absorption spectra of (A) 0.7 mg/ml of silymarin in dissolution medium pH 6.8, (B) blank solution: non-pariel sugar beads and PEG or PVP K30 in dissolution medium pH 6.8 (C) silymarin solid dispersion pellets in dissolution medium pH 6.8.
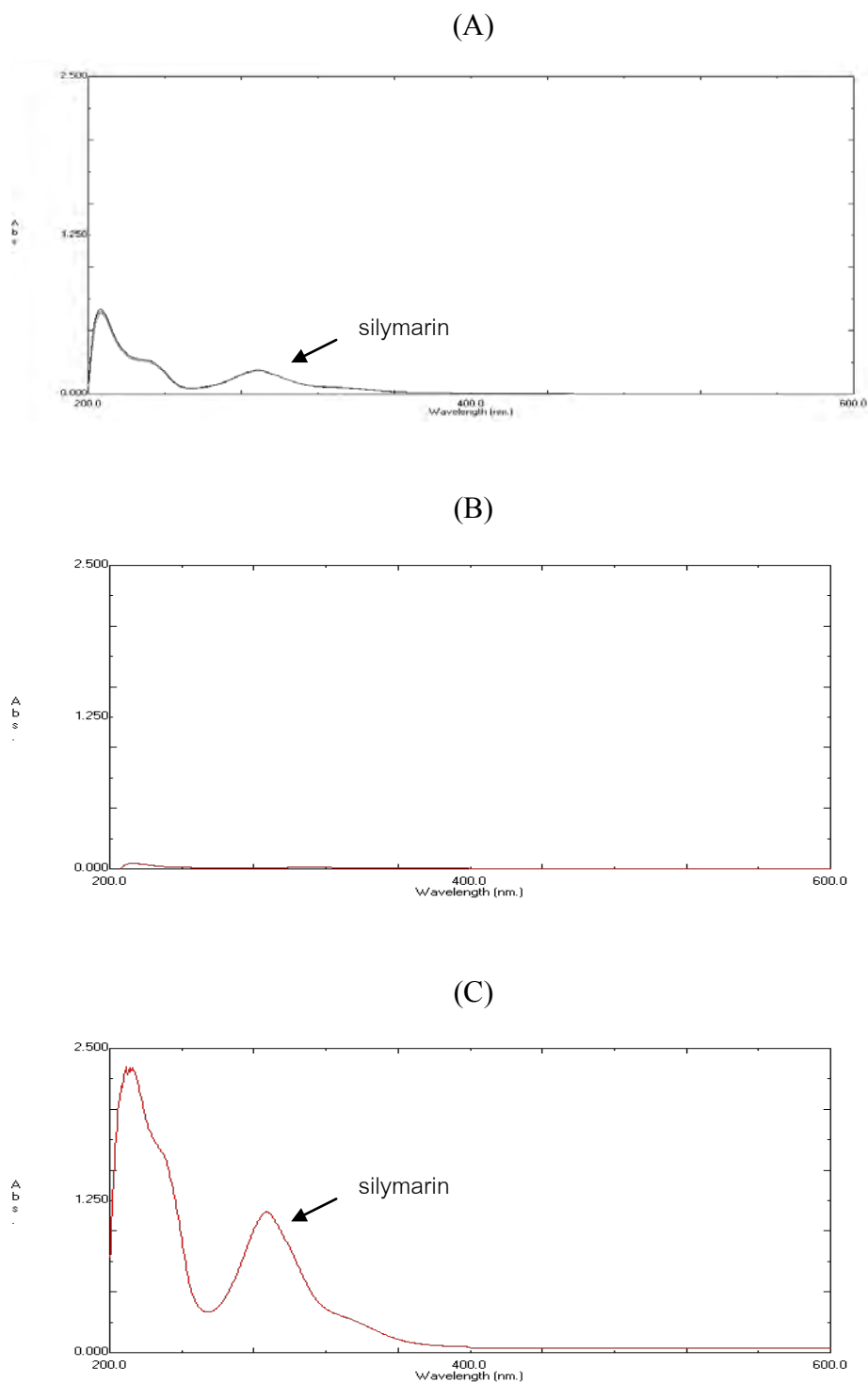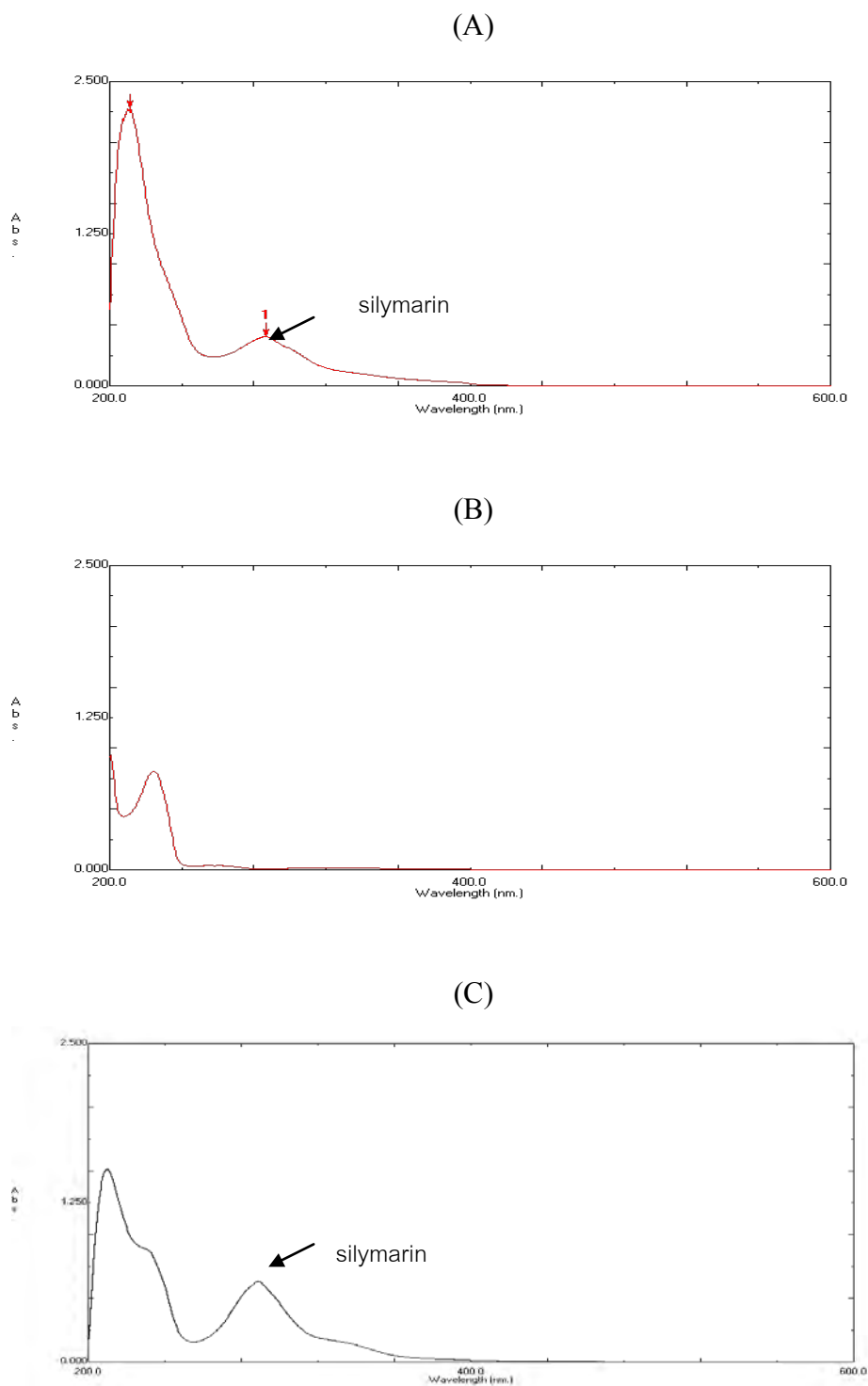
(A)

(B)

(C)
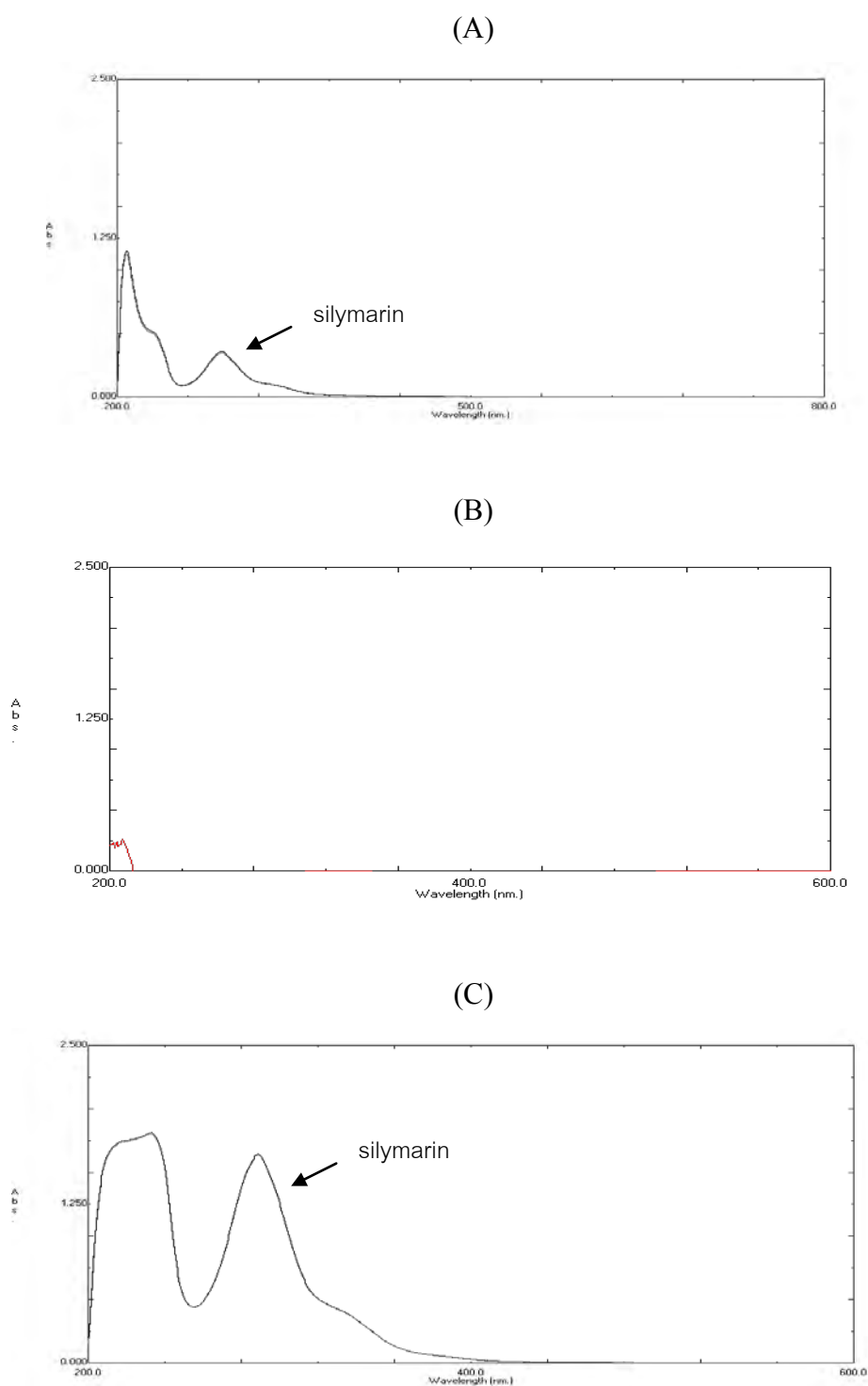
Figure 8 Absorption spectra of (A) 0.7 mg/ml of silymarin in dissolution medium pH 7.5 with 2% SLS, (B) blank solution: non-pariel sugar beads and PEG or PVP K30 in dissolution medium pH 7.5 with 2% SLS (C) silymarin solid dispersion pellets in dissolution medium pH 7.5 with 2% SLS.

2. Linearity

   The calibration curves of silymarin in methanol, dissolution media pH 1.2, 6.8 and 7.5 with 2% SLS are shown in Figures 9-12. The calibration data were found to be linear with coefficient of determination (R^2) of 0.9997, 0.9995, 0.9992 and 0.9999 in methanol, dissolution medium pH 1.2, 6.8 and 7.5 with 2% SLS respectively (Table 3) The acceptance criteria for coefficient of determination is more than 0.9990. These results indicated that UV-VIS spectrophotometric method was acceptable for the quantitative analysis of silymarin in the studied concentration range.
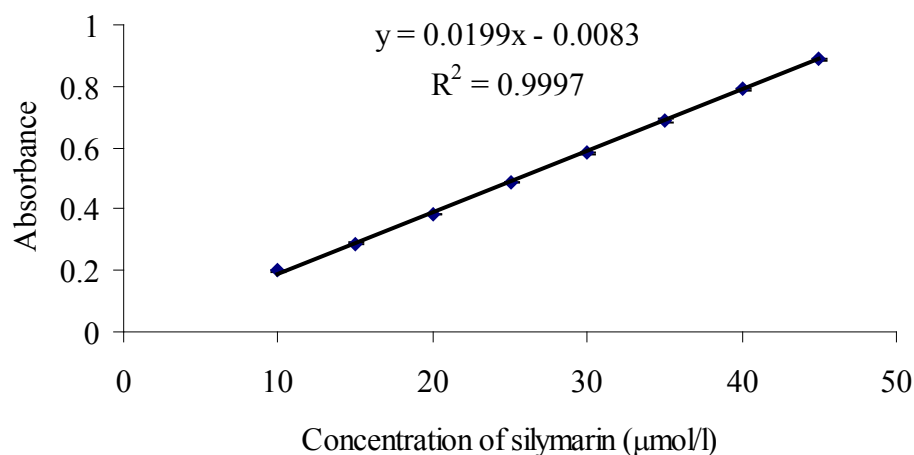
Figure 9 Calibration curve of silymarin in methanol. Data are represented as mean ± SD (n=3).

Figure 10 Calibration curve of silymarin in dissolution medium pH 1.2. Data are represented as mean ± SD (n=3).

Figure 11 Calibration curve of silymarin in dissolution medium pH 6.8. Data are represented as mean ± SD (n=3).

Figure 12 Calibration curve of silymarin in dissolution medium pH 7.5 with 2% SLS. Data are represented as mean ± SD (n=3).
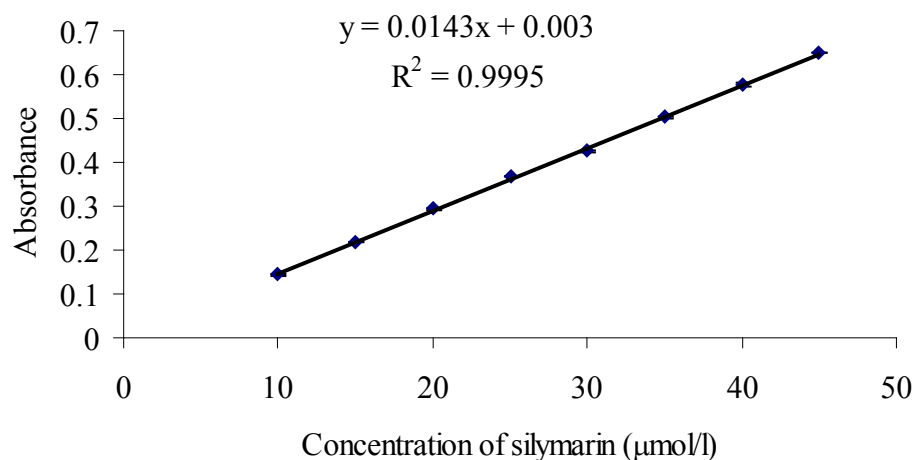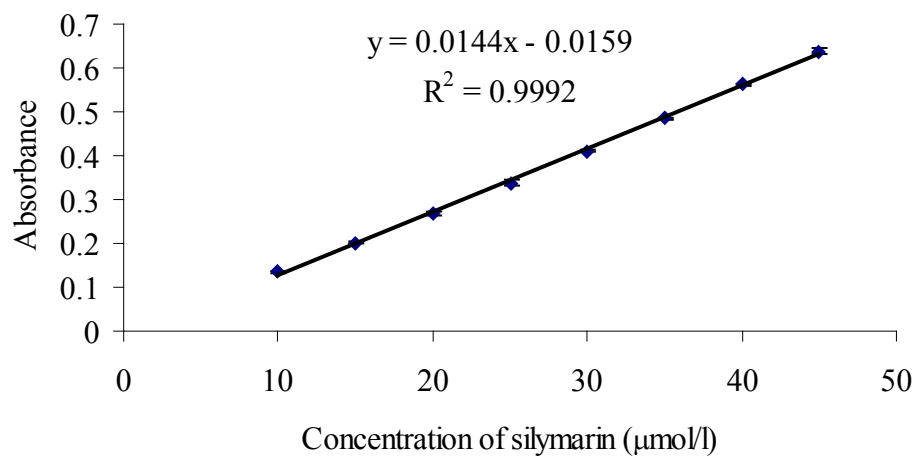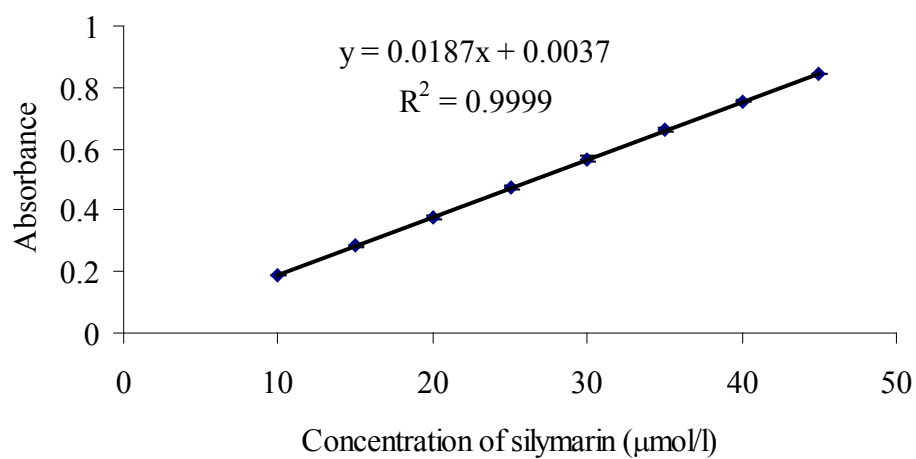
Table 3 UV absorption data of silymarin in methanol, dissolution media pH 1.2, 6.8 and 7.5 with 2% SLS.

Concentration (µmol/l)	Absorbance of silymarin at 288 nm			
	methanol	pH 1.2	pH 6.8	pH 7.5 with 2% SLS
10	0.1982 ± 0.003	0.1434 ± 0.002	0.1355 ± 0.002	0.1907 ± 0.0004
15	0.2879 ± 0.003	0.2176 ± 0.001	0.2011 ± 0.003	0.2841 ± 0.002
20	0.3833 ± 0.002	0.2934 ± 0.002	0.2671 ± 0.003	0.3767 ± 0.005
25	0.4879 ± 0.002	0.3670 ± 0.001	0.3382 ± 0.005	0.4741 ± 0.007
30	0.5835 ± 0.003	0.4259 ± 0.001	0.4106 ± 0.002	0.5662 ± 0.009
35	0.6891 ± 0.005	0.5028 ± 0.003	0.4850 ± 0.003	0.6605 ± 0.007
40	0.7901 ± 0.003	0.5759 ± 0.004	0.5619 ± 0.003	0.7551 ± 0.002
45	0.8877 ± 0.005	0.6486 ± 0.001	0.6368 ± 0.006	0.8435 ± 0.001
R^2	0.9997	0.9995	0.9992	0.9999

Data are represented as mean $\pm$ SD (n=3).

3. Accuracy

The accuracy of an analytical method is the closeness of test results obtained by the method to the true value. The recovery percentage of silymarin in methanol and dissolution media pH 1.2, 6.8 and 7.5 with 2% SLS are shown in Table 4. The acceptance criteria for the percentage of recovery should be within 98-102% (USP 29, 2006).The percentages of analytical recovery were in the range of 99.48-99.95%, 98.86-99.93%, 99.37-100.66% and 99.44-100.2% in methanol, dissolution media pH 1.2, 6.8 and 7.5 with 2% SLS respectively which indicated the high accuracy of the method. Therefore, this method could be used for the analysis of silymarin in all concentrations studied.

Table 4 The percentage of analytical recovery of silymarin in methanol and dissolution media pH 1.2, 6.8 and 7.5 with 2% SLS.

Concentration (μmol/l)	% Analytical recovery of silymarin			
	methanol	pH 1.2	pH 6.8	pH 7.5 with 2% SLS
17.5	99.48 ± 1.31	98.86 ± 0.58	99.37 ± 1.08	100.2 ±1.79
27.5	99.95 ± 0.35	99.93 ± 0.96	100.66 ± 1.03	99.46 ± 0.96
37.5	99.87 ± 0.39	99.33 ± 0.44	99.45 ± 0.85	99.44 ± 0.53

Data are represented as mean ± SD (n=5).

4. Precision

The precision of the analytical method of silymarin in methanol and dissolution media pH 1.2, 6.8 and 7.5 with 2% SLS were determined both within run precision and between run precision (Table 5 and Table 6). The coefficients of variation values were in range of 0.32-0.94%, 0.44-0.96%, 1.04-1.86% and 0.33-0.93% in methanol and dissolution media pH 1.2, 6.8 a nd 7.5 w ith 2% SLS respectively. The acceptance criteria of the coefficient of variation (CV) should generally be less than 2% (USP 29, 2006). Therefore, the UV-VIS spectrophotometric method was precise for quantitative analysis of silymarin in the studied range.

Table 5 The within run precisions of silymarin in methanol and dissolution media pH 1.2, 6.8 and 7.5 with 2% SLS.

Concentration (μmol/l)	% CV			
	methanol	pH 1.2	pH 6.8	pH 7.5 with 2% SLS
17.5	0.94	0.59	1.04	0.93
27.5	0.68	0.68	1.86	0.68
37.5	0.32	0.33	1.29	0.33

Data are represented as mean ± SD (n=5).

Table 6 The between run precisions of silymarin in methanol and dissolution media pH 1.2, 6.8 and 7.5 with 2% SLS.

Concentration	% CV			
(μmol/l)	methanol	pH 1.2	pH 6.8	pH 7.5 with 2% SLS
17.5	1.32	1.06	0.66	1.89
27.5	0.35	0.45	0.79	0.89
37.5	0.39	0.45	1.10	0.91

Data are represented as mean ± SD (n=5).

B. Dissolution Studies of Silymarin Solid Dispersion Pellets

All formulations of silymarin solid dispersion pellets were spherical and intact in shape with yellow color. The surface and cross-section of silymarin solid dispersion pellets were investigated using SEM (Figures 13-15). Under SEM, the silymarin solid dispersions pellets from PEG 4000 and PEG 6000 showed a smooth surface while the silymarin solid dispersion pellets from PVP K30 showed a coarse surface. The coarse surface from PVP K30 coating is similar with previous studied by Sun et al., 2008. The cross-section of pellets showed a layer of carriers and silymarin coating around the non-pariel core.

(1A)

(2A)

(1B)

(2B)

(1C)

(2C)

Figure 13 Scanning electron micrograph of the surface (1) and cross-section (2) of the silymarin/PEG 4000 solid dispersion pellets at ratio of PEG 4000 to silymarin of (A) 1 : 2, (B) 1 : 4 and (C) 1 : 8 respectively.

<center>(1A)</center>

<center>(2A)</center>

<center>(1B)</center>

<center>(2B)</center>

<center>(1C)</center>

<center>(2C)</center>

Figure 14 Scanning electron micrograph of the surface (1) and cross-section (2) of the silymarin/PEG 6000 solid dispersions pellet at ratio of PEG 6000 to silymarin of (A) 1 : 2, (B) 1 : 4 and (C) 1 : 8 respectively.
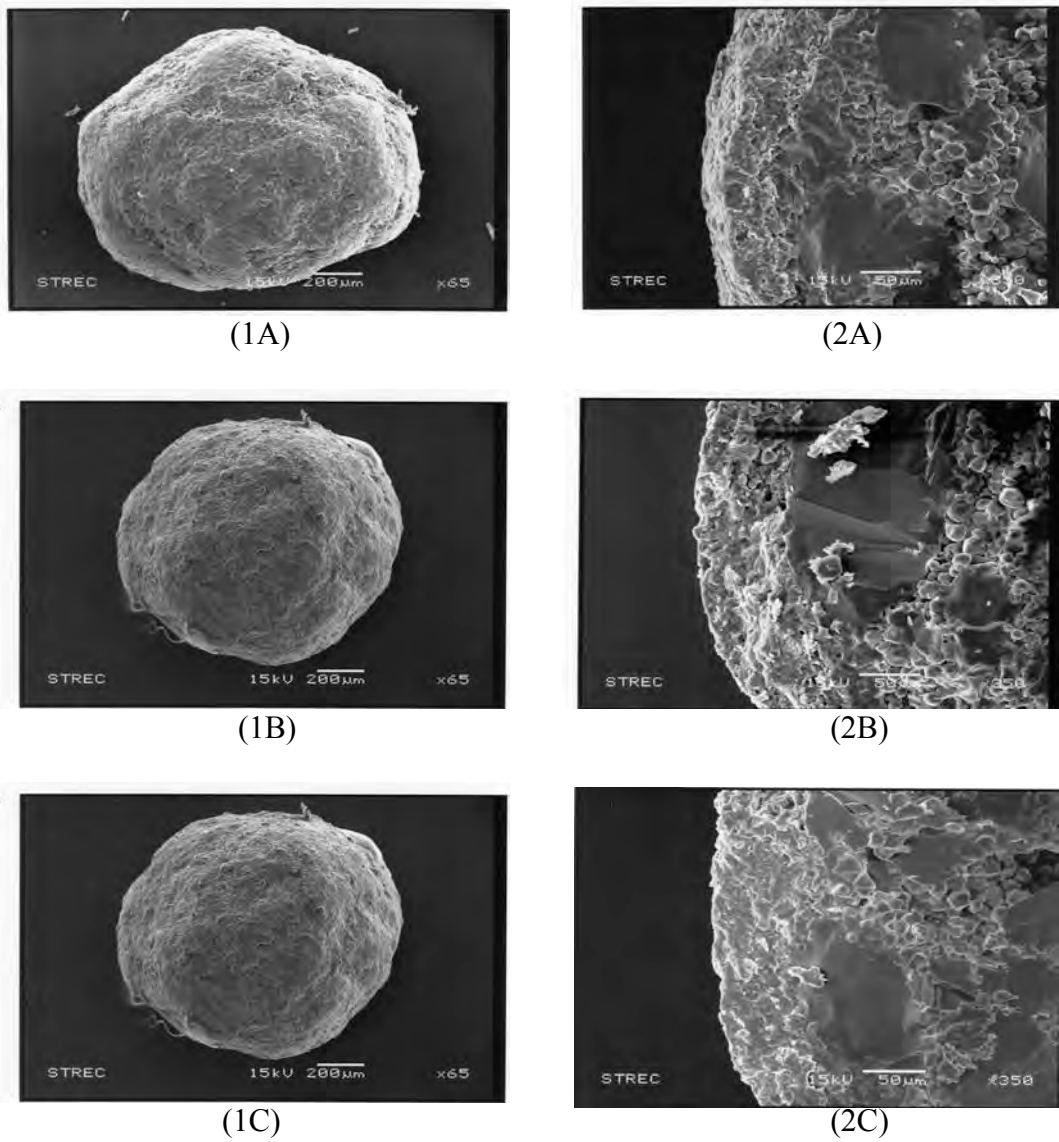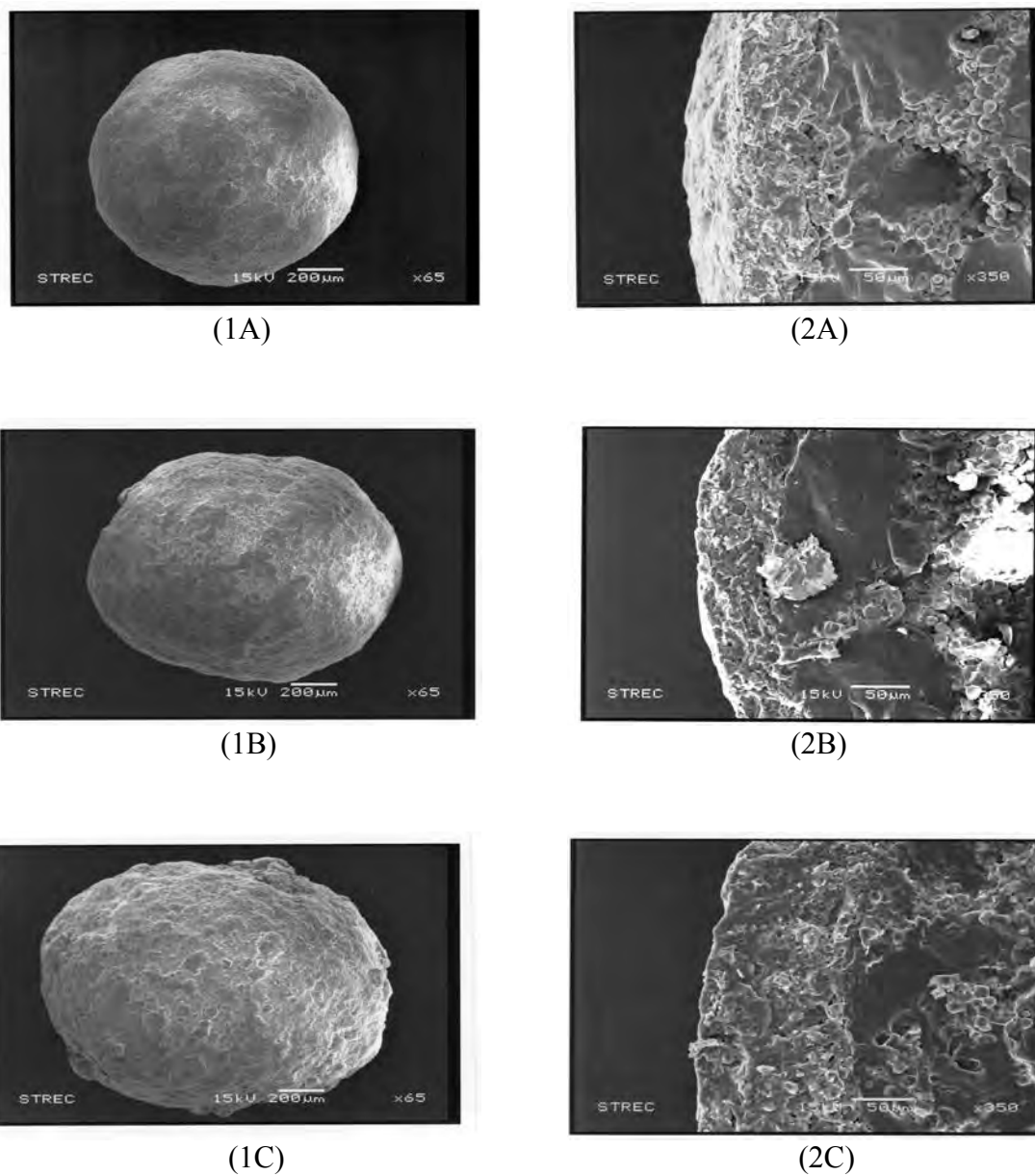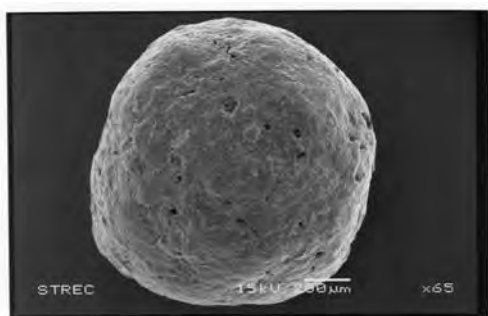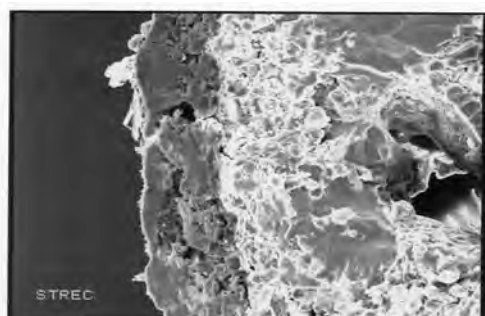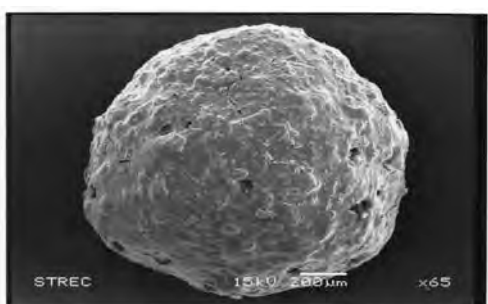
(1A)

(2A)

(1B)
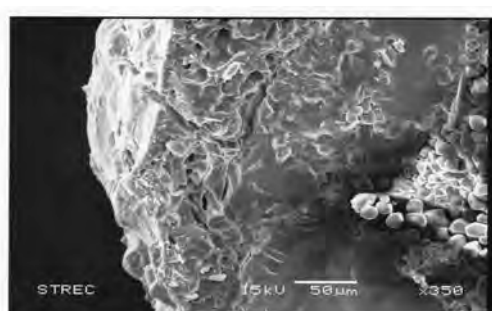
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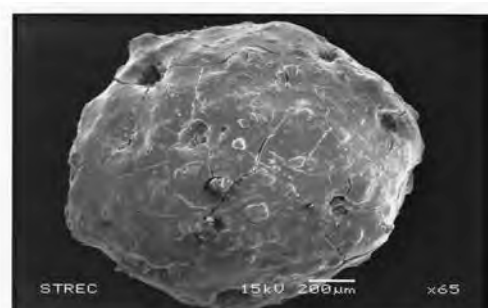
(1C)
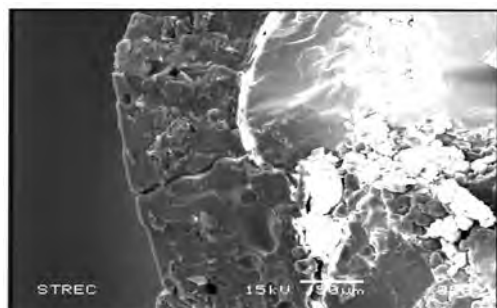
(2C)

Figure 15 Scanning electron micrograph of the surface (1) and cross-section (2) of the silymarin/PVP K 30 solid dispersion pellets at ratio of PVP K 30 to silymarin of (A) 1 : 2, (B) 1 : 4 and (C)1 : 8 respectively.

The recoveries of silymarin from all formulations were in range of 78%-83% (Table 7).

Table 7 Drug recovery (%) of silymarin from all formulations.

Formulations	Carrier	Ratio of silymarin:carrier (% w/w)	Percent recovery of silymarin (mean ± SD)
F1	PEG 4000	1 : 2	83.50 ± 2.00
F2	PEG 4000	1 : 4	79.80 ± 1.43
F3	PEG 4000	1 : 8	80.32 ± 2.12
F4	PEG 6000	1 : 2	81.83 ± 1.69
F5	PEG 6000	1 : 4	81.25 ± 1.40
F6	PEG 6000	1 : 8	78.75 ± 1.90
F7	PVP K 30	1 : 2	78.69 ± 1.35
F8	PVP K 30	1 : 4	78.58 ± 1.54
F9	PVP K 30	1 : 8	82.49 ± 2.00

Data are represented as mean ± SD (n=22).

Each silymarin solid dispersion pellets and silymarin powder was transferred to capsule for equivalent amount of 70 m g silymarin. The dissolution profiles of silymarin solid dispersion pellets and silymarin powder were performed in dissolution media pH 1.2, 6.8 and 7.5 with 2% SLS. $Qt_{x\ min}$, the amount of drug dissolved in that time, is used as an acceptance limit of the dissolution test. USP 29 determined the release of silymarin is not less than 75% in 45 min ($Qt_{45\ min} > 75\%$). The $t_{45\ min}$ of silymarin solid dispersion pellets and silymarin powder are shown in Table 8.

Table 8 Percent dissolved of silymarin solid dispersion pellets and silymarin powder in dissolution media pH 1.2, 6.8 and 7.5 with 2% SLS at time 45 min ($Qt_{45\,min}$).

Formulations	$Qt_{45\,min}$ (%) pH 1.2	$Qt_{45\,min}$ (%) pH 6.8	$Qt_{45\,min}$ (%) pH 7.5 with 2% SLS
Silymarin powder	54.43 ± 0.44	53.43 ± 0.22	90.49 ± 0.34
O1	43.36 ± 0.30	40.09 ± 0.40	94.71 ± 0.34*
O2	57.01 ± 0.33*	49.32 ± 0.36	90.23 ± 0.71
F1	81.50 ± 1.45*	61.44 ± 1.04*	75.25 ± 0.40
F2	91.37 ± 1.02*	75.99 ± 0.92*	93.71 ± 0.91*
F3	89.35 ± 0.98*	76.94 ± 1.80*	97.50 ± 2.36*
F4	100.71 ± 1.15*	87.43 ± 1.57*	97.22 ± 0.39*
F5	98.94 ± 0.49*	93.12 ± 1.60*	98.01 ± 0.60*
F6	98.93 ± 0.67*	99.37 ± 1.04*	99.04 ± 0.55*
F7	43.40 ± 0.92	84.98 ± 0.58*	99.32 ± 0.53*
F8	38.44 ± 2.18	98.24 ± 0.50*	98.44 ± 0.23*
F9	92.48 ±1.15*	84.14 ± 0.46*	80.11 ± 0.41

Data are represented as mean ± SD (n=6).

* significant difference from the control at a level of p-value < 0.05

The result suggested that percent dissolved of silymarin solid dispersion pellets in 45 min were more than 75% in all dissolution media excepted silymarin solid dispersion pellets at ratio of silymarin : PVP K 30 of 1 : 2 (F7) and 1 : 4 (F8) in dissolution medium pH 1.2. The results may be due to less effective surface area because of adsorption of bubble at the surface of pellets. In addition, this result showed that the percent dissolved of silymarin solid dispersions (F1-F9) were better than silymarin powder and two commercial products (O1 and O2) in dissolution medium pH 6.8. In dissolution medium pH 1.2, percent dissolved of silymarin solid dispersion pellets were better than silymarin powder and two commercial products excepted silymarin solid dispersion pellets at ratio of silymarin:PVP K 30 of 1 : 2 and 1 : 4. In dissolution medium pH 7.5 with 2% SLS, percent dissolved of silymarin

solid dispersion pellets were better than silymarin powder and two commercial products excepted silymarin solid dispersion pellets at ratio of silymarin:PEG 4000 for 1:2 (F1) and silymarin : PVP K 30 for 1 : 8. The enhancement of the percent dissolved of silymarin may increase the drug permeation and the bioavailability of silymarin. The results may be due to the increasing of surface area and the improvement of the wettability of silymarin solid dispersion pellets. According to the Noyes-Whitney equation, the higher surface area leads to improve the dissolution of the poor water soluble drug (Ho et al., 1996; Leuner and Dressman, 2000; Lheritier et al., 1995). In addition, the increase in the percent dissolved of silymarin solid dispersion pellets may be due to the reduction of the drug particle size in the carrier matrix and the solubilizing effect of the carrier (Rabasco et al., 1991). However, the differences in types, ratios of carrier and pH of dissolution medium could affect the percent dissolved of silymarin solid dispersion pellets.

1. Effect of ratios of carrier on the percent dissolved of silymarin

The result showed that the percent dissolved of silymarin depended on the ratios of carrier in all dissolution media (Figures 16-18). Increasing the ratio of silymarin to PEG 4000 and 6000 from 1 : 2 to 1 : 8 resulted in increased percent dissolved of silymarin in all dissolution mediums compared to silymarin powder. In contrast for PVP K 30, increasing the ratio of silymarin to PVP K 30 from 1 : 2 to 1 : 8 resulted in decreased percent dissolved of silymarin in dissolution medium pH 6.8 and 7.5 with 2% SLS. Meanwhile, increasing the ratio of silymarin to PVP K 30 only at 1 : 8 resulted in increased percent dissolved of silymarin in dissolution medium pH 1.2 compared to silymarin powder. This result may be due to the high percentage of the carriers which lead to the complete absence of crystallinity of the drug and thereby increase in the solubility and release rate of the drug (Lin and Cham, 1996; Nair et al., 2002; Torrado et al., 1996).

(A)

(B)

(C)

Figure 16 C umulative percent dissolved of silymarin powder, two commercial products and silymarin : PEG 4000 (A), silymarin : PEG 6000 (B) and silymarin : PVP K 30 (C) solid dispersion pellets at ratio of silymarin to carrier for 1 : 2, 1 : 4 and 1 : 8 in dissolution medium pH 1.2. Data are represented as mean ± SD (n=6).

(A)

(B)

(C)

Figure 17 C umulative percent dissolved of silymarin powder, two commercial products and silymarin:PEG 4000 (A), silymarin:PEG 6000 (B) and silymarin:PVP K 30 (C) solid dispersion pellets at ratio of silymarin to carrier for 1:2, 1:4 and 1:8 in dissolution medium pH 6.8. Data are represented as mean ± SD (n=6).
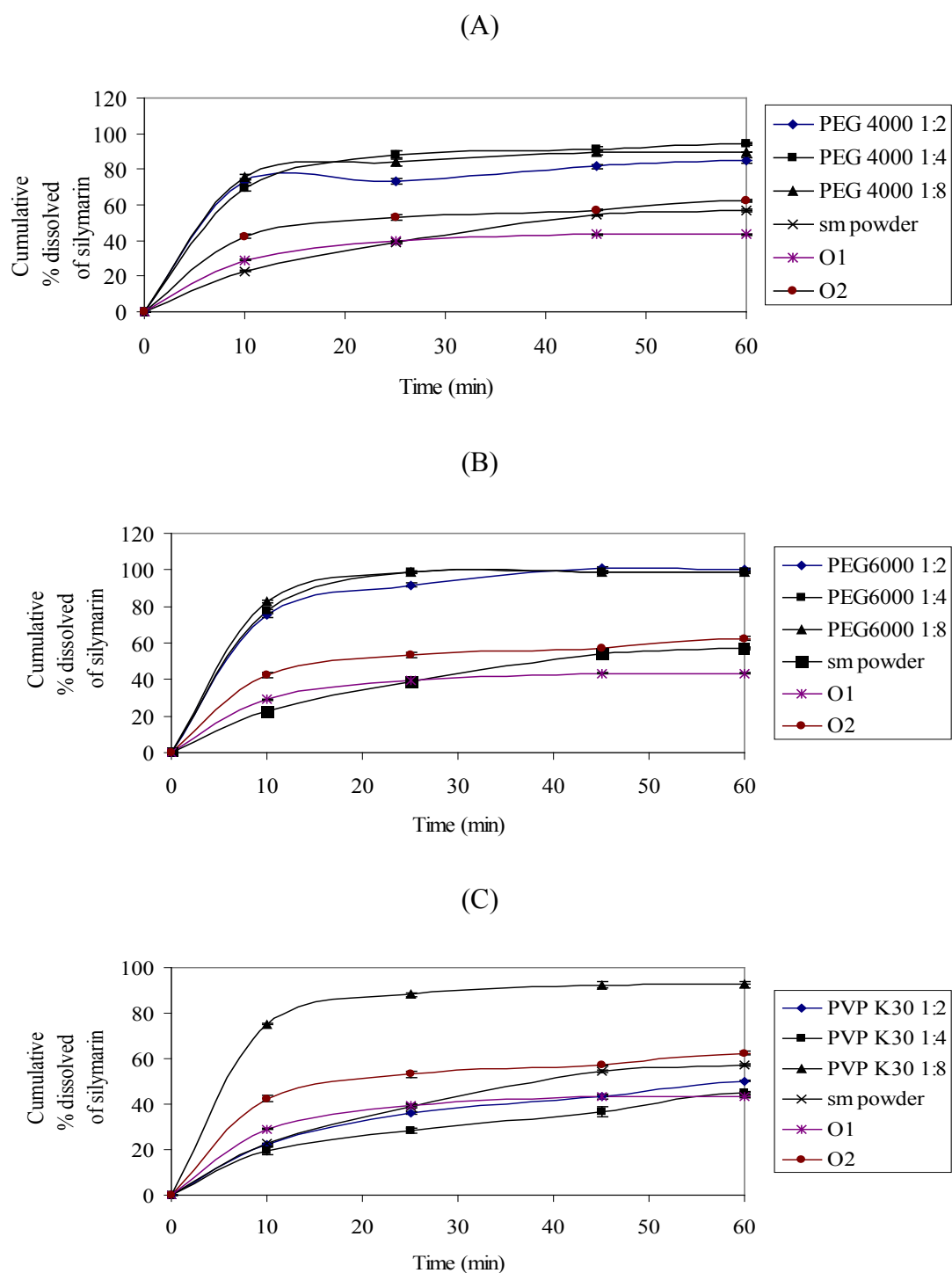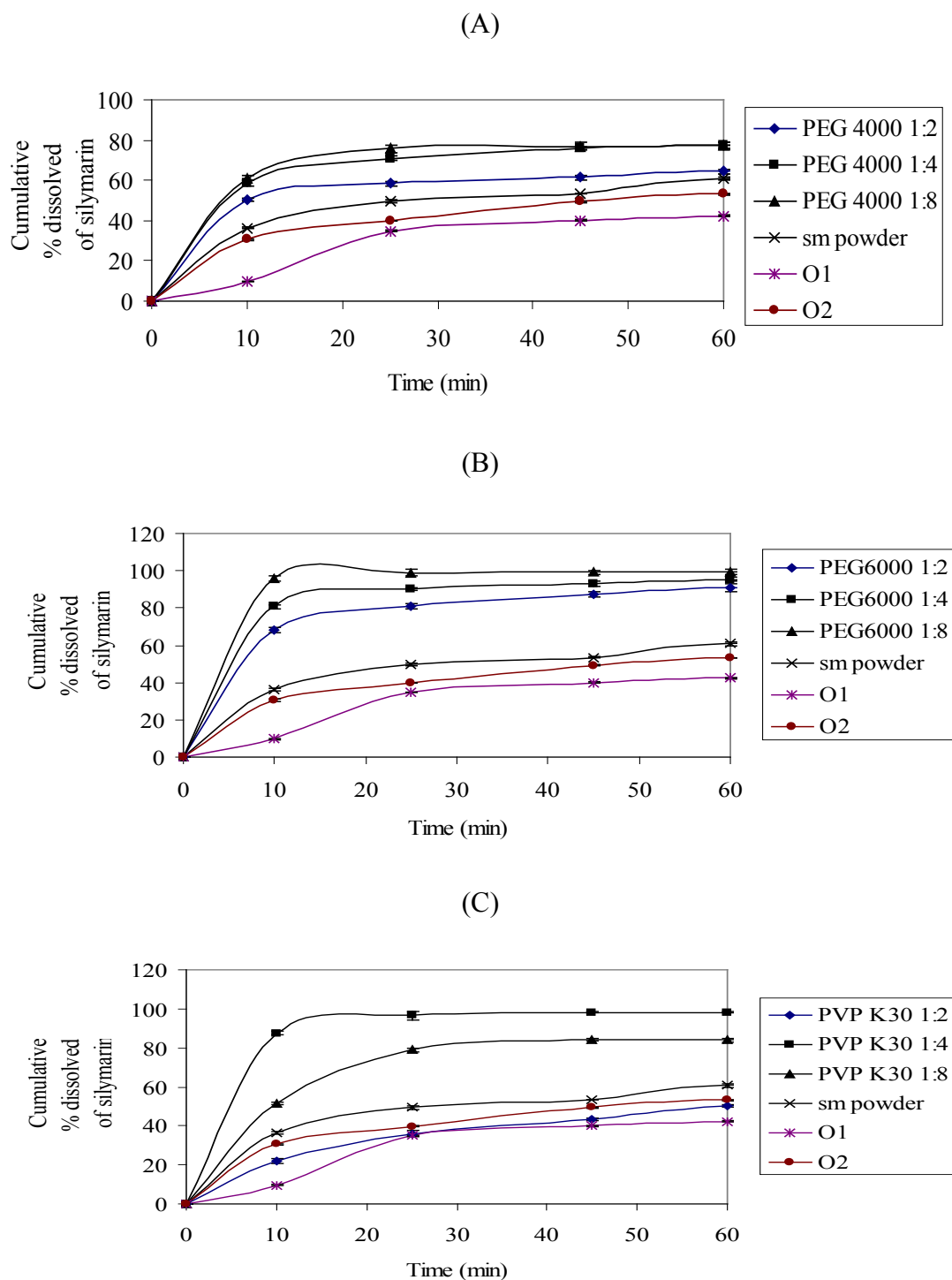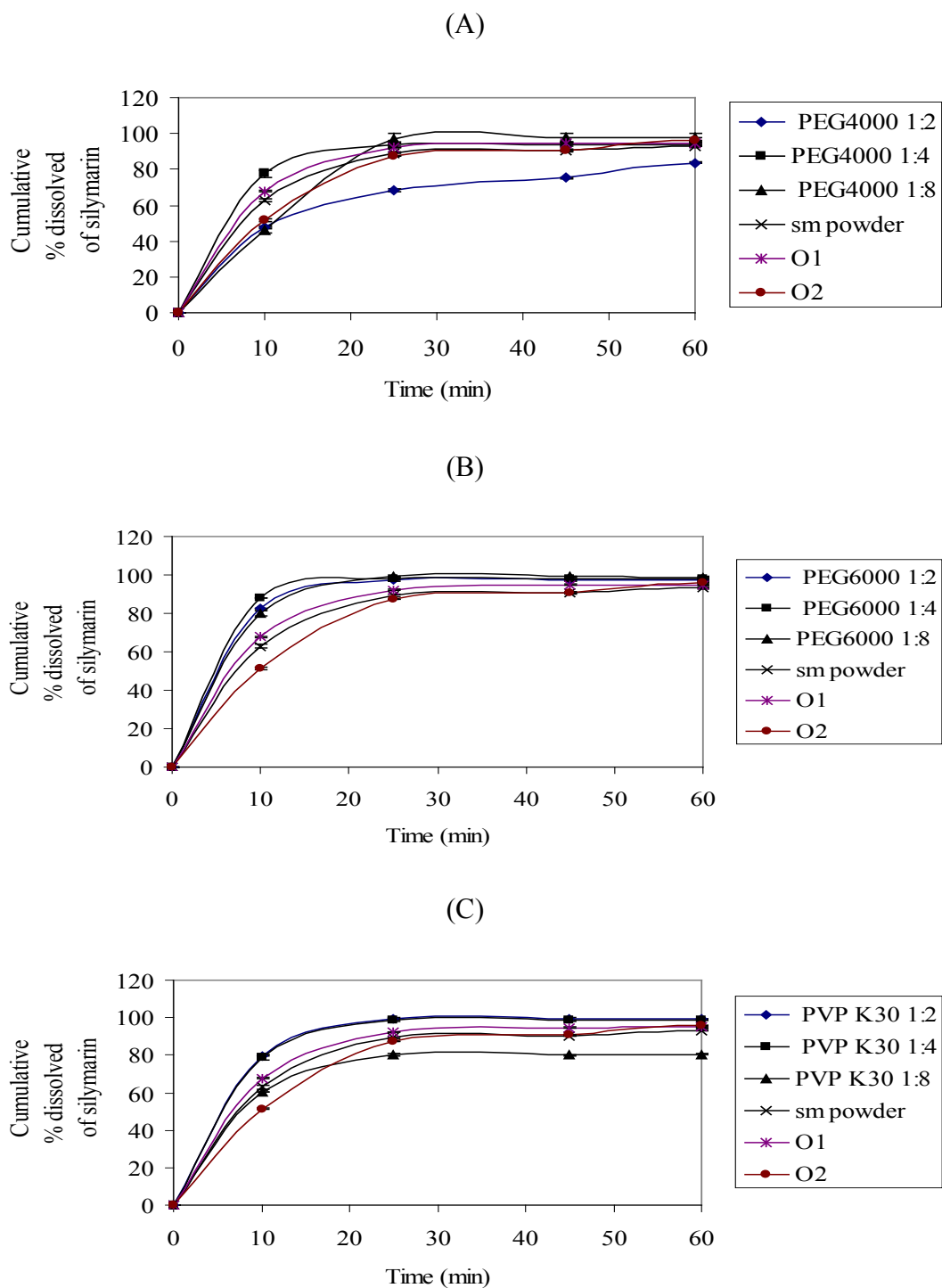
(A)

(B)

(C)

Figure 18 C umulative percent dissolved of silymarin powder, two commercial products and silymarin:PEG 4000 (A), silymarin:PEG 6000 (B) and silymarin:PVP K 30 (C) solid dispersion pellets at ratio of silymarin to carrier for 1:2, 1:4 and 1:8 in dissolution medium pH 7.5 with 2% SLS. Data are represented as mean ± SD (n=6).

2. Effect of carriers on percent dissolved of silymarin

The result suggested that carriers used in solid dispersion affected the percent dissolved of silymarin. At the same ratio of carrier to drug and the same dissolution medium, using PEG 6000 as a carrier showed the best percent dissolved of silymarin than other carriers and silymarin powder and two commercial products. PEG affects the diffusion layer surrounding the particle of poor water soluble drug that improve wettability and the dissolution (Fernandez et al., 1993; Verheyen et al., 2002). Ford et al. (1986) indicated that the dissolution rate is inversely proportional to the chain length of the PEG. However, these results found that increasing chain length of PEG from 4000 to 6000 resulted in increased dissolution. This result was in agreement with Betageri and Makarla (1995) that the dissolution rate from a solid dispersion in PEG 6000 was faster than a similar dispersion in PEG 4000 and PVP K 30. This result may be explained by the ability of PEG 6000 to dissolve more of drug than the PEG 4000 and PVP K 30, leading to a greater percentage of drug in the dispersed form. In addition, the high ratio of the PEG 6000 prevented precipitation of the drug (Leuner and Dressman, 2000). PVP K 30 gave the better enhancement of dissolution of silymarin as compared to PEG 4000 except in the dissolution medium pH 1.2. This may be due to the more solubilizing and wetting effect of PVP compared to PEG 4000 (Shah et al., 2009).

3. Effect of pH on percent dissolved of silymarin

The results found that the percent dissolved of silymarin solid dispersion pellets of all formulations were higher in pH 1.2 than pH 6.8 except for PVP at ratio of silymarin to carrier of 1 : 2 and 1 : 4. The results is in agreement with Qui et al., 2005 that silymarin was dissolved higher in pH 1.2 than pH 6.8 This result may be due to the solubility of silymarin in pH 1.2 is more than pH 6.8. The solubility of silymarin in pH 1.2 and pH 6.8 are 0.209 mg/ml and 0.148 mg/ml respectively (Nakhat et al., 2007). However, Yanyu et al., 2006 found that dissolution of silybin complex with phospholipid in pH 6.8 was significantly more than pH 1.2. This result may due to the complexation of phospholipid with silybin.

C. Cytoprotectivity of Silymarin against ROS in HK-2 Cells

The objective of this study was to investigate the cytoprotectivity of silymarin against ROS. The cytotoxicity of silymarin and ROS generators were firstly investigated. Non-cytotoxic concentration of silymarin and the half-maximal inhibitory concentration (IC_{50}) of ROS generators were used for determining the cytoprotectivity of silymarin against ROS.

1. Investigation of the cytotoxicity of silymarin

HK-2 cells were treated with silymarin at a concentration range of 25-400 μmol/l. The cytotoxicity of silymarin was evaluated after 24 and 48 hr of treatment. The results are shown in Figure 19 and Figure 20. The cytotoxicity of silymarin was similar to those reported elsewhere (Svobodova et al., 2006). Silymarin was non-toxic in the studied concentration range in HK-2 cells. Moreover, significant increase in the percent cell viability was observed in cells exposed to silymarin at concentration above 100 μmol/l for 24 hr (p-value < 0.05) and above 50 μmol/l for 48 hr (p-value < 0.05). The percentages of cell viability were approximately 8% higher than the control after 24 hr treatment with silymarin at concentration above 100 μmol/l and 13% higher than the control after 48 hr treatment with silymarin at concentration above 50 μmol/l. The result of this study suggested that silymarin not only non-toxic in HK-2 cells, but also increased the proliferation response of HK-2 cells. The proliferative effect of silymarin may be due to the stimulatory effect on biosynthesis of protein and DNA (Alidoost et al., 2006; Sonnenbichler et al., 1999).

Figure 19 The cytotoxicity of silymarin in HK-2 cells. Cells were incubated for 24 hr at 37°C with 25-400 μmol/l silymarin. Controls were untreated cells. Cell viability was examined using MTT assay. Data are represented as mean ± SD (n=3).

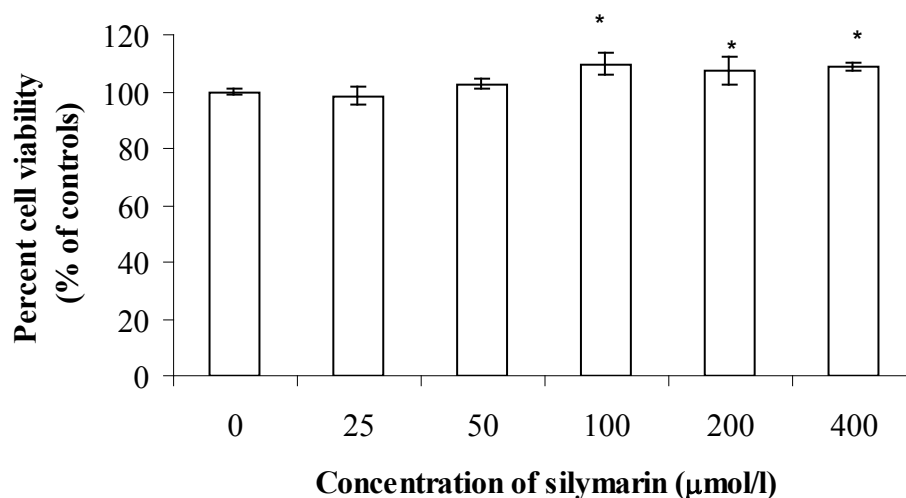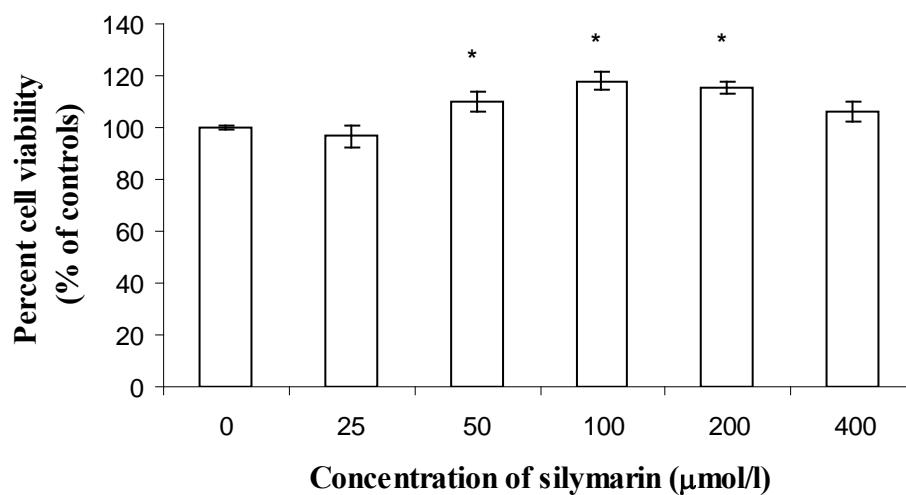∗ = significant difference from the control at a level of p-value < 0.05.

Figure 20 The cytotoxicity of silymarin in HK-2 cells. Cells were incubated for 48 hr at 37°C with 25-400 μmol/l silymarin. Controls were untreated cells. Cell viability was examined using MTT assay. Data are represented as mean ± SD (n=3).

∗ = significant difference from the control at a level of p-value < 0.05.

2. Investigation of the cytotoxicity of ROS generators

2.1 The cytotoxicity of DMNQ

HK–2 cells were treated with DMNQ at a concentration range of 10-30 μmol/l. The cytotoxicity of DMNQ was evaluated after 24 hr of treatment. The cytotoxicity of DMNQ was concentration dependent manner (Figure 21). As the concentration increased from 10 to 30 μmol/l, the percentages of cell viability after 24 of treatment were decreased from 91% to 22% respectively. The IC_{50} of DMNQ after 24 hr of DMNQ exposure was 20 μmol/l. DMNQ at concentration of 20 μmol/l was chosen for determining the cytoprotectivity of silymarin against DMNQ. DMNQ is one of the quinine analogues that can generate the superoxide anion radical (Ishihara et al., 2006). Several mechanisms have been proposed to explain the effects of superoxide anion radical on the induction of toxicity in cells. The superoxide anion radical may deplete the intracellular glutathione content (Park et al., 2007) or may increase the oxidative stress via redox cycle and arylation of nucleophile (Lee et al., 2001; Ishihara et al., 2006). Since DMNQ has no arylating moiety in its structure, its toxicity is mediated by redox cycle or glutathione depletion.
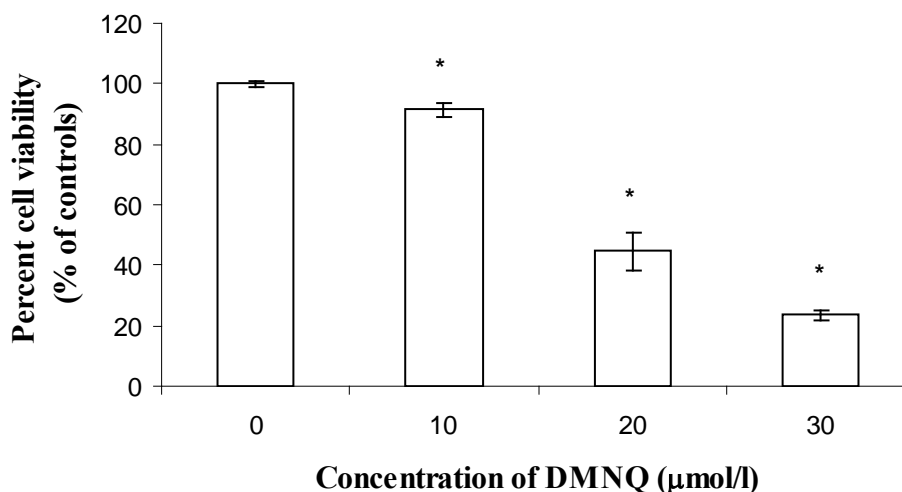

Figure 21 The cytotoxicity of DMNQ in HK-2 cells. Cells were incubated for 24 hr at 37°C with 10-30 μmol/l. Controls were untreated cells Cell viability was examined using MTT assay. Data are represented as mean ± SD (n=3).

∗ = significant difference from the control at a level of p-value < 0.05.

2.2 The cytotoxicity of hydrogen peroxide

HK-2 cells were treated with hydrogen peroxide at a concentration range of 200–1000 μmol/l. The cytotoxicity of hydrogen peroxide was evaluated after 24 hr of treatment. The result was shown in Figure 22. As the concentration increased from 200 to 1000 μmol/l, the percentages of cell viability after 24 hr of treatment decreased from 50% to 10% respectively. However, the percent cell viability at concentration of 800 μmol/l incubated for 24 hr was higher than lower concentrations. These results showed that hydrogen peroxide was cytotoxic to HK-2 cells in the studied concentration range. The result was in agreement with previous studied by Walker and Shah, 1991 that hydrogen peroxide was cytotoxic in pig's renal cells. The IC_{50} of hydrogen peroxide after 24 of hydrogen peroxide exposure was 400 μmol/l. Therefore, this concentration was chosen for determining the cytoprotectivity of silymarin against hydrogen peroxide. The cytotoxicity of the hydrogen peroxide may be due to DNA breakage (Cantoni et al., 1989) or may be due to an interaction with the iron forming the iron-oxygen species such as hydroxyl radical and ferryl radical. These iron-oxygen species play a critical role in hydrogen peroxide-mediated cytotoxicity (Walker and Shah, 1991).
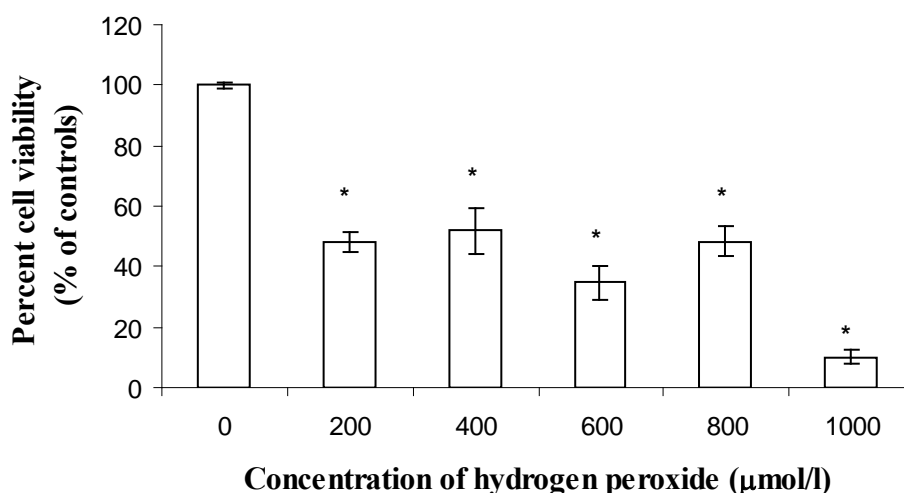

Figure 22 The cytotoxicity of hydrogen peroxide in HK-2 cells. Cells were incubated for 24 hr at 37°C with 200-1000 μmol/l. Controls were untreated cells. Cell viability was examined using MTT assay. Data are represented as mean ± SD (n=3).

∗ = significant difference from the control at a level of p-value < 0.05.

2.3 The cytotoxicity of hydrogen peroxide/ferrous sulphate

HK–2 cells were treated with hydrogen peroxide/ferrous sulphate at a concentration range of 600-1000 μmol/l. The cytotoxicity of hydrogen peroxide/ferrous sulphate was evaluated after 24 hr of treatment. The cytotoxicity of hydrogen peroxide/ferrous sulphate was concentration dependent manner (Figure 23). As the concentration increased from 600 to 1000 μmol/l, the percentages of cell viability after 24 hr of treatment decreased from 82% to 72% respectively. The IC_{25} of hydrogen peroxide/ferrous sulphate after 24 of incubation was 1000 μmol/l. The concentration of 1000 μmol/l was chosen for determining the cytoprotectivity of silymarin against hydrogen peroxide/ferrous sulphate. Hydrogen peroxide/ferrous sulphate is used as the hydroxyl radical generator. The cytotoxicity of hydroxyl radical may be due to its ability to withdraw an electron from atoms, organic and inorganic molecules. These molecules and atoms may crosslink with DNA and react with other nucleophiles such as glutathione, lipids and proteins, resulting in the disruption of cellular homeostasis followed by cell injury and cell death. In addition, hydroxyl radical may involve with p53 causing apoptosis cell death (Jiang et al., 2007; Wang et al., 2000).
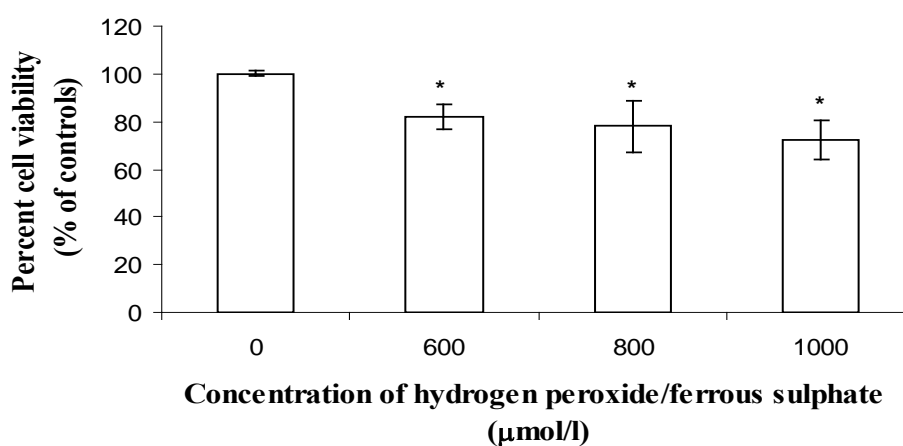
Figure 23 The cytotoxicity of hydrogen peroxide/ferrous sulphate in HK-2 cells. Cells were incubated for 24 hr at 37°C with 200-1000 μmol/l. Controls were untreated cells. Cell viability was examined using MTT assay. Data are represented as mean ± SD (n=3).

∗ = significant difference from the control at a level of p-value < 0.05.

3. Investigation of the cytoprotectivity of silymarin against ROS generators

3.1 The cytoprotectivity of silymarin against DMNQ

HK-2 cells were pre-treated with silymarin at concentration range of 25- 400 μmol/l for 30 min followed by DMNQ at concentration of 20 μmol/l (IC_{50}) for 24 hr. The cytoprotectivity of silymarin against DMNQ was evaluated after 24 hr of treatment. Pre-treatment with silymarin at concentration of 25 a nd 50 μmol/l provided significantly increased in the percent cell viability as compared to cells treated with DMNQ alone (p-value < 0.05) (Figure 24). As the concentration of silymarin increased from 25 t o 200 μmol/l, the percentages of cell viability were approximately 23.91%, 19.23%, 12.39% and 2.33% higher than cells treated with DMNQ alone. Meanwhile, at the concentration of 400 μmol/l, the percentage of cell viability was significantly lower than cells treated with DMNQ alone (p-value < 0.05).
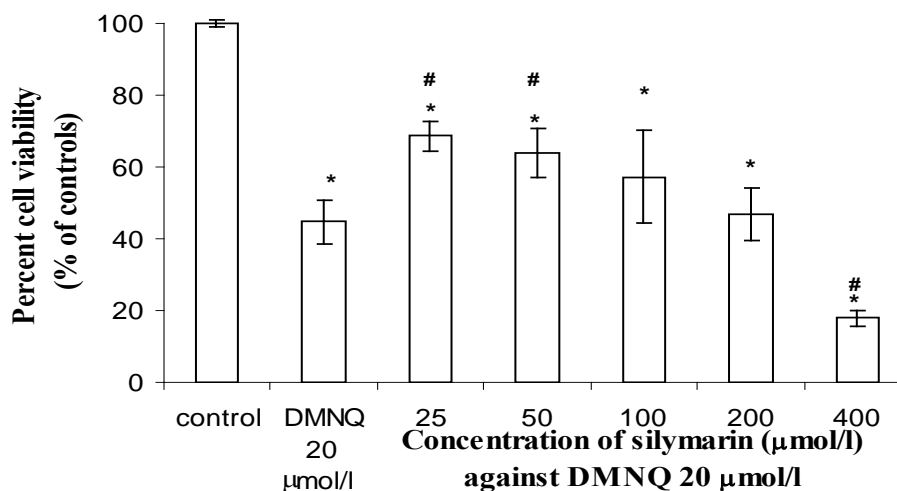
Figure 24 The cytoprotectivity of silymarin against DMNQ in HK-2 cells. Cells were incubated at 37°C with 25-400 μmol/l silymarin for 30 min followed by 20 μmol/l DMNQ for 24 hr. Controls were untreated cells. Cell viability was examined using MTT assay. Data are represented as mean ± SD (n=3).

∗ = significant difference from the control at a level of p-value < 0.05.

= significant difference from treated with DMNQ alone at a level of p-value < 0.05.

3.2 The cytoprotectivity of silymarin against hydrogen peroxide

HK-2 cells were pre-treated with silymarin solution at concentration range of 25-400 µmol/l for 30 m in followed by 400 µmol/l hydrogen peroxide. Cytoprotectivity of silymarin against hydrogen peroxide was evaluated after 24 hr of treatment. Cytoprotective of silymarin against hydrogen peroxide was concentration dependent (Figure 25). The percentages of cell viability were approximately 25% higher than cells treated with hydrogen peroxide alone after 24 hr treatment with silymarin at concentration above 100 µmol/l. The result suggested that silymarin was cytoprotective against hydrogen peroxide-induced cytotoxicity in HK-2 cells. Cytoprotectivity of silymarin against hydrogen peroxide induced cytotoxicity may be due to the antioxidant activity (Svobodova et al, 2006).
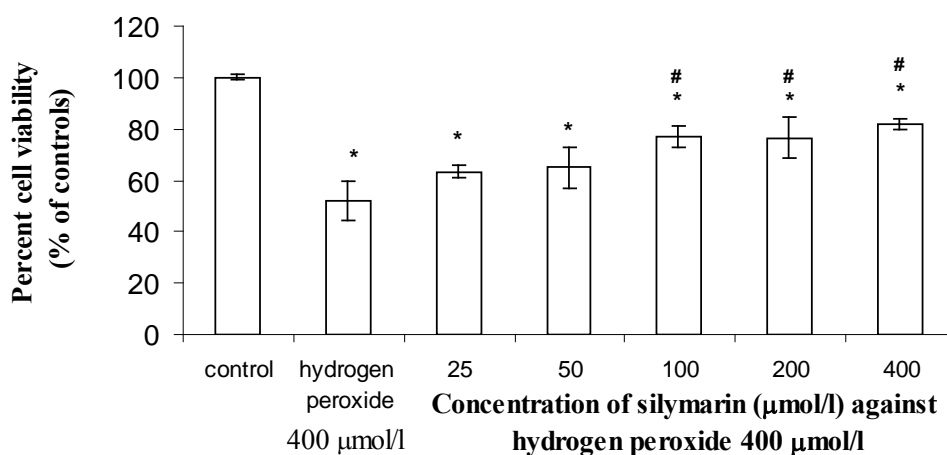
Figure 25 The cytoprotectivity of silymarin against hydrogen peroxide in HK-2 cells. Cells were incubated at 37°C with 25-400 µmol/l silymarin and 400 µmol/l hydrogen peroxide for 24 hr. Controls were untreated cells. Cell viability was examined using MTT assay. Data are represented as mean ± SD (n=3).

∗ = significant difference from the control at a level of p-value < 0.05.

= significant difference from treated with hydrogen peroxide alone at a level of p-value < 0.05.

3.3 The cytoprotectivity of silymarin against hydrogen peroxide / ferrous sulphate

HK-2 cells were pre-treated with silymarin solution at concentrations for 25- 400 μmol/l for 30 m in followed by hydrogen peroxide/ferrous sulphate solution at concentration of 1000 μmol/l for 24 hr. Cytoprotectivity of silymarin against hydrogen peroxide/ferrous sulphate was evaluated after 24 hr of treatment. The percentages of cell viability were approximately 30% higher than cells treated with hydrogen peroxide/ferrous sulphate after 24 hr treatment with silymarin at all concentrations (Figure 26). Moreover, the percentages of cell viability were approximately 12% and 20% higher than the control after 24 hr incubation with silymarin at concentration of 25 and 50 μmol/l respectively. The result suggested that silymarin was cytoprotective against hydroxyl radical generator-induced cytotoxicity in HK-2 cells. Cytoprotective of silymarin against hydroxyl radical may be due to the antioxidant activity and the prevention of antioxidant enzymes depletion.
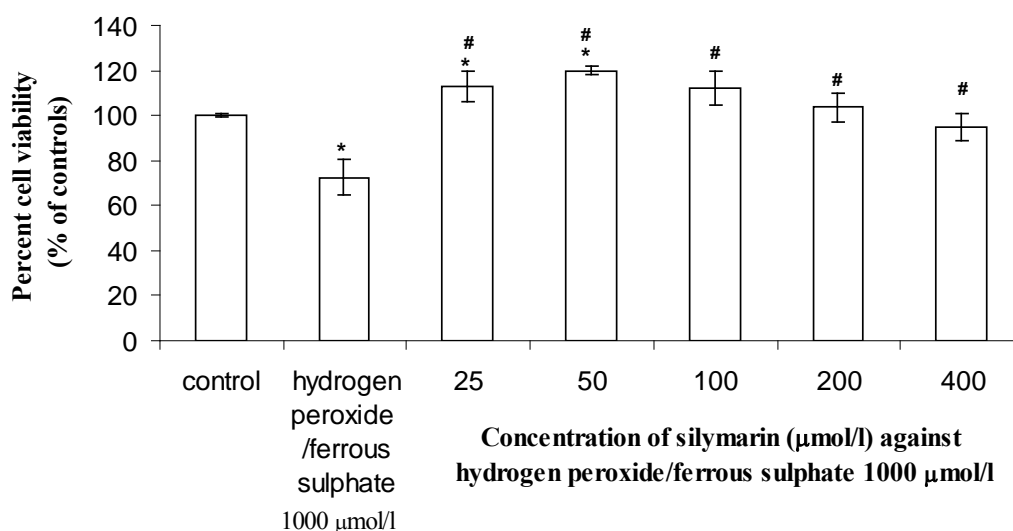
Figure 26 The cytoprotectivity of silymarin against hydrogen peroxide/ferrous sulphate on HK-2 cells. Cells were incubated at 37°C with 25-400 μmol/l silymarin followed by 1000 μmol/l hydrogen peroxide/ferrous sulphate for 24 hr. Controls were untreated cells. Cell viability was examined using MTT assay. Data are represented as mean ± SD (n=3).

∗ = significant difference from the control at a level of p-value < 0.05.

= significant difference from treated with hydrogen peroxide alone at a level of *p*-value < 0.05.

D. ROS Scavenging Activity of Silymarin against ROS Generators-Induced ROS Production in HK-2 Cells

The results from previous section suggested that silymarin was cytoprotective against hydrogen peroxide and hydroxyl radical. The result may be due to the scavenging activity of silymarin against these ROS. The objective of this study was to evaluate the scavenging activity of silymarin against ROS.

1. Investigation of intracellular ROS production from ROS generators

To investigate the intracellular ROS production, cells were treated with ROS generators at IC_{50}. ROS production was measured based on the DCFH-DA and DHE fluorescent intensity using reversed microscope and flow cytometer.

1.1 Intracellular ROS production in response to DMNQ

Cells were treated with DMNQ at concentration 20 μmol/l for 3 hr. The result showed that DHE and DCFH-DA fluorescent intensity increased after DMNQ exposure for 3 hr as compared to control (Figure 27 and Figure 28). After 3 hr exposure with 20 μmol/l DMNQ, no s igns of cell death were observed under the microscope. The result suggested that treatment with 20 μmol/l DMNQ for 3 hr increased the ROS production without any cytotoxic in HK-2 cells. Thus, this condition was used for evaluation the radical scavenging activity of silymarin against DMNQ.
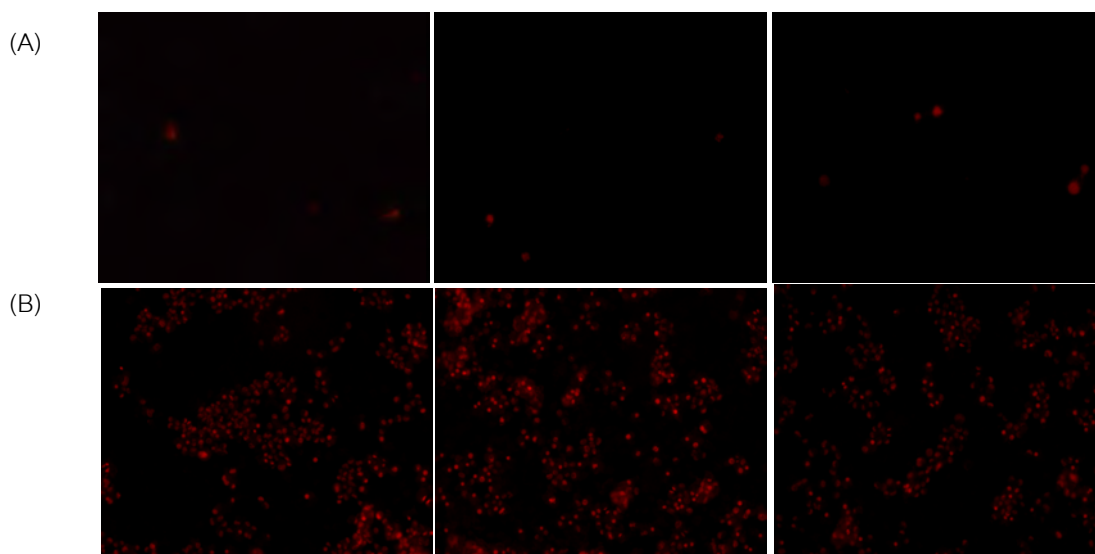
Figure 27 Fluorescent intensity of DHE after DMNQ exposure. Cells were treated with 20 μmol/l DMNQ for 3 hr. Controls were untreated cells. Cells were investigated the fluorescent intensity of DHE using reversed microscope (n=3). (A) control: 3 hr incubation time and (B) DMNQ: 3 hr incubation time.
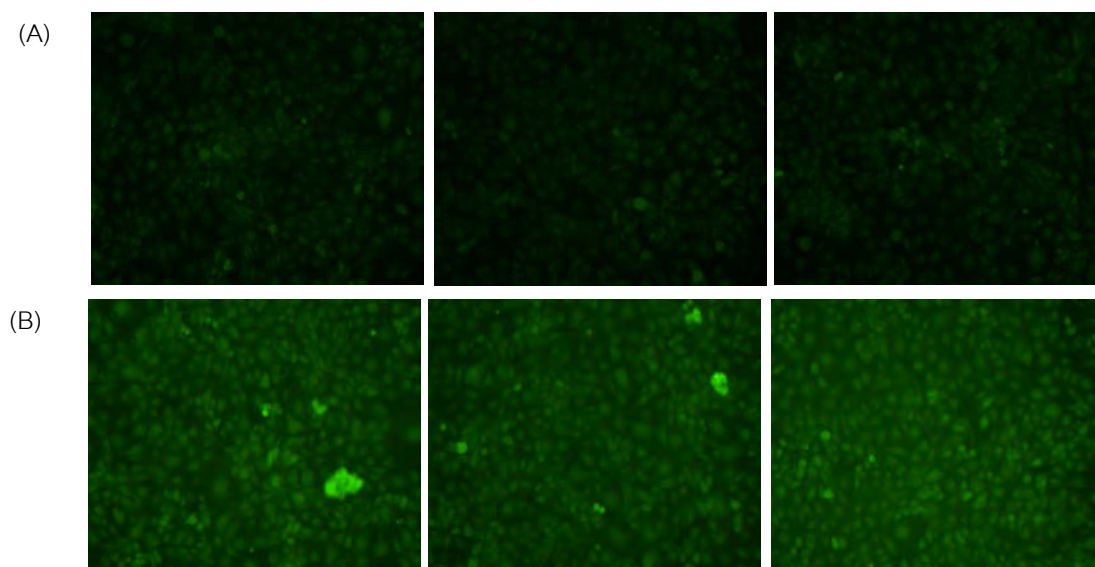
Figure 28 Fluorescent intensity of DCFH-DA after DMNQ exposure. Cells were treated with 20 μmol/l DMNQ for 3 hr. Controls were untreated cells. Cells were investigated the fluorescent intensity of DCFH-DA using reversed microscope (n=3). (A) control: 3 hr incubation time and (B) DMNQ: 3 hr incubation time.

1.2 ROS production from hydrogen peroxide and hydrogen peroxide/ferrous sulphate

Cells were treated with hydrogen peroxide at concentration of 400 μmol/l or hydrogen peroxide/ferrous sulphate at concentration of 1000 μmol/l for 30 min. The result showed that DCFH-DA fluorescent intensity increased after hydrogen peroxide or hydrogen peroxide/ferrous sulphate exposure for 30 min as compared to control (Figure 29 and Figure 30). After 30 min exposure to both ROS, no signs of cell death were observed under the microscope. The result suggested that treatment with 400 μmol/l hydrogen peroxide or 1000 μmol/l hydrogen peroxide/ferrous sulphate for 30 min increased the ROS production without any cytotoxic in HK-2 cells. Thus, this condition was used for evaluation the radical scavenging activity of silymarin against hydrogen peroxide and hydrogen peroxide/ferrous sulphate.
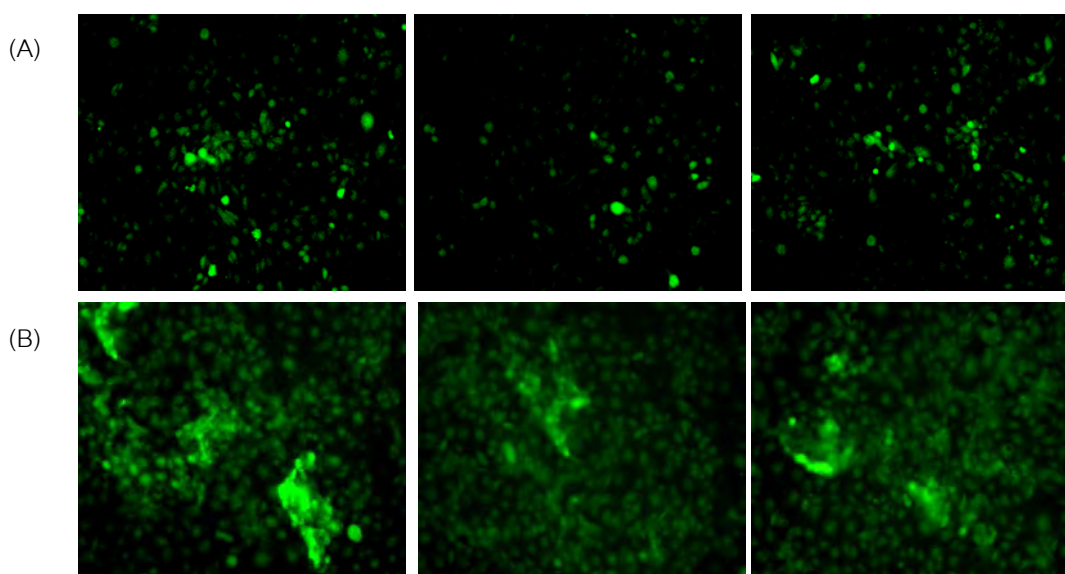
Figure 29 Fluorescent intensity of DCFH-DA after hydrogen peroxide exposure. Cells were treated with 400 μmol/l hydrogen peroxide for 30 min. Controls were untreated cells. Cells were investigated the fluorescent intensity of DCFH-DA using reversed microscope (n=3). (A) control: 30 min incubation time and (B) hydrogen peroxide: 30 min incubation time.
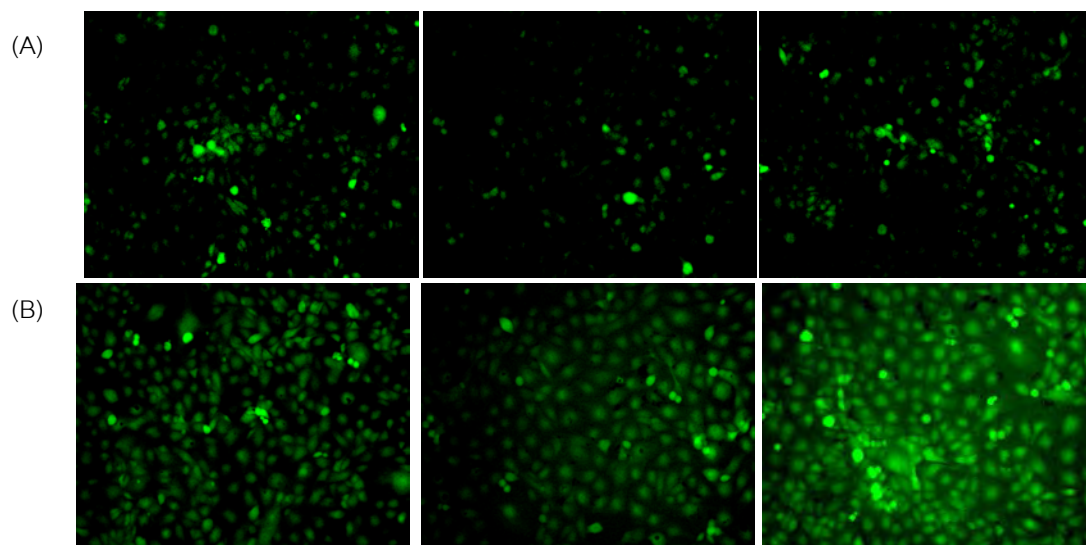
Figure 30 Fluorescent intensity of DCFH-DA after hydrogen peroxide/ferrous sulphate exposure. Cells were treated with 1000 μmol/l hydrogen peroxide/ferrous sulphate for 30 m in. Controls were untreated cells. Cells were investigated the fluorescent intensity of DCFH-DA using reversed microscope (n=3). (A) control: 30 min incubation time and (B) hydrogen peroxide/ferrous sulphate: 30 min incubation time.

2. Investigation of radical scavenging activity of silymarin against ROS

Cells were pre-treated with silymarin solution at a concentration of 100 μmol/l for 30 min followed by 20 μmol/l DMNQ for 3 hr or 400 μmol/l hydrogen peroxide for 30 m in or 1000 μmol/l hydrogen peroxide/ferrous sulphate for 30 min. The fluorescent intensity of DHE or DCF-DA was investigated in order to determine the ROS scavenging activity of silymarin using reversed microscope and flow cytometer.

2.1 Radical scavenging activity of silymarin against DMNQ

The fluorescent intensity of DHE, a specific fluorescence dye for superoxide anion detection, after DMNQ exposure was increased in the presence of silymarin (Figure 31). These results indicated that silymarin has no scavenging effect against DMNQ in HK-2 cells. The result was similar to previous reported (Dehmlow et al., 1996; Varga et al., 2006). This result may be due to the structure of silymarin. Silymarin has no the double bond between the C_2 and C_3 in the ring C. This structure

is important for the improvement of superoxide anion radicals scavenging activity (Cos et al., 1998; Varga et al., 2006). However, DMNQ significantly increased intracellular ROS which was detected by a p an ROS fluorescence dye DCFH-DA (Figure 32), suggesting that superoxide anion generated by DMNQ in these cells converted to another species of ROS. Interestingly, addition of sylimarin significantly reduced the DCFH-DA intensity. These results indicated that silymarin had an ability to scavenge other ROS species generated in these cells, but superoxide anion. The results is associated with the previous results that silymarin cannot protect against DMNQ-induced cytotoxicity in HK-2 cells.
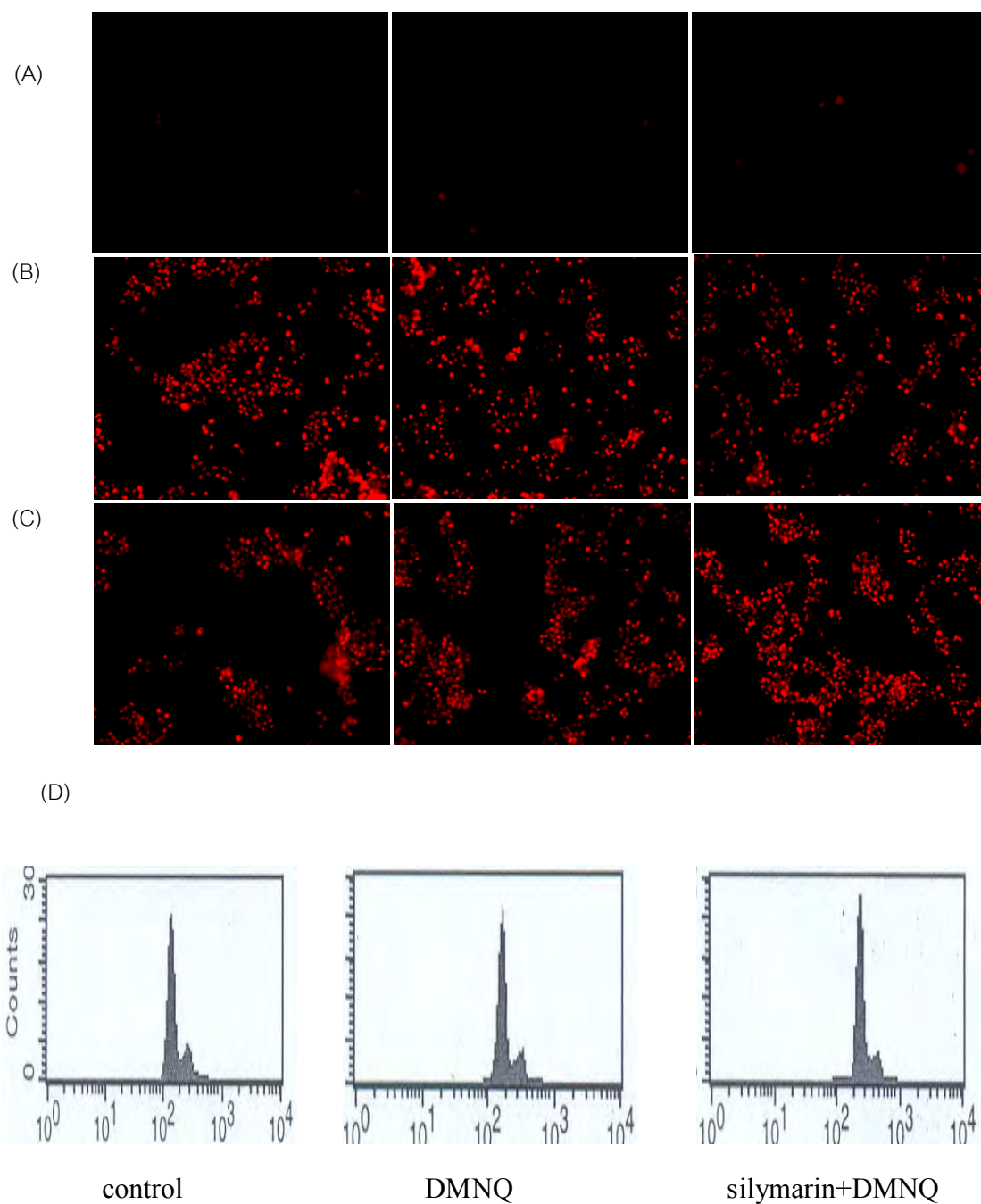
Figure 31 ROS scavenging activity of silymarin against DMNQ. Cells were incubated with 20 μmol/l DMNQ for 3 hr. Controls were untreated cells. Intensity of DHE was investigated using flow cytometry. (A) control, (B) DMNQ, (C) silymarin + DMNQ and (D) histogram charting the number of cells counted and the fluorescent intensity of DHE.
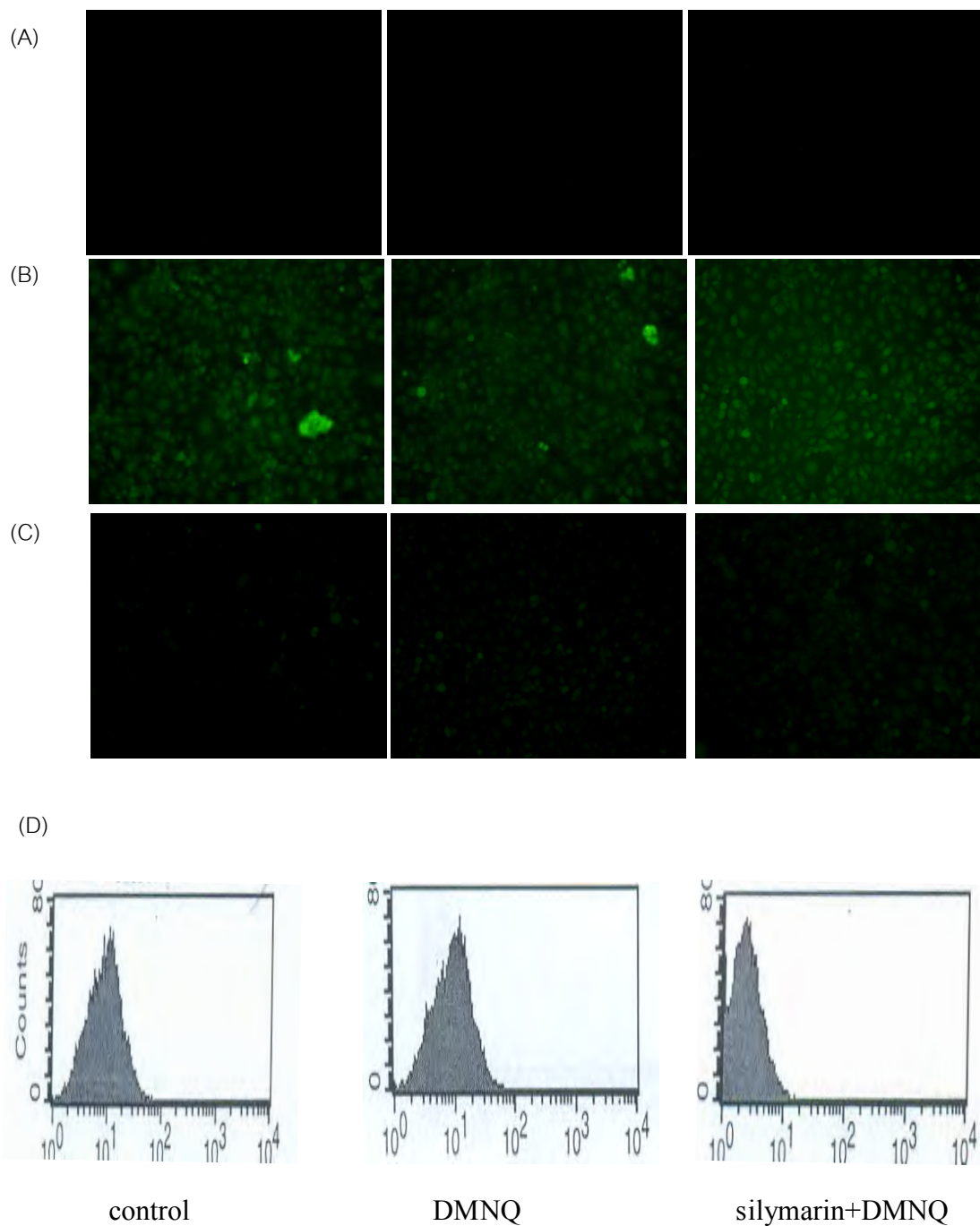
Figure 32 ROS scavenging activity of silymarin against DMNQ. Cells were incubated with 20 μmol/l DMNQ for 3 hr. Controls were untreated cells. Intensity of DCFH-DA was investigated using flow cytometry. (A) control, (B) DMNQ, (C) silymarin + DMNQ and (D) histogram charting the number of cells counted and the fluorescent intensity of DCFH-DA.

2.2 Radical scavenging activity of silymarin against hydrogen peroxide and hydrogen peroxide/ferrous sulphate

The DCFH-DA intensity after hydrogen peroxide or hydrogen peroxide/ferrous sulphate exposure was decreased in the presence of silymarin (Figure 33 and Figure 34). The result suggested that silymarin has ROS scavenging activity against hydrogen peroxide and hydroxyl radical in HK-2 cells. The antioxidant activity of silymarin against these ROS may be due to its structure that contains many hydroxyl groups. The 3, 5, 7-OH are important in the electron donating to hydroxyl radicals, stabilizing them and giving a stable flavonoid radical (Cos et al., 1998; Heim et al., 2002; Kiruthiga et al., 2007; Varga et al., 2006). The results are associated with previous studied that silymarin can prevent hydrogen peroxide or hydrogen peroxide/ferrous sulphate-induced cytotoxicity in HK-2 cells.

control   hydrogen peroxide   silymarin + hydrogen peroxide
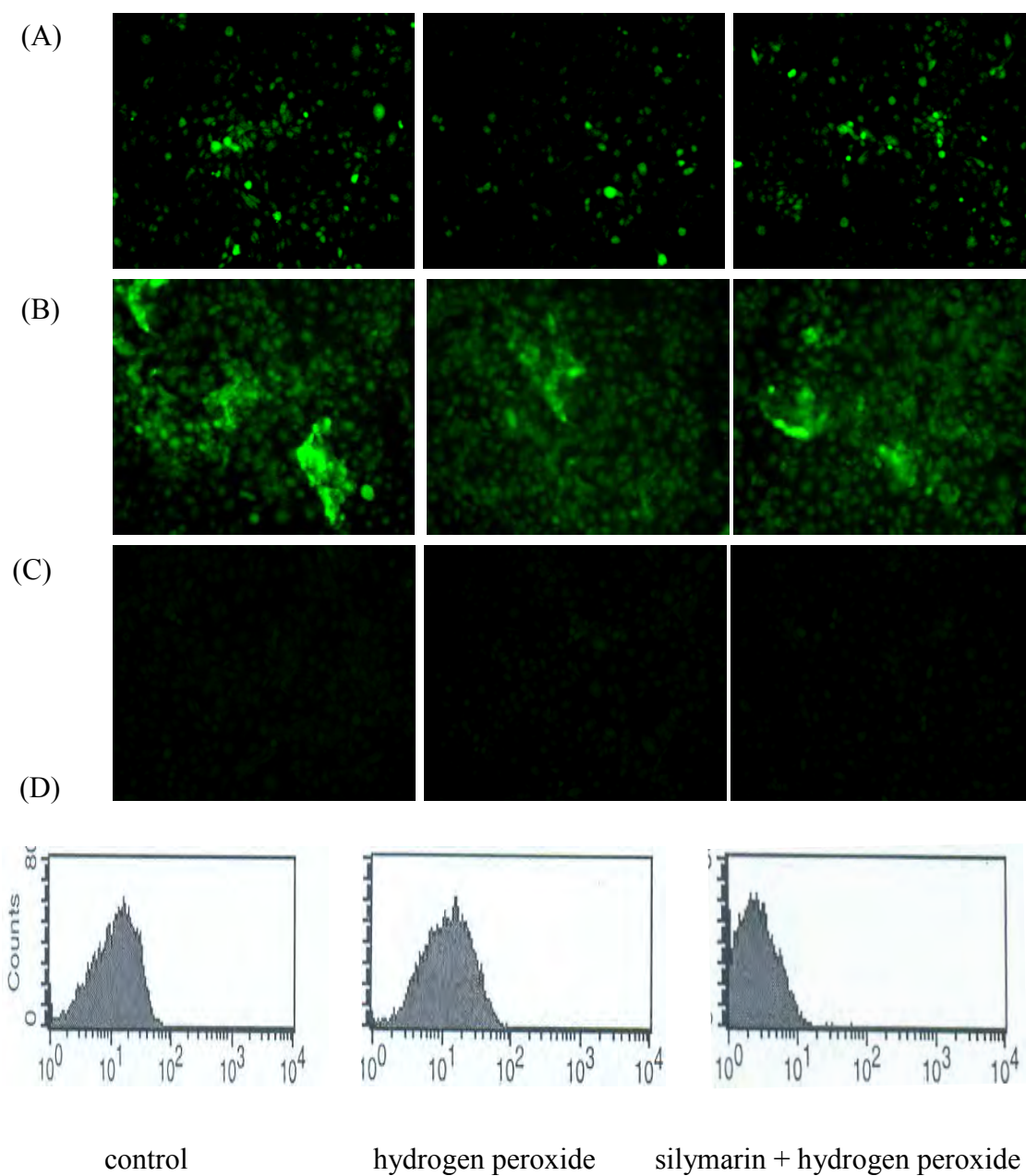
Figure 33 ROS scavenging activity of silymarin against hydrogen peroxide. Cells were incubated with 400 μmol/l hydrogen peroxide for 30 m in. Controls were untreated cells. Intensity of DCFH-DA was investigated using reveresd microscope and flow cytometry. (A) control, (B) hydrogen peroxide, (C) silymarin and hydrogen peroxide and (D) histogram charting the number of cells counted and the fluorescent intensity of DCFH-DA.
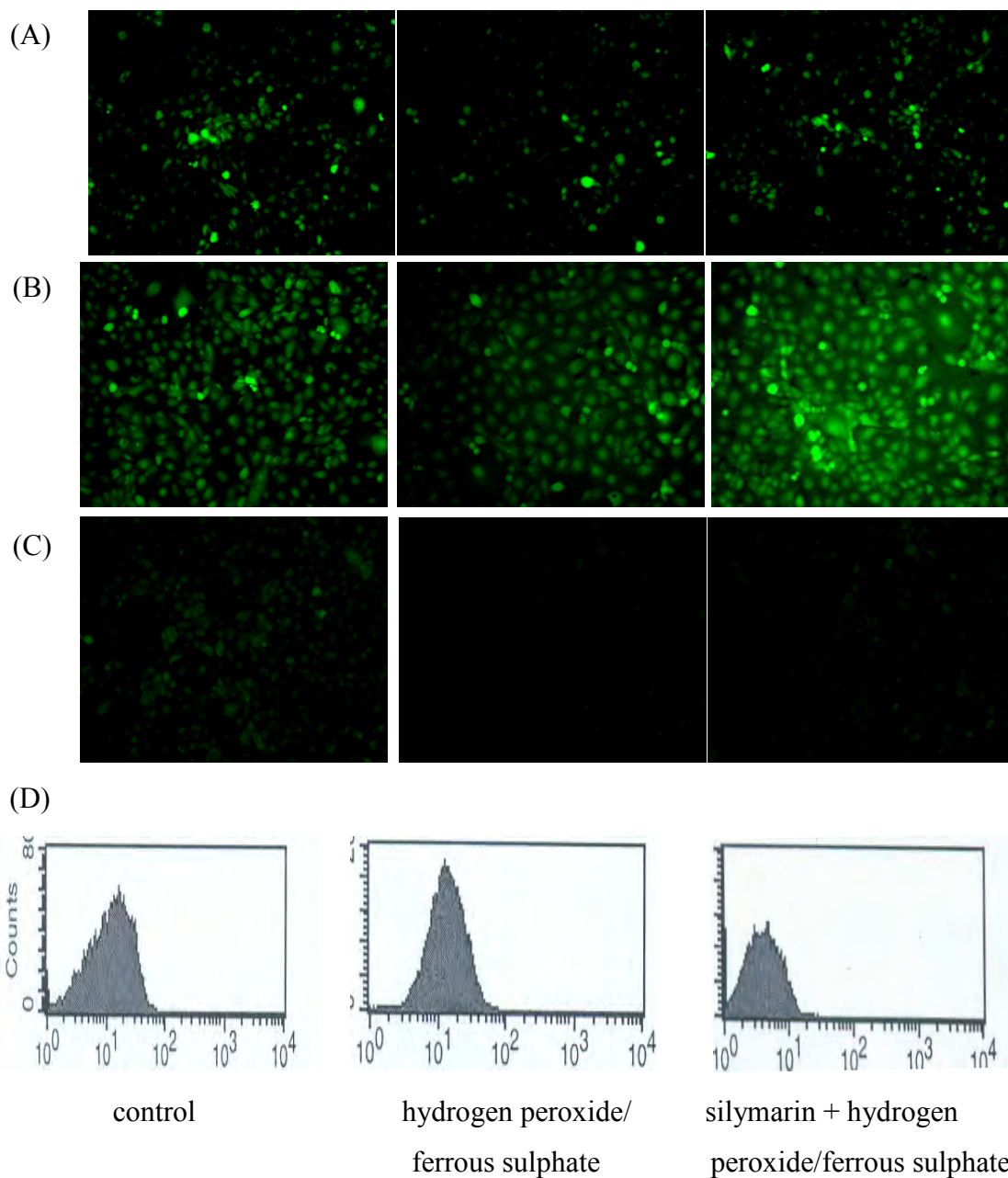
Figure 34 ROS scavenging activity of silymarin against hydrogen peroxide/ferrous sulphate. Cells were incubated with 1000 μmol/l hydrogen peroxide/ferrous sulphate for 30 min. Controls were untreated cells. Intensity of DCFH-DA was investigated using reveresd microscope and flow cytometry. (A) control, (B) hydrogen peroxide/ferrous sulphate, (C) silymarin and hydrogen peroxide/ferrous sulphate and (D) histogram charting the number of cells counted and the fluorescent intensity of DCFH-DA.

E. Cytoprotectivity of Silymarin against Cisplatin-Induced Cytotoxicity in HK-2 Cells

The results from the previous section suggested that silymarin showed the scavenging activity against hydrogen peroxide and hydroxyl radical in HK-2 cells. Therefore, silymarin may be cytoprotective against cisplatin-induced cytotoxicity since the cytotoxicity of cisplatin may be involved in ROS formation. The objective of this study was to evaluate the cytoprotectivity of silymarin against cisplatin in HK-2 cells. The cytotoxicity of cisplatin was firstly investigated and the IC_{50} of cisplatin was chosen for determining the cytoprotectivity of silymarin against cisplatin.

1. Investigation of the cytotoxicity of cisplatin

HK–2 cells were treated with cisplatin at a concentration range of 20–100 μmol/l. The cytotoxicity of cisplatin was evaluated after 24 hr of treatment. The percent cell viability significantly decreased at all concentrations studied after 24 hr of treatment as compared to the control (p-value < 0.05). The percentages of cell viability were 62.32%, 61.49%, 61.18%, 61.39% and 48.14% at concentration of 20, 40, 60, 80 a nd 100 μmol/l respectively (Figure 35). These results suggested that cisplatin was cytotoxic in HK-2 cells. The IC_{50} of cisplatin after 24 hr treatment was 100 μmol/l and was chosen for determining the cytoprotective of silymarin against cisplatin. The cytotoxicity of cisplatin is caused by DNA damage (Jordan and Fonesca, 2000) especially mitochondrial DNA damage (Singh, 1989). Mitochondrial damage is the primary event in renal injury with an overproduction of ROS, lipid peroxidation and enzyme leakage as a later consequence (Kruidering et al., 1997; Matsushima et al., 1998; Mcguinness and Ryan, 1994). In addition, cisplatin can lead to the depletion of antioxidants levels in the tissue (Sadzuka et al., 1994; Sueishi, et al., 2002).
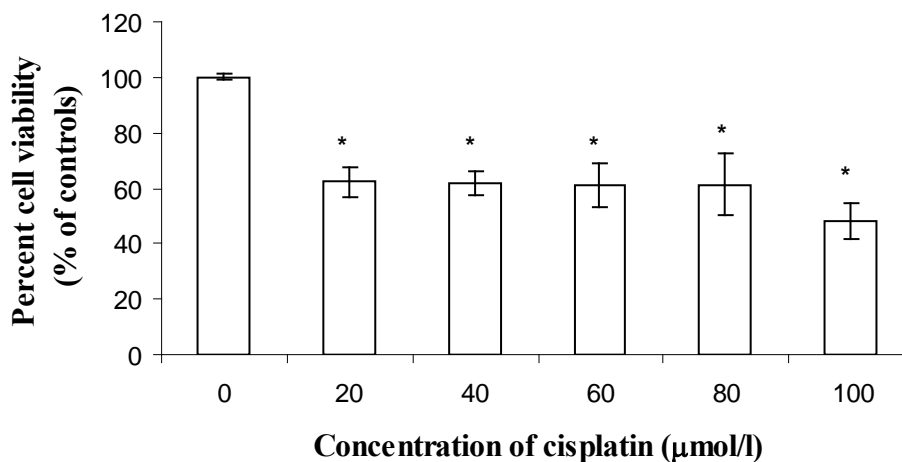
Figure 35 The cytotoxicity of cisplatin in HK-2 cells. Cells were incubated for 24 hr at 37°C with 20-100 μmol/l cisplatin. Controls were untreated cells. Cell viability was examined using MTT assay. Data are represented as mean ± SD (n=3).

∗ = significant difference from the control at a level of p-value < 0.05.

In order to investigate the types of cell death from cisplatin, cells were treated with cisplatin at concentrations of 20, 60 and 100 μmol/l. The types of cell death were investigated using hoescht 33342 and PI staining after 24 hr of treatment. The results showed that cisplatin led to both apoptosis and necrosis cell death (Figure 36 and 37). The effect of cisplatin on t he induction of apoptosis and necrosis cell death were similar to previous studies (Dunkern et al., 2001; Ramesh and Reeves, 2003; Faubel et al., 2004). The effect of cisplatin on the induction of apoptosis and necrosis in HK-2 cells depended on concentration. Low concentration of cisplatin led to more apoptosis than necrosis cell death (Figure 38). It has been reported that apoptosis cell death from cisplatin may be due to the ROS-activated apoptotic pathway including p53, caspase and tumor necrosis factor activation (Dunkern et al., 2001; Jiang et al., 2007). Necrosis cell death may be due to an overproduction of ROS and the induction of anti-inflammatory signal activation (Ali and Moundhri, 2006).
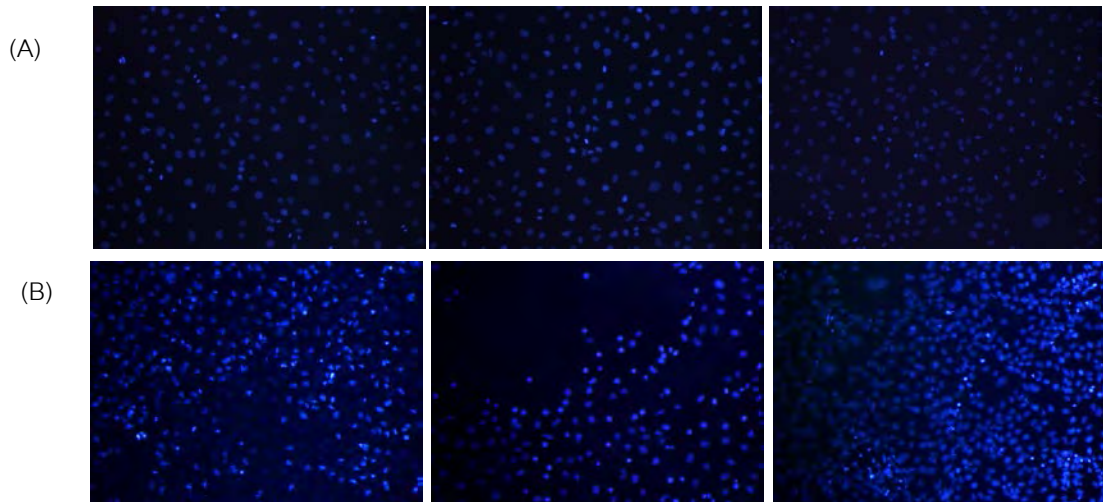
Figure 36 Cisplatin-induced apoptosis in HK-2 cells. Cells were incubated for 24 hr with 100 μmol/l cisplatin. Apoptosis cell death was investigated using hoescht 33342 staining (n=3). Controls were untreated cells; (A) control and (B) cisplatin.
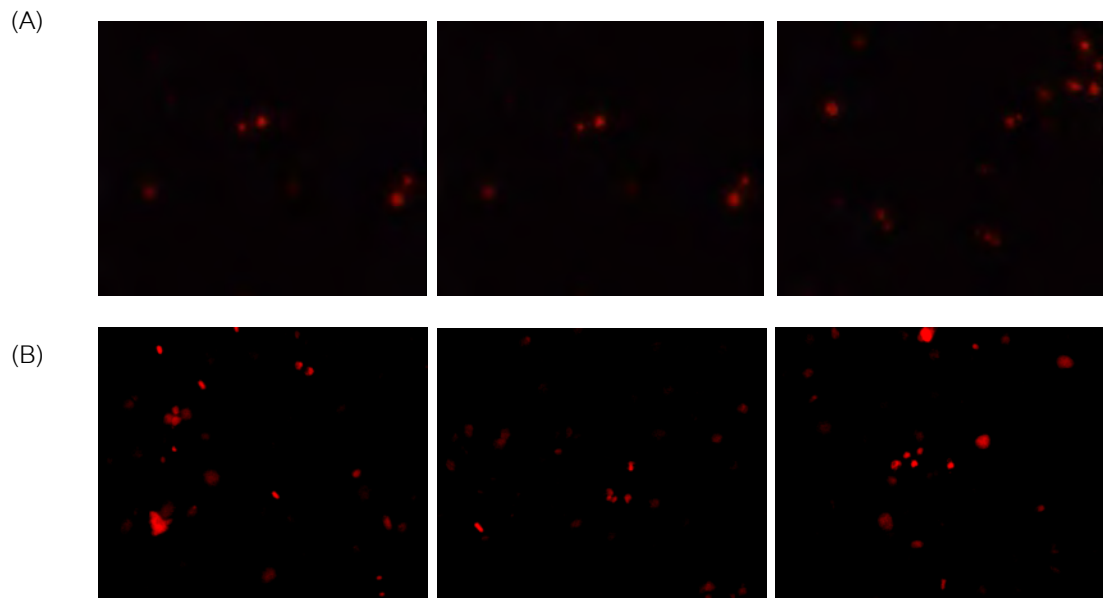
Figure 37 Cisplatin-induced necrosis in HK-2 cells. Cells were incubated for 24 hr with 100 μmol/l cisplatin. Necrosis cell death was investigated using PI staining (n=3). Controls were untreated cells. (A) control and (B) cisplatin.

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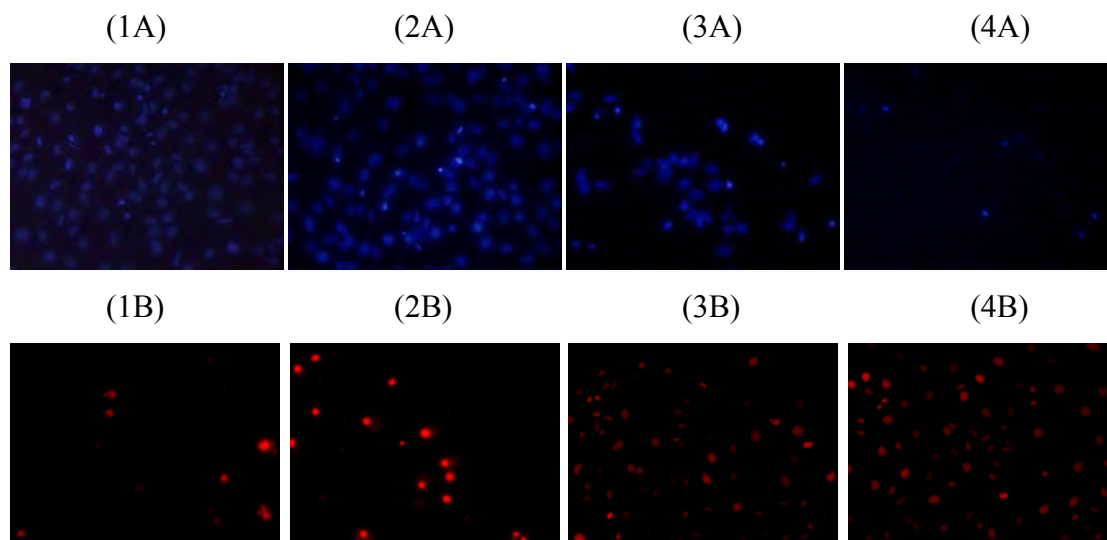
Figure 38 Cisplatin-induced apoptosis and necrosis in HK-2 cells. Cells were incubated for 24 hr with 20, 60 and 100 μmol/l cisplatin. Apoptosis and necrosis cell death were investigated using hoescht 33342 (A) and PI (B) staining, respectively. Controls were untreated cells. (1) control, (2) cisplatin 20 μmol/l, (3) cisplatin 60 μmol/l and (4) cisplatin 100 μmol/l.

2. Investigation of the cytoprotectivity of silymarin against cisplatin

HK-2 cells were pre-treated with silymarin at concentrations range of 25-400 μmol/l for 30 min followed by 100 μmol/l of cisplatin. Cytoprotectivity of silymarin against cisplatin was evaluated after 24 of treatment. All concentrations of silymarin significantly increased the percent cell viability in HK-2 cells compared to cells treated with cisplatin alone (p-value < 0.05) (Figure 39). For 24 hr incubation time, the percentages of cell viability were 83.58%, 82.59%, 86.77%, 92.16% and 103.18% at silymarin concentration of 25, 50, 100, 200 and 400 μmol/l respectively. The result suggested that silymarin had a protective effect against cisplatin-induced cytotoxicity in HK-2 cells. Cytoprotective effect of silymarin may be due to its antioxidant activity and its ability to stimulate protein and DNA synthesis (El-Shitany et al., 2008; Mansour et al., 2006; Pradeep et al., 2006; Sonnenbichler et al., 1999; Soto et al., 2003; Toklu et al., 2007).
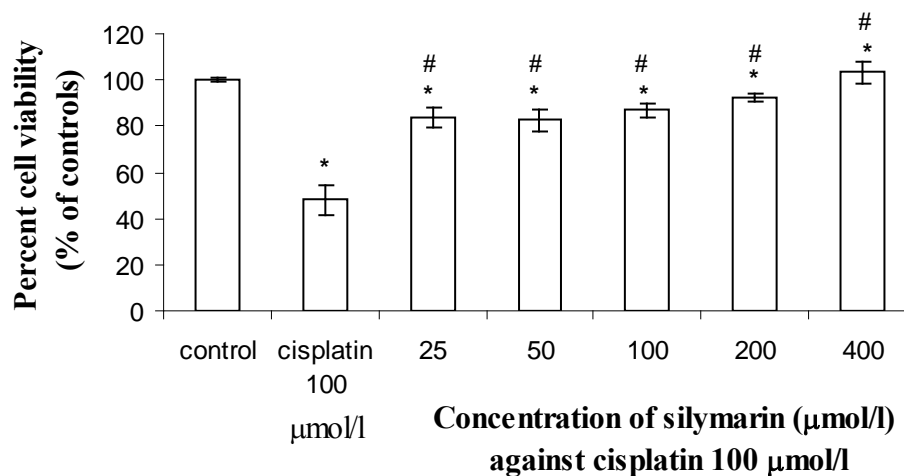
Figure 39 The cytoprotectivity of silymarin against cisplatin in HK-2 cells. Cells were pre-treated with 25-400 μmol/l silymarin for 30 min followed by 100 μmol/l cisplatin. Controls were untreated cells. Cell viability was examined using MTT assay after 24 of treatment. Data are represented as mean ± SD (n=3).

∗ = significant difference from the control at a level of p-value < 0.05.

= significant difference from treated with cisplatin alone at a level of p-value < 0.05.

In order to determine the effect of silymarin against the types of cell death induced by cisplatin, HK-2 cells were pre-treated with 100 μmol/l silymarin for 30 min followed by 100 μmol/l cisplatin. The effect of silymarin against cisplatin-induced apoptosis and necrosis in HK-2 cells were investigated using hoescht 33342 and PI staining after 24 hr of treatment. The number of apoptosis and necrosis in HK-2 cells pre-treated with silymarin significantly decreased as compared to cells treated with cisplatin alone (Figure 40, Figure 41 and Figure 42) (p-value < 0.05). The result from this study suggested that silymarin inhibited the effect of cisplatin on t he induction of both apoptosis and necrosis in HK-2 cells. The inhibitory effect of silymarin against cisplatin may be due to the inhibition of silymarin against tumor necrosis factor activation and caspase mediator (Manna et al., 1999; Kren and Walterova, 2005; Wang et al., 2005). In addition, reduction of cisplatin-induced cell death may be due to the antioxidant activity of silymarin (Wang et al., 2005).
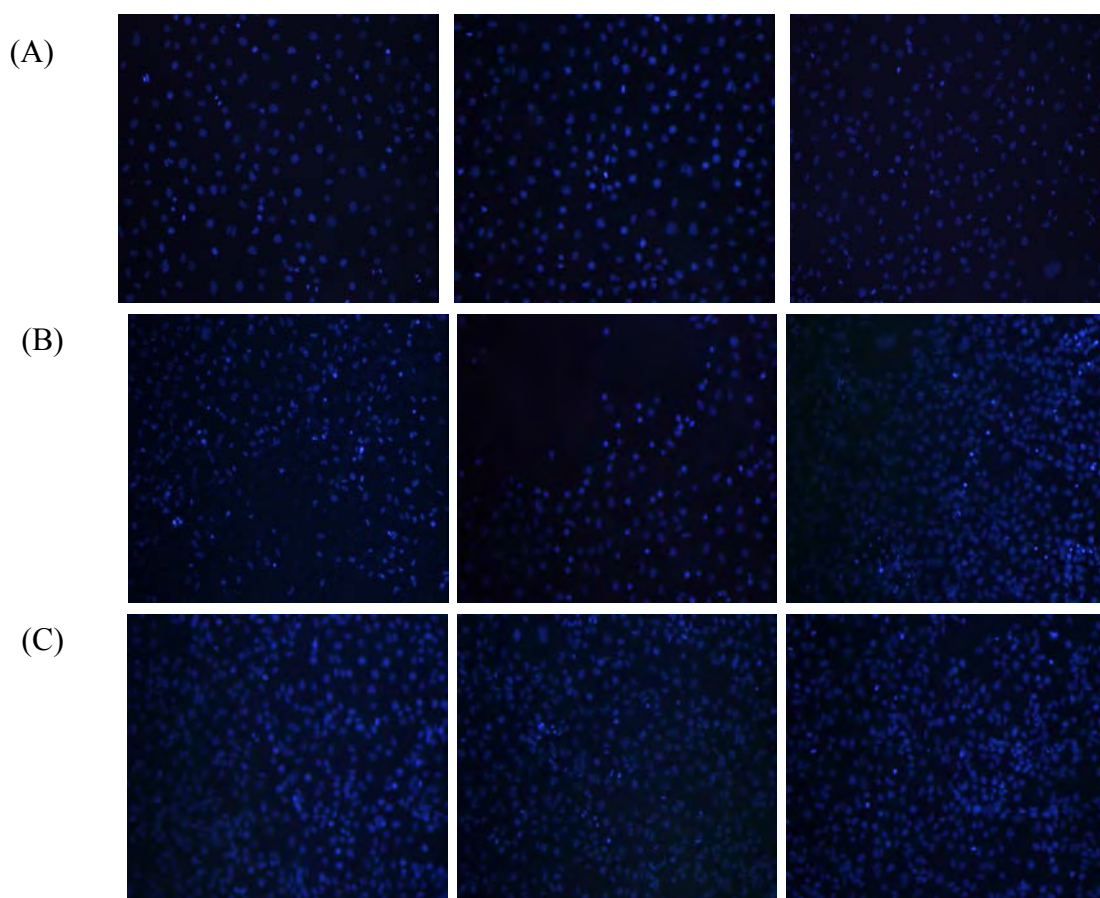
Figure 40 Effect of silymarin against cisplatin-induced apoptosis in HK-2 cells. Cells were pre-treated with 100 μmol/l silymarin for 30 min followed with 100 μmol/l cisplatin. Apoptosis cell death was investigated using hoescht 33342 staining after 24 incubation (n=3). Controls were untreated cells; (A) control, (B) cisplatin and (C) pre-treated with silymarin.
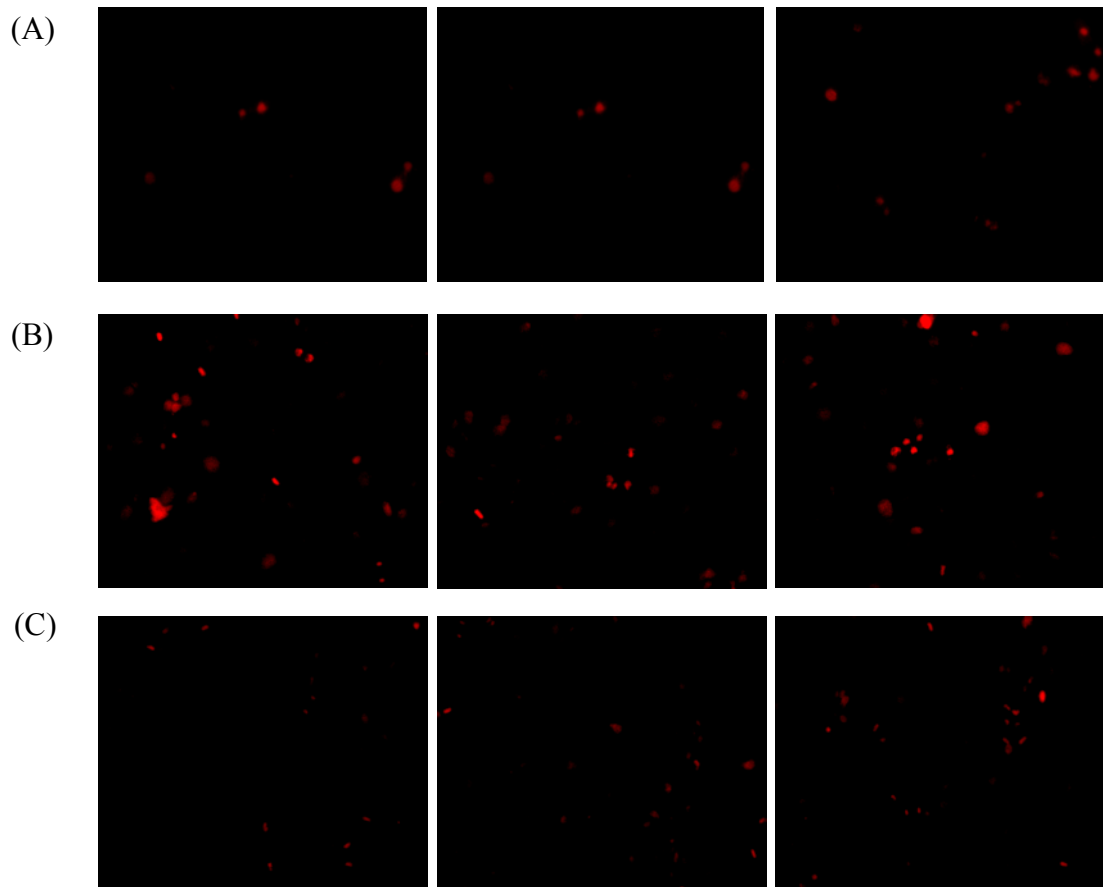
Figure 41 Effect of silymarin against cisplatin-induced necrosis in HK-2 cells. Cells were pre-treated with 100 μmol/l silymarin for 30 m in followed with 100 μmol/l cisplatin. Necrosis cell death was investigated using PI staining after 24 hr incubation (n=3). Controls were untreated cells; (A) control, (B) cisplatin and (C) pre-treated with silymarin.
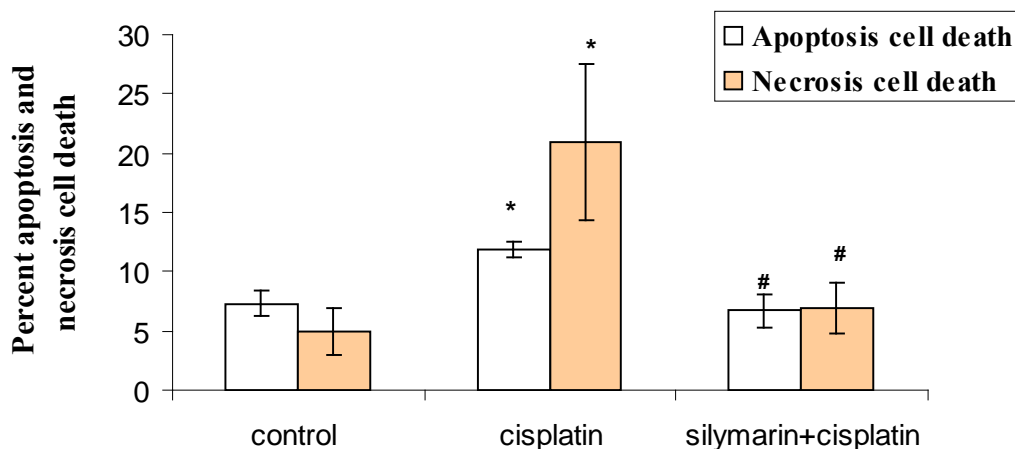
Figure 42 Percent apoptosis and necrosis cell death. Cells were pre-treated with 100 μmol/l silymarin for 30 min followed with 100 μmol/l cisplatin. Controls were untreated cells. Apoptosis and necrosis cell death was investigated using hoescht 33342 and PI staining after 24 hr incubation. Data are represented as mean ± SD (n=3).

∗ = significant difference from the control at a level of p-value < 0.05.

= significant difference from treated with cisplatin alone at a level of p-value < 0.05.

F. Antioxidant Activity of Silymarin against Cisplatin-Induced ROS Production in HK-2 Cells

The result from the previous section suggested that silymarin was cytoprotective against cisplatin-induced cytotoxicity. This result may be due to the antioxidant activity of silymarin. Therefore, the objective of this section was to evaluate the antioxidant activity of silymarin against cisplatin-induced cytotoxicity in HK-2 cells.

1. Investigation of cisplatin-induced ROS production

Cells were treated with 100 μmol/l cisplatin for 2 hr. The result showed that DCFH-DA fluorescent intensity increased after cisplatin exposure for 2 hr as compared to control (Figure 43). After 2 hr exposure with 100 μmol/l cisplatin, no signs of cell death were observed under the microscope. The result showed that

treatment with 100 μmol/l cisplatin for 2 hr increased the ROS production without any cytotoxic in HK-2 cells. Thus, this condition was chosen for evaluating the radical scavenging activity of silymarin against cisplatin.
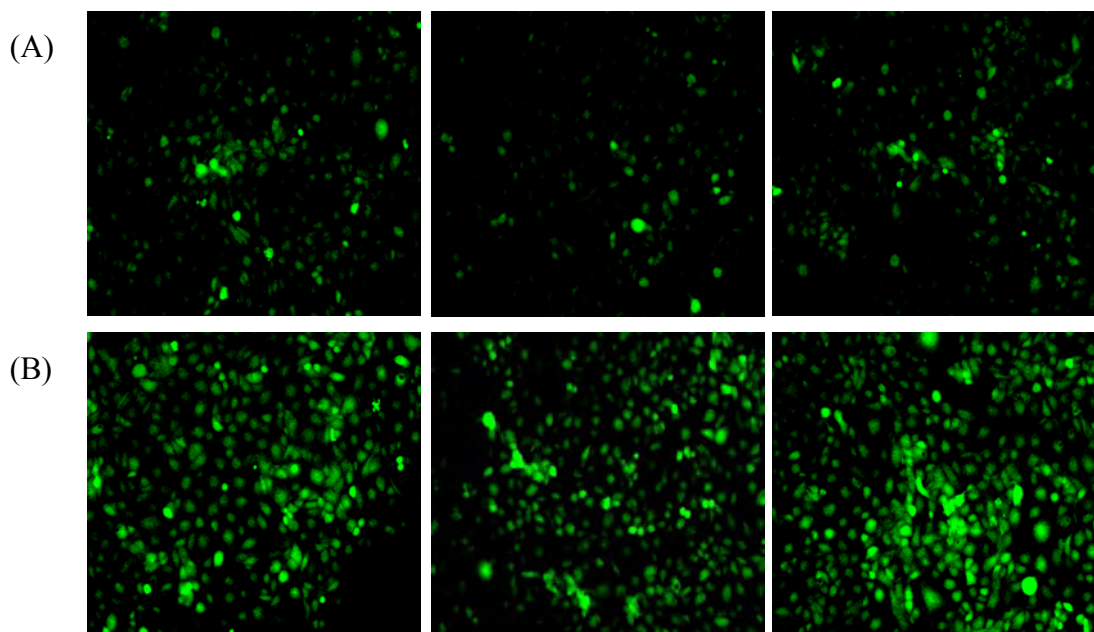
Figure 43 Fluorescent intensity of DCFH-DA after cisplatin exposure. Cells were treated with 100 μmol/l cisplatin for 2 hr. Control was untreated cells. DCFH-DA fluorescent intensity were investigated using microscope (n=3). (A) control and (B) cisplatin.

2. Investigation of the types of ROS production from cisplatin

ROS scavengers were used to determine the types of ROS production from cisplatin. To avoid the interferences between ROS scavengers and cisplatin on percent cell viability, cytotoxicity of ROS scavengers were investigated. HK-2 cells were treated with MnTBAP for 50 μmol/l, catalase for 1500 units/ml, sodium formate for 5 mmol/l and deferoxamine for 50 μmol/l. Cytotoxicity of these ROS scavengers were evaluated for 24 and 48 hr of treatment. Treatment with MnTBAP, catalase and sodium formate for 24 hr significantly increased the percent cell viability as compared to control (p-value < 0.05) (Figure 44). In addition, after treatment with all ROS scavengers for 48 hr, percent cell viability was similar to control (Figure 45). The

results suggested that all ROS scavengers at concentration studied were non-toxic to HK-2 cells.
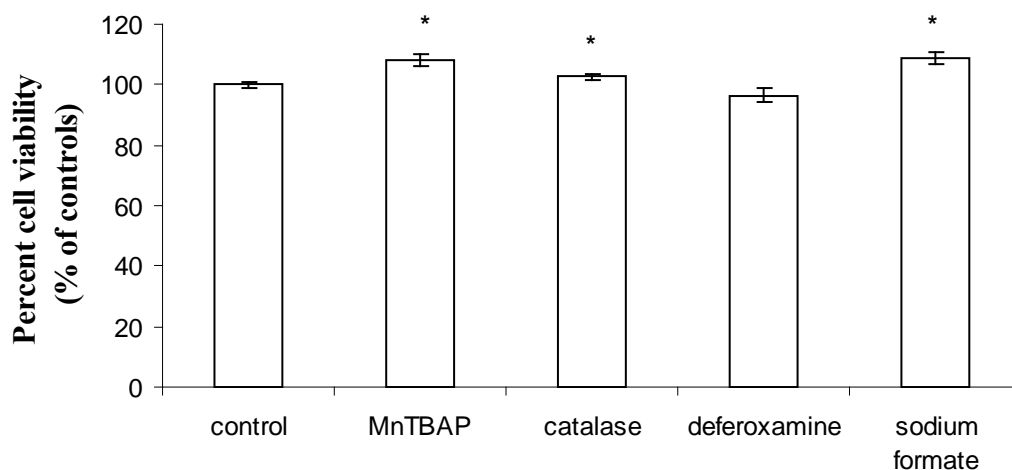
Figure 44 The cytotoxicity of ROS scavengers in HK-2 cells. Cells were incubated for 24 hr at 37°C with 50 μmol/l MnTBAP, 1500 units/ml catalase, 5 mmol/l sodium formate and 50 μmol/l deferoxamine. Controls were untreated cells. Cell viability was examined using MTT assay. Data are represented as mean ± SD (n=3).

∗ = significant difference from the control at a level of p-value < 0.05.
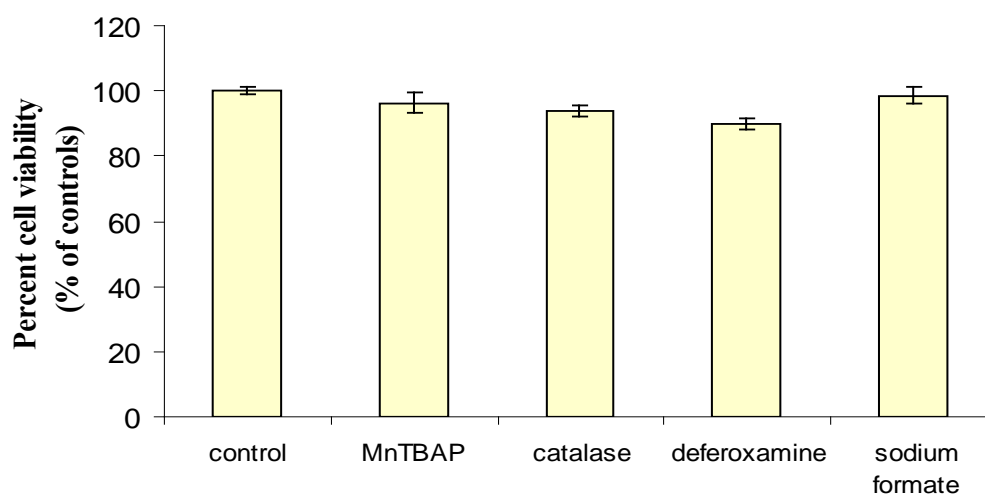
Figure 45 The cytotoxicity of ROS scavengers in HK-2 cells. Cells were incubated for 48 hr at 37°C with 50 μmol/l MnTBAP, 1500 units/ml catalase, 5 mmol/l sodium formate and 50 μmol/l deferoxamine. Controls were untreated cells. Cell viability was examined using MTT assay. Data are represented as mean ± SD (n=3).

In order to determine the type of ROS production from cisplatin using ROS scavengers, HK-2 cells were pre-treated with all ROS scavengers at concentration above for 30 min followed by 100 μmol/l cisplatin. Cytotoxicity of ROS scavengers were evaluated for 24 and 48 hr. All ROS scavengers significantly increased percent cell viability from cisplatin (p-value < 0.05) (Figure 46 and Figure 47). MnTBAP increased percent cell viability to 89.99% and 84.35% for 24 and 48 hr respectively. MnTBAP is a superoxide anion radical scavenger (Quagliaro et al., 2007). Increasing percent cell viability as scavenging the radical suggested that superoxide anion radical play an important role in cisplatin-induced cytotoxicity. This result was in consistent with Chirino et al. (2008) and Nishikawa et al. (2001) that cisplatin-induced superoxide anion radical production caused the cytotoxicity in the rat's renal. Sodium formate increased percent cell viability to 89.61% and 81.74% for 24 and 48 hr respectively. Sodium formate is a hydroxyl radical scavenger. Scavenging the radical resulted in the increasing of percent cell viability. The result suggested that hydroxyl radicals play an important role in cisplatin-induced cytotoxicity. This result was similar to previous studied by Beak et al. (2003) and Yoshida et al.(2003) that scavenging hydroxyl radical lead to prevention of cisplatin-induced cytotoxicity. Catalase combined with deferoxamine increased percent cell viability to 76.58% and 71.30% respectively. Deferoxamine is an iron chelator, prevents the conversion of hydrogen peroxide to hydroxyl radical. Catalase is a hydrogen peroxide scavenger. Increasing percent cell viability due to the hydrogen peroxide scavenger suggested that cytotoxicity of cisplatin involved with hydrogen peroxide production. This result was in agreement with Tsutsumishita et al.(1998) that cisplatin-induced hydrogen peroxide in proximal tubule cells and this cytotoxicity was reduced by catalase. The result suggested that cytotoxicity of cisplatin involved with ROS generation, particularly, superoxide anion radicals and hydroxyl radicals.
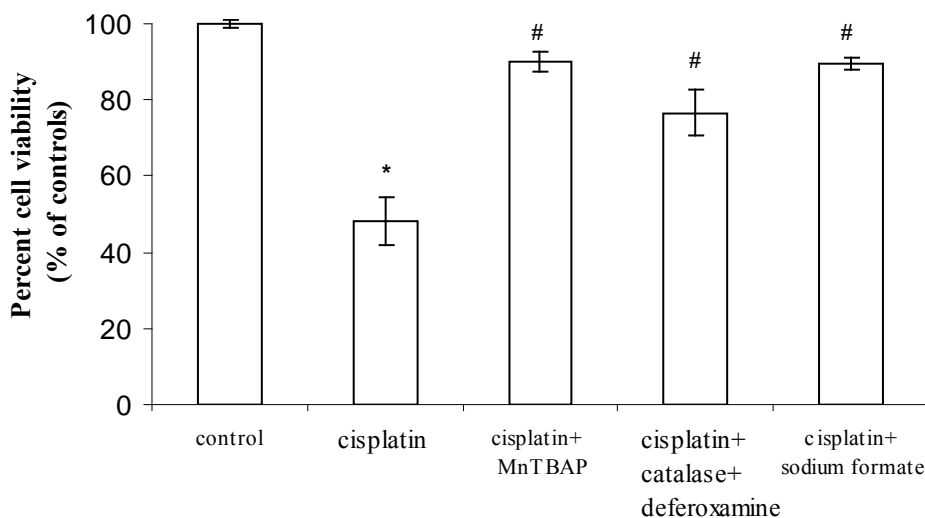
Figure 46 The cytotoprotectivity of ROS scavengers against cisplatin in HK-2 cells. Cells were pre-treated with 50 μmol/l MnTBAP, 1500 units/ml catalase, 5 mmol/l sodium formate and 50 μmol/l deferoxamine for 30 min followed by 100 μmol/l cisplatin. Controls were untreated cells. Cell viability was examined using MTT assay after 24 hr of treatment. Data is represented as mean ± SD (n=3).

∗ = significant difference from the control at a level of p-value < 0.05.

= significant difference from treated with cisplatin alone at a level of p-value < 0.05
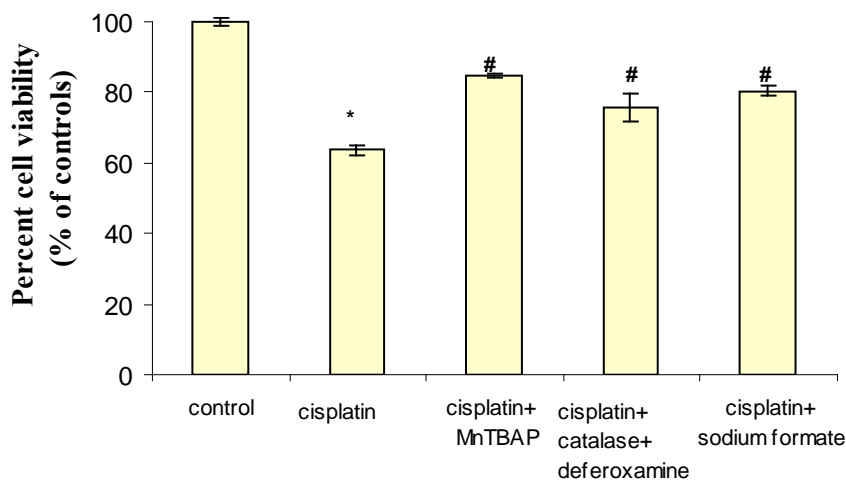
Figure 47 The cytotoprotectivity of ROS scavengers against cisplatin in HK-2 cells. Cells were pre-treated with 50 μmol/l MnTBAP, 1500 uni ts/ml catalase, 5 mmol/l sodium formate and 50 μmol/l deferoxamine for 30 m in followed by 100 μmol/l cisplatin. Controls were untreated cells. Cell viability was examined using MTT assay after 48 hr of treatment. Data is represented as mean ± SD (n=3).

∗ = significant difference from the control at a level of p-value < 0.05.

= significant difference from treated with cisplatin alone at a level of p-value < 0.05

3. Antioxidant activity of silymarin against cisplatin

Cells were pre-treated with 100 μmol/l silymarin for 30 min followed by 100 μmol/l cisplatin for 2 hr. The DCFH-DA fluorescent intensity after cisplatin exposure was decreased in the presence of silymarin (Figure 48). The result suggested that silymarin had an antioxidant activity against cisplatin-induced ROS production in HK-2 cells.
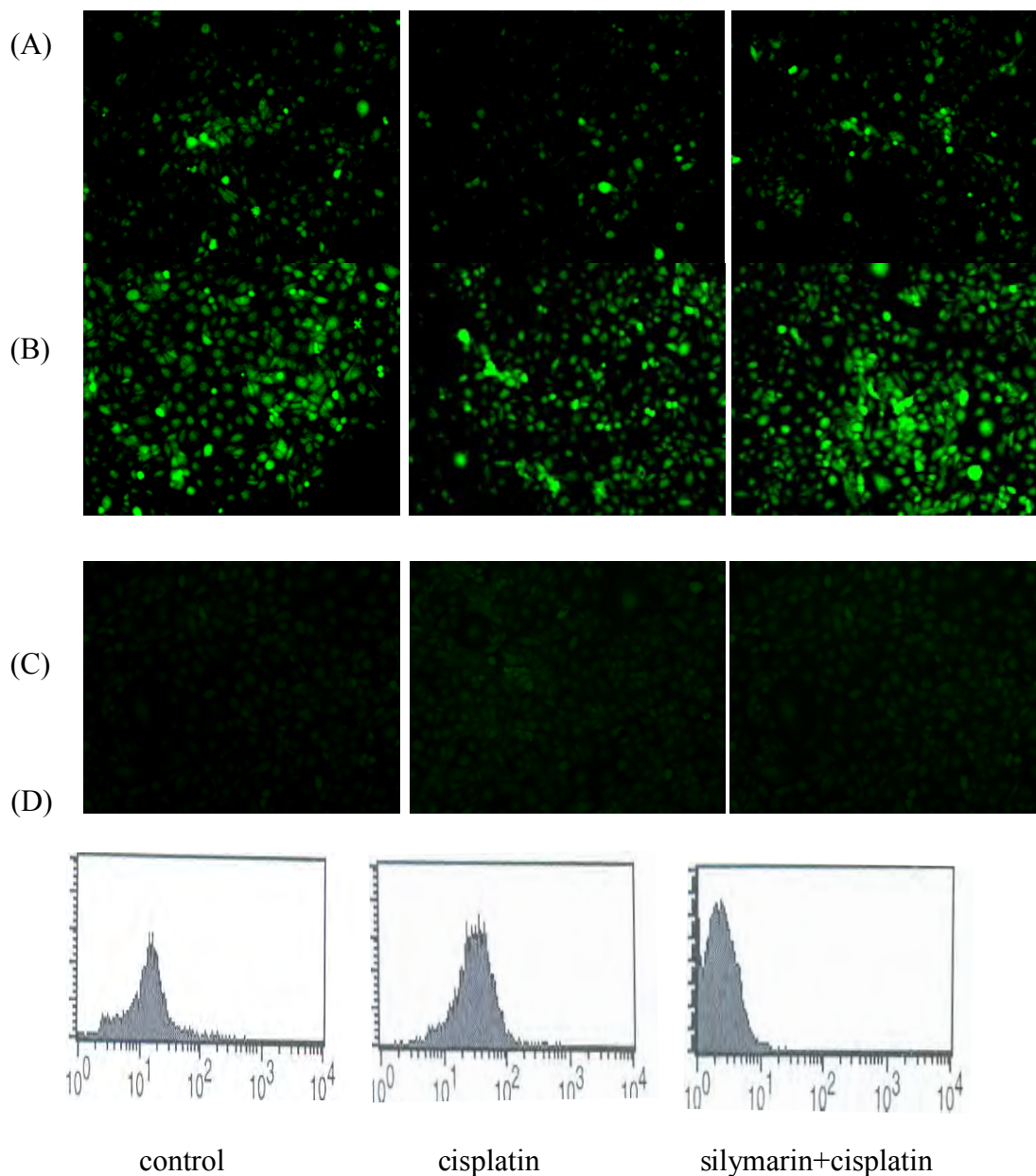
Figure 48 Antioxidant activity of silymarin against cisplatin. Cells were pre-treated with 100 μmol/l silymarin for 30 min followed by 100 μmol/l cisplatin for 2 hr. Controls were untreated cells. Intensity of DCFH-DA was investigated using reveresd microscope and flow cytometry. (A) ontrol, (B) cisplatin, (C) silymarin + cisplatin and (D) histogram charting the number of cells counted and the fluorescent intensity of DCFH-DA.

CHAPTER V

CONCLUSIONS

The purposes of this study were to investigate the effect of PEG 4000, PEG 6000 and PVP K 30 at various ratios on the dissolution of silymarin solid dispersion pellets prepared by fluidized-bed coating technique and the inhibition effect of silymarin against cisplatin-induced cytotoxicity in HK-2 cells. The results of this study could be concluded as follows:

All formulations of silymarin solid dispersion pellets prepared by fluidized-bed coating technique were intact in shape with yellow color. The carriers used in this study were PEG 4000, PEG 6000 and PVP K 30 at the ratio of carrier to silymarin for 1:2, 1:4 and 1:8. The percent dissolved of silymarin depended on the type of carriers, ratio of carrier and pH of the dissolution media. In the dissolution medium pH 7.5 with 2% SLS, the percent dissolved of silymarin solid dispersion pellets were comparable with silymarin powder. In the dissolution media pH 1.2 and 6.8, the percent dissolved of silymarin solid dispersion pellets in all formulations significantly increased as compared to silymarin powder except the ratio of silymarin to PVP K30 for 1:2 and 1:4 in dissolution medium pH 1.2. Increasing the ratio of PEG 4000 and PEG 6000 resulted in increasing the percent dissolved of silymarin solid dispersion pellets. Silymarin solid dispersion pellets using PEG 6000 as carriers provided the higher percent dissolved of silymarin than other carriers at the same ratio and the same dissolution medium.

Silymarin showed the protective effect against cisplatin-induced cytotoxicity in human kidney cells, HK-2 cells. In addition, the cytotoxicity of cisplatin caused apoptosis and necrosis cell death. Pre-treatment with silymarin decreased the number of apoptosis and necrosis cell death. Cisplatin-induced cytotoxicity in HK-2 is caused by the production of ROS, especially, superoxide anion radical, hydrogen peroxide and hydroxyl radical. Silymarin was cytoprotective against hydrogen peroxide and hydroxyl radical-induced cytotoxicity in HK-2 cells. The protective effect of

silymarin against cisplatin may be due to the hydroxyl radical scavenging activity of silymarin.

The results showed that the preparation of silymarin solid dispersion by fluidized-bed coating technique improved the dissolution of silymarin. Therefore the bioavailability of silymarin may be increased. In addition, silymarin was cytoprotective against cisplatin-induced cytotoxicity in HK-2 cells. The further study should investigate the protective effect of silymarin against cisplatin in in vivo study by using solid dispersion pellets compared to silymarin powder.

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APPENDICES

APPENDIX A

Evaluation of Antioxidant Activity of Silymarin by Determining DPPH Free Radical Scavenging Activity Using HPLC

(9th National Grad Research Conference: Poster Presentation 14-15 March 2008, Burapha University, Bangsaen, Chonburi, Thailand)

การประเมินฤทธิ์ต้านออกซิเดชันของซิไลมารินโดยวัดการกำจัดอนุมูลอิสระดีพีพีเอชโดยใช้โครมาโทกราฟีของเหลวแบบสมรรถนะสูง

Evaluation of Antioxidant Activity of Silymarin by Determining DPPH Free Radical Scavenging Activity Using HPLC

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บทนำ

silymarin เป็นสารที่สกัดได้จากเมล็ดของต้น Milk Thistle (*Silybum marianum*) วงศ์ Asteraceae และนำมาใช้ในการรักษาโรคตับ (Ulbricht and Basch, 2005) การลดความเป็นพิษต่อตับและอวัยวะอื่นๆ จากสารเคมีหรือยาบางชนิด เช่น ethanol (Singha, Ray and Dey, 2007) และการรักษาโรคต่างๆ เช่น โรคมะเร็ง (Yang et al., 2003; Hogan et al., 2007) เป็นต้น จากการศึกษาพบว่า silymarin มีกลไกในการออกฤทธิ์เป็นสารต้านออกซิเดชันด้วยหลายกลไก เช่น ยับยั้งการเกิดออกซิเดชันของไขมัน (Bindoli, Cavallini and Siliprandi, 1977) ช่วยในการกำจัดอนุมูลอิสระต่างๆ (Svobodova, Walterova and Psotova, 2006) จึงเป็นที่น่าสนใจในการประเมินฤทธิ์ต้านออกซิเดชันของ silymarin เปรียบเทียบกับสารต้านออกซิเดชันมาตรฐานต่างๆ เช่น L-ascorbic acid

การประเมินฤทธิ์ในการกำจัดอนุมูลอิสระของสารต้านออกซิเดชันด้วยวิธี DPPH นั้นเป็นวิธีที่น่าสนใจ เนื่องจากมีข้อดีคือ สะดวก รวดเร็ว มีหลักการคือเมื่อ DPPH สูญเสียอิเล็กตรอนจะกลายเป็นอนุมูลอิสระ (DPPH) เกิดเป็นสารละลายสีม่วง มีการดูดกลืนแสงที่ความยาวคลื่น 517 นาโนเมตร และเมื่อทำปฏิกิริยากับสารต้านอนุมูลอิสระจะเปลี่ยนแปลงสีของสารละลายเป็นสีเหลือง ทำให้ค่าการดูดกลืนแสงที่ความยาวคลื่นดังกล่าวหายไป (Brand-Williams, Cuvelier and Berset, 1995) โดยทั่วไปในการประเมินผลของวิธี DPPH จะใช้หลักวัดการเปลี่ยนแปลงของสี (Colorimetric method) ซึ่งถ้าสารที่นำมาประเมินฤทธิ์ต้านออกซิเดชันนั้นมีสี อาจทำให้เกิดการบดบังสีที่แท้จริง ทำให้ได้ผลที่ไม่น่าเชื่อถือ จึงมีการพัฒนาวิธีวิเคราะห์วิธีประเมินฤทธิ์ของสารต้านอนุมูลอิสระด้วยวิธี DPPH โดยใช้เทคนิค high performance liquid chromatography (HPLC) (Chandrasekar et al., 2006)

ดังนั้นในงานวิจัยนี้ จึงมีความสนใจในการประเมินและเปรียบเทียบฤทธิ์ต้านออกซิเดชันของซิไลมารินกับสารต้านออกซิเดชันมาตรฐานคือ กรดแอสคอร์บิก ด้วยวิธี DPPH และทำการวิเคราะห์ผลโดยใช้เทคนิค HPLC ซึ่งมีการตรวจสอบความถูกต้องของวิธีวิเคราะห์ดังกล่าวตามแนวทางของ ICH (International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use)

วิธีดำเนินงานวิจัย

1 ตรวจสอบความถูกต้องของวิธีวิเคราะห์ตามแนวทางของ ICH

1.1 *specificity* ตรวจสอบความจำเพาะเจาะจงของวิธีวิเคราะห์ โดยต้องไม่มีการรบกวนการวิเคราะห์ DPPH จากสารต่างๆ

1.2 *sensitivity* หาความเข้มข้นของ DPPH ที่น้อยที่สุดที่สามารถวิเคราะห์ได้โดยมีความถูกต้องแม่นยำ โดยพิจารณาอัตราส่วนระหว่าง signal : noise

1.3 *linearity* เตรียมสารละลาย DPPH ที่ความเข้มข้น 0.125, 0.25, 0.5, 1, 1.5 และ 2 มิลลิโมลาร์ (n=3) หาความสัมพันธ์ระหว่างความเข้มข้นของ DPPH ต่อ peak area โดยใช้สมการ linear regression ซึ่ง coefficient of determination (R²) ไม่น้อยกว่า 0.99

1.4 *accuracy* เตรียมสารละลาย DPPH ที่ความเข้มข้น 0.375, 0.75 และ 1.25 มิลลิโมลาร์ แล้วนำไปวิเคราะห์ (n=3) หาความถูกต้องของวิธีวิเคราะห์ โดยคำนวณเป็น % recovery และ ค่า % recovery ควรอยู่ในช่วง 80-120%

1.5 *precision* เตรียมสารละลาย DPPH ที่ความเข้มข้น 0.375, 0.75 และ 1.25 มิลลิโมลาร์ (n=3) หาความแม่นยำของวิธีวิเคราะห์โดยคำนวณเป็น % RSD และค่า %RSD ควรมีค่าไม่เกิน 10%

2. การประเมินฤทธิ์ต้านออกซิเดชันของ silymarin กับ L-ascorbic acid

ผสมสารละลาย silymarin กับ L-ascorbic acid ความเข้มข้น 0.5, 1 และ 2 มิลลิโมลาร์ (200 ไมโครลิตร) กับ สารละลาย DPPH ความเข้มข้น 2 มิลลิโมลาร์ (200 ไมโครลิตร) ผสมให้เข้ากัน ตั้งทิ้งไว้ในที่มืด 10 นาที วิเคราะห์ฤทธิ์การต้านออกซิเดชัน โดยคำนวณเปอร์เซ็นต์ในการกำจัดอนุมูลอิสระ

$$\%\text{การกำจัดอนุมูลอิสระ} = \frac{\text{peak area blank} - \text{peak area sample}}{\text{peak area blank}} \times 100$$

หมายเหตุ - การเตรียมสารละลาย DPPH, silymarin กับ L-ascorbic acid จะเตรียมใน methanol

- สภาวะของ HPLC ที่ใช้วิเคราะห์ DPPH ใช้คอลัมน์คือ C18 ขนาด 250×4.5 มิลลิเมตร เส้นผ่านศูนย์กลาง 5 ไมครอน, เฟสเคลื่อนที่คือ methanol : น้ำ (80: 20 %v/v), อัตราการไหล 1 มิลลิลิตร/นาที และ detector คือ UV-VIS spectrophotometer ที่ความยาวคลื่น 517 นาโนเมตร

สรุปและอภิปรายผลการทดลอง

จากการตรวจสอบความถูกต้องของวิธีวิเคราะห์ DPPH ในหัวข้อ specificity, sensitivity, linearity, accuracy และ precision พบว่าอยู่ในเกณฑ์ที่ยอมรับได้ ดังนั้นวิธีวิเคราะห์ดังกล่าวจึงมีความน่าเชื่อถือ และเมื่อนำวิธีดังกล่าวมาใช้ในการประเมินฤทธิ์ต้านออกซิเดชันของ silymarin โดยประเมินผลจาก % ในการกำจัด DPPH พบว่า silymarin มีฤทธิ์ในการต้านออกซิเดชันเพิ่มขึ้นตามความเข้มข้น ในขณะที่ L-ascorbic acid ที่ความเข้มข้นเริ่มต้นคือ 0.5 มิลลิโมลาร์ ก็ไม่พบ peak ของ DPPH แสดงให้เห็นถึงฤทธิ์ในการเป็นสารต้านออกซิเดชันที่แรง แต่อย่างไรก็ตามควรมีการประเมินฤทธิ์ต้านออกซิเดชันด้วยวิธีอื่นเพิ่มเติม และทำการศึกษาเทียบกับสารต้านออกซิเดชันมาตรฐานตัวอื่นๆ ด้วย เช่น vitamin E หรือสารในกลุ่ม flavonoids ต่างๆ เนื่องจาก silymarin อาจมีความจำเพาะเจาะจงในการกำจัดอนุมูลอิสระอื่นๆ ที่ต่างจากสารต้านออกซิเดชันมาตรฐานต่างๆ เพื่อการใช้ประโยชน์จาก silymarin ต่อไป

กิตติกรรมประกาศ

ขอขอบพระคุณ อาจารย์ ดร.อังคณา ตันติธุวานนท์ สำหรับคำปรึกษา อาจารย์ ดร.วิภาพร พนาพิศาล สำหรับคำปรึกษาและความอนุเคราะห์สาร silymarin รองศาสตราจารย์ ดร.ภาคภูมิ ตั้งอำนวย สำหรับคำปรึกษาและความอนุเคราะห์สาร DPPH และคณาจารย์รวมถึงเจ้าหน้าที่ภาควิชาเภสัชกรรม คณะเภสัชศาสตร์ จุฬาลงกรณ์มหาวิทยาลัยทุกท่านสำหรับคำปรึกษาแนะนำ

ผลการทดลอง

1. ผลการตรวจสอบความถูกต้องของวิธีวิเคราะห์

1.1 *specificity* วิธีวิเคราะห์ดังกล่าวมีความจำเพาะเจาะจงต่อ DPPH

รูปที่ 1 แสดง chromatogram ของ DPPH รูปที่ 2 แสดง chromatogram ของ silymarin และ L-ascorbic acid

1.2 *sensitivity* พบว่าวิธีวิเคราะห์ DPPH มี quantitation limit ของ DPPH เท่ากับ 0.125 มิลลิโมลาร์

1.3 *linearity* เมื่อ plot กราฟระหว่าง Peak area กับ DPPH พบว่าสามารถ fit กับ linear regression และมีค่า R² = 0.9964

1.4 *accuracy* และ *precision* ได้ผลดังตารางที่ 1

ตารางที่ 1 แสดง % recovery และ % RSD ของการวิเคราะห์ DPPH

ความเข้มข้นของ DPPH (มิลลิโมลาร์)	% recovery	% RSD
0.375	110.22 ± 9.37%	8.5
0.75	97.33 ± 7.06%	7.26
1.75	101.90 ± 1.44%	1.4
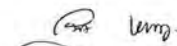
2. การประเมินฤทธิ์ต้านออกซิเดชันของซิไลมารินเทียบกับกรดแอสคอร์บิก

แผนภูมิที่ 1 แสดง% การกำจัด DPPH ของ silymarin และ L-ascorbic acid
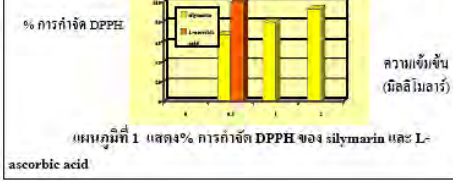
เอกสารอ้างอิง

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

Chandrasekar, D., Madhusudhana, K., Ramakrishna, S. and Diwan, P. (2006). Determination of DPPH free radicals scavenging activity by reverse-phase HPLC: a sensitive screening method for polyherbal formulation. Journal of Pharmaceutical Biomedical Analysis, 40, 460-464.

APPENDIX B

CERTIFICATE OF ANALYSIS OF SILYMARIN

Berlin

Berlin Pharmaceutical Industry Co.,Ltd.
63 Romklao Road, Bangkok 10520 Tel. 0-2737-6717 Fax. 0-2360-8730

CERTIFICATE OF ANALYSIS
RAW MATERIAL

Product	:	Silymarin Soluble (Powdered Milk Thistle Extract)			
Product Code	:	10-192-1			
Control Number	:	07R0595	Manufacturer	:	IVAX Pharmaceutical s.r.o.
Original Lot No.	:	17704SI926	Supplier	:	Thai Meochems Co.,Ltd
Lot Size	:	5 x 25 Kgs	Manufactured Date	:	04.2007
Invoice No.	:	3029	Retested Date	:	07.07.08
Receiving Date	:	04.07.07	Analysis Date	:	07.07.07

TESTS	REQUIREMENTS	RESULTS
Description	Microcrystalline brown to yellowish powder, tasteless or slightly bitter taste	Microcrystalline yellowish powder
Identification	Silymarin ; positive	Positive
Moisture Content (By KFR)	Not more than 1.5 % w/w	0.83
Assay	Min. 70% of Silymarin	83.4

Status	:	[X] Approved	[ ] Rejected	
Remarks	:			
Analyst	:		(Ms.Pitikan Kanjanapruk)	
QC Manager	:		(Ms.Kittima Ampawa)	

IVAX

Certificate of analysis

Page 1 from 2

Material code :	81323000001	No. of Certificate :	17704S1926 # 2767902/1/Q
Name :	SILYMARINE SOLUBLE		
Quality :	SV\QC\0177\V1		
Batch :	17704S1926	A. No. :	2767902
Manufacturing date :	04 2007	Retest date:	09 2008

Parameters	Specification	Results
Description	yellow, fine powder with characteristic odour	complies
Identity		
- HPLC	complies	complies
Water content	NMT 1,5 %	0,8 %
Sulphated ash	NMT 1,0 %	0,3 %
Heavy metals	NMT 100 ppm	<100 ppm
Assay		
- Silymarin assay in dried substance (DAB2001)	NLT 50,0 %	55,6 %
- Silybin in anhydrous substance (HPLC)	NLT 25,0 %	39,7 %
Bulk density	NLT 30 g/100 ml	37 g/100 ml
Dissolution in 30 min	NLT 75 %	93 %
Residual solvents		
- Ethanol	NMT 1,000 %	0,104 %
- Acetone	NMT 0,100 %	0,002 %
- Total amount of other solvents	NMT 0,100 %	0,012 %
Microbial contamination		
- Total aerobic microbial count	NMT 5 000 CFU/g	<5 CFU/g
- Fungi	NMT 500 CFU/g	<5 CFU/g
- Escherichia coli	absent	absent

The following residual organic solvents Class 1, as defined in the ICH Q3C(R3): benzene, carbon tetrachloride, 1,2-Dichloroethane, 1,1-Dichloroethene and 1,1,1-Trichloroethane are not present in the Active Pharmaceutical Ingredient.

IVAX

Certificate of analysis

Material code :	81323000001	No. of Certificate :	17704SI926 # 2767902/1/Q
Name :	SILYMARINE SOLUBLE		
Quality :	SV\QC\0177\V1		
Batch :	17704SI926	A. No. :	2767902
Manufacturing date :	04 2007	Retest date:	09 2008

The product has been produced and controlled in compliance with GMP rules and valid documentation. Tested parameters comply with the approved specification.

Date :   22.5.2007

Approved by :

Tomáš Kolašin
QA authorized person

Bohumir Biba
QC manager
spá. Jiří Hendrych

This document has been created electronically in system with electronic signature.

Storage terms: Temperature: to +25 °C. Protect from light.

IVAX Pharmaceuticals s.r.o.
OSTRAVSKÁ 29 , 747 70 OPAVA 9
CZECH REPUBLIC

APPENDIX C

PRODUCT INFORMATION OF HK-2 CELLS

ATCC®

Product Information Sheet for CRL-2190

Cell Line Designation: HK-2
ATCC® Catalog No. CRL-2190

Table of Contents:
- Cell Line Description
- Biosafety Level
- Use Restrictions
- Handling Procedure for Frozen Cells
- Handling Procedure for Flask Cultures
- Subculturing Procedure
- Medium Renewal
- Complete Growth Medium
- Cryoprotectant Medium
- References
- Replacement Policy
- Specific Batch Information

Cell Line Description
Organism: *Homo sapiens* (human)
Tissue: kidney, cortex; proximal tubule; human papillomavirus 16 (HPV-16) transformed
Age: adult
Gender: male
Morphology: epithelial
Growth properties: adherent
DNA profile (STR analysis)
    Amelogenin: X,Y
    CSF1PO: 13
    D13S317: 9
    D16S539: 11,12
    D5S818: 12
    D7S820: 10,11
    TH01: 9
    TPOX: 8,9
    vWA: 17,18
Depositors: R.A. Zager
Comments: HK-2 (human kidney 2) is a proximal tubular cell (PTC) line derived from normal kidney. The cells were immortalized by transduction with human papilloma virus 16 (HPV-16) E6/E7 genes. The recombinant retrovirus vector pLXSN 16 E6/E7 containing the HPV-16 E6/E7 genes was used to transfect the ectotropic packaging cell line Psi-2. Virus produced by the Psi-2 cells was used to infect the amphotropic packaging cell line PA317 (see ATCC CRL-9078). Virus produced by the PA317 cells was used to transduce primary PTCs. Although pLXSN 16 E6/E7 also confers resistance to neomycin, selection in G418 was not used to isolate transduced clones.
The cell line appears to be derived from a single cell based on Southern and FISH analysis.
The E6/E7 genes are present in the HK-2 genome as determined by PCR. The cells retain a phenotype indicative of well differentiated PTCs. They are positive for alkaline phosphatase, gamma glutamyltranspeptidase, leucine aminopeptidase, acid phosphatase, cytokeratin, alpha 3,beta 1 integrin, and fibronectin.
The cells are negative for factor VIII related antigen, -6.19 antigen and CALLA endopeptidase. HK-2 cells retain functional characteristics of proximal tubular epithelium such as Na+ dependent / phlorizin sensitive sugar transport and adenylate cyclase responsiveness to parathyroid, but not to antidiuretic hormone. The cells are capable of gluconeogenesis as evidenced by their ability to make and store glycogen. HK-2 cells are anchorage dependent. The cells will not grow in methylcellulose, soft agar or suspension. Cell growth is dependent on epidermal growth factor. HK-2 cells can reproduce experimental results obtained with freshly isolated PTCs.

Biosafety Level: 2
WARNING: This cell line is known to contain an agent that requires handling at Biosafety Level 2 containment [U.S. Government Publication *Biosafety in Microbiological and Biomedical Laboratories* (CDC, 1999)]. These agents have been associated with human disease. This cell line has **NOT** been screened for Hepatitis B, human immunodeficiency viruses or other adventitious agents. Cell lines derived from primate lymphoid tissue may fall under the regulations of 29 CFR 1910.1030 Bloodborne Pathogens.

Handle as a potentially biohazardous material under at least Biosafety Level 2 containment. This cell line is sent with the condition that you are responsible for its safe storage, handling and use. ATCC is not liable for damages or injuries resulting from receipt and/or use of an ATCC culture.
Detailed discussions of laboratory safety procedures are provided in **Laboratory Safety: Principles and Practices** (Fleming et al., 1995), the ATCC manual on quality control (Hay et al., 1992), the Journal of Tissue Culture Methods (Caputo, 1988), and the U.S. Government Publication, ***Biosafety in Microbiological and Biomedical Laboratories***, 4th ed. HHS Publication No. (CDC) 93-8395. U.S. Department of Health and Human Services, Centers for Disease Control and Prevention. Washington DC: U.S. Government Printing Office; 1999. The entire text is available online at www.cdc.gov/od/ohs/biosfty/bmbl4/bmbl4toc.htm.

Use Restrictions
These cells are distributed for research purposes only. ATCC recommends that individuals contemplating commercial use of any cell line first contact the originating investigator to negotiate an agreement. Third party distribution of this cell line is discouraged, since this practice has resulted in the unintentional spreading of cell lines contaminated with inappropriate animal cells or microbes.

Handling Procedure for Frozen Cells
To insure the highest level of viability, thaw the vial and initiate the culture as soon as possible upon receipt. If upon arrival, continued storage of the frozen culture is necessary, it should be stored in liquid nitrogen vapor phase and not at –70°C. Storage at –70°C will result in loss of viability.

SAFETY PRECAUTION: ATCC highly recommends that protective gloves and clothing always be used and a full face mask always be worn when handling frozen vials. *It is important to note that some vials leak when submersed in liquid nitrogen and will slowly fill with liquid nitrogen. Upon thawing, the conversion of the liquid nitrogen back to its gas phase may result in the vessel exploding or blowing off its cap with dangerous force creating flying debris.*
1.   Thaw the vial by gentle agitation in a 37°C water bath. To reduce the possibility of contamination, keep the O-ring and

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cap out of the water. Thawing should be rapid (approximately 2 minutes).

2. Remove the vial from the water bath as soon as the contents are thawed, and decontaminate by dipping in or spraying with 70% ethanol. *All of the operations from this point on should be carried out under strict aseptic conditions.*

3. Transfer the vial contents to a centrifuge tube containing 9.0 ml complete culture medium. and spin at approximately 125 xg for 5 to 7 minutes.

4. Resuspend cell pellet with the recommended complete medium (see the specific batch information for the culture recommended dilution ratio). and dispense into a **25 cm²** culture flask. *It is important to avoid excessive alkalinity of the medium during recovery of the cells. It is suggested that, prior to the addition of the vial contents, the culture vessel containing the complete growth medium be placed into the incubator for at least 15 minutes to allow the medium to reach its normal pH (7.0 to 7.6).*

5. Incubate the culture at 37°C in a suitable incubator. A 5% CO_2 in air atmosphere is recommended if using the medium described on this product.

Handling Procedure for Flask Cultures
The flask was seeded with cells (see specific batch information) grown and completely filled with medium at ATCC to prevent loss of cells during shipping.

1. Upon receipt visually examine the culture for macroscopic evidence of any microbial contamination. Using an inverted microscope (preferably equipped with phase-contrast optics), carefully check for any evidence of microbial contamination. Also check to determine if the majority of cells are still attached to the bottom of the flask; during shipping the cultures are sometimes handled roughly and many of the cells often detach and become suspended in the culture medium (but are still viable).

2. **If the cells are still attached,** aseptically remove all but 5 to 10 ml of the shipping medium. The shipping medium can be saved for reuse. Incubate the cells at 37°C in a 5% CO_2 in air atmosphere until they are ready to be subcultured.

3. **If the cells are not attached,** aseptically remove the entire contents of the flask and centrifuge at 125 xg for 5 to 10 minutes. Remove shipping medium and save. Resuspend the pelleted cells in 10 ml of this medium and add to 25 cm² flask. Incubate at 37°C in a 5% CO_2 in air atmosphere until cells are ready to be subcultured.

Subculturing Procedure
NOTE: the cells should not be allowed to become confluent, subculture at 80% of confluence
Volumes used in this protocol are for 75 cm2 flask; proportionally reduce or increase amount of dissociation medium for culture vessels of other sizes.

1. Remove and discard culture medium.

2. Briefly rinse the cell layer with 0.05% (w/v) Trypsin-0.53mM EDTA solution to remove all traces of serum which contains trypsin inhibitor.

3. Add 3.0 ml of Trypsin-EDTA solution to flask and observe cells under an inverted microscope until cell layer is dispersed (usually with 5 to 15 minutes).

   Note: To avoid clumping do not agitate the cells by hitting or shaking the flask while waiting for the cells to detach. Cells that are difficult to detach may be placed at 37°C to facilitate dispersal.

4. Add 6.0 to 8.0 ml of complete growth medium and aspirate cells by gently pipetting.

5. To remove trypsin-EDTA solution, transfer cell suspension to centrifuge tube and spin at approximately 125 xg for 5 to 10 minutes.

6. Discard supernatant and resuspend cells in fresh growth medium. Add appropriate aliquots of cell suspension to new culture vessels.
   Subcultivation Ratio: 1:4.

7. Place culture vessels in incubators at 37°C.

Note: For more information on enzymatic dissociation and subculturing of cell lines consult Chapter 10 in *Culture of Animal Cells, a manual of Basic Technique* by R. Ian Freshney, 3rd edition, published by Alan R. Liss, N.Y., 1994.

Medium Renewal
Two to three times weekly.

Complete Growth Medium
The base medium for this cell line is provided by Invitrogen (GIBCO) as part of a kit: Keratinocyte Serum Free Media (K-SFM), Kit Catalog Number 17005-042. This kit is supplied with each of the two additives required to grow this cell line (bovine pituitary extract (BPE) and human recombinant epidermal growth factor (EGF)).
To make the complete growth medium, you will need to add the following components to the base medium:
•0.05 mg/ml BPE - provided with the K-SFM kit
•5 ng/ml human r EGF - provided with the K-SFM kit.
NOTE: Do not filter EGF.
This medium is formulated for use with a 5% CO_2 in air atmosphere.

Cryoprotectant Medium
Complete culture medium described above supplemented with 7.5% (v/v) DMSO.
Cell culture tested DMSO is available as ATCC Catalog No. 4-X.

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Product Information Sheet for CRL-2190

Additional Information

Additional product and technical information can be obtained from the catalog references and the ATCC Web site at www.atcc.org, or by e-mail at tech@atcc.org.

References

(additional references may be available in the catalog description at www.atcc.org)

Ryan MJ et al. HK-2: **an immortalized proximal tubule epithelial cell line from normal adult human kidney.** Kidney Int. 45: 48-57, 1994 PubMed: 94172946

Hay, R. J., Caputo, J. L., and Macy, M. L., Eds. (1992), **ATCC Quality Control Methods for Cell Lines.** 2nd edition, Published by ATCC.

Caputo, J. L., **Biosafety procedures in cell culture.** J. Tissue Culture Methods 11:223-227, 1988.

Fleming, D.O., Richardson, J. H., Tulis, J.J. and Vesley, D., (1995) **Laboratory Safety: Principles and Practice.** Second edition, ASM press, Washington, DC.

Centers for Disease Control (1993), **Biosafety in Microbiological and Biomedical Laboratories** Human Health Service Publication No. (CDC) 93-8395. U.S. Dept. of Health and Human Services; 3rd Edition U.S. Government Printing Office Washington D.C.

ATCC Warranty

The viability of ATCC products is warranted for 30 days from the date of shipment. If you feel there is a problem with this product, contact Technical Services by phone at 800-638-6597 (U.S., Canada, and Puerto Rico) or 703-365-2700 (elsewhere) or by e-mail at tech@atcc.org.

Disclaimers

This product is intended for laboratory research purposes only. It is not intended for use in humans.

While ATCC uses reasonable efforts to include accurate and up-to-date information on this product sheet, ATCC makes no warranties or representations as to its accuracy. Citations from scientific literature and patents are provided for informational purposes only. ATCC does not warrant that such information has been confirmed to be accurate.

This product is sent with the condition that you are responsible for its safe storage, handling, and use. ATCC is not liable for any damages or injuries arising from receipt and/or use of this product. While reasonable effort is made to insure authenticity and reliability of strains on deposit, ATCC is not liable for damages arising from the misidentification or misrepresentation of cultures.

Please see the enclosed Material Transfer Agreement (MTA) for further details regarding the use of this product. The MTA is also available on our Web site at www.atcc.org.

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Product Information Sheet for CRL-2190

Lot number: 4738634

Designation: HK-2 Description: Kidney Proximal Tubule
Total Cells/mL: 1.0 x 10(6)
Expected Viability: 59.5% to 66.7%
Ampule Passage No.: Unknown
Population Doubling (PDL): N/A
Dilute Ampule Content: 1:10 (T-25) or 1:15 (T-75)
Volume/Ampule: 1 mL
Date Frozen: 05/25/07

A T-25 setup at a dilution of 1:10, using Keratinocyte-Serum Free
Medium supplemented with the supplied frozen EGF and BPE,
reaches approximately 30% to 40% confluence in 2 days.

American Type Culture Collection
10801 University Boulevard
Manassas, VA 20110-2209

Ordering info: 800 638-6597 (USA and Canada)
703 365-2700 (elsewere) Fax: 7033652750
email: sales@atcc.org web site: www.atcc.org

VITA

Miss Pacharaporn Jiamchaisri was born on M arch 2, 1983 in Bangkok, Thailand. She received her Bachelor Degree in Pharmacy from the Faculty of Pharmaceutical Sciences, Chulalongkorn University in 2005. She continued the enrollment to the Master degree program in Pharmacy at Chulalongkorn University in the same year.