ฤทธิ์ของหญ้าใต้ใบในการต้านออกซิเดชันและป้องกันความเป็นพิษต่อเซลล์หัวใจที่เกิดจากยา DOXORUBICIN

นางสาว ลินดา จุฬาโรจน์มนตรี

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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ANTIOXIDATIVE AND CYTOPROTECTIVE EFFECTS OF *PHYLLANTHUS URINARIA* L. ON DOXORUBICIN-INDUCED CARDIOTOXICITY

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ลินดา จุฬาโรจน์มนตรี : ฤทธิ์ของหญ้าใต้ใบในการต้านออกซิเดชันและป้องกันความเป็นพิษต่อเซลล์หัวใจที่เกิดจากยา DOXORUBICIN (ANTIOXIDATIVE AND CYTOPROTECTIVE EFFECTS OF *PHYLLANTHUS URINARIA* L. ON DOXORUBICIN-INDUCED CARDIOTOXICITY) อาจารย์ที่ปรึกษา : รศ.ดร.สุพัตรา ศรีไชยรัตน์, อาจารย์ที่ปรึกษาร่วม : ผศ.ดร.สุวรา วัฒนพิทยกุล, ดร. อังคณา หิรัญสาลี 113 หน้า. ISBN 974-17-6428-6.

ผลข้างเคียงที่สำคัญของการใช้ยา doxorubicin (DOX) คือ การเกิดพิษต่อหัวใจ ซึ่งเกิดจากการสร้างสารในกลุ่ม reactive oxygen species มากขึ้น งานวิจัยนี้ได้ศึกษาฤทธิ์ของสารสกัดจากสมุนไพรหญ้าใต้ใบ (*Phyllanthus urinaria* L.) ในการต้านออกซิเดชันและปกป้องความเป็นพิษต่อเซลล์หัวใจที่เกิดจาก doxorubicin โดยใช้ H9c2 cardiac myoblasts ในการทดสอบ พบว่า total antioxidant capacity ของหญ้าใต้ใบ (1 mg/mL) มีค่าเท่ากับ 5,306.75±461.62 μM FRAP value การทดสอบฤทธิ์ปกป้องเซลล์หัวใจจาก DOX ใช้การเปรียบเทียบค่า IC50 ของ DOX ในกรณีที่มีสารสกัดสมุนไพร (1 หรือ 10 μg/mL) และไม่มี เปรียบเทียบกับสารแอนติออกซิแดนซ์บริสุทธิ์ คือ วิตามินซี และ N-acetylcysteine (NAC) ที่ความเข้มข้น 100 μM สารสกัดสมุนไพรปกป้องเซลล์หัวใจจากความเป็นพิษของยาโดยแปรผันขึ้นกับความเข้มข้น โดยสมุนไพรทำให้ค่า IC50 ของ doxorubicin มีค่าเพิ่มขึ้น 2.8 เท่า และ 8.5 เท่า ตามลำดับ ในขณะที่วิตามินซี และ NAC ทำให้ค่า IC50 ของ doxorubicin มีค่าเพิ่มขึ้น 3.3 เท่า และ 4.2 เท่า ตามลำดับ การทดสอบต่อมาพบว่าสารทุกชนิดที่ใช้ทดสอบป้องกันการเกิด lipid peroxidation ได้อย่างสมบูรณ์ และยับยั้ง caspase-3 activation จากการเหนี่ยวนำของ DOX ที่ความเข้มข้น 1 μM การศึกษาด้าน endogenous antioxidant defense เช่น total glutathione (tGSH), catalase และ SOD activity พบว่าสารแอนติออกซิแดนซ์สามารถเปลี่ยนแปลงการทำงานของเอ็นไซม์เหล่านี้ได้ โดยสารสกัดสมุนไพรเดี่ยวๆ ทำให้ระดับ tGSH เพิ่มขึ้นแปรผันตามความเข้มข้นของสมุนไพรที่ใช้ และฤทธิ์นี้ยังคงอยู่แม้ว่าจะมี DOX ร่วมอยู่ด้วย พบผลลักษณะเช่นเดียวกันนี้เมื่อตรวจวัดการทำงานของเอ็นไซม์ catalase และ SOD การทดสอบการทำงานของ NFκB transcription factor แสดงให้เห็นว่าสารแอนติออกซิแดนซ์ทุกชนิดที่ศึกษายับยั้ง NFκB activation อย่างมีนัยสำคัญทางสถิติ ผลการศึกษานี้แสดงให้เห็นว่า สารสกัดสมุนไพรหญ้าใต้ใบปกป้องความเป็นพิษของเซลล์หัวใจที่เกิดจากการเหนี่ยวนำของ DOX โดยมีกลไกหลายอย่าง และสมุนไพรชนิดนี้อาจเป็นทางเลือกของแหล่งที่มาของแอนติออกซิแดนซ์ที่ได้จากธรรมชาติ ในการป้องกันความเป็นพิษที่หัวใจของผู้ที่ได้รับยา DOXORUBICIN

สาขาวิชาเภสัชวิทยา
ปีการศึกษา 2547

ลายมือชื่อนิสิต ลินดา จุฬาโรจน์มนตรี
ลายมือชื่ออาจารย์ที่ปรึกษา .....
ลายมือชื่ออาจารย์ที่ปรึกษาร่วม .....

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KEY WORDS: *Phyllanthus urinaria* L. / DOXORUBICIN / ANTIOXIDANTS/ REACTIVE OXYGEN SPECIES

LINDA CHULAROJMONTRI : ANTIOXIDATIVE AND CYTOPROTECTIVE EFFECTS OF *PHYLLANTHUS URINARIA* L. ON DOXORUBICIN-INDUCED CARDIOTOXICITY. THESIS ADVISOR : ASSOC. PROF. SUPATRA SRICHAIRAT Ph.D., THESIS COADVISOR : ASST. PROF. SUVARA K. WATTANAPITYAKUL, Ph.D., ANGKANA HERUNSALEE, Ph.D. 113 pp. ISBN 974-17-4428-6.

Cardiac toxicity is a major adverse effect caused by doxorubicin (DOX) therapy. DOX toxicity involves generation of reactive oxygen species (ROS). In this study, we investigated the antioxidative and cytoprotective effect of *Phyllanthus urinaria* (PU) against DOX toxicity using H9c2 cardiac myoblasts. Total antioxidant capacity of PU (1 mg/mL) was 5,306.75±461.62 μM FRAP value. DOX IC50s were used to evaluate cytoprotective effect of PU ethanolic extract (1 or 10 μg/mL) in comparison with ascorbic acid (VIT C, 100 μM) and N-acetylcysteine (NAC, 100 μM). PU treatments (1 or 10 μg/mL) dose dependently caused rightward-shifted of DOX IC50 to 2.8- and 8.5-fold, respectively, while treatments with VIT C and NAC increased DOX IC50 by 3.3- and 4.2-fold, respectively. Further evaluation of cytoprotective effect showed that PU and the pure antioxidants completely inhibited cellular lipid peroxidation and caspase-3 activation induced by DOX (1 μM). Endogenous antioxidant defense such as total glutathione (tGSH), catalase and SOD activity was also modulated by the antioxidants. PU treatment alone dose-dependently increased tGSH and this effect was retained in the presence of DOX. Similar effect was observed in catalase and SOD enzyme activity. The NFκB transcription factor assay demonstrated that all antioxidants significantly inhibited DOX-induced NFκB activation. Our results suggest that PU protected against DOX cardiotoxicity was mediated through multiple pathways and this plant may serve as alternative source of antioxidants for prevention of DOX cardiotoxicity.

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Advisor's signature .....
Co-advisor's signature .....

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LIST OF ABBREVIATIONS

ARE	=	Antioxidant Respond Element
Apaf-1	=	Apoptosis protease activating factor-1
Ab	=	Antiboby
ATP	=	Adenosine triphosphate
AngII	=	Angiotensin II
CAT	=	Catalase
CK	=	Creatinine kinase
DMEM	=	Dulbecco's modified Eagle's medium
DNA	=	Deoxyribonucleic acid
DOX	=	Doxorubicin
ELISA	=	Enzyme-linked immunosorbent assay
FRAP	=	Ferric Reducing/Antioxidant Power
FBS	=	Fetal Bovine Serum
GSH	=	Reduced Glutathione
GSSG	=	Oxidized Glutathione
GST	=	Glutathione S-transferase
GPx	=	Glutathione peroxidase
GR	=	Glutathione reductase
G6PD	=	Glucose 6-phosphate dehydrogenase
H_2O_2	=	Hydrogen Peroxide
IRPs	=	Iron-regulatory proteins
IREs	=	Iron-responsive elements
IκB	=	Inhibitory kappa B
IC50	=	Concentration giving 50 percent inhibition
IL-1	=	Interleukin one
LOOH	=	Lipid peroxide
LDL	=	Low density lipoprotein
MDA	=	Malondialdehyde
MI	=	Myocardial infarction

mL	=	milliliter
mg	=	milligram
M	=	Molar (mole per liter)
NADP	=	Nicotinamide adenine dinucleotide phosphate
NADPH	=	Nicotinamide adenine dinucleotide phosphate (reduced form)
NAC	=	N-acetylcysteine
$NO^{\bullet}$	=	Nitric Oxide
NOS	=	Nitric oxide synthase
iNOS	=	Inducible nitric oxide synthase
NFκB	=	Nuclear Factor Kappa B
nm	=	nanometer
nM	=	nanomolar (nanomole per liter)
$O^{2\bullet-}$	=	Superoxide anion
$OH^{\bullet}$	=	Hydroxy radical
$ONOO^{-}$	=	Peroxynitrite
PUFA	=	Polyunsaturated fatty acid
PARP	=	Poly(ADP-ribose) polymerase
pH	=	Potential of hydrogen
P. urinaria	=	*Phyllanthus urinaria*
ROS	=	Reactive oxygen species
RNS	=	Reactive nitrogen species
SOD	=	Superoxide Dismutase
SR	=	Sarcoplasmic reticulum
tGSH	=	Total Glutathione
TNF-α	=	Tumor necrosis factor-alpha
XO	=	Xanthine Oxidase

CHAPTER I

INTRODUCTION

Background and Significance

Reactive oxygen species (ROS) include the superoxide anion ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), the hydroxyl radical ($OH^{\bullet}$), lipid peroxides, nitric oxide ($NO^{\bullet}$), singlet oxygen (O_2^1), ozone (O_3), and hypochlorous acid (HOCl) (1). They are partially reduced products of oxygen; some are free radicals. They are known to cause oxidation of membrane phospholipids; lipid peroxidation; protein damage, including cleavage of amino acid bonds; DNA strand breaks, or base modifications leading to point mutations; inhibition of RNA and protein synthesis; protein cross-linking; impaired maintenance of membrane ion gradients; and depletion of cellular levels of ATP, leading to cellular dysfunction and eventually to disease (2). A number of major cellular antioxidant defense mechanisms exist to neutralize the damaging effects of ROS (3-5). Antioxidants, both enzymatic and non-enzymatic, limit oxidative damage to biological molecules by various mechanisms. Enzymatic antioxidant system (Cu,Zn- and Mn-superoxide dismutase (SOD), catalase, glutathione (GSH) peroxidase (GPX), and GSH reductase (GR) function by direct or sequential removal of ROS, thereby terminating their activities (6-8). Nonenzymatic antioxidant systems consist of scavenging molecules that are endogenously produced (GSH, ubichinol, uric acid) or those derived from the diet (vitamins C and E, carotenoids, lipoic acid, selenium, etc.). Thus, oxidative stress occurs when there is an imbalance between ROS generation and the scavenging capacity of antioxidative defense mechanism of the organism.

Cells are constantly being exposed to ROS. A large body of experimental evidence suggests that in many cases, a correlation exists between the degree of oxidative stress and the progression of a number of human diseases and conditions of most, if not all, human chronic diseases, including AIDS, cardiovascular diseases, arthritis, neurodegeneration, aging and cancer (9). Furthermore, drugs used to treat these diseases may themselves contribute to increased oxidative stress. It is well established that certain antineoplastic agents, such as doxorubicin (DOX), generate

ROS which primarily contribute to many adverse effects of the drug. Over 20 percent of patients who received doxorubicin exhibit life-threatening cardiac reactions or delayed cardiac dysfunction over a period of time. About 10-15% of patients will develop congestive heart failure with a mortality of 50% within 2 years. Several investigations have shown that tissue oxidation via increased levels of cellular superoxide anion/ oxidative stress are mechanisms by which doxorubicin-induced cardiotoxicity. The high level of oxidative stress during chemotherapy may overcome the antioxidant defenses of the patients. Currently, the antioxidant dexrazoxane is the only approved drug indicated for its clinical use in conjunction with doxorubicin to alleviate the toxic effects. Despite the usefulness of the antioxidant dexrazoxane, its cost is very high. According to Thailand economic crisis during the past few years, it is, therefore, logical to reduce imported drugs and promote the use of Thai medicinal plants that show antioxidant effects.

Among herbs screened for antioxidant effect, *P. urinaria* has shown very high antioxidant activity. The methanolic extract of the closely related species of *P. urinaria*, *P. amarus*, demonstrated antioxidant activity by inhibiting lipid peroxidation, scavenging hydroxyl and superoxide radicals *in vitro* (10). However, the antioxidant activity of *P. urinaria* has not been studied. Therefore, the goal of this proposal is to investigate the antioxidant effects *P. urinaria* against cardiotoxicity induced by doxorubicin. Our aim is to study the antioxidant effects of *P. urinaria* at several cellular oxidative pathways, including oxidative enzymes, oxidative pathways in cell death, alteration in endogenous antioxidant defenses, and alteration in lipid and protein metabolism/function. The insight in the balance of oxidant production, cellular defense mechanism, and antioxidant effects of exogenous substances may allow for new drug development from medicinal plants.

Hypothesis

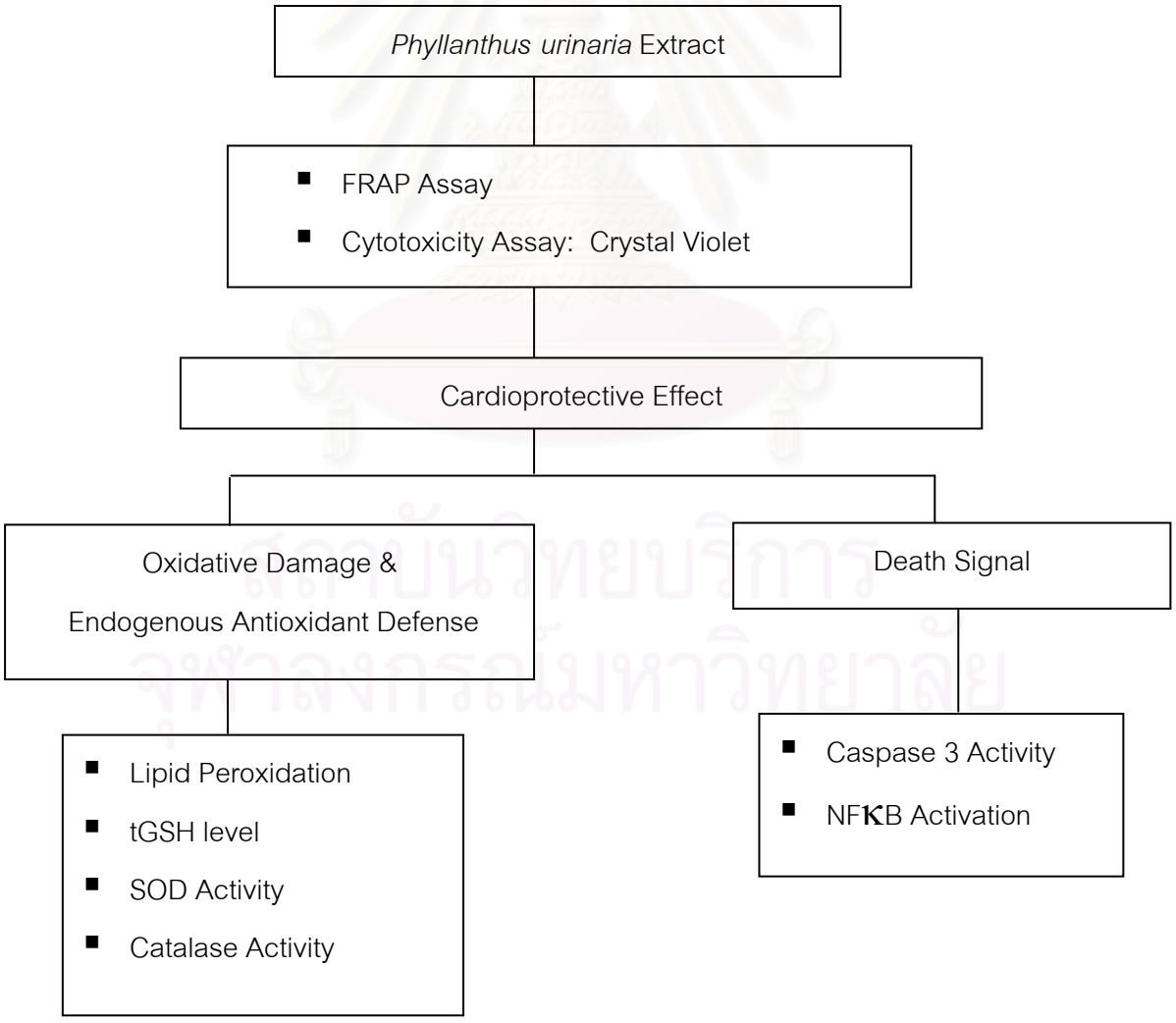
P. urinaria protects against DOX-induced cardiotoxicity by their antioxidant effect on different molecular levels, including decreases in oxidative damage and cell death signals

Objectives

The aim of the study is to identify the mechanisms of cytoprotective effect of *P. urinaria* in the model of ROS-induced cardiotoxicity in H9c2 cardiacmyoblast cell culture. Three aspects of antioxidant effect of *P. urinaria* will be studied as follows;

1. The total antioxidant capacity of *P. urinaria* evaluated by FRAP assay
2. The cytoprotective effect of *P. urinaria* against DOX-induced cardiac cell death (DOX IC50 rightward shift), lipid peroxidation, caspase-3, and NFκB activation.
3. The effect of *P. urinaria* on cellular antioxidant defense mechanism such as total glutathione levels, SOD and catalase activities

Scope



Limitations

This study used cardiac myoblast H9c2 and doxorubicin as a model of oxidative stress in cardiac cells. Therefore, suggestions or conclusions drawn from the experiments are limited to *in vitro* study.

Expected Outcomes

1. Identify the molecular mechanisms and oxidative pathways of cytoprotective effect of *Phyllanthus urinaria* for its therapeutic potential for the treatment of oxidative stress-induced cardiotoxicity
2. Provide scientific evidence of *Phyllanthus urinaria* for further studies in drug development as an alternative antioxidant therapy in a wide array of oxidative related chronic disease such as Alzheimer's disease, diabetes, AIDS, etc.

CHAPTER II

LITERATURE REVIEW

Phyllanthus urinaria

Phyllanthus urinaria L. (Euphorbiacae) [Synonyms: *P. lepidocarpus* Siebold & Zucc. (1843), *P. leprocarpus* Wight (1852), *P. verrucosus* Elmer (1915)] is usually an erect herb up to 80 cm tall with phyllanthoid branching, cataphylls scarious, their stipules ovate-lanceolate and conspicuously auriculate; decidous branchlets 3 to 13 cm long with 10 to 42 leaves; leaves oblong or ellipitical-oblong or elliptical-oblong to elliptical-obvate, 4 to 25 mm x 1 to 9 mm, obtuse to rounded and sometimes slightly unequal at base, apex rounded to obtuse and often apiculate, subsessile, stipules unequal, triangular-lanceolate; proximal 5 to 20 nodes of deciduous branchlets with solitary female flowers, succeeding nodes bearing monochasis of 5 to 7 male flowers; male flowers with 6 calyx lobes, disk segments 6, stamens 3, filaments connate, anthers free, dehiscing vertically; female flowers subsessile, calyx lobes 6, disk cup-shaped, margin sometimes crenulate, styles fused at base into a triangular plate; fruit a globular capsule, about 2 mm in diameter, usually rugose; seeds with sharp transverse ridges on the back and sides. *P. urinaria* is highly variable and two subspecies have been recognized. The rare subspecies *nudicarpus* Rossignol & Haricour. has creeping branches that root on the nodes and smooth-skinned capsules. *P. urinaria* is a common weed of waste places, clearing, gardens, along paths, but is also found in evergreen forest and bamboo forest. It grows on well drained, fertile, sandy soils, sometimes on limestone, often in humid places or even in marshy ground, up to 1,500 m altitude (11) (Figure 1).

Figure 1. *Phyllanthus urinaria*. (Photos are courtesy of Prof. Nantavan Bunyapraphatsara, Medicinal Plant Information Center, Faculty of Pharmacy, Mahidol University)

P. urinaria is widely distributed in Southern America and in many Asian countries, such as Thailand, India, China, Indonesia, Philippines and Japan. All parts of the plant are used medicinally. *Phyllanthus* has been used in Aryuvedic medicine for over 2,000 years and has a wide number of Thai traditional uses including diarrhea, tonic, common cold, swelling, diuresis, cough, diabetes, abdominal pain, kidney disease, hemorrhoids, and gonorrhea (12) *P. urinaria* is very similar to *P. amarus*. Its young leaves are for the treatment of cough in children and substitute for *P. amarus* in traditional Thai recipes.

The active constituents of *P. urinaria* contains lignans, alkaloids, tannin, flavonoids, triterpines (glochidonol and glochidone), and steroids (beta-sitosterol), methyl ester dehydrochebulic acid and methyl brevifolin carboxylate (12-14). Putative antioxidants from *P. urinaria* include compounds in the groups of lignans, tannins, flavonoids, other unknown structures. Recent studies have demonstrated that lignans from many natural products are very effective antioxidants (15, 16). They played a protective role against hepatotoxicity (17-19). Furthermore, several potential antioxidants from natural products protect cardiac cell damage induced by doxorubicin. For instance, arabic gum protected against doxorubicin cardiotoxicity in mice by significant reductions in serum CK and cardiac lipid peroxides. This study also indicated that arabic gum is a potent superoxide scavenger (20). Breitbart and coworkers have shown that water-soluble antioxidant from spinach protect doxorubicin-induced heart injury in mice. The spinach water extract protected doxorubicin-induced elevation in lipid peroxidation product, malondialdehyde, and hydroperoxides. Pretreatment with spinach water extract before DOX administration decreased catalase and increased superoxide dismutase activities compared to the DOX group (21). In addition, an organosulfur compound purified from garlic, S-allylcysteine, has shown to ameliorates toxicity in the heart and liver of mice injected with single dose of doxorubicin (15 mg/kg body weight) (22).

Recent research focus has been placed on antiviral, antiinflammatory, nociceptive, muscle relaxation effect, and anticancer activities of *Phyllanthus spp*. For *P. urinaria*, it has been shown *in vitro* that the aqueous extract inhibited viral DNA polymerase (DNAp) of hepadenoviruses (human hepatitis B virus and several animal

hepatitis viruses) (23). In chronic hepatitis patients, *P. urinaria* induced seroconversion of HBeAg from positive to negative as well as the seroconversion of HBeAb from negative to positive (24). The methanolic extracts obtained from callus cultures of *P. urinaria* significantly inhibited both neurogenic and inflammatory phases of the formalin test (13). The effect of the alcoholic extracts of *P. urinaria* induced guinea pig urinary bladder contraction involves direct action on smooth muscle and relies on the mobilization of extracellular calcium influx unrelated to activation of L- and N-type calcium channels or activation of protein kinase C mechanisms (25). Furthermore, the water extract of *P. urinaria* was shown to induce apoptosis and down regulate Bcl-2 in the Lewis lung carcinoma cells (26) and induced Fas receptor/ligand expression through ceramide-mediated apoptosis in HL-60 cells (27). Recent chemical investigation has isolated and identified several lignan from the root of *P. urinaria* (28) (Figure 2). Study by Giridharan et. al. demonstrated that methyllenedioxy substitution of a lignan from *P. urinaria*, 7'-hydroxy-3',4',5,9,9'-pentamethoxy-3,4-methylene dioxy lignan (Figure 3) suppressed cancer cells proliferation through telomerase and Bcl-2 inhibition, and activation of c-myc and caspases leading to apoptosis (29). Despite several pharmacological activities of *P. urinaria* have been tested, there is no report on the antioxidant effect of *P. urinaria* thus far.

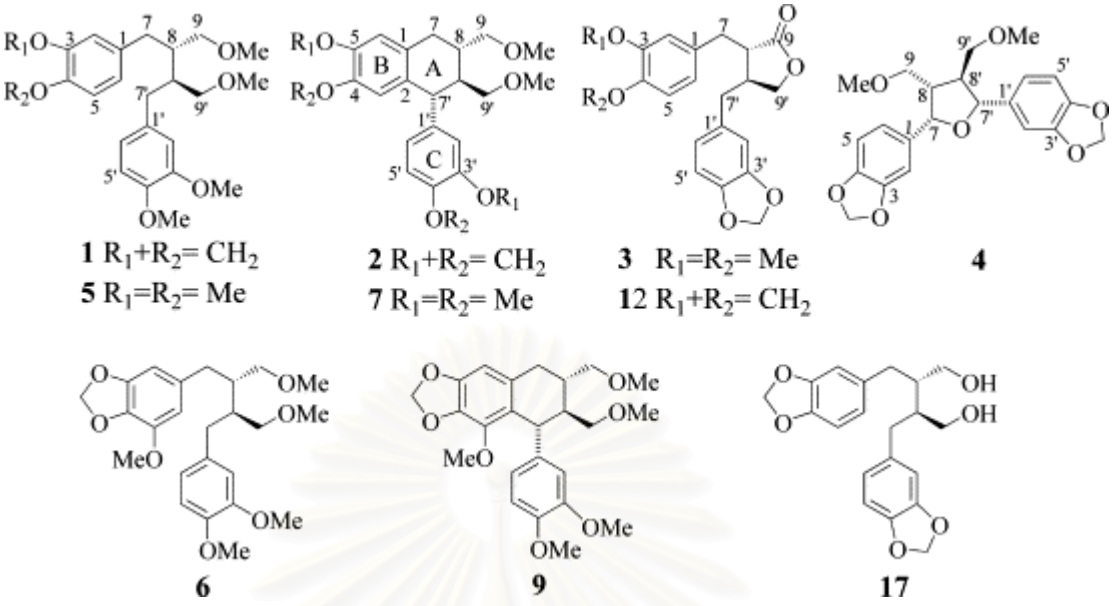


Figure 2. Lignans isolated from the root of *P. urinaria* (28)

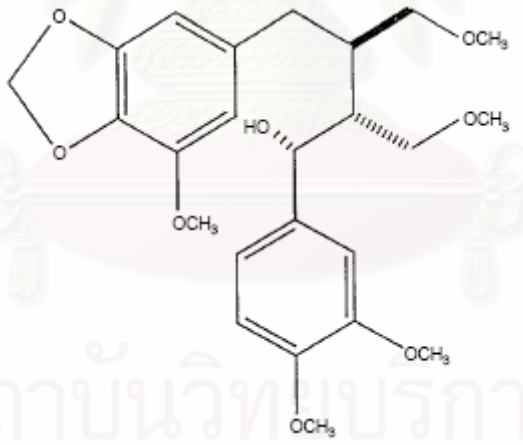


Figure 3. Structure of 7'-hydroxy-3',4',5,9,9'-pentamethoxy-3,4-methylene dioxy lignan (29)

1. ROS/RNS in health and disease

1.1 What is reactive oxygen species (ROS) and Reactive nitrogen species (RNS)?

Under normal physiological condition, biological systems are continuously exposed to oxidants, either generated endogenously by metabolic reactions (e.g. from mitochondrial electron transport during respiration or during activation of phagocytes) or exogenously, such as air pollutants, cigarette smoke, ultraviolet irradiation. Reactive oxygen species (ROS) is a collective term often refers to not only oxygen radicals – oxygen species capable of independent existence that contains one or more unpaired electrons, such as $O_2^{\bullet -}$ and $OH^{\bullet}$ but also some non-radical derivatives of O_2 such as H_2O_2, HOCl and O_3. The known biologically important radicals and non-radicals are described in (Table 1) (30). Similarly, the term "reactive nitrogen species" (RNS) has been introduced to indicate the reactive oxides of nitrogen (Table 2). Peroxynitrite ($ONOO^-$) is often included as both RNS and ROS. The word "reactive" is a relative term that describes the reactivities between intermediates.

Table 1 Reactive oxygen species [modified from (30)]

Radicals	Non-radicals
Superoxide, $O_2^{\bullet -}$	Hydrogen peroxide, H_2O_2
Hydroxly, $OH^{\bullet}$	Hypochlorous acid, HOCl[a]
Peroxyl, $RO_2^{\bullet}$	Ozone, O_3
Alkoxyl, $RO^{\bullet}$	Singlet oxygen, $^1\Delta g$
Hydroperoxyl, $HO_2^{\bullet}$	Peroxynitrite, $ONOO^-$[b]
Sulpher, $RS^{\bullet}$	
Transition metal, e.g., Fe, Cu, Zn, etc.	

[a] Could be equally called "reactive chlorinating species"
[b] Could be equally called "reactive nitrogen species"

Table 2 Reactive nitrogen species (30)

Radicals	Non-radicals
Nitric oxide, $NO^{\bullet}$	Nitrous acid, HNO_2
Nitrogen dioxide, $NO_2^{\bullet}$	Dinitrogen trioxide, N_2O_3
	Dinitrogen tetroxide, N_2O_4
	Nitronium (nitryl) ion, NO_2^+
	Peroxynitrite, $ONOO^-$
	Peroxynitrous acid, ONOOH
	Alkyl peroxynitrites, ROONO
	Nitroxyl anion, NO^-
	Nitroxyl cation, NO^+
	Nitryl chloride, NO_2Cl

Another term that is frequently used in the area of free radicals research is "oxidative stress". It refers to the situation of serious imbalance between production of ROS/RNS and antioxidant defense, leading to potential damage (often called "oxidative damage). Theoretically, oxidative stress can result from first, diminished antioxidant and second, increased production of ROS/RNS.

1.2 Sources of ROS/RNS

The general phenomenon of increased ROS production is well documented, but the actual sources of these species and mechanisms involved may not be identical in all conditions. For example, hydroxyl radical peak generation in myocardial ischemia-reperfusion did not coincide with cellular damage while doxorubicin-induced oxidative stress in the heart muscle was generated through reductive activation to its semiquinone form, and by the production of hydroxyl radicals mediated by Fenton reaction (31). There are several potential contributors to cellular ROS/RNS described as follows (2) (Figure 4 and 5).

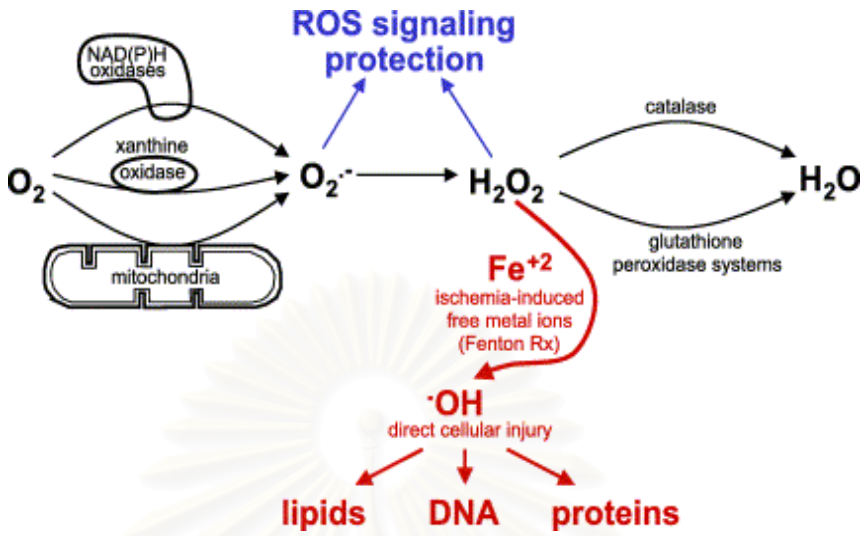


Figure 4. Reactive oxygen metabolism. Under normal condition, approximately 5% of respired oxygen is metabolized to water. Superoxide and hydrogen peroxide are produced normally and may have protective actions via signaling for preconditioning or oxidant stress induced gene products that activate multiple groups of protective proteins. However, with ischemic and reperfusion the normal balance is lost and hydroxyl radical can be produced via the Fenton reaction (31).

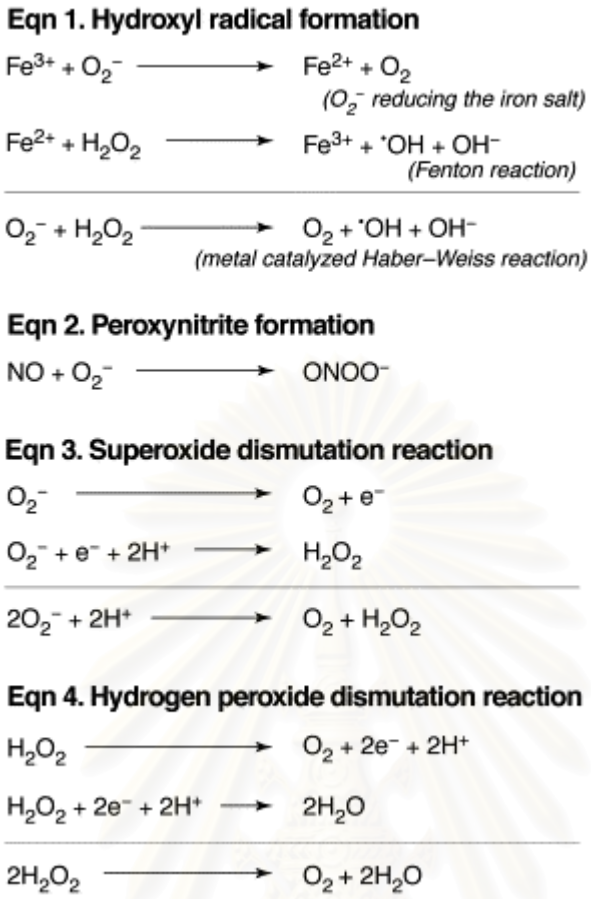


Figure 5. Chemical reactions by which some reactive oxygen species are formed and scavenged (32).

1.2.1 Uncoupling of mitochondrial electron transport:

The mitochondrial respiratory chain is a powerful source of ROS, primarily the superoxide radical and consequently hydrogen peroxide, either as a product of mitochondrial superoxide dismutase or by spontaneous disproportionation. It was calculated that 1–4% of oxygen reacting with the respiratory chain is incompletely reduced to ROS. There are two major respiratory chain regions where ROS are recognized to be produced, one being Complex I (NADH Coenzyme Q reductase) and the other complex III (ubiquinol cytochrome *c* reductase). In Complex III, antimycin is known not to completely inhibit electron flow from ubiquinol to cytochrome *c*. The antimycin insensitive reduction of cytochrome *c* is mediated by superoxide radicals. The source of superoxide in the enzyme may be either cytochrome b_{566}, or ubisemiquinone,

or Rieske's iron-sulfur center. The role of ubiquinone within ROS production deserves some comments because it has been described both as a pro-oxidant and as a powerful antioxidant (33).

This is a classical mechanism of intracellular oxidant production. Under normal physiological conditions, oxygen is essential for mitochondrial oxidative phosphorylation reactions and for production of ATP. ROS are formed intracellularly during mitochondrial electron transport, and are controlled by intracellular antioxidant defense. The lack of oxygen supply by either hypoxia or ischemia disrupts mitochondrial electron transport chain, resulting in an accumulation of toxic metabolites, acidosis, ATP depletion, intracellular Ca^{2+} overload, mitochondrial membrane depolarization, matrix swelling, and cell death. Interestingly, it is now well established that although restoration of blood flow prevents progression of ischemic cell necrosis, it also causes ``reperfusion injury'' in surviving cells. This phenomenon is associated with a massive production of ROS due to the resumption of oxygen supply to mitochondrial respiration. Several experimental hypoxia or ischemia/reperfusion models, both in vitro and in vivo, have suggested that injury in the myocardium is caused by oxygen radicals from mitochondrial electron transport. The generation of large amounts of ROS can overwhelm the intracellular antioxidant defense network, causing activation of neutrophils, lipid peroxidation, protein modification, and DNA breaks (2).

1.2.2 Metal ions

Iron and copper are essential in the human body for the synthesis of a huge range of enzymes and other proteins involved in respiration, O_2 transport, $NO^{\bullet}$ formation and other redox reactions. These metals are potentially dangerous because of their ability to undergo one-electron transfer enables them to be powerful catalysts of autoxidation reactions by the conversion of H_2O_2 to $OH^{\bullet}$ and decomposition of lipid peroxides to reactive peroxyl and alkoxyl radicals. It is not only the free metal ions that are catalytic, heme and certain heme proteins can decompose lipid peroxides and interact with H_2O_2 to cause damage. (30).

$$Fe\ (III) + e^{-} \rightleftharpoons Fe^{2+}$$

$$Cu^{2+} + e^{-} \rightleftharpoons Cu^{+}$$

Under normal condition, body is able to manage and sequester metal ions from producing oxidative damage through various protein and enzymes. For example, most intracellular iron is stored in ferritin which is a hollow protein shell. Iron enters the circulation in a bound form with transferrin. Transferrin also accepts iron released by the destruction of aged red blood cells, e.g. in the spleen. The cellular iron balance is regulated by cytoplasmic iron-regulatory proteins (IRPs) which bind to iron-responsive elements (IREs), special base sequences in the mRNAs of ferritin and transferin receptor proteins. Similarly, regulation of cupper metabolism is observed through many proteins. The copper absorbed from diet enters the blood by binding to albumin. Copper-albumin is taken up by liver, which incorporates copper into the protein caeruloplasmin. Caeruloplasmin is secreted into plasma and unwanted copper is excreted into bile.

In pathological conditions, iron overload (idiopathic hemochromatosis disease) or copper overload (Wilson's disease) occurs through the metabolic abnormality. It is likely (but not rigorously proven) that free radicals generated from these excess ions are involved in the pathology of the diseases.

1.2.3 Immune cell infiltration and cytokines:

It is now well established that many immune cells produce free radicals as a means of host defense and eradication of pathogens. As such, infiltration of activated immune cells into the area of cell injury is another potential mechanism of ROS/RNS production. The release of cellular content following injury triggers the expression of chemoattractant factors and cell adhesion molecules leads to the infiltration of the tissue by immune cells particularly neutrophils and monocytes. These activated neutrophils may cause more damage to the tissue by secretion of several mediators, including ROS/RNS, proteolytic enzymes, and pro-inflammatory cytokines.

1.2.4 Induction of oxidative enzymes:

Recent studies suggest that non-mitochondrial sources may be equally or perhaps more important in some oxidative conditions. Activation and/or induction of cytosolic oxidases (such as NADH/NADPH oxidase, xanthine oxidase [XO], and nitric oxide synthase [NOS] isoforms) has been demonstrated during several physiological stress conditions.

1.2.4.1 Xanthine oxidase:

Under normal physiological conditions, XO is a key enzyme in the purine degradation pathway. The enzyme generates the final product, uric acid (excreted by urine), and the byproduct, superoxide anion. Under normal physiological conditions, this enzyme is localized almost exclusively in the liver and the mucosa of the small intestine.

However, excess ROS production, during chronic hypoxia or in the presence of increased inflammatory cytokines, can enhance XO activity and its release into the plasma.

1.2.4.2 NADH/NADPH oxidase:

The enzyme NADPH oxidase generally is found in phagocytic cells. It plays a significant role in nonspecific host defense during infection by generating a large quantity of superoxide (millimolar). Recently, it has been demonstrated that vascular NADH/NADPH oxidase significantly contributes to superoxide production in all components of the vasculature, i.e., the endothelium, the medial smooth muscle, and the adventitia. Participants in cardiac disease such as angiotensin II (AngII) may activate superoxide production in vascular tissue via this enzyme. This enhanced production of superoxide has been implicated in the pathogenesis of AngII-induced vascular hypertrophy and endothelial cell dysfunction. Structure of neutrophil NAD(P)H oxidase is demonstrated in (Figure 6) .

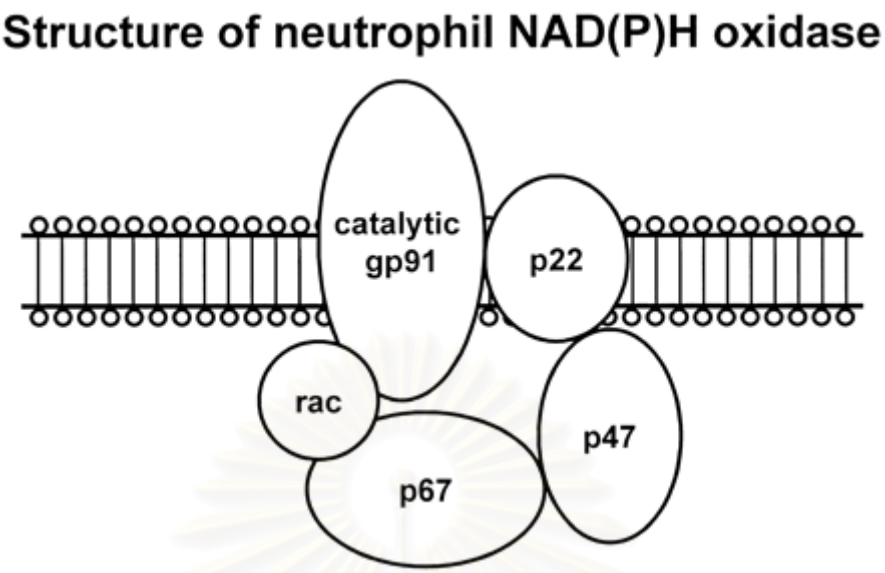


Figure 6. Structure of neutrophil NAD(P)H oxidase. The enzyme consists of the membrane-bound cytochrome b_{558} complex comprising $gp91^{phox}$ and $p22^{phox}$, the cytosolic proteins p47 and p67, and a low molecular-weight G protein of the rac family (9).

1.2.4.3 Nitric oxide synthases (NOS):

Increased nitric oxide ($NO^{\bullet}$) production via induction of NOS has been suggested and elevated levels are commonly found in the plasma of patients with heart disease, diabetes, AIDS, and Alzheimer's disease (34-37). In addition, other potent oxidants can be generated from reactions of $NO^{\bullet}$ with other ROS. For example, $NO^{\bullet}$ avidly reacts with superoxide anion at diffusion-limited rates to form the potent oxidant $ONOO^{-}$. The presence of $ONOO^{-}$ and its biological marker 3-nitrotyrosine has been associated with several oxidant related pathologic conditions, including diabetes, endothelial cell dysfunction, shock, inflammation, ischemia/reperfusion, and neurodegeneration (4, 34, 38). The actions of $NO^{\bullet}$ in vivo may be governed not only by production capabilities, but also by the setting and chemical environment in which it is formed (Figure 7).

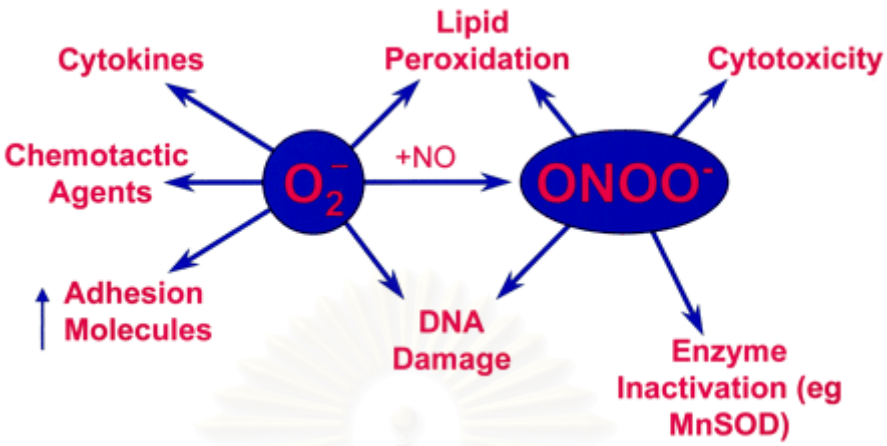


Figure 7. Biochemical impact of superoxide and peroxynitrite generation (4).

1.3 Consequences of oxidative stress

Oxidative stress can result in cellular adaptation and cell injury dependent on the concentrations, time, type of species exposed. Generally, cells are tolerable to mild oxidative stress, which often results in up-regulation of the synthesis of antioxidant defense systems in an attempt to restore the oxidant/antioxidant balance. A good example of adaptation is observed in the model of "ischemic preconditioning" in the myocardium where a brief period of occlusion (vary from 3 to 10 minutes) is introduced to render the myocardium resistance to the deleterious effects of ischemia or reperfusion (39). The mechanisms of adaptation frequently involve alterations in gene expression (increased or decreased in certain genes) that lead to elevated antioxidant defenses, heat shock proteins or changes in sensitivity of targets normally vulnerable to oxidative damage (40). In addition, oxidative stress can change cell behavior such as pro- or anti-proliferative effects, intercellular communication through gap junctions as well as signal transduction pathways. In any case, cell injury may occur in response to oxidative insult. The response to injury may be reversible and transient, or can be early events that eventually lead to irreversible injury leading to cell death.

The potential targets of oxidative damage include DNA, proteins and lipids which can cause direct cellular injury or induce a variety of cellular responses, through the generation of secondary metabolic reactive species. Accumulated evidence in the

literature has demonstrated consequences of oxidative stress in a wide array of experimental models. Potential targets of oxidative damage are summarized in (Table 3). Potential oxidative damage is discussed as follows:

Table 3 Target biomolecule damage induced by ROS/RNS

Target of damage	Event
DNA	- excessive DNA strand breakage (associated with depletion of cellular ATP and NAD^+ levels) - excessive activation of poly(ADP-ribose)polymerase (PARP) can deplete NAD^+ pool, leading to cell death.
Protein	- generation of carbonyls and other amino acid modifications - modification of protein function - protein fragmentation and cross-liked - increased susceptibility to proteolytic attack
Lipid and lipoprotein	- membrane lipid peroxidation - lipoprotein: oxidized LDL
Uric acid	- generation of allantoin, cyanuric acid, parabanic acid, oxonic acid and other products (levels are increased in chronic inflammatory/metal overload disease.

1.3.1 Cellular/organ dysfunction:

It is evident in many experimental models as well as in patients that oxidative damage may lead to cellular/organ dysfunction, and/or developmental abnormalities. For example, oxidative stress may play a role in the development of neuritic abnormalities since paired helical filaments are more often found in neurites with membrane abnormalities indicative of extensive lipid peroxidation (35). Impaired vascular function and decreased cardiac performance are mediated by $ONOO^-$ and other ROS via both direct impact on myocardial function through inhibition of the sarcoplasmic reticulum (SR) Ca^{2+} pump in the cardiac contraction and relaxation cycle. Cardiac depression (cardiac stunning) during ischemia/reperfusion and tissue damage

in acute MI was also observed (2). Endothelium dysfunction has been a consistent finding in animal models of diabetes and studies in humans with type 1 or type 2 diabetes. The role of free radicals generation the development of diabetic complications is well established (34).

1.3.2 DNA damage:

Free radical-mediated reactions can cause structural alterations in DNA (e.g., nicking, base-pair mutations, rearrangements, deletions, insertions, and sequence amplification). The endogenous reactions that are likely to contribute to ongoing DNA damage are oxidation, methylation, depurination, and deamination. Nitric oxide and its reactive products, such as NO_2, $ONOO_2$, N_2O_3 and HNO_2, are mutagenic agents, with the potential to produce nitration, nitrosation, and deamination reactions on DNA bases (4). The result of ROS attack on DNA--conversion of guanine to 8-hydroxyguanine-- has been found to alter the enzyme-catalyzed methylation of adjacent cytosines leading to altered methylation patterns. Different ROS affect DNA in different ways, e.g., H_2O_2 does not react with DNA bases at all, whereas $HO^{\bullet}$ generates various products from all four DNA bases, and this pattern appears to be a diagnostic "fingerprint" of $HO^{\bullet}$ attack. On the other hand, $O_2^{\bullet-}$ selectively attacks guanine. The most frequent found DNA base lesion and the one most often measured as an index of oxidative DNA damage is 8-hydroxyguanine.

Damage to DNA by ROS/RNS seems to occur naturally, in that low steady-state levels of base damage products have been detected in nuclear DNA from human cells and tissues. ROS/RNS can also damage mitochondrial DNA, and such damage has been suggested to be important in several human diseases and in the aging process (41). DNA damage by ROS/RNS can cause multiple lesions, including single and double strand breaks, apurinic/apyrimidinic sites and modified pyrimidines and purines. Repair of these lesions occurs primarily by base excision repair. A repair system for the abasic apurinic/apyrimidinic sites produced by spontaneous depurination also exists. Areas of current interest include the role of poly(ADP-ribose) polymerase (PARP) in the rejoining of DNA strand breaks, including those induced by ROS. Recent research has

focused on PARP as a mediator or marker of apoptosis (discussed in the following section).

1.3.3 Protein modifications:

In addition to their direct impact on cellular function, post-translational protein modification is another ROS-mediated protein regulation. ROS can oxidize amino acid side chains and the protein backbone, leading to protein-protein cross-linking and protein fragmentation. As a consequence of these modifications, signaling proteins may not function properly, which may lead to organ malfunction and even cell death. Two major protein derivatives have been used as markers of ROS-mediated protein modifications: protein carbonyl derivatives and protein nitrotyrosine derivatives. The presence of carbonyl groups in proteins has been referred to as ROS-mediated protein oxidation, while nitrated tyrosine residues have been identified as a product of peroxynitrite-dependent nitration ($ONOO^-$, mainly derived from the interaction of $O_2^{\bullet -}$ and $NO^{\bullet}$). Elevated levels of nitrated proteins are observed in cardiac tissue from animals and patients under various conditions of excess oxidant production, including ischemia/reperfusion, CHF, and myocardial sepsis. However, it is not clear whether protein nitration/oxidation causes cardiovascular dysfunction or if it simply represents excess oxidant production.

1.3.4 Lipid peroxidation:

The '*initiation'* of lipid peroxidation is induced by ROS/RNS attack upon a hydrogen atom from a methylene ($-CH_2-$) group. Since hydrogen atom has only one electron, abstraction of $H^{\bullet}$ from a ($-CH_2-$) group leaves behind an unpaired electron on the carbon. The carbon radical is usually stablilized by a molecular rearrangement to form a *conjugated diene*. The most likely fate of carbon radicals under aerobic conditions is to combine with O_2 leading to form peroxyl radical ($ROO^{\bullet}$ or $RO_2^{\bullet}$). Peroxyl radicals are capable of abstracting H from another lipid molecule, i.e. an adjacent fatty acid side chain. This step is called *'propagation stage'* of lipid peroxidation. The carbon radicals formed can react with O_2 to generate another peroxyl radical and then the *'chain reaction'* of lipid peroxidation can continue. The peroxyl

radical combines with the hydrogen atom to form *lipid hydroperoxide* or *lipid peroxide* (LOOH) (Figure 8) (30).

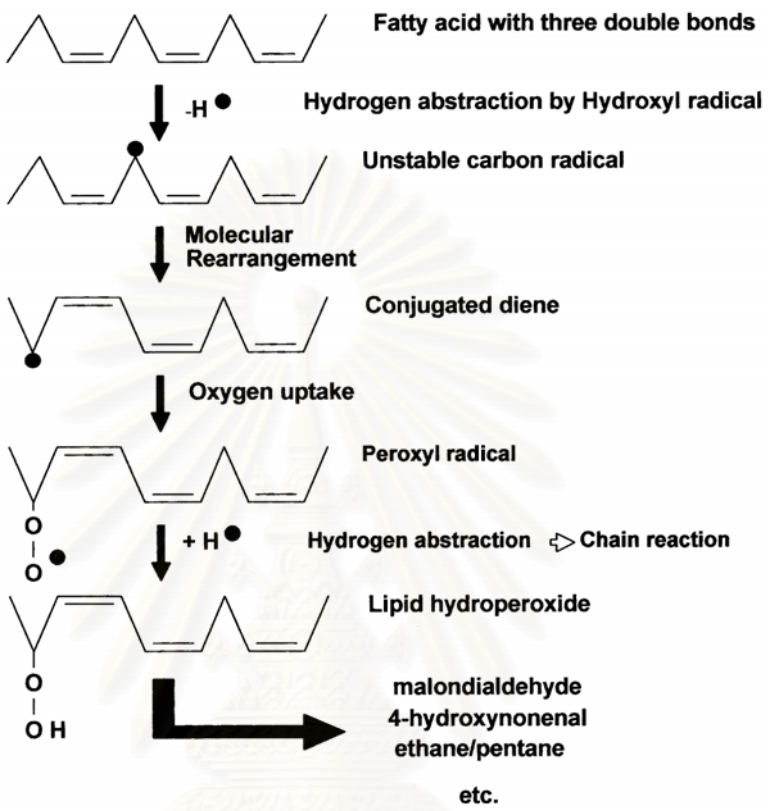


Figure 8. Basic reaction sequence of lipid peroxidation (42).

Similar events are observed in LDL oxidation (42). Once initiated, oxidation of LDL is a free-radical-driven lipid peroxidation chain reaction. Lipid peroxidation is initiated by free radical attack on a double bond associated with a polyunsaturated fatty acid (PUFA). This results in the removal of a hydrogen atom from a methylene (CH_2) group, the rate of which determines the rate of initiation, is a key step. Molecular rearrangement of the resulting unstable carbon radical results in a more stable configuration, a conjugated diene. The conjugated diene reacts very quickly with molecular oxygen, and the peroxyl radical thus formed is a crucial intermediate. A PUFA peroxyl radical in LDL may abstract a hydrogen atom from an adjacent PUFA to

form a hydroperoxide and another lipid radical, a reaction which results in chain propagation. Removal of hydrogen atoms by the peroxyl radical from other lipids, including cholesterol, eventually yields oxysterols. Lipid hydroperoxides fragment to shorter-chain aldehydes, including malondialdehyde and 4-hydroxynonenal. These reactive aldehydes in turn may bind to ε-amino groups of apo B-100, giving the protein an increased net negative charge. The classical LDL receptor recognizes a specific domain of positive charges from lysine, arginine and histidine residues on apo B. Alteration of this domain results in failure of binding by the apo B/E receptor, and an increase in negative surface charge on apo B-100 results in increased recognition by the scavenger receptor.

ROS are highly reactive and when generated close to cell membranes, oxidize membrane phospholipids (lipid peroxidation) which can lead to the generation and accumulation of lipid peroxidation products, such as malondialdehyde, 4-hydroxy-2-nonenal (4-HNE), acrolein and F2-isoprostanes (Figure 9). The peroxidative breakdown of polyunsaturated fatty acids impairs membrane function, inactivates membrane-bound receptors and enzymes and increases tissue permeability which has been implicated in the pathogenesis of many forms of cell injury. There is increasing evidence that aldehydes, generated endogenously during the process of lipid peroxidation, are involved in many of the pathophysiological events associated with oxidative stress in cells and tissues. In addition to their cytotoxic properties, lipid peroxides are increasingly recognized as being important in signal transduction for a number of important events in the inflammatory response (43).

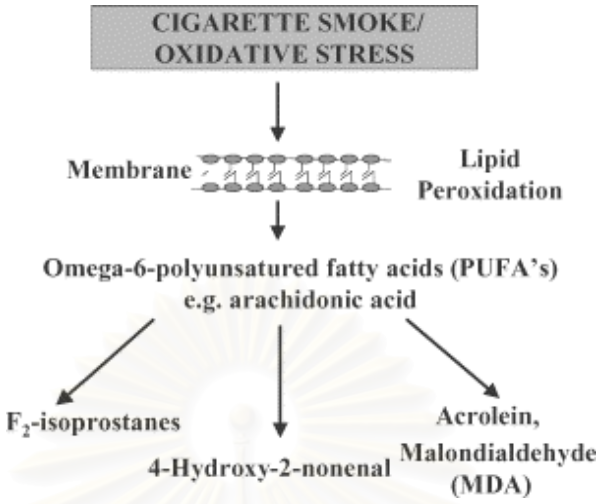


Figure 9. Membrane lipid peroxidation of polyunsaturated fatty acids leading to generation of various aldehydes (43)

1.3.5 Alteration of signal transduction and gene expression:

Recently, the area of "redox regulation" has gained increased interest. The term *redox signaling* is widely used to describe a regulatory process in which the signal is delivered through redox chemistry. Redox signaling is used by a wide range of organisms, including bacteria, to induce protective responses against oxidative damage and to reset the original state of "redox homeostasis" after temporary exposure to ROS (9). The oxidant-antioxidant balance is a key regulation of redox homeostasis that lead to changes in signal cascade and gene expression (Figure 10). The topic of antioxidants are discussed in the next section.

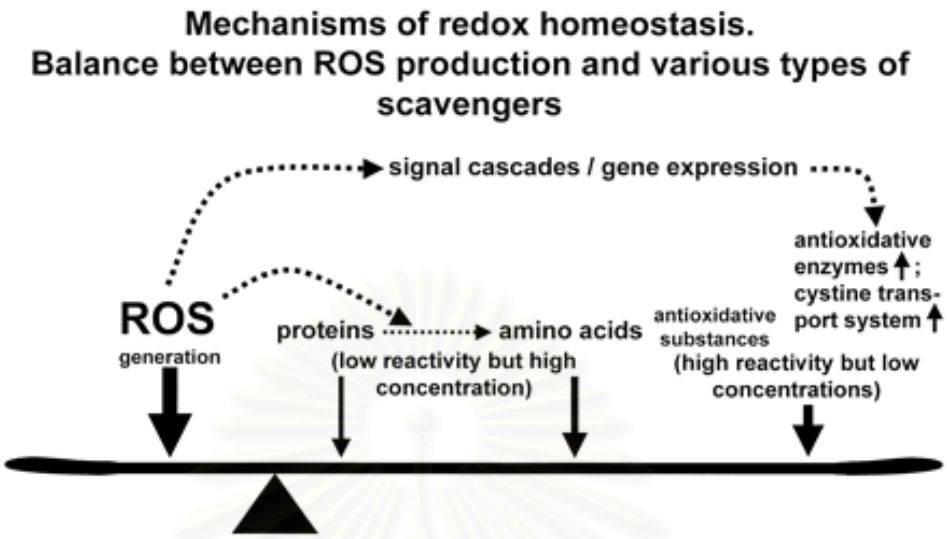


Figure 10. Mechanisms of redox homeostasis. Balance between ROS production and various types of scavengers. The steady-state levels of ROS are determined by the rate of ROS production and their clearance by scavenging mechanisms. Certain antioxidative enzymes including superoxide dismutase (SOD), glutathione peroxidase, catalase, and thioredoxin are potent ROS scavengers but occur in cells only at relatively low concentrations. The same is true for nonenzymic antioxidants. Amino acids and proteins are also ROS scavengers. Amino acids are less effective than the classical antioxidants on a molar basis, but their cumulative intracellular concentration is >0.1 M (9).

1.3.5.1 Signal Transduction:

Recent studies have demonstrated that ROS may indeed act as signal transduction molecules. For example, cellular H_2O_2 was transiently increased upon activation of platelet-derived growth factor (44). Several signaling pathways are affected by the platelet-derived growth factor-induced increase in H_2O_2 concentration, because of the effect of H_2O_2 on tyrosine phosphorylation, mitogen-activated protein kinase (MAPK) activation, DNA synthesis, and chemotaxis. Additionally, H_2O_2 activates hypertrophic and apoptotic signaling pathways in cardiac myocytes (45). ROS activate

a wide variety of cellular signaling molecules and pathways, including Ca^{2+} , protein tyrosine kinases, serine/threonine kinases, and phospholipases (46-48).

Protein phosphorylation regulates a wide array of cellular signaling pathways that carry signals through different functional proteins, including enzymes, receptors, transcription factors, and contractile elements. Generally, oxidant-related stimulation of protein phosphorylation acts through the enzymes that regulate the balance of phosphorylation (kinases) and dephosphorylation (phosphatases) of specific amino acid residues of signaling proteins. The overall increases in protein phosphorylation generally reflect stimulation of the signaling pathway. Two well defined subtypes of protein phosphorylation are tyrosine phosphorylation and serine/threonine phosphorylation. Most growth factor receptors are transmembrane tyrosine kinases, with the exception of transforming growth factor-β and insulin-like growth factor-II. These receptor kinases are sensitive to ROS stimulation, leading to activation of downstream signal transducers, such as the protein kinases of the MAPK cascade, phospholipase C, protein kinase C, and phosphatidylinositol-3-kinase. The MAPK superfamily of protein serine/threonine kinases is a group of enzymes that are involved in the regulation of cell growth, proliferation, and differentiation, as well as oxidative responses. There are three major subfamilies of MAPKs, including the extracellular responsive kinases (ERKs), the c-Jun N-terminal kinases (JNKs, also known as SAPKs), and the p38-MAPKs. The ERKs appear to be associated with cell growth and differentiation, while the JNKs and the p38-MAPKs are involved in responses to cytotoxic insults. In the heart, the ERKs are primarily activated by G-protein-coupling receptor agonists, such as (β-adrenergic receptor agonists, AngII, and endothelin-1, leading to activation of the phospholipase C cascade and ultimately activation of protein kinase C. The release of ROS, proinflammation cytokines, and humoral factors during ischemia/reperfusion activates ERKs, which are known to be responsible for cardiac hypertrophy in the non-ischemic zone of the heart to compensate for myocyte loss (ventricular remodeling). Additionally, JNKs and p38-MAPKs are activated during global cardiac ischemia and after incubation of H_2O_2, TNF-α, or IL-1β with cultured myocytes. Inhibition of p38-MAPK attenuated ischemia-induced cardiac apoptosis and decreased TNF-α production in cardiac tissue (49-51).

1.3.5.2 Gene Expression:

It has become increasingly evident that ROS are more than simply cellular toxicants and that they may be important modulators of cellular gene expression patterns. For example, redox cycling of cysteinyl residues is one of the important mechanisms of ROS-regulated activity of transcription factors and signaling molecules. Disruption (reduction) or formation (oxidation) of disulfide bonds plays a central role in determining a protein conformation. Conformation is critical for proper protein-protein and protein-DNA interactions, which, in turn, drive specific gene transcription.

1.3.5.2.1 NFκB and AP-1.

Perhaps, the finding of the production of ROS during the agonist-induced activation of NFκB provided the first concrete evidence for the role of ROS as second messenger. The NFκB/Rel family complex is a redox-sensitive transcription factor composed of several key regulatory molecules controlling expression of many inflammatory and protective/stress response genes. NFκB exists as a heterodimeric complex usually of p50 and p65/RelA subunits. In unstimulated cells NFκB is found in the cytoplasm as an inactive non-DNA-binding form, associated with an inhibitor protein called inhibitory κB (IκB) which masks the nuclear translocation signal and so prevents NFκB from entering the nucleus. Upon cell stimulation with various NFκB inducers, IκB-α is rapidly phosphorylated on two serine residues, which targets the inhibitor protein for ubiquination by the E3 ubiquitin-ligases (E3RSIkB) and subsequent degradation by the 26S proteasome. The released NFκB dimer can then be translocated into the nucleus and activate target genes by binding with high affinity to κB elements in their promoters. NFκB regulates the inducible expression of a number of genes involved in cell survival and execution. For example, NFκB has been found to control antiapoptotic gene, *Bcl-2*, and pro-apoptotic factors, *bax* and *p53*, in the ischemic/reperfused myocardium. Diverse extracellular signals from interleukin 1 (IL-1), tumor necrosis factor a (TNF-α), H_2O_2, etc. converge into development of oxidative stress, which leads to activation of NFκB. Such activation of NFκB can be blocked by

antioxidants such as vitamin E or α-lipoic acid. Antioxidants such as N-acetylcysteine can prevent NFκB activation by suppressing the generation of ROS (48).

In addition to NFκB, ROS-induced alterations in gene expression are mediated through activation protein-1 (AP-1). Changes in early response genes, such as egr-1, hsp70, c-fos, c-jun, and c-myc, are detected within 30 min after a hypertrophic stimulation. Furthermore, re-expression of fetal genes, such as β-major histocompatibility complex, α-skeletal actin, and atrial natriuretic peptide, was also observed within 6±12 hr. ROS-induced increases in intracellular Ca^{2+} homeostasis may be a significant initial step in the activation of NFκB (52). Lipid peroxidation products can activate NFκB. Thus, ROS-induced alterations in gene expression are a consequence of changes in cellular signaling pathways through modifications of enzyme activities and alterations of molecular structures of biomolecules (e.g., proteins, lipids, glycoproteins, nucleotides). At least two important transcription factors, NFκB and AP-1, are controlled by the intracellular redox state. These redox-regulated transcription factors bind to several promoter regions of genes that are directly responsible for the pathogenesis of atherosclerosis, complications of diabetes, cancer, and acquired immunodeficiency syndrome (53). Transcription activator NFκB is important in inflammatory responses since it regulates a number of cytokine genes and their receptors, including TNF-α, IL-1, IL-2, and major histocompatibility complex Class I. NFκB binding sites were also found in a variety of cell adhesion molecules, such as colony-stimulating factor-1, monocyte chemoattractant protein-1, and vascular cell adhesion molecule-1 (54). AP-1 proteins are a family of leucine zipper transcription factors that specifically regulate gene expression upon binding to cis-acting transcriptional control DNA element. AP-1 genes are inducible by a broad range of stimuli, including ROS. ROS act as mediators of transcriptional regulators in signal transduction processes, leading to cell proliferation and transformation (55). Examples of AP-1 transactivators are immediate-early response gene families, such as homodimers of c-jun proteins or heterodimers of c-fos and c-jun. The activity of AP-1 is regulated at transcriptional, post-transcriptional, and post-translational levels. Activation of c-fos/c-jun transcription is promoted by oxidants, cytokines, and growth factors. Redox regulation of AP-1 binding appears to occur at the post-

translational level. Oxidants can modify the redox state of cysteine residues located in the DNA-binding domain of each fos/jun protein that are crucial to their DNA binding activity and subject to redox regulation. Under oxidative stress, the loss of redox regulation by cysteine residues in AP-1 proteins inhibits their binding to DNA, whereas treatment with reducing agents, such as NADPH, GSH, or β-mercaptoethanol, increases AP-1 DNA binding (55). The field of oxidant-related stress genes and signaling is rapidly expanding, and a great deal of new insight into cellular responses to physiological stress has emerged. The regulation of transcriptional activators by oxidants and reductants is complex, and the ultimate biological effects are dependent upon the net results of interactions between these redox-mediated transcriptional activators.

1.3.5.2.2 ARE and Nrf.

The antioxidant response element (ARE) is a *cis*-acting enhancer sequence that mediates transcriptional activation of genes in cells exposed to oxidative stress and xenobiotics. Genes that are regulated by the ARE encode proteins that help control the cellular redox status and defend the cell against oxidative damage. Proteins that are encoded by the ARE gene battery include enzymes associated with glutathione biosynthesis, redox proteins with active sulfhydryl moieties, and drug-metabolizing enzymes (56).

In more recent studies, compelling evidence has been obtained from *in vitro* and in vivo studies suggesting that the bZIP transcription factor Nrf2 is intimately involved in mediating the ARE-driven response to oxidative stress and xenobiotics. The Nrf2 protein was first isolated by an expression cloning procedure using an oligonucleotide containing the NF-E2 DNA binding motif as probe to screen for closely related proteins. NF-E2 is a dimeric protein originally identified as involved in the regulation of globin gene expression in hematopoietic cell. The NF-E2 protein activates gene transcription following binding to its consensus DNA binding motif 50-TGCTGAGTCAC-30 as a heterodimer consisting of a 45-KDa and an 18-KDa subunit. The p45 subunit is a bZIP protein consisting of a transactivation domain within the N-terminal region and a basic DNA binding region/leucine zipper structure in the C-terminal region of the protein. The p18 subunit

was subsequently identified as a member of the small Maf proteins containing the basic region/leucine zipper but lacking any apparent transactivation (56-59) (Figure 11).

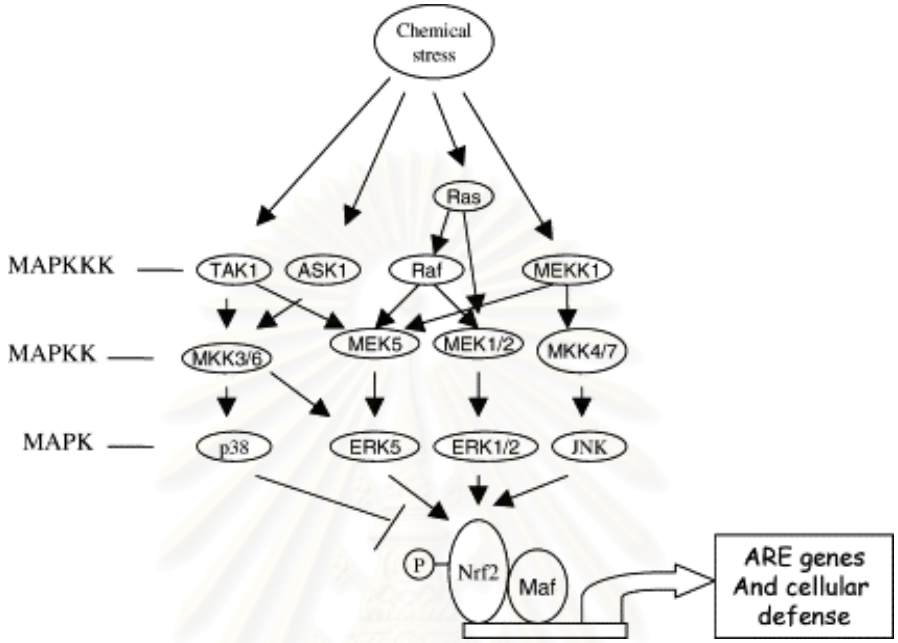


Figure 11. A model of drugs/chemicals/xenobiotics-induced chemical stress response leading to the activation of MAPK signaling pathway and subsequent activation of Nrf2/Maf-dependent anti-oxidant responsive genes including the phase II detoxifying enzymes as well as other stress enzymes, which result in the enhancement of detoxification of the xenobiotics and a potential homeostatic cell survival response. (60).

1.3.5.3 Redox modulation of chromatin remodeling.

ROS has also implicated in causing chromatin remodeling (histone acetylation/deacetylation) and gene expression in response to oxidants and pro-inflammatory mediators (43) (Figure 12). Many factors, including specific DNA sequences, histones, non-histone chromosomal proteins, transcriptional activators/repressors and the transcription machinery are all necessary for the establishment of an active transcription complex. Condensation of eukaryotic DNA in

chromatin suppresses gene activity through the coiling of DNA on the surface of the nucleosome core and the folding of nucleosome assemblies, thus decreasing the accessibility to the transcriptional apparatus. Tightly bound DNA around a nucleosome core (comprising the histone proteins H2A, H2B, H3 and H4), suppresses gene transcription by decreasing the accessibility of transcription factors, such as NFκB and AP-1 to the transcriptional complex. Acetylation of lysine residues on the N-terminal tails of the core histone proteins results in uncoiling of the DNA, allowing increased accessibility for transcription factor binding. Acetylation of lysine (K) residues on histone 4 (K5, K8, K12, K16) is thought to be directly related to the regulation of gene transcription. However, selective recognition of these acetylated lysines within the histone by other proteins containing bromodomains may add a further level of transcriptional regulation. Histone acetylation is reversible and is regulated by a group of acetyltransferases (HATs) which promote acetylation, and deacetylases (HDACs) which promote deacetylation. The nuclear receptor coactivators, steroid receptor coactivator 1 (SRC-1), CREB-binding protein (CBP)/adenoviral protein E1A (p300) protein, CBP/p300 associated factor (P/CAF), and ATF-2, all possess intrinsic HAT activity (Figure 13). Of these, CBP/p300 and ATF-2, which are regulated by the p38 MAP kinase pathway, are vital for the co-activation of several transcription factors, including NFκB and AP-1 in the transcription machinery. These activation complexes act with RNA polymerase II to initiate transcription. Thus, it is likely that histone acetylation of H4 via CBP/p300 and/or ATF-2 has a significant role in the activation of NFκB/AP-1-mediated gene expression for pro-inflammatory mediators, although the precise molecular mechanisms are still not fully understood (43).

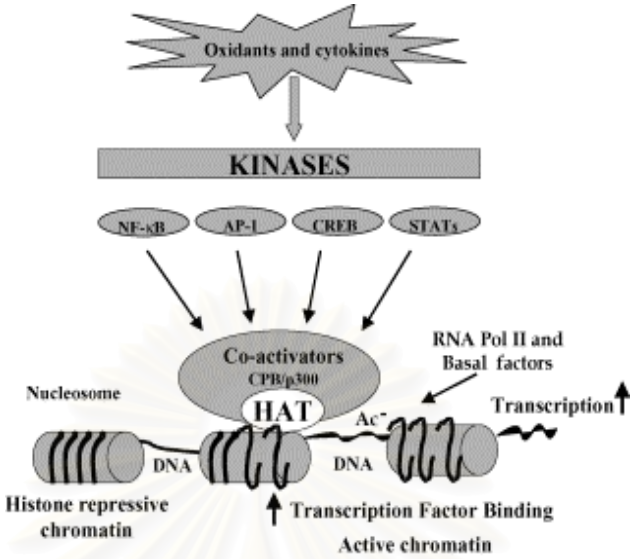


Figure 12. Histone acetylation and deacetylation: oxidative stress and other stimuli, such as cytokines, activate various signal transduction pathways (JAK, JNK and IKK) leading to activation of transcription factors, such as NF-κB, AP-1, CREB and STAT proteins. Binding of these transcription factors leads to recruitment of CBP and/or other co-activators to the transcriptional initiation complex on promoter region of various genes. Activation of CBP leads to acetylation (Ac) of specific core histone lysine residues by an intrinsic acetyltransferase activity (HAT). Histone acetylation (active chromatin) leads to loosening of the nucleosome which enables access to basal factors and RNA polymerase II for gene transcription to occur (43).

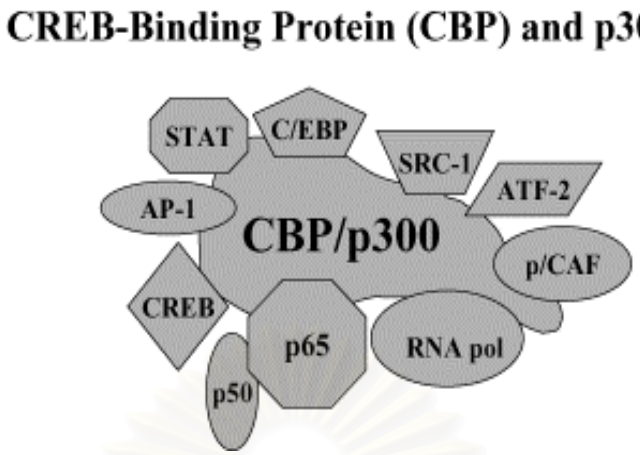


Figure 13. CBP/p300 co-activator transcription initiation complex. CBP recruits transcriptional co-activators and acts as an integrator of signaling pathways, links transcription factors and co-activators to the basal transcriptional machinery and stabilizes the transcriptional preinitiation complex (43).

1.3.6 Cell death:

Two types of cell death, necrosis and apoptosis, are both implicated in the pathology of oxidative-related cell death. Generally, necrotic cell death is associated with inflammatory cell infiltration and subsequent collagen deposition and scar formation, while apoptotic cell death is differentiated by ultrastructural and biochemical features, such as cytoplasmic and nuclear condensation, formation of membrane-bound apoptotic body, and DNA fragmentation (180-200 bp).

Apoptosis is an actively regulated process of cell death necessary for proper control of tissue growth. In humans, ~100,000 cells die every second by apoptosis and are replaced by new cells of the same number. Apoptotic death is a tightly controlled process, and the cellular pathways that regulate apoptosis have been well conserved in multicellular organisms from simple to complex animals. Two major pathways, extrinsic and intrinsic, for induction of apoptosis have been identified in recent years (61) (Figure 14).

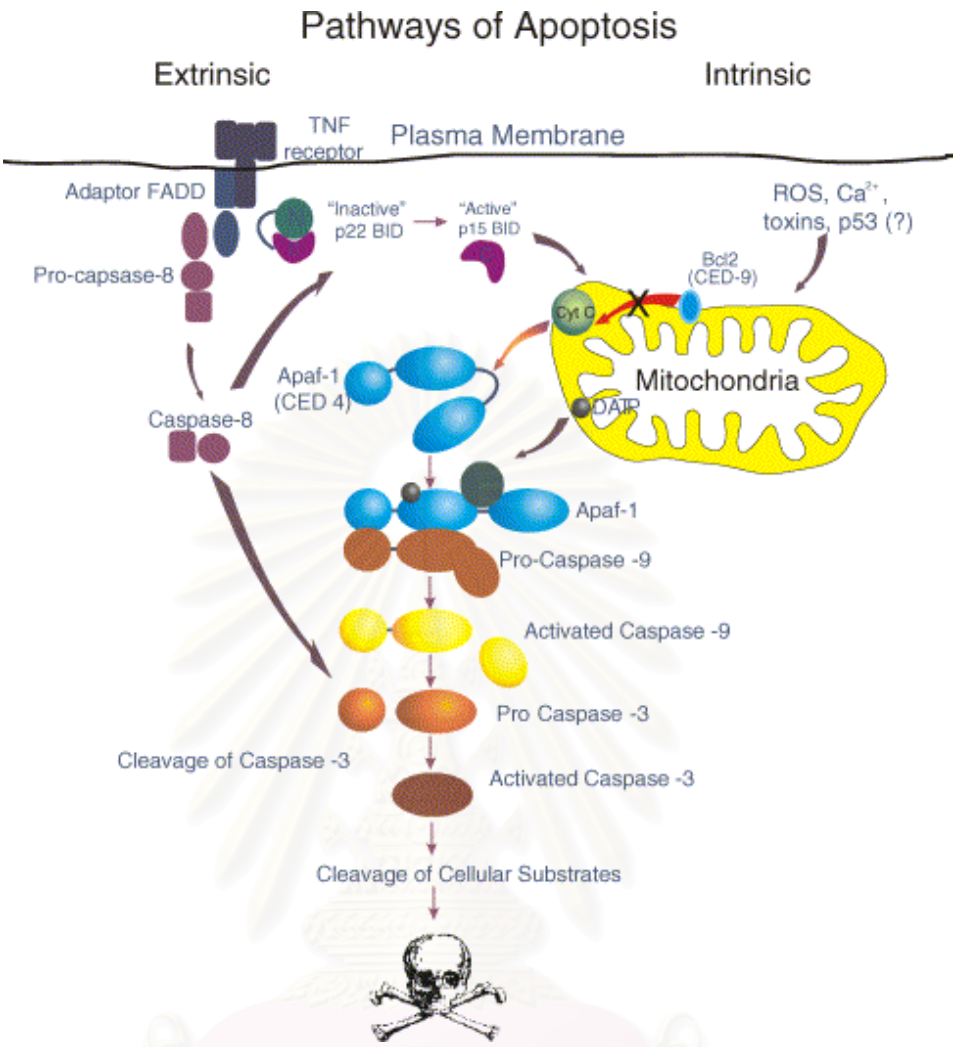


Figure 14. Pathways of apoptosis. Two general pathways have been described by which cells undergo apoptosis. The extrinsic pathway is triggered via cell surface receptors, leading to activation of initiator (i.e. caspase-8) and effector (i.e. caspase-3) proteolytic enzymes. The intrinsic pathway involves the mitochondria resulting in cytochrome *c* release and activation of the initiator caspase, caspase-9, with subsequent activation of effector caspases (i.e. caspase-3). The intrinsic pathway can be activated by agents that directly target the mitochondria, or indirectly via the extrinsic pathway through caspase-8-mediated cleavage of the inactive cytosolic protein BID. Once activated, BID translocates to the mitochondria where it stimulates cytochrome *c* release. Bcl-2, an anti-apoptotic protein that is localized to mitochondria, prevents cytochrome *c* release (61).

The extrinsic pathway is represented by tumor necrosis factor (TNF-α) family receptors that contain protein interaction modules known as death domains (DD) in their cytosolic regions. Following binding of ligand to these receptors, or when overexpressed in cells, DD-containing TNF receptor family members such as Fas (CD95) aggregate, resulting in recruitment of an adaptor protein Fadd, which contains both a DD and a similar protein interaction module known as the death effector domain (DED). The zymogen pro-forms of certain caspase family cell death proteases, namely procaspases-8 and -10, also contain DEDs in their N-terminal prodomains, allowing binding to Fas/Fadd complexes. Subsequent to Fadd recruitment, proteolytic processing and activation of the receptor associated proteases (i.e. caspase-8), initiate a cascade of additional processing and activation of downstream effector caspases (i.e. caspase-3). The intrinsic pathway of apoptosis involves mitochondria. A variety of key events in apoptosis focus on mitochondria including changes in electron transport, altered mitochondrial oxidation–reduction, loss of mitochondrial membrane potential ($\Delta\psi_m$), and release of cytochrome *c*. Numerous studies have demonstrated that induction of downstream caspase activity is dependent on dATP and cytochrome *c* release from mitochondria, and can be inhibited by anti-apoptotic Bcl-2 family members. Cytochrome *c* forms part of the "apoptosome" which also includes apoptosis protease activating factor-1 (Apaf-1) and procaspase-9. The extrinsic and intrinsic pathways can each stimulate apoptosis independent of one another. However, cross-talk between the two pathways can also occur. Recently, TNF-α treatment of cells has been found to induce caspase-mediated cleavage of cytosolic inactive BID to a smaller active BID protein that translocates to the mitochondria, integrates into the mitochondrial membrane causing cytochrome *c* release (41).

Generation of ROS causes cell death via apoptosis. Pro-oxidant agents, such as H_2O_2, diamide, etoposide and semiquinones, induce apoptosis. Other apoptotic stimuli, such as TNF-α and ceramide also elevate intracellular levels of ROS. Antioxidants, such as *N*-acetylcysteine, suppress apoptosis by acting as scavengers for ROS, and their actions provide evidence that ROS act as signaling molecules to initiate apoptosis (61, 62).

Poly(ADP) synthetase (PARS) [also known as PARP or poly(ADP-ribose) transferase] is a protein modifying and nucleotide-polymerizing enzyme that is present abundantly in the nucleus (4). The obligatory trigger of PARS activation is the nicks and breaks in the DNA strand, which can be induced by a variety of environmental stimuli and free radical (or oxidant) attacks; these include the oxidants $HO_2^{\bullet -}$, $HO^{\bullet}$, and $ONOO^-$, ionizing radiation, and genotoxic agents, such as *N*-methyl- *N*'-nitro-*N*-nitrosoguanidine. The physiological function of PARS and poly(ADP-ribosylation) is still under much debate. From studies using pharmacological inhibitors of PARS, poly(ADP-ribosylation) has been suggested to regulate gene expression and gene amplification, cellular differentiation and malignant transformation, cellular division, and DNA replication, as well as apoptotic cell death (63-65). However, recent studies using cells from PARS(–/–) mice have failed to demonstrate a role for PARS in the process of apoptosis induced by various apoptotic signals, such as the Fas ligand or dexamethasone (66, 67) . Advance in PARP research reveals that $NO^{\bullet}$ or peroxynitrite can trigger DNA single-strand breakage and PARS activation (65, 68). $NO^{\bullet}$ and peroxynitrite can also inhibit mitochondrial respiration and exert other cytotoxic effects on their own. Thus, it is likely that a synergistic relationship exists between the PARS-mediated pathways and PARS-independent pathways of cellular metabolic suppression. Furthermore, the observations that $NO^{\bullet}$ and peroxynitrite are important mediators of the cellular damage in various forms of inflammation and reperfusion injury suggest that the PARS-related suicide pathway might play a role in various pathophysiological conditions *in vivo*.

2. ROS/RNS and Doxorubicin Cardiotoxicity

Reactive oxygen species (ROS) are continuously produced in living cells naturally as a result of leakage of electrons on the electron transport chain in mitochondria. In addition, they can be produced in cells by various enzymatic mechanisms (xanthine oxidase, cytochrome P450, etc.), auto-oxidation of small molecules (catecholamines, etc), or in response to xenobiotics, exogenous environmental exposures, ischemia, inflammatory stimuli, or certain chemotherapeutic agents such as doxorubicin. Doxorubicin administration elicits both acute and chronic

cardiac dysfunction. Cardiotoxicity during chronic administration is dose-dependent, and the incidence increases at cumulative doses in excess of 550 mg/m^2 of body surface. The cardiotoxicity is mainly caused by enhanced generation of ROS and toxic lipid peroxides in cardiac myocytes that contain less enzymatic antioxidants, such as glutathione, catalase and superoxide dismutases than metabolic organs, such as liver and kidney. Furthermore, cancer patients have higher blood levels of lipid peroxidative degradation products and a higher lipid peroxide/(vitamin C + vitamin E) ratio than healthy controls, suggesting enhanced oxidative stress in cancer patients (69). Several antioxidants vitamins have shown protective role against doxorubicin cardiotoxicity. Vitamin E decreased cardiac lipid peroxidation and delayed the lethality of a single dose of doxorubicin at 24 hr (70). Daily vitamin E administration 4 days prior to a single i.v. dose of doxorubicin injection prevented cardiac damage and improved survival of rabbits, which was related to cardiac glutathione oxidation (71). However, other studies suggest that co-administration of vitamin E during chronic doxorubicin treatment improved survival of animals, but failed to protect animals from cardiotoxicity (72-74). Although, vitamin C also prevented cardiac lipid peroxidation and structural damage in mice and guinea pigs treated with one or multiple doses of doxorubicin, its effect on the prolongation of survival time of the animals is controversial (75, 76). The differences in the results are possibly due to the experimental setup, time course of measurement, and doses. Therefore, there are opportunities for the therapeutic implication of new antioxidants for the prevention of doxorubicin cardiotoxicity.

Doxorubicin may form a potentially harmful pro-oxidant complex with iron. For example, iron (III) readily interacts with doxorubicin. The complex is subsequently reduced by either glutathione or NADPH-dependent enzymes, thereby initiating a cascade of free radical generation reactions (77). Iron overload aggravated doxorubicin toxicity in rat heart cells and this effect is abrogated by the treatment of the iron chelator deferoxamine (78). However, cardioprotective effect did not occur when cells were not overloaded with iron. Currently, the iron chelator dexrazoxane is the only clinically used drug for reducing the incidence and severity of cardiomyopathy associated with doxorubicin administration in women with metastatic breast cancer who have received 3 cumulative doxorubicin dose of 300 mg/m^2 and who in their physician's opinion, would

benefit from continuing therapy with doxorubicin. It is not recommended for use with the initiation of doxorubicin therapy since it interferes with the antitumor efficacy of the regimen (Zinecard monograph). In addition, the drug may not be effective in protecting DOX-induced cardiac cell damage in young rat (79). Thus, the improvement of strategy for prevention of doxorubicin cardiotoxicity is awaited.

For the study of cardiotoxicity, H9c2 rat cardiac myoblasts elicit an excellent model of study since their electrophysiology properties and ion channels presented are similar to primary culture of cardiac myocytes (80, 81). H9c2 has been used to study the mechanisms of ATP metabolism, calcium signaling and cardiomyocyte apoptosis (82-84). A number of research has illustrated that DOX-mediated apoptosis reflect the role of oxidative stress and interactions with many intracellular signaling pathways (85-88). The complex cytotoxic mechanism involves: 1) increased pro-apoptotic signals such as membrane lipid peroxidation and DNA fragmentation, reduction or depletion of antioxidant defense mechanisms such as decreased total glutathione levels and SOD and catalase activities, and increased NO production; 2) lack of survival signal such as decreased Bcl-2/Bax ratio and NF-κB gene expression. It has been shown that a glutathione peroxidase and SOD mimetics protected cardiomyocytes toxicity induced by DOX (89). The principle ROS are superoxide radicals and hydroxyl radicals which have the potential to initiate the damage to various intracellular components such as DNA, lipids and proteins (90). Weak antioxidant capacity may be a major factor responsible for the high sensitivity of the heart to doxorubicin-induced oxidative damage. Kang et al. (91) demonstrated that overexperssion of catalase in the heart of transgenic mice exhibited a significant resistance to doxorubicin-induced cardiac lipid peroxidation, elevation of serum creatinine phosphokinase, and cardiac dysfunction. DOX-treated female rats increased cardiac apoptosis, as measured by the increased expression of the pro-apoptotic proteins Bax, caspase-3 activation, and oxidative stress (84). The elevation of $NO^{\bullet}$ production and increased expression of iNOS were associated with DOX-induced apoptosis in the rat cardiac cells. (83). Activated NFκB protects cells from apoptosis by inducing the expression of survival genes that promote resistance to apoptosis (92).

In summary, a large body of evidence suggests that ROS play a major role in DOX-induced cardiotoxicity. Although standard antioxidant vitamins show some benefits in animal model of DOX cardiotoxicity, it is still controversial. Moreover, recent studies have identified several oxidative pathways that are involved in the cellular oxidative damage at the molecular levels. Therefore, a better understanding of causes of DOX-induced cardiotoxicity and protective adaptive responses could lead to new target interventions to protect cardiomyocytes against apoptosis.

3. Antioxidants

Antioxidant is a term widely used but not well defined. Halliwell and Gutteridge (30) have proposed a broad definition of antioxidant as "any substance that, when present at low concentrations compared with those of an oxidizable substrate, significantly delays or prevents oxidation of that substrate". The term 'oxidizable substrate' includes every type of molecule found *in vivo*. Cellular antioxidants include agents from endogenous (e.g. glutathione) and exogenous sources (e.g. vitamin C and E), and enzymatic antioxidant defense (e.g. SOD and catalase). Under normal physiological conditions ROS is constantly produced and the levels are regulated primarily by these cellular defense mechanisms against oxidant stress (Figure 15). Some important antioxidants are discussed as follows:

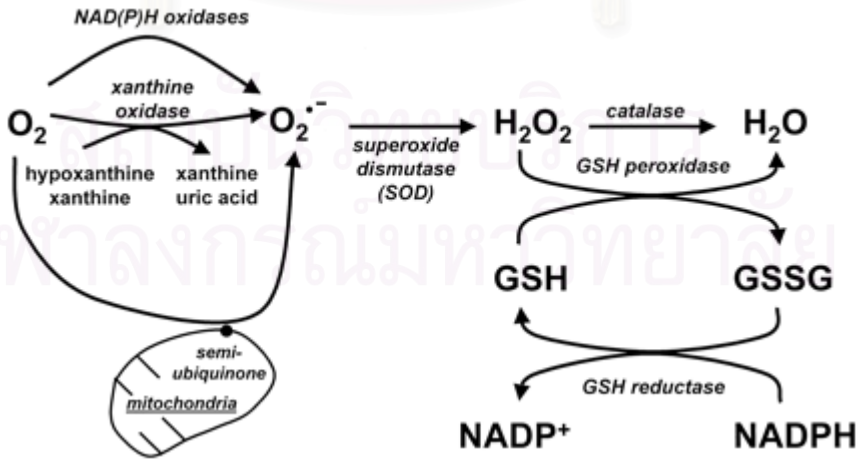


Figure 15. Pathways of reactive oxygen species (ROS) production and clearance. GSH, glutathione; GSSG, glutathione disulfide (9).

3.1 Exogenous antioxidants

Antioxidants are found in fruit, cereals, and vegetables, or they can be taken in the form of dietary supplements. There is some research suggesting that a diet rich in fruit, vegetables, and cereals has additional benefits, because of lesser-known but potent antioxidants in food that may have a combined effect greater than that of any single nutrient or individual antioxidant supplement. Antioxidant vitamins — E, C and beta carotene (a form of vitamin A) — have potential health-promoting properties. Though the data are incomplete, up to 30 percent of Americans are taking some form of antioxidant supplement.

3.1.1 Vitamin E

Antioxidants such as vitamin E act to protect your cells against the effects of free radicals, which are potentially damaging by-products of the body's metabolism. Free radicals can cause cell damage that may contribute to the development of cardiovascular disease and cancer. Studies are underway to determine whether vitamin E might help prevent or delay the development of those chronic diseases.

Vitamin E is a fat-soluble vitamin that exists in eight different forms. Each form has its own biological activity, the measure of potency or functional use in the body. Alpha-tocopherol is the most active form of vitamin E in humans, and is a powerful biological antioxidant. Vegetable oils, nuts, and green leafy vegetables are the main dietary sources of vitamin E. Fortified cereals are also an important source of vitamin E in the United States.

Vitamin E, as a scavenger of peroxyl radicals, is probably the most important (but not the only), inhibitor of the free radical chain reaction of lipid peroxidation in animals. However, it must be remembered that initiation of lipid peroxidation can be prevented, as an earlier line of defence, by enzymes that scavenger ROS/RNS and proteins that sequester transition metal ions. Sequestration of metal ion also prevents them from decompositing peroxides into chain-propagating peroxyl and alkoxyl radicals. The name ‘vitamin E’ does not refer to a particular chemical structure; it is a nutritional term. It was first used to refer to a fat-soluble ‘factor’ discovered in 1992 to be essential in the diet of rats to permit normal reproduction. Later work showed that

vitamin E is essential in the diets of all other animals. Lack of it causes a wide variety of symptoms including sterility in male rats, dogs, cocks, rabbits, guinea-pigs, crocodiles, snakes, lizards, elephants, monkeys, ducks, mice and minks, 'white-muscle disease' in lambs, flamingoes and claves, and degeneration of the cerebellum in chicks. Indeed, the dietary content of vitamin E is one factor that affects the sensitivity of laboratory animals to certain toxins or to tissue insults such as ischaemia-reperfusion.

Short-term absence of vitamin E from the human diet does not cause any specific deficiency disease, although low vitamin E levels in premature babies can predispose to haemolytic anaemia, propably due to increased fragility of the erythrocyte membrane. There is no evidence that human muscle dystrophy or multiple sclerosis respond to vitamin E administration. Sources of vitamin E in the human diet include wheat-germ, vegetable oils, margarines, nuts, grains and green leafy vegetables (30).

Most vitamin E supplements contain synthetic alpha-tocopherol, but unlike other vitamins, synthetic vitamin E is not identical to natural. Alpha-tocopherol (α-tocopherol) is present in nature in only one form, RRR-alpha-tocopherol. The chemical synthesis of α-tocopherol results in eight different forms, only one of which is RRR-alpha-tocopherol. Chemically synthesized vitamin E is known as all rac-alpha-tocopherol. These forms differ in that they can be "right" or "left" (R or S) at three different places in the alpha-tocopherol molecule. The most important place is what's known as the 2-position—half of the synthetic is 2R and half is 2S (Figure 16) (93). Vitamin E supplements are labeled d-alpha for natural and dl-alpha for synthetic.

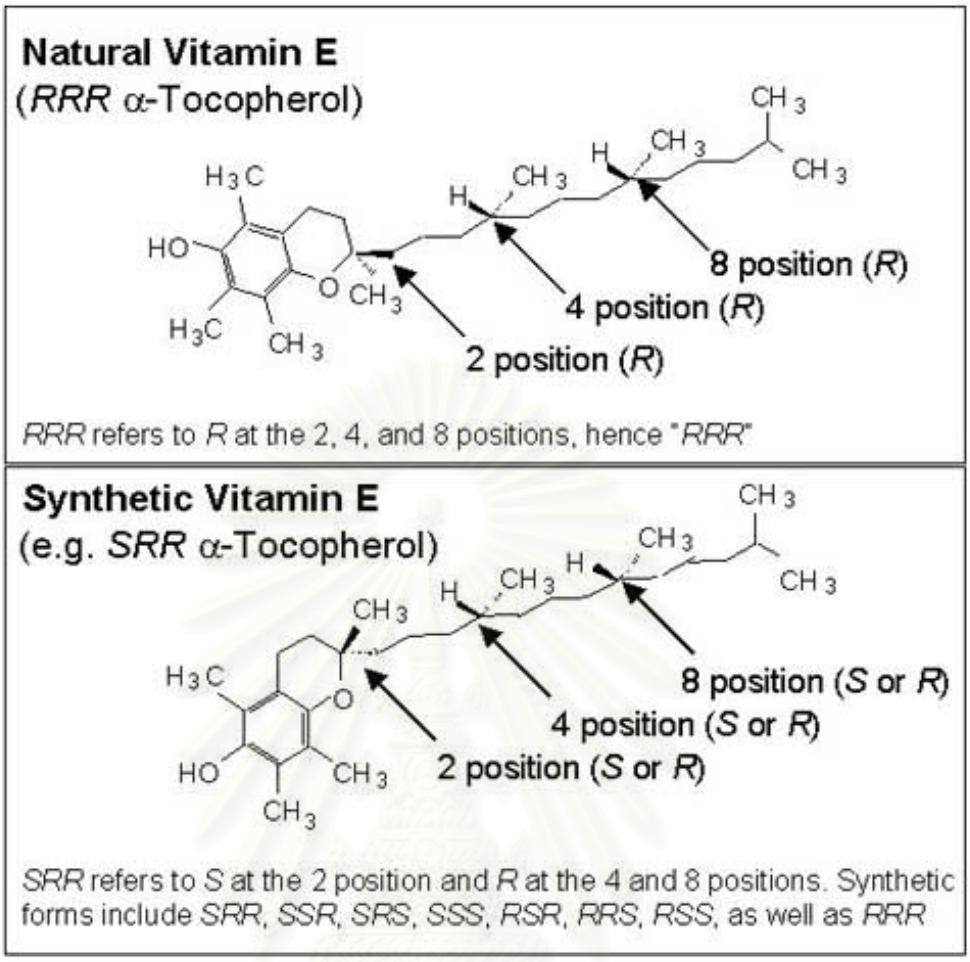


Figure 16. Structures of natural and synthetic vitamin E (93).

When equal amounts of deuterium-labeled natural and synthetic vitamin E were fed to humans, both plasma and tissues subsequently contained twice as much natural as synthetic vitamin E. This difference is thought to arise from differences of the affinity of the hepatic alpha-tocopherol transfer protein (alpha-TTP) for the various forms, with a preference for the 2R-alpha-tocopherol forms (94). In humans, all vitamin E forms are absorbed in the intestine. The majority of the absorbed vitamin E is delivered to the liver, where the naturally occurring form of vitamin E, RRR-alpha-tocopherol, is preferentially secreted into the circulation for delivery to tissues. Importantly, humans with ataxia from vitamin E deficiency have defects in the alpha-TTP. These people become vitamin E deficient because they are unable to secrete alpha-tocopherol into the circulation. However, because the amounts and activities of alpha-TTP have not

been measured in the patients' livers, the functions of alpha-TTP have remained incompletely understood. Moreover, the mechanisms for the regulation of vitamin E in tissues are not known

3.1.2 Vitamin C

Vitamin C or ascorbic acid, is the enolic form of 3-oxo-L-gulofuranolactone (Figure 17). It can be prepared by synthesis from glucose, or extracted from plant sources such as rose hips, blackcurrants or citrus fruits. Ascorbic acid has two ionizable –OH goups. Since PK_{a1} is 4.25 and PK_{a2} is 11.8, a mono-anion is the favoured form at physiological pH (hence ascorbate is more prevalence). Plants and most animals can synthesize ascorbate from glucose, but humans, other primates, guinea pigs and fruit-bats lost the enyzme required for the terminal step (gulonolactone oxidase) and so require ascorbate to be present in the diet, as the water-soluble vitamin, vitamin C.

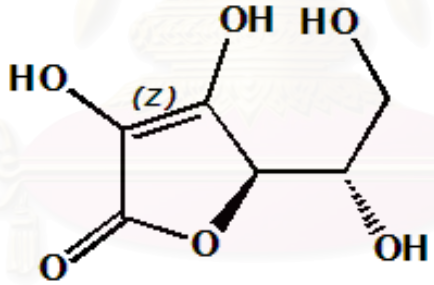


Figure 17. Chemical structure of L-ascorbic acid, $C_6H_8O_6$, MW 176.12.

Ascorbate is required *in vivo* as a cofactor for at least eight enzymes, of which the best known are proline hydroxylase and lysine hydroxylase, involved in the biosythesis of collagen. Both these enzymes contain iron at their active sites. Collagen sythesized in the absence of ascorbate is insufficiently hydroxylated and does not form fibers properly, giving rise to poor wound healing and fragility of blood vessels. Ascorbate is also required by the copper-containing enzyme dopamine-β-hydroxylase,

which converts dopamine into noradrenalin. The antioxidant effect of ascorbate demonstrated *in vitro* is a scavenger of $O_2^{\bullet-}$, $HO_2^{\bullet}$, $OH^{\bullet}$, thiyl, oxysulphur radicals, ergothioneine-derived radicals, hypochlorous acid, peroxynitrous acid and nitrosating agents, nitroxide radicals, O_3, $NO_2^{\bullet}$. Ascorbate also prevents damage by radicals arising by attack of $OH^{\bullet}$, or $RO_2^{\bullet}$ upon urate. It inhibits lipid peroxidation induced by haemoglobin- or myoglobin-H_2O_2 mixtures. Ascorbate reduces the heme Fe(IV) species back to Fe^{2+} state, preventing peroxide-dependent oxidation and heme breakdown (95).

Recycling of ascorbate: Oxidation of ascorbate by reaction with ROS/RNS in body fluids seems to lead to its depletion, probably by the reactions

Ascorbate $\xrightarrow{\text{radical attack}}$ semidehydroascorbate (SDA) radical

2SDA $\xrightarrow{\text{disproportionation}}$ ascorbate + DHA

DHA $\xrightarrow[\text{breakdown}]{\text{rapid non-enzyme}}$ oxalate, threonate, other oxidation products

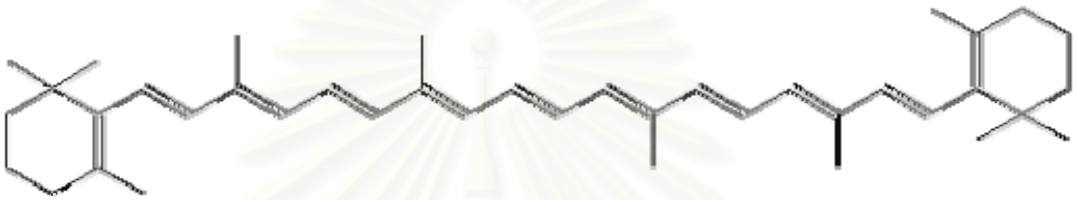
In addition, erythrocytes, neutrophils and probably some other cell types take up dehydroascorbic acid (DHA) rapidly and convert it back to (intracellular) ascorbate. Indeed, many ascorbate at the expense of GSH or of NADH. One example is NADH-semidehydroascorbate reductase enzymes. Glutathione dependent dehydroascorbate reductase enzymes have been identified in plants and in several mammalian tissues. However, their identity as unique enzymes is uncertain since some of the proteins involved in thiol-disulphide interchange within the cell such as protein-disulphide isomerase and glutaredoxin; show dehydroascorbate reductase activity.

3.1.3 β-carotene

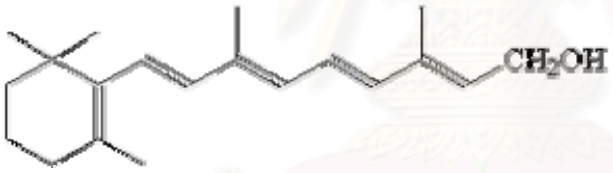
In animals and human, carotenoids particularly β-carotene and lycopene, play a role in the protection against photooxidative processes by acting as singlet molecular oxygen and peroxyl radicals scavengers and can interact synergistically with other antioxidants. The A group of vitamins are important metabolites of carotenoids. Vitamin

A1 (retinol) has a diterpene structure but it is derived in mammals by oxidative metabolism of tetraterpenoid, mainly β-carotene, taken in the diet (96).

Beta-carotene is also known as provitamin A, because it is one of the most important precursors of vitamin A in the human diet. Chemical structures of the two molecules clearly show that vitamin A (retinol) is very closely related to half of the beta-carotene molecule (Figure 18).



β-carotene



Vitamin A

Figure 18. Chemical structure of β-carotene and vitamin A

There are two ways in which beta-carotene can be converted to vitamin A: either by cleavage at the center or by breaking the molecule down from one end. The breakdown of beta-carotene occurs in the walls of the small intestine (intestinal mucosa) and is catalysed by the enzyme beta-carotene dioxygenase. The retinol formed is stored in the liver as retinyl esters. This is why cod liver oil used to be taken as a vitamin A supplement. It is also why you should never eat polar bear liver if you run out of food in the Arctic; vitamin A is toxic in excess and a modest portion of polar bear liver contains more than two years supply. Beta-carotene, on the other hand, is a safe source of vitamin A. The efficiency of conversion of beta-carotene to retinol depends on

the level in the diet. If too much beta-carotene is taken, less is converted, and the rest is stored in fat reserves in the body. Thus, hypervitaminosis may occur, usually elicited by yellow skin (96).

The antioxidant activity of beta-carotene has been shown both *in vitro* and *in vivo*. *In vitro* experiments suggest that beta-carotene inhibits peroxidation of simple lipid system at low O_2 concentration, but not at high O_2 concentration. However, it did not protect against LDL peroxidation regardless of O_2 concentration. Beta-carotene has potential to act as free-radical scavengers. Oxidized radicals can react with carotenoids by electron transfer, e.g. for nitrogen dioxide reacting with beta-carotene (written Car below) (30):

$$NO_2^{\bullet} + Car \longrightarrow Car^{\bullet+} + NO_2^{-}$$

Radicals such as $[OO\text{-}Car\text{-}OOR]^{\bullet}$, and $CarO_2^{\bullet}$ could propagate free-radical chain reactions such as lipid peroxidation by abstraction hydrogen.

3.1.4 N-acetylcysteine

N-acetylcysteine (NAC), a precursor of reduced glutathione (GSH), has been in clinical use for more than 30 years, primarily as a mucolytic. In addition to its mucolytic action, NAC is being studied and utilized in conditions characterized by decreased GSH or oxidative stress such as HIV infection, cancer, and heart disease. Because of its hepato-protective activity, intravenous and oral administration of NAC have been used extensively in the management of acetaminophen poisoning (97).

NAC is a thiol (sulfhydryl-containing) compound which has the chemical formula $C_5H_9NO_3S$ and a molecular weight of 163.2 (Figure 19). It is rapidly absorbed following an oral dose; however, extensive first pass metabolism by the cells of the small intestine and the liver results in the incorporation of NAC into protein peptide chains and the formation of a variety of metabolites of NAC. Only a small percentage of the intact NAC molecule arrives in the plasma, and subsequently in tissue.

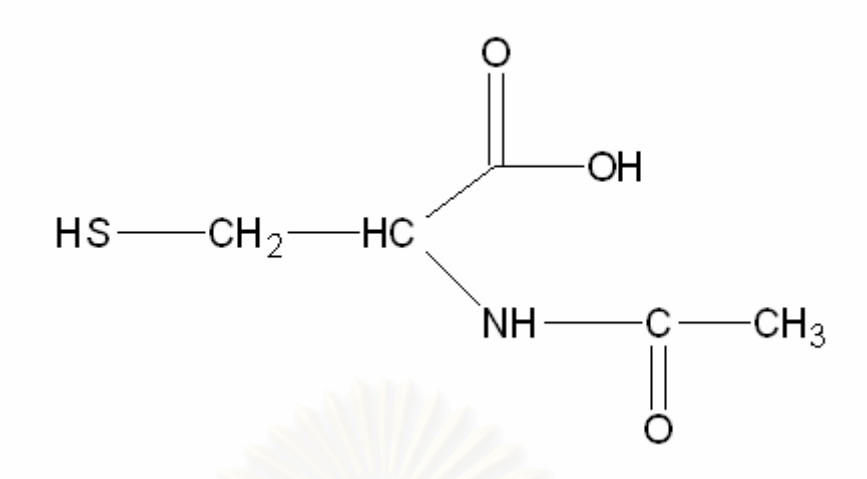


Figure 19. Chemical structure of N-acetylcysteine, $C_5H_9NO_3S$, MW 163.2.

As a source of SH groups, NAC can stimulate GSH synthesis, enhance glutathione-S-transferase activity, promote detoxification, and act directly on reactive oxidant radicals. NAC appears to support the synthesis of GSH under conditions when the demand for GSH is increased, such as during the metabolism of acetaminophen; however, in the absence of increased stress on the glutathione pools, NAC might have no effect on plasma GSH. After the administration of NAC (30 mg/kg) to healthy volunteers, no increase in total cysteine and free and total glutathione was observed in plasma. In contrast, following the co-administration of 2 grams NAC and 2 grams acetaminophen, an increase in circulating cysteine and GSH concentrations was observed (98).

NAC appears to exert protective effects by promoting GSH synthesis and metabolism, and by restricting the biotransformation of mutagenic/carcinogenic substances into more toxic compounds. Although NAC does not appear to affect the concentrations of cytochromes P-450 in hepatic and pulmonary microsomes, it can stimulate cytosolic enzyme activities involved in NADP reduction (glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase), in glutathione reduction (GSSG-reductase) and in the reductive detoxification of xenobiotics. NAC also appears to be able to increase cyclic guanosine monophosphate concentration under some experimental conditions. SH groups are essential for defense against reactive oxygen

species. So, it is not surprising that NAC is a powerful scavenger of hypochlorous acid, and is capable of reducing hydroxyl radicals and hydrogen peroxide. In animal experiments, NAC has been shown to be protective against oxygen toxicity to the lung caused by prolonged administration of 100 percent oxygen. (97, 98).

3.1.5 α-lipoic acid

Alpha-Lipoic acid (ALA) is a natural compound chemically named 1,2-dithiolane-3-pentanoic acid ($C_8H_{14}O_2S_2$). It is also referred to as thioctic acid. In humans, ALA is synthesized by the liver and other tissues, and functions as a cofactor within pyruvate dehydrogenase and α-keto-glutarate dehydrogenase. Recently ALA has been shown to be required for the oxidative decarboxylation of pyruvate to acetyl-CoA, the critical step bridging the gap between glycolysis and the citric acid cycle. α-Lipoic acid is both water and fat soluble, and therefore, is widely distributed in plants and animals in both cellular membranes and cytosol. In addition, ALA and its reduced dithiol form, dihydrolipoic acid (DHLA), are powerful antioxidants (Figure 20), their functions described by Biewenga et al. (99) include: *1*) quenching of reactive oxygen species, *2*) regeneration of exogenous and endogenous antioxidants such as vitamins C and E, and glutathione, *3*) chelation of metal ions, and *4*) reparation of oxidized proteins. In most cells containing mitochondria, ALA is reduced by an NADH-dependent reaction with lipoamide dehydrogenase to form DHLA. In cells that lack mitochondria ALA can be reduced to DHLA via NADPH with glutathione and thioredoxin reductases (100).

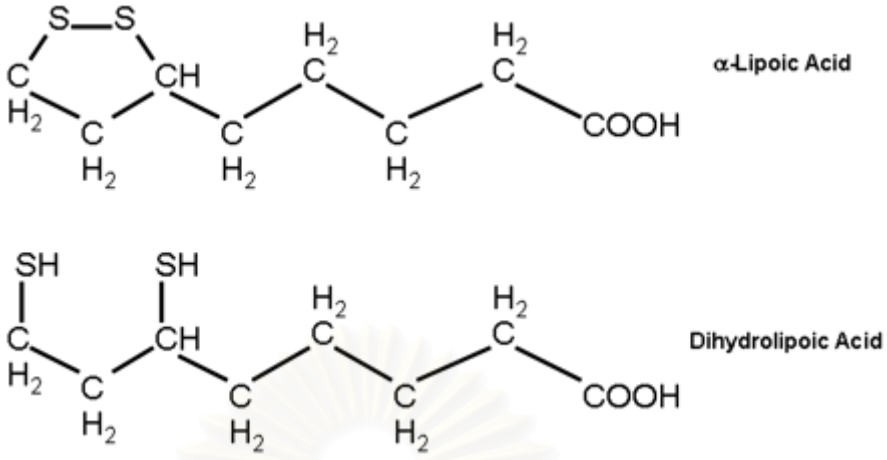


Figure 20. Simplified diagram of alpha-lipoic acid and its reduced form dihydrolipoic acid (100).

3.1.6 Other Antioxidants

Aside from antioxidant vitamins and minerals, there are several other types of antioxidants available both in foods and supplements. Many of these belong to a family of plant chemicals called flavonoids. There are also several herbs which have antioxidant activity. These include cayenne or chili pepper (*Capsicum annuum*), ginger (*Zingiber officinale*), garlic (*Allium sativum*), turmeric (*Curcuma longa*), ginkgo biloba (*Ginkgo biloba*) and bilberry (*Vaccinium myrtillus*). Grape seed extract and Pycnogenol®, from pine bark extract, are two of the most powerful antioxidant supplements available today. ORAC (Oxygen Radical Absorbance Capacity) is a test tube analysis that measures the total antioxidant power of foods and other chemical substances.

3.2 Endogenous antioxidants and antioxidant defense enzymes

3.2.1 Glutathione

Glutathione (GSH) is a water-soluble tripeptide composed of the amino acids glutamine, cysteine, and glycine. The thiol group is a potent reducing agent, rendering GSH the most abundant intracellular small molecule thiol, reaching millimolar concentrations in some tissues. As an important antioxidant, GSH plays a role in the detoxification of a variety of electrophilic compounds and peroxides via catalysis by glutathione S-transferases (GST) and glutathione peroxidases (GPx). The importance of GSH is evident by the widespread utility in plants, mammals, fungi and some prokaryotic organisms. In addition to detoxification, GSH plays a role in other cellular reactions, including, the glyoxalase system, reduction of ribonucleotides to deoxyribonucleotides, regulation of protein and gene expression via thiol:disulfide exchange reactions. The tripeptide can exist intracellularly in either an oxidized (GSSG) or reduced (GSH) state. Maintaining optimal GSH:GSSG ratios in the cell is critical to survival, hence, tight regulation of the system is imperative. A deficiency of GSH puts the cell at risk for oxidative damage. It is not surprising that an imbalance of GSH is observed in a wide range of pathologies, including, cancer, neurodegenerative disorders, cystic fibrosis (CF), HIV and aging (101).

GSH is involved in important cell functions including vitamin C metabolism, chelation of copper ions, and the Phase II biotransformation of foreign substances and intermediate oxygen metabolites. An adequate intracellular supply is essential for cell survival: reduction of cell concentration usually indicates a pathological state preceding apoptosis in viral infections (36). The liver contains high concentrations of catalase and GSHPx, the former mostly in peroxisomes and the latter in the cytosol and mitochondrial matrix. Both are involved in peroxide removal where GSH is the reducing agent. Reduced GSH (glutathiol), a nonprotein thiol synthesized primarily in the liver, is the main intracellular defense against ROS, free radicals, and electrophilic xenobiotics. It is essential, often in high concentrations, in all cells. Availability of cysteine, the unstable precursor of GSH synthesis in most cells, is a critical determinant of cellular GSH levels, virtually all intracellular cysteine being present in its oxidized form (cystine). The

clinically safe thiol antioxidants α-lipoic acid and NAC that enhance cellular GSH levels are beneficial (102).

3.2.1.1 Glutathione synthesis

GSH is synthesized de novo from the amino acids glycine, cysteine and glutamic acid. Synthesis of GSH requires the consecutive action of two enzymes, c-glutamylcysteine synthetase (c-GCS) and GSH synthetase (Figure 21). c-GCS is a heterodimer composed of a catalytically active heavy subunit c-GCS-HS (73 kDa) and a regulatory subunit, c-GCS-LS (30 kDa). The regulation of c-GCS is complex. Induction of c-GCS expression has been demonstrated in response to diverse stimuli in a cell specific manner. The bioavailability of cysteine is rate limiting for the synthesis of GSH. Cysteine and the oxidized form of the amino acid, cysteine, are transported into the cell via sodium dependent and independent transporters, respectively. Oxidants (including hyperoxide, H_2O_2 and electrophilic compounds) promote cystine uptake and a concomitant increase in expression of c-GCS. The c-GCS promotor region contains a putative AP-1 binding site, an antioxidant response element (ARE), and an electrophile responsive element. The AP-1 site is critical to constitutive expression of the c-GCS-HS subunit. Post-translational modifications of c-GCS also influence GSH synthesis. Specifically, phosphorylation of c-GCS leads to the inhibition of GSH synthesis. GSH itself regulates the activity of c-GCS via a negative feedback mechanism. Hence, GSH depletion increases the rate of GSH synthesis.

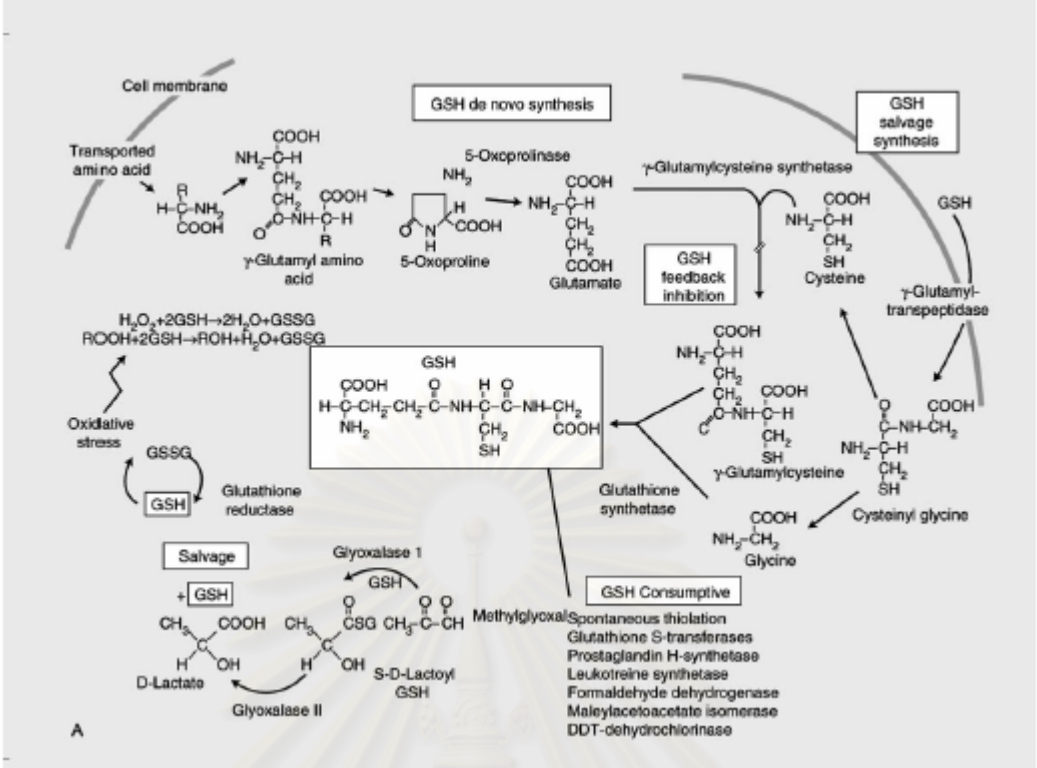


Figure 21. Homeostasis of glutathione is maintained intracellularly through a de novo and salvage synthesis pathway. Tight regulation of the GSH:GSSG ratio is maintained by glutathione reductase (101).

3.2.1.2 GSH redox cycle

The formation of excessive amounts of reactive O_2 species (ROS), including peroxide (H_2O_2) and superoxide anions ($O_2^{-\bullet}$) is toxic to the cell. Hence, metabolizing and scavenging systems to remove them are functionally critical and tightly controlled in the cell. GSH peroxidase (GPx) in concert with catalase and superoxide dismutase (SOD) function to protect the cell from damage due to ROS. GPx detoxifies peroxides with GSH acting as an electron donor in the reduction reaction, producing GSSG as an end product. The reduction of GSSG is catalyzed by GSH reductase (GR) in a process that requires NADPH. GR is a member of the flavoprotein disulfide oxidoreductase family and exists as a dimer. Under conditions of oxidative stress, GR is regulated at the level of transcription as well as by post-translational modifications. Alterations in GR expression and activity have been implicated in cancer and aging.

GPx is an 80 kDa protein that is composed of four identical subunits. Five distinct GPx isozymes have been characterized in mammals. While GPx's are ubiquitously expressed, individual isoforms are tissue specific. GPx expression is induced by oxidative stress and aberrant expression of GPx's has been associated with a wide variety of pathologies, including hepatitis, HIV, and a wide variety of cancers, including skin, kidney, bowel and breast (101).

In mitochondria, hydrogen peroxide, the product of $O_2^{\bullet -}$ dismutation and the main precursor of $OH^{\bullet}$ in the presence of reduced transition metals, is mostly decomposed by the enzyme glutathione peroxidase. In the liver, mitochondria account for about one third of the total glutathione peroxidase activity. A second glutathione peroxidase associated with the mitochondrial membrane, known as phospholipid-hydroperoxide glutathione peroxidase, is specifically involved in reducing lipid peroxides associated with the membrane (103).

3.2.2 SOD

At present, three distinct isoforms of SOD have been identified in mammals. Two isoforms of SOD have Cu and Zn in their catalytic center and are localized to either intracellular cytoplasmic compartments (CuZn-SOD or SOD1) or to extracellular elements (EC-SOD or SOD3). The gene structures are shown in (Figure 22). SOD1 has a molecular mass of about 32,000 Da and has been found in the cytoplasm, nuclear compartments, and lysosomes of mammalian cells. SOD3 is the most recently discovered and least characterized member of the SOD family. The enzyme exists as a homotetramer of molecular weight 135,000 Da with high affinity for heparin. SOD3 was first detected in human plasma, lymph, ascites, and cerebrospinal fluids. The expression pattern of SOD3 is highly restricted to the specific cell type and tissues where its activity can exceed that of SOD1 and SOD2. A third isoform of SODs has manganese (Mn) as a cofactor and has been localized to mitochondria of aerobic cells (Mn-SOD or SOD2). It exists as a homotetramer with an individual subunit molecular weight of about 23,000 Da. SOD2 has been shown to play a major role in promoting cellular differentiation and tumorgenesis and in protecting against hyperoxia-induced pulmonary toxicity (104).

Because SODs catalyze superoxide anions to oxygen and hydrogen peroxide, cells are protected against the toxic effects of oxygen metabolism. Cuprozinc SOD (Cu/ZnSOD) is present in the cytosol, nucleus, and peroxisomes of all cells. Manganese SOD is present in the intracisternal space of mitochondria. Extracellular cuprozinc SOD differs structurally from intracellular Cu/Zn SOD, the latter being apparent in extracellular fluids after cell disruption (36).

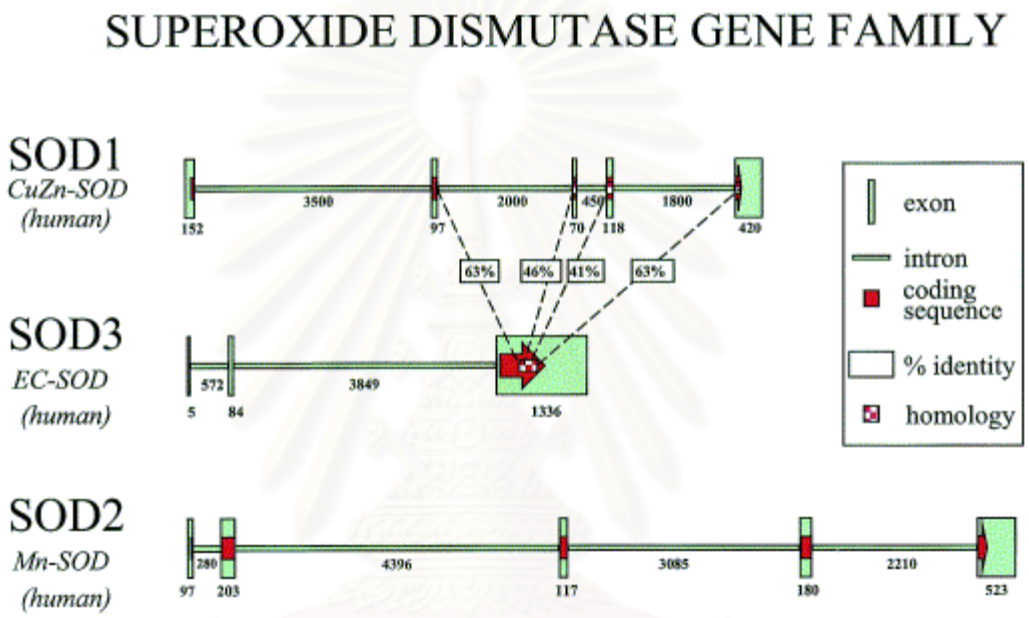


Figure 22. Superoxide dismutase gene family. Genomic organization of the three known members of the human SOD enzyme family (104).

The deleterious effects resulting from the formation of ROS in the mitochondrion are, to a large extent, prevented by various antioxidant systems. Superoxide is enzymatically converted to H_2O_2 by a family of metalloenzymes called SOD. Since $O_2^{\bullet -}$ may either reduce transition metals, which in turn can react with H_2O_2 producing $OH^{\bullet}$ or spontaneously react with $NO^{\bullet}$ to produce peroxynitrite, it is important to maintain the steady-state concentration of $O_2^{\bullet -}$ at the lowest possible level. Thus, although the dismutation of $O_2^{\bullet -}$ to H_2O_2 and O_2 can also occur spontaneously, the role of SODs is to increase the rate of the reaction to that of a diffusion-controlled process (103).

The mitochondrial matrix contains a specific form of SOD, with manganese in the active site which eliminates the $O_2^{\bullet -}$ formed in the matrix or on the inner side of the inner membrane. The expression of MnSOD is further induced by agents that cause oxidative stress, including radiation and hyperoxia, in a process mediated by the oxidative activation of the nuclear transcription factor NFκB (105).

3.2.3 Catalase

Catalase (EC 1.11.1.6), present in the peroxisomes of nearly all aerobic cells, serves to protect the cell from the toxic effects of hydrogen peroxide by catalyzing its decomposition into molecular oxygen and water without the production of free radicals. Dismutation of $O_2^{\bullet -}$ generates H_2O_2, a species also generated by several oxidase enzymes in vivo, including xanthine, urate and D-amino acid oxidases. Hydrogen peroxide is usually removed in aerobes by two types of enzyme. The catalases directly catalyse decomposition of H_2O_2 to ground-state O_2 (30).

$$2H_2O_2 \longrightarrow 2\,H_2O + O_2$$

Peroxidase enzymes remove H_2O_2 by using it to oxidize another substrate (shown as SH_2 below)

$$SH_2 + H_2O_2 \longrightarrow S + H_2O$$

Catalase, a major H_2O_2 detoxifying enzyme found in peroxisomes, is also present in heart mitochondria. However, this enzyme has not been found in mitochondria from other tissues, including skeletal muscle (103).

The protein exists as a dumbbell-shaped tetramer of four identical subunits (220,000 to 350,000 kD). Each monomer contains a heme prosthetic group at the catalytic center. Catalase monomers from certain species (e.g. cow) also contain one tightly bound NADP per subunit. This NADP may serve to protect the enzyme from oxidation by its H_2O_2 substrate. Catalase was one of the first enzymes to be purified to

homogeneity, and has been the subject of intense study. The enzyme is among the most efficient known, with rates approaching 200,000 catalytic events/second/subunit (near the diffusion-controlled limit). Catalase structure from many different species has been studied by X-ray diffraction. Although it is clear that all catalases share a general structure, some differ in the number and identity of domains. Despite a large number of studies on catalases, the only mammalian catalase structure available is that from beef liver, in which about 50% of the heme groups are degraded to bile pigments (106).

CHAPTER III

MATERIALS AND METHODS

Instruments

1. Carbon dioxide incubator (Nuaire model NU-4750E)
2. Laminar air flow hood (Nuaire model NU-440)
3. Inverted microscope (Olympus CKx41 Culture microscope)
4. Microscope digital camera (Olympus optical DP21)
5. Culture ware: cell culture dish diameter 60 mm, diameter 100 mm, 96-well microtiter plates, 6-well microtiterplates, cryotubes, conical tubes 15 mL, 50 mL
6. Microplate reader (Biorad Model 550)
7. Syringe membrane filter: 0.22 μm, 0.45 μm
8. Microliter pipette : 10-100 μL, 100-1000 μL
9. pH meter
10. Spectrophotometer (Shimadzu model UV-1601)
11. Refrigerated centrifuge (Beckman model J2-21)
12. Centrifuge (Jouan model MR23i)
13. Autoclave (Iwaki autoclave model ACV-3167)
14. Ultralow freezer (Nuarie model NU-6510E)

Chemicals

1. Cell : H9c2 cells (American Type Culture Collection-CRL 1446)
2. Cell culture: Dulbecco's Modified Eagle's Medium (DMEM) (Gibco BRL), Fetal Bovine Serum (FBS) (Gibco), Penicillin G (Gibco), Streptomycin sulfate (Gibco), Dulbecco's Phosphate Buffered Saline (DPBS) (Gibco BRL), Trypsin (Gibco), Dimethyl sulfoxide (DMSO), sodium bicarbonate (Sigma); EDTA (Sigma)
3. Crystal Violet Cytotoxic Assay: 37% Formalin (Sigma), Crystal violet (Sigma), Sodium citrate (Sigma), Methanol (Fluka), syringe membrane filter 0.45 μm
4. FRAP Assay: Ferrous sulfate heptahydrate (Merck), Ferric chloride (Merck), Ferric tripyridyltriazine, Hydrochloric acid, TPTZ (2,4,6-tripyridyl-s-triazine)

5. Lipid peroxidation: Tris-HCl, Triton X-100 (Sigma), HCl (Sigma), butylated hydroxytoluene (BHT) (Sigma), ethanol (Sigma), Thiobarbituric acid (Sigma), Malondialdehyde (Sigma)
6. Catalase assay kit (Cayman Chemical, Ann Arbor, MI, USA.)
7. Caspase-3 Colorimetric Activity assay kit (Chemicon International, Inc., Temecula, CA, USA)
8. NFκB p50/p65 Transcription Factor Assay kit (Chemicon International, Inc., Temecula, CA, USA)
9. Total Glutathione (tGSH) Determination Colorimetric Microplate Assay Kit (Oxford Biomedical Research, Inc., Oxford MI, USA) : Glutathione standard solution (reduced form GSH); 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), β–Nicotinamide adenine dinucleotide phosphate, reduced form (β–$NADPH_2$), Glutathione Oxidoreductase suspension, Metaphosphoric acid (MPA)
10. Protein assay: Standard Bovine Serum Albumin (Sigma), Bradford Protein assay Kit

Test Compound

1. Antioxidant standards: Ascorbic acid (Sigma), N-acetylcysteine, Ferous sulfate (Merck)
2. Plant extract: *P. urinaria* ethanolic extracts
3. Doxurubicin (Sigma)

Plant Extract

P. urinaria ethanolic extract and compound(s) was obtained from Medicinal Plant Research Institute, Department of Medical Science, Ministry of Public Health, Nonthaburi, Thailand. The aerial part of *P. urinaria* was dried and ground into powder. Ethanolic extract was obtained using Soxhlet apparatus (yield = 20.3%). The crude extract was standardized to control batch to batch variations.

Cell Culture

H9c2 cells, embryonic rat heart-derived cell line was obtained from the American Type Culture Collection (ATCC) and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C. The culture medium was replaced every 2 days and subcultured at 80% confluence.

Ferric Reducing/Antioxidant Power Assay (FRAP)

Concept of FRAP Assay

A biological antioxidant has been as "any substance that when present at low concentrations compared to those of an oxidisable substrate, significantly delays or prevents oxidation of that substrate." However, unless an antioxidant prevents the generation of an oxidizing species, for example, by metal chelation or enzyme-catalyzed removal of a potential oxidant, a redox reaction still generally occurs, even in the presence of an antioxidant. The difference is that the oxidizing species reacts with the antioxidant instead of the "substrate," i.e., the antioxidant reduces the oxidant. In simple terms then, electron-donating antioxidants can be described as redox reactions in which one reactive species is reduced while another is oxidized. In this context, therefore, "total antioxidant power" may be referred to analogously as total reducing power.

Priniciple of FRAP Assay

FRAP assay is a recently developed, direct measure of "total antioxidant power"(107). At low pH, reduction of a ferric tripyridyltriazine (Fe^{III}-TPTZ) complex to the ferrous form, which has an intense blue color, can be spectrophotometrically monitored at 593 nm. The change of absorbance is directly related to the "total" reducing power of the electron-donating antioxidants present in the reaction. The following antioxidants were used to compare the antioxidant power of *P. urinaria* extracts: solid L-(+)-ascorbic acid extra pure crystals; N-acetylcysteine.

Reaction of Fe(II) represents a one electron exchange reaction and is taken as unity, i.e., the blank corrected signal given by 100 μM solution of Fe(II) is equivalent to a FRAP value of 100 μM. To perform the FRAP microplate assay, working reagent, standards, control, and test samples are used to give a final volume of 200 μL of

reaction mixtures. Absorbance change is translated into a FRAP value (in μM) by relating to the ΔA_{593nm} of test sample to that of a standard solution of known FRAP value, [e.g.1000 μM Fe(II)] shown in the equation below:

$$\frac{\text{0-to 4-min } \Delta A_{593nm} \text{ test sample x FRAP value of standard } (\mu M)}{\text{0-to 4-min } \Delta A_{593nm} \text{ standard}}$$

Cytotoxicity assay (Crystal violet method)

Crystal violet cytotoxicity assay is an easy and rapid method in detecting cytotoxicity of many cell types such as the detection of the toxicity of tumor necrosis factor-alpha in L929 cells, and the doxorubicin-induced cytotoxicity in tumor cells (108, 109). Cell suspension at $5x10^4$ cells/mL was seeded to 96-well plate and allowed the cells to attach overnight. The standard or test compounds were assayed in triplicate. After 48-hr incubation, cells were washed with 200 μL PBS, and fixed with 10% buffered formalin. Prefiltered 0.1% crystal violet solution in water/MeOH will be used to stain the life cells. Cell survival is quantified by lysing the cells in 50 mM sodium citrate solution in water/EtOH and read absorbance at 595 nm. To determine the protective effect of *P. urinaria*, IC50 of doxorubicin was used to evaluate the H9c2 cytotoxicity in the absence or presence of *P. urinaria* extracts in comparison with antioxidant standards ascorbic acid (VIT C, 100 μM) or N-acetylcysteine (NAC, 100 μM).

Lipid peroxidation

Quantification of lipid peroxidation is essential to assess the role of oxidative injury in pathophysiological disorders. Lipid peroxidation results in the formation of highly reactive and unstable hydroperoxides of both saturated and unsaturated lipids. Traditionally, lipid peroxidation is quantified by measuring the lipid peroxide product malondialdehyde (MDA). Sensitive colorimetric assays have been developed to measure these aldehydes. MDA interacts with thiobarbituric acid to form an adduct thiobarbituric acid reactive substance (TBARS), which can be measured by fluorometry or spectrophotometry (110).

Approximately $2x10^6$ cells were used per assay. The cells were lysed by repetitive freezing/thawing procedure in distilled water. The 100 µL aliquot of standard MDA or sample was added to 50 µL of 0.2% butylated hydroxytoluene (BHT) in 95% ethanol, and 350 µL of Tris Buffer. The reaction of MDA and thiobarbituric acid produces chromogenic reagent TBARS at 95 °C. This product was monitored by reading absorbance at 532 nm.

Catalase assay

Catalase (CAT) is involved in the detoxification of hydrogen peroxide (H_2O_2), a reactive oxygen species (ROS), which is a toxic product of both normal aerobic metabolism and pathogenic ROS production. This enzyme catalyzes the conversion of two molecules of H_2O_2 to molecular oxygen and two molecules of water (catalytic activity). CAT also demonstrates peroxidatic activity, in which low molecular weight alcohols can serve as electron donors. While aliphatic alcohols serve as specific substrates for CAT, other enzymes with peroxidatic activity do not utilize these substrates.

$$2H_2O_2 \xrightarrow{\text{Catalase}} O_2 + 2H_2O$$

The catalase assay utilized the peroxidatic function of catalase for determination of enzyme activity. This method is based on the reaction of the enzyme with methanol in the presence of an optimal concentration of H_2O_2. The formaldehyde produced was measured spectrophotometrically with 4-amino-3-hydrazino-5-mercapto-1,2,4-triazole (Purpald) as the chromogen. Purpald specifically forms a bicyclic heterocycle with aldehydes, which upon oxidation changes from colorless to a purple color.

The cell samples were harvested using cell scrapers and sonicated on ice in 1-2 mL of cold buffer (50 mM potassium phosphate, pH 7.0, containing 1 mM EDTA). Then, cell lysate was centrifuge at 10,000 x g for 15 minutes at 4 °C. The supernatants were removed for assay and stored on ice or –80 °C for later use. The catalase assay was performed according to the manufacturer's instruction (Cayman Chemical™

catalog No. APT165). The final volume of the assay is 240 μL in all the wells. The samples and formaldehyde standards was assayed in duplicate. The assay was conducted as follows:

1. Preparation of the Formaldehyde Standards - Dilute 10 μL of formaldehyde standard with 9.99 mL of Sample Buffer (dilute) to obtain a 4.25 mM formaldehyde stock solution. Then, a serial dilution of standard formaldehyde was obtained by adding the amount of formaldehyde stock and Sample Buffer (dilute) to each tube as described in the following table (see below):

Tube	Formaldehyde stock (μL)	Sample Buffer (μL)	Final Concentration (μM formaldehyde)*
A	0	1,000	0
B	10	990	5
C	30	970	15
D	60	940	30
E	90	910	45
F	120	880	60
G	150	850	75

*Final formaldehyde concentration in the 170 μL reaction.

2. Formaldehyde Standard Wells -Add 100 μL of Assay Buffer (dilute), 30 μL of methanol, and 20 μL of standard (tubes A-G) per well in the designated wells on the plate.
3. Positive Control Wells (bovine liver CAT) - Add 100 μL of Assay Buffer (dilute), 30 μL of methanol, and 20 μL of diluted CAT (control) to two wells.
4. Sample Wells - Add 100 μl of Assay Buffer (dilute), 30 μL of methanol, and 20 μL of sample to two wells. To obtain reproducible results, the amount of CAT added to the well should result in an activity between 0.25-4 nmoL/min/mL. When necessary, samples should be diluted with Sample Buffer (dilute) or

concentrated with an Amicon centrifuge concentrator with a molecular weight cut-off of 100,000 to bring the enzymatic activity to this level.

5. Initiate the reactions by adding 20 µL of hydrogen peroxide (dilute) to all the wells being used. Make sure to note the precise time the reaction is initiated and add the hydrogen peroxide as quickly as possible.
6. Cover the plate with the plate cover and incubate on a shaker for 20 minutes at room temperature.
7. Add 30 µL of potassium hydroxide to each well to terminate the reaction and then add 30 µL of Purpald (chromogen) to each well.
8. Cover the plate with the plate cover and incubate for 10 minutes at room temperature on the shaker.
9. Add 10 µL of potassium periodate to each well. Cover with plate cover and incubate 5 minutes at room temperature on a shaker.
10. Read the absorbance at 540 nm (550 nm) using Biorad model 550 plate reader.
11. Generate the formaldehyde standard curve and calculate the formaldehyde concentration of the samples using the equation obtained from the linear regression of the standard curve substituting corrected absorbance values for each sample.
12. Calculate the Catalase activity of the sample using the following equation. One unit is defined as the amount of enzyme that will cause the formation of 1.0 nmol of formaldehyde per minute at 25°C.

$$\text{CAT Activity} = \frac{\mu M \text{ of sample}}{20 \text{ min}} \times \text{Sample dilution} = \text{nmoL/min/mL}$$

SOD assay

Superoxide dismutases (SODs) are metalloenzymes that catalyze the dismutation of the superoxide anion to molecular oxygen and hydrogen peroxide.

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

SOD catalyzes the conversion of superoxide anion radical ($O_2^{\bullet-}$) to hydrogen peroxide and molecular oxygen. The enzyme activity can be measure by the dismutation of $O_2^{\bullet-}$ generated by non-enzymatic system (111). All assay solutions were prepared at ambient temperature in glass vessels under subdued fluorescent room light. The Complete reaction system (in 0.25 mL total volume) consisted of 50 mM phosphate buffer, pH 7.4, containing 0.1 mM EDTA, 50 μM NBT, 78 μM NADH, and 3.3 mM PMS (final concentrations). Twenty five microlitters of samples or SOD standards (0 to 1,000 ng/mL, or 0 to 5.25 Units) were pipetted to microplate well containing 200 μL of freshly prepared reaction mixture (0.1 mM EDTA, 62 μM nitro blue tetrazolium (NBT), and 98 μM NADPH in 50 mM phosphate buffer, pH 7.4). The reaction was initiated by the addition of 25 μL 33 μM phenazine methosulfate in phosphate buffer, pH 7.4, containing 0.1 mM EDTA. After 5 min incubation, rate of NBT reduction was monitored at 550 nm. Cellular SOD activities were expressed in units SOD/mg protein (111).

Total Glutathione (tGSH) Determination

This assay employs a kinetic enzymatic recycling assay, based on the oxidation of GSH by 5,5'-dithiobis(2-nitrobenzoic acid), [DTNB] to measure the total glutathione (tGSH) content of biological samples. The glutathione standards or treated samples are added to the microtiter plate wells, followed by DTNB and glutathione reductase. Addition of $NADPH_2$ to the wells initiates the progressive reduction of DTNB by GSH, causing a color increase that is monitored at 405 nm. The rate of color change, typically followed over a 4-minute time period, is proportional to the tGSH concentration.

Assay Procedure

Prepare Standards:

Prepare the GSH standards within a concentration range from 0.5 to 20 µM in 5% MPA or anywhere between 0-100 µM as needed. Prepare and label standards as follows:

STANDARD 8 (S8): Add 40 µL of GSH standard solution into 3.96 mL of MPA and vortex. The concentration of S8 is 20 µM (20ng/mL).

STANDARD 7 (S7): Add 3 mL of S8 to 1 mL MPA and vortex. The concentration of S7 is 15 µM (15 ng/mL).

STANDARD 6 (S6): Add 2.67 mL of S7 to 1.33 mL MPA and vortex. The concentration of S6 is 10 µM (10 ng/mL).

STANDARD 5 (S5): Add 2 mL of S6 to 2 mL MPA and vortex. The concentration of S5 is 5 µM (5 ng/mL).

STANDARD 4 (S4): Add 2 mL of S5 to 2 mL MPA and vortex. The concentration of S4 is 2.5 µM (2.5 ng/mL).

STANDARD 3 (S3): Add 1.6 mL of S4 to 2.4 mL MPA and vortex. The concentration of S3 is 1 µM (1 ng/mL).

STANDARD 2 (S2): Add 2 mL of S3 to 2 mL MPA and vortex. The concentration of S2 is 0.5 µM (0.5 ng/mL).

STANDARD 1 (S1): This has only 2 mL MPA. The concentration of S1 is 0.

Reconstitute chemicals as follows:

1. Add 6 mL of assay buffer to the 3.0 mg DTNB and vortex. Leave at room temperature.
2. Add 6 mL of assay buffer to the 3.6 mg $NADPH_2$ and vortex. Leave on ice.
3. Transfer 11 µL of the Glutathione Oxidoreductase to 6 mL of assay buffer and vortex. Leave on ice.

Initiate the assay:

1. Add 50 µL of each GSH standard or diluted or undiluted sample extract to each well of the microtiter plate.
2. Add 50 µL each of DTNB and glutathione oxidoreductase solution to each well. The use of an eight-channel pipettor is recommended.
3. Incubate the plate for 5-10 minutes at room temperature.
4. Add 50 µL of $NADPH_2$ solution to each well to start the reaction.
5. Within 1 minute after the addition of $NADPH_2$, read the plate using a kinetic program which can monitor the reaction at 1 minute intervals for 4 minutes. The rate of color change is monitored in the 405-412 nm (depending on the wavelengths of filters available on your plate reader).

Note: If your plate reader does not have a kinetic reading function, then read the plate and record the OD value at 0 minutes and again at 4 minutes.

Sample Preparation

Cell Cultures in 96 well plates

1. After incubation, the cells (x104) are washed gently with phosphate buffered saline (PBS), pH 7.2.
2. Aspirate the washing solution and add 55-100 µL of 5% MPA to each well.
3. The culture plates are then frozen at -80 ^{o}C and subsequently thawed at 37 ^{o}C. This freeze/thaw cycle should be done twice and then the plate left on ice for 15 minutes.
4. Remove 50 µL of the MPA extract from each well and transfer it to the assay microtiter plate using a multiple-channel pipettor.
5. Transfer all the wells such that the plate configuration of the treated wells will be maintained in the assay microtiter plate. Leave space to run the GSH standard curve.

Calculation of Results

Plot the standard curve with Mean V versus GSH concentrations. The total GSH concentration of the samples can be calculated from the standard curve.

If a kinetic microplate reader is not available, the Mean V (the rate of color change) can be estimated by subtracting the OD value at 0 minutes from the OD value at 4 minutes minus and dividing the result by 4. That is:

Mean V = (OD at 4 minutes - OD at 0 minutes) / 4

The rate of color change represented as Mean V is proportional to the GSH standard concentrations.

Caspase-3 activity

These assays are based on spectrophotometric detection of the chromophore p-nitroaniline (pNA). The pNA is linked to the end of the caspase-specific substrate. Active caspase will cleave the substrate and release free pNA. The absorbance is then measured on a standard microplate reader. To perform the assay, cell pellet ($2x10^6$ cells) is suspended in cold lysis buffer, incubated for 10 minutes, and centrifuge for 1 minute at 10,000 x g. The supernatant is transferred to a fresh microtube and put on ice. The protein concentration of each sample is adjusted to 50-200 µg/50 µL cell lysate. Then, 5 µL of the labeled substrate solution is added and incubated at 37 °C for 2 hr. The enzyme products are measured by reading the absorbance at 405 nm.

Generating a pNA Standard Curve

Prepare a dilution series (1:2 is suggested) of pNA solutions in the concentration range of 10 µM – 1 mM by diluting the provided pNA stock solution in 1X Assay buffer. Add 100 µl of each dilution to a well. Include 100 µL of 1X assay buffer as a blank. Read OD at 405 nm.

Assay Instructions

1. Induce apoptosis in cells by desired method. Concurrently incubate a control culture *without* induction.
2. Count cells and pellet 0.5 – 2 X 10^6 cells (1500 rpm for 10 minutes).
3. Resuspend cells in 50-500 µL of chilled 1X Cell Lysis Buffer. Incubate cells on ice

for 10 minutes. Centrifuge for 5 minutes in a microcentrifuge (10,000 x g).

4. Transfer supernatant (cytosolic extract) to a fresh tube and put on ice. Assay the protein concentration for each sample set if necessary.

Sample	Assay Mixture				Caspase-3 Substrate	Total Volume
	5X Assay Buffer	Caspase-3 Sample	Inhibitor	DI H_2O		
Buffer Blank	20 µL	0 µL	0 µL	80 µL	0 µL	100 µL
Substrate Blank	20 µL	0 µL	0 µL	70 µL	10 µL	100 µL
Test Sample	20 µL	X µL	0 µL	(70-X) µL	10 µL	100 µL
Test Sample + Inhibitor (optional)	20 µL	X µL	Y µL	70-(X+Y) µL	10 µL	100 µL

5. Prepare assay mixture in a 96-well plate or standard microcentrifuge tubes, according to the above table. If using the optional inhibitor, pre-incubate inhibitor with caspase sample for 10 minutes at room temperature before adding caspase-3 substrate solution.
6. Incubate samples for 1-2 hours at 37 °C.
7. Read samples at 405 nm in a microtiter plate reader. Fold-increase in caspase-3 activity can be determined by comparing the OD reading from the induced apoptotic sample with the level of the uninduced control.

Note: Background reading from cell lysates and buffers should be subtracted from the readings of both induced and uninduced samples before calculating fold increase in caspase-3 activity.

Calculation of Results

Optical Density (OD) values obtained with the CHEMICON Caspase-3 Colorimetric Activity Assay Kit may be compared with known standards or other test samples to obtain relative activities.

Nuclear extract preparation

Nuclear and cytoplasmic extracts were prepared as described by Andriollo M et al. with little modification (112). Cells $5x10^6$ cells, treated or not, are washed twice in cold PBS and centrifuged (2 min, 2665 g, 4 °C). The pellet is resuspended in 250 μL of hypotonic buffer A (10 mM Hepes, pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM DTT, 0.2 mM PMSF) and centrifuged (2 min, 2665 g, 4 °C). Then, pelleted cells are resuspended in 300 μL of hypotonic buffer and incubated on ice for 15 min, 40 μL of NP-40 solution is added, and the cells are vigorously mixed for 10 s and centrifuged (30 s, 15,000 g, RT). The cytosolic supernatants are harvested and stored at 80 °C in aliquots for later use. Pelleted nuclei are suspended in 40 ll of hypertonic buffer (10 mM Hepes, pH 7.9, 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, 0.5 mM DTT, 0.2 mM PMSF, 10% (v/v) glycerol), incubated for 30 min on ice, mixed for 10 s, and centrifuged for 10 min at 15,000g at 4 °C. The supernatants that contained nuclear proteins are harvested and stored at 80 °C in aliquots for later use. Protein concentrations are determined with the Bradford protein assay kit (BioRad).

NFκB Activation Assay

Assay Principle:

The Non-Radioactive NFκB p50/p65 Transcription Factor Assay kit is provided in a 96-well format. During the assay, the Capture Probe, a double stranded biotinylated oligonucleotide containing the consensus sequence for NFκB binding (5'-GGGACTTTCC-3'), is mixed with cellular (nuclear) extract in the Transcription Factor Assay Buffer provided. When incubated together, the active form of NFκB contained in the nuclear extract binds to its consensus sequence. After incubation, the extract/probe/buffer mixture is then directly transferred to the streptavidin coated plate. The biotinylated double stranded oligonucleotide bound by active NFκB protein is immobilized and any inactive, unbound material is washed away. The bound NFκB transcription factor subunits, p50 and p65, are detected with specific primary antibodies, a Rabbit anti-NFκB p50 (active form) and a Rabbit anti-NFκB p65. An HRP-conjugated secondary antibody is then used for detection and provides sensitive

colorimetric detection that can be read in a spectrophotometric plate reader. Included in the kit are positive cell extract, a non-specific double stranded oligonucleotide, and a specific competitor double stranded oligonucleotide.

Assay Procedure:

A. Prepare 1x Transcription Factor Assay Buffer (TFA Buffer)

1. Calculate the volume of 1x TFA Buffer required: Each well requires 3 mL of 1x TFA Buffer (e.g. 8 wells require 24 mL of 1x TFA Buffer).
2. Dilute 1 part 5x TFA Buffer into 4 parts distilled water to obtain the desired amount of buffer.

B. Prepare Enhanced Transcription Factor Assay Buffer (Enhanced TFA Buffer)

1. Take a portion of the 1x TFA Buffer prepared in Step A and use to prepare 1x Enhanced TFA Buffer by adding blocking reagent.
2. Calculate the volume of Enhanced TFA Buffer required: Each well requires 1.8 mL 1x TFA Buffer supplemented with 0.06 g Blocking Reagent.
3. Mix Blocking Reagent with 1x TFA Buffer for 15 minutes with gentle agitation. Make sure that the blocking reagent is completely dissolved prior to use.
4. Enhanced TFA Buffer will be used for all steps with the exception of the final wash following secondary antibody incubation. For this wash, use the remaining 1x TFA Buffer prepared in Step A (approximately 1.2 mL per assay).

C. Sample Incubation

1. Thaw nuclear extract and place on ice.
2. Prepare the test sample, negative control sample, positive control sample, or competitive oligonucleotide control sample in a 0.5 mL microcentrifuge tube (1.5 mL microcentrifuge tube or 96-well plate for multiple tests) as described:

	Enhanced Transcription Factor Assay Buffer (1x)	NFκB Capture Probe (90269)	NFκB Competitor Oligonucleotide (90270)	TFA Negative Control Probe (90271)	Nuclear Extract	HeLa Whole Cell Extract (TNFα Treated) (90350)	Total Volume
Transcription Factor Assay (Normal)	44µL	1µL	_	_	5µL	_	50µL
Transcription Factor Assay—Positive Control	44µL	1µL	_	_	_	5µL	50µL
Transcription Factor Assay—Specific Competitor Control	42µL	1µL	2µL	_	5µL	_	50µL
Transcription Factor Assay—Negative Control	44µL	_	_	1µL	5µL	_	50µL

a. Positive Control:

The positive control assay utilizes TNF-α treated HeLa whole cell extract and should be performed to ensure that the assay is performing correctly. Under normal conditions, this assay will result in signal from the sample well(s). It is not necessary to add the two control probes (the NFκB Competitor Oligonucleotide and the TFA Negative Control Probe) to the positive control extract, unless desired.

b. Competitor Control:

The specific NFκB Competitor Control oligonucleotide is provided to ensure that the NFκB complex is binding the probe DNA in a sequence specific manner. It is an unlabeled competitor oligonucleotide containing the identical NFκB consensus sequence as the capture probe. This control will compete with the capture probe for NFκB binding. With a typical nuclear extract, this assay setup should greatly diminish the signal intensity. However, assays may be performed using varying amounts of the NFκB capture probe and/or specific competitor oligonucleotide as desired. Alternatively, the investigator may choose to incorporate other competitor oligonucleotides into the assay.

c. Negative Control:

The TFA Negative Control Probe is used without the addition of the NFκB Capture Probe. The TFA Negative Control Probe is used to ensure that the signal obtained from the normal assay setup is specific to NFκB binding to its

consensus sequence. Any signal obtained with this assay setup will reflect non-specific binding of NFκB to the capture probe.

3. Rotate sample cocktail for 1 hour at room temperature
4. Transfer the entire 50 µL reaction to an individual well on the plate.(or 50 µL per well when running samples in multiplex format.)
5. Incubate for 1 hour at room temperature. Note: While our best results were obtained with an one hour incubation, shorter incubation times may be sufficient. Optimal incubation time should be determined by the end user.
6. Wash wells three times with 150 µL of the 1x Enhanced TFA Buffer. Incubate each wash as follows: 1st wash, 30 seconds; 2nd wash, 2 minutes; 3rd wash, 4 minutes. Completely remove the buffer from the wells following the final wash.

D. Primary Antibody Incubation

1. Dilute primary antibody in 1x Enhanced Transcription Factor Assay Buffer.
   a. Prepare enough for 100 µL of diluted antibody per well tested.
   b. Dilute anti-NFκB p50 1:100 and anti-NFκB p65 1:1000.
2. Add 100 µL of diluted primary antibody to each assay well. Incubate for 60 minutes at room temperature. Note: While our best results were obtained with a one hour incubation, shorter incubation times may be sufficient. Optimal incubation time should be determined by the end user.
3. Wash wells three times with 150 µL of the 1x Enhanced Transcription Factor Assay Buffer (as in step A.6.). Incubate each wash as follows: 1st wash, 30 seconds; 2nd wash, 2 minutes; 3rd wash, 4 minutes. Completely remove the buffer from the wells following the final wash.

E. Secondary Antibody Incubation

1. Dilute the anti-rabbit IgG-HRP conjugated secondary antibody in 1x Enhanced Transcription Factor Assay Buffer at a 1:1000 dilution. Prepare enough for 100 µL of diluted secondary antibody per assay well tested.
2. Add 100 µL of the diluted secondary antibody to each well and incubate at room temperature for 30 minutes. Note: At this time the TMB/E should be removed

from 2-8°C and allowed to equilibrate to room temperature during this 30 minute incubation.

3. Wash wells four times with 250 µL of 1x Transcription Factor Assay Buffer (without blocking reagent) per well. Incubate each wash as follows: 1st wash, 30 seconds; 2nd wash, 2 minutes; 3rd wash, 3 minutes; 4th wash, 4 minutes. Note: Using 1x Enhanced TFA Buffer, which contains Blocking Reagent, in this step will increase background signal.

F. Color Development

1. Add 100 µL of the pre-equilibrated TMB/E substrate to each assay well being tested and incubate at room temperature for approximately 10 minutes. The development time may vary according to laboratory conditions. Therefore, we recommend that you closely monitor the speed at which color is generated during the 10 minute incubation. The assay may need to be stopped prior to 10 minutes to prevent signal saturation. Note: The color development reaction can be carried out at 37°C to increase sensitivity.
2. Add 100 µL of the Stop solution to each well.
3. Measure the absorbance of the samples at 450 nm using a microplate reader with the reference wavelength set to 650 nm.

Calculation of Results

Relative optical density (OD) values obtained using CHEMICON's® Transcription Factor Assay Kit may be compared with known standards or other test samples to obtain relative activities. The following graph represents unstimulated or TNFα-stimulated HeLa nuclear extracts. The data below is for reference use only and the data should not be used to interpret actual assay results.

Statistical analysis

Experiments were performed in duplicate or triplicate, and at least three separate assays. The data are presented as mean ± SEM. Statistical analyses among groups were tested by analysis of variance with Bonferroni post hoc comparison. Probability values of < 0.05 is considered to be significant.

CHAPTER IV

RESULTS

Total antioxidant power of *P. urinaria*. The characteristics of standard curve ($FeSO_4$) and pure antioxidants used in the FRAP assay was similar to previous study (107) (Figure 23). Generally, aqueous standard solutions of Fe (II) were used in the rage of 100-1000 μM. Reaction of Fe (II) represents a one electron exchange reaction and is taken as unity, i.e. the blank corrected signal given by 100 μM solution of Fe (II) is equivalent to a FRAP value of 100 μM. Ascorbic acid (VIT C) has a constant stoichiometric factor of 2.0 in the FRAP assay, i.e., direct reaction of Fe (II) gives a change in absorbance half that of an equivalent molar concentration of ascorbic acid. An ascorbic acid standard of 100 μM, therefore, is equivalent to 200 μM of antioxidant power as FRAP value.

The total antioxidant capacity of the *P. urinaria* (1 mg/mL) is presented as 5,306.75$\pm$461.62 FRAP value (μM). The FRAP value of 1 mg/mL plant extract was calculated as 0.455 mg/mL ascorbic acid (MW 176.1), based on the constant stoichiometric factor of 2.0 in the FRAP assay (107).

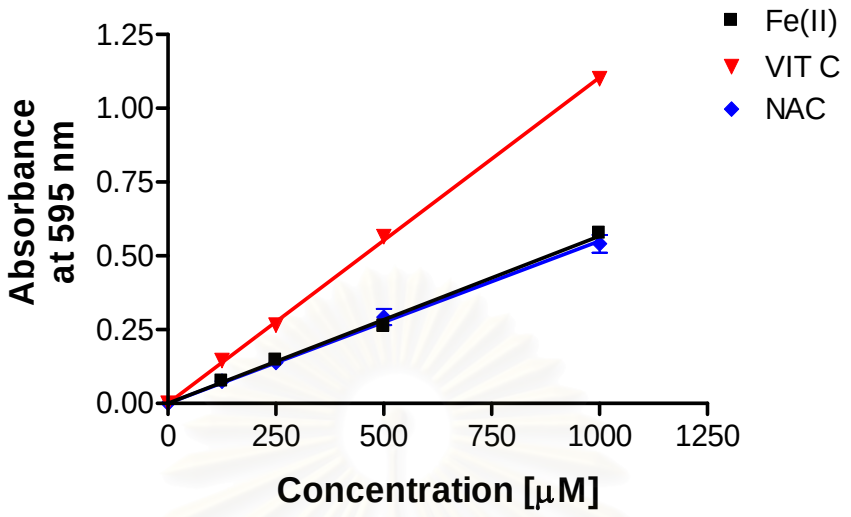
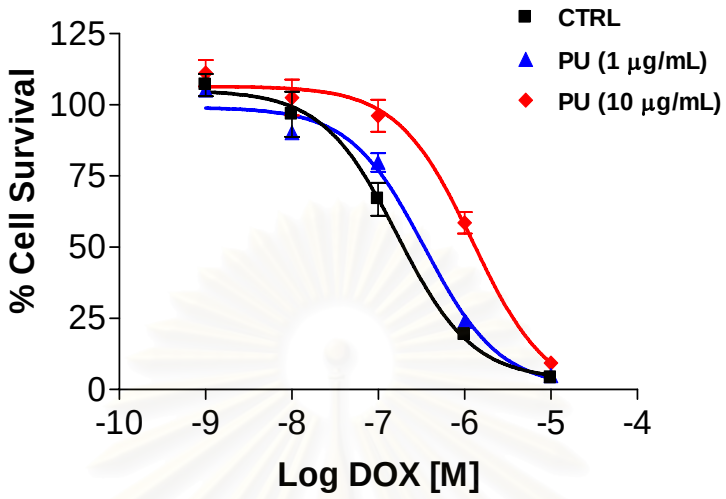


Figure 23. Linearity of FRAP assay. Concentration-response regression line of pure antioxidant solutions; Fe (II), ascorbic acid (VIT C), and N-acetylcysteine (NAC).

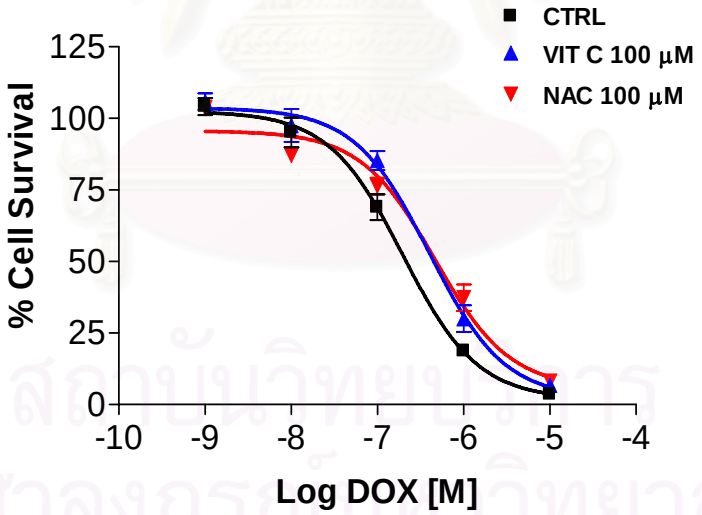
P. urinaria increased IC50 of DOX

Our preliminary studies have shown that *P. urinaria* dose-dependently caused cytotoxicity, i.e. 50-60% cell survival was observed in cells incubated with 100 µg/mL plant extract (data not shown). Therefore, in our study, we selected the concentrations 1 and 10 µg/mL where more than 80% cell survival was observed. Demonstrated in (Figure 24A-B) are the cardioprotective effects of the antioxidants *P. urinaria*, ascorbic acid, and NAC. The DOX IC50s of vehicle treated cells (CTRL), *P. urinaria* 1 and 10 µg/mL, VIT C (100 µM), and NAC (100 µM) were 0.171±0.014, 0.0481±0.068, 1.45±0.224, 0.569±0.034, and 0.711±0.027, respectively. *P. urinaria* dose-dependently protected against DOX cardiotoxicity. The significant rightward shifts of DOX IC50 observed in *P. urinaria* (10 µg/mL) treatments were higher (8.5-fold) than that were observed in VIT C and NAC treatments (100 µM) (3.3-fold and 4.2-fold, respectively). The morphological changes were also observed in cell treated with DOX in the presence and absence of *P. urinaria* extract (Figure 24C-F)

A)



B)



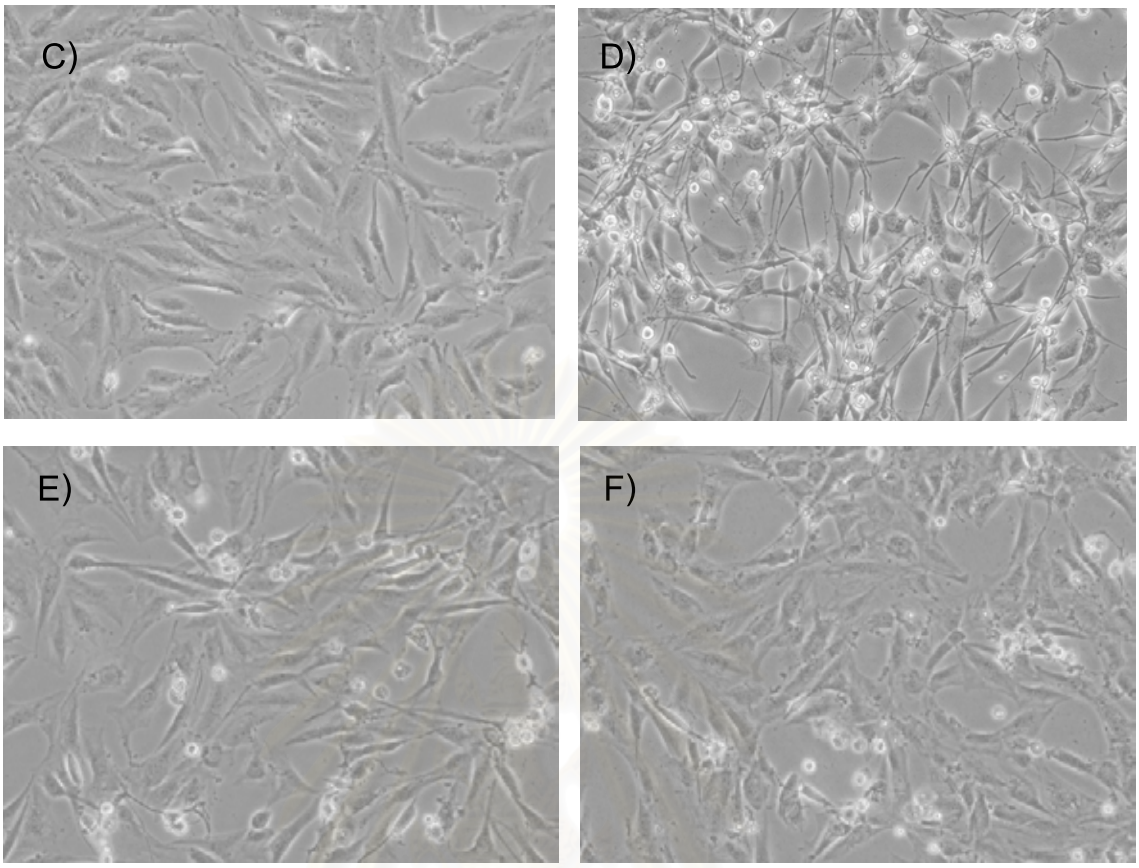


Figure 24. Cardioprotective effects of *P. urinaria* extract (PU), ascorbic acid (VIT C), and N-acetylcysteine (NAC) on DOX-induced cardiotoxicity (H9c2). The DOX IC50s were obtained from **A)** H9c2 cells were co-incubated with cumulative doses of DOX (10^{-9} to 10^{-5} M) and PU (1 or 10 µg/mL), and **B)** co-incubation with VIT C (100 µM) or NAC (100 µM). The morphological illustrations of H9c2 are shown in **C)** vehicle treated cells; **D)** 1 µM DOX; **E)** 1 µM DOX + 1 µg/mL PU; and **F)** 1 µM DOX + 10 µg/mL PU.

P. urinaria increased tGSH content

tGSH was determined to evaluate the effect of exogenous antioxidants on the cellular GSH reservior. Linearity of GSH standard curve was obtained (Figure 25). *P. urinaria* treatment alone (1 and 10 µg/mL) dose-dependently increased tGSH in the cardiac myoblasts 2.13- and 2.69-fold, respectively (Table 4, Figure 26) while VIT C and NAC at 100 µM had no significant effect. DOX (1 µM) markedly decreased tGSH content down to 0.05 fold of control. VIT C but not NAC significantly reversed DOX effect by restoring the amount of tGSH to the level that comparable to CTRL. Despite DOX was present in the culture medium, *P. urinaria* (1 and 10 µg/mL) markedly increased tGSH levels to 1.65 and 2.44-fold, respectively, compared to CTRL ($p<0.001$).

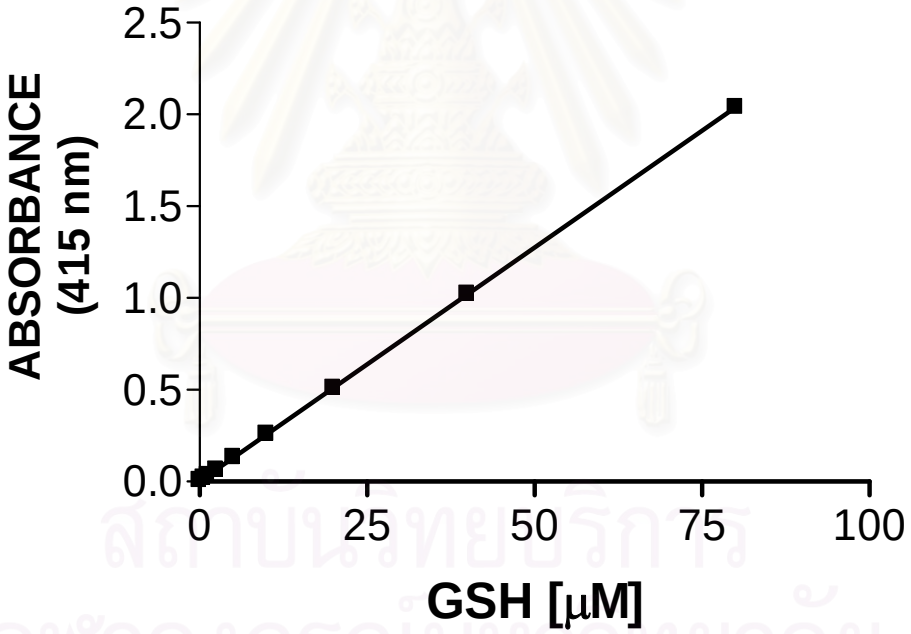


Figure 25. Glutathione standard curve.

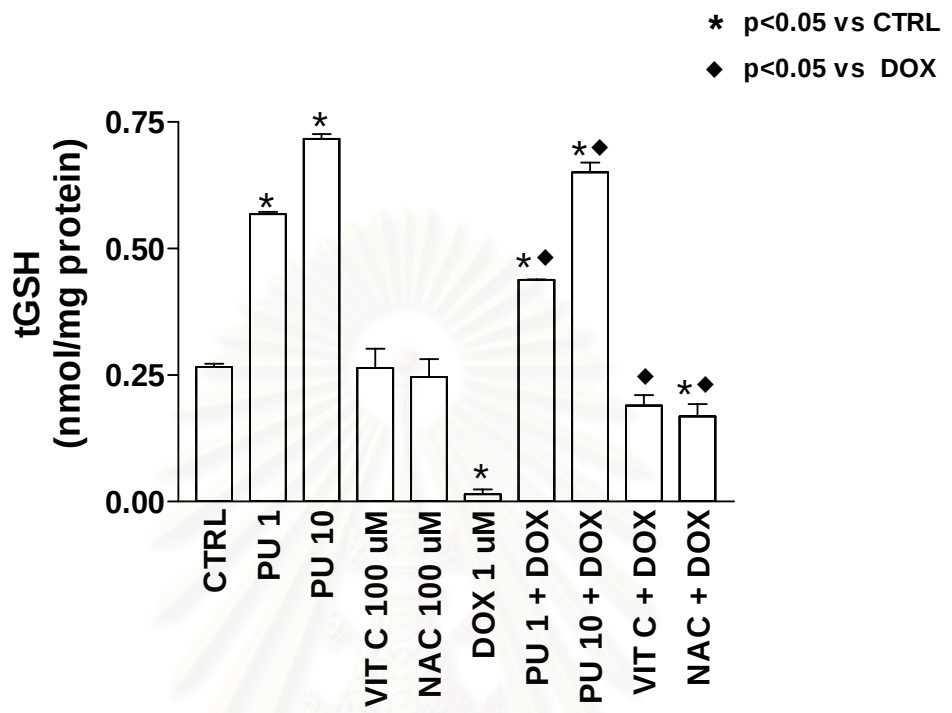


Figure 26. Effect of *P. urinaria* extract on total glutathione (tGSH) levels. H9c2 cells were treated with vehicle control (CTRL), *P. urinaria* extract (1 and 10 µg/mL, respectively), ascorbic acid (100 µM, VIT C), N-acetylcysteine (100 µM, NAC), doxorubicin (1 µM, DOX), or the combinations of *P. urinaria* extract, ascorbic acid, and N-acetylcysteine (PU 1 + DOX, PU 10 + DOX, VIT C + DOX, NAC + DOX, respectively). Following 48 hr incubations total glutathione (tGSH) was measured in H9c2 cell lysate as described in Materials and Methods. * significantly different from CTRL; ◆ significantly different from DOX.

***P. urinaria* increased SOD activity.**

The measurement of SOD activity is based on the generation of superoxide anion by PMS (phenazine methosulfate). NBT serves as a detector molecule for superoxide generation through a reduction to a stable formazan product that can be monitored at 450 nm. NBT reduction was linear throughout the 5-min incubation (Figure 27). In the presence of SOD, the formazan product is decreased according to SOD concentration. Superoxide generation DOX significantly decreased the endogenous antioxidant enzyme activities SOD and catalase. Treatment of each of the antioxidant *P. urinaria*, VIT C, or NAC alone significantly enhanced SOD activity ($p<0.001$), and this effect was sustained even in the presence of DOX except for NAC (Figure 28, Table 4).

P. urinaria increased catalase activity both in the presence and absence of DOX. In contrast, VIT C and NAC did not significantly reverse DOX effect.

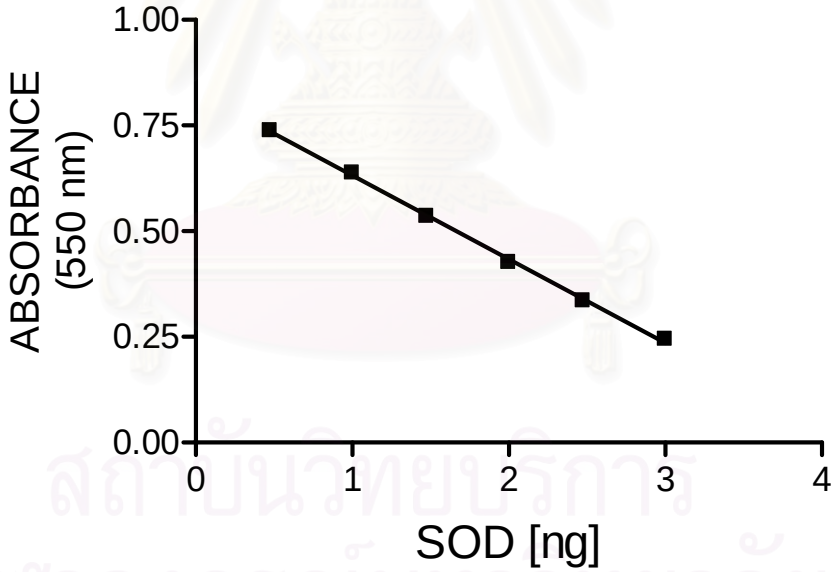


Figure 27. SOD standard curve.

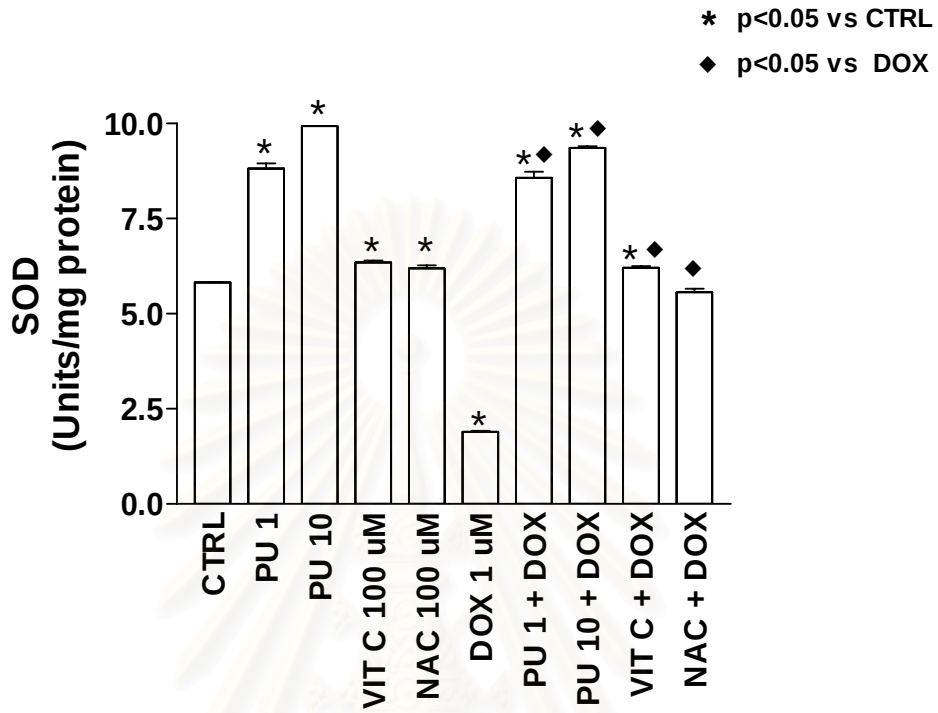


Figure 28. Effect of *P. urinaria* extract on superoxide dismutase (SOD) activity. H9c2 cells were treated with vehicle control (CTRL), *P. urinaria* extract (1 and 10 µg/mL, respectively), ascorbic acid (100 µM, VIT C), N-acetylcysteine (100 µM, NAC), doxorubicin (1 µM, DOX), or the combinations of *P. urinaria* extract, ascorbic acid, and N-acetylcysteine (PU 1 + DOX, PU 10 + DOX, VIT C + DOX, NAC + DOX, respectively). Following 48 hr incubations SOD activity was measured in H9c2 cell lysate as described in Materials and Methods. * significantly different from CTRL; ◆ significantly different from DOX.

P. urinaria increased CAT activity

The catalase assay in this study is based on its peroxidatic acitivity that converts methanol in the presence of an optimal concentration of H_2O_2 to form formaldehyde. The formaldehyde produced was measured according to the generation of Purpald at 550 nm. The linear regression equation of formaldehyde standard was used for calculation of CAT activity (Figure 29).

Shown in Figure 30 and Table 4 are the effect of *P. urinaria*, VIT C, and NAC on lipid peroxidation of cardiac myoblasts. Incubation of H9c2 with each antioxidant alone did not change basal level of the lipid peroxide derivative MDA. In contrast, DOX (1 μM) treatment significantly elevated MDA content 2.9-fold compared to CTRL ($p<0.001$). VIT C and NAC diminished DOX-induced lipid peroxidation down to the basal level. Interestingly, *P. urinaria* extract dose-dependently decreased lipid peroxidation to the level that was less than CTRL ($p<0.001$).

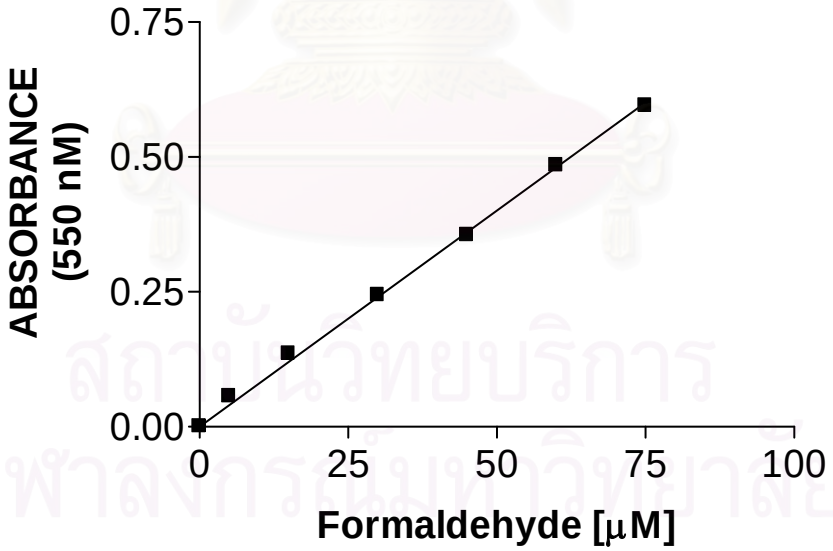


Figure 29. Formaldehyde standard curve.

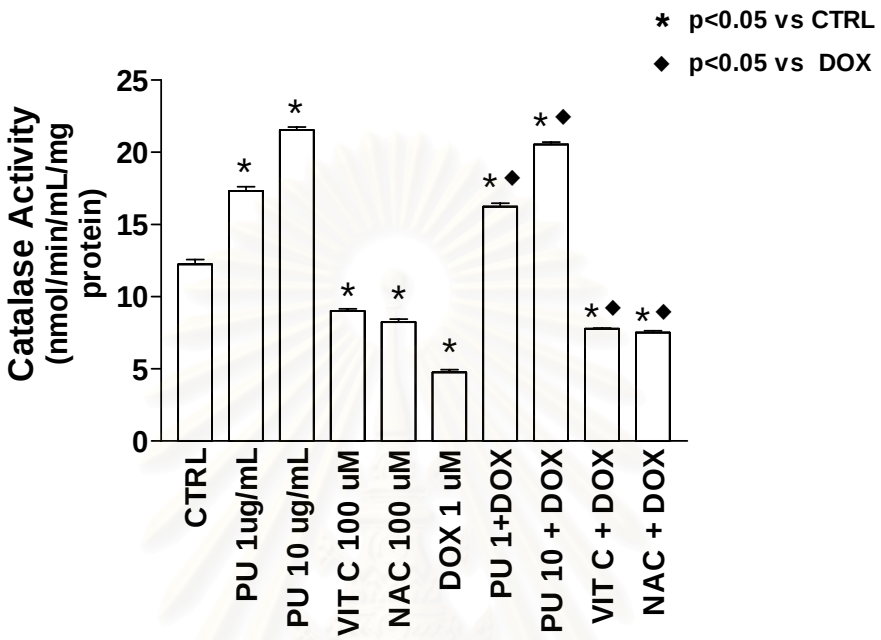


Figure 30. Effect of *P. urinaria* extract on catalase activity. H9c2 cells were treated with vehicle control (CTRL), *P. urinaria* extract (1 and 10 µg/mL respectively), ascorbic acid (100 µM, VIT C), N-acetylcysteine (100 µM, NAC), doxorubicin (1 µM, DOX), or the combinations of *P. urinaria* extract, ascorbic acid, and N-acetylcysteine (PU 1 + DOX, PU 10 + DOX, VIT C + DOX, NAC + DOX, respectively). Following 48 hr incubations catalase activity was measured in H9c2 cell lysate as described in Materials and Methods. * significantly different from CTRL; ◆ significantly different from DOX.

Inhibition of lipid peroxidation by *P. urinaria* extract

Malondialdehyde (MDA) is the stable product of lipid peroxidation. Standard curve of MDA was generated (Figure 31) to measure the extent of lipid peroxidation in H9c2 treated with DOX and antioxidants. (Figure 32 and Table 4) demonstrate the effect of *P. urinaria*, VIT C, and NAC on lipid peroxidation of cardiac myoblasts. Incubation of H9c2 with each antioxidant alone did not change basal level of the lipid peroxide derivative MDA. In contrast, DOX (1 μM) treatment significantly elevated MDA content 2.9-fold compared to CTRL ($p<0.001$). VIT C and NAC diminished DOX-induced lipid peroxidation down to the basal level. Interestingly, *P. urinaria* extract dose-dependently decreased lipid peroxidation to the level that was less than CTRL ($p<0.001$).

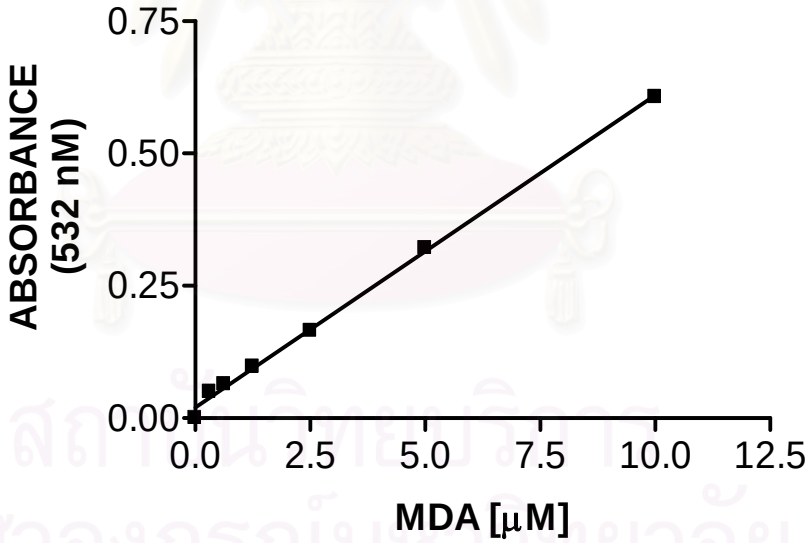


Figure 31. Malondialdehyde (MDA) standard curve.

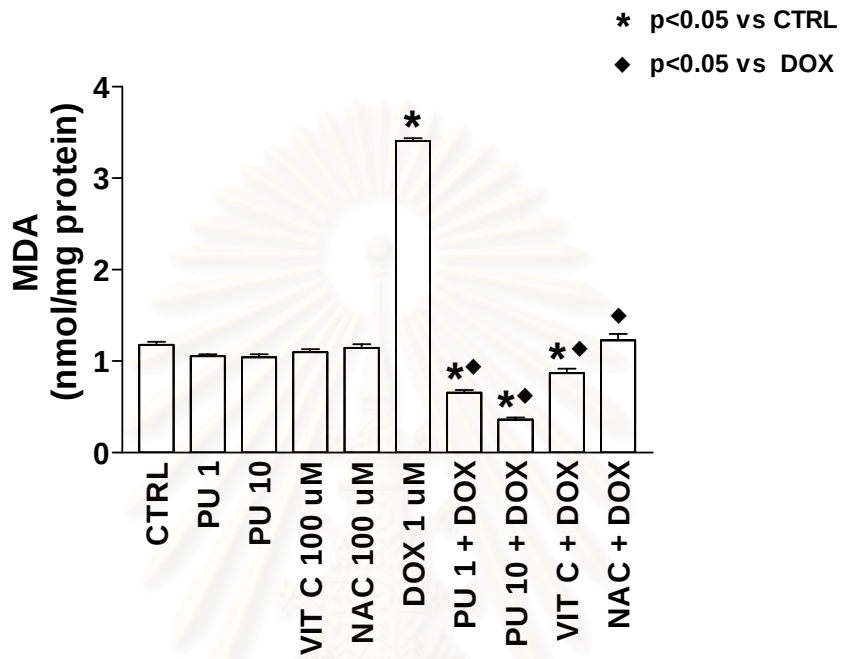


Figure 32. Inhibition of doxorubicin (DOX)-induced lipid peroxidation by *P. urinaria* extract (PU), ascorbic acid (VIT C), and N-acetylcysteine (NAC). H9c2 cells were incubated for 48 hr with a variety of treatments, i.e., CTRL (vehicle), DOX (1 μM); PU (1 or 10 μg/mL respectively), ascorbic acid (100 μM, VIT C), NAC (100 μM), or combinations of DOX and PU; VIT C or NAC (PU 1 + DOX, PU 10 + DOX, VIT C + DOX, NAC + DOX, respectively). The extent of lipid peroxidation was evaluated as described in Materials and Methods. * Significantly different from CTRL; ◆ Significantly different from DOX.

Reduction of caspase-3 activity by *P. urinaria* extract

One method to determine cardiac apoptosis is to evaluate activity of the key enzyme caspase-3. pNA is the product of caspase-3 cleavage and thus reflecting enzyme activity (Figure 33). DOX (1 μM) treatment significantly increased caspase-3 activity (2.3-fold). In the presence of DOX, VIT C and NAC significantly decreased the enzyme activity to the level that slightly higher but not significantly different from CTRL. On the other hand, H9c2 incubation with *P. urinaria* alone decreased the enzyme activity ($p<0.05$) and this effect was sustained even in the presence of DOX (Figure 34 and Table 4).

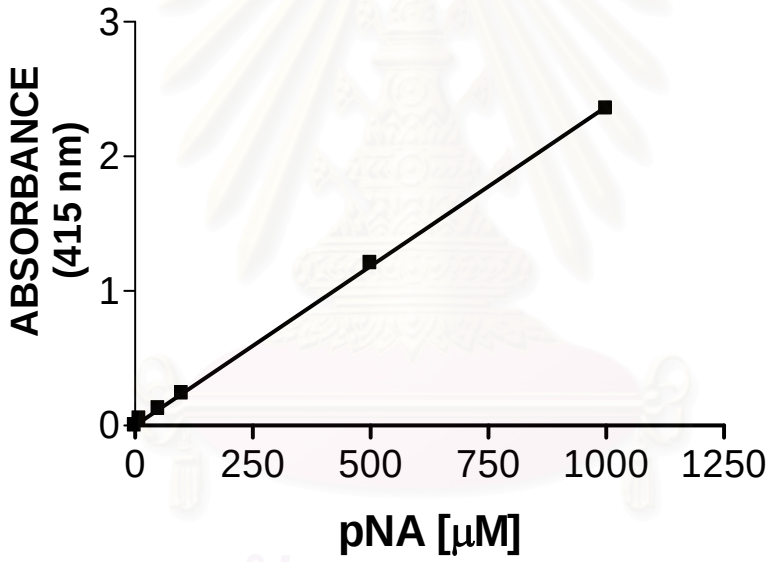


Figure 33. Standard curve of p-nitroaniline (pNA).

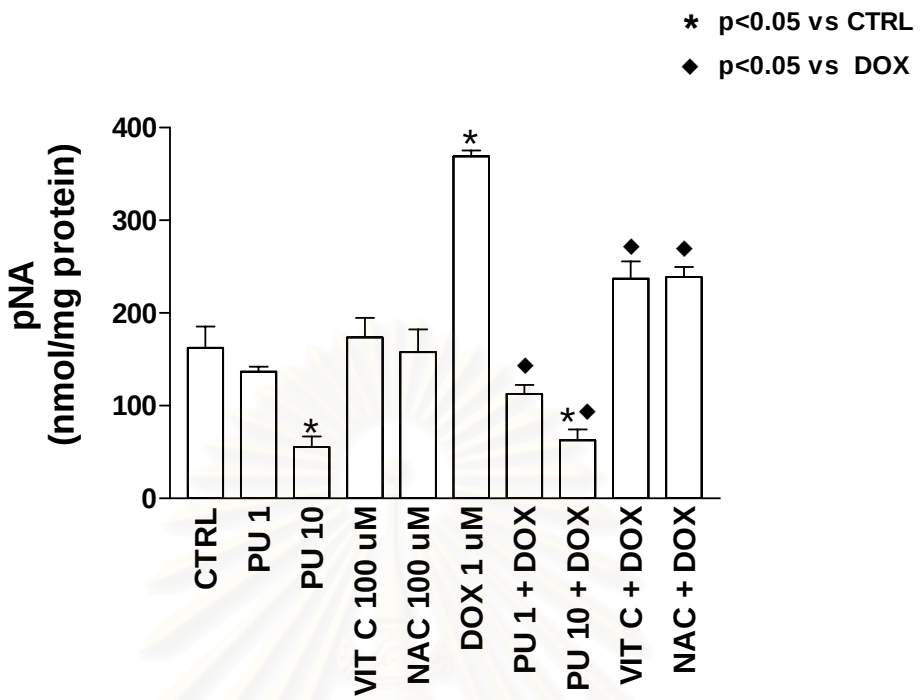


Figure 34. Effect of *P. urinaria* extract (PU), ascorbic acid (VIT C), and N-acetylcysteine (NAC) on caspase-3 activity. H9c2 cells were incubated for 48 hr with vehicle (CTRL), DOX (1 μM); PU (1 or 10 μg/mL, respectively), ascorbic acid (100 μM, VIT C), NAC (100 μM), or combinations of DOX and PU, VIT C or NAC (PU 1 + DOX, PU 10 + DOX, VIT C + DOX, NAC + DOX, respectively). Caspase-3 activity was measured as the amount of pNA generated (nmole/mg protein). * Significantly different from CTRL; ◆ Significantly different from DOX.

Antioxidants inhibit DOX-induced NFκB activation

Rel or NFκB proteins are eukaryotic transcription factors involving in the control of a large number of normal cellular processes, such as immune and inflammatory responses, developmental processes, cellular growth, and apoptosis. NFκB commonly refers to a p50/p65(RelA). The p50/p65 heterodimers are the most abundant dimers found in the NFκB signaling pathway. The activity of NFκB is tightly regulated by interaction with inhibitory IκB proteins. Upon activation by multiple inducers IκBk is rapidly phosphorylated and degraded, resulting in the release of the active NFκB complex. To investigate the effect of *P. urinaria* on DOX-induced NFκB activation, we employed the p50/p65 NFκB transcription factor assay (CHEMICON®) that detected the active NFκB. The amount of NFκB in antioxidants treated cells was compared relatively to vehicle treated cells.

DOX (1 μM) significantly activated NFκB by 1.85 fold ($p<0.05$). Treatments with the antioxidants *P. urinaria* (1 and 10 μg/mL), VIT C (100 μM), or NAC (100 μM) alone did not significantly change NFκB activity. As shown in Figure 35, DOX-induced NFκB activation was abolished by all antioxidants. These results along with caspase-3 activity assay recapitulate that NFκB activation involves in DOX-induced apoptosis.

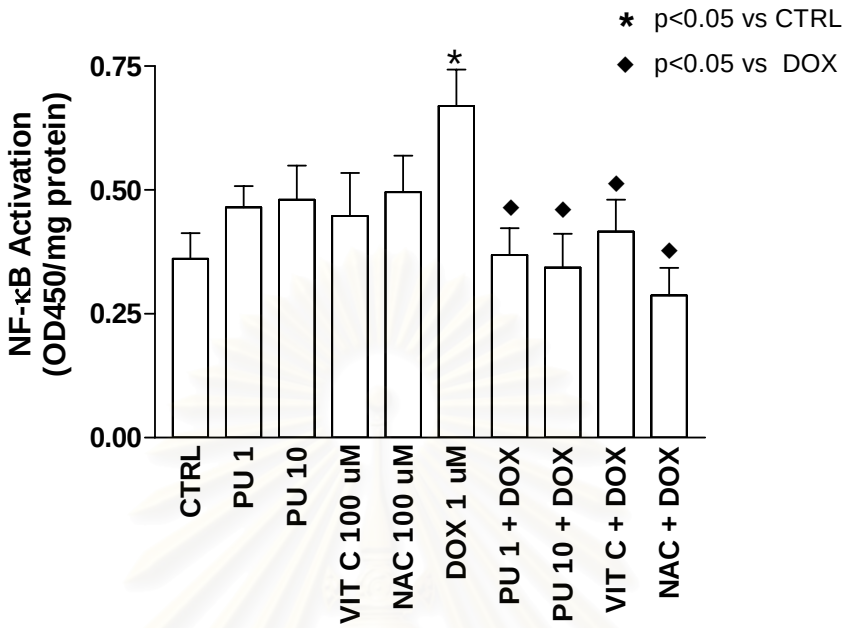


Figure 35. Inhibition of DOX-induced NFκB by antioxidants. H9c2 cells were incubated for 48 hr with a different treatments, i.e., CTRL (vehicle), DOX (1 μM); PU (1 or 10 μg/mL respectively), ascorbic acid (100 μM, VIT C), NAC (100 μM), or combinations of DOX and PU; VIT C or NAC (PU 1 + DOX, PU 10 + DOX, VIT C + DOX, NAC + DOX, respectively). The amount of active NFκB (p50/p65) in treated cells was presented as the absorbance of enzyme product based on enzyme-linked immunosorbent assay as described in Materials and Methods. * Significantly different from CTRL; ◆ Significantly different from DOX.

Table 4. Cardioprotective effect of ascorbic acid, N-acetylcysteine, and *P. urinaria* extract.

Treatment	MDA (nmol/mg protein) (n = 5)	tGSH (nmol/mg protein) (n = 5)	SOD activity (units/mg protein) (n=3-5)	Catalase activity (nmol/min/mL/mg protein) (n=6)	Caspase-3 activity (% Control) (n=4-8)
Vehicle (CTRL)	1.179 ± 0.034	0.266 ± 0.006	5.817 ± 0.014	11.854 ± 0.471	100.00 ± 14.29
P. urinaria 1 µg/mL (PU 1)	1.054 ± 0.021	0.568 ± 0.005*	8.816 ± 0.128*	16.211 ± 1.141*	84.19 ± 3.34
P. urinaria 10 µg/mL (PU 10)	1.043 ± 0.032	0.716 ± 0.010*	9.930 ± 0.001*	20.082 ± 1.465*	34.25 ± 7.00*
Ascorbic acid 100 µM (VIT C)	1.097 ± 0.034	0.264 ± 0.038	6.343 ± 0.056*	8.733 ± 0.302*	107.02 ± 13.01
N-acetylcysteine 100 µM (NAC)	1.146 ± 0.038	0.246 ± 0.035	6.188 ± 0.086*	7.943 ± 0.348*	97.26 ± 15.13
Doxorubicin 1 µM (DOX)	3.407 ± 0.031*♦	0.015 ± 0.009*	1.898 ± 0.023*	4.840 ± 0.177*	227.30 ± 3.88*
DOX + PU 1	0.655 ± 0.030*♦	0.438 ± 0.002*♦	8.571 ± 0.160*♦	15.246 ± 1.009*♦	69.47 ± 6.00♦
DOX + PU 10	0.362 ± 0.025*♦	0.650 ± 0.019*♦	9.357 ± 0.037*♦	19.156 ± 1.388*♦	38.78 ± 7.17*♦
DOX + VIT C	0.869 ± 0.049*♦	0.190 ± 0.021♦	6.205 ± 0.041*♦	7.690 ± 0.097*♦	146.01 ± 11.61♦
DOX + NAC	1.228 ± 0.069♦	0.169 ± 0.024*♦	5.560 ± 0.091♦	7.265 ± 0.259*♦	147.09 ± 6.80♦

Data are presented as Mean±SEM. * Statistically significant difference from Control (CTRL) ($p<0.05$); ♦ Statistically significant difference from doxorubicin (DOX) ($p<0.05$)

CHAPTER V

DISCUSSION AND CONCLUSION

Medicinal plants have long been valued as sources of new compounds with antioxidant activity. Our study has demonstrated that *P. urinaria* possessed high antioxidant capacity relative to other plants with known antioxidant activity, such as *Curcuma longa* and *Morus alba* (113). *P. urinaria* efficiently protected against DOX cardiotoxicity and the rightward shifts of DOX IC50s were more remarkable than that obtained from pure antioxidants at the concentrations that produced their maximal protective effects in this setting, i.e., ascorbic acid at 100 μM and NAC at 100 μM (113).

While most recent studies have focused on anticancer activity of *P. urinaria* we are the first who investigated the cardioprotective effect of *P. urinaria* in a hope to concurrently observe cardioprotective effect and synergistic antitumor effect with the present anticancer drug DOX. It is well established that significant role of antioxidants in protecting cells from ROS damage includes exogenous antioxidant activity and the ability to activate or enhance endogenous antioxidant defense.

Anthracyclines rank among the most effective anticancer drugs ever developed. The first anthracyclines were isolated from the pigment-producing *Streptomyces peucetius* early in the 1960s and were named DOX and daunorubicin (DNR). Whereas DOX is an essential component of treatment of breast cancer, childhood solid tumors, soft tissue sarcomas, and aggressive lymphomas, DNR shows activity in acute lymphoblastic or myeloblastic leukemias. As with any other anticancer agent, however, the clinical use of both DOX and DNR soon proved to be hampered by such serious problems as the development of resistance in tumor cells or toxicity in healthy tissues, most notably in the form of chronic cardiomyopathy and congestive heart failure (CHF). To avoid the latter, the maximum recommended cumulative doses of DNR and DOX were tentatively set at 500 or 450 to 600 mg/m^2, respectively.

The last two decades numerous attempts has placed on the search for a "better anthracycline" and resulted in more than 2000 analogs. Yet only few analogs have reached the stage of clinical development and approval; among them, epirubicin (EPI)

and idarubicin (IDA) are popularly used as alternatives to DOX or DNR, respectively. Nontheless, a significantly increasing risk of CHF was documented in patients who received cumulative doses of EPI greater than 950 mg/m^2, and the recommended maximum cumulative dose of EPI was cautiously adjusted to 900 mg/m^2. Thus, replacing DOX with EPI does not eliminate the risk of developing chronic cardiotoxicity. Only a few more anthracyclines have attained clinical approval; these include pirarubicin, aclacinomycin A (aclarubicin), and mitoxantrone (a substituted aglyconic anthraquinone). Both pirarubicin and aclarubicin demonstrate only modest improvements over DOX and DNR in terms of drug resistance. Pirarubicin, a 4-tetrahydropyranyl doxorubicin, has been reported to induce much less cardiotoxicity than DOX in animal models, but studies in women with metastatic breast cancer have indicated that it may cause significant decrease in LVEF or full-blown CHF at cumulative doses of 460 mg/m^2 or >500 mg/m^2, respectively. In elderly patients with non-Hodgkin's lymphoma, pirarubicin may cause severe cardiac dysfunction at cumulative doses as low as 360 mg/m^2. Aclarubicin, a trisaccharide anthracycline, was shown to be active and cardiac-tolerable in adult patients with acute myeloblastic leukemia. However, aclarubicin induced late cardiac events in a phase II study of adult patients with refractory acute myelogenous or lymphoblastic leukemia and proved to be inactive in women with metastatic breast cancer (114).

DOX currently remains extensively used in clinic practice because of its efficiency in tumor suppression. Many physicians continue to use Adriamycin despite the toxicities because it is one of the most effective treatments. Despite extensive clinical utilization, the mechanisms of action of anthracyclines in cancer cells remain a matter of controversy. In a seminal commentary the following mechanisms were considered: 1) intercalation into DNA, leading to inhibited synthesis of macromolecules; 2) generation of free radicals, leading to DNA damage or lipid peroxidation; 3) DNA binding and alkylation; 4) DNA cross-linking; 5) interference with DNA unwinding or DNA strand separation and helicase activity; 6) direct membrane effects; 7) initiation of DNA damage via inhibition of topoisomerase II; and 8) induction of apoptosis in response to topoisomerase II inhibition.

Cardioprotective effect of *P. urinaria* was evaluated by a significant increased in DOX IC50, inhibition of lipid peroxidation and abrogation of caspase-3 activation induced by DOX. It is well documented that oxidative stress commonly leads to accumulation of the key cytotoxic lipid peroxide MDA. The cytoprotective effect of antioxidants against DOX usually associated with inhibition of lipid peroxidation, regardless of types and structures of the antioxidants (115-119). Although the identification of active antioxidants in *P. urinaria* extract is still awaited, this predominant suppression of lipid peroxide formation is warranted further investigations.

In addition to inhibition of lipid peroxidation, cytoprotective effect of *P. urinaria* as well as VIT C and NAC may occur in part through the inhibition of DOX-induced caspase-3 activation. Caspase-3 has been identified as a key mediator of apoptosis in mammalian cells. Recent study revealed that activation of caspase-3 was crucial in DOX-induced apoptosis in non-tumor cells. Caspase-3 activation was preceded other apoptotic mediators such as p53 in DOX-induced apoptosis in endothelial and cardiac cells and the opposite observation was found in tumor cells (120). Furthermore, suppression of DOX-induced p53 activation by a p53 inhibitor prevented apoptosis only in tumor cell lines but not in endothelial cells and cardiomyocytes. In this study, *P. urinaria* alone at 10 µg/mL remarkably suppressed caspase-3 activity to the levels that lower than vehicle treated cells, and this effect remained even in the presence of DOX treatment. In contrast, this effect was not observed in VIT C and NAC treatment. This data suggest that markedly suppression of caspase-3 activity may contribute to the superior cytoprotective effect of *P. urinaria* over VIT C (100 µM) and NAC (100 µM) in this setting.

Major important intracellular antioxidant defenses are total reduced glutathione pool, and reactive oxygen species (ROS) scavenging enzymes such as catalase and SOD. Increased or activation of these endogenous antioxidants or enzymes have shown to protect cells against oxidative damage. For instance, the chemoprotective agent, 3 H -1,2-dithiole-3-thione protected against DOX-mediated injury in cardiac cells by induction of a battery of cellular antioxidants, including reduced glutathione (GSH), GSH peroxidase, GSSG reductase, GSH S-transferase (GST) and catalase (121). Cardioprotective effect was also found in the overexpressions of GST, CuZn-SOD /Mn-

SOD and catalase in both in vitro cardiac gene transfer and in transgenic mice models (122-124). It is well established that there are two major pathways that DOX generates ROS. First, the enzymatic pathway utilizes NADPH-dependent reductase activity to generate superoxide radicals through DOX semiquinone intermediate. Second, the non-enzymatic pathway generates H_2O_2 via the reaction of DOX with Fe^{3+} to form DOX-Fe^{2+} intermediate. Thus, the putative major antioxidant enzymes against oxidative species generated in this system are SOD and catalase, respectively.

Interestingly, *P. urinaria* alone dose-dependently increased tGSH, catalase and SOD activity to the levels that substantially higher than the control and these effects were retained in the presence of DOX. On the other hand, the antioxidant VIT C and NAC at the dose given did not have similar profile of tGSH enhancement and catalase/SOD enzyme activation. NAC is a thiol antioxidant and cysteine source for GSH synthesis. NAC appears to enhance the synthesis of GSH under conditions when the demand for GSH is increased. However, in the absence of increased stress on the glutathione pools, NAC might have no effect on GSH levels (98). However, in multidrug resistance-associated protein 1 (MRP1)-mediated drug resistance (MDR) against DOX, glutathione is the most abundant nonprotein that occurs intracellularly among thiol-containing molecules. Akan et al. (125) demonstrated in human embryonic kidney cells transfected with a plasmid encoding the whole MRP1 gene that N-acetylcysteine increased the resistance against doxorubicin. DL-buthionine (S,R)-sulfoximine decreased NAC-enhanced MRP1-mediated doxorubicin resistance, indicating that induction of MRP1-mediated doxorubicin resistance depends on GSH synthesis. Doxorubicin decreased the cellular GSH concentration and increased GPx activity. Glutathione S-transferase activity was decreased by NAC.

In this study, we found that VIT C and NAC at 100 μM suppressed CAT activity. Similar observation was reported in 3T6 cell culture experiments that the higher the concentration of ascorbate, the greater the decrease in catalase activity (126). Our results suggest that *P. urinaria* exert a better cytoprotective effect (higher degree of DOX IC50 rightward shifts) than the pure antioxidants may partially due to these particular properties.

Nevertheless, it can not be excluded that the cytoprotective effect of *P. urinaria* may be mediated in part by activation of NF-E2-related factor 2 (Nrf2), a redox-regulated transcription factor that binds to the antioxidant response element (ARE). ARE is a cis-acting enhancer sequence that regulates transcriptional activation of stress-induced genes, including SOD and phase II detoxifying enzymes such as glutathione S-transferase (GST) and UDP-glucuronyltransferase (127, 128). It has been shown that avicins – triterpenoid electrophiles isolated from Australian desert tree, *Acacia victoriae* – activated the translocation of Nrf2 into the nucleus and a time-dependent increase in ARE activity. These properties were sensitive to DTT, suggesting that avicins affect one or more critical cysteine residues, probably on the Keap1 molecule. Downstream of ARE, an activation of a battery of stress-induced proteins occurred. The implications of these findings were evaluated in vivo in mouse skin exposed to an ancient stressor, UV light. Avicins inhibited epidermal hyperplasia, reduced p53 mutation, enhanced apoptosis, decreased generation of 8-hydroxy-2'-deoxyguanosine, and enhanced expression of NADPH:quinone oxidoreductase 1 and heme oxygenase-1 (129).

NF-κB is a collective name for the complexes formed by the multigene NF-κB-Rel family. In mammalian cells, there are five NF-κB subunits RelA(p65), RelB, c-Rel, p105–p50 (NF-κB1) and p100–p52 (NF-κB2 or lyt-10). All contain an ~300-amino-acid region of extensive homology in their N-termini, termed the Rel homology domain (RHD), which mediates their DNA-binding and dimerization. Most combinations of homo- and heterodimers are possible. RelA(p65), RelB and c-Rel contain nonhomologous transactivation domains in their C-termini. p105 and p100 require proteolytic processing to generate their active nuclear-DNA-binding forms – p50 and p52. Typically, in unstimulated normal cells, NF-κB subunits are held in an inactive cytoplasmic form bound to a member of IκB protein family – IκB α, β or ε. The unprocessed forms of p100 and p105, which contain ankyrin repeats in their C-termini, similar to those seen in the IκBs, can also function as IκB-like proteins, retaining their dimeric NF-κB partners in the cytoplasm of the cell. The Bcl-3 protein has the appearance of an IκB but is actually a transcriptional coactivator for p52 and p50 (130).

The suggestion that the members of NF-κB family might behave as tumorigenic transcription factors was first put forward upon the cloning of the p50–p105 (NF-κB1) subunit. Analysis of the NF-κB1 sequence immediately revealed homology to v-rel, a potent transforming oncogene of the avian reticuloendotheliosis virus, and its cellular counterpart the protooncogene c-rel. Further support for a role of NF-κB subunits in cancer came with the discovery of the gene encoding p52–p100 (NF-κB2) – in some B- and T-cell lymphomas the gene encoding p100 is subject to rearrangement, resulting in truncated and constitutively nuclear forms of the protein. Furthermore, Bcl-3, a coactivator of the p50 and p52 subunits of NF-κB, is also a protooncogene and was originally identified as part of a translocation event in chronic lymphocytic leukemia. Loss-of-function mutations in IκB proteins, which result in constitutively active NF-κB, have also been found in cases of Hodgkin's lymphoma. Furthermore, rearrangements, amplifications and missense mutations have been reported for NF-κB subunits in both solid and hematopoietic tumors. Despite these findings, genetic alterations in NF-κB and IκB subunits are relatively rare phenomena, especially when compared with those in well-known oncogenes and tumor suppressors such as c-Myc, Ras and p53. What has emerged is that aberrant activation and nuclear localization of NF-κB in cancer is actually quite frequent but most often results from defects in the pathways regulating NF-κB.

DNA damage, oncogene activation and cellular stress are well-established activators of the p53 tumor suppressor; however, they also induce DNA-binding and transcriptional activity of NF-κB. Activation of p53 can result in either cell-cycle arrest or induction of apoptosis, whereas as discussed above, the induction of NF-κB is generally associated with resistance to apoptosis and proliferation. This suggested that mechanisms must exist within the cell to integrate the activities of these two divergent pathways. Consistent with this hypothesis, my laboratory reported that p53 and the RelA(p65) subunit of NF-κB could mutually inhibit the transcriptional activity of each other; similar results were reported by the laboratories of Bedi and Collins. In addition, a more recent study from the Culmsee laboratory indicates that, in neurons, reciprocal inhibition of transcriptional activity by p53 and NF-κB determines cell fate. These

observations provided a somewhat satisfactory explanation for the parallel induction of p53 and NF-ΚB, which was also consistent with the tumor suppressor status of the former and the role of the latter as a tumor promoter; each could fulfill its ascribed functions by inhibiting the activity of the other (130).

NFΚB is an inducible transcription factor that plays a significant role in regulation of gene transcription associated with both cell survival and apoptosis (Figure 36).

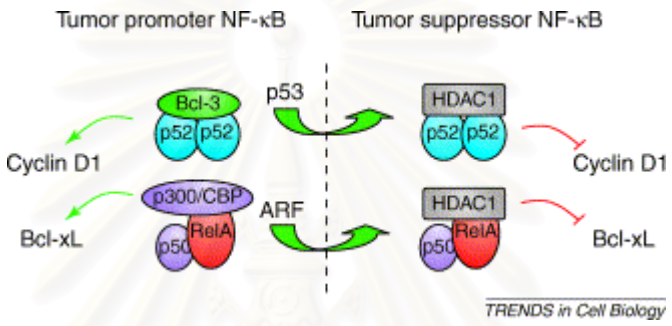


Figure 36. Regulation of NF-ΚB activity by tumor suppressors. NF-ΚB subunits are inherently oncogenic and tumor promoting because of their ability to induce the expression of genes that promote resistance to apoptosis (e.g. Bcl-xL) and induce cellular proliferation (e.g. cyclin D1). However, tumor suppressor proteins, such as p53 and ARF, can induce the association of NF-ΚB subunits with histone deacetylase 1 (HDAC1) co-repressor complexes. This results in NF-ΚB-dependent repression of target gene expression, providing a model through which NF-ΚB subunits can facilitate apoptosis and cell-cycle arrest, and thus functioning as tumor suppressors themselves (130).

It appears that NFκB induces multiple factors to suppress apoptosis at many steps along the cell death cascade including TRAF1 and 2, cIAP1 and 2, caspase-8, and Bcl-2 gene family (Bcl-2 and BclxL) (131, 132). In addition, NFκB can activate the expression of SOD that has been implicated in potential mechanism of cell survival from ROS (38, 133). On the other hand, the key determining factors in NFκB death signal are activation of RIP, TRAF2, FADD, JNK, p38, and ERK (134). Indeed, the role of NFκB in cell faith is contingent upon the balance of pro- and anti-apoptotic gene products, and the pathways of regulation are cell type- and stimulus-dependent. Recent studies have shown that NFκB is a key target for ROS, and its activation leads to cell dysfunction and death particularly in DOX-induced cell death (120, 135, 136). Like others, this study demonstrated that treatments with antioxidants, including *P. urinaria*, abolished DOX-induced NFκB activation and cardiac cell death.

In summary, we demonstrated that cardioprotective effect of *P. urinaria* extract against DOX cardiotoxicity was mediated through multiple pathways possibly due to the inhibition/scarvenger of DOX-generated ROS, or activation of cellular antioxidant defense. It also can not be excluded that the cytoprotective effect of PU may derived from its inhibition of cell death at the initial step thereby inhibiting subsequent signaling pathways and and other changes in paramerters observed in this study. The balance in the survival signals and death signals is crucial in the determination of cell faith. *P. urinaria* enhanced survival factors through elevation of tGSH, activation of catalase/SOD enzyme activity, and inhibition of lipid peroxidation while death signals such as caspase-3 and NFκB activation were suppressed. This study suggests that *P. urinaria* extract may serve as alternative antioxidants for prevention of DOX cardiotoxicity.

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