2011

# ATTENUATION OF OXIDATIVE AND NITROSATIVE STRESS BY PURE CONSTITUENTS OF TRADITIONAL CHINESE REMEDIES

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# ATTENUATION OF OXIDATIVE AND NITROSATIVE STRESS BY PURE CONSTITUENTS OF TRADITIONAL CHINESE REMEDIES

(spine title: antioxidant effects of traditional Chinese remedies)

(Thesis Format: Monograph)

by

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Graduate Program in Pathology

A thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science

School of Graduate and Postdoctoral Studies
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THE UNIVERSITY OF WESTERN ONTARIO
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titled:

**Attenuation of Oxidative and Nitrosative Stress by Pure Constituents of Traditional Chinese Remedies**

is accepted in partial fulfilment of the
requirements for the degree of

**Master of Science**

Date_____ _____

Chair of the Thesis Examination Board

## ABSTRACT

The formation and disposition of sulfamethoxazole hydroxylamine (SMX-HA), a metabolite of sulfamethoxazole (SMX), plays a critical role in the pathogenesis of adverse drug reactions (ADRs), and can generate cellular oxidative and nitrosative stress by forming reactive oxygen and nitrogen species (ROS/RNS). We investigated the potential attenuation of oxidative and nitrosative stress-mediated ADRs by baicalein, crocetin, resveratrol and schisanhenol alone and in combination using Jurkat *E*6.1 cells. These compounds are presented in herbs used as Traditional Chinese Medicine (TCM). Our results show that SMX-HA causes cell damage mediated by ROS/RNS. Low micromolar concentrations of these four chemicals attenuate SMX-HA-mediated LDH release, lipid hydroperoxide formation and carbonylated protein formation, as well as the formation of protein mixed disulfides, endpoints indicative of cytotoxicity. Treatment of cells with equimolar mixtures of these four phytochemical antioxidants was more protective of disulphide formation induced by SMX-HA., suggesting that defined mixtures of pure TCM constituents may better protect against oxidative and nitrosative stress than single chemicals *in vivo*.



**KEYWORDS:** baicalein, crocetin, disulfide proteome, LDH release, lipid peroxidation, oxidative and nitrosative stress, protein carbonylation, protein mixed disulfides, reactive oxygen species, reactive nitrogen species, resveratrol, schisanhenol, sulfamethoxazole hydroxylamine

## ACKNOWLEDGEMENTS

First, I would like to thank my supervisor Dr. Jack Bend and co-supervisor Dr. Michael Rieder for their continued assistance, knowledge and guidance throughout the duration of my Masters. I would also like to thank Dr. Subrata Chakrabarti, a member of my Advisory Committee for his advice and assistance during my committee meeting.

I wish to express thanks to the other members of the Bend and Rieder laboratories; Drs. Xueyan Xia and Garth Oakes; Abudi Awaysheh, Ewa Jaworski, Anda Marcu, and Kemi Adeyanju for their sharing of knowledge including experimental techniques and friendship. You made it a pleasant environment to work in.

I would like to acknowledge members of the Rieder and Freeman groups for their thoughtful discussions during our weekly laboratory meetings. I learned much about research during these regular, informal exchanges.

Finally, special thanks go to my family and friends, for their continued support, encouragement, and understanding.

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| | |
|---|---|
| $^{\bullet}NO$ | Nitric oxide |
| $^{\bullet}NO_2$ | Nitrogen dioxide |
| $^1O_2$ | Singlet Oxygen |
| ADRs | Adverse drug reactions |
| BE | Baicalein |
| CA | Corrected absorbance |
| CAM | Complementary and alternative medicine |
| CHM | Chinese herbal medicine |
| Cro | Crocetin |
| CYP | Cytochrome P450 |
| DMSO | Dimethyl sulfoxide |
| DNPH | 2,4-dinitrophenylhydrazine |
| DTT | Dithiothreitol |
| FBS | Fetal bovine serum |
| GSH | Glutathione (reduced) |
| GSH-Px | GSH peroxidase |
| GSH-*S*-T | Glutathione-*S*-transferase |
| GST | Glutathione S-transferase |
| $H_2O_2$ | Hydrogen peroxide |
| HEK | Human embryonic kidney |
| $HNO_2$ | Nitrous acid |
| $HO_2^{\bullet}$ | Hydroperoxyl |
| HOCl | Hypochlorous acid |
| HPLC | High performance liquid chromatography |
| IA | Idoacetamide |
| IDR | Idiosyncratic drug reaction |
| IL-6 | Interleukin-6 |
| iNOS | Inducible nitric oxide synthase |
| INT | Iodonitrotetrazolium chloride |
| LACTATE | L(+)-lactic acid |
| LDH | Lactate dehydrogenase |
| LDL | Low-density lipoprotein |

| | |
|---|---|
| LPO | Lipid hydroperoxide |
| MPO | Myeloperoxidase |
| $N_2O_3$ | Dinitrogen trioxide |
| $N_2O_4$ | Dinitrogen tetroxide |
| NAD | β-nicotinamide adenine dinucleotide hydrate from yeast |
| $NO^-$ | Nitrosyl anion |
| $NO^+$ | Nitrosyl cation |
| $NO^+_2$ | Nitronium cation |
| $NO_2Cl$ | Nitryl chloride |
| NOS2 | Nitric oxide synthase 2 |
| $O_2^{\bullet-}$ | Superoxide |
| $O_3$ | Ozone |
| $OH^\bullet$ | Hydroxyl |
| $ONOO^-$ | Peroxynitrite |
| ONOOH | Peroxynitrous acid |
| ox-LDL | Oxidized low density lipoprotein |
| PBMCs | Peripheral blood mononuclear cells |
| PCP | Pneumocystic jiroveci pneumonia |
| PMS | Phenazine methosulfate |
| Prx | Peroxiredoxin |
| $P\text{-}SO_2H$ | Sulphinic acid |
| $P\text{-}SO_3H$ | Sulphonic acid |
| Res | Resveratrol |
| RNS | Reactive nitrogen species |
| $RO^\bullet$ | Alkoxyl |
| $RO_2^\bullet$ | Peroxyl |
| ROONO | Alkyl peroxynitrites |
| ROS | Reactive oxygen species |
| Sal | Schisanhenol |
| SDS | Sodium dodecyl sulphate |
| SMX | Sulfamethoxazole |
| SMX-HA | Sulfamethoxazole hydroxylamine |
| SMX-NO | Sulfamethoxazole nitroso |
| SOD | Superoxide dismutase |
| *t*-BHP | *tert*-Butylhydroperoxide |

| | |
|---|---|
| TCA | Trichloroacetic acid |
| TCM | Traditional Chinese Medicine |

# CHAPTER 1: INTRODUCTION

## 1.1 TRADITIONAL CHINESE MEDICINE

Traditional Chinese medicine, also know as TCM, is a medical system which originated in China more than two thousands years ago (Ou, Huang et al. 2003). It involves traditional Chinese culture, TCM theory, and the collective experiences of a large number of TCM doctors. TCM evolved before the era of modern biomedical research and was a unique holistic system to diagnose and cure illness. Similar to present day medical practice, retaining a healthy status, preventing and healing diseases were the primary objectives of TCM. Moreover, abundant resources, unique curative methods (Zhang, Peng et al. 2010), combination intervention, personalized therapy (Zhang, Sun et al. 2010) are all characteristics of TCM, which have drawn more attention to TCM and popularized aspects of TCM throughout the world. TCM is a complex medical science which includes Chinese herbal medicine (CHM), acupuncture, Chinese food therapy, Chinese therapeutic massage (Tui na), and other techniques like moxibustion and tai chi chuan (Xue, Zhang et al. 2010).

Among these therapeutic methods and techniques, CHM is a well-organized and complete medical system that involves products of natural origin. It involves thousands of individual constituents, many of which have been documented systematically based on their medicinal efficacy. It includes complex prescriptions composed of preparations derived from natural plants, minerals and in some cases, parts of animals. It has been recently reported that CHM includes 11,146 kinds of medicinal plants, 1,581 kinds of medicinal animals, and 80 kinds of mineral drugs (Pan, Pan et al. 2010). In China, by 2007, CHM included 3,563 extracts, 64,715 formulations, and 5,000 single compounds (Pan, Pan et al. 2010). According to a patient's individual condition, prescriptions are various and served either as a boiled decoction, dried herbal extract, or pills (Flower, Lewith et al. 2010).

In Asian countries especially in China, CHM is considered as the front line and primary therapeutic modality in internal medicine. It was recently estimated that CHM products account for about $31 billion annually, more than a quarter of the total expense on all medicines in China (Flower, Lewith et al. 2010). Although CHM is a relative newcomer in the Western world, it has attracted much attention and is now widely accepted as complementary and alternative medicines. A report showed that the annual research funding spent on the study of complementary and alternative medicine (CAM), including its efficacy, in the United States' National Centre is more than US$120 million, which included more than 1200 projects since 2000 (Xue, Zhang et al. 2010).

Adverse drug reactions (ADRs) refer to an undesirable secondary effect which happens during the administration of medication (Zeng and Jiang 2010). ADRs are affected by many factors including the nature of different drugs, individuals, dosages, as well as the interactions of drugs. Similar to treatment with western drugs, ADRs are also an unavoidable problem for CHM, as has been recorded in ancient China. This issue is frequently identified but its study and subsequent clinical reports are infrequent. Despite the fact that most of the CHM are naturally derived and natural products are considered to exert few side effects, ADRs may happen with improper usage and drug abuse due to the duality of medicines. The most common ADRs of CHM are allergy and toxicity (Zeng and Jiang 2010). For example, Ginseng is a well-known tonic that can also produce ADRs in the nervous and digestive systems (Zeng and Jiang 2010). Side effects of CHM are also known to occur at the cellular and gene level. For example, it was recently reported that aristolochic acids administered with other herbal mixtures lead to gene mutation (after metabolic activation of the aristolochic acid) and thus caused nephrotoxic effects when they were used for slimming in European countries (Youns, Hoheisel et al. 2010). Adverse effects following the external use of herbal products have also received much attention. A survey in the United State showed that the population of usage of herbal treatments had reached to 42% until 1997

(Ernst 2000). This trend is still upwards and herbal products have gained more and more acceptance by the public in North America. Herbal remedies are known to improve dermatological conditions, on the other hand, some researchers have found that some herbal products (e.g. St John's Wort, Kava, aloe vera, eucalyptus, camphor, henna and yohimbine) can cause dermatological side effect including rashes and skin lesions (Ernst 2000). Compared with single drug therapy, the ADRs of natural product-derived drugs are more difficult to analyze due to many factors involved like individual factors, interactions with other drugs, as well as lack of standardized processing (Zeng and Jiang 2010).

According to the WHO Collaborating Centre for International Drug Monitoring, there has been an increasing trend of reported ADRs resulting from treatment with TCM in the past decade (Zeng and Jiang 2010). In general, the safety of CHM refers to the potential interaction among different herbs and adverse reactions. Based on the completed clinical trials, mild adverse reactions are more common than serious ADRs (Xue, Zhang et al. 2010). In this case, the safety of Chinese herbal products has received increasing attention and has become a major research area. Correspondingly, more and more new techniques have emerged which are intended to help decrease the incidence of ADRs of herbal medicines. Toxicogenomics is a multi-disciplinary and integrated field which combines toxicology, genomics, metabolomics and proteomic approaches (Afshari, Hamadeh et al. 2010). Nowadays, it has been widely used in evaluating the toxicity and ADRs of drugs and TCM. Briefly, the expression of gene will be changed after the cells are exposed to a drug or mixture used in medicine, and the encoded gene(s) will thus influence the proteins. By detecting the production and function of individual protein, toxicogenomics can be used for risk assessment (Youns, Hoheisel et al. 2010). In other words, toxicogenomics is a scientific technique that can investigate and predict the interactions and safety of TCM by analyzing gene expression profiles and molecular mechanism for toxicity based on cell signaling.

## 1.2 OXIDATIVE STRESS AND NITROSATIVE STRESS

Oxidative/nitrosative stress is caused by the production of highly reactive chemical species known as reactive oxygen species (ROS) or reactive nitrogen species (RNS). Toxicity resulting from ROS/RNS occurs when the amount of these species produced exceed the ability of cellular anti-oxidant defense mechanisms to neutralize and eliminate the reactive intermediates or easily repair the resulting damage (Dalle-Donne, Scaloni et al. 2005). This disturbance can lead to damage to major cellular components including lipids, proteins, carbohydrates, and DNA (Dalle-Donne, Scaloni et al. 2005), interfering with normal body functions. The rate of damage is determined by how fast ROS/RNS are generated and inactivated by anti-oxidants.

Oxidative/nitrosative stress has been implicated in many diseases including atherosclerosis (Chang, Kou et al. 2010), diabetes and its associated complications (Rains and Jain 2011), hepatitis (Ha, Shin et al. 2010), cancer (Bulteau and Bayot 2010) (Cobanoglu, Demir et al. 2010), respiratory disease (Gowdy, Krantz et al. 2010), and Alzheimer's disease (Barone, Cenini et al. 2011). Also, oxidative/nitrosative stress is involved in physiological phenomena like aging and longevity (Page, Robb et al. 2010). The chemical changes to lipids, proteins, carbohydrates, and DNA described above contribute to each of these pathologies.

How might the concept of anti-oxidant balance, a basis of homeostasis, be connected to TCM? Actually, a similar concept called yin-yang was recorded in the ancient medical book, *The Yellow Emperor's Classic of Internal Medicine*, 2,500 years ago (Ou, Huang et al. 2003). In this book, the author Su Wen illustrated that the imbalance of yin-yang was the main cause of various diseases. Briefly, yin refers to the counteractive activities like anti-oxidant activity, while yang represents the proactive properties like oxidation (Ou, Huang et al. 2003). Based on this theory, CHM can be divided into yin-tonic and yang-tonic herbs. Yin-tonic traditional Chinese herbs were regarded to possess six times more

anti-oxidant effect than yang-tonic herbs due to their higher anti-oxidant phenolic content (Ou, Huang et al. 2003).

### 1.2.1 Reactive Oxygen Species (ROS)

ROS are reactive molecules that contain the oxygen atom and are produced by single electron reductions of molecular $O_2$ (Bartz and Piantadosi 2010). They are formed either through exogenous source which can involve environmental agents, contaminants, ionizing radiation, therapeutic agents, and tobacco smoke (Klaunig, Wang et al. 2011) or endogenous sources, including mitochondria, peroxisomes, and inflammatory cell activation (Klaunig and Kamendulis 2004). In biological systems, ROS are mainly generated in mitochondria especially during mitochondrial respiration (Tiganis 2011). ROS refer both to free radicals and other non-radical oxygen derivatives (Table 1) (Dalle-Donne, Rossi et al. 2003).

The generation of ROS, such as singlet oxygen ($^{-1}O_2$), hydrogen peroxide ($H_2O_2$), superoxide anion radical ($O_2^{\bullet-}$) or hydroxyl radical ($OH^{\bullet}$), is related to respiration. $O_2^{\bullet-}$ is the primary metabolite of oxygen, while $H_2O_2$ is formed during dismutation of $O_2^{\bullet-}$ when catalyzed by superoxide dismutase (SOD), important cellular anti-oxidant enzyme in the body, or spontaneously. Effects of ROS on cellular metabolism have been widely documented. Under physiological conditions, ROS participate in the modulation of normal cellular functions which include gene expression, signal transduction, and defense against invading pathogens (Dalle-Donne, Scaloni et al. 2005). Taking the aging process as an example, ROS concentrations significantly increase in the early stages of embryonic development, and the metabolism and cell signaling initiated by ROS play an important role in early development (Page, Robb et al. 2010). Besides, ROS contribute to the pathogenesis and progression of several human chronic diseases like atherosclerosis (Chang, Kou et al. 2010), diabetes (Rains and Jain 2010), hepatitis (Ha, Shin et al. 2010), as well as traumatic brain injury (Hall,

Vaishnav et al. 2010). ROS are reactive molecules and most of them react with and affect intracellular proteins, lipids, and DNA (as described above). However, unrepaired damage to essential macromolecules or unabated formation of ROS may cause cell death through apoptosis and/or necrosis (Bartz and Piantadosi 2010). For example, incubation of 50 μM of hydrogen peroxide for 18 h caused 50% of cell death in Jurkat cells (Zhang, Stanley et al. 2006).

**Table 1. Reactive Oxygen Species**

| Radicals | Symbol | Non-Radicals | Symbol |
|---|---|---|---|
| Hydroxyl | $OH^{\bullet}$ | * Peroxynitrite | $ONOO^{-}$ |
| Superoxide | $O_2^{\bullet -}$ | Hypochlorous acid | HOCl |
| Peroxyl | $RO_2^{\bullet}$ | Hydrogen Peroxide | $H_2O_2$ |
| Alkoxyl | $RO^{\bullet}$ | Singlet Oxygen | $^1O_2$ |
| Hydroperoxyl | $HO_2^{\bullet}$ | Ozone | $O_3$ |

* Results from the reaction of superoxide anion radical with nitric oxide (NO) and responsible for both oxidative and nitrosative stress.

### 1.2.2 Reactive Nitrogen Species (RNS)

RNS are a family of nitrogen-based molecules. These radicals are derived from nitric oxide (·NO) and superoxide ($O_2^{\cdot-}$), the former of which is generated through the enzymatic activity of nitric oxide synthase (NOS). The form of NOS, most important in pathology is nitric oxide synthase 2 (NOS2). Superoxide is commonly formed from NADPH oxidase. (Wink, Miranda et al. 2001; Dalle-Donne, Scaloni et al. 2005; Hall, Vaishnav et al. 2010).

Excessive production of RNS (Table 2) (Dalle-Donne, Scaloni et al. 2005) cause nitrosative stress such as nitrosylation reactions that can interfere with normal body functions. Among these radicals, nitric oxide ($^{\cdot}NO$) has received a great deal of attention. At low concentrations (formed by NOS3 or NOS1), it is an important endogenous mediator and possesses many beneficial physiological actions. For example, $^{\cdot}NO$ is a bioactive and relatively unstable free radical involved in a number of biological process like modulation of signaling functions by working as a second messenger (Tillmann, Gow et al. 2011). However, $^{\cdot}NO$ may exhibit cytotoxicity at high concentration, attacking defense systems (Nathan and Shiloh 2000) and damaging proteins, lipids, and DNA through the reaction with superoxide or generation of powerful oxidizing and more reactive redox derivatives like peroxynitrite (Dalle-Donne, Scaloni et al. 2005; Powers, Talbert et al. 2011; Powers, Talbert et al. 2011). According to its mechanism of action in biological systems, nitric oxide has two effects which include direct and indirect effects. A direct effect refers to its direct reaction with other chemicals. However, nitric oxide has to be activated, as in the formation of peroxynitrite, and then continue further reaction to elicit its indirect effect (Wink, Miranda et al. 2001). Generally, nitrosative stress is induced by three kinds of RNS which derive from NO, including $NO^{\cdot}$ radical, nitrosonium cation, and nitroxyl anion (Tillmann, Gow et al. 2011). Another RNS that has received much attention is peroxynitrite ($ONOO^-$). Peroxynitrite is generated through the reaction of nitric oxide (·NO)

with superoxide ($O_2^{\cdot-}$). Peroxynitrite itself is a strong oxidizing radical which can enter cells via anion channels (Pacher, Beckman et al. 2007). Once into the cell, peroxynitrite oxidizes proteins, lipids, DNA, as well as low-molecular weight anti-oxidants (O'Donnell, Eiserich et al. 1999). Other RNS including nitrogen dioxide ($\cdot NO_2$) and dinitrogen trioxide ($N_2O_3$) are produced through the reaction between peroxynitrite and other molecules in the cell. Also, RNS have been reported to be involved in the pathogenesis of some diseases, including atherosclerosis (Chang, Kou et al. 2010), diabetes (Rains and Jain 2010), and Alzheimer's disease (Obulesu, Venu et al. 2011).

**Table 2. Reactive Nitrogen Species**

| | | | |
|---|---|---|---|
| Nitric oxide | $^{\bullet}NO$ | Dinitrogen tetroxide | $N_2O_4$ |
| Nitrogen dioxide | $^{\bullet}NO_2$ | Dinitrogen trioxide | $N_2O_3$ |
| Nitrous acid | $HNO_2$ | Peroxynitrite | $ONOO^-$ |
| Nitrosyl cation | $NO^+$ | Peroxynitrous acid | ONOOH |
| Nitrosyl anion | $NO^-$ | Alkyl peroxynitrites | ROONO |
| Nitronium cation | $NO^+_2$ | Nitryl chloride | $NO_2Cl$ |

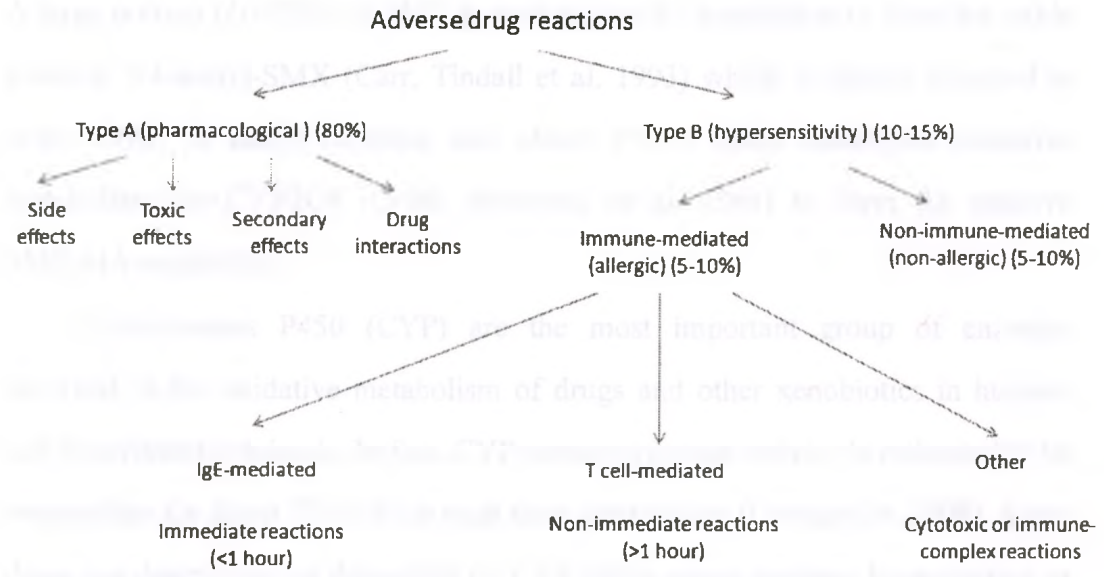
## 1.3 REACTIVE DRUG METABOLITES

Sulfamethoxazole N-hydroxylamine (SMX-HA) is the major cytochrome P450 (CYP) metabolite of sulfamethoxazole (SMX), an effective therapeutic drug for the treatment of opportunistic infections (Lavergne, Kurian et al. 2006). However, its use is largely limited in treatment of AIDS patients due to adverse drug reactions (ADRs) to SMX, a very common problem. It has been reported that the incidence of SMX induced ADRs is about 3%-8% of the exposed population, while this rate is increased rise to 50% in patients infected with HIV (Castrejon, Berry et al. 2010). These ADRs to SMX include fever, skin rash, lymphadenopathy, hepatotoxicity and hematological disorders (Summan and Cribb 2002) and occur in HIV-infected patients approximately 7-10 days after administration (Svensson 2003).

ADRs are a common problem caused by the use of medicine, and are major factors contributing to hospitalization and mortality. In all of the medicines, antibiotics, non-steroidal anti-inflammatory drugs, and antiepileptics are major sources of the development of ADRs (Porebski, Gschwend-Zawodniak et al. 2011). ADRs can be divided into two types: type A and type B reactions. Type A reactions are usually caused by the pharmacologic actions of the drugs, they possess the properties of being predictable and dose-dependent, and account for about 80% of ADRs. For examples, drug mediated toxicity, secondary effects, and drug interactions all belong to this type of adverse reaction (Thien 2006) . Type B reactions can not be predicted from the known primary pharmacology of drug and are related to genetic and environmental factors. It appears in normal people without drug overdoses and only comprises 15% of all ADRs (Posadas and Pichler 2007). In this case, type B reactions can be subdivided into immune-induced and nonimmune-induced reactions. Immune-mediated drug allergy includes

IgE-mediated, T cell-mediated as well as immune-complex mediated reactions (Fig 1) (Thien 2006).

**Figure 1. Classification of Common Adverse Drug Reactions**

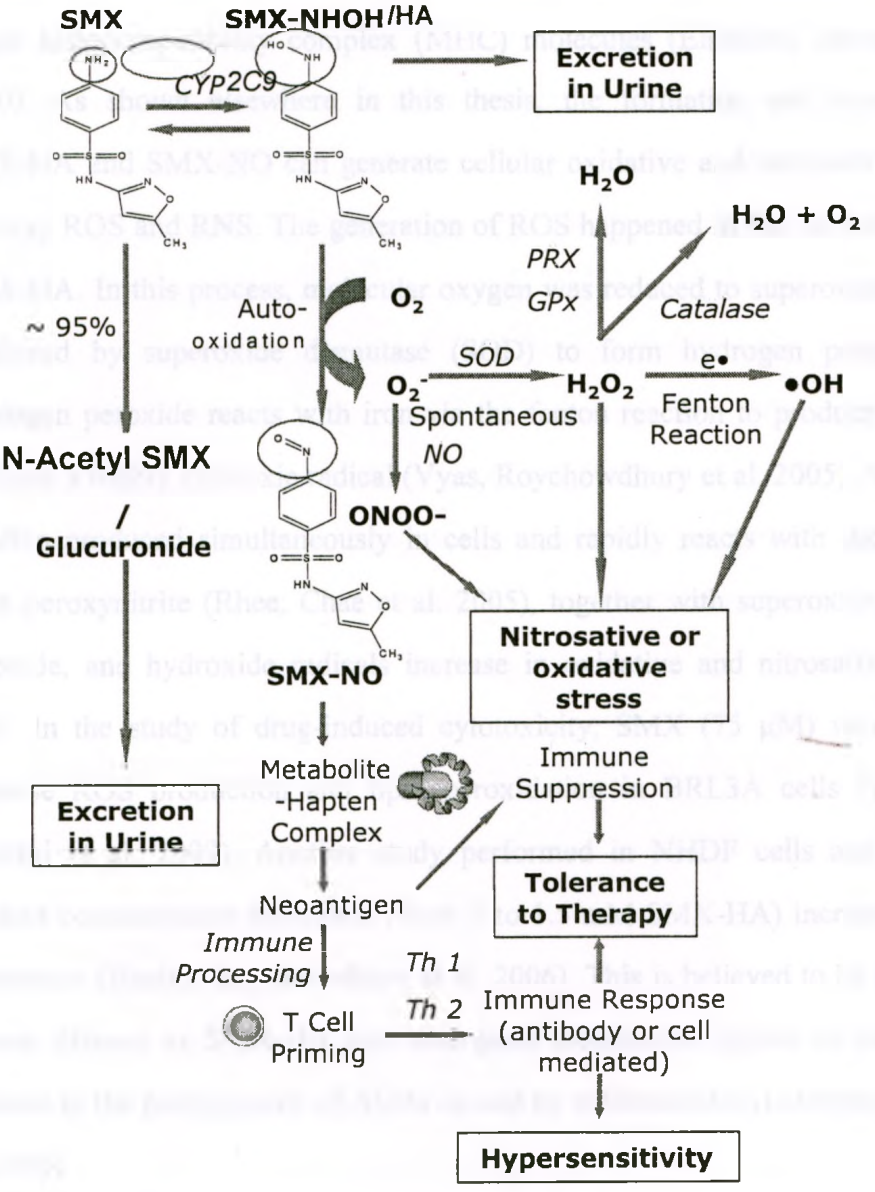

Adverse drug reactions
Type A (pharmacological ) (80%)
Type B (hypersensitivity ) (10-15%)
Side effects
Toxic effects
Secondary effects
Drug interactions
Immune-mediated (allergic) (5-10%)
Non-immune-mediated (non-allergic) (5-10%)
IgE-mediated
T cell-mediated
Other
Immediate reactions (<1 hour)
Non-immediate reactions (>1 hour)
Cytotoxic or immune-complex reactions

A large portion (50-70%) of SMX is metabolized by acetylation to form the stable product, N4-acetyl-SMX (Carr, Tindall et al. 1993) which is rapidly excreted in urine. SMX is easily oxidized and about 5% of SMX undergoes oxidative metabolism by CYP2C9 (Cribb, Spielberg et al. 1995) to form the reactive SMX-HA metabolite.

Cytochromes P450 (CYP) are the most important group of enzymes involved in the oxidative metabolism of drugs and other xenobiotics in humans and experimental animals, In fact, CYP monooxygenase activity is estimated to be responsible for about 75% of the total drug metabolism (Guengerich 2008). Some drugs are deactivated or detoxified by CYP while others undergo bioactivation or toxication by CYP. With drugs, particularly in combination, the activity of various CYP isozymes may be increased by the process of induction or inhibited due to inactivation of one or more CYP isozymes by a reactive drug metabolite. Such changes in the metabolism of a drug can contribute to ADRs.

The metabolism of SMX has received much attention due to the widespread use and high incidence of ADRs. After the reactive sulfamethoxazole hydroxylamine (SMX-HA) metabolite is generated by CYP2C9, an enzyme significant in drug metabolism in humans, it undergoes auto-oxidation to generate the protein-reactive, oxidative metabolite sulfamethoxazole-nitroso (SMX-NO) (Castrejon, Lavergne et al. 2010). In the absence of adequate detoxication, the powerful immunogen, SMX-NO can react covalently with cysteine thiol residues of cellular proteins, a preferred site, or other nucleophilic sites, to form adducts recognized as neo-antigens by the immune system. This, in turn, can provide an antigenic signal to T cells and elicit a T cell-mediated immune response which presents clinically as delayed-type hypersensitivity (Fig 2) (Rieder, Uetrecht et al. 1988; Sanderson, Naisbitt et al. 2007). In addition, the mechanism of T cell induced hypersensitivity was further studied and found to be closely related to the cross-reaction between the T cell receptor complexes and the haptenic immunogen (Castrejon, Berry et al. 2010). However, the initiation of an immune reaction is

**Figure 2. Proposed mechanism for SMX-HA induced ROS/RNS production and either tolerance to therapy or pathogenesis including drug hypersensitivity, an adverse drug reaction**


SMX
SMX-NHOH/HA
CYP2C9
Excretion in Urine
$H_2O$
$H_2O + O_2$
PRX
GPx
Catalase
~ 95%
Auto-oxidation
$O_2$
SOD
e•
$O_2^-$
$H_2O_2$
•OH
Spontaneous
NO
Fenton Reaction
N-Acetyl SMX / Glucuronide
ONOO-
Nitrosative or oxidative stress
SMX-NO
Metabolite -Hapten Complex
Immune Suppression
Excretion in Urine
Tolerance to Therapy
Neoantigen
Immune Processing
Th 1
Th 2
T Cell Priming
Immune Response (antibody or cell mediated)
Hypersensitivity

decided by a prerequisite which is the neo-antigens must be presented in specific major histocompatibility complex (MHC) molecules (Elsheikh, Lavergne et al. 2010). As shown elsewhere in this thesis, the formation and disposition of SMX-HA and SMX-NO can generate cellular oxidative and nitrosative stress by forming ROS and RNS. The generation of ROS happened in the auto-oxidation of SMX-HA. In this process, molecular oxygen was reduced to superoxide, which is catalyzed by superoxide dismutase (SOD) to form hydrogen peroxide, then hydrogen peroxide reacts with iron via the fenton reaction to produce hydroxide radicals, a highly cytotoxic radical (Vyas, Roychowdhury et al. 2005). Nitric oxide is often produced simultaneously in cells and rapidly reacts with superoxide to form peroxynitrite (Rhee, Chae et al. 2005), together with superoxide, hydrogen peroxide, and hydroxide radicals increase in oxidative and nitrosative stress in cells. In the study of drug-induced cytotoxicity, SMX (75 μM) was shown to increase ROS production and lipid peroxidation in BRL3A cells (Yoshikawa, Hosomi et al. 2009). Another study performed in NHDF cells and the result showed concentration dependent (from 0 to 1.5 mM SMX-HA) increases in ROS generation (Bhaiya, Roychowdhury et al. 2006). This is believed to be germane to human disease as SMX-HA and analogous metabolites appear to be a critical element in the pathogenesis of ADRs caused by sulfonamides (Lavergne, Kurian et al. 2006)

Many studies have been done to investigate SMX-HA induced cytotoxicity in T cells mainly due to the fact that lymphocytes can not generate reactive metabolites (Reilly, MacArthur et al. 1999). Besides lymphocytes, other cell types like peripheral blood mononuclear cells (PBMCs) were also found to be related to SMX-HA induced drug hypersensitivity reaction. To evaluate SMX-mediated delayed-type hypersensitivity, the in vitro measurement of cytotoxicity of SMX-HA towards PMBCs has been proposed as a biomarker (Rieder 1997). Timothy et al found that 3 h incubation with SMX-HA (from 0.1□1 mM) in PBMCs leads to the cell death in a dose-dependent manner (Reilly, MacArthur et

al. 1999). Several in vitro tests for evaluation of hypersensitivity mediated ADRs have been widely used. The lymphocyte transformation test (LTT) is a technique that measures the drug specific T cell clones in the peripheral circulation from patients who have suffered T cell mediated hypersensitivity reactions (Elzagallaai, Knowles et al. 2009). PBMCs from patients who had an ADR to a sulphonamide are more sensitive than cells from normal patients to the toxic effects of SMX-HA when challenged in vitro (Uetrecht 1989). Exposure of lymphocytes to SMX-HA causes ROS formation, leading to significant depletion of glutathione (GSH) (Rieder, Uetrecht et al. 1988; Vyas, Roychowdhury et al. 2005), and SMX-HA mediated ROS formation undoubtedly contributes to cytotoxicity following treatment with SMX-HA, but whether this is a major contribution to cell death remains unclear.

## 1.4 INDIVIDUAL CONSTITUENTS OF TCM SELECTED FOR DETAILED STUDY

### 1.4.1 Baicalein (BE)

BE is originally derived from the roots of *Scutellaria baicalensis* Georgi (Fig 3-A), a plant traditionally used in herbal medicine. The main bioactive constituents of *Scutellaria* are flavonoids (Woo, Cheng et al. 2005), a group of polyphenolic compounds that are found in the human diet in many fruits and vegetables as well as medicinal plants (Jiang, Su et al. 2010), flavonoids possess various biological activities and act as natural anti-oxidants in plants (Piao, Cho et al. 2008). It has been reported that some flavonoids possess anti-cancer, chemoprotective effects (Jiang, Su et al. 2010) and anti-microbial activities (Chandrashekaran, Adda et al. 2010). BE has long been extensively used in CHM as a remedy for treatment of respiratory tract infection, cancer (Wang, Ling et al. 2010) dysentery, jaundice, hepatitis and hypertension (Lai, Hsiu et al. 2003).

Recently, the anti-cancer properties of BE have received increasing attention. It has been reported that BE leads to the inhibition of cancer cell growth. For example, BE negatively regulates the binding of β-catenin/Tcf complexes, a pathway implicated in human carcinogenesis (Park and Choi 2010). Also, BE has been shown to induce apoptosis in a variety of human cancer cells including breast, hepatocellular, pancreatic, prostatic and urothelial carcinoma cell lines (Wang, Ling et al. 2010). Gao et al. recently reported that BE initiates apoptosis in prostate cancer cell lines via activation of caspase-3 and caspase-7 (Gao, Zhao *et al.* 2010). In addition, BE protects against adhesion, migration and invasion of MDA-MB-231 human breast cancer cells by suppressing the activation and expression of matrix metalloproteinases 2/9 (Wang, Ling et al. 2010).

Besides an anti-cancer effect, BE also exhibits many other biological properties like anti-viral, anti-inflammatory, anti-thrombotic and cytoprotective activities (Piao, Cho et al. 2008). Among its biological activities, one of the most important beneficial effects is its anti-oxidant effect. Liu and colleagues reported that BE inhibits the formation of ROS and decreases the level of vascular endothelial growth factor and matrix metalloproteinase-9, which are induced by $H_2O_2$ (Liu, Wann et al. 2010). Previous studies showed that BE can scavenge ROS including superoxide, hydrogen peroxide, and hydroxyl radicals (Hamada, Hiramatsu et al. 1993; Hanasaki, Ogawa et al. 1994). BE also acts as a neuroprotectant through downregulating of 12/15-lipoxygenase, p38 mitogen-activated protein kinase and cytosolic phospholipase A2, which are modulated by oxidative stress (Cui, Zhang et al. 2010). Also, Chao and co-workers demonstrated that BE can protect against ischemic-induced brain injury both *in vitro* and *in vivo.* This protective effect was achieved by scavenging free radicals and protecting the PI3K/Akt and PTEN pathways (Cui, Zhang et al. 2010). Similarly, Chang et al found that BE can protect against ischemia/reperfusion injury and decrease cell death in cardiomyocytes (Chang, Shao et al. 2007). In addition, Shieh et al indicated that, due to inhibition of xanthine oxidase activity, BE can prevent free radical production and attenuate

oxidative stress in cardiomyocytes (Shieh, Liu et al. 2000). To a great extent, the anti-oxidant activity of BE is related to its chemical properties. In general, the anti-oxidant capacity is related to the ability of donation of a hydrogen atom from its phenolic groups (Chang, Shao et al. 2007). BE (Fig 3-B) possesses an *o*-tri-hydroxyl structure in the A ring, a major contributor to its radical scavenging potential (Gao, Huang et al. 1999).

Besides these properties, BE was also found to inhibit the iron-mediated lipid peroxidation by acting as a strong iron chelator (Shieh, Liu et al. 2000). BE is reported to have a therapeutic effect on atopic dermatitis through regulation of molecular mediators and immune cells (Yun, Yang et al. 2010). *In vivo*, BE was shown to reduce the production of inducible nitric oxide synthase (iNOS) and the formation of excess nitric oxide (NO) by suppressing NF-κB activation (Cheng, Lee et al. 2007). *In vitro*, BE exhibits an important cytoprotective effect by scavenging the important ROS and RNS, peroxynitrite (Piao, Cho et al. 2008).

Previous work in our laboratory has shown that low concentrations of BE (5 or 10 μM) can attenuate *tert*-butylhydroperoxide (50 μM) induced cytotoxicity in HEK 293 cells by attenuating oxidative stress (Xueyan Xia, 2008, unpublished).

Collectively, recent studies support the chemoprotectant role of BE, a known anti-oxidant and immunomodulant, in protection against oxidation induced cell damage in vitro and in vivo. This is the reason it has been included as one of the test compounds in this thesis.

**Figure 3. Baicalein**

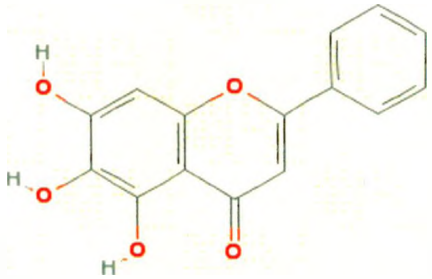
**A.** Photo of *Scutellaria baicalensis Georgi* .

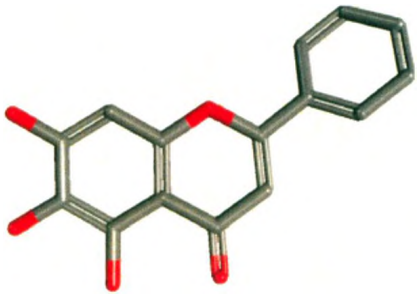
(Photo from Henriette Kress www.henriettesherbal.com)

**B**. Two and Three dimensional chemical structures of BE, IUPAC nomenclature: 5,6,7-trihydroxy-2-phenylchromen-4-one (From PubChem Public Chemical Database, http://pubchem.ncbi.nlm.nih.gov/)

**A**

**B**



### 1.4.2 Crocetin (Cro)

Cro, one of the major ingredients of saffron, is a natural carotenoid dicarboxylic acid found in *Crocus sativus* (Kanakis, Tarantilis et al. 2007) (Fig 4-A). Saffron, the red dried stigmas of *Crocus sativus L,* is the most expensive spice in the world. It is estimated that 75,000 flowers or 225,000 hand-picked stigmas are needed to obtain one pound of saffron (Aytekin and Acikgoz 2008). Saffron is used worldwide mainly due to its biological activities relevant to biology, pharmacology, and medicine. Saffron constituents have attracted the interest of researchers for their anti-cancer and anti-atherosclerotic activity and curative effects against ethanol-induced memory impairments (Ordoudi, Befani et al. 2009).

Cro, a member of the carotenoid family, has a dicarboxylic 20-carbon and symmetrical chemical structure (Fig 4-B) which includes seven double bond and four methyl groups. Carboxylic groups at each end of the carbon chain stabilize the molecule (Giaccio 2004). In mice, orally administered Cro is partially conjugated to the mono- and diglucuronides both in the intestine and in the liver. The free form of Cro is rapidly metabolized to these glucuronide conjugates (in 8 h), one of the reasons of its metabolic fate is different from other common $C_{40}$ carotenoids (Asai, Nakano et al. 2005).

Cro is reported to have protective and/or beneficial effects in various conditions including atherosclerosis, alveolar oxygen transport/pulmonary oxygenation, hemorrhage, arthritis and cancer, based on the ability of this compound to enhance oxygen transport and diffusivity (Giaccio 2004). Cro (in saffron) has been extensively used as traditional TCM for thousands of years and this compound shows potential as a drug and nutraceutical.

Several, but not all of these effects may be related to the antioxidant properties of Cro which are due to its conjugated diene structure. In human monocytes, Cro modulates intracellular oxidative stress by suppressing the intracellular production

of $O_2^{\bullet\square}$ (Ordoudi, Befani et al. 2009). In mitochondria, Cro protects against benzo[a]pyrene mediated oxidative damage by decreasing the levels of $O_2^{\bullet\square}$, $H_2O_2$, and hydroxyl radical (Venkatraman, Konga et al. 2008). In addition, Cro prevents ROS induced vascular endothelial cell apoptosis by acting as an antioxidant (Xiang, Yang et al. 2006).

The generation of intracellular ROS is a common cause of apoptotic cell death. It has been reported that Cro protects against $H_2O_2$ and tunicamycin induced retinal degeneration by inhibiting the activities of caspase-3 and caspase-9 (Yamauchi, Tsuruma et al. 2011). Also, ROS are involved in the stimulation of many signaling pathways, Cro inhibits the development of cardiac hypertrophy *in vitro* and *in vivo* by suppressing the mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase-1/2 (MEK/ERK1/2) pathway, a ROS-dependent pathway (Cai, Yi et al. 2009).

Among the beneficial biological activities of Cro, its anticarcinogenic property has attracted more and much attention. Cro is reported to inhibit cell proliferation and tumor progression in animal models of pancreatic cancer (Dhar, Mehta et al. 2009) and lung cancer (Magesh, DurgaBhavani et al. 2009).

Shen and co-workers reported, when evaluating the protective effects of Cro on norepinephrine-mediated injury of rat cardiac myocytes, that in addition to inhibiting ROS production Cro exhibited its sub-chronic effects by increasing superoxide dismutase (SOD) activity, elevating intracellular glutathione (GSH) content, and inhibiting $Ca^{2+}$ influx (Shen, Qian et al. 2009) although each of these endpoints could be positively affected by decreased ROS formation. *In vitro*, Cro has been found to increase glutathione S-transferase (GST) and GSH peroxidase (GSH-Px) activities (Giaccio 2004) but ROS is also known to inhibit the activities of some redox regulated GST and GSH-Px isozymes so these effects could be related to decreased intracellular ROS. In addition, Cro is reported to have a beneficial effect for human ulcerative colitis by reducing the levels of the enzyme inducible nitric oxide synthesis and its product, NO (Kazi and Qian 2009).

The presence of Cro (as saffron) in effective TCM prescriptions, the fact its antioxidant activity is not due to aromatic hydroxyl groups, and its reported chemoprotectant effects in an number of cellular and *in vivo* models explain why it was included for evaluation in this thesis.

**Figure 4. Crocetin**

**A**. Photo of *Crocus sativus L.*

(Photo from: http://somethingslovely.wordpress.com/2009/12/02/crocus/)

**B.** Two dimensional and three dimensional chemical structures of Cro.

IUPAC nomenclature:

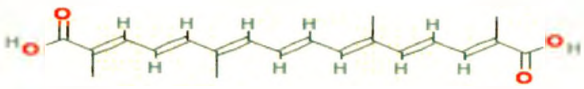
2E,4E,6E,8E,10E,12E,14E)-2,6,11,15-tetramethylhexadeca-

2,4,6,8,10,12,14-heptaenedioic acid. (From PubChem Public Chemical Database,

http://pubchem.ncbi.nlm.nih.gov/)

**A**

**B**



### 1.4.3 Resveratrol (Res)

Res has a long history as a constituent of CHM. This compound is commonly isolated from the root of white hellebore (Das and Das 2007) (Fig 5-A). It is a *trans*-stilbene polyphenol that occurs in many other food sources like grapes, peanuts, berries (Mercader, Palou et al. 2010) and Japanese knotweed (Jagdeo, Adams et al. 2010). In red wine, Res is one of the most important biologically active antioxidant components and it is present in concentrations from $0.1-15$ mg/L (Cai, Fang et al. 2003).

The term "*French Paradox*" was first used by Dr. Serge Renaud (Simini 2000) who found that despite a high cholesterol and saturated fat diet, the incidence of coronary heart disease (CHD) in France is still relatively low, which is attributed to the regular intake of red wine (Gresele, Cerletti et al. 2010). Res has attracted much attention recently based on the widespread consumption of red wine and the presumed safety of natural products, including CHD.

Although initial interest focused on the cardioprotective effects of Res, more recently it has been evaluated for many other conditions. Thus, Res has been recently and extensively studied for its beneficial health effects, including protection against aging, inflammation (Bereswill, Munoz et al. 2010), cancer, neurodegeneration (Liu, Shi et al. 2011), diabetes, viral infection and Alzheimers disease (Kapetanovic, Muzzio et al. 2010). In addition, Res has been reported to possess antioxidant and anti-apoptotic activities, to attenuate platelet aggregation and to modulate blood lipid metabolism (Liu, Shi et al. 2011).

Res (3,5,4'-trihydroxy-trans-stilbene) has two phenolic rings connected by a styrene double bond (Fig 5-B). It achieves its peak plasma concentration 30 min after oral administration in mice (Goldberg, Yan et al. 2003), however, this peak concentration in circulating blood is inadequate to achieve effective biological activity *in vivo* because of rapid metabolism to glucuronide and sulfate conjugates

(Kapetanovic, Muzzio et al. 2010). Such pharmacokinetic limitations can be potentially overcome by repeated administration or the use of prolonged release dosage forms in humans.

Among its large number of biological effects, its antioxidant activity has been particularly well documented. By decreasing the production of intracellular ROS, Res protects against oxidative stress induced death of neuron-like PC6.3-cells (Kairisalo, Bonomo et al. 2010). In human skin fibroblasts, the generation of ROS free radicals attenuated by Res in a dose-dependent manner (Jagdeo, Adams et al. 2010). As an antioxidant, its protective effect includes attenuation of the oxidation of lipid and protein. For example, 15 μM Res prevented *tert*-butyl hydroperoxide-induced lipid peroxidation and the damage of plasma membranes in human sperm and in rat germinal cells (Collodel, Federico et al. 2010). Also, Res showed significant attenuation of protein oxidation, as determined by decreased protein carbonyl content in red blood cells and plasma (Pandey and Rizvi 2009). Similar to ROS, RNS are also known to be involved in the pathogenesis of many diseases. Res also protects against nitrosative stress. For example, it has been reported that Res reduced *S. enterica serovar Typhimurium*-induced nitric oxide (NO) production in a time- and dose-dependent manner (Paolillo, Carratelli et al. 2011)

In some cases, the protective effect of Res is achieved through the combination of multiple mechanisms, for example, Liu et al reported that Res protects against the complications of spinal cord injury by upregulating SOD activity, inhibiting neutrophil infiltration and regulating the expression of caspase-3, an initiator of apoptosis (Liu, Shi et al. 2010). Res increases the intracellular concentration of the critical antioxidant, GSH. More specifically, 10-100 μM Res attenuated $H_2O_2$-induced oxidative damage and decreased GSH depletion (Yen, Duh et al. 2003). Xanthine oxidase is also a source of oxidative stress. Res has been reported both to scavenge superoxide as well as prevent its production by inhibiting xanthine oxidase activity (Jia, Zhu et al. 2008). Res was

included for evaluation in this thesis because it is active against both ROS and RNS stressors and it is a quantitatively important constituent of red wines and TCM prescriptions that have been widely used for centuries.

**Figure 5. Resveratrol**

**A**. Photo of white hellebore

(Photo from: images.mooseyscountrygarden.com/.../60/)

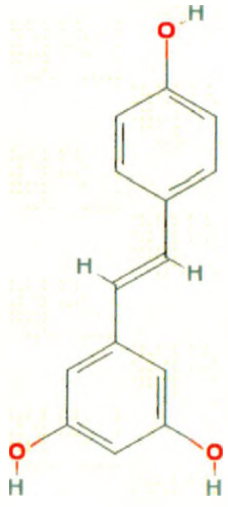
**B.** Two dimensional and three dimensional chemical structures of resveratrol.

IUPAC nomenclature: 5-[(E)-2-(4-hydroxyphenyl)ethenyl]benzene-1,3-diol.

(From PubChem Public Chemical Database, http://pubchem.ncbi.nlm.nih.gov/)

**A**

**B**




### 1.4.4 Schisanhenol (Sal)

Sal is isolated from the ripe fruit of *Schisandra rubriflora* Rhed (Fig 6-A) (Zhang, Lin et al. 1995). It was first recorded in the ancient pharmaceutical book "*Shen nong ben cao jing*" as a superior drug (Lu and Chen 2009) and has been used as a traditional CHM for 2,500 years. This CHM, which contains Sal, has long been used as a tonic, sedative, and astringent agent (Chen, Kilgore et al. 2006) as well as for the treatment of chronic cough and dyspnea, nocturnal emission, spermatorrhea, enuresis, frequent urination, protracted diarrhea, spontaneous sweating, impairment of body fluids with thirst, shortness of breath and feeble pulse, diabetes and wasting-thirst caused by internal heat, palpitation and insomnia (Lu and Chen 2009). Besides these effects, Sal is also reported to possess other beneficial activities including anti-tumor, anti-hepatitis (Chen, Kilgore et al. 2006), detoxicant, anti-HIV, and platelet-activating factor antagonistic activities (Wei, Sun et al. 2010).

The major and characteristic bioactive constituents of the fruit of *Schisandra rubriflora* are dibenzocyclooctene lignans (Chen, Kilgore et al. 2006; Lu and Chen 2009). Modern pharmacological research has found that lignans exhibit various beneficial pharmacological effects. Eight different dibenzocyclooctene lignans were isolated from *Schisandra rubriflora* (Wei, Sun et al. 2010) with Sal being the most biologically active (Lu and Liu 1992). Sal (Fig 6-B) possesses a structure with five methoxylated phenolic hydroxyl groups. This chemical structure increases its lipophilicity (Chen, 2008), relative to the same molecule containing 5 phenolic hydroxyl groups, facilitating the efficient absorption of Sal into cells. Our collaborator, Professor Daofeng Chen from Fudan University in Shanghai has isolated, purified and characterized Sal (Chen, Kilgore et al. 2006); (Lu and Chen 2009). From *in vivo* pharmacokinetic studies in rats, Dr. Chen and his colleagues found a prolonged biological half-life which is about 1.6 h of Sal because of its

methoxylated phenolic groups. These relatively stable ether groups will be O-demethylated by hepatic and extrahepatic microsomal cytochrome P450 isozymes to active antioxidant polyphenols, prolonging its antioxidant properties *in vivo*. Vitamin E is a well-known lipophilic antioxidant whose activity depends mainly upon its phenolic hydroxyl group (Lin, Liu et al. 1990). Sal has one unmethylated phenolic hydroxyl group in its chemical structure so that it has antioxidant properties even prior to P450-dependent metabolism (Lin, Liu et al. 1990). The potent antioxidant property of Sal has been attributed to its ability to scavenge $O_2^-$, $R^-$, $RO^-$, and $ROO^-$ radicals and the inhibition of ROS generation (Yu and Liu 2008)

Due to the inability to purchase or readily obtain pure Sal there are a paucity of studies that have carefully evaluated its biological activities. Yu and his colleagues reported that Sal inhibited the copper ion-ROS catalyzed oxidation of human low-density lipoprotein to form oxidized low density lipoprotein (ox-LDL) in a dose-dependent (from 10 μM to 100μM) manner (Yu, Liu et al. 2004). This group also demonstrated that Sal attenuated human ox-LDL-induced apoptosis in a bovine aorta endothelial cell model (Yu and Liu 2008). Sal is believed to be concentrated in mitochondria of cells after exposure. Lipid peroxidation is known to decrease membrane fluidity with the loss of ATPase activity in rat liver mitochondrial and microsomes, however, these oxidant effects were significantly inhibited by 1 mM Sal (Lin, Liu et al. 1992; Lu and Liu 1992). It has also been reported that 1 mM Sal prevented Vit C/NADPH induced lipid peroxidation in rat liver microsomes (Lu and Liu 1991), but these concentrations are very high for potential therapeutic use.

Sal has also been reported to have an effect on the CYP monooxygenase system. Liu et al (1985) reported that Sal can protect against $CCl_4$-mediated liver injury via induction of hepatic microsomal monooxygenases, although this seems to be an unlikely mechanism for chemoprotection. These authors noted an increase in aminopyrine demethylation activity (Liu and Wei 1985), a pathway catalyzed by

CYP isozymes and Lu et al (1990) also reported that Sal and Schizandrin, 200 mg/kg administered once daily by intragastric lavage, increased glutathione S-transferase and total CYP in mice and rats *in vivo* (Lu and Liu 1990). These are also high dose effects.

The protective effects of Sal also involve other mechanisms. For example, Schisanhenol can inhibit ethanol mediated malondialdehyde formation, a marker for lipid peroxidation, *in vivo* through increasing superoxide dismutase and catalase activities in the cytosol of rat liver (Lu and Liu 1991). Oral administration of Sal (200 mg/kg once daily for 3 days) in mice and rats can enhance liver glutathione-*S*-transferase (GSH *S*-T) level (Lu and Liu 1990).

Sal was selected as one of the TCM constituents in this thesis because it is reported to be concentrated in mitochondria, it has antioxidant activity both independent of and dependent upon metabolic activation to phenolic hydroxyl groups, and it has prolonged *in vivo* stability because of extensive methoxylation (5 of 6) of its phenolic hydroxyl groups. In addition, few carefully controlled experiments have been performed to evaluate the chemoprotectant properties of Sal at doses that might be used for chemoprotection.

**Figure 6. Schisanhenol**

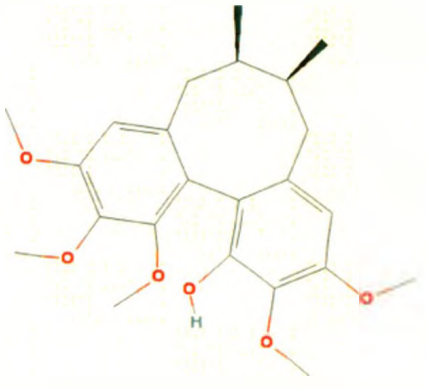
**A**. Photo of *Schisandra rubriflora* Rhed fruit

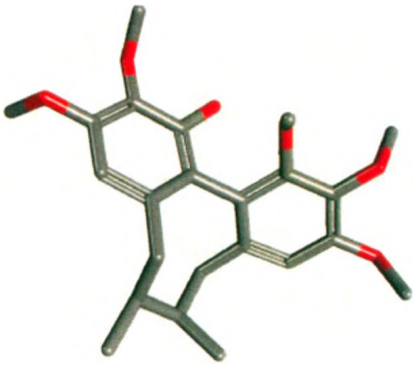
(Photo from: www.nutralegacy.com/wp-content/uploads/2008/1)

**B.** Two dimensional and three dimensional chemical structures of schisanhenol. IUPAC name: 5,6,7,8-tetrahydro-2,3,10,11,12- pentamethoxy-6,7-dimethyl-dibenzo(a,c)cycloocten-1-o1 ((From PubChem Public Chemical Database, http://pubchem.ncbi.nlm.nih.gov/)

**A**

**B**



### 1.4.5 Mixtures of purified active TCM constituents

In the clinical application of TCM, each remedy routinely contains hundreds of constituents and is seldom used alone. Most times, different natural products are combined into prescriptions to achieve better therapeutic effect. The combination of TCM preparations is based on practical experience and the basic theories of TCM. Although each different TCM product has its own biological properties, pharmacological activity and sphere of application, the interactions among the herbal medicines that can occur either alone or because of their combinatorial use has received more and more attention in the last two decades (Li, Wang et al. 2010). These herbal interactions produce positive and/or negative effects on the therapeutic efficacy which can enhance the curative effect or increase unwanted side effects. As with interactions between drugs used in Western medicine, induction or inhibition of drug-metabolizing enzymes and/or drug transporters are an important contributor to these interactions (Li, Wang et al. 2010). The formulated combinations of herbal medicines may lead to higher toxicity or better therapeutic effects by enhancing or reducing their plasma and tissue concentrations.

Among these herbal interactions, synergistic effects are of particular note. Synergism in medicine can be defined as two or more medicines working together to generate a more powerful positive effect not obtainable by any of the medicines singly (*from Wikipedia*). Many TCM medicines and constituents are known to possess antioxidant effects and each single constituent may function through a different mechanism, including direct antioxidant actions, increasing the level of endogenous antioxidants, attenuation or repair of oxidative damage (Aftab, Likhitwitayawuid et al. 2010). For example, resveratrol and curcumin are two well-known antioxidants. An equimolar (5 μM) combination of resveratrol and curcumin achieved two-fold greater antioxidant activity than either compound singly (Aftab, Likhitwitayawuid et al. 2010). Besides antioxidant effects,

synergism also occurs with other activities. *In vivo*, the mixture of emodin (2.5 mg/kg) and baicalein (20 mg/kg) was injected through the jugular vein into rats, and the pancreatic TNF-α, IL-6, and myeloperoxidase activity were measured 12 h later. The results showed that the combination of emodin and baicalein showed more powerful inhibition of acute pancreatitis than emodin or baicalein alone (Li, Xia et al. 2009). *In vitro*, a synergistic anti-cancer effect of baicalein and silymarin was detected in the alteration of cell cycle, especially in the G1/S transition in human hepatoma HepG2 cells (Chen, Huang et al. 2009). Research with several promising antioxidants has rarely attempted to characterize the effects of both single constituents of varying structure and function/mechanism and formulated combinations of these compounds. We have evaluated BE, Cro, Res and Sal individually and as mixtures, to determine if they might act synergistically to prevent or attenuate oxidative damage through complementary antioxidant or immunomodulant mechanisms. The premise of this work is that high nanomolar or low micromolar doses of these compounds will be required for effective protection against ADRs in humans, especially children.

## 1.5 INTEGRATED ASSAYS FOR OXIDATIVE AND NITROSATIVE STRESS INDUCED CELL TOXICITY

In our project, Jurkat T cells were selected to investigate the reactive drug metabolite (SMX-HA) induced cytoxicity. High level expression of T cells in blood and skin from patients with drug hypersensitivity reactions indicated that T cells, work as an vital immune mechanism to regulate the development of immune-induced ADRs (Naisbitt, Pirmohamed et al. 2007) We have evaluated the effects of SMX-HA-mediated oxidative and nitrosative stress in the Jurkat *E*6.1 cell line. These cytotoxic endpoints include lactate dehydrogenase (LDH) release, as an index of damage to the external membrane of Jurkat *E*6.1 cells treated with DMSO (vehicle control); SMX (negative control) or SMX-HA (the oxidative and nitrosative stressor) in the presence and absence of low micromolar concentrations of BE, Cro,

Res and Sal, alone or in combination. In addition, the amount of lipid peroxidation and protein carbonylation (endpoint for protein oxidation) that occurred under the same treatment conditions as outlined above were investigated to evaluate the ability of the various phytochemicals investigated to attenuate damage to these cells. In addition, we analyzed the contents of treated Jurkat *E*6.1 cells by redox-two dimensional polyacrylamide gel electrophoresis (R2DPAGE) to determine the types and amount of protein mixed disulphides formed as an index of the oxidation of proteins containing reactive protein thiol residues, that is cysteine residues that were ionized at physiological pH. This is an excellent assay for changes in the oxidation state of proteins that contain one or more reactive cysteine thiol residue, and are redox regulated.

## 1.6 DISULFIDE PROTEOME

Proteome refers to all of the proteins that are found in a particular cell type, and includes different molecular forms of the same protein formed by such well known post-translational modifications as acetylation, phosphorylation, ubiquitation, S-glutathionylation. The disulphide proteome describes only those proteins that contain cysteine residues with reactive protein thiols which are redox regulated . The thiol group of reactive cysteine residues is one of the most reactive amino acid side chains in protein and peptides (Perez, Pierce et al. 2010). The protein-protein disulphide bond is derived from pairs of thiol groups of cysteine residues and is the most common of the oxidized modifications of thiol group, after S-glutathionylation. The formation of mixed protein disulfides occurs because of the oxidation of sulphhydryl (-SH) groups and this reaction is promoted by many oxidants including iodine, hydrogen peroxide, and copper (Dalle-Donne, Scaloni et al. 2005). However, disulphide bonds are also produced in the endoplasmic reticulum (ER) in eukaryotic cells with the absence of exogenous oxidation (Cumming, Andon et al. 2004). The disulphide bond is a weak link in many molecules and unstable in the cytosol due to the reducing environment of

most cells (Hatahet, Nguyen et al. 2010). The property of thiol group made it sensitiv to oxidation. This redox regulation by oxidation of reactive protein thiol can induce structural changes in protein and lead to the modification of protein functions. Based on this, disulphide proteomics has been widely used to identify the proteins involved in redox regulation and signaling. For example, disulphide bond has been reported to possess various activities in protein regulation such as the control of protein folding, the stabilization of the native states of proteins via protection against the attack of ROS (Liang and Fernandez 2009; Arai, Dedachi et al. 2011).

Protein disulphide bonds are a reversible modification. Protein disulphides can be reduced to their thiol state, or be further oxidized by strong oxidants to protein sulphinic and sulphonic acids. Glutathione protein mixed disulphides may be involved in cell signaling (Lindahl, Mata-Cabana et al. 2011). The reduction of the disulfide bond at most times occurs through the thiol/disulfide exchange reaction. In this reaction, the original disulfide bond is attacked and replaced by a new disulfide bond which is formed between the attacking thiolate and the original protein sulfur atom (Gilbert 1995). *In vitro*, dithiothreitol (DTT) is a commonly used dithiol reducing agent which can reduce protein disulphides to their constituent thiols and also prevent the formation of additional disulfide bonds (Liang and Fernandez 2009). DTT was used in this project (2D-PAGE) to reduce protein disulphide bonds prior to resolution of cellular proteins in the second dimension of R2DPAGE.

Under physiological conditions, the reduction of protein disulphide bonds involves two systems in the cytoplasm, that includes cysteine-containing tripeptide glutathione (GSH) or thioredoxin. Both systems reduce disulphide bonds through a thiol-disulfide interchange reaction and a dithiol-disulfide exchange reaction, respectively (Cumming, Andon et al. 2004). As we mentioned above, protein cysteine are susceptible to attack by ROS and RNS (Mieyal, Gallogly et al. 2008).

Mild oxidation of protein reactive cystein cysteines thiols can generate protein mixed disulfides which are derived from protein thiol groups, or

low-molecular-mass disulphides resulting from reaction of an oxidized proteins cysteine thiol with the thiol of GSH. The first step in either S-glutathionylation of protein mixed disulphide formation is oxidation of protein reactive cysteine thiol to protein cysteine sulphenic (PSOH) acid. The PSOH derivative can either react non-enzymatically wither with a thiol of proteins or GSH, to form a protein-protein disulphide or a protein-glutathione disulphide (Giustarini, Rossi et al. 2004), respectiviely. The PSOH, in the formation of excess ROS or RNS can be further oxidized to form irreversible, highly oxidized states such as protein sulphinic ($PSO_2H$) and protein sulphonic ($PSO_3H$) acids (Fig 7). These post-translational modifications in the dithiol/disulfide state of protein can cause alterations in the functions of the protein (Jeong, Jung et al. 2011).

Redox two-dimensional SDS-PAGE is one of techniques that has been widely used in recent years to identify proteins that contain either intra- or intermolecular disulphide bonds in cells (Leichert and Jakob 2006). In our project, this technique was used to identify disulfide-bonded proteins. Overall, disulfide proteomics, although time consuming and not suitable for proteins that occur at low concentrations in cells, is an excellent endpoint to evaluate intracellular changes to proteins that occur as a result of oxidative or nitrosative stress.

### 1.6.1 Redox-regulated Peroxiredoxins

Peroxiredoxins (Prxs) belong to an ubiquitous family of antioxidant enzymes that are highly expressed in many cell types (Li and Fu 2010), including Jurkat cells (Cox, Pearson et al. 2009). Prxs are thiol-dependent peroxidases which control the level of toxic peroxides and peroxynitrites in cells (Tripathi, Bhatt et al. 2009). The first Prxs were found in *Saccharomyces cerevisiae* where these enzymes prevent the oxidation of glutamine synthase (Kim, Kim et al. 1988). The majority of the Prxs are located in the cytosol. Prxs are generally divided into three classes: typical 2-Cys Prxs, atypical 2-Cys Prxs, and 1-Cys Prxs. All of them contain at least one reactive cysteine thiol at the N-terminal region of the protein. In addition, 2-Cys Prxs contain

another reactive cysteine at the C-terminal region of the protein (Robinson, Hutchinson et al. 2010) and fulfill the catalytic activity through the oxidation of redox-active cysteine to a sulphenic acid (Abbas, Breton et al. 2008), as described above, by reacting with peroxides, the cysteine residue at its primary catalytic center of Prxs is oxidized to a sulphenic acid. The sulfenic acid is reduced through a next catalytic step which is the reaction with another thiols to form an intermolecular (2-Cys Prx) or intramolecular (1-Cys Prx) bond (Tripathi, Bhatt et al. 2009). The reaction of Prxs is a cycle which includes three steps: oxidation, derivatization, and regeneration (Hofmann, Hecht et al. 2002).

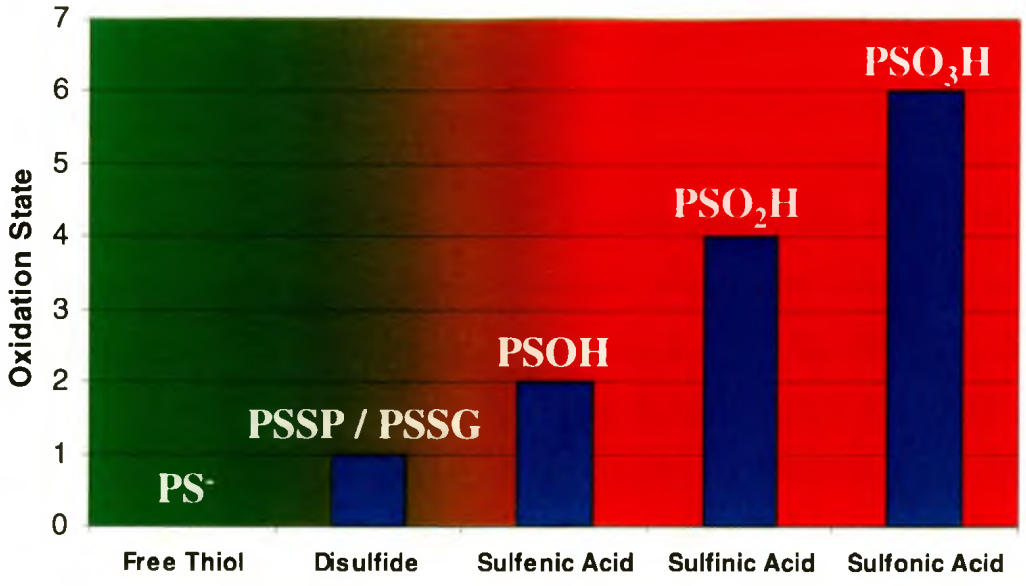
Prxs exhibit many important physiological properties mainly due to their relative abundance, which account for up to 1% of all soluble cellular protein (Cox, Winterbourn et al. 2010). More and more studies have shown the critical role that Prxs play to protect the cells of different organisms through controlling hydrogen peroxide signaling and oxidative and nitrosative stress (Shuvaeva, Novoselov et al. 2009). Also, Prxs have been reported to be involved in various cellular functions such as proliferation, cell cycle, apoptosis, as well as differentiation (Abbas, Breton et al. 2008). Most recently, Prxs was reported to be involved in the immune modulation via binding and activation of antigen presenting cells (Hall, Nelson et al. 2010).

In our project, we used redox two-dimensional SDS-PAGE to identify the change in oxidation state of proteins that form either intra- or intermolecular disulfide bonds in Jurkat *E*6.1 cells. The Prx enzymes are particularly good biomarkers to evaluate the oxidative effect of the reactive drug metabolite, SMX-HA, because some of these enzymes are present at high concentration and the normal catalytic cycle includes a dimeric disulphide that shows up on analysis. Excess oxidation of these Peroxiredoxins to their sulphinic or sulphonic acid derivatives results in their apparent disappearance from the gel because they are not reduced by DTT. The protein, Prx 1 and 2 were used as our key biomarkers in the R2DPAGE experiments because, among the redox regulated proteins, they occurred

at the highest concentration. This protein is also expressed at high concentrations in other cell types, including erythrocytes (Low, Hampton et al. 2008). Prx 2 also plays a critical role in the detoxication of ROS and RNS. An earlier proteomic study also noted that Prx 2 (along with GAPDH) was the most sensitive protein to oxidation in Jurkat cells following exposure to relatively low concentrations (20 μM for 10 min) of hydrogen peroxide (Baty, Hampton et al. 2005).

PSOOH
PSO2H
PSOH
PSSP / PSSG
PS

**Figure 7. Redox regulation of reactive protein cysteine thiol (PS-) residues**
(adapted from Biteau, B.et al. 2003. Nature 425, 980-984)


Oxidation State
7
6
5
4
3
2
1
0
PS⁻
PSSP / PSSG
PSOH
PSO2H
PSO3H
Free Thiol
Disulfide
Sulfenic Acid
Sulfinic Acid
Sulfonic Acid

# CHAPTER 2: OVER-RIDING RESEARCH HYPOTHESES AND SPECIFIC OBJECTIVES

Different cellular redox systems protect the reduced intracellular status in the face of a highly oxidizing extracellular environment. The *in vitro* cytotoxicity of SMX-HA in PBMCs has been used previously as a model for the study of delayed-type hypersensitivity reactions associated with sulphonamide drugs (Vyas, Roychowdhury et al. 2005). Exposure of lymphocytes to SMX-HA would be predicted to result in the formation of ROS and RNS (Rieder, Uetrecht et al. 1988). It has been shown that, under biological conditions, SMX-HA auto-oxidizes to its nitroso metabolite, which would be anticipated to produce downstream oxidative and nitrosative stress (Rieder, Krause et al. 1995).

Four pure constituents of traditional Chinese remedies, baicalein (BE), crocetin (Cro), resveratrol (Res), and schisanhenol (Sal), have antioxidant properties and are reported to attenuate cytotoxicity of chemicals associated with oxidative or nitrosative stress in different cell model systems. The reason for the selection of these four compounds is that they are structurally diverse, with the potential that they will have complementary mechanisms of chemoprotectant activity. These chemicals were evaluated individually, and as two equimolar mixtures, for their ability to counteract the toxic effects of SMX-HA in Jurkat *E*6.1 cells to test for enhanced activities of the equimolar (vs single constituent) mixtures. The TCM constituents were tested for their ability to acutely attenuate SMX-HA-mediated damage to the cell membrane, as well as increased cellular lipid peroxidation and protein oxidation, the latter assessed by the degree of protein carbonylation and oxidative changes to the disulphide proteome.

Jurkat *E*6.1 cells, a human leukaemic T cell lymphocyte line, were selected for the determination of SMX-HA cytotoxicity in this thesis as they are a cell line derived from lymphocytes; extensive earlier work by Spielberg and his colleagues (Rieder *at al.,* 1988; 1989; 1995) showed that peripheral blood mononuclear cells

(PBMCs), composed of lymphocytes, monocytes and macrophages, from patents with an ADR to SMX are more sensitive to SMX-HA that those from controls and that a useful model system for hypersensitivity ADRs can be based on cells of lymphocytic origin.

**Over-riding Research Hypothesis**

1. The administration of purified constituents of TCM in Jurkat *E*6.1 cells, in combination, at high nMolar or very low μMolar doses will attenuate the oxidative and nitrosative stress associated with administration of drugs such as sulphonamides that cause ADRs.
2. The attenuation of the oxidative and nitrosative stress mediated by drugs causing ADRs will, in turn, attenuate the severity of the ADR, providing chemoprevention under ideal conditions.

**Specific Objectives**

1. Determine the effects of BE, Cro, Res and Sal, alone and in combination, on Jurkat *E*6.1 cells in a time and concentration-dependent manner.
2. Determine the acute, concentration-dependent cytoprotective effects of the pre-exposure of Jurkat *E*6.1 cells to BE, Cro, Res and Sal, alone and in combination, on the amount of lactate dehydrogenase (LDH) released subsequent to treatment with SMX-HA.
3. Determine the acute, concentration-dependent cytoprotective effects of the pre-exposure of Jurkat *E*6.1 cells to BE, Cro, Res and Sal, alone and in combination, on the amount of lipid peroxide formed subsequent to treatment with SMX-HA.
4. Determine the acute, concentration-dependent cytoprotective effects of the pre-exposure of Jurkat *E*6.1 cells to BE, Cro, Res and Sal, alone and in combination, on the protein carbonyls formed subsequent to treatment with SMX-HA.

5. Determine the acute, concentration-dependent cytoprotective effects of the pre-exposure of Jurkat *E*6.1 cells to BE, Cro, Res and Sal, alone and in combination, on the oxidative changes to the disulphide proteome, especially at the level of the redox-regulated peroxiredoxins, subsequent to treatment with SMX-HA.

# CHAPTER 3: MATERIALS AND METHODS

## 3.1 MATERIALS AND CHEMICALS

High quality TCM constituents commercially available (>95% purity) were used throughout. Baicalein was purchased from Aldrich Chem. Co. (Milwaukee, WI); crocetin from MP Biomedicals, LLC (Solon, Ohio); and *trans*-resveratrol from Sigma-Aldrich (Oakville, ON, Canada). Schisanhenol (> 99%) was isolated and purified from plant sources by our collaborator within the Consortium for the Globalization of Chinese Medicine (CGCM), Professor Daofeng Chen, Fudan University, Shanghai (Lu and Chen 2009). Dimethyl sulfoxide (DMSO, Hybri-Max™), Triton® X-100, L(+)-lactic acid, iodonitrotetrazolium chloride (INT), phenazine methosulfate (PMS) and β-nicotinamide adenine dinucleotide hydrate from yeast (NAD) were purchased from Sigma-Aldrich (Oakville, ON, Canada).

Lipid Hydroperoxide (LPO) Assay kits (96 well) and Protein Carbonyl Assay kits were purchased from Cayman Chemical Company (Ann Arbor, MI). BD Falcon™ 75 or 150cm$^2$ tissue culture flasks, BD Falcon™ 5mL polypropylene round-bottom tubes, BD Falcon™ 5mL polystyrene round-bottom test tubes, Falcon® Blue Max™ 50mL polypropylene conical tubes, Falcon® Microtest™ tissue culture flat-bottom 96-well plates and Falcon® Multiwell™ tissue culture flat-bottom 12 well plates used for culture of cells were purchased from BD Biosciences (Mississauga, ON).

## 3.2 JURKAT *E*6.1 CELL LINE

The Jurkat cell line was initially established from the peripheral blood of a 14 year-old-boy (Schneider, Schwenk et al. 1977). The Jurkat *E*6.1 cells (human leukaemic T cell lymphocytes) used throughout this thesis are a clone of the Jurkat

–FHCRC line, derived from the original Jurkat cell line (Weiss, Wiskocil et al. 1984). The Jurkat *E*6.1 cell line was obtained from the American Type Culture Collection (ATCC), reference #TIB-152 (http://www.atcc.org).

## 3.3 CELL CULTURE AND CELL PREPARATION

The Jurkat *E*6.1 culture medium was prepared by dissolving 16.2 g RPMI medium 1640 (containing 2 mM L-glutamine, 10mM HEPES, 1mM sodium pyruvate, 4.5 g/L glucose, and 1.5 g/L sodium bicarbonate) powder in 1 L distilled water, then add 2g $NaHCO_3$ and adjust pH of medium to 7.2. Cell culture medium was filtered in the fume hood to remove undissolved particles. The prepared Jurkat *E*6.1 medium was kept in the 4 °C refrigerator until it is used for cell culture and experiments. The Jurkat *E*6.1 cell line was maintained in BD Falcon™ 75 $cm^2$ or 150 $cm^2$ tissue culture flasks in the incubator with humidified atmosphere at 37 ± 0.5 °C and 5% $CO_2$. Cell concentrations were maintained between $1\times10^5$ and $1\times10^6$ cells/mL. Cultures were maintained by adding the fresh medium or replacement of medium every 2 to 3 days.

All the cell culture work and cell preparation for experiments were performed in a sterile laminar flow hood at room temperature. Prior to the use of Jurkat *E*6.1 cells for experiments, cell medium was brought up for 30min until it reached to room temperature (20-26 °C). Cells were transferred to Falcon® Blue Max™ 50mL polypropylene conical tubes and centrifuged at 500 × g (1,500 rpm) using a S4180 Rotor with a Beckman GS-15R centrifuge for 10min. The supernatant was then discarded, and pellet was washed with 50mL phosphate buffered saline buffer (1×PBS; 1.15% $Na_2HPO_4$, 0.2% $KH_2PO_4$, 8.0% NaCl, 0.2% KCl, pH7.4) and centrifuged again in the same condition. After that, cells were resuspended in cell culture media to the desired cell density.

### 3.3.1 Trypan Blue Exclusion Assay

Trypan blue is a vital stain that used for viable cell counting (Phillips and Terryberry 1957). The principle of this assay is that live cells exclude the trypan blue dye using energy-dependent membrane pumps, while dead cells are stained blue due to dye inclusion, which is basis for the differential staining which enables visualization to determine viable versus non-viable cells.

0.4% trypan blue solution and a hemocytometer were used to determine total cell counts and viable cell number. A cell suspension-trypan blue mixture was prepared by mixing the same volume of cell suspension and trypan blue solution, and allowing the mixture to stand for 5 min. With the cover-slip in place, Pasteur pipette was used to transfer a small amount of the cell suspension-blue mixture to both chambers of a hemocytometer by carefully touching the edge of the cover-slip with the pipette tip, and allowing each chamber to fill by capillary action. Cells were then counted starting with the top and left at perimeter of each square, cells touching the middle line at bottom and right sides were excluded from the count. The procedure was repeated for the centre square and four corner squares of both chambers.

The cell concentration per mL was determined using the following calculation:

Cells per mL = the average count per square × dilution factor × $10^4$ (count 10 squares)

## 3.4 DRUGS

### 3.4.1 N-Hydroxyl-sulfamethoxazole

The synthesis of *N*-Hydroxyl-sulfamethoxazole (SMX-HA, IUPAC: 4-(hydroxyamino)-*N*-5-methyl-1,2-oxazol-3-yl)benzenesulfonamide, MW:269.2784) was according to the the method of (Rieder, Uetrecht et al. 1988). SMX-HA prepared by Dr. Michael Kerr at the University of Western Ontario

showed the purity ≥99% under high performance liquid chromatography (HPLC), mass spectrometry, and nuclear magnetic resonance analysis.

### 3.4.2 Baicalein

Baicalein (BE, IUPAC: 5,6,7-trihydroxy-2-phenylchromen-4-one, MW: 270.24) is a major flavonoid isolated from the root of *Scutellaria baicalensis* (Liu, Wu et al. 2010). BE is yellow crystalline solid and possess low water-soluble ability. Before each experiment, BE was dissolved in small amount of DMSO and then diluted with the appropriate cell culture medium. Before each experiment so that all TCM constituents could be treated identically. BE was freshly prepared for each experiment. The final concentration of DMSO in the cell culture medium did not exceed 0.2%.

### 3.4.3 Crocetin

Crocetin (Cro, IUPAC: (*2E,4E,6E,8E,10E,12E,14E*)-2,6,11,15-Tetramethyl- 2,4,6,8,10,12,14- hexadecaheptaenedioic acid, MW: 328.4) is an active carotenoid that is found in the crocus flower (Shen, Qian et al. 2009). Cro was freshly prepared before each experiment. Briefly, Cro was dissolved in small amount of DMSO and then diluted with the appropriate cell culture medium. The final concentration of DMSO in the cell culture medium was maintained at 0.2%.

### 3.4.4 Resveratrol

*Trans*-resveratrol (Res, IUPAC: (*E*)-5-(4-hydroxystyryl)benzene-1,3-diol, MW: 228.24) is a natural polyphenolic phytolexin isolated from the roots of white hellebore and found in red grapes and red wine (Das and Das 2007). The white powder with slight yellow cast was freshly dissolved in DMSO, and then diluted in

the appropriate cell culture medium or buffer prior to each experiment. The final concentration of DMSO used in the cell culture did not exceed 0.2%.

### 3.4.5 Schisanhenol

Schisanhenol (Sal, IUPAC:5,6,7,8-tetrahydro-2,3,10,11,12-pentamethoxy-6,7-dimethyldibenzo(a,c)cycloocten-1-o1, MW: 402.45) is a dibenzocyclooctene lignan derived from the ripe fruits of *Schisandra* plants (Zhang, Lin et al. 1995). A schisanhenol stock solution was freshly prepared by first dissolving the white powder in DMSO and then diluting with the appropriate cell culture medium prior to each experiment. The final concentration of DMSO in the cell culture medium was never more than 0.2%.

### 3.4.6 Mixture 1

Synthetic mixture 1 was an equimolar mixture prepared so that a 1 μM solution was 0.25 μM for each of BE, Cro, Res, and Sal, providing a final combined concentration of 1 μM. The ingredients were initially dissolved in DMSO, and then diluted in the appropriate cell culture medium. The final concentration of DMSO used in the cell culture medium was never more than 0.2%.

### 3.4.7 Mixture 2

Synthetic mixture 2 was an equimolar mixture of BE, Cro and Res prepared so that a 1 μM solution of final incubation mixture contained 0.33 μM BE, Cro and Res, equivalent to a final concentration of 1μM. This mixture was freshly prepared by first dissolving the powder in DMSO and then diluting with the appropriate cell culture media or buffer. The final concentration of DMSO in the cell culture medium was never more than 0.2%.

## 3.5 TREATMENT OF JURKAT *E*6.1 CELLS

One of the objectives of this thesis was to determine the effects of individual and combined constituents of TCM on the redox status of Jurkat *E*6.1 cells alone, to test for any cytotoxic effects. In addition, an important objective was to determine the cytoprotective activity of these TCM constituents, alone and in combination, against the cytotoxicty of the electrophilic and reactive metabolite of SMX, SMX-HA. In short, we set out to determine if antioxidant and immunomodulant BE, Cro, Res or Sal, which act by different antioxidant mechanisms, could protect against the cytotoxicity of SMX-HA, a known source of oxidative and nitrosative stress. We also evaluate the lowest concentration (range) over which these compounds are cytoprotective.

Jurkat *E*6.1 cells were transferred to Falcon® Blue Max™ 50 mL polypropylene conical tubes and centrifuged at 500 × g (1,500 rpm) using a S4180 Rotor with a Beckman GS-15R centrifuge for 10 min. The supernatant was then discarded, and pellet (cells) was washed with 50 mL PBS buffer and centrifuged again in the same condition. After that, the supernatant was discarded and the cells were resuspended in RPMI-1640 medium supplemented with P/S, and the cell density was adjusted to $5\times10^5$ cells/mL. Cells were routinely divided into multiple groups for individual experimental treatments and were seeded at $1\times10^5$ cells/well in triplicate for each group in Falcon® Microtest™ tissue culture flat-bottom 96-well plates. All test incubations were conducted at 37 °C in a 5% $CO_2$ humidified environment, and all experiments were repeated at least 3 times by using different cell cultures in each time.

To test the cytotoxicity effects of the various TCM constituents alone and in combination on Jurkat *E*6.1 cells, cells were incubated with different concentrations (from 6.25 μM to 400 μM) of BE, Cro, Res, Sal, mixture 1, and mixture 2 for 6 h and 24 h, then the level of released of LDH was compared to cells treated with RPMI-1640 medium supplemented with P/S (control) for 6 h and 24 h; with 0.2% DMSO in RPMI-1640 medium supplemented with P/S (vehicle

control) for 6 h and 24 h; and with 2% Triton X-100 (high control) for 6 h and 24 h.

Subsequently, individual group of cells were pretreated either with RPMI-1640 medium supplemented with P/S (control); with 0.2% DMSO in RPMI-1640 medium supplemented with P/S (vehicle control); with 2% Triton X-100 (high control); or with 1 or 5 μM of BE, Cro, Res, Sal, synthetic mixture 1 (equimolar mixture of BE, Cro, Res and Sal) or synthetic mixture 2 (equimolar BE, Cro and Res) for 30 min followed by incubation with 0 or 400μM SMX-HA for 2 h. As described before, all the TCM and drug solutions were freshly prepared in DMSO immediately before each experiment was performed. The individual assays are described in more detail below.

### 3.5.1 Measurement of Cell Membrane Damage by Release of Lactate Dehydrogenase

Lactate dehydrogenase (LDH) is a stable cytoplasmic enzyme that is present in many different types of cells, including plants and animals (Vanderlinde 1985). It is rapidly released into the cell culture supernatant if the cell membrane is damaged or ruptured, so that an increased release of LDH is generally attributed to cellular damage. The LDH release assay is a sensitive measure for cytotoxicity first used by Korzeniewski and Callewaert (1983).

Cytotoxicity was determined by measuring the LDH activity released into the cell free supernatants using a colorimetric assay. The increased level of LDH enzyme in the culture supernatant was mediated by the increased numbers of dead or plasma membrane-damaged cells under cytotoxicity effects. LDH leakage in the cell culture supernatant reduces iodonitrotetrazolium chloride (INT) to the formation of formazan via several steps of enzymatic reaction (Korzeniewski and Callewaert 1983). After a period of time, the formation of formazan induced the color change of cell culture supernatant. The amount of formed color is

proportional to the number of lysed cells. The formazan dye formed is water soluble and shows a broad absorption maximum near 500 nm.

The level of released of LDH into cell culture supernatant was used to evaluate the effects of BE, Cro, Res and Sal, alone and in combination on the membrane of Jurkat *E*6.1 cells in a time- and concentration-dependent manner. We use medium control incubations to measure any interfering factors contained in the assay medium. Also, medium control can be used to correct for other treatment groups to exclude any interfering factors which may influence the result. The low control incubation reflects spontaneous LDH release from untreated normal cells in the absence of any chemicals. The values of high control were obtained from cells that treated with 2% triton X-100 solution. Triton X-100 is a nonionic surfactant that can lyse cells. The incubation with 2% triton X-100 solution provided insight into the total LDH content of the cells (100% LDH release). Substrate controls (added at the end of the incubation period, immediately before analysis) provided information about any interference of the test substances (BE, Cro, Res and Sal) with the assay for LDH activity.

Jurkat *E*6.1 cells were washed in 1 × PBS and resuspended in assay medium [RPMI-1640 medium supplemented with 0.2% (v/v) BSA]. The cell seeding density has great effect on the absorbance difference between spontaneous LDH release and maximum LDH release. The optimal cell density was determined to be at $2\times10^5$ cells per mL for 6 h incubation, and $3\times10^5$ cells per mL for 24 h incubation experiments. For the leakage of LDH assay, cells were plated in triplicate into Falcon® Microtest™ tissue culture flat-bottom 96-well plates in assay medium. Cells were treated with BE, Cro, Res, Sal, mixture 1, and mixture 2 for both 6 and 24 h. The concentrations of constituents of TCM used were 6.25, 25, 50, 100, 200 and 400 μM. The whole incubations were performed at 37°C and in a 5% $CO_2$ humidified environment. After treatment for 6 or 24 h, Falcon® Microtest™ tissue culture flat-bottom 96-well plates were centrifuged at 500 × g for 10 min. 100 μL of cell culture supernatant were carefully transferred from each

well to a new Falcon® Microtest™ tissue culture flat-bottom 96-well plate. 100 µL of substrate mixture (LACTATE 54 mM, INT 660 µM, PMS 280 µM, NAD 1.3 mM) was freshly prepared (pH= 8.2) and added into the supernatant in each well. The mixtures were incubated at room temperature on rocking table for 20 min, protected from light. The absorbance of the samples was measured by a Tecan Microplate Reader at a wavelength of 490 nm. The absorbance values were collected using the XFluor 4 program.

To determine the percentage LDH release, the average absorbance values of triplicate incubations were calculated and corrected for background control absorbance. The resulting values are substituted in the following equation:

$$\text{LDH Release (\%)} = \frac{\text{exp. value} - \text{low control}}{\text{high control} - \text{low control}} \times 100\%$$

## 3.6 TREATMENT OF JURKAT E6.1 CELLS WITH BAICALEIN, CROCETIN, RESVERATROL OR SCHISANHENOL SINGLY AND IN EQUIMOLAR MIXTURES IN COMBINATION WITH THE CYTOTOXIC SULFAMETHOXAZOLE (SMX) METABOLITE, SMX-HA

### 3.6.1 Measurement of Cell Viability by Release of Lactate Dehydrogenase

One of the objectives of this thesis was to determine the effects of individual and combined constituents of TCM on the viability of Jurkat *E*6.1 cells also treated with the electrophilic and reactive metabolite of SMX, SMX-HA. Therefore, we were interested to determine if the anti-oxidants and immunomodulants BE, Cro, Res or Sal, which act by different complementary mechanisms, could protect against the cytotoxicity of SMX-HA, and the concentration range over which these compounds are cytoprotective.

Jurkat *E*6.1 cells were washed, resuspended in RPMI-1640 medium supplemented with P/S, and the density adjusted to $5\times10^5$ cells per mL. Cells were divided into individual groups and were seeded at $1\times10^5$ cells per well triplicate in each group in Falcon® Microtest™ tissue culture flat-bottom 96-well plates. A vehicle control group (0.2% DMSO in RPMI-1640 medium supplemented with P/S) was included in each experiment. Cells were pretreated with 5 or 20 μM of BE, Cro, Res or Sal, or synthetic mixture 1 or synthetic mixture 2 for 30 min. After that, some cells were treated with 0.2% DMSO in RPMI-1640 medium supplemented with P/S (vehicle control) for 2 h; others were treated with 400 μM of SMX-HA for 2 h; and the final group of cells was incubated with 400 μM of SMX-HA for 2 h. All incubations were at 37 °C and in a 5% $CO_2$ humidified environment. All drugs were freshly prepared in DMSO and RPMI-1640 medium supplemented with P/S as described above. The percentage of LDH release was also determined as described above.

### 3.6.2 Analysis of Lipid Peroxidation

Lipid is one of the important structural components of cell membrane and lipids are primary targets of oxidized modification by free radicals, mainly due to the unsaturated lipids in membranes (McIntyre and Hazen 2010). Lipid peroxidation changes the structure and function of membrane lipids. Also, lipid peroxidation results in the formation of highly reactive and unstable hydroperoxides of both saturated and unsaturated lipids. The Lipid Hydroperoxide Assay Kit (705003 with glass 96-well plate and 705002 without 96-well glass plate) supplied by the Cayman Chemical Company measures hydroperoxides directly (Mihaljevic, Katusin-Razem et al. 1996). Hydroperoxides are unstable and react readily with ferrous ions to produce ferric ions. The resulting ferric ions are detected using the thiocyanate ion as the chromogen. There were two factors that might influence the result. Firstly, samples may contain ferric ions and thus cause

the over-estimation of the result. In addition, $H_2O_2$ in biological samples also react with ferrous ions and cause the higher level of lipid hydroperoxide. These potential problems can be prevented by performing studies in chloroform.

Jurkat *E*6.1 cells were washed with 1 × PBS, resuspended in assay medium [RPMI-1640 medium supplemented with 0.2% (v/v) BSA]. Cell density was adjusted to $5\times10^5$ cells per mL. Cells were divided into individual groups and were seeded at $5\times10^5$ cells per well, in quadruplicate, for each group in Falcon® Multiwell™ tissue culture flat-bottom 12-well plates. A vehicle control group (0.2% DMSO in RPMI-1640 medium supplemented with P/S) was included in each experiment. Cells were pretreated with 5 or 20 μM of BE, Cro, Res or Sal, or synthetic mixture 1 or synthetic mixture 2 for 30 min. After that, some cells were treated with 0.2% DMSO in RPMI-1640 medium supplemented with P/S (vehicle control) for 2 h; others were treated with 400 μM of SMX-HA for 2 h; and the final group of cells was incubated with 400 μM of SMX-HA for 2 h.

After the 2.5 h incubation, cells from each well were transferred to corresponding 1.7 mL micro-centrifuge tubes, centrifugation at 500×g (F2402 Rotor with a Beckman GS-15R centrifuge) for 10 min, supernatant (drugs) were removed. Cells were resuspended in 1mL of RPMI-1640 medium supplemented with FBS and P/S, returned to the plates, and incubated for 18h at 37 °C and in a 5% $CO_2$ humidified environment.

After 18 h incubation, cells were transfered into glass test tubes and sonicated in 500μL HPLC-grade water. An equal volume of Extract R saturated methanol solution (100 mg of Extract R solid was added into 15 mL methanol and vortex thoroughly 2 min) was added to each tube and tubes were vortexed. One mL of cold deoxygenated chloroform (chloroform was deoxygenated by bubbling nitrogen for at least 30 min) was added to each tube and vortexed thoroughly. Tubes were then centrifuged at 500×g using the S4180 Rotor with a Beckman GS-15R centrifuge for 5 min at 0°C. The bottom chloroform layer was then

collected and transferred into a another glass test tube by carefully inserting a Pasteur pipette along the side of the test tube, and stored at -80 °C.

Standards for the assay were prepared by aliquoting Lipid Hydroperoxide Standards (HP) [containing 50 μM ethanolic solution of 13-hydroperoxy-octadecadienoic acid (13-HpODE)] and deoxygenated (2:1) chloroform-methanol mixture in triplicate to 12×75 mm borosilicate glass tubes. 500 μL of the chloroform extract of each sample were transferred to appropriately labeled 12×75 mm borosilicate glass tubes. Next, 450 μL of (2:1) chloroform-methanol solvent mixture were added to the sample test tubes. The chromogen was prepared by mixing equal volume of FTS Reagent 1 (containing 4.5mM ferrous sulphate in 0.2M hydrochloric acid) and FTS Reagent 2 (containing a 3% methanolic solution of ammonium thiocyanate) in test tube and vortex. We then prepared 50 μL of the freshly prepared chromogen for each assay tube and vortexed them thoroughly. The test tubes were closed tightly with polypropylene caps. The assay tubes were kept at room temperature for 5 min, letting the reaction to proceed. Finally, 300μL from each sample or standard tube was transferred into the glass 96-well plate. Each sample was assayed in triplicate. The plate was covered with aluminum foil cover to avoid evaporation from the wells and absorbance was read at 500 nm.

### 3.6.3 Analysis of Protein Carbonyl Content

Proteins are major targets for free radicals and other oxidants. Protein carbonylation to aldehydes or ketones is a major modification of proteins resulting from oxidative stress (Dalle-Donne, Rossi et al. 2003). Carbonyl groups are generated on protein side chains through oxidation of Lys, Arg, Pro or Thr residues, an irreversible modification that frequently results in loss of function (Dalle-Donne, Scaloni et al. 2005).

Protein carbonyl content was analyzed as a general biomarker for protein oxidation to evaluate the attenuation of preincubation of Jurkat *E*6.1 cells with TCM constituents, alone or in combination, on SMX-HA-mediated protein oxidation. The Caymen Chemical Protein Carbonyl Assay Kit (10005020) utilizes the 2,4-dinitrophenylhydrazine (DNPH) reaction to measure the protein carbonyl content. The DNPH reagent reacts with protein carbonyls, forming a Schiff base to produce the corresponding yellow 2.4-dinitrophenylhydrazones, which are analyzed spectrophotometrically (Levine, Williams et al. 1994). The amount of protein-hydrazones produced is quantified by their absorbance at 360nm.

Cell preparation and treatment were exactly as described for the assay of lipid hydroperoxides (above). After drug removal and subsequent incubation for 18 h at 37 °C in a 5% $CO_2$ humidified environment, cells were divided and transferred to two 100 µL aliquots in 1.7 mL microcentrifuge tubes. One tube was the sample tube and the other the control tube. 400 µL of DNPH regent was added to the sample tubes, while 400 µL of 2.5 M HCl was added to the control tubes. Both tubes were incubated in the dark at room temperature for 1 h. Each tube was vortexed briefly every 15 min during the incubation. After 1 h, 0.5 mL 20% trichloroacetic acid (TCA) was added to each tube and vortexed thoroughly. The tubes were placed on ice and incubated for 5 min. The tubes were then centrifuged at 10,000×g for 10 min at 4 °C to form protein pellet. The supernatant was then discarded and the pellet was resuspended in 0.5 mL 10% TCA solution. The tubes were then placed on ice for 5 min and were then re-centrifuged at 10,000× g for 10 min at 4 °C. The supernatant was removed, and the pellet was resuspended in 0.5 mL ethanol/ethyl acetate mixture (1:1). The pellet was manually suspended and vortexed thoroughly and then centrifuged at 10,000×g for 10 min at 4 °C. The wash step was repeated twice after the final wash, protein pellets were resuspended in 500 µL guanidine hydrochloride and vortexed. The tubes were again centrifuged at 10,000×g for 10 min at 4 °C to remove any remaining debris. 220 µL of the supernatant from the sample tubes and 200 µL of the supernatant from the control

tubes were transferred to a 96-well plate, and both sample tubes and control tubes were assessed in duplicate. The absorbance was measured at 360 nm using a Tecan Microplate Reader.

To determine the protein carbonyl content, the average absorbance of each sample and control was determined, and the corrected absorbance (CA) was calculated by subtracting the average absorbance of the controls from the average absorbance of samples. The concentration of the carbonyls was then determined using an extinction coefficient of 22,000 $M^{-1}$ (Dirmeier, O'Brien et al. 2004).

### 3.6.4 Analysis of the protein-protein mixed disulfides, a major component of the disulfide proteome by Redox Two-Dimensional Page (R2D SDS-PAGE)

Prior to treatment, Jurkat *E*6.1 cells were washed in 1 ×PBS, resuspended in RPMI-1640 medium supplemented with P/S, and the density adjusted to $8\times10^5$ cells per mL. Cells were seeded at $8\times10^5$ cells/mL (2 mL/well) in 6-well tissue culture plates to yield sufficient protein for analysis by R2D PAGE. Jurkat *E*6.1 cells were pretreated by incubation with 0, 1 or 5 μM of each of BE, Cro, Res or Sal, alone or in combination for 30 min, followed by treatment with 0 or 400 μM SMX-HA for 2 h. All incubations were conducted at 37 °C and in a 5% $CO_2$ humidified environment. All drugs were freshly prepared in 0.2% DMSO and RPMI-1640 medium supplemented with P/S just before each experiment.

After the 2 h incubation, cells were collected in 2 mL micro-centrifuge tubes, by centrifugation of cells and culture medium at 500×g (F2402 Rotor with a Beckman GS-15R centrifuge) for 5 min. Cell pellets were re-suspended in PBS and re-centrifuged for 5 min at 500 ×g. Then, cells were resuspended in cold PBS and treated with 40 mM iodoacetamide (IA) for 5 min to derivatize free cysteine protein thiol groups to prevent thiol-disulfide exchange and inhibit post-lysis oxidation of free cysteine thiols (Cumming, Andon et al. 2004). After IA incubation, samples were centrifuged again at 500 ×g for 5 min and cell pellets

were resuspended in 50µL lysis buffer (one protease inhibitor cocktail tablet from Roche Diagnostics GmbH, Indianapolis, IN, added to 9 mL lysis buffer, composed of 7.44 mg EDTA, 0.12 g Tris, 0.76 g NaCl, 0.159 g $NaH_2PO_4 \cdot 1H_2O$, 0.446 g $Na_4P_2O_7 \cdot 10H_2O$, 0.042 g NaF dissolved in 100 mL water) and quickly frozen in liquid nitrogen. The lysates were thawed in a water bath at room temperature, then centrifuged for 10 min at 14,000×g (F2402 Rotor with a Beckman GS-15R centrifuge), and the supernatant fractions were collected and transferred to new tubes. An aliquot was removed for assay of protein content by the Bradford assay, as described below.

The dye reagent was prepared by diluting 5x dye reagent concentrate 4:1 with water and filtrated through a filter paper. The protein standard was prepared by aliquot mixing the bovine serum albumin (BSA) (1mg/mL) and distilled water. 10 uL of protein standard and protein supernatant fractions were plated in triplicate in 96-well plate. 200 uL of diluted dye reagent was added to each well, which was then left on a moving table for 10 minutes. The absorbance was measured at 570 nm using a Tecan Microplate Reader. Finally, the standard curve was made and was used to calculate unknown protein concentration.

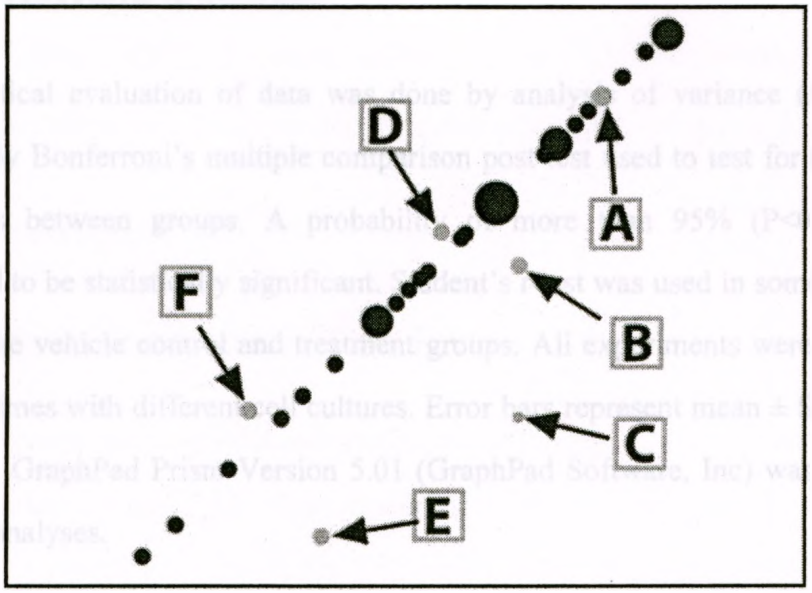
The gels for analysis in the first dimension (10% acrylamide, 1.0 mm thickness) were prepared using the procedure of (Cumming, Andon et al. 2004). The same volume (less than 50 µL) of protein extract (containing equal volume of SDS sample buffer, different volumes of distilled water, and 85 µg of the harvested cytosolic protein) were subjected to 10% non-reducing SDS-PAGE electrophoresis for 3 h, using a constant current of 24 mA/gel with a Bio-Rad Protean II apparatus. The different gel lanes contained proteins for each individual treatment. After the first-dimension electrophoresis, each gel lane containing the separated proteins for each treatment was then cut and put in an individual glass dish. Then each gel lane was immersed and reduced using 10 mL SDS sample buffer containing 100 mM DTT and the plates were left on the rocking table for 20 min at room temperature. Following 3 washes with 1×SDS running buffer (1 min/time), each gel lane was

incubated in 10 mL SDS sample buffer containing 100mM IA on the rocking table for 10 min at room temperature. Each gel lane was then placed horizontally on top of the second-dimension gel (10% acrylamide, 1.5 mm thickness), fixing and sealing each gel strip using 2 mL of boiled 2% low melt agarose buffer. Electrophoresis was then performed in the second dimension for 14 h at a constant current of 10 mA/gel (Cumming, Andon et al. 2004). After the second electrophoresis, the gels slabs were removed and put into a Dodeca small stainer (Bio-Rad) for silver staining. Each gel slab was initially fixed using a 50:50 mixture of water and methanol for 30 min. The gels were then washed twice (5 min/each) with distilled water to remove residual methanol. After that, the gels were incubated in sensitizer solution (0.02% sodium thiosulfate) for 5 min. Following two washing with distilled water for 1 min each, the gel slabs were immersed in cold 0.2% silver nitrate solution and incubated for 30 min. Then the gel slabs were rinsed with distilled water twice for 1 min, and were developed in solution containing 0.05% formaldehyde and 3% sodium hydroxide. The staining step was stopped after the developer solution turned yellow and the desired intensity of staining. After staining the gel, proteins that do not contain any disulfide bond form a diagonal line; proteins that are linked by an intermolecular disulfide bond are found at the right side of the diagonal line; proteins that form intramolecular disulfide bonds are found on the left side of the diagonal line (Fig 8). A shows the majority of proteins that does not form disulfide bonds which represents in diagonal line. B C E lie on the right of the diagonal line. These proteins that form intermolecular disulfide bonds exhibit a slower electrophoretic mobility under non-reducing conditions in the first dimension. D F appear to the left of the diagonal line represent proteins that exhibit a faster electrophoretic mobility under non-reducing conditions due to intra-molecular disulfide bonding. Here are some difference between B C and E, B and C are heterodimeric which will be separated in second dimension while E is homodimeric only represent one spot in second dimension.

The development step was stopped and followed by submerging the gel slabs in 5% acetic acid (Shevchenko, Wilm et al. 1996). Finally each gel slab was scanned prior to analysis of resolved proteins.

D
A
F
B
C
E

**Figure 8. Image of R2D-PAGE result**
(adapted from Biteau, B.et al. 2003. Nature 425, 980-984)


D
A
F
B
C
E

## 3.7 STATISTICAL ANALYSIS

Statistical evaluation of data was done by analysis of variance (ANOVA) followed by Bonferroni's multiple comparison post test used to test for statistical differences between groups. A probability of more than 95% ($P<0.05$) was considered to be statistically significant. Student's *t*-test was used in some cases to compare the vehicle control and treatment groups. All experiments were repeated at least 3 times with different cell cultures. Error bars represent mean ± SEM (N = at least 3). GraphPad Prism Version 5.01 (GraphPad Software, Inc) was used for statistical analyses.

# CHAPTER 4: RESULTS

## 4.1 CELL TREATMENTS-BAICALEIN, CROCETIN, RESVERATROL AND CHISANHENOL EXPOSURE ALONE AND IN COMBINATION

### 4.1.1 Cell Viability

#### 4.1.1.1 Lactate Dehydrogenase (LDH) Release

As a method to measure cell viability, LDH release assay was employed to determine concentration- and time-dependent effects of BE, Cro, Res and Sal singly and in combination on Jurkat *E*6.1 cells. Jurkat *E*6.1 cells were incubated for 6 and 24 h with indicated concentrations of these four chemicals.

Jurkat *E*6.1 cells were incubated with 6.25, 25, 50, 100, 200, or 400 μM of BE, Cro, Res, Sal, Mixture 1, and Mixture 2 for 6 h. The comparative ability of the various treatments to induce LDH in Jurkat *E*6.1 cells is summarized in Table 3.

At 6 h incubation, BE at concentration 50 μM showed a significant increase ($P<0.05$) in LDH release compared to control, and LDH leakage is further increased at higher concentration. Cro treated cells showed a significant increase ($P<0.05$) in LDH release starting at concentration above 25 μM, which further increased at higher concentrations. Res at concentration above 50 μM ($P<0.05$) causes significant increase in LDH release, and further increased with increasing concentrations. Sal induced significant LDH release starting at concentration 100 μM ($P<0.05$) compared to control, which further increased at 200 and 400 μM. Similar to Sal, mixture 1 starting at concentration 100 μM ($P<0.05$) showed significant increase in LDH release, which further increased at higher concentration. Among these single chemicals and mixtures, mixture 2 demonstrated the lowest cytotoxicity, with significant increases in LDH leakage starting at 200 μM.

**Table 3. Effect of Baicalein, Crocetin, Resveratrol, Schisanhenol and the mixtures on the Release of Intracellular Lactate Dehydrogenase from Jurkat *E*6.1 Cells Following Incubation for 6 h**

Mixture 1 is an equimolar mixture of Baicalein, Crocetin, Resveratrol and schisanhenol
Mixture 2 is an equimolar mixture of Baicalein, Crocetin and Resveratrol
*P<0.05, individual treatment vs 0 μM control
#P<0.05, within concentration comparisons to mixture 2, the lowest release of LDH at highest concentration tested.

| | **Release of Lactate Dehydrogenase (% Total Cellular LDH; Mean ± SEM, N=3)** | | | | | |
|---|---|---|---|---|---|---|
| Compound | **6.25 μM** | **25 μM** | **50 μM** | **100 μM** | **200 μM** | **400 μM** |
| **Baicalein** | 1.1 (.05)# | 1.6 (0.1)# | 3.1 (.06)*# | 3.9 (0.1)*# | 4.6 (0.1)*# | 6.9 (0.3)* |
| **Crocetin** | 1.2 (.03)# | 1.9 (.09)*# | 3.0 (0.1)*# | 4.6 (0.1)*# | 7.3 (.06)*# | 13.8 (0.2)*# |
| **Resveratrol** | -0.4 (.05) | 0.7 (.08) | 1.1 (.05)*# | 3.4 (0.1)*# | 4.7 (0.1)*# | 8.9 (.05)*# |
| **Schisanhenol** | -0.3 (.03) | -0.2 (.09) | 0.2 (0.1) | 2.8 (0.2)* | 14.9 (0.3)*# | 26.8 (0.3)*# |
| **Mixture 1** | .03 (.007) | 0.6 (0.1) | 2.3 (0.1)# | 3.6 (0.1)*# | 6.8 (0.1)*# | 12.8 (0.2)*# |
| **Mixture 2** | -0.5 (.02) | -0.3 (.03) | .04 (.002) | 0.4 (.03) | 1.5 (.01)* | 3.3 (0.1)* |

Next, Jurkat *E*6.1 cells were incubated with 6.25, 25, 50, 100, 200, or 400 μM of BE, Cro, Res, Sal, Mixture 1, and Mixture 2 for 24 h. The comparative ability of the various treatments to induce LDH in Jurkat *E*6.1 cells is summarized in Table 4.

At 24 h incubation, BE starting at concentration 50 μM caused a significant increase ($P<0.05$) in LDH release compared to control, and LDH leakage is further increased with increasing concentrations. Low concentrations of Cro also induced significant increases ($P<0.05$) in LDH release. Res at concentration higher 100 μM ($P<0.05$) causes significant increase in LDH release, which further increased at 200 and 400 μM. Sal induced significant LDH release starting at concentration 100 μM ($P<0.05$) compared to control, however, at 200 and 400 μM, Sal caused more release of LDH than other single chemicals. Mixture 1 starting at concentration 100 μM ($P<0.05$) produced significant leakage in LDH release, which further increased at higher concentration. Similar with 6 h incubation, mixture 2 at 24 h also exhibits the lowest cytotoxicity.

Antioxidants are in fact redox (reduction-oxidation) agents. They are normally studied at low concentrations to the goal of protecting against free radicals in some circumstances, thus avoiding their potential toxic (pro-oxidative) effect at higher concentrations. We used LDH release as the end-point and a biomarker to determine the concentration- and time-dependent cytotoxicity for these four chemicals alone and in combination. From these results, Sal produced higher cytotoxicity than the other single constituents at the highest concentration studied (400 μM). This property of Sal also exerts an influence on the mixtures, in that mixture 1 showed more cytotoxicity effect than mixture 2.

**Table 4. Effect of Baicalein, Crocetin, Resveratrol, Schisanhenol and the mixtures on the Release of Intracellular Lactate Dehydrogenase from Jurkat *E*6.1 Cells Following Incubation for 24 h**

Mixture 1 is an equimolar mixture of Baicalein, Crocetin, Resveratrol and Schisanhenol
Mixture 2 is an equimolar mixture of Baicalein, Crocetin and Resveratrol
*P<0.05, individual treatment vs 0 µM control
#P<0.05, within concentration comparisons to mixture 2.

| | Release of Lactate Dehydrogenase (% Total Cellular LDH; Mean ± SEM, N=3) | | | | | |
|---|---|---|---|---|---|---|
| Compound | **6.25 µM** | **25 µM** | **50 µM** | **100 µM** | **200 µM** | **400 µM** |
| **Baicalein** | 1.8 (0.2) | 3.3 (0.2) | 13.1 (0.8)*# | 18.0 (0.5)*# | 21.7 (0.5)*# | 27.4 (0.8)*# |
| **Crocetin** | 1.1 (.05)*# | 2.1 (.05)*# | 3.1 (0.1)* | 5.2 (.04)*# | 9.7 (0.1)*# | 20.8 (0.5)*# |
| **Resveratrol** | -0.9 (.03)# | -0.5 (0.1)# | 0.1 (.06)# | 0.7 (.02)*# | 3.9 (0.1)* | 27.6 (0.2)*# |
| **Schisanhenol** | -0.2 (.004) | 0.9 (.01) | 4.9 (.03)* | 10.2 (.06)*# | 23.6 (.08)*# | 35.6 (.03)*# |
| **Mixture 1** | 0.8 (.03)# | 1.6 (.07) | 2.7 (.06)* | 4.4 (.03)*# | 9.4 (0.1)*# | 19.5 (0.2)*# |
| **Mixture 2** | .01 (.004) | 0.9 (.004)* | 1.8 (.04)* | 3.0 (.06)* | 4.7 (.07)* | 11.4 (0.1)* |

## 4.2 CYTOTOXICITY OF SULFAMETHOXAZOLE N-HYDROXYLAMINE IN JURKAT E6.1 CELLS AND ITS ATTENUATION BY BAICALEIN, CROCETIN, RESVERATROL AND SCHISANHENOL, ALONE AND IN COMBINATION

### 4.2.1 Cell Membrane Damage

Following exposure of Jurkat *E*6.1 cells to 400 μM SMX-HA for 2 h between 35 and 40% of intracellular LDH was released to the medium (Figure 9A) presumably due to the combined effect of this reactive electrophilic metabolite and its associated oxidative stress. To evaluate the chemoprotection of the selected phytochemicals, Jurkat *E*6.1 cells were exposed to SMX-HA (400 μM) for 2 h with or without pretreatment with the pure TCM constituents and two synthetic mixtures for 30 min. The concentrations of the phytochemicals tested were in the range where there was no enhanced release of LDH relative to control cells after exposure to the TCM constituents (Tables 3 and 4).

Similar to the initial experiments performed, there was no increase of LDH release from Jurkat *E*6.1 cells incubated with 1 or 5 μM BE for 30 min compared to vehicle-treated control cells (Fig. 9-A). In contrast, treatment of cells with 400 μM SMX-HA damaged the cell membrane, resulting in the release of 38.9 % ± 4.7% (mean ± SEM, N=3) total cellular LDH, a significantly greater release than in vehicle treated control cells ($P<0.001$). On the other hand, BE at both 1 and 5 μM, partially protected Jurkat cells from SMX-HA-mediated cytotoxicity. More specifically, BE pretreatment for 30 min decreased the amount of LDH release subsequent to treatment with SMX-HA (400 μM) for 2 h by 31% and 58% ($P<0.05$, vs SMX-HA treated), at the lower and higher doses, respectively (Fig. 9-A). Only the attenuation at 5 μM was statistically significant but there appeared to be a dose-response for chemoprotection. Similar concentration-dependent protective

results were found with the other antioxidant chemicals selected for study, demonstrating that the strategy used for identification of these antioxidants was successful and the concentration range selected for study is relevant.

Following preincubation for 30 min at concentrations of 1 or 5 μM and an additional 2 h in the absence of SMX-HA (negative controls), neither Cro, Res nor Sal caused detectable membrane damage given that there was no increase in the amount of LDH released from Jurkat cells relative to vehicle controls (Fig. 9B-D), identical to the data obtained with BE (Fig. 9-A; Tables 3 and 4). These three latter compounds appeared to be better chemoprotectants for SMX-HA-mediated cytotoxicity than BE, given the attenuation of LDH release at the higher concentration studied (5 μM) was 85% ($P<0.01$), 80% ($P<0.01$), and 82% ($P<0.01$) for Cro (Fig. 9B), Res (Fig. 9-C) and Sal (Fig. 9-D), respectively compared to 58% for BE (Fig. 9-A). These three compounds were also more effective at 1 μM than was BE. Cro seemed to be slightly more effective than either Res or Sal, which were equipotent at both concentrations although the difference was not significant (Table 5). At the lower dose, the attenuation of LDH release in SMX-HA treated cells was 65% ($P<0.05$) for Cro, compared to 40%, 40% each for Res ($P<0.05$) and Sal ($P<0.05$). Of the compounds tested, only Sal resulted in a small, concentration-dependent decrease in the amount of LDH released compared to the solvent control treated cells (Fig. 9-D).

To test our hypothesis that mixtures of pure TCM constituents with diverse chemical structures and complementary mechanisms for antioxidant activity are more effective as chemoprotectants than single compounds at equimolar concentrations, we tested two synthetic mixtures of TCM, prepared based on two factors. First was the information collected in our cytotoxicity studies (Tables 3 and 4), which showed that Sal was more toxic than the other constituents at the highest concentration studied (400 μM). Second, based on the fact that Sal will be more effective as an *in vivo* antioxidant-immunostimulant than *in vitro* in Jurkat *E*6.1 cells due to cytochrome P450-dependent metabolic activation by

O-demethylation of 5 methoxylated phenolic groups to polyphenols (a reaction deficient in Jurkat cells) we wished to evaluate two similar mixtures, one with and one without Sal for eventual comparison *in vivo*. Mixture 1 was formulated to contain equimolar amounts (1:1:1:1) of BE, Cro, Res and Sal; and Mixture 2 to contain equimolar amounts (1:1:1) of BE, Cro and Res.

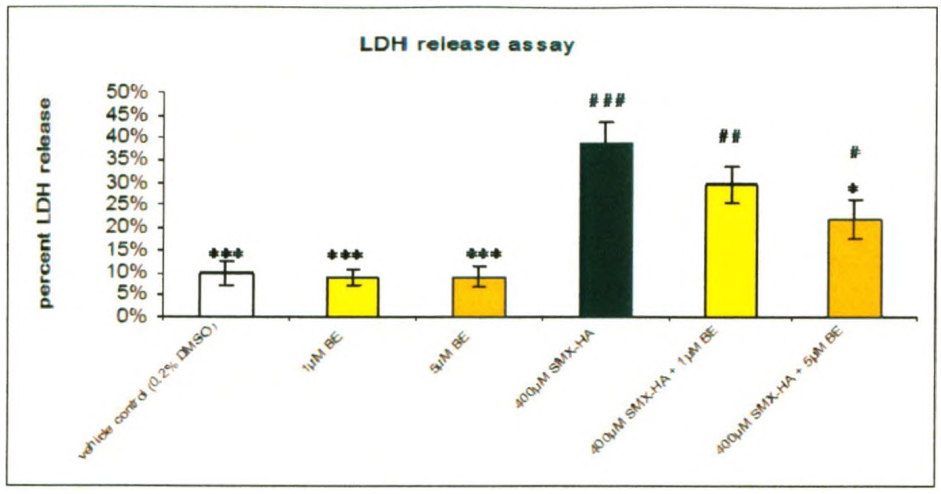
Treatment with 1 and 5 μM Mixture 1 or Mixture 2 for 30 min plus 2 h did not increase LDH leakage compared with vehicle control in Jurkat *E*6.1 cells, a result anticipated from the cytotoxicity experiments performed (Tables 3 and 4). These mixtures were comparable in their ability to attenuate SMX-HA-mediated cytotoxicity to Jurkat *E*6.1 cells. Thus, LDH release was decreased by pretreatment with 1 or 5 μM Mixture 1 by 55% ($P<0.05$) and 93% ($P<0.01$), respectively (Fig 9-E) and by 1 or 5 μM Mixture 2 by 53% ($P<0.05$) and 93% ($P<0.05$), respectively (Fig 9-F). The comparative ability of the various treatments to attenuate SMX-HA cytotoxicity in Jurkat *E*6.1 cells is summarized in Table 5. When we compared the attenuation effect of a single constituent, Cro either in 1 or 5 μM exhibit the best cytoprotective effect, which attenuated SMX-HA mediated LDH release to the most degree. In addition, pretreatment with 5 μM mixture 1 maximally decreased SMX-HA induce LDH leakage, which confirmed our hypothesis that the combination of pure TCM constituents can work through complementary mechanisms to have a greater protective effect than single component on an equimolar basis; also, the better chemoprotective effect of mixture 1 is likely attributable to the presence of Sal, an important contributor of the mixture.

**Figure 9. Effect of pretreatment of Jurkat *E*6.1 cells with baicalein, crocetin, resveratrol, schisanhenol, mixture 1, and mixture 2 on the SMX-HA mediated release of lactate dehydrogenase**.

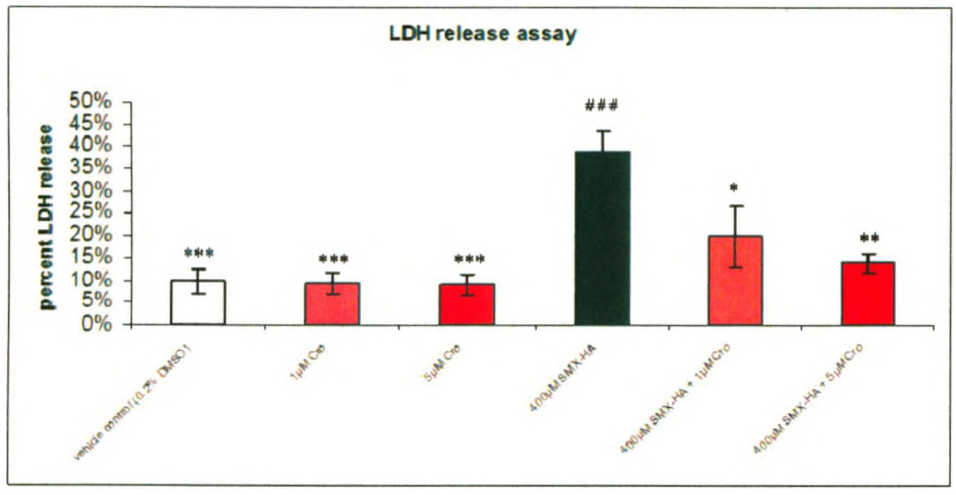
**A**. Jurkat *E*6.1 cells were preincubated with 0, 1 or 5 μM BE for 30 min followed by treatment for 2 h with 400 μM SMX-HA. Data are expressed as mean ± standard error, N=3 with experiments performed with three different samples of cultured cells on different days. Significant differences in LDH release were determined by comparing SMX-HA 400 μM treated cells with all other groups of cells. Levels of significance are designated by *P<0.05, **P<0.01, or ***P<0.001 relative to SMX-HA 400 μM. In addition, the amount of LDH release in each group was compared to the vehicle control to identify treatments that differed. Levels of significance are designated by #P<0.05, # #P<0.01, # # #P<0.001.

**B**. Jurkat *E*6.1 cells were preincubated with 0, 1 or 5 μM Cro for 30 min followed by treatment for 2 h with 400μM SMX-HA. Data are expressed as mean ± standard error, N= 3 with experiments performed with three different samples of cultured cells on different days. Significant differences in LDH release were determined by comparing SMX-HA 400 μM treated cells with all other groups of cells. Levels of significance are designated by *P<0.05, **P<0.01, or ***P<0.001 relative to SMX-HA 400 μM. In addition, the amount of LDH release in each group was compared to the vehicle control to identify treatments that differed. Levels of significance are designated by #P<0.05, # #P<0.01, # # #P<0.001.

A


LDH release assay
percent LDH release
50%
45%
40%
35%
30%
25%
20%
15%
10%
5%
0%
***
***
***
###
##
#
*
vehicle control (0.2% DMSO)
1µM BE
5µM BE
400µM SMX-HA
400µM SMX-HA + 1µM BE
400µM SMX-HA + 5µM BE

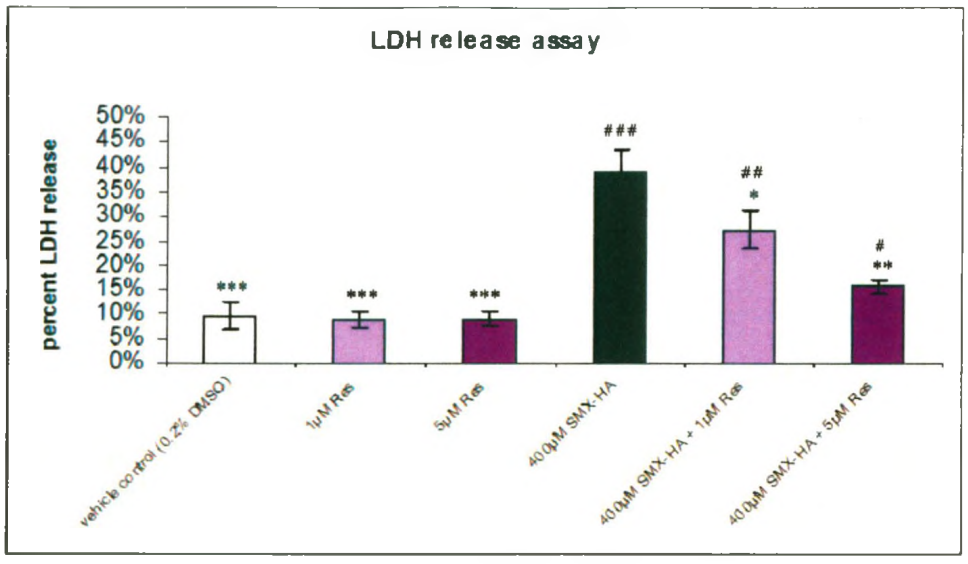
B


LDH release assay
percent LDH release
50%
45%
40%
35%
30%
25%
20%
15%
10%
5%
0%
***
***
***
###
*
**
1µM Cro
5µM Cro
400µM SMX-HA
400µM SMX-HA + 1µM Cro
400µM SMX-HA + 5µM Cro

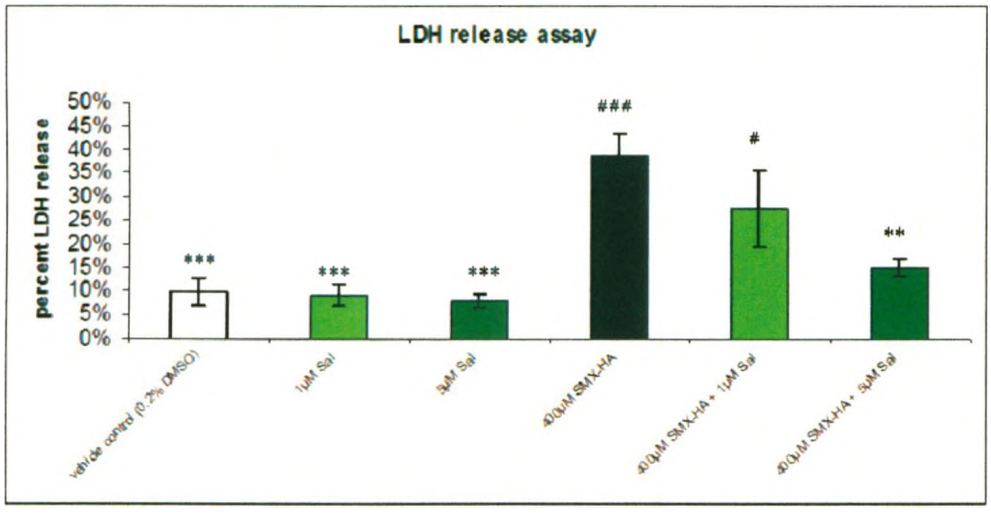
**C**. Jurkat *E*6.1 cells were preincubated with 0, 1 or 5 μM Res for 30 min followed by treatment for 2 h with 400μM SMX-HA. Data are expressed as mean ± standard error, N= 3 with experiments performed with three different samples of cultured cells on different days. Significant differences in LDH release were determined by comparing SMX-HA 400 μM treated cells with all other groups of cells. Levels of significance are designated by *P<0.05, **P<0.01, or ***P<0.001 relative to SMX-HA 400 μM. In addition, the amount of LDH release in each group was compared to the vehicle control to identify treatments that differed. Levels of significance are designated by #P<0.05, # #P<0.01, # # #P<0.001.

**D**. Jurkat *E*6.1 cells were preincubated with 0, 1 or 5 μM Sal for 30 min followed by treatment for 2 h with 400μM SMX-HA. Data are expressed as mean ± standard error, N= 3 with experiments performed with three different samples of cultured cells on different days. Significant differences in LDH release were determined by comparing SMX-HA 400 μM treated cells with all other groups of cells. Levels of significance are designated by *P<0.05, **P<0.01, or ***P<0.001 relative to SMX-HA 400 μM. In addition, the amount of LDH release in each group was compared to the vehicle control to identify treatments that differed. Levels of significance are designated by #P<0.05, # #P<0.01, # # #P<0.001.

C


LDH release assay
percent LDH release
50%
45%
40%
35%
30%
25%
20%
15%
10%
5%
0%
***
***
***
###
##
*
#
**
vehicle control (0.2% DMSO)
1μM Res
5μM Res
40.0μM SMX-HA
40.0μM SMX-HA + 1μM Res
40.0μM SMX-HA + 5μM Res

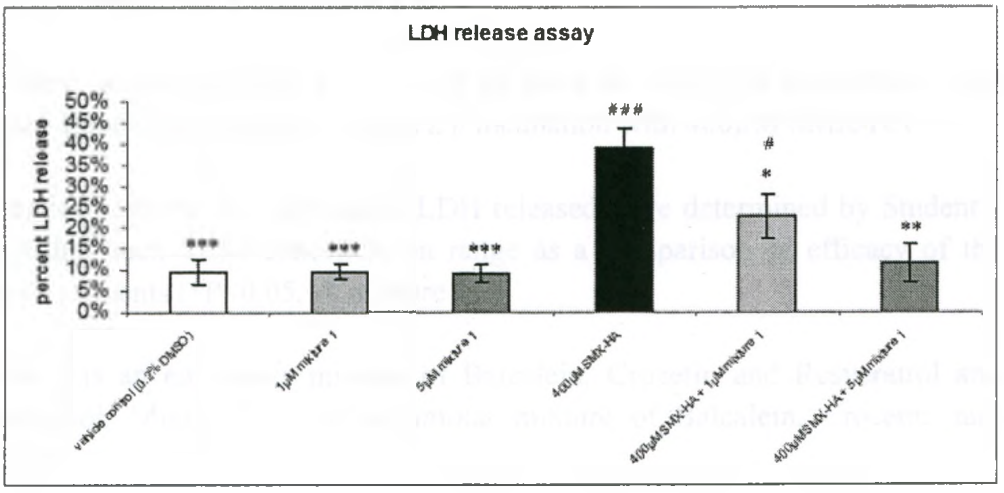
D


LDH release assay
percent LDH release
50%
45%
40%
35%
30%
25%
20%
15%
10%
5%
0%
***
***
***
###
#
**
vehicle control (0.2% DMSO)
1μM Sal
5μM Sal
40.0μM SMX-HA
40.0μM SMX-HA + 1μM Sal
40.0μM SMX-HA + 5μM Sal

**E**. Jurkat *E*6.1 cells were preincubated with 0, 1 or 5 μM Mixture 1 for 30 min followed by treatment for 2 h with 400μM SMX-HA. Data are expressed as mean ± standard error, N= 3 with experiments performed with three different samples of cultured cells on different days. Significant differences in LDH release were determined by comparing SMX-HA 400 μM treated cells with all other groups of cells. Levels of significance are designated by *P<0.05, **P<0.01, or ***P<0.001 relative to SMX-HA 400 μM. In addition, the amount of LDH release in each group was compared to the vehicle control to identify treatments that differed. Levels of significance are designated by #P<0.05, # #P<0.01, # # #P<0.001.

**F**. Jurkat *E*6.1 cells were preincubated with 0, 1 or 5 μM Mixture 2 for 30 min followed by treatment for 2 h with 400μM SMX-HA. Data are expressed as mean ± standard error, N= 3 with experiments performed with three different samples of cultured cells on different days. Significant differences in LDH release were determined by comparing SMX-HA 400 μM treated cells with all other groups of cells. Levels of significance are designated by *P<0.05, **P<0.01, or ***P<0.001 relative to SMX-HA 400μM. In addition, the amount of LDH release in each group was compared to the vehicle control to identify treatments that differed. Levels of significance are designated by #P<0.05, # #P<0.01, # # #P<0.001.

**E**


LDH release assay
percent LDH release
50%
45%
40%
35%
30%
25%
20%
15%
10%
5%
0%
***
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###
#
*
**
vehicle control (0.2% DMSO)
1μM mixture 1
5μM mixture 1
400μM SMX-HA
400μM SMX-HA + 1μM mixture 1
400μM SMX-HA + 5μM mixture 1

**F**

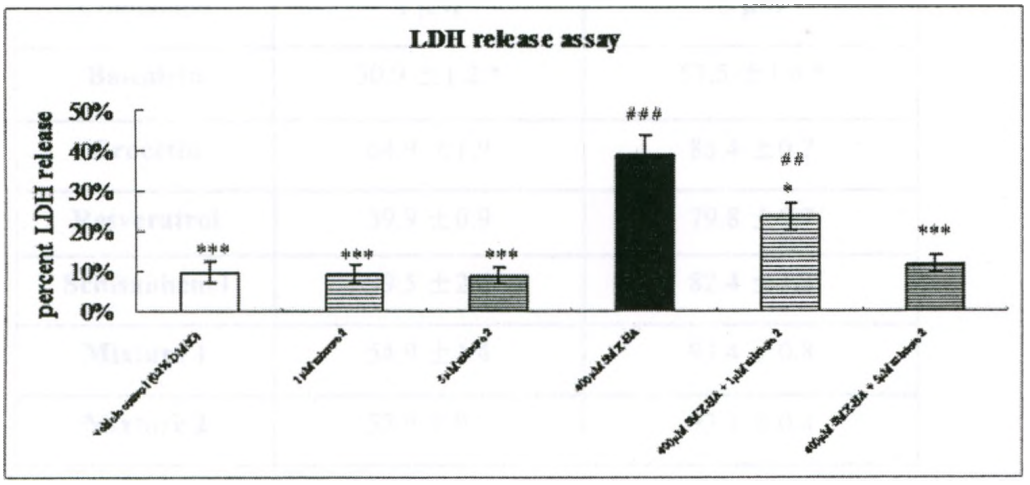
LDH release assay
percent LDH release
50%
40%
30%
20%
10%
0%
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**Table 5. Attenuation of the Release of Intracellular Lactate Dehydrogenase from Jurkat *E*6.1 Cells Following Treatment with 400 μM Sulphamethoxazole N-hydroxylamine (SMX-HA) for 2 h**

Cells were pretreated with 1 or 5 μM of each of the TCM constituents and Mixtures 1 and 2 for 30 min prior to a 2 h incubation with 400μM SMX-HA

Differences between the amounts of LDH released were determined by Student's t-test within each TCM concentration range as a comparison of efficacy of the various treatments (*P<0.05, vs mixture 1).

Mixture 1 is an equimolar mixture of Baicalein, Crocetin and Resveratrol and Schisanhenol; Mixture 2 is an equimolar mixture of Baicalein, Crocetin, and Resveratrol

| | **Attenuation of LDH Release caused by treatment with 400** μM SMX-HA for 2 h (% decrease; mean ± SEM, N=3) | |
|---|---|---|
| Compound | **1 μM** | **5 μM** |
| **Baicalein** | 30.9 ±1.2 * | 57.5 ±1.9 * |
| **Crocetin** | 64.9 ±1.9 | 85.4 ±0.7 |
| **Resveratrol** | 39.9 ±0.9 | 79.8 ±0.7 |
| **Schisanhenol** | 39.5 ±2.6 | 82.4 ±1.3 |
| **Mixture 1** | 54.9 ±1.4 | 93.4 ±0.8 |
| **Mixture 2** | 52.9 ±0.7 | 93.1 ±0.4 |

### 4.2.2 Lipid Hydroperoxide

The lipid hydroperoxide assay is an assessment of the formation of highly reactive hydroperoxide of lipid from lipid peroxidation. As an index of oxidative injury to cells from reactive compounds such as SMX-HA, the lipid hydroperoxide assay was also employed to evaluate potentially protective effects of anti-oxidants from natural herbs. To evaluate the effect of reactive drug metabolites and anti-oxidants, Jurkat *E*6.1 cells were exposed to SMX-HA 400 μM for 2 h with or without pretreatment for 30 minutes with BE, Cro, Res or Sal or mixture 1 and mixture 2.

Jurkat *E*6.1 cells were incubated with 400 μM SMX-HA for 2 h showed significant increased in lipid hydroperoxide formation (24.47 ± 3.59%) (Figure 10-A), which indicated that SMX-HA, the reactive electrophilic metabolite, cause oxidative stress to the lipid. Previous experiment (LDH release assay) has shown the chemoprotective effect of these four chemicals on the cellular membrane. To further confirm their antioxidant activities on lipid peroxidation, Jurkat *E*6.1 cells were incubated with 400 μM SMX-HA for 2 h with or without pretreatment with the pure TCM constituents and two synthetic mixtures for 30 min. We choose the concentrations of phytochemicals at 5 and 20 μM which are still in the safety range we established from the LDH release assay (Table 3 and Table 4).

There was no increase in lipid hydroperoxide formation compared to vehicle-treated control cells (Fig. 10-A) when Jurkat *E*6.1 cells were incubated with 5 and 20 μM BE for 30 min. However, treatment of cells with 400 μM SMX-HA induced the oxidation of lipid, resulting in the formation of 24.47 μM ± 3.59% (mean ± SEM, N=3) lipid hydroperoxide, which is significantly higher than in vehicle treated control cells ($P<0.01$). Conversely, pretreatment with BE at both 5 and 20 μM for 30 min followed by incubation of the cells with SMX-HA 400 μM 2 h, partly attenuated SMX-HA induced oxidative stress to the lipid. BE

pretreatment for 30 min decreased the content of lipid hydroperoxide subsequent to treatment with SMX-HA 400 μM for 2 h by 44.2% and 81.5% ($P<0.05$, vs SMX-HA treated), at the lower and higher concentrations, respectively (Fig 10-A). Although it was not significant at the 5 μM pretreatment, we can still see that the attenuation effect of BE exhibit concentration-dependent manner.

In this experiment, other selected antioxidant chemicals all showed the similar dose-response protective effect, indicating that the concentration range we choose for measurement of lipid peroxidation is relevant.

When Jurkat *E*6.1 cells were incubated with Cro 5 μM for 30 min there was a slight increase in lipid hydroperoxide content (Fig 10-B). However, neither Res nor Sal caused detectable increase in lipid hydroperoxide (Fig 10C-D), following preincubation for 30 min at concentrations of 5 or 20 μM and an additional 2 h in the absence of SMX-HA (negative controls). Although 5 μM Cro alone slightly increased lipid hydroperoxide concentration, together with Res and Sal, pretreatment with these three latter compounds for 30 min all attenuated SMX-HA induced lipid hydroperoxide formation. At the higher concentration studied (20 μM), the attenuation of lipid hydroperoxide formation was 84% ($P<0.05$), 93% ($P<0.01$), 91% ($P<0.05$) for Cro (Fig 10-B), Res (Fig 10-C) and Sal (Fig 10-D), respectively. These three compounds were also effective at 5 μM and showed better chemoprotective effects than BE. The attenuation of lipid hydroperoxide formation in SMX-HA treated cells was 72% ($P<0.05$) for Cro, compared to 72%, 66% each for Res ($P<0.05$) and Sal. The attenuation of Sal at 5 μM was not statistically significant due to sample variability, although the experimental data does suggest a trend (Fig 10-D).

In attenuation of LDH release caused by treatment with 400 μM SMX-HA, the mixtures of pure TCM constituents worked as more effective chemoprotectants than single compounds at equimolar concentrations. We further confirmed our hypothesis that anti-oxidants selected for the study can work through complementary mechanisms to produce a greater protective effect than single

components alone on an equimolar basis. Also, we compared the chemoprotective effects of mixture 1 and mixture 2 to evaluate the antioxidant property of Sal.

30 min treatment with 5 μM mixture 1, mixture 2 slightly increased lipid hydroperoxide content compared with vehicle control in Jurkat *E*6.1 cells (Fig 10 E-F). Nevertheless, these mixtures were comparable in their ability to attenuate SMX-HA-mediated lipid peroxidation in Jurkat *E*6.1 cells. Lipid hydroperoxide content was decreased by pretreatment with 5 or 20 μM Mixture 1 by 47% and 96% ($P<0.05$), respectively (Fig 10-E) and by 5 or 20 μM Mixture 2 by 42% and 93%, respectively (Fig 10-F). Related to sample variability, only the attenuation of mixture 1 at 20 μM was statistically significant but there was a trend for concentration-dependent chemoprotection. The comparative ability of the various treatments to attenuate SMX-HA cytotoxicity in Jurkat *E*6.1 cells is summarized in Table 6. Among these four single constituent of TCM, Cro, Res, and Sal exhibit better chemoprotective effects than was BE. Cro at 5 μM and Res at 20 μM showed more powerful attenuation in SMX-HA mediated lipid peroxidation. When overall comparison was done, we found that either mixture 1 or mixture 2 at higher concentration both were more effective than a single chemical. Moreover, mixture 1 at 20 μM maximally attenuated SMX-HA induced lipid peroxidation. From this result, we concluded that the better chemoprotective effects of mixtures were achieved through synergistic effects of TCM constituents. Also the presence of Sal appeared to enhance the antioxidant ability of the mixture.

**Figure 10. Effect of pretreatment of Jurkat *E*6.1 cells with baicalein, crocetin, resveratrol, schisanhenol, mixture 1, and mixture 2 on the SMX-HA mediated lipid peroxidation**

**A**. Jurkat *E*6.1 cells were preincubated with 0, 5 or 20 μM BE for 30 min followed by treatment for 2 h with 400 μM SMX-HA. Data are expressed as mean ± standard error, N= 3 with experiments performed with three different samples of cultured cells on different days. Significant differences in lipid hydroperoxide content were determined by comparing SMX-HA 400 μM treated cells with all other groups of cells. Levels of significance are designated by *P<0.05, **P<0.01 relative to SMX-HA 400 μM. In addition, the content of lipid hydroperoxide in each group was compared to the vehicle control to identify treatments that differed. Levels of significance are designated by #P<0.05, # #P<0.01.

**B**. Jurkat *E*6.1 cells were preincubated with 0, 5 or 20 μM Cro for 30 min followed by treatment for 2 h with 400 μM SMX-HA. Data are expressed as mean ± standard error, N= 3 with experiments performed with three different samples of cultured cells on different days. Significant differences in lipid hydroperoxide content were determined by comparing SMX-HA 400 μM treated cells with all other groups of cells. Levels of significance are designated by *P<0.05, **P<0.01 relative to SMX-HA 400 μM. In addition, the content of lipid hydroperoxide in each group was compared to the vehicle control to identify treatments that differed. Levels of significance are designated by #P<0.05, # #P<0.01.

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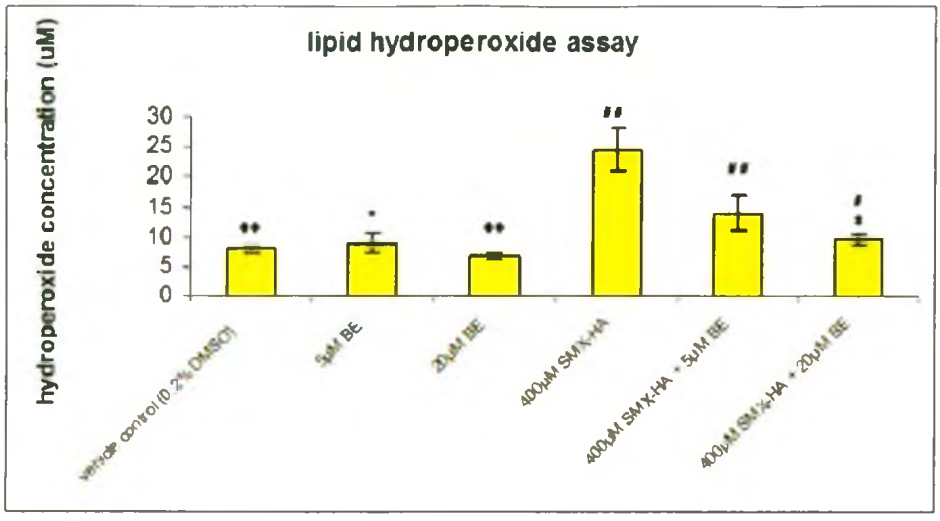
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20
15
10
5
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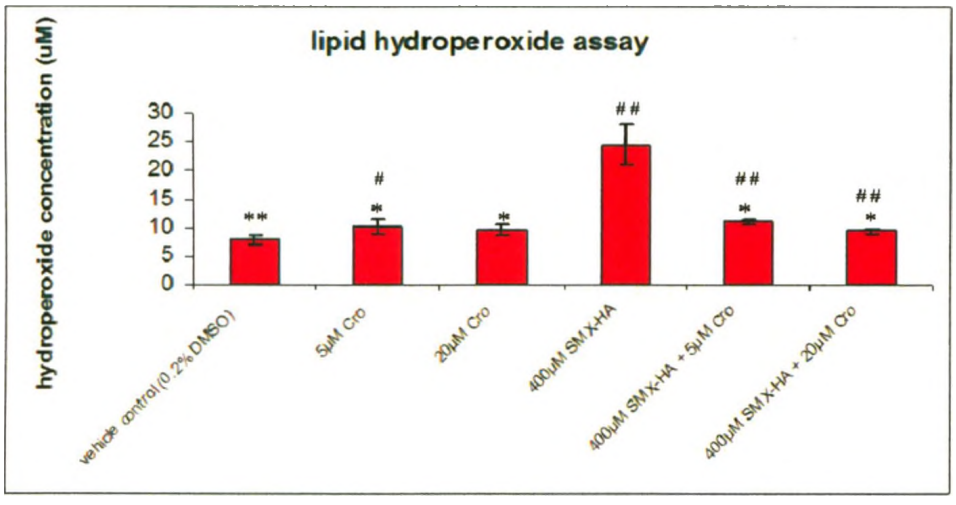
20μM BE
400μM SMX-HA
400μM SMX-HA + 5μM BE
400μM SMX-HA + 20μM BE

B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hydroperoxide concentration (uM)
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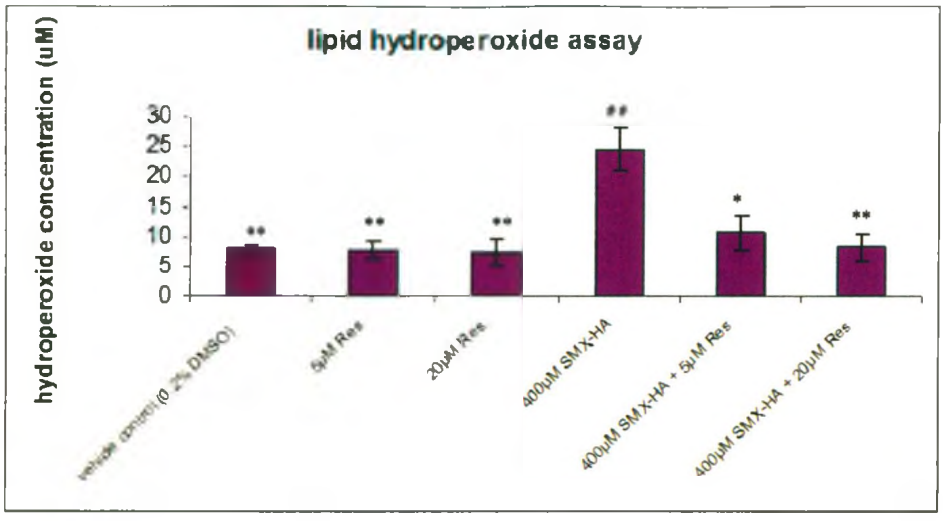
25
20
15
10
5
0
vehicle control (0.2% DMSO)
5μM Cro
20μM Cro
400μM SMX-HA
400μM SMX-HA + 5μM Cro
400μM SMX-HA + 20μM Cro

**C**. Jurkat *E*6.1 cells were preincubated with 0, 5 or 20 μM Res for 30 min followed by treatment for 2 h with 400 μM SMX-HA. Data are expressed as mean ± standard error, N= 3 with experiments performed with three different samples of cultured cells on different days. Significant differences in lipid hydroperoxide content were determined by comparing SMX-HA 400 μM treated cells with all other groups of cells. Levels of significance are designated by *P<0.05, **P<0.01 relative to SMX-HA 400 μM. In addition, the content of lipid hydroperoxide in each group was compared to the vehicle control to identify treatments that differed. Levels of significance are designated by #P<0.05, # #P<0.01.

**D**. Jurkat *E*6.1 cells were preincubated with 0, 5 or 20 μM Sal for 30 min followed by treatment for 2 h with 400 μM SMX-HA. Data are expressed as mean ± standard error, N= 3 with experiments performed with three different samples of cultured cells on different days. Significant differences in lipid hydroperoxide content were determined by comparing SMX-HA 400 μM treated cells with all other groups of cells. Levels of significance are designated by *P<0.05, **P<0.01 relative to SMX-HA 400 μM. In addition, the content of lipid hydroperoxide in each group was compared to the vehicle control to identify treatments that differed. Levels of significance are designated by #P<0.05, # #P<0.01.

C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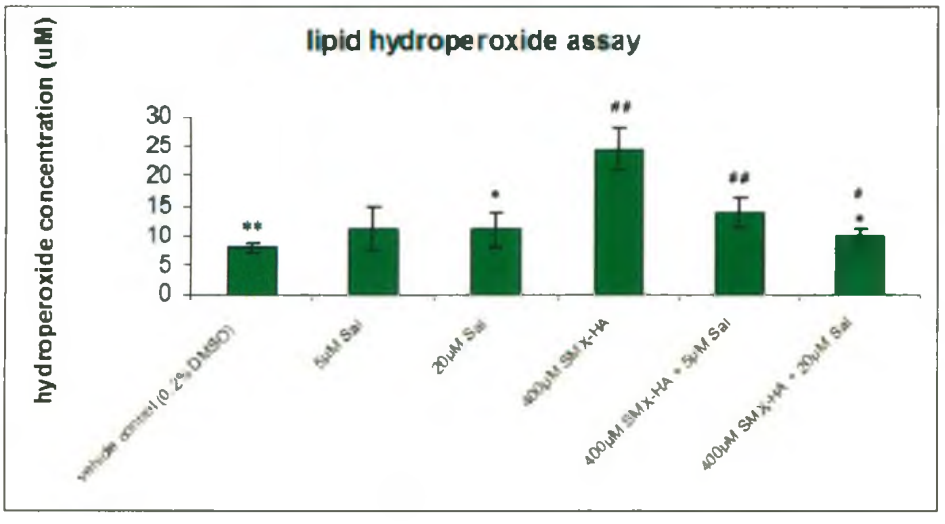
5μM Res
20μM Res
400μM SMX-HA
400μM SMX-HA + 5μM Res
400μM SMX-HA + 20μM Res

D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hydroperoxide concentration (uM)
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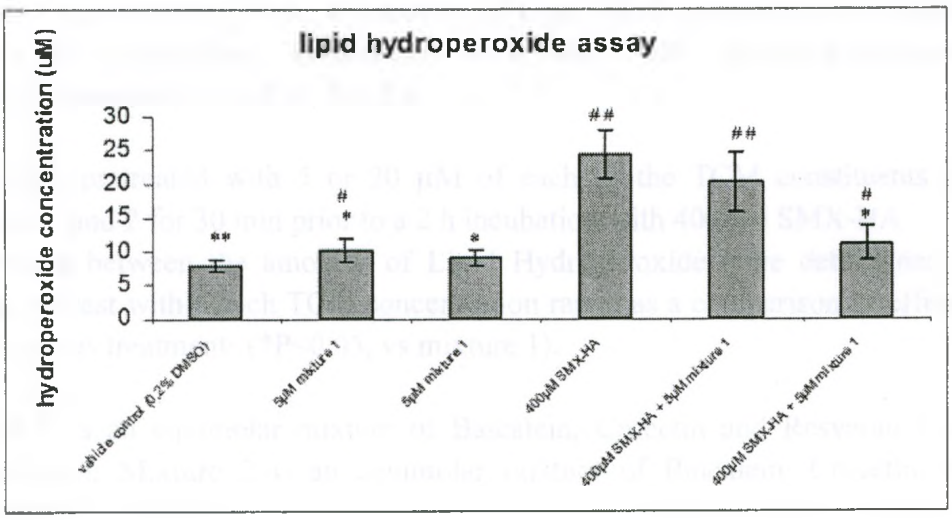
5μM Sal
20μM Sal
400μM SMX-HA
400μM SMX-HA + 5μM Sal
400μM SMX-HA + 20μM Sal

**E**. Jurkat *E*6.1 cells were preincubated with 0, 5 or 20 μM mixture 1 for 30 min followed by treatment for 2 h with 400 μM SMX-HA. Data are expressed as mean ± standard error, N= 3 with experiments performed with three different samples of cultured cells on different days. Significant differences in lipid hydroperoxide content were determined by comparing SMX-HA 400 μM treated cells with all other groups of cells. Levels of significance are designated by *P<0.05, **P<0.01 relative to SMX-HA 400 μM. In addition, the content of lipid hydroperoxide in each group was compared to the vehicle control to identify treatments that differed. Levels of significance are designated by #P<0.05, # #P<0.01.

**F**. Jurkat *E*6.1 cells were preincubated with 0, 5 or 20 μM mixture 2 for 30 min followed by treatment for 2 h with 400 μM SMX-HA. Data are expressed as mean ± standard error, N= 3 with experiments performed with three different samples of cultured cells on different days. Significant differences in lipid hydroperoxide content were determined by comparing SMX-HA 400 μM treated cells with all other groups of cells. Levels of significance are designated by *P<0.05, **P<0.01 relative to SMX-HA 400 μM. In addition, the content of lipid hydroperoxide in each group was compared to the vehicle control to identify treatments that differed. Levels of significance are designated by #P<0.05, # #P<0.01.

**E**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hydroperoxide concentration (uM)
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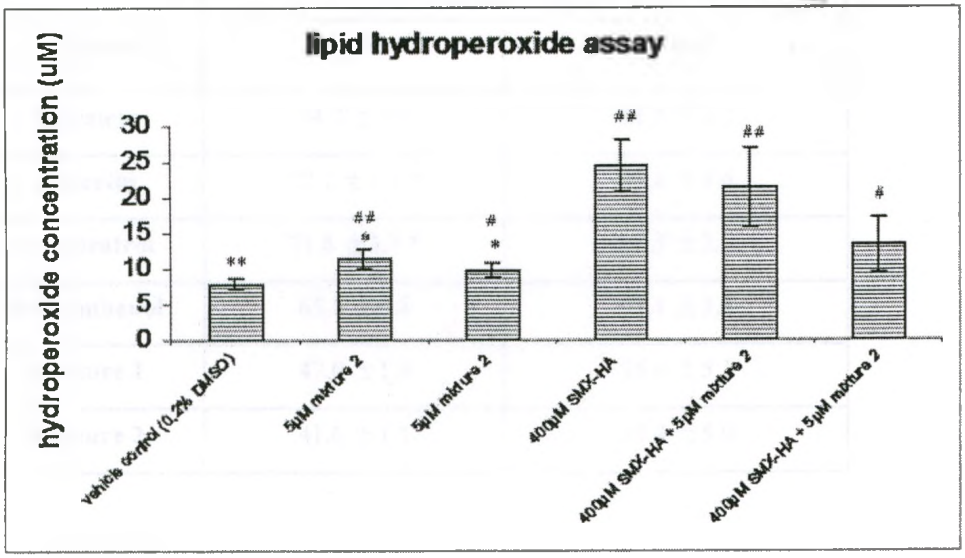
vehicle control (0.2% DMSO)
5µM mixture 1
5µM mixture 1
400µM SMX-HA
400µM SMX-HA + 5µM mixture 1
400µM SMX-HA + 5µM mixture 1

**F**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hydroperoxide concentration (uM)
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##
*
#
*
##
##
#
vehicle control (0.2% DMSO)
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5µM mixture 2
400µM SMX-HA
400µM SMX-HA + 5µM mixture 2
400µM SMX-HA + 5µM mixture 2

**Table 6. Attenuation of the Formation of Lipid Hydroperoxide from Jurkat *E*6.1 Cells Following Treatment with 400 µM Sulphamethoxazole N-hydroxylamine (SMX-HA) for 2 h**

Cells were pretreated with 5 or 20 µM of each of the TCM constituents and Mixtures 1 and 2 for 30 min prior to a 2 h incubation with 400µM SMX-HA. Differences between the amounts of Lipid Hydroperoxide were determined by Student's t-test within each TCM concentration range as a comparison of efficacy of the various treatments (*P<0.05, vs mixture 1).

Mixture 1 is an equimolar mixture of Baicalein, Crocetin and Resveratrol and Schisanhenol; Mixture 2 is an equimolar mixture of Baicalein, Crocetin, and Resveratrol

| | **Attenuation of Lipid Hydroperoxide formation caused by treatment with 400 µM SMX-HA for 2 h** (% decrease; mean ± SEM, N=3) | |
|---|---|---|
| Compound | **5 µM** | **20 µM** |
| **Baicalein** | 44.2 ±3.6 | 81.5 ±2.1 |
| **Crocetin** | 72.1 ±1.7 * | 83.8 ±1.6 |
| **Resveratrol** | 71.8 ±3.7 * | 92.5 ±3.1 |
| **Schisanhenol** | 65.8 ±4.7 | 91.1 ±3.4 |
| **Mixture 1** | 47.0 ±1.0 | 95.6 ±5.1 |
| **Mixture 2** | 41.6 ±1.1 | 93.4 ±5.9 |

### 4.2.3 Protein Carbonyl Content

The protein carbonyl content in Jurkat *E*6.1 cells was also conducted as a complementary assay for oxidative and nitrosative stress. Protein carbonyl assay was also used to evaluate the antioxidative effect of pure constituents of traditional Chinese remedies on oxidative stress-induced protein oxidation. In this experiment, the protein carbonyl content was expressed as percent of control.

Incubation of Jurkat *E*6.1 cells to 400 μM SMX-HA for 2 h about 245% protein carbonyl was formed in Jurkat *E*6.1 cells (Fig 11-A). This cytotoxicity effect is probably due to the combined effect of this reactive electrophilic metabolite and associated oxidative stress. To evaluate the chemoprotection of the selected phytochemicals on protein oxidation, Jurkat *E*6.1 cells were exposed to SMX-HA (400 μM) for 2 h with or without pretreatment with the pure TCM constituents and two synthetic mixtures for 30 min. The concentrations of the phytochemicals tested were same as in the lipid hydroperoxide assay.

Jurkat *E*6.1 cells were exposed to 5 or 20 μM of BE for 30 min, there was no increase of protein carbonyl formation from Jurkat *E*6.1 cells compared to vehicle-treated control cells (Fig 11-A). In contrast, SMX-HA 400 μM oxidized protein to form a significant concentration of protein carbonyl 245% ± 28% (mean ± SEM, N=3) which is significantly higher than in vehicle treated control cells ($P<0.05$). On the other hand, BE at both 5 and 20 μM, partially protected Jurkat cells from SMX-HA-mediated protein oxidation. Pretreatment with both lower and higher concentrations of BE effectively decreased SMX-HA induced protein carbonylation. Protein carbonyl content was decreased by pretreatment with 5 or 20 μM BE by 46% ($P<0.05$) and 74% ($P<0.01$), respectively (Fig 11-A).

Similar to BE, we did not find significant increase in protein carbonyl content compared with vehicle control when Jurkat *E*6.1 cells were incubated with Cro and Sal at 5 and 20 μM for 30 min (Fig 11-B 11-D). While, following

treatment with Res at 5 μM slightly increased protein carbonyl content (Fig 11-C) probably due to the sample variability. When each attenuation effect of these four constituents of TCM was analysis, BE, Cro, and Sal were apparently better chemoprotectants for SMX-HA-mediated protein oxidation than Res, for the attenuation of protein carbonyl formation at the higher concentration studied (20 μM) was 74% ($P<0.01$), 72% ($P<0.01$), and 71% ($P<0.01$) for BE (Fig. 11-A), Cro (Fig. 11-B) and Sal (Fig. 11-D), respectively compared to 63% for Res (Fig. 11-C).

At a lower concentration (5 μM), BE, Cro, Sal were also more effective than was Res. BE seemed to be slightly more effective than either Cro or Sal, which were equipotent at both concentrations but the difference is not significant (Table 6). At the lower dose (5 μM), the attenuation of protein carbonylation in SMX-HA treated cells was 55% ($P<0.01$) for Cro, compared to 6%, 46% ($P<0.05$) each for Res and Sal.

The same strategy was used to test the antioxidant effect of two synthetic mixtures of TCM on the protein level. Treatment with 5 and 20 μM Mixture 1 or Mixture 2 for 30 min plus 2 h did not increase protein carbonyl content compared with vehicle control in Jurkat *E*6.1 cells. Moreover, these mixtures were comparable in their ability to attenuate SMX-HA-mediated protein oxidation in Jurkat *E*6.1 cells. Thus, the content of protein carbonyl was decreased by pretreatment with 5 or 20 μM Mixture 1 by 75% ($P<0.01$) and 82% ($P<0.01$), respectively (Fig 11E) and by 5 or 20 μM Mixture 2 by 67% ($P<0.05$) and 59% ($P<0.05$), respectively (Fig 11-F). The comparative ability of the various treatments to attenuate SMX-HA induced protein oxidation in Jurkat *E*6.1 cells is summarized in Table 7.

When we compared the attenuation effect single constituent, the chemoprotective effects of BE, Cro, and Sal are quite close either in 5 or 20 μM. However, Res in this case exhibit the weakest cytoprotective effect, especially at lower concentration. In addition, pretreatment with mixture 1 maximally decreased SMX-HA induced protein carbonylation either at lower or higher doses. Again,

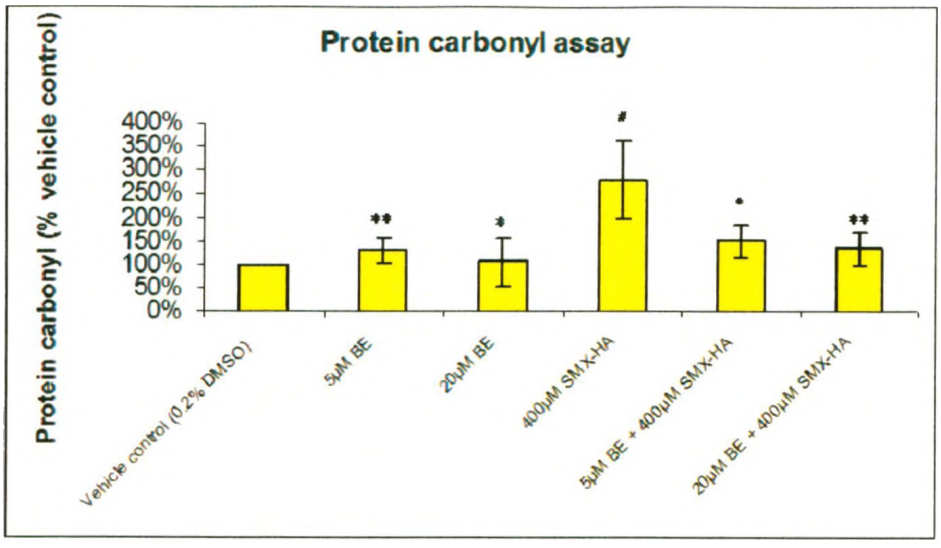
our hypothesis was proved in this experiment, which indicated that Sal plays a vital role in the combination of pure TCM constituents and they work through complementary mechanisms make the mixture to possess greater protective effect than single component on an equimolar basis.

**Figure 11. Effect of pretreatment of Jurkat *E*6.1 cells with baicalein, crocetin, resveratrol, schisanhenol, mixture 1, and mixture 2 on the SMX-HA mediated protein carbonylation**

**A**. Jurkat *E*6.1 cells were preincubated with 0, 5 or 20 μM BE for 30 min followed by treatment for 2 h with 400 μM SMX-HA. Data are expressed as mean ± standard error, N= 3 with experiments performed with three different samples of cultured cells on different days. Significant differences in protein carbonyl content were determined by comparing SMX-HA 400 μM treated cells with all other groups of cells. Levels of significance are designated by *P<0.05, **P<0.01 relative to SMX-HA 400 μM. In addition, the content of protein carbonyl in each group was compared to the vehicle control to identify treatments that differed. Levels of significance are designated by #P<0.05.

**B**. Jurkat *E*6.1 cells were preincubated with 0, 5 or 20 μM Cro for 30 min followed by treatment for 2 h with 400 μM SMX-HA. Data are expressed as mean ± standard error, N= 3 with experiments performed with three different samples of cultured cells on different days. Significant differences in protein carbonyl content were determined by comparing SMX-HA 400 μM treated cells with all other groups of cells. Levels of significance are designated by *P<0.05, **P<0.01 relative to SMX-HA 400 μM. In addition, the content of protein carbonyl in each group was compared to the vehicle control to identify treatments that differed. Levels of significance are designated by #P<0.05.

**A**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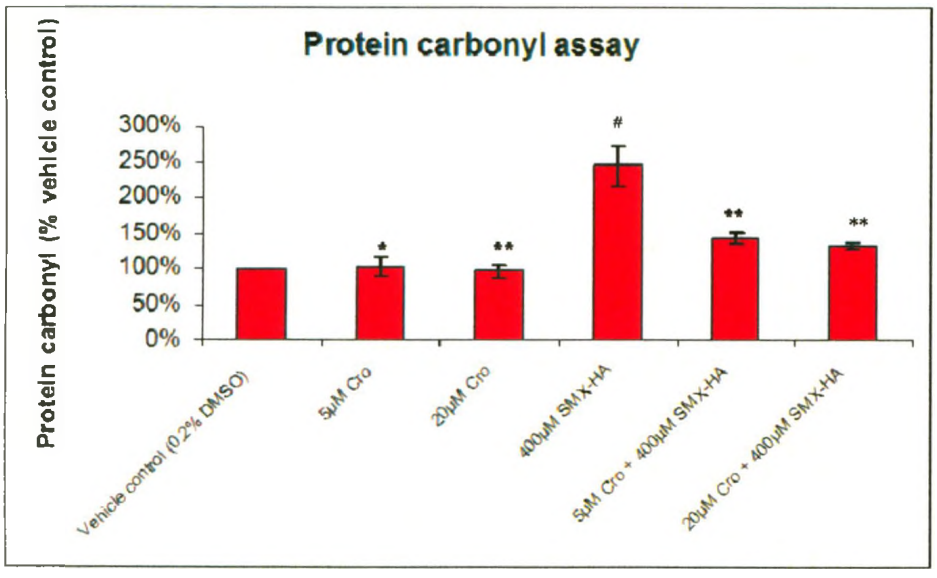
Protein carbonyl (% vehicle control)
400%
350%
300%
250%
200%
150%
100%
50%
0%
**
*
#
*
**
Vehicle control (0.2% DMSO)
5μM BE
20μM BE
400μM SMX-HA
5μM BE + 400μM SMX-HA
20μM BE + 400μM SMX-HA

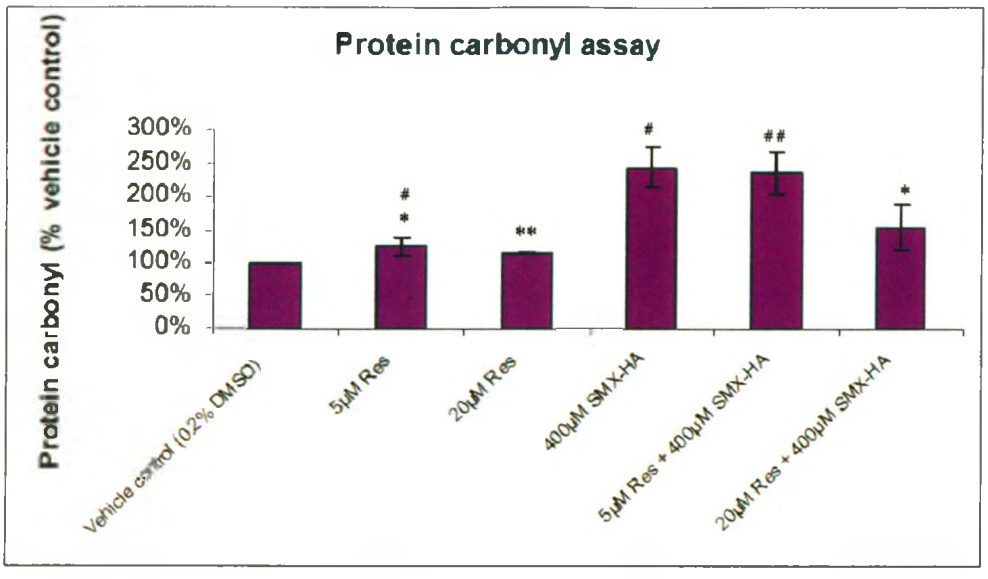
**B**


Protein carbonyl assay
Protein carbonyl (% vehicle control)
300%
250%
200%
150%
100%
50%
0%
*
**
#
**
**
Vehicle control (0.2% DMSO)
5μM Cro
20μM Cro
400μM SMX-HA
5μM Cro + 400μM SMX-HA
20μM Cro + 400μM SMX-HA

**C**. Jurkat *E*6.1 cells were preincubated with 0, 5 or 20 μM Res for 30 min followed by treatment for 2 h with 400 μM SMX-HA. Data are expressed as mean ± standard error, N= 3 with experiments performed with three different samples of cultured cells on different days. Significant differences in protein carbonyl content were determined by comparing SMX-HA 400 μM treated cells with all other groups of cells. Levels of significance are designated by *P<0.05, **P<0.01 relative to SMX-HA 400 μM. In addition, the content of protein carbonyl in each group was compared to the vehicle control to identify treatments that differed. Levels of significance are designated by #P<0.05, # #P<0.01.

**D**. Jurkat *E*6.1 cells were preincubated with 0, 5 or 20 μM Sal for 30 min followed by treatment for 2 h with 400 μM SMX-HA. Data are expressed as mean ± standard error, N= 3 with experiments performed with three different samples of cultured cells on different days. Significant differences in protein carbonyl content were determined by comparing SMX-HA 400 μM treated cells with all other groups of cells. Levels of significance are designated by *P<0.05, **P<0.01 relative to SMX-HA 400 μM. In addition, the content of protein carbonyl in each group was compared to the vehicle control to identify treatments that differed. Levels of significance are designated by #P<0.05.

C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100%
50%
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Vehicle control (0.2% DMSO)
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20μM Res
400μM SMX-HA
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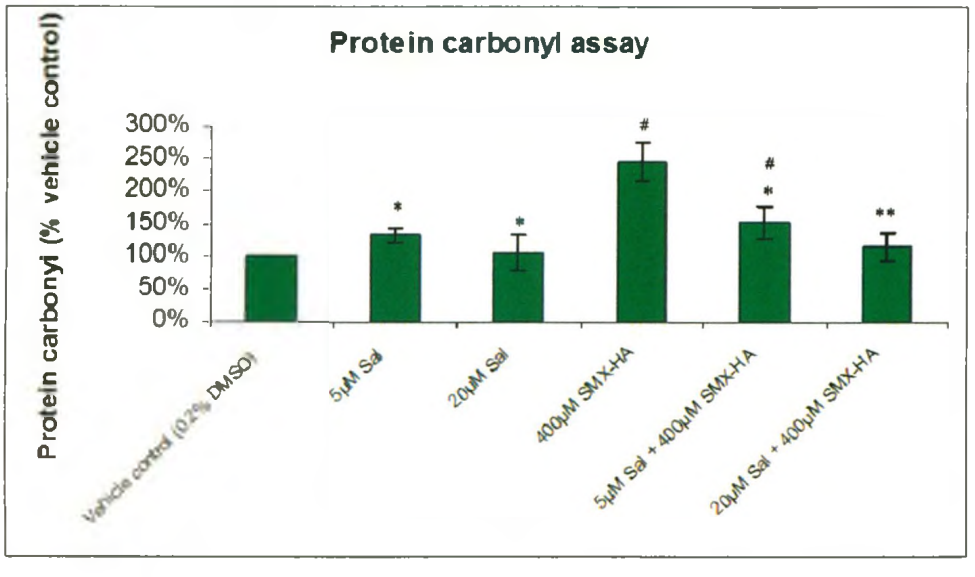
20μM Res + 400μM SMX-HA

D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Protein carbonyl (% vehicle control)
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200%
150%
100%
50%
0%
Vehicle control (0.2% DMSO)
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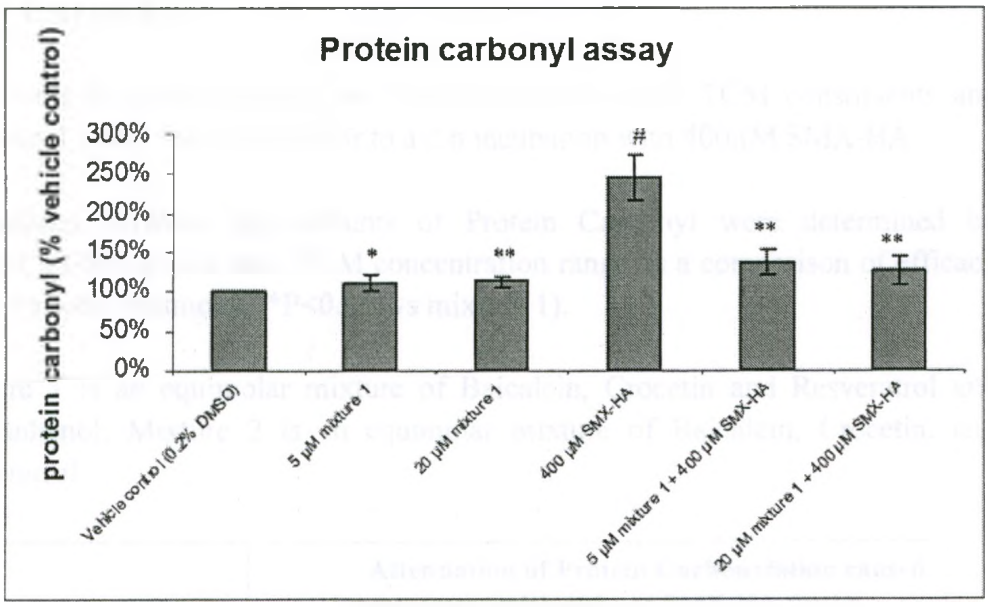
20μM Sal
400μM SMX-HA
5μM Sal + 400μM SMX-HA
20μM Sal + 400μM SMX-HA

**E**. Jurkat *E*6.1 cells were preincubated with 0, 5 or 20 μM mixture 1 for 30 min followed by treatment for 2 h with 400 μM SMX-HA. Data are expressed as mean ± standard error, N= 3 with experiments performed with three different samples of cultured cells on different days. Significant differences in protein carbonyl content were determined by comparing SMX-HA 400 μM treated cells with all other groups of cells. Levels of significance are designated by *P<0.05, **P<0.01 relative to SMX-HA 400 μM. In addition, the content of protein carbonyl in each group was compared to the vehicle control to identify treatments that differed. Levels of significance are designated by #P<0.05.

**F**. Jurkat *E*6.1 cells were preincubated with 0, 5 or 20 μM mixture 2 for 30 min followed by treatment for 2 h with 400 μM SMX-HA. Data are expressed as mean ± standard error, N= 3 with experiments performed with three different samples of cultured cells on different days. Significant differences in protein carbonyl content were determined by comparing SMX-HA 400 μM treated cells with all other groups of cells. Levels of significance are designated by *P<0.05, **P<0.01 relative to SMX-HA 400 μM. In addition, the content of protein carbonyl in each group was compared to the vehicle control to identify treatments that differed. Levels of significance are designated by #P<0.05.

**E**


Protein carbonyl assay
protein carbonyl (% vehicle control)
300%
250%
200%
150%
100%
50%
0%
*
**
#
**
**
Vehicle control (0.2% DMSO)
5 µM mixture 1
20 µM mixture 1
400 µM SMX-HA
5 µM mixture 1 + 400 µM SMX-HA
20 µM mixture 1 + 400 µM SMX-HA

**F**

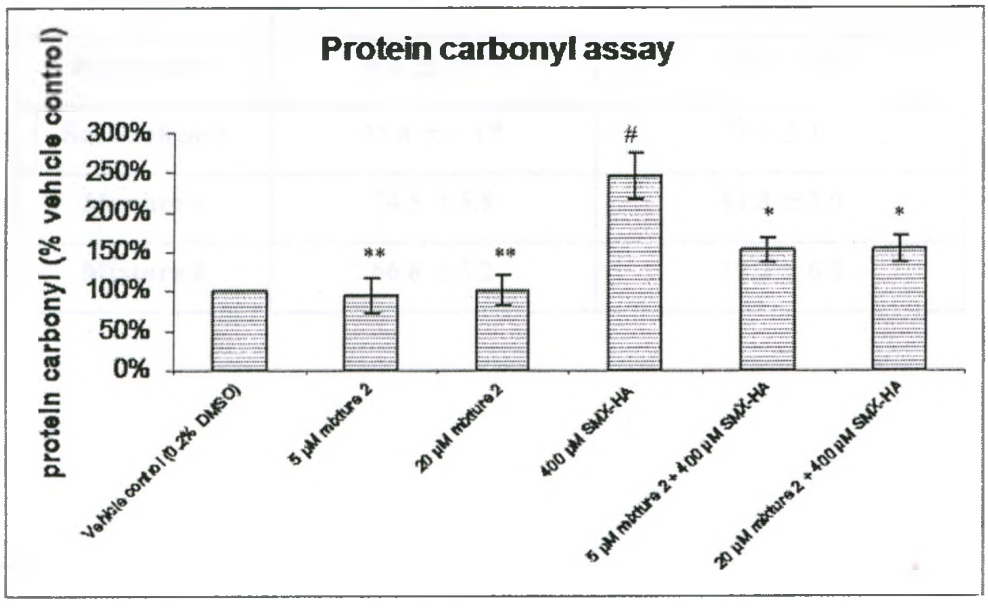

Protein carbonyl assay
protein carbonyl (% vehicle control)
300%
250%
200%
150%
100%
50%
0%
**
**
#
*
*
Vehicle control (0.2% DMSO)
5 µM mixture 2
20 µM mixture 2
400 µM SMX-HA
5 µM mixture 2 + 400 µM SMX-HA
20 µM mixture 2 + 400 µM SMX-HA

**Table 7. Attenuation of the Protein Carbonylation from Jurkat *E*6.1 Cells Following Treatment with 400μM Sulphamethoxazole N-hydroxylamine (SMX-HA) for 2 h**

Cells were pretreated with 5 or 20 μM of each of the TCM constituents and Mixtures 1 and 2 for 30 min prior to a 2 h incubation with 400μM SMX-HA.

Differences between the amounts of Protein Carbonyl were determined by Student's t-test within each TCM concentration range as a comparison of efficacy of the various treatments (*P<0.05, vs mixture 1).

Mixture 1 is an equimolar mixture of Baicalein, Crocetin and Resveratrol and Schisanhenol; Mixture 2 is an equimolar mixture of Baicalein, Crocetin, and Resveratrol

| | **Attenuation of Protein Carbonylation caused by treatment with 400** μM SMX-HA for 2 h (% decrease; mean ± SEM, N=3) | |
|---|---|---|
| Compound | **5 μM** | **20 μM** |
| **Baicalein** | 45.7 ±5.5 * | 73.6 ±1.8 |
| **Crocetin** | 55.0 ±4.3 | 71.8 ±1.9 |
| **Resveratrol** | 6.1 ±1.4 * | 62.6 ±8.6 |
| **Schisanhenol** | 45.8 ±5.3 * | 71.3 ±1.9 |
| **Mixture 1** | 74.5 ±3.8 | 81.8 ±3.9 |
| **Mixture 2** | 66.8 ±5.2 | 59.2 ±6.5 |

### 4.2.4 Disulfide Bonded Proteins

As a direct indication of oxidation, incubation of Jurkat *E*6.1 cells with 400 μM SMX-HA for 2 h (Fig 12-C) caused the virtual disappearance of the peroxiredoxin (Prx) band comprised of the 2-Cys dimeric disulphides of peroxiredoxins 1 and 2 (Prx 1 and 2; designated spot 1) compared to vehicle treated control cells (Fig 12-A) on R2D-PAGE gels. Prx 1-4 are dimeric proteins in which each monomer contains an active cysteine thiol residue. The disappearance of these protein is due to the oxidative effect of SMX-HA, which converts Prx 1 or 2 to its over oxidized sulfinic acid form. DTT is unable to reduce protein sulfinic acids so the over oxidized Prxs run on the diagonal line, causing the disappearance of Prx 1 and 2 from the right side and below the diagonal line of the reduced 2D-gel.

As a further sign of oxidative and/or nitrosative stress, SMX-HA-treated Jurkat *E*6.1 cells (Fig, 12-C) exhibit more protein spots derived from reduction of heterodimeric or homodimeric protein disulphides than the vehicle–treated control cells (Fig 12-A) or in Jurkat cells treated only with 5 μM BE (Fig. 12-B). These protein spots that occurred only after treatment with SMX-HA were labeled with letters (from a to l). The apparent molecular weights of these proteins are approximately 30, 35, 37, 39, 60, 65, 90, 112, 114, 115, 116, and 130 kDa, respectively. Most of these spots originated from proteins with intermolecular disulphide bonds and appeared below and to the right side of the diagonal line.

To evaluate the chemoprotective effects of the selected phytochemicals on the oxidation of reactive cysteine thiol containing proteins to protein mixed disulphides, Jurkat *E*6.1 cells were exposed to SMX-HA (400 μM) for 2 h with or without pretreatment with the pure TCM constituents and two synthetic mixtures for 30 min. The concentrations of the phytochemicals tested were in the range where there was no enhanced release of LDH relative to control cells, even 24 h after initial exposure to the TCM constituents (Tables 3 and 4). In other words, these concentrations

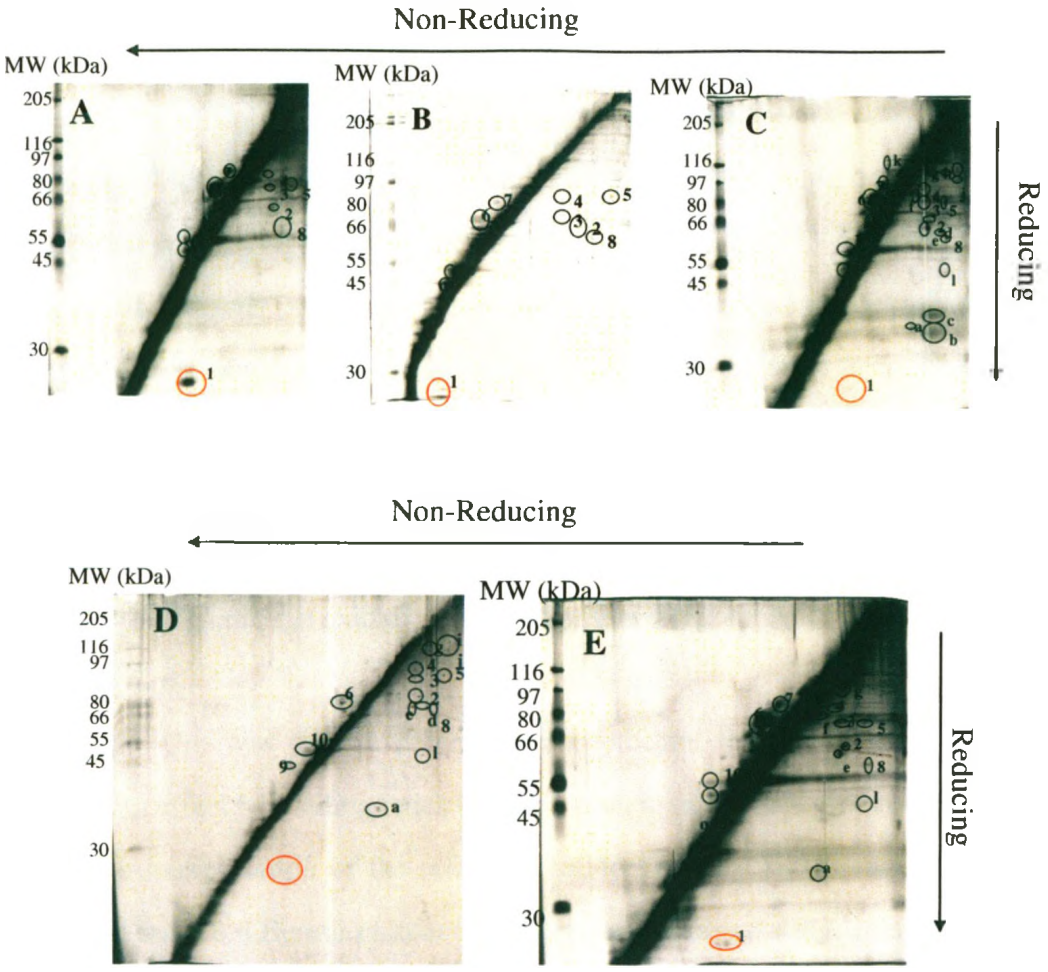
tested for chemoprotection did not cause even mild cytotoxicity to the cells.

Treatment of Jurkat *E*6.1 cells with 1 μM (Fig. 12-D) or 5 μM BE (Fig. 12-E) showed a concentration-dependent attenuation of the oxidation of Prx 1 and 2 best described as minor. However, pretreatment of Jurkat cells with 5 μM BE for 30 min caused the apparent loss of protein spots b, c, e, h and k, suggesting the oxidation of these proteins to intermolecular disulphide bonds was attenuated by pretreatment with 5 μM BE. Pretreatment with 1 μM BE did not attenuate the oxidation of Prxs 1 and 2 but it did attenuate the SMX-HA-mediated disappearance of proteins b, c, h, and k, verifying its antioxidant activity.

**Figure 12. Protective effects of Baicalein (BE: 1 and 5μM) on 400 μM SMX-HA-induced intercellular oxidation in Jurkat *E*6.1 cells evaluated by R2D-PAGE with silver staining.**

A = vehicle control (0.2% DMSO); B = 5 μM BE; C = 400 μM SMX-HA; D = 1 μM BE + 400 μM SMX-HA; E = 5 μM BE + 400 μM SMX-HA,

The protein spots seen in the vehicle-treated control cells are identified by numbers while the novel proteins found when the Jurkat *E*6.1 cells were incubated with SMX-HA are identified by letters. Supernatant proteins (85 μg) were loaded to the phase 1 gel in each case. All gels were run in triplicate, and the experiment was repeated three times, with a different batch of cells each time. A single representative gel is shown for each of the treatment conditions. The data from all 2D-dels for each experimental condition are summarized in tables 8 and 9.


Non-Reducing
MW (kDa)
205
116
97
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Reducing
Non-Reducing
MW (kDa)
D
E
Reducing

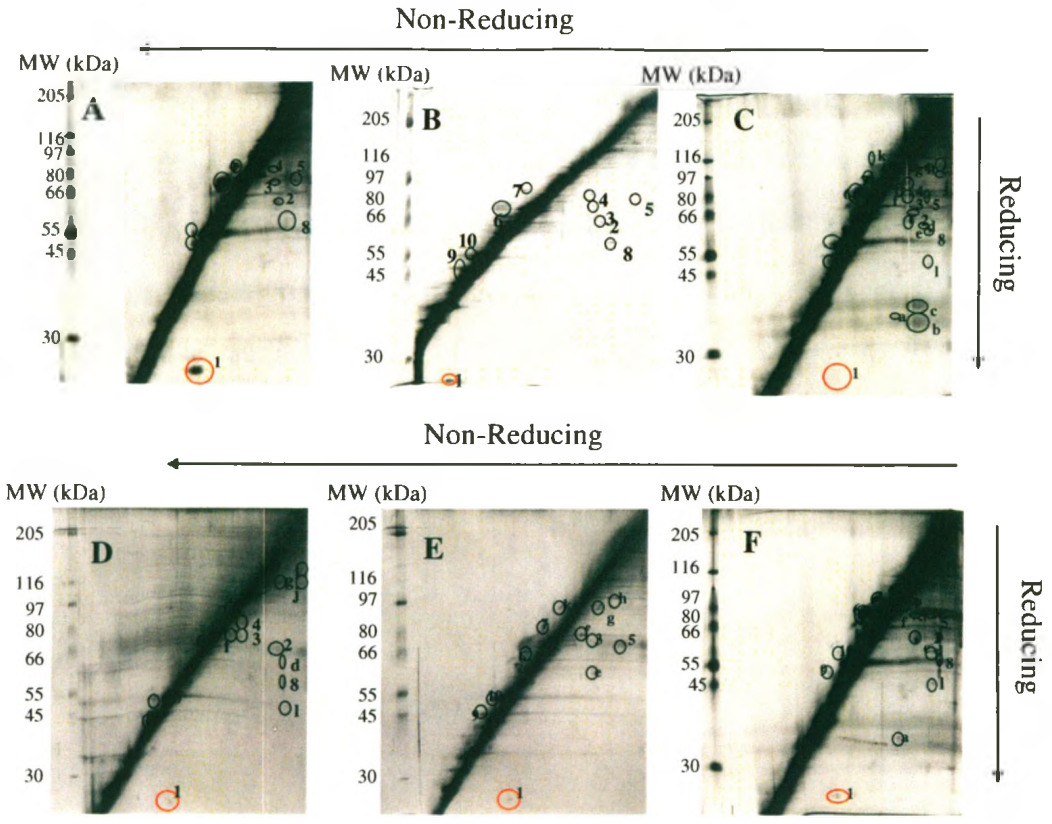
The second set of R2D-PAGE data shown (Figs. 13A-F) is for Cro. In all experiments, treatment of Jurkat *E*6.1 cells with 400 μM SMX-HA caused the complete loss of the Prx spot (Fig. 13-C). In contrast to the BE data, there was some detectable attenuation of Prxs 1 and 2 by the 1 μM Cro pretreatment (Figs. 13-D,E) that was slightly more pronounced at 5 μM Cro (Fig. 13-F). Although this protection is still only partial, there was concentration-dependent chemoprotection on SMX-HA-mediated oxidation of Prx according to the relative intensities of the Prx 1 and 2 spot. Besides, pretreatment with 5 μM Cro protected against SMX-HA-mediated loss of proteins b, c, h, i, j, k whereas pretreatment with 1 μM Cro lead to the loss of proteins b, c, i, l. In other words, the higher concentration of Cro protected against the oxidation of three proteins (h,j,k), an effect not observed with 1 μM Cro.

Because Cro was a more potent chemoprotectant for Prx 1 and 2, we also evaluated its effect by loading twice as much protein to the first dimension gel (Fig. 13-D). The interpretation of the protective effect, based on Prx 1 and 2 was not different at the t20 different protein concentrations. Consequently, gels were run at the higher protein concentrations for only the most effective chemoprotectans, based on the R2D gel analysis (Cro, and Mixtures 1 and 2).

**Figure 13. Protective effects of Crocetin (Cro; 1 and 5μM) on 400 μM SMX-HA-induced intercellular oxidation in Jurkat *E*6.1 cells evaluated by R2D-PAGE with silver staining.**

A = vehicle control (0.2% DMSO); B = 5 μM Cro; C = 400 μM SMX-HA; D = 1 μM Cro + 400 μM SMX-HA (170 μg protein); E = 1 μM Cro + 400 μM SMX-HA (85 μg protein); F = 5 μM Cro + 400 μM SMX-HA,

The protein spots seen in the vehicle-treated control cells are identified by numbers while the novel proteins found when the Jurkat *E*6.1 cells were incubated with SMX-HA are identified by letters. Supernatant proteins (85 μg) were loaded to the phase 1 gel in each case. All gels were run in triplicate, and the experiment was repeated three times, with a different batch of cells each time. A single representative gel is shown for each of the treatment conditions. The data from all 2D-dels for each experimental condition are summarized in tables 8 and 9..


Non-Reducing
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Non-Reducing
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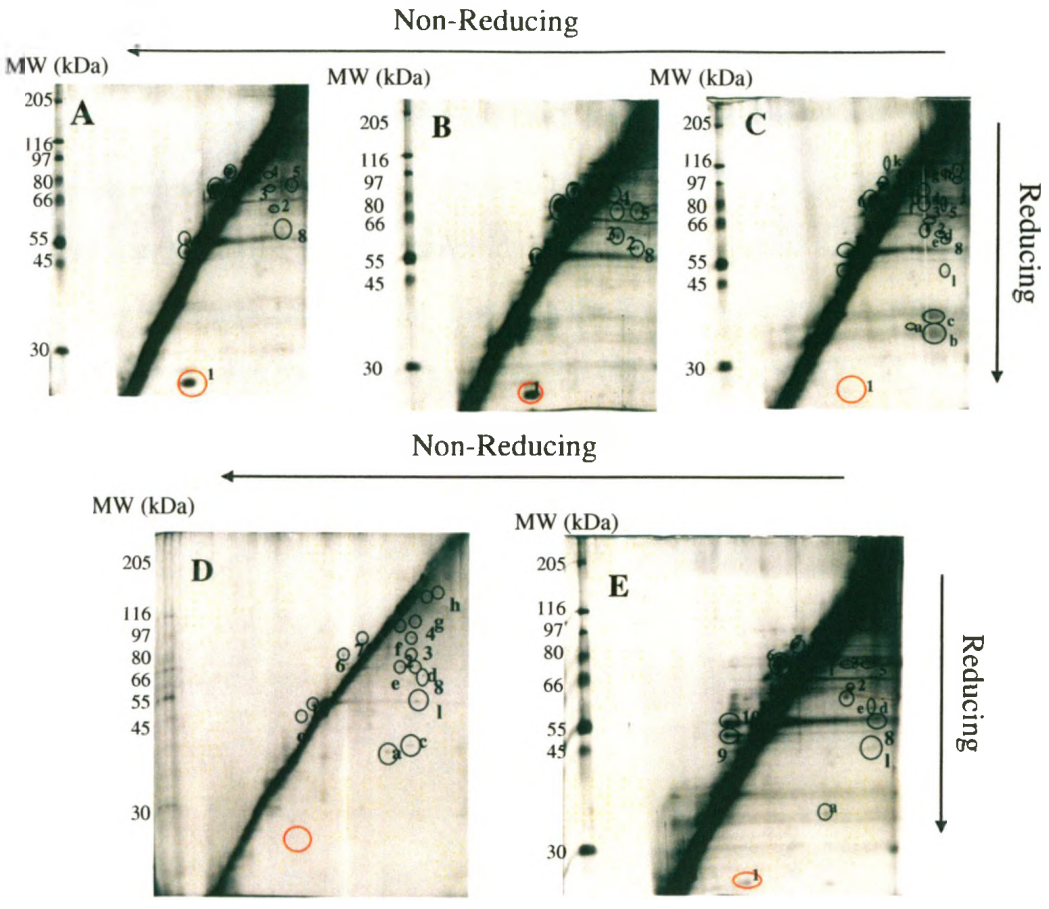
The third set of data presented (Fig. 14A-E) is for Res. Pretreatment with 5 μM (Fig 14-E) but not 1 μM Res (Fig. 14-D) partially prevented oxidation of Prx 1 and 2 and also prevented the oxidation of proteins b, c, h, i, j and k, which disappeared from the gel, clearly demonstrating the concentration-response of the Res effect. However, pretreatment with 1 μM Res for 30 min prior to SMX-HA prevented the oxidation of protein spots b, i, j k. Of interest, 5 μM Res (Fig. 14-B) appeared to increase the intensity of the Prx 1 and 2 spot, suggesting it attenuated the formation of Prx 1/2 sulfinic acids in Jurkat *E*6.1, normal incubation conditions for 2 h, in the absence of SMX-HA.

**Figure 14. Protective effects of Resveratrol (Res; 1 and 5 μM) on 400 μM SMX-HA-induced intercellular oxidation in Jurkat *E*6.1 cells evaluated by R2D-PAGE with silver staining**

A = vehicle control (0.2% DMSO); B = 5 μM Res; C = 400 μM SMX-HA; D = 1 μM Res + 400 μM SMX-HA; E = 5 μM Res + 400 μM SMX-HA

The protein spots seen in the vehicle-treated control cells are identified by numbers while the novel proteins found when the Jurkat *E*6.1 cells were incubated with SMX-HA are identified by letters. Supernatant proteins (85 μg) were loaded to the phase 1 gel in each case. All gels were run in triplicate, and the experiment was repeated three times, with a different batch of cells each time. A single representative gel is shown for each of the treatment conditions. The data from all 2D-dels for each experimental condition are summarized in tables 8 and 9.


Non-Reducing
MW (kDa)
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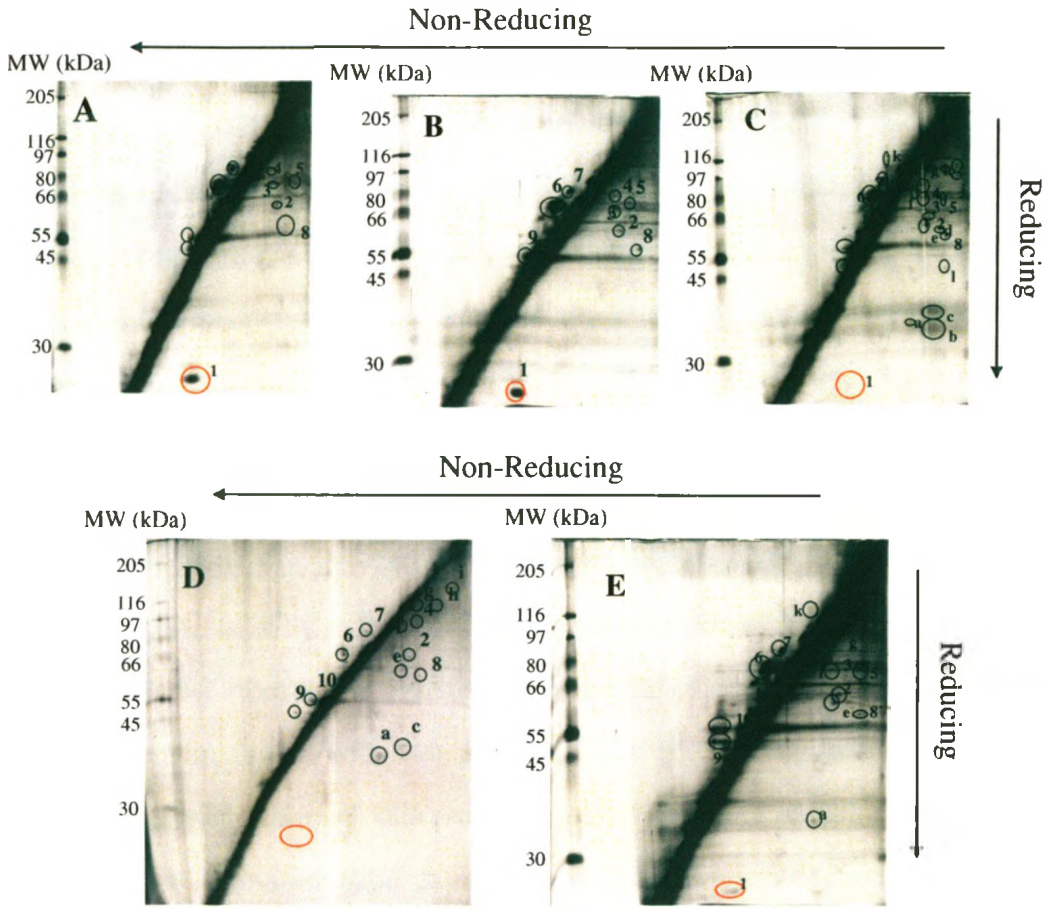
Reducing
Non-Reducing
MW (kDa)
D
E
Reducing

The final set of single compound data presented (Figs. 15A-E) is for Sal. Similar to the previous results described for BE and Res, treatment of Jurkat *E*6.1 cells with 1 μM Sal (Fig. 15-D) did not attenuate the oxidation of Prx 1/2, an effect observed with the higher concentration of Sal. Pretreatment for 30 min with 5 μM Sal partially attenuated SMX-HA-mediated oxidation of Prx 1/2 in Jurkat E6.1 cells and also protected against disulphide formation of proteins b, c, d, h, i, j and l (Fig, 15-E). Pretreatment of Jurkat cells with the lower concentration of Sal (1 μM; Fig 15-D) did prevent the oxidative conversion of proteins b, d, j, k and l to their homomeric or heterodimeric disulphides.

**Figure 15. Protective effects of Schishenol (Sal; 1 and 5 μM) on 400 μM SMX-HA-induced intracellular oxidation in Jurkat *E*6.1 cells evaluated by R2D-PAGE with silver staining**

A = vehicle control (0.2% DMSO); B = 5 μM Sal; C = 400 μM SMX-HA; D = 1 μM Sal + 400 μM SMX-HA; E = 5 μM Sal + 400 μM SMX-HA,

The protein spots seen in the vehicle-treated control cells are identified by numbers while the novel proteins found when the Jurkat *E*6.1 cells were incubated with SMX-HA are identified by letters. Supernatant proteins (85 μg) were loaded to the phase 1 gel in each case. All gels were run in triplicate, and the experiment was repeated three times, with a different batch of cells each time. A single representative gel is shown for each of the treatment conditions. The data from all 2D-dels for each experimental condition are summarized in tables 8 and 9.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In experiments described earlier in the Results, we compared the chemoprotectant effects of two synthetic mixtures with single antioxidant constituents of TCM. Mixture 1, in particular, was effective in attenuating SMX-HA-mediated LDH release, lipid hydroperoxide formation and protein carbonyl formation, the endpoints used to evaluate cytotoxicity. Here R2D PAGE analysis was used in an attempt to differentiate the relative antioxidant activities of the mixtures in comparison to the four individual the most reliable TCM constituents, at equimolar concentrations. Overall, in all R2D-PAGE experiments performed, attenuation of the disappearance of Prx 1/2 was the most sensitive indicator for antioxidant activity in cells treated with SMX-HA.

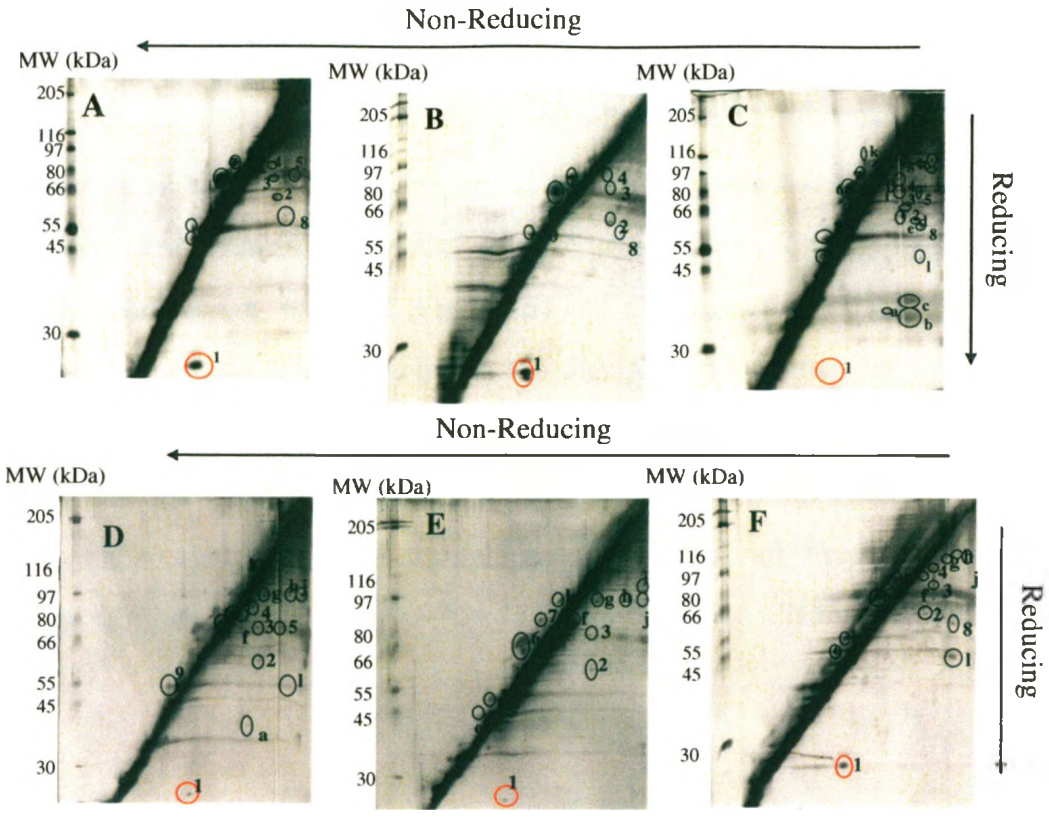
The mixture 1 (BE:Cro:Res:Sal; 1:1:1:1) data are shown below (Figs. 16A-F). Both 1 and 5 μM mixture 1 attenuated the oxidation of Prx 1/2 in Jurkat *E*6.1 cells resulting from treatment with 400 μM SMX-HA (Fig. 16-C) in a concentration dependent-manner. Although this protective effect was only partial, it is somewhat reassuring that the attenuation at the 1 μM concentration occurred in all gels loaded with rather 85 (Fig. 16-E) or 170 μg protein (Fig. 16-D). Moreover, the density of spot 1 (Prx 1/2) served as an effective dosimeter of the concentration-dependent antioxidant effects of mixture 1. Thus, the intensity of spot 1 increased from Fig. 16-E (1 μM mix 1; 85 μg protein) to Fig. 16-D (1 μM mix 1; 170 μg protein) to Fig. 16-F (5 μM mix 1; 85 μg protein). In addition, pretreatment with 5 μM mixture 1 (Fig. 16-F) attenuated the oxidation of proteins a, b, c, d, e, i, k compared to pretreatment with 1 μM mixture 1 (Fig. 16-E) which prevented the oxidation of proteins b, c, d, e, l.

**Figure 16. Protective effects of synthetic mixture 1 (mix 1; 1 and 5 μM) on 400 μM SMX-HA-induced intracellular oxidation in Jurkat *E*6.1 cells evaluated using R2D-PAGE followed by silver staining**

A = vehicle control (0.2% DMSO); B = 5 μM mix 1; C = 400 μM SMX-HA; D = 1 μM mix 1 + 400 μM SMX-HA (170 μg protein); E = 1 μM mix 1 + 400 μM SMX-HA (85 μg protein); F = 5 μM mix 1 + 400 μM SMX-HA (85 μg protein) ,

Mixture 1 is an equimolar ratio of baicalein, crocetin, resveratrol and schishenol.

The protein spots seen in the vehicle-treated control cells are identified by numbers while the novel proteins found when the Jurkat E6.1 cells were incubated with SMX-HA are identified by letters. Supernatant proteins (85 μg) were loaded to the phase 1 gel in each case. All gels were run in triplicate, and the experiment was repeated three times, with a different batch of cells each time. A single representative gel is shown for each of the treatment conditions. The data from all 2D-dels for each experimental condition are summarized in tables 8 and 9.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Non-Reducing
MW (kDa)
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F
Reducing

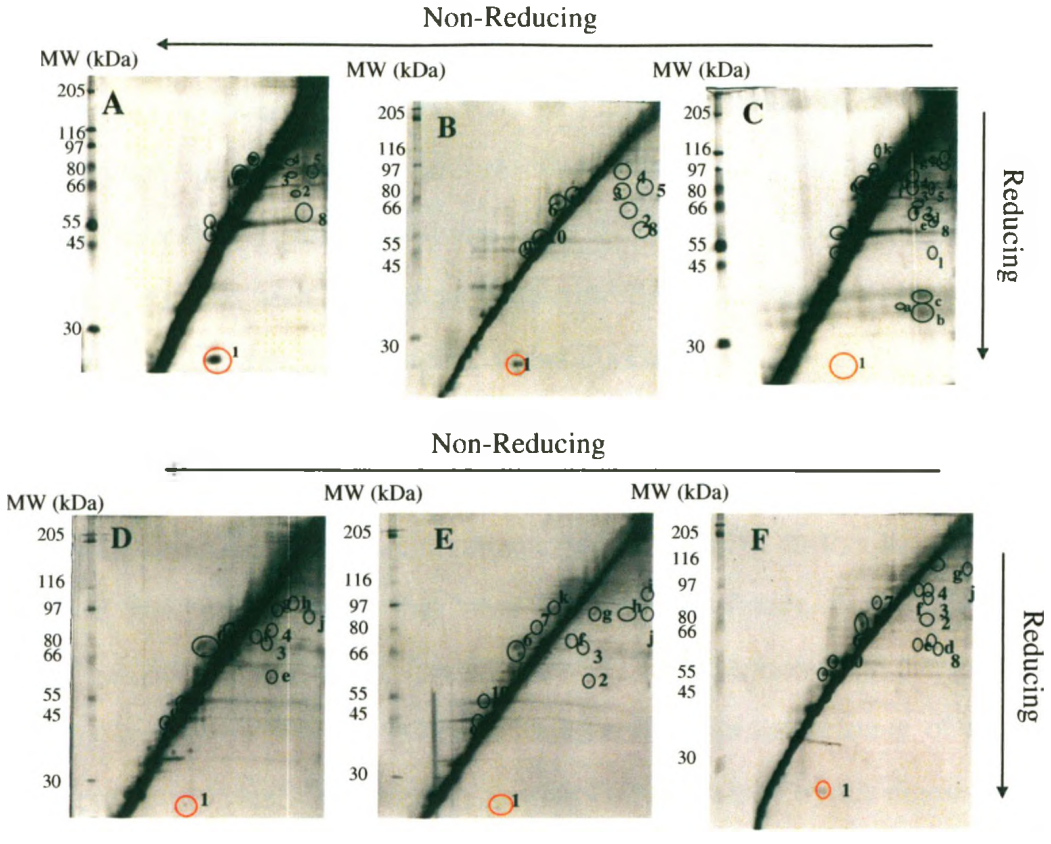
Finally, we tested the ability of synthetic mixture 2 (BE:Cro:Res; 1:1:1; Fig. 17-D, -E and –F) to attenuate the SMX-HA induced oxidative changes in Jurkat *E*6.1 cells (Fig. 17C). Based on the intensity of the Prx 1/2 spot, this mixture was more effective than any of the single antioxidants tested or mixture 1, at equivalent concentrations. Pretreatment with either 1 or 5 µM mixture 2 prevented the oxidation of proteins a, b, c, d, k, l (Fig. 17-E) and a, b, c, h, i, k, l (Fig. 17-F), respectively. The results of the changes in the disulphide proteome , attenuated (Fig. 17 D-F) partly reduced SMX-HA mediated disulphide proteome, which represent in the disappearance of protein spots a, b, c, h, i, k, l and respectively. The comparative changes in the disulphide proteome of all treatments are summarized in Table 8 and 9.

**Figure 17. Protective effects of synthetic mixture 2 (mix 2; 1 and 5 μM) on 400 μM SMX-HA-induced intracellular oxidation in Jurkat *E*6.1 cells evaluated using R2D-PAGE followed by silver staining**

A = vehicle control (0.2% DMSO); B = 5 μM mix 2; C = 400 μM SMX-HA; D = 1 μM mix 2 + 400 μM SMX-HA (170 μg protein); E = 1 μM mix 2 + 400 μM SMX-HA (85 μg protein); F = 5 μM mix 2 + 400 μM SMX-HA (85 μg protein)

Mixture 2 is an equimolar ratio of baicalein, crocetin, and resveratrol

The protein spots seen in the vehicle-treated control cells are identified by numbers while the novel proteins found when the Jurkat *E*6.1 cells were incubated with SMX-HA are identified by letters. Mitochondrial supernatant proteins (85 μg) were loaded to the phase 1 gel in each case. All gels were run in triplicate, and the experiment was repeated three times, with a different batch of cells each time. A single representative gel is shown for each of the treatment conditions. The data from all 2D-dels for each experimental condition are summarized in tables 8 and 9.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Non-Reducing
MW (kDa)
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Reducing

Treatment of Jurkat *E*6.1 cells with 5 μM BE, Cro, Res, Sal, mixture 1 or mixture 2 alone had no or little effect on the disulphide proteome in each case. The protein spots that are identified on the R2D gels and these data for each of the treatments with the phytochemical alone are summarized in Table 8. The numbers in this table indicate the number of times each spot was observed in the three different gels run for each individual treatment. Each number represents an individual treatment of a different batch of Jurkat *E*6.1 cells.

Of importance for our study, the effect of the phytochemicals on the intensity of the Prx spot(s) was small compared to that from untreated control cells. Other changes in the disulfide pattern were summarized in Table 9. In this table, there were several new protein spots that were labeled with letters. These proteins underwent oxidation process and formed inter- or in intra-molecular disulphide bonds under oxidative effect of SMX-HA. Among these proteins, some of them have been previously identified by mass spectrometric analysis of tryptic digests in a related study which evaluated the formation of protein-glutathione mixed disulphides by S-glutathionylation of proteins in Jurkat cells following treatment with cellular oxidants. Earlier unpublished work in our laboratory also identified HSP 70-pB; aldolase; HSP 60 and enolase using both matrix associated laser desorption/ionisation-time of flight-mass spectrometry (MALDI-TOF-MS) and/or electrospray ionisation-quadropole-time of flight-tandem mass spectrometry (ESQ-Q-TOF-MS/MS) analysis of proteins that formed covalent bonds when MOLT-3 cells were treated with SMX-HA, a reaction that occurs primarily at the thiolate ion of proteins with reactive cysteine residues .When we compared the attenuation effect of 5 μM single constituent on SMX-HA induced disulfide proteome, there was no significant difference shown either in the intensity of Prx or the numbers of disulfide proteome formation mediated by SMX-HA. However, in low concentration's (1 μM) comparison, mixtures showed better antioxidant effects than the single chemical. More specifically, when we compared the intensity of Prx, pretreatment with mixture 1 was associated with higher intensity of Prx than

pretreatment with mixture 2, suggesting that mixture 1 possess the most potent antioxidant effect in regulating the redox states of disulfide proteome. This conclusion was correlated to the previous results, suggesting that the combination of pure TCM constituents can work through complementary mechanisms to produce a greater protective effect than single component on an equimolar basis; also, the enhanced chemoprotective effect of mixture 1 is partly credited to the presence of Sal, as noted above an important contributor of the mixture.

**Table 8. Proteins formed from protein-protein disulphides that occurred on R2D gels of Jurkat *E*6.1 cells treated with 5 μM Baicalein, Crocetin, Resveratrol, Schisanhenol or Mixture 1 or 2, compared to vehicle-treated control cells**

Cells were incubated with 5 μM of each of the TCM constituents or Mixtures 1 or 2 for 30 min. Grey squares indicate the presence of the corresponding protein spots in Jurkat *E*6.1 cells under the different treatment conditions. The numbers in each square indicate the number of times these protein spots showed up in three independent experiments.

Mixture 1 is an equimolar mixture of baicalein, crocetin and resveratrol and schisanhenol; Mixture 2 is an equimolar mixture of baicalein, crocetin, and resveratrol.

The monomeric molecular weights of proteins 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 are 25, 64, 73, 82, 74, 75, 85, 58, 50, 55 kDa, respectively

| Protein spots | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
|---|---|---|---|---|---|---|---|---|---|---|
| control | 3 | 3 | 3 | 1 | 2 | 3 | 3 | 2 | 3 | 3 |
| 5μM BE | 3 | 2 | 1 | 2 | 0 | 3 | 3 | 2 | 3 | 3 |
| 5μM Cro | 3 | 3 | 3 | 2 | 2 | 3 | 3 | 1 | 3 | 3 |
| 5μM Res | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 2 | 0 | 3 |
| 5μM Sal | 3 | 3 | 3 | 2 | 3 | 3 | 3 | 2 | 3 | 0 |
| 5μM mix1 | 3 | 3 | 3 | 2 | 1 | 3 | 3 | 3 | 0 | 3 |
| 5μM mix2 | 3 | 3 | 3 | 3 | 2 | 3 | 3 | 2 | 3 | 3 |

**Table 9. Proteins formed from protein-protein disulphides that occurred on R2D gels of Jurkat *E*6.1 cells pretreated with 5 μM Baicalein, Crocetin, Resveratrol, Schisanhenol or Mixture 1 or 2 for 30 min prior to incubation with 400 μM SMX-HA for 2 h**

Cells were pretreated by incubation with 1 and 5 μM of each of the TCM constituents or Mixtures 1 or 2 for 30 min prior to exposure to 400 μM SMX-HA for 2 h. Grey squares indicate the presence of the corresponding protein spots in Jurkat *E*6.1 cells under the different treatment conditions. The numbers in each square indicate the number of times these protein spots showed up in three independent experiments.

Mixture 1 is an equimolar mixture of baicalein, crocetin and resveratrol and schisanhenol; Mixture 2 is an equimolar mixture of baicalein, crocetin, and resveratrol.

The monomeric molecular weights of proteins 1-10 are shown in Table 8. The monomeric weights of proteins a-l are: 30, 35, 37, 39, 60, 65, 90, 112, 114, 115, 116 and 130 kDa, respectively.

| Protein spots | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | a | b | c | d | e | f | g | h | i | j | k | l |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| control | 3 | 3 | 3 | 1 | 2 | 3 | 3 | 2 | 3 | 3 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 400μM SMX-HA | 0 | 3 | 2 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 2 | 2 | 3 | 3 | 2 | 3 | 2 | 3 | 3 | 2 | 2 |
| 1μM BE+HA | 0 | 3 | 3 | 2 | 2 | 3 | 2 | 1 | 3 | 3 | 3 | 0 | 0 | 3 | 3 | 1 | 3 | 0 | 2 | 2 | 0 | 1 |
| 5μM BE+HA | 3 | 3 | 2 | 3 | 2 | 3 | 3 | 2 | 3 | 3 | 2 | 0 | 0 | 1 | 0 | 1 | 3 | 0 | 2 | 2 | 0 | 1 |
| 1μM Cro+HA (170ug) | 3 | 2 | 2 | 3 | 0 | 3 | 3 | 2 | 3 | 3 | 1 | 0 | 0 | 2 | 0 | 2 | 3 | 0 | 3 | 2 | 0 | 1 |
| 1μM Cro+HA (85ug) | 3 | 0 | 3 | 0 | 3 | 3 | 3 | 0 | 3 | 3 | 1 | 0 | 0 | 0 | 3 | 3 | 3 | 3 | 0 | 1 | 2 | 0 |
| 5μM Cro+HA | 3 | 3 | 2 | 3 | 2 | 3 | 3 | 2 | 2 | 1 | 2 | 0 | 0 | 2 | 3 | 2 | 1 | 0 | 0 | 0 | 0 | 2 |
| 1μM Res+HA | 0 | 3 | 3 | 2 | 0 | 3 | 3 | 2 | 2 | 3 | 3 | 0 | 2 | 2 | 3 | 2 | 1 | 2 | 0 | 0 | 0 | 2 |
| 5μM Res+HA | 3 | 3 | 2 | 0 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 0 | 0 | 3 | 3 | 2 | 2 | 0 | 0 | 0 | 0 | 1 |
| 1μM Sal+HA | 0 | 3 | 1 | 3 | 0 | 3 | 2 | 1 | 2 | 3 | 3 | 0 | 1 | 0 | 2 | 3 | 3 | 2 | 3 | 0 | 0 | 0 |
| 5μM Sal+HA | 3 | 3 | 3 | 1 | 2 | 3 | 3 | 2 | 3 | 3 | 3 | 0 | 0 | 0 | 3 | 3 | 2 | 0 | 0 | 0 | 1 | 0 |
| 1μM mix 1+HA (170ug) | 3 | 2 | 2 | 3 | 1 | 3 | 1 | 0 | 2 | 1 | 2 | 0 | 0 | 0 | 0 | 3 | 3 | 2 | 0 | 3 | 2 | 2 |
| 1μM mix 1+HA (85ug) | 3 | 3 | 3 | 1 | 0 | 3 | 3 | 1 | 3 | 3 | 1 | 0 | 0 | 0 | 0 | 3 | 3 | 3 | 2 | 3 | 2 | |
| 5μM mix 1+HA | 3 | 3 | 3 | 3 | 0 | 3 | 3 | 2 | 3 | 3 | 0 | 0 | 0 | 0 | 0 | 3 | 3 | 3 | 0 | 2 | 0 | 3 |
| 1μM mix 2+HA (170ug) | 3 | 1 | 3 | 2 | 0 | 3 | 3 | 3 | 3 | 2 | 0 | 0 | 0 | 0 | 3 | 3 | 3 | 2 | 1 | 3 | 0 | 1 |
| 1μM mix 2+HA (85ug) | 3 | 3 | 3 | 1 | 1 | 3 | 3 | 0 | 3 | 3 | 0 | 0 | 0 | 1 | 1 | 3 | 3 | 3 | 2 | 2 | 1 | 0 |
| 5μM mix 2+HA | 3 | 3 | 3 | 3 | 1 | 3 | 3 | 2 | 3 | 3 | 0 | 0 | 0 | 3 | 3 | 3 | 3 | 0 | 0 | 3 | 0 | 0 |

# CHAPTER 5: DISCUSSION

The goal of our research was to pursue the hypothesis that anti-oxidant compounds of herbal origin, such as BE, Cro, Res and Sal, may mediate sulfamethoxazole hydroxylamine (SMX-HA) induced oxidative and nitrosative stress in Jurkat *E*6.1 cells. The compounds tested in our study have been used, along with other herbal medicines, for over two thousands years as part of Traditional Chinese Medicine.

SMX is a antimicrobial drug that has been used to treat a number of common and important infections. However, a major problem associated with sulphonamide therapy has been hypersensitivity reactions. The pathogenesis of hypersensitivity reactions to sulphonamides is complex and has been incompletely understood, but appears to involve initial bioactivation of the sulphonamide to a reactive intermediate followed by cellular injury and a misdirected immune response. Carr et al. have demonstrated that, in the context of HIV infection – known to be a risk factor for a sharp increase in risk for sulphonamide hypersensitivity - the pathogenesis of SMX-HA induced hypersensitivity ADRs, at least *in vitro,* appeared to be associated with decreased total GSH concentrations (Carr, Tindall et al. 1993) while in the same context it has been suggested that there are increases in activated form of caspase-3 (Adeyanju, Krizova et al. 2009). The role of oxidative stress in serious ADRs has been increasingly appreciated. With special relevance to serious ADRs, it has been reported that arylhydroxylamine metabolites of SMX induce ROS generation in normal human epidermal keratinocytes (Vyas, Roychowdhury et al. 2005). During the auto-oxidation process of SMX-HA, a succession of reactions occurs, which include reduction of molecular oxygen to superoxide, followed by conversion of superoxide to hydrogen peroxide either spontaneously or catalyzed by superoxide dismutase (SOD). $H_2O_2$ can be converted to the hydroxyl radical through a Fenton reaction

(Vyas, Roychowdhury et al. 2005). Additionally, superoxide rapidly reacts with nitric oxide which is often produced simultaneously in cells, to form peroxynitrite. In this series of reactions, the production of superoxide, hydrogen peroxide, hydroxide radicals, and peroxynitrite all produce oxidative and nitrosative stress. These alterations in ROS and RNS may form the biochemical basis for cellular injury leading to a misdirected immune response in sulphonamide hypersensitivity. ROS and RNS generated during the metabolic process can cause cell death and oxidation of protein and lipid, which we evaluated as increases of protein carbonyl content and lipid hydroperoxide.

We believe that attenuating oxidative stress will reduce cellular injury and damage, and may reverse or prevent drug hypersensitivity in patients at risk. Our hypothesis was that BE, Cro, Res, and Sal purified anti-oxidants, common constituents of TCM, would attenuate oxidative and nitrosative stress and maintain the redox states of cells. The first objective of our study was to determine the individual effect of BE, Cro, Res, and Sal for cell viability in Jurkat *E*6.1 cells, in a time- and concentration-dependent manner. The ability of these compounds to alter oxidative injury in these low concentrations has not been reported previously. These observations are potentially important and suggest non-toxic concentrations of herbal phytochemicals can serve as chemoprotectants. Moreover, our results suggested a possible duality between the pro-oxidant and anti-oxidant effects of BE, Cro, Res, and Sal. Many of the desired pharmacological and biochemical activities of natural products are believed to be related to their anti-oxidant effects. However, it is known that anti-oxidants can also act as reducing agents. They can thus exhibit protective effect at low concentration while act as pro-oxidants and show their potential toxic, pro-oxidative effect at higher concentrations (Woo, Cheng et al. 2005). Many studies focused on herbal medicines in isolation have ignored the potential pro-oxidative effects of these compounds at higher concentrations.

The antioxidant basis of BE has been illustrated by Woo and colleagues who applied BE on rat cardiomyocytes, and found that BE performed its anti-oxidant activities by forming stable semiquinone radicals (Woo, Cheng et al. 2005). The oxidation of flavonoids to form semiquinone radicals was accompanied with the generation of $O_2^{\cdot-}$, which can further oxidize the flavonoids, leading to the production of $H_2O_2$ (Woo, Cheng et al. 2005). The generation of ROS in this process may be the basis for the pro-oxidant effect of BE. Although this issue has not been investigated with Cro, the anti-oxidant and prooxidant activity of carotenoids has been reported due to be related to various factors including the structure, the location of carotenoid molecule in the cell, the interaction with other anti-oxidants, the partial pressure of oxygen, as well as its cellular concentrations (Young and Lowe 2001). High concentrations of carotenoids may increase the permeability of biological membrane (Lowe, Vlismas et al. 2003) and permit free radicals to enter cells easily. It has also been reported that high concentrations (10 μM) of β-carotene can enhance the pro-oxidant (Fotiou, Fotiou et al.) effect by inducing catalase and superoxide dismutase as well as depleting the amount of glutathione peroxidase (Lawlor and O'Brien 1995). In addition, the formation of a carotenoid peroxyl radical during the interaction with ROS can further induce lipoperoxidation (carotenoids as prooxidants 2003). Resveratrol has also been reported to possess pro-oxidant activities which may be part of the basis of its reported anti-proliferative, pro-apoptotic, and anticancer properties (Ruweler, Gulden et al. 2009). Moreover, Fotiou *et al.* found that resveratrol can cause neurotoxicity through up-regulation of nitric oxide synthase and inducing peroxynitrite and singlet oxygen formation (Fotiou, Fotiou et al. 2010). The prooxidant activity of schisanhenol has not been well characterized in the literature. However, the previous work that have been done in our lab showed that 100 μM of schisanhenol caused a significant increase in LDH release from HEK 293 cells when compared to the DMSO control, indicating toxicity to the cells at this concentration.

Our second objective was to determine the effect of pure constituents of traditional Chinese remedies on cell viability in cells treated with SMX-HA. Most current assays for measuring cytotoxicity are based on alterations in plasma membrane permeability and the subsequent release (leakage) of components into the supernatant, or the uptake of dyes, which are normally excluded by viable cells. This study using the LDH release assay demonstrated an attenuation of SMX-HA cytotoxicity by BE, Cro, Res, and Sal alone and in combination treatment at the concentration of 1 and 5 μM. At the concentration of 1 μM, Cro treatment inhibited LDH release to a greater degree than other chemicals alone. However, at a concentration of 5 μM, mixture 1 showed the best cytoprotective effect among these chemicals. In this experiment, low doses (1 and 5 μM) of these anti-oxidants singly and in combination significantly attenuated toxicity induced by 400 μM SMX-HA. This suggests that concurrent therapy with low dose anti-oxidants has the potential to reduce the toxicity of reactive drug metabolites. However, our data also suggest that these effects occur within a specific range with the potential for high doses to increase toxicity, suggesting that *in vitro* the dose must be carefully controlled to avoid toxicity or enhanced adverse drug effects.

A lipid hydroperoxide assay was used to study SMX-HA induced lipid peroxidation, an important marker for cellular injury occurring during oxidative stress. Lipids are a varied group of water insoluble compounds and one of the most important components of cells. Lipids involve in various functions of cells such as energy storage molecules, structural components of biological membranes, enzyme cofactors, as well as intracellular messengers. The properties of lipids make it become one of the main targets of free radicals like ROS or RNS. All lipids are derived from fatty acids which can be subdivided into saturated fatty acids, monounsaturated fatty acids, and polyunsaturated fatty acids (PUFA) based on the numbers of carbon-carbon double bonds. Saturated fatty acids have no carbon-carbon double bonds, while monounsaturated fatty acids contain one double bond. Using the membrane lipids as an example, the major component is

phospholipids which contain PUFA. PUFA have two or more double bonds which are susceptible to oxidation (McIntyre and Hazen 2010). Oxidative damage to lipids (lipid peroxidation) is regarded as an important process which is involved in various diseases. Their susceptibility to oxidation is mainly due to its structure which possesses a single methylene group (□$CH_2$□) group between two adjacent double bonds (McIntyre and Hazen 2010). ROS are able to abstract a hydrogen atom from the methylene group, forming a carbon-centered radical that reacts with molecular oxygen to form the lipid hydroperoxide (Podrez, Abu-Soud et al. 2000).

Generally, the process of lipid peroxidation involves three stages: initiation, propagation, and termination (Gutierrez, Reboredo et al. 2007). In initiation, ROS or RNS abstract a hydrogen atom from methylene group of unsaturated lipid and thus produce a lipid radical; the lipid radical is not stable and easily react with molecular oxygen to generate lipid peroxyl radicals, once a lipid peroxyl radical is generated, it will further react with neighbouring lipid radical by abstracting hydrogen from another lipid molecule, producing lipid hydroperoxides, which is called propagation. In this process, the resulting radical can then continue a free radical chain reaction. The propagation stage can be repeated many times and will be terminated when two radicals react and produce a non-radical species (Catala 2010; Klaunig, Wang et al. 2011). It has been reported that high dose of hydrogen peroxide can lead to Jurkat cell apoptosis through the membrane lipid peroxidation (Enukidze, Machavariani et al. 2009). One of the common characteristics of baicalein, crocetin, resveratrol, and schisanhenol is the ability of capturing free radicals, and these antioxidants can accelerate termination of lipid peroxidation via free radical capture and, therefore, protect the cell membrane against oxidation.

This work clearly demonstrated that biologically relevant concentrations of SMX-HA were associated with the production of significant amounts of oxidative stress. The concentrations of SMX-HA used were those that would be predicted to occur in vivo in patients treated with sulphonamides (Rieder et al. 1995). We

evaluated the anti-oxidant properties of these four chemicals using lipid peroxidation as a biomarker. It has been demonstrated that at concentration 100 μM and greater, SMX-HA has the ability to bind to the outer surface of neutrophils and lymphocytes, and thus cause the loss of membrane and show toxicity to lymphocytes (Naisbitt, Hough et al. 1999). In our project, we measured lipid hydroperoxide content to evaluate the oxidant effect of SMX-HA on lipids. There was a significant increase in lipid hydroperoxide content in Jurkar *E*6.1 cells treated with 400 μM SMX-HA compared to vehicle treated control cells. Thus, this suggests that the auto-oxidation of SMX-HA may occur on the plasma membrane, resulting in the oxidation of lipid, and SMX-HA dose cause oxidative stress on lipid cell membrane. 5 and 20 μM BE, Cro, Res, Sal, mixture 1, and mixture 2 pretreatment appeared to attenuate lipid hydroperoxide formation mediated by SMX-HA in a concentration-dependent manner. At the concentration of 5 μM, pretreatment with Cro for 30 min inhibited lipid hydroperoxide formation to a greatest degree which decreased lipid hydroperoxide content by 72%. However, pre-incubation for 30 min with 20 μM of mixture 1 showed the best cytoprotective effect among these chemicals, with lipid hydroperoxide content induced by SMX-HA decreased by 96%. We demonstrated that low concentrations (5 or 20 μM) of BE, Cro, Res and Sal attenuate SMX-HA-induced lipid hydroperoxide formation in concentration-dependent manner. Especially for Cro and mixture 1, the cytoprotective effect may occur through cytoprotection against lipid peroxidation.

SMX-HA undergoes auto-oxidation to form SMX-NO, a more reactive compound than SMX-HA (Rieder et al. 1995). It appears that binding of SMX-HA and SMX-NO to proteins is a key step in serious ADRs to sulphonamides. Proteins are essential for biological organisms. They participate in diffferent biological processes due to various activities and functions. ROS and RNS mediated oxidative and nitrosative stress may damage proteins and proteins are likely the most direct target for oxidative damage (Dalle-Donne, Rossi et al. 2003). Proteins

are composed of amino acids, some of which are susceptible to ROS and RNS and can form protein carbonyls (Dalle-Donne, Rossi et al. 2003). Protein carbonylation is an irreversible oxidative modification and usually refers to the formation of reactive ketones or aldehydes on protein side chains, especially those containing proline, argnine, lysine, and threonine (Suzuki, Carini et al. 2010). Carbonylated proteins either undergo proteolysis or accumulation as damaged proteins (Dalle-Donne, Scaloni et al. 2005). Thus, formation of carbonylated proteins is regarded as the most commonly modification during protein oxidation. For this reason protein carbonylation has been widely used in the assessment of cellular oxidative stress (Luo and Wehr 2009). Briefly, one common method of detecting protein carbonylation relies upon the reaction between 2,4-dinitrophenylhydrazine (DNPH) and DNPH detectable protein products like reactive ketone and aldehyde groups formed during oxidation of proteins (Suzuki, Carini et al. 2010). These protein aldehydes and ketones are formed either by oxidation of side chains of proteins, called primary protein carbonylation; or produced from the radicals formed during the lipid peroxidation process, called secondary protein carbonylation. In the primary protein carbonylation, ROS oxidize the side chains of protein, leading to inactivation, crosslinking, and/or degradation of proteins (Suzuki, Carini et al. 2010; D'Alessandro, Rinalducci et al. 2011). It has been reported that 100 μM of hydrogen peroxide leads to the protein carbonyl formation in a time-dependent manner in Jurkat cells (Cox and Hampton 2007).

We measured the carbonylation of total protein including three subcellular fractions- the mitochondria, the microsomes, and the cytosol. Microsomal proteins are a source of CYP (Neuman, Malkiewicz et al. 2000). Proteins within the cytosol play a key role in signal transduction pathway and glycolysis (Cumming, Andon et al. 2004). They also act as intracellular receptors and are a key component of ribosomes (Schwartz 2007). When quantifying protein carbonyl content in Jurkat *E*6.1 cells, treatment with 400 μM SMX-HA significantly increases protein carbonyl content compared to vehicle treated control cells, which suggests that

SMX-HA does oxidize protein carbonyls. 5 and 20 μM BE, Cro, Res, Sal, mixture 1, and mixture 2 pretreatment effectively attenuated protein carbonyl formation mediated by SMX-HA in a concentration-dependent manner. At the concentration of 5 μM, pre-treatment with mixture 1 for 30 min maximally decreased protein carbonyl content by 75% (Fig 4B). As same as 5 μM, pre-incubation for 30 min with 20 μM of mixture 1 also showed the best cytoprotective effect among these chemicals. Protein carbonyl content was reduced by 82%. As an important marker of protein oxidation, the measurement of protein carbonylation in our study indicated that SMX-HA induced oxidative damage. Low concentrations (5 or 20 μM) of mixture 1 attenuated SMX-HA mediated protein oxidation to a great extent, suggesting that mixture 1 was optimal for attenuation of SMX-HA induced oxidative stress, especially at the level of the proteins.

Modification of thiol-disulfide bonds of cysteine is regarded as a primary and important consequence of an altered oxidative states. The importance of this modulation is involved both in signal transduction in normal cell biology and in the defence of the proteins against oxidative stress. Redox-related processes can produce conformational changes to protein structures related to formation of disulfide bonds, sulfenic, sulfinic, and sulfonic acid. These can lead to the modification of protein properties and functions. The ionized thiol group is oxidized to a cysteine radical under high levels of oxidative stress in the cells. During this oxidation, the formation of sulphenic acids are readily reduced through reacting with GSH, glutaredoxin, or thioredoxin systems (Baty, Hampton et al. 2005). This reduction is regarded as one of several routes and is depended on the availability of free GSH which is the most abundant, non-enzymatic antioxidant in the cells. When this and other natural antioxidant systems are overwhelmed, the cysteine radicals can react with GSH, forming reversible protein mixed disulphides, intra- or inter-molecular disulphides bonds, protect the protein from further oxidation. However, during oxidative stress, the cysteine radical can be further oxidized to sulphinic and sulphonic acids. These two oxidation products are

irreversible and dysfunctional modification of proteins. R2D PAGE is a sophisticated technique that has recently been developed to visualize the cysteine modulations that undergo molecular changes during different conditions of redox or oxidative stress (Cumming, Andon et al. 2004). It has been reported that as many as 400 proteins with oxidized cysteine residues can be detected by R2D PAGE, these proteins being either in a disulphide state or in oxidized form (Baty, Hampton et al. 2005). We used this technique to investigate the effects of oxidative stress produced by SMX-HA treatment and alterations produced by anti-oxidants pretreatment of Jurkat *E*6.1 cells. The diagonal line represents all the proteins that were not altered or modified in the reducing step of the experiment. The proteins on the diagonal line are either not redox-regulated by reactive cysteine thiol groups or are irreversibly oxidized proteins that contain reactive cysteine thiol moieties. Proteins that have formed intramolecular disulfide bonds before the reduction appear slightly above and to the left of the diagonal line, while those that formed intermolecular disulphide bonds appear below and to the right of the diagonal line. Because of the relatively low sensitivity of this technique, only proteins that are found at high concentration will be visible on the gels, which may explain why there are so few distinct protein spots found on the gels (Cumming, Andon et al. 2004). This issue also plays a part in the slight variability in presence and intensity of protein spots between gels on different runs. In addition, while one may expect more differences between the SMX-HA treated cells and DMSO control, both show relatively few protein spots. Although this could indicate that there are few proteins that formed disulphide bonds, it could also indicate the oxidation of protein cysteine thiols to irreversible sulphonic acids (as may be the case in oxidant treated cells). In our studies, there were three proteins spots that highlighted in 25 kDa region were stood out on some anti-oxidant control. These spots were identified using mass spectrometry to be peroxiredoxins (prxs) 1, 2 and 3, respectively. In addition, our laboratory has identified this protein by mass spectrometry analysis of tryptic fragments in both

human kidney (HEK 293) and mouse liver (Hepa 1c1c7) cell lines but not in Jurkat cells.

Prxs are predominant targets of ROS that enter cells. However, in most of our studies, only one cluster of 25 kDa protein was seen, that being identified as prx 1 and 2. In addition, the R2D PAGE shown that Prx are located on the right side of the diagonal line, which represents Prx as having intermolecular disulfide bonded protein in untreated Jurkat cells. In this case, it has been reported that 2-Cys Prxs exist as homodimers in a head-to-tail conformation (Smith-Pearson, Kooshki et al. 2008) link by the peroxidatic cysteine residue in conserved NH2-terminal under physiological conditions. Prxs are known redox-regulated proteins and belong to the thioredoxin-peroxiredoxin system (Smith-Pearson, Kooshki et al. 2008). Prxs are encoded by distinct genes and include of six isoforms (Prx 1-6). The 2-Cys Prxs is the predominant Prxs subfamily, comprising Prx 1-4. Prxs have important anti-oxidant properties because they detoxify peroxides by transferring reducing equivalents from NADPH to peroxides (Smith-Pearson, Kooshki et al. 2008), and reduce hydrogen peroxide to water until their reactive thiol groups are oxidized to their sulfinic or sulfonic acid forms. Also, the antioxidant properties of Prxs may be due to the complementary function with glutathione peroxidases and catalases (Wood, Schroder et al. 2003). All the Prxs contain an active cysteine residue for catalysis, which can be oxidized by peroxides to reversible cysteine sulfenic acid. In some cases, Prx-sulfenic acid (-SOH) can be further oxidized by oxidant to sulfinic ($-SO_2H$) or sulfonic ($-SO_3H$) acid, causing the loss of function of Prxs. For some peroxiredoxins, the sulfinic acid derivatives can be enzymatically reduced back to functional form by a sulfiredoxin enzyme (Rhee, Chae et al. 2005). As there are no evident differences in prxs - in anti-oxidants controls as compared to DMSO control, it was concluded that the anti-oxidant treatments did not produce oxidative toxicity to these prx proteins. When Jurkat *E*6.1 cells treated with 400 µM SMX-HA, the disappearance of this protein spot suggested that Prxs underwent ROS-dependent oxidation to their sulfinic ($P-SO_2H$) and sulfonic

(P-$SO_3H$) acid derivatives. As these oxidation derivatives can not be reduced by DTT, they would run on the diagonal line with all other proteins that do not occur as disulphides since they are not altered in the reduction step, thus accounting for the shift of the Prxs and disappearance of the protein spot from the gel. In addition, peroxynitrite that generated during the auto-oxidation of SMX-HA also oxidizes thiols to form nitrite and sulfenic acid. As some of these forms of peroxiredoxins cannot be reduced by DTT, they would appear on the diagonal line as they do not have disulfide bonds and with all other proteins that do not occur as disulfides since they are not altered in the reduction step. When pretreated with 5 μM of BE, Cro, Res, and Sal, Prx 2 partly reversed and remained in their disulfide bonds form. This indicated that single anti-oxidant at this concentration all showed a slight protective effect although they could not completely attenuate SMX-HA mediated oxidative stress. However, Prx 2 was no longer visible when pretreated with 1 μM of BE, Res, and Sal. Only pretreatment with 1 μM of Cro slightly reversed the Prx 2. This suggested that these four chemicals possess dose-dependent anti-oxidant effects and Cro is most suitable to protect against the formation of sulfinic or sulfonic acid derivatives of Prx 2 induced by 400 μM SMX-HA treatment. When these anti-oxidants were combined to mixture 1 or mixture 2 and pretreated Jurkat *E*6.1 cells with mixture 1 or mixture 2, Prx 2 partly remained in their disulfide form. As with a single treatment, mixtures also showed concentration-dependent manner, which represent greater intensity of Prx 2 in 5 μM of mixtures pre-incubation and lower intensity of Prx 2 in 1 μM of mixtures pre-treatment. Finally, we compared the anti-oxidant effect of low concentration (1 μM) of single chemical, mixture 1, and mixture 2. In this case, the mixtures of anti-oxidants exhibited greater protection than single chemical. In addition, the mixture with Sal even showed better cytoprotective effect than the mixture without Sal, suggesting that Sal was a vital contributor to the mixture's effect. This result supports the theory that anti-oxidants can work through complementary mechanisms to achieve a greater protective effect than a single component alone on an equimolar basis,

permitting lower concentration of each to be used and thereby lowering the potential for adverse effects. In this experiment, less protein spots and the weak diagonal line were shown on the gels when we load 85 μg protein samples. It might be involved in several factors such as less protein samples or the time of silver staining. More protein spots were shown up when double protein samples were loaded on the gels.

In addition, SMX-HA treatment produced more protein spots visible either on left or on right side of the diagonal line. The increased numbers of proteins can be explained by oxidation of cysteine residues either into inter-molecular or into intra-molecular disulphides. Moreover, SMX-HA treatment led to decreased protein intensity in some cases, which can be explained by overoxidation of thiol groups. Other factors may be involved in this phenomenon, as an example the modification of protein produced by SMX-HA may be at a different site (e.g. phosphorylation); the electrophoretic mobility of proteins would then be changed in this process (Baty, Hampton et al. 2005).

# CHAPTER 6: CONCLUSIONS

In conclusion, our results suggest a duality between the pro-oxidant and anti-oxidant effects of BE, Cro, Res, and Sal. We have also demonstrated that ROS may be a key biochemical mediator of SMX hypersensitivity. As well, our results suggest that low concentrations of anti-oxidants therapy may reduce the toxicity of reactive drug metabolites that generate oxidative and nitrosative stress. Pretreatment with BE, Cro, Res, and Sal singly and in combination at low concentration conferred chemoprotection of Jurkat *E*6.1 cells from SMX-HA mediated membrane damage, lipid peroxidation, protein carbonylation, and oxidation of prx 2 protein. In the last case, the mixture of anti-oxidants exhibited greater anti-oxidant effect than a single anti-oxidant. There were significant differences between the mixture with and without Sal in these experiments especially in R2D PAGE, suggesting that Sal was a vital contributor to the mixture's effects. The presence of Sal in the mixture enhanced the anti-oxidant ability of the mixture. Together, the evidence supports the theory that anti-oxidants can work through complementary mechanisms to have a greater protective effect than single component alone on an equimolar basis. The potential benefits of complementary anti-oxidant therapy would include the use of lower concentrations of each with less chance of toxicity; however, the anti-oxidants used must be carefully selected in order to enhance drug safety and lower the potential for negative side effects.

# CHAPTER 7: FUTURE DIRECTIONS

The present study offered insight on the mechanisms of drug-induced oxidative and nitrosative stress with special reference to the effect of SMX-HA auto-oxidization on the redox changes in the cells and the potential for using complementary anti-oxidant therapy studies using an *in vitro* model. The use of BE, Cro, Res, and Sal seem to be ideal traditional Chinese remedies for the attenuation of oxidative and nitrosative stress by SMX-HA. Work from the present study should be extended to suitable animal models of oxidative stress, such as peroxiredoxin knock-out mouse models. An *in vivo* model will be critical to determine the reproducibility of the antioxidative effects and the potential effects of metabolism. The ultimate goal of this research is to use natural origin anti-oxidants such as BE, Cro, Res, and Sal in patients, in an ideal mixture of anti-oxidants that can synergistically attenuate oxidative and nitrosative stress via complementary mechanisms, and thereby prevent oxidative and nitrosative damage associated with many human diseases as well as reducing the risk of serious adverse drug effects.

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