Anti-oxidant Effect of a Traditional Chinese Medicinal Formula, *Wu-zi-yan-zong-wan*

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A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of

Master of Philosophy

in

Chinese Medicine

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June 2004

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Acknowledgements

Several people have particularly important roles in the development of this thesis and I would like to give them special thanks. I would first like to thank Professor C.T. Che for all the confidence you have shown in me and this project.

Since coming onto the project, Dr. S.P. Ip has played a major role in providing guidance and support to me. Thank you for all the helpful advices and kindness guidance on my research work. He is one of the most generous, insightful, and brightest people I have ever known. I'd really having a good time and love to learn from him.

In addition to being a dear friend, a special thanks is owned Miss W.H. Lee, student in School of Chinese Medicine, who was assembling support and care to me during my project. I am also grateful to the technical advices of Mr. M. Zhao, S.H. Tsui, Y.Y. Zong, and M.L. Chen.

I would also like to thank Prof. P.S. Leung, E. Wong and S.W. Tsang from Department of Physiology; Prof. W. K. Liu, from Department of Anatomy and Prof. W.P. Fong, from Department of Biochemistry.

I am especially grateful to Mr. K. Y. Au Yeung, Mr. H. Xe, Miss S. L. Cao, Miss S. K. Ng, Mrs. S. Lam, and Miss L. Y. Au to their assistance and friendship.

Abstract

Wu-zi-yan-zong-wan (WZ), a combination of *Fructus Lycii, Semen Cuscuta, Fructus Rubi, Semen Plantaginis, Fructus Schisandrae*, is generally indicated for promoting kidney functions and treating infertility. Evidence exists that some of the herbals ingredients in this traditional Chinese medicinal formula, such as *Fructus Lycii* [Gou-qi-zi], *Fructus Rubi* [Fu-pen-zi], and *Fructus Schisandrae* [Wu-wei-zi], possess antioxidant properties. However, relevant investigations on this formula and its modifications are rarely been reported. It is therefore hypothesized that WZ can produce protective effect on oxidative stress in the liver and the antioxidant properties of WZ may be eliminating improved by some of the component herbs.

An aqueous extract of WZ showed free radical scavenging activity in the (Trolox Equivalence Antioxidant Capacity) TEAC assay system. *Fructus Rubi* was determined to be the most significant antioxidant herb among the ingredient herbs. An *in vitro* study using HepG2 cells was then used to test the effect of WZ and its modified or simplified formulae on oxidative stress induced by hydrogen peroxide or menadione. It was found that the WZ extract and some of its simplified formulae displayed antioxidant effect in cultured HepG2 cells. WZ and its simplified formulae were further tested using an *in vivo* mouse model against menadione-induced

hepatotoxicity. The result indicated that WZ treatment could protect against menadione-induced hepatotoxicity in mice, as indicated by a significant decrease in serum alanine aminotransferase (ALT) activity. However, the biochemical index of liver injury was not paralleled by observation from histopathological assessment, which did not show any protective effect.

In summary, the aqueous extract of WZ and some of its simplified formulae were shown to possess antioxidant properties in both *in vitro* and *in vivo* assay systems. Further studies are warranted to determine the active ingredients of the formulae.

概論

五子衍宗丸是中國傳統方劑。從中醫角度，它能提升腎功能，治療腎虛和不育症。五子衍宗丸由枸杞子、菟絲子、覆盆子、車前子和五味子等五味藥物組成。事實上，有不少文獻曾提及枸杞子、覆盆子和五味子均有抗氧化功能。但很少就其全方和拆方做研究。本研究的目的就是要測定五子衍宗丸及其拆方在體外和體內的抗氧化能力，從而爲未來對全方治療機制的研究打下美好基礎。

體外實驗指出，五子衍宗丸的水提液能清除 TEAC 系統所產生的自由基；其中，覆盆子水提液擁有最大的抗氧化能力。而在 HepG2 人肝細胞試驗中，五子衍宗丸水提液和某些拆方均能增加細胞的生存能力，對抗由過氧化氫和甲萘醌所產生的氧化作用。在體內實驗，我們利用 ICR 種小鼠進行體內測定。以血清中的丙氨酸氨基轉移酶作爲指標，測定藥物對甲萘醌所構成肝損傷的作用。結果顯示，與甲萘醌組比較，五子衍宗丸組能減低甲萘醌所產生的肝損傷。可是，對上述實驗小鼠進行病理組織顯微觀察時，未能得出相同結論。而利用正交設計法也未能找出五種單味藥中，那一種對減低肝損傷起主導作用。總括而言，五子衍宗丸水提液擁有一定程度的抗氧化能力，但其抗氧化的機理和當中究竟那一味(或一組)藥起主導作用仍有待進一步研究。

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List of abbreviations

ABTS	2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic aicd)
AIDS	Acquired Immunodeficiency Syndrome
ALT	Alanine aminotransferase
ANOVA	Analysis of Variance
ARDS	Acute Respiratory Distress Syndrome
AST	Aspartate aminotransferase
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
CO_2	Carbon Dioxide
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribinucleic acid
DTNB	5, 5'-Dithiobis-(2-nitrobenzoic acid)
EDTA	Ethylenediaminetetraacetic acid
FL	Fructus Lycii
FR	Fructus Rubi
FS	Fructus Schisandrae
Fe^{2+}	Iron (II) ion
Fe^{3+}	Iron (III) ion

GR	Glutathione reductase
GSH	Glutathione
GST	Glutathione transferase
HBSS	Hank's balanced salt solution
HOCl	Hypochlorous acid
H_2O	Water molecules
H_2O_2	Hydrogen Peroxide
K^+	Potassium ion
LASEC	Laboratory Animal Service Centre
LDH	Lactate dehydrogenase
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
NADH	Nicotinamide adenine dinucleotide
NaCl	Sodium Chloride
Na^+	Sodium ion
OD	Optical density
• OH	Hydroxyl radical
$O_2 \bullet ^+$	Superoxide radical
PBS	Phosphate buffered saline
ROS	Reactive oxygen species

S.D.	Standard Deviation
S.E.	Standard Error
SC	Semen Cuscuta
SDS	Sodium dodecyl sulfate
SP	Semen Plantaginis
SPSS	Statistics Package for Social Science
TBA	Thiobarbituric acid
TCA	Trichloroacetic acid
TCM	Traditional Chinese Medicine
TEAC	Trolox Equivalence Antioxidant Capacity
TLC	Thin Later Chromatography
UV	Ultra-violet
WZ	Wu-zi-yan-zong-wan
WZ-e	Aqueous extract of WZ from raw materials
WZ-p	Aqueous extract of WZ from Sheng Cheung Pharmacological Ltd.

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Chapter 1

Introduction

Clinical and biomedical scientists are interested in antioxidants because they could retard the oxidative tissue damage by scavenging free radicals or lowering oxidative stress. There is also an increasing interest in the antioxidant effects of naturally occurring substances because of their potentials of being developed into nutritional supplements, health foods, and preventive medicines.

1.1 Oxidation stress

Oxidative stress has long been implicated in a number of neurodegenerative diseases, including Alzheimer's disease, Parkinson's disease, Huntington's disease and ischemic injury (Behl, 1994; Coyle, 1993; Markesbery, 1997; Simonian, 1996; Toufektsian, 2001). Overwhelming evidence also indicates that oxidative stress can lead to cell and tissue injury when the antioxidant status was altered by exposure to excessive oxidants. However, the same free radicals that are generated during oxidative stress are produced during normal metabolism (Montagnier, 1998). Thus, no one is able to avoid free radicals.

1.1.1 Free radicals

Free radicals are highly reactive compounds that are created in the body during normal metabolic processes or introduced from the environment. Free radicals are inherently unstable, since they contain “extra” energy, the unpaired electrons. Free radicals are molecules with an odd number of electrons. They are potentially reactive in order to gain or lose one electron (Muriel, 1997). These reactive free radicals can cause damages to cells and tissues in certain pathways. DNA damage leads to cell injury, destruction of nucleotide coenzymes, disturbance to SH-dependent enzymes, covalent binding, membrane damage and lipid peroxidation (Slater, 1985).

However, free radicals are involved in many activities in the body during oxidative metabolism and energy production. For example, free radicals are involved in:

- Enzyme-catalyzed reactions
- Electron transport in mitochondria
- Signal transduction and gene expression
- Activation of nuclear transcription factors
- Anti-microbicidal action of neutrophils and macrophages
- Aging and diseases

(Takahashi, 1998)

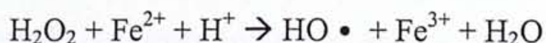
Oxidants or reactive oxygen species (ROS) is a collective term that includes oxygen-derived radicals, such as superoxide ($O_2 \bullet^-$) and hydroxyl radicals ($\bullet OH$), and non-radical derivatives of oxygen such as hydrogen peroxide (H_2O_2) and hypochlorous acid (HOCl).

1.1.1.1 Hydrogen peroxide

Hydrogen peroxide (H_2O_2) is thought to be an important precursor of highly reactive free radicals, and it has been reported to induce apoptosis in brain, liver, kidney and also other organs of the body (Ishikawa, 1999; Ratan, 1994; Bode, 1997; Duru 2000). Apoptosis, also known as programmed cell death, is a form of cell death that occurs during several pathological situations in mutlicellular organisms and constitutes a common mechanism of cell replacement, tissue remodeling, and removal of damaged cells. Though, apoptosis is a complex process characterized by cell shrinkage, chromatin condensation, internucleosonal DNA fragmentation, and formation of "apoptosis body". Hydrogen peroxide can readily cross the cell membranes and encounters transition metal ions, especially Fe^{2+} and Cu^+, to form the aggressive $\bullet OH$ radical as in the Fenton reaction. On the other hand, transition metal ions can act as catalysts to stimulate the

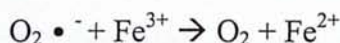
formulation of the $\bullet OH$ from $O_2 \bullet^-$ and H_2O_2, as in the Haber-Weiss reaction.

Fenton reaction:

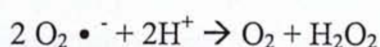


$$H_2O_2 + Fe^{2+} + H^+ \rightarrow HO\bullet + Fe^{3+} + H_2O$$

Haber-Weiss reaction:



$$O_2 \bullet^- + Fe^{3+} \rightarrow O_2 + Fe^{2+}$$



$$2\,O_2 \bullet^- + 2H^+ \rightarrow O_2 + H_2O_2$$

(Poli, 1994)

1.1.1.2 Menadione

Menadione (2-methyl-1, 4-naphthoquinone), also known as vitamin K3, is a potent oxidative stress inducer. It has been used as an effective and remarkably safe cytotoxic drug for treatment of several human tumors (Margolin,1995; Tetef, 1995). Menadione induces apoptotic cell death through a poorly understood mechanism. It promoted oxidation of protein sulfhydryl groups in cytoskeletal protein, DNA fragmentation and alteration of calcium homeostasis. (Czaja, 2003) The cytotoxic effect is thought to be related to ROS generated from the NADPH-mediated and cytochrome P-450 reductase-catalyzed redox cycling of menadione (Thor, 1982).

1.1.2 Diseases related to oxidative stress

Oxidative stress is an underlying factor in health and disease. There are many diseases related to oxidative stress. More and more evidence is accumulating that a proper balance between oxidants and antioxidants is involved in maintaining health and longevity, and that altering this balance in favor of oxidants may result in pathological responses causing functional disorders and disease. Oxidative stress, rather than being the primary cause of disease, is more often a secondary complication in many disorders. Oxidative stress-related diseases include inflammatory bowel disease, retinal ischemia, cardiovascular disease and restenosis, AIDS, ARDS, and neurodegenerative diseases such as stroke, Parkinson's disease, and Alzheimer's disease (Montagnier, 1998). Such ailments may prove amenable to antioxidant treatment because there is a clear involvement of oxidative injury in these disorders.

1.1.3 Liver Injury

The liver is the largest organ in human body. It performs a number of functions, such as detoxification of metabolic waste products, e.g. deamination of amino acids to produce urea; destruction of spent red cells and reclamation of their constituents (in conjunction with the spleen); synthesis and secretion of bile;

synthesis of the plasma proteins including the clotting factors; synthesis of plasma lipoproteins; glucogen synthesis, gluconeogenesis; storage of glucogen, some vitamins and lipid (Thomas, 1989).

However, it is very easy to cause damages to the liver. There is an endless list of different kinds of liver injury related to oxidative stress. It includes acute hepatitis, chronic hepatitis, granulomatous disease of the liver, hepatic manifestations, alcoholic liver injury, fatty liver, hyperbilirubinemia and cholestasis, hepatic pigment abnormalities, fibrosis and cirrhosis, cancer etc.

On the other hand, the liver is playing a key role in antioxidant defense. It contains nearly all physiological antioxidants in the highest amount in the body. This organ is the main site for detoxification of xenobiotics and the decomposition of surplus ROS or other free radicals (Olinescu, 1992; Sies, 1985; Yagi, 1983). It is to be expected that when chemical intoxication (involving ROS) occurs, the increased formation of peroxides and decrease of antioxidants will only occur when the antioxidants pool in the liver are exhausted. The chemical intoxication of liver always leads to increase formation of lipid peroxidation in the blood plasma. Liver failure caused by viral or bacterial infections produces similar outcomes (Olinescu, 1985; Parola, 1996).

1.1.4 Antioxidants

1.1.4.1 Importance of antioxidant

Antioxidants are a conspicuous first-line defense against free radicals and are able to counteract or, at least, slow down all major steps in the process of free radical induction of injury (Olinescu, 2002). Antioxidants work in several ways: they may reduce the energy of the free radical, stop the free radical from forming, or interrupt the oxidizing chain reaction to minimize the damage caused by free radicals (Williams, 1997). In the course of lipid peroxidation they can act as oxygen quenchers, radical scavengers (quenching initial radicals such as hydroxyl radicals as well as quenching intermediate radicals such as peroxy and alkoxy radicals), or metal ion chelators (Diaz, 1997). Therefore, antioxidants in foods and supplements are being widely promoted for their health benefits and prevention of diseases.

1.1.4.2 Examples of antioxidant

Many vitamins and minerals act as antioxidant in their own right, such as catechin, vitamin E, vitamin C, beta-carotene, lutein, lycopene, vitamin B2, coenzyme Q10, cysteine, flavonoids, and polyphenols. They can provide powerful antioxidant protection for the body (Ames, 1993).

Therefore consuming a wide variety of vitamins, minerals, and herbs that

have an antioxidant effect may be the best way to provide the body with the most complete protection against free radical damage.

1.2 Traditional Chinese Medicinal (TCM) formula Wu-zi-yan-zong-wan (WZ)

1.2.1 The WZ medicinal formula

Wu-zi-yan-zong-wan (WZ) is a traditional Chinese medicinal formula prescribed for promoting kidney functions, and treating infertility. The composition of the WZ recipe include *Fructus Luycii* (*Lycium barbarum or L. chinensis*) [Gou-qi-zi], *Semen Cuscutae* (*Cuscuta chinensis*) [Tu-si-zi], *Fructus Rubi* (*Rubus chingii*) [Fu-pen-zi], *Semen Plantaginis* (*Plantago asiatica or P. depressa*) [Che-qian-zi], and *Fructus Schisandrae* (*Schisandra chinensis*) [Wu-wei-zi].

The WZ medicinal formula has a long history of usage and its composition is described in the Chinese Pharmacopoeia as:

Frutus Lycii	400g
Semene Cuscutae (stir-fried)	400g
Frucuts Rubi	200g
Semen Plantaginis (stir-fried with salt)	100g
Frucuts Schisandrae (steamed)	50g

According to the Pharmacopoeia (2000), all five ingredients are grounded into powder, sieved and mixed. To prepare "water-honey pills", 35-50 g of honey is added for each 100 g of powder, with adequate amount of water. The pills are taken orally, 9 g/dose, twice daily for "large-honey pills".

1.2.2 Pharmacological actions of WZ

Traditionally, Wu-zi-yan-zong-wan (WZ) is generally indicated for male "kidney deficiency". It means that the weakness of the kidney causes the decline in sexual and reproductive functions (Zhang, 1988). According to the Pharmacopoeia, WZ has a replenishing effect toward the kidney and indicated for manifestation by sexual dysfunction such as impotence, sterility, premature ejaculation, seminal emission, and dribbling of urine after micturition (Pharmacopoeia, 2002).

Modern pharmacological studies indicated that WZ has the capacity of stimulating spermatogenesis and modulating the hormone levels in the rat model (Wang, 1993). Also, WZ has been demonstrated to regulate the liver function. It was reported that WZ prescription protected rat against alcohol induced liver damage by reducing lipid peroxidation. A reduction in alcohol-induced fatty liver and necrosis was also observed after WZ administration (Li, 1994). A preliminary clinical study also showed that WZ could improve the hepatotoxic conditions, as indicated by

decreased serum ALT and AST, activities in 28 out of 30 alcohol-intoxicated patients after a 20-month treatment (Liang, personal communication). This formula also inhibited alcohol induced liver damage in the rat model (Liang, personal communication).

1.2.3 Pharmacological actions of individual herbs

1.2.3.1 Fructus Lycii

Fructus Lycii (FL), known in Chinese as "Gou-qi-zi", is one of the main ingredients in the WZ formula. It is the dried fruits of *Lycium barbarum.* L. (Solanaceae). It benefits the liver and the kidney (according to the Chinese medical theory) and it is good for replenishing vital essence and improving eyesight.

Recent studies showed that FL could suppress lipid peroxidation in erythrocytes (Ren, 1995) and inhibit tumor growth in mice (Liu, 1996). The FL extract also has been shown to possess anti-fatigue (Luo, 1999, 2000; Zhan, 1989) and anti-hypoxic (Li, 1999a, 1999b) activities. FL contains polysaccharides that have the ability to scavenge hydroxyl radicals, decrease the generation of MDA, inhibit the decrease of mitochondria fluidity induced by hydroxyl radicals, and alleviate the swelling of mitochondria induced by hydroxyl radicals (Wang, 2001). The aqueous extract of the herb protected the animals from carbon tetrachloride-induced liver

damage, inhibited the fatty changes in the serum and liver, slightly inhibited fat deposition in the hepatocytes following liver damage induced by carbon tetrachloride, and fostered the regeneration of hepatocytes (Lu, 1964).

In the WZ prescription, FL serves as the "emperor" and contributes to the main therapeutic action of the formula.

1.2.3.2 Semen Cuscutae

Semen Cuscutae (SC) is the seeds of *Cuscuta chinensis* Lam or *C. australis* R.Br., known as "Tu-si-zi" in Chinese. SC is usually used in treating and strengthening both liver and kidney disorders (Dictionary of Chinese Medicine, 1993-1998).

The aqueous extract of SC has been shown to inhibit aldose reductase (Matsuda, 1995) and scavenge free radical (Kwon, 1997; Bao, 2002). *Semen Cuscutae* could protect the mice's liver from carbon tetrachloride-induced hepatotoxicity (郭, 1992). The activity of Na^+, K^+-ATP was increased by intragastric administration of aqueous extract of *Semen Cuscutae*.

In the WZ formula, SC serves as the "emperor" and it is responsible for the main therapeutic action.

1.2.3.3 Fructus Rubi

Fructus Rubi (FR), "Fu-pen-zi", is the fruit of *Rubus chingii* Hu (Rosaceae). It is traditionally used to benefit the kidney, and arrest seminal discharge and excessive urination. It is able to strengthen the kidney functions (Dictionary of Chinese Medicine, 1993-1998).

It was reported that *Fructus Rubi* was useful in protecting against t-BHP induced oxidative damage and might be capable of attenuating cytotoxicity of other oxidants (Yau, 2002). FR was also shown to increase the level of LH-releasing hormone and testosterone, whereas the levels of luteinizing hormone, follicle stimulating hormone and estradiol were decreased (Chen, 1996)

FR is included in the WZ formula as the "minister" which plays a role in enhancing thetherapeutic effects of FL and FC.

1.2.3.4 Semen Plantaginis

Semen Plantaginis (SP) is derived from the dried seed of *Platago astiatica* L. or *P. depressa* Willd. and is known as "Che-qian-zi". It removes heat, causes diuresis and eliminates dampness, relieves dysuria, clears the eye and promotes expectoration (Pharmacopoeia, 2000). It is also used for treating diarrhea in other countries (WHO, 1999).

The extract of SP showed hepatoprotective activity against carbon

tetrachloride induced toxicity in mice (Yun, 1977). Moreover, SP showed antioxidant activity in *in vitro* assay (Yamamoto, 1982).

SP serves as the "assistant and envoy" in the WZ formula that helps in assisting other ingredients to boost up the therapeutic effect.

1.2.3.5 Fructus Schisandrae

Fructus Schisandrae (FS), "Wu-wei-zi", is derived from the dried fruit of *Schisandra chinensis* Baill (Magnoliaceae). FS is often used as a tonic and sedative drug (Dictionary of Chinese Medicine, 1993-1998). It produces an effect to arrest discharges, replenish *qi*, promote fluid secretion, tonify the kidney and induce sedation (Pharmacopoeia, 2000).

FS exhibits a wide variety of pharmacological action including the anti-hepatotoxic effect. It is a strong antioxidant against carbon tetrachloride induced liver damage in rats (Ko, 1995a, 1995b). Its chemical components, such as Schisandrol A, can inhibit liver MDA formation in rats subjected to 50% ethanol induced hepatotoxicity (Lu, 1991). Schisandrin B enhances the activities of glutathione reductase and glutathione transferase in mice treated with carbon tetrachloride (Ip, 1995).

Since FS is the component of smallest amount in the WZ formula, it serves as the "minister" to enhance the therapeutic effects of the main ingredients, FL and

SC.

1.3 The relationship between the liver and the kidney

The interrelationship between liver disease and kidney dysfunction has been recognized. Basically, there are three major categories of disease states support such interrelationship: (i) disorders involving the liver and kidney directly; (ii) primary disorders of the kidney with secondary hepatic involvement, and (iii) primary disorders of the liver with secondary renal dysfunction (Epstein, 1994). Although the understanding of the ways in which the liver affects renal process is inevitably incomplete, there is evidence showing the pathophysiological relation between the kidney and liver. Many patients who have renal functional abnormalities have liver disease as well, such as the association of parlnchymal liver disease with secondary impairment of renal function, deranged renal sodium handling, impaired renal water excretion and, impaired renal concentrating ability, hepatorenal syndrome, acute renal failure, glomerulopathies with cirrhosis or hepatitis, and impaired renal acidification (Epstein, 1978). These illustrate that kidney and liver are really closely related to each other physiologically and pathologically.

In Traditional Chinese Medicine (TCM) theory, the liver stores blood and the kidney stores the essence of life. The blood of the liver depends on nourishment

from the essence in kidney, while the essence stored in the kidney depends on replenishment from the blood of liver. In TCM, it is believed that blood can develop into the essence of life, and vice versa. Therefore, it is widely said that the liver and the kidneys have a common source (Zhang, 1998). Pathologically, a deficiency of the essence in the kidney may lead to a deficiency of the blood in the liver. A long-term deficiency of the blood of the liver may cause a deficiency of the essence of life in the kidney. Therefore, in treating the deficiency of the *yin* of the liver or kidneys, a method is often used to reinforce the *yin* of both the liver and kidney.

1.4 Objectives of the study

Many herbal medicines for boosting kidney's function have been shown to delay the aging process, presumable through safe guarding kidney function and antioxidation. It is also proposed that the kidney function boosting medications might have an effect on regulating the function of other organs. As the theory of TCM goes, it is rational to hypothesize that kidney-targeting herbal medicines may produce a protective effect on the liver.

Wu-zi-yan-zong-wan (WZ) is a well-known traditional Chinese medicinal formula for its therapeutic effect on infertility. In TCM, infertility is related to the dysfunction of kidney. Moreover, previous studies also showed that WZ protected

against on liver toxicity (Li, 1992; Li, 1994). Among the ingredients in the formula, *Fructus Lycii* (FL), *Semen Cuscutae* (SC) and *Fructus Schisandrae* (FS) have been reported to have strong antioxidant protective effect on liver toxicity (Yun, 1980; Kim, 1994; Kim, 1997; Liu, 1991; Li, 1991; Sinclair, 1998; Hankcke, 1999). Therefore, it is proposed that WZ might have an antioxidant effect with great potentials of developing into a therapeutic or food supplement.

Moreover, it is proposed that simplifying the WZ traditional formula by eliminating one or more ingredients may be able to maintain or even improve the biological activity against oxidation. As a result of simplification, it will be more convenient and simplier to investigate the therapeutic effect as well as its mechanism by reducing any unnecessary interactions among the herbal ingredients.

In the present study, the primary objective is to investigate the hepatoprotective effect of the aqueous extract of the WZ formula. The abilities of WZ formula to scavenge free radicals *in vitro* and protect mouse liver and hepatocytes from oxidants-induced damage were examined. In addition, the simplified WZ formulae were examined for their *in vitro* and *in vivo* antioxidant effects.

Chapter 2

Preparation of Aqueous Extract of Wu-zi-yan-zong-wan

2.1 Introduction

Traditionally, most of the Chinese herbal medications are prepared by making decoction or formulating into pills. From ancient records and the official Chinese Pharmacopoeia, Wu-zi-yan-zong-wan is prepared in the form of "honey pills", in which the powdered herbs are mixed with honey and water. However, this kind of traditional dosage form is inconvenient to prepare and be examined in experiments. Alternatively, aqueous extraction is used in the present study to prepare the WZ formula from crude herbs.

In addition to the aqueous extracts in the form of freeze-dried powder prepared from crude materials, the ready-to-use herbal powders prepared from aqueous extract and supplied by Sheng Cheung Pharmaceutical Ltd. (a local herbal store) were also used for testing.

2.2 Materials and Methods

2.2.1 Preparation of Wu-zi-yan-zong-wan (WZ)

2.2.1.1 WZ extracts from raw materials (WZ-e)

The constituents of Wu-zi-yan-zong-wan are mentioned in section 1.2.1. All ingredients were obtained from a local vendor (as shown in Figure 2.1) and authenticated by Mr. H. Xie. After grounding with a grinder, each of the herbal ingredients was soaked in distilled water (10 ml water per gram of powder), ultra sonicated for 30 minutes, and boiled at 100 ℃ under reflux for 3 hours. Afterward, the samples were cooled down to room temperature and filtered successively through cheesecloth and filter paper. Finally, the aqueous extract of Wu-zi-yan-zong-wan was dried by freeze-drying to afford a powder.

For the simplified formulae, raw herbs were combined according to the scheduled shown in Table 2.1. The extraction process was the same as previously described.

Table 2.1. Composition of Wu-zi-yan-zong-wan (WZ) and its simplified formulae

	Fructus Lycii (FL)	Semen Cuscutae (SC)	Fructus Rubi (FR)	Semen Plantaginis (SP)	Fructus Schisandrae (FS)
% in formula	34.78%	34.78%	17.39%	8.70%	4.35%
WZ	✓	✓	✓	✓	✓
(4)-1		✓	✓	✓	✓
(4)-2	✓		✓	✓	✓
(4)-3	✓	✓		✓	✓
(4)-4	✓	✓	✓		✓
(4)-5	✓	✓	✓	✓	
(3)-1	✓	✓	✓		
(3)-2		✓	✓	✓	
(3)-3			✓	✓	✓
(3)-4	✓	✓		✓	
(3)-5	✓	✓			✓
(3)-6	✓		✓	✓	
(3)-7	✓		✓		✓
(3)-8	✓			✓	✓
(3)-9		✓	✓		✓
(3)-10		✓		✓	✓
(2)-1	✓	✓			
(2)-2	✓		✓		
(2)-3	✓			✓	
(2)-4	✓				✓
(2)-5		✓	✓		
(2)-6		✓		✓	
(2)-7		✓			✓
(2)-8			✓	✓	
(2)-9			✓		✓
(2)-10				✓	✓
FL	✓				
SC		✓			
FR			✓		
SP				✓	
FS					✓

2.2.1.2 WZ extracts from commercially available ready-to-use powders (WZ-p)

Rather than performing the aqueous extraction using raw herbs, five different ingredients in powder form, which were manufactured by Sheng Cheung Pharmaceutical Ltd. in Taiwan were obtained from a local store. (as shown in the Figure 2.2). According to the manufacturer, the powders were made by freeze-drying of the aqueous extracts of herbs. These powders were shown to be free of heavy metals and pathogenic bacteria by our in-house testings. As per the manufacturer's instruction, the powders were prepared from raw herbs at a yield of 20% (w/w).

In the experiments, powders of the ingredient herbs were mixed according to the schedule as shown in Table 2.1.

Figure 2.1. Raw materials of ingredients in WZ formula. (WZ-e)
Upper: Fructus Rubi, Fructus Lycii, Fructus Schisandrae
Lower: Semen Plantaginis, Semen Cuscuta

Figure 2.2 Aqueous extracts of ingredients in WZ formula from commercial source. (WZ-p)
Upper: Fructus Rubi, Fructus Lycii, Fructus Schisandrae
Lower: Semen Plantaginis, Semen Cuscuta

2.3 Results

2.3.1 Extraction Yield for WZ-e

The extraction efficiencies for WZ formula as well as its simplified formulae are shown in Table 2.2. The percentage yields of the extracts were estimated from the following equation:

Percentage yield (%) =

[Final weight after freeze-drying / raw material] x 100%

There was a wide range in extraction yield (1.2-50.2%) among various herbal combinations materials into dried powder. The formula of (3)-8 gave the highest amount of dried aqueous extract with an extraction yield of 50.2%, while the formula (2)-5 gave the least amount of dried extract (1.2%).

Table 2.2. **The table of percentage yield of WZ-e and its simplified formulae from raw materials.**

	Extraction Yield (%)
WZ-e	18.7
(4)-1	11.7
(4)-2	22.7
(4)-3	20.5
(4)-4	14.6
(4)-5	11.8
(3)-1	20.4
(3)-2	12.6
(3)-3	12.7
(3)-4	14.6
(3)-5	34.0
(3)-6	34.8
(3)-7	38.3
(3)-8	50.2
(3)-9	8.3
(3)-10	12.7
(2)-1	5.9
(2)-2	34.0
(2)-3	44.0
(2)-4	44.4
(2)-5	1.2
(2)-6	4.5
(2)-7	8.0
(2)-8	10.5
(2)-9	11.4
(2)-10	15.1
FL	40.5
SC	18.1
FR	13.8
SP	17.5
FS	27.5

Chapter 3

In vitro Total Antioxidant Capacity of Aqueous Extracts of Wu-zi-yan-zong-wan and its Components

3.1 Introduction

3.1.1 Trolox Equavalent Antioxidant Capacity (TEAC)

6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) is a water soluble vitamin E analogue (Mantle, 2000). Trolox equivalent antioxidant capacity (TEAC) assay was developed to assess the total antioxidant capacity of the testing samples. This is a commonly used method to evaluate *in vitro* antioxidant effects. The assay, served as a measure of antioxidant capacity, is based on the efficacy of the tested material to scavenge 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) derived radicals (Arts, 2001). The reaction involves the oxidation of ABTS to an intensely-colored nitrogen-centered radical cation, ABTS $\bullet^{+}$, which has an absorption maxima at 734 nm (Figure 3.1).

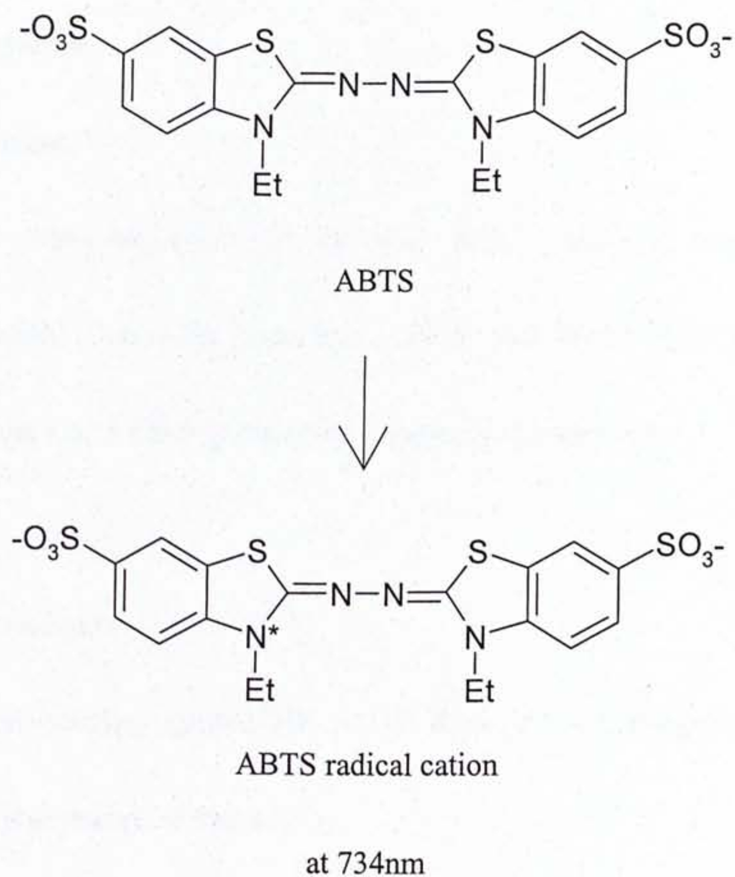


Figure 3.1 Oxidation of ABTS to ABTS • $^{+}$ radical

3.1.2 Objectives

In the present study, the TEAC assay was employed to measure the total antioxidant capacity of the aqueous extract of WZ formula as well as its simplified formulae.

3.2 Materials and methods

3.2.1 Materials

3.2.1.1 Reagents

The chemicals employed in these studies were of reagent grade or equivalent quality. Potassium persulfate, ABTS, and Trolox were obtained from Sigma Chemical Co.. Milli-pore distilled water system was used.

3.2.1.2 Instruments

A spectroscopy system HP 8453E from Hewlett Packard was used to determine the absorbance of the samples.

3.2.2 Methods

3.2.2.1 Total Antioxidants: Trolox Equivalent Antioxidant Capacity (TEAC)

A volume of 100 ml potassium persulfate (4.9 mM) was mixed with 100 ml ABTS solution in distilled water (14 mM). It is then stood still for 24 hours to produce ABTS • ions. After 24 hours, the solution was diluted around 100-fold to maintain an OD reading of about 0.7 at 734 nm (Schlerier, 2002). However, the stock solution of Trolox was prepared in ethanol to enhance its solubility.

The ABTS Reagent (1ml) was added to 0.1 ml sample or Torlox in a 25 mm

test tube. After standing at room temperature for one minute, optical density (OD) was taken using the UV-Vis Spectrophotometer.

The percentage of antioxidant activity was calculated according to the formula:

% of antioxidant activity = [A(sample) –A (Trolox) / A(sample)] x 100%

3.2.3 Statistical analysis

All analyses were performed for more than 3 times ($n > 3$). The percentage of antioxidant activity were estimated and expressed as mean ± S.E. One-way analysis of variance (ANOVA) was performed using SPSS version 12.0.1 for Windows (SPSS Inc., US). Significant differences between means were determined where P-values <0.05.

3.3 Results

3.3.1 Antioxidant Capacity of Trolox

The TEAC assay was calibrated with Trolox. As shown in Figure 3.2, Trolox caused a concentration-dependent decrease in absorbance.

3.3.2 Antioxidant capacity of WZ-e formula

Figure 3.3 shows the antioxidant capacity of the WZ-e formula. A concentration-dependent in absorbance was produced by the addition of WZ-e, with the inhibition concentration at 50% being 0.2-0.4 mg/ml.

3.3.3 Antioxidant capacity of WZ-p formula

Figure 3.4 shows the antioxidant capacity of WZ-p formula. WZ-p produced a concentration-dependent decrease in absorbance, with the inhibitory concentration at 50% being 4mg/ml.

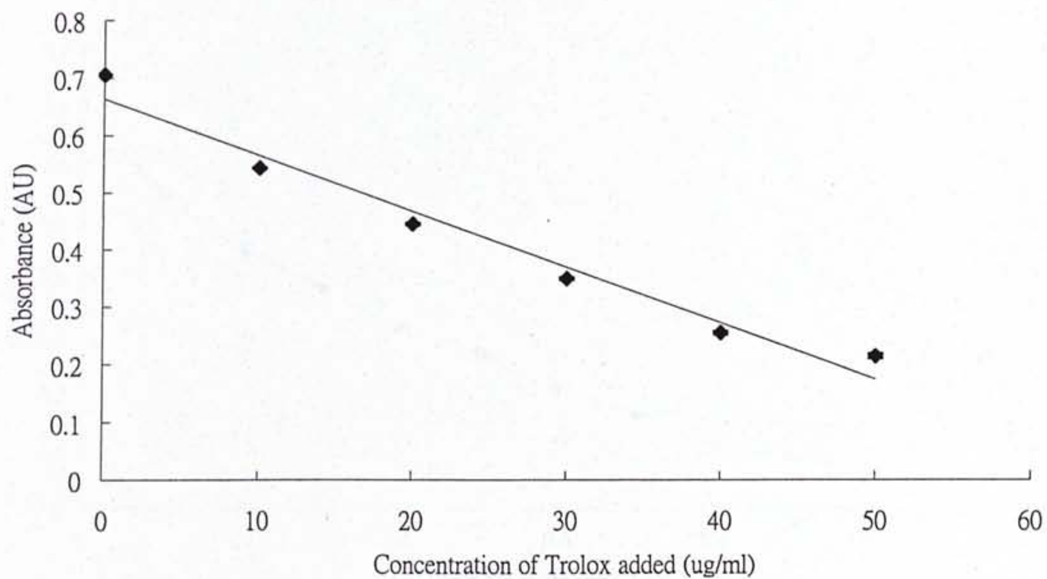


Figure 3.2 Trolox standard curve. Data are expressed as mean ± S.E., with n=3.

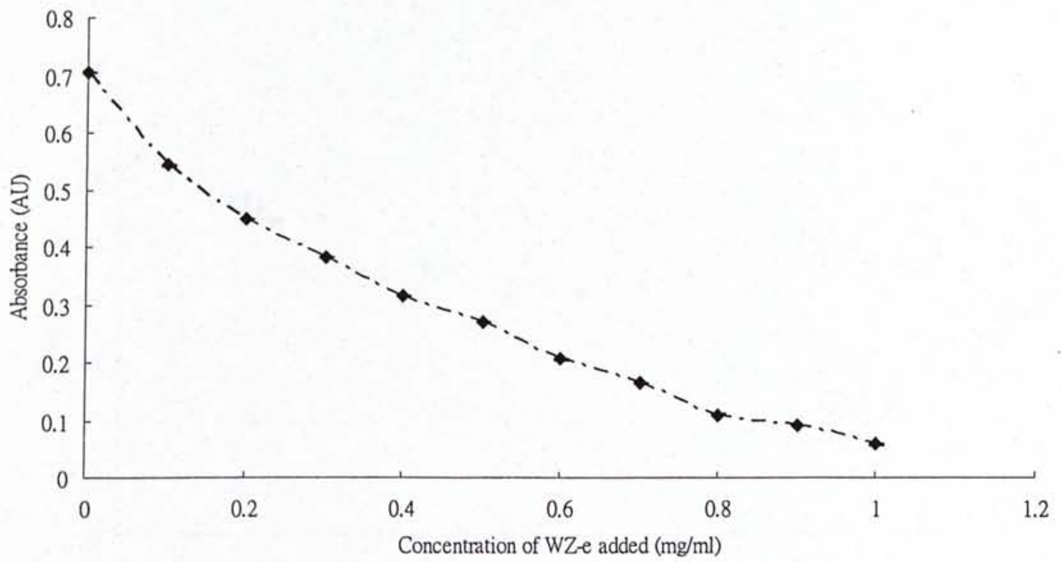


Figure 3.3 Antioxidant capacity of WZ-e formula. Data are expressed as mean ± S.E. with n=3.

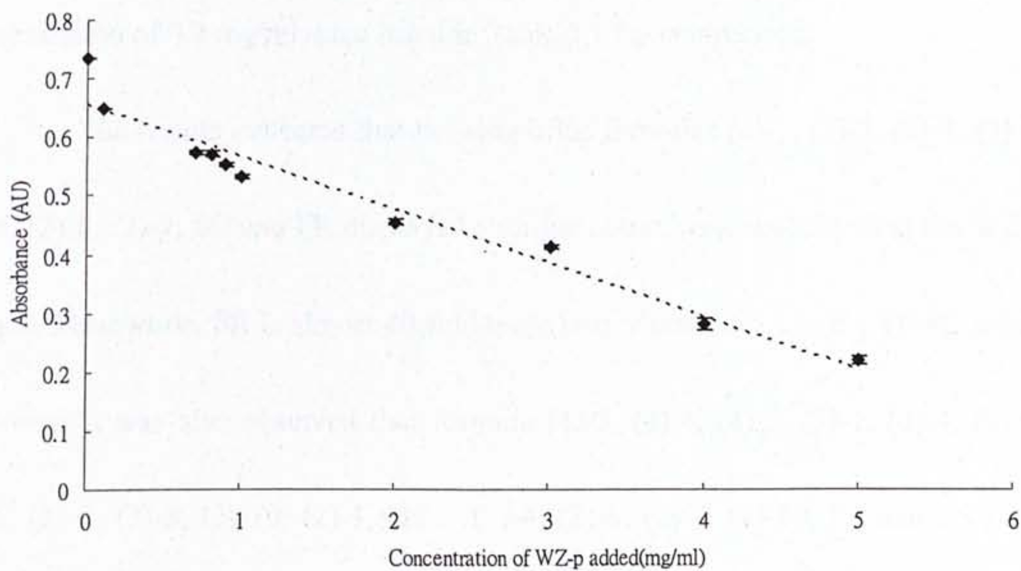


Figure 3.4 Antioxidant capacity of WZ-p formula. Data are expressed as mean ± S.E. with n=3.

3.3.4 The total antioxidant capacity of WZ-e and its simplified formulae

The *in vitro* antioxidant activity of WZ-e and its simplified formulae were quantified by TEAC assay (Table 3.1). The tests were performed at increasing concentrations of 0.2 mg/ml, 0.3 mg/ml, and 0.4 mg/ml. Data obtained at a test concentration of 0.2 mg/ml were listed in Table 3.1 for comparison.

The results indicated that the simplified formulae (4)-1, (3)-2, (3)-3, (3)-9, (2)-5, (2)-8, (2)-9, SC and FR displayed stronger antioxidant capacity than the WZ-e extract. Meanwhile, FR is almost 40-fold more potent than WZ-e in the TEAC assay. However, it was also observed that formula (4)-3, (4)-4, (4)-5, (3)-1, (3)-4, (3)-5, (3)-6, (3)-7, (3)-8, (3)-10, (2)-1, (2)-3, (2)-4, (2)-6, (2)-7, (2)-10, FL and FS only exhibited weak antioxidant activity in the assay, while (2)-10 had the lowest TEAC value (1.16%).

3.3.5 The total antioxidant capacity of WZ-p and its simplified formulae

The TEAC assays on WZ-p and its simplified formulae were performed. However, no significant difference in the total antioxidant capacity was observed among the formulae (data not shown). Among, the simplified formulae, they had more or less the same free radical scavenging ability as assessed by the TEAC assay (data not shown).

Table 3.1 The total antioxidant capacity of WZ-e and its simplified formulae

	% of antioxidant activity
WZ-e	14.7 ± 1.6
(4)-1	59.5 ± 7.8*
(4)-2	16.7 ± 1.7
(4)-3	1.8 ± 0.0*
(4)-4	6.5 ± 0.1*
(4)-5	3.3 ± 0.1*
(3)-1	5.3 ± 0.1*
(3)-2	134.0 ± 56.9*
(3)-3	67.9 ± 12.3*
(3)-4	2.1 ± 0.1*
(3)-5	2.2 ± 0.1*
(3)-6	9.8 ± 0.2*
(3)-7	10.2 ± 0.1*
(3)-8	1.4 ± 0.1*
(3)-9	123.2 ± 27.9*
(3)-10	2.6 ± 0.0*
(2)-1	3.3 ± 0.0*
(2)-2	11.9 ± 0.2
(2)-3	1.9 ± 0.0*
(2)-4	1.6 ± 0.0*
(2)-5	111.5 ± 22.4*
(2)-6	7.5 ± 0.1*
(2)-7	4.9 ± 0.0*
(2)-8	72.8 ± 10.3*
(2)-9	43.5 ± 5.5*
(2)-10	1.2 ± 0.0*
FL	2.4 ± 0.0*
SC	60.8 ± 5.6*
FR	584.4 ± 165.6*
SP	11.7 ± 0.2
FS	1.5 ± 0.0*

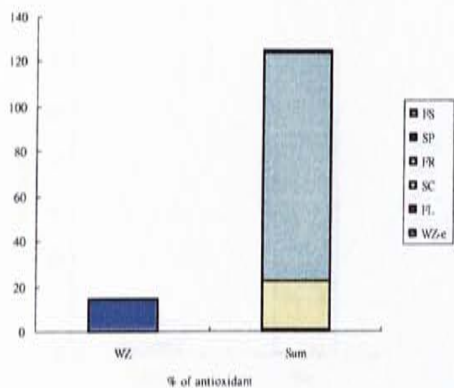
Data were obtained at a test concentration of 0.2mg/ml.

* $P < 0.05$ based on comparison with WZ-e.

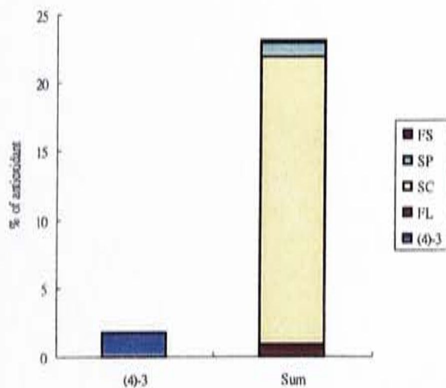
3.3.6 Synergistic effect of WZ-e and its simplified formulae

In order to examine the synergistic effect of various combinations of component herbs, the antioxidant capacity of each component herb was adjusted according to its weight percentage in a formula. Synergistic effect can be observed if the antioxidant capacity of a formula is greater than the sum of its component herbs.

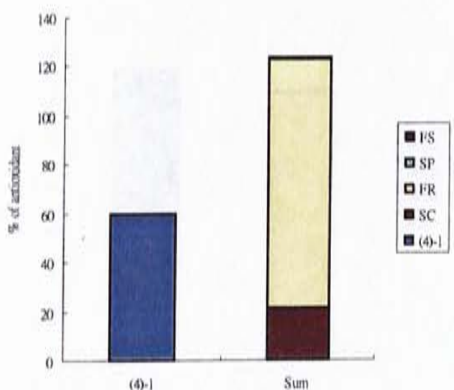
The results indicated that WZ-e and most of its simplified formulae did not exhibit significant synergistic effect except for formula (2)-4 which was comprised of FL and FS (Figure 3.5).



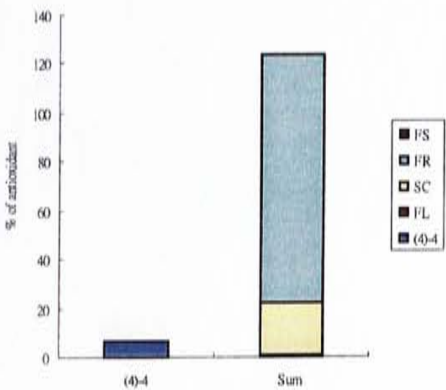
a): WZ-e



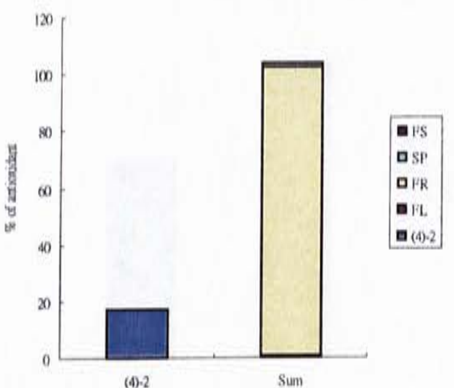
(d): (4)-3



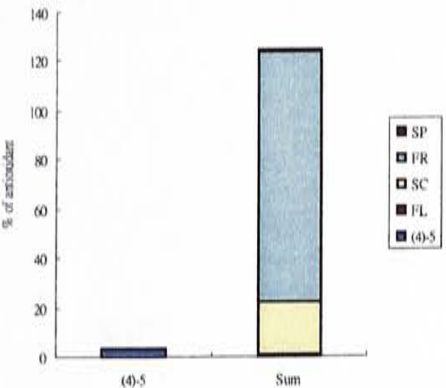
(b): (4)-1



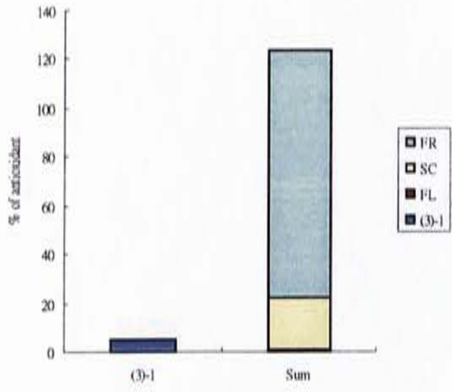
(e): (4)-4



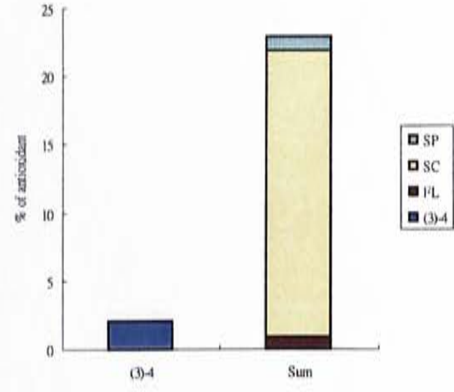
(c): (4)-2



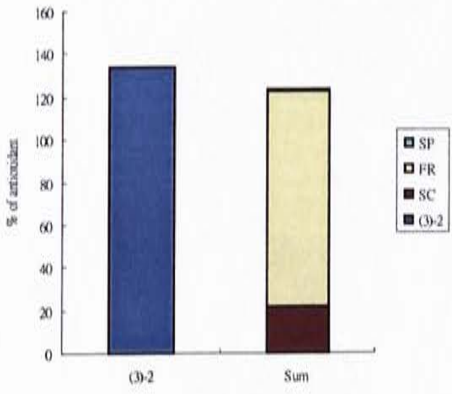
f): (4)-5



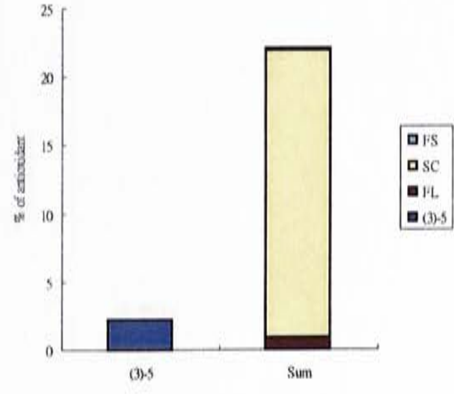
(g): (3)-1



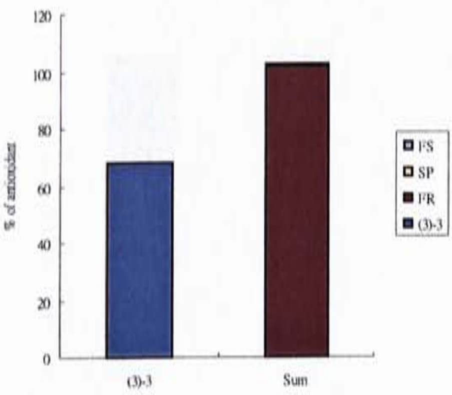
(j): (3)-4



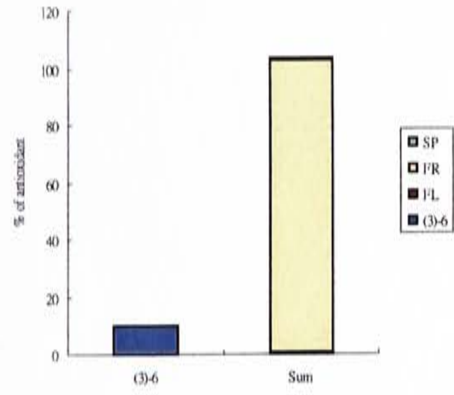
(h): (3)-2



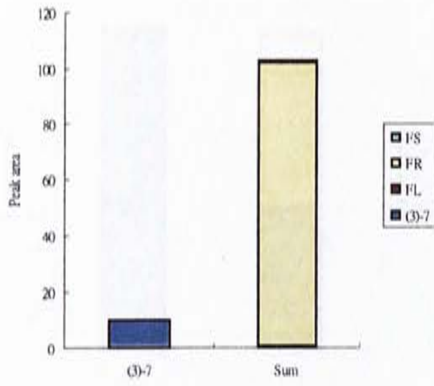
(k): (3)-5



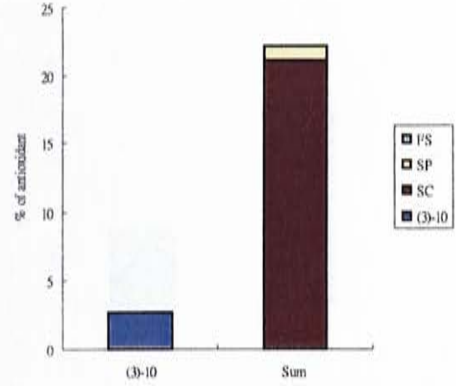
(i) (3)-3



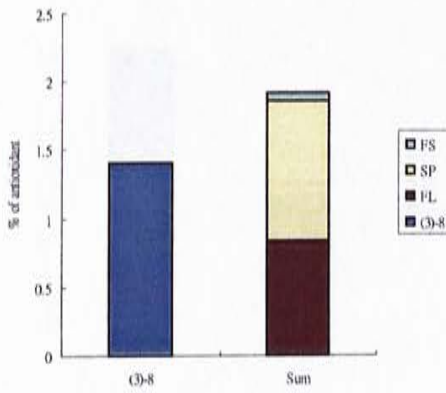
(l): (3)-6



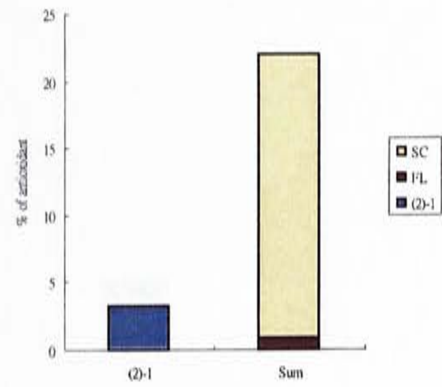
(m): (3)-7



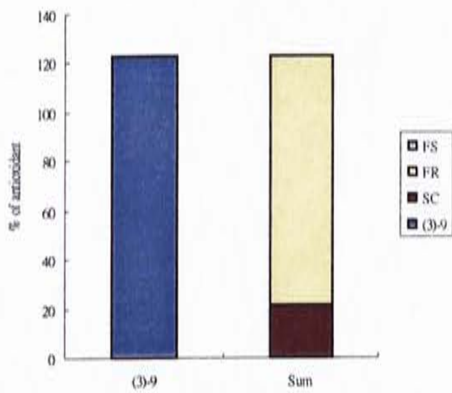
(p): (3)-10



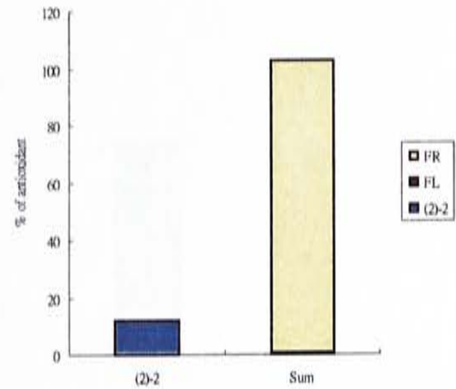
(n): (3)-8



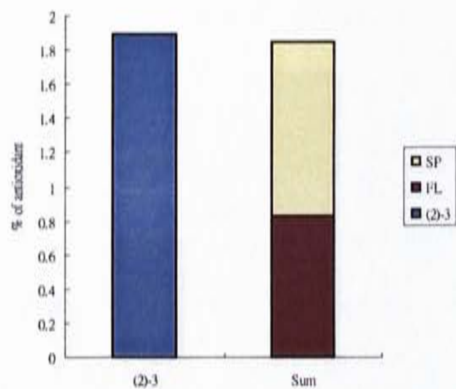
(q): (2)-1



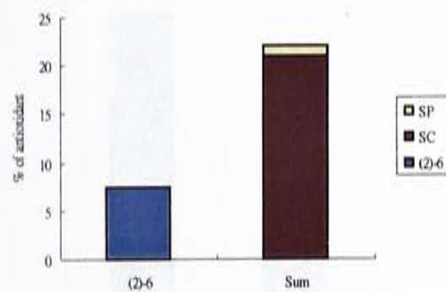
(o): (3)-9



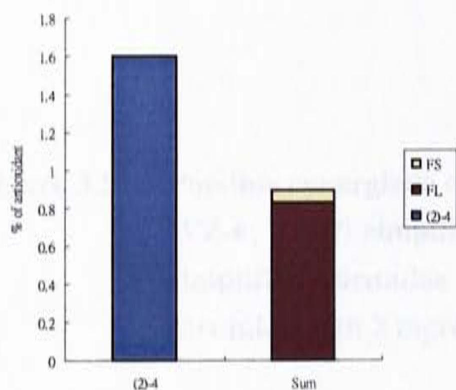
(r): (2)-2



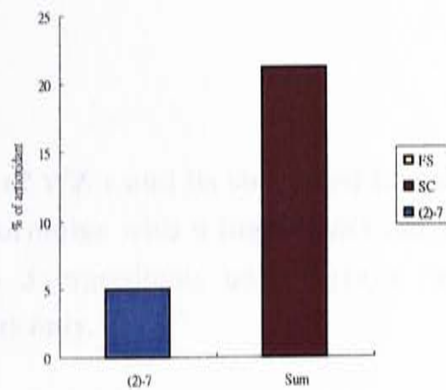
(s): (2)-3



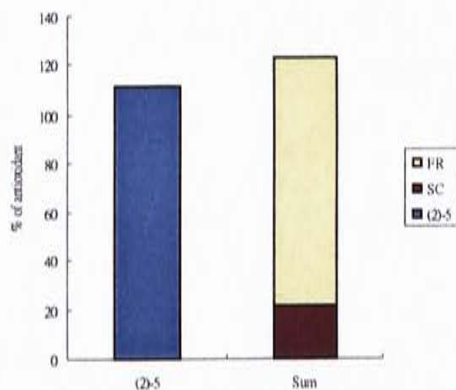
(v): (2)-6



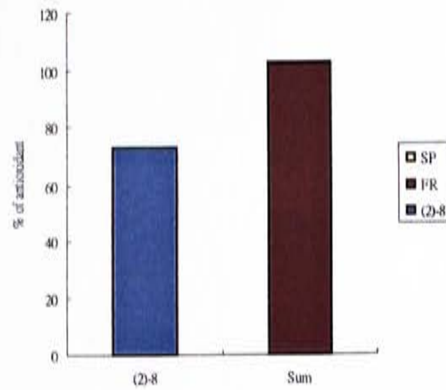
(t): (2)-4



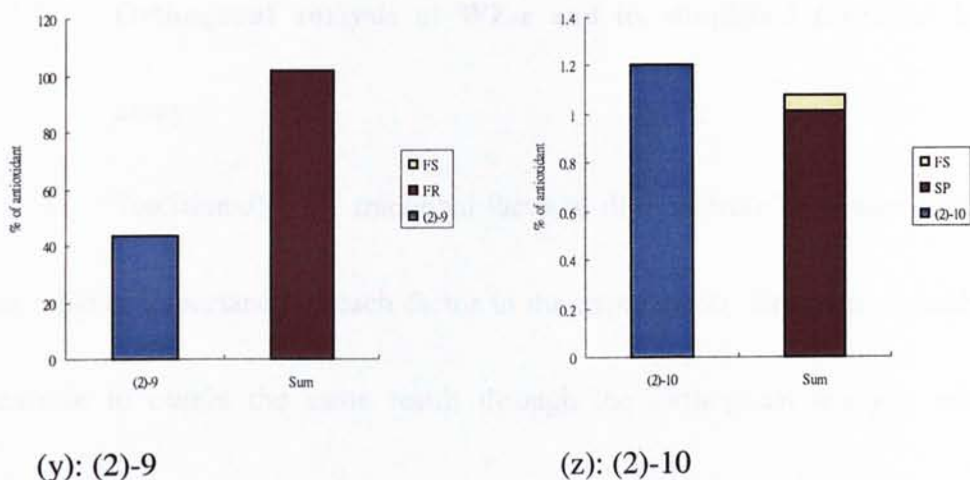
(w): (2)-7



(u): (2)-5



(x): (2)-8



(y): (2)-9 (z): (2)-10

Figure 3.5 Possible synergistic effect of WZ-e and its simplified formulae. (a) WZ-e; (b)-(f) simplified formulae with 4 ingredients only; (g)-(p) simplified formulae with 3 ingredients only; (q)-(z) simplified formulae with 2 ingredients only.

3.3.7 Orthogonal analysis of WZ-e and its simplified formulae in TEAC assay

Traditionally, 2^{n-k} fractional factorial designs have been used to determine the relative importance of each factor in the experiments. However, sometimes it is possible to obtain the same result through the Orthogonal analysis with fewer experimental groups.

In the present study, a commercial software, SPSS, is used to run this Orthogonal analysis. By simply enter the factors in the experiment, SPSS lists all experimental groups. By analyzing the results of these experimental groups, the computer program will identify the critical element among all factors. In the present case, there are 5 ingredients (factors) in the WZ formula and 8 experiment groups were suggested by the computer program. They are (2)-8, (2)-3, WZ, (2)-1, (2)-7, (3)-10, FS and (2)-5.

The results of Orthogonal analysis (Table 3.2) showed that only FR had a significant effect ($P < 0.05$) in the formulae. It suggested that FR was a critical component in the WZ formulae as far as the antioxidant capacity was concerned.

Table 3.2 Orthogonal analysis of antioxidant capacity data from WZ-e and selected simplified formulae

Source	Significance (P-value)
FL	0.410
SC	0.081
FR	0.011
SP	0.365
FS	0.093

3.4 Discussion

The Trolox equivalent antioxidant capacity (TEAC) assay was performed in order to determine *in vitro* antioxidant activity of the WZ formula. Both WZ-e and WZ-p were tested along with their simplified formulae.

Both WZ-e and WZ-p exhibited concentration-dependent antioxidant effect in the TEAC assay. By comparing the concentration-response curves for WZ-e and WZ-p, it is clearly shown that WZ-e is 10-fold stronger than WZ-p. Although the manufacturer claimed that the WZ-p ingredients were prepared from raw herbs at a yield of 20%, results obtained from the TEAC assay indicated that the aqueous extract of WZ-e was much more potent than WZ-p. One possible reason is that the antioxidant compounds present in the powder had at least partially, degraded over time.

Contradicting results were obtained when the antioxidant activity of WZ-e and its simplified formulae were compared. It is obvious that (3)-2, (3)-9, (2)-5, and FR displayed higher antioxidant activity than that of WZ-e. Interestingly, formulae (3)-2, (3)-9 and (2)-5 all contained both SC and FR. However, not all the combinations containing SC and FR are effective in scavenging free radicals. Therefore, it is premature to draw conclusion that SC and FR are the major component contributing to the antioxidant activity of WZ.

By comparing the antioxidant capacity of a formula and the arithmetic sum of those of its component herbs, synergistic effect was observed between FL and FS (formula (2)-4). However, both FL and FS had very weak antioxidant activity in the TEAC assay.

The result obtained from Orthogonal analysis (Table 3.2) indicated that FR is the most important ingredient in the WZ prescription as far as the antioxidant capacity is concerned. Therefore, this is consistent with the finding that FR has the greatest antioxidant capacity in the TEAC assay.

While the exact chemical composition of FR is still unclear, it is possible that phenolic compounds present in FR are responsible for the free radical scavenging activity. In this connection, aqueous extract of FR has been reported to protect against tert-butyl hydroperoxide-induced oxidative damage (Yau, 2002).

Chapter 4

Antioxidant Activity of Aqueous Extracts of Simplified Formulae of Wu-zi-yan-zong-wan *In Vitro*

4.1 Introduction

4.1.1 *In vitro* antioxidant

Based on the positive findings in the TEAC assay, further antioxidant assays were carried out using cellular system.

The cell line, HepG2, used in this study was obtained from ATCC, with the cells being originated from a 15-year-old male Caucasian. These human hepatocytes are used to carry out an *in vitro* cellular testing on the performance against oxidative stress. By using human hepatocytes, it is more reliable and reasonable to make predictions of *in vivo* studies.

4.1.2 Antioxidant effect of Catechins

Tea catechins and polyphenols are effective scavengers of reactive oxygen species *in vitro* and may also function indirectly as antioxidants through their effects on transcription factors and enzyme activities. Catechins can act as chain-breaking antioxidants by scavenging chain-propagating peroxyl radicals due to the presence of

a phenolic hydroxyl group at the 3'-position, and a hydroxyl group at the 5'-position, which both have the ability to scavenge free radicals *in vitro* (Nanjo, 1996; Rice-Evans, 1996; Guo, 1999). It has also been reported that green tea catechins have a cytoprotective effect against chemical-induced cell injury in primary cultured hepatocytes (Miyahawa, 1997; Yokozawa, 2000).

4.1.3 MTT assay

Determinations of cellular proliferation, viability and activation are key areas in a wide variety of cell biological approaches. The MTT assay is based on the cleavage of the yellow tetrazolium salt MTT (3-[4,5-dimethylthiazol2-yl]-2,5 -diphenyl tetrazolium bromide) to purple formazan crystals by metabolically active cells (Figure 4.1) (Vistica, 1991; Maehara Y, 1986; Slater, 1963). An increase in the number of viable cells would result in an increase in the total metabolic activity in the sample, thus the amount of purple formazan crystals formed, which could be quantified by measuring in the absorbance at 595nm.

Comparing with other labeling methods, MTT assay is safe, accurate, sensitive, fast and easy to operate. Therefore, it is widely used nowadays.

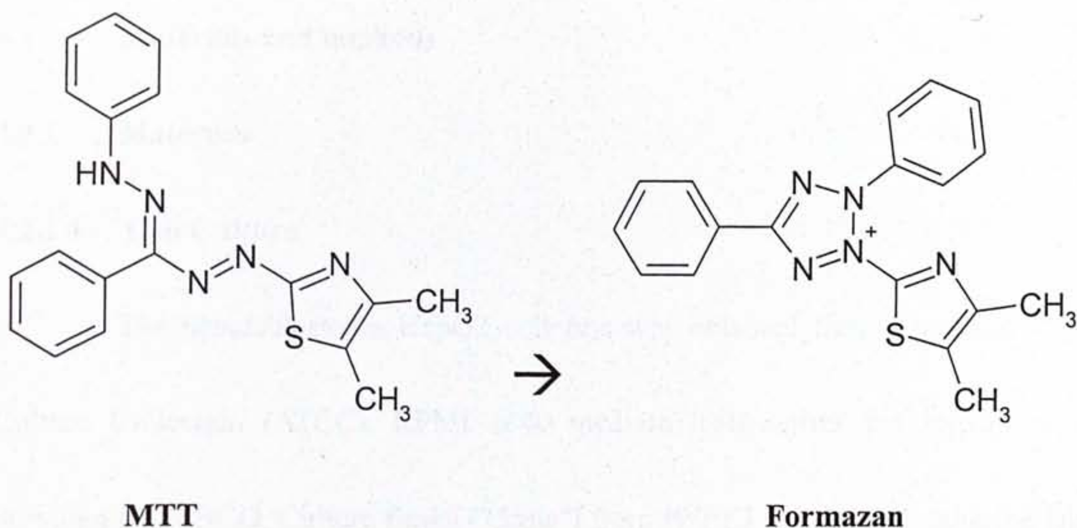


Figure 4.1 Metabolism of MTT to a formazan salt in viable cells.

4.1.4 Objectives

The present study was conducted to determine whether the aqueous extract of WZ-e and WZ-p have antioxidant activity in the cellular system. An oxidative stress inducing agent, such as hydrogen peroxide and menadione, was added to promote free radical production or even cause cell death. In addition, cell viability was determined in simplified formulae of WZ-e and WZ-p pretreated cells in order to investigate the effect between the original WZ formula and its components. It was hypothesized that the simplified formulae may have a even stronger antioxidant effect than the WZ formula.

4.2 Materials and methods

4.2.1 Materials

4.2.1.1 Cell Culture

The hepatoblastoma HepG2 cell line was obtained from American Type Culture Collection (ATCC). RPMI 1640 medium, calf serum and trypsin were provided by GIBCO. Culture flasks (75mm^2) from IWAKI Co. for cell culturing and 96-well cell culture cluster from Costar Corning Incorporated were used.

4.2.1.2 Reagents

Menadione, Sodium chloride, (Tris) Trisma Base, EDTA, Potassium chloride, Sodium phosphate, potassium phosphate, SDS were obtained from Sigma; catechin from Aldrich Chemical Co. and hydrogen peroxide from BDH Labotatory Supplies. MTT assay kit was provided by Amersham Life Science.

4.2.2 Methods

4.2.2.1 Cell Culture

HepG2 cells were cultured in RPMI 1640 medium (GIBCO) supplemented with 10% heat-inactivated calf serum at 37℃ in 5% CO_2 in a humidified cell incubator. The medium was changed every 2 days. For examining the effect of

Wu-zi-yan-zong-wan on cell death induced by H_2O_2, cells were pre-treated with WZ at increasing concentrations ranging from 0 ug/ml to 10000 ug/ml for 24 hours. Immediately after the treatment with WZ, H_2O_2 was applied at a concentration of 0.5 mM for 24 hours. The control group was left untreated.

4.2.2.2 MTT Cytotoxicity Assay

Cell viability was determined using an MTT assay kit according to the manufacturer's protocol. After drug treatment, 10 ul of MTT labeling reagent was added to each well, and the micro-titer plates were incubated for 4 hours. The cells were then incubated in 100 ul of the solubilization solution overnight. Absorbance was measured with a microtiter plate reader (Spectramax 250 microplate UV/Vis spectrophotometer) at 595 nm.

4.2.3 Statistical analysis

All analyses were performed for more than 3 times ($n > 3$). The data were expressed as mean ± S.E. One-way analysis of variance was performed by ANOVA procedures using SPSS version 12.0.1 for Windows (SPSS Inc., US). Significant differences between means were determined when P-values <0.05.

4.3 Results

4.3.1 The effect of WZ–e formula on HepG2 cells

A dose-response experiment of WZ-e was performed in order to obtain an optimum concentration for further investigation. A biphasic dose-response was observed, with a steep rising slope from 1 mg/ml to 4 mg/ml followed by an abrupt decline (Figure 4.2). As the concentration increased from 0mg/ml to 3mg/ml, the number of viable cells also increased. However, it is also observed that there was a decrease in number of viable cells as the concentration increased from 4mg/ml to 10mg/ml. The sub optimal concentration of WZ-e applied on HepG2 cell was determined to be 3 mg/ml.

4.3.2 The effect of hydrogen peroxide on HepG2 cells

Figure 4.3 shows a dose response curve of hydrogen peroxide on HepG2 cell. The lethal concentration at 50% (LC50) of hydrogen peroxide was found to be 0.5mM. This concentration of hydrogen peroxide was used in all subsequent experiments.

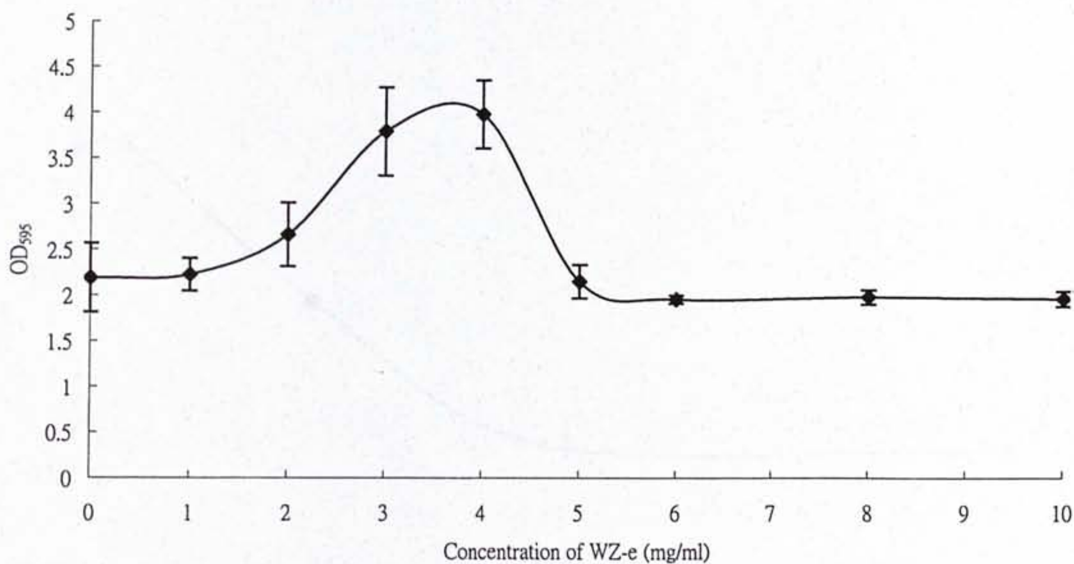


Figure 4.2 **The effect of aqueous WZ-e extract on HepG2 cells.** Increasing concentrations of WZ extract were added to hepatocytes and cultured for 24 hours. The viability of hepatocytes was determined by MTT assay. Data are expressed as mean ± S.E. (n = 8).

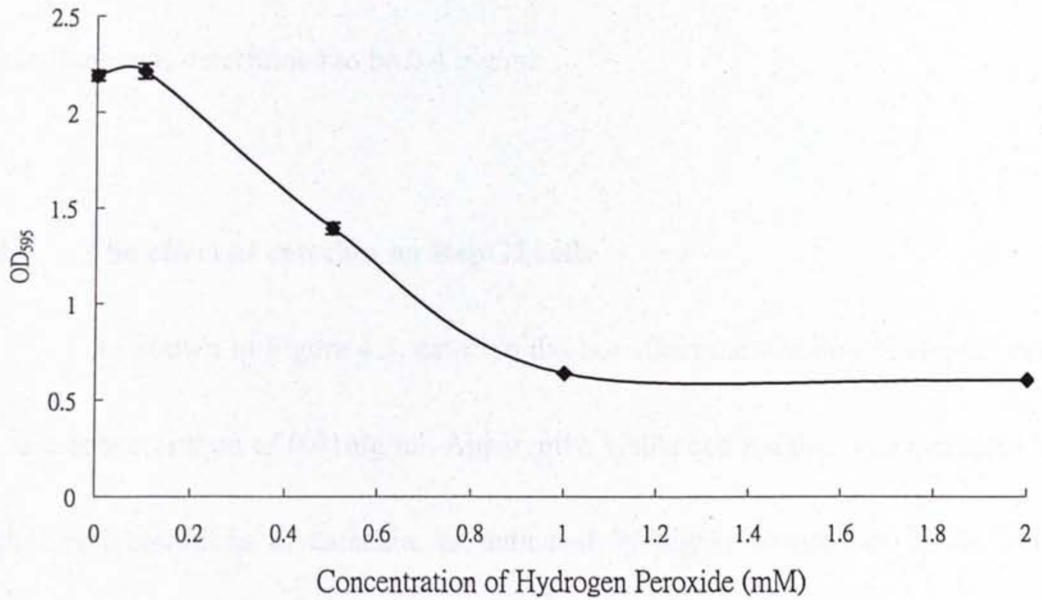


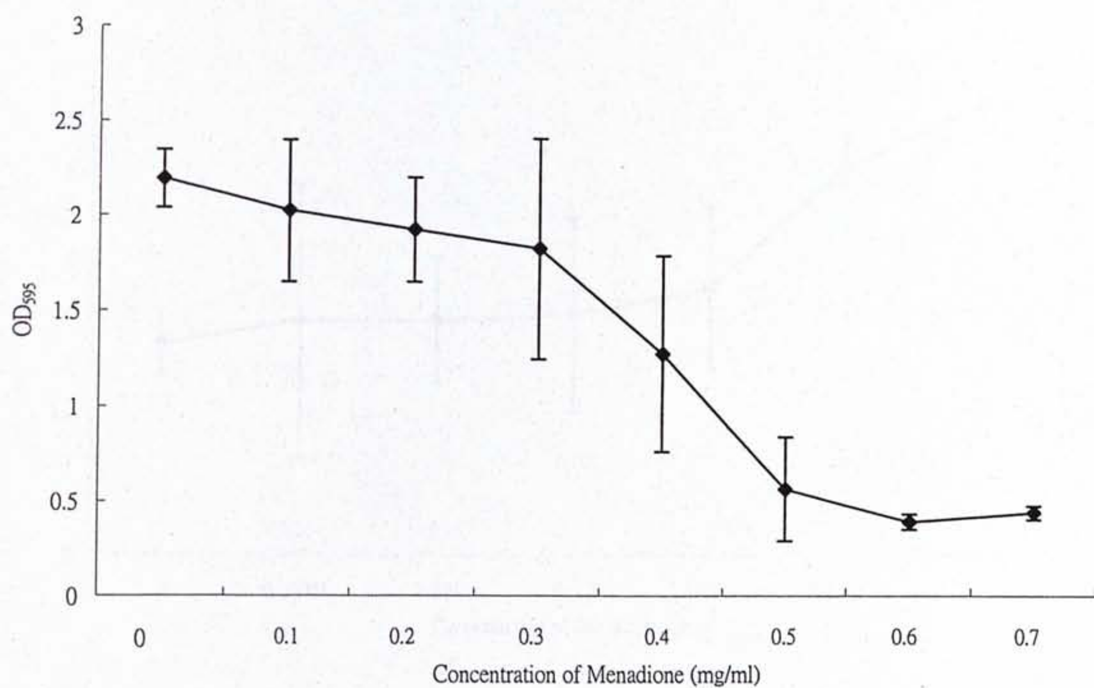
Figure 4.3 The effect of hydrogen peroxide (H_2O_2) on HepG2 cells. Increasing concentrations of hydrogen peroxide were added to hepatocytes and cultured for 24 hours. The viability of hepatocytes was determined with MTT assay. Data are expressed as mean ± S.E. (n = 8).

4.3.3 The effect of menadione on HepG2 cells

Increasing concentrations of menadione (dissolved in DMSO) were added to HepG2 hepatocytes. A dose response curve was shown in Figure 4.4. The LC $_{50}$ of meandione was determined to be 0.4 mg/ml.

4.3.4 The effect of catechin on HepG2 cells

As shown in Figure 4.5, catechin did not affect the viability of HepG2 cells up to a concentration of 0.01mg/ml. Apparently, viable cell number was increased by higher concentrations of catechin, as indicated by higher absorbance in the MTT assay. Thus, 0.1 mg/ml of catechin was used as a positive control in subsequent assays.



Figrue 4.4 **The effect of menadione on HepG2 cells.** Menadione was added to hepatocytes and cultured for 24 hours. The viability of hepatocytes was determined by MTT assay. Data are expressed as mean ± S.E. (n = 8).

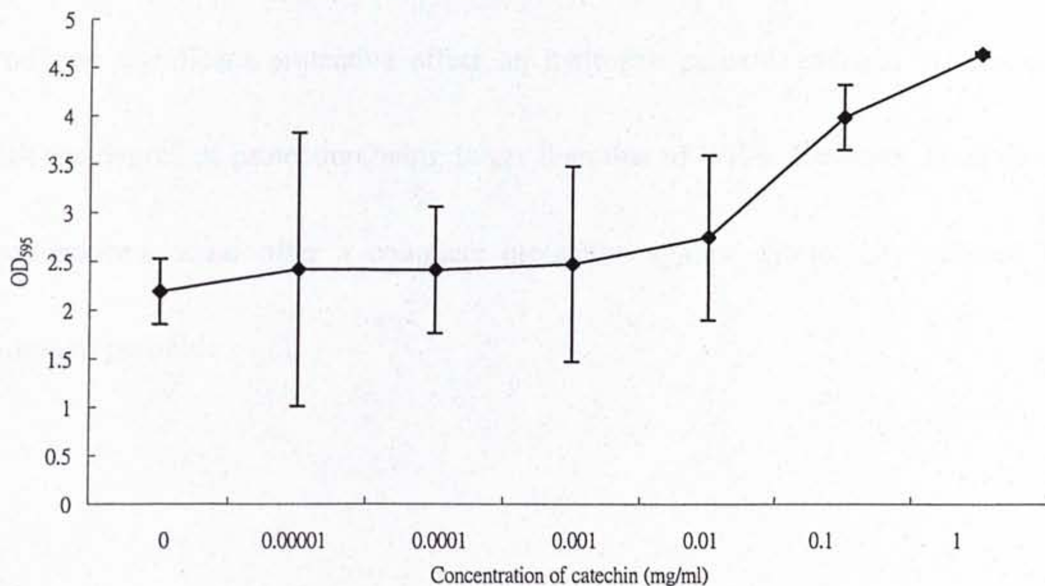


Figure 4.5 **The effect of catechin on HepG2 cells.** Catechin was added to hepatocytes and cultured for 24 hours. The viability of hepatocytes was determined by MTT assay. Data are expressed as mean ± S.E. (n = 8).

4.3.5 The effect of WZ-e and its simplified formulae on hydrogen peroxide -induced cytotoxicity in HepG2 cells

As shown in Figures 4.6 – 4.9, formulae (4)-1, (3)-2, (3)-4, FL and SC produced significant protective effect on hydrogen peroxide-induced cytotoxicity, with the degree of protection being larger than that of WZ-e. However, none of the combinations could offer a complete protection against cytotoxicity induced by hydrogen peroxide.

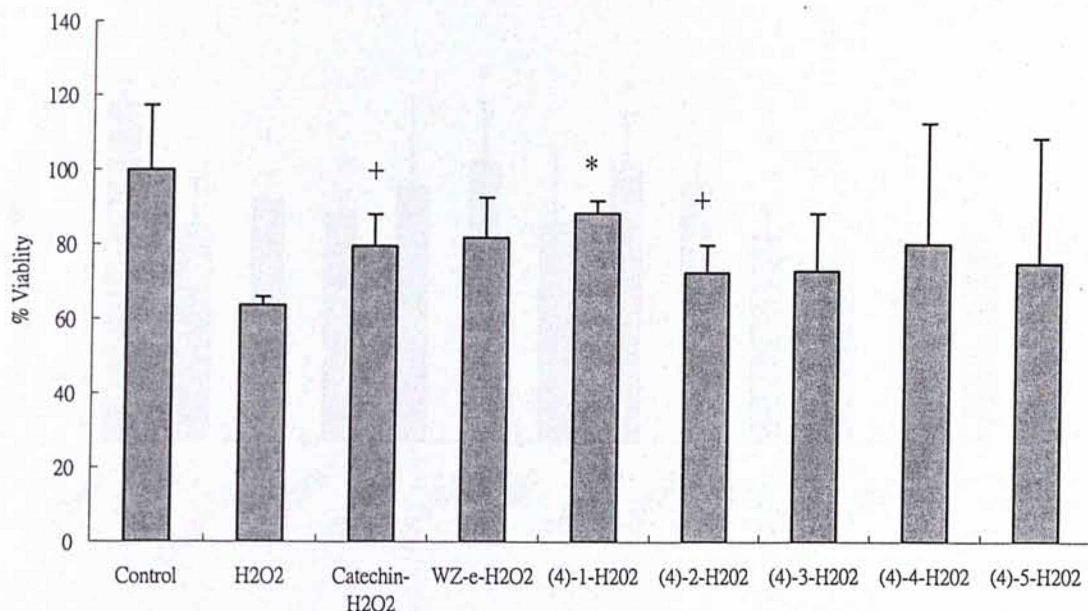


Figure 4.6 The effect of WZ-e and simplified formulae on hydrogen peroxide-induced toxicity in HepG2 cells. The HepG2 cells were treated with WZ-e or its simplified formulae (at a concentration of 3 mg/ml) for 24 hours at 37 ℃ with 5% CO_2. MTT assay was performed after treating with hydrogen peroxide at concentration 0.5 mM for 24 hours. Data are expressed as mean ± S.D. (n = 4)

+ $P < 0.05$ compared with control group.

* $P < 0.05$ compared with hydrogen peroxide-treated control group.

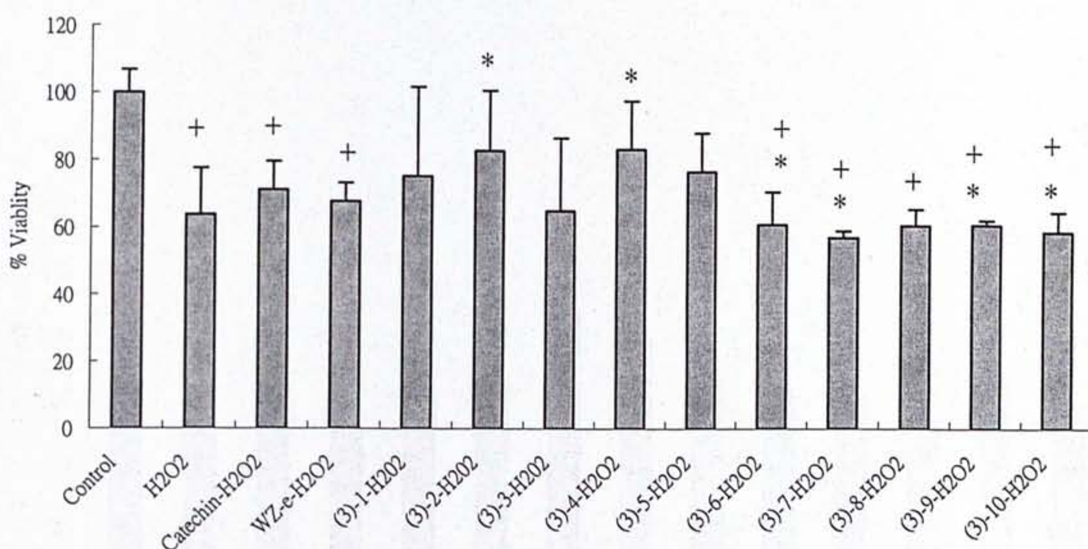


Figure 4.7 The effect of WZ-e and simplified formulae on hydrogen peroxide-induced toxicity in HepG2 cells. The HepG2 cells were treated with WZ-e or its simplified formulae (at a concentration of 3 mg/ml) for 24 hours at 37 ℃ with 5% CO_2. MTT assay was performed after treating with hydrogen peroxide at concentration 0.5 mM for 24 hours. Data are expressed as mean ± S.D. (n = 4)

+ $P < 0.05$ compared with control group.

* $P < 0.05$ compared with hydrogen peroxide-treated control group.

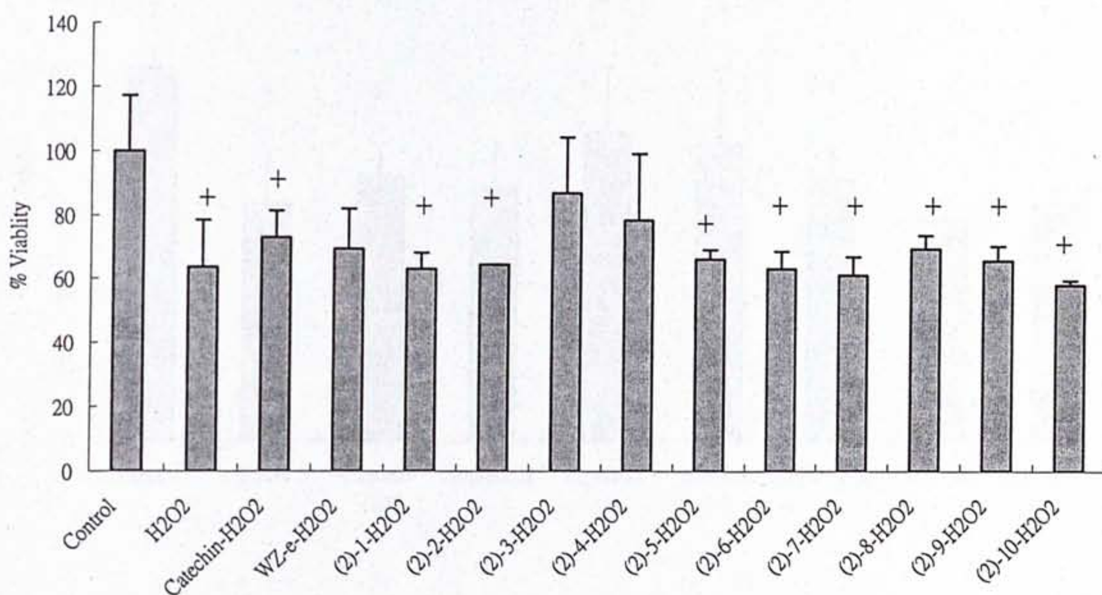


Figure 4.8 The effect of WZ-e and simplified formulae on hydrogen peroxide-induced toxicity in HepG2 cells. The HepG2 cells were treated with WZ-e or its simplified formulae (at a concentration of 3 mg/ml) for 24 hours at 37℃ with 5% CO_2. MTT assay was performed after treating with hydrogen peroxide at concentration 0.5 mM for 24 hours. Data are expressed as mean ± S.D. (n = 4)

+ $P < 0.05$ compared with control group.

* $P < 0.05$ compared with hydrogen peroxide-treated control group.

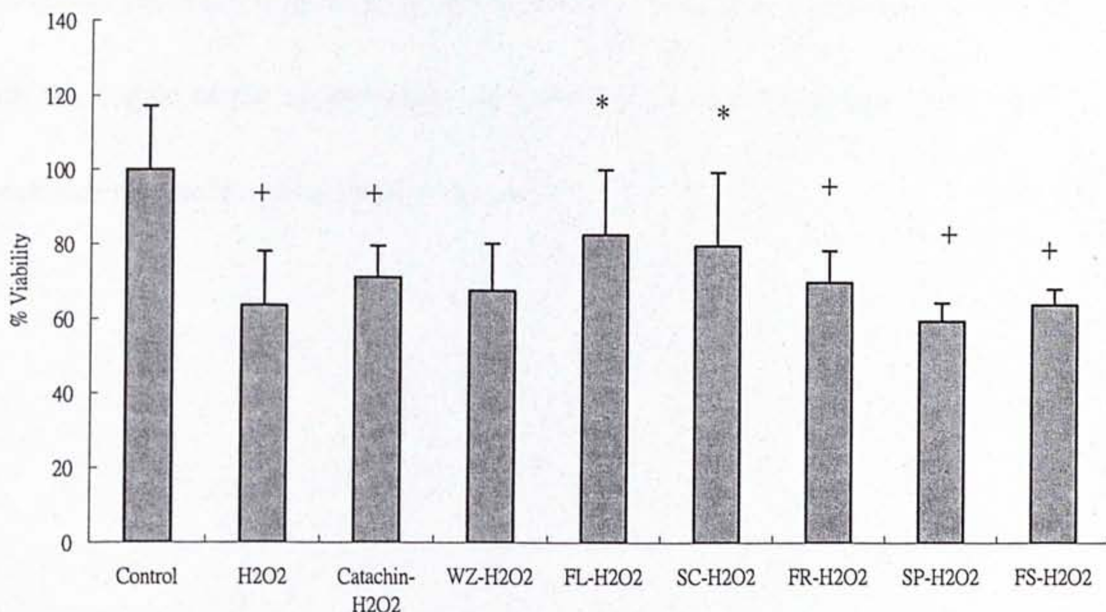


Figure 4.9 The effect of WZ-e and simplified formulae on hydrogen peroxide-induced toxicity in HepG2 cells. The HepG2 cells were treated with WZ-e or its simplified formulae (at a concentration of 3 mg/ml) for 24 hours at 37℃ with 5% CO_2. MTT assay was performed after treating with hydrogen peroxide at concentration 0.5 mM for 24 hours. Data are expressed as mean ± S.D. (n = 4)

+ $P < 0.05$ compared with control group.

* $P < 0.05$ compared with hydrogen peroxide-treated control group.

4.3.6 The effect of WZ-e and its simplified formulae on menadione-induced cytotoxicity in HepG2 cells

As shown in Figures 4.10 – 4.13, formulae (2)-1, (2)-5, (2)-6, (2)-8, (2)-9 and (2)-10 produced significant protective effect on menadione-induced cytotoxicity, with the degree of protection being larger than that of WZ-e. However, none of the combinations could offer a 100% protection.

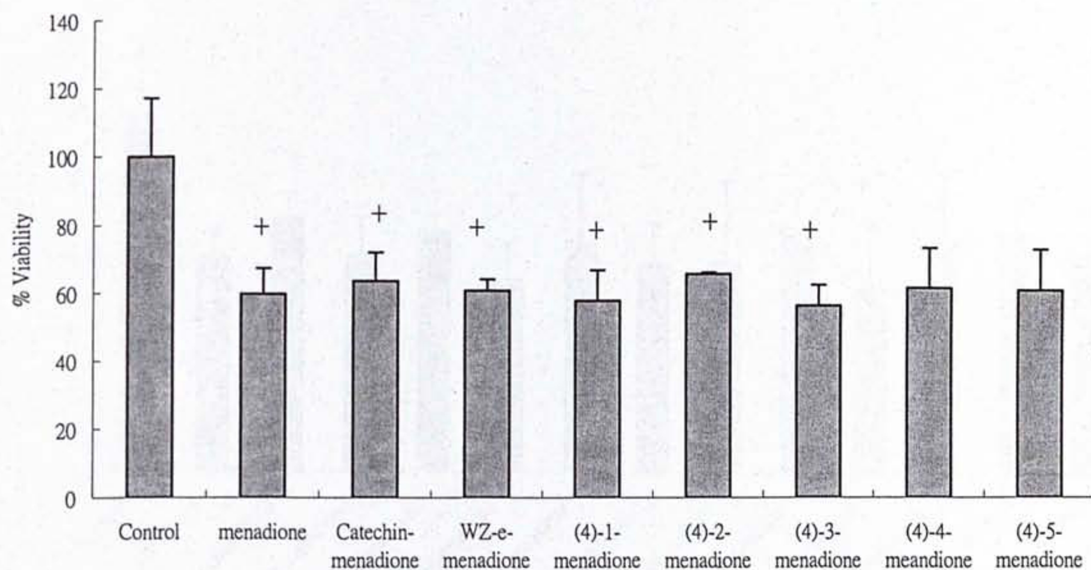


Figure 4.10 **The effect of WZ-e and simplified formulae on menadione-induced toxicity in HepG2 cells.** The HepG2 cells were treated with WZ-e or its simplified formulae (at a concentration of 3 mg/ml) for 24 hours at 37℃ with 5% CO_2. MTT assay was performed after treating with menadione at concentration of 0.5 mM for 24 hours. Data are expressed as mean ± S.D. (n = 4)

+ $P < 0.05$ compared with control group.

* $P < 0.05$ compared with menadione-treated control group.

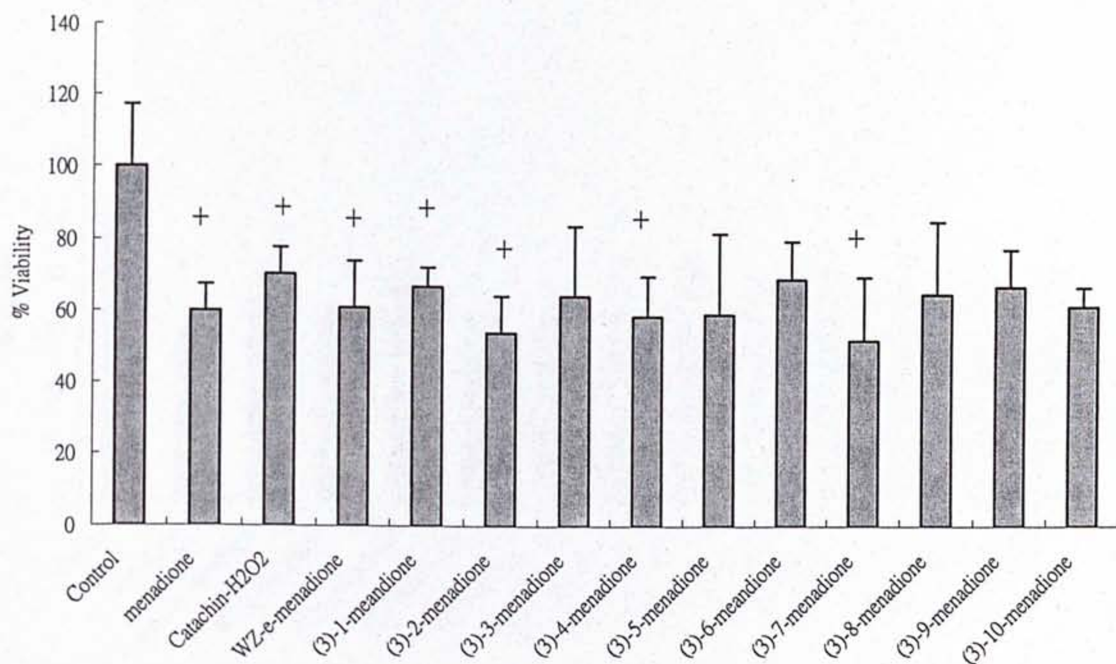


Figure 4.11 **The effect of WZ-e and simplified formulae on menadione-induced toxicity in HepG2 cell line.** The HepG2 cells were treated with WZ-e or its simplified formulae (at a concentration of 3 mg/ml) for 24 hours at 37℃ with 5% CO_2. MTT assay was performed after treating with menadione at concentration of 0.5 mM for 24 hours. Data are expressed as mean ± S.D. (n = 4)

+ $P < 0.05$ compared with control group.

* $P < 0.05$ compared with menadione-treated control group.

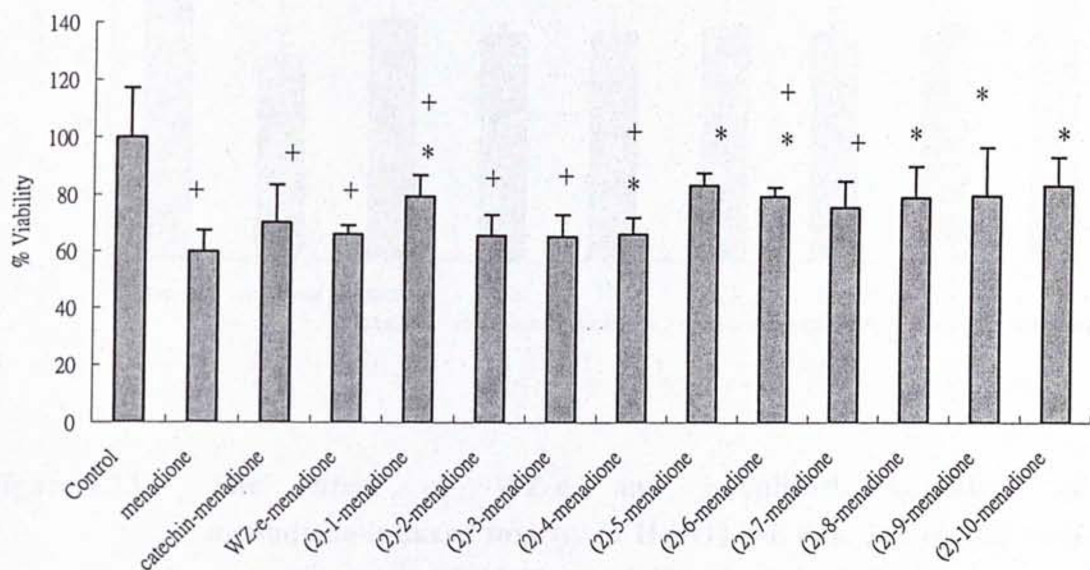


Figure 4.12 The effect of WZ-e and simplified formulae on menadione-induced toxicity in HepG2 cell line. The HepG2 cells were treated with WZ-e or its simplified formulae (at a concentration of 3 mg/ml) for 24 hours at 37℃ with 5% CO_2. MTT assay was performed after treating with menadione at concentration of 0.5 mM for 24 hours. Data are expressed as mean ± S.D. (n = 4)

+ $P < 0.05$ compared with control group.

* $P < 0.05$ compared with menadione-treated control group.

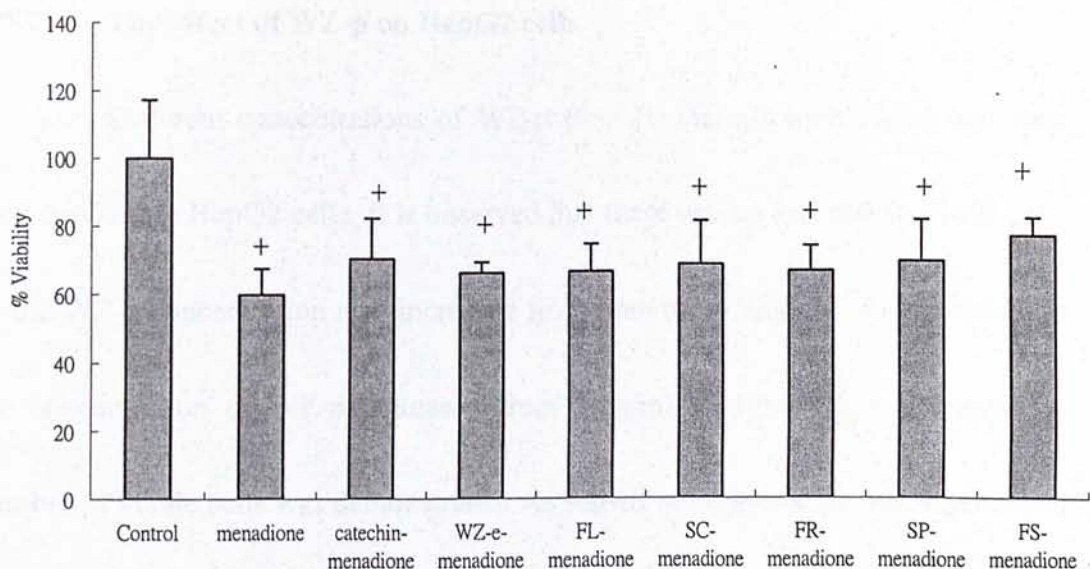


Figure 4.13 The effect of WZ-e and simplified formulae on menadione-induced toxicity in HepG2 cell line. The HepG2 cells were treated with WZ-e or its simplified formulae (at a concentration of 3 mg/ml) for 24 hours at 37℃ with 5% CO_2. MTT assay was performed after treating with menadione at concentration of 0.5 mM for 24 hours. Data are expressed as mean ± S.D. (n = 4)

+ $P < 0.05$ compared with control group.

* $P < 0.05$ compared with menadione-treated control group.

4.3.7 The effect of WZ-p on HepG2 cells

Different concentrations of WZ-p (0 – 10 mg/ml) were added into each well containing HepG2 cells. It is observed that there was an increase in viable cells as the WZ-p concentration also increased from 0mg/ml to 3mg/ml. Alternatively, as the concentration of WZ-p increased from 3mg/ml to 10mg/ml, a decreased in number of viable cells was demonstrated. As shown in Figures 4.14, WZ-p produced a biphasic effect on the viability of HepG2 cells, with the enhancement of cell survival being optimal at 3mg/ml.

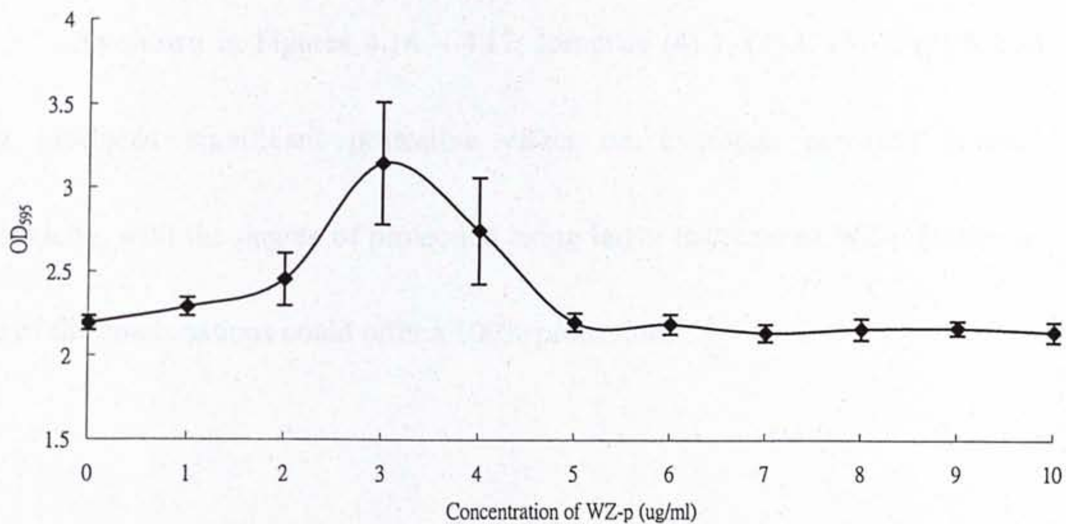


Figure 4.14 The effect of aqueous WZ-p extract on HepG2 cells. Different concentrations of WZ-p extract were added to hepatocytes and cultured for 24 hours. The viability of hepatocytes was determined with MTT assay. Data are expressed as mean ± S.E. (n = 8).

4.3.8 The effect of WZ-p and its simplified formulae on hydrogen peroxide-induced cytotoxicity in HepG2 cells.

As shown in Figures 4.14 – 4.17, formulae (4)-2, (3)-1, (3)-2, (2)-8, and (2)-9 produced significant protective effect on hydrogen peroxide induced cytotoxicity, with the degree of protection being larger than that of WZ-p However, none of the combinations could offer a 100% protection.

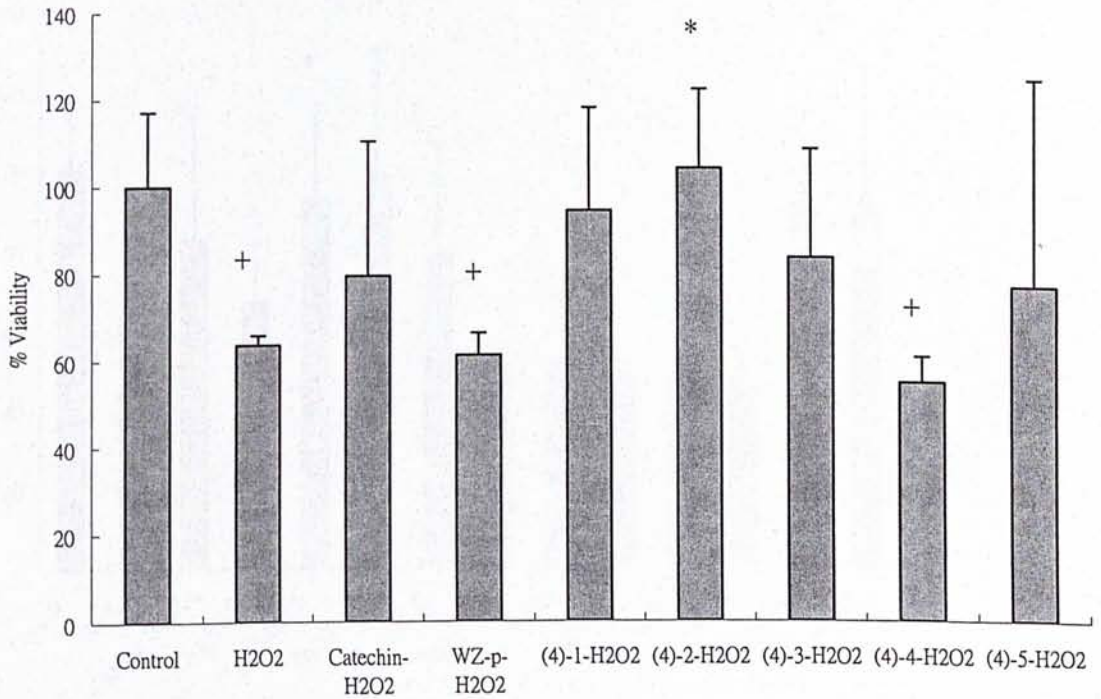


Figure 4.15 The effect of WZ-p and simplified formulae on hydrogen peroxide-induced toxicity in HepG2 cell line. The HepG2 cells were treated with WZ-p or its simplified formulae (at a concentration of 3 mg/ml) for 24 hours under 37℃ with 5% CO_2. MTT assay was performed after treating with hydrogen peroxide at concentration of 0.5 mM for 24 hours. Data are expressed as mean ± S.D. (n = 4)

* $P < 0.05$ compared with hydrogen peroxide treatment group

+ $P < 0.05$ compared with control group

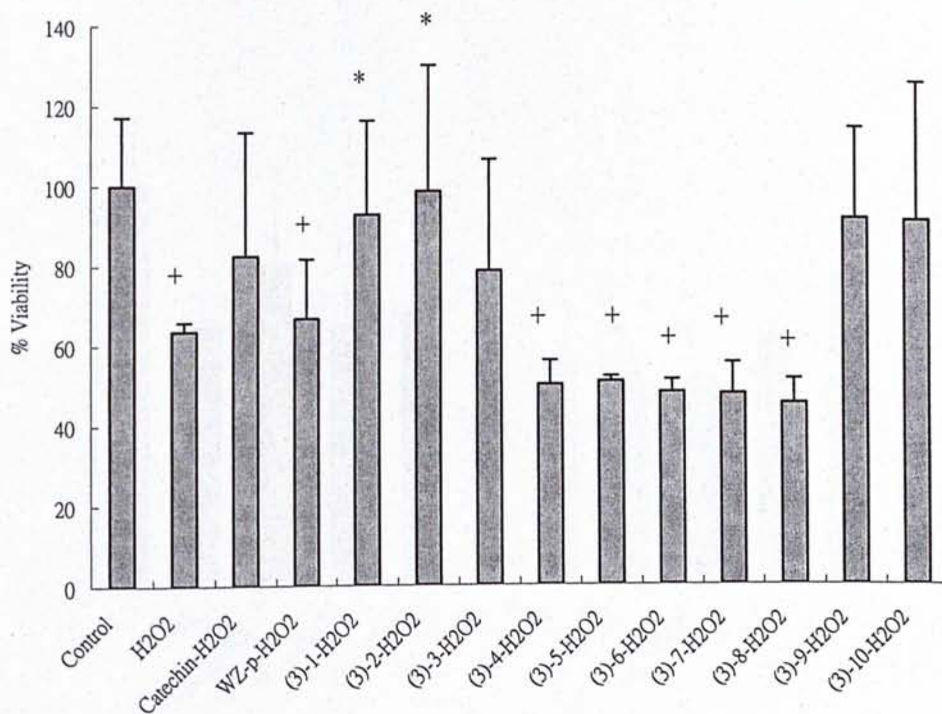


Figure 4.16 The effect of WZ-p and simplified formulae on hydrogen peroxide-induced toxicity in HepG2 cell line. The HepG2 cells were treated with WZ-p or its simplified formulae (at a concentration of 3 mg/ml) for 24 hours under 37℃ with 5% CO_2. MTT assay was performed after treating with hydrogen peroxide at concentration of 0.5 mM for 24 hours. Data are expressed as mean ± S.D. (n = 4)

* $P < 0.05$ compared with hydrogen peroxide treatment group

+ $P < 0.05$ compared with control group

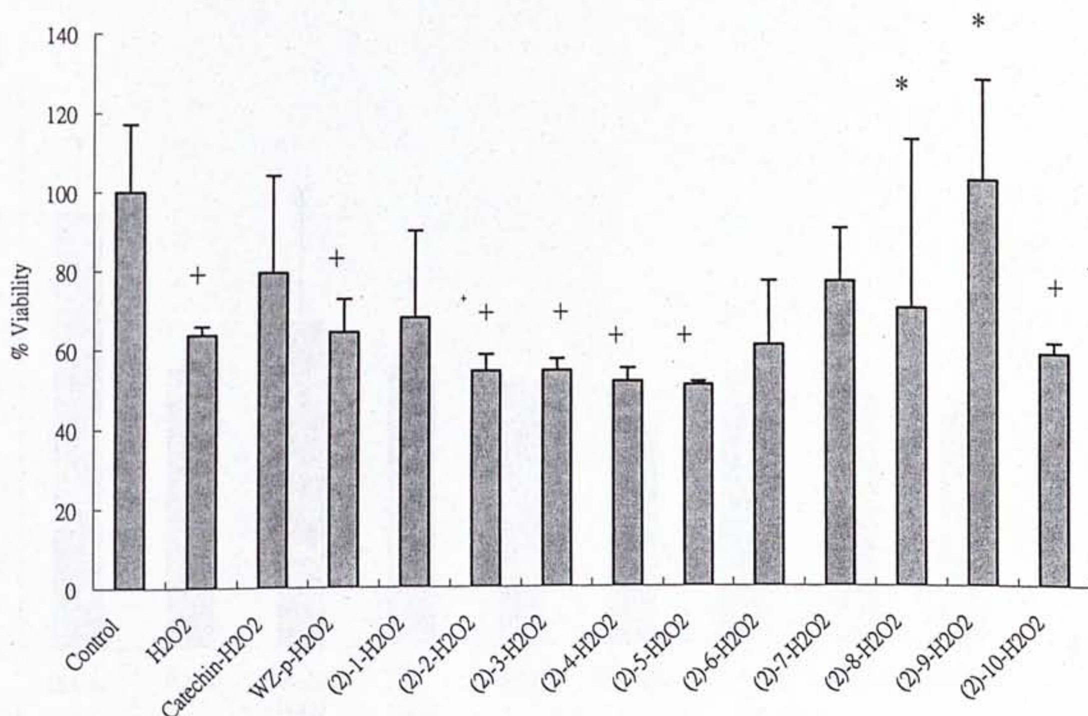


Figure 4.17 The effect of WZ-p and simplified formulae on hydrogen peroxide-induced toxicity in HepG2 cell line. The HepG2 cells were treated with WZ-p or its simplified formulae (at a concentration of 3 mg/ml) for 24 hours under 37℃ with 5% CO_2. MTT assay was performed after treating with hydrogen peroxide at concentration of 0.5 mM for 24 hours. Data are expressed as mean ± S.D. (n = 4)

* $P < 0.05$ compared with hydrogen peroxide treatment group

+ $P < 0.05$ compared with control group

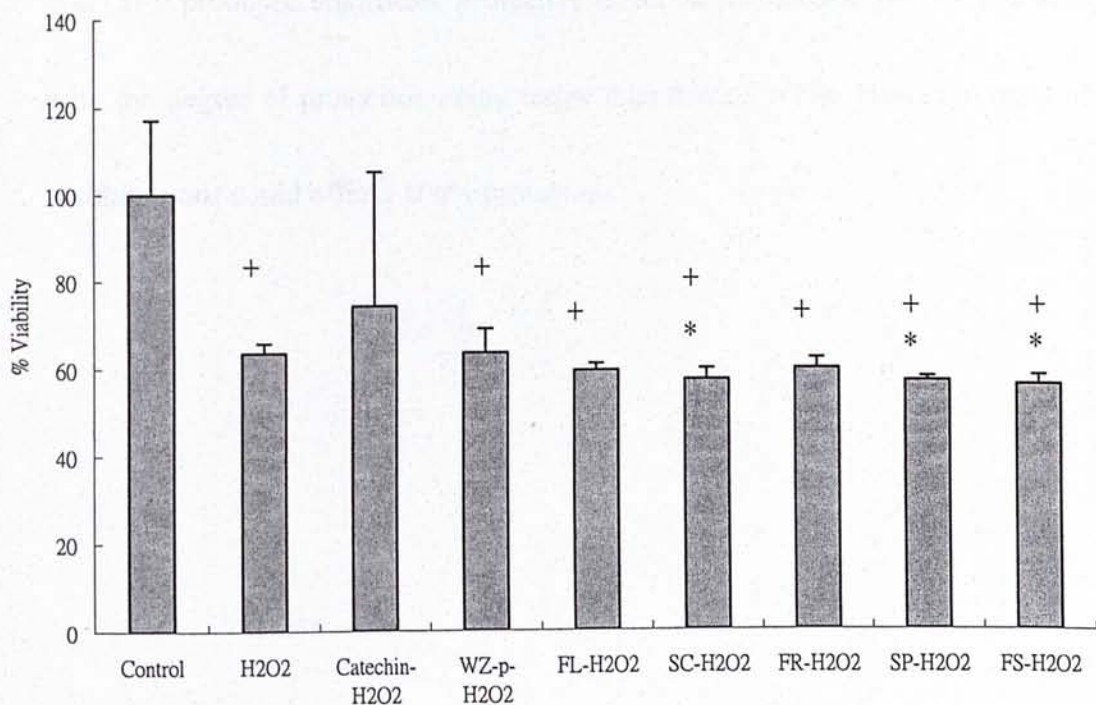


Figure 4.18 The effect of WZ-p and simplified formulae on hydrogen peroxide-induced toxicity in HepG2 cell line. The HepG2 cells were treated with WZ-p or its simplified formulae (at a concentration of 3 mg/ml) for 24 hours under 37℃ with 5% CO_2. MTT assay was performed after treating with hydrogen peroxide at concentration of 0.5 mM for 24 hours. Data are expressed as mean ± S.D. (n = 4)

* $P < 0.05$ compared with hydrogen peroxide treatment group

+ $P < 0.05$ compared with control group

4.3.9 The effect of WZ-p and its simplified formulae on menadione-induced cytotoxicity in HepG2 cells.

As shown in Figures 4.19 – 4.22, formulae (4)-1, (4)-4, (4)-5, (3)-6, (3)-7 and (3)-9 produced significant protective effect on menadione-induced cytotoxicity, with the degree of protection being larger than that of WZ-p. However, none of the combinations could offer a 100% protection.

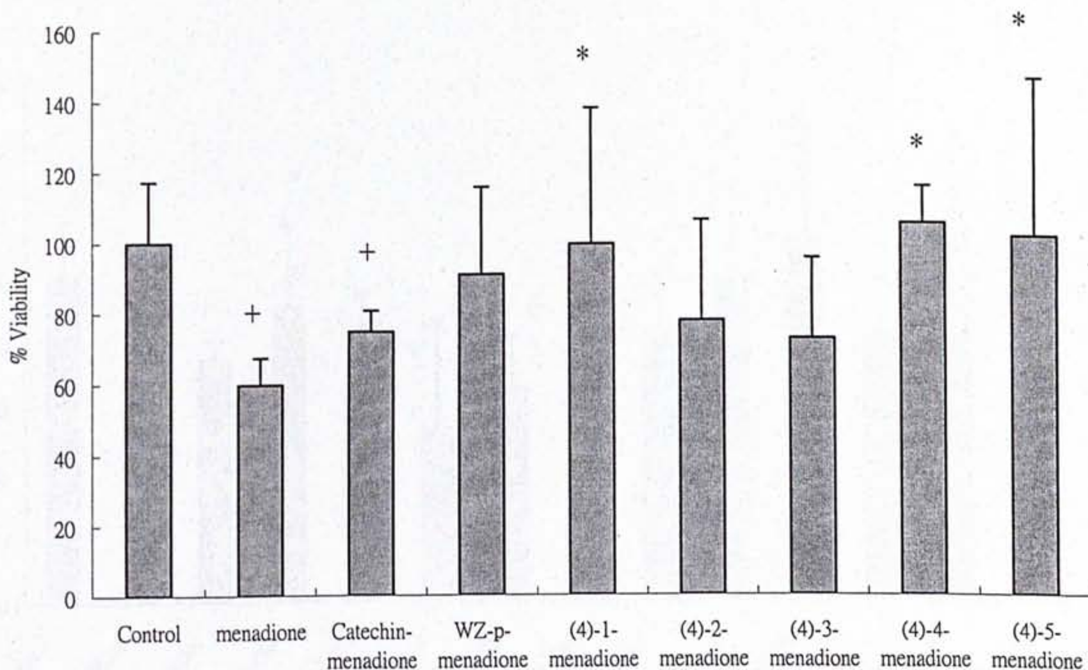


Figure 4.19 The effect of WZ-p and simplified formulae on menadione-induced toxicity in HepG2 cell line. The HepG2 cells were treated with WZ-p or its simplified formulae (at a concentration of 3 mg/ml) for 24 hours at 37℃ with 5% CO_2. MTT assay was performed after treating with menadione at concentration of 0.5 mM for 24 hours. Data are expressed as mean ± S.D. (n = 4)

* $P < 0.05$ compared with menadione treatment group

+ $P < 0.05$ compared with control group

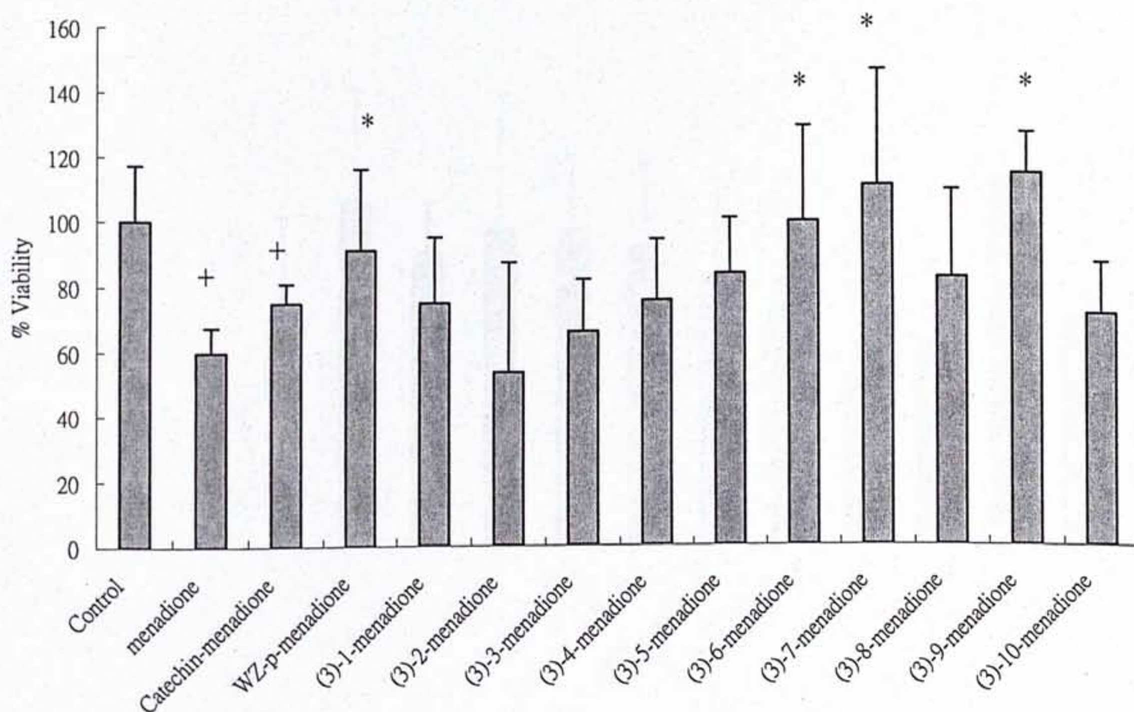


Figure 4.20 The effect of WZ-p and simplified formulae on menadione-induced toxicity in HepG2 cell line. The HepG2 cells were treated with WZ-p or its simplified formulae (at a concentration of 3 mg/ml) for 24 hours at 37℃ with 5% CO_2. MTT assay was performed after treating with menadione at concentration of 0.5 mM for 24 hours. Data are expressed as mean ± S.D. (n = 4)

* $P < 0.05$ compared with menadione treatment group

\+ $P < 0.05$ compared with control group

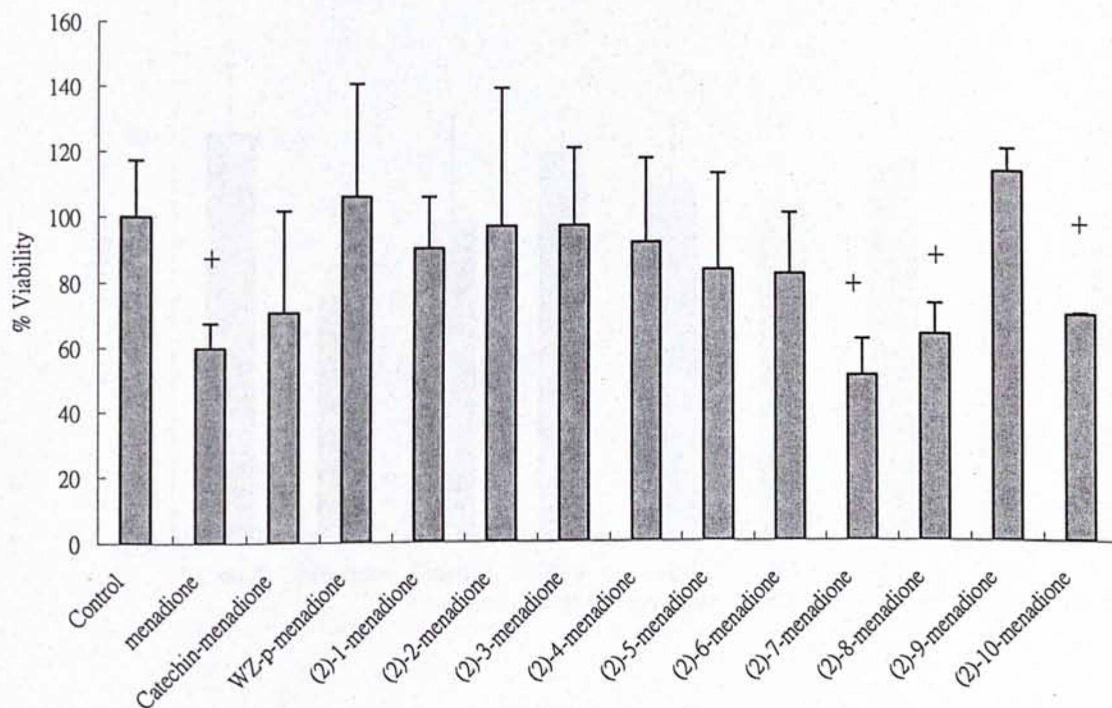


Figure 4.21 The effect of WZ-p and simplified formulae on menadione-induced toxicity in HepG2 cell line. The HepG2 cells were treated with WZ-p or its simplified formulae (at a concentration of 3 mg/ml) for 24 hours at 37℃ with 5% CO_2. MTT assay was performed after treating with mendaione at concentration of 0.5 mM for 24 hours. Data are expressed as mean ± S.D. (n = 4)

* $P < 0.05$ compared with menadione treatment group

+ $P < 0.05$ compared with control group

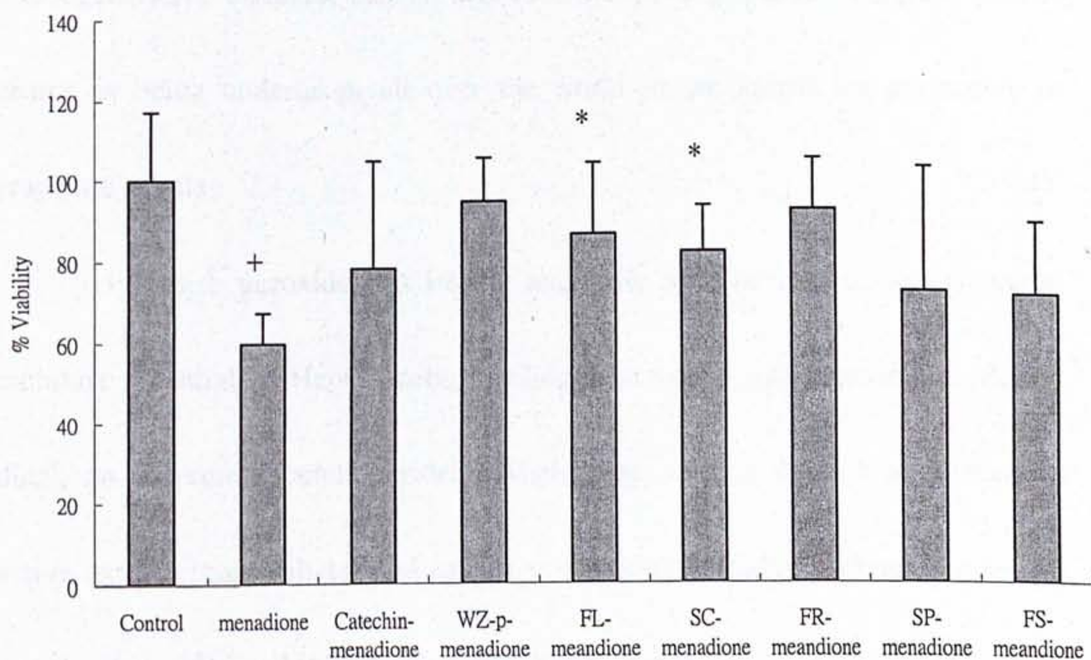


Figure 4.22 The effect of WZ-p and simplified formulae on menadione-induced toxicity in HepG2 cell line. The HepG2 cells were treated with WZ-p or its simplified formulae (at a concentration of 3 mg/ml) for 24 hours at 37℃ with 5% CO_2. MTT assay was performed after treating with menadione at concentration of 0.5 mM for 24 hours. Data are expressed as mean ± S.D. (n = 4)

* $P < 0.05$ compared with menadione treatment group
\+ $P < 0.05$ compared with control group

4.4 Discussion

Reactive oxygen species and other free radicals are considered to be important causative factors in the development of age-related diseases such as neurodegenerative diseases, cancer, and cardiovascular diseases. Therefore intense research is being undertaken all over the world in the search for preventive or therapeutic agents.

Hydrogen peroxide can induce apoptosis and the loss of mitochondrial membrane potential in HepG2 cells. Hydrogen peroxide could generate hydroxyl radical, an extremely potent oxidant. Menadione, on the other hand, generates reactive oxygen intermediates and causes cytotoxicity by redox cycling. Our results showed that the addition of menadione to HepG2 cells produced a concentration-dependent loss of cell viability. Both of H_2O_2 and menadione increase oxidative stress in HepG2 cells, leading to the decrease in cell viability or even cell death. The herbal samples, which were found to possess antioxidant activity, were studied to determine their ability to protect against oxidative stress.

Alternatively, catechin was prepared in different concentrations to construct achieve a concentration curve in order to determine an optimal concentration as a standard. However, it clearly shows a proliferation effect on HepG2 cells. Catechin has been widely studied as a strong and effective antioxidant on both *in vitro* and *in*

vivo assay. It was reported that tea catechins inhibit cellular lipid peroxidation and reduce cytotoxicity in HepG2 cells (Murakami, 2002; Huang, 2003; Feng, 2001; Liu, 2003). Thus, catechin was used as a positive control in the assays.

Results obtained from the present study indicated that there was no sample capable of providing a complete protection. However, a number of hernal combinations displayed stronger protective effect than the complete WZ-e formula itself. Similarly, some of simplified formulae of WZ-p protected against cytotoxicity induced by hydrogen peroxide or menadione. However, a critical component herb for the cytoprotective effect could not be identified from both studies.

It is believed that there are many unknown factors that may affect the results of WZ-e and WZ-p experiments. While WZ-e was extracted in our laboratory and the water extract was freeze-dried into powder, WZ-p was obtained from the market as a ready-to-use powder. Neither the quality of the raw herbs used, nor the processing procedure of these commercial products is known It is also uncertain about how long the products have been in the market. The antioxidant activity of the product may lose if it has been shelved at room temperature for a long period of time.

Chapter 5

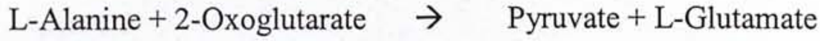
Effect of Aqueous Extract of Wu-zi-yan-zong-wan on Menadione -Induced Oxidative Damage in Mouse Liver

5.1 Introduction

Liver function tests, such as the measurement of plasma alanine aminotransferase (ALT) activity test, are used for the differential diagnosis of liver diseases or injuries and monitor for the treatment response. Although ALT is widely presented in a variety of tissues, it can be used as biochemical indicator for chronic liver disease and liver damage. The major source of plasma/ serum ALT is the liver; an increase in plasma ALT activity can be used for the assessment of the extent of liver damage. In this regard, elevated serum levels are, in particular, found most commonly in hepatitis, cirrhosis, and obstructive jaundice (Herny, 1974; Toyoda, 2004). Spectrophotometric measurement of ALT activity was based on the oxidation of NADH by lactate dehydrogenase (LDH). ALT catalyzes the transfer of the amino group from alanine to 2-oxoglutarate, to form glutamate and pyruvate. The pyruvate formed is then reduced to lactate in the presence of LDH, with simultaneous oxidation of the reduced nicotinamide adenine dinucleotide (NADH). The rate of decrease in absorbance at 340 nm is directly proportional to ALT activity (Bergmeyer,

1977).

ALT



L-Alanine + 2-Oxoglutarate → Pyruvate + L-Glutamate

LDH

Pyruvate + NADH + H^+ → D-Lactate + NAD+ + H_2O

The aqueous extract of WZ-e formula showed an antioxidant capacity on TEAC assay *in vitro*. Therefore, it is hypothesized that WZ-e can also produce a hepato-protective effect on menadione-induced toxicity. A mouse model was established to study the *in vivo* effect of the herbal preparation. Animal models would provide data more relevant to the situation in human. In animal experiments, test samples are usually administered through gastrointestinal tract or other parenternal routes; the active ingredients will be absorbed into the blood stream before exerting their effects on distal targets. The antioxidant activities of WZ-e extract as well as its simplified formulae were therefore examined using the mouse model of menadione hepatotoxicity.

5.2 Materials and methods

5.2.1 Materials

5.2.1.1 Animals

ICR mice (18-20 g) were supplied by the Laboratory Animal Services Center (LASEC), The Chinese University of Hong Kong. They were acclimated under the conditions of 22-25℃ and a 12-hour light-dark cycle before start of the experiments. They were supplied with standard rodent chow and allowed free access to tap water.

5.2.1.2 Reagents

Alanine aminotransferase (ALT) assay kit from Stanbio Laboartory was used. Menadione, heparin, and sodium chloride (NaCl) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals used were of analytical grade.

5.2.1.3 Apparatus

UV spectrophotometer from HP, microscopic illuminator was obtained from Leica Microsystem Ltd., Reicherj-Jung microtome at 5um thick.

5.2.2 Methods

5..2.2.1 Animal treatments

ICR mice were divided into 6 groups on day 0 and housed separately in the LASEC. Different concentrations of WZ extract as well as the selected simplified formulae according to Orthogonal analysis [(2)-8, (2)-3, WZ, (2)-1, (2)-7, (3)-10, FS, and (2)-5] were prepared in distilled water. The extract was administered daily to mice by intragastric feeding at 0.42 g/kg/day, 1.25 g/kg/day, and 3.75 g/kg/day dose levels for 7 days. For the control group, mice were given 0.2 ml saline each day. As for the positive control group, mice were treated with 0.2ml catechin (1mg/ml) daily (Yamaguchi, 1991; Hayek, 1997). All groups were allowed free access to standard rodent chow and tap water throughout the experiment. After 7 days of drug treatment, a single dosage (60 mg/kg in DMSO) of menadione was given intraperitonally to each mouse to induce hepatic oxidative damage. Animals were sacrificed 24 hours thereafter (Radjendirane, 1997).

5.2.2.2 Collection of samples

All mice were scarified 24 hours after menadione administration. Blood was collected for serum preparation while the liver was removed after perfusion with saline and 4% PFA. The liver samples were immersed in 4% PFA for fixation for 3

days. This was followed by dehydrating, processing, embedding, sliding and staining (Haematoxylin and Eosin) procedures. The histopathology of the liver was then investigated.

5.2.2.3 Marker enzyme measurement (ALT)

Plasma ALT activities was measured using Stanbio assay kit. To 1 ml of assay medium, 100 μl of plasma was added and the change in NADH concentration was detected by monitoring the decrease in absorbance at 340 nm for 120 seconds. Enzyme activity was calculated according to instructions of assay kit menu from the following equation:

$$\text{ALT activity (U/L)} = [(\Delta A \text{ at 340nm / Min}) / 0.0063] \times [1.10/0.10]$$

$$= (\Delta A \text{ at 340nm / Min}) \times 1746$$

5.2.3 Statistical analysis

All analyses were performed in triplicate. The data were recorded as mean ± S.E. One-way analysis of variance was performed by ANOVA procedures using the SPSS version 12.0.1 for Windows (SPSS Inc., US). Significant differences between means compared were determined when P-values <0.05.

5.3 Results

5.3.1 Dose-dependent effect of WZ-e on menadione hepatotoxicity

The plasma ALT activity in control group was estimated to be 12.24 ± 1.06 U/L. Menadione treatment caused a significant increase in plasma ALT activity (59.1 ±25.9) when compared with the control. The plasma ALT activity of WZ-e treated mice at low (0.42 g/kg/day), medium (1.25 g/kg/day) and high (3.75 g/kg/day) doses were found to be 50.08 ± 9.16 U/L, 31.88 ± 10.32 U/L, and 57.99 ± 20.63 U/L, respectively, following menadione treatment (Figure 5.1). As a dose of 1.25g/kg/day offered the best hepatoprotection, this dosage was chosen to carry out the subsequent Orthogonal analysis experiments even though the protective effect was not statistically significant.

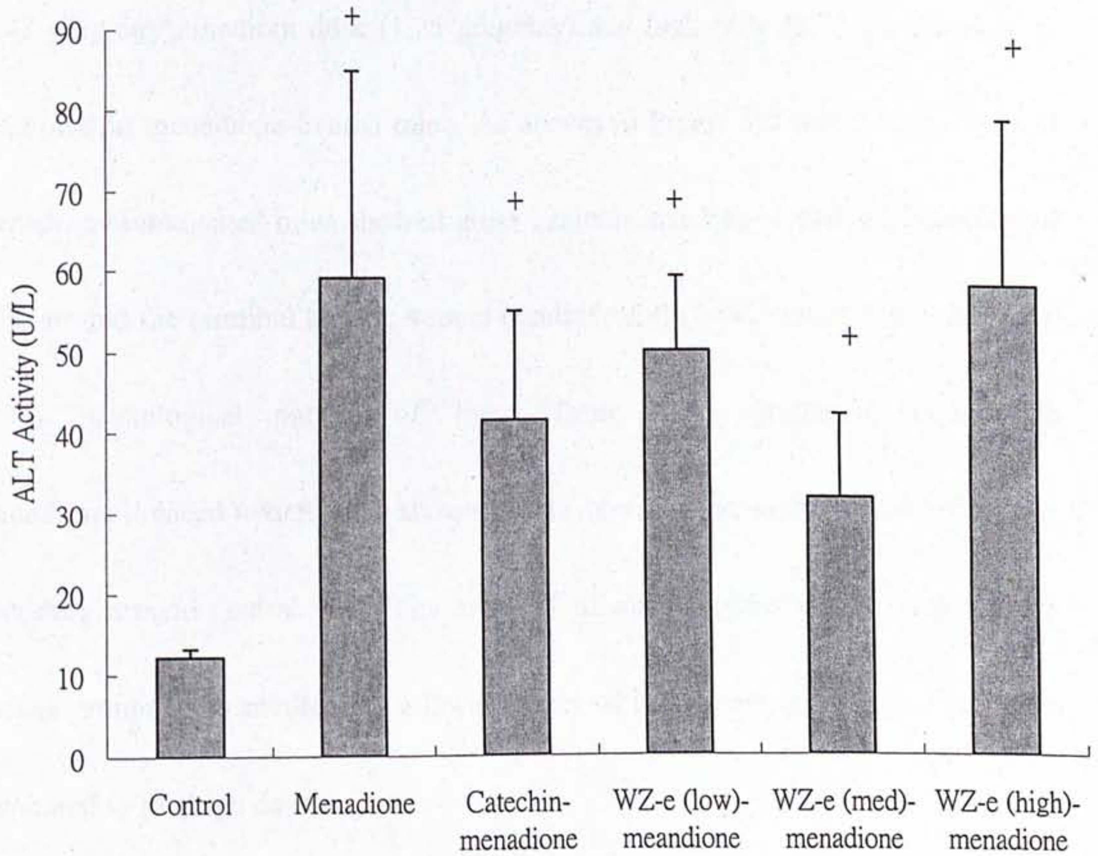


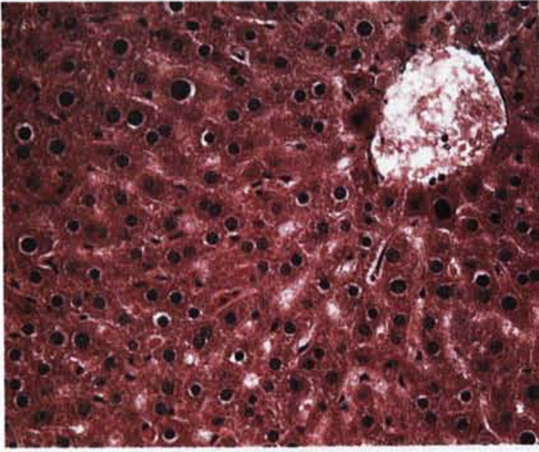
Figure 5.1 Effects of WZ-e on menadione-induced hepatotoxicity in mice. Female ICR mice were treated intragastrically with WZ-e at different doses for 7 days. Menadione was injected into mice at 24 hours after the last dose. All mice were sacrificed 24 hours after menadione injection. Plasma ALT activity was measured using a commercial assay kit. Data are expressed as mean ± S.D. (n = 5)

+ $P < 0.05$ compared with control group.

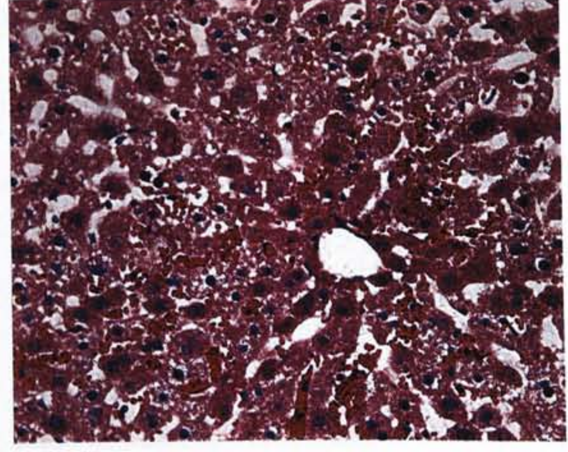
* $P < 0.05$ compared with menadione-treated control group.

5.3.2 Dose-dependent effect of WZ-e on menadione hepatotoxicity as illustrated by histopathological observations

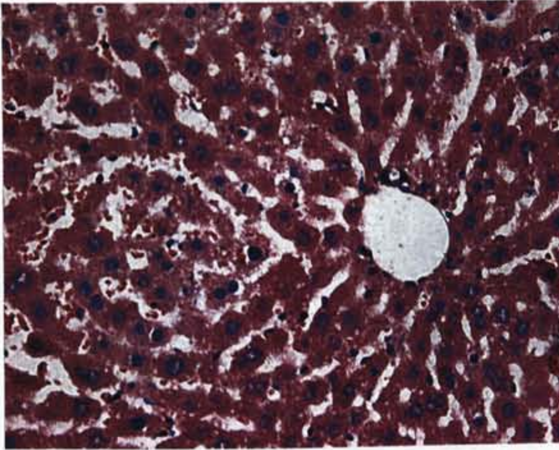
Histological changes associated with the treatment with WZ-e at low dose (0.42 g/kg/day), medium dose (1.25 g/kg/day) and high dose (3.75 g/kg/day) were examined in menadione-treated mice. As shown in Figure 5.2 and 5.3, the liver of menadione-intoxicated mice showed gross necrosis and loss of cellular boundary of cells around the terminal hepatic venule (central vein) (LefKowitch, 1989; Ruebner, 1991). Histological pattern of livers from WZ-e pretreated mice with menadione-induced toxicity also showed some degree of necrosis and loss of cellular boundary around central vein. The areas of affected regions in low and medium dosage groups were smaller and a lower degree of inflammation was observed when compared to the high dosage group.



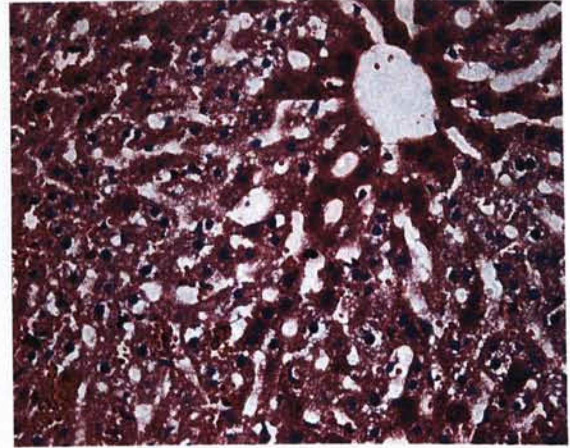
(a)



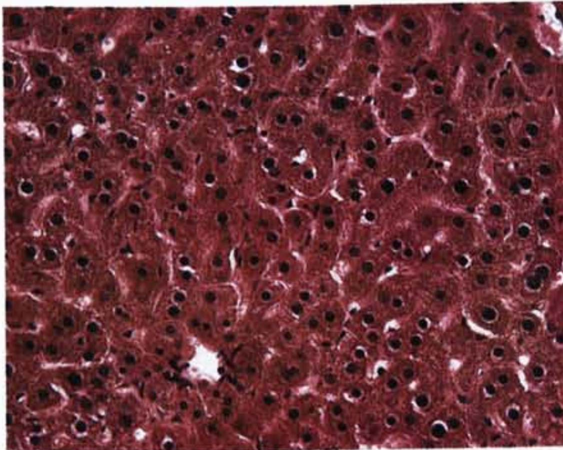
(d)



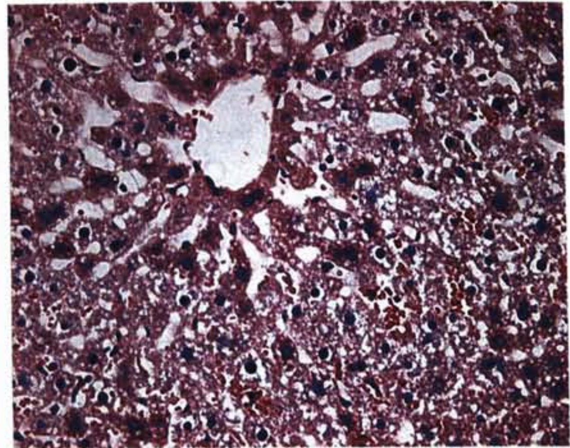
(b)



(e)



(c)



(f)

Figure 5.2 The histopathological conditions of the liver sections in menadione challenged mice pretreated with catechin and WZ-e (400x). (a) control; (b) menadione treatment; (c) catechin + menadione treatement; (d) WZ-e low dosage + menadione treatment; (e) WZ-e medium dosage + menadione treatment; (f) WZ-e + menadione treatment.

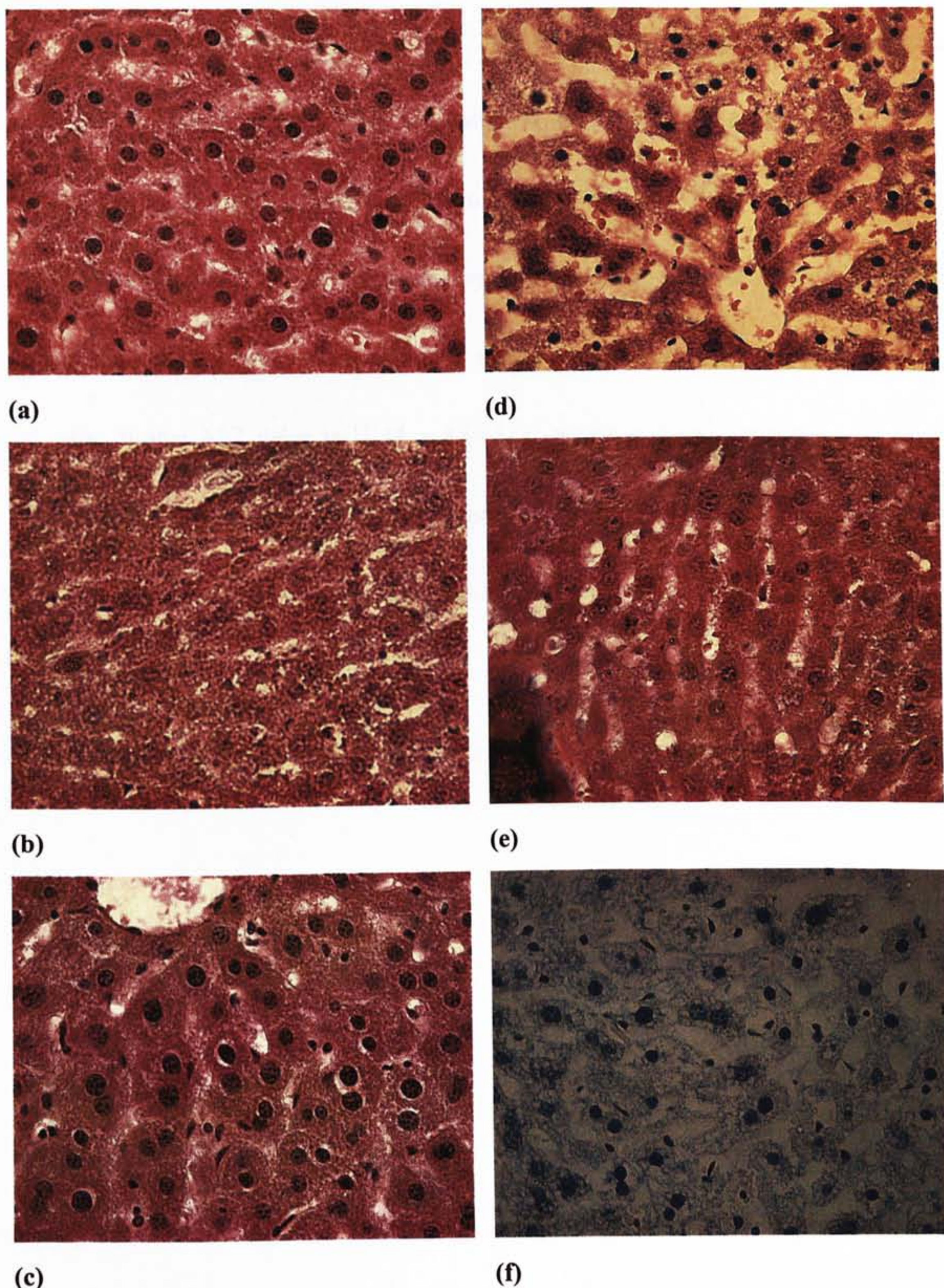


Figure 5.3 The histopathological conditions of the liver sections in menadione challenged mice pretreated with catechin and WZ-e (630x). (a) control; (b) menadione treatment; (c) catechin + menadione treatement; (d) WZ-e low dosage + menadione treatment; (e) WZ-e medium dosage + menadione treatment; (f) WZ-e + menadione treatment.

5.3.3 Analyzing the effect of WZ-e and its simplified formulae on menadione-induced hepatotoxicity by Orthogonal Analysis

Through Orthogonal analysis, formulae (2)-8, (2)-3, WZ-e, (2)-1, (2)-7, (3)-10, FS and (2)-5 were suggested by the computer program to be the experimental groups. The results of these groups are shown in Figure 5.2. For formulae (2)-5, the plasma ALT value is 11.44 ± 2.86 U/L, which is the lowest among all groups. Formula (2)-5 thus had a significant hepato-protective effect on menadione toxicity. On the other hand, it is interesting to note that formula (2)-8 displayed a high plasma ALT activity, indicating a hepatotoxic effect. However, Orthogonal analysis showed no significant difference among all 5 ingredients of WZ.

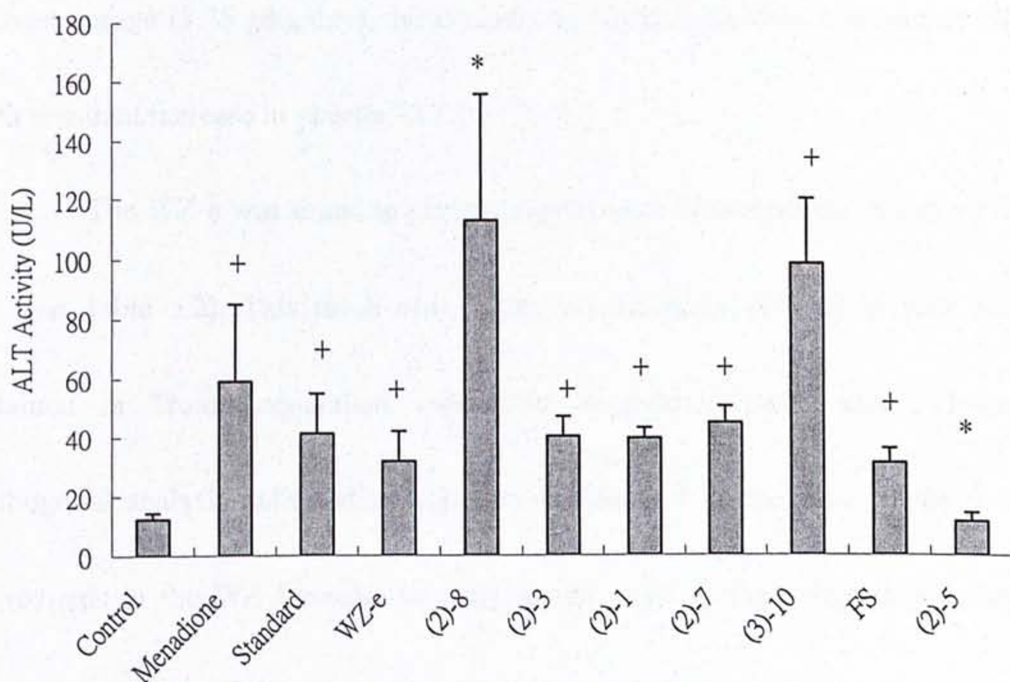


Figure 5.4 Hepatoprotective effect of WZ-e and selected simplified formulae on menadione-treated mice. Female ICR mice were treated intragastrically with WZ-e and selected simplified formulae for 7 days. Menadione was injected at 24 hours after the last dose. All mice were sacrificed 24 hours after menadione injection. Plasma ALT activity was measured using commercial assay kit. Results are expressed as mean ± S.D. (n = 5).

+ P < 0.05 compared with control group.
* P < 0.05 compared with menadione-treated control group.

5.4 Discussion

The results indicated that the protective effect of WZ-e was better at medium dose (1.25 g/kg/day) than at low dose (0.42 g/kg/day). However, at the highest dosage (3.75 g/kg/day), the extract was found to produce hepatotoxic effect, with resultant increase in plasma ALT.

The WZ-e was found to protent against menadione-induced toxicity (Table 5.1 and Table 5.2). This result observation is consistant with the *in vitro* results obtained in Trolox equivalent antioxidant capacity (TEAC) assay. However, Orthogonal analysis indicated no significant difference in the effect of the 5 main ingredients in the WZ formula. In other words, none of the 5 ingredients plays a leading role in the WZ prescription. Therefore, this finding is contradictory to the result of TEAC assay, in which FR is the most significantly important component in the formula.

The TEAC assay indicated that formulae (2)-8 and (2)-5 displayed strong antioxidant potential. In the *in vivo* test, however, (2)-8 was found to exhibit toxic effect in mice. In contrast, (2)-5 has an excellent protective effect on the menadione toxicity in mice. In this regard, SC and FR are the components in (2)-5.

It has been reported that WZ formula could produce a protective effect on oxidative damage of mDNA in aged men and aged rats (Wang, 2001). No chronic

toxicity was observed for Wu-zi-yan-zong pill being taken orally for 8 weeks in SD rats (Xu, 2001). However, the effect of WZ on the oxidative damage in mice produced by menadione has not been reported in literature. In the present study, we have demonstrated the protective effect of WZ-e on menadione induced hepatotoxicity, as evidenced by both biochemical and histological parameters.

It is worth nothing that aqueous extracts were used in all experiments, whereas the WZ formula, as recorded in Pharmacopoeia (2000), as used in pill form containing dried powders of raw plant materials. Since, aqueous extract was used in the present study, fat-soluble substances were not extracted from the herbal materials. As a result, the pharmacological effects of WZ-e used in the present study are deemed to be different from those of the traditional formulation.

Taken together, the ensemble of results, it can be indicates that WZ-e possesses an *in vivo* antioxidant activity in protecting against menadione-induced hepatotoxicity and a combination of SC and FR could be optional in producing the hepato-protective effect. However, further investigation should be performed to examine the hepatoprotective potential of the combined SC and FR formula.

Conclusion

Wu-zi-yan-zong-wan (WZ) is a tradition Chinese Medicinal formula which is indicated for kidney deficiency. In the present study, an aqueous extract of WZ was shown to possess antioxidant capacity in TEAC assay while *Fructus Rubi* (FR) was determined to be the strongest antioxidant component herb. This suggests that the simplification of WZ might optimize the antioxidant potential of WZ. On the other hand, both WZ-e and WZ-p enhanced cell viability in HepG2 cells in the presence of hydrogen peroxide. However, there is no evidence showing the relative importance of the ingredient herbs of WZ in terms of cytoprotection against H_2O_2-induced oxidation stress. *In vivo* study showed that WZ-e (1.25g/kg/day) produced hepato-protective effect against menadione-induced toxicity, as evidenced by a decrease in plasma ALT activity. However, through an Orthogonal Analysis, it was found that none of the ingredients in the WZ formula would have a critical role for the hepato-protecting action.

In summary, it is concluded that the aqueous extract of WZ possesses antioxidant activity in both *in vitro* and *in vivo* assay system. Further investigations should be carried out to delineate the role of the ingredient herbs in producing antioxidant action and the biochemical mechanism involved in the cyto-and

hepato-protection.

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