# Effects of Danshen and its Active Components on Rat CYP2E1 Expression and Metabolism of Model CYP2E1 Probe Substrate

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# Abstract

Danshen, the dried root and rhizome of *Salvia miltiorrhiza* of family *Labiatae*, is commonly used in traditional Chinese medicine (TCM) to promote blood circulation and has previously been shown to be involved in herb-drug interactions with warfarin, in which warfarin metabolism was reduced and the prothrombin time was prolonged in rats. Further studies revealed Danshen, and its tanshinones, inhibited rat and human cytochrome P450 (CYPs 1A2, 2C9 and 3A4) enzyme activities *in vitro* and *in vivo* in the rat. In this study, the effects of Danshen and its active components on the activities of CYP2E1 were investigated *in vitro* (in rat liver microsomes and pooled human liver microsomes), and *in vivo* in the rats by using a specific CYP2E1 model probe substrate. CYP2E1 is one of the members in cytochrome P450 superfamily, and along with other CYP isoforms, are responsible for the metabolism via oxidation of endogenous and exogenous compounds including drugs, environmental pollutants, dietary chemicals and carcinogens.

The effects of Danshen and its active components on the metabolism of a model CYP2E1 probe substrate chlorzoxazone (CZX) to its metabolite 6-hydroxychlorzoxazone were investigated using freshly prepared rat liver microsomes and commercially available pooled human liver microsomes. The enzyme kinetics of the probe drug chlorzoxazone (CZX) and the metabolite 6-hydroxychlorzoxazone (HCZX) were evaluated by high performance liquid chromatography (HPLC). The effects of whole Danshen extract, the ethanolic fraction and the aqueous fraction of Danshen, cryptotanshinone, dihydrotanshinone, tanshinone I and tanshinone IIA were studied. Sodium diethyldithiocarbamate, a

specific inhibitor of CYP2E1, was used as a positive control. Whole Danshen extract, the ethanolic fraction and the aqueous fraction of Danshen, cryptotanshinone, dihydrotanshinone and tanshinone I decreased 6-hydroxylation of CZX and formation of HCZX ($p < 0.05$). For rat microsomes, the K*i* values of whole Danshen extract, the aqueous fraction of Danshen, cryptotanshinone and tanshinone I were 1.46 mg/ml, 1.89 mg/ml, 87.86 μM and 78.74 μM, respectively. For human microsomes, the K*i* values of whole Danshen extract, the aqueous fraction of Danshen, cryptotanshinone and tanshinone I were 0.03 mg/ml, 0.08 mg/ml, 10.87 μM and 3.67 μM, respectively. The K*i* values of the ethanolic fraction of Danshen and dihydrotanshinone could not be determined as they had uncompetitive inhibitory modes. Whole Danshen extract and tanshinone I exerted non-competitive inhibition. The aqueous fraction of Danshen and cryptotanshinone exerted competitive inhibition. The inhibitory effect of Danshen on CYP2E1 enzyme activity was in a concentration-dependent manner except for tanshinone IIA, which did not inhibit CYP2E1 activity significantly.

For *in vivo* experiments, acute, 3-day and 14-day treatments of various concentrations of whole Danshen extract were given to group of 6 - 8 rats in two routes (i.p. or p.o.) followed by chlorzoxazone administration (i.v.). Blood samples were collected after chlorzoxazone administration for determination of plasma-concentration–time profile and pharmacokinetic parameters. Cimetidine and phenobarbitone were used as controls for CYP2E1 inhibition and induction, respectively. Acute treatment of whole Danshen extract (p.o) inhibited the formation of HCZX with altering $V_d$ of chlorzoxazone, while 3-day treatments did not produce significant effect on formation of HCZX. There was inhibition on HCZX formation after 14-day treatments.

Rat liver microsomes after 3-day and 14-day Danshen treatments were used to analyze CYP2E1 protein expression by Western Blotting. Acetone (5%) water was used as a positive control of enzyme up-regulation and dexamethasone was used as a negative control of enzyme down-regulation. There was no induction on CYP2E1 expression but it was decreased after 3-day i.p. of 400 mg/kg/day Danshen, 14-day i.p. of 400 mg/kg/day Danshen and 14-day p.o. of 200 mg/kg/day Danshen.

In conclusion, CYP2E1 activity was decreased by both the ethanolic fraction and the aqueous fraction of Danshen in rat and human *in vitro*. Dihydrotanshinone was the most potent inhibitor among the tanshinones and tanshinone IIA did not affect CYP2E1 activity. In rat *in vivo*, acute and 14-day treatments with high doses of Danshen reduced HCZX formation which is indicative of reduction in chlorzoxazone metabolism. The results in *in vivo* were in agreement with that in *in vitro*. CYP2E1 expression was reduced after high doses of 3-day and 14-day Danshen treatments and no induction was observed. However, the inhibitory effects of Danshen *in vivo* may not be caused by the reason of the *in vitro* inhibition and there was no enzyme induction after Danshen treatments.

# 論文摘要

丹參屬於唇形科鼠尾草屬植物 *Salvia miltiorrhiza* Bunge 的乾燥根，是一種用以促進血液循環的常用中藥。然而，在過往的研究當中發現在丹參和西藥華法林 (warfarin) 共用時會發生相互作用，以致減低華法林的新陳代謝循環而導致凝血酶原時間被延長。進一步的研究顯示丹參和其部份丹參酮抑制了大鼠和人類的細胞色素酶 P450 1A2, 2C9 和 3A4 (Cytochrome P450, CYPs 1A2, 2C9, 3A4) 在體內和體外的活性。在這個研究當中，CYP2E1 的探針藥物被用以研究丹參和其活性提取物在大鼠和人類體外(大鼠及人類肝微粒體) 及大鼠體內的 CYP2E1 活性上的影響。CYP2E1 屬於細胞色素酶總科之一成員，它和其他色素酶一起負責內外化合物，包括藥物、環境污染物、食物化學品和致癌物質等的氧化代謝循環。

透過用前準備的大鼠肝微粒體和匯集的人類肝微粒體以研究丹參提取物、丹參脂溶和水溶性部份、隱丹參酮、二氫丹參酮、丹參酮 I 及丹參酮 IIA 對 CYP2E1 探針藥物氯唑沙宗 (chlorzoxazone) 代謝循環的影響。高壓液相色譜儀 (HPLC) 在實驗中用以研究氯唑沙宗的藥物動力學。二乙基二硫代氨基甲酸鈉 (Sodium diethyldithiocarbamate) 為 CYP2E1 的特定抑制劑並用以作陽性控制組。丹參提取物、丹參脂溶和水溶性部份、隱丹參酮、二氫丹參酮、丹參酮 I 降低氯唑沙宗代謝循環及其代謝物的形成 ($p < 0.05$)。在大鼠肝微粒體實驗中，丹參提取物、丹參水溶性部份、隱丹參酮和丹參酮 I 的 K*i* 值分別為 1.46 mg/ml、1.89 mg/ml、87.86 μM 和 78.74 μM。在人體肝微粒體實驗中，丹參提取物、丹參水溶性部份、隱丹參酮和丹參酮 I 的 K*i* 值分別為 0.03 mg/ml、0.08 mg/ml、10.87 μM 和 3.67 μM。丹參脂溶性部份和二氫丹參酮的 K*i* 值由於其為反競爭型抑制劑，所以未能被測定。丹參提取物和丹參酮 I 的抑制型態為非競爭型，而水溶性部份

和隱丹參酮則為競爭型抑制劑。除了丹參酮 IIA 沒有顯著的抑制，丹參在 CYP2E1 酶的活性上的抑制都是濃度依賴性的。

在大鼠體內的實驗裏，6-8 隻大鼠為一組，在每組進行急性、三天及十四天的丹參腹腔注射和口服丹參處方後進行氯唑沙宗靜脈注射。所收集的血液樣本是用以分析血漿-濃度-時間剖面圖和藥物動力學參數的。甲氰咪胍 (cimetidine) 和苯巴比妥 (phenobarbitone) 分別為抑制控制組和誘導控制組。急性口服丹參處方抑制了氯唑沙宗代謝物的形成並改變了氯唑沙宗的分佈容積($V_d$)。三天的丹參處方對氯唑沙宗代謝和動力學參數沒有顯著影響，而十四天的丹參處方則抑制了氯唑沙宗代謝物的形成。

大鼠肝微粒體在經過以上三天和十四天的丹參處方後被用以以免疫印蹟技術作蛋白表達分析。丙酮水 (5%) 是作上調的陽性控制組而氟美松 (dexamethasone) 則是下調的陰性控制組。在三天和十四天的 400 mg/kg/day 丹參腹腔注射和十四天 200 mg/kg/day 口服丹參後，CYP2E1 的蛋白表達被抑制了。

總括而言，在大鼠和人體體外的 CYP2E1 的活性被丹參脂溶和水溶部份降低了。二氫丹參酮在丹參酮中是最有效的抑制劑，但丹參酮 IIA 則不影響 CYP2E1 的活性。在大鼠的體內實驗中，急性和十四天的高濃度丹參處方降低了氯唑沙宗代謝物的形成。大鼠的體內實驗結果和體外結果一致。而 CYP2E1 的蛋白表達在三天和十四天的丹參處方後降低了並沒有顯示任何誘導的程況。然而，丹參在大鼠體內的影響未必是由於體外實驗結果得出的結論所致。而丹參亦沒有對 CYP2E1 產生任何誘導作用。

# Acknowledgement

I feel an immense gratitude to my supervisor, Professor John HK Yeung, for his patient guidance and encouragement throughout these two years. His advice and support helped me re-establish my confidence once I met frustration in my research. I would also like to thank for his patience in helping me to accomplish this thesis. It is my pleasure to be his student that I have learnt a lot with joyfulness under his supervision.

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# Abbreviations

| | |
|---|---|
| AUC | Area under curve |
| β–NADPH | β-nicotinamide adenine dinucleotide phosphate in reduced form |
| $C_{initial}$ | Initial concentration |
| CL | Clearance |
| $C_{max}$ | Maximum concentration |
| CYP | Cytochrome P450 |
| CZX | Chlorzoxazone |
| DMSO | Dimethyl sulfoxide |
| DTT | Sodium diethyldithiocarbamate trihydrate |
| HCZX | 6-hydroxychlorzoxazone |
| HPLC | High performance liquid chromatography |
| K*i* | Inhibition constant |
| $K_m$ | Michaelis constant |
| INR | International Normalized Ratio |
| i.p. | Intraperitoneal |
| p.o. | Per oral |
| r.p.m. | Revolution per minute |
| SEM | Standard error of mean |
| $T_{1/2}$ | Half-life |
| TCM | Traditional Chinese medicine |
| $V_d$ | Apparent volume of distribution |
| $V_{max}$ | Maximal velocity |

# Chapter 1

# General Introduction

Traditional Chinese Medicine (TCM) has been gaining interest and acceptance in recent years worldwide as alternative treatment or adjunction treatment. About 1.5 billion people in different countries have used or are using herbal medicines (Dobos et al., 2005). TCM has been developed for more than 2000 years in China, in which different combinations of herbs are used to treat diseases. With advances of analytical methods, current interest in identifying the active ingredients in herbal medicine has substantially increased.

## 1.1 Danshen

Danshen, the dried root and rhizome of *Salvia miltiorrhiza* of family *Labiatae* (Fig. 1.1), is commonly used in traditional Chinese medicine (TCM) to promote blood circulation (Zhou et al., 2005). Danshen can improve the peripheral circulation by lowering the blood viscosity and accelerating red blood cells electrophoresis, hence it was used to treat cardiovascular diseases (Chen, 1981; Lam et al., 2006) such as coronary heart disease (Lei and Chiou, 1986), chronic renal failure (Lu and Foo, 2002) and atherosclerosis (Wu et al., 1998). It has also been suggested that Danshen has beneficial effects in patients with liver fibrosis and cirrhosis (Nan et al., 2001).

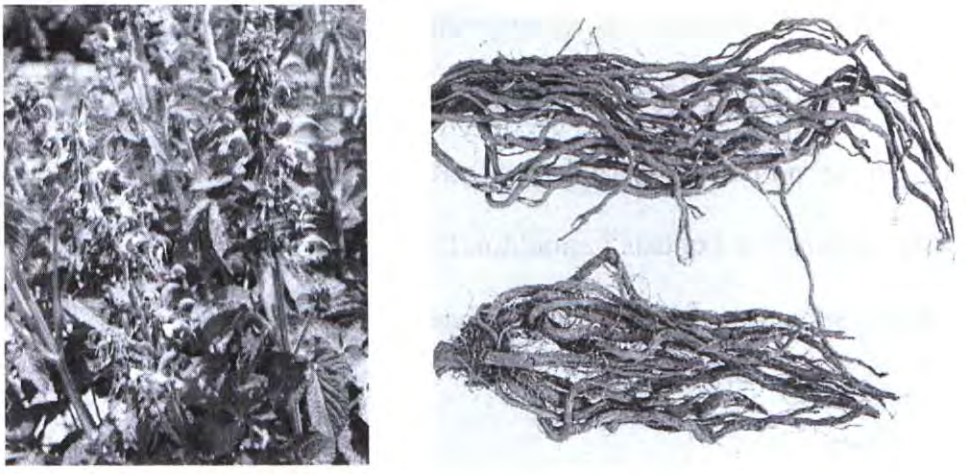


Fig. 1.1 (a) Flower of *Salvia miltiorrhiza.* (b) Dried root of *Salvia miltiorrhiza.(Zhou et al., 2005)*

The major bioactive constituents of Danshen can be classified into lipid-soluble compounds and water-soluble compounds, based on their partition when extracted in different solvents. There are more than 30 lipid-soluble compounds and more than 50 water-soluble compounds isolated from Danshen (Zhou et al., 2005).

### 1.1.1 Lipid-soluble compounds extracted from Danshen (Fig. 1.2)

Tanshinones are the major lipid-soluble compounds extracted from Danshen. Most of the tanshinones are diterpenes because of their furano-ortho-naphthaquinone or furano-para-naphthaquinone skeleton in their structures (Yang et al., 2006). Tanshinone I, tanshinone IIA, cryptotanshinone and dihydrotanshinone are the four main tanshinones that have been investigated.

#### 1.1.1.1 Tanshinone I

Tanshinone I is one of the active components in Danshen. It was recently found to have anti-cancer effects on macrophage-conditioned medium-stimulated human lung

adenocarcinoma cell line CL1-5 *in vitro* by inhibiting the migration, invasion and gelatinase activity and reducing the tumorigenesis and metastasis in CL1-5-bearing severe combined immunodeficient mice (Lee et al., 2008). It was suggested to be a phospholipide $A_2$ ($PLA_2$) inhibitor that may have contribution to the *in vivo* anti-inflammatory activity of Danshen. Tanshinone I also exerts cytotoxic effects by modulating the mutagenesis activity and inhibiting aldose reductase (Kim et al., 2002).

#### 1.1.1.2 Tanshinone IIA

Tanshinone IIA is one of the most investigated constituents of Danshen. It is the most abundant constituents of the Danshen root that can be used in improving the angina pain and the feeling of chest-tightness (Cao et al., 1996; Yin et al., 2008). It was found that sodium tanshinone IIA sulfonate reduce myocardial infarct size significantly *in vivo* (Wu et al., 1993). Tanshinone IIA has been shown to scavenge free radicals including superoxide anions and hydroxyl radicals. It is also believed to have ability to clear lipid free radicals (Cao et al., 1996) and inhibit low density lipoprotein (LDL) oxidation (Niu et al., 2000) and angiotensin II activity leading to attenuation of cardiac cell hypertrophy (Takahashi et al., 2002). The anti-oxidative effect of tanshinone IIA is even similar to vitamin E and butylated hydroxyl-toluene (BHT) (Cao et al., 1996).

#### 1.1.1.3 Cryptotanshinone

Cryptotanshinone is another most investigated components of Danshen. Its pharmacological activites include anti-inflammatory, anti-oxidative, anti-apoptosis and anti-platelet aggregation (Ng et al., 2000; Kim et al., 2002; Zhou et al., 2005; Park et al., 2007). Cryptotanshinone was reported to improve cognitive deficits in

mice, stroke and ischaemic diseases by passing through blood-brain barrier with minimum side effects (Adams et al., 2006). In addition, it was suggested that cryptotanshinone could be used as lead compounds for the development of new Alzheimer's disease drugs even though the mechanisms of its effects on Alzheimer's disease is unknown (Yu et al., 2007; Mei et al., 2009).

#### 1.1.1.4 Dihydrotanshinone

It has been reported that dihydrotanshinone has cytotoxicity function *in vitro*, inhibitory effects on mast cell degranulation, nitric oxide generation induced by lipopolysaccharide and osteoclast differentiation (Mosaddik, 2003; Choi et al., 2004; Choi and Kim, 2004; Lee et al., 2005). It was recently proved that dihydrotanshinone has relaxation effects on the 5-HT-precontracted coronary artery rings (Lam et al., 2008).

### 1.1.2 Water-soluble compounds extracted from Danshen (Fig. 1.3)

Phenolic acids, including Danshensu, salvianolic acids and protocatechuic aldehyde are the main water-soluble compounds in Danshen (Zhou et al., 2005). There were over 25 phenolic acid compounds isolated and identified from Danshen (Jiang et al., 2005). Danshensu and salvianolic acid B are the most abundant compounds (1% and 3-5% of total dried weight respectively) with reported pharmacological actions and clinical indications (Hu et al., 2005).

#### 1.1.2.1 Danshensu

Danshensu can dilate coronary artieries, inhibit platelet aggregation, improve microcirculation and protect myocardium from reperfusion injury of the ischemic heart. It was suggested that some of its mechanisms may be mediated by inhibiting

$Ca^{2+}$ aggregation in cardiac muscle cells which lead to overload of $Ca^{2+}$. It was also found to inhibit myocardial cell apoptosis and protect endothelial cells from homocsteinemia (Zhou et al., 2005).

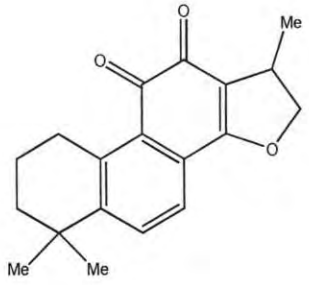
#### 1.1.2.2 Salvianolic acid B

Salvianolic acid B (lithospermate acid B) (Zhao et al., 2008) has been assigned as marker species for Danshen in Chinese Pharmacopoeia in 2005 edition (Lam et al., 2006). It possesses hepatoprotective and antifibrogenic effects which can inhibit platelet aggregation and improve cerebral blood flow (Tang et al., 2002; Lin et al., 2006). This as a result promotes the angiogenic processes and protects the skin, heart and brain from ischaemia-reperfusion-caused injury (Lay et al., 2003) by decreasing lipid peroxides, scavenging free radicals and reforming energy metabolism (Lam et al., 2006).

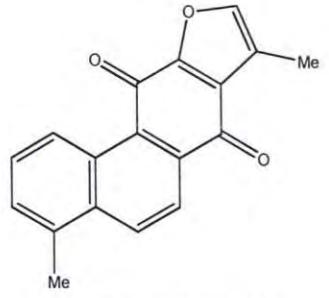
## 1.2 Drug-drug interactions

### 1.2.1 Problems associated with herbal administration

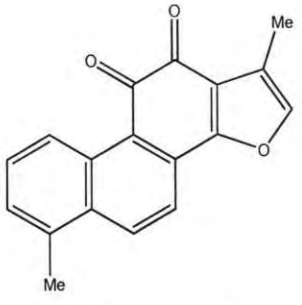
The use of complementary and alternative medicine has increased in last decade in United States. In 2004, there were about 30000 dietary supplements sold on the market. The U.S. Department of Health and Human Services revealed that about 62% of adults used complementary and alternative medicine including mind-body therapies, alternative medicine such as acupuncture, and biologically based therapies such as herbs and dietary supplements (Chavez, 2005). Due to the trend of self medication with complementary and alternative medicine especially with dietary supplements containing herbs, herb-drug interactions appear more frequently as a consequence.



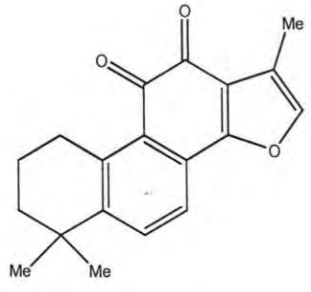

Cryptotanshinone




Dihydrotanshinone I

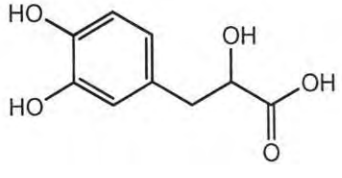



Tanshinone I

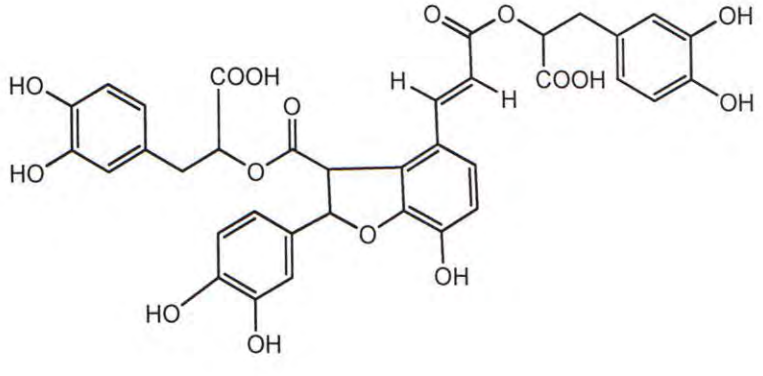



Tanshinone IIA

Fig. 1.2 Chemical structures of some important lipid-soluble compounds found in Danshen.




Danshensu




Salvianolic acid B

Fig. 1.3 Chemical structures of some water-soluble compounds found in Danshen

Between 1997 and 2002, self prescription of herbal medicine has augmented by 10% (Tindle et al., 2005). It has been estimated that about 18% of patients on medications also took herbal products or high-dose multivitamins. An estimation of 15 million adults was made to be at risk for dietary supplement-drug interactions. The increase in reports of drug interactions are also due to the disclosure of complementary and alternative medicine usage to physicians (Eisenberg et al., 1998). Natural products usually consist of complex mixture of chemical constituents. The bioactive compounds inside the natural products are often unknown as variable chemicals are contained in different parts of the plant. The process in the manufacturing of the natural product can further its complexity. The manufacturers of dietary supplements are not required to follow good manufacturing practices (GMPs) for drugs and the herbal products and/or dietary supplements are not under the regulation of FDA that FDA only takes responsibility on proving the harmfulness of a supplement instead of its safety or effectiveness without standards for herbal products (Chavez, 2005). All these factors increase the difficulties in determining the possibility of herb-drug interactions. One of the causes of herb-drug interactions is misidentification of the herbs. An example showed that some European women developed severe nephrotoxicity after taking a Chinese weight-loss product mistakenly containing *Aristolochia fangchi* instead of *Stephania tetranda*. This was caused by the confusion with the Chinese names *Guang fang ji* and *Han fang ji*, respectively (Martinez et al., 2002).

### 1.2.2 Herb-drug interactions

Adverse drug-drug interactions involving CYP system are mainly caused by induction and inhibition, in which inhibition appears to be more vital due to toxicological and clinical problems (Tanaka et al., 2007). This concern leads to

investigations on drug-drug interactions in the past two decades, especially on the herb-drug interaction studies that involve CYP enzymes. People nowadays tend to take herbal medicine as health supplementary. If they take it at the same time with western drugs, the potential adverse herb-drug interactions can occur. These herb-drug interactions happen through either pharmacodynamic or pharmacokinetic mechanisms. Adverse herb-drug interaction can be seen when herbals possessing antiplatelet effect are taken together with anticoagulant drugs that risk for bleeding will be highly increased, such as St. John's wort and warfarin. Also, herbals with function of depressing central nervous system (CNS), such as kava, should not be taken with the CNS depressant drugs. Likewise, the hepatotoxic herbal comfrey should be avoided to be administered with large and prolonged doses of paracetamol that could cause serious liver damage (Chavez, 2005). Other examples of herb-drug interactions are listed in Table 1.1.

#### 1.2.2.1 St. John's Wort-drug interactions

Drug interactions caused by St. John's Wort occur frequently, with 67 case reports of these drug interactions in 2004 alone (Mannel, 2004) as its metabolism involves main cytochrome P450 enzymes including CYP1A2 and CYP3A4. One of the examples of herb-drug interaction involving St. John's Wort is the concomitant of cyclosporine A, an immunosuppressive agent. It was reported that the concomitant of cyclosporine A and St. John's Wort would enhance the metabolism of cyclosporine A and a double dosage of cyclosporine A was required that might cause side effects (Karliova et al., 2000). Many drugs including, but not limiting to, warfarin, HIV protease inhibitor indinavir, midazolam, digoxin, simvastatin, theophylline and verapamil were also reported to have adverse drug interactions with St. John's Wort (Nebel et al., 1999; Markowitz et al., 2000; Piscitelli et al., 2000; Yue et al., 2000;

Sugimoto et al., 2001; Mueller et al., 2004; Tannergren et al., 2004). It was found that some constituents of St. John's Wort, such as hyperforin, were potent ligands for pregnane X receptor which regulates CYP3A (Watkins et al., 2003). Long term treatment of St. John's Wort could induce CYP3A4 and also CYP1A2 (Karyekar et al., 2002). As many drugs are metabolized by CYP1A2 and CYP3A4, caution should be carefully taken before taking St. John's Wort.

#### 1.2.2.2 Warfarin-herb interactions

Warfarin is a common oral anticoagulant drug that prevents blood from clotting via a mechanism interfering with the conversion of vitamin K-dependent clotting factors II (prothrombin), VII, IX and X in liver. Warfarin also suppresses the activation of vitamin K-dependent regulatory proteins C and S (Rosado, 2003; Chavez et al., 2006). It was found that metabolism of S-enantiomers of warfarin were more clinically essential than R-enantiomers due to the 5-time higher pharmacological activity of S-warfarin than R-warfarin (Hirsh et al., 1992; Greenblatt and von Moltke, 2005). Greenblatt et al. (2005) also revealed that the main enzyme for S-warfarin metabolism was CYP2C9 that effects on CYP2C9 expression and activity could directly affect the anticoagulant response. CYP1A2 and CYP3A4 were also involved in warfarin metabolism. CYP1A2 was responsible for R-warfarin and CYP3A4 was a minor metabolic pathway of S-warfarin. Some research showed that it was unlikely to interfere the herb-warfarin interactions even CYP1A2 and CYP3A4 were inhibited (Wittkowsky, 2001).

Warfarin had been reported to have drug interactions with Dong Quai (*Angelica sinensis*) that concomitant administration of Dong Quai with warfarin could lower prothrombin times (Lo et al., 1992). Also, it was reported that warfarin interacting

with large amount of garlic would inhibit platelet aggregation caused by a constituent, ajoene, of garlic oil (Lawson et al., 1992). In addition, warfarin interacted with Asian ginseng (P. ginseng) and even green tea. Asian ginseng was reported to have inhibitory effects on platelet aggregation and the conversion of fibrin from fibrinogen (Park et al., 1996). The INR of a patient had decreased after consuming a large amount of green tea during warfarin therapy (Taylor and Wilt, 1999). A study revealed the potent inhibitory activity on thrombin stimulated platelet thromboxane formation of green tea that might increase the risk of bleeding if it was used at the same time with warfarin (Ali and Afzal, 1987). One of the most important herb-warfarin interactions revealed in these two decades is Danshen-warfarin interactions.

#### 1.2.2.3 Danshen-warfarin interactions

Danshen is a prevalent herb used in mainland China for treating atherosclerosis-related disorders. Intake of Danshen can affect hemostasis such as inhibition of platelet aggregation, interference with extrinsic blood coagulation, antithrombin III-like activity and also promotion of fibrinolytic activity (Chan, 2001). Clinical reports on over-anticoagulation caused by Danshen-warfarin interaction had also been recorded. A 66-year-old man was discovered to have bleeding from gastric carcinoma with an increase in INR from 2.0 to 5.5 during a warfarin treatment for 14 months at 2-2.5 mg/day dosage. He admitted to Danshen consumption with medicated topical oil (methyl salicylate) for 3 to 5 days before admission (Tam et al., 1995; Yu et al., 1997). Another case showed that a 48-year-old woman had increased INR to 5.6 after taking Danshen and other herbs during a 4 mg/day warfarin treatment (Yu et al., 1997). Also, a 62-year-old man receiving chronic warfarin therapy at 5 mg/day dosage was reported to have decoction of Danshen daily for two

weeks before admission which caused an increase in INR from stable 3.0 to more than 8.4 (Izzat et al., 1998).

Our previous studies showed that if Danshen was administered with warfarin, the prothrombin time of warfarin would be prolonged. It has shown that 5 g/kg twice a day for 3 days of Danshen sole administration on rat did not affect prothrombin time. Therefore the interactions observed were suggested to be associated with altered warfarin metabolism by Danshen administration (Lo et al., 1992). The experiments being carried out in rats showed that the absorption rate constants, AUC, $C_{max}$ and eliminiation half–life were increased by Danshen treatment while clearance and volume of distribution of R- and S-warfarin involving CYP1A2, CYP2C9 and CYP3A4-mediated metabolism were decreased (Lo et al., 1992; Chan et al., 1995).

#### 1.2.2.4 Danshen-drug interactions

As Danshen interaction with warfarin may have involved CYP enzymes, the effects of Danshen on other drugs which are metabolized by CYP were investigated. Warfarin is metabolized by various CYP isoforms including CYP1A2, CYP2C9 and CYP3A4 and it is not certain if Danshen had affected the metabolic activity of one or more of these CYP isoforms. Previous experiments of Danshen and its active components such as cryptotanshinone, dihydrotanshinone, tanshinone I and tanshinone IIA were investigated and it was found to have inhibitory effects on CYP1A2-mediated phenacetin and caffeine metabolism, CYP2C9-mediated tolbutamide metabolism and CYP3A4-mediated testosterone metabolism *in vitro* and *in vivo* in rats, and also *in vitro* in human. Danshen and its active components inhibited the metabolisms with interfering the pharmacokinetic parameters, such as AUCs, volumes of distribution and half-lives of the probe substrates (Wang, 2007).

## 1.3 Cytochrome P450 enzymes (CYP)

Cytochrome P450 (CYP) contains a superfamily of hemethiolate isozymes which can be found on the membrane of endoplasmic reticulum (Zerilli et al., 1997). As these enzymes are bound to the membrane within a cell, a word "cyto" was put in the first part of the word; and, due to the heme pigment possessed by the enzymes with a wavelength of 450 nm when exposed to carbon monoxide, "chrome" and "P" were added into the word and "Cytochrome P450" was so named (Lynch and Price, 2007). They are important in the oxidation of endogenous and exogenous compounds including drugs, environmental pollutants, dietary chemicals and carcinogens. CYP isoenzymes (CYPs), also called as polysubstrate mono-oxygenases due to its ability to have multiple substrates by one isoenzyme, have mixed function oxidases and mono-oxygenases that a CYP uses one molecule of oxygen for forming an oxidized substrate and another molecule of oxygen is consumed to form water as a byproduct. CYPs are mostly found in liver but also appear in intestine, lungs, kidneys and brain. Drugs are transformed into ionic and more water-soluble compounds by CYPs in order to be excreted out of the body. However, this kind of biotransformation will decrease the bioavailability of the drugs (Zerilli et al., 1997; Kalra, 2007).

A typical CYP reaction:

$NADPH + H^+ + O_2 + RH \rightarrow NADP^+ + H_2O + R\text{-}OH$

where RH is parent substrate and R-OH is the oxidized product (Coon, 2005).

Up to 21 families, 20 subfamilies and 57 genes have been described in humans. CYP 1, 2 and 3 account for 70% of total hepatic CYPs content which are responsible for 94% of drugs metabolism in liver (Chang and Kam, 1999; Kalra, 2007). All genes of families 1 – 3 CYP enzymes are polymorphic that account for development of certain

adverse drug reactions (ADRs). Among the reported ADRs, about 56% of these drugs was predicted to be metabolized by polymorphic phase 1 enzymes that 86% was CYPs. It was found that CYP2C9, CYP2C19 and CYP2D6 were the dominant polymorphic forms responsible for about 40% of P450-mediated drug metabolism. Although CYP3A4 has high conservation among individuals, CYP3A activites vary over 10 fold inter-individually due to genetic origin (Ingelman-Sundberg, 2004).

### 1.3.1 Cytochrome P4502E1

CYP2E1 is one of the major cytochrome P450 enzymes that it is involved in a number of low molecular mass xenobiotics metabolism, including ethanol (Fig. 1.4 and 1.5), long chain fatty acids, paracetamol, chlorzoxazone, and the volatile anaesthetics such as enflurane, sevoflurane, methoxyflurane and isoflurane metabolisms (Cox et al., 2003; Wang et al., 2003; Caro and Cederbaum, 2004; Lee et al., 2006; Wan et al., 2006). The hydrophobic active site of CYP2E1 has been observed as the smallest compared with other CYP enzymes to complement low molecular mass substrates as decribed (Porubsky et al., 2008). Rat hepatic CYP2E1 is activated within a day after birth. The expression level of CYP2E1 remains relatively stable in the whole life but it can be induced by different substances easily (Lieber, 1999). CYP2E1 was found to comprise over 50% of total CYP mRNA in human adult liver (Bieche et al., 2007) and is responsible for 7% of hepatic metabolism (Kalra, 2007). Although mRNA amount of CYP2E1 is the highest in liver, it was shown to have no correlation between the amount of CYP2E1 mRNA transcription and CYP2E1 protein expression that is an opposite case to other CYP enzymes such as CYP3A4 (Sumida et al., 1999). Regulation of CYP2E1 expression was therefore thought to be related to pre-transcription, transcription, pre-translation, translation and post-translation (Sumida et al., 1999).

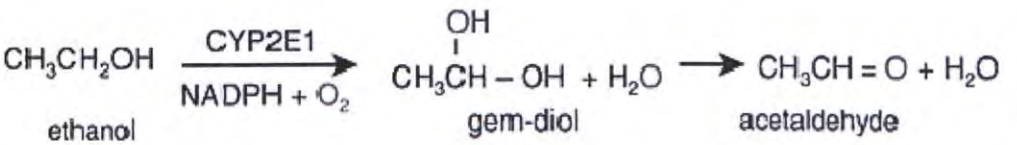



Fig. 1.4 Ethanol is metabolized by CYP2E1. The unstable intermediate gem-diol produced will decompose to produce acetaldehyde and water (Koop, 2006).

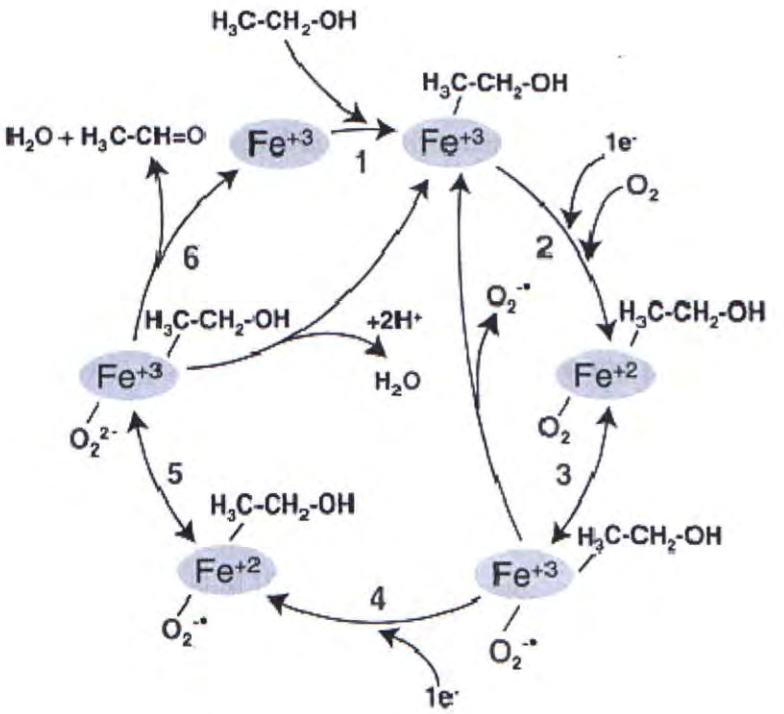



Fig. 1.5 Generation of reactive oxygen by cytochrome P450 2E1 (CYP2E1). The blue ovals with heme iron represents CYP2E1. Reactive oxygen species (ROS) is generated when CYP2E1 uses oxygen to metabolize alcohol. (1) Firstly, ethanol binds to the enzyme. (2) Then, the first electron is passed to the heme of CYP2E1 and binds to oxygen. (3) Superoxide generated is bound to the heme of CYP2E1. (4) The superoxide will break down to release free superoxide and the starting enzyme is generated. (5) If second electron is added to the enzyme, a peroxide will be produced. (6) When this product breaks down, two hydrogen atoms will be picked up to generate hydrogen peroxide. (6) The process generates reduced oxygen species (ROS) which is referred to as an "uncoupled reaction" since oxygen does not end up in substrate. If ROS remains bound, an unstable intermediate gem-diol will decompose to form acetaldehyde (Koop, 2006).

CYP2E1 is also found in other extraheptic tissues with lower expression that was thought to play role in main endogenous molecules metabolism (Bieche et al., 2007). CYP2E1*5B (RsaI) has been shown to be in association with alcoholic liver disease in Oriental population (Chinese and Japan) (Piao et al., 2003). It was reported that polymorphisms of minor allele of CYP2E1*5B happened much more frequently in Oriental population (20 – 30%) than in either Caucasians (1 – 4%) or Northern Indians (1%) (Khan et al., 2009). This polymorphism was suggested to have close relationship with the susceptibility of alcoholic liver cirrhosis occurred in an individual and the enzyme activity of CYP2E1 (Tsutsumi et al., 1994). Alcohols including ethanol, glycerol, phenol and *p*-nitrophenol are known to be substrates of CYP2E1 (Koop, 1986). Alcohol is converted into acetaldehye by CYP2E1 (Fig. 1.4) when the alcohol level is high enough to reach the saturation of alcohol dehydrogenase (ADH) (Lieber, 1999). The free radicals and peroxides produced in this step can cause oxidative stress that would damage the components of cells, such as protein, DNA and lipids (Conde de la Rosa et al., 2008) and this explains why chronic consumption of alcohol leads to liver damage.

Although hepatic CYP2E1 is only involved in 7% metabolism, it is seen as an important inducible enzyme. Ethanol is one of the inducers of CYP2E1 enzymes in rat and human that chronic treatment with ethanol can increase CYP2E1 activities and expression (Howard et al., 2001; Oneta et al., 2002). Nicotine was also found to be involved in the induction of CYP2E1 activity in African green monkeys (Lee et al., 2006). Other substrates such as paracetamol and isoniazid are among other CYP2E1 substrates that inhibit toxicological and clinical significance. Chlorzoxazone (CZX), a muscle relaxant, is the most common CYP2E1 probe substrate used to investigate the CYP2E1 activity *in vitro* and *in vivo* rat and human studies. It was shown that the

half-life of chlorzoxazone was about 1.0 – 1.2 hour with lower than 65% urinary recovery in human. The clearance of CZX was about 2 ml/min/kg (Kramer et al., 2003). Other substrates, inhibitors and inducers of CYP2E1 are listed in Table 1.2.

Table 1.2 Main substrates, inhibitors and inducers of CYP2E1 enzyme (Mizuno et al., 2000; Chavez, 2005; Kalra, 2007).

| Isoenzyme | Substrates | Inhibitors | Inducers |
|---|---|---|---|
| **CYP2E1** | Paracetamol<br>Chlorzoxazone<br>Ethanol<br>Halothane<br>Isoflurane<br>Dapsone<br>Enflurane<br>Halothane<br>Isoniazid<br>Sevoflurane<br>Theophylline<br>Venlafaxine | Disulfiram<br>Diethyldithiocarbamate | Ethanol<br>Isoniazid |

## 1.4 Aims of study

As self prescription of herbal supplements is more common nowadays, herb-drug interactions appear more frequently. Our previous studies showed the concurrent use of Danshen with warfarin could induce over-anticoagulation and consequently a series of investigations on CYP-mediated warfarin metabolisms were carried out. It was found that the clearance and volume of distribution of S- and R-warfarin were decreased after Danshen treatment in rat *in vivo* (Lo et al., 1992; Chan et al., 1995). It was also found that Danshen could affect CYP1A2, CYP2C9 and CYP3A4-mediated metabolisms of the respective specific probe substrates (Wang, 2007). CYP enzymes are important enzymes involved in most of the drug interactions. Exploring the herb-CYP interactions can improve the current understanding of potential herb-drug interactions involving the use of Danshen.

This study is mainly focused on the effects of Danshen and the tanshinones on rat and human CYP2E1 activity *in vitro* and *in vivo* in the rat. The activities of main CYP enzymes CYP1A2, CYP2C9 and CYP3A4 had been investigated with Danshen treatments, the study of CYP2E1, another crucial enzyme being responsible for ethanol metabolism, would be a logical continuation in an attempt to investigate the potential of Danshen to cause herb-drug interaction of the major CYP isoforms. CYP1A2, CYP2C9, CYP2D6, CYP3A4 and CYP2E1 are important CYP isoforms responsible for the metabolism of over 80% of drugs currently in clinical use. The protocols developed previously for CYPs 1A2, 2C9 and 3A4 and the current study of CYP2E1 can be useful as part of a screening tool to investigate the potential effects of other herbal medicines on causing herb-drug interactions.

Table 1.1 Herbal-drug interactions (Chavez, 2005).

| Herbal | Drug | Comment | Mechanism |
|---|---|---|---|
| Betel nut (*Areca catechu*) | Procyclidine | Betel nut has cholinergic activity | ↓ drug effect |
| Boldo (*Peumus boldus*) (in combination with fenugreek) | Warfarin | Boldo constituents have antiplatelet activity | ↑ bleeding risk |
| Capsicum (*Capsicum annuum*) | ACE inhibitor | Increased risk of cough | ↑ drug toxicity |
| Danshen (*Salvia miltorrhiza*) | Warfarin | Danshen decreases half-life of warfarin | ↑ drug effect |
| Dong quai (*Angelica sinensis*) | Warfarin | Dong quai contains coumarin derivatives; danshen decreases half-life of warfarin | ↑ drug effect |
| Fenugreek (*Trigonella* species) in combination with boldo | Warfarin | Fenugreek constituents have antiplatelet activity | ↑ bleeding risk |
| Fiddleheads | Warfarin | Fiddleheads contains vitamin K | ↓ drug effect |
| Garlic (*Allium sativum*) | Warfarin | Garlic has antiplatelet activity | ↑ drug effect |
| Garlic (*Allium sativum*) | Saquinavir | Induction of CYP3A4 enzymes | ↓ drug effect |
| Ginger (*Zingiber officinale*) | Phenprocoumon | Ginger can inhibit thromboxane synthetase and/or decreases platelet aggregation | ↑ bleeding risk |
| Ginkgo (*Ginkgo biloba*) | Aspirin | Ginkgo has antiplatelet activity | ↑ bleeding risk |
| Ginkgo (*Ginkgo biloba*) | Haloperidol | Ginkgo may scavenge free radicals produced by hyperdopaminergic activity | ↓ drug toxicity |
| Ginkgo (*Ginkgo biloba*) | Ibuprofen | Ginkgo has antiplatelet activity | ↑ bleeding risk |
| Ginkgo (*Ginkgo biloba*) | Omeprazole | Induction of CYP2C19 enzymes | ↓ drug effect |
| Ginkgo (*Ginkgo biloba*) | Trazodone | Ginkgo may have GABA-ergic activity | ↑ drug effect |
| Ginkgo (*Ginkgo biloba*) | Valproic acid | Contaminants of leaf/seed that may contain neurotoxins | ↑ drug toxicity |
| Ginseng, American (*Panax quinquefolius*) | Warfarin | Unknown | ↓ drug effect |
| Ginseng, Asia (*Panax ginseng*) | Phenelzine | Unknown | ↑ drug toxicity |
| Ginseng, Siberian (*Eleutherococcus senticosus*) | Digoxin | False elevation of digoxin by unknown mechanism | No effect |
| Green tea (*Camellia sinensis*) | Warfarin | Green tea contains vitamin K | ↓ drug effect |
| Kava (*Piper methysticum*) | Alprazolam | Additive CNS depressant effect | ↑ drug effect |
| Kava (*Piper methysticum*) | Levodopa | Kava may antagonize dopamine | ↓ drug effect |
| Lycium (*Lycium barbarum*) | Warfarin | Induction of CYP2C9 by Lycium | ↑ bleeding risk |

(Con't)

| | | | |
|---|---|---|---|
| Noni juice (*Morinda citrifolia*) | Warfarin | Noni juice contains vitamin K | ↓ drug effect |
| Papaya | Warfarin | Unknown | ↑ drug effect |
| Peppermint oil (*Mentha piperita*) | Nifedipine | Increases oral bioavailability | ↑ drug effect |
| Psyllium (*Plantago* species) | Carbamzepine | Psyllium decreases absorption | ↓ drug effect |
| Psyllium (*Plantago* species) | Lithium | Psyllium decreases absorption | ↓ drug effect |
| St. John's wort (*Hypericum perforatum*) | Alprazolam | Induction of CYP3A4 | ↓ drug effect |
| St. John's wort (*Hypericum perforatum*) | Amitriptyline | Induction of CYP3A4 | ↓ drug effect |
| St. John's wort (*Hypericum perforatum*) | Buspirone | Induction of serotonin syndrome | ↑ drug toxicity |
| St. John's wort (*Hypericum perforatum*) | Chlorzoxazone | Induction of CYP2C19 | ↓ drug effect |
| St. John's wort (*Hypericum perforatum*) | Cyclosporine | Induction of CYP3A4 and modulation of P-glycoprotein | ↓ drug effect |
| St. John's wort (*Hypericum perforatum*) | Digoxin | Modulation of P-glycoprotein | ↓ drug effect |
| St. John's wort (*Hypericum perforatum*) | Fenoxfenadine | Modulation of P-glycoprotein | ↓ drug effect |
| St. John's wort (*Hypericum perforatum*) | General anesthetic agents (fentanyl, propofol, sevoflurance) | Delayed emergence by unknown mechanism | ↑ drug toxicity |
| St. John's wort (*Hypericum perforatum*) | Imatinib | Induction of CYP3A4 | ↓ drug effect |
| St. John's wort (*Hypericum perforatum*) | Indinavir | Induction of CYP3A4 | ↓ drug effect |
| St. John's wort (*Hypericum perforatum*) | Irinotecan | Modulation of P-glycoprotein | ↓ drug effect |
| St. John's wort (*Hypericum perforatum*) | Loperamide | Induction of serotonin syndrome | ↑ drug toxicity |
| St. John's wort (*Hypericum perforatum*) | Mephytoin | Induction of CYP2C19 | ↓ drug effect |
| St. John's wort (*Hypericum perforatum*) | Methadone | Induction of withdrawal symptoms | ↑ drug toxicity |
| St. John's wort (*Hypericum perforatum*) | Midazolam | Induction of intestinal CYP3A4 | ↓ drug toxicity |
| St. John's wort (*Hypericum perforatum*) | Nefazodone | Induction of serotonin syndrome. | ↑ drug toxicity |
| St. John's wort (*Hypericum perforatum*) | Nevirapine | Induction of CYP3A4 and modulation of P-glycoprotein | ↓ drug effect |
| St. John's wort (*Hypericum perforatum*) | Omeprazole | Induction of both CYP3A4 and CYP2C19 | ↓ drug effect |
| St. John's wort (*Hypericum perforatum*) | Oral contraceptives | Induction of CYP3A4 and modulation of P-glycoprotein | ↓ drug effect |
| St. John's wort (*Hypericum perforatum*) | Paroxetine | Induction of serotonin syndrome | ↑ drug toxicity |

(Con't)

| | | | |
|---|---|---|---|
| St. John's wort (*Hypericum perforatum*) | Phenprocoumon | Decreased bioavailability | ↓ drug effect |
| St. John's wort (*Hypericum perforatum*) | Ritonavior | Induction of CYP3A4 and modulation of P-glycoprotein | ↓ drug effect |
| St. John's wort (*Hypericum perforatum*) | Rosiglitazone | Induction of CYP2C8 | ↓ drug effect |
| St. John's wort (*Hypericum perforatum*) | Sertraline | Induction of serotonin syndrome | ↑ drug toxicity |
| St. John's wort (*Hypericum perforatum*) | Simvastatin | Induction of CYP3A4 | ↓ drug effect |
| St. John's wort (*Hypericum perforatum*) | Tacrolimus | Induction of CYP3A4 | ↓ drug effect |
| St. John's wort (*Hypericum perforatum*) | Theophylline | Induction of CYP1A2 (only in female subjects) | ↓ drug effect |
| St. John's wort (*Hypericum perforatum*) | Trazodone | Induction of serotonin syndrome | ↑ drug toxicity |
| St. John's wort (*Hypericum perforatum*) | Venlafaxine | Induction of CYP3A4 and modulation of P-glycoprotein | ↓ drug effect |
| St. John's wort (*Hypericum perforatum*) | Verapamil | Induction of intestinal CYP3A4 | ↓ drug effect |
| St. John's wort (*Hypericum perforatum*) | Voriconazole | Induction of CYP2C19, CYP3A4, and CYP2C9 | ↓ drug effect |
| St. John's wort (*Hypericum perforatum*) | Warfarin | Induction of CYP2C9 | ↓ drug effect |
| Soy (*Glycine max*) | Warfarin | Unknown | ↓ drug effect |

# Chapter 2

# Effects of Danshen and some of its active components on chlorzoxazone metabolism in rat and human liver microsomes *in vitro*

## 2.1 Introduction

Most pharmacokinetic herb-drug interactions were related to perturbation of cytochrome P450 enzyme activity. One of these interactions includes St John's wort and midazolam, which is metabolized by CYP3A4 (Wang et al., 2001). Previous studies also showed that *Salvia miltiorrhiza* (Danshen) enhanced the anticoagulant effect of warfarin by prolonging its prothrombin time. It was suggested that CYP2C9 was involved in this metabolism and Danshen had inhibited CYP2C9 activities *in vivo* (Lo et al., 1992; Chan et al., 1995). Further interaction experiments between Danshen and other CYP enzymes such as CYP1A2, CYP2C9 and CYP3A4 have been established. The results showed that Danshen and its active components had inhibitory effects on CYP1A2, CYP2C9 and CYP3A4-mediated metabolisms (Wang, 2007; Wang et al., 2009).

Apart from CYP1A2, CYP2C9 and CYP3A4, CYP2E1 is also an important CYP isoform with its ethanol-inducible function (Zerilli et al., 1997). Although CYP2E1 is only responsible for 7% of total CYP metabolism in liver, it plays important roles in xenobiotic with low molecular weight and carcinogenic metabolisms (Guengerich et al., 1991; Koop, 1992; Kalra, 2007) such as ethanol, paracetamol, chlorzoxazone and long chain fatty acids (Wan et al., 2006). Rat CYP2E1 is found to be about 80% identical to human CYP2E1 through immune-inhibition, immune-quantitation and

structural studies (Wrighton et al., 1986; Wrighton and Stevens, 1992; Yue and Peng, 2009). Therefore, the rat CYP2E1 experimental model may be useful to investigate potential effects of CYP2E1 inhibitors or substrates.

Chlorzoxazone is probe substrate of CYP2E1 enzyme and is metabolized to 6-hydroxychlorzoxazone. Chlorzoxazone is a musculoskeletal pain killer (Ernstgard et al., 2007) and has been widely used for CYP2E1 phenotyping as the ideal probe (Bachmann and Sarver, 1996). It had been suggested that other hepatic CYP enzymes, including CYP1A1, CYP1A2 and CYP3A4, may contribute to chlorzoxazone metabolism (Carriere et al., 1993; Ono et al., 1995; Gorski et al., 1997), but this contribution was shown to be negligible (Yamazaki et al., 1995). Moreover, the use of chlorzoxazone as a probe substrate of CYP2E1 is reliable, since the inter-individual variation in CYP2E1 activity is small with chlorzoxazone (Ernstgard et al., 2007).

In this study, rat and human liver microsomes were used to investigate enzyme kinetics for Danshen-chlorzoxazone interaction. The effects of whole Danshen extract, the ethanolic fraction of Danshen, the aqueous fraction of Danshen and the tanshinones (cryptotanshinone, dihydrotanshinone, tanshinone I and tanshinone IIA) were studied. The effects on CYP2E1-mediated chlorzoxazone metabolism were compared between rat liver microsomes and human liver microsomes, and also compared to the results of previous studies on CYP1A2 and CYP3A4. Sodium diethyldithiocarbamate was chosen in the experiment as a specific inhibitor of CYP2E1 activity.

## 2.2 Materials and Methods

### 2.2.1 Chemicals and reagents

Chlorzoxazone, 6-hydroxychlorzoxazone, β-nicotinamide adenine dinucleotide phosphate in reduced form (β-NADPH), phenacetin, sodium diethyldithiocarbamate trihydrate (DTT), β-glucuronidase were from Sigma Chemical Co. (St Louis, MO, USA). Acetonitrile (HPLC Grade) and ethyl acetate (HPLC Grade) were from Labscan Analytical Sciences (Bangkok, Thailand). Ammoniumacetate and magnesiumchlorid-hexahydrat reinst were from Merck & Co., Inc. (US). Crude Danshen root was purchased from Eu Yan Sang (Hong Kong). Whole Danshen extract was kindly donated by Winsor Health Products Limited (Hong Kong). Cryptotanshinone, dihydrotanshinone, tanshinone I, tanshinone IIA, and danshensu were purchased from Chengdu Congon Bio-tech Co., Ltd. (China) and checked for purity by NMR and HPLC analysis methods.

### 2.2.2 Preparation of aqueous fraction of Danshen (Wang, 2007)

The crude Danshen root was cut into small pieces (200 g), boiled with 250 ml of water under reflux condition. The filtrate from filtered mixture was collect after 1 hour. Water (250 ml) was added to the residue to boil for another hour. The filtrate was collected and combined with the previous one followed by cooling down at room temperature. After freeze-dry process, the aqueous fraction of Danshen (35 g) was obtained with a yield of 17.5% (Wang, 2007). The constituents of the aqueous fraction of Danshen has been shown in Table 2.1.

### 2.2.3 Preparation of ethanolic fraction of Danshen (Wang, 2007)

Crude Danshen root (200 g) was boiled with 250 ml of 95% ethanol twice under

reflux. The filtrate after filtration was collected and dried by a rotator evaporator below 50 °C. A brown residue was obtained and re-dissolved in ethyl acetate. The solvent layer was collected and dried by rotary evaporator. A brown-red crystal (2 g) was obtained finally with a yield of 1% (Wang, 2007). The constituents of the ethanolic fraction of Danshen has been shown in Table 2.1.

Table 2.1 HPLC analysis of whole Danshen extract and Danshen root

| Major constituents | Whole Danshen extract | Aqueous fraction of Danshen | Ethanolic fraction of Danshen |
|---|---|---|---|
| **Crytotanshinone (μg/g)** | 45.61 ± 2.37 | 34.55 ± 0.86 | 36820 ± 559 |
| **Dihydrotanshinone (μg/g)** | 99.40 ± 2.03 | 12.69 ± 0.46 | 9287 ± 123 |
| **Tanshinone I (μg/g)** | 26.55 ± 1.55 | 10.36 ± 0.40 | 17930 ± 317 |
| **Tanshinone IIA (μg/g)** | 17.45 ± 1.15 | 22.56 ± 0.51 | 118400 ± 2900 |
| **Danshensu (mg/g)** | 28.70 ± 0.32 | 3.12 ± 0.03 | 2.68 ± 0.08 |
| **Salvianolic acid B (mg/g)** | 38.46 ± 0.73 | 37.31 ± 0.50 | 209.3 ± 5.5 |
| **Rosmarinic acid (mg/g)** | 6.05 ± 0.06 | 1.83 ± 0.02 | 14.90 ± 0.46 |
| **Protocatechuic aldehyde (mg/g)** | 2.75 ± 0.05 | 0.16 ± 0.001 | 0.64 ± 0.02 |
| **Caffeic acid (mg/g)** | Not determined | Not determined | 1.07 ± 0.04 |

### 2.2.4 Animals

Sprague-Dawley rats (male, 260 - 280 g) were used for experiments under licence from the Government of the Hong Kong SAR and endorsed by the Animal Experimentation Ethics Committee of the Chinese University of Hong Kong. All the rats were supplied by the Laboratory Animal Services Centre of the Chinese University of Hong Kong. The rats were bred and kept in animal holding room under

standard conditions with 12-h light-dark cycle. Food and water was provided to the rats *ad libitum*.

### 2.2.5 Preparation of rat liver microsomes

The rats were killed by cervical dislocation. The liver was excised and rinsed with ice-cold 0.9% NaCl (saline) solution. The liver was homogenized in 0.05 M Tris/KCl buffer at pH 7.4 with 0.25 M sucrose. The homogenate was centrifuged at 14,000 r.p.m at 4°C for 30 minutes. The supernatant was then ultra-centrifuged at 44,000 r.p.m at 4°C for one hour. Phosphate buffer (0.05 M, pH 7.4) was used to reconstitute the pellet which was then stored at -80°C.

### 2.2.6 Pooled human liver microsomes

Pooled human liver microsomes were purchased from BD Biosciences (USA) which contained liver portions of 24 donors. Protein concentration at 20 mg/ml was stored in 250 mM sucrose per 500 µl sample. Enzyme activity measured for CYP2E1 using chlorzoxazone metabolism was 2400 pmol/mg/min.

### 2.2.7 Protein Assay

BCA protein assay kit (Thermo. Co. Ltd.) was used to measure the protein concentration of the liver microsomes. Concentrations at 0, 25, 125, 250, 500, 750 and 1000 µg/ml of BSA were prepared with saline to plot the standard curve. The microsomes were diluted into 25 or 50 folds by 0.05 M Tris/KCl buffer. BCA Reagent A was mixed with BCA Reagent B (Pierce BCA Protein Assay Kit) at a ratio 1:50. each standard or diluted sample (25 µl) was added into each well of 96-well plate. Reagent A and B mixture (200 µl) was added into each well. The plate was incubated for 30 minutes at 37 °C. The absorbance was measured by Universal

Microplate Reader (Bio Tek Instrument ELx 800) at 560 nm. The protein concentration was obtained from the standard curve with absorbance against protein concentration.

### 2.2.8 Microsomal Incubation

#### 2.2.8.1 Rat liver microsomes

Rat liver microsome (1.2 mg) was incubated with incubation buffer (0.05 M phosphate buffer with 3.3 mM $MgCl_2$), 1 mM NADPH, and substrate (10, 25, 50, 100, 200 μM CZX) in a total volume of 200 μl. The incubation mixture was pre-incubated for 5 minutes at 37 °C before adding NADPH. One-hour incubation was initiated by NADPH at 37 °C thermomixer, at 800 r.p.m. The reaction was terminated by adding 200 μl ice-cold acetonitrile. The mixture was centrifuged and the supernatant was extracted with 400 μl of ethyl acetate. The organic layer was dried under a gentle stream of nitrogen. The residue was reconstituted by 60 μl methanol and 50 μl was injected into HPLC for analysis. Phenacetin (10 μl, 50 μg/ml) was used as internal standard.

#### 2.2.8.2 Human liver microsomes

Human liver microsome (0.8 mg) was incubated with incubation buffer (0.05 M phosphate buffer with 3.3 mM $MgCl_2$), 1 mM NADPH, and substrate (25, 50, 100, 150, 200 μM CZX) in a total volume of 200 μl. The incubation mixture was pre-incubated for 5 minutes at 37 °C before adding NADPH. One-hour incubation was initiated by NADPH at 37°C thermomixer, at 800 r.p.m. The reaction was terminated by adding 200 μl ice-cold acetonitrile. The mixture was centrifuged and the supernatant was extracted with 400 μl of ethyl acetate. The organic layer was dried under a gentle stream of nitrogen. The residue was reconstituted by 60 μl

ACN/$H_3PO_4$ mixture (500 µl ACN with 1.2 ml $H_3PO_4$) and 50 µl was injected into HPLC for analysis. Phenacetin (10 µl of 50 µg/ml) was used as internal standard.

### 2.2.9 Inhibition kinetics studies

#### 2.2.9.1 Rat liver microsomes

Whole Danshen extract (0.125 mg/ml – 2 mg/ml, dissolved in water), the ethanolic fraction of Danshen (5 µg/ml – 80 µg/ml, dissolved in methanol), the aqueous fraction of Danshen (0.25 mg/ml – 2 mg/ml, dissolved in water), cryptotanshinone (6.25 µM – 100 µM, dissolved in DMSO), dihydrotanshinone (1.56 µM – 50 µM, dissolved in DMSO), tanshinone I (1.56 µM – 25 µM, dissolved in DMSO) and tanshinone IIA (6.25 µM – 100 µM, dissolved in DMSO) were used for the inhibition kinetics studies, with the substrate chlorzoxazone (10 µM – 200 µM, dissolved in 400 µl of 0.5 M NaOH with 100 µl of 1 M HCl, diluted by normal saline to reach 10 mg/ml). Sodium diethyldithiocarbamate (1.56 µM – 100 µM, dissolved in water) was a selective inhibitor of CYP2E1 (Brady JF *et al.*, 1991; Newton DJ *et al.*, 1994; Ohashi Y *et al.*, 2005) which was used as a positive control for CYP2E1 inhibition.

#### 2.2.9.2 Human liver microsomes

Whole Danshen extract (0.125 mg/ml – 1 mg/ml, dissolved in water), the ethanolic fraction of Danshen (2.5 µg/ml – 20 µg/ml, dissolved in DMSO), the aqueous fraction of Danshen (0.25 mg/ml – 2 mg/ml, dissolved in water), cryptotanshinone (6.25 µM – 50 µM, dissolved in DMSO), dihydrotanshinone (0.78 µM – 6.25 µM, dissolved in DMSO), tanshinone I (6.25 µM – 50 µM, dissolved in DMSO) and tanshinone IIA (6.25 µM – 50 µM, dissolved in DMSO) were used for the inhibition kinetics studies, with the substrate chlorzoxazone (50 µM – 200 µM, dissolved in

400 µl of 0.5 M NaOH with 100 µl of 1 M HCl, diluted by normal saline to reach 10 mg/ml). Sodium diethyldithiocarbamate (25 µM – 200 µM, dissolved in water) was used as a positive control for CYP2E1 inhibition.

### 2.2.10 High performance liquid chromatography (HPLC) analysis

The HPLC system consisted of Aligent Technologies 1100 series pumping system. The eluate was analysed by passing through a column (Agilent ZORBAX Eclipse XDB-C18, 4.6 x 150 mm, 5 µm). The mobile phase containing 0.15% ammonium acetate in water with 1N HCl (pH 5.92) and 30% acetonitrile was eluated by isocratic mode at a flow rate of 0.5 ml/min and the u.v. absorbance was detected at 287 nm. A chromatogram was shown in Figure 2.1. Linear standard curves of chlorzoxazone and 6-hydroxychlorzoxazone lied between 0.1 and 10 µg. Precision tests showed a good reproducibility that the differences were less than 10%. The accuracy of the standard curve was greater than 90%.

### 2.2.11 Data analysis

Enzyme kinetics data were analyzed by GraphPad Prism 5.0 (GraphPad Software, CA, USA) designed for one-way ANOVA. A Lineweaver-Burk plot is a double reciprocal plot with various substrate concentrations plotted against velocities to obtain Michaelis constant ($K_m$) and maximal velocity ($V_{max}$) values. The inhibition constant ($K_i$) was obtained by secondary plot of Lineweaver-Burk plot. Dixon plot is a plot with concentration of inhibitor against reciprocal of velocity that is also used to determine the $K_i$ value. The $K_i$ values shown in the results were obtained from secondary plot as the values were close to those obtained from Dixon plots.

$$\text{Michaelis-Menten equation:} \quad V = V_{max}[S] / ([S] + K_m)$$

$$\text{Lineweaver-Burk plot:} \quad I/V = I/V_{max} + K_m/V_{max}[S]$$

The significant difference between control and treated groups was determined by unpaired Student's t-test by GraphPad Prism 5.0. It was determined as statistically significant when $p < 0.05$. $IC_{20}$ and $IC_{50}$ were the required concentrations of inhibitors to produce 20% and 50% inhibition of the CYP enzyme activities, respectively. The estimated $IC_{50}$ was obtained from the inhibition curve assuming the highest inhibitory effect of the inhibitor reached 100%.

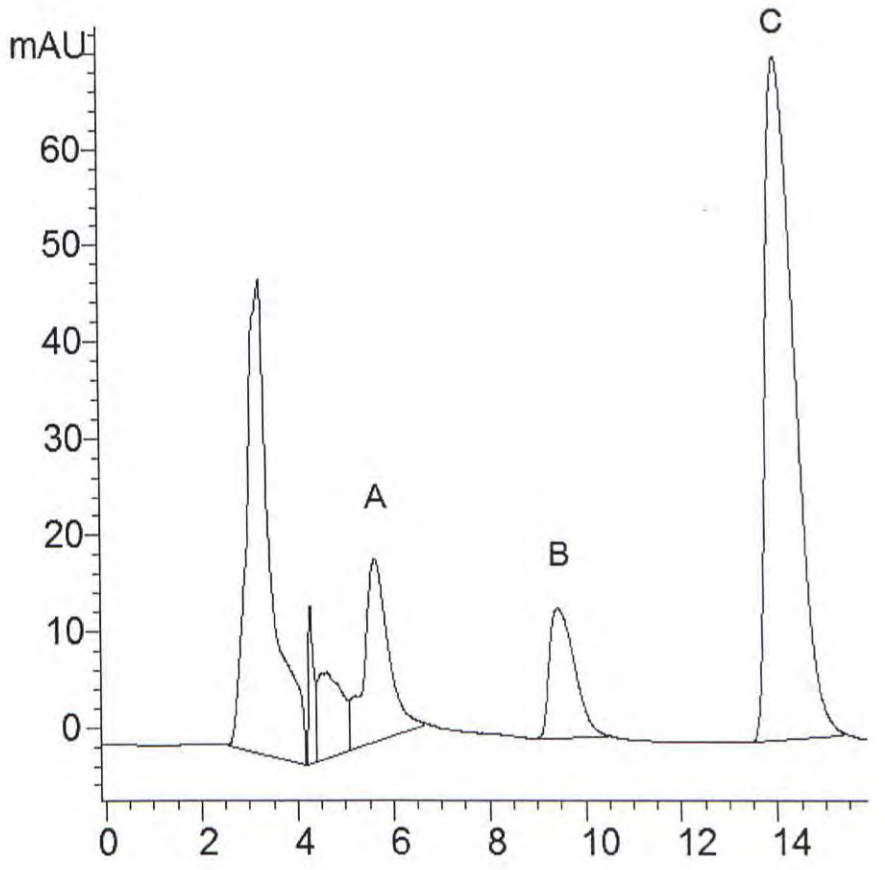



Fig. 2.1 Chromatogram of chlorzoxazone and 6-hydroxychlorzoxazone. A) 6-hydroxychlorzoxazone (5.6 min); B) Phenacetin (9.5 min); C) Chlorzoxazone (14.1 min).

## 2.3 Results

### 2.3.1 Effect of Danshen and tanshinones on rat CYP2E1 activity *in vitro*

Whole Danshen extract (0.125 mg/ml – 2 mg/ml, dissolved in water), the ethanolic fraction of Danshen (5 μg/ml – 80 μg/ml), the aqueous fraction of Danshen (0.25 mg/ml – 2 mg/ml), cryptotanshinone (6.25 μM – 100 μM), dihydrotanshinone (1.56 μM – 50 μM) and tanshinone I (1.56 μM – 25 μM) reduced the formation of 6-hydroxychlorzoxazone and decreased the 6-hydroxychlorzoxazone/chlorzoxazone ratio in a concentration-dependent manner (Fig. 2.2, 2.5, 2.8, 2.11, 2.14, 2.17). Tanshinone IIA (6.25 μM – 100 μM) did not decrease the formation of 6-hydroxychlorzoxazone and had no effect on the 6-hydroxychlorzoxazone/chlorzoxazone ratio (Fig. 2.20). The $IC_{50}$ of whole Danshen extract, the ethanolic fraction of Danshen and the aqueous fraction of Danshen were 1.88 mg/ml, 18.25 μg/ml and 1.03 mg/ml, respectively (Fig. 2.3 a, 2.6 a, 2.9 a). The $IC_{50}$ of cryptotanshinone and dihydrotanshinone were 20.47 μM and 6.71 μM (Fig. 2.12 a, 2.15 a), respectively while $IC_{20}$ of tanshinone I was 8.22 μM (Fig. 2.18 a). The $K_m$ and $V_{max}$ values were shown in Tables 2.2 and 2.3.

For whole Danshen extract and tanshinone I, Lineweaver-Burk Plots in Fig. 2.3 (b) and Fig. 2.18 (b) showed changes in slopes and y-intercept suggesting the inhibition mode was non-competitive. The respective K*i* of whole Danshen extract and tanshinone I from the secondary plot were 1.46 mg/ml and 78.74 μM (Fig. 2.4 a and Fig. 2.19 a). For the ethanolic fraction of Danshen and dihydrotanshinone, the slopes in Lineweaver-Burk Plot in Fig. 2.6 (b) and Fig. 2.15 (b) formed in parallel suggesting the inhibition mode was uncompetitive. The respective K*i* from secondary plots could not be determined because no change in primary slopes of

Lineweaver-Burk Plot (Fig. 2.7 a and Fig. 2.16 a). For the aqueous fraction of Danshen and cryptotanshinone, the slopes in Lineweaver-Burk Plots in Fig. 2.9 (b) and Fig. 2.12 (b) converged at a point on y-axis suggesting the inhibition mode was competitive. The respective K*i* of the aqueous fraction of Danshen and cryptotanshinone from secondary plots were 1.89 mg/ml and 87.86 μM (Fig. 2.10 a and 2.13 a). Tanshinone IIA did not exert inhibitory effects on CYP2E1 activities in rat liver microsomes.

Sodium diethyldithiocarbamate (1.56 μM – 100 μM) was used as a control for CYP2E1 inhibition. It reduced the formation of 6-hydroxychlorzoxazone significantly and decreased the 6-hydroxychlorzoxazone/chlorzoxazone ratio in concentration-dependent manner (Fig. 2.21). The $IC_{50}$ was 13.21 μM (Fig. 2.22 a). Lineweaver-Burk Plot in Fig. 2.22 (b) showed changes in slope and y-intercept that the inhibition mode of sodium diethyldithiocarbamate was proposed as non-competitive. The respective K*i* from secondary plot was 289.30 μM (Fig. 2.23 a).

Table 2.2 Enzyme kinetic parameters of the effects of whole Danshen extract, the ethanolic fraction of Danshen and the aqueous fraction of Danshen on the formation of 6-hydroxychlorzoxazone. Values are mean ± SE (n = 6).

| Whole Danshen Extract | | | | | | |
|---|---|---|---|---|---|---|
| **Enzyme Kinetic Parameters** | **Control** | **0.125 mg/ml** | **0.25 mg/ml** | **0.5 mg/ml** | **1 mg/ml** | **2 mg/ml** |
| **Vmax (nmol/mg protein/min)** | 15.83 ± 6.43 | 8.24 ± 0.99 | 6.40 ± 0.73 | 6.69 ± 1.50 | 3.57 ± 0.33 | 2.51 ± 0.85 |
| **Km (mg/ml)** | 360.00 ± 206.70 | 158.00 ± 34.02 | 114.40 ± 26.06 | 149.50 ± 61.51 | 88.88 ± 18.19 | 207.10 ± 115.90 |
| **Km/Vmax** | 22.74 | 19.17 | 17.88 | 22.35 | 24.90 | 82.50 |
| **The ethanolic fraction of Danshen** | | | | | | |
| **Enzyme Kinetic Parameters** | **Control** | **5 μg/ml** | **10 μg/ml** | **20 μg/ml** | **40 μg/ml** | **80 μg/ml** |
| **Vmax (nmol/mg protein/min)** | 83.16 ± 170.30 | 8.58 ± 1.76 | 9.47 ± 1.53 | 4.66 ± 0.69 | 2.05 ± 0.19 | 0.98 ± 0.070 |
| **Km (μg/ml)** | 3622.00 ± 7763.00 | 308.30 ± 92.95 | 377.90 ± 85.06 | 213.40 ± 51.40 | 100.60 ± 19.49 | 40.30 ± 8.32 |
| **Km/Vmax** | 43.55 | 35.92 | 39.89 | 45.79 | 49.03 | 41.27 |
| **The aqueous fraction of Danshen** | | | | | | |
| **Enzyme Kinetic Parameters** | **Control** | **0.25 mg/ml** | **0.5 mg/ml** | **1 mg/ml** | **2 mg/ml** | |
| **Vmax (nmol/mg protein/min)** | 369.10 ± 1773.00 | 12.74 ± 2.59 | 50.55 ± 38.09 | 2.91e+14 ± 2.98e+27 | 8.39 ± 10.04 | |
| **Km (mg/ml)** | 8362.00 ± 40990.00 | 244.30 ± 78.69 | 1269.00 ± 1073.00 | 1.28e+16 ± 1.31e+29 | 696.90 ± 1028.00 | |
| **Km/Vmax** | 22.66 | 19.18 | 25.10 | 43.93 | 83.06 | |

Table 2.3 Enzyme kinetic parameters of the effects of cryptotanshinone, dihydrotanshinone, tanshinone I and sodium diethyldithiocarbamate on the formation of 6-hydroxychlorzoxazone. Values are mean ± SE (n = 6).

| Cryptotanshinone | | | | | | |
|---|---|---|---|---|---|---|
| **Enzyme Kinetic Parameters** | **Control** | **6.25 μM** | **12.5 μM** | **25 μM** | **50 μM** | **100 μM** |
| **Vmax (nmol/mg protein/min)** | 118.00 ± 629.90 | 35.96 ± 46.17 | 24.41 ± 32.02 | 3.78e+13 ± 9.30e+25 | 3.57e+14 ± 9.04e+27 | 21.61 ± 32.80 |
| **Km (μM)** | 5431.00 ± 29913.00 | 1613.00 ± 2285.00 | 1330.00 ± 1962.00 | 2.77e+15 ± 6.82e+27 | 2.86e+16 ± 7.23e+29 | 1936.00 ±3205.00 |
| **Km/Vmax** | 46.03 | 44.86 | 54.49 | 73.33 | 80.02 | 89.59 |

| Dihydrotanshinone | | | | | | | |
|---|---|---|---|---|---|---|---|
| **Enzyme Kinetic Parameters** | **Control** | **1.56 μM** | **3.125 μM** | **6.25 μM** | **12.5 μM** | **25 μM** | **50 μM** |
| **Vmax (nmol/mg protein/min)** | 7.11 ± 1.46 | 9.35 ± 4.48 | 11.46 ± 7.92 | 3.10 ± 0.89 | 1.08 ± 0.16 | 21.61 ± 0.05 | 0.44 ± 0.03 |
| **Km (μM)** | 250.40 ± 80.68 | 523.70 ± 327.10 | 781.10 ± 657.90 | 212.50 ± 101.30 | 55.30 ± 22.93 | 20.27 ± 4.87 | 7.01 ± 3.14 |
| **Km/Vmax** | 35.24 | 56.02 | 68.16 | 68.61 | 51.30 | 28.78 | 16.06 |

| Tanshinone I | | | | | | |
|---|---|---|---|---|---|---|
| **Enzyme Kinetic Parameters** | **Control** | **1.56 μM** | **3.125 μM** | **6.25 μM** | **12.5 μM** | **25 μM** |
| **Vmax (nmol/mg protein/min)** | 9.32 ± 1.73 | 9.01 ± 1.27 | 6.08 ± 1.38 | 4.75 ± 1.29 | 1.86 ± 0.14 | 2.89 ± 0.53 |
| **Km (μM)** | 269.80 ± 76.50 | 349.10 ± 71.31 | 276.80 ± 96.30 | 238.10 ± 103.20 | 57.15 ± 10.37 | 131.40 ± 45.46 |
| **Km/Vmax** | 28.94 | 38.76 | 45.53 | 50.12 | 30.73 | 45.45 |

| Sodium Diethyldithiocarbamate | | | | | | |
|---|---|---|---|---|---|---|
| **Enzyme Kinetic Parameters** | **Control** | **1.56 μM** | **6.25 μM** | **25 μM** | **50 μM** | **100 μM** |
| **Vmax (nmol/mg protein/min)** | 14.89 ± 1.99 | 17.23 ± 2.65 | 18.96 ± 4.31 | 21.34 ± 7.99 | 31.94 ± 28.05 | 18.33 ± 7.46 |
| **Km (μM)** | 238.70 ± 50.18 | 276.80 ± 62.74 | 335.40 ± 107.40 | 499.50 ± 245.60 | 959.00 ± 987.70 | 557.80 ± 292.40 |
| **Km/Vmax** | 16.03 | 16.07 | 17.69 | 23.41 | 30.03 | 30.43 |

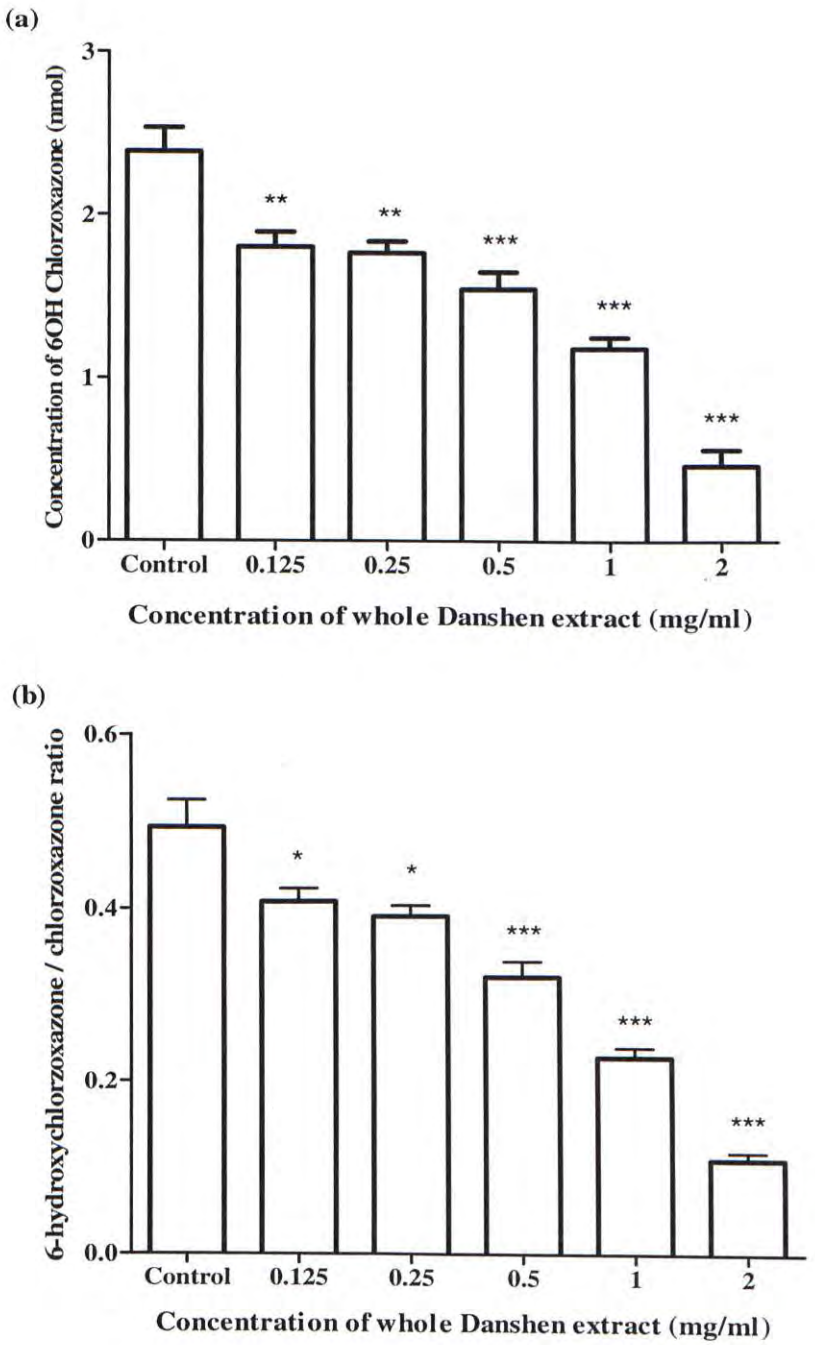



Fig. 2.2 Effects of whole Danshen extract (0.125 mg/ml – 2 mg/ml) on (a) formation of 6-hydroxychlorzoxazone and (b) ratio of 6-hydroxychlorzoxazone to chlorzoxazone in rat microsomes. Values are mean ± SEM (n = 6). *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ when compared with controls.

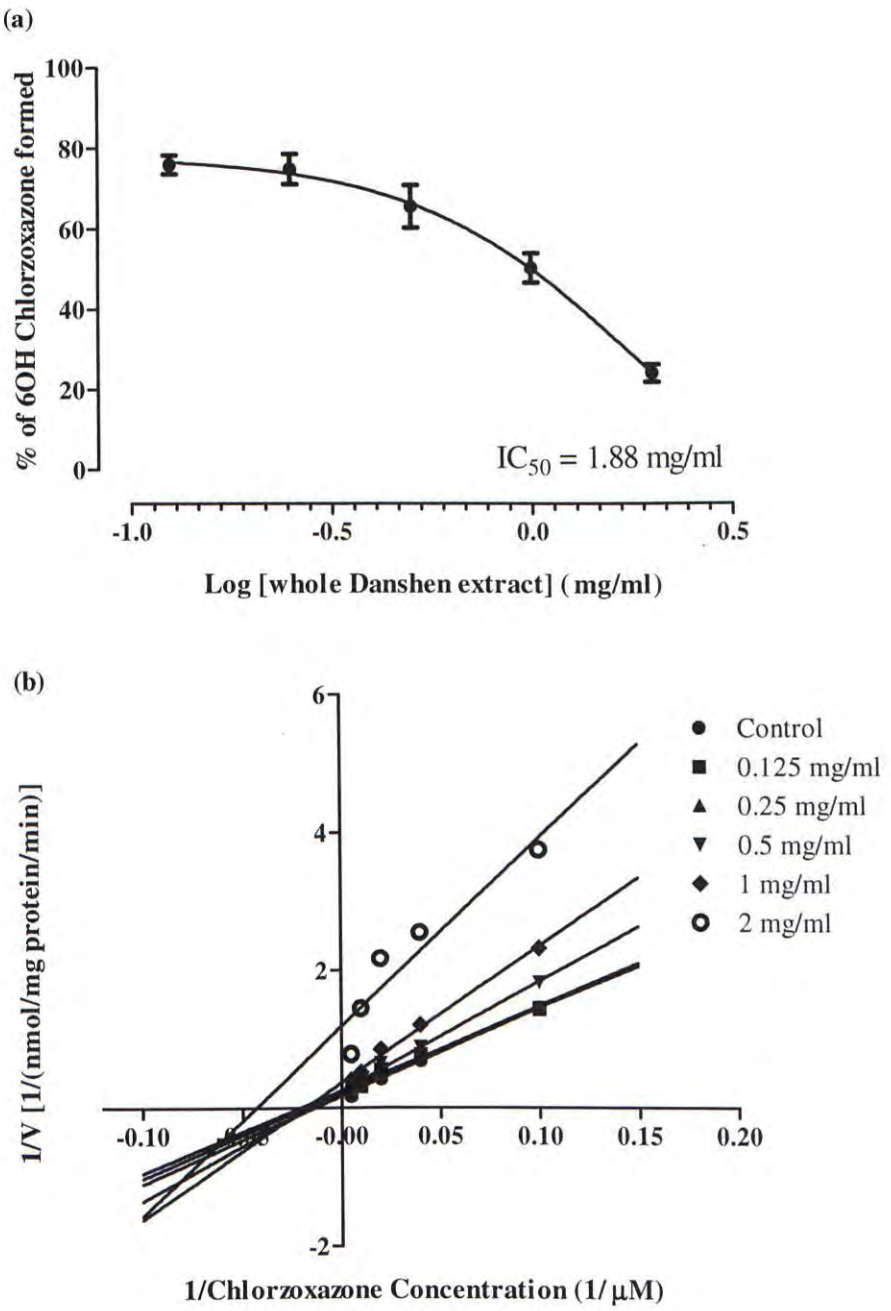



Fig. 2.3 (a) Inhibition curve showing effects of whole Danshen extract (0.125 mg/ml – 2 mg/ml) on formation of 6-hydroxychlorzoxazone in rat liver microsomes. Values are mean ± SEM (n = 6). (b) Lineweaver-Burk Plot for the inhibition of CYP2E1-mediated chlorzoxazone hydroxylation by whole Danshen extract (0.125 mg/ml – 2 mg/ml). Values are mean of 6 determinations.

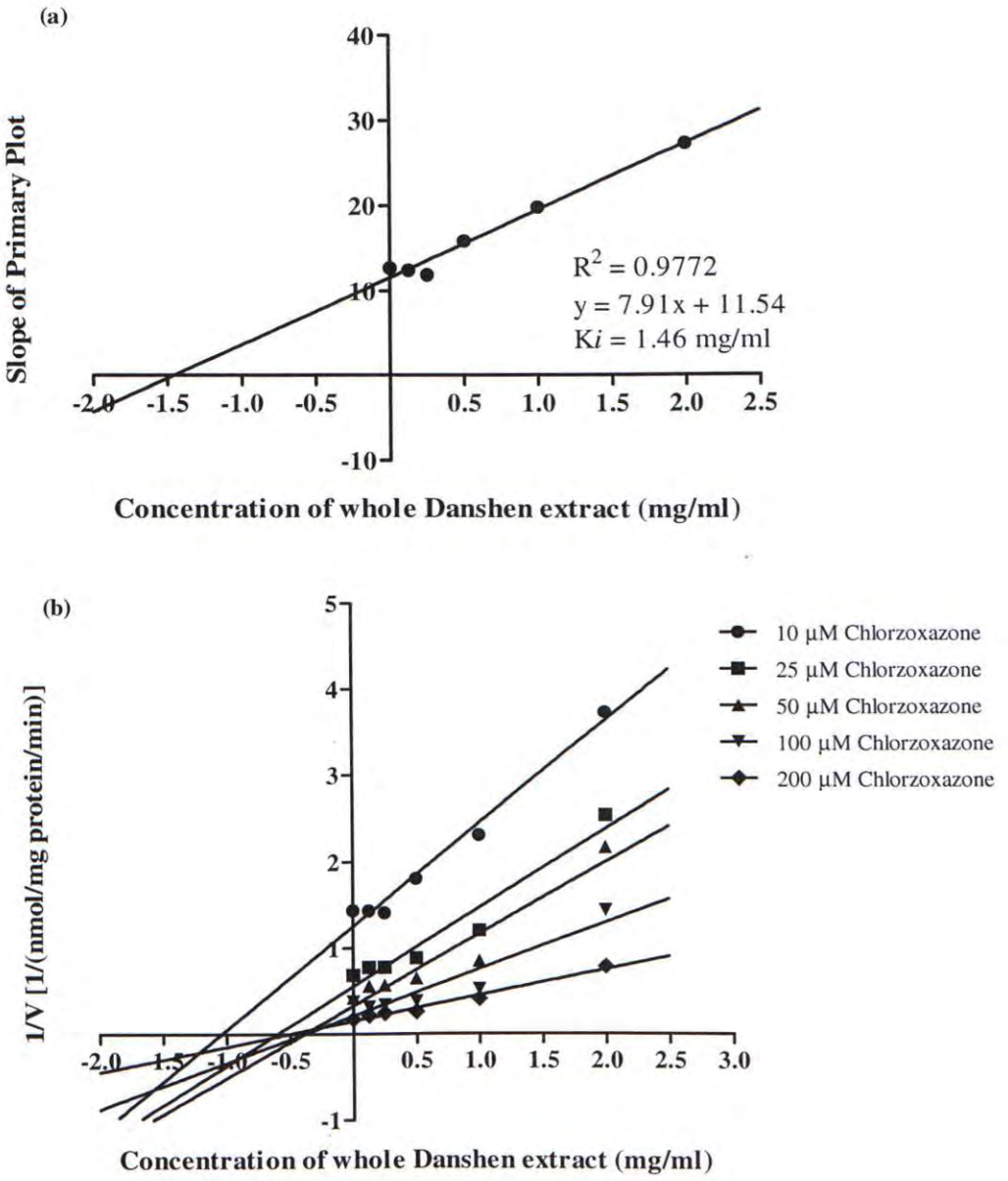



Fig. 2.4 (a) Secondary plot of Lineweaver-Burk Plot and (b) Dixon Plot show inhibitory effects of whole Danshen extract (0.125 mg/ml – 2 mg/ml) on CYP2E1-mediated chlorzoxazone hydroxylation. Values are mean of 6 determinations.

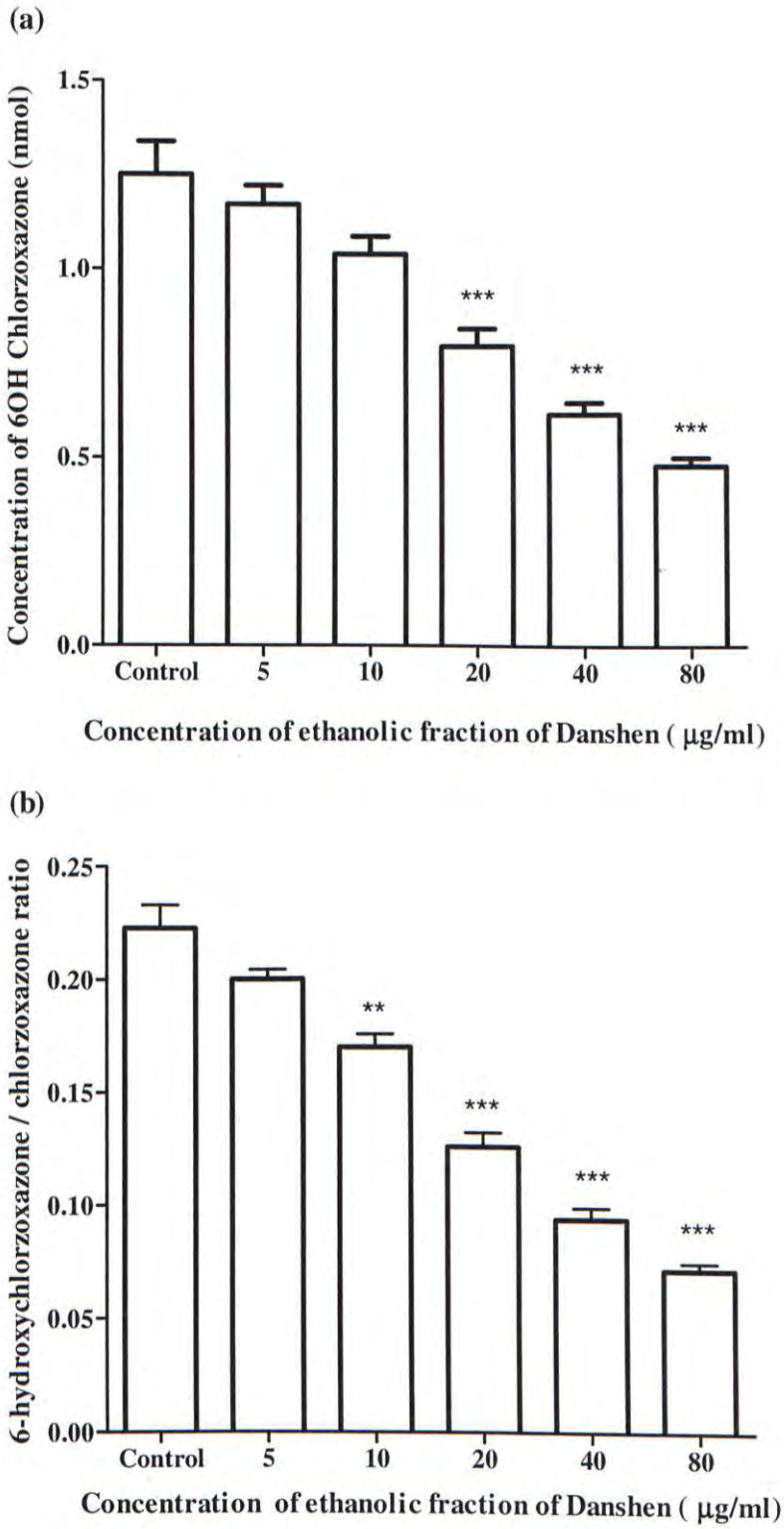



Fig. 2.5 Effects of ethanolic fraction of Danshen (5 μg/ml – 80 μg/ml) on (a) formation of 6-hydroxychlorzoxazone and (b) ratio of 6-hydroxychlorzoxazone to chlorzoxazone in rat microsomes. Values are mean ± SEM (n = 6). $^{**}p < 0.01$ and $^{***}p < 0.001$ when compared with controls.

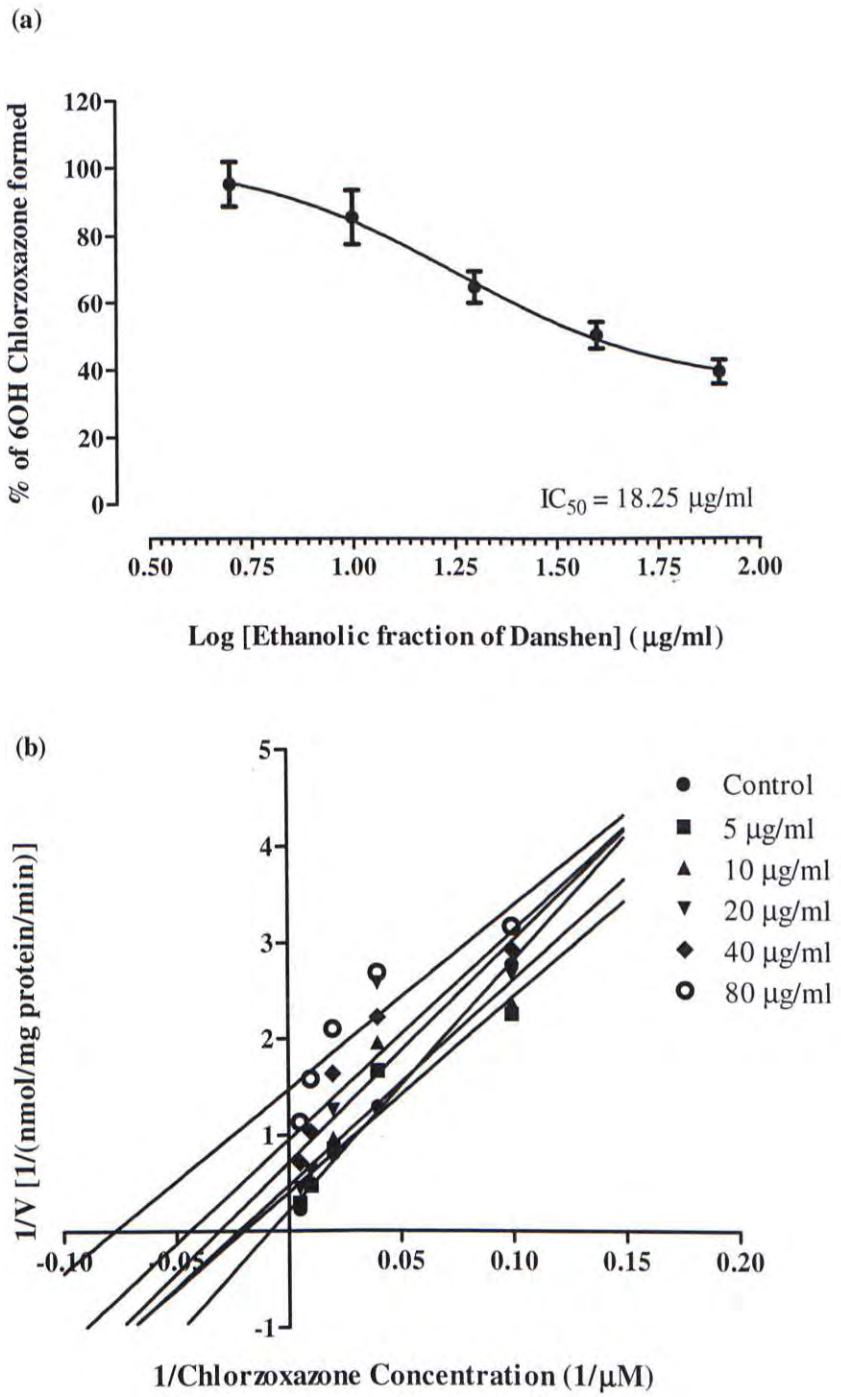



Fig. 2.6 (a) Inhibition curve showing effects of ethanolic fraction of Danshen (5 µg/ml – 80 µg/ml) on formation of 6-hydroxychlorzoxazone in rat liver microsomes. Values are mean ± SEM (n = 6). (b) Lineweaver-Burk Plot for the inhibition of CYP2E1-mediated chlorzoxazone hydroxylation by ethanolic fraction of Danshen (5 µg/ml – 80 µg/ml). Values are mean of 6 determinations.

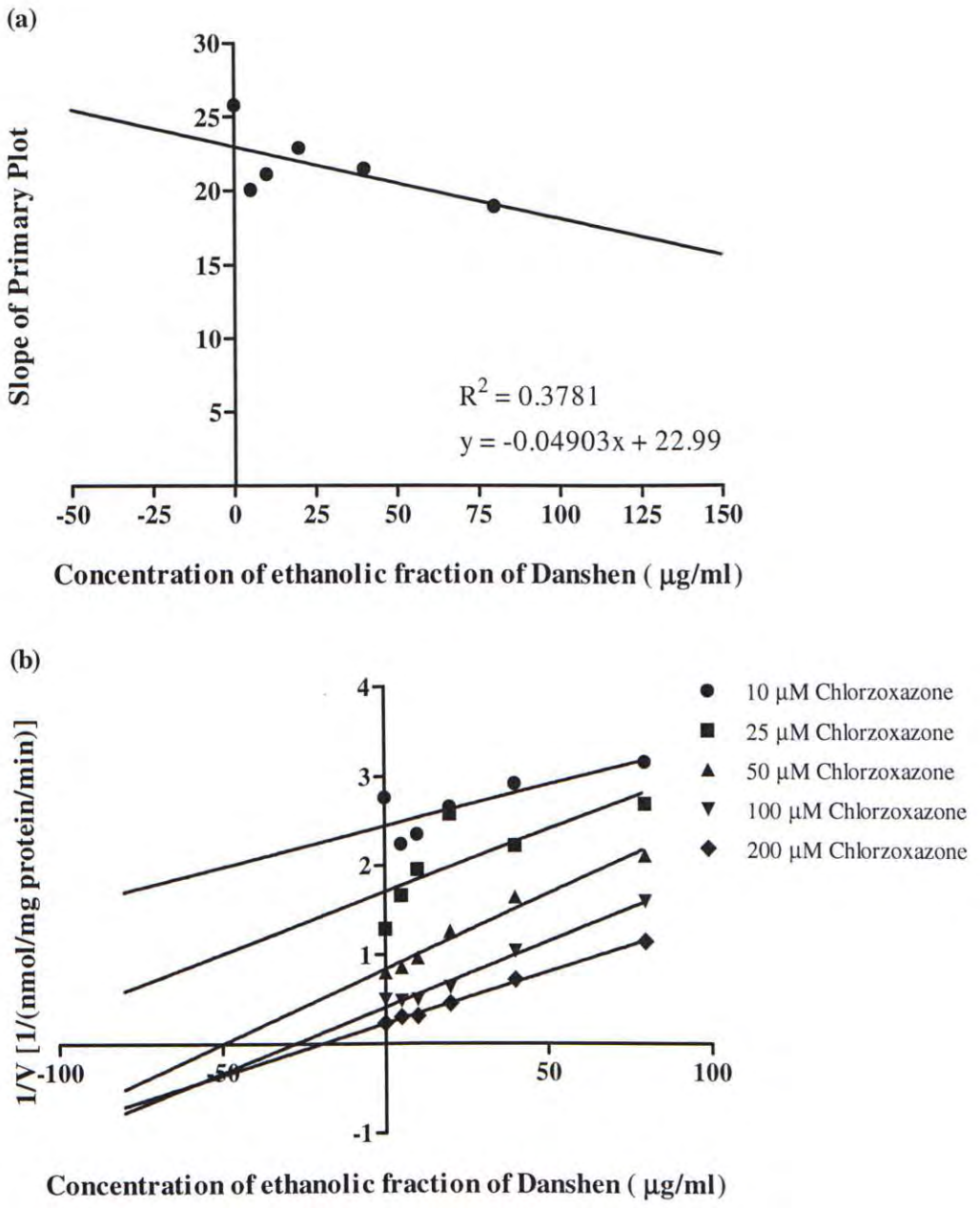



Fig. 2.7 (a) Secondary plot of Lineweaver-Burk Plot and (b) Dixon Plot show inhibitory effects of ethanolic fraction of Danshen (5 μg/ml – 80 μg/ml) on CYP2E1-mediated chlorzoxazone hydroxylation. Values are mean of 6 determinations.

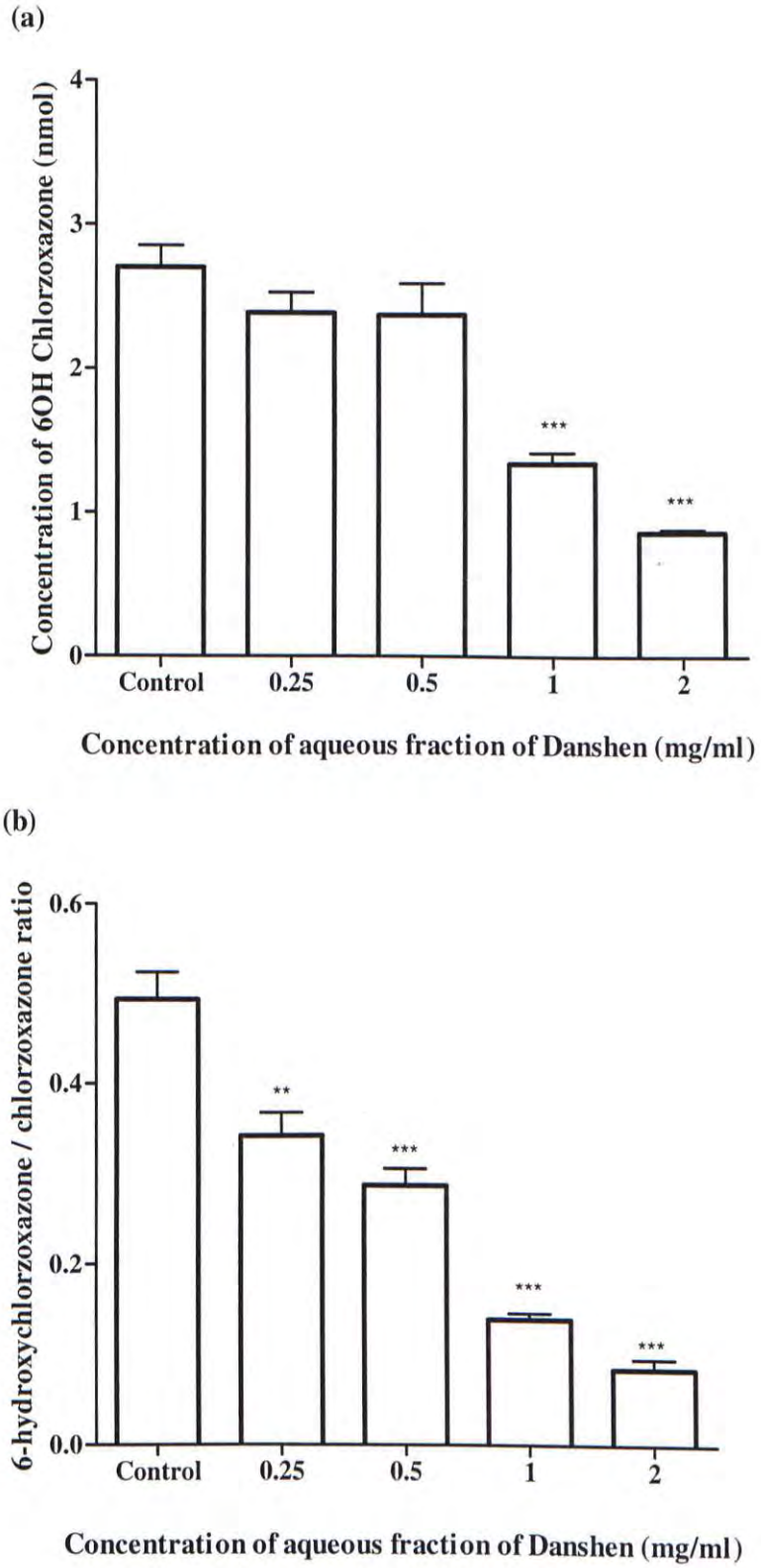



Fig. 2.8 Effects of aqueous fraction of Danshen (0.25 mg/ml – 2 mg/ml) on (a) formation of 6-hydroxychlorzoxazone and (b) ratio of 6-hydroxychlorzoxazone to chlorzoxazone in rat microsomes. Values are mean ± SEM (n = 6). $^{**}p < 0.01$ and $^{***}p < 0.001$ when compared with controls.

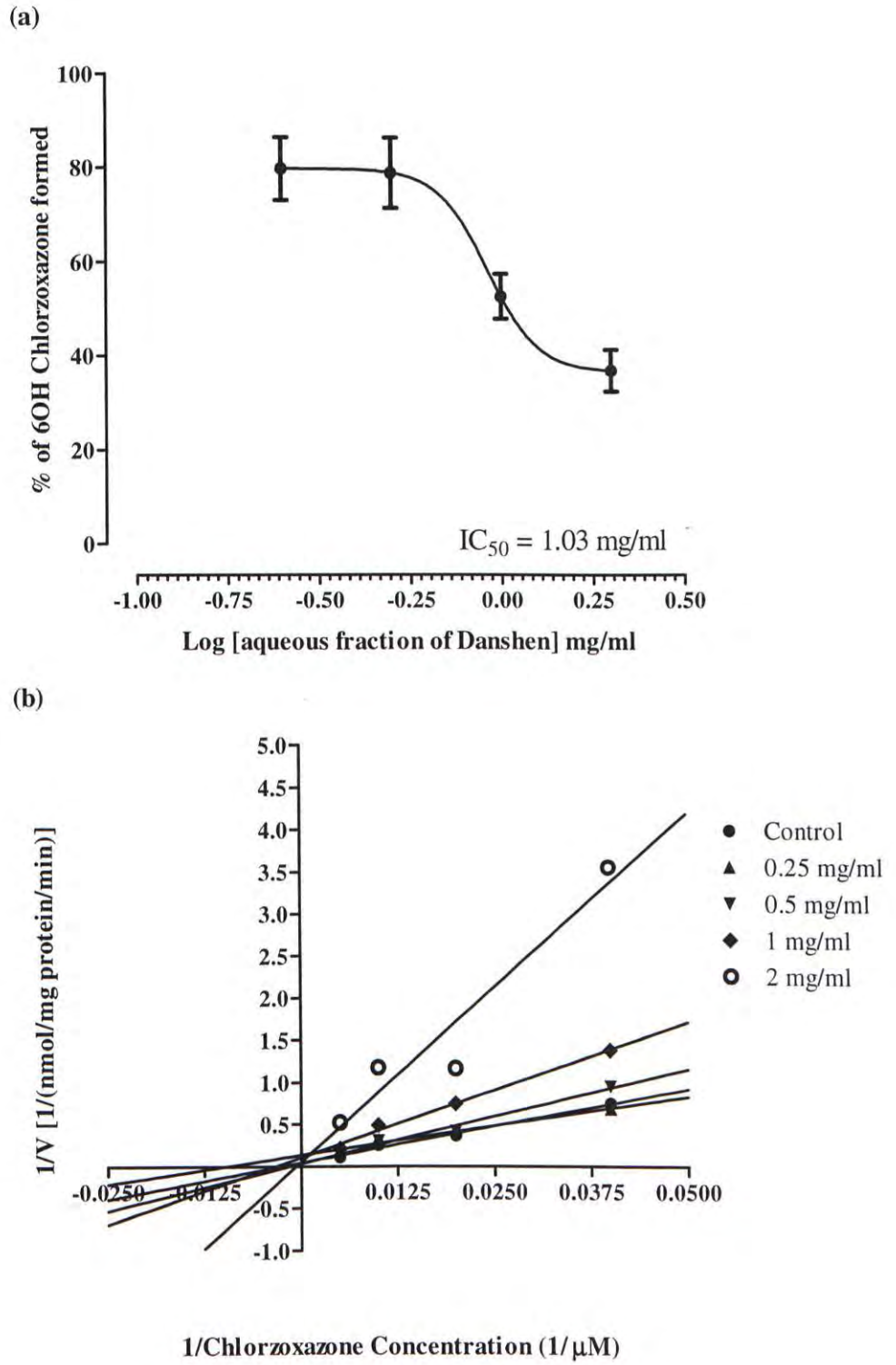
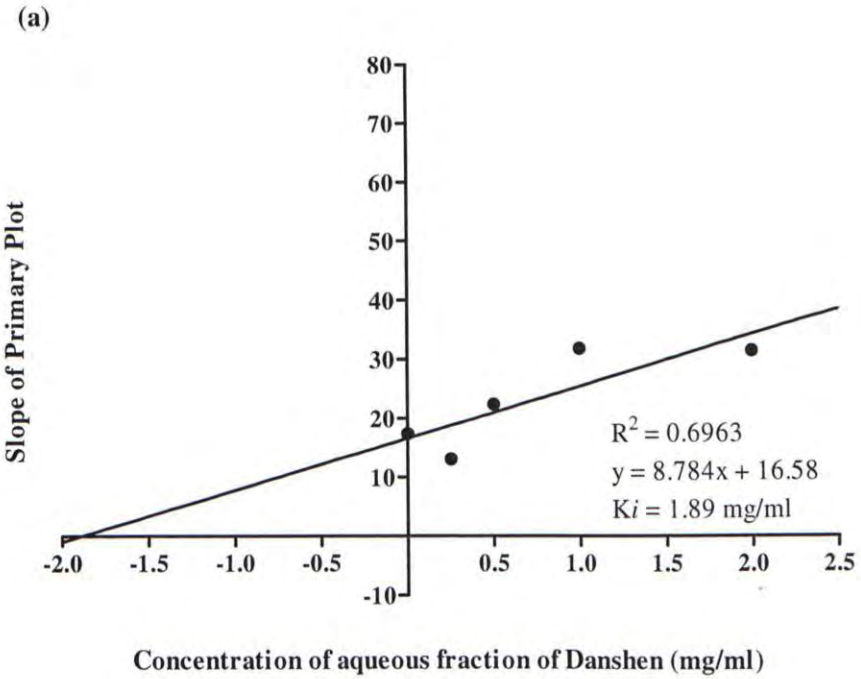



Fig. 2.9 (a) Inhibition curve showing effects of aqueous fraction of Danshen (0.25 mg/ml – 2 mg/ml) on formation of 6-hydroxychlorzoxazone in rat liver microsomes. Values are mean ± SEM (n = 6). (b) Lineweaver-Burk Plot for the inhibition of CYP2E1-mediated chlorzoxazone hydroxylation by aqueous fraction of Danshen (0.25 mg/ml – 2 mg/ml) in rat liver microsomes. Values are mean of 6 determinations.



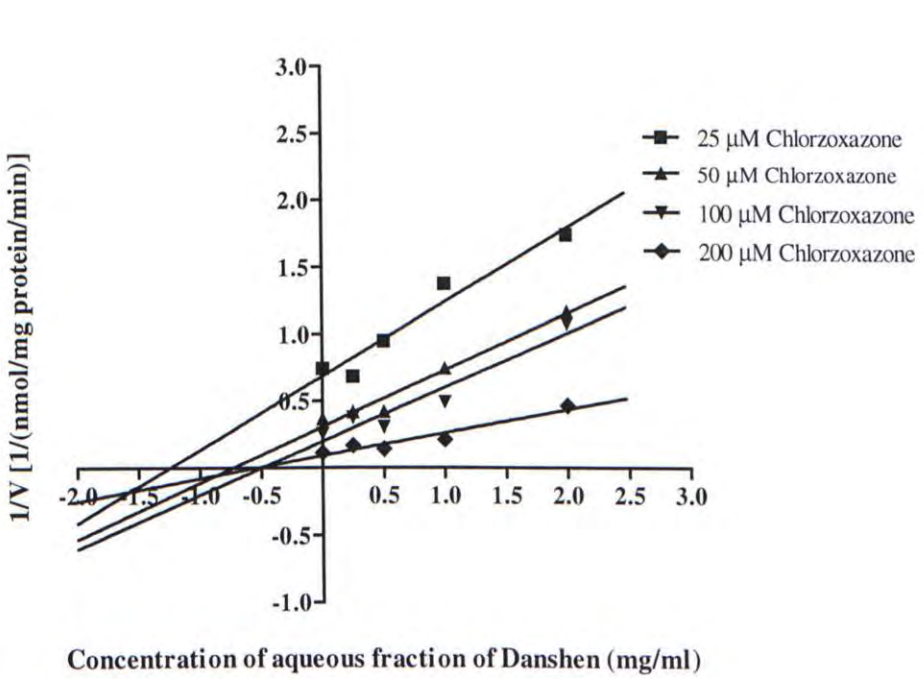




Fig. 2.10 (a) Secondary plot of Lineweaver-Burk Plot and (b) Dixon Plot show inhibitory effects of aqueous fraction of Danshen (0.25 mg/ml – 2 mg/ml) on CYP2E1-mediated chlorzoxazone hydroxylation in rat liver microsomes. Values are mean of 6 determinations.

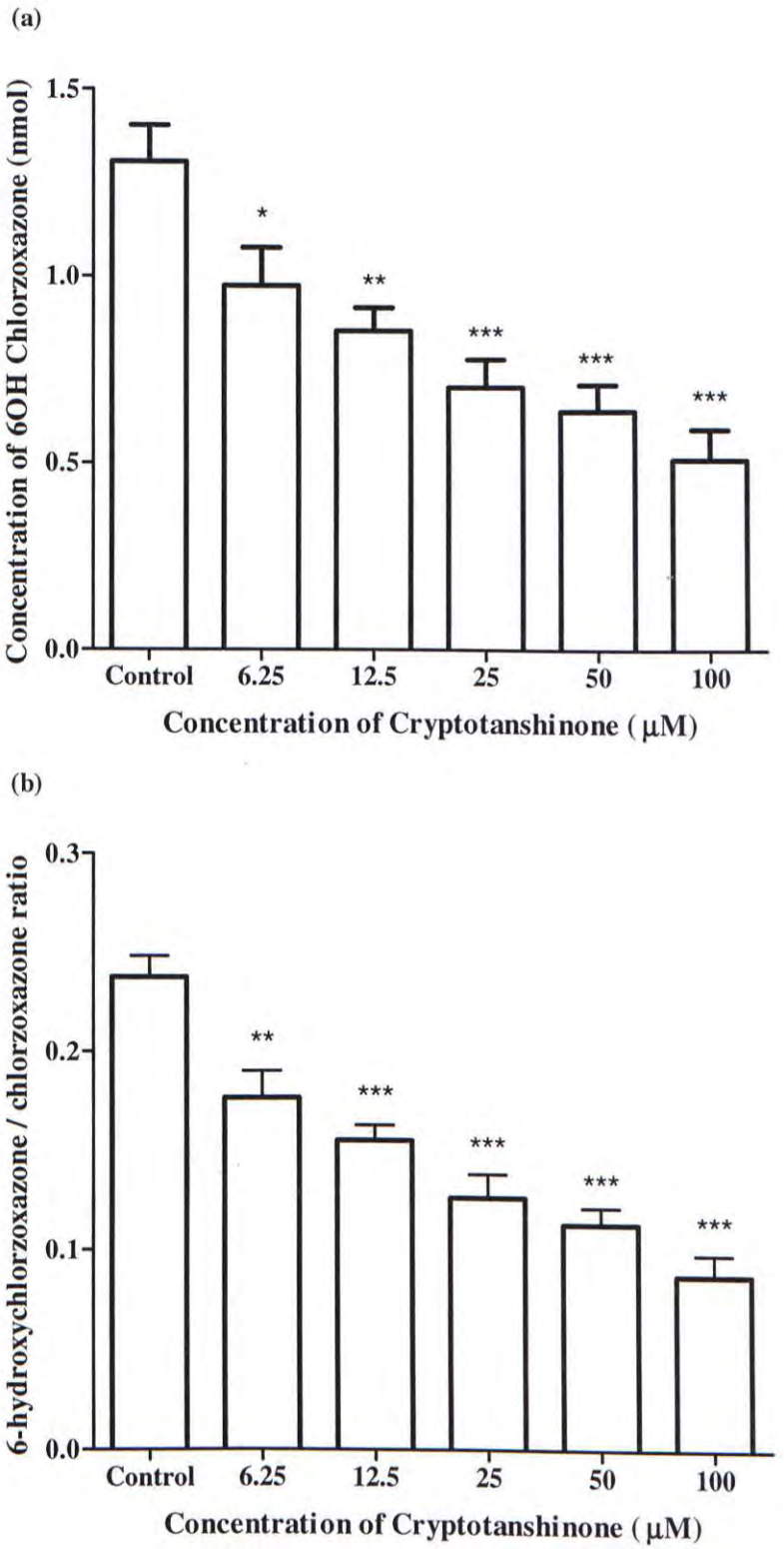



Fig. 2.11 Effects of cryptotanshinone (6.25 μM – 100 μM) on (a) formation of 6-hydroxychlorzoxazone and (b) ratio of 6-hydroxychlorzoxazone to chlorzoxazone in rat microsomes. Values are mean ± SEM (n = 6). *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ when compared with controls.

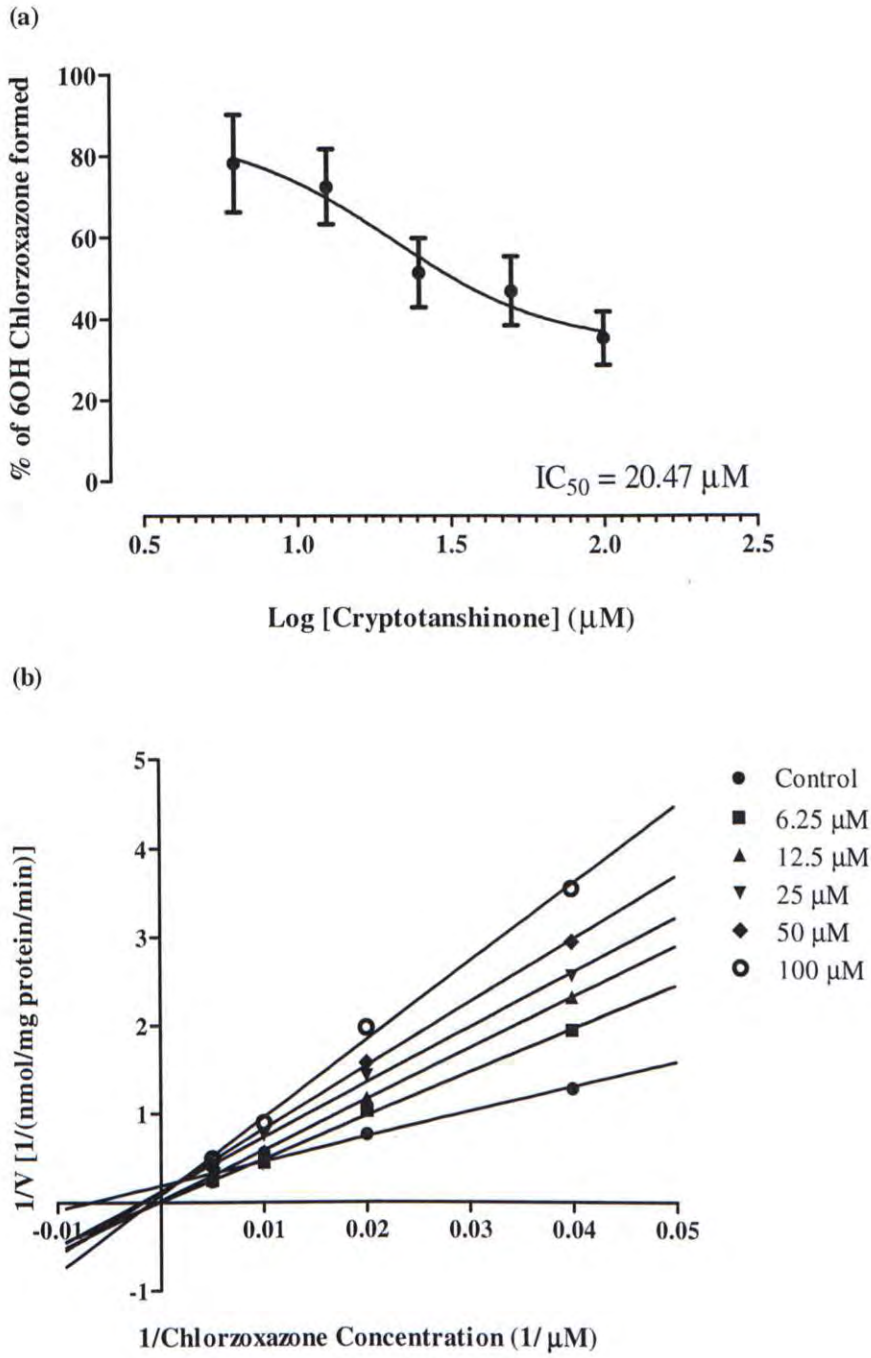



Fig. 2.12 (a) Inhibition curve showing effects of cryptotanshinone (6.25 μM – 100 μM) on formation of 6-hydroxychlorzoxazone in rat liver microsomes. Values are mean ± SEM (n = 6). (b) Lineweaver-Burk Plot for the inhibition of CYP2E1-mediated chlorzoxazone hydroxylation by cryptotanshinone (6.25 μM – 100 μM) in rat liver microsomes. Values are mean of 6 determinations.

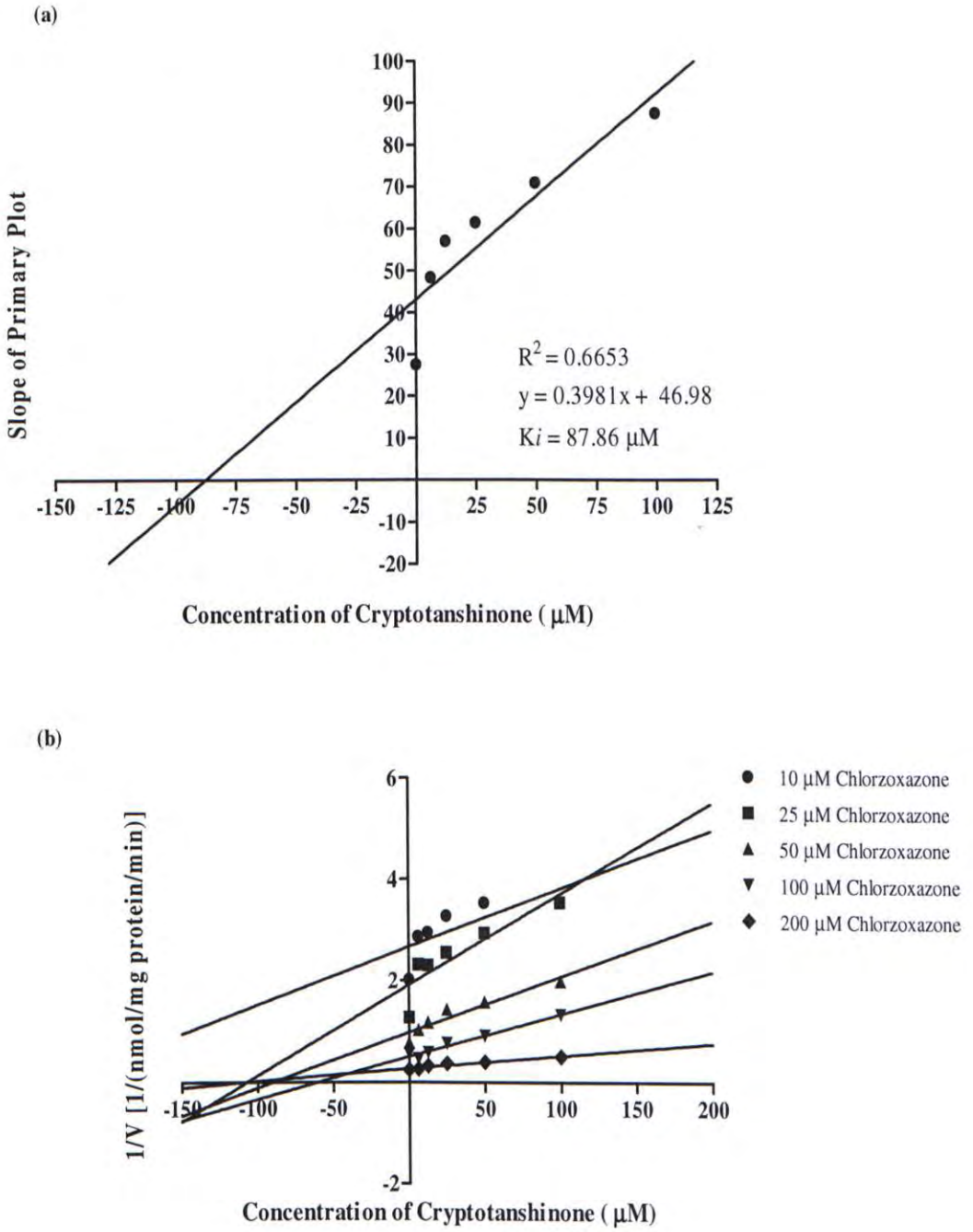



Fig. 2.13 (a) Secondary plot of Lineweaver-Burk Plot and (b) Dixon Plot show inhibitory effects of cryptotanshinone (6.25 μM – 100 μM) on CYP2E1-mediated chlorzoxazone hydroxylation in rat liver microsomes. Values are mean of 6 determinations.

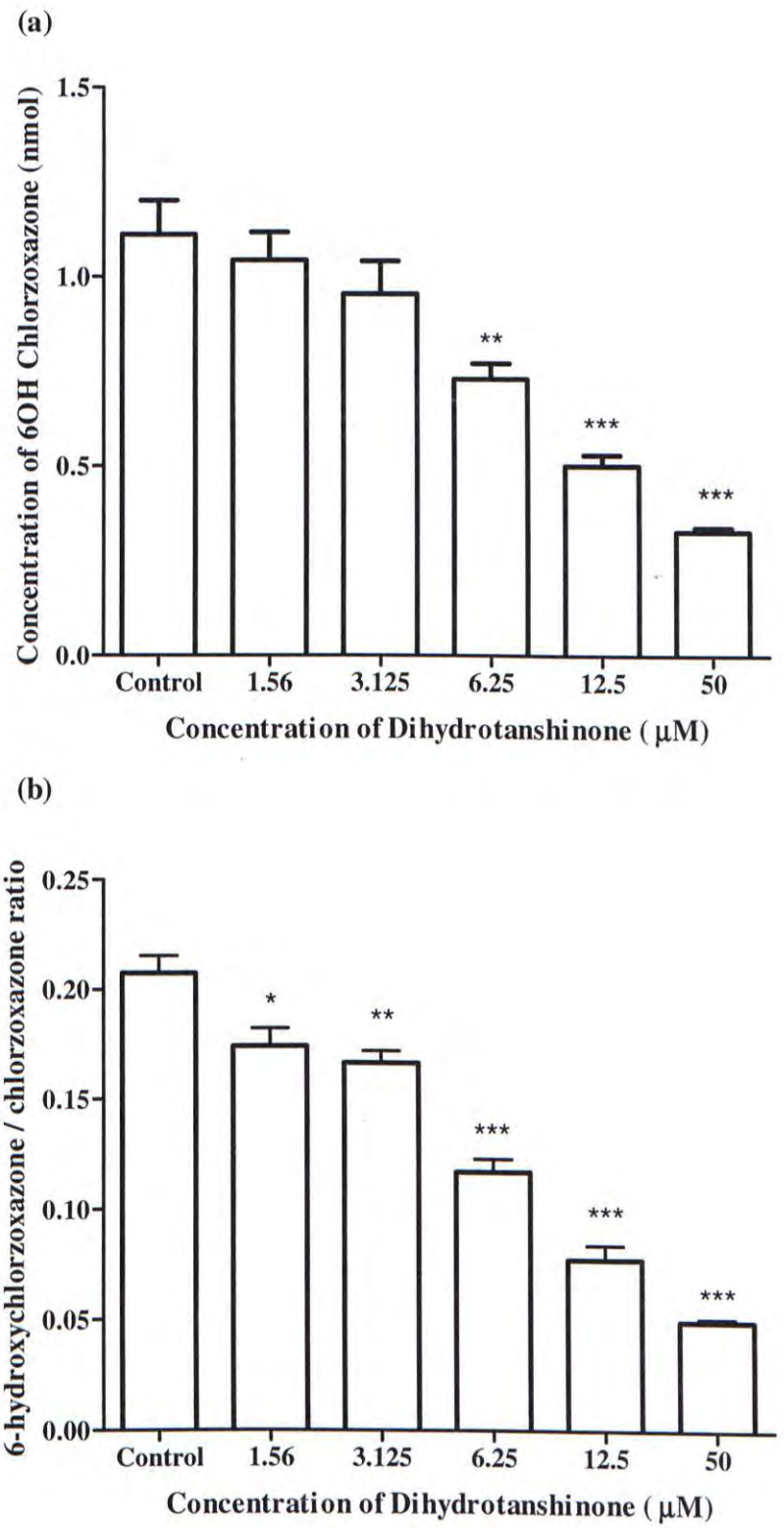



Fig. 2.14 Effects of dihydrotanshinone (1.56 μM – 50 μM) on (a) formation of 6-hydroxychlorzoxazone and (b) ratio of 6-hydroxychlorzoxazone to chlorzoxazone in rat microsomes. Values are mean ± SEM (n = 6). *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ when compared with controls.

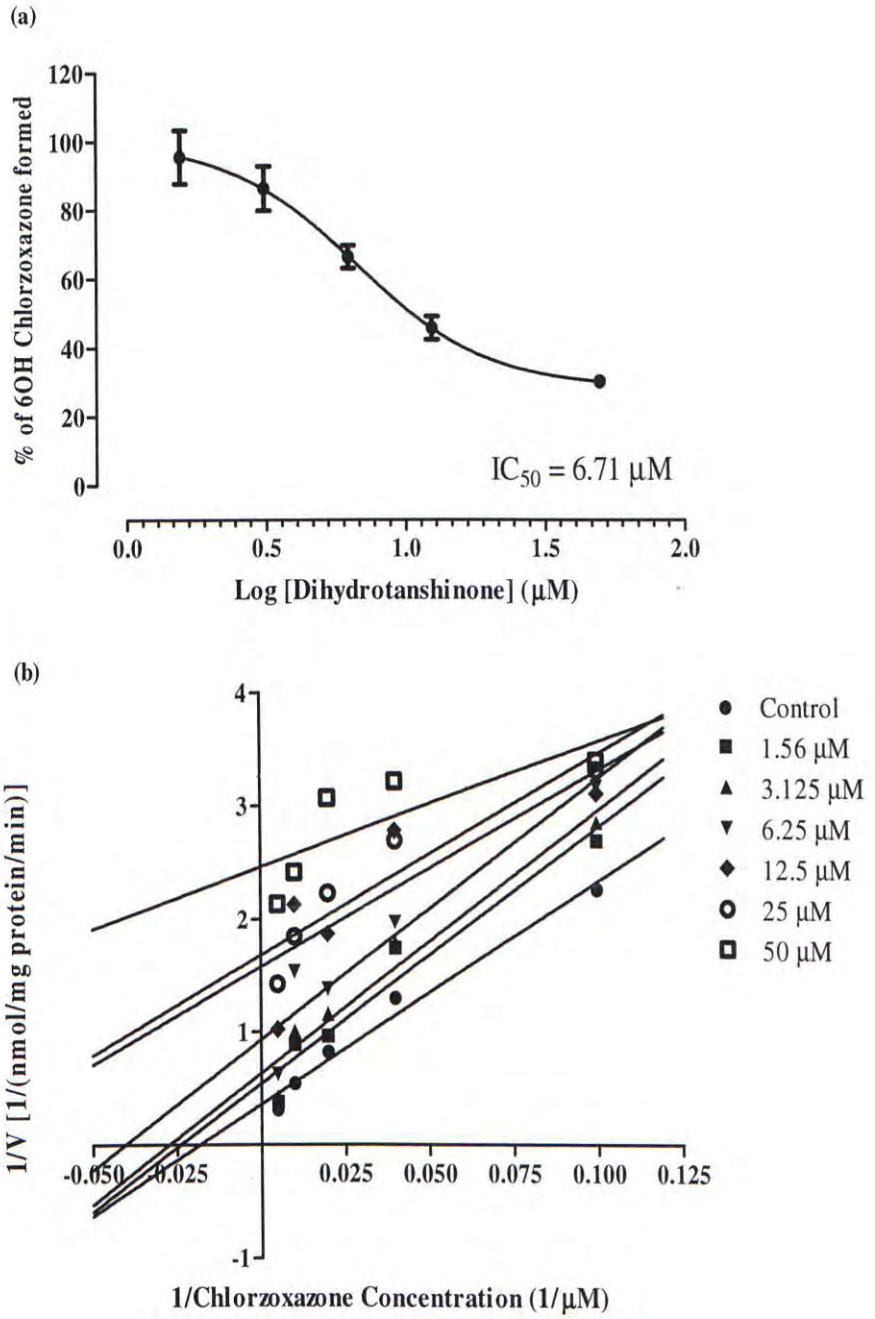



Fig. 2.15 (a) Inhibition curve showing effects of dihydrotanshinone (1.56 µM – 50 µM) on formation of 6-hydroxychlorzoxazone in rat liver microsomes. Values are mean ± SEM (n = 6). (b) Lineweaver-Burk Plot for the inhibition of CYP2E1-mediated chlorzoxazone hydroxylation by dihydrotanshinone (1.56 µM – 50 µM) in rat liver microsomes. Values are mean of 6 determinations.

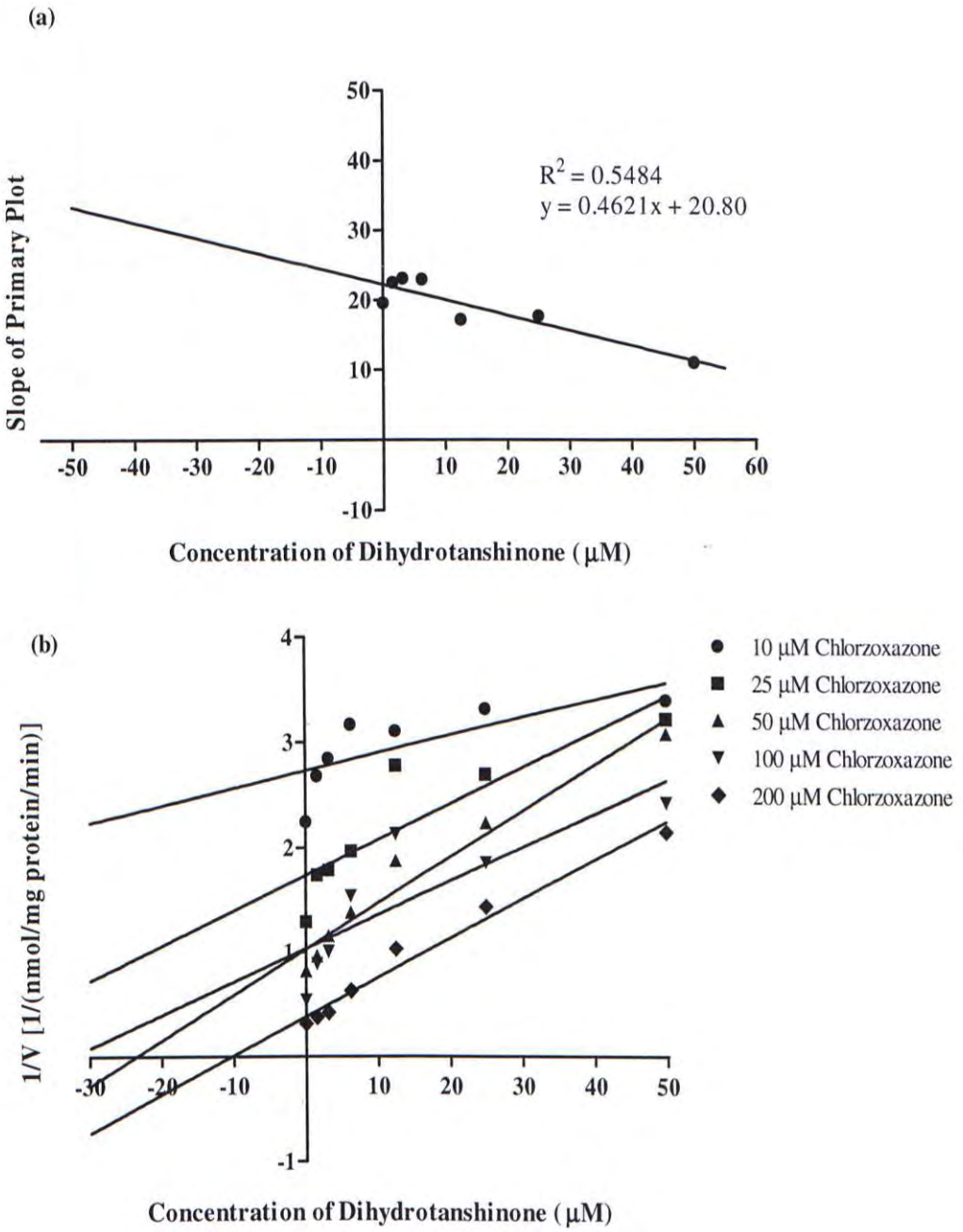



Fig. 2.16 (a) Secondary plot of Lineweaver-Burk Plot and (b) Dixon Plot show inhibitory effects of dihydrotanshinone (1.56 μM – 50 μM) on CYP2E1-mediated chlorzoxazone hydroxylation in rat liver microsomes. Values are mean of 6 determinations.

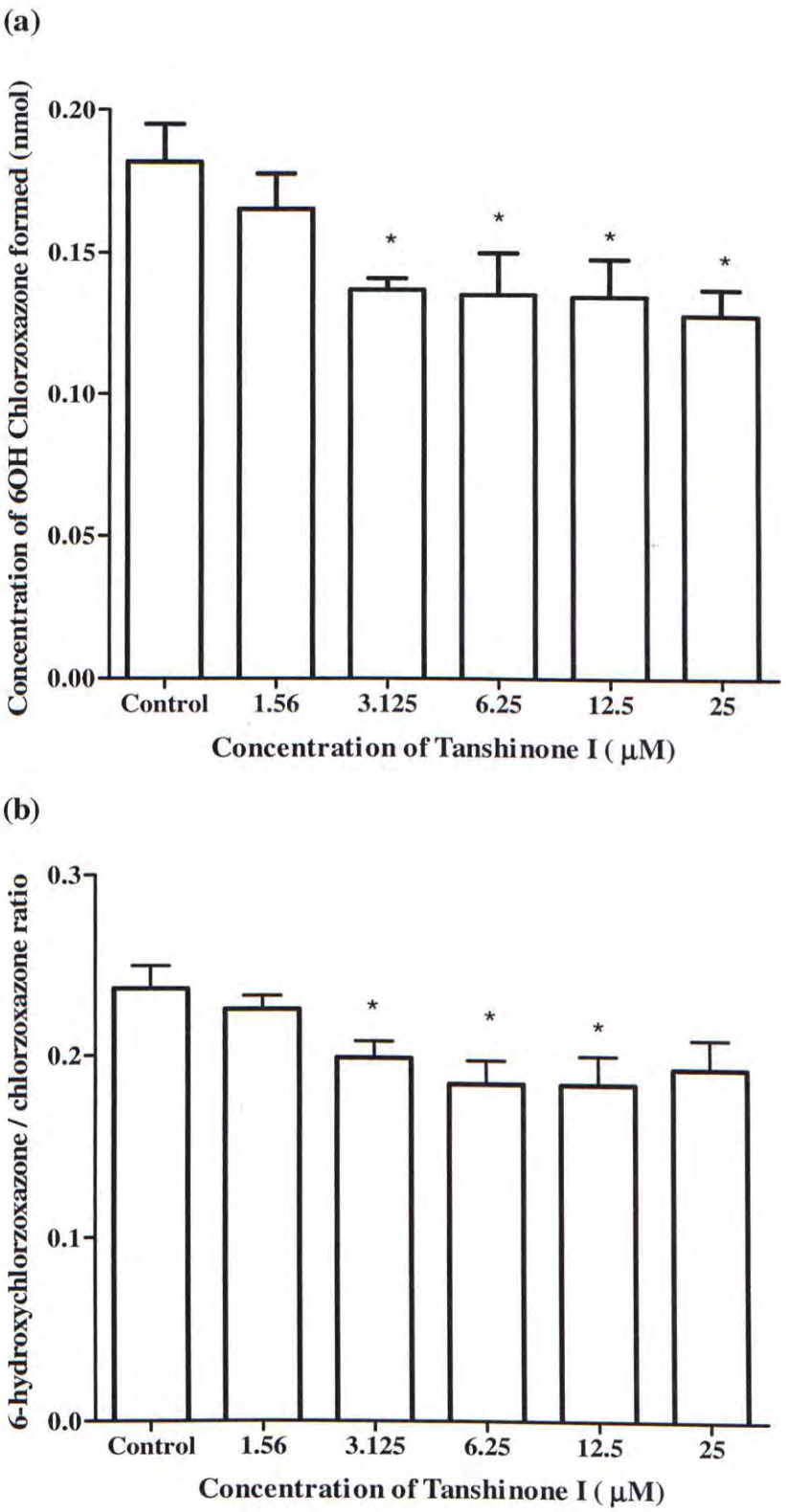



Fig. 2.17 Effects of tanshinone I (1.56 μM – 25 μM) on (a) formation of 6-hydroxychlorzoxazone and (b) ratio of 6-hydroxychlorzoxazone to chlorzoxazone in rat microsomes. Values are mean ± SEM (n = 6). *$p < 0.05$ when compared with controls.

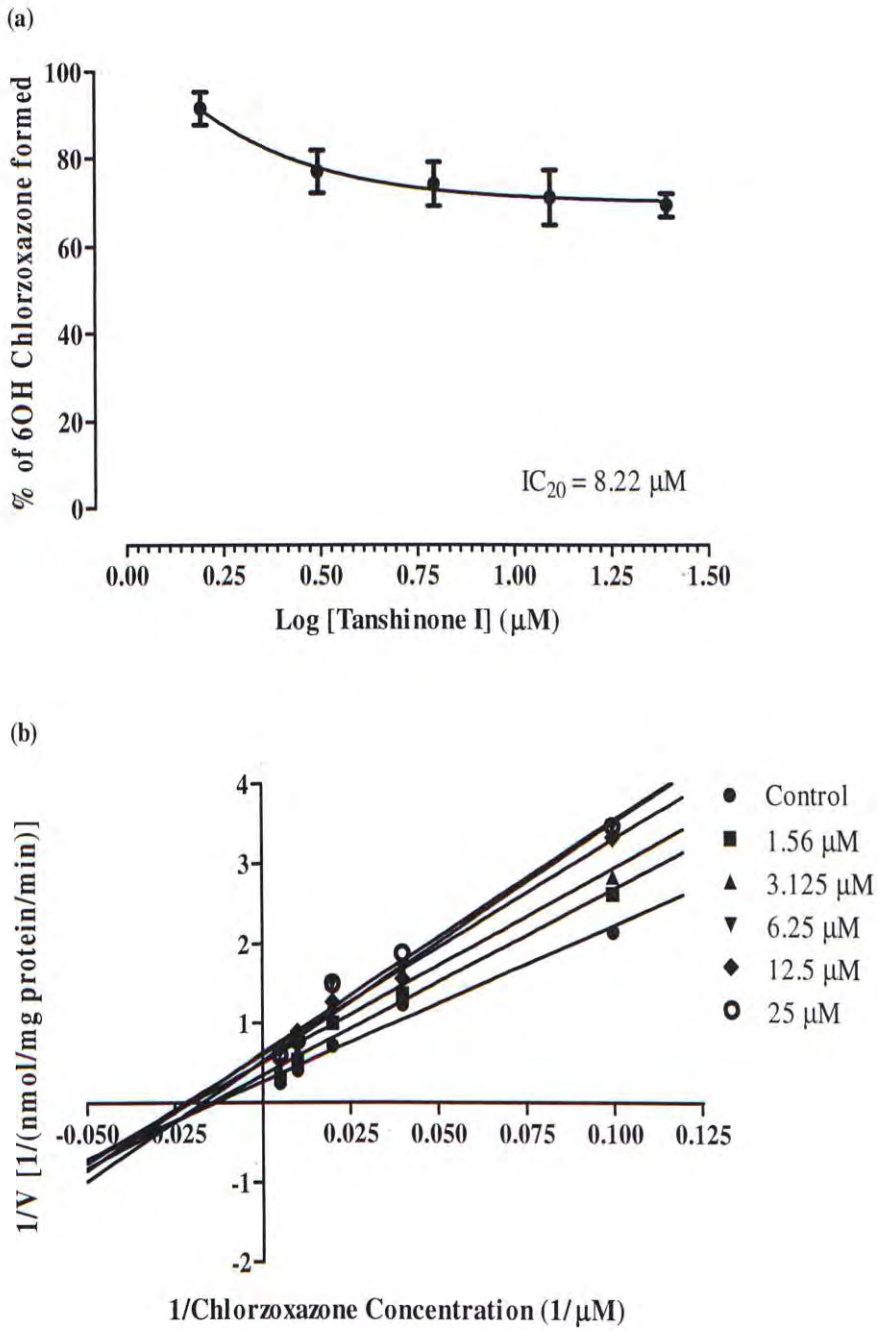



Fig. 2.18 (a) Inhibition curve showing effects of tanshinone I (1.56 μM – 25 μM) on formation of 6-hydroxychlorzoxazone in rat liver microsomes. Values are mean ± SEM (n = 6). (b) Lineweaver-Burk Plot for the inhibition of CYP2E1-mediated chlorzoxazone hydroxylation by tanshinone I (1.56 μM – 25 μM) in rat liver microsomes. Values are mean of 6 determinations.

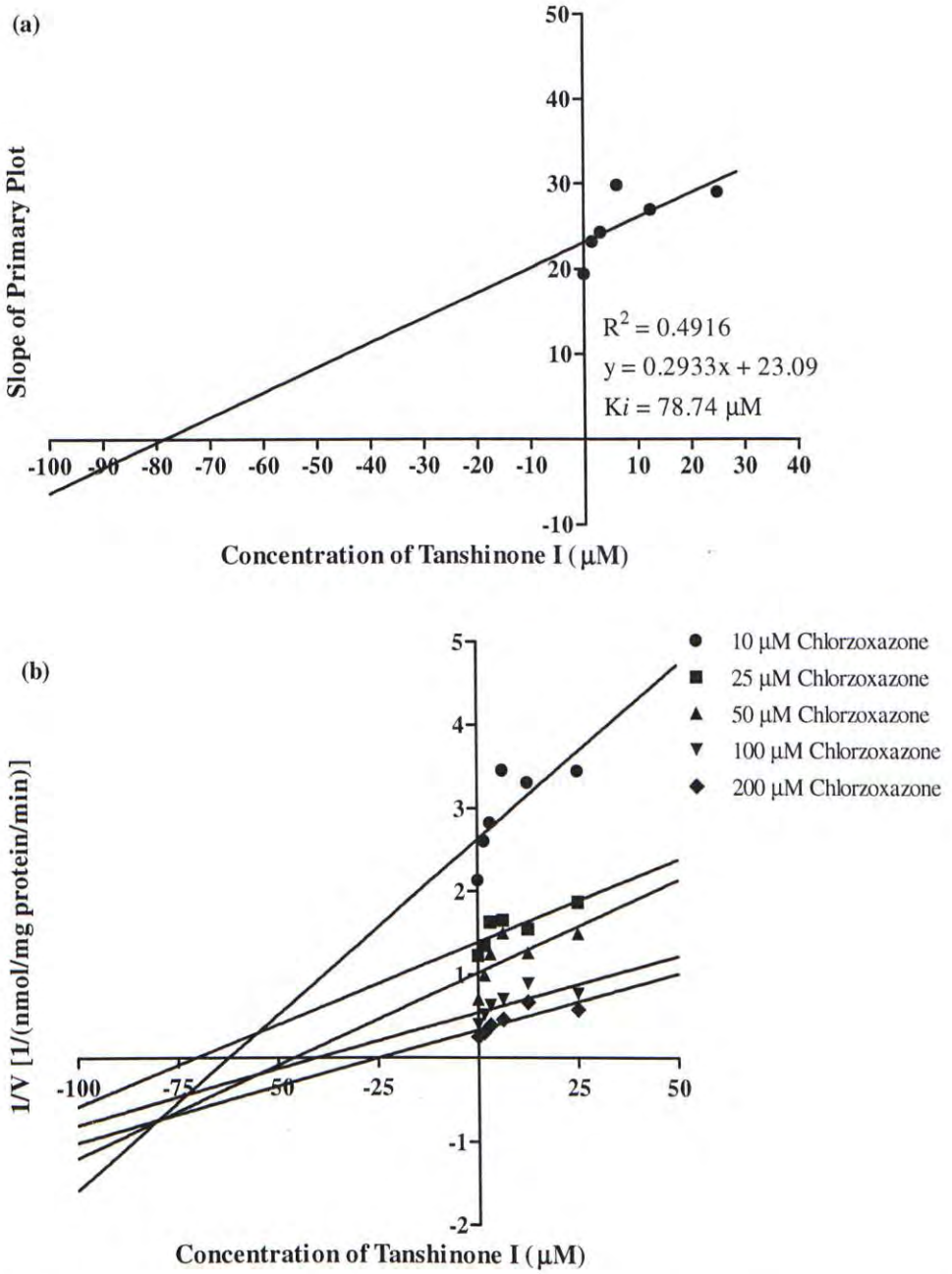



Fig. 2.19 (a) Secondary plot of Lineweaver-Burk Plot and (b) Dixon Plot show inhibitory effects of tanshinone I (1.56 μM – 25 μM) on CYP2E1-mediated chlorzoxazone hydroxylation in rat liver microsomes. Values are mean of 6 determinations.

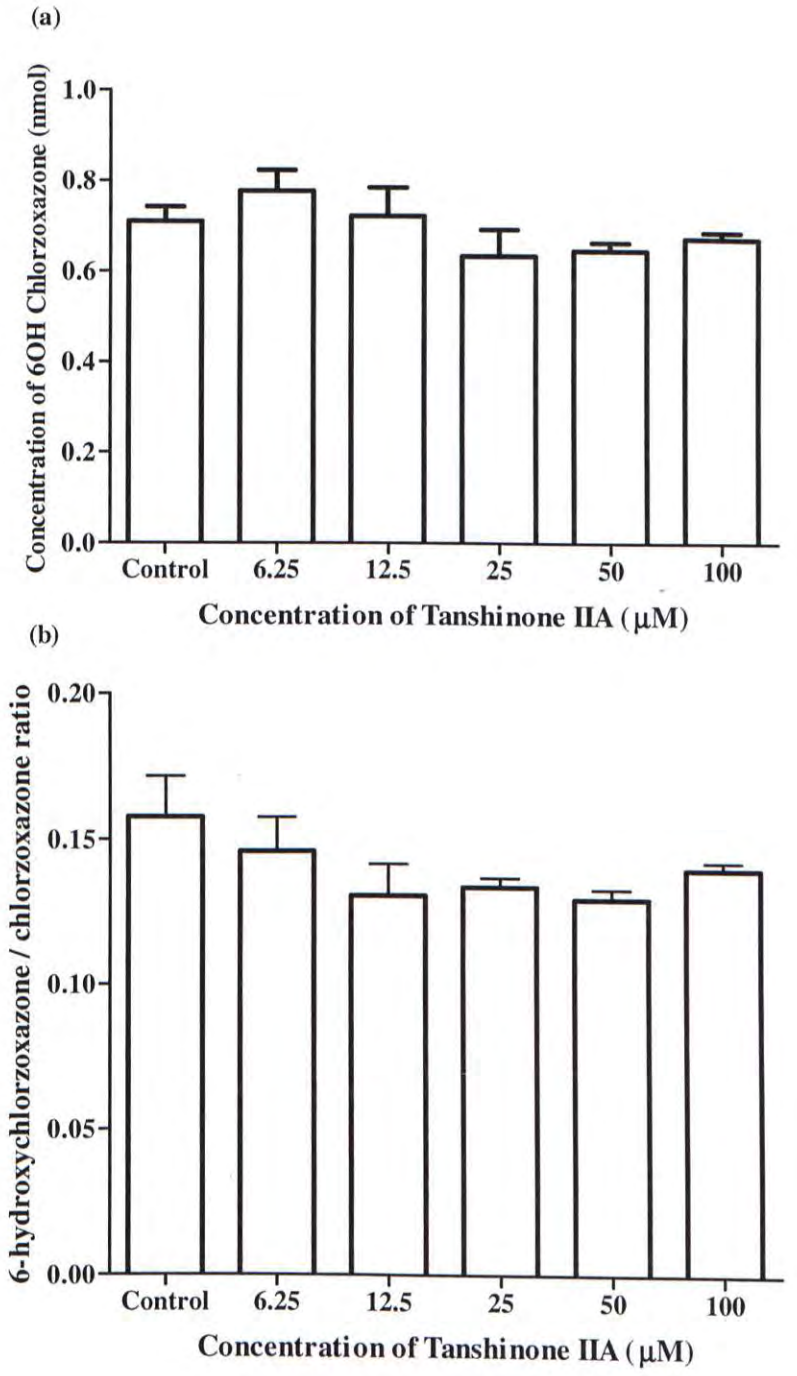



Fig. 2.20 Effects of tanshinone IIA (6.25 μM – 100 μM) on (a) formation of 6-hydroxychlorzoxazone and (b) ratio of 6-hydroxychlorzoxazone to chlorzoxazone in rat microsomes. Values are mean ± SEM (n = 6).

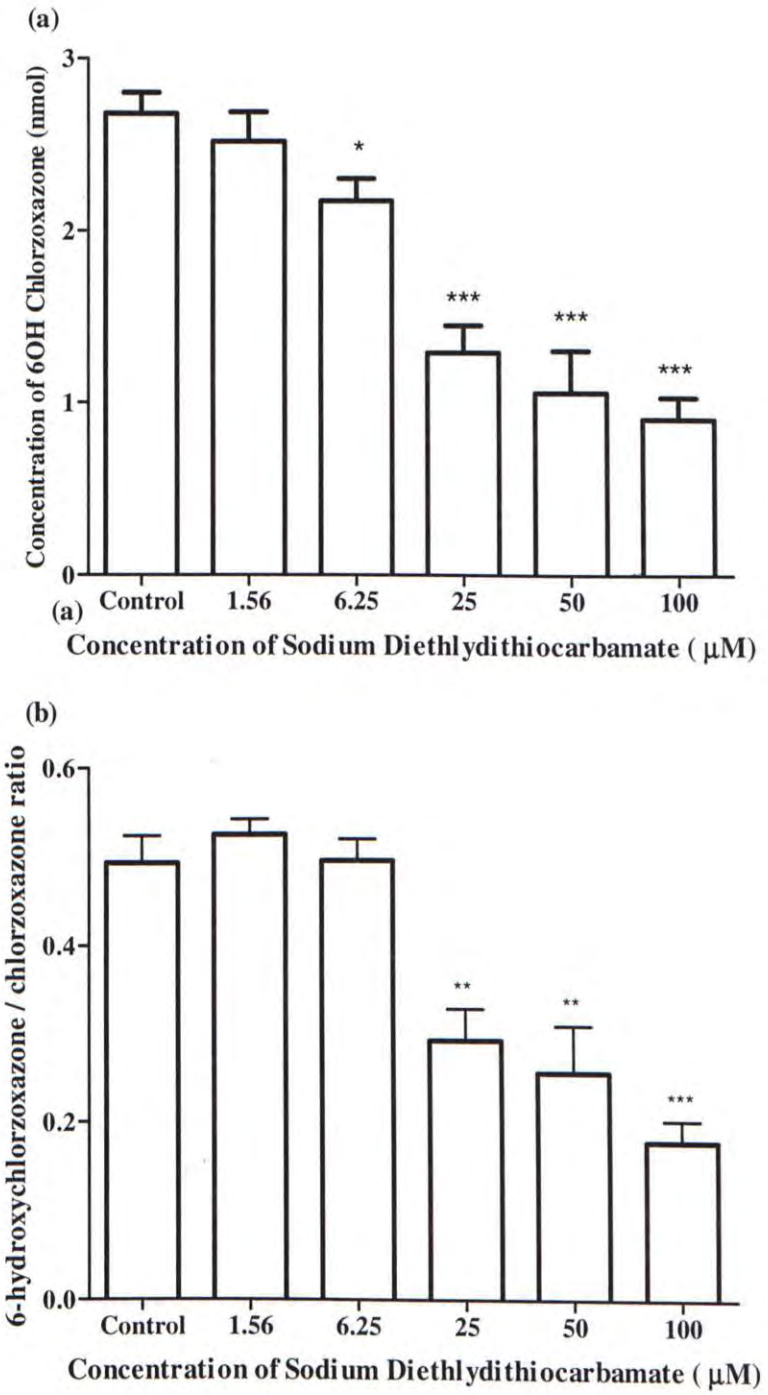



Fig. 2.21 Effects of sodium diethyldithiocarbamate (1.56 μM – 100 μM) on (a) formation of 6-hydroxychlorzoxazone and (b) ratio of 6-hydroxychlorzoxazone to chlorzoxazone in rat microsomes. Values are mean ± SEM (n = 6). *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ when compared with controls.

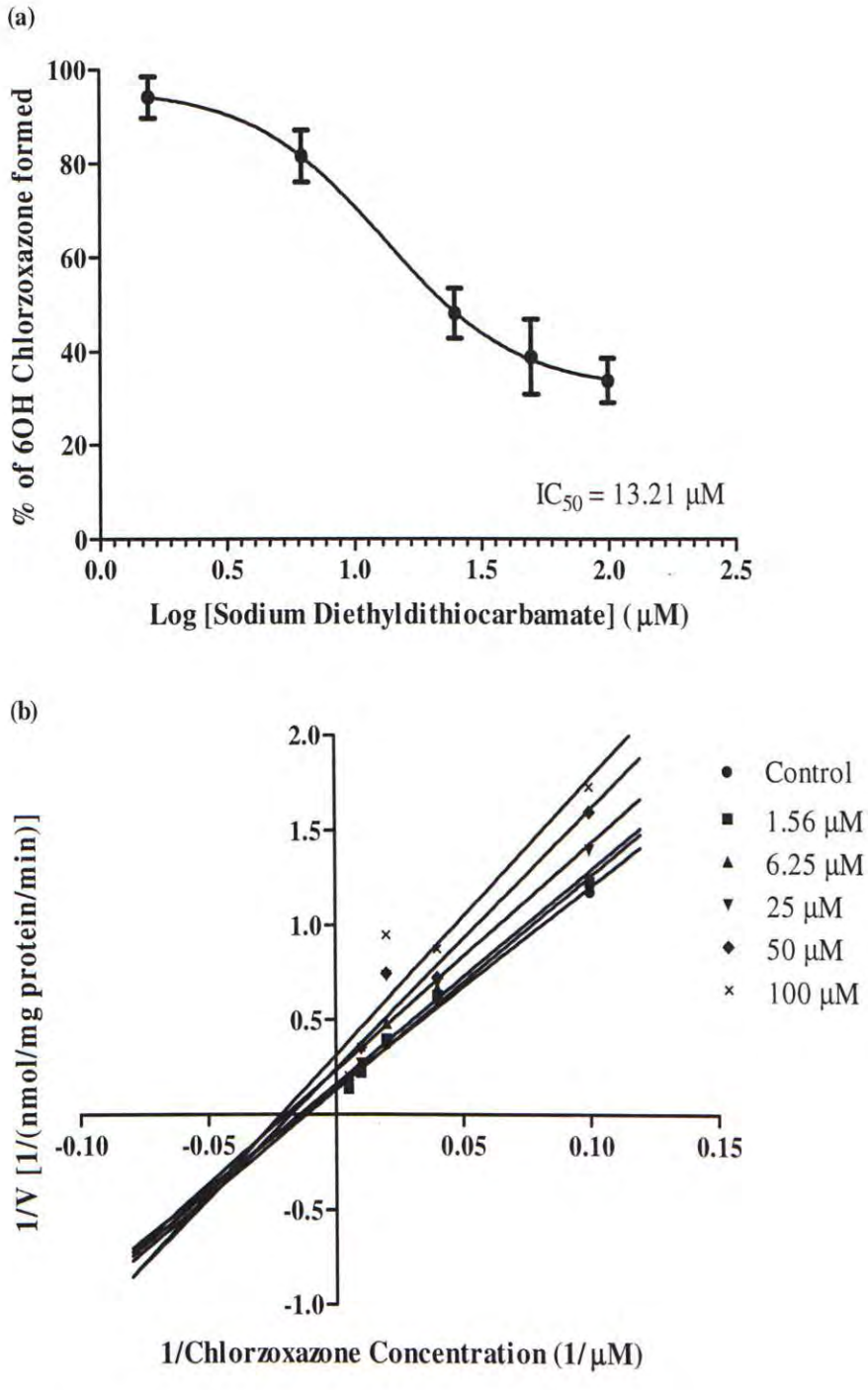



Fig. 2.22 (a) Inhibition curve showing effects of sodium diethyldithiocarbamate (1.56 µM – 100 µM) on formation of 6-hydroxychlorzoxazone in rat liver microsomes. Values are mean ± SEM (n = 6). (b) Lineweaver-Burk Plot for the inhibition of CYP2E1-mediated chlorzoxazone hydroxylation by sodium diethyldithiocarbamate (1.56 µM – 100 µM) in rat liver microsomes. Values are mean of 6 determinations.

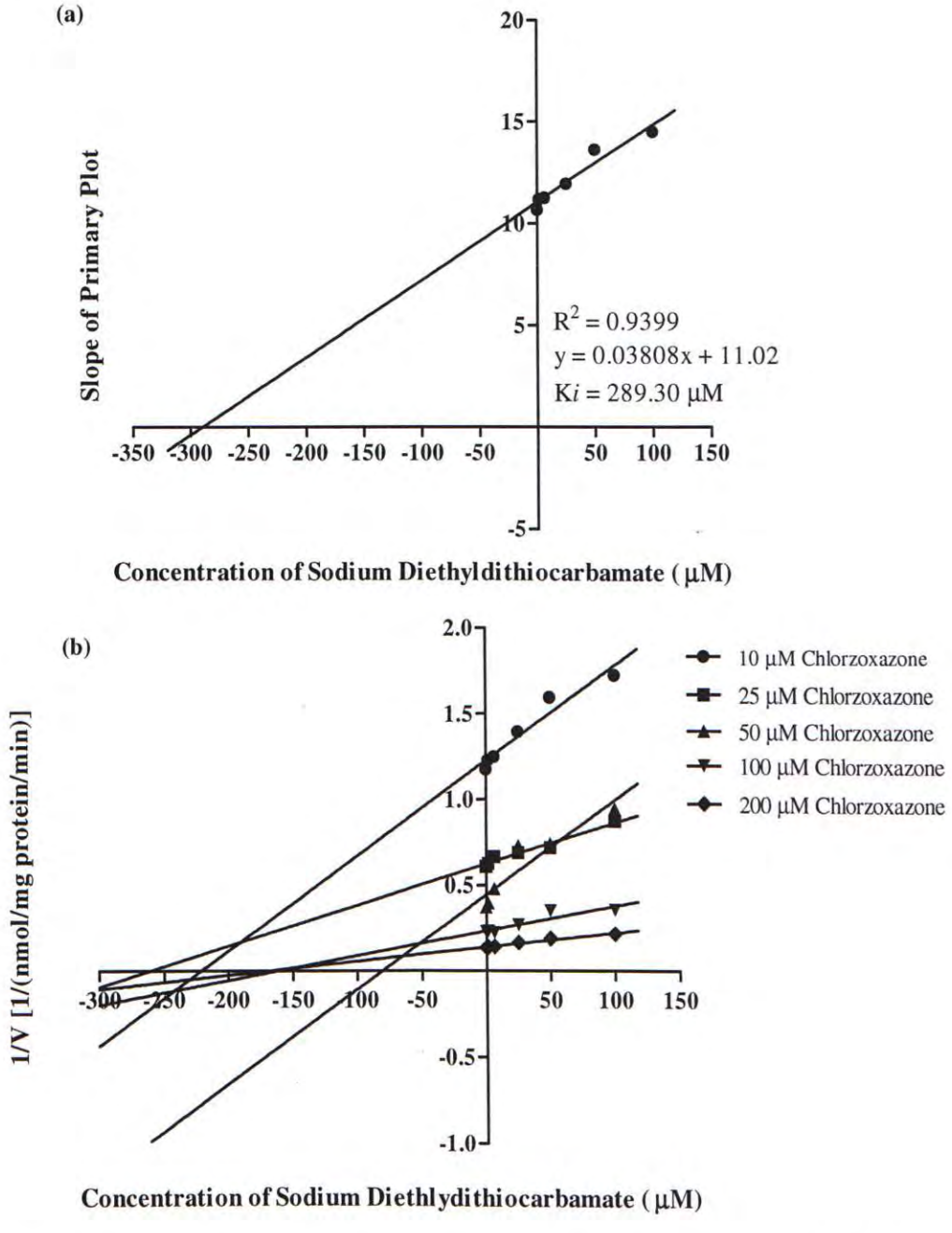



Fig. 2.23 (a) Secondary plot of Lineweaver-Burk Plot and (b) Dixon Plot show inhibitory effects of sodium diethyldithiocarbamate (1.56 µM – 100 µM) on CYP2E1-mediated chlorzoxazone hydroxylation in rat liver microsomes. Values are mean of 6 determinations.

### 2.3.1.1 Summary

Whole Danshen extract, the ethanolic fraction of Danshen and the aqueous fraction of Danshen exerted inhibitory effects on CYP2E1 activity, as indicated by a decrease in the 6-hydroxylation of the model CYP2E1 probe substrate, in rat liver microsomes with non-competitive, uncompetitive and competitive inhibitions, respectively. The ethanolic fraction of Danshen ($IC_{50}$ at 18.25 μg/ml) was more effective in decreasing CZX metabolism than the aqueous fraction of Danshen ($IC_{50}$ at 1.03 mg/ml). Dihydrotanshinone inhibited CYP2E1 activity most with $IC_{50}$ at 6.71 μM followed by tanshinone I and cryptotanshinone. Dihydrotanshinone showed uncompetitive inhibition and therefore K*i* cannot be determined from Secondary Lineweaver-Burk plot and Dixon plot. Cryptotanshinone competitively inhibited the chlorzoxazone metabolism while tanshinone I inhibited 6-hydroxylation of chlorzoxazone non-competitively. However, both compounds showed stronger inhibitory effects than sodium diethyldithiocarbamate which inhibited CYP2E1 non-competitively with a high K*i* value. Tanshinone IIA did not inhibit CYP2E1 activity in rat liver microsomes *in vitro*.

Table 2.4 Inhibition of Danshen, its active components and sodium diethyldithiocarbamate on CYP2E1 activity in rat liver microsomes *in vitro*.

| | $IC_{50}$ | K*i* | Inhibition Mode |
|---|---|---|---|
| **Whole Danshen Extract** | 1.88 mg/ml | 1.46 mg/ml | Non-competitive |
| **Ethanolic fraction of Danshen** | 18.25 μg/ml | Cannot be determined | Uncompetitive |
| **Aqueous fraction of Danshen** | 1.03 mg/ml | 1.89 mg/ml | Competitive |
| **Cryptotanshinone** | 20.47 μM | 87.86 μM | Competitive |
| **Dihydrotanshinone** | 6.71 μM | Cannot be determined | Uncompetitive |
| **Tanshinone I** | $IC_{20}$: 8.22 μM | 78.74 μM | Non-competitive |
| **Tanshinone IIA** | - | - | - |
| **Sodium diethyldithiocarbamate** | 13.21 μM | 289.30 μM | Non-competitive |

### 2.3.2 Effect of Danshen and tanshinones on human CYP2E1 activity *in vitro*

Whole Danshen extract (0.125 mg/ml – 1 mg/ml), the ethanolic fraction of Danshen (2.5 μg/ml – 20 μg/ml), the aqueous fraction of Danshen (0.25 mg/ml – 2 mg/ml), cryptotanshinone (6.25 μM – 50 μM), dihydrotanshinone (0.78 μM – 6.25 μM) and tanshinone I (6.25 μM – 50 μM) reduced the formation of 6-hydroxychlorzoxazone in a concentration-dependent manner (Fig. 2.24, 2.27, 2.30, 2.33, 2.36, 2.39). The $IC_{50}$ of whole Danshen extract, the ethanolic fraction of Danshen and the aqueous fraction of Danshen were 0.15 mg/ml, 4.50 μg/ml and 0.79 mg/ml, respectively (Fig. 2.25 a, 2.28 a, 2.31 a). The $IC_{50}$ of cryptotanshinone, and tanshinone I were 14.67 μM and 9.86 μM (Fig. 2.34 a, 2.40 a), respectively. Dihydrotanshinone produced a strong inhibition at 0.78 μM, with over 50% inhibition at the lowest concentration used. The $IC_{50}$ of dihydrotanshione was thus estimated to be 0.72 μM (Fig. 2.37 a). The $K_m$ and $V_{max}$ values were shown in Tables 2.5 and 2.6. Tanshinone IIA did not affect CYP2E1-mediated 6-hydroxylation of chlorzoxazone in human liver microsomes.

For experiments with whole Danshen extract and tanshinone I, Lineweaver-Burk Plots in Fig. 2.25 (b) and Fig. 2.40 (b) showed changes in slopes and y-intercept suggesting the inhibition mode was non-competitive. The respective K*i* from the secondary plots were 0.032 mg/ml and 3.67 μM for whole Danshen extract and tanshinone I, respectively (Fig. 2.26 a, 2.41 a). For experiments with the ethanolic fraction of Danshen and dihydrotanshinone, the slopes of Lineweaver-Burk Plots in Fig. 2.28 (b) and Fig. 2.37 (b) formed in parallel suggesting the inhibition mode was uncompetitive. The respective K*i* from the secondary plots could not be determined as the slopes of Lineweaver-Burk plot did not change (Fig. 2.29 a, 2.38 a). For experiments with the aqueous fraction of Danshen and cryptotanshinone, the slopes

of Lineweaver-Burk Plots in Fig. 2.31 (b) and Fig. 2.34 (b) had converged at y-axis suggesting the inhibition mode was competitive. The respective K*i* from the secondary plots for the aqueous fraction of Danshen and tanshinone I were 0.08 mg/ml and 10.87 μM, respectively (Fig. 2.32 a, 2.35 a).

Sodium diethyldithiocarbamate (25 μM – 200 μM) reduced the formation of 6-hydroxychlorzoxazone and decreased the 6-hydroxychlorzoxazone/chlorzoxazone ratio in a concentration-dependent manner (Fig. 2.43). The $IC_{50}$ was estimated at 3.16 μM (Fig. 2.44 a). The slopes of Lineweaver-Burk Plot in Fig. 2.44 (b) converged at x-axis that its inhibition mode was proposed to be non-competitive. The respective K*i* from the secondary plot was 122.70 μM (Fig. 2.45 a).

Table 2.5 Enzyme kinetic parameters of the effects of whole Danshen extract, the ethanolic fraction of Danshen and the aqueous fraction of Danshen on formation of 6-hydroxychlorzoxazone in human liver microsomes. Values are mean ± SE of triplicate determinations.

| **Whole Danshen extract** | | | | | |
|---|---|---|---|---|---|
| **Enzyme Kinetic Parameters** | **Control** | **0.125 mg/ml** | **0.25 mg/ml** | **0.5 mg/ml** | **1 mg/ml** |
| **Vmax (nmol/mg protein/min)** | 315.60 ± 64.63 | 201.80 ± 56.59 | 83.47 ± 15.51 | 29.02 ± 5.49 | 7.00 ± 1.22 |
| **Km (mg/ml)** | 340.20 ± 100.80 | 257.90 ± 113.90 | 204.60 ± 64.88 | 155.80 ± 55.95 | 116.90 ± 43.60 |
| **Km/Vmax** | 1.08 | 1.28 | 2.45 | 5.37 | 16.70 |
| **The ethanolic fraction of Danshen** | | | | | |
| **Enzyme Kinetic Parameters** | **Control** | **0.25 μg/ml** | **0.5 μg/ml** | **1 μg/ml** | **2 μg/ml** |
| **Vmax (nmol/mg protein/min)** | 7.34e+15 ± 2.22e+29 | 1.29e+16 ± 1.87e+30 | 27.21 ± 10.51 | 265.30 ± 1009.00 | 10.35 ± 2.31 |
| **Km (μg/ml)** | 3.63e+16 ± 1.10e+30 | 8.18e+16 ± 1.18e+31 | 113.60 ± 96.62 | 2498.00 ± 10135.00 | 97.57 ± 50.83 |
| **Km/Vmax** | 4.94 | 6.35 | 4.17 | 9.42 | 9.43 |
| **The aqueous fraction of Danshen** | | | | | |
| **Enzyme Kinetic Parameters** | **Control** | **0. 25 mg/ml** | **0.5 mg/ml** | **1 mg/ml** | **2 mg/ml** |
| **Vmax (nmol/mg protein/min)** | 315.60 ± 64.63 | 603.60 ± 525.70 | 297.50 ± 280.10 | 167.30 ± 210.60 | 7.18e+9 ± 8.79e+17 |
| **Km (mg/ml)** | 340.20 ± 100.80 | 672.20 ± 723.50 | 369.00 ± 491.30 | 495.20 ± 810.20 | 7.26e+10 ± 8.88e+18 |
| **Km/Vmax** | 1.08 | 1.11 | 1.24 | 2.96 | 10.11 |

Table 2.6 Enzyme kinetic parameters of the effects of cryptotanshinone, dihydrotanshinone, tanshinone I and sodium diethyldithiocarbamate on the formation of 6-hydroxychlorzoxazone in human liver microsomes. Values are mean ± SE of triplicate determinations.

| **Cryptotanshinone** | | | | | |
|---|---|---|---|---|---|
| **Enzyme Kinetic Parameters** | **Control** | **6.25 μM** | **12.5 μM** | **25 μM** | **50 μM** |
| **Vmax (nmol/mg protein/min)** | 7.34e+15 ± 2.22e+29 | 1.14e+16 ± 1.41e+30 | 1.19e+15 ± 4.11e+28 | 3.15e+15 ± 5.76e+29 | 3.13e+15 ± 5.57e+29 |
| **Km (μM)** | 3.63e+16 ± 1.10e+30 | 8.98e+16 ± 1.11e+31 | 1.36e+16 ± 4.68e+29 | 5.01e+16 ± 9.16e+30 | 7.02e+16 ± 1.25e+31 |
| **Km/Vmax** | 4.94 | 7.89 | 11.38 | 15.92 | 22.41 |
| **Dihydrotanshinone** | | | | | |
| **Enzyme Kinetic Parameters** | **Control** | **0.78 μM** | **1.56 μM** | **3.125 μM** | **6.25 μM** |
| **Vmax (nmol/mg protein/min)** | 7.34e+15 ± 2.22e+29 | 68.48 ± 59.30 | 6.73e+9 ± 1.55e+18 | 6.25 ± 1.96 | 1.53 ± 0.32 |
| **Km (μM)** | 3.63e+16 ± 1.10e+30 | 709.70 ± 757.10 | 1.20e+11 ± 2.77e+19 | 69.78 ± 60.48 | 17.11 ± 24.15 |
| **Km/Vmax** | 4.94 | 10.36 | 17.86 | 11.16 | 11.16 |
| **Tanshinone I** | | | | | |
| **Enzyme Kinetic Parameters** | **Control** | **6.25 μM** | **12.5 μM** | **25 μM** | **50 μM** |
| **Vmax (nmol/mg protein/min)** | 7.34e+15 ± 2.22e+29 | 1.95e+15 ± 5.24e+28 | 2.15e+15 ± 1.39e+29 | 5.76e+14 ± 2.97e+28 | 8.55e+15 ± 3.68e+30 |
| **Km (μM)** | 3.63e+16 ± 1.10e+30 | 1.97e+16 ± 5.29e+29 | 3.27e+16 ± 2.12e+30 | 1.14e+16 ± 5.89e+29 | 1.67=e+17 ± 7.19e+31 |
| **Km/Vmax** | 4.94 | 10.10 | 15.20 | 19.83 | 19.55 |
| **Sodium Diethyldithiocarbamate** | | | | | |
| **Enzyme Kinetic Parameters** | **Control** | **25 μM** | **50 μM** | **100 μM** | **200 μM** |
| **Vmax (nmol/mg protein/min)** | 315.60 ± 64.63 | 77.55 ± 28.18 | 33.56 ± 7.61 | 103.90 ± 120.00 | 19.78 ± 4.93 |
| **Km (μM)** | 340.20 ± 100.80 | 451.40 ± 220.40 | 160.10 ± 68.13 | 1169.00 ± 1529.00 | 176.10 ± 79.29 |
| **Km/Vmax** | 1.08 | 5.82 | 4.77 | 11.25 | 8.90 |

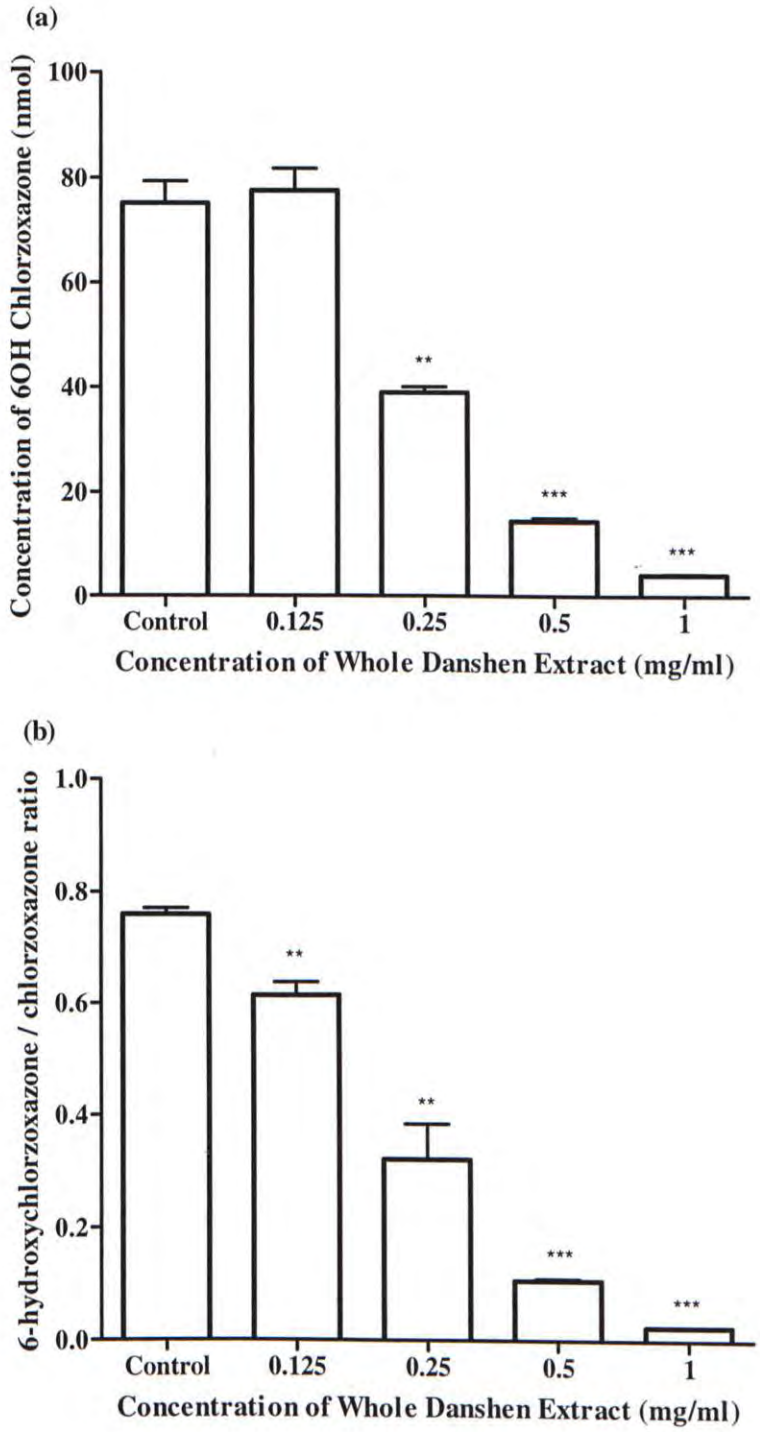



Fig. 2.24 Effects of whole Danshen extract (0.125 mg/ml – 1 mg/ml) on (a) formation of 6-hydroxychlorzoxazone and (b) ratio of 6-hydroxychlorzoxazone to chlorzoxazone in human microsomes. Values are mean ± SEM of triplicate determinations. $^{**}p < 0.01$ and $^{***}p < 0.001$ when compared with controls.

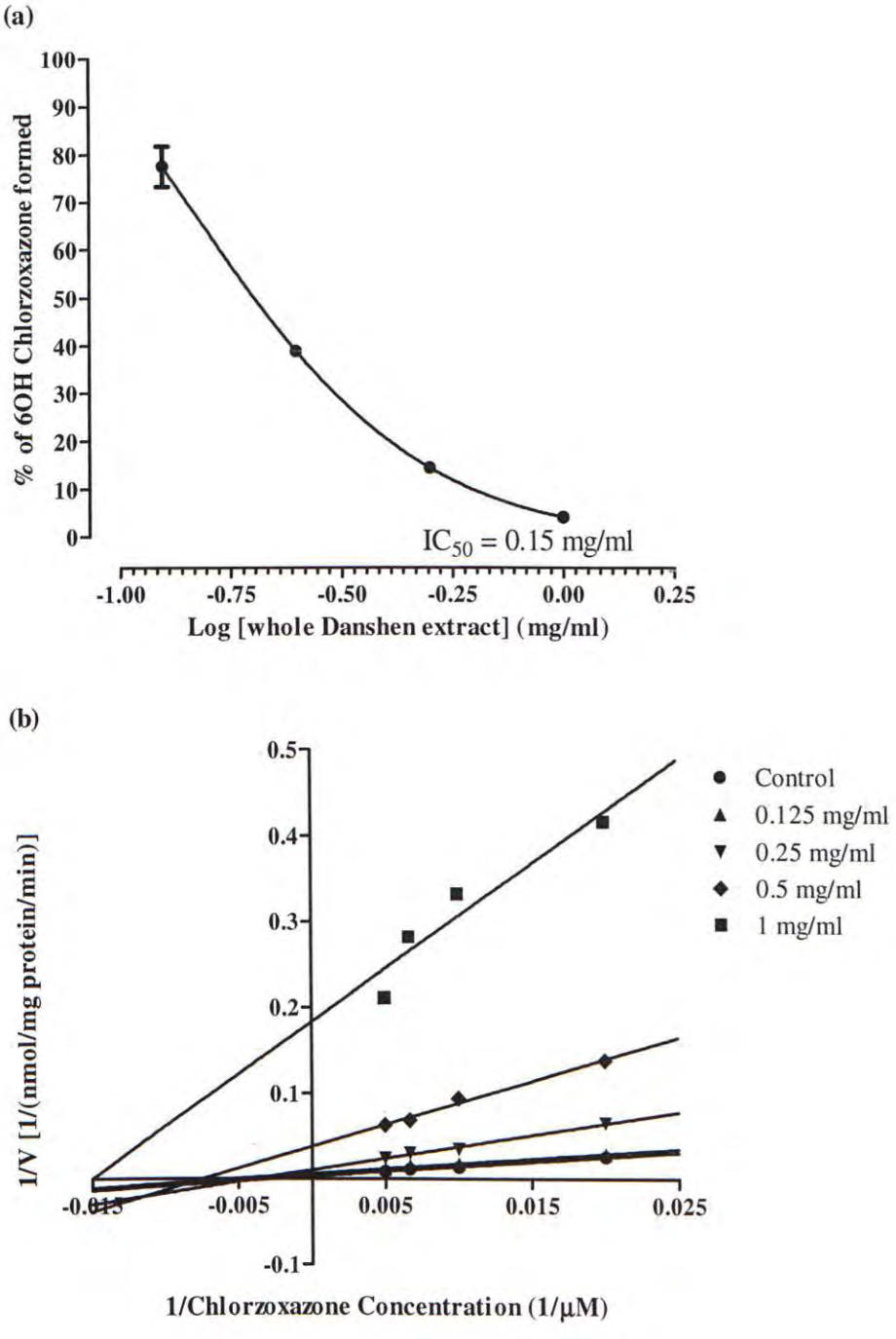



Fig. 2.25 (a) Inhibition curve showing effects of whole Danshen extract (0.125 mg/ml – 1 mg/ml) on formation of 6-hydroxychlorzoxazone in human liver microsomes. Values are mean ± SEM of triplicate determinations. (b) Lineweaver-Burk Plot for the inhibition of CYP2E1-mediated chlorzoxazone hydroxylation by whole Danshen extract (0.125 mg/ml – 1 mg/ml) in human liver microsomes. Values are mean of triplicate determinations.

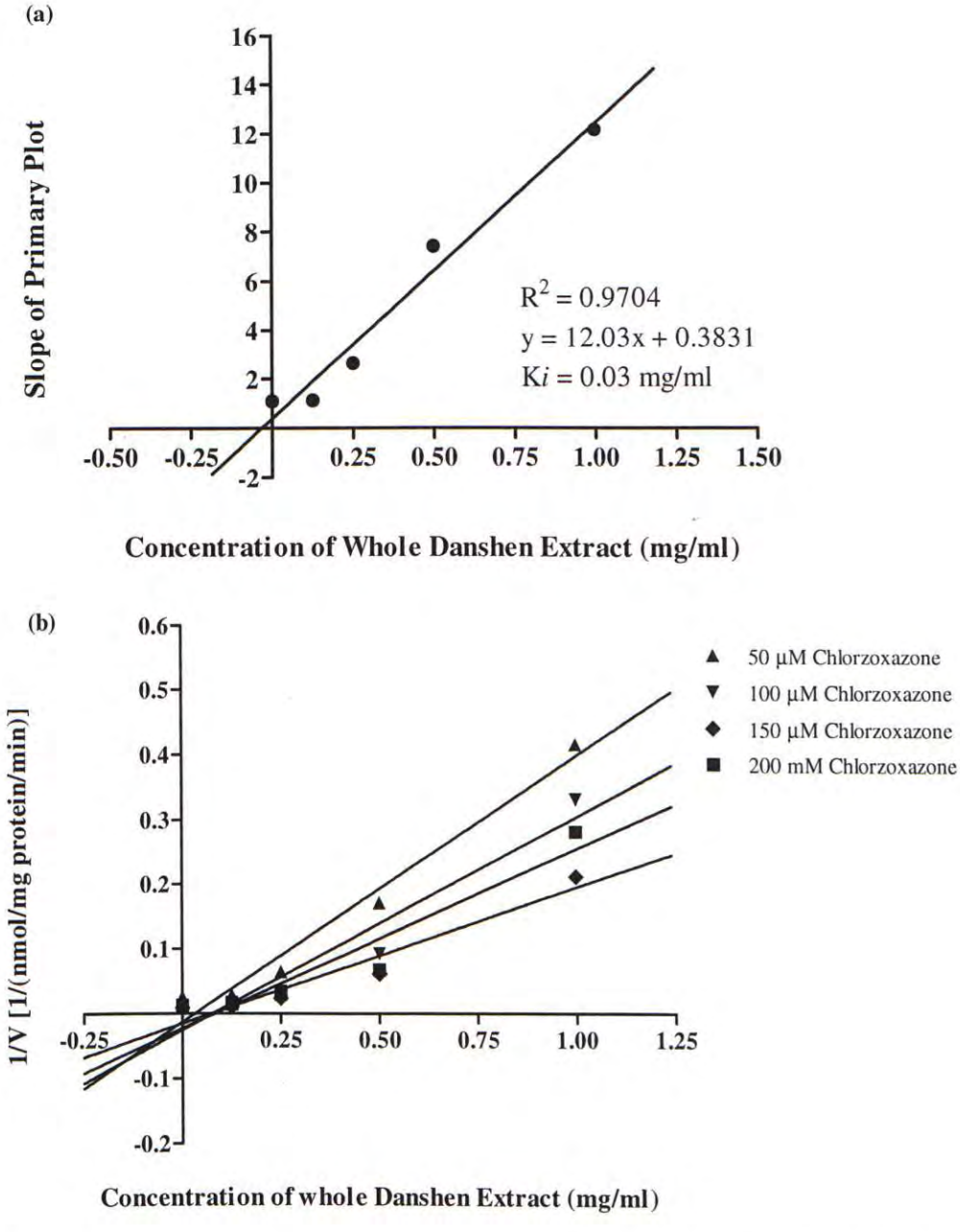



Fig. 2.26 (a) Secondary plot of Lineweaver-Burk Plot and (b) Dixon Plot show inhibitory effects of whole Danshen extract (0.125 mg/ml – 1 mg/ml) on CYP2E1-mediated chlorzoxazone hydroxylation in human liver microsomes. Values are mean of triplicate determinations.

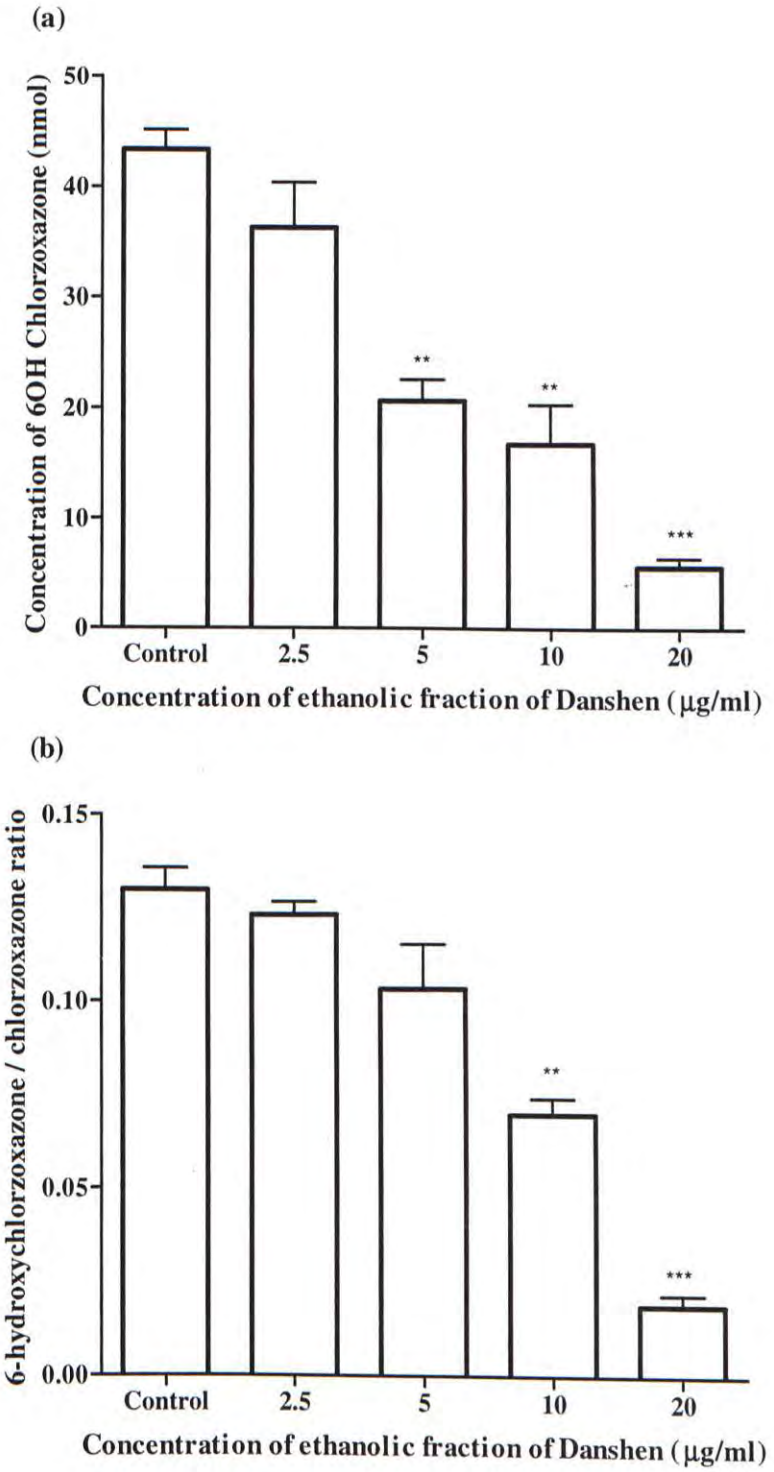



Fig. 2.27 Effects of ethanolic fraction of Danshen (2.5 μg/ml – 20 μg/ml) on (a) formation of 6-hydroxychlorzoxazone and (b) ratio of 6-hydroxychlorzoxazone to chlorzoxazone in human microsomes. Values are mean ± SEM of triplicate determinations. $^{**}p < 0.01$ and $^{***}p < 0.001$ when compared with controls.

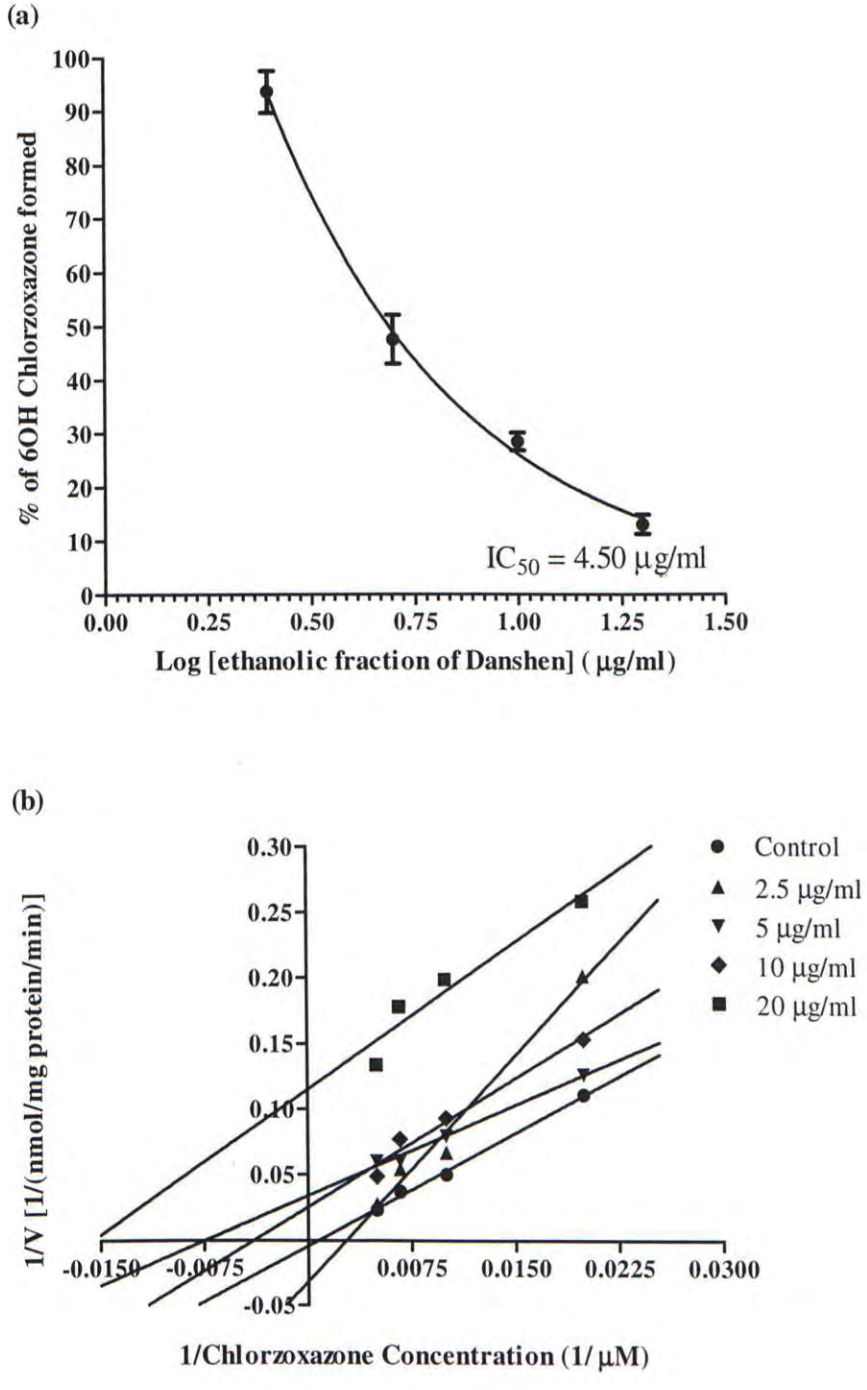



Fig. 2.28 (a) Inhibition curve showing effects of ethanolic fraction of Danshen (2.5 µg/ml – 20 µg/ml) on formation of 6-hydroxychlorzoxazone in human liver microsomes. Values are mean ± SEM of triplicate determinations. (b) Lineweaver-Burk Plot for the inhibition of CYP2E1-mediated chlorzoxazone hydroxylation by ethanolic fraction of Danshen (2.5 µg/ml – 20 µg/ml) in human liver microsomes. Values are mean of triplicate determinations.

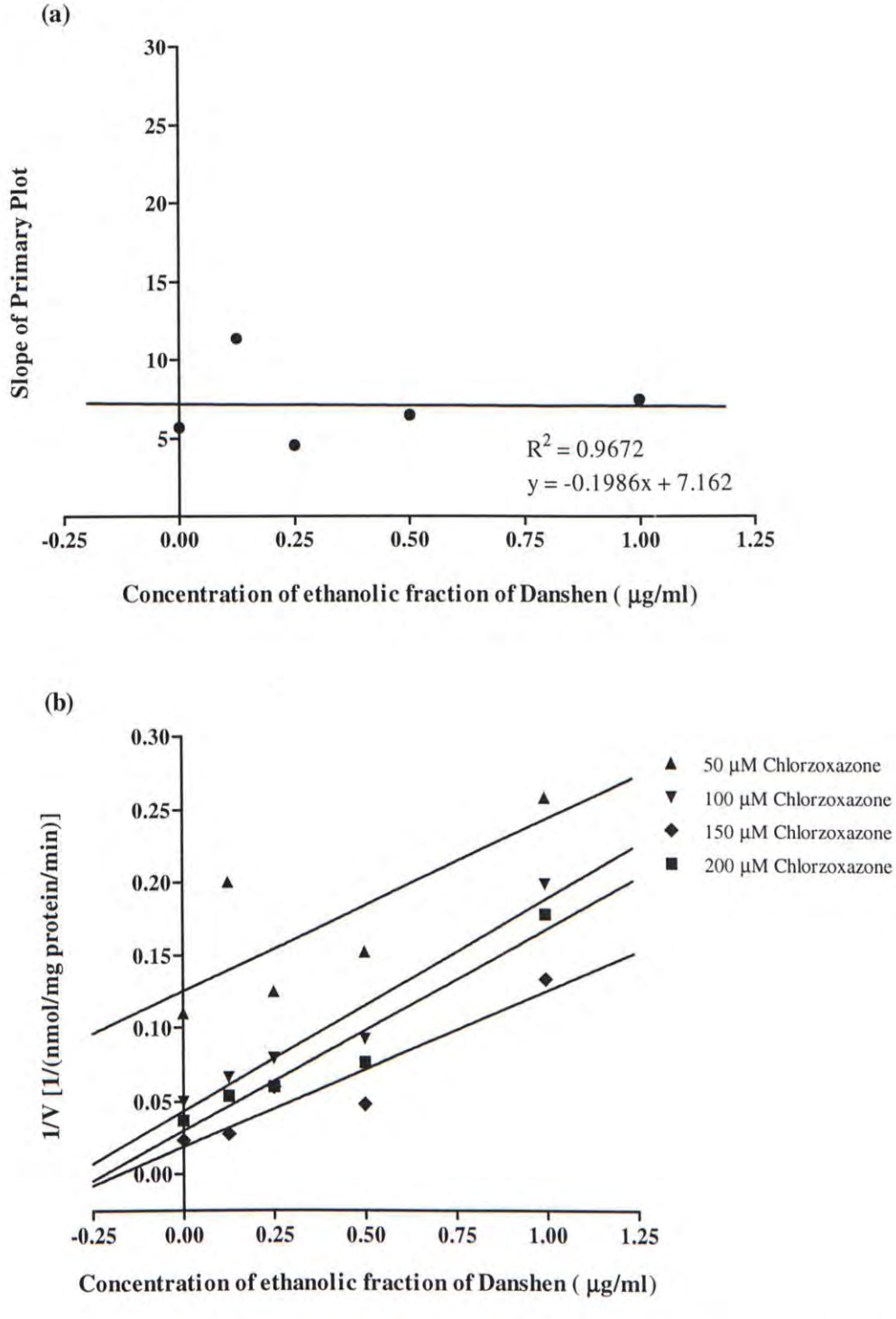



Fig. 2.29 (a) Secondary plot of Lineweaver-Burk Plot and (b) Dixon Plot show inhibitory effects of ethanolic fraction of Danshen (2.5 µg/ml – 20 µg/ml) on CYP2E1-mediated chlorzoxazone hydroxylation in human liver microsomes. Values are mean of triplicate determinations.

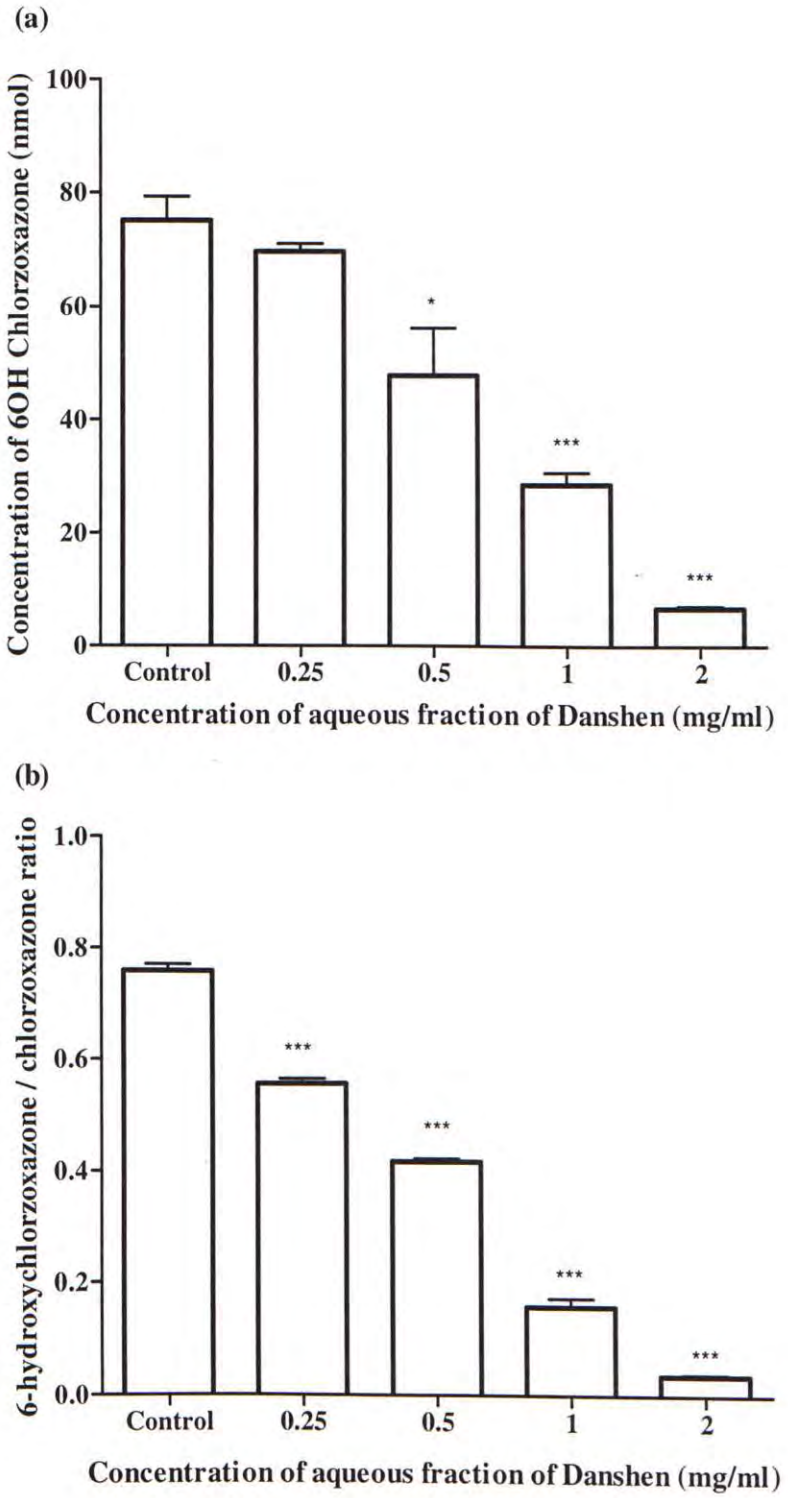



Fig. 2.30 Effects of aqueous fraction of Danshen (0.25 mg/ml – 2 mg/ml) on (a) formation of 6-hydroxychlorzoxazone and (b) ratio of 6-hydroxychlorzoxazone to chlorzoxazone in human microsomes. Values are mean ± SEM of triplicate determinations. *$p < 0.05$ and ***$p < 0.001$ when compared with controls.

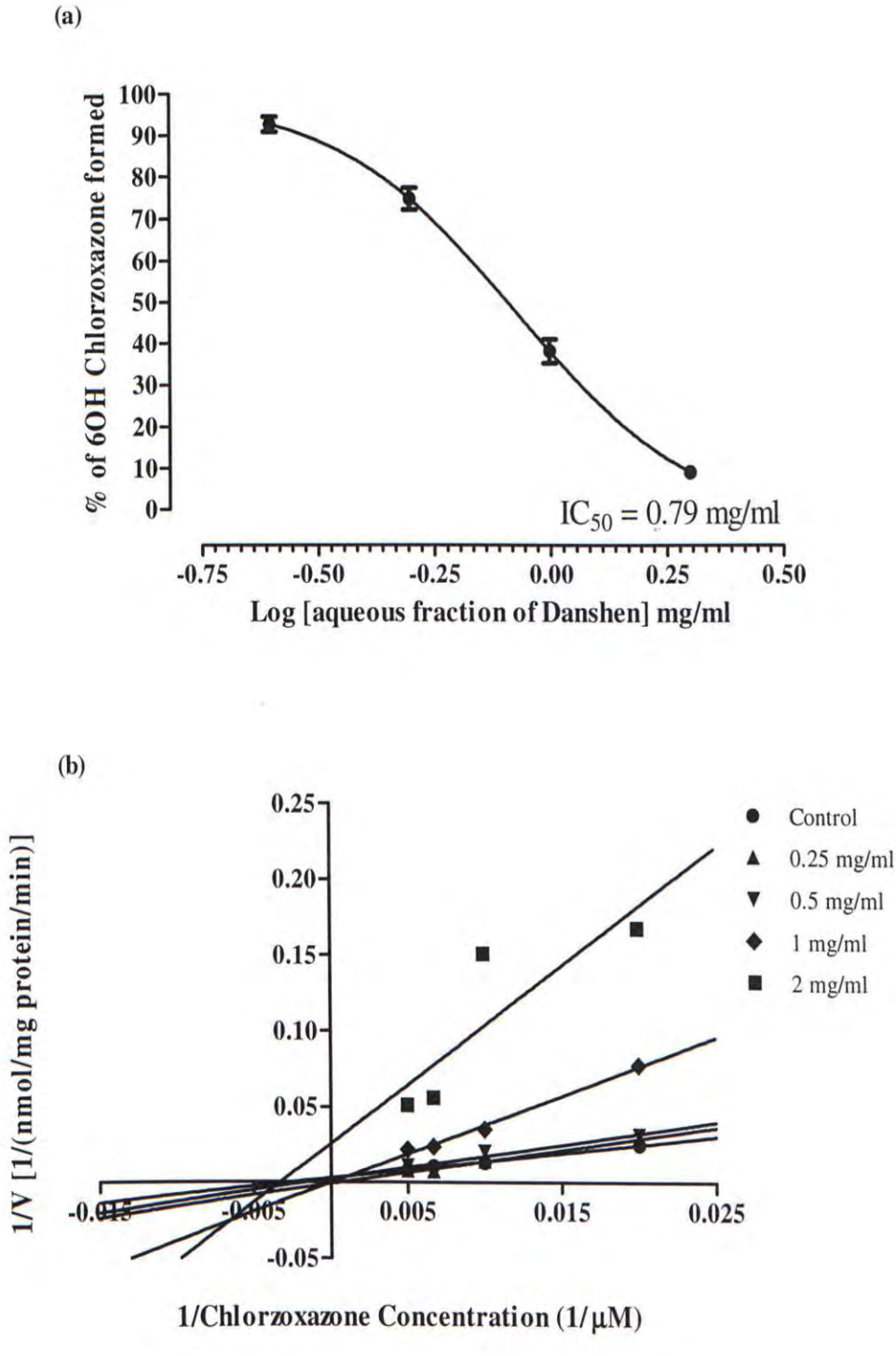



Fig. 2.31 (a) Inhibition curve showing effects of aqueous fraction of Danshen (0.25 mg/ml – 2 mg/ml) on formation of 6-hydroxychlorzoxazone in human liver microsomes. Values are mean ± SEM of triplicate determinations. (b) Lineweaver-Burk Plot for the inhibition of CYP2E1-mediated chlorzoxazone hydroxylation by aqueous fraction of Danshen (0.25 mg/ml – 2 mg/ml) in human liver microsomes. Values are mean of triplicate determinations.

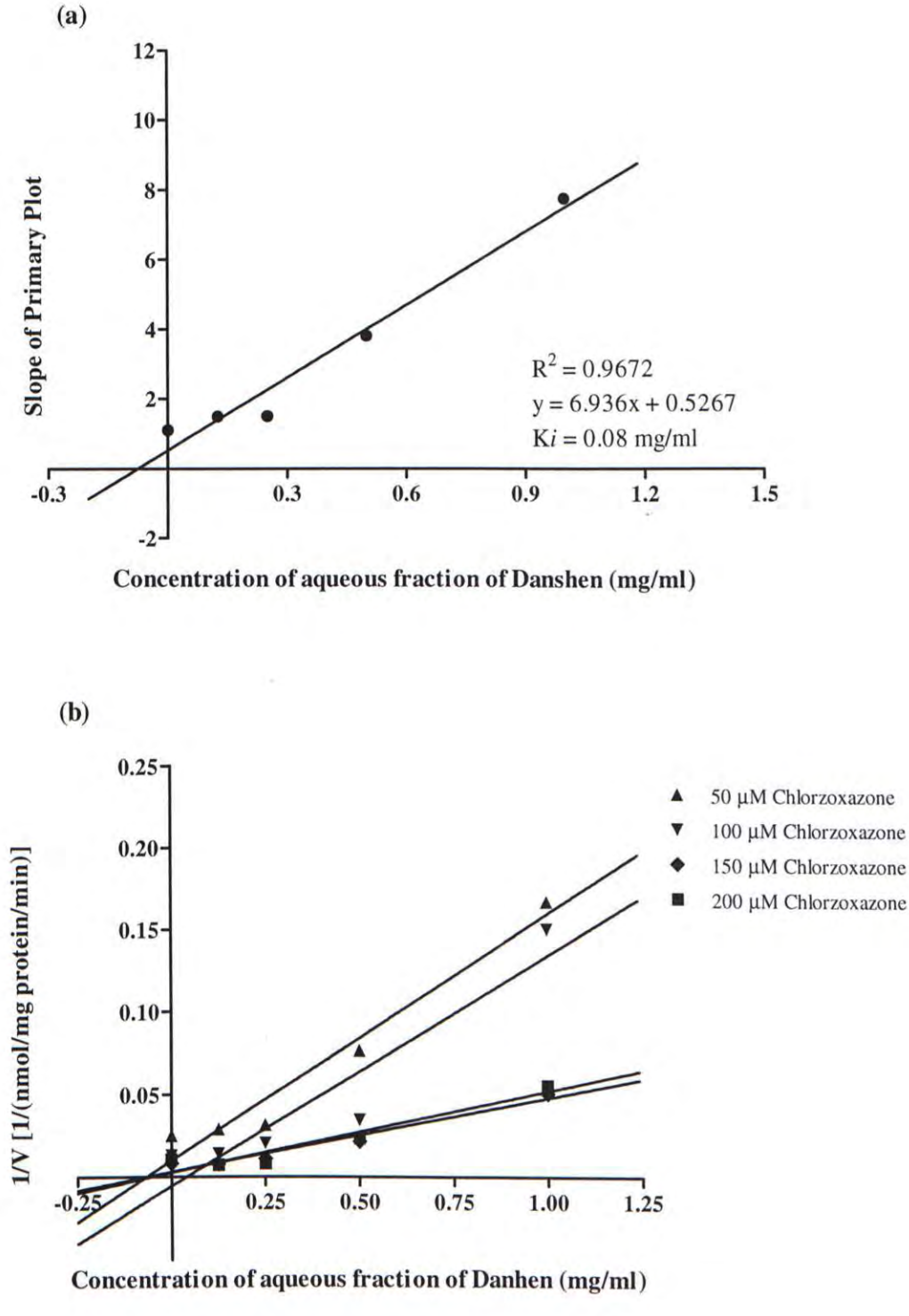



Fig. 2.32 (a) Secondary plot of Lineweaver-Burk Plot and (b) Dixon Plot show inhibitory effects of aqueous fraction of Danshen (0.25 mg/ml – 2 mg/ml) on CYP2E1-mediated chlorzoxazone hydroxylation in human liver microsomes. Values are mean of triplicate determinations.

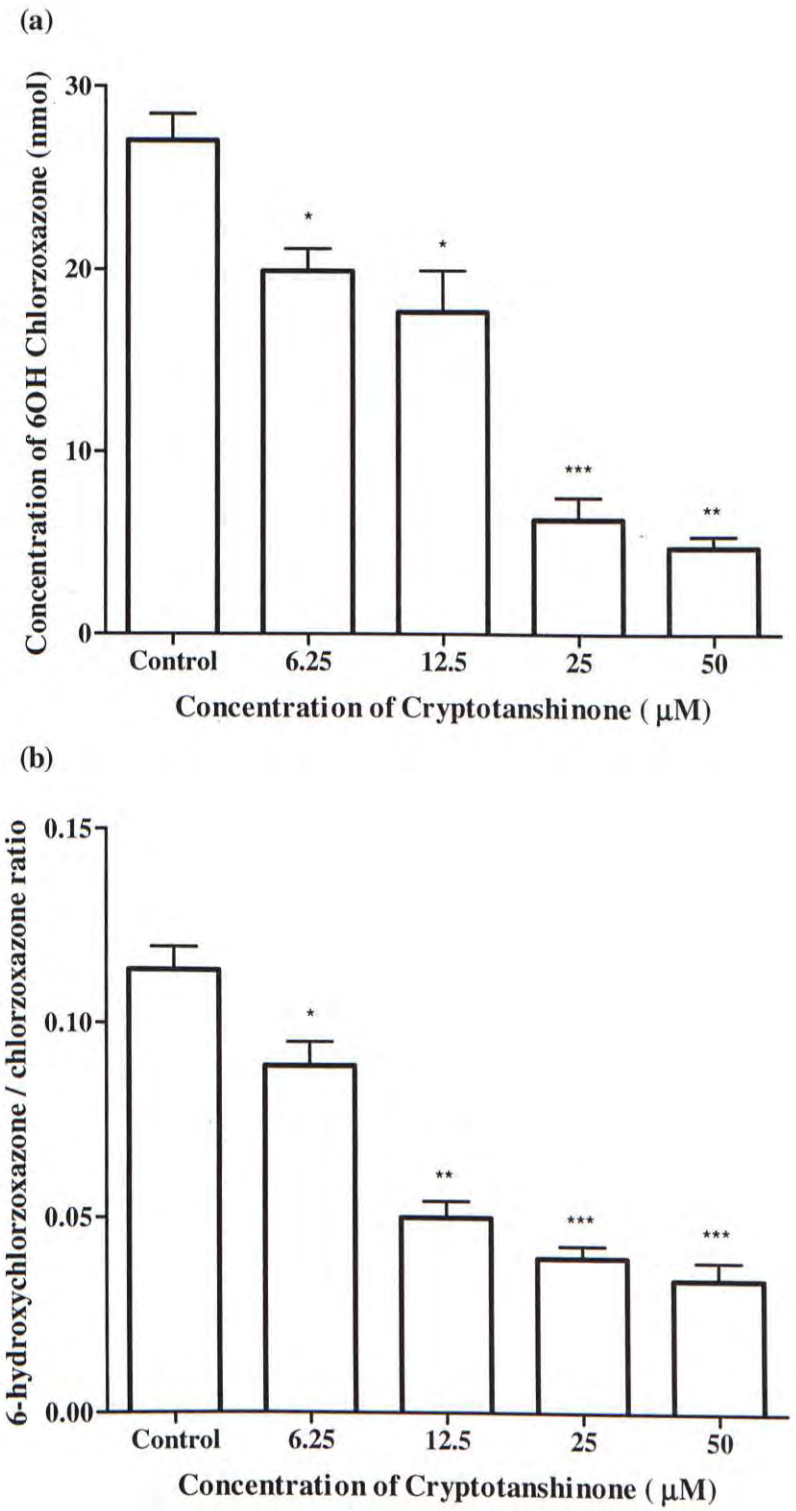



Fig. 2.33 Effects of cryptotanshinone (6.25 μM – 50 μM) on (a) formation of 6-hydroxychlorzoxazone and (b) ratio of 6-hydroxychlorzoxazone to chlorzoxazone in human microsomes. Values are mean ± SEM of triplicate determinations. *$p <$ 0.05, **$p < 0.01$ and ***$p < 0.001$ when compared with controls.

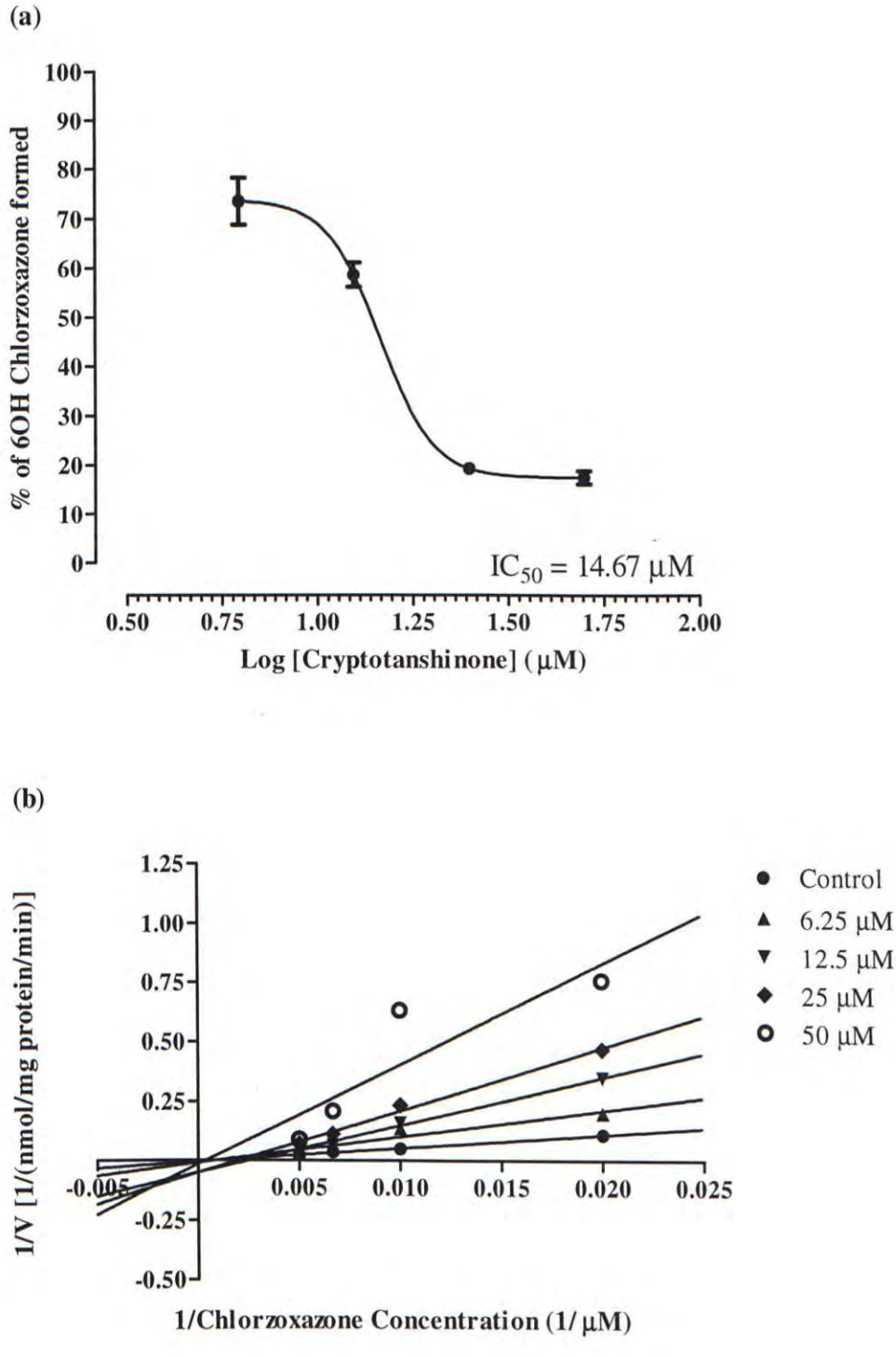



Fig. 2.34 (a) Inhibition curve showing effects of cryptotanshinone (6.25 µM – 50 µM) on formation of 6-hydroxychlorzoxazone in human liver microsomes. Values are mean ± SEM of triplicate determinations. (b) Lineweaver-Burk Plot for the inhibition of CYP2E1-mediated chlorzoxazone hydroxylation by cryptotanshinone (6.25 µM – 50 µM) in human liver microsomes. Values are mean of triplicate determinations.

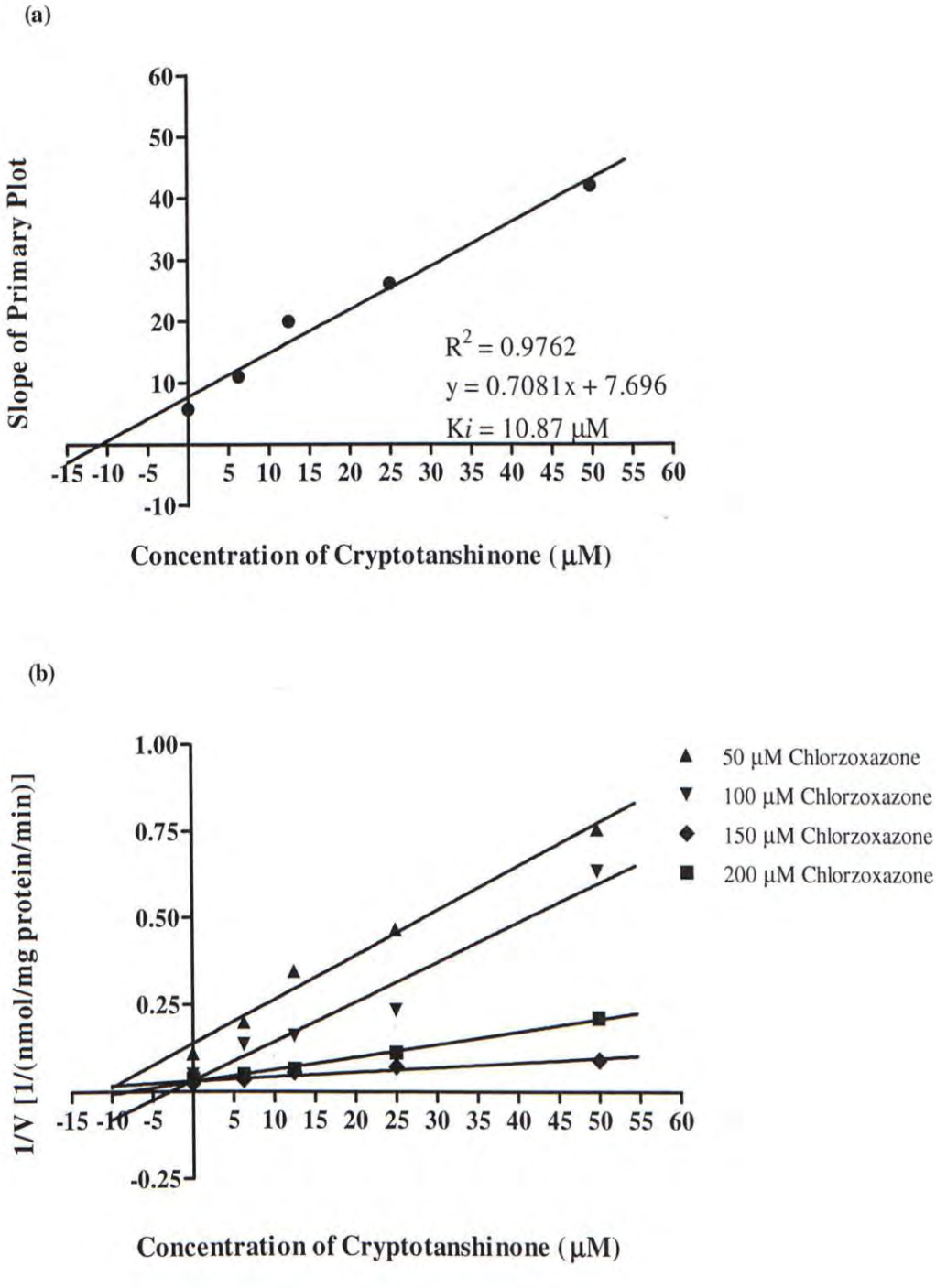



Fig. 2.35 (a) Secondary plot of Lineweaver-Burk Plot and (b) Dixon Plot show inhibitory effects of cryptotanshinone (6.25 µM – 50 µM) on CYP2E1-mediated chlorzoxazone hydroxylation in human liver microsomes. Values are mean of triplicate determinations.

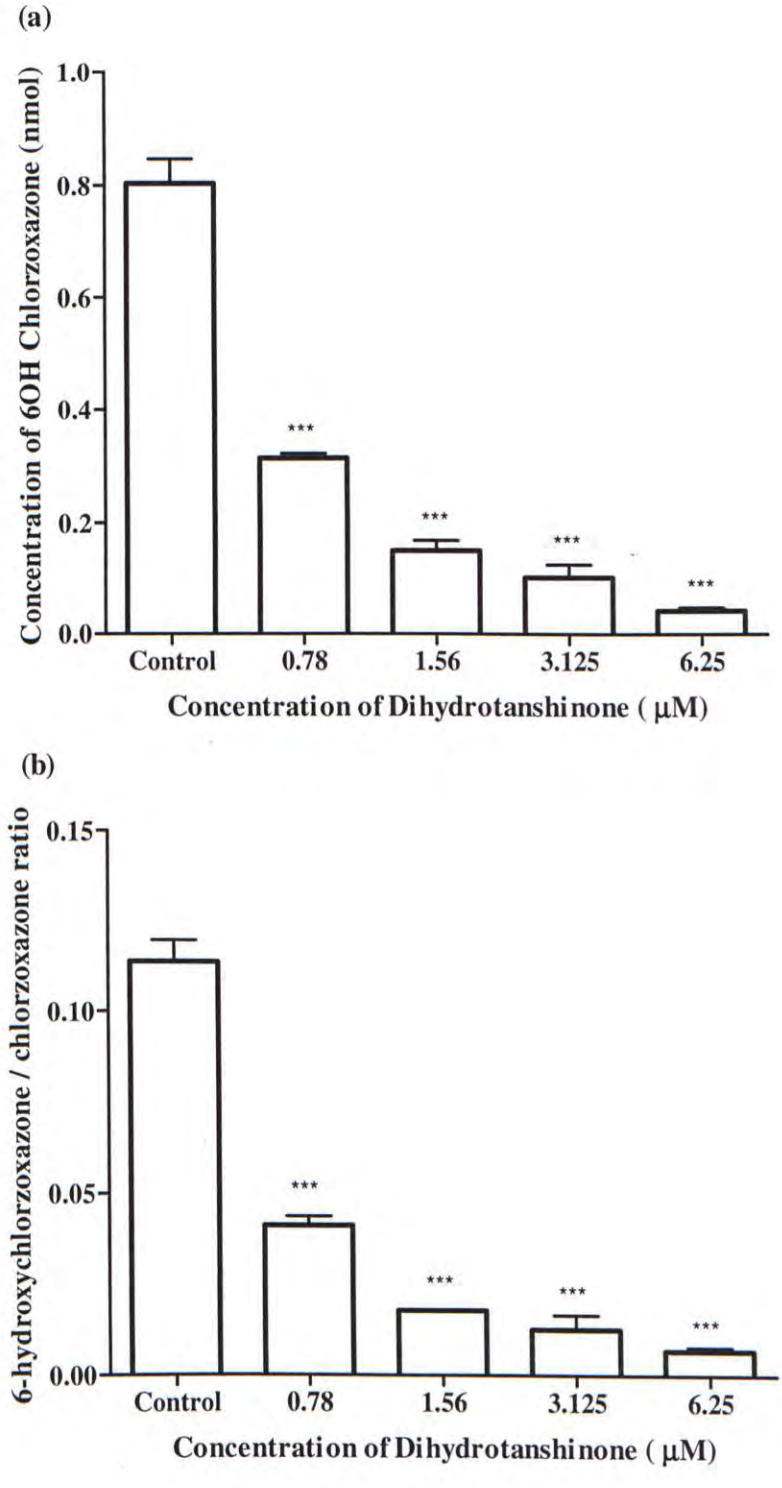
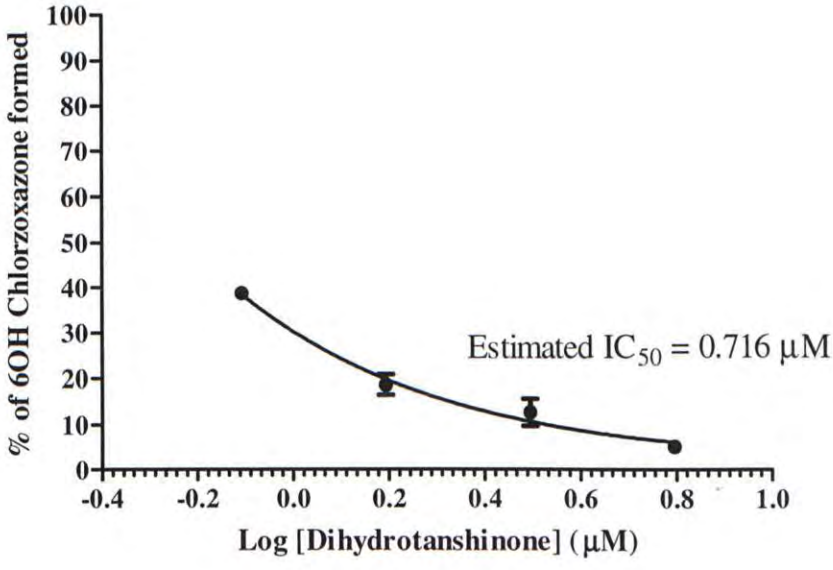



Fig. 2.36 Effects of dihydrotanshinone (0.78 μM – 6.25 μM) on (a) formation of 6-hydroxychlorzoxazone and (b) ratio of 6-hydroxychlorzoxazone to chlorzoxazone in human microsomes. Values are mean ± SEM of triplicate determinations. ***$p <$ 0.001 when compared with controls.

(a)




(b)

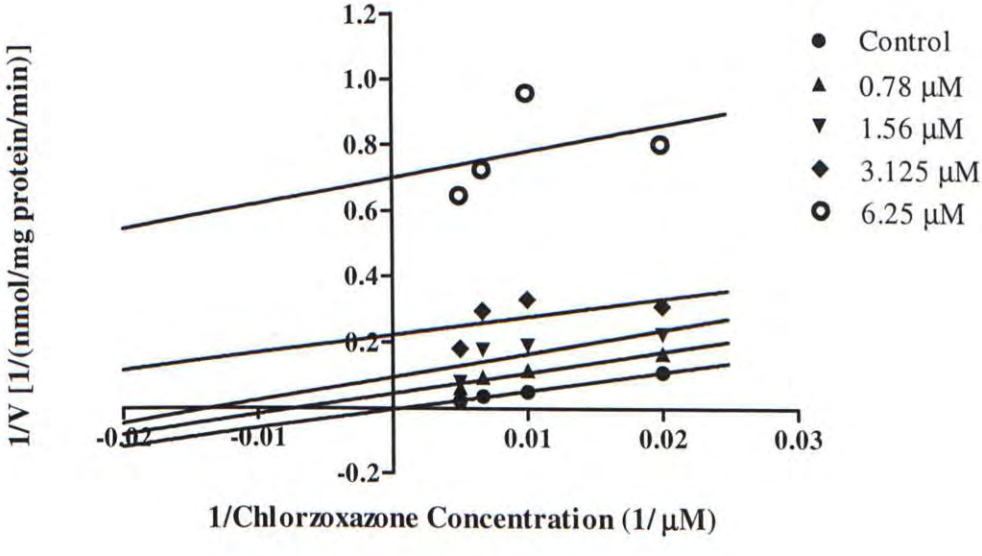



Fig. 2.37 (a) Inhibition curve showing effects of dihydrotanshinone (0.78 µM – 6.25 µM) on formation of 6-hydroxychlorzoxazone in human liver microsomes. Values are mean ± SEM of triplicate determinations. (b) Lineweaver-Burk Plot for the inhibition of CYP2E1-mediated chlorzoxazone hydroxylation by dihydrotanshinone (0.78 µM – 6.25 µM) in human liver microsomes. Values are mean of triplicate determinations.

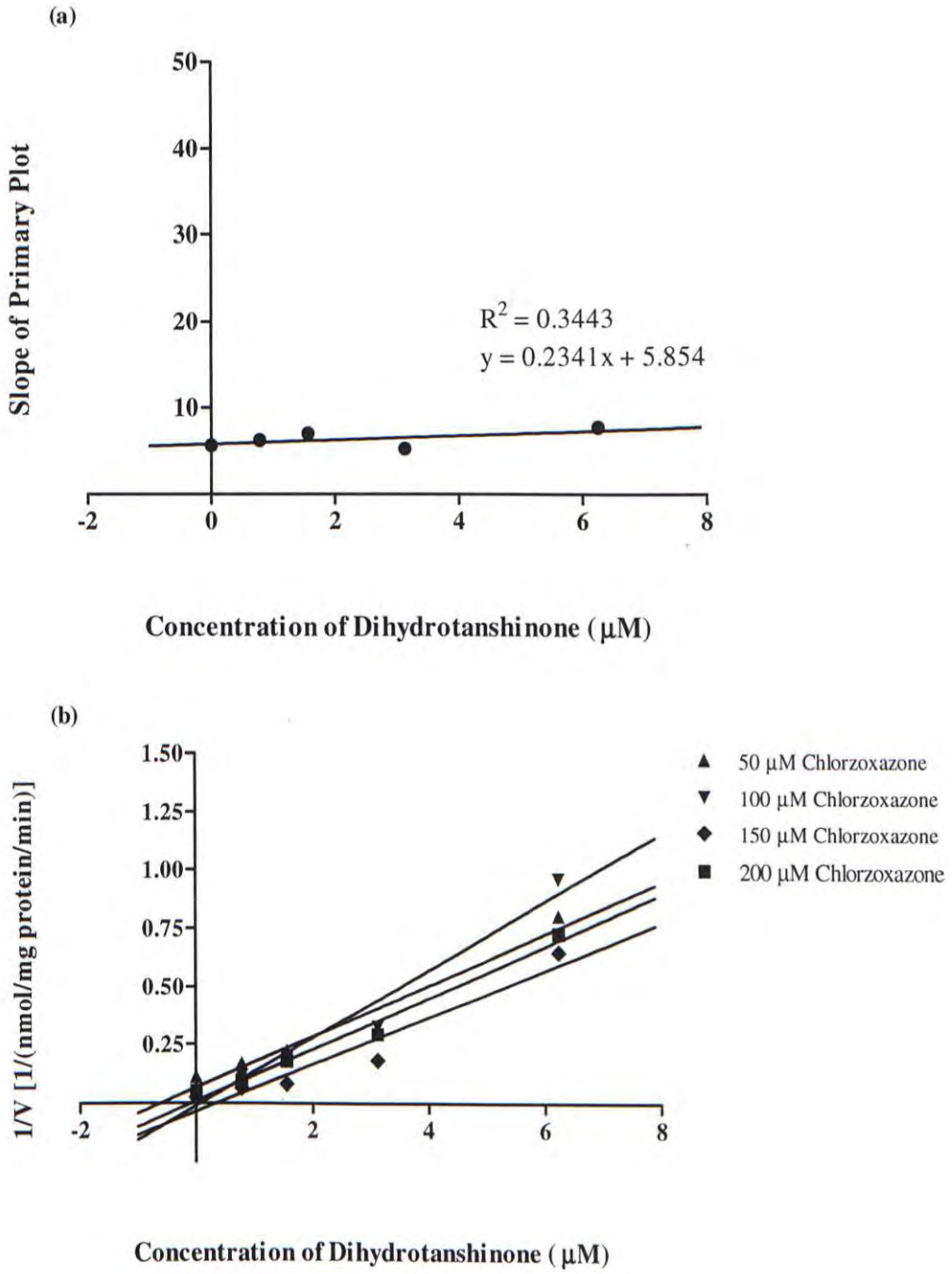



Fig. 2.38 (a) Secondary plot of Lineweaver-Burk Plot and (b) Dixon Plot show inhibitory effects of dihydrotanshinone (0.78 μM – 6.25 μM) on CYP2E1-mediated chlorzoxazone hydroxylation in human liver microsomes. Values are mean of triplicate determinations.

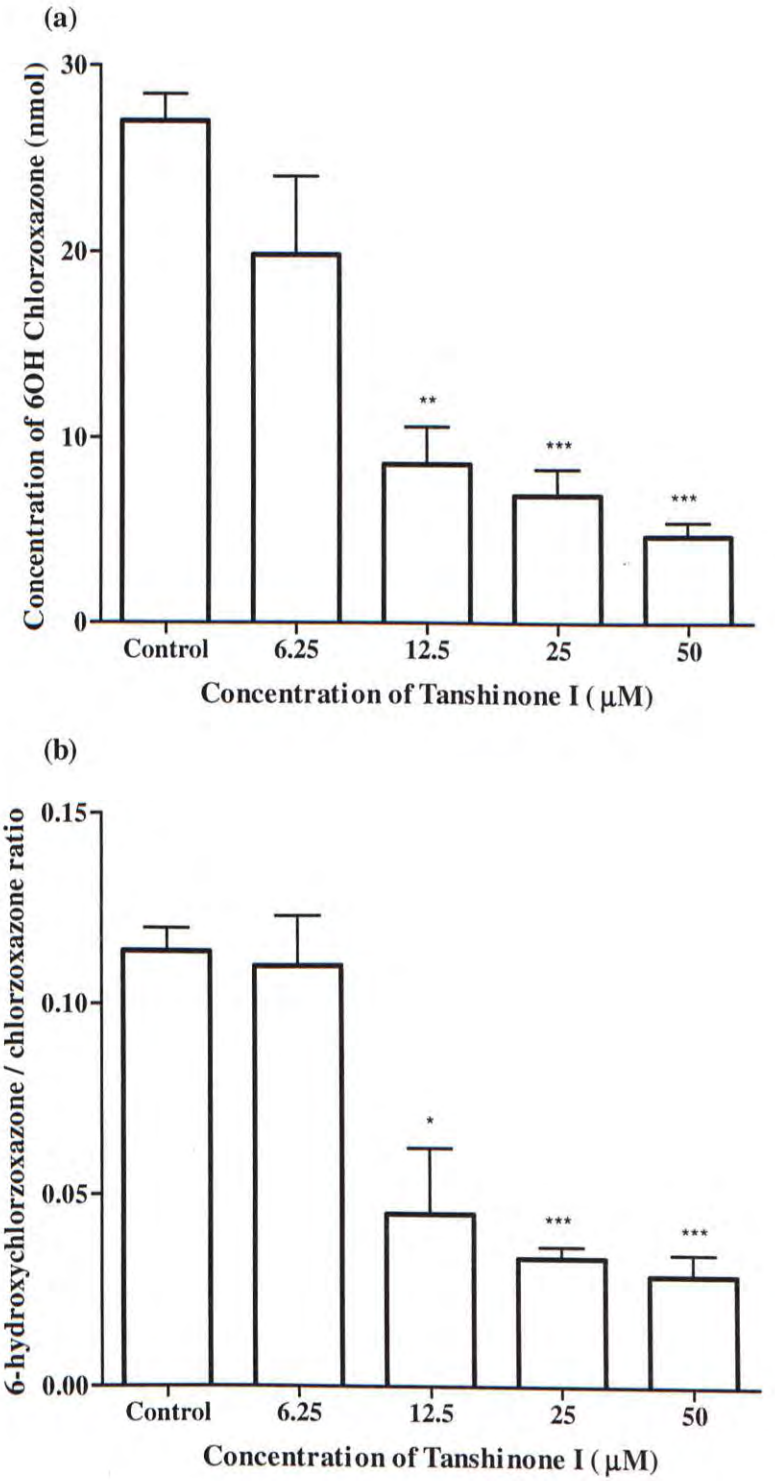



Fig. 2.39 Effects of tanshinone I (6.25 μM – 50 μM) on (a) formation of 6-hydroxychlorzoxazone and (b) ratio of 6-hydroxychlorzoxazone to chlorzoxazone in human microsomes. Values are mean ± SEM of triplicate determinations. *$p <$ 0.05, **$p < 0.01$and ***$p < 0.001$ when compared with controls.

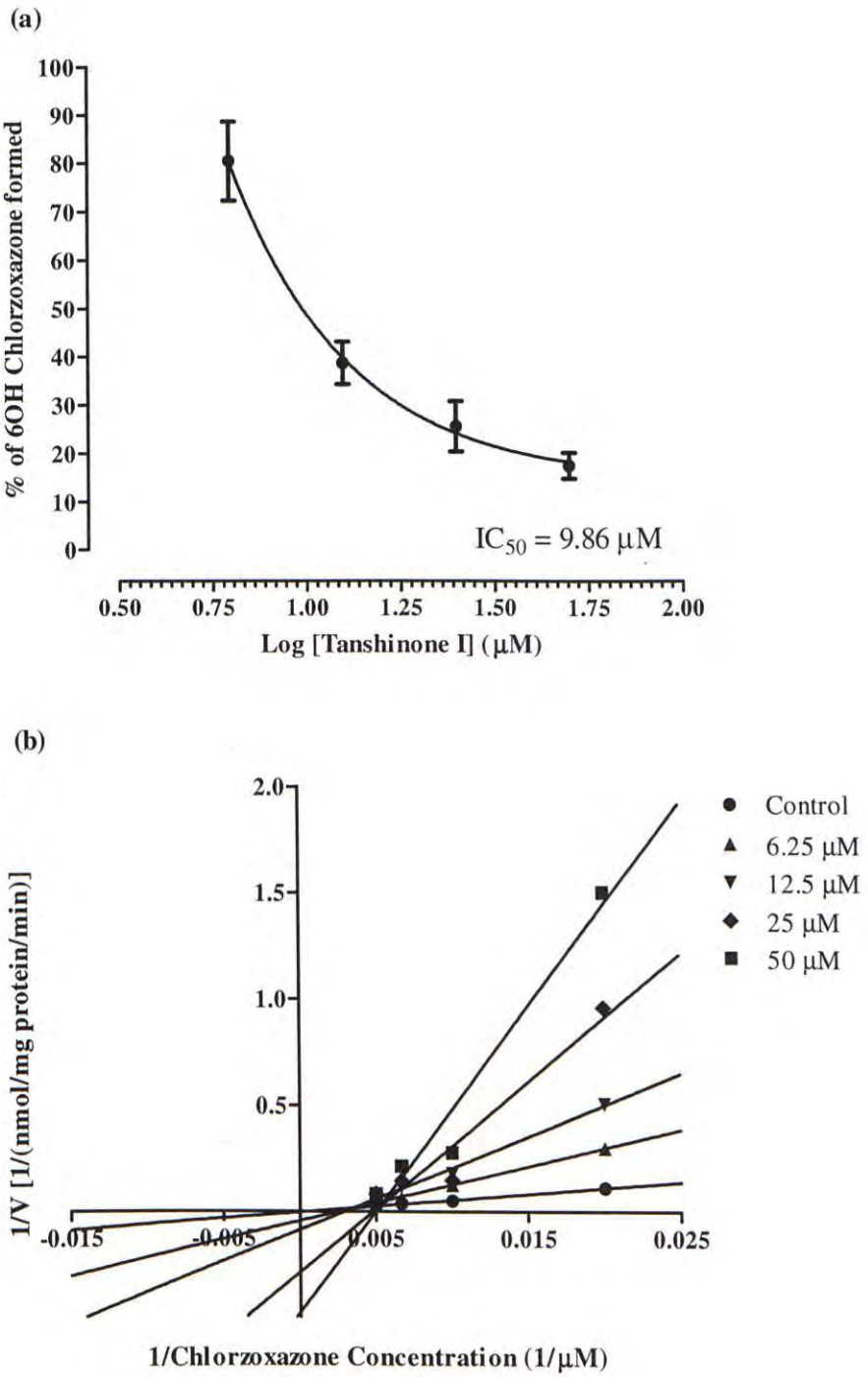



Fig. 2.40 (a) Inhibition curve showing effects of tanshinone I (6.25 µM – 50 µM) on formation of 6-hydroxychlorzoxazone in human liver microsomes. Values are mean ± SEM of triplicate determinations. (b) Lineweaver-Burk Plot for the inhibition of CYP2E1-mediated chlorzoxazone hydroxylation by tanshinone I (6.25 µM – 50 µM) in human liver microsomes. Values are mean of triplicate determinations.

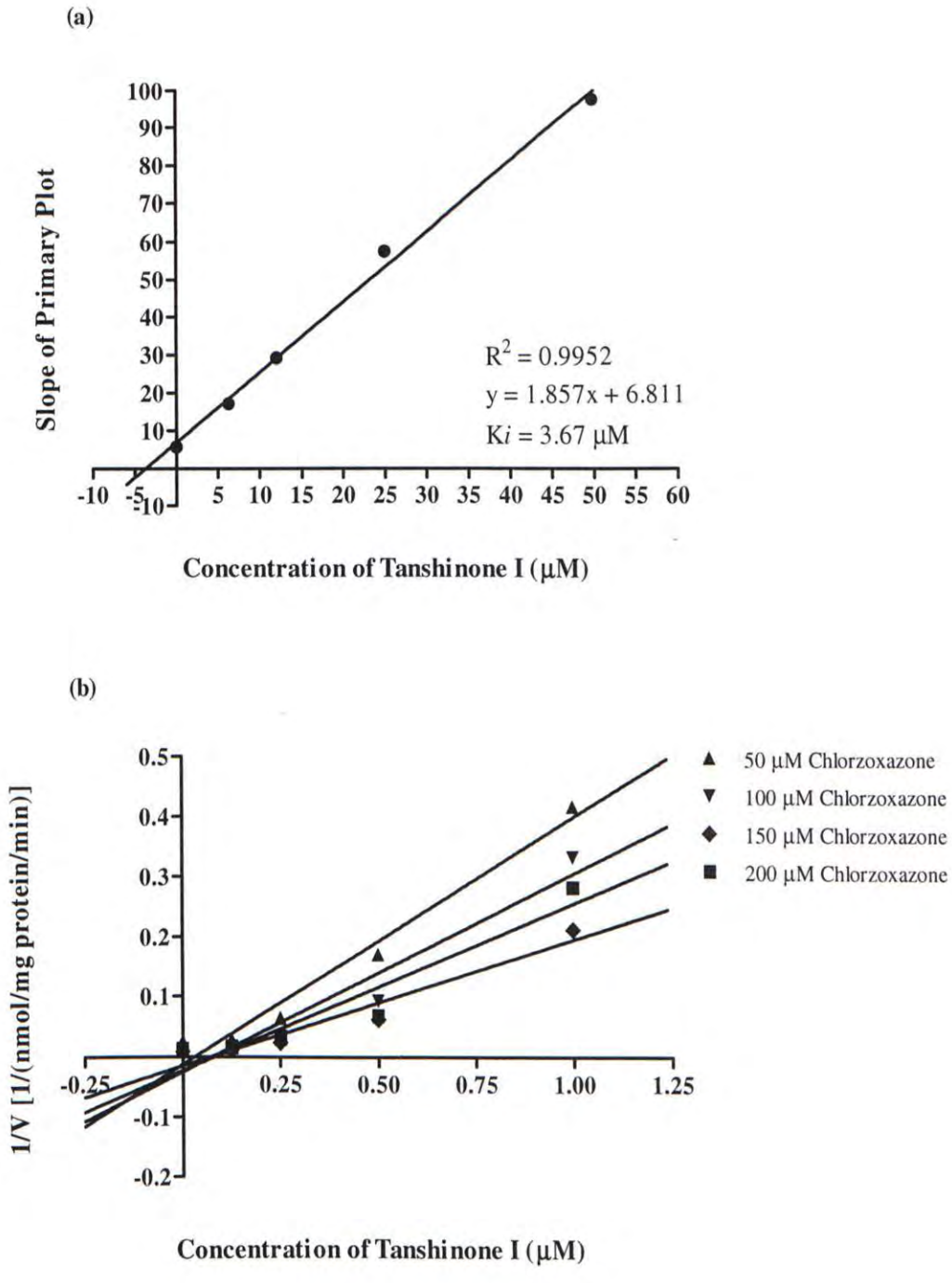



Fig. 2.41 (a) Secondary plot of Lineweaver-Burk Plot and (b) Dixon Plot show inhibitory effects of tanshinone I (6.25 µM – 50 µM) on CYP2E1-mediated chlorzoxazone hydroxylation in human liver microsomes. Values are mean of triplicate determinations.

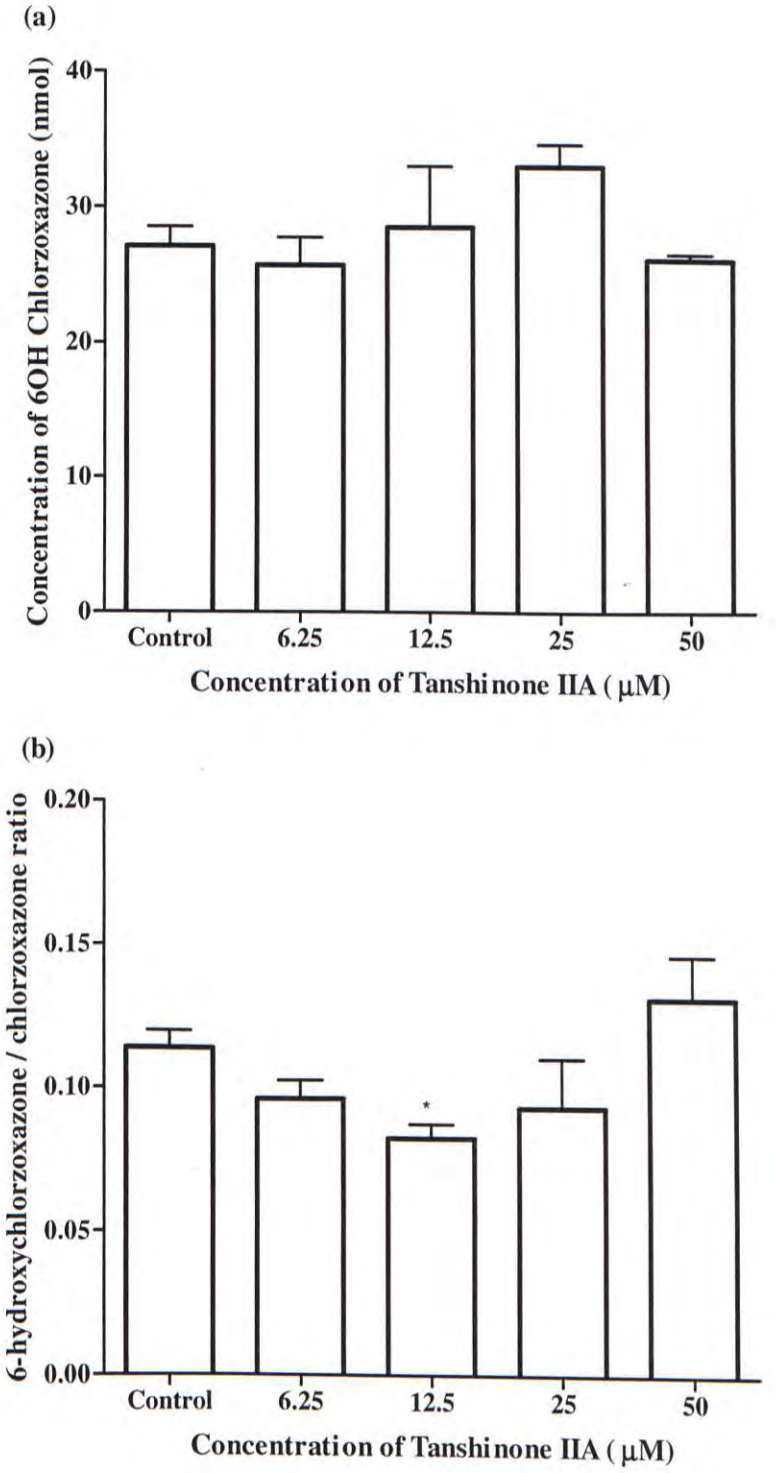



Fig. 2.42 Effects of tanshinone IIA (6.25 μM – 50 μM) on (a) formation of 6-hydroxychlorzoxazone and (b) ratio of 6-hydroxychlorzoxazone to chlorzoxazone in human microsomes. Values are mean ± SEM of triplicate determinations. *$p$ < 0.05 when compared with controls.

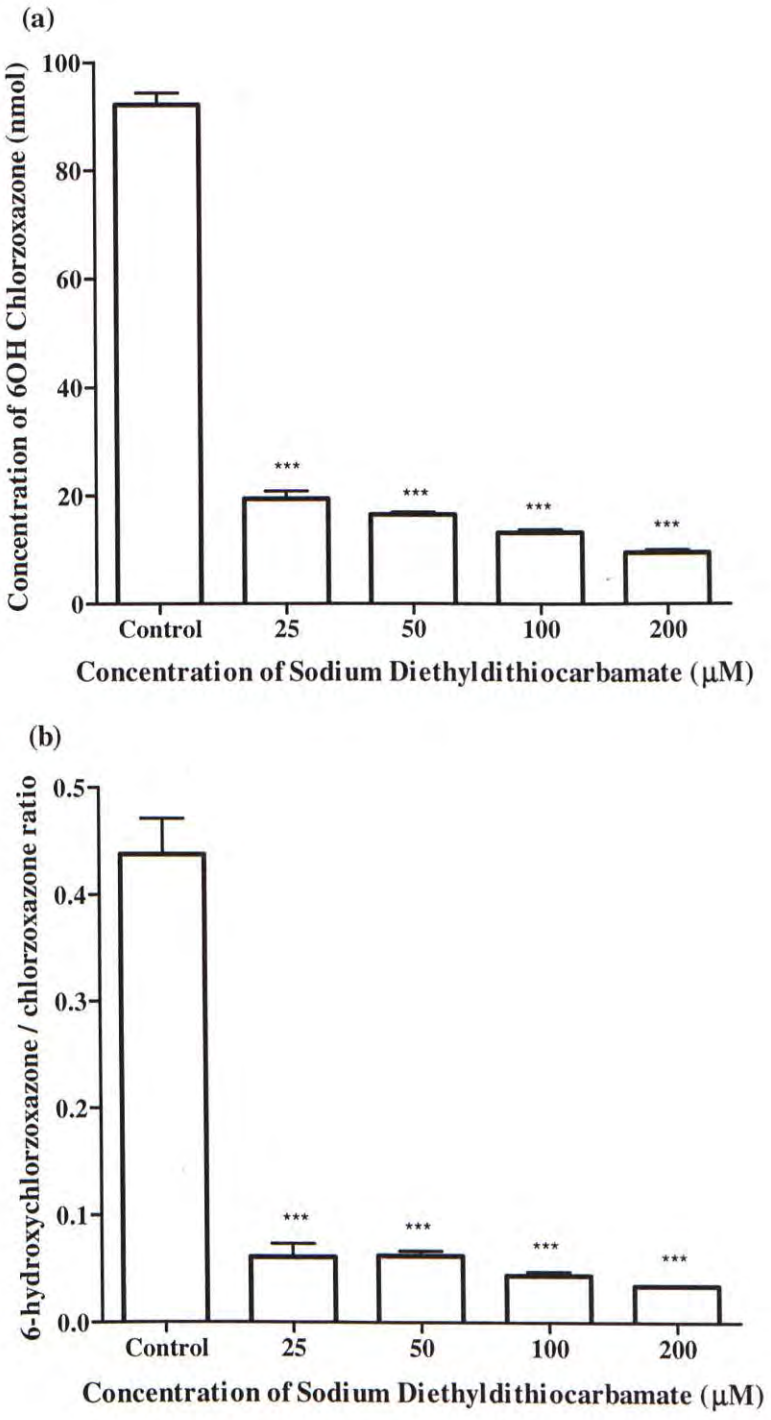



Fig. 2.43 Effects of sodium diethyldithiocarbamate (25 μM – 200 μM) on (a) formation of 6-hydroxychlorzoxazone and (b) ratio of 6-hydroxychlorzoxazone to chlorzoxazone in human microsomes. Values are mean ± SEM of triplicate determinations. ***$p < 0.001$ when compared with controls.

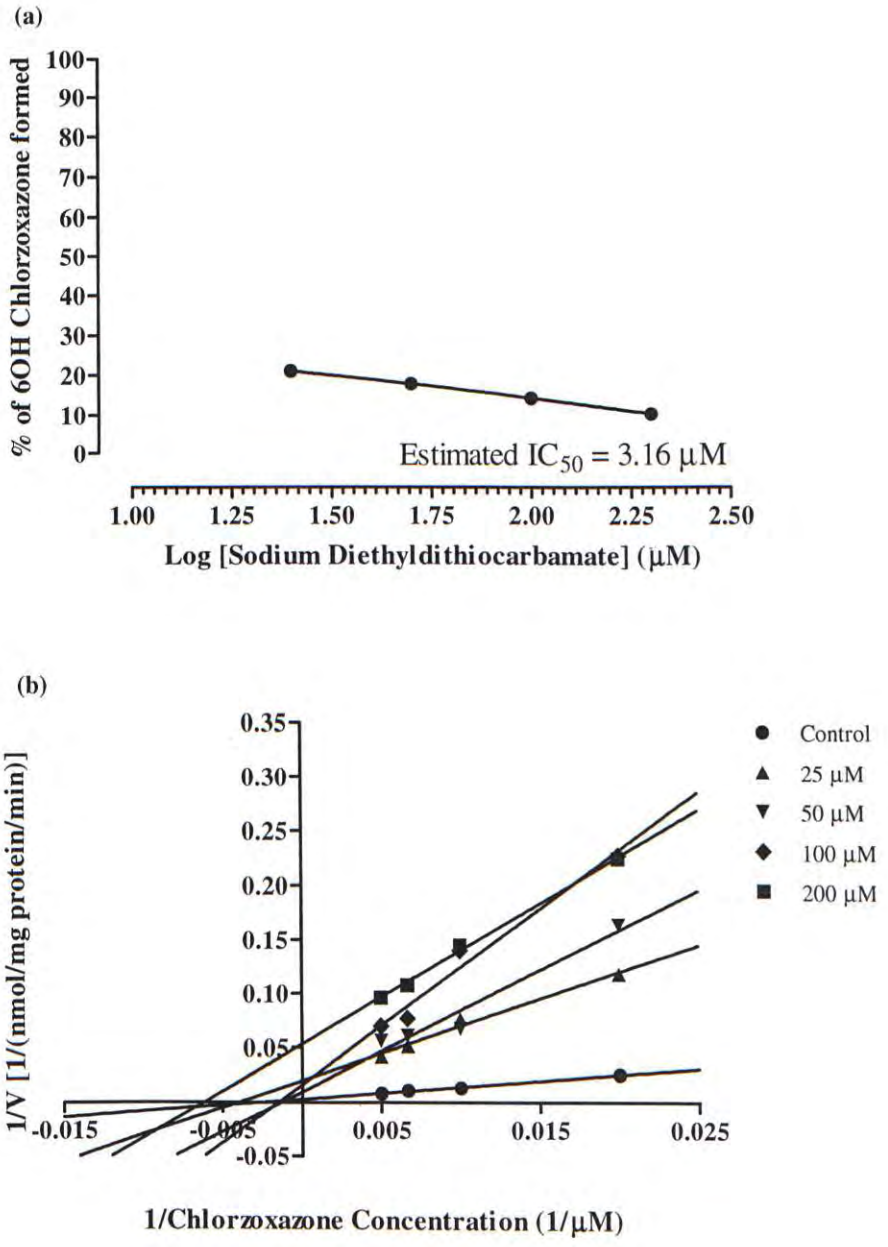



Fig. 2.44 (a) Inhibition curve showing effects of sodium diethyldithiocarbamate (25 µM – 200 µM) on formation of 6-hydroxychlorzoxazone in human liver microsomes. Values are mean ± SEM of triplicate determinations. (b) Lineweaver-Burk Plot for the inhibition of CYP2E1-mediated chlorzoxazone hydroxylation by sodium diethyldithiocarbamate (25 µM – 200 µM) in human liver microsomes. Values are mean of triplicate determinations.

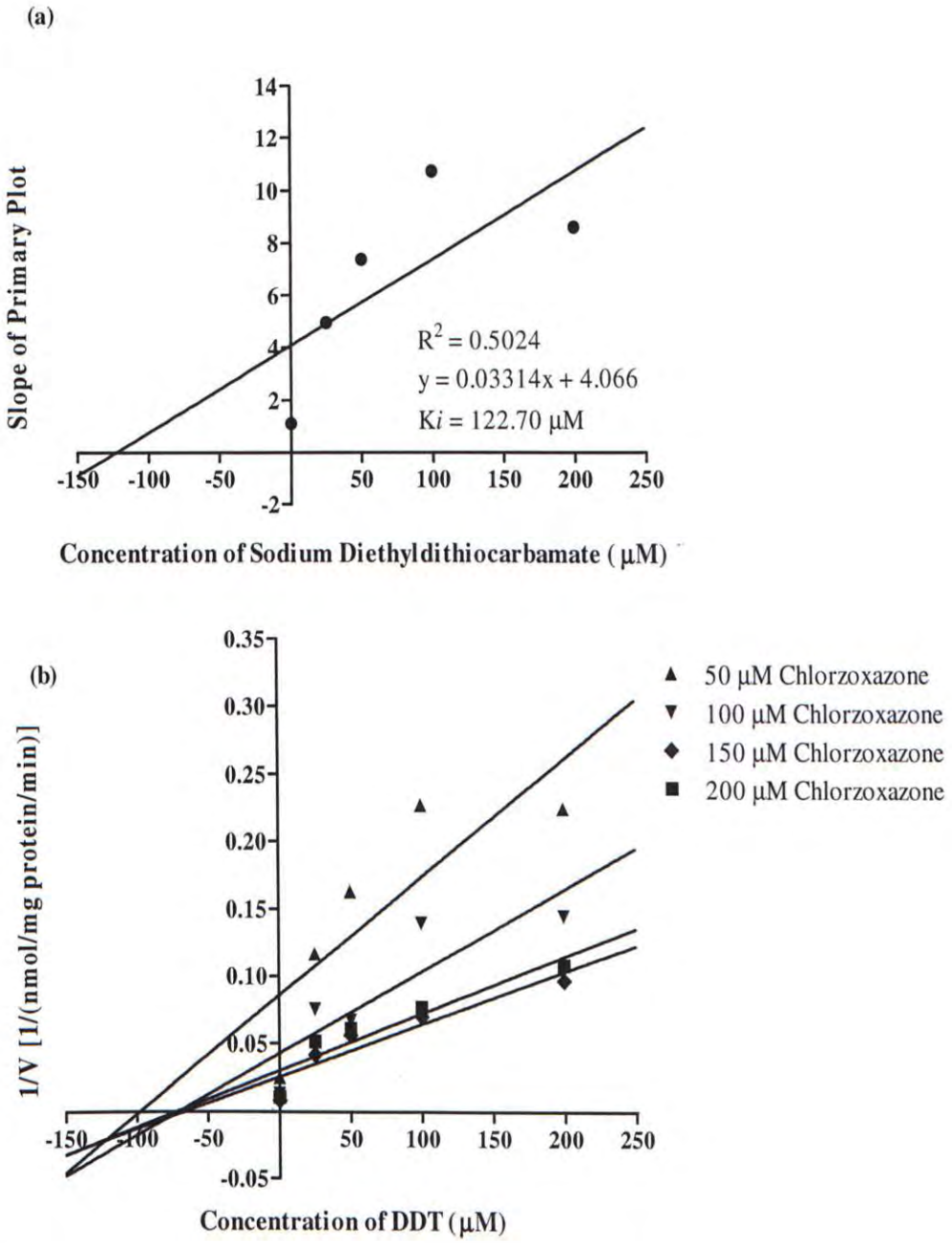



Fig. 2.45 (a) Secondary plot of Lineweaver-Burk Plot and (b) Dixon Plot show inhibitory effects of tanshinone I (6.25 µM – 50 µM) on CYP2E1-mediated chlorzoxazone hydroxylation in human liver microsomes. Values are mean of triplicate determinations.

### 2.3.2.1 Summary

Whole Danshen extract, the ethanolic fraction of Danshen and the aqueous fraction of Danshen exerted inhibitory effects on CYP2E1 activity in human liver microsomes with non-competitive, uncompetitive and competitive inhibitions, respectively. The ethanolic fraction of Danshen ($IC_{50}$ at 4.50 μg/ml) was more effective in decreasing CZX metabolism than the aqueous fraction of Danshen ($IC_{50}$ at 0.79 mg/ml). Dihydrotanshinone was the most effective in inhibiting CYP2E1 activity ($IC_{50}$ at 0.72 μM), followed by tanshinone I and cryptotanshinone. Dihydrotanshinone showed uncompetitive inhibition and therefore K*i* cannot be determined from the Secondary Lineweaver-Burk plot and Dixon plot. Cryptotanshinone competitively inhibited 6-hydroxylation of chlorzoxazone and tanshinone I showed non-competitive inhibition. Tanshinone IIA did not inhibit CYP2E1 activity in human liver microsomes *in vitro*.

Table 2.7 Inhibition of Danshen, its active components and sodium diethyldithiocarbamate on CYP2E1 activity in human liver microsomes *in vitro*.

| | $IC_{50}$ | K*i* | Inhibition Mode |
|---|---|---|---|
| **Whole Danshen Extract** | 0.15 mg/ml | 0.03 mg/ml | Non-competitive |
| **Ethanolic fraction fo Danshen** | 4.50 μg/ml | Cannot be determined | Uncompetitive |
| **Aqueous fraction of Danshen** | 0.79 mg/ml | 0.08 mg/ml | Competitive |
| **Cryptotanshinone** | 14.67 μM | 10.87 μM | Competitive |
| **Dihydrotanshinone** | 0.716 μM | Cannot be determined | Uncompetitive |
| **Tanshinone I** | 9.86 μM | 3.67 μM | Non-competitive |
| **Tanshinone IIA** | - | - | - |
| **Sodium diethyldithiocarbamate** | 3.16 μM | 122.70 μM | Non-competitive |

## 2.4 Discussion

This study showed that whole Danshen extract inhibited the CYP2E1 activites with a non-competitive mode of inhibition. The whole Danshen extract contains both the ethanolic and aqueous fractions of Danshen. The ethanolic fraction exerted a more potent inhibition ($IC_{50}$ at 18.25 μg/ml) on CYP2E1 activites than the aqueous fraction ($IC_{50}$ at 1.03 mg/ml). Tanshinones including cryptotanshinone, dihydrotanshinone and tanshinone I were found to have inhibitory effects on rat hepatic CYP2E1 activities while tanshinone IIA did not show any inhibitory effects. Dihydrotanshinone and cryptotanshinone were more potent than tanshinone I that the $IC_{50}$ of dihydrotanshinone was only 6.71 μM and cryptotanshinone was 20.47 μM. There are no previous study on the effects of Danshen on CYP2E1 enzymes, despite the effects of Danshen and its active components on several CYP enzymes, such as CYP1A2 and 3A4, had been studied. Previous studies have shown that whole Danshen extract competitively inhibited CYP1A2-mediated oxidation and the ethanolic components were more effective than the aqueous components. Dihydrotanshinone and cryptotanshinone were more potent to affect CYP1A2 activity compared to tanshinone I and tanshinone IIA (Wang et al., 2009). The aqueous fraction of Danshen did not produce inhibitory effects on rat hepatic CYP3A4 activities and only dihydrotanshinone exerted a potent inhibitory effects compared with the relatively weak inhibitions of cryptotanshinone, tanshinone I and tanshinone IIA (Wang, 2007). Taken together with previous studies, dihydrotanshinone plays an important role in inhibiting CYP-mediated hydroxylations involving CYP1A2, CYP2E1 and CYP3A4.

Table 2.8 $IC_{50}$ and K*i* values of cryptotanshinone, dihydrotanshinone, tanshinone I and tanshinone IIA on rat CYP2E1 and human CYP2E1 activities.

| Tanshinones | Rat CYP2E1 | | Human CYP2E1 | |
|---|---|---|---|---|
| | $IC_{50}$ | K*i* | $IC_{50}$ | K*i* |
| **Cryptotanshinone** | 20.47 μM | 87.86 μM | 14.67 μM | 10.87 μM |
| **Dihydrotanshinone** | 6.71 μM | Cannot be determined | 0.716 μM | Cannot be determined |
| **Tanshinone I** | $IC_{20}$: 8.22 μM | 78.74 μM | 9.86 μM | 3.67 μM |
| **Tanshinone IIA** | - | - | - | - |

In this experiment, pooled human liver microsomes have been used. From the data sheet of the supplier, most of the 24 donators of human liver microsomes had alcohol consumption habit for a long period of time. Therefore, hepatic CYP2E1 concentration would be prominent in the pooled human liver microsomes due to induction effect of ethanol. The effects of Danshen and tanshinones in human liver microsomes were similar to rat hepatic microsomes. The ethanolic fraction of Danshen ($IC_{50}$ at 4.50 μg/ml) was more effective in inhibiting CYP2E1 activities than the aqueous fraction of Danshen ($IC_{50}$ at 0.79 mg/ml). Dihydrotanshinone was again the most potent on inhibiting CYP2E1-mediated hydroxylation and tanshinone IIA did not inhibit human CYP2E1 activities. Table 2.8 showed that the inhibitory effects of the tanshinones were more prominent on human CYP2E1 than rat CYP2E1. It showed 9-fold lower concentration of dihydrotanshinone to give 50% inhibition on CYP2E1 activity in human than in rat. Tanshinone I had a higher inhibitory effect on human CYP2E1 than in rat with 21-fold difference in the K*i* value. Cryptotanshinone had a lower $IC_{50}$ value on human CYP2E1 activity with 8-fold difference K*i* value. This may indicate that Danshen decreased human chlorzoxazone metabolism more potently than rat chlorzoxazone metabolism.

As shown in Table 2.1, whole Danshen extract was mainly composed of water-soluble compounds such as danshensu, salvianolic acid B, rosmarinic acid and protocatechuic aldehyde. The aqueous fraction of Danshen mostly consisted of danshensu (3 mg/g) and salvianolic acid B (37 mg/g) that the inhibitory effects of aqueous fraction were suggested to be from these two components. Further study on danshensu and salvianolic acid B should be carried out in order to study their respective effects on CYP2E1 activities. In both rat and human liver microsomes experiments, dihydrotanshinone exerted the highest inhibitory effects on CYP2E1 activities uncompetitively that was the same inhibition mode as the ethanolic fraction of Danshen. However, the main compounds in the ethanolic fraction were tanshinone IIA (118 mg/g) and salvianolic acid B (209 mg/g) instead of dihydrotanshinone (9 mg/g) which had the lowest concentration compared with other three tanshinones. As tanshinone IIA did not exert inhibitory effects on CYP2E1 activity in the current study, cryptotanshinone, tanshinone I and dihydrotanshinone might have contributed to the inhibitory effects of the ethanolic fraction of Danshen on CYP2E1 activities.

Inhibition of CYP enzymes can be divided into reversible and irreversible inhibitions according to the enzymatic mechanism (McGinnity and Riley, 2001). Reversible inhibition is a common mechanism causing drug-drug interactions. It includes competitive inhibition, non-competitive inhibition and uncompetitive inhibition (Lin et al., 2000). The inhibition mode of the enzymatic metabolism can be predicted by Lineweaver-Burk plot, in which the axes are double-reciprocal that y-axis is 1/V and x-axis is 1/[S]. In competitive inhibition, the inhibitors bind at the active site of the enzyme to form E-I complex. In this case, $K_m$ will increase with unchanged $V_{max}$. Intersections of the lines on Lineweaver-Burk plot will form on

y-axis to have same y-intercept. For the non-competitive inhibition, the inhibitors bind at the allosteric site of the enzyme leaving the active site unblocked. Here, $K_m$ will increase with $V_{max}$ and the intersections of the lines will form after the y-axis and above or on the x-axis. Uncompetitive inhibition is a relatively rare case that the inhibitors bind to the E-S complex instead of the enzyme. In such situation, $K_m$ and $V_{max}$ will decrease at the same time. Therefore, the lines will all form in parallel on the Lineweaver-Burk Plot. The results shown in Tables 2.2, 2.3, 2.5 and 2.6 revealed a large variation on $K_m$ and $V_{max}$ values, especially for the control groups. This phenomenon was mainly caused by the experimental errors due to the small volume (200 μl) used for the reaction. This may be solved by increasing the reaction volume in order to minimize the pipetting errors. In the current experiments, the inhibition modes of whole Danshen extract and its active components in human liver microsomes gave agreement to that in rat liver microsomes. The results showed that whole Danshen extract was indicated as non-competitive inhibitor on CYP2E1 enzyme activities and the most potent inhibitor, dihydrotanshinone, caused uncompetitive inhibition to CYP2E1 while whole Danshen extract acted as competitive inhibitor on both CYP1A2 and CYP3A4 (Wang, 2007). Uncompetitive inhibitions are rare, an example of which was cotinine, a nicotine metabolite, which inhibited human CYP2E1-mediated *p*-nitrophenol metabolism by binding to CYP2E1-*p*-nitrophenol (E-S) complex with an estimated K*i* of 308 mM calculated by Hill model equation (Van Vleet et al., 2001).

Irreversible inhibitors bind to enzyme with covalent bonding or destroy a functional group of enzyme that is important in enzyme activity. Suicide inactivators, also named as mechanism-based inactivators, belong to a special class of irreversible inhibitors. Mechanism-based inactivators become a very reactive compound after

binding to the active site of an enzyme. This reactive compound will then combine with the enzyme irreversibly to inactivate the enzyme (Lehninger et al., 2005). The specific inhibitor of CYP2E1 used in this study was sodium diethyldithiocarbamate which was a metabolite of disulfiram and suggested as a mechanism-based inactivator (Brady et al., 1991; Guengerich et al., 1991; Ohashi et al., 2005). However, literatures on inhibitory mechanism of sodium diethyldithiocarbamate mostly depend on the research done by Guengerich (1991). Therefore, the mode of inhibition of sodium diethyldithiocarbamate on CYP2E1 still remains uncertain (Chang et al., 1994; Eagling et al., 1998). In this study, its inhibition mode was suggested to be non-competitive with K*i* values of 289 μM and 123 μM on rat and human chlorzoxazone metabolism, respectively.

Although Lineweaver-Burk Plot is a classical plot, it is prone to error due to the double reciprocal of the axes. Dixon plot can improve the problems faced in Lineweaver-Burk Plot as the concentration of inhibitors is directly plotted against 1/V. The intersecting point of the lines formed on Dixon plot determines the K*i* value of an inhibitor. However, the lines will form in parallel if the inhibition mode is uncompetitive and K*i* cannot be determined directly from Dixon plot. Also, $K_m$, $V_{max}$ and K*i*' cannot be determined by Dixon plot. Besides Dixon plot, K*i* values can be found by plotting Secondary plot of primary slopes of Lineweaver-Burk Plot. The x-intercept behind y-axis suggests the K*i* values of the inhibitor. Similarly, Secondary plot cannot predict K*i* values of uncompetitive inhibition because of the unchanged slope of Lineweaver-Burk Plot. K*i* value implies the affinity of inhibitor to enzyme, the lower the K*i* values the stronger the affinity. It also means the concentration needed for an inhibitor to decrease half of maximal rate of a reaction. K*i*' value indicates the affinity of inhibitor to enzyme-substrate complex instead of

enzyme alone and it is used in uncompetitive inhibition (Lehninger et al., 2005). In this case, Cornish-Bowden plot could be used as it can determine both K*i* and K*i*' in same graph. As K*i* value was the x-intercept on secondary plot and it was an intersecting point of all the lines on Dixon plot, standard errors could not be determined. In the current experiment, K*i* values obtained from secondary plot were used and Dixon plot was a reference to determine the accuracy of the K*i* values.

Table 2.9 K*i* values of cryptotanshinone, dihydrotanshinone, tanshinone I and tanshinone IIA on rat and human CYP2E1, CYP1A2 and CYP3A4 activities. (The data of CYP1A2 and CYP3A4 were published by Wang, 2007).

| **K*i* values** | **CYP2E1** | | **CYP1A2** | | **CYP3A4** | |
|---|---|---|---|---|---|---|
| | **Rat** | **Human** | **Rat** | **Human** | **Rat** | **Human** |
| **Whole Danshen extract** | 1.46 mg/ml | 0.03 mg/ml | 0.07 mg/ml | 0.05 mg/ml | 0.07 mg/ml | 0.08 mg/ml |
| **Cryptotanshinone** | 87.86 μM | 10.87 μM | 4.07 μM | 1.88 μM | 199.30 μM | 120.40 μM |
| **Dihydrotanshinone** | - | - | 6.40 μM | 0.53 μM | 127.20 μM | 2.10 μM |
| **Tanshinone I** | 78.74 μM | 3.67 μM | 22.58 μM | 2.16 μM | 199.40 μM | 86.90 μM |
| **Tanshinone IIA** | - | - | 23.81 μM | 1.45 μM | 243.20 μM | 218.70 μM |

The current results shown in Table 2.9, K*i* values of whole Danshen extract on CYP2E1 in rat liver microsomes was 1.46 mg/ml and tanshinone I has a relatively stronger affinity to the enzyme than cryptotanshinone due to lower K*i* value. The Ki values of tanshinone I and cryptotanshinone on human CYP2E1 were 8 to 21-fold less than on rat CYP2E1 metabolism. The K*i* values of whole Danshen extract on rat CYP1A2 and CYP3A4 were close (Wang, 2007). It may suggest that the inhibitory effect of whole Danshen extract on CYP3A4 and CYP1A2 was much higher than on CYP2E1 in rat liver microsomes. For human liver microsomes, K*i* value of whole Danshen extract on CYP2E1 was 0.03 mg/ml which was much lower than that in rat. To compare the K*i* values of whole Danshen extract on CYP2E1 with CYP1A2 and

CYP3A4, whole Danshen extract had the highest inhibitory effect on CYP2E1 followed by CYP1A2 and CYP3A4. Dihydrotanshinone had the strongest inhibitory effects on rat and human CYP1A2 and CYP3A4 with the lowest K*i* values. Although K*i* values of dihytrotanshinone on rat and human CYP2E1 could not be determined here, the low $IC_{50}$ values also showed its strong inhibitory effects on rat and human CYP2E1 activities. Cryptotanshinone, followed by tanshinone I, had the highest inhibitory effect on rat and human CYP1A2 with lowest K*i* values compared with that of CYP2E1 and CYP3A4. The range of K*i* values for most of the drugs in use lies between 100 nM to 10 μM. The K*i* values of cryptotanshinone and tanshinone I on human CYP1A2 and CYP2E1, K*i* values of dihydrotanshinone on human CYP1A2 and CYP3A4 are within the range and would be regarded as likely inhibitors of the CYP isoforms.

In conclusion, whole Danshen extract had inhibitory effects on CYP2E1 enzyme activities in both rat and human liver microsomes. The ethanolic fraction of Danshen exerted a more prominent inhibition on CYP2E1 than the aqueous fraction. Dihydrotanshinone inhibited CYP2E1 activities by uncompetitive inhibition, possibly binding to the CYP enzyme-substrate complex instead of CYP enzyme alone, and K*i* could not be obtained from both Secondary Lineweaver-Burk and Dixon plots. Cryptotanshinone and tanshinone I also inhibited CYP2E1 activities but not tanshinone IIA. Tanshinone IIA is commonly used for treating cardiovascular diseases. It was proved to have cardioprotective effects on myocardial ischemia rats by improving heart functions due to enhancement of hypoxia-inducible factor 1-α mRNA expression (Xu et al., 2009). It was even found to have anticancer effect on human breast cancer by inhibiting the cell proliferation and inducing apoptosis of ER-positive (MCF-7) and ER-negative breast cancer cell

*in vitro* (Zhang and Lu, 2009). The results in human liver microsomes agreed with that in rat liver microsomes. However, the *in vitro* experiments may not be able to predict effects of Danshen in *in vivo*. To further the investigation on effects of Danshen on CYP2E1-mediated chlorzoxazone metabolism, *in vivo* study would be carried out in rats with acute, 3-day and 14-day Danshen pre-treatments at different dosages.

# Chapter 3

# Effects of Danshen on cytochrome P450 protein expression and metabolism of model CYP2E1 probe substrate in the rat *in vivo*

## 3.1 Introduction

CYP1A2, CYP2C9, CYP2D6, CYP2E1 and CYP3A4 are important CYPs that are responsible for the metabolism of most of the drugs in clinical use. Previous studies in our laboratory have shown that Danshen and its active components had inhibited CYP1A2, CYP2C9 and CYP3A4 activities *in vitro* in rats and human microsomes and *in vivo* in rat (Wang, 2007). Therefore a similar approach was carried out in this study on CYP2E1. Paracetamol is one of the substrates of CYP2E1, investigations on drug interactions associating with CYP2E1 may have both pharmacological as well as toxicological significance. Paracetamol, also named as acetaminophen (APAP), is widely used as analygesic and antipyretic agent (Jemnitz et al., 2008). It reduces fever and mild-to-moderate pain effectively and safely. It has been reported that paracetamol overdose led to hepatotoxicity in both humans and experimental animals (Kaplowitz, 2005). About 40% of acute liver failure cases were due to paracetamol overdose (Lee, 2003). Paracetamol undergoes extrinsic phase II conjugation and CYP2E1 is considered as one of the main isozyme responsible for paracetamol metabolism. It was proposed that the hepatotoxicity was mainly caused by an increase in reactive intermediate metabolie *N*-acetyl-*p*-benzoquinone-imine (NAPQI) with depletion of cellular glutathione (GSH) (Hinson et al., 2004). CYP2E1 was discovered to activate the formation of NAPQI which is unstable and

is measured as a GSH conjugate (Morgan et al., 1983; Raucy et al., 1989; Gonzalez, 2007).

Chlorzoxazone and *p*-nitrophenol are model probe susbtrates of CYP2E1 used widely in *in vitro* activities. Some work showed that CYP3A had a significant involvement in 2-hydroxylation of *p*-nitrophenol metabolism *in vitro* in rats and human (Zerilli et al., 1997; Kobayashi et al., 2002). Chlorzoxazone metabolism in human liver microsomes was suggested to involve by CYP1A2 and CYP3A4 (Ono et al., 1995; Gorski et al., 1997). However, Lucas *et. al.* (1999) had shown that CYP1A2 did not play a prominent role in chlorzoxazone metabolism. Chlorzoxazone is used pharmaceutically in musculoskeletal pain relief (Ernstgard et al., 2007). In this part of study, chlorzoxazone was used as CYP2E1 probe substrate based on a study showing its selectivity to CYP2E1 isoform (Peter et al., 1990) and results from the *in vitro* study in Chapter 2. Ernstgard, et.al. (2007) reported that chlorzoxazone was the only *in vivo* CYP2E1 probe currently, used with its non-invasive and low toxicity (Lucas et al., 1999).

In Chapter 2, Danshen and its components effects on rat and human hepatic CYP2E1 activities *in vitro* had been studied. It was found that the tanshinones including cryptotanshinone, dihydrotanshinone and tanshinone I inhibited CYP2E1-mediated chlorzoxazone metabolism in rat and human liver microsomes. Further experiments in *in vivo* model would be necessary to substantiate the *in vitro* results. In this study, acute, 3-day and 14-day treatments were administered to the rats with whole Danshen extract. Single-dose treatment of Danshen was used for the study of pharmacokinetics of chlorzoxazone. Prolonged treatments (3-day and 14-day) were established to examine whether there was alteration in overall

pharmacokinetics after multiple dosages of Danshen. Chlorzoxazone was metabolized into 6-hydroxychlorzoxazone which is glucuronidated or sulphated before elimination (Lucas et al., 1999). The pharmacokinetic parameters for chlorzoxazone and area under curves of the metabolites were calculated and compared with the control to determine the effects of Danshen *in vivo*.

CYP2E1 is important in its association with alcohol oxidizing function. It has been reported that continuous consumption on alcohol can increase CYP2E1 activities by up to 20 folds (Khan et al., 2009). Enzyme induction usually requires 4 to 14 days to the peak (Tanaka and Hisawa, 1999). It has been shown that induction of CYP enzymes is mainly caused by transcriptional activation involving several nuclear receptors, such as aryl hydrocarbon receptor (Ah), constitutively active receptor (CAR), pregnane X receptor (PXR) and retinoid X receptor (RXR). When the inducer binds to one of the nuclear receptors, the transcription of CYP genes will be stimulated through interacting with the promoter region of the gene directly. The whole complex is then translocated to the nucleus and binds to other nuclear factors and promoter of the genes. The level of *m*RNA levels increase and the expression of the CYP is therefore increased (Whitlock, 1999; Ma, 2001; Yan and Caldwell, 2001; Gonzalez, 2007). However, induction of CYP2E1 does not involve nuclear receptors, but occurred at transcriptional, translational and post-translational levels with regulated CYP2E1 expression (Ronis and Ingelman-Sundberg, 1999; (Zhukov and Ingelman-Sundberg, 1999). CYP2E1 enzymes were suggested to be stabilized after inducers bound to the active site of enzyme that the protein was protected from proteolytic degradation, and therefore the protein expression level increases after induced by the inducers such as ethanol and acetone (Eliasson et al., 1992; McGehee et al., 1994). Since CYP2E1 is an inducible enzyme, its protein expression was

investigated by Western Blot in this study to examine the effect of Danshen on CYP2E1 expression.

## 3.2 Materials and Methods

### 3.2.1 Chemicals and reagents

Chlorzoxazone, 6-hydroxychlorzoxazone, cimetidine, heparin sodium salt, urethane, phenacetin, β-glucuronidase were from Sigma Chemical Co. (St Louis, MO, USA). Acetonitrile (HPLC Grade) and ethyl acetate (HPLC Grade) were from Labscan Analytical Sciences (Bangkok, Thailand). Phenobarbitone sodium was obtained from Universal Pharmaceutical Lab. (Hong Kong). Perchloric acid, ammoniumacetate and magnesiumchlorid-hexahydrat reinst were from Merck & Co., Inc. (USA). Sodium acetate trihydrate (HPLC grade) was from Scharlau Chemie (Barcelona, Spain). Formulated Danshen extract was kindly donated by Winsor Health Products Limited (Hong Kong). Primary antibodies for human and rat CYP2E1 were purchased from Chemicon (USA). Secondary antibodies (HRP-anti rabbit IgG) were purchased from Cell Signalling Technology (USA).

### 3.2.2 Animals

Sprague-Dawley rats (male, 260 - 280 g) were used to perform all experiments under licence from the Government of the Hong Kong SAR and endorsed by the Animal Experimentation Ethics Committee of the Chinese University of Hong Kong. All the rats were supplied by the Laboratory Animal Services Centre of the Chinese University of Hong Kong. The rats were bred and kept in animal holding room under standard conditions with 12-h light-dark cycle. Food and water was provided to the rats *ad libitum*.

### 3.2.3 Effects of Danshen treatments on pharmacokinetics of chlorzoxazone in rats *in vivo*

#### 3.2.3.1 Acute, 3-day and 14-day treatments with whole Danshen extract

For acute treatments, groups of 6 - 8 animals were treated with saline (control), whole Danshen extract (50 mg/kg, 100 mg/kg and 200 mg/kg, i.p. or 100 mg/kg, 200 mg/kg and 400 mg/kg, p.o.) or cimetidine (60 mg/kg, i.p., control for enzyme inhibition studies) 30 minutes before chlorzoxazone administration (25 mg/kg, i.v.).

For 3-day treatments, groups of 6 - 8 animals were treated with saline (control), whole Danshen extract (200 mg/kg/day and 400 mg/kg/day, i.p. or 200 mg/kg/day and 500 mg/kg/day, p.o.) or phenobarbitone (40 mg/kg/day, i.p., control for enzyme induction) for 3 days. Chlorzoxazone (25 mg/kg, i.v.) was administered on day 4.

For 14-day treatments, groups of 6 - 8 animals were treated with saline (control), or whole Danshen extract (200 mg/kg/day and 400 mg/kg/day, i.p. or 100 mg/kg/day and 200 mg/kg/day, p.o.) for 14 days. Chlorzoxazone (25 mg/kg, i.v.) was administered on day 15.

After each pre-treatment and right before chlorzoxazone administration, the rats were anaesthetized with urethane (20% w/v, i.p.). The carotid artery and jugular vein were cannulated. Heparinized saline (100 units/ml) was applied through carotid artery for preventing blood coagulation during blood sample collection. Saline (0.9% w/v) was replaced through jugular vein after each blood collection. Chlorzoxazone (25 mg/kg, i.v.) was administered via jugular vein and 0.5 ml blood was collected at 5, 10, 20, 40, 60, 90, 120, 150 and 180 minutes afterwards. The serum was analyzed by HPLC.

### 3.2.3.2 Plasma extraction

Blood sample (0.5 ml) was collected in 1.6 ml microcentrifuge tube. Serum was obtained by centrifugation at 10,000 r.p.m for 5 minutes. 100 µl serum was added to a 1.6 ml microcentrifuge tube with 300 µl of acetate buffer (0.2 M, pH 4.75) and 100 µl of β-glucuronidase (dissolved in 0.2% NaCl, 850 units). After 3-hour incubation at 37 °C thermomixer at 800 r.p.m., 100 µl of 2M perchloric acid was added, followed by 20 µl of phenacetin (50 µg/ml) and 600 µl ethyl acetate. The mixture was incubated at 25 °C for 30 minutes at 1400 r.p.m. The tubes were then centrifuged at 10,000 r.p.m. for 5 minutes. The solvent layer was transferred to clean conical tube and evaporated under a gentle stream of nitrogen at 40 °C. The residue was dissolved in 80 µl methanol and centrifuged at 10,000 r.p.m. for 10 minutes. Sample of 50 µl was then injected into the HPLC for analysis.

### 3.2.3.3 High performance liquid chromatography (HPLC) analysis

The HPLC system consisted of Aligent Technologies 1100 series pumping system. The eluate was analysed by passing through a column (Agilent ZORBAX Eclipse XDB-C18, 4.6 x 150 mm, 5 µm). The mobile phase containing 0.15% ammonium acetate in water with 1N HCl (pH 5.92) and 30% acetonitrile was eluated by isocratic mode at a flow rate of 0.5 ml/min and the u.v. absorbance was detected at 287 nm. The chromatogram was shown in Figure 3.1. Linear standard curves of chlorzoxazone and 6-hydroxychlorzoxazone lied between 0.1 and 10 µg. Precision tests showed a good reproducibility that the differences were less than 10%. The accuracy of the standard curve was greater than 90%.

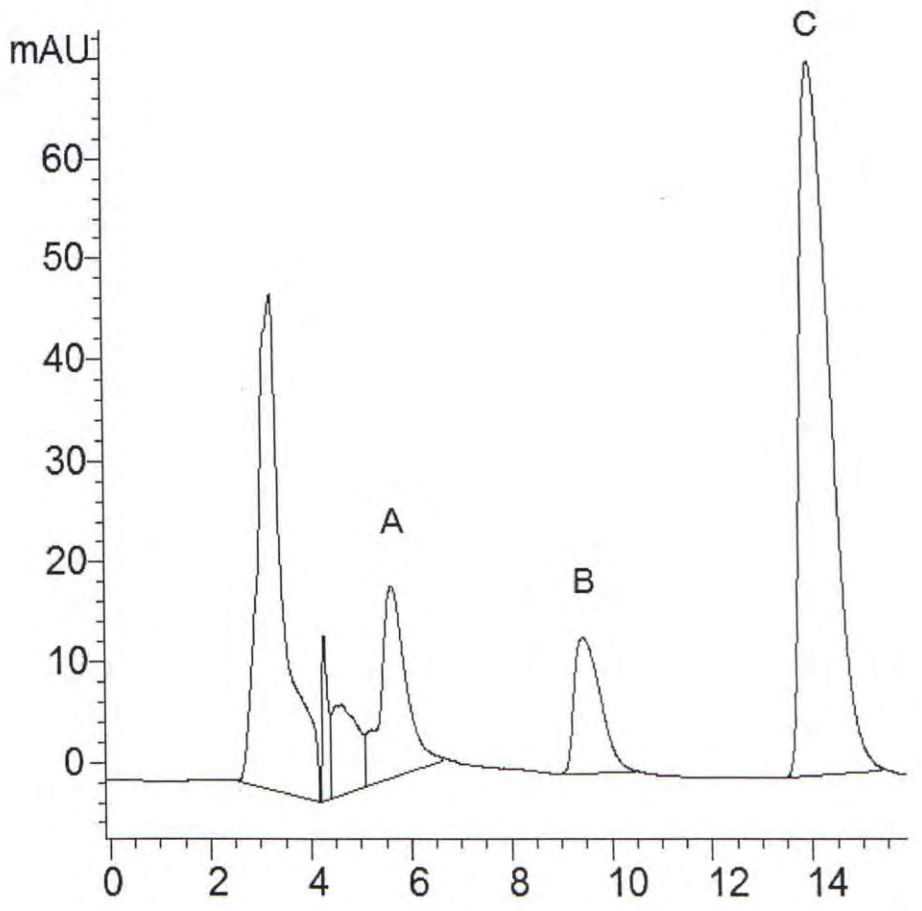



Fig. 3.1 Chromatogram of chlorzoxazone and 6-hydroxychlorzoxazone.
A) 6-hydroxychlorzoxazone (5.6 min); B) Phenacetin (9.5 min); C) Chlorzoxazone (14.1 min).

### 3.2.4 Effects of 3-day and 14-day Danshen treatments on CYP2E1 protein expression

#### 3.2.4.1 Preparation of rat liver microsomes for Western blotting

Sprague-Dawley rats (260 – 280 g, male) were treated with whole Danshen extract (200 mg/kg/day, 400 mg/kg/day, i.p. or 200 mg/kg/day, 500 mg/kg/day, p.o.) for 3 days or whole Danshen extract (200 mg/kg/day, 400 mg/kg/day, i.p. or 100 mg/kg/day, 200 mg/kg/day, p.o.) for 14 days. Control rats were treated with 5% acetone water for 7 days as control of induction and 75 mg/kg dexamethasone for 3 days as control of inhibition. After treatments, the rats were killed by cervical dislocation. The liver was excised and rinsed with ice-cold 0.9% NaCl (saline) solution. The liver was homogenized in 0.05 M Tris/KCl buffer at pH 7.4 with 0.25 M sucrose. The homogenate was centrifuged at 14,000 r.p.m at 4 $^{o}$C for 30 minutes. The supernatant was then ultra-centrifuged at 44,000 r.p.m at 4 $^{o}$C for one hour. Phosphate buffer (0.05 M, pH 7.4) was used to reconstitute the pellet which was then stored at -80 $^{o}$C.

#### 3.2.4.2 Protein assay

BCA protein assay kit (Thermo. Co. Ltd.) was used to measure the protein concentration of the liver microsomes. Concentrations at 0, 25, 125, 250, 500, 750 and 1000 μg/ml of BSA were prepared with saline to plot the standard curve. The microsomes were diluted into 25 or 50 folds by 0.05 M Tris/KCl buffer. Reagent A was mixed with Reagent B at a ratio 1:50. Each standard or diluted sample (25 μl) was added into each well of 96-well plate. Reagent A and B mixture (200 μl) was added into each well. The plate was incubated for 30 minutes at 37 $^{o}$C. The absorbance was measured by Universal Microplate Reader (Bio Tek Instrument ELx 800) at 560 nm. The protein concentration was obtained from the standard curve

with absorbance against protein concentration.

### 3.2.4.3 Western Blot

Tris/KCl buffer (0.05 M) and 2X loading dye were used to dilute the rat liver microsomes. A final concentration of 30 μg of protein in 15 μl volume was prepared and was incubated at 99 °C thermomixer for 5 minutes at 300 r.p.m. in order to denature the protein. The samples were cooled down by placing in ice and were given a flash spin to bring down the condensation. Sodium dodecyl sulfate-polyacrylamide (SDS-PAGE, 8%) gel was set with 10 wells by using 0.75 mm casting glass. Running buffer (1X; water: 10X running buffer = 9: 1) was poured to immerse the whole set of gel rig. Each sample (10 μl) was loaded into each well so that each well contained 20 μg of protein followed by electrophoresis (90 V, 90 minutes). Nitrocellulose membrane, filter paper and sponge were prewet in 1X transfer buffer (water: methanol: 10X transfer buffer = 7: 2: 1) for about 15 minutes. The gel was cut into membrane size and covered with filter, and bind together with two pieces of sponge into the "sandwich" cassette and 1X transfer buffer was poured to immerse the whole cassette (figure 3.2). The voltage was set at 90 V and about 1.5 hour was required to transfer the protein. Skimmed milk (5%) was prepared with Tris-buffered saline Tween-20 (TBST). After the transfer, skimmed milk (10 ml) was added onto the cut membrane and was blocked for 1 hour. CYP2E1 primary antibody (1: 4000) was added onto the membrane for incubating overnight at 4 °C. The membrane was washed with TBST for 3 times afterwards, each for about 15 minutes. Secondary antibody (1: 2000) was added and the membrane was incubated for at least 2 hours at room temperature. The membrane was then washed again with TBST for 3 times, each for about 5 minutes. Substrate Reagents A and B was mixed at a ratio of 1: 1. Add about 1 ml substrate mixture onto each membrane and the excess

substrate was removed by KimWipes paper. The bandings were filmed for 10 seconds.

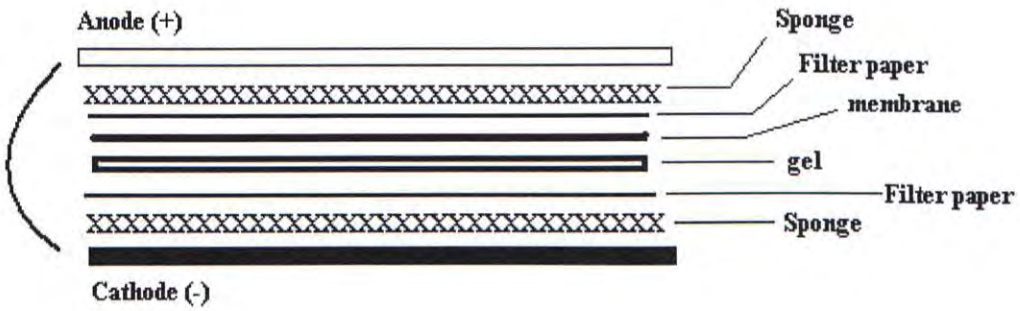



Fig. 3.2 shows the cassette setting for Western Blot.

### 3.2.5 Data analysis

The pharmacokinetic parameters were calculated by pharmacokinetics program PK solutions 2.0 software (Ashland, USA) with standard noncompartmental methods. $C_{initial}$ is the initial concentration extrapolated to time zero. AUC means the area under concentration-time curve. $V_d$ is the apparent volumne of distribution defined as the volume of fluid required to contain the total amount of drug in the body. CL is the total clearance of chlorzoxazone that involves all elimination processes in the body in unit time. The significance of difference of the pharmacokinetic parameters between treatments and control was analyzed by unpaired t-test. The significance of difference among groups was analyzed by two-way analysis of variance followed by Bonferroni post-test using GraphPad Prism 5.0 (GraphPad Software, CA, USA).

$$V_d = D / (AUC \times k)$$

$$CL = D / AUC$$

where D is the dose of chlorzoxazone and k is the elimination rate constant.

The intensities of the bandings on films of Western Blot were analyzed by Scion

Image Beta 4.0.3 (USA). The significant difference among groups was analyzed by one-way analysis of variance followed by Bonferroni's multiple comparison test. There is statistical significance when $p < 0.05$.

## 3.3 Results

### 3.3.1 Effects of whole Danshen extract on rat CYP2E1 activities *in vivo*

#### 3.3.1.1 Effects of acute treatments of whole Danshen extract on pharmacokinetics of chlorzoxazone

Figures 3.3, 3.4 and 3.5 show the plasma concentration-time profiles of chlorzoxazone and 6-hydroxychlorzoxazone after acute whole Danshen extract (50 mg/kg, 100 mg/kg and 200 mg/kg, i.p.) administration. Table 3.1 shows that the acute treatment via i.p. administration of Danshen did not alter the clearance, $C_{initial}$, AUC, and $T_{1/2}$ of chlorzoxazone although high dose (200 mg/kg) administration reduced $V_d$ by 14.1% and low dose (50 mg/kg) administration increased $T_{1/2}$ by 25.6%. As shown in Table 3.2, 6-hydroxychlorzoxazone formation was not significantly reduced in all Danshen concentrations by comparing AUC of the metabolites of treatments with that of control. When comparing the AUC ratio shown in Table 3.2, it was found that the ratio decreased for 24.8% at the lowest dose (50 mg/kg) of Danshen but increased for 4.8% at a higher dose (100 mg/kg) of Danshen. There was no change in AUC ratio at highest dosage (200 mg/kg). In Figure 3.6, cimetidine was used as a control for enzyme inhibition which showed significant changes in all pharmacokinetic parameters compared with control in Tables 3.1 and 3.2, also a significant decrease for 85.7% in AUC ratio.

Figures 3.7, 3.8 and 3.9 show the plasma concentration-time profiles of chlorzoxazone and 6-hydroxychlorzoxazone after acute p.o. administration of whole Danshen extract (100 mg/kg, 200 mg/kg and 400 mg/kg). As shown in Table 3.3, $V_d$ was the major pharmacokinetic parameter altered by all concentrations of Danshen, in which, $V_d$ was reduced by 33.5%, 34.4% and 32.4% respectively for 100 mg/kg,

200 mg/kg and 400 mg/kg of Danshen. $C_{initial}$ was increased by 33.0% and 34.8% respectively for 100 mg/kg and 200 mg/kg of Danshen. Danshen dosage at 200 mg/kg also reduced clearance for 22.3%. In Table 3.4, AUC of 6-hydroxychlorzoxazone of the Danshen treatments was compared with that of control. The AUC of metabolites after all Danshen treatments (100 mg/kg, 200 mg/kg and 400 mg/kg) were decreased significantly for 24.7%, 27.5% and 30.2% respectively. Lower dosage (100 mg/kg) of Danshen decreased AUC ratio for 28.6%, while higher dosages (200 mg/kg, 400 mg/kg) decreased AUC ratio for 35.7%.

#### 3.3.1.2 Effects of 3-day treatments of whole Danshen extract on pharmacokinetics of chlorzoxazone

The plasma concentration-time profiles of chlorzoxazone and 6-hydroxychlorzoxazone after i.p. treatments were shown in Figures 3.10 and 3.11. Table 3.5 shows that 400 mg/kg of Danshen administration reduced $C_{initial}$ by 29.5%, together with a significant increase in $V_d$ for 33.9%. Clearance, AUC and $T_{1/2}$ were not changed in all Danshen concentrations. There was no significant difference by comparing AUC of 6-hydroxychlorzoxazone of the Danshen treatments with the control as shown in Table 3.6, but it showed an 18.2% increase in 200 mg/kg Danshen treatment and 4.6% increase in 400 mg/kg Danshen treatment. However, it only showed increase in AUC ratio at lower dosage of Danshen treatment (200 mg/kg) and decrease in AUC ratio after 400 mg/kg Danshen treatment shown in Table 3.6. In Figure 3.12, phenobarbitone was used as a control for enzyme induction which showed changes in AUC, $V_d$, clearance and $T_{1/2}$ of chlorzoxazone and AUC of 6-hydroxychlorzoxazone compared with control in Tables 3.5 and 3.6.

The plasma concentration-time profiles of chlorzoxazone and

6-hydroxychlorzoxazone after oral treatments were shown in Figures 3.13 and 3.14. Although there was no significant difference in pharmacokinetic parameters of chlorzoxazone after all Danshen treatments shown in Table 3.7 and by comparing AUC of 6-hydroxychlorzoxazone of the Danshen treatments with the control in Table 3.8, it showed a 23.8% increase and a 28.8% decrease in AUC of 6-hydroxychlorzoxazone after 200 mg/kg and 500 mg/kg treatments, respectively. The AUC ratio in Table 3.8 also showed similarity to the AUC results that 200 mg/kg Danshen increased 14.3% of AUC ratio while 500 mg/kg Danshen decreased the AUC ratio for 14.3%.

#### 3.3.1.3 Effects of 14-day treatments of whole Danshen extract on pharmacokinetics of chlorzoxazone

The plasma concentration-time profiles of chlorzoxazone and 6-hydroxychlorzoxazone after i.p. treatments were shown in Figures 3.15 and 3.16. Table 3.9 shows that there was no change in the pharmacokinetic parameters of chlorzoxazone. A significant decrease (60%) in AUC of 6-hydroxychlorzoxazone has been shown in Table 3.10 if 400 mg/kg Danshen was applied everyday for 14 days although there was a 20% increase after 200 mg/kg Danshen treatment. AUC ratio in Table 3.10 also shows that a 33.3% increase and 66.7% decrease after 200 mg/kg and 400 mg/kg Danshen treatments, respectively.

The plasma concentration-time profiles of chlorzoxazone and 6-hydroxychlorzoxazone were after oral treatments shown in Figures 3.17 and 3.18. No significant difference in pharmacokinetic parameters shown in Table 3.11 but a significant decrease (40.5%) of AUC of 6-hydroxychlorzoxazone was shown in Table 3.12 after 400 mg/kg/day Danshen treatment was shown. There was a slight

increase (8.3%) in AUC of 6-hydroxychlorzoxazone after 100 mg/kg Danshen treatment. Table 3.12 showed that there was a 16.7% increase in AUC ratio after 100 mg/kg Danshen treatment but a 41.7% decrease after 200 mg/kg treatment.

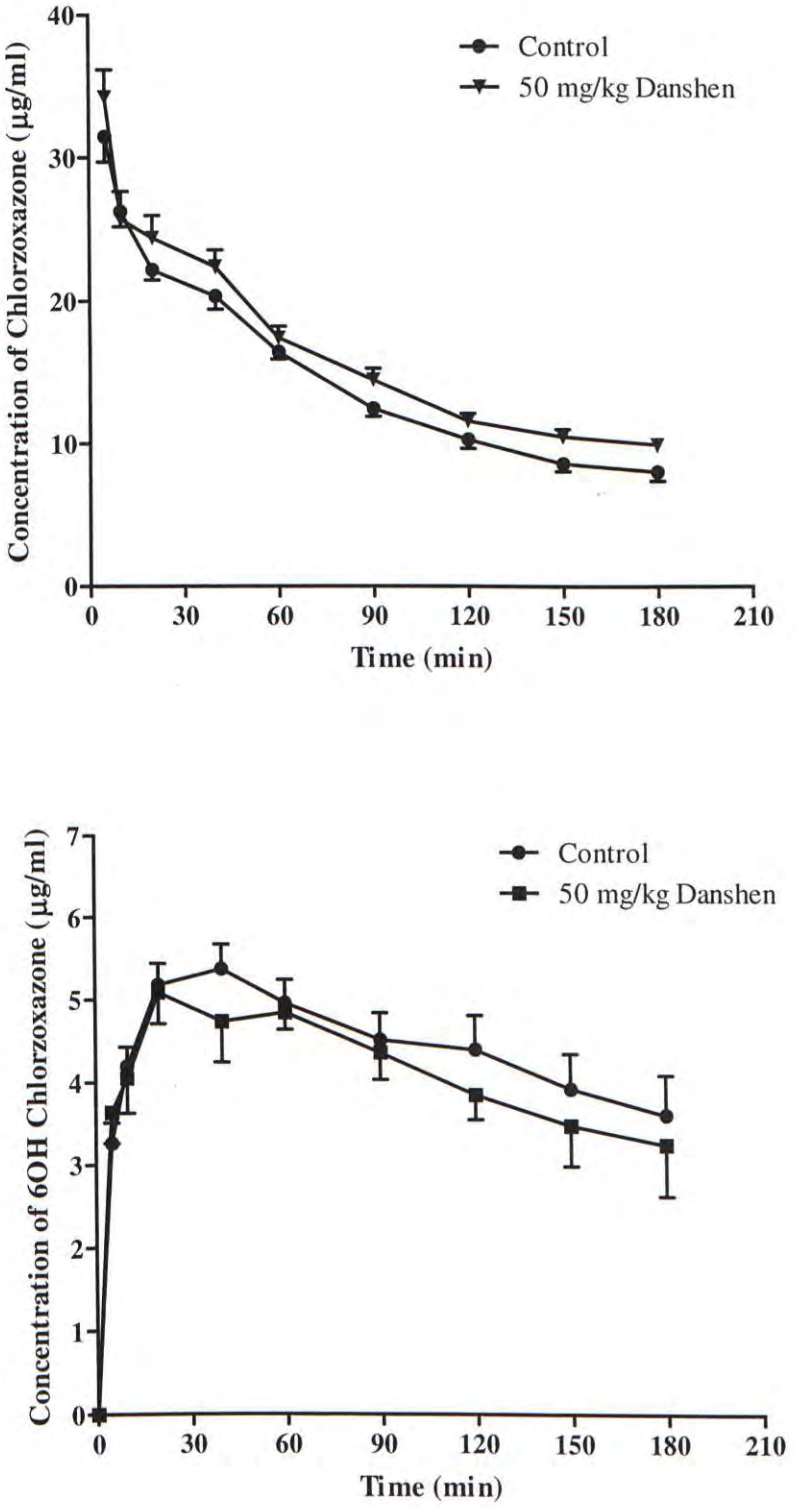



Fig. 3.3 Concentration-time profile of chlorzoxazone (25 mg/kg, i.v.) after i.p. acute treatment with saline (control) or 50 mg/kg of whole Danshen extract. Results were mean ± SEM of 6-8 rats.

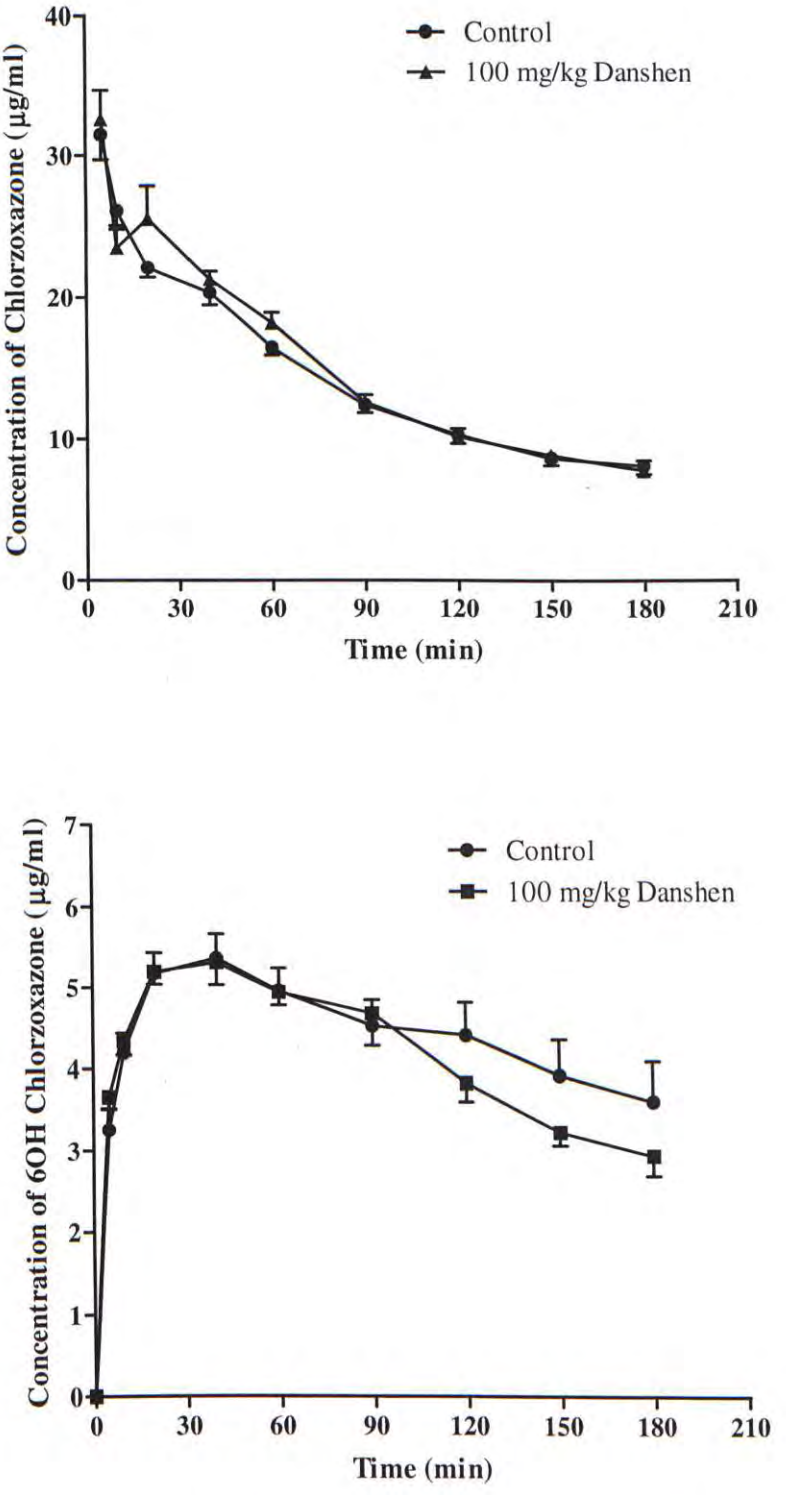



Fig. 3.4 Concentration-time profile of chlorzoxazone (25 mg/kg, i.v.) after i.p. acute treatment with saline (control) or 100 mg/kg of whole Danshen extract. Results were mean ± SEM of 6-8 rats.

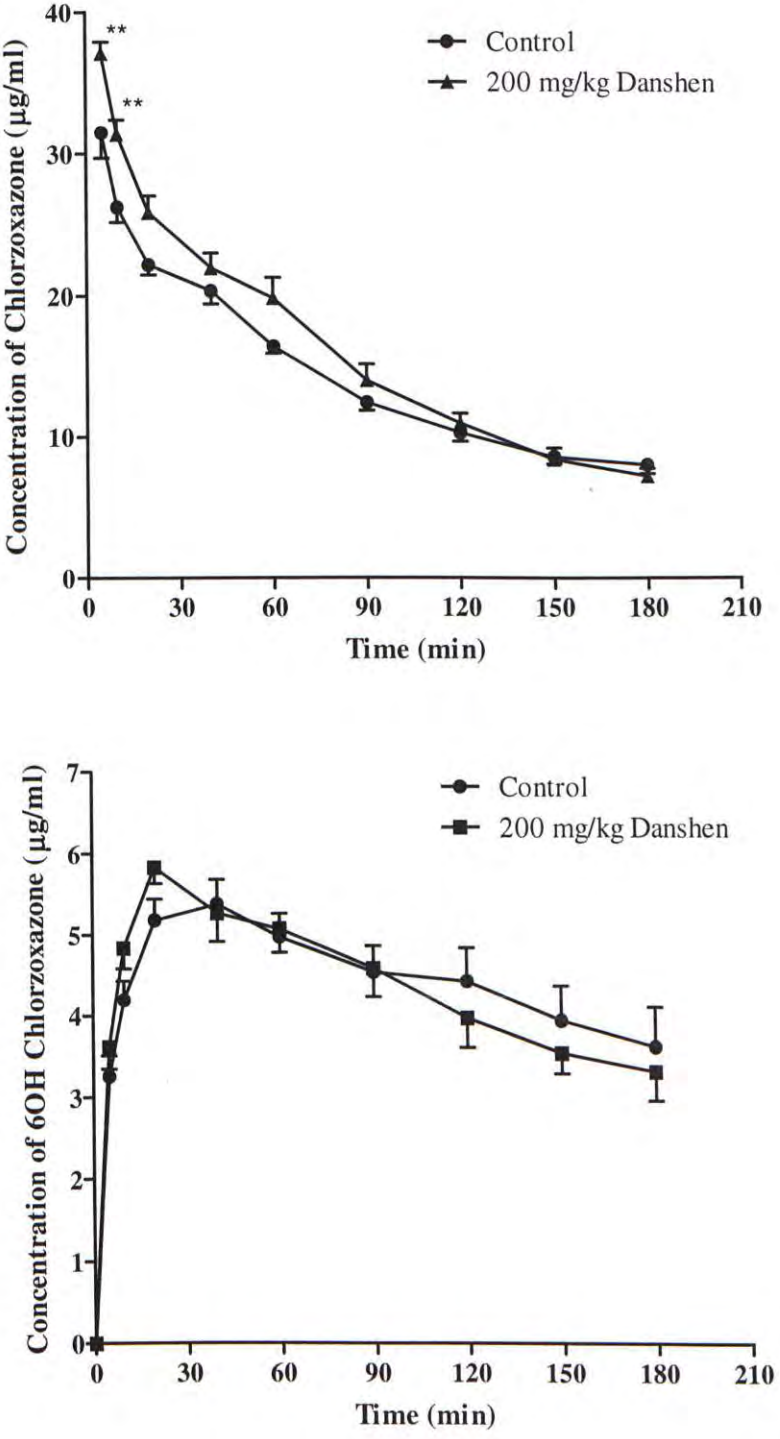



Fig. 3.5 Concentration-time profile of chlorzoxazone (25 mg/kg, i.v.) after i.p. acute treatment with saline (control) or 200 mg/kg of whole Danshen extract. Results were mean ± SEM of 6-8 rats. $^{**}p < 0.01$ when compared with controls.

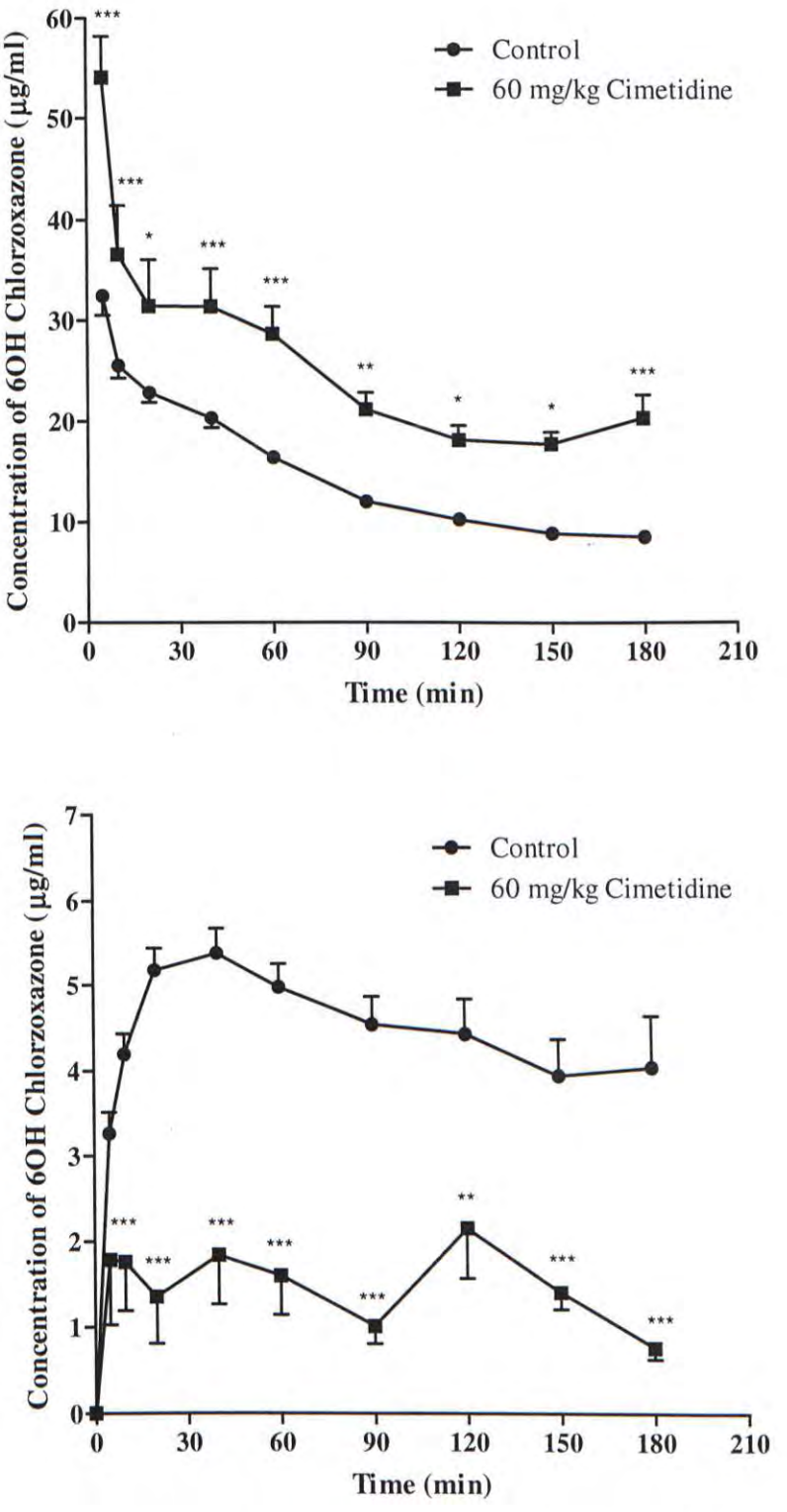



Fig. 3.6 Concentration-time profile of chlorzoxazone (25 mg/kg, i.v.) after i.p. acute treatment with saline (control) or 60 mg/kg of cimetidine. Results were mean ± SEM of 6-8 rats. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ when compared with controls.

Table 3.1 Pharmacokinetics of chlorzoxazone after acute pretreatment of whole Danshen extract (i.p.).

| Pharmacokinetic parameters | Control (saline) | Danshen (50 mg/kg) | Danshen (100 mg/kg) | Danshen (200 mg/kg) | Cimetidine (60 mg/kg) |
|---|---|---|---|---|---|
| $C_{initial}$ (g/ml) | 27.60 ± 1.234 | 27.69 ± 1.165 | 28.20 ± 1.770 | 32.49 ± 0.9200 | 39.52 ± 4.495** |
| AUC (g/min/ml) | 3761 ± 215.6 | 4293 ± 111.7 | 3457 ± 195.7 | 3683 ± 251.9 | 9217 ± 1259*** |
| $V_d$ (ml/kg) | 893.3 ± 32.58 | 887.0 ± 43.27 | 884.3 ± 52.88 | 767.7 ± 20.16* | 640.8 ± 107.3* |
| CL (ml/min/kg) | 6.867 ± 0.3729 | 5.666 ± 0.1332 | 7.213 ± 0.4550 | 6.899 ± 0.3957 | 2.748 ± 0.3712*** |
| $T_{1/2}$ (min) | 88.61 ± 5.147 | 111.3 ± 6.153* | 89.47 ± 5.107 | 82.62 ± 3.556 | 237.9 ± 62.21*** |

$T_{1/2}$: half-life, $C_{initial}$: initial concentration of chlorzoxazone, AUC: area under concentration-time curves, $V_d$: volume of distribution, CL: clearance. Results were mean ± SEM of 6 – 8 rats. *$p$< 0.05, **$p$ < 0.01 and *** $p$ < 0.001 when compared to control (saline).

Table 3.2 Area under curve of 6-hydroxychlorzoxazone after acute pretreatment of whole Danshen extract (i.p.).

| | Control (saline) | Danshen (50 mg/kg) | Danshen (100 mg/kg) | Danshen (200 mg/kg) | Cimetidine (60 mg/kg) |
|---|---|---|---|---|---|
| AUC (g/min/ml) | 805.1 ± 51.64 | 704.5 ± 47.72 (-12.5%) | 757.6 ± 19.89 (-6.0%) | 786.9 ± 43.12 (-2.3%) | 322.3 ± 87.94*** (-60.0%) |
| AUC ratio (HCZX/CZX) | 0.21 ± 0.002 | 0.16 ± 0.013 (-24.8%) | 0.22 ± 0.013 (+4.8%) | 0.21 ± 0.006 | 0.03 ± 0.010 (-85.7%) |

AUC: area under concentration-time curves. Results were mean ± SEM of 6 – 8 rats. *$p$< 0.05, **$p$ < 0.01 and *** $p$ < 0.001 when compared to control (saline).

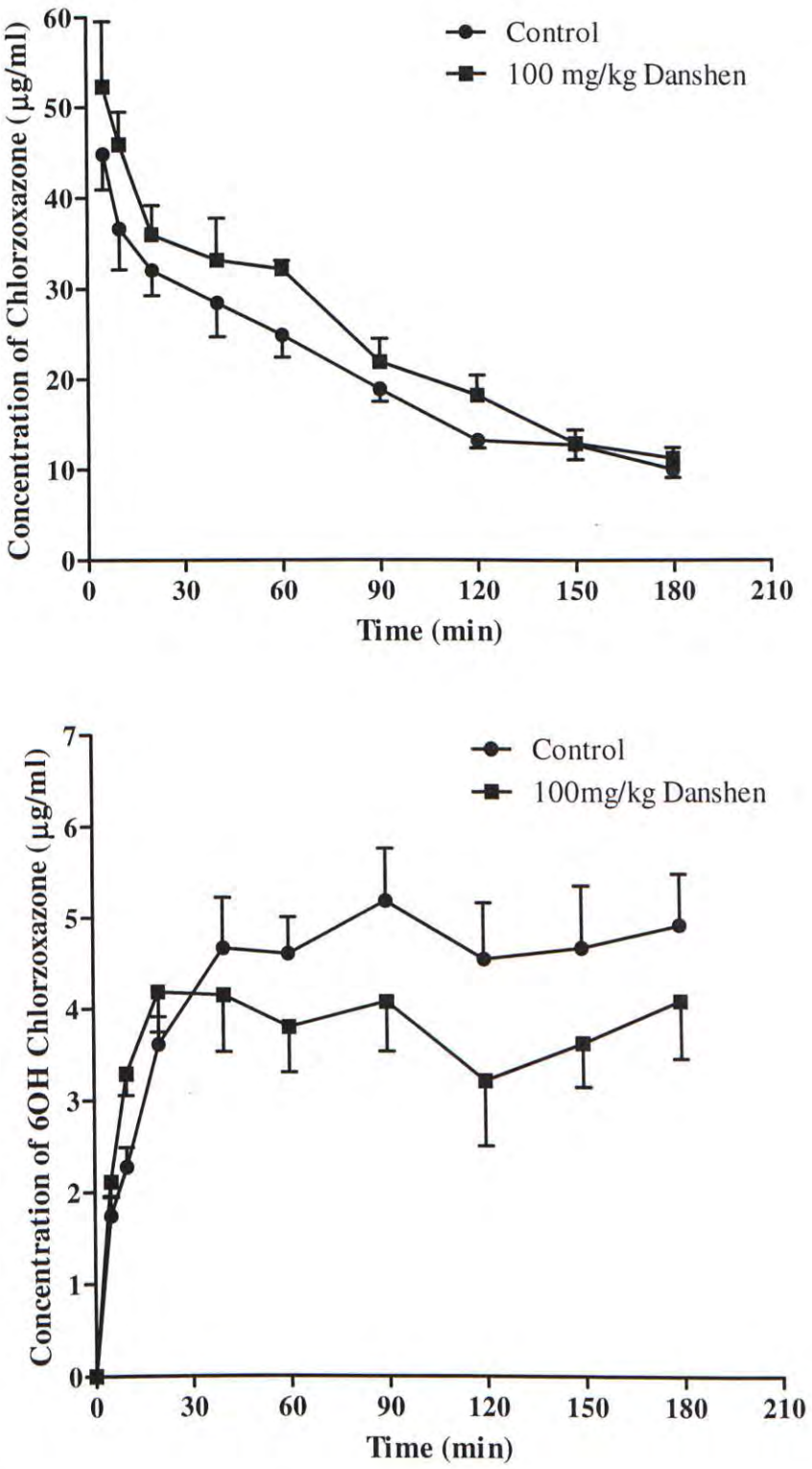



Fig. 3.7 Concentration-time profile of chlorzoxazone (25 mg/kg, i.v.) after p.o. acute treatment with saline (control) or 100 mg/kg of whole Danshen extract. Results were mean ± SEM of 6-8 rats.

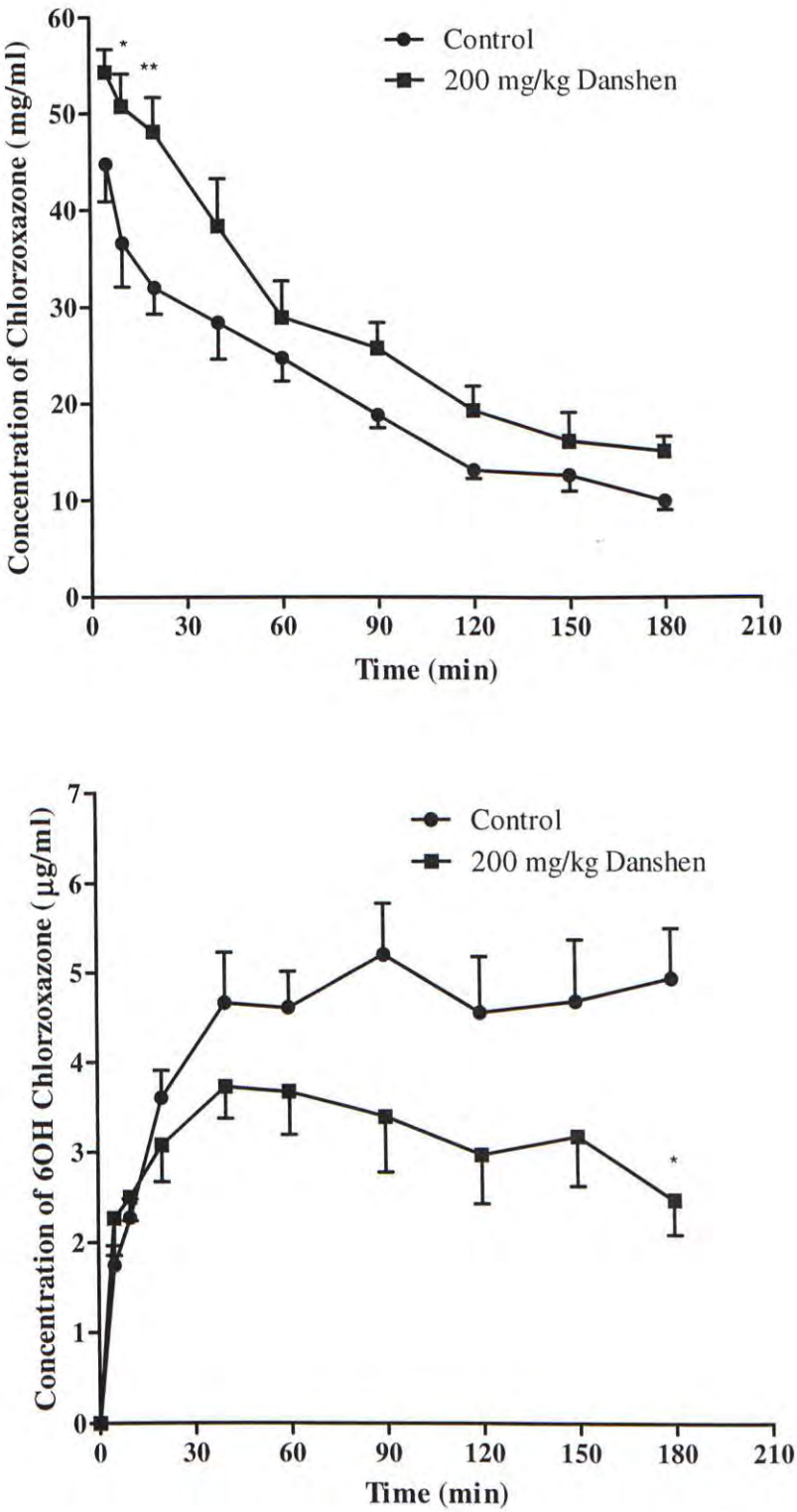



Fig. 3.8 Concentration-time profile of chlorzoxazone (25 mg/kg, i.v.) after p.o. acute treatment with saline (control) or 200 mg/kg of whole Danshen extract. Results were mean ± SEM of 6-8 rats. *$p < 0.05$ and **$p < 0.01$ when compared with controls.

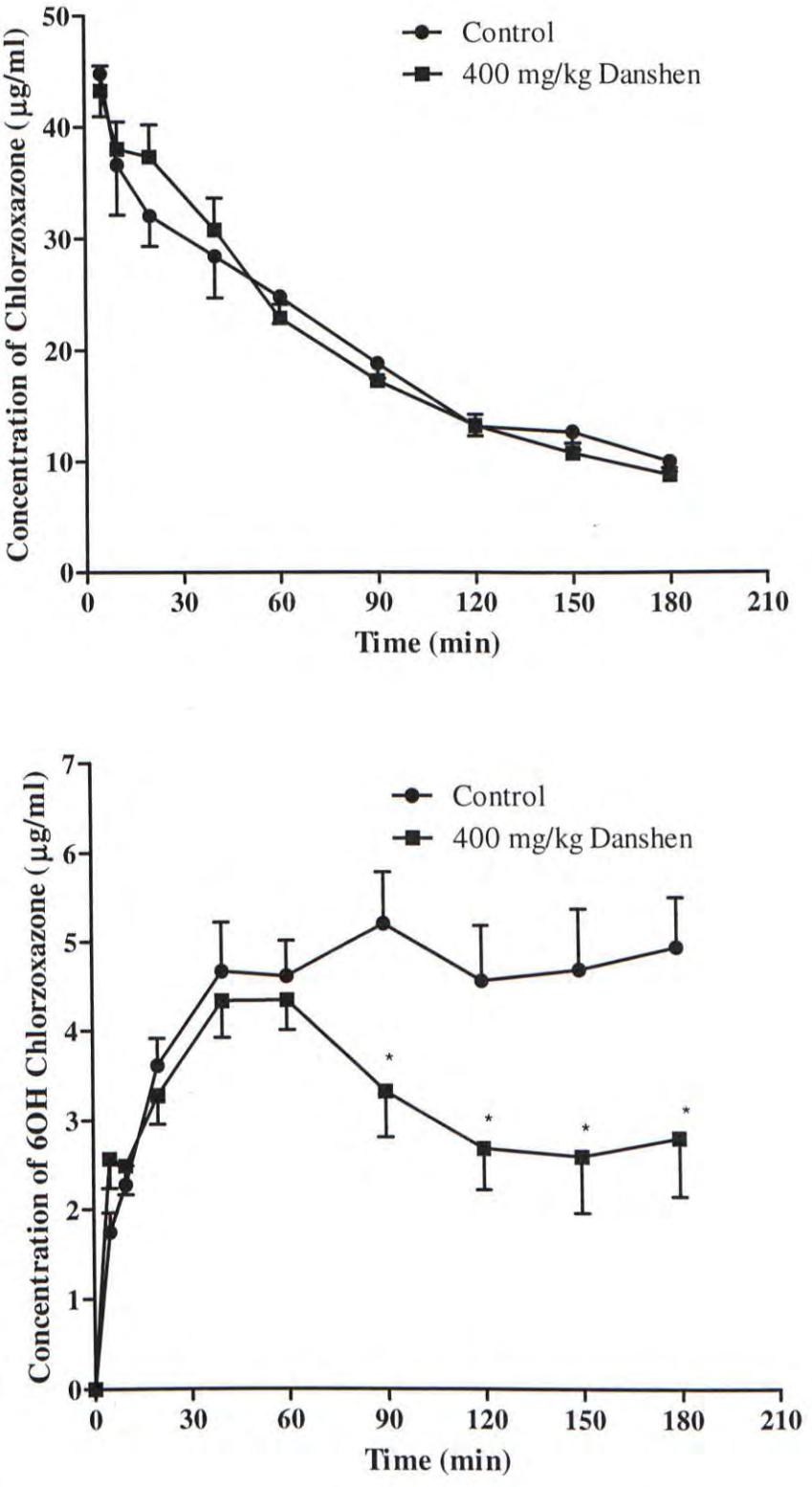



Fig. 3.9 Concentration-time profile of chlorzoxazone (25 mg/kg, i.v.) after p.o. acute treatment with saline (control) or 400 mg/kg of whole Danshen extract. Results were mean ± SEM of 6-8 rats. *$p < 0.05$ when compared with controls.

Table 3.3 Pharmacokinetics of chlorzoxazone after acute pretreatment of whole Danshen extract (p.o.).

| Pharmacokinetic parameters | Control (saline) | Danshen (100 mg/kg) | Danshen (200 mg/kg) | Danshen (400 mg/kg) |
|---|---|---|---|---|
| $C_{initial}$ (g/ml) | 40.86 ± 3.864 | 54.36 ± 5.028* | 55.08 ± 4.709* | 51.91 ± 4.063 |
| AUC (g/min/ml) | 5743 ± 500.0 | 6432 ± 656.5 | 6964 ± 583.0 | 6208 ± 603.8 |
| $V_d$ (ml/kg) | 706.8 ± 91.73 | 469.9 ± 41.25* | 463.6 ± 37.04* | 477.9 ± 31.22* |
| CL (ml/min/kg) | 4.296 ± 0.2967 | 3.977 ± 0.3285 | 3.340 ± 0.1286* | 3.907 ± 0.3126 |
| $T_{1/2}$ (min) | 92.10 ± 9.068 | 80.75 ± 4.996 | 89.42 ± 7.541 | 79.08 ± 5.916 |

$T_{1/2}$: half-life, $C_{initial}$: initial concentration of chlorzoxazone, AUC: area under concentration-time curves, $V_d$: volume of distribution, CL: clearance. Results were mean ± SEM of 6 – 8 rats. *$p$< 0.05 and **$p$ < 0.01 when compared to control (saline).

Table 3.4 Area under curve of 6-hydroxychlorzoxazone after acute pretreatment of whole Danshen extract (p.o.).

| | Control (saline) | Danshen (100 mg/kg) | Danshen (200 mg/kg) | Danshen (400 mg/kg) |
|---|---|---|---|---|
| AUC (g/min/ml) | 819.3 ± 64.14 | 617.0 ± 54.00* (-24.7%) | 594.1 ± 74.31* (-27.5%) | 572.1 ± 64.30** (-30.2%) |
| AUC ratio (HCZX/CZX) | 0.14 ± 0.003 | 0.10 ± 0.003 (-28.6%) | 0.09 ± 0.007 (-35.7%) | 0.09 ± 0.003 (-35.7%) |

AUC: area under concentration-time curves. Results were mean ± SEM of 6 – 8 rats. *$p < 0.05$ and **$p < 0.01$ when compared to control (saline).

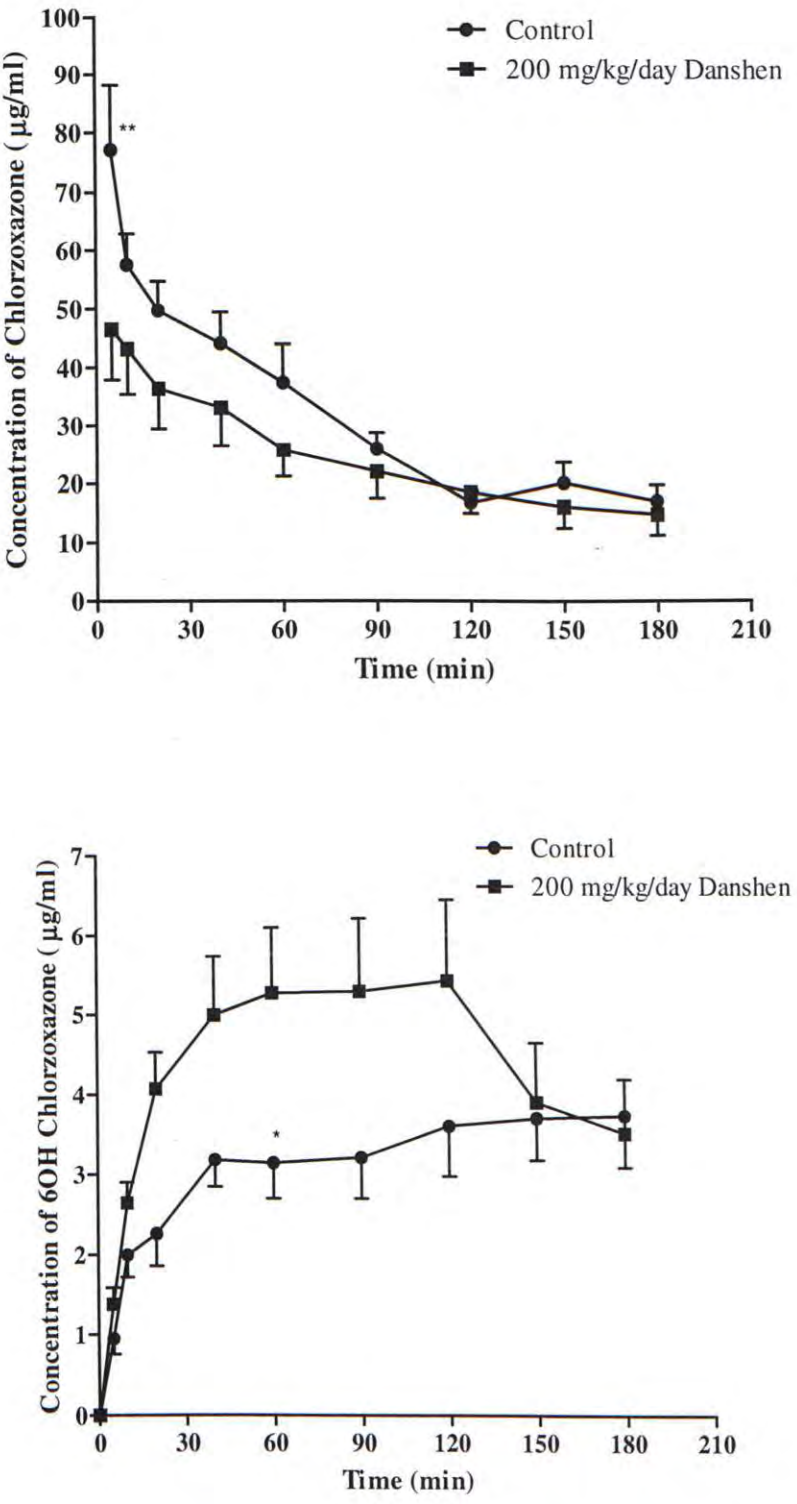



Fig. 3.10 Concentration-time profile of chlorzoxazone (25 mg/kg, i.v.) after i.p. 3-day treatment with saline (control) or 200 mg/kg/day of whole Danshen extract. Results were mean ± SEM of 6-8 rats. $*p < 0.05$ and $**p < 0.01$ when compared with controls.

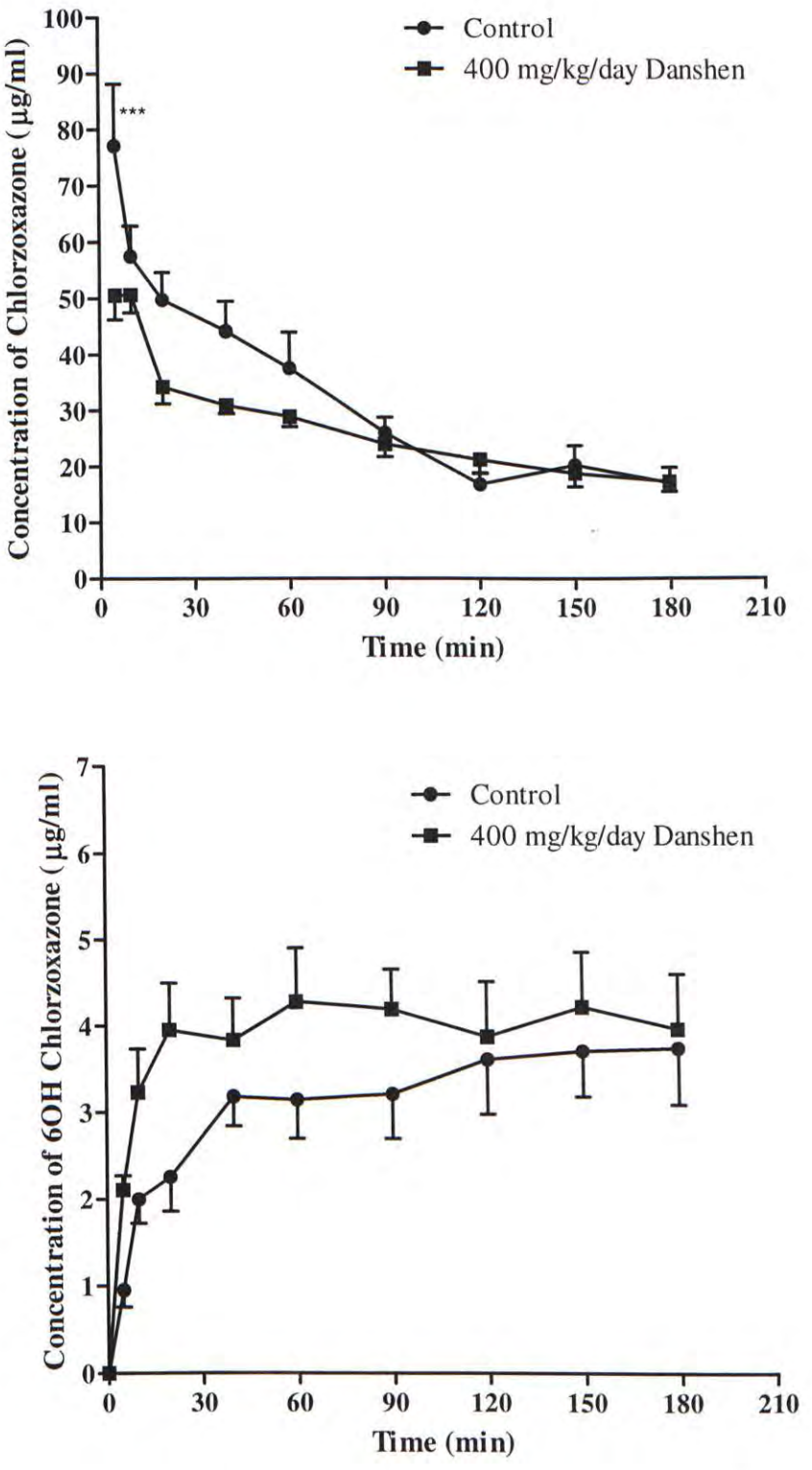



Fig. 3.11 Concentration-time profile of chlorzoxazone (25 mg/kg, i.v.) after i.p. 3-day treatment with saline (control) or 400 mg/kg/day of whole Danshen extract. Results were mean ± SEM of 6-8 rats. ***$p < 0.001$ when compared with controls.

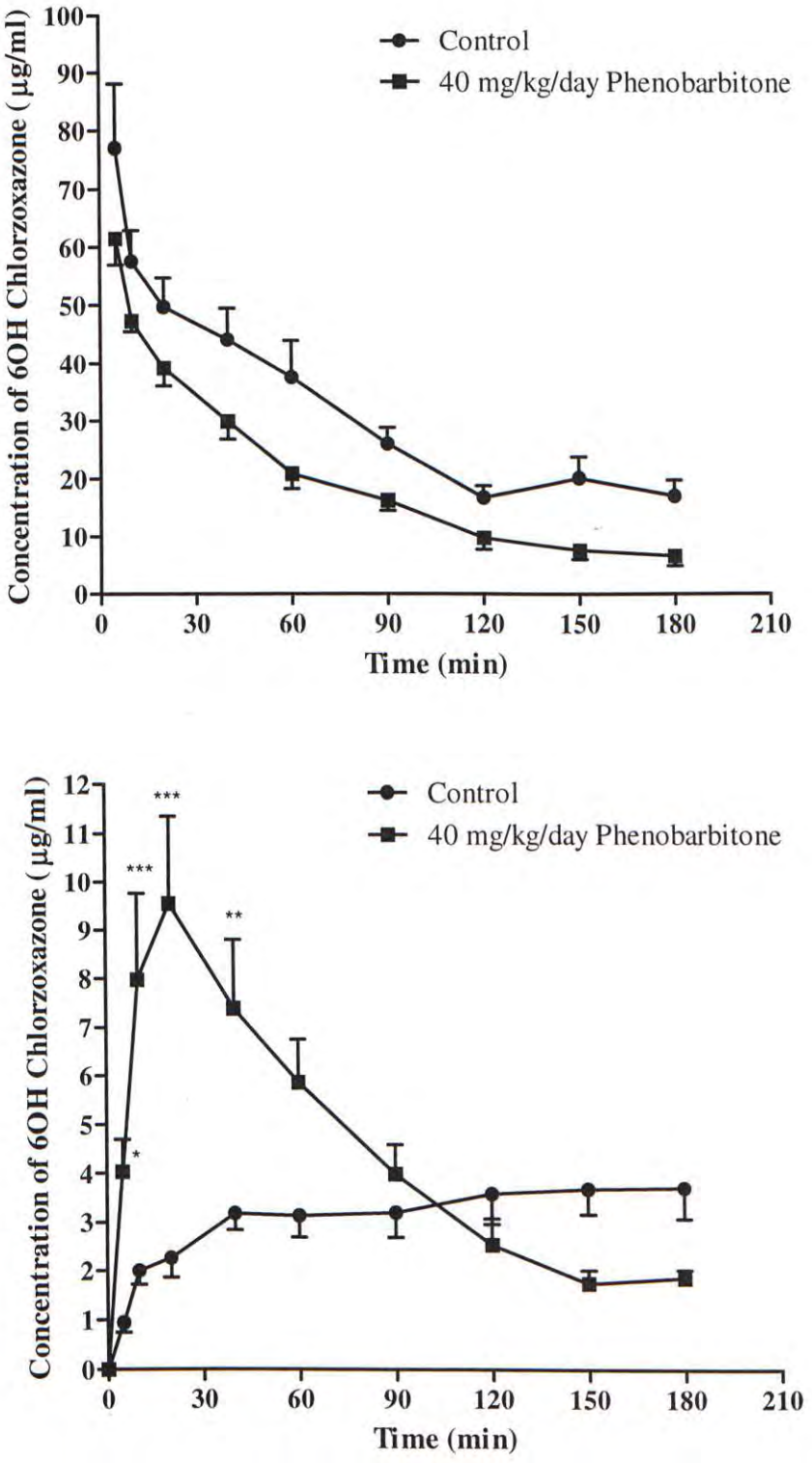



Fig. 3.12 Concentration-time profile of chlorzoxazone (25 mg/kg, i.v.) after i.p. 3-day treatment with saline (control) or 40 mg/kg/day of phenobarbitone. Results were mean ± SEM of 6-8 rats. $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$ when compared with controls.

Table 3.5 Pharmacokinetics of chlorzoxazone after 3-day pretreatment of whole Danshen extract (i.p.).

| Pharmacokinetic parameters | Control (saline) | Danshen (200 mg/kg/day, 3 days) | Danshen (400 mg/kg/day, 3 days) | Phenobarbitone (40 mg/kg/day, 3 days) |
|---|---|---|---|---|
| $C_{initial}$ (g/ml) | 62.80 ± 6.759 | 43.91 ± 7.394 | 44.25 ± 2.219* | 56.34 ± 2.620 |
| AUC (g/min/ml) | 6537 ± 851.3 | 6701 ± 1403 | 7499 ± 763.8 | 4018 ± 507.9* |
| $V_d$ (ml/kg) | 421.3 ± 50.17 | 705.3 ± 124.3 | 564.0 ± 27.63* | 452.7 ± 24.75 |
| CL (ml/min/kg) | 3.124 ± 0.3877 | 4.533 ± 0.8783 | 3.498 ± 0.3262 | 6.858 ± 0.8230** |
| $T_{1/2}$ (min) | 96.47 ± 5.189 | 95.46 ± 11.04 | 107.8 ± 6.800 | 49.36 ± 5.335*** |

$T_{1/2}$: half-life, $C_{initial}$: initial concentration of chlorzoxazone, AUC: area under concentration-time curves, $V_d$: volume of distribution, CL: clearance. Results were mean ± SEM of 6 – 8 rats. *$p$< 0.05, **$p$ < 0.01 and ***$p$ < 0.001 when compared to control (saline).

Table 3.6 Area under curve of 6-hydroxychlorzoxazone after 3-day pretreatment of whole Danshen extract (i.p.).

| | Control (saline) | Danshen (200 mg/kg/day, 3 days) | Danshen (400 mg/kg/day, 3 days) | Phenobarbitone (40 mg/kg/day, 3 days) |
|---|---|---|---|---|
| AUC (g/min/ml) | 598.9 ± 73.21 | 707.7 ± 144.4 (+18.2%) | 626.7 ± 59.00 (+4.6%) | 855.2 ± 118.2* (+50.5%) |
| AUC ratio (HCZX/CZX) | 0.09 ± 0.002 | 0.11 ± 0.001 (+22.2%) | 0.08 ± 0.001 (-11.1%) | 0.21 ± 0.005 (+133.3%) |

AUC: area under concentration-time curves. Results were mean ± SEM of 6 – 8 rats. *$p$< 0.05, **$p$ < 0.01 and ***$p$ < 0.001 when compared to control (saline).

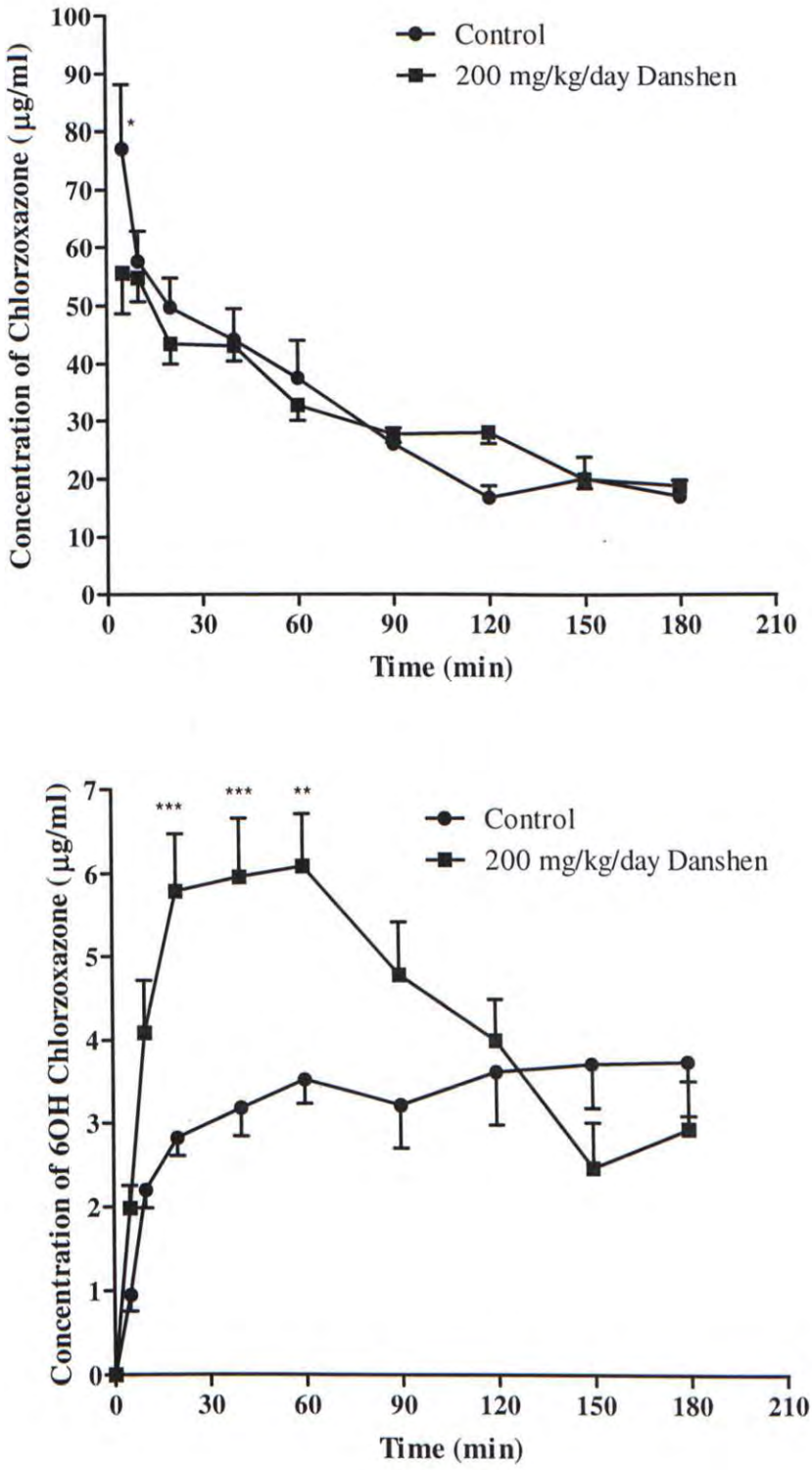



Fig. 3.13 Concentration-time profile of chlorzoxazone (25 mg/kg, i.v.) after p.o. 3-day treatment with saline (control) or 200 mg/kg/day of whole Danshen extract. Results were mean ± SEM of 6-8 rats. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ when compared with controls.

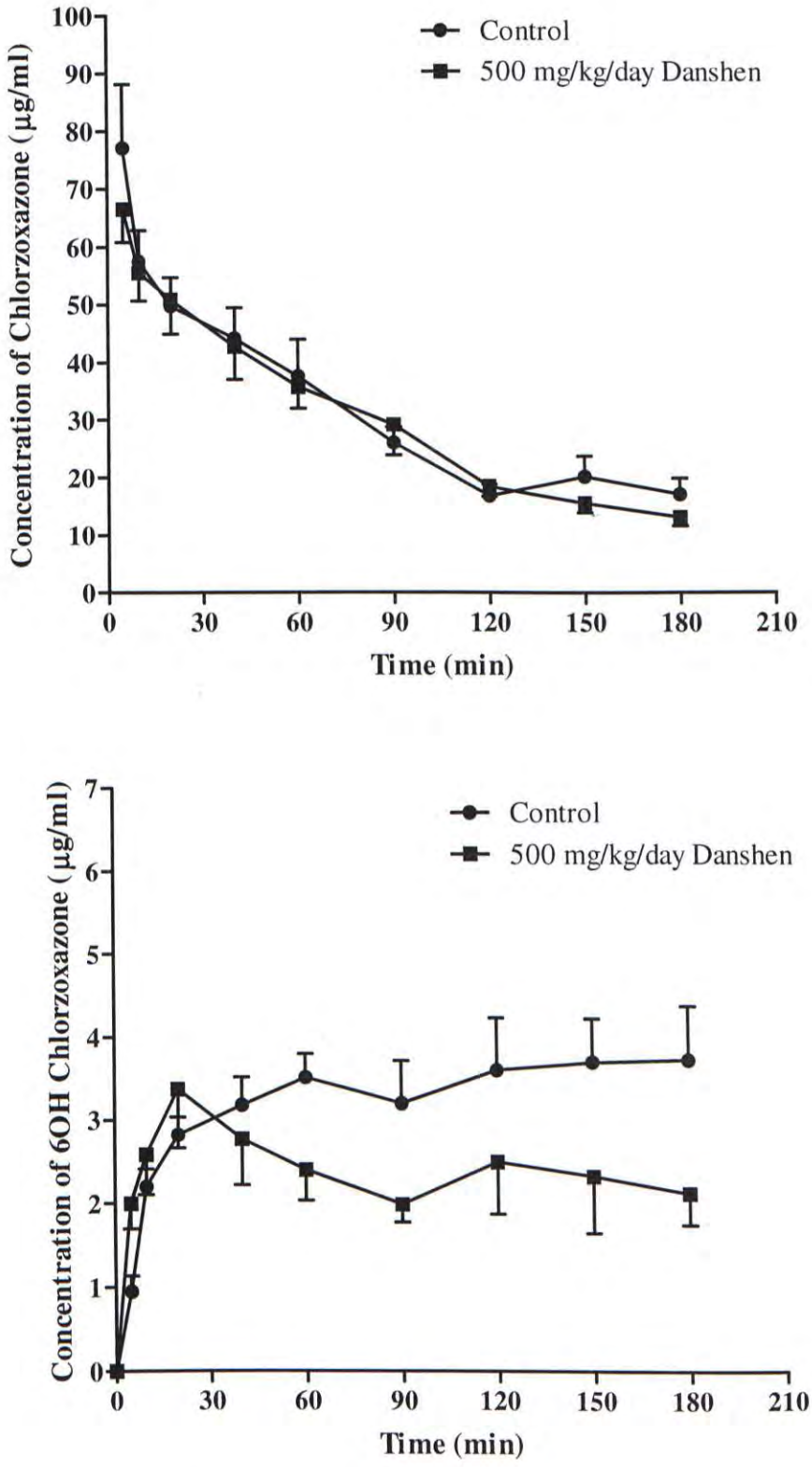



Fig. 3.14 Concentration-time profile of chlorzoxazone (25 mg/kg, i.v.) after p.o. 3-day treatment with saline (control) or 500 mg/kg/day of whole Danshen extract. Results were mean ± SEM of 6-8 rats.

Table 3.7 Pharmacokinetics of chlorzoxazone after 3-day pretreatment of whole Danshen extract (p.o.).

| Pharmacokinetic parameters | Control (saline) | Danshen (200 mg/kg/day, 3 days) | Danshen (500 mg/kg/day, 3 days) |
|---|---|---|---|
| $C_{initial}$ (g/ml) | 62.80 ± 6.759 | 55.64 ± 4.238 | 63.02 ± 7.040 |
| AUC (g/min/ml) | 8604 ± 764.3 | 8504 ± 644.0 | 6849 ± 698.3 |
| $V_d$ (ml/kg) | 419.6 ± 49.00 | 453.0 ± 31.35 | 444.1 ± 11.20 |
| CL (ml/min/kg) | 3.644 ± 0.5403 | 2.978 ± 0.2569 | 3.752 ± 0.3613 |
| $T_{1/2}$ (min) | 83.93 ±6.938 | 107.0 ± 8.306 | 76.24 ± 5.917 |

$T_{1/2}$: half-life, $C_{initial}$: initial concentration of chlorzoxazone, AUC: area under concentration-time curves, $V_d$: volume of distribution, CL: clearance. Results were mean ± SEM of 6 – 8 rats.

Table 3.8 Area under curve of 6-hydroxychlorzoxazone after 3-day pretreatment of whole Danshen extract (p.o.).

| | Control (saline) | Danshen (200 mg/kg/day, 3 days) | Danshen (500 mg/kg/day, 3 days) |
|---|---|---|---|
| AUC (g/min/ml) | 568.2 ± 70.45 | 703.6 ± 67.58 (+23.8%) | 404.4 ± 49.49 (-28.8%) |
| AUC ratio (HCZX/CZX) | 0.07 ± 0.005 | 0.08 ± 0.003 (+14.3%) | 0.06 ± 0.002 (-14.3%) |

AUC: area under concentration-time curves. Results were mean ± SEM of 6 – 8 rats.

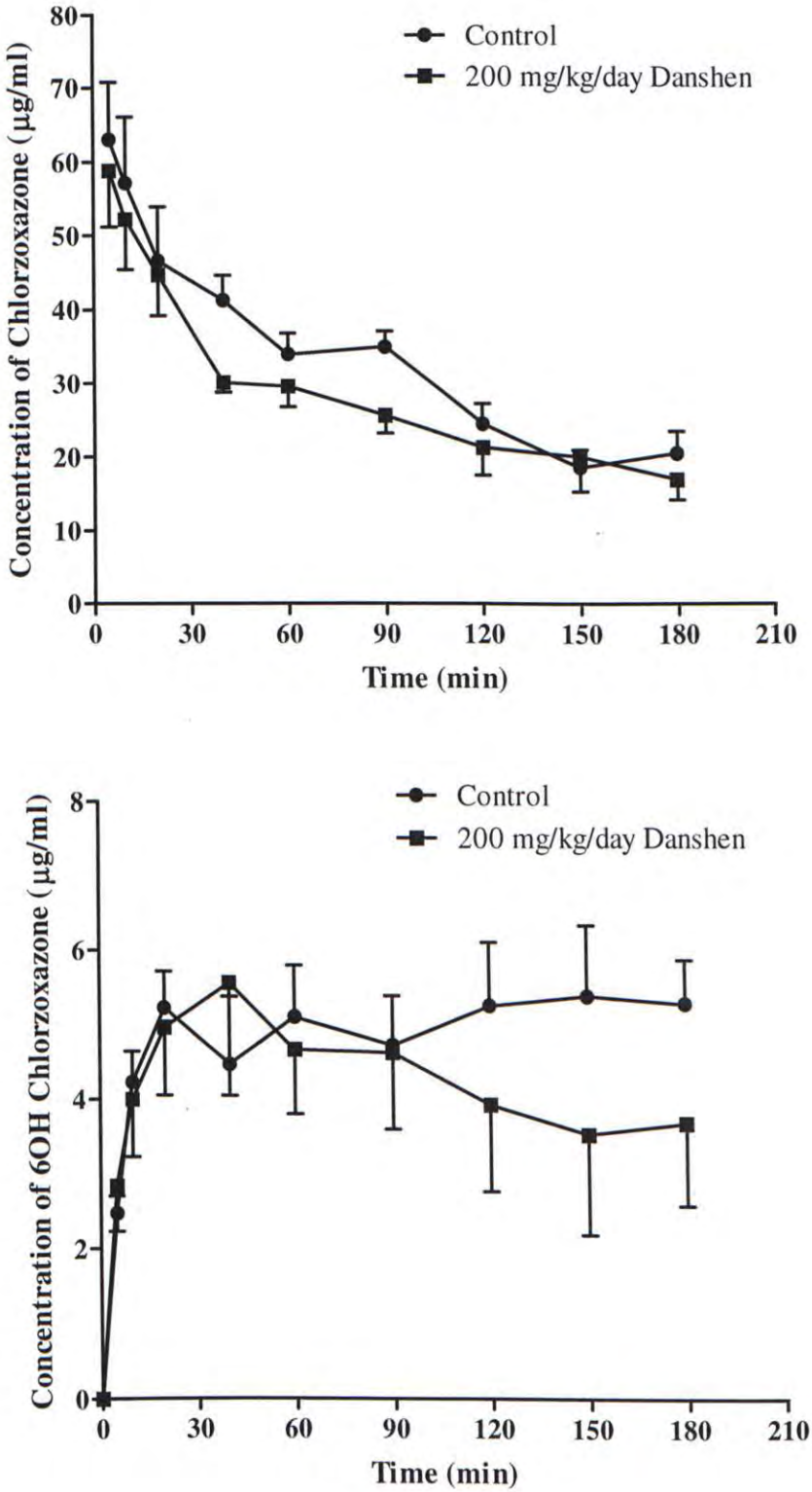



Fig. 3.15 Concentration-time profile of chlorzoxazone (25 mg/kg, i.v.) after i.p. 14-day treatment with saline (control) or 200 mg/kg/day of whole Danshen extract. Results were mean ± SEM of 6-8 rats.

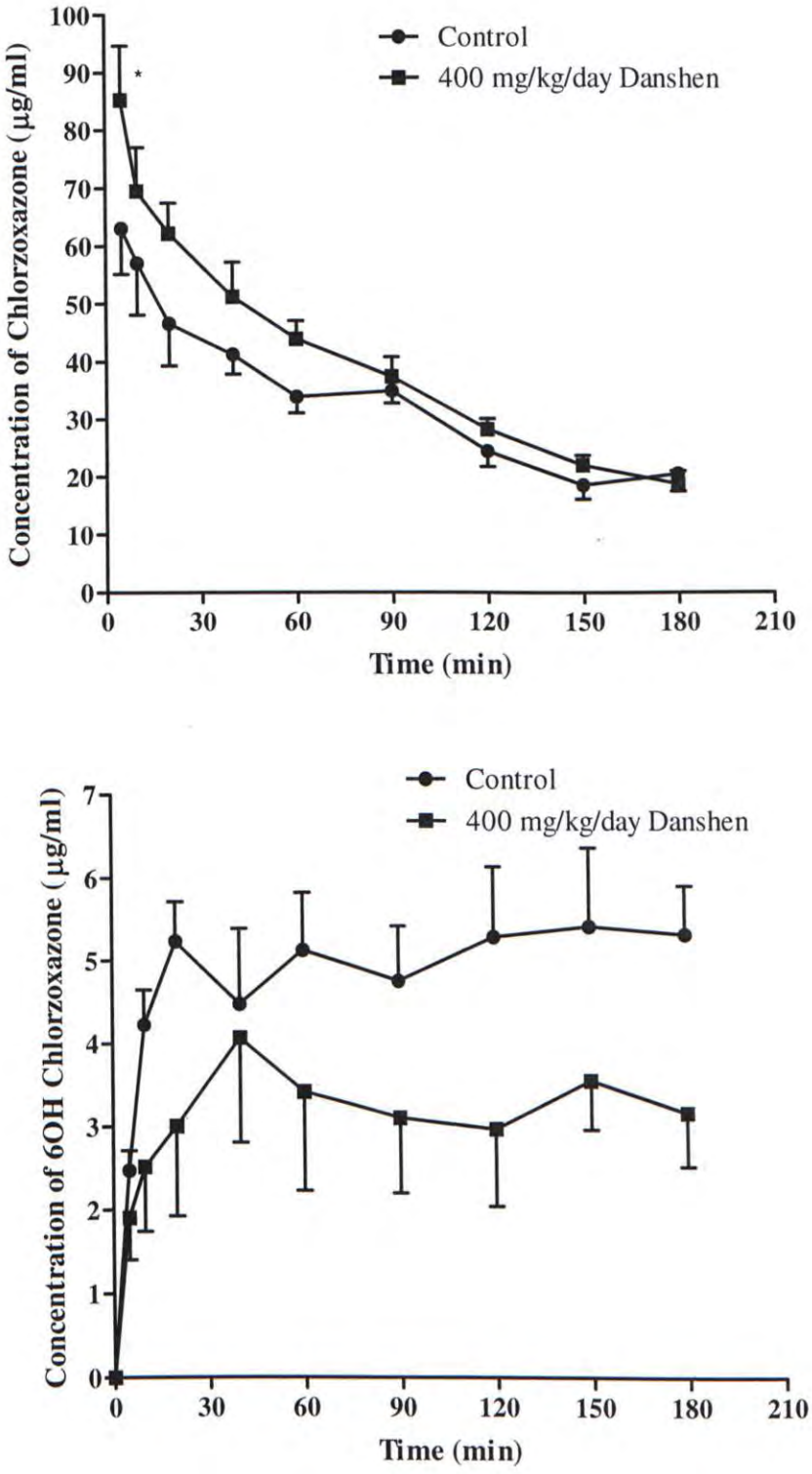



Fig. 3.16 Concentration-time profile of chlorzoxazone (25 mg/kg, i.v.) after i.p. 14-day treatment with saline (control) or 400 mg/kg/day of whole Danshen extract. Results were mean ± SEM of 6-8 rats. *$p < 0.05$ when compared with controls.

Table 3.9 Pharmacokinetics of chlorzoxazone after 14-day pretreatment of whole Danshen extract (i.p.).

| Pharmacokinetic parameters | Control (saline) | Danshen (200 mg/kg/day, 14 days) | Danshen (400 mg/kg/day, 14 days) |
|---|---|---|---|
| $C_{initial}$ (g/ml) | 55.94 ± 8.243 | 53.37 ± 5.940 | 79.05 ± 5.271 |
| AUC (g/min/ml) | 6941 ± 928.3 | 6610 ± 1003 | 8996 ± 636.9 |
| $V_d$ (ml/kg) | 458.6 ± 61.65 | 476.4 ± 36.48 | 385.0 ± 48.21 |
| CL (ml/min/kg) | 3.806 ± 0.5082 | 4.017 ± 0.4497 | 2.848 ± 0.2356 |
| $T_{1/2}$ (min) | 86.80 ± 3.420 | 84.64 ± 5.442 | 87.99 ± 6.802 |

$T_{1/2}$: half-life, $C_{initial}$: initial concentration of chlorzoxazone, AUC: area under concentration-time curves, $V_d$: volume of distribution, CL: clearance. Results were mean ± SEM of 6 – 8 rats. $^{**}p < 0.01$ when compared to control (saline) using unpaired Student's t-test.

Table 3.10 Area under curve of 6-hydroxychlorzoxazone after 14-day pretreatment of whole Danshen extract (i.p.).

| | Control (saline) | Danshen (200 mg/kg/day, 14 days) | Danshen (400 mg/kg/day, 14 days) |
|---|---|---|---|
| AUC (g/min/ml) | 853.9 ± 73.81 | 1025 ± 74.35 (+20%) | 341.9 ± 97.18** (-60%) |
| AUC ratio (HCZX/CZX) | 0.12 ± 0.012 | 0.16 ± 0.025 (+33.3%) | 0.04 ± 0.016 (-66.7%) |

AUC: area under concentration-time curve. Results were mean ± SEM of 6 – 8 rats. $^{**}p < 0.01$ when compared to control (saline).

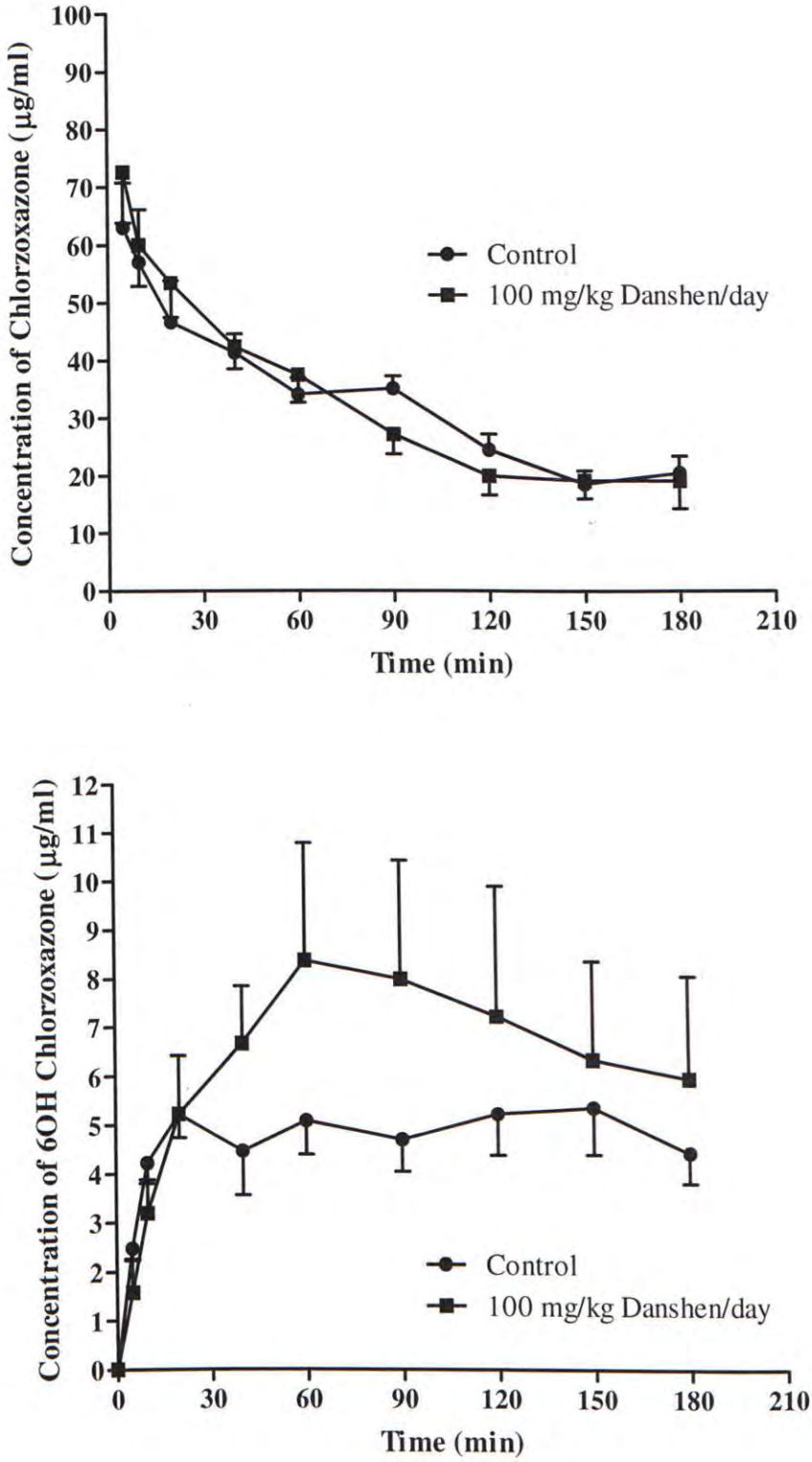



Fig. 3.17 Concentration-time profile of chlorzoxazone (25 mg/kg, i.v.) after p.o. 14-day treatment with saline (control) or 100 mg/kg/day of whole Danshen extract. Results were mean ± SEM of 6-8 rats.

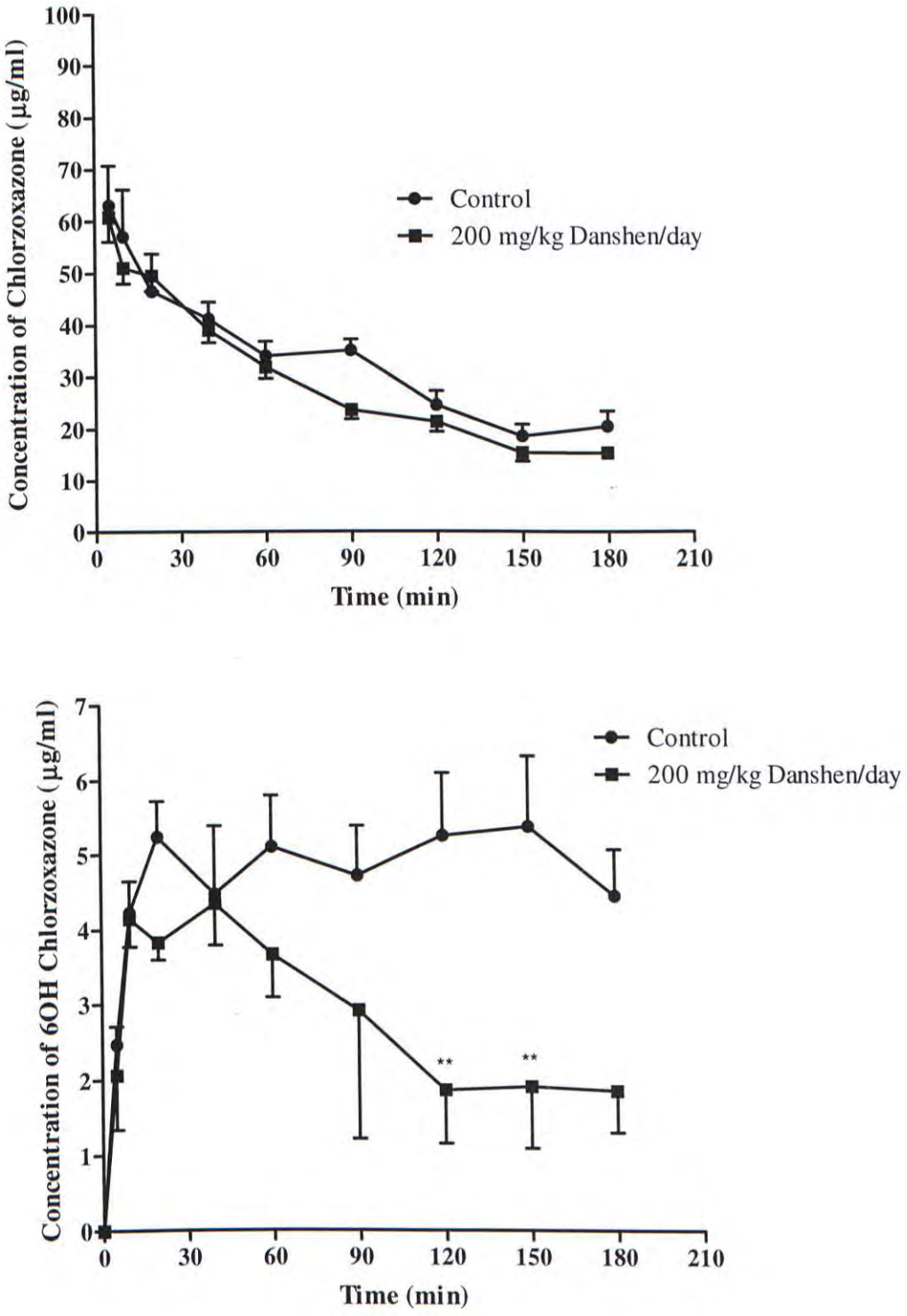



Fig. 3.18 Concentration-time profile of chlorzoxazone (25 mg/kg, i.v.) after p.o. 14-day treatment with saline (control) or 200 mg/kg/day of whole Danshen extract. Results were mean ± SEM of 6-8 rats. $^{**}p < 0.01$ when compared with controls.

Table 3.11 Pharmacokinetics of chlorzoxazone after 14-day pretreatment of whole Danshen extract (p.o.).

| Pharmacokinetic parameters | Control (saline) | Danshen (100 mg/kg/day, 14 days) | Danshen (200 mg/kg/day, 14 days) |
|---|---|---|---|
| $C_{initial}$ (g/ml) | 55.93 ± 8.243 | 62.68 ± 5.941 | 60.10 ± 4.493 |
| AUC (g/min/ml) | 6941 ± 928.3 | 6690 ± 598.8 | 6984 ± 485.3 |
| $V_d$ (ml/kg) | 458.6 ± 61.65 | 397.7 ± 45.24 | 410.6 ± 63.24 |
| CL (ml/min/kg) | 3.806 ± 0.5082 | 3.722 ± 0.3514 | 3.593 ± 0.2710 |
| $T_{1/2}$ (min) | 86.80 ± 3.420 | 87.57 ± 9.552 | 81.65 ± 7.459 |

$T_{1/2}$: half-life, $C_{initial}$: initial concentration of chlorzoxazone, AUC: area under concentration-time curves, $V_d$: volume of distribution, CL: clearance. Results were mean ± SEM of 6 – 8 rats. *$p < 0.05$ when compared to control (saline) using unpaired Student's t-test.

Table 3.12 Area under curve of 6-hydroxychlorzoxazone after 14-day pretreatment of whole Danshen extract (p.o.).

| | Control (saline) | Danshen (100 mg/kg/day, 14 days) | Danshen (200 mg/kg/day, 14 days) |
|---|---|---|---|
| AUC (g/min/ml) | 853.9 ± 73.81 | 924.9 ± 302.4 (+8.3%) | 508.0 ± 101.0* (-40.5%) |
| AUC ratio (HCZX/CZX) | 0.12 ± 0.012 | 0.14 ± 0.033 (+16.7%) | 0.07 ± 0.019 (-41.7%) |

AUC: area under concentration-time curves. Results were mean ± SEM of 6 – 8 rats. *$p < 0.05$ when compared to control (saline).

### 3.3.2 Effects of whole Danshen extract on rat CYP2E1 expression

For 3-day treatments, Figure 3.20 shows that only 400 mg/kg whole Danshen extract administered intraperitonealy had significant reduction on rat CYP2E1 expression and no effects were seen after either 200 mg/kg Danshen treatment or treatments via oral administration.

For 14-day treatments, 400 mg/kg Danshen i.p. treatment and 200 mg/kg Danshen p.o. treatment showed reduction on rat CYP2E1 expression but no effects caused by 200 mg/kg Danshen i.p. treatment and 100 mg/kg Danshen p.o. treatment.

Acetone water was used as the control of induction in CYP2E1 expression which showed a significant increase in CYP2E1 expression, while dexamethasone was a control of reduction in CYP2E1 expression but there was no significant difference in CYP2E1 expression when compared with the control.

Molecular weight of CYP2E1: 56 kDa (Tomaszewski et al., 2008)

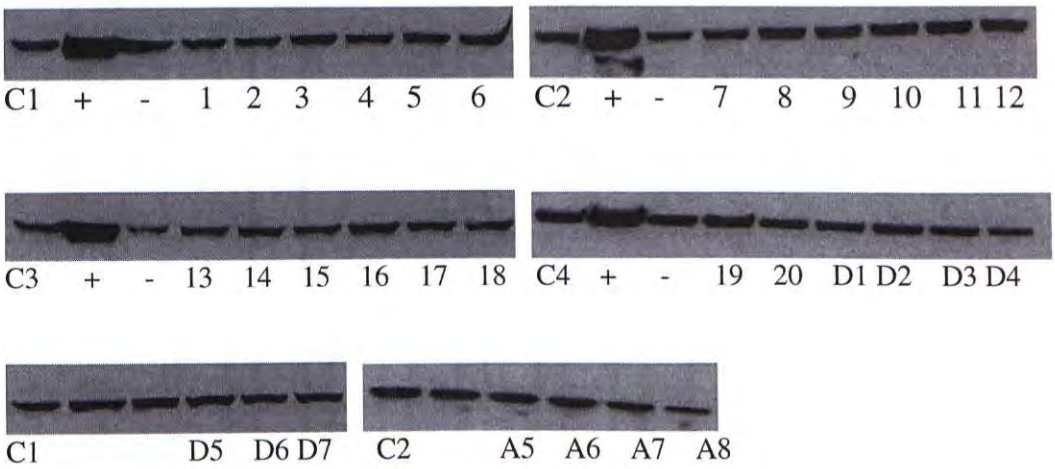



Figure 3.19 Films of Western blot.

C1-C4: Controls with saline only

+: Positive control (5% Acetone water for 7 days)

-: Negative control (75 mg/kg Dexamethasone for 3 days)

1-4: 14-day (i.p.) treatment with 400 mg/kg Danshen

5-8: 3-day (i.p.) treatment with 400 mg/kg Danshen

9-12: 3-day (p.o.) treatment with 200 mg/kg Danshen

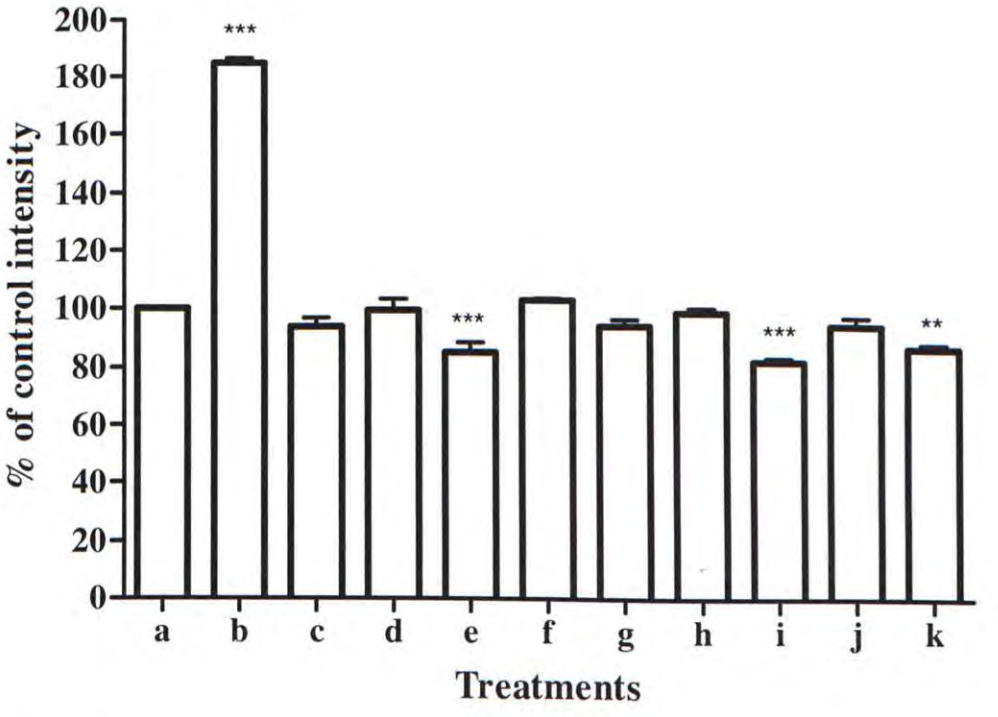
13-16: 14-day (i.p.) treatment with 200 mg/kg Danshen

17-20: 14-day (p.o.) treatment with 100 mg/kg Danshen

D1-D3: 3-day (i.p.) treatment with 200 mg/kg Danshen

D4-D7: 3-day (p.o.) treatment with 500 mg/kg Danshen

A5-A8: 14-day (p.o.) treatment with 200 mg/kg Danshen




a: Control
b: 5% acetone water
c: Dexamethasone
d: 3-day (i.p.) treatment with 200 mg/kg Danshen
e: 3-day (i.p.) treatment with 400 mg/kg Danshen
f: 3-day (p.o.) treatment with 200 mg/kg Danshen
g: 3-day (p.o.) treatment with 500 mg/kg Danshen
h: 14-day (i.p.) treatment with 200 mg/kg Danshen
i: 14-day (i.p.) treatment with 400 mg/kg Danshen
j: 14-day (p.o.) treatment with 100 mg/kg Danshen
k: 14-day (p.o.) treatment with 200 mg/kg Danshen

Fig. 3.20 Rat CYP2E1 expression after various concentrations of whole Danshen extract via intraperitoneal and oral administration. The results were expressed as percentage of control intensity. Results were mean ± SEM of 4 rats. $^{**}p < 0.01$ and $^{***}p < 0.001$ when compared to control (saline).

### 3.3.3 Summary

Table 3.13 summarizes the *in vivo* pharmacokinetic studies and CYP2E1 expression of this study. For acute treatment, Danshen decreased metabolite formation via oral administration but not intraperitoneal injection. Danshen had no effects on chlorzoxazone metabolism but reduced metabolite formation after 14-day treatments of Danshen via i.p. and p.o. route at 400 mg/kg/day and 200 mg/kg/day respectively. CYP2E1 expression was decreased after 3-day i.p. administration of 400 mg/kg/day Danshen, 14-day i.p. administration of 400 mg/kg/day Danshen and 14-day p.o. administration of 200 mg/kg/day Danshen.

Table 3.13 A summary to compare the effects of Danshen on CYP2E1 activity and protein expression.

| **Whole Dashen extract treatment (mg/kg)** | ***In vivo* Decrease in AUC of metabolite?** | **CYP2E1 expression Decrease in protein expression?** |
|---|---|---|
| **Intraperitoneal administration** | | |
| **Acute** | | |
| 50 | No | - |
| 100 | No | - |
| 200 | No | - |
| **3-day** | | |
| 200 | No | No |
| 400 | No | Yes |
| **14-day** | | |
| 200 | No | No |
| 400 | Yes | Yes |
| **Oral administration** | | |
| **Acute** | | |
| 100 | Yes | - |
| 200 | Yes | - |
| 400 | Yes | - |
| **3-day** | | |
| 200 | No | No |
| 500 | No | No |
| **14-day** | | |
| 100 | No | No |
| 200 | Yes | Yes |

## 3.4 Discussion

Whole Danshen extract, ethanolic and aqueous fractions of Danshen, cryptotanshinone, dihydrotanshinone and tanshinone I inhibited CYP2E1 activities in rat and human liver microsomes. To further the investigation on the effects of Danshen, current *in vivo* experiments were carried out. The doses of whole Danshen extract used were based on the dosages from previous studies (Wang, 2007; Wang et al., 2009). Apparent volume of distribution ($V_d$) was the most affected parameter by Danshen in acute studies. Plasma half-life of chlorzoxaone was increased after low dose (50 mg/kg) of Danshen i.p. treatment. $C_{initial}$ of chlorzoxazone had increased after 100 mg/kg and 200 mg/kg oral treatments. Clearance also decreased after 200 mg/kg oral treatment. The alteration of pharmacokinetic parameters after 3-day Danshen i.p. treatment at 400 mg/kg dosage can be seen that $C_{initial}$ had decreased and $V_d$ had increased. However, multiple Danshen treatments in the current experiment did not affect the pharmacokinetic parameters of chlorzoxazone.

In this study, AUC of 6-hydroxychlorzoxazone was also compared with the control. Danshen treatments had reduced AUC of metabolites in acute oral administration but not in intraperitoneal injection. AUC of metabolites after 14-day intraperitoneal injection and 14-day oral administration was reduced at 400 mg/kg/day and 200 mg/kg/day of Danshen, respectively. The results showed that an oral Danshen treatment may be more effective than intraperitoneal treatment on chlorzoxazone metabolism. Intraperitoneal administration is always thought to be an effective route as the drugs injected are directly absorbed through the blood capillaries surrounding peritoneum and go into liver through blood streams. For oral administration, drugs

will pass through the digestive system before entering the liver. The drugs may be chemically degraded or altered by the enzymes on the gut wall. The phenomenon of higher effectiveness of oral administration in the current experiment is uncommon. The difference between the effects of Danshen via i.p. and oral administrations may be due to the difference of the routes which could affect the absorption of Danshen. It has been shown that aqueous Danshen extraction was well absorbed in intestine, in which, danshensu was absorbed fast and well distributed (Zhou et al., 2005), but relevant literatures about absorption of Danshen and its components are limited. No significant effects could be seen in 3-day treatments although there was increase in AUC of 6-hydroxychlorzoxazone after i.p. treatment. Moreover, there was an interesting phenomenon that it showed some statistically insignificant increase in AUC of 6-hydroxychlorzoxazone after lower dose of Danshen treatment but decrease in higher dosage after 3-day and 14-day treatments. The AUC ratios gave a clearer picture to show the effects between low dose and high dose treatments of Danshen after 3 days and 14 days. However, the cause of these increase still remains unknown.

As CYP2E1 is an inducible enzyme, an experiment on CYP2E1 expression was carried out. There was no increase in CYP2E1 expression after 3-day and 14-day treatments implying there was no induction of CYP2E1 after multiple Danshen dosages. It also showed that the intensity of bandings decreased after 3 and 14-day i.p. treatments of Danshen at higher dosage and it only happened after 14-day p.o. treatment of higher Danshen dosage. This indicates that CYP2E1 expression may be down-regulated after the treatments. The results of CYP2E1 expression were in agreement with the *in vivo* experiment on reduction of chlorzoxazone metabolism.

Hakkola *et. al.*(2003) showed that the inflammatory cytokines IL-1β, TNFα and IL-6 down-regulated CYP2E1 expression in Fao rat hepatoma cell line. Down-regulation of CYP2E1 was found to be related to decrease in some transcription factors such as hepatocyte nuclear factor 1α (HNF-1α) to bind to promoter, acting on the CYP2E1 5'-upstream regulatory region, that reduced mRNA production. The reduction may be related to stabilization of mRNA (Hakkola et al., 2003). Another study found that insulin suppressed CYP2E1 expression through insulin-mediated signaling pathways involving PI3-kinase, p70 S6 kinase and Src kinase. The suggested mechanism increased in CYP2E1 mRNA turnover with inhibition of CYP2E1 gene transcription (Woodcroft et al., 2002). However, regulatory system in CYP2E1 is still under research.

In conclusion, the *in vivo* results in current experiments are in agreement with the *in vitro* studies in liver microsomes experiments that metabolism of chlorzoxazone was decreased by Danshen treatments. Danshen decreased CYP2E1-mediated chlorzoxazone metabolism, especially after acute and 14-day treatments. $V_d$ was the most affected pharmacokinetic parameter followed by $C_{initial}$, clearance and half-life after Danshen treatments. Oral administration is the most common pathway for Danshen intake. From the current results, an oral administration was more effective on reducing chlorzoxazone metabolism. This may be due to a fast absorption of Danshen after oral administration. There was some increase in AUC of 6-hydroxychlorzoxaone after 3-day and 14-day low dosages of Danshen treatments but it was not statistically significant. The reason leading to this increase was unknown. There was no induction in CYP2E1 expression after 3-day and 14-day Danshen treatments although there was decrease in CYP2E1 expression at high

Danshen dosages. The results of CYP2E1 expression were therefore in agreement with the *in vivo* experiment on reduction of chlorzoxazone metabolism.

The agreement of the current experiment with the previous experiments implied that CYP2E1-mediated metabolism could be affected by Danshen. Further investigation on interaction with ethanol and paracetamol should be carried out due to the importance of CYP2E1 on their metabolisms and the prevalence on ethanol and paracetamol consumption. Potential drug interaction may therefore be avoided when ethanol or paracetamol are taken with Danshen.

# Chapter 4
# General Discussion

The number of reports of herb-drug interactions has increased with increasing herbal consumption. Although concern of herb-drug interactions has been raised, their understanding and clinical and toxicological significance remain limited. The nature of herb-drug interactions may not be an interaction between chemical components of a drug and a herb to produce a toxic reaction (Lyna Irawati, 2007). Instead, the interactions may involve cytochrome P450 metabolisms affected by herbal components. Danshen is a prevalent herb used in mainland China for treating cardiovascular and cerebrovascular diseases. Previous studies showed that Danshen increased the bioavailability and decreased the clearance of warfarin, as well as prolonging prothrombin time (Chan et al., 1995). There were also reports on Danshen-warfarin interactions during operations with abnormal INR in patient (Tam et al., 1995; Yu et al., 1997). This herb-drug interaction between Danshen and warfarin was suggested to involve CYP enzymes (CYP1A2, CYP2C9 and CYP3A4) that mediate warfarin metabolism (Lo et al., 1992; Chan et al., 1995). Further studies found that Danshen and its active components affected the metabolism of specific model probe substrates of CYP1A2 (phenacetin and caffeine), CYP2C9 (tolbutamide) and CYP3A4 (testosterone) *in vitro* and *in viv*o in rats. In addition, Danshen also inhibited the CYP1A2 and CYP3A4 enzyme activities in human liver microsomes (Wang, 2007; Wang et al., 2009). CYP2E1 is one of the important CYPs which is responsible for ethanol metabolism. To accomplish the study on the effects of Danshen on CYP activities, the effects of Danshen and its active components on CYP2E1-mediated chlorzoxazone metabolism had been investigated *in vitro* and *in*

*vivo* in rats, and also *in vitro* in human in the current study.

In both rat and pooled human liver microsomes study, whole Danshen extract inhibited CYP2E1 activities non-competitively. The ethanolic fraction of Danshen had stronger inhibitory effect on CYP2E1 activities than aqueous fraction of Danshen and dihydrotanshinone was the most potent tanshinone to exhibit inhibitory effects on CYP2E1 activities uncompetitively followed by cryptotanshinone and tanshinone I. Although dihydrotanshinone showed the same inhibitory mode as the ethanolic fraction of Danshen, it was suggested that cryptotanshinone, dihydrotanshinone and tanshinone I might have contributed to the inhibitory effect of the ethanolic fraction of Danshen on CYP2E1 activities and tanshinone IIA did not show inhibitory effects. The results on rat liver microsomes agree with that using human liver microsomes except for the more prominent effects of Danshen on human liver CYP2E1 than in rat with much lower $IC_{50}$ and K*i* values. Previous studies showed that CYP1A2 and CYP3A4 activities in rat and human liver microsomes were inhibited competitively (Wang, 2007; Wang et al., 2009) instead of non-competively in CYP2E1 acitivites. Similarly, the stronger inhibitory effects of the ethanolic fraction of Danshen shows agreement with the previous studies on CYP1A2 and CYP3A4. The potent effects of dihydrotanshinone implied the importance of dihydrotanshinone on inhibiting CYP-mediated hydroxylations involving CYP1A2, CYP2E1 and CYP3A4 (Wang, 2007; Wang et al., 2009).

Table 4.1 Comparison among CYP2E1, CYP1A2 and CYP3A4 *in vivo* experiments via intraperitoneal administration of whole Danshen extract (results of CYP1A2 and CYP3A4 were from Wang, 2007 and Wang et al., 2009).

| **Intraperitoneal administration** | | | |
|---|---|---|---|
| **Dosages of Danshen (mg/kg/day)** | **CYP2E1** | **CYP1A2** | **CYP3A4** |
| **Acute treatment** | | | |
| **50** | No effect | No effect | No effect |
| **100** | No effect | No effect | No effect |
| **200** | No effect | Inhibition | No effect |
| **3-day treatment** | | | |
| **100** | - | Inhibition | No effect |
| **200** | No effect | Inhibition | Inhibition |
| **400** | No effect | - | - |
| **14-day treatment** | | | |
| **100** | - | Inhibition | No effect |
| **200** | No effect | - | - |
| **400** | Inhibition | - | - |

Table 4.2 Comparison among CYP2E1, CYP1A2 and CYP3A4 *in vivo* experiments via oral administration of whole Danshen extract (results of CYP1A2 and CYP3A4 were from Wang, 2007 and Wang et al., 2009)

| **Oral administration** | | | |
|---|---|---|---|
| **Dosages of Danshen (mg/kg/day)** | **CYP2E1** | **CYP1A2** | **CYP3A4** |
| **Acute treatment** | | | |
| **100** | Inhibition | - | - |
| **200** | Inhibition | No effect | No effect |
| **400** | Inhibition | - | - |
| **500** | - | Inhibition | No effect |
| **1000** | - | inhibition | Inhibition |
| **3-day treatment** | | | |
| **200** | No effect | No effect | No effect |
| **500** | No effect | Inhibition | Inhibition |
| **14-day treatment** | | | |
| **100** | Inhibition | - | - |
| **200** | Inhibition | Inhibition | No effect |

As inhibitory effects of Danshen and its active components were observed in *in vitro* experiments, *in vivo* study was carried out to investigate the effects of Danshen on pharmacokinetics of chlorzoxazone. $V_d$ of chlorzoxazone was the most affected pharmacokinetic parameter and was mostly altered after acute treatments. Danshen only had minor effects on $C_{initial}$, clearance and half-life of chlorzoxazone. Acute Danshen treatments reduced 6-hydroxychlorzoxazone formation via oral administration but not via intraperitoneal injection. This might be related to the different absorption routes. As shown in Tables 4.1 and 4.2, chlorzoxazone metabolism was reduced at 100 - 400 mg/kg Danshen dosages. There were no reduction on chlorzoxazone metabolism after 3-day treatments but a statistically insignificant increase in 6-hydroxychlorzoxazone was observed at low dosages. The cause of the increase has remained unknown. Danshen reduced 6-hydroxychlorzoxazone formation after 14-day treatments at high dosages. Lower concentration of Danshen (200 mg/kg/day) was required to decrease chlorzoxazone metabolism in 14-day p.o. treatment than in 14-day i.p. treatment (400 mg/kg/day). To compare with the previous studies, Danshen changed most of the pharmacokinetic parameters including $V_d$, clearance, AUC and half-life time of probe substrates of CYP1A2 and CYP3A4 in a dose-dependent manner. Danshen inhibited CYP1A2 metabolism at 200 mg/kg but showed no effect on CYP3A4 metabolism in acute i.p. treatments. At least 500 mg/kg Danshen was needed to inhibit CYP1A2 and CYP3A4 metabolism in oral acute treatments. Danshen did not show inhibitory effect on CYP3A4 metabolism after 3-day i.p. treatment but inhibited CYP1A2 at 200 mg/kg/day dosage. Dosage at 500 mg/kg/day also inhibited CYP1A2 and CYP3A4 metabolism after 3-day oral treatments. Danshen inhibited CYP1A2 at 100 mg/kg/day via i.p. treatments and 200 mg/kg/day via oral treatments after 14 days

while it did not show any effect on CYP3A4 metabolism (Wang, 2007; Wang et al., 2009).

Investigation on CYP2E1 expression after 3-day and 14-day Danshen treatment was also carried out. There was no induction of CYP2E1 after Danshen treatments but after 3-day i.p. and 14-day Danshen treatments the expression was decreased. The results of CYP2E1 expression were in agreement with the *in vivo* experiment. This indicates that the decrease in 6-hydroxychlorzoxazone in *in vivo* might be due to the decrease in CYP2E1 enzyme expression. Enzyme expression of CYP1A2 was also studied in previous research. Although Danshen had altered most of the pharmacokinetic parameters of CYP1A2 probe substrate, there was no effect on CYP1A2 expression after Danshen treatments for 3 and 14 days (Wang, 2007; Wang et al., 2009).

In conclusion, Danshen and its active components inhibited CYP2E1-mediated chlorzoxazone hydroxylation in rat and human liver microsomes. Danshen had also decreased chlorzoxazone metabolism *in vivo* by reducing the formation of 6-hydroxychlorzoxazone with changing $V_d$ of chlorzoxazone the most. Danshen did not induce but decreased CYP2E1 expression after 3-day and 14-day treatments. This may be concluded that the inhibitory effects of Danshen in *in vivo* were caused by the reduction in CYP2E1 expression rather than competing for the active sites on the enzymes with chlorzoxazone. The effects of Danshen on CYP2E1-mediated metabolisms are not reported in other literatures. Future studies could also investigate the effects of Danshen on other important CYP enzymes such as CYP2D6. By developing the protocols for CYP1A2, CYP2C9, CYP2D6, CYP2E1 and

CYP3A4, other potential herb-drug interactions caused by concurrent use of western drugs with other traditional Chinese medicine could be screened.

# References

Adams JD, Wang R, Yang J and Lien EJ (2006) Preclinical and clinical examinations of Salvia miltiorrhiza and its tanshinones in ischemic conditions. *Chin Med* **1:**3.

Ali M and Afzal M (1987) A potent inhibitor of thrombin stimulated platelet thromboxane formation from unprocessed tea. *Prostaglandins Leukot Med* **27:**9-13.

Bachmann K and Sarver JG (1996) Chlorzoxazone as a single sample probe of hepatic CYP2E1 activity in humans. *Pharmacology* **52:**169-177.

Bieche I, Narjoz C, Asselah T, Vacher S, Marcellin P, Lidereau R, Beaune P and de Waziers I (2007) Reverse transcriptase-PCR quantification of mRNA levels from cytochrome (CYP)1, CYP2 and CYP3 families in 22 different human tissues. *Pharmacogenet Genomics* **17:**731-742.

Brady JF, Xiao F, Wang MH, Li Y, Ning SM, Gapac JM and Yang CS (1991) Effects of disulfiram on hepatic P450IIE1, other microsomal enzymes, and hepatotoxicity in rats. *Toxicol Appl Pharmacol* **108:**366-373.

Cao EH, Liu XQ, Wang JJ and Xu NF (1996) Effect of natural antioxidant tanshinone II-A on DNA damage by lipid peroxidation in liver cells. *Free Radic Biol Med* **20:**801-806.

Caro AA and Cederbaum AI (2004) Oxidative stress, toxicology, and pharmacology of CYP2E1. *Annu Rev Pharmacol Toxicol* **44:**27-42.

Carriere V, Goasduff T, Ratanasavanh D, Morel F, Gautier JC, Guillouzo A, Beaune P and Berthou F (1993) Both cytochromes P450 2E1 and 1A1 are involved in the metabolism of chlorzoxazone. *Chem Res Toxicol* **6:**852-857.

Chan K, Lo AC, Yeung JH and Woo KS (1995) The effects of Danshen (Salvia miltiorrhiza) on warfarin pharmacodynamics and pharmacokinetics of warfarin enantiomers in rats. *J Pharm Pharmacol* **47:**402-406.

Chan TY (2001) Interaction between warfarin and Danshen (Salvia miltiorrhiza). *Ann Pharmacother* **35:**501-504.

Chang GW and Kam PC (1999) The physiological and pharmacological roles of cytochrome P450 isoenzymes. *Anaesthesia* **54:**42-50.

Chang TK, Gonzalez FJ and Waxman DJ (1994) Evaluation of triacetyloleandomycin,

alpha-naphthoflavone and diethyldithiocarbamate as selective chemical probes for inhibition of human cytochromes P450. *Arch Biochem Biophys* **311:**437-442.

Chavez ML (2005) Herbal-drug interactions. *InetEC* **9:**1-30.

Chavez ML, Jordan MA and Chavez PI (2006) Evidence-based drug--herbal interactions. *Life Sci* **78:**2146-2157.

Chen KJ (1981) Certain progress in the treatment of coronary heart disease with traditional medicinal plants in China. *Am J Chin Med* **9:**193-196.

Choi HS, Cho DI, Choi HK, Im SY, Ryu SY and Kim KM (2004) Molecular mechanisms of inhibitory activities of tanshinones on lipopolysaccharide-induced nitric oxide generation in RAW 264.7 cells. *Arch Pharm Res* **27:**1233-1237.

Choi HS and Kim KM (2004) Tanshinones inhibit mast cell degranulation by interfering with IgE receptor-mediated tyrosine phosphorylation of PLCgamma2 and MAPK. *Planta Med* **70:**178-180.

Conde de la Rosa L, Moshage H and Nieto N (2008) [Hepatocyte oxidant stress and alcoholic liver disease]. *Rev Esp Enferm Dig* **100:**156-163.

Coon MJ (2005) Cytochrome P450: nature's most versatile biological catalyst. *Annu Rev Pharmacol Toxicol* **45:**1-25.

Cox SK, Hamner T and Bartges J (2003) Determination of 6-hydroxychlorzoxazone and chlorzoxazone in porcine microsome samples. *J Chromatogr B Analyt Technol Biomed Life Sci* **784:**111-116.

Dobos GJ, Tan L, Cohen MH, McIntyre M, Bauer R, Li X and Bensoussan A (2005) Are national quality standards for traditional Chinese herbal medicine sufficient? Current governmental regulations for traditional Chinese herbal medicine in certain Western countries and China as the Eastern origin country. *Complement Ther Med* **13:**183-190.

Eagling VA, Tjia JF and Back DJ (1998) Differential selectivity of cytochrome P450 inhibitors against probe substrates in human and rat liver microsomes. *Br J Clin Pharmacol* **45:**107-114.

Eisenberg DM, Davis RB, Ettner SL, Appel S, Wilkey S, Van Rompay M and Kessler RC (1998) Trends in alternative medicine use in the United States, 1990-1997: results of a follow-up national survey. *JAMA* **280:**1569-1575.

Eliasson E, Mkrtchian S and Ingelman-Sundberg M (1992) Hormone- and

substrate-regulated intracellular degradation of cytochrome P450 (2E1) involving MgATP-activated rapid proteolysis in the endoplasmic reticulum membranes. *J Biol Chem* **267:**15765-15769.

Ernstgard L, Johanson G, Karlsson AS and Warholm M (2007) Phenotyping of cytochrome P450 2E1 in vitro and in vivo. *Curr Drug Metab* **8:**493-498.

Gonzalez FJ (2007) The 2006 Bernard B. Brodie Award Lecture. Cyp2e1. *Drug Metab Dispos* **35:**1-8.

Gorski JC, Jones DR, Wrighton SA and Hall SD (1997) Contribution of human CYP3A subfamily members to the 6-hydroxylation of chlorzoxazone. *Xenobiotica* **27:**243-256.

Greenblatt DJ and von Moltke LL (2005) Interaction of warfarin with drugs, natural substances, and foods. *J Clin Pharmacol* **45:**127-132.

Guengerich FP, Kim DH and Iwasaki M (1991) Role of human cytochrome P-450 IIE1 in the oxidation of many low molecular weight cancer suspects. *Chem Res Toxicol* **4:**168-179.

Hakkola J, Hu Y and Ingelman-Sundberg M (2003) Mechanisms of down-regulation of CYP2E1 expression by inflammatory cytokines in rat hepatoma cells. *J Pharmacol Exp Ther* **304:**1048-1054.

Hinson JA, Reid AB, McCullough SS and James LP (2004) Acetaminophen-induced hepatotoxicity: role of metabolic activation, reactive oxygen/nitrogen species, and mitochondrial permeability transition. *Drug Metab Rev* **36:**805-822.

Hirsh J, Dalen JE, Deykin D and Poller L (1992) Oral anticoagulants. Mechanism of action, clinical effectiveness, and optimal therapeutic range. *Chest* **102:**312S-326S.

Howard LA, Micu AL, Sellers EM and Tyndale RF (2001) Low doses of nicotine and ethanol induce CYP2E1 and chlorzoxazone metabolism in rat liver. *J Pharmacol Exp Ther* **299:**542-550.

Hu P, Luo GA, Zhao Z and Jiang ZH (2005) Quality assessment of radix salviae miltiorrhizae. *Chem Pharm Bull (Tokyo)* **53:**481-486.

Ingelman-Sundberg M (2004) Pharmacogenetics of cytochrome P450 and its applications in drug therapy: the past, present and future. *Trends Pharmacol Sci* **25:**193-200.

Izzat MB, Yim AP and El-Zufari MH (1998) A taste of Chinese medicine! *Ann Thorac Surg* **66:**941-942.

Jemnitz K, Veres Z, Monostory K, Kobori L and Vereczkey L (2008) Interspecies differences in acetaminophen sensitivity of human, rat, and mouse primary hepatocytes. *Toxicol In Vitro* **22:**961-967.

Jiang RW, Lau KM, Hon PM, Mak TC, Woo KS and Fung KP (2005) Chemistry and biological activities of caffeic acid derivatives from Salvia miltiorrhiza. *Curr Med Chem* **12:**237-246.

Johansson I, Lindros KO, Eriksson H and Ingelman-Sundberg M (1990) Transcriptional control of CYP2E1 in the perivenous liver region and during starvation. *Biochem Biophys Res Commun* **173:**331-338.

Kalra BS (2007) Cytochrome P450 enzyme isoforms and their therapeutic implications: an update. *Indian J Med Sci* **61:**102-116.

Kaplowitz N (2005) Idiosyncratic drug hepatotoxicity. *Nat Rev Drug Discov* **4:**489-499.

Karliova M, Treichel U, Malago M, Frilling A, Gerken G and Broelsch CE (2000) Interaction of Hypericum perforatum (St. John's wort) with cyclosporin A metabolism in a patient after liver transplantation. *J Hepatol* **33:**853-855.

Karyekar CS, Eddington ND and Dowling TC (2002) Effect of St. John's Wort extract on intestinal expression of cytochrome P4501A2: studies in LS180 cells. *J Postgrad Med* **48:**97-100.

Khan AJ, Ruwali M, Choudhuri G, Mathur N, Husain Q and Parmar D (2009) Polymorphism in cytochrome P450 2E1 and interaction with other genetic risk factors and susceptibility to alcoholic liver cirrhosis. *Mutat Res* **664:**55-63.

Kim SY, Moon TC, Chang HW, Son KH, Kang SS and Kim HP (2002) Effects of tanshinone I isolated from Salvia miltiorrhiza bunge on arachidonic acid metabolism and in vivo inflammatory responses. *Phytother Res* **16:**616-620.

Kobayashi K, Urashima K, Shimada N and Chiba K (2002) Substrate specificity for rat cytochrome P450 (CYP) isoforms: screening with cDNA-expressed systems of the rat. *Biochem Pharmacol* **63:**889-896.

Koop DR (1986) Hydroxylation of p-nitrophenol by rabbit ethanol-inducible cytochrome P-450 isozyme 3a. *Mol Pharmacol* **29:**399-404.

Koop DR (1992) Oxidative and reductive metabolism by cytochrome P450 2E1. *FASEB J* **6:**724-730.

Koop DR (2006) Alcohol metabolism's damaging effects on the cell: a focus on

reactive oxygen generation by the enzyme cytochrome P450 2E1. *Alcohol Res Health* **29:**274-280.

Kramer I, Dalhoff K, Clemmesen JO, Loft S and Poulsen HE (2003) Comparison of chlorzoxazone one-sample methods to estimate CYP2E1 activity in humans. *Eur J Clin Pharmacol* **59:**775-778.

Lam FF, Yeung JH, Chan KM and Or PM (2008) Dihydrotanshinone, a lipophilic component of Salvia miltiorrhiza (Danshen), relaxes rat coronary artery by inhibition of calcium channels. *J Ethnopharmacol* **119:**318-321.

Lam FF, Yeung JH, Kwan YW, Chan KM and Or PM (2006) Salvianolic acid B, an aqueous component of Danshen (Salvia miltiorrhiza), relaxes rat coronary artery by inhibition of calcium channels. *Eur J Pharmacol* **553:**240-245.

Lawson LD, Ransom DK and Hughes BG (1992) Inhibition of whole blood platelet-aggregation by compounds in garlic clove extracts and commercial garlic products. *Thromb Res* **65:**141-156.

Lay IS, Hsieh CC, Chiu JH, Shiao MS, Lui WY and Wu CW (2003) Salvianolic acid B enhances in vitro angiogenesis and improves skin flap survival in Sprague-Dawley rats. *J Surg Res* **115:**279-285.

Lee AM, Yue J and Tyndale RF (2006) In vivo and in vitro characterization of chlorzoxazone metabolism and hepatic CYP2E1 levels in African Green monkeys: induction by chronic nicotine treatment. *Drug Metab Dispos* **34:**1508-1515.

Lee CY, Sher HF, Chen HW, Liu CC, Chen CH, Lin CS, Yang PC, Tsay HS and Chen JJ (2008) Anticancer effects of tanshinone I in human non-small cell lung cancer. *Mol Cancer Ther* **7:**3527-3538.

Lee SY, Choi DY and Woo ER (2005) Inhibition of osteoclast differentiation by tanshinones from the root of Salvia miltiorrhiza bunge. *Arch Pharm Res* **28:**909-913.

Lee WM (2003) Acute liver failure in the United States. *Semin Liver Dis* **23:**217-226.

Lehninger AL, Nelson DL and Cox MM (2005) *Lehninger principles of biochemistry*. W.H. Freeman, New York.

Lei XL and Chiou GC (1986) Cardiovascular pharmacology of Panax notoginseng (Burk) F.H. Chen and Salvia miltiorrhiza. *Am J Chin Med* **14:**145-152.

Lieber CS (1999) Microsomal ethanol-oxidizing system (MEOS): the first 30 years (1968-1998)--a review. *Alcohol Clin Exp Res* **23:**991-1007.

Lin JH, Chen IW, Chiba M, Nishime JA and Deluna FA (2000) Route-dependent nonlinear pharmacokinetics of a novel HIV protease inhibitor: involvement of enzyme inactivation. *Drug Metab Dispos* **28:**460-466.

Lin YL, Wu CH, Luo MH, Huang YJ, Wang CN, Shiao MS and Huang YT (2006) In vitro protective effects of salvianolic acid B on primary hepatocytes and hepatic stellate cells. *J Ethnopharmacol* **105:**215-222.

Lo AC, Chan K, Yeung JH and Woo KS (1992) The effects of Danshen (Salvia miltiorrhiza) on pharmacokinetics and pharmacodynamics of warfarin in rats. *Eur J Drug Metab Pharmacokinet* **17:**257-262.

Lu Y and Foo LY (2002) Polyphenolics of Salvia--a review. *Phytochemistry* **59:**117-140.

Lucas D, Ferrara R, Gonzalez E, Bodenez P, Albores A, Manno M and Berthou F (1999) Chlorzoxazone, a selective probe for phenotyping CYP2E1 in humans. *Pharmacogenetics* **9:**377-388.

Lyna Irawati RJ (2007) Herbal-drug interactions: An Overview. *JASA* **2:**50-52.

Lynch T and Price A (2007) The effect of cytochrome P450 metabolism on drug response, interactions, and adverse effects. *Am Fam Physician* **76:**391-396.

Ma Q (2001) Induction of CYP1A1. The AhR/DRE paradigm: transcription, receptor regulation, and expanding biological roles. *Curr Drug Metab* **2:**149-164.

Mannel M (2004) Drug interactions with St John's wort : mechanisms and clinical implications. *Drug Saf* **27:**773-797.

Markowitz JS, DeVane CL, Boulton DW, Carson SW, Nahas Z and Risch SC (2000) Effect of St. John's wort (Hypericum perforatum) on cytochrome P-450 2D6 and 3A4 activity in healthy volunteers. *Life Sci* **66:**PL133-139.

Martinez MC, Nortier J, Vereerstraeten P and Vanherweghem JL (2002) Progression rate of Chinese herb nephropathy: impact of Aristolochia fangchi ingested dose. *Nephrol Dial Transplant* **17:**408-412.

McGehee RE, Jr., Ronis MJ, Cowherd RM, Ingelman-Sundberg M and Badger TM (1994) Characterization of cytochrome P450 2E1 induction in a rat hepatoma FGC-4 cell model by ethanol. *Biochem Pharmacol* **48:**1823-1833.

McGinnity DF and Riley RJ (2001) Predicting drug pharmacokinetics in humans from in vitro metabolism studies. *Biochem Soc Trans* **29:**135-139.

Mei Z, Zhang F, Tao L, Zheng W, Cao Y, Wang Z, Tang S, Le K, Chen S, Pi R and Liu P (2009) Cryptotanshinone, a compound from Salvia miltiorrhiza

modulates amyloid precursor protein metabolism and attenuates beta-amyloid deposition through upregulating alpha-secretase in vivo and in vitro. *Neurosci Lett* **452:**90-95.

Mizuno D, Tanaka E, Tanno K and Misawa S (2000) Chlorzoxazone: a probe drug whose metabolism can be used to monitor toluene exposure in rats. *Arch Toxicol* **74:**139-144.

Morgan ET, Koop DR and Coon MJ (1983) Comparison of six rabbit liver cytochrome P-450 isozymes in formation of a reactive metabolite of acetaminophen. *Biochem Biophys Res Commun* **112:**8-13.

Mosaddik MA (2003) In vitro cytotoxicity of tanshinones isolated from Salvia miltiorrhiza Bunge against P388 lymphocytic leukemia cells. *Phytomedicine* **10:**682-685.

Mueller SC, Uehleke B, Woehling H, Petzsch M, Majcher-Peszynska J, Hehl EM, Sievers H, Frank B, Riethling AK and Drewelow B (2004) Effect of St John's wort dose and preparations on the pharmacokinetics of digoxin. *Clin Pharmacol Ther* **75:**546-557.

Nan JX, Park EJ, Kang HC, Park PH, Kim JY and Sohn DH (2001) Anti-fibrotic effects of a hot-water extract from Salvia miltiorrhiza roots on liver fibrosis induced by biliary obstruction in rats. *J Pharm Pharmacol* **53:**197-204.

Nebel A, Schneider BJ, Baker RK and Kroll DJ (1999) Potential metabolic interaction between St. John's wort and theophylline. *Ann Pharmacother* **33:**502.

Ng TB, Liu F and Wang ZT (2000) Antioxidative activity of natural products from plants. *Life Sci* **66:**709-723.

Niu XL, Ichimori K, Yang X, Hirota Y, Hoshiai K, Li M and Nakazawa H (2000) Tanshinone II-A inhibits low density lipoprotein oxidation in vitro. *Free Radic Res* **33:**305-312.

Ohashi Y, Yamada K, Takemoto I, Mizutani T and Saeki K (2005) Inhibition of human cytochrome P450 2E1 by halogenated anilines, phenols, and thiophenols. *Biol Pharm Bull* **28:**1221-1223.

Oneta CM, Lieber CS, Li J, Ruttimann S, Schmid B, Lattmann J, Rosman AS and Seitz HK (2002) Dynamics of cytochrome P4502E1 activity in man: induction by ethanol and disappearance during withdrawal phase. *J Hepatol* **36:**47-52.

Ono S, Hatanaka T, Hotta H, Tsutsui M, Satoh T and Gonzalez FJ (1995) Chlorzoxazone is metabolized by human CYP1A2 as well as by human CYP2E1. *Pharmacogenetics* **5:**143-150.

Park EJ, Zhao YZ, Kim YC and Sohn DH (2007) PF2401-SF, standardized fraction of Salvia miltiorrhiza and its constituents, tanshinone I, tanshinone IIA, and cryptotanshinone, protect primary cultured rat hepatocytes from bile acid-induced apoptosis by inhibiting JNK phosphorylation. *Food Chem Toxicol* **45:**1891-1898.

Park HJ, Lee JH, Song YB and Park KH (1996) Effects of dietary supplementation of lipophilic fraction from Panax ginseng on cGMP and cAMP in rat platelets and on blood coagulation. *Biol Pharm Bull* **19:**1434-1439.

Peter R, Bocker R, Beaune PH, Iwasaki M, Guengerich FP and Yang CS (1990) Hydroxylation of chlorzoxazone as a specific probe for human liver cytochrome P-450IIE1. *Chem Res Toxicol* **3:**566-573.

Piao YF, Li JT and Shi Y (2003) Relationship between genetic polymorphism of cytochrome P450IIE1 and fatty liver. *World J Gastroenterol* **9:**2612-2615.

Piscitelli SC, Burstein AH, Chaitt D, Alfaro RM and Falloon J (2000) Indinavir concentrations and St John's wort. *Lancet* **355:**547-548.

Porubsky PR, Meneely KM and Scott EE (2008) Structures of human cytochrome P-450 2E1. Insights into the binding of inhibitors and both small molecular weight and fatty acid substrates. *J Biol Chem* **283:**33698-33707.

Raucy JL, Lasker JM, Lieber CS and Black M (1989) Acetaminophen activation by human liver cytochromes P450IIE1 and P450IA2. *Arch Biochem Biophys* **271:**270-283.

Rosado MF (2003) Thrombosis of a prosthetic aortic valve disclosing a hazardous interaction between warfarin and a commercial ginseng product. *Cardiology* **99:**111.

Sugimoto K, Ohmori M, Tsuruoka S, Nishiki K, Kawaguchi A, Harada K, Arakawa M, Sakamoto K, Masada M, Miyamori I and Fujimura A (2001) Different effects of St John's wort on the pharmacokinetics of simvastatin and pravastatin. *Clin Pharmacol Ther* **70:**518-524.

Sumida A, Kinoshita K, Fukuda T, Matsuda H, Yamamoto I, Inaba T and Azuma J (1999) Relationship between mRNA levels quantified by reverse transcription-competitive PCR and metabolic activity of CYP3A4 and

CYP2E1 in human liver. *Biochem Biophys Res Commun* **262:**499-503.

Takahashi K, Ouyang X, Komatsu K, Nakamura N, Hattori M, Baba A and Azuma J (2002) Sodium tanshinone IIA sulfonate derived from Danshen (Salvia miltiorrhiza) attenuates hypertrophy induced by angiotensin II in cultured neonatal rat cardiac cells. *Biochem Pharmacol* **64:**745-749.

Tam LS, Chan TY, Leung WK and Critchley JA (1995) Warfarin interactions with Chinese traditional medicines: Danshen and methyl salicylate medicated oil. *Aust N Z J Med* **25:**258.

Tanaka E and Hisawa S (1999) Clinically significant pharmacokinetic drug interactions with psychoactive drugs: antidepressants and antipsychotics and the cytochrome P450 system. *J Clin Pharm Ther* **24:**7-16.

Tanaka E, Nakamura T, Terada M, Shinozuka T and Honda K (2007) Metabolic interaction between ethanol, high-dose alprazolam and its two main metabolites using human liver microsomes in vitro. *J Forensic Leg Med* **14:**348-351.

Tang MK, Ren DC, Zhang JT and Du GH (2002) Effect of salvianolic acids from Radix Salviae miltiorrhizae on regional cerebral blood flow and platelet aggregation in rats. *Phytomedicine* **9:**405-409.

Tannergren C, Engman H, Knutson L, Hedeland M, Bondesson U and Lennernas H (2004) St John's wort decreases the bioavailability of R- and S-verapamil through induction of the first-pass metabolism. *Clin Pharmacol Ther* **75:**298-309.

Taylor JR and Wilt VM (1999) Probable antagonism of warfarin by green tea. *Ann Pharmacother* **33:**426-428.

Tindle HA, Davis RB, Phillips RS and Eisenberg DM (2005) Trends in use of complementary and alternative medicine by US adults: 1997-2002. *Altern Ther Health Med* **11:**42-49.

Tomaszewski P, Kubiak-Tomaszewska G and Pachecka J (2008) Cytochrome P450 polymorphism--molecular, metabolic, and pharmacogenetic aspects. II. Participation of CYP isoenzymes in the metabolism of endogenous substances and drugs. *Acta Pol Pharm* **65:**307-318.

Tsutsumi M, Takada A and Wang JS (1994) Genetic polymorphisms of cytochrome P4502E1 related to the development of alcoholic liver disease. *Gastroenterology* **107:**1430-1435.

Van Vleet TR, Bombick DW and Coulombe RA, Jr. (2001) Inhibition of human cytochrome P450 2E1 by nicotine, cotinine, and aqueous cigarette tar extract in vitro. *Toxicol Sci* **64:**185-191.

Wan J, Ernstgard L, Song BJ and Shoaf SE (2006) Chlorzoxazone metabolism is increased in fasted Sprague-Dawley rats. *J Pharm Pharmacol* **58:**51-61.

Wang X (2007) A study to investigate the mechanisms of Danshen-drug interactions using cytochrome P450 probe substrates. *PhD Thesis*:The Chinese University of Hong Kong.

Wang X, Lee WY, Or PM and Yeung JH (2009) Effects of major tanshinones isolated from Danshen (Salvia miltiorrhiza) on rat CYP1A2 expression and metabolism of model CYP1A2 probe substrates. *Phytomedicine*.

Wang Z, Gorski JC, Hamman MA, Huang SM, Lesko LJ and Hall SD (2001) The effects of St John's wort (Hypericum perforatum) on human cytochrome P450 activity. *Clin Pharmacol Ther* **70:**317-326.

Wang Z, Hall SD, Maya JF, Li L, Asghar A and Gorski JC (2003) Diabetes mellitus increases the in vivo activity of cytochrome P450 2E1 in humans. *Br J Clin Pharmacol* **55:**77-85.

Watkins RE, Maglich JM, Moore LB, Wisely GB, Noble SM, Davis-Searles PR, Lambert MH, Kliewer SA and Redinbo MR (2003) 2.1 A crystal structure of human PXR in complex with the St. John's wort compound hyperforin. *Biochemistry* **42:**1430-1438.

Whitlock JP, Jr. (1999) Induction of cytochrome P4501A1. *Annu Rev Pharmacol Toxicol* **39:**103-125.

Wittkowsky AK (2001) Drug interactions update: drugs, herbs, and oral anticoagulation. *J Thromb Thrombolysis* **12:**67-71.

Woodcroft KJ, Hafner MS and Novak RF (2002) Insulin signaling in the transcriptional and posttranscriptional regulation of CYP2E1 expression. *Hepatology* **35:**263-273.

Wrighton SA and Stevens JC (1992) The human hepatic cytochromes P450 involved in drug metabolism. *Crit Rev Toxicol* **22:**1-21.

Wrighton SA, Thomas PE, Molowa DT, Haniu M, Shively JE, Maines SL, Watkins PB, Parker G, Mendez-Picon G, Levin W and et al. (1986) Characterization of ethanol-inducible human liver N-nitrosodimethylamine demethylase. *Biochemistry* **25:**6731-6735.

Wu TW, Zeng LH, Fung KP, Wu J, Pang H, Grey AA, Weisel RD and Wang JY (1993) Effect of sodium tanshinone IIA sulfonate in the rabbit myocardium and on human cardiomyocytes and vascular endothelial cells. *Biochem Pharmacol* **46:**2327-2332.

Wu YJ, Hong CY, Lin SJ, Wu P and Shiao MS (1998) Increase of vitamin E content in LDL and reduction of atherosclerosis in cholesterol-fed rabbits by a water-soluble antioxidant-rich fraction of Salvia miltiorrhiza. *Arterioscler Thromb Vasc Biol* **18:**481-486.

Xu W, Yang J and Wu LM (2009) Cardioprotective effects of tanshinone IIA on myocardial ischemia injury in rats. *Pharmazie* **64:**332-336.

Yamazaki H, Guo Z and Guengerich FP (1995) Selectivity of cytochrome P4502E1 in chlorzoxazone 6-hydroxylation. *Drug Metab Dispos* **23:**438-440.

Yan Z and Caldwell GW (2001) Metabolism profiling, and cytochrome P450 inhibition & induction in drug discovery. *Curr Top Med Chem* **1:**403-425.

Yang M, Liu A, Guan S, Sun J, Xu M and Guo D (2006) Characterization of tanshinones in the roots of Salvia miltiorrhiza (Dan-shen) by high-performance liquid chromatography with electrospray ionization tandem mass spectrometry. *Rapid Commun Mass Spectrom* **20:**1266-1280.

Yin HQ, Kim YS, Choi YJ, Kim YC, Sohn DH, Ryu SY and Lee BH (2008) Effects of tanshinone IIA on the hepatotoxicity and gene expression involved in alcoholic liver disease. *Arch Pharm Res* **31:**659-665.

Yu CM, Chan JC and Sanderson JE (1997) Chinese herbs and warfarin potentiation by 'Danshen'. *J Intern Med* **241:**337-339.

Yu XY, Lin SG, Chen X, Zhou ZW, Liang J, Duan W, Chowbay B, Wen JY, Chan E, Cao J, Li CG and Zhou SF (2007) Transport of cryptotanshinone, a major active triterpenoid in Salvia miltiorrhiza Bunge widely used in the treatment of stroke and Alzheimer's disease, across the blood-brain barrier. *Curr Drug Metab* **8:**365-378.

Yue J and Peng R (2009) Does CYP2E1 play a major role in the aggravation of isoniazid toxicity by rifampicin in human hepatocytes? *Br J Pharmacol* **157:**331-333.

Yue QY, Bergquist C and Gerden B (2000) Safety of St John's wort (Hypericum perforatum). *Lancet* **355:**576-577.

Zerilli A, Ratanasavanh D, Lucas D, Goasduff T, Dreano Y, Menard C, Picart D and

Berthou F (1997) Both cytochromes P450 2E1 and 3A are involved in the O-hydroxylation of p-nitrophenol, a catalytic activity known to be specific for P450 2E1. *Chem Res Toxicol* **10:**1205-1212.

Zhang PR and Lu Q (2009) [A study on anticancer activity of tanshinone II A against human breast cancer]. *Sichuan Da Xue Xue Bao Yi Xue Ban* **40:**245-249.

Zhao GR, Zhang HM, Ye TX, Xiang ZJ, Yuan YJ, Guo ZX and Zhao LB (2008) Characterization of the radical scavenging and antioxidant activities of Danshensu and salvianolic acid B. *Food Chem Toxicol* **46:**73-81.

Zhou L, Zuo Z and Chow MS (2005) Danshen: an overview of its chemistry, pharmacology, pharmacokinetics, and clinical use. *J Clin Pharmacol* **45:**1345-1359.

Zhukov A and Ingelman-Sundberg M (1999) Relationship between cytochrome P450 catalytic cycling and stability: fast degradation of ethanol-inducible cytochrome P450 2E1 (CYP2E1) in hepatoma cells is abolished by inactivation of its electron donor NADPH-cytochrome P450 reductase. *Biochem J* **340 ( Pt 2):**453-458.