

Hepatoprotective effect of ketoconazole in chronic liver injury model

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Abstract: Ketoconazole is a first orally available anti-fungal drug which has been reported as a potent inhibitor of human cytochrome P-450. The present study was designed to examine the hepatoprotective effect of ketoconazole in both *in vitro* and *in vivo* liver injury models. Hepatocyte injury was induced by 8mM CCl₄ while hepatic fibrosis model was established by injecting 1 ml/kg CCl₄ followed by treatment with ketoconazole. Effect of ketoconazole treatment on injured hepatocytes was determined by lactate dehydrogenase release and trypan blue assay. Analysis of ketoconazole treatment and prevention on liver fibrosis was assessed by sirius red staining, masson trichome staining, PCR and liver function tests for bilirubin and alanine transaminase (ALAT). A significant reduction ($P < 0.05$) in LDH release and reduced number of dead cells was observed in hepatocytes treated with ketoconazole. Sirius red and masson trichome stainings showed reduced levels of collagen in both treated and preventive groups and down regulation of alpha smooth muscle actin was observed with up-regulations of MMP-2, CK-8 and CK-18. Hepatic functional assessment demonstrated reduced serum levels of bilirubin and ALAT. Treatment of fibrotic liver with ketoconazole improves hepatic microenvironment and enhanced reduction of liver injury after fibrosis. Cytochrome P-450 inhibitors seems a favored therapeutic option in attenuation of liver fibrosis.

Keywords: Ketoconazole, cytochrome P-450, liver fibrosis, hepatic stellate cells (HSCs).

INTRODUCTION

Liver fibrosis is the pathophysiologic process due to chronic liver injury, recognized by the accumulation of extra cellular matrix (Paik *et al.*, 2014). Chronic liver damage may be due to chronic infection with Hepatitis B virus (HBV) and Hepatitis C virus (HCV) both are well recognized causes of liver fibrosis worldwide (Perz *et al.*, 2006). More than 240million and 150million people die due to HBV and HCV, respectively (WHO, APRL 2014). Alcoholic and non-alcoholic steatohepatitis is the leading cause of liver fibrosis and hepatocellular carcinoma (Brunt, 2004). Chronic liver injury may be due to drug induced, metabolic, autoimmune disorders and carcinogens leads to progressive accumulation of extra cellular matrix proteins (ECM). Cirrhosis is categorized as end stage of liver fibrosis (Pinzani *et al.*, 2010).

The main role of cells in liver fibrosis includes hepatic stellate cells (HSCs) and kupffer cells to exert or induce intracellular and extra cellular levels of oxidants (Poli, 2000). In chronic liver injury when HSCs activate and release droplets of vitamin A also change their morphology and come in contact with the injury site and express mesenchymal markers e.g; α -SMA, collagen $\alpha 1$ and fibronectin. Then they differentiate into myofibroblasts and produce excessive extra cellular matrix (ECM) (Shirakami *et al.*, 2012). Kupffer cells play an important role in initiation of stellate cell activation and can enhance matrix synthesis, cell proliferation and loss of vitamin A from HSCs through reactive oxygen

species (oxidative stress) and cytokines (Gressner, 1995). Ketoconazole is first clinically oral administered anti fungal drug. It is different from triazoles on the basis of greater specificity to inhibit human cytochrome P-450 than fungal cytochrome P-450 (Trevor *et al.*, 2009). However, pharmacological actions, includes therapy for prostate cancer (Trachtenberg *et al.*, 1984), induce gynecomastia (Deepinder *et al.*, 2012), inhibits testicular and adrenal androgen synthesis (Pont *et al.*, 1982), inhibits 11 β -hydroxylation, CYP17 and cholesterol side chain (Ryan *et al.*, 2007; Santen *et al.*, 1983), hormone-refractory prostate cancer treatment (Trump *et al.*, 1989), down regulates cytokines induced and polycyclic aromatic hydrocarbons induced oxidative stress (Tsuji *et al.*, 2012), inhibition of the metabolism of all-trans-retinoic acid (RA) (Van Wauwe *et al.*, 1990), anti-inflammatory effect (Cutsem *et al.*, 1991), treatment of inflammatory skin disorders (Hegemann *et al.*, 1993).

The aim of the present study was to analyze ketoconazole therapeutic potential in preventing chronic liver disease by reducing the inflammatory response and fibrosis. We demonstrate that ketoconazole can enhance hepatic repair, reduce liver fibrosis and improve liver function *in vitro* and *in vivo*.

MATERIALS AND METHODS

Materials

Ketoconazole was gifted from Mass Pharma (Pvt.), Limited, Lahore, Pakistan and other chemicals were purchased from Sigma Aldrich, USA.

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Animals

The research conforms to the *Guide for the care and Use of Laboratory Animals* by the US National Institutes of Health (NIH publication No.85-23, revised 1985). All animals were treated according to the procedures approved by the Institutional Review Board (IRB) at the National Center of Excellence in Molecular Biology, Lahore, Pakistan.

Hepatocyte isolation

Hepatocytes were isolated from C57BL/6 mice (n=3) according to the two step perfusion method as described previously (Okubo *et al.*, 2002). Isolated hepatocytes were plated at a concentration of 1×10^4 cells/cm² in collagen coated plates (Becton Dickinson, USA) in RPMI 1640 medium (Sigma Aldrich, USA) supplemented with 100µg/ml streptomycin (MP Biomedicals, USA), 100units/ml penicillin (MP Biomedicals, USA) and 10% fetal bovine serum (Sigma Aldrich, USA) in a humidified incubator at 5% CO₂ and 37°C temperature. Medium was replaced after 24hrs followed by various treatments.

In vitro liver injury model & ketoconazole treatment

Hepatocytes were seeded on a 6-well collagen coated plate (Becton Dickinson, USA) at a concentration of 1×10^4 cells/cm² and were subjected to injury with 8mM Carbon tetrachloride (CCl₄, Merck, Germany) dissolved in DMSO (Merck, Germany) (Liu *et al.*, 2015).

Hepatocytes were divided into 6 groups as, non-treated, CCl₄ control, Ketoconazole control, CCl₄+Ketoconazole, Ketoconazole for 30min then CCl₄ (Pretreatment), CCl₄ for 30min then Ketoconazole (Post treatment). Co-culture lasted for 4 hours and hepatocytes were harvested for trypan blue assay and lactate dehydrogenase (LDH) cytotoxicity tests (Liu *et al.*, 2015; Zeng *et al.*, 2002).

LDH assay

Cytotoxicity was analyzed through lactate dehydrogenase assay according to manufacturer's protocol (Sigma Aldrich, USA). Assay was run in triplicate for each experimental group and absorbance was measured at 490nm (Cai *et al.*, 2005).

Trypan blue assay

Trypan blue exclusion method was used to check the cell viability after treating cultured cells in 6-well plates for 4 hours. The medium was transferred from the culture plate to the eppendorf, cells were washed with Phosphate buffer saline (PBS) and then trypan blue solution (Sigma Aldrich, USA) was added. The plates were incubated at 37°C for 8-10 minutes. Cells were again washed with PBS and analyzed under phase contrast microscope (Foresti *et al.*, 2001). Six high power fields of each well were selected. Total number of cells was counted. The number of trypan blue positive cells was divided by total number of cells examined and then multiplied by 100 for calculating dead cells in each well.

In vivo liver fibrosis model & ketoconazole treatment

Male C57BL/6 mice aged 6-8 weeks and weighing 22-25 g were used in experiments. All animals were housed in cages under controlled conditions of temperature (23±3°C) and relative humidity (50%±20%), with light illumination for 12h/day. The animals were allowed access to water and food. To induce liver fibrosis, CCl₄ (1 µl/g) was administered twice a week to animals as described (Mohsin *et al.*, 2011). After 4 weeks of CCl₄, 50mg/kg ketoconazole was dissolved in tween 20 (100 µl) and saline water (300µl) and administered orally thrice a week for 6 weeks. Mice were randomly divided (n=10) into vehicle, CCl₄ control, Ketoconazole, tween 20 groups.

Ketoconazole preventive treatment groups

As described above in liver fibrosis model that liver fibrosis was induced by CCl₄ (1µl/g) was administered twice a week to animals for four weeks. Likewise, ketoconazole, was administered 2 hours before the administration of CCl₄ for four weeks. Mice were randomly divided (n=10) into vehicle, CCl₄ control, Ketoconazole+CCl₄, tween 20+CCl₄ groups.

Measurement of liver fibrosis

Fixed liver tissues were embedded in paraffin and sections were cut from different lobes of the liver. 5µm thick sections of all experimental groups were stained with sirius red 31 to estimate the liver fibrosis. Images of the fibrotic area from 3 sections per animal and 3 animals per group were taken by an Olympus BX-61 microscope equipped with Digital Camera DP-70 (Olympus, Japan). Total area of each image was measured and the percentage of fibrotic area was calculated by using Image J software (<http://imagej.nih.gov/ij/>) (Mohsin *et al.*, 2011).

Blood biochemistry

Blood samples were taken from all experimental groups at 3 weeks and at 6 weeks for fibrosis treatment groups. Preventive groups animals were sacrificed after 4 weeks in each group and blood samples were taken. Serum was isolated and the amount of bilirubin (Diasyme Europe, GmbH) and alanine transaminase (ALAT) (Bioassay System, USA) was estimated using commercial kits according to the manufacturer's protocol.

Gene expression

Total RNA was extracted from liver tissues of all experimental groups by using TRIZOL kit (Invitrogen, USA). cDNA synthesis was carried out from 1µg of RNA samples using M-MLV reverse transcriptase kit (Invitrogen, USA). For analysis of gene expression in treated and injured groups, PCR was carried out for the expression of αSMA, CK18, CK8 and MMP2. Primer Sequences are given in table 1.

STATISTICAL ANALYSIS

Analysis for percentage of fibrosis area, bilirubin and ALAT between different treatment groups vs control was performed by one-way ANOVA with bonferroni post-hoc test. p-value of less than 0.05 was considered statistically significant.

RESULTS

Effect of ketoconazole on CCl₄ induced cell death

As a preliminary step in this work, we examined which concentration of CCl₄ is optimum for primary cultured hepatocytes to produce an in vitro liver injury model. Comparison of different concentrations showed that a dose of 8mM CCl₄ exposure for four hours gave an ideal in vitro liver injury model. Cells treated with both CCl₄ and ketoconazole together (Ketoconazole+CCl₄ group) showed a significant reduction in cell death (17.2±5.31%) compared to CCl₄ control group (52.9±11.3%). Cells pre-treated and post treated with ketoconazole also showed significant reduction in cell death (36.3±11.9% and 32.3±8.55% respectively) upon exposure to CCl₄ as determined by trypan blue staining (fig. 1A-B). LDH assay further confirmed reduction in cell injury as a significant reduction in LDH release was shown in Ketoconazole+CCl₄ (16±2%), pretreatment (20±2%) and post treatment (19±2%) groups as compared to CCl₄ control (26±2%) (fig. 1C).

Ketoconazole reduced CCl₄ induced liver fibrosis

Ketoconazole treatment resulted in significant reduction of liver fibrosis measured by sirius red and masson trichome staining. In ketoconazole treatment experiment, quantification of fibrotic area measured by image J software showed marked increase in percentage of fibrotic area after CCl₄ administration (8.53±3.25%) that was reduced significantly after 6 weeks of ketoconazole treatment (3.21±2.25%) compared to other treatment and control groups (fig. 2A). In ketoconazole preventive group, Fibrotic area (%) was reduced significantly CCl₄+Ketoconazole group (3.28±.87%) as compared to other controls (CCl₄ control=8.53±3.25%, CCl₄+Tween=7.71±2.93%) groups (fig. 2B).

Ketoconazole improved liver functions of CCl₄ induced liver fibrosis model

Biochemical functions to further evaluate the role of ketoconazole on augmentation of liver functions; serum levels of alanine transaminase (ALAT) and bilirubin were analyzed in all experimental groups. Serum levels of ALAT were significantly lower in mice receiving ketoconazole treatment for 6 weeks (87±10IU/L) as compared to CCl₄ control group (377±15IU/L) and tween20 for 6 weeks (332±10IU/L) groups (fig. 3A). Similarly, bilirubin level in ketoconazole treatment for 6 weeks (0.7±0.1mg/dl) was significantly lower than

tween20 (2.1±0.5mg/dl) and CCl₄ control (2.15±1mg/dl) groups (fig. 3C). Collectively, these results indicate that treatment with ketoconazole for 6 weeks results in higher recovery of hepatic functions. In preventive group, serum levels of ALAT were significantly lower in mice receiving CCl₄+ketoconazole (157±5IU/L) as compared to CCl₄ control (377±15IU/L) and tween20+CCl₄ (325±20IU/L) groups (fig. 3-B). Similarly, Bilirubin level in CCl₄+ketoconazole group (0.9±0.1mg/dl) was significantly lower than tween20+CCl₄ (1.2±0.2mg/dl) and CCl₄ control (1.8±0.2mg/dl) groups (fig. 3-D). Collectively, these results indicate that ketoconazole can prevent and treat liver from injury and ultimately fibrosis.

In vivo gene expression profiling

Gene expression profiling was conducted in experimental groups receiving ketoconazole treatment and preventive group. HSCs are considered to be the main source of ECM-production in the liver (Hernandez-Gea & Friedman, 2011) and express α -SMA. We observed an increase in mRNA levels of α -SMA after treatment with CCl₄. Ketoconazole treatment resulted in decrease α -SMA expression which is significantly lower than other experimental groups. Similar pattern was observed in preventive groups in which ketoconazole administered 2hours before CCl₄ to animals. An increased level was observed in mRNA levels of MMP-2, which is a contributing factor in remodeling of liver fibrosis, in ketoconazole treated groups. Increased expression of hepatic markers CK-8 and CK-18 were also observed in ketoconazole treatment groups and in preventive groups, depicting the improvement of liver structure and functions (fig. 4).

DISCUSSION

Worldwide major cause of morbidity and mortality associated with liver diseases is liver fibrosis which may be due to chronic liver injury such as chronic viral hepatitis and from fatty liver disease. Hepatic stellate cells upon activation play a critical role in liver fibrosis because upon injury, they are the main source of production of extracellular matrix. Advancement in culturing and animal models has increased our knowledge of the mechanism behind hepatic stellate cell activation on the basis of genetic regulations, role of immune signaling and the reversal of the disease. Different pathways of fibrogenesis are discovered, now the challenge will be to develop antifibrotic therapies for chronic liver disease patients (Hernandez-Gea et al., 2011).

In vitro model of hepatocytes injury induced by carbon tetrachloride was established to study hepatoprotective effect of cytochrome P-450 inhibitors (ketoconazole). It is recently believed that carbon tetrachloride is metabolized by the cytochrome P-450 in the liver to produce

Table 1: Primer sequences

Genes	Sequence	Product size	Temperature ($^{\circ}$ C)
α SMA (F)	ATAACCCTTCAGCGTTCAGCC	704	58
α SMA (R)	ACGAAGGAATAGCCACGCTC		
MMP2(F)	TCAACGGTCGGAATACAGC	875	58
MMP2(R)	CTGTGGGGCCTCATACACAG		
CK18 (F)	AAGACCTGAACGATCGCCTG	959	57
CK18 (R)	TTGCATGGAGTTGCTGGAGT		
CK 8 (F)	GCTCTCGAACCTCCGTCTTC	871	57
CK 8 (R)	CTCGTACTGGGCACGAACCTT		

trichloromethyl radicals and then these radicals react with oxygen to form trichloromethyl peroxy radicals, which causes cell damage by covalently binding to cellular macro-molecules and lipid peroxidation (Koneri *et al.*, 2008).

To measure the injury by CCl₄, cell viability was assessed by trypan blue uptake and LDH release. Results obtained from current study showed that hepatocytes treated with CCl₄ 8mM highly significantly raised the level of LDH as also reported by Yin *et al.* (2011) while in this study combine treatment of ketoconazole with CCl₄ significantly decreased the level of LDH release while pre- and post-treatment with ketoconazole bit significantly reduce LDH release while trypan blue assay showed highly significant decreased percentage of dead cell as compared to CCl₄ however pre- and post-treatment showed significant and bit significant decreased percentage of dead cells respectively (fig. 1). Dresser *et al.* (2000) also reported that ketoconazole inhibit cytochrome P-450 enzyme system and as studied by Wiseman *et al.* (1991) anti oxidant effect of ketoconazole which was stronger than other azoles miconazole and clotrimazole.

Ketoconazole showed its activity by inhibiting the activation of nuclear receptors, constitutive androstene receptor and human pregnenolone X receptor that are involved in regulation of *MDR-1* and *CYP3A4*. They suggest that ketoconazole can be used as antagonist of nuclear receptors which may improve drug effect and tolerance and lead to novel strategies (Huang *et al.*, 2007) these nuclear receptor are important in bio transformation of drugs and xenobiotics which is proven in our study that ketoconazole inhibit the metabolism of CCl₄ and prevent hepatocytes from injury.

Drug screening for anti-inflammatory and hepatoprotective effect, showing hepatic pathology similar to that of human is CCl₄ induced hepatic injury (Tsukamoto *et al.*, 1990). CCl₄ induced liver injury exerts effects on NK cells, T cells, macrophages, phagocytes and lymphatic organs besides increasing cytokines inflammatory (Delaney *et al.*, 1994). The present study used this model to analyze ketoconazole therapeutic

potential in preventing chronic liver disease by reducing the inflammatory response and fibrosis. Sirius red staining (fig. 2) showed reduced collagen level in treated group as well as masson trichome staining (fig. 3) also showed reduced level of collagen as compared to CCl₄ control. Serum bilirubin and ALAT level were also observed and showed significant reduction in serum bilirubin and ALAT level in ketoconazole+CCl₄ (Fig. 4). Same model was used by Rocha *et al.* (2014) to study the therapeutic potential of Diethylcarbamazine in mice model.

Ketoconazole 1 μ M synergize the apoptotic effects of nocodazole in COLO 205 cancer cells and also demonstrated the therapeutic effect of combination therapy of ketoconazole and nocodazole in mouse model. In mouse model, athymic mice were treated with both drugs bearing COLO 205 tumor xenografts. After six weeks treatment with both drugs, Ketoconazole (50mg/kg) potentiate the anti tumor effect of nocodazole. There is no gross toxicity observed in mouse model. So the apoptotic effects of ketoconazole make it very attractive and contributing agent for cancer treatment in future (Wang *et al.*, 2002). In current study same concentration of ketoconazole was used in hepatocytes, highly significant decreased percentage LDH release and percentage dead cells were observed and mice model with liver fibrosis was treated for six weeks with ketoconazole 50mg/kg reduced or reverse liver fibrosis significantly as compared to control. Hepatic stellate cells were the major cause of extra cellular matrix in liver fibrosis (Hernandez-Gea & Friedman, 2011) in current study treatment of mice with ketoconazole can reduce liver fibrosis and prevent liver from further advance stages of liver fibrosis through its apoptotic effect. Ketoconazole also inhibit the metabolism of retinoid and increase the level of endogenous retinoid concentration (Ahmad, 2011) as in liver fibrosis there is increase metabolism and loss of retinoic acid which causes activation of hepatic stellate cells to matrix producing cells (Senoo *et al.*, 2010). In current study ketoconazole may increase the level of endogenous RA and prevent further activation of hepatic stellate cells and reduce liver fibrosis. In present study ketoconazole may increase the level of endogenous RA and prevent further activation of hepatic stellate cells and reduce liver fibrosis.

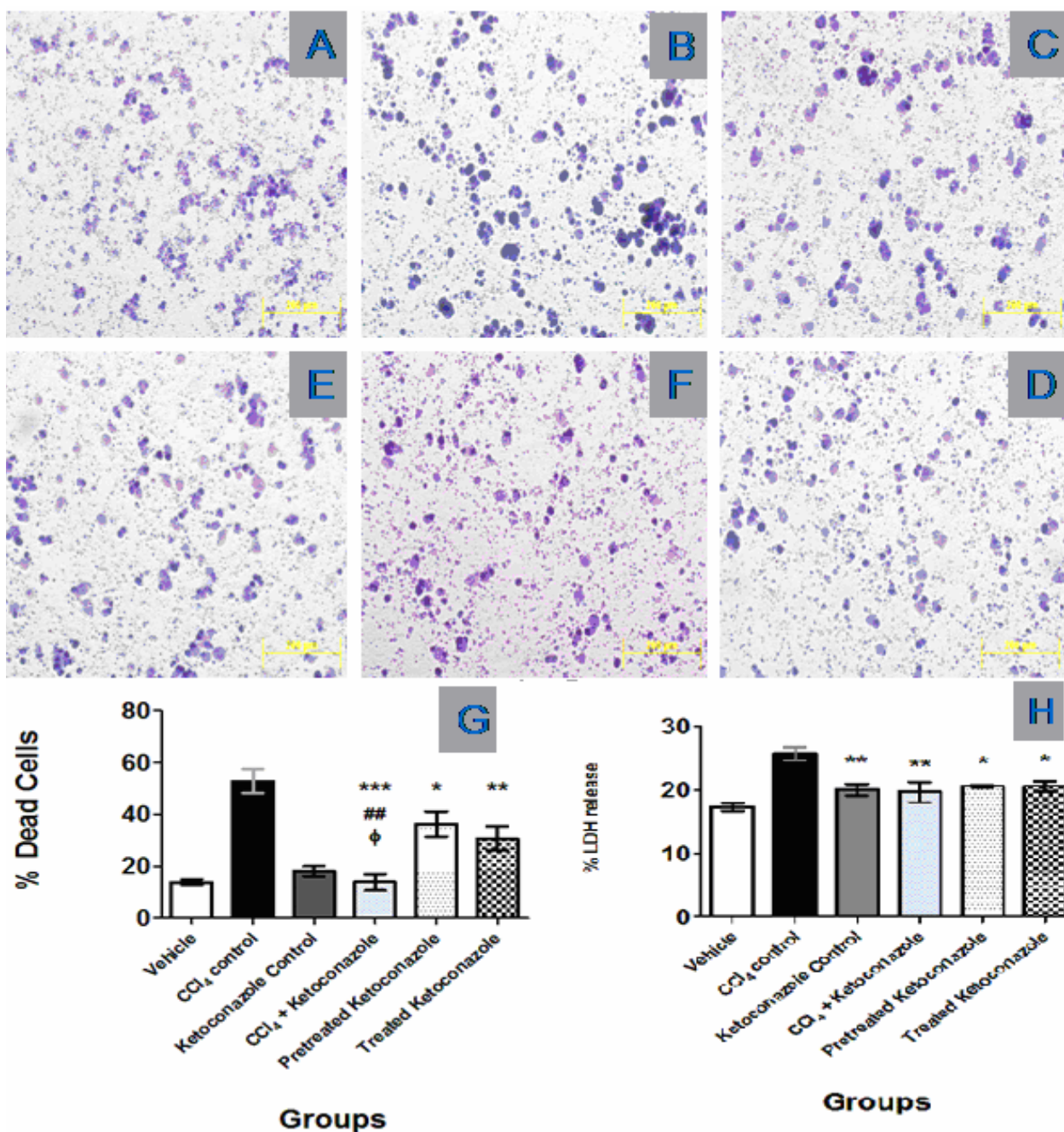


Fig. 1: Enhanced hepatocyte survival in different groups of Ketoconazole

A) Vehicle control B) CCl₄ control C) Ketoconazole control D) Ketoconazole + CCl₄ E) Pretreated ketoconazole F) Treated ketoconazole G) Quantitative analysis of dead cells (%) in different experimental groups. One way ANOVA was applied to check the significance of the data. All values are expressed as mean $\pm$ SEM. p -value < 0.05 was considered significant. *** $p < 0.001$ for CCl₄ + ketoconazole Vs CCl₄, ## $p < 0.01$ for CCl₄ + ketoconazole Vs pretreated ketoconazole; ϕ $p < 0.05$ for CCl₄ + ketoconazole Vs treated ketoconazole, * $p < 0.05$ for pretreated ketoconazole Vs CCl₄, ** $p < 0.01$ for treated ketoconazole Vs CCl₄ H) LDH (%) release levels in control and treatment groups. ** $p < 0.01$ for CCl₄ + ketoconazole Vs CCl₄, * $p < 0.05$ for pretreated ketoconazole Vs CCl₄, ** $p < 0.01$ for treated ketoconazole Vs CCl₄

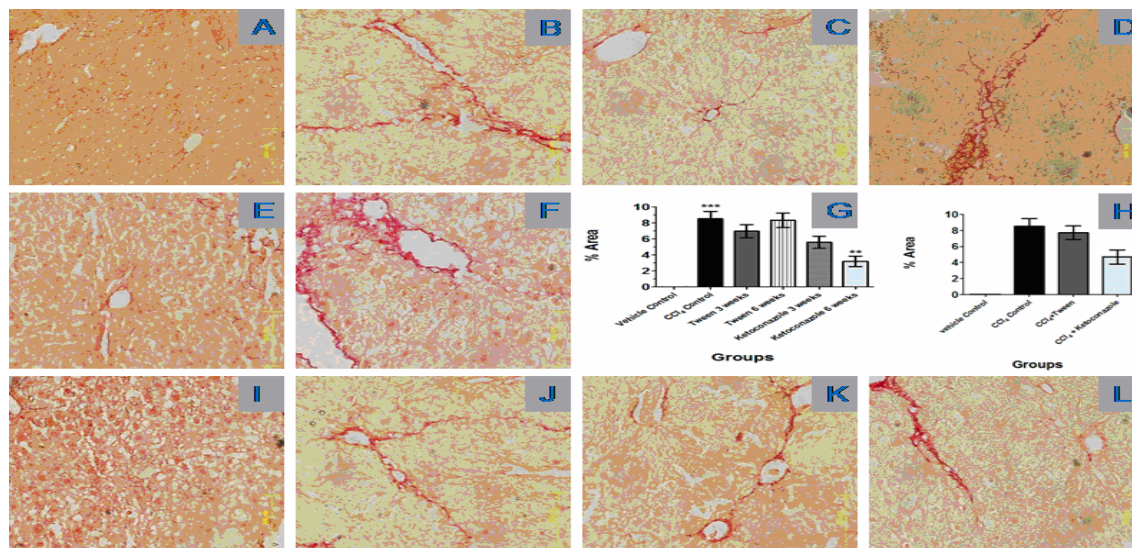


Fig. 2: Representative micrograph of hepatic tissue stained with sirius red showing collagen deposition in various treatment and preventive groups (A=Vehicle, B=CCl₄, C=Ketoconazole 3 weeks, D =Tween20 3 weeks, E =Ketoconazole 6 weeks, F=Tween20 6 weeks). G) Quantitative analysis of fibrosis in different treatment groups. One way ANOVA was applied to check the significance of the data. All values are expressed as mean $\pm$ SEM. p-value<0.05 was considered significant. **p<0.01 for Ketoconazole 6 weeks vs. CCl₄ control H) Quantitative analysis of fibrosis in different preventive groups. One way ANOVA was applied to check the significance of the data. All values are expressed as mean $\pm$ SEM. p-value<0.05 was considered significant I) Vehicle J) CCl₄, K) CCl₄+tween20, L) CCl₄+ketoconazole

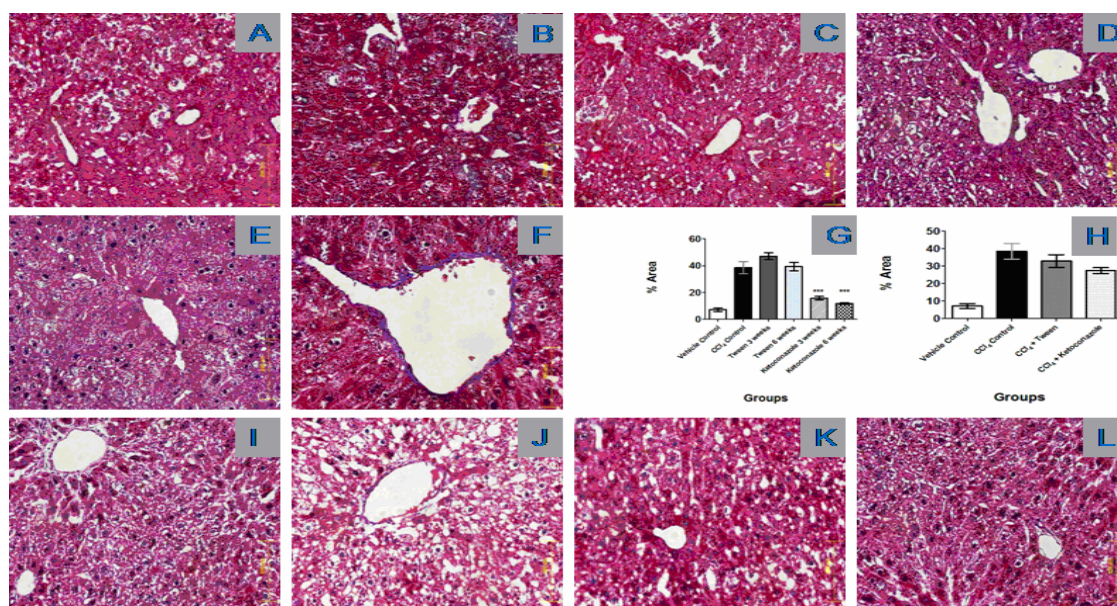


Fig. 3: Representative micrograph of hepatic tissue stained with masson trichrome showing collagen deposition in various treatment and preventive groups (A=Vehicle, B=CCl₄, C=Ketoconazole 3 weeks, D =Tween20 3 weeks, E =Ketoconazole 6 weeks, F=Tween20 6 weeks). G) Quantitative analysis of fibrosis in different treatment groups. One way ANOVA was applied to check the significance of the data. All values are expressed as mean $\pm$ SEM. p-value<0.05 was considered significant. ***p<0.001 for Ketoconazole 3 weeks vs. CCl₄ control, ***p<0.001 for Ketoconazole 6 weeks vs. CCl₄ control H) Quantitative analysis of fibrosis in different preventive groups. One way ANOVA was applied to check the significance of the data. All values are expressed as mean $\pm$ SEM. p-value<0.05 was considered significant I) Vehicle J) CCl₄, K) CCl₄+tween20, L) CCl₄+ketoconazole

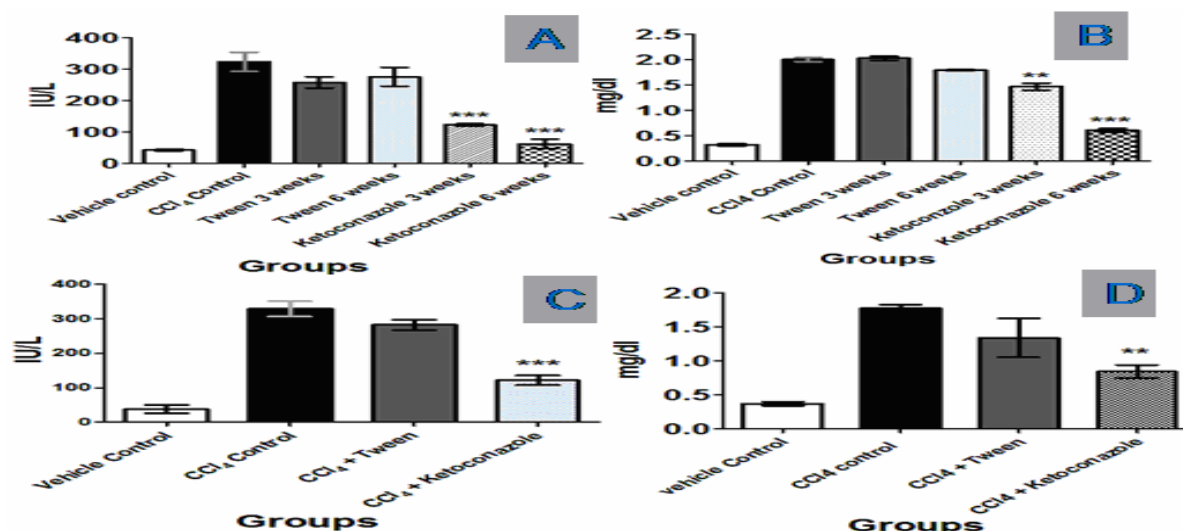


Fig. 4: Liver function tests. Treatment groups A) Alanine transaminase in serum of all animals groups, *** $p < 0.001$ for Ketoconazole 3 weeks vs. CCl_4 control, *** $p < 0.001$ for Ketoconazole 6 weeks vs. CCl_4 control B) Bilirubin serum levels in all animal groups ($n = 10/\text{group}$), ** $p < 0.01$ for Ketoconazole 3 weeks vs. CCl_4 control, *** $p < 0.001$ for Ketoconazole 6 weeks vs. CCl_4 control. Preventive groups C) Alanine transaminase in serum of all animals groups, *** $p < 0.001$ for CCl_4 +ketoconazole vs CCl_4 control D) Bilirubin serum levels in all animal groups ($n = 10/\text{group}$), ** $p < 0.01$ for CCl_4 +ketoconazole vs CCl_4 control

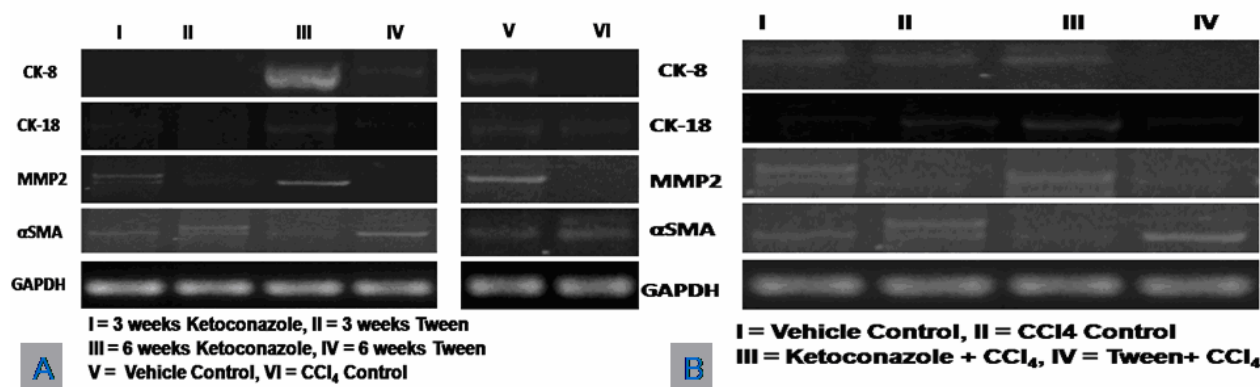


Fig. 5: A) In vivo PCR of treatment group B) In vivo PCR of preventive group

Carpino *et al.* (2005) concluded that for *in vivo* studies alpha smooth muscle actin expression is most consistent marker for HSCs activation and it is reliable to diagnosis of early stages of liver fibrosis and helpful in monitoring the efficiency of anti fibrotic drugs.

Our results showed increased expression of mRNA of alpha smooth muscle actin in CCl_4 induced liver fibrosis model (fig. 5) and in CCl_4 control of preventive group (fig. 5) while treatment with ketoconazole for 6 weeks reduced the expression (fig. 5) and also reduced in preventive group. In addition, MMP-2 stimulation and increased expression causes apoptosis of activated HSCs and increased resolution of liver fibrosis (Preaux *et al.*, 2002; Radbill *et al.*, 2011). Our results showed increased expression of MMP-2 in treated and prevented groups as compared to CCl_4 control group. Radbill *et al.* (2011)

investigated that increase expression of MMP-2 have anti fibrotic effects and supported by many other *in vivo* studies. While, hepatic markers CK-8, CK-18 in present study showed increased expression in treated groups as compared to CCl_4 control groups.

Chronic accumulation of kupffer cells, HSCs activation and collagen deposition is related to chronic inflammation and liver fibrosis (Iredale, 2007; Jarcuska *et al.*, 2010; Marra, 2002). The present study showed that there was highly significant increase in ECM deposition in CCl_4 control group. It has been believed that liver fibrosis is mainly the result of abnormality in homeostasis of the collagen including synthesis, deposition and degeneration. In liver fibrosis, HSCs activation characteristically has the ability to remodel ECM through the production of ECM proteins (Domitrović *et al.*, 2010; Ghazwani *et al.*, 2014).

After ketoconazole treatment, a significant decrease in collagen was assessed by masson trichome staining (fig. 3) and sirius red staining (fig. 2) with significant decrease in serum bilirubin and ALAT level (fig. 4). Therefore present study suggests that ketoconazole has an anti-fibrotic effect.

CONCLUSION

We have demonstrated that ketoconazole treatment can significantly reduce liver fibrosis. Ketoconazole induces apoptosis of activated HSCs which are considered as critical mediators of liver fibrosis. Activation of HSCs in the injured liver transforms these vitamin A storing cells into fibroblasts. Ketoconazole have also preventive effect on CCl₄ induced liver fibrosis. We have also demonstrated that ketoconazole treatment significantly increased cell viability in CCl₄ induced hepatocytes injury *in vitro*. Therefore, we report here a new treatment strategy to treat liver fibrosis by using cytochrome P-450 inhibitor.

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REFERENCES

- Ahmad M (2011). Study on cytochrome p-450 dependent retinoic Acid metabolism and its inhibitors as potential agents for cancer therapy. *Sci. Pharm.*, **79**(4): 921-935.
- Brunt EM. (2004). Nonalcoholic steatohepatitis. *Semin Liver Dis.*, **24**(1): 3-20.
- Cai, Yan and REN Jin (2005). Apoptosis initiated by carbon tetrachloride in mitochondria of rat primary cultured hepatocytes. *Acta Pharmacologica. Sinica.*, **26**(8): 969-975.
- Carpino G, Morini S, Corradini S, Ginanni, Franchitto A, Merli M, Siciliano M and Rossi M (2005). Alpha-SMA expression in hepatic stellate cells and quantitative analysis of hepatic fibrosis in cirrhosis and in recurrent chronic hepatitis after liver transplantation. *Dig. Liver Dis.*, **37**(5): 349-356.
- Cutsem, Jan Van, Gerven, Frans Van, Cauwenbergh, Geert, Odds, Frank and Janssen and Paul AJ (1991). The antiinflammatory effects of ketoconazole: A comparative study with hydrocortisone acetate in a model using living and killed *Staphylococcus aureus* on the skin of guinea pigs. *J. Am. Acad. Dermatol.*, **25**(2, Part 1): 257-261.
- Deepinder, Fnu and Braunstein and Glenn D (2012). Drug-induced gynecomastia: An evidence-based review. *Expert Opin. Drug Saf.*, **11**(5): 779-795.
- Delaney, Bryan, Strom, Stephen C, Collins, Sharon and Kaminski and Norbert E (1994). Carbon tetrachloride suppresses T-cell-dependent immune responses by induction of transforming growth factor-1. *Toxicol Appl Pharmacol.*, **126**(1): 98-107.
- Domitrovic, Robert, Jakovac and Hrvoje (2010). Antifibrotic activity of anthocyanidin delphinidin in carbon tetrachloride-induced hepatotoxicity in mice. *Toxicology*, **272**(1): 1-10.
- Dresser, George K, Spence, J David and Bailey and David G (2000). Pharmacokinetic-pharmacodynamic consequences and clinical relevance of cytochrome P450 3A4 inhibition. *Clin. Pharmacokinet.*, **38**(1): 41-57.
- Foresti, Roberta, Goatly, Helen, Green, Colin J and Motterlini, Roberto (2001). Role of heme oxygenase-1 in hypoxia-reoxygenation: requirement of substrate heme to promote cardioprotection. *Am. J. Physiol. Heart Circ. Physiol.*, **281**(5): H1976-H1984.
- Ghazwani, Mohammed, Zhang, Yifei, Gao, Xiang, Fan, Jie, Li, Jiang and Li and Song (2014). Anti-fibrotic effect of thymoquinone on hepatic stellate cells. *Phytomedicine*, **21**(3): 254-260.
- Gressner AM. (1995). Cytokines and cellular crosstalk involved in the activation of fat-storing cells. *J. Hepatol.*, **22**(2 Suppl): 28-36.
- Hegemann, Lutz, Toso, Susan M, Lahijani, Khosrow L, Webster, Guy F and Uitto and Jouni (1993). Direct interaction of antifungal azole-derivatives with calmodulin: a possible mechanism for their therapeutic activity. *J. Invest. Dermatol.*, **100**(3): 343-346.
- Hernandez-Gea V and Friedman SL (2011). Pathogenesis of liver fibrosis. *Annu. Rev. Pathol.*, **6**: 425-456.
- Huang H, Wang H, Sinz M, Zoeckler M, Staudinger J, Redinbo MR and Mani S (2007). Inhibition of drug metabolism by blocking the activation of nuclear receptors by ketoconazole. *Oncogene*, **26**(2): 258-268.
- Iredale and John P. (2007). Models of liver fibrosis: exploring the dynamic nature of inflammation and repair in a solid organ. *J. Clin. Invest.*, **117**(3): 539.
- Jarcuska, Peter, Janicko, Martin, Veseliny, Eduard, Jarcuska, Pavol and Skladany, Lubomir. (2010). Circulating markers of liver fibrosis progression. *Clinica Chimica Acta.*, **411**(15): 1009-1017.
- Koneri, Raju, Balaraman R, Firdous, Kumar MV and Kumar M (2008). Hepatoprotective effects of *Momordica cymbalaria* Fenzl. against carbon tetrachloride induced hepatic injury in rats. *Pharmacologyonline*, **1**: 365-374.
- Liu Ying-Juan, Du Jin-Liang, Cao Li-Ping, Jia Rui, Shen Yu-Jin, Zhao Cai-Yuan and Yin Guo-Jun (2015). Anti-inflammatory and hepatoprotective effects of *Ganoderma lucidum* polysaccharides on carbon tetrachloride-induced hepatocyte damage in common

- carp (*Cyprinus carpio* L.). *Int. Immunopharmacol.*, **25**(1):112-120.
- Marra F (2002). Chemokines in liver inflammation and fibrosis. *Frontiers in Bioscience*, **7**: d1899-1914.
- Mohsin, Sadia, Shams, Sulaiman, Ali Nasir, Ghazanfar, Khan, Mohsin, Javaid Awan, Sana, Khan, Shaheen N, and Riazuddin and Sheikh (2011). Enhanced hepatic differentiation of mesenchymal stem cells after pretreatment with injured liver tissue. *Differentiation*, **81**(1): 42-48.
- Okubo, Hisashi, Matsushita, Michiaki, Kamachi, Hirofumi, Kawai, Tomoaki, Takahashi, Manabu, Fujimoto, Tetsuya and Todo Satoru (2002). A novel method for faster formation of rat liver cell spheroids. *Artif. Organs*, **26**(6): 497-505.
- Paik Yong-Han, Kim Jonghwa, Aoyama Tomonori, De Minicis, Samuele, Bataller, Ramon, Brenner and David A (2014). Role of NADPH oxidases in liver fibrosis. *Antioxid. Redox Signal.*, **20**(17): 2854-2872.
- Perz Joseph F, Armstrong, Gregory L, Farrington, Leigh A, Hutin, Yvan JF and Bell and Beth P (2006). The contributions of hepatitis B virus and hepatitis C virus infections to cirrhosis and primary liver cancer worldwide. *J. Hepatol.*, **45**(4): 529-538.
- Pinzani, Massimo and Macias-Barragan Jose. (2010). Update on the pathophysiology of liver fibrosis. *Expert Rev. Gastroenterol. Hepatol.*, **4**(4): 459-472.
- Poli Giuseppe (2000). Pathogenesis of liver fibrosis: Role of oxidative stress. *Mol. Aspects Med.*, **21**(3): 49-98.
- Pont, Allan, Williams, Paul L, Azhar, Salman, Reitz, Richard E, Bochra, Charlotte, Smith, Erla R and Stevens and David A (1982). Ketoconazole blocks testosterone synthesis. *Arch. Intern. Med.*, **142**(12): 2137-2140.
- Pont, Allan, Williams, Paul L, Loose, David S, Feldman, David, Reitz, Richard E, Bochra, Charlotte and Stevens David A (1982). Ketoconazole blocks adrenal steroid synthesis. *Ann. Intern. Med.*, **97**(3): 370-372.
- Preaux, Anne-Marie, D'Ortho, Marie-Pia, Bralet, Marie-Pierre, Laperche, Yannick and Mavier and Philippe. (2002). Apoptosis of human hepatic myofibroblasts promotes activation of matrix metalloproteinase-2. *Hepatology*, **36**(3): 615-622.
- Radbill BD, Gupta R, Ramirez MC, DiFeo A, Martignetti JA, Alvarez CE, Friedman SL, Narla G, Vrabie R, Bowles R, Saiman Y and Bansal MB (2011). Loss of matrix metallo proteinase-2 amplifies murine toxin-induced liver fibrosis by upregulating collagen I expression. *Dig. Dis. Sci.*, **56**(2): 406-416.
- Rocha, Sura Wanessa Santos, França, Maria Eduarda Rocha de, Rodrigues, Gabriel Barros, Barbosa, Karla Patricia Sousa, Nunes, Ana Karolina Santana, Pastor, André Filipe, Peixoto and Christina Alves. (2014). Diethylcarbamazine reduces chronic inflammation and fibrosis in carbon tetrachloride-(CCl₄) induced liver injury in mice. *Mediators Inflamm.*, 2014:696383. doi: 10.1155/2014/696383. Epub 2014 Oct 13.
- Ryan, Charles J, Halabi, Susan Ou, San-San, Vogelzang, Nicholas J, Kantoff, Philip and Small Eric J (2007). Adrenal androgen levels as predictors of outcome in prostate cancer patients treated with ketoconazole plus antiandrogen withdrawal: Results from a cancer and leukemia group B study. *Clin. Cancer Res.*, **13**(7): 2030-2037.
- Santen RJ, Van den Bossche H, Symoens J, Brugmans J and DeCoster R (1983). Site of action of low dose ketoconazole on androgen biosynthesis in men. *J. Clin. Endocrinol. Metab.*, **57**(4): 732-736.
- Senoo H, Yoshikawa K, Morii M, Miura M, Imai K and Mezaki Y (2010). Hepatic stellate cell (vitamin A-storing cell) and its relative--past, present and future. *Cell Biol. Int.*, **34**(12): 1247-1272.
- Shirakami, Yohei, Lee, Seung-Ah, Clugston, Robin D Blaner and William S (2012). Hepatic metabolism of retinoids and disease associations. *BBA-Mol. Cell Biol. L.*, **1821**(1): 124-136.
- Trachtenberg John and Pont Allan (1984). Ketoconazole therapy for advanced prostate cancer. *The Lancet.*, **324**(8400): 433-435.
- Trevor, Anthony J, Katzung, Bertram G and Masters and Susan B (2009). Basic & Clinical Pharmacology: McGraw-Hill Medical.
- Trump, Donald L, Havlin, Kathleen H, Messing, Edward M, Cummings, Kenneth B, Lange, Paul H and Jordan V Craig. (1989). High-dose ketoconazole in advanced hormone-refractory prostate cancer: endocrinologic and clinical effects. *J Clin Oncol.*, **7**(8): 1093-1098.
- Tsuji G, Takahara M, Uchi H, Matsuda T, Chiba T, Takeuchi S, Yasukawa F, Moroi Y and Furue M (2012). Identification of keto conazole as an AhR-Nrf2 activator in cultured human keratinocytes: The basis of its anti-inflammatory effect. *J. Invest. Dermatol.*, **132**(1): 59-68.
- Tsukamoto, Hidekazu, Matsuoka, Masaki and French and Samuel W (1990). Experimental models of hepatic fibrosis: A review. Paper presented at the Seminars in Liver Disease.
- Van Wauwe JP, Coene MC, Goossens J, Cools W and Monbaliu J (1990). Effects of cytochrome P-450 inhibitors on the *in vivo* metabolism of all-trans-retinoic acid in rats. *J. Pharmacol. Exp. Ther.*, **252**(1): 365-369.
- Wang Ying-Jan, Jeng Jjiang-Huei, Chen Rong-Jane, Chen Li-Ching, Liang, Yu-Chih Lin, Chien-Huang Ho and Yuan-Soon (2002). Ketoconazole potentiates the antitumor effects of nocodazole: *In vivo* therapy for human tumor xenografts in nude mice. *Mol. Carcinog.*, **34**(4): 199-210.
- WHO. (APIRL 2014).
- Wiseman, Helen, Smith, Cheryl, Arnstein, Henry RV, Halliwell, Barry and Cannon Michael (1991). The antioxidant action of ketoconazole and related azoles: Comparison with tamoxifen and cholesterol. *Chem. Biol. Interact.*, **79**(2): 229-243.

- Yin Guojun, Cao Liping, Xu Pao, Jeney Galina, Nakao Miki and Lu Chengping. (2011). Hepatoprotective and antioxidant effects of Glycyrrhiza glabra extract against carbon tetrachloride (CCl₄)-induced hepatocyte damage in common carp (*Cyprinus carpio*). *Fish Physiol. Biochem.*, 37(1): 209-216.
- Zeng Qinghua, Chen Shaoqiong, You Zongbing, Yang Fan, Carey Thomas E, Saims Daniel and Wang Cun-Yu (2002). Hepatocyte growth factor inhibits anoikis in head and neck squamous cell carcinoma cells by activation of ERK and Akt signaling independent of NFκB. *J. Biol. Chem.*, 277(28): 25203-25208.