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Hepato-protective role of itraconazole mediated cytochrome p450 pathway inhibition in liver fibrosis

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Abstract: Liver is a vital organ and is routinely exposed to toxins. Carbon tetrachloride is one such noxious agent which cause toxicity in liver when CYP450 enzyme bio-activates it. Many hepatoprotective agents are available in market with severe side effects. Appropriate agent is required to combat such liver problems. Azole compounds have much therapeutic values in many diseases. Based upon this fact, present study is aimed to evaluate the repurposing of Itraconazole in the prevention of hepatic fibrosis via inhibition of cytochrome P450 pathway. For *in-vitro* evaluation of cyto-protective effects in HepG2 cells (untreated and treated groups), cell viability assays, antioxidant evaluation, enzyme linked immunosorbent assay (ELISA) and immunocytochemistry was used. For *in-vivo* evaluation, CCl_4 induced liver fibrotic rat model was used and post treated evaluation was done by blood biochemistry, hematoxylin and eosin (H&E) staining and gene expression profiling. Results of the current study indicated hepatoprotective role of itraconazole via inhibition of CYP450 pathway inhibition. Therefore, Itraconazole use could be a potential therapeutic approach to prevent liver fibrosis.

Keywords: Itraconazole, CCl_4, liver fibrosis, cytochrome P-450, CYP2E1, hepato-protective.

INTRODUCTION

Liver performs several important functions of body including removal of toxic substances including pathogens, xenobiotics, drugs, etc (Perazzoli *et al.*, 2017) by involving enzymes of cytochrome P450 system. Liver is exposed to toxic chemicals on daily basis due to its structure, biochemical and physiological role in body. Fibrosis of liver is regarded as major hazard of health in reference to the rate of morbidity and mortality (Rahmani *et al.*, 2019). Several compounds can affect liver and cause problems like hepatotoxicity, fatty liver and liver dysfunction. Although liver has great regeneration capacity but persistent injury can lead to inflammation, fibrosis, cirrhosis and even hepatic failure (Perazzoli *et al.*, 2017).

CYP2E1 gene is involved in liver injury when exposed to CCl_4 and as a result of bio-activation of CCl_4, liver injury occurs. Liver functional enzymes levels are also increased after injury as evidenced in *in-vivo* study (Dutta *et al.*, 2018), indicating anatomical damage of liver which can contribute pathology up to cellular level.

In a study, repurposing of Ketoconazole was explored and found protective against CCl_4 toxicity in both *in-vitro* and *in-vivo* models (Akhtar *et al.*, 2019). Similarly other azole related compounds could be explored for their efficacy as hepatoprotective. Itraconazole is one such azole family related compound which is available in market. It is not recommended with drugs which are administered for gastric acidity problems like proton pump inhibitors and histamine-II blockers. It is easily metabolized in liver with an absorption rate of up to 80% in oral solution and 55% in capsule form (Nett and Andes, 2016).

In the current study, repurposing of itraconazole was evaluated for its hepatoprotective potential against liver injury models as it is hypothesized that it may reduce fibrosis induced by CCl_4 involving CYP2E1 enzyme, related to cytochrome P450 system in both *in-vitro* and *in-vivo* models. It is to be noted that inhibition of CYP2E1 can be helpful in preventing liver from injury.

MATERIALS AND METHODS

Materials

Drug (Itraconazole) was obtained from pharmacy.

Animals

Treatment of female rats was according to methodology mentioned by parent institute & rules mentioned in NIH publication No. 85-23, revised 1985.

Culturing of HepG2 cell line

HepG2 is liver cell line which was cultured by the method as mentioned by (Maqbool *et al.*, 2019).

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In-vitro liver injury model & Itraconazole treatment
After culturing, cells (1×10^4 cells/cm^2) were shifted to 96-well plates (Corning) for treatment purposes. Injury was given to cells for 4 hours via carbon tetrachloride (CCl_4) at dose of 8mM (CCl_4, Merck, Germany). Study was conducted by making 4 groups (table 1). For dose evaluation, initially 0-200 mg/ml of Itraconazole was given in DMEM. Evaluation was conducted for one day period except injury group to estimate optimal dose for further studies. Then different assays were performed as mentioned below.

Cell viability assays
Both cell viability assays i.e., MTT (Invitrogen Inc., USA) & CV (crystal violet) assays were conducted on 96-well culture plates. For MTT assay, % viability was estimated via method mentioned by (Maqbool *et al.*, 2019). CV assay was conducted as described by Feoktistova in his study (Feoktistova *et al.*, 2016).

Antioxidant enzymes (GSH & SOD) estimation
To check oxidative stress, GSH and SOD antioxidant assays were performed in treated cells in 96 well plates as described by Shamim and Rehman in study (Shamim and Rehman, 2015).

Enzyme linked immunosorbant assay (ELISA)
ELISA (solid phase sandwich) was conducted as mentioned by (Maqbool *et al.*, 2019).

Immunocytochemistry
Immunocytochemistry was performed as mentioned by (Maqbool *et al.*, 2019).

Muse cell analysis
Muse cell analysis assay was done via kit known as count and viability kit (Cat. No MCH100102) using muse automated cell analyzer. After treatment, cells were underwent centrifugation for 5 minutes at 2000 rpm. Later, above liquid was thrown away and pellet was re-suspended in cell & viability reagent. Counting was done via "Muse"™ automated cell counter/ analyzer.

Gene expression analysis
For gene expression analysis, cells and tissue samples were lysed via Trizol reagent to obtain their RNAs, which later converted into their respective cDNAs. Real time PCR was done via real-time polymerase chain reaction machine (Bio-rad). Expression of certain apoptotic, proliferative and inflammatory markers (BAX, p53, caspase-3, Ki67, PCNA, TOP2A, TGF-β1, IGF-1 and CYP2E1) were analyzed. GAPDH was used as internal control.

In-vivo fibrotic model & treatment by Itraconazole
For *in-vivo* treatment, albino rats were used whose age range was 6-8 weeks and weighing range was 210-255g, which were kept in optimal conditions (12 hours/day light availability, 50%±20% humidity, 23 ± 3^0C temperature and clean cages). Rats were provided enough food and water and were separated into 4 groups (table 2). CCl_4 and Itraconazole were given to rats as mentioned in a study (Akhtar *et al.*, 2019). CCl_4 was administered at a dose of 1mg/kg body weight as 1:1 in olive oil. Itraconazole was administered at a concentration of 100mg/ml/kg body weight of rats.

STATISTICAL ANALYSIS

For statistical analysis, data was done in triplicates and expressed as mean ± SEM. One-way ANOVA along with Bonferroni test was used to compare group means. Graph-Pad Prism 5 software was used to statistically analyze quantitative data obtained from experiments. P value ≤0.05 was considered as significant.

RESULTS

Increased proliferative & cyto-protective effect of Itraconazole on CCl_4 induced cellular death
5-200 mg/ml concentrations of Itraconazole was used to evaluate maximum proliferation at minimum dose, which was found at 100 mg/ml dose (fig. 1A). Same dose was used to evaluate hepatoprotective activity in MTT and CV assays against injury (fig. 1B-1C). For four hours, 8 mM CCl_4 dose induced injury in cells. After the treatment, there was reduced death of cells in comparison with injured group as evidenced by cell viability assays (fig. 1B-1C).

Estimation of antioxidant potential of treated cells
Anti-oxidant assays (GSH & SOD) determine free radical scavenging activity, their levels were found reduced in treated group in comparison with injured group (fig. 1D-1E).

Decreased level of apoptosis in treated cells
p53 is regarded as apoptotic agent and level was found raised in CCl_4 group and decreased in treatment group in comparison. This predicted hepatoprotective role of Itraconazole (fig. 1F).

Enhanced angiogenesis level in treated HepG2 cells
VEGF is known to impart the angiogenic ability and its level was found to be decreased in injury group and raised in treatment group in comparison. This also predicted the hepatoprotective role of Itraconazole (fig. 1G).

Increased viability of Itraconazole in treated HepG2 cells
In muse analysis, 18.8% dead cells & 81.2% live cells were seen in untreated group, followed by 26.6% dead cells & 73.4% live cells in positive control group, followed by treatment group (41.2% & 58.8% live cells).

Table 1: shows different *in-vivo* groups.

Total Groups	Names of Groups	Treatments	Total Animals in each Group
Group 1	Negative-control group	Un-treated rats	10
Group 2	Injury group	CCl_4 treated rats	10
Group 3	Positive-control group	Itraconazole treated rats	10
Group 4	Treatment group	CCl_4 + Itraconazole treated rats	10

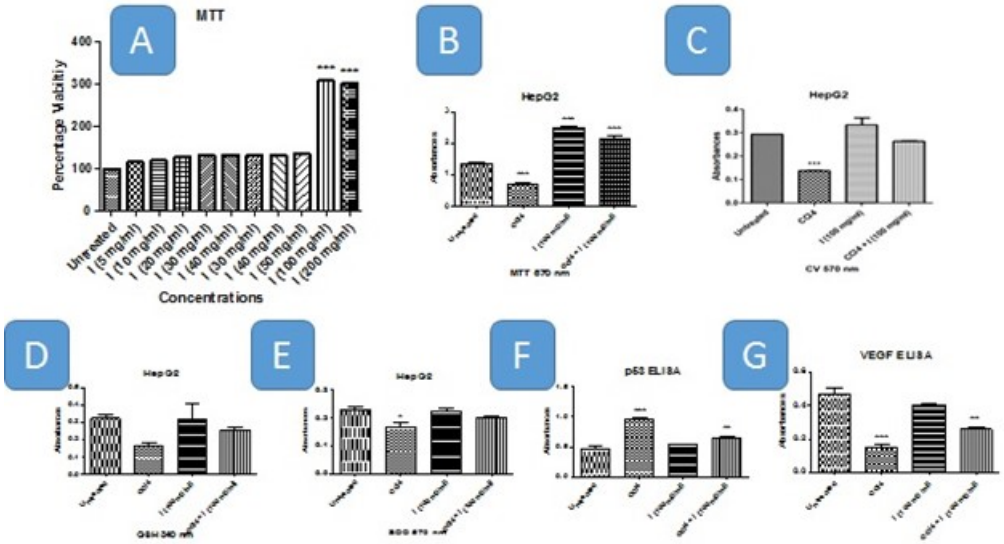

Fig. 1: Assays divided into untreated, CCl_4, Itraconazole and CCl_4 + Itraconazole groups. A: MTT assay of treatment on HepG2 cells with 5-200 mg/ml concentrations of Itraconazole. B-C: MTT and CV assays indicate that 100 mg/ml dose of Itraconazole was evaluated to check its protective activity in HepG2 cells against injury of CCl_4. D-E: Antioxidant assays (Superoxide dismutase, SOD & Glutathione Reductase, GSH) shows GSH and SOD levels after treatment. F-G: Expressions of apoptotic marker (p53) and angiogenic marker (VEGF) in all groups (blue colour indicated cells stained with DAPI and antibody stained in green). Data was calculated as mean ± SEM where $p \leq 0.05$ and *, ** & *** show significance levels between untreated vs treated groups.

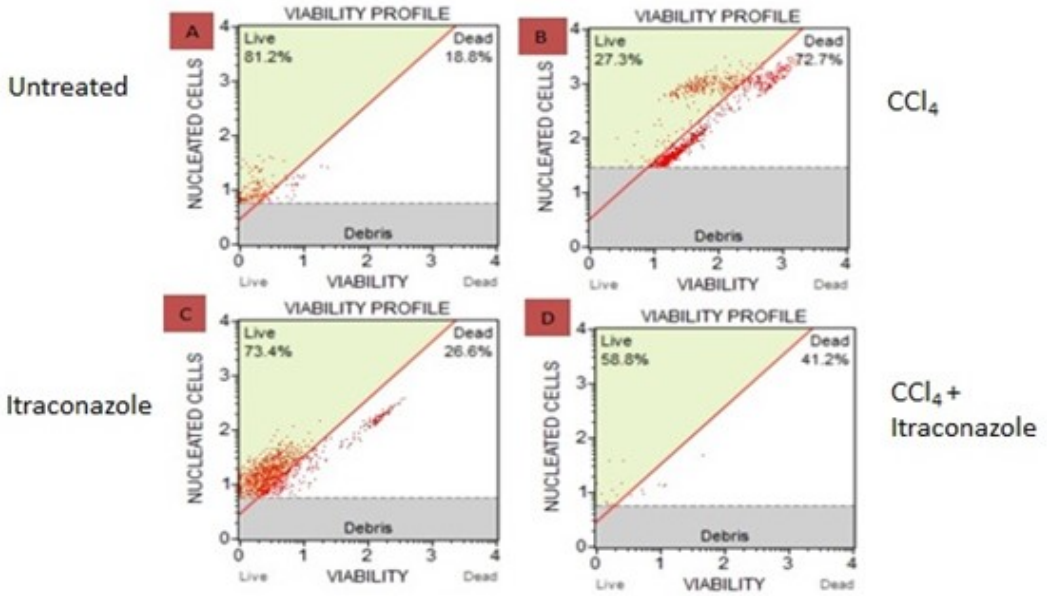

Fig. 2: Muse cell analysis percentages of live and dead cells in all groups. A: Untreated, B: CCl_4, C: Itraconazole, D: CCl_4 + Itraconazole.

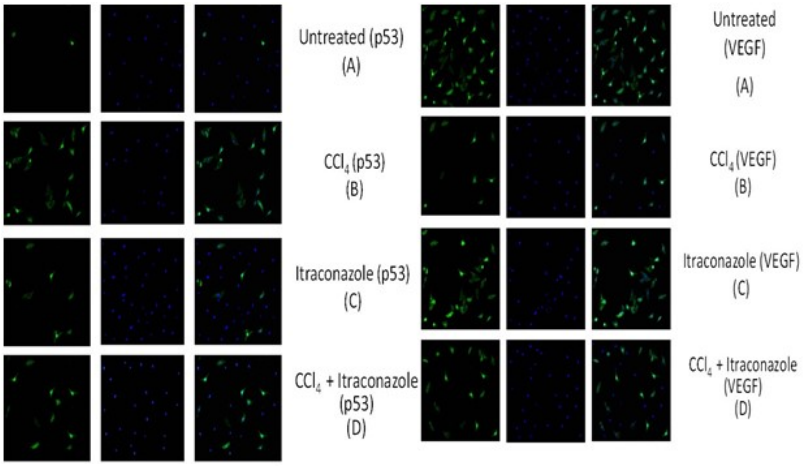

Fig. 3: Apoptotic and angiogenic markers expression analysis.

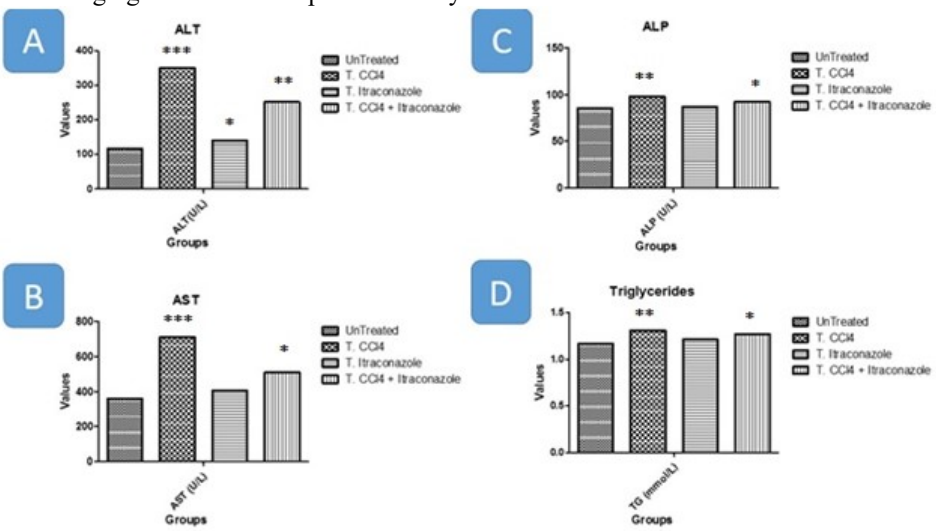

Fig. 4: Liver pathophysiology in all groups. A: ALT (alanine transaminase) levels. B: AST (aspartate aminotransferase) levels. C: ALP (alkaline phosphatase) levels. D: triglycerides level. Data was calculated as mean ± SEM where $p \leq 0.05$ and *, ** & *** show significance levels between untreated vs treated groups.

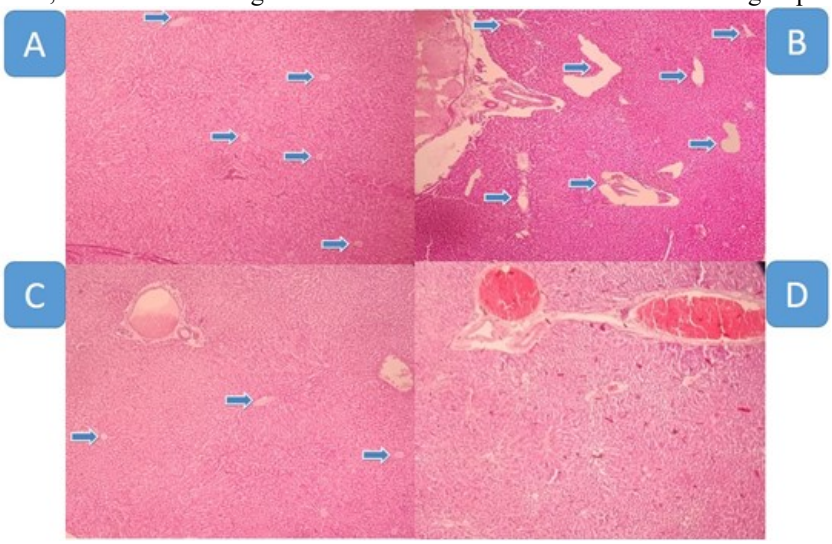

Fig. 5: H&E staining in all groups. (A) Shows untreated section (indicated with arrows). (B) Shows injured areas after administration of CCl_4 (indicated with arrows). (C) Shows Itraconazole treated sections (indicated with arrows). (D) Indicates liver sections of treatment group.

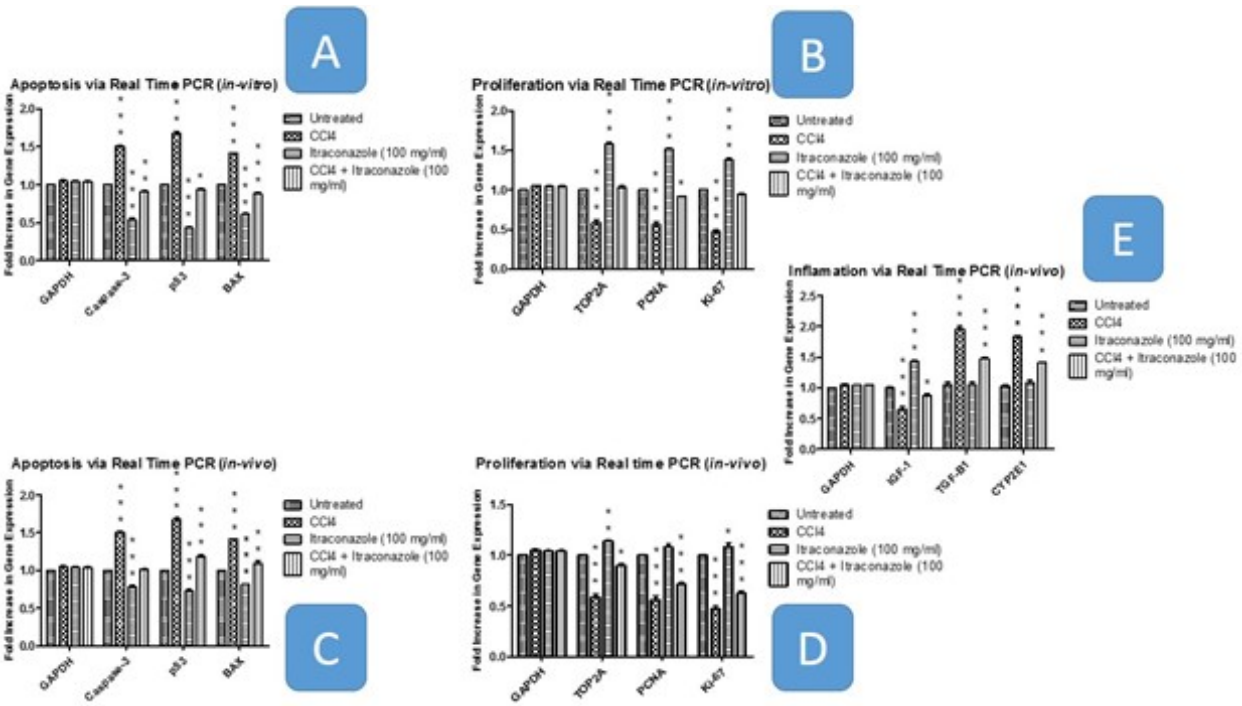

Fig. 6: *In-vitro* and *in-vivo* gene expression analysis in all groups in which GAPDH is kept as an internal control (house keeping gene). A: apoptotic markers (p53 + Caspase-3 + BAX) in *in-vitro* study. B: proliferative markers (PCNA + TOP2A + Ki-67) in *in-vitro* study. C: apoptotic markers (p53 + Caspase-3 + BAX) in *in-vivo* study. D: proliferative markers (PCNA + TOP2A + Ki-67) in *in-vivo* study. E: inflammatory markers (TGF-β1 + IGF-1 + CYP2E1) in *in-vivo* study.

Most of dead cells (72.7%) with least live cells (27.3%) were found in injury group. This study has demonstrated that Itraconazole succeeded to prevent cells from injury as less number of dead cells were found in treatment group in comparison with injury group (fig. 2).

Immunocytochemistry

Immunocytochemistry was conducted to evaluate levels of apoptosis and angiogenesis by applying p53 and VEGF antibodies respectively. In this procedure, increased levels of apoptosis was seen in cells of injury group in comparison with other groups and increased levels of angiogenesis in cells of treatment group was observed (fig. 3).

Functional improvement after treatment

After evaluating via biochemical tests, it was evidenced that all tests revealed increased serum levels of hepatic functions; such as of ALT (alanine transaminase), AST (aspartate aminotransferase), ALP (alkaline phosphatase) and triglycerides (fig. 4A-4D) in injury group as compared to treatment group. Collectively, these results indicated hepatoprotective role of Itraconazole against injury (fig. 4).

Itraconazole treatment reduces liver fibrosis

Hemotoxylin and eosin staining was used to stain tissues. Treatment of Itraconazole resulted in significant reduction of fibrosis. There was marked increase in fibrotic area observed in injury group. Different histopathological changes were observed in treated rats (fig. 5). Liver sections obtained from untreated group displayed normal hepatic architecture, whereas degenerated, ballooned and necrotic hepatocytes were seen in injury group with different morphology as compared to other groups. The severity and incidence of alterations were found less prominent in treatment group in comparison with CCl_4 group.

Gene Expression Analysis via Itraconazole treatment

Gene expression profiling was performed in both *in-vitro* and *in-vivo* samples obtained from all groups. GAPDH was regarded as internal control to compare. In *in-vitro* study, decreased expression of apoptotic markers (p53, BAX & caspase-3) (fig. 6A) and enhanced expression of proliferative markers (PCNA, TOP2A and Ki67) (fig. 6B) were noted in treatment group. Similarly, in *in-vivo* study, there were decreased levels of apoptotic markers (fig. 6C) and increased levels of proliferative markers (fig. 6D). As regarding inflammation to check, IGF-I & TGF-β1 were checked (fig. 6E). In *in-vivo* study, level of IGF-I was found decreased and of TGF-β1 was raised in injury group as compared to treatment group. CYP2E1 is the enzyme included in family of cytochome P450 system, which activates CCl_4 at cellular level. Inhibition of CYP2E1 can decrease CCl_4 bio-activation which can

ultimately prevent hepatic fibrosis. In treatment group, its level was found reduced in comparison with injury group, which shows that there was reduced bio-activation of CCl_4.

DISCUSSION

Hepatic fibrosis occurs in patients suffering from fatty liver disease or viral hepatitis. Hepatic stellate cells accumulate enough in liver tissue during hepatic injury and may be considered hallmark of fibrosis (Akhtar *et al.*, 2019). Itraconazole is effective against many fungal infections such as onychomycosis, sporotrichosis, histoplasmosis, coccidioidomycosis, cryptococcosis, candidiasis, blastomycosis and aspergillosis. Even, it is best suited in those patients who cannot tolerate voriconazole and amphotercin B (Nett and Andes, 2016). As compared to older azoles, Itraconazole is considered safer as evidenced in Taiwanese population (Kao *et al.*, 2014). CCl_4 is a potent hepto-toxin. During CCl_4 metabolism in liver cells, production of free radicals occurred (Abdel-Moneim *et al.*, 2015). Similarly, HepG2 cells are hepatic cells and have been used for evaluation of hepatoprotective potential of different compounds in previous studies (Wu *et al.*, 2016). In the present *in-vitro* study, proliferation of HepG2 cells was found to revert to normal by itraconazole when checked via cell viability assays. Hepatocytes show antioxidant systems related to enzymes such as glutathione peroxidase and superoxide dismutase (SOD), and their levels are first increased in order to combat during oxidative stress to cells when exposed to CCl_4 (Rahmani *et al.*, 2019, Abdelhafez *et al.*, 2018, Perazzoli *et al.*, 2017, Zahran *et al.*, 2018). But, as the injury proceeds, levels of antioxidant enzymes reduced as reported by different studies (Lu *et al.*, 2018). In current study, similar findings were observed that reduced levels of enzymes were found in injury group as compared to treatment group. Immunostaining procedure has been conducted to check CCl_4 induced apoptosis in hepatocytes world over (Lu *et al.*, 2018). ELISA and Immunostaining displayed decreased percentage of viable cells and more apoptotic cells in injury group in comparison with treatment group.

During injury, free fatty acids accumulate in liver cells (Ore and Akinloye, 2019). CCl_4 injury increased serum levels of ALP, ALT, AST and triglycerides (Lu *et al.*, 2018, Nguyen-Lefebvre *et al.*, 2018). Similar findings were observed in current study in injury group and low levels were observed in treatment group. Hemotoxylin and Eosin staining is used to check damage in liver tissues induced by CCl_4 (Lu *et al.*, 2018) and histopathological features were observed (Ore and Akinloye, 2019) as CCl_4 showed pathological changes in damaged tissues (Al-Seeni *et al.*, 2016) such as necrosis, inflammation, fatty accumulation (Mahmoodzadeh *et al.*, 2017), vacuolated cytoplasm, irregular nuclei and distorted microrganelles (Fahmy *et al.*, 2018). In current study, CCl_4 was administered as described by (Akhtar *et al.*, 2019) and less pathological changes were observed in itraconazole treatment group as compared to injury group. CCl_4 injury produced condition of DNA fragmentation due to gene expression of apoptotic markers such as p53 (Rahmani *et al.*, 2019), caspase-3 and BAX. During CCl_4 injury, raised levels of apoptotic markers were observed (Rahmani *et al.*, 2019). Similarly, up-regulation of proliferative markers such as TOP2A (Wu *et al.*, 2019), PCNA (Khalil *et al.*, 2015) and Ki67 (Miller *et al.*, 2018) were observed in in different studies. In current study, down-regulation of apoptotic markers (BAX, p53, caspase-3) and up-regulation of proliferative markers (PCNA, TOP2A, Ki67) were observed in Itraconazole treated group. Many studies indicated the relation of IGF-I with increased risk of liver diseases where fibrotic conditions were produced (Ore and Akinloye, 2019, Rocio *et al.*, 2017). In current study, less down-regulation of IGF-I in treatment group as compared to injury group showed somewhat less damage. TGF-β1 is a marker related to hepatic inflammation (Rocio *et al.*, 2017), as damaged liver cells show production of fibrogenic cytokines including TGF-β1 (Norona *et al.*, 2019). Many studies proved involvement of TGF-β1 in different liver injury studies (Nguyen-Lefebvre *et al.*, 2018, Li *et al.*, 2018, Liu *et al.*, 2018). In current study, up-regulation of TGF-β1 was more prominent in injury group as compared to treatment group. Cytochrome P450 (CYP450) enzyme family system operate in different parts of body including liver (Zhang *et al.*, 2017). Many compounds displayed hepatotoxicity in pre-treated studies of CCl_4 such as methanol, pyrazole, acetone and aliphatics, all showed increased level of CYP2E1, thus inhibitors of CYP450 were beneficial in reducing toxicity in liver (Zhang *et al.*, 2020). One study reported no significant effect of Itraconazole on CYP2E1 at low doses (less than 10 μM up to 50 μM) (Krasulova *et al.*, 2019), but we observed preventive effect at high doses. Reduced expressions of TGF-β1 and CYP2E1 were observed in treatment group as compared to injury group.

CONCLUSION

In conclusion, Itraconazole has capability to inhibit CYP450 enzyme and prevent bio-activation of CCl_4 in cellular metabolism, hence protecting liver as evidenced by both *in-vitro* and *in-vivo* studies.

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