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ORIGINAL PAPER

Evaluation of the Protective Effect of Silibinin in Rats with Liver Damage Caused by Itraconazole

Hamdi Sozen · Ozgür Ilhan Celik · Esin Sakalli Cetin · Nigar Yilmaz · Alper Aksozek · Yasar Topal · Ibrahim Hakkı Cigerci · Halil Beydilli

Abstract Itraconazole (ITZ) belongs to the triazole group of antifungals with potent keratinophilic and lipophilic features. Hepatotoxicity is one of its most remarkable features. Silibinin (SIL) is a plant used worldwide which is used in the treatment of many liver diseases and it is especially very well known for its hepatoprotective–cytoprotective effect. The aim of our study was to research the protective effect of SIL in ITZ-induced hepatotoxicity using biochemical and pathological tests. Liver enzymes and antioxidant enzyme activities were measured spectrophotometrically by using commercial kits. ALT and AST levels in ITZ group were significantly increased compared to the group, while the activities of GSH-Px and SOD had decreased ($p < 0.05$). When ITZ group was compared to ITZ + SIL group, AST, ALT, and levels of NO and MPO were significantly decreased, while the activities of GSH-Px and SOD were increased ($p < 0.05$). Histopathological evaluation showed that SIL significantly decreased periportal inflammation and parenchymal hepatocyte apoptosis in ITZ and ITZ + SIL groups ($p < 0.05$). Eventhough not statistically significant, partial improvement with the use of SIL has been detected ($p > 0.05$) in hepatocyte degeneration and multinuclear giant cell formation. According to the evaluation performed with comet assay method, ITZ leads to DNA damage, and the use of SIL significantly decreases DNA damage ($p < 0.05$). We have detected that the use of ITZ increases oxidative stress (MPO, NO), decreases antioxidant activity (SOD and GSH-Px), and leads to DNA damage and histopathological liver damage, whereas the use of SIL has a cytoprotective effect on the liver by increasing the antioxidant effect (SOD, GSH-Px) and by decreasing the oxidative stress (NO, MPO). ITZ causes the generation of ROS and leads to DNA damage and liver damage. SIL has a cytoprotective effect on the liver by increasing antioxidant enzyme activities, preventing the formation of ROS.

Keywords Itraconazole · Silibinin · Hepatotoxicity · Oxidative stress · Antioxidant activity · Comet assay

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Introduction

Itraconazole (ITZ) belongs to the triazole group of antifungals with potent keratinophilic and lipophilic features. Triazole drugs are broad spectrum antifungal agents used in the treatment of infections caused by various pathogenic yeast and fungi [1]. ITZ kills the fungal cell through inhibition of ergosterol synthesis, which is an important part of the fungal cell membrane [2]. Triazoles have higher antifungal activity, lower toxicity, and broader antifungal spectrum [3]. However, serious hepatotoxic events have been reported with the use of ITZ [4]. Latest studies indicate that one of the antimicrobial effects of some drugs is to stimulate cellular oxidative stress response in pathogens, contributing to higher microorganism death rates. This group of drugs is defined as oxidative stress drugs. ITZ is the best known drug in this group [5].

According to World Health Organization, the rate of traditional drug use such as silibinin (SIL) is 80 % [6]. SIL is pharmacologically active constitutive of Silybum marianum [7]. Silybum marianum is a plant used in various liver diseases since ancient times. Nowadays it is being used in cirrhosis, acute or chronic hepatitis, and alcohol- or toxin-related hepatitis treatment [8–10]. Antioxidant activity may probably have crucial role in the SIL effects. It is widely used for the treatment due to its safety and lack of adverse effects [11]. Recent studies have showed that SIL protects the liver against many drugs and chemicals-induced liver injury [12].

The antioxidant activity of SIL seems to be the key mechanism among cellular mechanisms [13, 14]. Thanks to its antioxidant features and elimination of free radicals: it is neuroprotective and prevents cancer, protects the skin from harmful UV effects, and it is used in the protection and treatment of cardiopulmonary and gastrointestinal systems [15]. Besides the strong antioxidant effect of SIL, cytoprotective, anti-inflammatory, and anticarcinogenic effects have been shown [16]. Inhibition of Kupffer cells functions and leukotriene production is reported to be effective in liver protection [17].

Single-cell gel electrophoresis or commonly known as "Comet Assay" is a non-invasive, fast and sensitive fluorescence microscopic method used for the detection of DNA damage on cellular level [18]. Comet assay is frequently used in the research of antioxidant agents efficacy [19], detection of pathogenic mechanisms of various diseases, in clinical researches [20, 21], evaluation of various biological and environmental changes such as nutrition, aging, hypoxia, ozone, and chemotherapy [22, 23], detecting DNA breaks due to necrosis or apoptosis [24], genotoxicity researches of known toxic agents [25], and different types of DNA damage and repair researches [26].

The present investigation was aimed to evaluate antioxidant and protective efficacies of SIL against ITZ-induced hepatotoxicity. In the current study, we investigate the effects of SIL on ITZ-induced liver toxicity in rats by evaluation levels of nitric oxide (NO) and myeloperoxidase (MPO), activities of superoxide dismutases (SOD) and glutathione peroxidase (GSH-Px), aspartate aminotransferase (AST), alanine aminotransferase (ALT), histological values, and comet assay method.

Materials and Methods

Ethics Statement

Experimental design and animal handling procedures were approved by the local authorities, The Chairmanship of Suleyman Demirel University Local Ethical Committee on Animal Experiment, in accordance with the recommendations of the Weatherall report. Every effort was done to minimize the number of animals and their suffering.

Chemicals

ITZ was purchased from Nobel Pharmaceutical Industry and Trade Co, Ltd.

SIL (2-[2R,3R-dihydro-3-(4-hydroxy-3-methoxyphenyl)-2-(hydroxymethyl)-1,4-benzodioxin-6-yl]-2R,3R-dihydro-3,5,7-trihydroxy-4H-1-benzopyran-4-one) was purchased from Sigma/Aldrich Chemicals, USA.

Animals

Thirty-two adult female Wistar Albino rats (200 ± 20 g) were obtained from the Süleyman Demirel University Laboratory for Research Animal Production and Experimental Research Turkey. Rats were acclimatized for 1 week before starting experiments. They were housed in stainless steel cages (three rats/cage) and kept at controlled temperature (23 ± 2 °C), humidity (60 ± 10 %), and light/dark (12/12 h) cycle. Rats were supplied with a commercial standard rat diet (Abalioglu Feed Industry, Denizli) and water ad libitum. The study was performed in 4 groups consisting of 8 rats.

Group 1 ($n = 8$): Control (CTL). The rats were given 0,3 ml oral saline, once in 24 h.
Group 2 ($n = 8$): SIL. The rats were given 100 mg/kg/day oral SIL for 14 days, once in 24 h.
Group 3 ($n = 8$): ITZ. The rats were given 100 mg/kg/day oral ITZ for 14 days, once in 24 h.

Group 4 ($n = 8$): ITZ + SIL. The rats were given 100 mg/kg/day SIL and 100 mg/kg/day ITZ once in 24 h for 14 days.

At the end of the experiment, all animals were anesthetized under intraperitoneal injection of ketamine/xylazine (60 and 6 mg/kg, respectively). Blood samples were taken from intracardiac to the sterile tubes for measuring the level of serum ALT, AST, MPO, NO, SOD, and GSH-Px, and they were obtained in EDTA tubes for comet assay. Blood samples were centrifugated and serum was separated. Livers were removed immediately, and washed with phosphate buffer (PBS) (pH 7.4), and then frozen promptly in a deep freezer for biochemical analysis. All samples were protected under −80 °C until analysis.

Determination of ALT and AST Activities

The activities of AST and ALT were calculated spectrophotometrically in serum using Beckman Coulter kits by autoanalyzer (Unicel D × C 800 Synchron, *Brea*, *California*, USA).

Determination of SOD Activity

The tissue was homogenized at 16,000 rpm on ice in 5–10 ml cold buffer, 20 m MHEPES buffer, pH 7,2, containing 1 mM EGTA, 210 mM mannitol, and 70 mM sucrose per g tissue. It was centrifugated at 1,500×*g* for 5 min at 4 °C. The supernatant was removed. The blood was centrifugated at 2,000×*g* for 15 min at 4 °C. The top yellow serum layer without disturbing the white buffy layer pipetted off. Serum should be diluted 1:5 with sample buffer. The SOD activity was measured in the supernatant and serum. SOD was determined via Cayman's Superoxide Dismutase assay kit (Cayman Chemical Co., Ann Arbor, MI, USA) in Bio-Tek ELx-800(Winooski, USA). The detection of superoxide radicals was generated by xanthine oxidase and hypoxanthine. One unit of SOD is defined as amount of enzyme needed to exhibit 50 % dismutation of the superoxide radical. The results were expressed as units per mg protein tissue for liver tissue and units per milliliter for serum.

Determination of GSH-Px Activity

The tissue was homogenized in 5–10 ml cold buffer, 50 mM Tris–HCl, pH 7.5, 5 mM EDTA ,and 1 mM DTT (Dithiothreitol) per tissue. Then it was centrifugated at 10,000×*g* for 15 min at 4 °C. The supernatant was removed. The blood was centrifugated at 700–1,000×*g* for 10 min at 4 °C. The plasma was removed. The GSH-Px activity was measured in liver tissue and plasma samples. The GSH-Px activity was determined via Cayman's GSH-Px assay kit (Cayman Chemical Co., Ann Arbor, MI, USA) in Bio-Tek ELx-800. The GSH-Px activity was measured indirectly by a coupled reaction with glutathione reductase. The oxidized glutathione was produced upon reduction of hydroperoxide by GSH-Px.

Determination of NO Level

The tissue was homogenized in PBS (pH 7.4) and centrifugated at 10,000×*g* for 20 min to create the supernatant. Total NO assay was performed by spectrophotometry at 540 nm using nirate/nitrite colorimetric assay kit (Cayman, Ann Arbor, Michigan USA) inBio-Tek ELx-800. The assay was based on nitrate and nitrite determinations. The nitrate and nitrite are the stable end products of the reaction of NO with molecular oxygen. It measured the total accumulation of nitrate and nitrite in serum and liver tissue. The results were expressed as uM/protein.

Determination of MPO Level

The quantitative detection of MPO was used by an enzyme-linked immunosorbent assay kit (MPO Instant Elisa, eBioscience, Vienna, Austria) in Bio-Tek ELx-800. The results were expressed as pg/ml/protein.

Histopathological Evaluation

The liver tissue was also removed for histopathological investigation. The specimens were fixed in 10 % formalin subsequent overnight and were dehydrated by immersion in a series of alcohol solutions of various concentrations, cleared in xylene, and paraffin-embedded tissue sections. The tissue samples were then infiltrated with paraffin as blocks, sectioned (5 µm-thick slides), and were obtained which afterward were dyed in hematoxylin-eosin. The preperates were evaluated for 9 different parameters on light microscope: (1) Centrilobular hepatocyte degeneration, (2) Ductular degeneration (bile duct hyperplasia), (3) Bile plugs, (4) Inflammation of the periportal area, (5) Eosinophilic infiltration, (6) Multinuclear giant cell formation, (7) The presence of xanthomatous cells in the portal area, (8) The presence of apoptotic cells, and (9) Necrosis. All parameters were semiquantitatively scored. (Scoring system: 0: Absent, 1: Mild, 2: Moderate, 3: Severe) [27, 28].

Comet Assay

Venous blood samples of 5–10 µl from the frogs were evaluated for any DNA damage. Whole blood was diluted with phosphate buffered saline (PBS) (1:200, v/v) and single-cell suspensions prepared. Then, the whole blood samples of 0.5 µl were mixed with 100 mL of 0.5 % low-melting agarose in PBS at 37 °C. Subsequently, 80 ml of

this mixture was layered onto a slide pre-coated with a thin layer of 1 % normal melting point agarose, covered immediately with a coverslip and stored for 5 min at 4 °C to allow the agarose to solidify. After removing the coverslips, the slides were immersed in freshly prepared cold (4 °C) lysing solution (2.5 M NaCl, 100 mM EDTA–Na_2; 1 % Na-laurylsarcosine, 10 mM Tris–HCl, pH 10–10.5; 1 % Triton X-100 with 10 % DMSO being added just before use) for at least 1 h. The slides were then electrophoresed (25 V/300 mA, 25 min) after which they were immersed in freshly prepared alkaline electrophoresis buffer (0.3 mol/L NaOH and 1 mmol/l EDTA–Na2, pH[13) at 4 °C for unwinding (40 min). Experimental treatments were carried out under minimal illumination. After the electrophoresis application, the slides were neutralized (0.4 M Tris–HCl, pH 7.5) for 5 min. Each slide was stained with ethidium bromide (20 μg/ml) and covered with a coverslip. Slides were stored at 4 °C in humidified sealed containers until analysis.

The alkaline comet assay allows for the detection of DNA damage occurring as single-strand breaks by measuring the migration of DNA fragments from the nucleoid, visually resembling a comet. The slides were examined at 400× magnification using a fluorescent microscope (BX51, Olympus, Japan). All slides were evaluated blindly by the observers (IHC and UCE). Fifty cells per specimen (25 cells/slide) were scored. Comets were randomly captured at a constant depth of the gel, avoiding the edges of the gel, occasional dead cells, cells near or in a trapped air bubble, and superimposed comets. For quantification of DNA damage, the parameters of comet length (CL; expressed in lm), tail intensity (TI; DNA% in comet tail), and olive tail moment (OTM; DNA% × tail moment length) were measured and calculated by Comet IV Computer Software (Perceptive Instruments, Steeple Bumpstead, UK) [29].

Statistical Analysis

Statistical analysis were performed using ''SPSS 20.0 for Windows'' packet program. In general, any significant differences between these groups were evaluated using the Kruskal–Wallis test. The Mann–Whitney *U* test was used to compare the groups with each other. Results are presented as mean + SD; $p < 0.05$ was regarded as statistically significant.

Results

AST values of group ITZ compared to group CTL were significantly higher ($p < 0.05$). SIL + ITZ groups compared to CTL group had significantly high AST levels ($p < 0.05$), while compared to ITZ group the values were significantly low ($p < 0.05$) (Table 1).

ALT values of group ITZ compared to group CTL were significantly increased ($p < 0.05$). While ALT values of SIL + ITZ group compared to CTL group were significantly increased, the values in group SIL were significantly decreased ($p < 0.05$) (Table 1).

Liver MPO values in group ITZ compared to group CTL were found to be significantly high ($p < 0.05$): however, no significant difference was found between group CTL and SIL + ITZ ($p > 0.05$). Liver MPO values in group SIL + ITZ compared to group ITZ were found to be significantly decreased ($p < 0.05$) (Table 1).

Liver NO values were significantly higher in group ITZ compared to group CTL ($p < 0.05$), while there was no significant difference between group CTL and group SIL + ITZ ($p > 0.05$). Liver MPO values were significantly lower in SIL + ITZ group compared to ITZ group ($p < 0.05$) (Table 1).

Liver SOD values were found to be significantly higher in group CTL compared to group ITZ and SIL + ITZ. In group SIL + ITZ compared to group ITZ, the values were significantly higher (each $p < 0.05$) (Table 1).

Liver GSH-Px values were found to be significantly higher in group CTL compared to group ITZ ($p < 0.05$). No significant difference was found between GSH-Px in group CTL compared to SIL + ITZ ($p > 0.05$). However, it has been found to be significantly decreased in group ITZ compared to SIL + ITZ ($p < 0.05$) (Table 1).

Pathology

Histopathological Results

Normal histological appearance of the liver was only found in the CTL group (Fig. 1a) and the group that used SIL (Fig. 1b). We detected that ITZ led to significant hepatocyte degeneration of the liver ($p = 0.000$), ductular reaction (bile duct hyperplasia) ($p = 0.001$), bile duct plugs ($p = 0.027$), inflammation of the portal/periportal area ($p = 0.000$), multinuclear giant cell formation ($p = 0.064$), the presence of xanthomatous cells in the portal area ($p = 0.144$), the presence of apoptotic cells ($p = 0.009$), and necrosis ($p = 0.317$) (Fig. 2a–c).

The use of ITZ and SIL significantly reduced the portal/periportal inflammation and apoptosis of parenchymal hepatocytes ($p < 0.05$). Furthermore, the use of SIL eventhough not significantly, partially improved hepatocyte degeneration and multinuclear giant cell formation ($p = 0.062$ and $p = 0.064$, respectively) (Fig. 3a–b).

Comet Assay

The decrease of DNA damage in SIL + ITZ group is statistically significant compared to ITZ group. The use of SIL has prevented DNA damage.

Table 1 AST, ALT, NO, MPO, SOD, and GSH-Px values of the four groups of the rats ($n = 8$)

	CTL	SIL	ITZ	SIL + ITZ
AST (U/L) (serum)	86.62 ± 16.09	94.25 ± 13.54	209.00 ± 58.28[a]	125.50 ± 58.19[a,b]
ALT (U/L) (serum)	30.12 ± 5.64	32.50 ± 4.40	70.25 ± 22.06[a]	41.75 ± 6.47[a,b]
MPO (U/g protein) (liver tissue)	1.70 ± 0.61	1.92 ± 0.45	3.48 ± 0.96[a]	2.21 ± 1.11[b]
NO (U/g protein) (liver tissue)	0.88 ± 0.08	0.90 ± 0.16	1.42 ± 0.40[a]	0.86 ± 0.19[b]
SOD (U/g protein) (liver tissue)	0.15 ± 0.04	0.18 ± 0.05	0.09 ± 0.02[a]	0.22 ± 0.04[a,b]
GPX (U/g protein) (liver tissue)	1.44 ± 0.44	1.61 ± 0.31	1.07 ± 0.63[a]	1.52 ± 0.15[b]

GSH-Px glutathione peroxidase, *SOD* superoxide dismutase, *NO* nitrite oxide, *MPO* myeloperoxidase

* Results are presented as mean ± SD

[a] $p < 0.05$ compared with CTL group

[b] $p < 0.05$ compared with ITZ group

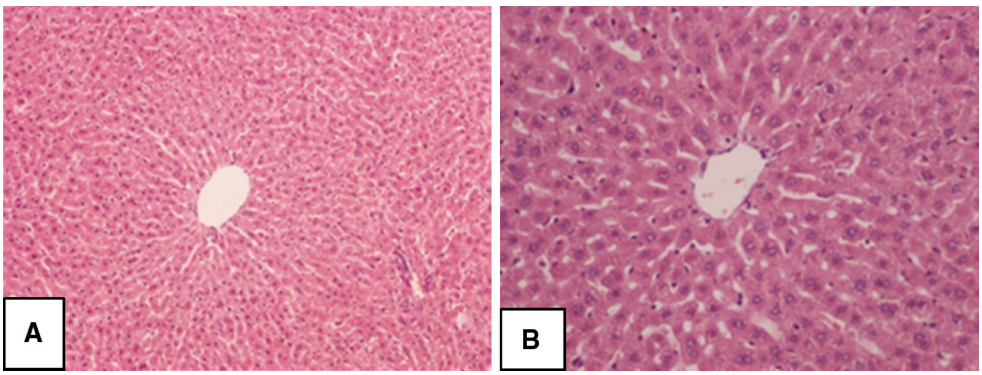

Fig. 1 CTL group: **a** (Hematoxylin-eosin ×100). **b** SIL group (hematoxylin-eosin ×200). Normal liver tissue-normal hepatocytes around central vein-normal portal areas

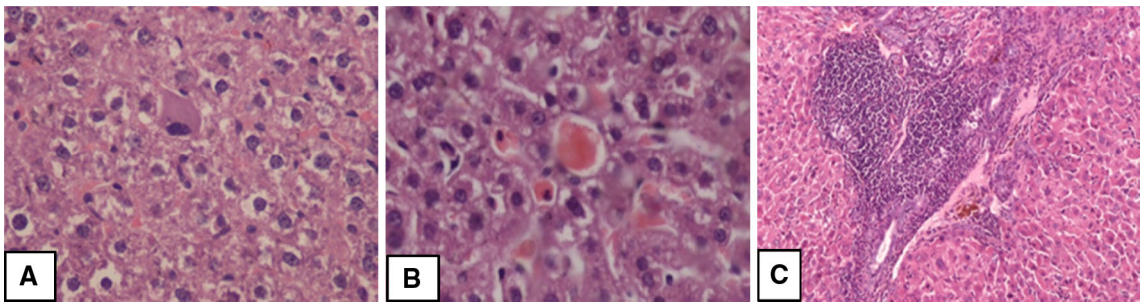

Fig. 2 ITZ group: **a** 3+ Hepatocyte degeneration (hematoxylin-eosin, ×400), **b** Formed in hepatocytes apoptosis (hematoxylin-eosin, ×400), **c** Portal/periportal in the area, 3+ inflammation, ductular proliferation, bile plugs (hematoxylin-eosin ×100)

Comet assay results showed that DNA damage was high at all concentrations compared to CTL group. While the highest genotoxic activity observed the ITZ (26.66 ± 2.88), the lowest one observed the CTL (12.66 ± 1.15). As it can be seen, exposure of ITZ and SIL + ITZ significantly increased the DNA damage. DNA damage was decreased after SIL + ITZ compare to ITZ group and these values was found statistical significant. Application of SIL and SIL + ITZ increased DNA damage, but these values were not found statistically significant (Table 2).

Discussion

AST, ALT, MPO, and NO levels in rats after ITZ administration were significantly increased compared to the CTL group, while activities of GSH-Px and SOD decreased. We suggest that ITZ caused a significant liver damage. AST, ALT, NO, and MPO levels decreased in SIL + ITZ group compared to ITZ group, whereas activities of GSH-Px and SOD significantly increased. SIL, given to rats with ITZ, showed a significant protective activity against liver damage induced by ITZ.

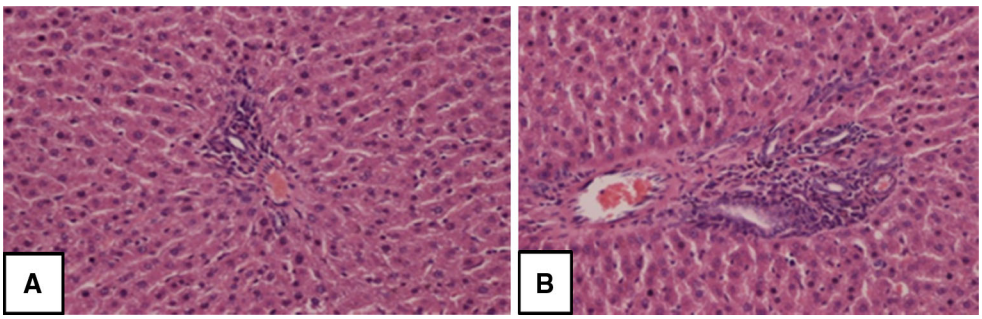

Fig. 3 SIL + ITZ group. **a** ve **b** decreased portal/periportal inflammation (hematoxylin-eosin ×100)

Table 2 Comet assay values of the four groups of the rats ($n = 3$)

Uygulama	*N*	DNA damage (arbitrary unit ± SD)*Mean ± SD
CTL	3	12.66 ± 1.15a
SIL	3	15.33 ± 1.15ab
ITZ	3	26.66 ± 2.88d
SIL + ITZ	3	18.72 ± 5.35ab

Different characters in the columns are $p < 0.05$ significant (Duncan test)

The liver, a very critical organ for detoxifying processes and oxidative stress, is thought to be a key of hepatocellular damage. The liver tissue was a major site of ITZ metabolism by assembling a great quantity of its metabolites [30].

Antiretroviral agent nelfinavir led to significant increase of reactive oxygen species (ROS) generation and suppression of cytosolic, but not mitochondrial SOD levels [31]. Similarly, in the study evaluating the protective effect of methylsulfonylmethane in liver damage caused by acetaminophen showed increased AST, ALT, MPO, MDA and decreased SOD and total GSH-Px levels [32]. In patients receiving ciprofloxacin therapy, lipid peroxide levels significantly increased; reduced activities of glutathione and SOD; also with the duration of treatment further increased lipid peroxide levels, activities of glutathione and SOD levels were significantly decreased [33].

ITZ can lead to liver damage through ROS such as superoxide anion radical O_2-, hydrogen peroxide (H_2O_2), and the highly reactive hydroxyl radical (OH). Enhanced ROS products lead to genomic DNA damage and oxidative degradation of lipid and proteins. ITZ may also lead to cell membrane permeability disruption. By inhibiting microsomal cytochrome P450 isoenzyme CYP3A4 may also cause increased toxicity. High doses lead to the autoinhibition of its metabolism leading to liver damage. It aggravates liver damage by inhibiting the antioxidant enzymes (SOD and GSH-Px) that deactivate itraconazole ROS's. Reactive nitrogen species formed after the increase of nitric oxide levels also contribute in the progress of liver damage [34, 35].

In addition, ALT and AST enzymes levels are a marker of hepatic function to determine hepatotoxicity. ITZ exposure resulted in the increasing levels of serum AST and ALT. As a result, it can lead to deterioration in transport function of hepatocytes. In earlier study, it was reported that SIL repaired the AST and ALT enzymes levels of hepatotoxicity [36].

In addition, increasing MPO, NO levels emphasizes that ITZ plays a role in pathogenesis of hepatic through oxidative stress mechanism. As one of the most important oxygen-dependent enzymes in neutrophils which if released into local tissues or the systemic circulation, MPO can induce oxidative stress, with variable degrees of cytotoxicity [37]. Estimation of tissue MPO activity has been confirmed to be reliable indicator of inflammation [38]. SIL repaired inflammatory process by decreasing MPO pathway and also via preventing free radical production.

Lipid peroxidation started as a consequence of ROS-induced isolation of hydrogen from PUFAs of cellular membrane, which results in the formation of relatively stable compounds like NO. Increasing the level of NO is a vital role in the modulation of oxidative stress and tissue damage. Prabu et al. reported that the level of NO decreased as a result of SIL treatment against arsenic-induced toxicity [39]. We found increased activity of liver tissue MPO and NO in ITZ group and decreased activity of liver tissue MPO and NO in ITZ + SIL group.

Topical use of SIL decreases oxidative stress in tissues. SIL prevents tumorogenesis and activation of inflammatory mediators; the chemopreventive effect of SIL was linked to the increase of endogenous cytoprotective mechanisms and the decrease of inflammatory mediators such as nitrous oxide, tumor necrosis factor-α, interleukin-6, interleukin-1β, COX-2, iNOS, and NF-κB [40].

SOD is assumed to be the most effective antioxidant. It is regarded the first step of defense against harmful effects of molecular oxygen radicals in cells. Superoxide is a vital

source of hydroperoxides and free radicals. GSH-Px is a pivotal selenocysteine-containing enzyme, which catalyzes the decrease of hydroperoxidase, including hydrogen peroxide, via reduced glutathione and functions to protect the cell from oxidative damage [41]. We found decreased activity of liver tissue antioxidant enzymes, SOD and GSH-Px, in ITZ group and increased activity of liver tissue SOD and GSH-Px in SIL + ITZ group. In a study with high-calory diet performed on rats with diabetes and various metabolic disorders such as hyperinsulinemia, hyperglycemia, and dyslipidemia, SOD level increased while GSH-Px levels decreased compared to the control group. Insulin resistance decreased and antioxidant levels were restored in the group receiving high-calory diet and SIL compared to the group receiving only high-calory diet [42].

SIL is natural antioxidant compound reversing ITZ-induced hepatic damage. SIL can act in many ways in halting and eradicating ROS production. It prevents liver damage by enhancing antioxidant enzyme activity (SOD and GSH-Px) and expression, decreasing MPO activity in hepatic inflammation, and inducing cytochrome P450 isoenzyme CYP3A4. Also, SIL may enhance cellular resistance and decrease the permeability by interacting with the cell membrane. It may transform ROS into less toxic products due to its structure [43].

The hepatoprotective activity of SIL on rats is through mitochondrial membrane stabilization, oxidative stress inhibition, and advanced insulin resistance mechanisms [44]. Esser-Nobis et al. have shown that SIL inhibits HCV-RNA. The drug should not be used solely and it is a promising drug particularly in treatment resistant patients as a part of combination therapy [45]. High-dose IV SIL has been shown to have a potent antiviral activity in previously unresponsive patients to conventional antiviral treatment; in cirrhotic patients, the use of IV SIL, ribavirin, and pegylated interferon alpha led to gradual decrease of HCV-RNA [46].

In our study, it has been detected that ITZ led to significant hepatocyte degeneration, bile duct hyperplasia, formation of bile duct plugs, inflammation of the portal/periportal area, and the presence of apoptotic cells. Even-though statistically insignificant, liver necrosis, formation of multinuclear giant cells, and increase in the presence of xanthomatous cells were detected. It was detected that SIL significantly reversed to ITZ-induced portal/periportal inflammation and hepatocyte apoptosis ($p < 0.05$). Even-though not significant, it partially reduced hepatocyte degeneration and multinuclear giant cell formation.

Somchit et al. have reported severe inflammation, granuloma, bile duct hyperplasia, and severe hepatic necrosis in the use of ITZ in rats [27]. Patel et al. showed that liver damage caused by Doxorubicin was generally limited to the centrilobular area leading to decrease of glycogen, balloon degeneration of hepatocytes, increase in inflammatory cells, and apoptotic and necrotic cells; however, in the group treated with doxorubicin and SIL, no major histopathological anomalies were seen and liver damage was prevented [47].

In the first place, chemical agents leading to DNA damage are alkylating agents, nitrous acid, crosslinkers such as platinum derivates, and xenobiotics which are metabolized by the cytochrome P450 system. These chemicals lead to DNA damage by alkylation, oxidation of bases, by forming cross-links between bases or causing strand breaks [48]. In our study, we demonstrated the DNA damage caused by ITZ and the significant preventive effect of SIL by comet assay. Comet assay is a reliable method used in the detection of DNA damage. The same method was used in a study evaluating acetaminophen dose-related DNA damage and cytotoxic effects, and its prevention by a triple (*N*-acetylcysteine-silibinin-alpha-tocopherol) antioxidant complex [49]. Similarly, the in vivo acute toxicity, mutageneity- and genotoxicity-related safety, and toxicology study of Rhizophora mucronata plant were performed using comet assay method, reporting that the plant is safety of all parameters [50].

As a result, ITZ leads to liver damage through ROS such as H_2O_2, O_2 and through enhanced ROS production to genomic DNA damage and oxidative breakdown of lipids and proteins. SIL is evaluated as a natural antioxidant compound against ITZ-induced liver damage. SIL acts against ROS production and elimination through inhibition of MPO activity, increasing the activity and production of antioxidant enzymes such as SOD and GSH-Px and induction of ITZ detoxifying agent cytochrome P450 isoenzyme CYP3A4. SIL also inhibits the DNA damage induced by ITZ and has a protective role against hepatotoxicity. Further and larger studies are needed to be performed in order for SIL to be used as hepatoprotective and cytoprotective agent.

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