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Preclinical Benefit of Silymarin in Ketoconazole-Induced Hepatotoxicity

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Abstract

Background: Ketoconazole (KT) use has raised safety concern regarding hepatotoxicity. Silymarin (SL) is a natural bioactive substance with activities on a wide range of human pathologies. The protective activity of SL against KT-induced hepatotoxicity in rats was determined in this study.

Methods: Thirty adult Wistar rats of both sexes (220-300g) of n= 5/group were used. Groups I (Control) and II were orally administered with normal saline (0.2mL/day) and SL (200 mg/kg/day), respectively, whereas group III was orally administered with KT (200 mg/kg/day) for 28 days. Groups IV-VI were orally supplemented with SL (50 mg/kg/day, 100 mg/kg/day, and 200 mg/kg/day) before the administration of KT (200 mg/kg/day) for 28 days, respectively. On day 29, the rats were anesthetized and blood samples were collected and examined for biochemical markers. Liver tissues were collected and assessed for oxidative stress markers and histology.

Results: KT significantly ($p<0.01$) increased liver weight, and significantly ($p<0.001$) increased serum bilirubin, amino transferases, lactate dehydrogenase, gamma-glutamyl transferase, alkaline phosphatase, and liver malondialdehyde levels when compared to the control. KT significantly ($p<0.01$) decreased body weight, and significantly ($p<0.001$) decreased liver catalase, glutathione peroxidase, superoxide dismutase, and glutathione levels when compared to the control. KT caused hepatocellular necrosis. However, body, and liver weights and the aforementioned biochemical and oxidative stress markers were significantly restored in a dose-related fashion by SL supplementation at 50 mg/kg ($p<0.05$), 100 mg/kg ($p<0.01$), and 200 mg/kg ($p<0.001$) when compared to KT. The various doses of SL restored liver histology.

Conclusion: SL may have clinical benefit in KT-induced hepatotoxicity.

Keywords: Ketoconazole, hepatoprotection, silymarin, liver, toxicity, rats

Introduction

Drug-induced liver injury (DILI) was first described in the 1960s as a term that explains the spectrum of pathological responses by liver after been exposed to potentially hepatotoxic chemical substances.[1] DILI remains a significant and serious challenge in clinical practice and is still a diagnosis of exclusion. It is an infrequent occurrence with an incidence of 14–19 cases per 100,000 population, causing less than 1% of acute liver injury. Nevertheless, it is the most and known frequent cause of liver failure in the West, with a fatality rate of 10–50%.[2] DILI usually occurs when drug metabolism is altered causing hepatic damage attributed to factors including oxidative stress, inflammation, necrosis, apoptosis, and mitochondrial membrane damage.[3] Its manifestations ranges from liver enzyme elevations without any symptoms to liver failure, or death within days of it beginning. Effective drug treatment is scarce, but novel drugs are been explored.[4]

Ketoconazole (KT) has been used for more than four decades for the treatment of fungal infections. It is used as the prototype of human cytochrome P450 3A inhibitor in research involving drug interaction and metabolism during drug development.[5] In 2013, the European Medicines Committee on Medical Products for Human Use and the United States Food and Drug Administration collectively gave safety warnings and admonished decreased oral KT use because of potential risk of causing hepatic injury, drug interactions, and increased risk of adrenal insufficiency.[6] Incidence of 3.6-17.5% liver injury due to KT was documented in some clinical studies.[6,7] Preclinical studies have documented features of KT –induced hepatotoxicity which includes liver inflammation, oxidative stress, altered serum liver biochemical marker and liver architecture.[8,9] A number of factors have been speculated to be associated with KT-induced hepatotoxicity, which include immune mediated response, and oxidative stress.[10, 11]

Silymarin (SL) is an extract obtained from the dried fruits and seeds of the milk thistle plant (*S. marianum*). It is a complex combination of plant-derived chemical compounds known mostly as polyphenols, flavonolignans, and flavonoids (taxifolin, and quercetin) molecules.[12] The four dominant flavonolignan isomers in silymarin are silibinin, silichristin, isosilibinin, and silidianin, but silibinin

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(also called silybin) is the most prevalent and biologically active of the four isomers.[12] SL has a lot of biologic activities such as antioxidant, anti-inflammatory, and anti-fibrotic properties. Its antioxidant activity includes free radicals scavenging, production and enhancement of antioxidant activities,[13] while its anti-inflammatory action includes the inhibition of inflammasomes, and NF-kB activation.[14] Prior to modern and recent discoveries in medicine, it was recognized as an important and useful therapeutic treatment for numerous liver diseases in Asian and European traditional systems.[15] In preclinical studies, it has remarkably prevented liver dysfunction by restoring normal liver function and structure in paclitaxel[16] anti-tuberculosis drug[17] and paracetamol[18] induced hepatotoxicity. In the light of this information, this study examined whether SL can prevent KT-induced hepatotoxicity in rats.

Materials and Methods

Animals and drugs

KT and SL were supplied by Sigma Chemical Co., St. Louis, MO, USA. All other chemicals used were of analytical grades. The study was performed according to the procedure for the Care and Use of Laboratory Animals, 8th edition, 2011.[19] Thirty adult Wistar rats of both sexes weighing 220–300 g of aged 10–11 weeks sourced from the experimental animal unit of the Faculty of Pharmacy, Madonna University, Nigeria were used. The rats were randomly grouped into 6 of 5 rats/group, and kept under laboratory conditions (55 ± 5% relative humidity, 37 ± 3 °C temperature, and 12-h day and 12-h night cycle) for 14 days before the study began. SL[20] and modified doses of KT[8] were used.

Drug administration and sample collection

Group I, the control was administered with normal saline (0.2mL/day) whereas groups II and III were administered with SL (200 mg/kg/day) and KT (200 mg/kg/day), respectively for 28 days. Groups IV to VI were supplemented with SL (50, 100 and 200 mg/kg/day) prior to the administration of KT (200 mg/kg/day) for 28 days. On day 29, the experimental rats were anesthetized using ketamine (75 mg/kg/ip) and blood samples were collected via cardiac puncture in heparinised tubes and assessed for serum biochemical markers. Then, the liver tissues were removed and placed in a 10% formalin solution for histological analysis. Also, liver tissues were collected, washed in physiological saline and stored at −80 °C for oxidative stress marker assay.

Biochemical evaluations

Alkaline phosphatase (ALP), conjugated bilirubin (CB), alanine amino transferase (ALT), total bilirubin (TB), gamma-glutamyl transferase (GGT), lactate dehydrogenase (LDH), and alanine amino transferase (AST) were evaluated using an auto analyser.

Determination of oxidative stress markers

Liver tissues were homogenized using 10% of 150 mM phosphate buffer (pH 7.4), with the aid of a homogenizer (IKA Overhead Stirrer, Staufen, Germany). The homogenates were centrifuged (Hettich Zentrifugen, Tuttlingen, Germany) at 12,000 rpm at 4°C for 10 min. The supernatants were decanted and assayed for oxidative stress markers. Glutathione peroxidase (GPX) and glutathione (GSH) were analysed using the processes described by Rotruck et al., 1973[21] and Sedlak and Lindsay, 1968,[22] respectively. Catalase (CAT) and Superoxide dismutase (SOD) activities were investigated as reported by Aebi, 1974[23] and Sun and Zigman, 1978[24] respectively. Malondialdehyde (MDA) was evaluated according to the protocol described by Buege and Aust, 1978.[25]

Histology of the liver

The collected liver tissues were passed through established histological procedures and were embedded in paraffin blocks. Sections of 4–6-μm-thick were prepared from the blocks using a microtome and stained with hematoxylin-eosin (H&E). Using a Leica DM500 microscope (Leica DFC295), the stained sections were examined and photographed.

Statistical analysis

This study used SPSS (Statistical Package for the Social Sciences; SPSS Inc., Chicago, IL, USA) for Windows Version 22 for data analysis. Two-ways analysis of variance (ANOVA) and Tukey's pair-wise multiple comparison tests were used for data analysis. The results were presented as mean ± standard error of mean (SEM). Significance was considered at $P < 0.05$; < 0.01; and < 0.001.

Results

Effects on body and liver weights

SL (200 mg/kg) did not produce significant ($p>0.05$) effects on the body and liver weights in comparison to control. In contrast, KT significantly ($p<0.01$) decreased body weight and significantly ($p<0.01$) increased liver weight in comparison to the control (Table 1). However, the altered body and liver weights were restored by SL supplementation at 50 mg/kg ($p<0.05$), 100 mg/kg ($p<0.01$), and 200 mg/kg ($p<0.01$) in comparison to KT (Table 1).

Table 1: Effects of silymarin on the body and liver weights of rats administered with ketoconazole.

Groups	Dose (mg/kg)	FBW (g)	ALW (g)	RLW(%)
I	Control	250.6± 14.8	4.18± 0.27	1.67±0.21
II	SL 200	256.2±16.3	4.23±0.13	1.65±0.18
III	KT 200	129.2±13.3*	8.22±0.18*	6.33±0.12*
IV	SL 50+ KT 200	177.3±15.7[a]	6.39±0.31[a]	3.60±0.09[a]
V	SL 100+ KT 200	228.6±14.4[b]	4.12±0.34[b]	1.79±0.20[b]
VI	SL 200+ KT 200	247.5±12.5[b]	4.05±0.35[b]	1.64±0.17[b]

Values are mean ± SEM, n = 5. S: NS: Normal saline, Silymarin, KT: Ketoconazole, *$p < 0.01$ when compared to control, [a]$p < 0.05$ and [b]$p < 0.01$ Differ significantly when compared to KT (ANOVA).

Table 2: Effect of silymarin on serum liver function markers of rats administered with ketoconazole

Groups	Dose (mg/kg)	AST (U/L)	ALT (U/L)	ALP (U/L)	TB (g/dL)	CB (g/dL)	LDH (U/L)	GGT (U/L)
I	Control	44.31±2.65	33.45±2.17	36.73±2.23	8.38±1.32	6.13±0.32	32.92±2.27	0.52±0.03
II	SL 200	41.94±4.79	32.71±3.08	37.24±6.65	8.18±0.57	5.82±0.68	32.53±5.12	0.58±0.07
III	KT 200	146.43±17.0*	132.55±12.3*	101.47±12.7*	20.37±3.07*	17.19±1.43*	126.72±14.3*	1.83±0.02*
IV	SL 50 + KT200	103.57±15.8[a]	83.62±14.2[a]	82.16±4.09[b]	16.24±2.16[a]	14.87±2.15[a]	70.14±5.14[a]	0.74±0.05[a]
VI	SL 100 + KT 200	74.46±7.07[b]	58.43±5.08[b]	61.44±3.53[c]	10.61±0.27[b]	10.21±0.64[b]	52.61±3.21[b]	0.65±0.04[b]
VI	SL 200 + KT 200	49.93±5.52[c]	37.92±4.16[c]	39.51±2.23[d]	8.81±0.39[c]	7.01±0.54[c]	35.81±2.42[c]	0.55±0.06[c]

Values are mean ± SEM, n = 5, SL: Silymarin, KT: Ketoconazole, AST: Aspartate aminotransferase, CB: Conjugated bilirubin, ALT: Alanine aminotransferase, LDH: Lactate dehydrogenase, TB: Total bilirubin, ALP: Alkaline phosphatase, GGT: Gamma-glutamyl tranferase,* $p < 0.001$ Differ significantly when compared to control. [a]$p < 0.05$; [b]$p < 0.01$ and [c]$p < 0.001$ Differ significantly when compared to KT (ANOVA).

Effect on serum liver biochemical markers

Serum ALT, ALP, AST, GGT, CB, LDH and TB levels remain unchanged (p>0.05) after SL (200 mg/kg) administration when compared to the control. But serum ALT, ALP, AST, CB, GGT, LDH and TB levels were significantly (p<0.001) elevated by KT when compared to the control (Table 2). Interestingly, serum ALT, ALP, AST, CB, GGT, LDH and TB levels were significantly restored in a dose-related fashion by SL supplementation at 50 mg/kg (p<0.05), 100 mg/kg (p<0.01), and 200 mg/kg (p<0.001) when compared to KT (Table 2).

Effect on liver oxidative stress markers

SL (200 mg/kg) administration had no significant (p>0.05) effects on liver GSH, SOD, CAT, GPX and MDA levels when compared to the control (Table 3). On the other hand, KT administration significantly (p<0.001) decreased liver GSH, SOD, CAT, and GPX and significantly (p<0.001) increased MDA levels when compared to the control (Table 3). But SL supplementation restored liver GSH, SOD, CAT, GPX and MDA levels in a dose-related fashion at 50 mg/kg (p<0.05), 100 mg/kg (p<0.01), and 200 mg/kg (p<0.001) when compared to KT (Table 3).

Effect on liver histology

Normal liver histology was observed in the control (Figure a) and SL (200 mg/kg) administered rats (Figure b). Hepatocellular necrosis was observed in the liver of KT administered rats (Figure c). More so, central vein congestion was observed in the liver of rats supplemented with SL (50 mg/kg) (Figure d), SL (100 mg/kg) (Figure e) and SL (200 mg/kg) (Figure f), respectively.

Discussion

The liver is a major organ which has array of functions. It metabolizes a wide range of drugs to water soluble compounds, which can be easily excreted.[26,27] Drug-induced hepatic injury is the most frequent reason known for the removal from the market of approved drugs, and it accounts for most cases of acute liver failure in the United States.[28,29] Despite the effectiveness of KT in treating fungal infections, the potential for endocrine dysregulation, and hepatotoxicity may undermine its benefits.[30] This study pre-clinically, examined the protective activity of SL against KT-induced hepatotoxicity. Administered SL had no deleterious impact on all evaluated indices in this study. Organ and body weights perturbation by drugs is imperative for toxicity assessment.[31] KT visibly decreased body and increased liver weights. This action may be a consequence of decreased body mass and liver inflammation. However, SL supplementation conspicuously restored body and liver weights. Biochemical markers are characteristic features, which can be objectively assessed and quantified as potential

Table 3: Effect of silymarin on liver oxidative stress markers of rats administered with ketoconazole

Groups	Dose (mg/kg)	SOD (u/mg protein)	CAT (u/mg protein)	GSH (µg/mg protein)	GPX (u/mg protein)	MDA (nmol/mg protein)
I	Control	45.74 ± 4.01	51.37± 6.01	38.67 ± 1.07	37.27 ± 3.41	0.18 ± 0.02
II	SL 200	47.07 ± 4.56	52.21 ± 5.03	39.16 ± 3.67	38.43 ± 1.39	0.17 ± 0.04
III	KT 200	19.85 ± 2.03*	20.31 ± 2.35*	11.21 ± 1.59*	11.27 ± 0.56*	0.92 ± 0.03*
IV	SL50 + KT 200	26.31 ± 3.12[a]	31.64 ± 3.07[a]	20.14 ± 0.43[a]	19.53 ± 0.76[a]	0.63 ± 0.09[a]
VI	SL 100 + KT 200	33.13 ± 4.12[b]	40.01 ± 5.31[b]	29.12 ± 2.78[b]	26.56 ± 2.58[b]	0.41 ± 0.06[b]
VI	SL 200 + KT 200	42.67± 4.01[c]	49.65± 6.61[c]	37.71 ± 3.54[c]	35.04 ± 3.18[c]	0.20 ± 0.03[c]

Values are mean ± SEM, n = 5, KT: Ketoconazole, SL: Silymarin, SOD: Superoxide dismutase, CAT: Catalase, GSH: Glutathione, MDA: Malondialdehyde, GPX: Glutathione peroxidase, * $p < 0.001$ Differ significantly when compared to control. [a]$p < 0.05$; [b]$p < 0.01$ and [c]$p < 0.001$ Differ significantly when compared to KT. (ANOVA).

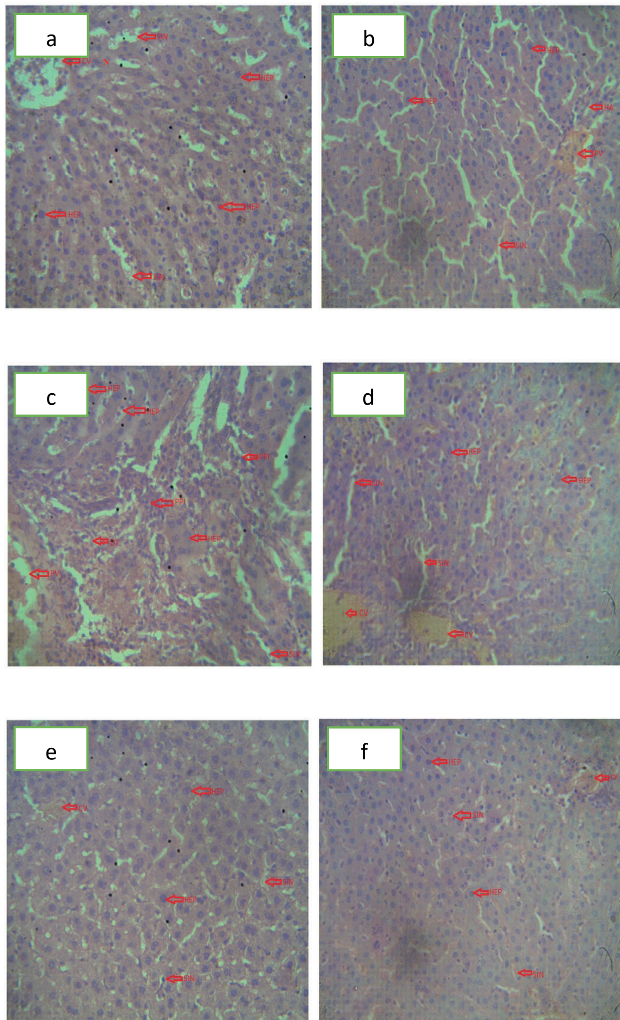

Figure 1: Liver histology of the control (Figure a) and SL (200 mg/kg) administered rats (Figure b). Liver of KT administered rats (Figure c). Liver histology of rats supplemented with SL; 50 mg/kg (Figure d), SL; 100 mg/kg (Figure e) and SL; 200 mg/kg (Figure f). HEP: Normal hepatocytes, CVN: Normal Central vein, PV: Portal vein, NEC: Necrosis, SIN; Sinusoids, HP: Hepatic artery, CV: Congested enteral vein.

indicators of any disease state or response to therapeutic regimen. Conventional indicators of liver function include AST, ALT, ALP, CB, GGT, LDH, and TB.[32,33] Altered levels of the aforementioned markers beyond the acceptable threshold especially in the presence of therapeutic agents is a pointer to an assault on the liver.[34,35] In this study, KT caused remarkable elevations in the serum levels of AST, ALT, ALP, CB, GGT, LDH, and TB. In agreement with our findings, Hamza et al., 2023[8] reported elevated levels of the aforementioned markers in adult rats administered with KT (100 mg/kg/day) for 5 days. Also, Rodriguez and Buckholz, reported similar findings in adult rats administered with KT (40 and 90 mg/kg/day).[36] The observed elevated levels of serum biochemical markers caused by KT may be related to the alterations of the permeability of the liver hepatocyte membrane causing the release of biochemical markers into the blood. In the current study, the assessment of the liver of KT administered rats showed altered liver architecture characterised by hepatocellular necrosis, which is similar to the findings reported by some scholars.[8] However, SL supplementation restored serum biochemical markers in a dose-related fashion. Also, various doses of SL restored liver histology.

Oxidative stress is the imbalance between the excess formation of reactive oxygen species and limited antioxidant defence. A direct consequence of excess reactive oxygen species production occurs due to its interaction with cellular biomolecules, such as proteins, DNA, and lipids causing structural alterations in the aforementioned biomolecules leading to cellular damage or death.[37] Oxidative stress has been considered as a primary conjoint pathological mechanism which detrimentally contributes to the initiation and progression of liver injury.[38] During liver injury, oxidative stress has been shown to cause notable depletions of liver antioxidant defence mechanism such as SOD, CAT, GPX and GSH. In the liver, SOD and CAT protect the cells from free radicals including superoxide radicals and hydrogen peroxide,[39] GSH, a thiols antioxidant detoxifies toxic compounds and heavy metals[39] whereas GPx reduces hydrogen peroxide and soluble lipid hydroperoxides.[40] The administration of KT caused oxidative stress marked by low liver levels of liver antioxidants (GSH, GPX, CAT and SOD). The observation is consistent with the induction of oxidative stress in the liver of adult rats administered with KT (100 mg/kg/day) for 5 days.[8] More so, in this study, KT caused elevation in the liver MDA level of rats. The observation indicates lipid peroxidation (LPO) caused by KT through the breakdown of liver poly unsaturated fatty acids which is consistent with previous findings.[8] LPO can disrupt membranes and produce reactive metabolites that can cause cellular dysfunction. LPO and its products can stimulate hepatic stellar cells and proinflammatory processes that can cause cell necrosis and apoptosis.[41] But this study found that SL supplementation inhibits KT-induced oxidative stress by restoring the liver levels of antioxidants and MDA in a dose-related manner. The observed hepatocellular necrosis caused by KT may be a consequence of KT-induced LPO, bimolecular damage and DNA fragmentation through oxidative stress. Despite the fact that the findings in this study showed that oxidative may be involved in KT-induced hepatotoxicity, immune-mediated mechanism was additionally suggested by some scholars.[8, 42]

In this study, perhaps SL prevented KT-induced hepatotoxicity by inhibiting oxidative stress. Studies showed that SL prevents oxidative stress by the inhibition of reactive oxygen species producing enzymes, scavenging of free radicals, antioxidant enzyme activation and the synthesis of protective molecules. Also, in drug/toxin-related hepatotoxicity, SL can protect against liver damage by preventing membrane permeability, chelation of intestinal ions and the inhibition of toxins at specific binding sites. This prevents the absorption and transportation of

harmful substances, especially in the hepatic phalloidin-transporting system.[14] Furthermore, the radical scavenging activity of SL can enhance hepatic lipid homeostasis by decreasing de novo lipogenesis through the down-regulation of acetyl-CoA carboxylase and peroxisome proliferator-activated receptor fatty acid synthase.[13] Due to the speculated involvement of immune mediated mechanism in KT associated hepatotoxicity as earlier mentioned, SL has immunomodulatory affects through the suppression of inflammasomes, TNF-α and the NF-κB signaling pathways.[14, 43]

Conclusion

SL inhibits KT-induced alterations in serum biochemical markers, liver oxidative stress markers and histology. This shows that SL may have clinical benefit against KT-related hepatotoxicity.

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