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Exploring the protective effect of silymarin against flutamide-induced hepatotoxicity in male rats

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Abstract

The current study was designed to explore the protective impact of silymarin (SIL) against the hepatotoxicity induced by daily treatment with flutamide (FLU), for one month, in intact male rats. Two different doses of FLU, one was low and corresponds to the human therapeutic dose (10 mg/kg) and the other was high and corresponds to ½ lowest lethal dose (500 mg/kg) were applied. Obtained results showed that oral administration of FLU in male rats significantly increased hepatic malonic dialdehyde (MDA), and markedly decreased hepatic reduced glutathione (GSH), superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione-s-transeferase (GST). In parallel, activities of alanine aminotranseferase (ALT), aspartate aminotranseferase (AST) and alkaline phosphatase (ALP), and level of total bilirubin in serum were significantly elevated; while serum total proteins, albumin and globulin, and hepatic total proteins were decreased in FLU-treated animals. Also, significant increases in serum and liver contents of total lipids (TL), total cholesterol (TC) and triglycerides (TG), and serum low density lipoproteins-cholesterol (LDL-c), which accompanied with decrease in serum level of high density lipoproteins-cholesterol (HDL-c) following administration of FLU. The adverse effects of FLU on measured parameters appeared to be dose-dependent. Treatment with SIL (50 mg/kg) in rats given FLU (10 mg or 500 mg/kg) greatly improved the levels of investigated biomarkers of liver status. However, the beneficial effect of SIL was more remarkable in rats receiving FLU at a dose of 10 mg/kg. Obtained biochemical changes induced by the two used doses of FLU and the ameliorative impact of SIL were confirmed by histopathological examinations of liver sections of treated rats. In conclusion, SIL can be used effectively in the protection against hepatotoxicity induced by FLU.

Keywords: Flutamide; silymarin; hepatotoxicity; lipid peroxidation; antioxidants hepatic markers

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Introduction

Flutamide (FLU) is a nonsteroidal antiandrogen drug which used in the treatment of prostatic cancer, and acne and hirsutism induced by female hyperandrogenism (Castelo-Branco and Del Pino, 2009). However, the therapeutic activity of FLU is compromised because of its potential liver toxicity (Wysowski and Fourcroy 1996). In clinical studies, FLU treatment caused severe hepatotoxicity in human

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patients which involved massive hepatic necrosis with fatal outcomes, cholestatic jaundice, hepatic necrosis and hepatic encephalopathy (Moghett et al., 1995; Crownover et al., 1996). Gomez et al. (1992) reported liver toxicity in prostate cancer patients treated with FLU which was manifested by increased levels of transaminases, and total bilirubin in serum corresponding to histopathological findings of a mixed pattern of cytotoxic and cholestatic changes. On the other hand, in young females treated with FLU against various hyperandrogenic conditions (acne and hirsutism), acute liver failure with laboratory data showed elevation of serum transaminases and bilirubin, and urgent liver transplantation were documented (Brahm et al., 2011).

In experimental animal studies, treatment of rats with FLU produced a highly significant increase in serum levels of liver enzymes and total bilirubin, the markers of hepatocellular injury (Mannaa et al., 2005). The drug also caused disturbance in the liver synthetic function as evidenced by decreased serum albumin and elevated lipid profile levels (Tavakkoli et al., 2011; Lateef et al., 2012). However, oxidative tissue damage was characterized by increased production of toxic product and decreased activity of antioxidant defence system was largely implicated in the mechanisms of FLU-induced hepatotoxicity (Leclerc et al., 2014; Teppner et al., 2016).

Available histopathological observations showed that treatment with FLU in male rats resulted in death of hepatocytes, increased eosinophilia of hepatocytes, which reflects cell injury, and highly congested central vein, portal vein and hepatic artery with blood (Sharaf et al., 2010). In clinical studies, therapy with FLU against prostate cancer in men caused cell destruction and induction of immunological reaction that would determine necrosis and cholestasis (Moller et al., 1990). Also, FLU treatment in prostate cancer patients and young females with hyperandrogenism caused acute hepatitis with massive necrosis, mononuclear inflammatory infiltrate and proliferation of small bile duct (Brahm et al., 2011). Moreover, a liver biopsy from FLU-treated patients showed intrahepatic cholestasis suggesting drug induced liver injury (Lübbert et al., 2004).

Phytochemicals including flavonoids are biologically active antioxidants that possess various protective and disease preventive actions (Karimi et al., 2005). Many populations rely on medicinal phytochemicals because they are easily available at an inexpensive price and their phototherapeutic properties in treating variety of diseases (Bellakhdar et al., 1991). Among phytochemicals, a mixture of flavonoids, extracted from the seeds of the herb *Silybum marianum* (milk thistle) has long been introduced as a dietary supplement for hepatoprotection, mainly, due to its antioxidant activity (Karimi et al., 2011). *Silybum marianum* (SIL) possesses several clinical properties such as immunomodulatory, antiproliferative, antifibrotic and antiviral activities (Saller et al., 2001).

In animal model studies, SIL was found to protect the rat liver against injury induced by various drugs and toxins such as CCl_4 and acetaminophen (Singh et al., 2012). It can protect liver against oxidative tissue injury that leads to adverse biochemical and histological alterations because of its ability, as antioxidant, to scavenge free radicals and hence causes decrease in lipid peroxidation (LPO) and increase in antioxidant capacity (Das and Vasudevan, 2006; Kaur et al., 2007), that ultimately lead to stabilization of plasma membranes (El- Maddawy and Gad, 2012; Zhang et al., 2013). Silibinin, the most biologically active component of SIL, has also been shown to be safe in animal models and no significant adverse reactions were reported in human studies (Hogan et al., 2007).

In view of above information, the present study was designed to explore, for the first time, the role of SIL as a potent antioxidant in the protection against FLU-induced hepatotoxicity in adult male rats at biochemical and histopathological levels. Two different doses of FLU, one was low (10 mg/kg) (Goto et al., 2005) and corresponds to the human therapeutic dose, and the other was high (500 mg/kg) and corresponds to ½ lowest lethal dose (½ LDL_O) (NIOSH 1987 and Martelli et al., 2000) were used. High dose was chosen in order to detect clearly the toxic effect of FLU on the liver. Investigated serum and hepatic parameters were selected to assess the adverse effects of FLU on oxidative stress and liver function. Histopathological changes in the liver tissue following treatment with FLU were also examined.

Materials and Methods

Drugs and preparation of used doses

Androxin tablets containing FLU (250 mg/tablet) and Legalon capsules containing SIL (150 mg/capsule) were purchased from local pharmacy in El-Mansoura city. Androxin tablets were produced by Egyptian Sigma Pharmaceutical Industries (SAE) Company, Egypt. While, Legalon capsules were manufactured by Chemical Industries Development (CID) Company, Giza, Egypt; under licence of Madaus GmbH, Germany. FLU tablets (250 mg/tablet) were grind until it became powder and mixed well with distilled water (w/v), and the two selective doses of FLU (10 mg or 500 mg/kg) were determined and given to the rats. In case of SIL (150 mg/capsule), the content of the capsules was also mixed well with distilled water (w/v), and the selective dose of SIL (50 mg/kg) was determined and introduced to the rats. The prepared suspension of FLU or SIL was shaken well before it was given to rats.

Experimental animals design

Adult male albino rats (*Rattus rattus*), weighing 130-150 g were obtained from Helwan Animal Farm, Cairo, Egypt, and maintained under controlled humidity, temperature and photoperiod (12 h light / 12 h dark). They were fed commercial rodent pellet diet, and tap water *ad libitum*. Care and use of the animals were conducted under supervision of the Animal Ethics Committee of Mansoura University, Egypt. After one week of adaptation, rats were randomly divided into six groups of six animals each as follows: control group received no chemical treatments; SIL-treated group received SIL in a dose of 50 mg/kg b.wt.; FLU (low dose) treated group received FLU in a dose of 10 mg/kg b.wt.; FLU (high dose) treated group received FLU in a dose of 500 mg/kg b.wt.; FLU (low dose) plus SIL-treated group received FLU (10 mg/kg b.wt.) and SIL (50 mg/kg b.wt.); and FLU (high dose) plus SIL-treated group received FLU (500 mg/kg b.wt.) and SIL (50 mg/kg b.wt.). The drugs were orally introduced to used rats using gastric tube, and the treatment was continued daily for one month.

Blood and tissue sampling

At the end of the experimental period, overnight fasted rats were anesthetized by diethyl ether, and the blood was collected from the jugular vein in non-heparinized tubes. The tubes, then, were centrifuged at 3000 rpm for 15 minutes and the blood sera were separated, labelled and kept at -20°C till biochemical analysis. On the other hand, the animals were immediately dissected and a portion of the liver of each rat was quickly separated, cleaned, weighed and homogenized in cold distilled water at 4°C forming 10% homogenate (1:9, w/v). The homogenates then were centrifuged for 10 minutes at 3000 rpm and the supernatants were kept at -20°C until biochemical investigations. For routine histological examination, portions of fresh tissue of the liver were removed from the dissected animals and fixed with 10% neutral formalin.

Biochemical analysis

The concentration of hepatic malonic dialdehyde (MDA) was measured following the method of Ohkawa et al. (1979), using kit from Biodiagnostic Company, Egypt. Hepatic content of reduced glutathione (GSH) was determined using the procedure of Prins and Loose (1969). Activities of antioxidant enzymes superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione-s-transeferase (GST) in the liver were estimated by the methods of Nishikimi et al. (1972), Bock et al. (1980), Paglia and Valentine (1967), Habig et al. (1974) respectively, using kits supplied by Bio-diagnostic Company, Cairo, Egypt. Activities of alanine aminotranseferase (ALT), aspartate aminotranseferase (AST), alkaline phosphatase (ALP) and total bilirubin in serum were determined according to methods described in Young (2001), using Spinreact diagnostics kit, Spain. Serum and liver total protein content was estimated using kit from Biodiagnostic Company, Egypt according to the method of Henry (1964). Serum albumin concentration was estimated by the method of Doumas (1971), using kit from Diamond Company, Egypt. Globulins concentration in the serum was calculated by subtracting the concentration of albumin from the total protein content. Serum and hepatic contents of total lipids (TL), total cholesterol (TC) and triglycerides (TG) were determined according to Zollner and Kirsch (1962), Young (1995), Fossati and Prencipe (1982), respectively using kit of Biodiagnostic Company, Egypt. The concentration of serum high density cholesterol (HDL-c) was measured by Grove (1979) method using Spinreact diagnostic kit, Spain. Serum low density cholesterol (LDL-c) was calculated from TG, TC and HDL-C concentrations using equation of Friedewald et al. (1972).

Serum LDL-C concentration = TC-HDL-C-TG/5 = mg/dl

Histopathological examinations

Fixed liver slices in 10% neutral formalin were dehydrated through the ascending series of ethyl alcohol, cleared in xylene and embedded in paraffin wax. Transverse sections of liver were cut at thickness of 5 µm and stained with Harris's alum hematoxyline according to Drury et al. (1976), and counter stained with eosin (H&E). The stained sections were then examined under electric microscope to detect the histopathological changes in different treated animal groups.

Statistical analysis

All the grouped data were statistically evaluated with SPSS 17.5 software. Differences among groups were evaluated by one-way analysis of variance (ANOVA) followed by Duncan's multiple range tests. *P* value equal or less than 0.05 was considered significant. All values were expressed as the mean ± SE of six animals per group. Percentage of changes in the treated groups were calculated.

Results

Obtained results in Table 1 show that administration of FLU (10 mg/kg and 500 mg/kg) to male rats produced significant increase in the content of MDA and marked decrease in the levels of antioxidant parameters (GSH, SOD, CAT, GPx and GST), when compared to control results. However, co-treatment of

FLU groups (10 mg/kg and 500 mg/kg) with SIL (50 mg/kg) significantly attenuated the elevated content of MDA and the lowered levels of antioxidants in the liver, in comparison with FLU-treated groups.

In Table 2, oral treatment of male rats with FLU (10 mg/kg and 500 mg/kg) caused marked increases in activities of enzymes ALT, AST and ALP; and levels of total bilirubin in the serum, compared to control group. On contrary, serum concentrations of total proteins, albumin and globulins and hepatic content of total proteins were significantly decreased following FLU administration, when compared to control results. However, combined treatment with FLU plus SIL showed remarkable reduction in the adverse effects of FLU on mentioned biomarkers of liver status, when compared to FLU-treated groups, suggesting hepatoprotective effect of SIL.

Obtained results in Table 3 showed marked elevation in the levels of TL, TC, TG in both serum and liver and in serum value of LDL-c following oral administration of FLU (10 mg/kg and 500 mg/kg), when compared to control group. In contrast, the same treatments of FLU lowered the concentration of serum HDL-c in treated rats. Oral administration of SIL (50 mg/kg) in combination with FLU at low and high doses to rats significantly lowered raised levels of lipid profile in both serum and liver, while it increased the serum concentration of HDL-c as compared to groups treated with FLU alone. As shown in Tables 1, 2 and 3, FLU-induced detrimental changes in all measured parameters appeared to be dose-dependent. However, treatment with SIL alone had no significant changes in the measured parameters, indicating no toxic effects of SIL at the level of applied dose.

Table 1: Effect of silymarin on flutamide-induced oxidative stress in the liver of male rats

Parameters	Control	SIL	FLU (10 mg)	FLU (10 mg)+ SIL	FLU (500 mg)	FLU (500 mg) + SIL
MDA (nmol/g)	53.17±2.34	49.83±2.37 (-6.28)	117.17±1.97^{a} (+120.37)	67.33±1.80^{ab} (+26.63)	168.17±2.44^{a} (+216.29)	119.17±3.06^{ac} (+124.13)
GSH (mg/g)	8.73±0.12	8.59±0.15 (-1.60)	4.45±0.13^{a} (-49.03)	7.80±0.14 ^{ab} (-10.65)	3.22±0.11^{a} (-63.12)	4.94±0.07^{ac} (-43.41)
SOD (U/g)	56.00±1.73	58.50±1.77 (+4.46)	28.00±1.44^{a} (-50.00)	49.17±1.17^{ab} (-12.20)	23.33±2.20^{a} (-58.34)	38.83±1.35^{ac} (-30.66)
CAT (U/g)	77.00±2.11	76.17±3.20 (-1.08)	40.00±1.32^{a} (-48.05)	68.50±1.09^{ab} (-11.04)	33.83±1.51^{a} (-56.06)	54.83±1.47^{ac} (-28.79)
GPx (U/g)	41.50±1.84	42.17±1.76 (+1.61)	28.67±0.56^{a} (-30.92)	38.50±0.67^{ab} (-7.23)	19.67±2.17^{a} (-52.60)	26.17±1.30^{ac} (-36.94)
GST (U/g)	0.63±0.01	0.63±0.01 (+0.00)	0.45±0.01^{a} (-28.57)	0.57±0.01^{ab} (-9.52)	0.35±0.01^{a} (-44.44)	0.41±0.02^{ac} (-34.92)

FLU = Flutamide, SIL = Silymarin; **a, b** and **c** = Significant difference at $P \leq 0.05$ comparing to control, FLU (10 mg) and FLU (500 mg) groups respectively. Values expressed as mean ± SE from 6 rats in each group, and % of changes from control are given in parentheses.

Table 2: Effect of silymarin on flutamide-induced changes in the liver function markers in male rats

Parameters	Control	SIL	FLU (10 mg)	FLU (10 mg)+SIL	FLU (500 mg)	FLU (500 mg)+SIL
Serum						
ALT (U/L)	26.83±0.28	25.83±0.33 (-3.72)	58.00±0.70^{a} (+116.17)	27.83±0.77^{b} (+3.72)	70.00±1.06^{a} (+160.90)	42.66±0.84^{ac} (+59.00)
AST (U/L)	37.83±0.66	39.16±0.82 (+3.51)	73.16±0.31^{a} (+93.39)	38.16±1.13^{b} (+0.87)	87.33±1.03^{a} (+130.84)	58.16±1.08^{ac} (+53.74)
ALP (U/L)	75.16±3.12	76.16±3.25 (+1.33)	140.33±4.33^{a} (+86.70)	77.00±3.53^{b} (+2.44)	195.16±7.22^{a} (+159.65)	138.66±4.06^{ac} (+84.48)
TB (mg/dl)	0.58±0.02	0.62±0.03 (+6.90)	0.82±0.03^{a} (+41.38)	0.62±0.02^{b} (+6.90)	0.91±0.03^{a} (+56.90)	0.75±0.03^{ac} (+29.31)
TP (mg/dl)	6.52±0.22	6.58±0.20 (+0.92)	4.52±0.11^{a} (-30.67)	6.45±0.11^{b} (-1.07)	3.50±0.14^{a} (-46.32)	4.82±0.08^{ac} (-26.07)
Albumin (mg/dl)	3.48±0.08	3.33±0.19 (-4.31)	2.58±0.21^{a} (-25.86)	3.20±0.14^{b} (-8.05)	2.40±0.13^{a} (-31.03)	2.95±0.07^{ac} (-15.23)
Globulin (mg/dl)	3.03±0.24	3.25±0.25 (+7.26)	1.94±0.25^{a} (-35.97)	3.25±0.24^{b} (+7.26)	1.10±0.18^{a} (-63.70)	1.87±0.09^{ac} (-38.28)
Liver						
TP (mg/g)	7.03±0.16	7.23±0.18 (+2.84)	4.37± 0.13^{a} (-37.84)	6.72±0.17^{ab} (-4.41)	3.60±0.22^{a} (-48.79)	5.52±0.13^{ac} (-21.48)

FLU = Flutamide, SIL = Silymarin, TB = Total bilirubin, TP = Total proteins; **a, b** and **c** = Significant difference at $P \leq 0.05$ comparing to control, FLU (10 mg) and FLU (500 mg) groups respectively. Values expressed as mean ± SE from 6 rats in each group, and % of changes from control are given in parentheses.

Table 3: Effect of silymarin on flutamide-induced changes in lipid profile in both serum and liver of male rats

Parameters	Control	SIL	FLU(10 mg)	FLU (10 mg)+SIL	FLU(500 mg)	FLU (500 mg)+SIL
Serum						
TL (mg/dl)	401.00±6.19	398.33±6.47 (-0.42)	537.83±12.56[a] +34.46	413.83±5.61[b] (+3.46)	703.67±30.60[a] (+75.92)	585.67±35.04[ac] (+46.05)
TC (mg/dl)	119.50±3.13	101.83±3.08[a] (-14.79)	183.17±2.09[a] +53.28	116.83±4.30[b] (-2.23)	235.00±3.77[a] (+96.[65])	169.33±4.39[ac] (+41.70)
TG (mg/dl)	105.83±2.14	107.67±2.22 (+1.74)	180.83±4.44[a] (+70.87)	116.67±5.97[b] (+10.24)	223.17±3.13[a] (+110.88)	165.83±4.57[ac] (+56.69)
LDL-C (mg/dl)	14.83±1.25	18.29±1.65 (+23.33)	89.66±3.69[a] (+504.58)	21.66±2.08[ab] (+46.05)	157.03±6.45[a] (+958.86)	75.16±4.73[ac] (+406.81)
HDL-C (mg/dl)	83.50±3.24	82.00±1.83 (-1.80)	47.33±1.58[a] (-43.31)	71.83±3.22[ab] (-13.98)	34.83±3.94[a] (-58.29)	46.00±2.34[ac] (-44.91)
Liver						
TL (mg/gm)	393.00±1.53	387.83±2.06 (-1.32)	653.17±3.37[a] (+43.30)	414.00±4.33[ab] (+5.34)	701.33±2.95[a] (+78.46)	531.00±14.62[ac] (+34.84)
TC (mg/gm)	176.17±5.50	172.83±5.75 (-1.90)	229.00±1.93[a] (+29.98)	191.33±2.39[ab] (+8.60)	269.83±7.90[a] (+53.16)	225.50±3.79[ac] (+28.00)
TG (mg/gm)	92.33±4.10	93.00±4.57 (+0.72)	129.67±4.10[a] (+40.44)	104.00±1.44[ab] (+12.63)	190.17±2.17[a] (+105.78)	154.50±0.76[ac] (+67.33)

FLU = Flutamide, SIL = Silymarin; **a, b** and **c** = Significant difference at P≤0.05 comparing to control, FLU (10 mg) and FLU (500 mg) groups respectively. Values expressed as mean ± SE from 6 rats in each group, and % of changes from control are given in parentheses.

Histopathological examinations of liver sections stained by H&E in both control and SIL-treated rats showed normal liver architecture (Fig. 1-A and B). However, treatment of rats with FLU (10 mg/kg) produced few lymphocytic infiltration and cytoplasmic vacuolation in the liver (Fig. 1-C). Administration of FLU at a dose level of 500 mg/kg exhibited highly inflammatory and degenerative changes since the results revealed more lymphocytic infiltration, pyknotic nuclei, necrotic area, congested blood vessel and dilated blood sinusoid (Fig. 1-E). Interestingly, combined treatment with SIL and FLU (10 mg or 500 mg/kg) partially ameliorated the histopathological alterations induced by each applied dose of FLU (Fig. 1-D and 1-F, respectively).

Discussion

The liver is a principal site of metabolism of almost all drugs and foreign substances. So, incidence of liver injury is a potential complication which could be expected with the use of various types of the drugs (Asgarshirazi et al., 2015). Flutamide is a non-steroidal antiandrogen drug which used primarily in the treatment of prostate cancer. Despite its therapeutic efficacy, it belongs to the drug group which is expected to induce idiosyncratic (non-predictable) hepatotoxicity (Al Maruf and O'Brien, 2014; Cetin et al., 1999). For hepato-protection against drug toxicity, several herbal compounds have been introduced as a complementary medicine. Among these herbal compounds, SIL, the main phytochemicals products of milk thistle (*Silybum marianum*), has been recognized as a safe and well-tolerated herb product with no or minimal side effects. Its protective efficacy against oxidative stress in various organs via its antioxidant properties raised the hope of its clinical impact against various types of hepatotoxicity (Loguercio and Festi, 2011). Present study was therefore planned to investigate the potential protective impact of SIL against hepatotoxicity induced by FLU at two different dose levels in male rats.

The results of the present study showed that oral administration of FLU (10 mg or 500 mg/kg) to adult male rats daily for one month caused significant elevation in the hepatic content of MDA accompanied with decreased hepatic antioxidant parameters including GSH, SOD, CAT, GST and GPx in a dose dependent manner. Increased MDA level indicated destruction of lipid macromolecules included in the biological membranes which led to structural and functional alterations of hepatic cells, that ultimately caused hepatotoxicity (Xie et al., 2014). Present finding is consistent with published *in vitro* and *in vivo* studies which demonstrated increase in LPO products in hepatocytes and plasma of rats after treatment with FLU (Al Maruf and O'Brien, 2014; Teppner et al., 2016). Also, treatment of rat primary hepatocytes with FLU resulted in a high reactive oxygen species (ROS) production and complete GSH depletion (Leclerc et al., 2014). Similarly, induction of LPO, as indicated by markedly increased serum levels of MDA, which was associated with a marked decline in serum GSH level had been reported following FLU treatment in rabbits (Ray et al., 2010). Moreover, current results derived support from the study of Mannaa et al. (2005) who reported that administration of FLU (100 mg/kg) to adult male rats for 15 days resulted in a highly significant decrease in the hepatic activities of SOD and GPx.

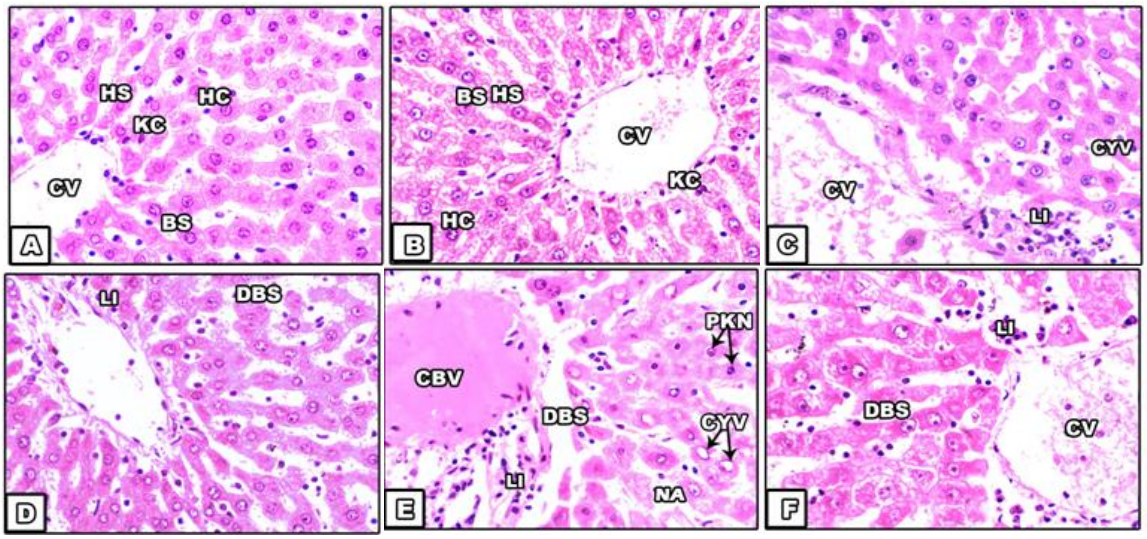

Fig. 1: Histopathological changes in liver sections of different groups. A: control, B: silymarin, C: flutamide (10mg/kg), D: flutamide (10mg/kg) plus silymarin, E: flutamide (500mg/kg), F: flutamide (500mg/kg) plus silymarin. HC: hepatic cell, KC: Kupffer cell, HS: hepatic strand, BS: blood sinusoid, CV: central vein, LI: lymphocytic infiltration, CYV: cytoplasmic vacuolation, PKN: pyknotic nuclei, CBV: congested blood vessel, DBS: dilated blood sinusoid, NA: necrotic area.

The exact mechanisms of FLU-induced hepatocyte injury remain unclear. However, formation of reactive metabolites and production of ROS were implicated in the pathogenesis of FLU-generated LPO and hepatocellular injury. Previous studies showed that FLU was found to induce a cytochrome P450 1A2 (CYP1A2), which involved in the bioactivation of the drug itself (Kang et al., 2008), resulting in the generation of ROS. Published data reported that FLU bioactivation using CYP1A2 generated a nitro anion free radical (Vazquez et al., 2004). These radicals can bind covalently to proteins and lipids, or remove hydrogen atoms from polyunsaturated fatty acids in lipids, leading to induction of LPO and subsequently hepatocyte injury (Williams and Burke, 2000; Teppner et al., 2016).

Moreover, the inhibitory effect of FLU on the hepatic antioxidant defence system, including GSH, SOD, CAT, GST and GPx, which protect the hepatic cells against damage induced by reactive metabolites of FLU and free radicals might also be implicated in the pathogenesis of FLU-induced oxidative stress and hepatotoxicity (Macpherson, 1994). It has been reported that GSH is an important antioxidant and plays a very important role in the defence mechanism for tissue against the ROS (Kosower and Kosower, 1976). The depletion of GSH is therefore associated with an increase in oxidative stress and/or detoxification (Snouber et al., 2013). Furthermore, SOD together with GPx and CAT form an important part of the cellular antioxidant defence system against damage caused by free radicals and hydroperoxides or lipoperoxides (Macpherson, 1994). SOD is the first line of antioxidant against ROS. It catalyzes the dismutation of $O_2^{\bullet-}$ to form H_2O_2 (Yu, 1994). The latter (H_2O_2) is then converted by CAT into H_2O and O_2 (Valko et al., 2006). GPx catalyzes the reduction of H_2O_2 and organic hydroperoxides with simultaneously oxidizing GSH into glutathione disulfide (GSSG). Thus, the reduction in hepatic activity of these antioxidative enzymes during treatment with FLU reflects oxidative stress and enhanced production of free radicals and ROS.

Mitochondrial damage and dysfunction during treatment with FLU has been documented and considered to be a risk factor which implicated in the mechanisms of FLU-induced cytotoxicity (Kashimshetty et al., 2009). Available data indicated marked inhibition of both mitochondrial respiration and adenosine-5-triphosphate formation (Fau et al., 1994), and increased superoxide anions, the marker of oxidative stress, following exposure to FLU (Kashimshetty et al., 2009), and this could contribute to aggravation of FLU cytotoxicity. In the present study, observed central vein congestion and stagnant blood flow can lead to tissue hypoxia that affects mitochondrial respiration which was reported in FLU hepatotoxicity (Scatena et al., 2007).

Hepatocyte integrity is often reflected by measurement of the activity of serum liver enzymes, particularly transaminases. Beside this, evaluation of serum level of ALP and bilirubin could provide evidence of bile duct injury where ALP is localized in the liver on the surface of bile duct epithelia (Moss, 1997; Giannini et al., 2005). In this context, present

study exhibited dose dependent marked elevations in serum levels of ALT, AST, ALP and total bilirubin in rats treated with FLU (10 or 500 mg/kg), when compared to control group, suggesting incidence of hepatocellular injury. These findings are compatible with data obtained by Mannaa et al. (2005) who found highly significant increase in the serum levels of ALT, ALP and bilirubin, in adult male rats treated with FLU. In human, several clinical studies on prostate cancer patients received FLU showed different patterns of hepatic injury characterized by induction of an immunologic reaction to necrosis and cholestasis, frequently associated with marked elevation in serum AST, ALT, ALT and total bilirubin (Gomez et al., 1992; Nakano et al., 2015).

On the other hand, a change in serum metabolic parameters such as total proteins and albumin levels could be associated with disturbance in the liver function. Obtained data of the present study displayed significantly decreased serum levels of total proteins, albumin and globulins, in addition to hepatic content of total proteins, in rats treated with FLU (10 or 500 mg/kg), in a dose- dependent manner. This result revealed that FLU has an inhibitory effect on the synthetic function of the liver. Available data in this concern are limited. In *in vitro* studies, Snouber et al. (2013) investigated the metabolic response of HepG2/C3a cells exposed to FLU and they found a reduction in the amino acid metabolism which was correlated with a reduction in the albumin production, suggesting an inhibitory effect of FLU on hepatic proteins synthesis. In clinical studies, FLU therapy in patients with prostatic adenocarcinoma also caused hypoalbuminemia in the serum (Tavakkoli et al., 2011).

Elevated serum levels of ALT, AST, ALP and total bilirubin, in addition to decreased serum concentration of total proteins, albumin and globulins could provide evidence that FLU induced hepatocellular injury and liver dysfunction. The mechanisms beyond this appeared to be involved production of active drug metabolites and free radicals which cause oxidative damage of macromolecules such membrane lipids, proteins and DNA. Furthermore, FLU-induced hepatic cell injury and dysfunction could be aggravated by decreased antioxidant activities such as GSH, SOD, CAT and GPx, which act as a free radical scavengers.

Many aspects of lipid metabolism are carried out predominantly by the liver. Most apolipoproteins, endogeneous lipids, and lipoproteins are synthesized in the liver, which depend on the cellular integrity and functions (Han et al., 2013). In the present study, treatment of male rats with FLU (10 mg or 500 mg/kg) exhibited significant increases in both serum and liver TL, TC and TG in addition to serum LDL-C levels, in a dose dependent manner, when compared to the control group. In contrast, a significant decrease in serum HDL-c level was seen in the same groups of FLU, compared to the control one. Such alterations in lipid profile following treatment with FLU are in agreement with several published studies (Smith et al., 2006; Ray et al., 2010; Lateef et al., 2012). For example, Lateef et al. (2012) recorded significant increase in the serum levels of TC, TG and LDL-C, accompanied with marked decrease in serum level of HDL-c in rats administered FLU (30 mg/kg/day, for 60 days).

The mechanisms beyond the disturbances in lipid profile by FLU are not fully understood. However, it has been known that under normal physiological conditions, the liver ensures homeostasis of lipid and lipoprotein metabolism. A bulk of the lipoproteins and large quantities of cholesterol and phospholipids are synthesized in the liver (Bertolotti et al., 1995; Nguyen et al., 2008). Thus any agent causes hepatocellular injury could disturb the synthetic and regulatory functions of liver toward various lipid components. In the present study, FLU-induced oxidative stress which resulted in hepatocellular damage and liver dysfunction could largely contribute to the mechanisms underlying the ability of this drug to adversely affect synthesis and metabolism of lipids in rats.

Another possible mechanism of FLU-induced adverse effects on lipid metabolism in rats could be explained on basis of the ability of FLU to induce androgen deprivation. Available data in this regard demonstrated that the levels of serum testosterone are inversely correlated with TC and LDL-c levels (Zhang et al., 2014). Also, animal studies have displayed marked increase in serum TC levels in testosterone-deficient male mice (Hatch et al., 2012). In addition, testosterone depletion led to increase in TC, TG and LDL-c levels in animals (Cai et al., 2015). Interestingly, present study displayed dose dependent decrease in the serum testosterone level (data not shown) accompanied with markedly enhanced concentrations of lipid profile (TC, TG and LDL-c) in FLU-treated rats. Thus, it seems likely that FLU-inhibited testosterone production and/or antagonized its androgenic action might partially contribute to observed disturbances in lipid metabolism.

FLU-induced hepatic oxidative stress accompanied with liver injury and dysfunction was confirmed in the present study by histopathological observations of liver sections stained by H&E. Microscopic examinations showed that treatment with FLU in a dose of 10 mg/kg produced mild inflammatory reaction and accumulation of fats in few hepatic cells. While, administration of high dose of FLU (500 mg/kg) in rats exhibited highly inflammatory reactions and degenerative changes, as the results revealed more lymphocytic infiltration, pyknotic nuclei and necrotic area, in addition to congested blood vessel and dilated blood sinusoids. These histopathological findings are in agreement with previously published data showing that treatment with

FLU in male rats resulted in death of hepatocytes and highly congested central vein, portal vein and hepatic artery, with blood (Sharaf et al., 2010). Also, cell destruction and induction of immunological reaction that would determine necrosis and cholestasis were observed during therapy with FLU against prostate cancer in men (Moller et al., 1990). Additionally, FLU treatment in prostate cancer patients and young females with hyperandrogenism caused acute hepatitis with massive necrosis, mononuclear inflammatory infiltrate and proliferation of small bile duct (Brahm et al., 2011). Moreover, treatment with FLU in patient with prostatic carcinoma was associated with hepatotoxicity which was characterized by mononuclear cells infiltration, centrilobular cholestasis and swollen hepatocytes with fatty changes (Hung et al., 2007).

Because of the FLU adverse side effects, the strategies of prostate cancer treatment using combined therapy that frequently involves natural products of antioxidative properties are considered more promising. SIL, a flavonoid compound, derived from the plant *Silybum marianum*, has been used effectively to treat liver disorders associated with alcohol consumption, acute and chronic viral hepatitis, and toxin-induced hepatic failures (Muriel and Mourelle, 1990; Thakur, 2002; Rambaldi et al., 2005). A large part from its hepatoprotective nature is due to its antioxidative and tissue regenerative properties. It can support the major line of cellular antioxidant defence system which includes GSH and SOD by scavenging free radicals and ROS (Fraschini et al., 2002). In the present study, combined treatment with SIL (50 mg/kg) and FLU (10 mg and 500 mg/kg) significantly decreased production of MDA and markedly increased activity of the antioxidant parameters including GSH, SOD, CAT, GPx and GST in the liver tissue as compared to FLU-treated groups. These results provide evidence that SIL can protect liver against FLU-induced oxidative stress and LPO.

Yet, there are no published data regarding the protective impact of SIL against FLU-induced hepatotoxicity. However, several published studies demonstrated the efficacy of SIL in the protection against hepatic injury induced by various diseases or exposure to other drugs and toxins. Of published data, co-administration of SIL to rats treated with either ethanol or CCl_4 significantly lowered the elevated MDA level, and markedly increased the reduced levels of GSH, SOD, CAT and GPx in the hepatic tissue (El-Maddawy and Gad, 2012; Zhang et al., 2013). Also, combined treatment with SIL and paracetamol-induced hepatotoxicity significantly increased the levels of antioxidant parameters including SOD, CAT and GSH in comparison with paracetamol group (Shelbaya, 2013). In addition, SIL can reduce the free radical load and lipid peroxidation, thus increasing GSH levels and stimulating SOD activity, leading ultimately to the increased membrane stability and organ function (Fraschini et al., 2002). On the other hand, extracts obtained from SIL can be used in chemoprevention against hepatocarcinogenesis caused by hepatitis C virus or chemicals (Mastron et al., 2015). In rats, for example, it protected the hepatocytes against hepatocarcinogenesis induced by N-nitrosodiethylamine (NDEA), as it ameliorated the hepatic markers of oxidative stress (LPO and antioxidants such as GSH, SOD and GPx) induced by this carcinogen (El Mesallamy et al., 2011). In view of above, SIL, due to its ability to protect against FLU hepatotoxicity in addition to its anti-carcinogenic activity, appeared to be a promising complementary therapeutic drug which may act in synergistic fashion with FLU in the treatment of prostatic carcinoma.

In the current study, combined treatment with SIL (50 mg/kg) and FLU (10 mg and 500 mg/kg) in male rats, significantly reduced FLU-elevated activities of ALT, AST, ALP and total bilirubin in the serum. In rats treated with FLU in a dose of 10 mg/kg, co-treatment with SIL can restore the mentioned biochemical parameters to almost the control values. This beneficial effect of SIL on markers of liver injury could be attributed to its ability to improve the oxidant-antioxidant imbalance induced by FLU in the liver, via reducing the LPO and elevating the antioxidant activity.

Available literatures showed lack of evidence regarding the beneficial impact of SIL on the FLU-induced hepatocellular damage. However, SIL effectively protected liver from alcohol-induced injury as evidenced by reducing ALT and AST activities, and total bilirubin level in serum (Wang et al., 1996; Zhang et al., 2013). Also, long-term co-administration of milk thistle extract with paracetamol significantly decreased the elevated level of transaminases and total bilirubin in the serum of rats (Shelbaya, 2013). In addition, SIL treatment exhibited significant hepatoprotection against CCl4-induced hepatotoxicity in the rats, by lowering the elevated serum activity of ALT, AST and ALP induced by the toxin (Salam et al., 2009; El- Maddawy and Gad, 2012). Moreover, extracts from *Silybum marianum* beneficially downregulated the increases in serum ALT, AST and γGT activities induced by the carcinogen NDEA (El Mesallamy et al., 2011). In clinical studies, therapy with SIL in patients with various patterns of liver diseases beneficially modulated the activities of transaminases and levels of total bilirubin in the serum (Feher et al., 1989; Dixit et al., 2007; Hajiani, 2009).

In the present study, treatment with SIL was also found to ameliorate the disturbances in hepatic synthetic function induced by FLU. The results showed that combined treatment of rats with SIL and FLU (10 mg or 500 mg/kg) significantly elevated the hepatic total proteins content and serum concentrations of total

proteins, albumin and globulin, when compared to the groups treated with FLU alone. Importantly, serum concentrations of these metabolic indices in rats treated with SIL plus FLU at 10 mg/kg dose were restored to values close to the control level. SIL-improved hepatic synthetic capability of proteins raised the possibility that SIL could stimulate tissue regeneration. In this context, it has been reported that SIL has the capacity to regulate nuclear expression by means of a steroid-like effect leading to enhancement of tissue regeneration (Saller et al., 2007). The herbal drug acts on RNA polymerase I enzymes and the transcription of ribosomal RNA resulting in increased DNA and protein synthesis (Sonnenbichler and Zetl, 1986; Pandey, 2014). Histochemical observations showed that pretreatment with SIL in rats injected with cisplatin caused restoration of reduced hepatic tissue content of proteins and DNA to normal level (Abouzeinab, 2013). In recent study, treatment with SIL (50 mg/kg) significantly elevated the lowered serum total protein level in thioacetamide-treated rats (Nada et al., 2015). Collectively, SIL showed high therapeutic efficacy not only because it acts either as a scavenger of the free radicals or a stimulator for the antioxidant system, but also because it enhanced the protein synthesis that facilitate antioxidative enzymes manufacture and tissue regeneration.

Importantly, combined treatment with SIL and FLU in male rats ameliorated FLU-induced adverse changes in lipid profile in both serum and liver. The results displayed elevated serum and hepatic levels of TL, TC and TG in rats treated with FLU (10 mg and 500 mg/kg) were reduced in response to concurrent administration of SIL (50 mg/kg) in these animals. However, obtained data from rats treated with both SIL and FLU (10 mg/kg) showed complete restoration of normal serum values of these lipids parameters. Present findings also showed that treatment with SIL in rats administered FLU partially decreased serum levels of LDL-c and increased HDL-c. Present results provided an additional evidence that SIL has a clinical benefit in the treatment of hyperlipidemia and this could encourage the use of SIL as a dietary supplement to improve lipid profiles associated with obesity and drugs therapy. In addition, obtained results also raised the potentiality of use of SIL in the prevention and therapy of hypercholesterolemia and atherosclerosis (Krecman et al., 1998).

There are no available literatures regarding the effect of combined treatment with SIL and FLU on lipid profile. However, several published data which demonstrated the effect of SIL on lipid metabolism under various conditions of hepatotoxicity were reported. Shelbaya (2013) recorded significant decreases in TC, TG, LDL-c and VLDL-c and significant increase in HDL-c in rats received milk thistle plus paracetamol when compared to paracetamol group. Also, in alcoholic fatty liver model of rats, treatment with SIL decreased hepatic content of TG which improved the degree of hepatic steatosis (Zhang et al., 2013). Treatment with silibinin in rats partly antagonized the increase in hepatic production of TC and TG in response to exposure to CCl4 (Pandey, 2014). Recently, long-term treatment of rats fed on high-fat diet (HFD) with SIL significantly lowered the levels of TC, TG and LDL-c in plasma compared with HFD group (Sayin et al., 2016).

Microscopic examinations of liver sections showed that SIL partially recovered the histopathological alterations induced by FLU. The herbal drug (SIL) reduced the inflammatory reaction and accumulation of fats in hepatic cells induced by FLU (10 mg and 500 mg/kg). Moreover, it ameliorated partially the degenerative changes (such as pyknotic nuclei and necrosis), in addition to the congested blood vessel and dilated blood sinusoids in rats treated with high dose of FLU (500 mg/kg). In previous studies, administration of SIL was found to reduce the histological changes in liver of rats treated with doxorubicin (Raskovic et al., 2011). Also, treatment with SIL in male rats treated with paracetamol or CCl_4 caused marked reduction in the liver histopathological changes induced by the drug and toxin, like inflammatory cells infiltration, fatty infiltration, centrilobular necrosis and sinusoidal dilation (Singh et al., 2012).

In view of available information and present findings, it seems likely that SIL could be used in combination with FLU in therapy of prostate cancer. This is because SIL could effectively protect against FLU-induced hepatotoxicity, in addition to its ability to prevent progression of cancer disease. The latter suggestion could derive support from published data which demonstrated the ability of SIL to inhibit cell proliferation of a human prostate cancer cell line by arresting the cell cycle at the G1 phase, and also by inhibiting androgen-stimulated cell proliferation at nuclear androgen receptor levels (Zhu et al., 2001). Furthermore, silybinin, the active component of SIL, was found to inhibit the growth of prostate cancer cells from human, mouse and rat origins (Singh and Agarwal, 2006).

Conclusion

The biochemical and histopathological findings of the present study reported, for the first time, that SIL can afford a hepatoprotective impact against FLU-induced detrimental changes in the markers of oxidative tissue damage and liver function. Thus, combined treatment with FLU plus SIL can offer promising therapeutic efficacy in patients suffering from prostate cancer, because: (1) SIL can protect against FLU-induced hepatotoxicity, and (2) SIL can exert a

synergistic preventive action with FLU against progression of prostate cancer cells.

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