Indian Journal of Experimental Biology
Vol. 56, March 2018, pp. 158-163

Protective effects of silymarin against isotretinoin induced liver and kidney injury in mice

Meltem Kumaş[1]*, Mukaddes Eşrefoğlu[2] & Eray Metin Güler[3]

[1]Department of Medical Laboratory Techniques, Vocational School of Health Services, Bezmialem Vakif University, 34093, Istanbul, Turkey

[2]Department of Histology and Embryology; [3]Department of Medical Biochemistry, Faculty of Medicine, Bezmialem Vakif University, 34093, Istanbul, Turkey

Received 28 June 2016; revised 25 October 2017

Isotretinoin (ISR), the common therapeuticagent for acne vulgaris, when used long term, leads to various side effects *viz*., oxidative toxicity, renal and hepatic dysfunction, depression, congenital abnormalities, aortic art defects, microcephaly, etc. Here, we explored the effects of silymarin (SLY), a flavonolignan from the seeds of the milk thistle *Silybum marianum* (L.) which has potential to protect the liver against chemical and environmental toxins and increase proliferation rate of tubule cells, against ISR induced liver and kidney injury. Thirty-two male Balb/c mice (3 months of age) were divided into four groups: control, isotretinoin (ISR, 40 mg/kg/day), silymarin (SLY, 200 mg/kg/day), and ISR+SLY group. We investigated liver and kidney injury by histopathological scoring system, and apoptotic cells labelled by TUNEL method. Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels were measured in serum samples biochemically. ALT and AST levels were increased in ISR group ($P = 0.025$, $P = 0.003$, respectively). SLY decreased those levels in ISR+SLY group ($P = 0.002$, $P = 0.013$, respectively). Liver tissues of ISR group showed interstitial edema and necrosis, alteration in shape and size of nuclei, mononuclear and kuppfer cell infiltration. Kidney tissues of ISR group showed tubular degeneration, necrosis, glomerular collapse, mononuclear cell infiltration, and hemorrhage. SLY improved those histopathological changes and suppressed apoptotic cell death. We suggest that silymarin might be beneficial to some extent by preventing the side effects induced by chronic ISR therapy in patients with acne vulgaris.

Keywords: 13-cis-Retinoic acid, Acne vulgaris, Apoptosis, Milk thistle, Oxidative stress, *Silybum marianum*

Isotretinoin (ISR), also known as 13-cis-retinoic acid, is a common agent in the treatment of acne vulgaris by suppressing proliferation of sebaceous glands, keratinization of hair follicles and activity of inflammatory cytokines[1,2]. Though it is one of the most effective therapeutic agents for acne vulgaris, its long term exposure causes oxidative toxicity renal and hepatic dysfunction in some of the patients[3-5]. As it decreases orbitofrontal cortex activity, some patients exhibit behaviors related to depression[6]. Moreover, it causes stillbirth or congenital abnormalities including craniofacial malformation, mental deficiency, microtia or anotia, micrognathia, cleft palate, aortic art defects and microcephaly[7].

Silymarin (SLY) is a flavonolignan concentrated in the seeds of the milk thistle *Silybum marianum* (L.). Traditionally, it has been used for the treatment of various disorders such as hepatitis, cirrhosis, type 2 diabetes, osteoarthritis, diabetic nephropathy, etc. It has been shown to protect the liver against poisoning from chemical and environmental toxins, including *Amanita phallodies* poisoning, alcohol and drugs[8-11]. Drug-induced kidney damage is associated with the release of high amounts of free oxygen radicals and thus damage of renal tubular cells. Further, following renal transplantation, SLY has been shown to increase proliferation rate of tubule cells, biosynthesis of protein and DNA[12]. Additionally, it has been reported to be protective against fibrosis by reducing transformation of stellate cells to myofibroblasts[13]. SLY is also known to decrease increased levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) induced by long term drug or alcohol administration[14].

Till date, there is no literature available on the effects of SLY on ISR-induced organ damage. In the present study, we investigated beneficial effects of silymarin against liver and kidney injury induced by

*Correspondence:
Phone: +90 555 596 2824
E-mail: kumasmeltem@gmail.com

high dose of isotretinoin by histopathological and biochemical methods.

Materials and Methods

Experimental design

Thirty-two male Balb/c mice (3 months of age) were divided into four groups: control group (received vehicle for 6 wk), isotretinoin-treated group (received ISR at a dose of 40 mg/kg/day for 6 wk), silymarin-treated group (received SLY at a dose of 200 mg/kg/day for 6 wk), and co-treated group (4 h following each ISR application, mice were treated with SLY for 6 wk at the same doses of the other groups). As we mentioned in the perivous section, isotretinoin is widely used for treatment of acne vulgaris. Patients who are treated with isotretinoin are generally young men and women. Therefore, we selected young Balb/c mice for experimental design, and we preferred male mice as well. Because metabolism of the female mice is more variable than males.

Silymarin-dose was adjusted based on the previous studies[15-17]. The dose of isotretinoin was also selected according to the Roxana *et al*.[18]. The animals were housed at 25°C under a 12 h light/dark cycle, with free access to water and food. All applications were made via oral gavage. All of the mice were anesthetized with 100 mg/kg ketamine (Ketalar, Pfizer, Turkey) and 10 mg/kg xylazine HCl (Rompun, Bayer Turkey).

The study procedure was approved by Decision No. 2013-237 by the Local Ethics Board for Animal Experiments, Bezmialem Vakif University Istanbul, Turkey.

Biochemically analysis

Blood samples were collected in tubes containing heparin by cardiac puncture. Plasma samples were removed by centrifugation for 10 at 1500 ×g. The remaining erythrocyte suspension was washed thrice an equal volume of cold 0.9% sodium chloride (NaCl). The samples were maintained at –80°C before performing assays (not longer than 7 days). Plasma ALT (Alanin aminotransferase), AST (Aspartate aminotransferase), BUN (Blood Urea Nitrogen) and creatinine (Cre) levels were measured by a biochemistry autoanalyzer (Abbott Architect ci16200) using commercial kits.

Histological analysis

For histopathological analysis, liver and kidney tissues were fixed in %10 neutral buffered formaldehyde and embedded in paraffin. About 5 µm cross sections were cut from paraffin blocks and stained with hematoxylin–eosin (H–E) and Masson trichrome staining methods. Samples were examined and scored by a blind observer using a Nikon Eclipse i5 light microscope with a Nikon DS-Fi1c camera, and Nikon NIS Elements version 4.0 image analysis systems (Nikon Instruments Inc., Tokyo, Japan).

Liver damage was scored in terms of inflammation and necrosis, perinuclear edema and cytoplasmic vacuolization, alterations in nuclear shape and size, mononuclear cell infiltration. Kidney damage was scored in terms of tubular degeneration (including dilatation, squamation of tubular epithelial cells), tubular vacuolization, necrosis, glomerulosclerosis, hemorrhage, and mononuclear cell infiltration. Each data was scored as: 0: absent, 1: minimal, 2: moderate, 3: severe with a maximum score of 12 for liver and 18 for kidney tissue.

TUNEL fluorescence (terminal deoxynnucleotidyl transferase-mediated dUTP-X nick end labelling assay, Roche- 11 684 795 910-kit) detection kit was used for determination of apoptotic cells. Twenty successive areas in each section were examined for the presence of TUNEL positive apoptotic cells at X20 magnification under fluorescence microscope. All of these analysis and imaging procedures was also performed by using Nikon Eclipse i5 microscope and Nikon NIS Elements version 4.0 imaging and analysis systems (Nikon Instruments Inc., Tokyo, Japan).

Statistical analysis

All statistical analyses and bar charts were done with SPSS 20.0 (IBM, New York, USA), MS Office Excel, and Graph Pad Prism 6. Normality of the data about biochemical analysis and histopathological scores was tested with Kolmogorov-Smirnov D test. Since they were not normally distributed (Kolmogorov–Smirnov D test, P <0.05), non-parametric Mann Whitney U-test was used for all comparisons. $P \leq 0.05$ was accepted as significant.

Results and Discussion

ISR is one the most effective treatment for acne vulgaris, but long term exposure causes renal and hepatic dysfunction in some of the patients[3-5]. It has been shown that ISR treatment results in increases in serum AST and ALT levels indicating liver damage[19-23]. Our results are compatible with those of previous studies. When compared with control group, ISR treatment increased the levels of ALT and AST

enzymes (P = 0.025, P = 0.003). Silymarin has been known to be a powerful antioxidant, free radical scavenger[14,24], it has hepatoprotective[15,25] and nephroprotective[16,26,27] effects against toxic agents, and also decreases levels of AST and ALT enzymes[22,28]. In ISR+SLY group, those of the enzyme levels decreased after SLY treatment (P = 0.002; P <0.05, respectively) (Fig. 1). The levels of the both ALT and AST of ISR+SLY group were similar to those of the control group (P >0.05).

In various studies, it was stated that SLY has regenerative effects on both liver and kidney tissues. SLY has been shown to be beneficial in improving methotrexatrexade-induced histopathological changes and induced apoptotic cell numbers[16]. Turgut *et al*.[28] demonstrated that SLY ameliorated histopathological alterations such as degeneration and necrosis of tubular cells caused by renal ischemia reperfusion injury. The kidney of obese animals also had serios damage including glomerular hypertrophy, mesengial expansion, and interstitial fibrosis[29]. CCl4 induced hepatotoxicity was reduced by silymarin administration[30].

In this study, SLY significantly protected both liver and kidney tissues against harmful effects of high dose administration of ISR (P ≤0.05). Histopathological changes in liver tissues such as inflammation (Fig. 2A), and necrosis (Fig. 2 A-C), alteration in shape and size of nuclei (Fig. 2B), mononuclear cell infiltration (Fig. 2D), increase in the number of Kuppfer cells (Fig. 2E) and cytoplasmic and nuclear vacuolization (Fig. 2 D and F) were observed in ISR group when compared the control group (P = 0.002). It was also detected that apoptotic hepatocytes around the portal area, which stained more dense than necrotic cells (Fig. 2C). The mean histopathological score of ISR group was 8.86±1.35, whereas 3.43±1.39 in ISR+SLY group. Histopathological findings in liver tissues were improved following SLY administration in ISR+SLY group (P = 0.002). In this group, only in one animal edema and necrosis were still obvious. Liver tissues of SLY groups showed the same histological features as the liver tissues of the control groups (Fig. 3).

Similarly, most significant histopathological changes were observed in the kidneys tissue of ISR group when compare to the other groups. The highest histopathological score was determined in ISR group. The mean histopathological score of ISR group was 15.57±1.13 whereas 4.85±1.46 in ISR+SLY group. Histological features of the kidneys of SLY group were similar to those of the control group (P >0.05). In ISR group, histopathological changes including

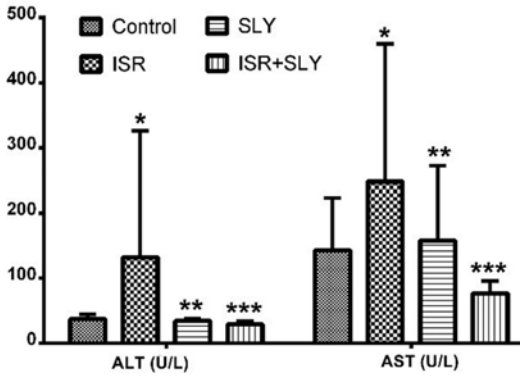

Fig. 1—Mean ALT and AST levels of all groups. [* P ≤0.05 *vs*. control group, ** P ≤0.05 *vs*. ISR group, ***P ≤0.05 *vs*. SLY group. (ALT: alanine aminotransferase, AST: aspartate aminotransferase, ISR: isotretinoin, SLY: silymarin, *vs*.: versus)]

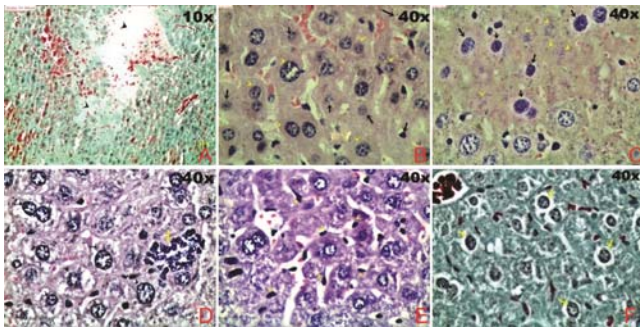

Fig. 2—Liver sections in ISR (isotretinoin) group. (A) Necrosis of hepatocytes around the portal area accompanied by inflammation (arrowheads). Masson's trichrome method; (B) Alteration in shape and size of hepatocyte nuclei (yellow arrowheads), and necrotic hepatocytes containing shrinking nucleus (black arrows) around the centrolobular area H-E (hematoxyline-eosin) method; (C) Necrotic hepatocytes (yellow arrowheads) and apoptotic hepatocytes (black arrows). H-E method; (D) Nuclear vacuolization (black arrowheads) and mononuclear cell infiltration (yellow arrows). H-E method; (E) Increase in the number of Kuppfer cells around the sinusoidal capillary (yellow arrowheads). H-E method; and (F) Cytoplasmic vacuolization especially in perinuclear area (yellow arrows). Masson's trichrome method.

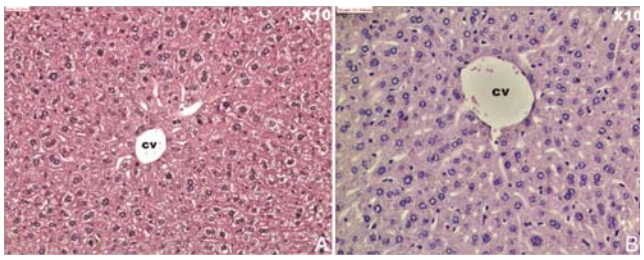

Fig. 3—Normal hepatic histology in SLY (silymarin), and ISR+SLY (isotretinoin+silymarin) groups, respectively (cv: central ven). H-E method.

proximal tubular degeneration and necrosis (Fig. 4A), glomerular collapse, and infiltration (Fig. 4 B and D), and hemorrhage in the interstitial space and within the glomeruli (Fig. 4C) were observed. In ISR+SLY group, tubular degeneration and glomerular collapse where nor prominent, whereas mononuclear cell infiltration, necrosis and hemorrhage were not present. SLY administration considerably improved the ISR-induced histological damage. (Fig. 5 A-B, P = 0.002). Whereas increasing levels of blood urea nitrogen (BUN) and creatinine (Cre) shows kidney injury, interestingly it was not measured or calculated any differences among groups in terms of serum concentration of BUN and Cre (P >0.05, Fig. 6) even though kidney tissues in ISR group were seriously damaged. Mean histopathological scores of liver and kidney tissues in all groups are summarized in Fig. 7A.

In liver tissues, the highest mean number of apoptotic cells was counted as 33.63±6.46 in ISR group. SLY administration decreased mean number of apoptotic cell in ISR+SLY group (15.88±3.04, P = 0.001). Same results were observed in kidney tissues of ISR and ISR+SLY groups. ISR caused increase of apoptotic cells (18.75±2.92), whereas SLY administration decrease number of apoptotic cells in ISR+SLY group (9.75±2.6, P = 0.001).

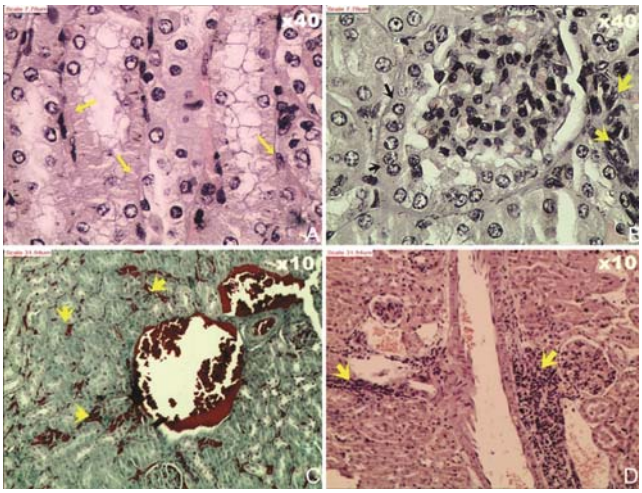

Fig. 4—Kidney sections from ISR (isotretinoin) group. (A) Necrosis and degeneration of proximal tubular epithelial cells (arrows) H-E method; (B) Glomerular collapse (black arrows) and infiltration (yellow arrows) H-E method; (C) Hemorrhage in the interstitial space and within the glomeruli. Masson's trichrome method; and (D) Mononuclear cell infiltration around the vessels. H-E method.

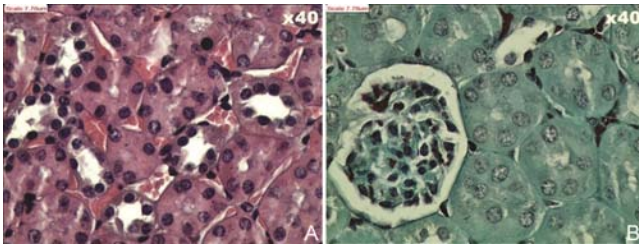

Fig. 5—Normal histological view of proximal and distal tubules and glomerulus in control (H-E) and ISR+SLY groups, respectively. [Masson's trichrome method. (ISR: isotretinoin, SLY: silymarin)]

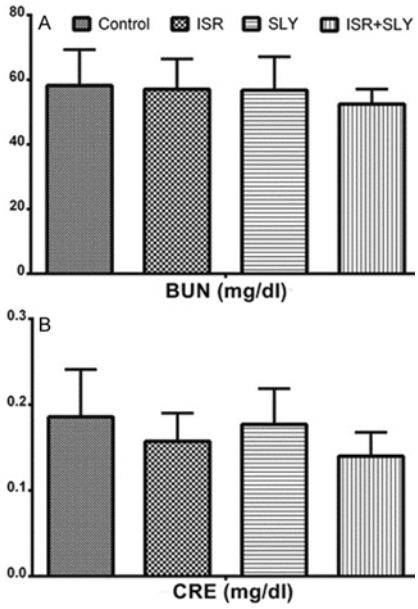

Fig. 6—Mean serum levels of BUN and Cre of all groups

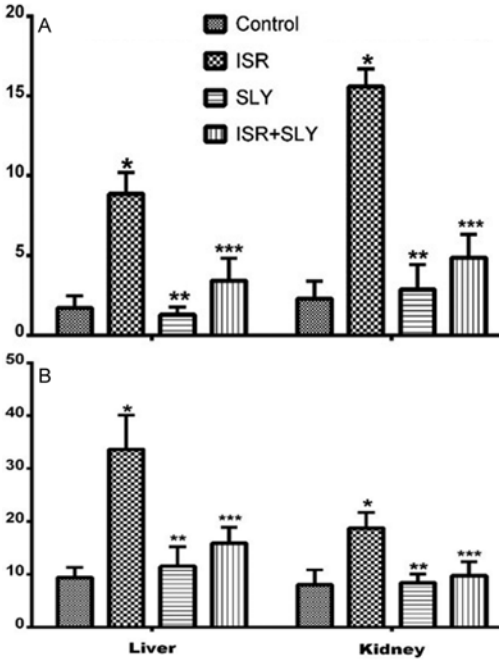

Fig. 7— (A) Mean histopathological scores; and (B) Mean number of apoptotic cells of liver and kidney of all groups. [* P ≤0.05 *vs*. control group; ** P ≤0.05 *vs*. ISR group; and *** P ≤0.05 *vs*. SLY group. (ISR: isotretinoin, SLY: silymarin, *vs*.: versus)]

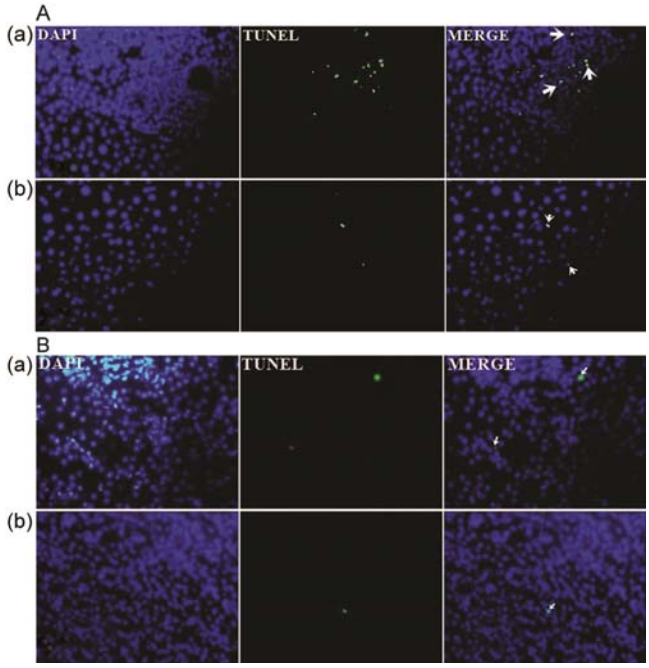

Fig. 8—TUNEL (terminal deoxynnucleotidyl transferase-mediated dUTP-X nick end labelling assay) detected apoptosis in (A) liver tissues; and (B) kidney tissues of (a) ISR; and (b) ISR+SLY groups, respectively. [Apoptotic nuclei are stained in green (indicated by arrow), while normal nuclei are stained in blue by DAPI (4',6-diamidino-2-phenylindole), magnification ×40. (ISR: isotretinoin, SLY: silymarin)]

Summary of the number of apoptotic cells in liver and kidney tissues are shown in Fig. 7B. TUNEL fluorescence stains of liver and kidney tissues from ISR and ISR+SLY groups were shown in Fig. 8 A and B, respectively.

As a conclusion, we detected that SLY is highly effective in improvement of hepatic and renal histopathological alterations induced by ISR. Additionally, it was effective in re-establishment of the homeostasis of cellular antioxidant enzyme system. In our previous study, we suggest that SLY is effective in normalization of the cellular antioxidant enzyme system[31]. SLY is effective in reducing ISR-induced hepatic and renal damage by suppressing cell death and providing the normalization of liver enzyme system. Thus, it may be beneficial in protection against the side effects of ISR in patients since no side effects have been detected so far.

Acknowledgement

This was supported by the Research Fund Accountancy of Bezmialem Vakif University, Istanbul/Turkey (Project number:12.2013/9).

References

1 Cunliffe WJ, Van Der Kerkhof PCM, Caputo R, Caicchini S, Cooper A, Fyrand OL, Gollnick H, Layton AM, Leyden JJ, Mascaró JM, Ortonne JP & Shalita A, Roaccutane treatment guidelines: results of an international survey. *Dermatology*, 194 (1997) 352

2 Goulden V, Layton AM & Cunliffe WJ, Current indications for isotretinoin as a treatment for acne vulgaris. *Dermatology*, 284 (1995) 285.

3 Erturan I, Naziroğlu M & Akkaya VB, Isotretinoin treatment induces oxidative toxicity in blood of patients with acne vulgaris: a clinical pilot study. *Cell Biochem Funct*, 30 (2012) 554.

4 Ozkol HU, Ozkol H, Karadag AS, Bilgili SG, Tuluce Y & Calka O, Oral isotretinoin therapy of acne patients decreases serum paraoxonase-1 activity through increasing oxidative stress. *Drug Chem Toxicol*, 38 (2015) 64.

5 Zaher A, Shhadeh H, Abdalla B, Mohammed A, Adel J, Yumna F & Zaid A**,** Acute kidney injury following isotretinoin treatment. *Am J Case Rep*, 14 (2013) 555.

6 Bremner JD, Fani N, Ashraf A, Votaw JR, Brummer ME, Cummins T, Vaccarino V, Goodman MM, Reed L, Siddiq S & Nemeroff CB. Functional brain imaging alterations in acne patients treated with isotretinoin**.** *Am J Psychiatry*, 165 (2008) 1614.

7 Goodman D, Vitamin A in health and disease. *N Engl J Med*, 310 (1984) 1023.

8 Desplaces A, Choppin J, Vogel G & Trost W, The effects of silymarin on experimental phalloidine poisoning. *Arzneimittelforschung*, 25 (1975) 90.

9 Choppin J & Desplaces A, The effects of silybin on experimental phalloidine poisoning**.** *Arzneimittelforschung*, 28 (1978) 637.

10 Ferenci P, Dragosics B, Dittrich H, Frank H, Benda L, Lochs H, Meryn S, Base W & Schneider B, Randomized controlled trial of silymarin treatment in patients with cirrhosis of the liver. *J Hepatol*, 9 (1989) 111.

11 Křena V & Walterováb D, Silybin and silymarin- new effects and applications. *Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub*, 149 (2005) 36.

12 Ahlenstiel T, Burkhardt G, Köhler H & Kuhlmann MK, Bio- flavonoids attenuate renal proximal tubular cell injury during cold preservation in Euro-Collins and University of Wisconsin solutions**.** *Kidney Int*, 63 (2003) 554.

13 Fuchs EC, Weyhenmeyer R & Weiner OH. Effects of silibinin and of a synthetic analogue on isolated rat hepatic stellate cells and myofibroblasts. *Arzneimittelforschung*, 47 (1994) 1384.

14 Valenzuela A & Garrido A, Biochemical bases of the pharmacological action of the flavonoid silymarin and of its structural isomer silibinin. *Biol Res*, 27 (1994) 107.

15 Cetinkunar S, Tokgoz S, Bilgin BC, Erdem H, Aktimur R, Can S, Erol HS, Isgoren A, Sozen S & Polat Y, The effect of silymarin on hepatic regeneration after partial hepatectomy: is silymarin effective in hepatic regeneration? *Int J Clin Exp Med*, 8 (2015) 2580.

16 Dabaka DO & Kocaman N, Effects of silymarin on methotrexate-induced nephrotoxicity in rats. *Ren Fail*, 37 (2015) 736.

17 Freitag AF, Cardia GF, da Rocha BA, Aguiar RP, Silva-Comar FM, Spironello RA, Grespan R, Caparroz-Assef SM, Bersani-Amado CA & Cuman RK, Hepatoprotective effect of silymarin (Silybum marianum) on hepatotoxicity induced by acetaminophen in spontaneously hypertensive rats. *Evid Based Complement Alternat Med*, 2015 (2015) 3.

18 Roxana AR, Nathan LM, Steven N, Xinran L, Paul AS & Gabriel HT, Treatment with isotretinoin inhibits lipofuscin accumulation in a mouse model of recessive Stargardt's macular degeneration. *PNAS*, 100 (2003) 4744.

19 Bozkurt B, Irkec MT & Atakan N, Lacrimal function and ocular complications in patients treated with systemic isotretinoin. *N Eur J Ophthalmol*, 12 (2002)173.

20 Zane LT, Leyden WA, Marqueling AL & Manos MM, A population-based analysis of laboratory abnormalities during isotretinoin therapy for acne vulgaris. *Arch Dermatol*, 142 (2006) 1016.

21 Vieira AS, Beijamini V & Melchiors AC, The effect of isotretinoin on triglycerides and liver aminotransferases. *An Bras Dermatol*, 87 (2012) 382-387.

22 Giannini EG, Testa R & Savarino V, Liver enzyme alteration: a guide for clinicians. *Can Med Assoc*, 172 (2005) 367.

23 Saied NM & Hamza AA, Selenium ameliorates isotretinoin-induced liver injury and dyslipidemia via antioxidant effect in rats. *Toxicol Mech Methods*, 24 (2014) 433.

24 Akkaya H & Yılmaz O, Antioxidant capacity and radical scavenging activity of *Silybum marianum* and *Ceratonia silique. Ekoloji*, 21 (2012) 9.

25 Luper S, A review of plants used in the treatment of liver disease: part 1. *Altern Med Rev*, 3 (1998) 410.

26 Momeni A, Hajigholami A, Geshnizjani S & Kheiri S, Effect of silymarin in the prevention of cisplatin nephrotoxicity, a clinical trial study. *J Clin Diagn Res*, 9 (2015) 11.

27 Tan J, Hu J, He Y & Cui F, Protective role of silymarin in a mouse model of renal Ischemia–Reperfusion injury. *Diagn Pathol*, 10 (2015) 1981.

28 Turgut F, Bayrak O, Catal F, Bayrak R, Atmaca AF, Koc A, Akbas A, Akcay A & Unal D, Antioxidant and protective effects of silymarin on ischemia and reperfusion injury in the kidney tissues of rats. *Int Urol Nephrol*, 40 (2008) 453.

29 Feng B, Meng R, Huang B, Bi Y, Shen S & Shu D, Silymarin protects against renal injury through normalization of lipid metabolism and mitochondrial biogenesis in high fat-fed mice. *Free Radic Biol Med*, 110 (2017) 240.

30 Clichici S, Olteanu D, Nagy AL, Oros A, Filip A & Mircea PA, Silymarin inhibits the progression of fibrosis in the early stages of liver injury in CCl4-treated rats. *J Med Food*, (2014) 1.

31 Kumas M, Esrefoglu M & Ozer OF, Protective effects of silymarin against cardiac tissue injury caused by a high-dose administration of isotretinoin in mice. *Bezmialem Sci*, 2 (2016) 43.