CYTOPROTECTIVE EFFECT OF SILYMARIN AGAINST SODIUM FLUORIDE-INDUCED OXIDATIVE STRESS IN RAT ERYTHROCYTES

Seyed Mohammad Nabavi,[a,b*] Seyed Fazel Nabavi,[a,b] Monica Rosa Loizzo,[c] Antoni Sureda,[d]
Mohammad Ali Amani,[a] Akbar Hajizadeh Moghaddam[e]

Tehran and Babolsar, Iran; Cosenza, Italy; and Illes Balears, Spain

SUMMARY: Silymarin is a potent antioxidant agent isolated from milk thistle seeds of *Silybum marianum* (L.) Gaertn (Family Asteraceae), comprised mainly of three isomeric flavonolignans: silibinin, silydianin, and silychristin. The aim of the present study was to evaluate the protective antioxidant effects of silymarin in erythrocytes of fluoride-treated rats. Fifty adult male Wistar rats were divided into five groups of ten each: a normal control group received low fluoride (F) drinking water (DW) and a standard diet throughout the study; a second group received only 600 ppm NaF (271 ppm F ion) in their DW for seven days. A third group received 10 mg silymarin/kg bw intraperitoneally (i.p.) for one week, followed by 600 ppm NaF in their DW for seven days. The fourth group received 20 mg silymarin/kg bw i.p. for one week, followed by 600 ppm NaF in their DW for seven days. The fifth group received 10 mg vitamin C/kg bw i.p. for one week, followed by 600 ppm NaF in their DW for seven days. At the end of the one-week pre-treatment period, the erythrocytes of the rats were isolated; and catalase and superoxide dismutase activities, reduced glutathione, and lipid peroxidation levels were determined. Pre-treatment with 20 mg silymarin (and, to a lesser extent, with 10 mg silymarin and 10 mg vitamin C), prior to NaF intoxication, maintained the basal levels of antioxidant parameters and restored the enzymatic antioxidant activities in the erythrocytes when compared with those in the control group. We conclude that silymarin has a potent ameliorative effect on NaF-induced oxidative stress in rat erythrocytes.

Keywords: Fluoride and erythrocytes; Oxidative stress; Rat erythrocytes; Silymarin antioxidant effects.

INTRODUCTION

Fluorosis induced by excessive consumption of fluoride (F) anions is a progressive degenerative disease in mammals, including humans, known to adversely affect the teeth, skeletal system, function and structure of the brain, skeletal muscle, kidney, heart, and red blood cells.[1] Recent reports have shown that sodium fluoride (NaF) induces oxidative stress through significant changes in the levels of superoxide dismutase, catalase, reduced glutathione, and lipid peroxidation markers, resulting in altered free-radical metabolism in several tissues and red blood cells of rats.[2-3]

Natural antioxidant agents can quench free radicals by electron or proton (hydrogen) donation and can protect body tissues against oxidative stress and therefore, mitigate various injuries induced by reactive oxygen species.[4] Silymarin

[a,b*]For correspondence: Seyed Mohammad Nabavi, Applied Biotechnology Research Center, Baqiyatallah University of Medical Sciences, Tehran, Iran, PO Box 19395-5487; E-mail: Nabavi208@gmail.com; [a]Applied Biotechnology Research Center, Baqiyatallah University of Medical Sciences, Tehran, Iran; [b]National Elites Foundation of Iran, Tehran, Iran; [c]Department of Pharmaceutical Sciences, Faculty of Pharmacy, Nutrition and Health Sciences, University of Calabria, Italy; [d]Departament de Biologia Fonamental i Ciències de la Salut, Universitat de les Illes Balears, Illes Balears, Spain; [e]Department of Biology, University of Mazandaran, Babolsar, Iran.

is a natural product with potent antioxidant activity that is present in the fruits and seeds of milk thistle *Silymarin marianum* Gaertn (Family Asteraceae).[5] It is a mixture of three compounds, silibinin, silydianin, and silychristin (Figure 1).[6]

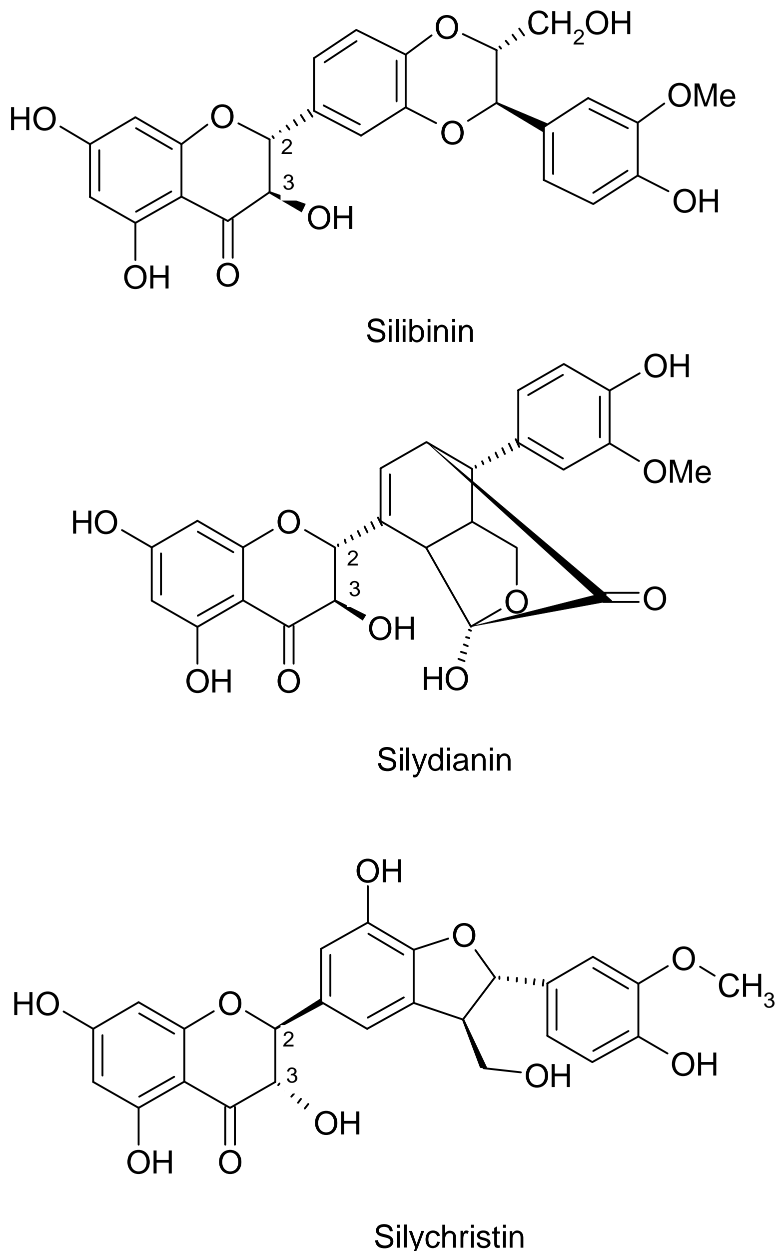

Figure 1. Components of silymarin.

Scientific reports indicate that silymarin has multiple pharmacological effects including anti-inflammatory, antioxidant, antiallergenic, antimutagenic, antibacterial, antiviral, antithrombotic, antineoplastic, and vasodilatory effects.[5] To the best of our knowledge, there is no report on protective effects of silymarin

against F-induced oxidative stress in erythrocytes. In the present study we have evaluated the extent silymarin has any protective action on the antioxidant parameters of erythrocytes of rats previously intoxicated with NaF.

MATERIALS AND METHODS

Chemicals: Bovine serum albumin and a kit for protein estimation were purchased from ZiestChem Company (Tehran, Iran). Silymarin, glacial acetic acid, 5,5-dithiobis(2-nitrobenzoic acid), heparin, reduced glutathione, sodium dihydrogen phosphate, thiobarbituric acid, nitro blue tetrazolium chloride, potassium dihydrogen phosphate, trichloroacetic acid, sodium fluoride, and hydrogen peroxide were purchased from Sigma-Aldrich chemical company, (St. Louis, MO USA). Other solvents and chemical reagents were of analytical grade quality or higher.

Animals: Male Wistar rats weighing 200–250 g, purchased from the Pasteur Institute of Iran-Amol Research Center (Amol, Mazandaran, Iran) were used for this study. All rats were kept in the animal room of the Department of Biology (Faculty of Sciences, University of Mazandaran, Babolsar, Iran) under standard conditions. The experiments were processed following the internationally accepted ethical guidelines for the care of laboratory animals (No.S-2009 UMZ). Rats were housed in ventilated animal rooms at a temperature of 24 ± 2ºC with a 12-hr light/dark cycle and 60 ± 5% relative humidity.

Animal treatments: The rats used in this study were divided into five sub-groups of ten each. The first group, considered as the normal control, only received only blank intraperitoneal (i.p.) injections during 7 days. The toxin control group similarly received blank injections during 7 days and then 600 mg NaF (271 mg F ion)/L through the drinking water (DW) during the following 7 days. The next two groups of rats were pre-treated with i.p. administration of silymarin at doses of 10 and 20 mg/kg bw for 7 days followed by 600 mg NaF/L through the DW for the next 7 days, respectively. A standard group of rats was also pre-treated with ascorbic acid at 10 mg/kg bw for the same period.

Preparation of hemolysates: Blood samples were collected by conventional cardiac puncture, kept in heparinized tubes, and then centrifuged at 1000 × g for 15 min. The upper layer of the centrifuged samples was removed, and the packed erythrocytes of the bottom were washed three times with phosphate buffer saline (0.01 M, pH 7.4). A known volume of red blood cells of rats were lysed with hypotonic phosphate buffer. After removing the red blood cell debris by centrifugation of the reaction mixtures (3000 × g for 15 min), the hemolysates were recovered and immediately used for biochemical analysis. The protein content of the hemolysates was measured by the method of Bradford using a commercial kit of ZiestChem Co. (Tehran, Iran) using bovine serum albumin as standard.[7] The hemoglobin content was determined evaluated by Drabkin method.[1]

Biochemical analyses: The level of lipid peroxidation in terms of the formation of thiobarbituric acid reactive substances (TBARS) was evaluated in the

hemolysate samples by the method of Nabavi et al.[4] TBARS levels were expressed using the molar extinction coefficient of malondialdehyde. The superoxide dismutase (SOD) activity was also evaluated according to the method of Nabavi et al.[8] The SOD unit was considered as the enzyme concentration needed to prevent the chromogen generation by 50% in 60 seconds under the assay conditions. The catalase (CAT) activity of the hemolysate was evaluated according to the method of Pari and Latha.[9] The CAT unit was expressed as the enzyme concentration that diminished 1 μmol of H_2O_2 under the assay conditions. The reduced glutathione (GSH) level was determined by the method of Ellman.[10] GSH levels of the hemolysate were calculated using the GSH calibration curve.

Statistical analysis: Data are presented as means ± S.D. Significant differences between groups were analyzed using one-way ANOVA followed by Duncan's multiple range tests. Differences were considered statistically significant when $p < 0.05$.

RESULTS AND DISCUSSION

Lipid peroxidation effects measured as TBARS levels in the rat erythrocytes are reported in Figure 2.

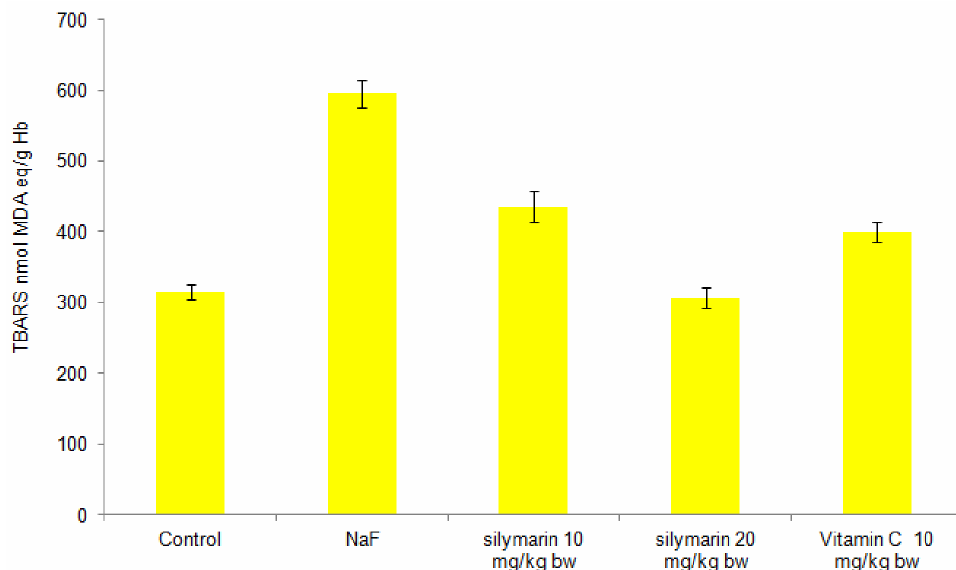

Figure 2. Effect of silymarin (10 and 20 mg/kg bw) and vitamin C (10 mg/kg bw) on TBARS levels in erythrocytes of rats treated during one week with 600 mg NaF/L through the DW. Data are expressed as mean ± S.D. (n = 10). [a] p<0.001 versus control group. [b] p<0.05 versus control group.

The levels of TBARS in the F group (595.13 ± 20.23 nmol eq MDA/g Hb) were significantly increased compared to the control group (315.44 ± 9.76 nmol eq MDA/g Hb). Rats pre-treated with silymarin and vitamin C for one week prior to NaF Intoxication from 600 mg NaF/L in the DW showed a significant reduction in the TBARS levels. The superoxide dismutase (SOD) activity in the erythrocytes is shown in Figure 3. NaF treatment induced a significant reduction in the SOD

(590.24 ± 17.11 U/g Hb) compared to the control group (1625.34 ± 40.62 U/g Hb). Administration of silymarin for seven days prior to intoxication with NaF significantly prevented the decrease in SOD activity. As seen in Figure 3, pre-treatment with vitamin C gave results similar to those observed with silymarin.

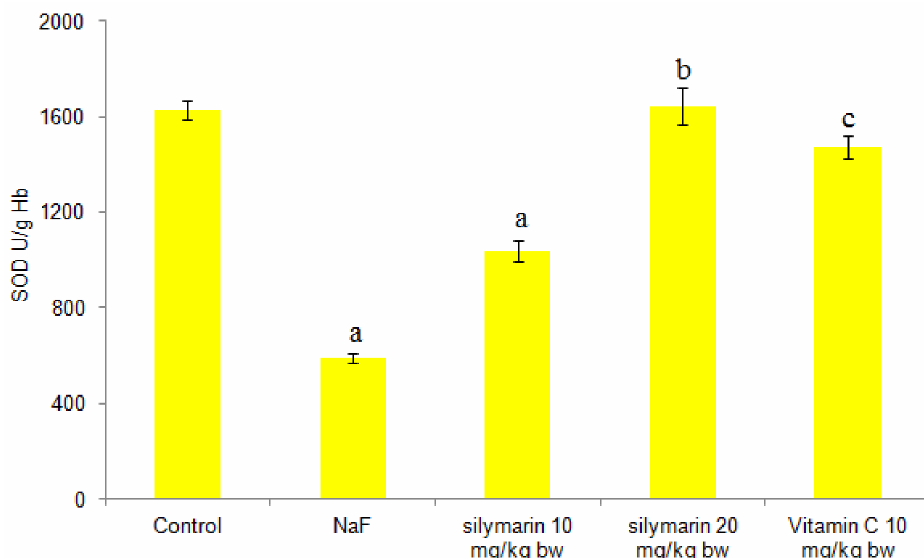

Figure 3. Effect of silymarin (10 and 20 mg/Kg bw) and vitamin C (10 mg/Kg bw) on SOD activity in erythrocytes of rats treated during one week with 600 mg NAF/L through DW. Data are expressed as mean ± S.D. (n = 10). [a] $p<0.001$ versus control group. [b] $p<0.05$ versus control group. [c] $p< 0.01$ versus control group.

Figure 4 shows catalase (CAT) activity in the erythrocytes.

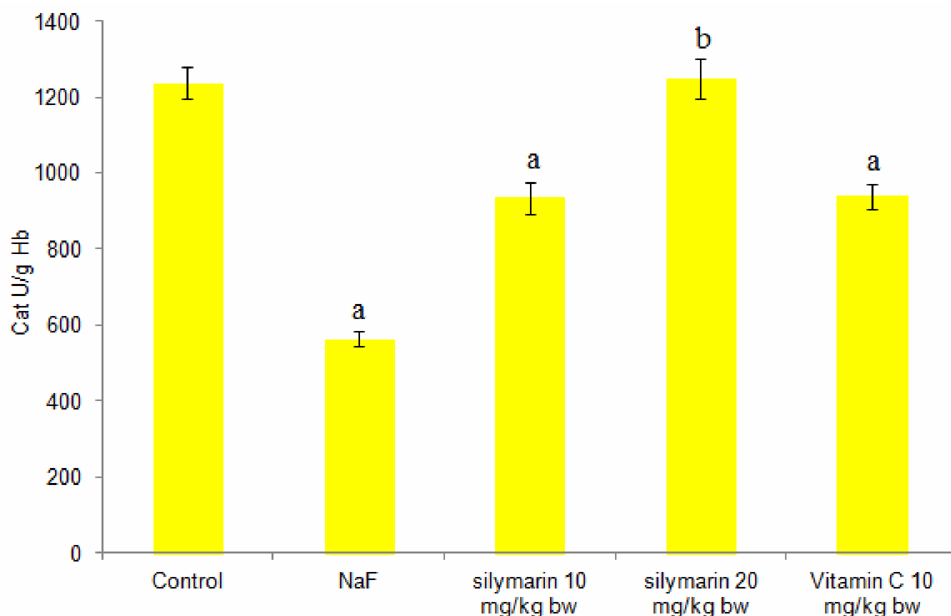

Figure 4. Effect of silymarin (10 and 20 mg/Kg bw) and vitamin C (10 mg/Kg bw) on catalase activity in erythrocytes of rats treated during one week with 600 mg NaF/L through the DW. Data are expressed as mean ± S.D. (n = 10). [a] $p<0.001$ versus control group. [b] $p<0.05$ versus control group.

CAT activity in the hemolysate (563.93 ± 18.47 U/g Hb) of the NaF-treated group was significantly reduced in comparison with the control group (1237.27 ± 0.002 U/g Hb). Silymarin treatment at doses of 10 and 20 mg/kg bw as well as vitamin C for one week before the one-week exposure to NaF in the DW preserved the CAT activity compared to the intoxicated group. Reduced glutathione (GSH) levels in hemolysates are shown in Figure 5.

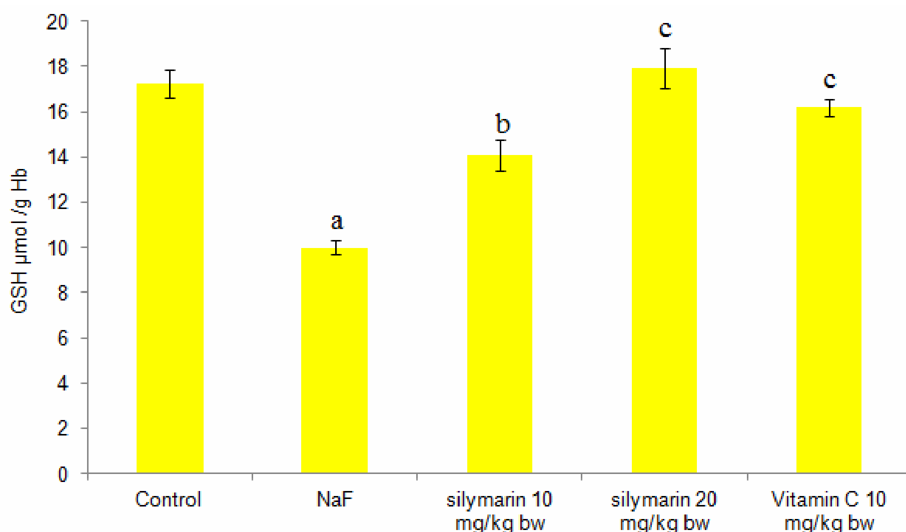

Figure 5. Effect of silymarin (10 and 20 mg/Kg bw) and vitamin C (10 mg/Kg bw) on reduced glutathione levels in erythrocytes of rats treated during one week with 600 mg NaF/L through DW. Data are expressed as mean ± S.D. (n = 10). [a] $p<0.001$ versus control group. [b] $p<0.01$ versus control group. [c] $p< 0.05$ versus control group.

NaF intoxication led to significant reduction in GSH levels in comparison with the control group. Treatment of silymarin before exposure to NaF preserved the level of GSH. Although rats can synthesize vitamin C in their hepatic tissue through the D-glucuronic acid pathway metabolism, prior administration of 10 mg vitamin C/kg bw prevented a decrease in GSH levels.[11]

As the present study has shown, F intoxication induced significant changes in antioxidant parameters of rat erythrocytes, reducing the antioxidant enzyme activities and glutathione levels, while increasing lipid peroxidation. Silymarin administration before exposure of the rats to NaF in the DW greatly normalized all the antioxidant parameters. In the cellular system, reactive oxygen species are continuously generated through sequential aerobic metabolism.[1] It is well established that cigarette smoke, environmental pollutants, excessive consumption of alcohol, exposure to ionizing radiation, etc., significantly increase the production of reactive oxygen species leading to oxidative damage to cells and tissues.[11] It is also known that oxidative stress plays a crucial role in the progression or initiation of many disorders such as cancer, diabetes, rheumatoid arthritis, thalassemia, and cardiovascular and neurodegenerative diseases.[1] Correlation between F intake and oxidative stress in erythrocytes has been reported previously.[11]

Silibinin (Figure 1) is the major constituent of the silymarin complex together with other flavonolignans, such as silychristin, silydianin, isosilybin, and the flavonoid taxifolin.[6] Studies based on a structure activity relationship survey have demonstrated that analogues of flavonoids with a hydroxymethyl group at C-3 showed potent hepatoprotective effects similar to coumarin derivatives.[12] Other studies indicate that different moieties of silibinin have an important role in the protection against over-generation of superoxide anions by stimulated neutrophils or xanthine oxidase activity, and in the inhibition of heme-mediated oxidative change of low density lipoproteins. There is evidence that the scavenging/ antioxidant potency of flavonoids may derive from the presence of hydroxyl group especially in the aryl ring.[6]

The present results reporting the protective effects of sylimarin to prevent NaF-induced oxidative stress are in accordance with previous studies that indicate the capability of sylimarin to ameliorate benzo(a)pyrene-induced oxidative stress in erythrocyte,[13] and to protect against phenylhydrazine-induced hemolysis.[14] Previous studies have demonstrated protective effects of sylimarin against arsenite[15], H_2O_2[16] and anthracycline-induced cytotoxicity,[17] a beneficial effect that was directly correlated with its potent antioxidant capacity. Recently, Kiruthiga et al. have found that silymarin administration normalized the activities of superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase, and glutathione-s-transferase as well as TBARS levels in hydrogen peroxide treated-erythrocytes.[13]

In conclusion, the present study has shown that silymarin has potent ameliorative effects on F-induced oxidative stress in erythrocytes of male rats. After appropriate clinical studies are conducted, these results are obviously of interest as a starting point for applications in the pharmaceutical industry. Meanwhile, additional investigations are needed for a better understanding of exactly how silymarin exerts its protective anti-oxidant effects.

REFERENCES

1 Nabavi SF, Nabavi SM, Abolhasani F, Moghaddam AH, Eslami S. Cytoprotective effects of curcumin on sodium fluoride-induced intoxication in rat erythrocytes. Bull Environ Contam Toxicol 2012;88:486-90.
2 Chlubek D. Fluoride and oxidative stress. Fluoride 2003;36:217-28.
3 Sharma A, Chinoy NJ. Role of free radicals in fluoride-induced toxicity in liver and kidney of mice and its reversal [abstract]. Fluoride 1998;31:S26.
4 Nabavi SM, Nabavi SF, Eslami S, Moghaddam AH. *In vivo* protective effects of quercetin against sodium fluoride-induced oxidative stress in the hepatic tissue. Food Chem 2012;132:931-5.
5 Kiruthiga PV, Shafreen RB, Pandian SK, Devi KP. Silymarin protection against major reactive oxygen species released by environmental toxins: exogenous H_2O_2 exposure in erythrocytes. Basic Clin Pharmacol Toxicol 2007;100:414-9.
6 Lee DY, Liu Y. Molecular structure and stereochemistry of silybin A, silybin B, isosilybin A, and isosilybin B, Isolated from *Silybum marianum* (milk thistle). J Nat Prod 2003;66:1171-4.
7 Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976;72:248-54.
8 Nabavi SF, Moghaddam AH, Eslami S, Nabavi SM. Protective effects of curcumin against sodium fluoride-induced toxicity in rat kidneys. Biol Trace Elem Res 2012;145:369-74.

9 Pari L, Latha M. Protective role of *Scoparia dulcis* plant extract on brain antioxidant status and lipid peroxidation in STZ diabetic male Wistar rats. BMC Complement Altern Med 2004;4:16.

10 Ellman GL. Tissue sulphydryl groups. Arch Biochem Biophys 1959;82:70-7.

11 Sinha, M, Manna P, Sil PC. A 43 kD protein from the herb, *Cajanus indicus* L., protects against fluoride induced oxidative stress in mice erythrocytes. Pathophysiology 2007;14:47-54.

12 Szilági I, Tétényi P, Antus S, Seligmann O, Chari VM, Seitz M, et al. Structure of silandrin and silymonin, two new flavanolignans from a white blooming *Silybum marianum* variety. Planta Med 1981;43:121-7. [in German].

13 Kiruthiga P, Pandian SK, Devi KP. Silymarin prevents the toxicity induced by benzo(a)pyrene in human erythrocytes by preserving its membrane integrity: an *in vitro* study. Environ Toxicol 2011;Doi: 10.1002/tox.20783.

14 Valenzuela A, Barría T, Guerra R, Garrido A. Inhibitory effect of the flavonoid silymarin on the erythrocyte hemolysis induced by phenylhydrazine. Biochem Biophys Res Commun 1985;126:712-8.

15 Soria EA, Eynard AR, Quiroga PL, Bongiovanni GA. Differential effects of quercetin and silymarin on arsenite-induced cytotoxicity in two human breast adenocarcinoma cell lines. Life Sci 2007;81:1397-402.

16 Svobodová A, Walterová D, Psotová J. Influence of silymarin and its flavonolignans on H_2O_2-induced oxidative stress in human keratinocytes and mouse fibroblasts. Burns 2006;32(8):973-9.

17 Chlopcíková S, Psotová J, Miketová P, Simánek V. Chemoprotective effect of plant phenolics against anthracycline-induced toxicity on rat cardiomyocytes. Part I. Silymarin and its flavonolignans. Phytother Res 2004;18(2):107-10.

Copyright © 2012 The International Society for Fluoride Research Inc.
www.fluorideresearch.org www.fluorideresearch.com www.fluorideresearch.net
Editorial Office: 727 Brighton Road, Ocean View, Dunedin 9035, New Zealand.