Advance Journal of Food Science and Technology 8(4): 278-282, 2015
DOI:10.19026/ajfst.8.1508
ISSN: 2042-4868; e-ISSN: 2042-4876

Submitted: December 20, 2014 Accepted: January 27, 2015 Published: May 20, 2015

Research Article

Evaluation of Immunomodulatory Activity of Silymarin Extract from Silybum Marianum in Mice of Health Food

Fei Zhao and Xinhua Li
College of Food Science, Shenyang Agricultural University, Shenyang 110866, China

Abstract: In the study of silymarin on the immunoregulatory effects of immunosuppressive mice, 80 mice were randomly divided into 4 groups, divided into negative control group, the silimary low-dose group (silymarin-LG), the middle-dose group (silymarin-MG) and the high-dose group (silymarin-HG), each group of 20 mice. Silymarin was given by gavage to the mice at doses of 100, 200 and 400 mg/kg for the silymarin-LG, silymarin-MG and silymarin-HG and the same volume of distilled water was given to mice in the CG. After 30 d, then observe the changes of each immunological indexes in mice. Purified silymarin showed dose-dependent immunomodulatory properties in vivo, as evidenced by the increase in acid phosphatase activity, lysozyme and nitric oxide content, macrophage phagocytosis and immune organ indexes. The results of this study could be used to further improve the purification of silymarin immunoactive fractions from *S. marianum* and other plant extracts.

Keywords: Immune regulation, immunosuppression, mice, silymarin

INTRODUCTION

Silymarin, derived from the plant *Silybum marianum* (milk thistle), has been widely used for centuries for its hepatoprotective properties. It has shown activity against toxic liver damage, hepatitis and cirrhosis (Flora *et al*., 1998; Mayer *et al*., 2005; Wellington and Jarvis, 2001; Saller *et al*., 2001; Křen and Walterová, 2005). Silymarin has also demonstrated antioxidant properties and remarkable anti-tumor activity (Lahiri-Chatterjee *et al*., 1999; Singh and Agarwal, 2004; Bhatia *et al*., 1999; Davis-Searles *et al*., 2005) and has been shown to prevent skin cancer (Katiyar, 2005). It primarily consists of a mixture of active structurally related flavonolignans, including silychristin, silydianin and two groups of diastereoisomers, silybins A and B and isosilybins A and B (Hahn *et al*., 1968; Wagner *et al*., 1974; Kim *et al*., 2003; Lee and Liu, 2003; Rickling *et al*., 1995; Lee *et al*., 2006).

In recent years, the study of silymarin immunity activity was rarely reported. In addition, the immunomodulatory properties of the purified silymarin, including acid phosphatase activity, lysozyme and nitric oxide release, macrophage phagocytosis and immune organ indexes, were evaluated in vivo. Our results could be useful for the development of an effective method for the purification of silymarin from *S. marianum* extracts.

MATERIALS AND METHODS

Materials: The seeds of *S. marianum* were obtained from Panjin Tianyuan Pharmaceutical Co., Ltd. (Liaoning, China). Silymarin is extracted from Silybum marianum by the ultrasonic assisted enzymatic method. Reagent kits for the determination of Acid phosphatase (ACP), lysozyme (LYSO) and Nitric Oxide (NO) measurements were purchased from Jiancheng Biotechnology Co. (Nanjing, China). Injection of cyclophosphamide (cyclophosphamide, Cy), Jiangsu Hengrui Medicine Co., Ltd.; India ink, Shanghai Changjiang Daily adhesive materials plant; sheep erythrocytes (sheep erythrocyte, SRBC, First Affiliated Hospital of Anhui Medical University, test subjects provided; other reagents were of analytical grade. Biochemical analyses were determined with use of an auto-analyzer (Hitachi 7060, Hitachi, Tokyo, Japan). A UV-1700 spectrophotometer (Shimadzu Corporation, Japan) was used for the analysis of silymarin and an RE-52AA rotary evaporator (Shanghai Yarong Biochemistry Instrument Factory, Shanghai, China) was used for concentration of the samples.

Experimental animals: healthy Kunming mice 80, single-sex male, SPF grade, weighing 18~22 g, were provided by the Liaoning longevity biotechnology companies (Liaoning, China). SPF level rat food was provided by the Experimental Animal Center of Shenyang Agricultural University.

Corresponding Author: Xinhua Li, College of Food Science, Shenyang Agricultural University, Shenyang 110866, China

Preparation of sample solutions: After peeling by rolling, *S. marianum* seeds were mixed with water. Seed coats were isolated by winnowing, collected, dried and crushed. The powder was passed through a 40-mesh sieve and used to extract silymarin by ultrasonic-assisted enzymatic digestion. For the extraction, 1 kg of dried powder was mixed with petroleum ether (boiling temperature range, 60-90°C), skimmed for 8 h, filtered and defatted for 4 h with petroleum ether. Then, 500 g of dry nonfat seed coat powder was weighed and dissolved in 100 mL of 50% ethanol in a beaker. The pH of the mixture was adjusted to 5.0 and the beaker was placed in a water bath at 35°C. The solution was then sonicated at the ultrasonic output power of 200 W for the duration of the extraction and the digestion with 3 U/g of the enzyme was performed for 1 h; after that, the enzyme was inactivated at 85°C for 20 min. The resultant solution was filtered by vacuum suction and each filtrate (about 250 mL) was concentrated to 100 mL in a rotary evaporator and used to determine the total silymarin content.

Determination of total silymarin: The total silymarin content was determined by the colorimetric method (Aliakbarian *et al*., 2012; Antoine *et al*., 2004; Karabegovic *et al*., 2011; Roux, 1957; Zhang *et al*., 2011) with some modifications. Briefly, 1, 2, 3, 4, or 5 mL of the diluted solution containing rutin in a 10 mL volumetric flask was mixed with 5 mL of 60% (v/v) ethanol and 0.3 mL of 5% (w/w) $NaNO_2$ for 6 min. Then, 0.3 mL of 10% $AlCl_3$ (w/w) was added and mixed for another 6 min followed by the addition of 4 mL of 1 mol/L NaOH and incubation for 15 min at room temperature. Afterward, 60% ethanol solution was added up to 10 mL and the absorbance was measured at 510 nm against the same mixture without the sample, as a blank. The calibration curve (y = 0.9787x+0.0021, where y is the absorbance of the sample and x is the sample concentration) ranged from 0 to 1 mg/mL (R^2 = 0.9974).

Evaluation of immunomodulatory activities of silymarin *in vivo*:

Experimental design: Will adapt to the 3 d in the laboratory of 100 mice after random points 5 groups, namely negative control group, the silimary low-dose group (silymarin-LG), the middle-dose group (silymarin-MG) and the high-dose group (silymarin-HG), each group of 20 mice. In the silymarin-LG, the silymarin-MG and the silymarin-HG, cyclophosphamide 40 mg/kg were orally intraperitoneal injection into mice once per day, for 3 d. Silymarin was given by gavage to the mice at doses of 100, 200 and 400 mg/kg, the blank control group given water filling and take each group were 0.1 mL/10 g, Samples were orally administered into mice using a feeding atraumatic needle once per day, at 8: 00 am, for 4 weeks. Detection the corresponding biochemical parameters assayed, including acid phosphatase activity, lysozyme and nitric oxide content, macrophage phagocytosis and immune organ indexes.

Acid phosphatase, lysozyme and nitric oxide measurements: After 28 days, 30 min after the last gavage administration of silymarin, 10 mice from each group were selected for analyses and blood samples were collected in heparinized tubes by removing the left eyeball. Serum was obtained by centrifugation at 1, 000 rpm, 4°C for 15 min. Acid phosphatase (ACP) activity and lysozyme and Nitric Oxide (NO) content were measured in the serum using commercial assay kits purchased from Jiancheng Biotechnology Co. (Nanjing, China) according to the manufacturer's instructions. After blood collection, the mice were immediately dissected and the livers were harvested, frozen in liquid nitrogen and kept at -80°C until the analysis.

Macrophage phagocytosis: Macrophage phagocytosis was determined using the carbon clearance method (Kawaguchi *et al*., 2006). India ink suspension diluted four times in saline was intravenously injected at 0.10 mL/10 g mouse body weight. Two and 20 min after the injection, 20 μL of blood was taken from the left and right eye, respectively, added to 2.5 mL of 0.1% Na_2CO_3 solution and the absorbance at 600 nm (OD) was measured using a UV-1700 spectrophotometer. The mice were sacrificed and the liver and spleen were weighed. Carbon dissection index (κ) and phagocytic index (α) were calculated according to the following equations:

$$\kappa = (\log OD_1 - \log OD_2)/t_2 - t \quad (1)$$

$$\alpha = \kappa^{1/3} \cdot W/WLS \quad (2)$$

where,

OD_1, OD_2 = The absorbance values of blood samples collected at 2 min and 20 min
t_1, t_2 = Blood collection times (2 and 20 min, respectively) after India ink injection
W = The body weight
WLS = The weight of the liver and spleen

Immune organ index: Ten mice randomly selected in each group were weighed and sacrificed. The thymus and spleen were removed, rinsed with saline, dried using filter paper and the weight ratio of immune organs to the body was calculated:

$$\text{Immune organ index} = \text{immune organs (g)} \times 10/\text{body weight (g)} \quad (3)$$

Statistical analysis: All data are represented as the mean±standard error in the tables and indicated by vertical bars in the figures. Differences between the groups were determined by the Analysis of Variance (ANOVA) and Student's t-test. Probability value (p) less than 0.05 was considered significant and p less than 0.01 was considered very significant.

Table 1: Silymarin effects on serum ACP activity in mice

	ACP activity	
Group	Blood serum (u/100 mL)	Hepatic tissue (u/g protein)
Negative-CG	56.21±5.97	2.12±0.13
Silymarin-LG	62.22±9.89[a]	2.88±0.15[a]
Silymarin-MG	64.99±7.22[a]	2.90±0.34[a]
Silymarin-HG	82.86±8.13[a]	3.22±0.46[a]

[a]: p<0.01, [b]: p<0.05, compared to the control group

Table 2: Silymarin effects on serum lysozyme and NO content in mice

Group	Lysozyme Blood serum (μg/mL)	No content Blood serum (μmol/L)
Negative-CG	8.7±1.2	2.62±0.9
Silymarin-LG	11.5±3.4[a]	4.26±1.1[a]
Silymarin-MG	15.7±3.6[a]	6.32±0.7[a]
Silymarin-HG	22.7±4.8[a]	8.01±0.8[b]

[a]: p<0.01; [b]: p<0.05; compared to the control group

Table 3: Silymarin effects on carbon dissection index and phagocytosis index in mice

Group	Carbon dissection index	Phagocytic index
Negative-CG	0.026±0.003	3.20±0.12
Silymarin-LG	0.031±0.007[b]	4.59±0.23[a]
Silymarin-MG	0.043±0.008[a]	5.00±0.41[a]
Silymarin-HG	0.054±0.091[a]	6.23±0.38[a]

[a]: p<0.01; [b]: p<0.05, compared to the control group

RESULTS AND DISCUSSIONE

E Valuation of silymarin immunomodulatory activity *in vivo*:

Silymarin effect on serum ACP activity: ACP activity is one of the reliable indicators of macrophage activation and, consequently, of nonspecific immune reactions in the body. Serum ACP levels presented in Table 1 indicate that serum ASP in the silymarin-LG, silymarin-MG and silymarin-HG groups were significantly higher, by 10.87% (p<0.05), 15.81% (p<0.01) and 47.41% (p<0.01), respectively, than those in the CG group. Similarly, ASP levels in hepatic tissues were significantly higher, by 35.84% (p<0.01), 36.79% (p<0.01) and 51.88% (p<0.01), respectively, than those in the CG group. These results indicate that ACP activity in the liver and blood of silymarin-treated mice was significantly increased compared to the control group (p<0.01); the effect was even more pronounced in the mice treated with silymarin-HG. The increase of ACP levels in the liver and serum of silymarin-treated mice suggests that silymarin enhances phagocytosis improving non-specific immune functions and resistance to infectious agents.

Silymarin effect on serum lysozyme and NO concentration: Lysozyme is one of the non-specific immune factors in the body, which participates in a variety of immune responses, including non-specific immunity and has an important role in the physiological balance. It can induce macrophage phagocytosis and digestion, activate the phagocytosis of white blood cells and enhance resistance to infection (Krusteva *et al*., 1997). Lysozyme serum levels in the silymarin-LG, silymarin-MG and silymarin-HG groups were significantly higher, by 32.18% (p<0.05), 80.46% (p<0.01) and 160.92% (p<0.01), respectively, than those in the CG group (Table 2). The results indicate that serum lysozyme in the silymarin-treated mice was significantly increased compared to the control group (p<0.01), confirming that silymarin enhances non-specific immune responses in mice.

NO is a free radical that has been established as an important cellular signaling molecule participating in many physiological and pathological processes (Macmicking *et al*., 1997). It has been demonstrated that macrophage anti-tumor activities and inhibition of intracellular pathogen proliferation are mediated via NO release, making NO level an important indicator of macrophage activation (Liew and Cox, 1991). Table 2 shows that NO release in the silymarin-LG, silymarin-MG and silymarin-HG groups was significantly higher, by 62.59% (p<0.01), 141.22% (p<0.01) and 205.73% (p<0.05), respectively, than that in the CG animals, confirming that silymarin could improve non-specific immune functions.

Silymarin effect on macrophage phagocytosis: Table 3 shows that carbon dissection index in the silymarin-LG, silymarin-MG and silymarin-HG groups was significantly higher, by 19.23% (p<0.05), 65.38% (p<0.01) and 107.6% (p<0.01), respectively, than that in the CG mice. Similarly, phagocytic index was also higher, by 43.44% (p<0.01), 56.25% (p<0.01) and 94.69% (p<0.01), respectively, than in the control mice.

The test results showed that purified silymarin can significantly improve carbon clearance index (κ) and phagocytic index (α), indicating that silymarin can induce macrophage phagocytosis and support the reticuloendothelial system in mice, further confirming its activity as an enhancer of non-specific immune responses.

Silymarin effect on the weight of immune organs: The weight of immune organs can reflect, to a certain extent, the number of lymphocytes in the organ and indirectly indicate the overall level of lymphoid cells in the body. The thymus and spleen are specialized organs of the immune system responsible for the generation of immune cells and thus, cellular and humoral immunity. Stressed animals demonstrate a reduction in the thymus and spleen weight; therefore, determination of the thymus and spleen weight index can reveal the functional status of the immune system.

Figure 1 shows that the spleen index in the silymarin-MG and silymarin-HG groups was significantly increased compared to the control group (p<0.05), but the increase in the silymarin-LG mice was not significant (p>0.05). Similarly, the thymus index in the silymarin-MG and silymarin-HG mice was significantly higher compared to the control group (p<0.01). These results are consistent with the data

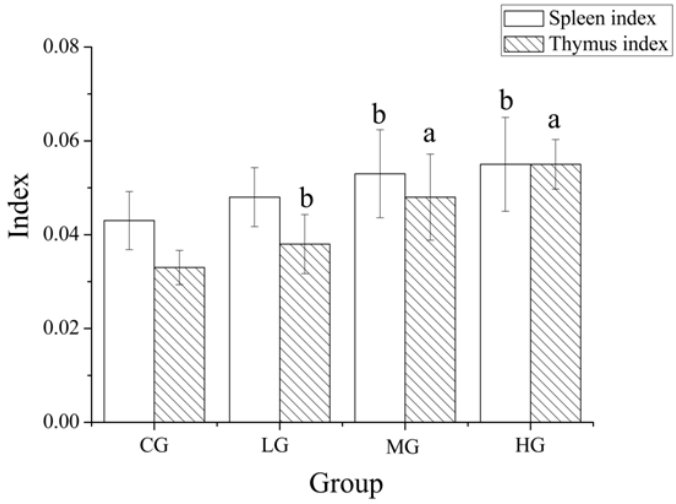

Fig. 1: The effect of silymarin on immune organ indexes in mice

presented in Table 1 to 3, confirming the immuno-modulatory activity of purified silymarin.

CONCLUSION

In the present study, we performed the immunomodulatory properties of purified silymarin *in vivo* were significant, as evidenced by ACP activity, lysozyme and NO content, carbon dissection and phagocytosis indexes and immune organ indexes, suggesting that silymarin can promote cellular and humoral immunity.

ACKNOWLEDGMENT

The authors are grateful to the Analysis and Testing Center of Shenyang Agricultural University.

REFERENCES

Aliakbarian, B., A. Fathi, P. Perego and F. Dehghani, 2012. Extraction of antioxidants from winery wastes using subcritical water. J. Supercrit. Fluid., 65: 18-24.

Antoine, M.L., C. Simon and A. Pizzi, 2004. UV spectrophotometric method for polyphe-nolic tannin analysis. J. Appl. Polym. Sci., 91: 2729-2732.

Bhatia, N., J. Zhao, D.M. Wolf and R. Agarwal, 1999. Inhibition of human carcinoma cell growth and DNA synthesis by silibinin, an active constituent of milk thistle: Comparison with silymarin. Cancer Lett., 147: 77-84.

Davis-Searles, P.R., Y. Nakanishi, N. Kim, T.N. Graf, N.H. Oberlies, M.C. Wani, M.E. Wall, R. Agarwal and D.J. Kroll, 2005. Milk thistle and prostate cancer: Differential effects of pure flavonolignans from *Silybum marianum* on antiproliferative end points in human prostate carcinoma cells. Cancer Res., 65(10): 4448-4457.

Flora, K., M. Hahn, H. Rosen and K. Benner, 1998. Milk thistle (*Silybum marianum*) for the therapy of liver disease. Am. J. Gastroenterol., 93: 139-143.

Hahn, G., H.D. Lehmann, M. Kürten, H. Uebel and G. Vogel, 1968. Pharmacology and toxicology of silymarin, an antihepatotoxic active principle from *Silybum marianum* (L.) gaertn. Arzneimittel-Forsch., 18: 698-704.

Karabegovic, I.T., V.B. Veljkovic and M.L. Lazic, 2011. Ultrasound-assisted extraction of total phenols and silymarin from dry tobacco (*Nicotiana tabacum*) leaves. Nat. Prod. Commun., 6: 1855-1856.

Katiyar, S.K., 2005. Skin photoprotection by green tea: Antioxidant and immunomodulatory effects. Curr. Drug Targets Immune Endocr. Metabol. Disord., 3(3): 234-242.

Kawaguchi, K., H. Maruyama and T. Kometani, 2006. Suppression of collageninduced arthritis by oral administration of the citrus flavonoid hesperidin. Planta Med., 72(5): 477-479.

Kim, N.C., T.N. Graf, C.M. Sparacino, M.C. Wani and M.E. Wall, 2003. Complete isolation and characterization of silybins and isosilybins from milk thistle (*Silybum marianum*). Org. Biomol. Chem., 1: 1684-1689.

Křen, V. and D. Walterová, 2005. Silybin and silymarin -new effects and applications. Biomed. Papers, 149: 29-41.

Krusteva, E., S. Hristova, D. Damyanov, A. Bogdanov, I. Altaparmakov and E. Pacelli, 1997. Clinical study of the effect of the preparation DEODAN on *Leukopenia*, induced by cytostatics. Int. J. Immunopharmacol., 19: 487-492.

Lahiri-Chatterjee, M., S.K. Katiyar, R.R. Mohan and R. Agarwal, 1999. A flavonoid antioxidant, silymarin, affords exceptionally high protection against tumor promotion in the SENCAR mouse skin tumorigenesis model. Cancer Res., 59(3): 622-32.

Lee, D.Y. and Y. Liu, 2003. Molecular structure and stereochemistry of silybin A, silybin B, isosilybin A and isosilybin B, isolated from silybum marianum (*Milk thistle*). J. Nat. Prod., 66(7-12): 1171-1174.

Lee, J.I., B.H. Hsu, D. Wu and J.S. Barrett, 2006. Separation and characterization of silybin, isosilybin, silydianin and silychristin in milk thistle extract by liquid chromatography-electrospray tandem mass spectrometry. J. Chromatogr. A, 1116: 57-68.

Liew, F.Y. and F.E. Cox, 1991. Nonspecific defence mechanism: The role of nitric oxide. Immunol. Today, 12(3): A17-21.

Macmicking, J., Q.W. Xie and C. Nathan, 1997. Nitric oxide and macrophage function. Annu. Rev. Immunol., 15: 323-350.

Mayer, K.É., R.P. Myers and S.S. Lee, 2005. Silymarin treatment of viral hepatitis: A systematic review. J. Viral Hepatitis, 12: 559-567.

Rickling, B., B. Hans, R. Kramarczyk, G. Krombiegel and R. Weyhenmeyer, 1995. Two high-performance liquid chromatographic assays for the determination of free and total silibinin deastereomers in plasma using column switching with electrochemical detection and reversed-phase chromatography with ultraviolet detection. J. Chromatogr. B, 670(2): 267.

Roux, D.G., 1957. Ultraviolet photometric methods of tannin extracts in relation to wattle extract utilisation. J. Am. Leather. Chem. As., 52: 319-329.

Saller, R., R. Meier and R. Brignoli, 2001. The use of silymarin in the treatment of liver diseases. Drugs, 61(14): 2035-2063.

Singh, R.P. and R. Agarwal, 2004. A cancer chemopreventive agent silibinin, targets mitogenic and survival signaling in prostate cancer. Mutat. Res., 555(1-2): 21-32.

Wagner, H., P. Diesel and M. Seitz, 1974. The chemistry and analysis of silymarin from Silybum marianum Gaertn. Arznei-Forschung, 24: 466-471.

Wellington, K. and B. Jarvis, 2001. A review of its clinical properties in the management of hepatic disorders. BioDrugs, 15(7): 465-489.

Zhang, G., L. He and M. Hu, 2011. Optimized ultrasonic-assisted extraction of silymarin from *Prunella vulgaris* L. and evaluation of antioxidant activities *in vitro*. Innov. Food Sci. Emerg., 12: 18-25.