Immunomodulatory effects of silymarin after subacute exposure to mice: A tiered approach immunotoxicity screening

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1. Introduction

The body is protected against foreign agents by the immune system. If the immune response is suppressed, the risks of infection and the development of specific types of cancers are increased. During the last few decades, evaluation of immunosuppression/stimulation of natural compounds has been of keen interest in treatment of special diseases [1-5]. From antiquity, people have sought treatment of their illnesses in nature. The use of natural medicines has been amplified in recent decades due to a low level of side effects and price, in addition to a good efficacy against several human diseases [6]. Silymarin is a flavonoid complex extracted from the Silybum marianum plant (Fig. 1) with a wide range of pharmacological and biochemical effects [7-9]. Silymarin contains some flavonolignans, including silybinin, isosilybinin, silychristin, and silydianin (Fig. 2). In traditional medicine, silymarin has been widely used as a hepatoprotective, anti-inflammatory, and antioxidant agent. However, its immunomodulatory effects have not been thoroughly investigated.

In the present study, the immunomodulatory effects of silymarin were investigated in BALB/c mice. Silymarin was administered daily by intraperitoneal injection at doses of 50, 100 and 150 mg/kg for 14 consecutive days. Following the exposure, host hematological parameters, spleen cellularity and histopathological examination, as well as delayed-type hypersensitivity (DTH) responses, hemagglutination titers (HA), splenocyte cytokine production and lymphoproliferation assay were studied in all of the test groups of animals. The results showed that the low dose of silymarin (50 mg/kg) could stimulate both cellular and humoral immune functions in the treated hosts. In addition, silymarin at 100 mg/kg appeared to impact on DTH responses and lymphoproliferation. Based on the finding here, it would seem that silymarin has efficient immunostimulant properties. As a recommendation, the application of silymarin along with acupuncture technique (herbal acupuncture) can be thought as a good plan to modulate and enhance the immune system for the management of several immunodeficiency disorders. However, further studies are required to demonstrate this hypothesis.

Key Words
Silymarin, Immunomodulatory, Cellular immunity, Humoral immunity

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Evidence has indicated that silymarin also may impart some immunomodulatory effects [3, 14-21]. For example, in an investigation of anti-inflammatory effects of silymarin, this extract attenuated allergic airway inflammation, atopic dermatitis, and allergic rhinitis [19-21]. The immunoprotective effects of silymarin, also have been assessed in UVB-exposed (ultraviolet type B) and sensitized to 2,4-dinitrofluorobenzene (DNFB) mice. Accordingly, they have suggested that silymarin acts to save dendritic cells (DCs) from UV radiation-induced DNA damage and improve UVB-induced DNA damage in DCs [18]. In another investigation, it has been reported that silibinin is a potent inhibitor of phenotypic and functional maturation of lipopolysaccharide (LPS) exposed dendritic cells [17]. This extract was also almost effective to inhibit the production of T helper-1 (TH1) related cytokines [Interleukin-2 (IL-2), Interferon- gamma (IFN-γ), and Tumor necrosis factor-alpha (TNF-α)] by activated peripheral blood mononuclear cells dose-dependently [14, 15]. In addition, in another study performed on male BALB/c mice, silymarin inhibited T-lymphocyte function at low doses (10 and 50 mg/kg) while stimulates inflammatory processes at, high dose (250 mg/kg) in a 5-day-regimen. They concluded that silymarin had a broad spectrum of immunomodulatory functions under different conditions [16]. Because the aforementioned studies only investigated the effects of silymarin on just a few aspects of the immune system, a study appeared necessary, one that is based on the tiered-approach to immunomodulatory screening [22]. Such studies are also needed in that, as noted above, some data were conflicting, thus limiting the potential wider use of silymarin as a therapeutic immunomodulant. Thus, due to this overall lack of information about the immuno-toxic effects of silymarin, the present study was carried out to investigate such effects following a set of subacute exposures in mice.

2. Materials and Methods

Animals

BALB/c inbred mice (female, 19-21 g, 6-8 weeks old) were purchased from the Pharmacy School of Mashhad, Iran and kept in large polystyrene cages and provided free access to rodent chow and water with an ambient temperature of 20-25°C with a 12 h light/dark lighting cycle. Animals were allowed to acclimate for at least one week prior to use. All protocols used in this study were approved by the Ethic Council of Mashhad University of Medical Sciences, Mashhad, Iran.

Chemicals

Phytohemagglutinin-A (PHA), lipopolysaccharide (LPS), cyclophosphamide (CYP) and silymarin were obtained from Sigma (UK) Company while fetal bovine serum (FBS) and RPMI-1640 medium were taken from Gibco (UK). Sheep red blood cells (SRBCs) were obtained from Razi Institute (Mashhad, Iran). IFNγ and IL4 ELISA kits were obtained from eBioscience Company.

Doses and exposure schedules

Dedicated sets of female BALB/c mice were used for each experiment. Five subsets of female BALB/c mice (n=6/group) were administered by three doses of silymarin, normal saline as negative and cyclophosphamide as positive controls. Mice in silymarin groups were injected intraperitoneally daily with a silymarin solutions (prepared in normal saline) to receive 50, 100 and 150 mg/kg of silymarin for 14 consecutive days. Mice in the negative and positive control groups received only normal saline and cyclophosphamide (20 mg/kg/day) injections for 14 days, respectively. All injection volumes were 100 μl.

Determination of the hematological parameters

Each mouse underwent blood sampling from the retro-orbital plexus (before being sacrificed) by capillary tubes. Two hundred microliters of blood from each mouse was dispensed in a sterile anti-coagulated Ethylene diamine tetra acetic acid dipotassium salt (K2-EDTA) tube to allow hematological indexes determinations. Furthermore, a smear of peripheral blood was also provided, stained with Giemsa, and then observed under a light microscope for differential count of leukocytes [23].

Histopathological examination

On day 15, different groups of animals were euthanized by cervical dislocation and spleen of each animal was then removed and stabilized in a solution of 10% formalin. Afterwards, the process of mounting of tissues was performed followed by staining of 5-μm thick sections of these tissues with Hematoxylin & Eosin (H&E). Furthermore, the femurs of mice were isolated and bone marrow smears provided and stained with H&E. Histopathological alterations of spleen and bone marrow in different groups

Figure 1 Silybum marianum

Figure 2 The major components of silymarin
of mice were then assessed through light microscopy [24].

**Preparation of single-cell suspension**

Each spleen removed was put into a small, clear and round dish containing 10 ml RPMI-1640 media supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 10% fetal bovine serum (FBS), and 2 mM glutamine. The spleen was disrupted between two frosted slides and the tissue dispersion formed was filtered via a nylon mesh with pore size of 40 μm and collected in a Falcon 50 ml centrifuge tube and then centrifuged at 1200 rpm at 4°C for 10 min. The supernatant was decanted and the pellet re-mixed in 3 ml of RBC lysis buffer (0.83% NH4Cl in 100 mM Tris buffer, pH 7.4) and incubated at room temperature for 3 min. Following three times washing of the cells with the media, the final pellet was suspended into 1 ml of the media containing 10% FBS. Spleen cell count was measured using the Neubauer chamber. Viability of cells was carried out using the trypan blue exclusion method [23].

**Hemagglutination assay**

On Day 10, the mice in each regimen were immunized by i.p. injection by 5×10^8 SRBCs in PBS. Injection of silymarin was resumed until day 14. At the termination of experiment, after preparing sera from peripheral blood samples, aliquots (50 μl) of two-fold dilutions of the sera (in PBS) were combined with 50 μl of a 2% [v/v] SRBCs suspension in a glass tubes. The tubes were kept at 37°C for 2 hours and then assessed for hemagglutination. The highest dilution giving hemagglutination was taken as the antibody titer [23].

**Lymphocyte proliferation test**

This experiment was performed as previously described [23]. Briefly, the 100 μl aliquots of the splenocytes isolated above at 2×10^6 cells/ml were dispensed into wells of a 96-well microtiter plate. Afterwards, complete media or Phytohemagglutinin-A (PHA at a final level of 5 μg/ml) or lipopolysaccharide (LPS at a final concentration of 1 μg/ml) was added to triplicate designated wells. The plates were then incubated for 48 h at 37°C in a CO2 humidified incubator and then cell proliferation was measured by an MTT [3-(4,5-dimethyl-2-thiazolyl)2,5-diphenyl-2H-tetrazolium] colorimetric assay. For this, 15μl MTT solution with a concentration of 5 mg/ml was added to each well at 37°C in CO2 humid incubator for a further 4 hours. The blue formazan formed was then dissolved by addition of 100 μl dimethylsulfoxide (DMSO) and the absorbance of the wells was measured at 570 nm by using Stat-FaxTM Elisa Reader. Proliferation index (PI) was calculated as follows:

PI = Absorbance of stimulated cells/Absorbance of unstimulated cells.

**Delayed-type hypersensitivity response (DTH)**

Delayed-type hypersensitivity response (DTH) was performed for experimental and control groups of mice using the method of Riahi et al [23]. On Day 9 of treatment, mice in each regimen were primed i.p. with 100 μl of a SRBCs solution containing 109 cells. Five days later, all mice were re-challenged with a booster dose of 108/50 μl SRBCs in the left hind footpad. As trauma control for elimination of non-specific swelling, the right hind footpad was injected with the same volume of PBS. Mean percentage increase in footpad thickness was calculated 24 h after the SRBCs challenge as following formula:

\[(\text{Left footpad challenged with SRBC} - \text{Right footpad}) \times 100 / \text{Right footpad}\]

**Cytokine production**

For this experiment, the supernatants harvested, subsequent to 48 h of splenocyte cultivation in the presence of PHA, were kept at 70°C until analysis. The levels of cytokines produced (IFNg and IL-4) by splenocytes in supernatants were determined using commercially available ELISA kits according to the manufacturer’s protocol [23].

**Statistical analysis**

Data were statistically analyzed by Student’s t-test to assess significant changes in the data of different groups. P values less than 0.05 were supposed significant. The values are expressed as means ± SEM.

| Parameter | Silymarin 50 mg/kg | Silymarin 100 mg/kg | Silymarin 150 mg/kg | Normal Saline | Cyclophosphamide 20 mg/kg |
|-----------|-------------------|-------------------|-------------------|---------------|-------------------------|
| WBC (count × 10^9/μl) | 14.1 ± 1.44 | 13.4 ± 1.89 | 14.8 ± 2.48 | 12.5 ± 2.30 | 4.1 ± 2.08* |
| RBC (count × 10^12/μl) | 8.3 ± 0.25 | 9.6 ± 0.10 | 9.1 ± 0.29 | 9.3 ± 0.29 | 8.7 ± 0.42 |
| Hct (%) | 43.1 ± 2.10 | 49.7 ± 0.71 | 47.4 ± 2.17 | 46.5 ± 2.56 | 47.0 ± 3.33 |
| HB (g/dl) | 13.25 ± 0.3772 | 15.30 ± 0.1975 | 14.20 ± 0.5845 | 14.02 ± 0.7358 | 14.18 ± 0.4191 |
| MCH (pg) | 96.7 ± 1.27 | 129.2 ± 7.4 | 122 ± 2.29 | 1026 ± 1.10 | 946 ± 2.67 |
| MCHC (g/dl) | 15.9 ± 0.40 | 15.9 ± 0.15 | 15.4 ± 0.77 | 14.8 ± 0.42 | 16.3 ± 0.44 |
| MCV (fl) | 32.05 ± 0.8884 | 31.02 ± 0.2059 | 29.95 ± 0.2872 | 30.10 ± 0.1844 | 30.20 ± 0.6621 |
| Neutrophil count (10^9/μl) | 4.96 ± 1.19 | 51.3 ± 0.48 | 51.6 ± 0.91 | 49.3 ± 1.43 | 53.7 ± 0.69 |
| Lymphocytes count (10^9/μl) | 8.0 ± 0.85 | 8.9 ± 1.38 | 10.5 ± 1.84 | 9.7 ± 1.70 | 3.3 ± 0.50 |
| MDO (count × 10^9/μl) | 0.57 ± 0.153 | 0.31 ± 0.033 | 0.37 ± 0.088 | 0.800 ± 0.142 | 0.17 ± 0.033 |
| Spleen cell content (10^9) | 3.6 ± 0.97* | 3.1 ± 3.48 | 3.0 ± 2.46 | 25.0 ± 3.12 | 17.3 ± 0.62*** |

WBC: White blood cell; RBC: Red blood cell; Hct: Hematocrit; HB: Hemoglobin; PH: Phlebot; MCH: Mean corpuscular hemoglobin; MCHC: Mean corpuscular hemoglobin concentration; MCV: Mean corpuscular volume; MXD: The sum of monocytes and eosinophils. Data shown as mean ± SEM. *p < 0.05 indicates significant changes compared to the negative control group. **p < 0.001 indicates significant changes compared to the negative control group.
Figure 3 Effect of subacute exposure to silymarin i.p. for 14 days on mice antibody response. Data shown as mean ± SEM. *p<0.05 indicates significant changes compared to the negative control group. **p<0.001 indicates significant changes compared to the negative control group.

Figure 4 Effect of subacute exposure to silymarin i.p. for 14 days on mice DTH response. Data shown as mean ± SEM. *p<0.05 indicates significant changes compared to the negative control group. **p<0.01 indicates significant changes compared to the negative control group. ***p<0.001 indicates significant changes compared to the negative control group.

Figure 5 Effect of subacute exposure to silymarin i.p. for 14 days on mice lymphoproliferation response to PHA and LPS. Data shown as mean ± SEM. *p<0.05 indicates significant changes compared to the negative control group. **p<0.001 indicates significant changes compared to the negative control group.

Figure 6 Effect of subacute exposure to silymarin i.p. for 14 days on cytokine produced (pg/ml) by isolated mice splenocytes. Data shown as mean ± SEM. *p<0.05 indicates significant changes compared to the negative control group. **p<0.01 indicates significant changes compared to the negative control group.
3. Results

Hematological parameters and spleen cellularity

None of the doses of silymarin caused mortality in the present study. The hematological findings and spleen cellularity in different groups of mice are shown in Table 1. Measures of the parameters in silymarin treated groups (except the neutrophil count) did not show any significant differences as compared to negative control. However, a significant increase in the neutrophil count of 50 mg/kg silymarin group was observed (p < 0.05). In addition, spleen cellularity changed significantly at dose of 50 mg/kg/day (p<0.05). Cyclophosphamide (CYP) caused significant reductions in leukocyte and lymphocyte levels (p<0.001) and also affected the levels of spleen cells in these hosts (p<0.001).

Histopathological examination

Spleen

Spleen was investigated in terms of any atrophy or hyperplasia in white pulp, white pulp: red pulp ratio, as well as the presence of any abnormality including necrosis, apoptosis, clumps, and debris in the white and red pulp regions. Also, any splenic trabecular abnormality was evaluated. The light microscopic evaluation of spleen tissue showed that silymarin at all doses did not have any significant side effect on spleen.

Bone marrow

Each bone marrow isolated was analyzed in terms of some important parameters such as maturation/presence of hematopoietic cell subtypes, cellularity, in addition to amount of the erythroid lineage relative to myeloid lineage. The observational analysis revealed that there was no significant pathologic change among the tissue samples obtained from the different silymarin groups as compared to negative controls.

Hemagglutination (HA) titer assay

Measures of serum anti-SRBC titer of 50 mg/kg silymarin group showed a significant increase as compared to negative control group whereas the amount of antibody against SRBCs in other groups of silymarin had no significant changes relative to negative controls (p < 0.05). Cyclophosphamide significantly (p < 0.001) decreased generation of anti-SRBC antibody (Figure 3).

Delayed-Type Hypersensitivity (DTH) response

With regard to evaluation of DTH response, there were significant suppression in 24h-DTH response of silymarin treated groups at doses of 50 mg/kg (p < 0.01) and 100 mg/kg (p < 0.05) when compared with negative controls (Figure 4). Cyclophosphamide group showed a significant decrease in DTH response (P < 0.001).

Proliferation responses to PHA or LPS

The results clearly demonstrated effects on the inducible proliferative responses of lymphocytes from mice treated with silymarin at doses of 50 and 100 mg/kg in comparison with values seen with cells from the control hosts (p < 0.05) (Figure 5). In contrast, PI (subsequent to LPS stimulation) values for the cells from mice treated with these three regimens were not found to have significant differences relative to negative control host (LPS-stimulated) PI levels. In addition, the positive control significantly decreased the proliferative response subsequent to PHA and LPS stimulation (p < 0.001).

Cytokine production

Host treatment with silymarin at 50 mg/kg/day significantly increased the ex vivo IFNγ production of their splenocytes in response to PHA (p < 0.05) relative to that by negative control mice splenocytes (Figure 6). On the other hand, IL-4 production by these same cells was not significantly affected by any doses of silymarin. Moreover, the positive control significantly reduced the production of IFNγ (p<0.01), while IL-4 generated by splenocytes did not show any changes in this group as compared to negative controls.

4. Discussion

Since the silymarin, as a traditional medicine or dietary supplement, has been believed that has a low toxicity and because it is critical to have a healthy immune system, this study was considered to evaluate potential immunomodulatory effects of silymarin after a subacute exposure. In this study, the potential immunomodulatory effects of silymarin in mice were explored. In the present study, a dose of 50 mg silymarin/kg caused significant stimulation in the immune endpoints examined. Specifically, silymarin at a dose of 50 mg/kg/day significantly increased the peripheral neutrophil and the spleen cell counts. In addition, a significant increase in PHA-induced splenocyte proliferation response (PI values) from the 50 mg/kg silymarin-treated mice suggested a stimulatory effect on the activation/proliferation of T-cells; these effects, in turn, appeared to manifest as enhanced DTH and HA response. Of course, silymarin at all doses did not show any changes in LPS-induced splenocyte proliferation response, suggesting that an increase in serum antibody against SRBCs (a T-dependent antigen) at a dose of 50 mg/kg may be secondary to T cells stimulation. On the other hand, a significant increase in the absolute numbers of spleen cells in the 50 mg silymarin/kg mice suggested that there was a stimulatory effect on the production of lymphocytes in primary lymphatic organs and/or on the deposition of blood lymphocytes in spleen. Moreover, the data here showed that silymarin at a level of 100 mg/kg caused stimulatory effects on some of the assessed endpoints, such as DTH responses after 24 h and lymphoproliferation assay in the presence of PHA. However, silymarin at a dose of 150 mg/kg was unable to induce any significant changes in immunological parameters.

As noted earlier, some of the immunomodulatory properties of silymarin observed here might be due to a direct effect on the activation/differentiation of lymphocytes.
T-cells arrange the acquired immune response and are essential for guarding and immunological memory. During the time naive T-cells are stimulated by foreign antigen, they experience an activation program in which they proliferate and then differentiate into effector subtypes [25].

There are studies showing that reactive oxygen species (ROS) can operate as signaling mediators in the above-mentioned processes [26-28]. Low levels of ROS are vital in inducing transcription of NF-κB and expression of genes for several essential cytokines and receptors needed for the proliferation of T-cell [29].

On the other hand, because unsaturated fatty acid side chains of phospholipids in cell membranes are vulnerable to free radicals attack, this effect could lead to reduced immune cell membrane fluidity. [30]. In normal circumstances the interaction of lymphocytes with antigens and other cell subsets needs the cell membrane integrity. Lower levels of intracellular antioxidants such as GSH in T-cells make them more susceptible to lipid peroxidation [31, 32]. Therefore in our study, silymarin at lower doses (50 and 100 mg/kg) may have acted as a potent antioxidant to remove any additional ROS that could partake in the disruption of T-cells membrane fluidity, ultimately resulting in overall immunostimulation. On the other hand, silymarin at dose of 150 mg/kg did not produce any significant change in non-functional/functional parameters of mice immune system. This may be related to the importance of the intracellular redox (reduction-oxidation) state in terms of maintaining correct function of immune cells. Indeed, the balance between oxidizing and reducing agents within immune cells controls their redox state. Temporary controlled changes of redox state such as increased formation of ROS is necessary for induction of various biological processes like activation and proliferation of immune cells [33, 34]. As a result, the absence of immunomodulatory effect of silymarin at high dose (150 mg/kg) may be due to scavenging both additional ROS and needed ROS for activation/proliferation of lymphocytes.

In addition, in our study, a significant increase in IFNγ and an insignificant decrease in IL-4 formation in cultures of spleen cells was observed with cells from mice treated with the lower dose (50 mg/kg) of silymarin, suggesting a shift in the host to a more T-helper TH-1 cell based response. While the expansion of IFNγ-producing TH1 cells would promote cell-mediated immunity, the expansion of IL-4-producing TH2 cells would increase generation of regulatory T-cells and, thus, weaken immune system reactions [35]. This shift may be an important factor in the overall observed immunostimulatory effects of silymarin; further study is underway to confirm this.

5. Conclusion

In conclusion, the current study showed that silymarin at low doses (i.e. 50 and 100 mg/kg/day for 14 days) imparted a stimulatory effect on mice immune system. At higher dose (i.e. 150 mg/kg/day), however, silymarin was unable to affect the immunological parameters. Further studies are needed to better determine the immunomodulatory properties of this natural product. Mechanistic studies can clarify precisely how this material is acting to impart the immunomodulatory effects demonstrated here. Particularly, studies should be performed to measure levels of ROS formed by mouse splenocytes after silymarin treatment and alterations in the activation of pathways in which ROS operate as a signal transduction intermediates in these cells.

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Conflict of interest

The authors declare that there are no conflicts of interest.
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