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Significant immunomodulatory and hepatoprotective impacts of Silymarin in MS patients: A double-blind placebo-controlled clinical trial

Faezeh Abbasirad [a], Vahid Shaygannejad [b,c], Fahimeh Hosseininasab [a], Omid Mirmosayyeb [d,e], Behzad Mahaki [f,g], Behjat Moayedi [a], Nafiseh Esmaeil [a,h,*]

[a] Department of Immunology, School of Medicine, Isfahan University of Medical Sciences (IUMS), Isfahan 81744, Iran
[b] Isfahan Neurosciences Research Center, Isfahan University of Medical Sciences, Isfahan 81744, Iran
[c] Department of Neurology, School of Medicine, Isfahan University of Medical Sciences, Isfahan 81744, Iran
[d] Universal Council of Epidemiology (UCE), Universal Scientific Education and Research Network (USERN), Tehran University of Medical Sciences, Tehran 14176, Iran
[e] Isfahan Neurosciences Research Center, Isfahan University of Medical Sciences, Isfahan 81744, Iran
[f] Department of Biostatistics, School of Health, Kermanshah University of Medical Sciences, Kermanshah 67148, Iran
[g] Social Development & Health Promotion Research Center, Health Institute, Kermanshah University of Medical Sciences, Kermanshah 67148, Iran
[h] Research Institute for Primordial Prevention of Non-Communicable Disease, Isfahan University of Medical Sciences, Isfahan 81744, Iran

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ABSTRACT

Interferon beta (IFN-β) has successfully been experimented with to treat multiple sclerosis (MS). However, patients sometimes do not respond effectively to treatment, and adverse effects, including liver toxicity, accompany this therapy. Accordingly, we decided to treat MS patients simultaneously with Silymarin (SM) as an immunomodulatory and hepatoprotective agent and IFN-β in a clinical trial study.

Complete blood count (CBC), liver enzyme levels, and the serum concentration of inflammatory and anti-inflammatory cytokines were measured. Also, the frequency of immune cells was determined by flow cytometry.

Liver enzyme levels were significantly lower in the intervention group ($p < 0.05$). The percentage of Th17 cells in the intervention group was significantly reduced compared to the placebo group ($P < 0.001$). Also, the frequency of Treg cells after treatment with SM plus IFN-β was significantly increased compared to the placebo group ($p < 0.05$). Furthermore, the IL-17 and IFN_{γ} cytokine levels were significantly reduced in the intervention group ($p < 0.05$). Moreover, the levels of anti-inflammatory cytokines IL-10 and TGF_{β} were significantly increased in the intervention group ($P < 0.05$). Overall, the results provide novel and supplementary information on SM's notable immunoregulatory effects on inflammatory response and liver function in MS patients.

Clinical Trial Identifier Number: IRCTID: IRCT20171220037977N1.

1. Introduction

Multiple sclerosis (MS) is a demyelinating inflammatory disease involving the central nervous system (CNS) [1]. There are four subtypes of MS: remitting-relapsing (RR), primary-progressive (PP), secondary-progressive (SP), and progressive-relapsing (PR) [2]. This disease initiates blood–brain barrier disruption, as well as the migration and infiltration of innate immune cells and autoreactive specific immune cells, including CD4+ T cells (especially Th1 and Th17 cells) from the periphery into the CNS. Th1 cells are a type of myelin-reactive T cell that release interferon γ (IFN-γ) and tumor necrosis factor-a (TNFa); they also activate innate immune cells such as macrophages. The other T cell subset, Th17, produces IL-17, IL-22, and IL-21. Both Th1 and Th17 exacerbate inflammatory immune responses in the CNS by producing inflammatory cytokines, increased antigen-presenting, and affecting the resident cells [3,4]. Moreover, macrophages and microglia cells induce oxidative stress, mainly in MS pathogenesis [5].

On the other hand, the functional impairment of regulatory CD4+, CD25+, FOXP3 + T cells (Tregs) has been indicated in MS and other autoimmune diseases [6]. Regulatory T cells play a prominent role in immune tolerance and autoreactive immune response inhibition. Treg induces its suppressor effects by releasing inhibitory cytokines such as IL-10 and TGF_{β}. Accordingly, an imbalance between auto-reactive T cells and Tregs and T cells' resistance to Tregs' suppressive effects has

* Corresponding author at: Department of Immunology, School of Medicine, Environment Research Center, Research Institute for Primordial Prevention of Non-communicable Disease, Isfahan University of Medical Sciences, Isfahan 81744-176, Iran.
E-mail address: n_esmaeil@med.mui.ac.ir (N. Esmaeil).

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been demonstrated in MS cases [4,7].

Various therapeutic strategies have indicated different impacts on autoreactive T cells and Tregs in MS disease. For example, studies have shown IFNβ therapy suppresses Th17 differentiation [8–10]. In other work, treating MS patients with Fingolimod and dimethyl fumarate caused Th17 responses to decrease [11,12]. However, patients sometimes show little or no response to treatment, and T cells' functions are not affected by treatment [13].

Cinovex is an Iranian type of beta interferon a-1 group that has been successfully used to treat RRMS in previous experiments [14,15]. Cinovex accompanies adverse effects like other beta interferons, including influenza-like syndrome and liver toxicity [16,17].

Silymarin(SM) is an herbal extract of the Silybum marianum (milk thistle) plant with hepatoprotective and antioxidant properties. The many facts about SM are related to its proven antioxidant effects [18]. However, recent studies have shown that SM represents immunoregulatory and anti-inflammatory impacts and modulates immune responses [19–23].

Based on the role of inflammatory immune responses and oxidative stress in the pathogenesis of MS, we decided to treat RRMS patients simultaneously with SM as an adjuvant therapeutic agent and Cinovex (an Iranian type of beta interferon a-1) in a double-blind clinical trial study.

2. Materials and methods

2.1. Study design

The trial was a double-blind randomized study of interferon beta-1 a (IFNβ) (Cinovex, Cinnagen. Iran) plus SM Vs IFNβ and placebo to treat RRMS.

Fifty-four RRMS patients who received IFNβ were recruited to take part in the present study based on referrals to the division of MS, Kashani Hospital, Isfahan University of Medical Sciences. These patients were treated either with SM (Meda Pharma, Germany) (27 patients, mean ages: 38.25 ± 12.55) or a placebo (27 patients, mean ages: 37.77 ± 7.90). Subjects were randomized to receive an intramuscular injection of IFNβ plus an oral dose of SM (420 mg, three 140 mg capsules per day, seven days a week) or a placebo. Follow-ups were conducted for up to six months.

To ensure the routine treatment in patients—and following ethical principles—all subjects received IFNβ. The inclusion criteria were as follows: a precise diagnosis of RRMS, no prior treatment with immunomodulatory or immunosuppressive drugs, an Expanded Disability Status Scale (EDSS) score of 0 to 3.5, no corticosteroid consumption within one month of enrollment, and no active infections or cancer. Subjects who had normal brain magnetic resonance (MR) imaging, other types of MS, systemic diseases, as well as those who had received concomitant therapy with β2-adrenergic agonists or antagonists, diuretics, tricyclic antidepressants, or monoamine oxidase inhibitors were excluded from the study.

A neurology specialist examined subjects at the beginning of the study and at three and six months, and blood collection was performed at baseline and six months. The physician recorded medication compliance and adverse events during each study visit. The study's protocol and potential risks were explained to participants, and written informed consent was obtained. The Ethics Committee approved the present study's protocol of the Isfahan University of Medical Sciences, Isfahan, Iran (IR.MUI.REC.1395.1.012). This trial was registered at www.clinicaltrials.gov as # (IRCTID: IRCT20171220037977N1).

2.2. Sample collection

Peripheral blood was collected from all participants just before and six months after SM or placebo treatment. Fifteen ml samples of blood were drawn from all subjects. Five ml samples of blood were allowed to clot at room temperature, and the serum was obtained by centrifugation and was stored at −20 °C until analysis. Also, 10 ml samples of blood were collected in EDTA tubes for flow cytometry analysis.

2.3. Safety and efficacy evaluation

A physician monitored adverse events, and none of the subjects reported any adverse reactions. Also, we assessed SM's safety by checking various laboratory parameters, including blood cell tests and liver enzyme detection. The efficacy of SM in reducing inflammation levels was determined as a primary objective in this study.

2.4. Blood markers disclosure

Complete blood count (CBC) was performed using an automated blood cell analyzer. The output included leukocyte, erythrocyte, and platelet counts and the hemoglobin, hematocrit, mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) in red blood cells. Liver enzyme levels, including Alanine transaminase (ALT), Aspartate transaminase (AST), and Alkaline phosphatase (ALP), were measured using the colorimetric method via an automated analyzer. According to the manufacturer's protocol, ferritin levels were detected using an ELISA kit (AccuBind, USA), and iron and NO levels were detected by a colorimetric method.

2.5. Enzyme-Linked immunosorbent Assay (ELISA) analysis

The serum concentrations of IL-10, IFN$^{\gamma}$, IL-17, and TGFβ were determined using the ELISA kits according to the manufacturer's instructions (PeproTech® EC Ltd., UK and R&D Systems, Minneapolis, MN, USA). Assay ranges were defined at 31.2–2,000 pg/mL for TGFβ, 47–3000 pg/mL for IFN$^{\gamma}$, 23–3000 pg/mL for IL-10, and 0.4–100 ng/ml for IL-17.

2.6. Isolation, Stimulation, Cell-Surface and intracellular staining of PBMCs

Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Paque gradient centrifugation (Inno-train, Germany). First, immunostaining was performed for CD4 as a surface marker using an FITC-conjugated anti-CD4 antibody. Then, fixation and permeabilization were performed according to the manufacturer's instructions (eBioscience, USA). Cells were then stained with PE-conjugated anti-IFNγ and PerCP-Cy conjugated anti-IL17 antibodies. Afterward, isotype controls were used for the compensation and confirmation of antibody specificity (all antibodies were bought from eBioscience, USA).

Treg cell immunostaining was carried out according to the manufacturer's instructions for a one-step staining human Treg flow TM kit (Biolegend, USA) using an anti-human CD4 PE-Cy5/CD25 PE cocktail and Alexa Fluor 488 anti-human FOXP3 antibody. Stained cells were assessed by a FACSCaliber flow cytometer (BD Bioscience Pharmingen, San Jose, CA, USA) and analyzed using FlowJo software (FlowJo, LLC).

2.7. Statistical analysis

The Kolmogorov-Smirnov test was used to analyze the normal distribution of data. A paired *t*-test or an unpaired *t*-test was performed to analyze normally distributed and parametric data. In contrast, Wilcoxon or Mann-Whitney was used to analyze non-normally distributed or non-parametric data. A value of $P < 0.05$ was considered statistically significant. Our data were analyzed using IBM SPSS version 22 (IBM, Armonk, NY, USA).

Table 1
Ages and sex differences between intervention and placebo groups.

Variable	Intervention Group	Placebo Group	P-value
Age(years,mean ± SD)	38.25 ± 12.55	37.77 ± 7.90	0.867
Sex (Female, n (%))	22(81.5)	24(88.9)	0.704

3. Results

3.1. Total blood cell counts and blood cell percentage

According to Table 1, there were no statistically significant differences in the age and sex between the two groups of patients treated with SM and the placebo. Thus, it is assumed that these two variables did not affect the results. Also, comparisons of blood cells, the number of white blood cells, red blood cells, platelets, hemoglobin level, hematocrit, and MCHE revealed no significant differences between the two groups

Table 2
Blood markers of study precipitants.

	Intervention group (mean ± SD)		Placebo group (mean ± SD)		F (1,41)	B(S.E)	Effect size	P-value
Variables	pre	post	pre	post				
WBC	5691.30 ± 15093	5669.56 ± 1303.6	5728.00 ± 847.30	5772.00 ± 794.26	2.78	352.38(211.28)	0.06	0.103
RBC	4.75 ± 0.47	4.71 ± 0.35	4.77 ± 0.47	4.73 ± 0.47	0.21	0.03(0.064)	0.005	0.642
Neut	55.88 ± 9.94	58.53 ± 10.44	56.25 ± 9.59	54.98 ± 7.74	2.50	4.19(2.65)	0.05	0.122
lymph	35.47 ± 9.09	32.17 ± 9.95	34.50 ± 9.28	34.38 ± 8.57	2.49	−3.81(2.41)	0.05	0.122
Hb	13.91 ± 1.13	13.51 ± 1.04	13.62 ± 1.63	13.58 ± 1.53	2.06	−0.17 (0.12)	0.04	0.15
Hct	41.14 ± 2.67	40.71 ± 2.25	40.63 ± 3.94	40.57 ± 3.87	0.10	−0.10(0.33)	0.003	0.746
MCH	29.33 ± 1.91	28.74 ± 2.48	28.86 ± 1.76	28.94 ± 1.83	4.15	−0.52(0.25)	0.09	**0.048**
MCHC	33.83 ± 1.91	33.16 ± 1.16	32.86 ± 1.90	32.77 ± 2.22	0.21	−0.17(0.38)	0.006	0.648
PLT	254217.39 ± 75076.90	244875.00 ± 61751.47	268360.00 ± 69436.94	314080.00 ± 222652.36	1.94	73045.67 (52357.05)		0.170

Values are showed as mean ± SD.
p values represent a test of crude differences between groups. WBC; White Blood Cells, RBC; Red Blood Cells, Neu; Neutrophil, Lymph; Lymphocyte, Hb; Hemoglobin, Hct; Hematocrit, MCH; mean corpuscular hemoglobin, MCHC; mean corpuscular hemoglobin concentration, PLT; Platelet.

Table 3
Liver enzymes, iron, and ferritin levels of study participants.

	Intervention group (mean ± SD)		Placebo group (mean ± SD)		F (1,41)	B(S.E)	Effect size	P-value
Variables	pre	post	pre	post				
AST(U/L)	26.04 ± 19.89	18.96 ± 4.94	24.38 ± 5.72	24.44 ± 5.90	10.65	−5.06(0.05)	0.19	**0.002**
ALT(U/L)	22.26 ± 16.89	15.62 ± 5.0.07	23.56 ± 13.04	23.94 ± 11.70	16.98	−4.69(1.55)	0.28	**0.001**
ALP(U/L)	170.90 ± 45.81	168.86 ± 35.99	177.76 ± 41.69	172.52 ± 38.29	3.23	−8.78(4.88)	0.07	0.080
Fe(*ug/dl*)	70.70 ± 27.58	91.43 ± 39.68	63.04 ± 35.77	64.74 ± 36.90	2.35	14.03(9.15)	0.05	0.133
Ferritin(*ug/ml*	81.95 ± 132.27	63.39 ± 62.89	74.80 ± 39.14	80.12 ± 34.65	2.57	8.84(5.51)	0.06	0.117
NO(*umol/L*)	11.19 ± 1.63	10.72 ± 1.27	11.50 ± 1.37	11.24 ± 1.21	2.42	−0.30(0.19)	0.04	0.126
EDSS	0.77 ± 0.75	0.77 ± 0.75	1.08 ± 0.71	1.2 ± 0.76	1.44	−0.09(0.08)	0.02	0.197

Values are showed as mean ± SD. *p* values represent a test of crude differences between groups. AST; Aspartate Aminotransferase, ALT; Alanine Aminotransferase, ALP; Alkaline Phosphatase, NO; Nitric Oxide.

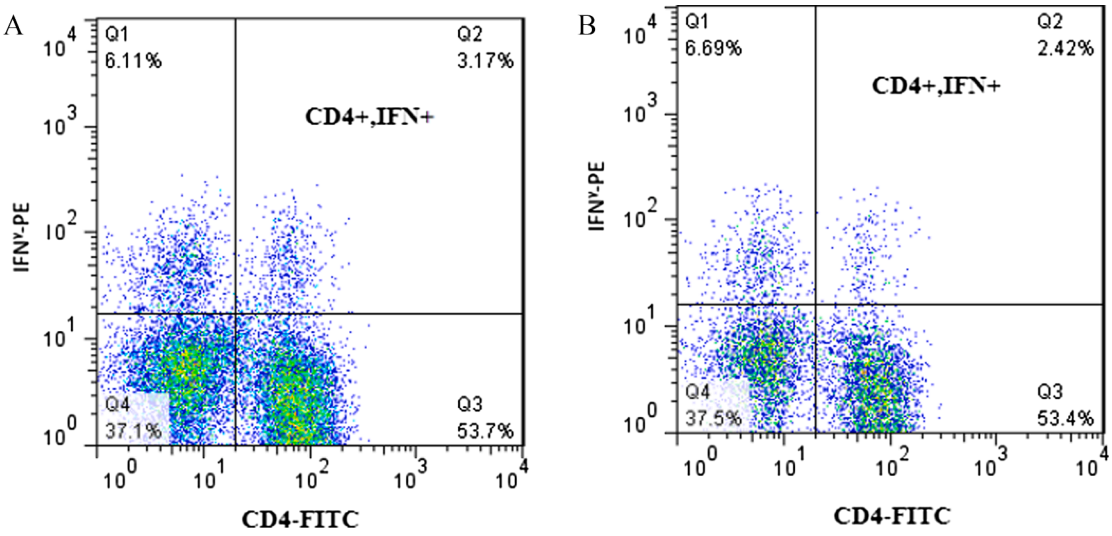

Fig. 1. Frequency of Th1 cells population 6 months after treatment with SM and placebo. A. Flow cytometry analysis of CD4+, IFN_{γ} + population in MS patients who treated with Placebo plus IFNβ. B. Flow cytometry analysis of CD4+, IFN_{γ} + population in MS patients who treated with SM plus IFNβ.

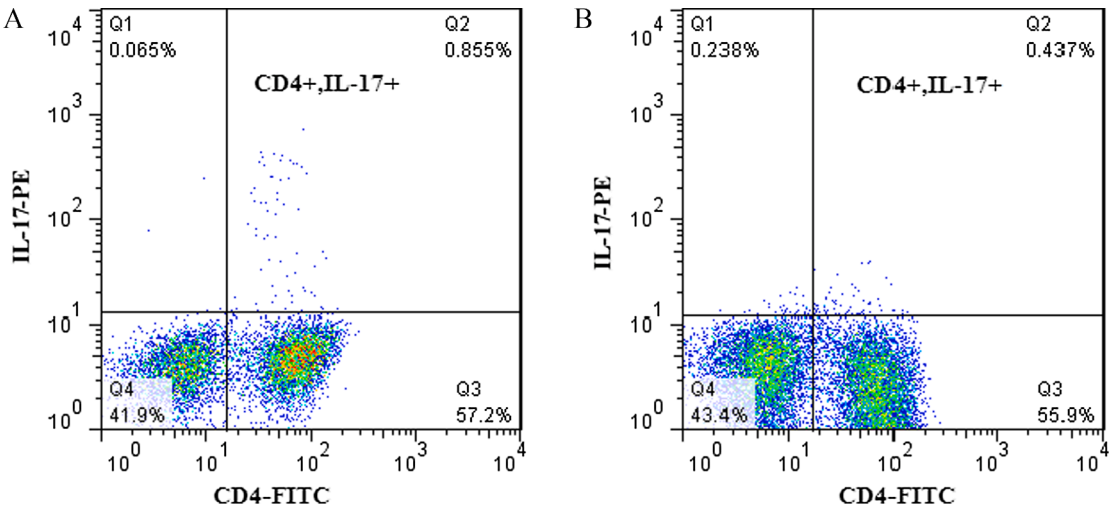

Fig. 2. Frequency of Th17 cells population 6 months after treatment with SM and placebo. A. Flow cytometry analysis of CD4+, IL-17 + population in MS patients who treated with Placebo plus IFNβ. B. Flow cytometry analysis of CD4+, IL-17 + population in MS patients who treated with SM plus IFNβ.

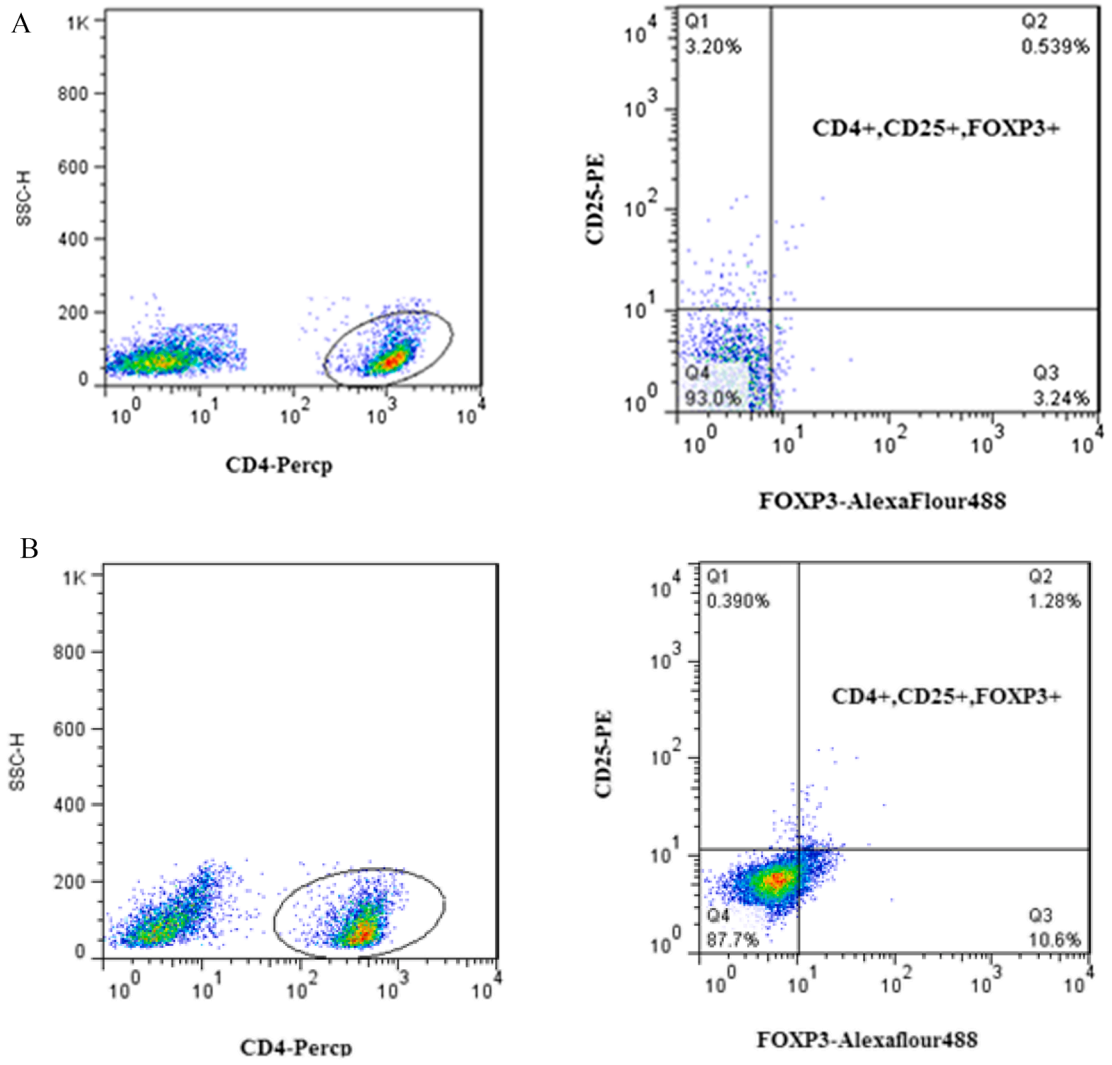

Fig. 3. Frequency of Treg cells population 6 months after treatment with SM and placebo. A. Flow cytometry analysis of CD4+, CD25, Foxp3 + population in MS patients who treated with Placebo plus IFNβ. B. Flow cytometry analysis of CD4+, CD25, Foxp3 + population in MS patients who treated with SM plus IFNβ.

Table.4
Percentage of Th1, Th17, and Treg cells in study participants.

	Intervention group (mean ± SD)		Placebo group (mean ± SD)		F (1,41)	B(S.E)	Effect size	P-value
Variables	pre	post	pre	post				
Treg%	0.40 ± 0.36	1.06 ± 0.75	0.54 ± 0.45	0.48 ± 0.32	21.38	0.67 (0.14)	0.30	**0.001**
Th1%	3.51 ± 2.61	3.21 ± 3.29	2.56 ± 1.83	2.89 ± 1.94	1.00	−0.51 (0.51)	0.01	0.320
Th17%	0.90 ± 0.61	0.61 ± 0.32	0.75 ± 0.48	0.88 ± 0.62	12.82	−0.36 (0.10)	0.20	**0.001**

Values are showed as mean ± SD. *p* values represent a test of crude differences between groups. Treg: Regulatory T cell, Th1: T helper1, Th17: T helper17

Table.5
Inflammatory and anti-inflammatory cytokine levels in study participants.

	Intervention group (mean ± SD)		Placebo group (mean ± SD)		F (1,41)	B(S.E)	Effect size	P-value
Variables	pre	post	pre	post				
IFN_{γ}(pg/ml)	4004.44 ± 4074.58	2769.44 ± 2898.19	47.44.76 ± 4578.28	4642.38 ± 3935.00	6.08	−1377.87 (558.64)		**0.018**
IL-10(pg/ml)	7.91 ± 4.51	12.47 ± 5.16	8.23 ± 3.28	8.23 ± 3.28	9.72	4.12(1.32)	0.18	**0.003**
TGFβ (pg/ml)	156.47 ± 111.39	281.49 ± 187.55	167.74 ± 125.37	179.85 ± 122.42	12.49	113.19 (32.01)	0.21	**0.001**
IL-17(ng/ml)	1.59 ± 0.39	1.10 ± 0.12	1.52 ± 0.48	1.53 ± 0.51	45.60	−0.46(0.06)	0.49	**0.001**

Values are showed as mean ± SD. *p* values represent a test of crude differences between groups. IFN^{γ}; Interferon gama, IL-10; Interleukin 10, TGFβ; Tumor growth factor beta, IL-17; Interleukin 17

(Table 2). However, as shown in Table 2, the mean corpuscular hemoglobin (MCH) level was significantly different between the SM-treated and placebo groups ($p = 0.048$).

3.2. Comparison of iron, ferritin, nitric oxide, liver enzyme levels, and EDSS between the two groups

As shown in Table 3, the comparisons of iron and ferritin levels in the two groups did not present any significant differences between the SM-treated and placebo groups (p = 0.133, P = 0.117).

The levels of liver enzymes, including aspartate aminotransferase (AST), alanine aminotransferase (ALT), and alkaline phosphatase (ALP), were lower in the intervention group than in the placebo group. These differences were statistically significant only for AST and ALT (P = 0.002, P = 0.001, respectively). Although the NO level decreased in the intervention group, this decrease was not statistically significant (P = 0.126). Moreover, the EDSS scores of patients treated with SM did not change, while they increased in the placebo group. However, the difference between groups was not significant.

3.3. Comparison of the percentage of Th1, Th17, and Treg cells between IFNβ plus SM treated and placebo plus IFNβ groups

Although the percentage of Th1 cells in patients treated with SM plus IFNβ decreased after six months compared to the placebo plus IFNβ group, this decrease was not statistically significant (P = 0.320) (Fig. 1).

The percentage of Th17 cells' population in the intervention group was significantly reduced after six months compared to the placebo group (Fig. 2) (P = 0.001). Also, the percentage of Treg cells after treatment with SM plus IFNβ was significantly increased when compared to the placebo plus IFNβ group (P = 0.001) (Fig. 3 and Table 4).

3.4. A comparison of mean concentrations of IFN_{γ}, TGF_{β}, Il-10, Il-17 cytokines between the two groups

Comparing IL-17 and IFN_{γ} cytokine levels in the two groups showed that these two cytokines were significantly reduced in the intervention group compared to the placebo group (P = 0.018, P = 0.018, respectively) (Table.5). Also, the level of anti-inflammatory cytokines IL-10 and TGF_{β} in the intervention group was significantly increased compared to the placebo group (P = 0.001, P = 0.003, respectively) (Table.5).

4. Discussion

Although different therapeutic agents, including cytokines, monoclonal antibodies, and corticosteroids, are used for MS treatment, none are definite treatments, and they all have various adverse effects. Therefore, using natural immunomodulatory compounds is recommended, as they may reduce the side effects of such drugs and improve their effectiveness. In the present study, we found that SM, as an adjuvant therapeutic drug, decreased liver enzyme levels in MS patients treated with IFNβ.

The treatment of MS patients with different forms of IFNβ has been associated with hepatic injuries. Also, increased aminotransferase levels have been found in the serum of many patients, and persistent ALT elevation in MS patients suggests the probability of chronic hepatitis [24,25]. Moreover, Durelli et al. detected autoantibodies that fight against organ-specific antigens and non-organ-specific antigens in MS patients treated with IFNβ—their presence is thought to be related to thyroid or liver function alterations [26].

Silymarin is probably the most applied natural compound for treating hepatic disorders worldwide [27,28]. The hepatoprotective effects of SM are exerted through the reduction of free radicals formed by toxins. The use of SM in non-alcoholic hepatic steatosis patients after six months significantly decreased the AST and ALT levels in patients while also stabilizing the hepatocyte membrane [29]. Therefore, considering the side effects of IFNβ on the liver and our study's findings, it seems that using SM in patients treated with IFNβ can improve hepatocytes' function.

We also found that co-treatment of MS patients with SM and IFNβ after six months decreased the percentages of Th1 and Th17 cells while increasing the percentage of Treg cells. Also, the levels of cytokines related to Th1 and Th17 cells—including IFN_{γ} and IL-17, respectively—were diminished after SM and IFNβ treatment, while the levels of anti-inflammatory cytokines, including IL-10 and TGF-β, increased.

Our findings agree with the results of our previous study, which assessed SM's effects on isolated Th1, Treg, and Th17 cells (data not published) in MS patients treated with IFNβ in vitro [20,21]. We found that SM significantly decreases Th1's specific transcription factor (T-bet) and the amount of IFN_{γ} produced by these cells. Also, enhanced

production of TGF-β and IL-10—accompanied by the up-regulation of FOXP3, JAK3, and STAT5 gene expression—were found in Treg cells isolated from IFNβ-treated RRMS patients [20,21].

Min et al. indicated that treating EAE mice (an animal model of MS) with Silibinin significantly diminishes the histopathological signs of demyelination in the spinal cord. Also, the presence of inflammatory cells in the CNS has significantly decreased after Silibinin treatment. Moreover, consistent with our findings, they have shown Th1-related cytokine levels were decreased after Silibinin treatment while the levels of Th2-related cytokines were upregulated [30]. The neuroprotective effects of SM have also been indicated in Alzheimer-like disease in rats [31]. Thus, the lack of significant changes in the EDSS of MS patients after SM treatment and the impacts of SM immunoregulation in our study seem to indicate its neuroprotective effects in MS patients. However, an assessment of brain plaques using MRI techniques is needed to confirm the neuroprotective effects of SM. In another study, SM's effects on chronic and acute activation of immune cells have been evaluated, and the researchers suggested SM significantly suppresses pathogen-associated molecular pattern (PAMP)-induced inflammations.

According to previous studies, SM suppresses inflammatory responses by inhibiting NF-κB and m-TOR signaling pathways. These immunomodulatory effects of SM are similar to those of other immunoregulatory drugs such as Rapamycin. Accordingly, studies have shown that the suppression of m-TOR increases FOXP3 expression, and Bagherpour et al. have found that *FOXP3* gene expression upregulates six months after treatment with Rapamycin [32]. Accordingly, all of the above findings indicate the suppression of m-TOR while JAK3/STAT5 signaling pathways increase the Treg cell population. So, SM exerts its immunoregulation activity both by decreasing inflammatory responses and increasing the Treg cell population.

5. Conclusion

The results present new supplementary findings on Silymarin's notable immunoregulatory effects on inflammatory responses and liver function in MS patients. In agreement with previous studies, SM therapy has no adverse effects. Therefore, it is recommended as an adjuvant treatment with acceptable anti-inflammatory and hepatoprotective impacts in MS patients.

Our results show that SM, at a dose of 420 mg per day, can significantly deplete inflammation by decreasing Th1 and Th17 while increasing the Treg cell population in MS patients under treatment with IFNβ. As such, it seems that SM diminishes the disease's severity and activity, likely due to its immunomodulatory impacts on Th1, Th17, and Treg cells. Also, a decrease in liver enzyme levels after treatment with SM in MS patients under therapy with IFNβ lessens the side effects of IFNβ on liver function. These virtues of SM might be correlated with its anti-inflammatory and hepatoprotective effects.

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