The Effect of Aqueous Extract of Tarragon on Clinical Symptoms and T Cell Differentiation in Experimental Autoimmune Encephalomyelitis

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ABSTRACT

Multiple sclerosis (MS) is one of the autoimmune diseases that affects the central nervous system (CNS) and causes myelin loss and axonal damage. Recent studies have shown the important role of autoreactive T cells in the pathogenesis of MS. One of the plants in the Astersa family, which has therapeutic benefits, is Artemisia dracunculus L. or Tarragon. In this study, the role of aqueous extract of Tarragon in suppressing Th1 and Th17 cell differentiation and ameliorating experimental autoimmune encephalomyelitis (EAE) was investigated.

EAE was induced in C57BL/6 female mice by Hook kit MOG35-55/CFA Emulsion PTX and one group was treated with Tarragon at a dose of 500 mg/kg. Mice were euthanized on day 33 post-immunization, spleens were removed for assessing Th1, Th17 and Treg cells by flow cytometry. We provided evidence that Tarragon (500 mg/kg) significantly ameliorated clinical scores of EAE.

We did not observe significant alterations in T cell differentiation to Th1, Th17 or Treg in the spleen of mice during EAE.

This is the first experimental evidence showing that administration of aqueous extract of Tarragon reduces the severity of EAE, but the protective effect of Tarragon is independent of alteration in T cells in the spleen. These results suggest other mechanisms for the effectiveness of this extract in improving the EAE process.

Keywords: Artemisia dracunculus; EAE; Tarragon; Th1 cells; Th17 cells; Treg cells

INTRODUCTION

Multiple sclerosis (MS) is one of the autoimmune diseases that affects the central nervous system (CNS) and causes myelin loss and axonal damage.[1] This chronic inflammatory disease affects millions of people worldwide and is on the rise.[2] The incidence of MS, like many other autoimmune diseases, is higher in women than men.[3] The exact cause of MS is still unknown, but many environmental and genetic factors have been suggested for it.[4] Among these, recent

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studies have shown the important role of autoreactive T cells in the pathogenesis of MS.[5] Experimental autoimmune encephalomyelitis (EAE) has been widely used among various animal models to understand the immunopathology of MS.[6] During EAE autoreactive T cells are activated in peripheral lymph organs and by clonal expansion a large reservoir forms from these cells.[5] Then, these cells enter the CNS by affecting the blood-brain barrier (BBB).[5] In this organ targeting myelin proteins and producing inflammatory cytokines leading inflammatory lesions, myelin destruction and loss of neuronal function.[7] Among autoreactive T cells, the destructive role of some of CD4 + T-cells has been identified in the pathogenesis of this disease. Interferon-gamma producing CD4 + T-cell has been shown to increase in the blood and CSF (Cerebro-spinal fluid) of MS patients.[8] Other studies have identified mice with deficiencies in IFNγ, the fundamental cytokines of Th1 cells and the STAT1 (Signal transducer and activator of transcription 1) signal transmission pathway indication an intensified EAE.[9] Another class of CD4 + T-cells is Th17, which secrete a series of proinflammatory cytokines including Interleukin 17, 21, and 22.[10] Reductions in these cells have also been reported in the blood and CSF of MS patients and the presence of these cells causes damage to the blood-brain barrier which is essential for the entry of other immune cells to the CNS.[8,11] Additionally, administration of anti-IL-17 inhibits the onset of EAE, as well as the transmission of Th17 cells, induces intense EAE.[12,13] Evidence suggests the presence of T-regulatory cells (Tregs) in the CNS during EAE and their increase in the final stages of the disease, which leads to EAE amelioration.[14] In confirmation of this, the removal of CD4 + CD25 + T cells prevents natural recovery from the EAE.[14] These FOXP3 + (forkhead box P3) cells play an important role in achieving immune balance and prevention the autoimmune disease by inhibition of the activity of other immune cells.[15] Considering these new findings, it appears that the population of CD4 + T cells is likely to be considered for the development of new therapeutic approaches for MS. Although pharmaceutical treatments control MS, they are not fully effective and are also associated with side effects.[16] Recently, attention has been drawn to the use of alternative and complementary therapies, including the use of medicinal herbs.[16]

One of the plants in the Astersa's family, which has therapeutic benefits is Artemisia dracunculus L. or Tarragon[17] This plant contains various compounds, including phenylpropanoids,[18] comarin[19,] and isocoumarin.[20] The plant has antibacterial,[21] antifungal,[22] antitumor,[23] antidiabetic[24] and antioxidant[25] effects. The extract of this plant has been able to reduce the secretion of interferon gamma from the PBMC (Peripheral Blood Mononuclear Cell) in the culture medium.[26] It has been shown that Tarragon inhibits the production of nitric oxide and also inhibits the production of interleukin 6 pro-inflammatory cytokine, which indicates the anti-inflammatory properties of this plant.[27]

Based on these results, we hypothesized that Tarragon may have a protective effect in MS/ EAE by impacting the immunopathological processes involved in the disease development. In this study, the role of aqueous extract of Tarragon in suppressing Th1 and Th17 cell differentiation and ameliorating EAE was investigated.

MATERIALS AND METHODS

Preparation of the Aqueous Extract of Tarragon

Briefly, the Tarragon (Artemisia dracunculus L.) was purchased from the herbal institute in Birjand, Iran. Aerial parts including stems and leaves were separated and dried under the shade in room temperature, then turned into powder and were macerated in distilled water (1000 mL) with constant stirring for 24 hours. Afterward, the mixture was centrifuged for 10 minutes at 1789 g. Then passed through filter paper (Pore size:<2 µm, Blue Ribbon, Grade 589, Germany), and concentrated under vacuum evaporator, subsequently lyophilized by freeze dryer (Dena Vacuum Industry, model FD-5005-BT, Iran) to produce the crude aqueous extract.

Animals

Female C57BL/6 mice (12 weeks) were purchased from the Pasteur Institute of Iran. Mice were maintained in control environment (12 h light/dark cycle and 21-25°C temperature) and allowed free access to water and diet. All mice were observed daily for general health and clinical signs of disease. At the end of the study, Mice were prepared for sampling using ketamine-xylazine (100:20 mg/kg) anesthetics. All conditions and handling of the animals in the present study were approved by the Ethics Committee of

Birjand University of medical science, Iran (N. ir.bums.REC.1396.318).

EAE Model and Treatment

EAE was induced using the Hooke Kit MOG35-55/CFA Emulsion PTX (cat. No. EK-2110, Hooke Laboratories, USA) according to the manufacturers' instructions.[28] According to the protocol, on day zero, MOG35-55/CFA Emulsion (0.1 mL/site) was injected subcutaneously at two places on the mouse's back, the upper back and lower back. Following a two-hour PTX (80 ng for each mouse) dissolved in Phosphate-Buffered Saline (PBS) (PH of 7.4), was injected for the first time subcutaneously. After 24 hours, the second injection of PTX was performed intraperitoneally. Recording of EAE symptoms began 7 days after immunization and mice were checked daily. The scoring criteria used were as follows: 0, no clinical signs; 0.5, partially limp tail; 1, paralyzed tail; 1.5, paralyzed tail and Mild hind limb weakness; 2, paralyzed tail and middle hind limb weakness; 2.5, Weight intolerance on the hind limbs with some movement in the limbs; 3, complete paralysis of both hind limbs; 3.5, complete paralysis of hind limbs and weakness in Front limbs; 4, complete paralysis of both hind and Front limbs; 4.5, moribund; and 5, Death.[28]

Mice were divided into three groups (6 mice per group) as follows: Group I (healthy control group): Mice in this group were considered as healthy normal without EAE and only treated with PBS as the vehicle. Group II (EAE model group): Mice in this group were considered as PBS-treated EAE group without receiving Tarragon extract. Group III (Tarragon-treated EAE group): The mice with EAE that received 500 mg/kg Tarragon extract[29] via oral gavage for 33 days (Figure 1).[30]

Sample Collection

The mice were sacrificed on Day 33 PI (post-immunization). The spleens of mice were removed and the splenocytes were obtained by mashing the organ through a cell strainer (40μm, BD Falcon) into culture media (RPMI 1640, Dacells) using a syringe plunger. Then, splenic mononuclear cells (SMCs) were isolated by Ficoll (Ficoll-Paque™ PREMIUM, GE Healthcare) gradient method and SMCs washed using RPMI-1640, subsequently. The cells were then frozen in a cryo-medium containing 90% FBS (Gibco) and 10% DMSO (PAN BIOTECH). The cryotubes were placed at -20ºC degrees for 2 hours, then transferred to -80ºC and finally transferred to the nitrogen tank (-196ºC) after 24 hours.
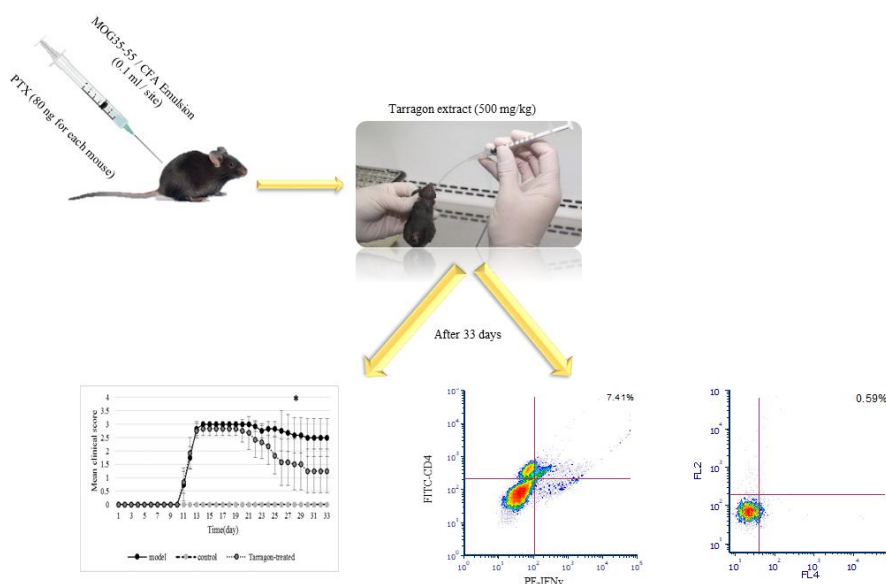

Figure 1. The protocol of research. Experimental autoimmune encephalomyelitis (EAE) induction in Female C57BL/6 mice for evaluation the effect of aqueous extract of Tarragon. EAE was induced using the Hooke Kit MOG35-55/CFA Emulsion PTX. Mice were divided into three groups: healthy control group, model group, and Tarragon-treated EAE group. The third group received 500 mg/kg Tarragon extract via oral gavage for 33 days. In the end, Flow cytometry analysis was performed.

Flow Cytometry Analysis

The cells were thawed and washed with RPMI 1640 supplemented with 10% fetal calf serum (FCS), 2 mM L-Glutamine (Gibco), and 100 U/mL Penicillin-Streptomycin. A single cell suspension of spleen mononuclear cells was first stimulated for 5–6 h at 37°C with cell Activation Cocktail containing brefeldin A (Biolegend, San Diego, CA, USA) according to the manufacturer's protocol. The activated cells were harvested from 24 well plates and washed in PBS (PH of 7.4). For blocking of Fc receptors TruStain fcX™ anti-mouse CD16/32 Antibody (Biolegend, San Diego, CA, USA) was used and incubated for 10 min in 4°C. Surface staining was then performed using anti-CD4 FITC (Biolegend, San Diego, CA, USA) and -anti-CD-25 PE (Biolegend, San Diego, CA, USA) at room temperature for 30 min. After 1 washing with ice-cold staining buffer, cells were fixed in 3% formaldehyde for 15 min followed by permeabilization using True-Nuclear™ Transcription Factor Buffer Set (Biolegend, San Diego, CA, USA). The cells were then stained intracellularly with anti-IL17 PE (Biolegend, San Diego, CA, USA), anti-IFN ɣ PE (Biolegend, San Diego, CA, USA) and anti-FoxP3 Alexa Fluor 647 (Biolegend, San Diego, CA, USA) for 60 min at room temperature. A control group stained with isotype control antibody containing: FITC Rat IgG2b k (Biolegend, San Diego, CA, USA), Alexa Fluor 647 Mouse IgG k (Biolegend, San Diego, CA, USA), PE Rat IgG1 k (Biolegend, San Diego, CA, USA). Cells were washed twice with permeabilization buffer and fixed in formaldehyde. Flow cytometry was performed using CyFlow Cube 6 cytometer (Sysmex Partec, Germany) and analyzed using FCS express 6 (DeNovo™ Software).

Statistical Analysis

Statistical analyses of data were performed using the SPSS 16 software (SPSS Inc. Chicago, Ill, USA). The Kolmogorov-Smirnov test (k-s) was used to examine the normal distribution of the parameters. One-way ANOVA test followed by Tukey's post hoc test was used to compare all groups. GraphPad Prism 6 software (GraphPad Inc., San Diego, CA, USA). was used to plot the graphs. Data were presented as mean±SD. A p-value of <0.05 was considered significant.

RESULTS

Tarragon Extract Ameliorate Severity of Clinical Scores in EAE Mice

In the present study to determine the effects of Tarragon extract on EAE, 12-week-old female C57BL/6 mice were subcutaneously injected with MOG35–55 peptide and were sacrificed on day 33 post-immunization. Tarragon extract was gavaged daily (at concentrations of 500 mg/kg) when the mice showed slight clinical symptoms (clinical scores ≤1.0). Symptoms of EAE appeared in experimental mice from Day 11 PI. The clinical scores of EAE mice reached a peak on Day 14 (Figure 2).
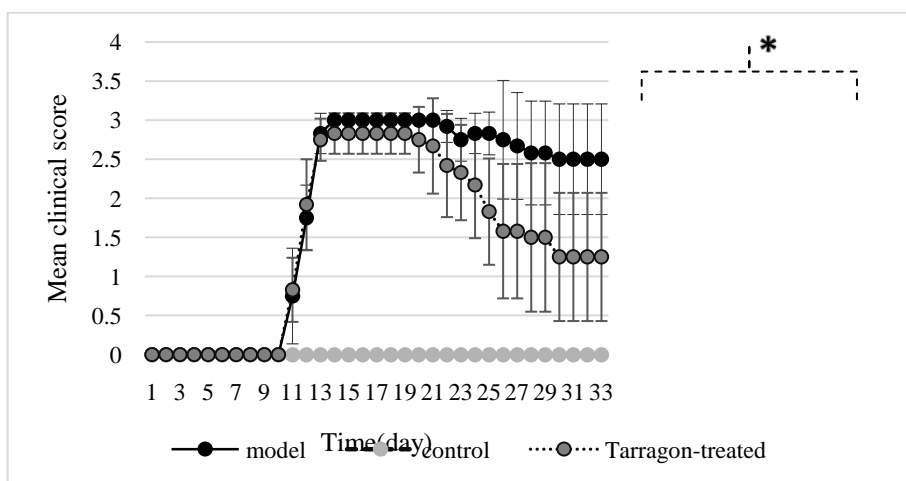

Figure 2. Tarragon reduced disease severity of experimental autoimmune encephalomyelitis (EAE). Mice (N=6) were treated with Tarragon (500 mg/kg) or phosphate-buffered saline (PBS) after EAE induction and scored daily (0: normal to 5: death). Data are expressed as mean clinical score±SD (One-way ANOVA test; *p<0.05 treatment vs model)

These scores were decreased significantly from Day 24 to Day 33 PI in Tarragon -treated mice, compared to the scores in EAE mice ($p<0.05$). Results showed that Tarragon treatment decreased the EAE scores and enhanced clinical recovery from EAE.

Tarragon Did Not Alter Pathogenic T Cell Subsets

Immunopathology of the EAE indicates the role of autoreactive CD4 + T cells in the formation of the disease. The important role of interleukin-17 (IL17) secreting CD4+ T cells and interferon γ (IFN- γ) secreting CD4+ T cells has been shown with resistant to EAE in mice which had defective Th1 and Th17 development (i.e., IL-23-specific p19 subunit-deficient mice).[31] In contrast to the pre-inflammatory activity of Th1 and Th17 cells, Treg cells play an inhibitory role in MS. Treg is suppressed the proliferation and function of autoreactive T cells by the use of multiple mechanisms including TGF-beta (Transforming growth factor beta) secretion and expressing CTLA-4 (cytotoxic T-lymphocyte-associated protein 4), resulting in self-tolerance in the EAE.[32] We examined the presence of Th17 and Th1 cells during EAE in the spleen of mice. The spleen of mice swas harvested on day 33 after immunization with MOG.[35–55] Intracellular cytokine staining showed that the frequencies of both Th1 and Th17 cells were higher in a model group than in controls (Figure 3.D, E). But the frequency of Th1 and Th17 cells did not decrease significantly in the splenocytes of Tarragon treated group. In the frequency of Treg cells, there was no significant difference between groups. We used intracellular cytokine staining for Foxp3, IFN-γ and IL-17A to identify Treg, Th1 and Th17 cells, respectively (Fig3.A-C), and found that Foxp3+, IFN-γ+, and IL-17A+ CD4 T cells were unaffected by Tarragon treatment. These results suggest other mechanisms for the effectiveness of this extract in improving the EAE process.

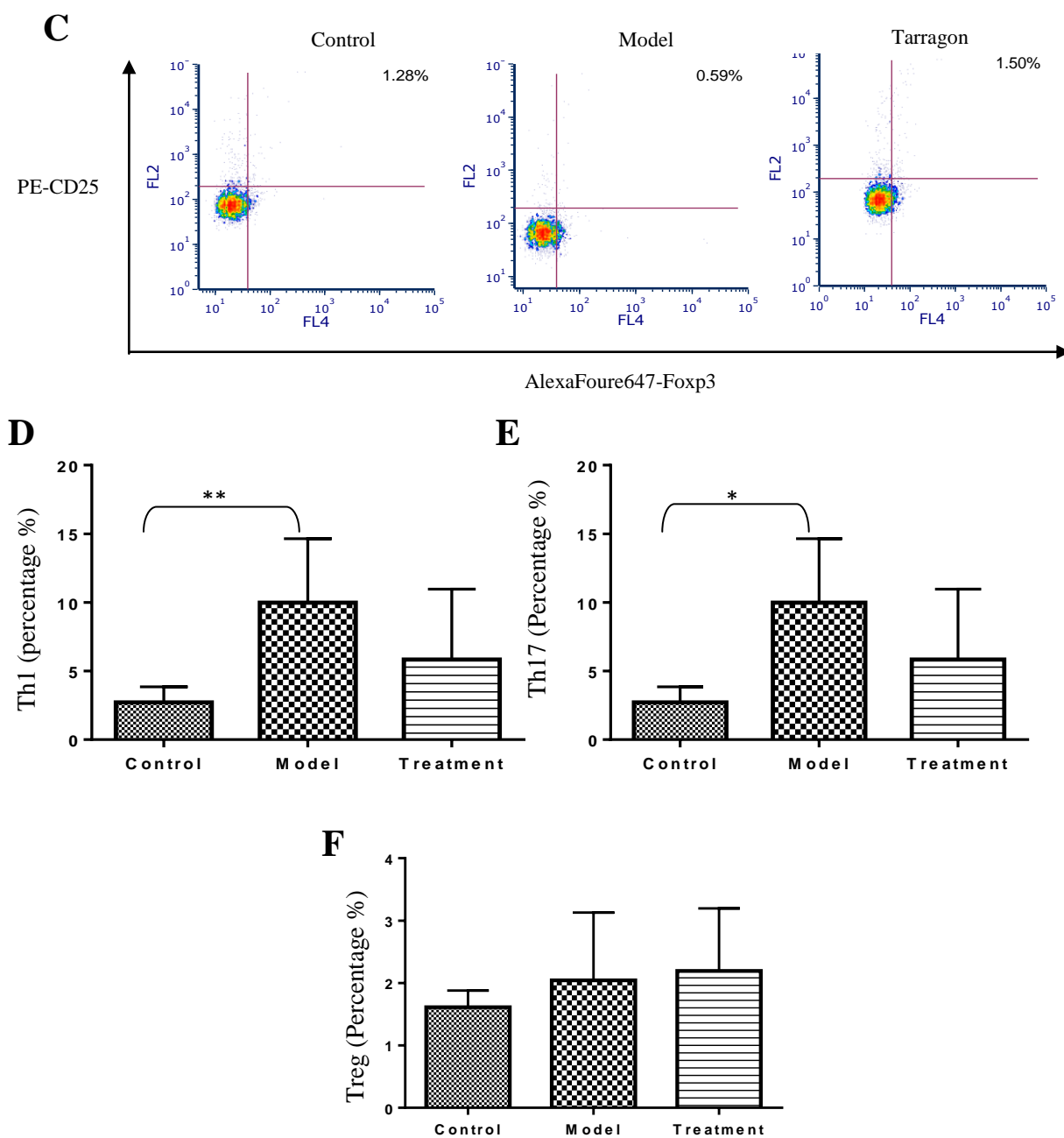**C**

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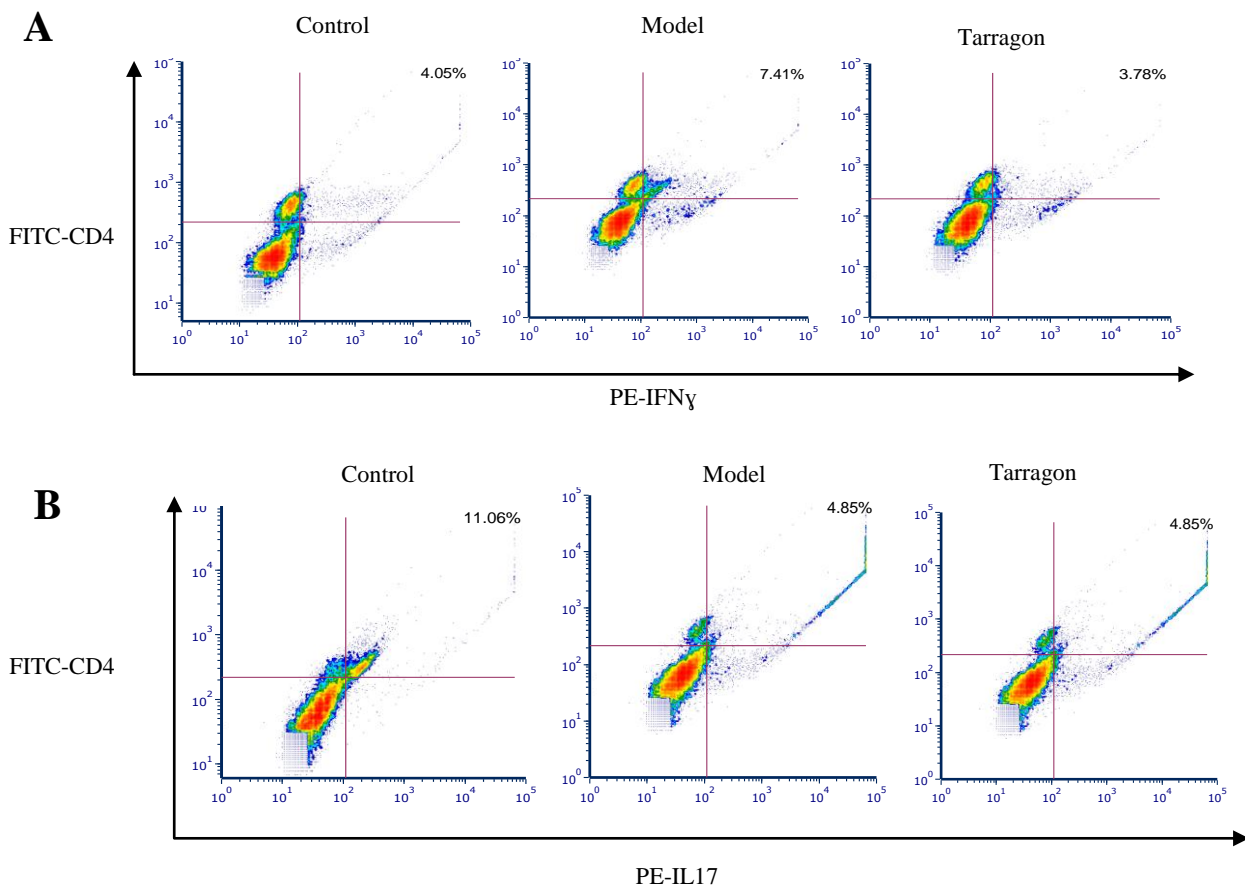
Figure 3. Tarragon did not alter T helper (Th) cell subsets in the spleen of mice. For evaluation the effect of aqueous extract of Tarragon on experimental autoimmune encephalomyelitis (EAE), the spleen of mice were removed and the cells were analyzed by intracellular staining of interferon γ (IFN-γ), Interleukin 17 (IL-17A), and forkhead box P3 (Foxp3) (A-C). Statistical analysis was performed using SPPSS software. Th1 cells (D), Th17 cells (E), T-regulatory (Treg) cells (F) frequencies in the spleen of mice. (One-way ANOVA test; *p <0.05, **p <0.01, N=6)

Tarragon and the Th17/Treg Cell ratio in EAE

The imbalance between Th17 cells expressing the RORγt (retinoic acid-related orphan receptor gamma) transcription factor and the regulatory T-cells that express Foxp3 plays an important role in inducing inflammatory immune responses.[33] This Th17/Treg imbalance is associated with various autoimmune and inflammatory diseases, including MS.[34] To study the effects of the Tarragon extract on EAE, we measured the Th1/Treg and Th17/Treg ratio in the splenocyte of
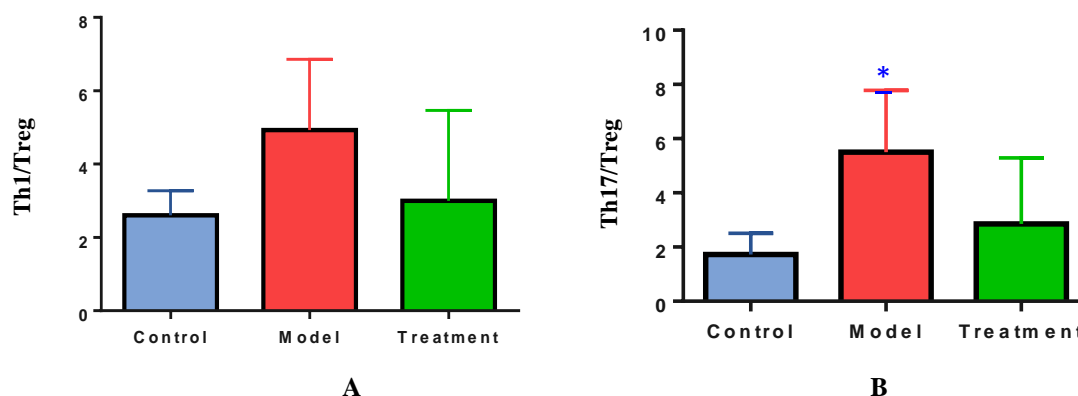
mice.

Figure 4. Tarragon does not improve the cell's ratio in experimental autoimmune encephalomyelitis (EAE). For evaluation the effect of aqueous extract of Tarragon on EAE, the spleen of mice were removed and the cells were analyzed by Flow Cytometry. Th1/Treg (A), Th17/Treg ratio (B). Data are presented as means ±SD. (One-way ANOVA test; *$p< 0.05$, N=6)

The Th17/Treg ratio in the mice in the model group was significantly higher than the control group, but there was no significant decrease in the Tarragon treated group (Figure 4.B). In the case of the Th1/Treg ratio, no significant difference was found between the studied groups (Figure 4.A).

DISCUSSION

EAE a well-known animal model for MS is an auto-reactive T-cell dependent disease. T cells include CD4+IL17+ and CD4+IFNγ+ by targeting myelin proteins and releasing inflammatory mediators, leading to myelin destruction and extensive axonal damage.[35] Current MS treatments include one of the following actions: inhibiting the inflammatory response, reducing certain classes of immune cells, stopping immune cells in the lymph nodes, or limiting immune cell access to the CNS.[36] In the Asteraceae's family, Artemisia dracunculus L. (Tarragon) with a widespread distribution in the west part of North America, Eastern Europe and most of temperate Asia has a long history of use as a spice.[18] It also has a wide range of health benefits and has therefore been widely used as herbal medicine. Previous studies on A. dracunculus indicate that this plant has pharmacological properties, including anti-inflammatory, antibacterial, antidiabetic and antioxidant.[18,21,24,25] Rezaei R, et al have found that the secretion of interferon gamma from the PBMC in the culture medium in the presence of this plant was decreased.[26] Also, studies have shown that Tarragon inhibits the production of nitric oxide and interleukin 6

pro-inflammatory cytokine, which indicates the anti-inflammatory properties of this plant.[27] The present study was conducted to evaluate the therapeutic potentials of Tarragon on the improvement of MOG-induced EAE in C57BL/6 mice. Tarragon markedly was able to reduce EAE. In our study, we did not observe significant alterations in T cell differentiation to Th1 and Th17 during EAE. In the study conducted by Haghmorad D, et al administration of hesperidin did not significantly decrease the percentage of Th1 cells in the spleen, while reducing the percentage of Th17 cells.[37] In another study by Yang C, et al for investigation of Betaine's effect also was shown similar results.[38] Contrary to our results, in other studies by Li W, et al using Arctigenin,[39] Hwang I, et al using acidic polysaccharide from Panax ginseng[30] as well as Shen R, et al using icariin,[40] Th1 and Th17 cells were significantly decreased in the spleen. In contrast to the pro-inflammatory action of Th1 and Th17 cells, Treg cells play an inhibitory role in EAE. Some of the mechanisms of immunosuppression by Treg cells include secretion of anti-inflammatory cytokines and expression of inhibitory receptors.[41] Our results showed that the Tarragon extract was not able to significantly increase Treg cells in the spleen and this result was similar to the study by Yang et al.[38] Other studies, including the study by Zheng et al. On the effects of Bu Shen Yi Sui capsule,[42] the study of Haghmorad et al. on the effects of hesperidine[37] and the study by Zarei et al.[43] On the clozapine drug, was showing a significant increase in Treg cells in the spleen of mice. This suggests that the protective effect of Tarragon does not

directly correlate with alteration of the T cell response. Some evidence suggests that proinflammatory monocytes are important in EAE. For example, the infiltration of monocytes into the CNS results in worsening of the intensity of EAE.[44] Removal of monocytes and/or macrophages in rodents reduces or prevents the clinical manifestation of EAE.[45] Also, depletion of monocytes and/or macrophages reduces entry of CD4+ T cells into the CNS.[46] In general, proinflammatory myeloid cells have multiple functions in EAE, including the production of cytokines and chemokines that can further boost inflammatory responses. These responses include the summons of more lymphocytes to the CNS,[47] phagocytosis of debris (degenerated myelin, for example) and production of oxidative stress and other mediators of injury.[48] In previous studies, the immunomodulatory effects of Tarragon had been shown. Tarragon reduces the production of IL-17 and IFN-γ pro-inflammatory cytokines, as well as the reduction of the ratio of INF-γ to IL-10 and IL-17 to IL-10 in splenocytes. In addition, it has been observed that the amount of respiratory burst and production of nitric oxide in peritoneal macrophages and the phagocytosis potential of macrophages in mice treated with Tarragon has been reduced.[49] Another study has shown that Tarragon causes inhibition LPS/INFγ-induced inflammation and inflammatory mediators in macrophages through AMPK (5' adenosine monophosphate-activated protein kinase) activation.[27] It is possible that Tarragon has an effect on the activation of macrophages and microglia cells in the CNS. In conclusion, our results indicate the protective effect of Tarragon independent of alteration in T cells in the spleen. We believe that the protective mechanism may include other immune pathways such as inhibiting the inflammatory response and alteration of myeloid activation in the CNS. Other mechanisms that may be related to the protective effect of Tarragon in EAE include stopping immune cells in the lymph nodes or limiting immune cells access to the CNS which should be investigated in the future. One of the limitations of our work was the inability to perform flow cytometry analysis immediately after the separation of the cells from the spleen. Another limitation is the impossibility of using a chemical drug for MS treatment as a positive control and using several doses of the extract, due to the high cost of the Hook kit so, we could not have more EAE mice. Finally, it has been suggested that therapeutics that are targeted specifically to a myeloid cell in the CNS and readily cross an intact BBB would be an advantage.[50] These findings support the possibility that Tarragon can be a potential treatment for MS.

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