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Beneficial effects of *Thymus vulgaris* extract in experimental autoimmune encephalomyelitis: Clinical, histological and cytokine alterations

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ABSTRACT

The imbalance between pro and anti-inflammatory cytokines plays an important role in the pathogenesis of multiple sclerosis (MS) and its animal model, experimental autoimmune encephalomyelitis (EAE). *Thymus vulgaris* (thyme) as a traditional medicinal plant has been reported to exert antimicrobial, antioxidant, and anti-inflammatory effects. Therefore, this study evaluated the modulatory effects of *Thymus vulgaris* on the clinical symptoms, histopathological scores, and the production of some anti-inflammatory (TGF-β, IL-4, and IL-10) and pro-inflammatory (IFN-γ, IL-6 and IL-17) cytokines in EAE model. EAE was induced by MOG35–55 peptide and mice were treated intra-peritoneally (i.p) with phosphate buffered saline (PBS) in the control group or thyme extract (50 or 100 mg/kg of body weight, every other day) in thyme-treated EAE groups, from day 0 to +21 of post MOG immunization. Mice were sacrificed at day 22, and splenocytes were isolated and re-stimulated *in vitro* with MOG in order to measure the cytokine production and proliferation of re-stimulated cells by enzyme linked immunosorbent assay (ELISA) method and WST-1 reagent, respectively.

The clinical symptoms and histopathological scores of the CNS were lower in thyme-treated than EAE control group. Furthermore, the production of IFN-γ and IL-6 by splenocytes was lower in thyme-treated EAE than in the control group. The production of IL-10 and TGF-β increased in mice treated with thyme extract compared to the control group. In this study, we showed for the first time that the immunomodulatory effects of *Thymus vulgaris* in EAE model. Thus, the possible therapeutic potential of thyme for treatment of MS could be considered in future research.

1. Introduction

Multiple sclerosis (MS) is a chronic neurodegenerative disease of the central nervous system (CNS), in which the neurological disabilities appear following the damage to the myelin, axons, and oligodendrocytes [1]. Experimental autoimmune encephalomyelitis (EAE) is an appropriate animal model for MS, which is usually induced in a susceptible rodent by the immunization with various myelin-derived components such as MBP, PLP, and MOG [2]. In MS and EAE, peripheral immune cells (neutrophils, DCs, macrophages, $CD4^+$ T cells, and $CD8^+$ T cells) infiltrate the CNS and play a critical role in the demyelinating process [3]. Moreover, some residential cells of the CNS such as microglia and astrocytes contribute to the pathogenic pathways through the production of inflammatory cytokine [4].

Abbreviations: AP-1, Activator protein 1; BBB, blood-brain barrier; CFA, Complete Freund's adjuvant; CNS, Central nervous system; CRP, C-reactive protein; EAE, Experimental autoimmune encephalomyelitis; H&E, Hematoxylin and eosin; IP, Intra-peritoneal; IFN-γ, Interferon gamma; MCP-1, Monocyte chemotactic protein-1; MMP-9, Matrix metalloproteinase; MOG, Myelin oligodendrocyte glycoprotein; MS, Multiple sclerosis; MMCS, Maximum mean clinical score; OVA, Ovalbumin; PHA, Phytohemagglutinin; PBS, phosphate buffered saline; SI, Stimulation Index; TGF-β, Transforming growth factor beta; VCAM-1, Vascular cell adhesion molecule-1

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Among leukocytes, the $CD4^+$ T helper (Th) cells play a pivotal role in the development or progression of EAE and MS. Depending on the cytokine milieu, T helper cells are differentiated into four main subtypes each possessing a unique function in the immune system. Differentiation of the Th1, Th2, Th17 and regulatory T cells (Treg) is induced in the presence of IFN-γ/IL-12, IL-4, IL-6/TGF-β, and TGF-β/IL-2, respectively [5].

Th1 cells mediate an essential function in the neuronal demyelination of MS and EAE [5]. The expression of Th1 cell-related cytokines such as IFN-γ is increased in the CNS of MS patients as well as EAE mice, causing activation of microglia, macrophages, and astrocytes [6].

In addition, Th17 cell-derived cytokines, especially IL-17 A, can induce the expression of chemokines such as CXCL1 and CXCL8, matrix metalloproteinases and ROS, which in turn promote leukocyte migration into the CNS and local lesions [7,8]. Also, elevated levels of IL-6 and IL-17 have been observed in the serum, CSF and CNS lesions of MS patients [7,9].

In contrast, Treg cells as key players in maintaining peripheral self-tolerance, secret immunosuppressive cytokines including TGF-β, IL-10 and IL-35 [5]. It has also been indicated that both TGF-β and IL-10 (signature cytokines of Treg) confer protection against EAE and MS through the inhibition of inflammatory T cell responses [5,10]. Likewise, the reduced numbers of Treg cells as well as IL10 and TGF-β cytokines has been reported in both MS and EAE [3].

Th2 cells inhibit the differentiation of Th1 cells by secreting IL4 and IL10, which exhibits a protective effect on EAE development [5,11]. However, in some studies, it has been observed that IL-4 could shift immune responses toward humoral immunity and enhance the production of myelin-specific autoantibodies [12,13].

So far, various immunomodulatory and anti-inflammatory agents, including β-interferon, glatiramer acetate, corticosteroids, and monoclonal antibodies have been used to improve MS symptoms [1]. Nevertheless, the low efficacy and relevant side effects of mentioned medications encourage scientists to research more effective and safer agents [14]. Hence, the indigenous herbs with a long history of use in traditional medicine could be a proper candidate to achieve this goal. *Thymus vulgaris* (thyme) contains active components, among them, thymol and carvacrol have beneficial therapeutic effects [15]. Experimentally, the antimicrobial, antioxidant, anti-nociceptive, antispasmodic and anti-inflammatory effects, as well as wound healing activities of the thyme extract and its active constitutes, were demonstrated [16–19]. The beneficial effects of *Thymus vulgaris* extract or its derivatives have also been demonstrated in a number of human inflammatory diseases and animal models [20–23].

To the best of our knowledge, there is no study concerning the effects of *Thymus vulgaris* extract or its components on MS or EAE. Thus, this study is aimed, for the first time, to evaluate the effects of *Thymus vulgaris* extract on the clinical and histopathological symptoms of EAE model. In addition, we evaluated the modulation of several important anti-inflammatory and pro-inflammatory cytokines.

2. Materials and methods

2.1. Extract preparation of Thymus vulgaris

Thymus vulgaris (thyme) was obtained from an Agricultural research farm (Isfahan, Iran) and the plant verification was done by Dr. M. Mirtadzadini with a Voucher Number of 2130, at the Department of Botany, ShahidBahonar University (kerman, Iran).

The aerial parts of fresh thyme were cleaned, washed, air-dried, and a fine powder was primed using a pestle and mortar. Then, 200 ml ethanol 70% was added to 100 g of prepared thyme powder in a suitable container, for 72 h in the dark, with continuous mild shaking for 15 h. After that, the liquid was isolated from solid using filtration and then it was concentrated using a rotary evaporator at 40 °C to obtain a semi-dried extract which was kept at a temperature of 4 °C.

2.2. HPLC analysis of Thymus vulgaris

High performance liquid chromatography (HPLC) analysis was performed using a Knauer system equipped with a vacuum degasser, quaternary solvent mixing, and a WellChrom K-2600 UV detector. Detection wavelength for carvacrol and thymol was set based on their standard solutions (purity > 98%) in 274 and 277 nm, respectively. EZ ChromElit software was utilized as instrument control, data collection and data processing. The column used was a Perfectsil Target ODS-3 C18 (4.6 × 250 mm, 5 µm). A gradient elution (methanol and water) with a flow rate of 1 ml/min was applied for this analysis. Injection volume for all samples and standard solutions was 20 µl.

2.3. Mice

Female C57BL/6 mice (8–12 weeks of age) were purchased from Royan Institute of Isfahan, Iran. All mice were kept in the animal house with a constant temperature and 12- hour light/dark cycle and allowed to free access to rodent food and water. The experiments were performed according to the guidelines of the Animal Ethics Committee of Mashhad University of Medical Sciences, Mashhad, Iran.

2.4. Induction and scoring of EAE

EAE was induced by subcutaneous injection of 200 ml emulsion containing MOG35–55 peptide (SBS Genetech) and complete Freund's adjuvant (CFA) (Sigma) at two sites of animal body: between the shoulder, and the other in the flank. Each mouse received 400 mg MOG 35–55 and 0.4 mg *Mycobacterium tuberculosis* (100 ml CFA). Two additional injections of pertussis toxin (250 ng) (Sigma, USA) were also performed intra-peritoneally (ip), on the day of MOG immunization and 48 h later.

Mice were allocated into 3 groups (5 mice in each group) as follows: group I was considered as an EAE negative control group which was subjected to EAE induction and treated with PBS vehicle. Mice in groups II and III were considered as thyme-treated EAE groups which were subjected to EAE induction and were treated with 50 mg/kg- or 100 mg/kg of thyme extract, respectively [24]. After EAE induction, the mice were treated intra-peritoneally (ip) with PBS in the control group or thyme extract (50 or 100 mg/kg of body weight, every other day) in thyme-treated EAE groups, from day 0 to +21 of post MOG immunization. The clinical symptoms of EAE and body weight were measured daily, until day 21.

The clinical EAE score was categorized as follows: 0: no clinical signs; 1: incomplete loss of tail tonicity; 2: complete loss of tail tonicity; 3: flaccid tail and abnormal movement; 4: hind leg paralysis; 5: hind leg paralysis with hind body paralysis; 6: hind and foreleg paralysis; and 7: death [25]. The mice were sacrificed at day 22, and then the spleen, brain, and lumbar spinal cord were removed for more experiments.

2.5. Histologic analysis

Lumbar spinal cords and brains were fixed in 10% formalin solution for 48 h and then embedded in paraffin. After that, the paraffin blocks were cut to make serial sections with 5 µm thickness. Hematoxylin and eosin (H&E) staining was performed after de-waxing and rehydration of paraffin sections. Five slices of each sample were employed to estimate leukocyte infiltration. Finally, semi-quantitative analysis of inflammation was reported for brains: 0, no inflammation; 1, cellular infiltrates only around the blood vessel and meninges; 2, mild cellular infiltration in the parenchyma; 3, moderate cellular infiltration in parenchyma; and 4, serious cellular infiltration in the parenchyma [26]. The histological score of spinal cords was also assigned as score 0 without inflammation to score 4 with the most severe inflammation [27].

2.6. Proliferation assay of splenocytes

The effects of thyme on lymphocyte proliferation response were assessed by WST-1reagent (Roche). After spleen isolation, the small pieces of tissue were passed through a cell strainer (70 μm) with the aid of a sterile syringe, and this was followed by washing with RPMI 1640 medium (shellmax). The cell suspension was centrifuged at 300 × *g* for 5 min at 4 °C and then the RBCs were lysed by the addition of a lysis buffer (150 mM NH4Cl, 1 mM KHCO3, 0.1 mM EDTA [pH, 7.2]) for 10 min. Afterward, the suspension was washed twice with RPMI as mentioned. The splenocytes with the minimal viability of 95% (determined by trypan blue staining) were cultured in 96-well flat-bottom plate (5×10^5 cells/well) in complete RPMI containing10% FBS (fetal bovine serum) (shellmax) in the presence of 100 μg/ml penicillin-streptomycin as antibiotics (Gibco).

The splenocytes from each mouse were cultured with 10 μg/ml PHA (phytohemagglutinin) (Gibco) as a positive control and with 10 μg/ml MOG as a specific stimulator, or without any stimulator as a negative control culture (in triplicate). The splenocyte cultures were incubated at 37 °C, in a condition of 5% CO_2 for 48 h. Then, the WST-1reagent (20 μl/well) was added to the wells and the splenocyte proliferation was assessed after 4 h using a microplate reader at 450 nm. The stimulation Index (SI) was calculated by dividing the optical density (OD) of the stimulated cells into the related un-stimulated cells as previously described [28].

2.7. Determination of the Cytokine production by splenocytes

Splenocytes were obtained as described at item 2.6, adjusted to 2×10^6 cells/well and cultured for 48 h. Then, the supernatants were removed and the amounts of IL-10, IL-17 A, IFN-γ, TGF-β, IL-4, and IL-6 cytokines were measured with commercial enzyme-linked immunosorbent assay (ELISA) kits (eBioscience). All samples were performed in duplicate.

2.8. Statistical analysis

All data were expressed as mean ± SEM and the differences between variables were analyzed using appropriate statistical tests, using one-way analysis of variance (ANOVA) or Kruskal–Wallis followed by Tukey post-hoc and Mann-Whitney test for multiple comparisons of groups. The repeated measure test followed by Bonferroni comparison was also utilized to analyze clinical scores and body weight during the study period. The differences were considered significant when the P values were less than 0.05. All data were analyzed using GraphPad Prism (6.01software).

3. Results

3.1. Chromatography

The standard for thymol has a retention time at 70.13 min with maximum absorbance in 277 nm (Fig. 1A). The standard of carvacrol also showed a retention time near thymol, approximately at 69.26 min, 274 nm (Fig. 1B). Based on the standard curves, the amount of thymol and carvacrol in the hydro alcoholic thyme extract were 10 and 4 mg / g (thyme) respectively as analyzed by HPLC. (Fig. 1C).

3.2. The effects of thyme extract on clinical score

The first clinical signs of EAE [24] were observed in untreated control group, 50 mg/kg- and 100 mg/kg thyme-treated EAE mice, on days 9, 10 and 10 post MOG immunization, respectively (Fig. 2). Overall, the EAE symptoms were observed in 100%, 100% and 80% of the EAE control group, 50 and 100 mg/kg thyme-administrated EAE mice, respectively. The clinical score in both thyme-administrated EAE group significantly decreased compared to the untreated EAE group at indicated time points following MOG immunization ($P < 0.05$ at days 10, and $P < 0.001$ at days 13–21) (Fig. 2A). The maximum mean clinical score (MMCS) was reduced in 50 mg/kg (1.7 ± 0.31) and 100 mg/kg thyme-treated EAE groups (1.5 ± 0.28) compared to the untreated EAE mice (3.6 ± 0.36) (both with $P < 0.05$). The MMCS in 100 mg/kg thyme-administrated EAE group was also lower than 50 mg/kg thyme-treated EAE mice, but the difference was not significant.

3.3. The effects of thyme extract on body weight

The body weight was significantly higher in the 50 mg/kg thyme-treated EAE group compared to the untreated EAE counterparts at the indicated time points post MOG immunization. ($P < 0.05$ at day 13 and $P < 0.001$ at days 14–21) (Fig. 2B). Moreover, 100 mg/kg thyme-treated EAE mice exhibited higher body weight than non-treated EAE group at some time points of post-immunization ($P < 0.05$ at day 16, $P < 0.01$ at day 17, and $P < 0.001$in 18–21 days) (Fig. 2B).

3.4. The effects of thyme extract on cellular infiltration in the CNS

the inflammation scores in the lumbar region of the spinal cords from 50 mg/kg (1.20 ± 0.199) and 100 mg/kg thyme-treated EAE (1.00 ± 0.316) groups decreased in comparison with untreated EAE mice (3.00 ± 0.316, both with $P < 0.01$) (Fig. 3D). Likewise, the inflammation scores in the brain of 50 mg/kg (1.5 ± 0.22) and 100 mg/kg thyme-treated EAE (1.25 ± 0.19) groups were lower than that in the untreated EAE control mice (3.5 ± 0.22; $P < 0.05$ and $P < 0.01$, respectively) (3.5 ± 0.22) (Fig. 3E).

3.5. The effects of thyme extract on MOG-specific T cell proliferation

The *ex vivo* MOG-induced proliferation of splenocytes isolated from EAE mice treated with 50 or 100 mg/kg thyme extract and untreated EAE control group is illustrated in Fig. 4. The SI in splenocytes from 50 and 100 mg/kg thyme extract-treated EAE mice (1.47 ± 0.26 and 1.45 ± 0.27, respectively) was decreased as compared with untreated EAE control group (1.72 ± 0.32), but the changes were not statistically significant (Fig. 4).

3.6. The effects of thyme extract on the secretion of pro-inflammatory cytokines by splenocytes

To explain the beneficial effects of thyme on the clinical and pathological signs of EAE, the pro-inflammatory (IFN-γ, IL-17 A and IL-6) and anti-inflammatory (TGF-β, IL-10, IL-4) cytokines were evaluated in splenocyte cultures after being re-stimulated with MOG. The production of IFN-γ significantly decreased in mice treated with 50 and 100 mg/kg thyme extract compared to the untreated EAE group ($P < 0.001$ for both thyme-treated groups) (Fig. 5A).

The production of IL-6 also decreased by splenocytes from mice treated with 50 and 100 mg/kg thyme extract compared to the untreated EAE control group ($P < 0.01$ and $P < 0.001$ for 50 mg/kg-and100 mg/kg thyme-treated groups, respectively) (Fig. 5B). Furthermore, the amount of IL-6 production (but not IFN-γ) was considerably reduced by splenocytes from 100 mg/kg thyme treated-EAE group in comparison with 50 mg/kg thyme treated group ($P < 0.05$). Both doses of thyme extract lead to a reduction in IL-17 A production, although the difference was not significant when compared with the untreated mice (Fig. 5C).

3.7. The effects of thyme extract on the secretion of anti-inflammatory cytokines by splenocytes

The amount of TGF-β production by splenocytes from EAE mice treated with 50 and 100 mg/kg of thyme extract was significantly

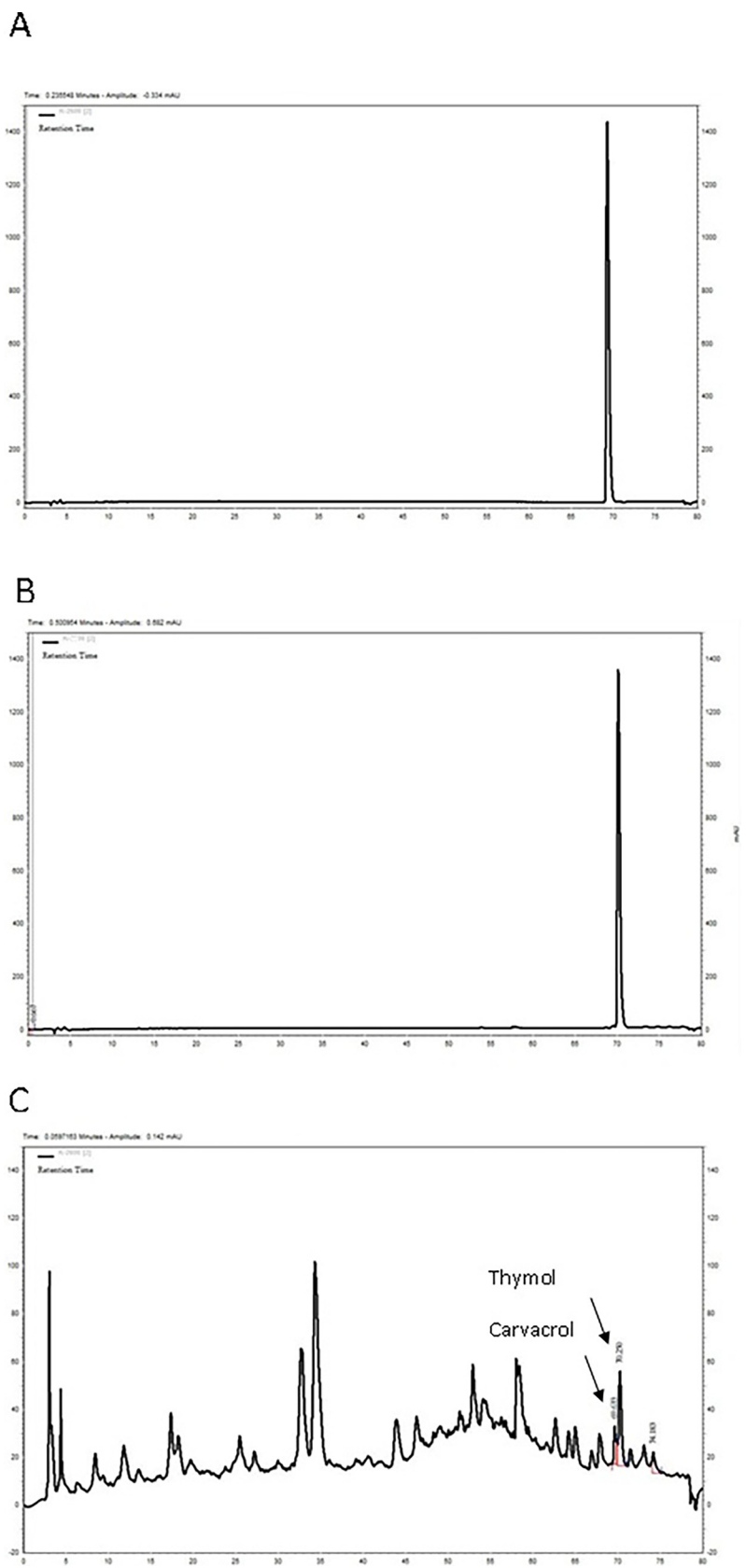

Fig. 1. HPLC chromatograms. (A) Carvacrol standard with retention time at 69.26 min (at 274 nm). (B) Thymol standard with retention time at 70.13 min (at 277 nm). (C) HPLC chromatogram of *Thymus Vulgaris* extract that two picks of carvacrol and thymol are identified on it.

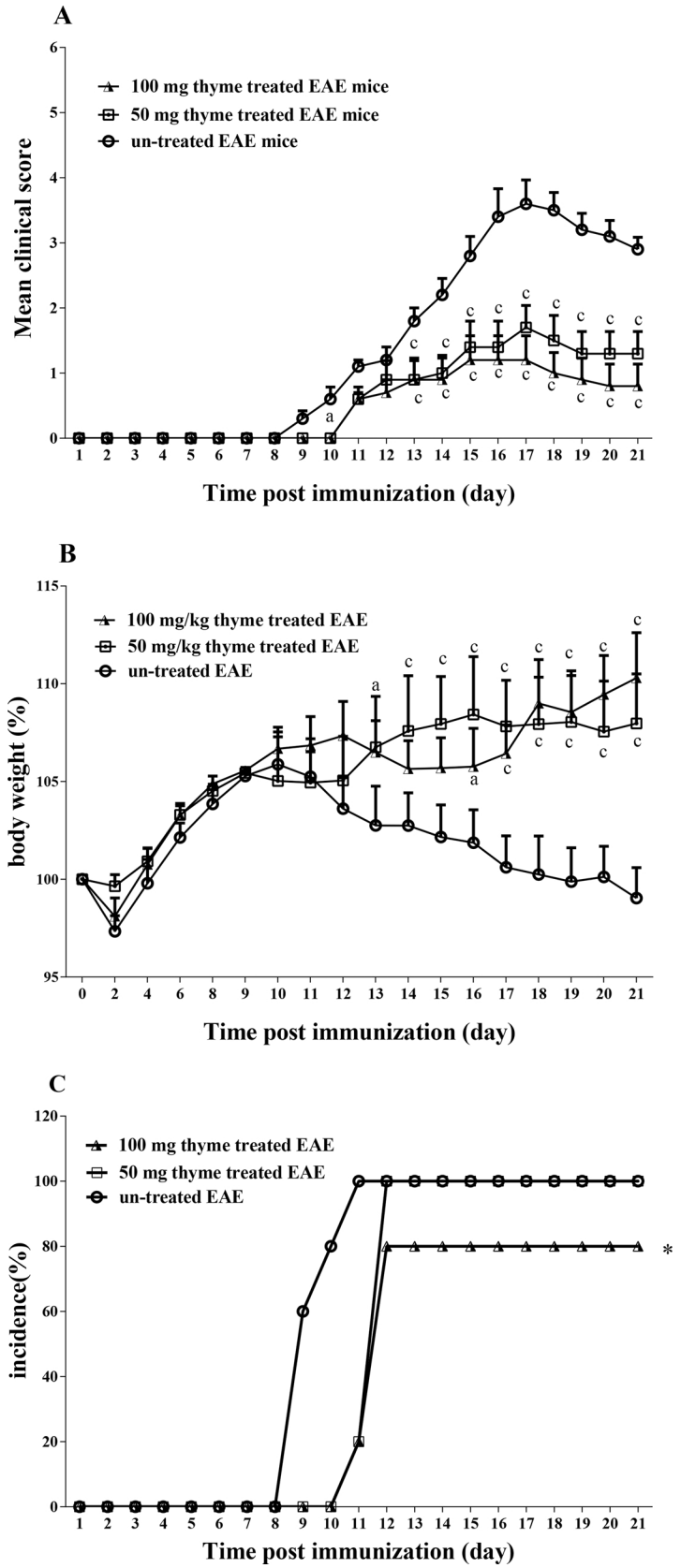

Fig. 2. Comparison of the clinical score, body weight and incidence between thyme-treated EAE groups and un-treated EAE control mice. The MOG- immunized mice were treated (i.p) with 50 mg/kg thyme, 100 mg/kg thyme, and PBS (control group) from the immunization day (every other day). (A) Decrease in the mean clinical score, (B) inhibitory effect of thyme on the weight loss and (C) incidence of disease in each group during 21 days. Results are presented as the mean ± SEM. [a,] *$p < 0.05$, [b,] ** $p < 0.01$, [c,] ***$p < 0.001$compared to the un-treated EAE control group.

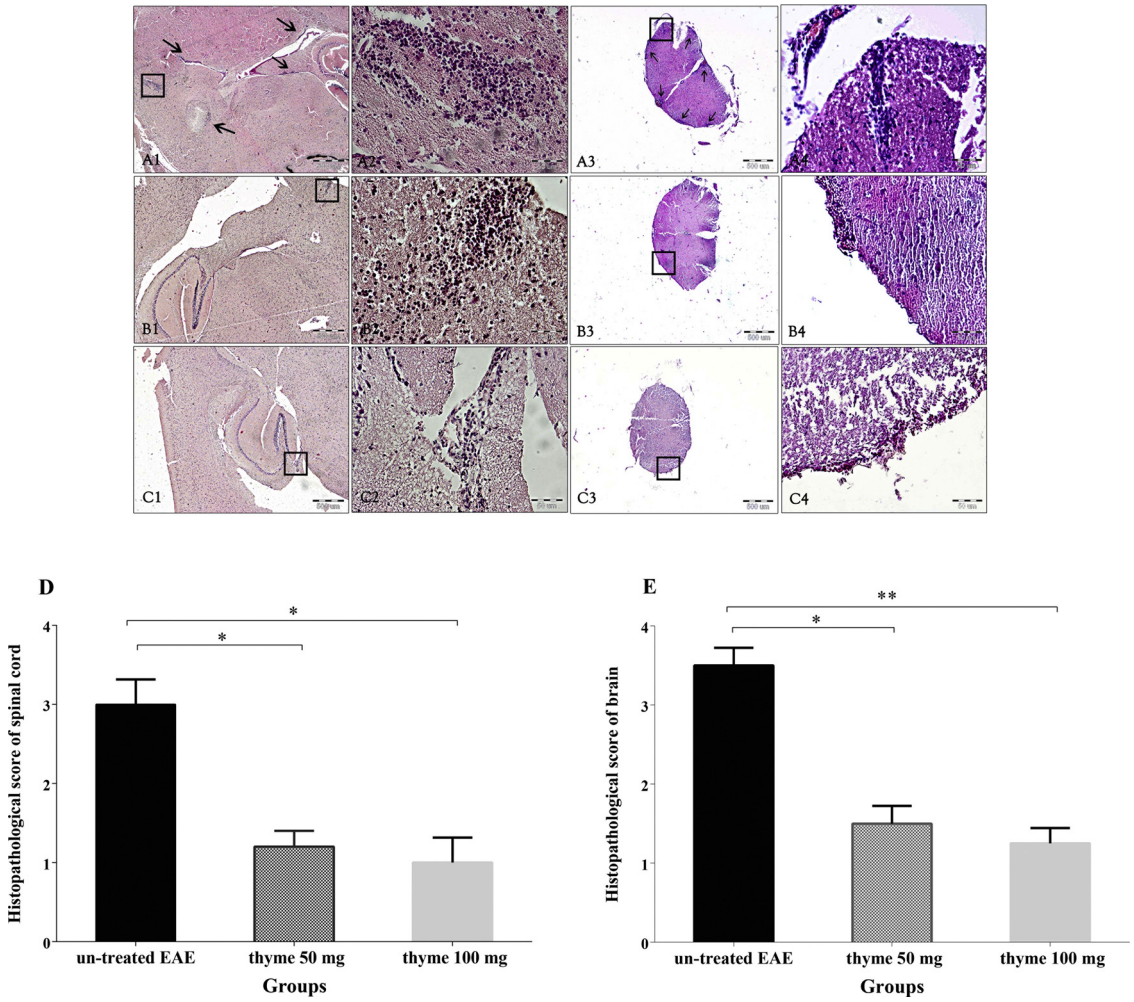

Fig. 3. Comparison of the leukocyte infiltration into the brains and spinal cords between thyme-treated EAE groups and un-treated EAE control mice. Treatment with thyme extracts reduces leukocyte infiltration into the brain and spinal cord. The brain and spinal cord section in untreated EAE control mice (A1-A4), in 50 mg/kg thyme-treated EAE groups PBS-treated EAE mice (B1-B4) and in 100 mg/kg thyme-treated EAE groups (C1-C4). Image A2, A4, B2, B4, C2, and C4 are inserted squares in images A1, A3, B1, B3, C1, and C3 respectively. The arrow heads show infiltrated leukocytes in different parts of tissue. (D) The histopathological scores in spinal cords. (E) The histopathological scores in brains. The comparison of cell infiltration severity as semi quantitatively between groups. Results are presented as the mean ± SEM and analyzed by Kruskal–Wallis test followed by Mann-Whitney test. *p < 0.05, ** p < 0.01, *** p < 0.001 compared to the un-treated EAE control group (n = 5).

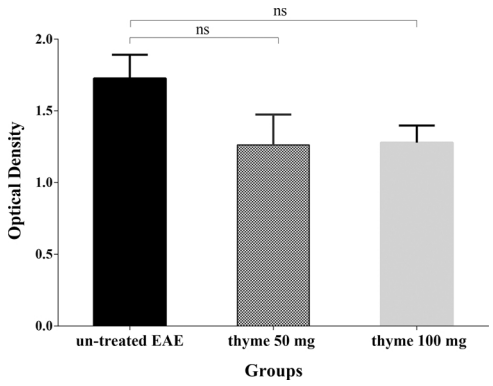

Fig. 4. Comparison of the splenocyte proliferation between thyme-treated EAE groups and un-treated EAE control mice. The splenocytes were cultured in the medium alone or at the presence of MOG. After 48 h, cell proliferation was assessed using WST-1 reagent and the Stimulation Index (SI) was evaluated. Proliferation assay was conducted in triplicate wells. The results expressed as mean ± SEM and was analyzed by one-way analysis of variance (ANOVA) followed by Tukey post-hoc test (n = 5). ns (not significant).

higher than the untreated EAE group (P < 0.001) (Fig. 5D). Furthermore, the amount of TGF-β secretion was significantly higher by splenocytes from100 mg/kg thyme-treated EAE mice in comparison with 50 mg/kg thyme-treated mice (P < 0.05) (Fig. 5D). The amount of IL-10 secretion by splenocytes of untreated EAE mice (863.715 ± 147.05 pg/mL) was significantly decreased compared to the 50 mg/kg thyme treated EAE mice (1228.35 ± 54.66 pg/mL, P < 0.05) and 100 mg/kg thyme administrated-EAE group (1429.64 ± 31.11 pg/mL, P < 0.01) (Fig. 5E). The difference in the production of IL-10 by splenocytes from 50 and 100 mg/kg thyme-treated mice was not significant, although it was discovered to be in EAE mice treated with 100 mg/kg of thyme (Fig. 5E).

In contrast to TGF-β and IL-10, the splenocytes from both 50 and 100 mg/kg thyme groups produced lower amounts of IL-4 compared to the untreated EAE group, but it reached a significant level only for 50 mg/kg thyme group (P < 0.01) (Fig. 5F). Interestingly, the IL-4/IFN-γ ratio in mice treated with 50 and 100 mg/kg of thyme was higher than those found in cultures from untreated control group (both with P < 0.05) (Fig. 5G).

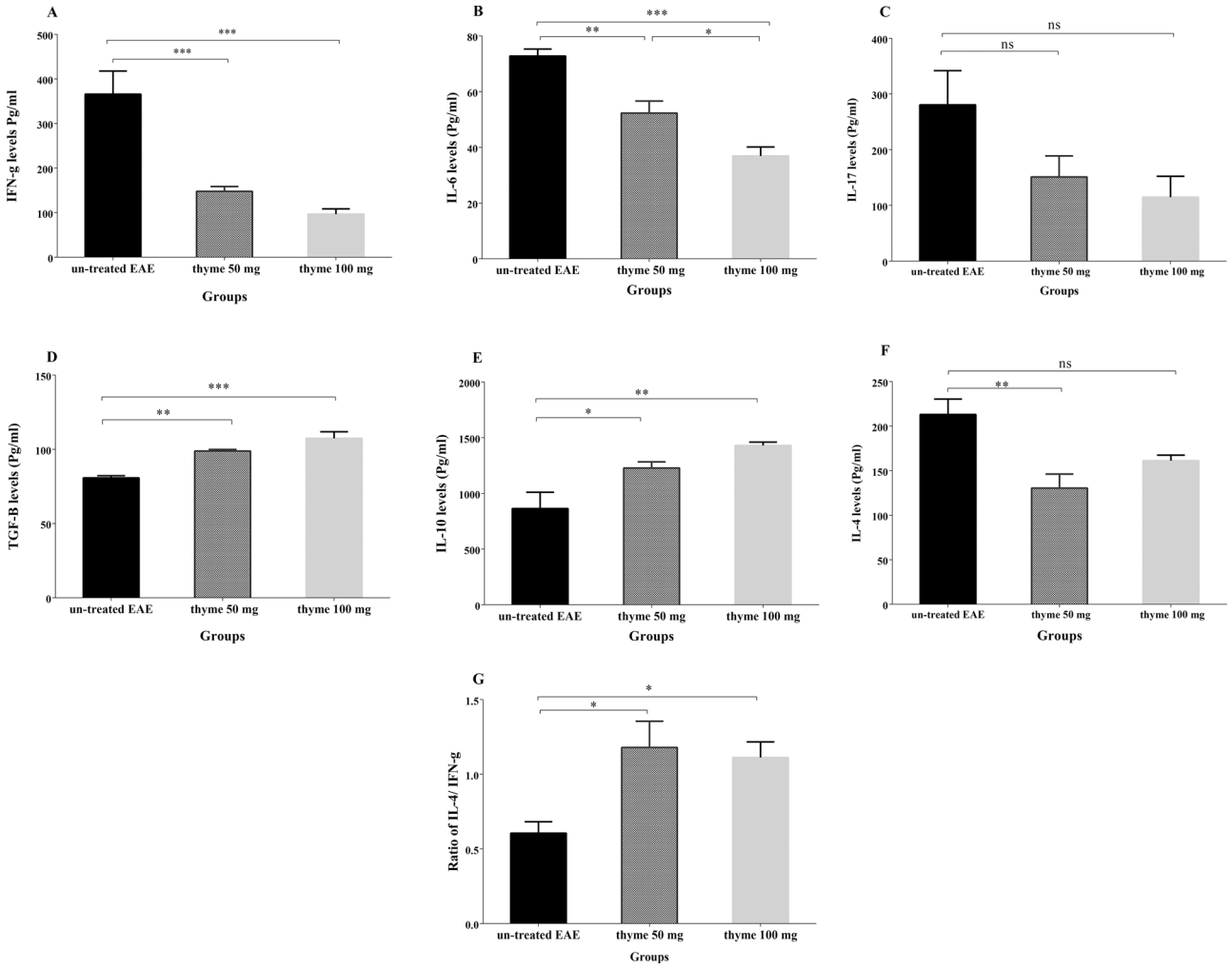

Fig. 5. Comparison of pro and anti-inflammatory cytokines production by splenocytes between thyme-treated EAE groups and un-treated EAE control mice. The splenocytes were cultured in the medium at the presence of MOG. After 48 h, pro-inflammatory cytokine (A–C), anti-inflammatory cytokines (D–F) and the ratio of IL-4/IFN-γ (G) were measured in supernatants. Cytokine assays were conducted in duplicate wells. The results are expressed as mean ± SEM and analyzed by one-way analysis of variance (ANOVA) followed by Tukey post-hoc test. The comparison of IL-17 was analyzed by Kruskal–Wallis test followed by Mann-Whitney test. ns (not significant), *$p < 0.05$, ** $p < 0.01$, *** $p < 0.001$compared to the un-treated EAE control group ($n = 5$).

4. Discussion

For many decades, the beneficial anti-oxidant properties of *Thymus vulgaris* (thyme) and its main constituents (thymol and carvacrol) have been documented; whereas the anti-inflammatory and immunomodulatory potentials of this herb are rarely examined and need more studies. In this regard, for the first time, we examined the anti-inflammatory and immunomodulatory effects of hydro-alcoholic extract of thyme on clinical symptoms and disease severity in EAE.

The results of the present study demonstrated that thyme reduced EAE clinical symptoms and also avoided body weight loss.

The beneficial effects of *Thymus vulgaris* extract and its derivatives have been demonstrated in a number of human inflammatory diseases and animal models. In an animal model of high fat diet-induced hyperlipidemia, it was demonstrated that thymol displayed down-regulating effects on various inflammatory parameters such as C-reactive protein (CRP), IL-6, IL-1, TNF-α, TNF-β, vascular cell adhesion molecule-1 (VCAM-1), monocyte chemotactic protein-1 (MCP)-1, and matrix metalloproteinase (MMP)-9 [29]. The beneficial activities of thymol in combination with diacerein were also indicated in a model of osteoarthritis [30]. Thymol also displayed anti-depressant effects in mice through increasing the central neurotransmitters and suppressing the expression of IL-1, IL-6, and TNF-α [31].

Our histopathological results indicate that CNS infiltration of leukocytes was lower in thyme-administrated group. In agreement with our findings, it has been demonstrated that thyme mitigates the histopathological score in an experimental animal model of colitis [22], in the liver of diabetic rats [32], and also in the brain of mice with hyperlipidemia [32].

As mentioned previously, the deleterious effects of IFN-γ, IL-6, and IL-17 may induce the expression of adhesion molecules, chemokines, and consequently the recruitment of encephalitogenic T cells into the CNS [5,33,34]. IL-17 also intensifies neural cell damage by enhancing ROS concentration in the brain endothelial cells and destruction of the blood-brain barrier (BBB) [5]. In this study, thyme extract administration reduced IFN-g, IL-6 and IL-17 secretion by splenocytes. In agreement with our findings, the inhibitory effects of *Thymus vulgaris*-derived components including thymol and carvacrol were demonstrated through reducing the production of Th1 cell-related parameters (IL-2, IFN-g, and T-bet) and Th17 cell-related parameters (IL-17 A and RORc) in ovalbumin-immunized mice [35]. Additionally, it has been shown that thymol and carvacrol also modulate T cell activation by suppressing the transcription factors AP-1 and NFAT-2 and preventing the production of IL-2 and IFN-γ [36]. Thymol and carvacrol also exhibit anti-inflammatory activities by reducing the expression of pro-inflammatory cytokines such as TNF-α, IL-1, and IL-6 in hepatotoxic

rats and myocardial infarction model [37,38]. Based on these findings, it seems that thyme extract can reduce the leukocyte infiltration into the CNS partly by reducing inflammatory cytokines.

The inhibitory effects of *Thymus vulgaris* on the IFN-γ, IL-6 and IL-17 expression may be directly performed via the modulation of signalling pathways and transcription factors associated with these cytokines in Th1, macrophages, and Th17 cells as shown in ovalbumin-immunized mice and Lipopolysaccharide-treated mouse macrophages [35,39].

The modulatory effects of *Thymus vulgaris* on the production of the mentioned cytokines may also be performed indirectly through the induction of immunosuppressive cytokines TGF-β and IL-10. The inhibitory effects of TGF-β and IL-10 on the activation of Th1, macrophages, and Th17 have been well documented [3]. The results presented here indicated that the TGF-β and IL-10 production by splenocytes from *Thymus vulgaris*-administered groups was higher than untreated EAE mice. In conformity with our results, the enhancing effects of *Thymus vulgaris*-derived carvacrol and thymol on IL-10 and TGF-β expression were supported by studies of CFA-induced paw edema and ovalbumin-induced asthmatic models [35,40]. TGF-B is produced mainly by Treg cells and IL-10 is secreted from a number of leukocytes such as Treg, Breg, and Th2 cells [5,41]. IL-10 reduces the secretion of inflammatory cytokines and increases the anti-inflammatory cytokines by astrocytes [41]. TGF-β also promotes the survival and differentiation of Tregs as well as maintaining peripheral self-tolerance [5]. It is postulated that the enhancing effects of *Thymus vulgaris* on TGF-B and IL-10 production may be performed directly via its inductive effects on Treg, Breg and Th2 cells.

The results of our study indicated that the IL-4 production by splenocytes from thyme-administrated groups was lower than the untreated control EAE mice. In this regard, in a mouse model of OVA-induced asthma, it has been reported that thymol ameliorates airway inflammation probably via preventing NF-κB activation and suppressing the expression of IL-4, IL-5 and IL-13 [42]. However, the IL-4/IFN-γ ratio was significantly higher in thyme-administrated groups compared to the untreated control EAE mice, which implies that *Thymus vulgaris* may improve the Th1/Th2 cell imbalances toward Th2 cells in EAE mice. Therefore, the net effects of treatments with *Thymus vulgaris* may lead to the attenuation of Th1 cell-related cytokines.

Moreover, our findings demonstrated that thyme could reduce the proliferation of MOG-specific splenocytes which was also observed in OVA-induced airways allergy and PHA-treated PBMC of healthy subjects [35,43]. Thus, it is possible that thyme acts by suppressing the initial auto-immune cells expansion in spleen and subsequently diminishes detrimental responses in the CNS.

5. Conclusion

The results presented here indicate for the first time that the treatment of EAE mice with *Thymus vulgaris* attenuates the clinical EAE symptoms, reduces the histopathological scores, decreases the pro-inflammatory cytokines (IFN-g, IL-6 and IL-17) and enhances the anti-inflammatory cytokines (TGF-B and IL-10). Our findings also encourage more studies evaluating the modulatory effects of *Thymus vulgaris* and its derivatives on EAE and MS development.

Conflict of interest

The authors declare no conflict of interest.

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