Life Sciences 219 (2019) 257–263

Contents lists available at ScienceDirect

Life Sciences

journal homepage: www.elsevier.com/locate/lifescie

Carvacrol ameliorates experimental autoimmune encephalomyelitis through modulating pro- and anti-inflammatory cytokines

Merat Mahmoodi[a,b], Houshang Amiri[c,d], Fatemeh Ayoobi[e], Mehdi Rahmani[f], Zahra Taghipour[g], Razieh Taghizadeh Ghavamabadi[g], Abdollah Jafarzadeh[h,*], Mojtaba Sankian[a,**]

[a] Immunology Research Center, Medical School, Mashhad University of Medical Sciences, Mashhad, Iran
[b] Mashhad University of Medical Sciences, Mashhad, Iran
[c] Neurology Research Center, Kerman University of Medical Sciences, Kerman, Iran
[d] Department of Radiology and Nuclear Medicine, VU University Medical Center, Amsterdam, the Netherlands
[e] Non-Communicable Diseases Research Center, Rafsanjan University of Medical Sciences, Rafsanjan, Iran
[f] Department of Immunology, School of Medicine, Rafsanjan University of Medical Sciences, Rafsanjan, Iran
[g] Department of Anatomy, Rafsanjan University of Medical Sciences, Rafsanjan, Iran
[h] Molecular Medicine Research Center, Rafsanjan University of Medical Sciences, Rafsanjan, Iran

ARTICLE INFO

Keywords:
Experimental autoimmune encephalomyelitis
Carvacrol
Cytokines
Inflammation

ABSTRACT

Aim: The inflammatory process is a key step in multiple sclerosis (MS) development. Carvacrol exhibits various anti-inflammatory properties. We aimed to assess the Carvacrol effects on clinical manifestations and production of pro-inflammatory (IFN-γ, IL-6 and IL-17) and anti-inflammatory (TGF-β, IL-4, and IL-10) cytokines in experimental autoimmune encephalomyelitis (EAE) as MS animal model.

Main methods: EAE mice were treated with 5, 10 mg/kg dose of Carvacrol or vehicle, as the control EAE group, every other day until day-21 post EAE induction. On day22, the leukocyte infiltration within the CNS was estimated using hematoxylin-eosin staining. The cytokine production by splenocytes was determined after in vitro stimulating with myelin oligodendrocyte protein (MOG).

Key findings: The EAE clinical scores in 5 and 10 mg/kg Carvacrol-treated mice were lower than untreated group ($P < 0.001$ and $P < 0.01$, respectively). The amounts of IFN-γ and IL-6 production by splenocytes of 5 and 10 mg/kg Carvacrol-administered mice were lower than control group ($P < 0.001$, and $P < 0.01$ for IFN-γ respectively; $P < 0.05$ for IL-6). Splenocytes of 5 and 10 mg/kg Carvacrol-treated mice produced higher levels of TGF-β than untreated mice ($P < 0.001$). in splenocytes of 5 mg/kg Carvacrol-treated group the IL-10 production was higher while IL-17 secretion was lower than control group (both with $P < 0.01$).

Significance: Carvacrol exhibits modulatory effects on expression of pro- and anti-inflammatory cytokines. It ameliorates EAE clinical and pathological consequences and therefore its potentials may be considered in treating MS patients.

1. Introduction

Multiple sclerosis (MS) is an autoimmune inflammatory disease of the central nervous system (CNS) that is characterized by demyelination and degeneration of neuronal cells [1]. The experimental autoimmune encephalomyelitis (EAE) is an animal model of MS, which can be induced in vulnerable rodents by immunization with myelin-based antigenic components, in particular myelin oligodendrocyte protein (MOG) [2]. During initial stages of MS, infiltration of a large number of various inflammatory cells such as dendritic cells (DCs), macrophages and lymphocytes (CD4+ and CD8+ T cells as well as B cells) occur within the CNS [3]. Both infiltrated and residential cells, e.g. microglia and astrocytes, contribute to the MS development through production of the various types of pro-inflammatory parameters such as cytokines, chemokines, reactive oxygen species (ROS) and nitric oxide (NO) [4]. DCs cross the damaged blood–brain barrier (BBB) and play a fundamental role in the differentiation of myelin specific CD4+ T cells into the pathogenic Th1 and Th17 cells, which mediate neuronal demyelination within the CNS [5–7].

Analysis of the serum and cerebrospinal fluid (CSF) of MS patients

* Correspondence to: A. Jafarzadeh, Immunology of Infectious Diseases Research Center, Rafsanjan University of Medical Sciences, Rafsanjan, Iran.
** Corresponding author.
E-mail addresses: Jafarzadeh14@rums.ac.ir (A. Jafarzadeh), SankianM@mums.ac.ir (M. Sankian).

https://doi.org/10.1016/j.lfs.2018.11.051
Received 10 July 2018; Received in revised form 21 November 2018; Accepted 22 November 2018
Available online 23 November 2018

has shown increase in IFN-γ (a potent inducer and a product of Th1 cells) and IL-17 (a Th17 cell type cytokine) [8–11]. Differentiation of naive $CD4^+$ lymphocytes to Th17 occur after antigenic stimulation at the presence of IL-6, a powerful pro-inflammatory cytokine [12]. The increased frequency of Th17 cells has also been reported in MS patients [11]. On the other hand, there are evidences indicating that Th2 and Treg cells are protective against MS and EAE [13,14]. In this context, previous studies have shown that IL-4 (a powerful inducer and a product of Th2 cells) could reduce the iNOS (inducible nitric oxide synthase) expression in glial cells and EAE severity [15]. Furthermore, Treg cells play an essential function in establishing the self-tolerance through production of immunomodulatory cytokines such as TGF-β and IL-10 [12]. These cytokines have shown a protective role in EAE and MS, through inhibition of pathogenic effector cell responses [5,16].

Different medications have been developed to regulate the activity of immune system in MS patients, although they only control progression of the disease [1]. Due to incomplete efficacy of current medication and their side effects in MS patients, complementary medicine such as herbal-derived components has become increasingly interesting. Carvacrol is a phenolic monoterpene constituent in the *Lamiaceae* plant family exhibiting various - antimicrobial [17], antifungal [18], antitumor [19], anti-inflammatory, and immunomodulatory properties [20]. Furthermore, in CNS disorders, Carvacrol exerts its modulatory effect on different targets including dopaminergic system in depression [21], aquaporins in cerebral edema [22], and acetylcholinesterase as an important enzyme in Alzheimer's disease [23].

To our knowledge, to date, there has been no investigation on the effects of Carvacrol on EAE-or MS-related immunologic, pathologic, and inflammatory parameters. Hence, we aimed to evaluate the anti-inflammatory and immunomodulatory effects of Carvacrol in an animal model of EAE focusing on the production of pro-inflammatory (IFN-γ, IL-6 and IL-17) and anti-inflammatory (TGF-β, IL-4, and IL-10) cytokines.

2. Materials and methods

2.1. Animal models

Female C57BL/6 mice (8 weeks old) were purchased from Royan Institute of Isfahan, Iran. All mice were kept in the animal house with constant temperature (24 °C) and 12-h light/dark cycle and access to rodent pellet and water ad libitum. The study protocol was approved by the institutional Animal Ethics Committee of Mashhad Medical University (No. IR.MUMS.sm.REC.1395.139).

2.2. EAE induction and scoring

For EAE induction, as described previously by Jafarzadeh et al. [24], each mouse was immunized with 400 μg MOG 35–55 (100 μl) (SBS Genetech Co. Ltd., Beijing, China) emulsified in equal volume of complete Freund's adjuvant containing 0.5 mg Mycobacterium tuberculosis (Sigma, USA). The emulsion (200 μl) was injected subcutaneously into the flank and between the shoulders of the mice. Then, 500 ng of pertussis toxin (Sigma, USA) was injected intra-peritoneally on the day of immunization (250 ng) and 48 h later (250 ng). The body weight and clinical score of all mice were recorded every other day from day-0 to day-21 post immunization. The clinical score was graded as follow: 0 = no symptoms; 1 = partial loss of tail tonicity; 2 = complete loss of tail tonicity; 3 = flaccid tail and abnormal gait; 4 = hind leg paralysis; 5 = hind leg paralysis with hind body paresis; 6 = hind and foreleg paralysis; and 7 = death [25].

2.3. Experimental groups

In this study, first five doses of 100, 50, 25, 10 and 5 mg/kg were selected based on various animal studies [22,26,27]. Each dose was injected daily into three mice for seven days. The 100 mg dose caused death after first 2–3 days and treatment with 50 and 25 mg Carvacrol led to obvious movement impairments. However, 5 and 10 mg Carvacrol did not affect normal activity and movement of the mice. Therefore, for treating EAE mice, two doses of 5 and 10 mg/kg Carvacrol were chosen in this study.

The mice were divided into 4 groups (5 mice in each group weighing between 16 and 20 g) as follows: Group I: EAE-induced mice were administrated 2% Tween 80 in PBS as vehicle and as EAE control group. Group II and III: Carvacrol-treated EAE mice that were injected intra-peritoneally with 5 mg/kg or 10 mg/kg of Carvacrol [22]. Group IV: healthy normal mice without EAE were administrated vehicle. Treatment protocol consisted of every other day injections from day-0 to day-21. Mice were sacrificed on day-22 post-immunization according to the recommendations of animal ethics. The lumbar spinal cord tissues were dissected for histologic analysis and the splenocytes were cultured for cytokine and proliferation assays.

2.4. Histopathological analysis

Lumbar spinal cords of the mice were dissected and fixed in 10% formalin. After 48 h, paraffin molds of specimens were prepared and sectioned via a microtome into 5 μm thickness and stained with hematoxylin and eosin (H&E). Then, 5 slides of each sample were evaluate for severity of inflammation. Analysis of cell infiltration in spinal cord was done semi-quantitatively by two specialized observers who were blinded to the study; as such scores of 0 to 4 were defined as without inflammation to severe inflammation, respectively [28].

2.5. Preparation of spleen cells and proliferation assay

After the isolation of spleen, a cell suspension was prepared in RPMI-1640 medium (shellmax, China) by passing the small pieces of tissue through a cell strainer (70 μm). Then, the red blood cells (RBCs) were lysed by addition of ammonium chloride buffer (150 mM NH4Cl, 1 mM KHCO3, 0.1 mM EDTA [pH, 7.2]) at 4 °C for 10 min. Afterward, the cell suspension was centrifuged at 300 ×*g* for 5 min at 4 °C and the cell pellet was washed twice with RPMI. Collected splenocytes were resuspended in complete RPMI containing 10% fetal bovine serum (FBS) (shellmax, China) in the presence of 100 μg/ml penicillin-streptomycin as antibiotics (Gibco, UK). The splenocytes of each mouse were cultured in 96-well flat-bottom plate (in triplicate, 5×10^5 cells/well) in the presence or absence of MOG (10 μg/ml) as a specific stimulator and negative control, respectively. After 48 h culturing at 37 °C and 5% CO_2, 20 μl/well WST-1-reagent (Roche Diagnostics GmbH, Mannheim, Germany) was added to the wells and incubated 4 h at 37 °C. The splenocyte proliferation was measured using a microplate reader at 450 nm and Stimulation Index (SI) was calculated for each sample.

2.6. Cytokine assay

For cytokine assessment, cells were cultured and stimulated as previously mentioned (2×10^6 cells/well) in 24-well culture plate. After 48 h, the supernatant was collected and the amounts of different cytokines (IL-10, IL-17A, IFN-γ, TGF-β, IL-4, and IL-6) was assess by ELISA (eBioscience, San Diego, CA, USA) according to the manufacturer's protocol. All samples were performed in duplicate.

2.7. Statistical analysis

Statistical analysis was performed using GraphPad Prism version 6.01. All data were reported as mean ± SEM. After evaluating the normality test (Kolmogorov–Smirnov with the Dallal-Wilkinson-Lilliefor corrected *P* value) of the samples, the parametric test one-way analysis of variance (ANOVA) followed by Tukey post-hoc was conducted for data with normal distribution. Kruskal–Wallis followed by

Mann-Whitney test was used to compare the differences between ordinal data. The repeated-measure test followed by Bonferroni comparison was also utilized to analyze clinical scores and body weight over the study period. *P*-values < 0.05 were considered significant.

3. Results

3.1. Carvacrol effect on the clinical score of EAE mice

To assess the effect of Carvacrol on EAE severity, mice were carefully monitored for clinical signs such as disease onset and maximum mean clinical score (MMCS). After 9 days of immunization, the first clinical signs started to appear in all three animal groups. The clinical score in both Carvacrol-administered EAE group significantly decreased (effect of time, F (20,80) = 126.2, $P < 0.0001$; effect of treatment F (2,8) = 8.193, $P = 0.0116$ and time × treatment interaction F (40, 160) = 3.917, $P < 0.0001$) as compared with untreated EAE group (for 5 mg/kg Carvacrol at days 15–21 ($P < 0.001$) and for 10 mg/kg on day-15 ($P < 0.01$), on days 16–18 ($P < 0.001$), on day-19 ($P < 0.05$) and on days 21–22 ($P < 0.01$)). Mice treated with 5 and 10 mg/kg Carvacrol showed lower MMCS (2.3 ± 0.2, $P < 0.001$ and 2.8 ± 0.3, $P < 0.01$ respectively) as compared with untreated control group (3.700 ± 0.36) on day 17 (Fig. 1A). The MMCS was lower in 5 mg/kg Carvacrol-treated group compared to the 10 mg/kg treated group but this was not significant. After day-18 post-immunization, the clinical score was significantly lower in mice treated with 5 mg/kg Carvacrol compared with 10 mg/kg Carvacrol-treated group ($P < 0.05$).

3.2. Carvacrol effect on body weight

Weight loss is an indicator of EAE onset [29] Percentage of body weight is reported in Fig. 1B. The body weight loss in untreated control group was more than that in both Carvacrol-administered EAE group (effect of time, F (17, 68) = 10.02, $P < 0.0001$; effect of treatment F (3, 12) = 6.474, $P = 0.0075$ and time × treatment interaction F (51, 204) = 5.125, $P < 0.0001$). The body weight loss in untreated control group was more than that in 5 mg/kg Carvacrol-treated EAE mice at both day-16 ($P < 0.01$) and days 17–21 ($P < 0.001$). Also, mice treated with 10 mg/kg Carvacrol showed lower body weight loss ($P < 0.05$, at day 18–19 and $P < 0.01$ at day 20–21) as compared with untreated control group. This difference was not significant between two treated groups (Fig. 1B).

3.3. Carvacrol effect on inflammation of CNS

The histological evaluation revealed that the entry of inflammatory cells into the spinal cord was significantly different between groups (H = 15.35, $P = 0.0015$). The inflammation score was significantly declined after administration of 5 mg/kg Carvacrol compared with untreated control group ($P < 0.05$). But the difference of inflammation score in 10 mg/kg Carvacrol-treated group, although lower, it was not statistically significant compared to untreated EAE mice, (Fig. 2).

3.4. Carvacrol effects on the proliferation of splenocytes

The splenocytes were cultured in the presence of MOG antigen to examine the possible modulatory effects of Carvacrol on cell proliferation. The results showed that the difference of cell proliferation between groups was significant ($P = 0.0001$and F (3, 16) = 13.02). Untreated EAE mice, 5 and 10 mg/kg Carvacrol-treated groups showed a significantly increased cell proliferation in the presence of the antigen compared to normal mice ($P < 0.0001$, $P < 0.05$ and $P < 0.01$ respectively). Also, data analysis demonstrated that treatment with Carvacrol decreased the MOG-induced proliferation of splenocytes compared to the untreated EAE mice, however it was not significant (Fig. 3).

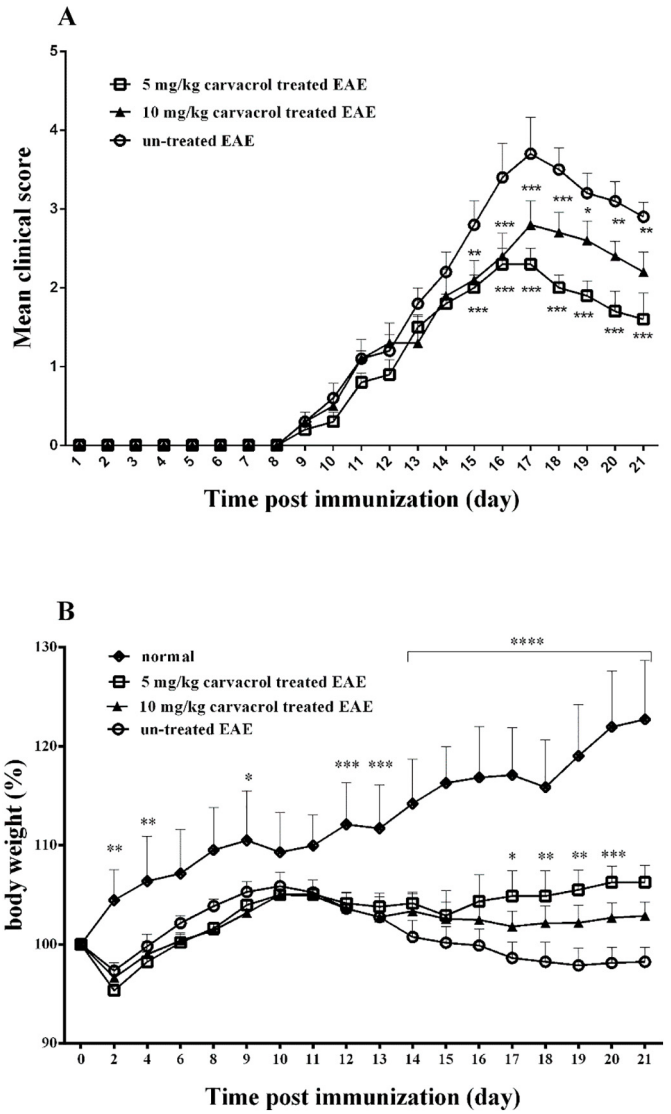

Fig. 1. Clinical score and body weight of Carvacrol-treated EAE mice. The MOG-immunized mice were treated (i.p.) with 5 mg/kg Carvacrol, 10 mg/kg Carvacrol, and 2% Tween 80 in PBS (control group) from the immunization day (every other day). (A) Decrease in the mean clinical score and (B) inhibitory effect of Carvacrol on the weight loss during 21 days. The repeated-measure test followed by Bonferroni comparison was used to analyze clinical scores and weight loss. Results are presented as the mean ± SEM. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ and ****$P < 0.0001$vs control group ($n = 5$).

3.5. Carvacrol effects on the production of inflammatory cytokines

To assess the mechanisms involved in the remission or progression of the disease, the pro-inflammatory cytokines (IFN-γ, IL-6, IL-17A) were detected in the supernatant of the MOG-stimulated splenocytes (Fig. 4).

The data analysis of IFN-γ, IL-6 and IL-17A indicated significant differences between groups (F (3, 16) = 21.25, $P < 0.0001$ for IFN-γ; F (3, 16) = 6.869, $P = 0.0035$ for IL-17; F (3, 16) = 18.74, $P < 0.0001$ for IL-6).

The production of IFN-γ, IL-17A and IL-6 were significantly increased in the untreated EAE group as compared with normal mice ($P < 0.0001$, $P < 0.01$ and $P < 0.0001$ respectively). The concentrations of IFN-γ in the supernatant of MOG-stimulated splenocytes from treated mice were significantly lower than equal cultures prepared from untreated EAE mice ($P < 0.001$ for both Carvacrol-treated groups) (Fig. 4A). In addition, MOG-stimulated splenocytes from mice

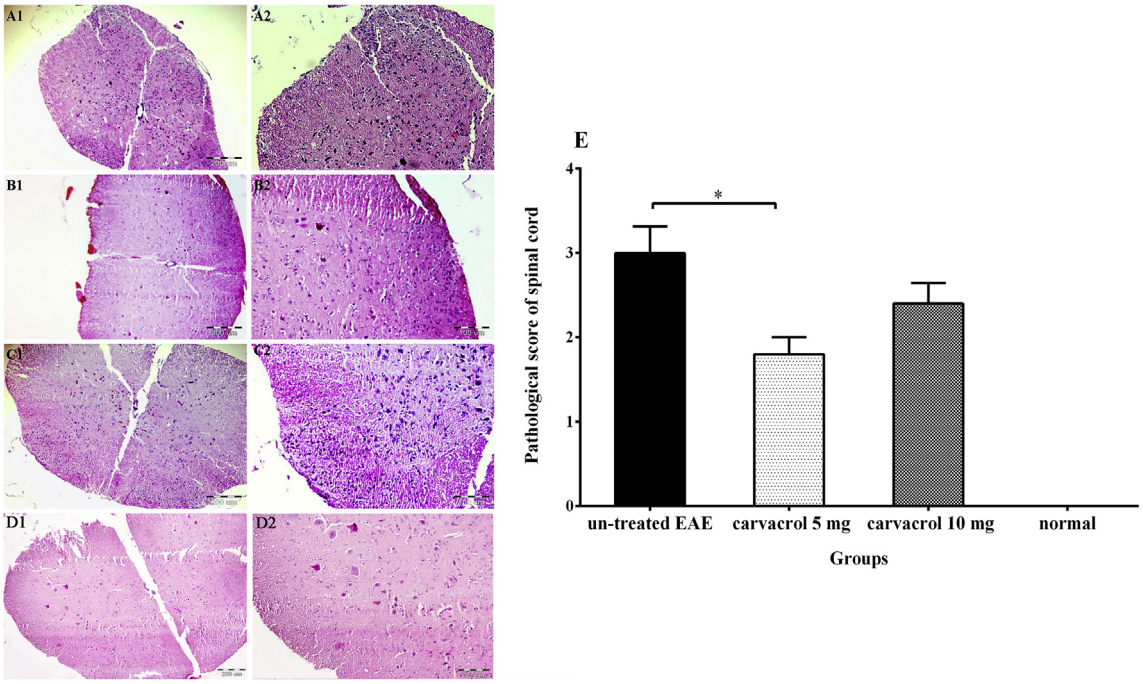

Fig. 2. H&E staining of the spinal cord in Carvacrol-treated EAE mice. Leukocytes infiltration into the spinal cord of, (A1, A2) EAE control group, (B1, B2) 5 mg/kg Carvacrol-treated group, (C1, C2) 10 mg/kg Carvacrol-treated group and (D1, D2) normal group. (E) The comparison of cell infiltration severity (as ordinal data) between groups was assessed by Kruskal–Wallis test followed by Mann-Whitney. Results are presented as the mean ± SEM.*$P < 0.05$ vs control group.

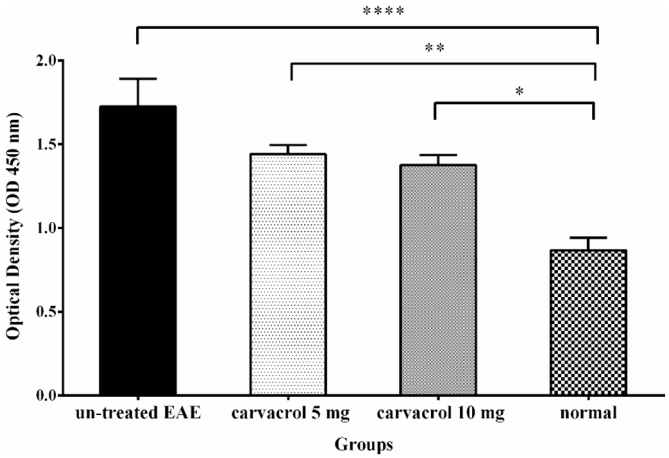

Fig. 3. Analysis of the splenocyte proliferation in Carvacrol-treated EAE mice. There was a trend in decreasing the proliferation of the MOG-stimulated splenocytes in treated groups but the difference was not significant. Proliferation assay was conducted in triplicate wells. One-way analysis of variance (ANOVA) followed by Tukey post-hoc test was conducted to compare the splenocyte proliferation in different groups. Results are presented as the mean ± SEM. *$P < 0.05$, ** $P < 0.01$ and ****$P < 0.0001$ vs control group ($n = 5$).

treated with 5 mg/kg of Carvacrol produced lower levels of IL-17 compared with those from untreated group ($P < 0.05$). The IL-17 production by splenocytes from mice treated with 10 mg/kg was lower than untreated group; however, this difference did not reach a significant level (Fig. 4B). The levels of IL-6 were also significantly lower in stimulated splenocytes from both Carvacrol-treated groups as compared with splenocytes from untreated EAE group ($P < 0.001$, and $P < 0.01$ for 5 and 10 mg/kg Carvacrol, respectively). Also, the IL-6 production by splenocytes from normal mice was lower than mice treated with 10 mg/kg ($P < 0.05$) (Fig. 4C).

3.6. Carvacrol effects on the production of anti-inflammatory cytokines

Overall, the differences of the TGF-β, IL-10 and IL-4 production were significant between groups ($F(3, 16) = 23.15$, $P < 0.0001$ for TGF-β; $F(3, 16) = 9.624$, $P = 0.0007$ for IL-10 and $F(3, 16) = 14.02$, $P < 0.0001$ for IL-6). The amount of TGF-β and IL-10 were not different between untreated EAE mice and normal mice although the amount of IL-4 was significantly lower in normal mice ($P < 0.001$).

The TGF-β concentrations in the supernatant of MOG-stimulated splenocytes from mice treated with 5 and 10 mg/kg Carvacrol were significantly higher than untreated EAE mice ($P < 0.0001$ and $P < 0.001$ respectively) and normal mice ($P < 0.0001$ and $P < 0.001$ respectively) (Fig. 5A). Furthermore, MOG-stimulated splenocytes from mice treated with 5 mg/kg Carvacrol secrete higher levels of IL-10 compared with those from untreated control and normal groups ($P < 0.01$). Likewise, the IL-10 production by splenocytes from mice treated with 10 mg/kg Carvacrol was higher than untreated control and normal groups but not significantly (Fig. 5B). The differences of IL-4 production were not significant between MOG-stimulated splenocytes from untreated and Carvacrol-treated groups but the amount of this cytokine was higher in 5 ($P < 0.001$) and 10 mg Carvacrol treated groups ($P < 0.01$) compare to normal group (Fig. 5C).

4. Discussion

This study aimed to examine the effects of Carvacrol on the clinical signs and production of a number of pro- and anti-inflammatory cytokines in EAE mice. Our results revealed that Carvacrol, especially in lower dose (5 mg/kg), can mitigate the progression course of EAE and prevent the body weight loss. In line with our findings, neuroprotective effects of Carvacrol have also-been demonstrated in a number of neurological diseases such as experimental models of focal cerebral ischemia [26], Parkinson's disease [27,30], epilepsy [31], and traumatic neuronal injury [32].

Although the precise etiology of MS is unmet, it is very likely that the inflammatory and autoimmunity-mediated process including the entrance of immune cells into the CNS play a major role in myelin

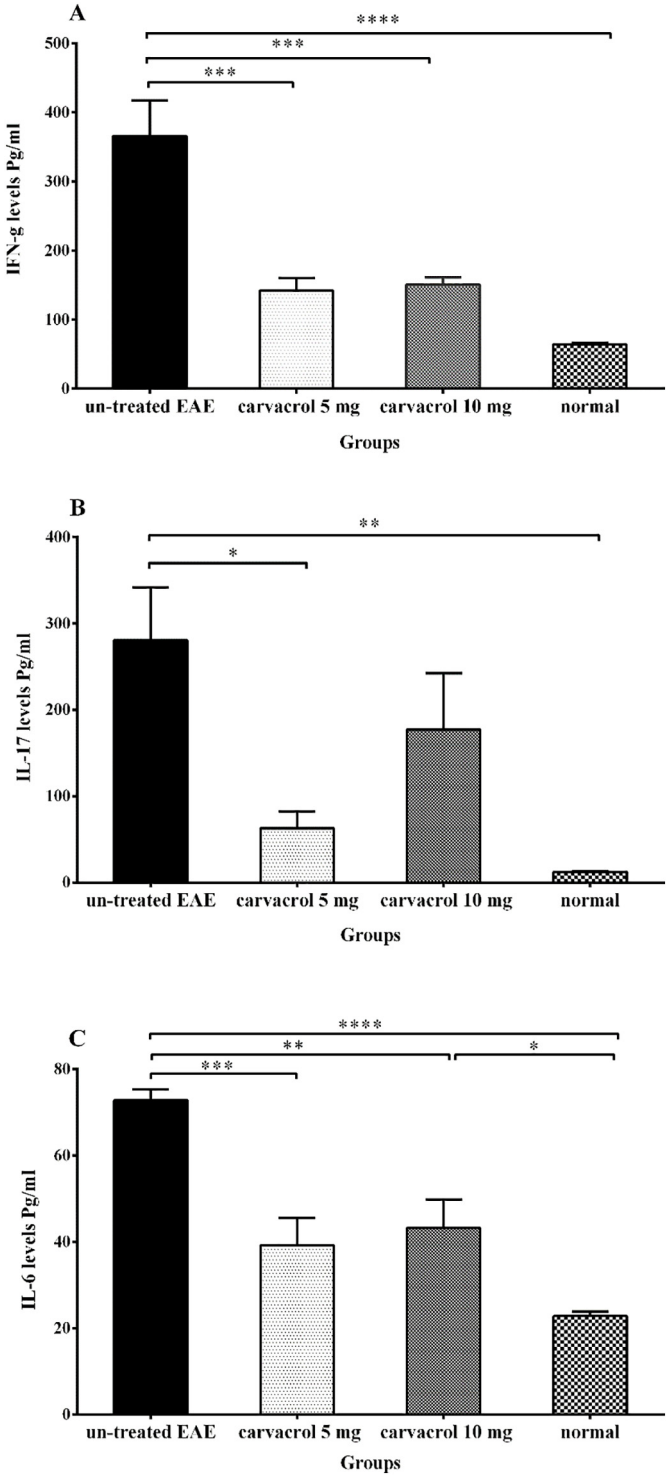

Fig. 4. Analysis of pro-inflammatory cytokine in Carvacrol-treated EAE mice. The average level of pro-inflammatory cytokines (A) IFN-γ, (B) IL-17, and (C) IL-6 in the supernatant of MOG-stimulated splenocytes. Cytokine assays were conducted in duplicate wells. One-way analysis of variance (ANOVA) followed by Tukey post-hoc test was used to compare the amount of cytokines in different groups. Results are presented as the mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ and $^{****}P < 0.0001$vs control group ($n = 5$).

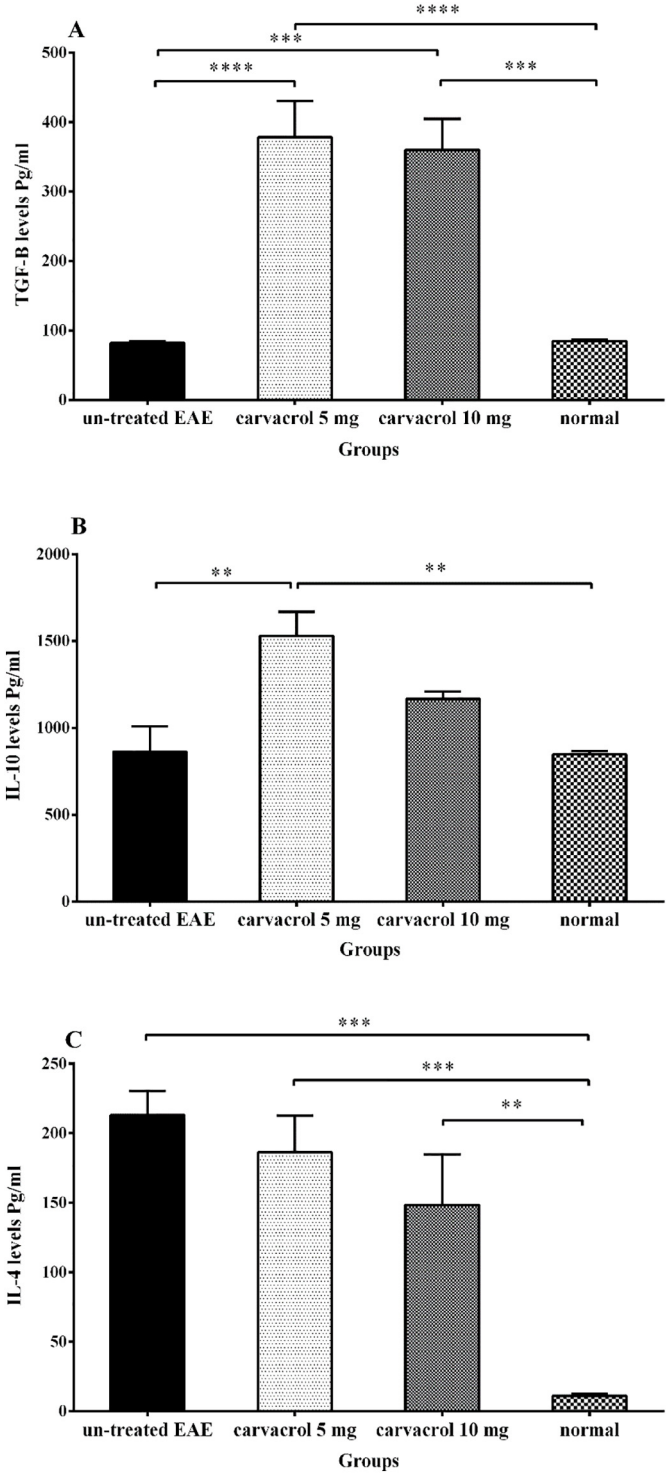

Fig. 5. Analysis of anti-inflammatory cytokine in Carvacrol-treated EAE mice. The average level of anti-inflammatory cytokines (A) TGF-β, (B) IL-10 and (C) IL-4 in the supernatant of MOG-stimulated splenocytes. One-way analysis of variance (ANOVA) followed by Tukey post-hoc test was used to compare the amount of cytokines in different groups. Results are presented as the mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ and $^{****}P < 0.0001$ vs control group ($n = 5$).

degeneration and damage to the axons [1]. In our study, histological staining of spinal cord demonstrated that Carvacrol, particularly at lower concentration, could decrease influx of the immune cells to the spinal cord. The principle role of adhesion molecules and chemokines in the leukocytes infiltration has been well documented [33]. Therefore, the effects of Carvacrol on reducing the leukocytes accumulation within the CNS may in part be due to its modulating effects on the expression of the adhesion molecules and/or pro-inflammatory chemokines.

We also observed that treatment with Carvacrol decreased the

production of IFN-γ, IL-6 and IL-17 by splenocytes. Interestingly, a landmark study has reported that administration of Carvacrol to mice with OVA-induced airways allergy and D-galactosamine induced hepatotoxicity is effective in reducing IFN-γ, IL-6, and IL-17A production [20,34]. Presence of IFN-γ contributes to the development and advancement of MS through several mechanisms, in particular triggering the expansion of M1 macrophages, elicitation of the ROS and NO synthesis by antigen-presenting cells (DCs and macrophages), apoptosis of oligodendrocytes and penetration of encephalitogenic T cells into the CNS [8,9,35]. On the other hand, the absence of IFN-γ or its receptor can aggravate EAE, which can be due to the inhibitory effect of IFN-γ on differentiation of pathogenic Th17 cells [36]. Thus, it has been suggested that imbalanced IFN-γ expression (excessive or complete absence) can aggravate the disease [37].

Th17 cells and its major cytokine IL-17 are involved in MS-related pathological processes [10,11]. IL-17 exerts its pathological influences through augmentation of chemokines and adhesion molecules participating in the movement of the neutrophils from periphery toward the CNS [12]. It also exacerbates the inflammatory responses by increasing the BBB rupture via inducing a number of matrix metallo proteinases and oxidative stress [38]. Therefore, the effects of Carvacrol on decreasing the production of IFN-γ, IL-6 and IL-17 may be performed in part through modulating auto-reactive Th1 and Th17 cells. Additionally, Carvacrol may also reduce severe influx of the leukocytes into the CNS by reducing the inflammatory cytokines that are involved in the local expression of chemokines and adhesion molecules.

The results of this study also indicated higher production of TGF-β and IL-10 by splenocytes in Carvacrol-treated mice. A well-executed study has depicted that Carvacrol acts as a potent suppressor of inflammatory agents (IL-1beta and prostaglandin E2) through enhancement of IL-10 which attenuated edema in a classical paw inflammatory model [39]. Moreover, Carvacrol has shown diminishing effects on OVA-induced airways allergy in mice by enhancing the IL-10 and TGF-β secretion but declining IL-4 production [20]. In autoimmune diseases, anti-inflammatory cytokines contribute to the modulation of immuno-inflammatory pathways. For instance, IL-4, IL-10, and TGF-β cooperatively bypass the generation of Th1/Th17-derived cytokines and consequently repress Th1/Th17 differentiation [12,37]. Additionally, IL-10 significantly participates in regulatory activities including astrocyte survival, releasing of TGF-β from astrocytes, and withholding the production of pro-inflammatory cytokines via reactive microglial cells [40]. Interestingly, prominent feature of immune regulation in terms of improving clinical symptoms in MS can be met through IL-10 [41]. Therefore, our data suggest that Carvacrol could inhibit the production of inflammatory cytokines via induction of anti-inflammatory cytokines or expansion of regulatory cells.

We observed that the secretion of IL-4 by splenocytes did not differ between Carvacrol-treated and control EAE mice. There are some controversies regarding the role of Th2 cells in the pathogenesis of MS. Contrary to our findings, vast majority of experiments imply to excessive levels of IL-4 in autoimmune conditions like EAE and have pointed out the importance of decreased Th1/Th2 ratio in disease improvement [37]. On the other hand, elevated level of IL-4 (as well as IFN-γ and TNF) in serum of MS patients has been reported during the acute stage of the disease but not in the remission phase [41]. One possible explanation can be the participation of IL-4 in shifting immune responses toward humoral immunity for secreting myelin-specific autoantibodies [42]. Therefore, the lower levels of IL-4 in Carvacrol-treated groups can be in favor of a permissive environment for alleviating of EAE.

Moreover, our findings showed a trend in reducing splenic cell proliferation in the Carvacrol-treated mice as reported in OVA-induced airways allergy model [20]. Thus, it is likely that Carvacrol suppresses initial auto-immune cells expansion in the spleen and subsequently diminishes detrimental responses in the CNS. Although in this study, only the severity of leukocyte infiltration into the CNS was evaluated and it would be better to evaluate the local levels of inflammatory and anti-inflammatory cytokines in future studies.

This study showed that the lower dose of Carvacrol (5 mg/kg) was more effective than higher dose (10 mg/kg). One of the possible reasons is likely that 10 mg dose is within the boundary between the toxic doses and the effective doses. Furthermore, investigation of doses lower than 5 mg in EAE model is warranted.

5. Conclusion

The results presented here illustrate that treatment of EAE mice with Carvacrol improves their clinical signs, reduces leukocytes infiltration into the CNS, and modulates production of pro- and anti-inflammatory cytokines. Nevertheless, to understand the precise effects of Carvacrol on other mechanisms involved in the pathogenesis of EAE/MS, more investigation is warranted.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

We would like to thank Dr. Ali Niapour for his kind supports in histopathological analysis of cell infiltration into the spinal cords. We also acknowledge that this study was extracted from a Ph.D. thesis (Merat Mahmoodi) and financially supported by a grant (No. 941744) from Mashhad University of Medical Sciences, Mashhad, Iran.

References

[1] M.M. Goldenberg, Multiple sclerosis review, P T. 37 (3) (2012) 175.
[2] R. Gold, C. Linington, H. Lassmann, Understanding pathogenesis and therapy of multiple sclerosis via animal models: 70 years of merits and culprits in experimental autoimmune encephalomyelitis research, Brain 129 (8) (2006) 1953–1971.
[3] M. Sospedra, R. Martin, Immunology of multiple sclerosis, Annu. Rev. Immunol. 23 (2005) 683–747.
[4] L.K. Peterson, R.S. Fujinami, Inflammation, demyelination, neurodegeneration and neuroprotection in the pathogenesis of multiple sclerosis, J. Neuroimmunol. 184 (1) (2007) 37–44.
[5] J. Fletcher, S. Lalor, C. Sweeney, N. Tubridy, K. Mills, T cells in multiple sclerosis and experimental autoimmune encephalomyelitis, Clin. Exp. Immunol. 162 (1) (2010) 1–11.
[6] M. Greter, F.L. Heppner, M.P. Lemos, B.M. Odermatt, N. Goebels, T. Laufer, R.J. Noelle, B. Becher, Dendritic cells permit immune invasion of the CNS in an animal model of multiple sclerosis, Nat. Med. 11 (3) (2005) 328.
[7] B. Serafini, B. Rosicarelli, R. Magliozzi, E. Stigliano, E. Capello, G.L. Mancardi, F. Aloisi, Dendritic cells in multiple sclerosis lesions: maturation stage, myelin uptake, and interaction with proliferating T cells, J. Neuropathol. Exp. Neurol. 65 (2) (2006) 124–141.
[8] F.O. Martinez, S. Gordon, The M1 and M2 paradigm of macrophage activation: time for reassessment, F1000prime rep 6 (2014).
[9] R.C. Gruber, D. Larocca, S.B. Minchenberg, G.P. Christophi, C.A. Hudson, A.K. Ray, B. Shafit-Zagardo, P.T. Massa, The control of reactive oxygen species production by SHP-1 in oligodendrocytes, Glia 63 (10) (2015) 1753–1771.
[10] S.A. Ghaffari, M. Nemati, H. Hajghani, H. Ebrahimi, A. Sheikhi, A. Jafarzadeh, Circulating concentrations of interleukin (IL)-17 in patients with multiple sclerosis: evaluation of the effects of gender, treatment, disease patterns and IL-23 receptor gene polymorphisms, Iranian J. Neurol. 16 (1) (2017) 15–25.
[11] M.S. Maddur, P. Miossec, S.V. Kaveri, J. Bayry, Th17 cells: biology, pathogenesis of autoimmune and inflammatory diseases, and therapeutic strategies, Am. J. Pathol. 181 (1) (2012) 8–18.
[12] I. Raphael, S. Nalawade, T.N. Eagar, T.G. Forsthuber, T cell subsets and their signature cytokines in autoimmune and inflammatory diseases, Cytokine 74 (1) (2015) 5–17.
[13] M.J. McGeachy, L.A. Stephens, S.M. Anderton, Natural recovery and protection from autoimmune encephalomyelitis: contribution of CD4+ CD25+ regulatory cells within the central nervous system, The J. Immunol. 175 (5) (2005) 3025–3032.
[14] K. Venken, N. Hellings, M. Thewissen, V. Somers, K. Hensen, J.L. Rummens, R. Medaer, R. Hupperts, P. Stinissen, Compromised CD4+ CD25high regulatory T-cell function in patients with relapsing-remitting multiple sclerosis is correlated with a reduced frequency of FOXP3-positive cells and reduced FOXP3 expression at the single-cell level, Immunology 123 (1) (2008) 79–89.
[15] A. Paintlia, M. Paintlia, I. Singh, A. Singh, IL-4-induced peroxisome proliferator-activated receptor gamma activation inhibits NF-kappaB trans activation in central

nervous system (CNS) glial cells and protects oligodendrocyte progenitors under neuroinflammatory disease conditions: implication for CNS-demyelinating diseases, J. Immunol. (Baltimore, Md.: 1950) 176 (7) (2006) 4385–4398.
[16] M.-L. Chen, B.-S. Yan, Y. Bando, V.K. Kuchroo, H.L. Weiner, Latency-associated peptide identifies a novel CD4+ CD25+ regulatory T cell subset with TGFβ-mediated function and enhanced suppression of experimental autoimmune encephalomyelitis, J. Immunol. 180 (11) (2008) 7327–7337.
[17] A. Nostro, T. Papalia, Antimicrobial activity of Carvacrol: current progress and future prospectives, Recent Pat. Anticancer Drug Discov. 7 (1) (2012) 28–35.
[18] A. Ahmad, A. Khan, F. Akhtar, S. Yousuf, I. Xess, L. Khan, N. Manzoor, Fungicidal activity of thymol and Carvacrol by disrupting ergosterol biosynthesis and membrane integrity against *Candida*, Eur. J. Clin. Microbiol. Infect. Dis. 30 (1) (2011) 41–50.
[19] M.V. Sobral, A.L. Xavier, T.C. Lima, D.P. de Sousa, Antitumor activity of monoterpenes found in essential oils, Sci. World J. 2014 (2014).
[20] N. Gholijani, Z. Amirghofran, Effects of thymol and Carvacrol on T-helper cell subset cytokines and their main transcription factors in ovalbumin-immunized mice, J. Immunotoxicol. 13 (5) (2016) 729–737.
[21] F.H.C. Melo, B.A. Moura, D.P. de Sousa, S.M.M. de Vasconcelos, D.S. Macedo, M.M.D.F. Fonteles, G.S.D.B. Viana, F.C.F. de Sousa, Antidepressant-like effect of Carvacrol (5-Isopropyl-2-methylphenol) in mice: involvement of dopaminergic system, Fundam. Clin. Pharmacol. 25 (3) (2011) 362–367.
[22] Z. Zhong, B. Wang, M. Dai, Y. Sun, Q. Sun, G. Yang, L. Bian, Carvacrol alleviates cerebral edema by modulating AQP4 expression after intracerebral hemorrhage in mice, Neurosci. Lett. 555 (2013) 24–29.
[23] M. Jukic, O. Politeo, M. Maksimovic, M. Milos, M. Milos, In vitro acetylcholinesterase inhibitory properties of thymol, Carvacrol and their derivatives thymoquinone and thymohydroquinone, Phytother. Res. 21 (3) (2007) 259–261.
[24] A. Jafarzadeh, M. Mohammadi-Kordkhayli, R. Ahangar-Parvin, V. Azizi, H. Khoramdel-Azad, A. Shamsizadeh, A. Ayoobi, M. Nemati, Z. Hassan, S. Moazeni, Ginger extracts influence the expression of IL-27 and IL-33 in the central nervous system in experimental autoimmune encephalomyelitis and ameliorates the clinical symptoms of disease, J. Neuroimmunol. 276 (1–2) (2014) 80–88.
[25] D. Haghmorad, M.B. Mahmoudi, Z. Salehipour, Z. Jalayer, M. Rastin, P. Kokhaei, M. Mahmoudi, Hesperidin ameliorates immunological outcome and reduces neuroinflammation in the mouse model of multiple sclerosis, J. Neuroimmunol. 302 (2017) 23–33.
[26] H. Yu, Z.-L. Zhang, J. Chen, A. Pei, F. Hua, X. Qian, J. He, C.-F. Liu, X. Xu, Carvacrol, a food-additive, provides neuroprotection on focal cerebral ischemia/reperfusion injury in mice, PLoS One 7 (3) (2012) e33584.
[27] J. Hassanshahi, M. Roghani, S. Raoufi, Protective effect of Carvacrol in 6-hydroxydopamine hemi-parkinsonian rat model, J. Basic and Clin. Pathophysiol. 2 (2) (2014) 29–34.
[28] Y. Guo, S.K. Chung, C.-W. Siu, S.-C. Kwan, P.W.-L. Ho, P.K.-K. Yeung, K.-H. Chan, Endothelin-1 overexpression exacerbate experimental allergic encephalomyelitis, J. Neuroimmunol. 276 (1) (2014) 64–70.
[29] S. Bittner, A. Afzali, H. Wiendl, S. Meuth, Myelin oligodendrocyte glycoprotein (MOG35-55) induced experimental autoimmune encephalomyelitis (EAE) in C57BL/6 mice, J. Vis. Exp. (86) (2014).
[30] H. Haddadi, Z. Rajaei, H. Alaei, S. Shahidani, Chronic treatment with Carvacrol improves passive avoidance memory in a rat model of Parkinson's disease, Arq. Neuropsiquiatr. 76 (2) (2018) 71–77.
[31] T. Baluchnejadmojarad, M. Roghani, The protective effect of Carvacrol on kainic acid-induced model of temporal lobe epilepsy in male rat, J. Basic Clin. Pathophysiol. 4 (2) (2016) 11–16.
[32] W.-T. Li, S.-Y. Zhang, Y.-F. Zhou, B.-F. Zhang, Z.-Q. Liang, Y.-H. Liu, Y. Wei, C.-K. Li, X.-J. Meng, M. Xia, Carvacrol attenuates traumatic neuronal injury through store-operated Ca^{2+} entry-independent regulation of intracellular Ca^{2+} homeostasis, Neurochem. Int. 90 (2015) 107–113.
[33] R.M. Ransohoff, P. Kivisäkk, G. Kidd, Three or more routes for leukocyte migration into the central nervous system, Nat. Rev. Immunol. 3 (7) (2003) 569.
[34] B. Aristatile, A.H. Al-Assaf, K.V. Pugalendi, Carvacrol suppresses the expression of inflammatory marker genes in D-galactosamine-hepatotoxic rats, Asian Pac. J. Trop. Med. 6 (3) (2013) 205–211.
[35] T. Vartanian, Y. Li, M. Zhao, K. Stefansson, Interferon-gamma-induced oligodendrocyte cell death: implications for the pathogenesis of multiple sclerosis, Mol. Med. 1 (7) (1995) 732.
[36] E.H. Tran, E.N. Prince, T. Owens, IFN-γ shapes immune invasion of the central nervous system via regulation of chemokines, J. Immunol. 164 (5) (2000) 2759–2768.
[37] J. Imitola, T. Chitnis, S.J. Khoury, Cytokines in multiple sclerosis: from bench to bedside, Pharmacol. Ther. 106 (2) (2005) 163–177.
[38] J. Huppert, D. Closhen, A. Croxford, R. White, P. Kulig, E. Pietrowski, I. Bechmann, B. Becher, H.J. Luhmann, A. Waisman, Cellular mechanisms of IL-17-induced blood-brain barrier disruption, FASEB J. 24 (4) (2010) 1023–1034.
[39] M. da Silva Lima, L.J. Quintans-Júnior, W.A. de Santana, C.M. Kaneto, M.B.P. Soares, C.F. Villarreal, Anti-inflammatory effects of Carvacrol: evidence for a key role of interleukin-10, Eur. J. Pharmacol. 699 (1–3) (2013) 112–117.
[40] D. Lobo-Silva, G.M. Carriche, A.G. Castro, S. Roque, M. Saraiva, Balancing the immune response in the brain: IL-10 and its regulation, J. Neuroinflammation 13 (1) (2016) 297.
[41] K. Hohnoki, A. Inoue, C.-S. Koh, Elevated serum levels of IFN-γ, IL-4 and TNF-α/unelevated serum levels of IL-10 in patients with demyelinating diseases during the acute stage, J. Neuroinflammation 87 (1–2) (1998) 27–32.
[42] A. Iglesias, J. Bauer, T. Litzenburger, A. Schubart, C. Linington, T-and B-cell responses to myelin oligodendrocyte glycoprotein in experimental autoimmune encephalomyelitis and multiple sclerosis, Glia 36 (2) (2001) 220–234.