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Hesperidin ameliorates immunological outcome and reduces neuroinflammation in the mouse model of multiple sclerosis

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ABSTRACT

Multiple sclerosis (MS) is the most abundant central nervous system (CNS) inflammatory disease, which is due to the reaction of auto reactive T cells with own myelin proteins, leading to physical disorder and paralysis among people suffering the disease. Hesperidin, a flavanone glycoside found abundantly in citrus fruits possesses a wide range of pharmacological properties including potential anti-inflammatory and anti-cancer effects. This study was designed to reveal the molecular and cellular mechanisms underlying the effect of hesperidin on MS alleviation. Female C57BL/6 mice were immunized with $MOG_{35–55}$. Clinical scores and other parameters were monitored daily for the 21 days. At the end of the period, brain/spinal cord histology was performed to measure lymphocyte infiltration; T-cell profiles were determined through ELISA, flow cytometry, and real-time PCR. Transcription factor expression levels in the CNS were assessed using real-time PCR; T cell differentiation was evaluated via flow cytometry. The results demonstrated that hesperidin inhibited development of EAE. Histological studies revealed limited leukocyte infiltration into the CNS. Hesperidin increased Treg cells production of interleukin IL-10 and transforming growth factor (TGF)-β, but concurrently resulted in a significant reduction in production of IL-17 and IL-6. Flow cytometry revealed there were also significant decreases in the percentages of Th17 cells, as well as significant increase in percentages of Treg cells in the spleen and lymph nodes. Real-time PCR results indicated hesperidin treatment reduced ROR-γt factor expression, but enhanced Foxp3 expression. Collectively, these results demonstrated that hesperidin could reduce the incidence and severity of disease.

1. Introduction

Multiple sclerosis (MS) is an inflammatory demyelinating disease of the central nervous system (CNS) characterized by demyelination, axonal degeneration and subsequent loss of motor function. The underlying mechanisms for disease development are not known and a complex interplay between genes and environmental factors are likely to be operating (Haghmorad et al., 2016). About 30% of the MS patients develop clinical paralysis and become wheel chair-bound for the rest of their lives. Although the etiology of MS is not known, it is generally viewed as an autoimmune disease of the CNS. The destruction of oligodendrocyte and myelin sheath in the CNS is the pathological hallmark of MS (Muthian and Bright, 2004; Nosratabadi et al., 2015). Experimental Autoimmune Encephalomyelitis (EAE) is a T cell mediated inflammatory disease of the CNS that clinical and pathological features show close similarity to human MS; therefore, it has been commonly used as an ideal animal model system to study the mechanisms of MS pathogenesis and to test the efficacy of potential therapeutic agents for the treatment of MS. It is generally accepted that auto-reactive, myelin-specific $CD4^+$ T cells are responsible for disease initiation. However, recent studies suggest that oxidative stress play a key role in the pathogenesis of EAE (Muili et al., 2012; Muthian and Bright, 2004).

The pathogenesis of CNS demyelination in EAE/MS is a complex process that involves activation of macrophage/microglial cells, differentiation of neural Ag-specific Th1 cells, and secretion of inflammatory cytokines in the CNS (Natarajan and Bright, 2002). The invasion of $CD4^+$ T cells into the CNS is thought to play a significant role in the pathology of the animal model EAE as well as the human disease MS.

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Previous studies in MS and EAE suggested that the cytokine profile of infiltrating $CD4^+$ T cells is vital in determining the extent of disease pathology (Lees et al., 2008).

It has been determined that Nitric Oxide (NO) and its various oxidative metabolites such as nitrite and nitrate are found at higher concentrations in the cerebral spinal fluid (CSF) of people with MS when compared to the CSF of healthy, non-MS controls. Although an increase in the levels of NO or metabolites and demyelination are both characteristic features of MS, it is not clear if the NO has a direct effect on the formation of these lesions or are a result of such lesions. Summarily, while much is understood about MS, the underlying causes remain unsolved (Letourneau et al., 2010).

Flavonoids are naturally occurring polyphenolic compounds that are present in the human diet. They can be found in a variety of fruits, vegetables, cereals, tea, wine, and fruit juices. Based on variations in their basic structure, flavonoids are divided into nine subclasses, of which the major three are flavones, flavonols and flavanones. Flavonoids are known for their strong anti-oxidant properties, protecting tissues against oxidative stress. Several reports have suggested that diseases associated with oxidative stress and inflammatory diseases may be beneficially influenced by flavonoids which can directly quench free radicals, inhibit enzymes of oxygen reduction pathways and sequester transient metal cations (Verbeek et al., 2005; Nones et al., 2012).

Hesperidin, a flavanone glycoside found abundantly in citrus fruits such as sweet orange and lemon, possesses a wide range of pharmacological properties including potential anti-inflammatory and anti-cancer effects. Hesperidin induces cell growth arrest and apoptosis in a large variety of cells including colon and pancreatic cancer cells (Ghorbani et al., 2012).

Recent data reveal that in vitro flavonoids inhibit antigen specific memory T cell proliferation and pro-inflammatory IFN-γ production and reduce the phagocytic activity by macrophages. As well as flavonoids could have beneficial effects during the pathogenesis of MS and EAE since some of them have a strong inhibitory effect in vitro on proliferation and IFN-γ production of auto antigen specific T cells (Verbeek et al., 2005).

Flavonoids have been described as important neuroprotective molecules in diverse neuronal insults such as ischemia, oxidative-induced damage, dopamine induced neurotoxicity and anti amyloidogenic in Alzheimer's disease. Hesperidin-primed astrocytes protect against neuronal cell death. Astroglia interactions play key roles in several events of brain development, such as the proliferation and differentiation of neuronal precursors and neuronal migration (Nones et al., 2012).

The neuroprotective efficacy of hesperidin is attributed to its ability of inhibiting Fe^{2+} induced linoleate peroxidation and auto oxidation of cerebral membranes, scavenging peroxynitrite radicals and inhibition of ROS generation, including hydroxyl radical. It protects the neurons against various types of insults associated with many neurodegenerative diseases (Raza et al., 2011). Flavonoids play an important role in platelet reactivity and in the induction of anti-inflammatory cytokines in circulating monocytes (Kim et al., 2011).

Accordingly, this paper aimed to evaluate therapeutic potentials of hespridin on the improvement of EAE induced mice and elucidate the possible mechanisms involved.

2. Material and methods

2.1. Animals

C57BL/6 mice (female, 8-week-old) were obtained from the Pasture Institute of Iran (Tehran, Iran). Mice were housed in animal facilities of the Bu-Ali Research Institute maintained at 25 °C with a 50% relative humidity and a 12 h light/dark cycle. All mice had ad libitum access to standard rodent chow and filtered tap water. All experiments were performed according to Mashhad University of Medical Science ethical guidelines.

2.2. Induction of EAE

EAE was induced by immunizing C57BL/6 mice subcutaneously in the flank with 250 μg $MOG_{35–55}$ (MEVGWYRSPFSRVVHLYRNGK) (SBS Genetech Co. Ltd., Beijing, China) emulsified in complete Freund's adjuvant (Sigma-Aldrich, St. Louis, MO, USA) containing 4 mg/mL M. tuberculosis H37RA (Difco Laboratories, Detroit, MI, USA). On the day of immunization, and again 48 h later, mice were injected intravenously with 250 ng pertussis toxin (Sigma-Aldrich, St. Louis, MO, USA) (Huehnchen et al., 2011; Haghmorad et al., 2014a). Clinical signs of EAE were monitored and weight of the mice was measured daily until 25 days post immunization. Mice were scored for neurologic malfunction, in accordance with the following scale: 0, no clinical sign; 1, partial loss of tail tonicity; 2, complete loss of tail tonicity; 3, flabby tail and abnormal manner of walking; 4, hind leg paralysis; 5, hind leg palsy with hind body partial immobility; 6, hind and foreleg paralysis; 7, moribund or death (Haghmorad et al., 2014b; Mosayebi et al., 2010). Mice were scored daily and were assessed for incidence, onset day of disease, maximal score (at the peak day), mean score (at the last day) and Cumulative Disease Index (total disease score over experiment duration).

2.3. Hesperidin treatment

Mice were divided into four groups: 1. Control group (CTRL; n = 12), 2. Low-dose hesperidin treatment group (T1; n = 11; 50 mg/kg hesperidin), 3. Middle-dose hesperidin treatment group (T2; n = 10; 100 mg/kg hesperidin) and 4. High-dose hesperidin treatment group (T3; n = 12; 200 mg/kg hesperidin) (Trivedi et al., 2011; Shagirtha and Pari, 2011; Ahmad et al., 2012). EAE were induced in all groups and treatment groups were treated with three different dose of hesperidin to earn maximum advantage.

Hesperidin was administered in treatment groups orally simultaneous with EAE induction every day for 25 days. Control group received PBS as a solvent orally every day for 25 days.

Table 1
Sequences of primer and probes which used in study.

Genes	Forward	Reverse	Probe
Foxp3	CAGAGTTCTTCCACAACA	CATGCGAGTAAACCAATG	TGAGTGTCCTCTGCCTCTCCG
GATA3	CTGCGGACTCTACCATAA	GTGGTGGTCTGACAGTTC	CTGCTCTCCTTGCTGCCGAC
IFN-γ	CCAAGTTTGAGGTCAACA	CTGGCAGAATTATTCTTATTGG	CCGAATCAGCAGCGACTCCT
IL-4	CTGGATTCATCGATAAGC	GATGCTCTTTAGGCTTTC	TGAATGAGTCCAAGTCCACATCACT
IL-6	CCAACAGACCTGTCTATA	GCATCATCGTTGTTCATA	CACAAGTCGGAGGCTTAATTACACATG
IL-10	CAGGTGAAGACTTTCTTTC	AACCCAAGTAACCCTTAA	ACAACATACTGCTAACCGACTCCTT
IL-17	CCTCAGACTACCTCAACC	CCAGATCACAGAGGGATA	ACTCTCCACCGCAATGAAGACC
IL-23	CGGGACATATGAATCTACTAA	TGTCCTTGAGTCCTTGTG	CAACCATCTTCACACTGGATACGG
TGF-β	CGGACTACTATGCTAAAGA	CTGTGTGAGATGTCTTTG	CGTTGTTGCGGTCCACCATT
T-bet	TGTGGTCCAAGTTCAACC	CATCCTGTAATGGCTTGTG	TCATCACTAAGCAAGGACGGCG
ROR-γt	GGATGAGATTGCCCTCTA	CCTTGTCGATGAGTCTTG	CTCATCAATGCCAACCGTCCTG
B2m	CCTGTATGCTATCCAGAA	GTAGCAGTTCAGTATGTTC	TATACTCACGCCACCCACCG

2.4. Histological analysis

To estimate the rate of CNS inflammation and demyelination at the time of sacrifice (at day 25), mice were anesthetized with Ketamine & Xylazine and perfused by intracardiac injection of PBS containing 4% paraformaldehyde. Paraffin embedded 5 mm sections of brain and spinal cord were stained with Hematoxylin and Eosin (H&E) for evaluation of inflammation and Luxol fast blue for demyelination. Sections are scrutinized by light microscopy in a blinded manner. Inflammation was determined as follows; 0. No inflammation; 1. Small number of inflammatory cells; 2. Existence of perivascular infiltrates; 3. Extending intensity of perivascular cuffing with expansion into contiguous tissue. Demyelination was scored as follows: 0. No demyelination thing; 1. Unique foci; 2. Few sections of demyelination; 3. Considerable sections of demyelination (Horstmann et al., 2013; Sloka et al., 2013).

2.5. Cell culture and BrdU proliferation assay

Peripheral lymph nodes (inguinal and axillary) and spleens were excised from C57BL/6 mice on day 25 post immunization. Red blood cells were depleted using ammonium chloride. Cell suspensions were prepared and cultured in RPMI 1640 medium containing 10% fetal bovine serum (FBS), 100 U/mL penicillin, 100 mg/mL streptomycin (all reagents bought from Sigma, St. Louis, MO) in round-bottom 24-well plates (2×10^6 cells/well) and 96-well plates (4×10^5 cells/well). Cells were cultured and then treated with 50 μL medium alone or medium + $MOG_{35–55}$ (20 μg/mL; Chengdu Kaijie Biopharm Co., Chengdu, Sichuan, China). Cultures were incubated for 72 h at 37 °C and 5%CO_2 and the last 24 h for 96-well plates in the presence of BrdU labeling solution (100 μL/mL). Proliferation was evaluated using a Cell Proliferation ELISA, BrdU kit (colorimetric) in compliance with the manufacturer's instructions (Roche Applied Science, Indianapolis, IN, USA). Final measures were taken in the microplate reader (Stat Fax 2100 Awareness, Phoenix, Arizona, USA).

2.6. Detection of nitric oxide (NO) production

For determination of nitric oxide (NO) concentration in spinal cord tissue, the stable conversion product of nitric oxide, nitrite (NO^{2-}) as the end product of reaction was measured using the nitrate/nitrite colorimetric assay (R&D Systems, Inc. Minneapolis, MN, USA). In brief, supernatant from homogenized and centrifuged spinal cord tissue was added to 25 μL NADH and Nitrate reductase and incubate at 37° for 30 min, after converting nitrate in the sample to nitrite with nitrate reductase, Griess Reagent (1% sulfanilamide, 0.1% N-(1-naphthyl)-ethylene diamine hydrochloride, 2.5% H3PO4) were added at room temperature to convert nitrite into a deep purple compound. The absorbance at 540 nm was read after 10 min using a microplate reader (Stat Fax 2100 Awarness, Phoenix, Arizona, USA). The concentrations of nitrite were derived from standard curve analysis using serial dilutions of sodium nitrite as a standard.

2.7. ELISA for cytokine detection

Supernatants from 24-well plates were gathered after 72 h, and cytokine concentrations (IL-4, IL-6, IL-10, IL-17, TNF-α, TGF-β and IFN-γ) were measured by ELISA according to the manufacturer's instructions (eBioscience, San Diego, CA, USA). Briefly, ELISA plates were coated with capture antibody diluted in coating buffer and incubate overnight at 4 °C. After washing wells were blocked with ELISA diluent for 1 h at room temperature. For cytokine detection, standards and supernatants were incubated at room temperature for 2 h, followed by 1 h of incubation with biotinylated secondary antibody and then 30 min incubation with Avidin-HRP. Plates were developed using tetra methyl benzidine (TMB). Reaction was stopped with stop solution and plates were measured at 450 nm using the microplate reader (Stat Fax 2100 Awarness, Phoenix, AZ, USA). Standard curves were calculated based on measurements of different concentrations of recombinant cytokines. The level of sensitivity of the kits was 2 pg IL-4/mL, 6.5 pg IL-6/mL, 5 pg IL-10/mL, 1.6 pg IL-17/mL, 3.7 pg TNF-α/mL, 12 pg TGF-β/mL, and 5.3 pg IFN-γ/mL.

2.8. Flow cytometry

Splenocytes were removed and suspended in complete RPMI 1640 with 10% FBS at a density of 5×10^6/mL. For intracellular cytokine

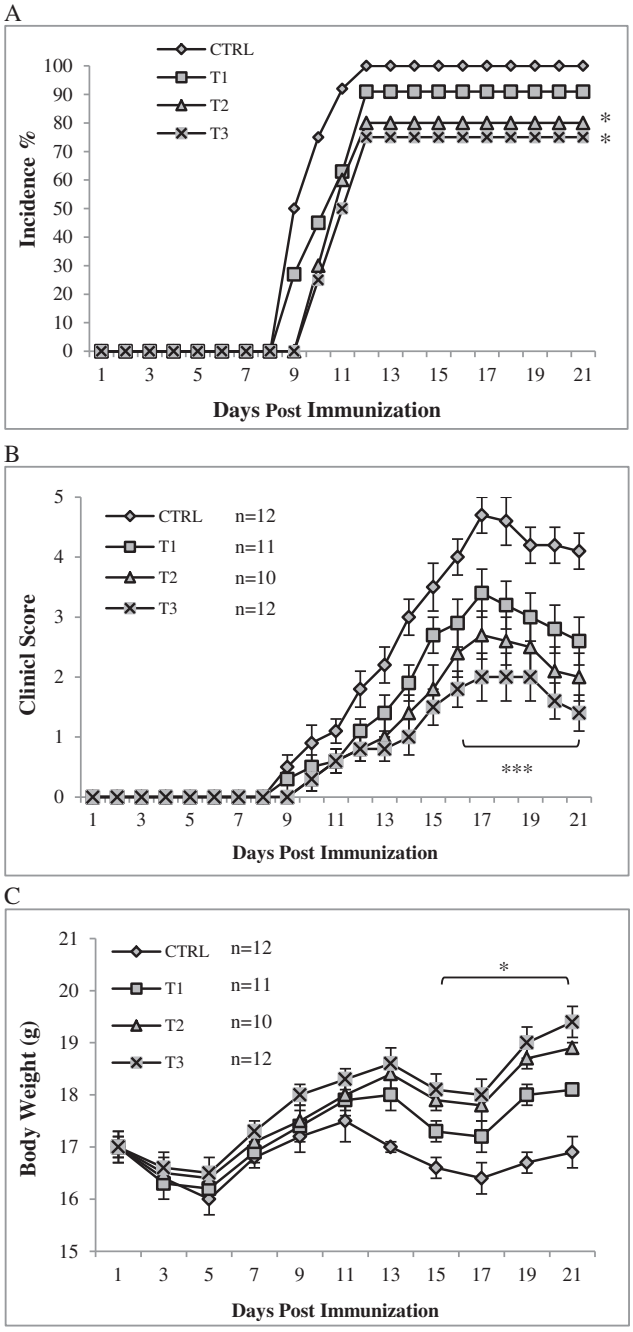


Fig. 1. Hespridin inhibited the development of EAE in MOG-immunized C57BL/6 mice. Female C57BL/6 mice were treated with 50, 100 and 200 mg/kg hesperidin simultaneous with EAE induction as detailed under Material and methods. Mice were monitored for signs of EAE, and the results for all mice, were presented as (A) incidence of disease, (B) mean clinical score ± SEM, and (C) body weight. Data are representative of 3 independent experiments with 2 month interval for each experiment. Results were expressed as mean ± SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, compared with control group. Mice were divided into four groups: 1. Control group (CTRL), 2. Low dose hesperidin treatment group (T1), 3. Middle dose hesperidin treatment group (T2) and 4. High dose hesperidin treatment group (T3).

Table 2
Clinical features of EAE in the administration of Hesperidin.

Group	Incidence %	Day of onset	Maximal score (Score at peak)	Mean score (Last Day)	Cumulative Disease Index (CDI)
CTRL[a]	100%	9.8 ± 1.03	4.7 ± 0.3	4.1 ± 0.3	38.8 ± 1.5
T1[b]	91%	10.7 ± 1.4	3.4 ± 0.4*	2.6 ± 0.3*	26.4 ± 1.11*
T2[c]	80%*	11.6 ± 1.2**	2.7 ± 0.3***	2 ± 0.4***	20.2 ± 1.06**
T3[d]	75%*	11.8 ± 1.06**	2 ± 0.4***	1.4 ± 0.3***	15.8 ± 0.8***

Data were expressed as mean ± SEM.
T1, T2 and T3 groups compared with CTRL group.
[a] Ctrl: Control group EAE induced.
[b] T1: Treatment group with low dose received 50 mg/kg hesperidin.
[c] T2: Treatment group with middle dose received 100 mg/kg hesperidin.
[d] T3: Treatment group with high dose received 200 mg/kg hesperidin.
* $p < 0.05$.
** $p < 0.01$.
*** $p < 0.001$.

staining, mononuclear cell suspensions were restimulated with PMA (phorbol 12-myristate 13-acetate) (50 ng/mL, Sigma-Aldrich, St. Louis, MO, USA) and ionomycin (1 μg/mL, Sigma-Aldrich, St. Louis, MO, USA) for 4 h in the presence of Brefeldin A, (10 mg/mL, BD Biosciences, San Jose, CA, USA). Cells were collected and washed in staining buffer. Following second wash step, cells were stained with a FITC *anti*-CD4 (*GK1.5*) and PE-Cy5 anti-CD8 (536.7) for 30 min at 4 °C. Cells were washed, fixed and permeabilized using fixation/permeabilization buffer

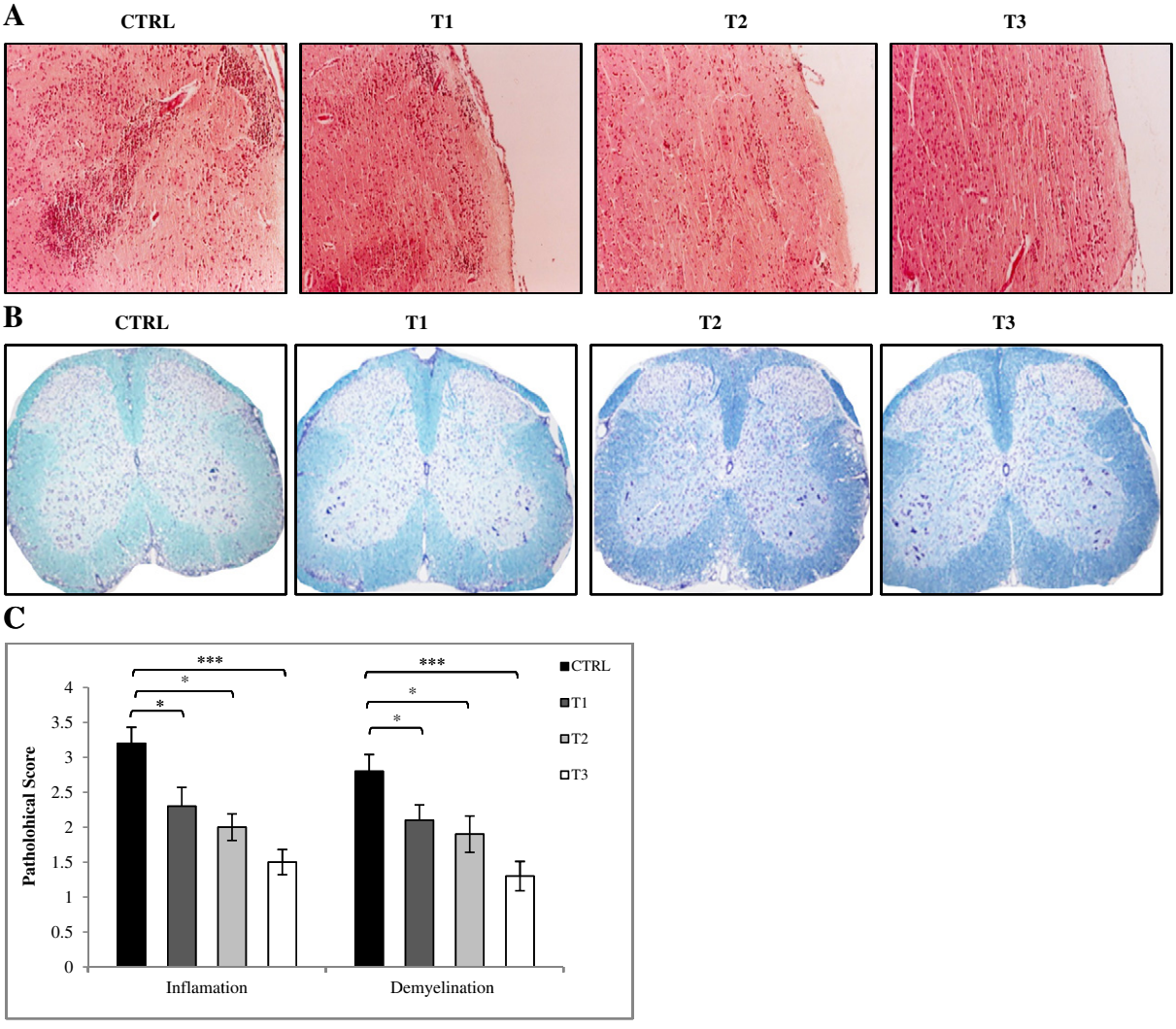


Fig. 2. Comparative histopathology of spinal cords demonstrated Hesperidin suppresses CNS inflammation and demyelination. Histopathological evaluation of spinal cords from treated groups (low, middle and high dose hesperidin) was performed. Spinal cords from each group, collected on day 25 post immunization, fixed in paraformaldehyde and embedded in paraffin. Five μm sections from different regions of the spinal cord from each of the groups were stained (A) with H&E to enumerate infiltrating leukocytes and (B) with Luxol fast blue to assess demyelination. (C) CNS inflammatory foci and infiltrating inflammatory cells were quantified. Pathological scores including inflammation and demyelination were analyzed and shown with bar graph as mean scores of pathological inflammation or demyelination ± SEM. Data are representative of 3 independent experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared with control group. Mice were divided into four groups: 1. Control group (CTRL), 2. Low dose hesperidin treatment group (T1), 3. Middle dose hesperidin treatment group (T2) and 4. High dose hesperidin treatment group (T3).

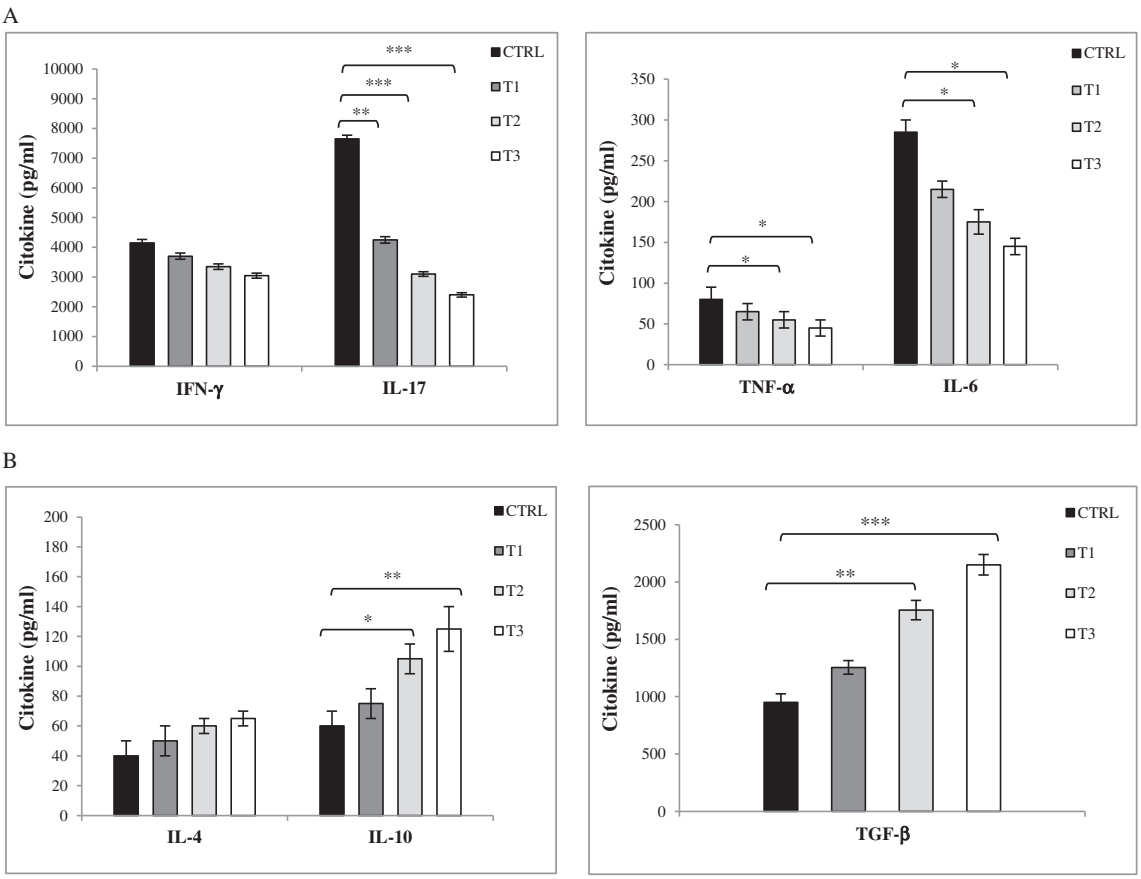


Fig. 3. High and Middle dose Hesperidin suppressed pro-inflammatory cytokines production except IFN-γ and enhanced anti-inflammatory cytokines production excluding IL-4 in splenocytes and lymph nodes from EAE mice. Splenocytes and lymph nodes from immunized mice from all groups (45 mice) were isolated on day 25 post immunization and restimulated with MOG_{35-55} (20 mg/mL) for 72 h. Culture supernatants were collected and indicated cytokine levels were measured by ELISA. Cytokine assays were conducted in duplicate wells. (A) Pro-inflammatory cytokines as IFN-γ, IL-17, TNF-α and IL-6 ± SEM and (B) Anti-inflammatory cytokines as IL-4, Il-10 and TGF-β were measured from supernatants of cultures from splenocytes and lymph nodes. (C) The average concentration ratio of IFN-γ or IL-17 to IL-4 and IL-10 was calculated for all mice. Results from lymph nodes were similar to splenocytes and data was not shown. Mice were divided into four groups: 1. Control group (CTRL), 2. Low dose hesperidin treatment group (T1), 3. Middle dose hesperidin treatment group (T2) and 4. High dose hesperidin treatment group (T3). Results were expressed as mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001 compared with control group.

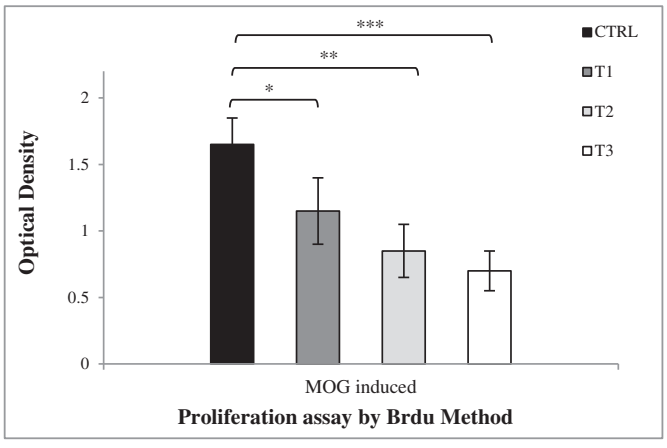


Fig. 4. Hesperidin suppresses T-cell proliferation. Spleen cells were harvested on day 25 post immunization and cultured in medium alone or with MOG (20 μg/mL) for 72 h on 96-well plates. Proliferation responses tested using a Cell Proliferation ELISA, BrdU (colorimetric) kit (Roche Applied Science, Indianapolis, USA). Proliferation assay were conducted in triplicate wells. Data presented as mean optical density ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001 compared with control group. Mice were divided into four groups: 1. Control group (CTRL), 2. Low dose hesperidin treatment group (T1), 3. Middle dose hesperidin treatment group (T2) and 4. High dose hesperidin treatment group (T3).

(BD Biosciences, San Jose, CA, USA). At last, cells were stained for intracellular cytokines with PE-conjugated rat anti-mouse IL-4 (11B11), IFN-γ (XMG1.2) and IL-17 (eBio17B7) antibodies. For intracellular staining

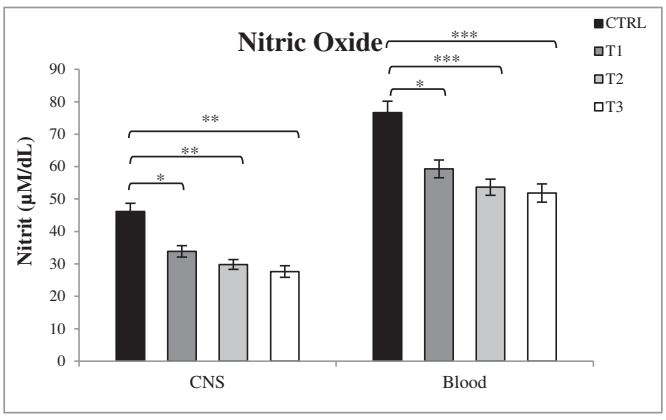
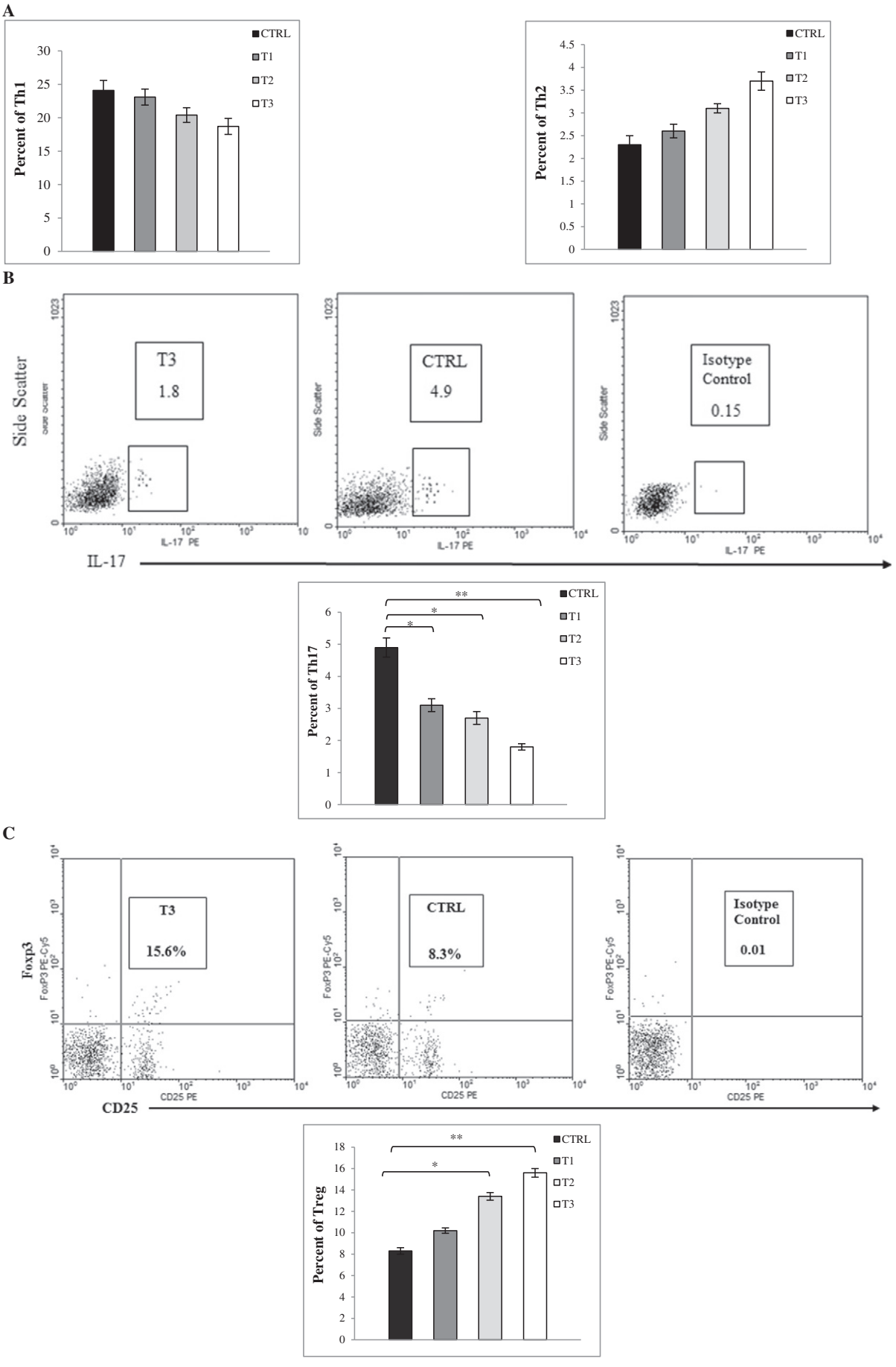


Fig. 5. Hesperidin suppressed Nitric Oxide (NO) production in both CNS and Blood. NO concentration in blood and spinal cord tissue was measured by the Griess reagent with using the nitrate/nitrite colorimetric assay. Data presented as mean optical density ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001 compared with control group. Mice were divided into four groups: 1. Control group (CTRL), 2. Low dose hesperidin treatment group (T1), 3. Middle dose hesperidin treatment group (T2) and 4. High dose hesperidin treatment group (T3).


A
Percent of Th1
CTRL
T1
T2
T3
Percent of Th2
B
Side Scatter
T3
1.8
CTRL
4.9
Isotype
Control
0.15
IL-17 PE
IL-17
Percent of Th17
C
Foxp3
FoxP3 PE-Cy5
T3
15.6%
CTRL
8.3%
Isotype
Control
0.01
CD25 PE
CD25
Percent of Treg

of Foxp3, mononuclear cells washed in staining buffer and stained with a FITC anti-CD4 (*GK1.5*) and PE anti-CD25 (PC61.5) for 30 min at 4 °C. Cells were washed and incubated in fixation/permeabilization buffer (BD Biosciences, San Jose, CA, USA). Foxp3 staining was performed using the PE-Cy5 anti-Foxp3 (FJK-16 s) antibody. All flow cytometric experiments were performed according to the manufacturer's instructions and using appropriate isotype controls. All antibodies were obtained from eBioscience (San Diego, CA, USA). Data were collected on a FACSCalibur (BD Biosciences, San Jose, CA, USA) and analyzed using CellQuest software. A minimum of 20,000 events/sample was acquired.

2.9. Real-time PCR

To determine cytokine expression and immune cells infiltration, both spinal cord and brain were removed from diseased mice at days 25 after EAE induction. The tissues of each mouse were separately pulverized in phosphate buffer using a nylon mesh to detach cells. The suspension was centrifuged at 3000 g for 10 min and the resulting cell pellet was suspended in TriPure Isolation Reagent (Roche Applied Science, Indianapolis, IN, USA) for RNA extraction. cDNA synthesis was conducted by PrimeScript™ RT reagent Kit (Takara Bio Inc., Otsu-Shiga, Japan) in compliance with manufacturer's guidance. As well as the mononuclear cells from spleen and lymph nodes were suspended in TriPure for RNA extraction and cDNA synthesis. Real-time PCR was accomplished by applying TaqMan PCR master mix Kit (Takara Bio Inc., Otsu-Shiga, Japan) with appropriate primers and probe (Table 1). Reactions were conducted on the Rotor Gene Q (Qiagen Hilden, Germany) to detect the quantity of mRNA expression compared with the β2 microglobulin (β2mG) reference gene. Based on expression of target genes normalized to β2mG, we calculated the relative quantification delta-delta CT and the results are presented as fold change compared to control.

2.10. Statistical analysis

One-way, non-parametric analysis of variance (ANOVA) (Kruskal-Wallis test) followed by Dunn's multiple comparison tests was conducted for analysis of clinical signs between groups. Comparison of the effect of hesperidin in treatment groups against control group, on the development of clinical signs conducted via two-way repeated measures ANOVA. Mann–Whitney non-parametric unpaired *t*-tests were used for two group comparisons. SPSS 21 was used to analyze the data. Data were presented as mean ± SEM. Statistical difference was accepted at the level of $p < 0.05$.

3. Results

3.1. Hesperidin treatment inhibited the development of EAE

It has been previously established that hesperidin can ameliorate cell-mediated autoimmune diseases such as rheumatoid arthritis and MS. As a result, hesperidin could be considered for the treatment of these diseases in animal model. The evaluation of hesperidin with different doses in MOG induced mice has not been investigated. The purpose of this study was to determine whether different doses of hesperidin could provide protection against EAE. In the current study, 8–12-week-old C57BL/6 mice ($N = 45$) were immunized with MOG_{35-55} peptide together with *Mycobacterium tuberculosis* and pertussis toxin to induce an encephalitogenic T-cell response and a chronic progressive form of EAE.

The doses of hesperidin chosen for these studies were low dose (50 mg/kg), middle dose (100 mg/kg) and high dose (200 mg/kg) of hesperidin. High and middle dose of hesperidin reduced the incidence and severity of clinical disease and more importantly, low dose hesperidin profoundly reduced the clinical manifestations of disease. All treatment group significantly reduced severity of paralysis when compared with control group. Likewise high dose treated mice (T3) almost completely protected against EAE through day 25 post immunization.

The clinical scores of treatment group with low dose hesperidin (T1: 2.3 ± 0.4), middle dose hesperidin (T2: 2 ± 0.4) and high dose hesperidin (T3: 1.4 ± 0.3) were significantly lower than control group (CTRL, 4.1 ± 0.3) (Fig. 1 and Table 2).

3.2. Hesperidin reduced immune cell infiltration into the CNS

To determine the effect of hesperidin therapy on CNS pathology, we evaluated inflammatory cell infiltration and demyelination of the spinal cord during EAE on day 25 post immunization. To evaluate mononuclear cell infiltration and perivascular cuffing, brain and spinal cord were analyzed by Hematoxylin and Eosin (H&E). Fewer infiltrating cells and areas of perivascular cuffing were observed in low, middle and high dose hesperidin treated groups as compared to CTRL group. High inflammation and cell infiltration on the brain and spinal cord have showed in CTRL group (Fig. 2A). To evaluate demyelination, brain and spinal cord sections were stained by Luxol fast blue. Here we observed significant demyelination in the areas of brain and spinal cord during the progression of disease in CTRL group (Fig. 2B). Treatment groups demonstrated reduction in brain and spinal cord demyelination during disease development. The lowest level of inflammation and cell infiltration on the brain and spinal cord as well as demyelination related to T3 group with high hesperidin therapy (Fig. 2C).

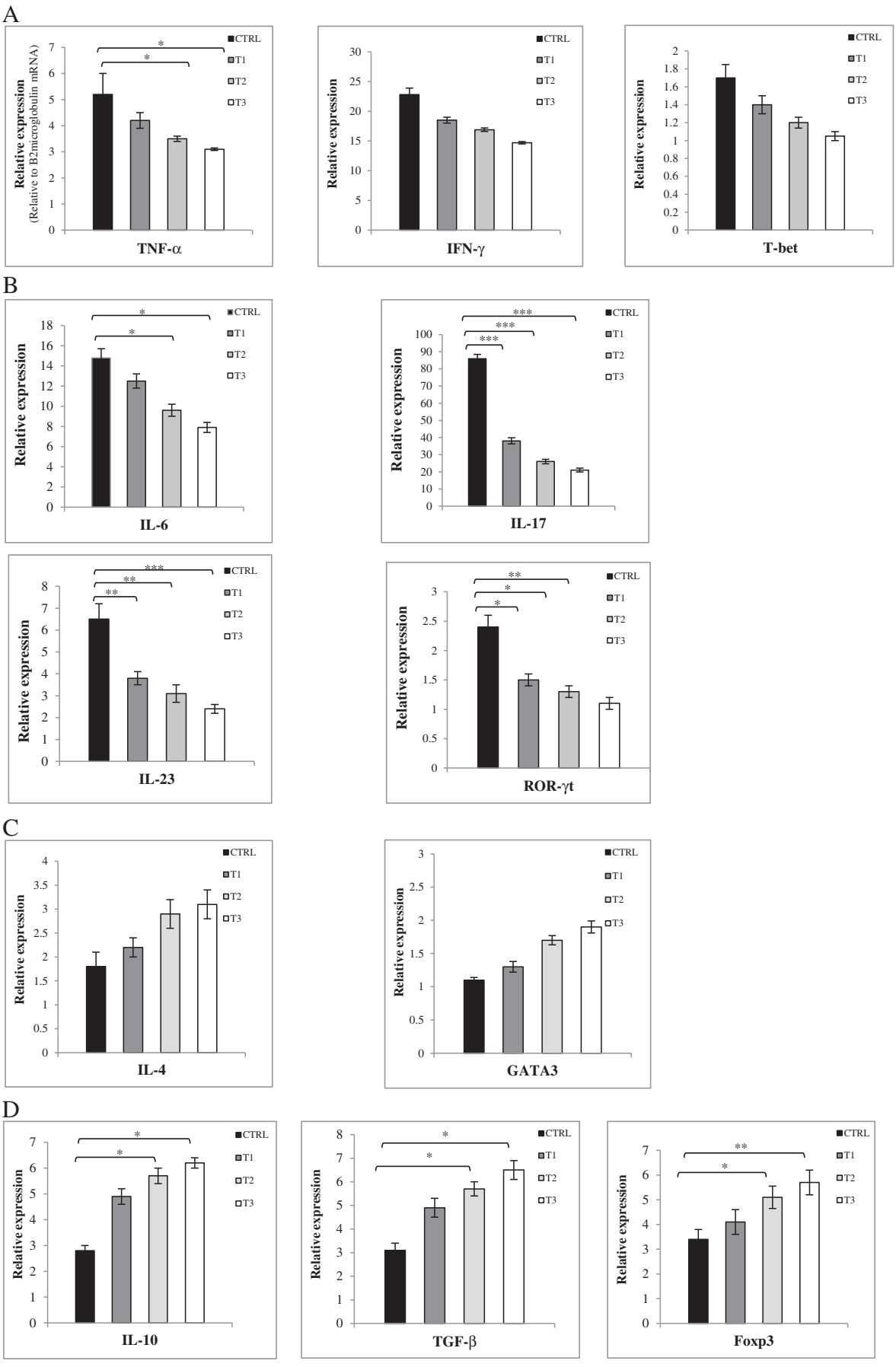
3.3. Hesperidin especially changes the balance of pro-inflammatory and regulatory cytokines

To determine the role of specific inflammatory mediators such as IL-6, TNF-α, IL-17 and IFN-γ we examined cytokine production in spleen and lymph node on day 25 post immunization. Splenocytes and lymph node cells were isolated from EAE-immunized mice, and then re-stimulated with the immunizing peptide MOG_{35-55} in vitro. Cells from all treated groups produced significantly less IL-17 relative to CTRL group; TNF-α and IL-6 also were found to be decreased in high and middle dose of hesperidin relative to CTRL group. Cells from low dose of hesperidin produced less TNF-α and IL-6 in comparison with CTRL group but is not significant (Fig. 3A).

However, at the RNA level, expression of IL-6, IL-17, and TNF-α were all down-regulated in high and middle dose hesperidin treated groups relative to CTRL group. Moreover, we found that splenocytes and lymph node cells from the high and middle dose hesperidin treated groups had increased production of IL-10 and TGF-β (Fig. 3B). Low dose hesperidin treated group had increased production of IL-10, TGF-β and decreased production of TNF-α, IL-6; however this differences is not significant relative to control group but it made to reduce severity of disease.

These data suggest that treatment with middle and high dose of hesperidin preferentially increase the production of Treg cytokines, and

Fig. 6. Flow cytometry profiles of spleen mononuclear cells. The percentage of Th1, Th2, Th17 and Treg cells in the $CD4^+$ gate were analyzed by flow cytometry. Mononuclear cells were isolated from spleen at the time of sacrifice on day 25 post immunization from mice induced EAE. Mononuclear cells were stimulated with PMA and ionomycin in the presence of the Golgi inhibitor brefeldin A for 4 h, then stained and analyzed by flow cytometry for intracellular production of (A) Th1 related marker, IFN-γ and Th2 related marker, IL-4, (B) Th17 related marker, IL-17. Values in the bar graphs are the mean ± SEM. (C) for intracellular staining of Foxp3, mononuclear cells washed and stained with *anti*-CD4 and anti-CD25 antibodies for 30 min at 4 °C. Cells were washed and incubated in fixation/permeabilization buffer. Foxp3 staining was performed using the anti-Foxp3 antibody. $CD4^+$ T cells from spleen were gated and their CD25 and Foxp3 expression were analyzed by flow cytometry. Values in the bar graphs are the mean ± SEM. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ compared with control group. Mice were divided into four groups: 1. Control group (CTRL), 2. Low dose hesperidin treatment group (T1), 3. Middle dose hesperidin treatment group (T2) and 4. High dose hesperidin treatment group (T3).


A
Relative expression (Relative to B2microglobulin mRNA)
TNF-α
IFN-γ
T-bet
B
IL-6
IL-17
IL-23
ROR-γt
C
IL-4
GATA3
D
IL-10
TGF-β
Foxp3
CTRL
T1
T2
T3

changes the balance of pro-inflammatory and regulatory cytokines in EAE model.

3.4. Hesperidin-treated mouse splenocytes demonstrate reduced T-cell proliferation ex vivo

MOG induced EAE is a T cell-mediated disease and auto-reactive T cells in EAE are activated within peripheral lymph nodes and spleen before they migrate into the CNS. To determine T cell proliferative capability, 25 day post-immunization lymph node cells and splenocytes were isolated and cultured upon stimulation with MOG_{35-55} peptide, and then BrdU incorporation assay was performed. The results clearly demonstrated that mononuclear cells of hesperidin treated mice had significantly lower proliferative capability in lymph nodes and spleen compared with CTRL group (Fig. 4).

3.5. Hesperidin inhibit production of nitric oxide in the CNS of EAE induced mice

We examined the pro-inflammatory molecule, nitric oxide (NO) secreted in the CNS. As shown in (Fig. 5.), the content of NO was significantly decreased in the brain and spinal cords of hesperidin treated groups relative to CTRL group. Administration of hesperidin (High, middle and low dose) obviously inhibited the production of NO in the central nervous system.

3.6. Hesperidin polarize $CD4^+$ T-cells to regulatory status

To further investigate the possible role of hesperidin in T helper polarization, we analyzed the $CD4^+$ T cell subsets in lymph nodes (inguinal and axillary) and spleens from hesperidin treated groups in comparison with CTRL group by flow cytometry. In compliance with cytokine ELISA results, there were significant decrease of the percentage of IL-17 producing $CD4^+$ T cell (Th17), and also significant increase of the percentage of $CD4^+$ $CD25^+$ $Foxp3^+$ T cells (Treg) in splenocytes and lymph node cells of high and middle dose hesperidin treated groups (Fig. 6). Furthermore, when we analyzed low dose hesperidin treated group, we found low levels of Th17 cells and high levels of Treg cells, however, these results were not statistically significant in contrast with CTRL group. Despite, remarkable decrease in IFN-γ producing T cell (Th1) and increase in IL-4 producing T cell (Th2) observed in treatment groups related to control group but these differences were not significant.

In addition, the expression of Th1, Th2, Th17 and Treg specific transcription factors in spleens were examined by Real-Time PCR. The results indicated that hesperidin treatment reduced ROR-γt (Th17) expression and enhanced Foxp3 (Treg) expression. Parallel to flow cytometry results, T-bet and GATA3 expression have remarkable changes related to control group but these differences were not significant. (Figure related to these data was similar to CNS mRNA expression results and did not shown)

3.7. T-cell infiltration and cytokine production in CNS upon EAE induction

To evaluate infiltration of activated T cells and also determine T helper polarization in the CNS microenvironment, mRNA expression levels of T cell related cytokines and transcription factors were assayed by real-time quantitative RT-PCR. At day 25 after EAE induction, all treatment groups exhibited decreased expression of the Th17 cytokines and transcription factors (ROR-γt, IL-17 and IL-23) related to control group. Moreover, high and middle dose hesperidin treated groups increased expression of Treg cytokines and transcription factor (Foxp3, IL-10 and TGF-β) when compared to control group (Fig. 7.). As well as these groups showed decreased expression of the inflammatory cytokines including TNF-α and IL-6. These changes in cytokines and transcription factors These consequences confirmed flow cytometry and cytokine ELISA results taken from peripheral lymphoid organs including spleen and lymph nodes.

4. Discussion

EAE, as a MS model, is a $CD4^+$ Th1 cell-mediated inflammatory demyelinating autoimmune disease of the CNS (Gaur and Kumar, 2010; Ciftci et al., 2015). The present study was conducted to evaluate therapeutic potentials of hesperidin on the improvement of MOG-induced EAE in C57BL/6 mice and elucidate the associated possible mechanisms in three different treatment groups as low, middle and high dosage of hesperidin. The results revealed that hesperidin treatment inhibited the development of EAE in a dose-dependent manner as compared with control group. So that, high and middle dose of hesperidin markedly reduced the incidence and severity of clinical disease and more importantly low dose hesperidin profoundly reduced the clinical manifestations of disease. Consistent with our results, a number of recent reports have shown that hesperidin mitigates CNS inflammations (Raza et al., 2011; Ciftci et al., 2015). It has been established that demyelination and CNS inflammation mainly correlated with immune responses which consistent with over expression of pro-inflammatory cytokines such as IFN-γ, TNF-α, IL-6, IL-17 and reduction of anti-inflammatory cytokines such as IL-4, Il-10 and TGF-β (Jiang et al., 2013; Zeng et al., 2007; Ma et al., 2010). The Pro-inflammatory cytokines promote the recruitment of inflammatory cells (Rotshenker, 2011; Nosratabadi et al., 2016) while the anti-inflammatory cytokine down-regulate secretion of pro-inflammatory cytokines (Glocker et al., 2011; Moore et al., 2001) which permits better regeneration of damaged axons that leads to greater myelination, thereby involved in nerve healing process (Sakalidou et al., 2011; Atkins et al., 2007). Our histological studies revealed that hesperidin reduced CNS demyelination during disease development, Moreover Real-time PCR results showed that the expression of Pro-inflammatory related cytokines (TNF-α, IL-6, IL-17 and IL-23) and Th17 cells transcription factor (ROR-γt) were profoundly decreased while Treg related cytokines (IL-10 and TGF-β) and FoxP3 transcription factor were increased in CNS tissues of EAE model. In this context, abnormal amounts of pro-inflammatory and anti-inflammatory cytokines in MOG-induced EAE mice suggesting the improvement effect of hesperidin demyelination and CNS inflammation may be in part, mediated through modulation of pro-inflammatory and anti-inflammatory cascades. These findings are corroborated with previous evidences. Ciftci et al. showed that hesperidin ameliorated EAE through reducing TNF-α in mouse model (Ciftci et al., 2015), whereas Raze et al. reported that hesperidin reduced neuroinflammation through reducing TNF-α in experimental stroke (Raza et al., 2011). Experimental research suggested that migration of T lymphocytes into the CNS is the one of the most important hallmarks in the pathogenesis of MS (Gaur and Kumar, 2010; Jiang et al., 2013). Based on our histological and cytokines assay, hesperidin reduces inflammatory cell infiltration into the CNS through modulating cytokines in CNS of EAE model. It has been established that during neuroinflammation, pro-inflammatory cytokines produced by pathogenic T-cells, macrophages and resident brain microglia mediate upregulation of adhesion molecules in the brain

Fig. 7. mRNA expression of cytokines and transcription factors in CNS. On day 25 post immunization, brains and spinal cords were collected and mRNA levels of cytokines and transcription factors were assessed by real time quantitative PCR as described in Material and methods. Assay was run in triplicate and relative expression of genes was determined compared to the housekeeping gene, β2microglobulin. (A) Th1 related cytokines and transcription factors; TNF-α, IFN-γ and T-bet (B) Th17 related cytokines and transcription factors; IL-6, IL-17, IL-23 and ROR-γt (C) Th2 related cytokines and transcription factors; IL-4 and GATA3 (D) Treg related cytokines and transcription factors; IL-10, TGF-β and Foxp3. Results were expressed as mean ± SEM. $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ compared with control group. Mice were divided into four groups: 1. Control group (CTRL), 2. Low dose hesperidin treatment group (T1), 3. Middle dose hesperidin treatment group (T2) and 4. High dose hesperidin treatment group (T3).

blood barrier that lead to influx of immune cells into the CNS (Holman et al., 2011; Ransohoff and Engelhardt, 2012).

Our other findings in consist with this result verified mechanism of hesperidin improvement effects on MOG-induced EAE model. In the spleen and lymph node cells of high and middle dose hesperidin treated groups, the expression of pro-inflammatory cytokines (IL-6, IL-17 and TNF-α) were decreased but expression of anti-inflammatory cytokines (IL-10 and TGF-β) were increased. These results were verified by flow cytometry analysis of spleen and lymph nodes $CD4^+$ T cell subsets. There were significant decrease in the percentage of Th17 as inflammatory cells, and also significant increase in the percentage of Treg as anti-inflammatory in splenocytes and lymph node cells of high and middle dose hesperidin treated groups. In consist with this result, recently Gamal et al. reported that hesperidin has differential effect on Th1, Th2, Th17, and production of pro-inflammatory cytokines from splenocytes (Allam and Abuelsaad, 2013).

In our study, proliferation of auto-reactive T cells was significantly reduced in peripheral lymph nodes and spleen of hesperidin treated mice that may be related to increase level of anti-inflammatory cytokines. It was established that Anti-inflammatory and pro-inflammatory cytokines control proliferation of $CD4^+$ T cells. Citing to this fact, hesperidin can inhibit proliferation of auto-reactive T cells through modulating cytokines that leads to reduction of inflammation.

It has been proved that there are NO, a pro-inflammatory mediator, and it's various oxidative metabolites such as nitrite and nitrate at higher concentrations in the cerebral spinal fluid (CSF) of MS patients when compared to the CSF of healthy people (Letourneau et al., 2010). In our study, the concentration of NO was evidently reduced in CSF of all hesperidin treated groups. Our result is consistent with other studies that confirm inhibitory effect of hesperidin against NO elevation (Gaur and Kumar, 2010). It was suggested that elevated NO concentration is maybe due to abate in IL-10 production (La Flamme et al., 2001). Mechanistically, hesperidin could attenuate NO production due to up-regulate in IL-10 production.

In conclusion, hesperidin could mitigate EAE in mice through motivating polarization of $CD4^+$ T cells toward regulatory T cells by induction of anti-inflammatory cytokines (IL-10 and TGF-β) and inhibition of pro-inflammatory cytokines (IL-6, IL-17 and TNF-α), thereby suppresses auto-reactive T cells proliferation and its infiltration into CNS.

Conflict of interest

The authors declare no financial or commercial conflict of interest. The authors alone are responsible for the content of this manuscript.

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References

Ahmad, S.T., Arjumand, W., Nafees, S., Seth, A., Ali, N., Rashid, S., Sultana, S., 2012. Hesperidin alleviates acetaminophen induced toxicity in Wistar rats by abrogation of oxidative stress, apoptosis and inflammation. Toxicol. Lett. 208 (2), 149–161.

Allam, G., Abuelsaad, A.S., 2013. Differential effect of hesperidin on Th1, Th2, Th17, and proinflammatory cytokines production from splenocyte of Schistosoma mansoni-infected mice. Cent. Eur. J. Immunol. 38 (1), 29–36.

Atkins, S., Loescher, A.R., Boissonade, F.M., Smith, K.G., Occleston, N., O'Kane, S., Ferguson, M.W., Robinson, P.P., 2007. Interleukin-10 reduces scarring and enhances regeneration at a site of sciatic nerve repair. J. Peripher. Nerv. Syst. 12 (4), 269–276.

Ciftci, O., Ozcan, C., Kamisli, O., Cetin, A., Basak, N., Aytac, B., 2015. Hesperidin, a citrus flavonoid, has the ameliorative effects against experimental autoimmune encephalomyelitis (EAE) in a C57BL/J6 mouse model. Neurochem. Res. 40 (6), 1111–1120.

Gaur, V., Kumar, A., 2010. Hesperidin pre-treatment attenuates NO-mediated cerebral ischemic reperfusion injury and memory dysfunction. Pharmacol. Rep. 62 (4), 635–648.

Ghorbani, A., Nazari, M., Jeddi-Tehrani, M., Zand, H., 2012. The citrus flavonoid hesperidin induces p53 and inhibits NF-kappaB activation in order to trigger apoptosis in NALM-6 cells: involvement of PPARgamma-dependent mechanism. Eur. J. Nutr. 51 (1), 39–46.

Glocker, E.O., Kotlarz, D., Klein, C., Shah, N., Grimbacher, B., 2011. IL-10 and IL-10 receptor defects in humans. Ann. N. Y. Acad. Sci. 1246 (1), 102–107.

Haghmorad, D., Amini, A.A., Mahmoudi, M.B., Rastin, M., Hosseini, M., Mahmoudi, M., 2014a. Pregnancy level of estrogen attenuates experimental autoimmune encephalomyelitis in both ovariectomized and pregnant C57BL/6 mice through expansion of Treg and Th2 cells. J. Neuroimmunol. 277 (1–2), 85–95.

Haghmorad, D., Mahmoudi, M.B., Mahmoudi, M., Rab, S.Z., Rastin, M., Shegarfi, H., Azizi, G., Mirshafiey, A., 2014b. Calcium intervention ameliorates experimental model of multiple sclerosis. Oman Med. J. 29 (3), 185–189.

Haghmorad, D., Salehipour, Z., Nosratabadi, R., Rastin, M., Kokhaei, P., Mahmoudi, M.B., Amini, A.A., Mahmoudi, M., 2016. Medium-dose estrogen ameliorates experimental autoimmune encephalomyelitis in ovariectomized mice. J. Immunotoxicol. 1–12.

Holman, D.W., Klein, R.S., Ransohoff, R.M., 2011. The blood-brain barrier, chemokines and multiple sclerosis. Biochim. Biophys. Acta 1812 (2), 220–230.

Horstmann, L., Schmid, H., Heinen, A.P., Kurschus, F.C., Dick, H.B., Joachim, S.C., 2013. Inflammatory demyelination induces glia alterations and ganglion cell loss in the retina of an experimental autoimmune encephalomyelitis model. J. Neuroinflammation 10, 120.

Huehnchen, P., Prozorovski, T., Klaissle, P., Lesemann, A., Ingwersen, J., Wolf, S.A., Kupsch, A., Aktas, O., Steiner, B., 2011. Modulation of adult hippocampal neurogenesis during myelin-directed autoimmune neuroinflammation. Glia 59 (1), 132–142.

Jiang, Y., Zou, Y., Chen, S., Zhu, C., Wu, A., Liu, Y., Ma, L., Zhu, D., Ma, X., Liu, M., 2013. The anti-inflammatory effect of donepezil on experimental autoimmune encephalomyelitis in C57 BL/6 mice. Neuropharmacology 73, 415–424.

Kim, S.W., Kim, C.E., Kim, M.H., 2011. Flavonoids inhibit high glucose-induced up-regulation of ICAM-1 via the p38 MAPK pathway in human vein endothelial cells. Biochem. Biophys. Res. Commun. 415 (4), 602–607.

La Flamme, A.C., Patton, E.A., Bauman, B., Pearce, E.J., 2001. IL-4 plays a crucial role in regulating oxidative damage in the liver during schistosomiasis. J. Immunol. 166 (3), 1903–1911.

Lees, J.R., Iwakura, Y., Russell, J.H., 2008. Host T cells are the main producers of IL-17 within the central nervous system during initiation of experimental autoimmune encephalomyelitis induced by adoptive transfer of Th1 cell lines. J. Immunol. 180 (12), 8066–8072.

Letourneau, S., Hernandez, L., Faris, A.N., Spence, D.M., 2010. Evaluating the effects of estradiol on endothelial nitric oxide stimulated by erythrocyte-derived ATP using a microfluidic approach. Anal. Bioanal. Chem. 397 (8), 3369–3375.

Ma, X., Jiang, Y., Wu, A., Chen, X., Pi, R., Liu, M., Liu, Y., 2010. Berberine attenuates experimental autoimmune encephalomyelitis in C57 BL/6 mice. PLoS One 5 (10), e13489.

Moore, K.W., de Waal Malefyt, R., Coffman, R.L., O'Garra, A., 2001. Interleukin-10 and the interleukin-10 receptor. Annu. Rev. Immunol. 19 (1), 683–765.

Mosayebi, G., Haghmorad, D., Namaki, S., Ghazavi, A., Ekhtiari, P., Mirshafiey, A., 2010. Therapeutic effect of EDTA in experimental model of multiple sclerosis. Immunopharmacol. Immunotoxicol. 32 (2), 321–326.

Muili, K.A., Gopalakrishnan, S., Meyer, S.L., Eells, J.T., Lyons, J.A., 2012. Amelioration of experimental autoimmune encephalomyelitis in C57BL/6 mice by photobiomodulation induced by 670 nm light. PLoS One 7 (1), e30655.

Muthian, G., Bright, J.J., 2004. Quercetin, a flavonoid phytoestrogen, ameliorates experimental allergic encephalomyelitis by blocking IL-12 signaling through JAK-STAT pathway in T lymphocyte. J. Clin. Immunol. 24 (5), 542–552.

Natarajan, C., Bright, J.J., 2002. Curcumin inhibits experimental allergic encephalomyelitis by blocking IL-12 signaling through Janus kinase-STAT pathway in T lymphocytes. J. Immunol. 168 (12), 6506–6513.

Nones, J., Spohr, T.C., Gomes, F.C., 2012. Effects of the flavonoid hesperidin in cerebral cortical progenitors in vitro: indirect action through astrocytes. Int. J. Dev. Neurosci.

Nosratabadi, R., Rastin, M., Sankian, M., Haghmorad, D., Tabasi, N., Zamani, S., Aghaee, A., Salehipour, Z., Mahmoudi, M., 2015. St. John's wort and its component hyperforin alleviate experimental autoimmune encephalomyelitis through expansion of regulatory T-cells. J. Immunotoxicol. 1-11.

Nosratabadi, R., Rastin, M., Sankian, M., Haghmorad, D., Mahmoudi, M., 2016. Hyperforin-loaded gold nanoparticle alleviates experimental autoimmune encephalomyelitis by suppressing Th1 and Th17 cells and upregulating regulatory T cells. Nanomed. Nanotechnol. Biol. Med. 12 (7), 1961–1971.

Ransohoff, R.M., Engelhardt, B., 2012. The anatomical and cellular basis of immune surveillance in the central nervous system. Nat. Rev. Immunol. 12 (9), 623–635.

Raza, S.S., Khan, M.M., Ahmad, A., Ashafaq, M., Khuwaja, G., Tabassum, R., Javed, H., Siddiqui, M.S., Safhi, M.M., Islam, F., 2011. Hesperidin ameliorates functional and histological outcome and reduces neuroinflammation in experimental stroke. Brain Res. 1420, 93–105.

Rotshenker, S., 2011. Wallerian degeneration: the innate-immune response to traumatic nerve injury. J. Neuroinflammation 8 (1), 109.

Sakalidou, M., Leibig, N., Boyle, V., Koulaxouzidis, G., Penna, V., 2011. Interleukin-10 and regeneration in an end-to-side nerve repair model of the rat. J. Peripher. Nerv. Syst. 16 (4), 334–340.

Shagirtha, K., Pari, L., 2011. Hesperetin, a citrus flavonone, protects potentially cadmium induced oxidative testicular dysfunction in rats. Ecotoxicol. Environ. Saf. 74 (7), 2105–2111.

Sloka, S., Metz, L.M., Hader, W., Starreveld, Y., Yong, V.W., 2013. Reduction of microglial activity in a model of multiple sclerosis by dipyridamole. J. Neuroinflammation 10, 89.

Trivedi, P.P., Kushwaha, S., Tripathi, D.N., Jena, G.B., 2011. Cardioprotective effects of hesperetin against doxorubicin-induced oxidative stress and DNA damage in rat. Cardiovasc. Toxicol. 11 (3), 215–225.
Verbeek, R., van Tol, E.A., van Noort, J.M., 2005. Oral flavonoids delay recovery from experimental autoimmune encephalomyelitis in SJL mice. Biochem. Pharmacol. 70 (2), 220–228.
Zeng, Y., Song, C., Ding, X., Ji, X., Yi, L., Zhu, K., 2007. Baicalin reduces the severity of experimental autoimmune encephalomyelitis. Braz. J. Med. Biol. Res. 40 (7), 1003–1010.