Original Article

Iranian Journal of Immunology

https://iji.sums.ac.ir

The Anti-inflammatory Effect of Ginger Extract on the Animal Model of Multiple Sclerosis

Fatemeh Kamankesh[1], Ali Ganji[1, 2], Ali Ghazavi[1, 3], Ghasem Mosayebi[1, 2*]

[1]Department of Immunology, School of Medicine, Arak University of Medical Sciences, Arak, Iran; [2]Molecular and Medicine Research Center, Arak University of Medical Sciences, Arak, Iran; [3]Traditional and Complementary Medicine Research Center (TCMRC), Arak University of Medical Sciences, Arak, Iran

ABSTRACT

Background: Experimental autoimmune encephalomyelitis (EAE), as an autoimmune disease in the central nervous system (CNS), is an animal model for multiple sclerosis (MS) mediated by T lymphocytes.

Objective: To investigate ginger extract's effect on reducing inflammation and improving the symptoms in the EAE model.

Methods: The EAE was induced by injecting MOG35-55 and pertussis toxin into eight-week-old female C57BL6 mice. The mice were treated with an intraperitoneal injection of 300 mg/kg/day of hydroalcoholic extract of ginger for 21 days. The disease severity and weight changes were measured daily. Then, the mice spleens were removed; the gene expressions of interleukin (IL)-17, transforming growth factor beta (TGF-β), interferon-γ (IFN-γ), and tumor necrosis factor α (TNF-α) were analyzed by Real-time PCR and the percentage of regulatory T lymphocytes (Treg cells) was determined by flow cytometry. Serum nitric oxide and antioxidant capacity were measured, and brain tissue sections were prepared to investigate the leukocyte infiltration and plaque formation.

Results: The severity of symptoms in the intervention group was lower than in the control. The gene expression levels of inflammatory cytokines, including IL-17 (P=0.04) and IFN-γ (P=0.01), were reduced. The Treg cells increased significantly, and the serum nitric oxide level was lower in the ginger-treated group. There was no significant difference in lymphocyte infiltration in the brain between the two groups.

Conclusion: The present study indicated that ginger extract could effectively reduce the inflammatory mediators and modulate immune responses in EAE.

Keywords: Cytokine; Experimental Autoimmune Encephalomyelitis; Ginger; Multiple Sclerosis

*Corresponding author:
Ghasem Mosayebi, Molecular and Medicine Research Center, Arak University of Medical Sciences, Arak, Iran
Email: ghasemmosayebi@arakmu.ac.ir

Cite this article as:
Kamankesh F, Ganji A, Ghazavi A, Mosayebi G. The Anti-inflammatory Effect of Ginger Extract on the Animal Model of Multiple Sclerosis. *Iran J Immunol.* 2023; 20(2):211-218, doi: 10.22034/iji.2023.97156.2482.

Received: 2022-11-02
Revised: 2023-03-14
Accepted: 2023-04-09

INTRODUCTION

Multiple sclerosis (MS) is an autoimmune disease that induces demyelination of neurons and axonal degeneration in the central nervous system (CNS) (1). Pathogenesis of MS indicates that produced inflammatory cytokines in the CNS, including interferon-γ (IFN-γ), interleukin (IL)-6, tumor necrosis factor α (TNF-α), and IL-1β from microglia, macrophages, and astrocytes, can increase the blood-brain barrier (BBB) permeability, the migration of leukocytes to the CNS, and ultimately, exacerbate inflammation (2). The primary leukocytes involved in MS pathogenesis include T $CD4^+$ (T helper cells), especially Th1 (IFN-γ secretion) and Th17 (IL-17 secretion); their cytokines have pivotal roles in chronic inflammation and demyelination (3). However, regulatory T lymphocytes (Treg cells) have protective roles in autoimmune diseases (4).

IFN-γ is one of the essential cytokines in MS demyelinated lesions, indicating its pathogenesis in MS and EAE. IFN-γ is critical in producing nitric oxide (NO), a pathogenesis and exacerbation factor in MS (5). IL-17 induces various inflammatory cytokines production such as IL-6, TNF-α, IL-8, IL-1β, and granulocyte colony-stimulating factor (G-CSF) and causes neutrophil infiltration to the inflammation site (6). Treg cells regulate the immune system that maintains tolerance, and their suppressive effect is mainly due to the production of IL-10 and transforming growth factor-β (TGF-β). Treg cells protect against the EAE progression in the mouse model by modulating Th1 responses and reducing lymphocyte accumulation in CNS (4, 7).

Various treatments are suggested to cure MS; however, each may have its own side effects. Since herbal compounds are effective in recovery from inflammatory diseases and have fewer side effects, such compounds, and their derivatives received attention for this purpose (8, 9). Ginger is a medicinal plant whose therapeutic effects are attributed to compounds such as gingerol, shogaols, zingerone, and paradols (10). Ginger has anti-inflammatory, antioxidant, anti-tumor, antibacterial, and antihypertensive activities (11, 12). Its therapeutic effects on rheumatoid arthritis, type 2 diabetes (13), liver and respiratory diseases are also studied (14). Investigation of the anti-inflammatory effects of ginger shows that it can relieve inflammation by reducing the TNF-α, IFN-γ, suppressing the IL-23 and IL-6 production (15). Hence, the present study aimed to investigate ginger extract's anti-inflammatory and immunomodulatory effects on inflammation relief, and facilitating the healing process in EAE.

MATERIALS AND METHODS

Herbal Extract

Hydroalcoholic extract powder of ginger was purchased from the Barij Essence Company, Kashan, Iran. The extract contained 4.7% gingerol.

Mice

For this purpose, female C57 BL/6 mice (20±2 g) with 6-5 week-old were received from the Pasteur Institute of Iran, Tehran. The mice were kept at 24-25 °C according to the protocol of the Animal Ethics Committee of Arak University of Medical Sciences [IR.ARAKMU.REC.1398.307].

Acute Toxicity Test

Eight mice were used to assess ginger extract's toxicity. First, the mice were weighed and assigned into two groups of the ginger extract receivers and the controls. The ginger extract dose was 300 mg/kg, and PBS and DMSO, with a ratio of 9:1, were used as a solvent. The control group received only the solvent intraperitoneally for 14 days in a 10 mL/kg volume. The mice were monitored daily for 14 days for weight changes, physical properties (skin and fur), mortality, behavioral patterns, respiratory changes, injury, pain, and disease symptoms (i.e., tremors, seizures,

diarrhea, and lethargy) (16).

The EAE Induction

The female C57BL/6 mice were divided into two groups of 6 to induce EAE. To induce the disease, myelin oligodendrocyte glycoprotein (MOG) peptide (200 µg) (MEVGWYRSPFS RVVHLYRNGK; Sigma-Aldrich, USA) and 200 µL complete Freund's adjuvant (Sigma-Aldrich, USA) were injected subcutaneously into their flank. Simultaneously 48 h after the MOG injection, pertussis toxin (250 ng in 400 µL PBS) (Sigma-Aldric, USA) was injected intraperitoneally into each mouse (17, 18).

Mice Treatment

The mice were assigned to two groups of six as the control and the ginger extract receivers. The control group received PBS, and the intervention group was injected 300 mg/kg of ginger extract daily intraperitoneally (19). The intervention period started with the appearance of the first paralysis symptom in the tail and lasted 21 days. Then all the mice were anesthetized using ketamine and xylazine, and a cardiac puncture was performed for blood collection. The removed spleens were disrupted and pipetted for splenocyte preparation. After washing the cell suspensions with RPMI, their cells were sorted to count Treg cells and measure the expression level of the desired genes. The brain tissue was removed for histopathological examination.

Clinical Evaluation

The mice were weighed every 2-3 days. The disease severity is graded as 0: no disease, 1:impaired tail movement, 2: paralysis of the tail, 3: mild gait disturbance, 4: severe gait disorder, 5: paralysis of one leg, 6: complete paralysis of both legs, 7: complete paralysis of arms and legs, and 8: death (20). The mice were evaluated for physical and clinical symptoms; daily disease severity changes were recorded (21).

Gene Expression Analysis of TNF-α, IFN-α, IL-17, TGF-β

The manufacturer's protocol was used for RNA extraction from splenocytes and cDNA synthesis (Yekta Tajhiz Azma, Iran). AlleleID 6.0 (Premier Biosoft., USA) was used for primers designing for IFN-γ, TNF-α, IL-17, TGF-β, and GAPDH (Table 1), and Real-time PCR was completed in triplicate using SYBR Green assay method (Yekta Tajhiz Azma, Iran) by the LightCycler system (Roche, Germany). Melting curves were drawn to remove the redundant fragments. The relative gene expression was measured using the Paffle method and reported as a ratio (22).

Treg Cells Counting by Flow Cytometry

For this purpose, 50 µl of the collected splenocytes from the mice were transferred into tubes for staining using surface antibodies, including CD4 (PE) and CD25 (PerCP). Following 40 min incubation, Lysing Solution

Table 1. The sequence of primers used for each gene.

Name	Sequence 5'-3'
GAPDH (224bp)	F: CGGTGTGAACGGATTTGG R: CTCGCTCCTGGAAGATGG
TGF-β (193bp)	F: AATTCCTGGCGTTACCTTGG R: GGCTGATCCCGTTGATTTCC
IFN-ɣ (259 bp)	F: GAGTGTGGAGACCATCAAGGAAG R: TGCTTTGCGTTGGACATTCAAGTC
TNF-α (201bp)	F: CCTCTTCTCATTCCTGCTTGTG R: ACTTGGTGGTTTGCTACGAC
IL-17 (174 bp)	F: GACTACCTCAACCGTTCCAC R: CCTCCGCATTGACACAGC

F: Forward; R:Reverse; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; TGF-β: transforming growth factor beta; IFN-ɣ: interferon-γ; TNF-α: tumor necrosis factor α; IL-17: interleukin-17

(1 ml) (BD Biosciences, Bedford, MA) was added for erythrocytes lysis. Then, the washed cells were fixed using a fixative solution (BD Biosciences, Bedford, MA), permeabilized by saponin (0.5%), and treated with Anti-FOXP3 (FITC). Isotype controls were included in flow cytometry analysis (FACSCalibur BD™, USA). All antibodies were purchased from BD Biosciences (Bedford, MA). The data were analyzed by counting 20,000 cells using FlowJo software. Lymphocytes were gated based on the side, and forward scatters and Treg cells were estimated based on CD4, CD25, and FoxP3 percentages.

Nitric Oxide Measurement

For this purpose, the Griess protocol was employed. The chemicals used in the experiment were purchased from Merck Company (Germany). In summary, 100 µL of serum samples with sulfanilamide (100 µL of 1% solution) were added to 96-well microplates. After 10 min of incubation, NED (100 µL of 0.1% solution) was added. After 10 min of incubation, the absorption was read at 540 nm with the ELISA Reader, and a standard curve was used for the concentration calculation of the samples (23).

Assessment of Serum Antioxidant Capacity by FRAP Method

The materials used in the study were purchased from Merck Company (Germany). In summary, the FRAP reagent was prepared by mixing acetate buffer (10 µL of 300 µM), TPTZ (1 µL of 10 µM), and ferric chloride (1 µL of 20 µM). 10 µL of the serum sample and then FRAP reagent (200 µL) were added to each well; after incubation (10 min at 37°C), the light absorption was read at 593 nm wavelength with an ELISA Reader, and a standard curve was used for the concentration calculation of the samples (24).

Histopathological Examination

The tissue of the brain's left hemisphere was removed and kept in 10% formalin. Then 5-8-µm thick tissue sections were prepared for histopathological examinations. For this purpose, the hematoxylin and eosin (H&E) dying technique was utilized, and the inflammatory areas were identified by mononuclear cells with dark nuclei and counted under a light microscope (25).

Data Analysis Method

The data were statistically analyzed in SPSS. The Kolmogorov-Smirnov normality test assessed the normal distribution of the data. The independent samples T-test was utilized to compare the mean. A P value of <0.05 was considered significant.

RESULTS

Monitoring Weight Changes and Disease Severity

No significant weight changes were observed in the intervention and the EAE-induced control groups (Figure 1 A). Although the severity of the symptoms in the treated

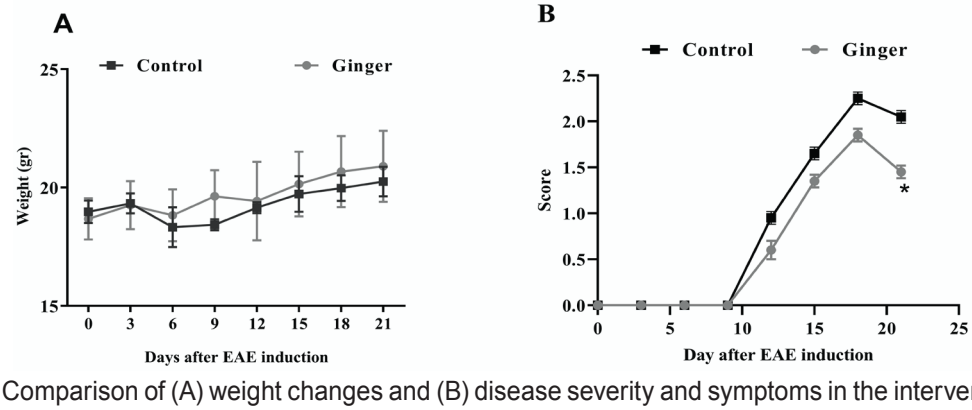

Figure 1. Comparison of (A) weight changes and (B) disease severity and symptoms in the intervention group and the EAE-induced control group in 21 days (P<0.05*). EAE: Experimental autoimmune encephalomyelitis

group was not significantly different on the days 9, 12, 15, and 18 of the treatment compared with that in the control one, it was reduced significantly on the day 21 of the treatment (P=0.034, Figure 1 B).

Gene Expression Evaluation

The comparison of the expression level of IFN-γ, TNF-α, IL-17, and TGF-β genes showed that the expression level of inflammatory cytokine genes, IFN-γ (P=0.01) and IL-17 (P=0.04), was significantly lower in the intervention group than in the EAE-induced control. In addition, the expression level of the TNF-α gene decreased in the ginger-treated EAE than in the control, although the difference was statistically insignificant (P=0.09). The gene expression of TGF-β between the studied groups also showed an insignificant difference (P=0.95, Figure 2).

Treg Cells Percentage

The percentage of Treg cells in the intervention group was significantly higher than in the control (P=0.01, Figure 3A-D).

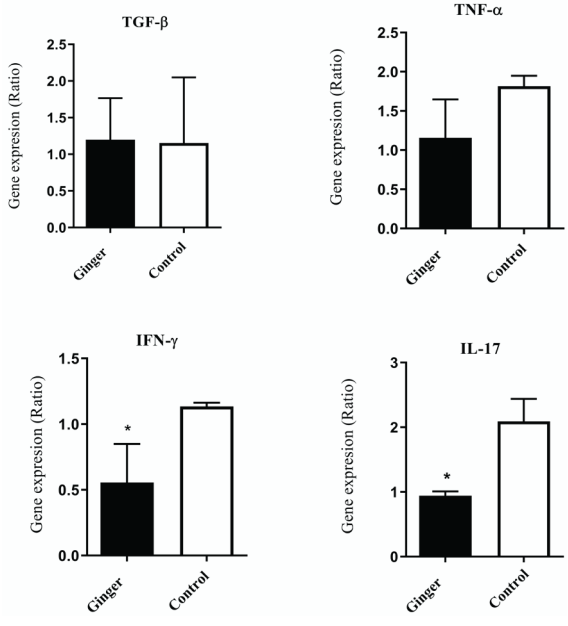

Figure 2. Comparison of TNF-α, IFN-γ, IL-17, and TGF-β expression in splenocyte supernatants between the intervention and the EAE-induced control group (P<0.05*). TNF-α: Tumor necrosis factor α; IFN-γ: Interferon-γ; IL-17: Interleukin-17; TGF-β: Transforming growth factor beta

Nitric Oxide Concentration and Antioxidant Capacity of Serum

A comparison of the serum NO levels between the groups showed that it was lower in the intervention group (P=0.04, Figure 4A). FRAP test comparison of the serum antioxidant capacity between the study groups was not statistically significant (P=0.3, Figure 4B).

Histological Examination of Brain Tissue

Microscopic examination of brain tissue sections showed no leukocyte infiltration and plaque formation in both groups, which might be attributed to the short intervention period (Figure 5).

DISCUSSION

Multiple sclerosis is an autoimmune neurological disorder. In MS, chronic inflammatory conditions cause neurons demyelination and nerve conduction disruption. TNF-α, IFN-γ, and IL-17 play a role in this inflammation. Th1 and Th17 cells produce these cytokines. However, Treg cells, by secreting TGF-β, can control autoimmune diseases such as MS by suppressing immune responses. The

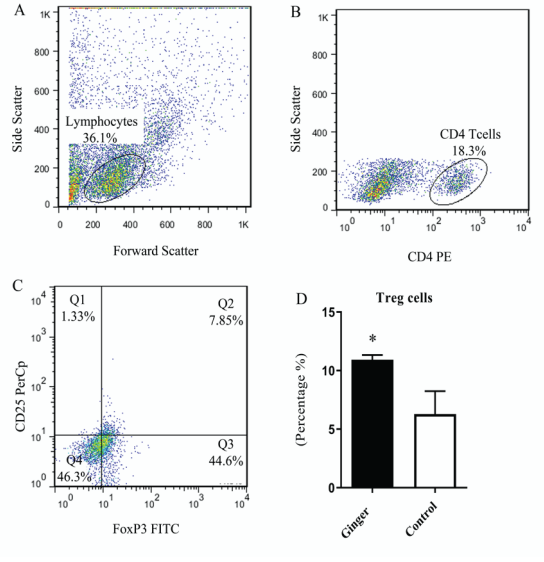

Figure 3. Flow cytometry analysis of Treg cells. (A) Gated lymphocytes. (B) Gated CD4 T cells. (C) Percentage of Treg cells. (D) Treg cells counting in prepared splenocytes between the intervention and the EAE-induced control groups (P<0.05*). Treg cells: Regulatory T lymphocytes

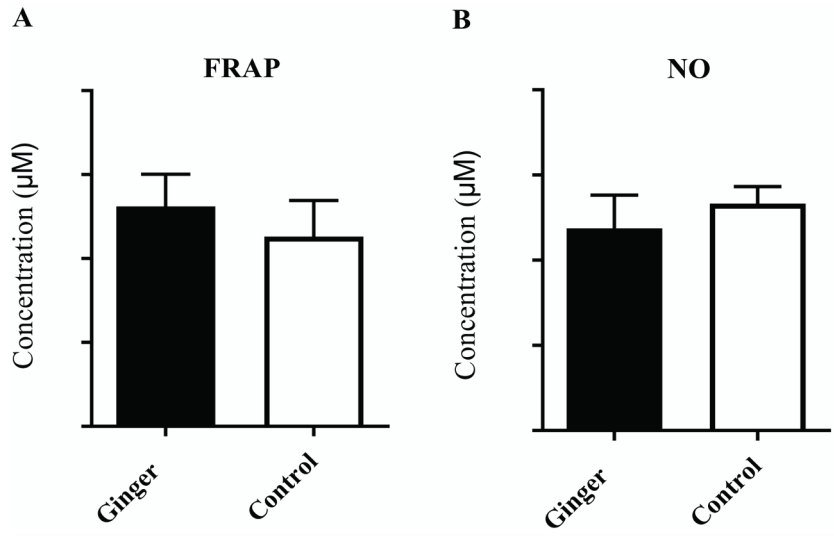

Figure 4. (A) The Griess method measured nitric oxide concentration between the intervention and the EAE-induced control groups. (B) Antioxidant capacity was measured by the FRAP reaction between the intervention and the EAE-induced control groups (P<0.05*); FRAP: Ferric reducing ability of plasma; NO: Nitric Oxide

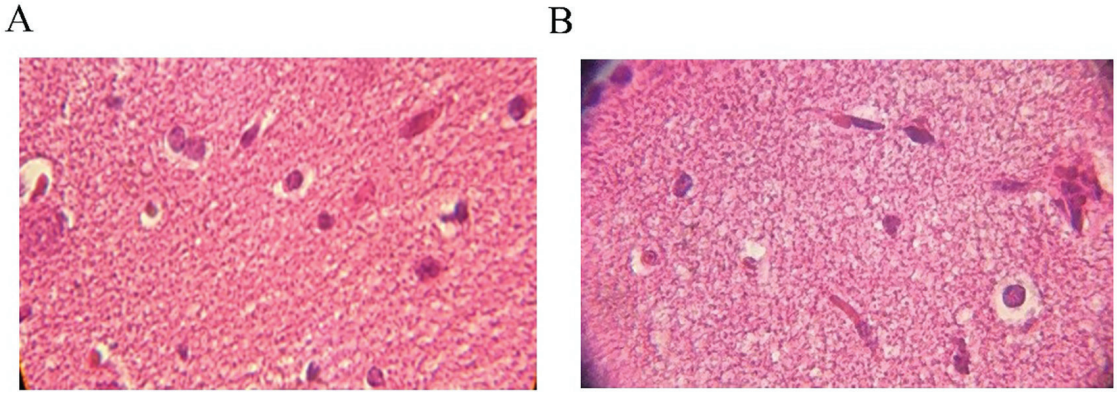

Figure 5. Microscopic examination of brain tissue for leukocyte infiltration and plaque formation in the (A) intervention and (B) the EAE-induced control groups.

present study evaluated ginger extract's anti-inflammatory and immunomodulatory effects on an animal model of MS. The present study showed that administering ginger extract to the EAE-induced mice was associated with improved clinical symptoms and a delay in the onset of disease attacks compared with the control group.

Studies show that ginger extract plays a role in modulating immune responses so that it can suppress IL-23 and IL-6 gene expression, as mentioned in similar studies. Since these cytokines are essential for differentiating Th17 cells, ginger extract can reduce Th17 cell count by suppressing their expression and consequently decreasing the production of IL-17 (19). Previous studies also showed ginger extract could suppress IL-6 and increase TGF-β production by differentiating Treg cells, balancing the Th17 / Treg cells ratio, and modulating immune responses (15).

Given that cellular immunity, especially Th1 cell responses, have pivotal roles in the pathogenesis of MS by inducing the secretion of IFN-γ and TNF-α, the administration of ginger extract for the treatment of MS drew the attention of researchers. As reported, treating EAE with ginger extract reduced IFN-γ and TNF-α production and decreased inflammation (26). According to the present study's findings, since IFN-γ is an inducing agent of inducible nitric oxide synthase (iNOS) in macrophages

and dendritic cells, the decrease in IFN-γ expression probably leads to a reduction in the NO production in the intervention group compared with the control one. As mentioned in other studies, ginger extract could suppress the expression of enzymes such as iNOS in autoimmune diseases such as rheumatoid arthritis (27). Previous studies also reported the antioxidant activity of ginger extract (28). Their results indicated an increase in the serum antioxidant capacity of mice receiving ginger extract compared with the controls; however, the effect mechanism is still unclear and needs further examination.

CONCLUSION

According to the present study, ginger extract reduces inflammation and modulates immune responses effectively in the EAE model by decreasing inflammatory cytokines TNF-α, IFN-ɣ, and IL-17 and increasing the percentage of Treg cells and TGF-β. Therefore, along with other classic and modern therapeutic effects, ginger extract effectively improves MS symptoms and reduces neurological complications.

FUNDING

The authors would like to thank the Research Council of the Arak University of Medical Sciences for the funding.

ACKNOWLEDGMENTS

This article is from the master's thesis in immunology at Arak University of Medical Sciences. The authors are grateful to the honorable professors at the Department of Immunology, Arak University of Medical Sciences, for cooperating with the study.

Conflict of Interest: None declared.

REFERENCES

1. Ömerhoca S, Akkaş SY, İçen NK. Multiple sclerosis: diagnosis and differential diagnosis. Archives of Neuropsychiatry. 2018;55:S1.
2. Ortiz GG, Pacheco-Moisés FP, Macías-Islas MÁ, Flores-Alvarado LJ, Mireles-Ramírez MA, González-Renovato ED, et al. Role of the blood–brain barrier in multiple sclerosis. Arch Med Res. 2014;45:687-697.
3. Dargahi N, Katsara M, Tselios T, Androutsou M-E, De Courten M, Matsoukas J, et al. Multiple sclerosis: immunopathology and treatment update. Brain Sci. 2017;7:78.
4. Danikowski K, Jayaraman S, Prabhakar B. Regulatory T cells in multiple sclerosis and myasthenia gravis. J Neuroinflammation. 2017;14:1-16.
5. Dungan LS, McGuinness NC, Boon L, Lynch MA, Mills KH. Innate IFN□γ promotes development of experimental autoimmune encephalomyelitis: a role for NK cells and M1 macrophages. Eur J Immunol. 2014;44:2903-2917.
6. Jadidi□Niaragh F, Mirshafiey A. Th17 cell, the new player of neuroinflammatory process in multiple sclerosis. Scand J Immunol. 2011;74:1-13.
7. Ebrahimimonfared M, Ganji A, Zahedi S, Nourbakhsh P, Ghasami K, Mosayebi G. Characterization of regulatory t-cells in multiple sclerosis patients treated with interferon beta-1a. CNS Neurol Disord Drug Targets. 2018;17:113-118.
8. Bahrami M, Mosayebi G, Ghazavi A, Ganji A. Immunomodulation in Multiple Sclerosis by Phytotherapy. Curr Immunol Rev. 2020;16:28-36.
9. Mirshafiey A, Aghily B, Namaki S, Razavi A, Ghazavi A, Ekhtiari P, et al. Therapeutic approach by Aloe vera in experimental model of multiple sclerosis. Immunopharmacol Immunotoxicol. 2010;32:410-415.
10. Zhang F, Ma N, Gao YF, Sun LL, Zhang JG. Therapeutic effects of 6□gingerol, 8□gingerol, and 10□gingerol on dextran sulfate sodium□induced acute ulcerative colitis in rats. Phytother Res. 2017;31:1427-1432.
11. Rahnama P, Montazeri A, Huseini HF, Kianbakht S, Naseri M. Effect of Zingiber officinale R. rhizomes (ginger) on pain relief in primary dysmenorrhea: a placebo randomized trial. BMC Complement Altern Med. 2012;12:1-7.
12. Saeedifar AM, Mosayebi G, Ghazavi A, Ganji A. Synergistic Evaluation of Ginger and Licorice Extracts in a Mouse Model of Colorectal Cancer. Nutrition and Cancer. 2021 2021/07/03;73:1068-1078.
13. Zhu J, Chen H, Song Z, Wang X, Sun Z. Effects of ginger (Zingiber officinale Roscoe) on type 2 diabetes mellitus and components of the metabolic

syndrome: A systematic review and meta-analysis of randomized controlled trials. Evid Based Complement Alternat Med. 2018;2018.
14. Kuo P-L, Hsu Y-L, Huang M-S, Tsai M-J, Ko Y-C. Ginger suppresses phthalate ester-induced airway remodeling. J Agric Food Chem. 2011;59:3429-3438.
15. Jafarzadeh A, Nemati M. Therapeutic potentials of ginger for treatment of Multiple sclerosis: A review with emphasis on its immunomodulatory, anti-inflammatory and anti-oxidative properties. J Neuroimmunol. 2018;324:54-75.
16. Saleem U, Amin S, Ahmad B, Azeem H, Anwar F, Mary S. Acute oral toxicity evaluation of aqueous ethanolic extract of Saccharum munja Roxb. roots in albino mice as per OECD 425 TG. Toxicol Rep. 2017;4:580-585.
17. Mosayebi G, Soleyman MR. Changes in synaptic transmission and long-term potentiation induction as a possible mechanism for learning disability in an animal model of multiple sclerosis. Int Neurourol J. 2016;20:26.
18. Ghazavi A, Mosayebi G, Salehi H, Abtahi H. Effect of ethanol extract of saffron (Crocus sativus L.) on the inhibition of experimental autoimmune encephalomyelitis in C57bl/6 mice. Pak J Biol Sci. 2009;12:690-695.
19. Jafarzadeh A, Azizi S-V, Nemati M, Khoramdel-Azad H, Shamsizadeh A, Ayoobi F, et al. Ginger Extract Reduces the Expression of IL-17 and IL-23 in the Seraand Central Nervous System of EAEMice. Iran J Immunol. 2015;12:288-301.
20. Ghazavi A, Mosayebi G. The mechanism of sesame oil in ameliorating experimental autoimmune encephalomyelitis in C57BL/6 mice. Phytother Res. 2012;26:34-38.
21. Mosayebi G, Ghazavi A, Aghili B, Mirshafiei A. Immunomodulating activity of Aloe Vera in animal model of multiple sclerosis. Journal of Arak University of Medical Sciences. 2009;12:109-115.
22. Yang J, Xu L. Elevated IL-23R Expression and Foxp3+ Rorγt+ Cells in Intestinal Mucosa During Acute and Chronic Colitis. Med Sci Monit. 2016;22:2785.
23. Mosayebi G, Ghazavi A. Effect of vitamin E on the inhibition of experimental autoimmune encephalomyelitis in C57BL/6 mouse. Journal of Arak University of Medical Sciences. 2006;9:68-75.
24. Palizvzn M, Khademi S, Ghazavi A, Mosayebi G. Correlation of two way active avoidance learning with Nitric Oxide and Ferric reduction/antioxidant power in rats. Journal of Arak University of Medical Sciences. 2006;9:1-8.
25. Peiris M, Monteith GR, Roberts-Thomson SJ, Cabot PJ. A model of experimental autoimmune encephalomyelitis (EAE) in C57BL/6 mice for the characterisation of intervention therapies. J Neurosci Methods. 2007;163:245-254.
26. Ha SK, Moon E, Ju MS, Kim DH, Ryu JH, Oh MS, et al. 6-Shogaol, a ginger product, modulates neuroinflammation: a new approach to neuroprotection. Neuropharmacology. 2012;63:211-223.
27. Al-Nahain A, Jahan R, Rahmatullah M. Zingiber officinale: A potential plant against rheumatoid arthritis. Arthritis. 2014;2014.
28. Stoilova I, Krastanov A, Stoyanova A, Denev P, Gargova S. Antioxidant activity of a ginger extract (Zingiber officinale). Food Chem. 2007;102:764-770.