

ORIGINAL ARTICLE

Iran J Allergy Asthma Immunol

December 2023; 22(6):575-587.

DOI: 10.18502/ijaa.v22i6.14646

Curcumin and Its Semisynthetic Derivative F-Curcumin Ameliorate the Expression of Cytokines in Autoimmune Encephalomyelitis Mouse Models of Multiple Sclerosis

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Received: 5 March 2023; Received in revised form: 22 August 2023; Accepted: 22 August 2023

ABSTRACT

Multiple sclerosis (MS) is an inflammatory disorder impacting the central nervous system, with cytokines significantly influencing its pathogenesis. This study investigates the effect of curcumin and its semisynthetic derivative F-curcumin on cytokine gene expression in autoimmune encephalomyelitis (EAE) mouse models of MS.

We assessed the expression levels of specific cytokines including interleukin (IL)-1 β , IL-4, IL-10, IL-17, interferon- γ (IFN- γ), and transforming growth factor- β (TGF- β), alongside key transcription factors for helper T cells (T-bet, GATA-3, ROR γ t, and FoxP3) in both the spinal cord and spleen.

Treatment with curcumin and F-curcumin significantly ameliorated the severity and onset of EAE. Notably, mice administered with either compound showed a substantial decrease in the expression of genes encoding IL-1 (2 folds), IFN- γ (2 and 4 folds), and IL-17 (2.5 and 3.5 folds), alongside a marked increase in TGF- β (7 folds) and IL-10 (4 and 6 folds) levels. Additionally, the gene expression of T cell-derived transcription factors nearly mirrored the changes observed in pro-inflammatory and anti-inflammatory cytokines across the groups. The F-curcumin-treated group exhibited more pronounced results.

In conclusion, curcumin and F-curcumin significantly modulate cytokine gene expression during EAE induction, potentially alleviating inflammation in MS, with F-curcumin showing a more substantial effect.

Keywords: Autoimmune encephalomyelitis; Curcumin; Inflammation; Multiple sclerosis

INTRODUCTION

Multiple sclerosis (MS) is a degenerative disorder

of the central nervous system (CNS) characterized by a heterogeneous chronic immune reaction. The disease is marked by the infiltration of lymphocytes and macrophages, axonal injury, and attenuated nerve function. MS shares features common to other neurodegenerative diseases such as Parkinson's disease, amyotrophic lateral sclerosis, and Alzheimer's disease. Experimental autoimmune encephalomyelitis (EAE) is

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the animal model most frequently used in MS studies.¹

Patients with MS and EAE mouse models exhibit varying degrees of axonal myelin degeneration due to immune reactions.² The etiology of demyelination in MS is multifaceted and entails the activation of macrophages and microglial cells as well as the release of cytokines that serve as key triggers of autoimmune attacks against myelin in the CNS.³⁻⁵ In MS, a multitude of pro-inflammatory cytokines (helper T [Th]1 type) such as interferon (IFN)- γ , and interleukin (IL)-2 are involved in the initiation and progression of the disease, while anti-inflammatory cytokines (Th2 type) such as IL-4 and transforming growth factor (TGF)- β demonstrate a reduction in expression during the active phase of the disease. Th2 cells are GATA3 positive.⁶⁻⁸

Another phenotype of Th cells is the Th17 cells involved in the production of the pro-inflammatory cytokine IL-17 which plays a more vital role in earlier stages of MS. On the other hand, Th1 cells play a more important role in the later stages of the disease.^{9,10} In addition, the exacerbation of EAE is associated with the suppression of regulatory T (Treg) cells. The activation of Treg cells has been found to prevent the development of EAE. Therefore, Th1, Th17, and Treg cells are considered significant contributors to the pathogenesis of MS. These cells are positive for Tbet, ROR γ t, and FoxP3, respectively.^{11,12}

Pro-inflammatory cytokines are found in a higher concentration in MS lesions, contributing to its pathogenesis through the accumulation of nitrogen and oxygen free radicals,¹³ ultimately leading to myelin destruction in the CNS.^{13,14}

Curcumin is the most active component of turmeric, whose anti-inflammatory and antioxidant properties can help alleviate inflammation, pain, and degenerative conditions.¹⁵⁻¹⁸ Nonetheless, curcumin's clinical efficacy is hindered by its poor bioavailability. Thus, efforts have been made to develop curcumin formulations with improved bioavailability and systemic tissue distribution.^{19,20}

This study aimed to assess the therapeutic potential of curcumin and a semisynthetic curcumin derivative, F curcumin, on phenotypic features of EAE mice. The evaluation encompassed weight, clinical score, enhanced forelimb strength, and the expression of genes coding for pro- and anti-inflammatory cytokines IL-1 β , IL-4, IL-10, IL-17, IFN- γ , and TGF- β , along with related transcription factors (Tbet, GATA-3, ROR γ t, and

FoxP3) in the spinal cord and spleen of C57BL/6 mice during EAE induction.

MATERIALS AND METHODS

Biological Models

Adult female C57BL/6 mice (10–12 weeks old, 18–20 g) were purchased from the Salari Institute of Cognitive and Behavioral Disorders (SICBD), Tehran, Iran. To ensure adequate acclimatization, all mice were domiciled in cages within the animal house for a period of 10 days under a 12-hour light-dark cycle.

Experimental Autoimmune Encephalomyelitis Induction

Mice were randomly assigned to 4 groups of EAE-induced mice (10 in each group) or a control group (n=10). The EAE models were produced at SICBD.²¹ In brief, female C57BL6 mice (aged 9–10 weeks) received an emulsion of myelin oligodendrocyte glycoprotein peptide (MOG35–55, SICBD; 150 μ g of the peptide was dissolved in phosphate-buffered saline [PBS]) and Freund's Complete Adjuvant (Sigma Chemical Co., St Louis, Mo., USA) containing 400 μ g of *Mycobacterium tuberculosis*. The emulsion was injected subcutaneously into both hind limbs of each animal. The mice were treated intraperitoneally with 200 ng of pertussis toxin from *Bordetella pertussis* (dissolved in PBS; List Biological Lab, USA) at the time of immunization and 48 hours after.

Treatment and Clinical Assessment

The mice received daily 20 mg/Kg intraperitoneal injections of either curcumin (Sigma-Aldrich, St. Louis, MO, USA) dissolved in ethanol or F curcumin dissolved in dimethyl sulfoxide (DMSO) for 21 days after immunization. The control groups received only ethanol or DMSO, as drug vehicles. The mice were subjected to daily weighing and clinical observation for symptoms of EAE. Each mouse was assigned a numerical score from 0 to 5 based on the degree of impairment, with score 0 indicating no symptoms, score 1 indicating complete paralysis of the tail, score 2 indicating weakness of hind legs and a limp tail, score 3 indicating complete paralysis of hind legs and limp tail, score 4 indicating partial paralysis of the forelimb along with complete paralysis of both hind legs and a limp tail, and score 5 indicating death. Mice exhibiting symptoms at the

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borderline of two scores were assigned an average score.²²

Grip Strength Test

The strength of the front legs of mice was tested using a grip dynamometer (Columbus Instruments, USA). Mice grabbed the triangular bar with their front paws and subsequently experienced a horizontal displacement. The grip strength was recorded based on the highest score obtained for each mouse. The experiment was repeated 5 times for each subject.

Semisynthetic Curcumin Derivative (F Curcumin)

Briefly, as described by Esmaeizadeh et al,²³ due to the diverse biochemical features of curcumin and its Knoevenagel derivatives, as well as the valuable

features of the 1,2,3-triazole ring system, a triazole tethered curcumin derivative has been successfully synthesized. This was achieved through the utilization of meta-propargyl ether of benzaldehyde in the Knoevenagel reaction of curcumin's middle carbon, followed by the Huisgen 1,3-dipolar cycloaddition. The resulting product, known as compound 1 with the International Union of Pure and Applied Chemistry (IUPAC) name (1E,6E)-4-(3-((1-(4-fluorobenzyl)-1H-1,2,3-triazol-yl) methoxy) benzylidene)-1, 7-bis (4-hydroxy-3-methoxyphenyl)hepta-1,6-diene-3,5-dione, was thoroughly characterized through the application of carbon-13 nuclear magnetic resonance (¹³C NMR), proton nuclear magnetic resonance (¹H NMR), and high-resolution electrospray mass spectrometry (HRESI-MS) (Figure 1).

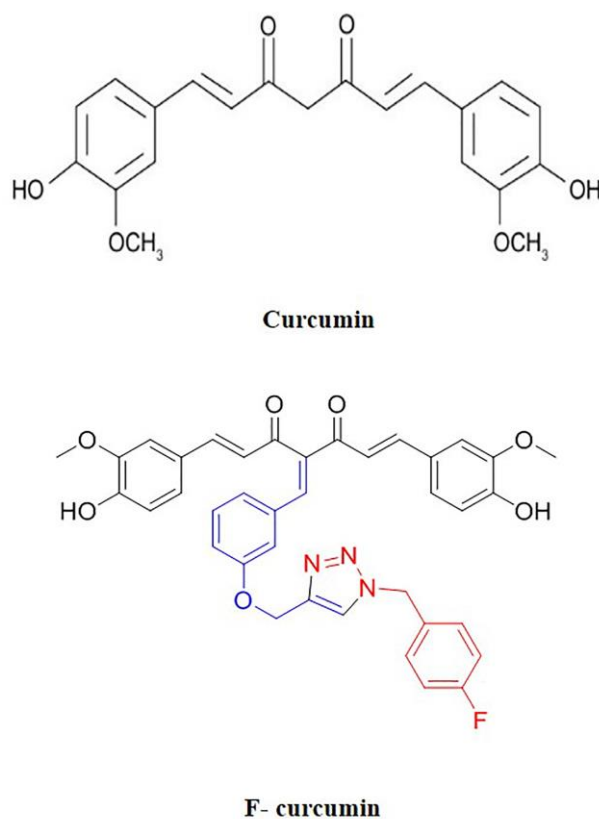


Figure 1. Chemical structures of curcumin and F Curcumin

Spinal Cord Tissue Preparation and Staining

The current procedure was performed based on two previous reports.²⁵⁻²⁶ Briefly, animals were anesthetized on day 15, which is considered the peak of the disease. Subsequently, they were perfused intracardially with 0.1 M PBS, followed by 4% paraformaldehyde in 0.1 M

PBS (pH 7.4). The lumbar spinal cord was immediately extracted and post-fixed overnight at 4°C. For hematoxylin and eosin staining, the tissues were dehydrated through a graded series of alcohol, cleared with xylene incubation, and then embedded in paraffin. A series of coronal sections with a diameter of 5 mm

were prepared using a rotary microtome (Leica Microsystems, UK). Sections were rehydrated and stained with hematoxylin for 4 minutes, followed by clearance with xylene. Then, the sections were washed and counterstained with eosin for 90 seconds, after which they were coverslipped and photographed. These procedures were conducted to facilitate subsequent analysis of inflammation severity by a pathologist, who was blinded to the experimental groups. For each animal's lesion area, 21 sections were evaluated for vacuolation and inflammatory cell count by the pathologist.

RNA Extraction, cDNA Synthesis, and Real-time Quantitative Polymerase Chain Reaction

RNA samples were isolated from the spinal cord and spleen tissues of all groups using the Hybrid-R RNA Isolation Kit (GeneAll, Korea). The purity and integrity of the RNA were assessed using a NanoDrop ND-2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, USA) and gel electrophoresis. Subsequently, cDNA was synthesized using the PrimeScript RT Reagent Kit (Takara, Japan). The expression of IFN- γ , IL-1, IL-17, TGF- β , IL-4, T-bet, GATA-3, ROR γ t, and FoxP3 were evaluated in each group employing SYBR Green-based real-time PCR. Gene expression levels from distinct samples were computed by normalizing against glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Following this, relative quantification levels were plotted. The PCR primer sequences are provided in the Supplementary Table. Real-time qPCR was conducted on an ABI 7500 real-time PCR system (Applied Biosystems, Foster City, CA) following Takara SYBR master mix instructions (Shiga, Japan). The PCR reaction was executed under the following thermal conditions: preheat, at 95°C for 5 minutes, followed by 40 cycles of denaturation at 95°C for 15 seconds, annealing for 20 sec, and elongation at 72°C for 20 seconds. Fold changes in gene expression were determined using the $2^{-\Delta\Delta C_t}$ method, with cycle threshold (Ct) values employed.

Statistical Analysis

The values are expressed as mean \pm SD or mean \pm SEM. The data were analyzed using one-way analysis of variance (ANOVA) followed by a post hoc Tukey test conducted to assess differences among groups using the GraphPad Prism 8.0 software (GraphPad, San Diego, CA). A p value <0.05 was considered significant.

RESULTS

The Effects of the Curcumin and F-curcumin on the Progression of EAE

Daily injections of both curcumin and F curcumin significantly reduced the severity of EAE and delayed the onset of clinical signs compared to untreated animals. The control group received daily injections of a vehicle (either ethanol or DMSO) used to dissolve curcumin or F curcumin over the same period, which did not reduce EAE scores or incidence significantly. Upon comparison, we observed that animals treated with curcumin and F curcumin experienced a significant reduction in EAE severity and delayed onset of scores ($p<0.01$ or $p<0.001$) in contrast to the vehicle groups (Figure 2). The utilization of DMSO in the study yielded similar results to alcohol; hence, in all figures in this article, a control group is presented under the name of the EAE-alcohol group.

Influence of F-curcumin and Curcumin Treatment on Spinal Cord Inflammation in EAE Mouse Models

To assess inflammatory parameters, spinal cord sections were stained with hematoxylin and eosin (Figure 3A). Treatment with F-curcumin or curcumin decreased immune cell infiltration ($p<0.01$; Figure 3B) and vacuolization ($p<0.005$; Figure 3C) in the CNS of the mice compared to EAE group animals

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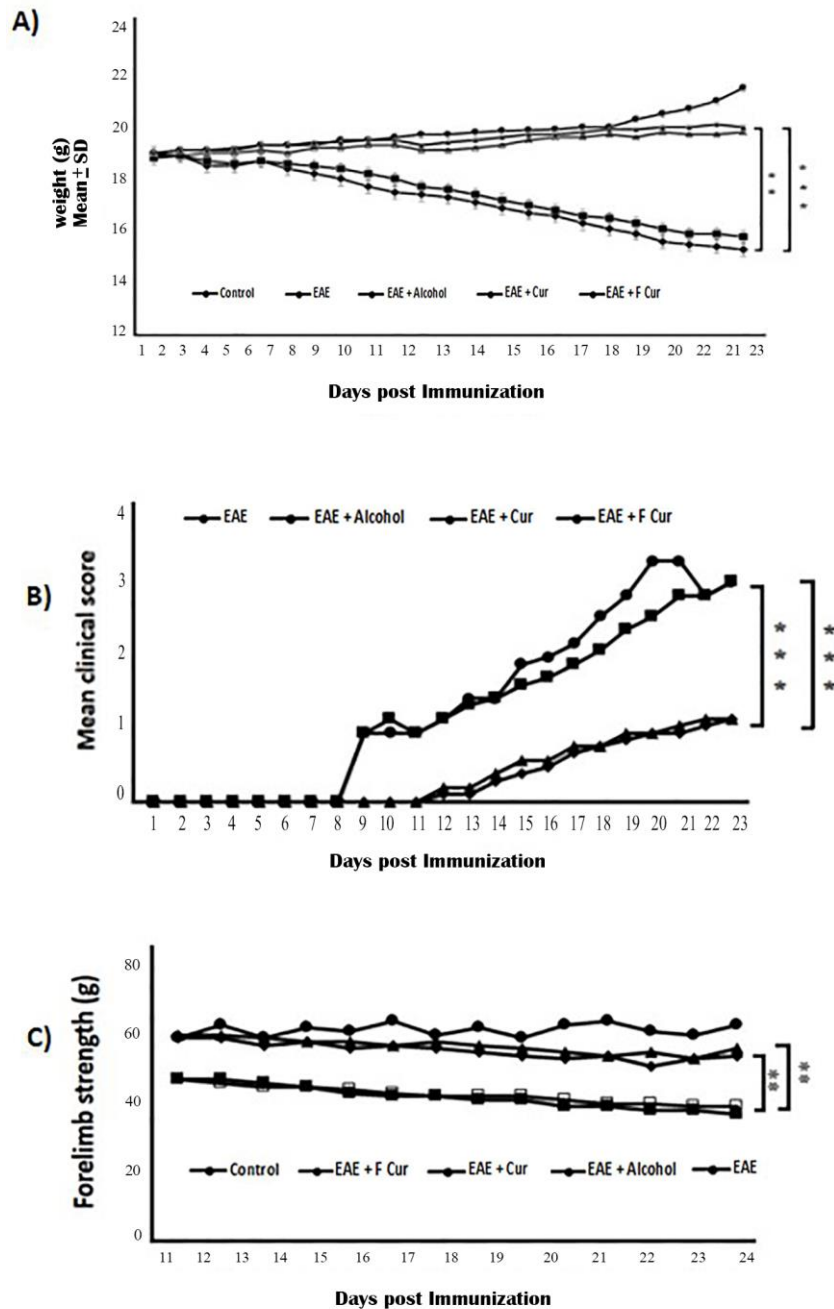


Figure 2. Impact of curcumin and F-curcumin on experimental autoimmune encephalomyelitis (EAE) progression and severity. (A) Mice's average body weight monitored during the experiment. Administration of either curcumin or F-curcumin markedly prevented weight reduction in the mice. (B) Average clinical score for EAE in mice. Intraperitoneal administration of curcumin or F-curcumin at a dose of 20 mg/kg daily for a duration of 21 days following immunization notably lowered the average clinical score in EAE mice. (C) Forelimb grip strength measurements starting from day 11. The use of curcumin and F-curcumin significantly improved forelimb grip strength in mice. Data presented as mean \pm SEM, with each group consisting of 10 mice (** $p < 0.01$, *** $p < 0.001$).

Effects of the F-curcumin and Curcumin on the Expression of Cytokine Genes and Helper T Cell Transcription Factors

The expression levels of 6 genes coding the cytokines TGF- β , IFN- γ , IL-1 β , IL-4, IL-10, and IL-17,

along with 4 genes coding the transcription factors T-bet, ROR γ t, GATA-3, and FoxP3 were determined on day 21 post-immunization in the spinal cord and spleen of the study groups (Figures 4, 5, 6, and 7).

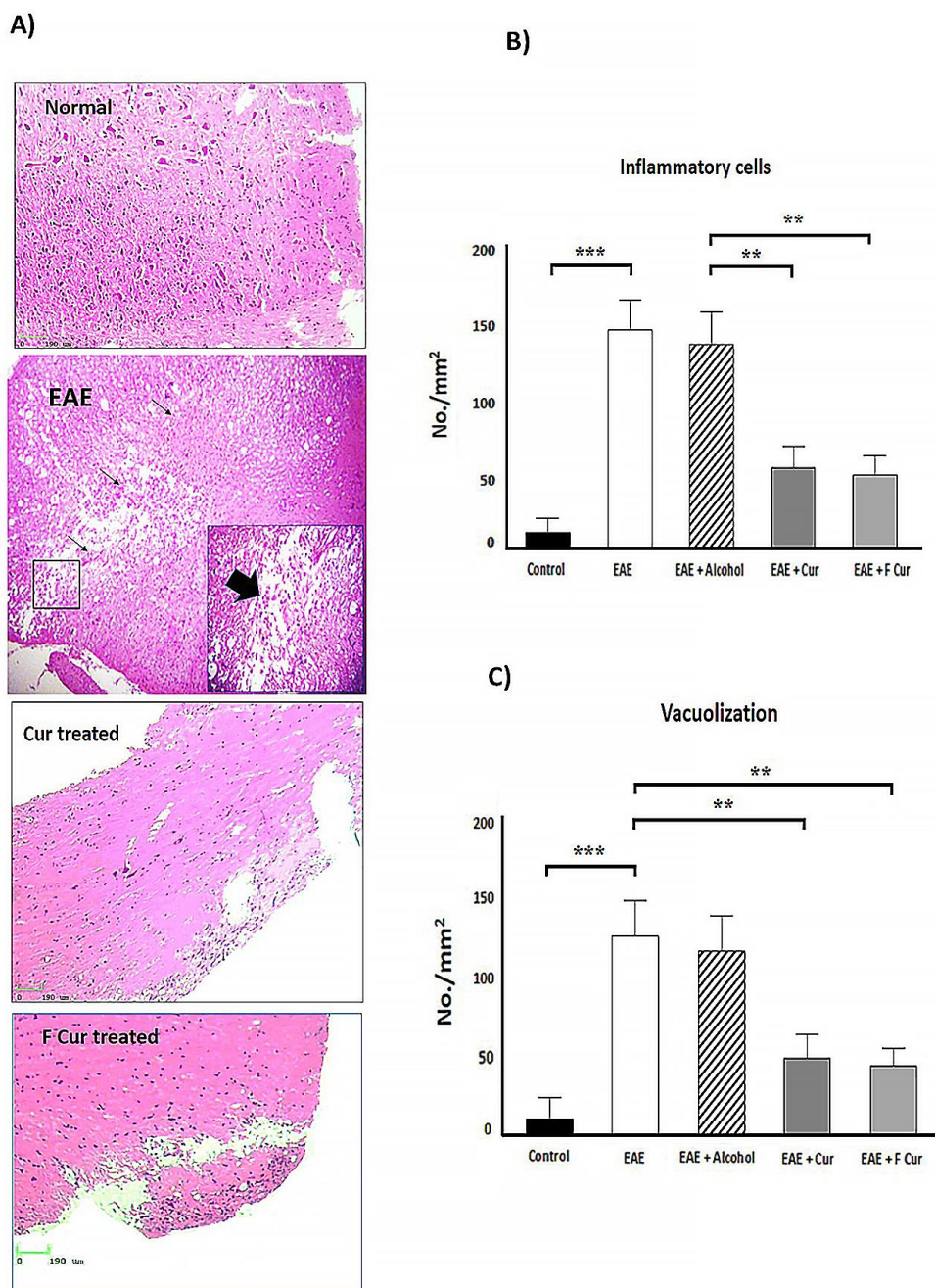
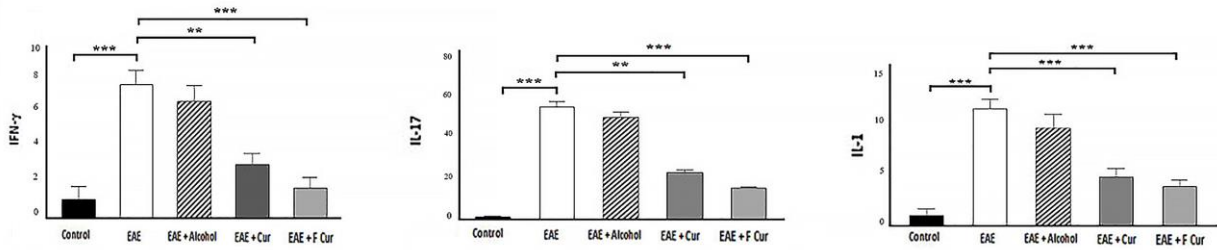


Figure 3. Analysis of inflammation through Hematoxylin and eosin staining. The group with experimental autoimmune encephalomyelitis (EAE) exhibited increased inflammation and vacuolization compared to EAE mice treated with curcumin (cur) or F-curcumin. In the EAE group sample image (A), vacuolization is indicated by a bold arrow. Statistical analysis of the number of inflammatory cells (B) and the extent of vacuolization (C) across different groups. A blinded pathologist evaluated twenty-one sections from each group. Significant differences indicated by (** $p < 0.01$, *** $p < 0.001$).

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A) Pro-inflammatory cytokines



B) Anti-inflammatory cytokines

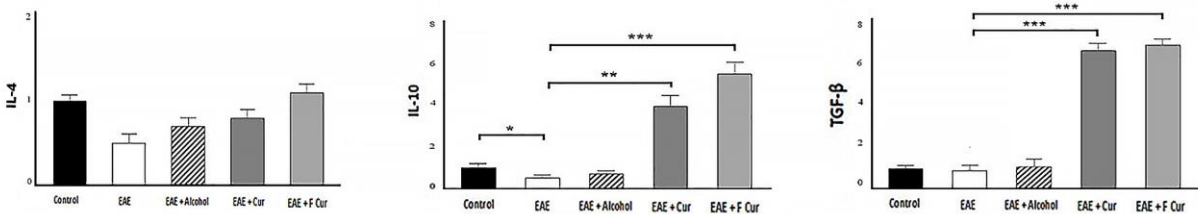
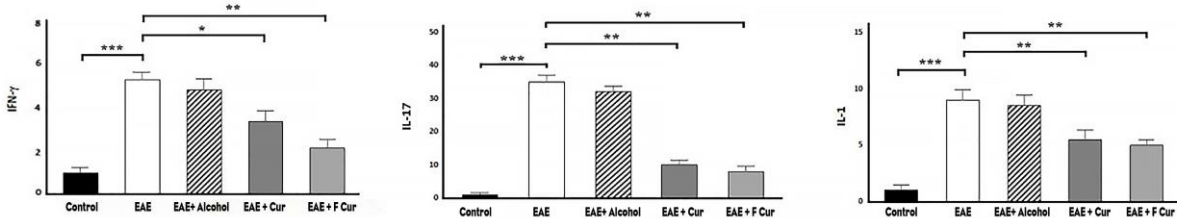


Figure 4. Comparative analysis of pro-inflammatory and anti-inflammatory cytokine levels in the spinal cords of different study groups. Both curcumin and, more effectively, F-curcumin substantially reduced the levels of (A) pro-inflammatory cytokines such as IL-1, IFN- γ , and IL-17 while notably elevating the levels of (B) anti-inflammatory cytokines TGF- β and IL-10. Statistical significance denoted by (* p <0.05, ** p <0.01, *** p <0.001).

A) Pro-inflammatory cytokines



B) Anti-inflammatory cytokines

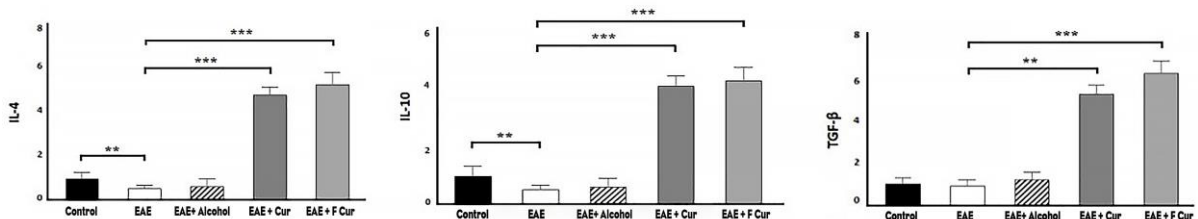


Figure 5. Assessment of pro-inflammatory and anti-inflammatory cytokine expression in the spleens of the study groups. The treatments with curcumin and F-curcumin markedly reduced the expression of (A) pro-inflammatory cytokines IL-1, IFN- γ , and IL-17, while they significantly elevated the expression of (B) anti-inflammatory cytokines TGF- β , IL-10, and IL-4. Levels of statistical significance are indicated by (* p <0.05, ** p <0.01, *** p <0.001).

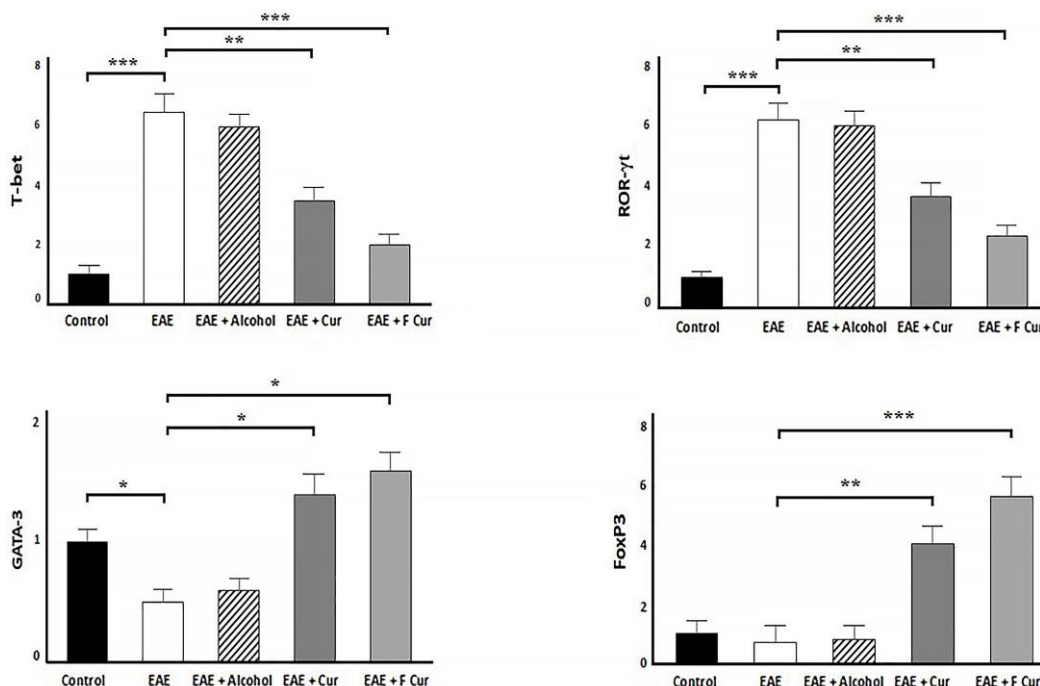


Figure 6. Relative quantification (RQ) of T helper cell subset transcription factors in the spinal cords of the study groups. Treatment with curcumin and F-curcumin resulted in a significant decrease in the expression of (A) Th1 and Th17 specific transcription factors, T-bet and ROR γ t, and a significant increase in the expression of (B) Th2 and Treg specific transcription factors, GATA3 and FoxP3. Statistical significance is denoted by (* p <0.05, ** p <0.01, * p <0.001).**

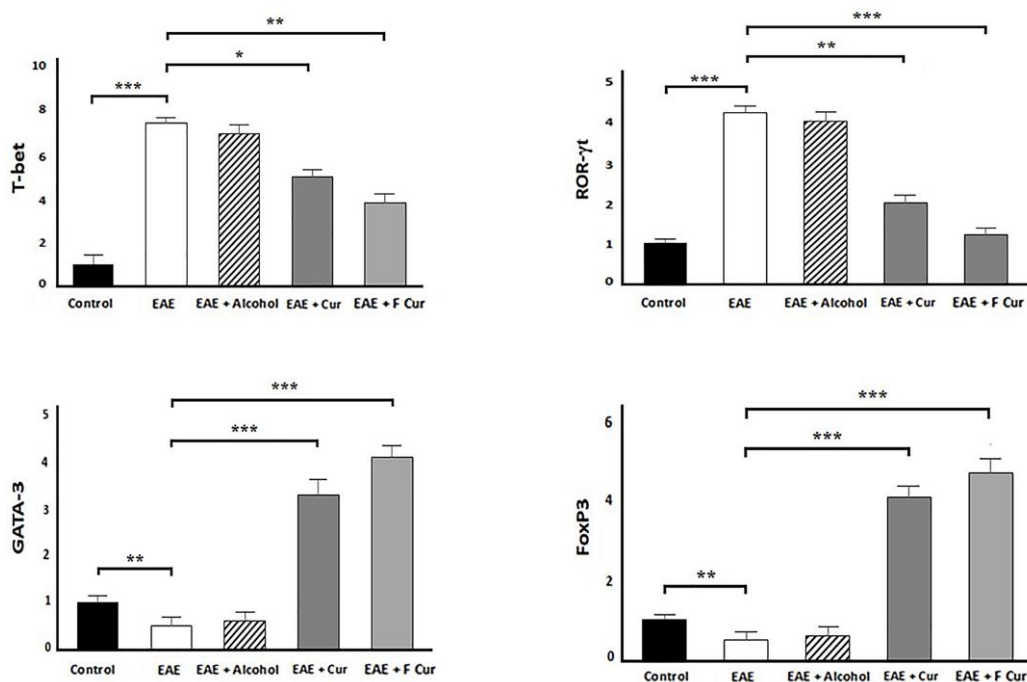


Figure 7. Relative quantification (RQ) of helper T cell subset transcription factors in the spleens of the study groups. The administration of curcumin and F-curcumin notably lowered the expression of (A) Th1- and Th17-specific transcription factors T-bet and ROR γ t, while markedly enhancing the expression of (B) Th2 and Treg specific transcription factors GATA3 and FoxP3. Levels of statistical significance are indicated by (* p <0.05, ** p <0.01, * p <0.001).**

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The findings of this study indicated that the expression of IL-10 and TGF- β in the spinal cord was elevated, while the expression of IL-1, IL-17, and IFN- γ was reduced in the animals treated with curcumin and its derivative compared to controls. However, the administration of both F-curcumin and curcumin did not elicit any effect on IL-4 coding gene expression. It is noteworthy that the results obtained from the group that received F-curcumin treatment were more significant in most of the experimental groups (Figure 4).

The findings of the study demonstrate that the gene expression of IL-4, IL-10, and TGF β in the spleen was increased, while the expression of IL-1, IL-17, and IFN- γ was diminished in the animals treated with curcumin and its derivative compared to the control group. Furthermore, the outcomes for TGF- β and IFN- γ were considerably more significant in the F-curcumin group (Figure 5).

Our analysis revealed that both compounds had significant effects on the upregulation of Foxp3 and Gata3 and the downregulation of T-bet and ROR γ t in the spinal cord (Figure 6). Thus, the compounds were found to have ameliorating effects on the immune response, specifically in the shift from Th1 and Th17 toward Treg and Th2. Furthermore, our analysis has shown a higher potency for F-curcumin compared to curcumin in the expression of the FoxP3, T-bet, and ROR γ t genes, as F-curcumin caused a more significant activation of Foxp3.

Our findings demonstrate that both F-curcumin and curcumin elicit significant upregulation of FOXP3 ($p < 0.001$) and GATA3 ($p < 0.001$) while down-regulating T-bet and ROR γ t in the spleen (Figure 7). These compounds, therefore, exert a direct impact on modulating the immune response from Th1 and Th17 towards Treg and Th2. Notably, F-curcumin appears to exert a greater influence than curcumin on the expression of T-bet and ROR γ t genes.

DISCUSSION

Previous studies have indicated that curcumin exhibits a median lethal dose (LD50) of approximately 2 g/Kg in both rats and mice.²⁴⁻²⁶ Administration of curcumin at a dose of 20 mg/Kg for a period of 15 days, as performed in our study, is both safe and nontoxic. Curcumin is a widely accepted natural product, widely studied, and has shown to have wide biological properties.²⁷ Hybridization of bioactive compounds with

heterocyclic rings tends to improve their bioavailability and receptor selectivity.^{28,29} The 1,2,3-triazole ring an active polymer that forms the amide group bioisoster and can increase the solubility of hybrid compounds with 3 nitrogen atoms. Therefore, we employed F-curcumin as a hybrid molecule of curcumin with a triazole ring. In this way, we exploited the nucleophilicity of the middle -CH₂ group of curcumin through successive reactions of Knoevenagel and Huisgen.

This study investigated several immunopathogenic mechanisms involved in the protection of demyelination by curcumin in EAE mice. Recent research has revealed that curcumin is effective in reducing inflammation.^{30,31} In this research, both curcumin and F curcumin were shown to have the potential to ameliorate inflammation, a crucial element involved in the pathogenesis of MS and EAE. However, the precise mechanisms by which these compounds exert their anti-inflammatory effects remain to be fully elucidated. In our study, we evaluated the expression levels of pro- and anti-inflammatory cytokines, particularly in the spinal cord and spleen, as these are two of the most affected sites in EAE and MS. These sites have received comparatively less attention in previous studies of gene expression in the CNS. Accumulation of pro-inflammatory cytokines cause inflammation, and subsequently demyelination, damage to oligodendrocytes, and neuronal death.³² Anti-inflammatory cytokines, on the other hand, have the potential to inhibit autoimmune responses and serve as a protective measure against inflammatory injury.³³ In the present study, curcumin and F-curcumin were found to mitigate inflammation. This was achieved through the reduction of pro-inflammatory cytokines, such as IFN- γ , IL-17, and IL-1, and the enhancement of anti-inflammatory cytokines, including TGF- β , IL-10, and IL-4. The difference in IL-4 gene expression between the spinal cord and spleen could be attributed to B lymphocytes that produce IL-4 especially prevalent in B-cell-rich organs, particularly the spleen.³⁴⁻³⁶ Previous studies have also shown that curcumin has a protective effect on the brain by counteracting lipid peroxidation.³⁷ and prevents NF- κ B-mediated transcription of inflammatory cytokines.³⁸

Furthermore, our study demonstrated that administration of curcumin and F-curcumin to EAE mice would markedly diminished the clinical symptoms in vivo. In other studies, the role of curcumin and F-

curcumin in alleviating neurodegenerative symptoms is often attributed to their anti-inflammatory effects in the CNS. Furthermore, apart from its potential efficacy in treating MS, curcumin's therapeutic benefits have been proposed in diverse models of inflammatory conditions. These include psoriasis, inflammatory bowel disease, systemic lupus erythematosus, Alzheimer's disease, and rheumatoid arthritis, as well as asthma and allergies.^{15,39,40}

Administration of 20 mg/Kg curcumin in EAE has been shown to significantly improve clinical symptoms. This observed improvement is primarily attributed to the suppression of Th17 cells and associated inflammatory mediators.⁴¹ The original association between the pathophysiology of MS and Th1/Th2 imbalance has been well documented. However, the identification of Th17 cells as key players in MS and EAE, as well as several other autoimmune diseases, has significantly contributed to a deeper understanding of the fundamental pathological processes involved.⁴² The development of CD4⁺ Th17 cells depend on the STAT3 pathway activation. Ultimately, this pathway regulates the expression of ROR γ t.⁴³ In addition, IL-1 cytokines have been recognized in their role in promoting the expansion of Th17. To explicate the impact of the compounds on Th17 production and consequently on MS and EAE, IL-1 and ROR γ t mRNA levels in the spleen and spinal cord tissues were measured. This yielded a significant decrease in comparison to control mice. Simultaneously, the cytokine product of Th17, IL-17, was also observed to be reduced. These findings imply that curcumin, and particularly F-curcumin, prevent EAE by curbing Th17. Apart from blocking STAT3 phosphorylation and reducing ROR γ t expression, there are multiple biochemical pathways through which curcumin may influence the treatment of MS and EAE. Ifergan et al. have indicated that dendritic cells produce IL-1, IL-6, and TGF- β which are crucial in the differentiation of Th17 lymphocytes. Curcumin interferes with the development and function of these cells by suppressing nuclear factor κ -light-chain-enhancer of activated B cell (NF- κ B) signaling.^{44,45}

In the context of Th17, it is noteworthy that unspecialized CD4⁺ T lymphocytes have the potential to differentiate into Th1, Th2, or Treg cells contingent upon their cytokine milieu. Regulation of inflammatory Th1 cells is facilitated by IL-12 and subsequent IFN- γ through T-bet mRNA expression. On the other hand, the secretion associated with pro-inflammatory Th2 cells is

connected to GATA-3 and IL-4 expression, as previously mentioned.^{46,47,48} We assessed key genes and cytokines linked to Th cell development in treated and control mice to further examine the processes involved with the prevention of MS and EAE. Our research focused on the effects of curcumin and F-curcumin, on upregulating pro-inflammatory Th2 and Tregs, and boosting the levels of IL-4 (particularly in the spleen), IL-10, and TGF- β , while downregulating pro-inflammatory Th1 and Th17 and their cytokine products, as measured by qRT-PCR. Our findings are consistent with those of an in vivo study in a lupus nephritis animal model, which showed that found curcumin increased Foxp3 expression and hence Tregs. We know that Treg overexpression can suppress the interaction between B-cell lymphocytes and Th2, thereby shutting down Th1 and Th2 activity. Consequently, curcumin can ameliorate the symptoms of autoimmune diseases by indirectly inhibiting autoantibodies.

The expression of *GATA3* and *FOXP3* genes was also significantly reduced in the EAE-induced model compared to control mice. However, in another case-control study, no link was found between genetic variants in *GATA3* and *FOXP3* and susceptibility to MS.⁴⁹ This implies that differences in gene expression mediators, rather than gene structure, exist between individuals with and without MS. In murine models of EAE and atherosclerosis, mice treated with curcumin exhibited immunomodulatory, neuroprotective, and atheroprotective effects.

There have been no investigations on the effect of curcumin on T-bet mRNA expression in MS models to date. However, our results are consistent with prior research that has shown that curcumin may decrease Th1 activity by suppressing IFN- γ production via the NF- κ B pathway and downregulating T-bet in systemic lupus erythematosus.⁴⁰

According to the above results, F-curcumin showed enhanced potency and efficacy compared to curcumin, which may be attributed to its higher solubility and bioavailability. As such, F-curcumin could represent a viable therapeutic method for the treatment of MS. Further research into the effects of this new compound on various aspects of MS, as well as other autoimmune disorders and human populations, is recommended. Furthermore, exploring the synergistic effects of this compound in combination with other anti-inflammatory medications could be an interesting approach for future research.

STATEMENT OF ETHICS

All the procedures used in this study were approved by the Committee of Ethics in Animal Research of Shahid Beheshti University of Medical Sciences (Tehran, Iran) (Ethics Committee Approval Code: IR.SBMU.MSP.REC.1398.595).

FUNDING

Not applicable

CONFLICT OF INTEREST

The authors have no relevant financial or non-financial interests to disclose.

ACKNOWLEDGEMENTS

Not applicable

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