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A potential therapeutic role in multiple sclerosis for stigmast-5,22-dien-3β-ol myristate isolated from *Capparis ovata*

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Abstract

Multiple sclerosis (MS) is an autoimmune disease of the human central nervous system. It is one of the most common neurological disorders around the world and there is still no complete cure for MS. Purification of a terpenoid from *Capparis ovata* was carried out and its structure was elucidated as stigmast-5,22-dien-3β-ol, myristate (3β,22*E*-stigmasteryl myristate; SDM) by NMR and mass spectral analyses. No information regarding its any health effect is available in the literature. In the present study, we have described its effects on inflammatory factors such as the expression levels of cytokines, chemokines and adhesion molecules as well as apoptosis/infiltration and myelination in SH-SY5Y cells. The expression levels of proinflammatory or inflammatory cytokines and chemokines such as NF-κB1, CCL5, CXCL9, CXCL10 and HIF1A along with T-cell activating cytokines such as IL-6 and TGFB1 were significantly downregulated with SDM treatment. Moreover, the expression levels of the main myelin proteins such as MBP, MAG and PLP that are essential for healthy myelin architecture were significantly up-regulated. The results presented in this study strongly suggest that the SDM offers a unique possibility to be used with autoimmune diseases, including MS due to its activity on the manipulation of cytokines and the promotion of myelin formation.

Introduction

Multiple sclerosis (MS) is a recurring disease of the human central nervous system (CNS) in which repeated occurrences of inflammatory demyelination result in the development of distinctly demyelinated plaques of gliotic scar tissue related to varying degrees of axonal loss (1,2). MS is presently viewed as an "unpredictable-complex characteristic" that is activated in hereditarily susceptible people by environmental components. The disease is likewise considered to contain an autoimmune factor where both the innate and adaptive systems have been involved in disease pathogenesis (3,4). It is one of the most common neurological disorders around the world. There is still no complete cure for MS and it remains entirely non-treatable disease with no effective treatment. Therefore, pharmaceutical companies and scientists are still searching for new drugs (2,5,6).

Recently, various studies portraying the valuable properties of extracts or constituents from plants utilized as a part of alternative and complementary treatments have been reported. The utilization of such extracts as complementary and alternative medicine has recently expanded (7,8). Furthermore, these plant extracts present an excellent source of drug candidates for both scientific and pharmaceutical industrial research.

The caper (*Capparis*) is a native Mediterranean plant and certain species of capers have been cultivated as an economically important plant. It has been reported that the genus *Capparis* consists of nearly 80 species. *C. ovata* and *C. spinosa* have wide natural distribution in Turkey, and they are consumed as pickles (9). In general, Capparaceae family members contain glucosinolates, alkaloids, and flavonoids. Caper flower buds, root bark, and fruits of the plant are used in folk medicine due to their analgesic, wound healing, cell regeneration, tonic, diuretic effects and treatment of Multiple Sclerosis (10-14).

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The water extract of *Capparis ovata* has been shown to be used as an alternative medicine for the treatment of MS (13,14). For this reason, *C. ovata* extract was further fractionated and studied for additional anti-neuroinflammatory effects in SH-SY5Y cells. Purification of the terpenoid was carried out and its structure was elucidated as stigmast-5,22-dien-3β-ol, myristate (SDM) based on 1D- and 2D-NMR and mass spectroscopic techniques. No information regarding its any health effect is available in the literature. In the present study, we have demonstrated that SDM significantly reduces the expression of the genes important in inflammation in SH-SY5Y cells. We have described its effects on inflammatory factors such as the expression levels of cytokines, chemokines and adhesion molecules as well as apoptosis/infiltration and myelination.

Materials and Methods

Plant materials, extraction and samples preparation

NMR spectra on a Varian VNMRS spectrometer operating at 600 MHz for^{1}H-NMR and 150 MHz for^{13}C-NMR (TMS as an internal standard) including APT, COSY, HMBC, HMQC spectra.

Capparis ovata Desf. parts (flowering buds, flowers and fruits) were supplied by Asci Murat Capers, Ice Cream, Dessert and Pickle Manufacturing & Export Co., Ltd. to prepare the extract. Fruits of *C. ovata* that had been collected from the beginning of May to the end of September in Denizli and Burdur (Southern Turkey) in 2015, and identified by Dr. Mehmet Çiçek. A voucher specimen was deposited in the Herbarium of the Faculty of Science and Letters, Pamukkale University, Denizli, Turkey (PAMUH2012000006300).

Fresh fruits of *C. ovata* (1.280 kg) were dried at room temperature, cut into pieces and ground in a grinder. They were macerated five times for 24 h at room temperature, with dichloromethane/hexane (50%, v/v). After filtration, the solvents were evaporated to dryness under vacuum.

Isolation and identification of stigmast-5,22-dien-3β-ol, myristate (3β,22*E*-stigmasteryl myristate; SDM)

The dichloromethane/hexane extract of fruits (CDHFr) (20 g) was subjected to a silica gel column (1.5 x 40 cm) and eluted with petroleum ether (40–60°) (5 x 150 mL), a gradient of dichloromethane was added by 10 mL increments into 100 mL petroleum ether up to reaching 100 %. Thus, 5 x 300 mL dichloromethane were used, followed by acetone by 10 mL increments up to 100% (5 x 200 mL) and finally, methanol was used by 10 mL increments up to 100% (5 x 200 mL). The similar fractions were combined by using TLC analysis and then further subjected to preparative thin layer chromatography to yield compound 3β,22*E*-stigmasteryl myristate from CDHFr (fraction 4) and purified from the solvent system (petroleum ether: dichloromethane, 3:2; v/v) as 15 mg. TLC plates were visualized by spraying with cerium (IV) sulphate dissolved in 10% sulphuric acid following UV light checking.

NMR spectra were recorded on a Varian VNMRS spectrometer operating at 600 MHz for ^{1}H-NMR and 150 MHz for ^{13}C-NMR (TMS as an internal standard), mass spectrum was taken by ESI technique on Tandem-Triple Quadrupole MS (Zivak Technology).

Cell Culture

The human neuroblastoma cell line SH-SY5Y was obtained from the American Type Culture Collection (ATCC, USA). The cells were cultured in DMEM-F12 supplemented with 10% FBS and 1% penicillin/streptomycin mixture in a humidified atmosphere of 95% air with 5% CO_2 at 37°C and were subcultured twice a week.

Cytotoxicity assay

SH-SY5Y cells were seeded in 96-well plates at a density of 1×10^3 cells/mL culture medium. After 24-h incubation, the cells were treated with varying concentrations (ranging from 4μM to 96μM) of SDM. An equal amount of medium without SDM was added to untreated cells (control). SDM-treated and control cells were incubated for 24 h at 37°C in humidified 5% CO_2 atmosphere. Following incubation, the medium was replaced by 0.5% crystal violet solution (w/v; in 50% ethanol). Dye absorbed by live cells was extracted with sodium citrate (0.1 M in 50% ethanol). Absorbance was read at 630 nm. Viability was expressed as a percentage of the control, untreated cells.

RNA isolation and cDNA synthesis

Total RNA was extracted from SH-SY5Y cells by using RNeasy Plus mini kit (Qiagen) according to the manufacturer's instruction with slight modifications. Elution was performed with 40μL RNase-free water. After elution, the RNA concentration was determined using a Nanodrop (MaestroNano micro-volume Spectrophotometer, USA), and the RNA was reverse transcribed using Easy Script cDNA Synthesis Kit (ABM). The reaction mixture was incubated for 50 min at 50°C followed by termination by heating at 5 min 85°C. cDNA was stored at -80°C for further use.

Quantitative RT-PCR

Quantitative Real Time PCR (qRT-PCR) analysis was performed using SYBR Green qPCR Master Mix (GM, Taiwan) in an Exicycler 96 Real Time Quantitative Thermal Block PCR System (Bioneer, Daejeon, Korea) for each gene. The mRNA levels of genes (APP, C1S, CCL5, CXCL9, CXCL10, GFAP, HIF1A, IL6, MAG, MBP, MMP9, NF-κB1, PLP, PTPN11, STAT3, SOD, TGFβ1, TNFα) were determined by qRT-PCR. Beta-actin was chosen from the group of housekeeping genes as the least varying reference gene. The qPCR using custom designed primers for the genes listed in **Table 1**.

Statistical analysis

Statistical analysis was performed using the Minitab 13 statistical software package (Minitab Inc. State College, PA, USA).

Table 1. Primer sequences of the selected human genes

GeneBank	Gene	F_Sequence (5'->3')	R_Sequence(5'->3')	Tm
NM_000533	PLP	GAAAGCCCTTTTCATTGCAGGA	GGCTAGTCTGCTTTGTGGCT	56°C
NM_002834	PTPN11	GACGTTCCCAAAACCATCCA	TCTTCTTCAATCCTGCGCTGT	56°C
NM_000600	IL6	ACTCACCTCTTCAGAACGAATTG	CCATCTTTGGAAGGTTCAGGTTG	59°C
NM_003998	NFKB1	TCGCGCTGAGTATAAAAGCC	GGCAAAGTTTCGTGGATGCG	61°C
NM_002416	CXCL9	GGCTCTTTCCTGGCTACTCC	TCCCTGGTCCCTGTAGTGAG	61°C
NM_000484	APP	GCCCTGCGGAATTGACAAG	CCATCTGCATAGTCTGTGTCTG	61°C
NM_002985	CCL5	CAGTCGTCTTTGTCACCCGA	AGAGCAAGCAGAAACAGGCA	62°C
NM_001565	CXCL10	ACCAGAGGGGAGCAAAATCG	GGAAGTGATGGGAGAGGCAG	62°C
NM_004994	MMP9	GGGACGCAGACATCGTCATC	TCGTCATCGTCGAAATGGGC	62°C
NM_002055	GFAP	GTGTCAGAAGGCCACCTCAA	TCAGGTCTGGGGAAATGTGC	62°C
NM_000454	SOD	TAAAGTAGTCGCGGAGACGG	CTTCGTCGCCATAACTCGCT	62°C
NM_000594	TNF	TGGGATCATTGCCCTGTGAG	GGTGTCTGAAGGAGGGGGTA	62°C
NM_002361	MAG	CCAAGTAGTCCACGAGAGCTT	CAGGTCCCCACGGAAGTAGT	62°C
NM_002385	MBP	TCGGCTCACAAGGGATTCAAG	TGATCCAGAGCGACTATCTCTTC	51°C
NM_001530	HIF1A	GGCGCGAACGACAAGAAAAA	GTGGCAACTGATGAGCAAGC	61°C
NM_001734	C1S	TTTGGCATGGGTTTATGCTGA	GGGTGAAGTAGAGGTGAATCCC	51°C
NM_000660	TGFβ1	TACCTGAACCCGTGTTGCTCTC	GTTGCTGAGGTATCGCCAGGAA	51°C
NM_003150	STAT3	AACAGGATGGCCCAATGGAA	GAAGCGGCTATACTGCTGGT	61°C
NM_001101	ACTB	GCCGCCAGCTCACCAT	GATGCCTCTCTTGCTCTGGG	59°C

All results were expressed as means including their Standard Error of Means (SEM). Comparison between groups was performed using Student's t-test, and $p < 0.05$ was selected as the level required for statistical significance. Statistical comparisons between three groups were assessed by one-way analysis of variance (ANOVA).

Results

Stigmast-5,22-dien-3β-ol, myristate (3β,22*E*-Stigmasteryl myristate = Stigmasta-5,22-dien-3β-ol, tetradecanoate)

It was isolated for the first time from the Capparaceae members and the structure of the compound is found to be 3β,22*E*-stigmasteryl myristate (SDM). The NMR and MS data are given below, and the spectra are given as supplementary material.

Waxy, yellowish.^{1}H-NMR (600 MHz, $CDCl_3$): δ 4.60 (1H, m, H-3α), 5.34 (1H, brd, J= 4.1 Hz, H-6), 5.02 (m, H-23), 5.75 (m, H-22), 2.26 (dd, J= 2.7;7.6 Hz, H-4α), 2.31 (dd, J= 6.5 Hz, H-4β), 0.68 (3H, s, Me-18), 1.02 (3H, s, Me-19), 0.92 (3H, d, J=6.5 Hz, Me-21), 0.81 (d, J=7.9 Hz, Me-27), 0.83 (d, J=6.8 Hz, Me-29), 1.25 (s, Me-28), 0.83 (3H, d, J=6.8 Hz, Me-26), 0.85 (3H, t, J=7.1 Hz, Me-29).^{13}C-NMR (150 MHz, $CDCl_3$): δ 173.28 (C-1′), 36.98 (C-1), 31.90 (C-2), 73.67 (C-3), 42.28 (C-4), 139.69 (C-5), 122.56 (C-6), 31.88 (C-7), 36.13 (C-8), 51.42 (C-9), 36.57 (C-10), 21.01 (C-11), 39.69 (C-12), 42.29 (C-13), 56.67 (C-14), 24.27 (C-15), 28.23 (C-16), 56.01 (C-17), 11.95 (C-18), 19.00 (C-19), 45.81 (C-20), 20.18 (C-21), 130.03 (C-22), 128.02 (C-23), 49.99 (C-24), 31.90 (C-25), 21.00 (C-26), 19.30 (C-27), 29.33 (C-28), 11.83 (C-29), 29.3 $(CH_2)_n$. $C_{43}H_{74}O_2$ APCI-MS (m/z): 622.5 [M^+] (4), 607.5, 448.2, 365.2, 333.2 (**Fig 1**).

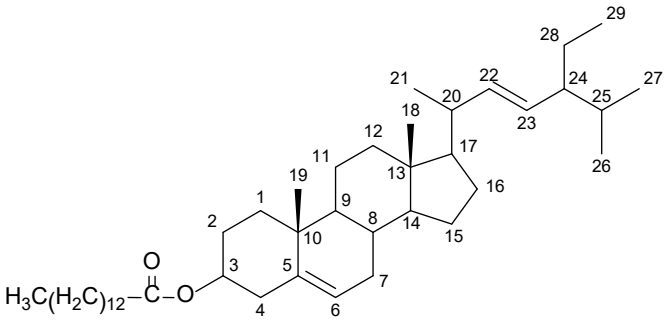

Figure 1. Chemical structure of stigmast-5,22-dien-3β-ol, myristate.

Effects of stigmast-5,22-dien-3β-ol, myristate on cell viability

The cytotoxicity of stigmast-5,22-dien-3β-ol, myristate (SDM) in SH-SY5Y cells was investigated by crystal violet staining. As shown in **Fig. 2**, SDM treatment revealed a concentration-dependent cytotoxic effect on SH-SY5Y cells in a dose-dependent manner. The EC5 and EC10 values of the SDM were found to be 8 and 12μM, respectively.

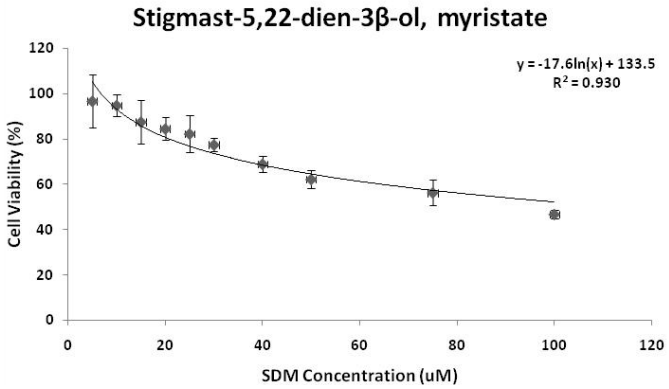

Figure 2. Cytotoxicity of stigmast-5,22-dien-3β-ol, myristate (SDM) on SH-SY5Y cells after 24h. The results are expressed as the means of two independent experiments, with each experiment performed in triplicate.

Effects of stigmast-5,22-dien-3β-ol, myristate on multiple sclerosis-related genes mRNA levels

The effect of SDM on the expression of the main MS-related genes participating in inflammation/cytokine/chemokine, myelination/demyelination and T cell activation was determined in this study (**Figs. 3–7**). All of the myelination/demyelination genes (MAG, MBP, PLP, SOD) were significantly increased by 8 and 12μM SDM treatment. CXCL10 and HIF1A mRNA levels were decreased by 8 and 12 μM SDM treatments; however, the reduction in mRNA levels due to 8 μM SDM treatment was not found to be statistically significant. Similarly, the other inflammation/chemokine/cytokine gene PTPN11 was significantly

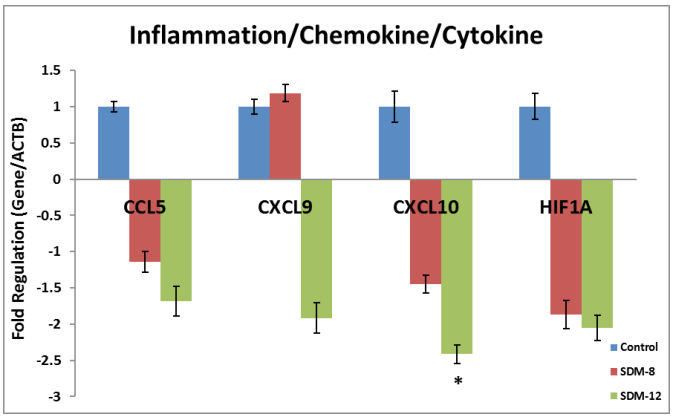

Figure 4. mRNA expression level of inflammation/chemokine/cytokine genes in the control and various treatment groups. *Significantly different from the respective control value (p<0.05).

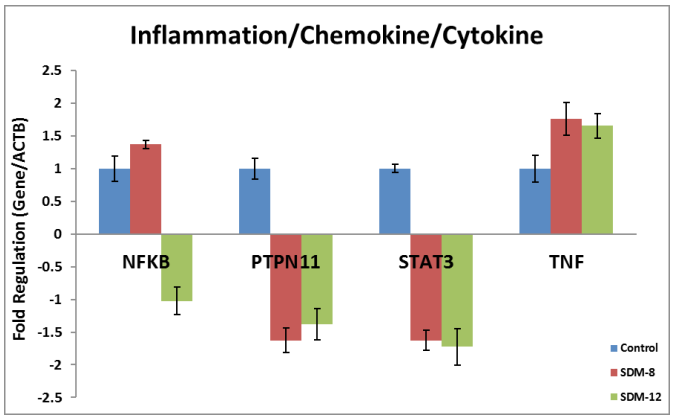

Figure 5. mRNA expression level of inflammation/chemokine/cytokine genes in the control and various treatment groups. *Significantly different from the respective control value (p<0.05).

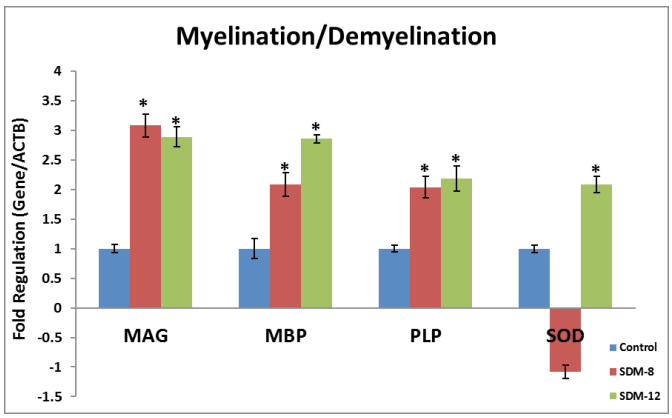

Figure 3. mRNA expression level of the myelination/demyelination genes in the control and various treatment groups. *Significantly different from the respective control value (p<0.05).

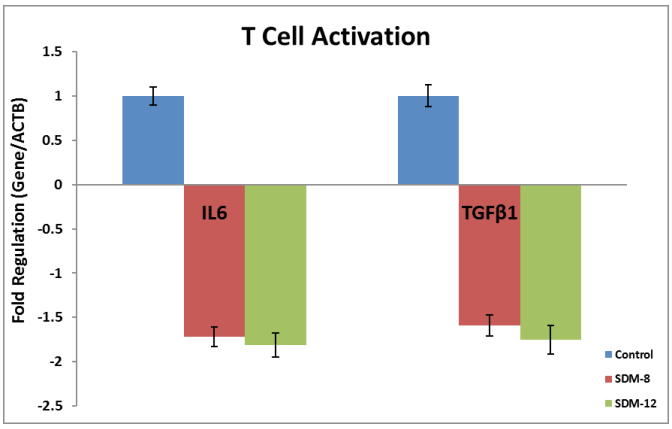

Figure 6. mRNA expression level of T cell activation genes in the control and various treatment groups.

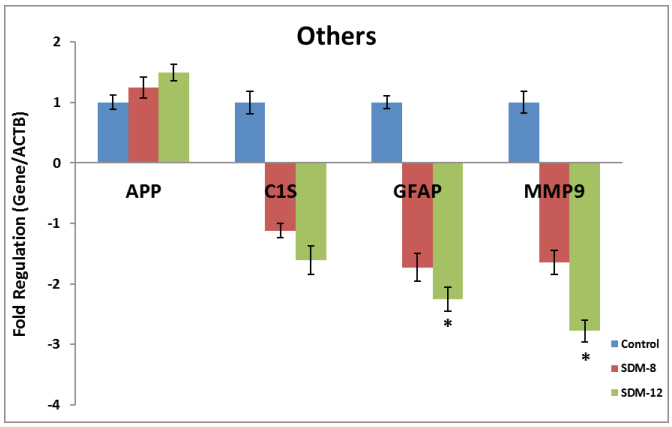

Figure 7. mRNA expression level of other groups of genes in the control and various treatment groups. *Significantly different from the respective control value ($p<0.05$).

down-regulated with SDM treatments. IL6, C1S, and TGFβ1 mRNA levels decreased slightly in response to SDM treatment but, while this decrease was not statistically significant. Finally, GFAP and MMP9 genes were downregulated with SDM treatment in SH-SY5Y cells.

Discussion

Despite several officially endorsed pharmaceuticals, the treatment choices in MS are restricted and no complete cure is presently known (15). Many individuals with MS investigate complementary and alternative solutions to help control their MS and treat their symptoms. It was reported that up to 70% of individuals with MS had attempted at least one CAM treatment for their MS (7). Turkish patients use *Capparis ovata* as CAM treatment and it has shown to be effective in MS treatment (14). To this end, we have focused on the further purification of *Capparis ovata* extract focusing on the phytosterols and terpenoids since several terpenoids such as ginkgolide B and tetrahydrocannabinol are known to be effective in MS (16-19).

The anti-neuroinflammatory and immunomodulatory activity of stigmast-5,22-dien-3β-ol, myristate were tested in SH-SY5Y cells which are often used as in vitro models of neuronal functions. Although it has been shown that stigmasterol exhibits some anti-inflammatory responses, no effects of stigmast-5,22-dien-3β-ol, myristate on inflammation and myelination have been reported (20). Therefore, we decided to evaluate the effect of SDM in SH-SY5Y cells at the two doses -both safe and exert no toxic effect- which were determined to be 8 and 12 μM.

In order to investigate the SDM treatment could be associated with a reduced immune-inflammatory reaction, we first examined the modulation of some inflammatory events, such as the expression of chemokines or cytokines in the SH-SY5Y cells. The genes to be considered were selected based on the previous animal model of inflammatory demyelinating disease that is experimental autoimmune encephalomyelitis (14, 21).

First, we have studied the expression levels of proinflammatory or inflammatory cytokines and chemokines such as NFKB1, CCL5, CXCL9, CXCL10, HIF1A after SDM treatment. The expressions of all these genes except TNF were significantly downregulated with SDM as compared to control. It strongly suggests that SDM exhibits powerful anti-inflammatory action by decreasing the expression levels of the major players in inflammation. The expression level of CCL5, CXCL10 and HIF1A are upregulated in either MS patient or EAE (Experimental Allergic Encephalomyelitis) animal model (22-24). Most of these genes are regulated by NF-κB1 and down-regulation of them may bring forward that SDM may act on NF-κB1 signaling pathway. It is well known that the NF-κB1 cascade integrates with many immunological pathways and can therefore effectively modulate immune response in MS patients and exert heterogeneous risk factors for MS (25), SDM as an inhibitor of this cascade would be a precious candidate for treatment of autoimmune diseases such as MS and may regulate the lymphocyte activation and trafficking as does the current drugs used to treat MS.

Furthermore, the effect of SDM on the expression of the genes that play a role in T-cell activation, cell adhesion and tissue infiltration or apoptosis were also determined (Figures 6 and 7). IL6 and TGFB1 are the cytokines that are involved in T-cell activation which is a critical parameter for the onset of MS (4). The down-regulation effect of SDM on the expression of both IL-6 and TGFB1 along with decreased expression of the tissue infiltration related gene (MMP9) would be recognized both anti-inflammatory and immune suppressive activities. These results further support the anti-inflammatory activity of the SDM.

In addition, as shown in Figure 3, the potential role of SDM in myelination or remyelination was studied since MS is a demyelinating disease and it is an essential challenge to target not only the inflammatory aspect of the MS, but also its neuroregenerative issues. In this aspect, SDM exhibited exceptionally well myelin recovery effect since MBP, MAG, PLP and SOD expression levels were significantly upregulated altogether (Figure 3). These are the main myelin proteins and are important for healthy myelin architecture (26). The increased expression of MBP, MAG and PLP by SDM may be acknowledged as signals that promoting myelin re-formation and repair. Thus, SDM displays not only anti-neuroinflammatory activity but also neuroprotective and neurodegenerative effects.

In conclusion, the manipulation of cytokines and the promotion of myelin formation by SDM offers a unique possibility to be used with autoimmune diseases, including MS. However, due to the complexity of the cytokine networks, side effects may occur and further animal studies and preclinical evaluations must be carried out to clarify the potency of SDM as a therapeutic agent.

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Conflict of Interest Statement

On behalf of all authors, the corresponding author states that there is no conflict of interest.

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