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Swertiamarin ameliorates inflammation and oxidative damage in autoimmune encephalomyelitis mouse model

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Abstract

Multiple sclerosis (MS) is a degenerative autoimmune disease attacks the myelin sheath of the central nervous system (CNS) neurons causing different disabilities. According to recent evidence, the main bioactive component in *Enicostema axillare*, the Swrtiamarin (SM) has been found to exert anti-inflammatory and antioxidant activities against several diseases. However, SM activities in treatment of autoimmune diseases remain to be explored. Herein, we used a murine model of MS, to show that SM treatment ameliorates the severity of experimental autoimmune encephalomyelitis (EAE). This occurs through reducing the levels of pro-inflammatory cytokines and infiltration of CD4+CD45+ cells into CNS. That was associated with a reduction in the expression of STAT3 and NFkB in CD4+ T cells under Th17 and LPS-stimulated macrophages. Furthermore, in silico studies revealed that SM interacts with NF-E2-related factor 2 (NRF2), and therefore, suppressed oxidative stress by inducing formation NRF2-antioxidant response element (ARE) complex. We found that SM is an agonist of NRF2 complex regulating the total CD4 population and antioxidant markers in EAE mice. Molecular docking analysis showed a stable and higher binding affinity between SM and NRF2. Results revealed that SM treatment increased the complex formation between ARE and NRF2 where immunoprecipitation methods showed a higher binding affinity of ARE to NRF2 in SM treated animals. Complex formation triggered ARE cascade of antioxidant gene clusters and reduces the MS pathological alterations in EAE mice model. Current data proposed SM as an effective biomolecule in treatment of MS and controlling neuronal damage through inhibiting oxidative stress markers and targeting NRF2.

Keywords: Multiple sclerosis, Demyelination, Anti-inflammatory, Oxidative stress, Sirt-1

Introduction

Swertiamarin (SM) is a secoiridoid glycoside extracted mainly from the Indian folk medicinal plant *Enicostema axillare* (Lam). SM is an active constituent and was suggested to be responsible for a wide range of biological activities such as anti-atherosclerotic, antidiabetic, anti-inflammatory and antioxidant effects [1]. SM was proposed to act as an anti-rheumatic agent through inhibiting the development of arthritis and modulating NF-κB and downstream signaling molecules [2, 3]. In addition, it is a natural Protein Kinase B (AKT) inhibitor induces a significant anti-inflammatory activity in vivo through regulating the AKT-PH domain and inhibiting the downstream inflammatory molecules [4]. Moreover, histopathological and radiological analyses evidenced the curative effect of SM on bone destruction [5]. Recently, we have shown that SM could work as an anti-inflammatory agent against the auto-immune disease and inhibits the development of arthritis, via modulating NF-κB/IκB and other transcription factors in animal models [2]. Moreover, it was found to attenuate IL-1β induced

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fibroblast-like synoviocytes (FLS) indicating an anti-inflammatory role. These previous evidences provoked us to carry out the effective role of SM on neuro inflammatory studies using MS autoimmune model. On the other hand, SM was suggested as a promising protective agent against cerebral injury through suppressing oxidative stress and activating the nuclear factor erythroid 2-related factor 2 (NRF2) protective pathways [6]. In the meantime, SM pre-treatment significantly reduced infarct volume, apoptotic neurons and oxidative damage and enhanced neurologic recovery in vivo. That was associated with a decrease reactive oxygen species (ROS) and an increase in cell viability in vitro. The Keap–NRF2–ARE pathway is a major regulator of pathway of cell sensitivity and response to oxidative stress [7]. In previous reports stated that, SM controls CCl4 induced liver damage by activating NRF-2 in liver tissue of mouse model. Meanwhile it also activates the hemeoxygenase for controlling ARE activation [8]. In this pathway three cellular components are involved, the adaptor protein, Kelch-like ECH-associated protein 1 (Keap1), and antioxidant response element (ARE) and the nuclear transcription factor, NRF2, the later was reported to contribute in the anti-inflammatory processes by regulating the inflammatory cells and gene expression as an upstream of the ARE [9].

Typical treatment of the chronic inflammatory autoimmune disease, multiple sclerosis (MS), focuses on reducing the disease progression and managing its symptoms. Therefore, finding a promising specific and efficient anti-inflammatory drug that could improve the disease treatment and speed recovery from MS disease attack is urgently needed. Similar previous evidences of SM interaction against NRF-2 provoked to evaluate its effect on MS model [8]. The MS disorder is associated with infiltration of macrophages and T lymphocytes, which induce focal areas of demyelination and neurodegeneration [10]. The autoimmune encephalomyelitis (EAE) mouse model, is a well characterized model of MS disease, has been extensively used to study the MS disease progression, pathological features and propose treatment. Typically, the EAE model could be induced via either passive transfer of activated myelin-specific CD4+ T lymphocytes or through active immunization with myelin-derived proteins/peptides [11].

In the present study, proposed SM as a potential anti-inflammatory agent against MS disease.

Materials and methods

Docking analysis

The interaction between NRF2 and SM was evaluated by protein–ligand docking method using Autodock tools (ADT) v1.5.4 and Autodock v4.2 program; http://www.scripps.edu/mb/olson/doc/ autodock as reported earlier [12]. The chemical structure of SM (CID_442435) was obtained from the pubchem compound database; http://www.ncbi.nlm.nih.gov/pccompound. The structure of NRF2 (pdb id: 6TYM) and SIRT1 (pdb id: 4ZZI) were recovered from the Protein Data Bank: http://www.pdb.org. Ligand was considered as a rigid body and the receptor was considered as flexible. The results were evaluated and sorted on the basis of predicted binding energy.

Induction and scoring of EAE

Female C57BL/6j mice (6–8 weeks old) were maintained at the animal facilities of Biological Sciences Department, College of Science, King Faisal University. Animal care committee of deanship of scientific research, King Faisal University (KFU-EA-DSR-123), approved the usage of experimental mice in this study. Mice were housed in pathogen free environment at 12 h dark/light cycles and given standard diet. Mice were randomly divided into three groups (six mice each group), including: EAE, EAE + SM and Placebo. The EAE mouse model was induced with two pre-filled syringes of MOG 35–55 in an emulsion with complete Freund's adjuvant and a vial of 400 µg/kg of lyophilized pertussis toxin (PTX). This model was induced according to the protocol [13]. The clinical symptoms of EAE and body weight were estimated daily, until day 21. The clinical EAE score was categorized as follows: [14]. The mice were sacrificed at day 22, and then the spleen, brain, and lumbar spinal cord were removed for further experiments.

Grip strength test

The grip-strength analysis was tested by cage grill hanging method for testing the forelimbs strength of the mice. Mice were allowed to grip a triangular bar with their forelimbs and were pulled back horizontally. This test was repeated five times for each mouse, and the highest score was recorded as the grip force timing for that mouse and recorded as milli sec.

Histological analysis

Lumbar spinal cords and brains were fixed in 10% formalin solution for 48 h and then embedded in paraffin. After that, the paraffin blocks were cut to make serial sections of 5 µm thickness. Hematoxylin and eosin (H&E) staining was performed after de-waxing and rehydration of paraffin sections. Five slices of each sample were employed to estimate leukocyte infiltration. Finally, semi-quantitative analysis of inflammation was reported for brains: 0, no inflammation; 1, cellular infiltrates only around the blood vessel and meninges; 2, mild cellular infiltration in the parenchyma; 3, moderate cellular infiltration in parenchyma; and 4, serious cellular infiltration in the

parenchyma [15]. The histological score of spinal cords was also assigned the score from 0 (without inflammation) to the score 4 (with the most severe inflammation) [16].

Isolation of spleen lymphoid cells (SPLC) and stimulation with anti-CD3/CD28

The spleen was smashed using cell strainer (8 μM) with 2 ml of RoSMll Park Memorial Institute (RPMI) 1640 media (Gibco, Grand Island, NY, USA) in a sterile 60 mm cell culture plate. The solution was left undisturbed and the supernatant containing single cells were collected to remove the insoluble cells. Cells were centrifuged at 1200 rpm for 5 min at 4 °C. To lyse the red blood cells (RBC), the cell pellet was treated with 1 ml of ammonium-chloride potassium (ACK) lysis buffer (Gibco) for 10 min at room temperature. After washing with RPMI-1640 twice, cells were resuspended in 2 ml of RPMI supplemented with 5% fetal calf serum (HyClone, Logan, UT, USA), 100 units/ml penicillin (Welgene, Gyeongsan, Korea), 100 μg/ml streptomycin (Welgene), and 50 μM of β-mercaptoethanol (Gibco). The isolated SPLC were seeded at a density of 1×10^6 cells/well in a 96-well plate and incubated for 30 min at 37 °C in 5% CO_2. Cells were pretreated with 0.1% dimethyl sulfoxide (DMSO; Amresco, Solon, OH, USA) as a vehicle (VH) or GR extract for 30 min and stimulated with 2 μg/ml of anti-mouse CD3 (BioLegend, San Diego, CA, USA) and 5 μg/ml of anti-mouse CD28 (BioLegend) for 3 days. Supernatants were collected for enzyme-linked immunosorbent assays (ELISAs) and the cells were treated with 5 μg/ml of brefeldin A (BioLegend), a protein transport inhibitor, for 4 h for flow cytometric analysis.

Proliferation assay of splenocytes

The effects of SM treatment on lymphocyte proliferation response were assessed by CCK-8 (Dogingo, US). After spleen isolation, the small pieces of tissue were passed through a cell strainer (70 μm) with the aid of a sterile syringe, and this was followed by washing with RPMI-1640 medium (UFC Biotech, KSA). The cell suspension was centrifuged at $300 \times g$ for 5 min at 4 °C and then the RBCs were lysed by the addition of a RBC lysis buffer (150 mM NH_4Cl, 1 mM $KHCO_3$, 0.1 mM EDTA [pH, 7.2]) for 5 min. The suspension was washed twice with RPMI as mentioned above. The splenocytes with the minimal viability of 95% (determined by trypan blue staining) were cultured in 96-well flat-bottomed plate (5×10^5 cells/well) with complete RPMI containing 10% FBS (fetal bovine serum, Gibco) in the presence of 100 μg/ml penicillin–streptomycin as antibiotics (Gibco).

The splenocytes from each mouse were cultured with 10 μg/ml PHA (phytohemagglutinin, Gibco) as a positive control and with 10 μg/ml MOG as a specific stimulator, or without any stimulator as a negative control culture (in triplicate). The splenocyte cultures were incubated at 37 °C, in a condition of 5% CO_2 for 48 h. Then, the CCK-8 reagent (10 μl/well) was added to the wells and the splenocyte proliferation was assessed after 1 h using a microplate reader at 450 nm. The stimulation Index (SI) was calculated by dividing the optical density (OD) of the stimulated cells into the related un-stimulated cells as previously described [17].

Determination of the cytokine production by splenocytes

Splenocytes were obtained as described as above and adjusted to 2×10^6 cells/well of 6 well plate and cultured for 48 h. Then, the supernatants were removed and the estimation of IL-10, IL-17 A, IFN-γ, TGF-β, IL-4, and IL-6 cytokines were measured with commercial enzyme-linked immunosorbent assay (ELISA) kits (Thermo Fisher Scientific, Inc., Waltham, MA, USA). All samples were performed in triplicate experiments.

Antioxidant estimation in SM treated mice

Briefly, mice neuronal tissues were decapitated at the end of MOG immunization. The tissues were washed in ice cold normal PBS, then pieces of brain tissue were choped and homoginated. The homogenate was centrifuged at 3000 rpm for 15 min to collect supernatant. The supernatant was stored at −80 °C for further analysis. The immune based lipid oxidative stress was measured by estimating Malondialdehyde (MDA) levels as described by Yoshioka et al. [18]. The results were expressed as nanomoles per milliliter (nmol/ml) for serum and nanomoles per milligram-tissue (nmol/mg-tissue) for tissue fluids. The activity of superoxide dismutase (SOD), and catalase (CAT) ratio was estimated by Biovision kit protocols. Commercially available assay kits were used to assess the concentration of SOD, CAT, (Nanjing Institute of Biological Engineering, Nanjing, China). Glutathione GSH was measured in the groups following the Ellman's method [19]. Briefly, homogenized spinal tissue mixed with 0.01 M DTNB and optical value was recorded at 412 nm and results are expressed as nmole GSH/mg of tissue protein. All analyses were performed according to kit instructions.

Flow cytometry analysis

Single-cell suspensions from CNS and spleen were incubated for 30 min at 4 °C with fluorochrome-conjugated anti-CD4, anti-CD8, anti-NRF2 (all purchased from BD Biosciences), for staining of surface markers. For intracellular staining of cytokines, the cells were stained with anti-CD4, followed by staining with anti-IFN-γ, anti-IL-17A (Invtirogen, CA, USA) and according to

the manufacturer's protocol. The cell count, viability and differentiation were noted on triple experimental procedures. IL17 cell pollution was gated in R1 against total CD4 cell populations. Total CD4 cells was gated against the stimulated anti-CD3/CD65L antigens in naïve splenocytes populations. Gated cell populations were expressed as fold units of cells.

RNA extraction, cDNA synthesis, and real-time qPCR

Total RNA was extracted from brain and spinal cord tissues in all groups using trizol reagent (Sigma). Purity and integrity of RNA were determined using Nano Drop ND-2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, USA). In the next step, cDNA was synthesized using High capacity RT reagent Kit (Applied biosystems, Foster City, CA). The relative expression levels of genes including NFκB, NRF2, MAPK6 and STAT3 with the internal control of GAPDH were analyzed in each group using SYBR green-based real-time PCR. The expression values of genes from different samples were calculated by relative quantitation values. The sequences of PCR primers are displayed in Table 1. The real-time qPCR was performed on an Vii7a real-time PCR system (Applied Biosystems, Foster City, CA) using AB SYBR Master Mix instructions (USA). The PCR conditions were under the following thermal condition: preheating, 95 °C, 5 min, 40 cycles of denaturation: 95 °C, 15 s, annealing: 60 °C, 20 s, and extension: 72 °C, 20 s. Cycle threshold (Ct) values were used to calculate fold changes in gene expression using $2-\Delta\Delta Ct$ method.

Western blot analysis

The proteins of tissues or cells were extracted by using radio immunoprecipitation assay (RIPA) buffer containing 1% proteinase and phosphatase inhibitor cocktails (Sigma-Aldrich). Protein lysates were separated by 11% sodium dodecyl sulfate-polyacrylamide (SDS-PAGE) gel electrophoresis, transferred onto polyvinylidene fluoride (PVDF) membrane, and separately immunoblotted with primary antibodies including NFKB-p65 (1:1000), iNOS (1:1000), MAPK6 (1:1000), NRF2 (1:1000), STAT3 (1:1000), and GAPDH (1:2000) following HRP-conjugated secondary antibodies (1:2000). The signals were detected by chemiluminescent ECL Select Kit (GE Healthcare, IL, USA), captured by Gel-Doc system (Licor, USA) and analyzed by ImageJ software.

Statistical analysis

All data were expressed as mean ± SEM and the differences between variables were analyzed using appropriate statistical tests, using one-way analysis of variance (ANOVA) multiple comparisons of groups. The repeated measure test followed by Bonferroni comparison was also utilized to analyze clinical scores and body weight during the study period. The differences were considered significant when the P values were less than 0.05. All data were analyzed using GraphPad Prism (6.01software).

Results

Effect of SM on EAE pathology

Physiological symptoms are predominant modification in multiple sclerosis model. Clinical score and disease index has been observed as macroscopic modification in MOG immunized mice. In this study, the protective effect of SM administration on the demyelination and paralysis symptoms of MS mice were evaluated using in vivo model. Naïve EAE group displayed MS clinical signs, including tail and hind limb paralysis, at 12 days after MOG immunization. The symptoms appeared gradually after immunization, peaked several days later and then persisted (Fig. 1a) until the end of the experiment. Treatment of EAE mice with 25 mg/kg SM showed insignificant symptoms recovery whereas, 50 mg/kg of SM showed potential attenuation of paralysis symptoms upto 22 days of immunization. During EAE induction, SM treatment 25 and 50 mg/kg showed a significant decrease in symptoms compared to naïve EAE mice (Additional file 1: Fig. S1a, b). During 24th and 26th day of observation, the clinical symptoms are attains normal and drastic improvement in SM 50 mg/kg group. These clinical signs reduced due to the auto recovery of demyelination and also with reduced infiltration of inflamed cells in spinal cord. Which was further correlated in tissue pathological as well as cytokine examinations. SM

Table 1 Primer list

Primer	Forward sequence	Reverse sequence	PCR product Size in bp
NFKB	CAGATGGCCCATACCTTCAAAT	CGGAAACGAAATCCTCTCTGTT	185
NRF-2	CCCGAAGCACGCTGAAGGCA	CCAGGCGGTGGGTCTCCGTA	190
MAPK-2	TAAAGCCATTGACATGTGGG	TCGTGCACAACAGGGATAGA	129
Stat-3	ACCCAACAGCCGCCGTAG	CAGACTGGTTGTTTCCATTCAGAT	193
GAPDH	AGACAGCCGCATCTTCTTGT	TGATGGCAACAATGTCCACT	142

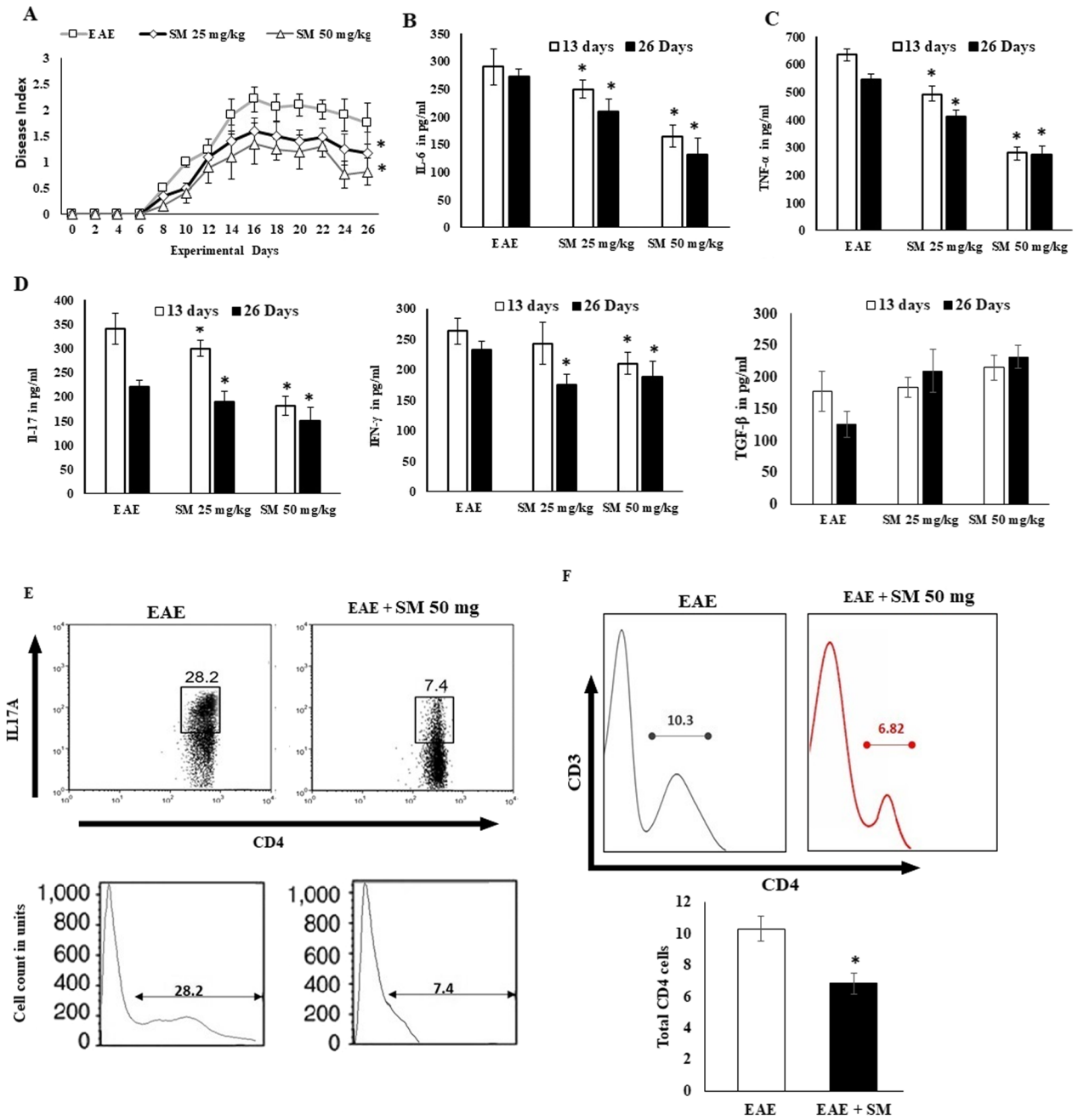

Fig. 1 SM ameliorates the experimental MS and regulates the inflammatory cytokines. **A** Clinical score of EAE after 26 days of MOG immunization and evaluated the experimental autoimmune symptoms. **B**–**D** Inflammatory cytokines such as IL-6, TNF-α, IFN-γ, IL-17, TGF-β were estimated in 13 and 26th days of MOG immunized mice and values are expressed in pg/ml. **E**, **F** SM regulates the total Th-17 population and CD4 population in EAE mice. The CD4 positive cells were counted using cell counter and it was prepared by miltenyi cell sorted system from the splenocytes. Total CD4 IFN-γ positive cells in SM treated EAE mice and EAE disease mice were evaluated using flow cytometry. The differences were considered significant when the P values were less than 0.05

caused a dose-dependent reduction in residual fore limb strength and showed under the different measurement of limb hanging strength values (Additional file 1: Fig. S1b). SM reduces of fore limb symptoms by a non-dose dependent fashion. The H&E staining of histopathological examination revealed that the demyelination and spinal recovery were reduced significantly (Additional file 1: Fig. S1c, d), where the histopathological alterations

decreased significantly in SM treated mice. The neuronal inflammation was reduced by SM dose of 50 mg/kg (Fig. 1b–d). The cytokine estimation in serum of EAE mice showed significant decrease at 26th days of immunization and its level was reduced in 50 mg/kg of SM concentration. The cytokines such as IL-6, TNF-α, IL-17, IFN-γ, and TGF-β were found to be significantly reduced in SM treated EAE mice at 13 and 26 days of tested intervals (Fig. 1b–d). Although cytokine markers were dysregulated, IFN-γ level did not show much changes using both concentrations at both intervals. The splenic lymphoid cells regulate the immune pathology in autoimmune diseases. Primarily, we examined the effect of SM treatment on T cell immunity and inflammation in mice. These T lymphoid cells were counted by flow cytometry gated with cumulative CD4 cell count in treated and naïve EAE mice (Fig. 1e). CD4 based IL-17 population lays a major role in autoimmune diseases. The CD4-IL-17 positive cell population was significantly decrease in SM treated group compared to naïve EAE mice. The significant reduction in the cell population lead an reduction of disease pathology and neuronal demyelination. Inflamed tissues expressed inflammatory cytokines for infiltration of lymphoid cells and neutrophils. However, the IFN-γ positive cells are drastically reduced in MOG stimulated lymphoid cells experiments (Fig. 1d). The results revealed that stimulating of splenic lymphoid cells (SPLC) using anti-CD3/CD28 resulted in polarized progression of TH1 and TC1 populations, as compared to naïve cells. SM treated (at dose of 25 μM of SM, Fig. 1e) EAE mice resulted in a significant decrease of TH1 population. However, the decrease in TC1 population was a dose dependent manner. The TH-17 specific population was significantly reduced in SM group compared to EAE group ($P \leq 0.042$) (Fig. 1e). The CD4 population was decreased in splenocytes of SM treated group compared to EAE group (Fig. 1f).

In addition, SM 25 μM potentiates the IFN-γ inhibition in stimulated lymphoid cells (Fig. 2e, f). These results, indicates that SM reduces the MOG reactive T-cells and it was co-related with IL-17-IFN-gamma cytokines estimations.

SM reduces the kinase mediated oxidative markers

Regulation of inflammatory cytokines leads positive cellular modifications and recover the oxidative mediated cell damage. The macrophagic markers showed in significant reduction in SM treated group and moreover, SM treatment significantly alter the pathology in macrophagic mediated response (Fig. 2a). The macrophage glial cells from naïve mice was stimulated with LPS showed increased secretion of IL-6 and TNF-α (Fig. 2a, b) whereas, SM treated LPS stimulated cells showed significant reduction ($P \leq 0.036$). In parallel, SM treatment aggregated the mononuclear cells and resulted in a reduction in infiltration of total mononuclear cells in inflamed tissues of neuronal regions. The total mononuclear cells from CSF of EAE and SM treated mice showed increased cell population ($P \leq 0.032$). This indicates the activation of lymphoid cells and cell polarization in the site of neuro inflammation was explored (Fig. 2c). Regulatory cytokines such as IL-17, IFN-γ and macrophagic infiltration marker GM-CSF were estimated in SM treated EAE mice (Fig. 2d, e). The CD4 cells from CD3/CD28 showed significant reduction of IL-17 from 315 to 3183 pg/ml concentration (Fig. 2d). The IFN-γ cytokines were altered significantly in SM treated group. The mRNA and protein markers of immune pathology in demyelination and SM bounded target of NFκB, MAPK6 and STAT3 signaling were altered significantly in SM treated group compared to naïve EAE mice (Fig. 2f, g). Except, MAPK6 markers, all the tested markers changed their regulation in SM treated group. This indicates that, SM alters the EAE pathology through NFκB binding (Fig. 2f, g). These results indicated that the neuroinflammation was positively controlled by reduction of these markers in brain CSF.

Effect of SM on ex vivo splenic lymphoid cells

The physical interaction between SM and NRF2 was confirmed by co-immunoprecipitation method (Fig. 3a). The splenocytes from naïve mice were treated with SM and results showed independent activation of KEAP1 protein as a benchmark in SM chemistry. The co-IP estimation showed SM (25 μM) treatment increases the ARE complex with NRF2 element in the nucleus and it was significantly decreased in control group (Fig. 3a). In addition, mRNA expression of NRF2 and ARE complex were overexpressed by the SM (25 μM) treatment (Fig. 3b). The effect of SM on oxidative NRF2 protein was potentially inhibited in MOG immunized mice (Fig. 3c). Moreover, the modification of NRF-2 in SM treated mice was analyzed by immunostaining of NRF2 markers in neuronal tissues. The results revealed that, SM treatment at both concentrations reduced the accumulation of NRF2, and that NRF2 engaged with ARE and made antioxidant responsive elements to attenuate the EAE pathogenesis (Fig. 3d). Moreover, the NRF2 binding triggers the ARE cascade of antioxidant gene clusters and reduced the disease in a dual role of inhibition of pathological markers as well as activation of ARE elements. These results showed that SM treatment reduced the oxidative damage in neuronal tissues and increase the recovery by prophylactic approach.

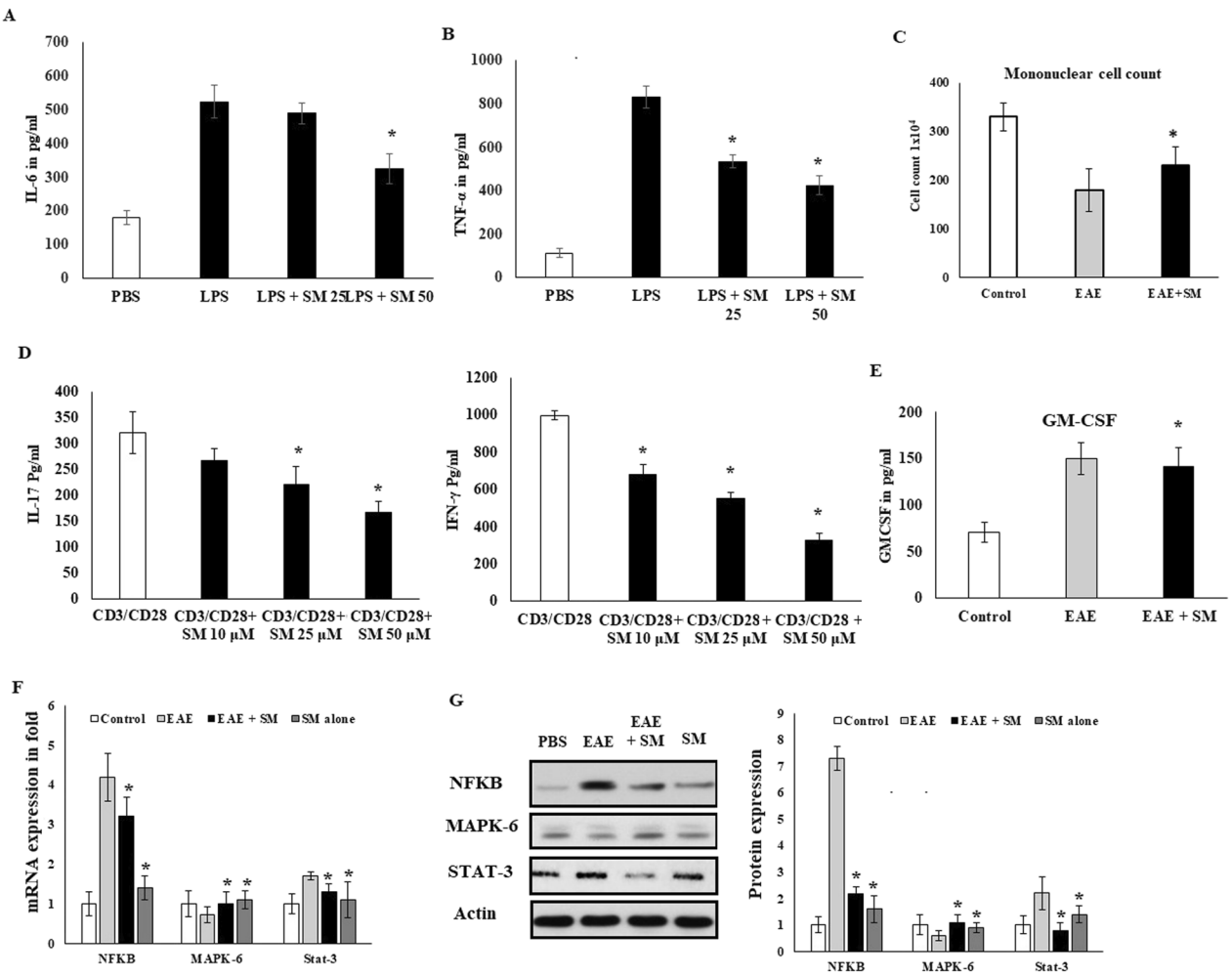

Fig. 2 SM reduced the induced inflammation in macrophages and CD4 cells. **A**, **B** SM regulates cytokines of neuroinflammation in EAE mice; IL-6, TNF-α from macrophage of glial cells. **C** Mononuclear cell count in CNS using hematological examination gives a total count of leukocytes in the EAE mice. **D** Cytokine estimation of IL-17, IFN-γ and IL-17 in CD3—CD28 activated CD4 cells were treated with SM in a concentration of 10, 25 and 50 µM. **E** GM-CSF cytokine were estimated in stimulated macrophages. CD3—CD28 activated CD4 cells were treated with SM in a concentration of 10, 25 and 50 µM. **F** mRNA expression of NFKB, MAPK6 and STAT3 were quantified using quantitative real time PCR and **G** immunoblot of NFKB, MAPK6 and STAT3 protein were estimated and β actin used as internal control. All data were expressed as mean ± SEM and the differences between variables were analyzed using student T test. The differences were considered significant when the P values were less than 0.05

Molecular docking of SM against NRF2 and Sirt-1

The 3D structure of SM is shown in Fig. 4a. The ligand receptor docking was done to determine the interaction between SM and NRF2 and between SM and SIRT1 is shown in Fig. 4b, c, respectively. Docking result showed that eight strong hydrogen bonds are formed (−7.5 kcal/mol binding energy) between SM and the active residues of NRF2 (Fig. 4b; Table 2). In addition, three hydrogen bonds were formed between SM and SIRT1 (Fig. 4c; Table 2). The non-covalent binding and the stable binding energy of SM to NRF2 and SIRT1 revealed that the SM may interact with both in vitro and in vivo.

SM downregulates neuro pathological markers and antioxidant mediators in EAE mice

T lymphocytes polarization and subtypes population plays a major role in autoimmune diseases. The above results revealed that, the CD4-IL-17 and CD8-IL-17 positive cell populations were comparatively reduced against naïve EAE mice. This was confirmed by estimation of oxidative stress and the oxidative markers. The lymphoid based oxidative regulation leads to reciprocal modifications in multiple sclerosis pathology. We measured the MDA (Fig. 5a) SOD/CAT (Fig. 5b), Glutathione synthase (GSH) (Fig. 5c) and neuroregulators ACHE/ACH (Fig. 5d) levels in spinal cord of EAE mice. In EAE mice,

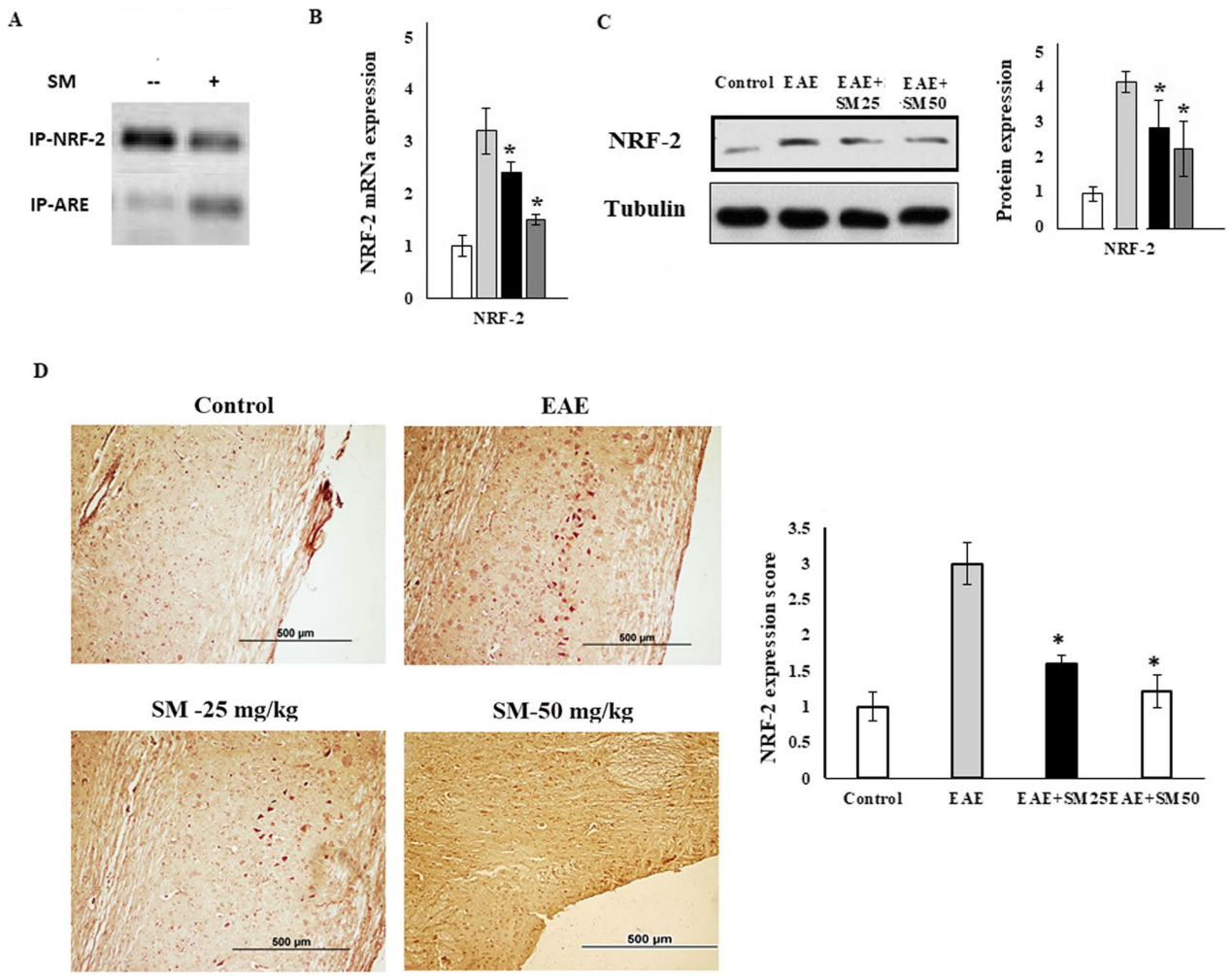

Fig. 3 SM is an agonist of NRF2 complex and CD4 mediators, **A**, **B** immunoprecipitation of NRF2 with ARE complex in SM treated CD3 activated CD4 cells were blotted using immuno blot. **B** mRNA expression of NRF2 was quantified using quantitative real time PCR and **C** SM modulates the nuclear oxidative protein and quantified using Immunoblot of NRF2 protein and Tubulin used as internal control. **D** Immunohistological score of NRF2 in SM treated EAE mice and naïve mice were evaluated and presented in NRF2 expression score. All data were expressed as mean ± SEM and the differences between variables were analyzed using student T test. The differences were considered significant when the P values were less than 0.05

the mean MDA level increased significantly compared to untreated and SM treated groups and SOD/CAT levels were decreased (Fig. 5a, b) significantly in SM treated EAE mice than naïve EAE mice ($P < 0.05$). In addition, SM reduced the oxidative stress by lowering the levels of SOD and CAT in either EAE-untreated or EAE-SM-treated groups than that of the healthy control group. However, we observed that SM treatment regulates the concentration of GSH and ACHE/ACH ratio in reciprocal behavior (Fig. 5c, d). The oxidative inducer activated nitric oxide synthase in inflamed tissues (Fig. 5e). The expression of iNOS in brain tissue was higher in EAE tissue, whereas the SM treated mice showed less iNOS expression (Fig. 5e, f). Meanwhile, SM treatment effectively prevented the elevation in oxidative stress and lipid peroxidation products. These results revealed that, SM abrogates the oxidative stress in neuronal cells through the inverse behavior on GSH/ACH axis markers.

Discussion

In the present study, molecular docking and CoIP data revealed a strong and stable binding affinity between SM and NRF2. The binding of SM to NRF2 enhances the later upstream action on ARE, which modulates the levels of the antioxidant agents and pro-inflammatory cytokines leading to reduction of the inflammatory response in

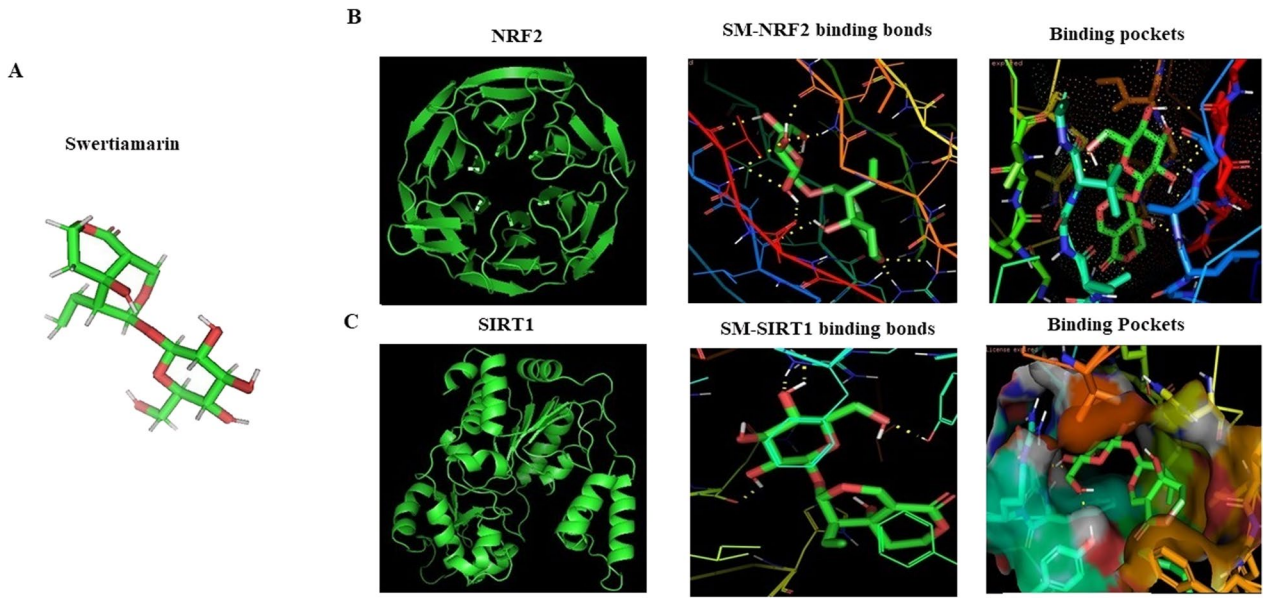

Fig. 4 SM is an agonist of NRF2 complex and CD4 mediators. **A** 3D structure of SM. **B** 3D structure of Mouse NRF2 and in-silico binding of SM with NRF2 and transparent binding pockets. **C** 3D structure of Mouse SIRT-1 and in-silico binding of SM with SIRT-1 and semitransparent binding pockets

Table 2 Insilico docking of ligand receptor interaction using autodock software

Sl. No.	Protein	Ligand	Binding energy	Ligand efficiency	Intermole energy	Ligand atoms (ring)	Docked amino acid residue (bond length)
1	NRF-2 (pdb id: 6TYM)	Swertiamarin	− 7.17	0.32	− 8.99	C-7′ O10 C-4′ H2 C-4′ H C-7 C-9 C-10 C-12 C-16	Glu Ser His Gln Lys-5 (1.8 A) Glu-290 (2.6 and 2.0 A) Lys-137 (2.1 A) Cys
2	SIRT1 (pdb id: 4ZZI)		− 5.22	0.83	− 3.52	C-3 C-7 C-9	His Cus Lys

EAE mice. That was associated with a decrease in the EAE paralysis symptoms at the pathological level, in EAE mice model to that of control group. Interestingly, the observed restoration of CNS-resident microglia in spinal cord of EAE mice could explain the amelioration of relapsing–remitting EAE and decreased CNS-inflammation after 20 days of disease induction [20].

Preclinical studies indicated that SM has a broad spectrum in treating many diseases including the autoimmune diseases [21, 22]. For instance, SM pre-treatment promoted recovery of ischemic injury in mice model in vivo through NRF2 nuclear translocation and formation of Keap1-NRF2 complex [7]. The Keap1–NRF2–ARE signaling system was found to regulate the transcription and subsequent expression of cellular cytoprotective proteins and plays a crucial role in preventing pathological conditions occur through the overproduction of oxidative stress [23]. Molecular virtual docking [6] confirmed the binding mode of Keap1 to SM. In the coding of these proteins, NRF2 can regulate response to a great diverse types of stress resulting in a stable internal environment. This protein has many cysteine those modified by electrophilic molecules blocking it. Then it cannot target NRF2 proteins in stressed conditions [24, 25]. Genes targeted by NRF2 have a versatile role in cytoprotection as anti-inflammation, detoxification and antioxidants therefore NRF2/KEAP1 system is the keystone for cell protection against several diseases in different organs including

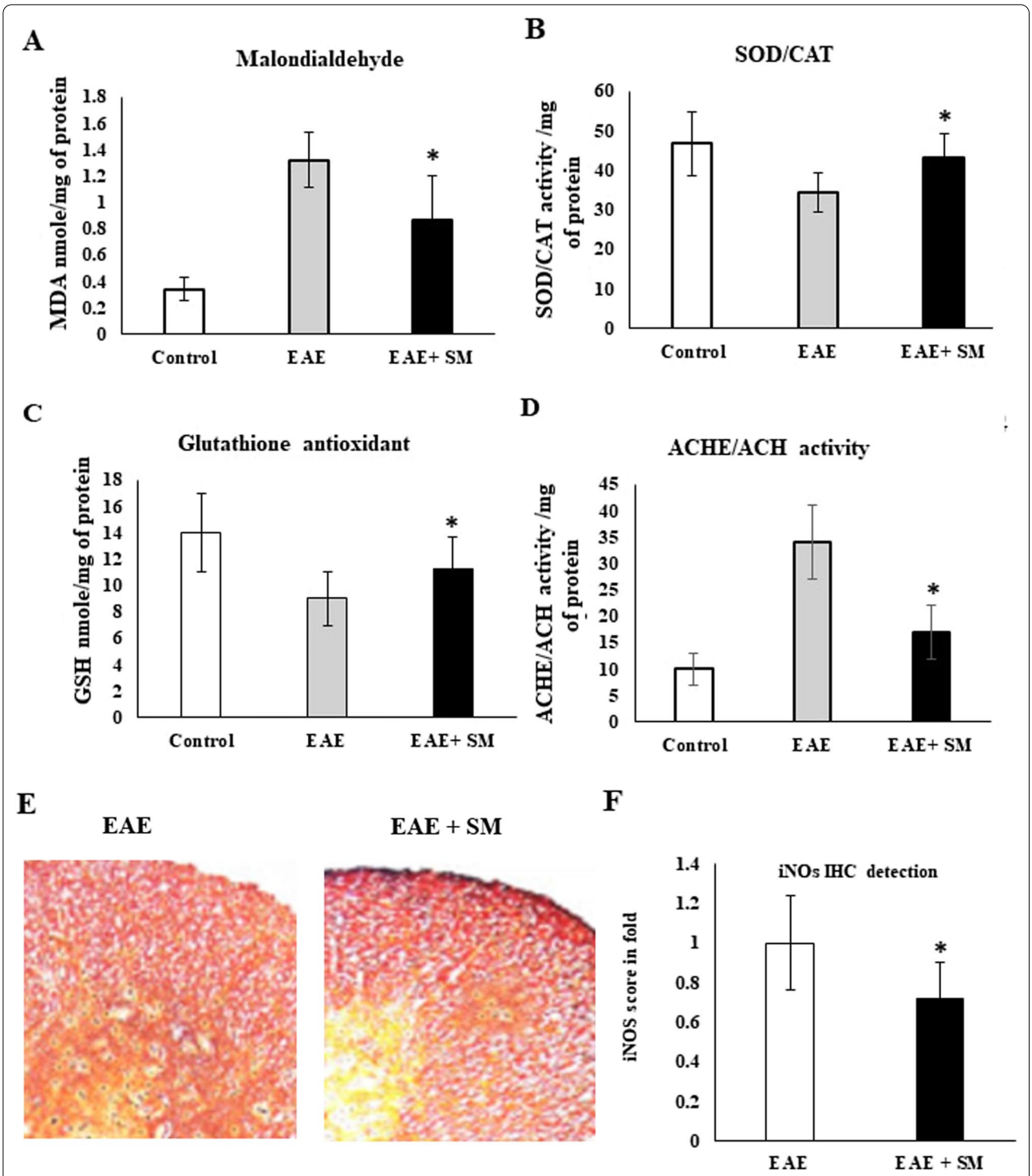

Fig. 5 Effect of SM on oxidative mediated neuropathological markers in EAE mice (**A**–**D**). Different markers were investigated in spinal tissue homogenates. **A** Malondialdehyde is an marker for inflammatory response as well as neutophile infilterations. This was raised in EAE mice compared with SM treated EAE mice. **B** SOD/CAT ratio was examined the primary oxidative stress of EAE and SM treated EAE mice. **C** Glutathione antioxidant was examined for the evaluation of total glutathione in the EAE mice and SM treated mice. **D** Acetylcholine esterase activity was evaluated the neuro communication interactions. It was secreted more in myelinated EAE disease conditions. **E**, **F** Immunohisto examination of iNOS in SM treated EAE mice and naïve EAE mice. All data were expressed as mean ± SEM and the differences between variables were analyzed using student T test. The differences were considered significant when the P values were less than 0.05

inflammatory autoimmune diseases [26] where as NRF2 trigger the regulatory region that encode proinflammatory mediators [8, 27]. Activation of NRF2 by pharmacological agents suppresses most of the proinflammatory cytokines and proteins [27–30]. Our data also indicate that SM significantly virtual binding with NRF2 protein and potentially modulates the increase in NFκB and STAT3 levels induced in EAE mice. In the same line, SM was reported to attenuates inflammation mediators via modulating NFκB/I-κB and JAK2/STAT3 transcription factors in adjuvant induced arthritis [2].

In the current EAE model, binding of NRF2 to SM lead to activation of both TH1 and TC1 population relative to control mice especially mice treated with a dose of 50 g/ml. The pro-inflammatory cytokines including IL-6, INF-γ, IL-17 and TNF-α significantly reduced in SM treated EAE mice which reflects the infiltration of TH-1 splenocytes and confirms the anti-inflammatory activity of SM. These proinflammatory cytokines supply oxygen and nutrients to infiltrate the inflammatory cells to the fluid resulting in enhancing inflammation [31, 32]. Consistent with that, SM was found earlier to ameliorate the inflammatory status of the cell and has a high potential as neurogenic, antioxidant, anti-nociceptive and TNF suppression [33–35].

The autoimmune diseases are controlled by pool of different cell types. Of these cells are CD4 based IL-17 population [35, 36]. Measuring CD4-IL-17 positive cells in the current study reveals that cell population is changed significantly by SM treatment. Reduction of CD4 positive cells in the SM treated mice confirms the amelioration effect of SM on the myelination of neuronal cells via CD4 dependent manner. Results of the current study revealed that a significant change occurs due to binding of SM to these markers. The oxidative stress in neuronal tissues bring diverse roles and activates the tissue damage and formation of free radicals induced neuro inflammation [37]. After MOG induced EAE mice investigated to MDA derived SOD and catalase were elevated and control the radicals emerged from inflamed tissue (Fig. 5a, b). SM actively controlled the generation of reactive oxygen species via suppression of MDA and SOD/CAT in EAE mice. Some earlier reports defined the MDA level was increased in MS tissue and confirming the nitric oxide in conjugation with other stress markers such as SOD, CAT and GSH. The cholinergic neurons are triggered through ACHE neurotransmitters [38, 39]. The demyelination leads by oxidative stress and activates the ROS in EAE mice, the acetylcholenergic level was potentially regulated to the accumulation of microglial and astrocytes in spinal tissue regions [40]. In this study, the SM treatment counteract with oxidative damage and regulates the secondary level of antioxidant GSH in EAE mice. Hereby, this study evidenced that, SM treatment was positively regulated the GSH expression and reduce the free neurotransmitters ratio (ACHE/ACH). The confirmation of EAE experiments suggest that SM actively regulates the ACHE increase the concentration of extracellular acetylcholine (ACH) and controls the induced nitric oxide level in EAE spinal cord tissue (Fig. 5e, f). Previous findings also supported that, ACHE inhibitors showed anti-inflammatory activity through inhibition of CD4 proliferation and inflammatory cytokines [41]. These experiments results point to SM treatment actively suppresses cholinergic to influence immunological reactions reflects the relations between the immune system and other mediators provide neuroprotection.

These results were confirmed by histopathological analysis, where tissues from EAE/SM treated mice showed a good tissue texture when compared with EAE mice where leucocytes infiltration was significantly lower at the former mice. This implies that, SM alters the EAE pathology through NFkB and NRF2 binding. Moreover, the NRF2 binding triggered the ARE cascade of antioxidant gene clusters and reduced the diseases symptoms via inhibiting the pathological markers and activating the ARE elements to prevent the neurological diseases [16, 17, 24].

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13765-022-00682-1.

Additional file 1: Figure S1. A. Body weight of SM treated and control mice were evaluated and presented in grams. B. Fore Limb strength was evaluated by hold and hanging method. Values are expressed in psi. C and D. Histopathological examination of SM treated EAE mice and control EAE and naïve mice were evaluated and presented in pathological score. All data were expressed as mean ± SEM and the differences between variables were analyzed using student T test. The differences were considered significant when the P values were less than 0.05.

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Authors' contributions
HIMI: conceptualization, funding acquisition, writing the discussion; AA: writing-original draft preparation, HH: conceptualization, methodology; EAA: methodology, formal analysis, writing-review and editing; AAS: methodology writing the discussion, writing-original draft preparation. All authors read and approved the final manuscript.

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Availability of data and materials
All available datasets will provide based on their request to corresponding author.

Declarations

Ethics approval and consent to participate
The animal experiments were conducted in accordance with the protocol approved by the Standing Research Ethics Committee, (KFU-EA-DSR-123) King Faisal University, Saudi Arabia. Informed consent given for drug administration, tissue collection/use of data or samples, and/or publication, as applicable.

Consent for publication
Not applicable to this manuscript.

Competing interests
The all authors declared that they have no competing interests.

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