Chapter 2

Evaluation of the immunological mechanism of dimethyl fumarate in the EAE model of multiple sclerosis

2 Immunological mechanism of dimethyl fumarate in EAE model of multiple sclerosis.

2.1 Introduction

Multiple sclerosis (MS) is a chronic inflammatory, demyelinating, chronic neurological disease of autoimmune origin, affecting mostly young adults. The hallmarks include the dissemination of demyelinated lesions throughout the white matter of the central nervous system (CNS) visualized histopathologically in the form of inflammation, demyelination, and gliosis [77]. The exact pathogenesis of MS is not well known, it is categorized as an autoimmune disease of CNS with hyperactivation of immune cells majorly T lymphocytes-mediated inflammation along with macrophages, antibody-mediated destruction of myelin sheaths and oligodendrocyte unit [78]. The CD4+ and CD8+ T lymphocytes present in demyelinating lesions are known to mediate demyelination and axonal damage. CD8+ T lymphocytes present in a higher proportion in CNS of MS patients, suggesting their selective infiltration. Moreover, an oligoclonal expansion of CD8+ T cells has been observed in the blood, CSF, and brain of MS patients [79]. CD8+T cells reactive to myelin components have also been found in the MS patients' blood [80]. CD8+ T cells may exert neuronal damage by directly inducing apoptosis to the neurons and the supporting glial cells causing demyelination and MS-related symptoms [81]. Targeting CD8+ by monoclonal antibody provided treatment and prevention against autoimmune diseases like rheumatoid arthritis and glomerulonephritis. Targeting CD8+ cells via monoclonal antibodies would certainly remain a vital element in the management of MS [82]. However, monoclonal antibodies cause a permanent inhibition of the target protein and therefore carry the risk of causing immune hyper-activation reactions like the generation of antibodies, acute anaphylaxis, and serum

sickness [83]. Enzymes are preferable drug targets and play an important role in drug discovery [84]. There are critical enzymes like cathepsins, granzymes and perforins in the immune cells which play pivotal roles in the apoptosis and pathogenesis of many diseases [85].

Granzyme B (GrB) mediated apoptosis is a critical pathway utilized by cytotoxic lymphocytes in the MS and experimental autoimmune encephalitis (EAE) [86]. GrB is a serine protease which is considered one of the most potent proapoptotic cytotoxins contained as a granule exocytosis in cytotoxic lymphocytes like CD8+ T cells, and NK cells [87]. GrB is biosynthesized as an inactive zymogen and it gets activated as a cytotoxic-granules by the action of lysosomal cysteine protease called dipeptidyl peptidase I or cathepsin C [88]. Cathepsin C is highly expressed in cytotoxic lymphocytes as cytotoxic granules with an acidic pH of 6 [89]. It has been recently reported that disinhibition of Cathepsin C aggravated demyelination in the EAE model [90]. Moreover, cathepsin C has been found in the demyelinating lesions in the CNS and plays a critical role in exacerbating the symptoms in the myelin oligodendrocyte protein $(MOG)_{35-55}$ induced EAE model [91].

In EAE, myelin antigen-reactive CD8+ T cells have been shown to induce MS-like symptoms in the C57BL/6 mice model [92]. MOG_{35-55}, the most common EAE model, induces CNS autoimmunity and increases disease onset and severity with the addition of pertussis toxin [93]. This model provides insight into the role of the mononuclear cells-CD8+, CD4+ T lymphocytes, B lymphocytes and monocytes in the pathogenesis of MS which can be counted with immunophenotyping using fluorescence assorted cell sorting (FACS) [94].

DMF is an FDA-approved drug for the treatment of MS due to its immunomodulating and neuroprotective properties. The concise mechanism of action of DMF is still not known however, DMF influences intracellular glutathione (GSH) levels by binding to its thiol unit [95]. Another reported mechanism includes activation of Nrf2 by DMF thus regulating cellular antioxidant responses and stimulation of cytoprotective and anti-inflammatory factors such as heme oxygenase-1 and NFκB [96]. CD8+ plays a crucial role in the pathophysiology of MS, but the mechanism of DMF on CD8+ has not been explored. Moreover, DMF has a crucial impact on the effector cells including microglia, dendritic cells, macrophages, astrocytes and neurons [97].

S-(2-succinyl) cysteine (2SC) is a chemical modification of proteins formed by a Michael addition reaction (**Figure 1-1**) between the Krebs cycle intermediate, fumarate, and thiol groups in a protein called as succination of protein which generally causes inactivation of the enzyme [75]. Interestingly, cathepsin C also contains cysteine (Cys-234) at its active site [76]. For standardization of covalent inhibitors, IC_{50} is not a much useful indicator rather k_{inact} and K_i are preferred tools against IC_{50} values for comparing covalent inhibitors against a particular drug target [98].

The exact immunomodulatory mechanism of DMF remains elusive, the objective of the study is to decipher the mechanism of action of DMF. Cathepsin C is an important protein target involved in neuronal apoptosis and demyelination. In this study, we have evaluated the *in-vitro* enzyme kinetics of cathepsin C with DMF and MMF (an active metabolite of DMF) and the *in-vivo* activity of DMF/MMF on cathepsin C and its downstream effector protein-granzyme B in the EAE model of MS. EAE model has been validated by clinical scoring,

demyelination of mice spinal cord using luxol fast blue staining and FACS analysis of CD8+ and CD4+ T lymphocytes isolated from mice CNS.

2.2 Materials and methods

2.2.1 Chemicals

MOG_{35-55}, from Genscript, CFA (complete Freund's adjuvant), Fingolimod standard drug, Pertussis toxin and Luxol fast blue stain from Sigma Aldrich, recombinant Mouse Active Cathepsin C (R& D systems, USA), Gly-Phe β-naphthylamide substrate (Santa cruz biotechnology), DTT MES buffer (Sigma Aldrich), Leica cryotome, OCT fluid Leica. For flow cytometric analysis, the following antibodies purchased from Thermo Fisher Scientific (USA) were used: rat anti-mouse CD3 FITC (Clone 17A2, Cat no. 11-0032-82), rat anti-mouse CD4 PerCP-Cy 5.5 (Clone RM4-5, Cat no. 45-0042-82), rat anti-mouse CD8a PE (Clone 53–6.7, Cat no. 12-0081-81). All other chemicals and reagents of high-performance liquid chromatography (HPLC) and analytical grade were procured from local suppliers.

2.2.2 *In-vitro* experiment

The enzyme assay was performed following [76] with certain modifications. The assay was carried out in black well plates in a final volume of 110 µL at 22°C. The assay conditions contained the following: MES buffer with pH 5.5, 50 mM NaCl, 5 mM DTT, 0.01% (v/v) Triton X100, recombinant mouse active cathepsin C (~50 pM). DMF and MMF were prepared in DMSO and then diluted in the assay buffer to give a final concentration not exceeding 1% (v/v) DMSO, DMF and MMF were preincubated with cathepsin C for 60 min prior to the addition of the 200 µM Gly-Phe β-naphthylamide substrate (Santa cruz biotechnology) prepared in the same assay buffer to start the reaction for a further 60 min at

22°C. Afterwards, the plates were read in a fluorescence plate reader using the fluorescence intensity at λ_{ex} = 350 nm and λ_{em} = 450 nm.

2.2.3 Time-dependent/ Irreversible enzyme kinetics and calculation of IC_{50}

The irreversible enzyme inhibition assay was performed based on the discussed method with some optimization [99]. DMF was preincubated with recombinant mouse active cathepsin C (R& D systems, USA) activated cathepsin c enzyme at concentrations of 1 µm, 10 µm, 25 µm, 50 µm and 100 µm for a period of 60 minutes, 120 minutes and 180 minutes respectively. A fluorescent reaction was initiated by the addition of 200 µM Gly-Phe β-naphthylamide to the black well plate. It was incubated for the last 30 minutes of the total reaction period. The plates were immediately read in a fluorescence plate reader using the fluorescence intensity at λ_{ex} = 350 nm and λ_{em} = 450 nm. The enzyme inhibition kinetics was analysed and various parameters were calculated.

2.2.4 Reversibility inhibition studies

For reversibility inhibition studies, the protocol was adopted from the literature with certain modifications [64, 100]. DMF/MMF was incubated with the Recombinant Mouse Active Cathepsin C (R& D systems, USA) activated cathepsin c enzyme at concentrations of 10 × K_i and 100 × K_i at 22°C for 60 min. A control group with no inhibitor was also taken. After a 60 min incubation period, the samples were subsequently diluted to 100-fold with the addition of 20 mM Gly-Phe β-naphthylamide substrate (Santa cruz biotechnology) to achieve final inhibitor concentrations of 0.1 x K_i and 1 x K_i value, respectively.

2.2.5 Experimental animals and drug treatment

Female C57BL/6 mice (6-8 weeks old) were procured from a laboratory animal facility, CDRI Lucknow. The experiments were performed by adopting guidelines (NIH publication number 85-23, revised 2011) and approved by the Institutional Animal Ethical Committee, Banaras Hindu University (BHU; Dean/2015/CAEC/1420). Animals were acclimatized for one week in the experimental lab before the experiments. The complete experimental protocol is illustrated in Figure 2.1.

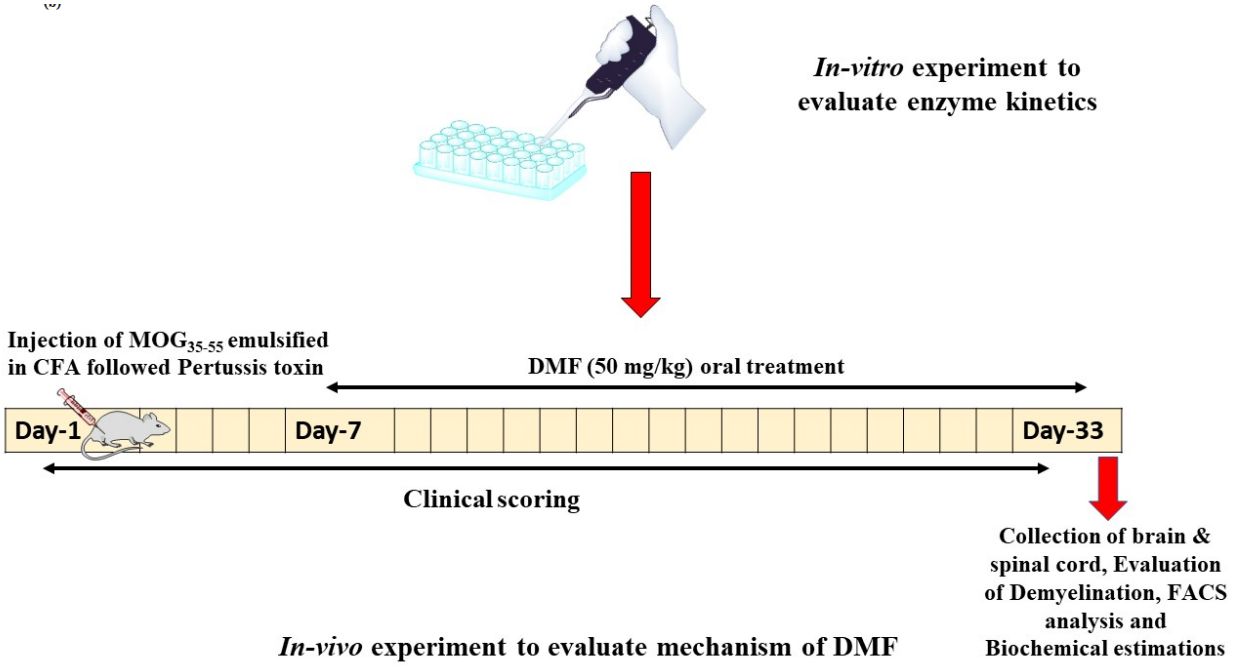

Figure 2-1 Experimental design

2.2.6 Preparation of MOG_{35-55} emulsion in CFA

It was prepared following [30] with slight modifications. A sufficient amount of MOG_{35-55} was taken and dissolved in double distilled water to make the concentration of 2 mg/ml taken in a plastic tube. To this, CFA containing *Mycobacterium tuberculosis* was added in a dropwise manner to bring the final concentration of 1 mg/ml and it was kept under

continuous vertexing. Once the color of the solution turned milky white, it was transferred to a 5 ml glass syringe with the help of a 18 gauge metallic syringe bridge by connecting it to another 5 ml glass syringe. It was further emulsified by pushing the syringes against each other for a few minutes until a homogenate emulsion was obtained, which was kept at 4°C for 12 hr, for any instability.

2.2.7 Preparation of Pertussis Toxin

Pertussis toxin is an essential adjuvant as it induces EAE by opening up the blood-brain barrier and thereby intensifies the migration of immune cells into the CNS [101]. It was prepared by reconstituting 50 µg of pertussis toxin in 500 µl double distilled water for a 100 µg/ml stock solution. It was stored at 4°C. It was diluted 1:50 using phosphate buffer solution to get the working solution of 400 ng in 200 µl PBS.

2.2.8 EAE induction

The experiment was performed following [30] with slight modifications as per the design shown in **Figure 2-1** The mice were grouped, taking nine mice in each group, namely control, EAE, and EAE + DMF (50 mg/kg**).** The mice were anaesthetized the mice using *intraperitoneal* injection of ketamine 100 mg/kg and xylazine 10 mg/kg. 100 µl CFA emulsion containing 100 µg of MOG_{35-55}, was injected subcutaneously on each hind flank of the mice. It was ensured that a bulbous mass was formed under the skin which persisted throughout the experiment. Two doses each containing 200 µl of pertussis toxin, one concurrently and another after 48 hr were injected intraperitoneally into the mice. CFA emulsified with saline alone was injected in the same manner as the control group. Clinical

scoring was done from day-1 onwards till day-33 as per the following clinical scoring pattern [102].

0. No clinical signs;

1. Affected tail tonus

2. Paresis of hind legs

3. Complete paralysis of the hind legs

4. Complete hind leg paralysis and foreleg paresis

5. Death or moribund

2.2.9 Histopathology to assess demyelination:

The spinal cord was isolated from the mice using the hydraulic extrusion method following [103]. Mice were decapitated and the spinal column was trimmed at both ends to ensure the spinal cord was visible at both ends. The spinal column was straightened and extruded by hydraulic pressure using a syringe fitted with a pipette tip. The spinal cord was kept in formalin for 24 hr and later treated with an incremental concentration of sucrose solution of 10, 20 and 30% respectively for 24 hr in each concentration before cryosectioning. The blocks of the brain were prepared and sections of the spinal cord were cut at a thickness of 10 µm on a freezing microtome (Leica Microsystems GmbH, Wetzlar, Germany). It was kept in -80°C till further processing. It was sectioned 10 microns thick at the lumbosacral region using a cryotome and a glass slide was prepared which is stained with luxol fast blue (LFB) which differentiates between myelination and demyelination. Four intact cross-sections from each mouse (four to five mice per group) were photographed on an Olympus BX40 microscope. Normally myelinated areas and areas of demyelination in the dorsal region were measured on each section and the percent demyelination was calculated [104].

2.2.10 Mononuclear cell isolation and preparation:

The mononuclear cells were isolated from the brains and spinal cords of anaesthetized mice and perfused with ice-cold saline. The brain and spinal cord were removed by dissecting them and a single-cell suspension in ice-cold RPMI 1640 containing 1% foetal calf serum (FCS) was prepared using a glass homogeniser. The cell suspension was layered over isotonic Ficoll (Ficoll: Hank's balanced salt solution 9:1) in 50 ml centrifuge tubes and centrifuged for 25 min at 640×g at 4°C. The supernatant layer was discarded and the cell pellet was retained. The cell pellet was resuspended, transferred to a conical 10 ml centrifuge tube, underlaid with 1 ml Ficoll and centrifuged for 20 min at 600×g at 4°C. The mononuclear cells were harvested from the interface and counted taking 10 µl aliquots of the sample were mixed with 10 µl of Trypan blue (0.4%) (Invitrogen, Italy). It was put into a Countess™ cell counting chamber slides and the count was done in Countess® Automated cell counter (Invitrogen, Italy) [105].

2.2.11 FACS analysis

The mononuclear cells obtained were resuspended in the FACS buffer. After cells were counted, $1x10^6$ cells per sample were stained with the following antibodies (purchased from Thermo Fisher Scientific) rat anti-mouse CD3 FITC (Clone 17A2, Cat no. 11-0032-82), rat anti-mouse CD4 PerCP-Cy 5.5 (Clone RM4-5, Cat no. 45-0042-82), rat anti-mouse CD8a PE (Clone 53-6.7, Cat no. 12-0081 81) as per manufacturer's instructions. The cells were then incubated in anti-mouse blocking solution Fc Block (eBiosciences, San Diego, CA) for 30 minutes at room temperature. After staining, cells were washed and fixed with 0.4% paraformaldehyde in PBS. The samples were analysed using the BD LSRII flow cytometer

and the data was acquired using BD FACS Diva software (BD Bioscience). Compensation was performed on the BD LSRII flow cytometer at the beginning of each experiment [106].

2.2.12 Cathepsin C Activity

Cathepsin C activity was assayed by the hydrolysis of gly-phe β-naphthylamide which is a specific substrate for cathepsin C activity. 200 uM gly-phe β-naphthylamide was added to the brain and spinal cord homogenate and incubated at 37°C and pH 5.5 (120 mM NaCl, 4 mM EDTA, 2 mM DTT) for 30 min. 500 µl of stop buffer (50 mM glycine-NaOH, pH 10.4) was added to cease the incubation. Fluorescence was measured at excitation and emission λ of 335 nm and 405 nm respectively [107].

2.2.13 Granzyme B activity

Following the procedure [108], 10 mM stock of the substrate N-acetyl-Ile-Glu-Pro-Asp-pNA or Ac-IEPD-pNA cat. No. 27139 procured from Cayman Chemicals (USA) was prepared in DMSO (~5 mg/ml) and stored as aliquots at -20°C. 100 µg protein lysate of each brain and spinal cord homogenate was diluted in the buffer to a final volume of 150 µl. 50 µl of the solution was transferred to each well in triplicate (i.e.~50 µg/well). 50 µl of diluted substrates (200 uM) to each set of triplicates was added using a multichannel pipette. The optical density of the resulting mixture was determined at 405 nm.

2.2.14 Statistical Analysis

All the values are expressed as the mean ± standard deviation (SD). One-way ANOVA followed by post-hoc student's Newman–Keuls test was performed for the analysis of all the data. Graph Pad Prism version 5 (San Diego, CA). Groups with $p < 0.05$ were considered

significantly different. Flowjo_V10 was used for FACS data analysis and image analysis and processing.

2.3 Results

2.3.1 Time-dependent and irreversible enzyme kinetics and calculation of IC_{50}

Irreversible or covalent inhibitors cannot be ranked in terms of effectiveness using an equilibrium dissociation constant, K_d, or K_i making IC_{50} irrelevant. Therefore, the parameters including k_{inact} and K_i, which are calculated from time-dependent experiments with varying DMF/MMF concentrations, where the rate constant, K_{obs}, represents the conversion from the initial complex to an irreversible covalent complex. Here, k_{inact} is the rate constant for inactivation of the enzyme whereas the K_i term describes the concentration of inhibitor required for one-half of the maximum rate of covalent bond formation. K_{inact} independently represents the maximum observed rate constant for inactivation at saturation of the enzyme [109]. These kinetic parameters (k_{inact} and K_i) can be derived from the rate of enzymatic activity decay observed at each concentration of inhibitor by using the Kitz-Wilson plot which is a double reciprocal plot of k_{obs} against DMF/MMF concentrations (**Figure 2-2 to Figure 2-5**). K_{obs} can be obtained from the loss of 50% of the enzymatic activity of the different concentrations of the DMF/MMF [110].

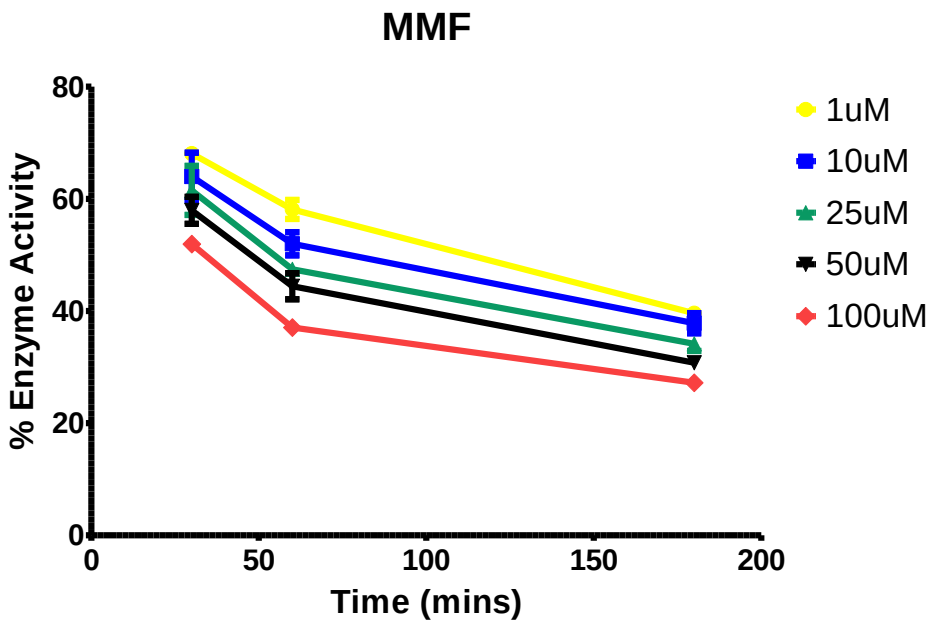

Figure 2-2 Percentage enzyme activity of cathepsin C with different concentrations of MMF

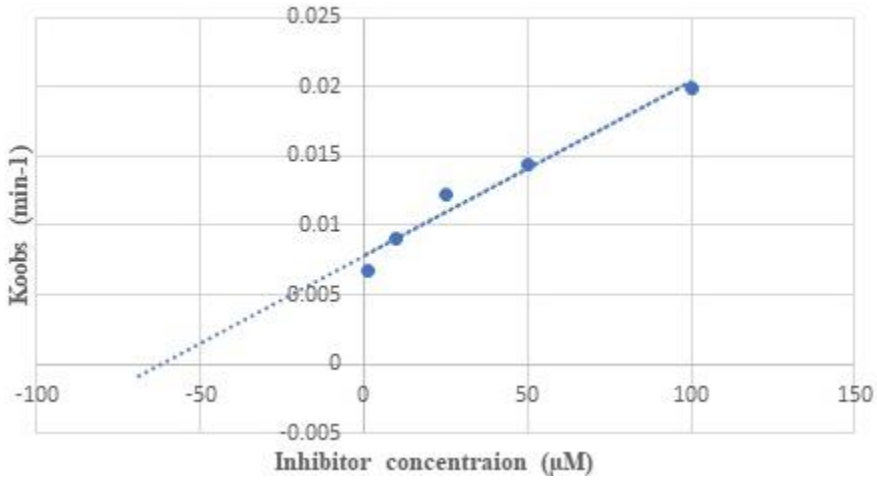

Figure 2-3 Kitz-Wilson Plot for MMF with cathepsin C

DMF gets rapidly metabolized by esterases into its active metabolite known as monomethyl fumarate (MMF) [111]. Therefore, it becomes important to evaluate the enzyme kinetics of cathepsin C with both DMF and MMF. The *in-vitro* enzyme kinetics was performed and K_{inact} for MMF was found to be 0.0078 min-1. and Ki was 58 µM moreover, K_{inact} for DMF

was found to be 0.0046 min-1 and K_i was found to be 40 µM. **Figure 2.2** to **Figure 2.5** and the obtained parameters proved that DMF and MMF caused time-dependent loss of enzyme activity of cathepsin C by forming Micheal adduct at the active site. In many previous instances, K_i and K_{inact} have been used as important parameters for characterizing the inhibitory potential of drugs with time-dependent or covalent enzyme inhibition [109]. There are drugs like mitomycin C which shows time-dependent and irreversible type of enzyme inhibition kinetics [112].

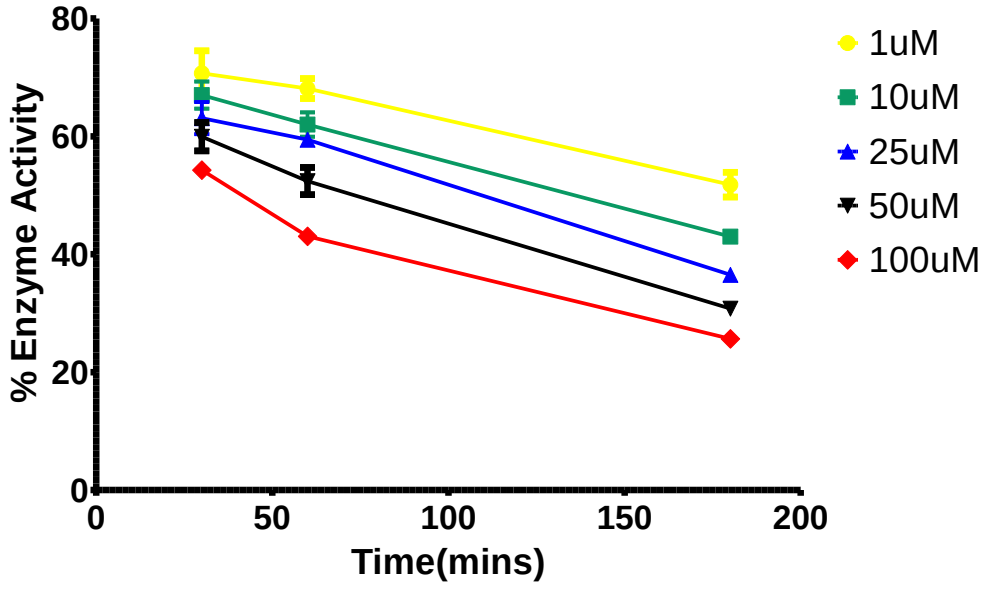

Figure 2-4 Percentage enzyme activity of cathepsin C with different concentrations of DMF.

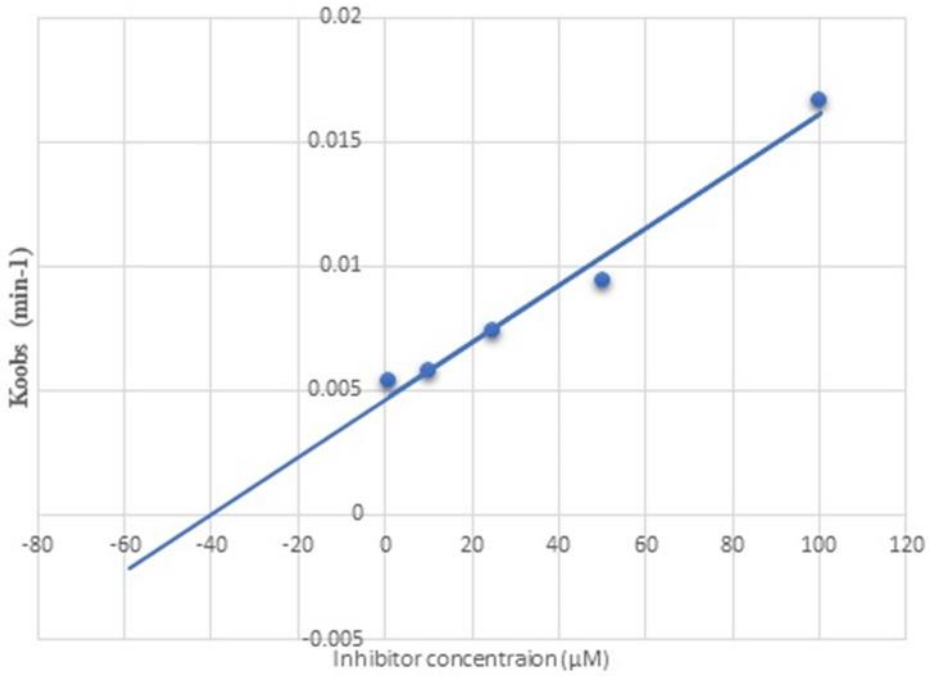

Figure 2-5 Kitz-Wilson Plot for DMF with cathepsin C.

2.3.2 Reversibility inhibition studies of DMF and MMF with cathepsin C enzyme:

One of the crucial criteria for an inhibitor to be regarded as an irreversible inactivator, its activity should not be reversed upon the addition of substrate into the inhibitor-enzyme system [113]. It was evaluated by incubation of cathepsin C with DMF and MMF at concentrations of 10 x K_i and 100 x K_i, (in order to saturate the enzymes completely excess concentrations of DMF and MMF were taken) the enzyme activity was reduced tremendously. However, even upon 100 times dilution of the drug-enzyme solution with the substrate solution, the recovery of enzyme activity was not significant as shown in the respective graphs shown in the **Figures 2-6** and **2-7** below, indicating that the enzyme inhibition is irreversible [100].

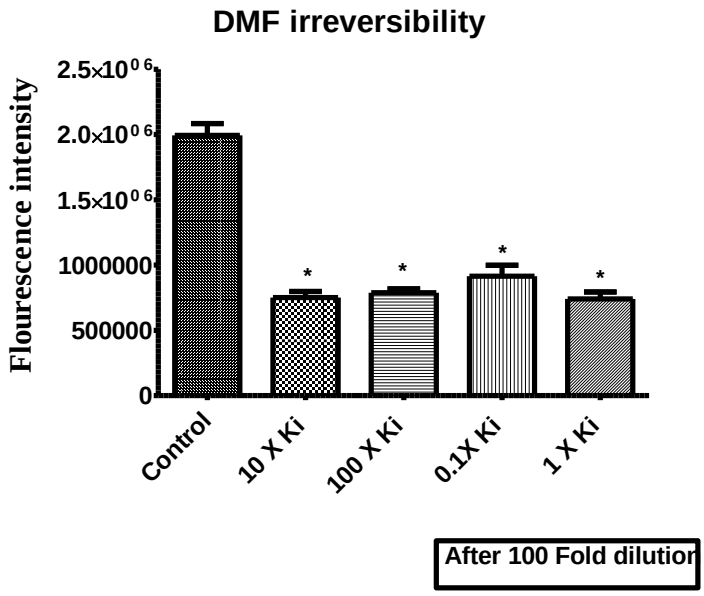

Figure 2-6 Reversibility inhibition studies of DMF with cathepsin C.

*All values are mean ± SD; n = 6; *p< 0.05 compared to control [One-way ANOVA followed by Student Newman-Keuls Post-hoc test].*

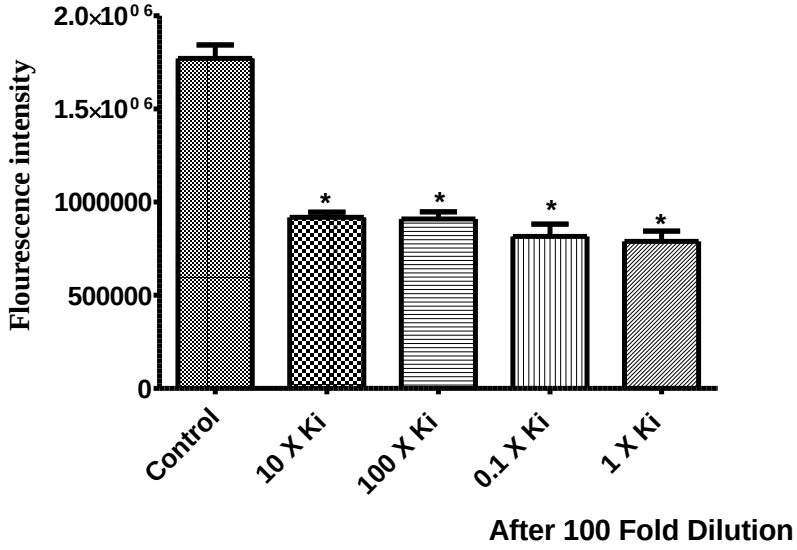

Figure 2-7 Reversibility inhibition studies of MMF with cathepsin C

*All values are mean ± SD; n = 6; *p< 0.05 compared to control [One-way ANOVA followed by Student Newman-Keuls Post-hoc test].*

The mechanism that we propose for the inhibition of cathepsin C by DMF/MMF starts by a conjugate addition of an electron from the cysteine (Cys-234) to the DMF to form the covalent complex (a Michael adduct). DMF is known to covalently modify the active cysteine residues causing an interaction defined as succination. In a study, DMF has succinated and inactivated catalytic cysteine in a cellular target glyceraldehyde 3-phosphate dehydrogenase (GAPDH) in both *in-vitro* and *in-vivo* conditions [64].

2.3.3 Effect of DMF on clinical scoring in MOG_{35-55} induced EAE mice

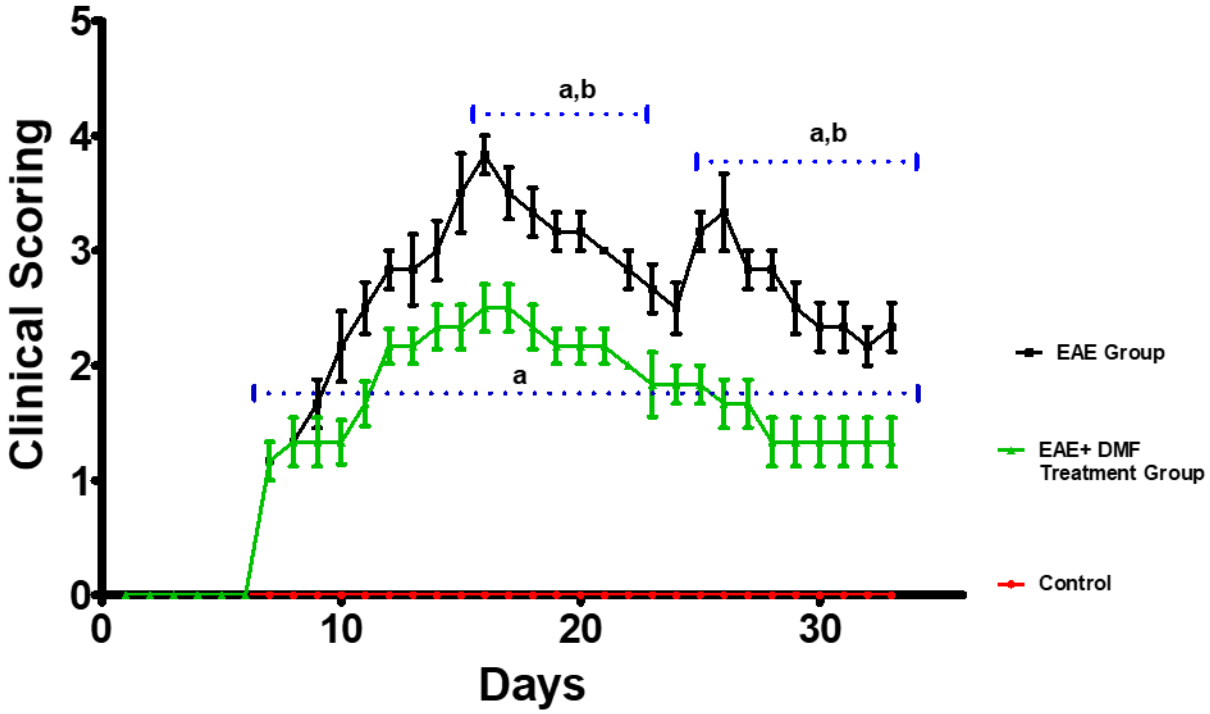

Figure 2-8 Clinical scoring of the control group, EAE group and EAE+DMF group.
*All values are mean ± SD; n = 9; *p< 0.05 compared to control. [Two-way ANOVA followed by Bonferroni Post-hoc test].*

One-way ANOVA revealed significant differences between control and EAE and EAE+DMF groups from day-7 up to day-33 [F (2, 6) = 34.02, p<0.05]. Also, we have found significant

differences between the EAE+DMF and EAE groups from day-15 up to the end of the study (day-33) as shown in **Figure 2-8.** The post-hoc test showed that the MOG_{35-55} injection significantly increased the clinical scoring which corresponds to the induction of disease in the mice. The induction of EAE in C57BL/6 mice by active immunization with MOG_{35-55} peptide results in the induction of multiple sclerosis-like symptoms by causing CNS inflammation and demyelination [114]. In a previous study, DMF significantly improved the symptoms and clinical scoring of EAE in the MOG_{35-55} mice model [115]. These effects of DMF may be through the activation of Nrf2-dependent and independent pathways [96].

2.3.4 Effect of DMF on the migration of CD4+ and CD8+ T cells migration into CNS of MOG_{35-55} induced EAE mice

The mononuclear cells from the mice CNS (brain and spinal cord) were isolated and counted using FACS. However, the number of mononuclear cells in the CNS of the control group mice was not sufficient enough to be detected by FACS therefore EAE group was compared with the EAE+Treatment group. **Figure 2-9** shows the FACS analysis of CNS infiltrating mononuclear cells in mice induced with EAE for 33-days.

As shown in **Figure 2-10**, one-way ANOVA revealed significant differences in CD8+ cells between control and EAE and EAE+DMF groups [$F(2, 32) = 1812$, $p<0.05$]. The post-hoc test showed that the MOG_{35-55} injection significantly increased the migration of CD4+ and CD8+ cells in the CNS which corresponds to the induction of disease in the mice. MOG_{35-55} induction causes an increase in the CD4+ and CD8+ T cells in the EAE model and their increase is associated with disease development and progression [116]. DMF is known to bring a reduction in the absolute count of T lymphocytes [117]. The exact mechanism

involved in the reduction of T lymphocytes count is still unclear but there is a recent study which supports the inactivation of GAPDH in the glycolysis pathway by succination by DMF which ultimately brings a shut to the energy demands of immune cells [64].

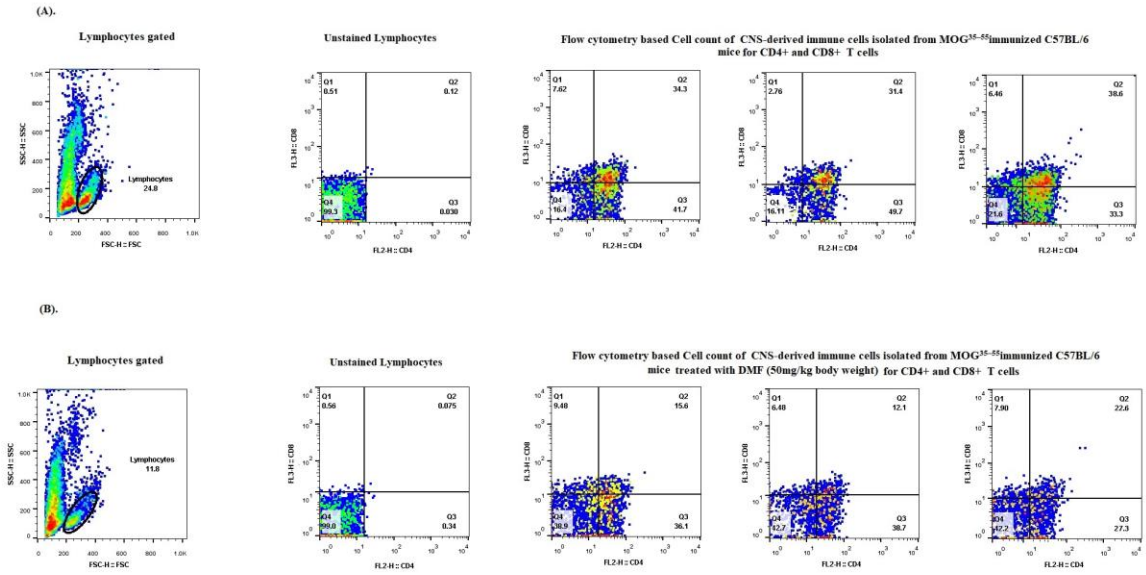

Figure 2-9 Flow cytometry data of CNS-derived immune cells isolated from MOG$_{35–55}$ immunized C57BL/6 mice.

(A) Percentage of CD4+ T cells (shown in the Q3 quadrant) and CD8+ T cells (shown in the Q2 quadrant) in the CNS of mice from the EAE groups. (B) Percentage of CD4+ T cells (shown in the Q3 quadrant) and CD8+ T cells (shown in the Q2 quadrant) in the CNS of mice from the EAE+DMF (50mg/kg) treatment group (3 panels representing the sample size of 3).

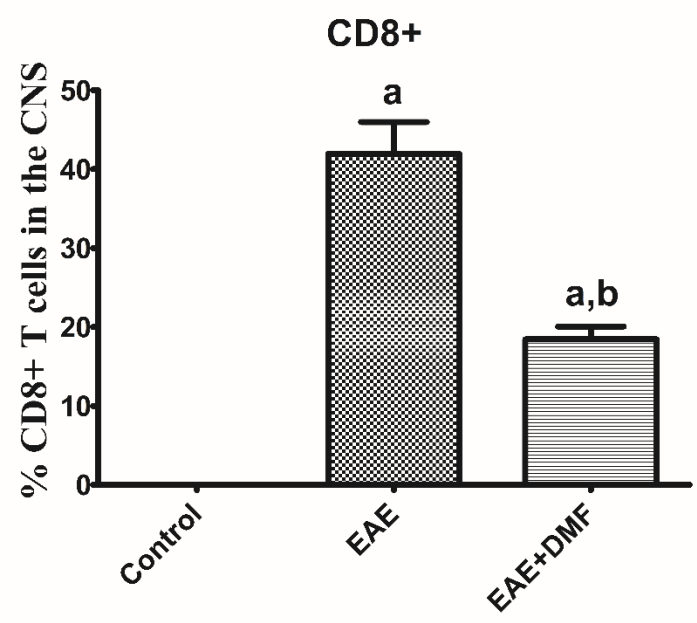

Figure 2-10 % CD8+ T cells count in the CNS.

All values are mean ± SD; n = 3; [a]p< 0.05 compared to control, [b]p< 0.05 compared to EAE. [One-way ANOVA followed by Tukey Post-hoc test].

One-way ANOVA revealed significant differences in CD4+ cells between control and EAE and EAE+DMF groups [F (2, 32) = 1812, p<0.05] as in the **Figure 2-11**. The post-hoc test showed that the MOG_{35-55} injection significantly increased the CD4+ cells migration in the CNS which corresponds to the induction of disease in the mice.

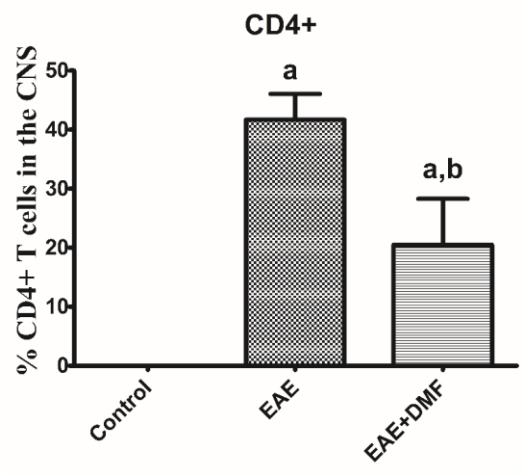

Figure 2-11 % CD4+ T cells count in the CNS.

All values are mean ± SD; n = 3; [a]p< 0.05 compared to control, [b]p< 0.05 compared to EAE. [One-way ANOVA followed by Tukey Post-hoc test].

2.3.5 Effect of DMF on cathepsin C activity in MOG_{35-55} induced EAE mice

With reference to **Figure 2-12**, one-way ANOVA revealed significant differences between control and EAE and EAE+DMF groups from day-7 up to day-33 [F (2, 12) = 73.47, p<0.05]. Also, there is a significant difference between EAE+DMF and EAE group. The post-hoc test showed that the MOG_{35-55} injection significantly increased the Cathepsin C activity, corresponding to the disease progression in the mice. Cathepsin C has been found to be expressed in the demyelinating CNS in the MOG_{35-55}-induced EAE model [91]. Upregulation of cathepsin C leads to aggravation of the demyelination process by increasing the expression of chemokines, in the myelin sheath in the CNS [90].

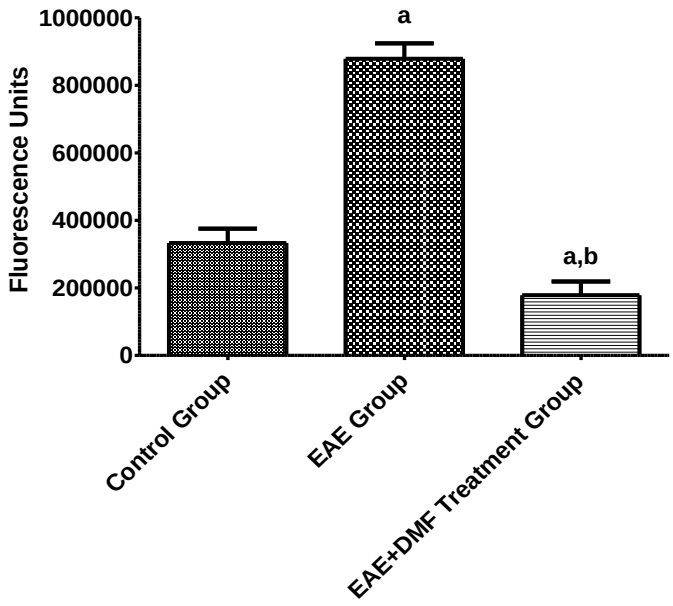

Figure 2-12 Cathepsin C activity.

All values are mean ± SD; n = 3; [a]p< 0.05 compared to control, [b]p< 0.05 compared to EAE. [One-way ANOVA followed by Tukey Post-hoc test].

2.3.6 Effect of DMF on Granzyme B activity in MOG_{35-55} induced EAE mice

Granzyme B (GrB) is a 32 kDa serine protease released from the granules of cytotoxic T lymphocytes and NK cells and is involved in the mediation of apoptosis. Inhibition of GrB inhibitor (serpina3n) treatment has ameliorated axonal and neuronal injury in the EAE model [118].

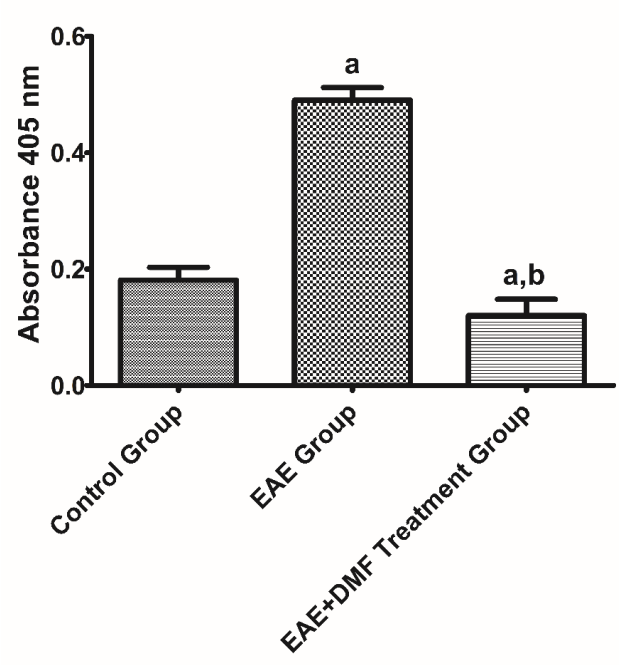

Figure 2-13 Granzyme B activity.

All values are mean ± SD; n = 3; [a]p< 0.05 compared to control, [b]p< 0.05 compared to EAE. [One-way ANOVA followed by Tukey Post-hoc test].

As shown in **Figure 2-13**, one-way ANOVA revealed a significant difference in the granzyme B activity in the CNS between control and EAE and EAE+DMF groups from day-7 up to day-33 [F (2, 14) = 61.89, p<0.05]. Also, there is a significant difference between EAE+DMF and EAE groups. The post-hoc test showed that the MOG_{35-55} injection significantly increased the GrB activity and it was alleviated by DMF (50 mg/kg) treatment. Cathepsin C has a critical role in activating GrB in the immune cells [119]. The covalent inhibition of Cathepsin C might have contributed in the reduction of GrB activity.

2.3.7 Effect of DMF on demyelination in MOG_{35-55}-induced EAE mice

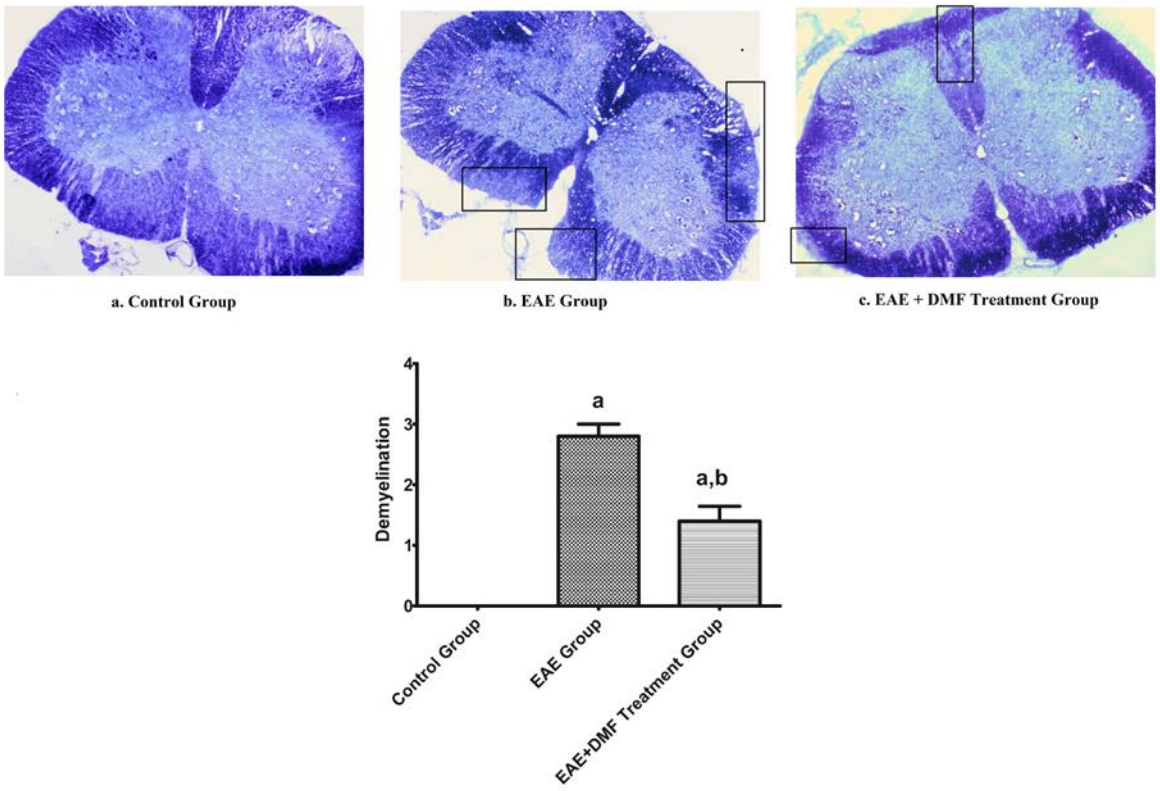

Figure 2-14 Demyelination in the spinal cord.

All values are mean ± SD; n = 3; [a]p< 0.05 compared to control, [b]p< 0.05 compared to EAE. [One-way ANOVA followed by Tukey Post-hoc test].

Lumbosacral region is a critical region controlling sexual functions, locomotion, and micturition which are major manifestations of MS [120]. Spinal cord sections from the lumbosacral region were sectioned and stained with LFB for demyelination and its scoring was done as described: score 0, no demyelination; score 1, mild demyelination; score 2, moderate demyelination and score 3, severe demyelination. The average score from the spinal cord sections of each animal was calculated [121]. One-way ANOVA revealed significant differences between control and EAE and EAE+DMF groups day-33 [$F(2, 12) = 58.81$, $p<0.05$] as shown in **Figure 2-14**. There is a significant difference between

EAE+DMF and EAE group as well. The post-hoc test showed that the MOG_{35-55} injection caused severe demyelination in the mice and this was significantly ameliorated by DMF treatment. The demyelination in the spinal cord can be correlated with the clinical scoring of the experimental animals as shown in the **Figure 2-8**. This signifiies axonal damage (demyelination) affecting the motor functions in the EAE mice and DMF mitigated severe demyelination to a state of mild to moderate demyelination.. MOG_{35-55} induced EAE in C57BL/6 mice causes demyelination which was evaluated by LFB staining in CNS sections to study the loss of myelin [122]. DMF suppresses demyelination due to its antioxidant effect on the neurons and glial cells [96]. In a recent research, DMF has been reported to succinate and inactivate the glycolytic enzyme GAPDH both *in vitro* and *in vivo* and therefore downregulating the critical aerobic glycolysis mechanism in myeloid as well as lymphoid cells which contributes to neuronal death followed by demyelination [64]. In our study, we have found that DMF (50 mg/kg) has reduced the infiltration and count of T lymphocytes when compared to the EAE group, thereby suggesting the implication of DMF in EAE by reducing the lymphocyte count. Moreover, in MS, the degree of axonal demyelination correlates directly with the presence of infiltrating T cells in the demyelinating white matter [123] and T cells contribute to neuronal damage through various pathways, including direct cell contact and the release of substances like cathepsin C and granzyme B [124]. Amelioration of demyelination by DMF can be attributed to its limiting effect on the infiltration of T lymphocytes into the CNS and inhibiting cathepsin C and granzyme B activities.

2.4 Summary

Our results indicate that DMF might function by targeting lymphocytes, exerting its immunomodulatory activity by binding and inactivating an important upstream target controlling versatile immune activations. The proven therapeutic effectiveness of DMF in the treatment of various autoimmune diseases is likely due to its mode of action which is the inactivation of prominent enzymes-cathepsin C and GrB, thus impacting several cellular events involved in immune activation. We have first found the effect of DMF on enzymatic activity and kinetics of cathepsin C. Moreover, we have evaluated the effect of DMF on the activity of cathepsin C and granzyme B in the EAE model and their role in demyelination. The research can help provide insight into understanding the probable mechanism of action of DMF in the treatment of RMMS.