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ROLE OF ASIATICOSIDE-A ON AXONAL PROTECTION IN EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS

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ABSTRACT

The aim of this study is to determine the action of Asiaticoside A against acute and chronic models of experimental autoimmune encephalomyelitis in mice. In this study, we investigated Asiaticoside A against acute and chronic models of EAE in mice. Asiaticoside A treated EAE animals shown reduced progression of the disease in both models. Gene expression studies revealed that the downregulation of IL-6, TNFα, IL-17a and CCL-5 in EAE mice were depending on the extend of treatment. The treatment with asiaticoside could not reverse the downregulation of NCAM1, FOXP3 and BDNF1 levels in EAE mice. Furthermore, histopathological studies revealed that the treatment with Asiaticoside A can able to control the demyelination, infiltrations and cellular changes in brain of EAE mice. Asiaticoside A delayed the EAE progression through the inhibition of inflammatory cytokines and chemokine and not by any other mechanisms of remyelination. The anti-inflammatory effect of this compound provides an alternative therapeutic management in multiple sclerosis.

KEYWORDS: EAE, Asiaticoside, MS, Cytokines, Multiple Sclerosis.

ABBREVIATIONS

MS, Multiple Sclerosis; CNS, Central Nervous System; EAE, Experimental Autoimmune Encephalomyelitis; MOG, Myelin Oligodendrocyte Glycoprotein; CFA, Complete Freund's Adjuvant; cDNA, complementary DeoxyriboNucleic Acid; IL-6, Interleukin-6; TNFα, Tumor Necrosis Factor α; IL-17, Interleukin-17; CCL-5, chemokine ligand 5; FOXP3, forkhead box P3; NCAM1, Neural Cell Adhesion Molecule1; BDNF1, Brain-Derived Neurotrophic Factor1; p.o., post orally.

INTRODUCTION

Multiple Sclerosis (MS) is a chronic inflammation of the myelin sheath, results in demyelination, astrogliosis, axonal loss and neuronal death in CNS. In 2013, around the world, more than 2.3 million people were diagnosed with MS (Browne P *et. al*., 2014). The incidence of MS is more reported in women than men. Autoimmunity, genetic predisposition, previous head trauma, viral infections, and environmental factors trigger MS. Autoimmunity is considered as the most common cause of this disorder. Experimental autoimmune encephalomyelitis is a well-known screening model for evaluating the autoimmune progression of MS in animals.

Current treatment strategies are relying on the disease-modifying agents. The critical problems with these agents are immunosuppression, poor tolerability, and other inflammatory disorders. In this scenario, an alternative medicine system can able to find out newer ways to inhibit the progression of MS. Asiaticoside-A is a triterpenoid glycoside obtained from *Centella asiatica*. Asiaticoside-A is reported for its anti-Alzheimer's activity due to the amyloid beta protein (Xing Y *et. al*., 2017) and blocked the oxidative stress, which produced during inflammation (Zhang Z *et. al*., 2017). This study focused on the mechanism behind the action of asiaticoside-A against EAE in mice.

MATERIALS AND METHODS

Animals

Female B6 mice of 7-8 weeks old were obtained from the Biogen Laboratory Animal Facility, Bengaluru, India. Mice were kept under 12 h dark/light cycle with water and food ad libitum. All procedures were performed in accordance with the guidelines of CPCSEA, India and authorized by the institutional animal ethics committee of Acharya & BM Reddy College of Pharmacy, Bengaluru, India. (Ref. no: IAEC/ABMRCP/2015-2016/05).

Chemicals

Asiaticoside-A 98% (Chengdu Biopurify phytochemicals Ltd, China), MOG_{35-55} (AnaSpec Inc.), and CFA (Sigma Aldrich).

Experimental Design

Acute model of EAE (aEAE): B6 mice were randomly divided into 4 groups, having 6 each. All animals were

administered with 100 µl of emulsion containing 1:1 ratio of MOG_{35-55} 2mg/ml and CFA intradermally to the base of tail on day 1 and 7 excluding Group I. On day 0 and 2 all mice except group I received 250 ng of pertussis toxin diluted with normal saline intravenously. Groups were denoted as I (negative control received normal saline p.o.), II (Positive control received normal saline p.o.), III (EAE mice treated with 40 mg/kg of asiaticoside-A p.o.), and IV (EAE mice treated with 80 mg/kg of asiaticoside-A p.o.). Treatments were started from day14 onwards after the confirmation of EAE in animals. The disease progression was assessed weekly after the development of disease up to day 35 (Furlan R *et. al*., 2001).

Chronic model of EAE (cEAE): B6 mice were randomly divided into 4 groups, having 6 each. All animals were administered with 100 µl of emulsion containing 1:1 ratio of guinea pig spinal cord homogenate (5%) emulsified with CFA subcutaneously near the hind leg on day 1 and 7 excluding Group I. Groups were denoted as I (negative control received normal saline p.o.), II (Positive control received normal saline p.o.), III (EAE mice treated with 40 mg/kg of asiaticoside-A p.o.), and IV (EAE mice treated with 80 mg/kg of asiaticoside-A p.o.). The treatments were started from day 14 after the confirmation of disease in animals. The disease progression was evaluated weekly after the development of disease up to day 70 (Hampton DW *et. al*., 2008).

Clinical Score

Normal= 0, partial tail paralysis= 1, complete tail paralysis= 2, hind limb paralysis = 3, forelimb paralysis= 4, and moribund/death= 5.

qPCR analysis

Total RNA of mouse brain was extracted by using TRIzol reagent (Invitrogen, CA, USA). The preparation of the reaction mix for cDNA synthesis was done per Thermo Scientific Verso cDNA Synthesis kit protocol. Synthesized cDNA was quantified using a fluorimeter Qubit 3.1 (Life Technologies, USA). The cDNA was diluted with water (nuclease free) to the desired concentration for qPCR assay. The qPCR assay was carried out using FastStart Essential DNA Green Master (Roche, Switzerland) with specific forward and reverse primers as shown in Table1. All the samples were performed in triplicates. The data was transferred, and fold amplification was calculated using Light cycler 96 analysis software.

Histopathological analysis

Mice were sacrificed (transcardially punctured and saline perfused) and their brains were rapidly excised and stored at -20°C. The brain sections were fixed in paraffin blocks and cut into sections of 8-µm thickness (-1.5 from bregma). Brain sections were stained with Luxol fast blue (LFB) or hematoxylin with eosin. The slides were visualized by light microscopy for the evaluation of demyelination, infiltrations and cellular changes.

Statistical Analysis

All data were represented as n=6, mean ± SEM. One-way ANOVA followed by Tukey's post hoc test was adopted for statistical evaluation (GraphPad Prism 5 software), $p<0.001$ considered as significant.

RESULTS

Asiaticoside-A reduced clinical score

In the acute model of EAE, treatment with asiaticoside A 40 mg/kg and 80 mg/kg showed a significant ($p<0.001$) reduction in the clinical score when compared to EAE mice during day 28 and 35 as shown in Fig.1. Even though, treatment with asiaticoside-A (40 mg/kg and 80 mg/kg) declined the development of disease during the investigation and a significant ($p<0.001$) inhibition was observed through day 42, 49, 56, and 70 against the chronic model of EAE (Fig.2).

Asiaticoside-A downregulated inflammatory cytokines and chemokine

In both models, the negative control mice showed lower levels of IL-6, IL-17a, CCL-5 and TNFα in the brain tissues (Fig.3, 4) The effect of asiaticoside-A on gene expression profile in an acute model of EAE is depicted in the Fig. 3. Treatment with asiaticoside-A significantly downregulated IL-6, TNFα, IL-17a, CCL-5 when compared to EAE mice ($p<0.001$). But, there was no upregulation of NCAM-1, BDNF1, and FOXP3 was observed in asiaticoside-A treated mice. The expression levels of these genes were decreased in the brain of rodents after the induction of EAE. Additionally, asiaticoside-A 40 mg/kg showed a significant downregulation of IL-6 ($p<0.01$), TNFα ($p<0.001$), IL-17a ($p<0.001$), and CCL-5 ($p<0.001$) when compared to mice treated with 80 mg/kg of asiaticoside-A. But, there was no improvement in the expression profiles of NCAM1, BDNF1, and FOXP3 after the increase in the dose of asiaticoside A.

Fig. 4 showed the effect of asiaticoside-A on the gene expression profile in a chronic model of EAE. Asiaticoside treatment significantly inhibited the levels of IL-6, TNFα, IL-17a and CCL-5 in EAE mice ($p<0.001$). The expression levels of NCAM-1, BDNF1, and FOXP3 were unimproved during the chronic medication with asiaticoside-A. The treatment with asiaticoside-A 40 mg/kg produced a better control over the expression of TNFα ($p<0.001$), and IL-17a ($p<0.001$) in cEAE mice than 80 mg/kg. The treatment with 80 mg/kg of asiaticoside A significantly down-regulated the level of CCL-5 ($p<0.001$) than the asiaticoside A 40 mg/kg treated EAE mice.

Asiaticoside-A reduced demyelination and neurodegeneration

The negative control mice showed intact cells with no cellular infiltrations or demyelination in brain sections. The acute induction of EAE in mice resuted in demyelination, profound neutrophil infiltration, and tissue damage in the brain sections (Fig.5). The treatment

with asiaticoside-A limited the demyelination, neutrophil infiltration, and tissue damage in EAE mice. There was no more adequate protection exhibited by asiaticoside-A at 80 mg/kg.

Fig.6 pointed that, the negative control animals showed intact cells without any infiltrations or demyelination. Meanwhile, chronic EAE in mice lead to in extensive demyelination, neutrophil infiltration and tissue degeneration. Asiaticoside-A 40 mg/kg prevented the neutrophil infiltration, demyelination, and tissue damage in the brain of EAE animals. But, treatment with asiaticoside A 80 mg/kg did not show much protection in the brain sections of EAE mice.

Table 1: Mouse primer sequences used for qPCR.

OLIGO NAME	FORWARD		REVERSE	
	SEQUENCE (5' ->3')	Tm	SEQUENCE (5' ->3')	Tm
GAPDH	TGCACCACCAACTGCTTAGC (20)	57.3	GGCATGGACTGTGGTCATGAG (21)	57.3
TNF-α	CCCAGGCAGTCAGATCATCTTC (22)	62.1	AGCTGCCCCTCAGCTTGA (18)	58.2
IL6	GGTACATCCTCGACGGCATCT (21)	61.8	GTGCCTCTTTGCTGCTTTCAC (21)	59.8
IL-17a	CTCAAAGCTCAGCGTGTCCAAACA (24)	62.7	TATCAGGGTCTTCATTGCGGTGGA (24)	62.7
CCL5	TGCCCACGTCAAGGAGTATTTC (22)	60.3	AACCCACTTCTTCTCTGGGTTG (22)	60.3
NCAM 1	TCAAGTACAAGGCTGAGTGGAA (22)	58.4	CCCACTGTGCTGTGACTAACAT (22)	60.3
FOXP3	CACCCAGGAAAGACAGCAACC (21)	61.8	GCAAGAGCTCTTGTCCATTGA (21)	57.9
BDNF I	AGTTGCTTTGTCTTCTGTAGTCGC (24)	61.0	CCTGGAGACTCAGTGTCTTA (20)	57.3

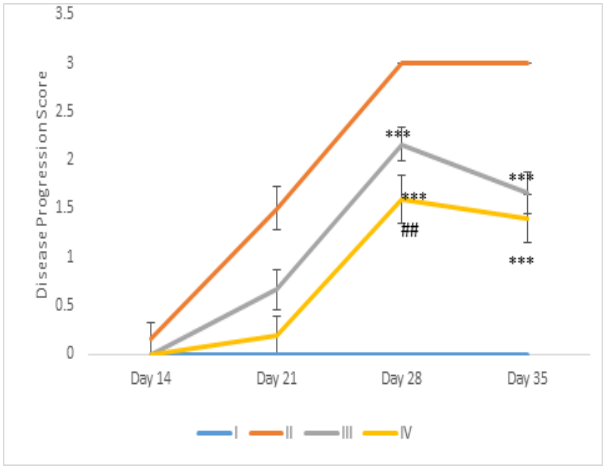

Fig.1: Disease progression score in acute model of EAE.

I -Normal mice (Negative Control), **II-** EAE mice (Positive control), **III** -EAE mice treated with 40 mg/kg of Asiaticoside A, **IV** -EAE mice treated with 80 mg/kg of Asiaticoside A. Each bar represents clinical score in mean ± SEM, n=6. Statistical analysis was carried out using ANOVA followed by Tukey's post hoc test. $p<0.01$ was considered as significant. ******* specifies $p<0.001$ compared to **II**, ## specifies $p<0.01$ compared to **III**.

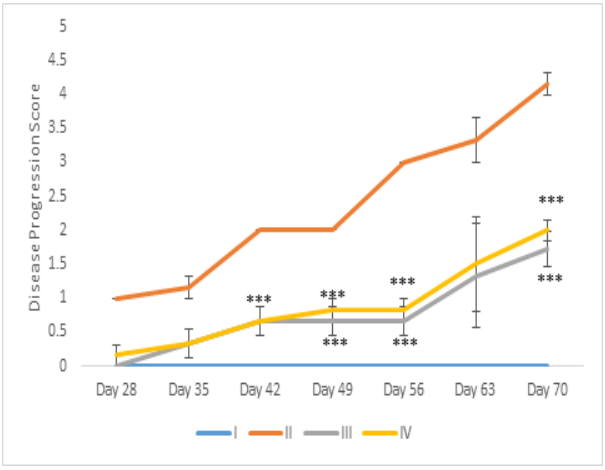

Fig. 2: Disease progression score in chronic model of EAE.

I -Normal mice (Negative Control), **II-** EAE mice (Positive control), **III** -EAE mice treated with 40 mg/kg of Asiaticoside A, **IV** -EAE mice treated with 80 mg/kg of Asiaticoside A. Each bar represents clinical score in mean ± SEM, n=6. Statistical analysis was carried out using ANOVA followed by Tukey's post hoc test. $p<0.05$ was considered as significant. ******* specifies $p<0.001$ compared to **II**.

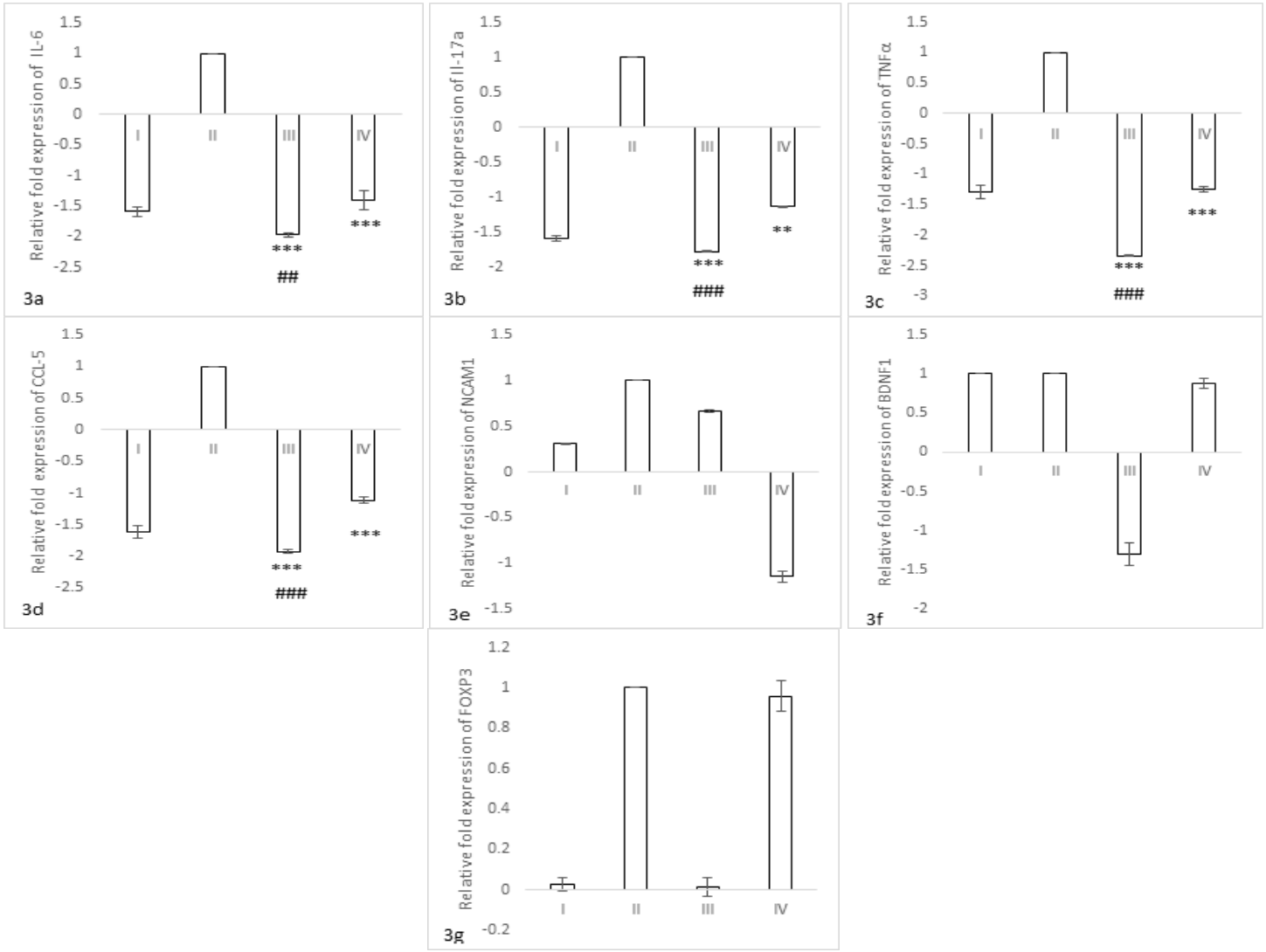

Fig. 3: Relative gene expression of various genes in acute model of EAE.

I -Normal mice (Negative Control), **II**- EAE mice (Positive control), **III** -EAE mice treated with 40 mg/kg of Asiaticoside A, **IV** -EAE mice treated with 80 mg/kg of Asiaticoside A. Each bar represents relative gene expression when compared to GADPH in mean ± SEM, n=6. Statistical analysis was carried out using ANOVA followed by Tukey's post hoc test. $p<0.05$ was considered as significant. ******* specifies $p<0.001$ compared to **II**, ## specifies $p<0.01$ compared to **IV,** ### specifies $p<0.001$ compared to **IV.**

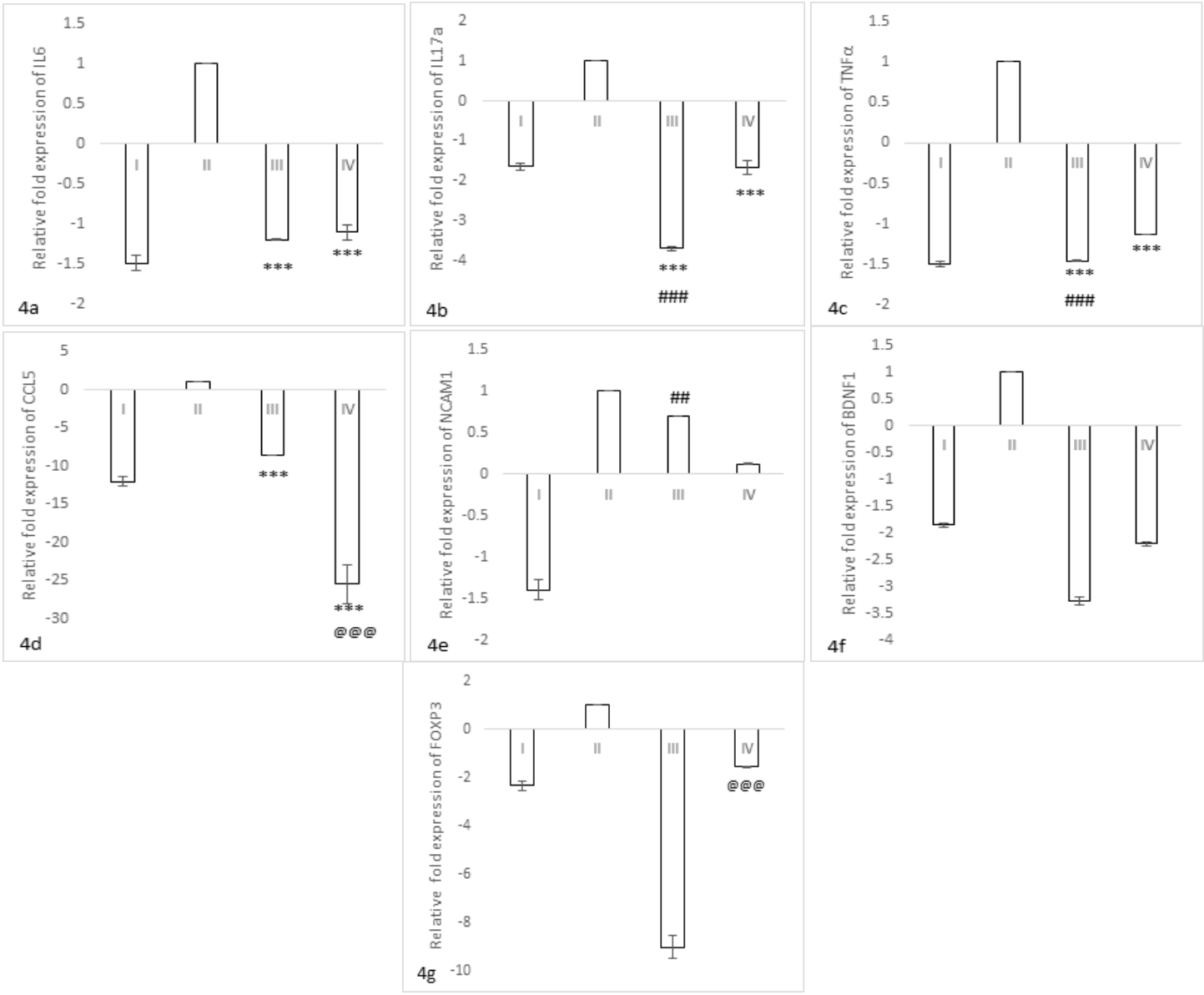

Fig. 4: Relative gene expression of various genes in chronic model of EAE.

I -Normal mice (Negative Control), **II-** EAE mice (Positive control), **III** -EAE mice treated with 40 mg/kg of Asiaticoside A, **IV** -EAE mice treated with 80 mg/kg of Asiaticoside A. Each bar represents relative gene expression when compared to GADPH in mean ± SEM, n=6. Statistical analysis was carried out using ANOVA followed by Tukey's post hoc test. $p<0.05$ was considered as significant. ******* specifies $p<0.001$ compared to **II**, **@@@** specifies $p<0.001$ compared to **III,** **###** specifies $p<0.001$ compared to **IV.**

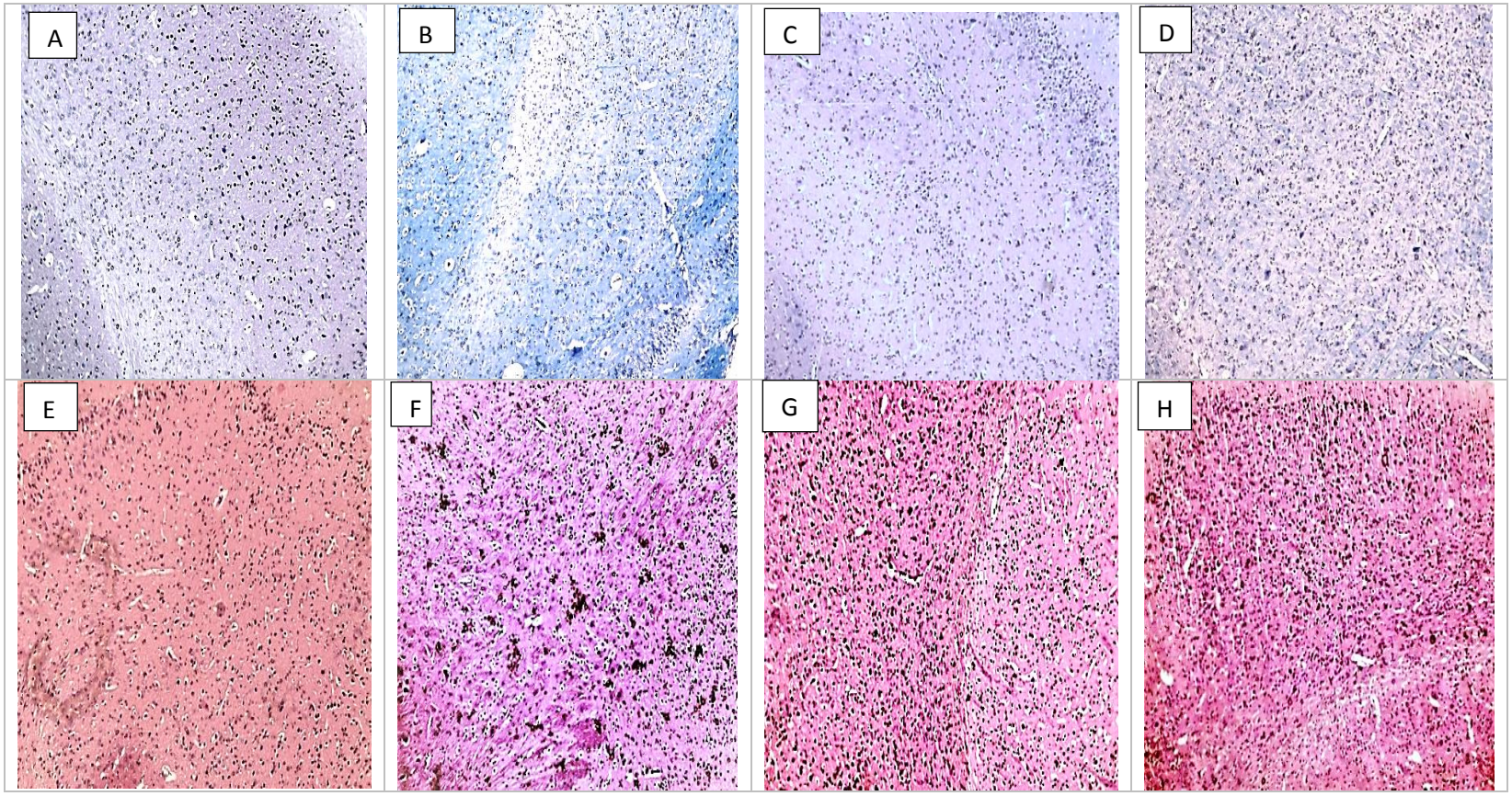

Fig. 5: Histopathology of brain sections LFB and H&E stain in acute model of EAE.

A, B, C, D represents LFB stained brain sections and E, F, G, H represents H&E stained brain sections. A, E represents negative control mouse brain section**,** indicates no demyelinations or infiltrations with intact cells. B, F represents positive control (EAE) mouse brain section, showed demyelinatory lesions, marked neutrophil infiltration and cellular damage. C, G represents the brain section of EAE mouse treated with Asiaticoside A 40 mg/kg, exhibited limited demyelination, mild neutrophil infiltration and cellular changes. D, H represents the brain section of EAE mouse treated with Asiaticoside A 80 mg/kg, displayed limited demyelination, moderate neutrophil infiltration and cellular changes.

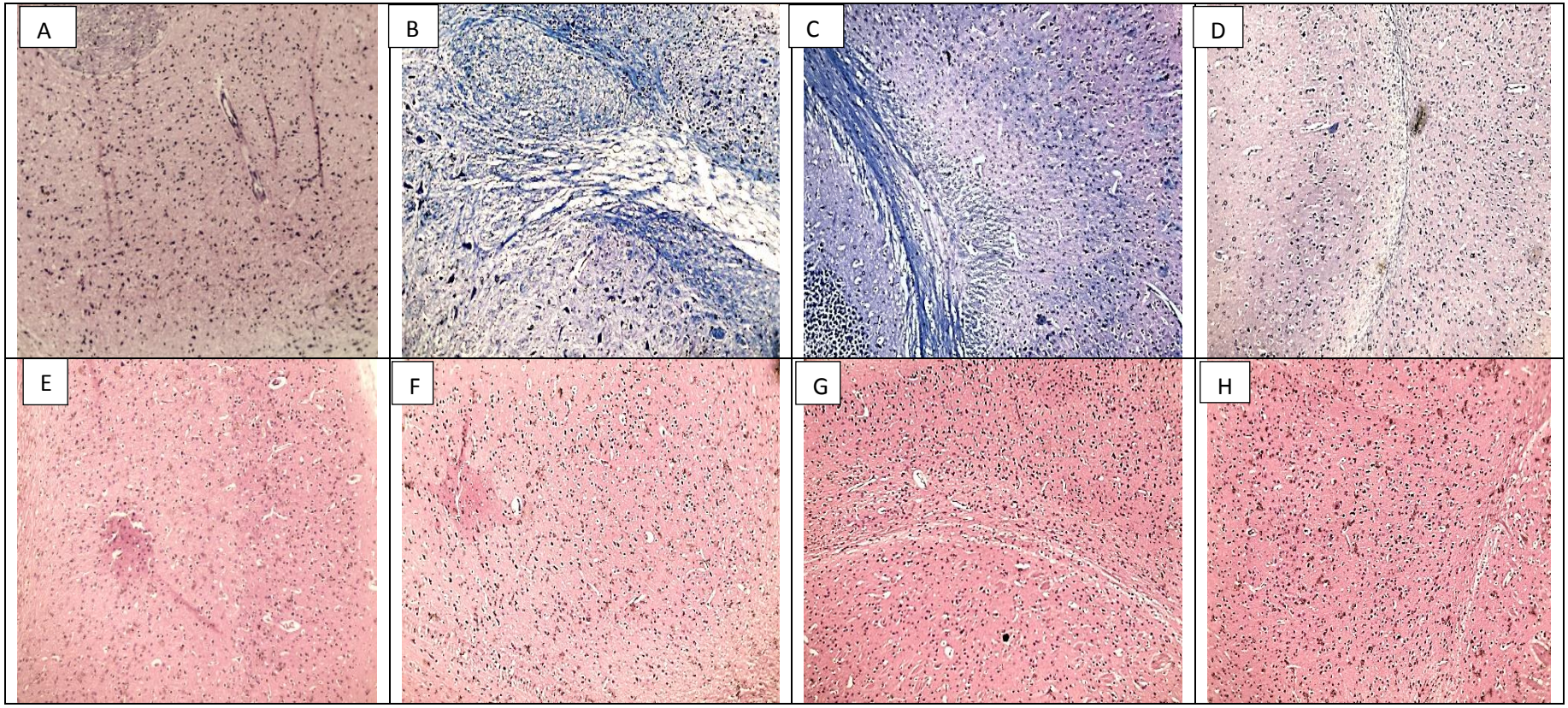

Fig. 6: Histopathology of brain sections LFB and H&E stain in chronic model of EAE. A, B, C, D represents LFB stained brain sections and E, F, G, H represents H&E stained brain sections. A, E represents negative control mouse brain section**,** indicates no demyelinations or infiltrations with intact cells. B, F represents positive control (EAE) mouse brain section, showed demyelinatory lesions, marked neutrophil infiltration and cellular damage. C, G represents the brain section of EAE mouse treated with Asiaticoside A 40 mg/kg, exhibited limited demyelination, mild neutrophil infiltration and cellular changes. D, H represents the brain section of EAE mouse treated with Asiaticoside A 80 mg/kg, displayed moderate demyelination and moderate neutrophil infiltration with cellular changes.

DISCUSSION

Autoimmunity is considered one of the key factors that trigger multiple sclerosis in the human population. EAE is a most commonly used screening model to consider the autoimmune aspects of MS in animals. It can be induced by the co-administration of myelin proteins like MBP, MOG with CFA or LPS. This hastens the process of T cell activation and results in enhanced BBB permeability (Ransohoff RM *et. al*., 2003), activate microglia and astrocytes (Brown DA *et. al*., 2007), and glutaminergic excitation induced axonal degeneration (Stojanovic IR *et. al*., 2014) results in neuronal damage. Currently, there was a shift in research happened to select the credible herbal constituents that can bring the potentials to manage the progression of MS. In this study, we utilized EAE models to discover the potential action of asiaticoside-A in mice.

In the acute model, the EAE was induced by the administration of MOG with CFA (1:1). EAE-induced mice exhibited the signs of disease from day 12 onwards, thereafter a gradual progression of illness was observed in animals and the clinical score was recorded. Treatment with asiaticoside A started after confirming the active induction of disease from the day 12. EAE mice indicated a rise in clinical score and treatment with asiaticoside A reduced during the investigation. Treatment with Asiaticoside-A reduced the clinical score in EAE animals, which indicate that the progression of the disease can be minimized during the therapy.

From the qPCR studies, we found that the asiaticoside-A treated mice showed downregulation of IL-6, TNF-α, IL-17a and CCL5. This suggested the inflammatory cytokines, and chemokine can be counteracted by the treatment with asiaticoside A in EAE mice. Meantime, treatment with asiaticoside-A neither improved the T cell regulation through the activation of Tregs+FOXP3 cells nor enhanced the growth factors like BDNF during EAE. NCAM-1levels were unimproved by the treatment with asiaticoside-A. This suggests the action of it was not mediated through NCAM-1 function or by any other cell adhesion molecules in CNS.

Inflammatory cytokines like IL-1b, IL-6, TNFα, and IL-17a have a critical role in any autoimmune process that was promoted by the T cell activation. IL-6 is considered as a major cytokine that promotes the MS due to its ability to enhance the BBB permeability and triggers the production of Th17 cells (Heink S *et. al*., 2016). This marks the differentiation of Th1 cells and leads to the active progression of the disease. The level of IL-6 is high near the active lesions, which were mainly synthesized from the astrocytes (Giralt M *et. al*., 2013). Similarly, elevated levels of IL-1β and TNFα were observed in the CSF of MS patients (Seppi D et al., 2014 and Rossi S *et. al*., 2013).

IL-6 is a pro-inflammatory cytokine released in response to antigen stimulus, activate the IL6Rs through JAK-STAT pathway results in the activation of naïve T cells. T cells on activation, release cytokines like IL-6, IL-1β, TNFα (Panzer S *et al*., 1993), leads to the promotion of Th22 and Th17. This causes the BBB disruption and which helps the entry of activated T cells to the CNS. This marks the progression of EAE through the production of IL-17a and IL-22 (Kebir H *et. al*., 2007). Concurrently, the transformation of T cells to Th17 cells results in the production of IL-17a-f, IL-22, and TNFα. This recruit the more isolated promotion of activated T cells and peripheral macrophages to the CNS consequences in extensive demyelination, microglia and astrocyte activation and neuronal degeneration (Prineas JW *et. al*., 1981). Further, the active demyelination results in the elevation of proteinases like MMPs in the CNS (Cammer W *et. al*., 1978). This marks the destruction of myelin proteins, these peptides, in turn, re-activates the autoimmune responses in EAE.

From the histopathological analysis, the treatment with asiaticoside-A reduced the inflammatory cell infiltration, demyelination, and cellular damage when compared to the brains of EAE animals, which correlates with the results obtained from gene expression studies. This indicates that asiaticoside-A has a potential role to counteract the changes produced by the EAE in the animals.

CONCLUSION

Asiaticoside-A treatment can able to inhibit the progression of EAE through the downregulation of inflammatory cytokines and chemokines. Further, the molecular studies are required to confirm the mechanism action and the specific targets of asiaticoside-A in EAE.

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Conflict of interest

We confirm that there are no known conflicts of interest associated with this publication.

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