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Bacoside-A inhibits inflammatory cytokines and chemokine in experimental autoimmune encephalomyelitis

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ABSTRACT

Chronic inflammation of the myelin sheath is the crucial event behind the progression of multiple sclerosis (MS). Bacoside-A is one of the major constituents obtained from *Bacopa monerii* (L.) Wettst., and possess neuroprotective as well as anti-inflammatory actions. The current study explores the effect of Bacoside-A in acute and chronic models of Experimental Autoimmune Encephalomyelitis (EAE). The results indicate that the Bacoside-A treated mice produced a significant reduction in disease score compared to disease control in both models. The treatment with Bacoside-A downregulated the inflammatory cytokines (IL-6, IL-17a, and TNFα) and inflammatory chemokine CCL-5 in EAE mice. On the other hand, Bacoside-A treated mice showed a nonsignificant effect on promoting the expressions of NCAM, BDNF1, and FOXP3 in acute and chronic models of EAE. Histopathological analysis revealed that the Bacoside-A treated mice at a dose of 10 mg/kg exhibited a significant reduction in cellular infiltrations, cellular changes, and demyelination in cerebral tissues, but unable to protect at a higher dose in both models. In conclusion, Bacoside-A can able to inhibit the progression of EAE may be by the inhibition of inflammatory cytokines and chemokine evolved during active EAE.

1. Introduction

Multiple Sclerosis (MS) is characterized as chronic inflammation of the central nervous system (CNS), lead to demyelination, axonal damage and neuronal apoptosis. Epidemiological studies suggest that autoimmunity have a crucial role in the pathology of MS. Experimental Autoimmune Encephalomyelitis (EAE) is one of the preclinical models of MS, helps to understand the autoimmune progression of this neurodegenerative disorder. The critical difference is that MS is mediated through the activation of Cluster of Differentiation (CD) 8 + T (T-lymphocyte) cells [1] while EAE progress via the triggering of CD4 + T cell [2]. However, EAE helps to explore the molecular mechanism of disease progression in humans and fosters the drug discovery process for MS therapy.

Current treatment focused on preventing the progression of MS in patients, but the tolerability of these drugs is still in doubt. So, the novel researchers are more inclined to reveal the pathological mechanism of disease and the possibility of herbal constituents in the management of MS. *Bacopa monerii* (L.) Wettst (Family: Scrophulariaceae), is a well-known nootropic herb, commonly used for longevity and to enhance cognition. The ethanolic extract of this plant possesses anti-convulsant [3], anti-inflammatory [4], cholinesterase inhibition [5] and prevents dementia by improving cerebral blood flow in rats [6]. Recent studies reported that extracts of *Bacopa monerii* (L.) protected against allodynia and hyperalgesia in neuropathic pain induced rats [7]. It also promoted learning-dependent hippocampal long-term synaptic potentiation [8] and protected against Type 2 diabetes mellitus induced brain aging and memory impairment in mice [9].

Bacoside-A is obtained from *Bacopa monerii*. (L.) Wettst. It consists of bacoside-A3, bacopaside-II, jujubogenin, and bacopasaponin-C. From the previous studies, we observed that it delays the aging-related neurodegeneration [10] and protects the brain through the modulation of Hsp70 in stress in rodents [11]. It also inhibits the membrane interactions of amyloid beta in neuronal cells results in neuroprotection during Alzheimer's disease [12]. In this study, we investigated the role of Bacoside-A in acute and chronic experimental autoimmune encephalomyelitis in mice.

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

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Table 1
Mouse primer 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

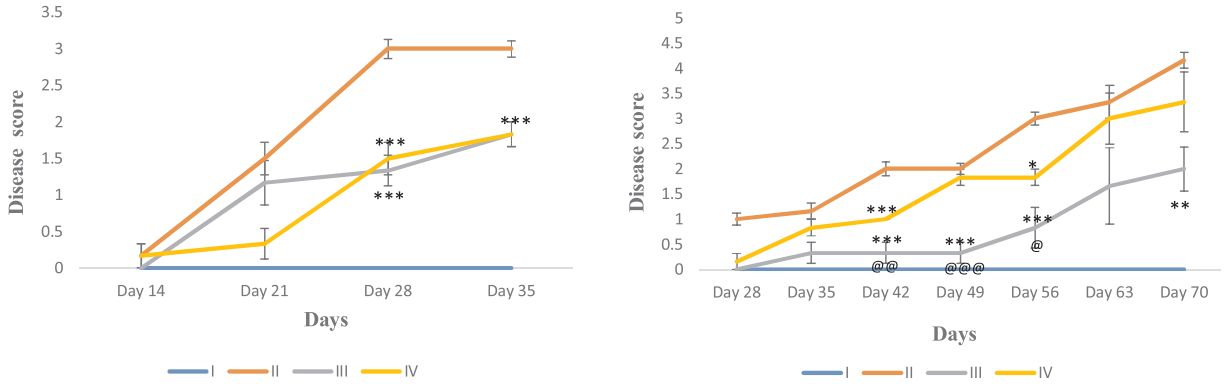

Fig. 1. Progression of disease score in acute and chronic model of EAE. **I**-Negative Control, **II**-Disease control/EAE mice, **III**-EAE mice treated with 10 mg/kg of Bacoside, **IV**-EAE mice treated with 20 mg/kg of Bacoside. Each bar represents clinical score in mean ± SEM, n = 6, Df = 23 (3,20). Statistical analysis was carried out using ANOVA followed by Tukey's post hoc test. $p < 0.001$ was considered significant. * specifies $p < 0.05$ compared to **II,** ** specifies $p < 0.01$ compared to **II,** *** specifies $p < 0.001$ compared to **II**. @ specifies $p < 0.05$ compared to **IV**. @@ specifies $p < 0.01$ compared to **IV**. @@@ specifies $p < 0.001$ compared to **IV**.

2. Materials and methods

2.1. Animals

C57BL/6 mice (female) of 7-8 weeks old procured from the Biogen Laboratory Animal Facility, Bengaluru, India. Mice kept under 12 h dark/light cycle with water and food *ad libitum*. All procedures were performed in agreement with the guidelines of CPCSEA, India and approved by Institutional animal ethics committee of Acharya & BM Reddy College of Pharmacy, Bengaluru, India. (Ref. no: IAEC/ABMRCP/2015-2016/05)

2.2. Chemicals

Bacoside-A 98% (Finetech Industry Limited, China), MOG35-55 (AnaSpec Inc., CA, USA), and CFA (Sigma Aldrich, St. Louis, MO, USA).

2.3. Experimental design

Acute model of EAE (aEAE): All mice were intradermally administered with 100 µl of an emulsion containing 1:1 ratio of Myelin Oligodendrocyte Glycoprotein$_{35\text{-}55}$ ($MOG_{35\text{-}55}$)2 mg/ml solution with Complete Freund's Adjuvant (CFA) to the base of the tail on day 1 and 7 [13]. After the disease induction, they were randomly divided into four groups, containing six animals each. Groups were represented as I (Negative control received normal saline containing 0.1% Carboxymethylcellulose (CMC)), II (Disease control/ EAE mice received normal saline containing 0.1% CMC), III (EAE mice treated with 10 mg/kg of Bacoside-A in normal saline containing 0.1% CMC orally), and IV (EAE mice treated with 20 mg/kg of Bacoside-A in normal saline containing 0.1% CMC orally). The treatment with Bacoside-A started from day 14 onwards after the confirmation of disease with the help of a change in locomotor coordination using open field apparatus. The disease score was assessed weekly throughout the experiment up to 35th day.

Chronic model of EAE (cEAE): On day 1 and 7, all mice were subcutaneously administered with 100 µl of an emulsion containing 1:1 ratio of guinea pig spinal cord homogenate (5%) with CFA near to the hind leg [14] except Group I. After the disease induction, they were randomly divided into four groups, containing six animals each. Groups were represented as I (Negative control received normal saline containing 0.1% CMC orally), II (Disease control/EAE mice received normal saline containing 0.1% CMC orally), III (EAE mice treated with 10 mg/kg of Bacoside-A in normal saline containing 0.1% CMC orally), and IV (EAE mice treated with 20 mg/kg of Bacoside-A in normal saline containing 0.1% CMC orally). Bacoside-A treatment started from the day 14th onwards after the confirmation of disease with the help of a change in locomotor coordination using open field apparatus. The disease progression was further evaluated weekly up to 70th day.

2.3.1. Disease score

The disease score was analyzed corresponding to the following criteria. Normal = 0, partial tail paralysis = 1, complete tail paralysis = 2, hindlimb paralysis = 3, forelimb paralysis = 4, and moribund/death = 5 [15].

2.3.2. Quantitative polymerase chain reaction (qPCR) analysis

Total RNA of mouse brain was extracted using TRIzol reagent; conferring on to manufacturer's manuals (Invitrogen, CA, USA). The cDNA synthesis was performed per Thermo Scientific Verso cDNA Synthesis kit protocol and quantified using a fluorimeter Qubit 3.1 (Life Technologies, USA). The cDNA was diluted with nuclease-free water to bring in the desired concentration for qPCR assay. FastStart Essential DNA Green Master (Roche, Switzerland) with specific forward and

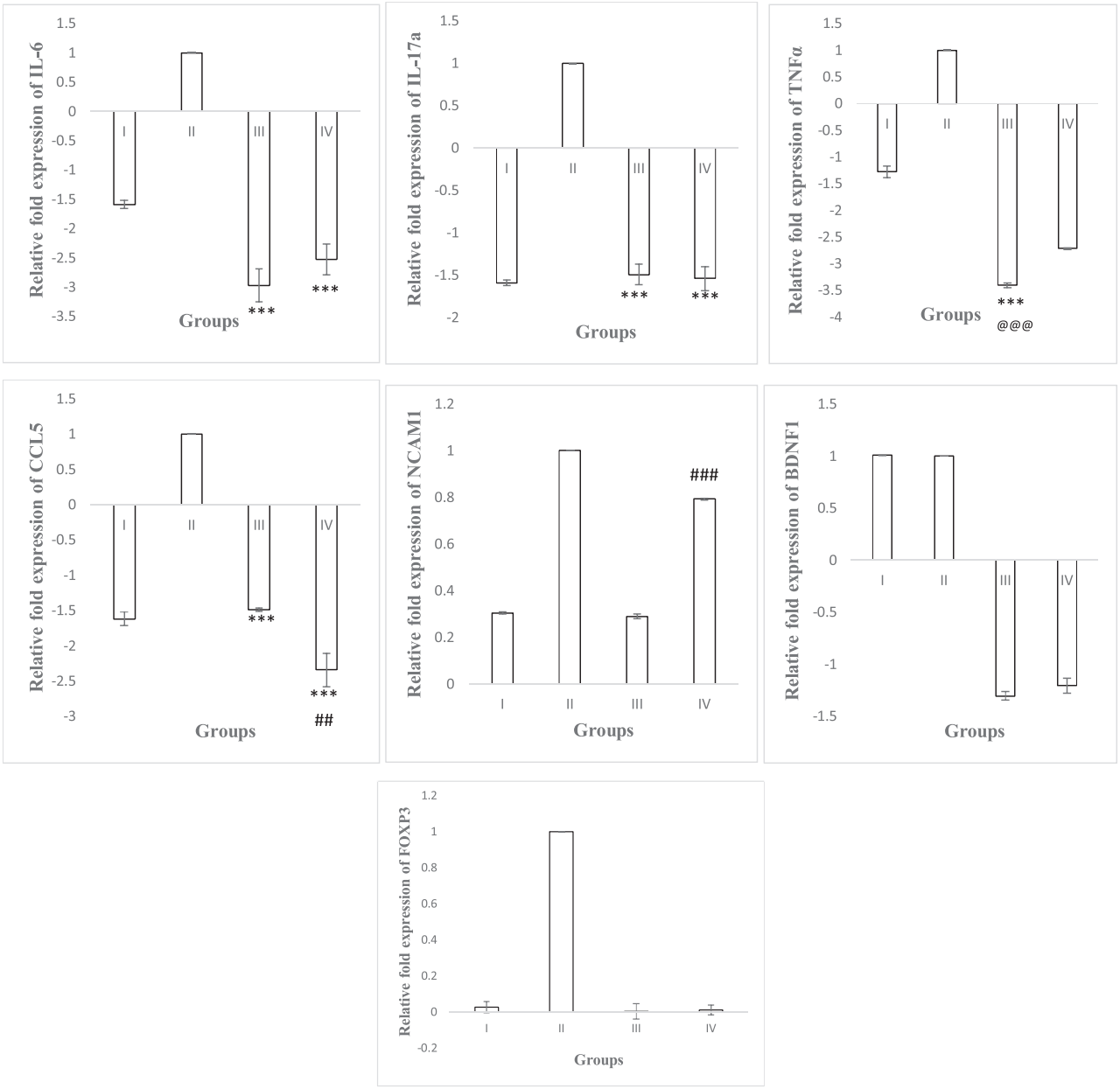

Fig. 2. Relative gene expression of IL-6, IL-17a, TNFα, CCL-5, NCAM1, BDNF1 and FOXP3 in an acute model of EAE. **I -** Negative Control, **II**- Disease control/EAE mice), **III**-EAE mice treated with 10 mg/kg of Bacoside-A, **IV**-EAE mice treated with 20 mg/kg of Bacoside-A. Each bar represents relative gene expression when compared to GADPH in mean ± SEM, n = 6, Df = 23 (3,20). Statistical analysis was carried out using ANOVA followed by Tukey's post hoc test. $p < 0.01$ was considered significant. *** specifies $p < 0.001$ compared to **II**. *##, ###* specifies $p < 0.01$, $p < 0.001$respectively compared to **III**. **@@@** specifies $p < 0.001$ compared to **IV**.

reverse primers (Table 1) were used to carry out the qPCR reaction. Each reaction performed in triplicates. The fold amplification was calculated using Light cycler 96 analysis software.

2.3.3. Histopathological analysis

Mice were anesthetized by 10 mg/kg of ketamine, intraperitoneally (NEON Laboratories Limited, India), sacrificed (transcardially punctured and saline perfused) and their brains were rapidly excised and stored at -20 ^{0}C. The paraffinized brain (cerebrum) tissues were cut (-1.5 from bregma) into sections of 8 µm thickness. The cerebral sections stained with Luxol fast blue (LFB) or hematoxylin with eosin. Each slide was visualized using light microscopy for the evaluation of demyelination and cell infiltration [16]. Neutrophil infiltrations were estimated using a scoring method: 0 = no cellular infiltration, 1 = occasional infiltration, 2 = focal infiltration, 3 = coalescing neutrophils without any loci, and 4 = distributed cellular infiltration.

2.4. Statistical analysis

All results represented as n = 6, Mean ± SEM. Statistical evaluation performed by one-way ANOVA followed by Tukey's post hoc test (GraphPad Prism 5.0 software), $p < 0.01$ considered as significant.

3. Results

3.1. Bacoside-A attenuated disease score

Fig. 1 showed that in acute EAE, disease control mice displayed a progressive rise in disease score from day 14th to day 35th. Treatment with Bacoside-A 10 mg/kg and 20 mg/kg showed a significant

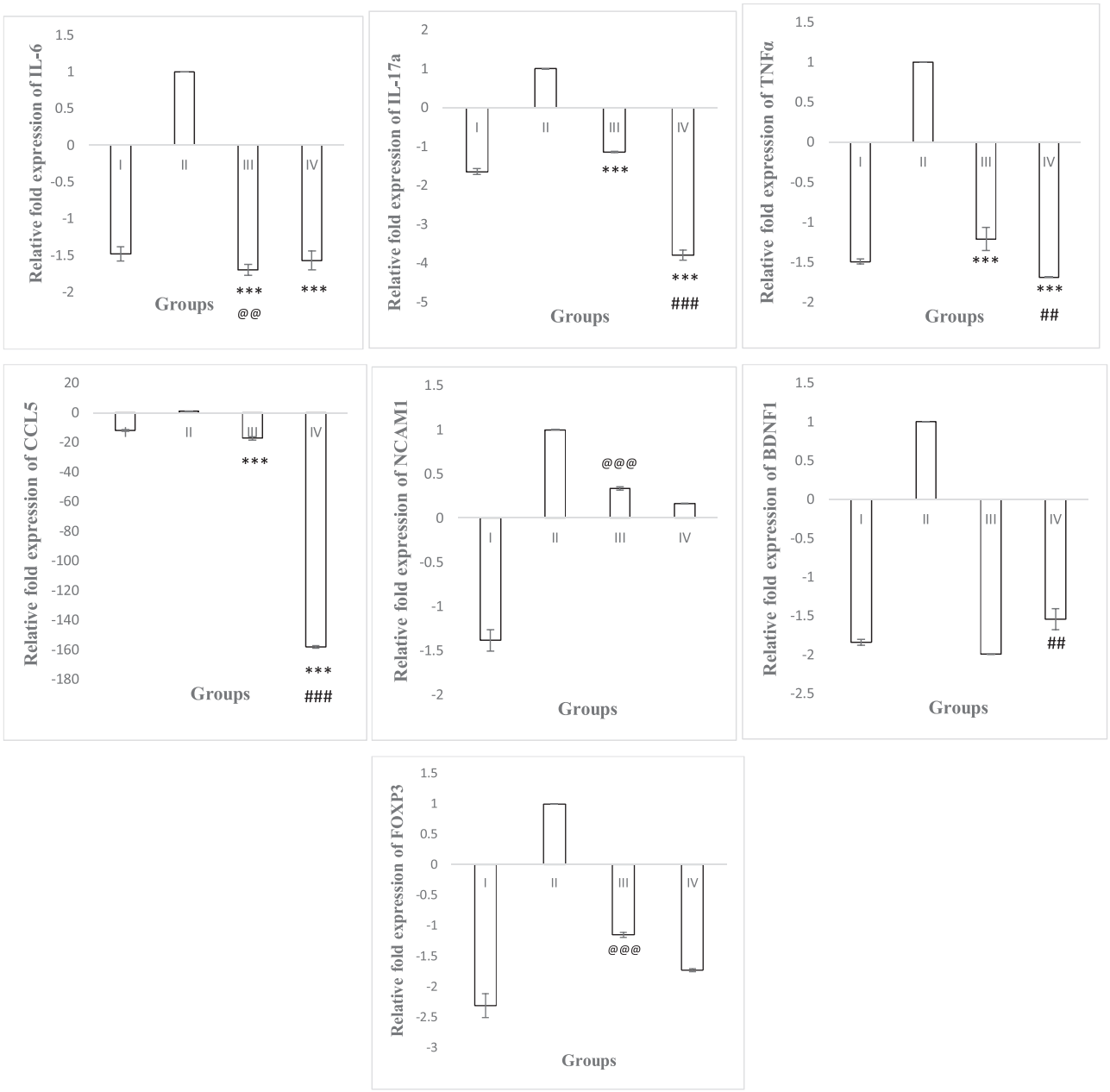

Fig. 3. Relative gene expression of IL-6, IL-17a, TNFα, CCL-5, NCAM1, BDNF1 and FOXP3 in a chronic model of EAE. **I -** Negative Control, **II**-Disease control/EAE mice, **III** - EAE mice treated with 10 mg/kg of Bacoside-A, **IV -** EAE mice treated with 20 mg/kg of Bacoside-A. Each bar represents relative gene expression when compared to GADPH in mean ± SEM, n = 6, Df = 23 (3,20). Statistical analysis was carried out using ANOVA followed by Tukey's post hoc test. $p < 0.01$ was considered significant. *** specifies $p < 0.001$ compared to **II**. ## specifies $p < 0.01$ compared to **III**. ### specifies $p < 0.001$ compared to **III**. **@@** specifies $p < 0.01$ compared to **IV**. **@@@** specifies $p < 0.00\ 1$ compared to **IV**.

($p < 0.001$, Df = 23 (3,20)) reduction in the disease score when compared to EAE mice during day 28th and 35th.

Fig. 1 shown that in chronic EAE, disease control mice exhibited gradual progression of disease score. Bacoside-A (10 mg/kg) treated mice displayed a significant ($p < 0.001$, Df = 23 (3,20)) reduction in the disease score throughout the investigation when compared to the disease control and Bacoside-A 20 mg/kg treated mice. Similarly, Bacoside-A (20 mg/kg) treated mice exhibited a significant ($p < 0.001$, Df = 23 (3,20)) decrease in the disease score when compared to disease control mice.

3.2. Bacoside-A downregulated inflammatory cytokines and chemokine

The Fig. 2 showed the effect of Bacoside-A on gene expression profile in the acute model of EAE. Treatment with bacoside-A significantly downregulated Interleukin-6 (IL-6), Tumor Necrosis factor α (TNFα), Interleukin-17a (IL-17a), and chemokine ligand-5 (CCL-5) when compared to EAE mice ($p < 0.001$, Df = 23 (3,20)). However, there was no upregulation of Neural Cell Adhesion Molecule-1 (NCAM-1), Brain derived neurotrophic factor-1 (BDNF1), and forkhead Box protein-3 (FOXP3) observed in Bacoside-A treated mice. The expression levels of these genes decreased in the brains of rodents after the induction of EAE. Additionally, Bacoside-A 10 mg/kg showed a significant downregulation of IL-6 ($p < 0.01$, Df = 23 (3,20)), TNFα ($p < 0.001$, Df = 23 (3,20)), IL-17a ($p < 0.001$, Df = 23 (3,20)), and CCL-5 ($p < 0.001$, Df = 23 (3,20)), when compared to mice treated with 20 mg/kg of Bacoside-A. But, there was no improvement in the expression profiles of NCAM1, BDNF1, and FOXP3 after the treatment

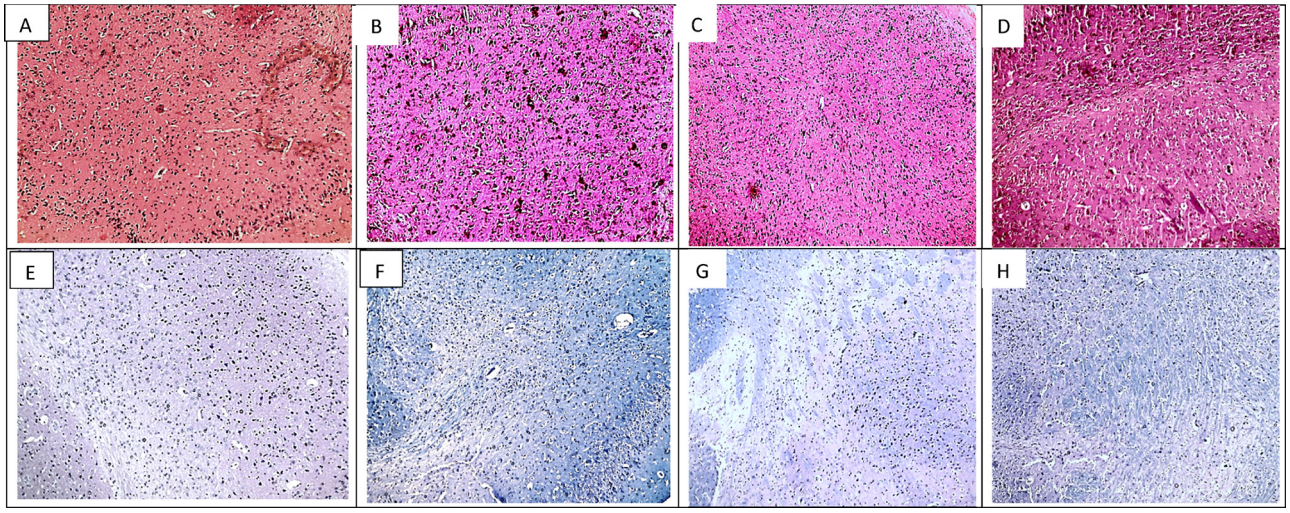

Fig. 4. Histopathology of brain sections LFB and H&E stain in an acute model of EAE. A, B, C, D represents LFB stained brain sections, and E, F, G, H represents H&E stained brain sections. The sections obtained from cerebrum (-1.5 from bregma). A, E represents negative control mouse brain section, indicates no demyelinations or infiltrations (score 1) with intact cells. B, F represents disease control (EAE) mouse brain section, showed demyelination, distributed neutrophil infiltration (score 4) and cellular damage. C, G represents the brain section of EAE mouse treated with Bacoside A 10 mg/kg, exhibited limited demyelination, coalescing neutrophil infiltration (score 3), and cellular changes. D, H represents the brain section of EAE mouse treated with Bacoside A 20 mg/kg, displayed marked demyelination, coalescing neutrophils (score 3), and cellular changes.

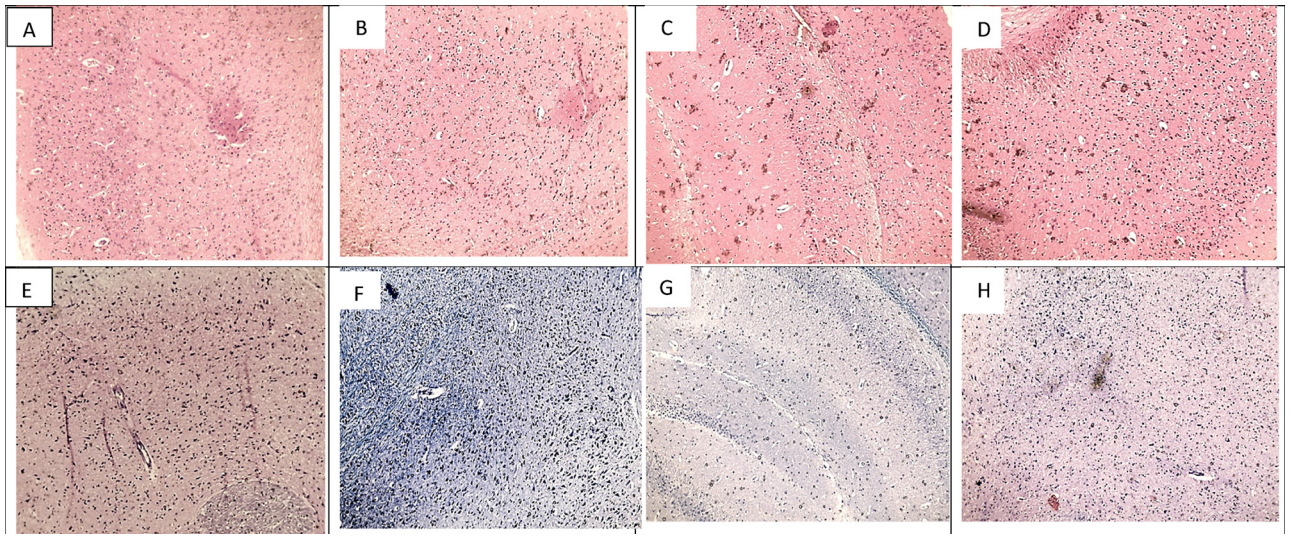

Fig. 5. Histopathology of brain sections LFB and H&E stain in a chronic model of EAE. The sections obtained from cerebrum (−1.5 from bregma). A, B, C, D represents LFB stained brain sections, and E, F, G, H represents H&E stained brain sections. A, E represents negative control mouse brain section, indicates no demyelinations or infiltrations with intact cells. B, F represents disease control (EAE) mouse brain section, showed demyelination, distributed neutrophil infiltration (score 4) and cellular damage. C, G represents the brain section of EAE mouse treated with Bacoside A 10 mg/kg, exhibited limited demyelination, focal neutrophil infiltration (score 2), and cellular changes. D, H represents the brain section of EAE mouse treated with Bacoside A 20 mg/kg, displayed marked demyelination and coalesing neutrophils (score 3) with cellular changes.

with a higher dose of Bacoside-A.

The Fig. 3 showed the effect of Bacoside-A on the gene expression profile in the chronic model of EAE. The treatment significantly inhibited the levels of IL-6, TNFα, IL-17a, and CCL-5 in EAE mice ($p < 0.001$, Df = 23 (3,20)). The expression levels of NCAM-1, BDNF1, and FOXP3 were unimproved during the chronic medication with Bacoside-A. The treatment with Bacoside-A 10 mg/kg showed better control on the expression of TNFα ($p < 0.001$, Df = 23 (3,20)) and IL-17a ($p < 0.001$, Df = 23 (3,20)) in cEAE mice. The treatment with 20 mg/kg of Bacoside-A significantly downregulated the level of CCL-5 ($p < 0.001$, Df = 23 (3,20)) than Bacoside-A 10 mg/kg treated EAE mice.

3.3. Bacoside-A reduced demyelination and neurodegeneration

The Fig. 4 specified that in acute EAE, the disease control mice produced profound demyelination, with distributed cellular infiltrations (score 4) and tissue damage in the cerebrum when compared to negative control mice. Meanwhile, Bacoside-A (10 mg/kg) treated mice shown a significant reduction in demyelination, neutrophil infiltration (score 2) and tissue damage in EAE mice. Bacoside-A (20 mg/kg) treated EAE mice displayed poor control over neutrophil infiltrates (score 3), cellular damage and demyelination.

Fig. 5 indicated that in chronic EAE, disease control mice showed extensive demyelination, dispersed cellular infiltrates (score 4), and cellular changes in cerebral sections when compared to negative control mice. Bacoside-A 10 mg/kg treated mice displayed a reduction in

neutrophil infiltration (score 2), demyelination and tissue damage on the cerebrum. Conversely, treatment with 20 mg/kg of Bacoside-A resulted in a minor decline in cellular infiltrates (score 3), cellular changes and demyelination in EAE mice.

4. Discussion

Autoimmunity is one of the critical factors behind multiple sclerosis in humans. EAE is a most widely used screening model to reveal the autoimmune characteristics of MS in animals, and which can be induced by the co-administration of myelin proteins like myelin basic protein (MBP) or MOG with CFA. These inducers initiate the process of T cell activation and eventually leads to enhanced BBB permeability [17], activation of microglia and astrocytes [18], excitotoxicity and axonal degeneration [19]. Novel researchers are trying to explore the potentials of herbal constituents to manage the ailments produced by the disease like MS. This study investigated the role of Bacoside-A against acute and chronic models of EAE in mice.

EAE induction resulted in a gradual progression of disease in animals. There was a gradual rise in clinical score from day 12 in both models. The treatment with Bacoside-A started after the confirmation of disease in animals. The therapy declined the progression of disease in EAE animals, can be correlated with the reduction in the disease score shown by Bacoside-A treated EAE animals during the investigation.

From the results of the qPCR analysis, we confirmed that the treatment with Bacoside-A could inhibit the promotion of inflammatory mediators like IL-6, TNF-α, IL-17a, and CCL5. Conversely, the procedure could not improve the anti-inflammatory mediators like FOXP3, NCAM1, and BDNF1 levels in the brain of the EAE animals. These actions enhanced on chronic treatment with the drug than the acute treatment. These actions suggest that the Bacoside-A have a significant role in regulating the inflammatory mediators especially CCL-5, IL-6, TNF-α, and IL-17a than promoting the anti-inflammatory mediators to counteracts the effect of EAE in mice.

During autoimmunity, the inflammatory cytokines like IL-6, IL-1b, IL-17a, and TNFα have a crucial role in T cell activation. In this, IL-6 has a vital role in disrupting the blood-brain barrier (BBB) and activates the production of Th17 cells [20], which results in the production of Th1 cells leading to the actual progression of MS. Moreover, elevated levels of IL-6 [21], IL-1 β [22] and TNFα [23] were observed in the CNS of MS patients, which indicates their importance in the promotion of disease.

IL-6 mainly mediates the activation of T cells, a pro-inflammatory cytokine released in response to antigen stimulus and activates the IL6Rs through JAK-STAT pathway. These reactive T cells release pro-inflammatory cytokines like IL-6, IL-1β, and TNFα [24]. These pro-inflammatory cytokines recruit the promotion of Th22 and Th17cells. These trigger the disruption of BBB and promote the entry of activated T cells into the CNS, which leads to the progression of EAE through the further production of IL-17a and IL-22 [25]. Simultaneously, T cells convert into Th17 cells and promote the synthesis of IL-17a-f, IL-22, and TNFα. These, in turn, recruit the promotion of activated T cells and peripheral macrophages into the CNS, this results in extensive demyelination, microglia, and astrocyte activation and neuronal degeneration [26]. Additionally, the active demyelination promotes the elevation of proteinases like MMPs in the CNS [27]. MMPs marks the destruction of myelin proteins, these peptides, in turn, re-activates the autoimmune responses in EAE.

The histopathological investigation revealed that the treatment with Bacoside-A lowered the neutrophil infiltration, demyelination, and cellular damages in CNS of EAE mice. These suggest that the treatment with Bacoside-A can counter the changes produced by the EAE in animals.

5. Conclusion

In conclusion, Bacoside-A inhibited the progression of EAE through the attenuation of CCL-5 and inflammatory cytokines, which limited demyelination, neuronal degeneration, and reduction of clinical progression EAE in mice. Further molecular explorations are required to elucidate the mechanism behind the inhibition of it in T cell activation during autoimmune processes.

Conflict of interest

Authors at this moment confirm that there are no known conflicts of interest related to this publication.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.biopha.2018.10.188.

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