Shikonin ameliorates Experimental Autoimmune Encephalomyelitis (EAE) via Immuno-modulatory, anti-apototic, and anti-oxidative activity

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Research

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Abstract

Background

Multiple sclerosis is a common auto-immuno-inflammatory diseases of the central nervous system in adults. There are several underlying mechanisms for pathogenesis of the disease, including inflammation, oligodendrocyte apoptosis, and oxidative stress.

Methods

We have investigated the mechanism of Shikonin action in C57BL/6 experimental autoimmune encephalomyelitis (EAE) model of multiple sclerosis.

Results

Our results revealed that EAE induction significantly increased the extent of demyelination in the corpus callosum tissues of the animals, while treatment of the mice with Shikonin, significantly decreased the extent of demyelination. Real-time PCR based analyzing the brain samples from the EAE mice, revealed a significant enhancement in the expression level of TNF-α , IFN-γ and Bax genes as well as a reduction in the expression level of TGF-β and Bcl2. Shikonin treatment significantly reduced the expression level of TNF-α , IFN-γ and Bax. On the other hand, the expression levels of TGF-β and Bcl2 as well as the Glutathione peroxidase-1 (GPX-1) enzyme were significantly increased following Shikonin treatment.

Conclusion

This study emphasizes the immune-modulatory, anti-apoptotic, and anti-oxitive effects of Shikonin, which may have an important healing influence on the severity of EAE.

Background

Multiple Sclerosis (MS) is a common auto-immuno-inflammatory and neurodegenerative disease in youth, affecting CNS (1). A combination of genetic, environmental and infectious factors are involved in the disease development (2). MS is described by central demyelinating in the white matter of the CNS and eventually lead to oligodendrocytes apoptosis (3).

Almost 2.5 million individuals worldwide suffering from this disease and the number is increasing (4). Cytokines are important components of immune system, mainly secreted by $CD4^+$ T helper cells and play a critical role in progression of inflammation that leads to axonal damage and brain lesions. $CD4^+$ T helper cells are mainly classified into Th1 and Th2 cells. The first group secretes pro-inflammatory cytokines including: *IL-6*, *IL-12, IFN-γ* and *TNF-α*. Th2 cells, on the other hand, secrete anti- inflammatory cytokines such as *IL-4*, *IL-5* and *TGF-β* (5, 6). The pro-inflammatory cytokines stimulate the triggering of excess free radicals species in active MS lesions (7). The excessive amount of free radicals result in damage to cellular components including DNA and protein (8). There are several studies representing

apoptosis as another pathological mechanism in MS and EAE(9, 10). Therefore, apoptotic pathway studies are essential for discovering new molecular targets and new drugs to treat neuro-inflammatory disorders, like MS. It has been reported that suppressing apoptosis may ameliorate the progression course of EAE (11). Shikonin, the main component of the red pigment from Lithospermum erythrorhizon, is a widely used pharmaceutical agent. Several studies have been reported the anti-inflammatory, anti-apoptotic, antioxidant and neuro-protective effects of Shikonin (12–14). However, more research is yet to be done for elucidating its exact therapeutic potential and mechanisms of action regarding its use in CNS diseases. Among several animal models for MS study, Experimental Autoimmune Encephalomyelitis (EAE) is a widely used model and share a lot of similarities with MS, including inflammation and axonal degeneration (15). Therefore, the aim of the present study was to evaluate the effects of Shikonin on inflammation and apoptosis in C57BL/6 mouse model of experimental autoimmune encephalomyelitis (EAE).

Methods

Animals

A total of 32 female *C57BL/6* mice (10–12 weeks old, 18–20 g) from Pasteur Institute of Iran were used in this study. Subjects were kept under standard circumstance (Controlled temperature and 12-hours light-dark cycle). The mice distributed in four experimental groups (8 mice per group): 1) Control; 2) Sham (EAE was induced and they received daily injection of drug vehicle); 3) EAE; 4) Shikonin treated (EAE was induced and they received 21 days 20 mg/kg Shikonin intraperitoneally). All experiment methods were approved by the Animal Research Ethics Committee of the AJA University of Medical Science, Tehran, Iran.

EAE induction and treatment

Hooke kit (Hooke Laboratories, Inc, USA) was used for induction of EAE. EAE was induced based on the kit protocol as described in our previous study (16). The Shikonin treatment group received daily injection of 20 mg/kg of Shikonin (Sigma-Aldrich, USA) for 21 days. Animals were daily monitored for clinical sign and graded according to 0–5 scoring scale (0, normal mouse; 1, Limp tail; 2, limp tail and hind limbs weakness; 3, paralysis of hind limbs; 4, full paralysis of the hind limbs and fore limbs weakness; 5, full paralysis or death). The mice with borders of two scores were given an average score.

RNA extraction, cDNA synthesis and Real-Time PCR

Isolation of total RNA was performed using the Hybrid-R™ RNA isolation kit (GeneAll, South Korea) from the brain tissues. Evaluation of the extracted RNA quantity and integrity was performed using NanoDrop ND-2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, USA) and RNase-free agarose gel electrophoresis, respectively. The RNA was reverse transcribed to cDNA using Maxima™ H Minus cDNA Synthesis Master Mix (Thermo Fisher Scientific, USA). Real-time PCR was performed based on the BioFACT SYBR Master Mix instructions on an ABI 7500 real-time PCR system (Applied Biosystems, Foster

City, USA). Table 1 shows the specific primers used for amplifying *Hprt-1, Tumor Necrosis Factor-α* (*TNF-α*), *Interleukin-6* (*Il-6*), *Interferon-γ* (*IFN-γ*), *Transforming Growth Factor-ß* (*TGF-β*), *Bax* and *Bcl-2 genes.* The relative expression values were calculated using *Hprt-1* normalizing and $2^{-\Delta\Delta Ct}$ method was used for representing fold change expression.

Table 1
Real time PCR primer sequences

Gene	Sequence (5'→3')
HPRT-1	F: AGCCCCAAAATGGTTAAGGT
	R: CAACGGCATATCCAACAACA
TGF-β	F: CAACAATTCCTGGCGTTACC
	R: GCTGAATCGAAAGCCCTGT
IL-6	F: ATGATGGATGCTACCAAACTGG
	R: TATCTCTCTGAAGGACTCTGGC
TNF-α	F: GCCCACGTCGTAGCAAACC
	R: GTCTTTGAGATCCATGCCGTTG
IFN-γ	F: TGAGTATTGCCAAGTTTGAGGTC
	R: CTGGATTCCGGCAACAGCT
Bax	F: GGAAGGCCTCCTCTCCTACTTC
	R: GAGGACTCCAGCCACAAAGATG
Bcl2	F: TTCGCAGCGATGTCCAGTCAGCT
	R: TGAAGAGTTCTTCCACCACCGT

Measurement of Anti-oxidant Enzymatic Activity

The level of NADPH oxidation is reflected an activity indicator for a group of peroxidase enzymes, including GPX-1. NADPH oxidation was determined based on a method, reported by St. Clair and Chow (17), that we have previously reported (13).

Tissue staining

To evaluate the effects of Shikonin on CNS pathology, the process of Luxol fast blue staining was performed. In summary, after anesthetizing the mice, perfusion was performed using intracardiac injection of 0.1 M PBS. The mice tissues then fixated using paraformaldehyde solutions. After collecting the brain tissues, overnight post-fixation at 4 °C was done. In the next step, samples were processed and embedded in paraffin blocks and coronal cross-sections of each brain sample obtained by rotary microtome. After hydration, the brain sections were stained with Luxol Fast Blue (British Drug House,

London, UK), for demyelination assessment. The extent of demyelination was analyzed using infinity v. 4.6 software (Lumenera Corporation, Canada).

Statistical analysis

Data analyzing was performed using GraphPad Prism software version 7 (La Jolla, Ca, USA).

Two experimental groups were comprised with student t-test. One way or two-way analysis of variance (ANOVA) were used for more than 2 group's comparison. The efficiency values of real-time PCR reactions calculated with The LinReg method and the expression levels were computed using REST 2009 software. The $p < 0.05$ was considered as minimum statistical significance.

Results

Shikonin treatment ameliorates EAE

To investigate the effect of Shikonin treatment, *C57BL/6* mice were immunized with MOG35-55 for EAE induction. For evaluating the disease progress in different groups, all animals were daily weighted and recorded. Shikonin treatment significantly reduced losing weight in animals (Fig. 1A, $p < 0.01$). The animals in the treatment group received Shikonin (20 mg/kg daily via IP injection) on the first day after model induction for a period of 21 days. For confirming the precise effects of Shikonin, a daily injection of ethanol with an equal dose for dissolved Shikonin was performed for sham group. Daily injection of Shikonin not only significantly declined mean clinical scores in comparison to EAE and the sham group (Fig. 1B, $p < 0.01$), but also delayed the onset of EAE sign, and tempered disease severity.

Effects of Shikonin treatment on the level of gene expression

Shikonin effect on the expression level of the genes was estimated in the brain tissues of the mice. The expression level of *IFN-γ*, *Il-6, TNF-α, and Bax* were significantly increased in EAE group in comparison to the control group (all $p < 0.01$). Significant decrease in pro-inflammatory cytokines *IFN-γ* and *TNF-α* as well as *Bax* gene, was observed following Shikonin treatment (Fig. 2).

Our results represented that EAE induction also significantly decreased the mRNA level of *TGF-β* and *Bcl2*. Shikonin administration significantly increased and reformed the expression levels of both genes ($p < 0.001$). Although *Il-6* expression did not remarkably decrease in the Shikonin treatment group (Fig. 2).

Assessment of peroxidase activity

Evaluating the intracellular peroxidase activities, revealed a significant reduction in the activity of peroxidase enzymes in the brain tissues of untreated EAE mice in comparison to controls ($p < 0.01$). Shikonin treatment significantly increased the peroxidase activity, reflecting the induction of various peroxidase enzymes including GPX-1 following Shikonin treatment (Fig. 3).

Histological analysis

Infinity software was used for analyzing the content of demyelinated areas within the tissue section. Histological analysis of the brains in the area of corpus callosum demonstrated a significantly reduction in demyelination in Shikonin treated mice when compared to the untreated EAE group ($p < 0.001$, Fig. 4).

Discussion

As most of the evidence suggests the role of inflammatory cytokines as one of the main causes of MS, the therapeutic approach has tended to anti-inflammatory drugs(18). On the other hand, studies have validated the function of oxidative stress and apoptosis on the pathophysiology of MS, and reduction in the activity of antioxidant enzymes has been clearly indicated in the blood of MS patients (19). It has been reported that among different cytokines, accumulation of TNF-α, IL-6, and IFN-γ may have an essential roles in the disease (20, 21). Accumulation of these cytokines can lead to demyelination, damage to oligodendrocytes and finally the loss of neurons (22). Our results showed that Shikonin greatly delayed incidence and reduced mean clinical score in the mouse model of EAE. In the present study, the expression level of TNF-α, IL-6, and IFN-γ were reduced in EAE group, which were consistent with previous reports (23, 24). TNF-α has a direct role in the induction of oligodendrocytes demyelination, apoptosis and the loss of oligodendrocyte progenitor cells (25, 26), so the enhancement in the percent of demyelination observed in the EAE group may partly due to the elevated TNF-α gene expression which was reduced in Shikonin treated group. IFN-γ is another pro-inflammatory cytokine with an activating role in the process of inflammation in EAE and MS which its Over-expression in the CNS causes progressive demyelinating disease(27). TGF-β is an anti-inflammatory cytokine, mainly produced by T cells, monocytes, astrocytes, microglia and prevents autoimmune response and protect against inflammatory damages (28). Treatment of EAE mice with a monoclonal antibody against TGF-β has reported to cause the worsening of EAE severity (29).

In our study, Shikonin treatment leaded to a significant increase in *TGF-B* mRNA level. The reduced severity of EAE in Shikonin treated group may be partly due to the *TGF-B* elevation.

The level of ROS is also identified to be elevated in MS and the anti-oxidant defense system was also impaired, which leading to enhanced permeability of the blood brain barrier (BBB) (30, 31). Our results showed that Shikonin treatment can restore the anti-oxidant defense system capacity through enhancing the GPX-1 enzyme activity. These results verified antioxidant capacity of Shikonin that is in concordance with previous reports (32, 33). The altered expression of Bax and Bcl2 in EAE mice were also reversed by Shikonin treatment that may due to the anti-apoptotic properties of this compound (33). Overall, a number of limitations exist for the present study. For example, although it was determined that Shikonin suppressed the apoptosis, no histological staining for the apoptosis in oligodendrocytes in the tissues was performed. In conclusion, the present study demonstrated that Shikonin can suppress the apoptosis progression by inhibiting the release of inflammatory mediators and reduction in *Bax* gene expression as

well as Bcl2 elevation. The promising therapeutic value of Shikonin for the treatment of MS was demonstrated, suggesting that it may have potential positive neuroprotective effects on patients with MS.

Conclusion

The present study verified that Shikonin can ameliorates EAE via its immunomodulatory, anti-apoptotic, and antioxidant activities.

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

The datasets analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

Authors declare that they have no conflict of interest.

Funding

Not applicable.

Authors' contributions

Mehrdad Nasrollahzadeh Sabet: contribution to the design and implementation of the work, analysis of data, drafting the work

Sajjad Biglari: contribution to the design of the work, analysis of data, revising the draft of work

Emran Esmaeilzadeh: supervisor and designer of the work, countable for all aspects of the work

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Figures

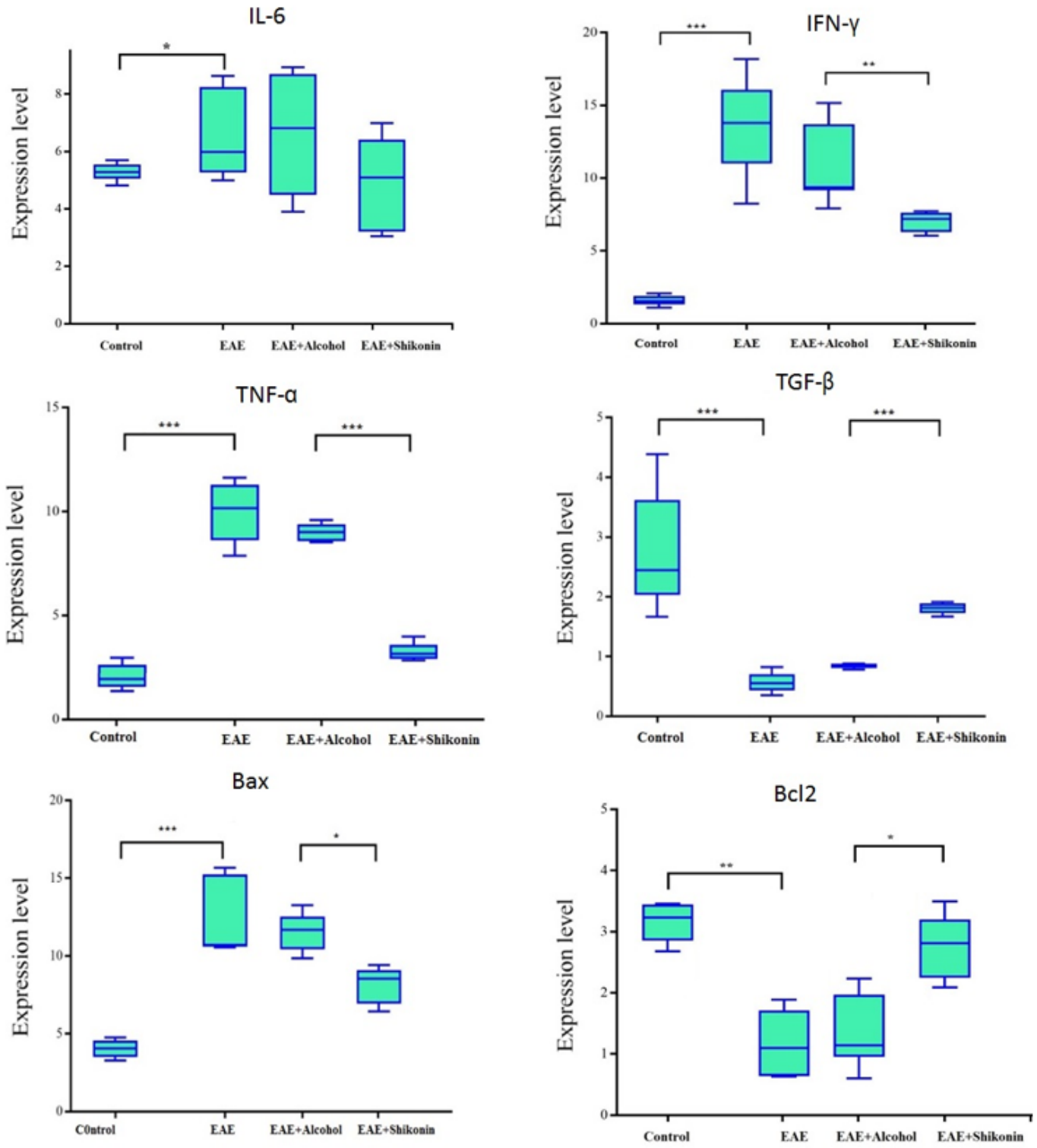

Figure 1

Fig. 2. The effects of Shikonin administration on the cytokine coding and apoptotic related genes expression. Shikonin remarkably declined the expression levels of IFN-γ, TNF-α and Bax as well as increase the expression of TGF-β and Bcl2. Shikonin administration did not have a significant effect on the expression level of IL-6 gene. (*P<0.05, **P<0.01, *** p<0.001). The results are the means ± SEM of data.

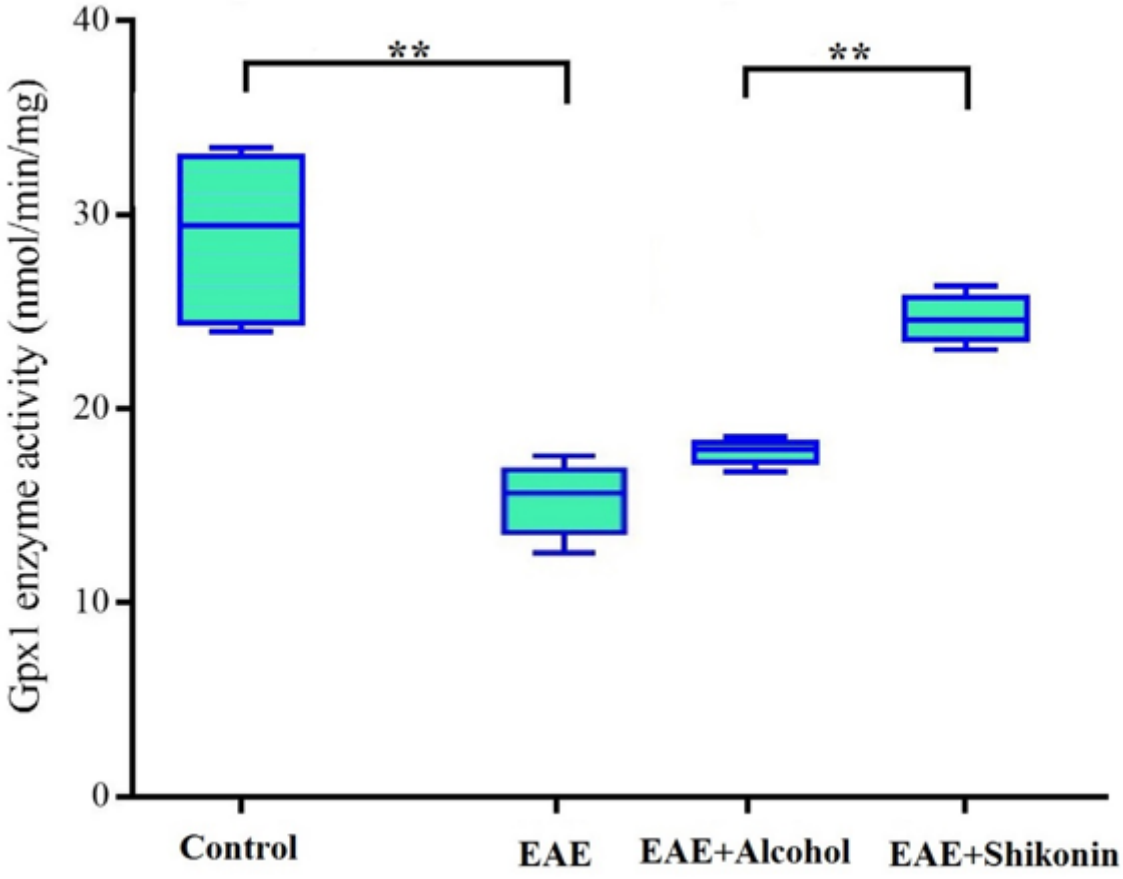

Figure 2

Fig. 3. Shikonin treatment affects Gpx-1 enzyme activity. Anti-oxidant enzyme activity was notably increased after Shikonin administration (*P<0.05, ** p<0. 01). The results are the means± SEM of data.

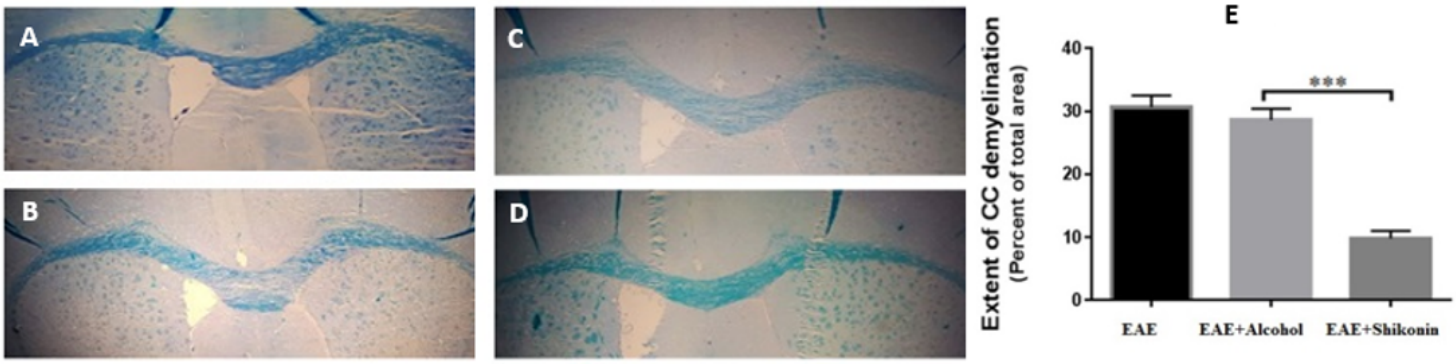

Figure 3

Fig. 4. Effects of Shikonin treatment on demyelination in Corpus callosum. A) Control, B) EAE, C) EAE+ Alcohol, D) EAE + Shikonin, E) percentage of demyelination area. Demyelination area in corpus callosum

was significantly extended in EAE and Sham groups; Shikonin treatment significantly preserved myelin sheath from destruction.