

# Myricitrin attenuates memory impairment in a rat model of sepsis-associated encephalopathy via the NLRP3/Bax/Bcl pathway

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## Abstract

**Introduction:** The present investigation determined the protective effect of myricitrin against sepsis-associated encephalopathy in rats.

**Material and methods:** Sepsis was induced by cecal ligation and puncture (CLP); rats were treated with 30 or 100 mg/kg of myricitrin for 5 days prior to the induction of CLP. The effect of myricitrin was observed by determining the neurological function using the open field test and step-down inhibitory avoidance test. Cerebral oedema and the levels of inflammatory and oxidative stress mediators were determined in brain tissues. Moreover, the expression levels of Bcl-2, Bax, I $\kappa$ B- $\alpha$ , nuclear factor  $\kappa$ B (NF- $\kappa$ B), caspase-3 and NLRP3 were estimated in brain tissues by Western blotting and the mRNA expression of NF- $\kappa$ B, caspase-3 and NLRP3 in brain tissues was estimated by real-time polymerase chain reaction. An immunofluorescence assay was performed to estimate inflammasome activity.

**Results:** The results suggest that treatment with myricitrin protects neuronal function in rats with CLP-induced sepsis. Decreases in inflammation and oxidative stress mediators were observed in the brain tissues of the myricitrin-treated group compared to the CLP group. Moreover, treatment with myricitrin ameliorated the altered Bcl-2, Bax, I $\kappa$ B- $\alpha$ , NF- $\kappa$ B, caspase-3 and NLRP3 protein and mRNA expression levels in the brain tissues of septic rats.

**Conclusions:** The data reveal that myricitrin ameliorated neuroinflammation and improved memory in rats with CLP-induced sepsis by regulating the NLRP3/Bax/Bcl signalling pathway.

**Key words:** myricitrin, neuroinflammation, sepsis, encephalopathy, oedema.

## Introduction

Sepsis is a systemic infection that leads to the development of sepsis-associated encephalopathy (SAE), which causes neurological dysfunction [10]. Several complications are associated with SAE, including delirium, cognitive dysfunction and coma. Patients suffering from SAE have a higher mortality rate, and patients who survive suffer from neurological dysfunction and impaired memory [20]. The

pathogenesis of SAE is unclear but several possible mechanisms have been postulated, including oxidative stress, loss of integrity of the blood-brain barrier, systemic inflammation and apoptosis of neuronal cells [14]. Managing SAE and its complication remains a challenge; thus, an alternative therapy is required for treatment.

The secretion of inflammatory mediators such as interleukin (IL)-6, IL-1 $\beta$ , tumour necrosis factor  $\alpha$

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(TNF- $\alpha$ ) and IL-18 enhances systemic inflammation [23]. Inflammation is indicated due to the presence of myeloperoxidase (MPO), which is abundant in neutrophils [17]. Moreover, oxidative stress damages cell membranes and causes organ dysfunction. NLRP3 inflammasomes become activated due to membrane damage and mitochondrial dysfunction, which further enhance the secretion of IL-1 $\beta$  [12]. Together, these events activate the cellular apoptosis pathway, leading to neuronal cell death in patients with SAE.

Molecules sourced from natural origins have shown potential for managing chronic disorders. Myricitrin is a flavonoid glycoside isolated from *Myrica cerifera* (family *Myricaceae*) [19]. Myricitrin has potential anti-inflammatory, antioxidant and anti-cancer activities [5,6,11]. Myricitrin also possesses an anti-nociceptive effect, free radical scavenging activity and can be used to treat coronary and neurodegenerative disorders [1,2,7]. Myricitrin prevents chronic inflammatory disorders by reducing inflammatory mediators such as ILs and TNF- $\alpha$  [11].

## Material and methods

### Animals

Male Wistar rats (350-400 g body weight) were housed under standard conditions, including a humidity of 60  $\pm$ 5%, temperature of 24  $\pm$ 3°C and a 12 h light/dark cycle. All study protocols were approved by the Institutional Animal Ethical Committee of the Affiliated Hospital of North Sichuan Medical College, Nanchong, China (IAEC/AH-NS-MC/2017/09).

### Chemicals

Myricitrin was procured from Sigma-Aldrich (St. Louis, MO, USA). Enzyme-linked immunosorbent assay (ELISA) kits were purchased from Thermo Fisher Scientific (Waltham, MA, USA). The antibodies used for Western blotting and immunohistochemistry were purchased from Thermo Fisher Scientific.

### Experimental conditions

All animals were anaesthetised by injecting 5 mg/kg of xylazine and 50 mg/kg of ketamine followed by an intravenous infusion of fentanyl at 2 mg/kg/h. The rats were intubated and mechanically ventilated at a tidal volume of 6.5 ml/kg and a respiratory rate of 100/min. Mean arterial pressure was monitored by

applying femoral venous and arterial catheters, and blood gas tension was estimated in blood samples. Isotonic sodium chloride solution was infused at a rate of 0.5 ml/h to maintain hydration. The cecal ligation and puncture (CLP) model was used to produce sepsis as per a previous study [18]. An incision was made at the midline of the lower abdomen, the cecum was ligated and 2-3 punctures were made to allow faeces to leak out. The abdomen was sutured after returning the intestines to the abdominal cavity.

The animals were separated into five groups: the Sham group, the CLP group and the Myricitrin 30 and 100 mg/kg groups, which received 30 or 100 mg/kg myricitrin p.o. for 5 days before and after the induction of sepsis by CLP. At the end of the protocol, blood was withdrawn and the animals were killed by decapitation. The brain from each animal was isolated for further study.

## Assessment of neurological function

### Open field test

Motor function was assessed during a training session and a retention session was performed to assess non-associative memory by the open field test (OFT). The open field apparatus was a square (60  $\times$  60 cm; height of 50 cm); 9 equal squares were formed on the floor of the apparatus with black lines. The TRU SCAN 2.0 system was used to determine movement of the animals in the open field. The number of times that an animal crossed the black lines and reared during the training and test sessions was counted.

### Step-down inhibitory avoidance test

Aversive memory was evaluated using the step-down inhibitory avoidance test. The test was performed on a 50  $\times$  25  $\times$  25 cm box containing stainless steel bars. An automatic device was used to measure the latency to step down on the grid with all four paws. Retention of the inhibitory avoidance memory was considered step-down latency.

### Determination of cerebral oedema

The wet/dry weight method was used to estimate the water content in the brain of each rat. The animals were killed and the wet weights of their brains were measured. The dry weight of the brain was measured

after drying the brain for 1 day. Cerebral oedema was estimated by determining the percentage of water:

$$\text{Percentage of water} = \frac{\text{Wet weight} - \text{dry weight}}{\text{Wet weight}} \times 100\%.$$

### Determination of inflammatory cytokines

Homogenates of the brain tissue samples were used to determine the levels of IL-1 $\beta$ , IL-16 and TNF- $\alpha$ . ELISA kits were used to estimate the levels of inflammatory cytokines according to the manufacturer's instructions.

### Determination of oxidative stress parameters

The cell supernatant was obtained from each isolated brain by sonication in phosphate-buffered saline (PBS) and the activities of catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPX) were determined. A UV spectrophotometer was used. The lipid peroxidation level was estimated in the brain tissue by determining the level of malondialdehyde (MDA) using the Bradford method. The brain tissue homogenate was prepared in KCl buffer and the supernatant was treated with thiobarbituric acid, sodium dodecyl sulphate (SDS), acetic acid and distilled H<sub>2</sub>O. Samples were observed at a wavelength of 532 nm and the level of MDA was expressed as nmol MDA/mg protein.

### Determination of MPO activity

MPO activity was estimated using the Bradford method. The supernatant from each brain tissue homogenate was separated by centrifugation for 40 min at 15,000 rpm. The supernatant was mixed with 1 mM H<sub>2</sub>O<sub>2</sub> and 1.6 mM tetramethylbenzidine. A SpectraMax microplate reader was used to determine MPO activity at a wavelength of 650 nm.

### Western blotting

The assessment of Bcl-2, Bax, I $\kappa$ B- $\alpha$ , NF- $\kappa$ B, caspase-3 and NLRP3 protein expression was done using brain tissue homogenates by Western blotting. A BCA assay kit was used to quantify the protein from each tissue homogenate and 10% SDS-polyacrylamide gel electrophoresis was used to separate the proteins, which were transferred to a nitrocellulose membrane by electroblotting. Subsequently, each membrane was blocked with 5% blocking solution (non-fat milk) and incubated in blocking buffer

with the following primary antibodies overnight at 4°C: Bcl-2 (1 : 200), Bax (1 : 200), I $\kappa$ B- $\alpha$  (1 : 100), p-I $\kappa$ B- $\alpha$  (1 : 100), NF- $\kappa$ B (1 : 200), caspase-3 (1 : 100), NLRP3 (1 : 500) and  $\beta$ -actin (1 : 100). Goat secondary antibodies conjugated with horseradish peroxidase were added to the blocking buffer and a chemiluminescence kit was used to detect the proteins.

### Real-time polymerase chain reaction analysis

RNA was isolated from the brain tissues using TRIzol reagent. A RevertAid First Strand cDNA Synthesis Kit (Fermentas, Burlington, ON, Canada) was used to reverse-transcribe the RNA. The primers listed below were mixed with RT2 SYBR Green Master Mix (Superarray, Frederick, MD, USA) to determine gene expression using quantitative SYBR Green PCR assays.

Primer	Sense	Antisense
NLRP3	5'-AGGAGGAAGAAGAAGAGAGGA-3'	5'-AGAGACCACGGCAGAAGC-3'
NF- $\kappa$ B	5'-GAGCAAATGGTGAAGGAG-3'	5'-TCTGGAAGTTGAGGAAGG-3'
Caspase-3	5'-CGTCTTGCCCTCATTATCTG-3'	5'-TCACCTCTTCACCATCTCC-3'

### Immunohistochemistry

Isolated brain tissues were fixed in formaldehyde (4%) and the tissues were washed with PBS. The tissue sections were incubated overnight at 4°C with primary antibodies, including anti-NLRP3 antibodies. The sections were treated with secondary antibodies for 60 min, and the tissue sections were stained with 4',6-diamidino-2-phenylindole. Fluorescence microscopy was used to capture images at 400 $\times$  magnification.

### Statistical analysis

All data are expressed as the mean  $\pm$  standard error ( $n = 10$ ). The statistical analysis was performed using a one-way analysis of variance. A post-hoc comparison of means was carried out by Dunnett's post-hoc test (GraphPad Prism 6.1.; GraphPad Software Inc., La Jolla, CA, USA). A  $p$ -value < 0.05 was considered significant.

## Results

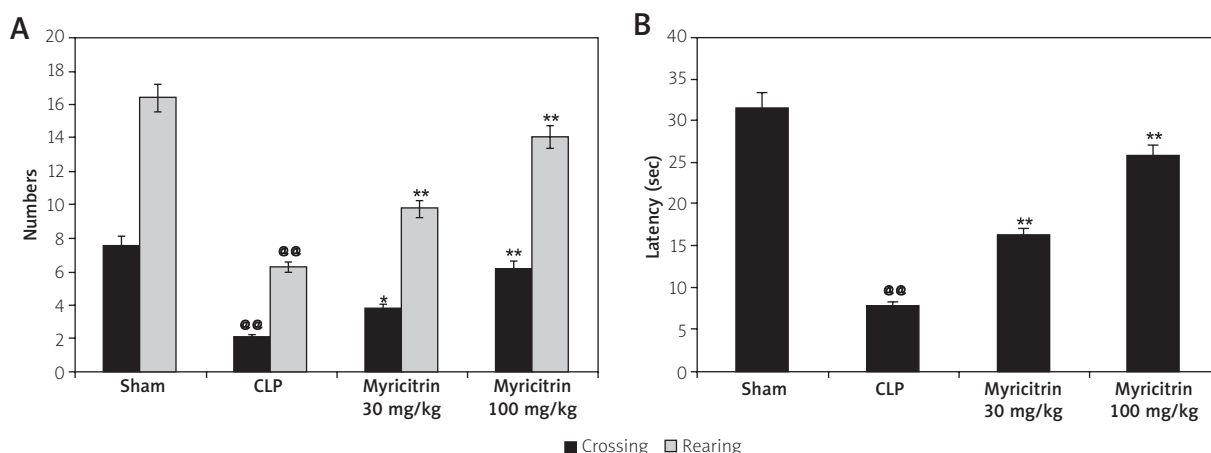
### Effect of myricitrin on neurological function

Neurological function and memory were assessed in myricitrin-treated rats with CLP-induced sepsis (Fig. 1). The OPT results suggest that the number of squares crossed and rearing

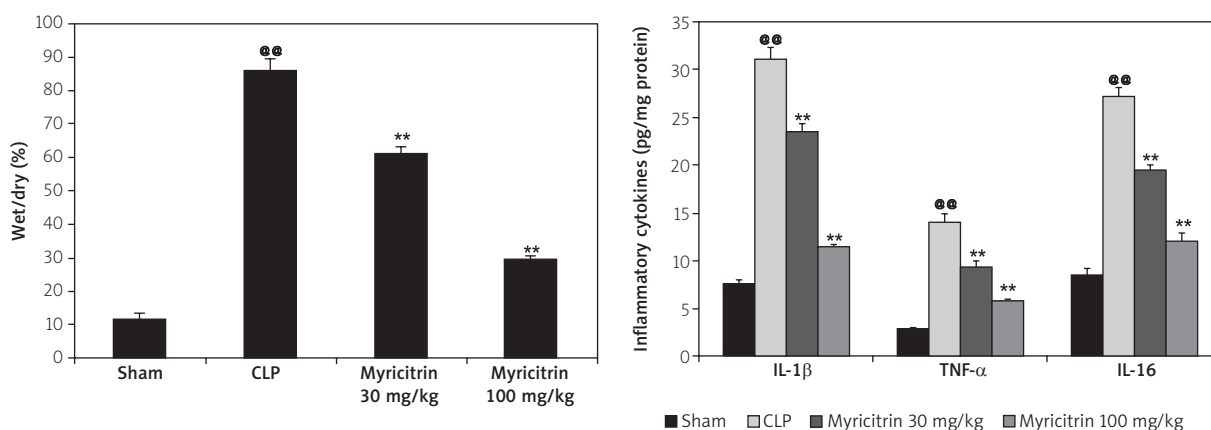
were reduced in the CLP group compared to the sham-operated rats. There was improvement in the number of rearings and crossings in the myricitrin-treated group compared to the CLP group (Fig. 1A). Moreover, latency to step down was significantly reduced in the CLP group compared to the sham-operated group. Latency to step down was enhanced in the myricitrin-treated group compared to the CLP group (Fig. 1B).

### Effect of myricitrin on cerebral oedema

Figure 2 shows the effect of myricitrin on cerebral oedema after estimating the wet/dry ratio in rats with CLP-induced sepsis. The percentage wet/dry weight ratio of brain tissue was significantly ( $p < 0.01$ ) enhanced in the CLP group compared to the sham-operated group. A reduction in the percentage wet/dry weight ratio of brain tissue was detected in the myricitrin-treated group compared to the CLP group.



**Fig. 1.** Effect of myricitrin on the neurological function in rats with CLP-induced sepsis. **A)** Effect of myricitrin on the number of crossings and rearings on the open field test. **B)** Effect of myricitrin on latency according to the step-down inhibitory avoidance test. Mean  $\pm$  standard error ( $n = 10$ ); @@ $p < 0.01$  compared to the sham group; \*\* $p < 0.01$  compared to the CLP group.



**Fig. 2.** Effect of myricitrin on cerebral oedema by determining the wet/dry ratio in rats with CLP-induced sepsis. Mean  $\pm$  standard error ( $n = 10$ ); @@ $p < 0.01$  compared to the sham group; \*\* $p < 0.01$  compared to the CLP group.

**Fig. 3.** Effect of myricitrin on inflammatory mediators in the brain tissue of rats with CLP-induced sepsis. Mean  $\pm$  standard error ( $n = 10$ ); @@ $p < 0.01$  compared to the sham group; \*\* $p < 0.01$  compared to the CLP group.

**Table I.** Effect of myricitrin on oxidative stress mediators in the brain tissues of rats with CLP-induced sepsis. Mean  $\pm$  standard error ( $n = 10$ );  $^{\text{@@}}p < 0.01$  compared to the sham group;  $^{**}p < 0.01$  compared to the CLP group.

Sr. no.	Groups	CAT (U/g)	SOD (U/g)	GPX (U/g)	MDA (nmol MDA/mg protein)	MPO (nmol MPO/mg protein)
1	Sham	2452 $\pm$ 183	67.8 $\pm$ 5.21	52.8 $\pm$ 4.27	21.8 $\pm$ 1.83	148.3 $\pm$ 7.29
2	CLP	984 $\pm$ 129 <sup>@@</sup>	13.6 $\pm$ 1.72 <sup>@@</sup>	9.45 $\pm$ 1.35 <sup>@@</sup>	76.29 $\pm$ 6.33 <sup>@@</sup>	682.4 $\pm$ 23.84 <sup>@@</sup>
3	Myricitrin 30 mg/kg	1396 $\pm$ 152 <sup>**</sup>	32.4 $\pm$ 2.65 <sup>**</sup>	28.16 $\pm$ 2.92 <sup>**</sup>	51.62 $\pm$ 4.76 <sup>**</sup>	458.7 $\pm$ 18.46 <sup>**</sup>
4	Myricitrin 100 mg/kg	2206 $\pm$ 206 <sup>**</sup>	56.2 $\pm$ 4.73 <sup>**</sup>	40.41 $\pm$ 5.38 <sup>**</sup>	29.36 $\pm$ 2.94 <sup>**</sup>	208.9 $\pm$ 9.74 <sup>**</sup>

### Effect of myricitrin on inflammatory mediators

The levels of inflammatory mediators were estimated in the brain tissue of septic rats by ELISA. Significant increases in the levels of IL-1 $\beta$ , IL-16 and TNF- $\alpha$  were observed in the CLP group compared to the sham-operated group. However, the levels of inflammatory mediators in the brain tissues of the myricitrin-treated group were lower than those in the CLP group (Fig. 3).

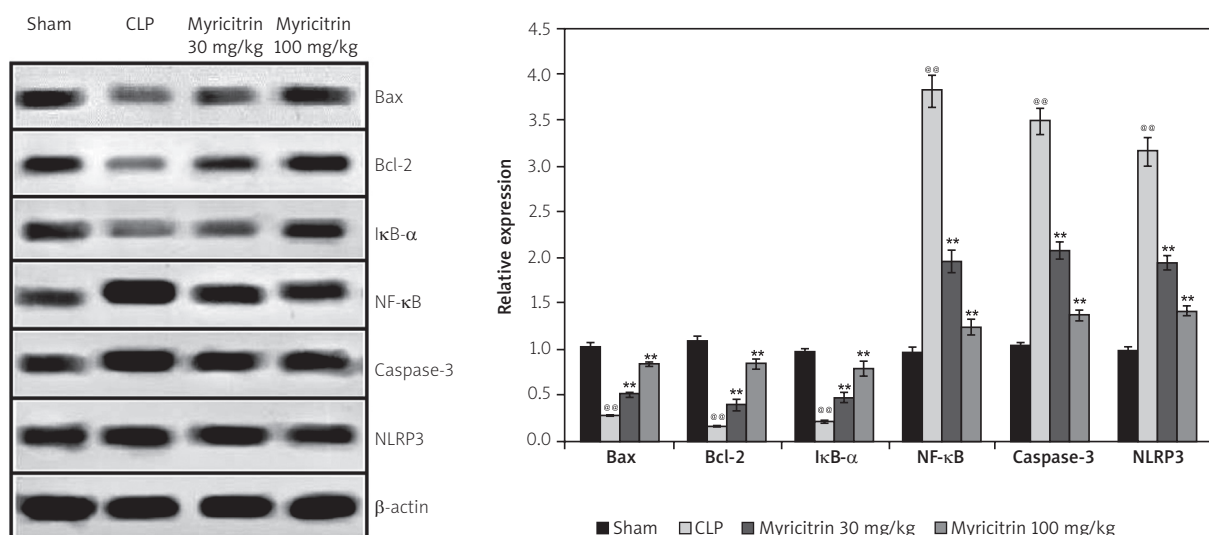
### Effect of myricitrin on oxidative stress mediators

Oxidative stress parameters (i.e., activity levels of CAT, SOD and GPX), and the levels of MPO and MDA were assessed in the brain tissues of the CLP and

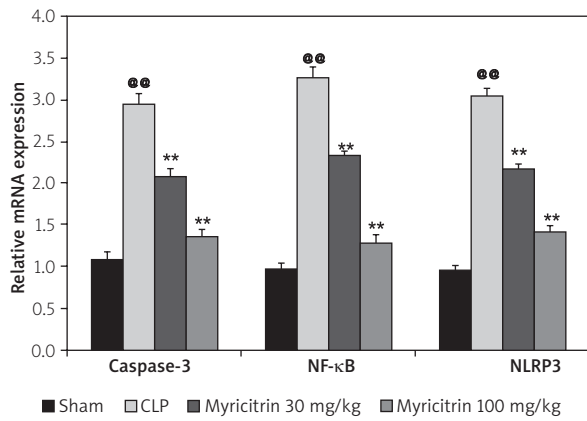
myricitrin-treated rats. SOD, CAT and GPX activity was decreased in the CLP group compared to the sham-operated group. However, the MPO and MDA levels were enhanced in the brain tissues of the CLP group compared to the sham-operated group. Treatment with myricitrin enhanced SOD, CAT and GPX activity in the brain tissue of septic rats compared to those in the CLP group. Moreover, the MPO and MDA levels decreased in the brain tissues of the myricitrin-treated group compared to the CLP group of rats (Table I).

### Effect of myricitrin on Bcl-2, Bax, I $\kappa$ B- $\alpha$ , NF- $\kappa$ B, caspase-3 and NLRP3 protein expression

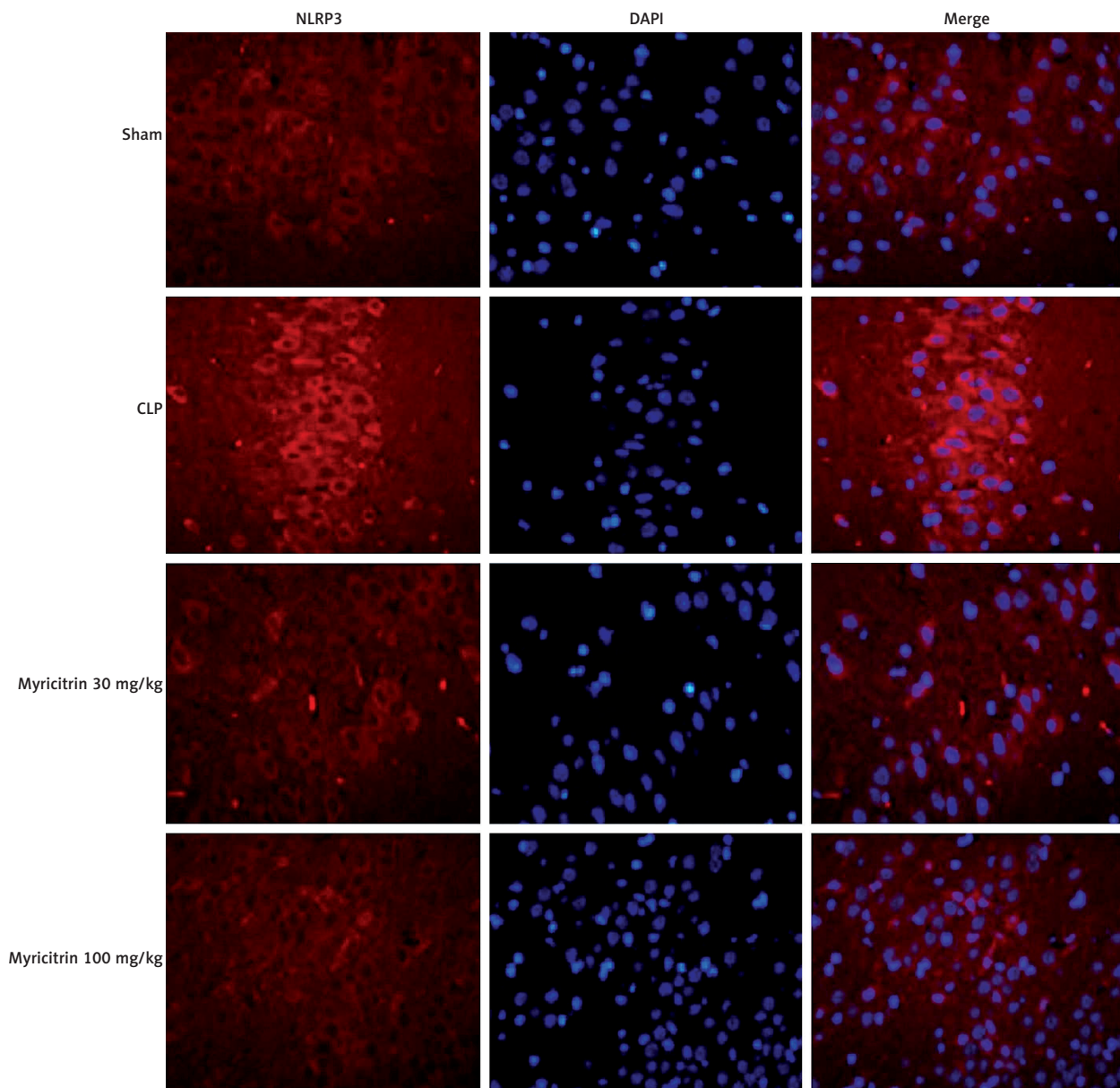
Bcl-2, Bax, I $\kappa$ B- $\alpha$ , NF- $\kappa$ B, caspase-3 and NLRP3 expression was estimated in septic rats treated



**Fig. 4.** Effect of myricitrin on Bcl-2, Bax, I $\kappa$ B- $\alpha$ , NF- $\kappa$ B, caspase-3 and NLRP3 expression in the brain tissues of rats with CLP-induced sepsis. Mean  $\pm$  standard error ( $n = 10$ );  $^{\text{@@}}p < 0.01$  compared to the sham group;  $^{**}p < 0.01$  compared to the CLP group.



**Fig. 5.** Effect of myricitrin on NF-κB, caspase-3 and NLRP3 mRNA expression in the brain tissues of rats with CLP-induced sepsis. Mean ± standard error (n = 10); <sup>@@</sup>p < 0.01 compared to the sham group; <sup>\*\*</sup>p < 0.01 compared to the CLP group.



**Fig. 6.** Effect of myricitrin on inflammasome activity in the brain tissues of rats with CLP-induced sepsis.

with myricitrin, as shown in Figure 4. Bax, Bcl-2 and  $\text{I}\kappa\text{B-}\alpha$  expression decreased, whereas the expression of NF- $\kappa\text{B}$ , caspase-3 and NLRP3 was enhanced in the brain tissues of the CLP group compared to the sham-operated group. Significant enhancement in the expression of Bax, Bcl-2 and  $\text{I}\kappa\text{B-}\alpha$  and reduced expression of NF- $\kappa\text{B}$ , caspase-3 and NLRP3 were observed in the brain tissues of the myricitrin-treated group compared to the CLP group.

### Effect of myricitrin on NF- $\kappa\text{B}$ , NLRP3 and caspase-3 mRNA expression

Figure 5 shows the effect of myricitrin on the mRNA expression of NF- $\kappa\text{B}$ , caspase-3 and NLRP3 in the brain tissues of rats with CLP-induced sepsis. NF- $\kappa\text{B}$ , caspase-3 and NLRP3 mRNA expression was significantly enhanced in the brain tissues of the CLP group compared to the sham-operated group. Treatment with myricitrin attenuated the changes in the mRNA levels of NF- $\kappa\text{B}$ , caspase-3 and NLRP3 in the brain tissues of the septic rats.

### Effect of myricitrin on inflammasome activity

The effect of myricitrin on inflammasome activity in the brain tissues of rats with CLP-induced sepsis was determined by immunofluorescence (Fig. 6). The number of NLRP3-positive cells in brain tissues of the CLP group increased compared to the sham-operated group. However, myricitrin suppressed the activation of microglia by reducing the number of NLRP3-positive cells in brain tissues compared to the CLP group of rats.

## Discussion

Sepsis is a systemic infection caused by the dysfunction of different organs, including the brain. Morbidity and mortality are enhanced in patients with SAE [16]. Several factors contribute to the development of SAE, including neurotoxicity, apoptosis and neuroinflammation, and SAE leads to complex pathological changes [3]. SAE causes many changes resulting in neuroinflammation, oxidative stress and apoptosis of neuronal cells [13]. The management of SAE is challenging and alternative medicines have shown potential to treat chronic disorders. Thus, the present investigation determined the protective effect of myricitrin against SAE in septic rats. Sepsis was produced by CLP and rats were treated with 30 or 100 mg/kg of myricitrin for 5 days prior to

the induction of CLP. The effects of myricitrin were observed by determining the neurological function using the OFT and step-down inhibitory avoidance test. Cerebral oedema and the levels of inflammatory and oxidative stress mediators were determined in brain tissues. Moreover, Bcl-2, Bax,  $\text{I}\kappa\text{B-}\alpha$ , NF- $\kappa\text{B}$ , caspase-3 and NLRP3 expression was estimated in brain tissues by Western blotting. Real-time polymerase chain reaction was used to estimate NF- $\kappa\text{B}$ , caspase-3 and NLRP3 mRNA expression in brain tissues. An immunofluorescence assay was performed to estimate inflammasome activity.

Neuroinflammation occurs during sepsis, resulting in an increased demand for energy and metabolism by neuronal cells [15]. This leads to mitochondrial dysfunction and increased oxidative stress in neuronal cells [8]. Our results suggest that myricitrin reduced the levels of inflammatory cytokines and oxidative stress parameters in brain tissues compared to those in the CLP group of rats. Several reports have suggested that inflammatory cytokines and neuronal inflammation activate apoptosis in neuronal cells by initiating mitochondria-dependent apoptosis [21]. Mitochondrial-dependent apoptosis is activated due to changes in the balance between anti-apoptotic and proapoptotic proteins [9]. Bcl-2 and Bax are anti-apoptotic proteins that inhibit the mitochondrial-dependent apoptosis pathway [4]. Moreover, caspase activity contributes to the apoptosis of neuronal cells. Our results show that myricitrin attenuated the altered expression of caspase-3, Bax and Bcl-2 in the brain tissues of septic rats.

Some studies have revealed that activated NLRP3 inflammasomes lead to the development of neurodegenerative disorders [22]. Moreover, NLRP3 promotes activation of the caspase pathway by activating caspases-1, -3 and -9. Activation of the caspase pathway causes apoptosis of neuronal cells [24]. The results of this study suggest that expression of caspase-3, NF- $\kappa\text{B}$  and NLRP3 decreased in the brain tissues of the myricitrin-treated group compared to the CLP group.

## Conclusions

In conclusion, our data reveal that myricitrin ameliorated neuroinflammation and improved memory in rats with CLP-induced sepsis. Myricitrin ameliorated neuronal apoptosis by regulating the NLRP3/Bax/Bcl signalling pathway in CLP-induced septic rats.

## Acknowledgements

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## Disclosure

The authors report no conflict of interest.

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