Original Article

# Effect of *Centella asiatica L.* Extract on Apoptosis and Bcl-2 Immunoexpression of Pyramidal Cells in Traumatic Brain Injury Rat Model

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## Abstract

**Background:** Traumatic brain injury therapy still has many shortcomings. The slow repair of post-traumatic pyramidal cells causes the need to find new alternatives to deal with the consequences of traumatic brain injury. This study aims to determine the effect of *Centella asiatica L.* extract in increasing pyramidal cells repair, assessed from the apoptosis and B-cell lymphoma 2 (Bcl-2) immunoexpression of pyramidal cells in traumatic brain injury rat model. **Methods:** This research was conducted during July 3–17, 2020 and used a true experimental research design with a posttest-only controlled group design. Rats were divided into five groups, that is, normal group, group treated with traumatic brain injury, and groups treated with traumatic brain injuries and given extracts of *C. asiatica (L.)* dose of 150, 300, and 600 mg/kg bw/day. Brains from each group were taken to examine the apoptosis and Bcl-2 immunoexpression of pyramidal cells at day 7. This study used Kruskal–Wallis test and post hoc Mann–Whitney test. **Results:** Based on the statistical analysis results, there was a significant correlation between the Bcl-2 immunoexpression and apoptosis of pyramidal cells in traumatic brain injury rat model with different doses of *C. asiatica (L.)* extract ($P < 0.05$). Dose of 600 mg/kgBW was the most effective in decreasing apoptosis and increasing Bcl-2 immunoexpression of pyramidal cells in traumatic brain injury rat model. **Conclusion:** This study proved that *C. asiatica (L.)* extract can decrease apoptosis and increase Bcl-2 immunoexpression of pyramidal cells in traumatic brain injury rat model.

**Keywords:** B-cell lymphoma 2 immunoexpression, *c. asiatica (l.)* extracts, pyramidal cells apoptosis, traumatic brain injury

## Introduction

Traumatic brain injury is brain damage resulting from an injury to the brain and is not the result of a degenerative or congenital process. Traumatic brain injury is the third cause of injury-related death, accounting for 30%.[1]

The mechanics of traumatic brain injury are complex. Traumatic brain injury results in cell death and neurological dysfunction due to primary and secondary physical impairment. Primary brain injury is caused by mechanical processes affecting the cerebral structures that cause distortion and damage in the early period of trauma.[2] Secondary brain injury occurs within hours, days, and months after the head injury and will cause neurochemical, metabolic, and cellular changes.[3] These secondary brain injuries will eventually lead to neuron cell apoptosis.[4]

Apoptosis is a form of programmed cell death. One of the regulators of apoptosis is a member of the family B-cell lymphoma 2 (Bcl-2). Currently, 18 members of the Bcl-2 family have been identified and are divided into two groups based on their structure, namely, the proapoptotic and antiapoptotic protein groups. The antiapoptotic protein group is represented by Bcl-2 and Bcl-xL.[5] The

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dominance of Bcl-2 and Bcl-xL can improve cell survival. In the nervous system, Bcl-2 protects nerve cells from various stimuli that cause neuronal apoptosis death.[6]

Several studies have shown that traumatic brain injury affects cerebral blood flow, causing global or focal cerebral ischemia. The grace period between the occurrence of trauma and the onset of ischemia provides time for us to provide neuroprotection therapy.[7] The goal of neuroprotection therapy in traumatic brain injury is to protect the brain and reduce secondary brain damage and prevent neurological death in the penumbra area.[8] Several drugs have been studied as neuroprotection but none have yet been shown to produce satisfactory results. Currently, research that focuses on herbal plants is widespread in the world. One of the many herbal plants used in research is Gotu Kola (*C. asiatica (L.)*).[9] *C. asiatica (L.)* has neuroprotective factors. So it is hoped that this plant can provide protection and improve pyramidal cells repair by decreasing apoptosis and increasing Bcl-2 immunoexpression in traumatic brain injury rat model.

# Methods

## Research design

The study was conducted during July 3–17, 2020. This study used a true experimental design with posttest-only controlled group design on the Wistar strain rat (*Rattus norvegicus*). The inclusion criteria used were white Wistar rats, male, 2 to 3 months old, weight between 150 and 200 g, healthy, and active. The exclusion criteria were sick and dead rats during acclimatization and during the study.

In this study, rats were divided into five groups, that is, normal group (P1), group treated with traumatic brain injury (P2), groups treated with traumatic brain injuries, and groups given extracts of *C. asiatica (L.)* dose of 150, 300, and 600 mg/kg bw/day (P3, P4, and P5). Each group consisted of seven rats. Figs. 1 and 2

All protocols related to experimental animals have been approved by the Research Ethics Commission of the Faculty of Medicine, Jenderal Soedirman University (038/KEPK/IV/2020).

As many as 35 rats were purchased and maintained at the Integrated Research and Testing Laboratory (LPPT) unit IV Gajah Mada University, Yogyakarta. The rats were acclimatized (adapted) for 7 days with the maintenance standard of the experimental animals.

## Traumatic brain injury model

The rats were anesthetized and then shaved their heads and cleaned with 70% alcohol. Then the scalp is opened. The mouse was placed on the flatbed with its four legs tied so that it was fixed with a hard base. Iron cylinder weighing 45 g (4 mm diameter) is dropped at an angle of 90° from a height of 25 cm one time.

## Extraction of *C. asiatica (L.)*

The *C. asiatica (L.)* plant was washed first and then dried in the sun and continued with heating. The simplicia is made into powder using a grinder. The fine powder is macerated with 96% ethanol solvent and then left for 24 hours. Furthermore, it was remacerated using 96% ethanol and allowed to stand again for 24 hours and then filtered using a Buchner. The filtrate obtained is concentrated using a rotary evaporator at a temperature of 40°C until a thick extract is obtained. The extract was dried in a water bath at 60–70°C. The extract was dissolved with 0.5% carboxymethyl cellulose (CMC) as a surfactant. *C. asiatica (L.)* therapy is given orally using probe every day for 7 days at a dose of 150, 300, and 600 mg/kg bw/day.

## Examination of Bcl-2 immunoexpression in pyramidal cells

The slides were washed using phospate buffer saline (PBS) with pH 7.4 once for 5 minutes. Endogenous peroxide blocking was performed using 3% HO for 20 minutes and then washed using PBS pH 7.4 three times for 5 minutes. Unspecified blocking using the 5% FBS containing 0.25% Triton X-100, which is then washed using PBS pH 7.4 three times for 5 minutes. Incubation was performed using anti-polyclonal rabbit Bcl-2 (Santacruz) for 60 minutes and then washed off using PBS pH 7.4 three times for 5 minutes. Furthermore, incubation was carried out using anti-rabbit horse readish peroksidase (HRP) conjugated for 40 minutes and washed using PBS pH 7.4 three times for 5 minutes, followed by drip with diaminobenzidine and incubation for 10 minutes. Washed using PBS pH 7.4 three times for 5 minutes and $dH_2O$ for 5 minutes. Counter staining was performed using Mayer Hematoxilin which was incubated for 10 minutes. Wash using tap water and rinse with $dH_2O$ and air dry. Mounting is done using mounting media and then covered with a glass cover. Observation was done on a light microscope.

Counting Bcl-2 immunoexpression in rat brain tissue by looking at a microscope at 100x magnification then increasing to 400x and counting pyramidal cells expressing Bcl-2 in the cytoplasm in five fields of view. The results of the assessment are given a score according to Allred's score.

## Observation of apoptosis in pyramidal cells with hematoxylin and eosin staining

The brain organs fixed with NBF were cut and inserted into the specimen holder. Dehydration and cleaning processes are carried out by inserting brain organs into alcohol with graded concentrations. Then the embedding process is carried out into paraffin. The results of the "embedding" are made of paraffin blocks (blocking) using iron molds. After the paraffin was frozen, the paraffin block was cut using a microtome with a thickness of 5 mm. The cut portions are put into a water bath at a temperature of 42 to 45°C and then dried. Then paraffin is removed using graded alcohol before

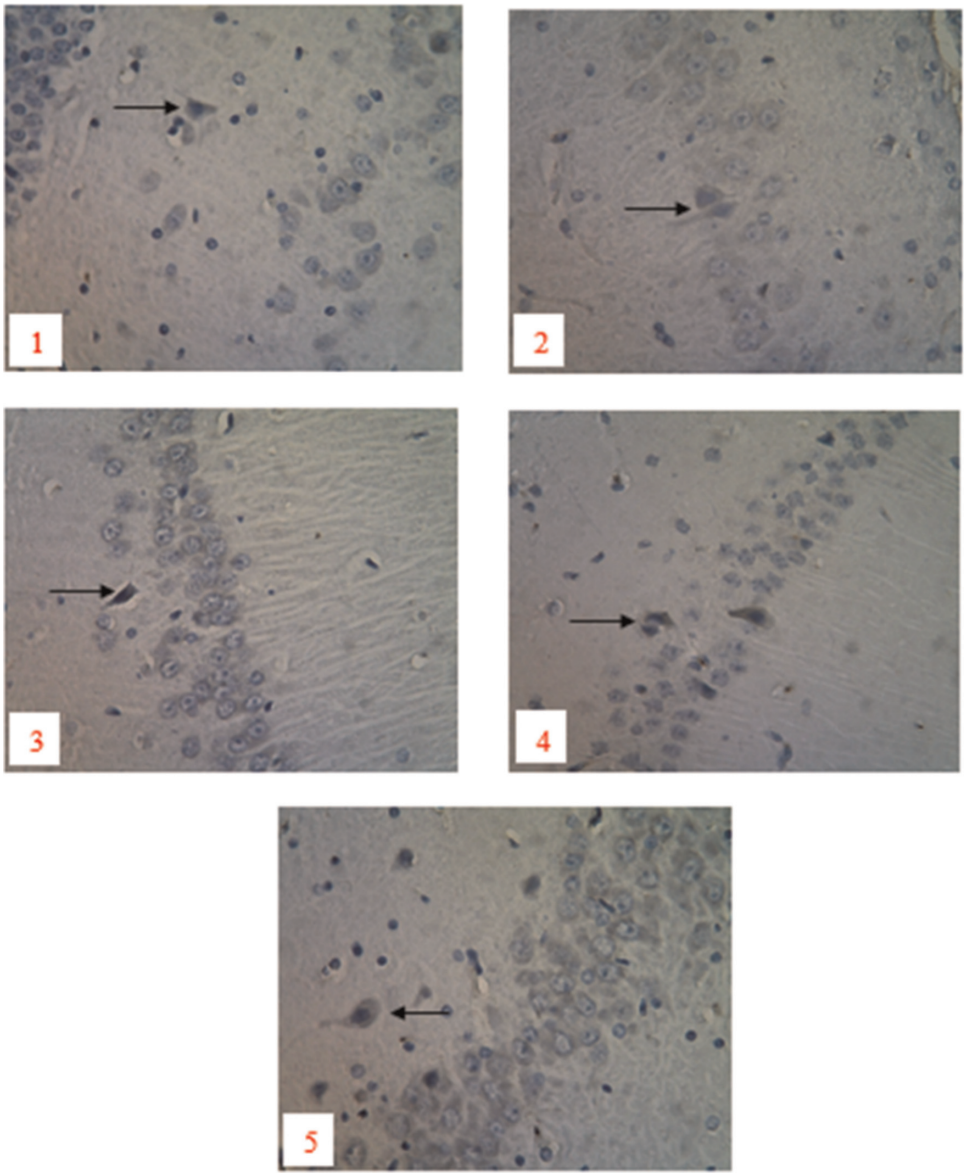


**Figure 1:** Bcl-2 immunoexpression in the control group and the treatment group (microscope magnification 400×). 1: P1; 2: P2; 3: P3; 4: P4; 5: P5. Black arrow: cells expressing Bcl-2. Bcl-2, B-cell lymphoma 2.

staining. Furthermore, the tissue is inserted into the hematoxylin and eosin (HE) dye. Finally, the mounting process is done by the closure of the object glass with a glass cover that was dripped using the mounting media.

Observation of apoptosis in pyramidal cells is performed by looking at the microscope at 100× magnification and then up to 400× and the pyramidal cells whose cell nucleus underwent picnosis, cariolisis, and cariorection were counted as five fields of view, then the average is calculated.

### Statistic analysis

Tests of normality showed that the normality test shows that the data is not normally distributed; therefore, Kruskal–Wallis was performed to compare the mean number of cells between groups, followed by a post hoc test to determine differences between specific groups. The critical level for rejection of the null hypothesis was considered to be a *P* value of 0.05.

## Results

### Effect of *C. asiatica* extract on Bcl-2 immunoexpression in rat brain

The mean expression of the antiapoptotic protein Bcl-2 in the normal group was 1.171 ± 0.382. Mean Bcl-2 immunoexpression in this group was the lowest compared to the other groups. Mean Bcl-2 immunoexpression in the group treated with traumatic brain injury (P2) was 1.314 ± 0.471, which was higher than that in the normal group but lower than in the groups receiving extracts of *C. asiatica (L.)* (groups P3, P4, P5). Groups P3, P4, and P5 receiving extracts of *C. asiatica (L.)* at a dose of 150, 300, and 600 mg/kg bw/day, respectively, had mean Bcl-2

immunoexpression values of 1.543 ± 0.471, 1.743 ± 0.700, and 1.914 ± 0.742, respectively. The results of the Kruskal–Wallis test showed significant differences in the Bcl-2 immunoexpression. To find out between which groups there was a significant difference, follow-up analysis using a post hoc Mann–Whitney test was performed (Table 1).

Results of post hoc analysis showed that the normal group (P1) was significantly different from the groups exposed to traumatic brain injury + *C. asiatica (L.)* dosage of 150 mg/kg bw/day (P3), traumatic brain injury + *C. asiatica (L.)* dosage of 300 mg/kg bw/day (P4), and traumatic brain injury + *C. asiatica (L.)* dosage of 600 mg/kg bw/day (P5). Group P2 was significantly different from the groups exposed to traumatic brain injury + *C. asiatica (L.)* dosage of 300 mg/kg bw/day (P4) and traumatic brain injury + *C. asiatica (L.)* dosage of 600 mg/kg bw/day (P5). Group P3 was significantly different from the group exposed to traumatic brain injury + *C. asiatica (L.)* dosage of 600 mg/kg bw/day (P5).

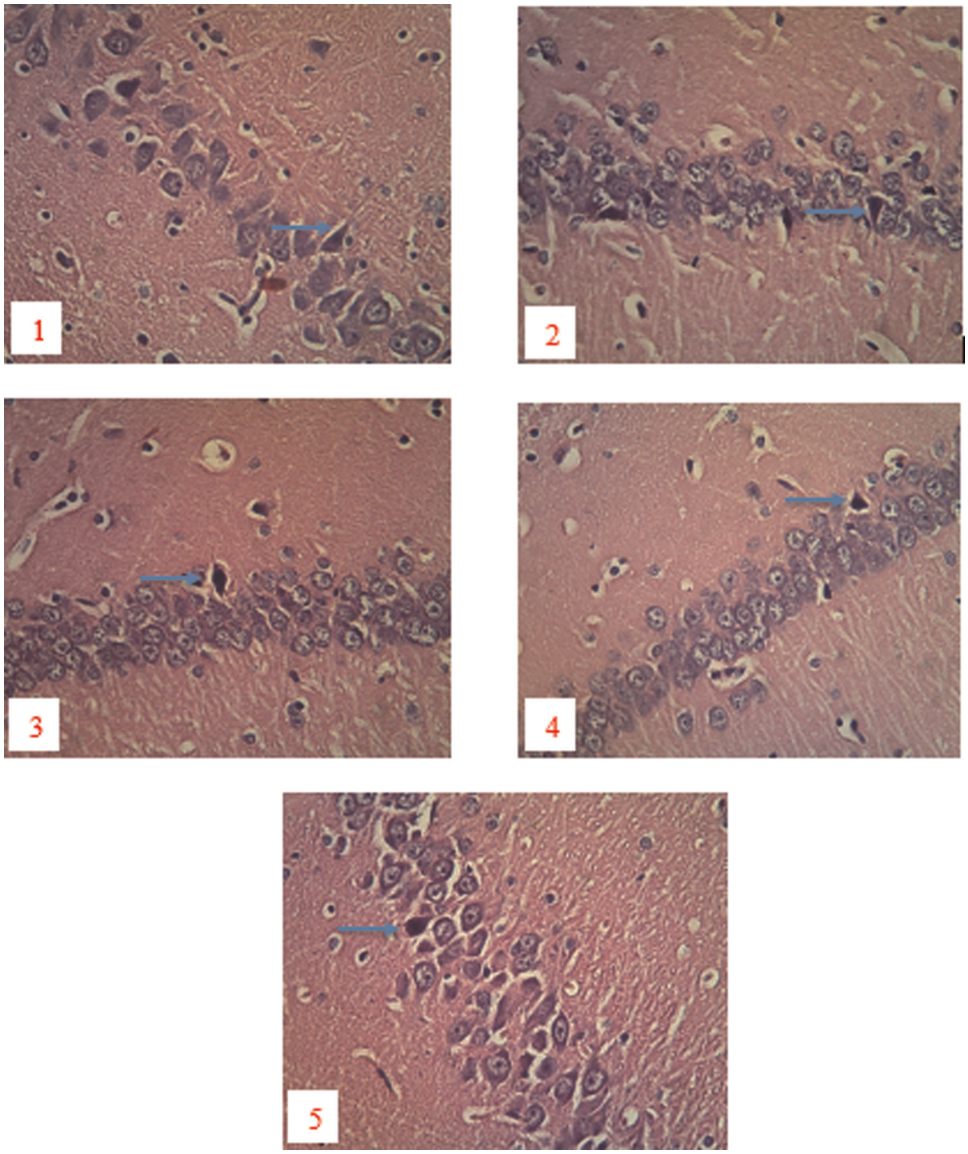


**Figure 2:** Apoptosis of Pyramidal Cells in the control group and the treatment group (microscope magnification 400×). 1: P1; 2: P2; 3: P3; 4: P4; 5: P5. Blue arrow: apoptosis of pyramidal cells.

**Table 1: Mean Bcl-2 immunoexpression by treatment groups**

| | Treatment groups | | | | | *P* |
|---|---|---|---|---|---|---|
| | P1 | P2 | P3 | P4 | P5 | |
| Bcl-2 immunoexpression | 1.171±0.382 | 1.314±0.471 | 1.543±0.657 | 1.743±0.700 | 1.914±0.742 | 0.000 |

Bcl-2, B-cell lymphoma 2.

### Effect of *C. asiatica* extract on apoptosis of pyramidal cells

The mean apoptosis of pyramidal cells in the normal group was 0.429 ± 0.558. Mean apoptosis of pyramidal cells in this group was the lowest compared to the other groups. Mean apoptosis of pyramidal cells in the group treated with traumatic brain injury (P2) was 1.514 ± 0.818, which was higher compared to the other groups. Groups P3, P4, and P5, receiving extracts of *C. asiatica (L.)* at a dose of 150, 300, and 600 mg/kg bw/day, respectively, had mean apoptosis of pyramidal cells values of 1.457 ± 0.700, 0.943 ± 0.725, and 0.600 ± 0.604, respectively. The results of the Kruskal–Wallis test showed significant differences in the mean apoptosis of pyramidal cells. To find out between which groups there was a significant difference, follow-up analysis using a post hoc Mann–Whitney test was performed (Table 2).

Results of post hoc analysis showed that the normal group (P1) was significantly different from the group exposed to traumatic brain injury (P2), traumatic brain injury + *C. asiatica (L.)* dosage of 150 mg/kg bw/day (P3), and traumatic brain injury + *C. asiatica (L.)* dosage of 300 mg/kg bw/day (P4). Group P2 was significantly different from the normal group (P1), traumatic brain injury + *C. asiatica (L.)* dosage of 300 mg/kg bw/day (P4), and traumatic brain injury + *C. asiatica (L.)* dosage of 600 mg/kg bw/day (P5). Group P3 was significantly different from the normal group (P1), traumatic brain injury + *C. asiatica (L.)* dosage of 300 mg/kg bw/day (P4), and traumatic brain injury + *C. asiatica (L.)* dosage of 600 mg/kg bw/day (P5).

## Discussion

In this study, Bcl-2 immunoexpression was in a low category because the mean was less than 2. This is probably due to various reasons, including the degree of severity of brain injury and the age of the rats. Brain injury that occurred in this study was included in the mild category; therefore, there was not much damage to brain cells. Minimal damage to brain cells that occurs will result in low expression of the antiapoptotic protein Bcl-2.[10]

Another thing that can influence the low expression of Bcl-2 is the age of the rat. The ages of the rats used in this study ranged from 2 to 3 months. According to research by Sengupta,[11] rats go through the transition to adulthood from the 8th week of postnatal life. Meanwhile, during adulthood, Bcl-2 immunoexpression is low in the central nervous system.[12] This can lead to low Bcl-2 immunoexpression in the rat brain in this study.

In this study, the group treated with traumatic brain injury showed higher Bcl-2 expression than the normal group. Differences in Bcl-2 immunoexpression between these two groups were not significant. Increase in Bcl-2 immunoexpression may be a compensatory mechanism for neuroprotection from stress.[13]

Bcl-2 immunoexpression in the traumatic brain injury group (P2) was lower than in the groups treated with *C. asiatica (L.)* (groups P3, P4, and P5). Treatment with extract of *C. asiatica (L.)* at a dose of 150, 300, and 600 mg/kg bw/day could increase Bcl-2 immunoexpression. This result shows that the extract of *C. asiatica (L.)* can provide neuroprotective effects by inhibiting apoptosis through increased Bcl-2 immunoexpression.

This result is consistent with research conducted Jazmi *et al.*,[14] who reported that ethanol extract of *C. asiatica (L.)* can improve the neurological function in rats induced by traumatic brain injury with the principle of weight drop injury. The research results of Kuswati *et al.*[15] show that the ethanol extract of *C. asiatica (L.)* can increase the expression of the antiapoptosis protein Bcl-2 in the prefrontal cortex of Sprague Dawley rats treated with chronic restraint stress.

*C. asiatica (L.)* is a herbal plant that contains various active substances. The known active substances include terpenoids, phenols, and saponins.[16] Asiaticosides, which are part of terpenoids, are considered to have antiapoptotic activity because they can affect the expression of Bcl-2 and Bax.[17] The results of research by Sun *et al.*[18] showed that asiaticosides can help protect hypoxic brain cells mediated by Bcl-2 protein. The content of flavonoids that provide neuroprotectant effects as reported in a study of focal cerebral ischemia mice, the results of which significantly decreased Bax, increased Bcl-2, and decreased caspase 3.[19] The presence of Bcl-2 will maintain the permeability and integrity of the mitochondrial membrane so that proteins that can activate the caspase cannot exit into the cytoplasm.[20] Bcl-2 contained in the mitochondrial membrane will regulate intracellular molecules, such as the release of cytochrome C and other proteins that induce apoptosis so that in the end it can inhibit cell apoptosis.[21]

Apoptosis of pyramidal cells was found to be the most in P2. The difference in P1 and P2 ranged very far. This is because P1 is a group of normal rats and is not given treatment, and so the number of pyramidal cells that experience apoptosis is less. Meanwhile, P2 was a group exposed to traumatic brain injury. Apoptosis that occurred in P1 was probably due to

**Table 2: Mean apoptosis by treatment groups**

| | Treatment groups | | | | | *P* |
|---|---|---|---|---|---|---|
| | P1 | P2 | P3 | P4 | P5 | |
| Apoptosis of pyramidal cells | 0.429 ± 0.558 | 1.514 ± 0.818 | 1.457 ± 0.700 | 0,943 ± 0.725 | 0.600 ± 0.604 | 0.000 |

normal apoptosis occurring in several cells in a network. This apoptosis aims to maintain homeostasis in the differentiation and proliferation of pyramidal cells, so that it can concluded that apoptosis can also occur under physiological conditions.[22]

Apoptosis occurred in P2 due to trauma to the brain. One of the things that can prevent traumatic brain injury from continuing is the provision of medication.[7] In the group with traumatic brain injury (P2), no treatment was given, and so the number of pyramidal cells experiencing apoptosis was more than the other groups.

Apoptosis that occurs can also be due to the administration of ketamine anesthesia. Ketamine is considered to induce changes in proteins associated with autophagy and apoptosis. Recent studies have shown that high doses of ketamine can induce the formation and accumulation of ROS in neurons.[23] Research by Li *et al.* [24] showed that ketamine anesthesia in pregnant rats can increase the process of autophagy and apoptosis in the fetal hippocampus. This study also states that rats given ketamine experienced increased levels of C-Caspase-3 and Bax and decreased levels of Bcl-2 protein.

Asiaticosides contained in the *C. asiatica (L.)* plant are thought to weaken the expression of N-methyl-d-aspartate (NMDA) receptors containing NR2B. Excitotoxicity is mediated by NR2B. Decreasing NR2B expression can prevent calcium from entering intracellularly, and so it will prevent further cell damage.[25]

The severity of traumatic brain injury can be affected by the buildup of free radicals called oxidative stress.[26] The *C. asiatica (L.)* plant contains many flavonoids, which are antioxidants that can immobilize free radicals. Research by Raza *et al.*[27] showed that *C. asiatica (L.)* extract could reduce levels of reactive oxygen species and oxidative stress in Sprague Dawley rats.

Asiatic acid can prevent apoptosis by inhibiting the production of free radicals so that it can accelerate axon regeneration.[28] Madecassoside besides having antioxidant effects can also increase the expression of brain-derived neurotrophic factor proteins.[29]

This study shows that increasing Bcl-2 immunoexpression will result in a tendency to decrease apoptotic cells in the rat brain traumatic injury model. The administration of *C. asiatica (L.)* extract in this study can increase the antiapoptotic protein, Bcl-2, and so the apoptosis process can be inhibited.

## Conclusion

Extract of *C. asiatica* could decrease apoptosis and increase Bcl-2 immunoexpression of pyramidal cells in traumatic brain injury rat model This study suggests that *C. asiatica* may be useful in the treatment of traumatic brain injury.

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## Conflicts of interest

There are no conflicts of interest.

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