

# Neuroprotective Effect of Olive Leaf Extract on Aif and Caspase-3 Expression in Chronic Spinal Cord Injury

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## Research Article

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# Abstract

## Background

Chronic spinal cord injury (SCI) remains a major neurological challenge due to limited regenerative capacity and persistent apoptosis. Olive leaf extract (OLE), rich in polyphenols such as oleuropein and hydroxytyrosol, has shown neuroprotective potential in various models. The aim of this study to evaluate the anti-apoptotic and neuroprotective effects of oral OLE administration on Apoptosis-inducing factor (AIF) and caspase-3 expression, as well as motor function outcomes in a rabbit model of chronic SCI.

## Methods

Thirty New Zealand white rabbits were divided into six experimental groups, including negative and positive controls, and three OLE-treated groups with different administration protocols. Chronic spinal cord compression was induced at C5 level. OLE was administered orally at a dose of 350 mg/kg. Histopathological and immunohistochemical analyses were conducted to assess AIF and caspase-3 expression. Motor function was evaluated using a modified Tarlov scale.

## Results

OLE significantly reduced the expression of AIF and caspase-3, particularly in the pre-treatment group (Treatment 3), with levels decreasing to  $4.6 \pm 2.07$  and  $5.4 \pm 1.82$  respectively. Correspondingly, motor function improved (Tarlov score: 3.0) compared to untreated controls (score: 2.0). Serum analysis also showed lower Tumor Necrosis Factor-alpha (TNF- $\alpha$ ) and Interleukin-6 (IL-6) levels and increased Brain-Derived Neurotrophic Factor (BDNF) expression.

## Conclusion

OLE exerts neuroprotective effects in chronic SCI by modulating apoptotic pathways and improving functional outcomes. These findings support its potential as an adjunct therapy in spinal cord injury management.

## INTRODUCTION

Spinal cord injury (SCI) is a devastating condition that results in long-term neurological impairment and imposes substantial physical, psychosocial, and economic burdens on patients and their families. In the United States, an estimated 288,000 individuals live with SCI-related symptoms, with an annual incidence of approximately 54 cases per one million people. Globally, the incidence ranges between 250,000 to 500,000 cases annually. While most therapeutic interventions target the acute or subacute phases—when salvageable neurons may still be preserved and glial scarring is not fully established—

approximately 95% of SCI patients are in the chronic phase, for which no effective curative therapies currently exist (Suzuki 2021).

Apoptosis, a regulated and energy-dependent form of cell death, plays a critical role in secondary damage following SCI. Apoptotic processes are initiated within hours and may persist for weeks post-injury, affecting glial cells and oligodendrocytes within and beyond the injury site. These processes are driven through intrinsic (mitochondrial) and extrinsic (death receptor) pathways, which are interlinked at various signaling checkpoints (Abbaszadeh 2020). Apoptosis, although considered immunologically silent, contributes significantly to progressive neuronal loss and functional decline in chronic SCI (Shi 2021).

Among the central mediators of apoptosis, caspase-3 is a critical executioner enzyme activated via both intrinsic and extrinsic pathways. It cleaves a variety of key substrates such as DNA fragmentation factor (DFF), nuclear lamins, and cytoskeletal proteins, thereby executing the morphological and biochemical hallmarks of apoptosis (Springer 1999; D'Amelio 2010). In SCI, caspase-3 has been shown to be activated in both neurons and oligodendrocytes, contributing to progressive neurodegeneration (Abbaszadeh 2020).

In parallel, apoptosis-inducing factor (AIF) is a mitochondrial flavoprotein that mediates a caspase-independent form of apoptosis. Upon cellular stress, AIF translocates from mitochondria to the nucleus, triggering large-scale DNA fragmentation and chromatin condensation (Cande 2002). While AIF serves protective roles in healthy mitochondria, its nuclear translocation under pathological conditions amplifies neuronal injury.

In recent years, considerable attention has been given to olive leaf extract (OLE) due to its rich content of polyphenols, particularly oleuropein and hydroxytyrosol, which exhibit potent antioxidant, anti-inflammatory, antimicrobial, antiviral, and neuroprotective properties (Coni 2000; Visioli 2000; Bazoti 2006). Polyphenols have been shown to modulate apoptotic pathways, influencing both caspase-dependent and caspase-independent cell death mechanisms depending on the disease context and concentration used (Giovannini 2012).

Previous animal studies have demonstrated that polyphenolic compounds from OLE can reduce caspase-3 expression in models of cerebral and spinal neuronal injury (Zhang 2018; Impellizzeri 2012). Furthermore, oleuropein significantly suppressed Bax and caspase-3 expression while increasing B-cell lymphoma 2 (Bcl-2), suggesting a protective anti-apoptotic role (Hassan 2022). Hydroxytyrosol, in particular, was shown to activate AIF translocation and promote autophagy, while reducing oxidative stress and cellular damage (Terzuoli 2017; Zhang 2020).

In this study, we aimed to evaluate the anti-apoptotic potential of olive leaf extract in a chronic spinal cord injury model by analyzing the expression of apoptotic markers caspase-3 and AIF, as well as assessing associated motor function outcomes in experimental animals.

# MATERIALS AND METHODS

## Animals

New Zealand white rabbits 12 weeks of age, weight: 2.6–3.0 kg (average: 2.9 kg), males were used in this study. Animals are given diet and water in the conventional laboratory. The room temperature is around 16–20 °C with a light-dark cycle of 12 hours. This study has obtained permission from the ethical committee of the Medical Faculty of Universitas Sumatera Utara, Medan, Indonesia.

## Olive Leaf Extract

This study used olive leaf extract (OLE) produced by Shaanxi Yongyuan BioTech Co.,Ltd. Extract contains 40% Oleuropein, the extract dose is 350 mg/kgBW or equivalent to 140 mg Oleuropein, suspended in distilled water and administered to animals via oral gavage in 4 cc solution, OLE was administered in the morning. The dose given is in accordance with previous studies in rats (Mohagheghi et al. 2011; Angeloni et al. 2017; Sarbishegi et al. 2017; Gao et al. 2020) converted from rat to rabbit doses (Laurence and Bacharach 1964).

## Experimental Group

This study used 30 rabbits divided into 6 groups. First group (n = 5) control negative (sham) group, performed a skin incision, paraspinal muscle dissection and lamina hole drill and no laminar screw was installed. The second group (n = 5) positive control-1, performed spinal cord compression with a screw and terminated on day 14. The third group (n = 5) positive control-2, performed spinal cord compression with a screw and terminated on day 21. Fourth group (n = 5) Treatment-1, performed spinal cord compression with a screw together with OLE administration and terminated on day 14. The fifth group (n = 5) Treatment-2, performed spinal cord compression with screws, after 14 days given OLE, terminated on day 21. Sixth group (n = 5) Treatment-3, OLE was given 7 days before spinal cord compression with screws, terminated on day 21.

## Surgery

The rabbit was anesthetized using 50 mg/kg of Ketamine hydrochloride (Pfizer) and 10 mg/kg of Xylazine (Bayer), profilactic antibiotic Cefazolin 50 mg/kg (Kanchiku et al. 2001). Rabbit in prone position, shaved in the posterior cervical area, disinfect with 10% betadine, sterilized with cloth cover, C4–C6 midline posterior cervical skin incision, small retractors was used, palpation of spinous processes, C5 paraspinal muscle dissection, identification of lamina. One hole is made in the lamina C5 midline position using a high speed diamond drill bur (3 mm in diameter), until it penetrates the lamina (2 mm thick lamina), the burr hole is tapered at 4 mm, then the lamina hole is inserted into a screw (stainless steel) with a diameter of 4 mm and a length of 10 mm, until the entire thickness of the lamina,

on the 1st day the compression is given 0.5 mm (by turning the screw 180°), on the 7th day, the screw is turned 180° again (total compression is 1 mm), on the 14th day the screw is rotated another 180° (the total compression is 1.5 mm), after the installation of screw the skin was sutured. The position of the screw head is 0.5 cm below the skin, easily felt, so that in the 2nd and 3rd procedures, it is enough to open one skin suture and the screw is turned, the repeated procedure is carried out by sterilization and the same anesthetics method. The macroscopical structural orientation and verification of this cervical compressive model are structurally illustrated in Fig. 1.

## Motor Function Evaluation

Motor function was evaluated by using the modification of Tarlov’s classification (Kanchiku.T et al. 2001) (Table 1). Evaluation was made before and immediately after the surgery and before animal termination.

Table 1  
Motor Function Evaluation using Modified Tarlov Classification

| Grade | Motor characteristics                                      |
|-------|------------------------------------------------------------|
| 0     | Unable to do voluntary movements                           |
| 1     | Perceptible movements at join, the hindlimbs follow        |
| 2     | Good movements at joins, but unable to stand up            |
| 3     | Can stand up and walk, but unable to start running quickly |
| 4     | Normal                                                     |

## Tissue Evaluation and Immunohistochemistry

The tissue of the C5 spinal cord area was taken and fixed with a buffer solution of 10% formalin. After that, dehydration was carried out using graded alcohol (30%, 50%, 70%, 80%, 96% and absolute) for 60 min each. Clearing was used with xylol 2 times for 60 min each. Then the soft paraffin embedding was carried out for 60 min at a temperature of 48 C°. Furthermore, the paraffin is allowed to stand for one day until it becomes a hard block. The next day it was attached to the holder and a 4 µm thick cut was made with a rotary microtome. Followed by the deparaffinization process; The glass object resulted from the paraffin block was immersed in xylol 2 times for 5 min each. After that, rehydration using serial alcohol (absolute, 96%, 80%, 70%, 50% and 30%) for 5 min each. Then rinsed in H2O for 5 min. Then the process of staining the slide was washed with PBS pH 7.4 for 5 min. Then stained with Hematoxylin for 10 min. After that, soak it in tap water for 10 min. Then rinsed with distilled water (dH2O). Dehydrated with alcohol 30% and 50% respectively for 5 min. Then stained with Eosin solution for 3 min. Then rinsed with 30% alcohol. Washed with H2O for 5 min and then dried. Then do the mounting with a stick and cover with a glass cover.

# Immunocytochemistry Protocol

## Immunocytochemistry Protocol

The distribution of microglia expressing AIF and Caspase-3 was observed by immunohistochemical techniques. Paraffin block containing spinal tissue was cut to a thickness of 4  $\mu$ m using a microtome, then deparaffinized with xylol. Subsequently, rehydration was carried out with a decreased concentration of ethanol, followed by rinsing with Phosphate Buffer Saline (PBS) for 3  $\times$  5 minutes. The tissue preparations were then incubated in DAKO® Buffer Antigen Retrieval in a microwave at a temperature of 94C for 20 min and followed by cooling at room temperature for 20 min. The next step, the preparation was washed with PBS for 3  $\times$  5 minutes, and incubated in a peroxidase block (Novocast®) for 20 min. Furthermore, the preparation was washed again with PBS for 3  $\times$  5 min and incubated in Protein Block for 20 min. After that it was washed again with PBS for 3  $\times$  5 min and incubated overnight (12–18 h) with primary antibody: anti AIF and anti caspase-3, for 1 h at room temperature, then washed with PBS pH 7.2 for 3  $\times$  5 min and incubated with a solution post primary antibody for 45 min and followed by incubation with Novolink® Horse Radish Peroxidase (HRP) for 60 min at room temperature. After incubation, the preparations were washed with PBS pH 7.2 for 3  $\times$  5 min. Then DAB (diamio benzidine) was applied for 10 min and the preparation was washed with PBS pH 7.2 for 3  $\times$  5 min, then counterstain with hematoxylin (Novocast®). Furthermore, dehydration was carried out using increased concentrations of ethanol. The next process is to do the purification with xylol, then do the mounting.

## Immunohistology Evaluation

Calculation of immunohistochemical results using techniques such as those in other study modified for spinal tissue (Pizem and Cor 2003; Soini et al. 1998). Examination of the number of brown cells in the nucleus or cytoplasm per 20 fields of view in the anterior horn compression area (C5) and cell counts were carried out separately between the two examiners (double blind). Examination and cell counts were performed on each slide in the field of view in the cortex of the spinal cord with 400 $\times$  and 1000 $\times$  magnification, for 20 fields of view respectively.

## Statistical Analysis

Statistical analyzes were performed using Statistical Package for the Social Sciences (SPSS) Version 21 for Windows (SPSS Inc., Chicago, IL, USA). To test the significance of differences of the variable expression between the two experimental groups, we performed ANOVA tests. The significance level was defined as  $p < 0.05$ .

## RESULTS

### Tissue Markers of Inflammation and Neuroprotection

The quantitative expression levels of various inflammation and neuroprotective tissue markers across all experimental groups are summarized in Table 2, with their corresponding statistical significance analyses detailed in Table 3. TNF-alpha, GFAP, and CD68 were significantly elevated in positive controls and reduced dose-dependently in treatment groups ( $p < 0.05$ ), indicating attenuated inflammation. BDNF increased significantly with treatment ( $p < 0.0001$ ), suggesting enhanced neuroprotection. Neurodegenerative markers P-Tau and TDP-43 were also reduced significantly in treated groups ( $p < 0.05$ ). IL-6 showed no significant difference ( $p = 0.7553$ ).

Table 2  
Tissue Markers of Inflammation and Neuroprotection

| Group              | IL-6       | TNF-α       | GFAP        | CD68        | BDNF        | P-Tau       | TDP-43      |
|--------------------|------------|-------------|-------------|-------------|-------------|-------------|-------------|
| Control Negative   | 3.0 ± 1.22 | 4.8 ± 1.92  | 3.8 ± 1.64  | 4.0 ± 1.58  | 9.4 ± 1.82  | 3.6 ± 2.41  | 2.4 ± 2.30  |
| Control Positive 1 | 4.6 ± 2.30 | 11.8 ± 2.17 | 8.0 ± 2.65  | 11.8 ± 2.49 | 4.0 ± 1.58  | 7.8 ± 3.56  | 9.0 ± 1.87  |
| Control Positive 2 | 4.2 ± 3.11 | 12.0 ± 3.54 | 12.2 ± 3.11 | 12.2 ± 2.49 | 3.8 ± 1.48  | 13.8 ± 2.59 | 12.8 ± 2.28 |
| Treatment 1        | 3.2 ± 1.30 | 6.6 ± 1.95  | 6.6 ± 1.95  | 10.0 ± 4.30 | 10.2 ± 1.64 | 7.8 ± 1.30  | 5.6 ± 2.19  |
| Treatment 2        | 3.4 ± 2.07 | 6.0 ± 1.58  | 5.2 ± 3.03  | 6.0 ± 2.24  | 12.2 ± 4.66 | 6.0 ± 2.55  | 5.0 ± 2.35  |
| Treatment 3        | 2.8 ± 0.84 | 5.4 ± 0.55  | 4.6 ± 2.51  | 5.0 ± 1.87  | 11.4 ± 1.14 | 5.2 ± 2.17  | 4.6 ± 1.95  |

Table 3  
Comparisson Tissue Markers of Inflammation and Neuroprotection between groups

| Marker | p-value |
|--------|---------|
| IL-6   | 0.7553  |
| TNF-α  | 0.0000  |
| GFAP   | 0.0001  |
| CD68   | 0.0177  |
| BDNF   | 0.0000  |
| P-Tau  | 0.0265  |
| TDP-43 | 0.0256  |

Expression of Apoptotic Markers in Tissue

Both Caspase-3 and AIF levels were significantly elevated in the positive control groups (Caspase-3 up to  $12.50 \pm 0.95$ ; AIF up to  $11.88 \pm 0.86$ ) compared to the negative control. Treatment with olive leaf extract reduced their expression in a dose-dependent manner, with the lowest levels observed in Treatment 3 (Caspase-3:  $5.40 \pm 1.82$ ; AIF:  $4.60 \pm 2.07$ ). These reductions were statistically significant ( $p < 0.0001$  for both markers), indicating a strong anti-apoptotic effect of the intervention, as documented in Table 4. The visual reduction of brown-stained positive neuronal cells expressing both apoptotic markers in the treatment groups is further demonstrated through immunohistochemical staining in Fig. 2.

Table 4  
Expression of Apoptotic Markers in Spinal Cord Tissue

| Group              | Caspase-3 (Mean ± SD) | p-value | AIF (Mean ± SD)  | p-value |
|--------------------|-----------------------|---------|------------------|---------|
| Control Negative   | $4.71 \pm 1.22$       | 0.0000  | $5.02 \pm 0.47$  | 0.0000  |
| Control Positive 1 | $11.05 \pm 1.06$      |         | $9.41 \pm 1.35$  |         |
| Control Positive 2 | $12.50 \pm 0.95$      |         | $11.88 \pm 0.86$ |         |
| Treatment 1        | $9.60 \pm 2.41$       |         | $9.20 \pm 1.30$  |         |
| Treatment 2        | $6.00 \pm 2.24$       |         | $5.20 \pm 1.92$  |         |
| Treatment 3        | $5.40 \pm 1.82$       |         | $4.60 \pm 2.07$  |         |

Immunohistochemical staining with AIF (apoptosis-inducing factor) marker, by counting the number of spinal cord tissue neuron cells expressing AIF (apoptosis-inducing factor) (brown color on the cell membrane, arrows in the image). An increase in AIF (apoptosis-inducing factor) was seen in spinal cord tissue neuron cells after receiving compression treatment (Control Positive 1 & 2) compared with the group without compression (Control Negative) and AIF (apoptosis-inducing factor) in neuronal cells decreased in the group given OLE (Treatment 1,2 & 3) compared to the group without OLE (Control Positive 1 & 2)

Immunohistochemical staining with Caspase-3 marker, by counting the number of spinal cord tissue neuron cells expressing Caspase-3 (brown color on the cell membrane, arrows in the image). An increase in Caspase-3 was seen in spinal cord tissue neuron cells after receiving compression treatment (Control Positive 1 & 2) compared with the group without compression (Control Negative) and Caspase-3 in neuronal cells decreased in the group given OLE (Treatment 1,2 & 3) compared to the group without OLE (Control Positive 1 & 2)

## Serum Biomarker Levels (ELISA)

As presented in Table 5, serum TNF-alpha and BDNF levels showed significant intergroup differences ( $p = 0.0001$  and  $p < 0.0001$ , respectively). TNF-alpha was markedly elevated in positive controls (up to  $515,000.0 \pm 109,843.1$  pg/mL) and decreased substantially in treated groups, particularly Treatment 1 ( $127,200.0 \pm 46,337.9$  pg/mL) and Treatment 3 ( $130,400.0 \pm 29,048.2$  pg/mL). Conversely, BDNF levels were drastically reduced in positive controls (~ 33,000 pg/mL) but increased significantly in Treatment 3

(339,128.0 ± 80,000.3 pg/mL), indicating restored neurotrophic signaling. IL-6 levels showed a decreasing trend with treatment but did not reach statistical significance (p = 0.0565).

Table 5  
Comparison of Serum ELISA Biomarkers by Experimental Group

| Group              | IL-6 (pg/mL)          | p-value | TNF-α (pg/mL)         | p-value | BDNF (pg/mL)         | p-value |
|--------------------|-----------------------|---------|-----------------------|---------|----------------------|---------|
| Control Negative   | 445,818.2 ± 61,390.9  | 0.0565  | 109,800.0 ± 40,517.9  | 0.0001  | 114,754.0 ± 69,082.8 | 0.0000  |
| Control Positive 1 | 578,943.4 ± 199,863.0 |         | 281,200.0 ± 36,465.1  |         | 32,876.0 ± 15,764.1  |         |
| Control Positive 2 | 451,390.0 ± 52,082.0  |         | 515,000.0 ± 109,843.1 |         | 33,252.0 ± 17,435.8  |         |
| Treatment 1        | 370,200.2 ± 192,251.3 |         | 127,200.0 ± 46,337.9  |         | 244,252.0 ± 39,075.9 |         |
| Treatment 2        | 336,200.2 ± 49,920.6  |         | 238,400.0 ± 46,634.8  |         | 123,752.0 ± 19,077.9 |         |
| Treatment 3        | 270,103.6 ± 35,405.8  |         | 130,400.0 ± 29,048.2  |         | 339,128.0 ± 80,000.3 |         |

## Motor Function Evaluation

Motor function, as measured by the Modified Tarlov Score, declined markedly in the positive control groups (mean score: 2.0–3.0), indicating impaired locomotor recovery following chronic spinal cord injury. In contrast, Treatment Groups 1 and 3 demonstrated improved motor scores (4.0 and 3.0, respectively), comparable to the negative control (4.0), suggesting partial restoration of motor function with olive leaf extract administration.

Table 6  
Motor Function Evaluation

| Group              | Mean Motoric Score |
|--------------------|--------------------|
| Control Negative   | 4.0                |
| Control Positive 1 | 3.0                |
| Control Positive 2 | 2.0                |
| Treatment 1        | 4.0                |
| Treatment 2        | 2.0                |
| Treatment 3        | 3.0                |

## Body Weight Evaluation

Body weight across all groups remained relatively stable before and after the intervention. Control groups, both negative and positive, exhibited minimal fluctuations (e.g., Positive Control 1: 2920.0 g to 2915.0 g), indicating that spinal cord injury and its consequences did not cause systemic weight loss. Treatment Groups 1 and 3 maintained identical pre- and post-treatment weights (2889.0 g and 2908.0 g, respectively), reflecting the metabolic safety of olive leaf extract. Although Treatment 2 showed a slight decrease (2830.0 g to 2774.0 g), the variation was within acceptable physiological range. Overall, the extract did not negatively affect general health or nutritional status during the study period. The longitudinal body weight measurements are systematically tracked in Table 7.

Table 7  
Body Weight Before and After Treatment

| Group              | BB Pre Mean (g) | BB Pre SD | BB Post Mean (g) | BB Post SD |
|--------------------|-----------------|-----------|------------------|------------|
| Control Negative   | 2880.0          | 130.38    | 2877.0           | 115.20     |
| Control Positive 1 | 2920.0          | 83.67     | 2915.0           | 80.78      |
| Control Positive 2 | 2850.0          | 111.80    | 2859.0           | 109.28     |
| Treatment 1        | 2889.0          | 105.50    | 2889.0           | 108.77     |
| Treatment 2        | 2830.0          | 57.01     | 2774.0           | 137.77     |
| Treatment 3        | 2908.0          | 91.31     | 2908.0           | 97.98      |

## DISCUSSION

In this study, we found that oral administration of olive leaf extract (OLE) significantly reduced the expression of apoptotic biomarkers, Apoptosis-Inducing Factor (AIF) and Caspase-3, and improved motor function outcomes in a rabbit model of chronic spinal cord injury (SCI). Quantitative analysis showed that the expression of AIF and Caspase-3 decreased notably in the treatment groups compared to the untreated injury groups, particularly in the group that received OLE prior to injury induction. These reductions were accompanied by improved motor scores based on the modified Tarlov scale, suggesting a protective effect of OLE on neuronal survival and functional preservation.

Our findings align with previous reports on the anti-apoptotic and neuroprotective properties of olive-derived polyphenols. Yao (2014) demonstrated that oleuropein, the major bioactive compound in OLE, induced apoptosis in HeLa cells through cell cycle arrest and activation of mitochondrial signaling cascades. Although this pro-apoptotic effect was shown in cancer models, other studies highlight the modulatory role of OLE in downregulating pathological apoptosis. For example, Katsouliris (2016) showed that OLE therapy decreased Caspase-3 expression and improved cell viability in neural cultures, while Zerrouh et al. (2017) confirmed that OLE inhibited Caspase-3 activity and prevented neuronal cell death in vitro.

Similarly, Pasban-Aliabadi et al. (2013) found that OLE reduced intracellular ROS levels and modulated apoptotic pathways—including Caspase-3 and the Bcl-2-associated X protein (Bax/Bcl-2) ratio—in models of Parkinson's disease, supporting its potential in chronic neurodegenerative conditions. In cardiac models, oleuropein reduced pro-inflammatory cytokines such as IL-6 and TNF-alpha, as well as Caspase-3 and phosphorylated ERK, thereby mitigating infarct development (Jin 2018). This anti-inflammatory component may also contribute to the neuroprotective mechanism observed in our study.

Relevant to spinal cord injury, Zhang et al. (2019) reported that hydroxytyrosol from olive extract, administered orally for 7 days in a T10–T12 spinal injury rat model, resulted in significant downregulation of apoptotic and inflammatory markers including Caspase-3, Bax, TNF-alpha, IL-1 beta, and Malondialdehyde (MDA), while simultaneously enhancing protective elements like Bcl-2, Superoxide Dismutase (SOD), and Glutathione (GSH). This is consistent with our findings, where we noted significant improvements in molecular and behavioral outcomes following OLE treatment.

Moreover, the inhibitory effects of OLE on AIF translocation were previously shown by Kim et al. (2017). They demonstrated that OLE prevented glutamate-induced neurotoxicity in hippocampal HT-22 cells by reducing Bax expression, increasing Bcl-2, and suppressing mitochondrial AIF release. This mechanism may partly explain our observed reduction of AIF expression in spinal cord tissues post-treatment.

Oleuropein has been reported to modulate multiple pathways involved in inflammation and cell death. It exerts anti-apoptotic effects by reducing Caspase-3 activity and preserving mitochondrial integrity, while simultaneously lowering oxidative stress markers such as Reactive Oxygen Species (ROS) and MDA. These effects support its potential neuroprotective properties, particularly in models of neurodegeneration and Central Nervous System (CNS injuries) (Singh et al. 2023).

Pretreatment with oleuropein significantly ameliorated the cognitive deficits and oxidative stress induced by colchicine in the hippocampal Cornu Ammonis 1 (CA1) region. The study demonstrated that oleuropein improved spatial learning and memory performance in the Morris Water Maze test, reduced lipid peroxidation (MDA levels), and restored antioxidant enzyme activities including glutathione peroxidase and catalase. Furthermore, oleuropein reduced nitrosative stress (nitrite levels) and inhibited apoptosis by decreasing Caspase-3 activity, suggesting its neuroprotective potential via antioxidant and anti-apoptotic pathways (Pourkhodad et al. 2016).

Treatment with olive leaf extract significantly reduced neuroinflammatory markers, including TNF-alpha and IL-1 beta, and suppressed microglial activation in spinal cord tissue after injury. Additionally, behavioral motor function scores improved notably in the treated group compared to untreated controls. The extract also inhibited apoptosis by downregulating Caspase-3 expression, highlighting its dual anti-inflammatory and anti-apoptotic properties in the context of spinal cord injury (Almeer and Abdel Moneim 2018).

Finally, the broader role of olive polyphenols in apoptosis modulation has also been explored in oncology. Numerous animal studies confirm that dietary olive extract exerts anti-cancer effects through

apoptotic regulation, particularly via Caspase-3 activation suppression in breast cancer models (Kossoy 2001; Stark 2003; Costa 2004; Chen 2007; Solanas 2010; Chimento 2014). These data further support the pleiotropic and context-dependent role of olive compounds in apoptosis, depending on the disease setting.

Taken together, our findings support the therapeutic potential of olive leaf extract in chronic spinal cord injury by attenuating apoptosis through reduction of AIF and Caspase-3 expression and by enhancing motor function. This adds to a growing body of literature suggesting that olive-derived bioactives could offer a complementary strategy for neuroregeneration and inflammation modulation.

## **CONCLUSION**

This study confirms that oral administration of olive leaf extract (OLE) effectively reduces the expression of apoptotic markers AIF and caspase-3, while improving motor function in a rabbit model of chronic spinal cord injury. These effects suggest that OLE exerts neuroprotective actions through modulation of apoptotic and inflammatory pathways. Given its safety and efficacy profile, OLE holds potential as an adjunctive therapy for chronic SCI. Further research is needed to validate these findings and explore clinical applications.

## **Declarations**

## **Competing Interests**

The authors have no relevant financial or non-financial interests to disclose.

## **Ethics Approval**

This study was performed in line with the principles of the ethical treatment of laboratory animals. Approval was granted by the Ethical Committee of the Medical Faculty of Universitas Sumatera Utara, Medan, Indonesia.

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## **Author Contribution**

S.I. and L. contributed to the study conception and design. Material preparation, data collection, and analysis were performed by S.I. and L. The first draft of the manuscript was written by S.I., and all authors commented on previous versions of the manuscript. S.I. and L. prepared the artwork, tables, and illustrations. All authors read and approved the final manuscript.

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## Data Availability

The statistical datasets generated and analyzed during the current study are included within this article. The raw data supporting the findings of this study are not publicly available due to institutional data privacy restrictions but are available from the corresponding author upon reasonable request.

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## Figures

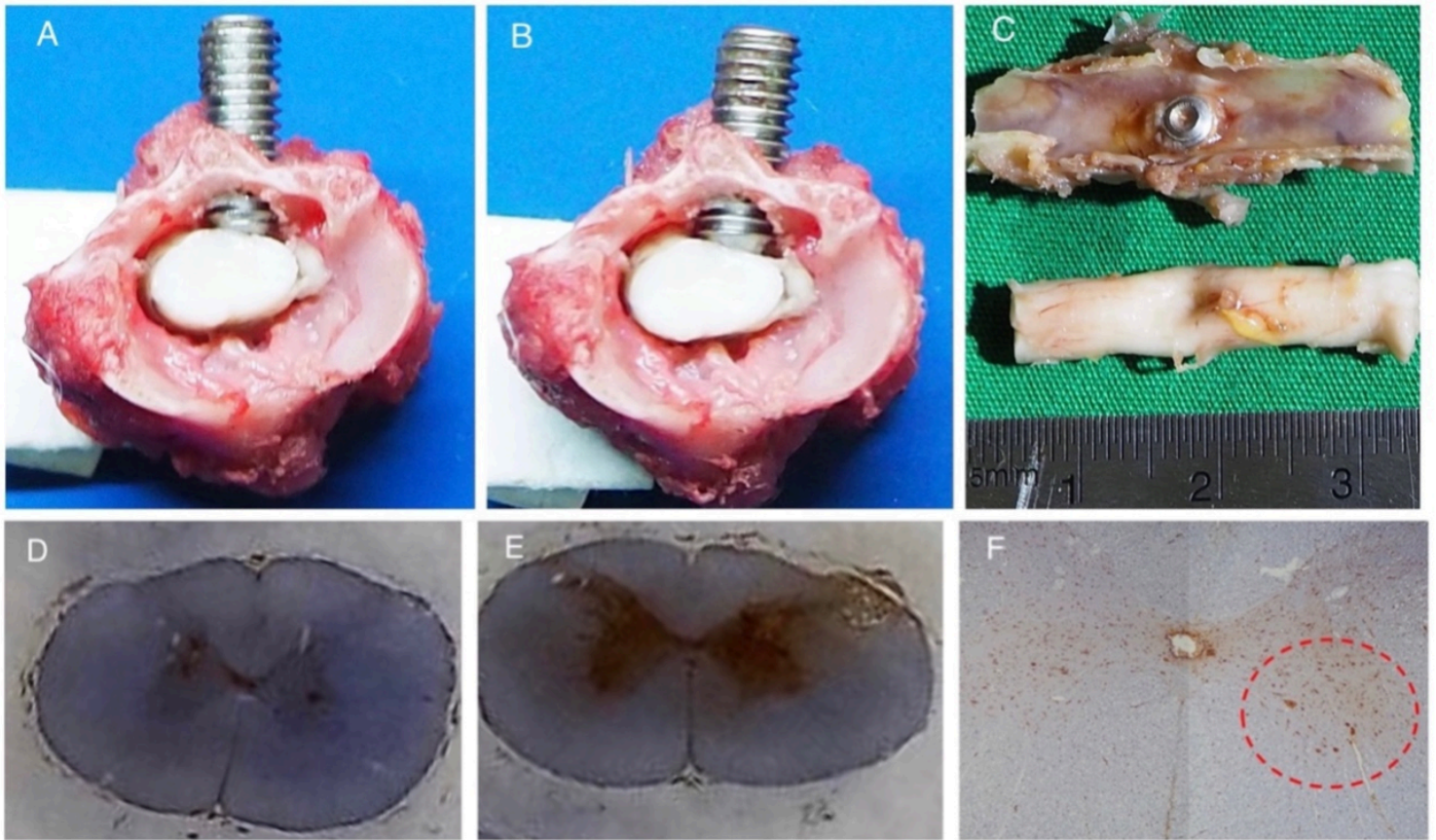


**Figure 1**

(a) Compression screw inserted 1.0 mm deep; rabbit terminated on Day 14.(b) Compression screw inserted 1.5 mm deep; rabbit terminated on Day 21.(c) Gross view of spinal cord tissue.(d) Axial section of C5 spinal cord in the control group.(e) Spinal cord with visible compression site.(f) Region of histological examination in the anterior horn of the spinal cord.

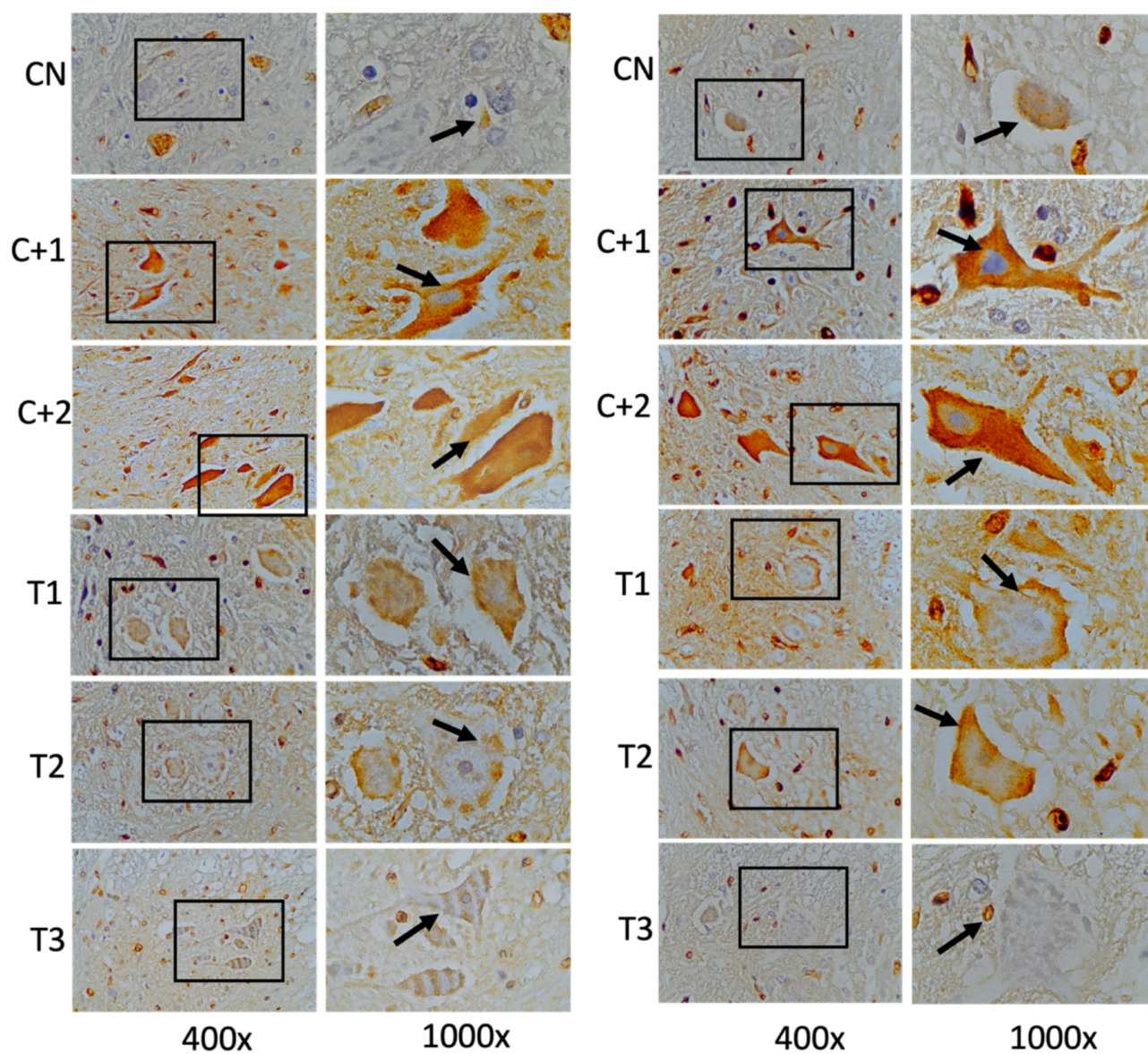


**Figure 2**

**Immunohistochemical staining with AIF marker and Caspase-3 marker**