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Original Article

HISTOLOGICAL ANALYSIS OF HEPATOPROTECTIVE EFFECTS OF *EUGENIA UNIFLORA* L. LEAF ETHANOLIC EXTRACT IN BALB/C MICE

DIAN AYU JUWITA[1*], FITRI RACHMAINI[2], HANSEN NASIF[3], ELVI MAYASARI[4], REDHA ILAHI[5]

[1,2,3]**Department of Pharmacology and Clinical Pharmacy, Faculty of Pharmacy, Universitas Andalas, Padang, Indonesia.** [4,5]**Undergraduate Pharmacy Study Program, Faculty of Pharmacy, Universitas Andalas, Padang-25175 Indonesia**

[*]**Corresponding author: Dian Ayu Juwita;** [*]**Email: dianayujuwita@phar.unand.ac.id**

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ABSTRACT

Objective: The plant *Eugenia uniflora* L. contains various bioactive compounds, including flavonoids, polyphenols, and terpenoids. These compounds can neutralize free radicals and reduce oxidative damage and inflammation in liver tissues, thereby potentially protecting the liver from damage caused by oxidative stress. The purpose of this study is to assess the effect of *Eugenia uniflora* L. Leaf Ethanolic Extract (EULEE) administration on the histopathological profile in male mice induced by carbon tetrachloride (CCl_4).

Methods: A total of 45 mice were assigned into 5 treatment groups, with 9 mice in each group. The negative control group was orally treated with Na-CMC suspension. The positive control group was treated orally with a 10% CCl_4 solution. The treatment groups were administered a 10% CCl_4 solution orally to induce hepatotoxicity, along with EULEE at doses of 200 mg/kg, 400 mg/kg, and 800 mg/kg BW. EULEE was administered for 7, 14, and 21 d. On days 8, 15, and 22, three mice from each treatment group were euthanized, and their liver organs were collected for histology analysis followed the Manja Roenigk Histopathology Scoring System. Statistical analysis was performed using two-way ANOVA.

Results: The administration of EULEE 200, EULEE 400, EULEE 800 resulted in a reduction of liver histology scores compared to the positive control group, indicating a decrease in the level of liver tissue damage. The average histology scores for the three dosage groups were 39.556±0.0555, 47.600±0.555, and 45.333±0.555.

Conclusion: The administration of EULEE results in an improvement in the liver's histological features in mice.

Keywords: *Eugenia uniflora* L, Ethanolic extract, Histopathology, Hepatoprotective, Carbon tetrachloride

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INTRODUCTION

The prevalence of liver diseases has markedly increased in recent decades, impacting populations in both developed and developing countries. Key factors contributing to this rise include excessive alcohol consumption, viral hepatitis infections, occupational exposure to hazardous chemicals, and improper medication use [1, 2]. Additionally, the accumulation of free radicals, particularly in the liver, results in substantial tissue damage [3]. This oxidative stress can lead to severe complications such as liver cirrhosis, hepatocellular carcinoma, and, ultimately, liver failure [4, 5].

Hepatoprotective agents are compounds that have the capacity to protect liver cells from the deleterious effects of toxic substances or free radicals [6]. While the body possesses natural defense mechanisms against free radicals through endogenous antioxidants (such as catalase, and glutathione peroxidase enzymes), these defenses may become inadequate under conditions of excessive free radical production [7]. In such cases, exogenous antioxidants are required to restore balance and mitigate oxidative damage. Exogenous antioxidants have the ability to modulate several molecular pathways associated with inflammation and apoptosis, thereby alleviating liver damage and restoring liver function impaired by toxic exposure [3]. The supplementation of exogenous antioxidants can inhibit oxidation processes and protect tissues from further injury, thereby supporting the body's defense mechanisms [8].

In the search for safer and more effective treatments, interest in medicinal plants has been steadily increasing. Medicinal plants are considered to have significant potential in terms of both efficacy and safety [9]. Many researchers are turning to natural products due to the limited availability of hepatoprotective drugs. Traditional medicine recommends various herbal remedies for the treatment of liver diseases through diverse mechanisms, such as free radical scavenging and antioxidant modulation [10, 11]. Therefore, the use of natural products offers potential as therapeutic agents in addressing liver damage.

Eugenia uniflora L. contains various antioxidant compounds, including flavonoids, polyphenols, and terpenoids, which have the ability to scavenge and neutralize free radicals, thereby reducing oxidative damage to liver cells [12, 13]. *Eugenia uniflora* L. extract also shows anti-inflammatory activity [14]. Chronic inflammation in the liver, often caused by toxin exposure or infections, can exacerbate liver damage and lead to liver diseases such as cirrhosis [15, 16]. The anti-inflammatory compounds in this leaves can help reduce inflammation and lessen the negative impact on liver tissues. Unlike other hepatoprotective plants, such as Silybum marianum, which contains a single active compound (silymarin), *Eugenia uniflora* L. exhibits a synergistic hepatoprotective effect through a range of bioactive compounds that work together to provide broad-spectrum protection for the liver [15]. The flavonoids and terpenoids in this leaves have a synergistic effect that enhances hepatoprotective activity [17]. Flavonoids, such as quercetin and kaempferol, are known to stabilize cell membranes, inhibit inflammation, and counteract oxidative damage, while terpenoids, such as saponins and tannins, also contribute to reducing liver cell damage and improving detoxification processes [18]. The purpose of this study is to evaluate the effect of EULEE on the histopathological profile of the liver in male mice induced by CCl_4.

MATERIALS AND METHODS

Plant materials

The Eugenia uniflora L. leaves were harvested from Nagari Kambang Timur, Lengayang District, Pesisir Selatan, West Sumatra, Indonesia. The specimen's identification was confirmed by Herbarium ANDA at Universitas Andalas, with the certificate number 002/K-ID/ANDA/I/2023.

Chemicals

Carbon tetrachloride (Merck, Germany), sodium carboxymethylcellulose (Na-CMC) 0.5% (PT. Brataco Indonesia), Mayer's albumine, paraffin (Leica), alcohol 96% (Sigma Aldrich,

Singapore), aqua injection (Otsuka, Indonesia) sodium chloride 0,9% for injection (Otsuka, Indonesia).

Extraction procedure

Four kilograms fresh leaves of *Eugenia uniflora* L. were cleaned and left to dry at 25 °C for 5 d. After drying, the leaves were crushed to a 60 mesh size and subjected to a 24 h extraction using ethanol 70%. The resulting mixture was filtered through filter paper. To eliminate any particle contaminants, the filtrate was centrifuged for 10 min at 10,000 g for. The extraction process was conducted again with the same type and amount of solvent to obtain a second macerate. The solution from the maceration process was filtered then concentrated using a vacuum rotary evaporator (Buchi) at 60 °C, yielding a thick extract of 306 g.

Qualitative phytochemical screening

Using the Indonesian Herbal Pharmacopeia's standard protocols, the plant extracts were evaluated for the presence of numerous phytochemicals such as saponin, alkaloids, phenols and flavonoids [19].

Animals and Experimental Design

The mice (27-35 g) were obtained from the Faculty of Pharmacy, Universitas Andalas, and allowed to acclimate to laboratory conditions for seven days, maintained at 25 °C±2, with a 12 h light/dark cycle and relative humidity of 55%±5%. They were fed a standard laboratory diet and had free access to water during the adaptation period. The protocol received approval from the Andalas University Research and Ethics Committee with number 173/KEP/FK/2023

A total of 45 mice were assigned into 5 treatment groups, with 9 mice in each group. The negative control group received an oral treatment with a 0.5% Na-CMC suspension. The positive control group was treated orally with a 10% CCl_4 solution. The treatment groups were administered a 10% CCl_4 solution orally to induce hepatotoxicity, along with *Eugenia uniflora* L. Leaf Ethanolic Extract at doses of 200 mg/kg BW (EULEE 200), 400 mg/kg BW (EULEE 400), and 800 mg/kg BW (EULEE 800). The extract was administered for 7, 14, and 21 d. The dose selection in this study was based on preliminary testing conducted earlier. Doses with incremental multiples were chosen to assess the differences in hepatoprotective effects resulting from a twofold increase in dosage. Additionally, administration durations of 7, 14, and 21 d were selected to evaluate the hepatoprotective effects at different stages of liver damage.

On days 8, 15, and 22, three mice from each treatment group were euthanized, and their liver organs were collected for histology analysis. Histopathological slides were then prepared using the paraffin method and examined under a microscope.

Histology examination of the liver

The liver was fixed in a 10% formalin solution with a pH buffer for 24 h, then progressively dehydrated using graded alcohols (70%, 80%, 90%, and 96%), followed by xylene and paraffin embedding. Next, the tissue was subjected to vacuum infiltration and embedding. Sections of the liver tissue, each 4-6 micrometers thick, were stained with hematoxylin and eosin. Histological analysis focused on evaluating central veins, hepatocytes, the arrangement of hepatocyte cells, and identifying any abnormal liver conditions such as parenchymal degeneration, hydropic degeneration, and necrosis of hepatocytes. Liver tissue samples were examined under a microscope with 40x magnification (Optilab®) across five different fields of view. ImageJ software (NIH-Bethesda, MD, USA) was used to measure the stained area and staining intensity. Each biological replicate was photographed separately, and the mean area of staining and the intensity of the stain across the five fields were measured. In each field, 20 cells were randomly assessed, ensuring that a total of 100 liver cells were observed per slide. The histopathological findings from the treatment group were compared with those from the control group. Subsequently, the average histopathological score for liver changes was calculated based on the five fields of view for each mice using the Scoring Histopathology Manja Roenigk Model [20, 21]. This scoring system evaluates morphological changes in liver tissue, including inflammation, necrosis, and fibrosis, by assigning scores based on the severity of damage. The degree of damage was assessed using a scoring system: score 1 (normal hepatocytes), score 2 (parenchymal degeneration), score 3 (hydropic degeneration), and score 4 (necrosis). Parenchymal degeneration is characterized by hepatocyte swelling and cloudy cytoplasm, indicating protein accumulation due to disrupted oxidation processes. Hydropic degeneration is characterized by cell swelling and clear cytoplasm, which occurs due to an increase in intracellular Na+ ions caused by impaired active transport, leading to water accumulation. Necrosis of hepatocytes refers to the death of liver cells due to severe cellular damage, leading to the loss of cellular function [3, 5]. Slides with higher histopathological scores indicated more severe tissue damage and a greater number of affected cells compared to those with lower scores.

Data analysis

All values are expressed as means±standard deviation (SDs). Statistical analysis was performed using two-way ANOVA, followed by Duncan's Multiple Range Test (DMRT).

RESULTS AND DISCUSSION

Table 1 presents the results of the phytochemical analysis of *Eugenia uniflora* L., which identifies the presence of saponins, phenols, and flavonoids. The findings reveal that the leaves of *Eugenia uniflora* L. are rich in flavonoids, which are recognized for their diverse biological activities, including antioxidant, anti-inflammatory, and antimicrobial effects [16, 18]. These flavonoids play a critical role in shielding cells from oxidative damage and may provide protective benefits against degenerative diseases [15]. The significant flavonoid content in the leaf extract emphasizes its potential as an important natural antioxidant source.

Table 1: Qualitative analysis of *Eugenia uniflora* L. leaves

Phytoconstituents	Result
Saponins	+
Alkaloids	-
Phenols	+
Flavonoids	++

Flavonoids, such as quercetin, and terpenoids, such as α-limonene, have been shown to enhance the activity of antioxidant enzymes in liver cells, including superoxide dismutase, catalase, and glutathione peroxidase. These enzymes are critical in mitigating oxidative stress, which can damage hepatocytes [8, 16]. Additionally, flavonoids and terpenoids reduce the production of pro-inflammatory cytokines, such as TNF-α, IL-6, and IL-1β, which contribute to liver injury through chronic inflammation. Moreover, these compounds can induce apoptosis in hepatocytes exposed to oxidative stress or infection, facilitating the removal of damaged cells and preventing the progression of liver fibrosis [12, 15].

The administration of EULEE at various doses demonstrated protective effects against CCl_4-induced liver damage. All treatment groups receiving different doses of the extract exhibited significant differences compared to the positive control group, indicating that extract administration at various doses can effectively reduce liver tissue damage. The EULEE 200, EULEE 400, and EULEE 800 groups resulted in a reduction in liver histology scores compared to the positive control group (table 2). These histopathological scores reflect the degree of tissue damage reduction. The mean histology scores for the three treatment groups were 39.556±0.0555, 47.600±0.555, and 45.333±0.555, respectively, suggesting that the 200 mg/kg BW dose provided significant protective effects compared to the higher doses ($p<0.05$), although all doses contributed to a reduction in liver damage.

A two-way ANOVA statistical test was conducted to analyze the effects of two independent variables, namely treatment groups and

duration of administration, on the dependent variable, which was the histopathological score. The analysis revealed that the histopathological score was significantly influenced by the dosage, with a significance value (Sig.) of 0.000 ($p<0.05$), and by the duration of administration, with a Sig. value of 0.001 ($p<0.05$). Furthermore, the interaction between dosage and duration of administration also had a significant effect on the degree of tissue damage with a Sig. Value of 0.006 ($p<0.05$).

Table 2: Liver histology scores of mice

Group	Liver histology scores on day-			Average
	7	14	21	
Negative control	30.600[a]	32.067[a]	33.067[a]	31.911[b]
Positive control	57.133[a]	59.667[a]	62.800[a]	59.867[b]
EULEE 200	38.000[a]	40.667[a]	40.000[a]	39.556[b]
EULEE 400	47.200[a]	46.800[a]	48.800[a]	47.600[b]
EULEE 800	43.867[a]	48.733[a]	43.400[a]	45.333[b]
average	43.360[c]	45.587[c]	45.613[c]	

Note: a = SE±0.962, b = SE±0.555, c = SE±0.430

The reduction in the degree of liver tissue damage observed in mice indicates that the active flavonoid and phenolic compounds present in the extract possess antioxidant activity. This activity plays a crucial role in mitigating liver cell damage, as evidenced by the decrease in histology scores [16]. The polyphenolic compounds in this extract exhibit antioxidant capabilities that can accelerate cellular regeneration and improve the structural integrity of liver tissue. The antioxidant activity prevents and repairs liver cell degeneration through mechanisms of oxidation inhibition by scavenging reactive oxygen species (ROS), such as peroxyl radicals, and neutralizing their electron deficiencies, thereby preventing lipid peroxidation. The mechanism involves the-OH groups on flavonoids that capture free radicals, rendering them inactive metabolites, while the active phenolic compounds bind to free radicals and transfer hydrogen atoms (H+) to achieve stability. This process effectively interrupts the chain reactions of free radicals, thus preventing more severe cellular damage [13, 22].

This study found that the administration EULEE durations of 7, 14, and 21 d showed a trend of decreasing liver histology scores in mice compared to the positive control group as the treatment period was extended. The average histology scores for each duration were 43.360±0.430, 45.587±0.430, and 45.613±0.430, respectively (table 2), indicating that liver damage was significant reduced after 7 d of EULEE administration ($p<0.05$), and no further reduction was observed after 14 and 21 d.

The significant improvement in liver histology observed on day 7 suggests that the hepatoprotective effects of EULEE are achieved within a relatively short timeframe. However, no further improvement was noted after 7 d. The processes of oxidative stress reduction, inflammation modulation, and hepatocyte recovery likely reach their maximum effect within the first week of treatment, after which the body's ability to further enhance these processes may diminish. One possible mechanism is the limitation of the liver's regenerative capacity, where hepatocyte regeneration, which begins in the early stages of recovery, reaches its peak. Liver regeneration is dependent on growth factors such as hepatocyte growth factor (HGF), which peak during the first few days following injury and subsequently decline. Additionally, the body may adapt to the active compounds in EULEE, leading to receptor desensitization or a diminished response to molecular pathways involved in reducing oxidative stress and inflammation. Once the optimal reduction in oxidative stress and inflammation is achieved, the body's ability to further enhance these processes becomes limited [17, 19].

This study revealed distinct variations in cellular conditions across the treatment groups, which included a range of normal, degenerative, and necrotic cells in differing proportions. The negative control group predominantly exhibited normal cells, whereas the positive control group showed a marked increase in cellular damage, including degeneration and necrosis. In the groups treated with EULEE, a higher proportion of normal cells and a reduced extent of cellular damage were observed compared to the positive control group.

The negative control group (fig. 1) predominantly exhibited normal hepatocytes arranged in an orderly manner, characterized by a clear appearance, centrally located round nuclei, normal sinusoid width, and central vein diameter. These features indicate that the liver tissue was in a healthy condition, with minimal cellular abnormalities detected [20, 21].

Fig. 2 revealed a predominance of various pathological abnormalities, particularly necrosis. Signs of necrosis were indicated by the shrinkage and condensation of cell nuclei (pyknosis) and the loss of cell nuclei (karyolysis), resulting in a blurred appearance of the cells. The toxicity induced by carbon tetrachloride was evident in the histological examination, showing severe damage to liver tissue, deformation of central veins, and central venous congestion. Damage to hepatocytes is thought to be caused by the formation of free radicals, lipid peroxidation, and a decrease in antioxidant enzyme activity [4, 21].

This research demonstrated that administering CCl_4 leads to significant acute liver damage in mice. This supports previous research indicating that CCl_4 is an effective chemical agent for inducing hepatic cell injury in animal models. The liver damage caused by CCl_4 is potentially reversible and closely replicates real-life liver cirrhosis by generating free radicals and triggering pro-inflammatory and pro-fibrotic cytokines [2, 23].

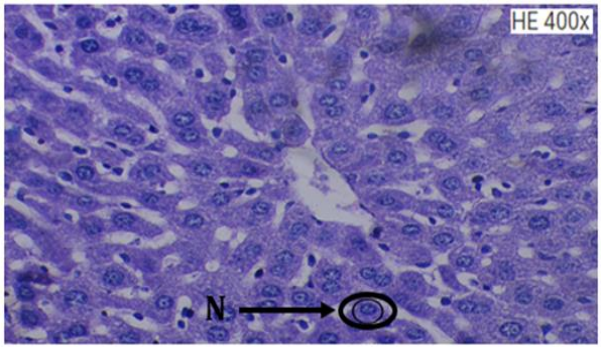

Fig. 1: Histology of liver mice cells in CCl_4-induced mice in negative control group (N = normal hepatocytes)

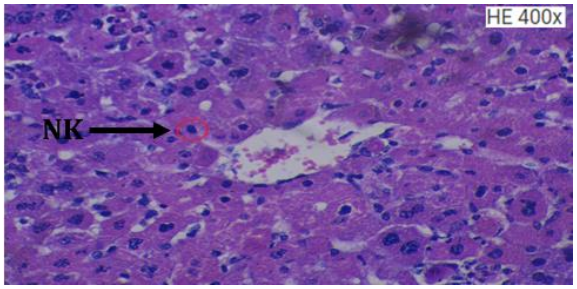

Fig. 2: Histology of liver mice cells in CCl_4-induced mice in positive control group (NK = necrosis)

The histological examination of the liver in mice treated with a dose of 200 mg/kg BW for 7 d revealed significant parenchymal degeneration. This degeneration became more pronounced on days 14 and 21 (fig. 3). Parenchymal degeneration is characterized by hepatocyte swelling and cloudy cytoplasm, indicating protein accumulation due to disrupted oxidation processes. This is likely caused by impairment in protein oxidation, which typically functions to maintain cellular balance and structural integrity [24]. The increasing parenchymal degeneration with prolonged administration suggests that hepatocytes are undergoing sustained oxidative stress. This oxidative stress has the potential to damage various cellular components, such as lipid membranes, proteins, and DNA. The accumulation of poorly oxidized proteins indicates that the liver's detoxification system, which is crucial for metabolizing harmful substances, is beginning to be compromised [25]. This may be due to reduced antioxidant enzyme activity or the cells' inability to repair damage caused by free radicals. The diminished capacity of hepatocytes to maintain protein synthesis and cellular metabolism can exacerbate cellular damage over time. If left uncontrolled, this process may lead to cell death or necrosis, ultimately affecting overall liver function [21, 26].

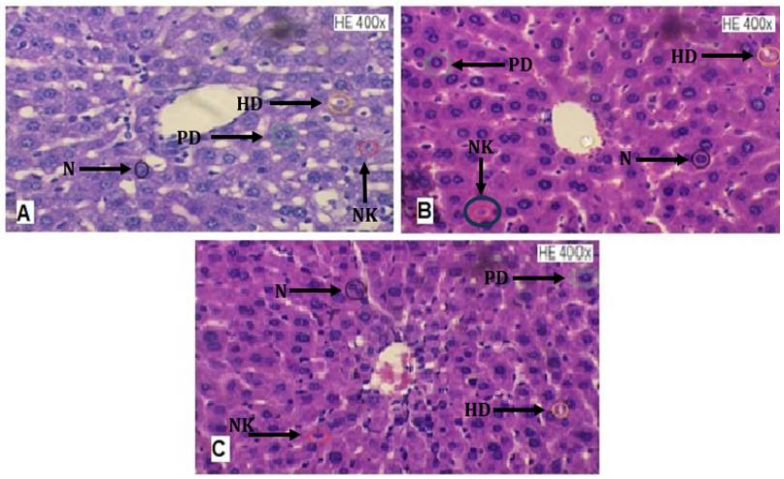

Fig. 3: Histology of liver mice cells in CCl_4-induced mice in EULEE 200 group on day 7 (A), EULEE 200 group on day 14 (B), EULEE 200 group on day 21, (N = Normal, PD = Parenchymal Degeneration, HD = Hydropic Degeneration, NK= Necrosis)

In the EULEE 400 group, the most commonly observed abnormality after 7 d of administration was parenchymal degeneration. An increase in the degree of parenchymal degeneration was evident on day 14, and this condition persisted until day 21, with a similar level of damage observed as on day 14 (fig. 4).

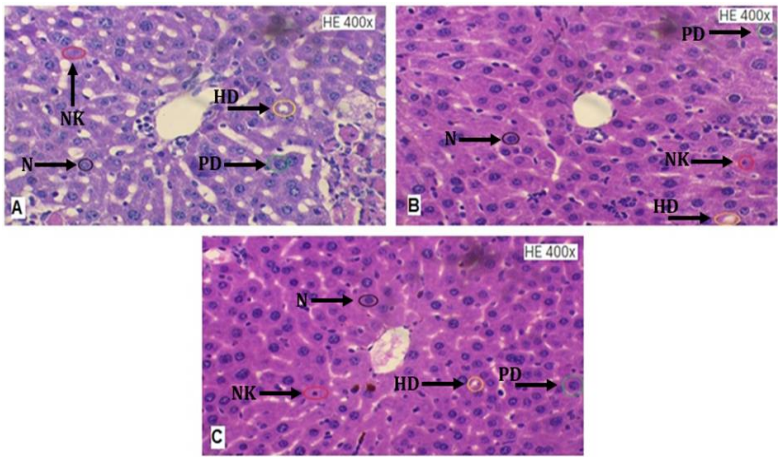

Fig. 4: Histology of liver mice cells in CCl_4-induced mice in EULEE 400 group on day 7 (A), EULEE 400 group on day 14 (B), EULEE 400 group on day 21, (N = Normal, PD = Parenchymal Degeneration, HD = Hydropic Degeneration, NK= Necrosis)

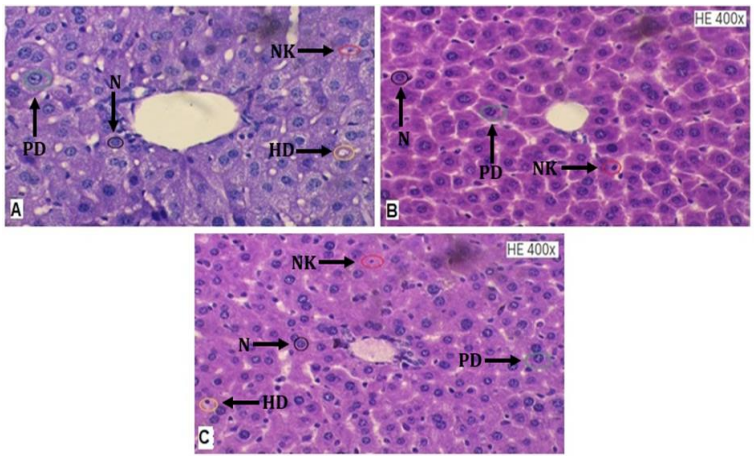

Fig. 5: Histology of liver mice cells in CCl_4-induced mice in EULEE 800 group on day 7 (A) EULEE 800 group on day 14 (B) EULEE 800 group on day 21 (N = Normal, PD = Parenchymal Degeneration, HD = Hydropic Degeneration, NK= Necrosis)

The most common abnormalities observed after administering an 800 mg/kg BW dose of the extract for 7 d were parenchymal degeneration and hydropic degeneration (fig. 4). In contrast, after 14 and 21 d of administration, parenchymal degeneration became more predominant (fig. 5).

The observed decrease in histopathological scores and liver damage histology in the treatment groups (EULEE 200, 400, and 800) indicates significant recovery of liver cells. This reduction in liver tissue damage is attributed to the active polyphenolic compounds present in the extract, which exhibit antioxidant properties and promote the regeneration process. EULEE is regarded as an effective hepatoprotective agent due to its antioxidant and anti-inflammatory properties, providing substantial protection to the liver against severe damage induced by CCl_4. The antioxidant activity of these compounds plays a critical role in preventing and repairing liver cell degeneration.

CONCLUSION

The administration of EULEE results in an improvement in the liver's histological features in mice.

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AUTHORS CONTRIBUTIONS

DAJ: provided supervision, contributed to the design concepts, and wrote the manuscript. FR and HN: contributed to the research design techniques. RI and EM: collected and analyzed the data. All authors reviewed and gave their approval for the final version of the manuscript.

CONFLICT OF INTERESTS

The authors confirm that there are no conflicts of interest associated with the publication of this research.

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