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Research Article

# Protective effect and potential natural antioxidant of *Cayratia trifolia* (L.) Domin. leaves extracts on nitrobenzene-induced hepatotoxic rats

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## ABSTRACT

*Cayratia trifolia* leaves contain polyphenols as a natural antioxidant that can dissolve in polar solvents. This study aims to evaluate the hepatoprotective and antioxidant activity of polar extracts (ethyl acetate and 70%-ethanol) were extracted sequentially from *C. trifolia* leaves on male rats induced by nitrobenzene. The extracts of *C. trifolia* leaves were given at a dose of 200 mg.Kg$^{-1}$ BW. Silymarin as a reference was given at a dose of 28.78 mg.Kg$^{-1}$ BW. The extracts and Silymarin were given orally once a day for 14 days. Rats that have received ethanol extract of *C. trifolia* leaves showed a significant decrease in Alkaline Phosphatase (ALP), Aspartate Transaminase (AST), Alanine Transaminase (ALT), total bilirubin, and direct bilirubin. A significant decrease in malondialdehyde (MDA) levels also occurred in the rats. On histopathological examination, the extract also showed a protective effect on rat liver. Thus, it can be concluded that the sequentially extracted ethanolic extract of *C. trifolia* leaves has the potential as a source of natural hepatoprotective and antioxidant activity.

**Keywords:**
Antioxidant, *Cayratia trifolia* L., Hepatoprotective, Nitrobenzene

## 1. INTRODUCTION

The liver is one of the vital organs in the body. The liver functions to help metabolism, detoxification, storage, and synthesis of certain substances such as amino acids, cholesterol, etc.[1]. The agent that can protect the liver from a hepatotoxic-induced radical substance is called hepatoprotective[2]. Herbal medicine is still used mainly by the world population for primary health care[3]. Herbal-based therapy for hepatoprotection is related to its phytoconstituent as an antioxidant[4]. One of those potential herbals is *Cayratia trifolia* (L.) Domin. (Vitaceae).

In Indonesia, *C. trifolia* is known as galing or lakum. This plant is called Fox grape[5]. Empirically, the leaves are used as medicine to cure headaches[1], boils, speed drying of the wound[6], muscle pains, and antidandruff[7]. *C. trifolia* leaves have been scientifically researched and proven to have several activities as antitumor[8], anti-implantation[9], anti-ulcer[10], larvicidal[11], antibacterial[12], antioxidant[13-14], antimitotic[15], and anti-hyperuricemia[16]. The purified ethanol extract of *C. trifolia* leaves at a dose <5,000 and 50,000 mg.Kg$^{-1}$ BW shows no liver cell damage[17].

Epifriedelanol is a compound found in ethanol extract of *C. trifolia*[8,18]. *C. trifolia* leaves contain stilbenes, several flavonoids[19], and linoleic acid[20]. The total flavonoids content in the ethanolic extract of *C. trifolia* leaves was 27.95±0.62 mgQE/g[21]. *C. trifolia* leaves also contain phytochemical compounds, such as alkaloids, polyphenols, terpenoids, steroids, and saponins. These compounds play a role in their biological activity, such as anticancer, anti-inflammatory, hepatoprotective, and antioxidant[22].

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The compounds can generally be dissolved using ethanol and ethanol-water[23]. Several other organic solvents (benzene, chloroform, dichloromethane, etc.) need to be considered as extraction solvents because of their possible toxic effects as residual solvents in the extracts[24].

Nitrobenzene (NB) is a chemical widely used in industry with an almond-like odor. It is a toxic substance and can be evaporated in the air. If exposed to high concentrations, it can cause liver damage. NB has methemoglobinemia, carcinogenic, neurotoxic, and hepatotoxic properties[25]. It produces several free radicals such as superoxide anion during its reductive metabolism[26]. NB-induced hepatotoxicity can be used as an *in vivo* model for hepatoprotective studies.

Previously, ethanol extract of *C. trifolia* leaves at a dose of 500 mg.Kg$^{-1}$ BW has hepatoprotective activity on paracetamol-induced rats[27]. Guru Kumar et al. (2011) reported that after administration of the *C. trifolia* whole plant extract (200 mg.Kg$^{-1}$) orally, there was hepatocyte regeneration in rats induced by nitrobenzene[28]. The present study evaluates the hepatoprotective and antioxidant effect of sequentially extracted polar extracts (ethyl acetate and ethanolic extracts) of *C. trifolia* leaves on nitrobenzene-induced rats. Thus, an extract containing active compounds with antioxidant and liver protection effects will be obtained.

## 2. MATERIALS AND METHODS

### 2.1. Plant Material

The *C. trifolia* leaves were collected from the Tuban, East Java, Indonesia. The plant was identified in Conservation Research Center and Botanical Garden, Indonesian Institute of Sciences/Lembaga Ilmu Pengetahuan Indonesia (LIPI), Bogor, Indonesia. The *C. trifolia* fresh leaves were washed under running tap water. Then, the leaves were dried for a few days in indirect sunlight and made into powder.

### 2.2. Extraction

The dried leaves powder (1.5 Kg) was extracted in various solvents such as *n*-hexane, ethyl acetate, and 70%-ethanol according to their increasing polarity by cold maceration (2 times, 24 hours). The extracts were filtered through filter paper. The residue was further repeated twice with the same solvent. Each filtrate was evaporated using a vacuum rotary-evaporator N-1200 BS series (EYELA, Shanghai, China) at 40°C. The percentage yield of *n*-hexane, ethyl acetate, and ethanolic extracts of *C. trifolia* leaves was calculated[29].

### 2.3. Phytochemical Identification

The presence of secondary metabolites (viz., alkaloids, flavonoids, phenolic, etc.) in the extracts were qualitatively identified using the standard procedures described in the[29-31]. Tests were carried out using reagents such as Dragendorff, Mayer, and Bouchardat (for alkaloids detection); Shinoda (for flavonoids detection); $FeCl_3$ 3% (for phenolics detection), 10% gelatine (for tannins detection), etc.

### 2.4. Total Phenolic Content Estimation

Total phenolic content (TPC) was measured by colorimetry according to the Folin-Ciocalteu method using gallic acid as the standard[32]. Extract (300 µL) were mixed vigorously with 1.5 mL of the Folin-Ciocalteu reagent. The reagent was diluted ten times in water. After 3 min, 1.2 mL of sodium carbonate solution (7.5%) was added to the mixture. The mixed solution was then incubated at room temperature (25°C) for 30 min. The absorbance was measured at 743.50 nm with a spectrophotometer UV-Vis Seri UV-1601 (Shimadzu, Kyoto, Japan). Gallic acid solution with concentrations ranging from 14-50 mg.L$^{-1}$ was used for calibration. A dose-response linear regression was generated by using the gallic acid standard absorbance. A linear calibration curve of gallic acid was obtained (y=0.0129x+0.0545) with the coefficient of determination (R) value of 0.99. The total phenolic content was expressed as gallic acid equivalent (mg of GAE.g$^{-1}$ of extract). The estimation was performed in triplicate, and the results were expressed as mean±SD.

### 2.5. Preparation of rats

Male white rats (*Rattus norvegicus* L.) of Wistar strain of 2-3 months old (bodyweight 140-160 g) were used in this study. The rats were maintained in a well-ventilated room with a natural day-night period. Rats were fed with standard pellets for rodents and water ad libitum throughout the experimental period. Before treatment, the rats were quarantined for seven days.

### 2.6. Experimental Design

This protocol (no.02/19.10/0230) was approved by the Health Research Ethics Commission of Universitas Muhammadiyah Prof. DR. HAMKA. Determination of the dose of the extracts in this study refers to Kumar et al. (2011), where used a methanol extract of the aerial part of *C. trifolia* at a dose of 200 mg/kg BW rats (28). Whereas the dose of Silymarin refers to its use in humans (280 mg.60 Kg$^{-1}$) which has been converted at a rat of 28.78 mg.Kg$^{-1}$ once a day. The extracts and Silymarin were prepared in 0.5% Na-CMC.

Rats were divided into five groups. All groups have received the treatments for 14 days. All groups were inducted with nitrobenzene (50 mg.Kg$^{-1}$BW) orally on

day 1, except the normal control group.

- Normal control group: rats have received standard pelleted rat feed without Na-CMC each day orally.
- Untreated control group: rats have received Na-CMC 0.5% each day orally.
- Silymarin control group: rats have received Silymarin at 28.78 mg.Kg$^{-1}$ each day orally.
- Ethyl acetate extract group: rats have received ethyl acetate extract at 200 mg.Kg$^{-1}$BW each day orally.
- Ethanolic extract group: rats have received ethanolic extract at 200 mg.Kg$^{-1}$BW each day orally.

### 2.7. Preparation of the Blood Serum

On day 15$^{th}$, after administration of the extracts, the rats were anesthetized using ketamine intramuscularly. The blood sample was taken through the orbital sinus and then collected in a microtube. Blood was then centrifuged for 15 minutes at 6,000 rpm to obtain the blood serum for biochemical analysis. After blood sampling, the rats were sacrificed by cervical dislocation, and the liver was dissected entirely and put into the 10% buffered formalin for histopathological evaluation.

### 2.8. Determination of Liver Enzymes Levels

The Alkaline Phosphatase (ALP) level in rat blood serum was determined using a diagnostic reagent (DiaSys, Holzheim, Germany) with a kinetic photometric test method according to the Germany Society of Clinical Chemistry procedure[33]. The absorbance was read at 405 nm. The Aspartate Aminotransferase (AST) level in rat blood serum was carried out following the protocol of GOT (ASAT) IFCC mod. liquiUV multipurpose reagent kit (HUMAN, Magdeburg, Germany), whereas the Alanine Aminotransferase (ALT) level in rat blood serum was carried out following the protocol of GPT (ASAT) IFCC mod. liquiUV multipurpose reagent kit (HUMAN, Magdeburg, Germany). The direct (conjugated) bilirubin and total bilirubin in rat blood serum were determined using Bilirubin D+T liquicolor photometric test kit (HUMAN, Magdeburg, Germany) modified from Jendrassik & Gróf, (1938) method[34]. The absorbance was read at 546 nm with an optical path is 1 cm. The measurement of absorbance from all liver enzyme levels was performed using a clinical spectrophotometer (Microlab-300).

### 2.9. Determination of Lipid Peroxidation Levels

The procedure was performed following the method reported by Esterbauer & Cheeseman (1990)[35]. The rat liver was added with phosphate buffer pH 7.4 (5 times liver weight) and homogenized in mortar. The homogenate of the liver (1 ml) was reacted with 0.5 ml of 20% trichloroacetic acid (TCA) solution (Sigma Aldrich, Darmstadt, Germany) and then centrifuged at 3,000 rpm for 10 minutes. The supernatant was added with 1 ml of 0.67% (w/v) Thiobarbituric acid (TBA) solution (Sigma Aldrich, Darmstadt, Germany), and then homogenized. The mixture was heated at 100°C for 10 minutes. The product of the reaction from two molecules of TBA and one molecule of MDA produces the pink-coloured solution. The absorbance can be measured at 532 nm using a spectrophotometer UV-Vis. The standard solution was used to make the standard curve is tetra ethoxy-propane (TEP) (Sigma Aldrich, Darmstadt, Germany). The blank solution contains the mixture of 1 ml of aqua distillate, 0.5 ml of 20% TCA solution, and 1 ml of 0.67% TBA solution.

### 2.10. Histopathological Studies

A small piece of the rat liver tissue from each group was prepared using the paraffin blocks method and transferred to a slide, and were stained using Hematoxylin and Eosin stain for histopathological observations using a biological microscope (Olympus CX23, Tokyo, Japan) at magnification 10×40. The liver cells of each rat were observed. The number of pyknotic nuclei was counted. Observations were made with five fields of view randomly for each sample.

### 2.11. Statistical Analysis

Data were analysed using one-way analysis of variant (ANOVA) and continued with the Tukey test ($\alpha$=0.05). The histopathological evaluation of the rat liver was analyzed descriptively.

## 3. RESULTS AND DISCUSSION

### 3.1. Characteristics of Extracts

In this study, the *C. trifolia* leaves were sequentially extracted using different solvent polarities (*n*-hexane, ethyl acetate, and 70%-ethanol). *C. trifolia* leaves are dominated by polar compounds found in the ethanolic extract (21.30 %, w/w) compared to ethyl acetate extract (1.73 %, w/w). Among the extracts, the *n*-hexane extract gave the lowest yield percentage (0.60%, w/w). The low percentage of yield indicates the most minor content of nonpolar compounds in *C. trifolia* leaves extracted in *n*-hexane.

Based on phytochemical screening, ethyl acetate extract of *C. trifolia* leaves contains alkaloids, flavonoids, phenolics, and steroids. Phytochemical compounds in ethanolic extract of *C. trifolia* leaves are alkaloids, flavonoids, phenolics, tannins, and steroids. *n*-Hexane extract contains steroids and triterpenoids. Sowmya et al. (2015) reported that phytochemical compounds in sequentially extracted ethyl acetate extract of *C. trifolia*

leaves are amino acids and proteins, carbohydrates, and polyphenols[22]. Sequentially extracted ethanolic extract of *C. trifolia* leaves contains amino acids and proteins, carbohydrates, polyphenols, flavonoids, cardio-glycosides, and steroids.

In this study, the *n*-hexane extract was not qualitatively detected to contain phenolics. Thus, TPC was only determined on ethyl acetate and ethanol extracts while the *n*-hexane extracts were not. TPC of the extracts was determined using gallic acid as a reference. Folin-Ciocalteu (F-C) was used as reagent. The colour product of reaction was measured at a maximum wavelength of 743.50 nm. Based on this study, sequentially extracted ethanolic extract of *C. trifolia* leaves has total phenolic content (23.79±0.09 mgGAE.g$^{-1}$**)** higher than ethyl acetate extract (18.28±0.20 mgGAE.g$^{-1}$). Anita et al. (2019) reported that ethanolic (80%) extract of *C. trifolia* leaves contain 3.1 mg.g$^{-1}$ dry weight of phenolic content higher than its stem (2.45 mg.g$^{-1}$ dry weight)[19].

### 3.2. Hepatoprotective activity

Evaluation of hepatoprotective activity in rats was determined by measuring liver enzyme levels parameters in rats' blood serum of each group, such as ALT, AST, ALP, bilirubin direct, and total bilirubin (Table. 1).

**Table 1.** Liver enzymes levels in each rat serum after 14 days treatment.

| Groups | ALP U.L$^{-1}$±SD | AST (U.I$^{-1}$)±SD | ALT (U.I$^{-1}$)±SD | Bilirubin Direct (mg.dL$^{-1}$)±SD | Total Bilirubin (mg.dL$^{-1}$)±SD |
|---|---|---|---|---|---|
| Normal control | 66.00±6.63$^{a}$ | 50.60±2.40$^{a}$ | 20.20±2.77$^{a}$ | 0.17±0.01$^{a}$ | 0.24±0.02$^{a}$ |
| Untreated control | 139.80±8.81$^{b}$ | 94.40±3.84$^{b}$ | 38.20±1.92$^{b}$ | 0.37±0.04$^{b}$ | 0.60±0.01$^{a,b}$ |
| Positive control | 76.00±6.30$^{a}$ | 62.40±3.84$^{a,b}$ | 23.80±2.58$^{a}$ | 0.22±0.01$^{a,b}$ | 0.33±0.03$^{a,b}$ |
| Ethyl acetate extract | 105.00±9.40$^{a,b}$ | 73.00±5.09$^{a,b}$ | 29.40±0.54$^{a,b}$ | 0.28±0.01$^{a,b}$ | 0.50±0.02$^{a,b}$ |
| Ethanolic extract | 89.00±7.31$^{a,b}$ | 65.20±1.92$^{a,b}$ | 24.20±2.77$^{a,b}$ | 0.24±0.01$^{a,b}$ | 0.38±0.04$^{a,b}$ |

Note: Data are reported as mean±SD, n=5. There was a significant difference compared to the untreated control group$^{a)}$ and the normal control group$^{b)}$ ($\alpha$=0.05) (through Tukey test analysis)

Based on Table 1, shows a decrease (sig 0.000, $\alpha$<0.05) in all liver enzymes parameters in the blood serum of nitrobenzene-induced rats after being given the test materials (both extracts of *C. trifolia* and Silymarin) for 14 days (through one-way ANOVA analysis). As compared to the Silymarin control group, the ethanolic extract-treated group of rats did not show any significant ($\alpha$>0.05) difference in all liver enzymes (through Tukey test analysis). It means that ethanolic extract of *C. trifolia* has a good potential as a source of hepatoprotective agent approached the silymarin activity as a positive control.

In the present study, the extracts and Silymarin were administered suspension form prepared in 0.5% Na-CMC. Rats in the normal group did not receive Na-CMC. Soldatova et al. (2020) reported that Na-CMC could affect hemostatic and provide an anti-adhesive effect in male Wistar rats at concentrations ranging from 3% to 6%[36]. To avoid things that are not expected in the normal control group, this group only receives standard pelleted rat feed and drink.

The liver is an essential organ in the body that plays an essential role in vascular function, metabolism (metabolism of carbohydrates, protein, fat, storage of vitamins, detoxification/excretion of drugs, hormones, and other substances), secretory, and excretory. Liver damage is generally associated with the occurrence of liver cell necrosis. The increased lipid peroxidation in tissues which will then have an impact on the liver function itself[37]. NB is one of the chemical compounds as an oxidant. It contains a single electrophilic nitro group attached to benzene[25]. Nitrobenzene can induce hepatotoxic conditions. It caused a significant increase in the serum liver marker enzymes and bilirubin in rats. As an oxidant, nitrobenzene also causes oxidative stress that damages to hepatic cells (necrosis)[38]. High levels of liver enzymes such as ALP, ALT, AST, and bilirubin are used to evaluate liver damage.

Silymarin (as a reference) has been used to treat liver disorders. It is a naturally occurring chemical compound obtained from *Silybum marianum* (or Milk thistle). Flavolignans, such as silybin, silydianin, and silychristine make up Silymarin. Not only as a liver protector, it is also capable of providing regenerative action. One of the mechanism action is that it decreases the production of free radicals, which may harm cell membranes[39]. In this study, it is suspected that the phenolic content in *C. trifolia* leaves extract can protect the liver. Identifying of the type of phenolics in this extract needs to be studied further to determine its mechanism of action clearly on its target of action.

### 3.3. Antioxidant Activity

Previously, Utami et al. (2013) reported that epifriedelanol (a terpenoid) isolated from nonpolar leaves and stem bark extract of *Elaeocarpus floribundus* did not provide antioxidant activity against DPPH radicals ($IC_{50}$>500 g/mL)[40]. It also strengthens why the *n*-hexane extract was not studied further in this study. The graph in Figure 1. shows that providing both extracts and Silymarin as test materials can reduce MDA levels in

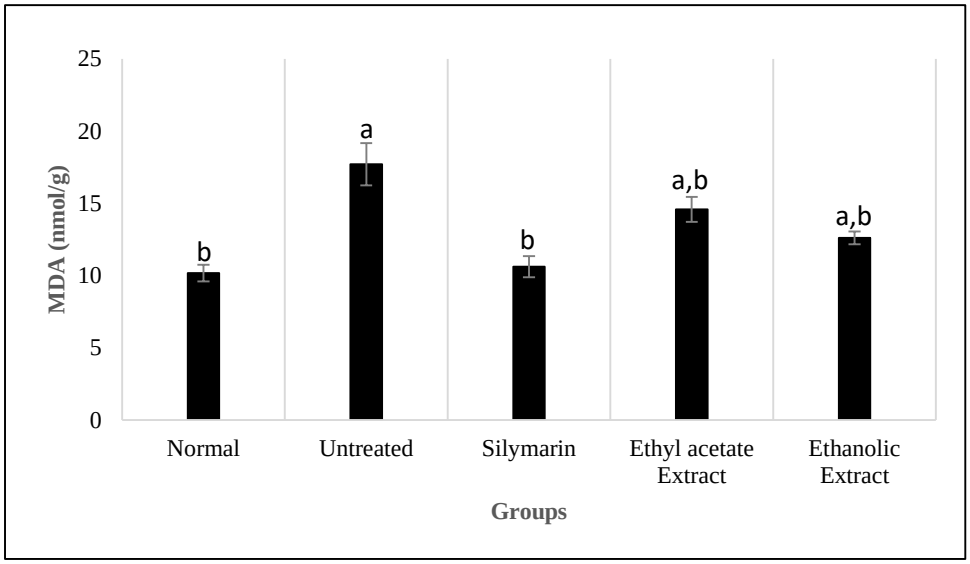


**Figure 1.** MDA level in each serum control and experimental group after 14 days of treatment. Data are reported as mean±SD, n=5. There was a significant difference ($p$<0.05) compared to the normal control groupa) and the untreated control groupb) rats.

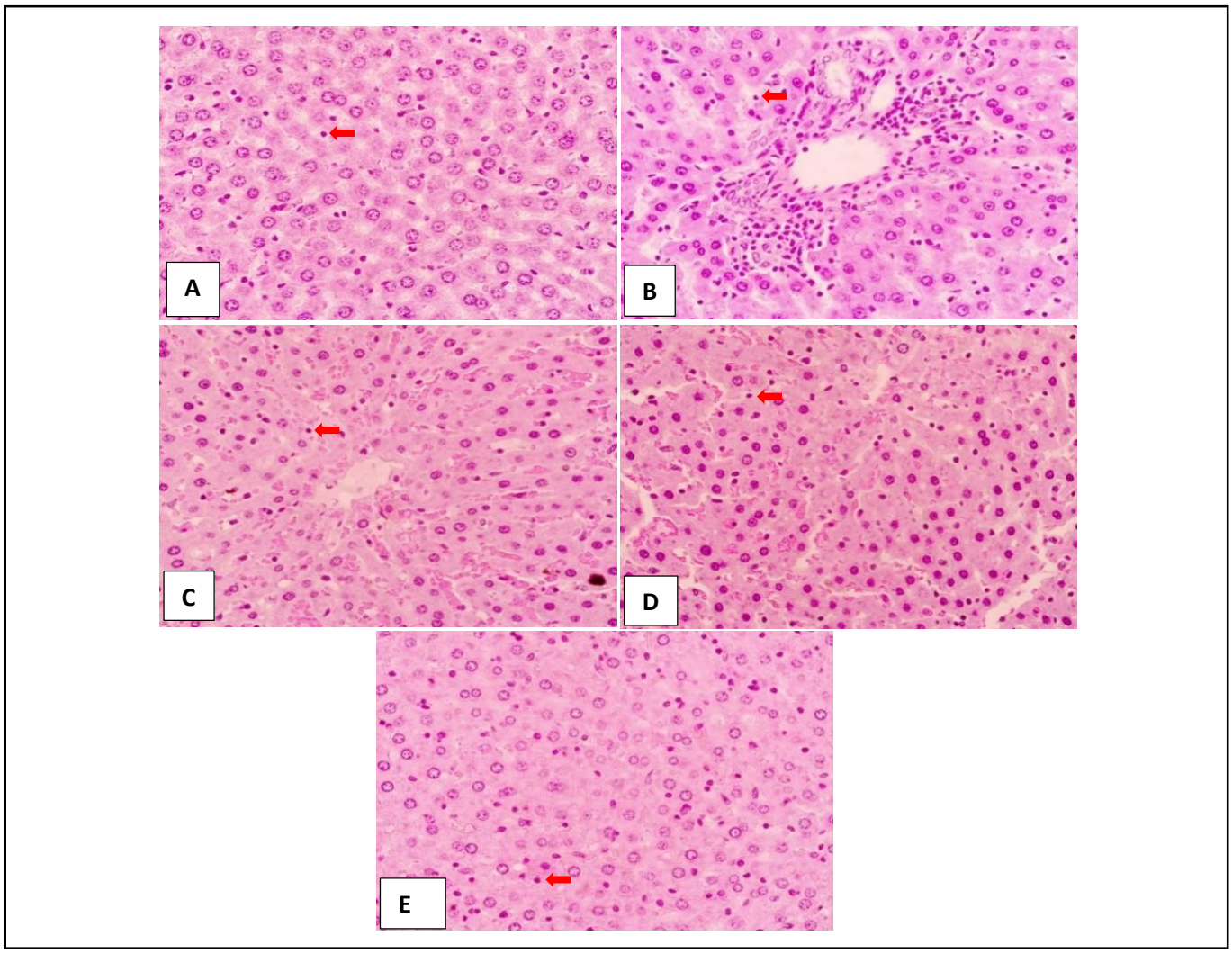


**Figure 2.** Histopathological of the liver in each control and experimental group after 14 days of treatment.
Note: (A) Normal control group (no nitrobenzene-induced, no drugs); (B) Untreated control group (nitrobenzene-induced+0.5% Na-CMC); (C) Silymarin control group (nitrobenzene-induced+Silymarin); (D) nitrobenzene-induced+ethyl acetate extract of *C. trifolia* leaves; (E) nitrobenzene-induced+ethanolic extract of *C. trifolia* leaves. Magnification at 10×40. The red arrow indicates the presence of pyknotic cells.

nitrobenzene-induced rats compared to the untreated (nitrobenzene-induced) control group after 14 days of treatment. In this study, the extracts treated groups showed a significant decrease ($p$<0.05) in malondialdehyde (MDA) levels compared to the untreated control group (through Tukey test analysis).

In the body, increased concentrations of free radicals and low enzymatic antioxidants cause oxidative stress, one of which is marked by the formation of malondialdehyde (MDA)[41]. Lipid peroxidation produces a small amount of MDA, as a major end degradation product[42] that causes hepatic cell damage and leads to the release of marker enzyme. The level of MDA can be determined using thiobarbituric acid (TBA) under acidic conditions[35]. TBA test was performed by extracting MDA using aqueous TCA and then reacting with TBA[42]. In this study, there was a decrease in liver enzyme levels in blood serum and MDA levels after administration of *C. trifolia* extract on nitrobenzene-induced rats.

Phenolic compounds (such as aglycon flavonoids, anthocyanin, catechins, phytoestrogens, and tannin) are important antioxidants in herbal medicine. In order to decrease the HO• radical, antioxidants attack it by giving an electron. It may function as an inhibitor of the production of MDA and MDA-TBA, as seen in the activity test result[43]. Thus, these compounds play a role in radical scavenging activity that leads to hepatoprotective activity[37]. Ethanol and aqueous ethanol are non-toxic extraction solvents that have suitable polarity to extract polar compounds, such as phenolic compounds (one of them is flavonoids). The compounds are effective as antioxidants[44].

### 3.4. Histopathological Studies in Liver

The presence or absence of pyknotic nuclei might indicate hepatic damage (hepatic necrosis). The pyknotic nuclei is the dead cells, shrunken in shape, with irregular borders, and dark in color[45]. In this study, the average total pycnotic nuclei in the normal, no-treated, Silymarin, ethyl acetate extract, and ethanol extract groups were 25, 72, 33, 52, and 42, respectively. The rat liver tissue from the normal control group shows normal hepatic cells (Figure 2A.). In the untreated control group (Figure 2B), severe hepatotoxicity was demonstrated by the appearance of severe hepatic necrosis. The rats which received ethyl acetate and ethanolic extracts of *C. trifolia* (Figure 2D. and 2E.) showed normal hepatocytes and more minor degree of inflammation, whereas the Silymarin control group (Figure 2C.) showed normal hepatic cells.

## 5. CONCLUSION

The ethanol extract of *C. trifolia* leaves (200 mg.Kg$^{-1}$) has good liver protective activity and antioxidant activity in nitrobenzene-induced rats compared to untreated rats. The phytochemical component in the extract responsible for its hepatoprotective mechanism of action using bio-assay guide isolation needs to be investigated further.

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**Conflict of interest**

None to declare

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**Ethics approval**

This protocol (no. 02/19.10/0230) was approved by the Health Research Ethics Commission of Universitas Muhammadiyah Prof. DR. HAMKA.

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