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In Vivo Study of Red Spinach Leaf Extract on the Excretory System of Wistar Rats Induced with a Toxic Dose of Paracetamol

Studi In Vivo Ekstrak Etanol Daun Bayam Merah terhadap Organ Ekskresi Tikus Wistar Yang Diinduksi Parasetamol Dosis Toksik

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Abstrak

Parasetamol adalah obat antipiretik-analgesik yang dapat menyebabkan efek toksik jika jika dikonsumsi dalam dosis tinggi. Antioksidan dalam bayam merah dapat berperan dalam menangkal radikal bebas dari efek toksik yang ditimbulkan oleh parasetamol. Penelitian ini bertujuan untuk menentukan dosis yang paling efektif ekstrak bayam merah dalam melindungi struktur histopatologi organ eksresi tikus Wistar yang diinduksi parasetamol dosis toksik. Metode penelitian ini menggunakan 24 ekor tikus Wistar jantan (bobot ±180 g) yang dibagi menjadi 4 kelompok terdiri dari K (aquadest), KN (Parasetamol 0,5 g/kg BB), P1 (ekstrak etanol bayam merah 0,4 g/kg BB dan Parasetamol 0,5 g/kg BB), dan P2 (ekstrak etanol bayam merah 0,8 g/kg BB dan Parasetamol 0,5 g/kg BB) dengan pemberian ekstrak selama 15 hari dan parasetamol diberikan dalam 5 hari terakhir. Pada hari ke-16, organ eskresi yang terdiri dari hati dan ginjal dibuat preparat histopatologi dengan metode parafin (Hematoxylin-Eosin). Parameter yang diamati meliputi indeks hepatosomatik, karakteristik hepatosit, sel nekrosis, sel inflamasi, glomerulus, dan hemoragi. Seluruh parameter penelitian dianalisis menggunakan *one way Anova* dengan uji lanjut DMRT ($p \leq 0,05$). Hasil penelitian menunjukkan dosis 0,4 g/kg BB ekstrak mengurangi luas area hepatosit, total sel nekrosis, area inflamasi, dan melindungi glomerulus ($p \leq 0,05$). Kesimpulan penelitian ini yaitu ekstrak etanol dari bayam merah pada dosis 0,4 g/kg BB menunjukkan efek protektif pada organ eksresi tikus Wistar yang diinduksi dengan parasetamol dosis toksik 0,5 g/kg BB.

Kata kunci: Bayam Merah; Histopatologi; Organ Ekskresi; Paracetamol; Tikus Wistar

Abstract

Paracetamol is an antipyretic-analgesic drug that can cause toxic effects when consumed in high doses. Antioxidants found in red spinach may counteract free radicals resulting from paracetamol's toxic effects. This study aims to determine the most effective dose of red spinach extract in protecting the histopathological structure of the excretory organs in Wistar rats induced with a toxic dose of paracetamol. This research used 24 male Wistar rats (weighing approximately 180 g), divided into four groups: K (aquadest), KN (Paracetamol 0.5 g/kg BW), P1 (ethanolic red spinach extract 0.4 g/kg BW and Paracetamol 0.5 g/kg BW), and P2 (ethanolic red spinach extract 0.8 g/kg BW and Paracetamol 0.5 g/kg BW). The extract was administered for 15 days, while paracetamol was given during the last 5 days. On the 16th day, the excretory organs, including the liver and kidneys, were prepared for histopathological examination using the paraffin method (Hematoxylin-

osin staining). Observed parameters included the hepatosomatic index, hepatocyte characteristics, necrotic cells, inflammatory cells, glomeruli, and hemorrhage. All parameters were analyzed using one-way ANOVA followed by the DMRT post hoc test ($p \leq 0.05$). The results showed that a dose of 0.4 g/kg BW of extract reduced the hepatocyte area, total necrotic cells, and inflammatory areas, and protected the glomeruli ($p \leq 0.05$). In conclusion, the ethanolic extract of red spinach at a dose of 0.4 g/kg BW exhibited a protective effect on the excretory organs of Wistar rats induced with a toxic dose (0.5 g/kg BW) of paracetamol.

Keywords: Excretory Organs; Histopathology; Paracetamol; Red Spinach; Wistar Rats

Introduction

Paracetamol is an antipyretic and analgesic drug that is relatively safe for oral consumption at appropriate doses and is available without a doctor's prescription. The percentage of paracetamol use as a self-medication drug in the community is 42.8%. According to the Indonesian Food and Drug Authority (BPOM RI), the maximum recommended oral dose of paracetamol is 4 g/day for adults, 2 g/day for children aged 6 to 12 years, 1 g/day for children aged 1 to 5 years, and 0.48 g/day for children aged 3 months to 1 year. Paracetamol taken in doses that exceed the recommended limit and used over a prolonged period can lead to the accumulation of a toxic compound called *N-acetyl-p-benzoquinoneimine* (NAPQI). This compound is produced through the metabolism of paracetamol via oxidation by cytochrome P450 (isoenzyme CYP2E1 in the liver) (Oktaviana *et al.,* 2017; Kurniadi *et al.,* 2018; Merdana *et al.,* 2018).

In general, NAPQI is detoxified by glutathione in the liver. When the amount of glutathione in the liver is insufficient to detoxify NAPQI, this compound reacts with cellular glutathione in the kidney cortex, which serves to reduce NAPQI and convert it into non-toxic mercapturic acid (Stollings *et al*., 2016). The use of paracetamol in doses that exceed the maximum limit and over a prolonged period can lead to the accumulation of toxic compounds and result in cellular glutathione depletion. This depletion allows NAPQI to bind to cellular proteins, initiating free radical formation, inducing oxidative stress, and potentially leading to cell death (El-Shafey *et al.,* 2015; Tittarelli *et al.,* 2017). Liver and kidney damage due to toxic NAPQI exposure is characterized by changes in histopathological structures such as necrosis, inflammation, hemorrhage, and a reduction in the number of hepatocytes and glomerular cells (Seok *et al.,* 2018; Dallak *et al.,* 2020).

Red spinach is one of the plant varieties cultivated in Indonesia that is rich in antioxidant activity. It contains secondary metabolites such as betacyanin pigments, flavonoids (quercetin), carotenoids, and vitamin C (Sarker *et al.,* 2019). The antioxidant activity of red spinach leaves is higher than that of green spinach leaves. The total flavonoid content in red spinach is 5.61% greater than that in green spinach (Guntarti and Ruliyani, 2020). The antioxidants present in red spinach leaves can serve as a source of exogenous antioxidants, which are needed by the body to enhance endogenous antioxidant levels and combat free radicals. Based on this background, further information is needed regarding the protective effects of red spinach leaves in preventing the adverse impacts of free radicals on excretory organs. Therefore, this study aims to determine the protective effect of ethanol extract from red spinach leaves on the histopathological structure of the liver and kidney in Wistar rats induced with a toxic dose of paracetamol.

Materials and Methods

This research is an in vivo experimental study using Wistar rats to preclinically assess the protective effect of ethanol extract of red spinach leaves on excretory organs induced by toxic doses of paracetamol. The research protocol was approved by the Ethics Committee of Universitas Ahmad Dahlan (approval number: 012212199). The red spinach plant was identified at the Laboratory of Plant Systematics, Faculty of Biology, Universitas Gadjah Mada (identification number: 0274/5.Tb./III/2023). The treatment and data analysis were carried out at the Laboratory of Animal Structure and Physiology, Faculty of Applied Science and

Technology, Universitas Ahmad Dahlan, from January to May 2023. Red spinach leaves (*Amaranthus tricolor* L.) were obtained from the Eduwisata Narakupu garden in Yogyakarta.

Preparation of Red Spinach Leaf Ethanol Extract

Red spinach leaves were dried in an oven at 50°C for 24 hours. The dried leaves were then mechanically crushed using a blender and subsequently extracted using the maceration method with 96% ethanol as the solvent for three days. After three days, the extract was filtered through filter paper, and the filtrate was evaporated using a Rotary Evaporator at 50°C until the extract was obtained in a paste form (Arif *et al.,* 2021).

Treatment of Wistar Rats

Wistar rats (*Rattus norvegicus* Berkenhout, 1769) (24 animals, body weight 180-250 g, and age 3-4 months) were divided into four groups, with each group consisting of six animals: K (Aquadest), KN (toxic dose of paracetamol 0.5 g/kg BW), P1 (ethanol extract of red spinach leaves 0.4 g/kg BW and toxic dose of paracetamol 0.5 g/kg BW), and P2 (ethanol extract of red spinach leaves 0.8 g/kg BW and toxic dose of paracetamol 0.5 g/kg BW). The extract was administered orally (1 ml via sonde) for 15 days, while the toxic dose of paracetamol (1 ml via sonde) was administered during the last 5 days, with a 2-hour interval after the administration of the extract (Mohammed and Sabry, 2020).

Preparation and Histopathology Observation

On day 16, the rats were anesthetized using 10% ether and then euthanized by cervical dislocation. The rats were dissected from the lower abdomen to obtain the liver and kidney organs. The organs were washed with 0.9% NaCl and weighed using a digital scale to calculate the Hepatosomatic Index. The Hepatosomatic Index was calculated based on Putri *et al.* (2021) by dividing the liver weight by body weight and multiplying by 100%.

Histopathological preparations were made using the paraffin embedding method. The organs were fixed in 10% formalin. The kidneys and liver were cut into pieces of approximately 1 cm in size, placed into cassettes, and processed in an automatic tissue processor (dehydration process with alcohol concentrations of 70%, 80%, 90%, 95%, and absolute) (Setiawan *et al.,* 2022). The tissue was then treated with xylene to dissolve the alcohol, facilitating paraffin infiltration. The next step was embedding the tissue in liquid paraffin, after which the paraffin blocks were stored in a refrigerator at 5°C. Each paraffin block containing tissue was sliced using a microtome to a thickness of 4-6 µm. Fragile tissue sections were placed on the surface of warm water to prevent shrinkage, and then mounted on glass slides and incubated for 24 hours to allow the tissue to adhere. Staining was performed using *Hematoxylin-Eosin* (HE) (Setiawan *et al.,* 2018).

The preparations were examined under an Olympus CX23 microscope, and images were captured using a Betaview Camera at 40x, 100x, and 400x magnification. The parameters observed in kidney tissue included the area of hemorrhage, the total number of tubular necrosis cells, and the area and number of glomeruli (Seok *et al.,* 2018; Dallak *et al.,* 2020). In liver tissue, the parameters observed included the total area of hepatocytes, the total number of necrotic cells, and the area of inflammation (Sari *et al.,* 2021). All histological observations were measured using the Image Raster application.

Data Analysis

All data parameters from the histopathological examination of the kidney and liver were analyzed using the Analysis of Variance (ANOVA) statistical test. If the results indicated a significant difference ($p < 0.05$), further analysis was performed using Duncan's Multiple Range Test (DMRT) with a 95% confidence level.

Results and Discussion

Liver Histopathology

The results showed that the administration of toxic doses of paracetamol and the ethanol extract of red spinach leaves had no significant effect on body weight, liver weight, or the hepatosomatic index ($p > 0.05$) (Table 1).

Table 1. Body weight and histopathological structure of liver tissue in treatments

Variable	K	KN	P1	P2
Day 0 body weight (g)	232.25 ± 16.87[a]	207 ± 18.38[a]	208.25 ± 15.47[a]	232.25 ± 24.14[a]
Day 11 body weight (g)	238.25 ± 22.85[a]	213 ± 17.16[a]	216.5 ± 10.84[a]	205.5 ± 35.56[a]
Day 15 body weight (g)	213.25 ± 26.56[a]	240.75 ± 43.77[a]	210.25 ± 3.59[a]	219.5 ± 30.25[a]
Liver weight (g)	7.58 ± 1.12[a]	8.07 ± 2.28[a]	6.89 ± 1.13[a]	7.69 ± 1.54[a]
Hepatosomatic Index (%)	3.55 ± 0.24[a]	3.31 ± 0.4[a]	3.48 ± 0,27[a]	3.48 ± 0.25[a]
Hepatocytes number	399.50 ± 34.14[b]	244,25 ± 24,46[a]	293.25 ± 11.24[a]	370 ± 54.12[b]
Hepatocyte area (μm^2)	197.44 ± 16.01[a]	323.38 ± 7.83[c]	300.55 ± 6[b]	286.48 ± 17.23[b]
Necrosis cells number	124.5 ± 40.09[a]	378 ± 23.02[c]	160.5 ± 25.59[a]	294.5 ± 31.63[b]
Inflammation area (mm^2)	1.26 ± 0.32[a]	7.67± 4.14[b]	3.90 ± 0.71[a]	2.17± 1.75[a]

Notes: K (aquadest), KN (Paracetamol toxic dose 0.5 g/kg BW), P1 (extract 0.4 g/kg BW + Paracetamol toxic dose 0.5 g/kg BW), P2 (extract 0.8 g/kg BW + Paracetamol toxic dose 0.5 g/kg BW). a,b,c Different superscripts in the same rows showed significant differences ($p<0.05$).

Histopathological examination of the rat liver revealed that administration of a toxic dose of paracetamol caused microscopic liver damage, characterized by the presence of inflammatory areas, altered hepatocyte structures, and a higher number of necrotic cells in the KN group ($p < 0.05$) (Table 1).

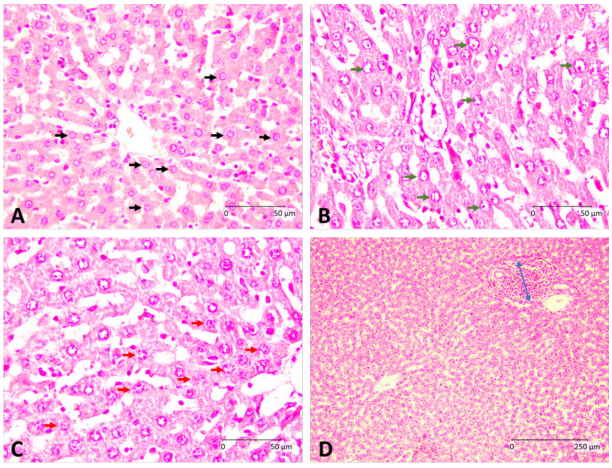

Figure 1. Histophatology liver after treatment. Description: A. Normal hepatocytes (black arrow); B. Cell swelling (green arrow); C. Cell necrosis (red arrow); D. Inflammatory area (blue arrow); Scale bar 50 μm and 250 μm; HE staining.

There is evidence of a preventive effect of ethanol extract of red spinach leaves in rats induced by toxic doses of paracetamol. Figure 1B shows swelling of hepatocyte cells, while Figure 1C indicates necrosis of hepatocytes caused by paracetamol toxicity. At a dose of 0.8 g/kg BW, a significant reduction in the number of necrotic cells was observed compared to the KN group ($p < 0.05$). This dose also resulted in a higher number of viable hepatocytes compared to KN ($p < 0.05$). The necrosis was attributed to accumulated NAPQI in the liver due to paracetamol overdose (Kwo *et al.,* 2017). The results further showed that both 0.4 and 0.8 g/kg BW doses led to a smaller hepatocyte cell area compared to KN ($p < 0.05$), indicating the absence of hepatocyte swelling.

NAPQI, a metabolite of paracetamol, generates reactive oxygen species (ROS) that stimulate the release of damage-associated molecular patterns (DAMPs), thereby triggering inflammatory responses in hepatocytes (Figure 1D). This mechanism recruits neutrophils, eosinophils, and macrophages, along with gamma-delta (γδ) T cells and dendritic cells, which secrete inflammatory cytokines and contribute to paracetamol-induced hepatic injury (Roh and Sohn, 2018; Xu and Wang, 2023). The results demonstrated that the lowest levels of inflammation were found in the K (Aquadest), P1, and P2 groups ($p < 0.05$), indicating that the ethanol extract of red spinach leaves at 0.4 g/kg BW can effectively reduce inflammation and necrotic cell formation caused by paracetamol toxicity.

The protective effect observed is attributed to the antioxidant activity of the ethanol extract of red spinach leaves administered over a 15-day period. Red spinach leaves are rich in four major flavonoids: quercetin, catechin, myricetin, and apigenin (Oba, 2019). Quercetin, in particular, can significantly reduce the levels of NAPQI produced from paracetamol metabolism (Pingli *et al.,* 2019). It also enhances antioxidant capacity, inhibits lipid peroxidation, and is effective in the treatment of liver injury (Kalantari *et al.,* 2018; Prasad *et al.,* 2013).

Moreover, quercetin functions as an anti-inflammatory agent, inhibiting TNF-α production induced by lipopolysaccharide (LPS) in macrophages. It also reduces other inflammatory cytokines including IL-1γ, IL-6, and TNF-γ (Li *et al.,* 2016). Based on these findings, the ethanol extract of red spinach leaves at 0.4 and 0.8 g/kg BW demonstrated significant improvements compared to the KN group, supporting its anti-inflammatory and hepatoprotective potential against paracetamol-induced toxicity.

Kidney Histopathology

The results demonstrated hemorrhage the preventive effects of the ethanol extract of red spinach leaves on several kidney histopathological parameters. The largest hemorrhagic area was observed in the KN group, whereas the K, P1, and P2 groups exhibited significantly smaller hemorrhagic areas (Table 2). The highest number of necrotic cells was also found in the negative control group, while the control group and the group treated with a 0.4 g/kg BW dose of the extract (P1) showed the lowest number of necrotic cells compared to other treatment groups (Table 2).

Observations of the glomerular area showed a significant difference ($p < 0.05$). The glomerular area in the KN group was the smallest compared to other groups (Figure 2B). In contrast, the control group and the groups treated with red spinach leaf extract at doses of 0.4 and 0.8 g/kg BW had larger glomerular areas than the KN group. Additionally, the KN group had the lowest number of glomeruli, whereas the 0.4 and 0.8 g/kg BW treatment groups exhibited the highest number of glomeruli among all groups ($p < 0.05$).

This study demonstrates that the induction of toxic doses of paracetamol can lead to the accumulation of NAPQI, a toxic metabolite that reduces the permeability of blood vessel cell membranes, resulting in vascular leakage

Table 2. Histopathologic structure of the kidney in the treatments.

Variable	K	KN	P1	P2
Hemorrhage area (mm^2)	1.75 ± 0.43^a	3.01 ± 0.88^b	1.74 ± 0.16^a	1.4 ± 0.63^a
Necrosis Cells number	199.00 ± 3.74^a	301.25 ± 20.69^c	201.50 ± 32.18^a	252.25 ± 22.81^b
Glomerulus area (mm^2)	5.12 ± 0.09^b	3.59 ± 0.83^a	6.10 ± 0.84^b	5.88 ± 0.57^b
Glomerulus number	199.00 ± 50.70^c	90.75 ± 56.86^a	153.75 ± 15.56^b	138.75 ± 19.09^b

Notes: K (Aquadest), KN (Paracetamol toxic dose 0.5 g/kg BW), P1 (extract 0.4 g/kg BW + Paracetamol toxic dose 0.5 g/kg BW), P2 (extract 0.8 g/kg BW + Paracetamol toxic dose 0.5 g/kg BW). a,b,c Different superscripts in the same rows showed significant differences ($p<0.05$)

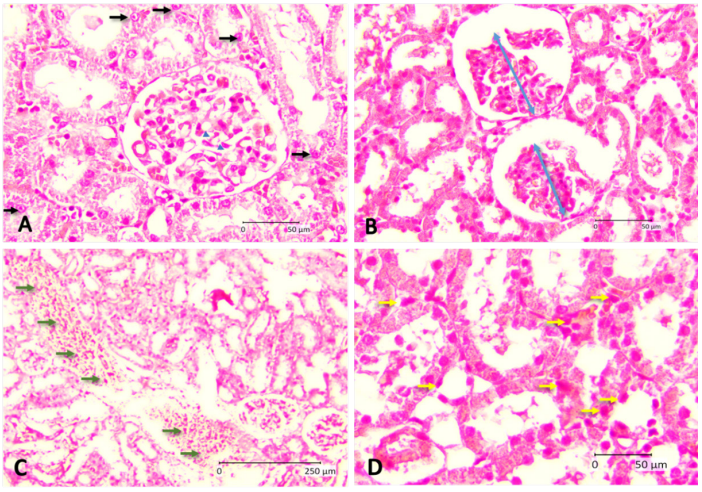

Figure 2. Histophathology of kidney after treatment. Description: A. Normal cell structure (black arrow), B. glomerular constriction (blue), C. hemorrhage (green arrow), D. cell necrosis (yellow arrow). Scale bar 50 µm and 250 µm. HE staining.

(hemorrhage) (Figure 2C). NAPQI can also cause vascular obstruction, increasing intravascular pressure beyond the pressure in surrounding tissues. This pressure imbalance may damage capillaries, leading to the escape of blood cells from the vessels (Merdana *et al.,* 2019; Rafe *et al.,* 2020). Furthermore, the accumulation of NAPQI in response to toxic doses of paracetamol can induce oxidative stress in tubular cells of the renal cortex, ultimately leading to cellular necrosis. The resulting damage, particularly necrosis in kidney tubules, is directly associated with the toxic effects of NAPQI and oxidative stress (Seok *et al.,* 2018).

Continuous elevation of toxic NAPQI levels can lead to the depletion of cellular glutathione and result in the covalent binding of NAPQI to cellular proteins, initiating the formation of Reactive Oxygen Species (ROS). This process triggers oxidative stress, which damages mesangial cells and glomerular endothelial cells, ultimately leading to a narrowing of the glomerular area. Moreover, persistent NAPQI accumulation and the presence of hemorrhage may cause partial destruction of the glomeruli, resulting in a reduced total number of glomeruli (El-Shafey *et al.,* 2015; Goncalves *et al.,* 2021; Kurniadi *et al.,* 2018; Stollings *et al.,* 2016; Xu *et al.,* 2020).

These results indicate that the antioxidant compounds in red spinach leaves provide protective effects against tissue damage induced by toxic doses of paracetamol, with the most optimal protection observed in the P1 group, which received the ethanol extract of red spinach leaves at a dose of 0.4 g/kg BW. The dose of 0.4 g/kg body weight shows the same result as the dose of 0.8 g/kg body weight, so it can be considered more optimal because it uses a lower extract. The P1 group was able to counteract free radicals that damage kidney cells, as evidenced by the number of necrotic tubular cells being lower and comparable to the K group. This is also consistent with the improved glomerular area and number observed in the P1 group, as well as the extent of hemorrhage, which was similar to that in the K group.

The administration of red spinach leaf extract plays a protective role in preventing kidney damage such as hemorrhage. Red spinach leaves contain quercetin, a flavonoid compound of the flavonol group. Quercetin acts as an antioxidant that can scavenge free radicals and enhance the body's antioxidant defense system. Therefore, quercetin can increase glutathione levels and facilitate the detoxification of NAPQI. Glutathione converts NAPQI into the non-toxic compound mercapturic acid, thereby preventing the accumulation of toxic substances in blood vessels. Additionally, quercetin improves the integrity of blood vessel membranes, reducing the likelihood of vascular leakage (Stollings *et al.,* 2016; Sarker *et al.,* 2019; Xu *et al.,* 2020; Chen *et al.,* 2022).

Conclusion

The results of this study conclude that the ethanol extract of red spinach leaves at a dose of 0.4 g/kg BW provides protective effects on the liver and kidneys of Wistar rats induced by toxic doses of paracetamol.

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