Shallot (Allium cepa L.) Peel Infusion Ameliorates Kidney Histopathological Damages in Diazinon-Induced Wistar Rats

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Abstract
Diazinon, an organophosphate pesticide, is used extensively in agricultural sector. Consumption of agricultural products containing diazinon residue may lead to harmful health consequences. Among these is nephrotoxicity, which includes lipid peroxidation, that can damage the kidney. Flavonoids in shallot peel can scavenge free radicals, inhibit necrosis, and activate bone marrow-derived cells for cell regeneration. This study aimed to determine the correlation between shallot peel infusion (SPI) dose and kidney damage amelioration to establish the maximum effective dose of SPI to ameliorate kidney histopathological damage in diazinon-induced rats. This study was performed at the Pharmacology Laboratory, Faculty of Medicine, University of Jember, Indonesia, in April 2021, on 35 rats that were divided into 7 groups—normal, diazinon, and five treatment groups. Diazinon 40 mg/kgBW was administered on day 1-7, while SPI 125, 250, 500, 1,000, 2,000 mg/kgBW were administered on day 8–14 according to the treatment group. Kidney histopathological slides with hematoxylin-eosin (H.E.) staining were assessed using Kocoglu scoring and Kidney damage scores of the treatment groups were analyzed using Pearson test. The maximum effective dose was determined using regression test. The damages found in diazinon-induced rats were tubular degeneration, necrosis, and inflammation with a higher damage score than normal rats (p<0.05). Pearson test showed moderate correlation (coefficient -0.594). Higher SPI doses presented lower kidney damage scores, with 1,359 mg/kgBW being the maximum effective dose. SPI dose and the kidney damage amelioration are moderately correlated with a SPI maximum effective dose to ameliorate kidney damage in diazinon-induced rats of 1,359 mg/kgBW.

Keywords: Flavonoid, kidney, oxidative stress, pesticide, red onion

Introduction
In agriculture sector, optimizing the usage of pesticide is an imperative aspect to do to increase the quality and quantity of agricultural products. Diazinon, an organophosphate pesticide, is extensively used by farmers.1 WHO estimates that 1–5 million cases of organophosphate poisoning occur each year, 80% coming from developing countries. The consumption of agricultural products containing diazinon residues due to its excessive use causes hazardous effects on humans.2 Diazinon has nephrotoxic effect related to renal function as a vital organ involved in the excretion of chemical substances.3,4 The previous study revealed that diazinon at dose of 40 mg/kgBW caused the increase of blood urea nitrogen (BUN) serum level and kidney histopathological damage in Wistar rats.5 Diazoxon, the active metabolite of diazinon, increases the level of free radicals which will bind to polyunsaturated fatty acids (PUFA) in cell membranes, including kidney nephrons, resulting lipid peroxidation.6 Natural ingredient is needed as an agent to repair tissue damage and prevent further organ damage. Shallot (Allium cepa L.), a horticultural commodity with almost evenly spread throughout Indonesia, contains antioxidant compounds i.e. flavonoids.7–9 Shallot peel generally considered as waste contains 3–5 times higher flavonoid compounds compared to the tuber which is commonly consumed.10 A phytochemical screening of shallot skin ethanol extract (SSEE) using aluminium chloride colorimetric method revealed that 1 g SSEE contained 228.1 mg QE total flavonoids.11 Flavonoids neutralize free radicals, increase prostaglandin production,
inhibit renal cell necrosis, and stimulate renal cell proliferation through activation of bone marrow-derived cells (BMDCs) leading to cell regeneration. It was discovered that engrafted BMDCs into the damaged cells plays an essential role in normal cell turnover to improve membrane integrity loss.12

Flavonoids are classified into the polar compounds dissolved in polar solvents such as water associated with their hydroxyl groups content.13 Shallot peel can be formulated using water as a solvent called infusion which can be provided using common tools and materials as well as simple procedure for people to apply. The antioxidant effect of shallot peel infusion (SPI) has not been widely studied. This study needs to be carried out to determine correlation between the SPI dose and the amelioration of kidney histopathological damage and establish the maximum effective dose of SPI to ameliorate kidney histopathological damage in diazinon-induced Wistar rats.

Methods

This study received ethical approval from The Ethical Committee for Research, Faculty of Medicine, University of Jember with reference number 1470/H25.1.11/KE/2021. This study was carried out at Pharmacology Laboratory Faculty of Medicine University of Jember, April 2021. The main material used was shallot peel Blue Lancor variety from Probolinggo, Allium cepa species L. var. ascalonicum Back. The other materials were rat feed pellets, liquid diazinon 600 g/L from farm shop Pasar Tanjung Jember, corn oil, aquadest, 10% neutral formalin buffer, xylol, paraffin, and hematoxylin-eosin (H.E.) dye. The shallot peels were cleaned and dried under the sunlight, subsequently crushed using a blender to produce a simplicial form. Simplicia were mixed with aquadest and heated in an infusion pan. The first pan was filled with water and heated and the second pan was placed on top of the first pan. To provide 20% SPI, as much as 10 g of shallot peel simplicia and 50 mL of distilled water were stirred and heated at 90°C for 15 minutes. The infusion is filtered using flannel and hot water is added to reach a volume of 50 mL.14 The diluted infusions were prepared using the serial dilution method to obtain 10% (5 g/50 mL), 5% (2.5 g/50 mL), 2.5% (1.25 g/50 mL), and 1.25% (0.625 g/50 mL) SPI concentrations.

The experimental animals used in this study were male white rats (Rattus norvegicus) Wistar strain, aged 2–3 months, and body weight of 150-250 g. As many as 35 male Wistar rats were randomly divided into normal group, diazinon group, and five treatment groups. On day 1-7, the normal group received 5 mL/kgBW corn oil, while the other groups received 40 mg/kgBW diazinon. On day 8-14, the normal and diazinon group received 10 mL/kgBW aquadest and the other group received SPI at doses of 125 mg/kgBW, 250 mg/kgBW, 500 mg/kgBW, 1,000 mg/kgBW, and 2,000 mg/kgBW. All treatments were administered orally.

Kidney histopathologic slides were made using paraffin method with H.E. staining and subsequently observed by anatomical pathologist using binocular microscope (Leica DMS500) in five fields with 400X magnification. Kidney histopathological damage score of each rat was determined based on Kocoglu scoring (0: no kidney damage such as tubular degeneration, tubular cell necrosis, accumulation of cell debris in the lumen, tubular cast formation, tubular dilatation, and neutrophil infiltration), 1: ≤10% kidney damage, 2: 11-25% kidney damage, 3: 26–45% kidney damage, 4: 46–75% kidney damage; 5: 76–100% kidney damage).15 Data were analyzed statistically using IBM SPSS Statistics 26. The average of kidney histopathological damage score of the normal and diazinon group were analyzed using independent T-test, while the treatment groups were analyzed using Pearson correlation test. The maximum effective dose was determined using regression test with quadratic curve.

Results

Based on the result, the highest kidney histopathological damage score was found in diazinon group (Table 1). Independent T-Test

Table 1 The Average of Kidney Histopathological Damage Score

<table>
<thead>
<tr>
<th>Group</th>
<th>The Average of Kidney Histopathological Damage Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>2.594 ± 0.556</td>
</tr>
<tr>
<td>Diazinon</td>
<td>3.413 ± 0.169</td>
</tr>
<tr>
<td>SPI 125 mg/kgBW</td>
<td>2.776 ± 0.233</td>
</tr>
<tr>
<td>SPI 250 mg/kgBW</td>
<td>2.658 ± 0.965</td>
</tr>
<tr>
<td>SPI 500 mg/kgBW</td>
<td>2.876 ± 0.309</td>
</tr>
<tr>
<td>SPI 1,000 mg/kgBW</td>
<td>2.079 ± 0.333</td>
</tr>
<tr>
<td>SPI 2,000 mg/kgBW</td>
<td>2.359 ± 0.373</td>
</tr>
</tbody>
</table>
Figure 1 Kidney Histopathological Damage with H.E. Staining (400X)

A: normal group, B: diazinon group, C: SPI 125 mg/kgBW, D: SPI 250 mg/kgBW, E: SPI 500 mg/kgBW, F: SPI 1,000 mg/kgBW, G: SPI 2,000 mg/kgBW. Black arrow: tubular degeneration, pink arrow: tubular cell necrosis, green arrow: accumulation of cell debris in the lumen, red arrow: tubular cast, yellow arrow: tubular dilatation, and blue arrow: neutrophil infiltration.
Table 2 Pearson Correlation Analysis

<table>
<thead>
<tr>
<th>Correlations</th>
<th>Kidney damage score</th>
<th>Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kidney damage score Pearson Correlation</td>
<td>1</td>
<td>-0.594</td>
</tr>
<tr>
<td>Sig. (2-tailed)</td>
<td></td>
<td>0.002</td>
</tr>
<tr>
<td>N</td>
<td>24</td>
<td>24</td>
</tr>
<tr>
<td>Group Pearson Correlation</td>
<td>-0.594**</td>
<td>1</td>
</tr>
<tr>
<td>Sig. (2-tailed)</td>
<td></td>
<td>0.002</td>
</tr>
<tr>
<td>N</td>
<td>24</td>
<td>24</td>
</tr>
</tbody>
</table>

The result between the normal and diazinon group showed a difference with significance value of 0.030 (p<0.05) indicating that diazinon is nephrotoxic.

The kidney histopathological damages found in microscopic observation were tubular degeneration, tubular cell necrosis, accumulation of cell debris in the lumen, tubular cast formation, tubular dilatation, and neutrophil infiltration (Figure 1). The result of Pearson correlation test showed moderate correlation with coefficient -0.594 (Table 2); the higher the SPI dose, the lower kidney histopathological damage score.

Based on the highest R² in regression test result, we used a quadratic curve with equation y = 3.21 - 1.62 \times 10^{-3}x + 5.96 \times 10^{-7}x^2 (Figure 2). The maximum effective dose of SPI is 1,359 mg/kgBW determined by calculating the x value from the derivative equation with y’=0.

Discussion

Diazinon causes damage to the histological structure of kidney through various pathophysiological pathways. Diazoxon, the active metabolite of diazinon, inhibits acetylcholine esterase (AchE) enzyme activity, thereby increasing acetylcholine (Ach) levels in the body. Ach accumulation will increase Ca²⁺ influx and nitric oxide (NO) production in endothelial cells. ⁶,¹⁶,¹⁷ These free radicals will bind to PUFA which are abundantly found in cell membranes, including kidney nephrons, resulting in lipid peroxidation. ⁶ The increase of Ca²⁺ influx and NO production disrupts mitochondrial respiration and cause adenosine triphosphate (ATP) depletion. It will interfere ATP dependent ion transport which subsequently increase Ca²⁺ influx and NO production. The excess of Ca²⁺ influx will also increase the levels of calcium-dependent enzymes such as calpain, endonuclease, and ATP-ase which will trigger cell damage through necrosis. ¹⁶,¹⁷

Tubular degeneration occurs due to the failure of the ATP-dependent ion pump on the cell membrane leading to disruption of fluid and ion...
homeostasis. Renal tubular cells are unable to pump sodium ions out which causes the increase of sodium ions and intracellular osmotic pressure. This condition triggers the entry of water to the tubular lumen, so that the tubules get swelling and lose their brush-border.\textsuperscript{18-20}

Necrosis is caused by loss of membrane integrity and leakage of cell contents related to diazinon toxic effect. Lipid peroxidation that occurs due to the increase of reactive oxygen species (ROS) induced by diazinon disrupts membrane permeability generating the impairment of kidney cell function which potentially triggers necrosis. The tubular cells undergoing necrosis will be shed and the cell components will be scattered in the lumen, hence there will be accumulation of cell debris in the lumen. The histopathological changes of nucleus during necrosis are shrinkage, irregular border, and color darkening.\textsuperscript{18}

Tubular casts are formed when there is a large leakage of protein in the glomerular filtrate, so that the tubules are no longer able to reabsorb proteins entering the lumen. It causes excessive accumulation and deposition of protein in vesicles related to tubular dilatation.\textsuperscript{18} In addition, oxidative stress in the kidney causes infiltration of immune cells through activation of proinflammatory cytokines and chemokines by ROS, demonstrated by the increase of neutrophil infiltration in kidney tissue.\textsuperscript{19}

Kidney histopathological appearance in the treatment groups showed amelioration along with the increase of SPI dose. The result of Pearson correlation test showed moderate correlation with coefficient correlation -0.594. It indicates that amelioration of kidney histopathological damage is affected 59.4% by SPI, while 40.6% is affected by other factors including self-repair mechanism of the rat body in 7 days which may also be influenced by foods and environment. The result of correlation did not get a strong correlation. This is due to the flavonoid level in infusion is not as high as the flavonoid level in extract. The impact of SPI administration is accelerating the repair of kidney histopathological damage; the higher the SPI dose, the lower kidney histopathological damage score. It indicates that kidney histopathological improvement depends on the dose of SPI. Flavonoids neutralize the toxic effects of free radicals by transferring hydrogen ions and the molecules become more stable. This condition will reduce oxidative stress in the tissues and cellular damage.\textsuperscript{21-23} Flavonoids also increase prostaglandin production resulting vasodilation and the increase of kidney perfusion. Furthermore, flavonoids promote BMDCs to be released from bone marrow into the peripheral blood and move into the damaged area after attracted by growth factors and inflammatory cytokines released from the injured area. It was discovered that engrafted BMDCs into the damaged cells plays an essential role in normal cell turnover to improve membrane integrity loss. In a previous study, quercetin induced bone marrow mesenchymal stem cells proliferation and osteogenic differentiation by increasing bone morphogenetic protein (BMP) signaling pathway activation and upregulates downstream genes expression such as \textit{OPN}, \textit{RUNX2}, and \textit{OSX}.\textsuperscript{12}

The administration of SPI exceeds the maximum effective dose is no longer effective to ameliorate diazinon-induced kidney damage. The kidney histopathological damage score at a dose of 2,000 mg/kgBW exceeds the damage score at a dose of 1,359 mg/kgBW. This is presumably because flavonoid contained in SPI above the maximum effective dose changes its properties from antioxidant to pro-oxidant so that it is unable to neutralize oxidative stress and the cell damage process continues.\textsuperscript{24} The previous study stated that the ethyl acetate extract of shallot peel doses of 1,600 mg/kgBW. and 2,900 mg/kgBW for 14 days caused liver damage in mice including sinusoidal dilatation, tissue bleeding, and lymphocyte aggregation.\textsuperscript{25}

It can be concluded that the SPI dose and the amelioration of kidney histopathological damage are moderately correlated; the higher the SPI dose, the lower the kidney histopathological damage. The maximum effective dose of SPI to ameliorate kidney histopathological damage in diazinon-induced Wistar rats is 1,359 mg/kgBW. Related to the limitations of this study, it is needed to measure the levels of flavonoids in SPI, analyze the structure of the kidney using quantitative stereology methods, and carry out kidney function test. In addition, it is also necessary to measure kidney malondialdehyde (MDA) as an indicator of oxidative stress.

**References**

2. Ardiwinata AN, Ginoga LN, Sulaeman E,


