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The effects of IR-Bagendit rice leaf infusions from Blora on renal tubular degeneration and necrosis: (Study on Wistar albino rats covered with plumbum acetate)

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Abstract: Plumbum exposure still becomes a health problem in Indonesia. Plumbum (Pb) is widely used in various industries; thus, it can cause health problems, including impaired kidney functions. The accumulation of Pb rise to degeneration and necrosis of renal tubule cells. Therefore, preventive efforts are necessary to inhibit renal function deterioration. Infused extract of IR Bagendit rice leaf from Blora contains protein metallothionein which could bind PB covalently. This study aims to determine the supplementation of IR Bagendit rice leaf infusion to inhibit degeneration and necrosis of renal tubular cells exposed to Pb. The experimental research method was conducted using Wistar albino rats divided into five groups: negative control group, positive group, and treatment groups 1, 2, and 3. Each group consisted of six rats. The negative control group only received a placebo, and the positive group only received Pb acetate 60 mg/kg BW/day. Meanwhile, treatment groups 1, 2, and 3 received 0.2 ml, 0.4 ml, and 0.8 ml IR Bagendit rice leaf infusions successively and Pb acetate 60 mg/kg BW/day. The experiment was conducted for 60 days. Histological preparations of tubular cells were colored using hematoxylin-eosin. Normal cell differences, degeneration, and necrosis of each group were tested using Kruskal Wallis and Mann Whitney tests.This study has revealed that the average normal cells in the positive control group are lower than those in the negative control group and treatment groups 1, 2, and 3. This difference is insignificant with the p = 0.477. Moreover, treatment groups 1, 2, and 3 show decreased cell degeneration and necrosis with significant differences of p = 0.000 and 0.001. IR Bagendit rice leaf infusions could inhibit degeneration and necrosis of renal tubular cells exposed to Pb.

Keywords: IR Bagendit; Degeneration; Necrosis; Pb; Metallothionein

INTRODUCTION

Plumbum (Pb) exposure still becomes a health problem in Indonesia and is widely used in various industries, such as battery, accumulator, paint, and coloring material industries. Thus, Pb could be toxic to humans. Pb enters the human body through three pathways: the gastrointestinal tract (gastrointestinal), respiratory tract (inhalation), and penetration through skin (topical). PB toxicity of 5 ug/dL could poison adults.[1] The accumulation of Pb in the body could cause loss of appetite, weak muscles, anemia, intellectual disability, behavioral disorders, renal physiology disorders, liver disease, and death. [2,3]

Pb toxicity can form free radicals and degrade endogenous antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT), and glutathione transferase (GSH), that cause organ dysfunctions. An imbalance number

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between free radicals and antioxidants will lead to oxidative stress.[4,5] Oxidative stress is the excessive production of reactive oxygen species (ROS), such as hydroxyl, superoxide, and hydroperoxyl, which react with fats, proteins, and cellular nucleic acids; this condition results in local damage (glomerulus dysfunction) and renal tubular cells.[6,7,8] Acute PB poisoning (Pb levels in the blood >80-100 µg/dL) disrupts the structure and functions of the renal tubules.[9] The kidney damage due to Pb toxicity is in the form of degeneration and necrosis.[10]

Cell degeneration refers to damage that occurs in cytoplasm, but not in nucleus cells, and could repair damage to be normal and reversible. Cell degeneration that lasts a long time and continuously will disable cells to conduct metabolism properly; consequently, the cells death or cell necrosis occurs. Cell necrosis can be characterized by damage in the form of irreversible karyolysis, pycnosis, or cariorexis.[11,10]

Various efforts through curative and preventive measures have been made to prevent Pb toxicity. Preventive measures are done with the chelating agent principles and are still considered the best choice. One of the chelating ingredients is IR Bagendit rice leaves that contain a lot of protein metallothionein. This protein is rich in sulfhydryl groups that could covalently bind heavy metals (Pb).[12,13] The content of metallothionein proteins in IR Bagendit rice leaves from various locations in Northern Java has been investigated, and the investigation has revealed that R Bagendit rice from Blora has the highest content.[14] The metallothionein gene is located on chromosome 3 of IR Bagendit rice leaves (*Oryza Sativa*) and functions as stress-inducible proteins in drought conditions as well as soil and water-containing metals. Therefore, metallothionein can be used as a biomarker of heavy metal exposure.[15] This study aims to determine the functions of IR-Bagendit rice leaf infusion from Blora to prevent degeneration and necrosis of renal tubular cells.

MATERIAL AND METHOD

1. Infusion Manufacturing Process

Rice leaves of various varieties had been cleaned and washed with running water before they were chopped into smaller pieces. Afterward, they were weighed as much as 100 g and put into pot A. Then, a liter of aquades was added. Finally, the pot was closed. Meanwhile, pot B (as a water bath) was added with sufficient water until the upper pot (A) was submerged partially. The pots were heated for 15 minutes until the temperature inside pot A reached 90°C while being stirred occasionally. The infusion was sprayed while it was hot using a fabric flannel, supernatants are infusions. This process is summarized Figure 1, the produced infusions were labeled according to their regions. Meanwhile, the infusions' protein metallothionein was examined using the Elisa method.

2. Metallothionein Examination:

Microplate coated with specific antibodies MT. Standards and samples were added to microwells and were corresponding to specific polyclonal antibodies-conjugated biotin for MT. Furthermore, peroxidase conjugated-avidin was added to each well and incubated. Then, substrate solution was added to each well. Discoloration occurs in wells containing MT, antibodies-conjugated biotin, and enzymes-conjugated avidin. Enzyme-substrate reactions end with adding a sulfuric acid solution and color changes that were measured spectrophotometrically with a wavelength of 450 2 nm. The concentration of MT in the samples was determined by comparing their OD to the standard curve.

3. Intervention on Research Animals

This study examined 30 male rats aged 2-3 months and weighed 170-200 grams. These rats were divided into five groups: the negative control group, the positive control group, and treatment groups 1, 2, and 3. Each group consisted of six rats. The negative control group only received a placebo while the positive control only received PB acetate 60 mg/kg BW/day for 60 days, that given in the form of a solution through a sonde. Treatment group 1 (P1) received 0.2 ml of IR-Bagendit rice leaf (*Oryza sativa L*) infusion, treatment group 2 (P2) received 0.4 ml of infusion, and treatment group 3 (P3) received 0.8 ml of infusion for 10 days. Afterward, the infusions were subsequently administered while exposing PB acetate of 60 mg/kg BW/day until the 60th day. Meanwhile, 100 g/L = 100,000 mg/1000 mL IR of Bagendit rice leaf infusion was produced from 100 mg/mL for various concentrations of 0.2 ml (P1), 0.4 ml (P2), and 0.8 ml (P3).

4. Veterinary Surgery

The surgical stage was performed on the 61st day. Moreover, at this stage, anesthesia was performed. The anesthetic induction used in this research was a combination of ketamine and xylazine with a dose of 40 mg/kg and 10 mg/kg; this induction was administered intraperitoneally (IP). Surgery was performed in the abdomen horizontally and vertically. Afterward, the kidney organ was taken to make histopathological preparations. The preparations were made using the paraffin method with the hematoxylin-eosin (HE).

5. Analysis of Normal Cells and Renal Tubular Degeneration and Necrosis

The quality of kidney tissue preparations resulting from staining hematoxylin-eosin (HE) was assessed macroscopically and microscopically. Then, the number of normal cells, degenerative cells, and renal tubular necrosis cells in the control and treatment groups was calculated. the number of normal, degenerative, and necrotic cells was calculated in percent units which were read using a microscope with a magnification of 400x. The data were analyzed by comparing the results of each group using the Kruskal-Wallis test.

6. Ethical Clearance

This research received the ethical clearance No: 503 503/KEPK-FKM/UNIMUS / 2021 from the Health Committee of the Faculty of Health, Universitas Muhammadiyah Semarang. Moreover, the head of the Integrated Research and Testing Laboratory (LPPT) of Universitas Muhammadiyah Semarang approved this research by considering research ethics.

RESULTS AND DISCUSSION

Metallothionein Content

The metallothionein protein levels of the obtained infusions were checked using the ELISA. The 0.321 absorbances with the obtained concentration have resulted in 380.636 ng/L. Based on this result, the treatment groups receiving rice leaf infusion of 100 g/L = 100,000 mg/1000 mL have produced 100 mg/mL with concentration variations of 0.2 ml, 0.4 ml, and 0.8 ml.

Microscopic Pictures of Renal Tubular Cells of Wistar Rats

The results of laboratory tests provide pictures of normal, degenerative, and necrosis renal tubular cells of all treatment groups can see at figure 2.

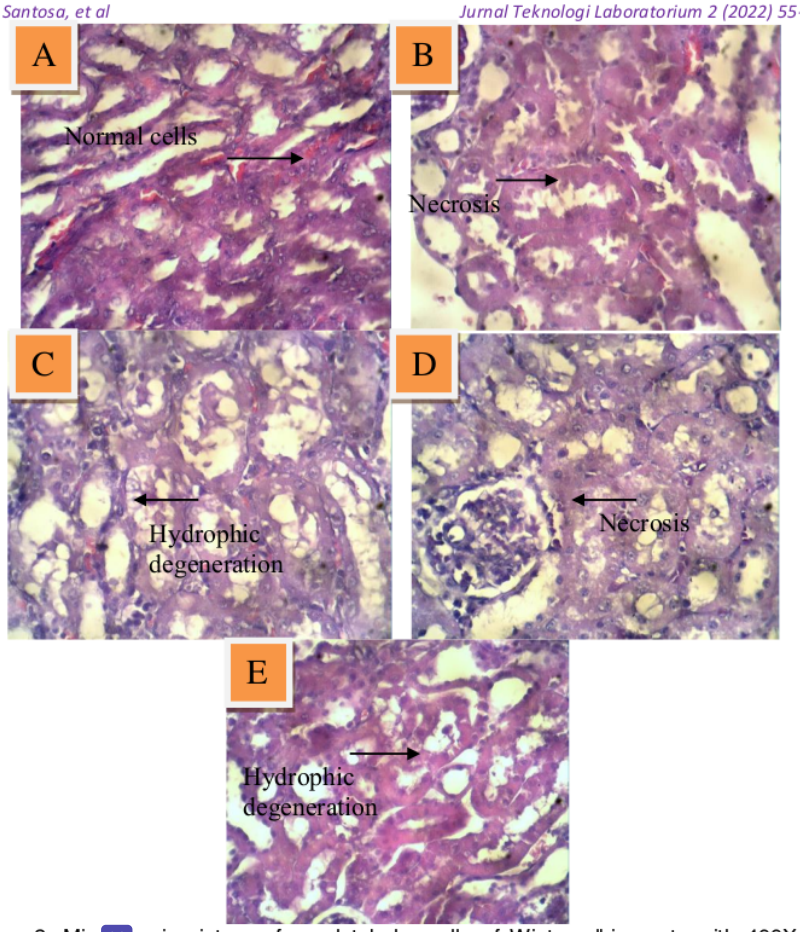

Figure 2. Microscopic picture of renal tubular cells of Wistar albino rats with 400X of magnification; A = Negative control; B = Positive control; C = Treatment 1; D = Treatment 2; E = Treatment 3

Meanwhile, the mean scores of normal, degenerative, and necrosis cells in the negative control group, positive control group, and treatment groups 1, 2, and 3 are summarized in Table 1. Table 1 shows that the body weights of rats in all treatment groups are not significantly different. All groups have gained weight from the beginning to the end of the treatment. The mean of normal cells of the positive control group has decreased, but that of the treatment groups 1, 2, and 3 has increased and is nearly equal to that of the negative control group. The highest mean of degenerative and necrosis cells is found in the positive control group. Meanwhile, P1 and P2 treatment groups have experienced a decrease in both cell groups, but the P3 treatment group has not. The data show significant differences between the positive group and the treatment control groups (Table 2). The calculation results in Table 2 are related to Figure 2 presenting degenerative and necrosis cells of renal tubules in Figures C, D, and E. The degenerative and necrosis cells are significantly different; to determine the differences between groups, the Mann-Whitney test was conducted. The results of this statistical test are presented in Table 2.

The results of the statistical test denote that the negative and positive control groups have significantly different degenerative cells from the treatment groups. The negative control group has significantly different necrosis from the

positive control group and treatment group 1. Meanwhile, the positive control group has significantly different necrosis from all treatment groups (P1, P2, and P3).

Moreover, the weight of Wistar albino rats has met the inclusion criteria and group average in each homogeneous group. The average number of normal cells in each group is not significantly different. However, the average number of normal cells in the positive control group is descriptively lower than that in the negative control group and the treatment groups 1, 2, and 3. In contrast, all groups show a significantly different average number of degenerative cells. This study has discovered that necrosis cells in the control groups 1, 2, and 3 decrease, but not in the positive control group. Moreover, their differences are significant.

This study has revealed that IR Bagendit rice leaves from Blora contain high metallothionein protein that meets the requirement for this research.[14] This study has deployed that Plumbum acetate is a toxic heavy metal that causes impaired functions characterized by the increasing amount of damage in renal tubule cells. Plumbum acetate can cause oxidative stress that can increase the formation of free radicals, such as superoxide, hydrogen peroxide, hydroxyl, and lipid peroxide, as well as decrease endogenous antioxidant systems, such as superoxide dismutase, catalase, glutathione peroxidase, and glutathione reductase. Such conditions could damage kidney cells.[15,5] The decrease in the body's antioxidants and the increase in free radicals will cause oxidation and escalate lipid peroxidation. Unstable lipid peroxidation can damage three types of compounds that significantly maintain cell integrity; they are cell membranes, proteins, and DNA.[7,8] Cell degeneration constitutes damage that occurs in cell membranes. This damage will trigger the oxidation of amino acids and increase the permeability of cell membranes. As a result, cellular reactions do not work perfectly and could become reversible and irreversible (cell death).[5,16] Degenerative cells could have the adaptability to reach new conditions and maintain their viability. To a certain extent, the cells return to a stable state that enables them to divide by responding to injury through hypertrophy. Afterward, adaptation and proliferation of the cells are performed with re-eptelization.[10] If the stress is severe or persistent, irreversible injury occurs, and the cells will die (necrosis).[5] The provision of IR Bagendit rice leaf infusion could reduce the degeneration of renal tubule cells.

Necrosis of renal tubule cells occurs due to a higher level of cell damage after the membrane permeability is disrupted by the emergence of turbid swelling followed by lysis.[17] Irreversible damage (necrosis) is characterized by the loss of nucleus and cell fragmentation as well as the release of cytoplasmic contents in the renal tubules.[18] Such conditions occur because tubule cells have a weak epithelium and leak easily.[19] This study has proven that necrosis is characterized by more basophilic and disappearing nucleus cells (karyolysis).

Protein metallothionein is mostly found in various plants, such as IR-Bagendit rice leaves (*Oryza sativa L*). The leaves of IR-Bagendit rice (*Oryza sativa L*) contain the highest protein metallothionein among other leaves of other types of rice.[20] Protein metallothionein in (*Oryza sativa L*) IR-Bagendit rice leaves has a sulfhydryl group and can bind PB acetate; thus, this protein metallothionein is stable for the detoxification process.[21,22] This study has found that the provision of IR-Bagendit rice (*Oryza sativa L*) leaf infusion of 0.2 ml/day, 0.4 ml/day, and 0.8 ml/day can successively decrease necrosis cell damage up to 75% in P1, 90% in P2, and 100% in P3. These numbers show that the intervention groups have significantly different improvements from the positive control group. The greater the dose of R-Bagendit rice (*Oryza sativa L*), the greater the decrease in degenerative and necrosis cell damage. It shows that protein metallothionein has a nephroprotective activity against nephrotoxicity induced by Plumbum acetate.

CONCLUSION

The provision of IR Bagendit rice leaf infusion from Blora can inhibit renal tubular cell damage (degeneration and necrosis) and has been proven effective for the treatment groups 2 (0.4 ml = 40 mg) and 3 (0.8 ml = 80 mg). The metallothionein protein could bind plumbum so that renal tubular degeneration and necrosis can be prevented.

AUTHORS' CONTRIBUTIONS

BS formulated the research design, conducted experiments, analyzed data, and compiled, revised, and reviewed the manuscripts critically. The EEA collected and analyzed the data. AHM prepared the reagents and materials and analyzed the data. All of the researchers read and approved the final manuscript.

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DATA AVAILABILITY STATEMENT

The utilized data to contribute to this investigation are available from the corresponding author on reasonable request.

DISCLOSURE STATEMENT

The data is the result of the author's research and has never been published in other journals and not any conflict of interest between authors and financial funding.

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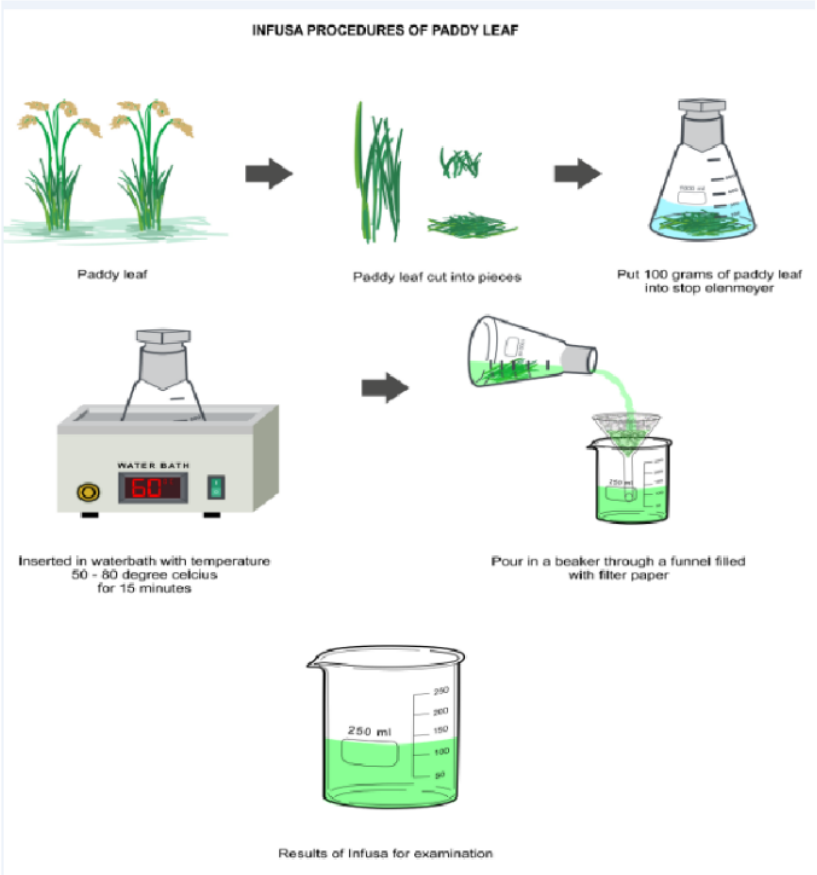

Figure 1. Stages of making IR Bagendit rice leaf infusion: 1) cleaning and washing rice leaves from several varieties with running water; 2) chopping the leaves into small parts; 3) weighing the small parts as much as 100 g and putting them into pot A; 4) adding a liter of aquades; 5) closing the pot; 6) adding sufficient water on pot B (as a *water bath)* until the upper pot (A) partially submerged; 7) heating the pots while stirring the water inside occasionally for 15 minutes until the temperature inside pot A reaching 90^{o}C; 8) spraying the infusion while hot using a fabric flannel, supernatants are infusions.

Table 1. Average numbers of normal, degenerative, and necrosis cells and differences between initial weight and final weight after the 60-day intervention in the control and treatment groups

Variables	Treatment					p-value
	Control -	Control +	P1	P2	P3	
Initial BW (g)	178 ± 7.07	177 ± 9.20	180 ± 10.71	176.3 ± 4.96	184 ± 10.91	0.575
Final BW (g)	212 ± 20.01	215.8 ± 25.38	209.6 ± 10.89	200 ± 9.18	202.5 ± 32.18	0.310
Normal cells	604 ± 9.49	483 ± 9.49	608 ±9.9	608 ± 6.04	609 ± 6.33	0.477
Degenerative cells	0	2 ± 1.43	1.71 ± 0.9	1.55 ± 0.73	1.05 ± 0.35	0.000
Necrosis cells	0	2 ± 1.41	0.5 ± 1	0.2 ± 0.4	0	0.001

Table 2. Results of difference test on degenerative cells and necrosis cells of two treatment groups

Groups	P-value				
	Control-	Control+	P1	P2	P3
Degenerative cells					
K-	-	0.004*	0.005*	0.004*	0.004*
K+	0.004*	-	0.007*	0.005*	0.015*
Necrosis cells					
K-	-	0.005*	0.018*	0.134	1.000
K+	0.005*	-	0.008*	0.007*	0.005*

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