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by Budi Santosa

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THE COMBINATION OF IR BAGENDIT RICE LEAF WATER EXTRACT AND COCONUT WATER REDUCES OXIDANT ACTIVITY AND IMPROVES THE MORPHOLOGY AND FUNCTION OF KIDNEY CELLS AFTER MERCURY EXPOSURE

Budi Santosa[1*], Tulus Ariyadi[2], Sufiati Bintanah[3], Arista Kurniasari Budi Fristiani[4], Fitriani Nur Damayanti[5]

Master Program of Medical Laboratory Science, Universitas Muhammadiyah Semarang[1]
Medical Laboratory Technology, Nursing and Health Faculty, Universitas Muhammadiyah Semarang[2,4]
Department of Nutrition, Faculty of Nursing and Health Sciences, Universitas Muhammadiyah Semarang[3]
Departement Midwifery, Faculty of Nursing and Health Sciences, Universitas Muhammadiyah Semarang[5]

Corresponding Author: 1*

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IR Bagendit rice leaves, coconut water, oxidants, degeneration and necrosis of kidney tubular cells.

ABSTRACT

Mercury chloride can cause nephrotoxicity, increase ROS, MDA, and decrease TGF-β resulting in tubular cell membrane damage. The metallothionein protein found in IR Bagendit rice leaves can bind mercury and coconut water contains lots of antioxidants. The aim of this study was to find out how the infusion of IR Bagendit rice leaves and coconut water improves kidney damage. The experimental study on Rattus nurvegicus rats used in the experiment was divided into five groups: negative, positive control, and treatment 1, 2, and 3. Each group contained six rats. The negative control group was given only a placebo. The positive control group received a daily dose of HgCl2 20 mg/day/kg BW. Treatments 1, 2, and 3 each received 0.2 ml, 0.4 ml, 0.8 ml of water extract of rice leaves of IR Bagendit and coconut water up to 4 ml/100 g BW, all treatment groups were exposed to mercury chloride 20 mg/kg body weight per day. All groups had their blood and kidney organs taken on the 15th day. ROS, MDA, TGF-β levels using Elisa, urea, and creatunu using enzymatic. Renal tubular necrosis and degeneration using HE staining. The results showed that when compared with the positive control group, the average ROS, MDA decreased, the average TGF- increased, and the average cell degeneration and cell necrosis decreased. The average levels of urea and creatinine are still within normal limits. In conclusion, rice leaf water extract can improve the morphology and function of kidney cells in Rattus nurvegicus exposed to HgCl2.

1. Introduction

In industry, agriculture, dentistry, hospitals, and research labs, mercury (Hg) is frequently used [1]. There are three different types of mercury: organic mercury (methylmercury and dimethylmercury), salts of mercury (Hg+ and Hg2+), and metallic mercury (element Hg) [2]. In its free state, elemental mercury is a liquid that can be used for thermometers, for instance. Such mercury vapor can harm the brain and the lungs [3]. Because it is not water soluble, mercury oxide is utilized as a topical antiseptic. Inorganic mercury salts include mercury chloride as an example, but methylmercury, an organic form of mercury, is frequently employed in fungicides, disinfectants, paint preservatives, and other products [4].

Due to the fact that they bind to sulfides and cell membranes, heavy metals' high affinity for sulfur (sulphur) might interfere with the transport of sulfur through cell membranes, preventing enzymes from functioning as intended [5]. [6]. The majority of the biological consequences are caused by covalent connections between mercury and sulfur, and when sulfur is present in the form of sulfhydryl, divalent mercury substitutes hydrogen atoms to create mercaptides. Because sulfhydryl enzymes are inhibited by organic Hg, metabolism and cell function can be affected [7]. [8]. Inorganic and ionic mercury (mercury chloride) can have severe acute toxicity [9- 11]. Nephrotoxicity, an adverse impact of inorganic mercury that affects proximal tubular cells, is one of these effects [12], [13]. When mercury attaches to membrane proteins in the sulfhydryl (SH) group, the integrity of the membrane is disrupted, which can lead to renal tubule necrosis [8]. Oliguria, anuria, uremia, and glomerular degeneration may accompany renal tubular necrosis [14], [15]. Oxidative stress in cells or tissues, which results in cell damage brought on by the creation of Reactive Oxygen Species (ROS) as a result of ongoing exposure to mercury, also leads to necrosis [16]. The emergence of degenerative cells as a symptom of stress on cells that can be reversible or irreversible can occur before necrosis takes place.

High mercury levels can raise MDA levels while decreasing SOD antioxidant levels, resulting in the appearance of ROS. The low antioxidant system is caused by thiol group reduction in antioxidant enzyme proteins as a result of mercury binding because these compounds have a high affinity for thiol groups [17], [18].

The elimination of ROS is critical for maintaining the integrity of cell function and can be accomplished by increasing antioxidant activity. Because synthetic antioxidants have undesirable side effects, natural antioxidants may be developed as an alternative. Natural antioxidants can protect the body from ROS-induced cell damage. Natural antioxidant-rich foods can be used as a strategy to reduce morbidity and mortality, particularly those caused by oxidative stress.

Transforming growth factor-β (TGF-β) is the primary cause of fibrosis in most chronic kidney diseases (CKD). The TGF-β isoform, TGF-β1, or their downstream signaling pathways, when inhibited, significantly reduces renal fibrosis. TGF-β deficiency is linked to nephropathy, and acute kidney injury [19-21]. Various studies have found a trend of decreasing TGF-β in rats exposed to mercury, though not significantly [22].

Urea and creatinine are biomarkers that can be used to assess kidney function [23]. Mercury exposure can impair kidney function because it increases MDA and ROS, resulting in nephrotoxicity, oxidative stress of cells or tissues, and degeneration and necrosis of kidney proximal tubular cells [17].

Chelating agents, which should be given at the beginning of treatment, are used to treat mercury toxicity. Using thiol groups, this substance will compete to bind the mercury. Some chelating agents include 2,3-dimercaptosuccinic acid (DMSA), dimercaprol (BAL) or d-penicylamine (DPCN). Even in cases of

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inorganic mercury poisoning, the use of DMSA is preferable to DCPN [24]. In addition to chelating agents, natural ingredients high in antioxidants can help reduce the effects of mercury exposure. The use of chelating agents in conjunction with antioxidant-rich ingredients is a novel approach to reducing the effects of mercury exposure. Natural ingredients such as Euterpe oleracea (EO) have recently been studied as chelating agents and have been shown to protect populations exposed to mercury in the Amazon [16]. In this study the chelating agent used was water extract of IR Bagendit rice leaves combined with natural ingredients rich in antioxidants, namely coconut water.

IR Bagendit Rice Leaves contain the protein metallothionein, which is high in sulfhydryl groups [25]. The ability of metallothionein to bind toxic Hg is related to its role in the Hg detoxification mechanism. Because it contains a high concentration of "thiol" (sulfidril, SH) groups, metallothionein can bind Hg very strongly and efficiently. Cys sulfhydryl residues can bind one metal ion to two or three sulfhydryl residues (SH). A tetrahedral tetrathiolate structure is formed by the coordination of the binding of each metal ion from Cys. To detoxify Hg, Cys residue is required [26]. According to recent research, the metallothionein gene is located on chromosome 3 and functions as a protein that is induced by environmental stress such as metal contamination [27].

Coconut water is a beverage made from coconuts found in tropical countries such as Indonesia. Vitamins, minerals, amino acids, enzymes, and antioxidants are all found in coconut water [28]. Recent research has shown that drinking coconut water can reduce oxidative stress and increase antioxidants, as evidenced by a decrease in MDA [29]. Based on the foregoing, this study aims to determine the effect of combining IR Bagendit rice leaf water extract with coconut water on reducing oxidants (ROS, MDA), increasing TGF-, and maintaining urea and creatinine within normal limits in order to prevent damage to tubular cell morphology (decreased cell degeneration and necrosis) and improving kidney function.

2. MATERIALS AND METHOD

2.1 Research design

Experimental design with a randomized post-test only control-group design was carried out on animal models that were kept and intervened at the Integrated Research and Testing Laboratory (LPPT), Muhammadiyah University Semarang.

The number of samples was calculated using the following formula: BS = (t – 1) (r – 1) ≥ 15. There were six mice in each group (1 negative control group, one positive control group, and three treatment groups), for a total of 30 Rattus norvegicus rats used in this study. All rats selected were male and 3 weeks old. Acclimatization lasted one week, and intervention lasted two weeks [30].

2.2 Infusion Production Process

According to previous research, the highest metallothionein levels were obtained from IR Bagendit rice leaves harvested in Blora [25]. Before being chopped into small pieces, the rice leaves are cleaned and washed under running water. Weighed 100 g into pot A and added 1 liter of distilled water before closing. Part B of the pot (as a water bath) is filled with enough water to partially submerge the top pot (A). Heated for 15 minutes, beginning when the temperature in pot A reaches 90oC while stirring occasionally. The infusion is added while still hot, using a flannel cloth. The supernatant is a type of infusion. The obtained infusion was tested for metallothionein protein using the Elisa method. Picture of the process of making an infusion is as follows.

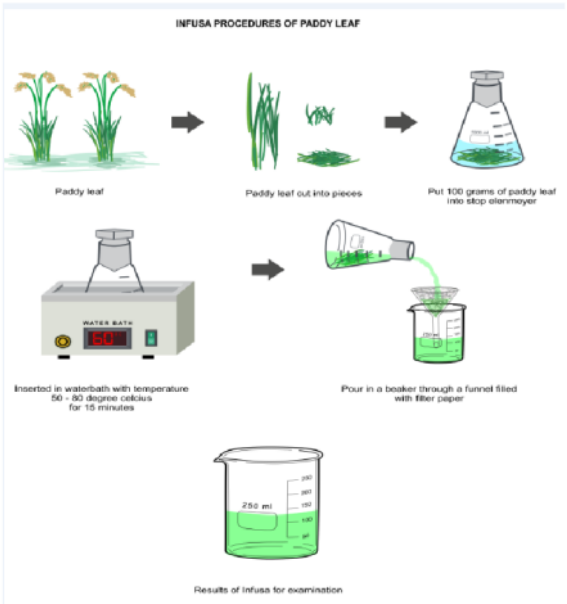

Figure 1. Infusion Production of IR Bagendit rice leaf water extract

2.3 Preparation of HgCl2 reagent

Mercury Chloride is made per ml of 20 mg of HgCl2 as much as 500 ml. the calculation formula is 20 mg / ml = 10,000 mg / 500 ml, converted into grams to 10,000mg = 10 g. From the results of these calculations, 10 g of HgCl2 ad 500 ml of Aquades is weighed.

2.4 Intervention in animal models

The negative control group received a placebo, while the positive control group received HgCl2 at a dose of 20 mg/kg BW per day, which was a toxic dose in previous studies [31]. Water extract of rice leaves IR Bagendit Blora location was given to treatment groups 1 (T1), 2 (T2), and 3 (T3) in the amounts of 0.2 ml, 0.4 ml, and 0.8 ml, respectively, and combined with administration of coconut water to each treatment group in the amount of 4 ml/100 g BW, all treatment groups were exposed to mercury chloride at a rate of 20 mg/kg BW per day for 4 weeks. The duration of the experiment also refers to previous studies involving the exposure of young coconut water to mercury [32]. The control and treatment groups had their blood drawn through the retro-orbital plexus on the 28th day (4 weeks), and termination was performed. The kidneys were taken to make preparations with Hematoxylin-Eosin staining, and cell degeneration and proximal tubular necrosis were observed under a 400x magnification. MDA, ROS, Transforming growth factor-, urea, and creatinine levels were measured in the blood.

2.5 Hematoxyline Eosin (HE) Stain

The paraffin tissue was cut into small pieces and placed on a glass slide. The tissue slides were then deparaffinized, rehydrated, stained with hematoxylin and eosin, and rinsed with water. The slides were then dehydrated, covered with a glass cover, and examined under a light microscope magnified 400 times [33]. Two histopathology experts performed manual observation.

2.6 Scoring and identification of proximal tubular cells of the kidney

The proximal renal tubule was examined from the middle to the periphery. For each preparation in all groups, 100 cells were observed and counted in each image of the proximal tubule. The number of normal

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cells, degeneration, and necrosis in the 100 cells was counted. Normal cells are those that reveal the entire cell wall and cytoplasm. Normal cells that swell are called degenerate cells. The criteria of lysed cells, loss of cell structure, karyolysis, and cariorexis were used to identify necrotic kidney cells [33].

2.7 Measurement of MDA, ROS, TGF-β, Ureum, Creatinine

MDA, ROS, TGF-β, Ureum, and Creatinine measurements were carried out based on the reagent kit, MDA (Catalog No. RK009070 ABclonal), ROS kit (Catalog No. SL2046Hu Solong Biotech), TGF-β (Catalog No. E0778Ra (Bioassay Technology laboratory), Ureum kit (Catalog No. 1 3101 99 10 021, Diasys Diagnostic System GmbH) and Creatinine kit (Catalog No. 1 1711 99 10 021, Diasys Diagnostic System GmbH) from the manufacturer. Sandwich ELISA is used to measure MDA, ROS, and TGF-. The urea measurement principle is based on urea hydrolysis by urease, which produces ammonia and carbon dioxide. The glutamate dehydrogenase (GLDH) enzyme uses ammonia to reduce α-Ketoglutarate (α-KG), and the two react to degrade and oxidize nicotinamide-adenine dinucleotide (NADH). NADH was measured using a bichromatic technique at 340 nm. NADH absorbance is proportional to urea concentration in the sample. The creatinine measurement principle is based on an alkaline environment, where creatinine reacts with picrates to form the Janousky complex. The rate of increase in absorbance to the creatinine-picrate complex at 510 nm is directly proportional to the sample creatinine.

2.8 Statistical Analysis

Data distribution was tested with kolmogorov-smirnov. Normal data is expressed as the mean ± SD and abnormal data is expressed as the median with a min-max value. The differences between the groups were calculated by the Friedman test. The difference between the 2 groups was quantified by the Mann-Whitney test. Statistical calculations were performed using SPSS version 16.0 (IBM Corporation, New York, USA).

2.9 Ethical clearance

The research obtained ethical clearance from the Medical/Health Research Bioethics Commission, Faculty of Medicine, UNISULA Semarang with No.203/VII/2020/Bioethics Commission and in accordance with the Declaration of Helsinki

3. HASIL RESULTS

Mice's weight measurements were carried out at the beginning and end of the study. Based on the results of weight measurements before and after treatment, the data on the difference in weight in the T1, T2 and T3 groups was higher than the positive and negative control groups (Table 1).

Table 1. The difference of body weight before and after treatment.

Group	Average Weight		
	Before(gram)	After (gram)	Difference
C-	186.17	201.67	15,5
C+	196.33	186.33	-10
T1	204.67	208.00	3,33
T2	224.17	220.50	-3,67
T3	223.50	217.50	-6

*The differences between multiple groups were calculated by Anova test.
Note: C -= Control negative, C+= Control Positive, T=Treatment

The results of the examination of oxidants (MDA, ROS) and TGF-β are presented in table 2 below:

Table 2. Average levels of MDA, ROS, and TGF-β in the control and treatment groups

Group	MDA		ROS		TGF-β	
	Average	*p*-value	Average	*p*-value	Average	*p*-value
C-	1.73±0.17		136.14±35.12		38.67±0.80	
C+	2.30±0.20		262,63±16.35		33.62±2.87	
T1	1.80±0.02	0.01	124.99±31.76	0.01	38.65±0.88	0.01
T2	1.61±0.07		112.25±14.57		38.56±1.68	
T3	1.51±0.11		121.02±24.77		39.07±1.84	

*The differences between multiple groups were calculated by Anova test.

Based on table 2, MDA and ROS levels in the treatment group 1 to 3 and the negative control group were lower when compared to the positive control group and there was a significant difference (p value: 0.01). TGF-β levels had the highest average in treatment group 3, followed by treatment group 2, 1 and the lowest in the positive control group, there was a significant difference (p value 0.01)

The morphology of kidney cells revealed that the positive control group, which received only mercury exposure, had the fewest normal cells, while the treatment and negative control groups had the most. The positive control group had degenerative cells and necrosis in the proximal tubules of the kidney, whereas the treatment and negative control groups had fewer degenerative cells and necrosis (table 3). With a p value of 0.001, the mean of the positive control group and the treatment group differed significantly.

Table 3. Average normal cells, degeneration, and proximal tubular necrosis of the kidney in the control and treatment groups

Group	Normal Cell		Degeneration		Necrosis	
	Average	*p*-value	Average	*p*-value	Average	*p*-value
C-	79.83±2.32		13.00±1.41		7.16±2.40	
C+	9.83±2.31		19.50±3.44		70.67±5.05	
T1	40.83±11.87	0.001	29.83±10.03	0.001	29.33±7.63	0.001
T2	60.67±5.57		24.33±3.77		15.00±5.58	
T3	57.50±1.18		20.16±3.31		22.33±5.85	

*The differences between multiple groups were calculated by Anova test.

An overview of the kidney histopathological results can be seen in Figure 2. According to this figure, there were many degenerative and necrotic cells in the positive control group, while normal cells were found in the negative control group and treatments 1, 2, and 3.

Urea and creatinine as parameters for measuring kidney function can be seen in table 4. There is no difference in the average levels of urea and creatinine, but if you look at the averages, the numbers are relatively the same and are still within normal limits.

Table 4. Average levels of urea and creatinine in the control and treatment groups

Group	Urea		Creatinine	
	Average	*p*-value	Average	*p*-value
C-	15.16±1.01		0.37±0.10	
C+	17.48±1.65		0.58±0.15	
T1	18.06±2.10	0.41	0.58±0.13	0.34
T2	17.03±1.51		0.45±0.19	
T3	17.50±1.53		0.60±0.13	

*The differences between multiple groups were calculated by Anova test.

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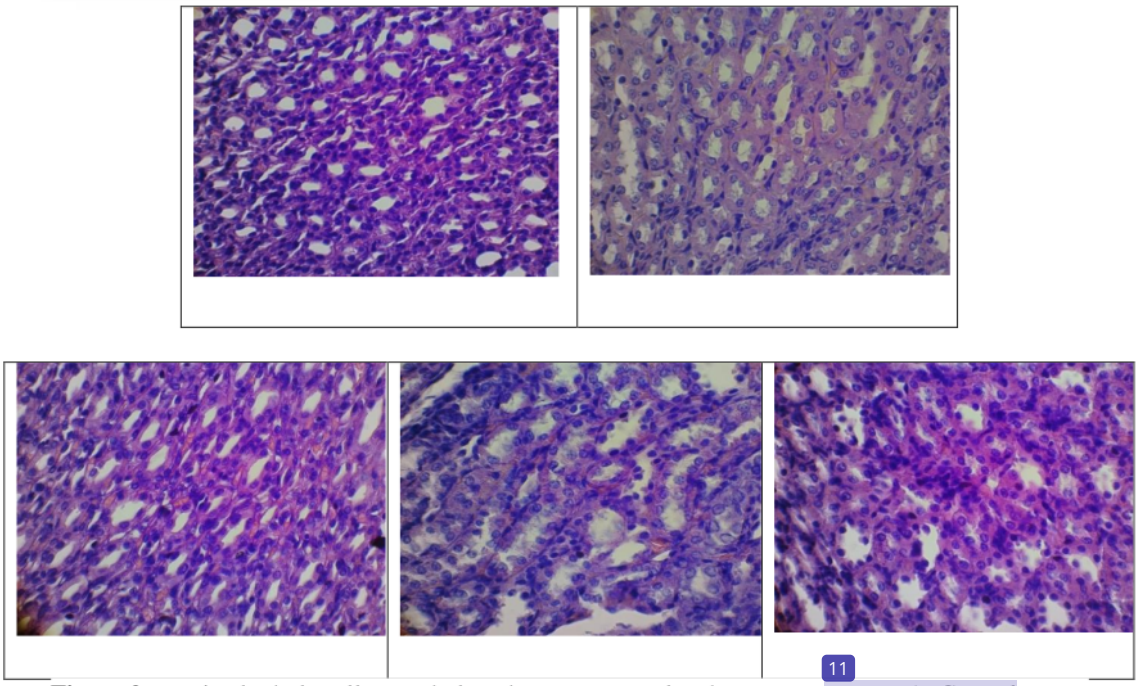

Figure 2. proximal tubule cells morphology between control and treatment groups. A: Control group negative; B: Control Group positive; C: Treatment T1 group; D: Treatment T2 group; E: Treatment T3 group. Black bar: 100 μm.

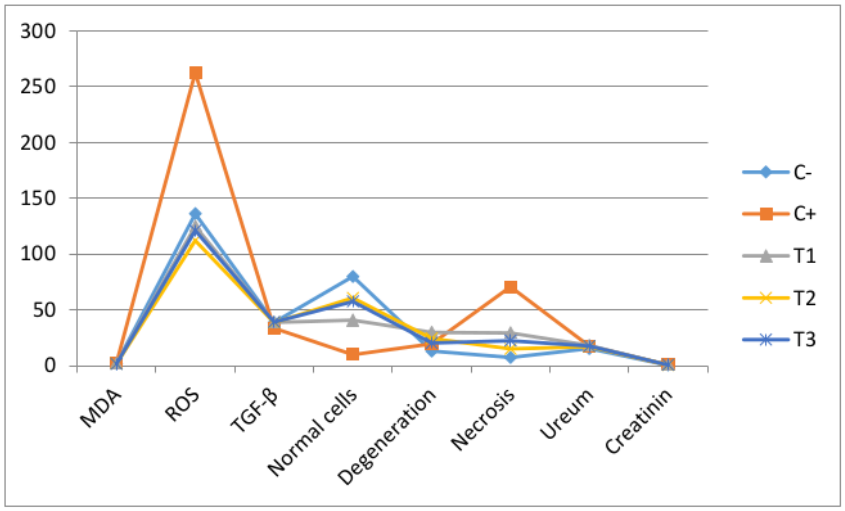

Figure 3. Graph of MDA, ROS, TGF-β, normal cells, degeneration, necrosis, urea, creatinine

4. Discussion

Mercury chloride is an inorganic form of mercury that has been linked to kidney damage [34]. Inorganic Hg has the most severe systemic effects as a nephrotoxicant that attacks proximal tubuus cells [35- 37]. This occurs because continuous exposure to mercury causes the formation of Reactive Oxygen Species (ROS), an increase in lipid peroxidation, oxidative stress in cells or tissues, the inhibition of enzymes, cell damage, protein structure damage, and DNA damage [7]. High mercury exposure can raise MDA levels, lowering

antioxidants [38]. In addition to ROS and high MDA, previous research has shown that mercury exposure can reduce TGF-β, albeit not significantly [22].

This study shows that giving rats exposed to mercury a combination of IR Bagendit rice leaf water extract and coconut water can reduce ROS, MDA, and increase TGF-β, as well as reduce degenerative cells and proximal tubular necrosis cells in the kidney. Urea and creatinine levels, which are used to assess kidney function, have not been affected. When compared to the positive control group, which was only exposed to mercury, this occurred in the negative control group, which was only given a placebo. The most recent finding from this study is that combining coconut water with water extract of rice leaves IR Bagendit was able to significantly reduce ROS, MDA, and increase TGF-β, as well as reduce degenerative cells and proximal tubular necrosis cells of the kidney. The levels of urea and creatinine are still normal.

The novelty results in this study can be explained by the fact that coconut water is a drink high in nutrients such as antioxidants, vitamins, minerals, amino acids, enzymes, and a source of L-arginine and vitamin C. [29]. Consuming coconut water can reduce oxidative stress and boost antioxidant levels. The water extract of IR Bagendit rice leaves contains a high concentration of metallothionein protein, which is rich in sulfhydryl groups (SH) [25], [27]. The metallothionein protein's sulfhydryl group will compete with the membrane protein's sulfidyl group to bind to mercury, resulting in no disruption of membrane integrity (no degeneration or necrosis of the proximal tumulus) [8], [39]. According to the findings of this study, the combination of coconut water and rice leaf water extract IR Bagendit is very effective at preventing kidney damage caused by mercury exposure in Rattus nurvegicus rats. Urea and creatinine levels were found to be within normal limits in both the control and treatment groups in this study. This can be explained by the fact that the exposure to mercury occurred in a short period of time, namely 14 days, so that even though there was an increase in ROS, MDA, and a decrease in TGF-β, as well as an increase in degradation and renal proximal tubular cell necrosis, it did not affect the expression of urea and creatinine, implying that more research with a longer exposure time to mercury is required to prove impaired kidney function through urea and creatinine biomarkers.

5. Conclusion

A combination of IR Bagendit rice leaf aqueous extract and coconut water was found to significantly reduce ROS, MDA, and increase TGF-β, as well as reduce degenerative cells and proximal tubular necrosis cells in mercury-exposed kidney cells. Urea and creatinine, two parameters used to assess kidney function, show no difference from the positive control and remain within normal limits. A combination of treatments was effective in improving tubular cell morphology and kidney function.

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