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RESEARCH ARTICLE OPEN ACCESS

Glutathione reductase, glutathione peroxidase, and catalase in rats exposed to mercury after the addition of ir bagendit rice leaf water infusion

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Abstract
Objective: The study objective was to determine the antioxidant levels of GSr, GPx, and CAT after adding rice leaf infusion to mice exposed to mercury.
Methods: Research method: Randomised post-test only control-group design in groups of experimental animals from male Rattus norvegicus according to inclusion and exclusion criteria. ANOVA, Kruskal-Wallis, and Bonferroni's Post Hoc statistical tests were used to test the differences between each group. The experimental animals consisted of 5 groups: negative control group, positive control group, and treatments 1, 2 and 3. Each group consisted of 8 experimental animals, totalling 40 experimental animals. The positive control group was only exposed to mercury. In contrast, the treatment group, apart from being exposed to mercury, was also exposed to IR Bagendit rice leaf infusion with various doses. The desired intervention was tried for 15 days. Examine GSr, GPx, and CAT using an experimental animal serum with the ELISA method.
Results: The results showed that the average levels of GSr, GPx, and CAT were higher in all treatment and negative control groups compared to the positive control group. Statistically, there was a significant difference in GPx (p=0.03) and CAT (0.02) levels. Furthermore, post hoc analysis using the Bonferroni test obtained significant differences in GPx and CAT levels between the positive and negative control groups and treatment 3.
Conclusion: In conclusion, there was an increase in antioxidant levels in the treatment group after adding water and IR Bagendit rice infusion.

Keywords: Antioxidant, IR bagendit rice leaf, Mercury, Oxidative stress

Plain English Summary

Metallothionein can bind mercury strongly and efficiently because it contains large amounts of "thiol" (sulfhydryl, SH) groups. The sulfhydryl residues of Cys can bind one metal ion to two or three sulfhydryl

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residues (SH). Therefore, the Cys residue is needed to detoxify mercury. Recent studies have shown that the metallothionein gene is located on chromosome three, which functions as a protein induced by environmental stress, such as metal contamination. This research is helpful as a preventive measure against exposure to Hg. Water extract from IR Bagendit rice leaves can prevent kidney damage, as evidenced by the levels of GSr, GPx, and CAT, which did not decrease in Rattus norvegicus rats exposed to Hg.

Background

Mercury (Hg) is a heavy metal widely used in various industries such as agriculture, cosmetics, dentistry, hospitals, research laboratories, traditional gold miners and others (1). Mercury poisoning can occur due to inhalation, ingestion and absorption through the skin. Mercury is a free radical which oxidises macromolecules in the body, such as fats, proteins and nucleic acids (2). Mercury in tissues can catalyse oxidation-reduction reactions to produce Reactive Oxygen Species (ROS) (3). The increase in ROS activity will cause oxidative stress. This condition would lead to biomolecule damage and several diseases, such as hyperglycemia, cancer, and atherosclerosis (4). Biomarker of oxidative stress was the decreased antioxidant enzymes such as superoxide dismutase (SOD), glutathione reductase Gsr), glutathione peroxidase (GPx), and catalase (CAT) (5).

Glutathione reductase is a flavoprotein that catalyses the reduction of glutathione disulfide (GSSG) to glutathione (GSH). This reaction is essential for maintaining glutathione levels (6). Glutathione is a significant reducing agent in the oxidation-reduction process and has a role in heavy metal detoxification (7). Glutathione peroxidase (GPx) is an endogenous antioxidant that plays a role in preventing the formation of free radical compounds that can form reactive molecules (8). The glutathione peroxidase enzyme plays a role in changing the H_2O_2 produced by superoxide dismutase into water. Catalase is an antioxidant enzyme that can be used as a biomarker of oxidative stress and heavy metal toxicity. Catalase is one of the most abundant antioxidant enzymes produced by the body (9). This enzyme is primarily located in the liver. Catalase enzyme is one of the antioxidants that play a role in detoxifying Reactive Oxygen Species (ROS) (10).

As a result of exposure to mercury, which is very dangerous for health, preventive efforts are needed to reduce the impact it causes (11). Therefore, using a chelating agent for the water extract of rice leaves, IR Bagendit, needs to be tested as a preventive agent against mercury toxicity (12, 13, 14).

Rice leaf water extract is proven to contain a lot of metallothionein protein as a chelating agent against heavy metals. IR Bagendit Rice Leaves contain metallothionein protein, which is rich in sulfhydryl groups (15). The role of metallothionein in the mercury detoxification mechanism is related to the ability of metallothionein to bind to toxic mercury (16). Metallothionein can bind mercury strongly and efficiently because it contains large amounts of "thiol" (sulfhydryl, SH) groups. The sulfhydryl residues of Cys can bind one metal ion to two or three sulfhydryl residues (SH). Therefore, the Cys residue is needed to detoxify mercury (17). Recent studies have shown that the metallothionein gene is located on chromosome three, which functions as a protein induced by environmental stress, such as metal contamination. The present study aimed to analyse the effect of IR Bagendit Rice Leaves extract on glutathione reductase Gsr), glutathione peroxidase (GPx), and catalase (CAT) in *Rattus norvegicus* rats exposed to Hg.

Materials and Methods

Research Design

This study employed the randomised post-test-only control-group design in a group of research animals from *Rattus Norvegicus* aged 15 weeks. It weighed 180-220 grams, was healthy, agile, and had no anatomical abnormalities. The maintenance and intervention of animals are carried out at the Integrated Research and Testing Laboratory (LPPT) of Universitas Muhammadiyah Semarang, certified by ISO 9001-2015. The total number of samples was 40 male *Rattus Norvegicus* was selected using the formula: BS = (T-1) (R-1) ≥ 15. This study employed one negative control group, one positive control group, and three treatment groups. Each group consisted of six rats and two more.

Process of Making Infusion

IR Bagendit rice leaves originate from Blora Regency because the results of previous studies show that it has the highest level of metallothionein (18). Rice leaves are cleaned and washed under running water before being chopped into small pieces. Then, 100 grams of chopped leaves were put into pot A, and 1 litre of water was added.

Afterwards, the pots were closed. In pot B (as a water bath), added water until the top of pot A was partially submerged. The pots were heated for 15 minutes (calculated starting when the temperature in pot A reaches a temperature of 90 °C) while stirred occasionally. The infusion was sprayed while it was hot through the flannel. Afterwards, the infusion's protein metallothionein was examined using the ELISA method.

Preparation of $HgCl_2$ Reagent
In preparing mercury chloride reagent, the following calculation formula is used: 20 mg/ml = 10,000 mg / 500 ml. The calculation results obtained 10 grams of $HgCl_2$, which was then dissolved in 500 ml of distilled water.

Intervention in Research Animals
The negative control group was given a placebo, and the positive control group received 20 mg/kg BW of $HgCl_2$ per day. Water extract of IR Bagendit rice leaves was given to treatment groups one (T1), two (T2), and three (T3), respectively, in the amounts of 0.2 ml, 0.4 ml and 0.8 ml. Afterwards, all treatment groups were exposed to 20 mg/kg BW of mercury chloride daily. On day 15, the control and treatment groups had their blood taken using the retro-orbital plexus. The blood was examined for GSr, GPx, and CAT levels at the Biomolecular Laboratory of Universitas Muhammadiyah Semarang.

GSr, GPc, and CAT analysis
The GSr, GPx, and CAT levels were analysed using the Sandwich ELISA method according to the reagent catalogue number: No. ER0631 for GSr level, No. CK-bio-14705 for GPx level, and No. RK03551 for the CAT level. The principle of measuring GSr, GPx, and CAT levels is that the Microtiter plate is coated with a specific antibody. Then, added standard was added to the appropriate microtiter plate wells with specific polyclonal conjugated biotin antibody preparations and Streptavidin conjugated to horseradish peroxidase (HRP) was added to each well of the microplate and incubated. The TMB substrate solution was then added to each well. Only wells containing the antibody of interest, biotin-conjugated antibody, and enzyme-conjugated Streptavidin will show a colour change. The substrate enzyme reaction was completed by adding sulfuric acid solution, and the colour change was measured spectrophotometrically at a wavelength of 450 nm. The concentration in the sample is then determined by comparing the O.D. sample with a standard curve.

Statistical Analysis
Data distribution was tested using the Shapiro-Wilk test. Normal data were presented as the mean ± standard deviation (SD), and abnormal data were presented as the median with a min-max value. Analysis of differences between the GSr groups using the Kruskal-Wallis Test, GPx and CAT using the ANOVA test.

Results
The negative control group, which was only given a placebo, experienced weight gain, whereas the positive control group, exposed to mercury, experienced weight loss. All treatment groups experienced an increase in body weight, present in Figure 1.

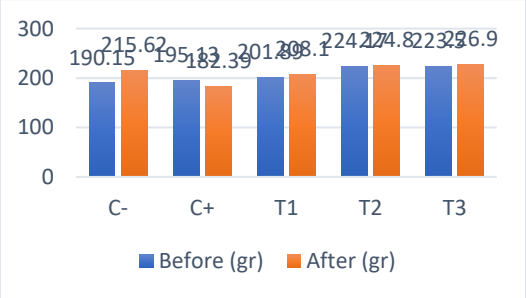

Figure 1. The difference in body weight before and after treatment.
Note:C -= Control negative, C+= Control Positive, T=Treatment

Based on Table 1, the average levels of GSr, GPx, and CAT were obtained in all treatment groups and the negative control compared to the positive control group. There was a significant difference in the levels of GPx (p=0.03) and CAT (0.02). The results of antioxidant examinations (GSr, GPx, CAT) are presented in Table 1 below:

Table 1: The average levels of GSr, GPx, and CAT in the control and treatment groups

Group	GSr		GPx		CAT	
	Mean ± SD	*p*-value	Mean ± SD	*p*-value	Mean ± SD	*p*-value
C-	34.06±6.15		26.64±7.63		56,.0±1.43	
C+	32.15±7.90		16.51±3.32		37.93±3.61	
T1	36.66±3.93	0.82*	26.39±8.10	0.03**	42.82±5.69	0.02**
T2	35.47±5.98		19.12±3.01		42.93±5.28	
T3	35.44±6.40		29.11±4.26		44.14±7.44	

**The differences between multiple groups were calculated by the Kruskal-Wallis Test*
***The differences between multiple groups were calculated by the ANOVA test.*

Based on the Post Hoc results using the Bonferroni test on Table 2, there was a significant difference in GPx and CAT levels between the positive control group and the negative control group and treatment 3 (T3).

Table 2. Post Hoc Tests GPx and CAT results

Variable	Group		Δ	p-value
GPx	C+	C-	-10.14	0.02
		T1	-9.88	0.06
		T2	-2.61	1.00
		T3	-12.60	0.00
CAT	C+	C-	-18.77	0.00
		T1	-4.89	1.00
		T2	-5.00	1.00
		T3	-6.20	0.04

Note: Bonferroni test

Discussion

Based on the research results, the GSr, GPx, and CAT levels descriptively showed an increase in treatment groups 1, 2, and 3 compared to the negative control group, which was only given a placebo. There was a statistically significant difference in GPx and CAT. GSr, GPx, and CAT were antioxidants that decreased exposure to Hg as an oxidant. It was proven that in all positive control groups exposed to Hg, the GSr, GPx, and CAT levels were lower than the negative control group, who were only given the placebo.

The results of this study can be explained that Mercury chloride is one of the pro-oxidants that induces oxidative stress (19). Mercury compounds have a high affinity for the thiol (-SH) group of biomolecules. Water infusion of rice leaves IR Bagendit, Blora location, contains a lot of metallothionein protein. Metallothionein protein can bind Hg strongly and efficiently because it contains large amounts of “thiol” (sulfhydryl, SH) groups. The sulfhydryl residues of Cys can bind one metal ion to 2 or 3 sulfhydryl residues (SH). The coordination of the binding of each metal ion from Cys forms a tetrahedral tetrathiolate structure to produce a covalent bond, namely a bond formed due to the sharing of two electrons by two atoms. This bond is stable and irreversible (20). In the following figure, Hg^{2+} covalent bonds occur with sulfhydryl groups in metallothionein proteins. Cys residue is needed for detoxification.

Glutathione reductase (GSr), Glutathione peroxidase (GPx), and Catalase (CAT) are antioxidants whose numbers will decrease if there is exposure to Hg, which is a pro-oxidant (21). However, the results of this study show the levels of GSr, GPx, and CAT have increased; This is evidence that the water infusion of IR Bagendit rice leaves containing protein metallothionein can bind Hg so that the levels of GSr, GPx, and not decrease.

As an antioxidant agent, Glutathione has a specific role in protecting the body from mercury toxicity. Glutathione binds to mercury by forming complexes that prevent mercury from binding to cellular proteins and causing damage to enzymes and tissues (22). Glutathione reductase catalyses the reduction of glutathione disulfide (GSSG) to the sulfhydryl glutathione (GSH) form, an essential molecule in resisting oxidative stress and maintaining cell reduction (23). Glutathione reductase plays a vital role in the metabolism and clearance of xenobiotics, acts as a cofactor in certain detoxifying enzymes, participates in transport, and regenerates antioxidants such as Vitamins E and C (24).

Glutathione peroxidase (GPx) is one of the body's natural antioxidants, which can be decreased if

oxidants increase. The primary biological role played by GPx is to protect organisms from oxidative damage (25). Glutathione peroxidase reduces lipid hydrogen peroxide to the corresponding alcohols and the reduction of free hydrogen peroxide to water (26).
Catalase (CAT) is an enzyme that protects cells from oxidative damage by reactive oxygen species (ROS). Catalase has one of the highest turnover rates of any enzyme; one molecule of catalase can convert millions of hydrogen peroxide molecules into water and oxygen every second (27). Catalase is a tetramer of four polypeptide chains, each more than 500 amino acids long (28). Four iron-containing heme groups allow the enzyme to react with hydrogen peroxide (29).
This study proves that giving IR Bagendit rice leaf infusion can prevent a decrease in the antioxidants GSr, GPx, and CAT upon mercury exposure. The results showed that the average levels of GSr, GPX and CAT in all treatment groups were higher than in the positive control group. IR bagendit rice leaves contain a lot of metallothionein protein, which is rich in sulfhydryl groups so that it can bind covalently to Hg exposure for further detoxification. This research is helpful as a preventive measure against exposure to Hg.

Conclusion

Water extract from IR Bagendit rice leaves can prevent kidney damage, as evidenced by the levels of GSr, GPx, and CAT, which did not decrease in Rattus norvegicus rats exposed to Hg.

Declarations

Ethical Consideration

This study has obtained ethical clearance from the Medical/Health Research Bioethics Commission, Faculty of Medicine, Universitas Islam Sultan Agung Semarang, with number:78/II/2023/Komisi Bioetik.

Consent for Publication

All the authors gave consent for the publication of the work under the Creative Commons Attribution Non-Commercial 4.0 license.

Availability of Data

Data for this work is available from the authors and may be provided upon reasonable request.

Conflict of interest

None

Competing interests

All authors do not have any competing interests.

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The authors provided the funding for this study.

Authors' contributions

Conceptualisation and data interception: SB; Study design, writing the original draft and data evaluation: SB, AT, BS, JFF, PS; Supervision: BS, SP; Final approval: All authors.

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