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Effect of *Moringa oleifera* L. Leaf Extract on GPx-3 Gene Expression in the Brain of Induced Ethylene Glycol on Rats (*Rattus norvegicus*)

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ABSTRACT

Ethylene glycol is one of the toxic substances commonly used as solvents and is known to induce oxidative stress in neural tissue. GPX-3 is an antioxidant enzyme that works to reduce ROS, maintain redox balance, and protect cells from damage caused by free radicals produced during oxidative stress processes. This study aims to evaluate the effect of administering Moringa oleifera L. leaf extract after ethylene glycol induction on the intensity of GPx-3 gene expression in the brain of white rats (Rattus norvegicus). This research employed a completely randomized design (RAL) using 20 rats divided into five groups: a normal control group, a 0.75% ethylene glycol group, a 150 mg/kgBW Mo group, a 300 mg/kgBW Mo group, and a 450 mg/kgBW Mo group. The research stages included brain RNA extraction, cDNA synthesis, PCR amplification, agarose gel electrophoresis, and band intensity analysis using ImageJ. The results showed that GPx-3 gene expression in rat brain tissue appeared at 300 bp. Administration of Moringa oleifera L. leaf extract increased GPx-3 gene expression intensity in ethylene glycol induced rats, with the most prominent increase observed in treatment 2 (300 mg/kgBW). The increase or decrease in measured expression after normalization to GAPDH indicates that the observed changes reflect a biological response to treatment rather than technical error. This study presents a novelty finding by demonstrating, for the first time, the dose-dependent modulation of GPx-3 gene expression in brain tissue following ethylene glycol induced oxidative stress and identifying 300 mg/kgBW as the most effective dose, thereby revealing a potential molecular mechanism underlying the neuroprotective effect of Moringa oleifera L. leaf extract.

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INTRODUCTION

Moringa oleifera L. is a South Asian medicinal plant widely recognized for its antioxidant and anti-inflammatory properties. The leaf extract contains high concentrations of bioactive compounds, including flavonoids, phenolics, vitamins, and essential minerals, which contribute to its cytoprotective effects. Polyphenolic compounds in Moringa leaves exhibit antifungal, antidiabetic, and anti-inflammatory activities and are reported to modulate oxidative stress pathways. Toxicological evaluations indicate that Moringa leaf extract is relatively safe for consumption, even at higher doses (Satriyani, 2021). Several studies have demonstrated that Moringa leaf extract can modulate the expression of genes involved in antioxidant defense. One of the key genes associated with cellular protection against oxidative stress is GPx-3. Glutathione Peroxidase (GPx) represents a major enzymatic antioxidant system in mammalian cells and plays a central role in maintaining redox balance. GPX-3 specifically catalyzes the reduction of hydrogen peroxide and lipid peroxides using glutathione as an electron donor, thereby preventing oxidative cellular damage (Pei et al., 2023).

Oxidative stress is defined as a pathological condition in which ROS production exceeds the capacity of endogenous antioxidant systems. This imbalance contributes to inflammation, cellular dysfunction, and tissue injury, particularly in the brain (Cueto-Ureña et al., 2023). Although previous studies have reported the antioxidant potential of Moringa oleifera, limited research has focused on its molecular effects on GPx-3 gene expression in brain tissue under toxin-induced oxidative conditions. Therefore, this study aimed to analyze the intensity of GPx-3 gene expression following the administration of Moringa oleifera L. leaf extract in the brains of white rats (Rattus norvegicus) induced by ethylene glycol. The findings are expected to determine the effective dose of Moringa leaf extract in enhancing endogenous antioxidant gene expression and to clarify its potential role as a neuroprotective agent against toxin-induced oxidative brain damage. Clinical examination revealed elevated levels of mercury in his blood and brain tissue, accompanied by increased biomarkers of oxidative stress, particularly in regions associated with motor control and cognitive function.

The patient had a history of consuming large quantities of tuna, leading to chronic mercury exposure. Mercury, a potent neurotoxic heavy metal, readily crosses the blood brain barrier and accumulates in neural tissue, where it stimulates excessive production of reactive oxygen species (ROS). This oxidative imbalance contributes to neuronal degeneration, synaptic dysfunction, and may accelerate the progression of neurodegenerative disorders (Ahn et al., 2018). Globally, heavy metal exposure remains a significant environmental and public health issue, particularly in populations with high seafood consumption. Through bioaccumulation and biomagnification processes, mercury concentrations increase along the food chain, reaching high levels in predatory fish. Chronic exposure, even at subacute doses, can disrupt mitochondrial function, impair antioxidant enzyme systems, and promote lipid peroxidation in neuronal membranes. These mechanisms collectively enhance neuronal vulnerability to oxidative injury. In addition to heavy metals, various chemical compounds such as ethylene glycol are known to induce systemic toxicity associated with oxidative stress. Although ethylene glycol is primarily recognized for its nephrotoxic effects, its metabolites can generate metabolic acidosis and promote ROS formation, indirectly affecting other organs, including the brain.

Experimental models have demonstrated that toxin-induced oxidative stress may alter endogenous antioxidant gene expression, thereby disturbing cellular redox homeostasis. The brain is particularly susceptible to oxidative damage due to its high oxygen consumption, abundant polyunsaturated fatty acids, and relatively limited antioxidant reserves. Excessive ROS production can initiate lipid peroxidation, protein oxidation, and DNA damage, ultimately leading to apoptosis or necrosis of neuronal cells. Therefore, strengthening endogenous antioxidant defense systems represents a critical strategy to mitigate toxin-induced neural damage. Oxidative stress can cause cellular degeneration, requiring antioxidants to protect the body against free radicals. Antioxidants inhibit oxidation reactions by neutralizing free radicals and reactive molecules (Sari, 2016). Adequate intake of antioxidant-rich foods has been associated with a reduced risk of degenerative diseases linked to oxidative damage. Despite the widespread commercialization of antioxidant supplements at relatively high prices, many potent antioxidant compounds are naturally abundant in medicinal plants. Commonly used natural antioxidant sources include turmeric, garlic, ginger, and one of the most widely recognized medicinal plants, moringa.

MATERIALS AND METHOD

All stages of this research were conducted in June-August 2025 at the Integrated Laboratory of Zoology and Genetics, State Islamic University of North Sumatra, Medan, as a place for maintaining and treating experimental animals until the analysis of GPx-3 gene expression. Twenty male white rats (*Rattus novergicus*) of the Wistar strain, aged 2-3 months, were placed in animal house cages. The rats were acclimatized by providing food and water for one week to reduce stress. The rats were divided into five groups: a normal control group, an ethylene glycol control group, and three treatment groups, each receiving Moringa leaf extract at 150 mg/kg, 300 mg/kg, or 450 mg/kg body weight. The purpose of this grouping was to observe differences in the results of the different treatments. The rats used in the study were required to be healthy (normal weight and no hair loss).

Necropsy and Organ Removal. The rats were euthanized by inhalation used the inhalation anesthetic ether. The rats were then placed in sealed glass jars filled with cotton soaked in ether and left to ambulate until unconscious. The mice were fixed using a needle in their feet in a paraffin bath, and their heads were surgically removed to remove the brains (Korban et al., 2023). The brains were washed with PBS (*Phosphate-Buffered Saline*) to remove blood and debris. Brain samples were stored in 1.5 ml tubes containing RNA preservation solution and stored at -20°C.

Brain RNA Extraction. This step involved weighing 30 mg of the entire tissue sample, grinding it to a powder in liquid nitrogen using a mortar and pestle, and transferring it to a microcentrifuge tube. 350 µl of *FARB Buffer* and 3.5 µl of ß-Mercaptoethanol were added. The mixture was then homogenized by passing it through a 20-G syringe 10 times and incubated at room temperature for 5 minutes. A filter column was placed in the collection tube, and the sample mixture was transferred to the filter column, which was then centrifuged at 18,000 x g for 2 minutes. Then the clarified supernatant was transferred from the collection tube to a new microcentrifuge tube, and the supernatant volume was measured. Discard the Filter Column and Collection Tube. Add 1 volume of 70% *RNase-free* ethanol and mix thoroughly by vortexing. Place the *FARB Mini Column* into the Collection Tube and transfer the added ethanol sample mixture (including sediment) to the *FARB Mini Column*. Then centrifuge at 18,000 x g for 30 seconds. Discard the flow through and return the FARB Mini Column to the collection tube. Continued with the RNA extraction process using the RNA isolation kit (FAVORGEN) according to the work procedures in the kit provided. The entire extraction process was carried out at cold temperatures and sterile conditions using a UVC (*Ultra Violet Cabinet*). Total RNA collected in 50 µl was measured for purity of the final RNA results using a UV-Vis spectrophotometer to equalize its concentration in the cDNA synthesis analysis. Sample storage must be at a temperature of -200 C finally using a UV-Vis spectrophotometer to equalize its concentration in cDNA synthesis analysis.

cDNA Synthesis. This step uses the reverse transcriptase II (SMOBIO) kit, which performs reverse transcription to convert RNA molecules into complementary DNA. This cDNA synthesis reaction is mediated by an enzyme called reverse transcriptase, or RT. The RNA extraction step, using the kit's procedure for one reaction, involves denaturing the first-strand cDNA as Mix A with 1 µl of *Oligo (di) Primer*, adding the calculated volume of RNA, and adding 10 µl of *DEPC-treated water* to the tube. Spin down the mixture and incubate it in a Thermal Cycler at 70°C. The cDNA strand buffer step uses Mix B, containing 4 µl of 5Xrt Buffer, 5 µl of DEPC Water, and 1 µl of RI Enzyme. Add 10 µL of each A and B mixture to a new tube, for a total volume of 20 µL. Then, spin down the tube using a vortex and perform PCR (*Polymerase Chain Reaction)* at 250°C for 10 minutes, 300°C -500°C for 50 minutes, 850°C for 5 minutes, and -200°C for long-term cDNA storage.

GPx-3 Gene PCR Amplification. GPx-3 gene PCR amplification was performed to increase the number of target copies for further analysis. As an initial step, GPx-3 gene expression was analyzed by RT-PCR using GAPDH as a reference gene. The primers used for the GPx-3 gene consisted of the forward (5'-CAGAACTCCTGGGCTCACCT-3') and reverse (5'-TCCATCTTGACGTTGAC-3') primers, while the primers for the reference GAPDH gene were the forward (5'-GCACCGTCAAGCTGAGAAC-3') and reverse (5'-ATGGTGGTGAAGACGCCAGT-3') primers. The PCR cycle conditions used for target gene analysis included an initial denaturation at 95°C for 42 seconds, followed by 33 cycles of denaturation (95°C, 10 seconds), annealing (50°C, 1 minute), extension (72°C, 1 minute), and a final extension at 72°C for 5 minutes. The PCR amplification results were then used for agarose gel electrophoresis analysis.

Agrose Gel Electrophoresis. agarose gel electrophoresis is used to separate PCR-derived DNA fragments ranging from 100 bp to 25 kb. The initial step of this process involves converting a 1-2% agarose gel using 1x TAE buffer, heating it on a hotplate, adding SYBR Safe dye, pouring the gel into a mold, and allowing it to

solidify. The printed wells are first inserted with a DNA ladder marker, followed by equal volumes of the sample in each well. The machine is then run at 85-100 volts for 30-60 minutes. The final DNA fragments are visible as bands under UV transillumination, and their intensities are quantified using ImageJ.

Data Analysis. Rat brain RNA analysis using the Glutathione Peroxidase-3 (GPx-3) gene is followed by cDNA synthesis analysis. The resulting cDNA is then used as a template in PCR. Amplification of mouse brain RNA using the GPx-3 gene with the GAPDH gene as a reference gene revealed the formation of a band during electrophoresis. This band indicates that all treated organs were successfully amplified through PCR. The results will be further analyzed using ImageJ software.

RESULTS AND DISCUSSION

GPx-3 Gene Expression Intensity. Rat brain RNA analysis using the Glutathione Peroxidase-3 (GPx-3) gene was followed by cDNA synthesis analysis. The resulting cDNA was used as a template in PCR. Amplification of rat brain RNA using the GPx-3 gene, with GAPDH as a control, yielded a single band on electrophoresis. This band indicated that PCR had successfully amplified all treated organs. The observations were further analyzed used ImageJ.

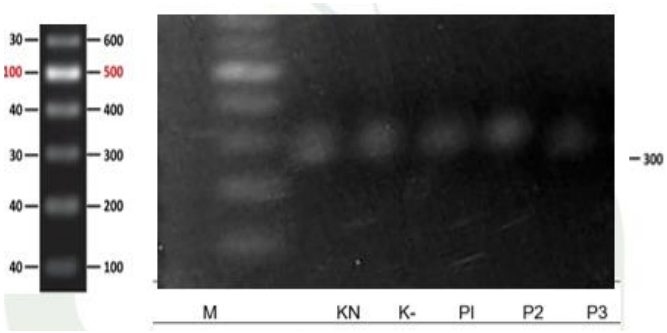

Figure 1. PCR Electrophoresis Results Used GPx-3 Primer

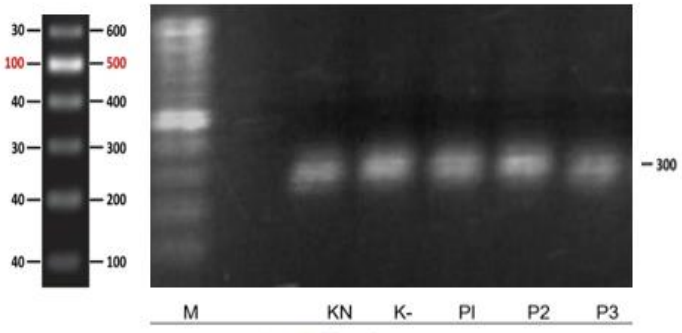

Figure 2. PCR Electrophoresis Results Using GAPDH Primer

explen:

M = DNA Marker 100-3kb; KN = Normal Control; K- = Negative Control (*Ethylene Glycol*); P1 = Ethylene Glycol + Moringa Leaf Extract 150; P2: Ethylene Glycol + Moringa Leaf Extract 300; P3 = Ethylene Glycol + Moringa Leaf Extract 45

Figure 1 and **Figure 2** shows that the GPx-3 and GAPDH expression results in this study show that the band size of 300 bp is consistent across treatments, both in the control group and the groups given induction and extract treatment, shows the target expression band of the GPx-3 gene, measuring 300 bp. This indicates that all five RNA samples successfully expressed the GPx-3 gene. Molecular movement during electrophoresis occurs in an electric field, influenced by the shape, size, molecular weight, and electrical charge of the macromolecule. When an electric current is passed through a support medium containing genetic code, the code will migrate. Therefore, based on these results, all samples have the same shape and chemical properties, as evidenced by the electrophoresis bands appearing in the same positions (Jurnal et al., 2019). Visually, the band intensity of the normal control appears very faint compared to the other bands. The brightest GPx-3 gene band was expressed in treatment sample 2, which consisted of mice given ethylene glycol (EG) and then induced with Moringa Leaf Extract at doses of 150 mg/kg and 300 mg/kg. The intensity of the visible DNA band is based on the GPx gene expressed in each sample treatment.

The results of this observation are in line with the theory that administering Moringa leaf extract at a dose of 300 mg/kgBW is more effective in relieving oxidative stress caused by Ethylene Glycol (EG) compared to a dose of 150 mg/kgBW and a dose of 450 mg/kgBW (Olurishe et al., 2016). The high intensity of the band in treatment 2, with Moringa leaf extract at a dose of 300 mg/kgBW, is due to antioxidant compounds that protect the brain from oxidative stress induced by the toxic substance ethylene glycol. This shows that there is a higher increase in the level of expression of the GPx-3 gene, which encodes a good antioxidant enzyme, in treatment 2 mice given Moringa leaf extract 300 mg/kgBW (Indrisari et al., 2023). Determining a good dose level for administering extracts such as Moringa leaves is not based solely on high or low doses. As seen in Figure 1, the highest band intensity, P3, at an extract dose of 450 mg/kgBW, is too thin. This could be due to several factors, such as technical errors during RNA extraction or too low RNA purity.

GAPDH Gene Expression Intensity was used as a reference gene to normalize technical variations that may arise during RNA isolation, reverse transcription, and PCR amplification. Normalization for housekeeping genes is crucial because the amount of total RNA obtained from each sample can vary due to differences in cell number, extraction efficiency, and cDNA quality. By comparing the expression of the target gene (GPx-3) with that of GAPDH, the resulting data is more valid and representative of actual biological conditions. **Figure 2** shows that the GAPDH expression results in this study show that the band size of 300 bp is consistent across treatments, both in the control group and the groups given induction and extract treatment. This consistency indicates that GAPDH is not significantly affected by the experimental treatment, making it a suitable reference for comparison. The stability of GAPDH expression also supports the reliability of the relative quantification method used to assess changes in GPx-3 gene expression. In this study, the data indicate that GAPDH is quite stable, making its use as a control gene justified. Normalization of GPx-3 gene expression values with the GAPDH gene show of the GPx-3 gene expression analysis were also analyzed semi-quantitatively used ImageJ software in Table 1.

Table 1. Net Intensity Normalization of GAPDH and GPx-3 Genes

GROUP	DOSE	RAT	NET INTENSITY GAPDH	NET INTENSITY GPX-3	RNA EXPRESSION	NORMALIZATION
KN	-	1	53.032	30.213	1.75527091	1
K-	EG	1	63.414	28.938	2.191374663	1.248453815
P1	150 mg/kgB	1	56.677	31.127	1.82083079	1.037350291
		2	69.342	38.271	1.811867994	1.032244073
		3	60.289	34.202	1.762733173	1.004251346
P2	300 mg/kgB	1	60.044	41.906	1.399594415	0.79736661
		2	59.881	41.803	1.46756366	0.836089547
		3	64.553	31.83	2.028055294	1.155408708
P3	450 mg/kgB	1	46.78	24.527	1.907285848	1.08660483
		2	69.59	34.684	2.006400646	1.143071781
		3	85.368	24.102	3.091195751	1.76109325

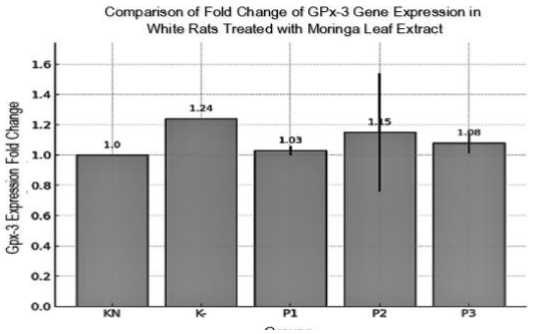

Figure 3. GPx 3 expression and normalized to the GAPDH

Image Caption:

KN = Normal Control; K- = Negative Control (Ethylene Glycol); P1 = Ethylene Glycol + Moringa Leaf Extract 150 mg; P2= Ethylene Glycol + Moringa Leaf Extract 300 mg; P3 = Ethylene Glycol + Moringa Leaf Extract 450 mg/kgBW.

Based on the results of the fold change calculation, it can be seen that the normal group (KN) was set as a reference value with a relative expression of 1. The negative group (K-), which was only given ethylene glycol (EG), showed an increase in GPx-3 gene expression to 1.24, which could occur due to an indication of a cellular compensatory response to oxidative stress in the brain triggered by ethylene glycol (EG) induction. This is also because GPx-3 is an antioxidant enzyme that plays a role in reducing peroxides, so this increase is in line with the defense mechanism (Ighodharo, 2018).

Figure 3 shows that the groups treated with moringa leaf extract showed varying GPx-3 gene expression results. The 150 mg/kgBW (P1) dose showed a relatively low fold change of 1.03 ± 0.03, approaching that of the normal control. This indicates that low doses of moringa leaf extract are beginning to provide a protective effect by suppressing GPx-3 induction induced by oxidative stress. The 300 mg/kgBW dose showed the highest expression, with a value of 1.15 ± 0.39, compared to the other treatment groups. This suggests that this dose triggers a more significant increase in GPx-3 activity, as the bioactive compounds such as flavonoids, polyphenols, and vitamin C in the Moringa leaf extract work optimally in inducing the endogenous antioxidant defense system. The 450 mg/kgBW dose (P3) showed decreased expression, with a value of 1.08 ± 0.07 compared to P2. This indicates that increasing the dose does not always correlate with increased GPx-3 expression. This could be due to a threshold effect or a negative cellular feedback mechanism that prevents overexpression of antioxidant enzymes. Moringa leaf extract can support the body's antioxidant defense mechanisms, including increasing the activity of genes that produce enzymes such as GPx-3, which then plays a role in reducing cell damage.

CONCLUSION

Based on the results and discussion of this study, it was concluded that the intensity of the GPx-3 gene band was 300 bp, and the brightest expression was in the treatment sample 2 induced by Moringa Leaf Extract at 300 mg/kgBW. The results of this observation are in line with the theory that administering Moringa leaf extract at

a dose of 300 mg/kgBW is more effective in reducing oxidative stress caused by Ethylene Glycol (EG) compared to a dose of 150 mg/kgBW and a dose of 450 mg/kgBW. Moringa leaf extract has the potential to modulate GPX-3 expression, with an optimal dose of 300 mg/kg showing the highest expression after normalization to GAPDH (1.15 ± 0.39) compared to other treatment groups. This suggests that this dose triggers a more significant increase in GPX-3 activity, possibly because the bioactive compounds (flavonoids, polyphenols, and vitamin C) in Moringa leaves function optimally in activate the endogenous antioxidant defense system.

AUTHORS CONTRIBUTION

N.M. Nasution conducted the research, analyzed and interpreted the data, and drafted the manuscript. Z. Idami, W. Leni and N.A. Ningrum designed the research, analyzed and interpreted the data, reviewed the manuscript draft, and supervised the entire process.

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