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Research Article

Effect of Ethanol Extract and Ethyl Acetate Fraction of Soursop Leaves administration on Malondialdehyde Levels of Alloxan-Induced Rat Endocrine Cells

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ABSTRACT

Soursop leaves (*Annona muricata* L.) contain many antioxidant compounds such as phenols and flavonoids that play an active role in inhibiting oxidative stress and can inhibit damage and death in cells, tissues, or organs. The objective of this study was to determine the effect of soursop leaf ethanol extract and ethyl acetate fraction effect on malondialdehyde levels and the quantity of pancreatic endocrine cells in male rats (*Mus musculus*) induced with alloxan. Method, the test animals were divided into seven groups (n=4). The normal control group was only given feed, the negative control group was given CMC-Na solution, the positive control group was given vitamin C dose of 50 mg/kg.BW, treatment group I was given soursop ethanol extract dose of 50 mg/kg.BW, treatment group II was given soursop ethanol extract dose of 100 mg./kg.BW, ethyl acetate fraction treatment group III was given soursop ethyl acetate fraction dose of 25 mg/kg BW, and treatment group IV was given soursop ethyl acetate fraction dose of 50 mg/kg BW. Results showed that the administration of ethyl acetate fraction of soursop leaves at a dose of 25 mg/kg BW showed the most effective potential significantly ($p<0.05$) in reducing malondialdehyde levels and cell death. In conclusion, administering ethanol extract and ethyl acetate fraction of soursop leaves to rats which experiencing oxidative stress in their pancreas can stimulate and increase regeneration of endocrine cell in all rat groups. This is proven by a decrease of malondialdehyde levels and the number of necrosis cell necrosis.

Keywords: *Annona muricata* L.; alloxan; malondialdehyde; pancreatic endocrine cells; reactive oxygen species

INTRODUCTION

The presence of reactive oxygen species (ROS) in the body is necessary to maintain physiological functions such as redox homeostasis and has an important role in proliferation, differentiation, migration, apoptosis, and necrosis in cells. However, excessive formation of ROS and the absence of balance between reactive ROS and antioxidants can disrupt redox homeostasis in the body which can cause oxidative stress (Vona et al., 2021). Alloxan is a toxic compound that has a highly reactive 5-carbonyl group on the thiol group. Intracellular thiol, specially from glutathione (GSH), leads to cyclic events in alloxan that generate ROS like hydroxyl radical (OH) and superoxide radical anion (O2) (Ighodaro et al., 2017).

The level of oxidative stress in individuals can be measured through the measurement of malondialdehyde (MDA) levels in plasma (Setiowati et al., 2018). MDA is the product of lipid peroxide radicals that have toxic properties to living cells. The

higher levels of malondialdehyde in plasma indicates the higher the oxidative stress that occurs in living cells (Prawitasari, 2019). The body has created a complex antioxidant system defense system to balance the level of oxidative stress in the body. The antioxidant system is formed by enzymatic antioxidants such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxide and thyroxine (Trx) (Jomova et al., 2023).

Soursop plants belong to the Annonaceae family which contains bioactive compounds such as acetogenins, phenol alkaloids, and flavonoids that have pharmacological activities in various diseases and metabolite disorders and natural antioxidant (Coria et al., 2018). Flavonoids are secondary metabolites which act as an organic antioxidant, found abundantly in Annona muricata leaves. Extracting flavonoids by using an appropriate solvent is crucial to obtaining the best target compounds. Flavonoid components in soursop leaf extract are effectively extracted using ethanol as solvent in the extraction procedure. Ethanol is a neutral, non-toxic solvent that works well at sucking to attract polar molecules, particularly flavonoids (Rustanti & Fatmawati, 2020). In addition, the use of ethyl acetate in the further separation process is also effective since the ethyl acetate is a semi-polar solvent with low toxicity, thus it is easier to attract and separate semi-polar compounds such as flavonoid aglycones (Rizky et al., 2023).

The antioxidant activity of the ethyl acetate, butanol, and water fractions of soursop leaves show good activity with IC50 values of 0.02± 0.55, 0.021± 0.01, and 9.28 ± 0.28 µg/mL, respectively, but the butanol fraction shows the best antioxidant activity to reduce MDA levels (Sanni, et al., 2020). Other study confirmed that the antioxidant activity of soursop leaf ethanol extract showed an inhibitory value of 23.61±0.42 mmol TE/g, while soursop leaf water extract showed a lower antioxidant capacity with an inhibitory value of 2.97±0.40 mmol TE/g (Carmona et al., 2020). The differences in antioxidant activity of these extracts can be influenced by the physical and chemical properties of the solvent used. Non-polar solvents will show better capacity in dissolving bioactive compounds as antioxidants (Carmona et al., 2020).

This study discusses the effect of giving ethanol extract and ethyl acetate fraction of soursop leaves on malondialdehyde levels and endocrine cells in male rats. The effect of giving ethanol extracts and ethyl acetate fractions on endocrine cells histopathologically in test animals has not been tested. The purpose of this study was to determine the effect and effectiveness of ethanol extract and ethyl acetate fraction of soursop leaves on malondialdehyde levels and pancreatic endocrine cells in male rats (Mus musculus) given alloxan.

METHODS

Tools and Materials

The tools used in this study are analytical scales, glass containers (glass jars), rotary evaporators, separating funnels, spatulas, oral syringes, injection syringes, surgical scissors, small tissue bottles, label paper, test animal cages, microscopes with digital cameras (Olympus Optilab CZ 23), rotary microtome.The materials used in this research include dried soursop leaves (Annona muricata L.), distilled water,70% ethanol, n-hexane (technical), ethyl acetate (technical), cotton, alcohol, CMC Na, n-butanol, sodium sulfate (Na_2SO_4), normal saline, 10% formalin, normal buffer, xylol, liquid paraffin, alloxan, material for staining pancreatic tissue (Haematoxylin-Eosin), and test animal feed. Alloxan is used for modeling the incidence of oxidative stress in test animals.

Procedures

The design used in this study was a laboratory experiment. The Health Research Ethics Committee of the Faculty of Medicine, Muhammadiyah University Surakarta, gave its approval for the use of experimental animals in this study with No.4899/A.1/KEPK-FKUMS/VI/2023. The animal test used were 28 male Wistar rats aged 2 months. All groups except the normal control group were induced alloxan dose of 150 mg/kg BW intraperitoneally. Test animals were divided into seven groups (n = 4). The extract and fraction were administered orally for 21 days. Blood samples were taken on days 0, 7, 14, and 21 through the tail vein.

The normal control group was given feed only, negative control group was given CMC- Na solution. Positive control group was administered vitamin C dose of 50 mg/kg BW. Group I was given soursop leaf ethanol extract dose of 50 mg/kg BW. Group II was given soursop leaf ethanol extract dose of 100 mg/kg BW. Group III was given soursop leaf ethylacetate fraction dose of 25 mg/kg BW and group IV was given soursop leaf ethylacetate fraction 50 mg/kg BW.

MDA Analysis

Plasma that had been collected in a vial containing EDTA solution was taken as much as 200 µL and put into a centrifugation tube and added 1 mL of 10% trichloroacetate and 2 mL of thiobarbituric acid (TBA). This solution was then homogenized, boiled for 10 minutes in a water bath, rapidly cooled in an ice bath to halt the reaction, extracted by adding up to 4 mL of n-butanol, then centrifuged once more for 10 minutes at 3000 rpm. Visible spectrophotometry was used to determine the absobance of the pink filtrate obtaines from centrifugation at a wavelength of 532 nm (Wulandari et al., 2020).

Panreatic Histopathology test

Tissue samples were fixed with 10% phosphate-buffered formalin (BNF) solution at room temperature for 24 hours. Sections were taken ± 3 mm thick for histopathological examination with Hematoxylin-Eosin staining. All sections were examined under a light microscope at 400 x magnification. Evaluation of histopathological changes observed included degeneration and necrosis of cells. Observation of the preparations was done by observing the entire field of view. Calculation of the number of cells was determined by the number of cells that reacted positively and had clear cell nuclei at low magnification (Nuralifah et al., 2022).

Data Analysis

Variables in the form of data obtained from the measurement of MDA levels and the results of cell count analysis were statistically analyzed using IBM SPSS Statistic 26 software with the One-way ANOVA test. If the analysis results showed a significant difference ($p<0.05$), the analysis was continued with the LSD test with confidence level ($p<0.05$).

RESULTS AND DISCUSSION

Malondialdehyde (MDA) Levels

Analysis of MDA levels in alloxan-induced rat blood samples is used to analyze the role of oxidative stress in organ morphology and physiology disorders in the pancreas.The analytical method used to analyze MDA levels using TBA which is able to detect MDA through spectophotometric analysis. The results of MDA levels in the alloxan-induced rat group after the administration of ethanol extract and ethylacetate fraction of soursop leaves for 21 days showed significant differences with the group without administration of the test sample (Figure 1).

The results of the analysis of MDA levels in each group induced by alloxan as a free radical are shown in Figure 1, where the MDA levels in groups of rats induced by alloxan and groups which given ethanol extract of soursop leaves (KP1 and KP2) and ethyl acetate fraction of soursop leaves (KP3 and KP4) showed a significant difference ($p<0.05$) with the negative control group. Administration of ethanol extracts and ethyl acetate fractions of soursop leaves was able to reduce MDA levels in rats were previously given radical compound (alloxan) through a neutralization process by donating one electron of the antioxidant compound in the test sample to the free radical compound so that it becomes a stable compound (Hairunisa et al., 2021).

Mechanism of MDA formation begins with the occurrence of lipid peroxidation reactions, especially in unsaturated fatty acids containing many double bonds (C-C), which are biomolecule that play a role in oxidative stress reactions in the body (Tuo et al., 2023). In this process, oxidants work by taking oxygen which is able to form unstable lipid radicals. The binding process of oxygen will cause the formation of lipid peroxyl radicals. The lipid peroxyl will rebind hydrogen from other lipid molecules to form stable lipid peroxides so that it will form secondary products, one of which is MDA (Ayala et al., 2014). In addition, the lipid peroxidation reactions also remove hydrogen atoms (H) from long-chain unsaturated lipid molecules by hydroxyl radical groups (*OH), so that lipids become a radical. Then the lipid reacts with an oxygen atom (O2) to form a peroxyl radical (*OO), which then produces MDA with more than three unsaturated bonds (Martemucci et al., 2022).

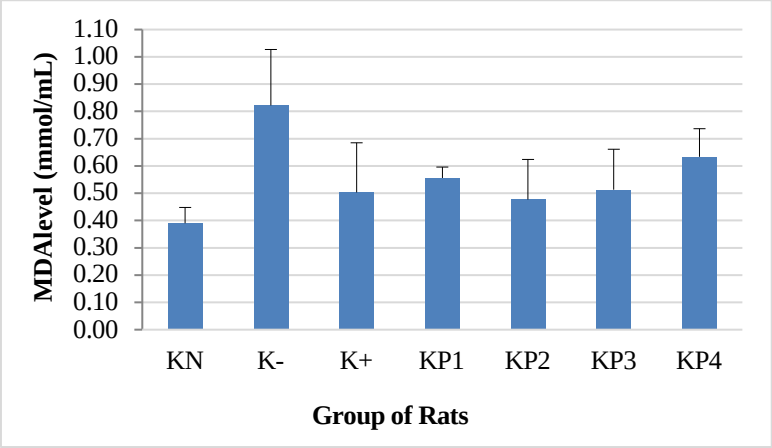

Figure 1. MDA levels of rat groups after 21days of treatment (n=4).

Note:Normal control (KN) (feed only), negative control (K-) (CMC Na), positive control (K+) (vitamin C), soursop leaf ethanol extract treatment group 50 mg/kg BW (KP1), soursop leaf ethanol extract treatment group 100 mg/kg BW (KP2), soursop leaf ethyl acetate fraction treatment group 25 mg/kg BW (KP3), soursop leaf ethyl acetate fraction treatment group 50 mg/kg BW (KP4). Data are expressed as mean and standard deviation (SD). *Significantly different from the negative control ($p<0.05$)

MDA is one of the most widely observed major compounds due to its active role in causing mutagenic and toxic effects. In addition, MDA can synthesize thromboxane A2 biosynthesis through enzymatic reaction. In general, lipids found in cell membranes will produce the enzyme phospholipase A2 (PLA2) which will release arachidonic acid through an enzymatic process. Arachidonic acid (AA) is an unsaturated fatty acid that plays an important role in carrying signals in cell function and plays a role in regulating the body's biological functions. When the body is exposed to free radicals and then experiences oxidative stress, the ROS formed, especially hydroxyl radicals (-OH), will attack arachidonic acid through a non-enzymatic process and will produce lipid endoperoxides. The lipid endoperoxide will spontaneously break down and will release MDA into the extracellular space and eventually into the blood circulation (Placin et al., 2023).

Pancreatic Histopathology Test

Animal models created under oxidative stress conditions can show physiological changes in tissues and cells. In eukaryotic cells, mitochondria are the main source in regulating the production of ROS in the cell. Several mechanisms indicate that the induction of toxic compounds such as alloxan can cause an increase in ROS and produce reactive nitrogen species (RNS) in the body. The presence of active nitrogen species can trigger damage to cells, tissues, and organs which can ultimately trigger various chronic diseases as the development of inflammatory diseases (Cristani et al., 2016).

Determination of MDA levels in rats experiencing oxidative stress is one indicator of the pathological condition of the rat pancreas by observing the histopathology of rat pancreas using the Hematoxylin-Eosin staining method which aims to facilitate observation of tissue under a microscope (Ozturk et al., 2015). In principle, staining of the cell nucleus found in acidic tissue will attract substances that have alkaline properties, so that it will produce a blue color when viewed under a microscope. The alkaline cytoplasm in cells when attracting acidic substance will produce a red (pink) color when viewed under a microscope (Nuralifah et al., 2022). Histopathology observation of the pancreas in this study used a microscope with a magnification of 400x. The group of test animals that experienced oxidative stress due to alloxan administration had a histopathological picture that was different from the normal group (Figure 1).

The data presented in Table 1 shows a significant difference ($p<0.05$) between the MDA levels of the four treatment groups (KP1, KP2, KP3, and KP4) with negative control. MDA levels in the negative control showed the highest MDA levels of all treatment groups. This is because the negative control group is only given CMC-Na, so it does not affect MDA levels in rats.

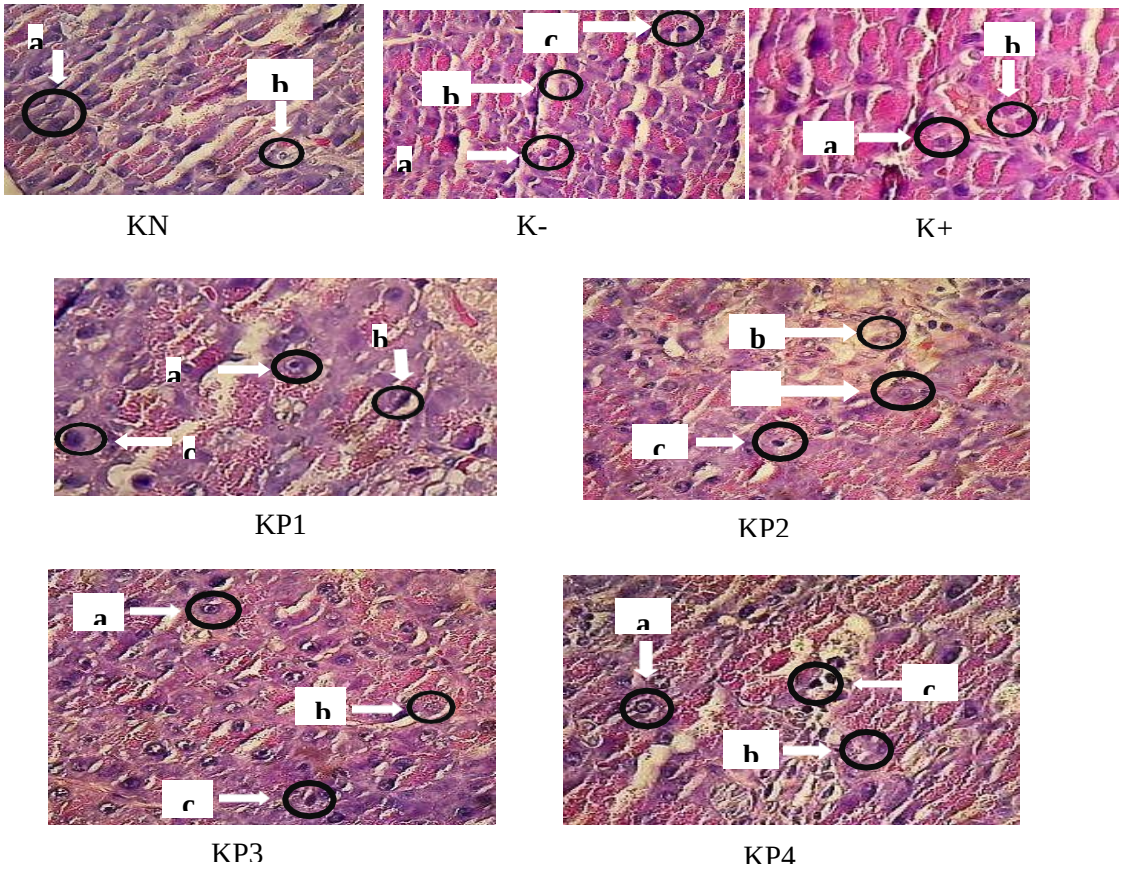

Figure 2. Histopathologic features of rat groups after 21 days treatment (n=4).

Note: KN (normal control), K- (negative control), K+ (positive control), KP1(ethanol extract dose of 50 mg/KgBW), KP2 (ethanol extract dose of 100 mg/kg BW), KP3 (ethylacetate fraction dose of 25 mg/kgBW), KP4 (ethyl acetate fraction dose of 50 mg/kg BW). Normal cells (a) look round and have a cell nucleus in the center, degenerated cells (b) look like vacuoles of varying sizes, and necrotizing cells (c) look dark in color with loss of cell nucleus.

Based on the results of histopathologycal examination of the pancreas (Figure 2.) in the normal control (KN) group, intact and dense endocrine cells and the number of living cells were clearly visible. In the normal control, there was no addition of toxic substances into the rat's body so that there was no necrosis in the cells. The positive control (K+) group showed dense and intact endocrine cells as in the normal control. This is due to the administration of vitamin C as an strong antioxidant in inhibiting and repairing cells continuously, so that the endocrine cells did not show any necrotic cells. In contrast, the negative control (K-) showed that the most cells in the islets of Langerhans experienced cell degeneration followed by necrosis. In the ethanol extract treatment group with a dose of 50 mg/kg BW and a dose of 100 mg/kg BW showed less cell degeneration and necrosis compared to the negative control group and had the same antioxidant potential as the positive control in inhibiting cell necrosis (Table 1). While in the ethyl acetate fraction treatment group with a dose of 25 mg/kg BW, the cells that experienced degeneration and necrosis were fewer compared to the negative control, but showed antioxidant activity like in the positive control. In the ethyl acetate fraction treatment group with a dose of 50 mg/kg BW, fewer cells experienced degeneration and necrosis to the negative control.

Table 1 Data analysis of Islets of Langerhans after 21days of treatment (n=4)

No	Trial Group	Observation Results	
		Degeneration	Necrosis
1	Normal control	89 ± 4.4	14[b] ± 1.3
2	Negative control	83 ± 13.1	101[a] ±16.2
3	Positive control	12 ± 3.3	0[b] ± 0.0
4	Soursop ethanol extract 50 mg/kg BW	200 ± 15.7	38[b] ± 2.5
5	Soursop ethanol extract 1000 mg/kg BW	167 ± 14.2	27[b] ± 3.3
6	Soursop ethylacetate fraction 25 mg/kg BW	102 ± 6.8	13[b] ± 1.9
7	Soursop ethylacetate fraction 50 mg/kg BW	84 ± 0.4	32[b] ± 0.9

Degeneration in the form of cell damage is reversible, necrosis is cell death characterized by loss of cell nucleus and is irreversible (Nuralifah et al., 2022).
[a]significantly different from normal control ($p<0.05$)
[b]significantly different from the negative control ($p<0.05$)

Table 1 explains the improvement of the Islets of Langerhans in each group against the histopathological picture can be seen from the number of cells that experience necrosis and degeneration. Cell degeneration is characterized by abnormalities that occur in endocrine cells that attract structures in cells that can result in a reduction in the number of cells and the arrangement of endocrine cells is irregular, becomes smaller and even destroyed and disappears. Degeneration is reversible and adaptive, where the damage done to the cell usually disappears in a short time according to the reduction of toxic substances (Mumtazah et al., 2021).

Necrosis is cell death characterized by the presence of empty spaces in the islets of Langerhans due to the loss of cell nuclei (Nuralifah et al., 2022). The mechanism of necrosis in cells occurs due to exposure to toxic compounds (alloxan) which can cause damage to cell membranes. Membrane damage is initially characterized by loss of membrane integrity, degradation of phospholipid arrangement, cytoskeleton damage, and the formation of reactive oxygen species (Caronni et al., 2023). The formation of reactive oxygen species results in the death of cells involved in inflammation (necroinflammation) (Maremonti et al., 2022).

The induction of alloxan as a stimulating agent that can damage cells in the tissue will previously cause apoptosis in cells which will then eventually cause permanent cell death (necrosis). The induction of alloxan will disrupt endogenous homeostasis in the cell it will result in cell shrinkage due to the stress on the outside and inside of the cell being different (Ighodaro et al., 2017). In addition, the imbalance of ATP in intracellular due to the induction of alloxan as a toxic compound will. Stimulate necrosis in cells, because in principle the high level of energy generated but not needed in the cell will cause apoptosis and will continuously eliminate the cell nucleus and cause cell necrosis (Peng et al., 2022).

The average group of test animals given ethanol extract and ethyl acetate fraction of soursop leaves showed good effectiveness in reducing the number of cell necrosis. Langerhans island repair is influenced by the content of secondary metabolites present in ethanol extracts and ethyl acetate fractions of soursop leaves in the form of phenolic compounds and flavonoids. Flavonoids are antioxidant compounds that have been believed to protect the body from damage caused by ROS and a reprotective against damage to pancreatic β cells (Nuralifah et al., 2022).

In previous studies, the content of antioxidant compounds, namely rutin from soursop leaf water extract, was able to repair and inhibit damage or cell death (necrosis) in rat heart tissue due to the presence of ROS (Haq, et al., 2022). In addition, the antioxidant compound content of the aqueous extract and ethanol extract of soursop leaves tested by DPPH and ABTS methods showed values of 23.61±0.42 and 24.91± 0.61 mmol equivalent to per gram of Trolox which has a positive effect in reducing oxidative stress in human erythrocytes and viral infections (Carmona et al., 2020). Trolox is an antioxidant analog of tocopherol. Ascorbic acid (vitamin C) and trolox have the same antioxidant mechanism by donating one electron to reactive molecules (Wulansari, 2018). So with this, antioxidant compounds contained in ethanol extracts and ethylacetate fractions of soursop leaves are ableto show their antioxidant activity through inhibition of oxidative stress as measured by looking at MDA levels and analyzing the number of cells that experience necrosis in alloxan-induced rats.

CONCLUSION

Ethanol extract and ethyl acetate fraction of soursop leaves (*Annona muricata* L.) can reduce oxidative MDA levels and can repair endocrine cells in the pancreas of rats that experience oxidative stress due to alloxan induction. This occurs due to the presence of flavonoids as antioxidant compounds in ethanol extract and ethyl acetate fraction of soursop leaves.

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CONFLICT OF INTEREST

This research has no affiliation or involvement in any organization or entity with any interest such as honoraria, educational grants, memberships, or other equity interests.

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