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Expression of Caspase 3 in Rat Liver Cells by Maximum Physical Exercise After Administration of Gagatan Harimau Nanoherbal (*Paraboea leuserensis* B.L.Burtt)

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Abstract

This research aims to analyse Expression of Caspase 3 in Rat Liver Cells With Maximum Physical Exercise After Administration of Gagatan Harimau Nanoherbal (Paraboea leuserensis B.L.Burtt). The research was conducted in April-June 2023. The research location was the Animal Physiology Laboratory and Experimental Animal House, Department of Biology, FMIPA USU, PT. Synergy of Indonesian Nanotech, South Tangerang, Banten and the Anatomic Pathology Laboratory, Faculty of Medicine, University of North Sumatra, Medan. There were 6 treatments in the study, namely control group (K-), maximum physical exercise (K+), maximum physical exercise + Vitamin C at a dose of 2 mg/Kg Body Weight (P1), maximum physical exercise + Gagatan Harimau nanoherbal 100 mg/Kg BW (P2), Maximum physical exercise + Gagatan Harimau nanoherbal dose 125 mg/Kg BW (P3), Maximum physical exercise + Gagatan Harimau nanoherbal dose 150 mg/Kg BW (P4). The data obtained from each observation parameter (variable) is recorded and presented in table form. The data was then analyzed using SPSS version 25 software with significant differences determined at $p<0.05$. The results of the study showed significant differences between all groups ($p<0.05$) in the expression of caspase 3 in the liver. A dose of 150 mg/Kg BW from the nanoherbal Gagatan Harimau leaves (Paraboea leuserensis B.L.Burtt) reduced the expression of caspase 3 in rat liver. The induction of gagatan harimau leaf nanoherbal to act as an antioxidant in male rats given swimming physical training showed improvements in liver histology and morphology. The dose of 150 mg/KgBW of nanoherbal gagatan harimau leaves was the best dose because it showed cell regeneration that was close to the normal treatment group.

Keywords : Caspase 3; Physical training; Nanoherbal, Gagatan Harimau

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1. Introduction

Indonesia is mostly rich in various traditional medicinal plants which can be used to treat various diseases [1]. Traditional medicines can be obtained from several plants in Indonesia, either passed down from generation to generation or through compounds. Gagatan Harimau species are part of the Gesnericeae family and belong to the *Paraboea* genus [2]. Traditionally, gagatan harimau leaves are known by the people of Tanah Karo as a medicine for stomach aches, an energy enhancer, a medicine for diarrhea and a stamina enhancer. Based on research [1], Gagatan Harimau leaves have been proven to be used as a stamina enhancer by testing the length of swimming in mice. Consuming herbal plants can be done to increase stamina. The use of herbs for consumption so far mostly comes from plant extracts.

Extract preparations have weaknesses, namely their solubility in water is low so their bioavailability is low and their functional properties can decrease due to the processing process and long storage time [3]. This can be overcome by increasing solubility and maintaining the

functional properties of the extract by formulating the extract in nanoparticle preparations [4]. Nanoparticles from herbs are called nanoherbal [3]. Systematic sports activities carried out repeatedly over a long period accompanied by a gradual and continuous increase in load according to each in individual's abilities is the definition of physical training [5]. Excessive physical activity will increase reactive oxygen species (ROS) in tissues and 2-5% of oxygen will be used. A high increase in oxygen consumption will result in increased production of free radicals and can cause cell damage. Oxidative stress is a condition where there is an imbalance in the amount of oxidants (free radicals) with the amount of antioxidants in the body, causing damage to cells such as liver and muscle cells [6]. Repair of liver cells and muscle cells can be seen by observing caspase 3 expression.

Caspases are a group of cysteine protease enzymes that play an important role in regulating and executing apoptotic cell death. Apoptosis is a programmed cell death mechanism and is used to get rid of cells that are no longer needed by the body. When cells lose their apoptotic ability or their apoptotic ability is inhibited by a virus, the cells grow uncontrollably and become cancerous [7]; [8]. Upstream caspases (2, 8, 9 and 10) are included in the Caspase domain which functions as an initiator (trigger) of apoptosis and the small domain or downstream Caspases (caspases 3, 6 and 7) function as effectors or executors of apoptosis and are the part involved direct cell destruction. Based on research [9] that the anticancer agent brusein-A increases caspase-3 expression in mouse breast cancer induced by dimethylbenzanthracene (DMBA).

The liver is an important organ in the body which functions to detoxify substances that enter the body. Clinically, liver damage in experimental animals can be examined by testing liver histology preparations which are characterized by degeneration and necrosis [10]. In order to prevent damage to liver cells, efforts are needed to provide external antioxidant intake. One external intake that has high antioxidant content is Gagatan Harimau [11]. Gagatan Harimau has the potential as a source of anti oxidants [12]. Based on this description, it can be concluded that research is needed to analyze the expression of caspase 3 in rat liver cells with maximum physical exercise after administering Nanoherbal Gagatan Harimau (*Paraboea leuserensis* B.L. Burtt) as an antioxidant and preventing the formation of free radicals in the body.

2. Materials and Methods

Research sites.

The research project was carried out from April to June 2023 at the Animal Physiology Laboratory and Experimental Animal House, Department of Biology, FMIPA USU, PT. Synergy of Indonesian Nanotech, South Tangerang, Banten and the Anatomic Pathology Laboratory, Faculty of Medicine, University of North Sumatra, Medan.

Preparation of nanoherbal Gagatan Harimau (*Paraboea leuserensis* B.L.Burtt).

Nanoherbal from Gagatan Harimau (*Paraboea leuserensis* BL. Burtt) collected from the University of North Sumatra.Gagatan Harimau (*Paraboea leuserensis* B.L Burtt) leaf samples used in this research came from Timbang Lawan Village, Pancur Batu District, Deli Serdang Regency, North Sumatra Province. The sample was then cleaned and air dried. After drying, the sample was cut into small pieces and then blended. Samples were sent to PT. Indonesian Nanotech Synergy to obtain nanoherbal.

Animal handling.

This study used 24 male mice and were divided into control and treatment groups. All mice were kept in groups in clean cages with light, humidity and given food and drink ad libitum for 2 weeks in the Animal House, Biology Laboratory, University of North Sumatra (USU).

Study Design.

This research included 6 treatments. Group K (-) is the control group, Maximum physical exercise (K+), Maximum Physical Exercise + Vitamin C at a dose of 2 mg/Kg BW (P1), Maximum physical exercise + Gagatan Harimau nanoherbal 100 mg/Kg BW(P2), Exercise maximum physical exercise + nanoherbal Gagatan Harimau dose of 125 mg/Kg BW (P3), Maximum physical exercise + nanoherbal Gagatan Harimau dose of 150 mg/Kg BW (P4) treatment group who swam in the morning between 08.00 – 09.00 for 20 minutes a day 4 times in 20 days. After 20 days, the mice were then dissect ed. The rat's neck was dislocated. and underwent surgeryin the liver. Paraffin blocks were performed for immunohistochemical staining of caspase 3 expression.

Immunohistochemical staining of caspase 3 expression.

Liver tissue preparations were stained with Hematoxylin–Eosin (HE). Liver tissue in paraffin blocks was cut using a microtome and mounted on polylysinecoated glass slides and dried at room temperature. Deparaffinization using xylol 1 and 2 for 4 minutes each. The rehydration stages were carried out using 100% alcohol (2x2 minutes), 95% (2 minutes), 90% (2 minutes), 80% (2 minutes), 70% (2 minutes) and cleaned with running water. The liver was stained immunohistochemically. Tissues were incubated with H2O2 in methanol for 15 min to remove endogenous peroxidase activity. Next, the tissue was incubated in 10% bovine serum albumin (BSA) for 45 minutes in an incubator at 37°C. After washing 3 times with PBS, the

tissue was then incubated in monoclonal caspase-3 primary antibody at room temperature for 1 hour. To bind secondary antibodies, the tissue was then incubated with biotinylated IgG for 30 minutes at room temperature. After washing 3 times with PBS, the tissue was incubated with avidin biotin HRP for 30 minutes. The tissue was washed again with PBS 3 times. After being washed 3 times with PBS, the results of the antigen-antibody reaction were visualized using diamino benzidine (DAB) at room temperature for 10 minutes. After being washed 3 times with PBS, then counterstained with hematoxylin until brown chromogenic bonds were formed, followed by the dehydration and clearing process and mounting which is dripped with Canada balsam on the tissue and then covered with a cover glass. In the final stage, the preparations are labeled and explained, then observed under a microscope with 400 times magnification.

Data analysis.

The research data used SPSS version 25 software using the ANOVA test which was significant at $p<0.05$, the Kruskal-Wallis test then continued with the Mann-Whitney test.

3. Results and Discussion

HE (Haematoxylin-Eosin) staining.

1. Histology of rat liver with HE (Haematoxylin-Eosin) staining.

The results of observations made after administering the nanoherbal Gagatan Harimau leaves (*Paraboea leuserensis* BLBurtt) on the liver histology of mice given maximum physical exercise (Figure 1.) Figure 1 is a histological picture of the liver of mice with HE staining at 40x10 magnification after administering the nanoherbal gagatan harimau leaves. K(-): Normal Rats, K(+): Rats that were given swimming training, P1: Rats that were given swimming training by administering Vit. C, P2: Rats were given swimming training by administering 100 mg/KgBW of gagatan harimau leaf nanoherbal, P3: Rats were given swimming training by administering 125 mg/KgBW of gagatan harimau leaf nanoherbal, P4: Rats were given swimming training by administering gagatan harimau leaf nanoherbal 150 mg/KgBW. Description: a. Central vein, b. Normal hepatocyte cells, c. Parenchymatous degeneration, d. Hydropic degeneration, e. Necrosis.

Based on Figure 1, the results of histological observations of hepatocytes in the livers of mice given the nanoherbal gagatan harimau leaves revealed cell changes in the form of parenchymal degeneration, hydropic degeneration and necrosis in each group. In normal K(-) treatment there were central veins, normal hepatocyte cells and cell changes were found including parenchymal degeneration, hydropic degeneration and necrosis. This can occur due to natural physiological conditions that can cause damage to hepatocytes. In the groups treated with K(+), P1, P2, and P3 who were given nanoherbal gagatan harimau leaves after maximum physical exercise by swimming, there was damage to hepatocytes due to treatment from maximum physical exercise which caused oxidative stress. Excessive physical activity will result in necrosis in liver cells due to the continuous metabolic process of hepatocytes [13]. Necrosis can occur due to several things, namely a decrease in blood supply to the liver, the presence of toxic compounds, mechanical trauma, the effects of exposure to radioactive rays, and temperature [14]. This statement is in accordance with the research results, the highest damage to hepatocytes in the form of necrosis occurred in the K(+) treatment group where the hepatocyte cells looked damaged, the cell nuclei and cell parts looked unclear. According to [15] Necrosis is often found around the central veins (centrilobular necrosis) which is caused by the large amount of toxic substances in the blood that empty into the central veins. Mild degeneration of liver cells has the ability to regenerate well. Cells in mild degeneration are stable and divide very slowly, however, if severe damage occurs, the liver structure cannot be reshaped to normal conditions, resulting in the liver not being able to work normally and showing abnormalities in its function [16]. In the treatment group P4 nanoherbal gagatan harimau leaves 150 mg/kgBW showed an improvement in the histological structure of the liver as seen from a decrease in the amount of degeneration and necrosis when compared to other treatment groups such as P1, P2 and P3.

This shows that gagatan harimau contains phenylethanoid and triterpenoid glycosides which are widely distributed in the genus *Paraboea*. *P. martinii* [17] has resulted in the isolation and characterization of various secondary metabolites, including phenylethanoid glycosides, triterpenoids, steroids, and organic acids. Gagatan harimau leaves can protect hepatocytes from damage caused by excessive physical activity because they have a natural source of antioxidants produced by secondary metabolites of gagatan harimau [12] [18].

2. Hepatocyte damage.

The results of observing the histology of hepatocytes in the livers of rats given the nanoherbal gagatan harimau leaves after swimming physical exercise for 20 minutes showed that there was a significant difference between the control group and the treatment group, $p<0.05$. The amount of reduction in hepatocyte damage in the P4 group of gagatan harimau leaf nanoherbal with a dose of 150mg/kgBW tended to increase better compared to the P1, P2 and P3 treatment groups, this is because the 150mg/kgBW dose of gagatan harimau leaf nanoherbal is the right dose and contains secondary

metabolite compounds. such as flavonoids and saponins which are very good at repairing hepatocyte damage. The results of observations of hepatocyte damage can be seen in Table 1.

Table 1. Number of Changes in Hepatocyte Cells in Rats Given Maximum Physical Exercise by Giving Gagatan Harimau Leaf Nanoherbal.

No	Treatment	Normal Hepatocytes	Parenchymal Degeneration	Degeneration Hydropic	Necrosis
1	K(-)	7.54 ± 0.82^{d}	6.50 ±1.44^{b}	5.28 ± 1.37^{a}	0.68 ± 0.72^{a}
2	K(+)	0.21 ± 0.43^{a}	4.51±1.80^{a}	5.64 ± 1.91^{a}	9.64 ±1.37^{d}
3	P1	1.03 ± 0.78^{ab}	4.95±1.84^{a}	7.33 ± 1.92^{c}	6.54 ± 1.97^{c}
4	P2	1.74 ± 0.97^{b}	4.90 ± 1.52^{a}	6.82 ± 1.77^{bc}	6.58 ± 1,70^{c}
5	P3	2.85 ± 0.99^{c}	5.08±1.52^{a}	6.45 ± 1.57^{b}	5.61 ±1.46^{b}
6	P4	7.59 ± 2.03^{d}	6.73±1.54^{b}	5.26 ± 1.43^{a}	0.44 ± 0.59^{a}

Information: Different letter notations in the same column for each damage indicate significant differences in each group.

The control (-) group of mice showed little damage to liver cells although necrosis also occurred in the control group, this is not considered a pathological event because necrosis can also occur under normal circumstances [19]. The process of aging and physiological cell death is experienced by all normal cells. Every cell in the body experiences aging and ends with cell death, but the cells will be replaced with new cells through the degeneration process. According to [20], flavonoid compounds are able to protect liver cells from oxidative damage from oxidative stress which causes cell damage and liver cell death. The mechanism of flavonoids as antioxidants is to capture free radicals directly. Flavonoid compounds that are oxidized by free radicals then become more stable and the nature of the previous free radicals becomes less reactive. This shows that flavonoids are able to stabilize reactive oxygen by reacting with free radical compounds.

The results of hepatocyte damage in the positive control (K+) treatment tended to be higher than other treatment groups. This is because mice that are given maximum physical exercise have a significant effect on hepatocyte cell damage. Damage to the liver that can be observed microscopically is degeneration and necrosis [21]. Degeneration is a change in cell morphology that is reversible because if the cause of the damage can be stopped, the cells will return to normal. Necrosis is cell death that cannot be recovered due to continuous damage. According to [22], Parenchymal degeneration is the lightest form of degeneration and is reversible (can be recovered) with a microscopic appearance in the form of cell swelling accompanied by turbidity in the cytoplasm. Hydropic degeneration is a continuation of parenchymatous degeneration with a microscopic appearance in the form of small vacuoles in the cytoplasm and is reversible. Necrosis is irreversible cell death (cannot be recovered) with a microscopic appearance in the form of small dark colored cell nuclei (pyknotic), karyorrhexis in the form of fragmentation of the cell nucleus into several parts and cell nuclei disappearing (karyolosis) [23].

3. Caspase 3 IHC (Immunohistochemistry) staining.

Figure 2: Overview of the results of caspase 3 immunohistochemical staining at 40x10 magnification. Description: Red arrow soil: +caspase 3.

In Figure 2, the caspase-3 protein is expressed very clearly in the K (+) treatment after maximum physical exercise. The K(+) treatment showed increased caspase expression and the expression of caspase-3 in the cell cytoplasm was quite strong as indicated by a clear brown color in the cell cytoplasm compared to the K(-), P1, P2, P3 and P4 treatments. So it is very likely that an apoptosis process (programmed cell death) will occur in liver cells induced by gagatan harimau nanoherbal. All apoptotic pathways, both intrinsic and extrinsic, go through caspase 3 which is the executioner caspase. Therefore, the presence of caspase 3 is necessary for apoptosis to occur. So, if the expression of caspase 3 is positive then the possibility of apoptosis will be greater and in this study this happened [7] ; [8]. The P4 treatment that can most reduce the activity of caspase 3 is characterized by a less obvious brown color in the cell cytoplasm.

Administration of nanoherbal gagatan harimau leaves at the highest dose of 150 mg/KgBW can improve liver histopathology given maximum physical exercise by swimming in mice because gagatan harimau leaves contain high antioxidants, secondary metabolites of alkaloids, flavonoids, saponins, tannins, especially phenylethanoid glycosides and have been proven as agents. curing degenerative diseases [12] ; [18] .

Based on these data, the expression of caspase 3 showed a significant difference in the liver after

administration of harimau gagatan nanoherbal. Box plots showed significant differences ($p<0.05$) by Kruskal-Wallis test and Mann-Whitney follow-up test between each treatment (Table 2 and Figure 3).

Table 2: Kruskal-Wallis and Mann-Whitney analysis of Caspase 3 expression in the liver.

G	Mean Rank	Kruskal-Wallis	Mann-Whitney					
			K-	K+	P1	P2	P3	P4
K-	48.75	0,000*		0,000*	0.375	0.034	0.423	0.055
K+	99.30				0,000*	0,000*	0,000*	0,000*
P1	55.63					0.182	0.806	0.044
P2	68.13						0.124	0.001*
P3	57.78							0.003*
P4	33.43							

Description: (*$p<0.05$), G : Groups

Figure 3 shows a box plot of caspase expression data in the liver when given gagatan harimau leaves, K-: Control, K+: Rats given swimming physical training, P1: Rats given swimming training with Vit. C, P2: Rats that were given swimming training by administering 100 mg/KgBW of gagatan harimau leaf nanoherbal. 150 mg/KgBW.

4. Conclusion

Maximum physical exercise by swimming can cause oxidative stress which causes microscopic damage characterized by damage to liver hepatocytes in the form of parenchymal degeneration, hydropic degeneration and necrosis. there was a significant difference between all groups ($p<0.05$) in the expression of caspase 3 in the liver. a dose of 150 Mg/ KgBW of nanoherbal Gagatan Harimau leaves (*Paraboea leuserensis* B.L.Burtt) Reduced The Expression Of Caspase 3 In Rat Liver. The Dose Of 150 Mg/ KgBW Of Nanoherbal Gagatan Harimau Leaves Was The Best Dose Because It Showed Cell Regeneration That Was Close To The Normal Treatment Group.

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