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Caspase 3 and Caspase 10 Expression in Rat Muscles Post Gagatan Harimau (*Paraboea leuserensis* B.L.Burtt) Nanoherbal and Exercise

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Abstract: This study aims to analyze the Expression of Caspase 3 and Caspase 10 in Rat Muscles Post Gagatan Harimau (*Paraboea leuserensis* B.L.Burtt) Nanoherbal and Exercise. Treatment consists of control group, maximum physical exercise, maximum physical exercise + Vitamin C at a dose of 2 mg/Kg Body Weight, maximum physical exercise + Gagatan Harimau nanoherbal with concentration 100, 125 and 150 mg/Kg BW. Data obtained from each observation parameter were recorded and presented in table and figure form. The data were analyzed using SPSS software version 25 with significant differences at 5%. The results of the study showed all treatments ($p<0.05$) in the expression of caspase 3 and caspase 10 in rat muscle. At dose of 150 mg/Kg BW can reduce the expression of caspase 3 and caspase 10 in rat muscle. Induction of Gagatan Harimau leaf nanoherbal as an antioxidant in male rats given swimming physical exercise showed improvements in muscle histology and morphology. A dose of 150 mg/Kg BW of nanoherbal Gagatan Harimau leaves was the best dose because it showed cell regeneration close to the normal treatment group.

Keywords: Caspase 3; Caspase 10; Gagatan harimau; Nanoherbal; Physical exercise

Introduction

Indonesia is largely rich in various traditional medicinal plants that can be used to treat various diseases (Pohan, 2023). Traditional medicine can be obtained from several plants found in Indonesia, either through inherited knowledge or through compounds. *Paraboea leuserensis* B.L. Burtt, also known as Gagatan harimau, belongs to the Gesnericeae family and the Paraboea genus (Pohan, 2023). This plant is a shrub and has been used as a medicinal plant by chewing it for treatments such as stomach pain and stamina enhancement (Ginting et al., 2015; Purba et al., 2016). The consumption of herbal plants can be done to boost stamina.

The utilization of herbs for consumption mostly comes from plant extracts. Extract preparations have drawbacks such as low water solubility, resulting in low bioavailability and functional properties that may deteriorate due to processing and storage time (Dwitarani et al., 2021). This can be overcome by increasing solubility and maintaining the functional properties of the extract by formulating it into nanoparticle preparations (Ardila et al., 2017). Nanoparticles from herbs are called nanoherbals (Dwitarani et al., 2021). Systematic physical activity performed repeatedly over a long period, accompanied by gradually increasing and continuous loads according to individual capabilities, is defined as physical exercise (Soesanto, 2018).

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Excessive physical activity will increase reactive oxygen species (ROS) in tissues and consume 2-5% of oxygen. High 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 between oxidants (free radicals) and antioxidants in the body, leading to cell damage such as muscle cells (Nurdyansyah, 2017). The repair of muscle cells can be observed by examining the expression of caspase 3 and caspase 10. Caspase is a group of cysteine protease enzymes that play a crucial role in regulating and executing cell death through apoptosis. Apoptosis is a programmed cell death mechanism used to remove unnecessary cells from the body. When cells lose the ability to undergo apoptosis or apoptosis is inhibited by a virus, cells proliferate uncontrollably and become cancerous (Hariwaluyo, 2015; Elmore, 2007). Initiator caspases (2, 8, 9, and 10) belong to the domain of caspases that function as apoptosis triggers, while executioner caspases (caspases 3, 6, and 7) function as effectors or executors of apoptosis and are directly involved in cell destruction. Based on research (Muhartono & Subeki, 2017), the anticancer activity of brusein-A increases the expression of caspase-3 in rat breast cancer induced by dimethylbenzanthracene (DMBA).

The Soleus muscle is located below the gastrocnemius muscle (Simorangkir & Andri 2018). Muscle damage is marked by necrosis. To prevent damage to muscle cells, efforts are needed to provide external antioxidant intake. One external intake with high antioxidant content is Gagatan Harimau (Prasetyawan et al., 2021). Gagatan Harimau has the potential as an antioxidant source (Wasnis et al., 2022). and will increasing and maintaining stamina for the community, especially for those who use their muscles and do heavy work Based on the description, it can be concluded that research is needed to determine the effectiveness of administering nanoherbal Gagatan Harimau as one of the antioxidants and preventing the formation of free radicals in the body on the expression of caspase 3 and caspase 10 in muscle histopathology in experimental animals (Rattus norvegicus) after maximum physical exercise. The use of nanoherbal Gagatan Harimau as a new therapeutic source for muscle recovery after physical exercise.

Method

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 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).

Immunohistochemical Staining of Caspase 3 and Caspase 10 Expression

Muscles tissue preparations were stained with Hematoxylin–Eosin (HE). Muscles 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 muscle stained immunohistochemically.

Table 1. Immunohistochemistry Scoring System (IHC) (Herwanto, 2015; Ilyas & Nadapdap, 2015)

Frequency of Appearance	Intensity of Appearance	Frequency of appearance x Intensity of appearance
0= Not visible	0= Not displayed	0= No response
1= <25% stained	1= Low	1-3= Small response
2= 25-50% stained	2= Moderate	4-6= Moderate response
3= >50% stained	3=Strong	7-9= Good response

Tissues were incubated with H_2O_2 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 and caspase 10 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.

Result and Discussion

HE (Haematoxylin-Eosin) Staining

Histology of Rat Muscle with HE (Haematoxylin-Eosin) Staining

The results of observations made after administering the nanoherbal Gagatan Harimau leaves (*Paraboea Leuserensis* B.L. Burtt) on the muscles histology of mice given maximum physical exercise (Figure 1.) Figure 1 is a histological picture of the muscles of mice with HE staining at 10x40 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. Inflammation, b. Fibrosis, Necrosis, d. Adipocytes (fat) Infiltration, Fiber Regeneration, f. Bleeding.

Based on Figure 1, the results of histological observations of soleus muscle tissue of mice given the nanoherbal gagatan harimau leaves revealed cell changes in the form are inflammation, fibrosis, necrosis, adipocyte (fat) infiltration, fiber degeneration and bleeding. In normal K(-) treatment there were fibrosis, necrosis, adipocyte (fat) infiltration, fiber regeneration and bleeding. This can occur due to natural response of the mammalian immune system to tissue damage. In the groups with K(+) maximum physical exercise by swimming, there are inflammation, fibrosis, necrosis, adipocyte (fat) infiltration, fiber regeneration and bleeding can cause damage of tissue damage in muscle cells. P1, P2, and P3 who were given by vitamin C and nanoherbal gagatan harimau leaves after maximum physical exercise by swimming, there was damage to muscles due to treatment from maximum physical exercise which caused oxidative stress.

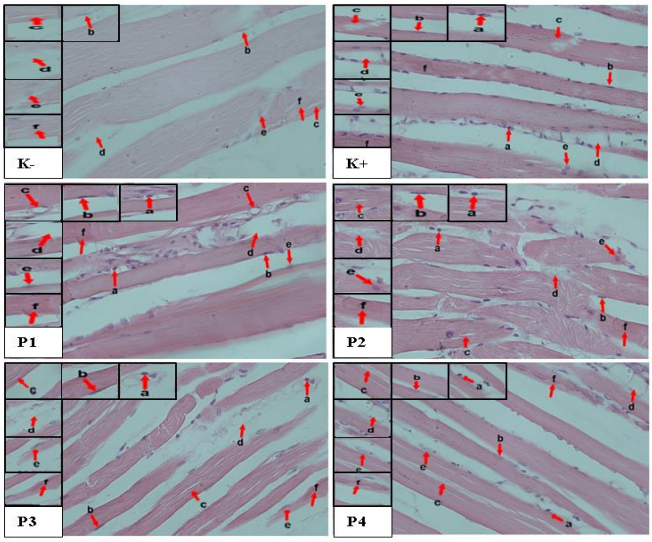

Figure 1. Histology of rat muscles

Excessive physical activity will result in inflammation, fibrosis, necrosis, adipocyte (fat) infiltration, fiber regeneration and bleeding especially necrosis in muscle cells due to the continuous metabolic process of tissue damage. Necrosis in mice is characterized by collections of highly fragmented, pitted muscle fibers, some dark red in color that are hyper eosinophilic, and pale. Necrosis can occur due to several things, namely a decrease in oxygen supply to the muscle, the presence of toxic compounds, mechanical trauma and excessive physical activity to muscle cells. In this study, P4 treatment was able to reduce muscle necrosis because it contains antioxidants and flavonoids which can improve muscle performance and recovery. The muscle healing process begins immediately after injury and involves phases of tissue destruction, repair, and remodeling, as well as regeneration of myofibers (Singh et al., 2017).

In the treatment group P4 nanoherbal gagatan harimau leaves 150 mg/kgBW showed an improvement in the histological structure of the muscle as seen from a decrease in the amount of inflammation, fibrosis, necrosis, adipocyte (fat) infiltration, fiber regeneration and bleeding when compared to other treatment groups such as P1, P2 and P3. Gagatan harimau leaves can protect muscle from damage caused by excessive physical activity because they have a natural source of

antioxidants produced by secondary metabolites of gagatan harimau such as flavonoid. Previous studies reported that Paraboea plants leaves have several activities, including anti-inflammatory, antibacterial, and antioxidant, which have the main content of flavonoids such as myricetin, myricitrin, quercetin, kaempferol, and ellagic acid which provides essential nutrients and oxygen to support tissue growth and repair (Heryani et al., 2024; Raina et al., 2021; Wang et al., 2011; Gong et al., 2019; Fu et al., 2022; Nanjala et al., 2022).

Histology of Soleus Muscle Fibers

Histology of Soleus Muscle Fibers can be seen in Figure 2. Figure 2 is a histological picture of the muscles of mice with HE staining at 10x40 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. K+ = 3652.68 μm², P4 = 123.66 μm².

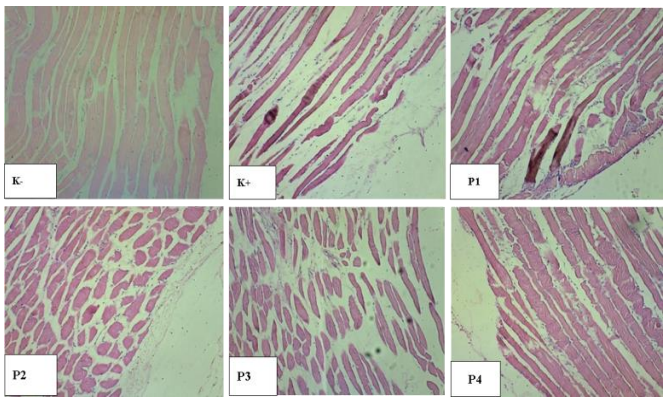

Figure 2. Histology of soleus muscle fibers

Based on Figure 2, the results of histological observations of soleus muscle tissue of mice given the nanoherbal gagatan harimau leaves revealed , it can be seen that the number of soleus muscle fibers in the K+ group was higher compared to other treatment groups. In particular, treatment P4 showed the lowest number of muscle fibers due to physical exercise after administration of Gagatan Harimau nanoherbal. Studies Hawke (2005) indicate that the physiological response to physical exercise increases the number and size of muscle fibers, which is a muscle adaptation to load and can cause hypertrophy. Maximal physical exercise can cause skeletal muscle damage through acute and repetitive mechanical trauma, which directly affects muscle fibers. This can result in secondary inflammation of muscle fibers and collagen, increased cell permeability, as well as impaired microcirculation, potentially damaging components of muscle contraction, leading to release of muscle contents and necrosis. Studies have also shown that repetitive exercise can result in loss of skeletal muscle fiber integrity, as demonstrated by loss of clear boundaries and pale cytoplasmic staining, as well as infiltration of inflammatory cells including neutrophils and macrophages, as well as microhemorrhages (Lio et al., 2020).

IHC (Immunohistochemistry) Caspase staining

IHC (Immunohistochemistry) muscle Caspase 3

Muscle caspase 3 immunohistochemical staining results at 10x40 magnification after administration tiger gagatan leaf nanoherbal can be seen in figure 3. Description: Red arrow: +caspase 3. 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 tiger leaf nanoherbal, P3: Rats were given swimming training by administering 125 mg/KgBB of tiger gagatan leaf nanoherbal, P4: Rats were given swimming training by administering gagatan leaf nanoherbal tiger 150 mg/KgBW.

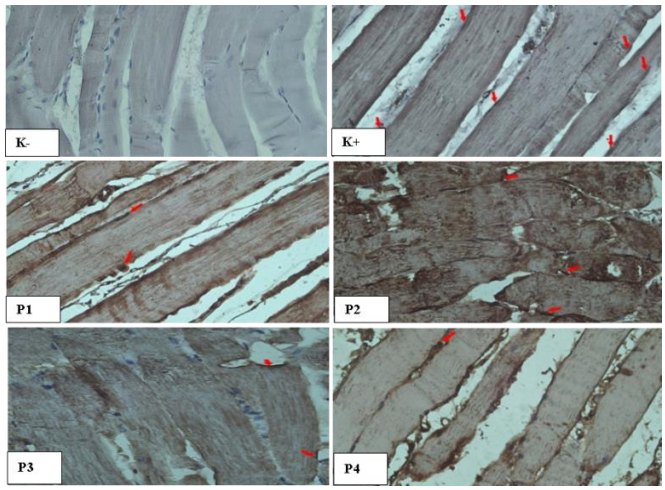

Figure 3. Immunohistochemistry of muscle caspase 3

Based on Figure 3, caspase-3 protein in muscle shows that P4 treatment is effective in reducing caspase-3 activity, indicated by the less obvious brown staining in the muscle cells compared to other treatments, indicating a decrease in caspase expression. In contrast, K+ treatment showed a significant increase in caspase-3 expression, characterized by clear brown staining in the muscle cells, indicating the possibility of apoptosis (programmed cell death) in muscle cells after maximal physical exercise. Apoptosis in muscle atrophy can

accelerate the loss of muscle mass and may be the main mechanism leading to decreased muscle performance. Caspase-3 has an important role in this process, with its ability to destroy the actomyosin complex and its degradation products, which are further degraded by other protein systems in the cell, causing a decrease in skeletal muscle mass and strength. The process of mitochondrial caspase- dependent apoptosis (endogenous apoptosis) is regulated by the Bcl-2 family of proteins. Bcl-2 and Bcl-XL act as anti-apoptotic proteins, while Bax is a pro-apoptotic protein (Ashkenazi et al., 2017). Reactive Oxygen Species (ROS) play a role in oxidative stress which is closely related to this process. Significant oxidative stress produces high levels of ROS, which disrupts the balance between oxidation and antioxidants, damages genetic material, alters mitochondrial membrane permeability, and ultimately induces the process of apoptosis. Based on Figure 3, it can be seen that the caspase-3 protein in muscle is clearly expressed in the K(+) treatment after maximal physical exercise.

The K(+) treatment showed a strong increase in caspase-3 expression in the tissue cells, characterized by clear brown staining compared to the K(-), P1, P2, P3, and P4 treatments. Administration of nanoherbal from Gagatan Harimau leaves at the highest dose of 150 mg/ kg BW (P4 treatment) was proven to improve the histopathology of muscles undergoing maximum physical exercise, such as swimming in mice. Studies have reported that a significant decrease in the protein expression of caspase-3 after aerobic exercise is associated with a decrease in pre-apoptotic factors such as Bax protein expression, a decreased Bax to Bcl2 ratio, as well as an increase in the anti-apoptotic protein Bcl2. The decrease in mitochondrial apoptotic potential after aerobic exercise in aged mice may be related to the decreased release of apoptotic agents such as cytochrome c and Apaf1 into skeletal muscle, which significantly reduces caspase-3 expression (Zarali et al., 2020). This explanation confirms that Gagatan Harimau nanoherbal, especially at certain doses, has the potential to protect muscles from the negative effects of maximum physical exercise by reducing the expression of caspase-3, which is the main marker in the process of cell apoptosis.

Based on these data, the expression of caspase 3 showed a significant difference in the msucle after administration of harimau gagatan nanoherbal. Boxplot results of Kruskal-Wallis and Mann-Whitney analysis of muscle Caspase 3 expression can be seen in Figure 4. Figure 4 shows that the expression of caspase-10 in muscle shows that the caspase-10 index between the control group (K-) and the treatment group (P4) is significantly different ($p < 0.05$) based on the Kruskal-Wallis test and the Mann-Whitney follow-up test between each treatment. Figure 4 also shows that administration of nanoherbal from Gagatan Harimau leaves in treatment P4 was most effective in reducing caspase 3 expression in muscles after maximum physical exercise. K+ treatment, on the other hand, showed a significant increase in caspase 3 expression compared with other treatments, which was due to increased Reactive Oxygen Species (ROS) which triggers the apoptosis process mediated by caspase 3.

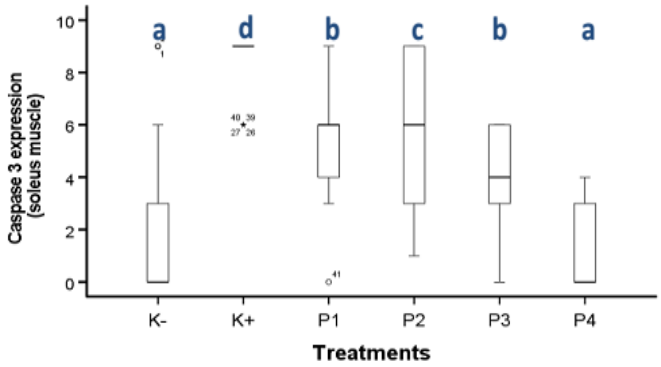

Figure 4. Boxplot of caspase 3 expression muscle (SPSS V.25)

IHC (Immunohistochemistry) Muscle Caspase 10

Muscle caspase 10 immunohistochemical staining results at 10x40 magnification after administration tiger gagatan leaf nanoherbal can be seen in figure 4. Description: Red arrow: +caspase 10. 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 tiger leaf nanoherbal, P3: Rats were given swimming training by administering 125 mg/KgBB of tiger gagatan leaf nanoherbal, P4: Rats were given swimming training by administering gagatan leaf nanoherbal tiger 150 mg/KgBW.

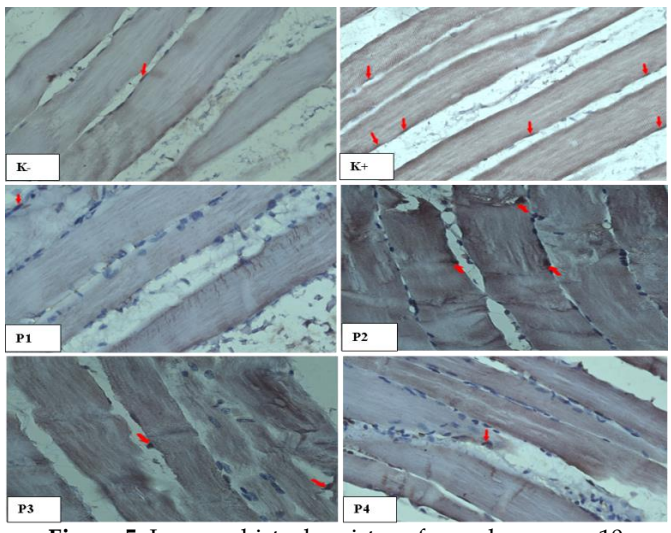

Figure 5. Immunohistochemistry of muscle caspase 10

Based on Figure 5, it can be seen that the caspase-10 protein in muscle is expressed significantly in the K(+) treatment after maximum physical exercise. The K(+) treatment showed a strong increase in caspase-10 expression in the muscle cell, marked by intense brown staining compared to the K(-), P1, P2, P3, and P4 treatments, indicating the possibility of an apoptotic process (programmed cell death) after maximum physical exercise. Weinstein et al. stated that moderate and low intensity aerobic exercise increases antioxidant capacity, reduces cellular oxidative stress, and provides the adaptations needed to inhibit or halt apoptosis induced by intense aerobic exercise. The results of this study indicate that long-term and moderate aerobic exercise can increase the expression of genes such as AIF (Apoptosis Inducing Factor) and caspase-10 in the mitochondrial pathway, which in turn can worsen skeletal muscle apoptosis in mice (Zarali et al., 2020). During exercise, increased muscle metabolism leads to the production of ROS.

High amounts of ROS can cause oxidative damage and trigger apoptosis through internal pathways, where the role of mitochondria is very important. P4 treatment, which was most effective in reducing caspase-10 activity was characterized by less brown staining in the muscle tissue cells. Administration of nanoherbal from Gagatan Harimau leaves at the highest dose of 150 mg/kg BW has been proven to improve the histopathology of muscle undergoing maximum physical exercise such as swimming in mice, because it contains high antioxidants which can reduce ROS and apoptosis. Gagatan Harimau nanoherbal also contains various secondary metabolites such as flavonoids, terpenoids, phenolic glucosides, quinones, xanthones, and lignin (Harfita, 2024).

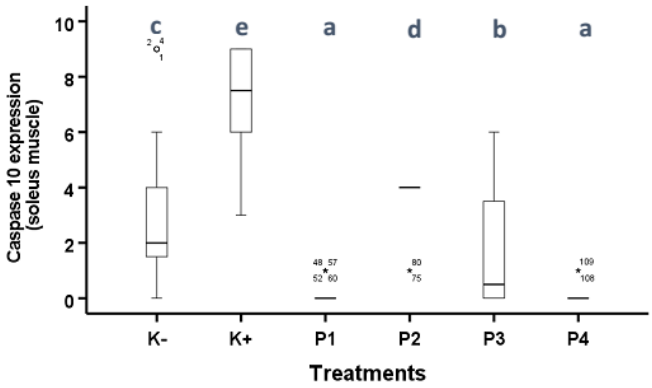

Figure 6. Boxplot of caspase 10 expression muscle (SPSS V.25)

Based on these data, the expression of caspase 10 showed a significant difference in the msucle after administration of harimau gagatan nanoherbal. Boxplot results of Kruskal-Wallis and Mann-Whitney analysis of muscle Caspase 10 expression can be seen in Figure 6. Figure 6 shows that the expression of caspase-10 in muscle shows that the caspase-10 index between the control group (K-) and the treatment group (P4) is significantly different ($p < 0.05$) based on the Kruskal-Wallis test and the Mann-Whitney follow- up testbetween each treatment. Figure 6 show P4 treatment with the administration of Gagatan Harimau nanoherbal showed the most significant reduction in caspase-10 in muscles after maximum physical exercise, due to its antioxidant content. On the other hand, K+ treatment increased caspase-10 expression which triggered an increase in ROS and finally induced apoptosis through the caspase-10 pathway.

Conclusion

Based on the results of the research and data processing that has been carried out, Gagatan Harimau leaf nanoherbal as an antioxidant in mice given maximum physical exercise showed a decrease in the histology and morphology of muscle of mice with HE staining. A dose of 150 mg/Kg BW from the leaves of nanoherbal gagatan tiger is the best dose because it shows cell regeneration that is close to the normal treatment group. Dose 150 mg/kgBB from nanoherbal Gagatan Harimau leaves (Paraboea leuserensis B.L. Burtt) reduces the expression of caspase 3 and caspase 10 in rat muscle. This is caused by the antioxidant and secondary metabolite content in Gagatan Harimau which reduces Reactive Oxygen Species (ROS) and inhibits the apoptosis process, thereby helping repair damage and optimizing muscle performance.

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Author Contributions
A; contributed as researcher and article writer, S.I; contributed as a research idea and article writing supervisor, and M.T; contributed as a supervisor in processing research data. All authors have read and agreed to publish versions of the manuscript.

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Conflicts of Interest
The authors declare no conflict of interest. The funders had no role in the design of the study.

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