Effect of Therapy on Mangosteen (Garcinia Mangostana L.) Bark Extract on Serum Blood Protease Activity and Expression of Malondialdehyde (MDA) on Rattus Norvegicus Traumatic Brain Injury Model

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Abstract: Traumatic brain injury is a cause of impaired cognitive and physical function that is permanent or temporary and is accompanied by loss or change of level of consciousness and cause damage or death of cells in the brain. Cell death or apoptosis can cause inflammation and oxidative stress and produce reactive oxygen species (ROS). The use of mangosteen peel extract serves to prevent inflammation and inhibit the production of oxidative stress. This study aims to determine the role of mangosteen peel extract therapy in brain organ expression and protease activity in rat blood serum. Besides that, minocycline is used as a test standard and comparison of mangosteen peel extract. The results of this study formed 4 treatment groups namely the negative group, the positive group TBI model, the TBI group with mangosteen skin extract therapy dose 0.5 mL/day for 5 days and the TBI group with minocycline therapy 0.5 mL/day dose for 5 days. The results showed that the treatment of mangosteen peel extract after brain injury could reduce the expression of malondialdehyde (MDA) by 32.29% and protease activity in rat blood serum by 47.62%. The results of statistical analysis of MDA exposure and protease activity showed that there were very significant differences between treatment groups (p < 0.01). This study can be concluded that mangosteen peel extract therapy can be used when a traumatic brain injury occurs.

1 INTRODUCTION

Brain injury is one of the highest causes of death in traffic accidents. Indonesia itself has a 50% increase in mortality rate every year. Brain injury is a major factor causing death in traffic accidents (Warpani, 2002).

Rats (Rattus norvegicus) are animals in the form of mammals. This white rat belongs to the Muridae, the genus Rattus with the order of Rodentia. Also, it is an experimental animal that is widely used in research. This is because rats have a high similarity of 95% of the same genes as humans. This white rat also has a short generation rate of 1-year rats equal to 30 years of humans with high reproduction and can manipulate genomes directly (Armitage, 2004). This then becomes the basis of research to observe brain injuries that occur in humans by making mice as a model of Traumatic Brain Injury. Mangosteen extract is known to contain active xanthones, flavonoids, etc. Where xanthone is a substance that has important benefits such as anti-inflammatory, antioxidant, anti-cancer, and also anti-cardioprotective (Miryanti et al., 2011). The anti-inflammation contained in the mangosteen extract is very important to be used in traumatic brain injury because it can prevent the onset of increased inflammation. Increasing inflammation will indicate the body's physiological response to an interruption by external factors (Murfu’ati et al., 2014). Besides that, the flavonoid content in mangosteen peel extract also has an important effect which can inhibit the work of enzymes involved in the formation of ROS (Redha, 2010). Based on the results of research that has been done, it is stated that flavonoids are a group of phenolic compounds that have antioxidative...
properties and play a role in preventing cell damage and cellular components caused by free radicals (Simamora, 2009).

Some things to be able to prove that mangosteen peel extract is a therapy that can provide positive changes for traumatic brain injury that is proven by using levels of malondialdehyde (MDA). This substance is widely known and is also often used as a biological marker of lipid peroxidation and oxidative indicators. Also, it is easily obtained in the blood circulation and is the main product of the reaction of free radicals with phospholipids. The results of these reactions are produced in constant accordance with the proportion of lipid peroxidation that occurs, so it is a good indicator to see the speed of lipid peroxidation in vivo. The higher levels of MDA are expected to increasingly show the level of damage that occurs in traumatic brain injury (Yigit et al., 1999).

Also, observations were made on protease activity. Protease activity itself is the ability of protease to break down proteins. Protease is secreted into tissues involved in the mechanism of tissue damage. Excessive protease activity can damage tissue cells found in the brain (Hantoko and Drajat, 2003). Increased uncontrolled protease activity causes an inflammatory process so it is expected to be inhibited by mangosteen peel extract therapy.

2 MATERIAL AND METHOD

2.1 Preparation of Animals Trying Mice (Rattus norvegicus)

Rats were divided into 4 treatment groups, each group consisted of 5 mice. Before being treated, mice were adapted to the laboratory environment for 7 days by standard feeding to all mice.

Group 1 was a negative control group of rats, namely the group of rats without TBI induction and the administration of minocycline therapy and mangosteen extract. Group 2 was a positive control rat group, namely the TBI induction rat group. Group 3 was a group of TBI induction mice and treated with minocycline at a dose of 0.5 mL/day. Group 4 is a group of TBI induction mice and mangosteen extract treated with a dose of 0.5 mL/day. The research scheme can be seen in appendix 1.

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>Repeat</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Negative control</td>
<td>1 2 3 4 5</td>
</tr>
<tr>
<td>2</td>
<td>Positive group (TBI induction mouse)</td>
<td>1 2 3 4 5</td>
</tr>
<tr>
<td>3</td>
<td>TBI induction group and minocycline therapy with a dose of 0.5 cc/day for 5 days</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>TBI induction group and mangosteen extract therapy with a dose of 0.5 cc/day for 5 days</td>
<td></td>
</tr>
</tbody>
</table>

The research sample used was a rat animal (Rattus norvegicus) male Wistar stain with a bodyweight of 300-350 grams. Calculation of the number of samples can use the Federer formula as follows [45]:

\[
t (n - 1) \geq 15 \\
4 (n - 1) \geq 15 \\
4n - 4 \geq 15 \\
4n \geq 19 \\
n \geq 4.75 \text{(be rounded 5)}
\]

Note:

- \( t \): number of treatment groups
- \( n \): number of repetitions needed

Based on the estimation calculation from the sample above, then for the four treatment groups required at least 5 replications in each group so that the total number of rat animals needed was 20.

Rats were kept by the treatment group and kept in a room temperature of 22-24°C and humidity 50-60% with adequate ventilation, where each cage consisted of 5 mice. The mouse cage is made of a plastic tub with a size of 17.5 x 23.75 x 17.5 cm which is equipped with a cover of wire.
The variables observed in this study are as follows:
1. Independent variable: treatment of falling brain load, therapeutic dose of mangosteen extract
2. Dependent variable: brain organ, serum protease activity, MDA immunohistochemistry
3. Control variables: sex, age, body weight of Rattus norvegicus strain Wistar rats.

2.2 Providing Mangosteen and Minocycline Skin Extract Therapy

Minocycline therapy was given to the KB TB post-treatment group at a dose of 0.5 mL/day for 5 days. While mangosteen therapy was given to the KC treatment group at a dose of 0.5 mL/day for 5 days.

2.3 Preparation of Animal Rats (Rattus norvegicus) TBI Models

Ketamine at a dose of 100 mg / KgBB and xyla at a dose of 10mg / kg BW are anaesthetized through intramuscular injection of the thigh muscle. Then the rats were placed face down on the surgical board and fixed the four extremities using a paper clip. The rat's head was disinfected using 70% alcohol and the rat's head was shaved. The scalp of the rat is opened with scissors from the middle to between the two ears toward the frontal until the skull is visible. Then the mouse head is positioned just below the cylinder sleeve with a distance of 1 cm. The iron cylinder weighing 40 grams and 4 mm diameter was dropped perpendicularly from a height of 180 cm 1 time. Then the scalp is cleaned, stitched back and given a topical 10% gentamicin ointment and intramuscular analgesic.

2.4 Intake of Rat Brain

Rats were treated with euthanasia using ketamine at a dose of 0.2 mL and placed on a surgical board. Next, cut the back of the rat's neck or cut in the direction of the back to the abdomen entirely so that you can see the boundary between the skull and skin.

The scalp of mice in the TBI lesion area is completely removed. Then the mouse skull is cut as needed from the direction of the neck intersection. The skull was opened with the power of a finger to open and obtained a cross-section of the brain and its limits. Nerves that are still connected to the brain are cut. Then the brain is carefully removed and placed in an organ bottle containing 10% formalin Solution.

2.5 Retrieval of Rat Blood Serum

Rats were placed in a dorsal lying position on the surgical board. Then dissected in the abdominal cavity and taken as much as 5 cc of blood plasma in the superior vena cava of the heart. Then put in a red vacutainer.

2.6 Making Slides of Brain Tissue Histopathology Preparations

The rats head was cut into 2 cm x 1 cm x 3 cm size and brain tissue was fixed using 10% formalin, then soaked for 18-24 hours. Then washed with running water for 15 minutes and dehydrated using acetone Solution for 1 hour 4 times. Then the stage of clearing (clearing) using xylol for 30 minutes as much as 4 times. Furthermore, the stage of immersion (impregnation) using liquid paraffin with a temperature of 55°C for 1 hour 4 times. Casting (blocking) is done on the paraffin block and sliced on a network that has been embedded in the paraffin block using a rotary microtome with a thickness of 3-5 microns and placed on a glass object.

2.7 MDA Measurement by Immunohistochemistry

Preparation slides before being deprived are heated at 60°C for 60 minutes. The preparations were depolished with xylol for 2 times 10 minutes, put into absolute ethanol for 2 times 10 minutes. Then put into multilevel ethanol (95%, 90%, 80%, and 70% and distilled water) for 5 minutes each. The slide preparation was immersed in a Chamber containing citrate buffer pH 6.0. Then the Chamber is heated in a water temperature of 95°C for 20 minutes. Slides are removed from the water bath, wait until the room temperature ± 20 minutes. Slides were washed with PBS for 6 minutes.

On the first day the slides were ready for IHC, 3% H2O2 in methanol was incubated for 15 minutes and washed with PBS pH 7.4 3 times 2 minutes. In Unspecific Blocking Protein Background Sniper drops, incubated 15 minutes at room temperature then washed with PBS pH 7.4 3 times 2 minutes. Primary antibody (MDA) drops were Solutioned in PBS + 2% BSA buffer, incubated overnight at 4oC. Then on the second day, the two slide preparations were removed, waited for room temperature, then
washed with PBS pH 7.4 3 times 2 minutes. Secondary antibody is dropped, incubated 30 minutes at room temperature and washed PBS pH 7.4 3 times 2 minutes. Furthermore, SA-HRP (StrepAvidin-Horse Radish Peroxidase) was added and incubated for 20 minutes at room temperature. Washed with PBS pH 7.4 3 times 2 minutes and rinsed with distilled water. Added DAB (Chromagen DAB: DAB buffer = 1:50) and incubated for 3-10 minutes at room temperature.

Washed with PBS pH 7.4 3 times 2 minutes and washed with aqua dest 3 times 2 minutes. Counterstain (Mayer's Hematoxylin) was given with a tap water in a ratio of 1:10 and incubated for 5-10 minutes at room temperature, then rinsed with tap water. Mounting glass cover. Furthermore, dried until dried. Furthermore, observations were made using a microscope. MDA levels in rat brain tissue can be calculated with a portrait microscope at the Anatomy Pathology Laboratory, Faculty of Medicine, Brawijaya University. The preparations are placed on a microscope with 10x ocular magnification and 10x objective magnification. After brain tissue is seen, the objective magnification is increased to 40x. Observations were made on 10 different fields of view so that the results obtained are objective.

2.8 Measurement of Protease Enzyme Activity (serum) Protein Isolation

Blood serum is prepared first and added a little quartz sand. After homogenate added with PBS-Tween: PSMF (9: 1) Solution as much as 1 mL and transferred into a sterile effendorf tube. Followed by vortexing for 15 minutes (6000 rpm), and 10 minutes sonicated with a sonicator. Then the supernatant is taken and added to absolute cold ethanol in a ratio of 1 and left overnight to form a precipitate. After that centrifuge for 15 minutes (10,000 rpm), the sediment is taken and dried until the ethanol odour disappears. Then the precipitate was added with a 0.02 M Tris-HCl pH 6.5 cold Solution with a volume ratio of 1: 1.

2.9 Making the Tyrosine Raw Curve

The first step in making the tyrosine standard curve is to prepare 10 volumetric flasks and each filled with 20 ppm tyrosine standard Solution 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 mL. Then the tube is closed with aluminium foil and shaken. Then the absorbance is measured in each standard Solution at the maximum wavelength. The blank used is aquades.

2.9 Measurement of Protease Activity in Protein Isolation

The first step that must be done is to mix 500 ppm casein as much as 200 µL, phosphate buffer Solution pH 7 as much as 300 µL and protease enzyme as much as 100 µL then let stand 60 minutes at 37°C in the incubator. Then a 400% TCA Solution of 400% was added and allowed to stand for 30 minutes at 27°C (room temperature). Then it is rotated with a centrifuge at 4000 rpm for 10 minutes. The supernatant was taken as much as 100 µL and diluted 5 times the sample volume with phosphate buffer then measured its absorbance value at a maximum tyrosine of 280 nm. The blank used was made by the same procedure as the determination of activity, but for the addition of TCA treatment was carried out as soon as possible after the addition of the enzyme Solution. One unit of activity is as much as tyros avoided the breakdown of 1 mL of the protease enzyme.

Measurement of protease enzyme activity was carried out based on the Walter (1984) method using the formula:

\[
\text{Enzyme Activity} = \frac{\text{q} \times \text{fp} \times \frac{\text{Mr}}{\text{q}}}{\text{p}}
\]

q = incubation time (mL) fp = dilution factor p = amount of enzymes (mL)

2.10 Data Analysis

Analysis of quantitative immunohistochemical data using MDA levels in rat brain and calculation of protease activity as an anti-inflammatory marker on brain tissue was performed statistically using a one-way analysis of variance (ANOVA) variance test.

Then the Honestly Significant Difference (BNJ) or Tukey test is performed to determine whether there is a significant difference with a significance level of 5% using Microsoft Office Excel and statistical package for the social science (SPSS) version 16.0 for Windows 7.
3 RESULT AND DISCUSSION

In this research, therapy on traumatic brain injury rats using mangosteen extract and minocycline as gold standard. Observations made were the expression of malondialdehyde (MDA) in rat brain organs and serum protease activity in the blood.

3.1 MDA Expression of White Rat (Rattus norvegicus) Brain TBI Model in Mangosteen Skin Extract Therapy

In rats, traumatic brain injury occurs an inflammatory process due to free radicals. Oxidative stress on the central nervous system is very deadly because the human brain mainly uses oxidative metabolism. Although the brain weighs only 2% of body weight, the brain uses about 50% of all body oxygen. Another very dangerous factor is oxidative stress in the brain with a high PUFA (polyunsaturated fatty acid) content, affecting almost 50% of all brain tissue structures. Poly Unsaturated Fatty Acid is degraded by free radicals which are ROS products such as hydroxyl radicals (-OH), superoxide radicals (O2-), hydrogen peroxide (H2O2) then form malondialdehyde (MDA) [48]. High MDA levels indicate that cells experience oxidative stress [49] and are indicated by changes in the appearance of mouse brain cells in brown colour. Mangosteen peel extract has antioxidant and anti-inflammatory properties. Antioxidants will inhibit the process of activation of inflammatory cells so that the activation of macrophages in producing cytokines is also reduced and inhibited the production of free radicals. Inhibition of free radicals by antioxidants by the bioactive content of mangosteen through the process of inhibiting the oxidation reaction to reduce the expression of MDA. The content of bioactive compounds possessed by mangosteen peel extract can reduce MDA expression better than minocycline because minocycline is only a derivative of tetracycline so that minocycline has only one benefit to reduce MDA expression in TBI model mice. Minocycline is also a drug that has long been used for the treatment of brain injuries. This can be proven in Table 4.1.

Table 4.1 Average number of cells expressing malondialdehyde (MDA) in TBI mouse brain organs.

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>MDA Expression Average (%)</th>
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<tbody>
<tr>
<td>Negative Control (Healthy) (A)</td>
<td>7,87 ± 0,09</td>
</tr>
<tr>
<td>TBI Positive Control (B)</td>
<td>12,48 ± 0,29</td>
</tr>
<tr>
<td>TBI Control and Mangosteen Skin Extract Therapy (C)</td>
<td>2,16 ± 0,37</td>
</tr>
<tr>
<td>TBI Control and Minocycline Therapy (D)</td>
<td>8,45 ± 0,53</td>
</tr>
</tbody>
</table>
The average MDA level in group A was 7.87 ± 0.09. These values indicate the standard value of MDA levels in mice under normal circumstances. The mean value of MDA levels in group B is the highest value of 12.48 ± 0.29 and testing by BNJ test shows that there are significant differences compared to treatment groups A, C, and D.

The results of statistical analysis (Appendix 11) indicate that group B provide a real influence on the increase in MDA expression in rat brain. The results showed that a drop of iron cylinder weighing 40 grams and a diameter of 4 mm can cause inflammation in the rat brain. Therapeutic groups namely group C and group D showed a decrease in MDA expression after administration of mangosteen and minocycline skin extract therapy. But in the therapy done, mangosteen skin therapy is better than minocycline therapy. This can be caused because there is a high antioxidant content in mangosteen peel extract which can counteract free radicals which is indicated by a decrease in MDA levels in the brains of mice that have been injured. Antioxidants in the content of mangosteen peel extract functions as a revenger (catcher) of free radicals so that it can help reduce high levels of free radicals in the rat brain due to traumatic brain injury. The mechanism of inhibition of free radicals by antioxidants mangosteen rind extract is inhibiting the oxidation process by inhibiting the initiation and propagation of oxidation reactions from free radicals and ROS. Antioxidants in mangosteen peel extract namely xanton can contribute hydrogen atoms to capture hydroxyl radicals (OH) so as not to become reactive so that it inhibits free radicals. The xanton component works through the capture of O-peroxide nitrite (ONOO-) formed from nitric oxide (NO) with superoxide (O2-) which is a free radical. The antioxidant content of mangosteen peel extract inhibits the initiation process so as to prevent the formation of lipid radicals that are unstable due to the loss of one hydrogen atom (H) from the lipid molecules due to hydroxyl radicals (OH-), prevent the propagation process so that free radicals will not react with oxygen and automatically does not directly reduce MDA levels in rat brain.

This is also strengthened by the presence of other bioactive compounds including flavonoids where these compounds can ward off free radicals by reducing free radical compounds so that they become stable compounds. Binding of free radicals by flavonoids will prevent chain radical reactions that damage protein function and normal tissue structure. Flavonoids are compounds that can be easily modified to stop radicals so that they can prevent oxidative stress in cells and increase the enzyme protease in tissues.

3.2 Effect of Mangosteen Skin Extract Therapy on TBI Model Blood Serum Protease Activity

Protease activity is the ability of proteases to hydrolyze peptide bonds in proteins. The method used in the analysis of protease activity is spectrophotometry. The method is based on enzymatic hydrolysis by proteases tested from a casein substrate Solution at pH 7, and followed by deposition of non-hydrolyzed substrates using trichloroacetic acid (TCA) 4% to stop the reaction. The result of hydrolysis from the casein substrate Solution is L-tyrosine. Therefore, in the measurement of protease activity, the tyrosine standard curve is used with $\lambda = 275$ nm because the final product (product) is measured by UV spectrophotometer at a maximum wavelength of 275 nm. The protease activity unit of rat blood serum (Rattus norvegicus) is defined as the number of tyrosine units produced by hydrolysis of peptide bonds in proteins by protease isolated from rat duodenum (Rattus norvegicus) under optimum conditions in pH 6.5, temperature 370 C, and time incubation of 60 minutes. On the tyrosine standard curve obtained by the equation of the line $y=0.015x - 0.013$. The line equation is used to calculate the measured tyrosine concentration from the study so that the protease activity can be calculated for each treatment. A complete calculation of protease activity is presented in Attachment 9.
4 CONCLUSIONS

Based on the results of research that has been done can be concluded that:

1. Mangosteen skin extract therapy can repair damage to the cortex of the rat brain.
2. Mangosteen peel extract therapy can reduce malondialdehyde (MDA) expression in rat brain by 2.16 ± 0.37.
3. Mangosteen peel extract therapy can reduce the activity of protease enzymes by 47.62%.

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REFERENCES


