Pengaruh Ekstrak Daun *Moringa oleifera* terhadap Presentase Necrosis Sel Purkinje pada Cerebellum Mencit (*Mus musculus*) yang dipapar Metilmerkuri

Effect of *Moringa oleifera* Leaf Extract on the Percentage of Necrotic Purkinje Cells in Mice (*Mus musculus*) Induced by Methylmercury

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Abstrak

Penelitian ini bertujuan untuk mengamati pengaruh pemberian ekstrak daun *Moringa oleifera* terhadap presentase nekrosis sel Purkinje pada cerebellum mencit (*Mus musculus*) yang dipapar metilmerkuri. Sejumlah 20 mencit jantan digunakan pada penelitian ini dan kemudian dibagi menjadi lima kelompok (C –, C +, T 1, T 2, T 3). C – diberi 0.2 ml CMC Na, C + diberi 0.4 mg/kg bb metilmerkuri, T 1 diberi 200 mg/kg bb ekstrak daun *Moringa oleifera* + 0.4 mg/kg bb metilmerkuri, T 2 diberi 400 mg/kg bb ekstrak daun *Moringa oleifera* + 0.4 mg/kg bb metilmerkuri, dan T 3 diberi 800 mg/kg bb ekstrak daun *Moringa oleifera* + 0.4 mg/kg bb metilmerkuri. Perlakuan terhadap kelompok percobaan dilakukan selama 21 hari, kemudian mencit dikorbankan dengan metode dislokasi servikalis. Cerebellum diambil dan dijadikan preparat histologi. Pengamatan mikroskopik dilakukan untuk menghitung jumlah total sel Purkinje dan sel Purkinje nekrosis. Hasil penelitian menunjukkan bahwa ekstrak daun *Moringa oleifera* menurunkan presentase nekrosis sel Purkinje pada cerebellum mencit (*Mus musculus*) yang dipapar metilmerkuri. Ekstrak daun *Moringa oleifera* dengan dosis 800 mg/kg bb memberikan efek protektif yang paling baik dibandingkan dengan dosis 200 dan 400 mg/kg bb.

Keywords: *Moringa oleifera*, Metilmerkuri, Cerebellum, Sel Purkinje.

Research Background

Among humans, the sole source of methylmercury exposure is the consumption of fish and sea mammals. Methylmercury is produced environmentally by biomethylation of the inorganic mercury present in aquatic sediments (Clarkson et al., 2003). In 1953 and 1960, the toxicity of methylmercury was recognized worldwide following epidemics of mercury poisoning in the Japanese inhabitants of Minamata and Niigata Bays due to consumption of fish caught in the region. Waste containing mercuric chloride had been released into the bays and became concentrated in the fish after conversion to methylmercury by plankton (Broussard et al., 2002). In Cipinang River Jakarta, mercury level in waters is about 0.03 mg/L while the
quality set by the government is 0.005 mg/L (Yudo, 2006).

Methyl mercury binds to cysteine and forming a very similar complex to methionine, this complex then will be carried across the blood brain barrier by L- methionine transporter (Fonnum et al., 2000). It is surprising that methyl mercury which readily reacts with –SH groups, leads to specific degeneration of the cerebellar cells (Shiraki, 1979 in Fonnum et al., 2000). In the brain, methylmercury causes focal necrosis of neurons and destruction of cells and is toxic to the cerebral and cerebellar cortex (Broussard et al., 2002).

MeHg has been thought to induce ROS and generation of oxidative events leading to cell damage. MeHg exposure increases the rate of ROS in the cerebellum and in the brain synaptosomes as well as in the cerebellum neuronal cultures (Do Nascimento et al., 2008). The Purkinje cells is one of the most important targets in cerebellum for toxic substances. It is sensitive to excitotoxic chemicals and also to an effect on DNA or its repair mechanisms (Fonnum et al., 2000). The clinical signs of exposure to methylmercury compounds in CNS are ataxia, tremors and unsteady gait (Broussard et al., 2002).

Some studies report that Moringa oleifera leaf extract has beneficial effect in protecting animals against alcohol-induced liver oxidative damage due to its free radicals scavenging capability (Saalu et al., 2012). The administration of Moringa oleifera leaf extract also increasing the levels of antioxidant profiles, SOD (Superoxide Dismutase) and Catalase by eliminating reactive free radicals that may affect the normal functions of cells (Paliwal et al., 2011). Moringa oleifera leaf extract is the potential neuroprotectant by decreasing oxidative stress (Kirisattayakul, 2013) and increasing antioxidant enzymes (Rahmath et al., 2015).

The aim of this research was to determine the effect of Moringa oleifera leaf extract in protecting cerebellum methylmercury toxicity by reducing the number of necrosis Purkinje cells in mice (Mus musculus) cerebellum. Hopefully this research can give a great contribution and benefit for the development of the veterinary world in the future.

**Research Material**

This research was conducted at the Animals Model Laboratory and Veterinary Pathology Department of Veterinary Medicine Faculty, Pharmacognosy and Phytochemistry Department of Pharmacy Faculty and Laboratory of Anatomy and Histology, Faculty of Medicine, Universitas Airlangga. Implementation of this research was carried out from March to April 2015. The object in this study are twenty healthy male mice (Mus musculus) strain BALB/C aged 16 weeks old with an average weight of 20-40 grams, maintained at the same place and were given the same feed.

The equipment used in this study include rotavapor, weight scale, mice cage, container for feed and drink, feeding tube, 1 ml tuberculin syringes, surgical scissor, scalpel, forceps, plastic pots, objects glass, cover glass, microscope, a series of dehydration apparatus, microtome, water bath, hot plate and camera.

Materials used in this study were Methylmercury(II)chloride (CH$_3$ClHg), aquadest, Moringa oleifera leaves, 96 % ethanol, 0.5 % Na CMC, 10 % Formalin, 70, 80, 90 and 96 % alcohol, xylol, paraffinand Hematoxylin Eosin.

**Research Methods**

Twenty male mice aged 12 weeks old, weighing 20-40 grams reared to receive treatments for 21 days in Animals Models Laboratory, Veterinary Medicine Faculty, Universitas Airlangga. Treatment started after the adaptation period for a week, then the animal models weighted and randomly divided into five groups:
C (-) : Control negative group given 0.2 ml of 0.5% Na CMC
C (+) : Control positive group 0.4 mg/kg bw of MeHg
T 1 : Treatment for group 1, 200 mg/kg bw of Moringa+ 0.4 mg/kg bw of MeHg*
T 2 : Treatment for group 2, 400 mg/kg bw of Moringa+ 0.4 mg/kg bw of MeHg*
T 3 : Treatment for group 3, 800 mg/kg bw of Moringa+ 0.4 mg/kg bw of MeHg*
*) The methylmercury administration is given an hour after the administration of Moringa oleifera leaf extract.

Preparation of Moringa oleifera leaf extract

Fresh leaf of Moringa oleifera were collected, shade dried and pounded into powder, then underwent the extraction process. 500 grams of Moringa oleifera leaf powder was taken and soaked in 3750 ml 96% ethanol with ultrasonic system bath for 10 minutes. Screening was done in order to separate the dregs and solution. The dregs was soaked, separated and re-soaked for 3 times. The macerate evaporated using rotavapor tools at 50 °C for 4-5 hours to obtain condensed Moringa oleifera leaf extract.

The dose of Moringa oleifera leaf extract used in this research would be 200, 400 and 800 mg/kg BW. Dose of 200, 400 and 800 mg/kg BW made from 2000, 4000 and 8000 mg of Moringa oleifera leaf extract dissolved in 100 ml Na CMC 0.5%

Preparation of Methylmercury

In this research 0.4 mg/kg bw was used as dosage of Methylmercury made from the mixture of 4 mg Methylmercury dissolved in 100 ml aquadest.

Microscopic Examination

Histological examination was done using microscope by magnification 1000 times. The examination and calculation of Purkinje cells was done on 5 different field of views, started from the left corner, right corner, upper part, bottom part and the central part of the histological slides. The necrotic Purkinje cells calculated to see the percentage of normal cell against the methylmercury toxicity. The calculation of necrotic Purkinje cells percentage using the following formula (Ausubel et al., 2003):

\[
\text{% Necrotic Purkinje Cells} = \frac{\text{Total Number of Necrotic Purkinje Cells}}{\text{Total Number of Purkinje Cells Counted}} \times 100\%
\]

Data Analysis

The data of necrotic Purkinje cell were taken on each group and would be analyzed statistically by One-way ANOVA to find the differences between treatments. The mean differences among treatments would be tested by Duncan Multiple Range. Data statistical analysis would be using the SPSS (Statistical Product and Service Solution) 22 for Windows software.

Research Result and Discussion

After 21 days of treatment was done, the observation and necrotic Purkinje cells calculation would be done. From the physical observation some mice from C+ group were showing a major clinical symptom of central nervous system disorder, specifically gait abnormalities.

While in other group treatments, it was not found any physical abnormality. Data were obtained from each of treatment groups with 4 repetition for each group treatments. According to the percentage of necrotic Purkinje cells calculations, the mean of necrotic Purkinje cells in mice (Mus musculus) cerebellum is as followed:
Table 1. Mean and Standard Deviation of Necrotic Purkinje Cells Percentage in Mice Cerebellum.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>C −</td>
<td>6.92 ± 2.21</td>
</tr>
<tr>
<td>C +</td>
<td>64.96 ± 9.07</td>
</tr>
<tr>
<td>T 1 (200 mg/kg bw)</td>
<td>41.55 ± 3.34</td>
</tr>
<tr>
<td>T 2 (400 mg/kg bw)</td>
<td>22.84 ± 1.88</td>
</tr>
<tr>
<td>T 3 (800 mg/kg bw)</td>
<td>17.76 ± 6.59</td>
</tr>
</tbody>
</table>

*Different superscript in the same column refers to significant difference (p<0.05).

Based on the table above, the difference necrotic percentage of Purkinje cells in mice cerebellum between each treatment groups can be clearly observed on the graphic below.

Graph 1. Mean of Necrotic Purkinje Cells Percentage in Mice Cerebellum.

It can be concluded from the data above that there is a significant difference between C+ (0.4 mg/kg bw MeHg) group, T1 (200 mg/kg bw Moringa oleifera+ 0.4 mg/kg bw MeHg) group, T2 (400 mg/kg bw Moringa oleifera+ 0.4 mg/kg bw MeHg) group and T3 (800 mg/kg bw Moringa oleifera+ 0.4 mg/kg bw MeHg) group (p<0.05). The comparison between T1 (200 mg/kg bw Moringa oleifera+ 0.4 mg/kg bw MeHg) group and T2 (400 mg/kg bw Moringa oleifera+ 0.4 mg/kg bw MeHg) group shows a significant difference, as well as the comparison between T1 (200 mg/kg bw Moringa oleifera+ 0.4 mg/kg bw MeHg) group
and T3 (800 mg/kg bw Moringa oleifera+ 0.4 mg/kg bw MeHg) group. However, T2 (400 mg/kg bw Moringa oleifera+ 0.4 mg/kg bw MeHg) group does not show a significant difference compared to T3 (800 mg/kg bw Moringa oleifera+ 0.4 mg/kg bw MeHg) group.

Histopathological observation and examination of necrotic Purkinje cells in cerebellum of mice (Mus musculus), will be demonstrated by the figure below.

Figure 1. Comparison of purkinje cells histopathological features in cerebellum of mice (Mus musculus) of each groups. C – (showed normal purkinje cells with open face type; round shape), C + (showed the abnormality in purkinje cells morphology with flattened purkinje cells), T 1 (showed apoptotic purkinje cells with nucleolus undistinguishable), T 2 (showed normal purkinje cells), and T 3 (showed normal purkinje cells) (Stain: H.E.; 1000× magnification).
Methylmercury the most toxic form between others (elemental mercury, inorganic mercury) because of its ability to cross the blood brain barrier due to its lipid solubility and actively bind with cysteine forming a methionine like substance, this substance will be carried across blood brain barrier by methionine transporter (Newland, 2002).

Methylmercury exposure increases the rate of ROS in cerebellum and lead to cell damage (Do Nascimento et al., 2008). Oxidative damage could contribute to the deletions and mutations in mitochondrial DNA. Damage to mitochondrial DNA could play a role in neurodegenerative diseases: mitochondrial deletions and increased steady-state mitochondrial oxidative DNA damage have been reported in Alzheimer’s diseases (Wiseman et al., 1996).

Methylmercury form very stable complexes with biomolecules, particularly with proteins that contain sulfhydryl group (-SH or thiol functionality). With the result that Methylmercury is capable of inactivating the sulfhydryl-containing enzymes at very low concentrations (Scarmoutzos et al., 2003). Glutathione is one of the crucial non protein thiol that plays an important role in intracellular defense against ROS- induced oxidative damage. When the stress increased, GSH concentrations dropped and redox state become more oxidized, which marked the degradation of the system (Sharma et al., 2012).

In this research, it is showed that there is a significant differences between groups C +, T1, T2 and T3, it demonstrated the reduction in necrotic Purkinje cells damagein cerebellum with Moringa oleifera leaf extract administration (p<0.05). However, there was no significant difference between groups T 2and T 3 (p>0.05), it might be due to the dose of 400 mg/kg bw and 800 mg/kg bw of the Moringa oleifera leaf extract had similar amount of active substance and gave identical effect in providing a protective effect against cerebellum toxicity induced by methylmercury.

Moringa oleifera leaves extract is the potential neuroprotectant which is underlying mechanism may occur partly via the decreased oxidative stress (Kirisattayakul et al., 2013). The ethanolic extract of the Moringa oleifera leaf, rich in phenolic components, showed increased GSH content (Luqman et al., 2011). Moringa oleifera improved memory of young Wistar albino rats probably by increasing antioxidant enzymes (Rahmath et al., 2015). Other studies also show that Moringa oleifera leaf extract significantly normalized the depleted antioxidant capacity in rat brain caused by chlorpyrifos (Oyewole et al., 2014).

Moringa oleifera has 3 major antioxidantsubstances, cryptochlorogenic, astragalin and isoquercetin. Chlorogenic acidand its isomers are esters of quinic and caffeic acids that have abilities to inhibit oxidationand also promote various pharmacological activities. Astragalin is reported as a natural antioxidant agent exhibiting some biological properties such as cellular protective effect (Vongsak et al., 2013). Isoquercetin is one of the major glycosidic forms of natural flavonol quercetin and have been known as powerful natural antioxidant (Day et al., 2000).

Conclusion

In this present research, it is found that Moringa oleifera leaf extract reduced the number of necrotic Purkinje cells in mice (Mus musculus) cerebellum induced by methylmercury. The dose of 800 mg/kg bw Moringa oleifera leaf extract could provide the best protective effect compared to dose of 200 mg/kg bw and 400 mg/kg bw.
References


