**ABSTRACT**

**Background:** Physiological aging and due to oxidative stress in long term will have an impact on cellular response disorders, can caused aging of hippocampus and senility. Brain weight is known to decrease with age and p16INK4a as aging biomarkers have been investigated. Andaliman is one of typical herbal plants from North Sumatra has been widely used as an antioxidant, anti-inflammatory and anti-aging. **Objective:** This study was evaluated effect of andaliman (Zanthoxylum achantopodium DC) fruit ethanol extract (AEE) on brain weight and p16INK4a expression in aging model rats. **Methods:** This study was carried out experimentally of 24 male wistar rats. The treatment group consisted of 4 groups; KN= negatif control (normal), KP= positif control (aging model rat), P1 and P2= aging model rat + AEE at dose 150 and 300mg/kgbw respectively. The aging model rats were D-galactose-induced at dose of 150mg/kgbw for 8 weeks. Brain weight were recorded by digital scales. p16INK4a expression using immunohistochemical methods. The data analysis with Anova test. **Results:** This study showed differences brain weight between groups (p=0.523). Brain weight in P1 (1.34±0,06) and P2 (1.30±0.09) tendency increased than KP (1.29±0.62). The p16INK4a expression between groups significant difference (p=0.041), continued with post hoc Least Significant Difference (LSD) showed p16INK4a expression in KN significant decreased than KP (p=0.027). Likewise, p16INK4a expression in P2 was significant decreased than KP (p=0.010). **Conclusion:** Andaliman ethanol extract at a dose 300mg/kgbw for 8 weeks was improved aging process caused D-galactose induced.

**Keywords:** Zanthoxylum achantopodium DC, brain weight, p16INK4a, aging model rat.

1. **BACKGROUND**

The Aging induced by chemical compounds, such as D-galactose, has been used as a model for aging in experimental animals such as mice, rats and dogs (1, 2). Excessive consumption of D-galactose caused conversion to D-galacto-hexodiadoses and hydrogen peroxide by galactose oxidase and to galactitol via axialdose reductase (3). These products accumulated in cells which in turn caused osmotic and oxidative stress which may explain accelerated aging. Many studies on experimental animals as aging models have been carried out. Ji et al. (2017) used a 2-year-old dog as a model of D-galactose-induced aging 50 mg/kg/day for 90 days, showed significant increased the expression of p16INK4a and p21 in brain tissue (1). Another study showed D-galactose-induced at a dose of 150 mg/kgbw/day for 6 weeks was increased oxidative stress and apoptosis in rat brain tissue (4, 5). The p16INK4a protein synonymous with cyclin-dependent kinase inhibitor 2A (CDKN2A), is a protein that slows cell cycle progression from G1 to S phase (6). The studies have investigated whether senescent astrocytes display aging markers (7). Human brain tissue, with age, show increased number of astrocytes positive for p16INK4a, matrix metalloproteinase 3 (MMP3) and SASP-associated proteases (8). Astrocytes in vitro response to ROS exposure, characterized by SAgal activity, growth arrest and increased expression of p16INK4a and p21 (9). Ex-
posure to ionizing radiation caused excessive DNA damage which is also capable caused aging and SASP production in human astrocytes (10). The study on transcriptional changes that accompany ROS-induced aging in human astrocytes has shown that genes associated with neurodevelopment and differentiation are downregulated whereas pro-inflammatory genes are up-regulated (11). Interestingly, senescent astrocytes also downregulated genes associated with astrocyte activation. Astrocytes become active or reactive as a result of various pathogens, including neurodegenerative diseases (12). If aging has affected astrocytes, they may not be able to achieve a normal response to this pathological condition (11). Brain derived neurotrophic factor (BDNF) expression, as a marker neuroprotective, has been shown decreased in aging tissues. Studies with human subjects showed that hippocampus volume decreased with decreasing plasma, suggested that association might explain some of cognitive decline that occurs during aging (13).

The andaliman fruit ethanol extract with a concentration of 300 mg/ml for 4 weeks showed anti-aging and antibacterial activity (14). Another study showed andaliman fruit ethyl acetate extract at a dose of 300 mg/kgbw for 9 days had a cardioprotective effect against doxorubicin-induced cardiomyopathy in rats (15). However, the effectiveness of andaliman fruit ethanol extract has never been reported on the decline aging process caused by oxidative stress.

2. OBJECTIVE
The aim of the study were threefold: a) to analyze effect of andaliman fruit ethanol extract on brain weight; b) to analyze effect of andaliman fruit ethanol extract on p16INK4a expression of hippocampus in aging model rats.

3. MATERIAL AND METHODS

Animal and Treatments
This study was experimental study with a post test only control group design, it was carried out on experimental animals with D-galactose-induced aging rats consisting of several treatment groups. A total of 24 male wistar rats, weighing 200-250g were divided into 4 groups, namely KN, KP, P1 and P2, 3 months of age rat, given a standard diet. KN: given a standard diet, as normal group; KP: aging model rats, injected D-galactose subcutaneously at dose 150mg/kgbw, as positive control and treatment group (P1 and P2): aging model rats + AEE at doses of 150 and 300mg/kgbw for 8 weeks, respectively. They were kept under controlled conditions (room temperature, 22 24˚C; relative humidity, 40% 60% and acclimatized to the housing environment for 1 week prior to the experiment with free access to feed and distilled water. Body weight from each group were recorded every 1 weeks. The rats were sacrificed on the last day of treatment, brain weight were assessed on each slide in the field of view in the hippocampus area with 400x magnification, 20 fields of view each, images of each slide were then photographed with microscope Olympus CX22 and calculation of cell number using the image J application (16).

Statistical analysis
The data were analyzed statistically via the SPSS software version 24.0 (SPSS Inc., Chicago, Illinois). All the variables in this sample of the study were tested by Shapiro–Wilk, the data was normally distributed (p > 0.05) were tested by parametric test (Anova one way), but the abnormal distribution variables (p < 0.05) were tested by Non Parametric test, Mann-Whitney test.

4. RESULTS

Sample Characteristics
Twenty four wistar rats, male, weighing 200-250g, grouping into 4: namely KN, KP, P1 and P2 groups. The rats were sacrificed after the last treatment. Brain weight assessment of experiment rats listed in the Table 1.

Table 1. Differences of brain weight between treatment groups

<table>
<thead>
<tr>
<th>Groups</th>
<th>Brain weight (g) (x ± SD)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>KN (n=6)</td>
<td>1.35 ± 0.96</td>
<td></td>
</tr>
<tr>
<td>KP (n=6)</td>
<td>1.29 ± 0.62</td>
<td>0.523</td>
</tr>
<tr>
<td>P1 (n=6)</td>
<td>1.34 ± 0.06</td>
<td></td>
</tr>
<tr>
<td>P2 (n=6)</td>
<td>1.30 ± 0.09</td>
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</tbody>
</table>

Table 2. Differences of p16INK4a expression between treatment groups

<table>
<thead>
<tr>
<th>Groups</th>
<th>p16INK4a expression of hippocampus (cell count) (x ± SD)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>KN (n=6)</td>
<td>36.11 ± 9.73</td>
<td></td>
</tr>
<tr>
<td>KP (n=6)</td>
<td>47.75 ± 8.02</td>
<td>0.041*</td>
</tr>
<tr>
<td>P1 (n=6)</td>
<td>42.18 ± 9.99</td>
<td></td>
</tr>
<tr>
<td>P2 (n=6)</td>
<td>33.77 ± 5.19</td>
<td></td>
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</tbody>
</table>

4.1 Measures
Immunostaining of p16INK4a of the hippocampus brain tissue was performed using 5-µm-thick with PBS containing 0.05 M EDTA followed by 4% formaldehyde fixed, parafin-embedded tissue sections, which were deparaffinized using xylol. As a p16INK4a polyclonal antibody (Thermo Fischer, PA5-20379) was used and detected using Powervision (DAKO A/S, Denmark) and peroxidase-DAB visualization. Two independent pathologist performed evaluations of the immunostained samples. The cell examination was carried out on each slide in the field of view in the hippocampus area with 400x magnification, 20 fields of view each, images of each slide were then photographed with microscope Olympus CX22 and calculation of cell number using the image J application (16).

Table 2. Differences of p16INK4a expression between treatment groups.
* = significantly (p<0.05), One Way Anova test.
different groups (p<0.05) were indicated by different lowercase letters (One Way Anova test with post hoc LSD); I=standard deviation; KN=normal group; KP=positive control; P1=AEE at 150mg/kgbw/day; P2=AEE at dose of 300mg/kgbw/day.

Based on figure 1, the p16INK4a expression showed difference between treatment group. The post hoc LSD test showed that administration of AEE at dose of 300mg/kgbw reduced p16INK4a expression compared with aging model groups (KP) significantly (p=0.010).

The figure 2 showed increased brown color in nucleus of hippocampus neuron cells after D-galactose-induced in KP group compared to normal group (KN). The brown color in nucleus of hippocampus neuron cells was significantly decreased in P2 group compared to KP group.

5. DISCUSSION

D-galactose is an aldohexose that occurs naturally in the body, included in brain, but when the dose of D-galactose is given over dosess, can induced of aging effects in several organs result of increased oxidative stress. This study showed brain weight decreased in aging model rats compared normal groups. Brain weight is known to decrease with age. This is line with Lessard-Beaudoin et al, (2015) showed brain weight in C57BL/6 mice increased between 3 and 12 months of age and decreased is observed from the age of 12 months (17). This is similar to studies on brain volume in mice. This decrease could be due to a loss of solid material of the brain as a 36% reduction of solid portion of the brain is observed. A reduction in the concentration of several lipids in the brain is also observed, while a weak increase of the concentration of water was detected. Thus, in ageing a majority of solid-brain material would be lost and a portion replaced by water (18).

This study showed was significant difference the p16INK4a protein expression between the treatment groups after 8 weeks (p<0.05), listed in table 2. The expression of p16INK4a protein was assessed by count number of brown stained cells in nucleus. This study showed that aging model rats (KP) was increased than normal group (KN) significantly (p=0.027), indicated that experimental animals were in an aging condition after D-galactose induced. The p16INK4a protein is one of the markers play a role in aging (19), inhibited the cell cycle in the G1 phase by preventing the association of CDK4 and CDK6 with D-type cyclins and is involved in the aging process (20). This is line with Anggraini et al, (2021) showed expression of p16INK4a in D-galactose 100 mg/kgbw was similar to naturally aging group and the highest expression compared to other groups (p>0.05) (21). The p16INK4a expression was detected at 1 year of age in various rodent tissues, including the neurogenic niche and increased further at 2 years of age (6, 19). Similarly in human tissues, p16INK4a levels increased with aging (21).

This study showed that administration of andaliman ethanol extract at dose 300mg/kgbw/day for 8 weeks reduced p16INK4a expression compared aging model groups, significantly (p<0.05), indicated that andaliman ethanol extract was proven to slow down the aging process caused by D-galactose-induced. Andaliman fruit used in this study was dried andaliman fruit from the Samosir Dolok area, Ronggu NIHuta district, Samosir district. Simplicia powder was extracted by maceration using 96% ethanol as solvent. The phytochemical test showed that AEE contain alkaloids, flavonoids, glycosides, tannins and triterpenoids/steroids. In this study, the total flavonoid content of AE showed 21.0174mgQE/g, with UV-Vis spectrophotometry at a maximum wavelength of 438nm. The antioxidant level test using the DPPH (1,1-diphenyl-2-picrylhydrazli) methods showed IC50 concentration of
108.11 ppm.

Another study showed the IC50 value of andaliman fruit methanol extract had the highest antioxidant activity 390.92 ppm (DPPH method) and 30.04 ppm (ABTS/2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic Acid methods) by using UV-Vis and infrared spectrophotometers. It is suspected that the main active compounds contained in the E2 fraction have the potential as antioxidants are flavonoid compounds of the auron and flavanol groups (22). Any results showed that ethanolic extract of fruit andaliman has the highest antioxidant activity among extracts of acetone, ethyl acetate, and a mixture of ethanol and ethyl acetate, with an IC50 of 344.75 ppm (23).

Flavonoids activated the ERK–CREB pathway and the P13-kinase-mTOR cascade that influence changes in synaptic plasticity and potentially angiogenesis and neurogenesis through eNOS activation. The flavonoids also inhibited pro-apoptotic signaling through inhibition of JNK (Jun N-terminal kinase) and ASK1 (Apoptosis signal-regulating kinase 1). Inhibition of this kinase together with activation of ERK1/2 (Extracellular signal-regulated kinase) suppressed apoptosis and neuroinflammation, thereby preventing neurodegeneration and aging of brain cells (24).

6. CONCLUSION

Andaliman fruit ethanol extract at dose 300mg/kgbw for 8 weeks contributed to slow down brain aging process caused D-galactose-induced.

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