

Anxiolytic Effect Evaluation of Methanol Extract of Ceplukan Leaves in the Elevated Plus Maze Test through IL-6 Level Changes in Ovariectomized Rat

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ABSTRACT

Ceplukan (*Physalis minima* L.) has long been used to treat various conditions in traditional medicine. This study aims to demonstrate the anxiolytic effects of Methanol Extract of Ceplukan Leaves (MECL) in the Elevated Plus Maze (EPM) test and correlate to IL-6 level of ovariectomized rat brain. Total of 24 Wistar rats were divided into six groups: one normal group, one group of 1 month ovariectomized (ovx), one group of 2 months ovx, three groups of 2 months ovx (each given with MECL 500; 1500 and 2500 mg/kg doses for 1 month). The anxiety-like behavior level was measured by EPM test. After EPM test, the brain was removed to measure level of IL-6 by ELISA. The data were processed and analyzed by one-way ANOVA and Pearson correlation. We found that the MECL-treated rats enter the opened-arm higher than the control rats. It indicates that the MECL-treated rats are less anxious than the control rats. The results also show the decreased of IL-6 level in MECL-treated rats.

Keywords: anxiety-like behavior, elevated plus maze, IL-6, *Physalis minima* L.

INTRODUCTION

Anxiety is a major symptom in menopausal women which showed by the high rates of anxiety in menopausal women [1,2]. Previous research shows the relationship between anxiety with increase of proinflammatory cytokine IL-6 [3-5]. At menopause, the production of IL-6 may increased which triggered by the state of estrogen deficiency [6,7]. Estrogen replacement therapy usually used to treat menopausal symptoms. However, HRT raises new problems such as increasing risk of breast cancer, endometrial cancer, venous thromboembolic disease, stroke and ovarian cancer [8]. To avoid such problems, the researchers begin to study the effectiveness of phytoestrogens from natural plants which has estrogen-like effects.

Ceplukan plant (*Physalis minima* L.) has long been used for traditional treatment in Indonesia for various diseases, such as purgative, antihelmintic, febrifuge, and vermifuge. Steroidal lactones from ceplukan believed to have antifertility effect, hypoglycemic, cytotoxic, antiulcer, anti-bacterial, anti-malarial and anti-

gonorrhea [9]. Mulchandani et al. (1979) showed that the plant contained of fisalin steroid A, fisalin B, fisalin C, fisalin D, wita fisalin A, wita fisalin C and 5.6 epoxy fisalin [10]. There were similarities between mechanism of *Physalis minima* L. extract and 17 β -estradiol, so *Physalis minima* L. can be classified as phytoestrogens [11]. Even so there was no further research whether ceplukan could reduce anxiety in menopausal women.

The aim of the study was to determine whether MECL has anxiolytic effect on EPM test through the changes of IL-6 level in the rat brain after ovariectomy.

MATERIALS AND METHODS

Animals

Three-month female Wistar rats (*Rattus norvegicus*) (195 \pm 3 g; n=24), weighing before and after experiment, kept in cages made of a plastic with a lid made of woven wire cage, with a cycle of 12 hours light/dark, fed and watered by ad libitum. After 7 days acclimatization, the experimental animals were divided into 6 groups (each consist of 4 rats): one normal group, one group of 1 month ovx, one group of 2 month ovx, 3 groups of 2 months ovx (each treated with MECL 500; 1500 and 2500 mg / kg doses for 1 month). All procedure were approved by Health Research Ethics Com-

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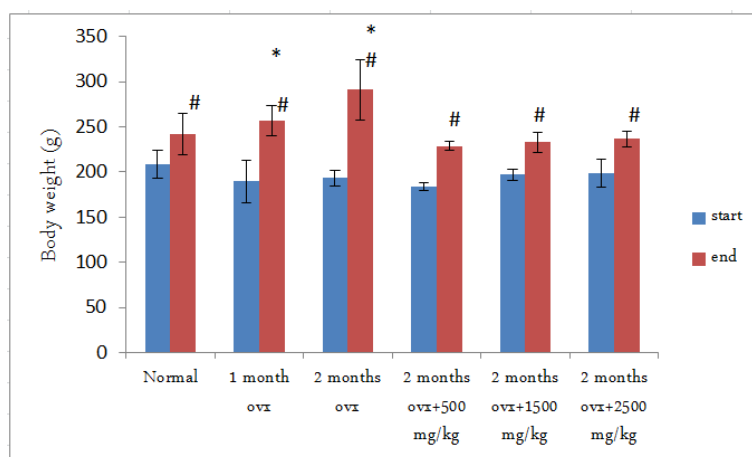


Figure 1. Body weight changes in normal and ovx rats with and without MECL treatment. Body weight was measured from the start to the end of experiment; # $p < 0.05$ compared with other group by oneway ANOVA analysis; * $p < 0.05$ compared with other group at Tukey Post Hoc analysis. The data were expressed as means \pm SD.

mittee of University of Brawijaya.

Sample Preparation

Ceplukan leaves were obtained from Balai Tanaman Obat Materia Medika, Batu, Indonesia. Ceplukan leaves (dry powder) that have been weighed and wrapped in filter paper, inserted in funnel extraction then soaked with methanol to obtain the compounds. The solution from immersion process then collected and precipitated. The solution was separated and collected from the precipitated product then dried in rotator evaporator at 70-80°C to obtain the thick extract. The product then heated in oven at 70°C to remove the remaining methanol.

Ovariectomy

The rats were anaesthetized by intramuscular (IM) injection of ketamine (40mg/kg). Hair at ventral was

shaved approximately 1 cm above the imaginary line ovaries. The site then cleaned with povidone-iodine and alcohol 70%. The paralumbar lateral incision was made using a sharp knife and the ovaries were removed. The wound was sutured using catgut and covered by sterile gauze. Each rat was injected with gentamicin (60-80 mg/kg, IM) and cleaned with povidone-iodine during 3 days after surgery to prevent post-operative infection.

Behavior Test

Behavioral assessment using EPM test, a standard test to measure anxiety-like behavior in rats [12,13]. The EPM apparatus was made of wood which has two open arms (50x10 cm), two contradictory close arms (50x10x40 cm) with an open square in the middle (10x10 cm), placed 50 cm above the floor. The open arms then given a 1 cm wall. The arms basis was put

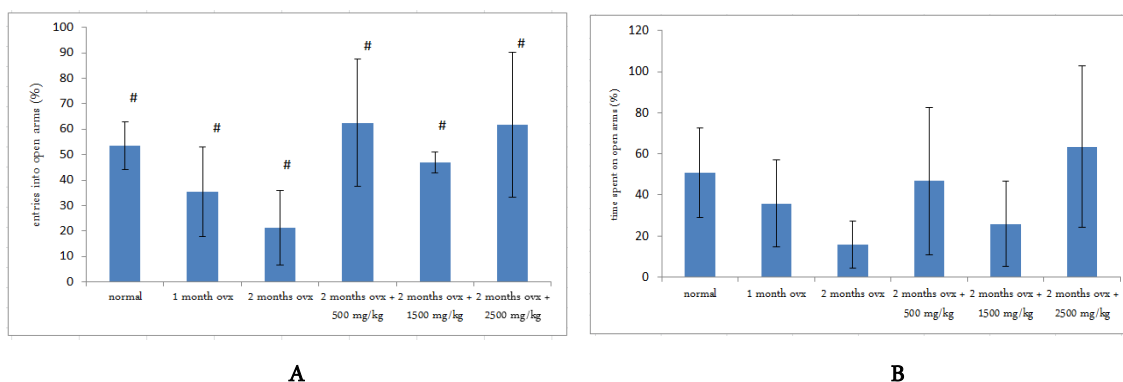


Figure 2. **A.** Open arm entry, **B.** Open arm time as determined by EPM test in normal and ovx rats with and without MECL treatment; # $p < 0.05$ compared with other group by oneway ANOVA analysis; * $p < 0.05$ compared with other groups at Tukey Post Hoc analysis. The data were expressed as means \pm SD.

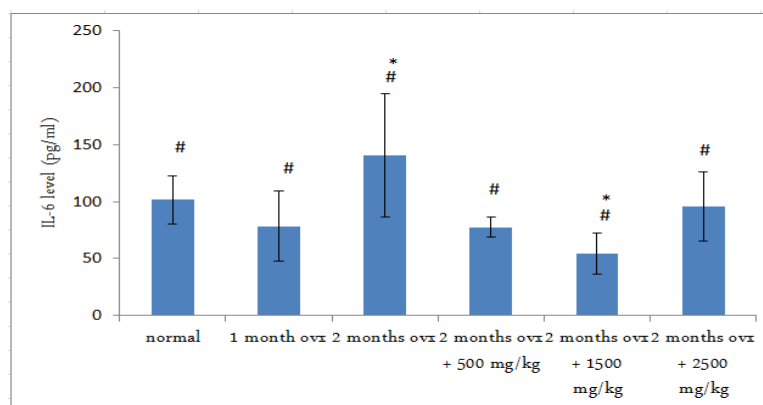


Figure 3. Level of IL-6 brain rats in normal and ovx rats with and without MECL treatment; # $p < 0,05$ compared with other groups by oneway ANOVA analysis; * $p < 0,05$ compared each other at Tukey Post Hoc analysis. The data were expressed as means \pm SD.

up rough plastic to facilitate the cleansing of rats feces [13-15]. The instrument placed at closed room which had low noise and low lighting. Tests carried out for each rat at the same hour every day (at 16:00 pm to 18:00 pm). During the EPM test, the rats were placed in the apparatus and let it explored the maze for 5 minutes. The rat activities were recorded by using video. The video then analyzed for: a) percentage of rat entered the open arms (total for each rat) b) percentage of total time spent in the open arms [16]. Rat which entered the arms was defined as all four paws being placed in the arms.

Measurement of IL-6 level

After the EPM test, the rats were exposed to ether vapor and their brains were removed after cardiac perfusion. The hippocampus were dissected out on an ice-bath plate B, based on [17], the hippocampus was taken off approximately 100 mg and washed with distilled water and 1 ml PBS. The protein of the tissue was extracted by homogenizing the tissue in lysis buffer PMSF (containing Tris base 0,1211 g; EDTA 0,0074 g; NaCl 0,8775 g; 0,009 g PMSF; NP 40 0,125 ml, 100 ml diionized, protease inhibitor cocktail 50 μ L) about 2 minutes. The mixture was then incubated 30 minutes at 40°C and cold centrifuged at 6000 rpm for 10 minutes. The supernatant was taken to measure IL-6 levels using Quantikine Rat IL-6 kit (R & D Systems, USA & Canada) according to the manufacture instructions.

Statistical Analysis

The results were expressed as means \pm SD. Multiple comparisons were analyzed by one-way analysis of variance (ANOVA) followed by post-hoc analysis with Tukey method. The relationship between two variables were examined using Pearson correlation method. The

level of significance was $p < 0,05$. All statistical tests were performed by SPSS 17.00.

RESULTS AND DISCUSSION

Body Weight

Ovariectomy significantly increased the body weight. Rats which given 1 month ovx and 2 months ovx respectively gained the body weight; 67 grams and 97,5 grams. Significantly different with normal rats which only gained 33 grams. The results of oneway ANOVA analysis shows that there are significant differences on body weight gain ($p = 0.000$) in all groups. Further analysis using Tukey's Post Hoc Test shows that there are significant differences on weight gain between 1 month ovx and 2 months ovx groups with the other groups ($p < 0,05$) (Fig.1).

Behavior Test

The EPM results show that anxiety-like behavior in ovx rats is increased. Treated rats for 1 month and 2 months ovx have lower percentage of open-arm entries and open-arm time than others. The MECL-treated rats has a greater number of percentage total rat entered the open arms ($p < 0,05$). Groups of Ov x + MECL more often entered the open arms, so they have greater percentage (respectively 62,5%, 46,95% and 61,67%) compared to 2 months ovx rats which only 21,4%. The results indicate an anxiolytic-like effect of MECL in rats. Dose of 500 mg/kg give the lowest levels of anxiety (Fig.2A).

The EPM results show that MECL-treated rats spent more time in the open arms, however the results of oneway ANOVA shows no significant differences between the six-groups of rats ($p = 0,190$) (Fig.2B).

IL-6 Levels

As shown in Fig.3, the level of IL-6 on 2 months ovx group is higher than normal group (140,63 pg/ml compared to 101,65 pg/ml). Interestingly, the level of IL-6 on 1 month ovx group is lower than normal rats. All three dosage MECL used in this study (500, 1500 and 2500 mg/kg/day) significantly decreased IL-6 level in the hippocampus of ovx rats ($p < 0,05$). Further analysis using Tukey's Post Hoc Test shows that there are significant differences between groups of rats which given MECL 1500 mg/kg with 2 months ovx rats ($p < 0,05$).

To determine the relationship between the anxiolytic effects of MECL with the levels of IL-6 in treated rats, we used Pearson correlation analysis. The results show no significant relationship between the percentage of the rat entered the open arms and level of IL-6 ($p > 0,05$), with a weak correlation coefficient ($r = 0,008$). Furthermore, Pearson correlation analysis was also used to determine the relationship between the percentage of rats spending time in the open arms and level of IL-6. The result shows no significant correlation between the percentage of rat spending time in the open arms and level of IL-6 ($p > 0,05$), with a weak correlation coefficient ($r = 0,019$).

The present study showed that the body weight in the 1 month and 2 months ovx rats increased significantly compared to the group without ovariectomy, it indicates that ovariectomy on this study is successful [18]. The results also show that anxiety-like behavior in each rat groups have a high standard deviation. The standard deviation shows the distribution of the average individual, in other words the higher value gives higher variation [19]. The high variations of anxiety-like behavior is caused by the parameters related to "trait", where animal has a wide range which reflected high variation in the dimensions of the human personality [13,20].

The increasing of anxiety-like behavior in ovx rats without MECL showed on EPM test, although only a percentage of total rat entering the open arms which gave statistically significant result ($p < 0,05$). While the result of total spending time percentage in the open arms showed not significance. Previous study showed indices of anxiety can vary independently [16]. This study not in accordance with previous study, which analysis factor of time spent in the open arms should contribute more to anxiety than the rat enter the open arms (-0,95 compared to -0,86) using EPM test [21]. The cause is most likely due to the manual apparatus in this study. This is consistent with previous references which show different laboratories will produce

varying EPM data test [22].

The MECL showed an anxiolytic effect. The effect is confirmed by all three dosage used in this study (500, 1500 and 2500 mg/kg/day) which reduced anxiety-like behaviour in EPM test. Dose of 2500 mg/kg showed consistency on reducing anxiety, both at percentage of total rat entered the open arms and percentage of total time spent in the open arms. There is no specific pattern of dose-response curve.

The result showed that the levels of IL-6 on brain in 2 months ovx rats is the highest within groups. Interestingly, the level of IL-6 in the brain of 1 month ovx rats group is lower than normal group. This is probably caused by the differences between natural menopause with surgical menopause related with acute changes in estrogen levels. Gradual changes in natural menopause causes the recovery of immune function through feedback mechanisms, but surgical menopause will alter immune function so that the cytokine levels could be lower than the normal value [23].

The present study demonstrate that MECL can reduce the levels of IL-6 in the brain. Level of IL-6 in the MECL-treated rats are lower than those of 2 months ovx rats. These results are consistent with previous studies in which level of IL-6 in 24 months ovx, decreased after treated by isoflavone that classified as phytoestrogens [24]. The ability of MECL to reduce level of IL-6 in the brain, indirectly prove estrogen-like effect of ceplukan leaves. The mechanism may through the estrogen receptor (ERs). Estrogens or phytoestrogens will show biological activity after binding to ERs. Activated ERs will block NF κ B gene by changing the conformation of protein and unable to activate the IL-6 gene promoter. Activated ER by estrogen will give biological effects on NF- κ B with protein conformational changes which lead to the inability to bind DNA [7].

Statistical analysis shows that there is no significant relationship between the anxiolytic effects of MECL with IL-6 level in brain of MECL-treated rats. The results show that the decreasing of anxiety-like behavior in MECL-treated rats is not through decreasing levels of IL-6, but through other pathway. The accepted path is serotonergic pathway. Estrogen affects the serotonin system by increasing the degradation of monoamine oxidase, an enzyme that breaks down serotonin, and affecting serotonin transport intraneuron, which result in increasing the availability of serotonin in the synapse, and changing the mood into good feeling [25].

Previous studies examining the potential of diosgenin, one of phytoestrogens, as an anxiolytic agent

leads to reversed U-shaped dose-response curve [26]. No previous studies which examined the anxiolytic effects of MECL. This study did not obtain a specific pattern of dose-response curves. It probably caused by a few amount of treatment doses. So further research is recommended to increase the treatment doses.

CONCLUSIONS

The methanol extract of ceplukan leaves (*Physalis minima* L.) shows anxiolytic effects by using EPM test. It does not through changes on the level of IL-6 in ovariectomized rat brain. Further research is suggested to investigate molecular mechanism of the anxiolytic effects.

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