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The Effect of Ethanol Extract of *Marsilea crenata* Presl. Leaves on Rotenone-Induced Zebrafish Locomotor Activity

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ABSTRACT

Neurodegenerative diseases are mostly experienced by postmenopausal women and are often caused by estrogen deficiency, so it is necessary to replace the hormone estrogen in the form of phytoestrogens. One of the plants that contain phytoestrogens is *Marsilea crenata*. The objective of this study is to see if giving rotenone-induced zebrafish a 96% ethanol extract of *M. crenata* leaves increases locomotor activity. This research was conducted by inducing 5 µg/L rotenone as a compound that will interfere with the locomotor activity of zebrafish. Next, treatment was given with 96% ethanol extract of clover in each group with a dose of 2.5; 5; 10; and 20 mg/mL to determine the effect of the extract on increasing locomotor activity of rotenone-induced zebrafish. Observations were made by looking at the quantity of zebrafish swimming every five minutes until day 28. The treatment of 96% ethanol extract of *M. crenata* leaves could significantly increase zebrafish motility at the optimum dose of 2.5 mg/mL, because every week the zebrafish locomotor activity increased compared to the negative control. *M. crenata* leaves extract is proven to prevent neurodegenerative diseases. However, further research needs to be done on the degenerative effects of rotenone every week.

INTRODUCTION

In the past decade, the prevalence of neurodegenerative disorders with dementia-like symptoms has increased globally (Prince *et al*., 2015). This is linked to an increase in women's life expectancy, which is now 70-80 years, and a relatively steady menopausal age of 50-51 years. Women spend more than a third of their lives in menopause as a result of estrogen insufficiency, which produces a variety of health concerns, one of which is a neurodegenerative disease (Ma'arif *et al*., 2021).

Decreased estrogen action in maintaining homeostasis of numerous mechanisms in the central nervous system (CNS), which can lead to dementia owing to neuronal cell death, is neurodegenerative in cases of estrogen insufficiency (Numakawa *et al*., 2011; Fiocchetti *et al*., 2012). Parkinson's disease is an example of a disease that frequently develops when dementia progresses uncontrolled (Engler-Chiurazzi *et al*., 2017). Several medications, such as celecoxib, ibuprofen, minocycline, and hormone replacement therapy (HRT), can be

used to treat neurodegenerative diseases (Radtke *et al*., 2017; Dong *et al.,* 2019). However, long-term use of these medications might cause nausea, gastritis, abdominal pain, hypertension, migraines, vertigo, and other side effects (Wixey *et al*., 2012; Garrido-Mesa *et al.,* 2013; Zheng *et al*., 2019). These negative side effects highlight the need for more research into new medication sources with low side effects, one of which being phytoestrogens (Ma'arif *et al*., 2019).

Phytoestrogens are plant-derived chemicals that have a structure comparable to estrogen or can replace estrogen's role in maintaining organ homeostasis (Chui *et al.,* 2013), making them a promising treatment option for postmenopausal women with neurodegenerative diseases (Yang *et al.*, 2012). They can be found in a variety of plants, including *Marsilea crenata* Presl. (*M. crenata),* which is one of the plants used as a particular cuisine for the local community in Surabaya, East Java, Indonesia. Phytoestrogen content and neuroprotective capabilities of *M. crenata* leaves have been demonstrated in multiple prior investigations, both in silico using the results of metabolite profiling of the estrogen receptor β (ERβ) and in vitro using various biomarkers on HMC3 microglia (Agil *et al*., 2021; Ma'arif *et al*., 2019; Ma'arif *et al*., 2021). The neuroprotective effects of *M. crenata* leaves on the locomotor function of zebrafish (*Danio rerio*), which can represent the level of central nervous system (CNS) health in people, were examined in vivo in this work. The utilization of zebrafish is representatively employed as a test model for neuroprotective treatment in humans due to the resemblance of genes that mimic humans or mammals (Becker *et al*., 2012; Stewart *et al*., 2015). Continued research into *M. crenata* will aid in the creation of neuroprotective herbal products to improve the health of post-menopause woman, as well as the attempt to maintain Indonesia's ethnocultural wealth.

METHODS

Plants and Chemicals

M. crenata leaves were collected in September 2019 in Benowo Village, Surabaya, East Java, Indonesia, and identified with a key of determination 1a-17b-18a-1 at UPT Materia Medica, Batu, East Java, Indonesia. To keep their green tint, the leaves were dried and powdered carefully. The chemicals used included 96% ethanol solvent (Merck). Rotenone (Sigma 8875), dimethyl sulfoxide (DMSO) (Sigma-Aldrich), and tween 80 (Sigma-Aldrich)

Zebrafish

Adult female zebrafish (six months) were obtained from the Faculty of Fisheries at Universitas Brawijaya. Zebrafish were acclimatized in a 7 L open tank and reared according to protocol prior to treatment. Tetra Bit and Color Tropical Flakes (Blacksburg, Germany) were fed three times per day, and the fish were kept in a 10:14 dark-light cycle with water temperatures between 24-26.5 ºC. The Research Ethics Commission of Brawijaya University gave its approval to all of the procedures (No. 107-KEP-UB-2021).

Extraction

Using ultrasonic-assisted extraction methods (UAE) with an ultrasonic bath (Sonica 5300EP S3), *M. crenata* leaves dry powder was extracted with 96% ethanol with a volume ratio of 1:20. This procedure was repeated to collect all of the supernatants, which were then evaporated (HEIDOLPH Hei-VAP G3) and dried at 40ºC in an oven (Memmert) to get an extract of *M. crenata* leaves (Ma'arif *et al.*, 2019).

Rotenone and *M. crenata* treatment

In this experiment, 5 µg/L rotenone (Sigma 8875) was used to cause PD in zebrafish, as described by Khotimah *et al*., (2015). Six fish were placed in one tank for each group (L x W x H: 25cm x 14.5cm x 22cm), fed 3 times per day, and every 48 hours the medium was changed. The sample consisted of a 96% ethanol extract of *M. crenata* in several dosages (2.5, 5, 10, and 20 mg/mL) given concurrently with rotenone for 28 days. The sample was previously prepared with 0.5 percent tween 80 in 0.5 percent DMSO to ensure the suspension's stability.

Motility observation

Locomotor activity impairment is the characteristic of Parkinson's disease (PD). The method used follows that of Khotimah *et al*., (2015) modified according to the research objectives. Adult zebrafish locomotor activity was monitored in a 1.5 L water system tank (LxWxH: 25cm x 14.5cm x 22cm). Fish have an innate need to swim back and forth across the tank's length. Simple observation was used to determine adult zebrafish locomotor activity and their movements were captured in a 5-minute video, which was then manipulated using Adobe Photoshop and Adobe Premiere software to create three vertical lines drawn at equal intervals on the tank, dividing it into four zones (the length of each zone was 5 cm) (Ma'arif *et al*., 2022). To determine locomotor activity, the

number of lines traveled by adult zebrafish was counted for five minutes. As a result, the adult zebrafish's overall distance traveled was proportionate to the total number of lines crossed. The zebrafish's total number of lines crossed divided by time, was then used to compute locomotor activity, which was given in the number of crossed lines/5 minutes. The findings of the calculation of locomotor activity were then examined using SPSS 20 program (IBM Corp., Armonk NY) with one-way ANOVA (p<0.05) used for statistical analysis.

RESULTS AND DISCUSSION

Extraction of *M. crenata* Leaves

A total of 39.4 g of 96% ethanol extract was obtained from 900 g of powder extraction, with a yield of 4.4%. *M. crenata's* chemical composition plays a key role in medical and nutraceutical uses, and this is thought to be due to its biologically active phytoestrogen components. Previous investigations have shown that using 96% ethanol as a solvent can extract the active components in *M. crenata* leaves. Kaempferol, an isoflavone group that serves as an estrogen-like chemical as well as an antioxidant and anti-inflammatory agent, is one of the phytoestrogens identified in *M. crenata* (Maarif *et al.*, 2020).

Motility assessment

The zebrafish (*Danio rerio*), a famous model organism for researching gene function and development, has grown in popularity. Because they are vertebrates, zebrafish are more closely related to humans than other genetic model species (Howe *et al.*, 2013). The zebrafish's central nervous system (CNS) patterning, pathfinding, and connectivity have all been deciphered and connected with the human CNS, touch as well as behavioral reactions that have human-like movement patterns, can be monitored (Khotimah *et al.*, 2015).

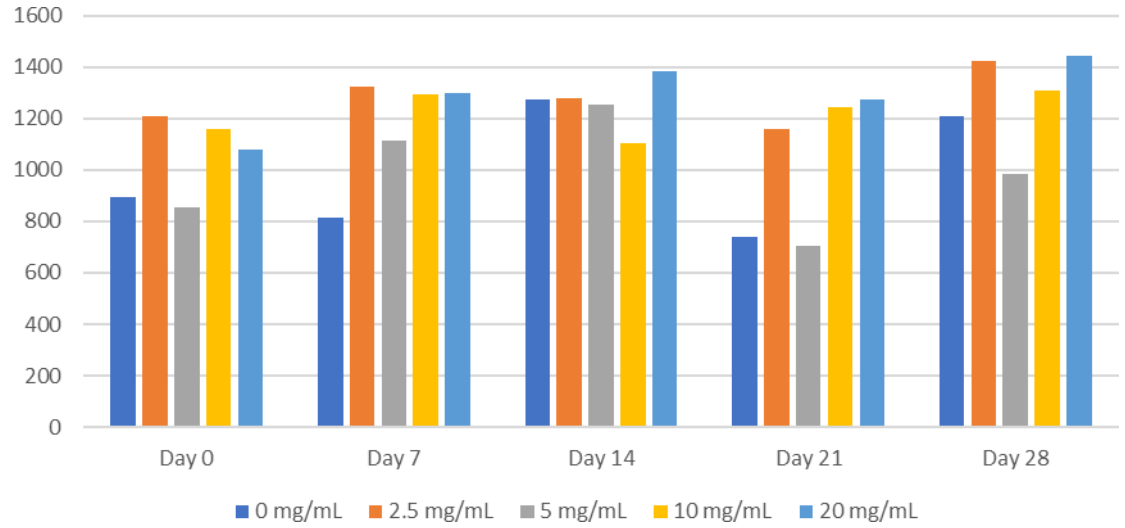


Figure 1. Locomotor activity of zebrafish by induction of 96% ethanol extract of *M. crenata*

Table 1. Zebrafish motility for each dosage group

Day	Mean ± SD of zebrafish motility for each dosage group (cm in 5 minutes)				
	0 mg/mL	2.5 mg/mL	5 mg/mL	10 mg/mL	20 mg/mL
0	896 ± 76.32	1208.33 ±106.96*	852.67 ± 56.13	1160.33 ± 75.43*	1080.25 ± 79.29*
7	814 ± 50.27	1325 ± 50*	1111.67 ± 27.54*	1294.33 ± 21.36*	1297.5 ± 92.96*
14	1276.33 ± 15.95	1281 ± 74.28	1252.67 ± 96.03	1104 ± 88.36*	1385.67 ± 57.76
21	740 ± 49.43	1158.75 ± 50.04*	705.33 ± 4.04	1242 ± 24.02*	1276.33 ± 78.7*
28	1207.67 ± 43.25	1425.33 ± 55.9*	985 ± 63.21*	1309.67 ± 58.40*	1446 ± 48.07*

**Each value is expressed as mean ± SD. Significant differences compared to negative control (0 mg/mL) at p<0.05.*

Rotenone (a naturally occurring toxin and widely used pesticide that inhibits the reduced form of nicotinamide-adenine dinucleotide dehydrogenase in mitochondria) appears to mirror the neuropathological, neurochemical, and behavioral aspects of Parkinson's disease (PD) in vertebrae. The rigidity or loss of capacity to move is a key symptom of PD (Subramaniam and Chesselet, 2013). In this study, giving rotenone did not show a decrease in locomotor activity in zebrafish every week, and this condition was possibly due to the influence of temperature and lighting. Rotenone is also very sensitive to light and oxygen, so it will be degraded to rotenolone, which is less active than rotenone. The decomposition rate of rotenone depends on several factors such as temperature, pH, sunlight, depth, dosage and the presence of organic debris (Radad *et al*., 2019).

To determine the effect of rotenone on locomotor activity, we measured zebrafish motility for five minutes. The decrease in zebrafish motility may be due to a decrease in motor nerve conduction velocity. Evidence of a link between dopaminergic impairment and peripheral motor neuron degeneration in rotenone-induced experimental animals supports this argument (Binienda *et al*., 2013).

The results are shown in Figure 1, where the sample dose of 2.5 mg/mL showed significantly different locomotor activity against the negative control (K. Rotenone) on days 0, 7, 21, and 28. Furthermore, the sample dose of 5 mg/mL showed significantly different locomotor activity against negative controls on days 7, and 28. Then, the administration of a sample dose of 10 mg/mL showed significantly different locomotor activity against negative controls every day. Finally, the administration of a sample at a dose of 20 mg/mL showed significantly different locomotor activity against negative controls on days 0, 7, 21, and 28.

From Table 1, it can be seen that on Day 7, a 2.5 mg/mL dose can increase locomotor activity compared to other doses with a significance value of 0.000 (p<0.05). However, on the 14th day, a dose of 10 mg/mL could increase locomotor activity compared to other doses with a significance value of 0.015 (p<0.05), on the 21st day the dose that could increase locomotor activity was 20 mg/mL compared to other doses with a significance value of 0.000 (p<0.05). Finally, on Day 28 it was found a dose of 20 mg/mL could increase locomotor activity compared to other doses with a significance value of 0.000 (p<0.05).

The optimum dose obtained was of 2.5 mg/mL because it showed the best increase in zebrafish motility compared to other doses and the zebrafish locomotor activity increased compared to the negative control every week. The results from Figure 1 and Table 1 revealed that rotenone could considerably? increase the motility of zebrafish. The treatment of a 96% ethanol extracts every week, as well as increasing the treatment dose, did not always result in an increase in fish motility. The non-monotonic dose-response (NMDR) response of zebrafish to a 96% ethanol extract of *M. crenata* leaves was the reason for this. The NMDR's properties at different points in a dose range are shown by the varied slope values. In this example, phytoestrogen chemicals in a 96% ethanol extract of *M. crenata* leaves caused NMDR, which is common in research with hormone treatment or samples used as hormone substitutes. Because of differences in affinity levels between the hormone or hormone replacement sample and the target, predicting the response that will occur with increasing doses will be challenging (Vandenberg *et al*., 2012).

Differences in receptor affinity and selectivity may cause phytoestrogens to produce an NMDR response. Phytoestrogens bind with high affinity to the estrogen receptors (ER) at low dosages, resulting in a large activity response. However, at greater dosages, phytoestrogens bind to additional receptors found in zebrafish, resulting in reduced activity responses due to antagonistic interactions between the two receptors (Vandenberg *et al.*,2012).

Phytoestrogens, particularly 17β-estradiol, are plant chemicals with a structure and function similar to estrogen. Phytoestrogens can replace estrogen in maintaining body homeostasis, have a higher degree of safety and activity than HRT, and can produce an estrogenic action either by binding to the ER (ER-dependent) or not binding to the ER (ER- independent) (ER-independent) (Alldredge *et al.,* 2013; Wells *et al.,* 2015)

Phytoestrogen compounds contained in *M. crenata* leaves are antineuroinflammatory (one of the neuroprotective mechanisms) and antioxidants through ER-dependent and ER-independent pathways. The presence of binding between phytoestrogens and ER in the ER-dependent pathway can activate ER in the nucleus and inhibit the activation of transcription factors of the inflammatory process. Inflammatory cytokines such as tumor necrosis factor- alpha (TNF-α), interleukin-1 (IL-1), interleukin-6 (IL-6), nitric oxide (NO), and

reactive oxygen species (ROS) are all reduced (Au *et al.,* 2016; Engler-Chiurazzi *et al.*, 2017).

CONCLUSIONS

The treatment of 96% ethanol extract of *M. crenata* leaves could significantly increase zebrafish motility at the optimum dose of 2.5 mg/mL, because every week the zebrafish locomotor activity increased compared to the negative control. *M. crenata* leaves extract is proven to prevent neurodegenerative diseases. However, further research needs to be done on the degenerative effects of rotenone every week.

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