ORIGINAL ARTICLE

Effect of polar fractions of *Marsilea crenata* C. Presl. leaves in zebrafish locomotor activity

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ABSTRACT

Neurodegenerative diseases (NDs) are pathological conditions initiated by the loss of neuronal cell structure and the progressive decline in function caused by prolonged neuroinflammation. Postmenopausal women are at a high risk of experiencing NDs due to estrogen deficiency in their bodies, necessitating the administration of phytoestrogens as a replacement for estrogen in the body. One alternative therapy is administering phytoestrogens, estrogen-like substances from plants, which can be obtained from *Marsilea crenata* C. Presl. leaves. The purpose of this study was to determine whether administration of the n-butanol fraction (BF) and water fraction (WF) of *M. crenata* leaves could increase locomotor activity in rotenone-induced zebrafish. Treatment was given to each group of zebrafish with BF and WF at doses of 2.5; 5; 10; and 20 ppm to determine the locomotor activity. Then an analysis was carried out by looking at each movement of the zebrafish swimming for 1 min at the time of observation on days 0, 7, 14, 21, and 28. The result showed that BF and WF significantly increased the locomotor activity of zebrafish at the optimum dose of 20 ppm for BF and 5 ppm for WF compared to the negative control. This concludes that the polar fraction of *M. crenata* leaves is proven to have the potential to prevent ND progressivity.

Key words: Locomotor activity, *Marsilea crenata*, neurodegenerative, phytoestrogen, zebrafish

INTRODUCTION

Women experiencing menopause have their bodies undergo an estrogen deficiency,[1] which triggers the body to be susceptible to various diseases, including neurodegenerative conditions. Neurodegenerative diseases (NDs) are pathological states caused by the loss of physiological function in neuron cells, resulting in negative impacts such as cognitive decline triggered by prolonged neuroinflammation.[2] Neuroinflammation occurs due to increased microglia activation in the brain, leading to the production of inflammatory cytokines that cause neurons to undergo apoptosis.[3-5]

A solution is needed to prevent ND progressivity due to estrogen deficiency, which can be achieved by providing a source of estrogen-like substances as estrogen substituents. *Marsilea crenata* Presl. is an aquatic plant that has been traditionally used as a traditional food in Surabaya, Indonesia.[6] In various previous studies, *M. crenata* has been proven to contain phytoestrogens and have anti-inflammatory and neuroprotective effects.[3-5,7] One of the

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studies conducted involved a locomotor activity test using 96% ethanol extract of *M. crenata*.[8-10]

In this study, the swimming speed of adult female zebrafish (*Danio rerio*) was used to measure the locomotor activity of fish after they were given the polar fraction of *M. crenata* leaves. The polar fractions used were n-butanol (BF) and the water fraction (WF). The zebrafish model has been extensively employed in *in vivo* investigations due to its demonstrated efficacy.[5,11] Zebrafish are easy to care for and share a lot of genes with mammals, which makes them a good model for studying ND.[12-14] The objective of the study was to demonstrate the potential of polar fractions derived from *M. crenata* leaves in enhancing locomotor activity in rotenone-induced zebrafish and to know their potential to prevent ND progressivity.

MATERIALS AND METHODS

Materials

Plant material

M. crenata leaves were obtained from Benowo Village, Surabaya, East Java, Indonesia, on November 24, 2021, and identified at the Materia Medica UPT in Batu, Indonesia (Determination Letter No: 074 / 133/102.20-A/2022). The leaves were processed into crude drugs through drying and grinding.

Chemical material

The chemicals used were 96% ethanol (Merck, Darmstadt, Germany) for solvent, dimethyl sulfoxide (DMSO) (Sigma-Aldrich, Missouri, USA), Tween 80 (Sigma-Aldrich, Missouri, USA), and Rotenone (Sigma-Aldrich, Missouri, USA).

Zebrafish

Adult female zebrafish (3 months old), wildtype with black color body stripes, measuring 3–3.5 cm in size were obtained from the Faculty of Fisheries and Marine Sciences, Universitas Brawijaya, Malang, Indonesia.

Methods

Extraction and fractionation

The ultrasonic-assisted extraction method was used for the extraction process. Up to 30 g of leaves powder from *M. crenata* were put into 500 ml of 96% ethanol and extracted for 3 min × 2 min. The extract was then filtered, and the filtrate was evaporated with a Heidolph G3 rotary evaporator. The 96% ethanol extract that was made was put into a separating funnel with 700 ml of distilled water and 700 ml of n-hexane. The mixture was then put into 700 ml of distilled water. After shaking the mixture and letting it settle, two phases formed. The n-hexane phase was not used, and 700 ml of ethyl acetate was used to further separate the water phase. The ethyl acetate phase was split off, and 700 ml of n-butanol was added to the water phase to further separate it into two phases. A rotary evaporator was used to collect the BF and WF phases and evaporate them.

Sample preparation

BF and WF were first suspended using 0.5% tween 80 and 0.5% DMSO. Then, the sample solution of each fraction was divided into several dose groups, namely 2.5, 5, 10, and 20 ppm,[9] which were administered together with the rotenone at 5 µg/L as an ND-inducing compound in fish for 28 days. The reason for selecting the above dosage variation groups is based on previous research.

Zebrafish preparation

Acclimatization was conducted for 1 week in tanks divided into six groups, each containing 10 fish, and fed three times a day (PF500 and frozen worms). They were maintained according to laboratory protocols with a light-dark cycle (14:10) and a water temperature of 24°C–26.5°C. The Ethics Committee of Brawijaya University approved all procedures (Ethical Clearance Letter No: 127-KEP-UB-2022).

Zebrafish locomotor activity test

The manifestation of ND includes disturbance in motor activity. The method used refers to the previous study by observing the swimming movements of adult zebrafish in an observation tank with a water volume of 1.5 L (L × W × H: 30 cm × 12.5 cm × 20 cm).[15] Zebrafish have a habit of swimming back and forth along the tank. The observation was carried out to determine the locomotor activity of zebrafish, which was recorded in a 1-min video. Then, the analysis was performed using video analysis tracker software to determine the average speed of zebrafish expressed in m/s [Figure 1]. The obtained data were statistically analyzed using Statistical Package for the Social Sciences (SPSS) version 26 (IBM (International Business Machines Corporation) from Armonk, N.Y.).

RESULTS AND DISCUSSIONS

Zebrafish are often used as model organisms to study genetic development and disease in humans. This is due to the relatively large similarity of the zebrafish gene with the human gene, thus making it an effective test animal model for studying human-related diseases.[15] In this study, rotenone was used as an ND inductor in zebrafish because it has a mechanism similar to the neuropathological causative and behavioral components of ND in rats.[16] Rotenone is a compound commonly used as a pesticide to eliminate harmful animals such as insects and disruptive fish in aquatic areas. Rotenone is highly lipophilic, freely crossing cell membranes, and the blood-brain barrier, and causing toxic effects on neurons through the inhibition of complex I of the mitochondrial electron transport chain, destroying specific dopaminergic neurons, nigral synuclein-positive inclusions, and motor deficits.[15] Based on several research studies, rotenone is used as a substitute agent to represent

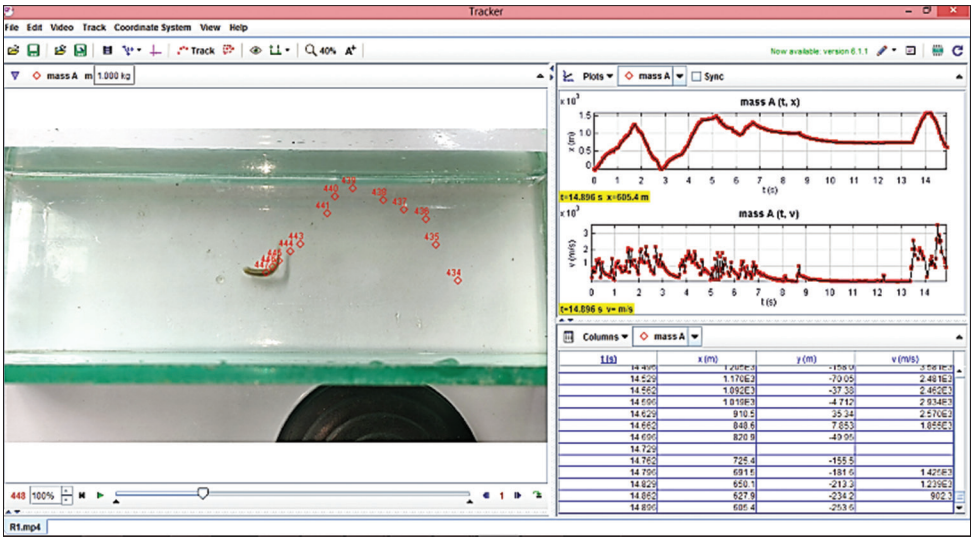

Figure 1: Video analysis using Tracker software

Table 1: Zebrafish locomotor activity as indicated by swimming speed after butanol fraction administration

Day	Speed average (m/s)				
	Negatif	Dose			
		2.5 ppm	5 ppm	10 ppm	20 ppm
0	5.002±0.263	5.372±0.185*	5.008±0.104	5.043±0.200	5.556±0.234*
7	2.399±0.086	3.176±0.150*	3.504±0.133*	3.547±0.229*	3.744±0.293*
14	1.876±0.124	2.604±0.141*	3.410±0.124*	4.166±0.151*	5.145±0.113*
21	1.469±0.120	2.828±0.188*	3.372±0.171*	4.193±0.200*	5.522±0.176*
28	1.711±0.102	3.347±0.068*	3.871±0.371*	4.098±0.139*	5.775±0.502*

*There is a statistically significant difference compared to the negative control group ($P<0.05$)

Table 2: Zebrafish locomotor activity as indicated by swimming speed after water fraction administration

Day	Speed average (m/s)				
	Negatif	Dose			
		2.5 ppm	5 ppm	10 ppm	20 ppm
0	4.084±0.913	3.847±0.277	4.177±0.519	3.595±0.394	3.889±0.620
7	2.549±0.535	3.475±0.445*	3.805±0.460*	3.292±0.454	3.390±0.593
14	1.947±0.404	2.949±1.014	3.097±0.458*	3.014±0.551	2.729±0.333
21	1.726±0.210	2.764±0.504*	3.239±0.236*	3.024±0.387*	3.232±0.359*
28	1.937±0.487	3.773±0.213*	4.041±0.226*	3.245±0.355*	3.933±0.422*

*There is a statistically significant difference compared to the negative control group ($P<0.05$)

ND models such as Parkinson's Disease in animals through its mechanism of inhibiting mitochondrial complex I (cI), reducing endogenous antioxidants, causing oxidative stress from complex I and III (cI and cIII) resulting in macromolecular oxidation.[17]

As shown in Table 1, when BF was given at a dose of 2.5 ppm, the speed average was significantly different from that of the negative control group on all days, with significance values of 0.015, 0.002, 0.000, 0.000, and 0.000 ($P < 0.05$). On days 7, 14, 21, and 28, the speed average was significantly different between the 5 ppm dose sample and the negative control, with significance values of 0.000, 0.000, 0.000, and 0.004 ($P < 0.05$). Furthermore, on days 7, 14, 21, and 28, when the 10 ppm dose sample was given, the speed average was significantly different from the negative control, with significance values of 0.005, 0.000, 0.000, and 0.000 ($P < 0.05$). Finally, when compared to the negative control group at a 20 ppm dose, the speed average was significantly different on all days, with significance values of 0.001, 0.009, 0.000, 0.000, and 0.002 ($P < 0.05$).

The results of the WF are shown in Table 2. On days 7, 21, and 28, the dose of 2.5 ppm demonstrated a significantly

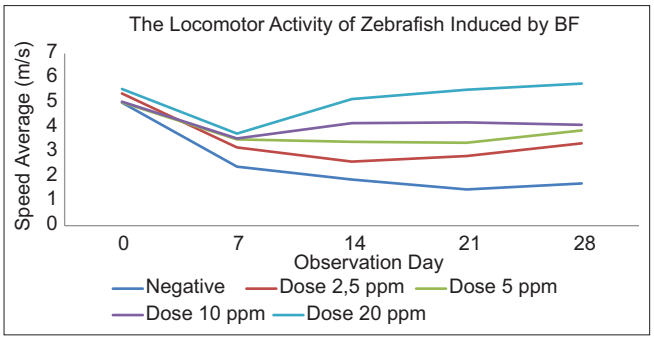

Figure 2: Speed average of zebrafish after n-butanol fraction administration. BF: N-butanol fraction

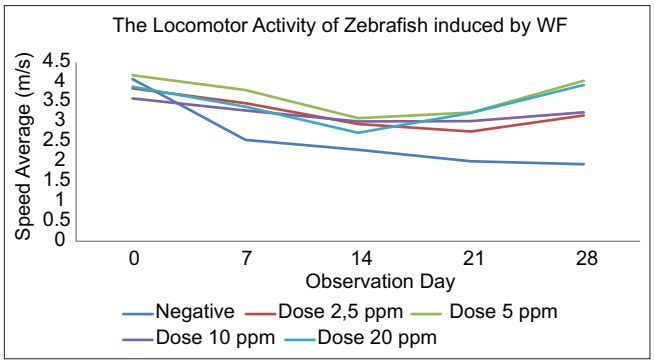

Figure 3: Speed average of zebrafish after n-butanol fraction administration. WF: Water fraction

different speed average value than the negative control, with significance values of 0.043, 0.000, and 0.000, respectively ($P < 0.05$). When compared to the negative control, the 5 ppm dose sample showed a significantly different speed average value on all 4 days, with significance values of 0.007, 0.038, 0.000, and 0.000 ($P < 0.05$). In addition, when the 10 ppm dose sample was given, the speed average was significantly different from the negative control on days 21 and 28 with significance values of 0.000 and 0.000 ($P < 0.05$). Finally, at the 20 ppm dose sample, significantly different speed average values on days 21 and 28 were observed as compared to the negative control group, with significance values of 0.000 and 0.000 ($P < 0.05$).

The optimum dose obtained based on the results of the data in Table 1 and Figure 2 for the BF was 20 ppm, as it represented the best increase in swimming speed, which demonstrated locomotor activity in zebrafish, compared to the negative control group and other treatment groups every day. Meanwhile, for the WF, based on Table 2 and Figure 3, the optimal dose obtained was 5 ppm, which increased the locomotor activity in zebrafish.

This proves and strengthens the argument that the phytoestrogen compounds contained in the *M. crenata* leaves have anti-inflammatory properties, which is one of the mechanisms of neuroprotection through estrogen receptor-dependent (ER-dependent) pathways.[18] The binding of phytoestrogens to estrogen receptors can activate ER in the nucleus and inhibit the activation of inflammation-related transcription factors,[19,20] thus increasing locomotor activity in zebrafish. The difference in the optimal dose between the two fractions is due to the nonmonotonic dose-response (NMDR) effect on the locomotor activity response induced by the polar fraction of *M. crenata* leaves on zebrafish. NMDR effect can occur due to differences in receptor affinities and selectivities and often occurs in studies using hormones, in this case, phytoestrogens contained in the polar fraction of *M. crenata* leaves. The difference in the affinity level between the hormone or hormone substitute sample and the target makes it difficult to predict the response that occurs with increasing doses.[20,21]

Phytoestrogens are chemical compounds produced by plants that have a structure and function similar to estrogen, especially 17 β-estradiol.[22] Phytoestrogens have estrogenic effects that can maintain body homeostasis as a substitute for estrogen. Phytoestrogens have been proven to have anti-inflammatory and antioxidant effects, and are considered as neuroprotective agents to prevent ND.[23-25] Phytoestrogens have a higher safety and activity level compared to hormone replacement therapy through binding to ER.[26] The bond that occurs between phytoestrogens and ER can induce an anti-inflammatory effect through the activation of microglia cells in M_2 polarity conditions, which results in a decrease in pro-inflammatory cytokines and an increase in anti-inflammatory cytokines.[27]

CONCLUSION

Administration of the polar fraction of *M. crenata* leaves significantly improved locomotor activity in rotenone-induced zebrafish, so it has been proven to have the potential to prevent ND both in the BF with an optimum dose of 20 ppm and in the WF with an optimum dose of 5 ppm.

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Nil.

Conflicts of interest
There are no conflicts of interest.

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