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Original Research Article

The Effect of 96% Ethanol Extract of Semanggi leaves (*Marsilea crenata* Presl.) on Locomotor Activity and Alpha-synuclein Aggregation in Zebrafish (*Danio rerio*) Parkinson's Disease Model

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ABSTRACT

Parkinson's Disease (PD) is a neurodegenerative condition caused by the loss of dopaminergic neurons in the substantia nigra pars compacta (SNc) of the brain. Various factors such as aging, genetic predisposition, and exposure to environmental toxins like rotenone causes PD. Current PD treatments primarily manage motor symptoms, but long-term use of such therapy comes with side effects, necessitating the search for a potential drug with minimal adverse effects. *Marsilea crenata* Presl., commonly known as "Semanggi," is a plant recognized for its anti-neuroinflammatory activity. This study aimed to evaluate the potential neuroprotective effect of *Marsilea crenata* Presl leaf extract by assessing alpha-synuclein levels and locomotor activity in a zebrafish (*Danio rerio*) model of PD. *Marsilea crenata* Presl. leaves were extract by ultrasonic-assisted extraction (UAE) method for 3 x 10 minutes. Zebrafish were induced with 5 ppb of rotenone and subsequently treated with 96% ethanol extract of *Marsilea crenata* Presl. leaves at various concentrations (2.5, 5, 10 and 20 ppm) for 28 days. Locomotor activity was observed weekly, while alpha-synuclein expression was measured using immunohistochemistry on day 28. The results indicate that 2.5 ppm of 96% ethanol leaf extract of *Marsilea crenata* Presl. was the optimal concentration that enhanced locomotor activity and reduced alpha-synuclein expression, with an average expression level of 42 ± 4-fold compared to 55 ± 6-fold in the rotenone group. Thus, 96% ethanol leaf extract of *Marsilea crenata* Presl. Exhibits antineurodegenerative activity, specifically in improving PD symptoms. Further study is required to confirm its molecular mechanism of action.

Keywords: Alpha-synuclein, *Danio rerio*, Locomotor activity, *Marsilea crenata* Presl., Parkinson's disease.

Introduction

Parkinson's disease (PD) is a neurodegenerative condition caused by the loss of dopaminergic neurons in the substantia nigra pars compacta (SNc) of the brain, leading to impaired dopaminergic activity.[1-4] The progressive degeneration of dopaminergic neurons reduces dopamine projections from the SNc to the striatum (nigrostriatal pathway), contributing to motor symptoms in PD patients.[5]

PD can be triggered by various factors, including aging as a primary risk factor, genetic predisposition, and exposure to environmental toxins such as rotenone, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), and Paraquat.[6] Rotenone, which is highly toxic to fish,[7] disrupts mitochondrial electron transport, inhibiting oxygen utilization for respiration, ultimately causing cell and organism death at high doses.[8]

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PD therapy primarily focuses on managing motor symptoms.[9] While dopaminergic drugs such as levodopa, monoamine oxidase-B (MAO-B) inhibitors, Catechol-*O*-methyl transferase (COMT) inhibitors, N-methyl-D-aspartate antagonists, and anticholinergics are effective, they may cause side effects such as gastrointestinal disturbances, anxiety, headaches, constipation, and hallucinations.[9-11] Long-term use can lead to complications such as dyskinesia and motor fluctuations,[12,13] highlighting the need for further research into alternative drug sources with minimal side effects.

Semanggi (*Marsilea crenata* Presl.), a specialty food in Surabaya, contains estrogen-like substances,[14] and has been proven to contain phytoestrogen compounds that help maintain body organ homeostasis.[15,16] The 96% ethanol extract of *Marsilea crenata* Presl. leaves contain three compounds (Prochlorperazine, 12-Aminododecanoic acid, and 1-methyl-2-[(4-methylpiperazin-1-yl)methyl]benzimidaol-5-amine hydrochloride) with activities similar to 17*β*-estradiol. It exhibits antineuroinflammatory properties by enhancing anti-inflammatory agents such as arginase-1 (Arg1) and reducing pro-inflammatory agents such as major histocompatibility complex II (MHC II) through the activation of estrogen receptor *β* (ER*β*) *in vitro* in human microglia clone 3 (HMC3) cells.[17-19] Phytoestrogen compounds such as daidzein, formononetin, and equol are known to bind to ER*α* and ER*β* in neurodegenerative disease pathology *in silico*.[20] This study aims to evaluate the *in vivo* expression of *α*-synuclein in zebrafish induced with rotenone and treated with a 96% ethanol extract of *Marsilea crenata* Presl. leaves. Additionally, the motility of the zebrafish was also analyzed.

Materials and Methods

Chemicals/Reagents and equipment

The following chemicals/reagents were used in the study: aquarium media, ice water, distilled water, glycerin, DMSO 0.5%, Tween 80 0.5%, paraffin, xylol, alcohol 95%, alcohol 80%, alcohol 70%, citric acid 0.01M, phosphate-buffered saline (PBS), and rotenone R8875 obtained from Sigma-Aldrich USA, with 99% purity. Additionally, hydrogen peroxide (H_2O_2) 3% and 30%, primary antibody α-synuclein anti-alpha, beta synuclein (AA 32-44) antibody, secondary antibody (Gt X Rb and X Ms IgG), streptavidin HRP, DAB, blocking reagent, hematoxylin, and HCL 1%from EMD Millipore Corp.

The equipment used were analytical balance (M214A Italy), glassware (IWAKI, Indonesia), ultrasonic bath (Sonica 5300EP S3 SOLTEC, Milano), rotary evaporator (HEIDOLPH Hei-VAP G3, Germany), micropipette (ThermoFisher Scientific), syringe, cell phone camera (Samsung, South Korea), tripod, volume pipette, thermometer, pH meter, and Eppendorf tube (Sigma Aldrich, Germany), Nikon Eclipse E400 microscope (Microscope marketplace, Sanford), and Optilab Advance camera microscope MTN004 (Wahana hilab, Indonesia).

Collection and identification of plant material

Marsilea crenata Presl. leaves were collected from the Benowo area (PJRM+3W Sememi, Surabaya, Jawa Timur), Surabaya City, East Java Province, Indonesia, and were identified at UPT Materia Medika, Batu City, East Java, using the determination key 1a-17b-18a-1 and letter number 074/133/102.20-A/2022. The leaves were dried, and subsequently pulverized to obtain crude powdered leaf sample. Moisture content of the powdered leaves was tested using a moisture content (MC) analyzer.

Extraction of crude powdered sample

The extraction of *M. crenata* Presl. leaf powder was carried out using a 96% ethanol solvent at a ratio of 1:20 of sample to solvent. Subsequently, the sample was subjected to ultrasonic-assisted extraction (UAE) using an ultrasonic bath (Sonica 5300EP S3) for 3 cycles of 10 minutes each. The resulting filtrate was evaporated using a rotary evaporator set at a speed of 70 rpm and a temperature of 50°C. Finally, the extract was dried in an oven (Memmert) to obtain the *M. crenata* leaf extract.

The UAE extraction technique is widely employed for extracting various types of natural products due to its numerous advantages, including cost-effectiveness, rapid extraction time, simple procedures, low energy requirements, reduced temperature, lower solvent consumption, and improved yields and extract quality. This makes it a preferable choice for commercial applications.[21,22]

Zebrafish maintenance

Zebrafish used in this study were obtained through independent breeding conducted at the Reproduction Laboratory of Brawijaya University. The parent zebrafish were also sourced from the Reproduction Laboratory of Brawijaya University and were raised until they reached the age of 3 months. The zebrafish were maintained in an aquarium with an efficient air circulation system, at a temperature of 28.5°C with a pH range of 7.9-8.3, and ensuring oxygen levels remained above 95%. Zebrafish were fed with tetramin and block worms three times a day, with the aquarium water changed every 48 hours.[23,24] During the preparation, zebrafish were subjected to a light and dark cycle in a 14:10-hour ratio.[25,26] Prior to their use in the research, zebrafish were acclimatized for 2 weeks. Ethical approval for this study was obtained from the Research Ethics Commission of Brawijaya University with reference number: 147-KEP-UB-2022.

Preparation of test sample

The preparation of the extract stock solution with a concentration of 5000 ppm was carried out by dissolving 500 mg of the extract in 0.5% DMSO.

Exposure of zebrafish to rotenone and extracts

The zebrafish were divided into six groups, each containing 10 animals. The zebrafish were treated according to the OECD guideline for testing of chemicals No. 203 on Fish, Acute Toxicity Testing. The zebrafish were exposed to rotenone at 5 ppb in 2 L of fish media to induce Parkinson's disease.[23] The groups consist of the normal control group (NC) (not treated with rotenone or extract), rotenone control group (RC) as negative control which was administered rotenone but not treated with extract, and the extract treatment groups which were treated with 96% ethanol extract of *Marsilea crenata* Presl. leaves at concentrations of 2.5 ppm (A1), 5 ppm (A2), 10 ppm (A3), and 20 ppm (A4) in 2 L of fish media after exposure to rotenone. The zebrafish were exposed to the various treatments (rotenone and extract) for 28 days. Rotenone and the extract were reintroduced following media change every 2 days.[25,26]

Test for locomotor activity

Locomotor activity of *zebrafish* was observed in a tank with dimensions (L × W × H: 25 cm × 14 cm × 15 cm). Normal locomotor behavior of the fish is characterized by free swimming along the tank, particularly near the surface of the aquarium. Simple observations were conducted by calculating the distance traveled and swimming speed of three zebrafish each, for 1 minute. The total distance covered by *the* zebrafish is directly proportional to the distance swum in 1 minute.[25] The higher the locomotor activity, the higher the activity in preventing the occurrence of Parkinson's disease. Locomotor analysis was performed using tracker software (https://physlets.org/tracker/). Observation of locomotor activity was conducted at intervals of 7 days until day 28.[25,26]

Measurement of alpha-synuclein level

Alpha-synuclein level was measured according to the guidelines specified in the product sheet for the anti-alpha, beta-synuclein (AA 32-44) antibody. Three zebrafish were sacrificed by immersion in ice water (5 parts ice:1 part water, 0-4°C), ensuring the cessation of opercular movement (gill movement), a process that takes approximately 10 minutes. The fish's head (cerebellum and midbrain) was then dissected and stored in a glycerin solution. Subsequently, the zebrafish brain was horizontally sliced with a thickness of 0.4 µm, and the alpha-synuclein measurement was carried out following the procedure in the referenced guidelines.

Statistical analysis

Data were presented as mean ± standard deviation (SD) of ten replicates. Statistical analysis was performed using IBM SPSS statistics version 24.0 software.

Results and Discussion

Extraction yield and moisture content of of Marsilea crenata Presl leaves

A total of 55.1651 g of 96% ethanol extract was obtained from 325 g of powder leaves of *Marsilea crenata* Presl. Corresponding to a percentage yield of 16.9%. the moisture content was determined as 7.48 ± 0.36%. The chemical composition of *M. crenata* especially its phytoestrogen components play a crucial role in its biological activity. Previous studies have demonstrated that using 96% ethanol as a solvent effectively extracts the active components in *M. crenata* leaves. Kaempferol, an isoflavone in *M. crenata*, acts as an estrogen-like compound, as well as an antioxidant and anti-inflammatory agent.[24]

Rotenone-induced Parkinson's disease

Parkinson's disease (PD) model in zebrafish was induced using 5 ppb rotenone, this has been proven in the studies of Ma'arif *et al*. (2022a and 2022b) and Gondokesumo *et al*. (2023).[24-26] Rotenone, found in various plant species, belongs to the isoflavone group, serving as a broad-spectrum insecticide, pesticide, and acaricide.[27] Rotenone toxicity disrupts mitochondrial electron transport, causing cell death and potentially leading to neurodegenerative diseases like Parkinson's disease.[8,27]

Effect of Marsilea crenata Presl. leaf ethanol extract on locomotor activity of zebrafish Parkinson's disease model

The administration of a 96% ethanol extract of *Marsilea crenata* Presl. leaves increased locomotor activity in zebrafish Parkinson's disease model compared to the negative control group. the extract exhibited a concentration-dependent increase in the locomotor activity of the zebrafish from day 7 to day 28. The increased concentration of the

extract correlated with greater distance traveled by the zebrafish, with coefficient of correlation of 0.467 (weakly correlated) on the 7th day, 0.866 (very strongly correlated) on the 14th day, 0.773 (very strongly correlated) on the 21st day, and 0.544 (strongly correlated) on the 28th day.[28] Meanwhile, for the swimming speed, the correlation coefficient were 0.467 (weakly correlated) on the 7th day, 0.866 (very strongly correlated) on the 14th day, 0.773 (very strongly correlated) on the 21st day, and 0.544 (strongly correlated) on the 28th day.[28] The non-linear increase in locomotor activity observed in zebrafish treated with 96% ethanol extract of *Marsilea crenata* Presl. leaves at intervals of 7 days may be attributed to the fish's response to the extract, acting as a substitute for levodopa and dopaminergic drugs.[29,30] The administration of the extract for 28 days continued to improve the Parkinson's disease condition in zebrafish, evident through increased locomotor activity compared to the negative control group. Non-linear progression in PD patients is common, indicating a faster progression in those with shorter disease durations. The irregularity occurs between movements, adjusting the precision of motor commands due to greater damage to the dopamine system in the posterior putamen at the onset of symptoms. PD is a neurodegenerative disease with no current cure. Treatment focuses on managing symptoms or halting disease progression. The administration of natural product compounds, with their multiple bioactive components, is beneficial in preventing and treating various diseases. Medicinal plant extracts, containing numerous bioactive compounds, act in synergy, achieving therapeutic effects through various mechanisms. The non-linear condition observed may guide the preparation of a 96% ethanol extract of *Marsilea crenata* Presl. leaves for PD therapy. Extended-release or controlled-release dosage forms can help maintain the plasma concentration of the extract; preventing non-linear locomotor responses in PD patients.

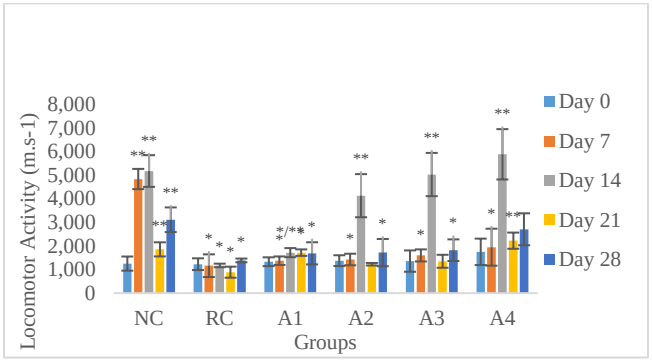

Figure 1: Locomotor activity of zebrafish Parkinson's disease model treated with 96% ethanol extract of *Marsilea crenata* Presl. leaves. Data represent mean ± standard deviation (SD), n = 10. *: Significant (P < 0.05) compared with normal control; **: Significant (P < 0.05) compared with rotenone control

Effect of Marsilea crenata Presl. leaf ethanol extract on the expression of α-synuclein in zebrafish Parkinson's disease model

In the measurement of α-synuclein aggregation, Image J software was employed. This widely used image analysis program is accessible to experts and non-experts alike. It is commonly used for various analyses, including colour intensity, particle size distribution, porosity, compound levels, and spatial or temporal features of biological elements.[31,32] Rotenone-induction resulted in a significant increase in α-synuclein expression in zebrafish model of Parkinson's disease compared to the normal control. However, treatment with 96% ethanol extract of *Marsilea crenata* Presl. leaves decrease α-synuclein expression in a concentration-dependent manner. There was a very strong negative correlation between the concentration of the 96% ethanol extract of *Marsilea crenata* Presl. leaves and α-synuclein expression (Pearson correlation coefficient = -0.820).[28] From the findings of the present study, 2.5 ppm of 96% ethanol extract of *Marsilea crenata* Presl. leaves was the least effective concentration that can increase locomotor activity and reduce α-synuclein expression in zebrafish Parkinson's disease model.

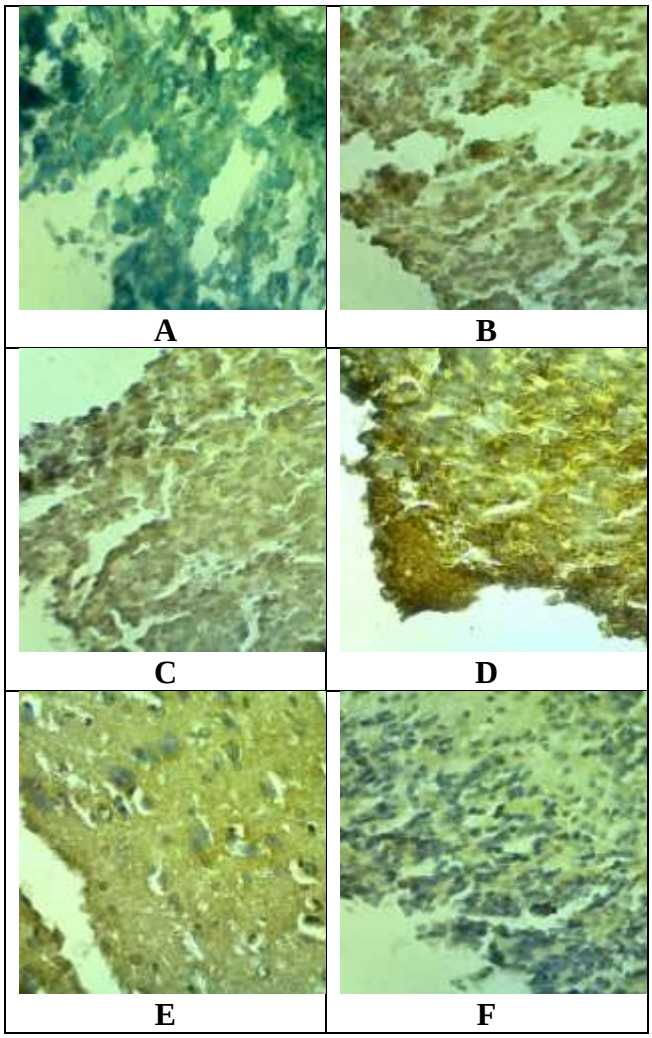

Figure 2: Expression of α-synuclein in zebrafiah Parkinson's disease model after administration of 96% ethanol extract of *Marsilea crenata* Presl leaves. **(A)** Normal Control; **(B)** Rotenone Control; **(C)** 2.5 ppm extract group; **(D)** 5 ppm extract group; **(E)** 10 ppm extract group; **(F)** 20 ppm extract group

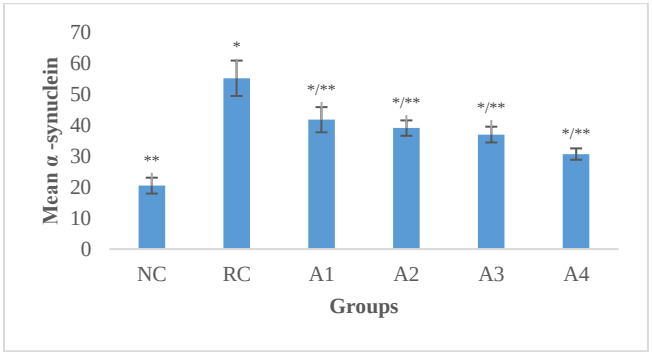

Figure 3: Expression of α-synuclein in zebrafish Parkinson's disease model after administration of 96% ethanol extract of *Marsilea crenata* Presl leaves. Data represent mean ± standard deviation (SD), n = 10. *: Significant (P < 0.05) compared with normal control; **: Significant (P < 0.05) compared with rotenone control

Conclusion

The findings from the present study have demonstrated that 96% ethanol extract of *Marsilea crenata* Presl. leaves have the potential to improve Parkinson's disease (PD) symptoms by increasing locomotor activity, and decreasing α-synuclein expression in zebrafish Parkinson's disease model. The extract at 2.5 ppm was shown to be the lowest concentration demonstrating significant improvement in PD conditions, both in terms of locomotor activity and the expression of α-synuclein in zebrafish PD model compared to the normal and negative control groups. Further molecular studies are necessary to ascertain the specific pharmacological role of *Marsilea crenata* Presl. leaves as antineurodegenerative agent, particularly in the management of Parkinson's disease.

Conflict of Interest

The author`s declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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