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Original Research

OPEN ACCESS

Decreasing α-synuclein Aggregation by Ethanol Extract of Keluwih (Artocarpus camansi) Leaves on Rotenone-Induced Adult Zebrafish as Parkinson's Diseases Model

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Abstract: The prevalence of Parkinson's disease is increasing every year. This progressive disease is characterized by the loss of neurons in the substantia nigra due to the presence of α-synuclein aggregates. Keluwih leaves (Artocarpus camansi) are known to have activity in inhibiting acetylcholinesterase, as well as being an antioxidant and anti-inflammatory. The aim of this study was to evaluate the effect of ethanol extract of A. camansi leaves on the levels of α-synuclein in male and female adult zebrafish induced with rotenone. The zebrafish were induced with rotenone at a concentration of 5 µg/L for 28 days, along with the administration of 96% ethanol extract of A. camansi leaves at doses of 2.5, 5, 7.5, or 10 mg/L. The media was changed every 48 hours to maintain the concentration of rotenone and extract. After 28 days, α-synuclein levels were examined using immunohistochemistry. The administration of ethanol extract of A. camansi leaves can reduce the average levels of α-synuclein in male and female adult zebrafish, with the optimum dose being 2.5 mg/L. Therefore, it can be concluded that the administration of ethanol extract of A. camansi leaves can be used as an alternative treatment for Parkinson's disease.

Keywords: *α-synuclein, Artocarpus camansi, Parkinson disease, Zebrafis.*

INTRODUCTION

The prevalence of Parkinson's disease (PD) has doubled over the last 25 years. Disability and mortality rates caused by Parkinson's disease are higher and grow faster than other neurodegenerative diseases. In 2019, Parkinson's disease morbidity rose to 5.8 million cases of disability and 329,000 cases of death.[1] This data places PD second-ranked as the most common disease, especially in people over 60 years old, then the cases are estimated to double in 2030 while increasing frequently each year.[2]

Parkinson's disease is characterized by the progressive loss of neurons in the substantia nigra, which is affected by decreasing dopamine production and Lewy bodies (LB) accumulation. Those conditions affect the presence of α-synuclein aggregates. The formation of LB impairs the ubiquitin-proteasome degradation process, causing mitochondrial dysfunction and then failure of adenosine triphosphate (ATP) production.[3] Mitochondrial dysfunction results in abnormal regulation of calcium ions involved with the increasing calcium ion level, thus it is toxic for neurons and accumulates α-synuclein aggregates.[4] Accordingly, mitochondrial dysfunction leads to dopaminergic damage and peripheral motor nerve degeneration in experimental animals.[5,6]

The common animal used for the PD model is the zebrafish (*Danio rerio*) due to the uniqueness of its ventral telencephalon, which is similar to the human striatum.[4,7] Parkinson's disease in zebrafish is induced by rotenone.[8] Rotenone,

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one of the commonly used pesticides, is a neurotoxin that penetrates the cell membrane and causes complex I mitochondrial dysfunction and oxidative stress production,[8] consequently dopaminergic damage and peripheral motor nerve degeneration.[4]
Traditionally, the plant *keluwih* (*Artocarpus camansi*) use in seizure treatment.[9] *Artocarpus camansi* that belongs to the Moraceae family spreads in various countries such as Indonesia, India, Malaysia, Africa, Australia, Brazil and others. *Keluwih* leaves have known potential as antioxidants, anti-inflammatory agents, antibacterial agents, and antivirals. The phytochemicals in ethanol extract of keluwih leaves show several active compounds, such as flavonoids, alkaloids, tannins, triterpenoids, and phenolics (Jangtap, 2010; Solichah et al., 2021).[10,11] Other studies also proved that several genera of Artocarpus including *A. camansi* contain artomunoisoxanthone, artocommunol CC, artochamin D, artochamin B, and dihydroartomunoxanthone from leaves extract, which have potential as antioxidants to fight oxidative damage.[10] *Keluwih* contains compounds such as stilbenoids, arylbenzofurans, and abundant flavonoids.[10] The flavonoid compounds from *keluwih* leaf extract confirmed inhibiting acetylcholinesterase (AChE) enzyme activity with anticholinergic and antioxidant effects.[12] Those properties are effective in neurodegenerative disease treatment. This study aims to evaluate the effectiveness of ethanol extract of *keluwih* leaves on α-synuclein levels in rotenone-induced adult zebrafish, both male and female, as PD patient models.

MATERIAL AND METHOD

Zebrafish

A wild type strain of black zebrafish, both male and female, were acquired from Tulungagung cultivators in East Java, Indonesia, and were identified by Airlangga University, Faculty of Fisheries and Maritime Affairs in Surabaya, East Java using identification letter number 074/ULMKILP/UA.FPK/12/2022. Late adulthood zebrafish, 9-12 months of age with 0.08 g body mass and 30.6 ± 0.95 mm length were chosen for the study to represent human brain of elderly [13], which is the age range commonly Parkinson's disease sufferers. The zebrafish were acclimatized for seven days and were maintained in accordance to the standard procedures set by the research ethics committee of Airlangga University (No: 3.KEH.159.11.2022).

A. camansi Leaf Extraction

Keluwih leaves (*A. camansi*) were obtained from the UPT Herbal Laboratory Materia Medika Batu in Malang, East Java. The study was conducted under the determination letter number 074/124/102.20-A/2022. To extract the active compounds from the *A. camansi* leaves, a dry powder weighing 200 grams was mixed with 96% ethanol (Merch) in a ratio of 1:10 and macerated for three cycles of 24 hours each. The resulting liquid extract was then concentrated into a thick extract using a Rotavapor® apparatus. Different concentrations of concentrated extract were prepared by weighing 2.5 grams of extract then dissolved in 500 ml, homogenized to obtain a stock solution of 5000 mg/L, then diluted to obtain Theconcentration of extract were 2.5 mg/L, 5 mg/L, 7.5 mg/L, and 10 mg/L.

Rotenone and *A. camansi* Treatment

To induce a Parkinson's disease model in zebrafish, a concentration of 5 µg/L rotenone in DMSO (Sigma R8875) was added to a 2L water volume in a 25 x 16.5 x 12.5 cm aquarium, simultaneously the extract of *A. camansi* was administered in different concentrations (2.5, 5, 7.5, and 10 mg/L). The zebrafish male and female were placed in difference aquarium to compare the effects. Then, the aquarium water was refreshed every 2 days to maintain the rotenone concentration. The water temperature in the tank was tightly controlled between 24-25.5℃, and a light-dark cycle of 14:10 was established.[14] Feeding the zebrafish was three times daily

with Tetra Bit and Color Tropical Flakes from Tetra Sales, Blackburg, Germany. This treatment conducted along 28 days.

Analysis of α-synuclein concentration with Immunohistochemistry (IHC) Technique

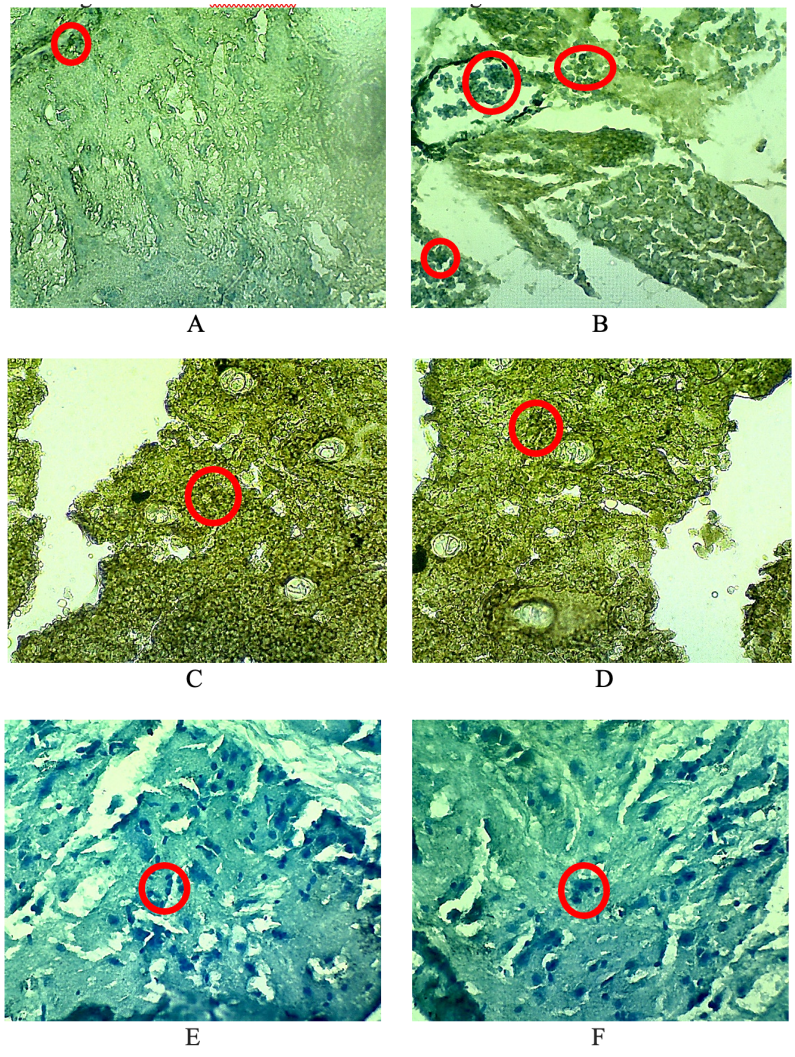
After 28 days, the animal was decapitated, the brain was isolated carefully and immediately immersed in a formalin buffer and prepared in paraffin blocks. The paraffin blocked samples were sectioned at a thickness of 0.4 mm for immunohistochemical staining. For staining, the slides were deparaffinized and subjected to the Millipore IHC procedure (Cat No. DAB 500). In this study, α-synuclein (Sigma) was used as the primary antibody, and the qualitative or quantitative expression of α-synuclein (brown color staining) was observed. Twenty fields of view in different areas were observed using a microscope on each slide at a magnification of 1000x, then quantified to obtain the average values of each treatment group were obtained from three replications (3 slides).

Data analysis

The data collected from each treatment group were subjected to statistical analysis using SPSS version 29. A one-way ANOVA test ($p < 0.05$) was employed for the analysis. The results are presented as the mean ± standard deviation (SD) for each respective treatment group.

RESULTS AND DISCUSSION

The image below shows where the positive cells are brownish (Qualitative), then quantified to obtain the average value of α-sinuclein levels shown in Figure 2.



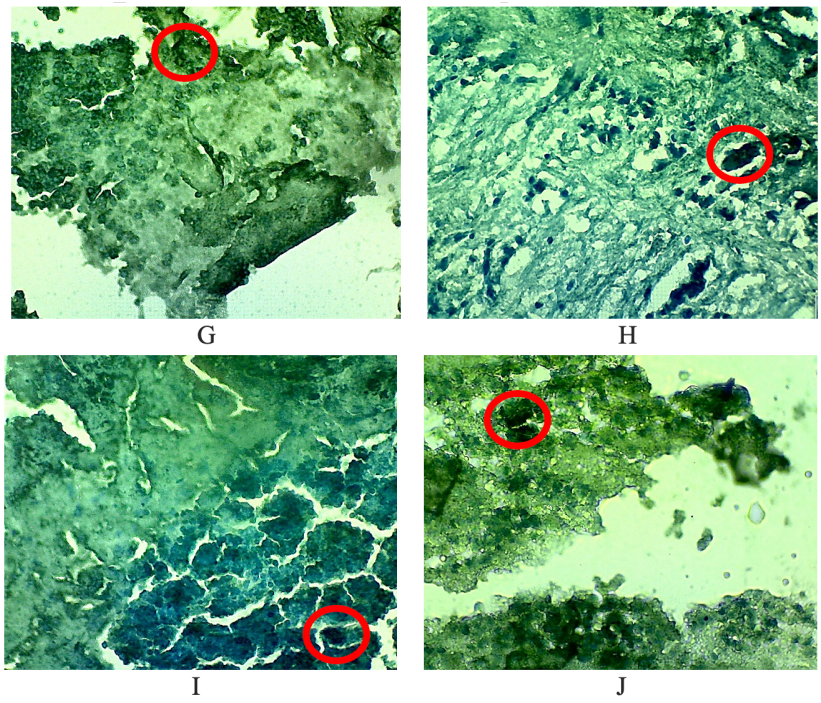


Figure 1. Staining Result of Brain Zebrafish to Evaluate Alpha-Synuclein Aggregation (brownish, in red circle). A: Control; B: Control Negative / Rotenone treatment; C: 2.5 mg/L leave extraction from adult male; D: 2.5 mg/L leave extraction from adult female; E: 5 mg/L leave extraction from adult male; F: 5 mg/L leave extraction from adult female; G. 7.5 mg/L leave extraction from adult male; H. 5 mg/L leave extraction from adult female; I. 10 mg/L leave extraction from adult male; J. 10 mg/L leave extraction from adult female

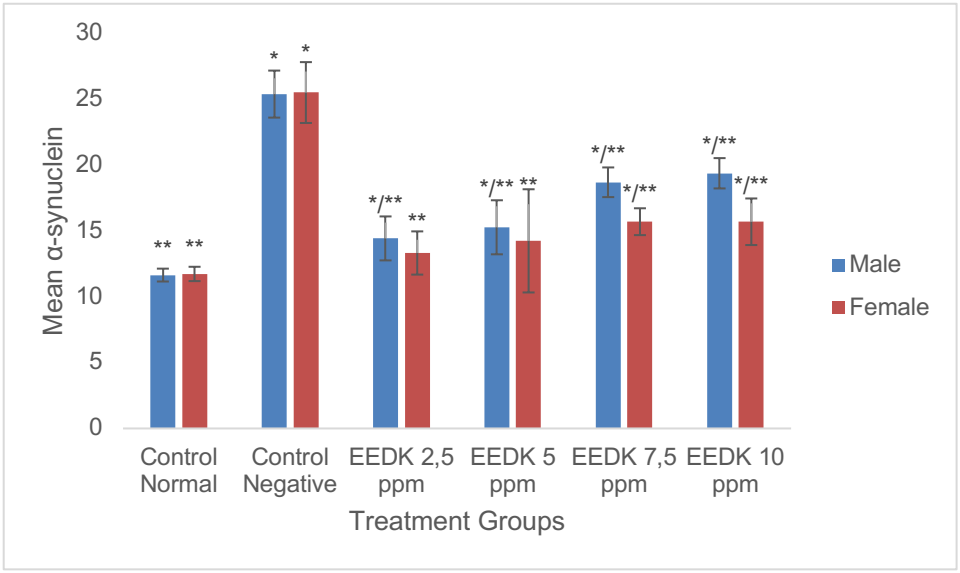


Figure 2. The Mean of α-synuclein Aggregation in Adult Zebrafish as Parkinson's Diseases Model with Ethanol Extract of *Keluwih* Leaves. **Significantly different with control normal, **Significantly different with control negative*

Upon administration, rotenone notably escalated the levels of α-synuclein in both male and female adult zebrafish, presenting a significant deviation from the control group ($p < 0.05$). Contrastingly, the introduction of *A. camansi* extract resulted in a marked decline in α-synuclein levels when juxtaposed with the control group. A comparison of α-synuclein aggregation between untreated and *A. camansi*-treated samples, as visually demonstrated in Figure 1, revealed a more subdued brownish color, indicative of α-synuclein aggregation, in the latter. Statistical evaluation substantiated this visual observation, revealing a significant improvement in α-synuclein levels following *A. camansi* treatment ($p<0.05$). These findings imply that *A. camansi* extract may have potential as a therapeutic agent in mitigating α-synuclein-related pathologies in rotenone-induced Parkinson's models in zebrafish.

Rotenone, owing to its potent lipophilic attributes, permeates cellular membranes with ease and speed, and infiltrates the brain with striking rapidity.[15] The inherent toxicity of rotenone incites a domino effect of damaging consequences, foremost among them being the impairment of mitochondrial electron transport. This, in turn, results in respiratory failure and the ultimate demise of the cell, or apoptosis.[16]

Rotenone's initial onslaught triggers oxidative stress, culminating in alterations to DNA, lipids, and protein folding, and subsequently sparking neurodegenerative changes.[17] These changes precipitate lipid modifications, thereby prompting mitochondrial dysfunction. The intricate interplay between mitochondrial damage and respiratory failure constitutes a hazardous cycle, spurred on by the dysfunction of electron transport.

Interestingly, respiration is intrinsically linked to ATP production, a cornerstone in facilitating axonal transport and cell metabolism.[18] This entire gamut of deleterious conditions inexorably leads to neuronal apoptosis, especially impacting the dopaminergic neurons of the substantia nigra. This, in turn, engenders a considerable reduction in dopamine production, a process vital to the neuron's survival.[19]

Moreover, the synergistic action of rotenone toxicity and oxidative stress induced by mitochondrial dysfunction has the potential to modify α-synuclein formation. α-synuclein, a presynaptic protein ubiquitous in various brain regions, under physiological conditions assumes a random formation, naturally unfolding rather than aggregating.[20] However, upon exposure to unfavorable conditions such as low pH, organic solvents, high temperatures, metal ions, oxidative stress, and pesticides like rotenone, α-synuclein tends to aggregate in cells. This is believed to play a crucial role in the pathogenesis of Parkinson's disease (PD), as such aggregation is predominantly found in neuronal cells.[21]

Investigations into the aggregation of α-synuclein in zebrafish models have revealed that it primarily takes place in axons and is highly toxic, often preceding neuronal death.[22,23] Such aggregation is associated with a reduced lifespan in fish models.[24] Further demonstrated that α-synuclein aggregation disrupts cellular microtubules and impairs mitochondrial axonal transport, owing to its high affinity binding to lipid structures such as cell membranes and organelles.[25] Thus, mitochondrial dysfunction in PD patients is influenced not only by rotenone exposure but also by α-synuclein aggregation.

Interestingly, the present study exhibits that the administration of an ethanol extract of *A. camansi* leaves can lower α-synuclein levels in rotenone-induced Parkinson's models in zebrafish. The improvement observed across all doses was significantly different from the control group, with the most optimal dose, based on α-synuclein measurements, being 2.5 mg/L. The constituents of the ethanol extract of *A. camansi* leaves flavonoids, alkaloids, tannins, triterpenoids, and phenolics known to be involved in the cellular pathology of Parkinson's, appear to have a synergistic effect on α-synuclein levels.

A. camansi leaves have been shown to inhibit acetylcholinesterase, the enzyme responsible for neuromelanin formation in the human brain. Neuromelanin, when increased significantly, can contribute to dopamine neurotoxicity and precipitate severe neurodegeneration. Therefore, the inhibition of neuromelanin by *A. camansi* might help stave off dopamine neurotoxicity and neurodegeneration.[26]

Additionally, the antioxidant and anti-inflammatory properties of the *A. camansi* leaf extract could potentially stabilize the synthesis, availability, and kinetics of dopamine.[18] This is further buttressed by the extract's capacity to prevent α-synuclein from aggregating, which would otherwise be neurotoxic, and inhibit the development of dopaminergic neuron degeneration.

CONCLUSION

Treatment of *keluwih* (*A. camansi*) leaves with 96% ethanol extract can be used as an alternative Parkinson's Disease ailment due to the ability of decreasing α-synuclein aggregation measurement, both male and female adult zebrafish. The lowest dose in this research of 2.5 mg/L is the optimum dose of 96% ethanol extract of *keluwih* leaves in adult male and female zebrafish. It is necessary to carry out further tests on other markers to ensure the pharmacological effects that contribute to PD pathology, and in silico tests can be carried out to determine compounds that have an effect.

AUTHORS' CONTRIBUTIONS

Marisca Evalina Gondokesumo: prepared the samples, designed the protocols, executed the protocols, wrote the manuscript, submit and revision the manuscript. Krisyanti Budipramana: reviewed and supervised the manuscript. Putu Dea Angelita Putri and Ni Putu Diah Nopitasari: data collection. Martanty Aditya and Liza Yudistira Yusan: data analytic and visualization statistically. All authors have read and approved the final manuscript.

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DATA AVAILABILITY STATEMENT

The utilized data to contribute in this research are available from the corresponding author on reasonable request.

DISCLOSURE STATEMENT

The views and opinions expressed in this article are those of the authors and do not necessarily reflect the official policy or position of any affiliated agency of the authors. The data is the result of the author's research and has never been published in other journals.

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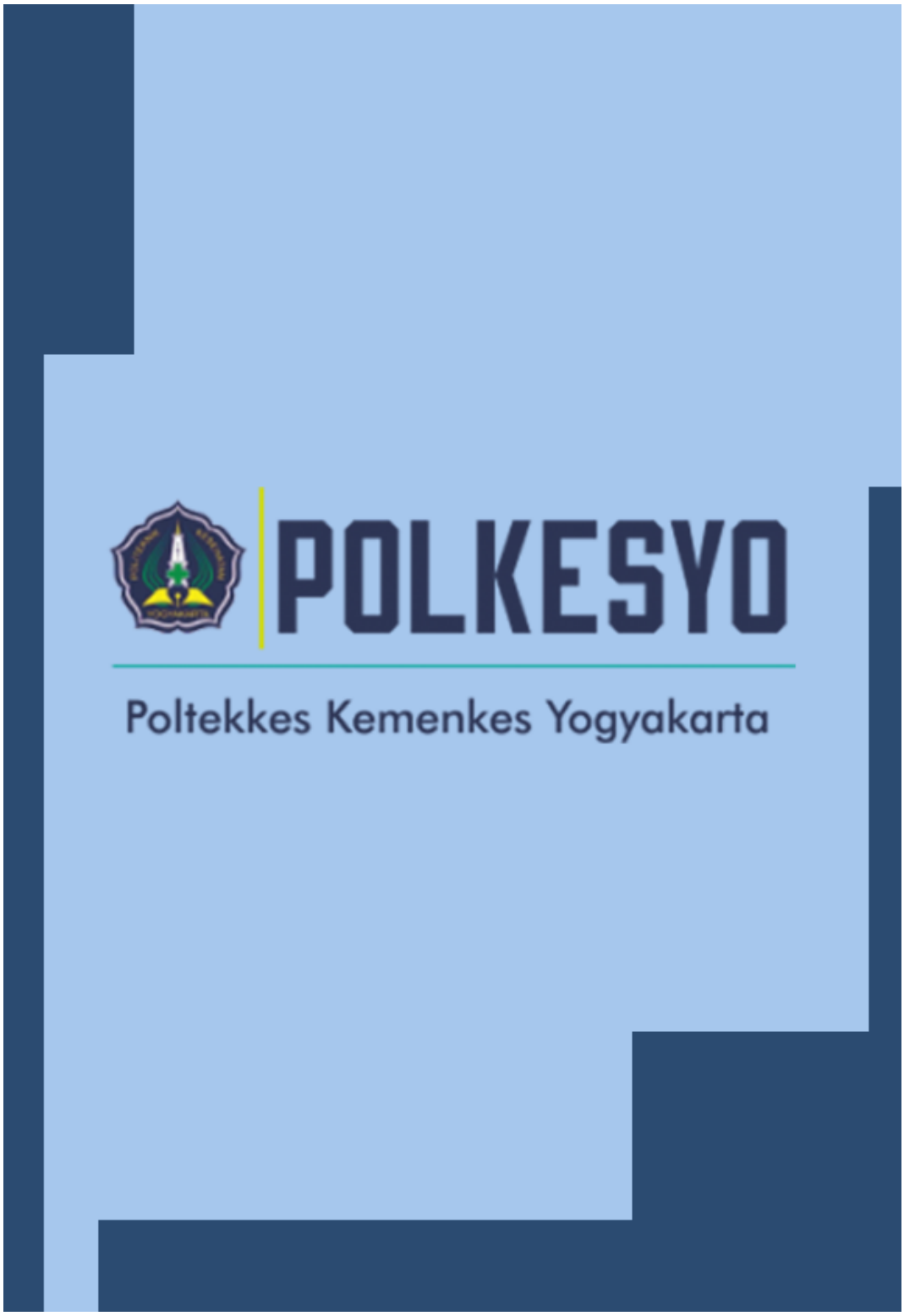

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Decreasing alpha-synuclein aggregation on rotenone-induced adult Zebrafish as Parkinson's Diseases model by ethanol extraction of Keluwih (Artocarpus camansi) leaves ›

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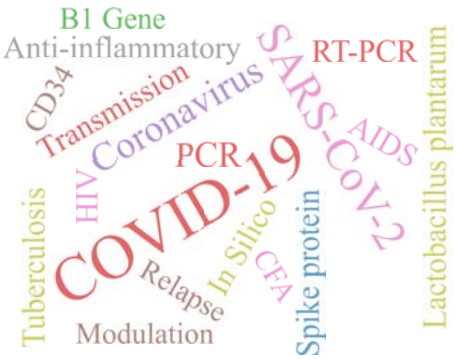

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