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# *Kluwih (Artocarpus camansi) leaves extract effects in zebrafish models of Parkinson's disease*

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**Abstract:** Parkinson's disease is a condition that affects the central nervous system in the brain and is brought on by a lack of dopamine. Uncontrollable tremors, uncoordinated movement, and stiffness characterize Parkinson's disease. Until now, the medication for Parkinson's disease is limited to relieve the symptoms and maintain the quality of life; thus, the progression of the disease can be delayed. In order to search for alternative therapy from herbs, *Kluwih* (*Artocarpus camansi*) has been used traditionally to relieve convulsants. This research aims to observe 96% ethanol extract of *A. camansi* leaves in dopamine and locomotor activity in adult male and female zebrafish (*Dario rerio*). The *A. camansi* extract concentration was 2.5; 5; 7.5; and 10 mg/ml for 28 days. Zebrafish locomotion was observed for 5 minutes on days 0; 7; 14; 21; and 28. ELISA measured the observations of dopamine after 28 days. The 96% ethanol extract of *A. camansi* leaves at 5 mg/ml can increase dopamine levels after induced with rotenone, but the dopamine level decreased at 7,5 and 10 mg/ml. The maximal concentration to increase locomotor activity is also at 5 mg/ml, along with dopamine concentration. Our findings revealed that 5 mg/ml of 96% ethanol extract of *A. camansi* leaves was the optimal dosage to stimulate dopamine release and enhance locomotor activity.

**Keywords:** Kluwih, Zebrafish, Parkinson, Rotenone, Dopamine.

## INTRODUCTION

In the last 25 years, the prevalence of Parkinson's Disease has doubled increase globally. Disability and death due to Parkinson's are rapidly rising more than any other neurological disorder. According to the latest figures, in 2019, Parkinson's disease caused 5.8 million years of life with disability and resulted in 329,000 deaths, an increase of more than 100% since 2000.[1] This disease occupies the second position as the most common cause affecting individuals over 60, and it is estimated that by 2030, it will continue to increase by more than two-fold, in line with the increasing population of early aging.[2]

Parkinson's disease is uncommon in individuals younger than 50s, but its prevalence increases and peaks at ages 60 to 75. While the preference is roughly the same for both sexes, men are more susceptible to its effects with a ratio of 3:2.[3] Parkinson's is a progressive neurodegenerative disease characterized by the loss of neurons in the substantia nigra resulting in decreased dopamine production and accumulation of Lewy bodies (LB) due to the formation of α-synuclein aggregates. The LB formation impairs the ubiquitin-proteasome degradation

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process, causing the failure of adenosine triphosphate (ATP) production, which results in mitochondrial dysfunction.[4] People with Parkinson's have mitochondrial dysfunction resulting in a malfunction of calcium ion regulation. More percentage of calcium in the body has a more toxic effect on neurons with α-synuclein aggregates accumulation.[5] This condition also activates the formation of free radicals due to oxidative stress that occurs when an imbalance between reactive oxygen species (ROS) production and cellular antioxidant activity. Increasing ROS production can inhibit the tyrosine hydroxylase (TSH) enzyme by decreasing dopamine levels.[6,7] Furthermore, the dopamine levels are inadequate to stimulate dopamine receptors in the striatum basal ganglia. It affects disturbances in locomotor activity characterized by slowed movements, tremors, stiffness, or balance problems.[8]

On the other hand, Zebrafish (*Danio rerio*) can be used as an experimental animal model for Parkinson's disease because it has a unique ventral telencephalon similar to the human brain striatum.[5,9] Another advantage of zebrafish is that many genes and proteins are similar to humans, transparent and large embryos.[10] While inducing Parkinson's disease in zebrafish models commonly use rotenone as a pesticide with neurotoxin.[11] Rotenone can penetrate cells, causing complex mitochondrial dysfunction and triggering the formation of oxidative stress,[12] which leads to dopaminergic and degenerative damage to peripheral motor nerves.[5]

Treatment of synthesis of Parkinson's disease uses several approaches, likes: 1) dopamine agonists such as levodopa, monoamine oxidase-b inhibitors such as selegiline, 2) anticholinergics such as trihexyphenidyl and N-methyl-D-aspartate (NMDA), 3) antagonists such as amantadine. All treatment is known to relieve and overcome the symptoms of Parkinson's due to improving the Activity of Daily Living (ADL) and Quality of Life (QQL), but they do not stop dopamine degradation.[3,8] This condition encourages research to develop better neuroprotective therapy strategies as supportive therapies for Parkinson's disease. The supportive therapy should be highly effective for the disease remedy and common side effects for patients. It could use herbs; thus, one of the herbs that have the potential to be developed as a supporting herb for Parkinson's therapy is *kluwih* (*A. camansi*). *Kluwih* (*A. camansi*) has traditionally been reported to be used to treat seizures.[13] *Kluwih* is a plant rich in compounds such as stilbenoids, aryl benzofurans, and flavonoids.[14] Flavonoid group from *A. camansi* leaves could inhibit the activity of the acetylcholinesterase (AChE) enzyme, anticholinergic, and antioxidant, which is effective against Alzheimer's disease. In this study, we wanted to investigate the effect of 96% ethanol extract of *A. camansi* leaves on the expression of dopamine levels and motility (locomotor) activity in adult male and female zebrafish, which had been induced by rotenone.[15]

## MATERIAL AND METHOD

### Zebra Fish

Adult male and female zebrafish, wild-type strain blackfish, were obtained from Tulungagung cultivators in East Java, Indonesia. Zebrafish identification was obtained from Airlangga University, Faculty of Fisheries and Maritime Affairs, Surabaya, East Java, with identification number 074/ULMKILP/UA.FPK/12/2022. The zebrafish has been ages group into three different groups, such as (1) early adulthood (3 - 6 months) has 0.42 ± 0.04 g in mass body and 28.4 ± 0.75 mm for a length, (2) middle adulthood (7 - 9 months) has mass and length body at 0.62 ± 0.09 g and 31.6 ± 1.17 mm, and (3) late adulthood (> nine months) calculates in 0.08 g for mass and 30.6 ± 0.95 mm for length body. This research took late adulthood zebrafish to figure out elderly human from the 60s until the 75 as the most preferred in Parkinson's patients. Acclimatization was carried out for seven days, and maintenance was according to standard procedures approved by the research ethics committee of Airlangga University (No: 3.KEH.159.11.2022).

**Chemical Material**
The chemicals used included ethanol 96% (Merck), rotenone (Sigma R 8875), dimethyl sulfoxide (DMSO) (Sigma-Aldrich), and Tween 80 (Sigma-Aldrich), 2N HCl, chloroform, NH4OH, dragendorf reagent, Mayer reagent, 10% NaCl, FeCl3 reagent, gelatin, chloroform, CH3COOH, concentrated H2SO4.

**Extraction**
*Kluwih* plants (*A. camansi*) were obtained and tested for termination at UPT Herbal Laboratory Materia Medika Batu, Malang, East Java, with letter of determination number 074/124/102.20-A/2022. *A. camansi* leaves dry powder (200gr) was extracted with 96% ethanol with a volume ratio of 1:10 and macerated for 3x24. The liquid extract was concentrated into a viscous extract using a Rotavapor® apparatus. The concentrated extract was made for several dosages, such as 2.5 mg/ml; 5mg/ml; 7.5 mg/ml; and 10 mg/ml.

**Phytochemical Screening of *A. camansi* Leaves Extract**
Phytochemical screening was carried out on the ethanol extract of *A. camansi* leaves, according to Sogandi & Amelia, 2020 which included testing the flavonoids, alkaloids, steroid-triterpenoids, phenolics, tannins, and saponins.

**Rotenone and *A. camansi* Treatment**
Zebrafish were induced to set Parkinsonis' disease model by adding 5 µg/L rotenone (Sigma R8875) to 2L of water in a 25 x 16.5 x 12.5 cm aquarium. The pool water is reversed every two days; thus, the concentration of rotenone in the aquarium is retained. Pool water temperature is maintained in the range of 24-25.5℃ with the darkest cycle of 14:10 (Khotimah et al., 2015). The zebrafish are fed thrice daily with Tetra Bit and Color Tropical Flakes; Tetra Sales; Blackburg, Germany. The sample consisted of a 96% ethanol extract of *A. camansi* leaves in several dosages (2.5; 5; 7.5; and 10 mg/ml) given concurrently with rotenone for 28 days.

**Analysis of Dopamine Levels with ELISA**
Zebrafish were anesthetized by immersion in ice water (5 parts ice to 1 part water at 0-4℃ for 30 seconds). The brain part of the fish head is carried by dissecting with the help of a dissecting microscope; then, the pure brain is extracted to obtain protein. The results of zebrafish brain extraction were tested for dopamine levels using the ELISA method (Fish Dopamine KIT Brand Biassay Technology Laboratory (BTLab) Cat.No EA0018FI).

**Motility Observation**
The locomotor activity test method was carried out by vertically dividing the aquarium into three zones (right sideline, middle line, and left side). Three vertical lines are drawn at identical intervals on the tank. Simple observations were made in this test to determine the locomotor activity of adult male and female zebrafish. Fish movements are captured in a 5-minute, then observed using the Tracker Video Analysis and Modeling software.

**Data analysis**
All data groups obtained from each treatment were analyzed using SPSS version 29 with one-way ANOVA ($p < 0.05$) for statistical analysis. These results are expressed as the mean ± SD for each treatment group.

## RESULTS AND DISCUSSION

*Kluwih* (*Artocarpus camansi*) is a species of the Moraceae family found in Indonesia, India, Malaysia, Africa, Australia, Brazil, and many other countries. Traditionally, breadfruit (*A. camansi*) has been effective in seizure treatment.[13] The flavonoid group from *keluwih* leaves has been reported to inhibit the activity of the enzyme acetylcholinesterase (AChE), anticholinergic affected, and high antioxidant against Alzheimer's disease effectively.[15] This study used breadfruit (*A. camansi*) as an antiparkinsonian agent for zebrafish rotenone-induced.

Phytochemical screening was carried out in this study to determine the class of compounds found in *A. camansi* leaves extract. The results of the phytochemical screening test showed that the 96% ethanol extract of *A. camansi* contained flavonoids, alkaloids, tannins, steroid-triterpenoids, and phenolic compounds (Table 1). Furthermore, dopamine levels and locomotor activity tests were evaluated on adult zebrafish of different sexes.

Table 1. Phytochemical Screening of 96% Ethanol Extract of *A. camansi* Leaves

| Phytochemical | Annotation | Result |
|---|---|---|
| Flavonoids | + | Orange red precipitation |
| Alkaloids | + | White and orange precipitation |
| Tannins | + | Green brownish change in color |
| Steroid | + | No blue-greenish ring |
| Triterpenoids | + | Brownish ring |
| Phenolic | + | Dark blue and greenish blue color |
| Saponin | - | No foam |

### Dopamine Concentration

To determine the effect of rotenone and *A. camansi* extract (ACE) on dopamine levels, after 28 days, dopamine levels were tested using the ELISA method from adult male and female zebrafish brains.

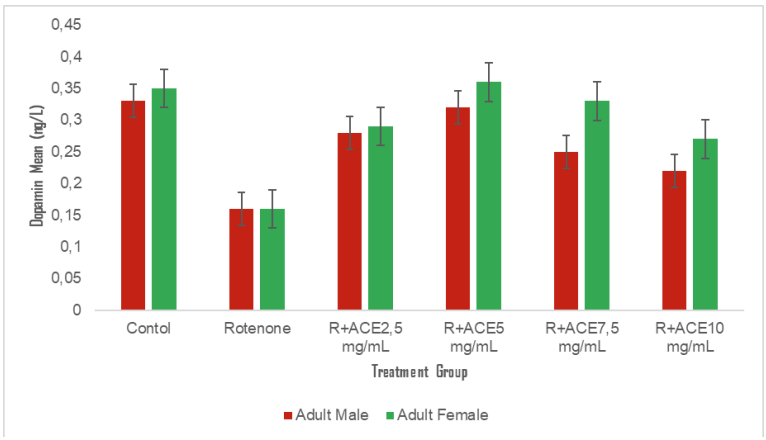


Fig 1. Dopamine levels of adult male and female zebrafish by induction of 96% ethanol extract of *A. camansi* leaves

Figure 1 shows that rotenone-induced significantly reduced dopamine levels compared to the control group ($p<0.05$). Giving 96% ethanol extract of *A. camansi* leaves levels of 2.5 and 5 mg/L to adult male and female zebrafish can increase dopamine levels. Improving the concentration of *A. camansi* leaves extract, which is higher than 5 mg/L, is not followed by an increasing dopamine level, otherwise decreasing dopamine levels. Hence, a concentration of 5 mg/ml is the maximum concentration in protecting neurons from rotenone-induced damage and is also a concentration to keep dopamine levels stable.

Dopamine (DA) is a brain hormone that acts as a neurotransmitter that regulates movement or motor systems, maintaining mood, memory, sleep, and cognitive processes.[16] Decreasing dopamine levels, cause increased Parkinson's symptoms with decreased motor activity and anxiety.[2]

Decreasing dopamine levels related with decrease locomotor activity is affected by the neurotoxin rotenone, which has a highly lipophilic structure. It encourages the molecules to quickly penetrates and cross the blood-brain barrier (BBB), then enters the central nervous system (CNS) and reach the position of the dopaminergic neurons.[17] Apart from the lipophilicity aspect, from a structural perspective, rotenone has a similar structure to dopamine, driving it easier to penetrate the dopaminergic nervous system of zebrafish. The improved dopamine level after 96% ethanol extract of *A. camansi* leaves treatment induced by flavonoid compound as antioxidant agent linked with locomotor effect.

**Locomotor Activity Assessment (*Tank Test*)**

The effect of rotenone-induce and 96% ethanol extract of *A. camansi* leaves treatment from 28 days onward on locomotor activity in adult male and female zebrafish was evaluated by motility observation with a tank test every seven days.

Table 2. The Motility of Male Adult Zebrafish for each group

| Day | Mortality means | | | | | |
|---|---|---|---|---|---|---|
| | **Control** | ***Rotenone*** | **2,5** | **5** | **7,5** | **10** |
| **0** | 436,23± 3,25 | 459,40± 3,67* | 472,57± 2,67** | 486,80± 3,95** | 437,73± 2,60** | 420,70± 2,42** |
| **7** | 459,17± 3,65 | 458,23± 4,83 | 409,77± 3,18** | 463,73± 3,98** | 429,03± 3,91** | 436,73± 10,93** |
| **14** | 472,23± 3,07 | 363,37± 2,95* | 426,03± 4,27** | 456,47± 4,74** | 389,17± 3,50** | 399,83± 7,35** |
| **21** | 463,17± 2,83 | 335,83± 3,73* | 412,57± 3,15** | 561,73± 1,84** | 456,83± 4,05** | 413,20± 2,75** |
| **28** | 500,97± 3,26 | 316,60± 4,00* | 578,87± 53,77** | 572,73± 2,80** | 536,27± 3,80** | 518,73± 60,63** |

*Each value is expressed as the mean ± SD. The significant difference compared to the control (without treatment) ($p<0.05$).
** Each value is expressed as the mean ± SD. The significant difference compared to rotenone ($p<0.05$).

In Table 2, the group of adult male zebrafish that received rotenone was shown to experience a decrease in locomotor activity compared to the control group ($p<0.05$). The 96% ethanol extract of *A. camansi* leaves treatment of 2.5 and 5 mg/ml in the adult male zebrafish group decreased motility activity. It affected rotenone induction, compared to higher concentrations of 7.5 and 10 mg/ml did not cause increased locomotor activity. The concentration of 5 mg/ml is the optimal concentration to maintain the locomotor activity of male zebrafish, and this is in line with the maximum dopamine level of 5 mg/ml. The decreasing dopamine levels were aline with decreasing adult male zebrafish locomotor activity.

Furthermore found a similar result, which is more of 5 µg/L rotenone inducing a lack of locomotor activity in zebrafish.[18] It seems that rotenone, a naturally occurring toxin and a widely used pesticide that inhibits the reduced form of nicotinamide-adenine dinucleotide dehydrogenase in mitochondria, imitates the neuropathological, neurochemical, and behavioral characteristics of Parkinson's disease in vertebrates.[19] Even though this rotenone effect depends on several factors, such as temperature, pH, sunlight, depth of the aquarium, and the

presence of organic debris,[20] the reduction in zebrafish movement could be attributed to a decline in the velocity of motor nerve conduction.[21]

Table 3. The Mortality of Female Adult Zebrafish for each group

| Day | Mortality means | | | | | |
|---|---|---|---|---|---|---|
| | **Control** | ***Rotenone*** | **2,5** | **5** | **7,5** | **10** |
| **0** | 545,03± 4,17 | 522,53± 4,44* | 519,07± 2,44 | 526,03± 2,48 | 519,97± 3,76 | 469,20± 78,53 |
| **7** | 535,90± 3,58 | 525,97± 2,63 | 457,17± 3,36** | 509,20± 3,37** | 478,43± 3,20** | 377,07± 53,92** |
| **14** | 496,83± 3,76 | 489,57± 2,97* | 577,50± 1,81** | 359,73± 57,27** | 447,10± 3,42** | 432,57± 3,00** |
| **21** | 593,00± 2,31 | 458,93± 2,93* | 415,40± 3,06** | 553,00± 2,89** | 586,40± 2,15** | 348,30± 2,95** |
| **28** | 575,53± 2,85 | 432,97± 2,55* | 429,87± 53,77** | 592,73± 1,10** | 535,80± 4,03** | 435,03± 1,16** |

*Each value is expressed as the mean ± SD. The significant difference compared to the control (without treatment) ($p<0.05$).
** Each value is expressed as the mean ± SD. The significant difference compared to rotenone ($p<0.05$).

In this research, we observed different motility between adult male and adult female zebrafish. Table 3 contains the motility of adult female zebrafish with rotenone-induced resulting in decreased motility. The increase of motility activity in adult female fish began to be seen as stable in 5 mg/ml *A. camansi* extract after being induced by rotenone. Along with increasing the concentration of leaves extract of 7.5 and 10 mg/ml group concentration was not followed by an increase in locomotor activity in adult female zebrafish. This pattern was also observed in adult male zebrafish, which had no increasing activity despite increasing extract concentrations. From these results, it can be seen that the maximum concentration to maintain dopamine levels in both adult male and female fish is 5 mg/ml. In addition, this research shows that male zebrafish have lower dopamine with lower locomotor activity than female zebrafish. It relates to the research on humans with Parkinson's disease that adult males have high risk than adult females.[22]

Several research has evaluated that 96% ethanol extract of *A. camansi* leaves dominated by a flavonoid, which is related to this research finding.[13,23,24] Moreover, flavonoid has significantly affected Parkinson's disease ailment, proven by clinical research by Gao *et al.* (2012).[25] The research evaluated 438 men and 367 women who developed PD during 20–22 years of follow-up consuming flavonoid-rich foods, resulting in a lower risk of Parkinson's disease. This result is supported by the flavonoid activity as an antioxidant that overcomes ROS levels in the brains of patients caused by mitochondrial damage accumulation.[26]

In several animal studies, flavonoids have been found to possess anti-inflammatory, antioxidant, and antidepressant properties.[27,28] These effects are believed to result from their ability to regulate neurotransmitter levels in the brain by interacting with transcription factors, enzymes, and kinases or by modifying neurotransmitters themselves.[29] Flavonoids also appear to inhibit the production of reactive oxygen and nitrogen species, which can lead to mitochondrial DNA damage and lipid peroxidation due to their potent antioxidant properties. When combined with endogenous scavengers, flavonoids have a synergistic and additive effect.[30] They can interfere with more than three free radical-producing systems at

a time and ultimately enhance the action of endogenous antioxidants, reducing cellular damage.

## CONCLUSION

The 96% ethanol extraction of *kluwih* (*A. camansi*) leaves treatment can increase dopamine levels and locomotor activity in adult male and female zebrafish. The maximum of *A. camansi* leaves extract in male and female zebrafish to maintain stable dopamine levels is 5 mg/ml. The results of the phytochemical screening showed that the *A. camansi* leaves extract positively possesses flavonoids, alkaloids, tannins, steroid-triterpenoids, and phenolics. Subsequent research continues to determine the active fractions and subfractions of the ethanol extract of *kluwih,* which can increase dopamine levels and locomotor activity.

## 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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