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The Effect of Keluwih (*Artocarpus camansi*) Leaves Extract On Pro-Inflammatory Expression, Growth Factors and Bodies in Zebrafish Larvae (*Danio rerio*) Stunting Model

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Stunting is a chronic nutritional problem that occurs during the first 1000 days of life which is considered a golden window of opportunity. Indonesia has the highest prevalence compared to other middle-income countries. Keluwih (Artocarpus camansi) is known to have various compounds that are beneficial for the body such as anti-inflammatory and antioxidant. This study was conducted to determine the potential of the ethanol extract of Keluwih leaves (Artocarpus camansi) zebrafish stunting model against inflammatory markers, growth factors and body size. Artocarpus camansi leaves were extracted using the maceration method for 3x24 hours with 96% ethanol solvent. Zebrafish larvae were obtained from male and female broodstock (2:1), then induced using rotenone and ethanol extract of Artocarpus camansi leaves, then immunohistochemical staining was performed using growh factor (VEGF and TGF-ß), inflammation (IL-6 and TNF-a) and body length measurements on day 9 dpf. The results showed that rotenone can provide a picture of stunting in zebrafish larvae from observations of growth factors, inflammation and body length, by administering ethanol extract of Artocarpus camansi leaves this can improve stunting conditions due to administration of rotenone, the concentration of ethanol extract of Artocarpus camansi leaves 2.5 ppm is the optimal concentration in improve stunting conditions. The ethanol extract of Artocarpus camansi leaves can improve stunting conditions by increasing the expression of growth factors, decreasing pro-inflammatory cytokines and improving body length in zebrafish larvae.

Keywords: Artocarpus camansi; Stunting, Growth factors; Malnutrition; pro-inflammatory cytokines.

Stunting represents a significant nutritional challenge encountered by developing nations [1]. Stunting is a persistent nutritional issue that manifests during the initial 1000 days of life, recognized as a critical window of opportunity [2]. Indonesia has one of the highest rates of stunting prevalence among middle-income countries [3].

Stunting can be caused by several factors, including carrying out malnutrition care and assessing the insufficient awareness of health

and nutrition among mothers prior to pregnancy, during pregnancy, and after the mother gives birth [3]. Stunting causes growth disorders, which are characterized by height that does not match age [4]. Children who undergo stunting may exhibit suboptimal intelligence levels, rendering them more vulnerable to diseases and, in the long term, exposing them to potential declines in productivity [5]. In addition, stunting also has an impact on cognitive development disorders and delays in motor development, most of which are irreversible [6].

The prevention of stunting is crucial to safeguard the quality of the next generation. By nurturing a superior next generation, Indonesia can enhance its competitiveness on the global stage and effectively tackle future challenges [7]. Family assistance teams have been deployed across all regions of Indonesia with the aim of decreasing the stunting rate to 14% by 2024. According to data from the 2021 Indonesian Toddler Nutrition Status Survey, the current prevalence of stunting stands at 24.4% [8].

Traditional medicine using herbal plants is still an option that can be used for treatment [9]. When compared to chemical drugs, traditional medicine has slower performance, but the use of herbal plants as the main raw material makes traditional medicine have milder side effects [10]. Keluwih (*Artocarpus camansi*) is a plant that is widely distributed in tropical and subtropical parts of Asia. Keluwih comes from Papua New Guinea, Indonesia, and the Philippines. One of the distribution areas of keluwih in Indonesia is Maluku, keluwih plants are also found in lowland areas [11]. Based on the results of phytochemical screening, simplicia and ethanol extracts of keluwih leaves contain alkaloids, flavonoids, tannins, glycosides, anthraquinone glycosides, and steroids/triterpenoids [10,11]. The rich content of compounds in keluwih leaves is expected to provide benefits to the Indonesian people, especially in existing stunting conditions. It is known that this keluwih has potential as an anti-inflammatory, antioxidant, antifungal, and antibacterial [12,13]. Therefore, this study was undertaken to assess the potential of keluwih leaf extract against zebrafish stunting models against inflammatory markers, growth factors, and body size. Zebrafish induced by rotenone 12.5 ppb can be used as a stunting model in previous studies [14].

METHODS

Animal Care

Adult male and female zebrafish sourced from the wild were identified at the reproduction laboratory of the Faculty of Fisheries and Marine Sciences, Brawijaya University. The zebrafish are housed in semi-static 60 L tanks, with temperature in water maintained between 24-26.5°C and a light cycle of 14:10 (dark:light) [15]. The fish received three daily feedings using Tetra Color ® Tropical Flakes from Blacksburg at Germany [16,17].

Embryos from zebrafish are collected after male and female fertilization in a 2:1 ratio. These embryos, aged 0-2 hours post-fertilization, are selected based on being round, clear, fertile, and free from mold. A total of 100 larvae are used in the study. The research is approved by the University of Brawijaya's Medical Faculty Ethics Committee (No. 149-KEP-UB-2023). The embryos are categorized into five different groups, including a normal control, a negative control with 12.5 ppb rotenone, and three experimental groups with varying levels of EEKL added to 12.5 ppb rotenone.

Embryo Media

The medium for embryo was prepared at a concentration of 10x, composed of 0.15 grams of CaCl, 0.15 grams of KCl, 5 grams of NaCl, 0.815 grams of MgSO4, and 500 milliliters of distilled water [14].

Extraction of Keluwih Leaves (*Artocarpus camansi*)

Artocarpus camansi has obtained certification from UPT (Unit Pelaksana Teknis) Materia Medica, with number 074/124/102.20-A/2022. The extraction procedure followed the maceration method, utilizing 96% ethanol (at a ratio of 1:10) and conducted over a period of 3 cycles, each lasting 24 hours. The extract obtained was evaporated until a concentrated ethanol extract of Keluwih Leaves (EEKL) was obtained.

Rotenone and EEKL Administration

Rotenone (R8875) purchased at sigma aldrich, with a purity of 95%, were dissolve in 1% DMSO to produce a stock solution. Rotenone was administered at a concentration of 12.5 ppb [14], and the EEKL concentration varied 2.5; 5; 10 ppm.

Body Length Measurement

On the 9th day post-fertilization (dpf),

measurements of zebrafish larvae's body length were conducted. The larvae were examined utilizing an Olympus SZ61 stereomicroscope and subsequently quantified using calibrated Image J software. The body's length is determined by measuring from the nose's tip (snout) to the tail fin's base [18].

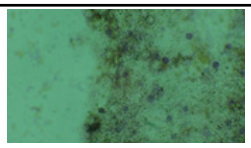
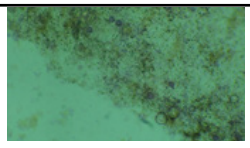
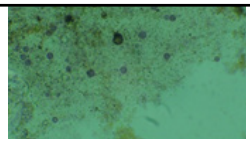
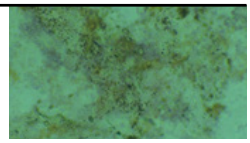
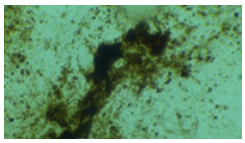
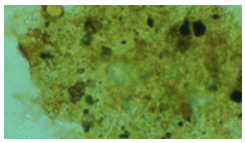
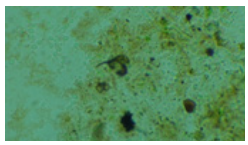
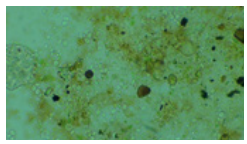
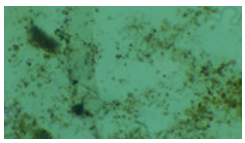
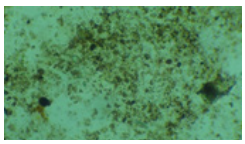
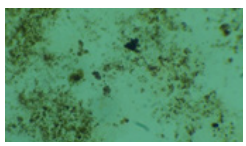
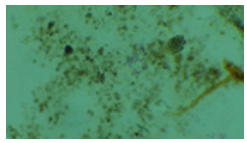
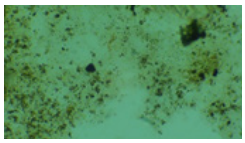
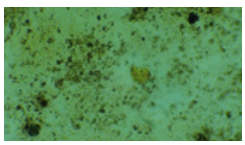
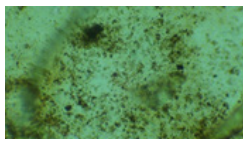
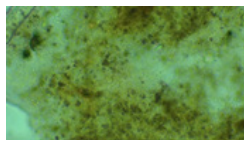
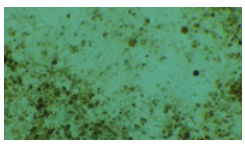
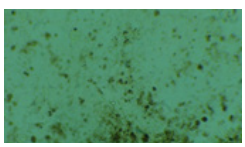
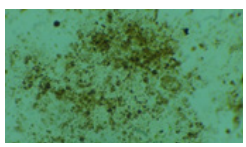
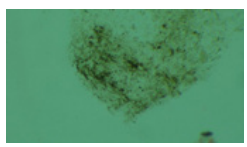
Growth factor (VEGF and TGF-â) and inflammation (IL-6 and TNF-á) measurements

Zebrafish larvae aged 9 dpf were euthanized based on the NIH protocol. Whole zebrafish larvae were placed in a microtube in ice water for at least 5 minutes and confirmed that there was no movement. Then rinsed and fixed with cold methanol 20 °C for 3-5 minutes, followed by inactivation using peroxide blocking solution at 25 °C for 10 minutes and under running water for 5 minutes. Whereupon incubated in prediluent blocking solution for 10 minutes at room temperature.

The next stage was by incubating 100µL of commercial monoclonal primary antibody per preparation in the refrigerator for 24 hours, then washing it using PBS for 5 minutes. Then added biotinylated universal secondary antibody for each IL-6 observation (100µL at 1:50 dilution, Sigma Aldrich, HPA035283); TNF-á (1-2µg/mL, Sigma Aldrich, SAB1404480); VEGF (at 1:20 to 1:100 dilution, Sigma Aldrich, AB1876-I); TGF-â (with 1:100 dilution, Termofisher, MA5-16949) was then incubated at 25 oC for 10 minutes. The incubated preparations were washed PBS (5 minutes) and again incubated using streptavidin/peroxide complex reagent (10 minutes) and washed PBS (5 minutes).

The next step is to detect the reaction by incubating with peroxide substrate solution (DAB) 100µL per preparation for 2-10 minutes, washing with running water and adding 100µL of Mayer's hemoxylin (counterstrain) reagent per preparation

Table 1. Visualization of positive cells containing observed proteins

Groups / markers	IL-6	TNF-α	TGF-β	VEGF
Normal controls				
Negative controls				
D1				
D2				
D3				

and incubating for 1-3 minutes then washing under water flow. The final stage is dehydration and mouting as usual. Afterward, it was scrutinized using a Nikon Eclipse type Ei light microscope, aided by an Optilab Microscope Camera that was connected to a computer.

Statistic analysis

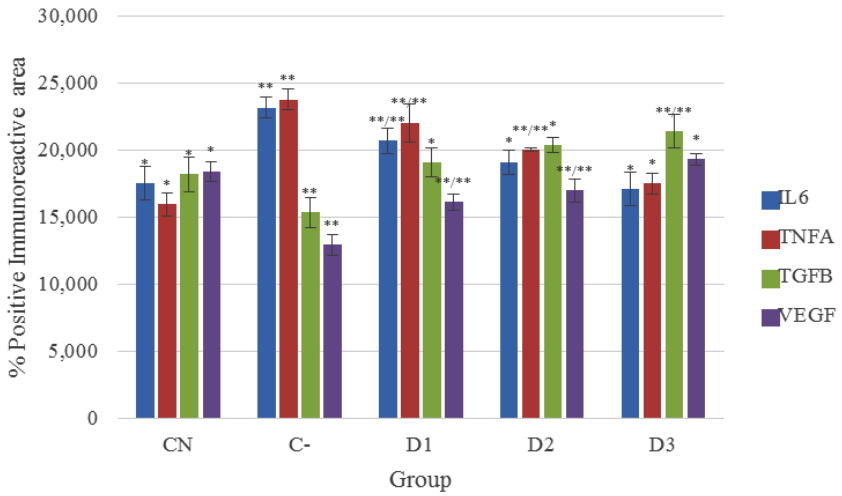
Statistical evaluation was conducted through IBM ANOVA SPSS version 23.0, followed by the LSD post hoc test at a 95% confidence interval. Shapiro-Wilk was used for the normality assessment and the Levene test was used for checking homogeneity.

RESULTS AND DISCUSSION

The use of pesticides as an environmental factor can trigger stunting. Rotenone is a pesticide with a concentration of 12.5 ppb, which can induce stunting [19]. Rotenone works to inhibit mitochondrial complex I and inhibit the process of ATP synthesis so that the amount of ATP decreases

Table 2. Body length measurements of zebrafish larvae

Groups	CN	C-	D1	D2	D3
Figures					
Means	2.118	1,854	2022	2040	2.113
SD	21.65641	61.29437	38	36.96395	15.27525



NB:
* : sig C- (p<0.05)
** : sig CN (p<0.05)

Fig. 1. Quantification of % immunoreactive positive areas

and induces an elevation in ROS (reactive oxygen species), leading to potential cell death, cellular damage, and other oxidative risks [20].

Giving rotenone with a concentration of 12.5 ppb to zebrafish larvae affected growth factors and inflammatory cytokines, which manifested in the body length of the larvae. Rotenone administration can increase levels of inflammatory cytokines (IL6 and TNFa) and decrease growth factors (TGF-â and VEGF). This can be seen from the many brown images in Figure 1, which show a positive immunohistochemical reactivity. Rotenone administration also showed a shorter body length in zebrafish larvae compared to the normal group (Table 2) [14].

The images obtained were measured quantitatively to determine the persentage positive area in immunohistochemical (immunoreactive) observations. The results of the quantification values obtained can be seen in Figure 1.

Giving EEKL can improve the condition of the stunting model in zebrafish. It has been proven to reduce inflammatory cytokine levels, increase growth factor levels, and improve body length in zebrafish stunting larvae. The optimal dose of EEKL is 2.5 ppm, because this is the lowest dose that has an effect on improving stunting conditions in zebrafish larvae.

Poor nutrition and ongoing inflammation driven by pro-inflammatory cytokines play a role in causing growth delays. At the initial stage, the levels of several highly inflammation mediators such as TNF-á, IL-6, and IL-12 were observed to be reduced in stunted children when compared to those in the normal control group [21]. Inflammatory cytokines observed in this study were IL6 and TNF-á. TNF consists of two related proteins mainly produced by mononuclear lymphocytes (TNF-â) and phagocytes (TNF-á). Assessing TNF-á levels in malnourished kids is crucial as low TNF-á can weaken the immune system, while high levels can worsen nutrition by causing anorexia and cachexia. IL-6 is key in triggering acute phase protein synthesis in liver cells. Excessive IL-6 in children can cause chronic inflammation and contribute to growth issues, including stunting. It negatively affects liver IGF-I gene activity and facilitates the reduction of IGFBP-3 (insulin-like growth factor-binding protein-3).

Sederquist and colleagues discovered that multiple inflammatory cytokines like TNF-á and IL-6 can singly or jointly impact child growth. These cytokines can operate through overall systemic pathways or specifically target the growth speed of long bones [22,23].

The cytokine Transforming Growth Factor â (TGF-â) holds a pivotal role in regulating cell growth and differentiation across diverse tissues. Additionally, it is involved in processes such as inflammation, autoimmunity, and tumor development [24]. Under normal circumstances, local sources maintain tissue homeostasis by preserving baseline levels of TGF-â signaling. Following tissue damage, TGF-â is extensively secreted by blood platelets and other stromal elements to aid in tissue repair, wound healing, and reducing inflammation. The interaction between TGF-â signaling and reactive oxygen species (ROS) metabolites is crucial [25].

Reduced ROS levels play crucial roles in determining cell fate and cellular responses affecting cell proliferation, differentiation, and death [26], similar to TGF-â signaling. If ROS levels surpass the body's antioxidant defenses, this imbalance leads to oxidative stress, harming proteins, nucleic acids, and lipids either directly or indirectly [27]. TGF-â is abundant in bones and cartilage. It promotes the growth, differentiation, and formation of osteoblasts from osteoprogenitor cells [28].

Under normal circumstances, increased ROS also causes hypoxia, which stimulates VEGF expression. Hypoxia boosts VEGF through enhanced mRNA transcription and stability, triggering blood vessel formation to sustain oxygen levels. Excessive ROS from oxidative stress damages cells by mutating VEGF via intricate signaling routes. Chronic hypoxia lowers VEGF expression, impairs tissue vascularization, and causes endothelial dysfunction. Such dysfunction disrupts angiogenesis, regulated by VEGF's interaction with VEGFR-2, affecting cell proliferation and growth [29].

CONCLUSION

Artocarpus camansi leaves ethanol extract (EEKL) can improve stunting conditions through increasing growth factor expression, decreasing pro-inflammatory cytokines and increasing body

length in zebrafish larvae induced by rotenone of 12.5 ìg/mL significantly, optimal concentration of *Artocarpus camansi* leaves ethanol extract in overcoming stunting conditions it's 2.5 ppb.

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Conflict of Interest

The authors declare no conflict of interest.

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