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Research Reports

Effect of *Artemisia vulgaris* Supplementation on Zebrafish Embryo Under Heat Stress Condition during In Vitro Culture

Efek Sumplementasi *Artemisia vulgaris* pada Embrio Zebrafish di bawah Pengaruh Kondisi Stres Panas Pada Saat Kultur In Vitro

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ABSTRACT

Background: *Artemisia vulgaris* contains flavonoids, which play a vital role in counteracting free radicals. **Purpose:** To determine the effect of *Artemisia vulgaris* extract supplementation on embryo development, heart rate and survival of zebrafish under heat stressed and non-heat stressed conditions. **Methods:** The research used a completely randomized design. Zebrafish embryos (n=240) were divided into heat stressed (36ºC) and non-heat stressed (28ºC) groups, while for each group were divided into three subgroups, namely T1/control (without *Artemisia vulgaris* supplementation); and supplemented group T1 and T2, with 2 µL and 4 µL of *Artemisia vulgaris* supplementation, respectively. The efficacy of *Artemisia vulgaris* supplementation was determined by observing the embryo development, heart rate, and survival rate of zebrafish up to 96 hours post fertilization (hpf). **Results:** The development of zebrafish embryos under heat stressed treated with *Artemisia vulgaris* extract gave the same quality as the control treatment without heat stressed exposure. Zebrafish embryos exposed to heat stressed with 4 µL *Artemisia vulgaris* supplementation gave the highest survival rate on the heat stressed group. *Artemisia vulgaris* supplementation improved the heart rate of zebrafish exposed to heat stressed as in the non-heat stressed group. **Conclusion:** *Artemisia vulgaris* extract can reduce the detrimental effects of heat stressed induction on zebrafish embryos, as evidenced by the improvement in embryonic development, heart rate, and survival rate of zebrafish embryos after supplementation.

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ABSTRAK

Latar Belakang: *Artemisia vulgaris* mengandung flavonoid, yang memainkan peran penting dalam menangkal radikal bebas. **Tujuan:** Untuk menentukan efek suplementasi ekstrak *Artemisia vulgaris* pada perkembangan embrio, denyut jantung dan kelangsungan hidup ikan zebra di bawah kondisi stres panas dan non-stres panas. **Metode:** Penelitian ini menggunakan desain acak lengkap. Embrio ikan zebra (n=240) dibagi menjadi kelompok stres panas (36°C) dan non-stres panas (28°C), sedangkan untuk masing-masing kelompok dibagi menjadi tiga subkelompok, yaitu T1/kontrol (tanpa suplementasi *Artemisia vulgaris*; dan melengkapi kelompok T1 dan T2, dengan 2 µL dan 4 µL suplementasi *Artemisia vulgaris*. Efikasi suplementasi *Artemisia vulgaris* ditentukan dengan mengamati perkembangan embrio, denyut jantung, dan tingkat kelangsungan hidup ikan zebra hingga 96 jam pasca fertilisasi. **Hasil:** Perkembangan embrio ikan zebra di bawah stres panas yang diobati dengan ekstrak *Artemisia vulgaris* memberikan kualitas yang sama dengan perlakuan kontrol tanpa paparan stres panas. Embrio ikan zebra yang terpapar stres panas dengan suplementasi 4 µL *Artemisia vulgaris* memberikan tingkat kelangsungan hidup tertinggi pada kelompok stres panas. Suplementasi *Artemisia vulgaris* meningkatkan denyut jantung ikan zebra yang terpapar stres panas seperti pada kelompok non-stres panas. **Kesimpulan:** Ekstrak *Artemisia vulgaris* dapat mengurangi efek merugikan dari induksi stres panas pada embrio ikan zebra, yang dibuktikan dengan peningkatan perkembangan embrio, denyut jantung, dan tingkat kelangsungan hidup embrio ikan zebra setelah suplementasi.

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Kata kunci: *Artemisia vulgaris*; Detak Jantung; Perkembangan Embrio Zebrafish; Stres Panas; *Survival Rate*

INTRODUCTION

Currently, zebrafish *(Danio rerio)* are used to study about molecular biology, developmental biology, neurology, genetics, cancer, nervous system, physiology, drug discovery, as well as reproduction and embryology (Reed and Jennings, 2010). For example, a pair of testicles in males and ovaries in females make zebrafish a good animal model to study reproduction and embryology (Ningsih, 2018). Furthermore, the transparent and fast developing zebrafish embryo has a fusiform body shape with a body length of less than 40 mm. This fish has a terminal oblique mouth that points upward with a more prominent lower jaw. The lateral line in this fish is incomplete and extends to the base of its pelvic fin. Two pairs of antennae and five to seven dark blue lines running from behind the operculum to the caudal fin. The anal fin has the same line, while the dorsal fin has a dark blue upper border, bordered with white (Spence *et al.*, 2008). According to Harper and Lawrence, (2011), the zebrafish is often referred to as an ideal organism for studying human gene function. The use of zebrafish as an animal model began to increase along with the development of the field of molecular biology during the 1960s and continued to grow rapidly after 1996.

The advantages of using zebrafish as experimental animals are that they are easy to care for and their embryos multiply rapidly within zero to 72 hours post fertilization (hpf). Zebrafish are animals that absorb chemical substances easily, making them well suited for research that examines the effect of an ingredient in liquid form (extract) that is added to the fish's aquatic environment for manipulation and experimental observation. Zebrafish are ideal as a model because they have 70% to 80% homologous gene similarity with mammals, including humans (Ningsih, 2018).

Artemisia vulgaris, also known as beungkar kucing, suket gajahan, kolo, and Chinese goro, are found in all regions in Indonesia. This plant is an herb that grows wild and abundantly in temperate and cold zones in the world, with a height of about 30 to 90 cm. *Artemisia vulgaris* contains flavonoids, tannins, saponins, quinones, and steroids/terpenoids (Dayana, 2020).

Heat stress (HS) is a condition that occurs when the body begins to lose control of its internal temperature. Temperature plays an important role in physical and chemical processes that determine how biological systems function. The susceptibility of animals to HS varies with species, genetic potential, life stage, production, and nutritional status. Exposure to HS can cause basic activities such as cell proliferation to stop cease due to cell damage, particularly to DNA. At severe acute stress levels, all cells can become damaged and trigger the induction of the apoptotic pathway (Logan and Somero, 2011). Our previous study indicatres that HS induced multiple adverse effects on bovine embryos exposed to HS, which can be repaired by lycopene supplementation due to lycopene's high antioxidant level (Residiwati *et al.*, 2021). In this study, we would like to observe the supplementation effect of another high flavonoid source, *Artemisia vulgaris,* on zebrafish *(Danio rerio)* embryos induced by HS, as a mimic (at least) of the effect of HS on human embryos as well as the potential effect of *Artemisia vulgaris* supplementation as one of the solutions to face the global warming.

MATERIAL and METHOD

Preparation of the Artemisia vulgaris Extract

Artemisia vulgaris was prepared according to Handoyo and Pranoto (2020). A. vulgaris leaves were washed, chopped, and dried in an oven for six to eight hours. The extract was made by mixing 1 gr of dried *Artemisia vulgaris* leaf added with 10 ml of distilled water, then allowed to stand for five hours and filtered using filter paper.

Preparation of Experimental Animals

Zebrafish embryos (n = 240) were collected from zebrafish broodstock obtained from the Faculty of Fisheries and Marine Sciences, Universitas Brawijaya. The embryos were then divided into HS (36°C) and NHS (28°C) group, while for each group were divided into three subgroups, namely P1 / control (without *Artemisia vulgaris* supplementation); and supplemented group P2 and P3, with 2 µL and 4 µL of *A. vulgaris* supplementation, respectively. The efficacy of *A. vulgaris* supplementation was determined by observing embryo development, heart rate, and survival rate of zebrafish up to 96 hours post fertilization (hpf).

Heat Stress Exposure

Heat stress was performed by transferring the zebrafish embryos to a glass beaker and placing it in a water bath (Memmert®, Germany). Meanwhile, the HS temperature was slowly increased from 28°C (normal temperature) to 36°C, and then kept constant for one hour. The heat stress temperature was then slowly decreased to the normal temperature of 28°C.

Treatment

Embryos were placed in six-well plates. Each well containing two ml of culture medium for 10 embryos (to study the survival rate and heart rate in group culture). For each treatment, we also gave an individual culture with one embryo on one plate to observe the embryo development. For supplemented group T1 and T2, 2 µL and 4 µL of A. vulgaris supplementation, were added respectively. The embryos were then incubated at normal temperature (28°C) and the embryo development, heart rate, and survival rate were observed at 48, 72, and 96 hours post fertilization (hpf). Every 24 hours, the medium in each well were replaced using the same solution.

Data Analysis

In this study, qualitative and quantitative data was obtained. The zebrafish embryo development was collected and then described as qualitative data. The quantitative data obtained, namely heart rate and survival rate of zebrafish embryos, were analyzed using SPSS 23 (International Business Machines Corporation, United States) with ANNOVA variance.

RESULTS

Zebrafish Embryo Development

The development of zebrafish embryos at gastrula, neurula, organogenesis, and hatching stages is described on **Figure 1, 2, 3, and 4**, respectively.

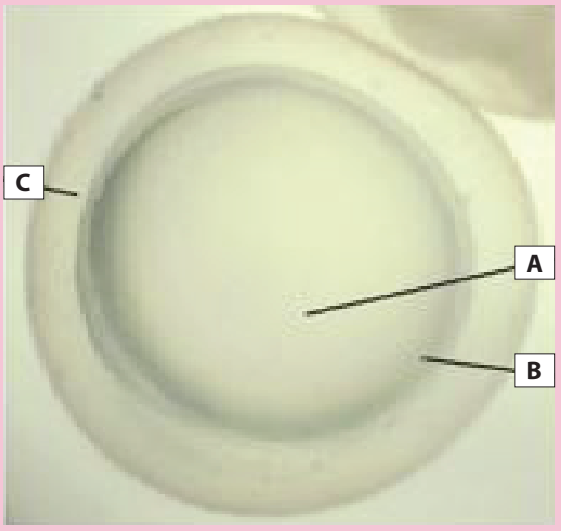

Figure 1. Gastrula phase of the zebrafish embryo development. A= Yolk Sack; B=Blastoderm; C= Perivitelline Space.

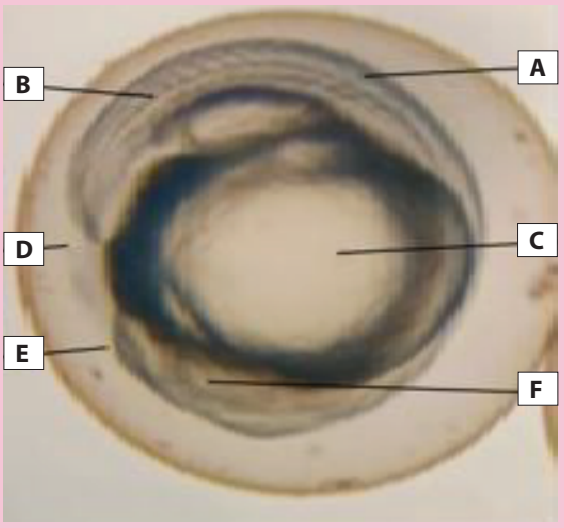

Figure 2. Neurula phase of the zebrafish embryo development. A= Somites; B=Notochord; C=Yolk Sack; D=Perivitelline Space=; E=Head; F=Eye.

Heart Rate and Survival Rate on Zebrafish Embryo

The average of heart rate and the percentage of survival rate on zebrafish embryo on 48, 72, and 96 hpf are shown in **Table 1 and 2**, respectively. The Least Squares Means (LSM) graph of heart rate on zebrafish embryo at 48, 72, and 96 hpf are presented in Table 3. The Least Squares Means (LSM) graphic of survival rate on zebrafish embryo at 48, 72, and 96 hpf are shown in **Table 4**.

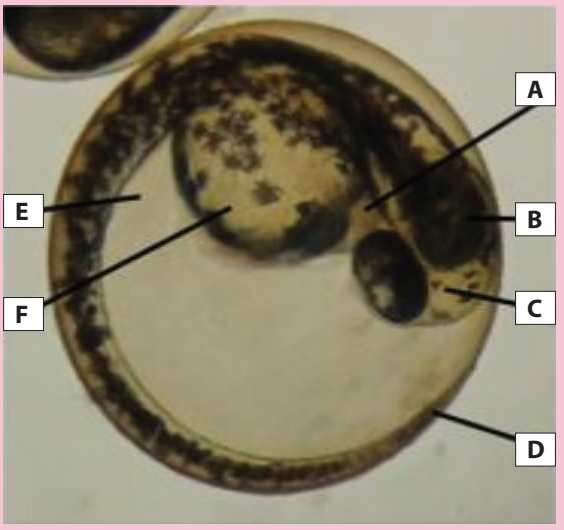

Figure 3. Organogenesis phase of the zebrafish embryo development. A = Heart; B= Eyes; C=Head; D=Tail; E=Perivitelline Space; F=Yolk Sack.

Figure 4. Hatching phase of the zebrafish embryo development. A=Eyes; B=Head; C= Heart; D=Yolk Sack; E=Tail

DISCUSSION

A. vulgaris Supplementation on Embryo Development

The development of zebrafish embryos can be observed after fertilization. According to Sholiha (2018), embryogenesis is the process of formation and progressive growth of a cell towards the organ period, which is called organogenesis. The development of the zebrafish embryo is divided into several phases, namely cell division (cleavage), morula, blastula, gastrula, neurula and organogenesis. In this study, the development of zebrafish embryos began to be observed after HS exposure, when they entered the gastrula phase. During the gastrula phase, the eyes, the tail, and the width of perivitelline space were observed. According to Bensch *et al.*, (2013), during the gastrula phase, blastomeres move to cover the surface of the yolk and there are three coordinated cell movements that then form the three primary germ layers, namely epiboly, embolism, and convergence. Epiboly is the spreading movement of embryonic cells over the yolk mass and produc-

Table 1. Heart Rate of Zebrafish Embryos at 48, 72 and 96 hpf.

Group	Average (bpm)		
	48 hpf	72 hpf	96 hpf
NHSP1	126	145	160
NHSP2	128	145	154
NHSP3	125	146	159
HSP1	148	160	170
HSP2	142	151	164
HSP3	133	148	157

Table 2. Survival Rate of Zebrafish Embryos at 48, 72 and 96 hpf.

Group	Average (%)		
	48 hpf	72 hpf	96 hpf
NHSP1	100	92.5	92.5
NHSP2	100	95	95
NHSP3	100	97.5	97.5
HSP1	92.5	87.5	82.5
HSP2	95	90	87.5
HSP3	100	95	95

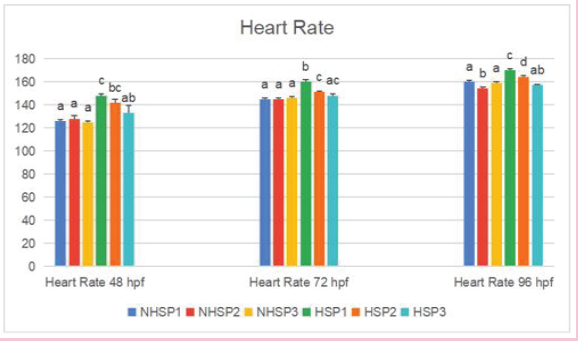

Figure 2. The results of the LSM graphic heart rate of zebrafish embryos at 48, 72 and 96 hpf.

Figure 3. The results of the LSM graphic survival rate of the zebrafish embryos at 48, 72 and 96 hpf.

es an ectoderm layer to cover the embryo. Embolism is a movement that relocates mesendoderm to the inside of the embryo. Meanwhile, convergence is the elongation of the embryo shield. The development of the embryo in the gastrula phase on this study can be seen in **Figure 1**. In the neurula phase **(Figure 2)**, the somites have been formed, the eyeballs are clearly visible, the perivitelline space is smaller, and the notochord is formed. This is consistent with the study from Sholiha (2018), who revealed that in the neurula phase, a central nervous system is formed and there are segments in the embryo such as the notochord, somites and eye spots.

The organogenesis phase is the phase after the neurula phase, where in this phase the eyes will become enlarged eye spots, while the heads and tails have also developed, and the hearts that have started to beat. Sholiha (2018) stated that the neurula phase is the phase where body segments appear, the formation of the spine becomes the future tail, the body of the embryo elongates, and the embryo can move in the egg yolk. The organogenesis phase of zebrafish embryos in this study is shown in **Figure 3**. Furthermore, hatching is the final process of embryogenesis where the embryo emerges from the chorion layer and begins to show clearer morphogenesis of its organs (Kimmel *et al.*, 1995). These hatched embryos then become free-swimming fish larvae. The hatching phase of zebrafish embryos in this study is shown in **Figure 4.**

In this study, there was no significant difference in zebrafish embryos development between HSP2, HSP3 and NHS group. It showed that *Artemisia vulgaris* supplementation can maintain the development of Zebrafish embryos under HS conditions to have the same conditions as in the control treatment (without HS exposure). We hypothesized that *Artemisia vulgaris* can alleviate abnormalities caused by HS induced at 36°C by activating heat shock proteins (HSPs) which can protect the embryos. According to Wu *et al.*, (2012), HSPs play an important role in protein folding, intracellular transport, protein degradation, and signalling, which are responsible for maintaining cell viability by preventing protein loss and promoting cell regeneration. On HSP3, there was one embryo with an abnormality in the form of curved vertebrae, which may be caused by the internal factors in the culture medium. This was consistent with the study of Harper and Lawrence, (2011), which stated that internal factors such as heredity, immunity, as well as for external factors such as temperature, water pH, alkalinity, and carbon dioxide levels on culture medium give a vital contribution on embryo development. Further studies should be done to know the molecular interaction.

A. vulgaris Supplementation on Embryo Heart Rate

In this study, zebrafish embryos with HS induction had a higher heart rate compared to those with NHS, but was still within the normal range of 125 to 170 bpm. This was consistent with the study of Sampurna *et al.*, (2018), which indicates that the normal range of zebrafish embryos was between 120 and 180 bpm. Fish are ectothermic animals, which means

that their body temperature can be affected by the ambient temperature. As a result, their ability to adapt to fluctuations in ambient temperature depends on changes in biochemical and metabolic processes to maintain their homeostasis. Iftikar and Hickey, (2013) stated that the metabolic rate of ectothermic animals usually increases with an acute increase of habitat temperature. If the metabolic rate increases drastically, it will affect the growth, physiological balance, and ultimately survival of the fish. Therefore, HS has complex and integrative effects on circulation, respiration, digestion, growth, reproduction, and ectothermic locomotor capacity. Ectothermic animals such as fish have a temperature sensitive cardiovascular system, and in most cases the critical temperature for heart failure (THF) is only a few degrees above the upper habitat temperature or maximum temperature (Tmax).

Acute heart failure in ectotherms is the result of decreased oxygen availability because increased habitat temperature decreases blood oxygen solubility in the blood while increasing metabolic rates (Pörtner *et al.,* 2004). Mitochondrial dysfunction can lead to heart failure(HF). Mitochondria are at the centre of HF in several heart diseases, and as temperature approaches Tmax, the concentration of succinate, or a tricarboxylic acid cycle intermediate, increases in the blood of fish experiencing HS (Pörtner and Knust, 2007). In vertebrates, the presence of succinate in the blood indicates mitochondrial dysfunction. Mitochondria occupy 20% to 40% of the vertebrate cardiomyocytes volume. Cardiac mitochondria differ from mitochondria of other tissues in that they are sensitive to heat stress, ischemic damage, and oxidative stress. Cardiac mitochondria have been studied in the context mammals exposed to heat stress. Thermally dysfunctional cardiac mitochondria can also increase the release of reactive oxygen species (ROS) and promote apoptosis. Apoptosis may contribute to heart failure through intrinsic or mitochondria pathways (Iftikar and Hickey, 2013). *A. vulgaris* extract has an antioxidant activity which is very good at confining ROS and other free radicals (Febrina *et al.,* 2017). The antioxidant content of *A. vulgaris* can reduce free radicals by balancing reactive oxygen species (ROS) and improve the body functions of fish exposed to HS, so there is no damage to the fish hearts mitochondria and it also prevents apoptosis.

A. vulgaris Supplementation on Zebrafish Embryo Survival Rate

The survival rate of zebrafish embryos exposed to HS after *A. vulgaris* supplementation (HSP2 and HSP3) had higher values than the control group (NHSP1), which indicates that the administration of A. vulgaris improved the survival rate of zebrafish embryos. According to Lee *et al.,* (2014), water temperature is one of the most important environmental factors affecting the survival of aquatic animals and the growth of aquaculture. In teleost fish, any change in culture water temperature can affect fish survival, physiological conditions, and immune responses. Fish survival refers to the ability of fish to adapt to certain conditions in an optimal conditions. Environmental conditions that can affect fish survival depend on pH, temperature, chemicals, and pollutants. When survival rate is low, it indicates that the organism is unable to survive in its environment (Thiagarajan *et al.,* 2019). Heat stress can disrupt animal body homeostasis of animals by mediating oxidative stress and the formation of reactive oxygen species (ROS). The balance between ROS production and the antioxidant defense system is disrupted by environmental stress, leading to excessive ROS production, which has toxic effects on cells (Qiu *et al.,* 2011). Under normal conditions, ROS play many important roles in cellular metabolism such as cell growth and apoptosis (Luo *et al.,* 2014). Normal cellular defense systems, such as enzymatic defence systems and natural antioxidant systems, counteract the negative effects of oxidative stress and are the first intracellular defense against ROS. Heat stress can induce excessive ROS production, which causes damage to cellular biomolecules such as proteins, lipids, and DNA, ultimately leading to impaired cellular function (Meng *et al.,* 2014). Extensive oxidative stress leads to impaired cell signaling, severe DNA damage, and apoptosis. Apoptosis can be triggered by two main mechanisms, the first is the extrinsic pathway and the second is the intrinsic apoptotic pathway or the mitochondrial pathway (Mariana *et al.,* 2019).

CONCLUSION

In general, this study indicates that *Artemisia vulgaris* has a potential ability to increase the embryo development, survival rate, and heart rate of zebrafish embryos under heat stress exposure to the same quality as those under non-heat stress. However, further studies should be conducted to investigate molecular interaction between A. vulgaris, culture medium, and zebrafish embryos.

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CONFLICT of INTEREST

The author declares no conflict of interest.

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ETHICAL APPROVAL

This study received approval from Brawijaya University with a certificate number 152-KEP-UB-2022.

AUTHORS' CONTRIBUTIONS

HSAT: conceptualization, writing the original draft, writing a review, & editing. NK: resources. RY: resources, writing the review, & editing. MAL: resources. VFH: resources. B: formal analysis & methodology. GR: resources, writing the review, & editing.

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