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Effects of purwoceng plant (*Pimpinella alpina*) extract on masculinization of guppy (*Poecilia reticulata*)

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ABSTRACT

In the context of guppies (*Poecilia reticulata*), male fish are considered more attractive and valuable. The majority of mono-sex production in male fish employs the use of 17α-methyltestosterone, an expensive hormone with limited supply. It is therefore necessary to explore the potential of natural ingredients as an alternative replacement, such as purwoceng (*Pimpinella alpina*). The objective of this study was to determine the optimal immersion duration for the masculinization of *P. reticulata*. This study employed a completely randomized design with four treatments of immersion duration (0, 8, 10, and 12 h), with three replications allocated to each treatment. Following immersion, the guppy larvae were reared for a period of 45 days and fed with *Daphnia* sp. and *Artemia* cysts. The sex of each fish was determined through surgical procedure at the conclusion of the larval rearing period. The findings revealed that the duration of larval immersion in *P. alpina* extract had a notable impact ($P < 0.05$) on the proportion of male fish. In this study, the 10-h immersion treatment yielded the highest number of male *P. reticulata*, at 83.33%. The results of this study are highly valuable for the application of sex reversal techniques using phytosterol as a replacement for synthetic hormones.

1. INTRODUCTION

Guppies (*Poecilia reticulata*) are ornamental fish. They have a high commercial value. This is true in both domestic and international markets. Male guppies exhibit more attractive morphology than females, including body shape, body color patterns, and fins [1-5]. Therefore, male guppies are often raised with the monoculture method, due to more profitable than female guppies [1,2,6,7]. Increasing the opportunity to produce male *P. reticulata* can be achieved through the application of sex reversal techniques [5,8].

The process of sex reversal in fish can be achieved through two distinct mechanisms: Masculinization and feminization. Masculinization generates male mono-sex fish [3,9]. Synthetic hormones are often required to implement the sex reversal techniques [10]. For masculinization process, the synthetic hormones utilized are 17α-methyltestosterone (17α-MT), androstenedione, 19-norethynyl testosterone, 17α-ethyl testosterone, 17α-methyl-dihydrotestosterone, and dihydrotestosterone [2,3,11,12]. The 17α-MT is the most commonly-used hormone for fish masculinization [9]. However, the synthetic hormone 17α-MT is classified as a hard drug and prohibited for further usage in fish culture activities in Indonesia [13]. Consequently, it is necessary to select other alternative substances for synthetic hormones. An alternative material that has been found to help increase the number of male fish in the masculinization process is the purwoceng (*Pimpinella alpina*) [14-19].

Purwoceng (*P. alpina*) is an indigenous plant of Indonesia [20-23]. This plant belongs to *Pimpinella* genus that contains phytosterols, including α-spinasterol, campesterol, stigmasta-5,7,2-trien-3-ol, Δ7-avenasterol, and Δ5-avenasterol. In another study, *P. anisum* contains campesterol, stigmasterol (stigmasta-5,22-dien-3β-ol), β-sitosterol (stigmast-5-en-3β-ol), and α-spinasterol [24]. Phytosterols have been demonstrated to affect endocrinal reproductive functions in fish [25-27]. Phytosterol (β-sitosterol) has been shown to reduce low-density lipoprotein cholesterol and triglyceride levels in trout (*Salvelinus fontinalis*) [28]. Consequently, β-sitosterol inhibits the synthesis of estrogen hormones, while stimulating the synthesis of androgen hormones. Androgen hormones influence the differentiation of sex toward the male phenotype.

Studies on the use of *P. alpina* for fish masculinization have been conducted on Betta fish (*Betta splendens*) [15], rainbowfish (*Iriatherina werneri*) [14], Synodontis catfish (*Synodontis* sp.) [16], and Nile tilapia (*Oreochromis niloticus*) [19]. These studies indicate that the *P. alpina* can be employed as an alternative material to synthetic hormones in the male monosex fish production. The immersion of Nile tilapia larvae in *P. alpina* extract for 10 h obtained 79.22% male fish [19]. The immersion method on pregnant females of the *P. reticulata* in *P. alpina* extract for 8 h produced 63.98% of male

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offspring [17]. Nevertheless, the impact of the immersion method on *P. reticulata* larvae in *P. alpina* extract has yet to be investigated. Therefore, this study determined the effect of the immersion duration on the success of *P. reticulata* masculinization with *P. alpina* extract. The findings of this study are anticipated to be instrumental in the field of sex reversal in fish, with a focus on the utilization of naturally occurring compounds, such as phytosterols, as a substitute for synthetic hormones.

2. MATERIALS AND METHODS

2.1. Period and Location

This study was conducted at the Laboratory of Fisheries, Laboratory of Aquaculture, Laboratory of Microbiology and Biotechnology for Fishery Products, Department of Fisheries, Faculty of Agriculture, Universitas Sriwijaya, and the Batanghari-9 Indralaya Breeding Unit from October to December, 2023.

2.2. Fish Spawning

This study used 24 *P. reticulata* broodstocks (12 males and 12 females, with a 1:1 sex ratio) from the *black-Moscow* strain at 3–4 months old with a total length of 3–4 cm. Broodstocks were fed with *Tubifex* sp. and *Daphnia* sp. at 2–3 times/day. The mass-spawning was conducted in a box container (50 × 30 × 32 cm^3). After the mating period for 14 days, the larvae were produced. The larvae were subsequently collected in different box containers (49 × 33 × 27 cm^3) for further treatments.

2.3. *P. alpina* Extraction

The 100 g of *P. alpina* powder were dissolved in 1 L of ethanol (70%) and macerated for 24 h, equipped with stirring every 2 h. After 24 h, the solution was filtered using a filter bag with a pore size of 200 µm. The filtrate was subsequently stored in an Erlenmeyer flask and evaporated using a rotary evaporator at 60 rpm in 48°C for 3 h and 30 min. This process produced a liquid *P. alpina* extract, which was ready for further treatments.

2.4. Immersion and Rearing

Larvae were immersed in 12 different jars with a volume of 1.5 L. Each jar was filled with 1 L of water. Before immersion, 0.2 mg/L of *P. alpina* extract was mixed with the water and aerated for 24 h. The concentration of *P. alpina* extract in the present study was determined on the basis of the findings of previous research. The present study utilized a total of 120 *P. reticula* larvae. A total of 10 larvae were subjected to a 24-h immersion period in each jar, with the immersion duration applied for 8, 10, and 12 h. A group of larvae was not subjected to the *P. alpina* solution and served as the control treatment. Subsequently, the larvae were transferred to 12 distinct jars (5 L). The *P. reticulata* larvae were reared for 45 days and fed with *Daphnia* sp. and *Artemia* cysts (Polar Red Artemia®) at *ad libitum* condition. The survival rate was calculated from the duration of the immersion and rearing periods, namely:

$$SR=\frac{Nt}{No}\times 100$$

Where:
SR: Survival rate (%)
Nt: Number of fish at the final treatment and rearing (fish)
No: Number of fish at the initial treatment and rearing (fish)

2.5. Sex Determination

This study has been approved by the Dean of the Faculty of Agriculture, Sriwijaya University, Number 4612/UN9.1.5/AK.16/2022. The sex determination was conducted through the surgical method. The fish were dissected to obtain their gonads using forceps. Subsequently, the gonads were placed on an object glass and minced with a scalpel. Two drops of acetocarmine solution were added and allowed to remain for a minute. The object glass was then covered with a cover glass. The gonads were observed under a binocular microscope (Olympus CX 23®) at 40× magnification. The male fish gonads were identified by the presence of developing sperm cells. The percentage of male fish was calculated using the following formula:

$$PM=\frac{MF}{TF}\times 100$$

Where:
PM: Percentage of male fish (%)
MF: Number of male fish at the initial rearing period (fish)
TF: Total number of fish at the final rearing period (fish)

2.6. Water Quality

The water quality parameters were measured using the following instruments: A pH meter, a thermometer, a DO meter, and a spectrophotometer. The parameters included pH, water temperature, dissolved oxygen, and ammonia. The temperature and pH were recorded on a daily basis, while the dissolved oxygen and ammonia levels were monitored on a weekly basis.

2.7. Data Analysis

All data were tabulated and calculated using the Microsoft Excel 2019. Subsequently, a statistical analysis was conducted using the analysis of variance with the assistance of the Statistical Package for the Social Sciences 20.0 version. The percentage of male fish and survival rate data were presented in bar charts, while the water quality data were presented in tabular form and analyzed descriptively.

3. RESULTS AND DISCUSSION

3.1. Percentage of Male Fish

The present study indicates that the immersion duration with *P. alpina* extract has a significant effect ($P < 0.05$) on the percentage of male fish. In the 0-h treatment, the percentage of males was 43.33%. In the 8-h treatment, the percentage increased by 70.00%. In the 10-h treatment, the percentage elevated to 83.33%. Yet, in the 12-h treatment, the percentage reduced to 73.33%. The 0-h treatment exhibited a significant difference ($P < 0.05$) from all other treatments. The 8-h treatment exhibited a statistically significant difference ($P < 0.05$) from the 10-h treatment. Meanwhile, the 12-h treatment had no statistically significant difference ($P > 0.05$) from the 8- and 12-h treatments [Figure 1].

This study yielded higher results from a previous study that reported a male fish percentage of 63.98% [17]. This condition has occurred as thought to be due to the presence of phytosterol compounds in *P. alpina*, which are known to influence gonadal differentiation toward males. The phytosterol contents in *P. anisum* are 551.9 mg/100 g sample [24]. In addition, the level of stigmasterol compounds in 10 mg of *P. alpina* extract is 0.538 mg [18]. Hypothetically, this condition occurred as phytosterol compounds may result in a reduction of low-density lipoprotein cholesterol and triglycerides in *P. reticulata*.

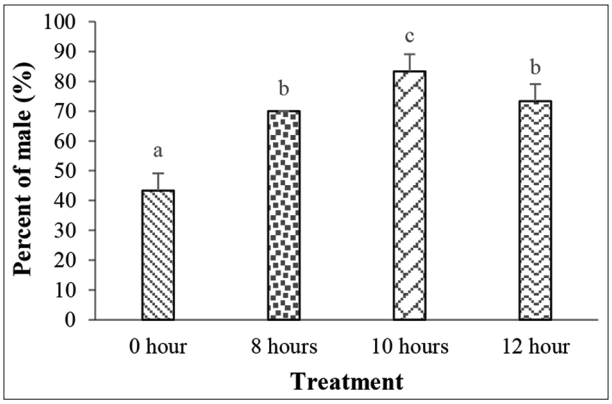

Figure 1: Percentage of male guppy fish (*Poecilia reticulata*) after immersion in purwoceng (*Pimpinella alpina*) extract. Different letters indicate significant differences between treatments ($P < 0.05$).

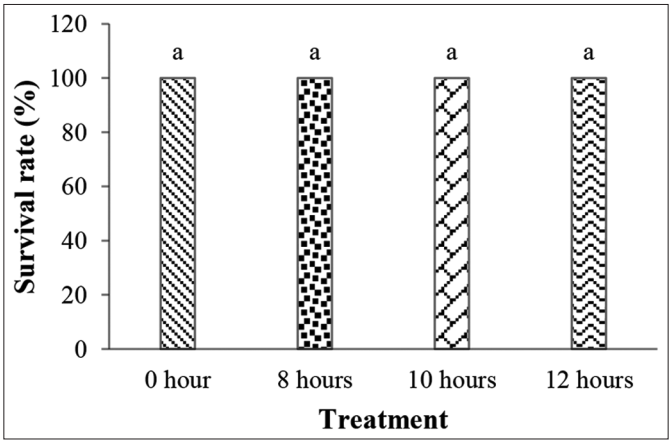

Figure 2: Survival of guppy fish (*Poecilia reticulata*) larvae during immersion in purwoceng (*Pimpinella alpina*) extract. The same letters indicate non-significant differences between treatments ($P > 0.05$).

Consequently, phytosterol compounds inhibit estrogenic hormone synthesis, while stimulating androgen hormone synthesis. The success of sex reversal is influenced by several factors, including the fish species of fish, treatment initiation time [29], the type of hormone and hormone dosage [5], treatment duration [30], and environmental factors (water temperature) [31].

Sex determination serves as the primary regulatory mechanism for the initial determination of bipotential gonads that initiate differentiation pathways [32]. The differentiation of fish sex is influenced by numerous factors, including hormonal systems and environmental factors. Environmental factors exert a direct influence on biochemical pathways that regulate the differentiation and maturation of gonads [33]. Gonadal differentiation in *P. reticulata* can occur up to the larval stage [34]. The vulnerability period in the poeciliids is limited to 30-day post-birth, while in *P. reticulata*, it encompasses both the embryonic and post-birth periods [35,36].

3.2. Survival Rate

The immersion of *P. reticulata* larvae in *P. alpina* extract had no significant effect on their survival rate during the immersion and post-immersion periods. During the immersion period, the survival rate of the larvae was 100% in all treatments [Figure 2]. The survival rate data of *P. reticulata* during the rearing period are presented in Figure 3.

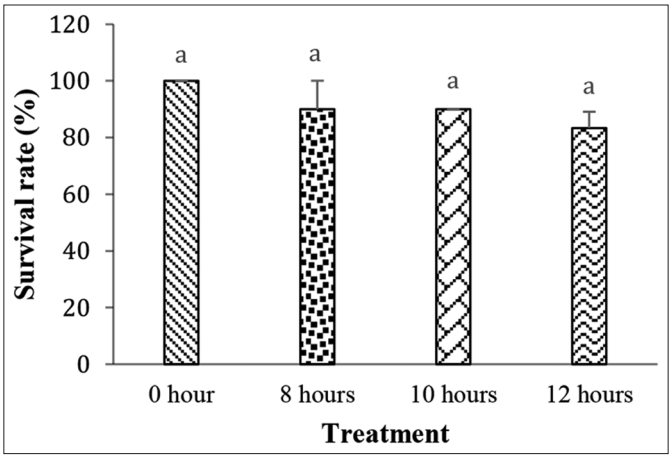

Figure 3: Survival of guppy fish (*Poecilia reticulata*) during a 45-day rearing period. The same letters indicate non-significant differences between treatments ($P > 0.05$).

Table 1: Water quality in the guppy (*Poecilia reticulata*) larval immersion media.

Treatment	Water quality parameters			
	pH (unit)	Temperature (°C)	DO (mg/L)	Ammonia (mg/L)
0 h	7.6–6.8	24.7–27.0	4.4–4.7	0.028–0.091
8 h	7.7–6.5	26.9–27.0	4.6–4.9	0.026–0.052
10 h	7.7–6.4	26.8–27.0	4.4–4.7	0.024–0.060
12 h	7.6–6.5	26.9–27.0	4.4–4.6	0.038–0.060

Table 2: Water quality in the guppy fish (*Poecilia reticulata*) rearing media.

Treatment	Water quality parameters			
	pH (unit)	Temperature (°C)	DO (mg/L)	Ammonia (mg/L)
0 h	7.0–7.8	24.4–27.5	4.4–4.6	0.024–0.060
8 h	7.0–7.8	24.3–27.6	4.6–4.9	0.06–0.072
10 h	7.2–7.8	24.4–27.7	4.4–4.8	0.030–0.072
12 h	7.1–7.8	24.4–27.8	4.4–4.6	0.030–0.091

The findings of the study indicate that immersing *P. reticulata* larvae in *P. alpina* extract for 8–12 h at a dosage of 0.02 mL obtained a zero mortality level among the *P. reticulata* larvae. This condition was occurred as *P. alpina* contains limonene and γ-himachalene, which have been demonstrated to enhance the immune system of *P.reticulata* [23].

The survival rate of *P. reticulata* after 45 days of rearing was 100% in the 0-h treatment, 90.00% (8 h), 90.00% (10 h), and 83.33% (12 h). These results present a declining trend in the survival rate of guppy fish. This decline is presumed to be due to inappropriate feeding and water quality exchange from the immersion medium to the rearing environment. In addition, high levels of ammonia are toxic to fish and pH levels that are too low or too high can cause fish tissue damage [37]. The survival rate of the fish larvae is highly influenced by feeding [38], whereas initial feeding stage is particularly susceptible to suboptimal conditions [39].

3.3. Water Quality

The water quality parameters during the immersion period and larval rearing period are presented in Tables 1 and 2, respectively.

The results showed that the *P. alpina* extract impacted the water's pH and ammonia levels. Before the addition of *P. alpina* extract to the immersion media, the pH was among 7.6–7.7, while the ammonia levels were among 0.024–0.038 mg/L. After mixing the immersion water with *P. alpina* extract, the pH level decreased from 6.4 to 6.8, while ammonia levels increased by 0.052–0.091 mg/L. Despite the observed changes in pH and ammonia levels, they remain within the established safe limits for *P. reticulata* larval rearing. The optimal water quality for guppy rearing is pH of 7.0–8.3 and temperature of 24–32°C [40]. In this study, the temperature was 24.3–27.8°C, while the pH was 7.0–7.8. The dissolved oxygen levels were among 3–6.8 mg/L, ammonia levels were 0.01–0.09 mg/L, and temperature was 20.1–28.9°C [41]. The pH of the rearing media exhibited at 7.6–7.8. The optimal pH for fish rearing is between 6.5 and 9.0 [42].

4. CONCLUSION

The immersion duration of *P. reticulata* larvae in *P. alpina* extract had no significant effect on fish survival rate. However, a significant effect has occurred on the percentage of male fish. For an effective treatment, an immersion duration of 10 h obtained the highest percentage of male fish at 83.33%. The findings of this research are expected an assist for reversal techniques, as they demonstrate the potential for the use of natural substances as alternatives to synthetic hormones.

5. AUTHORS' CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be an author as per the International Committee of Medical Journal Editors (ICMJE) requirements/guidelines.

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7. CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

8. ETHICAL APPROVAL

Ethical approvals details are given in the 'Materials and Methods' Section.

9. DATA AVAILABILITY

All the data is available with the authors and shall be provided upon request.

10. PUBLISHER'S NOTE

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11. USE OF ARTIFICIAL INTELLIGENCE (AI)-ASSISTED TECHNOLOGY

The authors declares that they have not used artificial intelligence (AI)-tools for writing and editing of the manuscript, and no images were manipulated using AI..

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