Hindawi
Aquaculture Research
Volume 2023, Article ID 8252139, 6 pages
https://doi.org/10.1155/2023/8252139

Research Article

The Effects of *Mucuna pruriens* and *Basella alba* Extracts on Sex Reversal in Rainbow Trout (*Oncorhynchus mykiss*)

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Received 12 April 2023; Revised 6 September 2023; Accepted 14 September 2023; Published 10 October 2023

Academic Editor: Hamed Ghafarifarsani

The use of synthetic steroids for sex reversal in various fish is widespread, but due to the potential dangers of these types of compounds, the use of phytochemicals has been explored as a suitable natural alternative. In this experiment, the effects of extracts of *Mucuna pruriens* seeds and *Basella alba* leaves on sex reversal in *Oncorhynchus mykiss* were investigated. Fifteen-days-old rainbow trout larvae (mean weight: 0.123 ± 0.001 g) were fed experimental diets containing 17 α-methyltestosterone (MT) (60, 90 mg/kg diet), ethanol extract of *B. alba* leaves (1.0, 2.0 g/kg diet), and methanol extract of *M. pruriens* seeds (0.1, 0.2 g/kg diet) for 90 days. After 90 days, the experimental diets were replaced with the basal diet until 10 months of age. The control group was fed the basal diet. At the end of the experiment, growth factors, survival (%), intersex (%), and masculinization (%) were measured. The feed conversion ratio and specific growth rate in all experimental groups were not significantly different from those in the control group ($P > 0.05$). The highest significant percentage of intersex ($6.03 \pm 3.53\%$) and the lowest percentage of survival ($72.33 \pm 2.51\%$) were observed in the treatment with 90 mg MT/kg compared with the other treatments ($P > 0.05$). The percentage of intersexuality was significantly lower in the groups fed with plant extract than in the groups fed with MT ($P < 0.05$). The percentage of intersexuality was significantly lower in the groups fed with plant extract than in the groups fed with MT. The highest percentage of masculinization was observed in the groups fed with *M. pruriens* extract (0.2 g/kg) ($61.75 \pm 17.16\%$) and MT (90 mg/kg) ($68.62 \pm 1.82\%$). Moreover, masculinization was significantly higher in all experimental groups than in the control group ($P > 0.05$). There were significantly more males (%) in all experimental groups than in the control group. In conclusion, extracts of *M. pruriens* and *B. alba* can be used as an environmentally friendly and safe alternative to MT to induce masculinization of rainbow trout.

1. Introduction

Recently, there has been a significant increase in demand for rearing mono-sex fish populations, particularly for aquaculture of all-female rainbow trout (*Oncorhynchus mykiss*). Because rainbow trout have an XY sexual system with male heterogamy, it is possible to produce female trout by gynogenesis. In other words, fish with a female genotype are obtained from eggs fertilized with neo-male (XX) milt [1]. The use of methyltestosterone (MT) is more widespread in neo-male production [2, 3]. However, synthetic steroids are often expensive, suppress growth, and can cause infertility, especially at high doses [4]. They also pose a public health problem due to consumer concern about residues of antibiotics and carcinogenic chemicals in fish and environmental disruption in natural populations [5]. In many countries, the use of synthetic steroids and chemical compounds is strictly prohibited in the aquaculture industry [6], while natural alternatives for sex reversal are attractive [4].

Nonhormonal compounds or plant extracts are potential alternatives to MT, which induce sex reversal and produce an all-male fish population [7]. Plant extracts block estrogen and biosynthesis by acting as aromatase inhibitors and nuclear estrogen receptor antagonists in the germ cells of the gonads [8], providing an effective source of masculinizing agents [2]. However, the efficacy of different phytochemicals to produce all-male populations varies widely, and the potential masculinizing effects of plant extracts remain to be documented.

Several studies have shown that the plant extract of *Basella alba* has masculinizing properties [9], so it can increase testosterone levels in older men. Studies have shown that

Mucuna pruriens has antipyretic effects in mammals [10] and has great potential as an alternative to protein sources in fish feed [11]. Induction of sex reversal in rainbow trout by powders or extracts of medicinal plants has also been associated with promising results [4]. For example, dietary supplements containing soy isoflavones increased the percentage of masculinization in rainbow trout [4].

Sex reversal in rainbow trout by *Tribulus terrestris* extract (50–100 mg/kg diet) compared to MT (3.6 mg/kg diet) was studied within 90 days and showed that treatment with *T. terrestris* plant extracts (50 and 100 mg/kg diet) resulted in masculinization of 55%–63%, while MT was 51%–53% [12]. In addition, sex reversal in tilapia was investigated with four plant extracts compared to MT (10 mg/kg diet), ethanol extract of *B. alba* leaves (1 g/kg diet), methanol extract of *M. pruriens* seeds (0.2 g/kg diet) and methanol extract of *Asparagus racemosus* root (0.2 g/kg diet) in a 30-day trial. The results showed that the percentage of masculinization of plant extracts was significantly higher than that of MT, with efficiency higher than 80% [6].

Natural substitution of MT in rainbow trout should reverse sex but should not adversely affect growth, immunity, hematology, and other conditions, nor reduce fish survival. Therefore, the present study investigated the possibility of sex reversal in rainbow trout by adding *B. alba* extract and *M. pruriens* extract to the diet and compared the results with the traditional method, MT.

2. Methods and Procedures

2.1. Plant Extract Preparations. Fresh leaves of *B. alba* and seeds of *M. pruriens* were purchased from the plant market in Golestan, Iran, washed in distilled water, air-dried in the shade at approximately 37°C, and finally ground well. The dried powders were soaked in glass flasks containing 70% ethanol (sample to solvent ratio 1 : 2 w/v) for 48 hr and then filtered through a Wathman Paper Filter No. 1 twice [2, 12]. The slurry was concentrated at 40°C (to 1/2 volume) in a rotary evaporator (IKA®RV 05 basic, Germany) and converted to a dry extract using a freeze-drying system (Alpha-2 LD plus, Germany), which was then stored at 4.0°C until further use [13, 14].

2.2. Experimental Design. The experiment was conducted in a commercial rainbow trout farm in Golestan Province, Iran. The water source was a spring that had slight temperature variations (11.5–13°C) throughout the year. All-female rainbow trout-eyed eggs were obtained from Aqualand Company, France. Fifteen-days-old, all-female larvae (mean weight 0.123 ± 0.001 g) were randomly divided into seven groups (100 larvae per tank); a control group (group 1) and six orally treated groups with three replicates, each receiving a diet containing 60 mg MT/kg diet (group 2), 90 mg MT/kg (group 3), 1.0 mg *B. alba* extract/kg (group 4), 2.0 mg *B. alba* extract/kg (group 5), 0.1 mg *M. pruriens* extract/kg (group 6) and 0.2 mg *M. pruriens* extract/kg (group 7) were administered for 90 days from the first feeding. After the sex reversal trial, all experimental groups were fed the same commercial trout diet (21-Beiza Company, Shiraz, Iran) until 10 months of age [6, 12]. The fish were then starved for 24 hr before sampling. At the end of the experiment, body weight (g), weight gain (g), specific growth rate (SGR, %/day), and feed conversion ratio (FCR) were analyzed for the experimental groups using standard formulas [15]. At 10 months of age, all fish in the different groups were anesthetized with a lethal dose of 2-phenoxyethanol (1 : 20,000, v/v) [16], slaughtered, and dissected to determine sex by visual observation of the gonads. Fish were classified as female if ovaries were visible and as intersex if testicular tissue was also found. Fish with testicular tissue were classified as male. The total phenolic content of the plant extracts was estimated by the spectrophotometric method using the Folin–Ciocalteu reagent, and the total flavonoid content of the plant extracts was determined by a colorimetric assay using standard protocols [17].

2.3. Statistical Analysis. All statistical analyses were performed using SPSS version 16.0 for Windows. Data were analyzed by one-way analysis of variance, and Duncan's test was used to detect significant differences between experimental groups after confirming the normality of the data with the Kolmogorov–Smirnov test. Significance was also set at the 5% level.

3. Results and Discussion

3.1. Growth Performance. B. alba extract, *M. pruriens* extract, and MT had no adverse effects on growth at the concentrations used in this study. The results showed that growth factors, including FCR and SGR, were not significantly different in the experimental and control groups ($P > 0.05$). The survival rate was not significantly different from the control ($P > 0.05$) group except in the 90 mg MT/kg group (Table 1). Therefore, both plants studied could be used as substitutes for MT.

Among the compounds found in plant extracts are polysaccharides. They have prebiotic properties that improve the digestive system, increase the digestibility of nutrients, and enhance the absorption and assimilation capacity of fish [18]. In addition, plants were found to have a positive effect on the secretion of digestive juices and total feed intake [19]. Based on the results of the present study, it can be said that the polysaccharides of *B. alba* extract and *M. pruriens* extract are unlikely to have negative effects on the induction of fish growth.

In this study, the lowest survival rate was found fish fed with 90 mg MT/kg diet ($72.33 \pm 2.51\%$) ($P > 0.05$) (Table 1). According to the results, high concentrations of MT might have negatively affected the survival rate. The results of the present study are confirmed by the fact that an increase in the concentration of MT leads to a decrease in the survival rate of swordtail [20], *Salmo salar* [21], zebra fish [22], and *Barbus conchonius* [23]. Apparently, the aromatization of MT to estrogens stimulates high vitellogenin production, which leads to liver enlargement and subsequently liver damage [23]. On the other hand, the survival rate of fish treated with different amounts of extracts of leaves of *B. alba* and seeds of *M. pruriens* was high, indicating that both plant

Table 1: The results of comparison of average growth performance in all-female rainbow trout fed with MT, *B. alba* leaf extract, and *M. pruriens* seed extract.

Treatment	Initial weight (g)	Final weight (g)	SGR (%)	FCR	Survival (%)
Control	0.124 ± 0.003	905.0 ± 49.24	2.96 ± 0.02	1.21 ± 0.06	84.33 ± 4.93^{a}
MT (60 mg/kg)	0.123 ± 0.004	953.33 ± 72.34	2.98 ± 0.01	1.15 ± 0.09	85.33 ± 5.50^{a}
MT (90 mg/kg)	0.122 ± 0.008	896.66 ± 55.07	2.96 ± 0.03	1.23 ± 0.07	72.33 ± 2.51^{b}
B. alba (1.0 g/kg)	0.126 ± 0.008	900.0 ± 133.32	2.95 ± 0.05	1.23 ± 0.17	83.00 ± 5.29^{ab}
B. alba (2.0 g/kg)	0.122 ± 0.006	815.0 ± 73.99	2.93 ± 0.03	1.35 ± 0.12	85.33 ± 5.03^{a}
M. pruriens (0.1 g/kg)	0.123 ± 0.005	840.0 ± 52.91	2.94 ± 0.03	1.31 ± 0.08	83.66 ± 7.63^{a}
M. pruriens (0.2 g/kg)	0.124 ± 0.008	918.33 ± 90.04	2.96 ± 0.05	1.20 ± 0.11	84.66 ± 9.71^{a}

Note: Value is expressed as the mean ± SD. Different superscripts mark significant differences in means within columns.

Table 2: Total phenol and total flavonoid content of plant extracts.

Treatment	Total phenol (mg of GAE/g dry weight)*	Total flavonoid (mg of RE/g dry weight)*
B. alba	258 ± 20	33.37 ± 4.85
M. pruriens	584 ± 26	0.34 ± 0.13

Note: *The concentration of total phenol expressed as milligram of gallic acid equivalents (GAE) per gram dry weight of the plant and total flavonoid content expressed as milligram of rutin equivalents (RE) per gram dry weight of the plant as described by Mukherjee et al. [16].

extracts did not have harmful effects on the survival of rainbow trout. Therefore, the proper concentration of the compounds in the diet is necessary to increase the percentage of masculinization. Similar results were obtained in Nile tilapia treated with extracts of leaves of *B. alba* and seeds of *M. pruriens*, which did not affect fish survival [24, 25]. The higher survival rate in the plant extract groups can also be attributed to the therapeutic properties of the extract and various compounds such as vitamins A, C, and E, fatty acids, and essential amino acid content [25].

3.2. Total Phenol and Total Flavonoid Content. Since the type and amount of plant constituents in the extracts depend on the different solvents, different results can be obtained in the induction of masculinization [16]. Ghosal and Chakraborty [2] reported that the methanol extract of *M. pruriens* and ethanol extract of *B. alba* had greater efficacy in the masculinization of tilapia fish. In the current study, the amounts of total phenolics and flavonoids of the methanol extract of *M. pruriens* and the ethanol extract of *B. alba* were listed in Table 2, which is in agreement with the results of Ghosal and Chakraborty [2]. The methanol extract from the seeds of *M. pruriens* and the ethanol extract from the leaves of *B. alba* contains various bioactive compounds such as tannins, alkaloids, saponins, glycosides, flavonoids, and steroids/terpenoids, which can induce the androgenic activity of the extract [16, 26].

3.3. Sex Reversal. The percentage of masculinization was significantly higher in the group with 90 mg MT/kg diet ($68.62 \pm 1.82\%$) and 0.2 g *M. pruriens* extract/kg diet ($61.75 \pm 17.16\%$) compared with the other groups ($P > 0.05$) (Table 3). These results are in agreement with those of Ghosal et al. [26], who reported that increasing the concentration of ethanol extract of *T. terrestris* seeds increased the percentage of masculinization in Nile tilapia. These results are also in agreement with the results reported in other literature [27–30].

In addition, Khiabani et al. [31] found that masculinization in swordtails was dose-dependent and that the number of males increased with increasing concentration of MT. The results showed that the highest percentage of masculinization was observed in MT (90 mg/kg diet) and *M. pruriens* extract (0.2 g/kg diet) groups ($P > 0.05$) (Table 3). The percentage of intersex was significantly higher in the MT (60 and 90 mg/kg diet) groups than in the other experimental groups ($P > 0.05$) (Table 3). These results indicate that plant extracts, especially *M. pruriens* extract (0.2 g/kg), can induce masculinization without significant differences from MT (90 mg/kg). In addition, the low percentage of intersex in the plant extracts compared to the groups MT (60 and 90 mg/kg diet) showed that the fish treated with plant extracts had a high rate of masculinization and less intersex. These results are in agreement with the study of Sarker et al. [32], who obtained 94.44% sex-reversed males in *O. niloticus* treated with 60 mg MT/kg diet for 90 days. Moreover, the results of *O. mykiss* support our conclusion, as *O. mykiss* fed with *T. terrestris* extract produced 55%–63% males, while MT-fed *O. mykiss* produced 51%–53% males [12].

Based on the results, these two plants have an effect on masculinization. This potential could be due to the increase in androgen levels, although this was not measured in this experiment. The plant compounds contain natural ingredients that have estrogenic/androgenic effects. There are several theories about the mechanism of phytochemical compounds in sex reversal in fish [33]. Phytochemicals, especially phenols and steroidal saponins in the extracts, may affect the endocrine system of fish, alter the process of sexual differentiation toward masculinization, and stimulate fish growth [33]. In addition, the compounds in plant extracts with aromatase inhibitory activity may be a suitable source for the induction of masculinization [2, 8]. The methanol extract of *B. alba* is used in traditional medicine to treat sexual impotence and infertility in men [33]. It has been reported that the methanol

Table 3: The results of the comparison of the mean percentage of masculinization and intersex in all-female rainbow trout fed with MT, *B. alba*, and *M. pruriens*.

Treatment	N^*	Masculinization (%)	Intersex (%)	Female (%)
Control	300	00.00^{d}	00.00^{c}	100^{a}
MT (60 mg/kg)	300	40.45 ± 8.21^{c}	5.73 ± 2.38^{a}	56.41 ± 5.43^{b}
MT (90 mg/kg)	300	68.62 ± 1.82^{a}	6.03 ± 3.53^{a}	25.34 ± 2.71^{d}
B. alba (1.0 g/kg)	300	47.48 ± 6.54^{bc}	1.20 ± 1.23^{bc}	48.02 ± 4.55^{bc}
B. alba (2.0 g/kg)	300	50.28 ± 6.43^{bc}	3.00 ± 1.36^{b}	47.77 ± 6.93^{bc}
M. pruriens (0.1 g/kg)	300	50.94 ± 9.42^{bc}	1.51 ± 1.64^{bc}	48.19 ± 4.12^{bc}
M. pruriens (0.2 g/kg)	300	61.75 ± 17.16^{ab}	2.00 ± 0.78^{bc}	36.24 ± 3.31^{cd}

Note: *The number of fish for which sex was determined value is expressed as the mean ± SD. Different superscripts mark significant differences in means within columns.

extract of *B. alba* stimulates testosterone production in testicular fractions and Leydig cell cultures in male albino rats [34, 35]. In another study, an increase in serum testosterone levels was observed across gastric intubation in male rats treated with aqueous *B. alba* extract [9].

As mentioned earlier, the highest percentage of intersex was observed in the MT groups (60 and 90 mg/kg diet). In these fish, body color and some phenotypic characteristics were similar to those of males, but they exhibited the structure of female gonads. Intersexed fish were also observed in the *B. alba* extract and *M. pruriens* extract groups, but their proportion was significantly lower than in the MT groups ($P > 0.05$) (Table 3).

Although all experimental groups had a high percentage of masculinization, the overall masculinization (%) of fish-fed plant-extract-containing diets was better compared to fish-fed MT. Pandian and Sheela [36] also reported that high concentrations of MT can cause sterility in tilapia fish, and even lower doses lead to the induction of intersexuality. This study highlights the importance of research in the field of finding nature-friendly combinations, including herbal extracts, due to the low survival and high percentage of intersex in the groups of MT compared to herbal treatments and the lack of significant differences in the ratio of MT to masculinization compared to some herbal compounds. Because the extraction of sperm from neo-male broodstocks often involves killing and dissecting the fish, the carcasses of these fish are frequently used in people's food baskets. Consumption of the meat of these fish can cause fertility problems in females due to the amount of MT. Because the persistence and fate of synthetic hormones and hormone metabolites in water, fish, and sediments pose potential environmental and health risks, hormonal sex control technology must be used with greater sensitivity [12]. Therefore, it seems necessary to pay attention to growth, survival, masculinization (%), and proper concentration of compounds when evaluating the effectiveness of percent masculinization.

4. Conclusion

Plant extracts are often environmentally friendly and have fewer side effects on humans and nature. With this in mind, this study presented for the first time the results of using extracts of *B. alba* extract and *M. pruriens* extract to induce masculinization of *O. mykiss* and compared them with the results of MT. Evaluation of the data in terms of growth parameters, survival (%), masculinization (%), and intersex (%) showed that plant extracts, especially the methanol extract of *M. pruriens* extract (0.2 g/kg), could be a suitable alternative to MT. These results can be used in sex reversal and neo-male production programs in *O. mykiss*.

Data Availability

The authors confirm that the data supporting the findings of this study are available within the article.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

A.H. was the supervisor of the project, methodology, laboratory, and statistical analysis, investigation, writing (and editing); M.S. has done the methodology and statistical analysis, writing (and editing); F.A.J. has done the methodology and laboratory analysis (and writing); K.A. has done the reviewing (and writing).

Acknowledgments

The authors wish to express their support through the University of Agricultural Sciences and Natural Resources of Gorgan, IRAN.

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