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# Effects of methyltestosterone, tamoxifen, genistein and *Basella alba* extract on masculinization of guppy (*Poecilia reticulata*)

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**ABSTRACT**

The effects of different anti-estrogenic chemicals were evaluated in guppy, *Poecilia reticulata* on sex differentiation and survival. New-born fry of the fish were fed diets containing genistein (1 gm $kg^{-1}$), tamoxifen (100 mg $kg^{-1}$), methanol extract of *Basella alba* (1 gm $kg^{-1}$) and 17α-methyltestosterone (60 mg $kg^{-1}$) for 30 days. The treated groups showed no significant difference ($P>0.05$) in survival percentage while a significant increase ($P<0.05$) in percentage of males were observed in all the treatment groups compared to that in control. Control fish showed the lowest percentage (44.49%) of males while 17α-methyltestosterone treatment provided maximum (81.90%) males, followed by tamoxifen (80.09%), genistein (70.59%) and B. alba (63.55%). Intersex fish with male like colouration and gonopodium development, but female gonad structure was observed in all the treated groups. The nonsteroidal compounds evaluated in this study showed potential for affecting sex ratios although their potency was lower compared to 17α-methyltestosterone. Further studies are needed to determine an optimum treatment regime with these agents for induction of 100% sex reversal in guppy.

## INTRODUCTION

One of the major constraints to aquaculture development is the reliability and sustainability of seed organisms for culture. Once a fish species has been identified to be of interest for aquaculture production, specific biological aspects need to be considered to determine the best production technology at high scale for a particular fish. Ornamental fishes are nowadays rapidly gaining importance because of their aesthetic value and also due to their immense commercial value in the export trade world over. Attractive colouration determines the commercial value of ornamental fish and pigmentation in the skin is responsible for colouration in the fish (Ahilan *et al*., 2008). In many groups of ornamental fish, the sexes differ in their colour patterns, with males being the more brightly coloured sex (Cogliati *et al*., 2010). Because of the price discrepancy, the culture of monosex (all-male) stocks of ornamental fish might be of a significant economic advantage. A lot of research has focused on the manipulation of phenotypic sex in ornamental fish in order to optimize the production of more valuable gender of the fish (Piferrer and Lim, 1997). Synthetic steroids are commonly used to induce sex reversal in ornamental fish but because of the potential hazards of such steroids; the use of new chemicals is a potential alternative to be explored. Phytochemicals are a large group of plant-derived compounds that are commonly found in fruits, vegetables, beans, cereals and plant-based beverages like tea and wine (Arts and Hollman, 2005). Based on their chemical structure, the plant active principles can principally be categorized into alkaloids, flavonoids, pigments, phenolics, terpenoids, steroids and essential oils and have been reported to promote various activities like antistress, growth promotion, appetite stimulation, tonic and immunostimulation, and antimicrobial properties in fish culture (Citarasu, 2010; Chakraborty and Hancz, 2011). Phytochemicals are also reported to block biosynthesis as well as action of estrogen by acting as aromatase inhibitors and antagonists to nuclear estrogen receptor in gonad germ cells (Rempel and Schlenk, 2008) and hence may be considered as potential mean for inducing sex reversal in fish. However, there are significant variations regarding the efficacy of different phytochemicals for production of all-male fish population and the potential anabolizing and virilizing effects of such plant extracts needs to be clearly documented. Tamoxifen, an estrogen antagonist has been found to induce masculinization in the gonads of genetic female fish (Nakamura *et al*., 2003).

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Genistein, a well-characterized isoflavone, is an important secondary plant metabolite that has been reported to be present in soybean meal and pulp mill effluents and exert estrogenic and/or antiestrogenic effects (Green and Kelly, 2009). Methanol extract from the dry leaves of Malabar spinach, *Basella alba* has been reported to possess active components that increase testosterone production in adult male rat testes during an *in vitro* study (Moundipa *et al*., 2005).

The live-bearing guppy, *Poecilia reticulata* is a popular freshwater species among aquarium hobbyists. Maintenance and breeding of male populations of guppy has generated good amount of commercial interest as the males of this species are more attractive than the females. Masculinization of guppy may be induced by direct synthetic hormonal treatment (Takahashi, 1975; Kavumpurath and Pandian, 1992; 1993). However, within the contemporary atmosphere of increasing governmental regulations on the use of chemicals on fish, dependency on steroid induced monosexing of fish may place the culture of guppy in a precarious position. Thus the development of reliable, effective and non-hazardous techniques to control the gonadal sex of fish and according produce all-male guppy populations in controlled conditions would be an advantage to breeders. Considering these aspects, the present study was focused to evaluate the efficacy of some non-steroidal compounds such as tamoxifen, genistein and methanol extract of *B. alba* as potent alternatives for induction of sex reversal and production of all-male population in guppy.

## MATERIAL AND METHODS

### Extraction procedure for *B. alba*

Fresh leaves of *B. alba* were collected from farmland in West Bengal, India, washed with water and dried under shade at about 35-40°C for several days. Air-dried leaves of *B. alba* (0.2 kg) were then grounded using an electric grinder and the powder was subjected to an organic solvent extraction by maceration under gentle agitation in a glass vessel for 48 h at room temperature using successively hexane (200 ml for 5 h, three times), methylene chloride (200 ml for 5 h, three times) and methanol (200 ml for 5 h, three times). The methanol extract was evaporated to dryness under pressure at 45ºC using a rotary evaporator and stored under nitrogen at -20ºC in amber glass bottle until it was used.

### Preparation of experimental diets

The control, 17α-methyltestosterone (MT)-treated and tamoxifen-treated diets were prepared following the alcohol evaporation method (Guerrero, 1975; Navarro-Martín *et al*., 2009). The control diet was treated with ethanol only and the alcohol was left to evaporate at room temperature. Genistein was added to finely ground diets using 10 ml of a 1:1 mixture of dimethylsulfoxide (DMSO) and ethanol for 1 kg feed (Green and Kelly, 2009) while methanol extract of *B. alba* was added after dissolving in DMSO only (Moundipa *et al*., 2005). The feed was then wetted with deionized water, mixed thoroughly, formed into pellets with a pelleter (diameter 2mm), and dried at room temperature. Pelleted feed was pulverized before feeding to newborn guppy.

### Experimental set up

Wild-type adult guppies were collected from the local market at Kaposvár, Hungary, 1 month before the onset of the experiment and stocked in a 500 lit. glass tank. From these brooders, newly born first feeding guppy were separately collected and randomly distributed into glass aquaria (10 lit) at a constant temperature of 24±2°C, and a density of minimum 35 fish per tank with four replicates per treatment. Experimental diets were formulated and designated as follows: Control (CON), 17α-methyltestosterone (MT) 60 mg $kg^{-1}$, tamoxifen (TAM) 100 mg $kg^{-1}$, genistein (GEN) 1 gm $kg^{-1}$ and methanol extract of *B. alba* (BAS) 1 gm $kg^{-1}$. Fish were fed ad libitum thrice daily for 30 days with the experimental diets and for another 30 days with commercial basal diet. The survival (%) was evaluated and final sex of the fish was determined by microscopic analysis of gonad tissue using the standard acetocarmine gonad squash technique at the end of the experiment (Guerrero and Shelton, 1974).

### Statistical analysis

All data are expressed in terms of mean ± standard error (SE). Treatment effects on different parameters were analyzed by one-way analysis of variance (ANOVA) after checking normality by Shapiro-Wilk's test. Where significant differences were found, a Tukey's test was performed for separating treatment means. All statistical analysis was performed using the SPSS version 11.5 for Windows.

## RESULTS AND DISCUSSION

There was no significant difference ($P>0.05$) in the survival of guppies among various treatment categories (Figure 1). The highest survival (64.9±8.9%) was observed in MT group, while the lowest survival (47.7±8.9%) was observed in GEN group. The CON group showed 44.5±1.3% males, which was significantly lower ($P<0.05$) compared to all other treatment groups (Table 1). The percentage of males in BAS and GEN groups were 63.5±2.2 and 70.6±0.8% respectively, while TAM group showed 80.1±1.0% males. However, the highest percentage (81.9±1.1%) of males was observed in MT group, though it was not significantly different ($P>0.05$) from the TAM group (Table 1).

**Table 1:** Percentage of males, females and intersex during treatment with different chemicals. Different superscripts mark significant differences in means within columns.

| Treatment category | Male (%) | Female (%) | Intersex (%) |
|---|---|---|---|
| CON | $44.49 \pm 1.28^{a}$ | $55.5 \pm 1.28^{d}$ | $0.0 \pm 0.0^{a}$ |
| BAS | $63.55 \pm 2.19^{b}$ | $29.85 \pm 3.16^{c}$ | $6.61 \pm 2.95^{a}$ |
| GEN | $70.59 \pm 0.85^{c}$ | $24.83 \pm 2.47^{c}$ | $4.58 \pm 1.71^{a}$ |
| TAM | $80.09 \pm 0.97^{d}$ | $15.38 \pm 1.79^{b}$ | $4.53 \pm 1.56^{a}$ |
| MT | $81.9 \pm 1.12^{d}$ | $0.0 \pm 0.0^{a}$ | $18.1 \pm 1.13^{b}$ |

Interestingly, 18.1±1.1% fish in MT group were found to be intersex showing male like colouration and gonopodium development, but female gonad structure. Such intersex fish were observed in BAS, GEN and TAM groups as well but their percentage was significantly lower (P<0.05) compared to that in MT group; and there was no intersex in the CON group (Table 1). Gonadal sex differentiation in guppy, an ovoviviparous cyprinodont, has been reported to occur at some period during the prenatal life (Takahashi, 1975). This limits the number of approaches to masculinization that can be considered in this species. Treatment with natural or synthetic androgens has been suggested in inducing masculinization of guppies (Piferrer and Lim, 1997). However, determination of ideal treatment regime and mode of application of the hormone under commercial condition requires more detailed analysis. This study addresses the possibility of dietary administration of MT and some non-steroidal compounds such as genistein, tamoxifen and methanol extract of *B. alba* to induce the required hormonal imbalance needed to observe an effect on sex differentiation in guppy.

Though there was no significant difference (P>0.05) in survival among the different treatment groups, fish fed diets containing genistein and tamoxifen showed a comparatively higher mortality than the others (Figure 1). Similar high mortality was observed in Nile tilapia, *Oreochromis niloticus* after dietary treatment with tamoxifen (Nakamura *et al*., 2003; Chikae *et al*., 2004). A dose-dependent cumulative mortality was observed in bagrid catfish, *Pseudobagrus fulvidraco* fed diets treated with tamoxifen (Park *et al*., 2003; 2004). In Japanese medaka, *Oryzias latipes* as well, the hatchability of fertilized eggs and time of hatching were significantly delayed after exposure to high concentration of tamoxifen (Sun *et al*., 2007). On the other hand, survival percentage of fish fed diets containing methanol extract of *B. alba* indicated that the plant extract might have no adverse affect on fish survival.

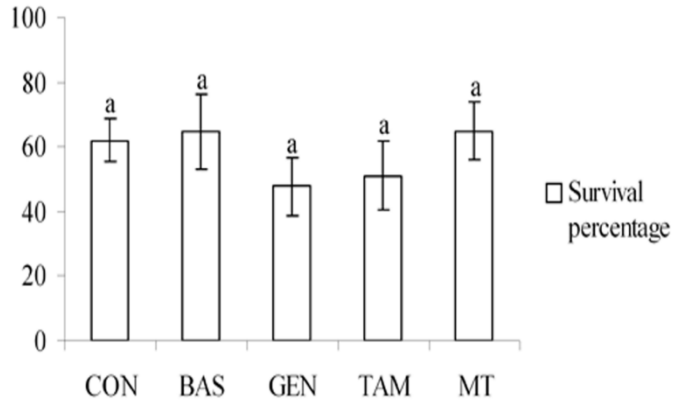


**Fig. 1:** Survival percentage of guppy during treatment with different chemicals. Similar alphabets denote homogenous means. CON: Control, MT: 17α-methyltestosterone, TAM: tamoxifen, BAS: methanol extract of *B. alba*, GEN: genistein.

All the treatment categories showed significantly higher (P<0.05) percentage of males compared to the control group (Table 1). However, the efficacy of the nonsteroidal chemicals at the applied concentrations to induce sex reversal was found to be less compared to MT. Tamoxifen, a nonsteroidal triphenylethyle derivative, is referred to as a selective estrogen-receptor modulator (Sun *et al*., 2007). It has been reported to be effective in inducing female-to-male sex reversal in Japanese flounder (*Paralichthys olivaceus*) (Kitano *et al*., 2007). Liu *et al*. (2007) obtained 70% sex reversed males in Southern catfish, *Silurus meridionalis* fed diets containing 25 mg $kg^{-1}$ for 20 days. A dose-dependent increase in percentage of males was observed in bagrid catfish fed diets treated with tamoxifen where the highest dose of 200 ppm produced 90% males (Park *et al*., 2004). However, no intersex was found during the treatment. On the other hand, dietary administration of tamoxifen at a dose of 2 mg $g^{-1}$ diet to 8 DAH *O. niloticus* fry for 150 days have resulted in gonads with both testicular and ovarian tissue (Nakamura *et al*., 2004). In an immersion experiment, tamoxifen treatment of Nile tilapia juveniles with 200 µg $l^{-1}$ for 60 days produced 90% male (Singh *et al*., 2012). Moreover, at high concentration tamoxifen has been found to inhibit the normal vitellogenin induction in female medaka (*O. latipes*) during oral administration (Chikae *et al*., 2004) and immersion experiments (Sun *et al*., 2007). Such masculinizing affect of tamoxifen may be presumably associated with blockage of estrogen function as it competes with endogenous estradiol for binding with estrogen receptor (Liu *et al*., 2010), and suppression of *cyp19a* expression (Kitano *et al*., 2007). Endocrine disrupting chemicals (EDCs) such as genistein has been reported to alter gonadal development and sexual differentiation in wild fish populations (Kiparissis *et al*., 2003). The interpretation of the effect of genistein must be done considering it to be both an in vivo agonist and an antagonist of estrogen (Adlercreutz, 1990). Several studies have reported increased level of vitellogenin in males of different fish species such as rainbow trout, *Oncorhynchus mykiss* (Bennetau-Pelissero *et al*., 2001) , striped bass, *Morone saxatilis* (Pollack *et al*., 2003) and medaka, *Oryzias latipes* (Inudo *et al*., 2004) during oral or immersion treatment with genistein. But, in yellow perch, *Perca flavescens*, Ko *et al*. (1999) reported no apparent estrogenic effects of genistein (0.75 and 7.5 mg $g^{-1}$ diet) on reproductive function and exposure of sexually mature male fighting fish, *Betta splendens* to pharmacological concentrations (1000 µg $l^{-1}$) of genistein and environmentally relevant (1 µg $l^{-1}$) concentrations of genistein showed no significant effect on circulating levels of androgen 11-ketotestosterone, estrogen E2, gonadosomatic index, sperm concentration and motility, or fertilization success (Stevenson *et al*., 2011). However, increased proportions of male and intersex individuals were observed in Channel catfish fed diets containing genistein at 4 and 8 mg $g^{-1}$ concentration between 5 and 140 days posthatch, while there was no significant (P>0.05) difference in EROD activity between the control and treated groups (Green and Kelly, 2009). Phenotypic sex was found to be significantly dependent on dietary phytoestrogen concentration (P=0.01) and a significant relation existed between genistein concentration in the diet and gonadal sex (P=0.02). This paradoxical sex reversal might have resulted from the dual role of genistein as not only an

estrogen agonist but also as an antagonist blocking estrogen's action. In vitro, this dual effect seems to be affected by the ratio between estradiol and genistein at the target organ (Adlercreutz *et al*., 1995). A genistein / estradiol ratio between 10 to 100 would lead to a competition for the estradiol receptor (ER) which fails to induce gene transcription and an anti-estrogenic effect is then observed (Verdeal *et al*., 1980). Below, the estrogenic effect of estradiol would mask genistein effect and above genistein exerts an overall estrogenic effect. This can be explained, at least partly, by the difference in affinity between estradiol and genistein for the ER. It was also shown in vitro, that genistein affinity is 50 times less than that of estradiol for rainbow trout ER (Latonnelle *et al*., 2002). *B. alba* has been reported to be used in traditional medicine to treat sexual asthenia and infertility in man (Adhikari *et al*., 2012). The methanol extract of its leaves was found to stimulate testosterone production in testicular fractions and Leydig cell cultures, and in normal adult albino male rats (Moundipa *et al*., 2005; Nantia *et al*., 2011).

The plant extract contain phytoconstituents such as saponins, kaempherol, betalin and terpenoids or sterols and these might render the androgenic activity of the extract (Moundipa *et al*., 2005; Adhikari *et al*., 2012). However, no previous report was found regarding its efficacy to induce sex reversal in guppy and usage of a higher dose may increase the percentage of males in the fish population. Of all the chemicals used in this study for induction of sex reversal in guppy, the synthetic steroid, MT was observed to be the most potent masculinizing agent with the highest percentage of males in the population (Table 1). Administration of an optimum dose of this sex steroid during the labile period has been found to result in sex reversal in fish (Pandian and Kirankumar, 2003). In guppy, MT has been reported to produce 100% male population during the treatment period but the percentage of males decrease once the treatment is stopped (Low *et al*., 1994). In the present study, MT treated fish were observed to be either males or intersex. As sexing of fish was performed 30-days after the treatment period, it might have been speculated that a 100% sex reversal was induced by MT treatment and the fish reversed back to their original sex when the effects of MT diminished (Piferrer and Lim, 1997). Indeed, sex reversal in guppy has been reported to be transitory as genotypic females-phenotypic males may reverse back into females once the treatment with masculinizing agent is stopped (Piferrer and Lim, 1997). Moreover, none of the treatment groups showed 100% male population, which may be due to the fact that as gonadal differentiation in guppy occurs in prenatal stage, the efficacy of these androgenic compounds to affect the process in new-born fry is limited.

## CONCLUSION

The phytochemicals evaluated in available literature on fish are mostly related to their activity in vitro using gonadal cells and measuring the coefficients of inhibitions of synthesis of estrogens. But the mechanism of the steroidal activity of the phytochemicals and their in vivo effects in fish remain unclear. Testing four different compounds in the same study allows placing the effects of one compound in light of the effects of the others. The findings of this study indicate the probability of using non-steroidal compounds as an alternative method for production of all-male guppy population. However, further studies will be required to determine the optimum treatment regime for induction of 100% sex reversal with these chemicals in guppy.

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