

A comparison with *Tribulus terrestris* extract fish immersion and bioincapsulation of enriched artemia on sex reversing of fighter fish (*Betta splendens*)

Janalizadeh E.¹; Manoucheri H.^{1*}; Changizi R.¹

Received: February 2018

Accepted: June 2018

Abstract

Since male fighter fish is more beautiful and commercially worthier than female one, the aim of this research was to achieve a new and appropriate safe method for masculinization in *B.splendens* fish with herbal extract of *Tribulus terrestris*. Juvenile fighter fish were examined by two methods, including immersion fish in the diluted extract, and enriched Artemia napeus bioencapsulation by *Tribulus terrestris* extract with abbreviation name of *TT*. The extraction was performed by ethanolic method and four different doses of *TT* (0.0, 0.01, 0.05, and 0.1 gL⁻¹) were examined. Results showed that, first method, immerse the fish in the water containing *TT* extract, was not effective on fighter fish and all the larvae died after 9-10 days, but in the second experimental method (feeding the treatments through Artemia enriched by *TT* extract), in terms of the value of masculinization and survival percentage the dose of 0.05 gL⁻¹ was the best dose which were 78.96% and 87.77%, respectively ($p<0.05$). In this study, *TT* had not a positive effect on survival of *B. splendens*. In these treatments, *TT* with a concentration of 0.05gL⁻¹ improved significantly the growth rate in *B. splendens* to some extent ($p<0.05$).

Keywords: Herbal extract of *Tribulus terrestris*, Masculinization, Fighter fish.

1-Department of Fisheries, Babol Branch, Islamic Azad University, Babol, Iran.

*Corresponding author's Email: manouchehri@baboliau.ac.ir

Introduction

Tribulus Terrestris with abbreviation name of *T.T.* (Zygophyllacea species) is an annual plant which is widely distributed in China, Japan, western areas of Asia and southern Europe and Africa. It was shown that this plant causes to increase testosterone levels naturally and as harmless in human – beings and creatures as well as it was indefinitely rumored that it caused high success of Bulgarian athletics lifters (Bucci, 2000). *TT* causes to improve lipid and spermatogenesis in humans and creatures (Grigorova *et al.*, 2008). Also, it is used in sexual disabilities experiments as well as for increasing testosterone levels and improvement of athletes, performance and efficiency (Adimoelja, 2000; Bucci, 2000; Gauthaman *et al.*, 2000; Adaikan *et al.*, 2009). Testosterone is a sexual hormone which plays a role in sexual cells and exists in male larvae during the distinction of embryonic cells and subsequently, it is used during or physiological maturity of testosterone in the body. Testosterone exists as biosynthetic pioneer both in androgen and estrogen of Teleostei fish (Borg, 1994; Baroillera *et al.*, 1999). Androgens are often important in order to study the physiological actions in males (Borg, 1994).

It was determined that *TT* increases testosterone levels (Adimoelja, 2000; Gauthaman *et al.*, 2000; Adaikan *et al.*, 2009). *TT* contains some different known materials similar to steroid materials. The effect if performance of materials in *TT* on protodioscin similar

testosterone levels was known (Ganzera *et al.*, 2001; Adaikan *et al.*, 2009). *B. splendens* fighter is a common aquarium fish. There are new shapes with different colors of them in aquariums. The males of this species are more interesting than females. Therefore, protection and production of male population provide abundant commercial profit. *B. splendens* masculinization can be performed by production of synthetic hormones through direct experiments, which is the most efficient and evident method of production (Kavumpurath and Pandian, 1992; 1993) . However the synthetic hormones are much more expensive than herbal extracts and ultimately, the risks of pressures resulted from using of synthetic hormones in the water and sediment are disclosed. Therefore, in this study, the effects of *TT* on *B. splendens* sex change are examined. The goal of this research is to introduce a new appropriate bioenvironmental method for masculinization in *B. Splendens* fish and study the possibility for sex change in Siamese fighter fish by two methods including immersion fish in the diluted extract, and enriched Artemia napleus bioencapsulation by *Tribulus terrestris* extract (*T.T.*) and produce more males compared to females because of their beauty and commercial value.

Materials and methods

Extraction

The extraction was performed by simple distillation. For this purpose, the powders prepared by from the plant

were poured in an Erlenmeyer and then for each 10g powder, 100 cc ethanol, 100 cc diethyl ether and 100 cc N-Hexane were added into each Erlenmeyer and their opening were closed by foil and were kept for 72 hours. After that, they were filtered. Then, ethanol solvent in the extract was extracted by Rotary Evaporator RE300 system. Then two steps of fat extraction were performed. In the first stage, 100^{cc} ethanol was added for ethanolic extracts and it was placed in the freezer at the temperature of -28 °c. All the impurities were precipitated in the freezer and the impurities attached to the body of Erlenmeyer and then, in the second stage, the extracts were filtered again and then, they were distilled and 100cc ethanol solvent was added again and it was placed in the freezer at the temperature of -28°C and then its solvent was by re-distillation and the extract was obtained (Erturk, 2006). The brood fish were provided from ornamental fish farm in Babol city (Mazandaran province) in November 1, 2011. 8 fishes were transferred into 8 aquariums (with the sex ratio of 1:1) after sex identification through size of fin and body color. Male fish had longer fins as well as more beautiful colors. Mean length of male fish was 4.12 cm that of female fish was 3.81 cm. The temperature was about 26°C, the hardness was 8 degrees and pH was kept at 7 during the experiment (Kavumpurath and Pandian, 1992; 1993; Kavumpurath and Pandian, 1994;

Gauthaman *et al.*, 2000; Adaikan *et al.*, 2009).

Artemia Hatching Method

Artemia Franciscana cysts with 5g in value were put into 1 liter water at temperature of 28-29°C with appropriate ventilation and they were hatched within 24 hours (Lim *et al.*, 2002). Enrichment of *Artemia* for 12 h was done with T.T. extract (Støttrup and McEvoy, 2008).

Feeding method of fish in treatments

2 days after hatching, initial feeding of all treatments was performed by green water for one week and after that, they were fed by *Artemia Napleus* until maturation. Fighters were divided in to 2 experimental and 1 control group with 3 levels of T.T. including 0.01, 0.05 and 0.1 g/L (Fig. 1, Table 1).

Control treatments

Tribulus terrestris extract was not used.

Treatments of fish immersion, in the extract

Tribulus terrestris extract with different doses, including 0.01, 0.05 and 0.1 were poured into the water of separated aquariums.

Treatments of fish fed by Napleus enriched by Tribulus terrestris extract

After one week feeding by green water, treatments were fed through *Artemia* enriched by *TT* extract up to maturation age (Table 1).



Figure 1: Fighter fish reproduction, bobble nest is seen on the water surface.

Table 1: Experimental methods in different treatments for masculinization of Fighter fish with *TT* extract within 20 days.

Treatments	Methods	Consumption dose of <i>TT</i> extract (g/l)	Food material	Number of food meals (In dormitory)
1	Control	-	Artemia and non-enriched Feeding	2meal 8 A.M. And 4 P.M. each meal : (0.3 g)
2	Dipping	0.01, 0.05 and 0.1	Ordinary Artemia in rearing water containing <i>Tribulus terrestris</i> extract	2meal 8 A.M. And 4 P.M.
3	Feeding just by the enriched Artemia with <i>Tribulus terrestris</i> extract	0.01, 0.05 and 0.1	The enriched Artemia	2meal 8 A.M. And 4 P.M.

Statistical method

After data collection, data normality was first determined using of the Kolmogorov – Smirnov test. Then data were analyzed using of SPSS software, version 18. Duncan and ANOVAs mean variance analysis was used to determine the significance levels of treatments. Tables and diagrams were drawn using of Microsoft software, Excel version 2007.

Results

After 3-4 days when the male and female were put together and in an aquarium, the reproduction was

performed. The number of fries resulted from broods reproduction was 100-120 for each pair of parents. The highest number of masculinization was recorded in dose of 0.05g/L-1 in treatment fed by Artemia enriched by *TT* extract. The results related to the effect of *TT* extract on sex change of fighter fish in different treatment is shown in Fig. 2.

In the present research, the required time of sexual maturity of the male treated in an experiment there was no significant change in a treatment which was fed by the enriched Artemia during rearing period and it increased to 128 days form 120 days.

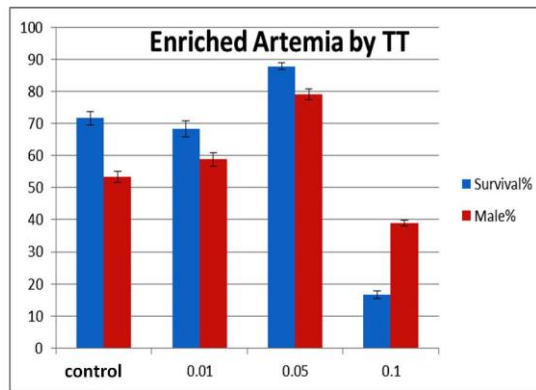


Figure 2: survival rate and male percentage in treatments fed by *Napleus* enriched by *TT* extract during 20 days.

Discussion

The use of herbal medicine has long been prevalent in the community and in recent years the use of medicine herbs is expanding. Researchers showed that extracts of *Tribulus terrestris* increases testosterone levels and improve athletic performance (Adimoelja, 2000; Bucci, 2000; Gauthaman *et al.*, 2000; Adimoelja *et al.*, 2005; Adaikan *et al.*, 2009). That is because of the presence of a material named as protodioscin into this plant. *TT* contains some different known materials similar to steroid materials. Testosterone is the biosynthetic precursor to both androgens and estrogens in Teleostei (Baroillera *et al.*, 1999; Devlin and Nagahama, 2002). Androgens are often important in order to study the physiological actions in males (Borg, 1994). It was determined that *TT* increases testosterone levels (Gauthaman *et al.*, 2000; Adimoelja *et al.*, 2005; Adaikan *et al.*, 2009).

According to the results of application of immersion method to

create sex change in fighter fish, it was not effective and resulted in some mortality after 9-10 days. Therefore, immersion method was useless in respect of fighter fish which can be due to high concentration, while the hormone immersion method was performed on fighter fish (*B. splendens*) by Pandian and Kirankumar (2002) assign of the growth and reproduction hormone (17 α -methyl testosterone) and resulted in 98% male construction (production) and 71% survival at dose of 900 mg L⁻¹.

In hormone immersion method of fighter (Kirankumar and Pandian, 2002), in dose of 1000 mg L⁻¹, the newly hatched treated fries suffer from deformities, and they died of 1000 mg L⁻¹ resulted 2-3 days. Therefore, the dose of 1000 mg L⁻¹ resulted in less survival and was not able to increase the percentage of immersion while in the experiment performed by the method of feeding, the treatments with the enriched Artemia with the extract of *TT* at the highest dose (0.1g L⁻¹), the fish

developed deformation to some extent but all survived and there was no death, and change of sexual was observed (Fig. 1), also were observed that survival ratio decreased. When the does increases, the survival level decreases, and among the doses including 0.01, 0.05, and 0.1g/L-1, the dose of 0.05g/L-1 was the best does.

In fighter fish by a hormone immersion method when the dose of 17 α -methyl testosterone increases, the survival of *B.splendens* decreases and in dose of 900 mg L-1, in reaches %76 and it reduced to %71 at the end of experiment and sexual maturation. In the present research, the sex reversing was observed in all doses, while in hormonal immersion method, it was not able to change the sexual at the least dose of 100 mg L-1 (Kirankumar and Pandian, 2002).

In this research, male grew slowly and there was no significant difference compared to control group. In the present study, the required time for sexual maturation of the treated male in an experiment in which the feeding of fighter fish was performed by the enriched Artemia during the rearing period increased to 128 days form 120 days, and there were no significant ($p<0.05$). So it was the best dose to pull off maturity, because this time gradually expanded from 110 days to 145 days in research which was performed by hormonal immersion method of fighter fish (Kirankumar and Pandian, 2002). The fertilization cycle became approximately two times and for the males of experimental series, with male

construction of 1% and above 1%, it became three times. Consequently hatching time (3-6 seconds), and the number of successful reproduction (8-19 times) and spawning pulses (steps of 4 and 5) decreased up to %50 while there was no significantly decreased in the performed research ($p<0.05$) (Kirankumar and Pandian, 2002).

In hormonal immersion method (Kirankumar and Pandian, 2002) both the ordinary males and the sex reverse of males created bubble nests and displayed the long fins when they saw a female. Female displayed her superiority to the ordinary male. These characteristics were observed in experiments performed in this research (Treatments fed by Artemia enriched). Also in a study by Cek *et al.* (2007) in *Poecilia reticulate* (Guppy) fish, they achieved to %80 of masculinization by immersing in the water containing 0.15 g/L-1 of the extract of *TT* for 0-60 days. The sexual ratio in 92 fish taken from the experimental series with the extract of *TT* 53:38 (male: female) and there were no statistically significant changes ($p<0.05$). the sexual ratio in the experimental series at the concentration of 0.1g/L-1 was 52:35 (male: female) this group had also no statistically significant changes ($p<0.05$), but the sexual ratio observed in 90 fish taken from the experimental series with the extract of *TT* at the concentration of 0.15 g/L-1 was 72: 18 (male: female) which this difference represents statistically significant changes ($p<0.05$), while no inter –sex-fish was

recorded in this research. The survival level increased equally to 87% from 83% in all treatments and control groups ($p>0.05$).

In all the experimental groups with different doses the extract of *TT*, the effects of masculinization are shown during a period of 60 days. At the end of the experiment, some statistically significant changes were observed ($p<0.001$) (Çek *et al.*, 2007). In another research, masculinization was obtained from *Cichlasoma nigrofasciatum* fish by immersion method into the extract of *TT* for 0-60 days in the water containing 0.30g/L-1 (Çek *et al.*, 2007).

The sexual ratio of the fish in the first experimental series (0.01 g extract of *TT*) was 1:1 compared to the control group. The sexual ratio in 93 fish in the second experimental group (0.10 g/L-1 extract of *TT*) was observed as 74:19 (female: male) and in the third experimental series, the sexual ratio of female: male was 80:14.

The highest ratio of male fish was observed in 95 series of the experimental fish with a dose of 0.30 g/L-1 extract of *TT*. The sexual ratio of inter-sex fish was 82: 11: 2 (female: male). These results show that increase in the dose of extract of *TT* causes an increase in the production of male's population (Çek *et al.*, 2007).

In a research performed, we concluded this same result, but when the dose increases, the percentage of survival significantly increases ($p<0.05$). In histological experimental of testicle and ovary of the experimental

groups with the extract of *TT*, the structure of testicle and ovary of the fish was observed undamaged. All spermatogenesis steps in both experimental groups, control groups and the experimental groups with the extract of *TT* were observed and compared to each other (Cek *et al.*, 2007; Çek *et al.*, 2007). In histology responses, testicles in the experimental groups with extract of *TT*, there was an increase in the number of spermatogenesis cysts and frequency of its steps. These testicles include a high number of spermatozoa into lobular cellular cavity. All spermatogenesis steps include the separation of spermatozoa and finding sperm channels site. Lobules have a numerous number of spermatosite from the initial steps (spermatogonia) to the complete spermatogenesis (spermatosites, spermatides, and spermatozoa) which in comparison to the control groups free spermatozoas are just sometimes seen and the testicles include more spermatogonia and spermatosites (Çek *et al.*, 2007). In experimental series of 0.30g/L-1 the extract of *TT*, two inter - sex fish were recorded. One male fish tasted by the extract of *TT*, had two testicle lobes which possessed a high number of lobules and a numerous number of sperms and a little primary ovules into the sperm channels and they were located just in one of the testicle lobes (Cek *et al.*, 2007), while in the present research, no inter - sex fish was observed.

In a research the survival level was equally high in all treatments and experiment and increased to 90.47% from %88.57 (Çek *et al.*, 2007), but in this research in which the feeding of fish was performed by Artemia enrichment method, it was not effective on the survival of fish during the breeding period, but significantly caused the color variety in the experimental treatments and more importantly, it caused the highest percentage of masculinization (87.77%) at the dose of 0.05g/L. also, in the above - mentioned experiments, it was observed that the survival has meaning significantly been decreased at the highest experimental dose (0.1g/L).

In the present research, the highest percentage of survival at the dose of 0.05 g/L-1, was observed that they were 87.77% in treatments which were fed through Artemia enriched by the extract during the breeding period.

References

- Adaikan, P., Gauthaman, K. and Prasad, R., 2009.** Proerectile pharmacological effects of *Tribulus terrestris* extract on the rabbit corpus cavernosum. *Ann Acad Med Singapore*, 29, 22-26.
- Adimoelja, A., 2000.** Phytochemicals and the breakthrough of traditional herbs in the management of sexual dysfunctions. *International Journal of Andrology*, 23, 82-84.
- Adimoelja, A., Sartono, S. and Soedjono, J., 2005.** Phyto-dhea treatment, an alternative option for aging men. . In *8th international congress of andrology*. Seoul, Korea: Blackwell.
- Baroillera, J.F., Guiguenb, Y. and Fostierb, A., 1999.** Endocrine and environmental aspects of sex differentiation in fish. *Cellular and Molecular Life Sciences*, 55, 910-931.
- Borg, B., 1994.** Androgens in teleost fishes. *Comparative Biochemistry and Physiology Part C: Pharmacology, Toxicology and Endocrinology*, 109, 219-245.
- Bucci, L.R., 2000.** Selected herbals and human exercise performance. *The American Journal of Clinical Nutrition*, 72, 624s-636s.
- Cek, S., Turan, F. and Atik, E., 2007.** The effects of Gokshura, *Tribulus terrestris* on sex reversal of guppy, *Poecilia reticulata*. *Pakistan Journal of Biological Sciences*, 10, 718-725.
- Çek, Ş., Turan, F. and Atik, E., 2007.** Masculinization of Convict Cichlid (*Cichlasoma nigrofasciatum*) by immersion in *Tribulus terrestris* extract. *Aquaculture International*, 15, 109-119.
- Devlin, R.H. and Nagahama, Y., 2002.** Sex determination and sex differentiation in fish: an overview of genetic, physiological, and environmental influences. *Aquaculture*, 208, 191-364.
- Erturk, O., 2006.** Antibacterial and antifungal activity of ethanolic extracts from eleven spice plants. *Section Cellular and Molecular Biology*, 61, 275-278.

- Ganzera, M., Bedir, E. and Khan, I., 2001.** Determination of steroidal saponins in *Tribulus terrestris* by reversed-phase high-performance liquid chromatography and evaporative light scattering detection. *Journal of Pharmaceutical Sciences*, 90, 1752–1758.
- Gauthaman, K., Adaikan, P., Prasad, R. and Goh, V., 2000.** Changes in hormonal parameters secondary to intravenous administration of *Tribulus terrestris* extract in primates. *International Journal of Impotence Research*, 12, 20-24.
- Grigorova, S., Kashamov, B., Sredkova, V., Surdjiiska, S. and Zlatev, H., 2008.** Effect of *Tribulus terrestris* extract on semen quality and serum total cholesterol content in white plymuth rock-mini cocks. *Biotechnology in Animal Husbandry* 24, 139-146.
- Kavumpurath, S. and Pandian, T.J., 1992.** Effects of induced triploidy on aggressive display in the fighting fish, *Betta splendens* Regan. *Aquaculture Research*, 23, 281-290.
- Kavumpurath, S. and Pandian, T.J., 1993.** Determination of labile period and critical dose for sex reversal by oral administration of estrogens in *Betta splendens*. *Indian Journal of Experimental Biology*, 31, 16-26.
- Kavumpurath, S. and Pandian, T.J., 1994.** Masculinization of fighting fish, *Betta splendens* Regan, using synthetic or natural androgens. *Aquaculture Research*, 25, 373-381.
- Kirankumar, S. and Pandian, T.J., 2002.** Effect on growth and reproduction of hormone immersed and masculinized fighting fish *Betta splendens*. *Journal of Experimental Zoology*, 293, 606-616.
- Lim, L.C., Cho, Y.L., Dhert, P., Wong, C.C., Nelis, H. and Sorgeloos, P., 2002.** Use of decapsulated *Artemia* cysts in ornamental fish culture. *Aquaculture Research*, 33, 575-589.
- Støttrup, J. and McEvoy, L., 2008.** *Live Feeds in Marine Aquaculture*: Wiley.