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SHORT COMMUNICATION

Effects of *Tribulus terrestris* extract on masculinization, growth indices, sex determination and steroid hormones level in Zebra fish (*Danio rerio*)

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Abstract *Tribulus terrestris* is a plant that has been introduced in aquaculture as a inducer for masculinization and stimulating growth in fish. This study has investigated the effect of the different levels of *T. terrestris* extract (TE) on growth indices, sex ratio and steroid hormones of Zebra fish (*Danio rerio*) that fed with four concentrations of 0 (T0), 50 (T1), 100 (T2) and 200 (T3) mg TE/kg of food during 60 days. 180 Zebra fish larvae (0.01±0.003 g average weight) were distributed into 12 aquaria (15 fish/aquarium) in four treatment groups and three replicates per each group. The results indicated fishes receiving higher dose of TE were in better conditions rather than the control group in terms of the growth rate ($p<0.05$). The highest male to female ratio has been achieved in fish fed with T2 diet (70.83±3.818). The values of testosterone and dehydroepiandrosterone were considerably different in the treatment groups compared with the control group and the maximum amount of these hormones were measured in fish fed with T3 and T2 diets, respectively ($p<0.05$). According to the histology results, the gonads have been completely formed and the growth of body performance and masculinization percentage showed a significant increase in fish fed with T2 diet compared with the other treatment groups.

Keywords Growth indices . Masculinization . *Tribulus terrestris* . Zebra fish

Introduction

Synthetic steroids are mostly used to induce sex reversal in fish but deu to the potential risks of such steroids (Jiménez-Badillo and Arredondo-Figueroa 2000; Pandian and Kirankumar 2003), investigating the use of new drugs and herbs is of high importance (Fung and Linn 2017). In fact, changing in the natural trend of the sexual differentiation is influenced by these hormones, or the stimulant compounds (steroidal saponins) as a result the male and female sex cells grow in the fishes which are genetically female and vice versa, but the genotype of the sex chromosomes remains unchanged (Rempel and Schlenk 2008).

Polluted aquatic environments cause changes in their physiological processes such as sex differentiation in the early stages of fish life period. If sufficient amounts of sex steroids are introduced to fishes in the embryonic development stages, change in the sexual differentiation might occur (Paul-Prasanth et al. 2011). The estrogen treatments in fishes have been very effective in most of the cases and led to the fish feminization (Rempel and Schlenk 2008).

Synthetic hormones are more expensive than the herbal extracts (Cek et al. 2007a). In addition, synthetic

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hormones have been reported to be capable of being stored in sediments and in the body of the aquatic organisms (Conterras– Sanchez et al. 2001, Cek et al. 2004).

Overall, herbal compounds have not (or rarely have) harmful side effects in comparison with chemical compounds, synthetic Steroids and antibiotics, they are cheaper and they do not show harmful accumulation in living tissue, decomposable and able to return to the environment (Karimi et al. 2015). On the other hand, medicinal plants with the least side effects on living beings, including aquatic organisms, can be extensively used in the diseases treatment or as food supplements for increasing the growth or prolife ration and survival rates of the eggs and larvae, as well as the creation of a population with a specific sex (Citarasu et al. 2003).

Tribulus terrestris from Zygophyllaceae family, is a annual plant with main distribution in Japan, China, west Asia and southern Europe and Africa. The application of this plant's exctract powder on human beings has been investigated and its positive results have been reported on reproduction and synthesis of the male sex hormones (Cek et al. 2007b). *T. terrestris* naturally and safely increases the testosterone in human and animals and improve lipid and spermatogenesis levels (Bucci 2000; Tomova et al. 1981). It contains various known substances such as flavonoids, flavonol glycosides, steroidal saponins, and alkaloids (Chhatre et al. 2014). The effect of the performance of the chemical compounds of *T. terrestris* is thought to be due to the presence of steroidal saponines (including protodioscin) on the testosterone levels (Yeganeh et al. 2017).
Zebra fish (*Danio rerio*) (Hamilton 1822) is an oviparous fish and pleasant to the ornamental growers and it is considered as one of the most beautiful ornamental fishs in the freshwater and tropical zones whose habitats are east India, Bangladesh, Pakistan, Myanmar and Nepal (Talwar and Jhingran 1991). This species has been able to attract the attention of many scientists to use it in varous experiments as the model due to the ease of reproduction, resistance to desease and omnivorous feeding behevior (Saddhe et al. 2013; Grunwald and Eisen 2002; Spitsbergen and kent 2003; Alestrom et al. 2006).
As our knowledge is little about the effects of *T. terrestris* on steroid hormones level and sex reversal in fish. The aim of this study was investigating the effect of *T. terrestris* extract on the masculinization, survival rate and growth indices of the Zebra fish.

Materials and methods

The *T. terrestris* (TE) plant was collected from the Hashem Abad, Gorgan, Iran and washed with sterile distilled water, air-dried in shade and grinded. 250 g of whole grinded plant is added to 500 ml of 70% ethanol (Kavitha and Subramanian 2011) and exposed to air and dried. Finally, 3.4 g of pure resin-type material was obtained and dissolved in 200 ml of distilled water (Cheema et al. 2005).

To perform the experiment, broodstocks of Zebra fishes have been prepared from the center of ornamental fishes of Golestan province, Iran. The males and females were placed in separate tanks with the temperature of 24.5±0.5 °C and pH value equal to 7.2±0.2, after two weeks of adaptation to the physical and chemical conditions they were transferred into spawning tanks (volume 45 l). After 48 hours, broodstocks fishes were brought out from the spawning special reservoir and the eggs were hatched after 30-48 hours. Larvae have been fed egg yolk till 75% of sac was adsorbed (one month). Then, larvea were fed with commercial food on the market (manufactured by Biomar france) that was enriched with the extract of the *T. terrestris* plant for next 60 days. In detail, 180 larvea of Zebra fish with an average weight of 0.01±0.003 g were randomly distributed in 12 aquaria (each volume 30 l) and were fed with four diets supplemented with 0 (T0), 50 (T1), 100 (T2) and 200 (T3) mg TE/kg of food and three replications per diet treatment during 60 days at a rate of 4% of the body weight per day, spread across two feeding times. Besides, 3% of gelatin was included in order to diminish the dissolving rate of food plates in water. The aeration of aquariums was carried out using an air stone attached to the aerated pump. During this period, siphoning was performed every two days and 50 percent of the tanks' water replaced by the dechlorinated tap water.

at the beginning and the end of the trial, weight of each fish was measured using a digital balance (AND EK610i). weight gain (WG), specific growth rate (SGR), condition factor (CF), survival rate (SR), Gonadosomatic index (GSI) and feed conversion ratio (FCR) are the factors which have been investigated

according to the following formula (Luz et al. 2008).

SGR (%)=((Ln W_f- Ln W_i)/experimental days)×100

CF=average weight/(average standard length)^3

FCR=feed intake/weight gain

WG(%) = (W_f−W_i)/W_i × 100

In which, W_i and W_f denote the initial and final weights (mg), respectively and WG indicates the weight growth.

Survival rate (%) = (Nt/N0) × 100

Here, Nt and N0 stand for the initial and final numbers, respectively.

GSI (%) = (body weight/gonad weight)×100

The male to female ratio has been also estimated through histology of the gonad (Leal et al. 2009). In order to evaluate gonads histology, three fishes of each replicate were randomly sampled at the end of the trial, and then embedded in paraffin. The paraffin embedded specimens were sectioned in 5 µm thickness. Then tissue sections were stained with Mayer's Hematoxylin-eosin and observed under a light microscope (Sequeira et al. 2015).

The testosterone and dehydroepiandrosterone (DHEA) hormones were extracted from whole fish following a solid-phase extraction protocol (modified from Lorenzi et al. 2012). Briefly, three fish tissue samples from each treatment group was weighed, each sample transferred to borosilicate vials, and homogenized in 350 µl of 0.1 M borate buffer in an ice-cold water bath (pH 7.5). Then, 1.5 ml of methanol has been added to each sample, and vigorously vortexed and put in ice-cold water bath again. After this step, all samples were shaken for 60 min and stored at 4°C overnight. The next day, samples were shaked for 20 min at room temperature, and centrifuged at 1000×*g* for 10 min at 4°C. The supernatant was decanted, and 16 ml of water was added to dilute the methanol. To separate the water and methanol phases, microtube was placed inside the liquid nitrogen (for 20 seconds) until the water phase was frozen and then supernatant containing methanol was transferred to another tube and dried. 200µl of phosphate buffered saline-Gly (PBS-G) was added to the supernatant and stored in the freezer under -80 °C. Both hormones were measuredvia EIA kits (Cayman Chemicals, Inc.) which used to quantify hormones in the samples; kit protocols were strictly followed.

Quantitative variables are presented as the mean ± SD and the normality of data was first investigated using Shapiro–Wilk test.The effect of treatment on different factors were analyzed by one-way analysis of variance (ANOVA), and for differences, Duncan's multiple range tests was used by using the SPSS (version 18) software.

Results

The effect of TE supplemented diets on growth indices and survival rate during 60 days are presented in Table 1. According to the results, it is clear that the different levels of the TE have no significant influence on the survival rate of the larvae ($p>0.05$). FCR in fish fed with TE supplemented diets improved significantly ($p<0.05$) compared to the control group, so that the minimum FCR has been achieved in the fish fed with T2

Table 1. The effect of the alcoholic extract of edible *Tribulus terrestris* on the average growth indices and survival rate of Zebra fish (n=6) after 60 days

Treatment / index	Control (T0)	Diet containing 50 mg of extract (T1)	Diet containing 100 mg of extract (T2)	Diet containing 200 mg of extract (T3)
condition factor (CF) %	1.58±0.21^a	1.56±0.15^a	1.93±0.14^b	1.84±0.14^b
feed conversion ratio (FCR)	2.09±0.11^b	1.88±0.25^b	1.25±0.05^a	1.41±0.12^a
specific growth rate (SGR) (%/day)	4.29±0.08^a	4.51±0.19^a	5.16±0.06^b	5.01±0.14^b
survival rate (SR) %	99.1±0.9	100±0.00	100±0.00	100±0.00

Means marked by different letters are significantly different (p<0.05).

diet. Also, the SGR of the fishes increases with an increment in TE amount of the diet during the trial period ($p<0.05$). The optimal SGR has been achieved in T2 diet. The maximum and minimum values of WG index have been obtained in the fish fed with T2 diet and the control group ($p<0.05$), respectively. Furthermore, the maximum and minimum CF values in the present research have been recorded in the fish fed with T2 (0.21±0.008) and T0 (0.12±0.007) diets, respectively.

The comparison of the sex differentiations of the Zebra fish base on histology data (Figs. 1-2) in the different treatment groups showed that almost equal male and female proportions appeared in the control group, but in the experimental treatments, the number of male fishes were higher than the female ones after 60 days, the highest male sex ratio was in the fish fed with T2 diet with a percentage of 70.83 (Table 2). The highest GSI was observed in the control treatment (0.15±0.018).

As shown in Table 3, the amounts of testosterone and DHEA hormones were significantly different in fish fed with TE supplemented diets compared with the control group ($p<0.05$). The maximum amounts of testosterone and DHEA hormones were reported in the fish fed withT3 and T2 diets, respectively (Table 3).

Table 2. The effect of the alcoholic extract of edible *Tribulus terrestris* on the average reproduction indices in Zebra fish (n=10)

Treatment / index	Control (T0)	Diet containing 50 mg of extract (T1)	Diet containing 100 mg of extract (T2)	Diet containing 200 mg of extract (T3)
Gonadosomatic index	0.15±0.018^{c}	0.12±0.007^{b}	0.10±0.005^{a}	0.10±0.002^{a}
Sex ratio percentage (male)	43.33^{a}	50.83^{ab}	70.83^{c}	54.16^{b}

Means marked by different letters are significantly different ($p<0.05$).

Table 3. The effect of the alcoholic extract of edible *Tribulus terrestris* on the average amount of testosterone and dehydroepiandrosterone hormones of Zebra fish (n=6) during 60 days

Treatment/index	Control ($\mathbf{T_0}$)	Diet containing 50 mg of extract ($\mathbf{T_1}$)	Diet containing 100 mg of extract ($\mathbf{T_2}$)	Diet containing 200 mg of extract ($\mathbf{T_3}$)
Testosterone (ng/mL)	0.05±0.004^{a}	0.25±0.05^{ab}	0.35±0.05^{b}	0.70±0.11^{c}
Dehydroepiandrosterone (µg/dL)	88.10±1.058^{a}	89.23±6.26^{ab}	96.35±1.47^{b}	94.41±3.44^{b}

Means marked by different letters are significantly different ($p<0.05$).

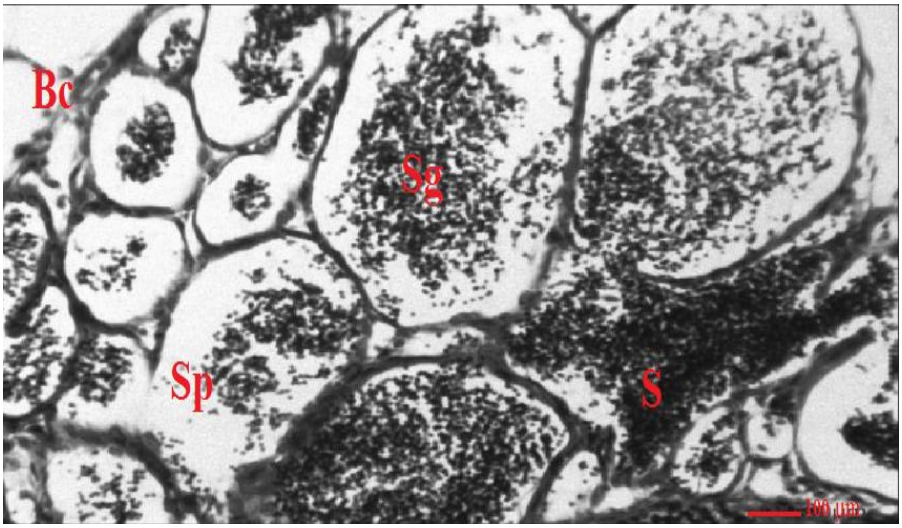

Fig. 1. The testicle histology of the male Zebra fish exposure by *Tribulus terrestris* (100 mg) after 60 days at a magnification of 40X. Body cavity (Bc), spermatogonium (Sg), spermatozoids (Sp), spermatid (S), each of which inside a sac of globules by hematoxylin-eosin (H&E) staining.

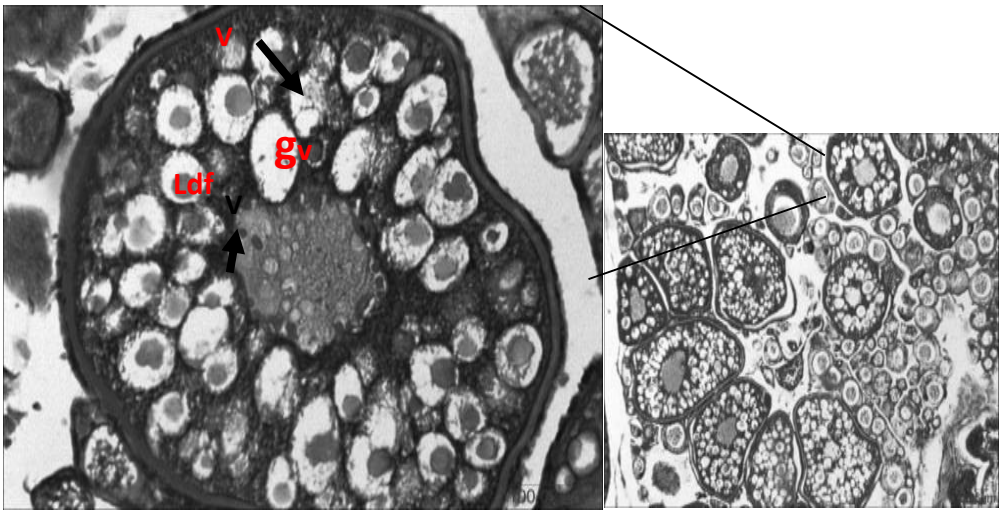

Fig. 2. Cross section of the ovary tissue of Zebra fish exposure by *Tribulus terrestris* (50 mg) after 60 days at the magnifications of 10X and 40X, Vitellogenesis (V) stage, lipid droplet follicle (Ldf), germinal vesicles (gv) towards the animal pole.

Discussion

The use of herbal supplements is highly recommended in aquaculture as areplacement to synthetic steroids. Because they have low cost and are more environmental friendly, many plants have been used in diet as immunostimulant, antibacterial, antiviral and growth enhancer (Yeganeh et al. 2017). According to the results, the use of TE had no significant influence on the survival of the zebra fish ($p>0.05$). In contrast, the FCR, SGR, WG and CF significantly ($p>0.05$) improved in fish fed with TE supplemented diets compared to the control group. The achievements indicated that TE does not have toxic and harmful effects on the fishes health because no loss has been observed during the trial period. Many studies have focused on commercial fish, but current knowledge is limited for ornamental fish. In this regard, researchers have studied the effect of *T. terrestris* plant on the growth and survival indices of fishes including *Cichlasoma nigrofasciatum* (Cek et al. 2007b), *Clarias gariepinus* (Turan and Cek 2007), *Poecilia latipinna* (Kavitha and Subramanian, 2011), *Oncorhynchus mykiss* (Yılmaz et al. 2013) and *Oreochomis mossambicus* (Omitoyin et al. 2013). The results of these studies on the specific growth rate and survival rate are similar to present study, which may be due to the fact that the TE contains different compounds, including vitamins A, C, E, fatty acid and essential amino acids so that several therapeutic effects and properties have been reported for this plant (Pérez-Sánchez et al. 2017) and it is used as a disinfectant of the digestive system, stimulant herb, appetite and sexual performance enhancer (Akhtari et al. 2014; Yeganeh et al. 2017). In addition, the presence of vitamin A in the extract which is one of the growth factors of the animals, may cause the fat storage in the body in the form of triglycerides and increase the body weight by converting to retinoid (Karimi et al. 2012).

Histology results of present study, indicated that the number of female fishes in the control group is more than the male ones (56.67 %) which is normal in this species (Foster and Hanford Brown 2018), but the number of females in fish fed with T1 diet is nearly the same as males (49.17 %). Also, the maximum male number was seen in fish fed with T2 diet (70.83 %) and the number of males in T3 treatment was less than that of T2. Several studies have been reported positive results on fish masculinization by *T. terrestris* plant such as *Poecilia reticulate* and *Cichlasoma nigrofasciatum* (Cek et al. 2007a,b), *Clarias gariepinus* (Turan and Cek 2007), *Poecilia reticulata* (Kavitha and Subramanian 2011), *Oncorhynchus mykiss* (Yilmaz et al. 2013), *Oreochromis niloticus* (Omitoyin et al. 2013; Ghosal and Chakraborty 2014; Ghosal et al. 2015).

The testosterone hormone is the primary male androgen in teleost and the precursor of the 11-Ketotestostrone which is mostly produced by testis. Testosterone is a sex hormone which plays role in the germ cells and it is existent at the time of the embryonic cells differentiation the male larvae and subsequently used in the body during the maturity or physiological development (Devlin and Nagahama 2002).

The *T. terrestris* plant increases the testosterone level due to containing estradiol glycosides (the most important of which is protodioscin) and as the inductor, the natural estradiols in this compound may facilitate the production of androgens from estradiol and thereby increase the testosterone levels (Tadayon et al. 2018).

Masculinization by the *T. terrestris* plant is probably due to the presence of unsaturated fatty acids (Masia et al. 1998) which increases the activity of 17-beta-hydroxydehydrogenase and it is involved in the production of testosterone and the testosterone hormone increases as a consequence (Chung et al. 2001). These available acidic compounds in the *T. terrestris* plant inhibits the activity of aromatase enzyme. Due to convertion of androgen to estrogen by aromatase enzyme, its inhibition increases the amount of androgen (testosterone) in the blood (Stocco 2012). The influence of the treatment with aromatase inhibitor (estrogen synthesis inhibitor) on the induction and creation of the male sex is much more than that with 17α-methyltestosterone hormone (Kwon et al. 2000). Saponins in the *T. terrestris* plant lead to an increment in the luteinizing hormone secretion from the pituitary gland which is a stimulus hormone especially for the testosterone production (Tsai et al. 2003). It has been demonestrated that the extract of the *T. terrestris* plant increases the testosterone levels in fish (Gauthaman et al. 2000;Yeganeh et al. 2017; Adaikan et al. 2000) which is probably due to the presence of several known substances similar to steroids in the extract of this plant. The protodioscin is one of the substances in the *T. terrestris* which affects on testosterone levels (Ganzera et al. 2001; Joshi et al. 2005). It has been specified that protodioscin increases the levels of DHEA (Adimoehja and Adaikan 1997), dihydrotestosteron and dehydroepiandrosterone sulfate. These increments in the testosterone and DHEA levels were measured in this study and based on the results of Table 3, it was found that the *T. terrestris* increase these hormones. This was in agreement with the results reported by Kavitha and Subramanian (2011) where the *T. terrestris* led to an increment in the testosterone level of the treated groups. Gauthaman et al. (2002) showed that the use of *T. terrestris* in castrated mice may also increase the blood's testosterone level which is similar to the present achievements.

According to the data given Table 2, the GSI in the control treatment (due to the great number of female fishes in) showed a higher increase than the other treatment groups. Since, the use of *T. terrestris* has increased the number of male fishes. These results are similar to those reported by (Wang et al. 2010) where they examined the effect of two other medicinal plants (*Froctos lissi, Froctos ligostri*) on the reproductive performance of yellowhead catfish (*Pelteobagrus fulvidraco)* for both of sexes . They reported that the GSI was significantly higher for the female fishes fed with the diets containing these plants.

In general, the results of the research indicate that the use of the TE with a concentration of 100 mg TE/kg of food, has a positive and significant effect on masculinization, growth and nutrition in Zebra fish. It is considered as a convenient and affordable option in order to reduce the cultural costs and a suitable alternative for the chemical food supplements. Therefore, it is recommended to use the TE in order to masculinize and to enhance the growth of the ornamental fishes (Zebra fish), especially for those needing the fully male population.

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