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PROOF

Influence of Fennel Foeniculum Vulgar Extract on Fertility, Growth rate and Histology of Gonads on Guppy *Poecilia reticulata*

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Abstract

Ornamental fish breeding and growing are an important sector of aquaculture, which has increased income and occupations in the country in recent years. Presenting the new methods and producing the new food supplements could have effects on efficiency and sustainable development in this new technology. In the present study, the effect of ethanolic extract of *F.vulgar* having phytoestrogenic compounds on growth performance and fertility rate and gonadal tissue changes of guppy was tested for offering it as a food supplement in the aquaculture. 360 juvenile guppies were distributed into 12 glass aquaria at 30 fish/ tank in triplicate. Four diets containing varying levels of alcoholic extract of *F.vulgar* seed at 0. 75, 100, 125 µl/ g were prepared and fed to the fish twice daily at 3% of their body weight for 133 days. Biometry of the groups was done every 15days. The born fry were separated from the mothers and counted. One fish from all groups were fixed in formalin buffered 10% every 15days for histology. 5 microns sections were prepared and stained with H&E technique. The results for each group were compared statistically with those for the control. Analysis of the results showed a significant increase in weight gain and fertility rate in group T2 (100µl/g). Gonad cells increased in the test groups comparing with control group simultaneously with raise in phytostrogenes in tests groups. In the follicles, enlarge granulose layer with increase in number of ovules in different evolution stage (O_1- O_7), were seen in test groups. The increase in fertility rate in tests groups may be due to the increase in the level of estrogenic compound which in turn could be due to the action of the extract present in the diet. The observation made in the present investigation show that *F.vulgar* can act as a phytoestrogenic compound at low concentration and hence *F. vulgar* can be considered as a potential as well as a natural additive to fish feed in commercial aquaculture to augment the fertility rate.

Keywords: Guppy, viviparous, phytostrogen, ornamental fish.

Introduction

Ornamental fish reproduction and breeding has important role in increasing the fish farming industry benefit. Ornamental fish farmers breed and raise many of species of aquarium fish, using a variety of techniques. Some species will spawn after simple environmental changes have been made, such as changes in water temperature, pH, or conductivity. Other species require more advanced methods, including administration of hormone products by injection for induced spawning (Roy *et al*., 2009).

The culturist utilized carp pituitary for induce artificial maturation in fishes particularly in genus Cyprinids (Kahkesh *et al*., 2010). Furthermore, human chronic gonadotropin (HCG), and gonadotropin releasing hormone (GnRH) and other harmonic compounds still use for fish reproduction (Mylonas *et al*., 2009). Also, ovaprim contains a salmon gonadotropin-releasing hormone analog and a dopamine antagonist used as a spawning inducer in fishes since 2005 (Jeffrey *et al*., 2009).

However, synthetic hormones are more expensive than plant extracts and their administration in fish is time-consuming, labor-intensive and requires specialist expertise. Moreover, the synthetic hormones have been reported to have the potential to accumulate in the sediment water and aquatic biota (Yilmaz *et al*., 2009).

Replacement of herbal medicine instead of hormones could be as new approach in fish breeding. Phytoestrogens are plant compounds that are structurally similar to animal estrogens (Clotfelter and Rodriguez, 2006). Recent years have seen increased interest in the human health benefits of phytoestrogen consumption, particularly in the treatment of hormone dependent cancers, menopausal symptoms, and cardiovascular disease (Adlercreutz,

1995; Setchell and Cassidy, 1999; Beck *et al*., 2005). The three classes of phytoestrogens (isoflavones, coumestans, and lignans) are found at high concentrations in plants such as legumes (Dixon, 2004) and fennel (Choi and Hwang, 2004). While terrestrial animals are exposed to phytoestrogens primarily through herbivory (Bennetts *et al*., 1946; de Man and Peeke, 1982), aquatic animals are exposed to waterborne phytoestrogens from several sources (Clotfelter and. Rodriguez, 2006). Fennel *Foeniculum vulgar* extracts proved to have antiinflammatory, antispasmodic, carminative, diuretic, expectorant, laxative, analgesic, stimulant of gastrointestinal mobility and are used in treatment of nervous disturbances (Choi and Hwang, 2004). Moreover, the effect of fennel extract on reproductive system and tissues of male rats has been investigated (Torkezahrani *et al*., 1997). The effects of fennel extract on polycystic ovary syndrome (PCO) have also been investigated (Citarasus, 2010). The fennel extract impacts on growth and reproduction system of fishes have not been reported so far.

The guppy *Poecilia reticulata* is one of the viviparous ornamental fishes considered as a suitable tool in the histological studies of gonads (Georgescu, 2012). On the other hand, the hormonal control of reproduction in guppy is similar to other vertebrates and pituitary gland influence ovary by gonadotropic hormones (Hogarth, 1976).

In addition, ovary in *P.reticulata* has the ability to produce steroids like ovary of other vertebrates. This ability is due to the presence of synthesized enzymes of steroids inside the granulose cells that have surrounded the growing follicles (Georgescu, 2012). Other features of the guppy fish that short period of pregnancy (between 21 to 30 days) by an average of 28 days (Lam, 1983). All these properties along with comfortable conditions for maintaining this species in the aquarium make it as a suitable fish species for various tests (Woodhead, 1979).

The histological effects of the Fennel extract on fish gonads as a replacement for synthetic estrogens has not been studied yet. The purpose of this study is microscopic investigation on the female guppy reproduction system treated by different dosage of ethanol extract of fennel.

Materials and Methods

Fish

360 Juvenile guppies with mean weight (0.017±0.035 gr) originally from Singapore were purchased from commercial supplier. The fishes were acclimatized in clean aquaria for two weeks before the experiment and fed with commercial ornamental fish feed (Tetramin, Germany) twice daily. The experiment was conducted in glass aquaria each containing 30 L of water. Then, the guppies were divided in four groups, three test groups and one control group in triplicate and distributed into 12 glass aquaria at 30 fish/ aquaria. The temperature was maintained at 28ºC and the aquaria were aerated.

Extract Preparation and Diet

The seed of Fennel were purchased from Herbal medicine shop in Isfahan, Iran. The seed was identified by Department of Botany of Science at Faculty of Biology, Azad University ,Falavarjan branch, Isfahan, Iran. Fennel seed was pulverized with a blender. Obtained seed powder (50g) was mixed with ethanol 96º in ratio of 1:1 and stirred on the room temperature for three times each time 24 hours. Subsequently, it was filtered over Whatman No.1 paper. The obtained filtrated extract was dried in room temperature. The final liquid was prepared by diluted the dried extract in distilled water in ratio of .01g/ml.

Four diets prepared containing 0, 75, 100, 125 µl/g extract and mixed with food and dried. The diets were fed to the fish twice daily at 3% of their body weight for 133 days. Biometry of the all groups was done every 15days. The born offspring was separated from the mothers and counted.

Histology

One fish from all groups were fixed in formalin buffered 10% every 15days for histology. The fixed fishes then dehydrated and mounted in paraffin blocks. Five-µm thick sections of fish blocks were cut, mounted on glass slides and stained with Haematoxylin and Eosin for histopathological examination using bright field light microscope.

Results

The guppies fed with variety of diets in three test groups for 133days shown considerable growth rate comparing with control group (Figure 1). The first group (T1) consists of 90 fishes in triplicate with average weight of 0.002 ± 0.051fed with 75µl/g fennel extract. This group obtained average weight of 0.144 ± 0.297g\after 133days of experiment. The second treatment (T2) in triplicates, with average weight of 0.002 ± 0.024g received 100µl/g fennel extract. The average weight gain in this treatment reached to 0.172 ± 0.52g after 133 days. In the third treatment (T3) as in the previous treatment with 90 fishes in triplicates with an average weight of 0.001 ± 0.035g, after 133 days of feeding with diet number three containing 125µl/g fennel extract reached to an average weight of 0.117 ± 0.466 g. The control group in triplicate and 90 fishes with a mean weight 0.002 ± 0.029 g fed with no fennel extract reached to 0.010 ± 0.238 g. Comparison of weight changes in test groups and control during experiment was shown in Figure 2.

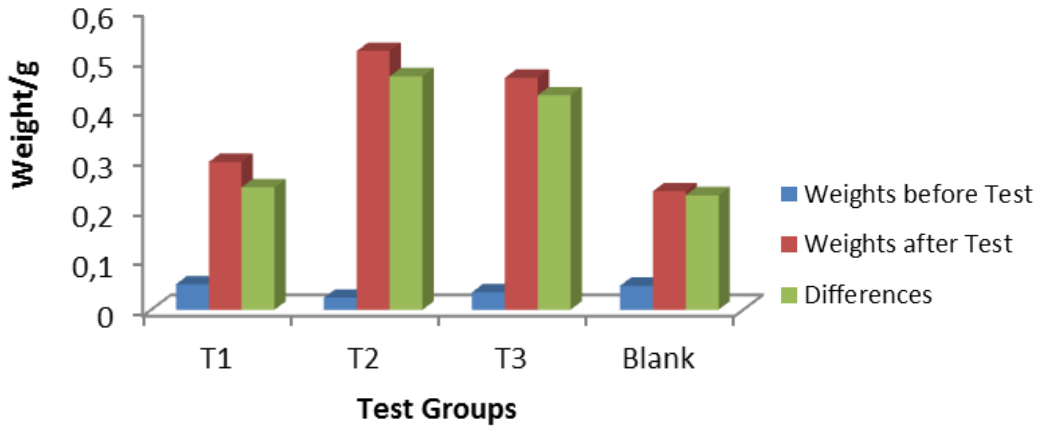


Figure 1. Comparison of growth rate in test groups with control group.

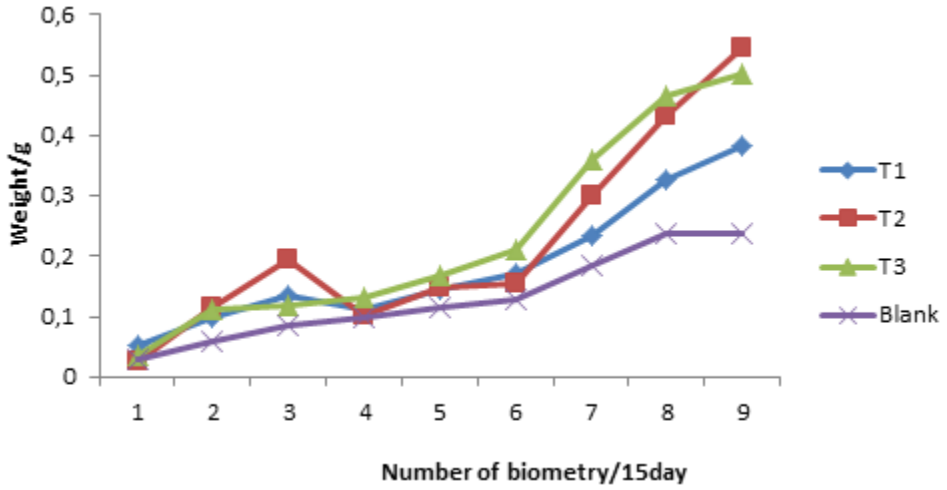


Figure 2. Comparison of weight changes in test groups and control during experiment.

According to this graph the daily growth rate in the group T2 is comparable with the other groups (M =0.0039) while in the control group the mean of daily growth was measured 0.0019 g/day. Based on the statistical analysis of results, the difference between the growth rate in the T2 group and control group is significant ($P<0.05$).

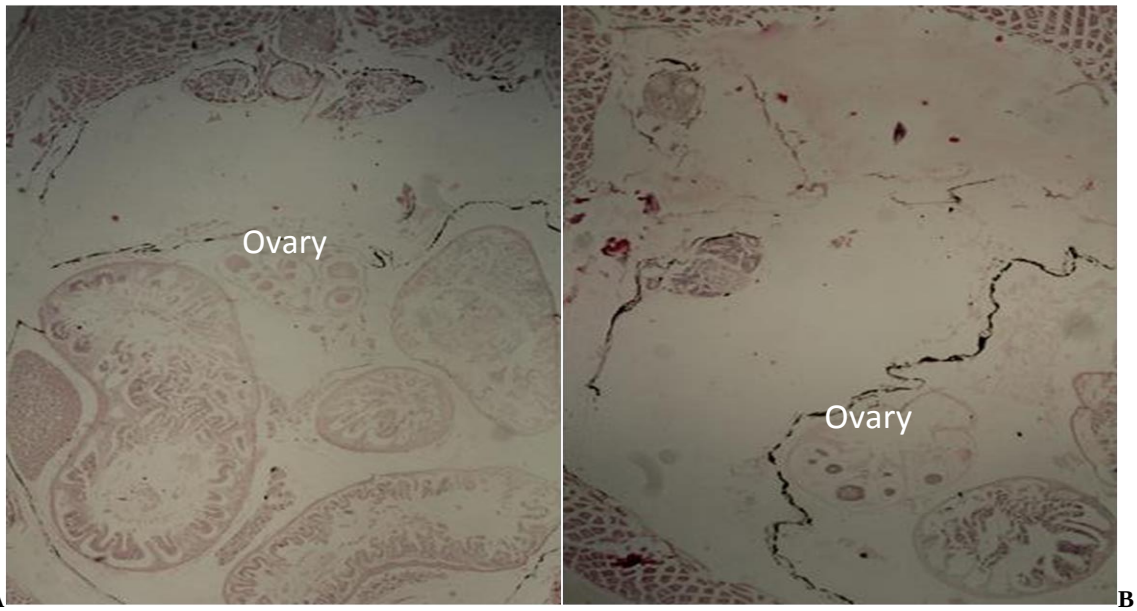
Histopathology

Figure 3A shows cross section of female guppy 15d after treatment with fennel extract in group 2 in compare with group control (B) after 30d. These figures shows no develop in ovaries at the beginning of the test. Immature follicles are seen in the ovaries.

After many days, ovary and follicles and seminal vesicles are appear inside the abdominal cavity of female's guppies in test groups. (Figure 4A) This process in control group that did not receive *F.vulgare* extract is specified in the longer and with fewer follicles (Figure 4B). Also, in the samples that received *F.vulgare* extract the granulose layer around follicles has had further development (Figure 5). Follicles containing oocytes in the different stages of development (O_1 to O_7) as well as with more number can be seen in Figure 5. Figure 6 indicates the sperm holder bag of female. As well as lipid droplets in the yolk oocytes can be seen in the Figure 4. The complete fetus in the day 133 after the start of the experiment can be seen in Figure 4. Figure 7 indicates the comparison of the average fertility in the different test groups. According to this diagram T2 group had the highest rate of regeneration among different groups by receiving 100 mg per liter of extract.

Discussion

The internal breeding control's pattern of poeciliidae and vertebrate is comparable. In this pattern, gonadotropic hormone controlled maturation of immature ovum. Increasing in hormones' concentration enhance evaluation of ovum and function of cell surface receptors (Hogarth, 1976). The aim of this study was to evaluate the influence of different value of ethanolic extract of fennel on development procedure of genital tissue of female guppy. Microscopically investigation and statistical results obtained from this research indicate that fennel



A **B**

Figure 3. A) Cross section of female guppy fed with fennel extract after 15d in group T2. **B)** cross section of female guppy in control group after 30d. X40, H&E.

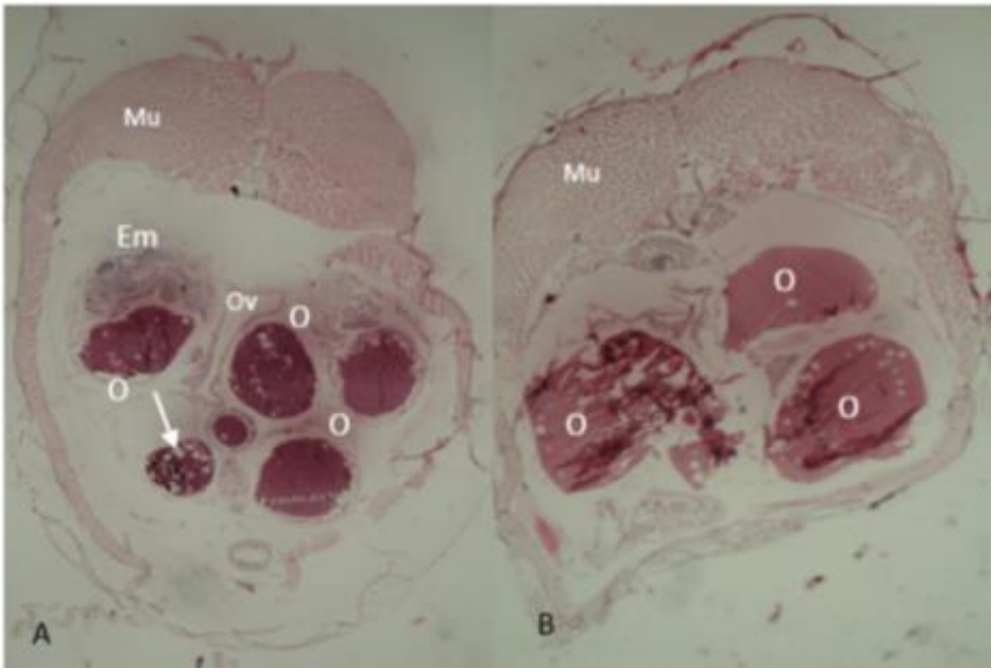


Figure 4. L Cross section of female Guppy 133d after treatment with fennel in Group 2. R: Cross section of female Guppy 133d after treatment with fennel in Group control. White arrow shows vitellus of follicules. Mu: Mussels, Em: Emberyo, Ov: Ovary, O: oocyte.

extract influenced of maturity of ovum in shorter time (Figure 4). Accordingly, in the treatment groups in comparable with control group the offspring born earlier and with more quantity. Findings in the present study are consistent with the findings of Thiago *et al.,* (2011). Their research was focused on poeciliidae family and indicates that increasing of vitellus in the liver of teleost has related to response to increase of 17-β estradiol. Thus, the estrogenic hormones level was increased in treatment groups during experiment by feeding. Increase in estrogenic hormones in fish enhanced reproductive activity and the offspring was born in shorter time. Pellisero *et al.,* (1991) indicated that Phytostrogens has same effect but weaker than 17-β estradiol. The histology figures in different stages were indicated that maturation of the ovum on the ovary would increased concurrently with increasing in the fennel extract on the diet but lesser in comparable with the synthetic hormones. Therefore, it is necessary to using the phytostrogens

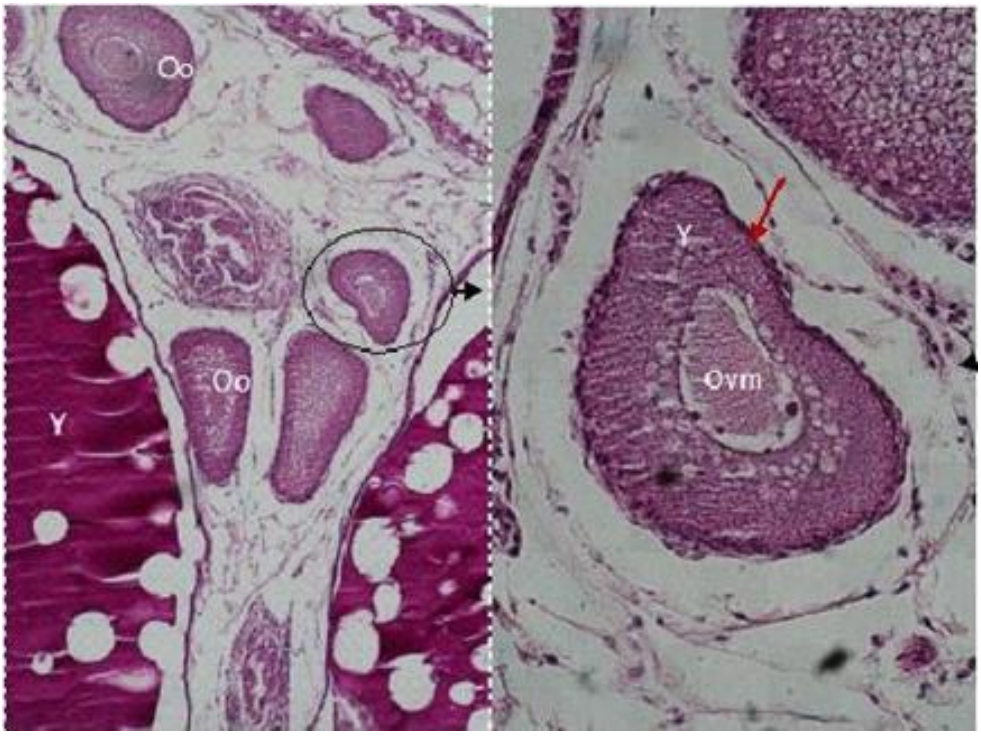


Figure 5. Cross section of female Guppy in ovary 133d after treatment with fennel. (L); Oocyte in different stage. X10. E&H. (R): Follicles with oocyte. Red arrow show granulose layer. OO: Oocyte, Y: Yolk, O: Ovum, x100, H&E.

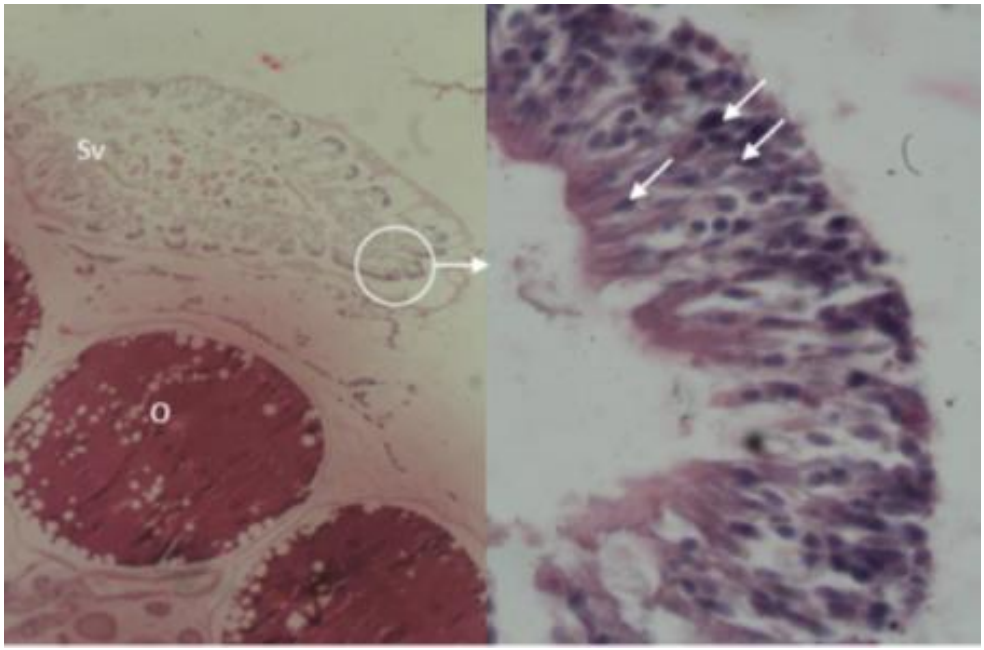


Figure 6. Cross section of female Guppy 118d after treatment with Fennel. (L); seminal vesicle, x10, H&E. (R); High magnification of seminal vesicle with sperms (white arrows), x100, H&E.

such as the fennel extract before beginning of reproduction season for more influencing on reproductive system. The time and quantity of fennel extract applied in fish diet has related to breeder weight, environmental factors, salinity and water temperature. After maturation, the follicles in the ovary of viviparous teleost would not be sensitive in equal level to the induction effect of gonodotropin and estrogens. Therefore, mature follicles, fertilize follicles and embryo ready to birth are exist together in the ovary simultaneously (Hogarth, 1976). Figure 4 shows these follicles in the ovary of the guppy exposed by fennel and indicted that fennel extract influenced the guppy same the estrogenic hormones whiles in the other species of oviparous teleost, the follicles have synchronic stages of maturation. In a

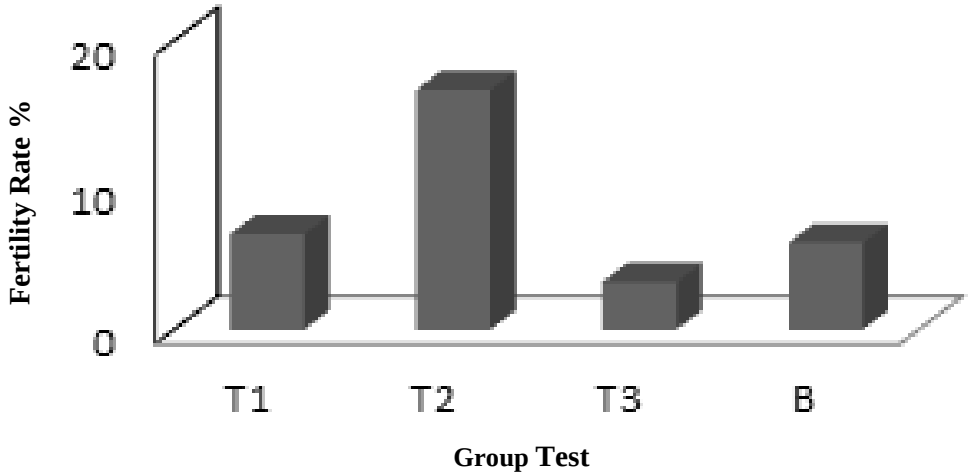


Figure 7. Comparison of fertility rate in test groups and control during experiment.

research on *Epinephelus Coiodes* an oviparous fish (Abbasi *et al*., 2006) indicated that all follicles will developed and matured concurrently. These species will spawn annually and in the same season. In the other research on *Thunnus albacore* which (Oryan *et al.*, 1993) evaluated that in this species same the other species belonging to family Scombridae the ovum in different stages occurred in the ovary simultaneously. There are similarities in results between the present study and those described by (Yilmaz *et al*., 2009). They evaluated that genesis including of phytostrogens increased growth rate and sex differentiation in the Sharptooth catfish *Clarias gariepinus*.

Previous study on phytostrogens compound showed that phytostrogens have different effect on the different species of fish spatially on ornamental species. Brown *et al.* (2002) indicated that Genistein as a phytostrogen has limited effect on reproductive endpoints in a female fighting fish *Betta splenden* while other researches showed contrast results. Phytostrogens increased vitellogenin synthesis in Tilapia primary hepatocytes (Turker and Takemura, 2011). Furthermore, phytoestrogens are able to stimulate vitellogenin synthesis *in vivo* in Siberian sturgeon *Acipenser baeri* (Pelissero *et al.*, 1991) and common carp, *Cyprinus carpio*, (Turker and Bozcaarmutlu 2009), *in vitro* in rainbow trout, *Oncorhynchus mykiss*, hepatocyte (Pelissero *et al.*, 1993) and *in vivo / in vitro* in Siberian sturgeon, *Acipenser baeri*, and rainbow trout (Latonnelle *et al.*, 2002). Turker and Takemura (2011) in their study indicated that plant extract such as fennel, fenugreek, aniseed, safflower, flaxseed, liqurice, pomegranate and soybean were considerably less potent than estradiol. In this regard it is recommended to applying phytostrogens in long duration for stronger effect on fish breeder. However, further research should be done to evaluate the effective dosage on different species by establishing an *in vivo* experiment in both male and female.

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