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Note

Effects of *Tribulus terrestris* and *Ferula communis* extracts on growth and gonad histology of red zebra cichlid *Maylandia estherae* (Konings, 1995)

SEVAL BAHADIR KOCA[1], HASAN BATUHAN EMRE OZDOGAN[1], OZLEM OZMEN[2], MUHAMMED BIYIKLI[3] AND NALAN OZGUR YIGIT[1]

[1]*Faculty of Egirdir Fisheries, Isparta University of Applied Sciences, 32100 Isparta, Turkey*
[2]*Department of Pathology, Faculty of Veterinary Medicine, Burdur Mehmet Akif Ersoy University, 15100 Burdur, Turkey*
[3]*Faculty of Agriculture, Isparta University of Applied Sciences, 32100 Isparta, Turkey*
e-mail: sevalkoca1@hotmail.com

ABSTRACT

The study was conducted to determine the effect of dietary supplementation of plant extracts from *Tribulus terrestris* and *Ferula communis* on gonad histology of the red zebra cichlid *Maylandia estherae* (Konings, 1995). The fish were fed dietary additions of 0.2% *T. terrestris* (TT); 0.2% *F. communis* (FC) and *F. communis* 0.1% + *T. terrestris* 0.1% (FC+TT) along with a control diet, each treatment in triplicates. A total of 36 females with a mean weight of 3.54 g and 36 males with an average weight of 3.54 g were randomly stocked (3♀:3♂) in 12 aquaria. The fish were fed *ad libitum* with treatment diets for 90 days. At the end of the experiment, the final weight, weight gain, feed conversion ratio (FCR), specific growth rate (SGR) and survival rate of fish fed with diets supplemented with FC and TT showed no significant differences from the fish fed the control diet. Histological examination revealed increase in number of mature oocytes in FC and TT groups. However, number of mature oocytes decreased in FC+TT group. The seminiferous lobules in FC and TT groups were found to be comparatively larger with much more spermatozoa compared to the control groups. Spermatozoa decreased in FC+TT group but increased spermatids were seen in seminiferous tubules. Degenerative changes were not seen in any group. The findings indicated that FC and TT extracts added to the diets of fishes helped to improve reproductive performance and FC addition may be more effective for increased ovarian and testicular capacity.

Keywords: *Ferula communis*, Fish, Histology, Reproductive performance, *Tribulus terrestris*

In aquaculture, hormones are used for inducing sterility, production of monosex population, sex reversal and for artificial reproduction (Hoga *et al.,* 2018) for which synthetic hormones are usually used. But synthetic hormones can pose risks to human and environmental health (Contreras-Sanchez *et al.,* 2001; Hoga *et al.,* 2018). In recent years, various researchers have studied on the effect of plant extracts on reproduction of fish. Studies have indicated that several plants inhibited reproduction in fishes such as: pawpaw seeds in *Oreochromis niloticus* (Ekanem and Okoronkwo, 2003); leaf meal of *Hibiscus rosa sinensis* in *O. niloticus* (Jegede, 2010); *Carcica papaya* seeds in *O. niloticus* (Abbas and Abbas, 2011; Khalil *et al.,* 2014; Waweru *et al.,* 2019); *Azadirachta indica* saponin in *O. niloticus* (Obaroh and Nzeh, 2014); Aloe vera latex in *O. niloticus* (Kushwaha, 2013); extract of *Psidium guajava* in *O. niloticus* (Obaroh *et al.,* 2018); *C. papaya* seeds, hibiscus leaves and sweet potato leaves in female of *Clarias gariepinus* (Ekpo *et al.,* 2018) and turmeric powder in *Pseudotropheus acei* (Koca *et al.,* 2019).

Plants found successful in monosex production or masculinisation of fish were; Aloe vera in *O. niloticus* (Gabriel *et al.,* 2017); aqueous extracts of *Glycyrrhiza glabra* in *Poecilia reticulata* (Turan, 2017), *C. papaya* seeds meal in *O. niloticus* (Ugonna *et al.,* 2018) and extract of *Tribulus terrestris* (TT) in *C. gariepinus* (Turan and Cek, 2007), in *P. reticulata* (Cek *et al.,* 2007a), in *Cichlasoma nigrofasciatum* (Cek *et al.,* 2007b), in *Betta splendens* (Janalizadeh *et al.,* 2018), in *O. niloticus* (Omitoyin *et al.,* 2013; Ghosal *et al.,* 2015; Ghosal *et al*., 2016; Ghosal and Chakraborty, 2020). and in rainbow trout (Yılmaz *et al.,* 2013).

T. terrestris is a medicinal plant and is reported to increase testosterone or testosterone precursor levels by affecting androgen metabolism (Neychev and Mitev, 2005). It is known to elevate the testosterone levels in humans and animals (Adaikan *et al.,* 2000; Gauthaman *et al.,* 2002). Positive effects of TT extracts on masculinisation of fish were reported by Turan and Cek (2007); Cek *et al.* (2007a, b); Omitoyin *et al.* (2013); Ghoal *et al.* (2015, 2016); Janalizadeh *et al.* (2018) and Ghosal and Chakraborty (2020).

Extracts of *Ferula* plant have been reported to improve sperm count, motility, viability and morphology

(Lohiya *et al.,* 2016). Kassis *et al.* (2009) reported that water-ethanol extracts of *Ferula asafoetida* added to diets of rats improved sperm counts. Onal and Guzey (2020) indicated supplementation with *F. communis* root to the diet of Awassi sheep may enhance ovulation. Studies in fish are limited and Yılmaz *et al.* (2006) did not find significant differences between gonadosomatic index (GSI) in *Cyprinus carpio* fed with diets supplemented with *Ferula coskunii* root. Similarly, Balci and Aktop (2019) did not detect significant differences between gonad histology of *Carassius auratus* fed *Ferula elaeochytris* root powder incorporated diets.

In the present study, the effect of *T. terrestris* and *F. communis* extracts, both individually and in combination on gonad histology of the red zebra cichlid, *Maylandia estherae* (Konings, 1995) was investigated.

The experimental diets were formulated to be isonitrogenous (37% crude protein) and isocaloric (4509 kcal). The plant extracts were added to the basal diet to preapare the treatment diets, FC: *F. communis* extract 0.2%, TT: *T. terrestris* extract 0.2%, FC+TT: *F. communis* 0.1% + *T. terrestris* extract 0.1%) along with C: Control diet. Composition of the diets is shown in Table 1. The feed ingredients were supplied by a local fish feed manufacturer. All ingredients were ground into small particles (0.5 mm) in a mill and dietary ingredients were blended in a mixer. Micro ingredients were first mixed and then slowly added to the macro ingredients to ensure a homogenous mixture. Water was added to obtain 30% moisture level. The diets were passed through a mincer with a 1 mm sieve. The pellets were fan-dried and stored at -20°C until use. Moisture, crude protein, crude fibre and ash contents of the diets were determined according to standard methods (AOAC, 2000). Total lipids of all samples were determined by chloroform-methanol extraction method (Bligh and Dyer, 1959).

Table 1. Formulation and proximate composition of experimental diets (%)

Ingredients	C	FC	TT	FC+TT
Fish meal	35.00	35.00	35.00	35.00
Soybean	25	25	25	25
Corn starch	0.2	-	-	-
F. communis extract	-	0.2	-	0.1
T. terrestris extract	-	-	0.2	0.1
Wheat	28.3	28.3	28.3	28.3
Fish oil	8.50	8.50	8.50	8.50
Vitamin mix[1]	1.00	1.00	1.00	1.00
Mineral mix[2]	1.00	1.00	1.00	1.00
Pellet binder	1.00	1.00	1.00	1.00
Proximate composition				
Crude protein (%)	37.79	37.79	37.79	37.79
Crude lipid (%)	11.78	11.78	11.78	11.78
Crude fiber (%)	2.51	2.51	2.51	2.51
Crude ash (%)	9.54	9.54	9.54	9.54
Energy (kcal kg^{-1})	4509.078	4509.078	4509.078	4509.078

[1]Vitamin premix (per kg) : 4,000,000 IU vitamin A, 480,000 IU vitamin D3, 40,000 mg vitamin E, 2400 mg vitamin K3, 4,000 mg vitamin B1, 6,000 mg vitamin B2, 40,000 mg niacin, 10,000 mg calcium D-pantothenate, 4,000 mg vitamin B6, 10 mg vitamin B12, 100 mg D-biotin, 1,200 mg folic acid, 40,000 mg vitamin C and 60,000 mg inositol.
[2]Mineral premix. (per kg): 23,750 mg Mn, 75,000 mg Zn, 5,000 mg Zn, 2,000 mg Co, 2,750 mg I, 100 mg Se, 200,000 mg Mg.

Dry *F. communis* and *T. terrestris* plants were obtained from an established spice supplier in Isparta, Turkey. Firstly, root of *F. communis* and shoot portion of *T. terrestris* were powdered using a blender. The ground powder (50 g) was soaked in 150 ml 95% ethanol in 500 ml round bottom flask. The flask was placed on a shaker waterbath at 24°C temperature for 24 h. The crude extract was then filtered using a filter paper (Whatman no. 1) and filtrates were collected and then ethanol was removed using a rotary evoporator at 40°C. The concentrated extract was then stored in refrigerator at 4°C (Deshwal and Vig, 2011).

M. estherae broodstocks were obtained from Isparta, University of Applied Sciences, Isparta, Turkey. A total of 36 females with a mean weight of 3.54 g and 36 males with an average weight of 3.54 g were randomly stocked (3♀:3♂) in 12 aquaria (30 x 40 x 100 cm), provided with shelters. The fish were fed *ad libitum* with treatment diets for 90 days. Each treatment was replicated three times. Water in the aquaria was well aerated and temperature was maintained at 26^0C using thermostat heaters. Dissolved oxygen levels in the tanks ranged from 6.5 to 7.2 mg l^{-1}. Experimental groups were fed by hand *ad libitum* twice daily at 08:30 and 20:30 hrs. Aquaria were cleaned daily and residual feed and faeces were removed by siphoning.

On termination of the feeding trial, ovary and testes samples were collected and fixed in 10% neutral buffered formalin for histological examination. Tissue samples were routinely processed in an automatic tissue processor (Leica ASP300S; Leica Microsystems, Germany); embedded in paraffin and 5 µm sections were made using a rotary microtome (Leica RM 2155, Leica Microsystem, Germany). Sections were then stained with hematoxylin and eosin (H&E) and examined under a light microscope (Olympus CX41, Tokyo, Japan). Oocyte numbers according to stages (stages I, II, III, IV and V) were calculated in randomly selected 5 areas of each ovaryof fishes belonging to all dietary groups under x400 magnification. Diameter of seminiferous tubules in 5 different randomly selected areas in fishes of all dietary groups were also determined under x200 magnification using Life Science Imaging Software System (Olympus Corporation, Tokyo, Japan).

SPSS 15.0 software (SPSS Inc., Chicago, IL, USA) was used for statistical analyses of the data. Variables were presented as mean±standard deviations and were analysed by one-way ANOVA and Duncan's multiple range tests were used to compare groups ($p<0.05$).

The growth performance of *M. estherae* broodstocks (male and female) fed with the control and test diets are shown in Table 2. The final weights, weight gain, feed conversion ratio (FCR), specific growth rate (SGR) and survival rate of fish fed with diets supplemented with FC and TT showed no significant differences from the fish fed the control diet ($p>0.05$).

Histopathological examination of the ovaries revealed normal vitelloegenic oocytes at different stages of development in control group. There was marked increase in the number of mature oocytes in FC and TT groups and interestingly, decreased number of mature oocytes compared to FC +TT group (Fig. 1, Table 3).

Examination of ovaries revealed five follicular stages. Stage I (immature) oocytes were irregular in shape without a zona radiata and were between 58 and 64 µm in diameter. Stage II, oocytes had an ellipsoid shape with a granular cytoplasm and were 160 to 182 µm in diameter throughout the long axis. In stage III, the oocytes had wavy margins with large cortical vacuoles and were 402 to 425 µm in diameter. In these oocytes, 2 to 4 µm thick zona radiata was observed. In stage IV, the oocytes were 755 to 827 µm in diameter, globular in shape and 7 to 9 µm thick zona radiata were noticed. At stage V (mature) oocyte diameter ranged between 830 and 875 µm, without nucleus/granules and the thickness of the zona radiata was less than that of the earlier development stages which ranged from 5 to 6 µm. Increase in mature oocyte number was observed in FC and TT groups compared to the control group. Marked increase in oocytes ($p<0.05$) in stage I was observed in FC +TT group.

In the testes of the sampled fishes, amount of primary spermatocytes, secondary spermatocytes, spermatids and spermatozoa were semiquantitatively analysed. In addition, sertoli cells were also evaluated for any possible damages. Each lobule of the testes contains numerous spermatogenic cells in all stages of spermatogenesis, which is an indication of healthy reproductive condition of testes. In FC and TT groups seminiferous lobules became larger and with much more spermatozoa compared to the control groups. In FC+TT group decreased spermatozoa but increased spermatids were seen in seminiferous

Table 2. Growth performance and survival rate of different dietary groups of *M. estherae*

Growth performance	Sex	C	FC	TT	FC+TT	df	F	P
Initial mean weight (g)	Male	4.98±0.01	4.72±0.15	4.94±0.21	5.11±0.03	3	0.17	0.91
	Female	3.36±0.23	3.69±0.20	3.63±0.14	3.27±0.12	3	1.31	0.29
Final mean weight (g)	Male	8.81±0.17	8.64±0.49	8.87±0.47	8.85±0.01	3	0.09	0.96
	Female	6.77±0.80	7.89±0.72	6.92±0.65	6.10±0.31	3	1.41	0.27
Weight gain (%)	Male	76.76±3.61	82.81±4.60	79.41±2.07	73.01±1.33	3	1.71	0.30
	Female	106.81±25.34	113.71±9.74	90.15±17.05	76.97±1.68	3	1.07	0.46
SGR (% day^{-1})	Male	0.63±0.02	0.67±0.03	0.65±0.01	0.61±0.01	3	1.73	0.30
	Female	0.80±0.14	0.84±0.05	0.71±0.10	0.63±0.01	3	1.10	0.45
FCR		8.07±0.88	7.22±0.83	8.54±0.28	8.45±0.70	3	0.71	0.59
Survival rate (%)		91.67±8.33	91.64±8.33	91.67±8.33	100±8.33	3	0.33	0.80

Specific growth rate (SGR) (% day^{-1}) = [(ln FBW – ln IBW)/T] *100
Weight gain (%) = [(FBW- IBW)/IBW]*100
Feed conversion ratio (FCR) = Feed intake/Weight gain
Survival rate (%) = Final fish number/Initial fish number)*100
T: time (days); FBW: Final body weight; IBW: Initial body weight

Table 3. Count of mature oocytes in ovary and in seminiferous tubules of the testes in different dietary groups of *M. estherae*

Groups	C	TT	FC	FC+TT	p value
Stage I	5.16 ± 0.98^{a}	4.83 ± 0.98^{a}	5.50 ± 1.37^{a}	7.16 ± 0.75^{b}	<0.01
Stage II	4.83 ± 0.98^{a}	5.16 ± 1.16^{ab}	6.83 ± 1.60^{c}	6.50 ± 1.04^{bc}	<0.05
Stage III	6.33 ± 1.21^{a}	7.66 ± 1.50^{a}	7.00 ± 2.28^{a}	7.16 ± 1.16^{a}	>0.05
Stage IV	3.16 ± 1.16^{a}	4.33 ± 0.51^{b}	5.66 ± 0.51^{c}	4.00 ± 0.89^{ab}	<0.001
Stage V	1.50 ± 0.54^{a}	2.50 ± 1.04^{ab}	4.00 ± 0.89^{c}	2.66 ± 0.81^{b}	<0.001
Tubule diameter (µm)	57.50 ± 4.23^{a}	59.16 ± 3.48^{a}	67.16 ± 1.94^{b}	55.66 ± 3.32^{a}	<0.001

*: The differences between the means of groups carrying different letters in the same row are statistically significant.
**: Values expressed as mean±standard deviation.

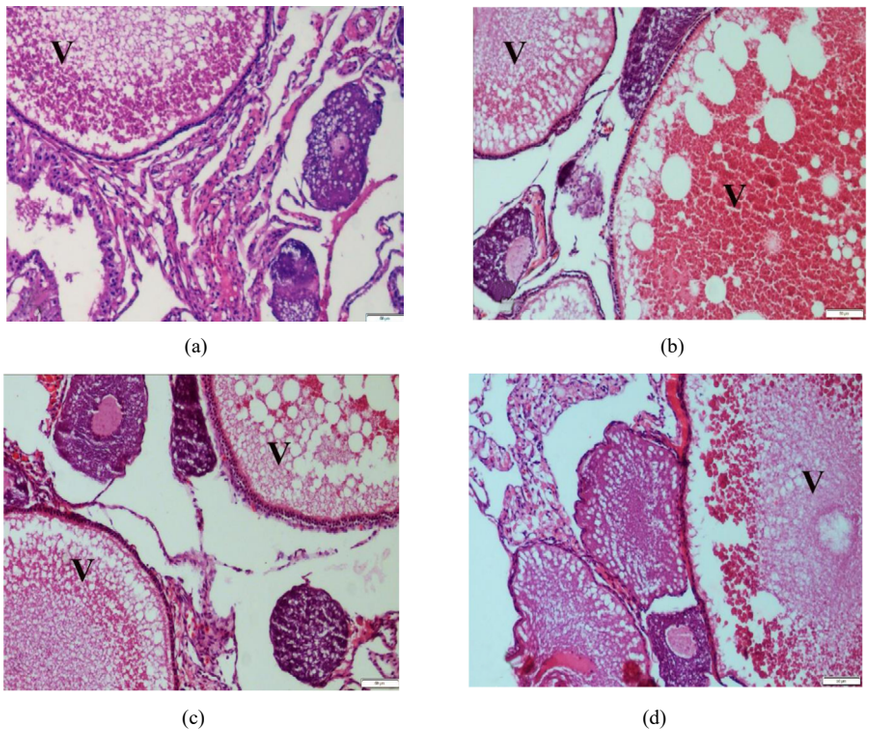


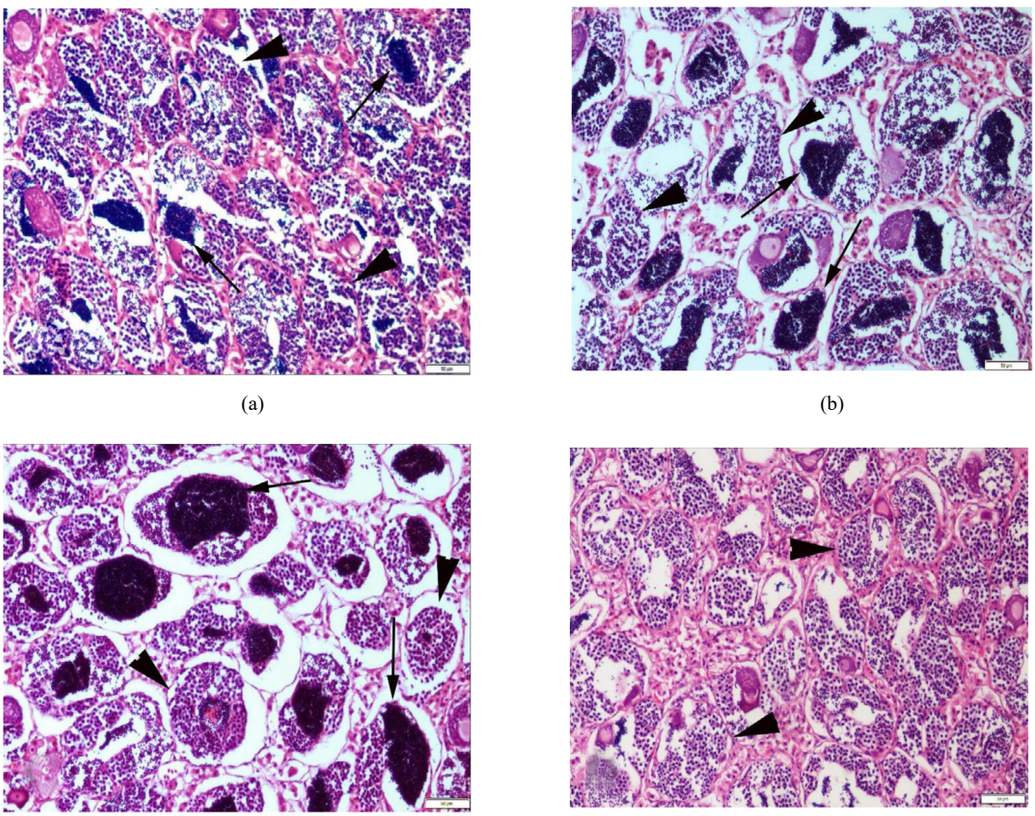
Fig. 1. Photomicrographs of histological sections of ovaries from different dietary groups of *M. estherae*. (a) Normal ovarian histology in control group, (b) Increased number of mature oocytes in TT group, (c) Increased numbers of mature oocytes in FC group, (d) Decreased numbers of mature oocytes in the ovarian parenchyma in FC+TT group (H&E), Bars = 50 µm. v: vitellogenic oocytes

tubules (Fig. 2, Table 3). Degenerative changes were not seen in any of the dietary group.

Histological examination of the gonads of different dietary groups indicated that FC and TT groups showed marked imporovement ($p<0.05$) in the reproductive organs, with FC being more effective than TT. However, combination of FC+TT did not cause significant change in the reproductive organs.

The results of the study showed that the final weights, weight gain, FCR, SGR and survival rate of fish fed with control diets and diets supplemented with FC and TT were similar in all groups. On the contrary, Omitoyin *et al.* (2013) reported that the best growth values were found in *O. niloticus* fed with 2-2.5% TT extract diets. Yılmaz *et al.* (2013) indicated that the best weight gains were obtained in male *Oncorhynchus mykiss* fed diets containing TT 100 mg kg^{-1} and in control group females. Yeganeh *et al.* (2017) reported highest weight gain in *C. nigrofasciatum* fed diets containing 1 g kg^{-1} TT extract. Turan and Cek (2007) observed best growth in *C. gariepinus* in 9 g per 30 l^{-1} TT extract to culture water. Cek *et al.* (2007a) determined better growth in *P. retuculata* in 0.15 g l^{-1} TT. Cek *et al.* (2007b) reported that fish treated 0.30 g l^{-1} TT exhibited successful growth. These variations may be due to different fish species, growing conditions of TT plant or extraction process of TT.

In the present study, histological evaluation of the gonads showed statistically significant difference in FC and TT treated fishes. Addition of FC and TT separately to diets caused significant ($p<0.05$) improvement in the count of mature oocytes in females (Table 3). In FC and TT groups, seminiferous lobules were found to have higher diameter with more spermatozoa compared to the control groups. But, combination of these plant extracts did not cause synergistic action in the reproductive organs. Cek *et al.* (2007a,b) observed an increased number of spermatogenic cysts and abundance of the late stages of spermatogenesis in *P. retuculata* and *C. nigrofasciatum* in all of the TT treatment groups, respectively. Similarly, in the present study, the testes contained preponderance of spermatozoa in the lobular lumen in TT group. On the contrary, Turan and Cek (2007) indicated that ovaries, and testes of *C. gariepinus* treated with TT extract (0, 3, 6, or 9 g 30 l^{-1}) were histologically similar to fish from the control groups. Yılmaz *et al.* (2013) determined that there were no marked differences in the gonad structures



(a) (b)

(c) (d)

Fig. 2. Histological sections of testes from different dietary groups of *M. estherae*. (a) Normal testicular histology in control group showing spermatids (arrow heads) and spermatozoa (arrows), (b) Increased number of spermatozoa (arrows) in seminiferous tubules in TT group, (c) Marked increase in spermatozoa (arrows) in seminiferous tubules in FC group, (d) Marked decrease in spermatids (arrow heads) in seminiferous tubules in FC+TT group (H&E), Bars = 50 µm

of rainbow trout between all TT (50 and 100 mg kg^{-1}) and control groups. Yeganeh *et al.* (2017) observed highest hatching rate in 1 g kg^{-1} TT extract group and the lowest hatching rate in group treated with 2 g kg^{-1} TT extract. Mode of treatment with TT extract (through water or diet) could be the reason for variation in the results. In addition, researchers have achieved best masculinisation results with TT extract such as, Janalizadeh *et al.* (2018) in *B. splendens* fed by Artemia enriched with TT extract (0.05 g l^{-1}); Ghosal *et al.* (2015) in *O. niloticus* fed with diet supplemented with 1.5 g kg^{-1} TT extract; Ghosal *et al.* (2016) 15.0 g kg^{-1} TT feed and 0.15 g l^{-1} aqueous extract immersion administrations in *O. niloticus* and Ghosal and Chakraborty (2020) in *O. niloticus* fed diet containing 2.0 g kg^{-1} TT extract.

The effect of *Ferula* plant extract has not been examined on the reproduction of fish and only its root in the powder form has been tested. Kassis *et al.* (2009) reported that 17 and 60% water-ethanol extracts of *Ferula asafoetida* added to diets of rats improved sperm counts quantitatively and qualitatively. Onal and Guzey (2020) indicated that supplementation of *F. communis* root to the diet of awassi sheep may enhance ovulation rate and luteal activity. Similarly, in the present study, seminiferous tubule diameter, spermatozoa and mature oocyte numbers increased in fish fed with FC incorporated diets compared to control group. On the contrary, Balcı and Aktop (2019) observed that there was no statistical difference between groups in terms of gonadal histology, growth and FCR of female gold fish fed diets supplemented with *F. elaeochytris* root powder (0.1, 0.5 and 1%). Yılmaz *et al.* (2006) reported that carp diets containing *F. coskunii* root powder (0.15, 0.30, 0.45%) did not affect gonadosomatic index values of male and female *C. carpio*, but negatively affected the growth and feed conversion. In addition, Filik (2009) determined that *F. eleaochytris* powder (0, 0.2, 0.4 and 0.8%) had no effect on egg production, egg mass and egg weight in Nick Brown chicken. Homady *et al.* (2002) indicated that intragastric application of *F. hormonis*

extracts of 3 mg kg^{-1} day^{-1} led to significant reduction in female mice fertility and decreased the number of mated females. The ova showed degeneration while most of the ovarian follicles suffered follicular atresia. Khleifat *et al.* (2001) reported that the numbers of epididymal sperm and motility were dramatically reduced in male mice fed 3 mg kg^{-1} of aqueous extract of *F. hormonis*.

Findings of the present study indicated that FC and TT extract may be added to the diets of fishes for improving reproductive performance. Further the results also showed that FC addition may be more effective for increased ovarian and testicular capacity.

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