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The effect of gokshura (*Tribulus terrestris)* and nettle root (*Urtica dioica*) extracts on growth rate and sex reversal in convict cichlid (*Cichlasoma nigrofasciatum)*

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Introduction

There are many differences between sex of fishes in many traits before and after puberty. Differences in growth rate, size, behavior patterns, and breeding time between males and females have great economic importance (Turan and Cek, 2007). Therefore, many breeders prefer to delay or prevent puberty or to produce unisex populations. Exogenous steroids can be effective in sex reversal of fishes (Al-ablani and Phelps, 2002). Two hormones, estradiol-17β (female-specific hormone) and 17α-methyltestosterone (male-specific hormone) are the most commonly used hormones for sex reversal (Stepherd and Bromage, 1995). Feminization (Liu *et al.*, 1996) and musculinization (Gale *et al.*, 1999) of catfish and Nile tilapia can be performed by direct synthetic hormonal treatment. Previous studies suggested that steroid hormones had disadvantages such as being costly and suppressing growth in teleosts (Hunter and Donaldson, 1983), producing sterile populations and occasional paradoxical feminization with prolonged duration at early stages of gonadal development in African catfish (Van Den Hurk *et al.*, 1989), channel catfish (Goudie *et al.*, 1983), rainbow trout (Solar *et al.*, 1984) and cichlid (Varadaraj *et al.*, 1994). On other hand, steroid hormones are more expensive than plant extracts, and their application in fish is time-consuming and labour-intensive and requires professional skills (Cek *et al.*, 2007a). Medical herb extracts are used as an alternative to steroid hormones for sex reversal in different species of fishes.

Gokshura *(Tribulus terrestris)* significantly caused masculinization in

African catfish (Turan and Cek, 2007), convict cichlid (Cek *et al.*, 2007a) and guppy (Cek *et al.*, 2007b). Gokshura could increase testosterone levels and improve athletic performance in humans (Adimoelja and Adaikan, 1997, Adimoelja, 2000; Bucci, 2000; Gauthaman *et al.*, 2002) and promote the libido and spermatogenesis in animals and humans (Gauthaman *et al.*, 2003). Gokshura contains a number of substances known as steroidal saponins. Protodioscin, the most dominant saponin in gokshura, is thought to be the main substance responsible for the enhancement of testosterone production (Ganzera *et al.*, 2001). Protodioscin has been reported to increase the levels of dehydroepiandrosterone (Adimoelja and Adaikan, 1997), dihydrotestosterone and dehydroepiandrosterone sulfate (Gauthaman *et al.*, 2000).

Nettle root (*Urtica dioica*) has a long history of use in folkloric and science based herbal medicine. Nettle root has substantial pharmacological effects such as an immune stimulator, anti-carcinogenic, anti-inflammatory, antioxidant and antiallergenic activities (Glusker and Rossi, 1986; Akbay *et al.*, 2003). The importance of nettle root lignans (such as 3,4-divanillyltetrahydrofuran) in benign prostatic hyperplasia (BPH) and other androgen- and estrogen-sensitive conditions may be due to interference with binding of sex hormone binding globulin (SHBG) to testosterone, the testosterone receptor, and/or the SHBG receptor (Hryb *et al.*, 1995; Schottner *et al.*, 1997).

Convict cichlid (*C. nigrofasciatum*) is a medium-sized cichlid from Central America (Axelrod *et al.*, 1971). Convict cichlid was used as a model fish in the current research because of its broad range of resistance to water hardness, pH and temperature. Convict cichlid is also efficiently attainable and can be proliferated cheaply (Wisenden, 1994; Fraser, 1996). It produces large numbers of viable eggs (100–800 per batch) under optimal conditions. The development period from fertilization to hatching is about 3 days at 27˚C (Cek *et al.*, 2007a).

The objectives of this study were to investigate the possible effects of gokshura (*T. terrestris)* and nettle root on sex reversal and growth rate in convict cichlid as an alternative technique.

Materials and methods

Laboratory conditions and experimental design

Six pairs of convict cichlid with a mean weight (±standard deviation) of 8.3 (±0.4) g and mean length of (± standard deviation) 7.3 (±0.3) cm were procured from an ornamental fish dealer (Langrood City, Guilan). Parent pairs were kept in an aquarium containing recirculation water (26±1˚C) and exposed to a 12:12 (light:dark) photoperiod. Once a female spawned in a mussel shell, the eggs hatched within approximately 3 days. The newly hatched larvae were immediately taken out of the hatching tank, counted, measured and transferred to 15 small glass aquaria, each containing 10L water that were regularly aerated with a

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4-cm air stone. A total of 525 fry were randomly assigned to five different treatments with three replicates each of which received one of five immersion doses of goskhura and nettle root tested: 0 (T1), 200 mgL^{-1} nettle root (T2), 300 mgL^{-1} nettle root (T3), 10 mgL^{-1} gokshura (T4) and 200 mgL^{-1} nettle root plus 10 mgL^{-1} gokshura. The larvae were fed three times a day with commercial flake food supplemented with freshly hatched artemia and tubifex throughout the experimental period of 2 months. Hatchlings were subjected to immersion in water containing gokshura and nettle root extracts from the age of day one to 8 weeks. The aquarium system was immobile, and bathing medium was changed 2 times in a week with the same concentration of gokshura and nettle root in each treatment.

Plants extraction

250 grams of whole washed and shade dried gokshura whole plant and nettle root (3 times) were powdered and extracted with 500 mL 70% ethanol (Hussain *et al.*, 2009).

Measurement of parameters

At the end of the experiment, all fish (60 days old) were anesthetized with 2-phenoxethanol (0.04%) and stored at -20ºC for assessing whole body weights (BW) and lengths (BL). Then growth parameters including specific growth rate (SGR) (Clark *et al.*, 1990), daily growth rate (DGR), percent of body weight gain (BWG%) (Ergun *et al.*, 2003), condition factor (CF) ((Weight/(length)^3)*100) and survival rate (SR) were calculated. Secondary sexual characteristics were used to distinguish males from females. Also, the structure of the testis and ovaries were observed by naked eye and examined under a light microscope (Olympus BX 50) at the end of the experiment.

Statistical analysis

The Chi Square test was used to test whether the observed sex ratios were significantly different from the expected sex ratios. Differences among groups were evaluated by one-way analysis of variance (ANOVA) using the SAS software (SAS 9.2). Duncan testing was performed for intergroup comparisons; $p<0.05$ was considered statistically significant.

Results and discussion

The aim of the present study was to evaluate the effect of gokshura and nettle root on the sex reversal and growth rate in convict cichlid. The effects of different immersion concentrations of plant extracts on sex reversal of convict cichlid for 60 days are presented in Table 1. The observed sex ratio in the control treatment (T1) was not significantly different. Nettle root treated fish (T2 and T3) yielded 100 % female fish, while in the gokshura treatment (T4), there were no statistically significant differences between male and female counts ($p>0.05$). We achieved significantly higher (68.76%) masculinization by immersing 0-day-old fry for 60 days in water containing gokshura and nettle root (T5) ($p<0.05$).

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Table 1: Effect of gokshura and nettle root treatment on sex reversal in convict cichlid.

Treatments	Sex ratio (%)		Sex distributions		χ^2 (*p*-value)
	Female	Male	Female	Male	
Control (T1)	46.65	53.85	30	35	NS
T2	100	0	87	0	***
T3	100	0	76	0	***
T4	39.73	60.27	29	44	NS
T5	37.14	62.86	26	44	*

NS: not significant (p>0.05), *: significant (p<0.05), ***: significant (p<0.001)

The results indicated that gushura (10 mgL^{-1}) plus nettle root (200 mgL^{-1}) were significantly effective in masculinization of convict cichlid. Masculinization of fish which received 10 mg L^{-1} gokshura was non-significantly effective. Previous studies showed the positive effects of gokshura (100, 200 and 300 mgL^{-1}) on masculinization of convict cichlid (Cek *et al.*, 2007a) and African catfish (Turan and Cek, 2007). In contrast, 50 and 100 mgL^{-1} of gokshura had no effect on sex reversal in *Poeciliata reticulata* (Cek *et al.*, 2007b). Fish studies suggested that higher doses may have generated better results on masculinization; we cannot support this conclusion from our results. In the current study, first we used the same amount of aqueous or/and alcoholic gokshura extract (200, and 300 mgL^{-1}). However after a few turns around (about 3 minutes), the treated larvae died out. Therefore, we examined another amount (90, 80 to 10 mgL^{-1}) of gokshura extract and all levels, except 10 mgL^{-1} led to the death of treated larvae. Finally, our results showed that gokshura is toxic to convict cichlid in doses higher than 10 mgL^{-1}. Studies in human (Adimoelja, 2000; Bucci, 2000) and rat (Gauthaman *et al.*, 2002; Gauthaman *et al.*, 2003) demonstrated that gokshura treatments strongly affected androgen metabolism, and significantly increased testosterone or testosterone precursor levels. Kavitha and Subramanian (2011) showed that the activities of testicular functional enzyme levels were increased in gokshura treated groups compared with that of the control in *Poecilia latipinna* fishes. A high rate of masculinization was observed in our study (in T4 and T5) but whether this potency is caused by increases in androgens or testosterone cannot be figured out, because we did not measure the plasma testosterone level during the experiment.

The effects of different immersion concentrations of gokshura and nettle root on growth indices of convict cichlid for 60 days are presented in Table 2. Mean of BW, BL, SGR, DGR, BWG% and CF for the 60-day experimental period differed significantly among treatments (p<0.05). BW and BL decreased in immersion treatments compared to control fish. In T5 treatment, the lowest BW, BL, SGR, DGR and BWG% were seen. CF index was substantially higher

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in T3 and T5 treatments than in other treatments. Higher doses of nettle root (T3) led to higher BW, BL, SGR, DGR, BWG%, CF parameters compared to lower doses (T2). SR in the extract-treated groups did not significantly differ from the control indicating that Gokshura and nettle root had no negative effect on survival rate.

Table 2: Effect of gokshura and nettle root treatment on growth parameters in convict cichlid.

	T1	T2	T3	T4	T5
BW (g)	2.19±0.05^{a}	0.83±0.021^{c}	1.42±0.02^{b}	1.73±0.02^{ab}	0.66±0.01^{c}
BL (cm)	4.61±0.05^{a}	2.84±0.01^{c}	2.87±0.03^{c}	3.97±0.02^{b}	1.99±0.01^{d}
DGR	0.017±0.00^{a}	0.006±0.00^{c}	0.011±0.00^{b}	0.014±0.00^{b}	0.005±0.00^{c}
BWG (%)	98.60±0.05^{a}	96.22±0.08^{b}	97.83±0.04^{a}	98.23±0.02^{a}	95.31±0.091^{c}
SGR	3.55±0.02^{a}	2.75±0.01^{c}	3.20±0.01^{b}	3.37±0.01^{ab}	2.56±0.01^{c}
CF	2.18±0.103^{c}	3.69±0.10^{c}	6.22±0.21^{b}	2.79±0.05^{c}	8.64±0.29^{a}
SR (%)	81. 9±2.51	82.85±9.89	82.85±5.94	89.52±7.43	87.62±10.73

The values with different letters are significantly different (p<0.05).

Significant decreases in body weight parameters (BW, BL, SGR, DGR and BWG) were seen in all extract treated fish compared to control fish in the current study. Contrarily, Cek *et al.* (2007a, 2007b), Kavitha and Subramanian (2011) and Yılmaz *et al.* (2013) found that the growth rate of fish treated with gokshura was faster than that of the controls in *Cichlasoma nigrofasciatum*, *P. reticulata*, *P. latipinna* and *Oncorhynchus mykiss*. On the other hand, Turan and Cek (2007) suggested that gokshura had no substantial effect on growth rates in *Clarias gariepinus*. Similar to our results, survival ratios in the gokshura-treated fish were similar to that in controls (Cek *et al.*, 2000a, 2007b; Turan and Cek, 2007; Yılmaz *et al.*, 2013).

Disagreement of the results of the current study with previous mentioned studies can be attributed to extract type and related compounds. In these studies, the industrial gokshura extract was used, whereas in our study, whole plants were purchased from local markets and then gokshura extract was prepared.

The current study was the first attempt yielding complete feminization of convict cichlid immersed in Nettle root (200 and 300 mgL^{-1}) compared to other experimental treatments. Growth rate parameters (BW, BL, SGR, DGR and BWG) had decreased in fish receiving nettle root in comparison with the control group, but survival rates were not significantly different among treatments as nettle root had no effect on survival rates. There are no published data for evaluating the effect of nettle root on sex reversal or growth rate in fish species. However the results of a study in rats showed that the body weight was reduced intraperitoneally in rats that received 300 $mgkg^{-1}day^{-1}$ nettle root compared to the control group and the mean number of spermatogonia per tubular tubules, leydig cells mm^{-2} in diameter tubules (µm) were decreased in the experiment group treated with 300 $mgkg^{-1}day^{-1}$ Nettle root (Pourahmadi *et al.*, 2014). The effect of nettle root on the body

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weight is due to ingredients such as flavonoids that induce lipid hydrolysis by competitive blockage of phosphodiesterase, and also by inhibition of 3-hydroxy-glutaril CoA that is the key to cholesterol biosynthesis in the liver that can reduce body weight or flavonoids that regulate the energy metabolism and body weight by binding to the site where ATP binds to enzymes and receptors (Kandaswmi, 1999).

In another report, Pourahmadi *et al.* (2013) showed that the nettle root extract led to a significant increase in LH, whereas the effect on FSH was variable and non-significant. We did not asses the effect of nettle root on gonadotropines or sex hormones in convict cichlid. Nettle extract contains linoleic acid, which can decrease cholesterol. It may reduce testosterone by lowering cholesterol. Diminishing testosterone levels, increases LH and FSH. LH affects the leydig cells and leads to an increase in their number and activity (Morovvati *et al.*, 2013). Nettle root contains compounds such as sterols, flavonoids and polysaccharides that have antiandrogen effects and deal with the testosterone function (Akbay *et al.*, 2003; Ganzera *et al.*, 2005). Nettle root extract has at least 18 types of sterols and 8 types of lignan (Kraus and Spiteller, 1990). It prevents the formation of the active form of testosterone, dihydrotestosterone, by inhibition of the enzyme 5-alpha reductase (Koch, 2001; Nahata and Dixit, 2012). Nettle root extract inhibits aromatase, therefore, it prevents the conversion of testosterone to estrogen, and also prevents androgen binding to androgen receptors (Chrubasik *et al.*, 2007). These descriptions may be possible causes of the feminization of convict cichlid in our study.

In conclusion, the results of the current study do not support the enhancement effect of gokshura on masculinization and the growth rate of convict cichlid. On the other hand, our results add a support to the effect of nettle root on the feminization property. Future studies subjected at measuring the amount of sexual hormone levels after nettle root treatment in convict cichlid supply more deterministic evidence about the effects of nettle root on the sex ratio and whether it can be successfully used as an agent in fish culture. Further investigations are necessary to determine the effects of nettle root on other cultivable fish species.

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