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Effect of Red Clover Extract on Sex Reversal and Gonadal Development in the African Catfish, *Clarias gariepinus* (Burchell, 1822)

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Authors' Contribution
FT, SC-Y and IA presented the concept of the study, designed methodology and administered the project. SC-Y and FT analysed the data. FT wrote the manuscript.

Key words
African catfish, *Clarias gariepinus*, Red clover, Sex reversal, *Trifolium pretense*

ABSTRACT

Monosex populations are in demand in many finfish species with sexual dimorphism, e.g., better growth performance, higher economic value. Synthetic steroids are commonly used to induce monosex population in fish but because of the potential hazards of such steroids; the use of new phytochemicals is a potential alternative to be explored. We evaluated hypothesis that red clover extract to sexually undifferentiated fry of African catfish (*Clarias gariepinus*) would affect their sex ratio. Five different concentrations (0, 25, 50 and 75 mg L^{-1}) of red clover extracts and 50 µg L^{-1} 17 β-Estradiol serving as positive control were applied by immersion method on *C. gariepinus* larvae for 30 days, and the effects of red clover extract on sex reversal and gonadal development were also examined at the end of 120 days. At the end of experiment, highest feminization (89%) was observed at 50 mg L^{-1} red clover group. Morphological and histological examinations of the gonads in all groups revealed no intersex fish. Histological examination of fish treated with red clover extract revealed no damage to the testes or ovaries. Therefore, this study suggested that red clover extract are effective in feminization of African catfish.

INTRODUCTION

African catfish cultured in various regions in the world is an important aquaculture species. Nigeria followed by the Netherlands, Brazil, Hungary, Kenya, Syrian Arab Republic, South African, Cameroon, and Mali are top African catfish culturing countries (FAO, 2016). The officially reported total production of African catfish is 246.476 tons in 2015 (FAO, 2016). There is globally an increasing trend and spread in African catfish culture as the total aquaculture production also increases (FAO, 2017). Male African catfish, *Clarias gariepinus*, grow faster and reach a larger final size than females. They have better feed conversion, protein efficiency ratios, apparent net protein utilization, lower fat and energy deposition, and higher gutted weight than females (Beardmore *et al*., 2001; Turan and Akyurt, 2005). Production of all male African catfish population might be achieved in two ways: (i) direct method: masculinization and (ii) indirect method: sex-reversal with estrogens. Sexually undifferentiated African catfish are applied with estrogens in order to obtain heterogametic (XY) neo-females. The neo-females are crossed with normal males, and then super-males (YY) are crossed with normal females (XX) in the next step and generation (Piferrer, 2001). The direct synthetic hormonal treatment can be used to produce feminized African catfish which is efficient and straightforward way (Liu *et al*., 1996). On the other hand, the synthetic steroids usage generally caused to different ecological and health-related hazards, and therefore the use of natural compounds should be studied as a potential alternative (Papoulias *et al*., 2000). Plant extracts comprising alkaloids, flavonoids, pigments, phenolics, terpenoids, steroids and essential oils have been reported to promote various activities such as growth promotion, antistress, immunostimulation and antimicrobial properties in aquaculture (Chakraborty *et al*., 2011). Phytochemicals may occur as having effects similar to estrogens although it may not be structurally gonadal estradiol-17β, these groups are called phytoestrogens (Lotke, 1998). Phytoestrogens have been used to production monosex culture in fish (Çek *et al*., 2007; Turan and Çek, 2007; Enyidi and Nduh-Nduh, 2016; Adenigba *et al*., 2017; Turan, 2017; Mukherjee *et al*., 2018). On the other hand, the effects of such plant extracts should be clearly investigated though significant variations regarding the efficacy of different phytochemicals for production of monosex population are exist.

Isoflavones derived from soy and soy derivatives

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are the most common phytoestrogens of which genistein and daidzein are the most abundant and well-studied. This group of phytoestrogens can be found in red clover. Red clover *Trifolium pratense* L. is a botanical dietary supplements and there are important studies which report their potential safe use in the treatment of menopausal symptoms, improvement and maintenance of cardiovascular health (Kawakita *et al*., 2009). The estrogenic activity present in red clover is mainly due to isoflavones (formononetin, biochanin A, genistein, and daidzein) and coumestans (Vishali *et al*., 2011). Isoflavones and phytoestrogens mimic estrogen, and their estrogenic effects in biological systems are believed to be related to their structural similarity to estrogen (Shakeri *et al*., 2015; Kang *et al*., 2015). Most of the established uses of red clover extracts are based on their estrogenic activity, which has been extensively documented in several in vitro and in vivo models (Liu *et al*., 2001).

Considering these aspects, the objective of the present study was to investigate the potential effects of red clover extract on sex reversal and gonadal development of African catfish, *Clarias gariepinus*.

MATERIAL AND METHODS

Experimental fish

The larvae of African catfish were obtained by artificial dry fertilization of catfish brooders. The fertilized eggs were incubated at 25±1ºC, in a rectangular plastic tanks connected to a flow through system. The incubators were subjected to 12-h light: 12-h dark period. Light intensity was obtained with black nylon screening of hatchery tanks. Photoperiod was maintained at 12-h light and 12-h dark. The water flow rate was 0.6 l per h. The eggs hatched after 36 h. Density of stocking in catfish was 100 larvae per each of the three replicate aquariums.

Experimental procedures and conditions

Red clover powder (Menoflavon®) was obtained from Melbrosin (Vienna, Austria). The aqueous extracts of red clover were prepared by boiling fine powder of red clover in 1500 ml distilled water for 30 min, filtered with a Whatman filter paper twice (Gauthaman and Adaikan, 2005) and added to the aquaria. The catfish larvae were exposed to the solution for three days, and then every 3 days of 30 days. The water in the aquaria was changed entirely. The red clover extract solution was freshly prepared every 3 days. The larvae were exposed to red clover for a total experimental period of 120 days. The newly hatched African catfish larvae (averaging 2.8±0.03 mg) were randomly removed from the hatching tank, and placed in 12 glass aquaria, each containing 30 L of water 12-h light: 12-h and 25±1ºC. Each aquarium contained 100 larvae and a total 1200 larvae were used for the experiment. Five different treatments were done in 3 replicates in a completely randomized design for 30 days. T1 served as negative control containing no red clover extract; T2, T3 and T4 contained 25, 50, 75 mg of red clover extract L^{-1}. T5 contained 50 µg L^{-1} 17 β-Estradiol, serving as positive control. The larvae were fed five times a day with freshly hatched *Artemia salina* (Subreme Bay Brand INC., San Francisco, USA) and live tubifex for the first five days after yolk absorption then weaned to commercial trout starter (58% crude protein, CP) and pelleted diets (45%, CP) (Akuamaks, Turkey).

Sampling procedure and statistical analysis

At the end of the experiment, all fish (120 days old) were anaesthetized with 5 mg L^{-1} quinaldine sulphate (Sigma Chemical Company, Germany) (Yanar and Genç, 2004). Gonads in male and female catfish can clearly be distinguished by naked eye and examined under light microscopy (Olympus BX 50). In addition, secondary sexual characteristics (especially genital papilla) were used to distinguish males from females. Then, 50 randomly sampled fish from each group were weighed, measured and sexed again by removing the gonad. The gonads of catfish were classified such as testes or ovaries. The extracted gonads were weighed using a digital scale (±0.01) to calculate the gonadosomatic index (Sturm, 1978). For histological examination, the gonads were fixed in 10% neutral formalin, processed by routine dehydration and paraffin embedding procedures. Cross-sections (4-6 µ thick) were stained with Mayer's hematoxylin and eosin phloxine B solution. Ovaryum was classified by developmental stage based on histological criteria adapted from Grier (1981).

Statistical analysis

Chi-square ($\chi 2$) test was used to determine whether the observed sex ratios differed from an expected 1:1. Differences in growth were assessed by oneway ANOVA. The Duncan test was used to analyze significant effect of different doses (Norusis, 1993).

RESULTS

The proportion of male and female catfish in the untreated groups was 43% and 57%, respectively (Table I). In contrast, red clover extract groups resulted in a significantly higher proportion of sex reversal (79 to 89%) than those in the control group ($P<0.05$); the proportion did not significantly vary between the pine pollen treated groups ($P>0.05$). At the end of experiment, highest

feminization (89%) was observed at 50 mg L^{-1} red clover group. Positive control containing the sex hormones 17 β-Estradiol exhibited a significantly higher proportion of females (96%) compared to the other treatment groups ($P<0.05$) (Table I).

Final survival rate was significantly lower ($p < 0.05$) in the positive control (17 β-Estradiol) group than red clover extract and negative control groups. But, survival rate in the red clover extract-treated groups did not differ from the negative control. The results on the survival rate of the catfish are recorded in Table I.

At the end of the experiment, there were highly significant overall differences between groups at gonadosomatic index values for males and females ($P<0.05$). The highest gonadosomatic index values for males and females were detected from 75 mg L^{-1} red clover group as 0.021% and 0.130%, respectively. Although gonadosomatic index values of females treated with different levels of red clover extract were similar, there was significant difference between gonadosomatic index values of males treated with different levels of red clover extract ($P<0.05$) (Table II).

Morphological and histological examination of the gonads revealed no intersex fish and histological examination of the extract treated testes and ovaries revealed no damage to the organ structure. The histology of the definitely male testes in catfish treated with the red clover extract showed normal testicular development. The tubules contained spermatids, with no indication of primary oocytes. In the ovaries of female catfish there were only oocytes in different developmental stages (Fig. 1). Despite differences in sex ratio, ovaries, and testes of fish treated with the red clover extract were histologically similar to fish from the control groups.

DISCUSSION

Sex reversal in African catfish can be obtained with direct synthetic hormonal treatment which is straightforward and effective (Liu *et al*., 1996) though is being increasingly criticized. There is increasing interest in decreasing the usage of synthetic hormones for sex reversal

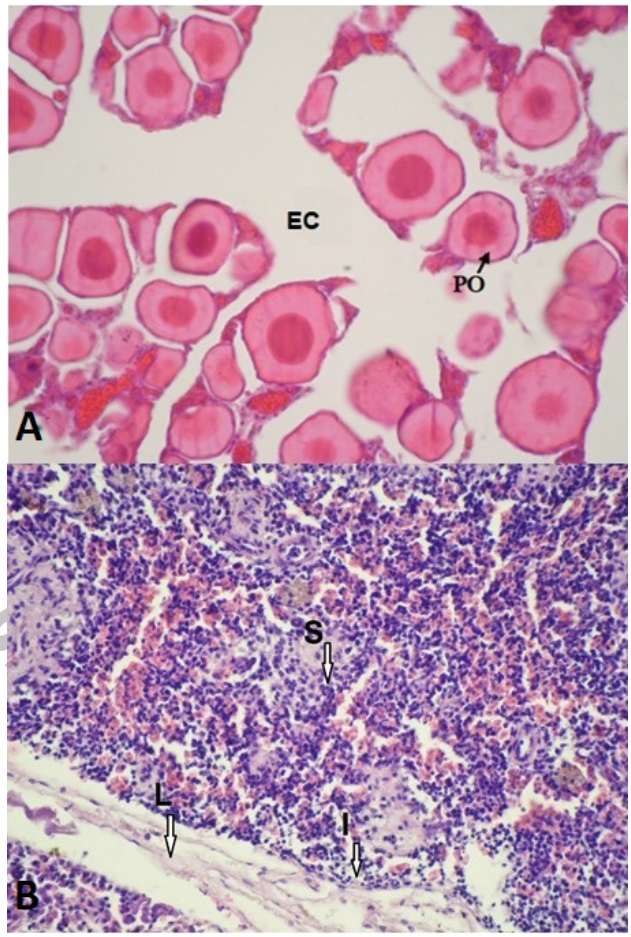

Fig. 1. Histological structure of ovary (A) and tests (B) of *Clarias gariepinus*. S, spermatids; L, Lumen; I, Interstitial tissue; PO, Primary oocytes; EC, Endoovarian canal) Stain: H & E. Magnification: A, 10X; B 40X scale bar: A, 50 µm; B, 100 µm.

Table I. Effects of red clover extract on the sex ratio of African catfish (*Clarias gariepinus*).

Phytoestrogen treatment	Dose (mg L^{-1})	Survival rate (%)	Sex reversal (%) ♀ : ♂	χ^2
T1	0	79.00±3.00^{ab}	57 : 43	1.960
T2	25	76.67±3.28^{ab}	79 : 21	33.640***
T3	50	82.00±1.73^{b}	89 : 11	60.840***
T4	75	81.33.±3.28^{b}	85 : 15	49.000***
Positive control (17 β-Estradiol)	Dose (µg L^{-1})			
T5	50	69.33±3.18^{b}	96 : 4	84.640***

Table II. Effect of different concentrations of red clover extract on body weight and gonadosomatic index of the African catfish, *Clarias gariepinus* for 120 days*.

Treatment	Dose (mg L^{-1})	Treatment time (days)	Mean body weight (g)	Mean gonad weight (mg)	GSI (%)
Female					
Phytoestrogen treatment					
T1	0	2-32	7.95 ± 0.36^{a}	8.32 ± 0.73^{ab}	0.104 ± 0.004^{a}
T2	25		9.52 ±0.8^{a}	9.19 ± 0.59^{bc}	0.097 ±0.002^{a}
T3	50		7.59 ±0.86^{a}	7.72 ± 0.39^{ab}	0.103 ±0.006^{a}
T4	75		9.44 ±1.21^{a}	10.36 ±0.30^{c}	0.130 ±0.027^{a}
Positive control (17β-Estradiol)	Dose (µg L^{-1})				
T5	50		7.90 ± 0.72^{a}	7.47 ± 0.27^{a}	0.095 ± 0.005^{a}
Male					
Phytoestrogen treatment					
T1	0	2-32	8.30 ± 1.13^{a}	1.22 ± 0.39^{a}	0.014 ± 0.002^{a}
T2	25		8.52 ± 0.51^{a}	1.44 ± 0.31^{a}	0.016 ± 0.002^{ab}
T3	50		9.24 ± 0.62^{a}	1.16 ± 0.05^{a}	0.012 ±0.001^{a}
T4	75		8.30 ± 0.73^{a}	1.83 ± 0.25^{a}	0.021 ±0.001^{b}
Positive control (17 β-Estradiol)	Dose (µg L-1)				
T5	50		8.79 ±1.53^{a}	1.56 ± 0.28^{a}	0.017 ± 0.001^{ab}

* Values (mean ± standard error of triplicate) superscripted by different alphabets within the same column are significantly different ($P< 0.05$). Statistical test (Duncan) was done separately for male and female groups. Initial live mean length and weight of the larvae were 5.98±0.10 mm and 2.80±0.03 mg, respectively. GSI, gonadosomatic index = gonad weight/body weight x 100.

treatments. The accumulation of hormones in wild fish populations has urged the researchers to produce environmentally friendly fish, using alternate techniques such as phytochemicals (Contreras-Sanchez *et al*., 2001; Müller and Hörstgen, 2007; Nguyen *et al*., 2007; Leet *et al*., 2011). Production of super males (YY) is another alternative technique (Mair *et al*., 1997). The present study provides environmentally friendly and more effective feminization method for the African catfish.

Recently, red clover botanical dietary supplements have received significant attention for their potential use in the treatment of menopausal symptoms (Kang *et al*., 2015). The estrogenic activity of red clover is mainly due to isoflavones, which are known to improve metabolic symptoms in menopause. Isoflavonoids exert a weak estrogen-like effect by binding to ER-α and -β in various tissues (Cornwell *et al*., 2004). Moreover, the flavonoids interact with estrogen receptors and control the activity of CYP19, and/or steroid dehydrogenases. These effects induce various alterations causing a change in the overall hormonal balance, resulting in protection against other menopausal symptoms (Hodek *et al*., 2002). Genistein, a well-characterized isoflavone, is an important secondary plant metabolite that has been reported to be present in red clover (Liu *et al*., 2001), and there are numerous reports of genistein exerting oestrogenic effects in fish. Moreover, the functional group of phytoestrogens are reported to have a variety of hormonal and gametic effects in a range of fish species (Chakraborty *et al*., 2013).

Since in the study the highest feminization (89%) was observed at 50 mg L^{-1} red clover group, further experiments with increased concentration might be required to achieve 100% sex reversal with red clover. We cannot conclude that this potency was caused by an increase in estradiol level since no levels were measured in plasma. Therefore, future studies should measure estrogen levels after red clover treatment to provide more conclusive evidence of the effects of this herb on the sex ratio. Most of the literature on red clover treatments in humans (Kang *et al*., 2015) and rats (Cornwell *et al*., 2004) demonstrate that it strongly affects estrogen metabolism, significantly increasing estradiol levels. Ko *et al*. (1999) used genistein on the growth and reproductive performance of yellow perch, *Perca flavescens* that genistein enriched diets on the endocrine process of gametogenesis and reproduction efficiency. Bennetau-Pelissero *et al*. (2001) reported increased plasma vitellogenin concentrations in male and female fish.

Immersion of newly hatched *C. gariepinus* larvae in a commercial mixture of phytoestrogens at a concentration

of 1500 mg mixture 30 L^{-1} water every 3 days for 30 days produced ~70% females, while lower concentrations (210, 420, 630 and 750 mg mixture 30 L^{-1} water) had no effect on the sex ratio (Yılmaz *et al*., 2009). Also, Turan and Akyurt (2005) explained results from an experiment evaluating the effects of a red clover, *Trifolium pratense*, on growth and body composition of *C. gariepinus*. They said that the presence of phytoestrogen in red clover extract may enhance nutrient utilization and stimulate growth hormones leading to an increase in the body growth of fish. To the best of our knowledge, this is the first report regarding the potential of red clover as feminization agent in African catfish.

The GSI is an indicator of sexual maturity of the fish and consequently of their health and nutritional status. Recent studies have shown that based on an observed reduction of GSI as well as morphological and histological changes undergone by the gonads, continuous exposure to synthetic compounds, including hormones, can induce a decrease in gonadal development (Marchand *et al*., 2008; Louiz *et al*., 2009). Similarly, Marín-Ramírez *et al*. (2016) reported that gonadal examination of the tilapia fed with diethylstilbestrol (synthetic hormone) at different concentrations in comparison with the control group showed a significant reduction in the values of GSI and an increase in the proportion of alterations of gonadal structure. Song *et al*. (2014) reported negative effects in the GSI and serious atrophy of the gonads in several treatments in goldfish (*Carassius auratus*) using individual and binary mixtures of synthetic estrogens.

In our experiment, GSI values of females treated with different levels of red clover extract were similar, there was significant difference between GSI values of males treated with different levels of red clover extract. The negative effect of red clover extract (phytoestrogens) in the GSI observed in our experiment couldn't have been the result of a direct effect on tissue development and gonadal structure. Also, gonadal development was normal in all fish analyzed, indicating that the dosage used in the present study was physiologically adequate. These results with GSI showed that red clover treatment is a better and safer/harmless method than synthetic hormonal treatments in African catfish. Similarly, Kang *et al*. (2015) reported that red clover (*Trifolium pratense* L.) botanical dietary supplements have received significant attention for their potential safe use in the treatment of menopausal symptoms in human. Most literature on red clover treatments in humans (Shakeri *et al*., 2015) and rats (Cornwell *et al*., 2004; Kawakita *et al*., 2009) demonstrate that it strongly affects estrogen metabolism and can be used safely.

There was no significant difference in survival rate and mean body weight between red clover extract groups and control group. These results for the treated fish indicated no adverse effects of treatment with red clover extract on the general health and growth of the fish. In our previous experiment as well, oral administration of red clover extract was found to have no adverse effect on survival in African catfish (Turan and Akyurt, 2005). Furthermore, Turan and Akyurt (2005) explained significantly improved growth for African catfish treated the 75 mg red clover kg^{-1} diet. Similar to results obtained by Adenigba *et al*. (2017) and Envidi and Nduh-Nduh (2016) in African catfish, Mukherjee *et al*. (2018) in Tilapia and Turan and Yigitarslan (201p) in rainbow trout.

CONCLUSION

Red clover significantly enhanced sex reversal in African catfish that can successfully be used as a feminization agent in fish culture. However, there are some gaps to be filled that the knowledge of the functional mechanism behind the physiological activity, determination of an ideal treatment dose and duration regime for this extract to obtain the maximum benefit require future research. Moreover, research on bioavailability and detailed metabolic processes associated with the potential sex-reversing phytochemicals need to be expanded.

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Ethical approval

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed by the authors.

Statement of conflict of interest

The authors have declared no conflict of interest.

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