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EVALUATION OF AQUEOUS SEED EXTRACT OF *Tribulus terrestris* AS ANDROGENIC AGENT FOR PRODUCTION OF ALL MALE RED-BELLY TILAPIA, *Coptodon zillii* (GERVAIS 1848)

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Abstract
The aim of this study was to evaluate the effect of *Tribulus terrestris* aqueous seed extract on sex reversal in *Coptodonzillii*. Three days old fry of *C. zillii* (mean weight 0.03 ± 0.0g) from the Federal University of Technology Akure fish farm, were randomly selected and assigned to 15 glass tanks (70cm x 45cm x 45cm) of 20 litres water capacity to five different treatments with three replicates, with each treatment representing varying concentrations levels of ASET. The graded levels of aqueous seed extract of *T. terrestris* were (0.00, *0.10, 0.15, 0.20 and 0.25 g/l for* each treatment group denoted as T1, T2, T3, T4 and T5 respectively. Each glass tank was stocked with 15 fry and a total of 225 fry was used for the experiment. The fry were immersed in the ASET for three days, and then every 3 days of 30 days, the water in all glass tank was changed entirely. The *T. terrestris* extract solution was freshly prepared 10 times every 3 days along the immersion period. The fry was fed finely ground artificial diet containing 35% crude protein three times daily between 08:00 and 09:00 and 12:00 and 01:00 and 16:00 and 17: 00h GMT to apparent satiation. The highest survival percentage (84.7%) was observed in control group, but there was no significant difference (P>0.05) in survival percentage among the different treatment groups. Immersion treatment with ASET caused significant increase (P<0.05) in percentage of males compared to that in untreated control. The highest percentage of males (80.2) was observed in 0.25 g/l group and it was significantly higher (P<0.05) than all other treatment categories. The extract showed presence of phytochemicals such as tannins, saponin, alkaloids, steroids and terpenoid which might be associated with its androgenic property.

Keywords: *Sex reversal, Tribulus terrestris, Coptodon zillii*

Introduction
In order to reduce the pressure on the wild stocks of fish, aquaculture is a viable alternative to fisheries throughout the world (Marra, 2005). Manipulation of phenotypic sex in fish farming is generally desirable since one gender, depending on the fish species, grows faster than the other (Uguz *et al.,* 2003). Tilapia fish is generally accepted and eaten by many, but most aquaculturists especially in Nigeria, are discouraged to culture tilapia because of their high fecundity rate, overpopulate ponds (Tidwell, 2012), they mature sexually at 20 - 30g which results in over spawning and reduced growth rate (Shahjahan, *et al.,* 2015). For this reason all male population of tilapia are desirable to female because of their fast growth. The use of 17α-methyltestosterone is by far the most common practice for many aquaculturist in view of the fact that it is efficient and relatively a cheap means of sex reversed fry of at least 95% for various tilapia specie (Phelps and Popma, 2000; El-Sayed, 2006). However, Synthetic hormones are more expensive and not readily available in Nigeria. Also, experienced level of expertise is required for handling the hormone when compared to that of plant materials. The alternatives that can be considered to reduce the use of synthetic steroid hormone for sex reversal of tilapia is the use of *T. terrestris* which are available from local markets in Nigeria. Plant sources with potential sex reversing mechanism and growth-promoting effects could be used to produce all male tilapia population since they have not been subjected to licensing for use in food animals. *T. terrestris* contains a number of substances known as steroidal saponins (Dhas*et al.,* 2015). Saponin in *T. terrestris* thought to be responsible for its effect on testosterone levels is known as protodioscin (Ganzera *et al.,* 2001). Oral administration of *T. terrestris* increased male populations of African Catfish (*Clarias gariepinus)* Turan and Çek (2007). Considering these qualities, the objective of this study is to evaluate the effect of aqueous seed extract of *T. terrestris* seed powder in production of all male of *C. zillii*.

Materials and Methods
Experimental Fish
Three days old mixed sex fry of C. zillii with mean weight 0.03 ± 0.0 g was collected from the Fish Hatchery of The Federal University of Technology Akure. Fry were transferred into glass tanks of dimension (70cm x 45cm x 45cm) and acclimatized for 24 hrs without feeding before the experiment.

Preparation of Aqueous Extract of *Tribulus terrestris* Seed

T. terrestris seeds were procured from a local plant market at Bode Ibadan, Oyo State, Nigeria. It was identified and authenticated at the Department of Crop, Soil and Pest Management, The Federal University of Technology, Akure. The seeds were removed from the nutlets, washed in distilled water and dried at room temperature to prevent loss of volatile compounds. *T. terrestris* seeds were ground into fine powder using an electric blender (Model ES 242) and stored at 4°C in a container until used. The extraction was done according to the modified method of (Kavumpurath and Pandian, 1993; Gauthaman and Adaikan, 2005) as follows: The aqueous seed extract of *T. terrestris*(ASET) was prepared by boiling 250 g of dry powdered seeds in 1500 ml distilled water for 30 minutes cooled and then filtered using What man No. 1 filter paper twice. The filtrate were collected in a 2000 ml beaker and concentrated with the aid of rotary evaporator (Resona, Germany) with rotor speed of 50 rpm at 45°C.The extract was dried at room temperature for 24 hours and then stored at 4°C in a well labelled airtight bottles prior to use in immersion treatment. Chemical tests were carried out on the ASET for the qualitative determination of phytochemical constituents as described by Malpani*et al.,* (2011); Kumar and Bhardwaj, (2012); Ray *et al.,* (2013).

Immersion Treatment of Fish with Plant Extracts

C. zillii from the above described stock were randomly selected and assigned to 15 glass tanks (70cm x 45cm x 45cm) of 20 litres water capacity to five different treatments with three replicates, with each treatment representing varying concentrations of aqueous seed extract of *T. terrestris*. The graded levels of aqueous seed extractof *T. terrestris* were (0.0, 0.10, 0.15, 0.20, and 0.25 g/l for each treatment group denoted as T1, T2, T3, T4 and T5. Each glass tank was stocked with 15 fry and a total of 225 fry of *C. zillii* was used for the experiment. The fry were immersed in the aqueous seed extract of *T. terrestris (*ASET) for three days, and then every 3 days of 30 days, the water in all glass tank was changed entirely. The *T. terrestris* extract solution was freshly prepared 10 times every 3 days along the immersion period. The immersion treatment in all fry groups were stopped and reared at the same condition for 90 days in a total experimental period of 120 days. The fry was fed finely ground artificial diet containing 35% crude protein three times daily between 08:00 and 09:00 and 12:00 and 01:00 and 16:00 and 17: 00h GMT to apparent satiation.

Weighing of Experimental Fish

Fish were weighed between 06:00 and 08:00h GMT in plastic bowls containing water using an electronic weighing balance (Model PB3002). The weight of water and plastic bowls were first taken without the fish, recorded as initial weight and then the fish were introduced and the new weight taken and recorded as final weight. The mean weights of fish per tank were recorded every two weeks until the experiment was terminated.

Quality Parameters

Water quality parameters (temperature, dissolved oxygen and pH) were determined twice a week. Temperature was measured using mercury in glass thermometer while pH was measured with a pH meter (Jenway model 9060). Dissolved oxygen (DO) was measured using dissolved oxygen test kit (Hanna model: HI-9142).

Growth Performance and Nutrient Utilization

Calculation of the growth performance data was according to Takeuchi (1988) and Tacon (1990). At the end of the experiment, fish were counted and weighed. The growth parameters and feed utilization indices were calculated as follows:

Weight gain (g) = Final weight – Initial weight

Specific growth rate

This was calculated from data on changes of body weight over given time intervals;

$$\text{SGR (\% per day)} = \frac{\text{Ln final weight - Ln initial weight}}{\text{days}} \times 100$$

$$\textbf{Feed intake (g)} = \frac{\text{Total feed intake}}{\text{Number of fish survived}}$$

Feed conversion ratio = Feed intake/Weight gain

$$\textbf{Survival (\%)} = \frac{\text{Number of fish that survived}}{\text{Number of fish stocked}} \times 100$$

Sexing of Experimental Fish

Six fish specimens from each group were dissected in fresh condition and gonad were removed from the body cavity. Sexing of the juvenile fish was done by the standard acetocarmine squash technique of gonadsas validated for *O. niloticus* by Wassermann and Afonso (2002). The thin gonad (thread-like structure lying along the dorsal side of the abdominal cavity) was extracted very carefully, placed in a formalin-solution made of equal volumes of 10% formalin and 0.9% sodium chloride solution for 24 hours. The gonads were placed on a clean glass slide and stained with a drop of aceto-carmine, then it was lightly squashed with a glass cover slip and examined under a microscope (Model CX40RF200) at 100 x magnification.

Statistical Analysis

The experiments was conducted in triplicate (n=3) and one-way analysis of variance (ANOVA) was used to compare the mean values of each treatment as described by Steel and Torrie (1980). Significant differences in mean values at the levels of ($P<0.05$) were established by using Duncan's New Multiple Range Test (Duncan, 1955). All statistical analyses were performed with the aid of the computer software SPSS (Statistical Package for Social Science Version 20). The optimum concentration of ASET required by the fish for best performance was determined by polynomial regression analysis using (Microsoft Office Excel Programme, 2010).

Results and Discussion

Phytochemical screening of the aqueous extract of *T. terrestris* seed revealed the presence of tannins, saponins, steroids, Terpinoid and alkaloids in ASET, while flavonoids, glycosides and carbohydrates are not present in the extract. These phyto constituents might be responsible for the masculinisation effect of the extract. A variety of pathways have been postulated to be associated with functional mechanisms of phyto-compounds causing both masculinisation and feminization at different concentrations (Chakraborty *et al.*, 2014).

The water quality parameters measured during the feeding trial for 60 days varied as follows; temperature; 26.8 - 27.3°C, dissolved oxygen; 6.82 -7.14 mg/l and hydrogen ion concentration; 6.93 to 7.08 respectively. These recorded water quality parameters of the experimental set up compared favourably with those reported by (Popma and Masser 1999; Phelps and Popma, 2000).

Table 1 :Phyto-Chemical composition of aqueous seed extract of *T. terrestris*

Phyto-Chemical constituents	ASET
Tannin	+
Saponin	+
Glycosides	-
Alkaloid	+
Carbohydrate	-
Flavonoid	-
Steroid	+
Terpenoid	+

Key: + = Present, - = Absent

Data on growth performance and survival rate of *C. zillii* is presented in Table 2a and 2b. There was no significant difference ($p>0.05$) in the initial weight of the fish at the beginning of the experiment. Total survival in all treatments and control were uniformly high ranging from 84.7 to 80.5% ($P>0.05$). However, survival of fish in all the groups was not 100%. All group of ASET treated fish exhibited accelerated growth compared to the control group, but only ASET treatment at the concentration of 0.15 g/l significantly improved growth rate of *C. zillii* ($P<0.05$), 4th degree polynomial regression analysis (Figure 1) revealed the optimum level as (0.14 g/l). The high survival of fish in different treatments indicates that immersion treatment with *T. terrestris* extract have no adverse effect on the experimental fish. This report is in conformity with the work of (Ghosal and Chakraborty 2014) where no significant difference was observed in survival of *O. niloticus* during immersion treatment with *B. alba*leaf aqueous extract. All group of *Tt* treated fish exhibited increase in growth compared with the control group, but only *T. terrestris* extract at the concentration of 0.15 g/l significantly improved growth ($P<0.05$).

Table 2a:Effect of *T. terrestris* on growth and survival of *C. zillii* at varying concentrations of ASE T for30 days

Parameters	ASET1	ASET2	ASET3	ASET4	ASET5
IW	0.03±0.00^{a}	0.03±0.00^{a}	0.03±0.00^{a}	0.03±0.00^{a}	0.03±0.00^{a}
FW	0.21±0.01^{a}	0.30±0.02^{c}	0.35±0.01^{d}	0.27±0.02^{bc}	0.26±0.01^{b}
WG	0.18±0.01^{a}	0.27±0.02^{c}	0.32±0.01^{d}	0.24±0.01^{bc}	0.23±0.01^{b}
SGR(%/day)	6.49±0.53^{a}	7.68±0.43^{b}	8.19±0.25^{c}	7.32±0.51^{b}	7.20±0.26^{b}
FI	0.31±0.01^{a}	0.38±0.04^{bc}	0.44±0.02^{c}	0.36±0.05^{bc}	0.35±0.02^{b}
FCR	1.72±0.16^{c}	1.41±0.16^{ab}	1.38±0.03^{a}	1.50±0.02^{b}	1.52±0.11^{b}
Survival (%)	84.7±6.00^{a}	81.7±4.30^{a}	78.4±5.00^{a}	80.7±3.67^{a}	80.5.0±6.00^{a}

Mean in the same row with different letter are significantly different at P<0.05
Key: IW=Initial weight, FW=Final weight, WG=Weight gain, SGR=Specific growth rate(%/day), FI=Feed intake, FCR=Feed conversion ratio

Table 2b:Effect of *T. terrestris* on growth and survival of *C. zillii* at various concentration level of ASET for 60 days

Parameters	ASET1	ASET2	ASET3	ASET4	ASET5
IW	0.21±0.01^{a}	0.30±0.00^{c}	0.35±0.01^{d}	0.27±0.00^{b}	0.26±0.01^{b}
FW	1.27±0.01^{a}	1.48±0.03^{bc}	1.53±0.03^{c}	1.42±0.0^{b}	1.38±0.02^{ab}
WG	1.06±0.01^{a}	1.17±0.02^{bc}	1.18±0.02^{c}	1.15±0.06^{b}	1.12±0.04^{ab}
SGR(%/day)	3.04±0.11^{c}	2.64±0.02^{ab}	2.45±0.02^{a}	2.78±0.11^{bc}	2.80±0.12^{bc}
FI	1.33±0.04^{a}	1.40±0.00^{bc}	1.41±0.01^{bc}	1.39±0.00^{b}	1.37±0.02^{ab}
FCR	1.25±0.04^{b}	1.20±0.02^{a}	1.19±0.02^{a}	1.21±0.06^{ab}	1.22±0.02^{ab}
Survival (%)	84.7±6.00^{a}	81.7±4.30^{a}	78.4±5.00^{a}	80.7±3.67^{a}	80.5±6.00^{a}

Mean in the same row with different letter are significantly different at P<0.05
Key: IW=Initial weight, FW=Final weight, WG=Weight gain, SGR=Specific growth rate(%/day), FI=Feed intake, FCR=Feed conversion ratio

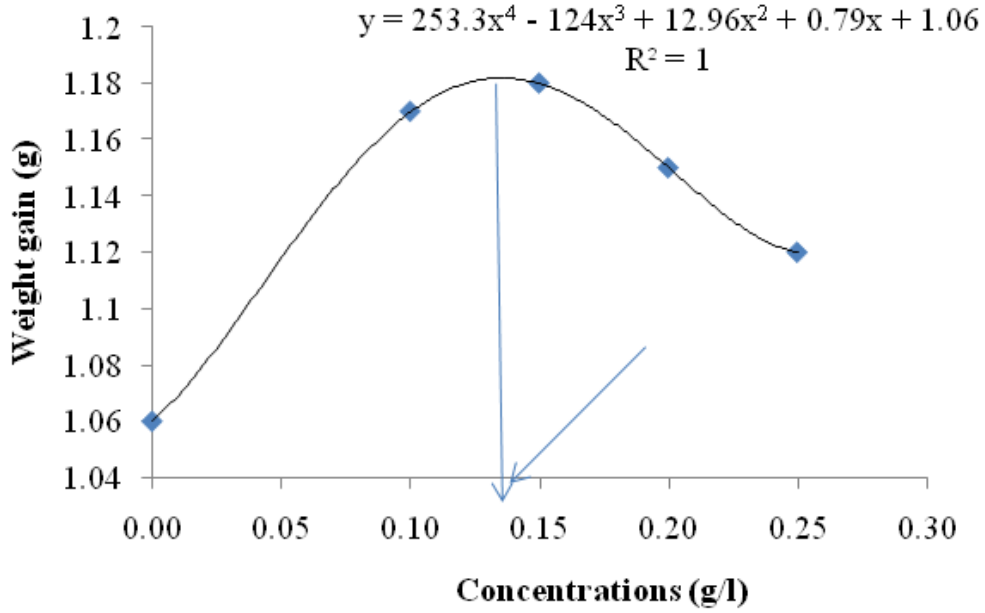

Figure 1: Fourth order polynomial regression analysis showed the relationship between the aqueous seeds extract of *T. terrestris* and male percentage of *C. zillii*

All the treatment categories showed significantly higher (P<0.05) percentage of males than with the control (Table 3). The maximum percentage of males (80.2%) was observed at the concentration of 0.25 g/l, which was significantly higher (P<0.05) than the males produced in other treatments. The percentage of females in the 0.25 g/l group was also significantly lower (P<0.05) than to the other groups. All the treatment categories except control groups which do not immersed in ASET showed intersex in both male and female gonadal tissue. The highest percentage of intersex fish was observed in 0.10 g/l treatment group (11.7%), which was significantly higher (P<0.05) than intersex percentage in other treatments. The population of male obtained in this present study may be due to the age of the fry. It is an established fact that fries at lower age respond better than at advanced age (Popma and Green, 1990). It is more likely that *T. terrestris* was absorbed by yolk which was in term simulated by the fry since the sole nutrition of the fry is dependent on the yolk. This result agrees with that of Ana *et al.* (2011) which reported that yolk sac fry exposed for a longer period to hormone feed led to higher number of male conversion with very few intersex observed in *O. niloticus*. This current work revealed the presence of intersex fish (11.7%) that was associated with low concentration of *Tt* used in immersion. This is in agreement with the work of Nakamura *et al.* (1998) in which they reported that addition of steroid hormone at lower dose was unable to perfectly form male sex, leading to intersex tilapias. The percentage of intersex recorded in this study was lower than that reported by Putra (2011) that immersion of 8-days tilapia (at a dose of 20 mg/l for 8 hrs) using *Pimpinella alpina* extract resulted in intersex of 13.3%. Ghosal and Chakraborty (2014) reported higher intersex fish of 7.2% as consequence of *Tt* immersion at 0.05 g/l in 3 days old *O. niloticus*. The percentage of male population (80.2%) that emanated from this study was higher than that reported by Ghosal and Chakraborty (2014) who obtained 81.4% male population in *O. niloticus* by immersing 3-days-old fry for 60 days in 0.15 g/l of *Tt* extract. Percentage of male population (80.2%) that emanated from this study was lower than that reported by Kavitha and Subramanian (2011) who obtained 97% male population in *Poecilia latipinna* by immersing 0-day-old fry for 60 days in water containing 50 ppm *Tt* extracted in 70% ethanol. This report disagreed with that of Omitoyin*et al.* (2013) who obtained 84% male population in *O.niloticus treated with* commercial feed containing *Tt* extract at a concentration of 2.5 g/kg.

Table 3: Effect of *Tt* on the sex ratio of *C. zillii* during immersion period

Treatment	Male (%)	Female (%)	Intersex (%)
Control (0.00 g/l)	44.5±4.73^{a}	55.5±4.73^{d}	00.0±00.0^{a}
0.10 g/l	63.9.±2.27^{b}	24.4±2.47^{c}	11.7±0.20^{d}
0.15 g /l	75.4±2.10^{bc}	15.1±2.77^{b}	9.5±0.74^{c}
0.20 g /l	78.3±3.52^{c}	14.2±4.16^{b}	7.5±0.65^{bc}
0.25 g /l	80.2±2.14^{d}	12.8±2.06^{a}	7.0±0.10^{b}

Mean in the same row with different letter are significantly different at P<0.05

Conclusion

In this study, the weight gain, specific growth rate, feed intake and feed conversion ratio improved progressively up to the inclusion level of 0.15 g/l of ASET and then declined indicating that 0.15 g/l of ASET is the optimum level that can promote the growth performance of the fish. It could be concluded that 0.14–0.15 g/100kg of ASET leave can be included in the diets of *C. zillii* fingerlings for optimum growth performance. The highest treatment concentration of 0.25 g/l produced the highest percentage of males (80.2%) among the different treatment categories. The highest percentage of males produced by the plant material was found to a little below the expected 100% male population. Thus, further studies would be required to establish an ideal treatment regime for production of the all-male tilapia population using the plant material and to provide conclusive evidence regarding the efficacy to be used as a sex-reversal agent in tilapia culture. Therefore, the use of ASET can be a natural alternative to synthetics hormones which is cheap and readily available in the country and also be of great relevance to organic tilapia production. Investigation can also be carried out to find the effects of *T. terrestris* on sex reversal of other culturable fish species.

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