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Potentials of *Azadirachta indica* leaves in masculinisation, growth enhancement and survival of *Oreochromis niloticus* fry

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Abstract
The present study was conducted to evaluate the potentials of *Azadirachta indica* leaves (AIL) in the masculinisation of *Oreochromis niloticus* fry of undifferentiated sex. Fresh plant leaves of *A. indica* were cut, washed, shaded dried and grinded to powder. 100 g of the powdered leaves was soaked in 500 ml ethanol for 24 hours with constant shaking. Five dietary concentrations of AIL in three replicates at 0.0g/kg (TR1), 0.5g/kg (TR2), 1.0g/kg (TR3), 1.5g/kg (TR4) and 2.0g/kg (TR5) were weighed, mixed (with Nutra0 Skretting Tilapia feed) and fed to one thousand five hundred *O niloticus* fry stocked at a density of 100 fry/m^2 for the first 30 days. Feeding after 30 days was with feed containing no AIL until their sex were determined macroscopically through their genital papillae. Results shows that *Azadirachta indica* leaves feed significantly induced masculinisation in *Oreochromis niloticus* fry as increase in the concentration level of *Azadirachta indica* leaves from TR1 to TR5 resulted in significant higher male percentage (72.97%) and survival rate (93.67%) in TR4 against the lowest male percentage (43.13%) and survival rate (90.33%) in TR1. TR4 and TR5 gave the best weight gain (115g and 11,7g), specific growth rate (4.77g and 4.79g), daily weight gains (0.56g and 0.57g) feed conversion ratio (0.53 and 0.57) and condition factor (2.13 and 2.17) respectively. This study suggested that *Azadirachta indica* leaves contain phytochemicals that were beneficial in the masculinisation of *Oreochromis niloticus* fry.

Keywords: Masculinisation, growth rate, survival, *Oreochromis niloticus*, fry, phytochemical, *Azadirachta indica*

Introduction
Monosex tilapia culture that constitutes males only can be employed to control reproductive activity, inbreeding, unhealthy competition for resources vital for growth, stunted growth and increase production because males grow faster than females (Phelps and Popma, 2000) [1]. Polyculture with Predatory species, sterilization, heat shock and hormonal sex reversal are applied in reducing prolific reproduction in Tilapia aquaculture (Fortes, 2005) [2]. However, masculinized *O. niloticus* are currently cultured because they grow faster and larger compared to the female stock hence leading to shortened culture cycle and better production output (Megbowon and Mojekwu, 2014) [3]. The most efficient method of producing all-male stock is the use of hormones feed (Guerrero and Guerrero, 1988) [4] fed to newly hatched fry with unknown sex (Ajiboye, *et al*., 2015) [5]. Oral administration of 17 Methyltestosterone to newly hatched tilapia fry (3–12days old) for 28 consecutive days results in populations composed of >90% males (David, *et al*., 2013) [6]. The use of synthetic drugs in aquaculture appears to be only profit-driven and unsustainable, as they cause several other constraints such as carcinogenicity, adverse effects on human (Jegede 2010) [7], fish pathogen drug resistance, immunosuppression, environmental pollution, and accumulation of chemical residues, which can be potentially harmful to public health (Bulfon, *et al*., 2013; WHO, 2006) [8, 9]. Plant extracts containing diverse bioactive compounds such as alkaloids, flavonoids, pigments, phenolics, terpenoids, steroids and essential oils which have been reported to promote various activities like antistress, growth promotion, appetite stimulation, immunostimulation, masculinisation and antimicrobial properties in cultured fish (Citarasu, 2010; Chakraborty and Hancz, 2011) [10, 11]. Medicinal herbal extracts are easily available, not expensive and more biodegradable in nature compared to synthetic drugs (Olusola, *et al*., 2013; Reverter, *et al*., 2014) [12, 13]. Some plants used for this purpose include *Carica papaya* seeds (Kareem, *et al*., 2016; Yadav, *et al*., 2021; Christopher, *et al*., 2021) [14, 15, 16], *Azadirachta indica* leaves (Saadony, *et al*., 2020) [17], *Moringa oleifera* (Gad, *et al*., 2019) [18], *Mangifera indica* (Obaroh and Nzeh, 2013) [19], *Tribulus terrestris* (Turan and Cek, 2007) [20] and *Lepidium meyenii* tuber meal (Lee, *et al*., 2004) [21]. Among the different medicinal plants, *A indica* had been reported to be promising in controlling prolific breeding of *O. niloticus* juvenile (Obaroh and Achionye-Nzeh, 2011, 2012, Obaroh, *et al*., 2014; Obaroh, *et al*., 2015; Kapinga, *et al*., 2018a) [22, 23, 24, 25, 26] but with little report on its effect on newly hatched fry. Hence, this study was aimed to investigate potentials of *Azadirachta indica* leaves feed in masculinisation, growth enhancement and survival of *Oreochromis niloticus* fry.

Materials and Methods
Phytochemical analysis of *A. indica* leaves
Phytochemical screening of ethanol solvent extract of the plant leaves was carried out to determine qualitative and quantitative analysis of alkaloids, tannins, flavonoids, saponins, glycoside, phytate, steroids and phenols present in the extract according to the method described by Airaodion, *et al*. (2019) [27].

Preparation of Experimental Diet

Fresh plant leaves of *A. indica* was obtained from Ejide farms, Abeokuta and authenticated in the herbarium section of the Department of Pure and Applied Botany, Federal University of Agriculture, Abeokuta, Ogun State. Preparation of neem leaves were done adopting the procedure describe by Obaroh and Achionye-Nzeh (2011). The plant leaves were thoroughly washed and then shade dried for two weeks in a ventilated room (Kapinga, *et al.*, 2018a) [26] before grinding with an electric blender (Model Number MC-B142). 100 g of ground dried leaves was soaked in 500 ml ethanol for 24 hours with constant shaking at intervals as described by Musa, *et al*. (2000) [28]. The dietary concentration levels of *A. indica* extract (TR1= 0.0g/kg, TR2 = 0.5g/kg, TR3 = 1.0g/kg, TR4 = 1.5g/kg and TR5 = 2.0g/kg) were weighed, mixed separately with 1 kg of feed (Nutra 0, Skretting Tilapia feed, containing 48% crude protein, 6% crude fat, 4.39% crude fibre, 10% moisture and 7.5% Ash) and fed experimental fish for 30 days.

Experimental Fish

One thousand five hundred newly hatched *O. niloticus* post yolk sac fry of undifferentiated sex was enumerated, stocked in fifteen treatment hapa nets of 1 × 1m at a density of 100 fry/m^2 (Bhujel, 2013) [29] and fed with the experimental diet for the first 30 days. However, feeding after 30 days was with feed containing no *A. indica* extract until their sex can be determined macroscopically through their genital papillae. The masculinisation efficiency was determined by observing the gonads of the experimental fish macroscopically (Jean-François and D'Cotta, 2019; Hussain, 2004) [30, 31]. Growth parameters of the experimental fish like specific growth rate, mean weight gain, daily weight gains, survival rate (%), feed conversion ratio and condition factor were calculated using the formulae expressed by Heidarieh, *et al*. (2012); Gabriel, *et al*. (2017) [32, 33]. Water quality parameters during the culture period were monitored for temperature, dissolved oxygen, pH and total ammonia. pH and total ammonia were monitored using Screen water test that is conforms to AWWA method while temperature and dissolved oxygen were monitored using YSI Professional ODO Water Quality Instrument Model No. 17C102135.

Statistical Analysis

Descriptive analysis was used to calculate the mean and standard error, inferential statistics using One-way Analysis of Variance (ANOVA) to test for significant ($p < 0.05$) difference among the treatments and Duncan multiple range test (DMRT) separate significant means. A binary logistic regression analysis was performed to determine the effect of AI feed in masculinising newly hatched *O. niloticus* fry on the sex ratio (1 = male; 0 = female) and Chi – square test to ascertain the level of deviation from expected 1:1 (M: F) ratio. Analysis was done using SPSS version 20 statistical package.

Results

The results of the phytochemical analysis of *Azadirachta indica* leaves to determine qualitative analysis indicating presence or absence of the phytochemical and quantitative analysis show the present of alkaloid, tannin, flavonoid, phytate, glycoside, saponin, steroid and phenol at different concentration. High concentration was recorded for flavonoid (18.326) and saponin (18.003) while tannin (13.572) and alkaloid (5.157 % g^{-1}) had a moderate concentration. Steroid (0.344) and phenol (0.031 % g^{-1}) recorded the lowest concentration among the phytochemicals analysed. Based on the logistic regression results, *A. indica* leaves significantly induced masculinisation in *Oreochromis niloticus* fry (Table 1). Increase in the concentration level of *A. indica* leaves from TR1 to TR5 resulted in a significantly higher percentage of male. The highest male percentage of 72.97 and 72.53 % were recorded in TR4 and TR5 respectively. TR3 and TR2 has a male percentage of 62.34 and 52.38 % respectively against the lowest value of 43.13 % in TR1. Based on the chi-square analysis, TR1 had a sex ratio of 0.80:1 which exhibit a significant deviation from expected 1:1 sex ratio of male to female recorded in TR2 (1.10:1). However, a significant deviation from expected 1:1 sex ratio was noticed in TR3, TR4 and TR5 at P values < 0.05. However, negative logistic regression coefficients were recorded for TR1 (-1.30), TR2 (-0.87), TR3 (-0.44) while positive coefficients were recorded for TR4 (0.004) and TR5 (0.96) which indicate that the male percentage increases as *Azadirachta indica* leaves concentration increases (higher concentration are associated with higher possibility of event occurring). Growth performance results for weight gain, specific growth rate, percentage weight gain, daily weight gain, feed conversion ratio and condition factor were presented in Table 2. The result of weight gain showed a significant difference among the treatment groups. The value of weight gain increased from TR1 to TR5. The weight gain was found highest in TR5 (117.66±3.67) which is not significantly different from what were recorded in TR3 (114.99±4.04) and TR4 (115.99±6.66). Specific growth rate showed a significant difference among the treatment groups with increased values from TR1 to TR5. The specific growth rates were found highest in TR5 (4.79±0.03) which is not significantly different from what were recorded in TR3 (4.77±0.03) and TR4 (4.77±0.06). The percentage weight gain values for TR1, TR2, TR3, TR4 and TR5 are 6800±57.73%, 8900±1154.70%, 11400±401.15%, 11500±665.83% and 11666.67±366.67% respectively. The lowest significant value was observed in TR1 and the highest value in TR5 while TR3, TR4 and TR5 showed no significant difference in their percentage weight gain values. There are significant differences in the values of the daily weight gains in the treatments. Calculated daily weight gain values for TR1, TR2, TR3, TR4 and TR5 are 0.33, 0.43, 0.55, 0.56 and 0.57 g respectively. TR1 (0.33g) had the lowest daily weight gain followed by TR2 (0.43g). The daily weight gain was found highest in TR5 (0.57g) which is not significantly different ($P > 0.05$) from what was recorded in TR3 (0.55) and TR4 (0.56 g). Food conversion ratio (FCR) value showed significant different among the treatment. No significant difference in the value recorded in TR3 (0.57±0.03), TR4 (0.53±0.03) and TR5 (2.17±0.03). The values recorded for TR1 (0.90±0.00) and TR2 (0.73±0.09) are significantly different from each other. The significant highest and lowest values were recorded in TR5 and TR1 respectively. The condition factor among the treatments varied significantly. The highest significant values were recorded in TR4 (2.13±0.03) and TR5 (2.17±0.03) which are not significantly different from each other but significantly different from TR1. No significant

difference between the condition factor values of TR2 (1.90±0.06) and TR3 (2.00±0.00). TR1 had the lowest value of 1.43±0.03. Mean ± Standard error of Water quality parameters during the culture period were 25.7± 0.11°C, 4.6± 0.50 mg/l, 8.0± 0.12, 0.03±0.10mg/l for water temperature, dissolve oxygen, pH and total ammonia respectively.

Table 1: Logistic regression model and survival rate of newly hatched *O. niloticus* fry fed varying concentration levels of *A. indica* leaves

	TR1	TR2	TR3	TR4	TR5
Initial number stocked	300	300	300	300	300
Number of fish examined	271	268	273	281	266
Survival rate	90.33±4.91 [a]	89.33±4.67 [a]	91.00±2.65 [a]	93.67±0.88 [a]	88.67±1.33 [a]
Male percentage (%)	43.13±0.27 [d]	52.38±1.45 [c]	62.34±1.14 [b]	72.97±0.71 [a]	72.53±2.08 [a]
Sex ratio (M: F)	0.80:1	1.10:1	1.67:1	2.70:1	2.67:1
P value	0.006	0.464	0.000	0.000	0.000
b + SE[a]	-1.297±0.185	-0.872±0.184	-0.441±0.186	0.004±0.191	0.962±0.138
Exponentiation of *b*	0.273	0.418	0.643	1.004	2.616
Wald[b]	49.315	22.447	5.605	0.001	48.858

[a]Logistic regression model in response to male occurrence: log(p/1-p) = – 1.29 – 1.29· (*A. indica* 0.0%) – 0.87 (*A. indica*, 0.5%) – 0.44· (*A. indica*, 1.0%) + 0.004· (*A. indica*, 1.5%) + 0.96· (*A. indica*, 2.0%), where p is the probability of male occurrence. [b]Wald values are Chi-square values (χ^2 = 57.339, df = 1) from binomial logistic regression analysis. Chi-square values with P < 0.05 indicated significant deviation from the expected sex ratio of 1:1. Male percentage and survival rate data are expressed as mean ± SE; within a given row. Lowercase letters indicate significant differences (P < 0.05) within a given row.

Table 2: The effect of *A. indica* leaves feed on the growth performance of *O. niloticus*

Parameter	P value	TR1	TR2	TR3	TR4	TR5
Mean initial weight (g)		0.01±0.00	0.01±0.00	0.01±0.00	0.01±0.00	0.01±0.00
Mean final weight (g)	0.001	69.00±0.58[c]	90.00±11.55[b]	115.00±4.04[a]	116.00±6.66[a]	117.67±3.67[a]
Weight gain (g)	0.001	68.99±0.58[c]	89.99±11.55[b]	114.99±4.04[a]	115.99±6.66[a]	117.66±3.67[a]
Specific Growth Rate (% per day)	0.001	4.26±0.01[c]	4.51±0.13[b]	4.77±0.03[a]	4.77±0.06[a]	4.79±0.03[a]
Percentage Weight Gain (%)	0.001	6800±57.73[c]	8900±1154.70[b]	11400±401.15[a]	11500±665.83[a]	11666.67±366.67[a]
Daily Weight Gain (g)	0.001	0.33±0.00[c]	0.43±0.06[b]	0.55±0.02[a]	0.56±0.03[a]	0.57±0.02[a]
Feed Conversion Ratio	0.001	0.90±0.00[a]	0.73±0.09[b]	0.57±0.03[c]	0.53±0.03[c]	0.57±0.03[c]
Condition factor	0.000	1.43±0.03[c]	1.90±0.06[b]	2.00±0.00[b]	2.13±0.03[a]	2.17±0.03[a]

[a b c] Means ± SE (Standard error) with different superscripts across the same row are significantly different at p < 0.01. TR1; 0.0g/kg, TR2; 0.5g/kg, TR3; 1.0g/kg, TR4; 1.5g/kg, TR5; 2.0g/kg dietary concentration levels of *A. indica* leaf extract

Discussions

The results of the phytochemicals were in agreement with Nwali, *et al*. (2018) [34] who studied comparative screening of leaf, stem, bark and root of *A. indica* in ethanol and aqueous extracts and reported the presence of alkaloids, flavonoids, saponins, tannins, phenols and glycosides in all plant parts studied. Likewise, the results of the phytochemical analysis from this study were similar to what was documented on phytochemical of methanol leaves extract of *Azadirachta indica* by Madaki, *et al*. (2016) [35] and reported the presence terpenes, cardiac glycosides, flavonoids, alkaloids and saponin, steriods, tannins, phenols and reducing sugar. Kapinga, *et al*. (2018a) [26] reported that dietary *Aspilia mossambicensis* and *Azadirachta indica* supplementation alter gonadal characteristics and histology of juvenile Nile tilapia (*Oreochromis niloticus*) where highest percentages of total flavonoids and alkaloids from *A. indica* ethanol extract were 23.7 and 14.2 % respectively. However, same trend was observed in the male percentage of this study by Sadek, *et al.* 2022 [36] who studied the effect of *Tribulus terrestris* powder on sexual transformation of *Oreochromis niloticus* and observed significant increase (P≤0.05) in percent male of 44.8, 71.53 and 83.50% with increasing *Tribulus terrestris* powder in the diets for 0.00,100 and 200 g/kg respectively. The result of this study was in agreement with Susmitha, *et al.* (2013) [37] who document reduction of hatchlings in *Oreochromis niloticus* fed with phytochemical extracts from Neem plant and suggested that it could be attributed to the antifertility properties of phytocompounds like flavonoids, saponins and alkaloids found in the plant.

Conclusion

This study reveals that *Azadirachta indica* leaves contain phytochemicals that help in the masculinisation of new hatched *Oreochromis niloticus* of undifferentiated sex. However, it reveals that *Azadirachta indica* leaves extract at 1.5 and 2.0 g/kg diet concentrations are the most effective in masculinisation. Moreso, the study has proofed that the extract has a positive and significant impact on the weight gain, specific growth rate, percentage weight gain, daily weight gain, feed conversion ratio, condition factor and survival of the experimental fish. Moreover, the discoveries of this study will help to increase Nile tilapia production and minimize the cost of sex reversal hormone during the production of mono-sex Nile tilapia populations.

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