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Piper guineense aqueous extract supplemented diet improves reproductive performance of female *Clarias gariepinus* brood stock

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Abstract
The present study was carried out to determine the effects of dietary supplementation of *Piper guineense* on the reproductive parameters of *Clarias gariepinus* female broodstock. Aqueous extract of *P. guineense* was added to the feed of *C. gariepinus* at the inclusions levels of 0.6, 1.2, 1.6 and 2.4 g/kg feed to form diets D2, D3, D4, D5 and the control diet D1 was not supplemented with *P. guineense.* 75 fish of mean weight of ± 407.5 g were stocked into 15 concrete tanks (1 x 2 x 1.5m^3) at the density of 5 fish per tank. The fish were fed two times daily at 3% body weight for 70 days at the end of which the reproductive parameters were evaluated. The results revealed that the fish fed the supplemented diets of *P. guineense* showed significantly improved reproductive indices over the fish fed control diet. Fish fed D5 had the highest gonadosomatic index (13.34 ± 1.84) which was significantly different ($P < 0.05$) to fish fed control diet. The fecundity, percentage fertilization and hatchability was highest in fish fed D4 which was significantly higher ($P < 0.05$) when compared to fish fed control diet. This result revealed that supplemented diets with *P. guineense* fruit aqueous extract improved gonadosomatic index, fecundity, percentage fertilization, hatchability and survival, egg size and estrogen level of female *C. gariepinus* broodstocks and has a potent fertility enhancing property which can be exploited in fish seed production by hatchery operators.

Keywords: *Piper guineense, Clarias gariepinus,* estrogen, gonadosomatic index

1. Introduction
African Catfish *Clarias gariepinus* (Burchell, 1822) [18] is a major popular fish species in Nigeria among fish farmer and consumers. This is because of its high quality meat in different processed forms, which include its smoked form, cooked and fried forms, [1]. It is widely cultivated by fish farmers due to some its qualities. The qualities includes; its ability to grow fast in captivity, ability to strive under unfavourable conditions, ability to resist disease, ability to breed in captivity, it has high fecundity, very high meat quality and taste, has high market value, and can tolerate high stocking density as reported by [2]. Attempts have been made in aquaculture, to obtain high quality sperm and to produce a very high numbers of good quality fingerlings. Good quality eggs and sperm increase fish production and all year round availability of fish [3].

The use of plant extracts and phytochemicals in animal production system to boost growth and to enhance fertility is becoming popular worldwide. [4], reported that there are many sources of safer and cheaper chemical compounds in plants, which include alkaloids, flavonoids, pigments, phenols, terpenoids, steroids and essential oils that possess diverse range of bioactivity. These compounds have particular physiological actions in the body. Now in aquaculture, attention is shifting from the use of synthetic drugs to natural plant products in order to enhance growth and reproductive performance [5]. Many plant products have been tested for the improvement of breeding of *C. gariepinus* in captivity; *Piper guineense* is one of such plants.

Piper guineense, commonly known as African black pepper or hot leave belongs to the family Piperaceae or Sapotaceae [6]. It is widely consumed in some part of West Africa especially Nigeria and Ghana on account of its nutritional and medicinal properties [7]. According to [8], the seeds are consumed by women that just put to bed in order to increase uterine contraction for the expulsion of placenta and other remains from the womb after delivery.

Its seeds are also used for weight control [9]. The leaves of *P. guineense* are used by local medical practitioners for the treatment of respiratory diseases and correction of female infertility problems, as reported by [10] and [11], and the seeds as an aphrodisiac. The phytochemical analysis of the leaves of *P. guineense* showed that it contains; alkaloids, flavonoids, saponins, tannin, resins and essential oil, [12]. The proximate composition of *P. guineense* was reported by [13] to contain crude protein, fat, carbohydrate, vitamins and minerals. Aqueous extract of *P. guineense* also was reported by [11] to improve male reproductive function because it stimulated the secretions of the testes, epididymis and seminal vesicles. Despite the fact that *P. guineense* leaves are widely consumed by people in the Southern and Eastern Nigeria and other parts of West Africa in addition to their versatile ethno medical usage, there is limited or no information on their effect on fish reproductive index. This study is therefore targeted at investigating the effect of the aqueous extract of *P. guineense* dry fruit on the reproductive performance of female *C. gariepinus.*

2. Materials and Methods

Collection, authentication and preparation of Plant materials

Fresh fruits of *Piper guineense* were purchased from a local market in Ado-Ekiti, Ekiti State, Nigeria. Identification and authentication was done at the Department of Crops, Soil and Pest Management, Federal University of Technology, Akure, Ondo State, Nigeria. The fruits of *P. guineense* were air dried and ground into powdery form using electric blender (Marlex Electroline Excella). One- hundred grams of the dried powder were macerated in 200 ml of distilled water for 12 hours at room temperature and was filtered with a muslin bag to obtain a final extract concentration of 28 mg/ml.

2.1 Experimental Fish

Seventy five broodstocks with mean weight ± 407.5 g were obtained from a fish farm in Akure, Ondo state. The brood stocks were stocked at the density of 5 fish per tank and conditioned for two weeks in the outdoor concrete tanks (1 x 2 x 1.5m^3) at the Department of Fisheries and Aquaculture Technology Research farm, Federal University of Technology Akure. During the acclimation, they were fed with commercial diet of 40% crude protein.

2.2 Experimental Procedure

The experiment is a completely randomized design in which five treatments (diets) were applied in three replicates. The following treatments were used: control diets one (D1) without any supplementations, D2 (containing 0.6g of *Piper guineense* extract), D3 (containing 1.2g of *P. guineense*), D4 (containing 1.8g of *P. guineense*) and D5 (containing 2.4g of *P. guineense*). Fish with an initial mean weight of ± 407.5g were stocked into 15 concrete tanks (1 x 2 x 1.5m^3) at a density of 5 fish per tank, at the Department of Fisheries and Aquaculture Technology Fish Farm, Federal University of Technology, Akure. The experimental diets containing aqueous extract of *P. guineense* dry fruits at different graded levels were used to feed the fish twice daily (08.00-09.00 h and 17.00-18.00 h) at 3% body weight for 70 days.

2.3 Formulation of experimental diets

The experimental diet was formulated based on the formulation determined for Africa catfish, *C. gariepinus* for 40% crude protein basal feed [14] as given in the Table 1. All dietary ingredients were ground into small particles size and weighed with a top load scale. Ingredients including protein sources, oil and vitamin premix were thoroughly mixed in a Hobbart A-2007 pelleting and mixing machine (Hobart Ltd London,) to obtain a homogenous mass, cassava starch was added as a binder. The resultant mash was then pressed without steam through a mixer with 6mm die attached to the Hobart pelleting machine. The pellets produced were dried and kept in a cool and dry place until the start of experiment.

Table 1: Ingredient Composition (g/kg) of Experimental diets fed to experimental fish

Ingredients	Experimental Diets				
	Diet 1	**Diet 2**	**Diet 3**	**Diet 4**	**Diet 5**
Fishmeal (65%cp)	250	250	250	250	250
Soy bean (45%cp)	400	400	400	400	400
Yellow Maize	150	150	150	150	150
Blood meal (85%cp)	50	50	50	50	50
Fish oil	40	40	40	40	40
Vegetable Oil	60	60	60	60	60
Vit premix	30	30	30	30	30
Binder	20	20	20	20	20
P. guineense	0	0.6	1.2	1.8	2.4

2.4.1 Fecundity Estimation

This was evaluated from fifteen randomly selected females. Fecundity estimation was done using gravimetric method. The females were dissected and the ovaries removed, the ovaries were weighed. 1g of eggs was taken and counted. This was used to extrapolate the number of eggs.

2.4.2 Determination of egg size

The eggs diameter was determined using micrometer, viewed at 100x microscope (at 0.01mm sensitivity).

2.4.3 Gonadosomatic Index (GSI)

The Gonadosomatic Index (GSI) was determined as described by [15] as:

GSI = gonad weight (g) x 100/ fish weight (g).

2.4.4 Determination of Estrogen level

Estrogen level in the serum of the experimental fish was determined using the blood collected from the fish. Blood samples were taken from the caudal vein of experimental fish, using a sterile syringe containing one drop of heparin. The blood collected was put into clean, dry centrifuge tubes. The tubes were centrifuged at 2500 rpm for 45 min using Uniscope Laboratory Centrifuge (model SM800B, Surgi friend Medicals, England). The serum was thereafter aspirated using Pasteur pipettes into clean and dry sample bottles as

described by [16]. The serum was obtained after centrifuging for 15 minutes, it was then analyzed for quantitative determination of estrogen (oesterone E1 and oestradiol E2) concentration by a Microplate Enzyme Immunoassay (MIA) according to [17].

2.4.5 Breeding

After the feeding trials, artificial reproduction of the broodstocks was carried out at the fish hatchery of the Department of Fisheries and Aquaculture, Federal University of Technology Akure. Fifteen pairs of matured male and female from all the treatments were collected and breeding was carried out using ovaprim, (a synthetic hormone, Aqualife Syndel International Inc.) at the rate of 0.5ml/kg of body weight. Ovulation occurred at 11hrs after injecting them. Stripping of the females was done by pressing their abdomen with a thumb from the pectoral fin towards the papilla. The ovulated eggs were collected into a dry plastic bowl. Male spawners were sacrificed to remove their testes. Eggs from the females were weighed and put into different bowls 1g each for fertilization with the milt that was collected from each of the male testes. The milt was squeezed out on the eggs batches, and dry fertilization was performed. The fertilized eggs were rinsed with fresh water after 1 minute of gentle stirring to remove excess milt. The bowls were filled with water (2liters) and aerated for incubating the eggs.

2.4.6 Fertilization

Fertilization was determined at 20 min after fertilization. Translucent eggs containing embryonic eyes at the time of polar cap formation, were considered fertilized while opaque eggs (the once that turned whitish in colour) were considered unfertilized and counted to estimate percentage fertilization. The percentage fertilized eggs as well as the percentage number of eggs hatched and percentage survival were calculated according to the method described by [18]:

$$\% \text{ Fertilization} = \frac{\text{Number of fertilized eggs}}{\text{Total number of eggs counted}} \times 100$$

$$\% \text{ Hatchability} = \frac{\text{Number of eggs hatched}}{\text{Total number of eggs in a batch}} \times 100$$

$$\% \text{ Survival} = \frac{\text{Number of hatchlings alive up to larvae stage}}{\text{Total number of hatchlings}} \times 100$$

2.5 Water quality parameters

Water quality parameters like temperature, pH, and dissolved oxygen concentration were monitored weekly during the period of the study using mercury-in-glass thermometer, pH meter (Hanna H198106 model) and dissolved oxygen meter (JPP- 607 model) as described by [19].

2.6 Histology of the testis and ovary of fish fed the experimental diets

After 70 days of feeding, a total of ten males were randomly taken from all the five treatments. They were dissected and the testes removed for sectioning and histological examination. The testes were placed in a formalin-solution made of equal volumes of 10% formalin and 0.9% sodium chloride solution for 24 hours [20]. Following dehydration in increasing ethanol concentrations (50-99.9), the tes444tes were cleared in xylene and embedded in paraffin wax. Section of 10 µ was cut and stained in haematoxylin and eosin. The stained specimens were observed under a light microscope fitted with camera. Photograph of the stained specimens were taken for interpretation.

2.7 Statistical analysis

All values were recorded as mean ± standard deviation and were subjected to a one-way ANOVA test to analyze the variance in the test groups and the control using the SPSS 15 for window software package. Duncan's multiple range tests was used to compare differences among individual means [21].

3. Results

3.1 The reproductive performance of female *C. gariepinus* fed dietary supplementation of *P. guineense* fruit

Data on growth and reproductive performances of the female *Clarias gariepinus* brood stocks fed on supplementary diet of aqueous extract of *P. guineense* fruit are presented in Table 2. The results showed that weight gain was highest in fish fed D5 followed by D4, D3, and D1 while the lowest weight gain was in D2. There was no significant difference ($P > 0.05$) in the weight of ovaries and GSI in all the diets, but the values increased in the fish fed with the experimental diets. Fecundity of the fish fed supplemented diets showed significant difference when compare to fish fed control diet (D1). There was significant improvement in percentage fertilization of the fish fed supplementary diets when compared to fish fed D1, the improvement increased with increase in the concentration of *P. guineense* in the diets. There was significant difference ($P < 0.05$) in percentage hatchability of the fish fed D3, D4 and D5 when compared to fish fed D1. Likewise, there was significant difference ($P < 0.05$) in the percentage survival of the fish fed the experimental diet when compared to fish fed D1 with D5 having the highest followed by D4, D3, D2 while D1 had the lowest. Egg size showed significant difference ($P < 0.05$) in the fish fed with the supplementary diets D4 and D5 when compared to fish fed D1 with D4 having the highest value followed by D5, while D3, and D2 are not significantly different from D1. Estrogen level showed significant difference between the fish fed control diet (D1) and the fish fed supplementary diets with the highest value (3144.20) occurring in fish fed D4.

Table 2: Reproductive performance of female *Clarias gariepinus* broodstocks fed dietary supplementation of aqueous extract of *P. guineense* fruit (mean ± S.E)

Parameter	D1 (control)	D2	D3	D4	D5
Initial fish weight (g)	395.33 ± 2.19a	399.67 ± 2.60a	395.67 ± 3.48a	400.67 ± 0.67a	399.67 ± 0.88a
Final fish weight (g)	539.73 ± 4.89ab	511.90 ± 13.75a	618.93 ± 48.03ab	640.40 ± 55.06b	651.33 ± 10.51b
Weight gain (g)	144.40 ± 7.27ab	112.23 ± 12.22a	223.27 ± 44.55b	239.73 ± 55.49b	252.30± 10.47b
Weight of ovaries (g)	41.67 ± 3.13a	46.40 ± 5.54a	46.90 ± 9.15a	50.10 ± 10.01a	65.00 ± 7.66a
GSI (%)	8.94 ± 1.25a	8.90 ± 1.08a	8.40 ± 0.98a	9.91 ± 1.04ab	13.34 ± 1.84b
Fecundity/fish(No)	43819.33±7587.77a	40521.00±2019.2a	43656.33±3647.47a	57523.67±5770.35b	40142.78±20045.94a
% Fertilization	72.84 ± 4.50a	85.03 ± 342b	87.08 ± 1.75b	92.54 ± 1.96b	89.92 ± 1.40b

% Hatchability	52.16 ± 5.04a	63.16 ± 2.52ab	69.42 ± 2.69b	75.73 ± 2.70b	74.77 ± 6.25b
% Survival	33.64 ± 8.03a	50.26 ± 3.69b	56.02 ± 1.72ab	64.54 ± 0.76ab	65.45 ± 4.08cb
Egg diameter (mm)	1.43 ± 0.67a	1.50 ± 0.00ab	1.50 ± 0.00ab	1.50 ± 0.00ab	1.57 ± 0.03b
EG level (pg/ml)	1475.00±2.13a	2965.50±71.86b	2641.61±.46b	3144.20±13.67b	2750.4±1.51b

Mean in a given row with the same letter were not significant different at $P < 0.05$
GSI = Gonado somatic index = Gonads weight (g) × 100/ fish weight
EG = Estrogen.

3.2 Water Quality Parameter Result

The water quality parameters measured varied as follows: temperature; 26 to 26.40°C, dissolved oxygen; 6.10 - 6.60 mg/l, and hydrogen ion concentration (pH); 9.60 - 9.80.

3.3 Effects of Dietary Supplementation of aqueous extract of *P. guineense* fruit on the Histology of Ovaries

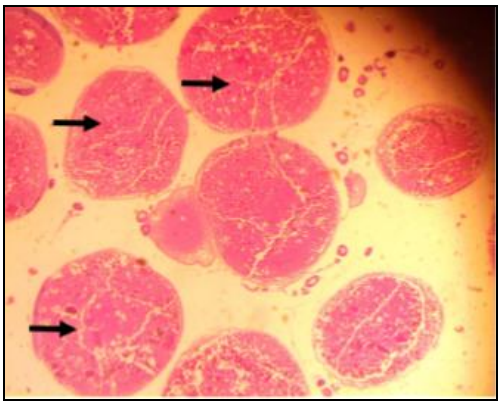

Plate 1: Photo-micrograph of a cross section of the ovary of C. *gariepinus* fed D1 (0g/kg of P. *guineese*.) Most stages seen are the vitellogenic stages (V). Mag x 200

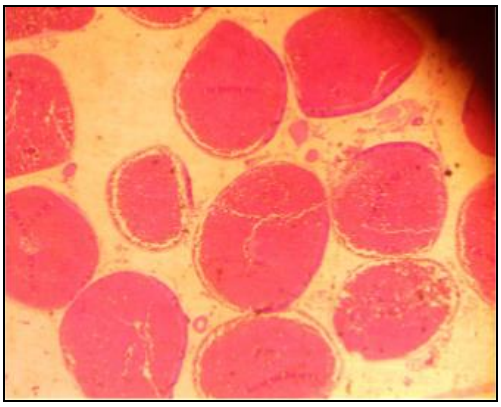

Plate 2: A cross section of the ovary of C. *gariepinus* fed D2 (0.6 g P. *guineese*/kg feed.) Most stages seen are the vitellogenic stages (V). Mag x 200

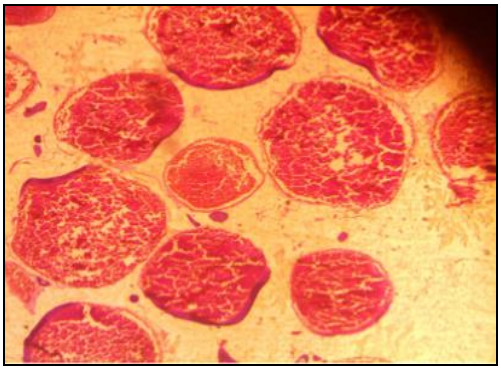

Plate 3: A cross section of the ovary of C. *gariepinus* fed D3 (1.2g P. *guineese*/kg feed) Most stages seen are the vitellogenic stages (V). Ripended and about to disperse. Mag x 200

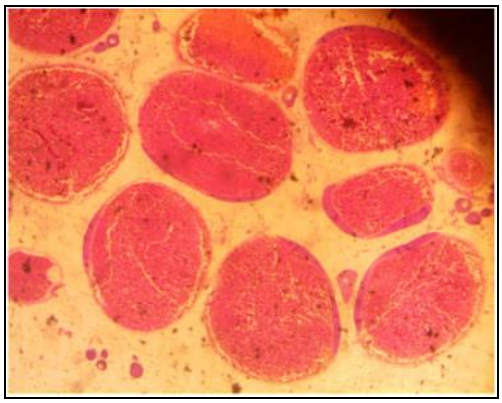

Plate 4: A cross section of the ovary of C. *gariepinus* fed D4 (1.8g/kg of *Piper guineese*) Mostly Vitellogenic stages seen. Mag x 200

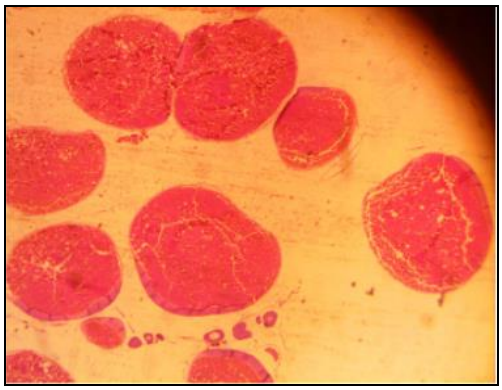

Plate 4: A cross section of the ovary of C. *gariepinus* fed D5 (2.4g/kg of *Piper guineese*) Vitellogenic stages. Mag x 200

4. Discussion

The water quality parameters measured varied as follows: temperature; 26 to 26.40 °C, dissolved oxygen; 6.10 - 6.60 mg/l, and hydrogen ion concentration (pH); 9.60 - 9.80. These levels fell with the range recommended for catfishes [22], and it was well tolerated by the experimental fish.

From the results, it could be said that *P. guineense* treatment enhanced reproductive performance, which was seen in improved ovary weight, GSI, fecundity, egg size, percentage fertilization, and also in percentage hatchability of the eggs and percentage survival of the *C. gariepinus* larvae. The estrogen level was also observed to be increased in the fish fed the supplementary diets. Generally, high values of the measured parameters were obtained in all the treatments supplemented with *P. guineense*. Better fecundity values were obtained in the fish treated with *P. guineense* when compared to the control and there was significant difference ($p < 0.05$) between the fish fed D4 and the control. The improvement in the reproductive indices (weight of ovaries, fecundity, gonado somatic index, and percentage fertilization), percentage hatchability and percentage survival was as a result of the *P. guineense* fruits extract which had a positive effect on the reproductive performance of *C. gariepinus*. This is in agreement with the report [11] who reported that *P. guineense* increased reproductive indices in male rats. Similar results

were also reported by [23] who used *Telfairia occidentalis* as fertility enhancing agent for catfish *C. gariepinus.* The increase in the fecundity of *C. gariepinus* observed in this study could be attributed to the presence of flavonoids and steroids in the plant which are potent antioxidants capable of increasing the production of estrogen. Estrogen is the key hormone involved in the production and maturation of eggs in the ovaries. This also explains the increase in the estrogen level in the fish fed the supplemented diets. The increase in the values of this sex hormone, estrogen in fish fed Diets (2, 3, 4 and 5) was evident in high fecundity, percentage fertilization and hatchability obtained in the text fish. *P. guineense* leaf was found to have a high carbohydrate content, contains vitamin C in considerable amount, contains vitamin A and traces of vitamin B1 and B2 [24]. It also contains vitamin E which plays a significant role as an antioxidant [25]. Most of these active ingredients have well documented ovulatory and activities. Zinc, for instance, promotes sexual maturation and reproduction, and vitamins C and E, also increase the development of egg in the ovary and sperm in the testes, thereby leading to a better ovulation and spermiation [26].

The egg diameter was 1.50 mm in group of fish fed diet D4 which had a positive effect on the fecundity of the fish and fertilization of the eggs. It is generally accepted that there is an inverse relationship between fecundity and egg size in which fish produce more eggs of smaller size or fewer eggs of larger size [27] and [28]. This is also supported in this study where fish that received the second to the highest dosage of *P. guineense* (1.80 g/kg) had the highest fecundity and lowest egg size.

In this study, the larval of the broodstocks fed supplementary diets of *P. guineense* survived well than the ones in control with the highest performance occurring in D4 followed by D5. This shows that *P. guineense* at inclusion level of 1.80 and 2.40 g/kg could improve larval survival. Since most of the losses in hatchery are recorded at the critical transitional period of moving from endogenous feeding to exogenous feeding, any effort made to improve the quality of the egg will surely equip the fry for survival [29]. The significant difference ($p<0.05$) in percentage fertilization and hatching observed in the fish fed supplementary diets of *P. guineense* agrees with [30] who reported similar results with effect of heated soybean on egg and sperm quality of *C. gariepinus*. [31] Also reported that species of the genus *Clarias* with larger eggs also have a higher viability and endurance to starvation than those with smaller eggs and that larger female catfish produce larger eggs.

The result of the histology of the ovaries revealed that most of the oocytes were at late vitellogenic stage (stages 4 and 5) gonadal developments, ripened and about to disperse. This shows the effect of *P. guineense* aqueous extract supplemented diet on them.

5. Conclusion

The results of the present showed that the inclusion of *P. guineense* up to 2.4g/kg feed enhanced fertility of female *C. gariepinus* without having any deleterious effect on the health of the fish. Hence, it could be included in the fish feeds, but caution should be taken because higher concentration may not give the desired result, as seen in the result of the present research where inclusion level of 1.8g/kg feed gave the highest fecundity, percentage fertilization and percentage hatchability of the fed fish. However, growth and reproduction may be negatively affected in fish if plant materials are not processed according to recommended methods to reduce anti-nutrients.

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