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Phytochemical screening and masculinization of Nile tilapia (*Oreochromis niloticus* Linnaeus) using the needle and root crude extracts of Benguet pine (*Pinus kesiya* Royle ex Gordon)

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Abstract This study evaluated the effect of Benguet pine (*Pinus kesiya*) needle and root extracts in the masculinization of Nile tilapia (*Oreochromis niloticus*) fry. The crude extracts of Benguet pine were phytochemically screened and found positive for steroid for root extracts while chlorophyll and xantene were present in needle extracts. Toxicity assay was conducted to evaluate the median lethal concentration (LC_{50}) after 96 hours. Probit analysis revealed LC_{50} was 150 ppm and 960 ppm for Benguet pine needles and roots, respectively. Temperature, dissolved oxygen (DO) and pH did not differ statistically in all treatments ($P>0.05$). For the sex-reversal, treatments were as follows; I-fry booster only, II- diet with 50 mg/kg methyltestosterone, III- diet with 100 ppm Benguet pine needle extract and IV-diet with 500 ppm Benguet pine root extracts. Results revealed no significant difference was observed in the survival and growth rate of different treatments ($P>0.05$). No sex inversion was observed in the diet with 100 ppm Benguet pine needle crude extract and diet with 500 ppm Benguet pine root crude extracts 62.27±7.02% and 61.51±5.88%, respectively. Percent males were significantly different at 50 mg/kg methyltestosterone compared to other treatments. No intersex gonads were observed in all treatments. Temperature, dissolved oxygen (DO) and pH did not differ statistically in all treatments ($P>0.05$) after 28-day sex-reversal in outdoor tanks.

Keywords: masculinization, methyltestosterone, phytochemical screening, sex-reversal

Introduction

Aquaculture is one of the main food production sectors to deal with the high demand for food due to human population explosion. Aquaculture, probably the fastest growing food-producing sector, now accounts for nearly 50 percent of the world's food fish (Food and Agriculture Organization, 2011). Tilapia is one of the major fish species for aquaculture. Farming of tilapia is

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fast expanding in many countries not only in the tropics but also in sub-tropical and temperate areas (Guerrero, 1997). Growth of tilapia production can be attributed to the different technologies adapted by the hatchery and grow-out operators including production of monosex tilapia (Fitzsimmons and Alanis, 2008). According to Mbahinzireki *et al.* (2001), all-male populations are desirable in tilapia aquaculture because males demonstrate superior growth characteristics. In addition, Phelps and Popma (2000) said that all-male populations divert less energy into reproduction.

One method commonly used to induce sex reversal is the oral administration of 17 α-methyltestosterone during the period of gonadal differentiation of the fish (Green *et al.*, 1997; Abucay and Mair, 1997; Gale *et al.*, 1999). However, the use of hormones has been under increasing public criticism due to its possible health and environmental impacts. With these risks, alternative methods using new and safe chemicals to produce monosex population should be considered.

A study conducted by Dabrowski *et al*. (2004) used phytochemicals (flavonoids and/or isoflavonoids) as alternative means to affect sex differentiation. There are only few studies conducted evaluating the use of phytochemicals in masculinizing tilapia fry. This present study aims to investigate the potential of locally available plants for phytochemical properties which can be used in the masculinization of tilapia. Phytoandrogen compounds are produced in plants that mimic the effects of androgen, the class of male hormones to which testosterone belongs. Phytoandrogens have been implicated in sex-reversal in fish (Godwin *et al*., 2003). This study evaluated the effect of Benguet pine needle and root extracts in the masculinization of Nile tilapia. Specifically, it aimed to determine the phytochemical profile of the crude extract of pine needle and root using thin-layer chromatography (TLC); and determine the efficacy of crude extract of pine needles and roots in producing all-male Nile tilapia fry.

Materials and methods

The study involved two experiments. Experiment 1 focused on the phytochemical profiling of needle and roots of Benguet pine extracts by using TLC. Experiment 2 involved masculinizing phenotypic males of tilapia and determine their influence on the growth and survival rate of Nile tilapia fry on a 28-day treatment period in outdoor tanks.

Phytochemical Profiling of Needle and Roots of Benguet Pine Extracts Using TLC

Experiment 1 involved sourcing the Benguet Pine (*Pinus kesiya*) (Figure 1) needle and roots and preparation of the plant extracts based on the methods by Aguinaldo *et al.* (2005). The raw materials were collected from the Ecosystems Research Development Service (ERDS) of the Department of Environment and Natural Resources (DENR), Loakan Road, Baguio City. There were two extraction methods done adopted from Aguinaldo *et al.* (2005) and the filtrates were labeled as shown in Table 1.

Figure 1. (a) Benguet pine (*Pinus kesiya*) (b) needles (c) roots (Photosource: FAC-CLSU (Phytoandrogen Project)

Screening by TLC was done in order to test for the presence of phytochemical compounds in the extracts. Following the procedure of Aguinaldo *et al.* (2005), about 0.1 to 0.2 ml of Solutions A and B were spotted on the plates (Table 2).

Masculinizing Phenotypic Males of Tilapia

Experiment 2 include both sex-reversal of Nile Tilapia in net enclosures (1 x 1 x 1 m) installed in outdoor tanks and further rearing in hapa nets in pond. Twelve (12) outdoor tanks, each with 3 m^3 capacity were used in this study arranged in Complete Randomized Design (CRD). Nile tilapia strain, *O. niloticus* fry from the GIFT Foundation, Inc. was used in the study. Prior to stocking, initial length (mm) and weight (mg) were measured using Vernier caliper and digital balance, respectively. The MT-treated diet purchased from

GIFT II was prepared by dissolving 50 mg of 17 α-methyltestosterone in 1,000 ml of ethyl alcohol. The feed was air-dried by placing it in a feed drying pan. The crude extract was prepared by weighing 0.1 g of pine needles and 0.5 g pine roots and was soaked separately in a 1 L ethyl alcohol for 8 hours. Feed samples with crude extracts from pine needle and roots from different treatments were sent to the Pampanga Agricultural College Feed Testing Center in Magalang, Pampanga for proximate analysis. Feeds containing the extracts and MT were given by oral administration for 28 days at the rates of 30, 20 and 10% of the fish body weight per day for the first, second, third and fourth week, respectively, with feeding frequency of 5 times a day. Sampling of the 10% of fish population was done weekly to determine the gain in weight and survival rate by bulk weighing and individual counting of fish. The Nile tilapia fingerlings (Table 3) were further reared for 60 days so that the gonads of the fish are big enough to be seen under the microscope using gonad squash technique (Phelps and Popma, 2000). Commercial feeds were given to the fish (10 to 8% of ABW) four times per day. After 60 days, sex of fish was determined using 100 pieces of the fish population in each hapa following the protocol of Guerrero and Shelton (1974) (Figure 2).

Table 1. Group of plant constituents assumed to be distributed in different extracting solutions (Aguinaldo *et al*., 2005)

Test Solution	Extracting Solvents	Plant Constituents
A	Mixture of chloroform and acetic acid	Anthraquinones, phenols, flavonoids, steroids, coumarins
B	Mixture of chloroform, methanol and acetic acid	Flavonoid glycosides, cardenolides, anthaquinone glycosides, tannins, saponins, coumarins, indoles

Table 2. Developing solvent system for TLC for phytochemical screening

Test Solution	Adsorbent (Stationary Phase)	Solvent System (Mobile Phase)
A	Silica Gel $60F_{254}$	Toluene:acetone:chloroform (40:25:35)
B	Silica Gel $60F_{254}$	Chloroform:aceticacid:water (50:45:5)

Table 3. Number of Nile tilapia fry stocked in hapa in pond

Treatment	Number of Stocks
Fry booster only	266-367
Fry booster + Methyltestosterone	417-500
100 ppm Pine needle incorporated in the diet	246-500
500 ppm Pine root incorporated in the diet	241-335

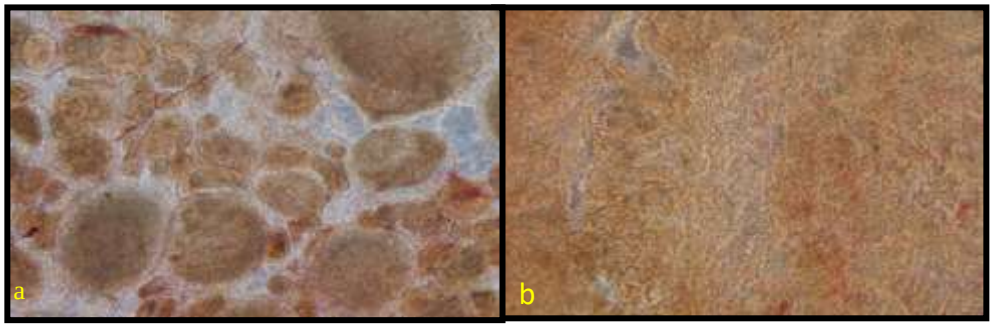

Figure 2. Tilapia gonads using squash method for the determination of sexes: (a) female oocyte and (b) male spermatozoa

Statistical Analysis

Statistical analysis was done using Statistical Analysis System (SAS) version 9. Sex ratio data were analyzed using Chi-square test ($\alpha \leq 0.05$) to determine efficacy of the treatments. Sample distributions violating assumptions were log-transformed before analysis. Data, expressed as percentages, were arc sine-transformed before analysis. Differences were regarded as significant at $P < 0.05$.

Results

Phytochemical Screening of the Benguet Pine Crude Extracts

Result of the phytochemical screening of the crude extract of Benguet pine is presented in Table 4. The solvent system toluene:acetone:chloroform (40:25:35) produced 1 spot for methyltestosterone with R_f value of 0.46, 7 spots for Benguet pine needle extracts (Figure 3) and 11 spots for Pine roots. The chloroform:acetic acid:water (50:45:5) produced 1 spot for methyltestosterone (Figure 4). The R_f values are shown in Table 5.

Steroid and Coumarins Compound

The concentrated crude extract of Benguet pine roots was found positive for steroid the extract has dark spots when sprayed with vanillin-sulfuric acid (Fig. 5). The retention factor (R_f) values of pine roots was positive for steroid and these are 0.18 and 0.48 while methyltestosterone has an R_f value of 0.46. The concentrated crude extract of pine roots was also found positive for coumarins; having blue-white fluorescence under UV light when sprayed with

methanolic potassium hydroxide (Figure 5). R_f values of pine roots positive for coumarins are 0.43 and 0.91.

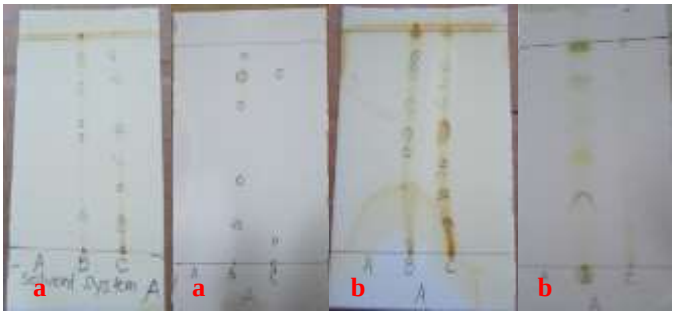

Figure 3. TLC profile of crude extracts of Benguet pine (*P. kesiya*) needle (a) and roots (b) and methyltestosterone using Toluene:acetone:chloroform as solvent

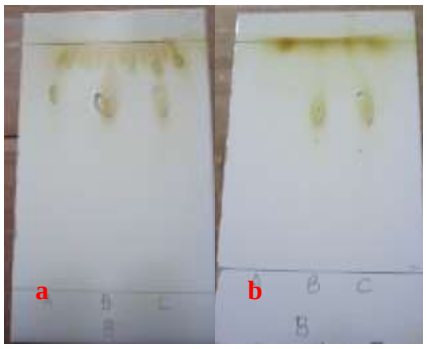

Figure 4. TLC profile of crude extracts of Benguet pine (*P. kesiya*) needle and roots and Methyltestosterone using Chloroform:acetic acid: water as solvent.

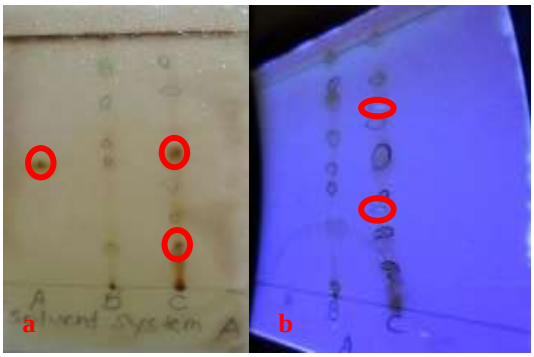

Figure 5. The encircled in red are indications of the presence of steroid (A) and coumarins (B)

Table 4. Qualitative analysis of needle and root crude extracts of Benguet pine (*P. kesiya*)

Compounds	Name of test	Needles	Roots
Steroid	Vanillin-sulfuric acid	-	+
Flavonoid	Potassium ferricyanide-ferric chloride	-	-
Phenols	Potassium ferricyanide-ferric chloride	-	-
	Methanolic potassium hydroxide (Borntager reagent)	-	-
Tannins	Potassium ferricyanide-ferric chloride	-	-
Coumarins	Methanolic potassium hydroxide (Borntager reagent)	-	+
Anthrones	Methanolic potassium hydroxide (Borntager reagent)	-	-
Anthraquinones	Methanolic potassium hydroxide (Borntager reagent)	-	-
	Magnesium acetate	-	-

Note: (+) Positive (-) Negative

Table 5. Retention factors (R_f) for each of the crude extracts of needle and roots of Benguet pine (*P. kesiya*)

Plate	Retention factor (R_f) for Solvent System A			Retention factor (R_f) for Solvent System B		
	MT	Needles	Roots	MT	Needles	Roots
I	0.46	0.27, 0.48, 0.61, 0.68, 0.81, 1.00	0.02, 0.15, 0.18, 0.19, 0.24, 0.37, 0.48, 0.69, 0.74, 0.80, 1.00	1.00	0.66	0.66
II		0.02, 0.39, 0.45, 0.59, 0.74, 0.80, 1.00	0.08, 0.12, 0.20, 0.29, 0.33, 0.43, 0.45, 0.57, 0.63, 0.74, 1.00	0.76	0.73	0.75, 0.89, 0.91
III		0.17, 0.38, 0.50, 0.73, 0.87, 0.96	0.02, 0.10, 0.13, 0.27, 0.38, 0.54, 0.77, 0.87, 0.96	0.87	0.83, 0.88, 0.98	0.87, 0.94, 1.00
IV		0.17, 0.50, 0.58, 0.75, 0.92	0.13, 0.17, 0.33, 0.42, 0.58,0.79, 0.92	0.80	0.63	0.77, 0.89, 0.91

Percent Males

Data on percent males of Nile tilapia fry after the 28-day treatment period are shown in Table 6. Results show that there was significant difference ($P<0.05$) among treatments at 5% probability level. Tilapia fry fed with MT-treated diet (Treatment II) obtained the highest percentage male with a mean of 97.87±2.11%. Results showed that MT-treated group was significantly higher ($P<0.05$) compared to untreated group (Treatment I), fish fed with 100 ppm pine needle extract (Treatment III) and fish fed with 500 ppm pine root extract (Treatment IV) with means of 62.5±3.54, 62.27±7.02, and 61.51±5.88%, respectively. Following the Chi-square test ($\alpha \leq 0.05$), the MT-treated group significantly skewed towards males (Fig. 6). No intersex gonads were observed in Nile tilapia fry fed with different treatments. No significant difference was observed between Fry booster, diet with 100 ppm Benguet pine needle extracts and diet with 500 ppm Benguet pine root extracts ($P<0.05$).

Table 6. Summary of results on the sex-reversal of Nile tilapia (*O. niloticus*) fry

Treatments	Males (%)	Specific growth rate (%)	Absolute growth rate (%)	Survival rate (%)
Fry booster	62.5 ± 3.5b[2]	6.00 ± 0.28[1]	0. 014 ± 0.0007[1]	63.10 ± 14.00[1]
MT diet	97.87 ± 2.11[1]	5.93 ± 0.20[1]	0. 0105 ± 0.0026[1]	90.87 ± 8.42[1]
Diet with 100 ppm Benguet pine needle extract	62.27 ± 7.02[2]	6.22 ± 0.29[1]	0.016 ± 0.0042[1]	67.40 ± 28.29[1]
Diet with 500 ppm Benguet pine root extract	61.51 ± 5.88[2]	6.08 ± 0.19[1]	0.012 ± 0.0015[1]	65.13 ± 10.00[1]

Note: Means with the same number are not significantly different at 5% level by DMRT

Water Quality During Sex-reversal

Water quality parameters such as temperature, pH and dissolved oxygen were all found to be within the desirable optimum range. Statistical analysis revealed that there were no significant differences ($P>0.05$) found among the treatments in terms of water quality monitored during the 28-day treatment period (Table 7).

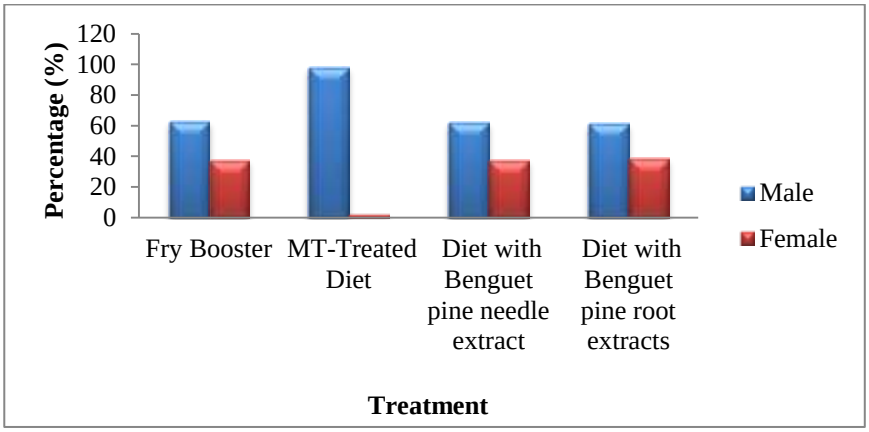

Figure 6. Percentage of male and female Nile tilapia (*O. niloticus*) fry

Table 7. Means ± standard deviations of water quality parameters during the 28-day sex-reversal treatment

Treatments	Temperature (℃)		DO (mg/L)		pH	
	AM	PM	AM	PM	AM	PM
Fry booster	27.12 ± 0.28	29.46 ± 0.51	4.67 ± 0.58	7.81 ± 0.28	8.10 ± 0.07	8.30 ± 0.15
MT diet	27.47 ± 0.12	29.20 ± 0.45	4.46 ± 0.21	8.63 ± 0.61	8.10 ± 0.10	8.40 ± 0.21
Diet with Pine needles	27.6 ± 0.25	29.36 ± 0.21	4.82 ± 0.98	7.34 ± 0.88	8.20 ± 0.10	8.20 ± 0.15
Diet with pine roots	27.32 ± 0.47	29.31 ± 0.32	4.21 ± 0.80	8.15 ± 0.60	8.2 ± 0.10	8.20 ± 0.06

Discussion

The positive sign in roots indicated presence of phytochemical compounds such as steroids and tannins. This finding conforms to the studies of Junzentiene *et al*. (2006), Zhang (2011), and (Pavlutskaya *et al.* (1984) that pine needles have pigments and one of which is chlorophyll. The MT-treated group was found to attain the highest percentage of males among the group. This result confirmed that oral administration of testosterone-treated feed (30-60mg/kg feed) to tilapia fry during a three to four-week period yields populations composed of ≥ 95% males (Shelton *et al.*, 1978; Guerrero and Guerrero, 1988; Jo *et al.*, 1988; Vera Cruz and Mair, 1994). Synthetic androgens are generally more potent than natural androgen for masculinizing fish (Yamamoto, 1969; Hunter and Donaldson, 1983).One possible factor why

no sex inversion was observed on the diet treated with Benguet pine needle and root extract can be due to the low conce ntration of extract incorporated in the diet. Another factor can be attributed in the time of extraction of Benguet pine needle and root extracts. It was only extracted for 8 hours, while it should be 48 hours for natural products. The thin layer chromatography provided the compounds present in the Benguet pine needle and root extracts, specifically, the Benguet pine root extract was found positive for steroid, however, there should be another investigation for the identification of phytoandrogen or phytoestrogen through High Performance Liquid Chromatography (HPLC).

Readings on the water quality parameters during the experimental period demonstrated desirable levels suitable for sex-reversal of Nile tilapia fry. The optimal average temperature recorded was 27℃ in the morning. However, it was also found to be relatively high during the afternoon with an average of 29℃. Phelps and Popma (2000) stated that optimum temperature suitable for sex reversal of tilapia fry falls between 26-28℃. Readings on dissolved oxygen and pH recorded were all within favorable conditions appropriate for sex reversal. Phelps and Popma (2000) suggested that dissolved oxygen concentrations should remain above 4 mg/l to ensure a strong feeding response. In terms of pH, it was mentioned that tilapia can best survive in pH of 6.0-9.0 (Popma and Masser, 1999). Based on the results of this study, the following can be concluded Based on the qualitative analysis using TLC, pine root extracts have phytochemical compounds like steroid and coumarins while pine needles have pigments such as chlorophyll and xanthene. The solvent system selected for better chromatography was the mixture of toluene:acetone:chloroform. Growth and survival rates of Nile tilapia were not affected by the incorporation of methyltestosterone, pine needle and root extracts in the diet. Nile tilapia fry diet incorporated with Benguet pine needle and root extracts were not effective in the masculinizing Nile tilapia fry compared to methyltestosterone.

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