ISSN: 2347-5129
(ICV-Poland) Impact Value: 5.62
(GIF) Impact Factor: 0.352
IJFAS 2016; 4(1): 358-363

www.fisheriesjournal.com
Received: 14-11-2015
Accepted: 16-12-2015

Indranath Ghosal
Fish Endocrinology Research Unit, Department of Zoology, University of Calcutta, Kolkata.

Debosree Mukherjee
Fish Endocrinology Research Unit, Department of Zoology, University of Calcutta, Kolkata.

Csaba Hancz
Faculty of Agricultural and Environmental Sciences, Kaposvár University, Kaposvár, Hungary.

Suman Bhusan Chakraborty
Fish Endocrinology Research Unit, Department of Zoology, University of Calcutta, Kolkata.

Correspondence
Suman Bhusan Chakraborty
Fish Endocrinology Research Unit, Department of Zoology, University of Calcutta, Kolkata.

Production of monosex Nile tilapia, *Oreochromis niloticus* by dietary and immersion treatment with *Basella alba* leaves and *Tribulus terrestris* seeds

Indranath Ghosal, Debosree Mukherjee, Csaba Hancz, Suman Bhusan Chakraborty

Abstract
In the present study mixed sex juveniles of Nile tilapia were subjected to dietary treatment with powdered *Basella alba* leaves and *Tribulus terrestris* seeds (0.0, 5.0, 10.0, 15.0 g/kg feed) and immersion treatment with aqueous extracts of both plant materials (0.05, 0.1, 0.15 g/l). There was no significant difference ($P>0.05$) in survival percentage among different treatment categories for both dietary and immersion experiments. Dietary treatment with both plant materials produced significantly higher percentage ($P<0.05$) of males compared to that of control. There was no significant difference ($P>0.05$) in male percentage between treatments with *B. alba* and *T. terrestris* during feeding and immersion experiments. For dietary treatment, the highest percentage of males (76.6±0.5) was observed with *T. terrestris* at the concentration of 15.0 g/kg, while treatment with *T. terrestris* aqueous extract at a concentration of 0.15 g/l showed the highest percentage of males (81.4±0.5) during immersion experiment.

Keywords: Phytochemicals, Dietary treatment, Immersion treatment, Sex reversal, Methyl testosterone

1. Introduction
The Nile tilapia, *Oreochromis niloticus* (Linnaeus) is a well-studied, fast-growing and widely cultured fish species. It is currently ranked second only to carps in global production and is likely to be the most important cultured fish in the 21st century [1]. Rapid growth, high tolerance to low water quality, efficient food conversion, resistance to disease, ease of spawning and good consumer acceptance makes tilapia a suitable fish for culture [2]. Females of tilapiine species have a high fecundity, generally reproducing at a small size and exhibiting stunted somatic growth at higher densities, while male tilapias exhibit faster growth rates and are often the preferred gender for monosex aquaculture [3]. Synthetic steroids are commonly used to induce sex reversal in tilapia but because of the potential hazards of such steroids; the use of new chemicals is a potential alternative to be explored [4]. Plant extracts containing diverse bioactive principles 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, tonic and immunostimulation, and antimicrobial properties in fish culture [5, 6]. Phytochemicals are also reported to block biosynthesis as well as action of estrogen by acting as aromatase inhibitors and antagonists to nuclear estrogen receptor in gonad germ cells [7] and hence may be considered as potential mean for inducing sex reversal in fish. However, there are significant variations regarding the efficacy of different phytochemicals for production of all-male fish population and the potential anabolizing and virilizing effects of such plant extracts needs to be clearly documented.

Aqueous and methanol extracts from the dry leaves of *Basella alba*, a fast growing vegetable, probably originating from India [8], has been reported to possess active components that increase testosterone production in adult male rat testes during *in vitro* studies [9, 10]. This edible plant has been found to possess nutritional values including androgenicity in traditional medicines of several countries [11]. Moreover, few studies have indicated a positive effect of the methanol extract of *B. alba* leaves on sex reversal, growth and immunostimulation in *Poecilia reticulata* and *O. niloticus* [12, 13]. The herb, *Tribulus terrestris* has been reported to

raise testosterone levels [14] and to induce sex reversal in fish when administered through immersion technique [15, 16]. *T. terrestris* has been observed to be effective for production of monosex *Poecilia latipinna* population [17]. The plant extract has also been found to stimulate growth in *Cichlasoma nigrofasciatum* and *P. reticulata* [15, 16]. Both *B. alba* and *T. terrestris* have been reported to possess medicinal values [18]. But, use of these plant extracts for sex reversal and growth induction in tilapia during its culture under Indian perspective is not documented. Various methods such as oral administration and immersion technique have been adapted for *in vivo* application of phytochemicals with medicinal values [19]. Therefore, the ideal method of application for *B. alba* and *T. terrestris* for commercially feasible induction of sex reversal and growth in tilapia must be determined. Considering these aspects, the objective of the present study was to investigate the potential effect of these two plants on the masculinisation of *O. niloticus*, to compare direct feeding and immersion techniques as methods for *in vivo* application of the plant material and to determine an ideal concentration for each method with the plants that might produce maximum percentage of males in tilapia.

2. Materials and Methods

1. Collection of fish seed

Hatched juveniles of mixed-sex Nile tilapia *Oreochromis niloticus* (Linnaeus) was collected from the Fish Hatchery of West Bengal Government, oxygen packed and transported to the laboratory.

2. Plant extracts preparation

B. alba leaves and *T. terrestris* seeds were procured from the local plant market, washed in sterile distilled water, air-dried in shade and powdered. These powdered plant materials (250 gm) were extracted with 500 ml water in a Soxhlet apparatus and the extracts were evaporated to dryness under pressure at 45 ºC using a rotary evaporator and stored under nitrogen at -20 ºC in amber glass bottle until those were used.

3. Determination of plant extract yield

The yield of evaporated dried extract based on dry weight basis was calculated from the following equation:

$$\text{Yield (\%)} = (W_1 \times 100) / W_2$$

Where W_1 was the weight of extract after evaporation of the solvent and W_2 was the dry weight of the fresh plant sample.

4. Dietary treatment of fish with powdered plant material

Three days old mixed sex juveniles of Nile tilapia (mean weight 0.025±0.009g; mean length 1.25±0.012cm) were randomly allocated into eight groups (40 fish/group). Three groups were fed diets containing powdered *Basella* leaves at different concentrations of 5.0, 10.0, 15.0 g/kg feed, three groups were fed diets containing powdered *Tribulus* seeds at concentrations of 5.0, 10.0, 15.0 g/kg feed, one group was fed control diet without *Basella* and *Tribulus* powder, while the last group was fed diet containing 17α methyltestosterone (MT) with a dose of 10 mg/kg. The powdered plant materials were mixed thoroughly with the finely ground (< 500-1000 µm) artificial diet containing 30% crude protein (Tokyu, Japan). It was then wetted with deionized water, mixed thoroughly, formed with a pelleter (diameter 2 mm), and dried at room temperature. Pelleted feed was pulverized before feeding to the juvenile fish. Hormone treated diet was prepared by the alcohol evaporation technique. The experiment was conducted for 30 days and the fish were fed with respective diets at a rate of 20% body weight / day. The aquaria were continuously aerated and maintained in heated (T = 27±2 ºC) static systems. Water in all aquaria was replaced manually and the fish was kept under similar photoperiod (14 L: 10 D). The entire experimental set up was conducted simultaneously in triplicate.

5. Immersion treatment of fish with plant aqueous extracts

Three days old mixed sex juveniles of Nile tilapia from the above described stock were randomly assigned in 18 glass aquaria to three different treatment groups (0.05, 0.1 and 0.15 g/l) for each of the two extracts. The experiment was conducted for 30 days and the fishes were exposed to the plant extracts four times (once weekly) during the study period. The aquaria were continuously aerated and maintained in heated (T= 27±2 ºC) static systems. Water in all aquaria was replaced manually and the fish was kept under similar photoperiod (14 L: 10 D). Each aquarium was stocked with 40 fish. The fishes were fed finely ground (< 500-1000µm) artificial diet containing 30% crude protein (Tokyu, Japan) at a rate of 20% body weight/day. The experiment was conducted simultaneously in triplicate.

6. Sexing of fish

Sexing of the juvenile fish was done by the standard acetocarmine squash technique of gonads [20]. Histological studies of the gonads were also performed.

7. Statistical analysis

Data were analyzed by IBM SPSS Statistics Version 20.0 software. Normality of variables was checked before conducting T-probe or ANOVA. Treatment means were compared by Tukey's HSD test.

8. Qualitative phytochemical studies

Qualitative phytochemical analysis of the aqueous extracts of the *Tribulus* seed and *Basella* leaves were carried out using standard procedures [21-23].

3. Results

The yield percentage for aqueous extracts of *B. alba* leaves and *T. terrestris* seeds were 28.35% and 13.4%, respectively. No significant difference ($P>0.05$) was observed for survival percentage among the various treatment groups of fish fed with powdered plant materials, MT and control feed (Figure 1). The fish fed diets containing powdered *B. alba* leaves showed the highest survival percentage (94.4±2.2), while the survival percentage was the lowest in the untreated control group (88.3±1.7). The percentage of males (44.4±1.6) in the untreated control diet fed fish was significantly lower ($P<0.05$) as compared to other treatment groups (Figure 1). Fish fed diets containing powdered *B. alba* leaves and *T. terrestris* seeds showed 61.6±2.5% and 65.5±3.1% males, respectively, which are significantly lower ($P<0.05$) compared to percentage of males in fish fed MT treated diet (95.6±0.9). The MT treated fish group showed the lowest percentage of females (2.7±0.03), while the control group showed the highest (55.6±1.6). The percentage of females and intersex in control and MT treated groups showed significant difference ($P<0.05$) compared to those in both *B. alba* and *T. terrestris* treated fish (Figure 1). The control diet fed group showed no

intersex fish while the highest percentage of intersex fish (12.5±1.6) was observed in tilapia fed diets containing powdered *T. terrestris* seeds.

In fish fed diets containing powdered *B. alba* leaves, there is no significant difference (P>0.05) for survival, female and intersex percentage among various concentrations (Table 1). However, fish fed diets with 10.0 g/kg concentration showed significantly higher (P<0.05) percentage of males compared to 5.0 g/kg and 15.0 g/kg groups (Table 1). In fish fed diets containing powdered *T. terrestris* seeds also, no significant difference (P>0.05) was observed among various concentration categories for survival percentage (Table 2). The maximum percentage of males (76.6±0.5) was observed at the concentration of 15.0 g/kg, which was significantly higher (P<0.05) than the other two concentration categories. The percentage of females in the 15.0 g/kg group was also significantly lower (P<0.05) as compared to the other groups. The highest percentage of intersex fish was observed in 5.0 g/kg treatment group (18.03±1.4), which is significantly higher (P<0.05) than intersex percentage in the other two concentration categories (Table 2).

There was no significance difference (P>0.05) in survival, male, female and intersex percentage between the *B. alba* and *T. terrestris* treated fish during immersion treatment (Figure 2). Though fish immersed in *B. alba* aqueous extract showed higher survival percentage (90.6±2.1) compared to fish immersed in *T. terrestris* aqueous extract (81.9±1.0), the percentage of males was higher in *T. terrestris* treatment (75.0±1.8) than that in *B. alba* treatment (63.2±2.4).

In fish immersed in aqueous extracts of *B. alba* leaves, there was no significant difference (P>0.05) for survival and intersex percentage among various concentrations (Table 3). However, fish treated with immersion in aqueous extracts of *B. alba* leaves at a concentration of 0.1 g/l showed significantly higher (P<0.05) percentage of males compared to 0.05 g/l and 0.15 g/l treatment groups. On the other hand, treatment group of 0.15 g/l showed significantly higher (P<0.05) percentage of females than the lower two concentration categories (Table 3). During immersion treatment with aqueous seed extract of *T. terrestris* at various concentration, no significant difference (P>0.05) was observed among the different concentration groups for survival and intersex percentage (Table 4). The highest percentage of males (81.4±0.5) was found in 0.15 g/l treatment category and this was significantly higher (P<0.05) compared to the two lower concentration groups. The 0.15 g/l concentration category also showed the lowest percentage of females (13.4±0.7), which is significantly lower (P<0.05) compared to the 0.1 g/l category, while the percentage of females in 0.05 g/l group was homogenous to that in both 0.1 g/l and 0.15 g/l groups (Table 4).

Qualitative analysis for phytochemicals revealed the presence of tannins, alkaloids, saponin and steroids in both *Basella* and *Tribulus* water extracts while flavonoids, glycosides and carbohydrates were not present in any of the extracts.

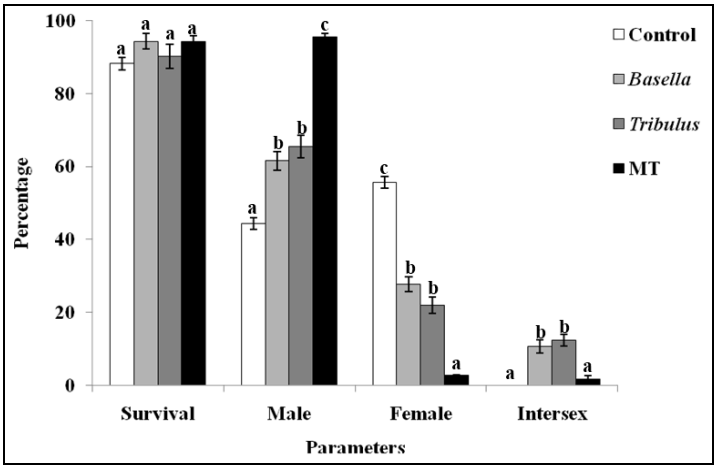

Fig 1: Percentage of survival, male, female and intersex in tilapia fed diets containing untreated control, powdered *B. alba* leaves, *T. terrestris* seeds, and MT. Different alphabets above columns mark significant difference (P<0.05) in means.

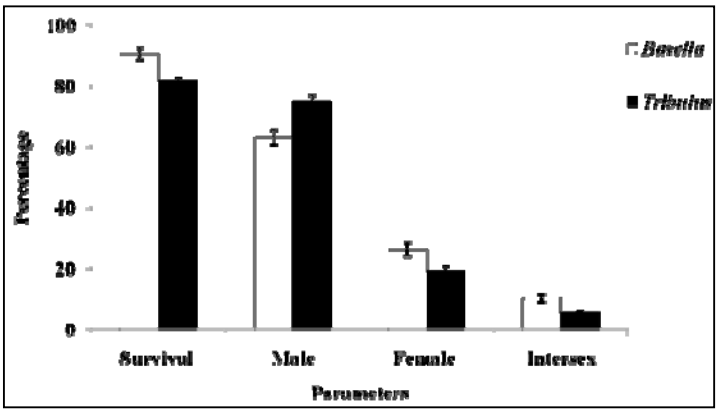

Fig 2: Percentage of survival, male, female and intersex during immersion treatment with aqueous extracts of *B. alba* leaves and *T. terrestris* seeds.

4. Discussion

The androgenic effects of two important plants *B. alba* and *T. terrestris* have been investigated in the present study. High yields were obtained for the aqueous extracts from both *B. alba* leaves and *T. terrestris* seeds. Similar high yield from *B. alba* leaves has also been reported with methanol in successive extraction with petroleum ether, ethyl acetate and methanol [11].

The result indicates that treatment with either powdered *B. alba* leaves and *T. terrestris* seeds or their aqueous extracts has no adverse effects on general fish health. Similar results were found in other studies, where immersion treatment of *P. reticulata* and *P. latipinna* with *T. terrestris* extract showed no significant difference in survival compared to that of untreated control [16, 17].

B. alba has been reported to be used in traditional medicine to treat sexual asthenia and infertility in man [24]. The methanol extract of its leaves was found to stimulate testosterone production in testicular fractions and Leydig cell cultures, and in normal adult albino male rats [10, 25]. Similar increase in serum testosterone level was also reported in male rats treated with aqueous extract of *B. alba* through gastric intubation [9]. Dietary treatment with methanol extract of *B. alba* was reported to cause significant increase in percentage of males in guppy, *Poecilia reticulata* [12]. Interestingly, the highest treatment concentration of 15.0 g/kg and 0.15 g/l produced the lowest percentage of males among the different treatment categories in the present study (Table 1 and 3). Reduced masculinisation and paradoxical feminization has been observed in fish treated with high concentration of synthetic steroids as well [26, 27].

Dietary inclusion of commercially available *T. terrestris* extract at a concentration of 2.5 g/kg basal diet have resulted in 84% male population in *O. niloticus* [28]. In another experiment, 97% masculinisation was achieved in *P. latipinna* by immersing 0-day-old fry for 60 days in water containing 50 ppm *T. terrestris* extracted in 70% ethanol [17]. Results emanating from this study indicate a dose dependent masculinisation effect of *T. terrestris* extract on Nile tilapia, which corroborates with other studies, where percentage of males increased with increase in the *T. terrestris* concentration in *P. latipinna*, *P. reticulata*, *Cichlasoma nigrofasciatum* and *Clarias gariepinus* [17, 29, 16, 15, 30]. As the highest treatment concentration of 15.0 g/kg feed and 0.15 g/l produced the maximum percentage of males among the different treatment categories in this study (Table 2 and 4), further analysis with increased concentration might be required to achieve 100% sex reversal with *T. terrestris*. The plant has been reported to be used in traditional medicine to treat sexual asthenia and infertility in man [31]. Oral treatment with *T. terrestris* extract was found to significantly increase body weight, intracavernous pressure, mount and intromission frequencies while to decrease mount latency and postejaculatory interval in Sprague-Dawley rat [32]. *T. terrestris* extract containing a steroid saponin protodioscin, was reported to increase sex hormone concentration in rat [33, 34]. However, it was observed that the steroid saponins in *T. terrestris* possess neither direct nor indirect androgen-increasing properties in young men [35]. Although the present work has indicated that dietary and immersion treatment with powder and aqueous extract, respectively, of both the plants might induce high rate of masculinisation, whether this potency is caused by increase in androgen level cannot be deduced as the serum testosterone level was not measured during the study. Qualitative analysis for phytochemicals revealed the presence of tannins, saponins, steroids and alkaloids in the aqueous extract of *B. alba* leaves and *T. terrestris* seeds, while flavonoids, glycosides and carbohydrates are not present in the extract. These phytoconstituents might render the androgenic activity of the extracts. A variety of pathways have been postulated to be associated with functional mechanisms of phyto-compounds causing both masculinisation and feminization at different concentrations [36]. Further analysis is required to deduce the functional mechanisms behind the androgenic potency of these two plants.

The results emanating from this study indicate that both investigated plants might be used as an alternative method to produce all-male tilapia population in an environment-friendly manner using a natural product. However, *T. terrestris* might be regarded to be more potent for induction of masculinization in Nile tilapia as it produced higher percentage of males compared to *B. alba* by both treatment methods. Both plants showed higher percentage of males during immersion treatment with aqueous extracts compared to dietary treatment with powdered plant materials. But, no conclusive remark can be made regarding the best mode of application for the plant materials as the concentrations used for both methods were not same. Dietary treatment might seem more practical approach for large scale production of monosex tilapia under field condition. Moreover, the highest percentage of males produced by the plant materials was found to be well below the ideal requirement of 100% male population. Thus, further studies would be required to establish an ideal treatment regime for production of all-male tilapia population using the plant materials and to provide conclusive evidence regarding their efficacy to be used as a sex-reversal agent in tilapia culture.

Table 1: Percentage of survival, male, female and intersex during feeding treatment with powdered *B. alba* leaves at different concentrations. Different superscripts mark significant difference ($P<0.05$) in means within columns.

Treatment category	% survival	% of male	% of female	% of intersex
Basella 5.0 g/kg	94.2±4.6^a	61.1±0.6^b	27.1±4.2^a	11.8±3.7^a
Basella 10.0 g/kg	98.3±1.7^a	70.3±1.2^c	22.9±1.1^a	6.8±2.3^a
Basella 15.0 g/kg	90.8±4.6^a	53.3±1.4^a	33.2±1.6^a	13.5±2.5^a

Table 2: Percentage of survival, male, female and intersex during feeding treatment with powdered *T. terrestris* seeds at different concentrations. Different superscripts mark significant difference ($P<0.05$) in means within columns.

Treatment category	% survival	% of male	% of female	% of intersex
Tribulus 5.0 g/kg	97.5±2.5^a	55.8±2.4^a	26.2±3.6^b	18.0±1.4^b
Tribulus 10.0 g/kg	89.2±5.5^a	64.1±0.8^b	25.6±1.0^b	10.3±1.5^a
Tribulus 15.0 g/kg	84.2±6.8^a	76.6±0.5^c	14.2±2.0^a	9.2±1.7^a

Table 3: Percentage of survival, male, female and intersex during immersion treatment with aqueous seed extract of *B. alba* at different concentrations. Different superscripts mark significant difference ($P<0.05$) in means within columns.

Treatment category	% Survival	% of male	% of female	% of intersex
Basella 0.05 g/l	85.8±5.5^{a}	61.1±1.4^{a}	25.0±3.9^{a}	13.9±2.9^{a}
Basella 0.1 g/l	91.7±1.7^{a}	71.9±1.9^{b}	20.8±1.4^{a}	7.3±0.9^{a}
Basella 0.15 g/l	94.2±0.8^{a}	56.7±0.8^{b}	33.6±1.6^{b}	9.7±0.9^{a}

Table 4: Percentage of survival, male, female and intersex during immersion treatment with aqueous seed extract of *T. terrestris* at different concentrations. Different superscripts mark significant difference ($P<0.05$) in means within columns.

Treatment category	% Survival	% of male	% of female	% of intersex
Tribulus 0.05 g/l	83.3±1.7^{a}	71.5±2.1^{a}	21.3±3.2^{ab}	7.2±1.1^{a}
Tribulus 0.1 g/l	80.8±2.2^{a}	72.0±0.8^{a}	23.0±0.5^{b}	5.0±1.0^{a}
Tribulus 0.15 g/l	81.7±1.7^{a}	81.4±0.5^{b}	13.4±0.7^{a}	5.2±1.2^{a}

5. Conclusion

The results emanating from this study indicate that both the plants might be used as an alternative method to produce all-male tilapia population in an environment-friendly manner using a natural product. However, *T. terrestris* might be regarded to be more potent for induction of masculinization in Nile tilapia as it produced higher percentage of males compared to *B. alba*. However, the highest percentage of males produced by the plant materials was found to be well below the ideal requirement of 100% male population. Thus, further studies would be required to establish an ideal treatment regime for production of all-male tilapia population using the plant materials and to provide conclusive evidence regarding their efficacy to be used as a sex-reversal agent in tilapia culture.

6. Acknowledgement

The research is financed by the University Grants Commission (Major Research Project, F. No. 42-519/2013(SR)). The Vice Chancellor, University of Calcutta is also acknowledged for giving permission for conducting the research work.

7. References

1. Ridha MT. Comparative study of growth performance of three strains of Nile tilapia, *Oreochromis niloticus*, L. at two stocking densities. Aquaculture Research. 2006; 37(2):172-179.
2. El-Saidy DMSD, Gaber MMA. Effect of dietary protein levels and feeding rates on growth performance, production traits and body composition of Nile tilapia, *Oreochromis niloticus* (L.) cultured in concrete tanks. Aquaculture Research. 2005; 36(2):163-171.
3. Hines GA, Watts SA. Nonsteroidal chemical sex manipulation of tilapia. Journal of World Aquaculture Society. 1995; 26(1):98-102.
4. Papoulias DM, Noltie DB, Tillitt DE. Effects of methyl-testosterone exposure on sexual differentiation in medaka, *Oryzias latipes*. Marine Environmental Research. 2000; 50:181-184.
5. Citarasu T. Herbal biomedicines: a new opportunity for aquaculture industry. Aquaculture International. 2010; 18:403-414.
6. Chakraborty SB, Hancz C. Application of phytochemicals as immunostimulant, antipathogenic and antistress agents in finfish culture. Reviews in Aquaculture. 2011; 3:103-119.
7. Rempel MA, Schlenk D. Effects of environmental estrogens and antiandrogens on endocrine function, gene regulation, and health in fish. International Review of Cell and Molecular Biology. 2008; 267:207-252.
8. Bamidele O, Akinnuga AM, Olorunfemi JO, Odetola OA, Oparaji CK, Ezeigbo N. Effects of aqueous extract of *Basella alba* leaves on haematological and biochemical parameters in albino rats. African Journal of Biotechnology. 2010; 9(41):6952-6955.
9. Moundipa PF, Kamtchouing P, Koueta N, Tantchou J, Foyang NPR, Mbiapo FT. Effects of aqueous extracts of *Hibiscus macranthus* and *Basella alba* in mature rat testis function. Journal of Ethnopharmacology. 1999; 65:133-139.
10. Moundipa PF, Beboy NSE, Zelefack F, Ngoula S, Tsamo E, Schill WB *et al*. Effects of *Basella alba* and *Hibiscus macranthus* extracts on testosterone production of adult rat and bull Leydig cells. Asian Journal of Andrology. 2005; 7(4):411-417.
11. Siriwatanametanon N, Fiebich BL, Efferth T, Prietoa JM, Heinrich M. Traditionally used Thai medicinal plants: In vitro anti-inflammatory, anticancer and antioxidant activities. Journal of Ethnopharmacology. 2010; 130:196-207.
12. Chakraborty SB, Molnár T, Hancz C. Effects of methyltestosterone, tamoxifen, genistein and *Basella alba* extract on masculinization of guppy (*Poecilia reticulata*). Journal of Applied Pharmaceutical Science. 2012; 2:048-052.
13. Chakraborty SB, Molnár T, Ardó L, Jeney G, Hancz C. Oral administration of *Basella alba* leaf methanol extract and genistein enhances the growth and non-specific immune responses of *Oreochromis niloticus*. Turkish Journal of Fisheries and Aquatic Sciences. 2015; 15:167-173.
14. Bucci LR. Selected herbals and human exercise performance. The American Journal of Clinical Nutrition. 2000; 72(2 Suppl):624S-636S.
15. Çek Ş, Turan F, Atik E. Masculinization of Convict Cichlid (*Cichlasoma nigrofasciatum*) by immersion in *Tribulus terrestris* extract. Aquaculture International 2007a; 15(2):109-119.
16. Çek Ş, Turan F, Atik E. The effects of gokshura, *Tribulus terrestris* on sex differentiation of guppy, *Poecilia reticulata*. Pakistan Journal of Biological Sciences. 2007b; 10(5): 718-725.
17. Kavitha P, Subramanian P. Effect of *Tribulus terrestris* on monosex production in *Poecilia latipinna*. Current Science. 2011; 101(1):100-104.
18. Ignacimuthu S, Ayyanar M, Sankarasivaraman K. Ethnobotanical study of medicinal plants used by Paliyar tribals in Theni district of Tamil Nadu, India. Fitoterapia, 2008; 79:562-568.
19. Gurib-Fakim A. Medicinal plants: Traditions of yesterday and drugs of tomorrow. Molecular Aspects of Medicine 2006; 27:1-93.
20. Guerrero RD, Shelton WL. An aceto-carmine squash

method for sexing juvenile fishes. The Progressive Fish-Culturist, 1974; 36(1):56.
21. Malpani MO, Rajput PR, Mane VD, Dhabe PS. Phytochemical screening, anifungal activity and curative impact on *Channa punctatus* fish of *Butea monosperma* (Lam): flower, leaves and gum. Der Pharmacia Lettre. 2011; 3(5):271-280.
22. Kumar A. Bhardwaj A. Comparative, qualitative and quantitative chemotypic characterization among north Indian *Tribulus terrestris*. International Research Journal of Pharmacy. 2012; 3(6):212-218.
23. Ray S, Chatterjee S, Chakrabarti CS. Antiprolifertive activity of allelochemicals present in aqueous extract of *Synedrella nodiflora* (L.) Gaertn. In apical meristems and Wistar rat bone marrow cells. IOSR Journal of Pharmacy. 2013, 3(2):1-10.
24. Adhikari R, Naveen HNK, Shruthi SD. A Review on Medicinal Importance of *Basella alba* L. International Journal of Pharmaceutical Sciences and Drug Research. 2012; 4:110-114.
25. Nantia EA, Travert C, Manfo FPT, Carreau S, Monsees TK, Moundipa PF. Effects of the Methanol Extract of *Basella alba* L (Basellaceae) on Steroid Production in Leydig Cells. International Journal of Molecular Sciences. 2011; 12(1):376-384.
26. Beardmore JA, Mair GC, Lewis RI. Monosex male production in finfish as exemplified by tilapia: applications, problems, and prospects. Aquaculture 2001; 197:283-301.
27. Devlin RH, Nagahama Y. Sex determination and sex differentiation in fish: an overview of genetic, physiological, and environmental influences. Aquaculture 2002; 208:191-364.
28. Omitoyin BO, Ajani EK, Sadiq HO. Preliminary investigation of *Tribulus terrestris* (Linn., 1753) extract as natural sex reversal agent in *Oreochromis niloticus* (Linn., 1758) larvae. International Journal of Aquaculture. 2013; 3(23):133-137.
29. Kavitha P, Ramesh R, Subramanian P. Histopathological changes in *Poecilia latipinna* male gonad due to *Tribulus terrestris* administration. In Vitro Cellular and Developmental Biology-Animal. 2012; 48(5):306-312.
30. Turan F, Çek. Ş. Masculinization of African catfish (*Clarias gariepinus*) treated with gokshura (*Tribulus terrestris*). Israeli Journal of Aquaculture – Bamidgeh. 2007; 59(4):224-229.
31. Adaikan PG, Gauthaman K, Prasad RNV. History of herbal medicines with an insight on the pharmacological properties of *Tribulus terrestris*. The Aging Male. 2001; 4(3):163-169.
32. Gauthaman K, Ganesan AP, Prasad RNV. Sexual effects of puncturevine (*Tribulus terrestris*) extract (Protodioscin): an evaluation using a rat model. The Journal of Alternative and Complementary Medicine. 2003; 9(2):257-265.
33. Gauthaman K, Adaikan PG, Prasad RNV. Aphrodisiac properties of *Tribulus terrestris* extract (Protodioscin) in normal and castrated rats. Life Sciences. 2002; 71(12):1385-1396.
34. Gauthaman K, Ganesan AP. The hormonal effects of *Tribulus terrestris* and its role in the management of male erectile dysfunction – an evaluation using primates, rabbits and rat. Phytomedicine. 2008; 15:44-54.
35. Neychev VK, Mitev VI. The aphrodisiac herb *Tribulus terrestris* does not influence the androgen production in young men. Journal of Ethnopharmacology. 2005; 101(3):319-323.
36. Chakraborty SB, Horn P, Hancz C. Application of phytochemicals as growth-promoters and endocrine modulators in fish culture. Reviews in Aquaculture, 2014; 6:1-19.