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Efficacy of *Basella alba* and *Tribulus terrestris* extracts for production of monosex Nile tilapia, *Oreochromis niloticus*

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ABSTRACT

The present study was aimed to evaluate the efficacy of *Basella alba* and *Tribulus terrestris* for induction of masculinisation in Nile tilpia. *B. alba* leaves and *T. terrestris* seeds were extracted with water, ethanol, methanol, dichloromethane, hexane and successive methanol and mixed sex juveniles of Nile tilapia were subjected to dietary treatment with the extracts at the concentration of 0.5, 1.0 and 1.5 gm/kg feed. Treatment with both the plants showed no adverse effect on general fish health. There was no significant interaction effects ($P>0.05$) of solvent and concentration, and solvent and plant material for percentage of males. But, significant interaction effect ($P<0.05$) of concentration and plant material was observed for percentage of males. Also, there was significant interaction effect ($P<0.05$) of solvent, concentration and plant material for percentage of males. For dietary administration of *B. alba* leaves, the highest percentage of males (83.2±0.7) was obtained by treatment with ethanol extract at the concentration of 1.0 gm/kg feed. For all the solvents, the highest percentage of males was observed at the concentration of 1.0 gm/kg. But, in treatment with *T. terrestris* seeds, the highest percentage of males (88.9±1.1) was obtained with ethanol extract at the concentration of 1.5 gm/kg feed, which was also the highest percentage of males for all the treatment categories.

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 (Ridha, 2006). Rapid growth, high tolerance to low water quality, efficient food conversion, resistance to disease, ease of spawning and good consumer acceptance make tilapia a suitable fish for culture (El-Saidy and Gaber, 2005). Females of tilapine 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 (Hines and Watts, 1995). 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 (Papoulias *et al.,* 2000). Plant extracts containing diverse bioactive principles such as alkaloids, flavonoids, pigments, phenolics, terpenoids, steroids and essential oils have been reported to promote various activities like antistress, growth promotion, appetite stimulation, tonic and immunostimulation, and antimicrobial properties in fish culture (Citarasu, 2010; Chakraborty *et al.,* 2011). 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 (Rempel *et al.,* 2008) 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 (Bamidele *et al.*, 2010), has been reported to possess active components that increase testosterone production in adult male rat testes during *in vitro* studies (Moundipa *et al.*, 1999; Moundipa *et al.*, 2005). This edible plant has also been described to possess nutritional values including androgenecity in traditional medicines of several countries (Siriwatanametanon *et al.*, 2010).

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However, no studies have been reported related to its *in vivo* effect on sex reversal, growth and immunostimulation of fish. The herb, *Tribulus terrestris* has been reported to raise testosterone levels (Bucci, 2000) and to induce sex reversal in fish while administered through immersion technique (Çek *et al.,* 2007a; Çek *et al.,* 2007b). *T. terrestris* has been observed to be effective for production of monosex *Poecilia latipinna* population (Kavitha and Subramanian, 2011). The plant extract has also been found to stimulate growth in fish (Çek *et al.*, 2007a, Çek *et al.*, 2007b). Both *B. alba* and *T. terrestris* have been reported to possess medicinal values (Ignacimuthu *et al.,* 2008). But, use of these plant extracts for sex reversal and growth induction in tilapia during its culture under Indian perspective is not documented. The type and amount of phytoconstituents in the plant extracts may vary with different solvents used for extraction, thereby showing variable results with respect to induction of masculinity (Tiwari *et al.*, 2011). 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 find the most potent solvent for extraction of the phytochemicals from the two plants that would yield the highest androgenic action and to determine an ideal treatment regime for each plants that might produce maximum percentage of males in tilapia.

METHODOLOGY

Collection of fish seed

Just 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.

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 solvents such as water, methanol, ethanol, dichloromethane and hexane in a Soxhlet apparatus and the extracts were evaporated to dryness under pressure at 45ºC using a rotary evaporator and stored under nitrogen at -20 ºC in amber glass bottle until those were used (Hussain et al. 2009). For successive extraction with solvents, plant powders (200 gm) were subjected to extraction by maceration under gentle agitation in a glass vessel for 48 h at room temperature using successively hexane (200 ml for 5 h, three times), dichloromethane (200 ml for 5 h, three times) and methanol (200 ml for 5 h, three times) (Moundipa *et al.,* 2005). The methanol extract was evaporated to dryness under pressure at 45 ºC using a rotary evaporator and stored under nitrogen at -20 ºC in amber glass bottle until it was used.

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.

Dietary treatment with different solvent extracts of the plants

This experiment had 2x6x3 factorial design: the first factor was plant materials (*B. alba* leaves and *T. terrestris* seeds), the second factor was related to solvents used for extraction (aqueous, methanol, ethanol, dichloromethane, hexane and successive methanol), the third factor was related to concentrations of extracts used for dietary treatment (0.5, 1.0 and 1.5 gm/kg feed). Three days old mixed sex juveniles of Nile tilapia (mean weight 0.025 ± 0.009 gm; mean length 1.25 ± 0.012 cm) were randomly assigned in glass aquaria (40 fish / aquaria) and three aquaria were assigned for each treatment category. Plant extracts at desired concentrations were dissolved in dimethyl sulfoxide (DMSO) and added to finely ground (<500-1000 μm) artificial diet containing 30 % crude protein (Tokyu, Japan) (Moundipa et al. 2005). The feed was then wetted with deionized water, mixed thoroughly, formed into pellets with a pelleter (diameter 2 mm), and dried at room temperature. Pelleted feed was pulverized before feeding to the juvenile fish.

Sexing of fish

Sexing of the juvenile fish was done by the standard acetocarmine squash technique of gonads (Guerrero and Shelton, 1974). Histological studies of the gonads were also performed.

Statistical analysis

Data were analyzed by IBM SPSS Statistics Version 20 software. Normality of variables was checked before conducting T-probe or ANOVA in GLM where solvent and concentration were considered as fixed and plant as random factors. Treatment means were compared by Tukey's HSD test for fixed factors. For variables not normally distributed nonparametric median tests were applied to evaluate treatment effects.

Qualitative phytochemical studies

Qualitative phytochemical analysis of the extracts of *T. terrestris* seed and *B. alba* leaves were carried out using standard procedures (Malpani *et al.*, 2011; Kumar and Bhardwaj, 2012; Ray *et al.,* 2013).

RESULTS AND DISCUSSION

For both plants, the maximum yield of evaporated dried extracts based on dry weight basis was obtained with water as extracting solvent (Figure 1). The yield for aqueous extract of *B. alba* leaves was 28.35 % while that for *T. terrestris* seeds was 13.4 %. Interestingly, yield for *B. alba* leaves with more polar solvents such as water, methanol and ethanol were comparatively higher than those for *T. terrestris* seeds, but with solvents such as dichloromethane and hexane, the yield percentage is higher for *T. terrestris* seeds (Figure 1). Moreover, the yield percentage with successive methanol was found to be higher for *T. terrestris* seeds (4.3%) compared to that for *B. alba* leaves (3.47 %). However, the yield percentage decreased with decreased polarity of the solvents for both the plants (Figure 1). In order to eliminate the influence of the moisture content of the plant, the yield of the extracts from *B. alba* leaves and *T. terrestris* seeds with different solvents were calculated depending on a dry weight basis and the yields were found to be high. Similar high yield from *B. alba* leaves has been reported with methanol in successive extraction with petroleum ether, ethyl acetate and methanol (Siriwatanametanon *et al.*, 2010). Maximum yield percentage for T. terrestris seeds was obtained with aqueous extraction in a study using solvents such as petroleum ether, chloroform, acetone, alcohol and water (Asadulla 2011).

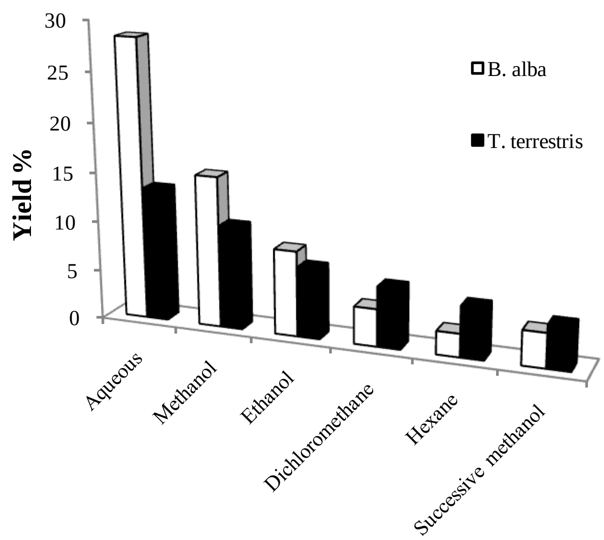

Fig. 1: Yield percentage of evaporated dried extracts based on dry weight basis with different solvents for *B. alba* leaves and *T. terrestris* seeds.

During treatment with different solvent extracts of *B. alba* leaves and *T. terrestrsis* seeds, the survival percentage was 94.96±0.6. This high survival indicates that the treatment with both plant extracts has no adverse effects on the general health of the fish. In these groups, the percentage of males was 70.6±0.8, females 22.9±0.7%, while 6.5±0.5% of the treated fish was found to be intersex. However, the variables except the percentage of males in different treatment categories are not normally distributed and could not be transformed to achieve normal distribution. Effects of plant materials (*B. alba* leaves and *T. terrestris* seeds), solvents used for extraction (aqueous, methanol, ethanol, dichloromethane, hexane and successive methanol), and concentrations of extracts used for dietary treatment (0.5, 1.0 and 1.5 gm/kg feed) on percentage of males in tilapia are given in Table 1. The percentage of males was the lowest in hexane and dichloromethane extraction, aqueous extract showed significantly higher (P<0.05) percentage of males compared to that in those two groups, but significantly lower (P<0.05) percentage of males than that in ethanol, methanol and successive methanol categories (Table 1). Extraction with ethanol showed the highest percentage of males (74.4±2.8) among the solvents.

Percentage of males was significantly different (P<0.05) for all concentration categories (Table 1). The highest percentage (75.0±0.8) of males was observed in 1.0 gm/kg feed concentration category, while the lowest percentage of males (66.1±1.0) was observed in 0.5 gm/kg feed group (Table 1). Plant material showed no significant effect (P>0.05) on percentage of males. However, treatment with *T. terrestris* seeds yielded higher percentage of males than *B. alba* leaves (Table 1).

Table 1: Factorial ANOVA comparing percentage of males in tilapia between treatments with extraction with different solvents (aqueous, methanol, ethanol, dichloromethane. Hexane and successive methanol), concentrations (0.5, 1.0, 1.5 gm/kg) and plant materials (*B. alba* leaves and *T. terrestris* seeds). Notations a, b and c are to compare between the means of solvent extraction category, notations x, y and z are to compare between the means of concentration and notation p is to compare between the means of plant materials. Values with different superscripts are significantly different (P<0.05). NS: Not significant. S: Significant.

		Male (%)
Solvent (S)	Aqueous	70.2 ± 1.5^{b}
	Methanol	72.2 ± 1.6^{bc}
	Ethanol	74.4 ± 2.8^{c}
	Dichloromethane	66.8 ± 1.8^{a}
	Hexane	66.5 ± 2.2^{a}
	Successive methanol	73.3 ± 1.6^{c}
Concentration (gm/kg) (C)	0.5	66.1 ± 1.0^{x}
	1.0	75.0 ± 0.8^{y}
	1.5	70.7 ± 1.9^{z}
Plant material (P)	*Basella alba* leaves	68.3 ± 1.1^{p}
	Tribulus terrestris seeds	72.8 ± 1.2^{p}
S X C		NS
S X P		NS
C X P		S
S X C X P		S

There was no significant interaction effects (P>0.05) of solvent and concentration, and solvent and plant material for percentage of males (Table 1). But, significant interaction effect (P<0.05) of concentration and plant material was observed for percentage of males (Table 1, Figure 2).

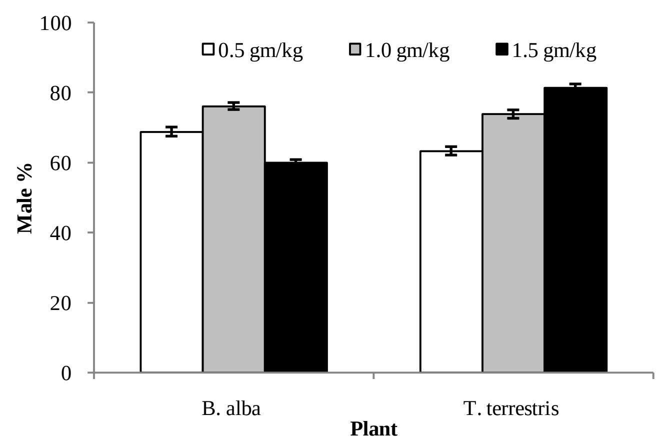

Fig. 2: Percentage of males in tilapia fed diets containing different concentrations of *B. alba* leaves and *T. terrestris* seeds extracts. Different alphabets above column indicates significant difference (P<0.05) in means.

In treatment with *B. alba* leaves, the percentage of males for every concentration differed significantly (P<0.05) from each other, and the highest percentage of males (76.1±1.0) was observed at the concentration of 1.0 gm/kg feed (Figure 2). Similar significant difference (P<0.05) in male percentage for every concentration was also observed in treatment with *T. terrestris* seeds, but the highest percentage of males (81.3±1.1) was found at the concentration of 1.5 gm/kg feed (Figure 2). There was significant interaction effect (P<0.05) of solvent, concentration and plant material for percentage of males (Tables 1, 2). For dietary administration of *B. alba* leaves, the highest percentage of males (83.2±0.7) was obtained for treatment with ethanol extract at the concentration of 1.0 gm/kg feed followed by treatment with successive methanol extract at the same concentration (Table 2). For all the solvents, the highest percentage of males was observed at the concentration of 1.0 gm/kg (Table 2). But, in treatment with *T. terrestris* seeds, the highest percentage of males (88.9±1.1) was obtained with ethanol extract at the concentration of 1.5 gm/kg feed, which was also the highest percentage of males for all the treatment categories (Table 2). For *T. terrestris* treatment with all the solvents, the highest percentage of male was observed at the concentration of 1.5 gm/kg feed (Table 2).

Table 2: Percentage of males in tilapia fed diets containing extraction of *B. alba* leaves and *T. terrestris* seeds with different solvents and at different concentrations. Different superscripts mark significant difference (P<0.05) in means within columns.

Plant material	Solvent	Concentration	Male %
B. alba leaves	Aqueous	0.5 gm/kg	65.4 ± 0.7^{cdefg}
		1.0 gm/kg	72.9 ± 0.8^{ghijklmn}
		1.5 gm/kg	62.1 ± 1.1^{abcde}
	Methanol	0.5 gm/kg	73.7 ± 1.9^{ijklmno}
		1.0 gm/kg	74.7 ± 1.0^{klmno}
		1.5 gm/kg	63.3 ± 1.7^{bcdef}
	Ethanol	0.5 gm/kg	74.2 ± 1.4^{jklmno}
		1.0 gm/kg	83.2 ± 0.7^{pq}
		1.5 gm/kg	54.9 ± 1.0^{a}
	Dichloromethane	0.5 gm/kg	65.5 ± 0.9^{cdefgh}
		1.0 gm/kg	71.3 ± 1.6^{fghijklm}
		1.5 gm/kg	58.4 ± 0.9^{abcd}
	Hexane	0.5 gm/kg	60.7 ± 0.4^{abcde}
		1.0 gm/kg	76.2 ± 1.0^{lmnop}
		1.5 gm/kg	58.3 ± 1.0^{abcd}
	Successive methanol	0.5 gm/kg	73.5 ± 0.8^{hijklmno}
		1.0 gm/kg	78.2 ± 0.3^{lmnop}
		1.5 gm/kg	63.3 ± 0.4^{bcdef}
T.terrestris seeds	Aqueous	0.5 gm/kg	67.2 ± 1.5^{efghijk}
		1.0 gm/kg	73.0 ± 1.5^{ghijklmno}
		1.5 gm/kg	80.8 ± 0.8^{nop}
	Methanol	0.5 gm/kg	66.2 ± 1.9^{defghij}
		1.0 gm/kg	74.2 ± 3.0^{jklmno}
		1.5 gm/kg	81.0 ± 3.0^{opq}
	Ethanol	0.5 gm/kg	65.7 ± 0.5^{cdefghi}
		1.0 gm/kg	79.5 ± 0.3^{nop}
		1.5 gm/kg	88.9 ± 1.1^{q}
	Dichloromethane	0.5 gm/kg	57.8 ± 1.5^{abc}
		1.0 gm/kg	70.2 ± 0.4^{fghijkl}
		1.5 gm/kg	77.4 ± 1.4^{lmnop}
	Hexane	0.5 gm/kg	56.0 ± 1.9^{ab}
		1.0 gm/kg	67.7 ± 1.5^{efghijk}
		1.5 gm/kg	79.9 ± 0.1^{nop}
	Successive methanol	0.5 gm/kg	66.7 ± 1.7^{efghijk}
		1.0 gm/kg	78.3 ± 1.7^{mnop}
		1.5 gm/kg	79.8 ± 2.7^{nop}

Results of non-parametric tests for percentage of survival, females and intersex, which showed no normal distribution, indicated that only the medians of female percentage differed significantly (P<0.05) across categories of plant, solvent and concentration (Table 3). Qualitative analysis for phytochemicals revealed the presence of alkaloids and steroids in all the solvent extracts for both *B. alba* leaves and *T. terrestris* seeds (Table 4). Tannins and saponins were present in aqueous, ethanol and methanol extracts for both the plants, and in hexane extract of *B. alba*. Flavonoids were present in all solvent extracts except aqueous for both the plants, while glycosides were not found in any extracts. Carbohydrates were found in ethanol and methanol extracts of *B. alba* leaves and only in methanol extract for *T. terrestris* seeds (Table 4). The result indicates that treatment with different solvent extracts of both *B. alba* leaves and *T. terrestris* seeds has no adverse effects on general fish health. Similar results were obtained in other studies with *Poecilia reticulata* and *P. latipinna* as well where immersion treatment with *T. terrestris* extract showed no significant difference in survival of fish compared to that of untreated control (Çek *et al.*, 2007b; Kavitha & Subramanian, 2011). Immersion treatment with *B. alba* leaf aqueous extract resulted in no significant difference in survival of tilapia (Ghosal and Chakraborty 2014). *B. alba* has been reported to be used in traditional medicine to treat sexual asthenia and infertility in man (Adhikari *et al.*, 2012). 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 (Moundipa *et al.*, 2005; Nantia *et al.*, 2011). Similar increase in serum testosterone level was also reported in male rats treated with aqueous extract of *B. alba* through gastric intubation (Moundipa *et al.*, 1999). Dietary treatment with methanol extract of *B. alba* was reported to cause significant increase in percentage of males in guppy, *Poecilia reticulata* (Chakraborty *et al.*, 2012). Interestingly, during treatment with *B. alba* leaf extracts for all the solvents, the highest treatment concentration of 1.5 gm/kg produced the lowest percentage of males among the different treatment categories in the present study (Table 2). Reduced masculinisation and paradoxical feminization has been observed in fish treated with high concentration of synthetic steroids as well (Beardmore *et al.*, 2001; Devlin and Nagahama, 2002). Dietary inclusion of commercially available *T. terrestris* extract at a concentration of 2.5 gm/kg basal diet have resulted in 84 % male population in *O. niloticus* (Omitoyin *et al.*, 2013).

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 (Kavitha and Subramanian, 2011). Results emanating from this study indicate a dose dependent masculinisation effect of *T. terrestris* extracts 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* (Kavitha and Subramanian, 2011; Kavitha *et al.*, 2012; Çek *et al.*, 2007b; Çek *et*

Table. 3: Non-parametric tests for survival percentage, female percentage and intersex percentage for plants, solvents and concentrations. Asymptotic significances are displayed. The significance level is 0.05.

Hypothesis Test Summary			
Null Hypothesis	**Test**	**Significance**	**Decision**
The medians of survival percentage are the same across categories of plant	Independent Samples Median Test	0.120	Retain the null hypothesis
The medians of female percentage are the same across categories of plant	Independent Samples Median Test	0.034	Reject the null hypothesis
The medians of intersex percentage are the same across categories of plant	Independent Samples Median Test	0.178	Retain the null hypothesis
The medians of survival percentage are the same across categories of solvent	Independent Samples Median Test	0.850	Retain the null hypothesis
The medians of female percentage are the same across categories of solvent	Independent Samples Median Test	0.029	Reject the null hypothesis
The medians of intersex percentage are the same across categories of solvent	Independent Samples Median Test	0.615	Retain the null hypothesis
The medians of survival percentage are the same across categories of concentration	Independent Samples Median Test	0.860	Retain the null hypothesis
The medians of female percentage are the same across categories of concentration	Independent Samples Median Test	0.001	Reject the null hypothesis
The medians of intersex percentage are the same across categories of concentration	Independent Samples Median Test	0.641	Retain the null hypothesis

Table. 4: Qualitative analysis of phytochemicals in different solvent extracts of *B. alba* leaves and *T. terrestris* seed.

Plant	**Solvent for extraction**	**Phytochemical groups**						
		Tannin	**Saponin**	**Alkaloid**	**Carbohydrate**	**Glycoside**	**Flavonoid**	**Steroid / Terpenoid**
B. alba leaves	Aqueous	+	+	+	-	-	-	+
	Ethanol	+	+	+	+	-	+	+
	Methanol	+	+	+	+	-	+	+
	Dichloro methane	-	-	+	-	-	+	+
	Hexane	+	-	+	-	-	+	+
	Successive methanol	-	-	+	-	-	+	+
T. terrestris seeds	Aqueous	+	+	+	-	-	-	+
	Ethanol	+	+	+	-	-	+	+
	Methanol	+	+	+	+	-	+	+
	Dichloro methane	-	-	+	-	-	+	+
	Hexane	-	-	+	-	-	+	+
	Successive methanol	-	-	+	-	-	+	+

al., 2007a; Turan and Çek, 2007). As the highest treatment concentration of 1.5 gm/kg feed produced the maximum percentage of males for all solvent extracts of *T. terrestris* seeds (Table 2) however further experiments with increased concentration might be required to achieve 100% sex reversal with the plant. The plant has been reported to be used in traditional medicine to treat sexual asthenia and infertility in man (Adaikan *et al.*, 2001). 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 (Gauthaman *et al.*, 2003). *T. terrestris* extract was reported to contain steroid saponin protodioscin and found to increase concentration of some of the sex hormones in rat (Gauthaman *et al.*, 2002; Gauthaman and Ganesan, 2008). However, Neychev and Mitev (2005) observed that the steroid saponins in *T. terrestris* possess neither direct nor indirect androgen-increasing properties in young men. Successful determination of biologically active compounds from plant material is largely dependent on the type of solvent used in the extraction procedure (Ugochukwu *et al.*, 2013, Pandey and Tripathi 2014). Although the present work has indicated that treatment with extracts of different solvents 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 steroids in all the extracts of *B. alba* leaves and *T. terrestris* seeds, which 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 (Chakraborty *et al.*, 2014). Further investigations are required to deduce the functional mechanisms behind the androgenic potency of these two plants.

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* with all the solvents. Dietary treatment with ethanol extracts of both the plants resulted in production of the highest percentage of males, but the concentrations observed for such production were different. 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.

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