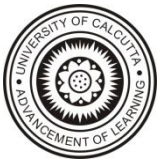
Evaluation of plants extracts on sex reversal and growth of Nile tilapia

Final Progress Report
(2013-2016)

Submitted by:

Dr. Suman Bhusan Chakraborty

Principal Investigator



Department of Zoology

University of Calcutta

Annexure –VIII

UNIVERSITY GRANTS COMMISSION

BAHADUR SHAH ZAFAR MARG

NEW DELHI – 110 002

Final Report of the work done on the Major Research Project (Report to be submitted within 6 weeks after completion of each year)

1. Project Report No.	:	Final
2. UGC Reference No.	:	F. 42-519/2013 (SR) dated 22.03.2013
3. Period of report	:	From 01.04.2013 to 31.03.2016
4. Title of research project	:	Evaluation of plants extracts on sex reversal and growth of Nile tilapia
5.		
(a) Name of the Principal Investigator	:	Dr. Suman Bhusan Chakraborty
(b) Dept.	:	Department of Zoology
(c) University where work has progressed:		The University of Calcutta
6. Effective date of starting of the project:		01.04.2013

7. Grant approved and expenditure incurred during the period of the report:

a. Total amount approved	:	Rs.10,45,300/- (Ten lakh forty five thousand three hundred only)
b. Total expenditure	:	Rs. 10,45,300/- (Ten lakh forty five thousand three hundred only)

8. Report of the work done : Separate sheet attached with brief objectives of the project and details of the performed work and achieved results

i) Publications, if any, resulting from the work : Four

1. Ghosal I, Chakraborty SB. 2014. Effects of the aqueous leaf extract of *Basella alba* on sex reversal of Nile tilapia, *Oreochromis niloticus*. IOSR Journal of Pharmacy and Biological Sciences 9(2): 162-164.
2. Ghosal I, Chakraborty SB. 2014. Effects of the aqueous seed extract of *Tribulus terrestris* on sex reversal of Nile tilapia, *Oreochromis niloticus*. Indian Journal of Applied Research 4(9): 459-461.
3. Ghosal I, Mukherjee D, Hancz C, Chakraborty SB. 2015. Efficacy of *Basella alba* and *Tribulus terrestris* extracts for production of monosex Nile tilapia, *Oreochromis niloticus*. Journal of Applied Pharmaceutical Science 5 (8): 152-158.
4. Ghosal I, Mukherjee D, Hancz C, Chakraborty SB. 2016. Production of monosex Nile tilapia, *Oreochromis niloticus* by dietary and immersion treatment with *Basella alba* leaves and *Tribulus terrestris* seeds. International Journal of Fisheries and Aquatic Studies 4(1): 358-363.

ii) Has the progress been according to original plan of work and towards achieving the objective. If not, state reasons :

Yes, the progress has been according to the original plan of work and towards achieving the objectives.

iii) Please indicate the difficulties, if any, experienced in implementing the project:

No difficulties worth mentioning were experienced in implementing the project.

iv) If project has not been completed, please indicate the approximate time by which it is likely to be completed. A summary of the work done for the period (Annual basis) may please be sent to the Commission on a separate sheet :

Not applicable.

v) If the project has been completed, please enclose a summary of the findings of the study. One bound copy of the final report of work done may also be sent to University Grants Commission :

Separate sheet has been enclosed as the summary of the study. One bound copy of the final report of the work done is also attached.

vi) Any other information which would help in evaluation of work done on the project. At the completion of the project, the first report should indicate the output, such as (a) Manpower trained (b) Ph. D. awarded (c) Publication of results (d) other impact, if any :

The JRF appointed in the project has been registered at the University of Calcutta as Ph.D. student (Indranath Ghosal, Registration No.: 0074/Ph.D.(Sc.)Proceed/2016, 6th January 2016). The candidate will be ready for thesis submission in 2018. Four publications have already been achieved from the project. Two more manuscripts are being prepared to be communicated for publication.

Suman Bhusan Chakraborty 7/7/17

Signature of Principal Investigator

Dr. Suman Bhusan Chakraborty
Assistant Professor
Department of Zoology
University of Calcutta

Signature of Registrar

10 JUL 2017

(Seal)

REGISTRAR
Calcutta University

10/7/17

Annexure – IX

UNIVERSITY GRANTS COMMISSION

BAHADUR SHAH ZAFAR MARG

NEW DELHI – 110 002

PROFORMA FOR SUBMISSION OF INFORMATION AT THE TIME OF SENDING THE FINAL REPORT OF THE WORK DONE ON THE PROJECT

1. Title of the project : Evaluation of plants extracts on sex reversal and growth of Nile tilapia.

2. Name and Address of the Principal Investigator :

Dr. Suman Bhusan Chakraborty, Department of Zoology, The University of Calcutta. 35 Ballygaunge Circular Road, Kolkata - 700019, West Bengal, India.

3. Name and address of the institution :

Department of Zoology, The University of Calcutta, 35 Ballygaunge Circular Road, Kolkata - 700019, West Bengal. India.

4. UGC approval letter no. and date : F. 42-519/2013 (SR) dated 22.03.2013.

5. Date of implementation : 01.04.2013

6. Tenure of the project : 3 Years

7. Total grant allocated : 10,45,300/- (Ten lakh forty five thousand three hundred only).

8. Total grant received : 9,62,200/- (Nine lakh sixty two thousand two hundred only).

9. Final expenditure : 10,45,300/- (Ten lakh forty five thousand three hundred only).

10. Title of the project : Evaluation of plants extracts on sex reversal and growth of Nile tilapia.

11. Objectives of the project :

Phytochemicals present in many plants have several reported biological properties. This study was intended to explore the possible utilization of two such plant extracts containing different phytochemicals as potential *in vivo* enzymatic inhibitors of aromatase and of nuclear estrogen receptors' antagonist in gonad germ cells. Such response might modulate the sex differentiation process of the gonad in sexually undifferentiated Nile tilapia and affect the sex ratio of the tilapia populations. Thus, the major objectives of the present study were:

i. Determination of the potentiality of *Basella alba* and *Tribulus terrestris* to induce sex reversal in tilapia under the ecological condition of the Gangetic plains in West Bengal, India.
ii. Determination of ideal method of *in vivo* application of the plant material for induction of sex reversal in tilapia.
iii. Determination of ideal solvent for extraction of phytochemicals from *Basella alba* and *Tribulus terrestris* for induction of sex reversal in tilapia.
iv. Determination of ideal concentration of the plant extract for induction of sex reversal in tilapia.
v. Determination of ideal treatment duration with plant extracts for induction of sex reversal in tilapia.
vi. Analysis of the growth-promoting, immunolostimulating and antioxidant properties of the plant extracts during *in vivo* application in tilapia.
vii. Identification of the bioactive principles in the plant extracts.

12. Whether objectives were achieved : Yes, the details of the results are provided in separate "Final Report".

13. Achievements from the project :

Tilapias are important foodfish in many tropical and subtropical countries. Despite promising expectations in tilapia aquaculture, there are a series of factors that are considered to be detrimental in the expansion of production. Female organism 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. Monosex population of male tilapia is produced by treating spawn with a synthetic male hormone 17α-methyltestosterone (MT). However, the increased use of synthetic steroid hormones to produce monosex populations of tilapia for intensive productive systems may lead to environmental and public health concerns. Therefore, alternative methods and new, safe chemicals to produce monosex tilapia populations should be considered.

Our experiments reveal that dietary administration of ethanol extract of *Basella alba* leaves at concentration of 1 gm/Kg feed and *Tribulus terrestris* seeds at concentration of 2 gm/Kg feed for 30 days can be used to produce all-male Nile tilapia (*Oreochromis niloticus*) in aquaculture practice. It has been found that fish fed plant extract fortified diet showed higher growth and better health compared to fish fed control diet. The plant extracts were found to contain stigmasterol, lupeol and quercetin as bioactive phytoconstituents, which may be responsible for the androgenic and immunostimulating efficacy of the plants. Thus, the outcomes of this study would help to establish tilapia culture in a scientific, economic and eco-friendly manner with maximum yield in minimum duration.

14. Summary of the findings :

The following aspects of efficacy of *Basella alba* and *Tribulus terrestris* for production and csustainable culture of all-male monosex Nile tilapia can be put forward from this study:

i. Both the leaves of *Basella alba* and seeds of *Tribulus terrestris* has the potential to induce sex reversal in tilapia. *Tribulus terrestris* is more potent than *Basella alba* in this context.

ii. The ideal method of in vivo application of the plant material for induction of sex reversal in tilpaia is the solvent based extraction method and addition of the extraction to the fish feed.

iii. The ideal solvent for extraction of phytochemicals from *Basella alba* and *Tribulus terrestris* for induction of sex reversal in tilapia is ethanol.

iv. The ideal concentration of the plant extract for induction of sex reversal in tilapia is 1 gm/Kg feed for *Basella alba* and 2 gm/Kg feed for *Tribulus terrestris*.

v. The ideal treatment duration with plant extracts for induction of sex reversal in tilapia is 30 days of feeding.

vi. *Basella alba* has more growth promoting and immunostimulating capacities than *Tribulus terrestris*. Both the plant proved that they are potent adpatogen in aquaculture which provide the fish a good non specific immunity, strengthen the hematological attributes, provides antioxidants and hepatoprotection. *Basella alba* has more antiradical or radical scavenging activity than *Tribulus terrestris*.

vii. HPTLC analysis of fractions, which gave the highest percentage of males revealed that- *Basella alba* contained stigmasterol, lupeol and quercetin; *Tribulus terrestris* contained lupeol. Further analysis with different standards would be required to pinpoint the phytoconstituents in both plant materials in this regard.

15. Contribution to the society :

Aquaculture has become the world's fastest growing food-producing sector, with a growth rate of 10% annually since 1984. Asia produces about 91% of the world's total aquaculture production, with India as one of the top producers. Freshwater aquaculture is a major source of growth for the whole Asian fishery sector and India contributed ~9.90% of the total worldwide freshwater aquaculture products in 1999. Freshwater fisheries are a priority area in our country with the general objectives of increasing fish production, improving export earnings, providing more animal protein and expanding employment opportunities in the sector. Culture of monosex all-male tilapia has proven to be beneficial for increased production during intensive tilapia culture in ponds throughout the country. However, the increased use of synthetic steroid hormones for such culture has evoked considerable environmental and public health concerns.

It is against this background that our study dealing with the production of sex reversed, massive grown all male tilapia population using biodegradable natural plant was both timely and important. The broad objective of our study was also to examine the growth inducing and immunostimulating efficacy of the plant materials during intensive pond culture of tilapia. This might reduce the cost of all-male tilapia production and thereby would benefit the poor rural communities of our country, enhancing food security by supplying cheap animal protein for household consumption. Our study demonstrated that, both leaves of *Basella alba* and seeds of *Tribulus terrestris* can be used as a substitute of 17α-Methyl testosterone to produce all-male Nile tilapia. Replacement of the synthetic steroid with plant materials during aquaculture exert an environment friendly method to achieve higher profit by lowering the environment degradation.

This method may also minimize the potential health hazards for the consumers associated with the residual effect, if any, of the synthetic steroid. Therefore, in developing nations like India, where a good number of natural open water systems is available for utilization, our study can lead to a low cost sustainable aquaculture method for the production of a major animal protein source. In this respect also, the introduction of phytochemical treated, sex reversed monosex tilapia population for culture in the natural systems deserves to be advocated and large scale field trials of our samples are awaited in India before they are marketed.

16. Whether any Ph.D. enrolled/ produced out of the project : Yes, one Ph.D. student is enrolled at University of Calcutta.

17. No. of publications out of the project : 4 (Reprints attached).

1. Ghosal I, Chakraborty SB. 2014. Effects of the aqueous leaf extract of *Basella alba* on sex reversal of Nile tilapia, *Oreochromis niloticus*. IOSR Journal of Pharmacy and Biological Sciences 9(2): 162-164.
2. Ghosal I, Chakraborty SB. 2014. Effects of the aqueous seed extract of *Tribulus terrestris* on sex reversal of Nile tilapia, *Oreochromis niloticus*. Indian Journal of Applied Research 4(9): 459-461.
3. Ghosal I, Mukherjee D, Hancz C, Chakraborty SB. 2015. Efficacy of *Basella alba* and *Tribulus terrestris* extracts for production of monosex Nile tilapia, *Oreochromis niloticus*. Journal of Applied Pharmaceutical Science 5 (8): 152-158.
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Signature of Principal Investigator 7/7/17

Dr. Suman Bhusan Chakraborty
Assistant Professor
Department of Zoology
University of Calcutta

Signature of Registrar

(Seal) 10 JUL 2017
REGISTRAR
Calcutta University

Annexure – X

UNIVERSITY GRANTS COMMISSION
BAHADUR SHAH ZAFAR MARG
NEW DELHI – 110 002

EVALUATION REPORT

1. It is certified that the final report of the Major Research Project entitled "Evaluation of plants extracts on sex reversal and growth of Nile tilapia" by Dr. Suman Bhusan Chakraborty, Deptt. of Zoology, University of Calcutta has been assessed by the 'Evaluation Committee' consisting the following members for final submission to the University Grants Commission, New Delhi:

Details of Expert Committee:

1. Prof. Debangshu Narayan Das, Rajib Gandhi University, Arunachal Pradesh
2. Prof. Ashim Kumar Nath, Sidhu Kanho Birsha University, Purulia, West Bengal

The Final Report is found to be satisfactory.

Prof. Debangshu Narayan Das

Dr. Debangshu Narayan Das
Professor Zoology
Rajiv Gandhi University
Ronohills,Itanagar-791112
Arunachal Pradesh

Prof. Ashim Kumar Nath

Dr. Ashim Kr. Nath
Professor in Zoology
Sidho-Kanho-Birsha Univ.
Purulia, W.B. - 723104

(REGISTRAR/ PRINCIPAL)
(Seal)

10 JUL 2017
REGISTRAR
Calcutta University

UNIVERSITY OF CALCUTTA

Dr. SUMAN BHUSAN CHAKRABORTY, Ph.D.
Assistant Professor
Department of Zoology
University of Calcutta
35 Ballygunge Circular Road
Kolkata - 700 019

Residence : 279/2, A. P. C. Road
Kolkata - 700 009
Phone : (033) 2351 5084
Mobile : 09831189211
E-mail : sumanbc76@gmail.com

Ref. No. : .....

OFFICE OF THE VICE CHANCELLOR
25 APR 2017
Letter No. 2102
UNIVERSITY OF CALCUTTA

Date : 24/04/2017

To
The Vice-Chancellor
University of Calcutta
Kolkata, India

Sub: Request for approval of evaluation committee for UGC MRP final report submission

Respected Sir,

I would like to inform you that I have completed a **UGC Major Research Project** titled "Evaluation of plants extracts on sex reversal and growth of Nile tilapia"' [Ref: F. No. 42-519/2013(SR) dt. 22.03.2013]. Before submission of the final report to the funding agency, the report is required to be evaluated by two experts not belonging to the implementing institution.

In this regard, I am proposing the names of two expert members for evaluation of the final project report.

I would be obliged if you approve the same and do the needful in this regard.

With warm regards,
Yours sincerely,

Suman Bhusan Chakraborty

Dr. Suman Bhusan Chakraborty
Assistant Professor
Department of Zoology
University of Calcutta

Forwarded
24/4/17

DR. PARTHIBA BASU
Head of the Department
Department of Zoology
University of Calcutta

Names of Expert:
1. **Prof. Debangshu Narayan Das**, Rajib Gandhi University, Arunachal Pradesh
2. **Prof. Ashim Kumar Nath**, Sidhu Kanho Birsha University, Purulia, West Bengal.

Approved
27/4/17

Summary of the project report (F. 42-519/2013 (SR) dated 22.03.2013)

EVALUATION OF PLANT EXTRACTS ON SEX REVERSAL AND GROWTH OF NILE TILAPIA

Despite promising expectations in tilapia aquaculture, there are a series of factors that are considered to be detrimental in the expansion of production. Female organism 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. Monosex population of male tilapia is produced by treating spawn with a synthetic male hormone 17α-methyltestosterone (MT). However, the increased use of synthetic steroid hormones to produce monosex populations of tilapia for intensive productive systems may lead to environmental and public health concerns. Chronic exposure of consumers to synthetic steroids may cause adverse health effects and its use in fish culture is associated with potential release to the environment and contamination of the biota. Therefore, alternative methods and new, safe chemicals to produce monosex tilapia populations should be considered.

Phytochemicals are a large group of plant-derived compounds that are commonly found in fruits, vegetables, beans, cereals and plant-based beverages like tea and wine. The use of medicinal plants as fertility enhancers and sex reversal agents in fish has been receiving some attention. Plant bioactive compounds might provide a useful source of new medicines and pharmaceutical entities for enhancing fish production and health; and food safety and quality, while conserving the aquatic environment. A possible alternative approach to the use of steroid hormones for sex reversal in tilapia may involve the use of plant extracts containing phytochemicals such as isoflavonoids, flavonoids and saponins, which are natural compounds characterized by estrogenic/androgenic activity. Besides, with their potent antioxidant and immunostimulating properties, phytochemicals have been found to stimulate fish growth (Makkar *et al* 2007, Citarasu 2010).

Basella alba, a fast growing vegetable native to tropical Asia, has been found to have anabolizing and virilizing effects and may be used to boost low levels of testosterone in aging males. The herb, *Tribulus terrestris* has been reported to raise testosterone levels and to induce sex reversal in fish. However, further experimentation is required to validate the possibility of the use of these plant extracts as sex reversal agents in tilapia, in order to ensure that they are as effective as the

common technique to produce monosex populations that involves the use of steroid hormones such as MT.

Objectives

Phytochemicals present in many plants have several reported biological properties. This study is intended to explore the possible utilization of two such plant extracts containing different phytochemicals as potential *in vivo* enzymatic inhibitors of aromatase and of nuclear estrogen receptors' antagonist in gonad germ cells. Such response might modulate the sex differentiation process of the gonad in sexually undifferentiated Nile tilapia and affect the sex ratio of the tilapia populations. Thus, the major objectives of the present study are:

i. Determination of the potentiality of *Basella alba* and *Tribulus terrestris* to induce sex reversal in tilapia under the ecological condition of the Gangetic plains in West Bengal, India.
ii. Determination of ideal method of *in vivo* application of the plant material for induction of sex reversal in tilapia.
iii. Determination of ideal solvent for extraction of phytochemicals from *Basella alba* and *Tribulus terrestris* for induction of sex reversal in tilapia.
iv. Determination of ideal concentration of the plant extract for induction of sex reversal in tilapia.
v. Determination of ideal treatment duration with plant extracts for induction of sex reversal in tilapia.
vi. Analysis of the growth-promoting, immunolostimulating and antioxidant properties of the plant extracts during *in vivo* application in tilapia.
vii. Identification of the bioactive principles in the plant extracts.

Achievements

Tilapias are important foodfish in many tropical and subtropical countries. Despite promising expectations in tilapia aquaculture, there are a series of factors that are considered to be detrimental in the expansion of production. Female organism 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. Monosex population of male tilapia is produced by treating spawn with a synthetic male hormone 17α-methyltestosterone (MT). However, the increased use of synthetic steroid hormones to produce monosex populations of tilapia for intensive productive systems

may lead to environmental and public health concerns. Therefore, alternative methods and new, safe chemicals to produce monosex tilapia populations should be considered.

Our experiments reveal that dietary administration of ethanol extract of *Basella alba* leaves at concentration of 1 gm/Kg feed and *Tribulus terrestris* seeds at concentration of 2 gm/Kg feed for 30 days can be used to produce all-male Nile tilapia (*Oreochromis niloticus*) in aquaculture practice. It has been found that fish fed plant extract fortified diet showed higher growth and better health compared to fish fed control diet. The plant extracts were found to contain stigmasterol, lupeol and quercetin as bioactive phytoconstituents, which may be responsible for the androgenic and immunostimulating efficacy of the plants. Thus, the outcomes of this study would help to establish tilapia culture in a scientific, economic and eco-friendly manner with maximum yield in minimum duration.

Major findings

The following aspects of efficacy of *Basella alba* and *Tribulus terrestris* for production and csustainable culture of all-male monosex Nile tilapia can be put forward from this study:

i. Both the leaves of *Basella alba* and seeds of *Tribulus terrestris* has the potential to induce sex reversal in tilapia. *Tribulus terrestris* is more potent than *Basella alba* in this context.

ii. The ideal method of in vivo application of the plant material for induction of sex reversal in tilpaia is the solvent based extraction method and addition of the extraction to the fish feed.

iii. The ideal solvent for extraction of phytochemicals from *Basella alba* and *Tribulus terrestris* for induction of sex reversal in tilapia is ethanol.

iv. The ideal concentration of the plant extract for induction of sex reversal in tilapia is 1 gm/Kg feed for *Basella alba* and 2 gm/Kg feed for *Tribulus terrestris*.

v. The ideal treatment duration with plant extracts for induction of sex reversal in tilapia is 30 days of feeding.

vi. *Basella alba* has more growth promoting and immunostimulating capacities than *Tribulus terrestris*. Both the plant proved that they are potent adpatogen in aquaculture which provide the fish a good non specific immunity, strengthen the hematological attributes, provides antioxidants and hepatoprotection. *Basella alba* has more antiradical or radical scavenging activity than *Tribulus terrestris*.

vii. HPTLC analysis of fractions, which gave the highest percentage of males revealed that- *Basella alba* contained stigmasterol, lupeol and quercetin; *Tribulus terrestris*

contained lupeol. Further analysis with different standards would be required to pinpoint the phytoconstituents in both plant materials in this regard.

Contribution to the society

Aquaculture has become the world's fastest growing food-producing sector, with a growth rate of 10% annually since 1984. Asia produces about 91% of the world's total aquaculture production, with India as one of the top producers. Freshwater aquaculture is a major source of growth for the whole Asian fishery sector and India contributed ~9.90% of the total worldwide freshwater aquaculture products in 1999. Freshwater fisheries are a priority area in our country with the general objectives of increasing fish production, improving export earnings, providing more animal protein and expanding employment opportunities in the sector. Culture of monosex all-male tilapia has proven to be beneficial for increased production during intensive tilapia culture in ponds throughout the country. However, the increased use of synthetic steroid hormones for such culture has evoked considerable environmental and public health concerns.

It is against this background that our study dealing with the production of sex reversed, massive grown all male tilapia population using biodegradable natural plant was both timely and important. The broad objective of our study was also to examine the growth inducing and immunostimulating efficacy of the plant materials during intensive pond culture of tilapia. This might reduce the cost of all-male tilapia production and thereby would benefit the poor rural communities of our country, enhancing food security by supplying cheap animal protein for household consumption. Our study demonstrated that, both leaves of *Basella alba* and seeds of *Tribulus terrestris* can be used as a substitute of 17α-Methyl testosterone to produce all-male Nile tilapia. Replacement of the synthetic steroid with plant materials during aquaculture exert an environment friendly method to achieve higher profit by lowering the environment degradation. This method may also minimize the potential health hazards for the consumers associated with the residual effect, if any, of the synthetic steroid. Therefore, in developing nations like India, where a good number of natural open water systems is available for utilization, our study can lead to a low cost sustainable aquaculture method for the production of a major animal protein source. In this respect also, the introduction of phytochemical treated, sex reversed monosex tilapia population for culture in the natural systems deserves to be advocated and large scale field trials of our samples are awaited in India before they are marketed.

Appendix 1

Final Report of the Work

Project Title:

EVALUATION OF PLANT EXTRACTS ON SEX REVERSAL AND GROWTH OF NILE TILAPIA

Introduction

Tilapia, a member of the family Cichlidae, represents an important dietary protein source in many countries of the world. This expression is derived from the African native Bechuana word "thiape", meaning fish. They are endemic to Africa and Middle East but have been transplanted to and stocked in waters of nearly every country of the world for aquaculture (Eknath *et al* 1993). More than 20 species of tilapia have been cultured in developing countries where animal protein is lacking (Guerrero 1982). Among these, the Nile tilapia, *Oreochromis niloticus* (Linnaeus, 1758) is one of the largest species reaching 40 to 50 cm in length and it has been spread throughout the tropics and subtropics for aquaculture (Alvendia-Casauay and Carino 1988, Lin *et al* 2008).

The Nile tilapia is a widely cultured species because it grows and reproduces in a wide range of environmental conditions and tolerates stress induced by handling (Tsadik and Bart 2007). The fish 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). The fish has considerable potential for aquaculture in many tropical and subtropical regions in the world (Fitzsimmons 2000). In 2003, the global production of tilapia was around 1.5 million mt (Fitzsimmons 2004). Farmed tilapia production increased dramatically in recent years, increasing from 383,654 mt in 1990 to 2,326,413 mt in 2006 (FAO 2007).

Being euryhaline, tilapias can be cultured in fresh as well as brackish water impoundments. The growth rates of the fish are influenced by a variety of factors like water temperature, dissolved oxygen (DO_2), free carbon-di-oxide (free CO_2), pH, sex, supplemental feeding and stocking

density. Rapid growth rates, high tolerance to low water quality, efficient food conversion, resistance to disease, good consumer acceptance and ease of spawning make tilapia a suitable fish for culture (El-Saidy and Gaber 2005). The fish is reported to sexually mature at a small size of around 6 cm and a young age of around 3 months (Chapman 1992).

Sex plasticity is a well documented phenomenon in teleost fish, given that gonadal sex differentiation can be re-directed by many other factors, besides genetic sex determination mechanisms, such as environmental conditions and exogenous endocrine-active chemicals (Blazquez *et al* 1995, Kitano *et al* 2000, Bertolla-Afonso *et al* 2001, Galbreath *et al* 2003). Effects of environmental parameters such as temperature (Baroiller and D'cotta 2001, Devlin and Nagahama 2002, Nakamura et al. 2003) and pH (Zelennikov 1997) are well documented. This issue is of considerable relevance for species of interest for aquaculture. Sex inversion is used to minimize reproduction in tilapia, given that when this species is cultured in mixed sex populations it begins to reproduce before reaching marketable size (Teichert-Coddington *et al* 2000).

Despite promising expectations in tilapia aquaculture, there are a series of factors that are considered to be detrimental in the expansion of production. Female organism 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). The success of the utilization of steroid hormones to produce monosex populations of tilapia is well documented (Baroiller and D'cotta 2001, Strussmann and Nakamura 2002). Monosex population of male tilapia is produced by treating spawn with a synthetic male hormone 17α-methyltestosterone (MT). However, the increased use of synthetic steroid hormones to produce monosex populations of tilapia for intensive productive systems may lead to environmental and public health concerns. MT is known to be associated with gonad malformation problems when administered at high doses or over a prolonged time period. MT induces gonadal intersexuality and paradoxical feminization (van den Hurk *et al* 1982, Goudie *et al* 1983, Solar *et al* 1984, Blazquez *et al* 1995, Rinchard *et al* 1999, Papoulias *et al* 2000). Chronic exposure of consumers to synthetic steroids may cause adverse health effects and its use in fish culture is associated with potential release to the

environment and contamination of the biota. Consequently, MT release and environmental concerns are a major reason behind a search for alternative, environment-friendly chemicals. Within the contemporary atmosphere of increasing governmental regulation on the use of chemicals on food fish, continued dependency on steroid-induced monosexing places the culture of tilapias in a precarious position. Therefore, alternative methods and new, safe chemicals to produce monosex tilapia populations should be considered.

Phytochemicals are a large group of plant-derived compounds that are commonly found in fruits, vegetables, beans, cereals and plant-based beverages like tea and wine (Arts and Hollman 2005). In general, the action of phytochemicals when administered to fish is a recent subject of study. Phytochemicals have been reported to enhance various activities like growth, feed consumption, are acting as tonic in immunostimulation, antistress, and promote antimicrobial properties of fish (Citarasu 2010). The use of medicinal plants as fertility enhancers and sex reversal agents in fish has been receiving some attention. Moreover, as the components of fish diet and/or compounds present in the aquatic environment, phytochemicals may induce biological responses in fish including estrogenic effects and reproductive retardance, and hence are sometimes regarded as endocrine disrupting chemicals (EDCs) (Ng *et al* 2006, Cheshenko *et al* 2008). Plant bioactive compounds might provide a useful source of new medicines and pharmaceutical entities for enhancing fish production and health; and food safety and quality, while conserving the aquatic environment. Intensive efforts must be made in exploiting plants, plant extracts or natural plant compounds as potential natural alternatives to synthetic steroids for enhancing fish productivity. The present work would be of broad general interest since it will not only deal with basic science but also act as a boon for the socioeconomically stressed third world populations where cheap animal protein sources are scarce.

Recent research has begun to demonstrate positive impacts of application of phytochemicals and herbal products in fish culture (Rawling *et al* 2009). A possible alternative approach to the use of steroid hormones for sex reversal in tilapia may involve the use of plant extracts containing phytochemicals such as isoflavonoids, flavonoids and saponins, which are natural compounds characterized by estrogenic/androgenic activity (Citarasu 2010). There is evidence that chemicals naturally occurring in plants could provide a useful source of masculinizing agents (Lohiya *et al*

1999). Among the known actions of phytochemicals that suggest estrogenic/androgenic activity, are the inhibition of several steroid metabolizing enzymes such as aromatase, and phytochemical ability to interact with nuclear estrogen receptors and consequently the modulation of the genomic response to estrogenic hormones (Thomas 2000, Eng *et al* 2001, Saarinen *et al* 2001). Therefore, these phytochemicals may direct the sex differentiation of the ovary in gonochoristic fish species towards testis (Piferrer 2001). Besides, with their potent antioxidant and immunostimulating properties, phytochemicals have been found to stimulate fish growth (Makkar *et al* 2007, Citarasu 2010).

Several reports provide evidence that extracts of *Basella alba*, a fast growing vegetable native to tropical Asia, has anabolizing and virilizing effects (Moundipa *et al* 1999, Moundipa *et al* 2005, Moundipa *et al* 2006) and may be used to boost low levels of testosterone in aging males. Addition of *Basella alba* leaves as part of daily diet has been reported to reduce anaemia and maintain general health in rat (Bamidele *et al* 2010). 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). The plant extract has also been found to stimulate growth in fish (Çek *et al* 2007a, Çek *et al* 2007b). However, further experimentation is required to validate the possibility of the use of these plant extracts as sex reversal agents in tilapia, in order to ensure that they are as effective as the common technique to produce monosex populations that involves the use of steroid hormones such as MT.

Herbs have long been used in India to promote health and prevent and treat diseases. Indian medicinal plants are a rich source of immune-enhancing substances. Nowadays herbs or herbal products also have a significant role in aquaculture (Jeney *et al* 2009, Citarasu 2010). *Basella alba* and *Tribulus terrestris* have been reported to possess medicinal values (Ignacimuthu *et al* 2008). *Tribulus terrestris* have been found to be effective for production of monosex *Poecilia latipinna* population (Kavitha and Subramanian 2011). But, use of these plant extracts for sex reversal and growth induction in tilapia during its culture under Indian perspective is not documented.

Significance of the study

In spite of their many biological uses in traditional medicines, there is dearth of information encountered in the literature on the effect of *Basella alba* and *Tribulus terrestris* on sex reversal and growth parameters in tilapia. Also, in view of the increasing use of plant extracts as potential alternative for synthetic hormones and chemotherapeutics, it is necessary to scientifically investigate the effects of these two plant extracts in tilapia culture. Various methods such as oral administration and immersion technique have been adapted for *in vivo* application of phytochemicals with medicinal values (Gurib-Fakim 2006). Therefore, the ideal method of application for *Basella alba* and *Tribulus terrestris* for commercially feasible induction of sex reversal and growth in tilapia must be determined. The overarching problem in respect to tilapia is to produce monosex fish for stocking and intensive rearing purposes using environmentally-sound and acceptable methods. Using natural substances in that process will be a great benefit to both farmers and the general public. The present study is aimed to develop a synthetic steroid-free method for producing all-male populations of tilapia using these two plant extracts containing specific phytochemicals and may add new aspects to the use of these plant materials in aquaculture.

The use of phytochemicals as an alternative method to produce monosex populations of tilapia will address human and environmental safety issues. Fish offered to the consumer will not be treated with MT and producers may have an alternative method for producing all-male populations of tilapia based on natural products. The low cost of using phytochemicals or plant extracts should be also attractive alternative to producers. Moreover, several phytochemicals have proven antioxidant activity (Lee *et al* 2005) and enhance immune resistance of fish. Thus, the use of plant extracts may stimulate general health in fish leading to growth increase that may be conferred to higher production and economic gain for the fish farmers of the country.

Objectives

Phytochemicals present in many plants have several reported biological properties. This study is intended to explore the possible utilization of two such plant extracts containing different phytochemicals as potential *in vivo* enzymatic inhibitors of aromatase and of nuclear estrogen

receptors' antagonist in gonad germ cells. Such response might modulate the sex differentiation process of the gonad in sexually undifferentiated Nile tilapia and affect the sex ratio of the tilapia populations. Thus, the major objectives of the present study are:

i. Determination of the potentiality of *Basella alba* and *Tribulus terrestris* to induce sex reversal in tilapia under the ecological condition of the Gangetic plains in West Bengal, India.
ii. Determination of ideal method of *in vivo* application of the plant material for induction of sex reversal in tilapia.
iii. Determination of ideal solvent for extraction of phytochemicals from *Basella alba* and *Tribulus terrestris* for induction of sex reversal in tilapia.
iv. Determination of ideal concentration of the plant extract for induction of sex reversal in tilapia.
v. Determination of ideal treatment duration with plant extracts for induction of sex reversal in tilapia.
vi. Analysis of the growth-promoting, immunolostimulating and antioxidant properties of the plant extracts during *in vivo* application in tilapia.
vii. Identification of the bioactive principles in the plant extracts.

Methodology

1. 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.

2. Plant extracts preparations

Basella alba leaves and *Tribulus terrestris* seeds (Figure 1) 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 45^0C using a rotary evaporator and stored under nitrogen at -20^0C 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). The methanol extract was evaporated to dryness under pressure at 45ºC using a rotary evaporator and stored under nitrogen at -20ºC in amber glass bottle until it was used.

A.

B.

Figure 1: A. *Tribulus terrestris* seeds, B. *Basella alba* leaves.

3. Determination of plant extract yield

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

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. Determination of antioxidant activity

The free radical-scavenging activity of *B. alba* and *T. terrestris* extracts with different solvents were evaluated using the stable radical DPPH, according to the method of Masuda et al. (1999) with modifications (Maisuthisakul et al., 2007). A series of extract concentrations with different ratios of the extract to methanol, i.e. 1:10, $1:10^2$, $1:10^3$, $1:10^4$, $1:10^5$, $1:10^6$, $1:10^7$, were prepared. Then, 4.9 ml of each diluted plant extract was mixed with 100 µl of 5 mM DPPH in methanol. The mixtures of different extract concentrations and DPPH were placed in the dark at 37ºC for 30 min. The absorbance of each sample of plant extract containing DPPH (A_1) was read at 517 nm using a spectrophotometer (UV–Vis Model 1601, Shimadzu, Kyoto, Japan). The absorbance of each sample of plant extract dilution without DPPH (A_s), and only DPPH solution without plant extract (A_o, called control) were also recorded. All determinations were performed in triplicate. The percentage of DPPH radical-scavenging activity of the plant extract determined at these seven concentrations within the range of dose response (at least 10–90% reduction in absorbance) was calculated as shown:

DPPH radical scavenging activity (%) = $[\{A_0 - (A_1 - A_s)\} / A_0] \times 100$

The percentage of DPPH radical-scavenging activity was plotted against the plant extract concentration (µg/ml) to determine the amount of extract necessary to decrease DPPH radical concentration by 50% (called EC_{50}). The EC_{50} value of each extract was estimated by sigmoid non-linear regression using SigmaPlot 2000 Demo (SPSS Inc., Chicago, IL, USA). The unit of EC_{50} was later converted to µg/µg DPPH. These values were changed to antiradical activity (A_{AR}) defined as $1/EC_{50}$.

5. Dietary treatment of fish with powdered plant material

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 allocated into 8 groups (40 fish / group). Three groups were fed diets containing powdered *Basella* leaves at concentrations of 5.0, 10.0, 15.0 gm/kg food, three groups were fed diets containing powdered *Tribulus* seeds at concentrations of 5.0, 10.0, 15.0 gm/kg food, one group was fed control diet with neither *Basella* nor *Tribulus* and another group was fed diet containing 17α methyltestosterone (17αMT) 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 into pellets with a pelleter (diameter 2mm), 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 (Shelton *et al* 1978). The treatment 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 ± 2^0C) static systems. Water in all aquaria was replaced manually and the fish was kept under similar photoperiod (14 L: 10 D).

6. Immersion treatment of fish with plant aqueous extracts

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 18 glass aquaria to 3 different treatment groups (0.05, 0.1 and 0.15 gm/l) for each of the two extracts. The treatment was conducted for 30 days and the fish were exposed to the plant extracts 4 times (once weekly) during this period. The aquaria were continuously aerated and maintained in heated (T = 27 ± 2^0C) 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 fish was fed finely ground (< 500-1000 μm) artificial diet containing 30% crude protein (Tokyu, Japan) at a rate of 20% body weight / day. The experiment was conducted simultaneously in triplicate.

7. 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 2mm), and dried at room temperature. Pelleted feed was pulverized before feeding to the juvenile fish.

8. Sexing of fish

Sexing of the juvenile fish was done by the standard acetocarmine squash technique of gonads (Guerrero and Shelton 1974) (Figure 2). Histological and scanning electron microscopic studies of the gonads were also performed (Figure 3).

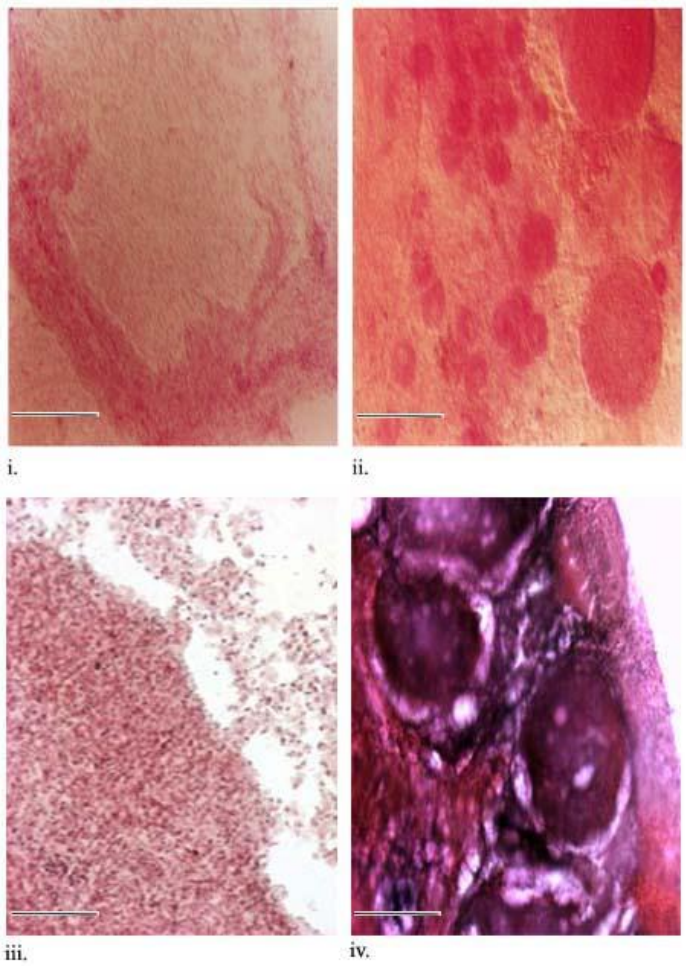


Figure 2: Light micrographs of i) control male, ii) control female, iii) treated male and iv) intersex gonads of tilapia after acetocarmine squash processing. Bar indicates 20 µm.

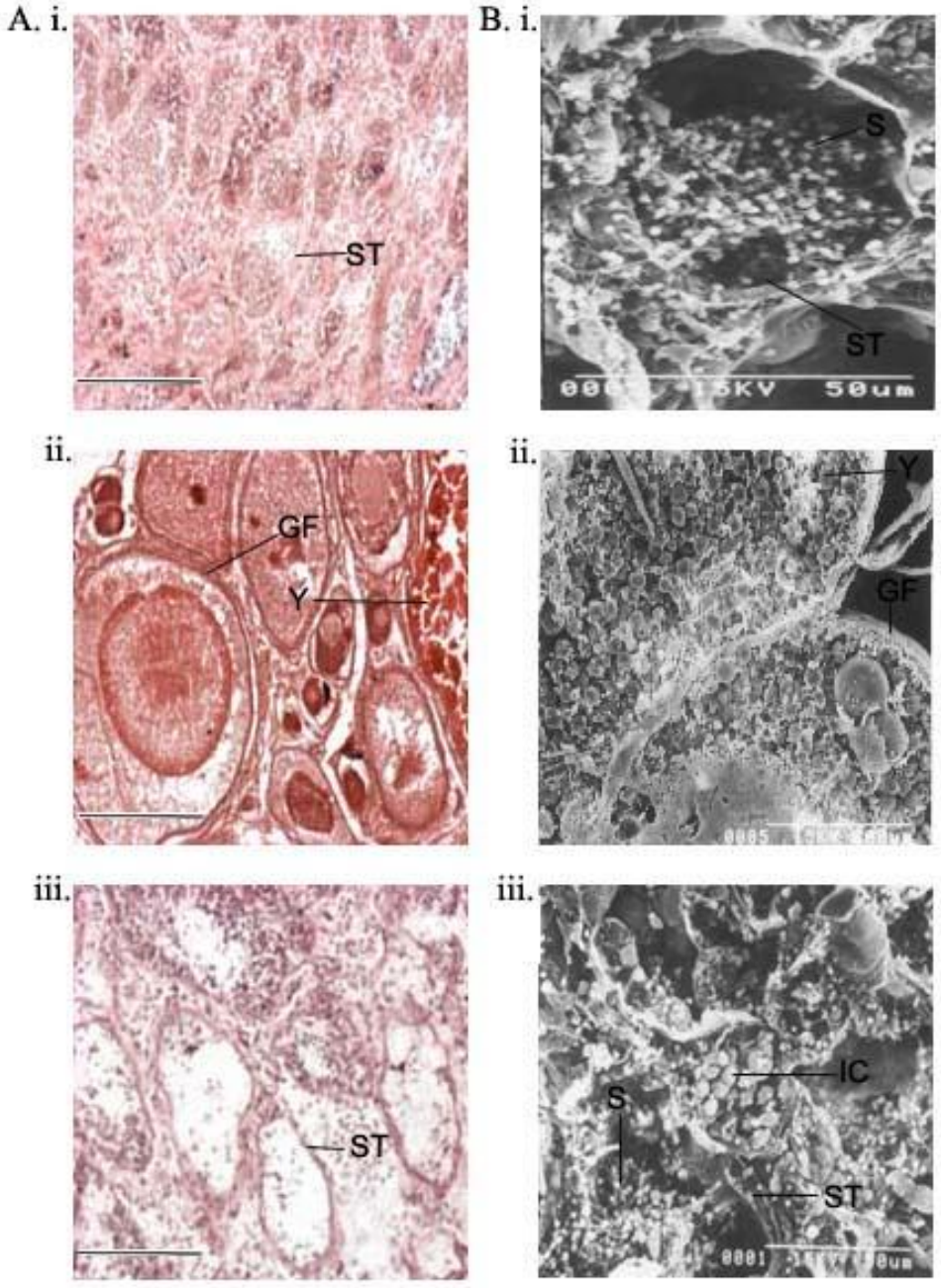


Figure 3: Light micrographs (A) and electron micrographs (B) of i) control male, ii) control female and iii) treated male gonads of tilapia. ST: seminiferous tubule; S: sperm; IC: interstitial cell; GF: Graafian follicle; Y: yolk. For light micrographs bar indicates 20 µm.

9. Acetocarmine squash technique

The gonads were located with the aid of a dissecting microscope and a portion of it was placed on a glass slide. A few drops of acetocarmine were added and the tissue was squashed with a cover slip. Finally the mounts were observed under a compound microscope.

10. Histological analysis

Some fishes were processed properly for histological examination of the gonad using the standard protocols. Cross sections of the gonadal tissue were cut at 6 µm, stained with Delafield's haematoxylin and eosin, examined and documented by using an Olympus photographer microscope.

11. Scanning electron microscopic study

The gonads were cut to small pieces and fixed at 4^0C in 0.1 M phosphate buffer (pH 7.2) with 4% gluteraldehyde. Then the tissue was processed accordingly and critical point drying (CPD) was performed. Finally, after gold coating, the tissue was examined under the electron microscope (Figure 4).

Figure 4: The scanning electron microscope apparatus.

12. Qualitative phytochemical studies

Qualitative phytochemical analysis of the aqueous extracts of the *Tribulus* seed and *Basella* leaves were carried out using standard procedures (Malpani *et al* 2011, Kumar and Bhardwaj 2012, Ray *et al* 2013).

- Ferric chloride test for tannin: To 1ml aqueous extract, 1ml 5% $Fecl_3$ solution was added. Appearance of blue-black precipitate indicates the presence of tannins.
- Frothing test for saponin: Few drop of $NaHCO_3$ was added to 1ml extract and the mixture was shaken for few seconds. Presence of persistent froth indicates presence of saponins.
- Flavonoid Test: Two tests were performed to determine the presence of flavonoids in the aqueous extracts of the two plants.
- 10-20 drops of dilute HCl was added to 1ml of extract, followed by a small piece of Zn. Development of pink or reddish pink colour precipitate indicates the presence of flavonoids.
- Few drop of dilute H_2SO_4 was added to 1ml of extract, development of yellow to crimson colour shows the presence of flavonoids.
- Steroid Test: To 1ml extract, 1ml glacial acetic acid and 1ml of concentrate H_2SO_4were added through the wall of the test tube kept in ice cubes. Development of brown colour indicates terpenoids, green colour indicates steroids and red colour indicates triterpenoids.
- Mayer's test for alkaloids: Mayer's reagent was added to 1ml extract, formation of white or pale yellow precipitate indicates presence of alkaloids.
- Wagner's test for alkaloids: Wagner's reagent was added to 1ml extract. Formation of reddish brown precipitate indicates the presence of alkaloids.
- Carbohydrate Test: To 5ml of Benedict's solution, 1ml extract was added and then boiled for 5 minutes. Development of coloured precipitate indicates the presence of carbohydrates.
- Glycosides Test: To 1ml extract, 1ml aqueous solution of NaOH was added; formation of yellow precipitate confirms the presence of glycosides.

13. Growout

A four-month grow-out trial was conducted to determine the effect of plant extract treatment on tilapia growth in pond culture systems. The culture system consisted twelve 0.01-ha earthen ponds. For the entire duration of the grow-out experiment, the various water quality parameters like temperature, dissolved oxygen, pH, and total alkalinity were regularly monitored (APHA 1998) and maintained within tolerable limits for tilapia culture. During this period, water temperature ranged from 29.0–31.7ºC, pH from 7.0–8.0, alkalinity from 120.1–148.2 mg/l, and DO_2 from 4.5–7.4 mg/l. Mixed-sex juvenile Nile tilapia (mean weight 0.025±0.004 gm; mean length 0.056±0.002 cm) was stocked randomly in 12 ponds at a density of 20,000 fingerlings/ha. Fish in 3 ponds were fed with control diet throughout the entire culture period. Fish in another 3 ponds were given 17α-MT-treated diet with a dose of 10 mg/kg for the first 30 days and control diet for the rest of the culture duration. Fish in another 3 ponds were provided diet containing ethanol extract of *B. alba* leaves at a concentration of 1.0 gm/kg (EB1) feed for the first 30 days and control diet for the rest of the culture duration, while fish in the remaining 3 ponds were provided with ethanol extract of *T. terrestris* seeds at a concentration of 2.0 gm/kg (ET2) feed for the first 30 days and control diet for the rest of the culture duration. The fish were fed twice daily at a constant rate of 20% body weight/day for the first month, 10% body weight/day for the next 2 months, and 5% body weight/day for the last month of the culture period. Fish from each pond were measured individually for weight and length every 15 days and at the end of the trial. Growth parameters like specific growth rate (SGR), weight gains (WG) and daily weight gains (DWG) were calculated at the end of the culture period as follows (Pechsiri and Yakupitiyage 2005):

SGR (% / day) = [(ln final weight - ln initial weight)/time (days)] × 100

WG = mean final weight (g) - mean initial weight (g)

DWG (g/day) = mean final weight (g) - mean initial weight (g)/ days

Secondary sexual characteristics (especially genital papilla) and macroscopic examination of gonads were used to distinguish males from females.

14. Study of immunostimulating properties

Blood samples were collected from fish (n=6) at the end of 4 months' culture in earthen ponds from each treatment categories and key factors of the non-specific immune response such as respiratory burst activity, phagocytosis assay, lysozyme assay, plasma total protein, plasma total immunoglobulin were measured using standard protocol (Ardó et al. 2008). In brief, blood samples (6 fishes/group) were collected from the caudal vein using heparin as an anticoagulant. Leukocytes were separated from each blood sample by density gradient centrifugation. One ml of histopaque 1.119 (Sigma) containing 100 µl of Bacto haemagglutination buffer, pH 7.3 (Difco, USA) was dispensed into siliconised tubes. One ml of a mixture of histopaque 1.077 and haemagglutination buffer was layered on the top. One ml of blood sample was then layered carefully on the top of the gradient. Sample preparations were centrifuged at 700 x g for 30 min at 4ºC. After centrifugation, plasma was collected and stored at −20°C for future analysis. Separated leukocytes were gently removed and dispensed into siliconised tubes, containing phenol red free Hank's balanced salt solution (HBSS). Cells were then washed in HBSS and adjusted to 10^7 viable cells/ml. Respiratory burst activity of isolated leukocytes was quantified by the nitroblue tetrazolium (NBT) assay which measures the quantity of intracellular oxidative free radicals. Phagocytosis activity of blood leukocytes was determined spectrophotometrically after digesting the macrophages using trypsin EDTA. Plasma lysozyme activity was measured spectrophotometrically after adding *Micrococcus lysodeikticus* and repeating the procedure again after 20 minutes. The total protein concentration of the plasma was determined by a colorimetric assay based on the Biuret reaction, using a protein diagnostic reagent kit. The assay was performed in 96-well microtiter plates. 10 µl plasma, standard solution and 300 µl diluted Biuret solution was added to the wells in triplicates. After 20 min of incubation at room temperature, absorbance was measured with a multi scan spectrophotometer at 550 nm. The protein concentrations of the samples were calculated by the following formula: $y=A_m/A_{st} \times x$, where y is the protein concentration of the sample, A_m is the absorbance of the sample, A*st* is the absorbance of the standard and x is the standard's known protein concentration. The assay of total immunoglobulin was similar to the previous one. 50 µl plasma and 50 µl polyethylene glycol (PEG) was added to each well of a 96-well microtiter plate. After 2 h of incubation at room temperature, plates were centrifuged at 1000 G for 15 min. The protein content of the supernatant was determined by the assay described above. This value was subtracted from the

total protein level, and the result was equal to the total immunoglobulin concentration of the plasma.

15. Study of haematological parameters

Haematological parameters such as total count, differential count, haemoglobin concentration, hematocrit, total plasma glucose and albumin were also analyzed for different treatment groups (n=6) using standard protocols (Min and Kang 2008) at the end of 4 months' culture in earthen ponds. Blood samples (6 fishes /group) were collected from the caudal vein using heparin as an anticoagulant. The RBC and WBC counts were obtained using a hemocytometer (Improved Neubauer, Germany) with RBC diluting solution and WBC diluting solution respectively (Sigma Chem. Co., St. Louis, MO). The Hb level was measured using a diagnostic kit (Sigma-Aldrich), This assay is based on the improved Triton®/NaOH method in which hemoglobin is converted to a colorimetric product measured at 400 nm. For Ht determination, three-quarters of micro-Ht capillaries were filled with blood, sealed at one side with a capillary sealer, and centrifuged at 13,000 rpm for 5 min in a micro-Ht centrifuge (Remi, RM-12C BL). From the results obtained, the mean corpuscular volume (MCV), the mean corpuscular Hb (MCH), and the mean corpuscular Hb concentration (MCHC) were calculated as follows:

$MCV\ (\mu m^3) = (Ht\ (\%) \times 10) / RBC\ count\ (10^6\ mm^{-3})$

$MCH\ (pg) = (Hb\ (g\ dL^{-1}) \times 10) / RBC\ count\ (10^6\ mm^{-3})$

$MCHC\ (\%) = Hb\ (g\ dL^{-1}) / Ht\ (\%) \times 100$

Differential count (lymphocytes, monocytes, neutrophils, eosinophils) was performed by staining blood films with Giemsa stain. Total serum glucose was measured using a colorimetric kit (Abcam, ab65333) at 570nm. Total serum albumin was estimated using another kit from Sigma-Aldrich and was measured colorimetrically at 620nm.

16. Study of biochemical parameters

Serum was isolated from blood collected from fish (n= 6 / group) at the end of 4 months' culture in earthen ponds and biochemical parameters such as lipid peroxide (LPO), reduced glutathione (GSH), glutathione reductase (GRD), glutathione S-transferase activity (GST), alkaline phosphatase activity (ALP), glutamic oxaloacetic transaminase activity (GOT), glutamic pyruvate transaminase (GPT) activity were analyzed for different treatment groups using standard protocols (Min and Kang 2008, Padmini et al. 2009, Bamidele et al. 2010). The level of lipid peroxide was determined at 540 nm by measuring the amount of thiobarbituric acid reacting substance (TBARS) formed. The reduced glutathione (GSH) was measured at 412 nm using 5, 5' dithiobis-(2-nitrobenzoic acid) (DTNB) reagent. The activity of GRD was determined by monitoring the glutathione-dependent oxidation of NADPH at 340 nm, in a reaction mixture containing 950 µl of 0.15 mM NADPH, 0.5 mM glutathione, and 3 mM $MgCl_2$ in 50 mM Tris (pH 7.5) and 50 µl extract. Activity of glutathione S-transferase (GST) was assayed at 340 nm by measuring the increase in absorbance using 1-chloro-2,4-dinitrobenzene (CDNB) as the substrate. The ALP was measured using marketed kit (Abcam, ab83369) at the wavelength of 405nm. GOT was measured using kit from Sigma-Aldrich at the wavelength of 450nm. GPT was measured using a diagnostic kit (Abcam, ab105134) at the wavelength of 570nm.

17. Fractionation of plant extracts and identification of bioactive principles

The ethanol extracts of both *B. alba* leaves and *T. terrestris* seeds, which have showed the highest sex reversal potency were subjected to chromatographic analysis for fractionation of the extract (Moundipa et al. 2005). In column chromatographic separation, 10gm silica was packed in 10 x 2 cm column. Ethanolic extract of *Tribulus* (10 mL) and ethanolic extract of *Basella* (6 mL) were dried and dry weights were taken as 0.6 gm and 0.06 gm respectively. Both the dried products were dissolved in chloroform. Mobile phase for the column chromatographic separation was $CHCl_3$:CH_3OH. Fractions were collected at every 15 minutes interval. A total of 71 fractions and 136 fractions were collected from respective columns for *Tribulus* and *Basella*. TLC studies of these fractions were carried out by using commercially available pre-coated plates with standardized adsorption layers, i.e., Silica gel 60 F254, (Merck, Germany). All the solvents systems for mobile phase were selected by trial and error basis. Mobile phase for ethanolic *Tribulus* extract was 85:15 $CHCl_3$: CH_3OH and ethanolic *Basella* extract was 75:25 $CHCl_3$:

CH_3OH. The chromatograms were developed in twin through glass chambers on 10 × 10 cm plates till the mobile phase travelled up to a distance of 8 cm from starting point. After development, the plates were dried at room temperature for 5-10 minutes and observed using iodine as developer. Photographs were taken and the Rf values were recorded. By measuring the Rf ultimately 8 sub fractions were obtained from *Tribulus* and 6 from *Basella*. Subsequently, juvenile fish was treated with such sub-fractions to determine the sub-fraction with the highest potency for induction of sex reversal. The sub-fractions were then subjected to high performance thin layer chromatographic analysis to find the bioactive principles present in it.

17. Statistical analysis

Data were analyzed by IBM SPSS Statistics Version 20.0 software. For dietary treatment with powdered plant materials and immersion treatment with aqueous plant extracts, normality of variables was checked before conducting T-probe or ANOVA. Treatment means were compared by Tukey's HSD test. In case of dietary treatment with plant extracts of different solvents, 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.

Results

For both the plants, the maximum yield of evaporated dried extracts based on dry weight basis was obtained with water as extracting solvent (Figure 5). The yield for aqueous extract of *B. alba* leaves was 28.353% 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 5). 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 5). Similar decrease in yield percentage with decreased

polarity of solvents was observed for another plant, *Limnophila aromatica* as well (Do et al. 2014).

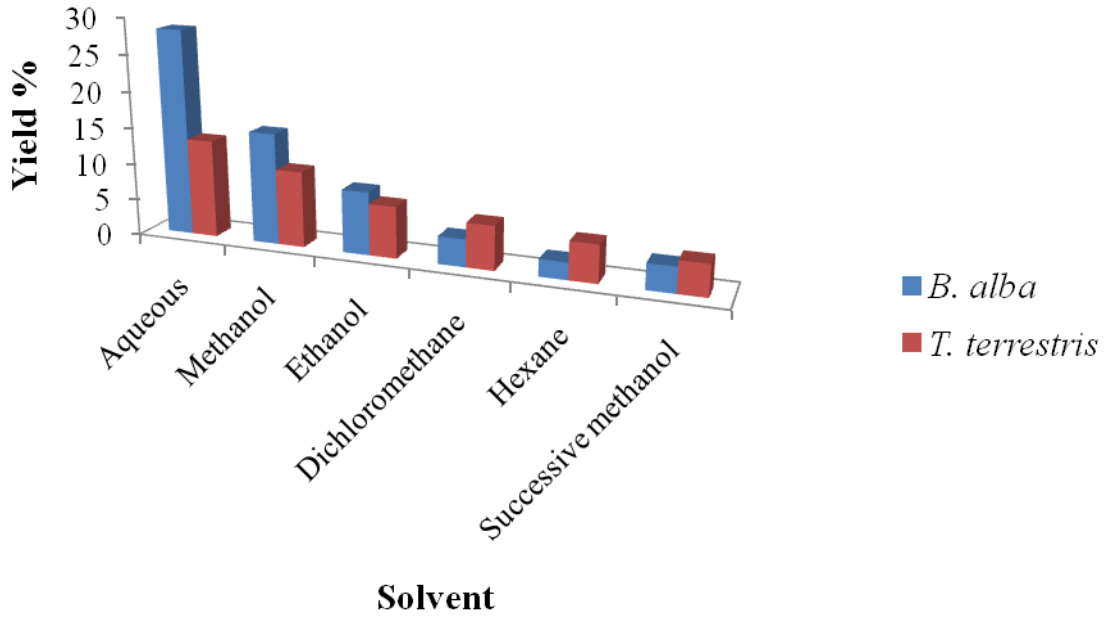
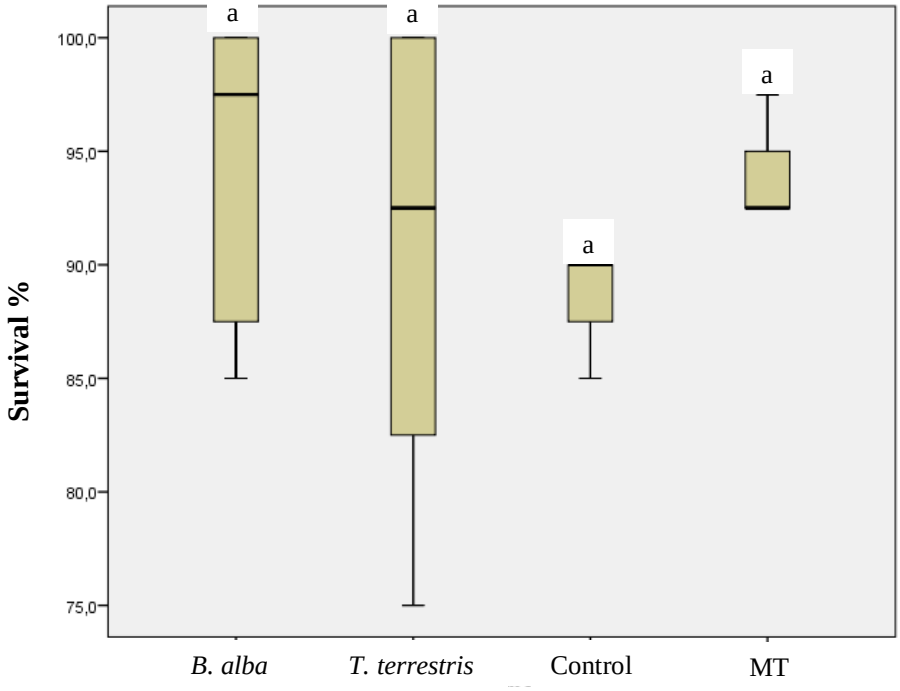


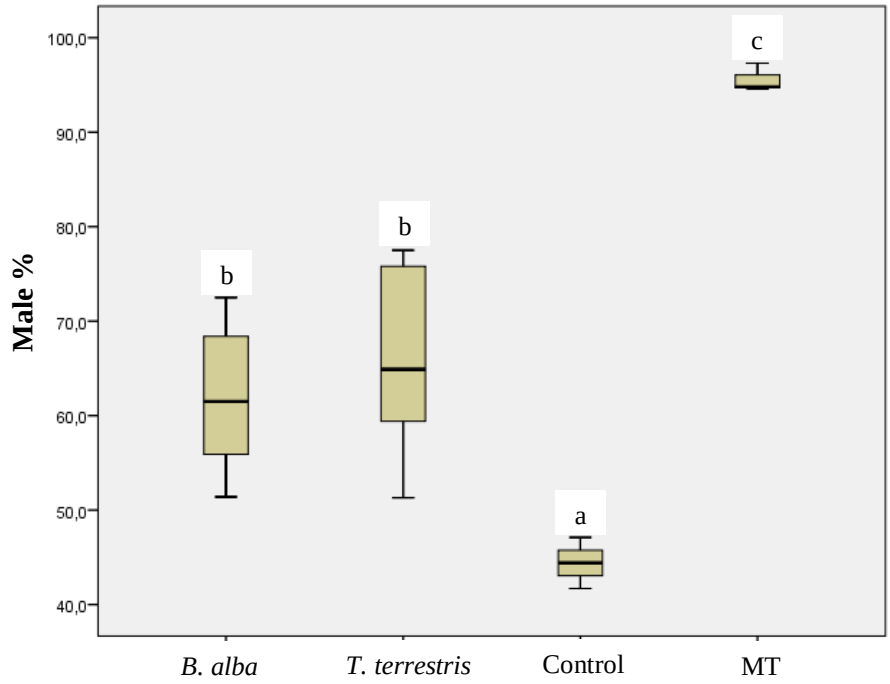
Figure 5: Yield percentage of evaporated dried extracts based on dry weight basis with different solvents for *B. alba* leaves and *T. terrestris* seeds.

No significant difference (P>0.05) was observed among the various treatment groups of fish fed diets containing the powdered plant materials, MT and control feed (Figure 6a). 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) compared to other treatment groups (Figure 6b). 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 6c and 6d). The control diet fed group showed no

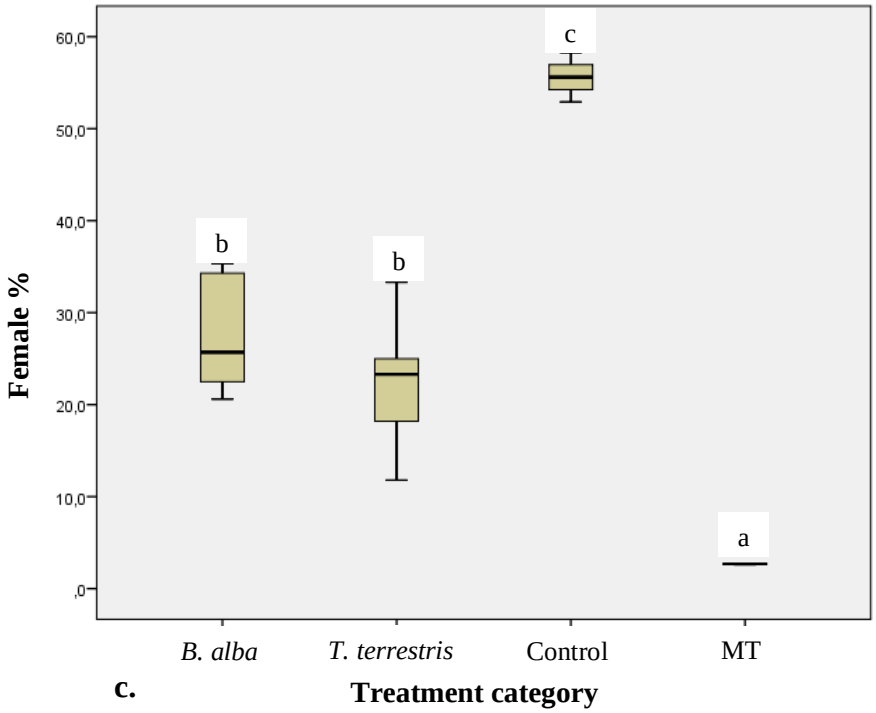
intersex fish while the highest percentage of intersex fish (12.5±1.6) was observed in tilapia fed diets containing powdered *T. terrestris* seeds.



a.

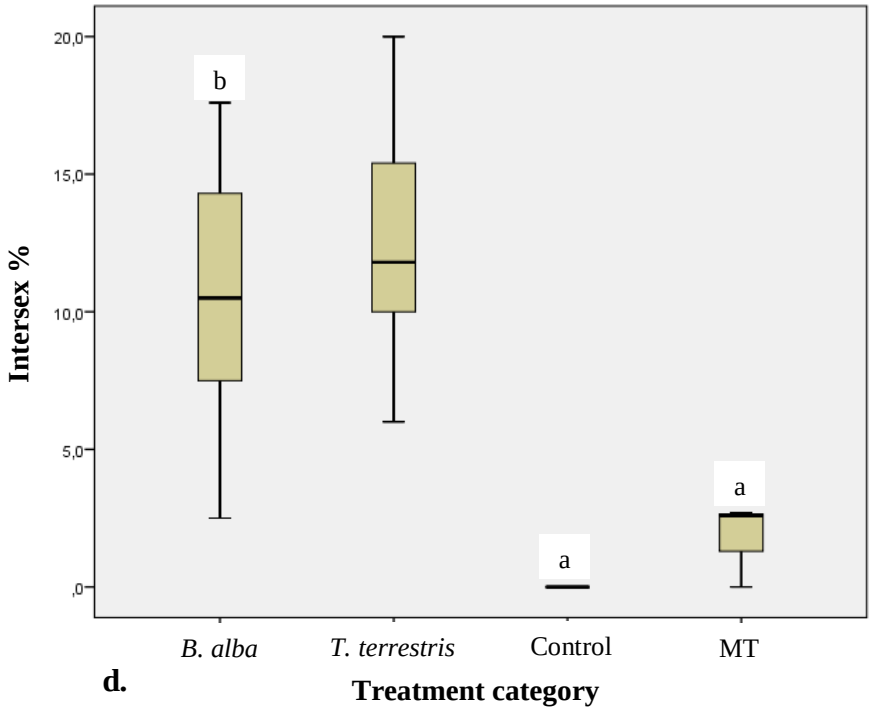

100,0
90,0
80,0
70,0
60,0
50,0
40,0
Male %
b
b
a
c
B. alba
T. terrestris
Control
MT
Treatment category

b.


60,0
50,0
40,0
30,0
20,0
10,0
,0
Female %
b
b
c
a
B. alba
T. terrestris
Control
MT
Treatment category

c.

b



d.

Figure 6: Percentage of survival (a), male (b), female (c) and intersex (d) in tilapia fed diets containing powdered *B. alba* leaves, *T. terrestris* seeds, untreated control and MT. Different alphabets above boxes mark significant difference (P<0.05) in means.

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 gm/kg concentration showed significantly higher (P<0.05) percentage of males compared to 5.0 gm/kg and 15.0 gm/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). Here, the maximum percentage of males (76.6±0.5) was observed at the concentration of 15.0 gm/kg, which was significantly higher (P<0.05) than the other two concentration categories. The percentage of females in the 15.0 gm/kg group was also significantly lower (P<0.05) compared to the other groups. The highest percentage of intersex fish was observed in 5.0 gm/kg treatment group (18.03±1.4), which is significantly higher (P<0.05) than intersex percentage in other two concentration categories (Table 2).

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 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
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 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.

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 7). 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).

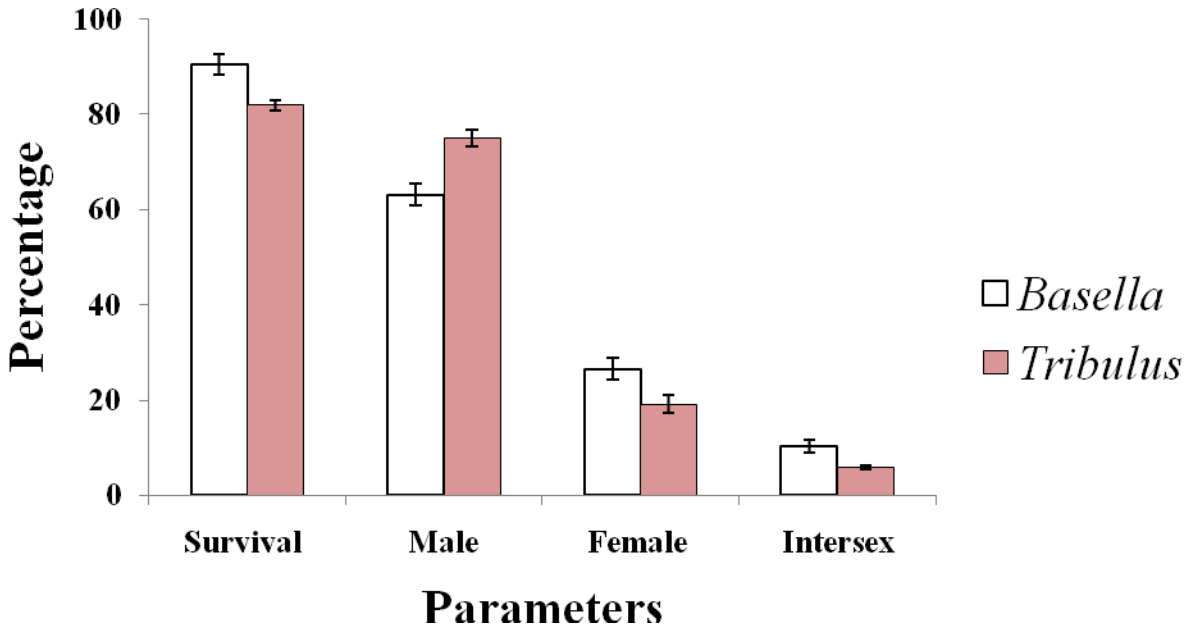


Figure 7: Percentage of survival, male, female and intersex during immersion treatment with aqueous extracts of *B. alba* leaves and *T. terrestris* seeds.

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 gm/l showed significantly higher ($P<0.05$) percentage of males compared to 0.05 gm/l and 0.15 gm/l treatment groups. On the other hand, treatment group of 0.15 gm/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 gm/l treatment category and this was significantly higher ($P<0.05$) compared to the lower two groups. The 0.15 gm/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 gm/l category, while the percentage of females in 0.05 gm/l group was homogenous to that in both 0.1 gm/l and 0.15 gm/l groups (Table 4).

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 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
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]

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.

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 percentage 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 5. 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 5). 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 5). 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 5). 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 5).

There was no significant interaction effects ($P>0.05$) of solvent and concentration, and solvent and plant material for percentage of males (Table 5). But, significant interaction effect ($P<0.05$) of concentration and plant material was observed for percentage of males (Table 5, Figure 8). 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 8). 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 8).

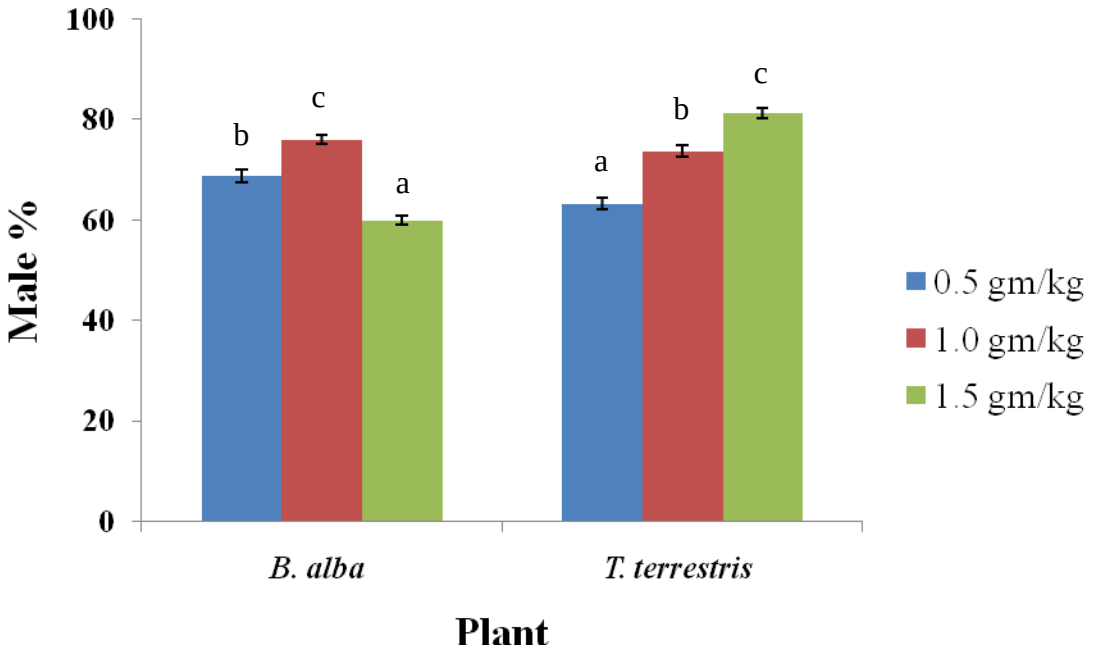


Figure 8: Percentage of males in tilapia fed diets containing different concentrations of *B. alba* leaves and *T. terrestris* seeds extracts. Different alphabets above column indicates significant difference ($P<0.05$) in means.

There was significant interaction effect ($P<0.05$) of solvent, concentration and plant material for percentage of males (Tables 5, 6). 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 6). For all the solvents, the highest percentage of males was observed at the concentration of 1.0 gm/kg (Table 6). 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 6). 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 6).

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}

Table 6: 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.

As those observed results indicated a dose dependent masculinisation effect of *T. terrestris* extract on Nile tilapia and extraction with ethanol provided the highest percentage of males, subsequent treatment of 3 days old juvenile tilapia (mean weight 0.025 ± 0.009 gm; mean length 1.25 ± 0.012 cm) were performed with ethanol extract of *T. terrestris* seeds at higher concentrations of 2.0, 2.5 and 3.0 gm/kg feed. There was no significant difference ($P>0.05$) in survival percentage among the different concentration groups and all showed 100% survival. Fish fed diets containing *T. terrestris* seeds ethanol extract at a concentration of 2.0 gm/kg feed showed 90.91±1.2% males, which was significantly higher ($P<0.05$) compared to the other two concentration categories. Interestingly, treatment with the higher concentrations of 2.5 and 3.0 gm/kg resulted in only 59.1±2.4% and 58.34±1.2% males, respectively. However, none of these groups showed any intersex fish.

Considering the results, it may be concluded that dietary treatment with *B. alba* leaves ethanol extract at a concentration of 1.0 gm/kg feed and with *T. terrestris* seeds ethanol extract at a concentration of 2.0 gm/kg feed for 30 days might be implemented for production of monosex tilapia population.

Both *B. alba* leaves and *T. terrestris* seeds showed a moderate degree of antiradical activity with an A_{AR} value ranging from 0.06 to 0.53 (Table 7). Successive methanol extract of *B. alba* leaves showed the highest antiradical activity, which was significantly higher ($P<0.05$) compared to all other extracts. The lowest antiradical activity was shown by dichloromethane extract of *T. terrestris* seeds (Table 7). Interestingly, among the *B. alba* extracts, aqueous extract showed the lowest antiradical activity, while for *T. terrestris* extracts, the aqueous extract showed the highest antiradical activity (Table 7). Variations in pattern of antioxidant activity of different solvent extracts were observed for other plants as well. The aqueous extract of *Linum usitatissimum* showed lower antioxidant activity than ethanol extract (Waszkowiak et al. 2015). Aqueous extract of *Rubus kuleszae* showed highest antiradical activity than the methanol and methanolic-water extracts (Gawron-Gzella et al. 2012).

Qualitative analysis for phytochemicals revealed the presence of steroids and alkaloids in extracts with all the solvents for both *B. alba* leaves and *T. terrestris* seeds (Table 8). Saponin

was found in aqueous, ethanol and methanol extracts of both the plants. *B. alba* leaves showed presence of tannins in aqueous, methanol, ethanol and hexane extracts while *T. terrestris* seeds showed presence of tannins in aqueous, methanol and ethanol extracts only. Flavonoids were found to be present in all solvent extracts except aqueous for both the plant materials (Table 8). Glycosides were not found in any of the extracts while carbohydrates were present in only ethanol and methanol extracts for *B. alba* leaves and methanol extracts for *T. terrestris* seeds (Table 8). In previous studies, phytochemical analysis revealed the presence of tannin, saponin, alkaloid, terpenoid, flavonoid, glycoside and phenolic compounds at varied composition in both aqueous and ethanol extracts of *Basella alba* (Ibrahim et al. 2014), while the phytochemical analysis revealed the presence of flavonoids, alkaloids, saponins, tannins and carbohydrates in both methanolic and aqueous extracts of *Tribulus terrestris* (Sharma et al. 2013).

Plant	Solvent	Antiradical activity (A_{AR})
B. alba leaves	Aqueous	0.08 ± 0.003^{ab}
	Methanol	0.48 ± 0.03^{g}
	Ethanol	0.23 ± 0.03^{de}
	Dichloromethane	0.25 ± 0.009^{ef}
	Hexane	0.16 ± 0.006^{cd}
	Successive methanol	0.53 ± 0.02^{g}
T. terrestris seeds	Aqueous	0.33 ± 0.01^{f}
	Methanol	0.25 ± 0.007^{e}
	Ethanol	0.13 ± 0.01^{abc}
	Dichloromethane	0.06 ± 0.003^{a}
	Hexane	0.17 ± 0.009^{cd}
	Successive methanol	0.15 ± 0.007^{bc}

Table 7: Antiradical activity ($1/EC_{50}$) of different solvents' extracts from leaves of *B. alba* and seeds of *T. terrestris*. Different superscripts mark significant difference ($P<0.05$) in means.

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

Table 8: Qualitative analysis of phytochemicals in different solvent extracts of *B. alba* leaves and *T. terrestris* seed.

During the grow-out experiments in the earthen ponds, at the end of 4 months' culture, fish fed diets containing 17α-MT (10 mg/Kg feed) showed significantly higher (158.90±1.67 gm) final

weight (P<0.05) compared to all other treatment categories. At this time, ET2 showed significantly higher (147.80±1.83 gm) final weight (P<0.05) compared to both EB1 (147.20±0.33 gm) and control (142.60±0.99 gm) treatment categories. Interestingly, till 105 days of culture it was found that EB1 has significantly (P<0.05) higher weight compared to the control and ET2 categories (Table 9, Figure 9).

Treatment	30 Days	45 Days	60 Days	75 Days	90 Days	105 Days	120 Days
Control	11.31 ± 0.74^{a}	25.03 ± 1.02^{a}	55.60 ± 0.91^{a}	87.20 ± 0.85^{a}	111.90 ± 0.46^{a}	125.80 ± 0.42^{a}	142.60 ± 0.99^{a}
17α-MT	17.35 ± 0.66^{c}	36.38 ± 0.68^{c}	74.20 ± 0.57^{c}	102.30 ± 0.40^{c}	125.30 ± 0.87^{b}	140.40 ± 1.02^{c}	158.90 ± 1.67^{c}
EB1	14.29 ± 0.89^{b}	28.90 ± 0.74^{b}	65.60 ± 1.01^{b}	94.80 ± 1.02^{b}	123.10 ± 1.20^{b}	136.70 ± 0.60^{b}	147.20 ± 0.33^{ab}
ET2	12.93 ± 0.80^{ab}	25.75 ± 1.21^{ab}	56.90 ± 2.30^{a}	88.00 ± 1.46^{a}	113.90 ± 0.78^{a}	126.70 ± 0.83^{a}	147.80 ± 1.83^{b}

Table 9: Final weight (gm) at every 15 days interval up to 4 months' culture in earthen ponds. Data are expressed as mean ± sem. Different superscripts mark significant difference (P<0.05) in means within columns. n= 10 fishes, Initial weight = 0.025±0.004 gm.

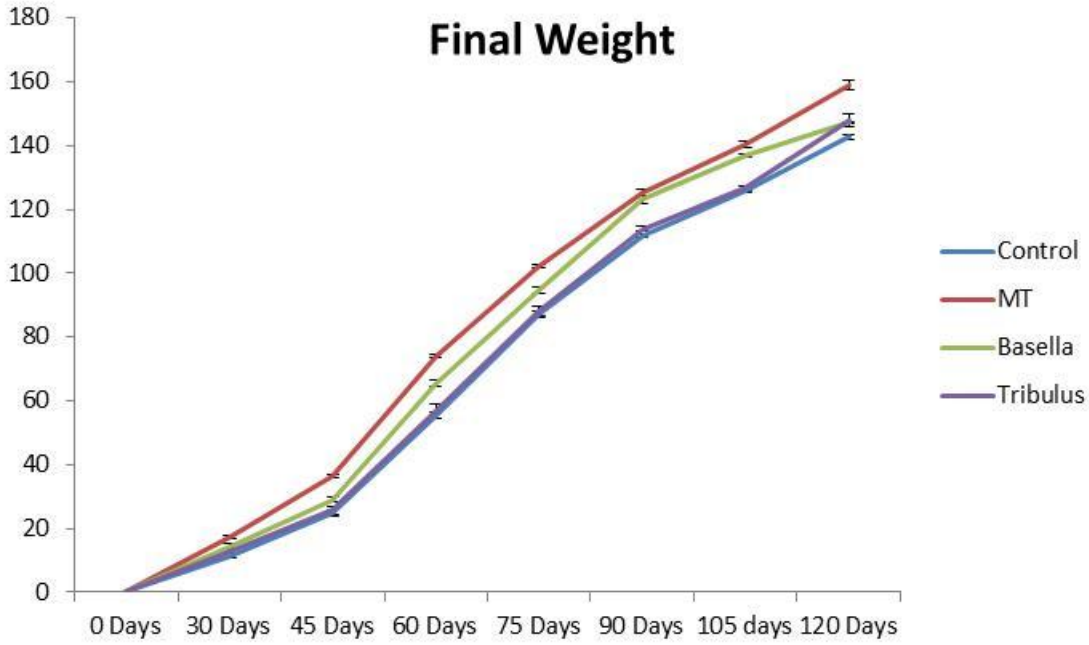


Figure 9: Day wise final weight (gm) of tilapia of different treatment categories during the 4 months' culture in earthen ponds.

Treatment with 17α-MT (10mg/Kg) showed significantly higher (205.30±0.42 cm) final length ($P<0.05$) compared to all other treatment categories. EB1 showed significantly higher final length ($P<0.05$) compared to both control and TB2 throughout the treatment regime (Table 10, Figure 10).

Treatment	30 Days	45 Days	60 Days	75 Days	90 Days	105 Days	120 Days
Control	21.50 ± 1.47^{a}	41.65 ± 2.00^{a}	76.30 ± 0.77^{a}	123.20 ± 0.76^{a}	161.30 ± 0.49^{a}	175.90 ± 0.67^{a}	183.40 ± 0.90^{a}
17α-MT	30.61 ± 1.16^{c}	65.31 ± 0.90^{d}	93.00 ± 0.63^{c}	136.30 ± 0.60^{c}	178.10 ± 1.12^{b}	199.70 ± 0.84^{d}	205.30 ± 0.42^{c}
EB1	27.27 ± 1.81^{bc}	58.54 ± 1.33^{c}	85.10 ± 0.98^{b}	126.00 ± 0.52^{b}	174.80 ± 0.94^{b}	194.60 ± 0.70^{c}	195.80 ± 0.66^{b}
ET2	24.40 ± 1.42^{ab}	51.16 ± 2.22^{b}	74.60 ± 2.17^{a}	123.40 ± 0.70^{a}	164.10 ± 1.01^{a}	184.40 ± 1.15^{b}	186.20 ± 1.41^{a}

Table 10: Final length (cm) at every 15 days interval up to 4months' culture in earthen ponds. Data are expressed as mean ± sem. Different superscripts mark significant difference ($P<0.05$) in means within columns. n= 10 fishes, Initial length = 0.056±0.002 cm.

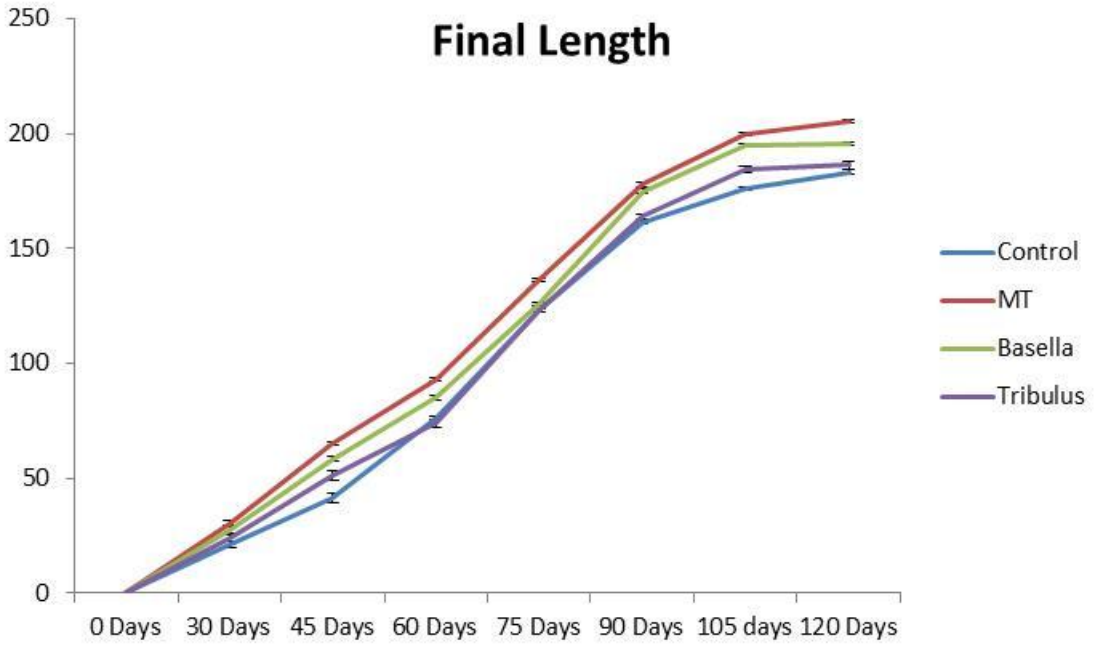


Figure 10: Day wise final length (cm) of tilapia of different treatment categories during the 4 months' culture in earthen ponds.

Weight gain (gm) and daily weight gain (gm / day) of fish fed diets containing 17α-MT showed significantly higher ($P<0.05$) values compared to other three treatment categories. EB1 and ET2 showed significantly higher ($P<0.05$) weight gain compared to control, while there was no significant difference ($P>0.05$) in DWG between EB1, ET2 and control at the end of the culture period. No significant difference ($P>0.05$) was observed for SGR among the four treatment categories (Table 11).

Treatment	Growth Parameters		
	WG (gm)	DWG (gm)	SGR (%)
Control	142.71 ± 0.13^{a}	1.192 ± 0.004^{a}	7.63 ± 0.32^{a}
17α-MT	159.48 ± 0.62^{c}	1.343 ± 0.019^{b}	7.48 ± 0.49^{a}
EB1	147.53 ± 0.35^{b}	1.251 ± 0.021^{a}	7.60 ± 0.26^{a}
ET2	148.06 ± 0.28^{b}	1.237 ± 0.006^{a}	7.92 ± 0.54^{a}

Table 11: Comparative growth parameters of the four treatment categories at the end of 4months' culture in earthen ponds. Data are expressed as mean ± sem. Different superscripts mark significant difference ($P<0.05$) in means within columns.

Thus it may be concluded that among the plant materials, though dietary administration of ethanol extract of *T. terrestris* at the concentration of 2.0 gm /kg feed produced higher percentage of males, ethanol extract of *B. alba* leaves at a concentration of 1.0 gm/kg feed has better growth promoting effect (Figure 11).

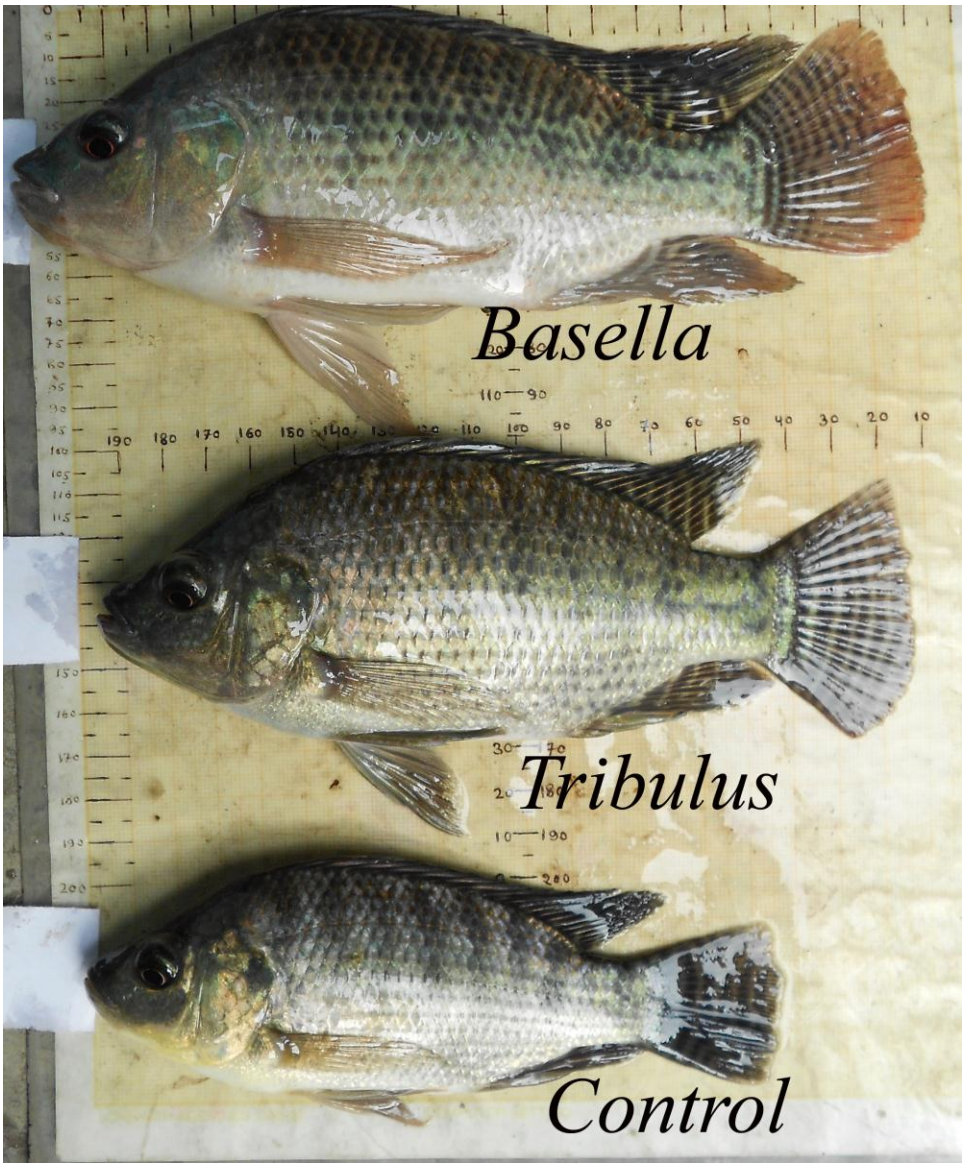


Figure 11: The growth differences of plant extracts treated fish at the end of 4 months' culture in earthen ponds.

At the end of 4 months' culture in earthen ponds, non-specific immune responses showed that EB1 has significantly ($P<0.05$) higher immunostimulatory activity compared to control, MT, and ET2 treatment categories. EB1 has significantly higher values of phagocytotic activity (0.695±0.011), respiratory burst activity (0.106±0.001), sera lysozyme activity (31.49±1.23 µg/mL) and total immunoglobulin production (0.098±.005 mg/mL) compared to other treatment categories. But in terms of total protein production ET2 showed significantly higher value

(0.224±.011 mg/mL).17α-MT treatment decreased significantly the phagocytotic activity (0.247±0.009) and total immunoglobulin (0.060±.002 mg/mL) production compared to control (Table 12).

Treatment	Phagocytotic activity	Respiratory burst	Sera lysozyme activity (µg/mL)	Total protein (mg/mL)	Total Ig (mg/mL)
Control	0.385 ± 0.016^{b}	0.084 ± 0.005^{a}	23.97 ± 1.23^{a}	$0.116\pm.004^{a}$	$0.075\pm.003^{ab}$
MT	0.247 ± 0.009^{a}	0.082 ± 0.004^{a}	25.47 ± 0.77^{ab}	$0.163\pm.002^{b}$	$0.060\pm.002^{a}$
EB1	0.695 ± 0.011^{c}	0.106 ± 0.001^{b}	31.49 ± 1.23^{c}	$0.145\pm.006^{b}$	$0.098\pm.005^{b}$
ET2	0.428 ± 0.019^{b}	0.091 ± 0.002^{a}	28.91 ± 0.98^{bc}	$0.224\pm.011^{c}$	$0.081\pm.010^{ab}$

Table 12: Immunological parameters at the end of 4 months' culture in earthen ponds. Data are expressed as mean±sem. Different superscripts mark significant difference (P<0.05) in means within columns.

Hematological studies of the same treatment categories showed that 17α-MT has significant detrimental issue on hematological parameters. It reduced the total RBC, hemoglobin concentration, hematocrit value, MCHC, total WBC, lymphocytes, monocytes, neutrophils and eosinophils significantly (P<0.5), but has no significant effect on MCH and MCV values (Table 13). Both plant extracts enhanced the hematological parameters significantly (P<0.5), which proved their adaptogenic activity. EB1 has slightly higher values of total RBC ($1.75\pm0.020 \times 10^{6}$/mL), hemoglobin concentration (8.54±0.04 g/dL), hematocrit (25.36±0.12 %), MCHC (33.660±0.003 g/dL), neutrophil (5.13±0.395%) and eosinophil (0.51±0.073%) compared to other treatment categories (Table 13). EB1 showed significantly (P<0.5) higher values of total WBC ($20.35\pm0.312 \times 10^{3}$/mL), lymphocyte (92.62±0.232%) and monocyte (6.99±0.216%) in comparison to control and ET2 treatment groups. Both MT (1.65±0.040 mg/mL) and EB1 (1.16±0.060 mg/mL) significantly (P<0.5) decreased the serum glucose level but only EB1 decreased the serum albumin level (0.59±0.031 mg/mL). ET2 significantly has higher serum albumin value (2.66±0.079 mg/mL) compared to other treatment groups (Table 13).

Treatment	RBC Indices					
	Total RBC (10^6/mL)	Hemoglobin (g/dL)	Hematocrit (%)	MCHC (g/dL)	MCH (pg)	MCV (μm^3)
Control	1.62±0.032^b	8.27±0.02^b	24.59±0.06^b	33.645±0.000^b	50.99±0.93^a	151.58±2.76^a
MT	1.46±0.043^a	7.80±0.06^a	23.20±0.18^a	33.606±0.006^a	53.60±1.28^a	159.50±3.84^a
EB1	1.75±0.020^b	8.54±0.04^b	25.36±0.12^b	33.660±0.003^b	48.81±0.36^a	144.97±1.08^a
ET2	1.67±0.031^b	8.32±0.18^b	24.73±0.53^b	33.650±0.009^b	49.80±1.99^a	147.99±5.89^a

a)

Treatment	WBC Indices				
	Total WBC (10^3/mL)	Lymphocyte (%)	Monocyte (%)	Neutrophil (%)	Eosinophil (%)
Control	17.23±0.147^b	90.51±0.205^b	5.80±0.247^b	4.36±0.095^b	0.42±0.055^b
MT	14.42±0.296^a	85.42±0.266^a	4.02±0.139^a	2.85±0.206^a	0.07±0.040^a
EB1	20.35±0.312^c	92.62±0.232^c	6.99±0.216^c	5.13±0.395^b	0.51±0.073^b
ET2	18.22±0.410^b	91.65±0.439^{bc}	5.96±0.110^b	4.93±0.209^b	0.43±0.049^b

b)

Treatment	Serum Glucose (mg/mL)	Serum Albumin (mg/mL)
Control	4.77±0.353^b	0.98±0.063^b
MT	1.65±0.040^a	1.29±0.117^b
EB1	1.16±0.060^a	0.59±0.031^a
ET2	4.51±0.200^b	2.66±0.079^c

c)

Table 13: Hematological parameters viz. a) RBC indices, b) WBC indices and c) Serum indices at the end of 4 months' culture in earthen ponds. Data are expressed as mean±sem. Different superscripts mark significant difference (P<0.05) in means within columns.

All the three treatments has significantly (P<0.5) lower value of LPO compared to control. Fish fed diets containing MT showed the lowest LPO level (6.65±0.25 U/mg protein) followed by ET2 (7.70±0.10Umg protein). ET2 showed significantly lower GSH (47.70±0.10 U/mg protein) and GST (1.135±0.015 U/mg protein) level, and higher GRD (259.95±0.15 U/mg protein) level compared to other treatment categories. These results clearly indicated that ethanol extract of *T. terrestris* seed has potent antioxidant potential in *Oreochromis niloticus* (Table 14a). Serum assessment of liver enzymes clearly showed that MT treatment has a hepatotoxic activity because it significantly (P<0.5) increased the serum ALP (0.395±0.015 U/mg protein), GOT (25.75±0.35U/mg protein) and GPT (191.64±0.895 U/mg protein) compared to control. Both EB1 and ET2 possess hepatoprotective activity. ET2 showed significantly lower value of ALP (0.145±0.025 U/mg protein) compared to other treatment categories and EB1 decreased both GOT (14.30±0.10 U/mg protein) and GPT (174.85±0.050 U/mg protein) significantly compared to other groups (Table 14b). It may thus be postulated that ethanol extract of *B. alba* leaves possesses more potent hepagtoprotective activity. The activities of ALP, GOT and GPT are useful marker enzymes of damage to the liver (Akanji et al. 1993). Hepatotoxic drugs or chemicals like carbamazepine (Eswaran et al. 2014), paracetamol (Krishna et al. 2012), and carbon tetrachloride (Nasir et al. 2013) were generally found to increase serum level of ALP, GOT and GPT enzymes in treated rats. Bi-herbal ethanolic extracts of leaves of *Aerva lanata* and *Achyranthes aspera* (Krishna et al. 2012); aqueous leaves extract of *Andrographis paniculata* (Nasir et al. 2013) were observed to decrease the elevated liver enzymes in rats and were considered as hepatoprotective.

Treatment	LPO (U/mg protein)	GSH (U/mg protein)	GRD (U/mg protein)	GST (U/mg protein)
Control	9.80 ± 0.10^{d}	54.90 ± 0.10^{c}	252.45 ± 0.15^{a}	1.865 ± 0.015^{c}
MT	6.65 ± 0.25^{a}	58.05 ± 0.45^{d}	249.75 ± 0.35^{a}	2.180 ± 0.050^{d}
EB1	8.60 ± 0.10^{c}	49.90 ± 0.10^{b}	254.35 ± 1.85^{a}	1.335 ± 0.015^{b}
ET2	7.70 ± 0.10^{b}	47.70 ± 0.10^{a}	259.95 ± 0.15^{b}	1.135 ± 0.015^{a}

a)

Treatment	ALP (U/mg protein)	GOT (U/mg protein)	GPT (U/mg protein)
Control	0.315 ± 0.025^{bc}	22.90 ± 0.50^{c}	187.60 ± 0.100^{c}
MT	0.395 ± 0.015^{c}	25.75 ± 0.35^{d}	191.64 ± 0.895^{d}
EB1	0.195 ± 0.025^{ab}	14.30 ± 0.10^{a}	174.85 ± 0.050^{a}
ET2	0.145 ± 0.025^{a}	17.85 ± 0.05^{b}	182.70 ± 0.100^{b}

b)

Table 14: Biochemical parameters viz. a) antioxidant enzymes and b) liver enzymes at the end of 4 months' culture in earthen ponds. Data are expressed as mean ± sem. Different superscripts mark significant difference ($P<0.05$) in means within columns.

The sequential column-chromatographic and thin layer chromatographic separation of ethanol extract of *B. alba* leaves and ethanol extract of *T. terrestris* seeds gave total 6 (BA1-BA6) and 8 (TT1-TT8) fractions, respectively (Figure 12). These fractions were then tested to evaluate their sex-reversal potential. Just hatched juveniles of Nile tilapia were fed diets containing the sub-fractions of *B. alba* ethanol extract at the concentration of 1.0 gm/kg feed, and containing the sub-fractions of *T. terrestris* at the concentration of 2.0 gm/kg feed for 30 days. Fraction TT7 was observed to have significantly ($P<0.5$) higher survival percentage (96.67±0.00) among all *T. terrestris* fractions and fraction BA4 showed the highest survival percentage (91.90±5.23) among *B. alba* fractions (Figure 13). Control showed 84.00±0.00 % of survival. Thus, it might be inferred that the fractions of the plants had no adverse effect on the survival of fish. The

highest male was produced in TT2 treatment category (94.12±5.88). TT2 (94.12±5.88) and TT7 (87.92±1.72) showed significantly (P<0.5) higher values compared to the control group (38.09±4.76) in *Tribulus* fractions, while BA2 (88.54±6.19), BA3 (86.40±3.06) and BA4 (85.52±8.60) showed higher values in *Basella* fractions (Figure 13). These fractions were chosen for HPTLC analysis to identify the bioactive components.

a) 

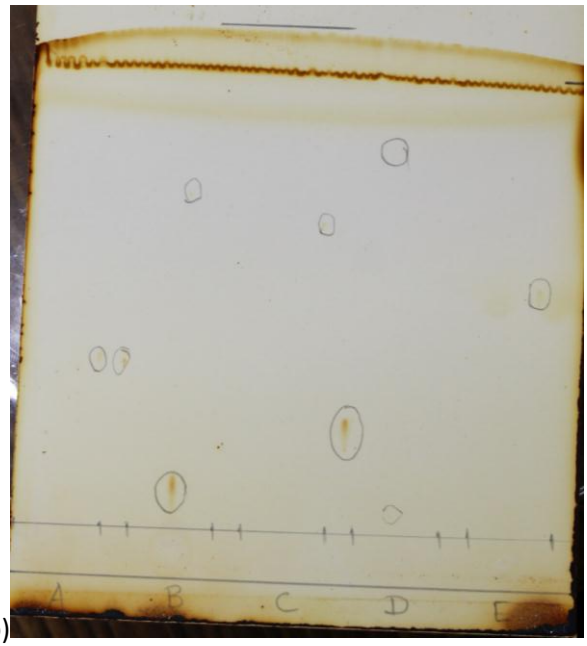
b) 

Figure 12: Chromatographic separation of plant extracts using a) column chromatographic technique and b) thin layer chromatographic technique.

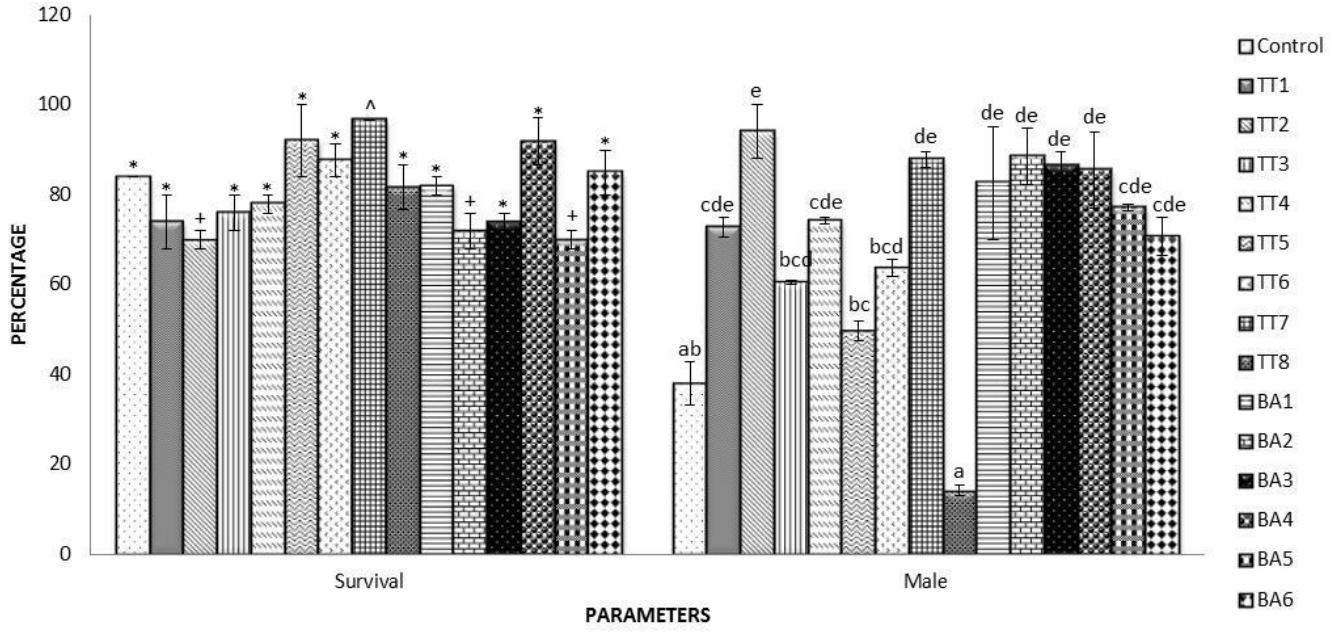


Figure 13: Percentage of survival and males in tilapia fed diets containing different fractions of ethanolic extracts of *B. alba* leaves and *T. terrestris* seeds. Different alphabets above column indicates significant difference ($P<0.05$) in means.

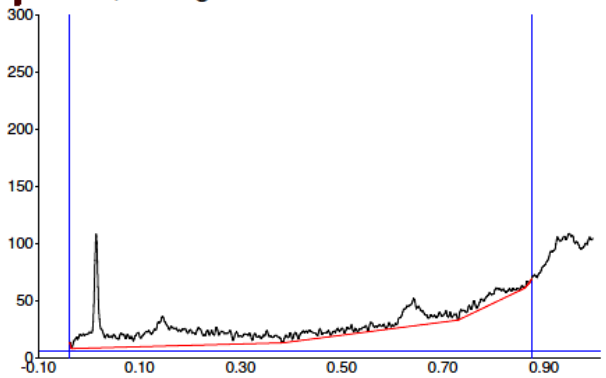
HPTLC analysis of BA2, BA3, BA4, TT2 and TT7 revealed that- BA2 contained stigmasterol, lupeol and quercetin; both BA3 and BA4 contained stigmasterol; and TT2 contained lupeol whereas no phytochemical was identified using the performed protocol in TT7 (Table 15, Figure 14, 15, 16). Further analysis with different standards would be required to identify the phytoconstituents in TT7.

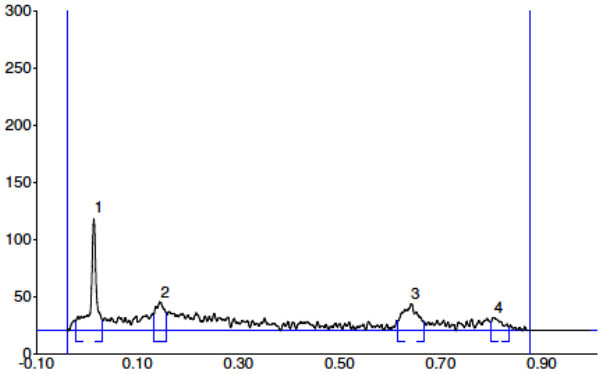
HPTLC Plate: Silica gel 60 F 254, Size: 10cm × 10 cm, Samples dissolve in methanol			
Detection of Sterol group (using Stigmasterol as standard) and Triterpenoid group (using Lupeol as standard), Mobile Phase: Toluene : Methanol= 9.5 : 0.5, Detection using D2 lamp (λ= 254nm)			
Track	Standard/ Fraction	Stigmasterol	Lupeol
1	Stigmasterol	+	-
2	Lupeol	-	+
3	TT2	-	-
4	TT7	-	-

5	BA2	+	+
6	BA3	-	-
7	BA4	-	-
Detection of Sterol group (using Stigmasterol as standard) and Triterpenoid group (using Lupeol as standard), Mobile Phase: Toluene : Methanol= 9.5 : 0.5, Detection using D2 lamp (λ= 209nm)			
Track	Standard/ Fraction	Stigmasterol	Lupeol
1	Stigmasterol	+	-
2	Lupeol	-	+
3	TT2	-	+
4	TT7	-	-
5	BA2	-	-
6	BA3	+	-
7	BA4	+	-
Detection of Phenolic acid group (using Gallic acid as standard) and Flavonoid group (using Quercetin as standard), Mobile Phase: Toluene : Ethayl acetate : Formic acid= 7 : 5 : 1, Detection using D2 lamp (λ= 280nm)			
Track	Standard/ Fraction	Gallic acid	Quercetin
1	Gallic acid	+	-
2	Quercetin	-	+
3	TT2	-	-
4	TT7	-	-
5	BA2	-	+
6	BA3	-	-
7	BA4	-	-

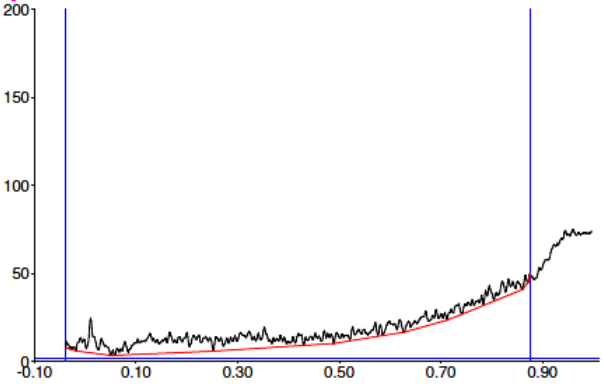
Table 15: HPTLC analysis of the fractions showing highest males percentage (two form ethanolic seeds extract of *T. terrestris* and three from ethanolic leaves extract of *B. alba*).

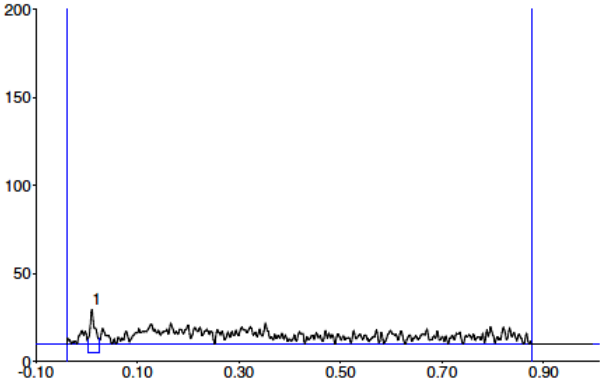
Track 1, ID: Stigmasterol



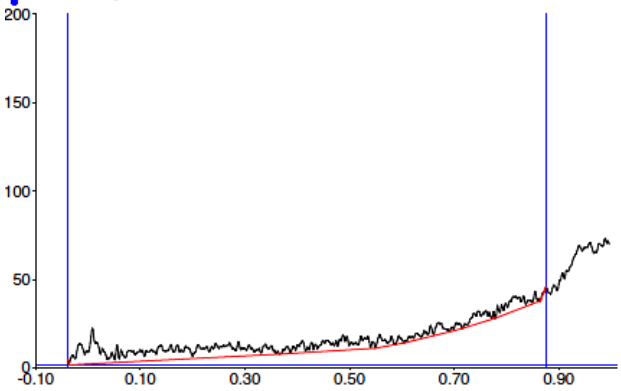


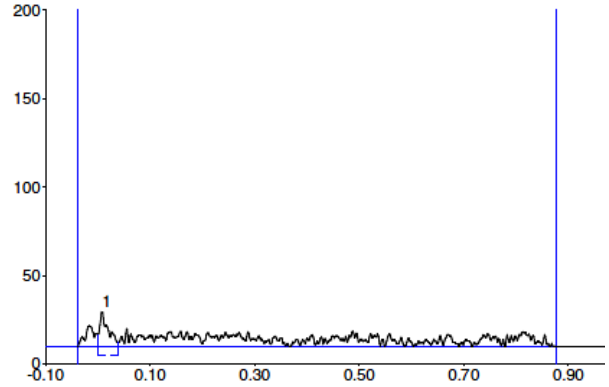
Track 2, ID: Lupeol



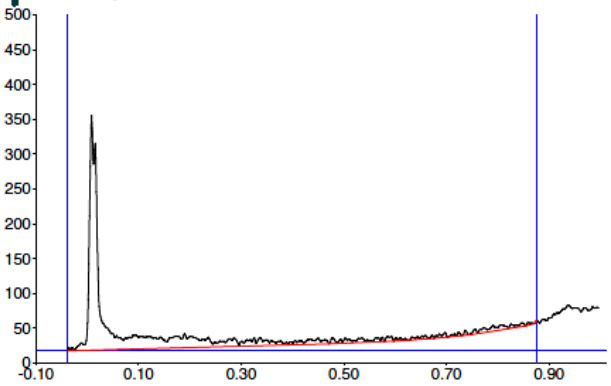


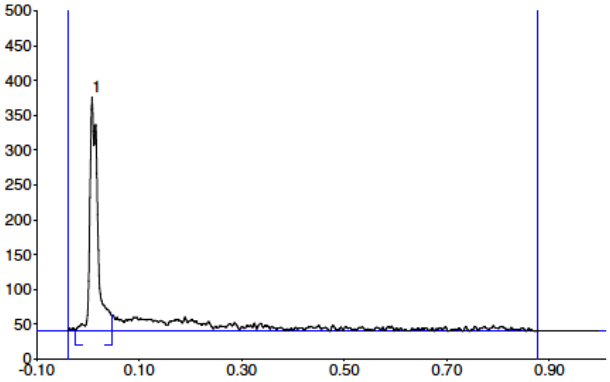
Track 3, ID: TT2



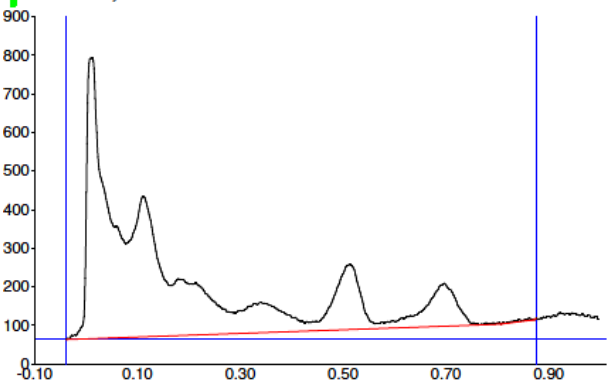


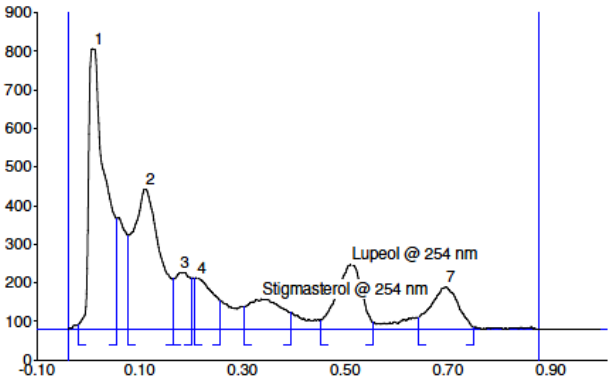
Track 4, ID: TT7



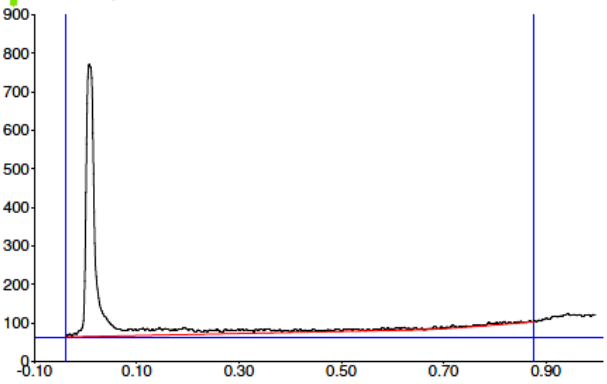


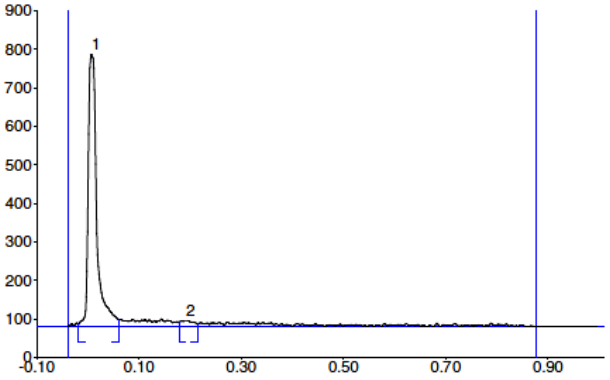
Track 5, ID: BA2





Track 6, ID: BA3





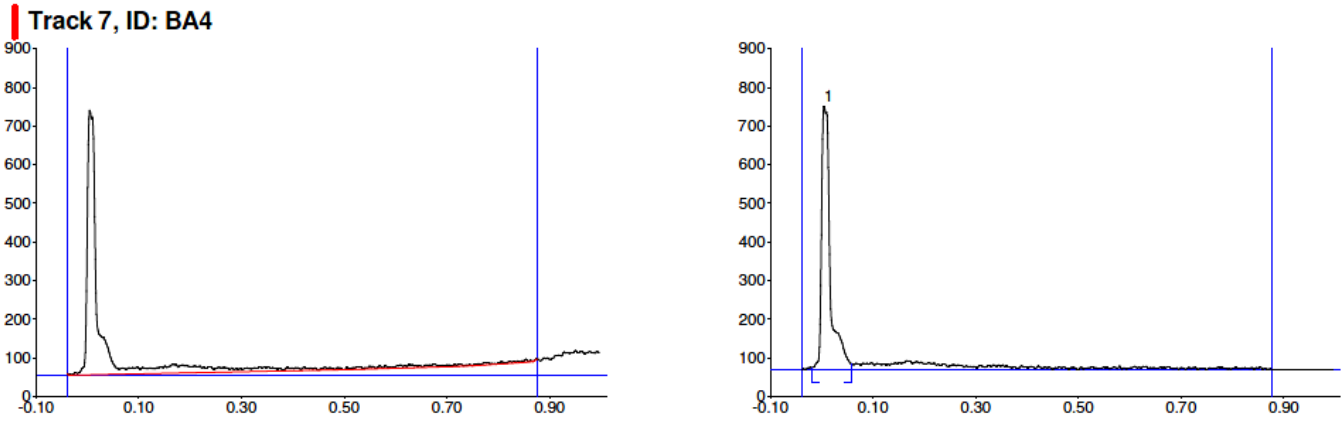
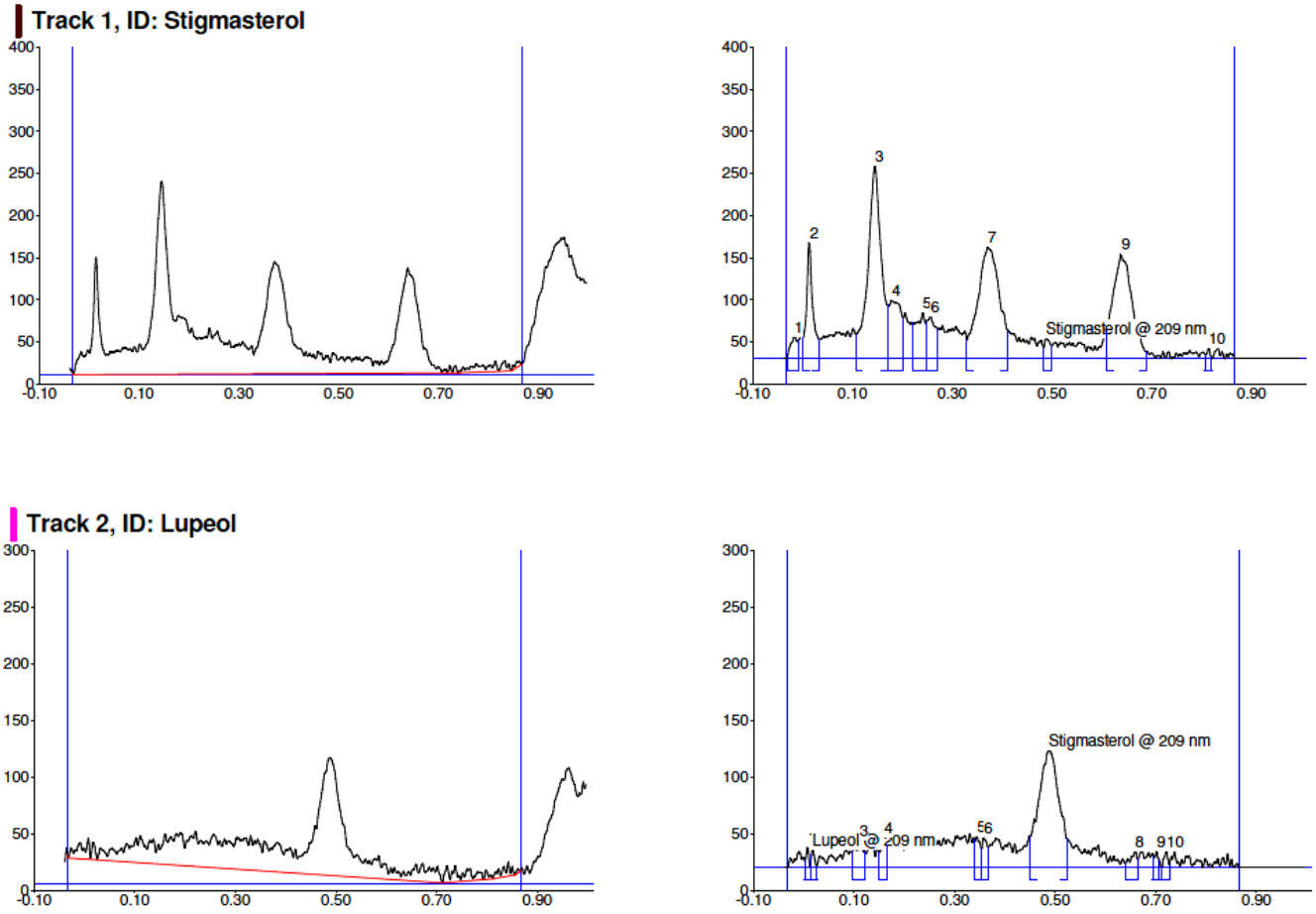


Figure 14: Chromatograms of fractions in reference to Stigmasterol and Lupeol as standards at λ=254nm using toluene : methanol= 9.5 : 0.5 as mobile phase.



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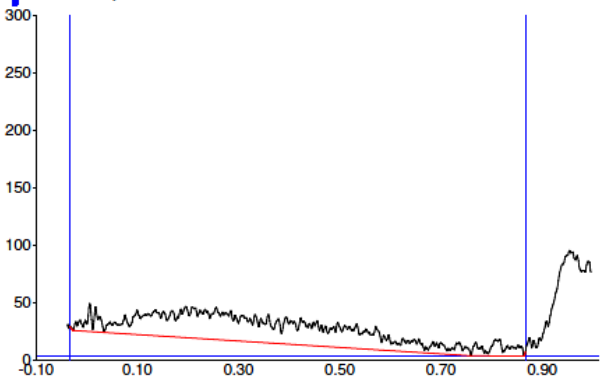
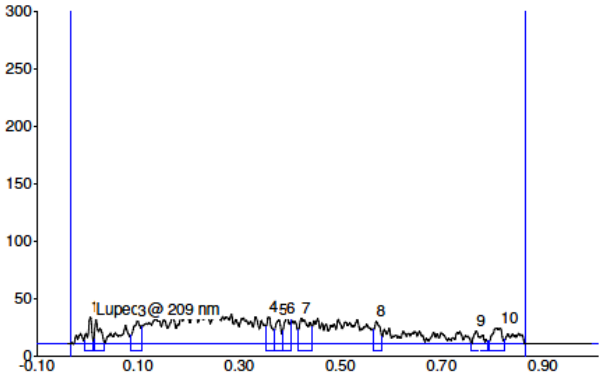
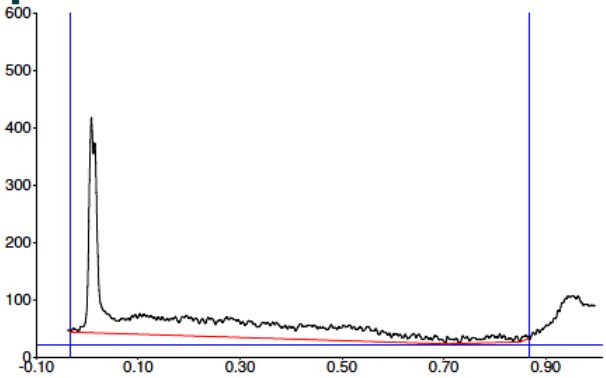
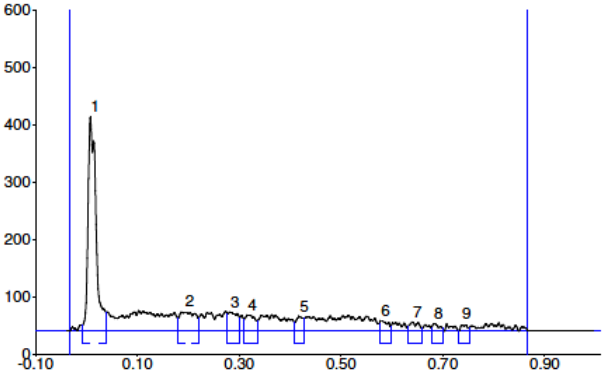


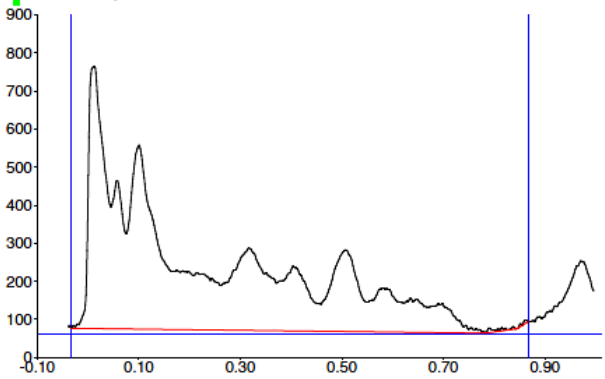


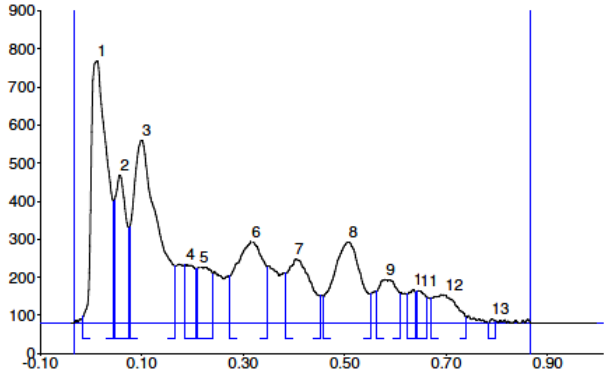
Track 4, ID: TT7





Track 5, ID: BA2





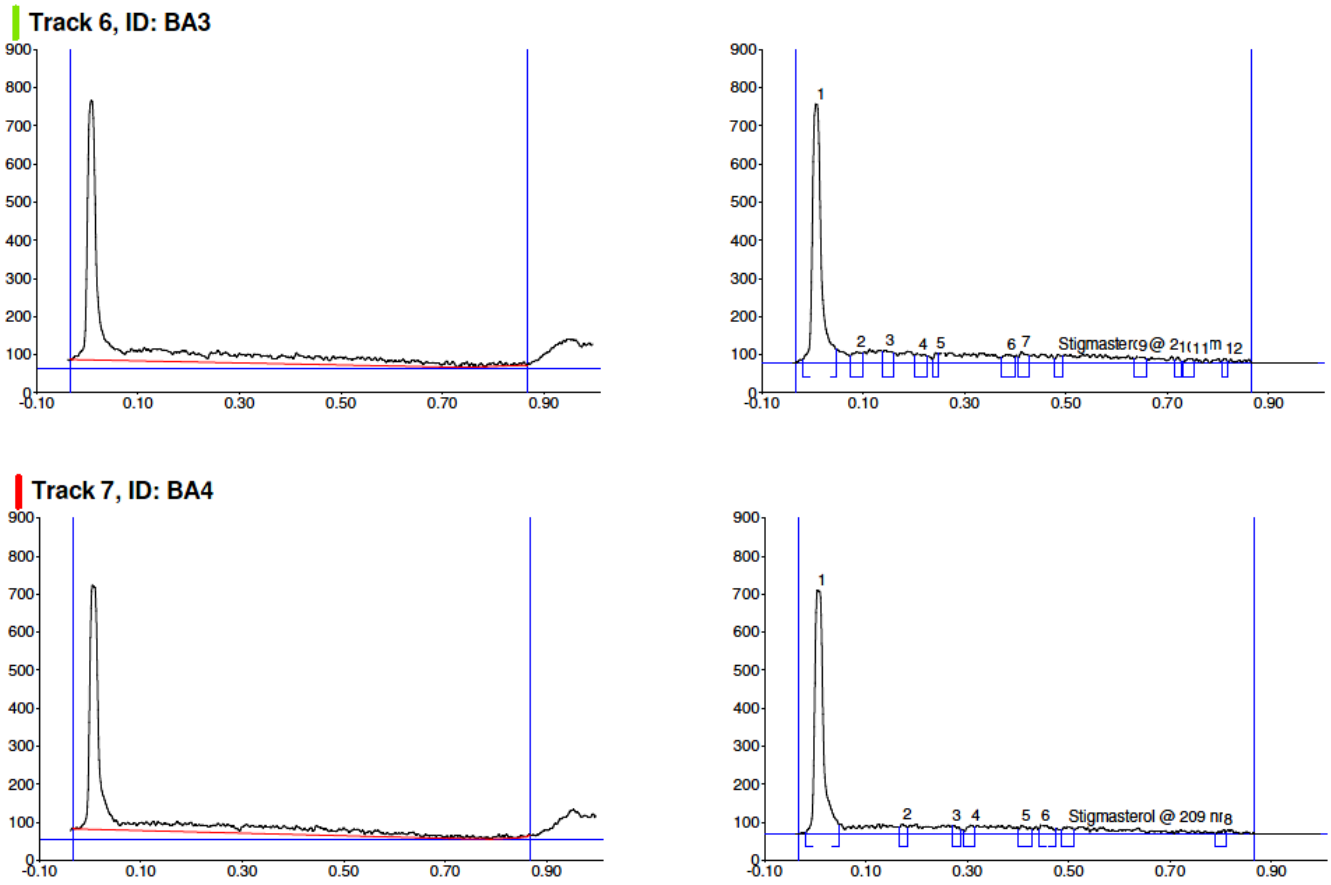
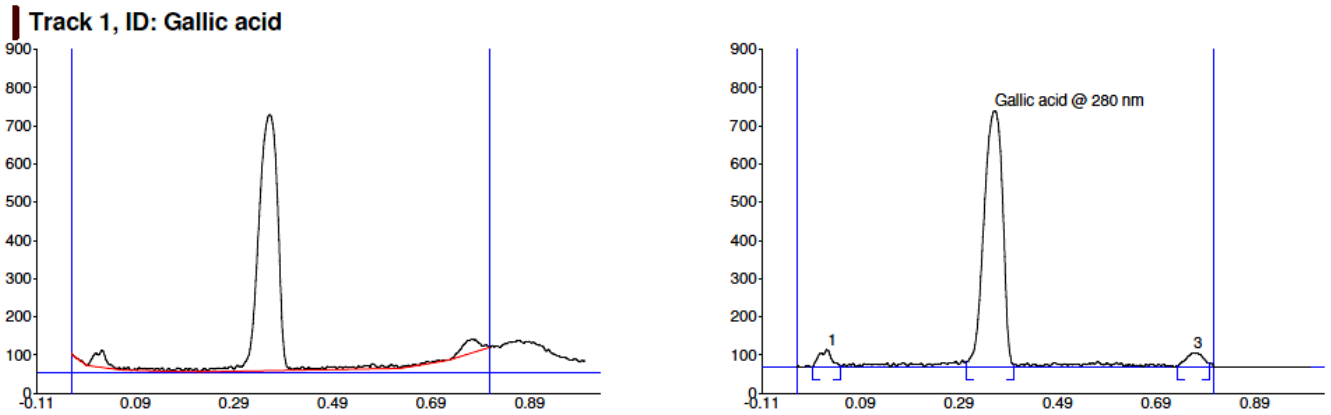
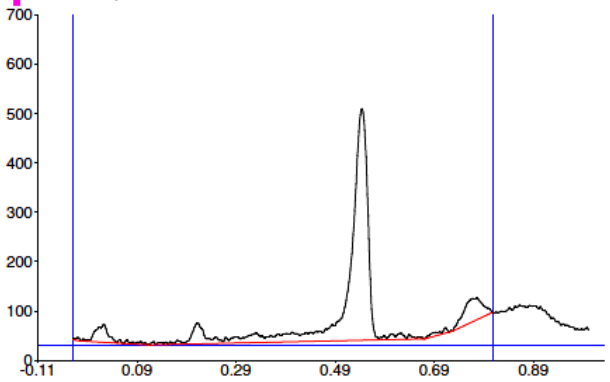
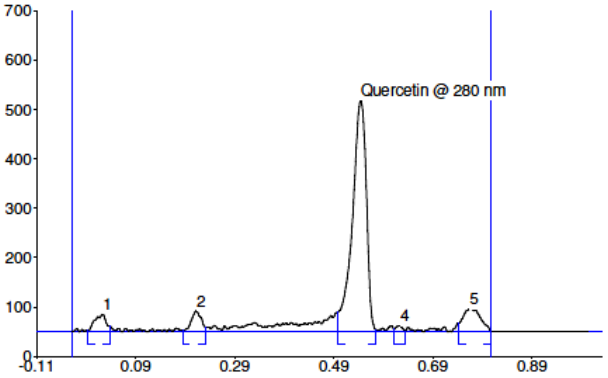


Figure 15: Chromatograms of fractions in reference to Stigmasterol and Lupeol as standards at λ=209nm using toluene : methanol= 9.5 : 0.5 as mobile phase.

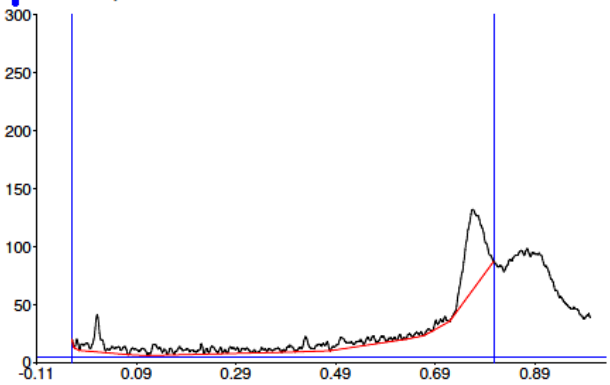


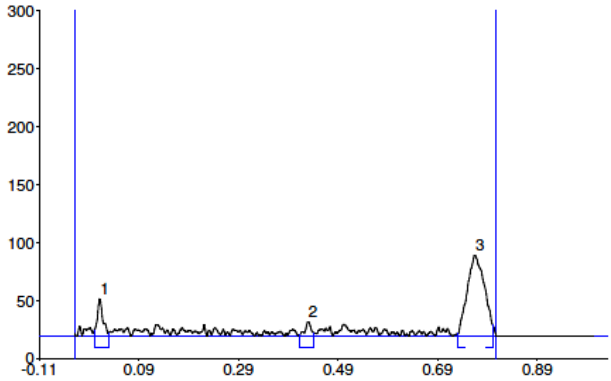
Track 2, ID: Quercetin



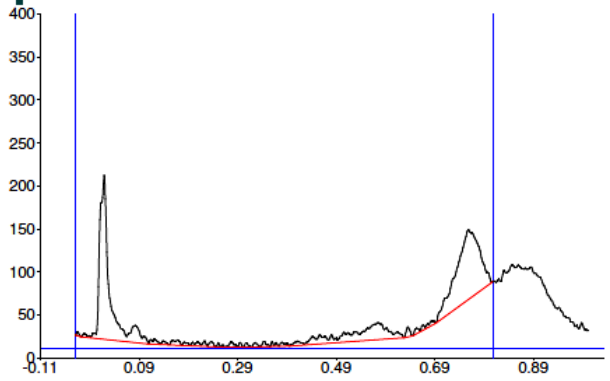


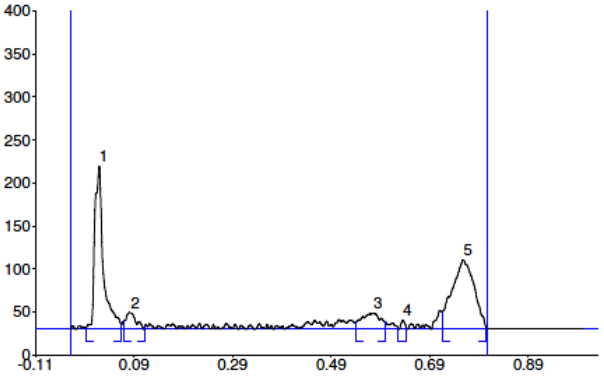
Track 3, ID: TT2





Track 4, ID: TT7





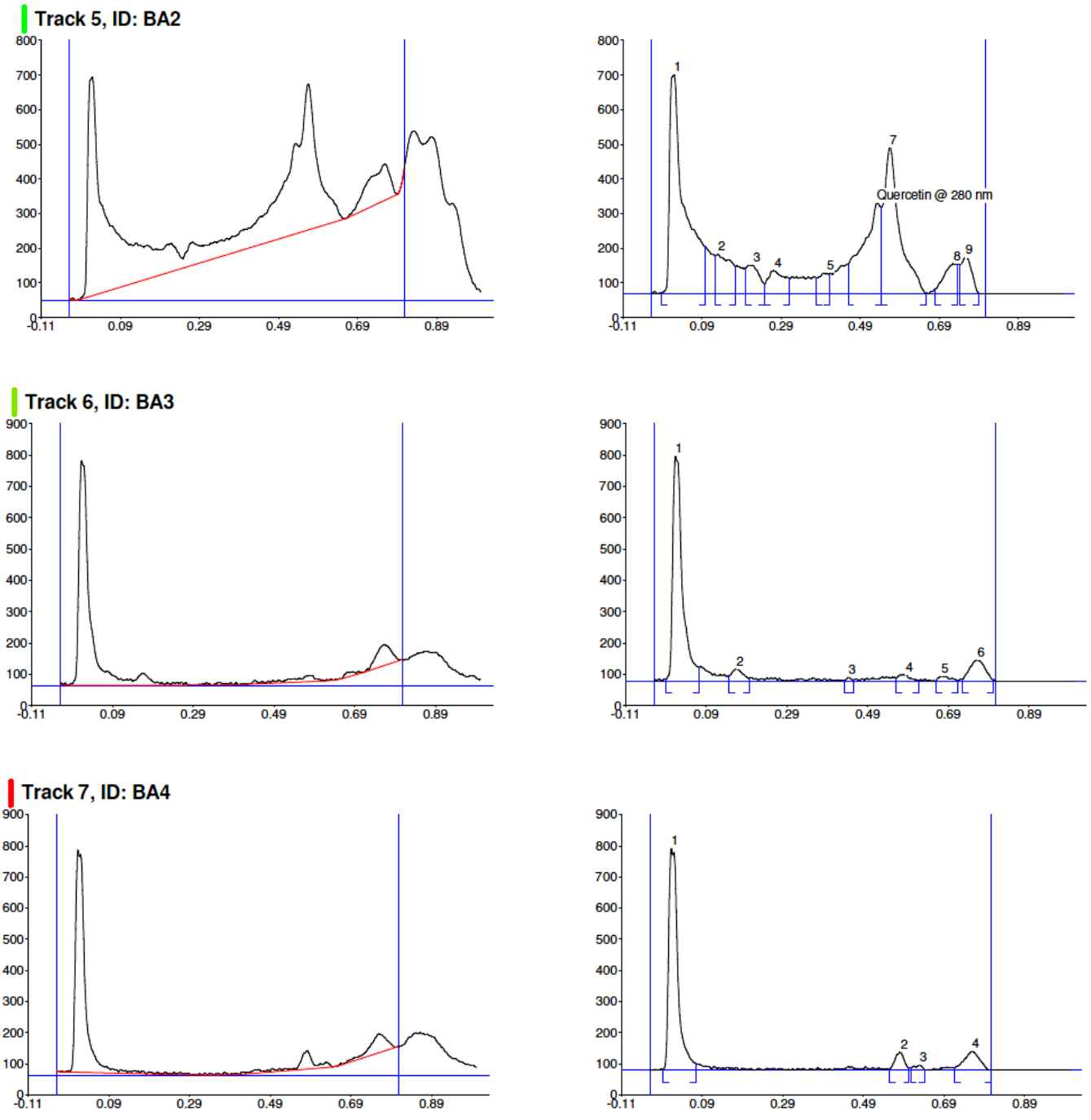


Figure 16: Chromatograms of fractions in reference to Gallic acid and Quercetin as standards at λ=280nm using toluene : ethyl acetate : formic acid= 7 : 5 : 1 as mobile phase.

Conclusion:

The results of the study may be summarised as follows-

1. Both the leaves of *Basella alba* and seeds of *Tribulus terrestris* has the potential to induce sex reversal in tilapia. *Tribulus terrestris* is more potent than *Basella alba* in this context.
2. The ideal method of in vivo application of the plant material for induction of sex reversal in tilpaia is the solvent based extraction method and addition of the extraction to the fish feed.
3. The ideal solvent for extraction of phytochemicals from *Basella alba* and *Tribulus terrestris* for induction of sex reversal in tilapia is ethanol.
4. The ideal concentration of the plant extract for induction of sex reversal in tilapia is 1 gm/Kg for *Basella alba* and 2 gm/Kg for *Tribulus terrestris*.
5. The ideal treatment duration with plant extracts for induction of sex reversal in tilapia is 30 days of feeding.
6. *Basella alba* has more growth promoting and immunostimulating capacities than *Tribulus terrestris*. Both the plant proved that they are potent adpatogen in aquaculture which provide the fish a good non specific immunity, strengthen the hematological attributes, provides antioxidants and hepatoprotection. *Basella alba* has more antiradical or radical scavenging activity than *Tribulus terrestris*.
7. HPTLC analysis of fractions, which gave the highest percentage of males revealed that- *Basella alba* contained stigmasterol, lupeol and quercetin; *Tribulus terrestris* contained lupeol. Further analysis with different standards would be required to pinpoint the phytoconstituents in both plant materials in this regard.

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Suman Bhusan Chakraborty 7/7/17

SIGNATURE OF THE PRINCIPAL INVESTIGATOR

Dr. Suman Bhusan Chakraborty
Assistant Professor
Department of Zoology
University of Calcutta

REGISTRAR/PRINCIPAL

(Seal)

REGISTRAR
Calcutta University

19/07/17

University of Calcutta

Senate House, Kolkata - 700073

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Dear Sir,

I am desired to inform you that you have been granted registration for the Ph.D. programme under this University in

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This registration shall remain valid for next five years with effect from the date of registration as indicated above.

You are to comply with the usual rules of migration in case you have passed the qualifying examinations for the Ph.D. programme from a University/Institute other than the University of Calcutta.

Title of Thesis

Assessment Of The Potential Use Of *Basella alba* And *Tribulus* Terrestris For Production And Culture Of Monosex Nile Tilapia.

Name of the Supervisor : **Dr. Suman Bhusan Chakraborty**

Name of the Joint Supervisor : **X**

Name of the Associate Supervisor : **X**

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Effects of the Aqueous Leaf Extract Of *Basella Alba* on Sex Reversal of Nile Tilapia, *Oreochromis Niloticus*

Indranath Ghosal[1], Suman Bhusan Chakraborty[1]
[1]*Department of Zoology, University of Calcutta*

Abstract: *Three days old mixed sex juveniles of Nile tilapia (mean weight 0.025 ± 0.009 g; mean length 1.25 ± 0.012 cm) were treated by immersion method with aqueous leaf extract of Basella alba (ALEB) at the concentration of 0.0, 0.05, 0.10 and 0.15 g/l for four weeks. The highest survival percentage (93.8±1.3) was observed in 0.15 g/l group, but there was no significant difference (P>0.05) in survival percentage among the different treatment groups. The highest percentage of males (70.3±1.9) was observed in 0.1 g/l group and it was significantly higher (P<0.05) compared to all other treatment categories. Immersion treatment with ALEB caused significant increase (P<0.05) in percentage of males compared to that in untreated control. The extract showed presence of phytochemicals such as tannins, saponins, steroids and alkaloids, which might be associated with its androgenic property.*

Keywords: *Aqueous extract, Basella alba, Orochromis niloticus, Phytochemicals, Sex reversal*

I. Introduction:

The Nile tilapia, *Oreochromis niloticus* (Linnaeus) is a well-studied, fast-growing and widely cultured fish species that 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 rates, high tolerance to low water quality, efficient food conversion, resistance to disease, good consumer acceptance and ease of spawning make tilapia a suitable fish for culture[2]. Female organism 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[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 and 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 also been described to possess nutritional values including androgenecity in traditional medicines of several countries[11]. However, no studies have been reported related to its *in vivo* effect on sex reversal, growth and immunostimulation of fish. Considering these aspects, the objective of the present study was to investigate the potential effect of the aqueous leaf extract of *B. alba* (ALEB) on the masculinisation of *O. niloticus* by immersion technique.

II. Materials And Methods

2.1 Collection of fish seed

Just hatched juveniles of mixed-sex Nile tilapia was collected from the Fish Hatchery of West Bengal Government, oxygen packed and transported to the laboratory.

2.2 Plant extracts preparations

B. alba leaves were procured from the local plant market, washed in sterile distilled water, air-dried in shade and powdered. The aqueous leaf extract of *B. alba* (ALEB) was prepared by boiling 18 g powder in 1500 ml distilled water for 30 minutes and then filtering it with a Whatman filter paper twice[12]. The solution was prepared weekly.

2.3 Immersion treatment of fish with plant extracts

Three days old mixed sex juveniles of Nile tilapia (mean weight 0.025 ± 0.009 g; mean length 1.25 ± 0.012 cm) were randomly assigned in 24 glass aquaria to four different treatment groups (0.0 or control, 0.05,

0.1 and 0.15 g/l). The treatment was conducted for 30 days and the fish were exposed to the ALEB 4 times (once weekly) during this period. The aquaria were continuously aerated and maintained in heated ($T = 27\pm2^0C$) 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 fish was fed finely ground (< 500-1000 μm) artificial diet containing 30% crude protein (Tokyu, Japan) at a rate of 20% body weight / day. The experiment was conducted simultaneously in triplicate.

2.4 Sexing of fish

Sexing of the juvenile fish was done by the standard acetocarmine squash technique of gonads[13]. Histological studies of the gonads were also performed.

2.5 Statistical analysis

All data are expressed in terms of mean ± standard error (SE). Treatment effects on different parameters were analyzed by one-way analysis of variance (ANOVA) after checking normality by Shapiro-Wilk's test. Where significant differences were found, a Tukey's test was performed for separating treatment means. All statistical analysis was performed using the SPSS version 11.5 for Windows.

2.6 Qualitative phytochemical studies

Qualitative phytochemical analysis of the ALEB was carried out using standard procedures[14,15,16].

III. Results And Discussion

Survival percentage in controls was similar to those observed in the ALEB treated groups, where no significant dose-related inter-group differences were noted ($P>0.05$) (Table 1). The highest survival percentage (93.8±1.3) was observed in treatment with ALEB at the concentration of 0.15 g/l while the lowest survival percentage was observed in treatment with ALEB at the concentration of 0.05 g/l (83.75±8.75) (Table 1). The result indicates that immersion treatment with ALEB has no adverse effects on general fish health. All the treatment categories showed significantly higher ($P<0.05$) percentage of males compared to the control. The highest percentage of males (70.3±1.9) was observed in treatment with ALEB at the concentration of 0.1 g/l, which is significantly higher ($P<0.05$) compared to all other groups (Table 1). Interestingly, all the treatment categories except the control group showed intersex with both male and female gonadal tissue. The highest percentage of intersex (12.4±4.3) was observed in ALEB 0.05 g/l treatment group (Table 1). *B. alba* has been reported to be used in traditional medicine to treat sexual asthenia and infertility in man[17]. 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[10,18]. Similar increase in serum testosterone level was also reported in male rats treated with aqueous extract of *B. alba* through gastric intubation[9]. Although the present work indicates that immersion treatment with ALEB could 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 ALEB, while flavonoids, glycosides and carbohydrates are not present in the extract (Table 2). These phytoconstituents might render the androgenic activity of the extract. Interestingly, the highest treatment concentration of 0.15 g/l produced the lowest percentage of males among the different treatment categories (Table 1). Reduced masculinisation and paradoxical feminization has been observed in fish treated with high concentration of synthetic steroids as well [19,20]. A variety of pathways have been postulated to be associated with functional mechanisms of phyto-compounds causing both masculinisation and feminization at different concentrations [21]. The results emanating from this study indicates that ALEB might be used as an alternative method to produce all-male tilapia population in an environment-friendly manner using a natural product. However, the highest percentage of males produced by immersion in ALEB is 70%, which is 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 ALEB and to provide conclusive evidence regarding its efficacy to be used as a sex-reversal agent in tilapia culture.

Table 1: Percentage of survival, male, female and intersex during immersion treatment with ALEB at different concentrations. Different superscripts mark significant differences in means within columns.

Treatment category	% Survival	% of male	% of female	% of intersex
Control	87.5 ± 2.5^a	45.75 ± 1.35^a	54.25 ± 1.35^b	0.0 ± 0.0^a
Basella 0.05 g / l	83.75 ± 8.75^a	59.75 ± 0.25^b	27.85 ± 4.55^a	12.4 ± 4.3^a
Basella 0.1 g / l	84.6 ± 5.4^a	70.3 ± 1.9^c	21.55 ± 2.15^a	8.15 ± 0.25^a
Basella 0.15 g / l	93.8 ± 1.3^a	55.35 ± 1.45^b	34.15 ± 1.75^a	10.5 ± 0.3^a

Table 2: Preliminary phytochemical screening of aqueous extract of B. alba leaves. '+' = present, '-'= absent.

PHYTOCHEMICAL	*Basella alba* aqueous extract
Tannin	+
Saponin	+
Flavonoid	-
Steroid	+
Alkaloid	+
Carbohydrate	-
Glycoside	-

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Effects of the Aqueous Seed Extract of *Tribulus terrestris* on Sex Reversal of Nile Tilapia, *Oreochromis niloticus*

KEYWORDS: Aqueous extract, *Tribulus terrestris*, *Orochromis niloticus*, Sex reversal

Indranath Ghosal
Department of Zoology, University of Calcutta

*** Suman Bhusan Chakraborty**
Department of Zoology, University of Calcutta
*Corresponding Author

ABSTRACT *Three days old mixed sex juveniles of Nile tilapia (mean weight 0.025 ± 0.009 g; mean length 1.25 ± 0.012 cm) were treated by immersion method with aqueous seed extract of Tribulus terrestris (ASET) at the concentration of 0.0 (control), 0.05, 0.10 and 0.15 g/l for four weeks. The highest survival percentage (87.5±2.5) was observed in control group, but there was no significant difference (P>0.05) in survival percentage among the different treatment groups. The highest percentage of males (81.4±0.5) was observed in 0.15 g/l group and it was significantly higher (P<0.05) compared to all other treatment categories. Immersion treatment with ASET caused significant increase (P<0.05) in percentage of males compared to that in untreated control. The extract showed presence of phytochemicals such as tannins, saponins, steroids and alkaloids, which might be associated with its androgenic property.*

Introduction

The Nile tilapia, *Oreochromis niloticus* (Linnaeus) is a well-studied, fast-growing and widely cultured fish species that 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 rates, high tolerance to low water quality, efficient food conversion, resistance to disease, good consumer acceptance and ease of spawning make tilapia a suitable fish for culture (El-Saidy & Gaber, 2005). Female organism 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 & 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, Noltie, & Tillitt, 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 & Hancz. 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 & Schlenk, 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. 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, Turan, & Atik, 2007a; Çek, Turan, & Atik, 2007b). The plant extract has also been found to stimulate growth in fish (Çek et al., 2007a; Çek et al., 2007b). However, no studies have been reported related to its in vivo effect on sex reversal, growth and immunostimulation of tilapia. Considering these aspects, the objective of the present study was to investigate the potential effect of the aqueous seed extract of *T. terrestris* (ASET) on the masculinisation of *O. niloticus* by immersion technique.

Materials And Methods

Collection of fish seed

Just hatched juveniles of mixed-sex Nile tilapia was collected from the Fish Hatchery of West Bengal Government, oxygen packed and transported to the laboratory.

Plant extracts preparations

T. terrestris seeds were procured from the local plant market, washed in sterile distilled water, air-dried in shade and powdered. The aqueous seed extract of *T. terrestris* (ASET) was prepared by boiling 18 g powder in 1500 ml distilled water for 30 minutes and then filtering it with a Whatman filter paper twice (Çek et al., 2007b). The solution was prepared weekly.

Immersion treatment of fish with plant extracts

Three days old mixed sex juveniles of Nile tilapia (mean weight 0.025 ± 0.009 g; mean length 1.25 ± 0.012 cm) were randomly assigned in 12 glass aquaria to four different treatment groups (0.0 or control, 0.05, 0.1 and 0.15 g/l). The treatment was conducted for 30 days and the fish were exposed to the ASET 4 times (once weekly) during this period. The aquaria were continuously aerated and maintained in heated (T = 27±2^{0}C) 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 fish was fed finely ground (< 500-1000 mm) artificial diet containing 30% crude protein (Tokyu, Japan) at a rate of 20% body weight / day. The experiment was conducted simultaneously in triplicate.

Sexing of fish

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

Statistical analysis

All data are expressed in terms of mean ± standard error (SE). Treatment effects on different parameters were analyzed by one-way analysis of variance (ANOVA) after checking normality by Shapiro-Wilk's test. Where significant differences were found, a Tukey's test was performed for

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separating treatment means. All statistical analysis was performed using the SPSS version 11.5 for Windows.

Qualitative phytochemical studies

Qualitative phytochemical analysis of the ASET was carried out using standard procedures (Malpani, Rajput, Mane, & and Dhabe, 2011; Kumar & Bhardwaj, 2012; Ray, Chatterjee, & Chakrabarti, 2013).

Results and Discussion

Survival percentage in control was similar to those observed in the ASET treated groups, where no significant dose-related inter-group differences were noted (P>0.05) (Table 1). The highest survival percentage (87.5±2.5) was observed in control fish while the lowest survival percentage was observed in treatment with ASET at the concentration of 0.1 g/l (80.8±2.2) (Table 1). The result indicates that immersion treatment with ASET 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).

All the treatment categories showed significantly higher (P<0.05) percentage of males compared to the control. The highest percentage of males (81.4±0.5) was observed in treatment with ASET at the concentration of 0.15 g/l, which is significantly higher (P<0.05) compared to all other groups (Table 1). Interestingly, all the treatment categories except the control group showed intersex with both male and female gonadal tissue. The highest percentage of intersex (7.2±1.1) was observed in ASET 0.15 g/l treatment group (Table 1). 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* (Omitoyin, Ajani, & Sadiq, 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 & Subramanian, 2011). Results emanating from this study indicate a dose dependent masculinisation effect of *T. terrestris* extract on Nile tilapia. This result 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 & Subramanian, 2011; Kavitha, Ramesh, & Subramanian, 2012; Çek et al., 2007b; Çek et al., 2007a; Turan & Çek, 2007). As the highest treatment concentration of 0.15 g/l produced the maximum percentage of males among the different treatment categories in this study (Table 1), further analysis with increased concentration might be required to achieve 100% sex reversal with ASET.

Table 1: Percentage of survival, male, female and intersex during immersion treatment with ASET at different concentrations. Different superscripts mark significant differences in means within columns.

Treatment category	% Survival	% of male	% of female	% of intersex
Control	87.5±2.5[a]	45.75±1.35[a]	54.25±1.35[c]	0.0±0.0[a]
Tribulus 0.05 g / l	83.3±1.7[a]	71.5±2.1[b]	21.3±3.2[ab]	7.2±1.1[a]
Tribulus 0.1 g / l	80.8±2.2[a]	72.0±0.8[b]	23.0±0.5[b]	5.0±1.0[a]
Tribulus 0.15 g / l	81.7±1.7[a]	81.4±0.5[c]	13.4±0.7[a]	5.2±1.2[a]

T. terrestris has been reported to be used in traditional medicine to treat sexual asthenia and infertility in man (Adaikan, Gauthaman, & Prasad, 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, Ganesan, & Prasad, 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, Adaikan, & Prasad, 2002; Gauthaman & 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. Although the present work indicates that immersion treatment with ASET could induce high rate of masculinisation in tilapia, whether this potency is caused by increase in androgen level cannot be deduced as the serum testosterone level was not measured during the study. Thus, extensive studies would be required to clarify the probable mode of action of *T. terrestris* steroid saponin in fish. Qualitative analysis for phytochemicals revealed the presence of tannins, saponins, steroids and alkaloids in ASET, while flavonoids, glycosides and carbohydrates are not present in the extract. These phytoconstituents might render the androgenic activity of the extract. 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, Horn, & Hancz, 2014).

The results emanating from this study indicates that ASET might be used as an alternative method to produce all-male tilapia population in an environment-friendly manner using a natural product. However, the highest percentage of males produced by immersion in ASET is 81%, which is 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 ASET and to provide conclusive evidence regarding its efficacy to be used as a sex-reversal agent in tilapia culture.

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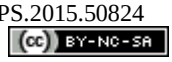
ISSN 2231-3354 

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

Indranath Ghosal[1], Debosree Mukherjee[1], Csaba Hancz[2], Suman Bhusan Chakraborty[1*]

[1]Fish Endocrinology Research Unit, Department of Zoology, University of Calcutta, Kolkata.
[2]Faculty of Agricultural and Environmental Sciences, Kaposvár University, Kaposvár, Hungary.

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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).

* *Corresponding Author*
Suman Bhusan Chakraborty, Department of Zoology, University of Calcutta. Email: sumanbc76@gmail.com

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).

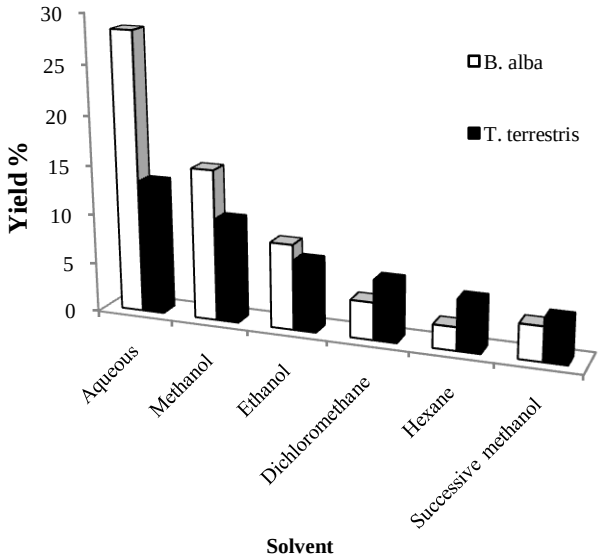

Fig. 1: Yield percentage of evaporated dried extracts based on dry weight basis with different solvents for *B. alba* leaves and *T. terrestris* seeds.

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).

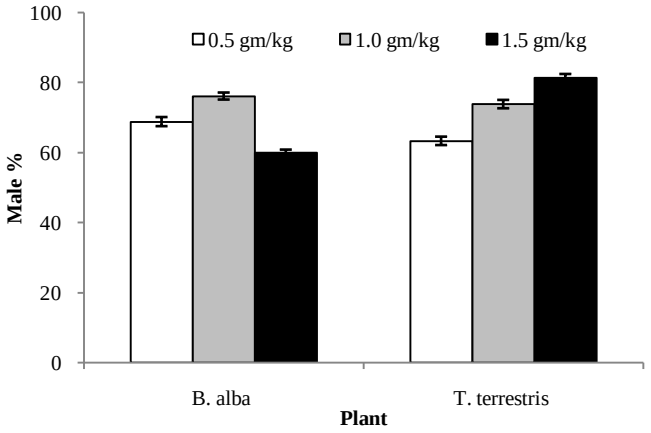

Fig. 2: Percentage of males in tilapia fed diets containing different concentrations of *B. alba* leaves and *T. terrestris* seeds extracts. Different alphabets above column indicates significant difference (P<0.05) in means.

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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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.

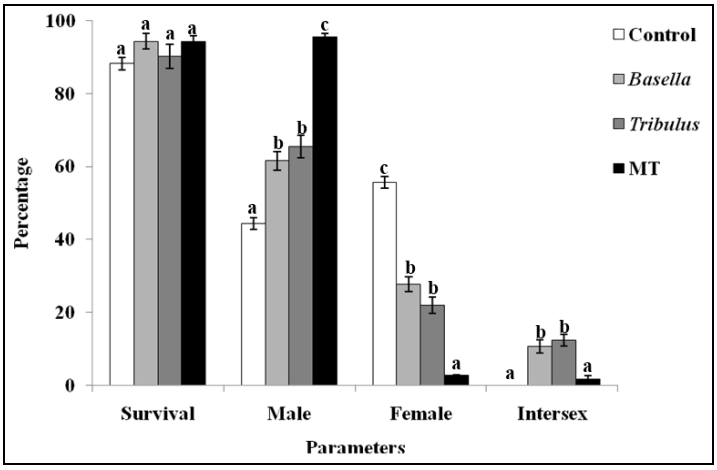

Fig 1: Percentage of survival, male, female and intersex in tilapia fed diets containing untreated control, powdered *B. alba* leaves, *T. terrestris* seeds, and MT. Different alphabets above columns mark significant difference (P<0.05) in means.

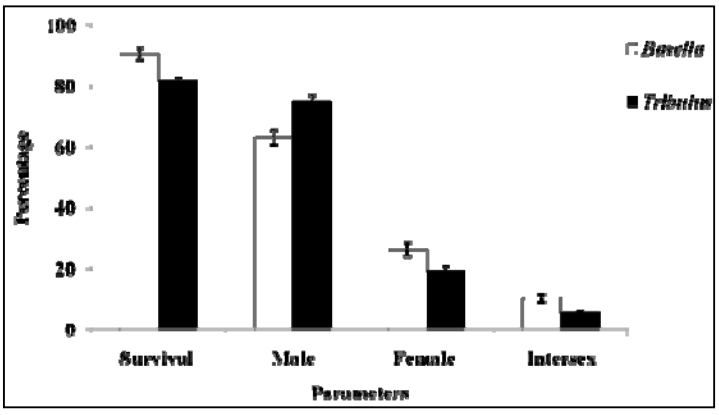

Fig 2: Percentage of survival, male, female and intersex during immersion treatment with aqueous extracts of *B. alba* leaves and *T. terrestris* seeds.

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.

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Summary of the project report (F. 42-519/2013 (SR) dated 22.03.2013)

EVALUATION OF PLANT EXTRACTS ON SEX REVERSAL AND GROWTH OF NILE TILAPIA

Despite promising expectations in tilapia aquaculture, there are a series of factors that are considered to be detrimental in the expansion of production. Female organism 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. Monosex population of male tilapia is produced by treating spawn with a synthetic male hormone 17α-methyltestosterone (MT). However, the increased use of synthetic steroid hormones to produce monosex populations of tilapia for intensive productive systems may lead to environmental and public health concerns. Chronic exposure of consumers to synthetic steroids may cause adverse health effects and its use in fish culture is associated with potential release to the environment and contamination of the biota. Therefore, alternative methods and new, safe chemicals to produce monosex tilapia populations should be considered.

Phytochemicals are a large group of plant-derived compounds that are commonly found in fruits, vegetables, beans, cereals and plant-based beverages like tea and wine. The use of medicinal plants as fertility enhancers and sex reversal agents in fish has been receiving some attention. Plant bioactive compounds might provide a useful source of new medicines and pharmaceutical entities for enhancing fish production and health; and food safety and quality, while conserving the aquatic environment. A possible alternative approach to the use of steroid hormones for sex reversal in tilapia may involve the use of plant extracts containing phytochemicals such as isoflavonoids, flavonoids and saponins, which are natural compounds characterized by estrogenic/androgenic activity. Besides, with their potent antioxidant and immunostimulating properties, phytochemicals have been found to stimulate fish growth (Makkar *et al* 2007, Citarasu 2010).

Basella alba, a fast growing vegetable native to tropical Asia, has been found to have anabolizing and virilizing effects and may be used to boost low levels of testosterone in aging males. The herb, *Tribulus terrestris* has been reported to raise testosterone levels and to induce sex reversal in fish. However, further experimentation is required to validate the possibility of the use of these plant extracts as sex reversal agents in tilapia, in order to ensure that they are as effective as the

common technique to produce monosex populations that involves the use of steroid hormones such as MT.

Objectives

Phytochemicals present in many plants have several reported biological properties. This study is intended to explore the possible utilization of two such plant extracts containing different phytochemicals as potential *in vivo* enzymatic inhibitors of aromatase and of nuclear estrogen receptors' antagonist in gonad germ cells. Such response might modulate the sex differentiation process of the gonad in sexually undifferentiated Nile tilapia and affect the sex ratio of the tilapia populations. Thus, the major objectives of the present study are:

i. Determination of the potentiality of *Basella alba* and *Tribulus terrestris* to induce sex reversal in tilapia under the ecological condition of the Gangetic plains in West Bengal, India.
ii. Determination of ideal method of *in vivo* application of the plant material for induction of sex reversal in tilapia.
iii. Determination of ideal solvent for extraction of phytochemicals from *Basella alba* and *Tribulus terrestris* for induction of sex reversal in tilapia.
iv. Determination of ideal concentration of the plant extract for induction of sex reversal in tilapia.
v. Determination of ideal treatment duration with plant extracts for induction of sex reversal in tilapia.
vi. Analysis of the growth-promoting, immunolostimulating and antioxidant properties of the plant extracts during *in vivo* application in tilapia.
vii. Identification of the bioactive principles in the plant extracts.

Achievements

Tilapias are important foodfish in many tropical and subtropical countries. Despite promising expectations in tilapia aquaculture, there are a series of factors that are considered to be detrimental in the expansion of production. Female organism 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. Monosex population of male tilapia is produced by treating spawn with a synthetic male hormone 17α-methyltestosterone (MT). However, the increased use of synthetic steroid hormones to produce monosex populations of tilapia for intensive productive systems

may lead to environmental and public health concerns. Therefore, alternative methods and new, safe chemicals to produce monosex tilapia populations should be considered.

Our experiments reveal that dietary administration of ethanol extract of *Basella alba* leaves at concentration of 1 gm/Kg feed and *Tribulus terrestris* seeds at concentration of 2 gm/Kg feed for 30 days can be used to produce all-male Nile tilapia (*Oreochromis niloticus*) in aquaculture practice. It has been found that fish fed plant extract fortified diet showed higher growth and better health compared to fish fed control diet. The plant extracts were found to contain stigmasterol, lupeol and quercetin as bioactive phytoconstituents, which may be responsible for the androgenic and immunostimulating efficacy of the plants. Thus, the outcomes of this study would help to establish tilapia culture in a scientific, economic and eco-friendly manner with maximum yield in minimum duration.

Major findings

The following aspects of efficacy of *Basella alba* and *Tribulus terrestris* for production and csustainable culture of all-male monosex Nile tilapia can be put forward from this study:

i. Both the leaves of *Basella alba* and seeds of *Tribulus terrestris* has the potential to induce sex reversal in tilapia. *Tribulus terrestris* is more potent than *Basella alba* in this context.

ii. The ideal method of in vivo application of the plant material for induction of sex reversal in tilpaia is the solvent based extraction method and addition of the extraction to the fish feed.

iii. The ideal solvent for extraction of phytochemicals from *Basella alba* and *Tribulus terrestris* for induction of sex reversal in tilapia is ethanol.

iv. The ideal concentration of the plant extract for induction of sex reversal in tilapia is 1 gm/Kg feed for *Basella alba* and 2 gm/Kg feed for *Tribulus terrestris*.

v. The ideal treatment duration with plant extracts for induction of sex reversal in tilapia is 30 days of feeding.

vi. *Basella alba* has more growth promoting and immunostimulating capacities than *Tribulus terrestris*. Both the plant proved that they are potent adpatogen in aquaculture which provide the fish a good non specific immunity, strengthen the hematological attributes, provides antioxidants and hepatoprotection. *Basella alba* has more antiradical or radical scavenging activity than *Tribulus terrestris*.

vii. HPTLC analysis of fractions, which gave the highest percentage of males revealed that- *Basella alba* contained stigmasterol, lupeol and quercetin; *Tribulus terrestris*

contained lupeol. Further analysis with different standards would be required to pinpoint the phytoconstituents in both plant materials in this regard.

Contribution to the society

Aquaculture has become the world's fastest growing food-producing sector, with a growth rate of 10% annually since 1984. Asia produces about 91% of the world's total aquaculture production, with India as one of the top producers. Freshwater aquaculture is a major source of growth for the whole Asian fishery sector and India contributed ~9.90% of the total worldwide freshwater aquaculture products in 1999. Freshwater fisheries are a priority area in our country with the general objectives of increasing fish production, improving export earnings, providing more animal protein and expanding employment opportunities in the sector. Culture of monosex all-male tilapia has proven to be beneficial for increased production during intensive tilapia culture in ponds throughout the country. However, the increased use of synthetic steroid hormones for such culture has evoked considerable environmental and public health concerns.

It is against this background that our study dealing with the production of sex reversed, massive grown all male tilapia population using biodegradable natural plant was both timely and important. The broad objective of our study was also to examine the growth inducing and immunostimulating efficacy of the plant materials during intensive pond culture of tilapia. This might reduce the cost of all-male tilapia production and thereby would benefit the poor rural communities of our country, enhancing food security by supplying cheap animal protein for household consumption. Our study demonstrated that, both leaves of *Basella alba* and seeds of *Tribulus terrestris* can be used as a substitute of 17α-Methyl testosterone to produce all-male Nile tilapia. Replacement of the synthetic steroid with plant materials during aquaculture exert an environment friendly method to achieve higher profit by lowering the environment degradation. This method may also minimize the potential health hazards for the consumers associated with the residual effect, if any, of the synthetic steroid. Therefore, in developing nations like India, where a good number of natural open water systems is available for utilization, our study can lead to a low cost sustainable aquaculture method for the production of a major animal protein source. In this respect also, the introduction of phytochemical treated, sex reversed monosex tilapia population for culture in the natural systems deserves to be advocated and large scale field trials of our samples are awaited in India before they are marketed.